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[Continued on next page]

(54) **Title**: BISPECIFIC TETRAVALENT ANTIBODIES AND METHODS OF MAKING AND USING THEREOF

(57) **Abstract**: A bispecific tetravalent antibody comprising an IgG having a pair of heavy chains and a pair of light chains, and two scFv components being connected to either C or N terminals of the heavy or light chains. The bispecific tetravalent antibody may have a binding specificity for two different epitopes on HER2 receptor.

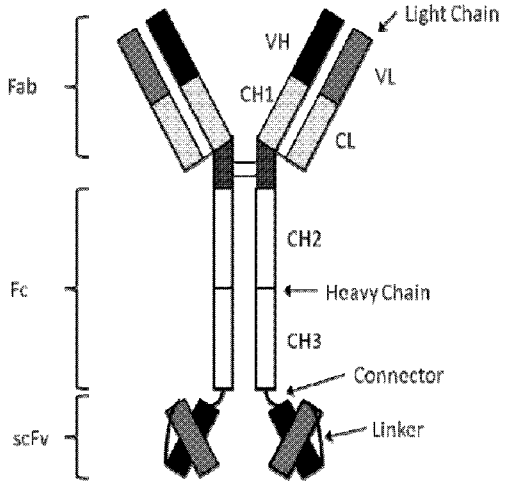

FIG. 1

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BISPECIFIC TETRAVALENT ANTIBODIES AND METHODS OF MAKING AND USING THEREOF

CROSS REFERENCE TO RELATED PATENT APPLICATIONS

This application claims priority over U.S. Provisional Application No. 62095348, filed December 22, 2014, titled "BISPECIFIC ANTIBODIES," which is hereby incorporated by reference in its entirety.

SEQUENCE LISTING

The Sequence Listing associated with this application is provided in text format in lieu of a paper copy, and is hereby incorporated by reference into the specification. The name of the text file containing the Sequence Listing is Sequence Listing_ST25_0003PCT2.txt. The text file is about 164 KB, was created on December 18, 2015, and is being submitted electronically via EFS-Web.

TECHNICAL FIELD

The present disclosure generally relates to the technical field of antibodies, and more particularly relates to bispecific antibodies.

BACKGROUND

HER2, a member of the ErbB/HER receptor family, is overexpressed and/or deregulated in several cancers of the breast and ovary (King, Kraus and Aaronson, *Science* 1985; 229: 974-976; Slamon et al., *Science* 1989; 244:707-712). Therapeutics targeting HER2 have been used successfully in the clinic and have been approved by the US FDA. Such antibody therapeutics includes trastuzumab (Horton, *Cancer Control* 2001: 8(1), 103-110) and pertuzumab (Badache and Hynes, *Cancer Cell*; 5(4): 299-301). Several studies have indicated that therapeutic enhancement may be achieved by combining two or more epitope-distinct anti-HER2 antibodies such as trastuzumab and pertuzumab

compared to a single antibody monotherapy (Kasprzyk et al., *Cancer Res* 1992; 52: 2771–2776, Ben-Kasus et al., *Proc Natl Acad Sci USA*, 106(9) 3294-3329). Trastuzumab which binds to the extracellular domain 4 of HER2 inhibits ligand independent signaling, stimulates ADCC, blocks HER2 shedding but does not inhibit HER2 dimerization. Pertuzumab which binds to the extracellular domain 2 inhibits HER2 dimerization and dimerization with other HER family receptors, inhibits multiple ligand-dependent HER mediated signaling pathways and stimulates ADCC (O'Sullivan and Connolly, *Oncology* 2014; 28(3): 186-194).

A combination of Pertuzumab and Trastuzumab for the treatment of HER2-positive metastatic cancer has been approved by FDA in 2013 as a new treatment for HER2-positive breast cancer, based on substantial clinical benefit seen over Trastuzumab alone (Baselga et al. *N Engl J Med.* 2012 Jan 12; 366(2):109-19). However, the efficacy of the use of the simple combination of two or more monoclonal antibodies is sub-optimal. In addition, the cost of producing two or more monoclonal antibodies separately is high.

Therefore, there is a need to improve the efficacy of cancer treatment by combining monoclonal antibodies and reduce the cost associated with the monoclonal antibody productions.

SUMMARY

The disclosure provides bispecific tetravalent antibodies. The bispecific tetravalent antibody may include two IgG1 heavy chains; two kappa light chains; and two single chain Fv (scFv) domains. The two IgG1 heavy chains and kappa light chains may form an IgG moiety with a binding specificity to a first domain of HER2. The two scFv domains may have a binding specificity to a second domain of HER2. The IgG moiety and two scFv domains are covalently connected to be functional as a bispecific tetravalent antibody. The objectives and advantages of the disclosure may become apparent from the following detailed description of preferred embodiments thereof in connection with the

accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Preferred embodiments according to the present disclosure may now be described with reference to the FIGs, in which like reference numerals denote like elements.

FIG. 1 shows the tetravalent bispecific antibody structure in accordance with one embodiment of the present invention.

FIG. 2 shows the functional block diagrams of example tetravalent bispecific antibodies 4X1, 4X2, 4X3 and 4X4 in accordance with embodiments of the present invention.

FIG. 3 shows the functional block diagram of example monospecific antibodies, 4C1 and 4C2

FIG. 4 shows the functional block diagram of example Fc-scFv antibodies 4C3, 4C4, 4C5, 4C6, 4C7, 4C8, 4C10 and 4C11.

FIG. 5 shows effect of SI-4X and SI-4C antibodies on BT-474 cell proliferation.

FIG. 6 shows effect of extending connector length from 10 amino acids to 30 amino acids on BT-474 cell proliferation.

FIG. 7 shows effect of extending connector length up to 40 amino acids on BT-474 cell proliferation

FIG. 8 shows effect of SI-4X antibodies on HER2 internalization on BT-474 cell.

DETAILED DESCRIPTION

This disclosure provides bispecific tetravalent antibodies. The antibodies may have advantage of targeting both extracellular domains 2 and 4 of HER2 simultaneously.

It must be noted that as used herein and in the appended claims, the

singular forms “a”, “and”, and “the” in Throughout this specification and claims, the word “comprise,” or variations such as “comprises” or “comprising,” will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers include plural referents unless the context clearly dictates otherwise.

“Antibody fragments” comprise a portion of an intact antibody, preferably the antigen-binding or variable region of the intact antibody. Examples of antibody fragments include Fv, Fab, Fab’, F(ab’)2, Fab’-SH; diabodies; linear antibodies (see U.S. Pat. No. 5,641,870, Example 2; Zapata et al., Protein Eng. 8(10): 1057-1062 (1995)); single-chain antibody molecules (e.g. scFv). While in the present description, and throughout the specification, reference is made to antibodies and various properties of antibodies, the same disclosure also applies to functional antibody fragments, e.g. dual action Fab fragments.

In one aspect, the bispecific tetravalent antibody may include two IgG1 heavy chains; two kappa light chains; and two single chain Fv (scFv) domains. The two IgG1 heavy chains and kappa light chains may form an IgG moiety with a binding specificity to a first domain of HER2. The two scFv domains may have a binding specificity to a second domain of HER2. Each scFv domain may be connected to the C-terminal residue of either of the IgG1 heavy chains by a connector having an amino acid sequence of $(gly\text{-}gly\text{-}gly\text{-}gly\text{-}ser)_n$ $((G_4S)_n)$. Each scFv domain may have a structure order of N terminus–variable heavy–linker–variable light–C terminus or N-terminus-variable light-linker-variable heavy-C-terminus, and the linker may include an amino acid sequence of $(gly\text{-}gly\text{-}gly\text{-}gly\text{-}ser)_m$ $((G_4S)_m)$. Both n and m are integrals. n may be an integral of at least 2. In one embodiment, n is from 1 to 10 or 2 to 9. In some embodiments, n is at least 9. In some embodiments, n is from 2 to 20. m may be an integral of at least 2. In some embodiment, m may be 2, 3, 4, or 5. In some embodiments, m may an integral selected from 2, 3, 4, 5, 6, 7, 8, 9, and 10.

In some embodiments, at least one of the IgG1 heavy chains or the kappa light chains is humanized or human. In some embodiments, both IgG1 heavy chains are humanized or human. In some embodiments, both kappa light chains are humanized or human.

In some embodiments, the domain of HER2 is independently selected from domain 2 and domain 4 of HER2. In some embodiments, the bispecific tetravalent antibody may include an IgG moiety with scFv connecting to either C or N terminals of heave or light chains via a peptide linker. The IgG moiety may have binding specificity to extracellular domain 2 or 4 of HER2 (human epidermal growth factor 2) expressing cells while the scFv domains may have binding specificity to ectodomain 4 or 2 of HER2 expressing cells, respectively. The binding may be bivalent.

The peptide linker may vary in length. In some embodiments, the peptide linker may include from about 15 to about 45 amino acids. In some embodiments, the peptide linkers may include from about 20 to about 50 amino acids. In some embodiments, the peptide linkers may include from about 10 to about 30 amino acids.

In some embodiments, the IgG moiety may have a binding specificity for domain 2 of HER2. In some embodiments, the scFv domains may have a binding specificity for domain 4 of HER2. In some embodiments, the IgG moiety may have a binding specificity for domain 4 of HER2. In some embodiments, the scFv domains may have a binding specificity for domain 2 of HER2.

In some embodiments, at least one of or both the IgG1 heavy chains comprises an amino acid sequences of or with at least 95%, 98%, or 99% similarity to SEQ ID NO 7, 15, 30, 40, 50 and 58. In some embodiments, the IgG1 heavy chain, connector, and scFv domain have an amino acid sequence of or with at least 95%, 98%, or 99% similarity to SEQ ID NO 30, 50, 40, and 58. In some embodiments, at least one of or both the kappa light chains comprises an amino acid sequence of or with at least 95%, 98%, or 99% similarity to SEQ ID

NO 3, 11, 25, 35, 45, and 53. In some embodiments, at least one of or both variable light chain comprises an amino acid sequence of or with at least 95%, 98%, or 99% similarity to SEQ ID NO 4, 12, 26, 36, 46, and 54. In some embodiments, at least one of or both variable heavy chain comprises an amino acid sequence of or with at least 95%, 98%, or 99% similarity to SEQ ID NO 8, 16, 31, 41, 79 and 59. In some embodiements, at least one of or both scFv domain comprises an amino acid sequence of or with at least 95%, 98%, or 99% similarity to SEQ ID NO 19, 22, 32, 42, 80, 60, 63, 66, 69, 72, 75, 78.

In some embodiments, the IgG moiety may have a binding specificity for domain 2 of HER2, and the scFv domains may have a binding specificity for domain 4 of HER2. In one embodiment, the IgG1 heavy chain, connector, and scFv domain may have an amino acid sequence of or with at least 95% similarity to SEQ ID NO 30, and the kappa light chain may have an amino acid sequence of or with at least 95% similarity to SEQ ID NO 25. In one embodiment, the IgG1 heavy chain, connector, and scFv domain may have an amino acid sequence of or with at least 95% similarity to SEQ ID NO 50, and the kappa light chain may have an amino acid sequence of or with at least 95% similarity to SEQ ID NO 45.

In some embodiments, the IgG moiety may have a binding specificity for domain 4 of HER2, and the scFv domains may have a binding specificity for domain 2 of HER2. In one embodiment, the IgG1 heavy chain, connector, and scFv domain may have an amino acid sequence of or with at least 95% similarity to SEQ ID NO 40, and the kappa light chain has an amino acid sequence of or with at least 95% similarity to SEQ ID NO 35. In one embodiment, the IgG1 heavy chain, connector, and scFv domain may have an amino acid sequence of or with at least 95% similarity to SEQ ID NO 58, and the kappa light chain may have an amino acid sequence of or with at least 95% similarity to SEQ ID NO 53.

The bispecific tetravalent antibodies have the activity of inhibiting cancer cell growth. In certain embodiments, an antibody of the invention has a dissociation constant (Kd) of ≦80 nM, ≦50 nM, ≦30 nM, ≦20nM, ≦10 nM, or

≦0.1 nM for its target EGRF or HER3. The antibody may bind to both targets simultaneously. In some embodiments, the antibody may bind to domain 2 of HER2 with a Kd less than 1nM, 10nM, 20nM, 50nM, or 100nM. In some embodiments, the antibody may bind to domain 4 of HER2 with a Kd less than 5nM, 10nM, 20nM, 50nM, or 100nM. In some embodiments, the antibody may bind to domain 4 of HER2 with a Kd less than 30nM and binds to domain 2 of HER2 with a Kd less than 30nM. In some embodiments, the antibody may bind to domain 4 of HER2 with a Kd less than 50nM and binds to domain 2 of HER2 with a Kd less than 20nM simultaneously.

In some embodiments, the IgG moiety may provide stability to the scFv domains. In addition and alternatively, the IgG moiety may provide specificity to the epitope. In some embodiments, the bispecific antibody may mediate ADCC (antibody dependent cell-mediated cytotoxicity) towards cells expressing HER2. In some embodiments, the antibody may be capable of binding at least two domains (i.e. epitopes) on the HER2 antigen. In some embodiments, the antibody may bind multiple domains on the HER2 antigen simultaneously.

In some embodiments, the antibody may provide stronger tumour inhibition in proliferation assays in vitro and in vivo than the mono-specific antibody parental controls or combination of mono-specific antibody parental controls. Not wanting to be bound by theory, it is believed that, by acting against the same antigen of two different epitopes, the bispecific tetravalent antibody disclosed herein may enhance internalization of the receptor (HER2) and down regulate the signalling pathway more efficiently than each of the individual mono-specific antibody or combination of the two mono-specific antibodies.

In some embodiments, the bispecific tetravalent antibody may inhibit a cancer cell growth. In some embodiments, the cancer cell may express HER2. In some embodiments, the bispecific tetravalent antibody may inhibit a cancer cell growth. In some embodiments, the cancer cell may express HER2+.

In another aspect, the disclosure provides isolated nucleic acids encoding

the bispecific tetravalent antibodies, a fragment or a subcomponent disclosed herein.

In a further aspect, the disclosure provides expression vectors having the isolated nucleic acids encoding the bispecific tetravalent antibody, a fragment or a subcomponent disclosed herein. The vectors may be expressible in a host cell. The host cell may be prokaryotic or eukaryotic.

In a further aspect, the disclosure provides host cells having the isolated nucleic acids encoding the bispecific tetravalent antibody, a fragment or a subcomponent disclosed herein or the expression vectors including such nucleic acid sequences.

In a further aspect, the disclosure provides methods for producing bispecific tetravalent antibodies. In one embodiment, the method may include culturing the above-described host cells so that the antibody is produced.

In a further aspect, the disclosure provides immunoconjugates including the bispecific tetravalent antibodies described herein and a cytotoxic agent.

In a further aspect, the disclosure provides pharmaceutical compositions. The pharmaceutical composition may include the bispecific tetravalent antibodies or the immunoconjugates described herein and a pharmaceutically acceptable carrier. In some embodiments, the composition may further include radioisotope, radionuclide, a toxin, a therapeutic agent, a chemotherapeutic agent or a combination thereof.

In a further aspect, the disclosure provides methods of treating a subject with a cancer. In one embodiment, the method includes the step of administering to the subject an effective amount of a bispecific tetravalent antibody described herein. The cancer may include cells expressing HER2, a domain, an epitope, a fragment or a derivative thereof. The cancer may be HER2+ breast cancer, colorectal cancer, ovarian cancer, gastric cancer, esophageal cancer, head and neck cancer and non small cell lung cancer.

In one embodiment, the method may further include co-administering

an effective amount of a therapeutic agent. The therapeutic agent may be, for example, an antibody, a chemotherapy agent, an enzyme, or a combination thereof. In some embodiments, the therapeutic agent may be an anti-estrogen agent, a receptor tyrosine inhibitor, or a combination thereof. In some embodiments, the therapeutic agent may be capecitabine, cisplatin, trastuzumab, fulvestrant, tamoxifen, letrozole, exemestane, anastrozole, aminoglutethimide, testolactone, vorozole, formestane, fadrozole, letrozole, erlotinib, lafatinib, dasatinib, gefitinib, imatinib, pazopinib, lapatinib, sunitinib, nilotinib, sorafenib, nab-palitaxel. In some embodiments, the subject in need of such treatment is a human. In some embodiments, the therapeutic agent may be a biologics. In some embodiments, the therapeutic agent may be a checkpoint inhibitor including but not limited to PD1, PDL1, CTLA4, 4-1BB, OX40, GITR, TIM3, LAG3, TIGIT, CD40, CD27, HVEM, BTLA, VISTA, and B7H4.

In one embodiment, the disclosure provides methods for treating a subject by administering to the subject an effective amount of the bispecific tetravalent antibody to inhibit a biological activity of a HER2 receptor.

In one embodiment, the disclosure provides solutions having an effective concentration of the bispecific tetravalent antibody. In one embodiment, the solution is blood plasma in a subject.

A diagram of the general structure of the bispecific tetravalent antibodies is shown in FIG. 1. In one embodiment, the bispecific tetravalent antibody includes two human IgG1 heavy chains, two human kappa light chain, and two single chain Fv (scFv) domains. The two human IgG1 heavy chains and human kappa light chains form an IgG moiety. The two scFv domains are respectively connected to the C-terminal residue of human IgG1 heavy chains with a connector with an amino acid sequence of repeats of gly-gly-gly-gly-ser- also known as $(G_4S)_n$. n can be integral. In one embodiment, n is from 2 to 10. In some embodiments, n may be from 1 to 15. The scFv may be in the order: N terminus–variable heavy–linker–variable light–C terminus. The scFv linker may

include amino acid sequence of repeat of gly-gly-gly-gly-ser, also known as $(G_4S)_m$. m is an integral. For example, m may be 3 or 4. For all of the constructs, CH1, CH2, CH3 and CL amino acid sequences may be identical. There are 4 bispecific antibodies designated 4X1, 4X2, 4X3 and 4X4. These are depicted in FIG. 2.

Each bispecific tetravalent antibody may bind specifically to extracellular domain 2 of HER2 on one end and to extracellular domain 4 of HER2 on the other end. These 2 anti-HER2 binding domains are termed 4C1 and 4C2 respectively. Structure 4X1 has the 4C1 binding domain at the amino terminal end of the bispecific antibody in a conventional IgG1/kappa heavy and light chain format, with 4C2 added at the carboxyl terminal end as a single chain Fv. 4X2 is in the opposite orientation with 4C2 located at amino terminal end and 4C1 as the carboxyl terminal single chain Fv. There are a variety of additional types of bispecific antibody structures that could be created using these binding pairs, including changes to the linker and connector sequences and alternate location and/or format of these binding domains. For example, 4X3 can be created by extending the connector of 4X1 from (G_4S)x2 to (G_4S)x6 and 4X4 can be created by extending the connector of 4X2 from (G_4S)x2 to (G_4S)x6.

To study the effect of the length of the connecter, multiple Fc-scFv constructs designated 4C3, 4C4, 4C5, 4C6, 4C7, 4C8, 4C10 and 4C11 have been generated. 4C3 contained scFv from 4C1 whereas 4C4 contained scFv from 4C2. Connector variants from (G_4S)x3 to (G_4S)x8 were generated for 4C4 and shown on FIG. 4 for example, 4C5 has connector length of 15 amino acids (G_4S)x3 whereas 4C11 has connector length of 40 amino acids (G_4S)x8. TABLE 1 shows the connector length for different variants.

TABLE 1. Variant designation and connector lengths

Variant Name	Connector length
4C4	(G_4S) x 2
4C5	(G_4S) x 3

4C6	(G_4S) x 4
4C7	(G_4S) x 5
4C8	(G_4S) x 6
4C10	(G_4S) x 7
4C11	(G_4S) x 8

Variable light chain, variable heavy chain and single chain Fv (scFv) DNA fragments were generated by gene synthesis. Human Gamma-1 heavy chain and human kappa light chain DNA fragments were generated by gene synthesis. The fragments were assembled together by DNA ligation using restriction sites and cloned into a vector that is designed for transient expression in mammalian cells. The vector contains a strong CMV-derived promoter, and other upstream and downstream elements required for transient expression. The resulting IgG expression plasmids were verified as containing the expected DNA sequences by DNA sequencing.Transient expression of the antibody constructs was achieved using transfection of suspension-adapted HEK293F cells with linear PEI as described in CSH Protocols; 2008; doi:10.1101/pdb.prot4977. Briefly, add DNA to each tube containing F17 expression medium that has been pre-warmed at 37° C followed by PEI. Incubate for 15 minutes at room temperature and add the DNA/PEI mixture to the flask containing HEK293 cells at a density of around 1 x 10^6 cells/ml in F17 Complete Medium. Incubate for 5 days at 37° C with shaking after which the sample was centrifuged and the supernatant was collected and stored at 4° C for purification.

Antibodies were purified from the resulting transfection supernatants using protein an affinity chromatography and Size Exclusion Chromatography when needed. Protein quality is analysed by Superdex 200 column. Protein used for all the assays have a purity of greater than 90%.

The bispecific antibodies specific to two different epitopes of HER2 can be used for the treatment of many HER2 expressed cancers such as breast, ovary, stomach, esophageal, prostate, lung and neuroendocrine cancers.

In one embodiment, the bispecific antibody is of tetravalent dual specificity. It includes an IgG and two scFv, which provides two different binding specificities compared to mono-specific antibody IgG. The IgG component provides stability over other bispecific antibodies used only scFv such as BiTE technology (Lutterbuese et al, *Proceedings of the National Academy of Sciences of the United States of America* 107.28 (2010): 12605–12610. *PMC*. Web. 2 Dec. 2014) and others (US Pat. No. 7332585). It is also capable of mediating ADCC while those without Fc component cannot (US Pat. No. 7332585). The tetravalent dual specificity nature provides the bispecific antibody a simultaneous binding capability over some other bispecific antibodies, which may only bind one antigen at a time (Kontermann, *MAbs.* 2012 Mar-Apr; 4(2):182-97; Schanzer et al, *Antimicrob. Agents Chemother.* 2011, 55(5):2369; EP272942).

For the convenience of narration, the sequences of or related to the bispecific antibodies are summarized in TABLE 2 herein-below.

TABLE 2. Summary of nucleotide and amino acid sequences of or related to the bispecific antibodies

SI-4C1 SEQUENCES	
SEQ ID NO 1	SI-4C1 Light Chain full-length nucleotide sequence
SEQ ID NO 2	SI-4C1 Light Chain variable light chain nucleotide sequence
SEQ ID NO 3	si-4c1 light chain full-length amino acid sequence. human kappa constant domain is underlined
SEQ ID NO 4	si-4c1 light chain variable light chain amino acid sequence. complementarity determining regions are underlined
SEQ ID NO 5	si-4c1 heavy chain full-length nucleotide sequence
SEQ ID NO 6	SI-4C1 heavy Chain variable heavy chain nucleotide sequence
SEQ ID NO 7	si-4c1 heavy chain full-length amino acid sequence. human

	gamma-1 domain is underlined
SEQ ID NO 8	si-4c1 heavy chain variable heavy chain amino acid sequence. complementarity determining regions are underlined
SI-4C2 SEQUENCES	
SEQ ID NO 9	SI4C2 Light Chain full-length nucleotide sequence
SEQ ID NO 10	SI-4C2 Light Chain variable light chain nucleotide sequence
SEQ ID NO 11	si-4c2 light chain full-length amino acid sequence. human kappa constant domain is underlined
SEQ ID NO 12	si-4c2 light chain variable light chain amino acid sequence. complementarity determining regions are underlined
SEQ ID NO 13	si-4c2 heavy chain full-length nucleotide sequence
SEQ ID NO 14	SI-4C2 heavy Chain variable heavy chain nucleotide sequence
SEQ ID NO 15	si-4c2 heavy chain full-length amino acid sequence. human gamma-1 domain is underlined
SEQ ID NO 16	si-4c2 heavy chain variable heavy chain amino acid sequence. complementarity determining regions are underlined
SI-4C3 SEQUENCES	
SEQ ID NO 17	SI-4C3 full-length nucleotide sequence
SEQ ID NO 18	SI-4C3 FULL-LENGTH PROTEIN SEQUENCE. human gamma-1 domain is underlined, connector is in italics, scfv is in bold
SEQ ID NO 19	SI-4C3 scfv amino acid sequence. order: vh – linker – vl. complementarity determining regions are underlined. linker is in bold italice

SI-4C4 SEQUENCES	
SEQ ID NO 20	SI-4C4 full-length nucleotide sequence
SEQ ID NO 21	SI-4C4 FULL-LENGTH PROTEIN SEQUENCE. human gamma-1 domain is underlined, connector is in italics, scfv is in bold
SEQ ID NO 22	SI-4C4 scfv amino acid sequence. order: vh – linker – vl. complementarity determining regions are underlined. linker is in bold italice
Si-4X1 sequences	
SEQ ID NO 23	SI4X1 Light Chain full-length nucleotide sequence
SEQ ID NO 24	SI-4X1 Light Chain variable light chain nucleotide sequence
SEQ ID NO 25	si-4X1 light chain full-length amino acid sequence. human kappa constant domain is underlined
SEQ ID NO 26	si-4X1 light chain variable light chain amino acid sequence. complementarity determining regions are underlined
SEQ ID NO 27	si-4X1 Bispecific heavy chain full-length nucleotide sequence
SEQ ID NO 28	SI-4X1 bispecific heavy Chain variable heavy chain nucleotide sequence
SEQ ID NO 29	SI-4X1 bispecific heavy chain scfv nucleotide sequence
SEQ ID NO 30	si-4x1 bispecific heavy chain full-length amino acid sequence. human gamma-1 domain is underlined, connector is in italics, scfv is in bold
SEQ ID NO 31	si-4x1 bispecific heavy chain variable heavy chain amino acid sequence. complementarity determining regions are underlined
SEQ ID NO 32	si4x1 bispecific heavy chain scfv amino acid sequence.

	order: vh – linker – vl. complementarity determining regions are underlined. linker is in bold italics
Si-4X2 sequences	
SEQ ID NO 33	SI4X2 Light Chain full-length nucleotide sequence
SEQ ID NO 34	SI-4X2 Light Chain variable light chain nucleotide sequence
SEQ ID NO 35	si-4X2 light chain full-length amino acid sequence. human kappa constant domain is underlined
SEQ ID NO 36	si-4X2 light chain variable light chain amino acid sequence. complementarity determining regions are underlined
SEQ ID NO 37	si-4X2 Bispecific heavy chain full-length nucleotide sequence
SEQ ID NO 38	SI-4X2 bispecific heavy Chain variable heavy chain nucleotide sequence
SEQ ID NO 39	SI-4X2 bispecific heavy chain scfv nucleotide sequence
SEQ ID NO 40	si-4x2 bispecific heavy chain full-length amino acid sequence. human gamma-1 domain is underlined, connector is in italics, scfv is in bold
SEQ ID NO 41	si-4x2 bispecific heavy chain variable heavy chain amino acid sequence. complementarity determining regions are underlined
SEQ ID NO 42	si4x2 bispecific heavy chain scfv amino acid sequence. order: vh – linker – vl. complementarity determining regions are underlined. linker is in bold italics
Si-4X3 sequences	
SEQ ID NO 43	SI4X3 Light Chain full-length nucleotide sequence
SEQ ID NO 44	SI-4X3 Light Chain variable light chain nucleotide sequence

SEQ ID NO 45	si-4X3 light chain full-length amino acid sequence. human kappa constant domain is underlined
SEQ ID NO 46	si-4X3 light chain variable light chain amino acid sequence. complementarity determining regions are underlined
SEQ ID NO 47	si-4X3 Bispecific heavy chain full-length nucleotide sequence
SEQ ID NO 48	SI-4X3 bispecific heavy Chain variable heavy chain nucleotide sequence
SEQ ID NO 49	SI-4X3 bispecific heavy chain scfv nucleotide sequence
SEQ ID NO 50	si-4x3 bispecific heavy chain full-length amino acid sequence. human gamma-1 domain is underlined, connector is in italics, scfv is in bold
SEQ ID NO 79	si-4x3 bispecific heavy chain variable heavy chain amino acid sequence. complementarity determining regions are underline
SEQ ID NO 80	si4x3 bispecific heavy chain scfv amino acid sequence. order: vh – linker – vl. complementarity determining regions are underlined. linker is in bold italics
Si-4X4 sequences	
SEQ ID NO 51	SI4X4 Light Chain full-length nucleotide sequence
SEQ ID NO 52	SI-4X4 Light Chain variable light chain nucleotide sequence
SEQ ID NO 53	si-4X4 light chain full-length amino acid sequence. human kappa constant domain is underlined
SEQ ID NO 54	si-4X4 light chain variable light chain amino acid sequence. complementarity determining regions are underlined
SEQ ID NO 55	si-4X4 Bispecific heavy chain full-length nucleotide sequence

SEQ ID NO 56	SI-4X4 bispecific heavy Chain variable heavy chain nucleotide sequence
SEQ ID NO 57	SI-4X4 bispecific heavy chain scfv nucleotide sequence
SEQ ID NO 58	si-4x4 bispecific heavy chain full-length amino acid sequence. human gamma-1 domain is underlined, connector is in italics, scfv is in bold
SEQ ID NO 59	si-4x4 bispecific heavy chain variable heavy chain amino acid sequence. complementarity determining regions are underlined
SEQ ID NO 60	si4x4 bispecific heavy chain scfv amino acid sequence. order: vh – linker – vl. complementarity determining regions are underlined. linker is in bold italics
SI-4C5 SEQUENCES	
SEQ ID NO 61	SI-4C5 full-length nucleotide sequence
SEQ ID NO 62	SI-4C5 FULL-LENGTH PROTEIN SEQUENCE. human gamma-1 domain is underlined, connector is in italics, scfv is in bold
SEQ ID NO 63	SI-4C5 scfv amino acid sequence. order: vh – linker – vl. complementarity determining regions are underlined. linker is in bold italice
SI-4C6 SEQUENCES	
SEQ ID NO 64	SI-4C6 full-length nucleotide sequence
SEQ ID NO 65	SI-4C6 FULL-LENGTH PROTEIN SEQUENCE. human gamma-1 domain is underlined, connector is in italics, scfv is in bold
SEQ ID NO 66	SI-4C6 scfv amino acid sequence. order: vh – linker – vl. complementarity determining regions are underlined. linker is in bold italice
SI-4C7	

SEQUENCES	
SEQ ID NO 67	SI-4C7 full-length nucleotide sequence
SEQ ID NO 68	SI-4C7 FULL-LENGTH PROTEIN SEQUENCE. human gamma-1 domain is underlined, connector is in italics, scfv is in bold
SEQ ID NO 69	SI-4C7 scfv amino acid sequence. order: vh – linker – vl. complementarity determining regions are underlined. linker is in bold italice
SI-4C8 SEQUENCES	
SEQ ID NO 70	SI-4C8 full-length nucleotide sequence
SEQ ID NO 71	SI-4C8 FULL-LENGTH PROTEIN SEQUENCE. human gamma-1 domain is underlined, connector is in italics, scfv is in bold
SEQ ID NO 72	SI-4C8 scfv amino acid sequence. order: vh – linker – vl. complementarity determining regions are underlined. linker is in bold italice
SI-4C10 SEQUENCES	
SEQ ID NO 73	SI-4C10 full-length nucleotide sequence
SEQ ID NO 74	SI-4C10 FULL-LENGTH PROTEIN SEQUENCE. human gamma-1 domain is underlined, connector is in italics, scfv is in bold
SEQ ID NO 75	SI-4C10 scfv amino acid sequence. order: vh – linker – vl. complementarity determining regions are underlined. linker is in bold italice
SI-4C11 SEQUENCES	
SEQ ID NO 76	SI-4C11 full-length nucleotide sequence
SEQ ID NO 77	SI-4C11 FULL-LENGTH PROTEIN SEQUENCE. human gamma-1 domain is underlined, connector is in italics, scfv is in bold

SEQ ID NO 78	SI-4C11 scfv amino acid sequence. order: vh – linker – vl. complementarity determining regions are underlined. linker is in bold italice

EXAMPLES

Example 1

To assess the growth inhibitory potential of anti-HER2 antibodies, the effect on proliferation of BT-474 cells (ATCC HTB-20, Manassas, Va.) which are a mammary ductal carcinoma tumor line was tested. Cells were seeded into 96-well tissue culture plates at a density of 6000 cells/well in 100µl RPMI-1640 medium containing 1% fetal bovine serum. After 4 hours, test antibodies were added at various concentrations, ranging from 0.0061nM to 400nM. Cells were cultured in the presence of test antibodies for 7 days. To each well, 20µl of MTS reagent (Promega, Madison, WI) was added and cells were incubated at 37°C for 2 hours. MTS is readily taken up by actively proliferating cells, reduced into formazan (which readily absorbs light at 490nm), and then secreted into the culture medium. Following incubation, OD490 values were measured using a BioTek (Winooski, VT) ELx800 absorbance reader. OD490 values for control cells (treated with medium only) were also obtained in this manner at the time of antibody addition in order to establish baseline metabolic activity. Proliferation may be calculated by subtracting the control baseline OD490 from the 72 hour OD490. Data from antibody titrations was expressed at % of control population according to the following formula: % of control proliferation = (test proliferation /control proliferation)*100.

The effect of SI-4X1 and SI-4X2 on BT-474 proliferation is shown in FIG. 5. Both molecules had anti-proliferative effect, but neither was as efficacious as the control antibody SI-4C2 or the combination of the control antibodies SI-4C1 and SI-4C2. Increasing the length of the connector G_4S linker which separates the C-terminal scFv from huIgG from 2 repeats to 6 repeats increased the

efficacy, as can be seen from SI-4X3. It is suspected that the lower efficacy of the bispecific antibodies could be the result of pro-proliferative activity supplied by the C-terminal scFv. There is precedence in the literature for anti-Her2 antibodies showing agonistic activity depending on their structure. To investigate this, we create a series of control molecules containing the same anti-Her2 scFv, but with progressively longer G_4S linkers. As can be seen in FIG. 6, anti-proliferative effect was directly proportional to the number of G_4S elements in the linker, with SI-4C8 (6 repeats) showing the highest degree of anti-proliferative activity, while SI-4C4 (2 repeats) exhibited agonistic activity. This effect is even more pronounced when the linker is increased to 7 (SI-4C10) and 8 (SI-4C11) repeats and can be seen in FIG. 7.

Example 2

The ability of anti-Her2 antibodies to be internalized by BT-474 cells was tested. One milligram aliquots of antibody in standard PBS were allowed to react with Alexa Fluor 488 carboxylic acid, TFP ester (Thermo Fisher #A-10235, Waltham, MA) for one hour at room temperature. Unincorporated dye was removed by gel filtration using a Bio-Gel P-30 column. Following conjugation, aliquots of $3x10^5$ BT-474 cells were incubated with 50nM each Alexa 488 labeled antibody in complete medium (RPMI-1640 + 10% FBS) for 1 hour at either 37°C or 4°C (ice). Following incubation, cells were washed twice in a cold centrifuge with ice cold PBS. Cells were then resuspended in either 500nM quenching rabbit-anti-Alexa488 antibody (Thermo Fisher #A-11094, Waltham, MA) or 500nM rabbit IgG isotype control antibody (Jackson ImmunoResearch Laboratories #011-000-003, West Grove, PA) and incubated on ice for 30 minutes. Two volumes of 2% paraformaldehyde were added to each sample and incubated for 10 minutes at room temperature. Cells were then washed once with 1ml ice cold PBS, resuspended in 200µl PBS and analyzed using a FACScalibur flow cytometer. Geometric mean fluorescence (GMFI) from $2x10^4$ events per sample was used to calculate the percentage of internalized antibody.

Since no internalization should occur at 4°C, the fluorescence measured in samples incubated on ice followed by incubation with the anti-Alexa488 antibody was considered to be unquenchable background surface fluorescence and was subtracted from GMFI values obtained samples incubated at 37°C prior to quenching. Internalization was calculated as follows: % internalization = (GMFI quenched/GMFI unquenched)*100.

The results may be seen in FIG. 8. The bispecific antibody, SI-4X2 internalized to a greater degree (39.3%) than the monospecific control antibodies SI-4C1 (18.29%) and SI-4C2 (14.97%) as well as the combination of SI-4C1 + SI-4C2 (29.19%).

Pharmaceutical Compositions

The term "effective amount" refers to an amount of a drug effective to achieve a desired effect, e.g., to ameliorate disease in a subject. Where the disease is a caner, the effective amount of the drug may inhibit (for example, slow to some extent, inhibit or stop) one or more of the following example characteristics including, without limitation, cancer cell growth, cancer cell proliferation, cancer cell motility, cancer cell infiltration into peripheral organs, tumor metastasis, and tumor growth. Wherein the disease is a caner, the effective amount of the drug may alternatively do one or more of the following when administered to a subject: slow or stop tumor growth, reduce tumor size (for example, volume or mass), relieve to some extent one or more of the symptoms associated with the cancer, extend progression free survival, result in an objective response (including, for example, a partial response or a complete response), and increase ovral survival time. To the extent the drug may prevent growth and/or kill existing cancer cells, it is cytostatic and/or cytotoxic.

With respect to the formulation of suitable compositions for administration to a subject such as a human patient in need of treatment, the antibodies disclosed herein may be mixed or combined with pharmaceutically

acceptable carriers known in the art dependent upon the chosen route of administration. There are no particular limitations to the modes of application of the antibodies disclosed herein, and the choice of suitable administration routes and suitable compositions are known in the art without undue experimentation.

Although many forms of administration are possible, an example administration form would be a solution for injection, in particular for intravenous or intra-arterial injection. Usually, a suitable pharmaceutical composition for injection may include pharmaceutically suitable carriers or excipients such as, without limitation, a buffer, a surfactant, or a stabilizer agent. Example buffers may include, without limitation, acetate, phosphate or citrate buffer. Example surfactants may include, without limitation, polysorbate. Example stabilizer may include, without limitation, human albumin.

Similarly, persons skilled in the art have the ability to determine the effective amount or concentration of the antibodies disclosed therein to effective treat a condition such as a cancer. Other parameters such as the proportions of the various components in the pharmaceutical composition, administration does and frequency may be obtained by a person skilled in the art without undue experimentation. For example, a suitable solution for injection may contain, without limitation, from about 1 to about 20, from about 1 to about 10 mg antibodies per ml. The example dose may be, without limitation, from about 0.1 to about 20, from about 1 to about 5 mg/Kg body weight. The example administration frequency could be, without limitation, once per day or three times per week.

While the present disclosure has been described with reference to particular embodiments or examples, it may be understood that the embodiments are illustrative and that the disclosure scope is not so limited. Alternative embodiments of the present disclosure may become apparent to those having ordinary skill in the art to which the present disclosure pertains.

Such alternate embodiments are considered to be encompassed within the scope of the present disclosure. Accordingly, the scope of the present disclosure is defined by the appended claims and is supported by the foregoing description.

5

CLAIMS

What is claimed is:

1. A bispecific tetravalent antibody, said bispecific tetravalent antibody comprising:

two IgG1 heavy chains;

two kappa light chains; and

two single chain Fv (scFv) domains;

wherein the two IgG1 heavy chains and kappa light chains form an IgG moiety with a binding specificity to a first domain of HER2;

wherein the two scFv domains have a binding specificity to a second domain of HER2, and each scFv domain is connected to the C-terminal residue of either of the IgG1 heavy chains by a connector having an amino acid sequence of $(gly\text{-}gly\text{-}gly\text{-}gly\text{-}ser)_n$ $((G_4S)_n)$; wherein n is an integral of at least 2; and

wherein each scFv domain has a structure order of N terminus–variable heavy domain–linker–variable light domain–C terminus or N-terminus-variable light domain-linker-variable heavy domain-C terminus, and wherein the linker is comprised of amino acid sequence of $(gly\text{-}gly\text{-}gly\text{-}gly\text{-}ser)_m$ $((G_4S)_{m)}$; wherein m is an integral of at least 3.

2. The bispecific tetravalent antibody of Claim 1, wherein n is an integral less than 20.

3. The bispecific tetravalent antibody of Claim 1, wherein n is an integral between 1 to 15.

4. The bispecific tetravalent antibody of Claim 1, wherein n is an integral between 1 to 9.

5. The bispecific tetravalent antibody of Claim 1, wherein m is an integral less than 10.

6. The bispecific tetravalent antibody of Claim 1, wherein m is an integral less than 7.

7. The bispecific tetravalent antibody of Claim 1, wherein m is 3, 4, 5 or 6.

8. The bispecific tetravalent antibody of Claim 1, wherein at least one of the IgG1 heavy chains is a humanized or human IgG1 heavy chain.

9. The bispecific tetravalent antibody of Claim 1, wherein both IgG1 heavy chains are humanized or human IgG1 heavy chains.

10. The bispecific tetravalent antibody of Claim 1, wherein at least one of the kappa light chains is a humanized or human kappa light chain.

11. The bispecific tetravalent antibody of Claim 1, wherein both kappa light chains are humanized or human kappa light chains.

12. The bispecific tetravalent antibody of Claim 1, wherein the first or the second domain of HER2 is independently selected from domain 2 and domain 4 of HER2.

13. The bispecific tetravalent antibody of Claim 1, wherein the IgG moiety has a binding specificity for domain 2 of HER2.

14. The bispecific tetravalent antibody of Claim 1, wherein the scFv domains have a binding specificity for domain 4 of HER2.

15. The bispecific tetravalent antibody of Claim 1, wherein the IgG moiety has a binding specificity for domain 2 of HER2, and the scFv domains have a binding specificity for domain 4 of HER2 simultaneously.

16. The bispecific tetravalent antibody of Claim 1, wherein the IgG moiety has a binding specificity for domain 4 of HER2.

17. The bispecific tetravalent antibody of Claim 1, wherein the scFv domains have a binding specificity for domain 2 of HER2.

18. The bispecific tetravalent antibody of Claim 1, wherein the IgG moiety has a binding specificity for domain 4 of HER2, and the scFv domains have a binding specificity for domain 2 of HER2 simultaneously.

19. The bispecific tetravalent antibody of Claim 1, wherein at least one of the IgG1 heavy chains comprises an amino acid sequences selected from SEQ ID NO 7, 15, 30, 40, 50 and 58.

20. The bispecific tetravalent antibody of Claim 1, wherein the IgG1 heavy chain, connector, and scFv domain have an amino acid sequence selected from SEQ ID NO 30, 50, 40, and 58.

21. The bispecific tetravalent antibody of Claim 1, wherein at least one of the kappa light chains comprises an amino acid sequence selected from SEQ ID NO 3, 11, 25, 35, 45, and 53.

22. The bispecific tetravalent antibody of Claim 1, wherein at least one of variable light chain comprises an amino acid sequence selected from SEQ ID NO 4, 12, 26, 36, 46, and 54.

23. The bispecific tetravalent antibody of Claim 1, wherein at least one of variable heavy chain comprises an amino acid sequence selected from SEQ ID NO 8, 16, 31, 41, 79 and 59.

24. The bispecific tetravalent antibody of Claim 1, wherein at least one of scFv domain comprises an amino acid sequence selected from SEQ ID NO 19, 22, 32, 42, 60, 63, 66, 69, 72, 75, 78, and 80.

25. The bispecific tetravalent antibody of claim 1, wherein the IgG moiety has a binding specificity for domain 2 of HER2, and the scFv domains have a binding specificity for domain 4 of HER2;

wherein the IgG1 heavy chain, connector, and scFv domain have an amino acid sequence of SEQ ID NO 30, and the kappa light chain has an amino acid sequence of SEQ ID NO 25.

26. The bispecific tetravalent antibody of claim 1, wherein the IgG moiety has a binding specificity for domain 2 of HER2, and the scFv domains have a binding specificity for domain 4 of HER2;

wherein the IgG1 heavy chain, connector, and scFv domain have an amino acid sequence of SEQ ID NO 50, and the kappa light chain has an amino acid sequence of SEQ ID NO 45.

27. The bispecific tetravalent antibody of claim 1, wherein the IgG moiety has a binding specificity for domain 4 of HER2, and the scFv domains have a binding specificity for domain 2 of HER2;

wherein the IgG1 heavy chain, connector, and scFv domain have an amino acid sequence of SEQ ID NO 40, and the kappa light chain has an amino acid sequence of SEQ ID NO 35.

28. The bispecific tetravalent antibody of claim 1, wherein the IgG moiety has a binding specificity for domain 4 of HER2, and the scFv domains have a binding specificity for domain 2 of HER2;

wherein the IgG1 heavy chain, connector, and scFv domain have an amino acid sequence of SEQ ID NO 58, and the kappa light chain has an amino acid sequence of SEQ ID NO 53.

29. The bispecific tetravalent antibody of one of Claims 1-10, wherein the antibody inhibits a cancer cell growth.

30. The bispecific tetravalent antibody of Claim 31, wherein the cancer cell expresses HER2.

31. The bispecific tetravalent antibody of Claim 1, wherein the antibody binds to domain 2 of HER2 with a Kd less than 50nM.

32. The bispecific tetravalent antibody of Claim 1, wherein the antibody binds to domain 4 of HER2 with a Kd less than 50nM.

33. The bispecific tetravalent antibody of Claim 1, wherein the antibody binds to domain 4 of HER2 with a Kd less than 50nM and binds to domain 2 of HER2 with a Kd less than 50nM simultaneously.

34. The bispecific tetravalent antibody of Claim 1, wherein the antibody binds to domain 4 of HER2 with a Kd less than 30nM and binds to domain 2 of HER2 with a Kd less than 30nM.

35. An IgG1 heavy chain for the bispecific tetravalent antibody of Claim 1, comprising an amino acid sequences selected from SEQ ID NO 7, 15, 30, 40, 50 and 58.

36. A kappa light chain for the bispecific tetravalent antibody of Claim 1, comprising an amino acid sequence selected from SEQ ID NO 3, 11, 25, 35, 45, and 53.

37. A variable light chain for the bispecific tetravalent antibody of Claim 1, comprising an amino acid sequence selected from SEQ ID NO 4, 12, 26, 36, 46, and 54.

38. A variable heavy chain for the bispecific tetravalent antibody of Claim 1, comprising an amino acid sequence selected from SEQ ID NO 8, 16, 31, 41, 79 and 59.

39. A scFv domain for the bispecific tetravalent antibody of Claim 1, comprising an amino acid sequence selected from SEQ ID NO 19, 22, 32, 42, 60, 63, 66, 69, 72, 75, 78 and 80.

40. An isolated nucleic acid encoding the antibody of Claim 1, the IgG1 heavy Chain of Claim 35, the kappa light chain of Claim 36, the variable light chain of Claim 37, the variable heavy chain of Claim 38, or the scFv domain of Claim 39.

41. An expression vector comprising the isolated nucleic acid of Claim 40.

42. The expression vector of Claim 41, wherein the vector is expressible in a cell.

43. A host cell comprising the nucleic acid of Claim 40.

44. A host cell comprising the expression vector of Claim 41.

45. The host cell of one of Claims 43 and 44, wherein the host cell is a prokaryotic cell or a eukaryotic cell.

46. A method of producing an antibody comprising culturing the host cell of one of Claims 43-45 so that the antibody is produced.

47. An immunoconjugate comprising the antibody of Claim 1 and a cytotoxic agent.

48. A pharmaceutical composition, comprising the bispecific tetravalent antibody of Claim 1 and a pharmaceutically acceptable carrier.

49. The pharmaceutical composition of Claim 48, further comprising radioisotope, radionuclide, a toxin, a therapeutic agent, a chemotherapeutic agent or a combination thereof.

50. A pharmaceutical composition, comprising the immunoconjugate of Claim 47 and a pharmaceutically acceptable carrier.

51. A method of treating a subject with a cancer, comprising administering to the subject an effective amount of the bispecific tetravalent antibody of Claim 1.

52. The method of Claim 51, wherein the cancer comprises cells expressing HER2 on their surface.

53. The method of Claim 51, wherein the cancer comprises cells expressing HER2+ on their surface.

54. The method of Claim 51, wherein the cancer is one selected from the group consisting of HER2+ breast cancer, colorectal cancer, ovarian cancer, head and neck cancer, gastric cancer, esophageal cancer and non small cell lung cancer.

55. The method of Claim 51, further comprising co-administering an effective amount of a therapeutic agent.

56. The method of Claim 55, wherein the therapeutic agent comprises an antibody, a chemotherapy agent, an enzyme, or a combination thereof.

57. The method of Claim 55, wherein the therapeutic agent comprises an anti-estrogen agent, a receptor tyrosine inhibitor, or a combination thereof.

58. The method of Claim 55, wherein the therapeutic agent comprises capecitabine, cisplatin, trastuzumab, fulvestrant, tamoxifen, letrozole, exemestane, anastrozole, aminoglutethimide, testolactone, vorozole, formestane, fadrozole, letrozole, erlotinib, lafatinib, dasatinib, gefitinib, imatinib, pazopinib, lapatinib, sunitinib, nilotinib, sorafenib, nab-palitaxel, a derivative or a combination thereof.

59. The method of Claim 55, wherein the therapeutic agent comprises a biologics.

60. The method of Claim 55, wherein the therapeutic agent comprises a checkpoint inhibitor.

61. The method of Claim 55, wherein the therapeutic agent comprises PD1, PDL1, CTLA4, 4-1BB, OX40, GITR, TIM3, LAG3, TIGIT, CD40, CD27, HVEM, BTLA, VISTA, B7H4, a derivative or a combination thereof.

62. The method of Claim 51, wherein the subject is a human.

63. A method of inhibiting a biological activity of a HER2 receptor in a subject, comprising administering to the subject an effective amount of the antibody of Claim 1 to inhibit a biological activity of a HER2 receptor.

64. A solution comprising an effective concentration of the bispecific tetravalent antibody of Claim 1, wherein the solution is blood plasma in a subject.

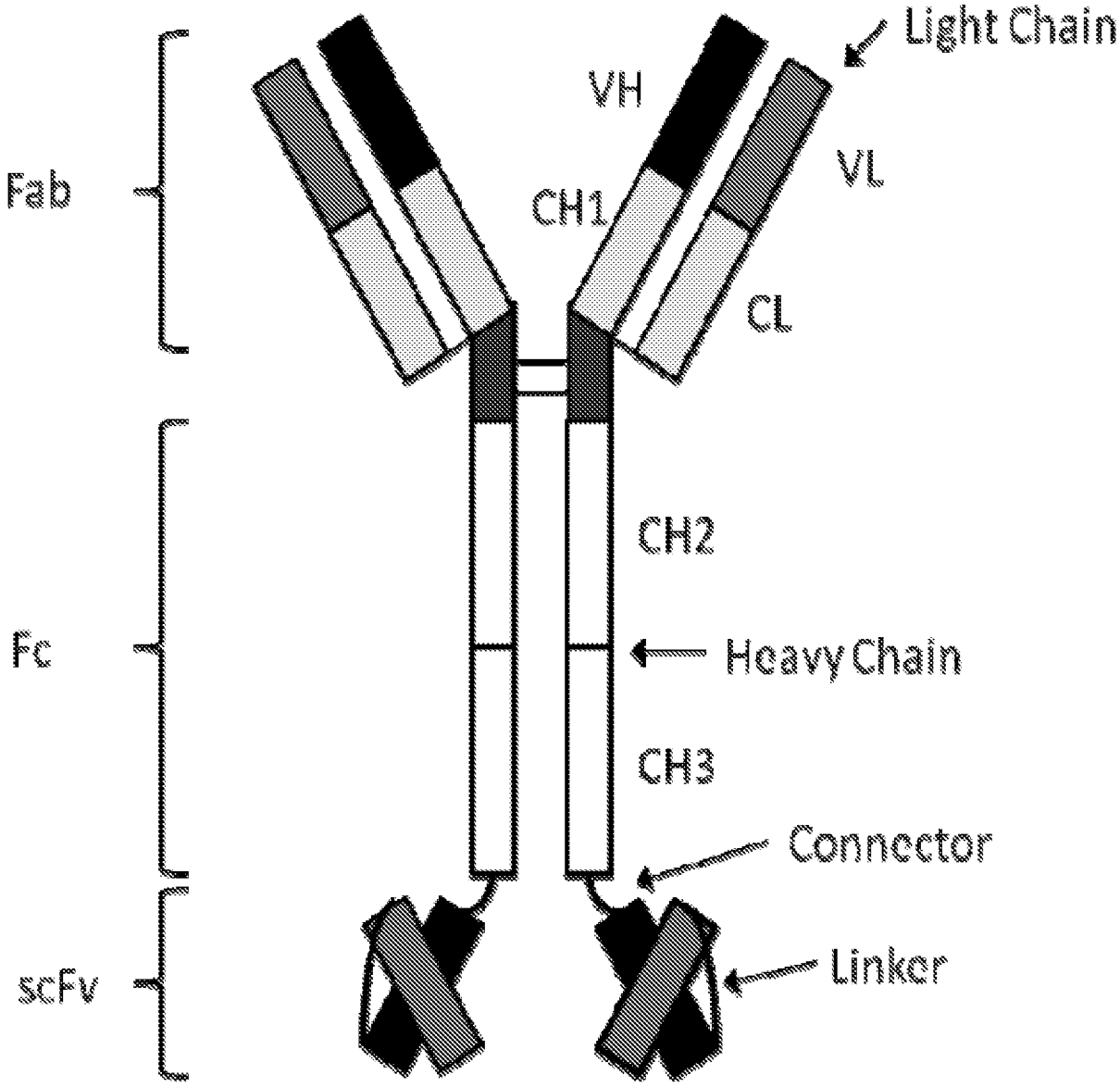
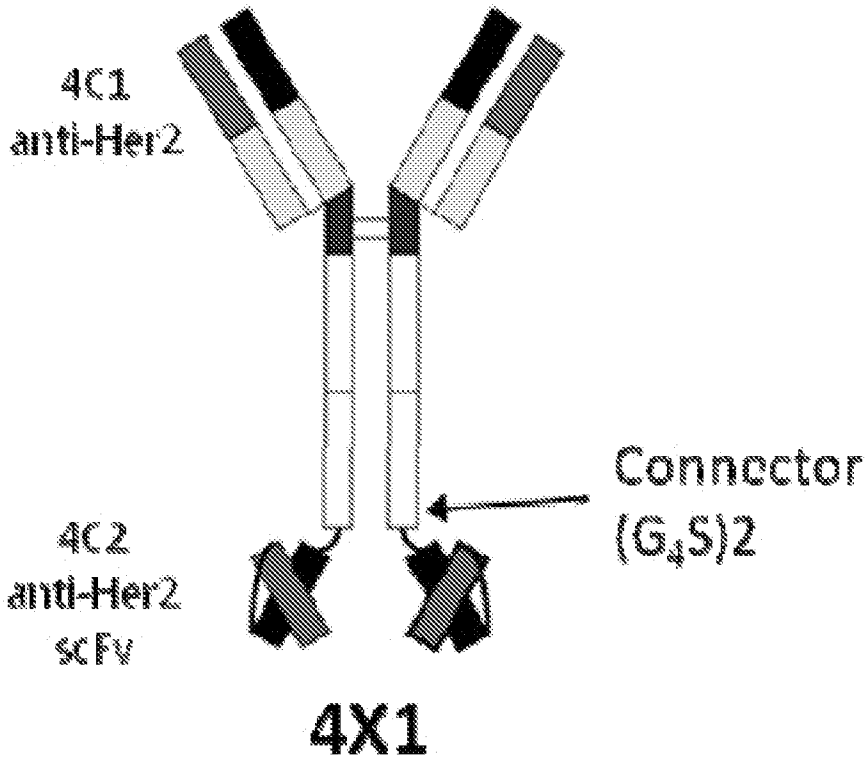

Light Chain
VH
VL
CH1
CL
Fab
CH2
Fc
Heavy Chain
CH3
Connector
Linker
scFv

FIG. 1


4C1
anti-Her2
Connector
(G4S)2
4C2
anti-Her2
scFv
4X1

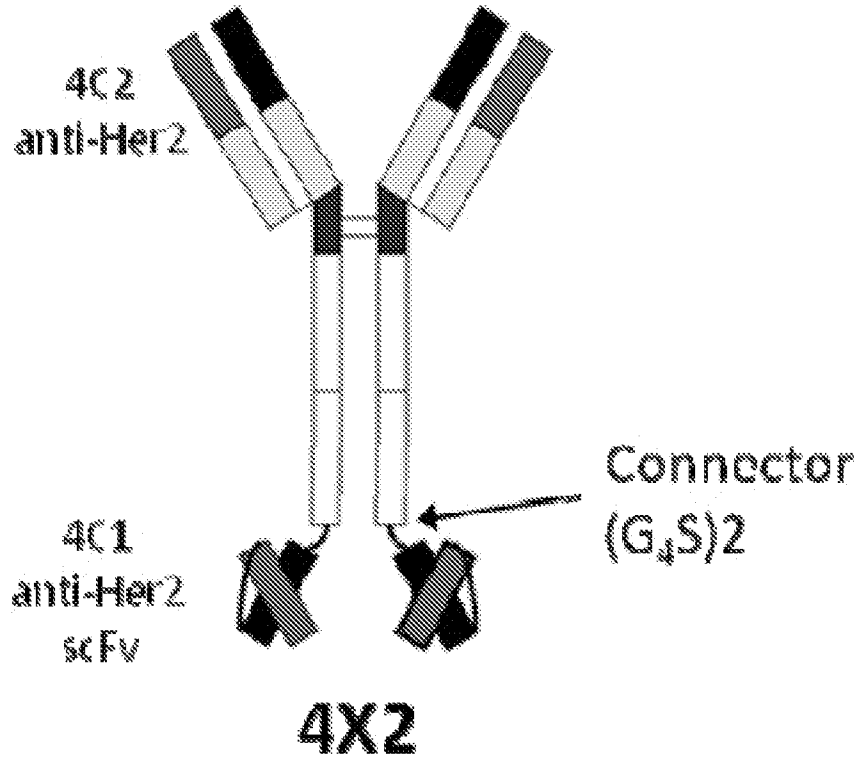
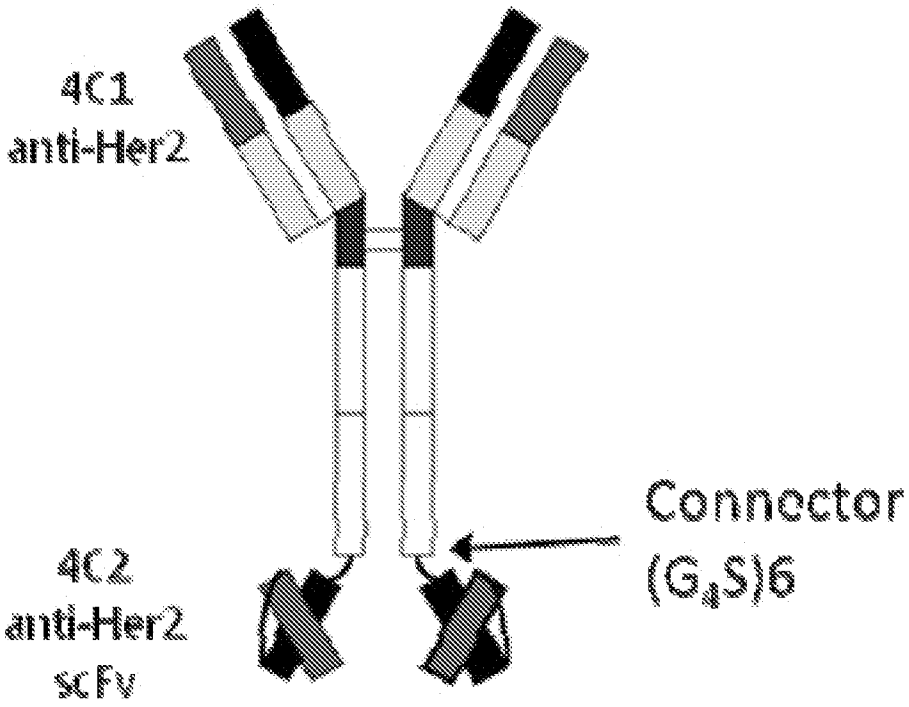
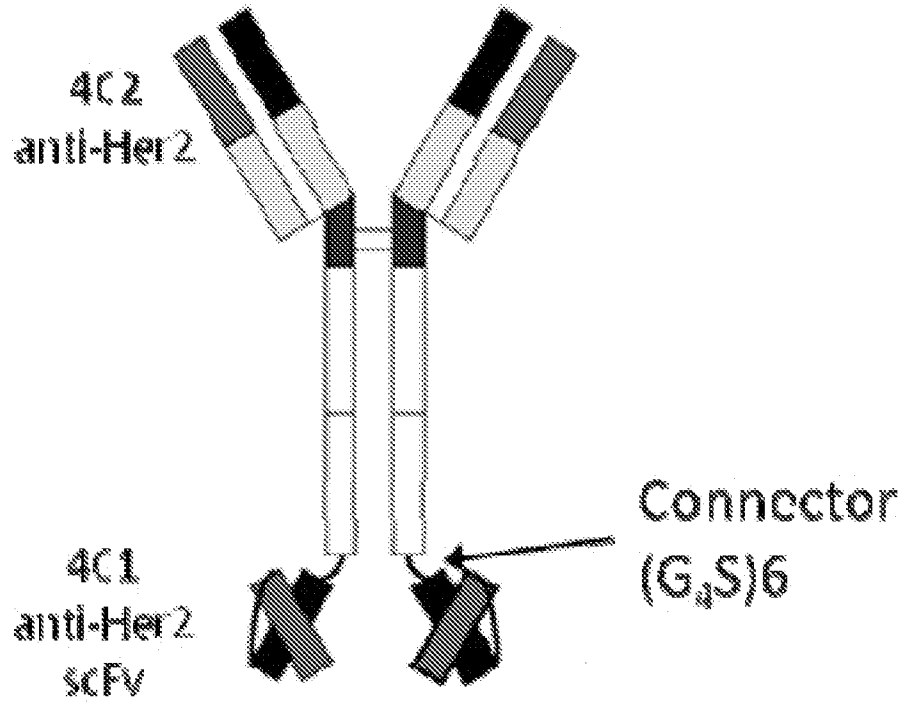

4C2
anti-Her2
Connector
(G4S)2
4C1
anti-Her2
scFv
4X2

FIG. 2a

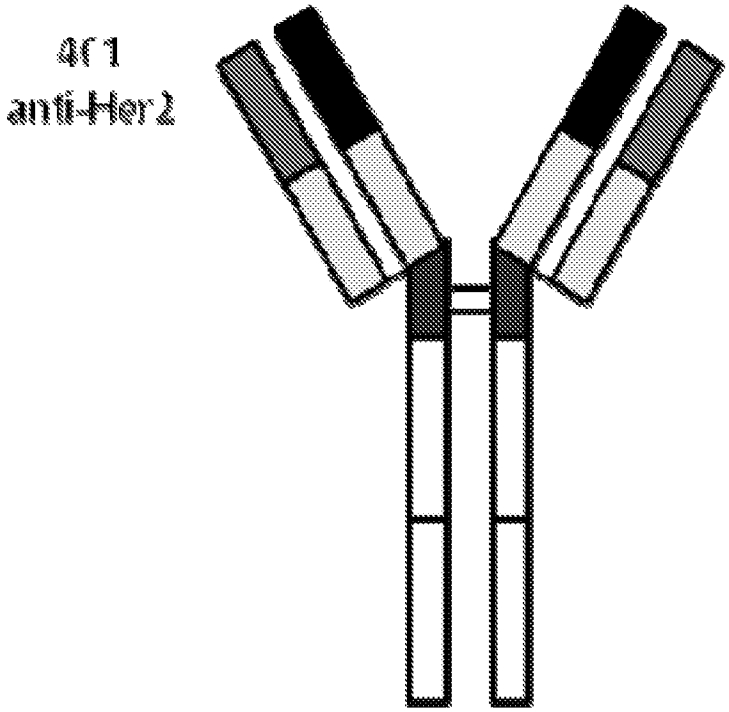


4X3



4X4

FIG. 2b



4C1

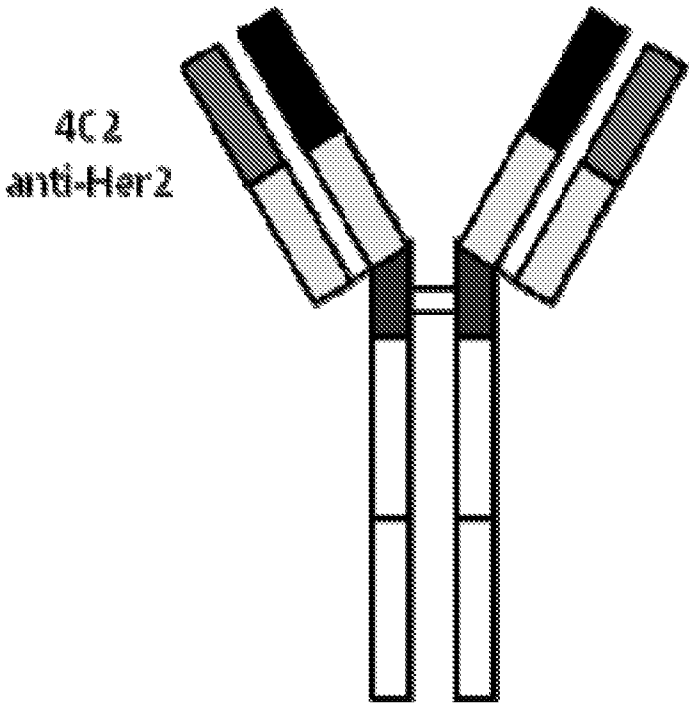
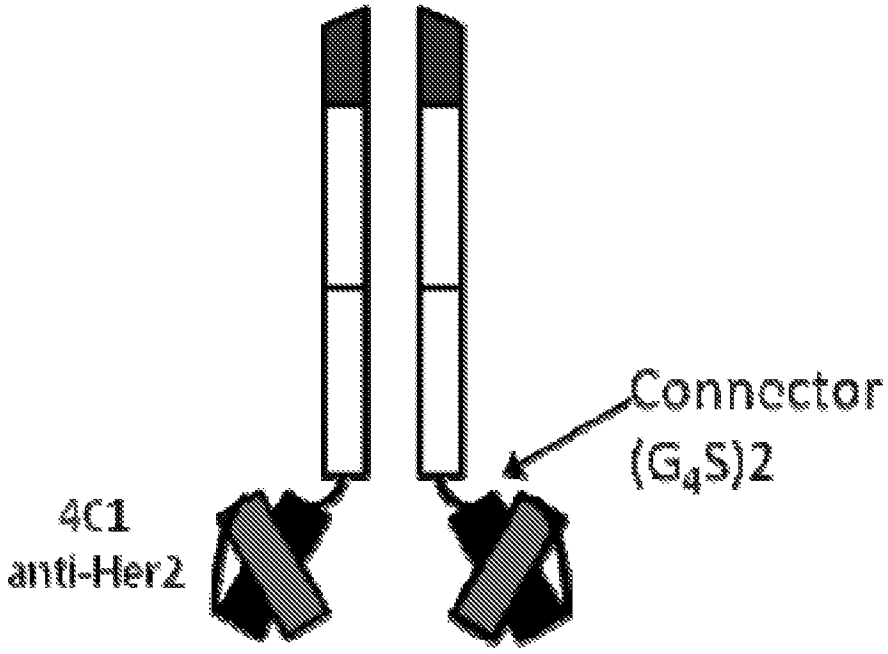
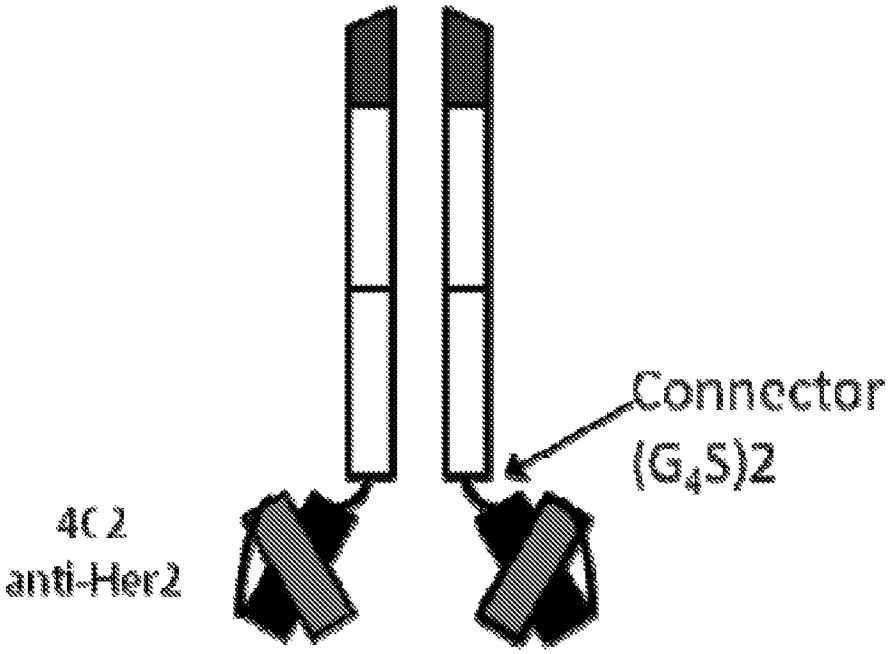


FIG. 3

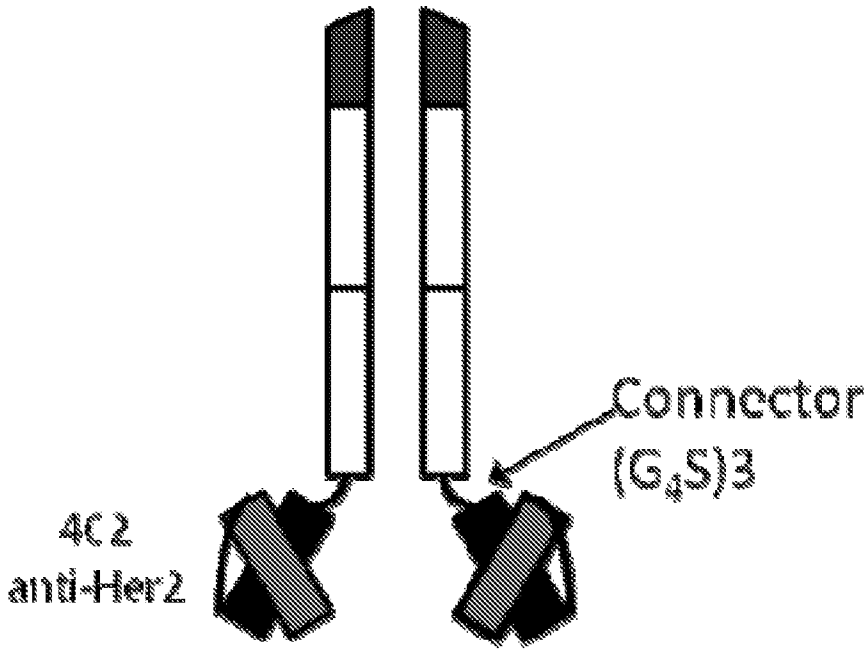


4C3

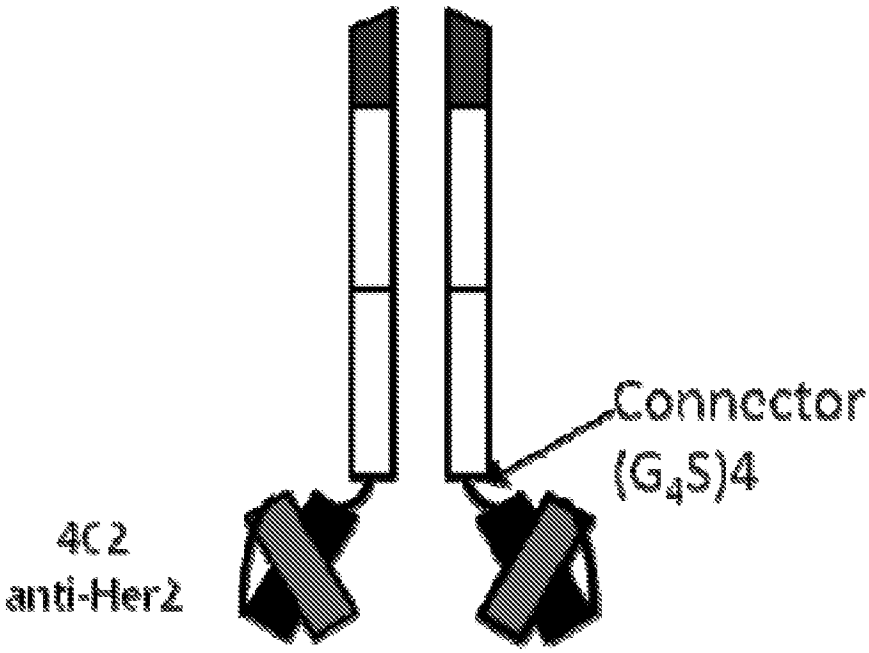


4C4

FIG. 4a

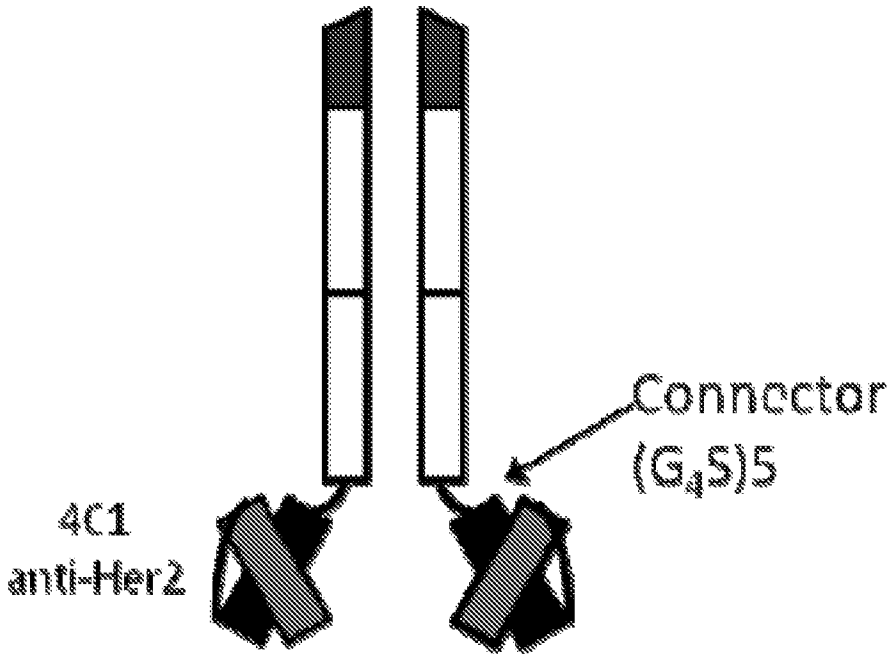


4C5

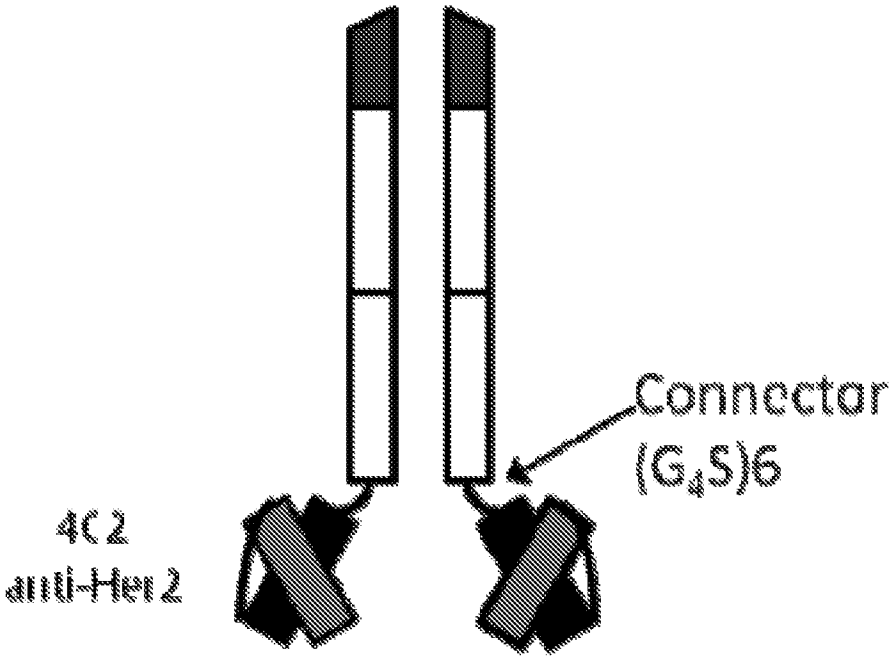


4C6

FIG. 4b

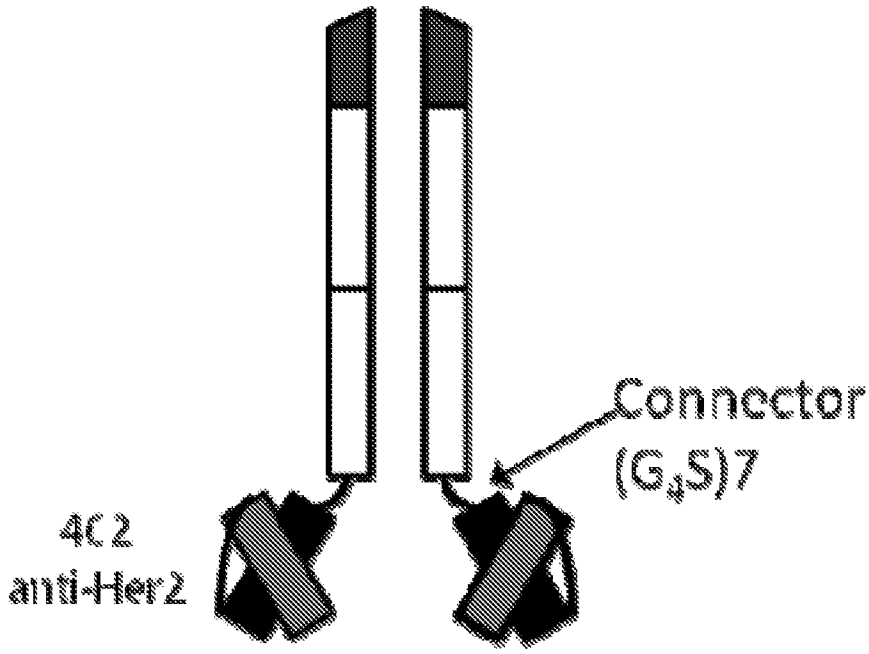


4C7

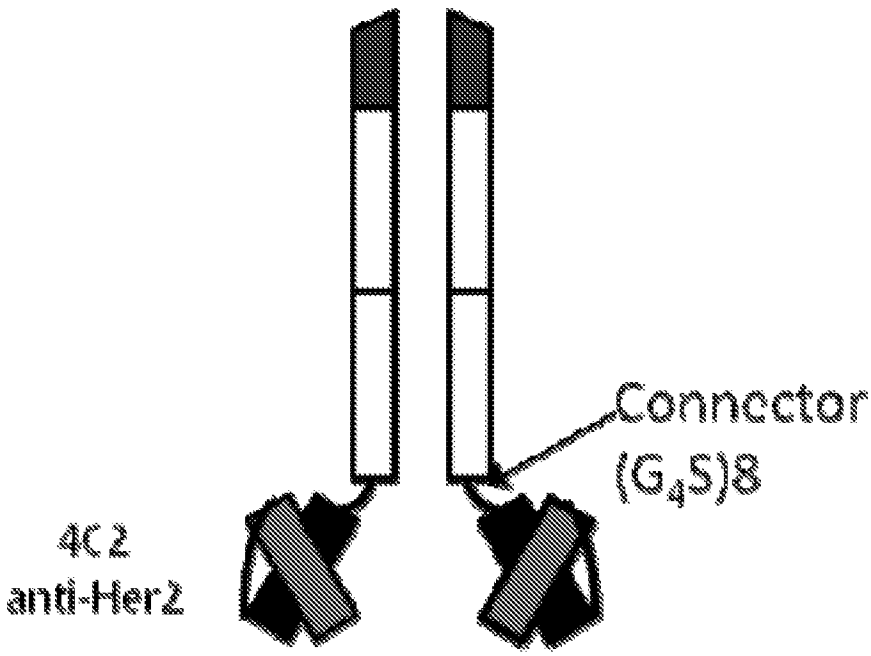


4C8

FIG. 4c

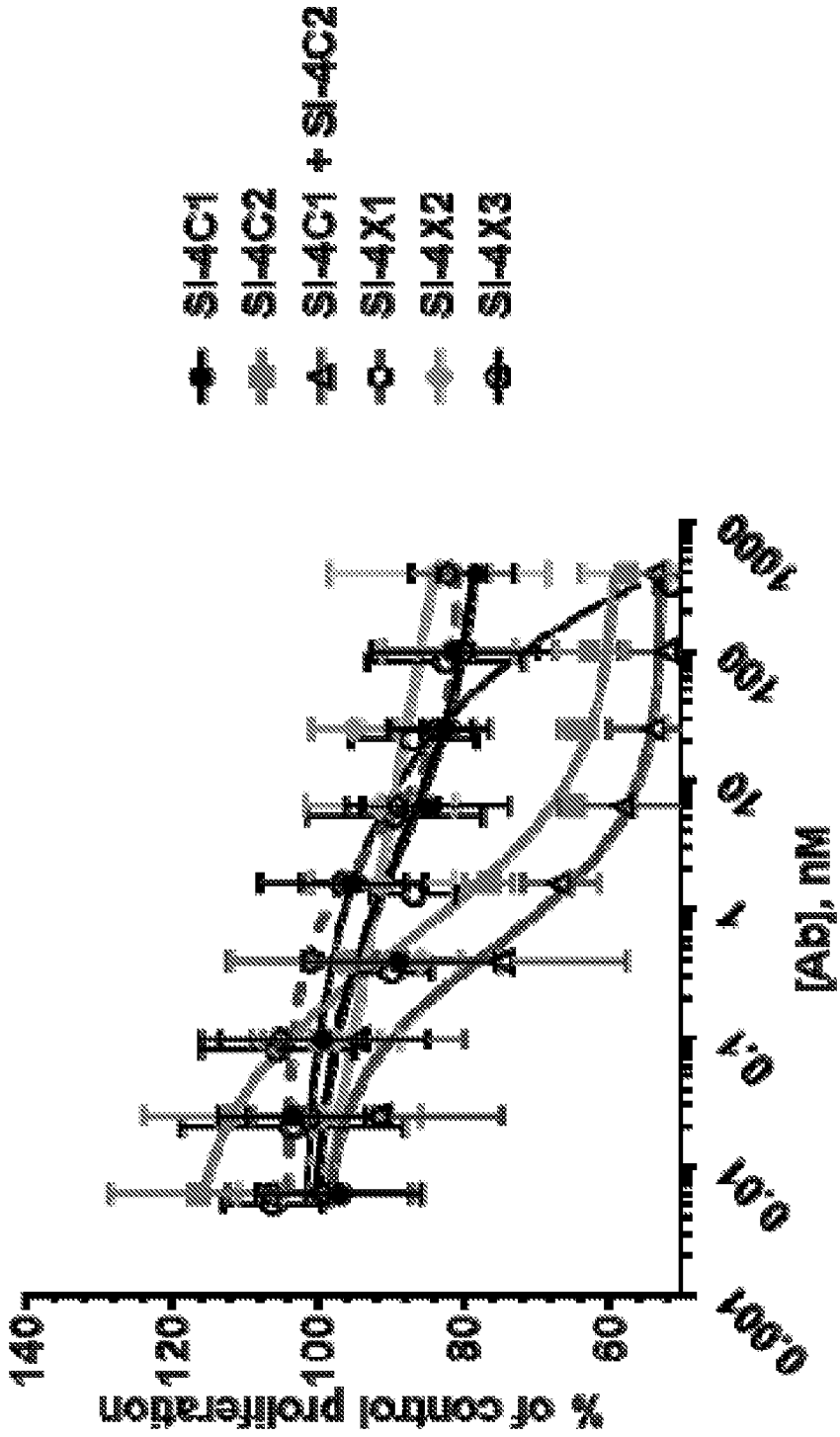


4C10



4C11

FIG. 4d



IC50 (nM)					
SI-4C1	SI-4C2	SI-4C1 + SI-4C2	SI-4X1	SI-4X2	SI-4X3
2.63	0.51	0.48	3.28	N/A	N/A

FIG. 5

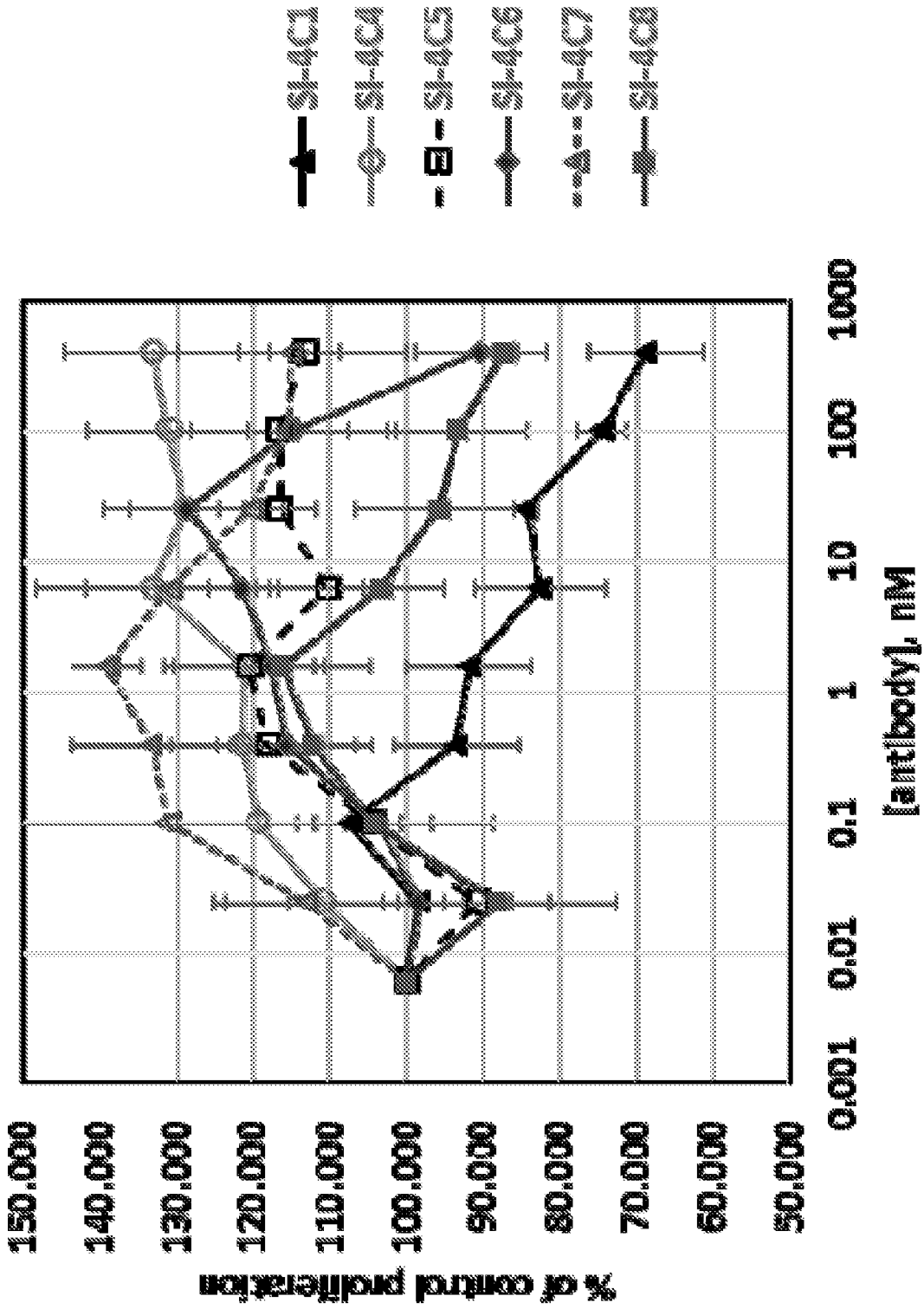

SI-401
SI-404
SI-405
SI-406
SI-407
SI-408
% of control proliferation
150.000
140.000
130.000
120.000
110.000
100.000
90.000
80.000
70.000
60.000
50.000
0.001
0.01
0.1
1
10
100
1000
[antibody], nM

FIG. 6

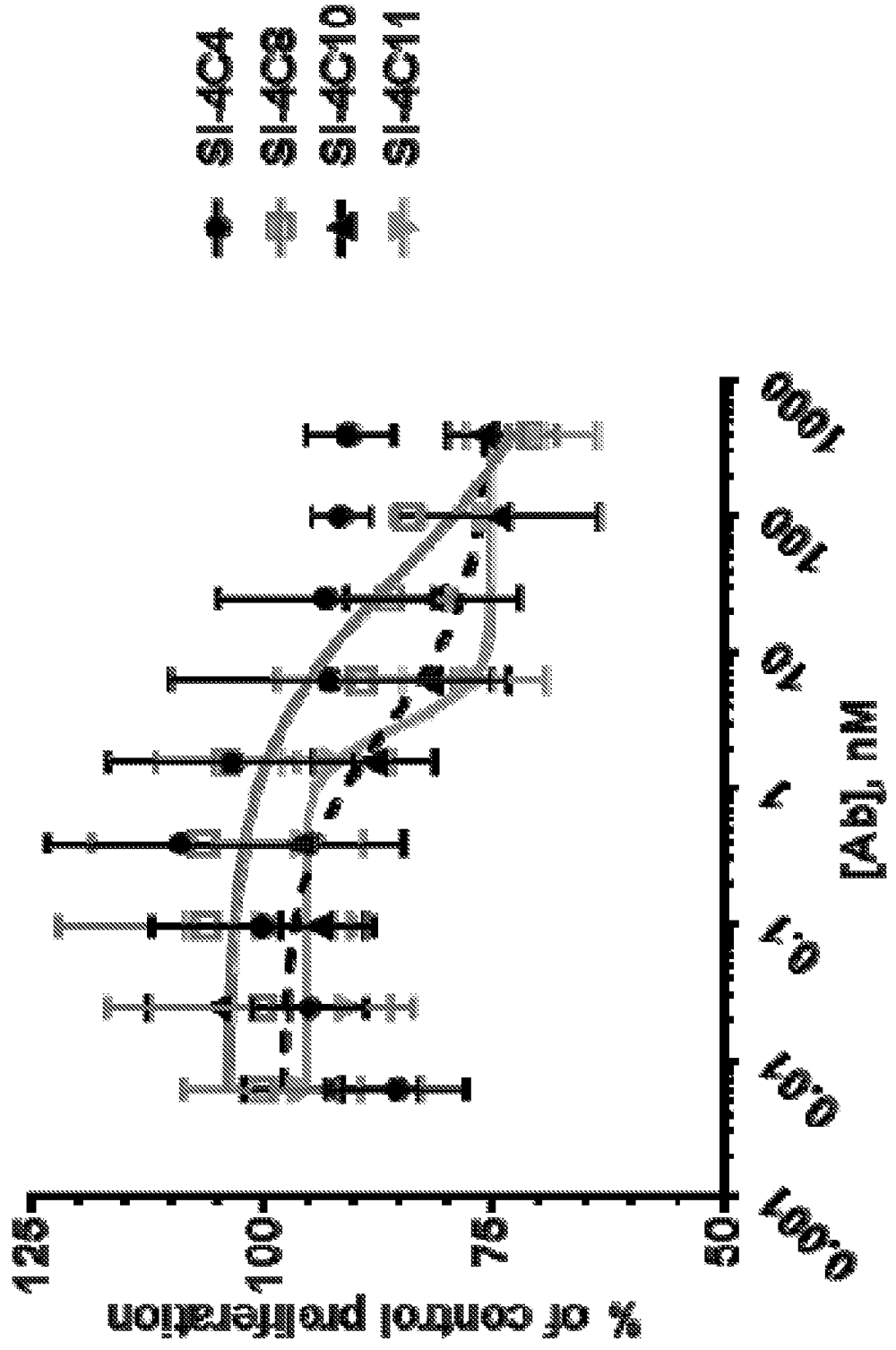

sl4C4
sl4C8
sl4C10
sl4C11
% of control proliferation
125
100
75
50
0.001
0.01
0.1
1
10
100
1000
[Ab], nM

FIG. 7

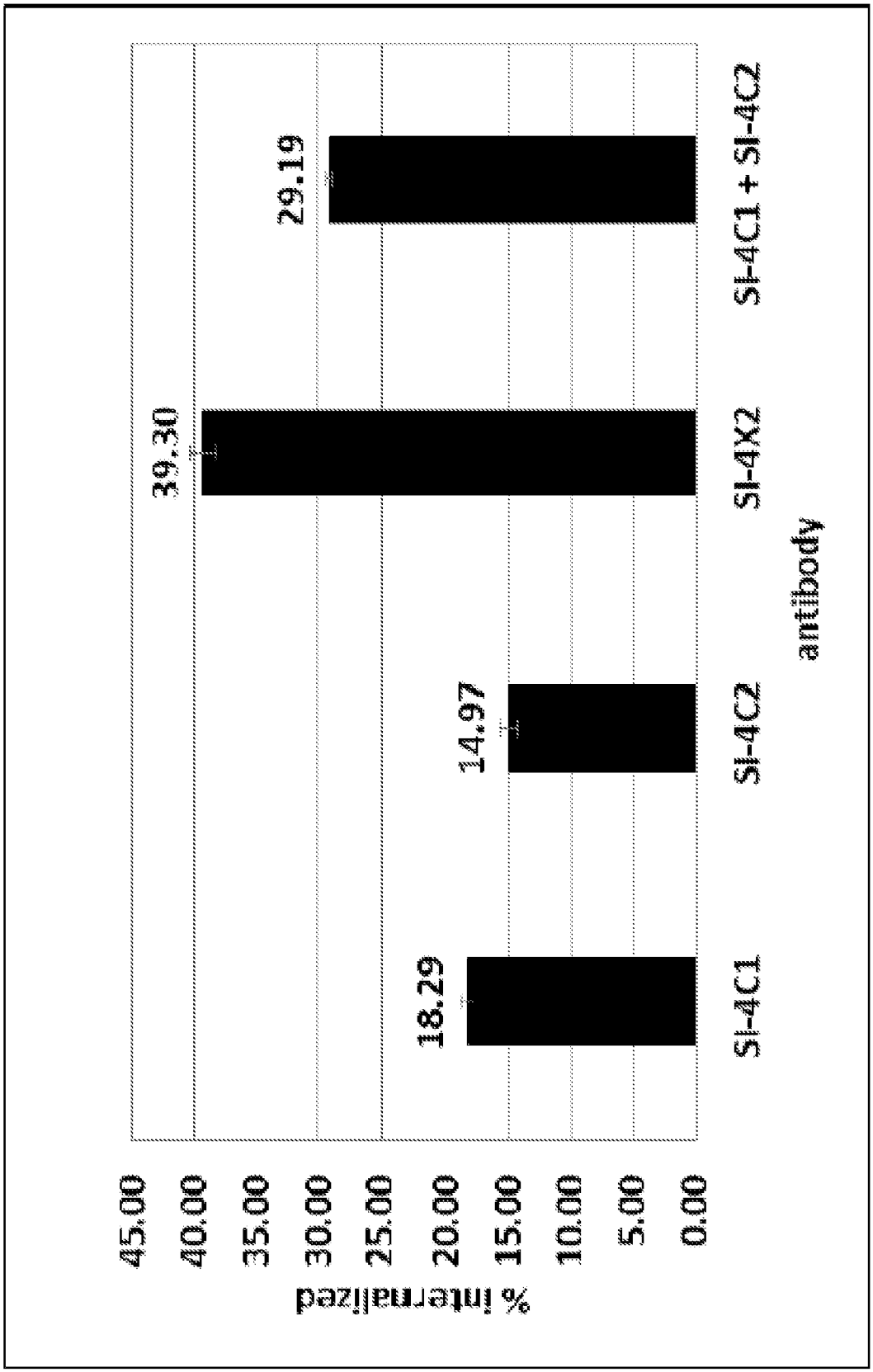

% internalized
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
18.29
14.97
39.30
29.19
SI-4C1
SI-4C2
SI-4X2
SI-4C1 + SI-4C2
antibody

FIG. 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/066952

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 39/395 (2016.01)
CPC - A61K 39/39558 (2016.02)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - A61K 39/395; A61P 35/00, 35/02; C07K 16/18, 16/22, 16/28, 16/46 (2016.01)
CPC - A61K 39/39558; C07K 16/2803, 16/283, 16/2863, 16/2887, 16/2893, 16/32, 16/468, 2317/31, 2317/35, 2317/522, 2317/ (2016.02)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 424/130.1, 135.1, 136.1, 143.1; 530/388.1, 388.22, 388.24 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Patbase, Google Patents, PubMed, Google.

Search terms used: IgG1kappa heavy chain light chain linker connector GGGGS HER2 domain 2 domain 4

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2014/0056895 A1 (BAURIN et al) 27 February 2014 (27.02.2014) entire document	1, 4-15, 18, 21, 22, 25, 26, 33-35, 37, 40-45, 47-64
A	US 2010/256338 A1 (BRINKMANN et al) 07 October 2010 (07.10.2010) entire document	1, 4-15, 18, 21, 22, 25, 26, 33-35, 37, 40-45, 47-64
A	HU et al. "Four-in-One Antibodies Have Superior Cancer Inhibitory Activity against EGFR, HER2, HER3, and VEGF through Disruption of HER/MET Crosstalk," Cancer Res. 04 November 2014 (04.11.2014), Vol. 79, Pgs. 159-170. entire document	1, 4-15, 18, 21, 22, 25, 26, 33-35, 37, 40-45, 47-64
A	WO 2014/144357 A1 (MERCK PATENT GMBH et al) 18 September 2014 (18.09.2014) entire document	1, 4-15, 18, 21, 22, 25, 26, 33-35, 37, 40-45, 47-64
A	US 2014/0135482 A1 (ROCHE GLYCART et al) 15 May 2014 (15.05.2014) entire document	1, 4-15, 18, 21, 22, 25, 26, 33-35, 37, 40-45, 47-64

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

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

Date of the actual completion of the international search	Date of mailing of the international search report
20 April 2016	05 MAY 2016
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/066952

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOs: 3, 11, 30, and 50 were searched.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/066952

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.: 46
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

see Extra Sheet(s).

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

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1, 4-15, 18, 21, 22, 25, 26, 33-35, 37, 40-45, and 47-64 restricted to a bispecific tetravalent antibody, wherein the antibody comprises a first moiety selected to be a first IgG1 heavy chain, connector, and scFv domain encoded by SEQ ID NO: 30, and a first kappa light chain encoded by SEQ ID NO: 3; and a second moiety selected to be a second IgG1 heavy chain, connector, and scFv domain, encoded by SEQ ID NO: 50, and a second kappa light chain encoded by SEQ ID NO: 11, where the first moiety binds domain 2 of HER2 and the second moiety binds domain 4 of HER2.

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/066952

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

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

Group I+: claims 1-45 and 47-64 are drawn to a bispecific tetravalent antibody that binds HER2.

The first invention of Group I+ is restricted to a bispecific tetravalent antibody, wherein the antibody comprises a first moiety selected to be a first IgG1 heavy chain, connector, and scFv domain encoded by SEQ ID NO: 30, and a first kappa light chain encoded by SEQ ID NO: 3; and a second moiety selected to be a second IgC1 heavy chain, connector, and scFv domain, encoded by SEQ ID NO: 50, and a second kappa light chain encoded by SEQ ID NO: 11, where the first moiety binds domain 2 of HER2 and the second moiety binds domain 4 of HER2. It is believed that claims 1, 4-15, 18, 21, 22, 25, 26, 33-35, 37, 40-45, and 47-64 read on this first named invention and thus these claims will be searched without fee to the extent that they read on the above embodiment.

Applicant is invited to elect additional IgG1 heavy chains, kappa light chains, scFv, connectors, and/or linkers each with a corresponding specific SEQ ID NO to be searched in a specific combination by paying additional fee for each set of election. An exemplary election would be a bispecific tetravalent antibody, wherein the antibody comprises a first moiety selected to be a first IgG1 heavy chain, connector, and scFv domain encoded by SEQ ID NO: 40, and a first kappa light chain encoded by SEQ ID NO: 25; and a second moiety selected to be a second IgG1 heavy chain, connector, and scFv domain, encoded by SEQ ID NO: 58, and a second kappa light chain encoded by SEQ ID NO: 35, where the first moiety binds domain 2 of HER2 and the second moiety binds domain 4 of HER2. Additional antibodies will be searched upon the payment of additional fees. Applicants must specify the claims that read on any additional elected inventions. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined.

The inventions listed in Groups I+ do not relate to a single general inventive concept under PCT Rule 13.1, because under PCT Rule 13.2 they lack the same or corresponding special technical features for the following reasons:

The Groups I+ formulas do not share a significant structural element for the the bispecific tetravalent antibody, requiring the selection of alternatives for the IgG1 heavy chain, kappa light chain, and scFv domains, where "a the two IgG1 heavy chains and kappa light chains form an IgG moiety with a binding specificity to a first domain of HER2; wherein the two scFv domains have a binding specificity to a second domain of HER2, and each scFv domain is connected to the C-terminal residue of either of the IgG1 heavy chains by a connector having an amino acid sequence of (gly-gly-gly-gly-ser)n ((G 4 S)n); wherein n is an integral of at least 2; and wherein each scFv domain has a structure order of N terminus-variable heavy domain-linker-variable light domain-C terminus or N-terminus-variable light domain-linker-variable heavy domain-C terminus, and wherein the linker is comprised of amino acid sequence of (gly-gly-gly-gly-ser)m ((G 4S)m); wherein m is an integral of at least 3".

The Groups I+ share the technical features of a bispecific tetravalent antibody, said bispecific tetravalent antibody comprising: two IgG1 heavy chains; two kappa light chains; and two single chain Fv (scFv) domains; wherein the two IgG1 heavy chains and kappa light chains form an IgG moiety with a binding specificity to HER2; wherein the two scFv domains have a binding specificity to a second member of the EGFR family, and each scFv domain is connected to the C terminus of either of the IgG1 heavy chains by a connector with an amino acid sequence of (gly-gly-gly-gly-ser)n, to provide a IgG1-connector connection, wherein n is an integral of at least 2; and wherein each scFv domain has a structure order of N terminus – variable heavy chain - linker - variable light chain - C terminus or N-terminus – variable light chain- linker- variable heavy chain- C-terminus, and wherein the linker is comprised of amino acid sequence of (gly-gly-gly-gly-ser)m. However, these shared technical features do not represent a contribution over the prior art.

Specifically, US 2010/0256338 A1 to Brinkmann et al. discloses a bispecific tetravalent antibody (In the following as one embodiment of the invention tetravalent bi specific antibodies, Para. [0160]), said bispecific tetravalent antibody comprising: two IgG1 heavy chains (the current application denotes a constant heavy chain region of a human antibody of the subclass IgG 1,Para. [0080]; with two pairs of heavy and light chain which comprise variable and constant domains in a typical order, Para. [0121]); two kappa light chains (and/or a constant light chain kappa, Para. [0080]; with two pairs of heavy and light chain which comprise variable and constant domains in a typical order, Para. [0121]); and two single chain Fv (scFv) domains (full length antibody to which two scFv fragments are fused, Para. [0018]); wherein the two IgG1 heavy chains and kappa light chains form an IgG moiety with a binding specificity to a first member of the EGFR family (The antigen-binding sites that specifically bind to the desired antigen (e.g. EGFR) can be derived a) from known antibodies to the antigen (e.g. anti-EGFR antibodies), Para. [0039]); wherein the two scFv domains have a binding specificity to a second member of the EGFR family (single chain formats (scFv, Bis-scFv), which are capable of binding two or more antigens, have been developed, Para. [0005]; full length antibody to which two scFv fragments are fused, Para. [0018]), and each scFv domain is connected to the C terminus of either of the IgG1 heavy chains by a connector with an amino acid sequence of (gly-gly-gly-gly-ser)n, to provide a IgGl-connector connection, wherein n is an integral of at least 1 (which two scFv fragments are fused via a peptide linker at the C-terminus of the heavy chain, Para. [0009]; via a peptide connector at the C- or N-terminus of the heavy...chain, Para. [0011]; In one embodiment the peptide connector is (GxS)n or (GxS)nGm with G=glycine, S=serine...In one embodiment the peptide connector is (G4 S)2, Para. [0034]); and wherein each scFv domain has a structure order of N terminus – variable heavy chain - linker - variable light chain - C terminus or N-terminus – variable light chain- linker- variable heavy chain- C-terminus, and wherein the linker is comprised of amino acid sequence of (gly-gly-gly-gly-ser)m (following orders in N-terminal to C-terminal direction: a) VH-CL-linker-VL-CH1 or b) VL-CH1 -linker-VH-CL, Para. [0030]; In one embodiment the peptide connector is (GxS)n or (GxS)nGm with G=glycine, S=serine...In one embodiment the peptide connector is (G4 S)2, Para. [0034]).

Further, "Four-in-One Antibodies Have Superior Cancer Inhibitory Activity against EGFR, HER2, HER3, and VEGF through Disruption of HER/MET Crosstalk," entitled to Hu et al. discloses an anti-Her2 antibodies (anti-HER2 antibodies, Pg. 159, left-hand column, first paragraph).

The inventions listed in Groups I+ therefore lack unity under Rule 13 because they do not share a same or corresponding special technical features.