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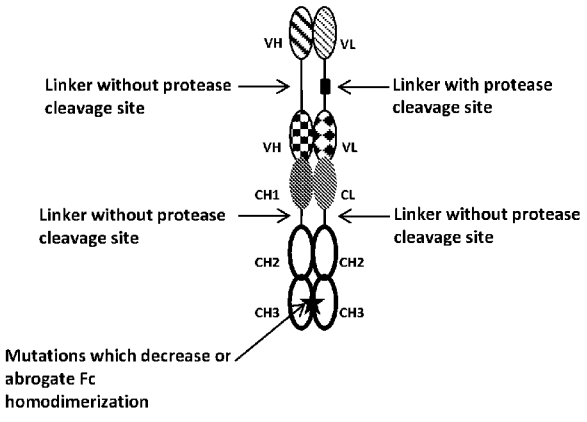
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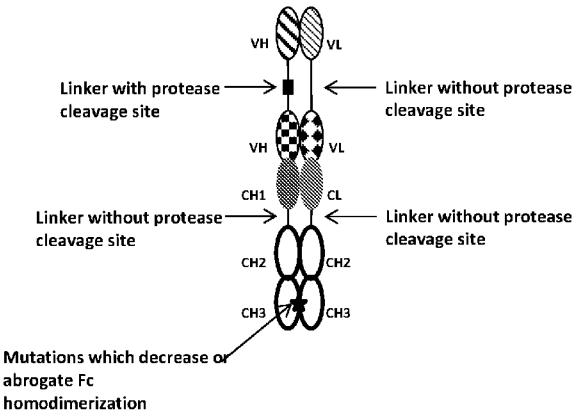
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(54) Title: PROTEASE CLEAVABLE BISPECIFIC ANTIBODIES AND USES THEREOF

Figure 1A





(57) Abstract: The present invention provides recombinant bispecific antibodies designed to bind to a surface antigen on target cells and to an activating component on immune cells such as T cells. The bispecific antibodies comprise two polypeptide chains that contain Fv and Fab as antigen¬ binding fragments and a modified Fc region to facilitate heterodimer formation. In one embodiment, the bispecific antibodies further comprise protease cleavage sites and/or motifs that would cause steric occlusion of the antigen binding sites so that the antibodies would be activated only in a specific environment, e.g. in the vicinity of a tumor. In another embodiment, the bispecific antibodies comprise modified sequences that confer reduced binding affinity to CDS and/or cross-reactive binding to CDS on cells from different species.

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INTERNATIONAL PATENT APPLICATION UNDER THE PATENT COOPERATION TREATY

PROTEASE CLEAVABLE BISPECIFIC ANTIBODIES AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This International Application claims the benefit of priority of U.S. Provisional Patent Application No. 62/783,411, filed on December 21, 2018, and U.S. Provisional Patent Application No. 62/815,132, filed on March 07, 2019.

BACKGROUND OF THE INVENTION

[0002] Antibodies are gammaglobulin proteins, predominantly referred to as immunoglobulins (Ig). A monomeric antibody is composed of two heavy chains and two light chains. The amino terminal ends of the polypeptide chains show considerable variation in amino acid composition and are referred to as the variable (V) domains/regions to distinguish them from the relatively constant (C) domains/regions. Each light chain has a variable domain and a constant domain. Each heavy chain has four domains: a variable domain, and constant domains 1, 2, and 3. The antigen binding site is located in the Fab (Fragment antigen binding) region which includes a light chain variable domain (VL), a heavy chain variable domain (VH), a light chain constant domain (CL) and a heavy chain constant domain 1 (CH1). The combination of a light chain variable domain (VL) and a heavy chain variable domain (VH) is called a Fv (Fragment variable) region. The Fc (Fragment, Crystallizable) region of an antibody includes heavy chain constant domains 2 and 3 (CH2 and CH3).

[0003] Each variable domain contains three hypervariable loops, known as complementarity determining regions (CDRs), evenly distributed between four less variable framework (FR) regions. It is the CDRs that provide a specific antigen recognition site on the surface of the antibody and the hypervariability of these regions enables antibodies to recognize an almost unlimited number of antigens. The heavy and light chains are held together by a combination of non-covalent interactions and covalent interchain disulfide bonds, forming a bilaterally symmetric structure. The hinge region is the area of the heavy chains between the first and second constant domains (CH1 and CH2) and is held together by disulfide bonds. This flexible hinge region allows the distance between the two antigen-binding sites to vary.

[0004] Selective destruction of an individual target cell or a specific target cell type is often desirable in a variety of clinical settings such as cancer therapy. One way of achieving this is by inducing an immune response against the tumor, for example, by making immune effector cells such as natural killer (NK) cells or cytotoxic T lymphocytes (CTLs) attack and destroy tumor cells. In this regard, bispecific antibodies designed to bind to a surface antigen on target cells and to an activating, invariant component of the T cell receptor (TCR) complex have become of interest in recent years. Simultaneous binding of such an antibody to both of its targets will force a temporary interaction between target cells and T cells, causing activation of cytotoxic T cells and subsequent lysis of the target cells. Hence, the immune response is re-directed to the target cells and is independent of peptide antigen presentation by the target cell or the specificity of the T cell as in normal MHC-restricted activation of CTLs.

[0005] With the recent advance of genetic and protein engineering technologies, bispecific antibodies (BsAb) such as BiTE has emerged to show promising applications. BiTE (Bi-specific T cell engager) is a type of fusion proteins with two single-chain antibody variable fragments (scFvs), one of which targets CD3 and the other targets a tumor antigen, joined by a (G4S)3 polypeptide linker. In the absence of Fc, these antibodies cannot be purified with protein A and G, and have short *in vivo* half-lives. Consequently, continuous infusion is required in clinical use. Also, bispecific antibodies in single-chain format (scFvs) and variants thereof have the problem of becoming aggregated easily.

[0006] There are other bispecific antibody technologies. Roche CrossMab contains Fc and therefore has much longer half-life *in vivo* than BiTE. CrossMab uses the knob-in-hole technology for Fc heterodimerization, but it could not yield 100% heterodimeric antibodies. In CrossMab, a wild-type IgG1 Fc and hinge are used which enable CrossMab to bind to all Fc receptor (FcR)-expressing cells such as macrophages. As a consequence, T cells will not only kill cancer cells but also kill the FcR-expressing cells leading to a side effect called lymphopenia.

[0007] Bispecific antibodies from Xencor Inc. and Synimmune GMBH comprise two antigen-binding domains linked to the N terminals and C terminals of Fc polypeptides respectively. The long distance between the two binding sites at the N terminals and C terminals

is less favorable for inducing an immune synapse like those formed in the course of natural cytotoxic T cell recognition.

[0008] Protease-activatable bispecific proteins developed by Amgen (U.S. patent application publication No. 2017/0247476) use two scFvs as antigen-binding fragments, CD3ε domain to mask CD3 binding, CH1-CL for heterodimerization, and wild-type IgG Fc or Fc with heterodimerizing alterations such as knob-in-hole mutations to extend half-lives. The use of scFvs, however, could cause aggregation and instability of the proteins. Although CH1-CL had been used as a heterodimerization scaffold to generate bispecific antibodies or multivalent fusion proteins, cooperation between the VH-VL and CH1-CL interface is requred for mutual stabilization. In the absence of VH-VL, it has been reported that CH1-CL is insufficient to yield heterodimeric products. Furthermore, Fc with currently known heterodimerizing alterations has failed to form heterodimers completely.

[0009] The protease-activated T cell bispecific molecules developed by Roche (international application publication No. WO2017/162587) are based on the above-mentioned CrossMab platform and uses an idiotype-specific scFv as a masking moiety to block binding of the CD3 antibody. This design inherits the shortcomings associated with CrossMab platform and scFv fragments described above.

[0010] International Application No. PCT/US18/43232 disclosed a bispecific antibody targeting both FLT3 and CD3, wherein the bispecific antibody includes a single chain variable fragment (scFv) of an anti-CD3 antibody fused to the Fab of an anti-FLT3 antibody. However, the binding domains to CD3 and FLT3 are not masked and no protease-sensitive polypeptide linkers are incorporated.

[0011] Bispecific antibodies with improved pharmacokinetic properties would be desirable to eliminate the need for continuous dosing. However, longer half-life could cause prolonged or poorly localized T cell activation, leading to undesirable side effects. Hence, there is a need in the art for bispecific antibody formats that have reasonably long half-life, but are activated specifically in a disease microenvironment, for example, in the vicinity of a tumor.

SUMMARY OF THE INVENTION

[0012] The present invention provides recombinant bispecific antibodies designed to bind to a surface antigen on target cells and to an activating component on immune cells such as T cells. In one embodiment, the bispecific antibodies of the present invention comprise Fv and Fab as antigen-binding fragments and a modified Fc region which result in excellent properties of final products including 100% heterodimer, high yield, high stability, low aggregation propensities and extended half-lives. In another embodiment, the present bispecific antibodies may also comprise protease cleavage sites and/or motifs that would cause steric occlusion of the antigen binding site so that the antibodies would be activated only in a specific environment, e.g. in the vicinity of a tumor.

[0013] In one embodiment, the present invention provides a recombinant bispecific antibody, comprising

[0014] (i) a first polypeptide comprising from N- to C-terminus a first light chain variable domain, a human light chain constant region (CL), a human heavy chain constant region 2 (CH2) and a human heavy chain constant region 3 (CH3);

[0015] (ii) a second polypeptide comprising from N- to C-terminus a first heavy chain variable domain, a human heavy chain constant region 1 (CH1), a human heavy chain constant region 2 (CH2) and a human heavy chain constant region 3 (CH3); and

[0016] (iii) a second light chain variable domain connected to the first light chain variable domain by a first linker, and a second heavy chain variable domain connected to the first heavy chain variable domain by a second linker; or

[0017] a second light chain variable domain connected to the C-terminus of the CH3 of the first polypeptide by a first linker, and a second heavy chain variable domain connected to the C-terminus of the CH3 of the second polypeptide by a second linker,

[0018] wherein the first light chain variable domain and the first heavy chain variable domain confer binding specificity to a first antigen, the second light chain variable domain and the second heavy chain variable domain confer binding specificity to a second antigen.

[0019] In one embodiment, the recombinant bispecific antibody of the present invention further comprises a heterodimerization motif or a long flexible motif, wherein the heterodimerization motif or long flexible motif is connected to the N terminus of the second light chain variable domain and the second heavy chain variable domain via one or more linkers.

[0020] In one embodiment, the recombinant bispecific antibody of the present invention further comprises modified sequences that confer reduced binding affinity to CD3 on T cells. In another embodiment, the recombinant bispecific antibody disclosed herein possesses cross-reactive binding to CD3, e.g. bindng to human and cynomolgus CD3.

[0021] The present invention also provides polynucleotides encoding one or both polypeptides of the recombinant bispecific antibody disclosed herein. In another embodiment, the present invention also includes expression vectors comprising the above polynucleotides. In yet another embodiment, the present invention also includes host cells comprising the above expression vectors.

[0022] The present invention also provides a method of using the above polynucleotides or expression vectors to prepare the recombinant bispecific antibody disclosed herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0023] Figure 1A shows schematic representation of examples of one embodiment of protease cleavable bispecific antibodies of the present invention.

[0024] Figure 1B shows schematic representation of examples of another embodiment of protease cleavable bispecific antibodies of the present invention.

[0025] Figure 2A shows a mechanism of action of the bispecific antibodies of the present invention in the absence or presence of tumor-associated proteases. Stars denote mutations which decrease or abrogate Fc homodimerization. All unlabeled modules have the same meanings as in Figures 1A-B.

[0026] Figure 2B shows mechanisms of action of the bispecific antibodies of the present invention in circulation (left panel) and in tumor tissue (right panel). Stars denote mutations which decrease or abrogate Fc homodimerization. All unlabeled modules have the same meanings as in Figures 1A-B.

[0027] Figure 3 shows schematic representation of bispecific antibodies of the present invention that can bind to EGFR and CD3. The antibody polypeptide chain containing anti-

CD3 VH-CH1 sequence is designated heavy chain and the one with anti-CD3 VL-CL sequence is designated light chain, regardless of their molecular weights. Stars denote mutations which decrease or abrogate Fc homodimerization. proBiTE: protease cleavable bispecific T cell engagers; HBiTE: H-shaped bispecific T cell engagers; iBiTE: I-shaped bispecific T cell engagers.

[0028] Figure 4 shows expression and purification of bispecific antibody EGFR×CD3 proBiTE. Molecular masses of standards are shown on the left.

[0029] Figure 5 shows cleavage of proBiTE with tumor-associated proteases. Molecular masses of standards are shown on the left. uPA and the isolated VL domain of anti-EGFR antibody are indicated by arrows on the right.

[0030] Figure 6 shows size-exclusion chromatography of proBiTE. The arrows at the top indicate the elution volumes of the molecular mass standards in PBS (pH7.4): ferritin (440 kDa), aldolase (158 kDa) and conalbumin (75 kDa).

[0031] Figure 7 shows binding affinity of bispecific antibodies for cell surface-associated EGFR and CD3 as measured by flow cytometry. MFI, mean fluorescence intensity.

[0032] Figure 8 shows binding of proBiTE to cell-surface EGFR and CD3 as measured by flow cytometry. The tracings on the left in each panel represent reference cells which were incubated with secondary antibody only (FITC-conjugated anti-human IgG (Fc-specific)). The tracings on the right in each panel represent experimental groups, in which the cells were first incubated with bispecific antibodies at a concentration of 2 μg/mL and then with the secondary antibody.

[0033] Figure 9 shows binding of pre-cleaved proBiTE to cell-surface EGFR and CD3 as measured by flow cytometry. The tracings on the left in each panel represent reference cells which were incubated with secondary antibody only (FITC-conjugated anti-human IgG (Fc-specific)). The tracings on the right in each panel represent experimental groups, in which the cells were first incubated with bispecific antibodies at a concentration of 2 μg/mL and then with the secondary antibody.

[0034] Figure 10 shows binding of intact and cleaved proBiTE-1 to cell surface EGFR and CD3 as measured by flow cytometry.

[0035] Figure 11 shows binding of pre-bound and then cleaved proBiTE-1 to cell-surface EGFR as measured by flow cytometry. The tracings on the left in each panel represent reference cells which were incubated with secondary antibody, FITC-conjugated anti-human IgG (Fc-specific), only. The tracings on the right in each panel represent experimental groups, in which the cells were first incubated with intact or pre-cleaved bispecific antibodies at a concentration of 2 μg/mL and then with the secondary antibody in the absence or presence of uPA.

[0036] Figure 12 shows characterization of several human colon cancer cell lines for EGFR expression on cell surface by flow cytometry.

[0037] Figure 13 shows T cell activation by iBiTE. The ability and specificity of iBiTE to activate human T cells in the presence of EGFR-expressing cells were evaluated by Promega T cell activation bioassay systems according to the manufacturer's instructions.

[0038] Figure 14 shows short-term activation of T cells by proBiTE. The ability of proBiTE to activate human T cells during a short period (5 h) of incubation with EGFR-expressing human colon cancer cells were evaluated by Promega T cell activation bioassay systems according to the manufacturer's instructions.

[0039] Figure 15 shows killing of HCT116 cells by the bispecific antibodies in the presence of human PBMC. Target cells (HCT116) and effector cells (PBMC) are at a ratio of 1:5. Representative images were taken after 72 h of incubation.

[0040] Figure 16 shows killing of HT29 cells by the bispecific antibodies in the presence of human PBMC. Target cells (HT29) and effector cells (PBMC) are at a ratio of 1:5. Representative images were taken after 72 h of incubation.

[0041] Figure 17 shows killing of HT29 cells stably transfected with red firefly luciferase by the bispecific antibodies in the presence of human PBMC. Target cells (HT29)

and effector cells (PBMC) are at a ratio of 1:5. Luciferase activity was measured after 72 h of incubation.

[0042] Figure 18 shows ELISA binding of proBiTE-1 to recombinant human Fc gamma receptors (FcγR).

[0043] Figure 19 shows pharmacokinetics of the bispecific antibodies in NOD/SCID mice.

[0044] Figures 20A-B show anti-tumor activity of proBiTE-12 in NOD/SCID mice engrafted with human PBMC and colon cancer cell line HCT116. Figure 20A shows macroscopic appearance of tumor tissues. Figure 20B shows average tumor weight.

[0045] Figure 21 shows cleavage of proBiTE-1 variants with uPA. Molecular masses of standards are shown on the left. uPA and the isolated VL domain of anti-EGFR antibody are indicated by arrows on the right.

[0046] Figure 22 shows size-exclusion chromatography of proBiTE-1 variants. The arrows at the top indicate the elution volumes of the molecular mass standards in PBS (pH7.4): ferritin (440 kDa), aldolase (158 kDa) and conalbumin (75 kDa).

[0047] Figure 23 shows binding of proBiTE-1 variants to cell-surface EGFR and CD3 as measured by flow cytometry. The tracings on the left in each panel represent reference cells which were incubated with secondary antibody, FITC-conjugated anti-human IgG (Fc-specific), only. The tracings on the right in each panel represent experimental groups, in which the cells were first incubated with bispecific antibodies at a concentration of 2 μg/mL and then with the secondary antibody.

[0048] Figure 24 shows killing of HCT116 and HT29 cells stably transfected with red firefly luciferase by proBiTE-1 variants in the presence of human PBMC. Target cells and effector cells (PBMC) are at a ratio of 1:5. Luciferase activity was measured after 72 h of incubation.

[0049] Figure 25 shows schematic representation of examples of various embodiments of protease cleavable bispecific antibodies with sterically restricted access of dual antigen binding sites (dproBiTE).

[0050] Figure 26 shows mechanism of action of dproBiTE. All unlabeled modules have the same meanings as in Figures 25A-B.

[0051] Figure 27 shows schematic representation of EGFR×CD3 dproBiTE. The antibody polypeptide chain containing anti-CD3 VH-CH1 sequence is designated heavy chain and the one with anti-CD3 VL-CL sequence is designated light chain, regardless of their molecular weights. Stars denote mutations which decrease or abrogate Fc homodimerization.

[0052] Figures 28A-B show results of SDS-PAGE of dproBiTE-HE and dproBiTE-GS. Figure 28A shows intact dproBiTE, whereas Figure 28B shows protease-cleaved dproBiTE. Molecular masses of standards are shown on the left.

[0053] Figure 29 shows size-exclusion chromatography of dproBiTE-HE and dproBiTE-GS. The arrows at the top indicate the elution volumes of the molecular mass standards in PBS (pH7.4): ferritin (440 kDa), aldolase (158 kDa) and conalbumin (75 kDa).

[0054] Figure 30 shows binding of dproBiTE-HE and dproBiTE-GS to cell-surface EGFR and CD3 as measured by flow cytometry. The tracings on the left in each panel represent reference cells which were incubated with secondary antibody, FITC-conjugated anti-human IgG (Fc-specific), only. The tracings on the right in each panel represent experimental groups, in which the cells were first incubated with bispecific antibodies at a concentration of 2 μg/mL and then with the secondary antibody.

[0055] Figure 31 shows binding of the selected anti-EGFR antibodies (SMET5 Fab) to cell surface EGFR as measured by flow cytometry.

[0056] Figure 32 shows affinity maturation of SMET5 Fab variants by light chain shuffling.

[0057] Figure 33 shows results of SDS-PAGE of SMET5 variants.

[0058] Figure 34 shows size-exclusion chromatography of SMET5 variants. The arrows at the top indicate the elution volumes of the molecular mass standards in PBS (pH7.4): ferritin (440 kDa), aldolase (158 kDa) and conalbumin (75 kDa).

[0059] Figure 35 shows ELISA binding of SMET5 variants to recombinant human and cynomolgus EGFR.

[0060] Figure 36 shows binding of SMET5 variants to cell surface EGFR as measured by flow cytometry.

[0061] Figure 37 shows competition of EGF and SMET5 variants for binding to cell surface EGFR as measured by flow cytometry.

[0062] Figure 38 shows pharmacokinetics of SMET5.2 IgG1 in NOD/SCID mice.

[0063] Figure 39 shows antitumor activity of SMET5.2 IgG1 in NOD/SCID mice engrafted with human PBMC and A431 cells.

[0064] Figure 40 shows a schematic representation of protease cleavable or non-cleavable bispecific EGFR×CD3 antibodies that have cross-reactive binding to CD3. The star in the IgG1 mFc CH3 domains denotes the T366L and Y407H substitutions. The star in the anti-CD3 VH domain denotes the V105A and N111.1S substitutions.

[0065] Figure 41 shows an analysis of hSP34 heavy chain CDR3 (HCDR3) sequence using IMGT/V-QUEST. The upper panel shows alignment of the nucleotide sequences of hSP34 HCDR3 V, D and J regions with the sequences of the closest germline human antibody sequences. The lower panel shows alignment of the amino acid sequence of hSP34 HCDR3 with that of the germline human antibody. Mutated nucleotide and amino acid sequences compared to the corresponding germlines sequences are underlined.

[0066] Figure 42 shows expression and purification of bispecific EGFR×CD3 antibodies. Molecular masses of standards are shown on the left.

[0067] Figure 43 shows cleavage of bispecific EGFR×CD3 antibodies with tumor-associated protease uPA. Molecular masses of standards are shown on the left. uPA and the isolated VL domain of anti-EGFR antibody are indicated by arrows on the right.

[0068] Figure 44 shows size-exclusion chromatography of the bispecific EGFR×CD3 antibodies. The arrows at the top indicate the elution volumes of the molecular mass standards in PBS (pH7.4): ferritin (440 kDa), aldolase (158 kDa) and conalbumin (75 kDa).

[0069] Figure 45 shows ELISA binding of the bispecific EGFR×CD3 antibodies to recombinant human EGFR (hEGFR) and HER4 (hHER4), an irrelevant antigen.

[0070] Figure 46 shows results of ELISA assays demonstrating cross-reactive binding of the bispecific EGFR×CD3 antibodies to recombinant human CD3 (hCD3) and cynomolgus CD3 (cCD3).

[0071] Figure 47 shows binding of the bispecific EGFR×CD3 antibodies to cell-surface EGFR and CD3 as measured by flow cytometry. The tracing on the left in each diagram represents binding to cells which were incubated only with the secondary antibody, FITC-conjugated anti-human IgG (Fc-specific). The tracing on the right in each diagram represents binding in the experimental groups, in which the cells were first incubated with the bispecific antibodies at a concentration of 2 μg/mL and then with the secondary antibody.

[0072] Figure 48 shows binding of the bispecific EGFR×CD3 antibodies at different concentrations to cell surface CD3 as measured by flow cytometry. The EC_{50} of iBiTE-sp was calculated by fitting the data to the Sigmoidal isotherm.

[0073] Figure 49 shows killing of HCT116 and CHO cells infected with Ad5-Luc by the bispecific EGFR×CD3 antibodies in the presence of human PBMC. Target cells and effector cells (PBMC) are at a ratio of 1:2.5. Cell viability was measured after 48 h of incubation using Promega Bright-Glo Luciferase Assay System according to the manufacturer's instructions.

[0074] Figure 50 shows killing of HCT116 and HT29 cells by the bispecific EGFR×CD3 antibodies in the presence of human PBMC. Target cells and effector cells

(PBMC) are at a ratio of 1:5. Cell viability was measured after 48 h of incubation using Promega CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay according to the manufacturer's instructions.

DETAILED DESCRIPTION OF THE INVENTION

Distinctive Features of The Present Invention

[0075] Compared to published monovalent bispecific antibodies developed by others, the bispecific antibodies of the present invention have the following advantageous and distinctive features. Firstly, in one embodiment, the bispecific antibodies of the present invention use monomeric human IgG1 Fc (mFc) to extend *in vivo* half-life. In addition, the present bispecific antibodies contain only two polypeptide chains and employ both Fab and Fv for heterodimerization and antigen recognition. The present mFc exists as a monomer at low concentrations but tends to form a dimer at high concentrations. Therefore, when the heavy and light chains of the present bispecific antibodies are assembled, the mFc forms a dimer due to high local concentrations and further stabilize the bispecific antibodies in cooperation with the heterodimerization strength from Fab and Fv. A combination of the features mentioned above results in excellent properties for the bispecific antibodies such as 100% heterodimer, high yield, high stability and low aggregation propensities.

[0076] In contrast, many bispecific antibodies in the art have problems in yield and homodimerization. For example, many bispecific antibodies in the art contain three or four polypeptide chains, thereby resulting in low yield of antibodies. Moreover, a number of bispecific antibodies in the art use the knob-in-hole technology in the Fc region to prevent Fc homodimerization. However, the knob-in-hole technique cannot completely prevent Fc homodimerization; there still remains 5-10% homodimers that could cause toxic side effects in humans. It should be noted that the bispecific antibodies of the present invention can be made to have 100% heterodimer due to the use of monomeric Fc (mFc) domains disclosed herein.

[0077] In some embodiments of the current bispecific antibodies in the art, a single-chain Fv (scFv), which is a single polypeptide chain containing a light chain variable domain (VL) and a heavy chain variable domain (VH), is used as the antigen binding domain. A problem in the uses of scFv is that it would typically lead to instability of the bispecific antibodies. It should be noted that the bispecific antibodies of the present invention do not contain any scFv, thereby avoiding the instability problem of scFv.

[0078] Another distinctive feature of the present bispecific antibodies is that long flexible motifs (e.g., repeats of the G4S motif or naturally occurring short heterodimerization peptides) which have small molecular size and low or no immunogenicity are used to block antigen binding sites by creating steric hindrance. Such steric hindrance can be removed by specific enzymes in certain microenvironment, for example, tumor-associated proteases. Hence, the bispecific antibodies disclosed herein can be configured to be activated specifically in a disease microenvironment, for example, in the vicinity of a tumor. In contrast, other bispecific antibodies in the art use non-human antibody binding peptides or anti-antibodies to create steric hindrance. Non-human antibody binding peptides could be highly immunogenic, whereas anti-antibodies could create problem in the making and uses of the bispecific antibodies due to their relatively large molecular size.

[0079] In one embodiment, the present invention provides bispecific antibodies having monomeric Fc (mFc) polypeptides or fusion molecules comprising human CH2 and CH3 domains, wherein the CH3 domain comprises one or two amino acid substitutions. The amino acid substitutions significantly reduce the ability of the mFc polypeptides to form homodimers. In one embodiment, the reduction in dimerization is 40%, 50%, 60%, 70%, 80%, 90% or 100%. For example, the present invention includes compositions comprising the present bispecific antibodies or mFc-fusion molecules wherein the amount of Fc-Fc homodimerization exhibited by the antibodies or mFc-fusion molecule is less than 60%, less than 20%, less than 15%, less than 14%, less than 13%, less than 12%, less than 11%, less than 10%, less than 9%, less than 8%, less than 7%, less than 6%, less than 5%, less than 4%, less than 3%, less than 2%, or less than 1%. Examples of such modified CH3 domains include, but are not limited to, one of SEQ ID NOs:155-171.

[0080] In one embodiment, an Fc polypeptide comprising an antibody CH3 domain with one or two amino acid substitutions/mutations has decreased ability to form homodimers compared to a polypeptide comprising a wild-type CH3 domain. In one embodiment, the substitutions are within the CH3-CH3 homodimerization interface. In another embodiment, the substitutions/mutations could also be in other regions that could induce conformational changes of CH3 leading to abrogation of CH3-CH3 homodimerization. In one embodiment, the Fc polypeptides could have less immunogenicity compared to an Fc polypeptide comprising three or more substitutions.

[0081] Antibody heavy chain is a polypeptide composed of VH-CH1-CH2-CH3 fragments from N- to C- terminus, and the amino acid positions are numbered according to the Kabat numbering system that is widely used in the antibody area. Various substitutions or mutations to the Fc portion of an antibody are contemplated. Such variations are designated by the amino acid at that position in the wild-type antibody heavy chain based on the EU numbering scheme of Kabat followed by the amino acid substituted at that position. For example, when the tyrosine at EU position 407 is substituted with methionine, it is designated "Y407M." By "Fc," it is meant a wild-type sequence of amino acids that occurs naturally within a species of animals, e.g., humans. Wild-type sequence may vary slightly between individuals within a population, e.g., different alleles for the various immunoglobulin chains are known in the art.

[0082] It is also contemplated that the creation of monomeric Fc-containing polypeptides is not limited to those based on IgG1 Fc but are also applicable to the Fc region of IgG3, IgG4 and other immunoglobulin subclasses including IgA, IgE, IgD, and IgM.

[0083] Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by one of ordinary skill in the art to which this invention belongs.

[0084] As used herein, the term “antibody” refers to a protein or polypeptide containing at least one VH or VL region, in many cases a heavy and a light chain variable region. Thus, the term "antibody" encompasses molecules having a variety of formats, including single chain Fv antibodies (scFv, which contain VH and VL regions joined by a linker), Fab, F(ab)2', Fab', scFv:Fc antibodies etc (as described in Carayannopoulos and Capra, Ch.9 in FUNDAMENTAL IMMUNOLOGY, 3rd ed., Paul, ed., Raven Press, New York, 1993, pp. 284-286). The terms “antibody” and “immunoglobulin” can be used synonymously throughout the specification.

[0085] As used herein, the term “cross-reactive binding” of an antibody refers to antibody binding to the same antigen from various species, e.g. cross-reactive binding to

antigen X refers to antibody binding to antigen X in various species such as humans, monkeys, mice and rats etc.

[0086] In one embodiment, the bispecific antibodies disclosed herein having cross-reactive binding to CD3, e.g. binding to human and cynomolgus CD3, which enables toxicology studies in commonly used pharmacologically relevant species such as cynomolgus monkeys in preclinical development of the bispecific antibodies. In another embodiment, amino acid mutations can be introduced to both the light and heavy chains of the anti-CD3 antibody to reduce binding to CD3, thus minimizing localization of the bispecific antibodies to T cells before engagement with antigen-expressing target cells. Bispecific antibodies with decreased affinity for CD3 could potentially diminish unwanted side effects *in vivo*. These side effects include, but are not limited to, nonspecific activation of T cells, interference with normal immune responses of T cells, and Fc receptor (FcR)-mediated killing of T cells by other cytotoxic cells such as macrophage and NK cells if the bispecific antibodies contain Fc.

[0087] The bispecific antibodies of the present invention, whether attached to other sequences or not, can also include insertions, deletions, substitutions, or other selected modifications of particular regions or specific amino acids residues, provided the activity of the bispecific antibodies is not significantly altered or impaired compared to non-modified antibodies. These modifications can provide some additional properties, such as to remove/add amino acids capable of disulfide bonding, to increase its bio-longevity, to alter its secretory characteristics, etc. Methods for generating amino acid sequence variants are readily apparent to one of ordinary skill in the art and can include, for example, site-specific mutagenesis or random mutagenesis. Both naturally occurring and non-naturally occurring amino acids (e.g., artificially-derivatized amino acids) may be used to generate amino acid sequence variants.

ABBREVIATIONS

[0088] As used herein, "proBiTE" refers to protease cleavable bispecific T cell engager. proBiTE-1, proBiTE-2, and proBiTE-12 are three variants of proBiTE. proBiTE-1 contains a cleavable polypeptide linker comprising the GGGGSLSGRSDNHGGGGS sequence (SEQ ID NO:58) (underlined is the substrate of uPA, matriptase and legumain). proBiTE-2 contains a cleavable linker comprising the GGGGSGPLGLARKGGGGS sequence (SEQ ID NO:59) (underlined is the substrate of MMP-7). The substrate sequences for both

classes of proteases were combined in proBiTE-12, resulting in a dual cleavable linker comprising the GGGGSLSGRSDNHGPLGLARK sequence (SEQ ID NO:60).

[0089] As used herein, "proBiTE-1s", "proBiTE-1s1", "proBiTE-1s2" are three proBiTE-1 variants. proBiTE-1s contains a shortened cleavable polypeptide linker comprising the GSLSGRSDNHGGGGS sequence (SEQ ID NO:61) (underlined is the substrate of uPA, matriptase and legumain). proBiTE-1s1 contains linker sequence GSGSGRSDNHGGGGS (SEQ ID NO:62). proBiTE-1s2 contains linker sequence GSGGSRSDNHGGGGS (SEQ ID NO:63).

[0090] As used herein, "proBiTE-1s1sp" refers to a bispecific antibody in which the VH and VL domains of anti-EGFR antibody SMET5.2 were fused to the N terminus of the VH and VL domains of anti-CD3 antibody hSP34 Fab. As used herein, bispecific antibody "proBiTE-1s1spg" was derived from proBiTE-1s1sp.

[0091] As used herein, "HBiTE" refers to H-shaped bispecific T cell engager; "iBiTE" refers to I-shaped bispecific T cell engager. "iBiTE-sp" refers to a bispecific antibody in which the scFv of anti-EGFR antibody was fused via a G4S linker to the N terminus of the VH-CH1 of hSP34 and therefore, binding to CD3 is not sterically restricted by the linker. As used herein, "HBiTE-spg" was derived from proBiTE-1s1spg, in which a protease cleavable linker in proBiTE-1s1spg was replaced by a protease non-cleavable linker.

[0092] As used herein, "dproBiTE" refers to protease cleavable bispecific T cell engager in which an antigen binding site is sterically restricted by flexible motifs or heterodimerization motifs. "dproBiTE-HE" and "dproBiTE-GS" are two variants of dproBiTE.

POLYNUCLEOTIDES, VECTORS, HOST CELLS

[0093] Also provided herein are nucleic acid sequences that encode the bispecific antibodies described herein. Nucleic acids include single stranded and double stranded nucleic acids of any type (e.g., DNA, RNA, DNA/RNA hybrid). Such nucleic acids can be used therapeutically or in methods of producing the present bispecific antibodies.

[0094] The nucleic acid sequences encoding the present bispecific antibodies can be part of a vector. Vectors include nucleic acid vectors, such as naked DNA and plasmids, and

various viral vectors and hybrid or chimeric viral vectors generally known in the art (see, e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory, NY (1989). The vectors can comprise any suitable promoter and other regulatory sequences (e.g., transcription and translation initiation and termination codons which are specific to the type of host cells) to control the expression of the nucleic acid sequence encoding the polypeptides. The promoter can be a native or normative promoter operably linked to the nucleic acid sequences described herein. The selection and construction of promoters, including various constitutive and regulatable promoters, is within the skill of an ordinary artisan.

[0095] The present invention also encompasses amino acid or nucleic acid sequences that are functionally equivalent to the amino acid or nucleic acid sequences disclosed herein. Functionally equivalent sequences refer to sequences that are the results of spontaneous or induced modifications, e.g. substitution, deletion, and/or insertion of nucleotides or amino acids. In other words, sequences functionally equivalent are sequences that are "substantially the same" or "substantially identical" to the amino acid or nucleic acid sequences disclosed herein. As it is generally understood in the art, two sequences are generally considered to be "substantially identical" if they contain identical residues in corresponding positions. Amino acid or nucleic acid sequences may be compared using any of a variety of algorithms, including those available in commercial computer programs such as BLASTN for nucleotide sequences and BLASTP, gapped BLAST, and PSI-BLAST for amino acid sequences. In addition to identifying identical sequences, these and other similar programs typically provide an indication of the degree of identity. In some embodiments, two sequences are considered to be substantially identical if at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more of their corresponding residues are identical over a relevant stretch of residues. The relevant stretch can be a complete or full-length sequence, or a partial sequence thereof.

[0096] Also provided herein are cells (e.g., isolated host cells) comprising the above nucleic acid sequences. Such cells are useful, for example, as therapeutic agents or for producing the present bispecific antibodies. A person of ordinary skill in the art would readily use any suitable host cells, including prokaryotic and eukaryotic cells. Alternatively, cells from a mammal, such as a human, to be treated in accordance with the methods described herein can be used as host cells. Methods of introducing nucleic acids and vectors into isolated host cells

and the culture and selection of transformed host cells *in vitro* are generally known in the art. In one embodiment, the nucleic acid sequence is transcribed and translated efficiently by the host cells.

[0097] In one embodiment, the bispecific antibodies described herein can be produced by a method comprising (a) transforming host cells with a nucleic acid or vector encoding the bispecific antibodies; (b) culturing the cells in culture medium under conditions sufficient to express the bispecific antibodies; and (c) harvesting the bispecific antibodies from the cells or culture medium. The bispecific antibodies can be expressed in prokaryotic cells (e.g., bacteria) or eukaryotic cells (e.g., yeast, insect, or mammalian cells), and subsequently harvested and purified, as necessary, using well-known methods (see, e.g., Sambrook et al. Molecular Cloning: a Laboratory Manual, Cold Spring Elarbor Laboratory Press (1989). Techniques for transforming, culturing, and harvesting polypeptides expressed by a nucleic acid sequence are generally known in the art. For example, one of ordinary skill in the art could readily express the bispecific antibodies of the present invention in a bacterial system, such as *E. coli*, or fungal systems, such as yeast. Alternatively, the bispecific antibodies can be produced in mammalian, avian, or plant systems. If the bispecific antibodies have poor solubility, the antibodies can be expressed in the form of insoluble inclusion bodies and refolded *in vitro* using methods generally known in the art.

PHARMACEUTICAL COMPOSITION AND METHODS OF ADMINISTRATION

[0098] The present bispecific antibodies, nucleic acid molecules, vectors, or host cells can be administered to a mammal alone, or in combination with a carrier (i.e., a pharmaceutically acceptable carrier). By pharmaceutically acceptable is meant a material that is not biologically or otherwise undesirable, i.e., the material can be administered to a mammal without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained. As would be well-known to one of ordinary skill in the art, the carrier is selected to minimize any degradation of the bispecific antibodies or polynucleotides and to minimize any adverse side effects in the mammal. For example, suitable carriers and their formulations are described in Remington: The Science and Practice of Pharmacy (19th ed.) ed. A. R. Gennaro, Mack Publishing Company, Easton, Pa. (1995).

[0099] The above pharmaceutical compositions comprising the bispecific antibodies, polynucleotides, vectors, or host cells can be administered (e.g., to a mammal, a cell, a tissue, or a tumor) in any suitable manner depending on whether local or systemic treatment is desired. For example, the composition can be administered topically (e.g. ophthalmically, vaginally, rectally, intranasally, transdermally, and the like), orally, by inhalation, or parenterally (including by intravenous drip or subcutaneous, intracavity, intraperitoneal, intradermal, or intramuscular injection). Topical intranasal administration refers to delivery of the compositions into the nose and nasal passages through one or both of the nares. The composition can be delivered by a spraying mechanism or droplet mechanism, or through aerosolization of the nucleic acid or vector. Delivery can also be directed to any area of the respiratory system (e.g., lungs) via intubation. Alternatively, administration can be intratumoral, e.g. local or intravenous injection.

[0100] If the composition is to be administered parenterally, the administration is generally by injection. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for suspension in liquid prior to injection, or as emulsions. Additionally, parental administration can involve preparation of a slow-release or sustained-release system so as to maintain a constant dosage.

[0101] In one embodiment, the present bispecific antibodies, polynucleotides, vectors, or host cells can be delivered to a mammal *in vivo* and/or *ex vivo* by a variety of mechanisms well-known in the art. If *ex vivo* methods are employed, cells or tissues can be removed from the mammal and maintained outside the body according to standard protocols known in the art. The nucleic acid molecules or vectors can be introduced into the cells or tissue via any gene transfer mechanism well-known in the art. The transduced cells are then infused or homotopically transplanted back into the mammal according to standard methods.

[0102] The bispecific antibodies disclosed herein may be used in therapeutic methods. In one embodiment, the bispecific antibodies of the present invention can be used as immunotherapeutic agents, for example in the treatment of cancers. In one embodiment, the bispecific antibodies of the present invention can be used alone or in combination with other anti-cancer therapies, such as chemotherapy and radiotherapy. The present bispecific antibodies can be administered to the mammal directly, or by administering to the mammal a

nucleic acid sequence encoding the bispecific antibodies, such nucleic acid sequence may be carried by a vector.

[0103] The exact amount of the bispecific antibodies, polynucleotides, vectors, or host cells or compositions thereof required to elicit the desired effects will vary from mammal to mammal, depending on the species, age, gender, weight, and general condition of the mammal, the particular bispecific antibodies, nucleic acid molecules, vectors, or cells used, the route of administration, and whether other drugs are included in the regimen. Thus, it is not possible to specify an exact amount for every composition. However, an appropriate amount can be determined by one of ordinary skill in the art using routine experimentation. Dosages can vary, and the antibodies can be administered in one or more (e.g., two or more, three or more, four or more, or five or more) doses daily, for one or more days. Guidance in selecting appropriate doses for antibodies can be readily found in the literature.

[0104] The present invention also provides kits comprising the bispecific antibodies, polynucleotides, vectors, or host cells, or compositions thereof. The kit can include a separate container containing a suitable carrier, diluent, or excipient. The kit also can include an adjuvant, cytokine, active agent, immunoassay reagents, PCR reagents, radiolabels, and the like. Additionally, the kit can include instructions for mixing or combining ingredients and/or methods of administration.

[0105] In one embodiment, the present invention provides a recombinant bispecific antibody, comprising

[0106] (i) a first polypeptide comprising from N- to C-terminus a first light chain variable domain, a human light chain constant region (CL), a human heavy chain constant region 2 (CH2) and a human heavy chain constant region 3 (CH3);

[0107] (ii) a second polypeptide comprising from N- to C-terminus a first heavy chain variable domain, a human heavy chain constant region 1 (CH1), a human heavy chain constant region 2 (CH2) and a human heavy chain constant region 3 (CH3); and

[0108] (iii) a second light chain variable domain connected to the first light chain variable domain by a first linker, and a second heavy chain variable domain connected to the first heavy chain variable domain by a second linker,

[0109] wherein the first light chain variable domain and the first heavy chain variable domain confer binding specificity to a first antigen, the second light chain variable domain and the second heavy chain variable domain confer binding specificity to a second antigen.

[0110] In one embodiment, the present invention provides a recombinant bispecific antibody, comprising

[0111] (i) a first polypeptide comprising from N- to C-terminus a first light chain variable domain, a human light chain constant region (CL), a human heavy chain constant region 2 (CH2) and a human heavy chain constant region 3 (CH3);

[0112] (ii) a second polypeptide comprising from N- to C-terminus a first heavy chain variable domain, a human heavy chain constant region 1 (CH1), a human heavy chain constant region 2 (CH2) and a human heavy chain constant region 3 (CH3); and

[0113] (iii) a second light chain variable domain connected to the C-terminus of the CH3 of the first polypeptide by a first linker, and a second heavy chain variable domain connected to the C-terminus of the CH3 of the second polypeptide by a second linker,

[0114] wherein the first light chain variable domain and the first heavy chain variable domain confer binding specificity to a first antigen, the second light chain variable domain and the second heavy chain variable domain confer binding specificity to a second antigen.

[0115] In one embodiment, the amino acid sequences of the heavy or light chain variable domains of the present bispecific antibody are derived from human. Alternatively, the heavy or light chain variable domains are derived from non-human species. In yet another embodiment, the heavy or light chain variable domains can be derived from humanized antibodies. As used herein, “humanized antibody” refer to a type of recombinant antibody resulted from combining a human antibody with a small part of a mouse or rat monoclonal antibody. The mouse or rat part of the antibody binds to the target antigen, whereas the human part makes it less likely to be destroyed by the body's immune system. One of ordinary skill in the art would readily construct humanized antibodies using standard techniques in the art.

[0116] In one embodiment, the present bispecific antibodies are designed to bind to a first antigen on target cells and to a second antigen on immune cells such as T cells. Examples of the first antigen on target cells include, but are not limited to, EpCAM, HER2, PSMA, gpA33, CD276, EGFR, CEA, CD19, CD20, CD22, CD30, CD33, CD123, FLT3 and BCMA.

Examples of the second antigen on immune cells include, but are not limited to, CD3, FcγRI, and FcγRIIIa.

[0117] In one embodiment, one or both of the human CH3 domains of the present bispecific antibodies comprise one or more amino acid substitutions that confer decreased or abrogated homodimerization between the first and second polypeptides. For example, amino acid T366 of the one or both CH3 is substituted with D (Aspartic Acid), L (Leucine), W (Tryptophan) or N (Asparagine). In another embodiment, amino acid Y407 of the one or both CH3 is substituted with I (Isoleucine), F (Phenylalanine), L (Leucine), M (Methionine), H (Histidine), K (Lysine), S (Serine), Q (Glutamine), T (Threonine), W (Tryptophan), A (Alanine), G (Glycine) or N (Asparagine). In one embodiment, one or both of the CH3 domains of the present bispecific antibodies comprise an amino acid sequence of one of SEQ ID NOs:155-171.

[0118] In one embodiment, the first or second linker of the present bispecific antibodies comprises amino acid sequence of SEQ ID NO:125, 126 or 127. One of ordinary skill in the art would readily understand that the linkers need to have length, flexibility and orientations to a degree that allows stable interactions between the VH and VL domains that bind to a first antigen, and simultaneously, efficient blockade of binding to a second antigen.

[0119] In another embodiment, the first or second linker of the present bispecific antibodies comprises a protease cleavage site. For example, the protease cleavage site comprises amino acid sequence of one of SEQ ID NOs:58-63. Further examples of protease cleavage sites include, but are not limited to, for matrix metalloproteases (MMP): PLGLWA (SEQ ID NO:128), GPLGLWA (SEQ ID NO:129), GPLGLWAQ (SEQ ID NO:130), GVPDLGRFQTFE (SEQ ID NO:131), GVPDVGHFSLFP (SEQ ID NO:132), GVPDVGEFSLFP (SEQ ID NO:133), GVPDVGNFSLFP (SEQ ID NO:134), GVPDVGRFSLFP (SEQ ID NO:135), GVPDVGHYSLFP (SEQ ID NO:136), GVPDVGEYSLFP (SEQ ID NO:137), GVPDVGNYSLFP (SEQ ID NO:138), GVPDVGRYSLFP (SEQ ID NO:139); for urokinase (uPA): PRFKIIGG (SEQ ID NO:140), PRFRIIGG (SEQ ID NO:141); for TGFβ: SSRHRRALD (SEQ ID NO:142); for plasminogen: RKSSIIIRMRDVVL (SEQ ID NO:143); for staphylokinase: SSSFDKGKYKKGDDA (SEQ ID NO:144); for factor Xa: IEGR (SEQ ID NO:145), IDGR (SEQ ID NO:146), GGSIDGR (SEQ ID NO:147); for human liver collagen: GPLGIAGI (SEQ ID NO:148); for human α2M:

GPEGLRVG (SEQ ID NO:149); for human PZP: YGAGLGVV (SEQ ID NO:150), AGLGVVER (SEQ ID NO:151), AGLGISST (SEQ ID NO:152).

[0120] In one embodiment, the CH1 and CL of the present bispecific antibodies are connected to the CH2 of the first and second polypeptides via a linker that comprises a shortened hinge sequence as compared to a hinge sequence in normal human IgG.

[0121] In one embodiment, the recombinant bispecific antibody of the present invention comprises a polypeptide comprising amino acid sequence of one of SEQ ID NOs: 1-16. In another embodiment, the recombinant bispecific antibody of the present invention comprises a polypeptide that is at least 85%, 90%, 92%, 94%, 96%, 98% or 99% identical to one of the amino acid sequences of SEQ ID NOs: 1-16.

[0122] In one embodiment, the recombinant bispecific antibody of the present invention further comprise a heterodimerization motif or a long flexible motif, wherein the heterodimerization motif or long flexible motif is connected to the N terminus of the second light chain variable domain and the second heavy chain variable domain via one or more linkers. In one embodiment, such one or more linkers comprise a protease cleavage site. In one embodiment, the heterodimerization motif comprises a N-terminal heterodimerization motif of human HER2 transmembrane domain or a N terminal heterodimerization motif of human EGFR transmembrane domain. In another embodiment, the heterodimerization motifs must have fully human sequences for minimized immunogenicity, are short for small molecular size, and have sufficient heterodimerization strength to ensure efficient blockade of antigen binding activity. One of ordinary skill in the art would readily recognize heterodimerization motifs from human proteins other than EGFR and HER2.

[0123] In one embodiment, the long flexible motif comprises an amino acid sequence GGGGSGGGGS (SEQ ID NO:153). One of ordinary skill in the art would understand that the long flexible motifs must have length, flexibility and orientations to a degree that allows efficient interruption of antigen binding activity. Further examples of such flexible motifs include, but are not limited to, any repeats of the G4S sequence or other unstructured recombinant polypeptide such as XTEN (Schellenberger et al., Nature Biotechnology 2009, 27: 1186-1190).

[0124] In one embodiment, the recombinant bispecific antibody comprising such heterodimerization motif or long flexible motif contains a polypeptide comprising amino acid sequence of one of SEQ ID NOs: 17-20. In another embodiment, the recombinant bispecific antibody of the present invention comprises a polypeptide that is at least 85%, 90%, 92%, 94%, 96%, 98% or 99% identical to one of the amino acid sequences of SEQ ID NOs: 17-20.

Bispecific Antibodies With Modified Binding To CD3

[0125] In one embodiment, a light chain variable domain and a heavy chain variable domain of the recombinant bispecific antibody comprise modified sequences that confer reduced binding affinity to CD3 as compared to that mediated by unmodified sequences. In another embodiment, a light chain variable domain and a heavy chain variable domain of the recombinant bispecific antibody confer binding specificity to human CD3 and cynomolgus CD3. In one embodiment, such bispecific antibody comprises a polypeptide comprising amino acid sequence of one of SEQ ID NOs:50-57.

[0126] In one embodiment, the present invention also provides a polynucleotide encoding one or more polypeptides disclosed above. In another embodiment, the present invention also provides an expression vector comprising one or more of the above polynucleotides. In another embodiment, the present invention also provides a host cell comprising such expression vector.

[0127] In one embodiment, the present invention also provides a method of preparing the recombinant bispecific antibody disclosed herein, the method comprises the steps of: (a) culturing host cells comprising one or more polynucleotides encoding the present recombinant bispecific antibody under conditions wherein such recombinant bispecific antibody is expressed; and (b) recovering the recombinant bispecific antibody from the host cells.

Anti-EGFR Antibodies

[0128] In one embodiment, the present invention also provides an isolated anti-human EGFR (epidermal growth factor receptor) antibody comprising a heavy chain and a light chain. In one embodiment, the heavy chain comprises a complementarity determining region 1 (CDR1) comprising amino acid sequence SEQ ID NO:22, a CDR2 comprising amino acid sequence SEQ ID NO:23, and a CDR3 comprising amino acid sequence SEQ ID NO:24. In one embodiment, the heavy chain comprises amino acid sequence SEQ ID NO:21 (the VH

domain). In another embodiment, the heavy chain comprises amino acid sequence SEQ ID NO:44 (a full length heavy chain). The above sequences are derived from antibody designated as SMET5.

[0129] In another embodiment, the light chain of the anti-human EGFR antibody comprises a CDR1 comprising amino acid sequence SEQ ID NO:26, a CDR2 comprising amino acid sequence SEQ ID NO:27, and a CDR3 comprising amino acid sequence SEQ ID NO:28. In one embodiment, the light chain comprises amino acid sequence SEQ ID NO:25 (the VL domain). In another embodiment, the light chain comprises amino acid sequence SEQ ID NO:45 (a full length light chain). The above sequences are derived from antibody designated as SMET5.

[0130] In another embodiment, the light chain of the anti-human EGFR antibody comprises a CDR1 comprising amino acid sequence SEQ ID NO:31, a CDR2 comprising amino acid sequence SEQ ID NO:32, and a CDR3 comprising amino acid sequence SEQ ID NO:33. In one embodiment, the light chain comprises amino acid sequence SEQ ID NO:30 (the VL domain). In another embodiment, the light chain comprises amino acid sequence SEQ ID NO:47 (a full length light chain). The above sequences are derived from antibody designated as SMET5.2.

[0131] In another embodiment, the light chain of the anti-human EGFR antibody comprises a CDR1 comprising amino acid sequence SEQ ID NO:36, a CDR2 comprising amino acid sequence SEQ ID NO:37, and a CDR3 comprising amino acid sequence SEQ ID NO:38. In one embodiment, the light chain comprises amino acid sequence SEQ ID NO:35 (the VL domain). In another embodiment, the light chain comprises amino acid sequence SEQ ID NO:49 (a full length light chain). The above sequences are derived from antibody designated as SMET5.3.

[0132] In another embodiment, the light chain of the anti-human EGFR antibody comprises a CDR1 comprising amino acid sequence SEQ ID NO:41, a CDR2 comprising amino acid sequence SEQ ID NO:42, and a CDR3 comprising amino acid sequence SEQ ID NO:43. In one embodiment, the light chain comprises amino acid sequence SEQ ID NO:40 (the VL domain). The above sequences are derived from antibody designated as SMET5.4.

[0133] In another embodiment, the present invention also provides a composition comprising a physiologically acceptable carrier and a therapeutically effective amount of any one of the anti-human EGFR antibodies described above.

[0134] The invention being generally described will be more readily understood by reference to the following examples that are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

[0135] Throughout this application, various references or publications are cited. Disclosures of these references or publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

EXAMPLE 1

Materials And Methods

Cells, Proteins, Plasmids And Other Reagents

[0136] 293 free style (293FS) cells and protein A agarose were purchased from ThermoFisher Scientific. HCT116, HT29 and SW480 colon cancer cell lines were purchased from Sigma. Other cell lines were purchased from ATCC. Recombinant human EGFR, Fc gamma receptor (FcγR), and urokinase (uPA) were products of Sino Biological and recombinant human MMP-7 protease was a product of Biolegend. The pDin1 vector used for mammalian expression was synthesized and assembled by overlapping PCR and ligation. The vector contains two transcription units and a built-in human IgG1 Fc gene fragment without introns that facilitates cloning of IgG1 or Fc-fusion proteins. Horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Fc-specific) antibody was a product of Sigma. Goat anti-human IgG (Fc-specific)-FITC conjugate was purchased from ThermoFisher Scientific.

Cloning Of EGFR×CD3 Bispecific Antibodies

[0137] The following primers were used:

[0138] OKTVLF, (sense) (SEQ ID NO:68); OKTVLR, (antisense) (SEQ ID NO:69)

[0139] CKF1, (sense) (SEQ ID NO:70); CKR1, (antisense) (SEQ ID NO:71)

[0140] MFCPAF, (sense) (SEQ ID NO:72); AAAR, (antisense) (SEQ ID NO:73)

[0141] OKT3VHF, (sense) (SEQ ID NO:74); OKT3VHR, (antisense) (SEQ ID NO:75)

[0142] CHMFCF, (sense) (SEQ ID NO:76); CHMFCR, (antisense) (SEQ ID NO:77)

[0143] bnIgG20H1, (sense) (SEQ ID NO:78)

[0144] 12VHF, (sense) (SEQ ID NO:79); 12VHR, (antisense) (SEQ ID NO:80)

[0145] 12VLF, (sense) (SEQ ID NO:81); 12VLR, (antisense) (SEQ ID NO:82)

[0146] bnIgG20L1, (sense) (SEQ ID NO:83)

[0147] CHF, (sense) (SEQ ID NO:84); CHF1, (sense) (SEQ ID NO:85); CHF2, (sense) (SEQ ID NO:86)

[0148] H12LR, (antisense) (SEQ ID NO:87)

[0149] 1S1F, (sense) (SEQ ID NO:88); 1S2F, (antisense) (SEQ ID NO:89)

[0150] HEHR, (antisense) (SEQ ID NO:90); HEHF, (sense) (SEQ ID NO:91)

[0151] HEHR1, (antisense) (SEQ ID NO:92); HELR, (antisense) (SEQ ID NO:93)

[0152] HELF, (sense) (SEQ ID NO:94); HELR1, (antisense) (SEQ ID NO:95)

[0153] GSHR, (antisense) (SEQ ID NO:96); GSHF, (sense) (SEQ ID NO:97)

[0154] GSLR, (antisense) (SEQ ID NO:98); GSLF: (sense) (SEQ ID NO:99)

Cloning of iBiTE

[0155] iBiTE was cloned as follows. To clone the light chain of iBiTE, the VL domain of humanized anti-CD3 antibody OKT3 (hOKT3), the human antibody kappa light chain constant domain (CK), and a monomeric human IgG1 Fc (mFc7.2)-poly A signal sequence were PCR amplified with primer pairs OKTVLF/OKTVLR, CKF1/CKR1 and MFCPAF/AAAR, and their encoding plasmids as templates, respectively. hOKT3 VL was joined to the 5' end of CK gene fragment by using overlapping PCR with the two gene fragments in the same molarities for 7 cycles in the absence of primers and 15 additional cycles in the presence of primers OKTVLF and CKR1. In the same way, a gene fragment (Hleader) encoding a leader peptide was fused to the 5' end of the hOKT3 VL-CK fragment by overlapping PCR with primers bnIgG20H1 and CKR1. The Hleader-hOKT3 VL-CK was further linked to the 5' end of the mFc7.2-poly A by overlapping PCR with primers bnIgG20H1 and AAAR. The final PCR product was digested with XbaI and SalI and cloned into pDin1.

[0156] To clone the heavy chain of iBiTE, the hOKT3 VH and human IgG1 CH1-mFc7.2 gene fragments were PCR amplified with primer pairs OKT3VHF/OKT3VHR and CHMFCF/CHMFCR, respectively. The OKT3VHF primer carries the HindIII and SacI restriction sites which will be used for subcloning. The hOKT3 VH gene fragment was fused

to the 5' end of CH1-mFc7.2 by overlapping PCR with primers OKT3VHF and CHMFCR. The product was digested with HindIII and EcoRI and cloned into the pDin1 construct containg iBiTE light chain. A gene fragment encoding a leader peptide (Lleader) and an anti-EGFR scFv was obtained by digestion of a previously constructed plasmid with HindIII and SacI, and then cloned into the plasmid containing the full-length light chain and partial heavy chain of iBiTE, leading to the final iBiTE construct.

Cloning of HBiTE

[0157] To clone HBiTE, the VH and VL domains of anti-EGFR antibody were PCR amplified with primer pairs 12VHF/12VHR and 12VLF/12VLR, respectively. The PCR products were fused to the 3' end of Lleader and Hleader by overlapping PCR with primer pairs bnIgG20L1/12VHR and bnIgG20H1/12VLR, respectively. The Hleader-VL gene fragment was digested with XbaI and BamHI and cloned into an iBiTE-derived vector with HindIII and BamHI restriction sites introduced into the 5' end of the hOKT3 VL domain. The Lleader-VH gene fragment was then further cloned into the construct containing the Hleader-VL insert via the HindIII and SacI restriction sites.

Cloning of proBiTE Variants

[0158] The proBiTE variants were cloned by using HBiTE as a template. For cloning of proBiTE-1, proBiTE-2 and proBiTE-12, the hOKT3 VL-CK-mFc7.2-poly A gene fragments were PCR amplified with HBiTE as a template and primer pairs CHF/AAAR, CHF1/AAAR and CHF2/AAAR, respectively. The PCR products were digested with BamHI and SalI and cloned into HBiTE.

Cloning of proBiTE-1s

[0159] For cloning of proBiTE-1s, the Hleader-VL fragment was PCR amplified with primers bnIgG20H1 and H12LR. The PCR product was digested with XbaI and BamHI and cloned into proBiTE-1 linearized with the same restriction enzymes. proBiTE-1s1 and proBiTE-1s2 were cloned the same way as proBiTE-1 was generated except that primer pairs 1S1F/AAAR and 1S2F/AAAR were used for PCR amplification of the hOKT3 VL-CK-mFc7.2-poly A gene fragments.

Cloning of dproBiTE-HE

[0160] To clone dproBiTE-HE, Lleader and anti-EGFR VH domain were PCR amplified with primer pairs bnIgG20L1/HEHR and HEHF/HEHR1, respectively. Lleader gene fragment was then fused to the 5' end of anti-EGFR VH by overlapping PCR with primers bnIgG20L1 and HEHR1. The full-length Lleader-anti-EGFR VH gene fragment was digested with HindIII and SacI and cloned into proBiTE-1s1. The Hleader-anti-EGFR VL gene fragment was obtained and cloned in the same way except the use of bnIgG20H1/HELR and HELF/HELR1 primer pairs for primary amplification and XbaI and BamHI restriction sites for cloning.

Cloning of dproBiTE-GS

[0161] To clone dproBiTE-GS, Lleader and anti-EGFR VH domain were PCR amplified with primer pairs bnIgG20L1/GSHR and GSHF/HEHR1, respectively. Lleader gene fragment was then fused to the 5' end of anti-EGFR VH by overlapping PCR with primers bnIgG20L1 and HEHR1. The full-length Lleader-anti-EGFR VH gene fragment was digested with HindIII and SacI and cloned into proBiTE-1s1. The Hleader-anti-EGFR VL gene fragment was obtained and cloned in the same way except the use of bnIgG20H1/GSLR and GSLF/HELR1 primer pairs for primary amplification and XbaI and BamHI restriction sites for cloning.

Protein Expression And Purification

[0162] All bispecific antibodies were expressed in 293FS cells as described previously (Chen et al., Proc Natl Acad Sci USA 2008, 105: 17121-17126) and purified from the 293FS culture supernatant using Protein A Sepharose 4 Fast Flow column chromatography (GE Healthcare) according to the manufacturer's instructions.

Protease Cleavage

[0163] For cleavage with urokinase (uPA), five μg antibodies were mixed with or without 1 μg recombinant human uPA (Sino Biological) in 15 μl PBS (pH7.4) and incubated at room temperature for 1 h. For cleavage with MMP-7, recombinant human MMP-7 (Biolegend) was diluted in the assay buffer (50 mM Tris, 10 mM CaCl2, 150 mM NaCl, 0.05% Brij-35, pH7.5) and activated by adding 4-aminophenylmercuric acetate (APMA) to a final concentration of 1 mM and incubating the mixture for 30 min at 37°C. Then, 4 μg antibodies were mixed with or without 0.5 μg activated MMP-7 in 20 μl assay buffer and incubated at 37°C for 1 h.

ELISA

[0164] ELISA was performed according to standard protocols. Briefly, recombinant human FcγRs (Sino Biological) were coated on Corning EIA/RIA high-binding 96-well plates (Corning Inc.) at 50 ng per well overnight at 4°C and blocked with 3% nonfat milk in PBS (pH7.4). Fivefold serially diluted biotinylated antibodies were added and incubated at room temperature for 2 h. The plates were washed with PBS containing 0.05% Tween 20. Bound antibodies were detected by HRP-conjugated streptavidin (ThermoFisher Scientific). The assay was developed at room temperature with TMB substrate (Sigma-Aldrich) and monitored at 450 nm with a microplate reader. The half-maximal binding (EC_{50}) was calculated by fitting the data to the Langmuir adsorption isotherm.

Flow Cytometry

[0165] About 5×10^5 cells were incubated with antibodies on ice for 1 h. The cells were washed once with PBS containing 0.1% bovine serum albumin (PBSA) and resuspended in 100 µl PBSA. Then 1 µl goat anti-human IgG (Fc-specific)-FITC conjugate (Invitrogen) was added and incubated for 30 min. The cells were washed once with PBSA and then used for flow cytometry analysis.

Size-Exclusion Chromatography

[0166] A Superdex200 10/300 GL column (GE Healthcare) was calibrated with protein molecular mass standards of carbonic anhydrase (29 kDa), ovalbumin (44 kDa), conalbumin (75 kDa), aldolase (158 kDa) and ferritin (440 kDa). Purified proteins at a concentration of 1 mg mL^{-1} in PBS (pH7.4) were loaded onto the pre-equilibrated column and eluted with PBS (pH7.4) at 0.5 mL/min.

T Cell Activation Assay

[0167] Target cells were plated on 96-well plates at a density of 2×10^4 cells in 25 µl RPMI1640 complete medium per well. Fifty µl antibodies 5-fold serially diluted were added into each well. Then effector cells (Jurkat NFAT-Luc2, Promega) were added at a density of 1×10^5 cells in 25 µl RPMI1640 complete medium per well to make a target : effector cell ratio of 1 : 5. The assay was developed after 5-h incubation by using the Promega Bio-Glo Luciferase Assay System according to the manufacture's instructions.

In Vitro Killing Assay

[0168] Target cells (2×10^4) stably transfected with red firefly luciferase gene were seeded in 100 µl RPMI 1640 complete medium overnight. Meanwhile, 2.5×10^7 frozen PBMC purchased from STEMCELL Technologies were revived and inoculated in 10 mL RPMI 1640 complete medium containing 50 U/mL IL-2 (Sigma) overnight. The second day, the medium was removed from target cells and 2×10^5 PBMC in 50 µl RPMI 1640 complete medium (actual target : effector ratio = 1 : 5 because target cells duplicate overnight) were added. Then, 50 µl antibodies 5-fold serially diluted from 10 nM were added into each well. 72 h after incubation, cell killing activity was measured by using the Promega Bio-Glo Luciferase Assay System according to the manufacture's instructions.

Pharmacokinetic Measurment In Mice

[0169] NOD/SCID mice were administered intravenously with 500 µg antibody on day 0. Plasma samples were collected on day 1, 3, 5 and 6 or 7 or 8 and used for measurement of antibody serum concentrations by ELISA with standard curves generated using the original antibody stocks.

In-Vivo Tumor Growth Inhibition

[0170] SCID mice were inoculated with a mixture of 2×10^6 HCT116 cells and 10^7 freshly isolated human PBMC subcutaneously into the left flank of the mice. The mice were dosed intravenously with PBS (control) or 20 µg antibody 6 h later and then every other day for 6 doses. After two weeks of treatment, mice were sacraficed and tumor weights were measured. Tumor growth inhibition rates were calculated by using the following formula: average weight of PBS group – average weight of antibody treated group/average weight of PBS group.

EXAMPLE 2

Rational Design Of Protease Cleavable Bispecific T Cell Engagers (proBiTE) With Sterically Restricted Access Of One Antigen Binding Site

[0171] Bispecific T cell engager (BiTE) is a novel class of bispecific antibodies that can guide cytotoxic T cells to kill cancer cells by simultaneously binding to a tumor antigen and the CD3 molecule on T cell surface. However, expression of some tumor antigens in normal tissues manifests dose-limiting on-target toxicity. Off-target toxicity such as cytokine release syndrome also limits their clinical use. It is hypothesized that multiple strategies aimed

at increasing tumor selectivity could be combined to minimize such toxicities and enlarge therapeutic windows of this class of bispecific antibodies. These strategies include optimization of antibody affinity for both tumor antigen and CD3, introduction of second tumor specificity, and minimization of FcR binding activity when Fc is included in BiTE for long half-life *in vivo*.

[0172] Some of the above goals could be accomplished by rational design of molecular structures of bispecific antibodies. For example, some proteases are highly up-regulated in a variety of tumors and play a critical role in cancer invasion and metastasis. Expression of these proteases and their proteolytic activities in healthy tissues are minimal. Therefore, employing tumor-associated proteases in the design of BiTE could be helpful to increase tumor selectivity leading to the generation of prodrugs for cancer therapy.

[0173] To test this possibility, a novel class of proBiTE with sterically restricted access of one antigen binding site was designed, in which the VH and VL domains of the first antibody are fused to the N terminus of the VH and VL domains of a second antibody Fab via a protease non-cleavable polypeptide linker or a protease cleavable polypeptide linker. The second antibody Fab is further fused to the N terminus of a human antibody Fc region which contains mutations capable of decreasing or abrogating Fc homodimerization via a polypeptide linker without protease cleavage site (Figure 1A).

[0174] In another embodiment of proBiTE, the VH and VL domains of the first antibody are fused to the C teminus of a human antibody Fc region which contains mutations capable of decreasing or abrogating Fc homodimerization via a polypeptide linker with or without protease cleavage site. The human Fc region is further fused to the C terminus of a second antibody Fab via a polypeptide linker without protease cleavage site (Figure 1B). proBiTE exhibits monovalent binding for each antibody and therefore, should not nonspecifically activate T cells before engagement with tumor cells.

[0175] A mechanism of action of the proBiTE is proposed as follows: in the absence of tumor-associated proteases, proBiTE binds to one antigen (antigen 1) but not or only weakly to the other antigen (antigen 2) due to steric hindrance caused by the polypeptide linkers connecting the two antibodies (Figure 2A). In the tumor microenvironment where protease expression is up-regulated, efficient cleavage of the polypeptide linker by the proteases

resolves the steric hindrance allowing proBiTE to bind to the other antigen (antigen 2) and exert its functions. Some tumor-associated proteases are secreted into the circulation as active soluble proteins. In one design of proBiTE, the VH and VL domains of the tumor antigen-binding antibody are fused to the N terminus of the VH and VL domains of the Fab of a CD3 antibody via a protease non-cleavable and cleavable polypeptide linker, respectively. Because cooperation between VH-VL and CH1-CL interface is required for mutual stabilization of the domains, the integrity and activity of proBiTE should be compromised when the antibody is cleaved in the circulation due to unstable VH-VL interaction of the tumor antigen-binding antibody (Figure 2B, left panel). In tumor tissues, however, proBiTE bound to tumor antigens could be stabilized due to cooperation between the VH-VL and antibody-antigen interface, leading to retained full function of the bispecific antibodies even after cleavage by proteases (Figure 2B, right panel). It is therefore hypothesized that this unique proBiTE design could further decrease on-target toxicity of this class of bispecific antibodies.

[0176] In summary, as compared to published monovalent bispecific antibodies developed by others, the bispecific antibodies of the present invention have the distinctive features of using (i) monomeric Fc domain, for example, human IgG1 Fc, to extend *in vivo* half-life, and (ii) only two polypeptide chains and employ both Fab and Fv for heterodimerization and antigen recognition. A combination of the features mentioned herein results in excellent properties for the present bispecific antibodies such as 100% heterodimer, high yield, high stability and low aggregation propensities.

EXAMPLE 3

Generation And Initial Characterization Of EGFR×CD3 Bispecific Antibody

[0177] To provide a proof-of-concept and test the above hypothesis, a proBiTE targeting EGFR and CD3 was generated. EGFR and CD3 were used as examples here and below. One of ordinary skill in the art would recognize that other T and non-T cell target antigens can be readily employed as described herein.

[0178] In one embodiment, the VH and VL domains of an anti-EGFR antibody were fused to the N terminus of the VH and VL domains of an anti-CD3 antibody Fab via a protease non-cleavable (G4S)3 linker and a protease cleavable linker, respectively. The anti-CD3 Fab was further fused to the N terminus of a monomeric human IgG1 Fc (mFc7.2) (SEQ ID NO:172) which contained two amino acid mutations (T366L/Y407H) capable of decreasing Fc

homodimerization (Figure 3). mFc7.2 exists as a monomer at low concentrations (<0.5 mg/mL) but tends to form a dimer at higher concentrations. It is therefore conceivable that when the heavy and light chains of proBiTE are assembled, mFc7.2 could form a dimer due to high local concentrations and further stabilize the bispecific antibodies.

[0179] In one embodiment, three proBiTE variants were generated. proBiTE-1 contains a cleavable polypeptide linker composed of the GGGGSLSGRSDNHGGGGS sequence (SEQ ID NO:58) (underlined is the substrate of uPA, matriptase and legumain). proBiTE-2 contains a cleavable linker composed of the GGGGSGPLGLARKGGGGS sequence (SEQ ID NO:59) (underlined is the substrate of MMP-7). The substrate sequences for both classes of proteases were combined in proBiTE-12, resulting in a dual cleavable linker composed of the GGGGSLSGRSDNHGPLGLARK sequence (SEQ ID NO:60).

[0180] As a negative control, an H-shaped BiTE (HBiTE) was generated, in which the cleavable linker was replaced with the non-cleavable (G4S)3 linker (see Figure 3). For positive control, an i-shaped BiTE (iBiTE) was designed where the scFv of anti-EGFR antibody was fused via the (G4S)3 linker to the N terminus of the VH-CH1 of anti-CD3 antibody (Figure 3). In this configuration, binding to CD3 is not sterically restricted by the linker.

[0181] All the bispecific antibodies were well expressed in transiently transfected 293 free style (293FS) cells and secreted into the culture supernatants. On a non-reducing SDS-PAGE, a vast majority of the purified antibodies migrated as a heterodimer with apparent molecular weight (aMW) of approximately 115 kDa (Figure 4). On a reducing SDS-PAGE, the two polypeptide chains of iBiTE were well separated while those of other bispecific antibodies overlapped with each other with apparent molecular weight of approximately 70 kDa.

[0182] proBiTE-1 and proBiTE-12 were efficiently cleaved by uPA, resulting in an isolated VL domain of the anti-EGFR antibody while other bispecific antibodies were not sensitive to the protease (Figure 5, left panel). Similarly, proBiTE-2 and proBiTE-12 were cleavable when incubated with MMP-7 while others did not appear to be specifically cleaved at the linker position (Figure 5, right panel). Several unexpected products were observed on the reducing SDS-PAGE, suggesting there could be other cleavage sites for MMP-7 in the antibodies. Size-exclusion chromatography analysis showed that the vast majority of purified

bispecific antibodies migrated as a monomer with aMW of approximately 120 kDa, similar to their calculated molecular weight (cMW) (Figure 6).

EXAMPLE 4

Binding Affinity Of Bispecific Antibody For Cell Surface-Associated EGFR And CD3

[0183] The monovalent and bivalent cell-binding affinities of the anti-EGFR and anti-CD3 antibodies were measured. The monovalent bispecific antibody iBiTE bound to an EGFR-expressing human colon cancer cell line HCT116 with EC_{50} of 97 nM and to a CD3-expressing human T cell line Jurkat with EC_{50} of 30 nM, suggesting moderate affinities of the EGFR and CD3 antibodies (Figure 7). The bivalent monospecific antibodies, anti-EGFR IgG1 and anti-CD3 scFv-Fc, bound much more strongly to HCT116 and Jurkat cell lines with EC_{50}s of 6.9 and 0.38 nM, respectively, suggesting high avidity effect of bivalent binding.

[0184] Next, the bindings of bispecific antibodies with different structures and composition of linkers to EGFR and CD3 were examined. At a concentration of 2 μg/mL, none of the bispecific antibodies interacted with CHO cells which do not express human EGFR and CD3, whereas iBiTE bound to both EGFR-expressing HCT116 and CD3-expressing Jurkat cells, suggesting specific binding activity of the anti-EGFR and anti-CD3 antibodies (Figure 8). In contrast, HBiTE bound to HCT116 cells as well as iBiTE while no binding to Jurkat cells was observed, suggesting that binding activity of the anti-CD3 antibody was completely abrogated at the concentration tested. The three proBiTE variants exhibited various binding activities with both cell lines, probably due to different flexibility and orientations of the cleavable linkers.

[0185] The bindings of pre-cleaved proBiTE-1 to EGFR and CD3 were then examined. The results showed that the binding of iBiTE, which has fully open antigen binding sites, to HCT116 and Jurkat cells was not affected by uPA. proBiTE-1 pre-cleaved by uPA exhibited decreased binding to EGFR-expressing HCT116 cells while restored binding activity with CD3-expressing Jurkat cells compared to iBiTE at an antibody concentration of 2 μg/mL (Figure 9). When different antibody concentrations were tested, proBiTE-1 bound to EGFR-expressing HCT116 cells slightly better than iBiTE at concentrations lower than 200 nM (Figure 10). After cleavage by uPA, binding of proBiTE-1 to HCT116 cells was decreased by approximately two-fold and was similar to that of iBiTE. In contrast, proBiTE-1 bound to CD3-

expressing Jurkat cells about 30-fold more weakly than iBiTE. Protease cleavage restored the binding activity of proBiTE-1 with Jurkat cells.

[0186] Whether the VH and VL domains of the EGFR antibody in proBiTE-1 pre-bound to cells could be stabilized was tested. As expected, pre-cleaved proBiTE-1 had decreased binding to HCT116 cells compared to the intact proBiTE-1 while proBiTE-1 pre-bound to the cells and then cleaved by uPA restored binding activity at a concentration of 2 μg/mL (Figure 11). These results are in agreement with the hypothesis that employing naturally unstable VH-VL domains as the tumor antigen binding antibody could further decrease on-target toxicity of this class of bispecific antibodies.

EXAMPLE 5

Bispecific Antibody-Mediated T Cell Activation

[0187] The ability and specificity of proBiTE to activate human T cells in the presence of EGFR-expressing cells were evaluated by using Promega T cell activation bioassay systems. Of the 6 target cell line tested, CHO cells do not express human EGFR while A431 cells has a high level of EGFR expression and has been frequently used as a model human cell line for drug discovery (Figure 12). The three human colon cell lines, HCT116, HT29 and SW480, express a similar level of EGFR while EGFR expression was not detectable on SW620 cells at an antibody concentration of 2 μg/mL.

[0188] In the presence of EGFR-positive A431 cells, human T cells were efficiently activated by iBiTE with EC_{50} of approximately 80 pM (Figure 13). With EGFR-negative CHO cells, however, human T cells were not or only marginally activated at high concentrations, suggesting high specificity of the bispecific antibody. In the presence of HCT116 cells, iBiTE efficiently activated human T cells whereas HBiTE did not have an effect on the T cells likely due to blockade of CD3 binding (Figure 14). proBiTE-1 gave a low level of T cell activation. Surprisingly, protease cleaved proBiTE-1 was less efficient in T cell activation than intact proBiTE-1 likely because of deteriorated interaction between the VH and VL domains of the EGFR-binding antibody in cleaved proBiTE-1. Results with proBiTE-2 and proBiTE-12, for which CD3 binding was not or only partially blocked, showed better T cell activation activity than proBiTE-1. Similar results were observed with HT29 cells except that proBiTE-1 failed to activate the T cells while apparent activation was seen with cleaved proBiTE-1. This is in

contrast to the result from HCT116 cells, suggesting that HCT116 cells might express a higher level of proteases than HT29 cells.

EXAMPLE 6

Bispecific Antibody-Mediated Killing Of Human Colon Cancer Cell Lines

[0189] HCT116 and HT29 cells are adherent and fibroblastic, and grow in a monolayer in RPMI 1640 medium while PBMC cells are round-shaped and grow in suspension. Engagement with the cancer cells caused aggregation and activation of T cells, eventually leading to killing of the cancer cells. Under a microscope, iBiTE and proBiTE-12 appeared to completely kill HCT116 cells at a concentration of as low as 3.2 pM (Figure 15). HBiTE was much less potent exhibiting apparent killing only at high concentrations. The killing efficiency of proBiTE-1 and proBiTE-2 was comparable and was about 5-fold lower than that of iBiTE and proBiTE-12. Similar results were obtained with HT29 cells (Figure 16).

[0190] To quantify cell killing efficiency, HT29 cells stably transfected with red firefly luciferase gene were used as target cells. The results showed that iBiTE and proBiTE-12 exhibited comparably potent killing efficacy with EC_{50} of approximately 6 pM (Figure 17). HBiTE was able to kill the cancer cells at high concentrations but was about 300-fold less potent (EC_{50}, 2000 pM) than iBiTE. With a protease cleavage site in the linker, proBiTE-1 gave an EC_{50} of about 90 pM, 15-fold higher than that of iBiTE likely due to inefficient cleavage and degradation of the former during the long period of incubation. The pre-cleaved proBiTE-1 was more potent than the intact proBiTE-1 but was still several-fold less potent than iBiTE, likely due to deteriorated interaction between the VH and VL domains of the EGFR-binding antibody in pre-cleaved proBiTE-1. None of the bispecific antibodies significantly inhibited the proliferation of SW620 cells at the concentrations tested, suggesting EGFR expression-dependent killing of HT29 cells (data not shown).

EXAMPLE 7

Binding Of Bispecific Antibody To Recombinant Human FcγRs

[0191] BiTE containing Fc could cause unwanted cytotoxicity of FcR-expressing cells. Because the hinge region of human IgG1 is directly or indirectly involved in Fc binding to FcγRs, it is hypothesized that the use of a shortened hinge sequence as the linker between the anti-CD3 Fab and mFc could lead to decreased FcγR binding due to steric hindrance and/or other mechanisms. To evaluate such a possibility with proBiTE, part (CPPCP (SEQ ID

NO:154)) of the human IgG1 hinge sequence (DKTHTCPPCP) (SEQ ID NO:64) was used as the linker (see Figure 3) and the resulting proBiTE-1 was compared with anti-EGFR IgG1 for binding to different FcγRs. The former showed decreased interactions with FcγRIIIa (by 10-fold) and FcγRI (by 3-fold) (Figure 18). Neither anti-EGFR IgG1 nor proBiTE-1 bound to FcγRIIa and FcγRIIb as measured by ELISA. These results indicate that proBiTE could be less toxic against FcγRIIIa- and FcγRI-expressing cells than those BiTEs which use conventional IgG1 Fc and full-length hinge sequence.

EXAMPLE 8

Inhibition Of Tumor Growth In NOD/SCID Mice Engrafted With Human PBMC

[0192] To test how proBiTE could inhibit tumor growth in vivo, NOD/SCID mice engrafted with human PBMC were used as a model. Pharmacokinetics analysis showed that iBiTE and HBiTE had relatively higher serum concentrations than proBiTE-1 and proBiTE-12 at almost all time points with proBiTE-12 showing the fastest clearance (Figure 19). The level of proBiTE-12, which has two different protease substrate sequences in the linker, declined at a higher rate than that of proBiTE-1 with only a single protease substrate sequence. These results suggest that introduction of protease cleavable linkers could decrease the stability of the bispecific antibodies in the circulation. In the tumor challenge experiments, proBiTE-12 potently inhibited the growth of HCT116 cells in the animals (Figure 20).

EXAMPLE 9

Generation And Characterization Of Bispecific Antibody Variants With Altered Length And Composition Of Cleavable Linkers

[0193] To test whether alteration of the length and composition of the cleavable linker could affect the protease cleavage efficiency, cell killing activity and other properties of the bispecific antibodies, three proBiTE-1 variants were generated. proBiTE-1s contains a shortened cleavable polypeptide linker composed of the GSLSGRSDNHGGGGS sequence (SEQ ID NO:61) (underlined is the substrate of uPA, matriptase and legumain). Substitution of the L residue in the substrate sequence of proBiTE-1s with the G residue led to the second variant, proBiTE-1s1 (linker sequence, GSGSGRSDNHGGGGS (SEQ ID NO:62)). The SG residues in the substrate sequence of proBiTE-1s can also be substituted by the GS residues, leading to the third variant, proBiTE-1s2 (linker sequence, GSGGSRSDNHGGGGS (SEQ ID NO:63)).

[0194] It was found that proBiTE-1s and proBiTE-1s1 were cleaved by uPA as efficiently as proBiTE-1 whereas proBiTE-1s2 was not sensitive to the protease (Figure 21). These results suggest that the second and third, but not the first, amino acid residues of the substrate sequence were critical for protease sensitivity. Size-exclusion chromatography revealed that the vast majority of the above purified proBiTE-1 variants migrated as a monomer with aMW of approximately 120 kDa, similar to their calculated molecular weight (cMW) (Figure 22). In flow cytometry analysis, all three proBiTE-1 variants showed slightly decreased binding activity with CD3-expressing Jurkat cells while their interactions with EGFR-expressing HCT116 were not affected (Figure 23). These results suggest that the length but not the amino acid residue substitutions could have an impact on the flexibility and orientations of the cleavable linker.

[0195] proBiTE-1 and proBiTE-1s showed similar killing efficiency, suggesting that shortening the cleavable linker does not have an effect on antibody function (Figure 24). proBiTE-1s1 killed HCT116 as efficiently as proBiTE-1s but the former showed slightly decreased (by 2-fold) efficiency with HT29 cells. In both cases, the activity of proBiTE-1s2 was significantly reduced compared to other variants, suggesting that the SG residues in the substrate sequence are crucial to the sensitivity of proteases, in agreement with the results from uPA cleavage (Figure 21).

EXAMPLE 10

Rational Design Of Bispecific Antibody With Sterically Restricted Access Of Dual Antigen Binding Sites (dproBiTE)

[0196] Having demonstrated protease-dependent functionality of proBiTE, dproBiTE with sterically restricted access of dual antigen binding sites was designed and evaluated. It is hypothesized that addition of a long flexible motif, for example, naturally occuring short heterodimerization peptides, to the N terminus of the antigen binding site which is not guarded in proBiTE, could cause steric occlusion of the antigen binding site (Figure 25). Protease cleavage of the cleavable linkers would reduce or eliminate the steric occlusion, leading to protease-dependent interaction with antigens (Figure 26).

EXAMPLE 11

Generation And Characterization Of EGFR×CD3 dproBiTE

[0197] To test the above hypothesis, two EGFR×CD3 dproBiTE, designated dproBiTE-HE and dproBiTE-GS were generated. dproBiTE-HE carries the N terminal GXXXG-like heterodimerization motif (LTSIISAVVG, (SEQ ID NO:65)) of human HER2 transmembrane domain and the N terminal GXXXG-like heterodimerization motif (SIATGMVG, (SEQ ID NO:66)) of human EGFR transmembrane domain, fused to the VH and VL domains of anti-EGFR antibody in proBiTE-1s1 via a non-cleavable and cleavable linker composed of the (G4S)3 and GGGGSGGGGSGRSDNH (SEQ ID NO:67) (underlined is the mutated substrate of uPA, matriptase and legumain) sequences, respectively (Figure 27). In dproBiTE-GS, the HER2 and EGFR heterodimerization mitifs were replaced with (G4S)2 sequence.

[0198] The two dproBiTEs were well expressed in transiently transfected 293 free style (293FS) cells and secreted into the culture supernatants. On a non-reducing SDS-PAGE, a vast majority of the purified antibodies migrated as a heterodimer with apparent molecular weight (aMW) of approximately 115 kDa (Figure 28A). On a reducing SDS-PAGE, the two polypeptide chains of dproBiTE overlapped with each other with apparent molecular weight slightly larger than those of proBiTE-1s1. Similar with proBiTE-1s1, dproBiTE-HE and dproBiTE-GS were efficiently cleaved by uPA, resulting in an isolated VL domain of the anti-EGFR antibody (Figure 28B). Size-exclusion chromatography showed that the vast majority of the purified bispecific antibodies migrated as a monomer with aMW of approximately 120 kDa, similar to their calculated molecular weight (cMW) (Figure 29). In flow cytometry analysis, dproBiTE-HE bound to EGFR-expressing HCT116 cells less strongly than proBiTE-1s1 and their binding to CD3-expressing Jurkat cells was not affected, as expected (Figure 30).

EXAMPLE 12

Generation of Anti-EGFR Antibodies

[0199] This example describes one embodiment of generating anti-EGFR (epidermal growth factor receptor) antibodies.

MATERIALS AND METHODS

Cells, Proteins, Plasmids And Other Reagents

[0200] 293 free style (293FS) cells and protein A agarose were purchased from ThermoFisher Scientific. CHO and A549 cells were purchased from ATCC. Recombinant human and cynomolgus EGFR were products of Sino Biological. Recombinant human EGF

was a product of AcroBiosystems. The pDin1 vector used for mammalian expression was synthesized and assembled by overlapping PCR and ligation. The vector contains two transcription units and a built-in human IgG1 Fc gene fragment without introns that facilitates cloning of IgG1 or Fc-fusion proteins. Horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Fc-specific) antibody and HRP-conjugated anti-FLAG antibody were products of Sigma. Anti-His-PE conjugate was purchased from Miltenyi Biotec. Goat F(ab')2 anti-human IgG (γ)-FITC conjugate was purchased from ThermoFisher Scientific.

Panning And Screening Of A Phage-Display Naïve Human Fab Library For Identification Of EGFR Antibodies

[0201] A large size (10^{11}) phage-display naïve human Fab library was constructed with peripheral blood B cells from about 100 healthy individuals according to previously published protocols (de Haard et al., J Biol Chem 1999, 274: 18218-18230). This library was used for selection of antibodies against recombinant human EGFR conjugated to magnetic beads (Dynabeads M-270 epoxy; DYNAL Inc.) as described previously (Zhu et al., J Virol 2006, 80:891-899) except 5, 1 and 0.1 μg of antigen were used in the first, second and third round of panning, respectively. Clones that bound to the antigen were identified from the third round of biopanning by using monoclonal phage ELISA as described (Zhu et al., J Virol 2006, 80:891-899).

Affinity Maturation Of SMET5

[0202] For affinity maturation of SMET5, a phage-display light-chain shuffling Fab library was constructed and panned, and SMET5 variants with higher binding activity were selected according to previously reported protocol (Zhu et al., J Infect Dis 2008, 197: 846-853).

Cloning Of Human IgG1 Of SMET5 Variants

[0203] The following primers were used:

[0204] ER1HF, (sense) (SEQ ID NO:100); ER1HR, (antisense) (SEQ ID NO:101)

[0205] ER1LF, (sense) (SEQ ID NO:102); ER1LR, (antisense) (SEQ ID NO:103)

[0206] ER12LF1, (sense) (SEQ ID NO:104); ER12LR1, (antisense) (SEQ ID NO:105)

[0207] ER12LF2, (sense) (SEQ ID NO:106); ER13LF, (sense) (SEQ ID NO:107)

[0208] bnIgG20H1, (sense) (SEQ ID NO:108); bnIgG20L1, (sense) (SEQ ID NO:109).

[0209] For cloning of SMET5 IgG1, SMET5 VH gene fragment was PCR amplified with primers ER1HF and ER1HR. A gene fragment (Hleader) encoding a leader peptide was fused to the VH by overlapping PCR with the two gene fragments in the same molarities for 7 cycles in the absence of primers and 15 additional cycles in the presence of primers bnIgG20H1 and ER1HR. The overlapping PCR product Hleader-VH was digested with XbaI and SacI and cloned into pDin1 vector with built-in gene sequence encoding the human IgG1 heavy chain constant regions. SMET5 L chain gene fragment was PCR amplified with primers ER1LF and ER1LR. A gene fragment (Lleader) encoding a leader peptide was fused to the L chain by overlapping PCR with primers bnIgG20L1 and ER1LR. The overlapping PCR product Lleader-L was digested with HindIII and EcoRI and cloned into the pDin1 construct containing the heavy chain of SMET5 IgG1.

[0210] For cloning of SMET5.2 IgG1, the N and C terminal portions of SMET5.2 L chain gene fragment were PCR amplified with primer pairs ER12LF1/ER12LR1 and ER12LF2/ER1LR, respectively. The whole-length SMET5.2 L chain was assembled by overlapping PCR with primers ER12LF1 and ER1LR. The Lleader gene fragment was then fused to the L chain by overlapping PCR with primers bnIgG20L1 and ER1LR. The overlapping PCR product Lleader-L was digested with HindIII and EcoRI and cloned into the pDin1 construct containing the heavy chain of SMET5 IgG1.

[0211] For cloning of SMET5.3 IgG1, the L chain of SMET5.3 was PCR amplified with primers ER13LF and ER1LR. The Lleader gene fragment was then fused to the L chain by overlapping PCR with primers bnIgG20L1 and ER1LR. The overlapping PCR product Lleader-L was digested with HindIII and EcoRI and cloned into the pDin1 construct containing the heavy chain of SMET5 IgG1.

Protein Expression And Purification

[0212] Fab antibodies were expressed in *E. coli* HB2151 cells and IgG1s were expressed in 293FS cells as described previously (Chen et al., Proc Natl Acad Sci USA 2008, 105: 17121-17126). His-tagged Fab antibodies were purified from the soluble fraction of HB2151 periplasm by using the Ni-NTA resin (Qiagen) according to the manufacturer's protocol. IgG1s were purified from the 293FS culture supernatant by using Protein A

Sepharose 4 Fast Flow column chromatography (GE Healthcare) according to the manufacturer's instructions.

ELISA

[0213] ELISA was performed according to standard protocols. Briefly, recombinant human or cynomolgus EGFR was coated on Corning EIA/RIA high-binding 96-well plates (Corning Inc.) at 50 ng per well overnight at 4°C and blocked with 3% nonfat milk in PBS (pH7.4). Fivefold serially diluted antibodies were added and incubated at room temperature for 2 hours. The plates were washed with PBS containing 0.05% Tween 20. Bound Fab and IgG1s were detected by HRP-conjugated anti-FLAG tag antibody and HRP-conjugated anti-human IgG (Fc-specific) antibody, respectively. The assay was developed at room temperature with TMB substrate (Sigma-Aldrich) and monitored at 450 nm with a microplate reader. The half-maximal binding (EC_{50}) was calculated by fitting the data to the Langmuir adsorption isotherm.

Flow Cytometry

[0214] To measure the binding of SMET5 Fab to cell surface EGFR, about 5×10^5 cells were incubated with 100 nM antibody on ice for 1 h. The cells were washed once with PBS containing 0.1% bovine serum albumin (PBSA) and resuspended in 200 µl PBSA. Then 5 µl anti-His-PE conjugate was added and incubated for 30 min. The cells were washed once with PBSA and then used for flow cytometry analysis.

[0215] To measure the binding of IgG1s of SMET5 variants to cell surface EGFR, about 5×10^5 cells were incubated with 2 µg mL^{-1} antibodies on ice for 1 h. The cells were washed once with PBSA and resuspended in 200 µl PBSA. Then 5 µl goat F(ab')2 anti-human IgG (γ)-FITC conjugate was added and incubated for 30 min. The cells were washed once with PBSA and then used for flow cytometry analysis.

[0216] To measure the competitive binding of SMET5.2 IgG1 and EGF to cell surface EGFR, about 5×10^5 cells were incubated with SMET5.2 IgG1 at 2 µg mL^{-1} in the absence or presence of EGF at 40 µg mL^{-1} on ice for 30 min. The cells were washed with PBSA once and resuspended in 200 ul PBSA. Then 4 µl goat F(ab')2 anti-human IgG (γ)-FITC conjugate were added and incubated for 30 min. The cells were washed once with PBSA and then used for flow cytometry analysis.

Pharmacokinetic Measurment In Mice

[0217] NOD/SCID mice were administered intravenously with 500 μg antibody on day 0. Plasma samples were collected on day 1, 3, 5 and 6 and used for measurement of antibody serum concentrations by ELISA with standard curves generated using the original antibody stocks.

In-Vivo Tumor Growth Inhibition

[0218] SCID mice were inoculated with 10^6 A431 cells subcutaneously into the left flank of the mice. Once tumors reached a volume of 100-150 mm^3, mice were randomized into groups of 5 mice/group of equal average tumor volume and reconstituted intravenously with 10^7 human PBMCs. One day later, the mice were dosed intravenously with PBS, 0.5 mg SMET5.2 IgG1, or 0.5 mg control antibody every 3-4 days for 6 doses. After one month of treatment, mice were sacrificed and tumor weights were measured. Tumor growth inhibition rates were calculated by using the following formula: average weight of PBS group – average weight of antibody treated group/average weight of PBS group.

RESULTS AND DISCUSSION

Selection And Affinity Maturation of Fully Human Anti-EGFR Antibodies

[0219] Panning and screening a large phage-display naïve human Fab library led to the identification of three antibodies against EGFR. One of the antibodies, designated SMET5, significantly bound to EGFR-expressing human lung cancer cell line A549 but not to human EGFR-negative CHO cells, hence indicating specificity of the antibody (Figure 31). To improve its affinity, a light chain shuffling Fab library was constructed with the heavy chain of SMET5. Panning and screening the new library resulted in the identification of three SMET5 variants, namely SMET5.2, SMET5.3 and SMET5.4. In an ELISA, SMET5.2 and SMET5.3 showed higher binding activity against recombinant human EGFR than SMET5 whereas SMET5.4 had slightly decreased binding activity (Figure 32).

Generation And In Vitro Characterization of IgG1 of SMET5, SMET5.2 and SMET5.3

[0220] To evaluate the potential of the EGFR antibodies to be developed as candidate therapeutics, full-length SMET5, SMET5.2 and SMET5.3 antibodies were generated in the IgG1 format, which confers bivalency (avidity) and long half-life in vivo. The IgG1 antibodies were expressed and purified from transiently transfected 293FS cell culture supernatants by using protein A with yield of 10-20 mg L^{-1} (Figure 33). Size-exclusion chromatography

revealed that the vast majority (>95%) of the purified IgG1s in PBS (pH7.4) were monomers with apparent molecular weights of approximately 150 kDa, comparable to their calculated molecular weights (Figure 34). In an ELISA, they showed cross-reactivity with human and cynomolgus EGFR with SMET5.2 IgG1 having highest binding activity (EC_{50}s, 1.4 nM for human EGFR and 0.76 nM for cynomolgus EGFR) (Figure 35). At a concentration of 2 μg mL^{-1}, SMET5 and SMET5.2 IgG1s had comparable binding to A549 cells while SMET5.3 IgG1 showing relatively lower binding activity (Figure 36). No binding was detected with CHO cells suggesting high specificity of the IgG1s. All the IgG1s efficiently competed with EGF for binding to cell surface EGFR (Figure 37).

Inhibition of Tumor Growth In NOD/SCID Mice Engrafted With Human PBMC

[0221] To test how the antibodies could inhibit tumor growth in vivo, NOD/SCID mice engrafted with A431 cells and human PBMC were used as a model. Pharmacokinetics analysis showed that SMET5.2 IgG1 was cleared slowly from the mouse circulation (Figure 38). In the tumor challenge experiment, the antibody significantly inhibited the growth of A431 cells in the animals (Figure 39).

EXAMPLE 13

Cloning Of EGFR×CD3 Bispecific Antibodies With Reduced Binding Affinity To CD3

Cells, Proteins, Plasmids And Other Reagents

[0222] 293 free style (293FS) cells and protein A agarose were purchased from ThermoFisher Scientific. HCT116 and HT29 colon cancer cell lines were purchased from Sigma. Other cell lines were purchased from ATCC. Recombinant human EGFR, human CD3 (hCD3), cynomolgus CD3 (cCD3), and urokinase (uPA) were products of Sino Biological. The pDin1 vector used for mammalian expression was synthesized and assembled by overlapping PCR and ligation. The vector contains two transcription units and a built-in human IgG1 Fc gene fragment without introns that facilitates cloning of IgG1 or Fc-fusion proteins. Horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Fc-specific) antibody was a product of Sigma. Goat anti-human IgG (Fc-specific)-FITC conjugate was purchased from ThermoFisher Scientific.

Cloning Of EGFR×CD3 Bispecific Antibodies With Reduced Binding Affinity To CD3

[0223] The following primers were used:

[0224] SP34HF, (sense) (SEQ ID NO:110); SP34HR, (antisense) (SEQ ID NO:111)

[0225] CFCF, (sense) (SEQ ID NO:112); MFc7.2R1, (antisense) (SEQ ID NO:113)

[0226] SP34LF, (sense) (SEQ ID NO:114); SP34LR, (antisense) (SEQ ID NO:115)

[0227] CFCPAF, (sense) (SEQ ID NO:116); AAAR, (antisense) (SEQ ID NO:117)

[0228] bnIgG20H1, (sense) (SEQ ID NO:118)

[0229] SFPAF, (sense) (SEQ ID NO:119)

[0230] SFF, (sense) (SEQ ID NO:120); VNFR, (antisense) (SEQ ID NO:121)

[0231] bnIgG20L1, (sense) (SEQ ID NO:122)

[0232] VNFF, (sense) (SEQ ID NO:123); HSPGF, (antisense) (SEQ ID NO:124)

Cloning of iBiTE-sp

[0233] Humanized SP34 (hSP34) antibody scFv gene (see U.S. patents US 8,236,308 and US 9,493,563) was chemically synthesized by Integrated DNA technologies. To clone the heavy chain of iBiTE-sp (see Figure 40), hSP34 VH gene fragment was PCR amplified with the synthesized hSP34 scFv gene as a template and primers SP34HF and SP34HR. A gene fragment encoding human IgG1 CH1 and monomeric Fc (CH1-mFc7.2) was PCR amplified with previously constructed plasmid iBiTE (U.S. provisional patent application #62/783,411) as a template and primers CFCF and MFc7.2R1. hSP34 VH gene fragment was fused to the 5' end of CH1-mFc7.2 by overlapping PCR with the two gene fragments in the same molarities for 7 cycles in the absence of primers and 15 additional cycles in the presence of primers SP34HF and MFc7.2R1. The overlapping product hSP34 VH-CH1-mFc7.2 was digested with SacI and EcoRI and cloned into the iBiTE plasmid with inserted anti-EGFR antibody SMET5.2 scFv.

[0234] To clone the light chain of iBiTE-sp (see Figure 40), hSP34 VL gene fragment was PCR amplified with the synthesized hSP34 scFv as a template and primers SP34LF and SP34LR. A gene fragment encoding human antibody CL domain, monomeric Fc and a poly A signal sequence (CL-mFc7.2-polyA) was PCR amplified with previously constructed plasmid iBiTE as a template and primers CFCPAF and AAAR. A gene fragment (Hleader) encoding a leader peptide was then fused to the 5' end of hSP34 VL by overlapping PCR with primers bnIgG20H1 and SP34LR. Hleader-hSP34 VL was further linked to the 5' end of CL-mFc7.2-polyA by overlapping PCR with primers bnIgG20H1 and AAAR. The product was digested with XbaI and SalI and cloned into the previous plasmid containing hSP34 VH-CH1-mFc7.2, resulting in the final construct iBiTE-sp.

Cloning of proBiTE-1s1sp

[0235] A gene fragment encoding hSP34 VL-CL-mFc7.2-polyA was PCR amplified with iBiTE-sp as a template and primers SFPAF and AAAR. The PCR product was digested with BamHI and SalI and cloned into a previously constructed plasmid proBiTE-1s1 (U.S. provisional patent application #62/783,411). Then, a gene fragment encoding hSP34 VH-CH1-mFc7.2 was PCR amplified with iBiTE-sp as a template and primers SFF and MFc7.2R1. The PCR product was digested with SacI and EcoRI and cloned into the previous plasmid containing hSP34 VL-CL-mFc7.2-polyA, resulting in the final construct proBiTE-1s1sp.

Cloning of proBiTE-1s1spg

[0236] A gene fragment encoding a leader peptide, anti-EGFR antibody SMET5.2 VH and the N terminal portion of hSP34 VH was PCR amplified with proBiTE-1s1sp as a template and primers bnIgG20L1 and VNFR. Another gene fragment encoding the C terminal portion of hSP34 VH and CH1-mFc7.2 was PCR amplified with proBiTE-1s1sp as a template and primers VNFF and MFc7.2R1. The two gene fragments were fused to each other by overlapping PCR with primers bnIgG20L1 and MFc7.2R1. The product was digested with HindIII and EcoRI and cloned into proBiTE-1s1sp, resulting in the final construct proBiTE-1s1spg.

Cloning of HBiBody-spg

[0237] A gene fragment encoding hSP34 L chain, mFc7.2 and polyA signal sequence was PCR amplified with proBiTE-1s1spg as a template and primers HSPGF and AAAR. The PCR product was digested with BamHI and SalI and cloned into proBiTE-1s1spg linearized by BamHI and SalI.

Protein Expression And Purification

[0238] All bispecific antibodies were expressed in 293FS cells as described previously (Chen et al., Proc Natl Acad Sci USA 2008, 105:17121-17126) and purified from the 293FS culture supernatant using Protein A Sepharose 4 Fast Flow column chromatography (GE Healthcare) according to the manufacturer's instructions.

Protease Cleavage

[0239] For cleavage with urokinase (uPA), 5 μg antibodies were mixed with or without 1 μg recombinant human uPA (Sino Biological) in 15 μl PBS (pH7.4) and incubated at room temperature for 1 hour.

ELISA

[0240] ELISA was performed according to standard protocols. Briefly, recombinant antigens were coated on Corning EIA/RIA high-binding 96-well plates (Corning Inc.) at 100 ng per well overnight at 4°C and blocked with 3% nonfat milk in PBS (pH7.4). Fivefold serially diluted biotinylated antibodies were added and incubated at room temperature for 2 hours. The plates were washed with PBS containing 0.05% Tween 20. Bound antibodies were detected by goat anti-human IgG (Fc-specific) antibody. The assay was developed at room temperature with TMB substrate (Sigma-Aldrich) and monitored at 450 nm with a microplate reader. Half-maximal binding (EC_{50}) was calculated by fitting the data to the Sigmoidal adsorption isotherm.

Flow Cytometry

[0241] About 5×10^5 cells were incubated with antibodies on ice for 1 hour. The cells were washed once with PBS containing 0.1% bovine serum albumin (PBSA) and resuspended in 100 μl PBSA. Then 1 μl goat anti-human IgG (Fc-specific)-FITC conjugate (Invitrogen) was added and incubated for 30 min. The cells were washed once with PBSA and then used for flow cytometry analysis. EC_{50} was calculated by fitting the data to the Sigmoidal isotherm.

Size-Exclusion Chromatography

[0242] A Superdex200 10/300 GL column (GE Healthcare) was calibrated with protein molecular mass standards of carbonic anhydrase (29 kDa), ovalbumin (44 kDa), conalbumin (75 kDa), aldolase (158 kDa) and ferritin (440 kDa). Purified proteins at a concentration of 1 mg/mL in PBS (pH7.4) were loaded onto the pre-equilibrated column and eluted with PBS (pH7.4) at 0.5 mL/min.

In-Vitro Killing Assay

[0243] For the assay using luciferase as a reporter gene, 10^4 target cells were seeded in 100 μl RPMI 1640 complete medium overnight. Meanwhile, 2.5×10^7 frozen human peripheral blood mononuclear cells (PBMCs) purchased from STEMCELL Technologies were revived and cultured in 10 mL RPMI 1640 complete medium containing 50 U/mL IL-2 (Sigma)

overnight. The medium was removed from the target cells on the next day and 5×10^4 PBMCs in 50 µl RPMI 1640 complete medium were added (actual target:effector ratio = 1:2.5 because the target cells duplicated overnight). Then, 50 µl antibodies (5-fold serially diluted from 1 nM) were added into each well. Twenty four hours later, 20 µl of adenovirus encoding luciferase gene (Ad5-Luc) was added at a multiplicity of infection (MOI) of 100, which effectively infects target cells but not effector cells. Twenty four hours later, cell viability was measured using Promega Bright-Glo Luciferase Assay System according to the manufacturer's instructions. Cell killing was calculated using the following formula: 1 - (average reading of antibody and Ad5-Luc treated group – average reading of cell only group) / (average reading of PBS and Ad5-Luc treated group – average reading of cell only group).

[0244] For the assay using MTS tetrazolium compound as a reporter reagent, 10^4 target cells were seeded in 100 µl RPMI 1640 complete medium overnight. Meanwhile, 2.5×10^7 frozen human peripheral blood mononuclear cells (PBMCs) purchased from STEMCELL Technologies were revived and cultured in 10 mL RPMI 1640 complete medium containing 50 U/mL IL-2 (Sigma) overnight. The medium was removed from the target cells on the next day and 10^5 PBMCs in 50 µl RPMI 1640 complete medium were added (actual target:effector ratio = 1:5 because target cells duplicated overnight). Then, 50 µl antibodies (5-fold serially diluted from 10 nM) were added into each well. Forty eight hours later, the plates were gently shaked and resuspended PBMCs were removed together with used medium. Each well of the plates was washed once with 100 µl fresh RPMI 1640 complete medium to completely remove resuspended PBMCs. Then 100 µl fresh RPMI 1640 complete medium was added into each well and cell viability was measured using Promega CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay according to the manufacturer's instructions. Cell killing was calculated using the following formula: 1 - (average reading of antibody treated group – average reading of medium only group) / (average reading of PBS treated group – average reading of medium only group).

EXAMPLE 14

Design Of New Bispecific EGFR×CD3 Antibodies With Reduced Binding To CD3

[0245] To provide a proof-of-concept, anti-EGFR and anti-CD3 antibodies were used as examples here and below. One of ordinary skill in the art would recognize that other T and non-T cell target antigens can be readily employed as described herein.

[0246] A series of novel protease-activatable bispecific EGFR×CD3 antibodies that demonstrated excellent drug-related properties and potent T cell-mediated killing of EGFR-expressing human colon cancer cells have been generated (U.S. provisional patent application #62/783,411). The anti-CD3 antibody used in the previous invention, the humanized OKT3 (hOKT3), does not cross-react with commonly used pharmacologically relevant species such as mice and cynomolgus monkeys. In the present invention, therefore, new bispecific EGFR×CD3 antibodies were designed using engineered humanized SP34 (hSP34) which is cross-reactive against CD3 molecules of humans and several non-human primates such as cynomolgus monkeys.

[0247] A new bispecific antibody, termed proBiTE-1s1sp, was generated in which the VH and VL domains of the anti-EGFR antibody SMET5.2 were fused to the N terminus of the VH and VL domains of the anti-CD3 antibody hSP34 Fab via a protease non-cleavable (G4S)3 linker and a protease cleavable linker GSGSGRSDNHGGGGS (SEQ ID NO:62) (underlined is the substrate sequence of uPA, matriptase and legumain), respectively. The anti-CD3 Fab was further fused to the N terminus of a monomeric human IgG1 Fc (mFc7.2) which contains two amino acid mutations (T366L/Y407H) capable of decreasing Fc homodimerization (Figure 40).

[0248] It is well known in the literature that T cells can be efficiently activated by low affinity binding (in the range of 1-90 μM) of T cell receptors (TCR) to peptide-MHC complexes on antigen-presenting cells. It is therefore conceivable that bispecific antibodies binding to the TCR/CD3 complex and a cancer-related antigen might also not need high affinities for efficient activation of T cells. In addition, low affinity binding to CD3 may be advantageous because it will reduce localization of bispecific antibodies to T cells that could potentially cause unwanted side effects. These include, but are not limited to, nonspecific activation of T cells, interference with normal immune responses of T cells, and Fc receptor (FcR)-mediated killing of T cells by other cytotoxic cells such as macrophage and NK cells if bispecific antibodies contain Fc.

[0249] In a preliminary experiment, it was found that hSP34 Fab had an affinity for CD3-expressing Jurkat cells of approximately 20 nM. It is believed that the affinity of hSP34 could be further reduced to enlarge therapeutics windows of bispecific antibodies. Analysis of the junction region of hSP34 heavy chain CDR3 (HCDR3) using IMGT/V-QUEST revealed that compared to the closest human antibody germline sequences, there are three amino acid

residue mutations, one (A105V) in the V region and two (S111.1N and Y112.2S) in the D region (Figure 41). It is hypothesized that, due to similar properties of the A and V, and S and N amino acids, reverse mutation of the V105 and N111.1 residues to their germline sequences might retain but reduce binding affinity of hSP34 while potentially minimizing immunogenicity in humans. To test this hypothesis, a new bispecific antibody, designated proBiTE-1s1spg, was generated by introducing the V105A and N111.1S substitutions to the proBiTE-1s1sp construct (Figure 40). In addition, two amino acid residues in the FR2 of VL were also reverse mutated to their germline sequences (E44Q and F50P, IMGT numbering scheme).

[0250] It has been shown that the polypeptide linkers connecting the VH and VL domains of anti-EGFR and anti-CD3 antibodies could decrease binding activity of the anti-CD3 antibody by creating steric hindrance (U.S. provisional patent application #62/783,411). To test this possibility with proBiTE-1s1sp and proBiTE-1s1spg, a control construct, iBiTE-sp, was designed in which the scFv of anti-EGFR antibody was fused via the G4S linker to the N terminus of the VH-CH1 of hSP34 and therefore, binding to CD3 is not sterically restricted by the linker (Figure 40). Another construct, termed HBiTE-spg, was generated by replacing the protease cleavable linker (GSGSGRSDNHGGGGS (SEQ ID NO:62)) in proBiTE-1s1spg with a protease non-cleavable linker (GSGGGGSGGGGS (SEQ ID NO:127)) (Figure 40).

EXAMPLE 15

Generation And Initial Characterization Of EGFR×CD3 Bispecific Antibodies With Reduced Binding To CD3

[0251] All the bispecific antibodies were well expressed in transiently transfected 293 free style (293FS) cells and secreted into the culture supernatants. On a non-reducing SDS-PAGE, a vast majority of the purified antibodies migrated as a heterodimer with apparent molecular weight (aMW) of approximately 120 kDa (Figure 42). On a reducing SDS-PAGE, the two polypeptide chains of iBiTE-sp were well separated while those of other bispecific antibodies overlapped with each other with apparent molecular weight (aMW) of approximately 60 kDa.

[0252] proBiTE-1s1sp and proBiTE-1s1spg were efficiently cleaved by uPA, resulting in an isolated VL domain of the anti-EGFR antibody while other bispecific antibodies were not sensitive to the protease (Figure 43). Size-exclusion chromatography analysis showed that the

vast majority of purified bispecific antibodies migrated as a monomer with aMW similar to or slightly larger than their calculated molecular weights (cMW) (Figure 44).

EXAMPLE 16

Cross-Reactive Binding Of Bispecific Antibodies To CD3

[0253] ELISA binding of the bispecific antibodies to recombinant human EGFR (hEGFR) and human CD3 (hCD3) and cynomolgus CD3 (cCD3) was first measured. The four bispecific EGFR×CD3 antibodies bound to hEGFR equally well (Figure 45). None of them interacted with recombinant human HER4 (hHER4), an irrelevant control antigen. They also strongly bound to hCD3 (EC_{50}s, 1.3, 1.9, 3.4 and 4.5 nM for iBiTE-sp, proBiTE-1s1sp, proBiTE-1s1spg and HBiTE-spg, respectively) and cCD3 (EC_{50}s, 1.6, 2.4, 4.5 and 3.8 nM for iBiTE-sp, proBiTE-1s1sp, proBiTE-1s1spg and HBiTE-spg, respectively) (Figure 46). A control HBiTE, which is a bispecific FLT3×CD3 antibody, did not interact with hEGFR, hCD3 and cCD3. These results suggest high specificity of the bispecific EGFR×CD3 antibodies.

[0254] How the bispecific antibodies with different structures and composition of linkers could bind to cell surface EGFR and CD3 was tested next. At a concentration of 2 μg/mL, none of the bispecific antibodies interacted with CHO cells which do not express human EGFR and CD3, while iBiTE-sp bound to both EGFR-expressing HCT116 and CD3-expressing Jurkat cells, suggesting specific binding activity of the anti-EGFR and anti-CD3 antibodies (Figure 47). proBiTE-1s1sp bound to HCT116 cells as well as iBiTE-sp whereas binding to Jurkat cells was decreased by approximately twofold, suggesting that binding activity of the anti-CD3 antibody hSP34 was only slightly affected by the polypeptide linkers connecting the anti-EGFR and anti-CD3 antibodies. This result is in contrast to the finding in the previous invention that binding activity of the anti-CD3 antibody hOKT3 in proBiTE-1s1 was completely abrogated in the same experimental setting (U.S. provisional patent application #62/783,411).

[0255] As expected, reverse mutation of the V105 and N111.1 residues of hSP34 HCDR3 to their germline sequences further decreased binding of proBiTE-1s1spg and HBiTE-spg to Jurkat cells, suggesting that the two substitutions could indeed retain but reduce binding affinity of hSP34. When the bispecific antibodies were tested at different concentrations, iBiTE-sp bound to Jurkat cells with an EC_{50} of 25 nM (Figure 48). In comparison, binding

affinity (EC_{50}, 105 nM) of proBiTE-1s1sp was decreased by approximately fourfold. Binding affinities of proBiTE-1s1spg and HBiTE-spg were further decreased with EC_{50} of > 500 nM.

EXAMPLE 17

Bispecific Antibody-Mediated Killing Of Human Colon Cancer Cell Lines

[0256] HCT116 and HT29 cells are human colon cancer cell lines with KRAS (G13D) and BRAF (V600E) mutations, respectively. They are not sensitive to the treatment with FDA-approved anti-EGFR monoclonal antibody CETUXIMAB. Therefore, there is an unmet need for novel therapeutic strategies for colorectal cancers with such mutations. HCT116 and HT29 cells are adherent and fibroblastic, and grow in a monolayer in RPMI 1640 medium.

[0257] In one killing assay, EGFR-expressing HCT116 and EGFR-negative CHO cells, which were infected with adenovirus type 5 encoding the luciferase reporter gene (Ad5-Luc), were used as target cells. The results showed that although the bispecific antibodies had substantially different binding to CD3-expressing Jurkat cells (Figure 48), they exhibited comparably potent killing of HCT116 cells with EC_{50} of approximately 1-3 pM (Figure 49). The anti-EGFR IgG1 antibody did not have an effect on HCT116 cells and none of the antibodies significantly inhibited the growth of CHO cells. These results suggest that indeed, low affinity binding to CD3 (>500 nM, HBiTE-spg) could activate T cells as effectively as high affinity CD3 binding (25 nM, iBiTE-sp).

[0258] In another killing assay, MTS tetrazolium compound was used as a reporter reagent. Both proBiTE-spg and HBiTE-spg with mutated HCDR3 and light chain FR2 on the anti-CD3 antibody killed HCT116 and HT29 cells with similar efficiency (Figure 50).

What is claimed is:

1. A recombinant bispecific antibody, comprising
   (i) a first polypeptide comprising from N- to C-terminus a first light chain variable domain, a human light chain constant region (CL), a human heavy chain constant region 2 (CH2) and a human heavy chain constant region 3 (CH3);
   (ii) a second polypeptide comprising from N- to C-terminus a first heavy chain variable domain, a human heavy chain constant region 1 (CH1), a human heavy chain constant region 2 (CH2) and a human heavy chain constant region 3 (CH3); and
   (iii) a second light chain variable domain connected to said first light chain variable domain by a first linker, and a second heavy chain variable domain connected to said first heavy chain variable domain by a second linker; or
   a second light chain variable domain connected to the C-terminus of said CH3 of said first polypeptide by a first linker, and a second heavy chain variable domain connected to the C-terminus of said CH3 of said second polypeptide by a second linker,
   wherein said first light chain variable domain and said first heavy chain variable domain confer binding specificity to a first antigen, said second light chain variable domain and said second heavy chain variable domain confer binding specificity to a second antigen.

2. The recombinant bispecific antibody of claim 1, wherein one or both of said CH3 comprise one or more amino acid substitutions that confer decreased or abrogated homodimerization between said first and second polypeptides.

3. The recombinant bispecific antibody of claim 2, wherein amino acid T366 of said one or both CH3 is substituted with D (Aspartic Acid), L (Leucine), W (Tryptophan) or N (Asparagine).

4. The recombinant bispecific antibody of claim 2, wherein amino acid Y407 of said one or both CH3 is substituted with I (Isoleucine), F (Phenylalanine), L (Leucine), M (Methionine), H (Histidine), K (Lysine), S (Serine), Q (Glutamine), T (Threonine), W (Tryptophan), A (Alanine), G (Glycine) or N (Asparagine).

5. The recombinant bispecific antibody of claim 2, wherein one or both of said CH3 comprise an amino acid sequence of one of SEQ ID NOs:155-171.

6. The recombinant bispecific antibody of claim 1, wherein said first or second linker comprises amino acid sequence of SEQ ID NO:125, 126 or 127.

7. The recombinant bispecific antibody of claim 1, wherein said first or second linker comprises a protease cleavage site.

8. The recombinant bispecific antibody of claim 7, wherein said protease cleavage site comprises amino acid sequence of one of SEQ ID NOs:58-63.

9. The recombinant bispecific antibody of claim 1, wherein said CH1 and CL are connected to said CH2 of said first and second polypeptides via a linker that comprises a shortened hinge sequence as compared to a hinge sequence in normal human IgG.

10. The recombinant bispecific antibody of claim 1, wherein said bispecific antibody comprises a polypeptide comprising amino acid sequence of one of SEQ ID NOs:1-16.

11. The recombinant bispecific antibody of claim 1, further comprising a heterodimerization motif or a long flexible motif, wherein said heterodimerization motif or long flexible motif is connected to the N terminus of said second light chain variable domain and said second heavy chain variable domain via one or more linkers.

12. The recombinant bispecific antibody of claim 11, wherein said one or more linkers comprise a protease cleavage site.

13. The recombinant bispecific antibody of claim 11, wherein said heterodimerization motif comprises a N terminal heterodimerization motif of human HER2 transmembrane domain or a N terminal heterodimerization motif of human EGFR transmembrane domain.

14. The recombinant bispecific antibody of claim 11, wherein said long flexible motif comprises an amino acid sequence of SEQ ID NO:153.

15. The recombinant bispecific antibody of claim 11, wherein said bispecific antibody comprises a polypeptide comprising amino acid sequence of one of SEQ ID NOs:17-20.

16. The recombinant bispecific antibody of claim 1, wherein said first light chain variable domain and said first heavy chain variable domain comprise modified sequences that confer reduced binding affinity to CD3 as compared to that mediated by unmodified sequences.

17. The recombinant bispecific antibody of claim 1, wherein said first light chain variable domain and said first heavy chain variable domain confer binding specificity to human CD3 and cynomolgus CD3.

18. The recombinant bispecific antibody of claim 1, wherein said bispecific antibody comprises a polypeptide comprising amino acid sequence of one of SEQ ID NOs:50-57.

19. A polynucleotide encoding one or both polypeptides of the recombinant bispecific antibody of claims 1, 11, 16 or 17.

20. An expression vector comprising the polynucleotide of claim 19.

21. A host cell comprising the expression vector of claim 20.

22. A method of preparing the recombinant bispecific antibody of claims 1, 11, 16 or 17, said method comprises the steps of: (a) culturing host cells comprising one or more polynucleotides encoding said recombinant bispecific antibody under conditions wherein said recombinant bispecific antibody is expressed; and (b) recovering said recombinant bispecific antibody from said host cells.

23. An isolated anti-human EGFR (epidermal growth factor receptor) antibody, said antibody comprises a heavy chain and a light chain, wherein said heavy chain comprises a complementarity determining region 1 (CDR1) comprising amino acid sequence SEQ ID NO:22, a CDR2 comprising amino acid sequence SEQ ID NO:23, and a CDR3 comprising amino acid sequence SEQ ID NO:24.

24. The anti-human EGFR antibody of claim 23, wherein said heavy chain comprises amino acid sequence of SEQ ID NO:21 or SEQ ID NO:44.

25. The anti-human EGFR antibody of claim 23, wherein said light chain comprises a CDR1 comprising amino acid sequence SEQ ID NO:26, a CDR2 comprising amino acid sequence SEQ ID NO:27, and a CDR3 comprising amino acid sequence SEQ ID NO:28.

26. The anti-human EGFR antibody of claim 23, wherein said light chain comprises amino acid sequence SEQ ID NO:25 or SEQ ID NO:45.

27. The anti-human EGFR antibody of claim 23, wherein said light chain comprises a CDR1 comprising amino acid sequence SEQ ID NO:31, a CDR2 comprising amino acid sequence SEQ ID NO:32, and a CDR3 comprising amino acid sequence SEQ ID NO:33.

28. The anti-human EGFR antibody of claim 23, wherein said light chain comprises amino acid sequence SEQ ID NO:30 or SEQ ID NO:47.

29. The anti-human EGFR antibody of claim 23, wherein said light chain comprises a CDR1 comprising amino acid sequence SEQ ID NO:36, a CDR2 comprising amino acid sequence SEQ ID NO:37, and a CDR3 comprising amino acid sequence SEQ ID NO:38.

30. The anti-human EGFR antibody of claim 23, wherein said light chain comprises amino acid sequence SEQ ID NO:35 or SEQ ID NO:49.

31. The anti-human EGFR antibody of claim 23, wherein said light chain comprises a CDR1 comprising amino acid sequence SEQ ID NO:41, a CDR2 comprising amino acid sequence SEQ ID NO:42, and a CDR3 comprising amino acid sequence SEQ ID NO:43.

32. The anti-human EGFR antibody of claim 23, wherein said light chain comprises amino acid sequence SEQ ID NO:40.

33. A composition comprising a physiologically acceptable carrier and a therapeutically effective amount of the anti-human EGFR antibody of any one of claims 23-32.

Figure 1A

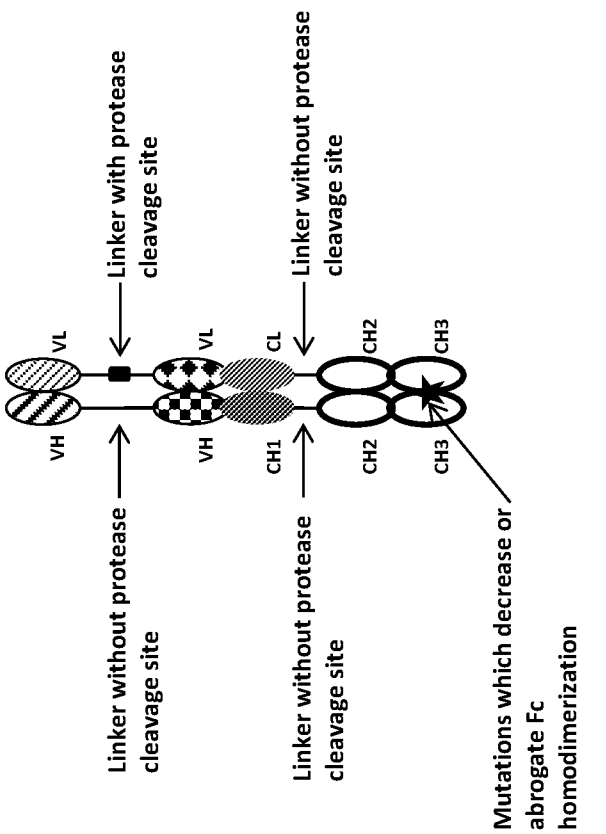

VL
VH
VL
VH
CL
CH1
CH2
CH2
CH3
CH3
Linker without protease cleavage site
Linker with protease cleavage site
Linker without protease cleavage site
Linker with protease cleavage site
Linker without protease cleavage site
Mutations which decrease or abrogate Fc homodimerization

Figure 1B

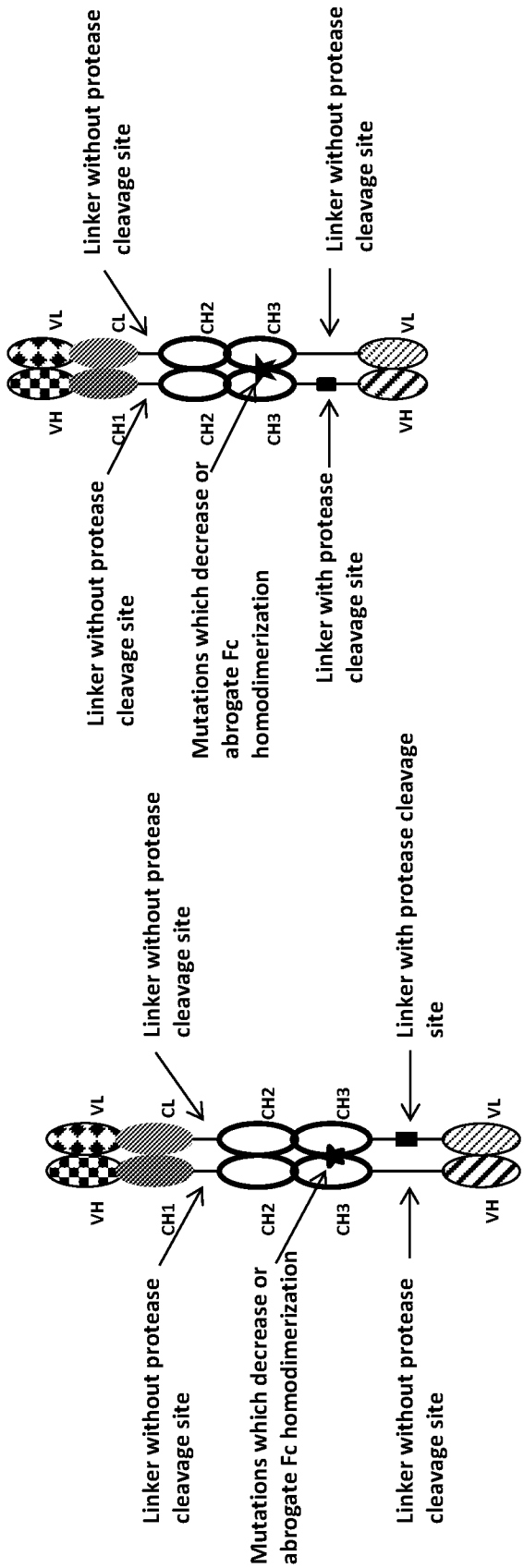

Linker without protease cleavage site
Linker without protease cleavage site
Linker with protease cleavage site
Mutations which decrease or abrogate Fc homodimerization
Linker without protease cleavage site
VL
CL
CH2
CH3
VL
VH
CH1
CH2
CH3
VH
Linker without protease cleavage site
Linker with protease cleavage site
Mutations which decrease or abrogate Fc homodimerization
Linker without protease cleavage site
VL
CL
CH2
CH3
VL
VH
CH1
CH2
CH3
VH

Figure 2A

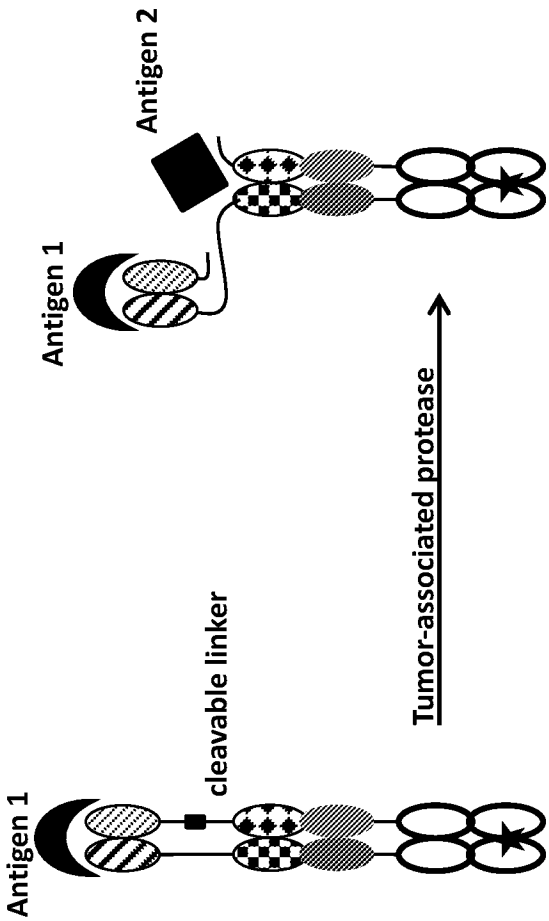

Antigen 2
Antigen 1
Tumor-associated protease
cleavable linker
Antigen 1

Figure 2B

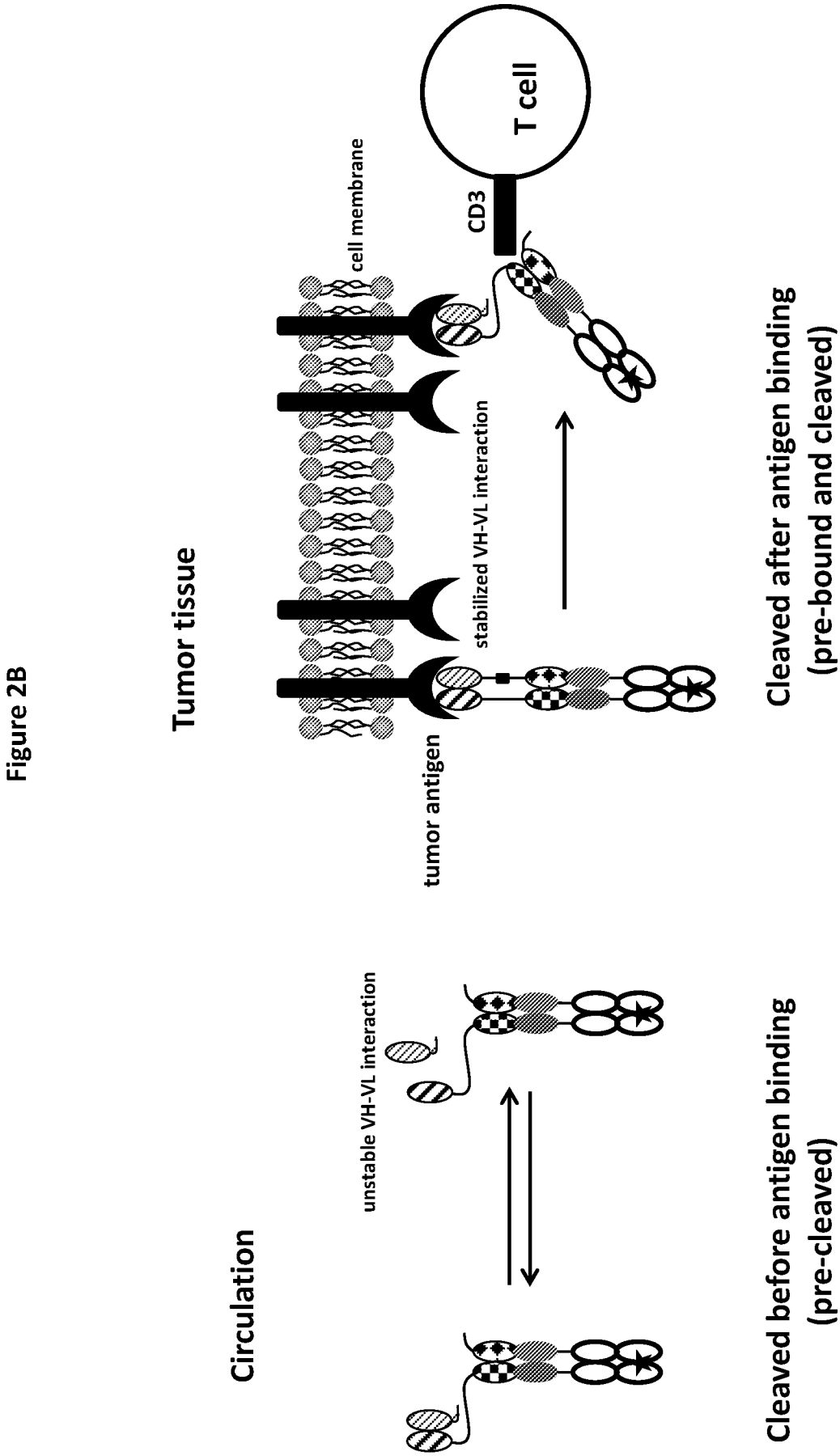

Tumor tissue
T cell
CD3
cell membrane
stabilized VH-VL interaction
tumor antigen
Cleaved after antigen binding
(pre-bound and cleaved)
Circulation
unstable VH-VL interaction
Cleaved before antigen binding
(pre-cleaved)

Figure 3

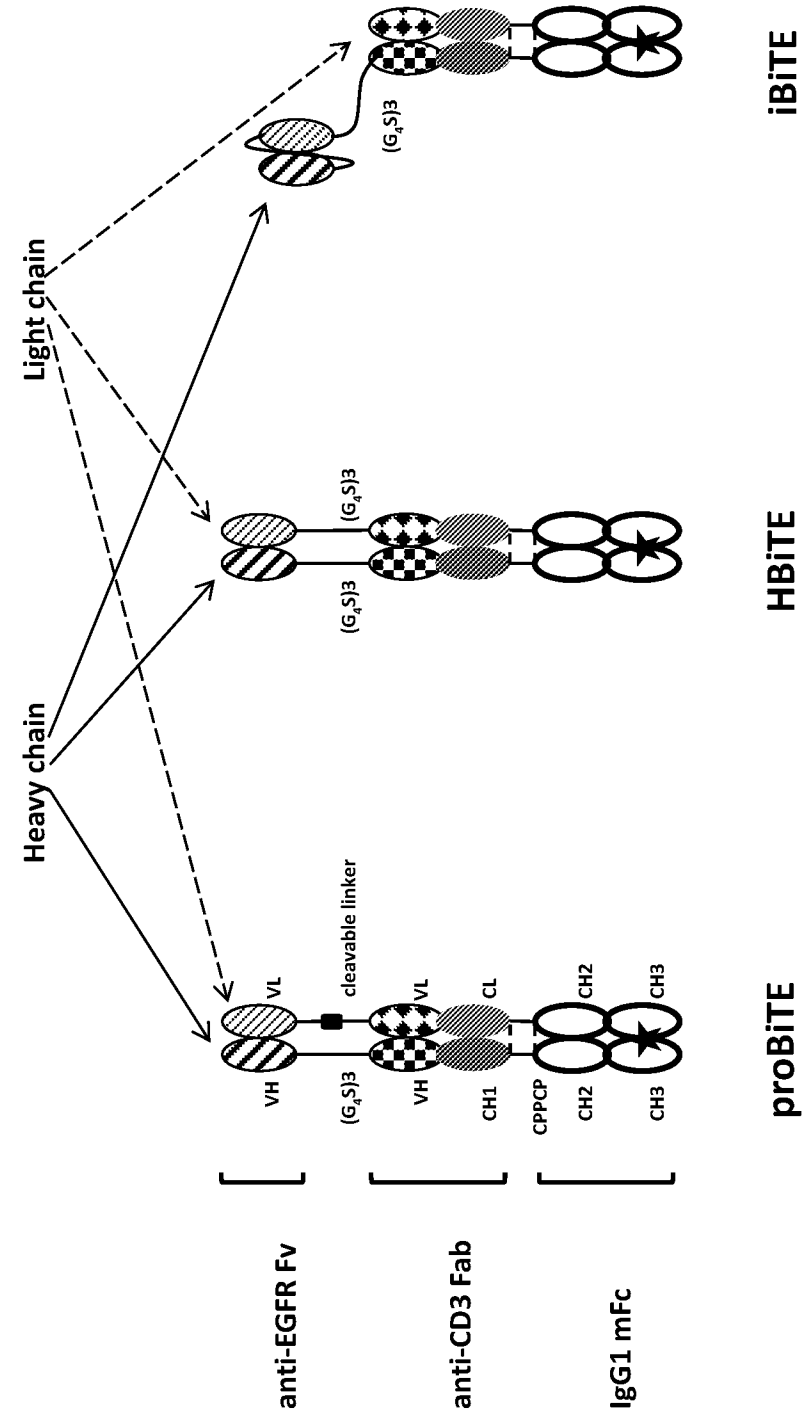

Light chain
Heavy chain
VL
cleavable linker
VH
(G4S)3
VH
CH1
CL
CPPCP
CH2
CH3
CH2
CH3
(G4S)3
(G4S)3
(G4S)3
anti-EGFR Fv
anti-CD3 Fab
IgG1 mFc
iBiTE
HBiTE
proBiTE

Figure 4

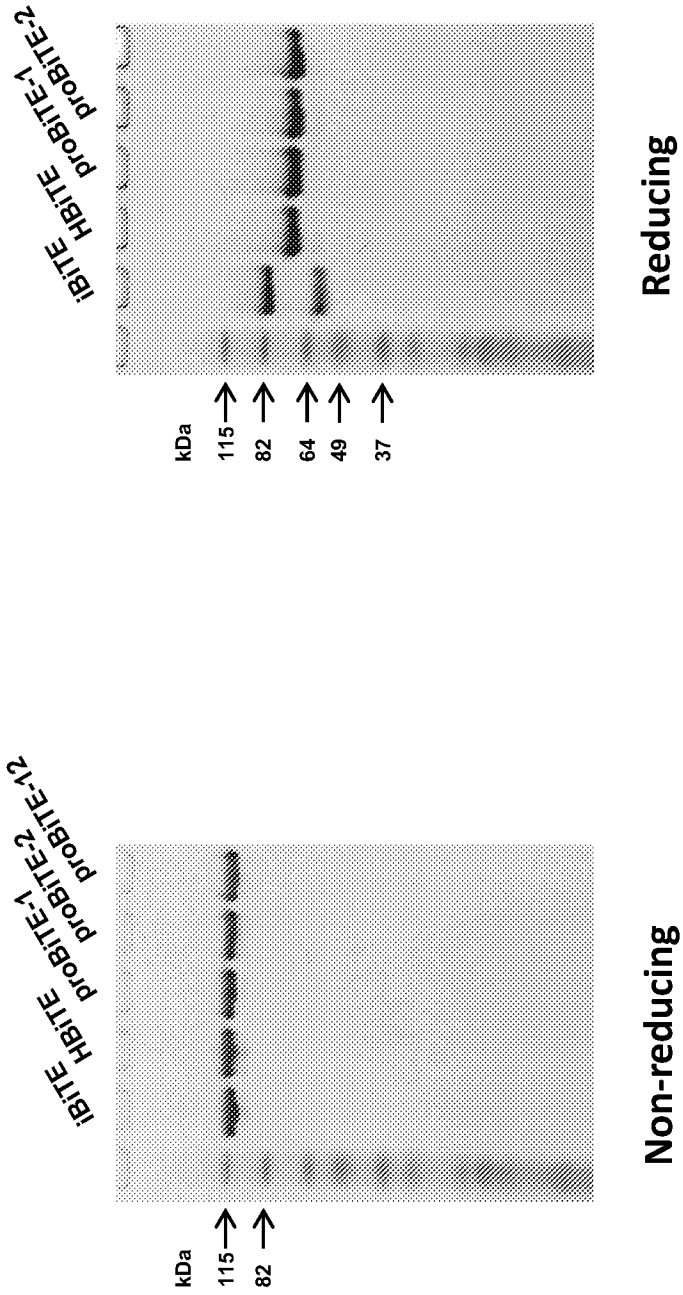
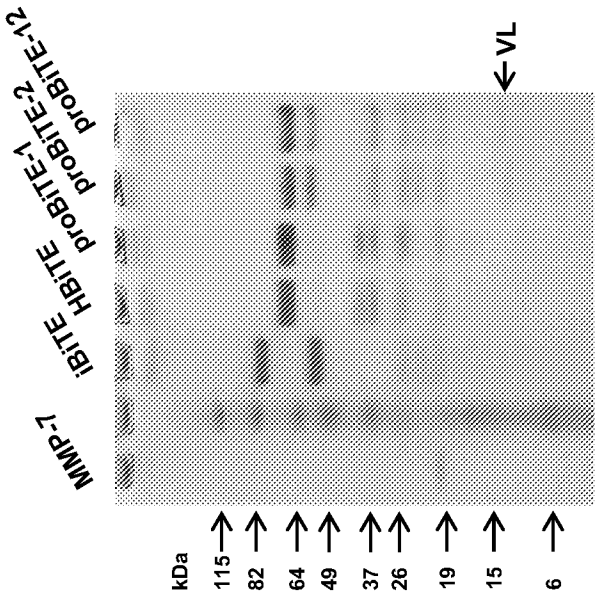

iBiTE HBiTE proBiTE-1 proBiTE-2
kDa
115
82
64
49
37
Reducing
iBiTE HBiTE proBiTE-1 proBiTE-2 proBiTE-12
kDa
115
82
Non-reducing

Figure 5


MMP-1
iBiTE HBiTE proBiTE-1 proBiTE-2 proBiTE-12
VL
kDa
115
82
64
49
37
26
19
15
6

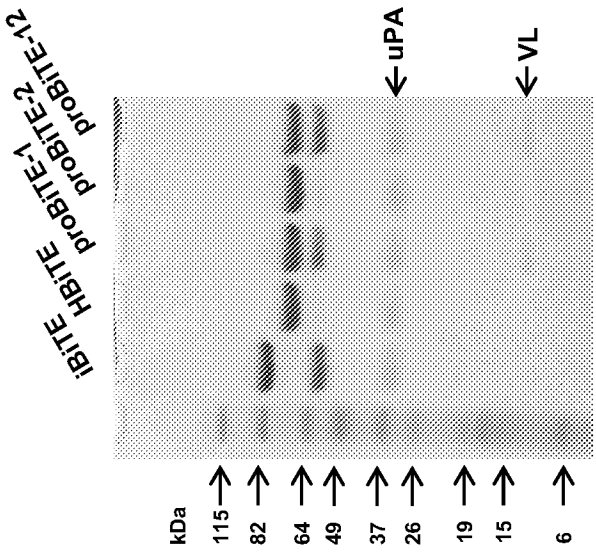

iBiTE HBiTE proBiTE-1 proBiTE-2 proBiTE-12
uPA
VL
kDa
115
82
64
49
37
26
19
15
6

Figure 6

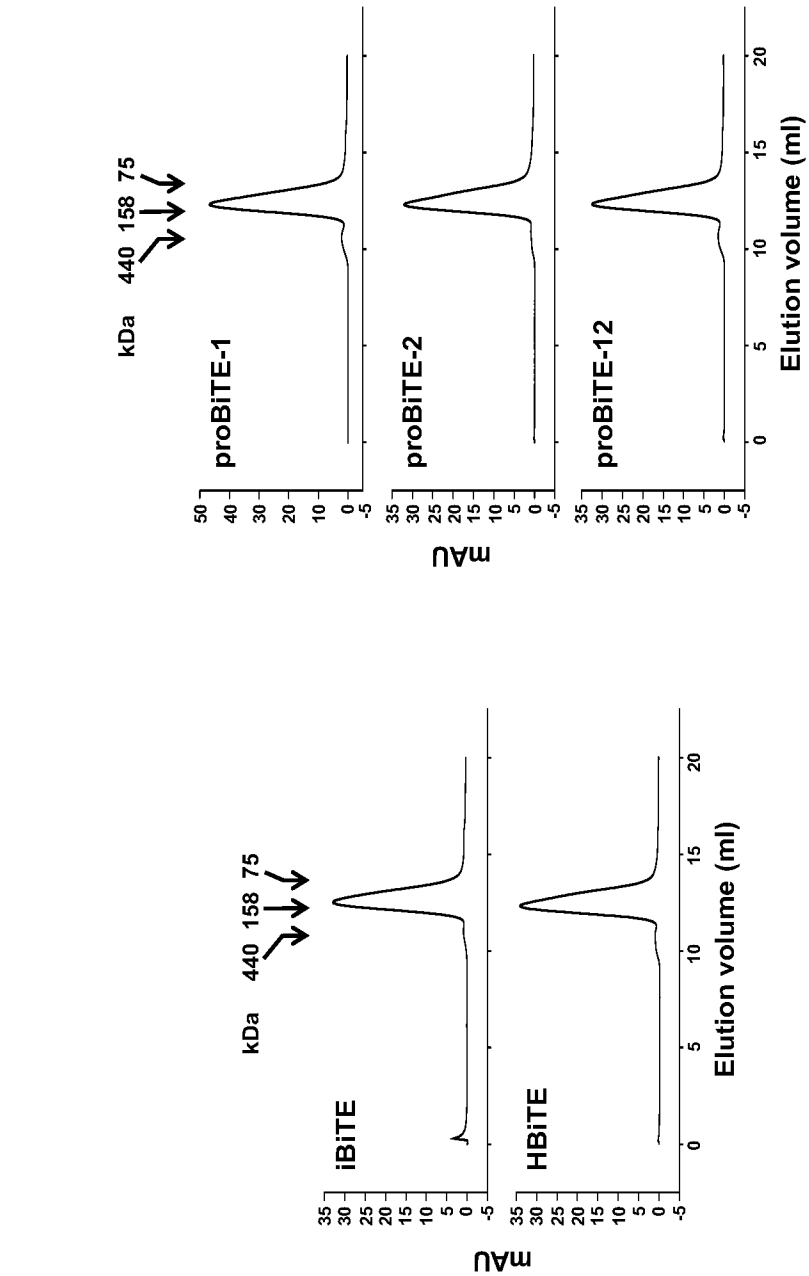

kDa
440
158
75
proBiTE-1
proBiTE-2
proBiTE-12
mAU
Elution volume (ml)
iBiTE
HBiTE

Figure 7

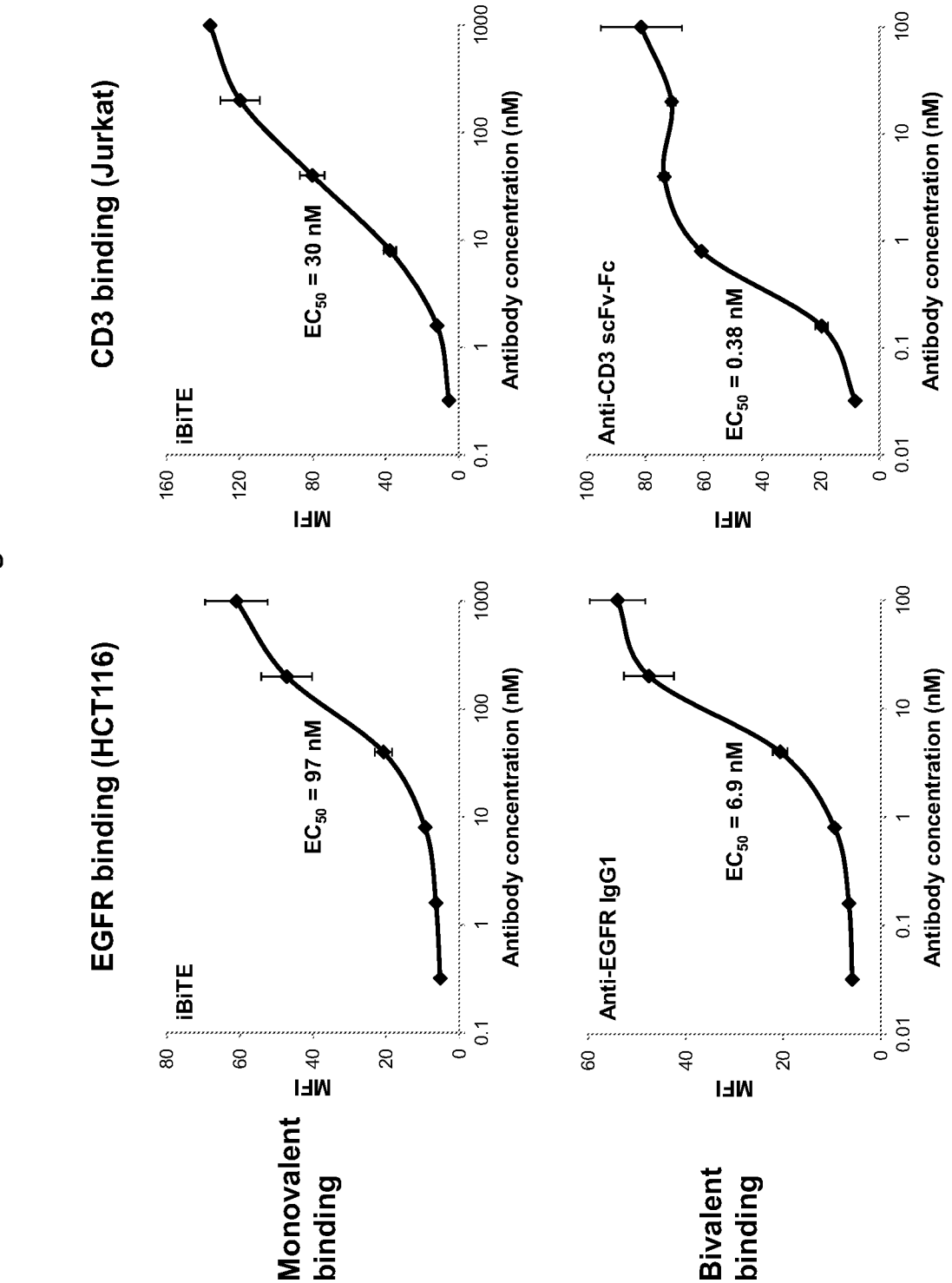

EGFR binding (HCT116)
CD3 binding (Jurkat)
Monovalent binding
Bivalent binding
iBiTE
Anti-EGFR IgG1
Anti-CD3 scFv-Fc
EC50 = 97 nM
EC50 = 30 nM
EC50 = 6.9 nM
EC50 = 0.38 nM
MFI
Antibody concentration (nM)

Figure 8

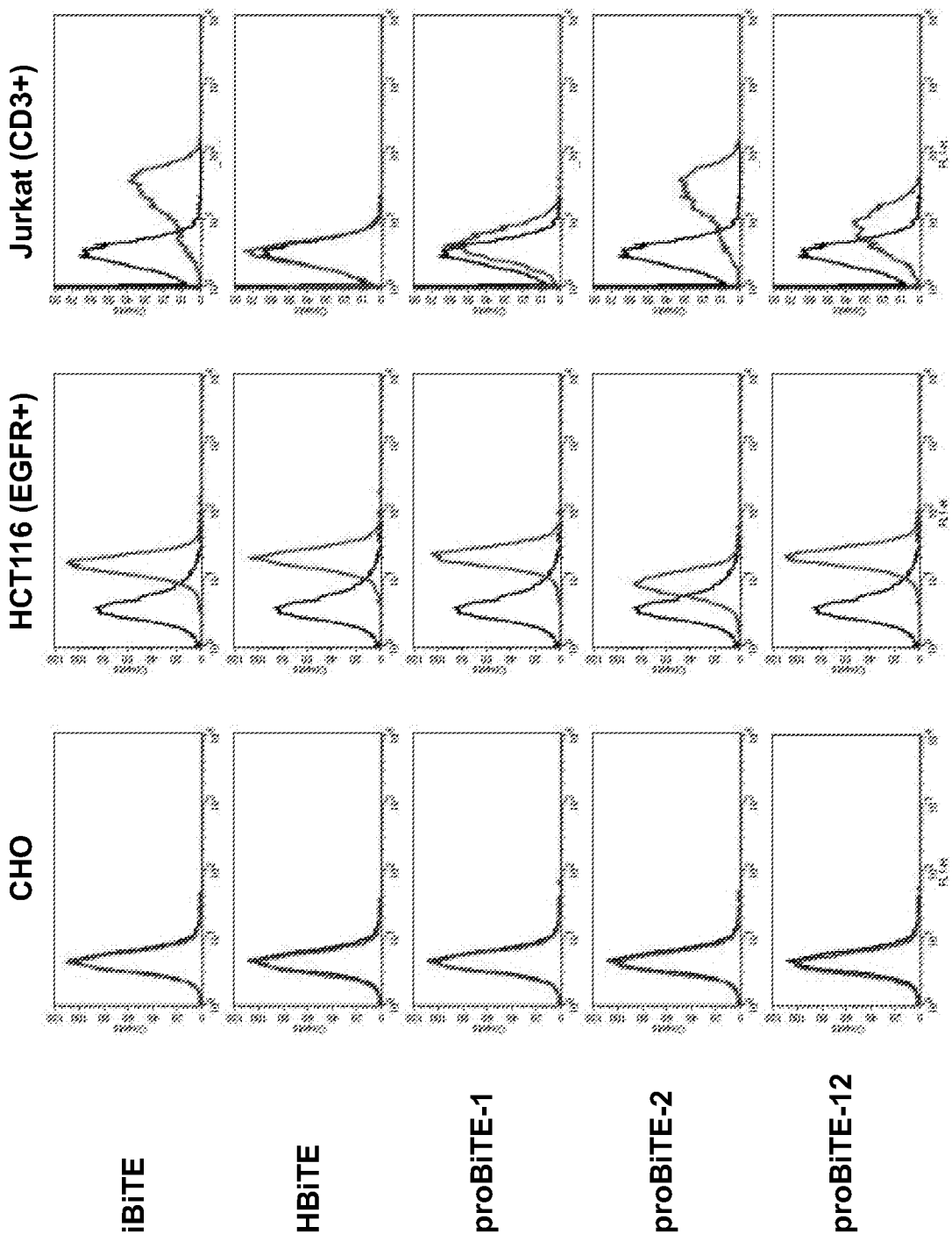

CHO
HCT116 (EGFR+)
Jurkat (CD3+)
iBiTE
HBiTE
proBiTE-1
proBiTE-2
proBiTE-12

Figure 9

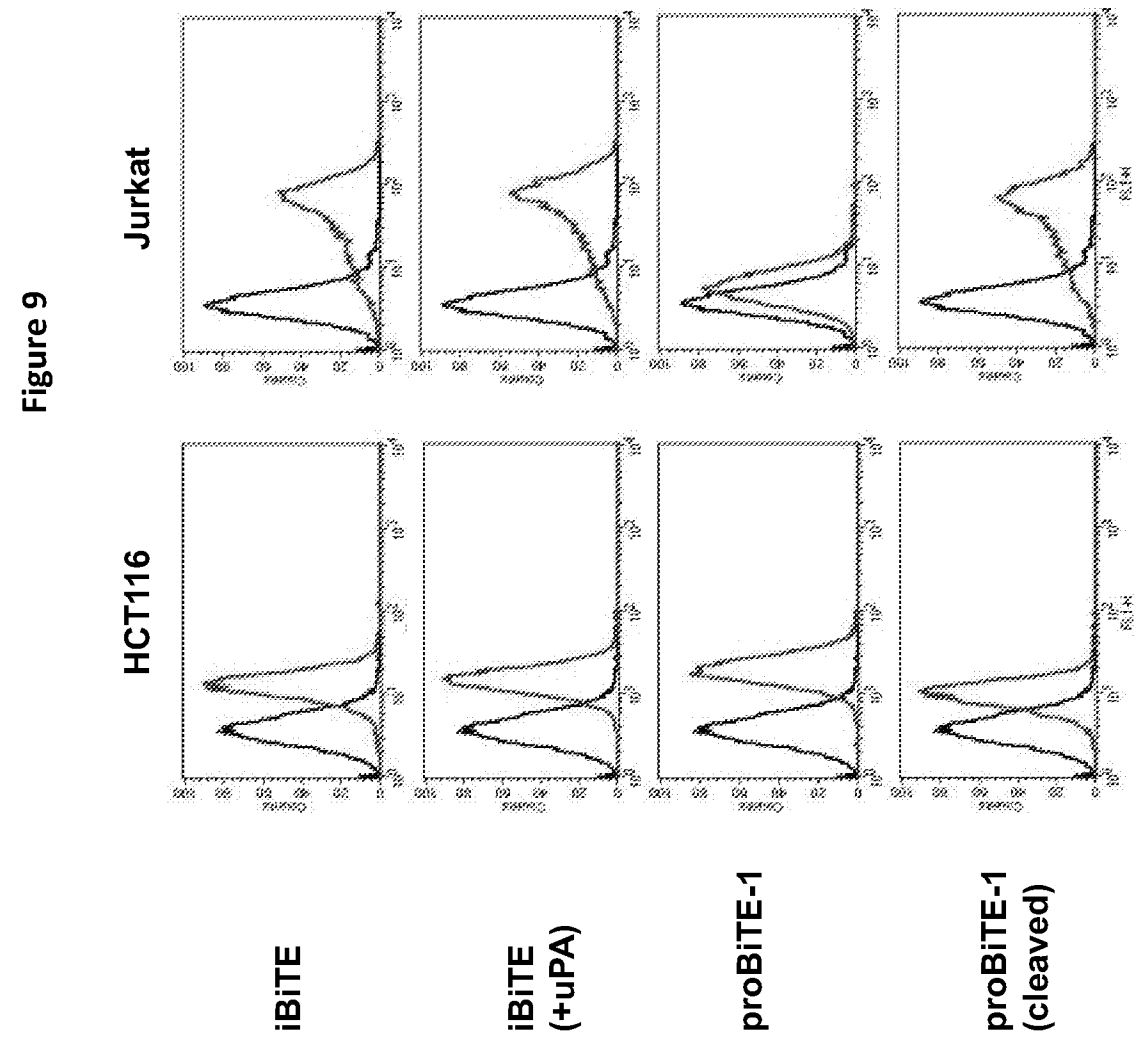


Figure 10

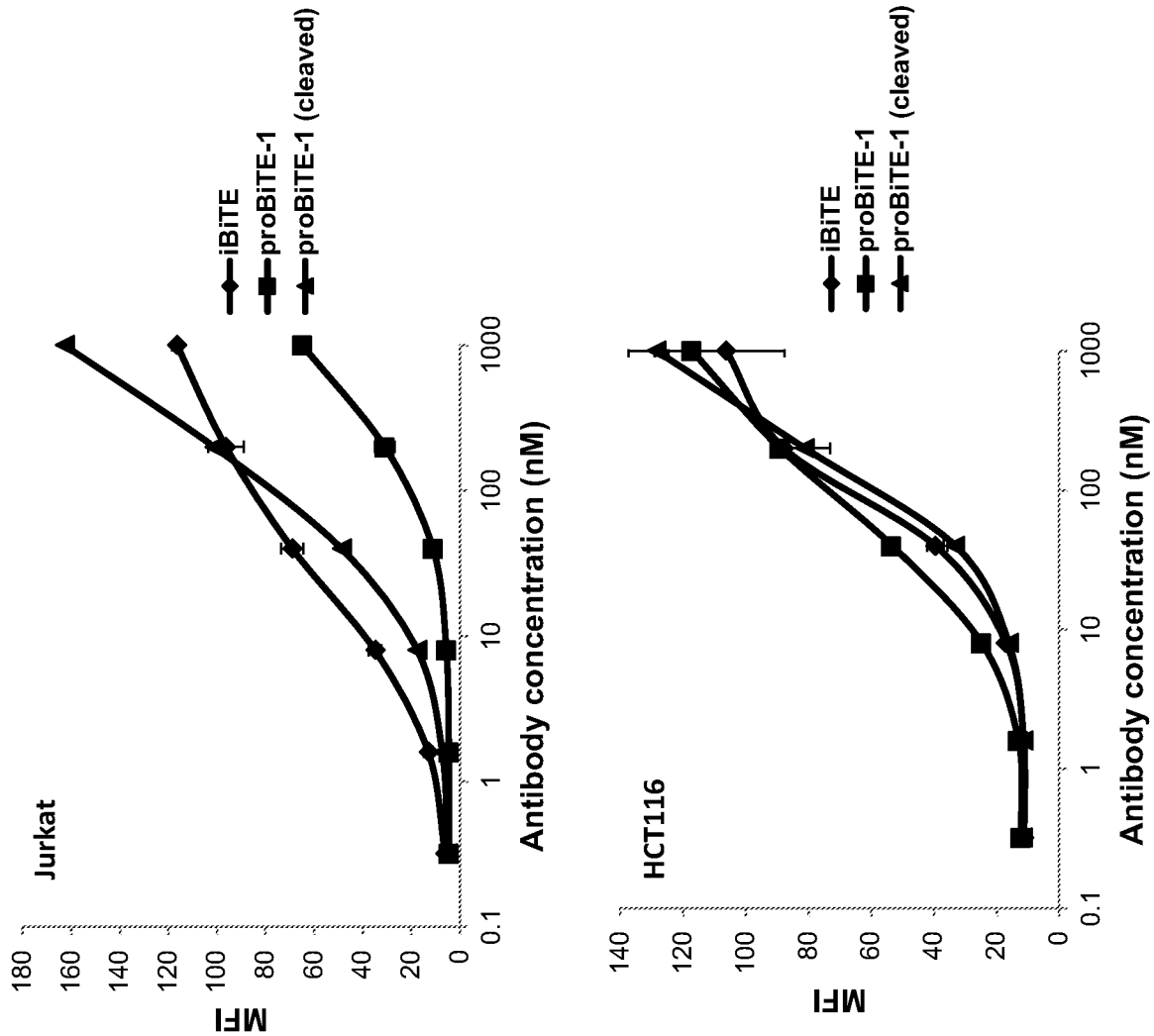

Jurkat
HCT116
MFI
Antibody concentration (nM)
iBiTE
proBiTE-1
proBiTE-1 (cleaved)
0
20
40
60
80
100
120
140
160
180
0.1
1
10
100
1000

Figure 11

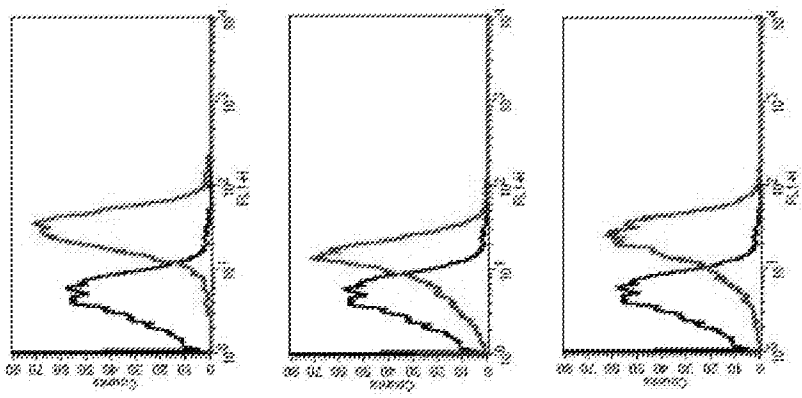


Figure 12

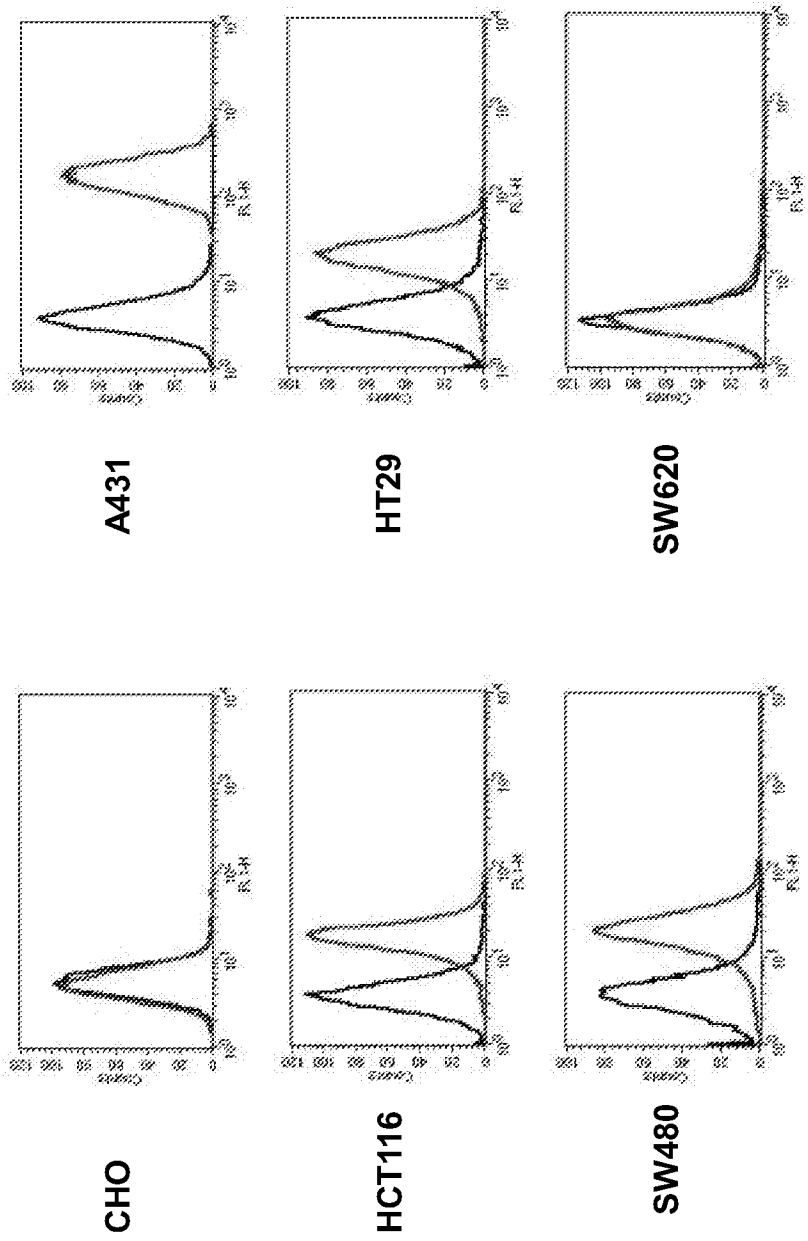


Figure 13

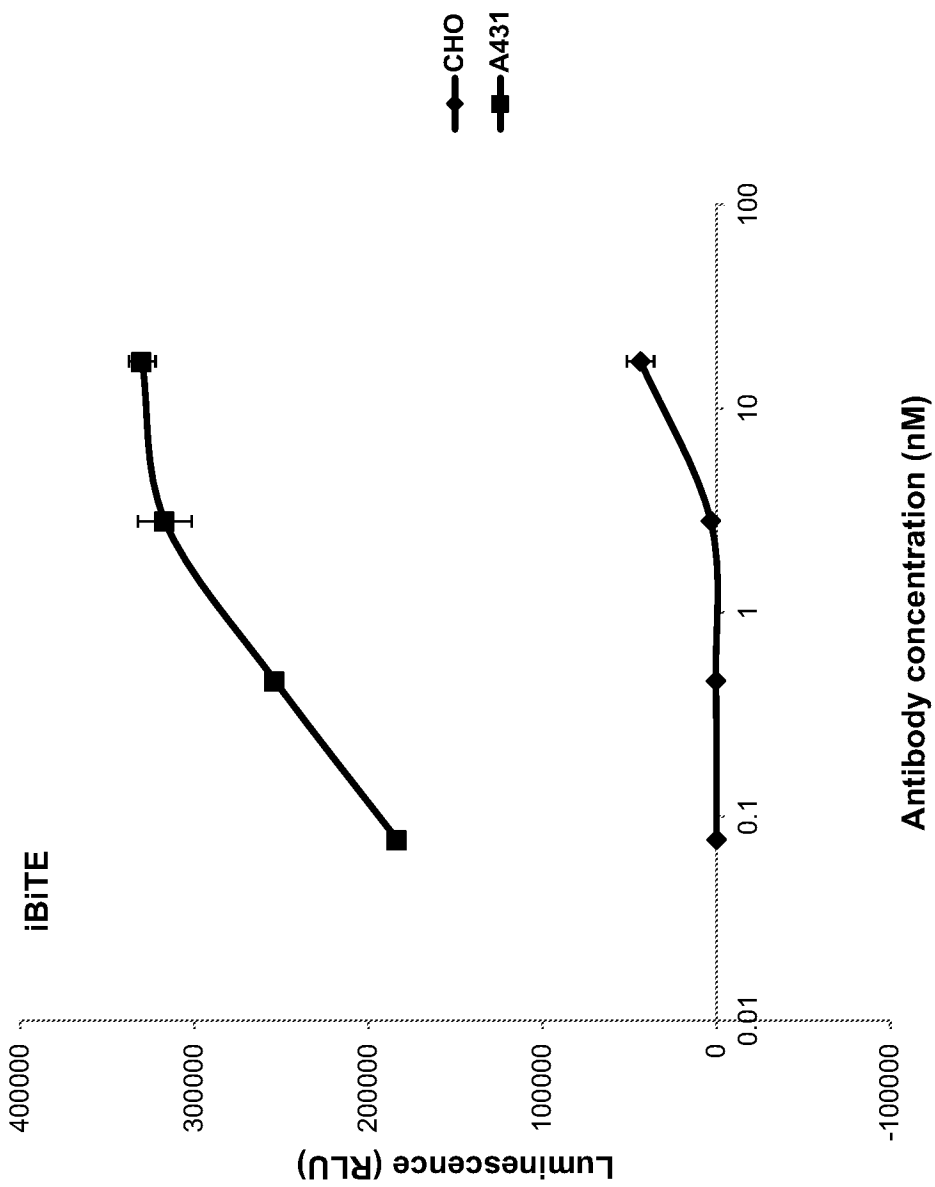

iBiTE
CHO
A431
Luminescence (RLU)
400000
300000
200000
100000
0
-100000
0.01
0.1
1
10
100
Antibody concentration (nM)

Figure 14

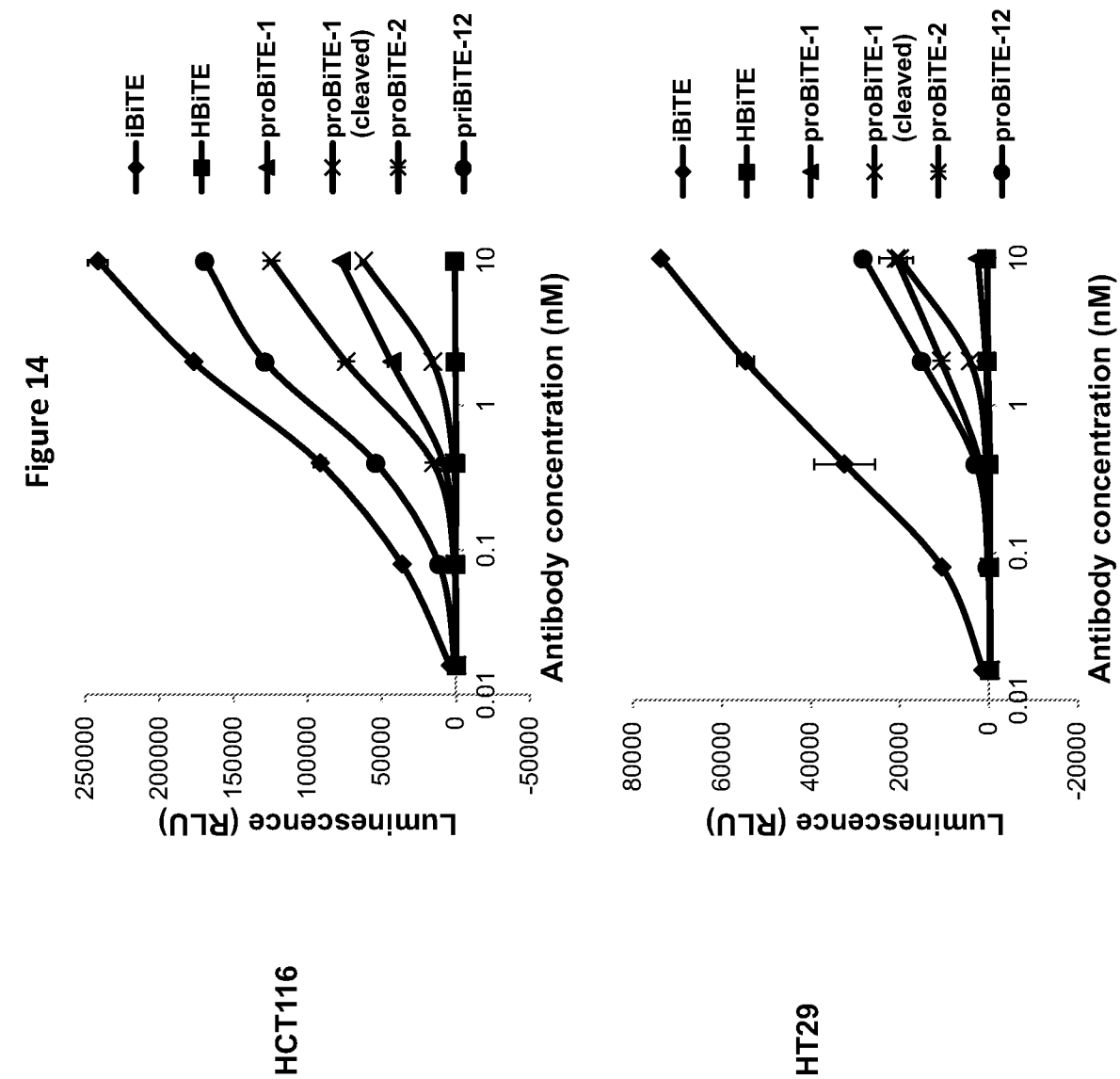

HCT116
HT29
iBiTE
HBiTE
proBiTE-1
proBiTE-1 (cleaved)
proBiTE-2
priBiTE-12
proBiTE-12
Luminescence (RLU)
Antibody concentration (nM)
250000
200000
150000
100000
50000
0
-50000
80000
60000
40000
20000
-20000
0.01
0.1
1
10

Figure 15

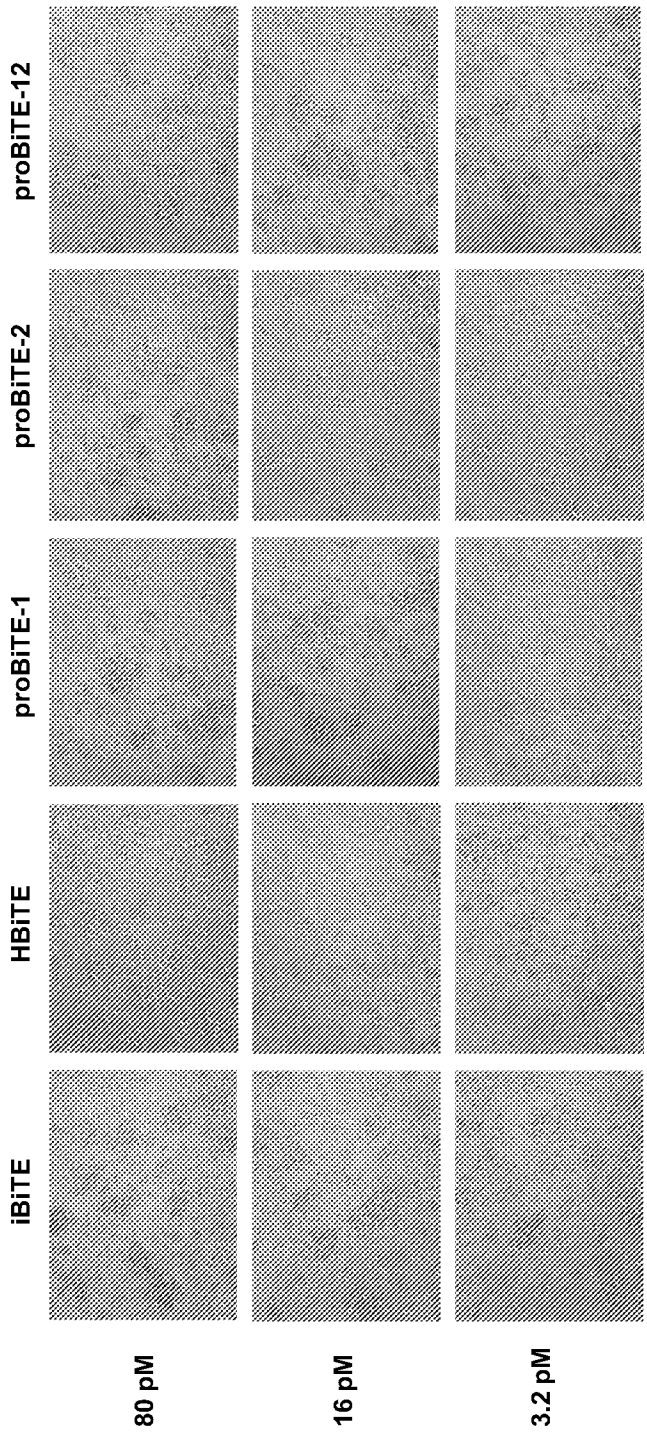


Figure 16

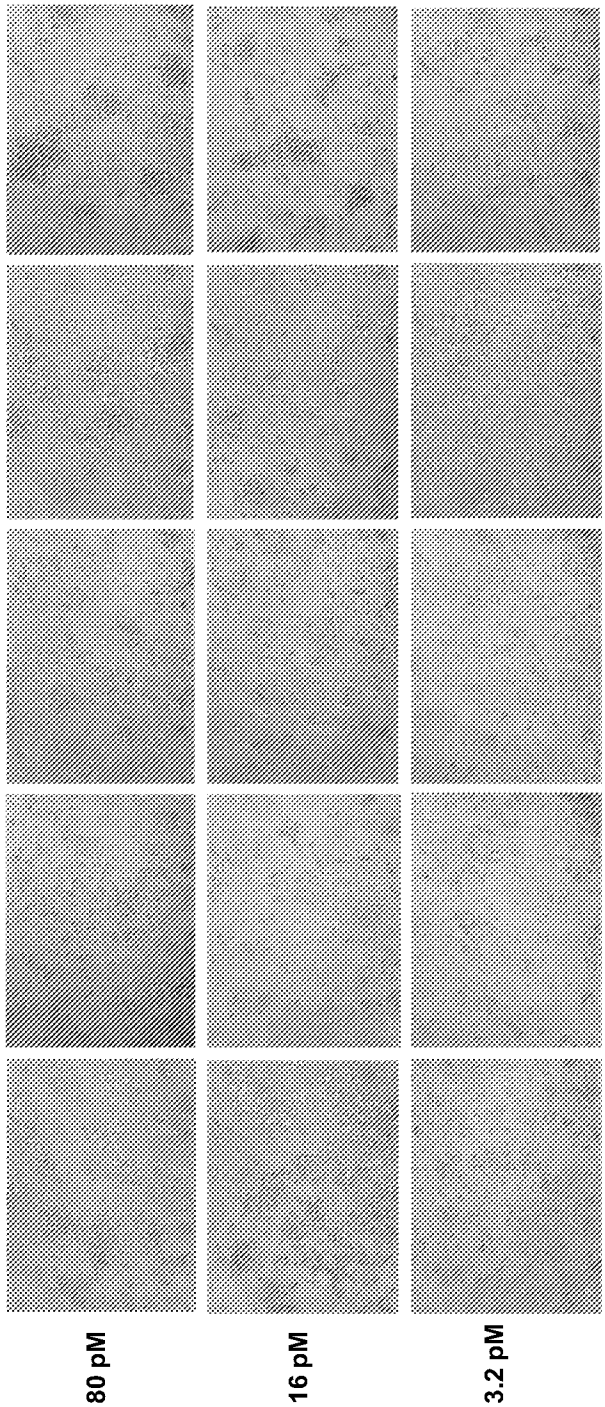


Figure 17

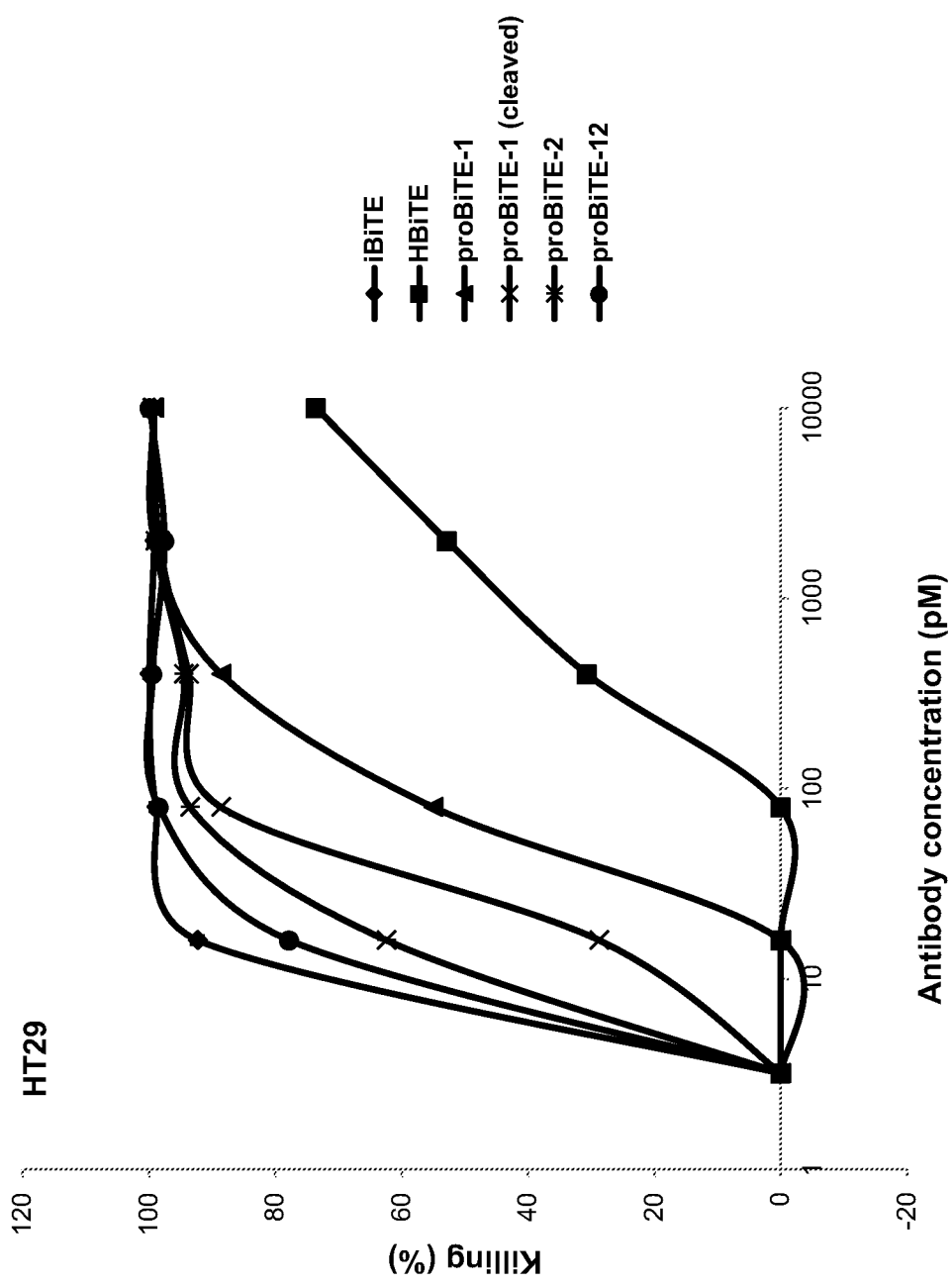

HT29
iBiTE
HBiTE
proBiTE-1
proBiTE-1 (cleaved)
proBiTE-2
proBiTE-12
Killing (%)
120
100
80
60
40
20
0
-20
Antibody concentration (pM)
10000
1000
100
10
1

Figure 18

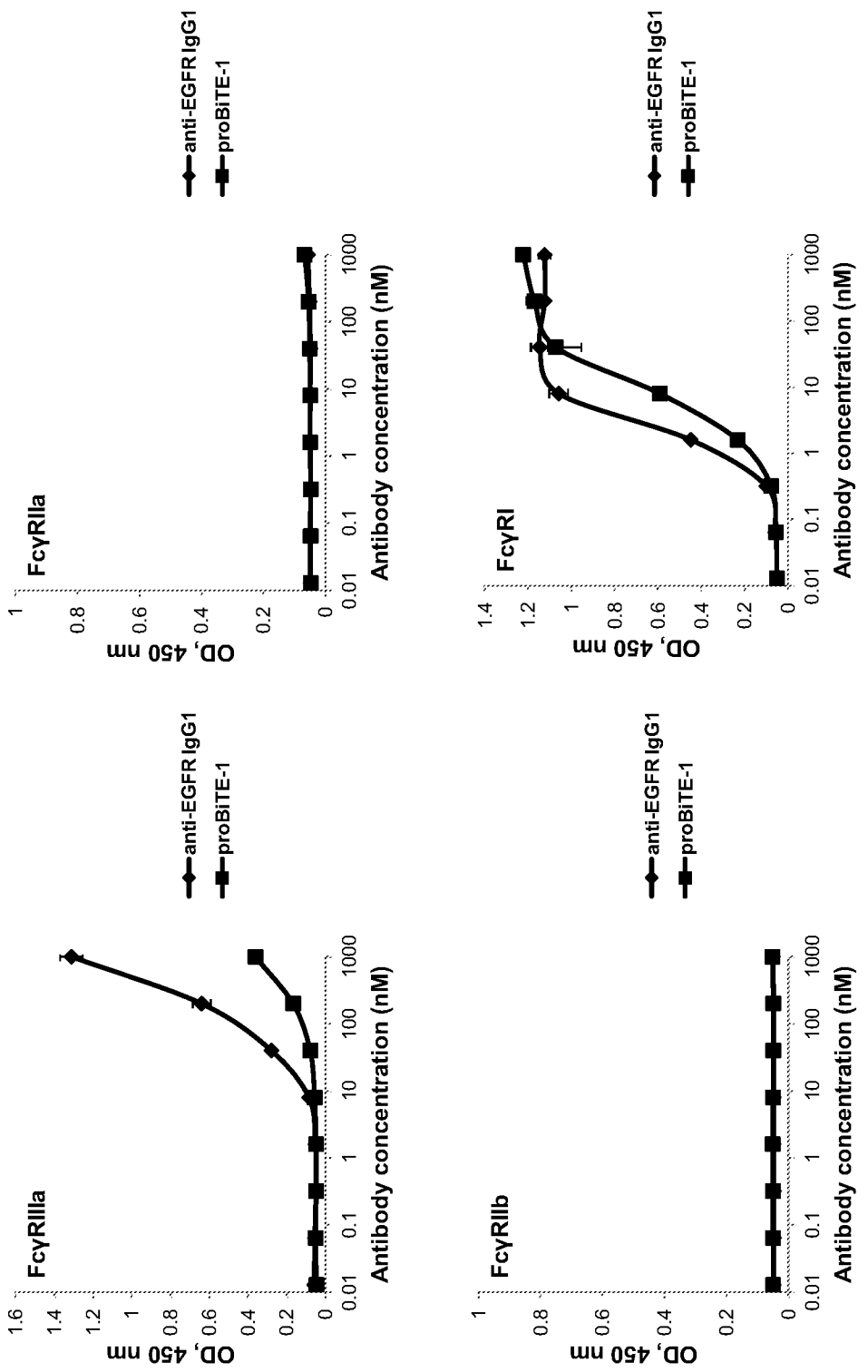
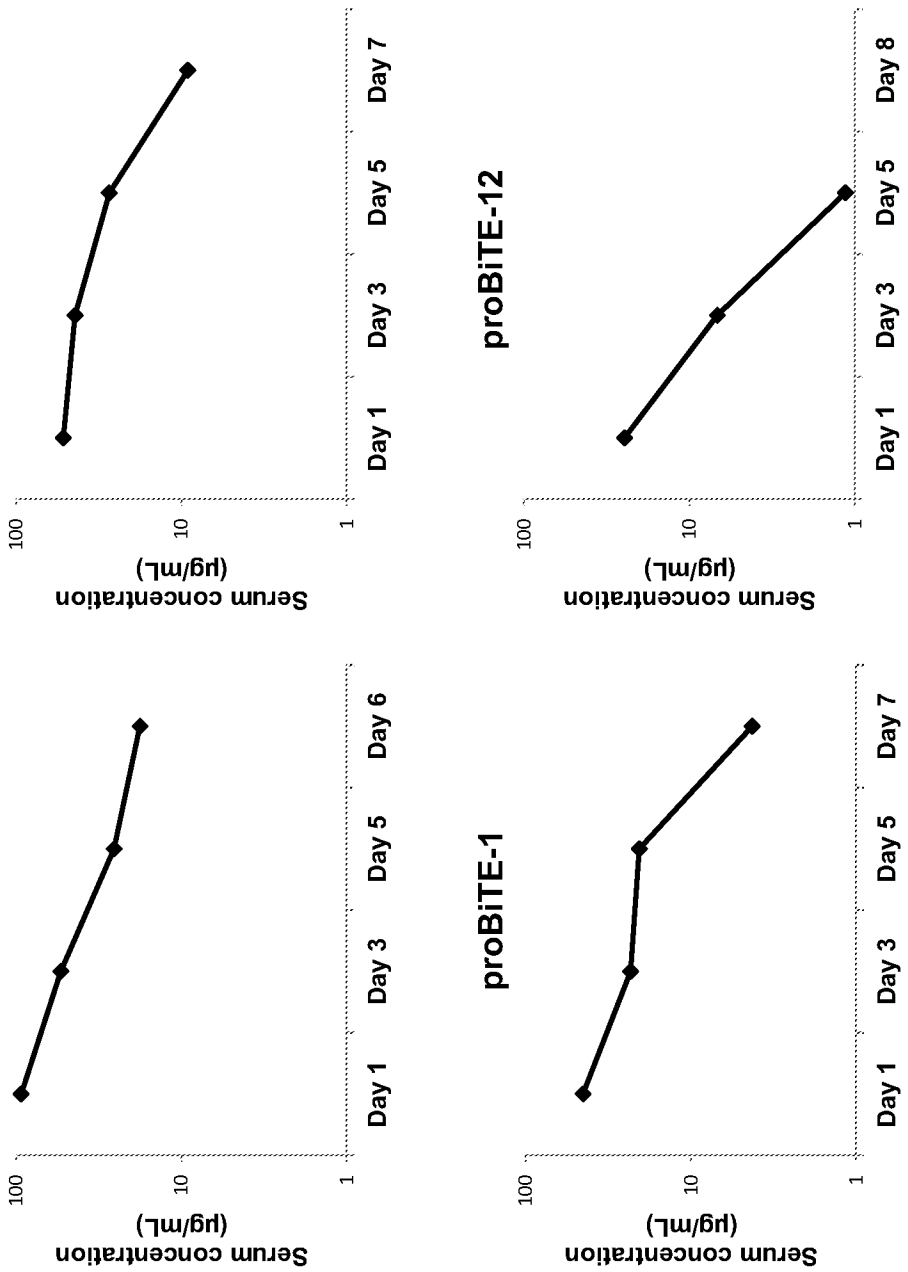

FcγRIIIa
FcγRIIa
FcγRI
FcγRIIb
OD, 450 nm
Antibody concentration (nM)
anti-EGFR IgG1
proBiTE-1

Figure 19


HBiTE
Serum concentration (µg/mL)
100
10
1
Day 1
Day 3
Day 5
Day 7
proBiTE-12
Serum concentration (µg/mL)
100
10
1
Day 1
Day 3
Day 5
Day 8
iBiTE
Serum concentration (µg/mL)
100
10
1
Day 1
Day 3
Day 5
Day 6
proBiTE-1
Serum concentration (µg/mL)
100
10
1
Day 1
Day 3
Day 5
Day 7

Figures 20A-B

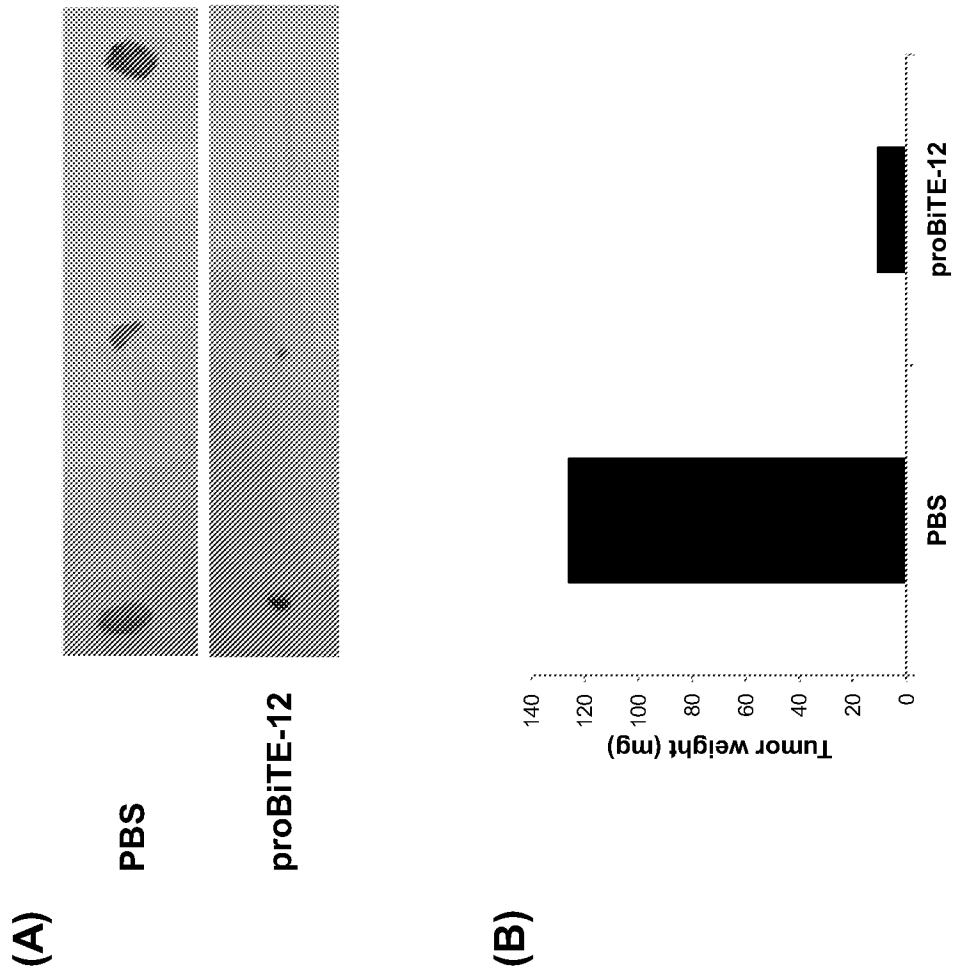


Figure 21

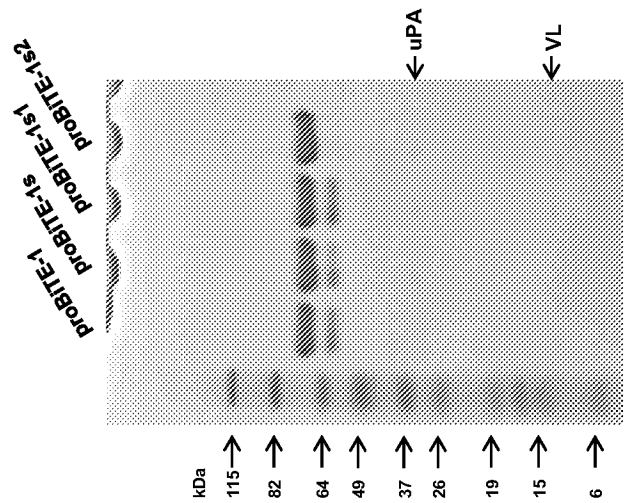

proBiTE-1
proBiTE-1s1
proBiTE-1s2
uPA
VL
kDa
115
82
64
49
37
26
19
15
6

Figure 22

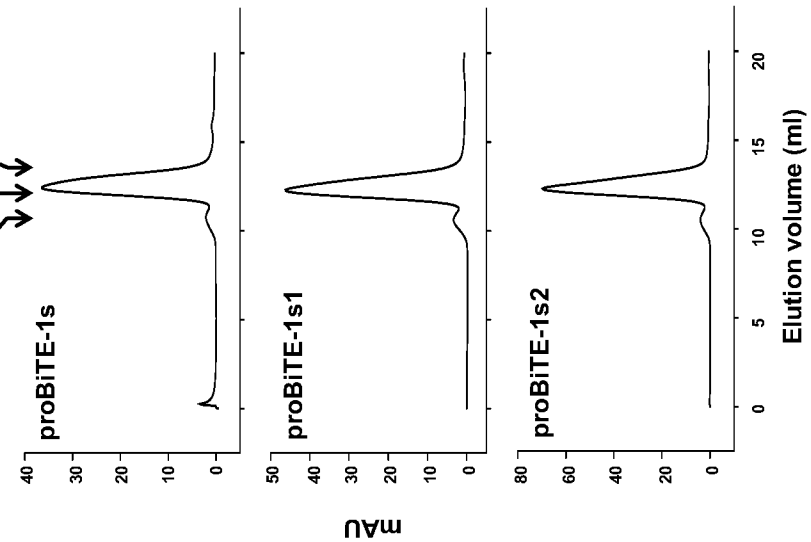

kDa
440
158
75
proBiTE-1s
proBiTE-1s1
proBiTE-1s2
mAU
Elution volume (ml)

Figure 23

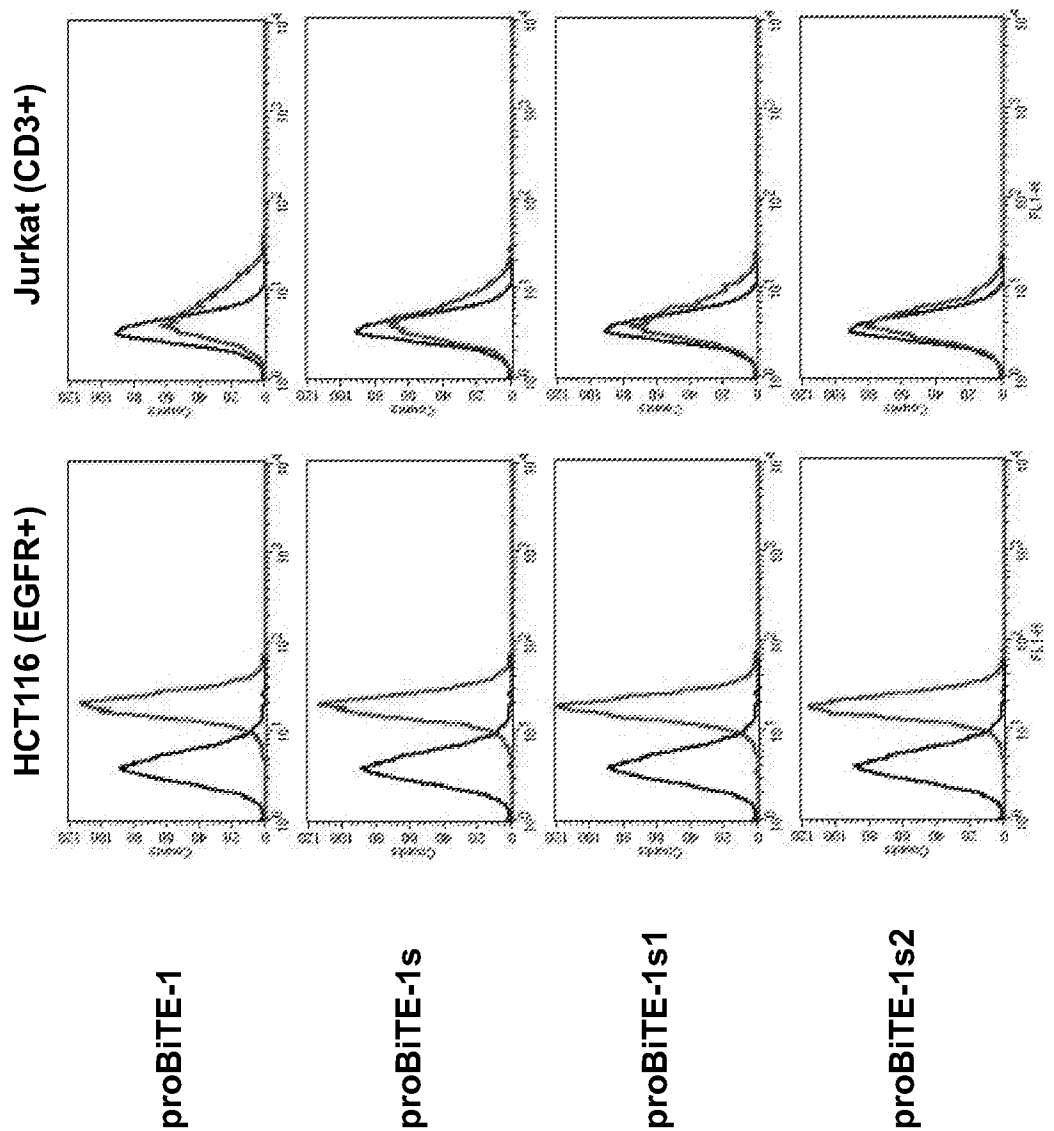

Jurkat (CD3+)
HCT116 (EGFR+)
proBiTE-1
proBiTE-1s
proBiTE-1s1
proBiTE-1s2

Figure 24

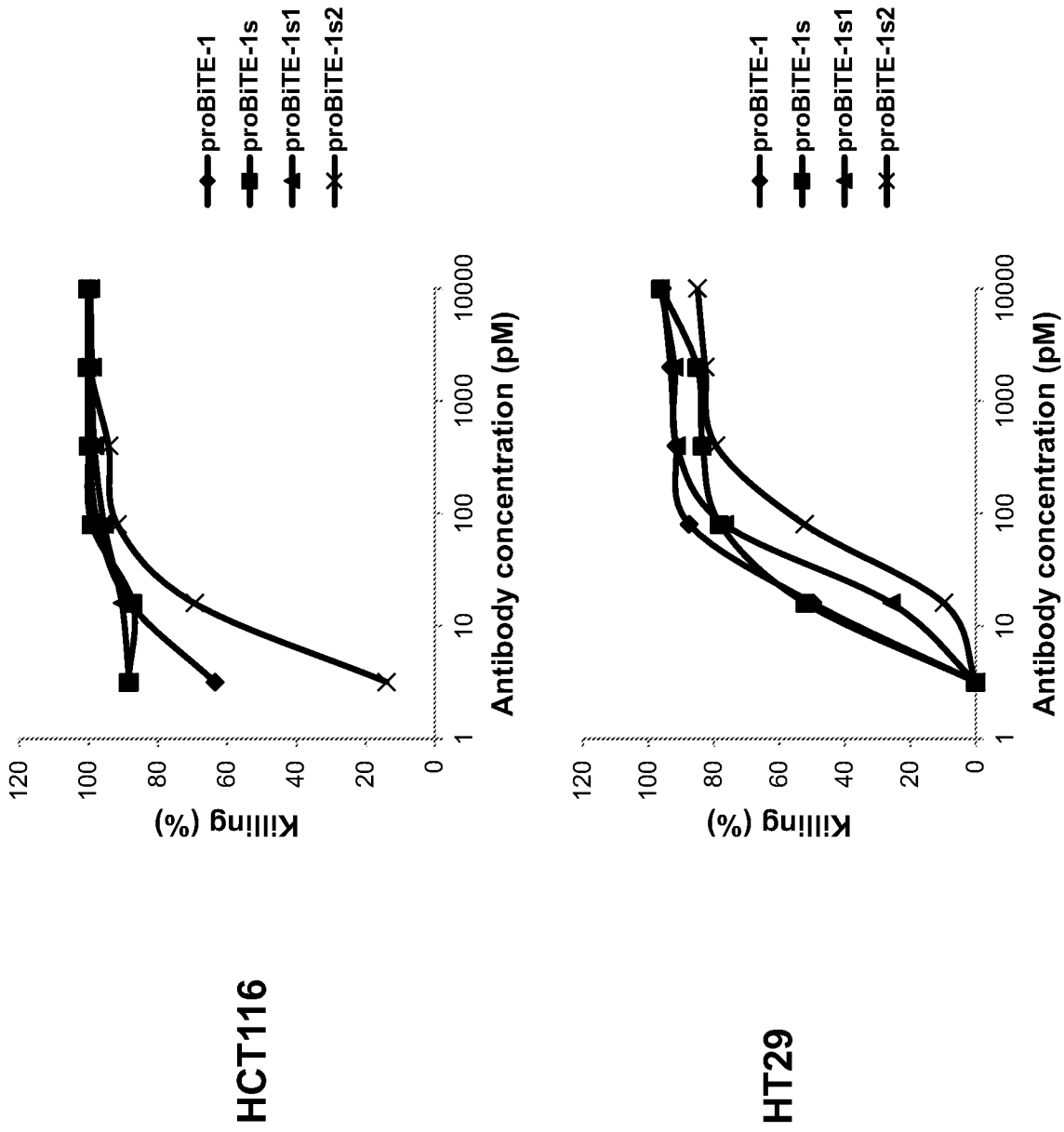

HCT116
Killing (%)
120
100
80
60
40
20
0
1
10
100
1000
10000
Antibody concentration (pM)
proBiTE-1
proBiTE-1s
proBiTE-1s1
proBiTE-1s2
HT29
Killing (%)
120
100
80
60
40
20
0
1
10
100
1000
10000
Antibody concentration (pM)
proBiTE-1
proBiTE-1s
proBiTE-1s1
proBiTE-1s2

Figure 25

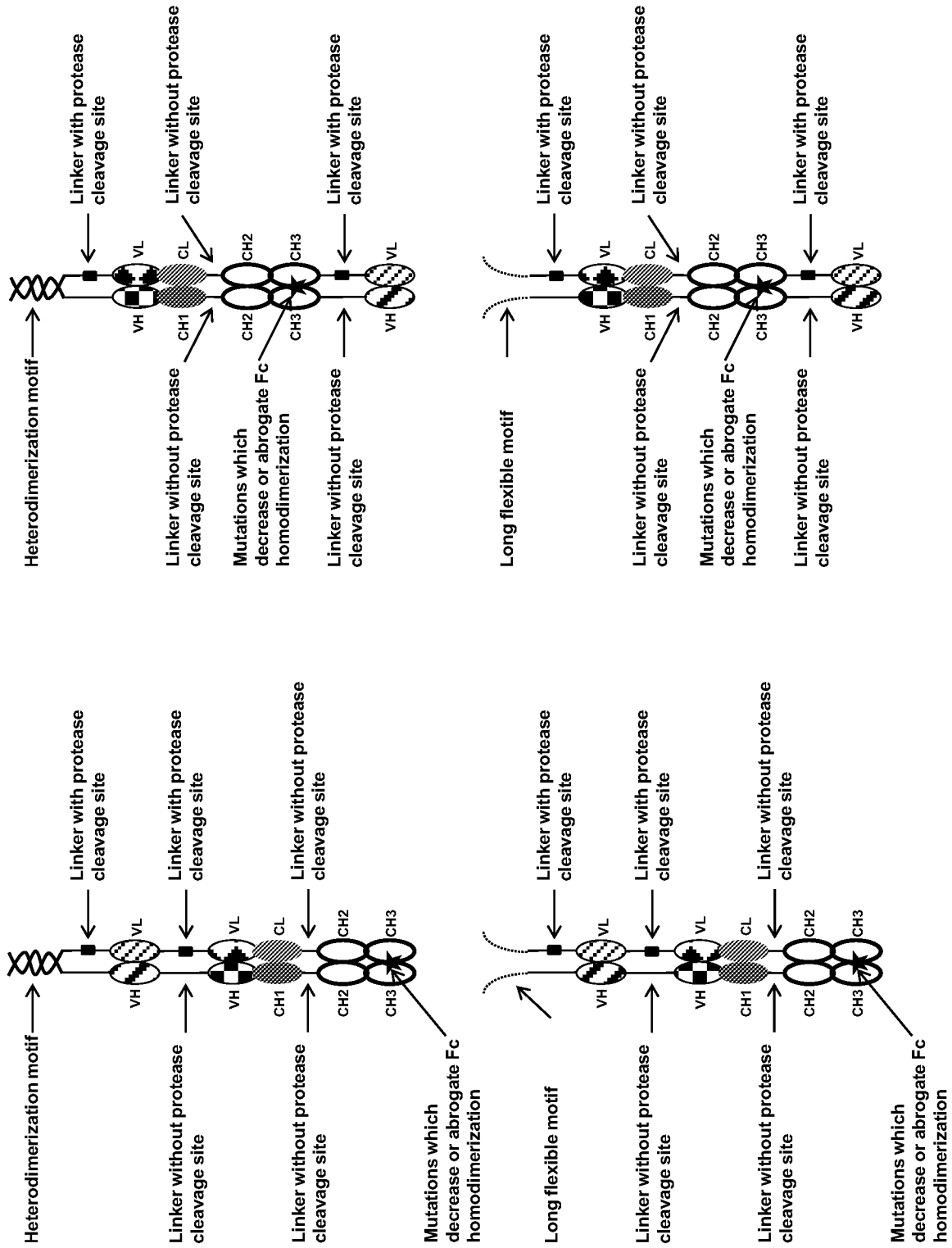

Heterodimerization motif
Linker with protease cleavage site
Linker without protease cleavage site
Mutations which decrease or abrogate Fc homodimerization
Linker without protease cleavage site
Linker with protease cleavage site
VH
VL
CH1
CL
CH2
CH3
Long flexible motif
Linker without protease cleavage site
Mutations which decrease or abrogate Fc homodimerization
Linker without protease cleavage site
Linker with protease cleavage site

Figure 26

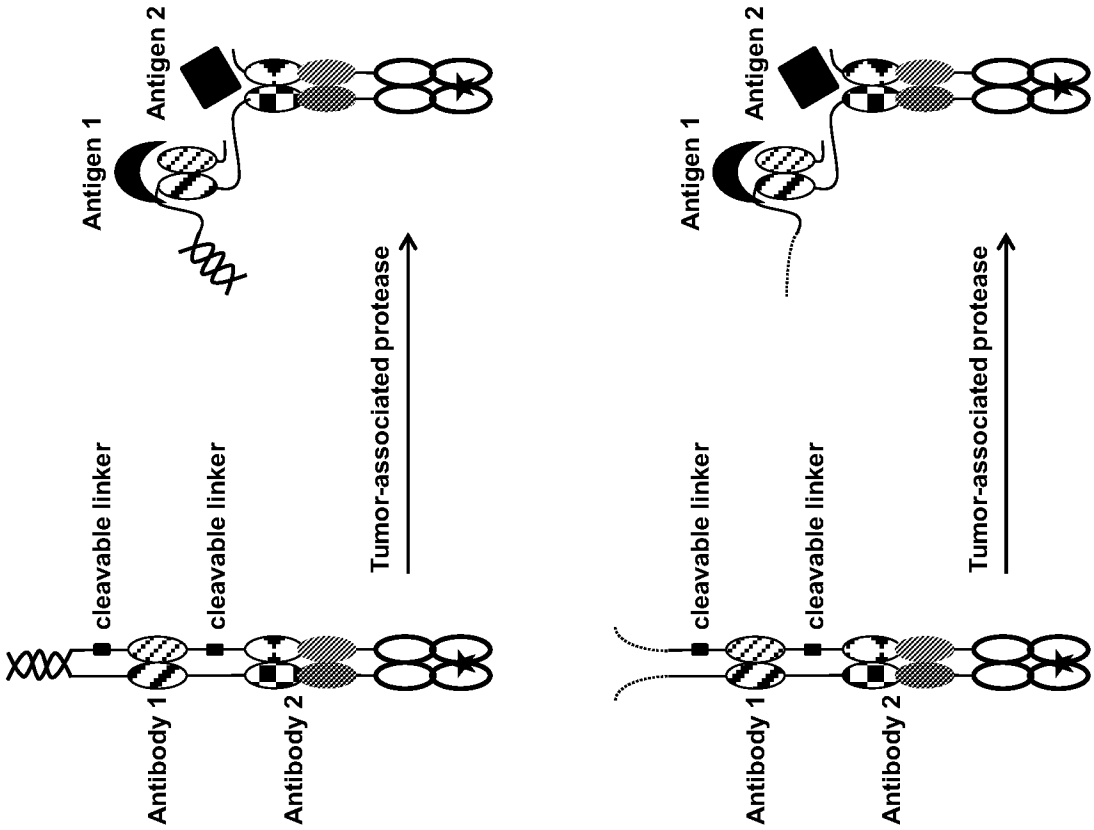
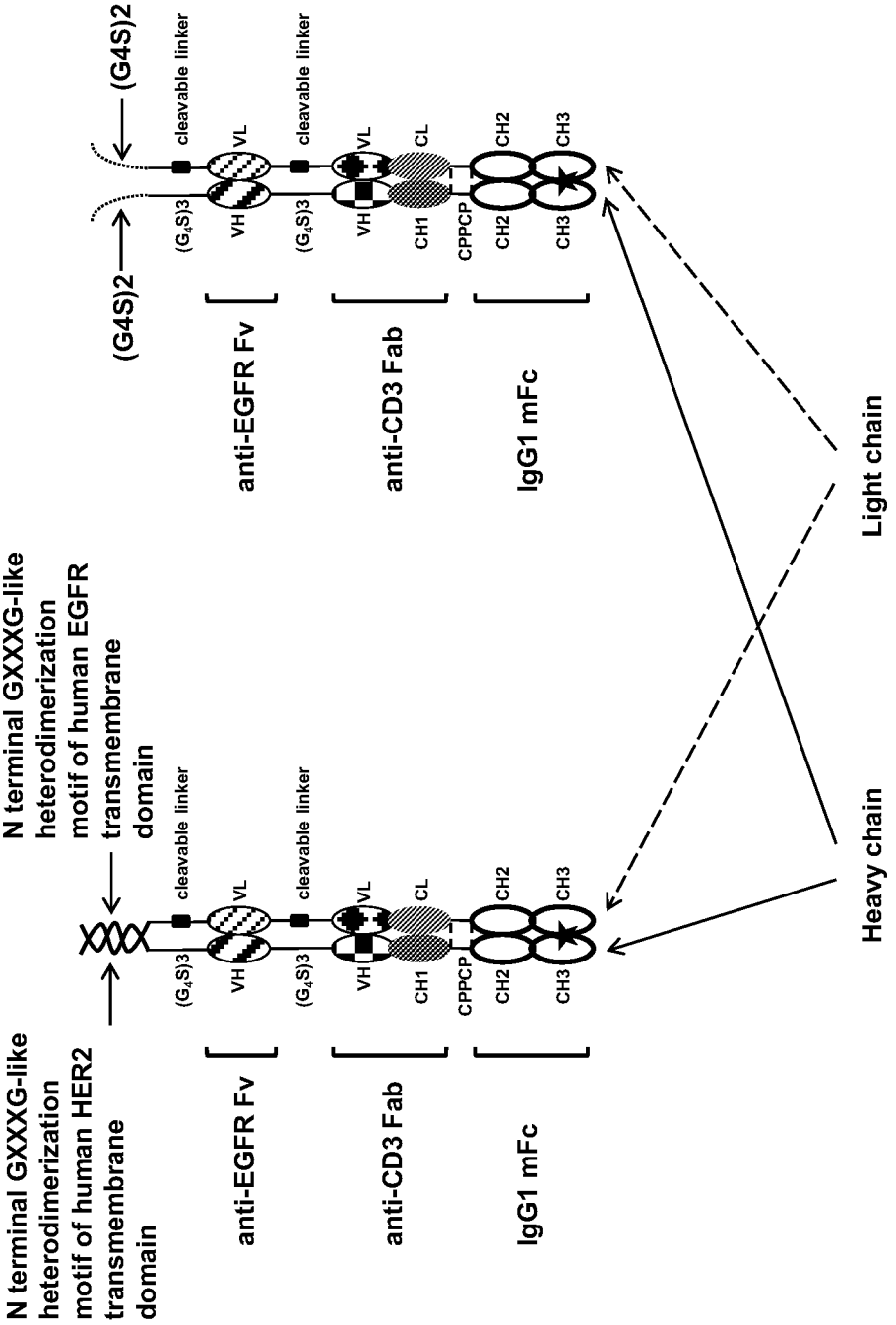

Antigen 1
Antigen 2
cleavable linker
cleavable linker
Tumor-associated protease
Antibody 1
Antibody 2
Antigen 1
Antigen 2
cleavable linker
cleavable linker
Tumor-associated protease
Antibody 1
Antibody 2

Figure 27


dproBiTE-HE
N terminal GXXXG-like heterodimerization motif of human HER2 transmembrane domain
N terminal GXXXG-like heterodimerization motif of human EGFR transmembrane domain
cleavable linker
(G4S)2
(G4S)3
VH
VL
CH1
CL
CPPCP
CH2
CH3
anti-EGFR Fv
anti-CD3 Fab
IgG1 mFc
dproBiTE-GS
Heavy chain
Light chain

Figures 28A-B

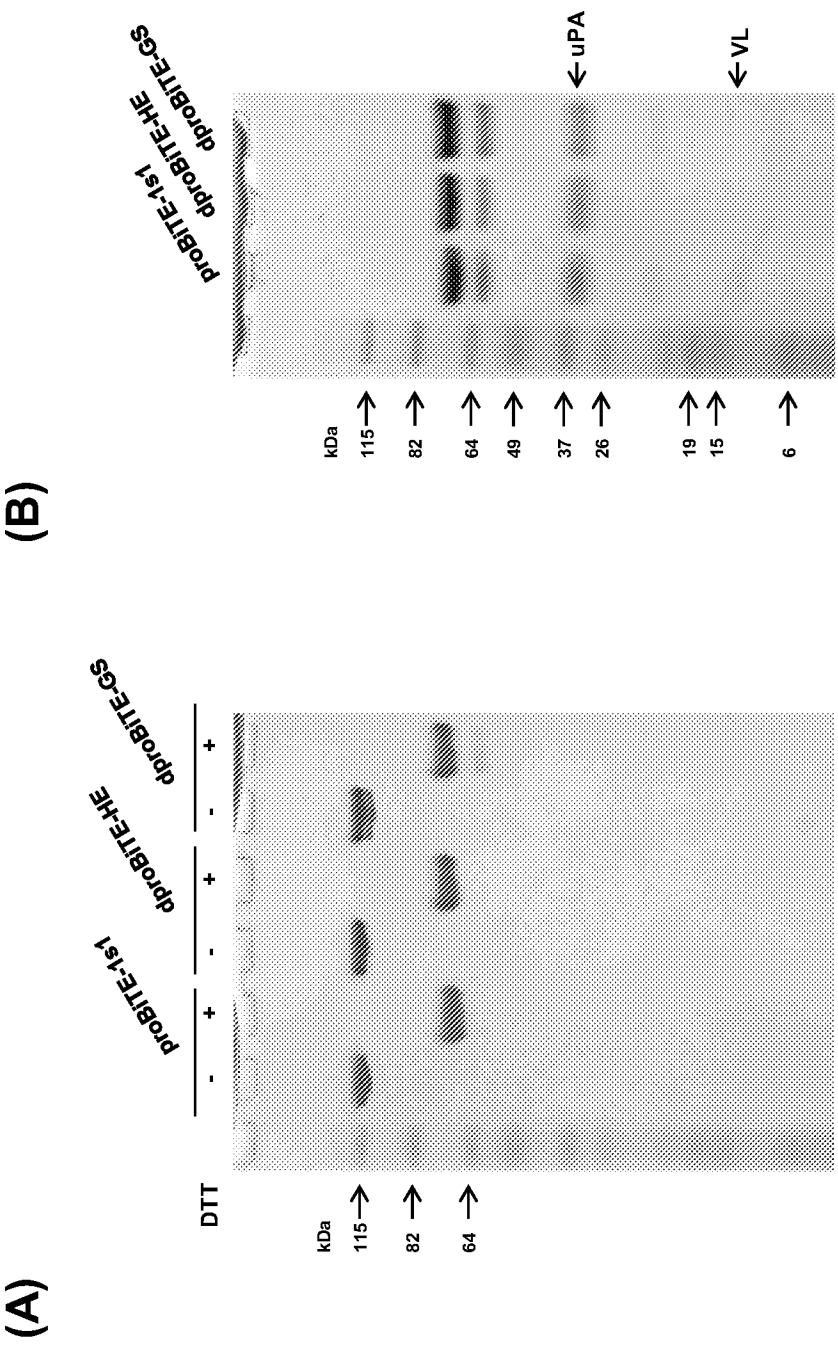


Figure 29

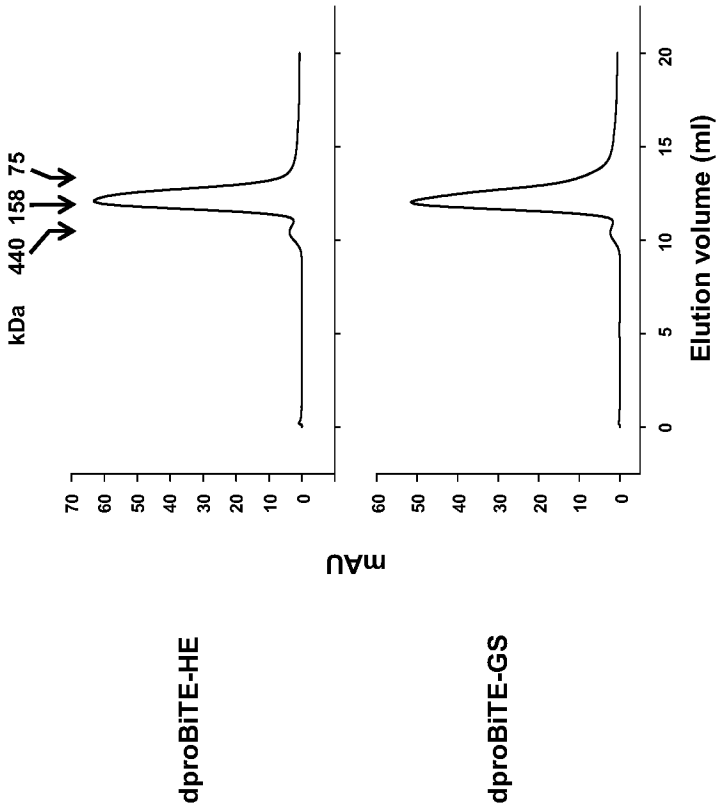

kDa
440
158
75
70
60
50
40
30
20
10
0
mAU
dproBiTE-HE
60
50
40
30
20
10
0
dproBiTE-GS
0
5
10
15
20
Elution volume (ml)

Figure 30

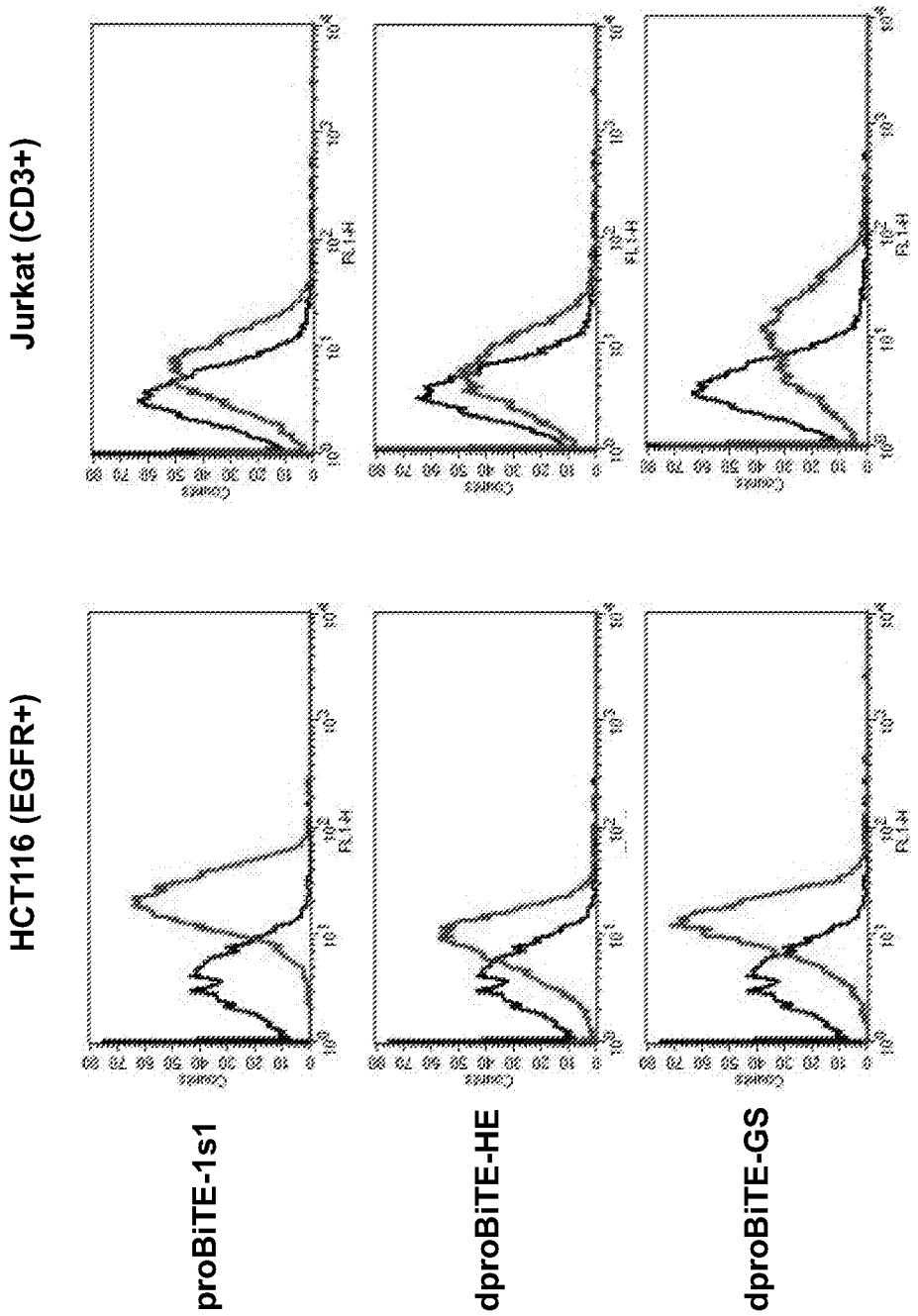
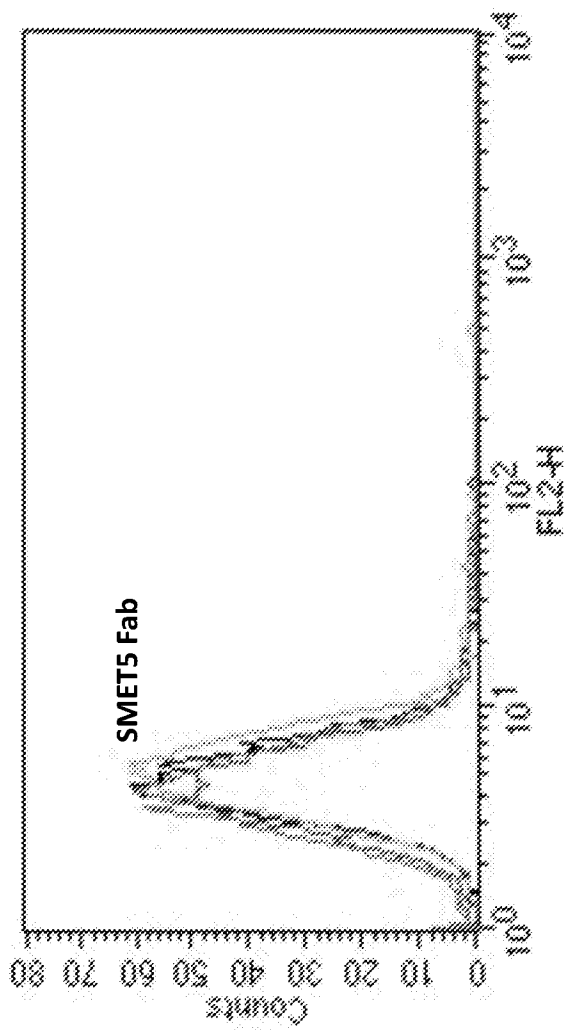
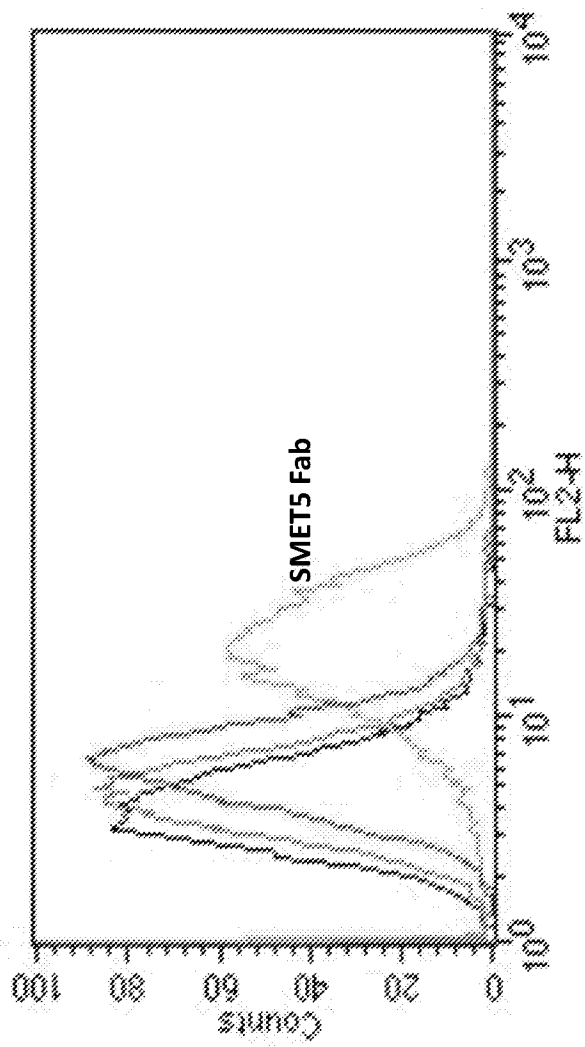


Figure 31



CHO (EGFR-)



A549 (EGFR+)

Figure 32

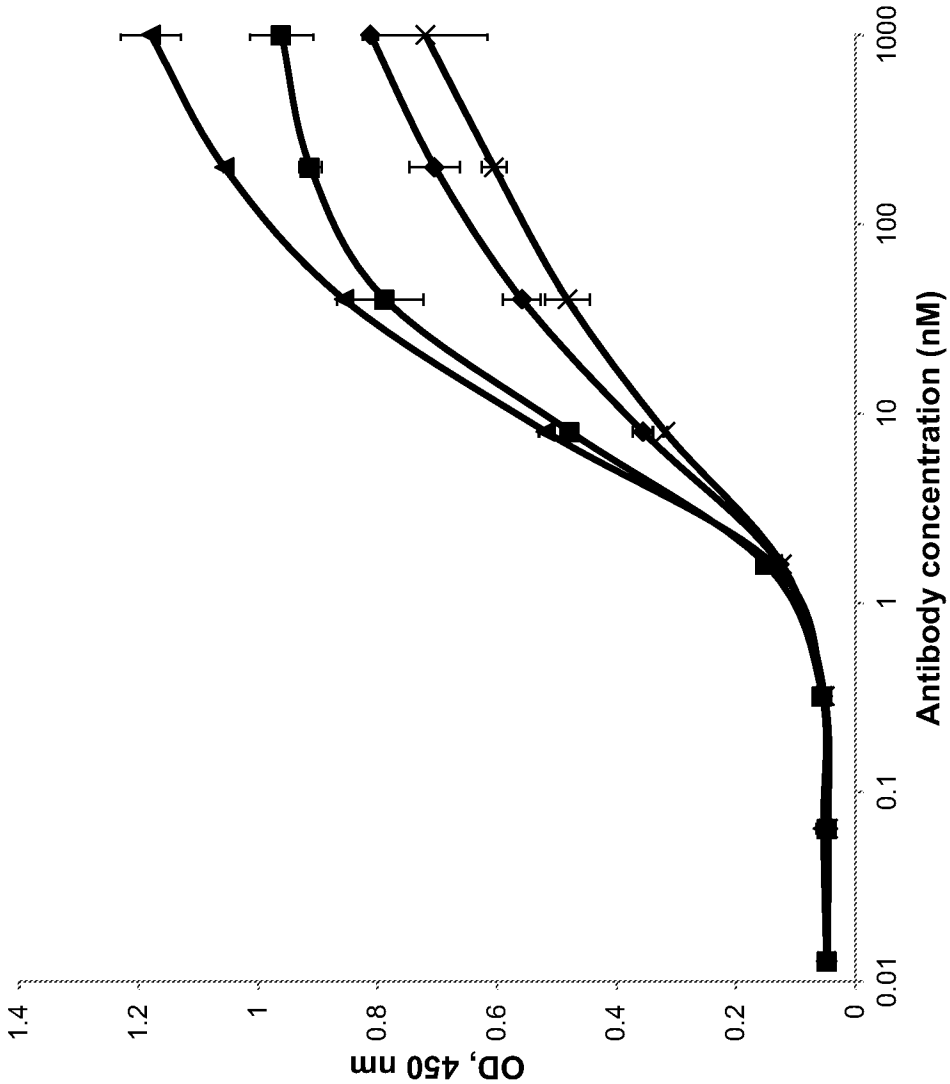
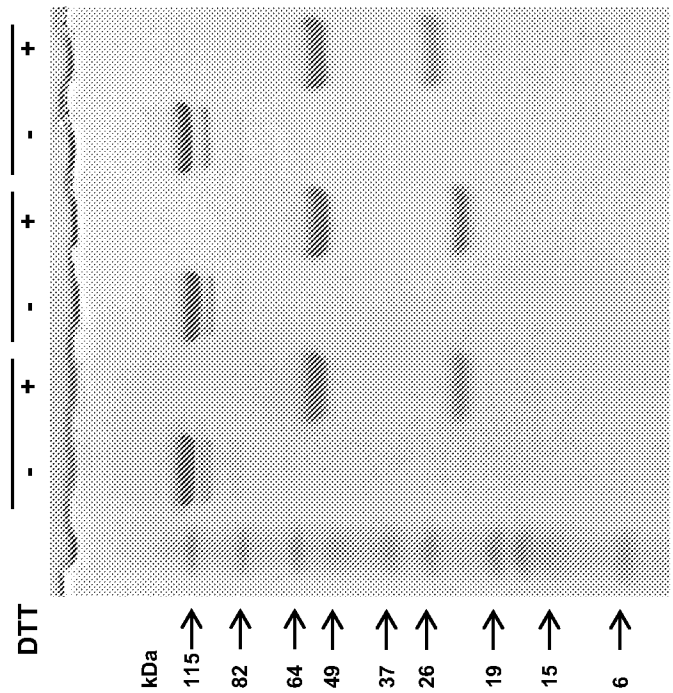

SMET5 Fab
SMET5.2 Fab
SMET5.3 Fab
SMET5.4 Fab
OD, 450 nm
0
0.2
0.4
0.6
0.8
1
1.2
1.4
0.01
0.1
1
10
100
1000
Antibody concentration (nM)

Figure 33



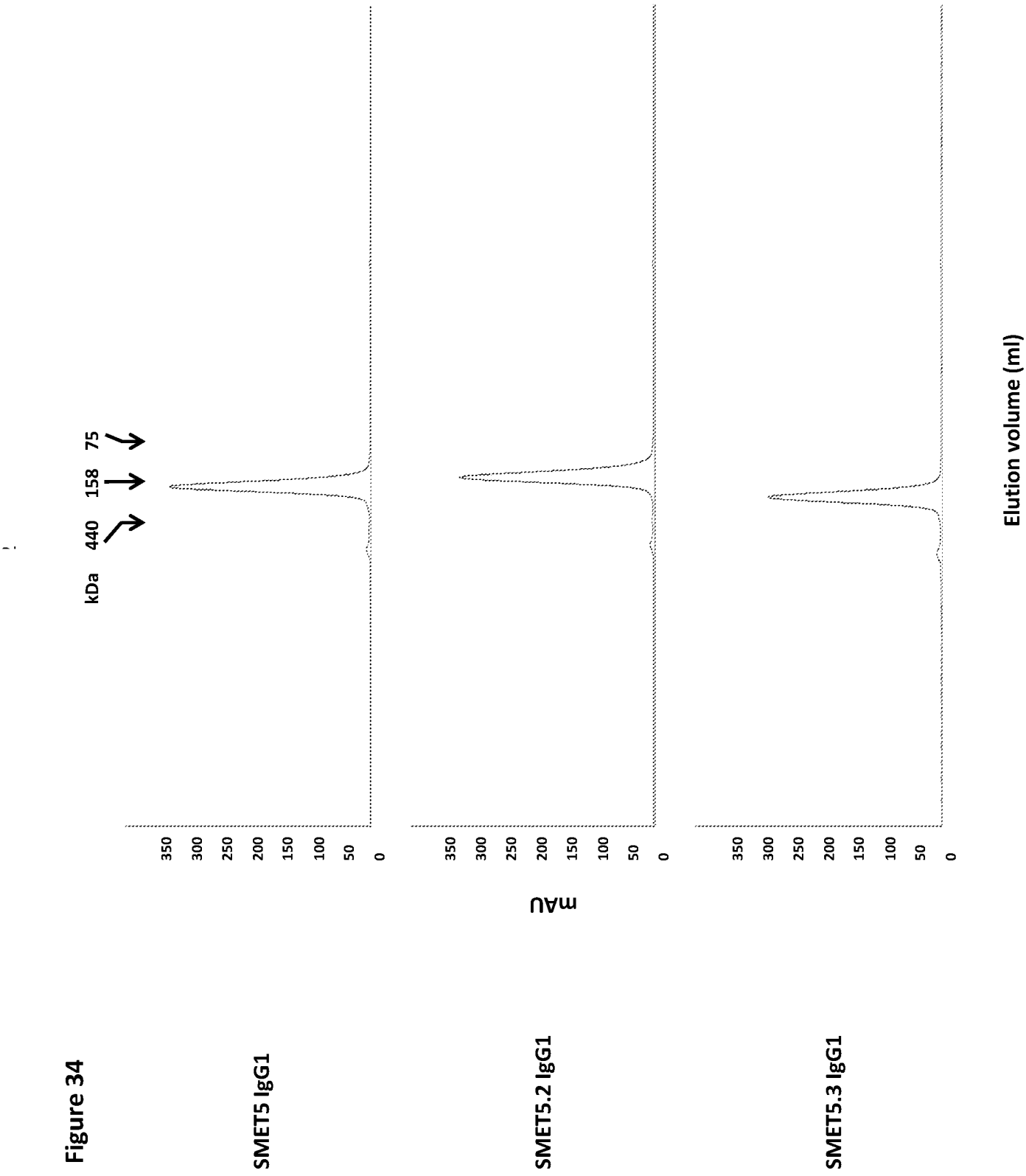

kDa
440
158
75
SMET5 IgG1
SMET5.2 IgG1
SMET5.3 IgG1
mAU
350
300
250
200
150
100
50
0
Elution volume (ml)

Figure 34

Figure 35

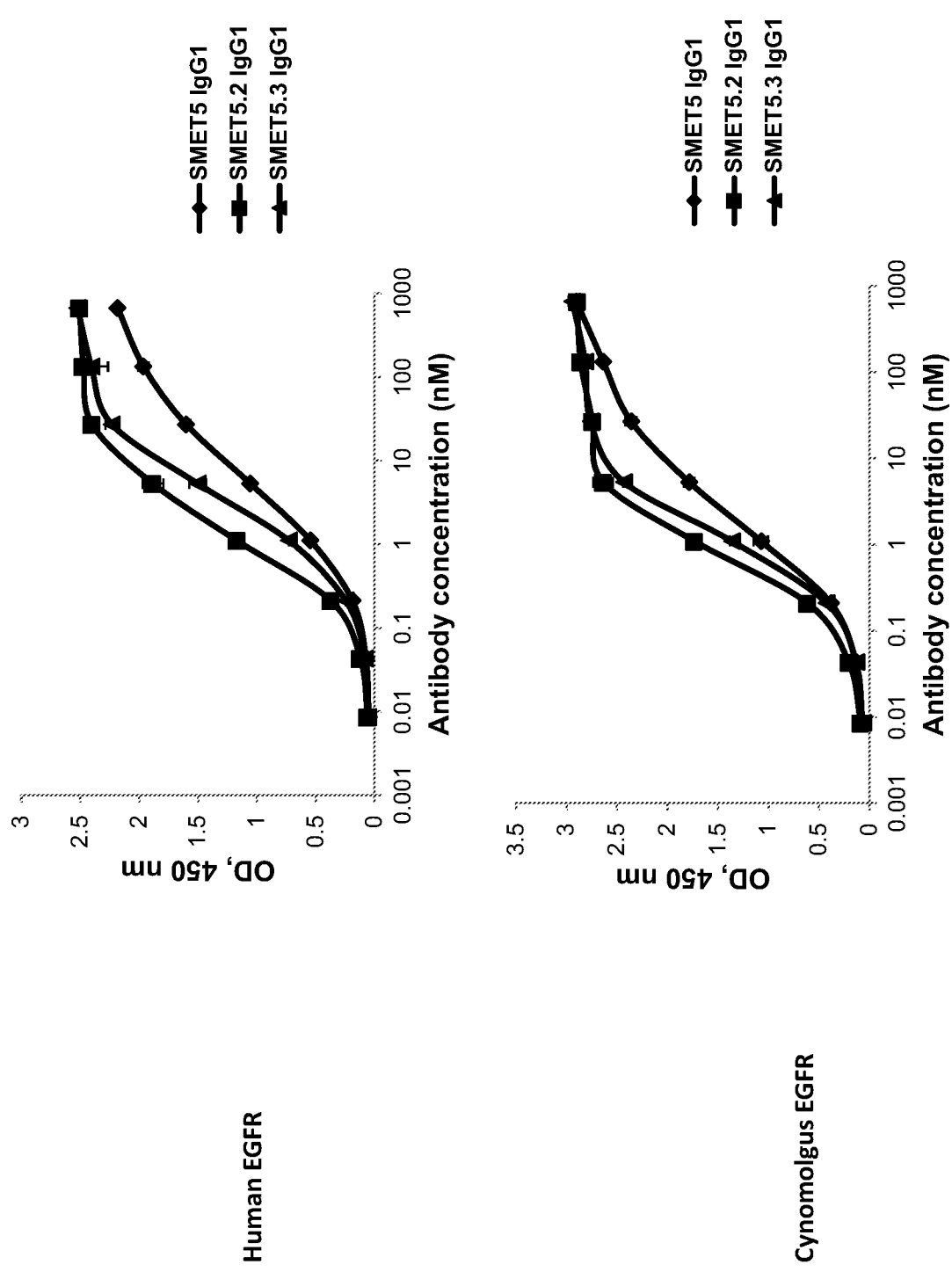
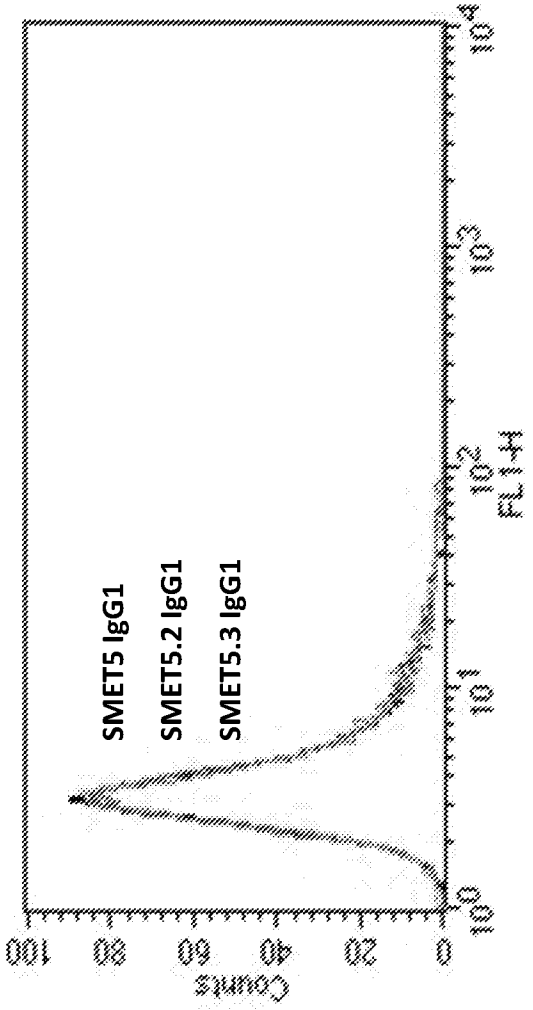
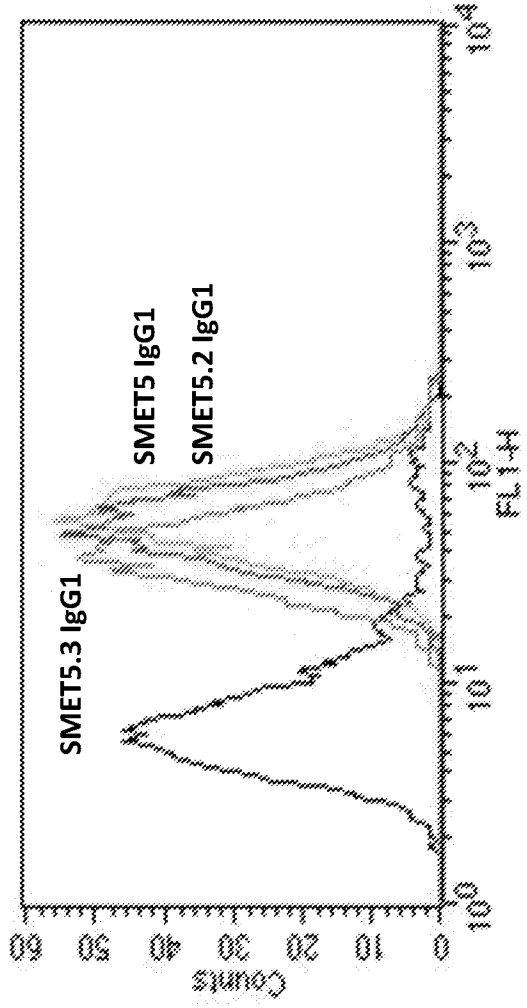

Human EGFR
OD, 450 nm
Antibody concentration (nM)
SMET5 IgG1
SMET5.2 IgG1
SMET5.3 IgG1
Cynomolgus EGFR
OD, 450 nm
Antibody concentration (nM)
SMET5 IgG1
SMET5.2 IgG1
SMET5.3 IgG1

Figure 36



CHO (EGFR-)



A549 (EGFR+)

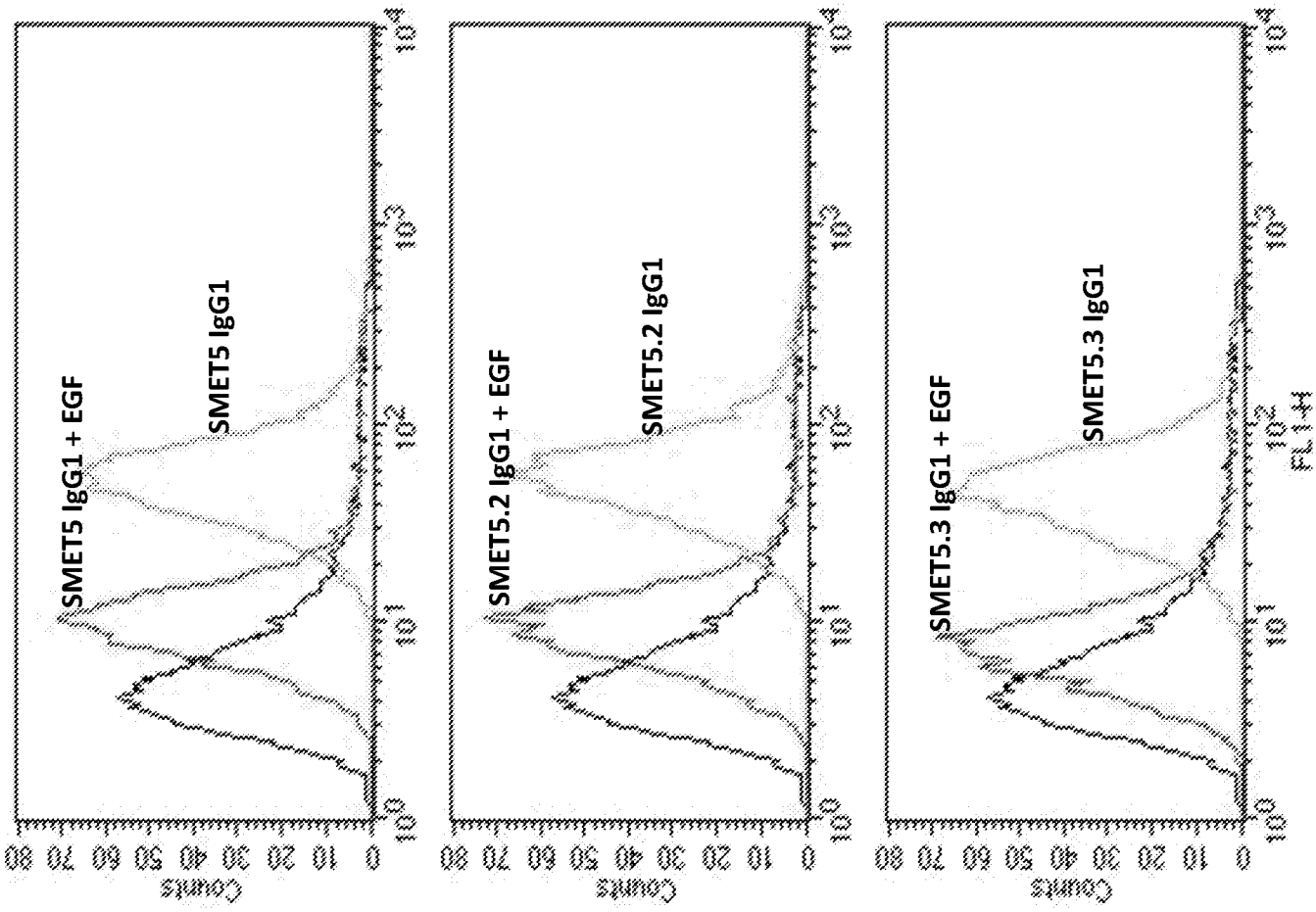

SMET5 IgG1 + EGF
SMET5 IgG1
SMET5.2 IgG1 + EGF
SMET5.2 IgG1
SMET5.3 IgG1 + EGF
SMET5.3 IgG1
Counts
FL1-H

Figure 37

Figure 38

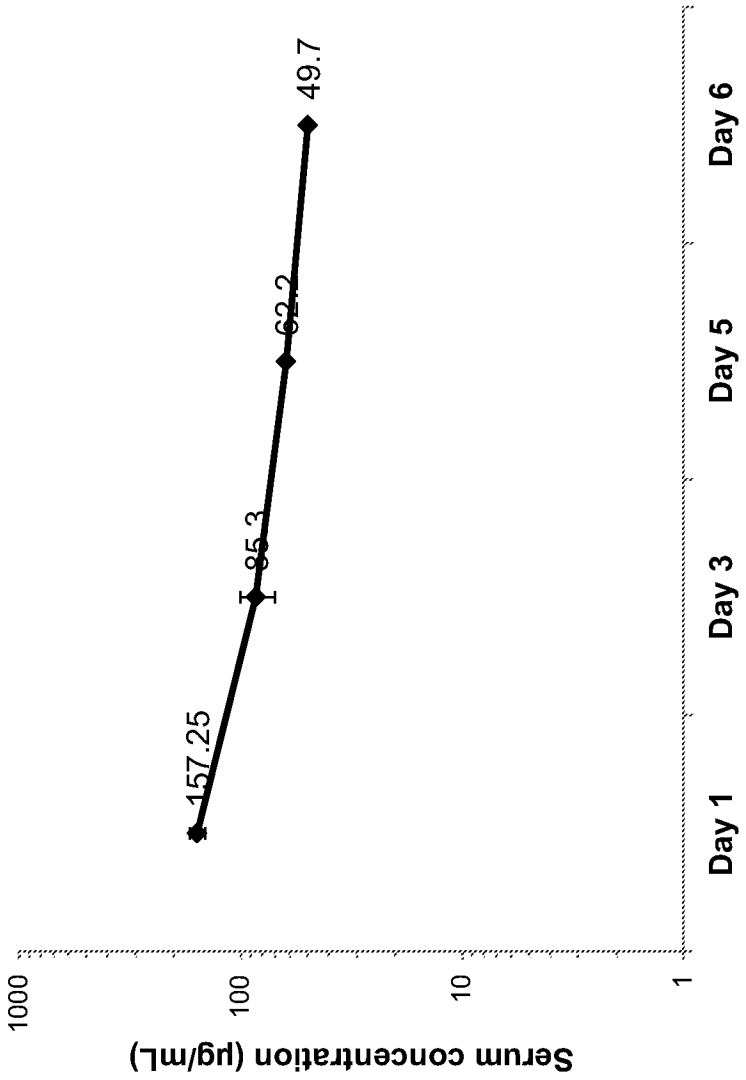

Serum concentration (µg/mL)
1000
100
10
1
Day 1
Day 3
Day 5
Day 6
157.25
85.3
62.2
49.7

Figure 39

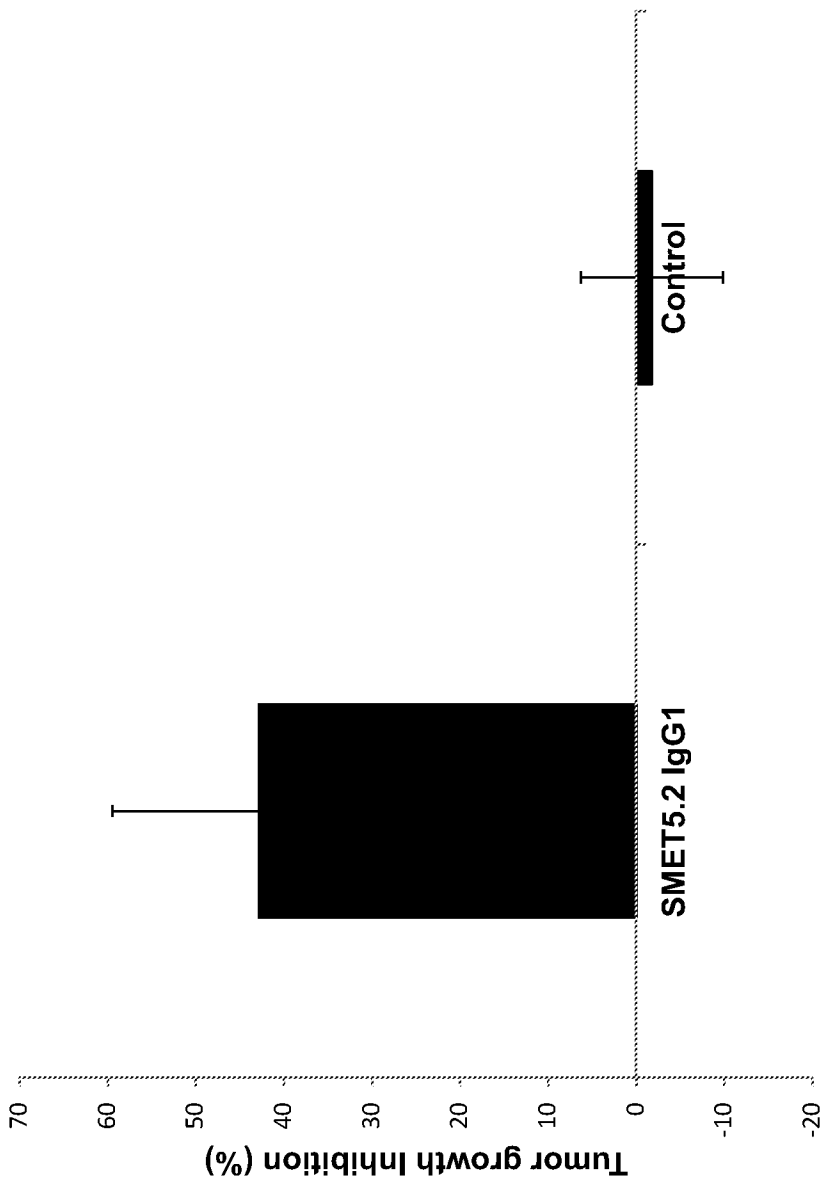

Tumor growth Inhibition (%)
70
60
50
40
30
20
10
0
-10
-20
SMET5.2 IgG1
Control

Figure 40

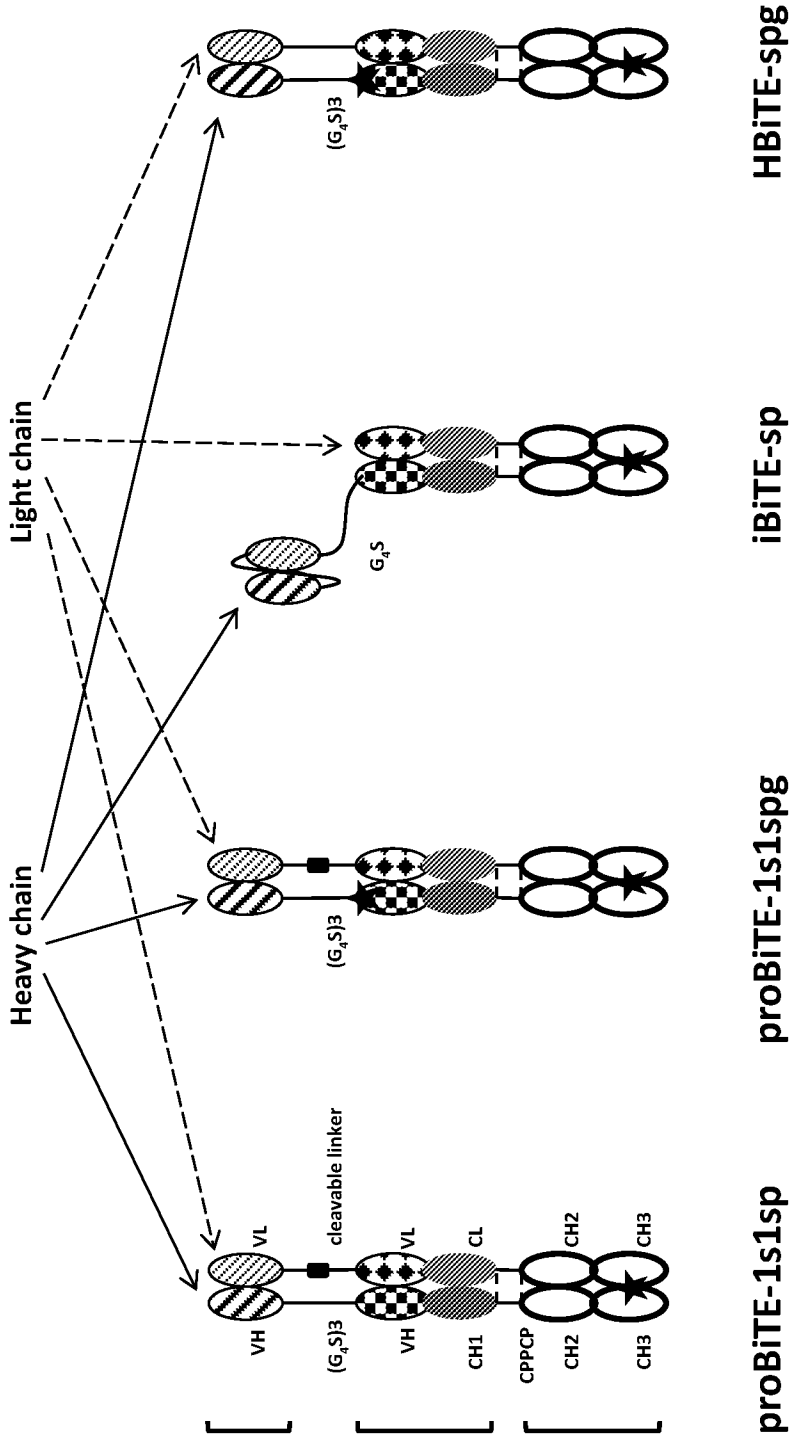

Heavy chain
Light chain
anti-EGFR Fv
(SMET5.2)
anti-CD3 Fab
(hSP34)
IgG1 mFc
(mFc7.2)
VH
(G4S)3
VL
cleavable linker
VH
CH1
CPPCP
CH2
CH3
VL
CL
CH2
CH3
(G4S)3
(G4S)3
G4S
proBiTE-1s1sp
proBiTE-1s1spg
iBiTE-sp
HBiTE-spg

Figure 41

Germline	hSP34 HCDR3					
	V region	N1	D region	P	N2	J region
	tgtgtga….gacacggaaacttc….gggaactcctac			gt	gtcttacttcgca….tactgg	
IGHV3-23*04	tgtgcga					
IGHD1-26*01			gggagctactac			
IGHJ4*03						tactgg

	IMGT numbering																	
hSP34 HCDR3	C	V	R	H	G	N	F	G	N	S	Y	V	S	Y	F	A	Y	W
Germline	C	A	R	H	G	N	F	G	S	Y	Y	V	S	Y	F	A	Y	W

Figure 42

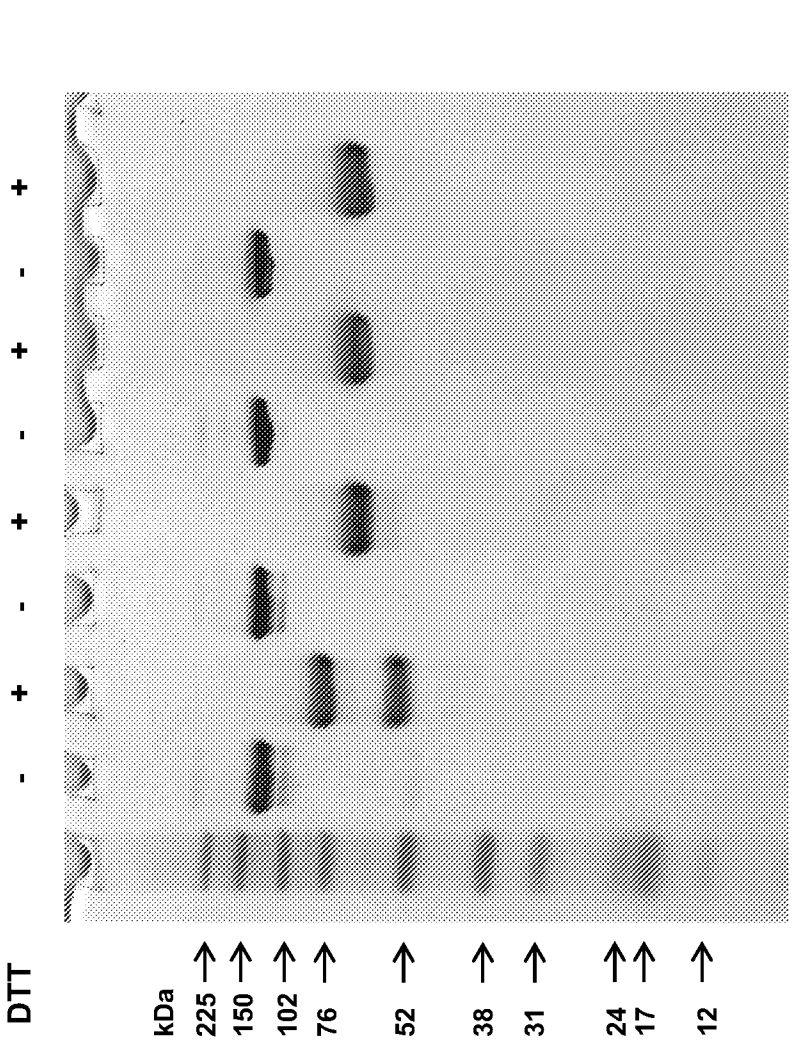

HBiTE-spg
proBiTE-1s1spg
proBiTE-1s1sp
iBiTE-sp
DTT
- + - + - + - +
kDa
225
150
102
76
52
38
31
24
17
12

Figure 43

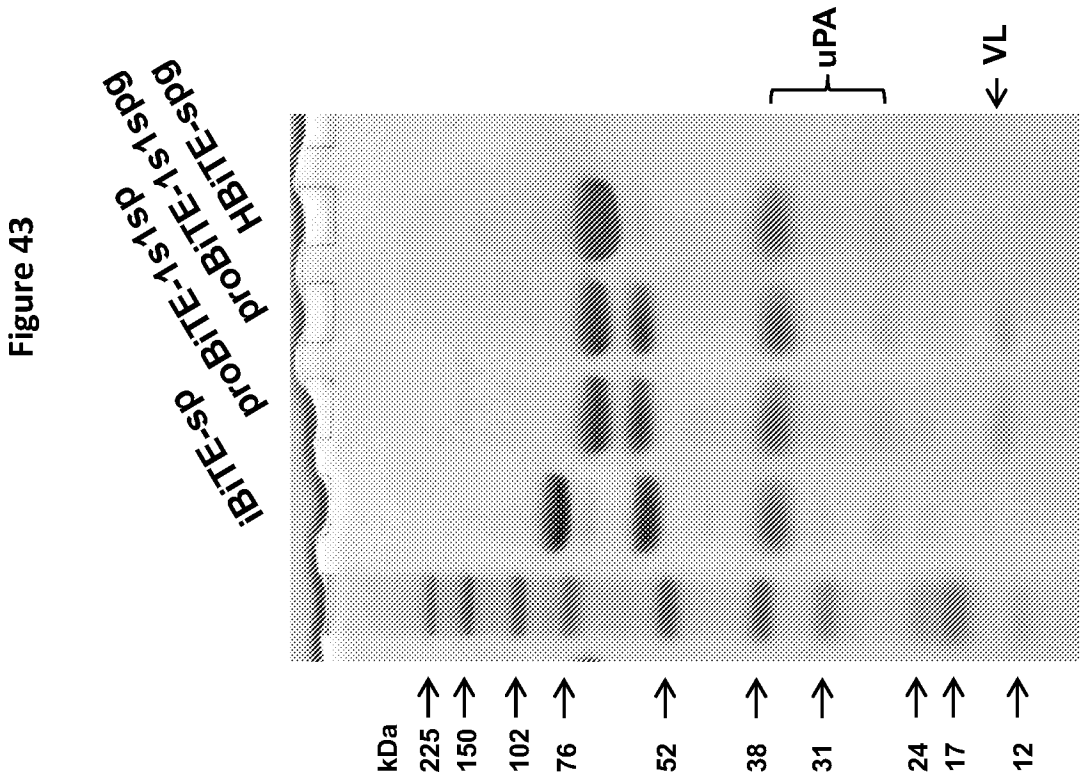
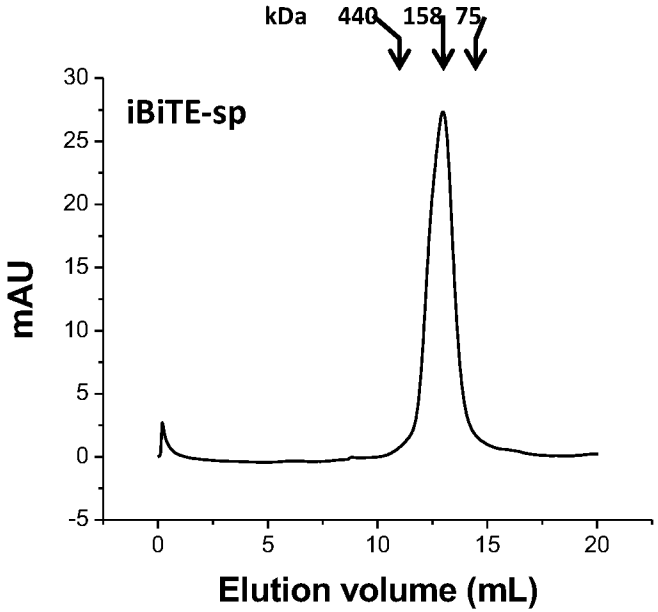
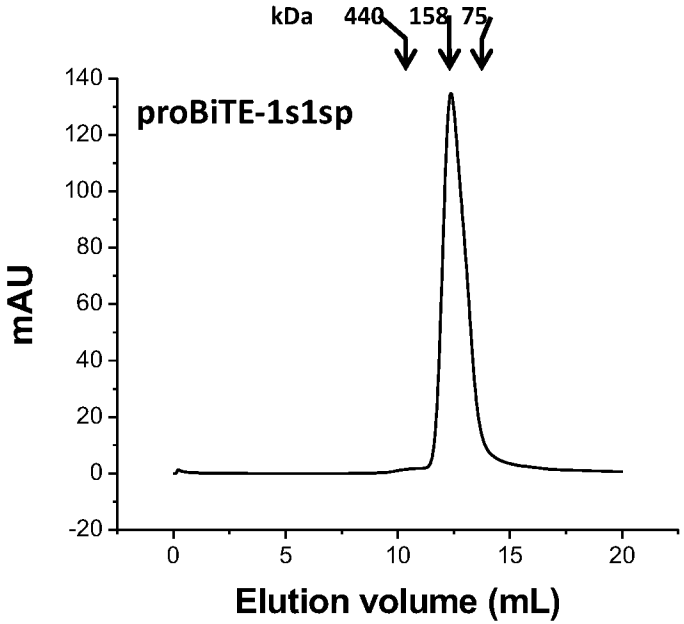
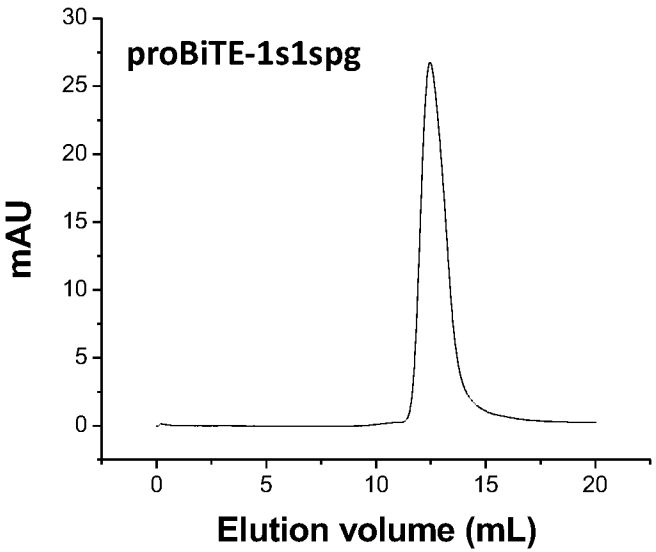

HBiTE-sp
proBiTE-1s1sp
proBiTE-1s1s-spg
HBiTE-spg
uPA
VL
kDa
225
150
102
76
52
38
31
24
17
12

Figure 44


kDa
440
158
75
iBiTE-sp
mAU
Elution volume (mL)


kDa
440
158
75
proBiTE-1s1sp
mAU
Elution volume (mL)


proBiTE-1s1spg
mAU
Elution volume (mL)

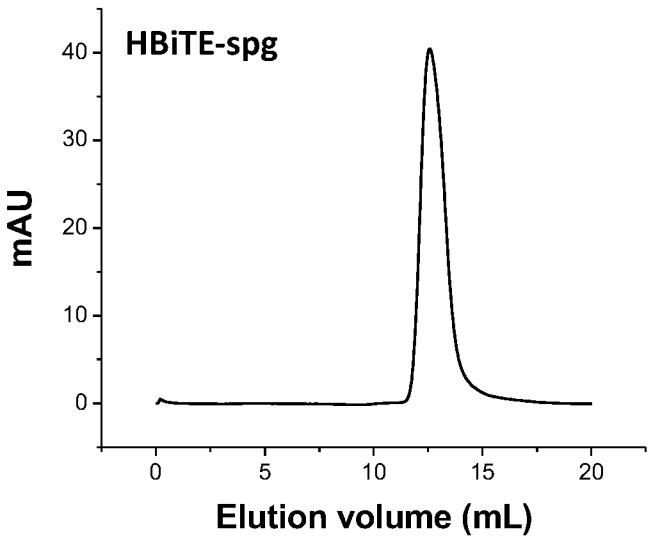

HBiTE-spg
mAU
Elution volume (mL)

Figure 45

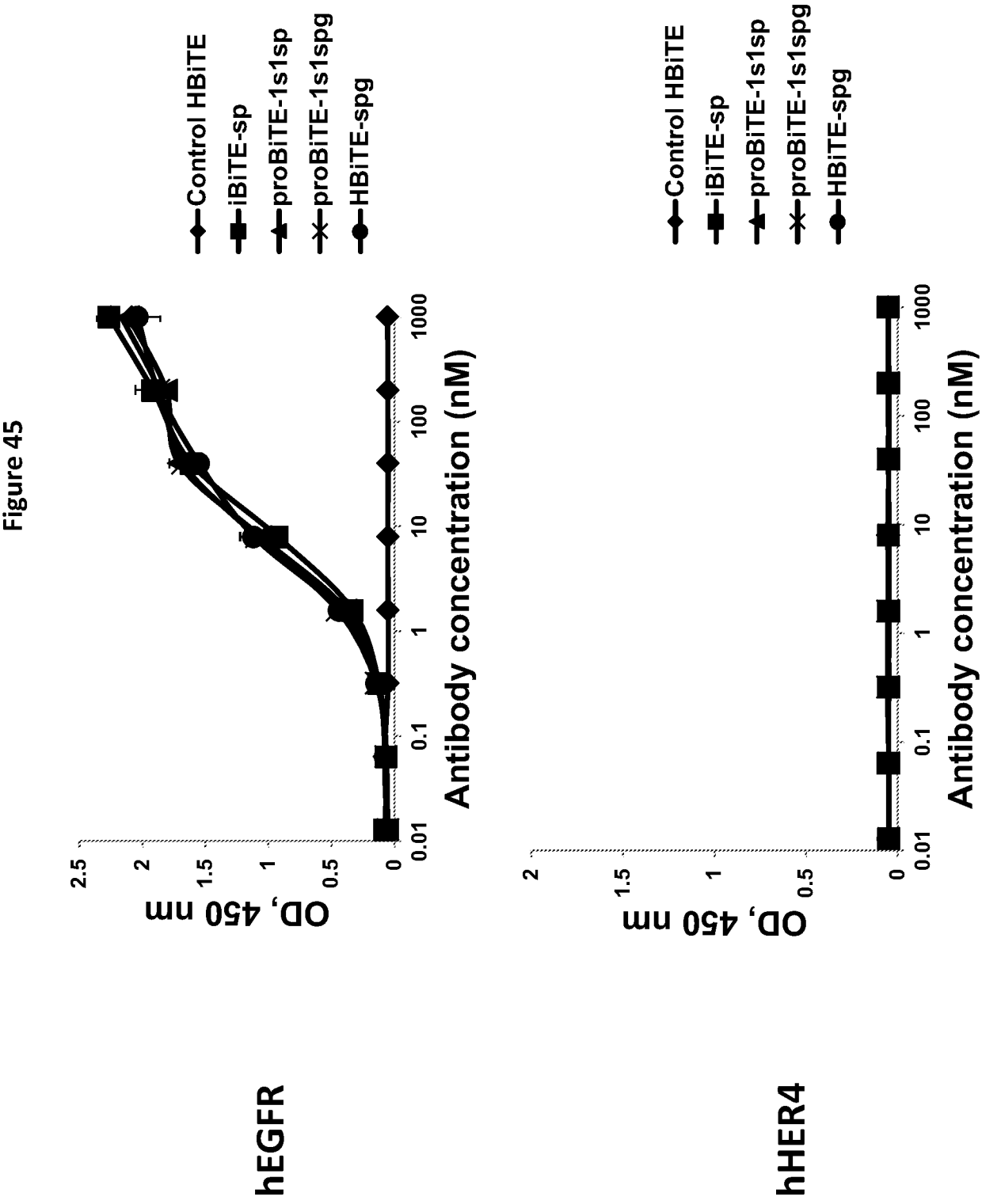

hEGFR
OD, 450 nm
Antibody concentration (nM)
Control HBiTE
iBiTE-sp
proBiTE-1s1sp
proBiTE-1s1spg
HBiTE-spg
hHER4
OD, 450 nm
Antibody concentration (nM)
Control HBiTE
iBiTE-sp
proBiTE-1s1sp
proBiTE-1s1spg
HBiTE-spg

Figure 46

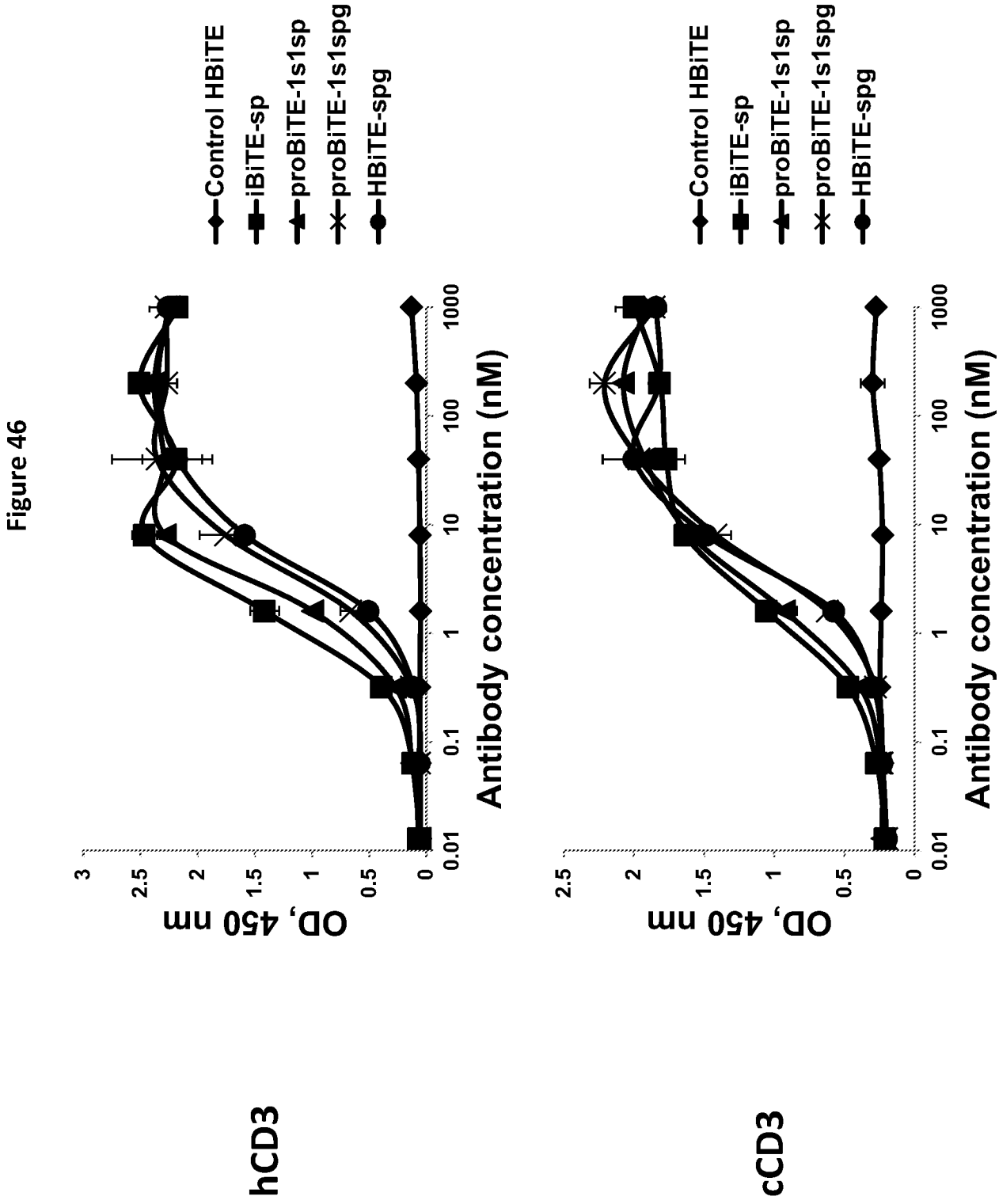

hCD3
cCD3
OD, 450 nm
Antibody concentration (nM)
Control HBiTE
iBiTE-sp
proBiTE-1s1sp
proBiTE-1s1spg
HBiTE-spg
0
0.5
1
1.5
2
2.5
3
0.01
0.1
1
10
100
1000

Figure 47

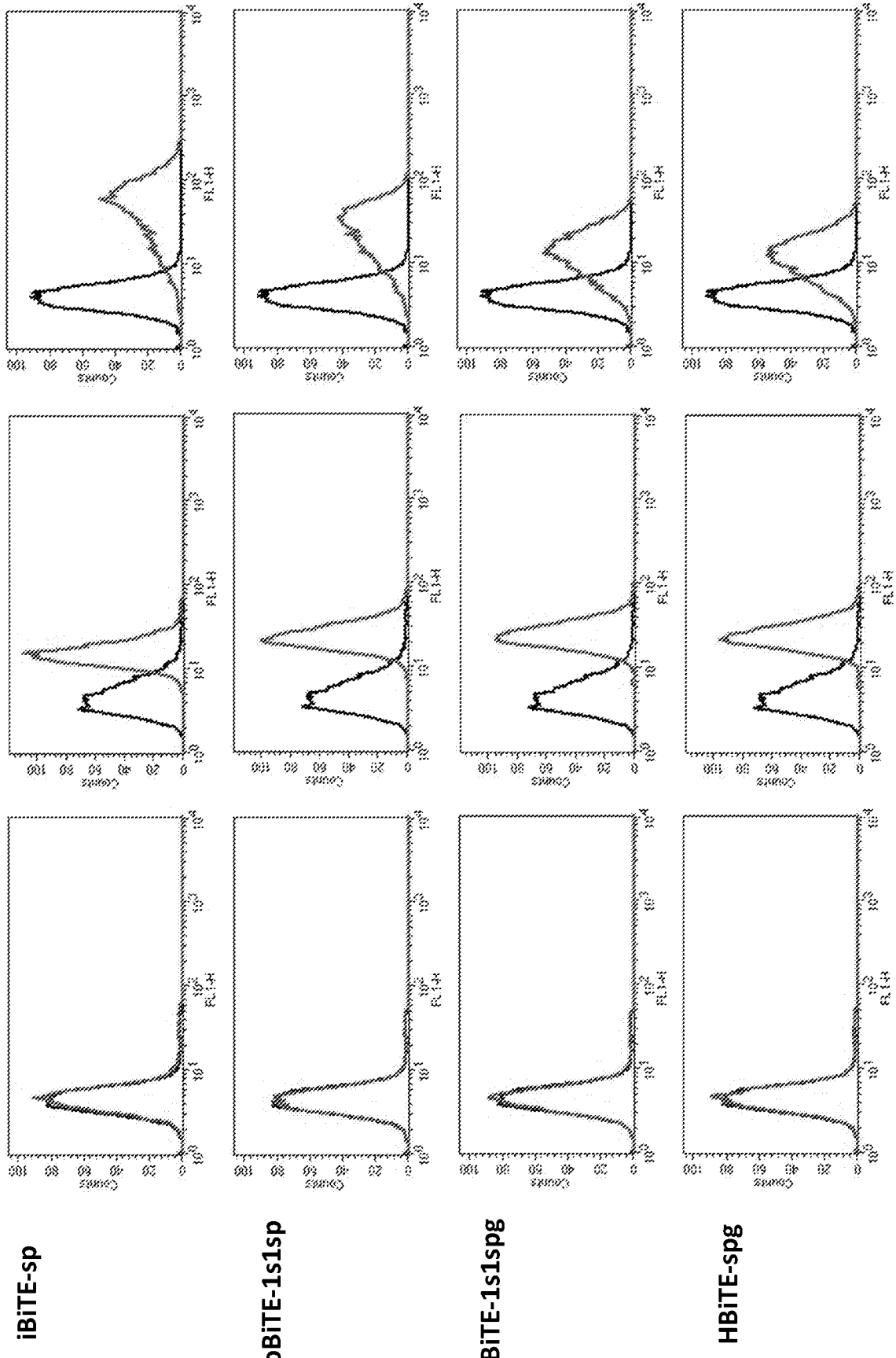

CHO
HCT116
Jurkat
iBiTE-sp
proBiTE-1s1sp
proBiTE-1s1spg
HBiTE-spg

Figure 48

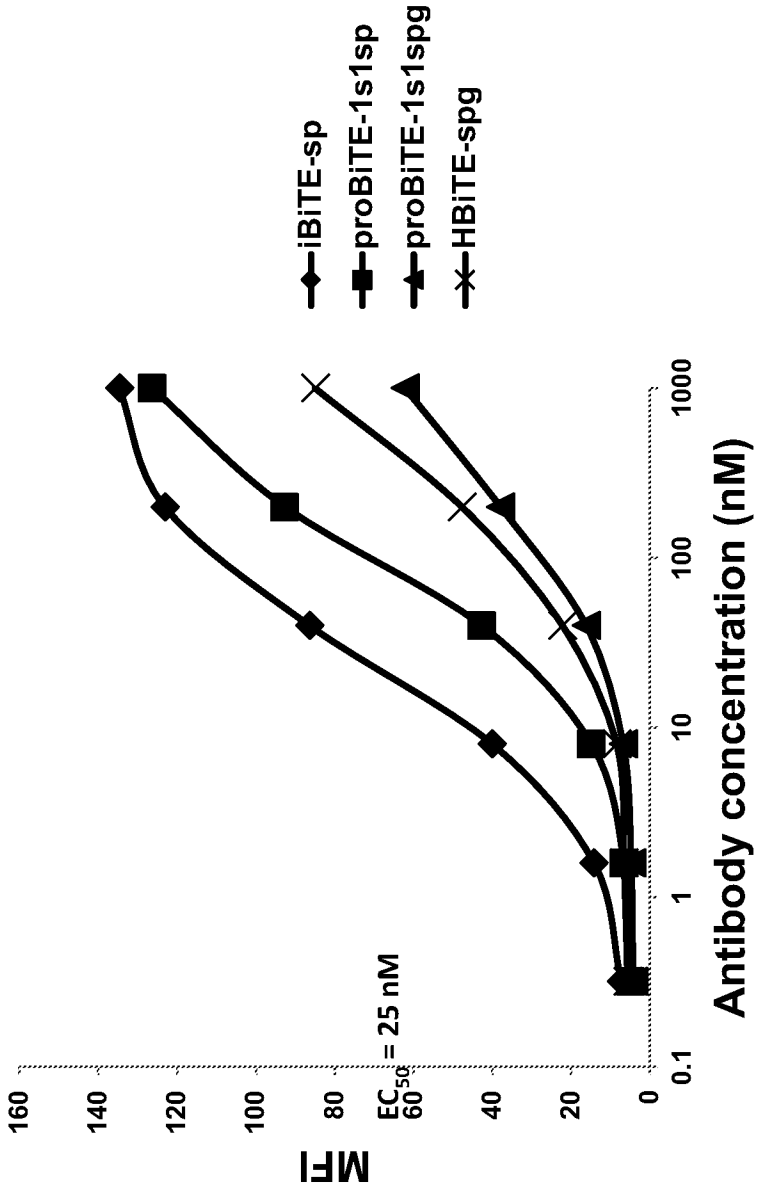

iBiTE-sp
proBiTE-1s1sp
proBiTE-1s1spg
HBiTE-spg
MFI
160
140
120
100
80
60
40
20
0
EC50 = 25 nM
0.1
1
10
100
1000
Antibody concentration (nM)

Figure 49

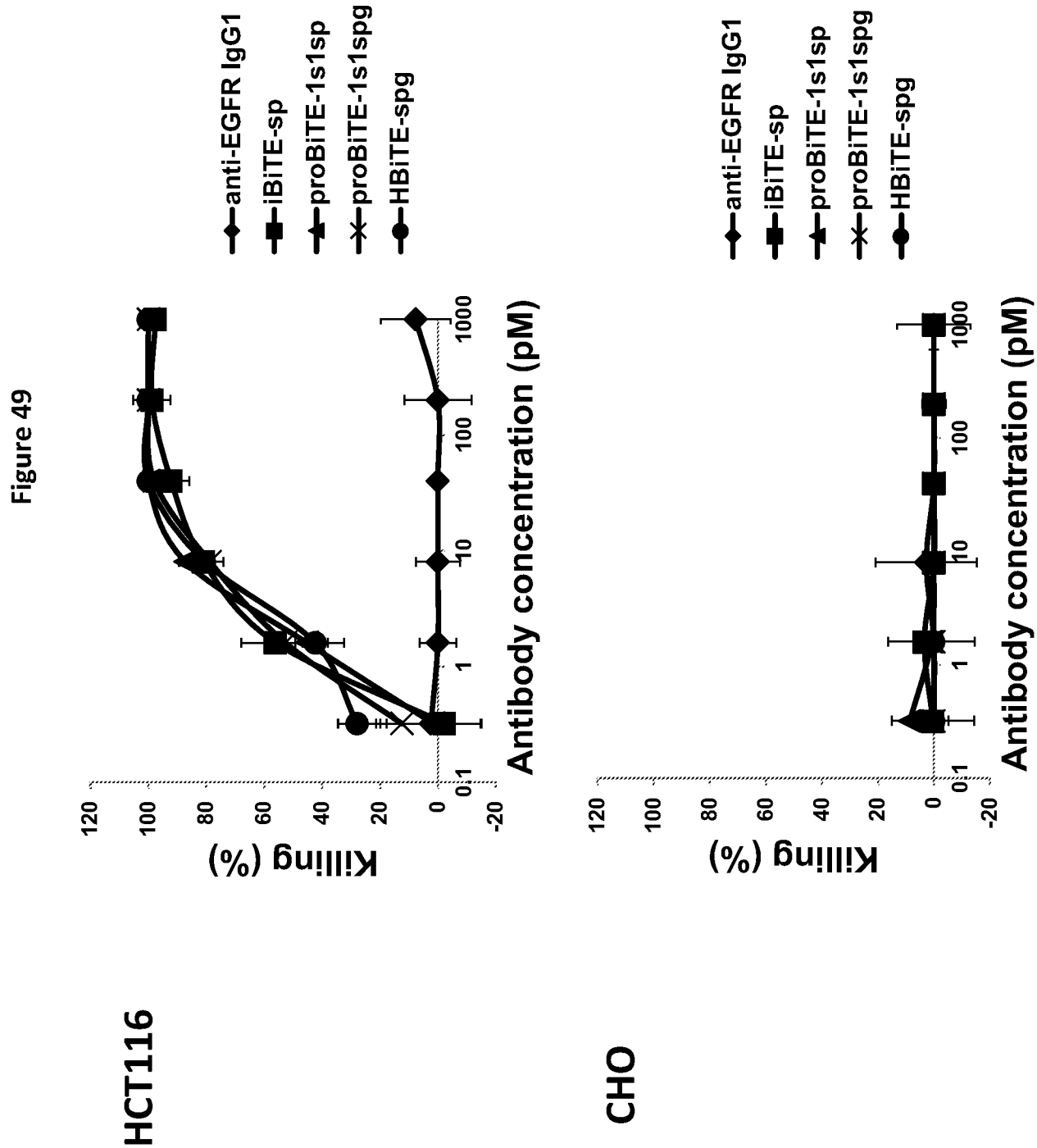
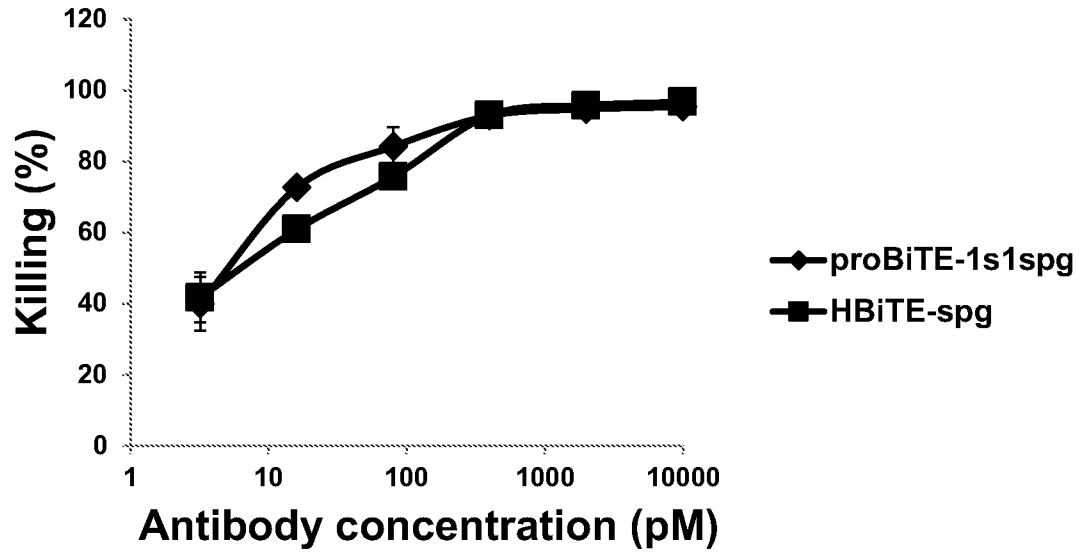
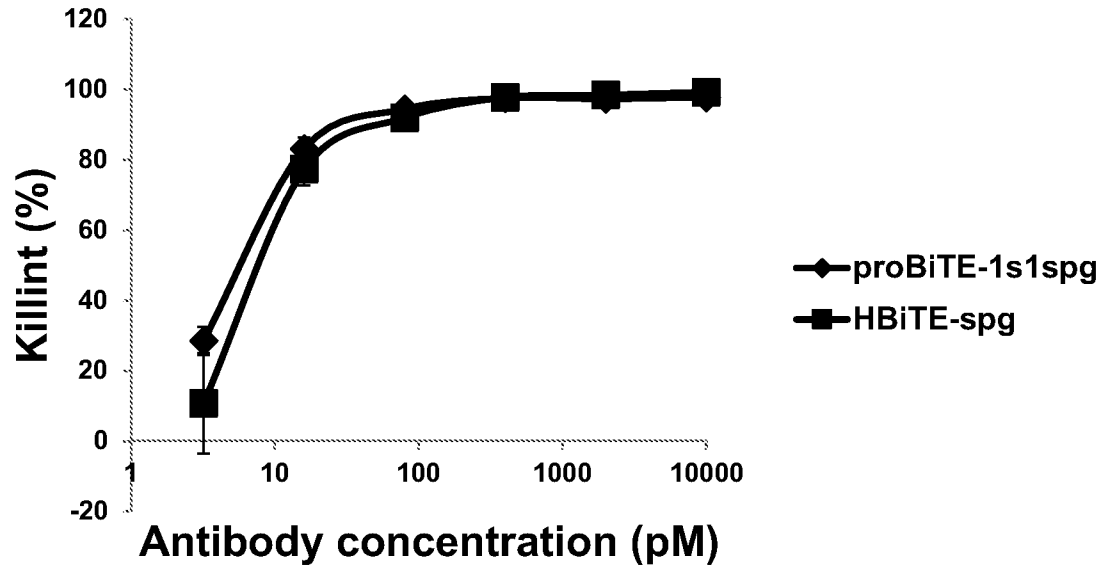

HCT116
Killing (%)
120
100
80
60
40
20
0
-20
0.1
1
10
100
1000
Antibody concentration (pM)
anti-EGFR IgG1
iBiTE-sp
proBiTE-1s1sp
proBiTE-1s1spg
HBiTE-spg
CHO
Killing (%)
120
100
80
60
40
20
0
-20
0.1
1
10
100
1000
Antibody concentration (pM)
anti-EGFR IgG1
iBiTE-sp
proBiTE-1s1sp
proBiTE-1s1spg
HBiTE-spg

Figure 50

HCT116



HT29



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 19/68063

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 39/395, C07K 16/28 (2020.01)

CPC - C07K 16/28, A61K 39/395, C07K 2317/522, C07K 2317/524, C07K 2317/526, C07K 2317/56, C07K 2319/00

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

B. FIELDS SEARCHED

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

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y -- A	WO 2018/144784 A1 (SMET PHARMACEUTICAL INC.) 09 August 2018 (09.08.2018) abstract, para [0005], [00014], [0098], [000157], Claim 10, Claim 11, Claim 12, Claim 19, Fig. 11	1-9, (19-22)/1 ---- 10
Y -- A	US 2013/0266568 A1 (ROCHE GLYCART AG) 10 October 2013 (10.10.2013) abstract, para [0047], [0052], [0098], [0276], Claim 1,	1-9, (19-22)/1 ---- 10
Y	US 2018/0125972 A1 (CHUGAI SEIYAKU KABUSHIKI KAISHA) 10 May 2018 (10.05.2018) SEQ ID NO: 27	8
A	US 2013/0315911 A1 (BRISTOL-MYER SQUIBB COMPANY) 28 November 2013 (28.11.2013) abstract, para [0092], SEQ ID NO: 77	10

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

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

Date of the actual completion of the international search	Date of mailing of the international search report
27 April 2020	13 MAY 2020
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Lee Young Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 19/68063

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

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

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

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

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

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

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

- see extra sheet for Box No. III Observations where unity of invention is lacking -

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.:
1-10, (19-22)(in part) limited to SEQ ID NO: 1

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US 19/68063

Continuation of:
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 searched, the appropriate additional search fees must be paid.

Groups I+: Claims 1-22, drawn to a recombinant bispecifc antibody and a method of preparation, thereof. The composition/method will be searched to the extent that the bispecifc antibody encompasses SEQ ID NO: 1. It is believed that claims 1-10, (19-22)(in part) encompass this first named invention, and thus these claims will be searched without fee to the extent that they encompass SEQ ID NO: 1. Additional bispecifc antibod(ies) will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected bispecifc antibod(ies). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be a bispecifc antibody encompassing SEQ ID NO: 17 (Claims 1-9, 11-15, (19-22)(in part).). Another exemplary election would be a bispecifc antibody encompassing SEQ ID NO: 50 (Claims 1-9, 11-18, (19-22)(in part).).

Groups II+: Claims 23-33, drawn to an isolated anti-human EGFR antibody. Group II+ will be searched upon payment of additional fees. The composition may be searched, for example, to the extent that the anti-EGFR antibody encompasses VHCDR1-3 of SEQ ID NOs: 22, 23, 24, respectively; VH of SEQ ID NO: 21, VLCDR1-3 of SEQ ID NOs: 26, 27, 28, respectively; and VL of SEQ ID NO: 25 for an additional fee and election as such. It is believed that claims 23-26 read on this exemplary invention. Additional anti-EGFR antibod(ies) will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected anti-EGFR antibod(ies). 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. Another exemplary election would be an anti-EGFR antibody comprising VHCDR1-3 of SEQ ID NOs: 22, 23, 24, respectively; VH of SEQ ID NO: 44, VLCDR1-3 of SEQ ID NOs: 31, 32, 33, respectively; and VL of SEQ ID NO: 30 (Claims 23-24, 27-28, 33(in part).)

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

Groups I+ include the special technical feature of a second light chain variable domain and a second heavy chain variable domain, not required by Groups II+.

Groups II+ include the special technical feature of an anti-EGFR antibody, not required by Groups I+.

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

Additionally, even if Groups I+ were considered to share the technical features of including: a recombinant bispecific antibody, these shared technical features are previously disclosed by WO 2018/144784 A1 to SMET Pharmaceutical Inc. (hereinafter "SMET") and US 2013/0266568 A1 to Roche Glycart AG. (hereinafter "Roche").

SMET teaches (instant claim 1) a recombinant bispecific antibody (para [00014], a bispecific antibody format iBiBody, comprising a binding protein, a Fab and one or more monomeric Fc polypeptide.), comprising:
(i) a first polypeptide comprising from N- to C-terminus a first light chain variable domain, a human light chain constant region (CL), a human heavy chain constant region 2 (CH2) and a human heavy chain constant region 3 (CH3) (para [0098], bispecific antibodies (iBiBody) comprises two monomeric Fc (mFc) fusion proteins (FIG. 11). In certain embodiments, one of the monomeric Fc fusion proteins comprises a binding protein, an mFc domain, and a VL-CL domain.; see Fig. 11, the antibody located at upper left.);
(ii) a second polypeptide comprising from N- to C-terminus a first heavy chain variable domain, a human heavy chain constant region 1 (CHI), a human heavy chain constant region 2 (CH2) and a human heavy chain constant region 3 (CH3) (para [0098], The other monomeric Fc fusion protein comprises an mFc domain and a VH-CH1 domain.);
wherein said first light chain variable domain and said first heavy chain variable domain confer binding specificity to a first antigen (para [0098], The said VL, CL, VH and CHI of two monomeric Fc fusion proteins form a Fab.).

SMET does not specifically teach a second light chain variable domain and a second heavy chain variable domain. Roche teaches activatable bispecific antibodies (abstract, bispecific antibodies wherein the binding affinity to one of the two antigens is reduced and which can be activated by tumor- or inflammation-specific proteases, and the preparation and use of such bispecific antibodies.) comprising an antibody linked to:
(iii) a second light chain variable domain connected to the C-terminus of said CH3 of said first polypeptide by a first linker, and a second heavy chain variable domain connected to the C-terminus of said CH3 of said second polypeptide by a second linker (para [0098], the bispecific antibody according to the invention is characterized in comprising from C- to N-terminus the following polypeptide chains one VH1-peptide linker-CH3-CH2-CH1-VH2 chain; ... one VL1-peptide linker-CH3-CH2-CH1-VH2 chain (see also FIG. 2 a for an exemplary scheme).),
wherein said second light chain variable domain and said second heavy chain variable domain confer binding specificity to a second antigen (Claim 1, A bispecific antibody comprising a) a first antibody that binds to a first antigen comprising a VH1 domain and a VL1 domain, and b) a second antibody that binds to a second antigen.). Given that a second pair of VH/VL that recognizes a second antigen is used in constructing a bispecific antibody, it would have have obvious to one of ordinary skill in the art to have replaced the binding protein (see SMET, Fig. 11, the antibody located on upper left) with the VH/VL domains of Roche, since Roche teaches that said bispecific antibody construct can bind to specific targets simultaneously (para [0047], They can simultaneously interfere with separate targets or pathways of tumors and regions of inflammation where tumor-specific proteases are secreted or expressed. Therefore, they mediate e.g. better suppression of such phenotypes in cancer or inflammatory diseases.).

----continued on next sheet----

Continuation of:
Box No. III. Observations where unity of invention is lacking

Some inventions of Groups I+ share the technical features of a heterodimerization motif or a long flexible motif (see instant claim 11), which is disclosed by SMET (para [000150], As in FIG. 8, human antibody kappa light chain constant region (CK) and human IgGI constant region I (CHI) were used for heterodimerizai.ion and mFc7.x was used to extend the half-life of the bispecific antibody. Specifically, in one arm, scFv I was fused to the N terminus of CK via a polypeptide linker composed of a single copy of the G4S motif ... the G4S linker provide sufficient flexibility for scFvs to exert their functions.).

Some inventions of Groups I+ share the technical features wherein said first light chain variable domain and said first heavy chain variable domain comprise modified sequences that confer reduced binding affinity to CD3 as compared to that mediated by unmodified sequences (see instant claim 16), which is disclosed by WO 2017/009442 A1 to Genmab A/S (hereinafter "Genmab") (pg 3, para 2, a humanized or ch chimeric antibody binding to human CD3, wherein said antibody comprises a binding region comprising a heavy chain variable (VH) region, wherein said VH region comprises a mutation in one of the three CDR sequences of a reference antibody having the VH CDR sequences.; pg 3, para 3, In one embodiment of the invention, the antibody has a reduced or increased binding affinity to human CD3 com pared to the reference antibody.).

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

Additionally, even if Groups II+ were considered to share the technical features of including: an anti-EGFR antibody, these shared technical features are previously disclosed by WO 2018/197502 A1 to Glenmark Pharmaceuticals S.A. (hereinafter "Glenmark").

Glenmark teaches (instant claim 23) an isolated anti-human EGFR (epidermal growth factor receptor) antibody, said antibody comprises a heavy chain and a light chain, wherein said heavy chain comprises a complementarity determining region 1 (CDRI), a CDR2, and a CDR3 (pg 27, In 25 to pg 28, In 10, Humanization of the anti-human EGFR mouse antibody 3A6 including selection of human acceptor frameworks that substantially retain the binding properties of human CDR-grafted acceptor frameworks is described herein ... Selection of heavy and light chain variable sequences (VH and VL) within these subfamilies to be used as acceptor may be based upon sequence homology and/or a match of structure of the CDR1 and CDR2 regions to help preserve the appropriate relative presentation of the six CDRs after grafting.).

As said technical features were known in the art at the time of the invention, these cannot be considered special technical features that would otherwise unify the groups.

Groups I+ and II+ therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.