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(71) Applicant: **SYSTIMMUNE, INC.** [US/US]; 2023 120th Ave. NE, Bellevue, Washington 98005 (US).

(72) Inventors: **GAO, Zeren**; 2023 120th Ave. Ne, Bellevue, Washington 98005 (US). **TAN, Phil**; 2023 120th Ave. Ne, Bellevue, Washington 98005 (US). **KOVACEVICH, Brian**; 2023 120th Ave. Ne, Bellevue, Washington 98005 (US). **RENSHAW, Blair**; 2023 120th Ave. Ne, Bellevue, Washington 98005 (US). **ADAMO, Jeffrey**; 2023 120th Ave. Ne, Bellevue, Washington 98005 (US). **MAK, Nga Sze Amanda**; 2023 120th Ave. Ne, Bellevue, Washington 98005 (US). **ZHUO, Shi**; Zhonghaijincheng, No.8, Jinlv Road, Wuhou Avenue, Wuhou District, Chengdu, SICHUAN (CN). **CHEN, Lan**; Youkelianbang Iii, No.1, Dongjiawan North Road, Wuhou District, Chengdu, SICHUAN (CN).

(74) Agent: **TIAN, Le**; 14150 NE 20th St F1-55, Bellevue, Washington 98007 (US).

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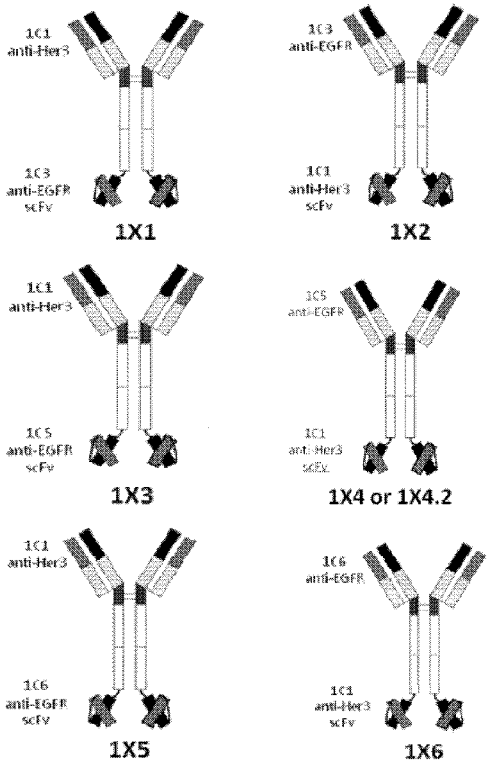

FIG. 3

(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 members of EGFR family.

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BISPECIFIC TETRAVALENT ANTIBODIES AND METHODS OF MAKIING 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 227 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 antibody therapeutic agents, and more particularly relates to bispecific tetravalent antibodies against two different members of EGFR family.

BACKGROUND

Overexpression and/or deregulation of members of the ErbB/HER receptor family such as EGFR, HER2, HER3, HER4 have been shown to play an important role in tumorigenesis in cancers. Mutation and amplification of EGFR or HER2 produce aberrant growth signal which activates downstream signaling pathway contributing to tumorigenesis. Therapeutic antibodies and small-molecule inhibitors directed against EGFR and HER2 have been approved for use in the treatment of cancer (Arteaga et al., *Nature Reviews Clinical Oncology* 9 16-32, January 2012). Monoclonal antibodies against members of EGFR family

such as EGFR and HER2, have demonstrated good clinical responses in colon cancer (Price et al., *The Lancet Oncology* 15(6), Pages 569-579, May 2014), squamous cell carcinoma of head and neck (Cohen, *Cancer Treatment Reviews* 40 (2014) 567–577), breast and gastric cancers (Arteaga et al., *Nature Reviews Clinical Oncology* 9 16-32, January 2012). Several therapeutic anti-EGFR antibodies, including cetuximab, panitumumab and nimotuzumab are approved therapeutics for several cancers including metastatic colorectal cancer, head and neck squamous cell carcinoma and glioma (Price and Cohen, *Curr Treat Options Oncol.* 2012 Mar;13(1):35-46; Bode et al., *Expert Opin Biol Ther.* 2012 Dec;12(12):1649-59). Unfortunately, many tumors that initially respond to these therapeutic agents eventually progress due to an acquired resistance to the agents (Jackman et al. *J Clin Oncol* 2010; 28:357–60). Therefore, there exists a need for better cancer therapeutics.

SUMMARY

The disclosure provides bispecific tetravalent antibodies. The bispecific tetravalent antibodies may include an immunoglobulin G (IgG) moiety with two heavy chains and two light chains, and two scFv moieties being covalently connected to either C or N terminals of the heavy or light chains. The IgG moiety may have a binding specificity to a first member of EGFR family. The scFv moiety may have a binding specificity to a second member of the EGFR family. The IgG moiety and two scFv moieties are covalently connected to be functional as a bispecific tetravalent antibody. The objectives and advantages of the disclosure will 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 will now be described with reference to the FIGs, in which like reference numerals denote

like elements.

FIG. 1 is a diagram showing the domain structures of an example bivalent monospecific immunoglobulin G (IgG) antibody.

FIG. 2 is a diagram showing the domain structure of an example tetravalent bispecific antibody comprising an IgG moiety and two scFv moieties in accordance with one embodiment of the present invention.

FIG. 3 shows the domain structure diagrams of example tetravalent bispecific antibodies 1X1, 1X2, 1X3, 1X4, 1X4.2, 1X5 and 1X6.

FIG. 4 shows the VH domain sequence comparison between SI-1X4 and SI-1X4.2 showing the 5 amino acid differences.

FIGS 5 and 6 are graphs showing monomeric EGFR binding by BLI.

FIGS 7, 8, and 9 are graphs showing bispecific ELI binding.

FIG. 10 is a graph showing dimeric EGFR ELISA.

FIGS 11 shows binding kinetics of SI-1C5.2 and SI-1X4.2 with monomeric EGFR as analyzed by Octet.

FIG. 12 shows flow cytometric analysis of SI-1X antibodies binding to A431 cells.

FIG. 13 shows flow cytometric analysis of SI-1X antibodies binding to BxPC3 cells.

FIG. 14 shows flow cytometric analysis of SI-1X4.2 antibody binding to Fadu cells.

FIG. 15 shows flow cytometric analysis of SI-1X4.2 antibody binding toA431 cells.

FIG. 16 shows effect of SI-1X antibodies on A431 cell proliferation.

FIG. 17 shows effect of SI-1X antibodies on A431 cell proliferation.

FIG. 18 shows effect of SI-1X antibodies on BxPC3 cell proliferation.

FIG. 19 shows effect of SI-1X antibodies on BxPC3 cell proliferation.

FIG. 20 shows effect of SI-1X4.2 antibodies on Fadu cell proliferation.

FIG. 21 shows effect of SI-1X4.2 antibodies on A431 cell proliferation.

FIG. 22 shows ADCC activity of SI-1X antibodies on Fadu cell.

FIG. 23 shows ADCC activity of SI-1X antibodies on NCI-H1975 cells.

FIG. 24 shows the thermal melting of SI-1X antibodies to demonstrate their stability.

FIG. 25 shows the serum stability of SI-1X antibodies over 7 days period.

FIG. 26 is a graph showing the results of EGFR coated ELISA for the PK study in rat.

FIG. 27 is a graph showing the results of HER3 coated ELISA for the PK study in rat.

FIG. 28 is a graph showing the results of sandwich ELISA for the PK study in rat.

FIG. 29 is a graph showing a plot of mean tumor volume vs days in the mouse xenograft study

FIG. 30 is a graph showing a plot relative body weight vs weeks in the mouse xenograft study.

DETAILED DESCRIPTION

The present disclosure This disclosure provides bispecific tetravalent antibodies with superior therapeutic properties or efficacies over the currently known anti-EGFR antibodies. In one embodiment, the antibodies target two members of EGFR family including, without limitation, EFFR and HER3. The bispecific tetravalent antibodies may inhibit both EGFR and HER3 mediated signaling simultaneously therefore overcome resistance in EGFR inhibitor or monoclonal antibody treatment.

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 antibodies may include an immunoglobulin G (IgG) moiety with two heavy chains and two light chains, and two scFv moieties being covalently connected to either C or N terminals of the heavy or light chains. The IgG moiety may have a binding specificity to a first member of EGFR family. The scFv moiety may have a binding specificity to a second member of the EGFR family. The IgG moiety may provide stability to the scFv moiety. The bispecific tetravalent antibody may block signalling for both AKT and MAPK/ERK pathways and may mediate antibody dependent cell-mediated cytotoxicity (ADCC) towards cells expressing either one or both antigens. In one embodiment, the bispecific tetravalent antibody is capable of binding both antigens simultaneously. In some embodiments, the bispecific tetravalent antibody provides stronger tumour inhibition in proliferation assays in vitro and in vivo than the mono-specific antibody parental control or combination of the mono-specific antibody parental controls.

In one embodiment, the disclosure provides a bispecific tetravalent antibody having two IgG1 heavy chains, two kappa light chains, and two single chain Fv (scFv) domains. 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 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 (gly-gly-gly-gly-ser)$_n$, also known as $(G_4S)_n$, to provide a IgG1-connector connection. n is an integral of at least 1. For example, n may be 2, 3, 4, 5, 6, 7, 8, 9, 10, or 17. Each scFv domain has a structure order of N terminus–variable heavy–linker–variable light–C terminus. The linker may have an amino acid sequence of (gly-gly-gly-gly-ser)$_m$, also known as $(G_4S)_m$. m may be an integral of at least 2 or at least 3. For example, m may be 3, 4, 5, 6, 11, or12. In some embodiments, at least one or both of the IgG1 heavy chains are humanized or human. In some embodiments, at least one or both of the kappa light chains are humanized or human.

The EGFR family members may include EGFR, HER2, HER3, a fragment or a derivative thereof. In some embodiments, the first member of the EGFR family may be EGFR, HER2, a fragment or a derivative thereof. In some embodiments, the second member of the EGFR family may be HER3, a fragment or a derivative thereof. In one embodiment, the IgG moiety may have a binding specificity for HER3. In one embodiment, the scFv domains may have a binding specificity for EGFR. In one embodiment, the IgG moiety may have a binding specificity for HER3, and the scFv domains may have a binding specificity for EGFR. In one embodiment, the IgG moiety may have a binding specificity for EGFR. In one embodiment, the scFv domains may have a binding specificity for HER3. In one embodiment, the IgG moiety may a binding specificity for EGFR, and the scFv domains may have a binding specificity for HER3.

In some embodiments, the C terminus of one or both of the IgG1 heavy chains misses an amino acid residue. For example, the lysine reside may be deleted from the C terminus of the IgG1 chain before the connector is fused onto the C-terminus. The deletion of the lysine residue makes the IgG1-connector connection resistant to protease activity.

In some embodiments, one or both of the IgG1 heavy chains contain two

mutations in the CH3 domain. For example, the two mutations may be reversion to the common residues in human CH3 domain.

In some embodiments, the IgG1 heavy chains may an amino acid sequences of or with at least 95%, 98% or 99% similarity to SEQ ID NO 7, 15, 23, 31, 39, 47, and 127. In some embodiments, the IgG1 heavy chain, connector, and scFv domain may have an amino acid sequence of or with at least 95%, 98% or 99% similarity to SEQ ID NO 56, 66, 76, 86, 98, 108, 118, and 136. In some embodiments, the kappa light chains may have an amino acid sequence of or with at least 95%, 98% or 99% similarity to SEQ ID NO 3, 11, 19, 27, 35, 43, 51, 61, 71, 81, 92, 103, 113, 123, and 131. In some embodiments, the variable light chain may an amino acid sequence of or with at least 95%, 98% or 99% similarity to SEQ ID NO 4, 12, 20, 28, 36, 44, 52, 62, 72, 82, 93, 104, 114, 124, and 132. In some embodiment, the variable heavy chain may have an amino acid sequence of or with at least 95%, 98% or 99% similarity to SEQ ID NO 8, 16, 24, 32, 40, 48, 57, 67, 77, 87, 99, 109, 119, 128, and 137.

In some embodiments, the IgG moiety has a binding specificity for HER3, and the scFv domains have a binding specificity for EGFR. In one embodiment, the IgG1 heavy chain, connector, and scFv domain have an amino acid sequence of SEQ ID NO 56, and the kappa light chain has an amino acid sequence of SEQ ID NO 51. In one embodiment, the IgG1 heavy chain, connector, and scFv domain have an amino acid sequence of SEQ ID NO 76, and the kappa light chain has an amino acid sequence of SEQ ID NO 71. In one embodiment, the IgG1 heavy chain, connector, and scFv domain have an amino acid sequence of SEQ ID NO 108, and the kappa light chain has an amino acid sequence of SEQ ID NO 103.

In some embodiments, the IgG moiety has a binding specificity for EGFR, and the scFv domains have a binding specificity for HER3. In one embodiment, the IgG1 heavy chain, connector, and scFv domain have an amino acid sequence of SEQ ID NO 66, and the kappa light chain has an amino acid sequence of SEQ

ID NO 61. In one embodiment, the IgG1 heavy chain, connector, and scFv domain have an amino acid sequence of SEQ ID NO 86, and the kappa light chain has an amino acid sequence of SEQ ID NO 81. In one embodiment, the IgG1 heavy chain, connector, and scFv domain have an amino acid sequence of SEQ ID NO 98, and the kappa light chain has an amino acid sequence of SEQ ID NO 92. In one embodiment, the IgG1 heavy chain, connector, and scFv domain have an amino acid sequence of SEQ ID NO 118, and the kappa light chain has an amino acid sequence of SEQ ID NO 113. In one embodiment, the IgG1 heavy chain, connector, and scFv domain have an amino acid sequence of SEQ ID NO 136, and the kappa light chain has an amino acid sequence of SEQ ID NO 131.

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 binds to EGRF and HER3 with a Kd less than 50nM. In some embodiments, the antibody binds to EGRF and/or HER3 with a Kd less than 40, 30, 25, 20, 19, 18 or 10nM. In one embodiment, the antibody binds to EGRF with a Kd less than 30nM and binds to HER3 with a Kd less than 30nM. In one embodiment, the antibody binds to EGRF with a Kd less than 50nM and binds to HER3 with a Kd less than 50nM simultaneously.

In another aspect, the disclosure provides isolated nucleic acids encoding the bispecific tetravalent antibodies or its sub-component disclosed herein. The sub-component may be the IgG1 heavy chain, the kappa light chain, the variable light chain, or the variable heavy chain.

In a further aspect, the disclosure provides expression vectors having the isolated nucleic acids encoding the bispecific tetravalent antibody or its sub-component 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 antibodies 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 at least two members of EGFR family including, for example, EGFR, HER2, HER3, a fragment or a derivative thereof. The cancer may be breast cancer, colorectal cancer, pancreatic cancer, head and neck cancer, melanoma, ovarian cancer, prostate cancer, and non-small lung cell cancer, glioma, esophageal cancer, nasopharyngeal cancer, anal cancer, rectal cancer, gastric cancer, bladder cancer, cervical cancer and brain 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, a cytotoxic 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 biologics. In one embodiment, the therapeutic agent may be a checkpoint inhibitor. In some embodiments, the therapeutic agent may include PD1, PDL1, CTLA4, 4-1BB, OX40, GITR, TIM3, LAG3, TIGIT, CD40, CD27, HVEM, BTLA, VISTA, B7H4, a derivative, a conjugate, or a fragment 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, or a derivative thereof. In some embodiments, the subject in need of such treatment is a human.

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 HER 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 IgG is shown in FIG. 1.

A diagram of the representative structure of the bispecific tetravalent antibodies according to some embodiments is shown in FIG. 2. In this example, the bispecific tetravalent antibody includes two human IgG1 heavy chains, two human kappa light chains, and two single chain Fv (scFv) domains. The two human IgG1 heavy chains and human kappa light chains form an IgG moiety with a binding specificity to one member of the EGFR family, and each of the two scFv domains is connected to the C-terminal residue of either of the human IgG1 heavy chains by a connector with an amino acid sequence of gly-gly-gly-gly-ser-gly-gly-gly-gly-ser ($(G_4S)_2$). Each scFv domain is in the order: N terminus–variable heavy–linker–variable light–C terminus. The linker is comprised of amino acid sequence of gly-gly-gly-gly-ser-gly-gly-gly-gly-ser-gly-gly-gly-gly-ser,

also known as $(G_4S)_3$. For some embodiments of the bispecific tetravalent antibodies, the CH1, CH2, CH3, CL, Connector and Linker amino acid sequences are identical. Each bispecific tetravalent antibody has a bivalent anti-HER3 binding specificity on one end of the antibody and a bivalent anti-EGFR binding specificity on the other end. One pair of anti-HER3 variable heavy chain and variable light chain is designated as 1C1, and four pairs of anti-EGFR variable heavy chains and variable light chains are designated as 1C3, 1C5, 1C5.2, 1C6 and 1C6.4, respectively. The bispecific tetravalent antibodies are designated as 1X1, 1X2, 1X3, 1X4, 1X4.2, 1X5, 1X5.2, 1X6, and 1X6.4

In addition, a control molecule 1C4 (also designated as SI-1C4) was used in some of the studies. 1C4 is a bispecific antibody against EGFR and HER3 built on the two-in-one platform described by Schaefer et. al., 2011 (Schaefer et al., *Cancer Cell.* 2011 Oct 18; 20(4):472-86). IC4 has a similar structure to a monoclonal antibody. The molecule can bind to either EGFR or HER3 on each Fab arm, but cannot engage both targets simultaneously on each Fab arm.

Variable light chain, variable heavy chain and single chain Fv (scFv) DNA fragments were generated by gene synthesis through an outside vendor. Human Gamma-1 heavy chain and human kappa light chain DNA fragments were generated by gene synthesis through an outside vendor. 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 elsewhere (see CSH Protocols; 2008; doi:10.1101/pdb.prot4977). Antibodies were purified from the resulting transfection supernatants using protein affinity

chromatography and size exclusion chromatography if needed. Protein quality is analysed by Superdex 200 column. Protein used for all the assays have a purity of greater than 90%.

The bispecific antibody may be used for the treatment of cancer types with EGFR and HER3 co-expressions, including without limitation colon cancer, head and neck squamous cell carcinoma, lung cancer, glioma, pancreatic cancer, nasopharyngeal cancer and other cancer types.

The bispecific antibody is of tetravalent dual specificity. The example antibody may include an IgG and two scFv, which provides two different binding specificities compared to mono-specific antibody IgG. The IgG component provides stability and improved serum half-life over other bispecific antibodies that 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 (for example, US7332585B2). It is also capable of mediating ADCC while those without Fc component cannot (for example, US7332585B2). 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 (Schanzer et al, *Antimicrob. Agents Chemother.* 2011, 55(5):2369; EP272942A1).

For the convenient of narration, the sequences of or related to the bispecific antibodies are summarized in TABLE 1 herein below.

TABLE 1. Summary of nucleotide and amino acid sequences of or related to the bispecific antibodies.

SI-1C1 SEQUENCES	
SEQ ID NO 1	SI-1C1 LIGHT CHAIN FULL-LENGTH NUCLEOTIDE SEQUENCE
SEQ ID NO 2	SI-1C1 LIGHT CHAIN VARIABLE LIGHT CHAIN NUCLEOTIDE SEQUENCE
SEQ ID NO 3	SI-1C1 LIGHT CHAIN FULL-LENGTH AMINO ACID SEQUENCE. HUMAN KAPPA CONSTANT DOMAIN IS UNDERLINED

SEQ ID NO 4	SI-1C1 LIGHT CHAIN VARIABLE LIGHT CHAIN AMINO ACID SEQUENCE. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED
SEQ ID NO 5	SI-1C1 HEAVY CHAIN FULL-LENGTH NUCLEOTIDE SEQUENCE
SEQ ID NO 6	SI-1C1 HEAVY CHAIN VARIABLE HEAVY CHAIN NUCLEOTIDE SEQUENCE
SEQ ID NO 7	SI-1C1 HEAVY CHAIN FULL-LENGTH AMINO ACID SEQUENCE. HUMAN GAMMA-1 DOMAIN IS UNDERLINED
SEQ ID NO 8	SI-1C1 HEAVY CHAIN VARIABLE HEAVY CHAIN AMINO ACID SEQUENCE. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED
SI-1C3 SEQUENCES	
SEQ ID NO 9	SI-1C3 LIGHT CHAIN FULL-LENGTH NUCLEOTIDE SEQUENCE
SEQ ID NO 10	SI-1C3 LIGHT CHAIN VARIABLE LIGHT CHAIN NUCLEOTIDE SEQUENCE
SEQ ID NO 11	SI-1C3 LIGHT CHAIN FULL-LENGTH AMINO ACID SEQUENCE. HUMAN KAPPA CONSTANT DOMAIN IS UNDERLINED
SEQ ID NO 12	SI-1C3 LIGHT CHAIN VARIABLE LIGHT CHAIN AMINO ACID SEQUENCE. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED
SEQ ID NO 13	SI-1C3 HEAVY CHAIN FULL-LENGTH NUCLEOTIDE SEQUENCE
SEQ ID NO 14	SI-1C3 HEAVY CHAIN VARIABLE HEAVY CHAIN NUCLEOTIDE SEQUENCE
SEQ ID NO 15	SI-1C3 HEAVY CHAIN FULL-LENGTH AMINO ACID SEQUENCE. HUMAN GAMMA-1 DOMAIN IS UNDERLINED
SEQ ID NO 16	SI-1C3 HEAVY CHAIN VARIABLE HEAVY CHAIN AMINO ACID SEQUENCE. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED
SI-1C4 SEQUENCES	
SEQ ID NO 17	SI-1C4 LIGHT CHAIN FULL-LENGTH NUCLEOTIDE SEQUENCE
SEQ ID NO 18	SI-1C4 LIGHT CHAIN VARIABLE LIGHT CHAIN NUCLEOTIDE SEQUENCE
SEQ ID NO 19	SI-1C4 LIGHT CHAIN FULL-LENGTH AMINO ACID SEQUENCE. HUMAN KAPPA CONSTANT DOMAIN IS UNDERLINED
SEQ ID NO 20	SI-1C4 LIGHT CHAIN VARIABLE LIGHT CHAIN AMINO ACID SEQUENCE. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED
SEQ ID NO 21	SI-1C4 HEAVY CHAIN FULL-LENGTH NUCLEOTIDE SEQUENCE

SEQ ID NO 22	SI-1C4 HEAVY CHAIN VARIABLE HEAVY CHAIN NUCLEOTIDE SEQUENCE
SEQ ID NO 23	SI-1C4 HEAVY CHAIN FULL-LENGTH AMINO ACID SEQUENCE. HUMAN GAMMA-1 DOMAIN IS UNDERLINED
SEQ ID NO 24	SI-1C4 HEAVY CHAIN VARIABLE HEAVY CHAIN AMINO ACID SEQUENCE. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED
SI-1C5 SEQUENCES	
SEQ ID NO 25	SI-1C5 LIGHT CHAIN FULL-LENGTH NUCLEOTIDE SEQUENCE
SEQ ID NO 26	SI-1C5 LIGHT CHAIN VARIABLE LIGHT CHAIN NUCLEOTIDE SEQUENCE
SEQ ID NO 27	SI-1C5 LIGHT CHAIN FULL-LENGTH AMINO ACID SEQUENCE. HUMAN KAPPA CONSTANT DOMAIN IS UNDERLINED
SEQ ID NO 28	SI-1C5 LIGHT CHAIN VARIABLE LIGHT CHAIN AMINO ACID SEQUENCE. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED
SEQ ID NO 29	SI-1C5 HEAVY CHAIN FULL-LENGTH NUCLEOTIDE SEQUENCE
SEQ ID NO 30	SI-1C5 HEAVY CHAIN VARIABLE HEAVY CHAIN NUCLEOTIDE SEQUENCE
SEQ ID NO 31	SI-1C5 HEAVY CHAIN FULL-LENGTH AMINO ACID SEQUENCE. HUMAN GAMMA-1 DOMAIN IS UNDERLINED
SEQ ID NO 32	SI-1C5 HEAVY CHAIN VARIABLE HEAVY CHAIN AMINO ACID SEQUENCE. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED
SI-1C5.2 SEQUENCES	
SEQ ID NO 33	SI1C5.2 LIGHT CHAIN FULL-LENGTH NUCLEOTIDE SEQUENCE
SEQ ID NO 34	SI-1C5.2 LIGHT CHAIN VARIABLE LIGHT CHAIN NUCLEOTIDE SEQUENCE
SEQ ID NO 35	SI-1C5.2 LIGHT CHAIN FULL-LENGTH AMINO ACID SEQUENCE. HUMAN KAPPA CONSTANT DOMAIN IS UNDERLINED
SEQ ID NO 36	SI-1C5.2 LIGHT CHAIN VARIABLE LIGHT CHAIN AMINO ACID SEQUENCE. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED
SEQ ID NO 37	SI-1C5.2 HEAVY CHAIN FULL-LENGTH NUCLEOTIDE SEQUENCE
SEQ ID NO 38	SI-1C5.2 HEAVY CHAIN VARIABLE HEAVY CHAIN NUCLEOTIDE SEQUENCE

SEQ ID NO 39	SI-1C5.2 HEAVY CHAIN FULL-LENGTH AMINO ACID SEQUENCE. HUMAN GAMMA-1 DOMAIN IS UNDERLINED
SEQ ID NO 40	SI-1C5.2 HEAVY CHAIN VARIABLE HEAVY CHAIN AMINO ACID SEQUENCE. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED
SI-1C6 SEQUENCES	
SEQ ID NO 41	SI-1C6 LIGHT CHAIN FULL-LENGTH NUCLEOTIDE SEQUENCE
SEQ ID NO 42	SI-1C6 LIGHT CHAIN VARIABLE LIGHT CHAIN NUCLEOTIDE SEQUENCE
SEQ ID NO 43	SI-1C6 LIGHT CHAIN FULL-LENGTH AMINO ACID SEQUENCE. HUMAN KAPPA CONSTANT DOMAIN IS UNDERLINED
SEQ ID NO 44	SI-1C6 LIGHT CHAIN VARIABLE LIGHT CHAIN AMINO ACID SEQUENCE. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED
SEQ ID NO 45	SI-1C6 HEAVY CHAIN FULL-LENGTH NUCLEOTIDE SEQUENCE
SEQ ID NO 46	SI-1C6 HEAVY CHAIN VARIABLE HEAVY CHAIN NUCLEOTIDE SEQUENCE
SEQ ID NO 47	SI-1C6 HEAVY CHAIN FULL-LENGTH AMINO ACID SEQUENCE. HUMAN GAMMA-1 DOMAIN IS UNDERLINED
SEQ ID NO 48	SI-1C6 HEAVY CHAIN VARIABLE HEAVY CHAIN AMINO ACID SEQUENCE. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED
SI-1X1 SEQUENCES	
SEQ ID NO 49	SI1X1 LIGHT CHAIN FULL-LENGTH NUCLEOTIDE SEQUENCE
SEQ ID NO 50	SI-1X1 LIGHT CHAIN VARIABLE LIGHT CHAIN NUCLEOTIDE SEQUENCE
SEQ ID NO 51	SI-1X1 LIGHT CHAIN FULL-LENGTH AMINO ACID SEQUENCE. HUMAN KAPPA CONSTANT DOMAIN IS UNDERLINED
SEQ ID NO 52	SI-1X1 LIGHT CHAIN VARIABLE LIGHT CHAIN AMINO ACID SEQUENCE. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED
SEQ ID NO 53	SI1X1 BISPECIFIC HEAVY CHAIN FULL-LENGTH NUCLEOTIDE SEQUENCE
SEQ ID NO 54	SI-1X1 BISPECIFIC HEAVY CHAIN VARIABLE HEAVY CHAIN NUCLEOTIDE SEQUENCE
SEQ ID NO 55	SI-1X1 BISPECIFIC HEAVY CHAIN SCFV NUCLEOTIDE SEQUENCE
SEQ ID NO 56	SI-1X1 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 57	SI-1X1 BISPECIFIC HEAVY CHAIN VARIABLE HEAVY CHAIN AMINO ACID SEQUENCE. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED
SEQ ID NO 58	SI1X1 BISPECIFIC HEAVY CHAIN SCFV AMINO ACID SEQUENCE. ORDER: VH – LINKER – VL. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED. LINKER IS IN BOLD ITALICS
SI-1X2 SEQUENCES	
SEQ ID NO 59	SI-1X2 LIGHT CHAIN FULL-LENGTH NUCLEOTIDE SEQUENCE
SEQ ID NO 60	SI-1X2 LIGHT CHAIN VARIABLE LIGHT CHAIN NUCLEOTIDE SEQUENCE
SEQ ID NO 61	SI-1X2 LIGHT CHAIN FULL-LENGTH AMINO ACID SEQUENCE. HUMAN KAPPA CONSTANT DOMAIN IS UNDERLINED
SEQ ID NO 62	SI-1X2 LIGHT CHAIN VARIABLE LIGHT CHAIN AMINO ACID SEQUENCE. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED
SEQ ID NO 63	SI-1X2 BISPECIFIC HEAVY CHAIN FULL-LENGTH NUCLEOTIDE SEQUENCE
SEQ ID NO 64	SI-1X2 BISPECIFIC HEAVY CHAIN VARIABLE HEAVY CHAIN NUCLEOTIDE SEQUENCE
SEQ ID NO 65	SI-1X2 BISPECIFIC HEAVY CHAIN SCFV NUCLEOTIDE SEQUENCE
SEQ ID NO 66	SI-1X2 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 67	SI-1X2 BISPECIFIC HEAVY CHAIN VARIABLE HEAVY CHAIN AMINO ACID SEQUENCE. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED
SEQ ID NO 68	SI-1X2 BISPECIFIC HEAVY CHAIN SCFV AMINO ACID SEQUENCE. ORDER: VH – LINKER – VL. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED. LINKER IS IN BOLD ITALICS
SI-1X3 SEQUENCES	
SEQ ID NO 69	SI-1X3 LIGHT CHAIN FULL-LENGTH NUCLEOTIDE SEQUENCE
SEQ ID NO 70	SI-1X3 LIGHT CHAIN VARIABLE LIGHT CHAIN NUCLEOTIDE SEQUENCE
SEQ ID NO 71	SI-1X3 LIGHT CHAIN FULL-LENGTH AMINO ACID SEQUENCE. HUMAN KAPPA CONSTANT DOMAIN IS UNDERLINED

SEQ ID NO 72	SI-1X3 LIGHT CHAIN VARIABLE LIGHT CHAIN AMINO ACID SEQUENCE. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED
SEQ ID NO 73	SI-1X3 BISPECIFIC HEAVY CHAIN FULL-LENGTH NUCLEOTIDE SEQUENCE
SEQ ID NO 74	SI-1X3 BISPECIFIC HEAVY CHAIN VARIABLE HEAVY CHAIN NUCLEOTIDE SEQUENCE
SEQ ID NO 75	SI-1X3 BISPECIFIC HEAVY CHAIN SCFV NUCLEOTIDE SEQUENCE
SEQ ID NO 76	SI-1X3 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 77	SI-1X3 BISPECIFIC HEAVY CHAIN VARIABLE HEAVY CHAIN AMINO ACID SEQUENCE. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED
SEQ ID NO 78	SI-1X3 BISPECIFIC HEAVY CHAIN SCFV AMINO ACID SEQUENCE. ORDER: VH – LINKER – VL. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED. LINKER IS IN BOLD ITALICS
SI-1X4 SEQUENCES	
SEQ ID NO 79	SI-1X4 LIGHT CHAIN FULL-LENGTH NUCLEOTIDE SEQUENCE
SEQ ID NO 80	SI-1X4 LIGHT CHAIN VARIABLE LIGHT CHAIN NUCLEOTIDE SEQUENCE
SEQ ID NO 81	SI-1X4 LIGHT CHAIN FULL-LENGTH AMINO ACID SEQUENCE. HUMAN KAPPA CONSTANT DOMAIN IS UNDERLINED
SEQ ID NO 82	SI-1X4 LIGHT CHAIN VARIABLE LIGHT CHAIN AMINO ACID SEQUENCE. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED
SEQ ID NO 83	SI-1X4 BISPECIFIC HEAVY CHAIN FULL-LENGTH NUCLEOTIDE SEQUENCE
SEQ ID NO 84	SI-1X4 BISPECIFIC HEAVY CHAIN VARIABLE HEAVY CHAIN NUCLEOTIDE SEQUENCE
SEQ ID NO 85	SI-1X4 BISPECIFIC HEAVY CHAIN SCFV NUCLEOTIDE SEQUENCE
SEQ ID NO 86	SI-1X4 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 87	SI-1X4 BISPECIFIC HEAVY CHAIN VARIABLE HEAVY CHAIN AMINO ACID SEQUENCE. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED
SEQ ID NO 88	SI-1X4 BISPECIFIC HEAVY CHAIN SCFV AMINO ACID SEQUENCE. ORDER: VH – LINKER – VL.

	COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED. LINKER IS IN BOLD ITALICS
SI-1X4.2 SEQUENCES	
SEQ ID NO 89	SI-1X4.2 LIGHT CHAIN FULL-LENGTH NUCLEOTIDE SEQUENCE
SEQ ID NO 90	SI-1X4.2 LIGHT CHAIN VARIABLE LIGHT CHAIN NUCLEOTIDE SEQUENCE
SEQ ID NO 91	SI-1X4.2 LIGHT CHAIN VARIABLE LIGHT CHAIN NUCLEOTIDE SEQUENCE CODON OPTIMIZED FOR CHO EXPRESSION
SEQ ID NO 92	SI-1X4.2 LIGHT CHAIN FULL-LENGTH AMINO ACID SEQUENCE. HUMAN KAPPA CONSTANT DOMAIN IS UNDERLINED
SEQ ID NO 93	SI-1X4.2 LIGHT CHAIN VARIABLE LIGHT CHAIN AMINO ACID SEQUENCE. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED
SEQ ID NO 94	SI-1X4.2 BISPECIFIC HEAVY CHAIN FULL-LENGTH NUCLEOTIDE SEQUENCE
SEQ ID NO 95	SI-1X4.2 BISPECIFIC HEAVY CHAIN VARIABLE HEAVY CHAIN NUCLEOTIDE SEQUENCE
SEQ ID NO 96	SI-1X4.2 BISPECIFIC HEAVY CHAIN VARIABLE HEAVY CHAIN NUCLEOTIDE SEQUENCE CODON OPTIMIZED FOR CHO EXPRESSION
SEQ ID NO 97	SI-1X4.2 BISPECIFIC HEAVY CHAIN SCFV NUCLEOTIDE SEQUENCE
SEQ ID NO 98	SI-1X4.2 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 99	SI-1X4.2 BISPECIFIC HEAVY CHAIN VARIABLE HEAVY CHAIN AMINO ACID SEQUENCE. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED
SEQ ID NO 100	SI-1X4.2 BISPECIFIC HEAVY CHAIN SCFV AMINO ACID SEQUENCE. ORDER: VH – LINKER – VL. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED. LINKER IS IN BOLD ITALICS
SI-1X5 SEQUENCES	
SEQ ID NO 101	SI-1X5 LIGHT CHAIN FULL-LENGTH NUCLEOTIDE SEQUENCE
SEQ ID NO 102	SI-1X5 LIGHT CHAIN VARIABLE LIGHT CHAIN NUCLEOTIDE SEQUENCE
SEQ ID NO 103	SI-1X5 LIGHT CHAIN FULL-LENGTH AMINO ACID SEQUENCE. HUMAN KAPPA CONSTANT DOMAIN IS UNDERLINED

SEQ ID NO 104	SI-1X5 LIGHT CHAIN VARIABLE LIGHT CHAIN AMINO ACID SEQUENCE. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED
SEQ ID NO 105	SI-1X5 BISPECIFIC HEAVY CHAIN FULL-LENGTH NUCLEOTIDE SEQUENCE
SEQ ID NO 106	SI-1X5 BISPECIFIC HEAVY CHAIN VARIABLE HEAVY CHAIN NUCLEOTIDE SEQUENCE
SEQ ID NO 107	SI-1X5 BISPECIFIC HEAVY CHAIN SCFV NUCLEOTIDE SEQUENCE
SEQ ID NO 108	SI-1X5 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 109	SI-1X5 BISPECIFIC HEAVY CHAIN VARIABLE HEAVY CHAIN AMINO ACID SEQUENCE. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED
SEQ ID NO 110	SI-1X5 BISPECIFIC HEAVY CHAIN SCFV AMINO ACID SEQUENCE. ORDER: VH – LINKER – VL. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED. LINKER IS IN BOLD ITALICS
SI-1X6 SEQUENCES	
SEQ ID NO 111	SI-1X6 LIGHT CHAIN FULL-LENGTH NUCLEOTIDE SEQUENCE
SEQ ID NO 112	SI-1X6 LIGHT CHAIN VARIABLE LIGHT CHAIN NUCLEOTIDE SEQUENCE
SEQ ID NO 113	SI-1X6 LIGHT CHAIN FULL-LENGTH AMINO ACID SEQUENCE. HUMAN KAPPA CONSTANT DOMAIN IS UNDERLINED
SEQ ID NO 114	SI-1X6 LIGHT CHAIN VARIABLE LIGHT CHAIN AMINO ACID SEQUENCE. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED
SEQ ID NO 115	SI-1X6 BISPECIFIC HEAVY CHAIN FULL-LENGTH NUCLEOTIDE SEQUENCE
SEQ ID NO 116	SI-1X6 BISPECIFIC HEAVY CHAIN VARIABLE HEAVY CHAIN NUCLEOTIDE SEQUENCE
SEQ ID NO 117	SI-1X6 BISPECIFIC HEAVY CHAIN SCFV NUCLEOTIDE SEQUENCE
SEQ ID NO 118	SI-1X6 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 119	SI-1X6 BISPECIFIC HEAVY CHAIN VARIABLE HEAVY CHAIN AMINO ACID SEQUENCE. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED
SEQ ID NO 120	SI-1X6 BISPECIFIC HEAVY CHAIN SCFV AMINO ACID SEQUENCE. ORDER: VH – LINKER – VL.

	COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED. LINKER IS IN BOLD ITALICS
SI-1C6.2 SEQUENCES	
SEQ ID NO 121	SI-1C6.2 LIGHT CHAIN FULL-LENGTH NUCLEOTIDE SEQUENCE
SEQ ID NO 122	SI-1C6.2 LIGHT CHAIN VARIABLE LIGHT CHAIN NUCLEOTIDE SEQUENCE
SEQ ID NO 123	SI-1C6.2 LIGHT CHAIN FULL-LENGTH AMINO ACID SEQUENCE. HUMAN KAPPA CONSTANT DOMAIN IS UNDERLINED
SEQ ID NO 124	SI-1C6.2 LIGHT CHAIN VARIABLE LIGHT CHAIN AMINO ACID SEQUENCE. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED
SEQ ID NO 125	SI-1C6.2 HEAVY CHAIN FULL-LENGTH NUCLEOTIDE SEQUENCE
SEQ ID NO 126	SI-1C6.2 HEAVY CHAIN VARIABLE HEAVY CHAIN NUCLEOTIDE SEQUENCE
SEQ ID NO 127	SI-1C6.2 HEAVY CHAIN FULL-LENGTH AMINO ACID SEQUENCE. HUMAN GAMMA-1 DOMAIN IS UNDERLINED
SEQ ID NO 128	SI-1C6.2 HEAVY CHAIN VARIABLE HEAVY CHAIN AMINO ACID SEQUENCE. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED
SI-1X6.4 SEQUENCES	
SEQ ID NO 129	SI-1X6.4 LIGHT CHAIN FULL-LENGTH NUCLEOTIDE SEQUENCE
SEQ ID NO 130	SI-1X6.4 LIGHT CHAIN VARIABLE LIGHT CHAIN NUCLEOTIDE SEQUENCE
SEQ ID NO 131	SI-1X6.4 LIGHT CHAIN FULL-LENGTH AMINO ACID SEQUENCE. HUMAN KAPPA CONSTANT DOMAIN IS UNDERLINED
SEQ ID NO 132	SI-1X6.4 LIGHT CHAIN VARIABLE LIGHT CHAIN AMINO ACID SEQUENCE. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED
SEQ ID NO 133	SI-1X6.4 BISPECIFIC HEAVY CHAIN FULL-LENGTH NUCLEOTIDE SEQUENCE
SEQ ID NO 134	SI-1X6.4 BISPECIFIC HEAVY CHAIN VARIABLE HEAVY CHAIN NUCLEOTIDE SEQUENCE
SEQ ID NO 135	SI-1X6.4 BISPECIFIC HEAVY CHAIN SCFV NUCLEOTIDE SEQUENCE
SEQ ID NO 136	SI-1X6.4 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 137	SI-1X6.4 BISPECIFIC HEAVY CHAIN VARIABLE HEAVY CHAIN AMINO ACID SEQUENCE. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED
SEQ ID NO 138	SI-1X6.4 BISPECIFIC HEAVY CHAIN SCFV AMINO ACID SEQUENCE. ORDER: VH – LINKER – VL. COMPLEMENTARITY DETERMINING REGIONS ARE UNDERLINED. LINKER IS IN BOLD ITALICS

EXAMPLES

While The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of non-critical parameters that could be changed or modified to yield essentially the same or similar results.

Example 1: Sequence differences between SI-1X4 and SI-1X4.2

SI-1X4.2 is a modification of SI-1X4 molecule and contained 5 amino acid changes as follows: V71A, T75S, N76S, A93T and S107T using the Kabat numbering system. Some of these changes especially positions 75, 76 and 93 potentially made interaction with antigen even though these are not in the CDR loops and are essential for binding and activity. FIG. 4 shows the 5 amino acid differences between SI-1X4.2 and SI-1X4.

Example 2: Characterization of Antibodies against Epidermal Growth Factor Receptor using BLI

Monomeric EGFR extracellular domain binding was measured in a biolayer interferometry (BLI) binding assay on a BLItz instrument (ForteBio, Inc.). 25µg/mL of SI-1C3, SI-1C4, SI-1C6, SI-1X1, SI-1X2, SI-1X5, and SI-1X6 were diluted in PBS and captured on anti-huIgG Fc BLItz biosensor tips for 120 seconds. Tips were washed for 30 seconds in PBS and moved to an EGFR (ProSpec Bio, PKA-344) sample for binding at 588nM. Binding of EGFR ECD to the tips was recorded as biolayer interferometry signals (Δnm) over an association time of 120 seconds. Tips were moved to PBS and dissociation was observed for 240 seconds (*SI-1C6 dissociation time of only 120 seconds observed). FIGS 5 and 6 report data starting at the association step of EGFR to the antibody-loaded biosensor. Each Figure shows comparison to SI-1C4 as a benchmark antibody.

Since SI-1C3 and SI-1X2 share their EGFR binding domain displayed as a Fab, their binding profiles are similar and stronger than the scFv form displayed

on SI-1X1 (FIG. 6). Each has a very slow off-rate to EGFR compared to SI-1C4 and is not affected by their on-rate. SI-1X1 may show weaker on-rate binding to EGFR, but stays bound very strongly. The same trend is observed in FIG. 5, where the Fab versions of the EGFR binding domains displayed on SI-1C6 and SI-1X6 bind at a faster rate than their representative scFv displayed on SI-1X5. Having the EGFR binding domain on the Fab side of the bispecifics antibody appears to bind with faster on-rates than the scFv versions, yet exhibit similar off-rates. SI-1X3 and SI-1X4 do not exhibit monomeric EGFR binding in this assay (data not shown) and dimeric EGFR binding is investigated in an ELISA below.

Example 3: Characterization of Antibodies against EGFR and Her3 using BL1

Bispecific binding to EGFR and Her3 extracellular domains was measured in a biolayer interferometry (BLI) binding assay on a BLItz instrument (ForteBio, Inc.). 200nM of SI-1C1, SI-1C3, SI-1C4, SI-1C6, SI-1X1, SI-1X2, SI-1X3, SI-1X4, SI-1X5, and SI-1X6 were diluted in 1X Kinetics Buffer (ForteBio, Inc.) and captured on anti-huIgG Fc BLItz biosensor tips for 120 seconds. Tips were washed in KB for 30 seconds and moved to an EGFR sample (ProSpec Bio, PKA-344) for binding at 200nM. Binding of EGFR ECD to the tips was recorded as biolayer interferometry signals (Δnm) over an association time of 120 seconds. Tips were moved to KB and dissociation was observed for 60 seconds. The process was repeated with Her3 ECD sample (Sino Biological, 10201-H08H-10) at 200nM for 120 seconds and a similar dissociation step of 60 seconds in KB. FIGS 8-10 7-9 report data starting at the association step of EGFR to the antibody-loaded biosensor. Antibodies are able to exhibit simultaneous bispecific binding of EGFR and Her3 while being bound by the Fc to the sensor. As observed in FIG. 7 and FIG. 8, the display of the EGFR binding domain as Fab (SI-1X2, SI-1X6) has stronger on-rate binding than their scFv forms (SI-1X1, SI-1X5, respectively). Here, both EGFR and Her3 exhibit the same Fab>>scFv on-rate trend. SI-1X3 and SI-1X4 do not exhibit binding to monomeric EGFR, however each has the ability to bind Her3, as expected since each molecule uses the same αHer3

binding domain as SI-1X1, SI-1X2, SI-1X5, and SI-1X6. SI-1X3 and SI-1X4 are investigated for dimeric EGFR binding in an ELISA below.

Example 4: Dimeric EGFR ELISA Assay

As observed earlier, SI-1X3 and SI-1X4 were unable to bind a monomeric form of EGFR in a BLI assay (FIG. 9). It has been suggested that in order for the αEGFR binding domain used in SI-1C5, SI-1X3, and SI-1X4 to bind to EGFR *in vitro*, bivalent binding is required (Perez et al, *Chin Clin Oncol* 2014;3(1):5). To observe this, we utilized ELISA for antibody binding relative to other EGFR binding antibodies using a dimeric form of EGFR.

ELISA was performed using dimeric EGFR ECD reagent, SI-2C1, fused to rabbit Fc created in house. EGFR was coated onto Maxisorp immunoplates (Nunc) at 3 µg/mL in PBS at 4°C overnight. Plates were blocked in PBS with 3% BSA and 0.05% Tween20 for 2 hours at room temperature. Antibodies were captured at starting at 10ug/mL except for SI-1C5, SI-1X3, and SI-1X4 which started at 50 µg/mL for (reported in nM), all with 3X dilutions in PBST (1% BSA) for 1 hour at room temperature. Goat αhuman IgG-HRP antibody (Jackson ImmunoResearch, 109-035-098) was used for detection of the Fc portion of the antibodies at 1:2000 dilution in PBST (1% BSA) and developed in TMB (Thermo Scientific) for 5 minutes with 2M H_2S0_4 as a stop solution. 3 washes with PBST (1% BSA) were performed between each step. All data points were performed in triplicate and collected at 450 nm (FIG. 10). SI-1C5, SI-1X3, and SI-1X4 all bound to the dimeric EGFR ECD in this ELISA format at high concentrations as compared to the other molecules.

Example 5: Binding Kinetics of 1C5.2 and 1X4.2 using Octet

Kinetics determined using ForteBio Octet Red96 instrument with anti-human Fc sensors (ForteBio, AHC #18-5060). Binding experiments performed at 30° C with 1000 RPM mixing. EGFR protein is extracellular domain (Met 1-Ser 645) of human EGFR with a C-terminal polyhistidine tag. All samples diluted in 10X Kinetics Buffer (ForteBio #18-5032). 1C5.2, 1X6 and 1X4.2 were loaded

onto 8 sensors at 10 µg/ml each for 300 seconds followed by a Baseline for 60 seconds in 10X Kinetics Buffer. Association with EGFR protein was performed for 300 seconds with each sensor in a single concentration of EGFR protein (300, 100, 33.33, 11.11, 3.705, 1.235, 0.4116 and 0 nM). Dissociation was then performed in 10X Kinetics Buffer for 900 seconds. A typical association and dissociation trace for 1C5.2 and 1X4.2 is shown in FIG. 11.

Data analysis was performed using ForteBio Data Analysis Software v9.0. Software curve-fitting was performed and the four most optimal curve fits for each 1C5.2 (TABLE 2), 1X4.2 (TABLE 3) and 1X6 (TABLE 4) were used and averaged to determine KD, k(on) and k(dis). The average KD for SI-1C5.2 and SI-1X4.2 were 19.2 nM and 18.4 nM respectively. The average KD for SI-1C6 was 3.04 nM 1C5.2 and 1X4.2 contained five amino acid changes as compared to 1C5 and 1X4 as described in example 1. These changes accounted for improved binding to EGFR ECD when compared to data generated for 1C5 and 1X4 in FIG. 10.

TABLE 2. Summary of KD, KON and KDIS for 1C5.2

	EGFR (NM)	**KD (M)**	**KON(1/MS)**	**KDIS(1/S)**
SI-1C5.2	300	3.74E-08	4.61E+04	1.72E-03
SI-1C5.2	100	2.23E-08	7.89E+04	1.76E-03
SI-1C5.2	33.3	9.94E-09	1.60E+05	1.59E-03
SI-1C5.2	11.1	7.08E-09	2.12E+05	1.50E-03
AVERAGES		**1.92E-08**	**1.24E+05**	**1.64E-03**

TABLE 3. Summary of KD, KON and KDIS for 1X4.2

	EGFR (NM)	**KD (M)**	**KON(1/MS)**	**KDIS(1/S)**
SI-1X4.2	300	3.69E-08	4.63E+04	1.71E-03
SI-1X4.2	100	2.10E-08	7.88E+04	1.65E-03
SI-1X4.2	33.3	9.44E-09	1.58E+05	1.49E-03

SI-1X4.2	11.1	6.19E-09	2.18E+05	1.35E-03
AVERAGES		**1.84E-08**	**1.25E+05**	**1.55E-03**

TABLE 4. Summary of KD, KON and KDIS for 1X6

	EGFR (NM)	**KD (M)**	**KON(1/MS)**	**KDIS(1/S)**
SI-1C6	300	3.04E-09	4.11E+05	1.25E-03
SI-1C6	100	3.04E-09	4.11E+05	1.25E-03
SI-1C6	33.3	3.04E-09	4.11E+05	1.25E-03
SI-1C6	11.1	3.04E-09	4.11E+05	1.25E-03
AVERAGES		**3.04E-09**	**4.11E+05**	**1.25E-03**

Example 6: Binding tests of example bispecific antibodies to tumor cell lines

The bispecific antibodies SI-1X1, SI-1X2, SI-1X3, SI-1X4, SI-1X5, and SI-1X6, as well as an isotype control were tested for binding to the tumor cell lines, A431 (epidermoid carcinoma, ATCC CRL-1555) and BxPC3 (pancreatic adenocarcinoma, ATCC CRL-1687) by flow cytometry. Cells were grown in RPMI-1640 medium containing 10% fetal bovine serum and were harvested for analysis while in exponential growth phase. Aliquots of 5 x 10^6 cells were washed once in PBS, then resuspended in 250 µl of PBS + 1% bovine serum albumin (BSA) and incubated at 4°C for 15 minutes to block membranes from non-specific binding. 250 µl of antibody, diluted to 10 µg/ml in PBS/1%BSA, was added to each sample for a final antibody concentration of 5 µg/ml. Cells were incubated in primary antibody for 1 hour at 4°C with mixing. Cells were then washed twice with 1ml PBS/1%BSA and then resuspended in 500 µl of PE-conjugated mouse-anti-human IgG-Fc and incubated at 4°C with mixing for 45 minutes. Samples were again washed twice with 1ml PBS/1%BSA, resuspended in 300ml PBS and analyzed using a FACScalibur flow cytometer. For each sample, 10000 events were collected in the FL-2 channel. Histograms were generated using FCS Express software and SI-1X histograms were overlaid with histograms

from the isotype control staining. All six bispecific antibodies displayed histogram shifts with respect to control staining indicating cell binding. This data is displayed in FIG. 12 (A431 cell binding) and FIG. 13 (BxPC3 cell binding).

Example 7: Characterization of SI-1C5.2 and SI-1X4.2 by cell binding assays

The bispecific antibody, SI-1X4.2, monospecific antibodies, SI-1C5.2 and SI-1C1, as well as an isotype control were tested for binding to the tumor cell lines, A431 (epidermoid carcinoma, ATCC CRL-1555) (FIG. 14) and FaDu (hypopharyngeal squamous cell carcinoma, ATCC HTB-43) (FIG. 15) by flow cytometry. Cells were grown in RPMI-1640 medium containing 10% fetal bovine serum and were harvested for analysis while in exponential growth phase. Cells were washed once in PBS, then resuspended in PBS + 5% fetal bovine serum albumin (FBS) at a concentration of $5x10^6$ cells/ml and incubated at 4°C for 15 minutes to block membranes from non-specific binding. 100µl aliquots of cells were added to 100µl aliquots of antibody (also diluted in PBS + 5% FBS) in a 96-well plate. Samples were incubated in primary antibody for 45 minutes on ice. Cells were then washed twice with 200µl of PBS + 5% FBS and then resuspended in 100µl of PE-conjugated mouse-anti-human IgG-Fc and incubated on ice 30 minutes. Samples were again washed twice with 200µl of PBS + 5% FBS, resuspended in 200µl PBS and analyzed using a FACScalibur flow cytometer. For each sample, 10000 events were collected in the FL-2 channel. Histograms were analyzed using FCS Express software and the geometric mean fluorescence intensity (GMFI) was determined for each data set. EC50 binding values were determined by plotting the GMFI versus antibody concentration using Graphpad Prism software. The bispecific antibody, SI-1X4.2 displayed similar binding profile as the monospecific anti-EGFR antibody, SI-1C5.2 with similar EC50 in both cell lines. The other monospecific anti-Her3 antibody, SI-1C1 binds weakly to the two cell lines probably due to low level of expression of Her3 on the surface of the cells. 1C5.2 and 1X4.2 contained five amino acid changes as compared to 1C5 and 1X4 as described in example 1. These changes accounted

for improved binding to target cells when compared to the parental molecule, 1X4.

Example 8: Anti-proliferative effect of SI-1X antibodies on tumor cell lines

To assess the growth inhibitory potential of anti-Her3/EGFR bispecific antibodies, the effect on proliferation of A431 cells (ATCC CRL-1555, Manassas, Va.) which are an epidermoid carcinoma tumor line was tested. The effect on proliferation of BxPC3 (ATCC CRL-1687, Manassas, Va.), a pancreatic adenocarcinoma tumor line was also tested. For each line, cells were seeded into 96-well tissue culture plates at a density of 6000 cells/well in 100 µl RPMI-1640 medium containing 1% fetal bovine serum. After 4 hours, test antibodies were added at various concentrations, ranging from 0.0015nM to 100nM. Cells were cultured in the presence of test antibodies for 72 hours. To each well, 20 µl 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 effects of various bispecific anti-Her3/anti-EGFR antibodies on A431 cell proliferation are shown in FIG. 16 and FIG. 17. SI-1X2 demonstrated more efficacious antiproliferative effect than the control antibodies SI-1C1 (anti-Her3), SI-1C3 (anti-EGFR), or SI-1C1 and SI-1C3 applied together. SI-1X1 exhibited antiproliferative effects, although not to the degree seen with SI-1C3 and the combination of SI-1C1 and SI-1C3. Inhibition plots as well as IC50 values are

shown in FIG. 17. Similar results were observed for SI-1X5 and SI-1X6, where SI-1X6 is more potent than SI-1X5 and the control antibody SI-1C1 (anti-Her3), however it displayed similar antiproliferative potential as the control antibody SI-1C6 (anti-EGFR) and the combination of SI-1C1 and SI-1C6. This may be seen along with IC50 values in FIG. 17.

These molecules were also tested for antiproliferative effects in the BxPC3 cell line (FIG. 18 and FIG. 19). Again, SI-1X2 demonstrated more efficacious antiproliferative effect than the control antibodies SI-1C1 (anti-Her3), SI-1C3 (anti-EGFR), or SI-1C1 and SI-1C3 applied together. SI-1X1 was more efficacious than SI-1C1, but weaker than SI-1C3 and the combination of SI-1C1 and SI-1C3. Inhibition curves and IC50 values are displayed in FIG. 19. BxPC3 proliferation was more strongly inhibited by both SI-1X5 and SI-1X6 than with the control antibodies SI-1C1 (anti-Her3), SI-1C6 (anti-EGFR), or SI-1C1 and SI-1C6 in combination. This data along with IC50 values is shown in FIG. 19.

Example 9: Anti-proliferative effect of SI-1C5.2 and SI-1X4.2 on tumor cell lines

To assess the growth inhibitory potential of anti-Her3/EGFR bispecific antibodies, the effect on proliferation of FaDu (nasopharyngeal squamous cell carcinoma line, ATCC HTB-43) and A431 (epidermoid carcinoma, ATCC CRL-1555) cells were 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 0.0015nM to 100nM. Cells were cultured in the presence of test antibodies for 72 hours. To each well, 11µl of alamar blue reagent (Thermo Scientific) was added and cells were incubated at 37°C for 2 hours. Alamar blue is readily taken up by actively proliferating cells, reduced, and then secreted into the culture medium. The reduced form of alamar blue is strongly fluorescent. Following incubation, fluorescence was measured using a Molecular Devices (Sunnyvale, CA) FilterMax F5 multi-mode plate reader using an excitation wavelength of 535nm and an emission wavelength of 595nm.

Fluorescence 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 fluorescence from the 72-hour fluorescence values. Data from antibody titrations was expressed at % of control population according to the following formula: % of control proliferation = (test proliferation /control proliferation)*100.

The effects of SI-1C5.2 and SI-1X4.2 on Fadu and A431 cell proliferation are shown in FIG. 20 and FIG. 21 respectively. In both cell lines, SI-1X4.2 demonstrated improved efficacious anti-proliferative effect than the control antibodies, SI-1C5.2 (anti-EGFR Mab), SI-1C1 (anti-Her3 Mab) or SI-1C1 and SI-1C7 applied together.

Example 10: ADCC activities of SI-1X bispecific antibodies

The ability of SI-1X antibodies to mediate cellular cytotoxicity against several tumor cell lines was tested. Whole blood was obtained from normal, healthy volunteers. Blood was diluted with an equal volume of phosphate buffered saline (PBS). 20ml aliquots of diluted blood were carefully layered over 15ml Ficol Pacque PLUS (GE Life Sciences cat# 17-1440-02; Pittsburgh, PA). Tubes were centrifuged at 300g for 40 minutes with no brake. Following centrifugation most of the plasma layer was carefully aspirated and the buffy coat (containing PBMC) was carefully removed with a pipet in the smallest possible volume. PBMCs were pooled in 50ml tubes and PBS added to bring each tube up to 50ml. Tubes were centrifuged at 1300RPM for 10 minutes and the supernatant was carefully aspirated. Cells were resuspended in 40ml PBS and centrifuged again. The process was repeated for a total of 2 washes. Following the final wash, cells were resuspended in 30ml RPMI-1630 + 10% FBS and incubated overnight at 37°C, 5% CO_2.

Target cells tested were the head and neck squamous cell carcinoma line, FaDu (ATCC HTB-43, Manassus, VA) and the non-small cell lung adenocarcinoma

cell line, NCI-H1975 (ATCC CRL-5908, Manassus, VA). Target cells were labeled with calcein as follows. Cells were grown as monolayers and were detached by incubation with accutase. Cells were washed twice in RPMI with no serum. 1ml of cells at $4x10^6$ cells/ml was mixed with 1ml RPMI (no serum) + 20µM calcein AM (Sigma cat# C1359; St. Louis, MO). Cells were incubated at 37°C for 30minutes, with gentle mixing every 10 minutes. Following labeling, cells were washed twice with 14ml RPMI + 10% FBS + 2.5mM probenecid (assay medium). Probenecid (Sigma cat# P8761; St. Louis, MO) is an anionic transporter inhibitor and is known to reduce spontaneous release of intracellular calcein. Cells were resuspended in 20ml assay medium and allowed to recover for 2 hours at 37°C, 5% CO_2. Cells were then washed once with assay medium and diluted to 200,000 cells/ml. Aliquots of 50µl (10,000 cells) calcein-labeled cells were aliquoted to 96-well round-bottom plates. 50µl of antibody (at 3X final concentration) was added to cells and allowed to bind for 40 minutes on ice. PBMCs from the previous day were centrifuged at 300g for 5 minutes, resuspended in 20ml fresh assay medium, counted, and diluted to $6x10^6$ cells/ml. 50µl PBMC (300,000) were added to each well and plates incubated at 37°C, 5% CO_2 for 4 hours. Each antibody was titrated in triplicate via 10-fold serial dilutions, starting at 50nM and going down to 0.00005nM. Control wells were also set up containing labeled target cells in the absence of antibody and effector cells in order to measure maximal and spontaneous calcein release.

At the end of the 4-hour incubation, 50µl of assay medium containing 8% IGEPAL CA-630 (Sigma cat# I8896; St. Louis, MO) was added to control wells containing labeled target cells only (to measure the maximal calcein release). 50µl of assay medium was added to all the other wells to bring the total volume to 200µl per well. Plates were centrifuged at 2000RPM for 10 minutes and 150µl supernatant was carefully transferred to V-bottom 96-well plates. These plates were centrifuged at 2000RPM for an additional 10 minutes and 100ml supernatant was carefully transferred to black, clear-bottom 96-well plates.

Calcein in the supernatant was quantitated by measuring the fluorescence of each sample using an excitation wavelength of 485nM and an emission wavelength of 535nM. The percentage of specific lysis was calculated as follows:

% specific lysis = [(test sample value – spontaneous release)/(maximal release – spontaneous release)]*100

The data is shown in FIG. 22 and FIG. 23. For both cell lines, SI-1X6.4 mediated cellular cytotoxicity, but was not particularly more effective than the control antibodies, SI-1C6.2, SI-1C7, or the combination of SI-1C6.2 + SI-1C7. SI-1X6.4 did mediate cytotoxicity with a lower EC50 than our benchmark antibody, SI-1C4. For both cell lines, SI-1X4.2 mediated cellular cytotoxicity at about the same degree as the control antibodies. However, it was not as effective as mediating cellular cytotoxicity as the benchmark, SI-1C4. This is likely due to the lower affinity of SI-1X4.2.

Example 11: Thermal stability of SI-1X bispecific antibodies

Protein Thermal Shift Study was performed for protein thermal stability analysis. Protein melt reactions were set up using Protein Thermal Shift Buffer ™ and the Protein Thermal Shift Dye ™ (Applied Biosystems). In brief, the 20ul reaction mixture contains 5ug protein, 5ul Protein Thermal Shift Buffer ™ and 2.5µ 8X diluted Protein Thermal Shift ™ Dye. For the negative control, PBS was used instead. The reaction mixture was added into MicroAmp Optical Reaction Plate and sealed with MicroAmp Optical Adhesive Film. Each sample consisted of 4 repeats. The protein melt reactions were run on Applied Biosystem Real-Time PCR System from 25 – 90 °C in 1% increment and then analyzed by Protein Thermal Shift Software ™. FIG. 24 shows the thermal curve of SI-1X2, SI-1X4.2, SI-1X6.4, SI-1C3, SI-1C3, SI-1C6.2, SI-1C5.2 and SI-1C7. TABLE 5 shows Tm for these molecules. Tm is defined as the temperature needed to unfold 50% of the protein. The bispecific molecules, 1X2, 1X4.2 and 1X6 all have Tm around 66 °C which are comparable to all the MAbs (1C3, 1C6.2, 1C5.2) and the Fc-scFv (1C7) molecules.

TABLE 5

Protein Name	Tm (°C)
SI-1X2	66.52
SI-1C3	70.06
SI-1X4.2	66.94
SI-1C5.2	70.26
SI-1X6.4	66.50
SI-1C6.2	70.12
SI-1C7	66.40

Example 12: Serum stability of SI-1X bispecific antibodies

Serum stability of the molecules SI-1C5.2, SI-1C6.2, SI-1X4.2, and SI-1X6.4 was determined by comparative binding to monomeric EGFR ECD by ELISA after incubation at 100µg/mL in 95% human serum (Atlanta Biologics, S40110) at 37°C for Days 0, 3, and 7 time points with an extra time point of 55°C on Day 7 to provide a known condition where degradation occurs. ELISA plates were coated with monomeric EGFR ECD (SI-2R4) at 3µg/mL in PBS at 4°C overnight. Coated ELISA plates were blocked with 3% BSA PBST for 2 hours at 25°C and then washed 3 times with PBST. SI-1C6.2 and SI-1X6.4 were diluted 1:10 with 1% BSA PBST and diluted 4x across the plate. SI-1C5.2 and SI-1X4.2 were diluted 1:2 with 1% BSA PBST and diluted 4x across the plate and incubated at 25°C for 1 hour. 3 more washes with PBST were performed before antigen capture with 1µg/mL Her3 ECD Rabbit IgG1 (SI-1R1) for 1 hour at 25°C in 1% BSA PBST. 3 more washes with PBST were performed before goat anti-rabbit IgG-HRP (Bio-Rad 172-1019) secondary antibody was applied at 1:5000 dilution in 1% BSA PBST at 25°C for 1 hour. 3 final washes with PBST before development with 100µl Pierce 1-step Ultra TMB ELISA (Pierce, 34028) for 10 minutes with a final quench of 100µl 2M H_2S0_4. Plates were read at 450nm. ELISA data was plotted and curves created using GraphPad Prism 6.

Results of the ELISA are reported by EC50 on FIG. 25 and indicate a favorable profile of minor degradation when held at 37°C. When placed in 55°C, the EC50 shifts roughly a log as the molecules are subjected to degradation conditions. EC50 values for SI-1C5.2 shift from 589.7 pM on Day 0 to 755.2 pM on Day 7 at 37°C (Δ165.5 pM) with a shift to 6.522 nM on Day 7 at 55°C (Δ5932.3 pM). EC50 values for SI-1C6 shift from 218.2 pM on Day 0 to 226.6 pM on Day 7 at 37°C (Δ8.4 pM) with a shift to 1.322 nM on Day 7 at 55°C (Δ1103 pM). EC50 values for SI-1X4.2 shift from 429.3 pM on Day 0 to 466.7 pM on Day 7 at 37°C (Δ37.4 pM) with a shift to 4.248 nM on Day 7 at 55°C (Δ3818.7 pM). EC50 values for SI-1X6 shift from 209.3 pM on Day 0 to 237.3 pM on Day 7 at 37°C (Δ28 pM) with a shift to 4.112 nM on Day 7 at 55°C (Δ3902.7 pM).

Example 13: PK half-life of SI-1X molecules

To test their half-life in vivo, pharmacokinetic experiments were performed in SD rats. A single, intravenous tail vein injection of bispecific Abs (1C6 10mg/kg, 1X6 10mg/kg, 1X2 10mg/kg, 1X4 32mg/kg) were given to groups of 4 female rats randomized by body weight (190-212g range). Blood (~150µL) was drawn from the orbital plexus at each time point, processed for serum, and stored at -80℃ until analysis. Study durations were 28 days.

Antibody concentrations were determined using three ELISA assays. In assay 1 (EGFR ECD coated ELISA), recombinant EGFR-rabbit Fc was coated to the plate, wells were washed with PBST (phosphate buffered saline with 0.05% Tween) and blocked with 1% BSA in PBST. Serum or serum diluted standards were then added, followed by PBST washing, addition of HRP labeled rabbit-anti-human IgG (BOSTER), and additional PBST washing. TMB was then added and the plates were incubated 2.5 minutes in the dark. Color reaction was stopped by adding 2M sulfuric acid. Plate was read at 450nm wavelength. For assay 2 (Her3 coated ELISA), serum was detected using a similar ELISA , but recombinant HER3-His was used as capture reagent. For assay 3 (Sandwich

ELISA), recombinant HER3-His was coated, serum or serum diluted standard were added, followed by PBST washing, addition of EGFR-rabbit Fc in PBST, and additional PBST washing. HRP labeled goat-anti-rabbit IgG (BOSTER) was then added. PK parameters were determined with a non-compartmental model.

FIGS 26-28 show serum concentration data for four antibodies with three different assays respectively. Fitted PK parameters from in vivo PK studies are provided in TABLE 6. PK data include half-life, which represents the beta phase that characterizes elimination of antibody from serum and Cmax, which represents the maximal observed serum concentration, AUC, which represents the area under the concentration time curve.

TABLE 6

Assay	Sample	Half-Life (h)	Cmax (μg/ml)	AUC (μg/ml*h)
EGFR Coated ELISA	SI-1X6	159	326.5	18250.6
	SI-1X2	130	280.3	18889.8
	SI-1X4.2	146	627.8	31317.0
	SI-1C6	130	196.4	3790.3
Her3 Coated ELISA	SI-1X6	142	236.7	14213.6
	SI-1X2	136	264.8	19012.2
	SI-1X4.2	124	715.6	40063.4
Sandwich ELISA	SI-1X6	136	301.6	14182.6
	SI-1X2	123	297.6	17203.9
	SI-1X4.2	211	518.9	34874.6

Example 14: Mouse Xenograft studies

The example tested the activity of SI-1X2, SI-1X4.2 and SI-1X6 of concomitant blockade of EGFR, HER3 in preclinical models of Fadu (head and neck squamous cell carcinoma xenograft model) and compared their potency with cetuximab and cetuximab in combination with an anti-HER3 antibody.

All mouse studies were conducted through Institutional Animal care and

used committee-approved animal protocols in accordance with institutional guidelines. Six-week-old female Balb/c Nude mice were purchased from Beijing Vital River Laboratories and housed in air-filtered laminar flow cabinets with a 12 - hour light cycle and food and water ad libitum. The size of the animal groups was calculated to measure means difference between placebo and treatment groups of 25% with a power of 80% and a P value of 0.01. Host mice carrying xenografts were randomly and equally assigned to either control or treatment groups. Animal experiments were conducted in a controlled and non-blinded manner. For cell line–derived xenograft studies, mice were injected subcutaneously with 2×106 Fadu suspended 150µl of culture medium per mouse.

Once tumors reached an average volume of 100-250mm3, mice were randomized into 9 groups, with 6 mice per group. Vehicle Control, 1C6 (25mg/kg), 1C4 (25mg/kg), 1C6 + 1C1 (25mg/kg+50mg/kg), SI-1X2 (25mg/kg), SI-1X6 (10mg/kg), SI-1X6 (25mg/kg), and SI-1X4.2 (10mg/kg) , SI-1X4 (25mg/kg) . All test articles were administered once weekly via intravenous injection. Tumors were measured by digital caliper over the entire treatment period every 3 days and the volume was determined using the following formula: 1/2×lenth×width2. The body weight of mice were recorded before the first dose and followed by every week during the treatment period and recovery period.

All the test groups of SI-1X2, SI-1X6 and SI-1X4.2 and SI-1X6 combination yielded significantly tumor growth inhibition compared to positive control of SI-1C6 excluding the low dose SI-1X4.2 10mg/kg group (FIGS 29-30). Moreover, no relapses were observed 2 weeks after treatment cessation excluding the low dose SI-1X4.2 10mg/kg group.

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 overall 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 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.

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 member of the EGFR family;

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 $(gly\text{-}gly\text{-}gly\text{-}gly\text{-}ser)_n$, to provide a IgG1-connector connection, wherein n is an integral of at least 1; 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 $(gly\text{-}gly\text{-}gly\text{-}gly\text{-}ser)_m$, wherein m is an integral of at least 3.

2. The bispecific tetravalent antibody of Claim 1, wherein n is an integral between 1 to 10.

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

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

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

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

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

8. The bispecific tetravalent antibody of Claim 1, wherein the first or the second member of the EGFR family comprises HER3, EGFR, a fragment or a derivative thereof.

9. The bispecific tetravalent antibody of Claim 1, wherein the first or the second member of the EGFR family is independently selected from a group consisting of HER3, EGFR, a fragment or a derivative thereof.

10. The bispecific tetravalent antibody of claim 1, wherein the IgG moiety has a binding specificity for HER3.

11. The bispecific tetravalent antibody of claim 1, wherein the scFv domains have a binding specificity for EGFR.

12. The bispecific tetravalent antibody of claim 1, wherein the IgG moiety has a binding specificity for HER3 and the scFv domains have a binding specificity for EGFR simultaneously.

13. The bispecific tetravalent antibody of claim 1, wherein the IgG moiety has a binding specificity for EGFR,

14. The bispecific tetravalent antibody of claim 1, wherein the scFv domains have a binding specificity for HER3.

15. The bispecific tetravalent antibody of claim 1, wherein the IgG moiety has a binding specificity for EGFR and the scFv domains have a binding specificity for HER3 simultaneously.

16. The bispecific tetravalent antibody of Claim 1, wherein the C terminus of at least one of the IgG1 heavy chains misses an amino acid residue.

17. The bispecific tetravalent antibody of Claim 16, wherein the amino acid residue is a lysine.

18. The bispecific tetravalent antibody of Claim 1, wherein the IgG1-connector connection is resistant to protease activity.

19. The bispecific tetravalent antibody of Claim 1, wherein at least one of the IgG1 heavy chains comprises two mutations in the CH3 domain, and wherein the two mutations are reversion to the common residues in human CH3 domain.

20. The bispecific tetravalent antibody of Claim 1, wherein at least one of the IgG1 heavy chains comprises an amino acid comprising SEQ ID NO 7, 15, 23, 31, 39, 47, and 127.

21. The bispecific tetravalent antibody of Claim 1, wherein the IgG1 heavy chain, connector, and scFv domain have an amino acid comprising SEQ ID NO 56, 66, 76, 86, 98, 108, 118, and 136.

22. The bispecific tetravalent antibody of Claim 1, wherein at least one of the kappa light chains comprises an amino acid sequence comprising SEQ ID NO 3, 11, 19, 27, 35, 43, 51, 61, 71, 81, 92, 103, 113, 123, and 131.

23. The bispecific tetravalent antibody of Claim 1, wherein at least one of variable light chain comprises an amino acid sequence comprising SEQ ID NO 4, 12, 20, 28, 36, 44, 52, 62, 72, 82, 93, 104, 114, 124, and 132.

24. The bispecific tetravalent antibody of Claim 1, wherein at least one of variable heavy chain comprises an amino acid sequence comprising SEQ ID NO 8, 16, 24, 32, 40, 48, 57, 67, 77, 87, 99, 109, 119, 128, and 137.

25. The bispecific tetravalent antibody of claim 1, wherein the IgG moiety has a binding specificity for HER3, and the scFv domains have a binding specificity for EGFR;

wherein the IgG1 heavy chain, connector, and scFv domain have an amino acid sequence of SEQ ID NO 56, and the kappa light chain has an amino acid sequence of SEQ ID NO 51.

26. The bispecific tetravalent antibody of claim 1, wherein the IgG moiety has a binding specificity for HER3, and the scFv domains have a binding specificity for EGFR;

wherein the IgG1 heavy chain, connector, and scFv domain have an amino acid sequence of SEQ ID NO 76, and the kappa light chain has an amino acid sequence of SEQ ID NO 71.

27. The bispecific tetravalent antibody of claim 1, wherein the IgG moiety has a binding specificity for HER3, and the scFv domains have a binding specificity for EGFR;

wherein the IgG1 heavy chain, connector, and scFv domain have an amino acid sequence of SEQ ID NO 108, and the kappa light chain has an amino acid sequence of SEQ ID NO 103.

28. The bispecific tetravalent antibody of claim 1, wherein the IgG moiety has a binding specificity for EGFR, and the scFv domains have a binding specificity for HER3;

wherein the IgG1 heavy chain, connector, and scFv domain have an amino acid sequence of SEQ ID NO 66, and the kappa light chain has an amino acid sequence of SEQ ID NO 61.

29. The bispecific tetravalent antibody of claim 1, wherein the IgG moiety has a binding specificity for EGFR, and the scFv domains have a binding specificity for HER3;

wherein the IgG1 heavy chain, connector, and scFv domain have an amino acid sequence of SEQ ID NO 86, and the kappa light chain has an amino acid sequence of SEQ ID NO 81.

30. The bispecific tetravalent antibody of claim 1, wherein the IgG moiety has a binding specificity for EGFR, and the scFv domains have a binding specificity for HER3;

wherein the IgG1 heavy chain, connector, and scFv domain have an amino acid sequence of SEQ ID NO 98, and the kappa light chain has an amino acid sequence of SEQ ID NO 92.

31. The bispecific tetravalent antibody of claim 1, wherein the IgG moiety has a binding specificity for EGFR, and the scFv domains have a binding specificity for HER3;

wherein the IgG1 heavy chain, connector, and scFv domain have an amino acid sequence of SEQ ID NO 118, and the kappa light chain has an amino acid sequence of SEQ ID NO 113.

32. The bispecific tetravalent antibody of claim 1, wherein the IgG moiety has a binding specificity for EGFR, and the scFv domains have a binding specificity for HER3;

wherein the IgG1 heavy chain, connector, and scFv domain have an amino acid sequence of SEQ ID NO 136, and the kappa light chain has an amino acid sequence of SEQ ID NO 131.

33. The bispecific tetravalent antibody of Claims 1, wherein the antibody inhibits cancer cell growth.

34. The bispecific tetravalent antibody of Claim 1, wherein the antibody binds to EGRF and HERS with a Kd less than 50nM.

35. The bispecific tetravalent antibody of Claim 1, wherein the antibody simultaneously binds to EGRF with a Kd less than 50nM and binds to HER3 with a Kd less than 50nM.

36. An IgG1 heavy chains for the bispecific tetravalent antibody of Claim 1, comprising an amino acid sequences selected from SEQ ID NO 7, 15, 23, 31, 39, 47, and 127

37. A kappa light chain for the bispecific tetravalent antibody of Claim 1, comprising an amino acid sequence selected from SEQ ID NO 3, 11, 19, 27, 35, 43, 51, 61, 71, 81, 92, 103, 113, 123, and 131.

38. A variable light chain for the bispecific tetravalent antibody of Claim 1, comprising an amino acid sequence selected from SEQ ID NO 4, 12, 20, 28, 36, 44, 52, 62, 72, 82, 93, 104, 114, 124, and 132

39. A variable heavy chain for the bispecific tetravalent antibody of Claim 1, comprising an amino acid sequence selected from SEQ ID NO 8, 16, 24, 32, 40, 48, 57, 67, 77, 87, 99, 109, 119, 128, and 137.

40. An isolated nucleic acid encoding the antibody of Claim 1, the IgG1 heavy Chain of Claim 36, the kappa light chain of Claim 37, the variable light chain of Claim 38, or the variable heavy chain 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 43.

45. The host cell of Claim 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 at least two members of EGFR family.

53. The method of Claim 51, wherein the cancer comprises breast cancer, colorectal cancer, pancreatic cancer, head and neck cancer, melanoma, ovarian cancer, prostate cancer, non-small lung cell cancer, glioma, esophageal cancer, nasopharyngeal cancer, anal cancer, rectal cancer, gastric cancer, bladder cancer, cervical cancer, or brain cancer.

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

55. The method of Claim 54, wherein the therapeutic agent comprises an antibody, a chemotherapy agent, an enzyme, or a combination thereof.

56. The method of Claim 54, wherein the therapeutic agent comprises an anti-estrogen agent, a receptor tyrosine inhibitor, or a combination thereof.

57. The method of Claim 54, 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.

58. The method of Claim 54, wherein the therapeutic agent comprises a check point inhibitor.

59. The method of Claim 54, 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.

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

61. A method of inhibiting a biological activity of a HER 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 HER receptor.

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

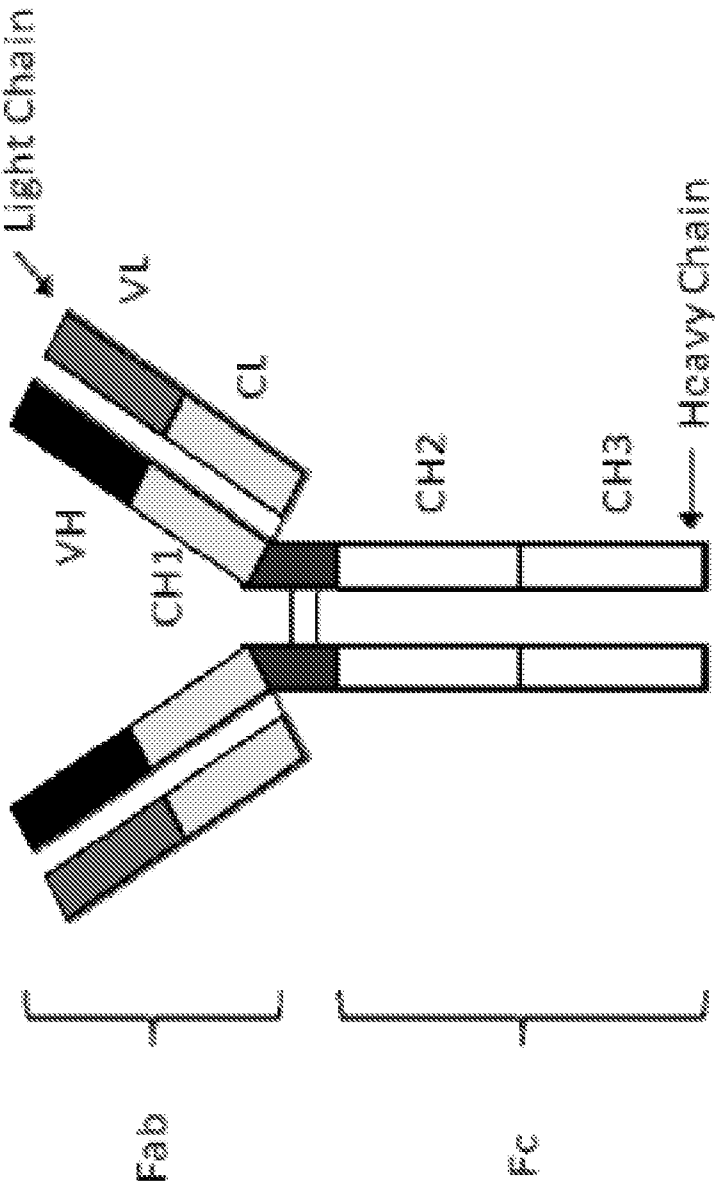


FIG. 1 (Prior Art)

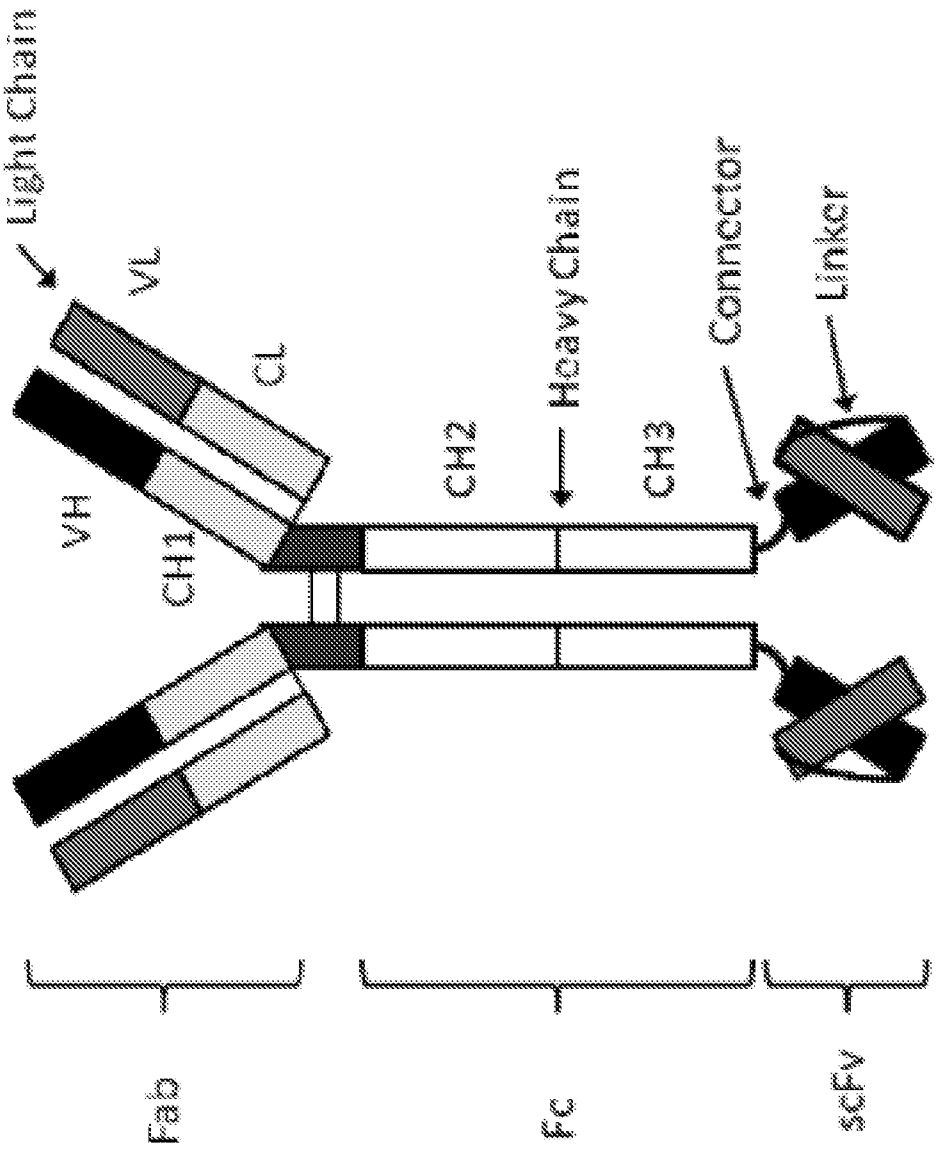

Light Chain
VL
CL
VH
CH1
CH2
Heavy Chain
CH3
Connector
Linker
Fab
Fc
scFv

FIG. 2

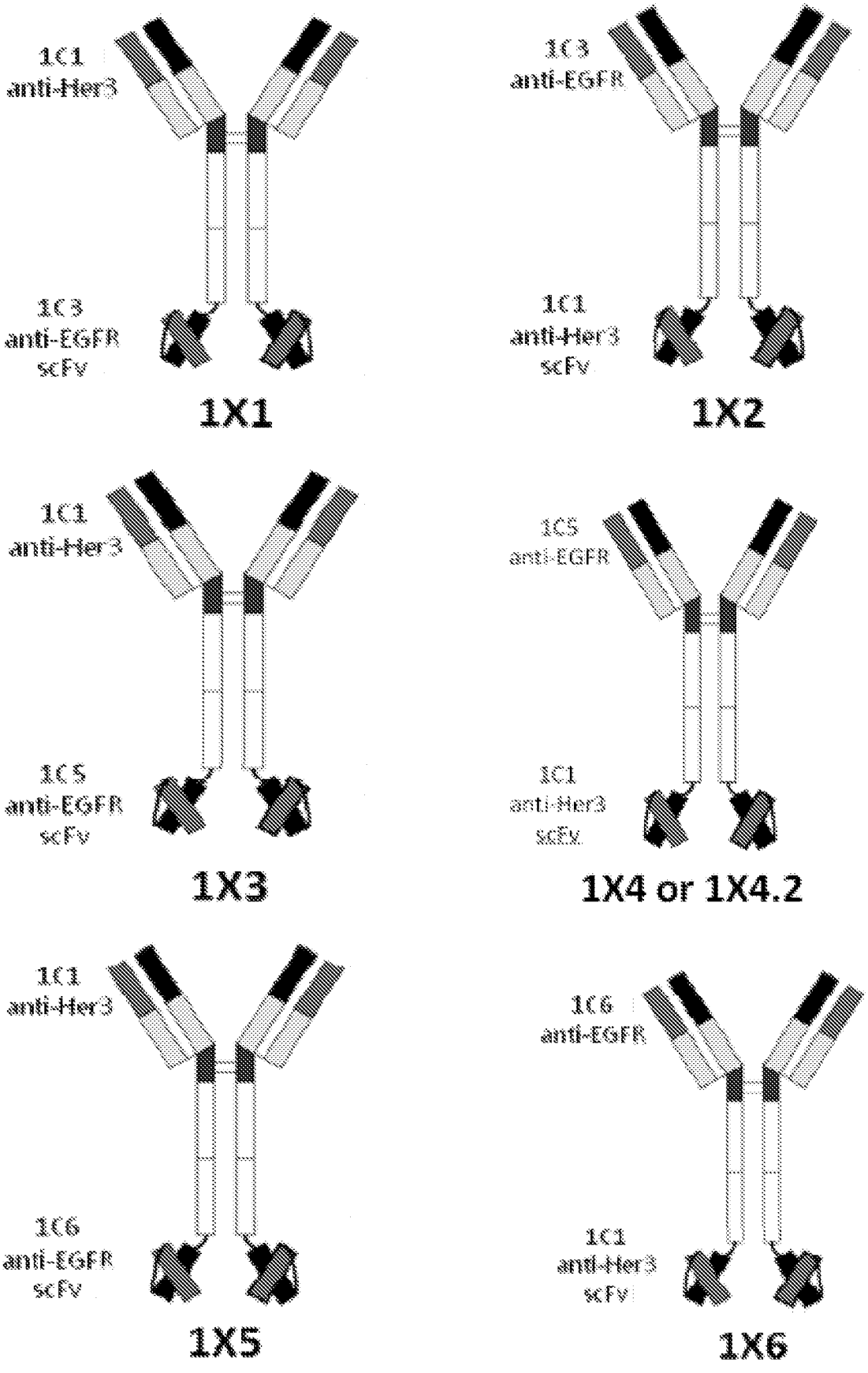

1C1
anti-Her3
1C3
anti-EGFR
scFv
1X1
1C3
anti-EGFR
1C1
anti-Her3
scFv
1X2
1C1
anti-Her3
1C5
anti-EGFR
scFv
1X3
1C5
anti-EGFR
1C1
anti-Her3
scFv
1X4 or 1X4.2
1C1
anti-Her3
1C6
anti-EGFR
scFv
1X5
1C6
anti-EGFR
1C1
anti-Her3
scFv
1X6

FIG. 3

WO 2016/106157

PCT/US2015/066951

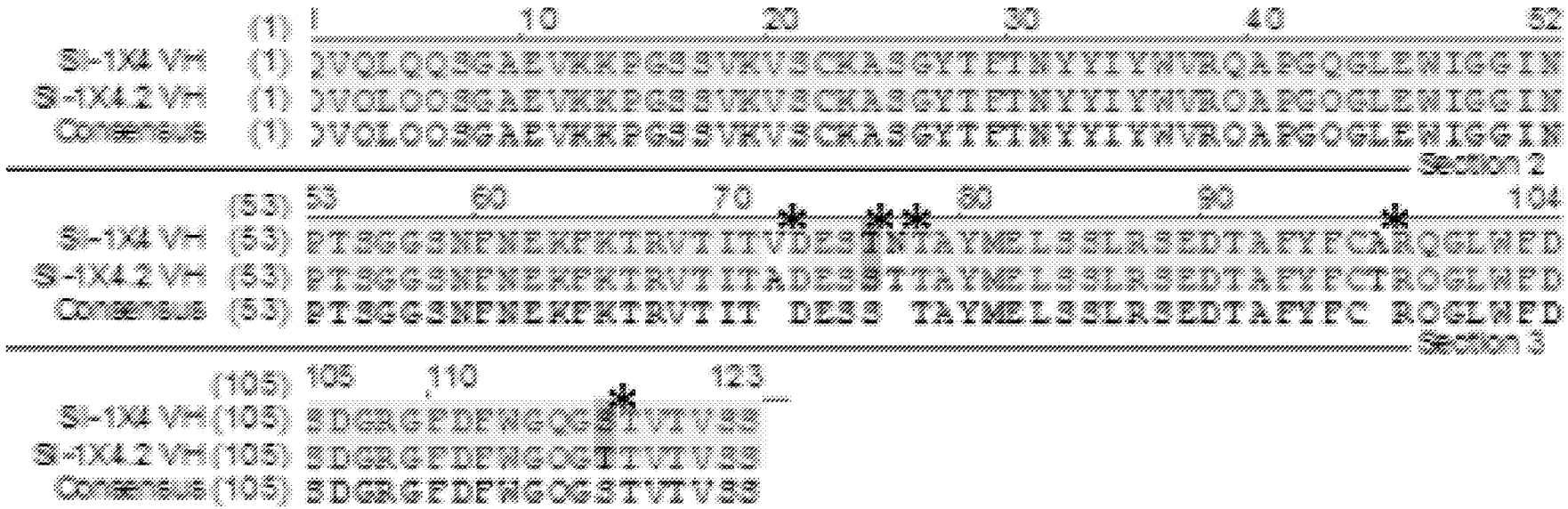


FIG. 4

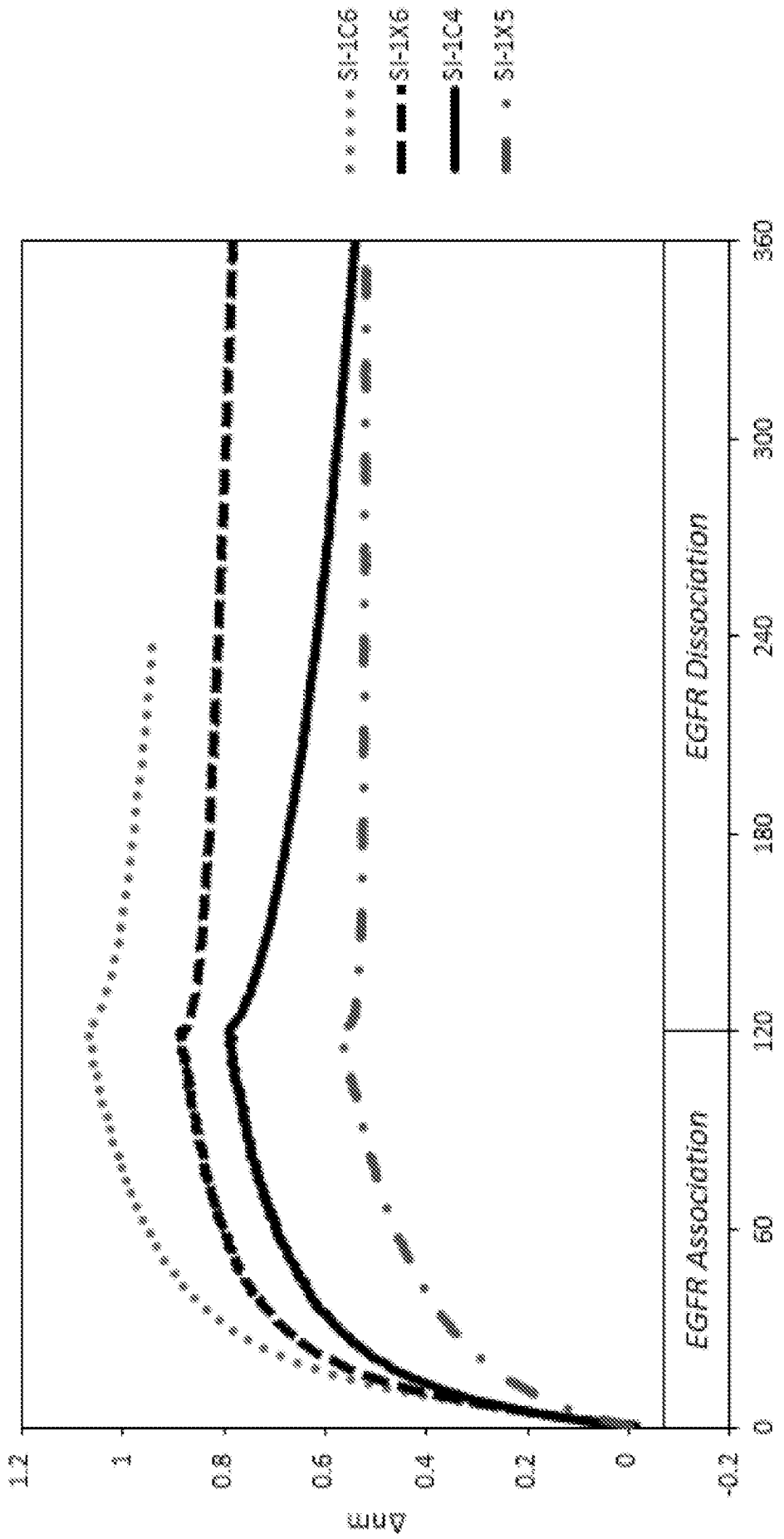

Si-1C6
Si-1X6
Si-1C4
Si-1X5
EGFR Association
EGFR Dissociation
Δnm
Time (s)
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
60
120
180
240
300
360

FIG. 5

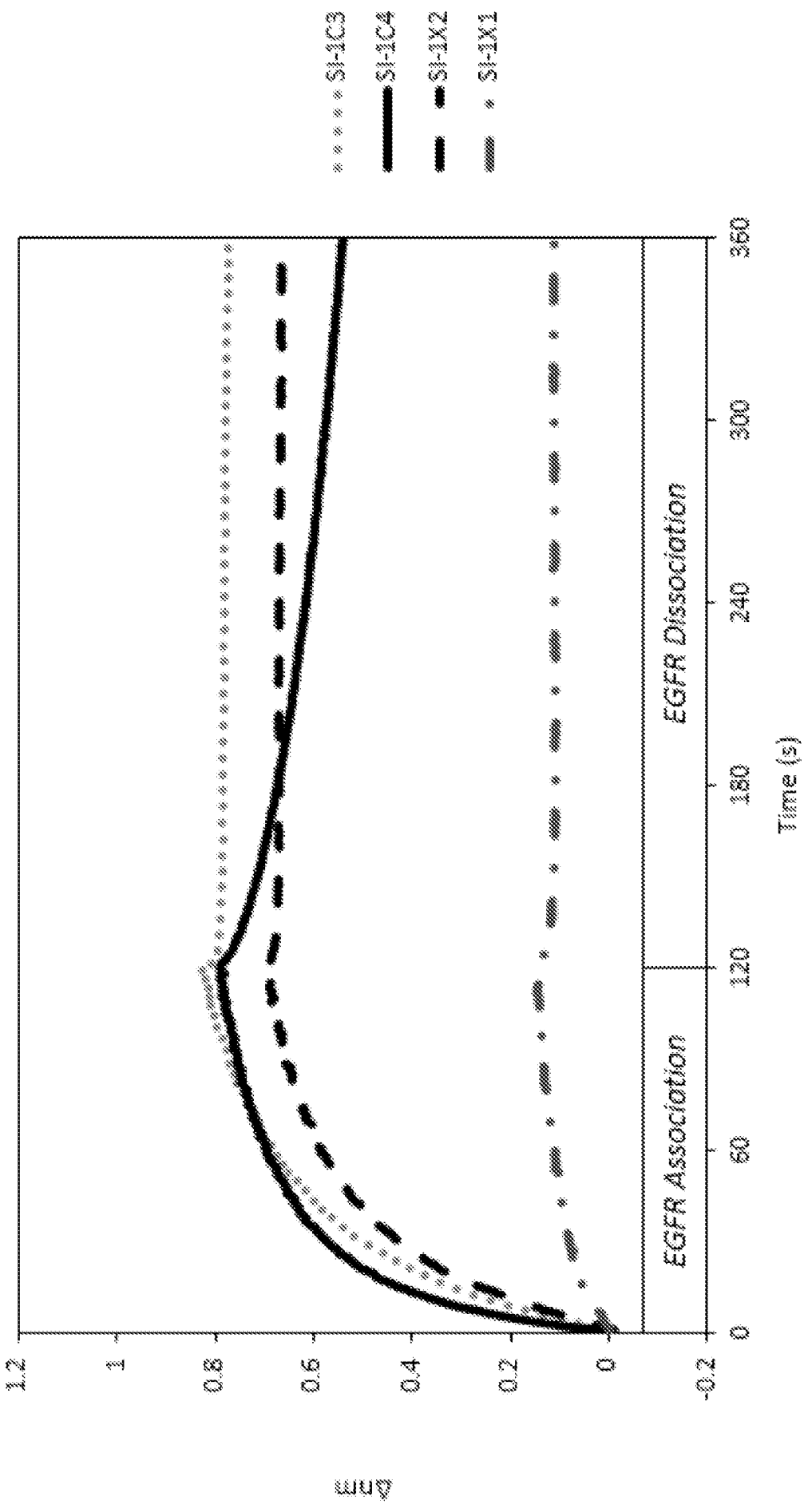

SI-1C3
SI-1C4
SI-1X2
SI-1X1
EGFR Association
EGFR Dissociation
Time (s)
Δnm
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
60
120
180
240
300
360

FIG. 6

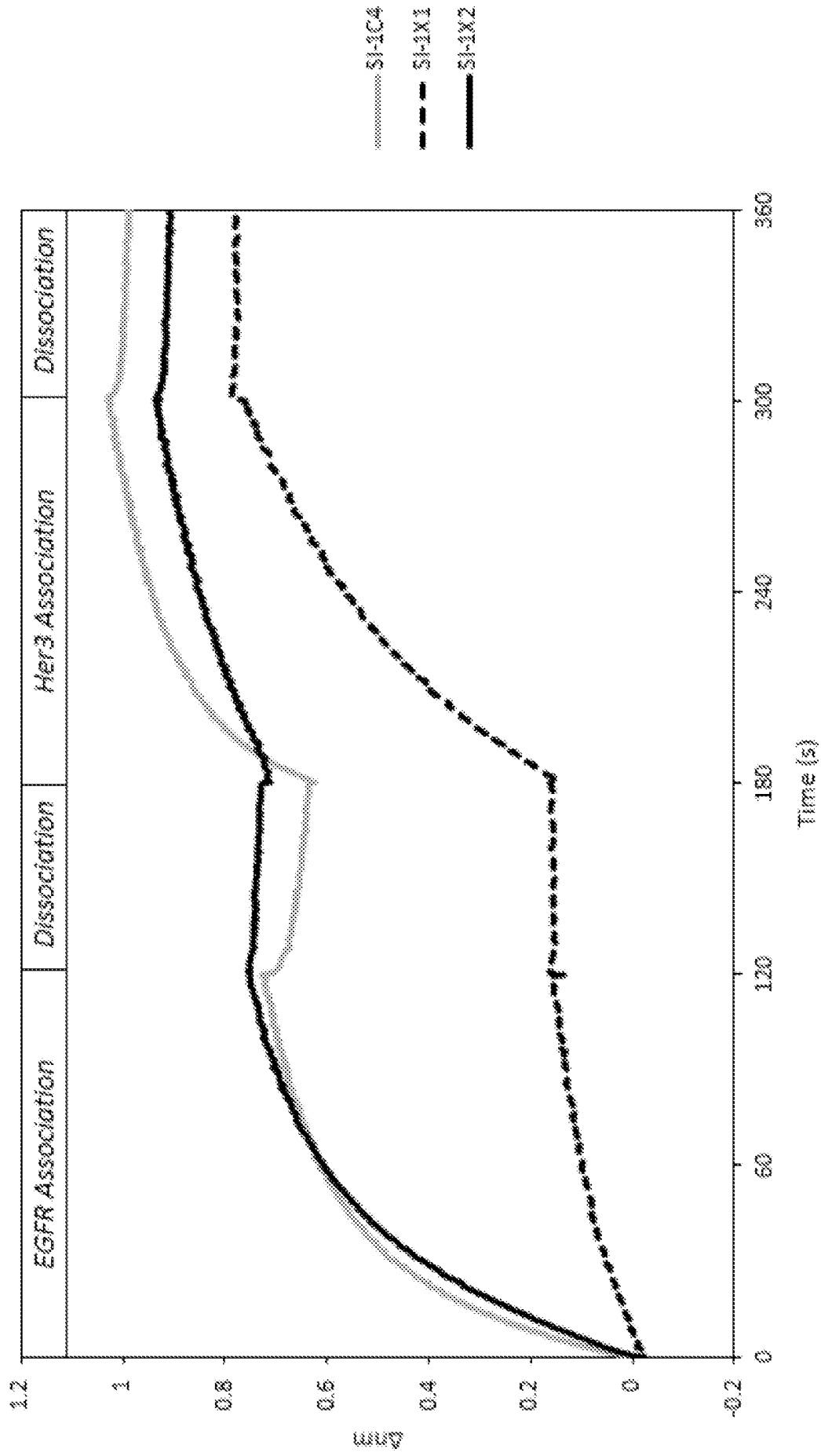

SI-1C4
SI-1X1
SI-1X2
EGFR Association
Dissociation
Her3 Association
Dissociation
nm
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
60
120
180
240
300
360
Time (s)

FIG. 7

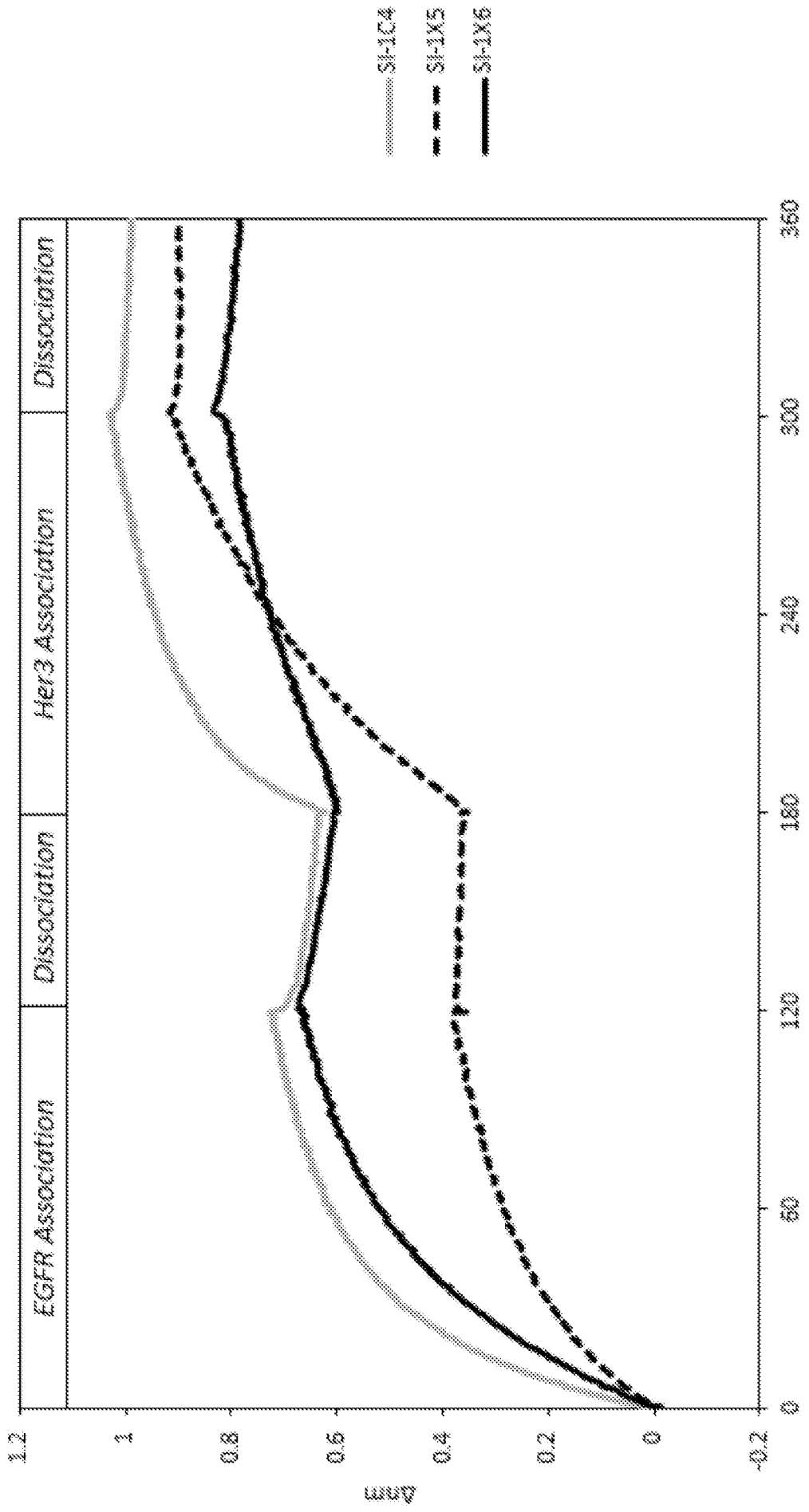

SH-1C4
SH-1X5
SH-1X6
EGFR Association
Dissociation
Her3 Association
Dissociation
nm
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
60
120
180
240
300
360
Time (s)

FIG. 8

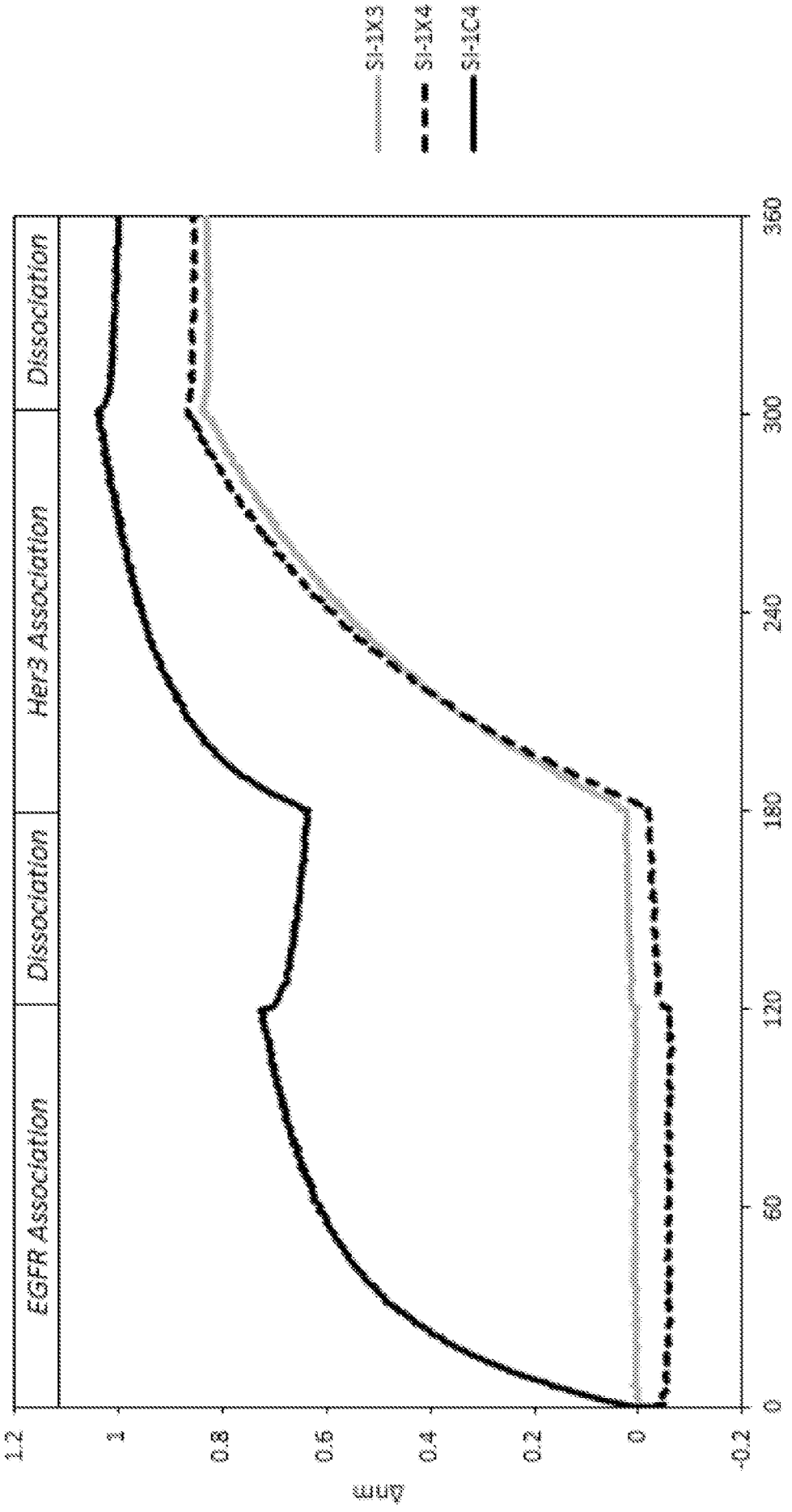

S1-1X3
S1-1X4
S1-1C4
EGFR Association
Dissociation
Her3 Association
Dissociation
dnm
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
60
120
180
240
300
360
Time (s)

FIG. 9

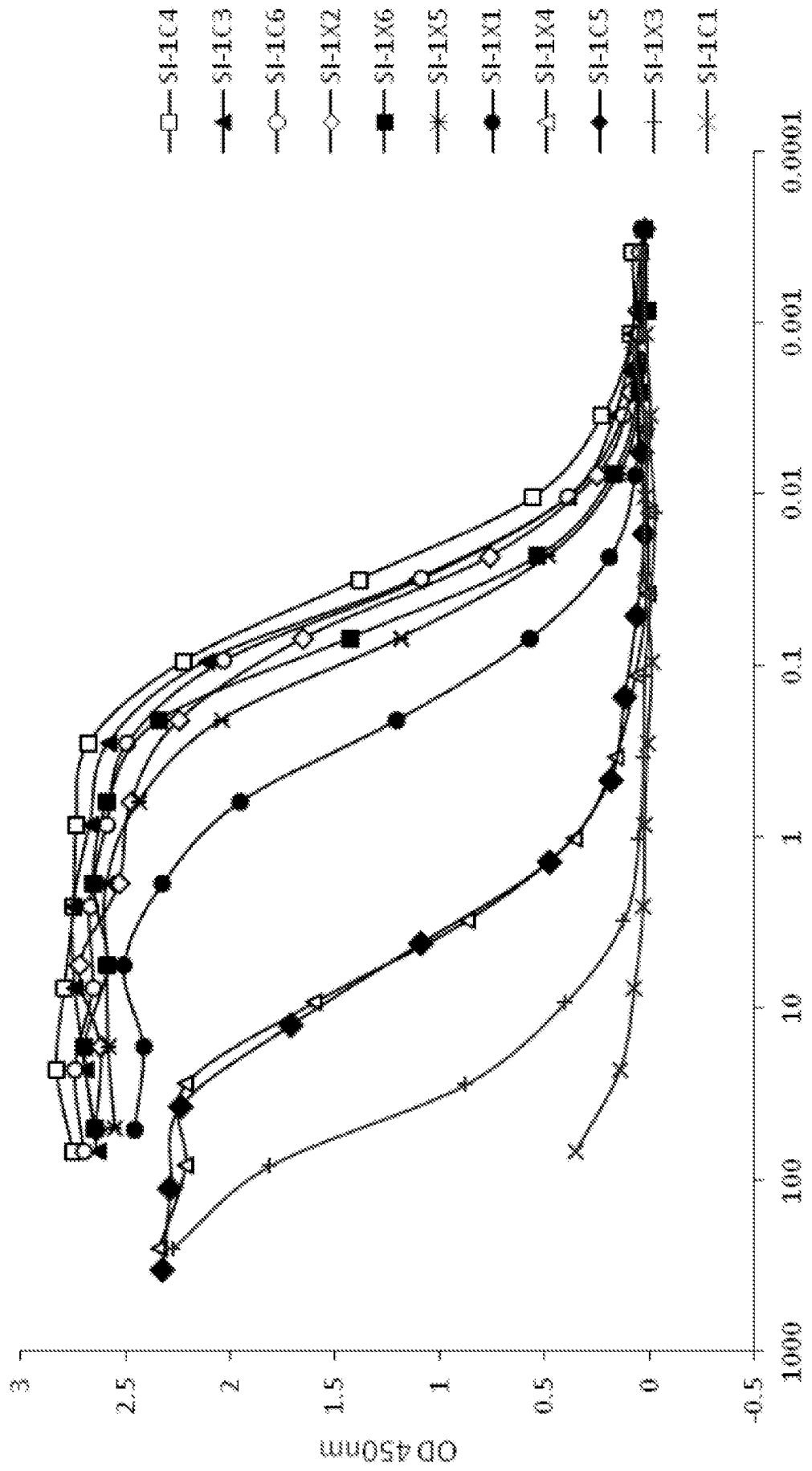

SI-1C4
SI-1C3
SI-1C6
SI-1X2
SI-1X6
SI-1X5
SI-1X1
SI-1X4
SI-1C5
SI-1X3
SI-1C1
OD 450nm
3
2.5
2
1.5
1
0.5
0
-0.5
1000
100
10
1
0.1
0.01
0.001
0.0001
Ab [nM]

FIG. 10

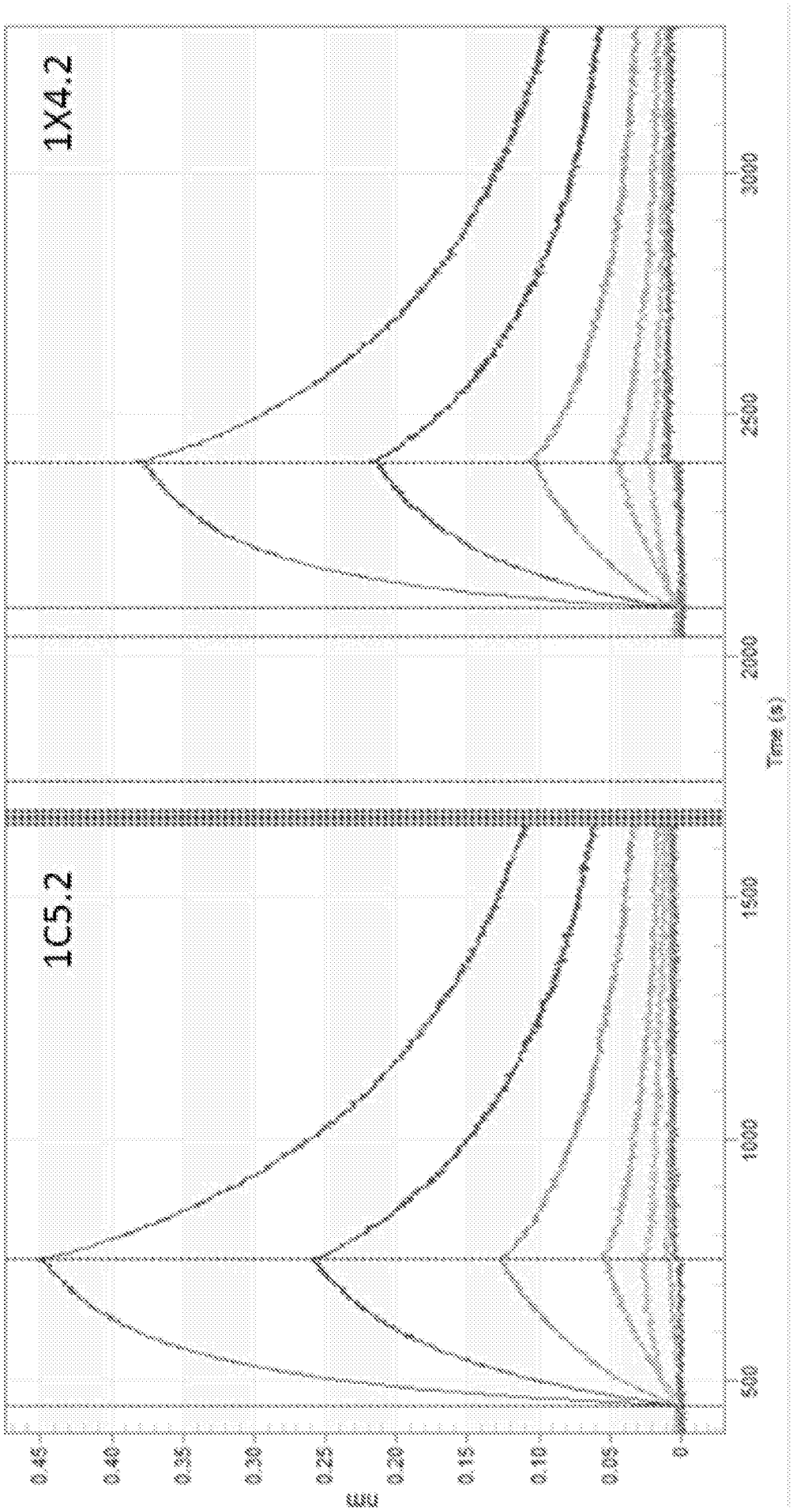

1X4.2
1C5.2
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
500
1000
1500
2000
2500
3000
Time (s)

FIG. 11

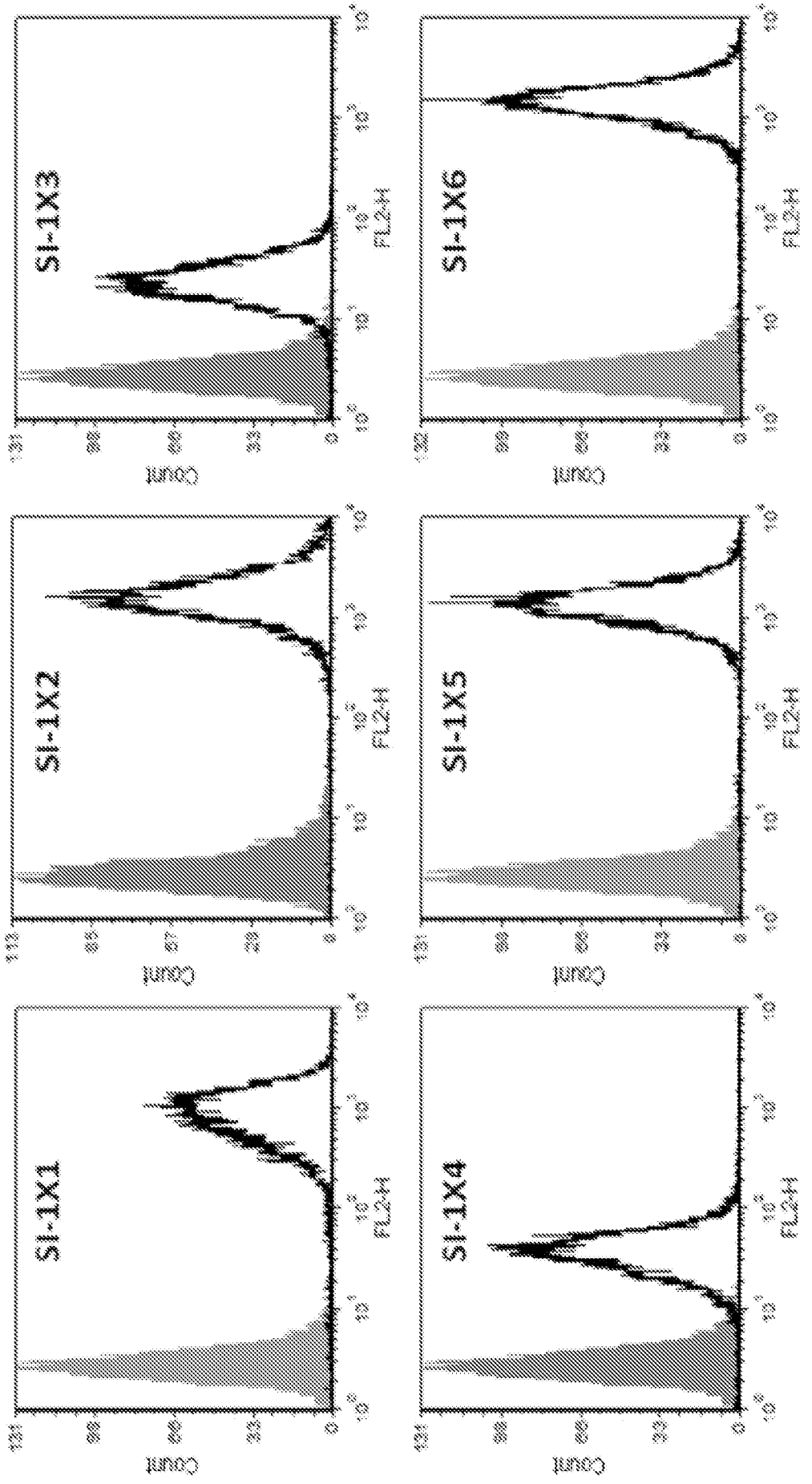

SI-1X1
SI-1X2
SI-1X3
SI-1X4
SI-1X5
SI-1X6
Count
FL2-H
grey, filled histogram = isotype control

FIG. 12

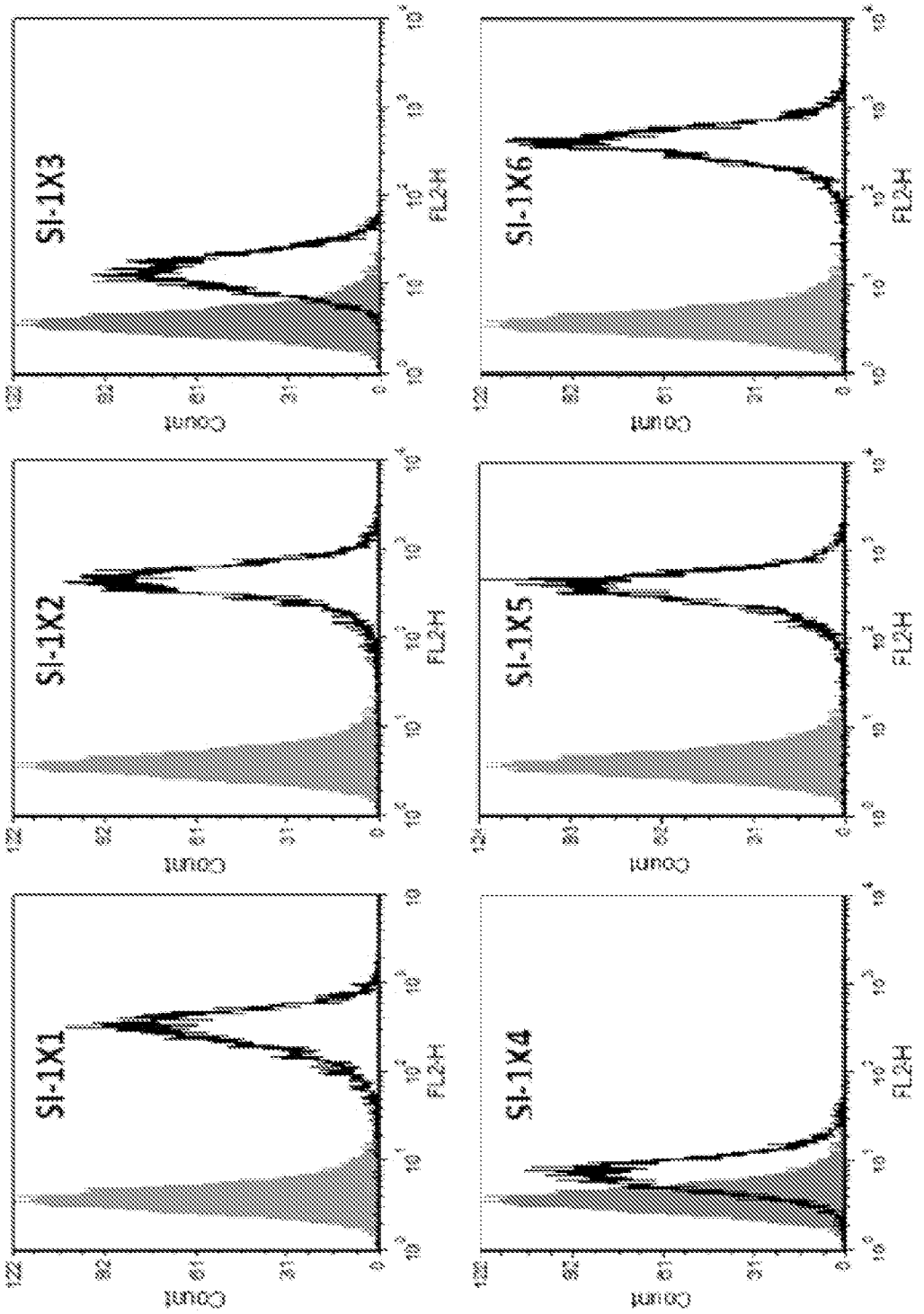
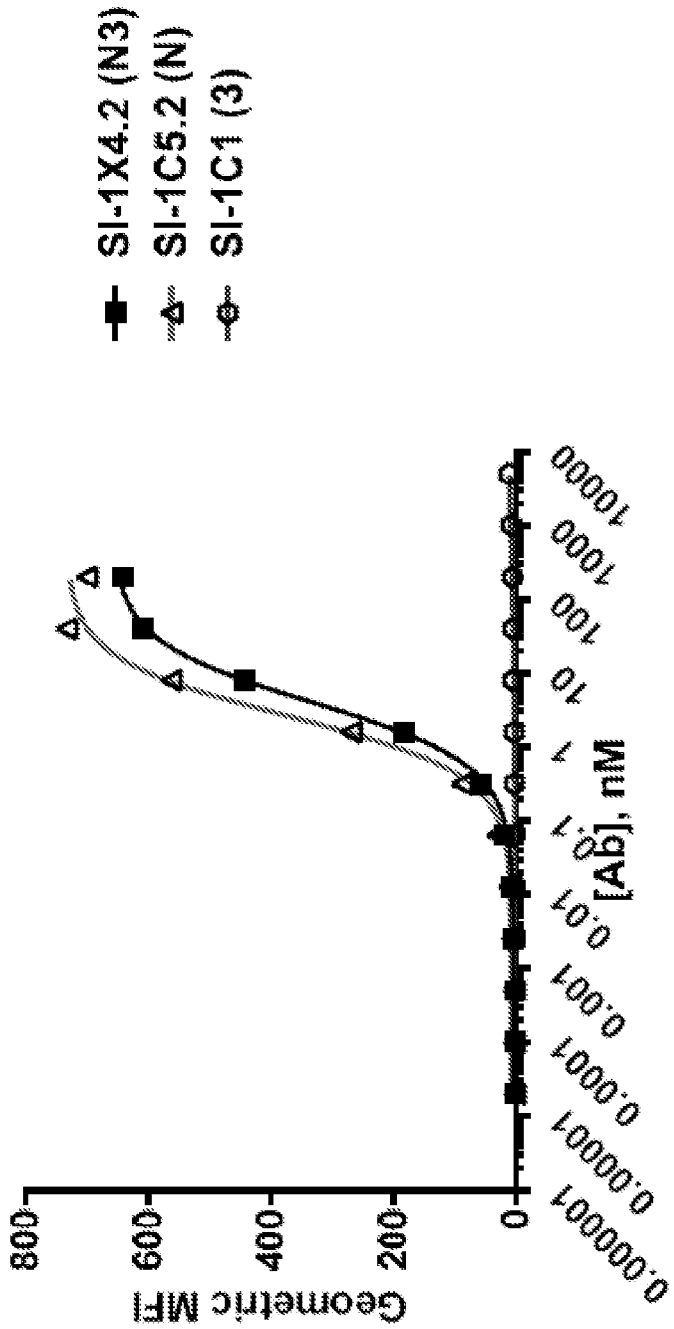

SI-1X1
SI-1X2
SI-1X3
SI-1X4
SI-1X5
SI-1X6
Count
FL2-H
grey, filled histogram = isotype control

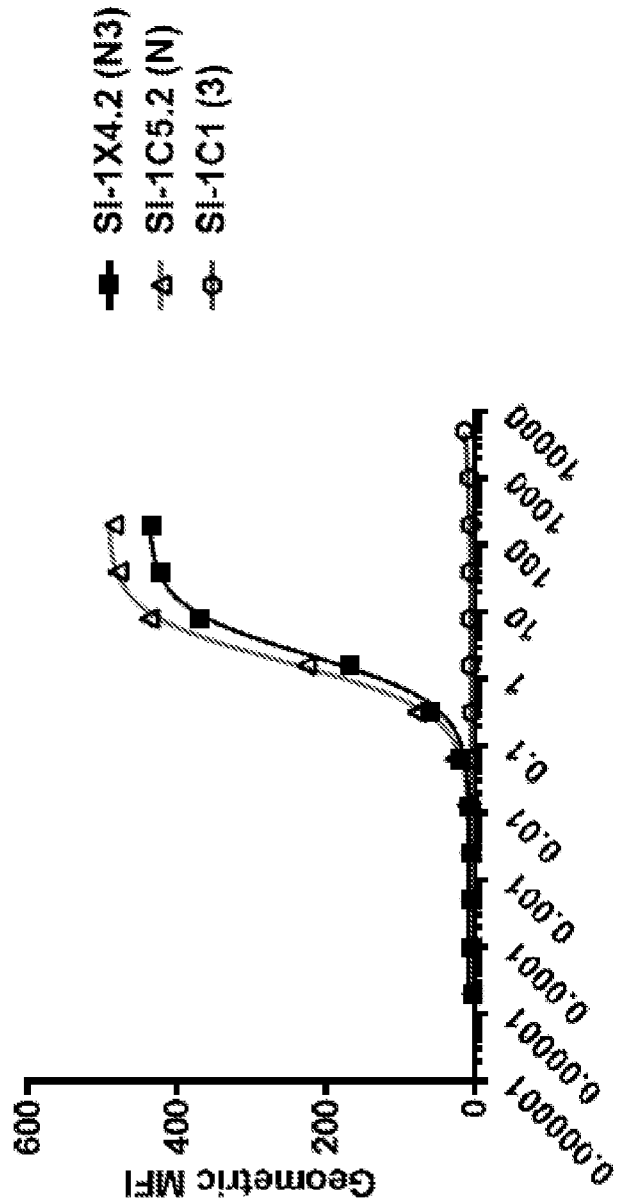
FIG. 13



Antibody	IC50 (nM)
SI-1X4.2 (N3)	4.02
SI-1C5.2 (N)	2.62
SI-1C1 (3)	N/A*

* IC50 not calculated - plateau not reached

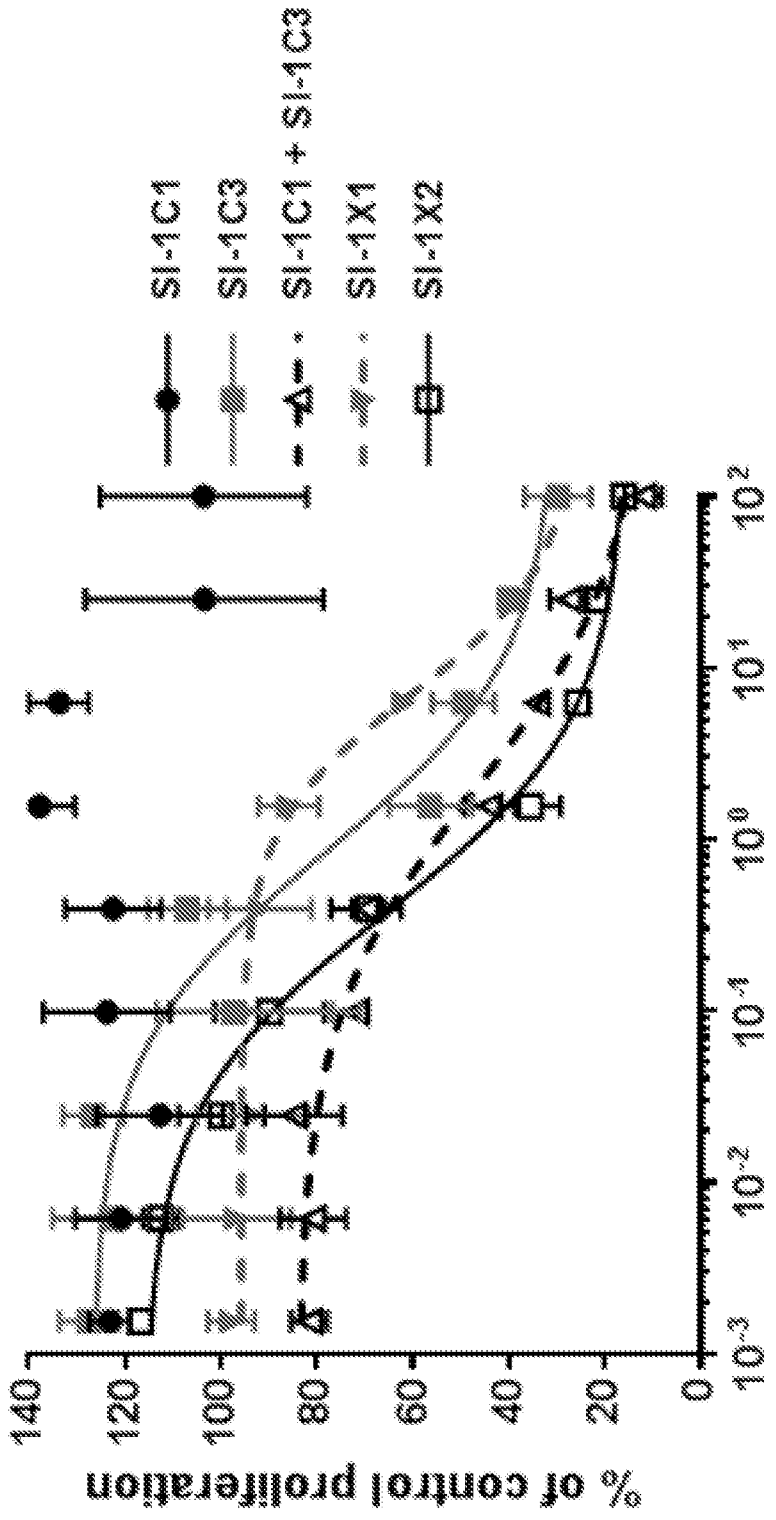
FIG. 14



Antibody	IC50 (nM)
SI-1X4.2 (N3)	2.28
SI-1C5.2 (N)	1.75
SI-1C1 (3)	N/A*

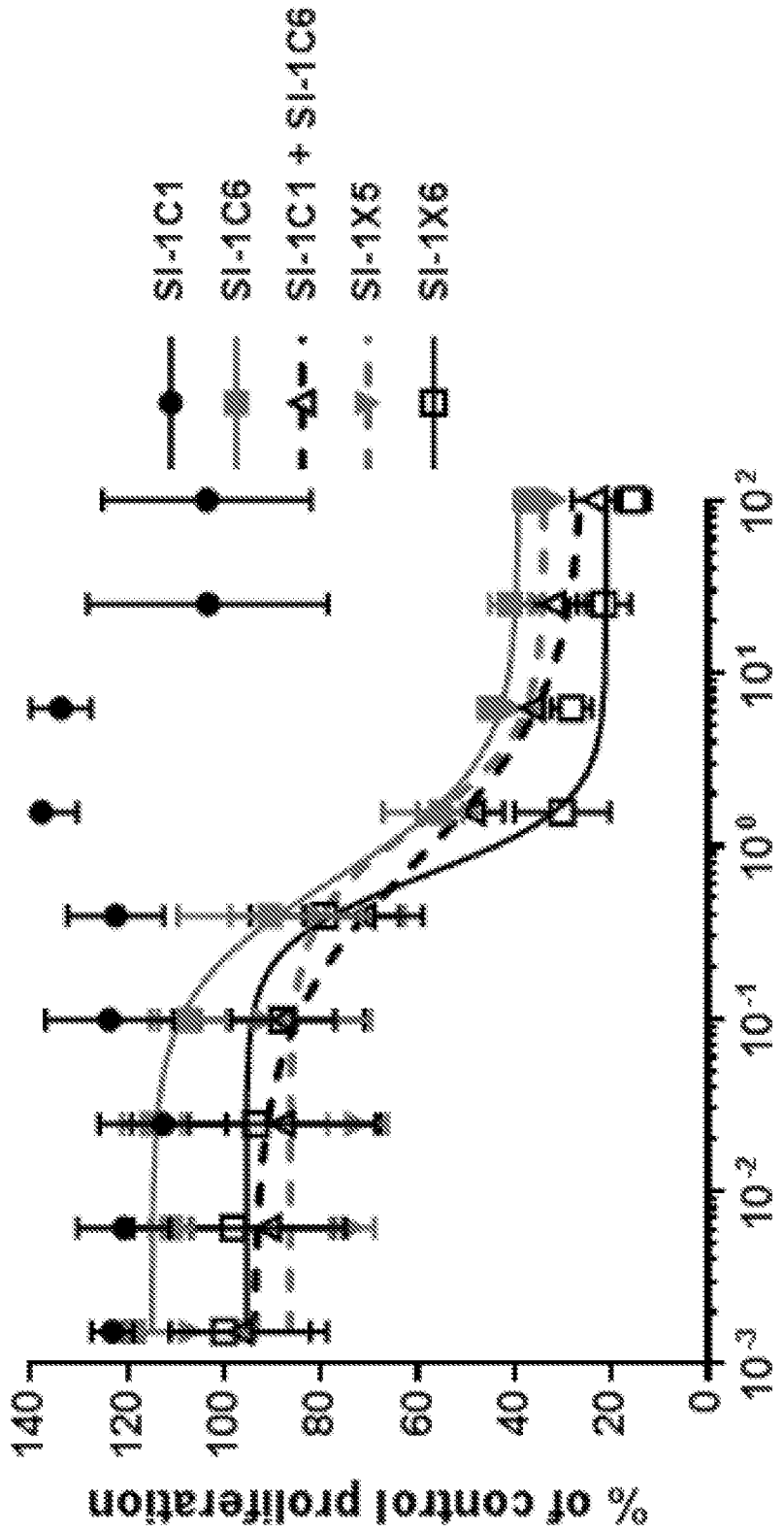
* IC50 not calculated - plateau not reached

FIG. 15



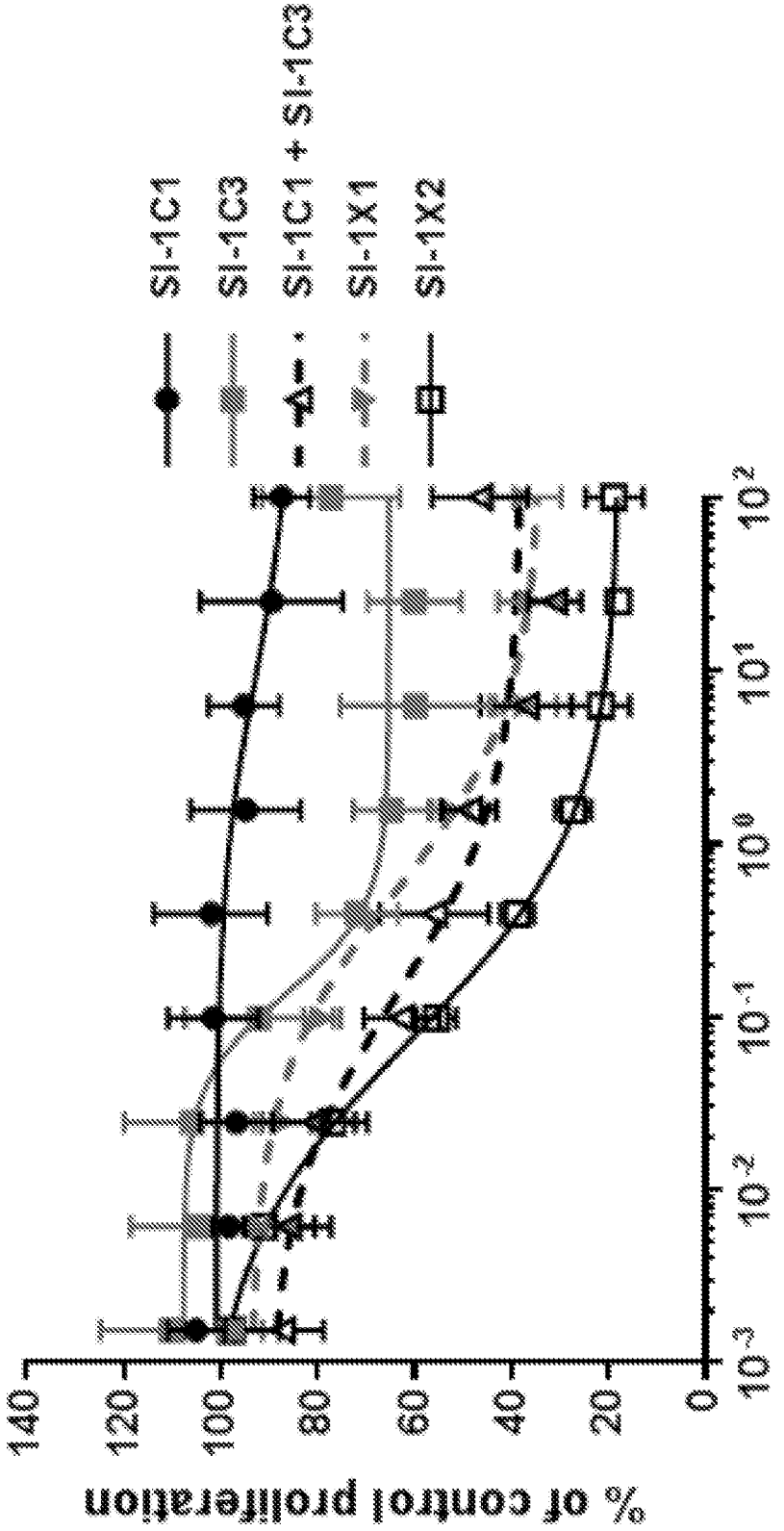
A431 IC50 values (nM)				
SI-1C1	SI-1C3	SI-1C1 + SI-1C3	SI-1X1	SI-1X2
N/A	0.83	1.91	6.84	0.37

FIG. 16



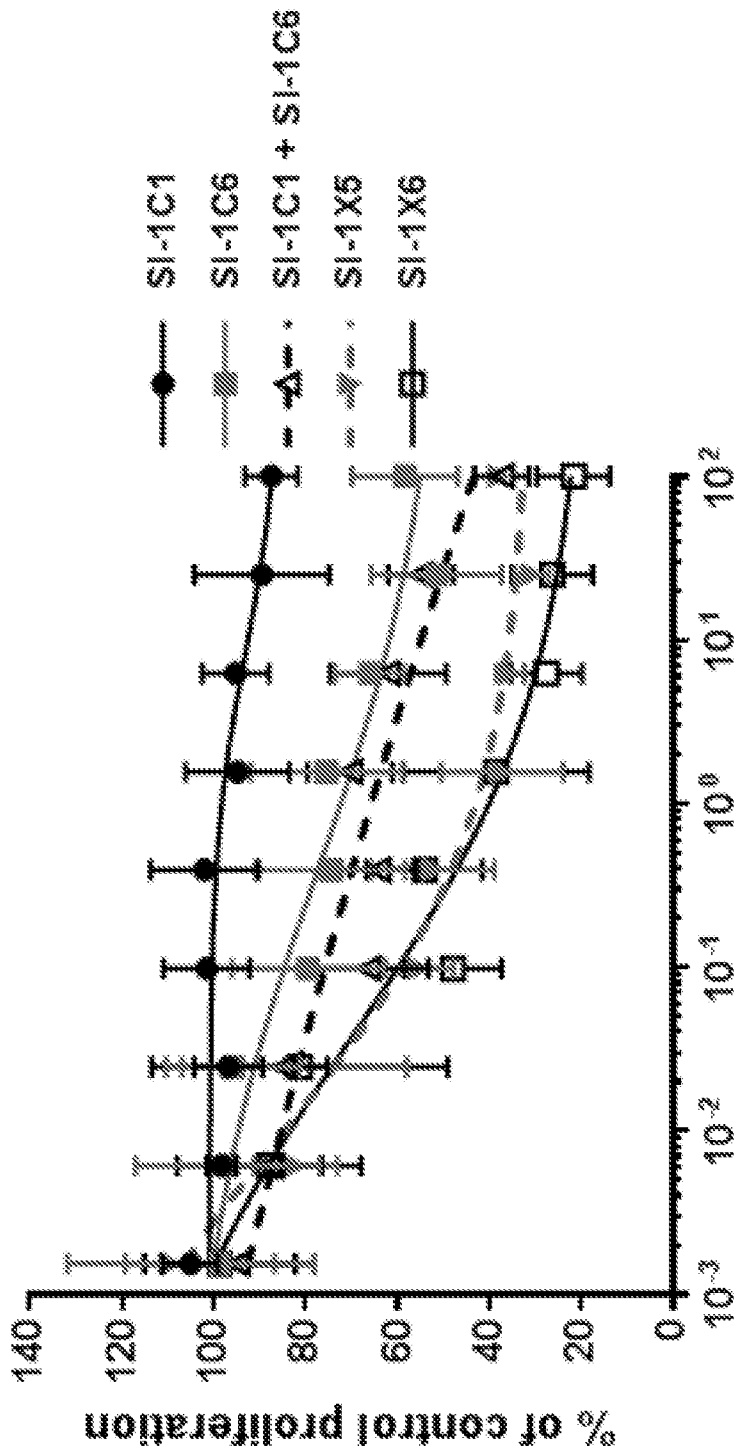
A431 IC50 values (nM)				
SL1C1	SL1C6	SL1C1 + SL1C6	SL1X5	SL1X6
N/A	0.64	0.84	1.30	0.68

FIG. 17



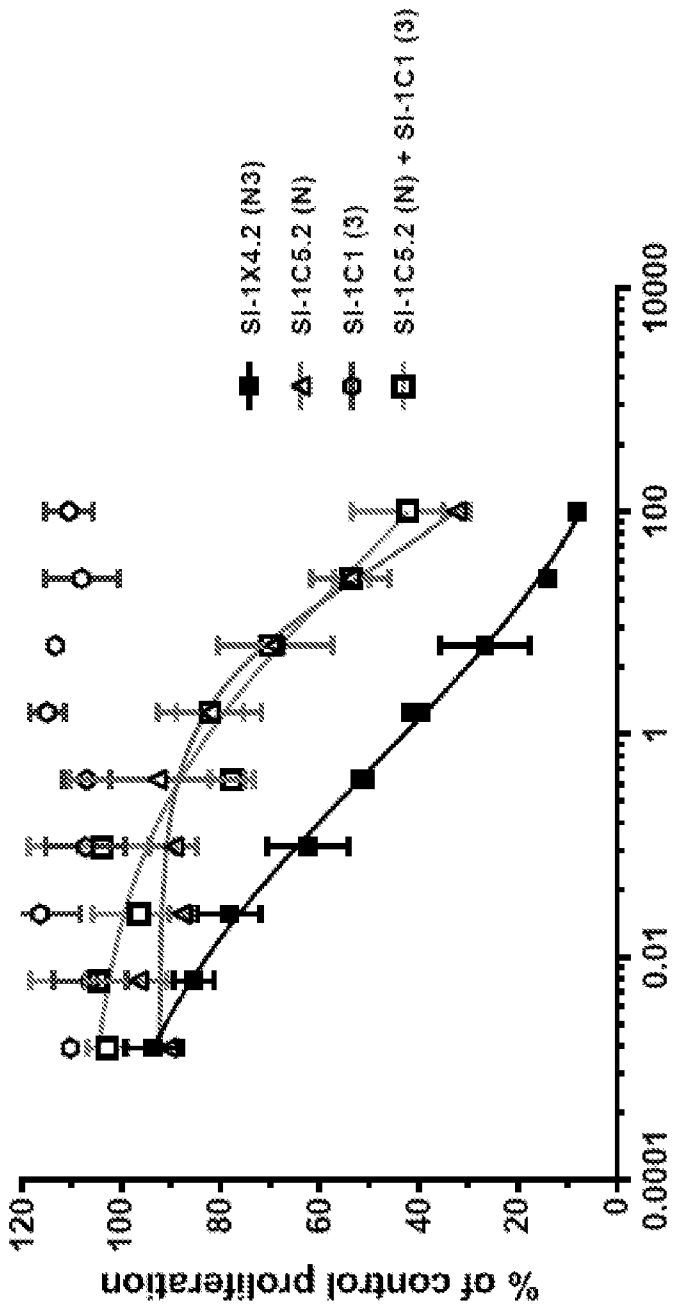
BxPC3 IC50 values (nM)				
SI-1C1	SI-1C3	SI-1C1 + SI-1C3	SI-1X1	SI-1X2
8.48	0.14	0.13	0.53	0.07

FIG. 18



BxPC3 IC50 values (nM)				
SL1C1	SL1C6	SL1C1 + SL1C6	SL1X5	SL1X6
8.48	0.37	ambiguous	0.01	0.02

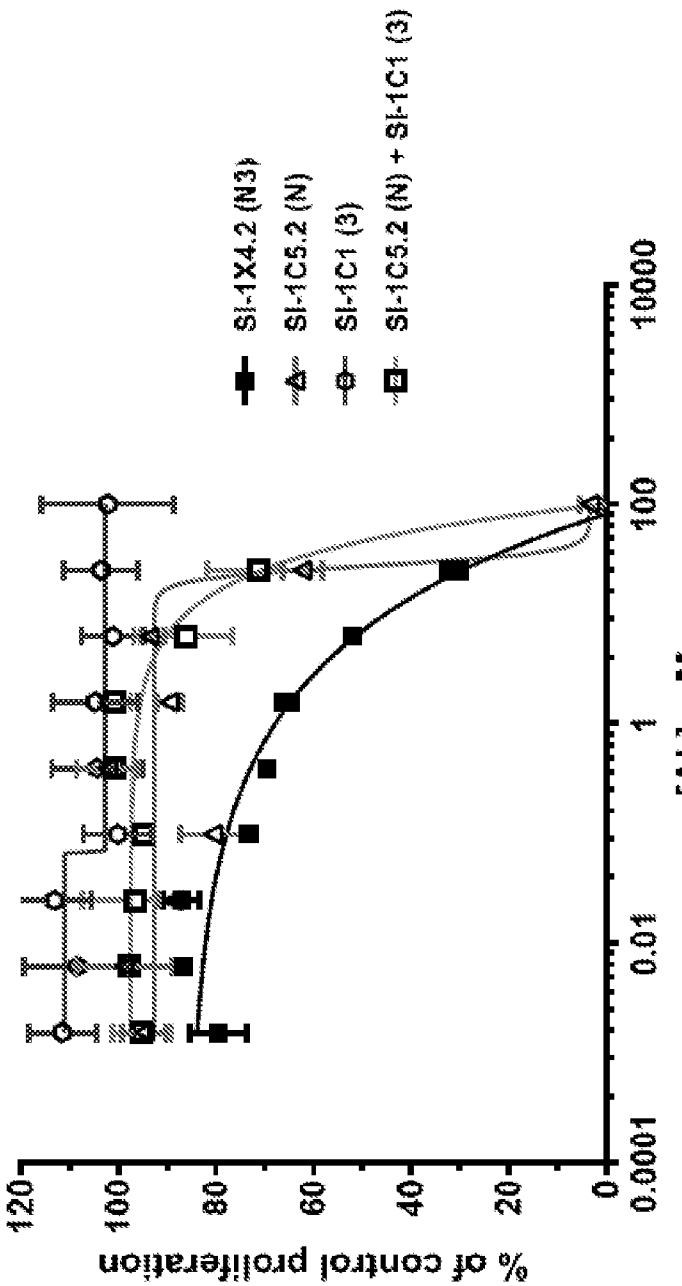
FIG. 19



Antibody	IC50 (nM)
SI-1X4.2 (N3)	0.54
SI-1C5.2 (N)	27.73 *
SI-1C1 (3)	N/A
SI-1X5.2 (N) + SI-1C1 (3)	35.04 *

* IC50 approximate - baseline not reached

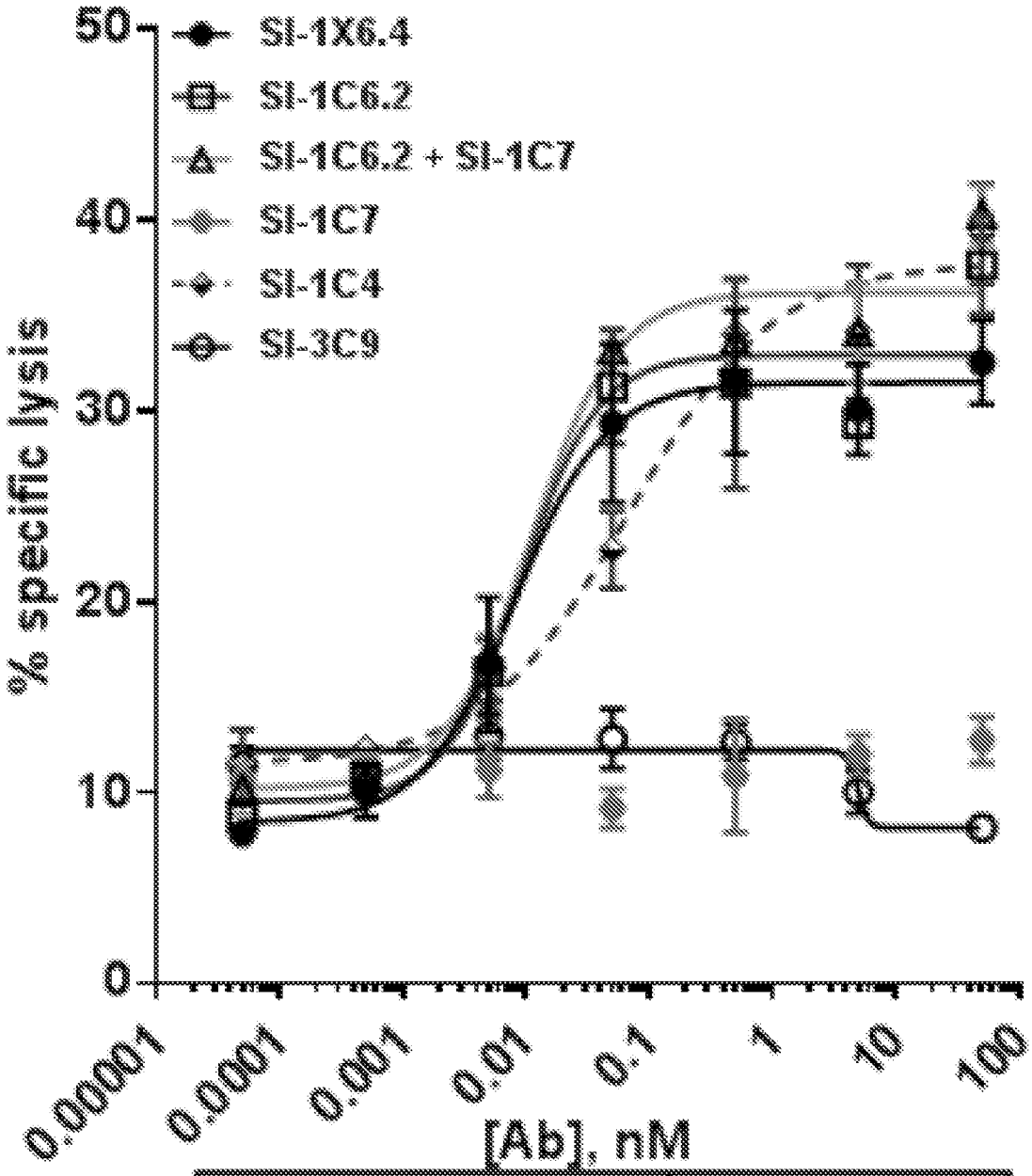
FIG. 20



Antibody	IC50 (nM)
SI-1X4.2 (N3)	no baseline
SI-1C5.2 (N)	no baseline
SI-1C1 (3)	N/A
SI-1X5.2 (N) + SI-1C1 (3)	no baseline

* IC50 approximate - baseline not reached

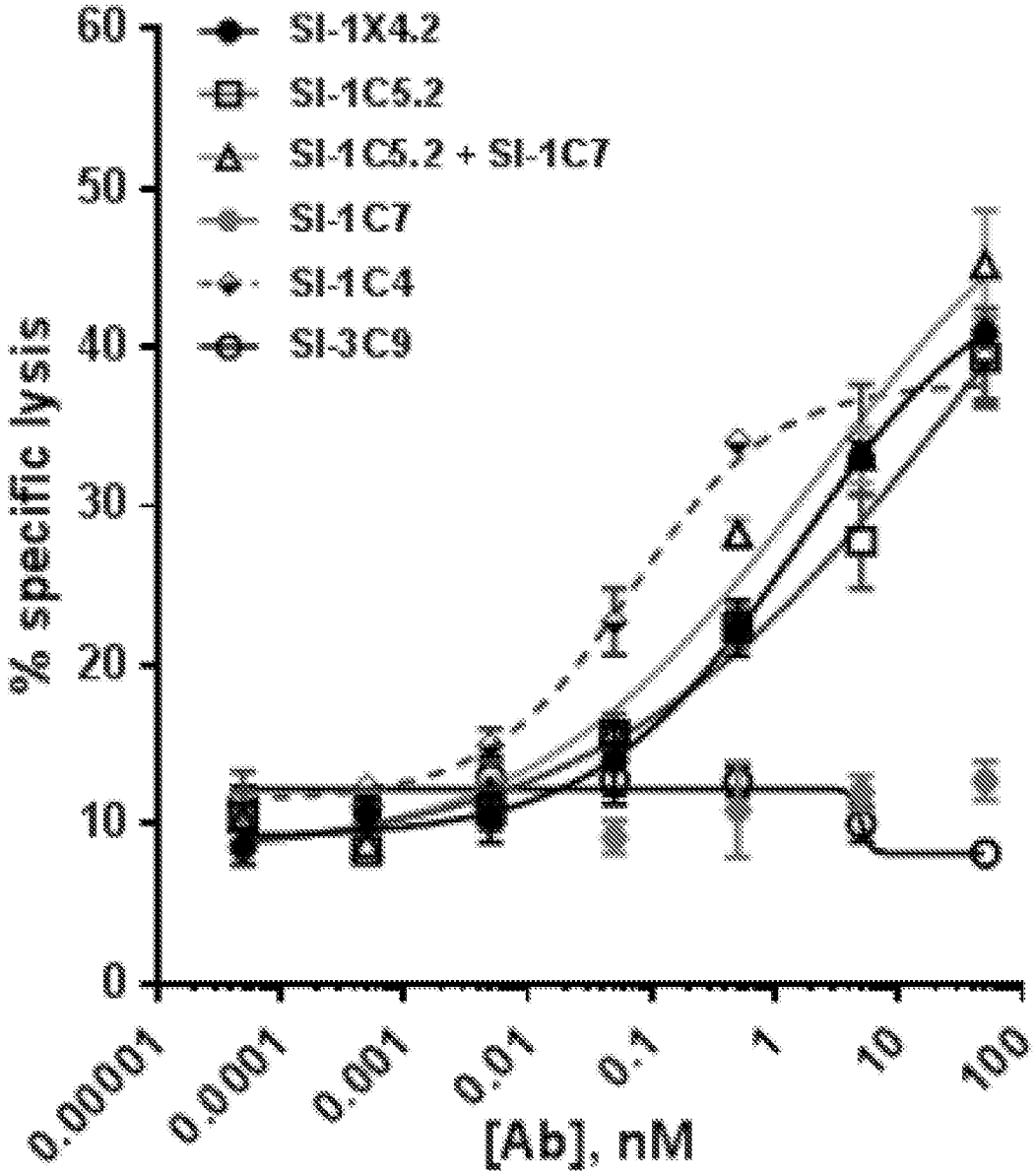
FIG. 21



Antibody	EC50 (nM)
SI-1X6.4 (C3)	0.008
SI-1C6.2 (C)	0.009
SI-1C6.2 (C) + SI-1C7 (3)	0.011
SI-1C7 (3)	N/A*
SI-1C4 (2in1)	0.068
SI-3C9 (control)	-

* EC50 not calculated - plateau not reached

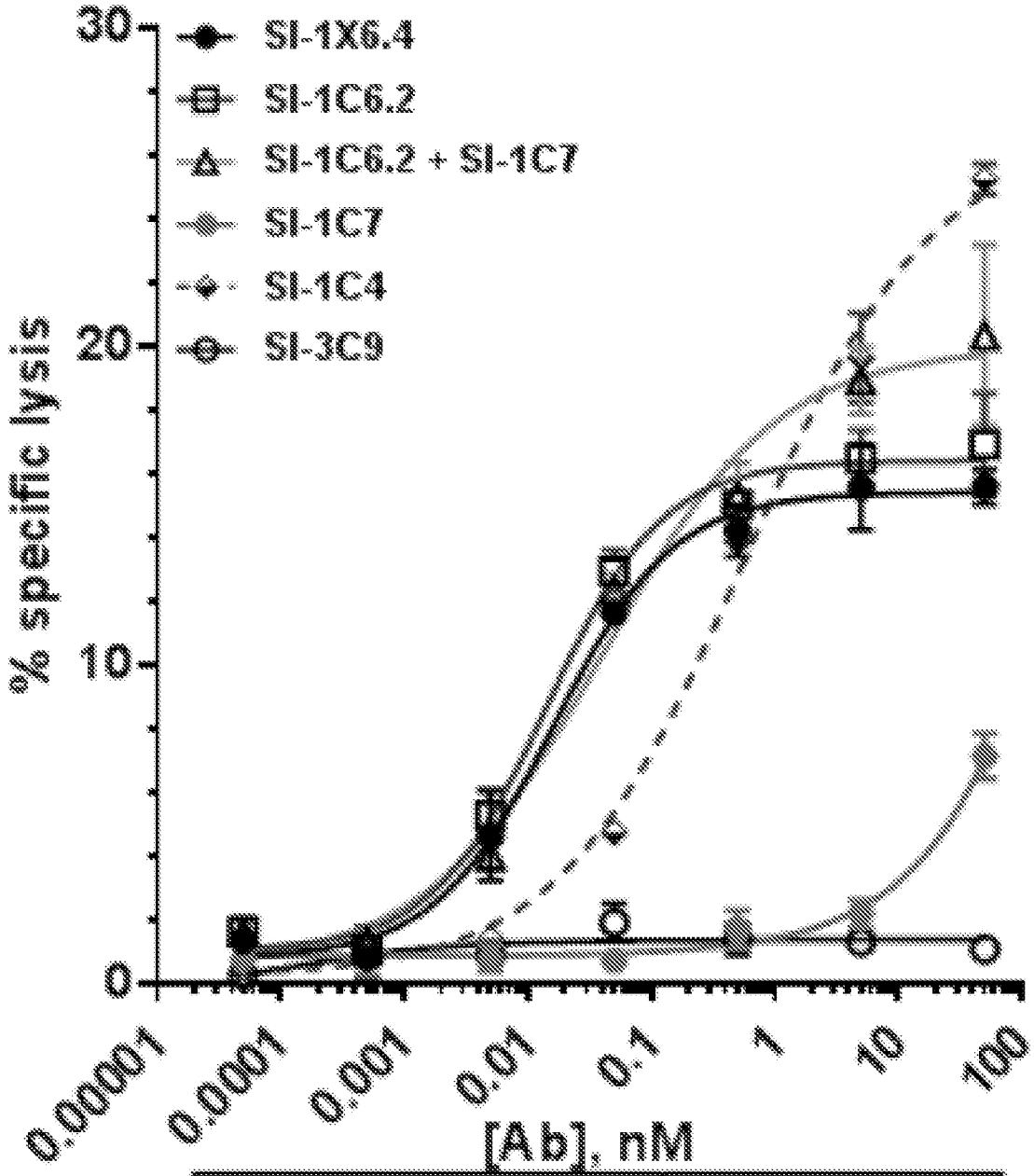
FIG. 22a



Antibody	EC50 (nM)
SI-1X4.2 (N3)	1.49
SI-1C5.2 (N)	N/A*
SI-1C5.2 (N) + SI-1C7 (3)	2.16
SI-1C7 (3)	N/A*
SI-1C4 (2in1)	0.068
SI-3C9 (control)	-

*EC50 not calculated - plateau not reached

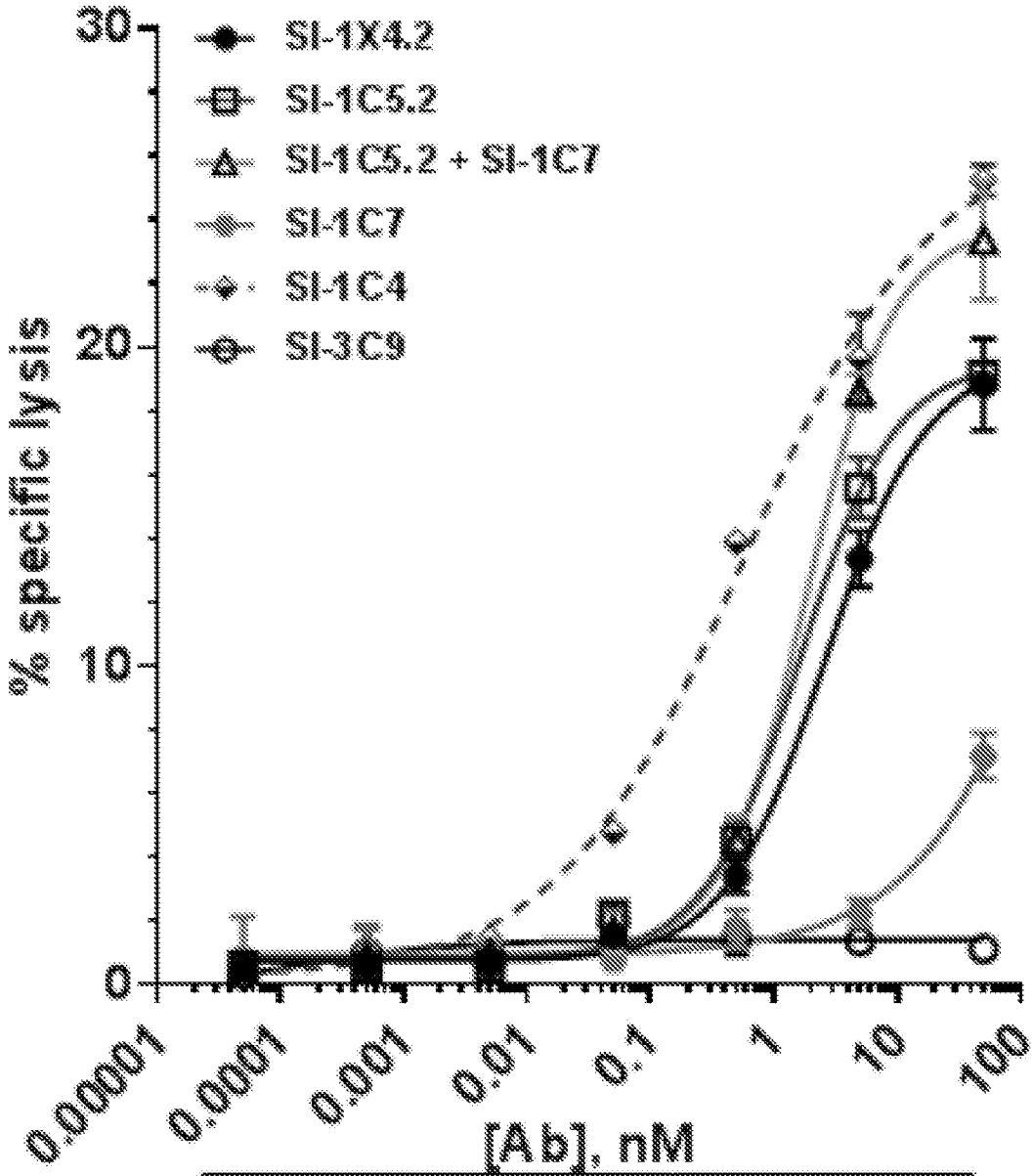
FIG. 22b



Antibody	EC50 (nM)
SI-1X6.4 (C3)	0.016
SI-1C6.2 (C)	0.014
SI-1C6.2 (C) + SI-1C7 (3)	0.039
SI-1C7 (3)	N/A*
SI-1C4 (2in1)	0.589
SI-3C9 (control)	-

* EC50 not calculated - plateau not reached

FIG. 23a



Antibody	EC50 (nM)
SI-1X4.2 (N3)	2.62
SI-1C5.2 (N)	1.63
SI-1C5.2 (N) + SI-1C7 (3)	1.87
SI-1C7 (3)	N/A*
SI-1C4 (2in1)	0.589
SI-3C9 (control)	-

*EC50 not calculated - plateau not reached

FIG. 23b

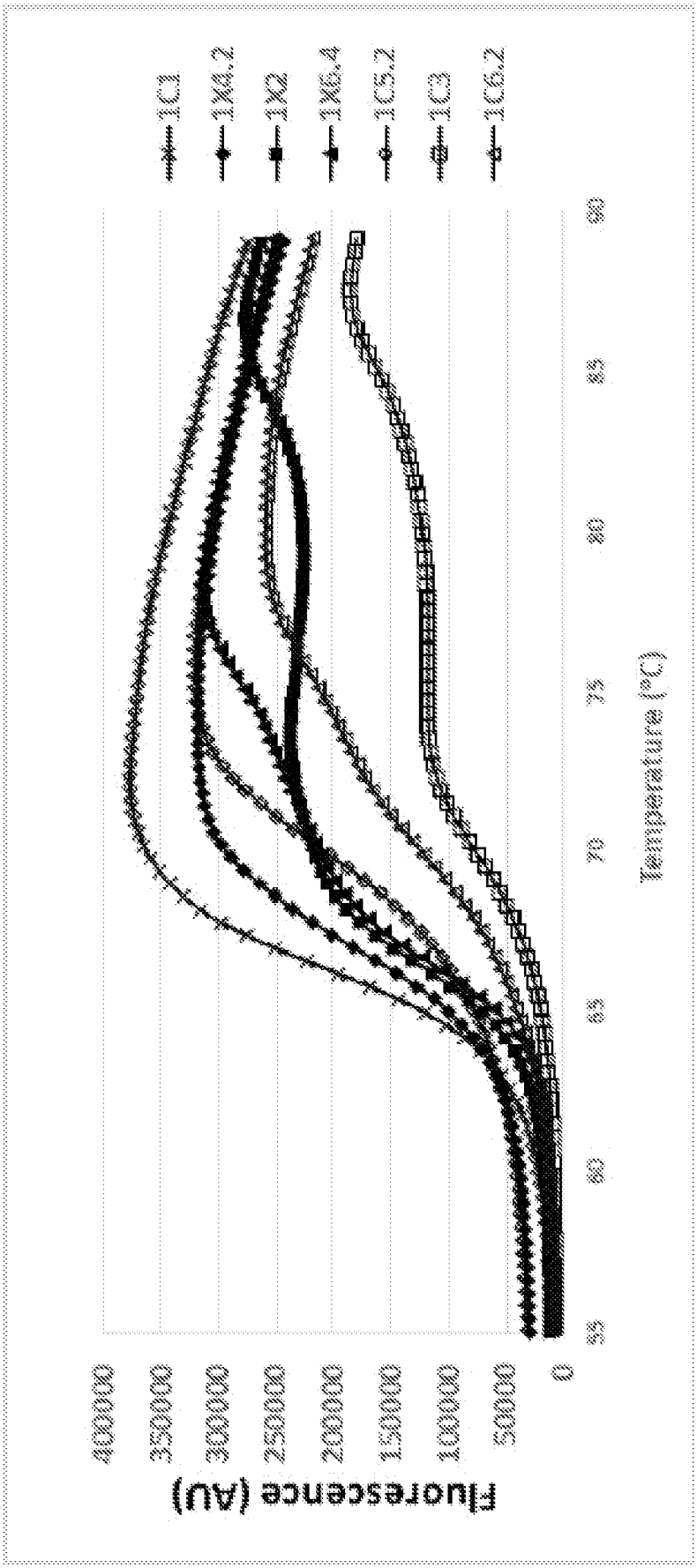
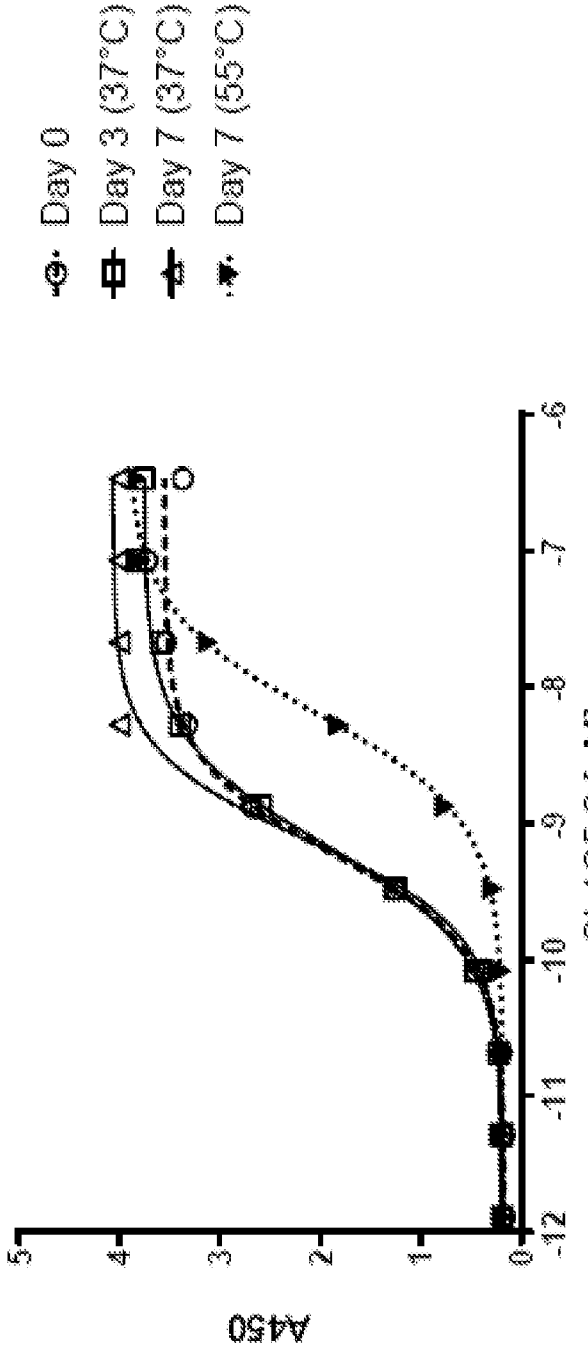
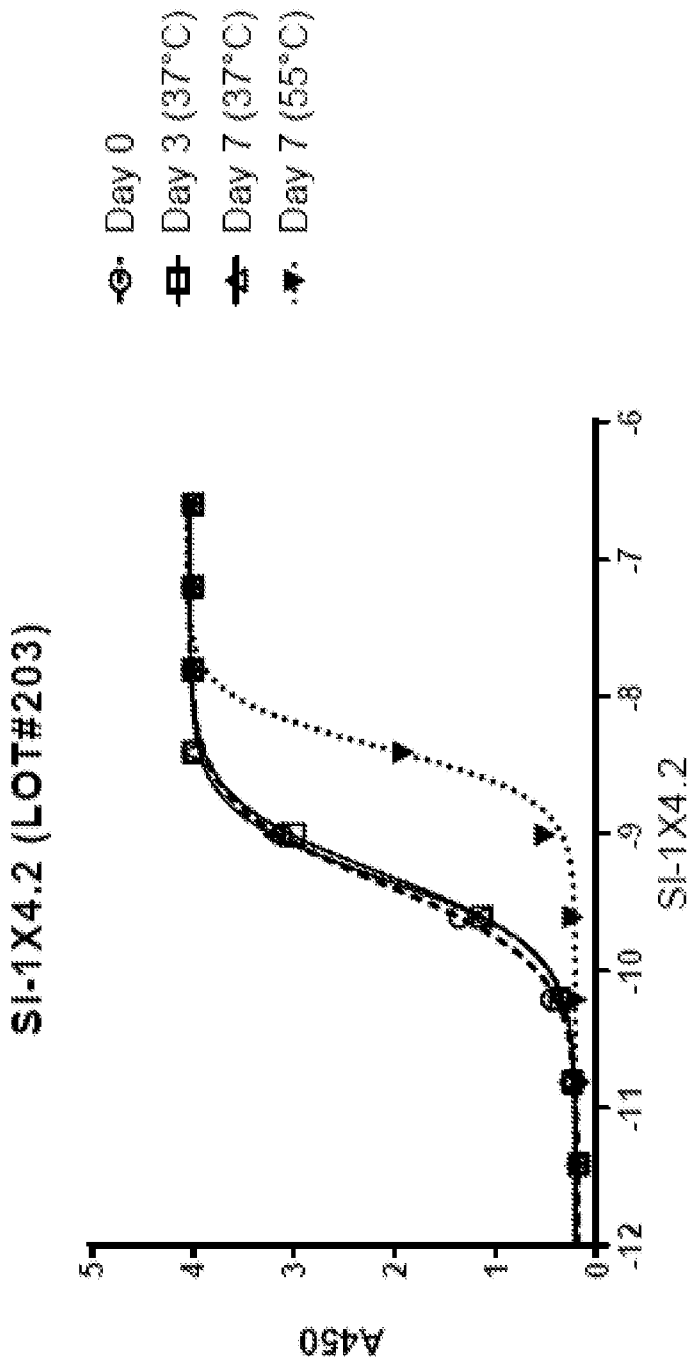

1C1
1X4.2
1X2
1X6.4
1C5.2
1C3
1C6.2
Fluorescence (AU)
400000
350000
300000
250000
200000
150000
100000
50000
0
55
60
65
70
75
80
85
90
Temperature (°C)

FIG. 24



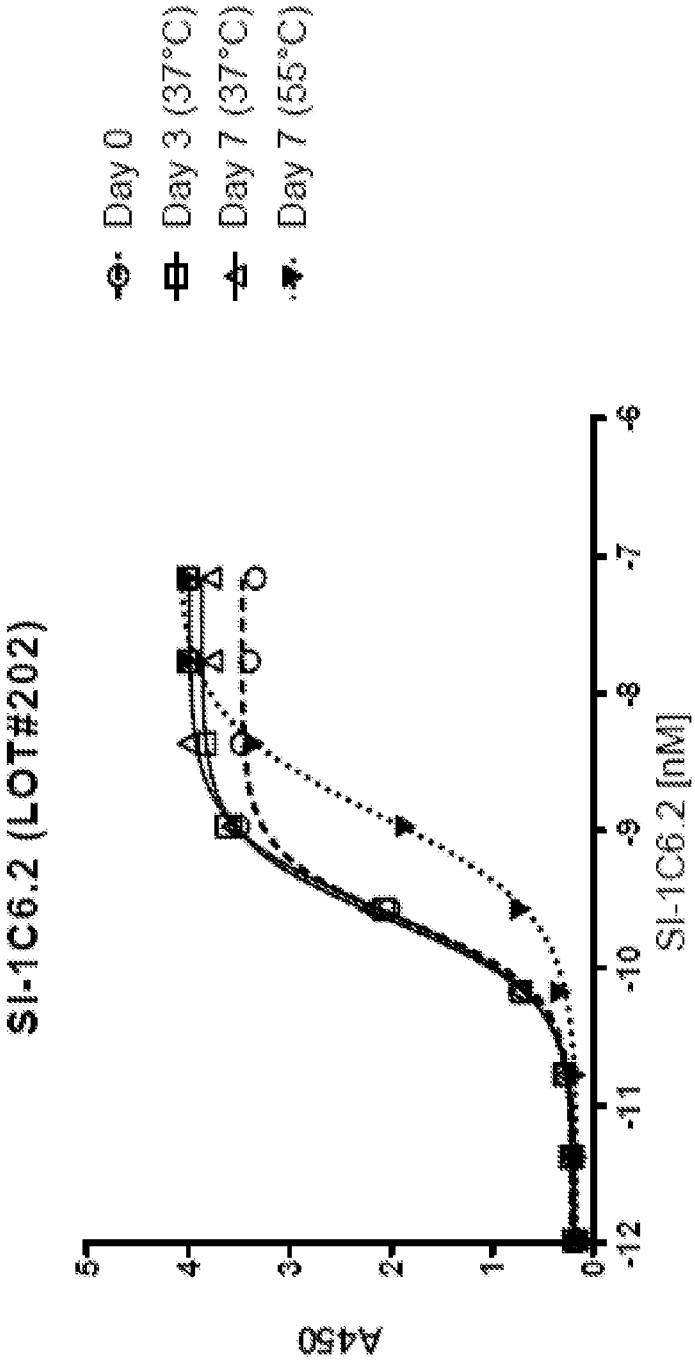
	Day 0	Day 3 (37°C)	Day 7 (37°C)	Day 7 (55°C)
EC50	5.897e-010	7.021e-010	7.552e-010	6.522e-009

FIG. 25a



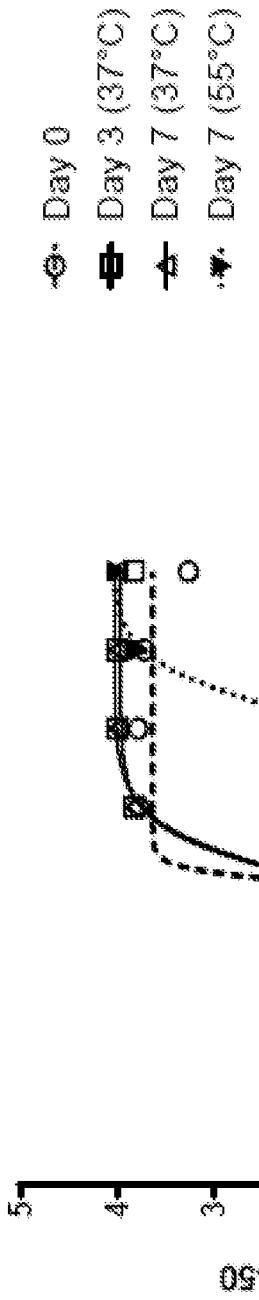
	Day 0	Day 3 (37°C)	Day 7 (37°C)	Day 7 (55°C)
EC50	4.293e-010	5.089e-010	4.667e-010	4.248e-009

FIG. 25b



	Day 0	Day 3 (37°C)	Day 7 (37°C)	Day 7 (55°C)
EC50	2.182e-010	2.651e-010	2.266e-010	1.322e-009

FIG. 25c



	Day 0	Day 3 (37°C)	Day 7 (37°C)	Day 7 (55°C)
EC50	~ 2.093e-010	2.300e-010	2.373e-010	4.112e-009

FIG. 25d

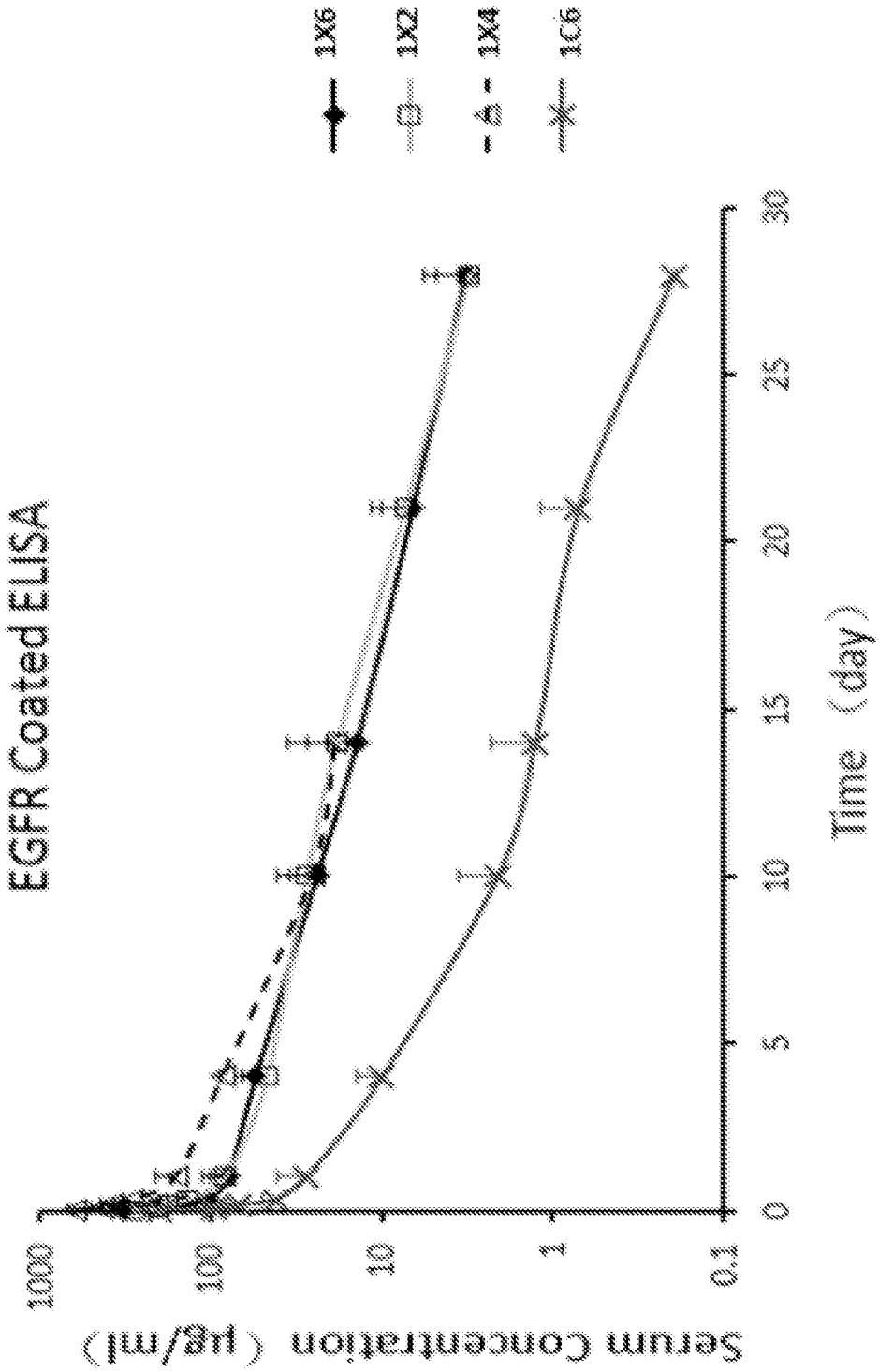

EGFR Coated ELISA
1X6
1X2
1X4
1C6
Serum Concentration (μg/ml)
1000
100
10
1
0.1
0
5
10
15
20
25
30
Time (day)

FIG. 26

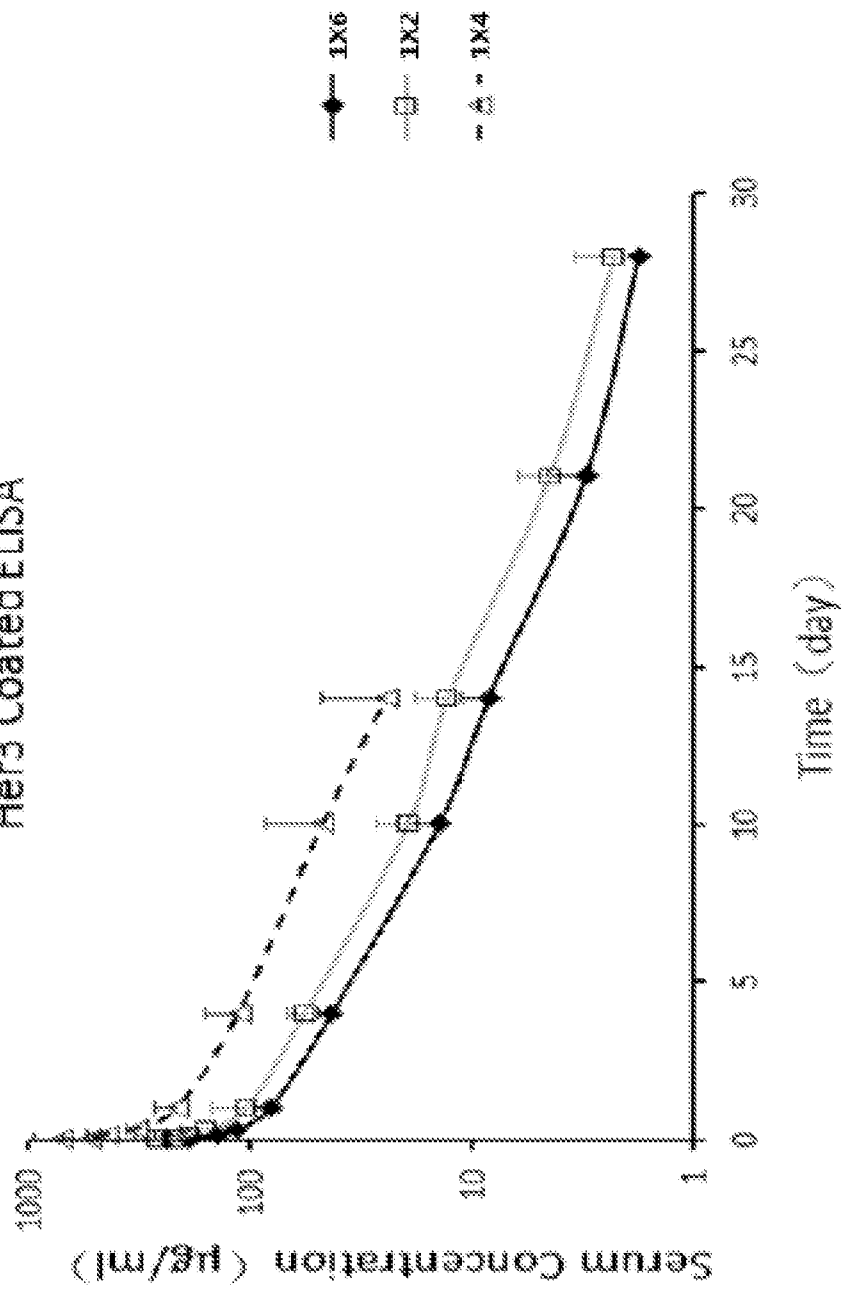

Her3 Coated ELISA
1X6
1X2
1X4
Serum Concentration (μg/ml)
1000
100
10
1
0
5
10
15
20
25
30
Time (day)

FIG. 27

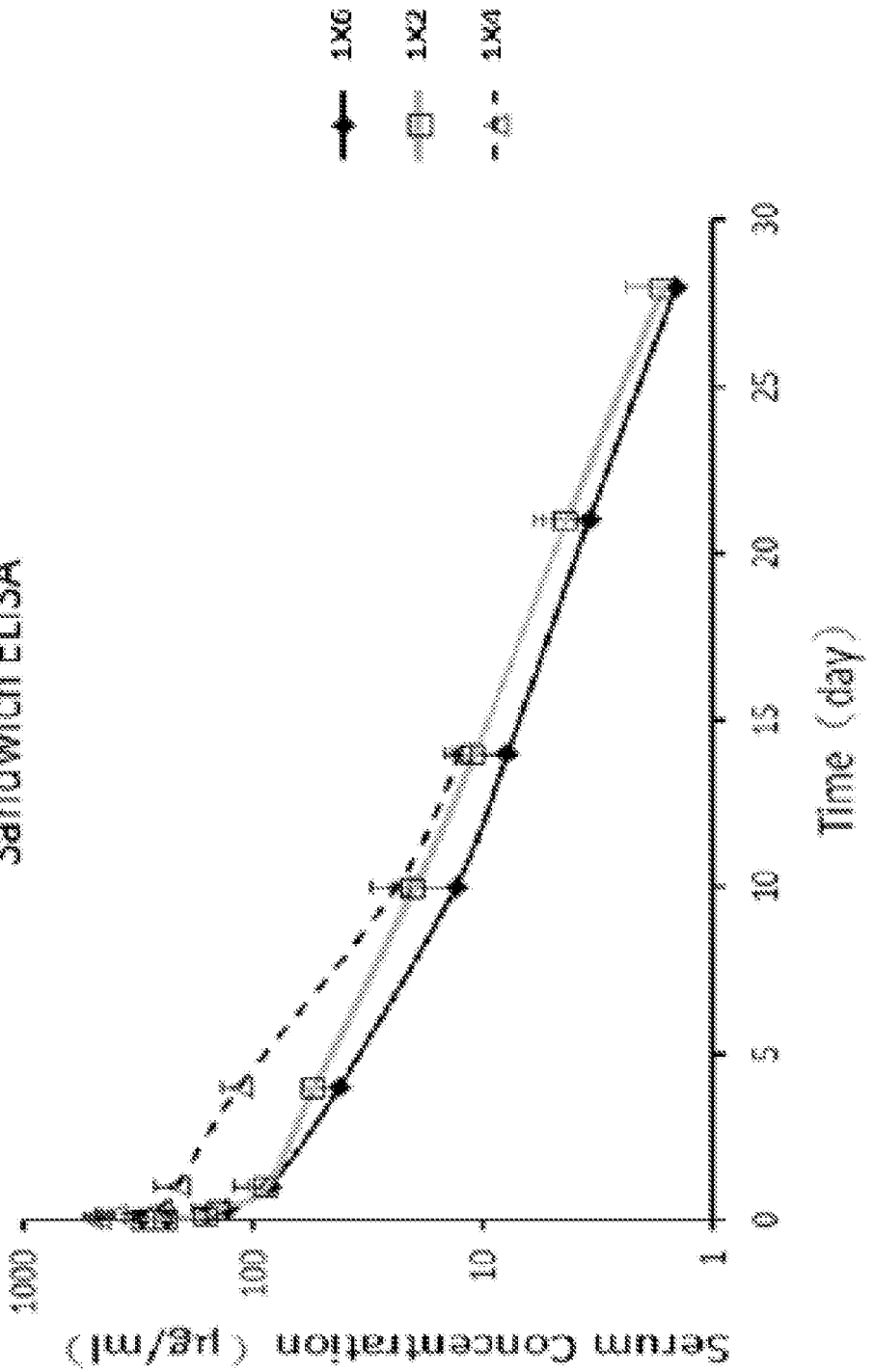

Sandwich ELISA
1X6
1X2
1X4
Serum Concentration (μg/ml)
1000
100
10
1
0
5
10
15
20
25
30
Time (day)

FIG. 28

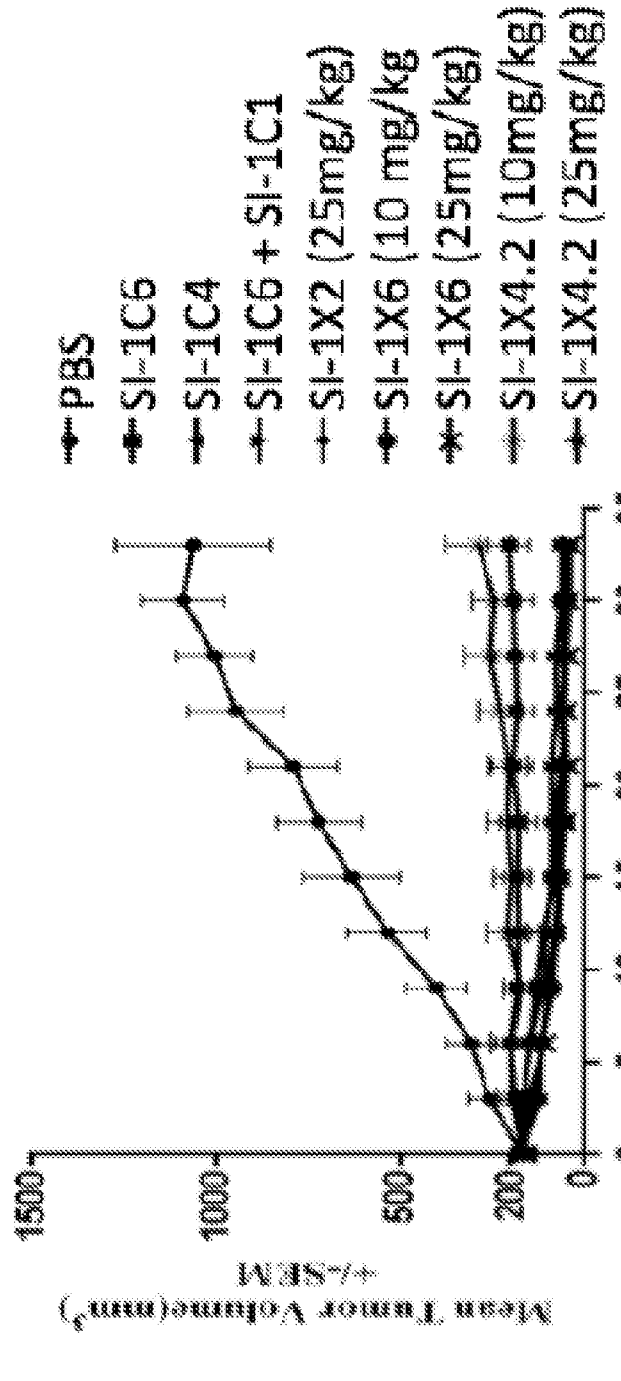

PBS
SI-1C6
SI-1C4
SI-1C6 + SI-1C1
SI-1X2 (25mg/kg)
SI-1X6 (10 mg/kg
SI-1X6 (25mg/kg)
SI-1X4.2 (10mg/kg)
SI-1X4.2 (25mg/kg)
Mean Tumor Volume(mm³) +/-SEM
1500
1000
500
200
0
Treatment (IV)
0
5
10
15
20
25
30
35
Day

FIG. 29

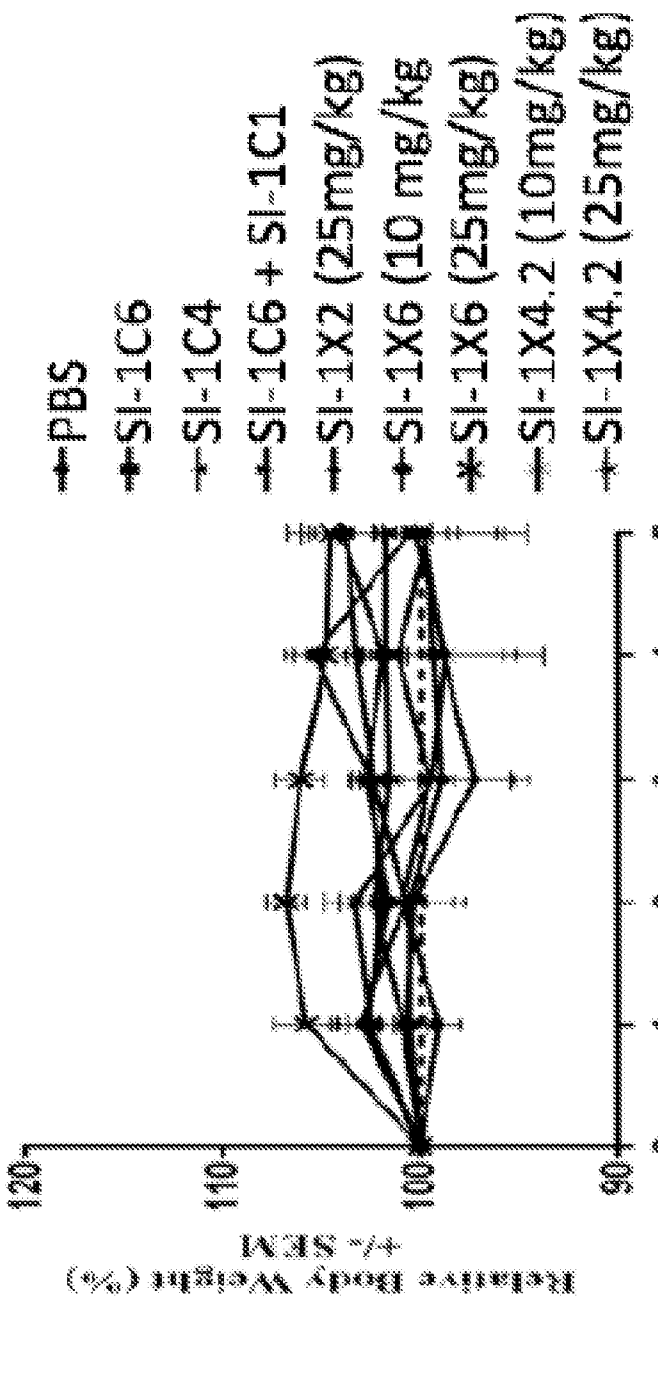

PBS
SI-1C6
SI-1C4
SI-1C6 + SI-1C1
SI-1X2 (25mg/kg)
SI-1X6 (10 mg/kg
SI-1X6 (25mg/kg)
SI-1X4.2 (10mg/kg)
SI-1X4.2 (25mg/kg)
Relative Body Weight (%)
+/- SEM
120
110
100
90
0
1
2
3
4
5
Week
Treatment (IV)

FIG. 30

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/066951

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: HER3 EGFR ErbB-1 tetravalent bispecific antibody kappa light chain heavy chain IgG1

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, 47-62
A	US 2010/0256338 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, 47-62
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, 47-62
A	US 2012/0134993 A1 (PAN et al) 31 May 2012 (31.05.2012) entire document	1, 4-15, 18, 21, 22, 25, 26, 33-35, 37, 40-45, 47-62
A	US 2014/0135482 A1 (ROCHE GLYCART AG et al) 15 May 2014 (15.05.2014). entire document	1, 4-15, 18, 21, 22, 25, 26, 33-35, 37, 40-45, 47-62

☐ 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
19 April 2016	06 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/US2015/066951

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, 56, and 66 were searched.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/066951

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 47-62 restricted to a bispecific tetravalent antibody, wherein the antibody is selected to be an antibody comprising first moiety selected to be a first IgG1 heavy chain, connector, and scFv domain encoded by SEQ ID NO: 56, 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: 66, and a second kappa light chain encoded by SEQ ID NO: 11, where the first moiety binds EGFR and the second moiety binds HER3.

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/US2015/066951

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 47-62 are drawn to a bispecific tetravalent antibody which binds members of the EGFR family.

The first invention of Group I+ is restricted to a bispecific tetravalent antibody, wherein the antibody is selected to be an antibody comprising first moiety selected to be a first IgG1 heavy chain, connector, and scFv domain encoded by SEQ ID NO: 56, 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: 66, and a second kappa light chain encoded by SEQ ID NO: 11, where the first moiety binds EGFR and the second moiety binds HER3. It is believed that claims 1, 4-15, 18, 21, 22, 25, 26, 33-35, 37, 40-45, and 47-62 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, single chain Fv domains, connectors, and/or and linkers each with corresponding 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 is selected to be an antibody comprising first moiety selected to be a first IgG1 heavy chain, connector, and scFv domain encoded by SEQ ID NO: 76, and a first kappa light chain encoded by SEQ ID NO: 19; and a second moiety selected to be a second IgG1 heavy chain, connector, and scFv domain, encoded by SEQ ID NO: 86, and a second kappa light chain encoded by SEQ ID NO: 27, where the first moiety binds EGFR and the second moiety binds HER3. Additional VH, VL, point mutations, and their corresponding SEQ ID Nos 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 bispecific tetravalent antibody, requiring the selection of alternatives for the IgG1 heavy chain, the kappa light chain, and the scFv, where "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; 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 Cterminus 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; 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, 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 a first member of the EGFR family; 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 IgGl-connector connection, wherein n is an integral of at least 1; 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]).

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