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(54) Title: BISPECIFIC HER2 ANTIBODIES

(57) **Abstract:** The present invention relates to anti-HER2 binding molecules (e.g., antibodies and antigen binding fragments thereof), derived HER2-binding molecules (e.g., bispecific anti-HER2 antibodies), and antibody-drug conjugates (ADC) that bind the extracellular domain of the HER2 receptor. Also provided are pharmaceutical formulation comprising the disclosed compositions and method for the treating diseases associated with HER2-mediated signal transduction.



BISPECIFIC HER2 ANTIBODIES

[0001] This application claims benefit of U.S. Provisional Patent Application No. 61/978,516, filed April 11, 2014 and U.S. Provisional Patent Application No. 62/107,050, filed January 23, 2015, the disclosures of which are incorporated by reference herein in their entirety.

BACKGROUND

[0002] The present invention provides compositions that specifically bind to HER2 and methods for the use of such compositions for the treatment of cancer.

[0003] The HER family of receptor tyrosine kinases are important mediators of cell growth, differentiation and survival. The receptor family includes four distinct members including epidermal growth factor receptor (EGFR, ErbB1, or HER1), HER2 (ErbB2 or p185neu), HER3 (ErbB3) and HER4 (ErbB4 or tyro2).

[0004] HER2 was originally identified as the product of the transforming gene from neuroblastomas of chemically treated rats. Amplification of the human homolog has been observed in breast and ovarian cancers and correlates with a poor prognosis. Overexpression of HER2 (frequently but not uniformly due to gene amplification) has also been observed in other carcinomas including carcinomas of the stomach, endometrium, salivary gland, lung, kidney, colon, thyroid, pancreas and bladder. HER2 can also be overexpressed in prostate cancer.

[0005] The HER receptors are generally found in various combinations in cells and heterodimerization is thought to increase the diversity of cellular responses to a variety of HER ligands (Earp *et al.* Breast Cancer Research and Treatment 35: 115-132 (1995)). While EGF and TGF α do not bind HER2, EGF stimulates EGFR and HER2 to form a heterodimer, which activates EGFR and results in transphosphorylation of HER2 in the heterodimer. Dimerization and/or transphosphorylation appear to activate the HER2 tyrosine kinase. See Earp *et al.*, supra. Likewise, when HER3 is co-expressed with HER2, an active signaling complex is formed and antibodies directed against HER2 are capable of disrupting this complex (Sliwkowski *et al.*, J. Biol. Chem., 269:14661-14665 (1994)).

[0006] Numerous antibodies targeting HER2 have been described in the art (see, e.g., Hudziak *et al.*, Mol. Cell. Biol. 9:1165-1172 (1989); U.S. Pat. No. 5,677,171; Fendly

et al. Cancer Research 50:1550-1558 (1990); Kotts *et al.* *In vitro* 26(3):59A (1990); Sarup *et al.* Growth Regulation 1:72-82 (1991); Shepard *et al.* J. Clin. Immunol. 11: 117-127 (1991); Kumar *et al.* Mol. Cell. Biol. 1:979-986 (1991); Lewis *et al.* Cancer Immunol. Immunother. 37:255-263 (1993); Pietras *et al.* Oncogene 9:1829-1838 (1994); Vitetta *et al.* Cancer Research 54:5301-5309 (1994); Sliwkowski *et al.* J. Biol. Chem. 269(20):14661-14665 (1994); Scott *et al.* J. Biol. Chem. 266:14300-5 (1991); D'souza *et al.* Proc. Natl. Acad. Sci. 91:7202-7206 (1994); Lewis *et al.* Cancer Research 56:1457-1465 (1996); and Schaefer *et al.* Oncogene 15:1385-1394 (1997).

[0007] Other HER2 antibodies with various properties have been described in Tagliabue *et al.* Int. J. Cancer 47:933-937 (1991); McKenzie *et al.* Oncogene 4:543-548 (1989); Maier *et al.* Cancer Res. 51:5361-5369 (1991); Bacus *et al.* Molecular Carcinogenesis 3:350-362 (1990); Stancovski *et al.* PNAS (USA) 88:8691-8695 (1991); Bacus *et al.* Cancer Research 52:2580-2589 (1992); Xu *et al.* Int. J. Cancer 53:401-408 (1993); WO94/00136; Kasprzyk *et al.* Cancer Research 52:2771-2776 (1992); Hancock *et al.* Cancer Res. 51:4575-4580 (1991); Shawver *et al.* Cancer Res. 54:1367-1373 (1994); Arteaga *et al.* Cancer Res. 54:3758-3765 (1994); Harwerth *et al.* J. Biol. Chem. 267:15160-15167 (1992); U.S. Pat. No. 5,783,186; and Klapper *et al.* Oncogene 14:2099-2109 (1997).

[0008] Trastuzumab (HERCEPTIN®; see U.S. Pat. No. 5,821,337), a recombinant humanized version of the murine HER2 antibody 4D5, is clinically active in patients with HER2-overexpressing metastatic breast cancers that have received extensive prior anti-cancer therapy (Baselga *et al.*, J. Clin. Oncol. 14:737-744 (1996)). To target the HER signaling pathway, Pertuzumab (PERJETA™; see Patent Publication WO2001/00245) was developed as a humanized version of the murine antibody 2C4 that inhibits the dimerization of HER2 with other HER receptors, thereby inhibiting ligand-driven phosphorylation and activation, and downstream activation of the RAS and AKT pathways. Ado-trastuzumab emtansine (T-DM1; KADCYLA®) is an antibody drug conjugate of trastuzumab linked to the cytotoxic agent mertansine approved for use in patients with HER2-overexpressing metastatic breast cancers resistant to trastuzumab.

[0009] Although, the therapeutic efficacy of trastuzumab in breast carcinoma is well demonstrated, it is strictly limited and only approved for 30% of breast cancer patients

whose tumor overexpress HER2. 70% of the breast cancer patients do not or insufficiently respond to trastuzumab because their individual tumor do not overexpress or do not sufficiently express HER2. In other cancers and/or individual cancers, HER2 is overexpressed in a significant percentage of cases ranging from 43 to 69%, However, as a rule, the levels of HER2 expression are in principle low in the majority of tumors. Furthermore, the-overexpression of HER2 receptors is often caused by encoding gene amplification (Hynes *et al.*, Nat Rev Cancer 5:341 (2005)). Thus, the present day consensus is that anti-HER2 monoclonal antibody therapy is inefficient in tumors with low HER2 expression or missing overexpression. Furthermore, resistance to these anti-HER2 antibodies is a significant problem.

[0010] Given the lack of an effective anti-HER2 therapy in specific cancers expressing low levels of HER2 and resistance to the current therapies, there is a need for improved antibodies capable of effectively binding to cancer cells expressing a wider range of levels of HER2 and inhibiting their growth via, for example, (i) Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) and/or (ii) cytotoxic action due to payloads conjugated to the antibodies as Antibody Drug Conjugates (ADC) and/or (iii) inhibiting receptor mediating signaling (e.g. by inhibiting receptor dimerization and/or mediating receptor internalization).

[0011] Thus, it is an object of the present disclosure to provide improved immunotherapeutic agents that effectively inhibit HER2-mediated cell signaling that can be used for the treatment of HER2-expressing cancers, including cancers where HER2 is not expressed at high levels.

BRIEF SUMMARY

[0012] The present disclosure provides an anti-HER2 binding molecule comprising an immunoglobulin heavy chain (VH) and an immunoglobulin light chain (VL), wherein the VH comprises the amino acid sequence of SEQ ID NO:15. Also provided is an anti-HER2 binding molecule comprising a VH and a VL, wherein the VL comprises the amino acid sequence of SEQ ID NO:16. In some aspects, the VH comprises the amino acid sequence of SEQ ID NO:15 and the VL comprises the amino acid sequence of SEQ

ID NO:16. In some aspects, the anti-HER2 binding molecule comprises an antibody, or antigen-binding fragment thereof.

[0013] The present disclosure also provides a bispecific anti-HER2 antibody comprising a first immunoglobulin antigen-binding domain and a second immunoglobulin antigen-binding domain, wherein (i) the first and second immunoglobulin antigen-binding domains specifically bind to distinct HER2 antibody binding sites, (ii) the first immunoglobulin antigen-binding domain binds to a first HER2 antibody binding site which comprises an epitope within domain II of HER2, and (iii) the first HER2 antibody binding site is distinct from the antibody binding site of pertuzumab. In some aspects, the second immunoglobulin antigen-binding domain binds to a second HER2 antibody binding site which comprises an epitope within domain IV of HER2. In some aspects, the second HER2 antibody binding site is identical the HER2 antibody binding site of trastuzumab. In some aspects, the second HER2 antibody binding site partially overlaps with the HER2 antibody binding site of trastuzumab. In other aspects, the second HER2 antibody binding site is distinct from the HER antibody binding site of trastuzumab.

[0014] The present disclosure also provides a bispecific anti-HER2 antibody comprising a first immunoglobulin antigen-binding domain and a second immunoglobulin antigen-binding domain, wherein the first and second immunoglobulin antigen-binding domains specifically bind to distinct HER2 epitopes; and wherein the first immunoglobulin antigen-binding domain binds HER2 at an epitope comprising one or more amino acid residues in SEQ ID NO: 52.

[0015] In some aspects, the first immunoglobulin antigen-binding domain binds HER2 at an epitope comprising one or more amino acid residues in SEQ ID NO: 52 and the second immunoglobulin antigen-binding domain specifically binds HER2 at an epitope within domain IV. In some aspects, the second immunoglobulin antigen-binding domain binds HER2 at an epitope comprising one or more amino acid residues in SEQ ID NO: 53.

[0016] In some aspects, the first immunoglobulin antigen-binding domain comprises a heavy chain variable region (VH) and a light chain variable region (VL) comprising:
(i) a variable heavy chain CDR-1 (VH-CDR1) sequence identical to SEQ ID NO: 1 or identical to SEQ ID NO: 1 except for up to 1, 2, 3, or 4 amino acid substitutions;

- (ii) a variable heavy chain CDR-2 (VH-CDR2) sequence identical to SEQ ID NO: 2 or identical to SEQ ID NO: 2 except for up to 1, 2, 3, or 4 amino acid substitutions;
- (iii) a variable heavy chain CDR-3 (VH-CDR3) sequence identical to SEQ ID NO: 3 or identical to SEQ ID NO: 3 except for up to 1, 2, 3, or 4 amino acid substitutions;
- (iv) a variable light chain CDR-1 (VL-CDR1) sequence identical to SEQ ID NO: 4 or identical to SEQ ID NO: 4 except for up to 1, 2, 3, or 4 amino acid substitutions;
- (v) a variable light chain CDR-2 (VL-CDR2) sequence identical to SEQ ID NO: 5 or identical to SEQ ID NO: 5 except for up to 1, 2, 3, or 4 amino acid substitutions; and,
- (vi) a variable light chain CDR-3 (VL-CDR3) sequence identical to SEQ ID NO: 6 or identical to SEQ ID NO: 6 except for up to 1, 2, 3, or 4 amino acid substitutions.

[0017] In certain aspects, the first immunoglobulin antigen-binding domain comprises at least one heterologous variable domain framework region (FW) relative to the immunoglobulin antigen-binding domain comprising a VH comprising the amino acid sequence SEQ ID NO:43 and a VL comprising the amino acid sequence of SEQ ID NO:44.

[0018] In certain aspects, the first immunoglobulin antigen-binding domain comprises an scFv antibody fragment.

[0019] In certain aspects, the second immunoglobulin antigen-binding domain comprises an scFv antibody fragment.

[0020] In some aspects, the at least one heterologous FW region of the bispecific anti-HER2 antibody comprises a first immunoglobulin antigen-binding domain further comprising (i) a variable light chain framework 1 (VL-FW1) amino acid sequence comprising SEQ ID NO:11; (ii) a VH-FW2 amino acid sequence comprising SEQ ID NO:12; (iii) a VH-FW3 amino acid sequence comprising SEQ ID NO:13; (iv) a VH-FW4 amino acid sequence at comprising SEQ ID NO:14; or (vi) any combination thereof.

[0021] In some aspects, the bispecific anti-HER2 antibody comprises a first immunoglobulin antigen binding-domain and a second immunoglobulin antigen-binding domain, wherein the first immunoglobulin antigen-binding domain comprises a VH and a VL, wherein the VH amino acid sequence comprises SEQ ID NO:15; wherein the first or the second immunoglobulin antigen-binding domain comprises an scFv antibody

fragment, and wherein the first and second immunoglobulin antigen-binding domains specifically bind to distinct HER2 epitopes.

[0022] In some aspects, the bispecific anti-HER2 antibody comprises a first immunoglobulin antigen binding-domain and a second immunoglobulin antigen-binding domain, wherein the first immunoglobulin antigen-binding domain comprises a VH and a VL, wherein the VL amino acid sequence comprises SEQ ID NO:16; wherein the first or the second immunoglobulin antigen-binding domain comprises an scFv antibody fragment, and wherein the first and second immunoglobulin antigen-binding domains specifically bind to distinct HER2 epitopes.

[0023] In some aspects, the bispecific anti-HER2 antibody comprises a VH and a VL, wherein the VH amino acid sequence comprises SEQ ID NO:15; and wherein the VL amino acid sequence comprises SEQ ID NO: 16, wherein the first or the second immunoglobulin antigen-binding domain comprises an scFv antibody fragment, and wherein the first and second immunoglobulin antigen-binding domains specifically bind to distinct HER2 epitopes.

[0024] In some aspects, the bispecific anti-HER2 antibody disclosed herein comprises a first immunoglobulin antigen binding domain comprising or consisting of (a) a VH further comprising a heavy chain constant region or a fragment thereof and a VL comprising a light chain constant region or a fragment thereof; (b) a single chain Fv ("scFv"); (c) a diabody; (d) a minibody; (e) an F(ab')₂; or (f) F(ab). In same aspects, the heavy chain constant region or fragment thereof is an IgG constant region. In some aspects, the IgG constant region or fragment thereof is an IgG1 constant region. In some aspects, the LC constant region is a kappa constant region.

[0025] In some aspects, the LC constant region is a lambda constant region. In some aspects, the first immunoglobulin antigen-binding domain is a monoclonal antibody. In some aspects, the first immunoglobulin antigen-binding domain is a humanized antibody. In some aspects, the first immunoglobulin antigen-binding domain is a human antibody. In some aspects, the first immunoglobulin antigen-binding domain is a chimeric antibody. In some aspects, the first immunoglobulin antigen-binding domain is an affinity optimized antibody. In some aspects, the first immunoglobulin antigen-binding domain does not compete with trastuzumab or pertuzumab for epitope binding. In some aspects,

the first and second immunoglobulin antigen binding domains specifically bind to distinct non-overlapping HER2 epitopes.

[0026] In some aspects, the bispecific anti-HER2 antibody comprises a first and a second immunoglobulin antigen-binding domain, wherein (a) the second immunoglobulin antigen-binding domain specifically binds to the same epitope in domain IV of HER2 as the trastuzumab antibody; (b) the second immunoglobulin antigen-binding domain competitively inhibits HER2 binding by the trastuzumab antibody; and/or (c) the second immunoglobulin antigen-binding domain comprises at least one, at least two, at least three, at least four, at least five, or at least six complementarity determining regions (CDRs) having amino acids selected from the group consisting of SEQ ID NOs: 54 to 59.

[0027] In some aspects, the second immunoglobulin antigen-binding domain comprises an scFv comprising: (i) a VH-CDR1 comprising the amino acids of SEQ ID NO:54; (ii) a VH-CDR2 comprising the amino acids of SEQ ID NO:55; (iii) a VH-CDR3 comprising the amino acids of SEQ ID NO:56; (iv) a VL-CDR1 comprising the amino acids of SEQ ID NO:57; (v) a VL-CDR2 comprising the amino acids of SEQ ID NO:58; and (vi) a VL-CDR3 comprising the amino acids of SEQ ID NO:59. In some aspects, the scFv is a disulfide stabilized scFv. In some aspects, the scFv comprises a VH comprising the amino acids of SEQ ID NO:17, and a VL comprising the amino acids of SEQ ID NO:18 .

[0028] In some aspects, the VH and VL of the scFv are covalently linked via a peptide linker. In some aspects, the peptide linker comprises the amino acid sequence of SEQ ID NO:19.

[0029] In some aspects, the second immunoglobulin antigen-binding domain of the bispecific anti-HER2 antibody is covalently linked to the carboxy-terminus of the HC of the first immunoglobulin antigen-binding domain. In some aspects, the bispecific anti-HER2 antibody comprises a linker interposed between the second immunoglobulin antigen binding domain and the carboxy-terminus of the HC of the first immunoglobulin antigen-binding domain. In other aspects, the second immunoglobulin antigen-binding domain of the bispecific anti-HER2 antibody is covalently linked to the amino-terminus of the HC of the first immunoglobulin antigen-binding domain. In some aspects, the bispecific anti-HER2 antibody comprises a linker interposed between the second

immunoglobulin antigen-binding domain and the amino-terminus of the HC of the first immunoglobulin antigen-binding domain.

[0030] In some aspects, the second immunoglobulin antigen binding domain of the bispecific anti-HER2 antibody is covalently intercalated in the polypeptide chain of the HC of the first immunoglobulin antigen-binding domain. In some aspects, the second immunoglobulin antigen binding domain is covalently intercalated between the CH1 region and CH2 region of the HC of the first immunoglobulin antigen-binding domain. In some aspects, the bispecific anti-HER2 antibody comprises a linker interposed between the CH1 region of the HC of the first immunoglobulin antigen binding domain and the second immunoglobulin antigen binding domain, and a second linker interposed between the second immunoglobulin antigen binding domain and the CH2 region of the HC of the of the first immunoglobulin antigen-binding domain.

[0031] In some aspects, the first linker and the second linker are identical. In other aspects, the first linker and the second linker are different. In some aspects, one or more of the linkers comprise a peptide linker. In some aspects, the peptide linker comprises at least one, at least two, at least three, at least four, at least five, at least 10, at least 20, or at least 30 amino acids. In other aspects, the peptide linker comprises a peptide having the formula $\text{Ser}_x[(\text{Gly})_y\text{-Ser}_4]_z$ where x is from 0 to 1, y is from 1 to 4, and z is from 1 to 10. In some aspects, the peptide linker comprises SEQ ID NOS:19, 20, 21, or 22.

[0032] In some aspects, the bispecific anti-HER2 antibody comprises a heavy chain which can include an Fc domain comprising a CH2 and a CH3 region. In some aspects, the Fc domain is an IgG1 Fc domain. In other aspects, the IgG1 Fc domain is a native IgG1 Fc domain. In some aspects, the native IgG1 Fc domain comprises the amino acid sequence of SEQ ID NO: 23.

[0033] In some aspects, the Fc domain is a mutant IgG1 Fc domain. In other aspects, the mutant IgG1 Fc domain comprises at least one mutation capable of reducing the ADCC activity of the bispecific antibody. In some aspects, at least one mutation capable of reducing the ADCC activity of the bispecific antibody is an amino acid substitution. In some aspects, at least one amino acid substitution comprises L234F, S239A, S239C, or any combination thereof.

[0034] In some aspects, the mutant IgG1 Fc domain comprises at least one amino acid substitution introducing a derivatizable group. In some aspects, the derivatizable group is a sulfhydryl group. In some aspects, the at least one amino acid substitution comprises S239C, 248C, 254C, 273C, 279C, 282C, 284C, 286C, 287C, 289C, 297C, 298C, 312C, 324C, 326C, 330C, 335C, 337C, 339C, 350C, 355C, 356C, 359C, 360C, 361C, 375C, 383C, 384C, 389C, 398C, 400C, 413C, 415C, 418C, 422C, 440C, 441C, S442C, 443C and 446C, or any combination thereof. In some aspects, the mutant Fc domain comprises the amino acids of SEQ ID NO: 24 or SEQ ID NO:25.

[0035] Also provided is a bispecific anti-HER2 antibody comprising a first and a second polypeptide chain associated with each other, wherein the first polypeptide chain comprises a sequence selected from:

- (1) [TZ_S]-[L₁]-[_BVH]-[_BCH]-[Fc_X]
- (2) [_BVH]-[_BCH]-[Fc_X]-[L₂]-[TZ_S]
- (3) [_BVH]-[_BCH]-[L₃]-[TZ_S]-[L₄]-[Fc_X]

wherein

TZ_S is an scFv that binds the same epitope as trastuzumab;

L₁, L₂, L₃, and L₄ are peptide linkers;

Fc_X is an Fc domain;

_BVH and _BCH are the VH and CH1 regions, respectively, of an antibody capable of binding to an HER2 epitope distinct from the epitope recognized by the trastuzumab antibody. In certain aspects the distinct epitope comprises one or more amino acid residues in SEQ ID NO: 52.

[0036] In some aspects a hinge polypeptide links [_BCH] and [Fc_X]. In a specific aspect the hinge polypeptide comprises or alternatively consists of the amino acids of SEQ ID NO: 26.

[0037] In some aspects, the second chain of the bispecific anti-HER2 antibody comprises the sequence [_BVL]-[CL] wherein _BVL is the VL region of an antibody capable of binding to an HER2 epitope distinct from the epitope recognized by the trastuzumab antibody, and CL is an IgG light chain constant region. In some aspects, CL is selected from the group consisting of a human kappa constant region and a human lambda constant region. In some aspects the _BVL comprises (i) a variable light chain CDR-1

(VL-CDR1) identical to SEQ ID NO: 4 or identical to SEQ ID NO: 4 except for up to 1, 2, 3, or 4 amino acid substitutions; (ii) a variable light chain CDR-2 (VL-CDR2) identical to SEQ ID NO: 5 or identical to SEQ ID NO: 5 except for up to 1, 2, 3, or 4 amino acid substitutions; and, (iii) a variable light chain CDR-3 (VL-CDR3) identical to SEQ ID NO: 6 or identical to SEQ ID NO: 6 except for up to 1, 2, 3, or 4 amino acid substitutions. In some aspects, _BVL comprises the amino acids of SEQ ID NO:16 or SEQ ID NO:44.

[0038] In other aspects, CL is a kappa light chain comprising the amino acid sequence of SEQ ID NO:27A.

[0039] In other aspects, CL is a lambda light chain comprising the amino acid sequence of SEQ ID NO:27B.

[0040] In some aspects, [TZ_S] comprises (i) a VH-CDR1 comprising the amino acids of SEQ ID NO:54; (ii) a VH-CDR2 comprising the amino acids of SEQ ID NO:55; (iii) a VH-CDR3 comprising the amino acids of SEQ ID NO:56; (iv) a VL-CDR1 comprising the amino acids of SEQ ID NO:57; (v) a VL-CDR2 comprising the amino acids of SEQ ID NO:58; and (vi) a VL-CDR3 comprising the amino acids of SEQ ID NO:59. In some aspects, [TZ_S] is a disulfide stabilized scFv. In some aspects, [TZ_S] comprises a VH comprising the amino acid sequence of SEQ ID NO:17 and a VL comprising the amino acid sequence of SEQ ID NO:18, covalently linked by a peptide linker. In some aspects, the peptide linker comprises the amino acid sequence of SEQ ID NO:19. In some aspects, [TZ_S] comprises of the amino acid sequence of SEQ ID NO:28.

[0041] In some aspects, the amino acid sequence of [Fc_x] is selected from the group consisting of SEQ ID NOs: 23, 24A, 24B, 24C, 25A, 25B and 25C. In some aspects, the amino acids of [L₁], [L₂], [L₃], and [L₄] is independently selected from the group consisting of SEQ ID NOs: 19, 20, 21, and 22. In other aspects (i) [L₁] comprises the amino acids of SEQ ID NO:20; (ii) [L₂] comprises the amino acids of SEQ ID NO:20; (iii) [L₃] comprises the amino acids of SEQ ID NO:21; and, (iv) [L₄] comprises the amino acids of SEQ ID NO:22.

[0042] In some aspects, [_BVH] comprises (i) a variable heavy chain CDR-1 (VH-CDR1) identical to SEQ ID NO: 1 or identical to SEQ ID NO: 1 except for up to 1, 2, 3, or 4 amino acid substitutions; (ii) a variable heavy chain CDR-2 (VH-CDR2) identical to SEQ

ID NO: 2 or identical to SEQ ID NO: 2 except for up to 1, 2, 3, or 4 amino acid substitutions; and (iii) a variable heavy chain CDR-3 (VH-CDR3) identical to SEQ ID NO: 3 or identical to SEQ ID NO: 3 except for up to 1, 2, 3, or 4 amino acid substitutions. In some aspects, [B^{VH}] comprises SEQ ID NO:15 or SEQ ID NO:43. In other aspects, [B^{CH}] comprises the amino acid sequence of SEQ ID NO: 29.

[0043] In some aspects, [B^{VL}] comprises (i) a variable light chain CDR-1 (VL-CDR1) identical to SEQ ID NO: 4 or identical to SEQ ID NO: 4 except for up to 1, 2, 3, or 4 amino acid substitutions; (ii) a variable light chain CDR-2 (VL-CDR2) identical to SEQ ID NO: 5 or identical to SEQ ID NO: 5 except for up to 1, 2, 3, or 4 amino acid substitutions; and (iii) a variable light chain CDR-3 (VL-CDR3) identical to SEQ ID NO: 6 or identical to SEQ ID NO: 6 except for up to 1, 2, 3, or 4 amino acid substitutions. In other aspects, [B^{VL}] comprises the amino acids of SEQ ID NO:16.

[0044] In some aspects, the first polypeptide chain of the bispecific anti-HER2 antibody comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 30, 31A, 31B, 32A, 32B, 32C 33A, 33B, 33C, 34, 35A, 35B, 36A, 36B, 36C 37A, 37B, 37C 38, 39A, 39B, 40A, 40B, 40C, 41A, 41B and 41C, and the second polypeptide chain of the bispecific anti-HER2 antibody comprises the amino acids of SEQ ID NO:42A or 42B.

[0045] In some aspects, the second immunoglobulin antigen-binding domain of the bispecific anti-HER2 antibody induces internalization upon binding to the HER2 target. In some aspects, the second immunoglobulin antigen-binding domain of the bispecific HER2 antibody promotes effective lysosomal trafficking following internalization. In some aspects, the second immunoglobulin antigen-binding domain of the bispecific HER2 antibody induces HER2 target degradation. In some aspects, the second immunoglobulin antigen-binding domain of the bispecific HER2 antibody blocks ligand-induced AKT phosphorylation in low HER2-expressing cancer cells. In some aspects, the first immunoglobulin antigen-binding domain of the bispecific HER2 antibody disrupts ligand-induced HER2:HER3 dimerization.

[0046] The present disclosure also provides an ADC comprising an anti-HER2 binding molecule disclosed herein, or an anti-HER2 bispecific antibody disclosed herein, further comprising at least one therapeutic moiety. In some aspects, the ADC further comprises

at least one optional spacer. In some aspects, the at least one spacer is a peptidic spacer. In some aspects, the at least one spacer is a nonpeptidic spacer.

[0047] Conventional conjugation strategies for ADCs rely on randomly conjugating the payload (e.g., therapeutic moiety) to the antibody through lysines or cysteines. Accordingly, in some aspects the ADC is randomly conjugated to a therapeutic moiety. In particular aspects, site-specific conjugation of therapeutic moieties to antibodies using reactive amino acid residues at specific positions yields homogeneous ADC preparations with uniform stoichiometry. In some aspects, the ADC comprises two, three, or four or more therapeutic moieties. In some aspects, all therapeutic moieties are the same. In some aspects, each therapeutic moiety is chemically conjugated to the side chain of an amino acid at a specific Kabat position in the Fc region of the bispecific antibody. In some aspects, the specific Kabat positions are 239, 442, or both. In some aspects, the specific positions are Kabat position 442, an amino acid insertion between Kabat positions 239 and 240, or both. In some aspects, the amino acid side chain is a sulfhydryl side chain. In some aspects, the therapeutic moiety comprises a cytotoxin, a radioisotope, an auristatin, a maytansinoid or a pyrrolobenzodiazepine (PBD), or combinations thereof. In certain aspects, the cytotoxin is tubulysin. In certain aspects, the tubulysin is *Compound T32* (also referred to herein as “tubulysin 1508” or simply “1508”).

[0048] The present disclosure also provides an ADC comprising a bispecific anti-HER2 antibody, wherein said antibody comprises:

- (i) a first polypeptide chain comprising the amino acids of SEQ ID NO: 32 and a second polypeptide chain comprising the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises a tubulysin molecule covalently linked to a cysteine amino acid at Kabat position 239.
- (ii) a first polypeptide chain comprising the amino acids of SEQ ID NO:33 and a second polypeptide chain comprising the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises two tubulysin molecules covalently linked to cysteine amino acids respectively located at Kabat positions 239 and 442.
- (iii) a first polypeptide chain comprising the amino acids of SEQ ID NO:40 and a second polypeptide chain comprising the amino acids of SEQ ID NO:42A, wherein the first

polypeptide chain comprises a tubulysin molecule covalently linked to a cysteine amino acid at Kabat position 239.

(iv) a first polypeptide chain comprising the amino acids of SEQ ID NO:41 and a second polypeptide chain comprising the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises two tubulysin molecules covalently linked to cysteine amino acids respectively located at Kabat positions 239 and 442.

[0049] The instant disclosure also provides an isolated nucleic acid molecule or a set of nucleic acid molecules encoding an anti-HER2 binding molecule (*e.g.*, a bispecific anti-HER2 antibody disclosed herein), or a complement thereof. Also provided is a vector or a set of vectors comprising such nucleic acid molecule or set of the nucleic acid molecules, or a complement thereof. Also provided is a host cell comprising the isolated nucleic acid molecule or a set of nucleic acid molecules, or the vector or set of vectors. Also provided is a host cell expressing an anti-HER2 binding molecule or bispecific anti-HER2 antibody disclosed herein. Also provided is a method for producing an anti-HER2 binding molecule or bispecific anti-HER2 antibody comprising culturing the host cell and recovering the antibody from the culture medium.

[0050] The instant disclosure also provides a pharmaceutical composition comprising an anti-HER2 binding molecule or anti-HER2 bispecific antibody, and a pharmaceutically acceptable carrier. Also provided is a method of treating a HER2-expressing cancer comprising administering an anti-HER2 binding molecule disclosed herein, an anti-HER2 bispecific antibody disclosed herein, an ADC disclosed herein, or a pharmaceutical composition disclosed herein to a subject in need thereof. In some aspects, the cancer is a low HER2-expressing cancer. In some aspects, the method further comprises administering at least one additional therapeutic agent. In some aspects, the at least one additional therapeutic agent is a radionuclide or a chemotherapeutic agent. Also provided is a method to target a therapeutic or prophylactic agent to the surface of cells expressing HER2 comprising conjugating the agent to an anti-HER2 binding molecule disclosed herein, or an anti-HER2 bispecific antibody disclosed herein. Also provided is a method to increase the activity of a therapeutic moiety comprising conjugating the agent to an anti-HER2 binding molecule disclosed herein, or an anti-HER2 bispecific antibody disclosed herein. Also provided is a method to improve the pharmacokinetic properties of

a therapeutic or prophylactic agent comprising conjugating the agent to an anti-HER2 binding molecule disclosed herein, or an anti-HER2 bispecific antibody disclosed herein. In some aspects, the therapeutic moiety is a cytotoxin. In some aspects, the cytotoxin is tubulysin. In some aspects, the tubulysin is *Compound T32*, also referred to herein as “tubulysin 1508” or simply “1508”. Also provided is a method to treat resistance to a HER2-targeting therapeutic agent comprising administering a bispecific HER2 antibody disclosed herein, an anti-HER2 binding molecule disclosed herein, or an ADC disclosed herein to a patient in need thereof. In some aspects, the patient is resistant to a HER2-targeting therapeutic agent comprising trastuzumab and/or the maytansinoid DM1.

BRIEF DESCRIPTION OF THE DRAWINGS/FIGURES

- [0051] **FIG. 1** shows a sequence alignment corresponding to the amino acid sequences of the VL and VH regions of the lead optimized 39S antibody (LO) and the parent AZ 1.39.1 antibody (WT). The location of CDR1, CDR2, and CDR3 is indicated. Amino acid residues which differ with respect to the parent antibody are highlighted. Replaced framework regions in VL region are also highlighted.
- [0052] **FIG. 2.** shows that changing the light chain frameworks to germline IGKV2D resulted in a doubling of expression.
- [0053] **FIG. 3.** Shows a ribbon representation of the structure of the extracellular domain of HER2. The binding sites of the 39S, pertuzumab and the domain IV scFv are indicated with arrows. The binding site of 39S includes amino acids within domain II that are distinct from those of pertuzumab.
- [0054] **FIG. 4** shows FACS competition assays using DL-680-labeled 39S (2 µg/ml) and varying concentrations of unlabeled monoclonal antibodies (R347 control, trastuzumab, pertuzumab, AZ 1.39.1, and 39S).
- [0055] **FIG. 5** shows ligand-dependent proliferation assays using different antibody combinations and the cell line MCF-7, demonstrating that 39S synergistically inhibits the growth of the assayed cell lines when administered in combination with trastuzumab and/or pertuzumab. For each experiment, the antibody samples plotted are: R347 control, trastuzumab, pertuzumab plus trastuzumab, 39S, 39S plus trastuzumab, and 39S plus pertuzumab.

- [0056] **FIG. 6** shows ligand-dependent proliferation assays using different antibody combinations and the cell line NCI-N87 demonstrating that 39S synergistically inhibits the growth of the assayed cell lines when administered in combination with trastuzumab and/or pertuzumab. For each experiment, the antibody samples plotted are: R347 control, trastuzumab, pertuzumab plus trastuzumab, 39S, 39S plus trastuzumab, and 39S plus pertuzumab.
- [0057] **FIG. 7** depicts bispecific constructs in which an HER2-binding moiety (a cysteine-stabilized scFv that binds within domain IV) has been genetically fused to different locations within the structure of the 39S antibody. The scFv was fused to the carboxy-terminus of each one of the heavy chains of the 39S antibody (referred to as “Bs3Ab-39SH”) (FIG. 7B), fused to the amino-terminus of each one of the heavy chains of the 39S antibody (referred to as “Bs2Ab-39SH”) (FIG. 7A), or intercalated between the CH1 and CH2 regions of each one of the heavy chains of the 39S antibody (referred to as “Bs4Ab-39SH”) (FIG. 7C).
- [0058] **FIG. 8A** shows the sequences of the heavy chains of Bs2 antibodies, indicating the amino acids at Kabat locations 234, 239, 239-ins, 330, 332 and 442 in wild-type heavy chains and heavy chains which may be optimized for ADCC activity or for the production of ADCs that have reduced ADCC activity and/or site specific conjugation.
- [0059] **FIG. 8B** shows the sequences of the heavy chains of Bs3 antibodies, indicating the amino acids at Kabat locations 234, 239, 239-ins, 330, 332 and 442 in wild-type heavy chains and heavy chains which may be optimized for ADCC activity or for the production of ADCs that have reduced ADCC activity and/or site specific conjugation.
- [0060] **FIG. 8C** shows the sequences of the heavy chains of Bs4 antibodies, indicating the amino acids at Kabat locations 234, 239, 239-ins, 330, 332 and 442 in wild-type heavy chains and heavy chains which may be optimized for ADCC activity or for the production of ADCs that have reduced ADCC activity and/or site specific conjugation.
- [0061] **FIG. 9A** shows ligand-dependent proliferation assays using different bispecific antibody constructs and MDA-MB-361 cells demonstrating that Bs2Ab-39SH, Bs3Ab-39SH and Bs4Ab-39SH have similar potency in MDA-MB-361 and MCF-7 cells (FIG. 9A and 9B, respectively), which is also comparable to the activity of parental antibody

combination (39S plus trastuzumab). For each experiment, the antibody samples plotted are: R347 control, Bs2Ab-39SH, Bs3Ab-39SH, Bs4Ab-39SH, and 39S plus trastuzumab.

[0062] **FIG. 9B** shows ligand-dependent proliferation assays using different bispecific antibody constructs and MCF-7 cells demonstrating that Bs2Ab-39SH, Bs3Ab-39SH and Bs4Ab-39SH have similar potency in MDA-MB-361 and MCF-7 cells (FIG. 9A and 9B, respectively), which is also comparable to the activity of parental antibody combination (39S plus trastuzumab). For each experiment, the antibody samples plotted are: R347 control, Bs2Ab-39SH, Bs3Ab-39SH, Bs4Ab-39SH, and 39S plus trastuzumab.

[0063] **FIG. 10** shows immunoprecipitation (IP) of HER2 from T47D cells followed by Western (WB) detection of HER2 and HER3 to measure the disruption of HER2:HER3 dimerization induced by heregulin (HRG1), in the presence or absence of anti-HER2 antibodies. T47D cells were incubated under control conditions (with no antibody and with no HRG1; with no antibody but with HRG1; or, with control antibody R347 and with HRG1), and after incubation with HRG1 plus (i) trastuzumab, (ii) pertuzumab, (iii) 39S antibody, or (iv) the Bs2Ab-39SH bispecific construct.

[0064] **FIG. 11** shows the representative HPLC size-exclusion chromatography profiles showing the size separation of immune complexes derived from antibody:HER2 molar ratio of 1:1. Bs2Ab-39SH can cross-link many HER2 molecules to form complexes as large as 1716 kDa in size, while trastuzumab can only bind to two HER2 molecules in maximal to form a 320 kDa complex.

[0065] **FIG. 12** presents FACS-based receptor internalization assays showing that the three formats of HER2 bispecific antibodies presented in FIG. 6 internalized rapidly in the cell line BT-474. Plotted on the graph are: R347 control, trastuzumab, 39S, trastuzumab plus 39S, Bs2Ab-39SH, Bs3Ab-39SH, and BsAb-39SH. Similar results were seen in MCF-7 , T47D , RT-112 , MDA-MB-361, and NCI-N87 cell lines (data not shown)..

[0066] **FIG. 13** shows the kinetics of Bs2Ab-39SH internalization by BT-474 cells. Microscopy images were taken at 0, 30, 60, 120, and 360 minutes after addition of the Bs2Ab-39SH construct, trastuzumab or the R347 control. The cytoplasm of the BT-474 cells was stained with CELLTRACKER™ Green (Life Technologies Corp.), and the antibodies were labeled with ALEXA FLUOR® 647 (Life Technologies Corp.).

[0067] **FIG. 14** is a Western immunoblot showing the degree of degradation of HER2, or lack thereof, 2 hours, 6 hours, and 24 hours after incubation of BT-474 cells with antibody samples. GAPDH was used as control. The tested antibody samples were: (lane 1) R347 control antibody, (lane 2) trastuzumab, (lane 3) pertuzumab, (lane 4) trastuzumab and pertuzumab, (lane 5) 39S, (lane 6) trastuzumab and 39S, (lane 7) pertuzumab and 39S, (lane 8) trastuzumab, pertuzumab and 39S, (lane 9) Bs2Ab-39SH, (lane 10) Bs2Ab-39SH_aFuc (homogenously afucosylated Bs2Ab-39SH), (lane 11) Bs3Ab-39SH, (lane 12) Bs3Ab-39SH_aFuc (homogenously afucosylated Bs3Ab-39SH_aFuc), (lane 13) Bs4Ab-39SH, and (lane 14) Bs4Ab-39SH_aFuc (homogenously afucosylated Bs4Ab-39SH).

[0068] **FIG. 15** is a detailed cartoon representations of exemplary ADCs derived from the Bs2Ab-39SH (panel A), the Bs3Ab-39SH (panel B) and the Bs4Ab-39SH (panel C) anti-HER2 constructs. Two potential engineered cytotoxic agent conjugation sites in the CH2 and CH3 domains of the ADC constructs are indicated by circles. Where the desired drug-to-antibody ratio (DAR) is 2 to 1 either site 1 or site 2 may be used. Where a DAR of 4 is desirable both sites 1 and 2 are utilized. Alternative and/or additional sites may be engineered. The bispecific anti-HER2 ADCs are abbreviated herein as “Bs2Ab-2T”, “Bs3Ab-2T” and “Bs4Ab-2T” (or simply as “Bs2-2T”, “Bs3-2T” and “Bs4-2T”) for the ADCs having a DAR of ~2 and as “Bs2Ab-4T”, “Bs3Ab-4T” and “Bs4Ab-4T” (or simply as “Bs2-4T”, “Bs3-4T” and “Bs4-4T”) for the ADCs having a DAR of ~4. The bispecific constructs lacking any cytotoxic agent are referred by the designations provided in Figure 7 and may be identified as “unarmed.” As provided herein (see, e.g., Figure 8) the engineered conjugate sites may be selected to reduce or ablate ADCC functions. Alternatively or additionally Fc portion of the antibody may further comprise additional mutations which reduce or ablate ADCC activity. Alternatively, an ADC may be generated using a classic conjugation method such as conjugating to antibodies through the often-numerous lysine residues or native cysteine residues of an antibody, generating a heterogeneous antibody-drug conjugate mixture.

[0069] **FIG. 16A** presents immunofluorescence images of SKOV-3 cells treated with 1 μ g/ml of the antibody indicated under each image, showing the disruption of the intracellular microtubule network or lack thereof. Microtubule staining is shown. The

following samples were tested: media control without any antibody (image i), R347 control antibody (image ii), trastuzumab (image iii), Bs2Ab-39SH (image iv), R347-DM1 (R347 control antibody conjugated to the cytotoxic agent maytansinoid DM1) (image v), R347-4T (R347 control antibody conjugated to 4 molecules of the cytotoxic agent tubulysin) (image vi), T-DM1 (trastuzumab conjugated to maytansinoid DM1) (image vii), and Bs2-4T (Bs2Ab-39SH conjugated to 4 molecules of the cytotoxic agent tubulysin) (image viii).

[0070] **FIG. 16B** presents immunofluorescence images of JIMT-1 cells treated with 1 µg/ml of the antibody indicated under each image, showing the disruption of the intracellular microtubule network or lack thereof. Microtubule staining is shown. The following samples were tested: media control without any antibody (image i), R347 control antibody (image ii), trastuzumab (image iii), Bs2Ab-39SH (image iv), R347-DM1 (R347 control antibody conjugated to the cytotoxic agent maytansinoid DM1) (image v), R347-4T (R347 control antibody conjugated to 4 molecules of the cytotoxic agent tubulysin) (image vi), T-DM1 (trastuzumab conjugated to maytansinoid DM1) (image vii), and Bs2-4T (Bs2Ab-39SH conjugated to 4 molecules of the cytotoxic agent tubulysin) (image viii).

[0071] **FIG. 16C** presents immunofluorescence images of RT-112 cells treated with 1 µg/ml of the antibody indicated under each image, showing the disruption of the intracellular microtubule network or lack thereof. Microtubule staining is shown. The following samples were tested: media control without any antibody (image i), R347 control antibody (image ii), trastuzumab (image iii), Bs2Ab-39SH (image iv), R347-DM1 (R347 control antibody conjugated to the cytotoxic agent maytansinoid DM1) (image v), R347-4T (R347 control antibody conjugated to 4 molecules of the cytotoxic agent tubulysin) (image vi), T-DM1 (trastuzumab conjugated to maytansinoid DM1) (image vii), and Bs2-4T (Bs2Ab-39SH conjugated to 4 molecules of the cytotoxic agent tubulysin) (image viii).

[0072] **FIG. 17A** shows the cytotoxic activity of the Bs2Ab format with a DAR of 2 or 4 relative to the T-DM1, non conjugated (unarmed) Bs2Ab-39SH and trastuzumab on the SKBR-3 human breast cancer cell line. Also shown are the curves for R347 (R347 control antibody), R347 conjugated with 2 or 4 tubulysins (R347-2T and R3474T

respectively) R347 conjugated to DM1. Both Bs2-2T and Bs2-4T are more potent than T-DM1.

[0073] **FIG. 17B** shows the cytotoxic activity the Bs4Ab format with a DAR of 2 or 4 (Panel B) relative to the T-DM1, non conjugated (unarmed) Bs4Ab-39SH and trastuzumab on the SKBR-3 human breast cancer cell line. Also shown are the curves for R347 (R347 control antibody), R347 conjugated with 2 or 4 tubulysins (R347-2T and R3474T respectively) R347 conjugated to DM1. Both Bs4-2T and Bs4-4T are more potent than T-DM1.

[0074] **FIG. 18A** shows the cytotoxic activity of the Bs2Ab format with a DAR of 2 or 4 relative to the T-DM1, non conjugated (unarmed) Bs2Ab-39SH and trastuzumab on the JIMT-1 human breast cancer cell line. Also shown are the curves for R347 (R347 control antibody), R347 conjugated with 2 or 4 tubulysins (R347-2T and R3474T respectively) R347 conjugated to DM1. Both Bs2-2T and Bs2-4T are very potent in killing JIMT-1 cells, while T-DM1 shows no activity.

[0075] **FIG. 18B** shows the cytotoxic activity of the Bs4Ab format with a DAR of 2 or 4 (Panel B) relative to the T-DM1, non conjugated (unarmed) Bs4Ab-39SH and trastuzumab on the JIMT-1 human breast cancer cell line. Also shown are the curves for R347 (R347 control antibody), R347 conjugated with 2 or 4 tubulysins (R347-2T and R3474T respectively) R347 conjugated to DM1. Both Bs4-2T and Bs4-4T are very potent in killing JIMT-1 cells, while T-DM1 shows no activity.

[0076] **FIG. 19A** shows the cytotoxic activity of the Bs2Ab format with a DAR of 2 or 4 relative to the T-DM1, non conjugated (unarmed) Bs2Ab-39SH and trastuzumab on the ZR-75-1 human breast cancer cell line. Also shown are the curves for R347 (R347 control antibody), R347 conjugated with 2 or 4 tubulysins (R347-2T and R3474T respectively) R347 conjugated to DM1. Bs2-4T is the most active in killing ZR-75-1 cells, while Bs2-2T has a lower level of activity and T-DM1 shows no or limited cytotoxic activity.

[0077] **FIG. 19B** shows the cytotoxic activity of the Bs4Ab format with a DAR of 2 or 4 (Panel B) relative to the T-DM1, non conjugated (unarmed) Bs4Ab-39SH and trastuzumab on the ZR-75-1 human breast cancer cell line. Also shown are the curves for R347 (R347 control antibody), R347 conjugated with 2 or 4 tubulysins (R347-2T and

R3474T respectively) R347 conjugated to DM1. Bs4-4T is the most active in killing ZR-75-1 cells, while Bs4-2T has a lower level of activity and T-DM1 shows no or limited cytotoxic activity.

[0078] **FIG. 20** shows the cytotoxic activity of the Bs2Ab format with a DAR of 2 or 4 (Panel A) relative to the T-DM1, non conjugated (unarmed) Bs2Ab-39SH and trastuzumab on the MDA-MB-468 human breast cancer cell line. Also shown are the curves for R347 (R347 control antibody), R347 conjugated with 2 or 4 tubulysins (R347-2T and R3474T respectively) R347 conjugated to DM1. Data indicate that neither Bs2-2T nor Bs2-4T is active in MDA-MB-468 cells, indicating that the cytotoxic activity of Bs2-2T and Bs2-4T is target (HER2)-dependent. Similar results were observed with Bs4-2T and Bs4-4T (data not shown)

[0079] **FIG. 21A** shows the activity of anti-HER2 ADC activity in the MDA-MB-361 tumor model. Shown are the tumor growth curves corresponding to mice treated with (1) vehicle control, (2) R347-4T (R347 control antibody conjugated to 4 tubulysin molecules) at 3 mg/kg, (3) T-DM1 (trastuzumab-DM1 ADC) at 3 mg/kg, (4) Bs2 ADMix (Bs2Ab-39SH-(FCC) construct mixed with tubulysin) at 3 mg/kg, and (5) Bs2-4T (Bs2Ab-39SH-(FCC) construct conjugated to 4 tubulysins) at 0.3, 1 and 3 mg/kg. Concentrations are indicated between parenthesis.

[0080] **FIG. 21B** shows the activity of anti-HER2 ADC activity in the MDA-MB-361 tumor model. Shown are the tumor growth curves corresponding to mice treated with (1) vehicle control, (2) R347-4T (R347 control antibody conjugated to 4 tubulysin molecules) at 3 mg/kg, (3) T-DM1 (trastuzumab-DM1 ADC) at 3 mg/kg, (4) Bs4 ADMix (Bs4Ab-39SH-(FCC) construct mixed with tubulysin) at 3 mg/kg, and (5) Bs4-4T (Bs4Ab-39SH-(FCC) conjugated to 4 tubulysins) at 0.3, 1 and 3 mg/kg. Concentrations are indicated between parenthesis.

[0081] **FIG. 22** shows the activity of anti-HER2 ADC in the ST996 triple-negative (ER-/PR-/HER2-1+) PDX tumor model. Shown are the tumor growth curves corresponding to mice treated with (1) vehicle control (CTRL), (2) isotype R347-2T CTRL (R347 control antibody conjugated to 2 tubulysin molecules), (3) isotype R347-4T CTRL (R347 control antibody conjugated to 4 tubulysin molecules), (4) T-DM1 (trastuzumab-DM1 ADC), (5) ADMix (construct mixed with tubulysin), (6) Bs2-2T, i.e., Bs2Ab-39SH-(FCC) construct

conjugated to 2 tubulysins, or (7) Bs2-4T, i.e., Bs2Ab-39SH-(FCC) construct conjugated to 4 tubulysins. Concentrations are indicated between parenthesis. Time of dosing is also indicated by arrows. Tumor growth curves in response to the various treatments are presented as the mean tumor volume (mm^3) $\pm$ SEM.

[0082] **FIG. 23** shows the activity of anti-HER2 ADC activity in the ST225 (HER2-3+) BrCa PDX tumor model. Shown are the tumor growth curves corresponding to mice treated with (1) vehicle control, (2) R347-4T (R347 control antibody conjugated to 4 tubulysin molecules), (3) T-DM1 (trastuzumab-DM1 ADC), (4) BS2 ADMix (Bs2Ab-39SH-(FCC) construct mixed with tubulysin), and (5) Bs2-4T (Bs2Ab-39SH-(FCC) construct conjugated to 4 tubulysins. Concentrations are indicated between parenthesis.

[0083] **FIG. 24** shows the activity of anti-HER2 ADC activity in the T-DM-1 non-responder JIMT-1 (HER2-3+) BrCa CBX tumor model. Shown are the tumor growth curves corresponding to mice treated with (1) vehicle control, (2) R347-4T (R347 control antibody conjugated to 4 tubulysin molecules), (3) T-DM1 (trastuzumab-DM1 ADC), (4) BS2 ADMix (Bs2Ab-39SH-(FCC) construct mixed with tubulysin), and (5) Bs2-4T (Bs2Ab-39SH-(FCC) construct conjugated to 4 tubulysins. Concentrations are indicated between parenthesis.

[0084] **FIG. 25** shows the activity of anti-HER2 ADC activity in the ST455B triple-negative (ER-/PR-/HER2-1+) BrCa PDX tumor model. Shown are the tumor growth curves corresponding to mice treated with (1) vehicle control (CTRL), (2) isotype CTRL (R347 control antibody conjugated to 4 tubulysin molecules), (3) T-DM1 (trastuzumab-DM1 ADC), (4) BS2 ADMix (Bs2Ab-39SH-(FCC) construct mixed with tubulysin), and (5) Bs2-4T (Bs2Ab-39SH-(FCC) construct conjugated to 4 tubulysins. Concentrations are indicated between parenthesis.

[0085] **FIG. 26A** shows the cytotoxic activity Bs2-4T and T-DM1 on a parental NCI-N87 cell line (left panel) and an NCI-N87 cell line with acquired resistance to T-DM1 (right panel). Bs2-4T has more potent activity in the parent cell line and is still active in killing the T-DM1 resistant cells.

[0086] **FIG. 26B** shows the activity of anti-HER2 ADC in the T-DM1 resistant NCI-N87 tumor model. Shown are the tumor growth curves corresponding to untreated mice and mice treated with (1) ADMix (construct mixed with tubulysin), (2) T-DM1 (trastuzumab-

DM1 ADC), (3) T-DM1 (trastuzumab-DM1 ADC) plus pertuzumab, or (4) Bs2-4T, i.e., Bs2Ab-39SH-(FCC) construct conjugated to 4 tubulysins. Concentrations are indicated between parenthesis. Tumor growth curves in response to the various treatments are presented as the mean tumor volume (mm^3) $\pm$ SEM (n=7). *P < 0.001 by Student's t test as compared to the untreated control group.

[0087] **FIG. 27** shows the activity of anti-HER2 ADC after pretreatment with trastuzumab. Shown are the tumor growth curves corresponding to mice treated with (1) vehicle CTRL (control), (2) trastuzumab followed by Bs2-4T, (3) vehicle followed by Bs2-4T, (4) trastuzumab followed by Bs2-4T, or (5) vehicle followed by Bs2-4T. Concentrations and dosage regimens are indicated between parentheses. Tumor growth curves in response to the various treatments are presented as the mean tumor volume (mm^3) $\pm$ SEM (n=10).

[0088] **FIG. 28A** shows a schematic for an assay to evaluate the bystander effect of the Bispecific ADCs.

[0089] **FIG. 28B** shows FACS analysis results of cells treated with (i) media alone; (ii) R347-T4 control; (iii) T-DM1; (iv) Bs2Ab-39SH admix; and (v) Bs2-4T. The reduction in cell numbers in both quadrants indicates that Bs2-4T can kill both HER2-expressing and HER2-null cells in a co-culture, suggesting Bs2-4T has bystander effect. In contrast, T-DM1 cannot kill HER2-null cells in a co-culture, suggesting it does not have bystander effect.

[0090] **FIG. 29** shows the activity of Bs2-4T on cancer stem cell (CSC) sphere formation (left panel) relative to T-DM1 and R347-4T (R347 control antibody conjugated to 4 tubulysin molecules); and on CSC in xenograph tumors (right panel) relative to R34-4T (R234 control antibody conjugated to 4 tubulysin molecules).

[0091] **FIG. 30A** shows ligand-dependent proliferation assays using the cell line MDA-MB-361 (human ductal breast epithelial adenocarcinoma derived from brain metastasis), which showed that all of the ADCC enhanced afucosylated bispecific antibodies retain *in vitro* anti-proliferative activity. In each experiment, the antibody samples used were: R347 control, 39S plus trastuzumab, Bs2Ab-39SH_aFuc, Bs3Ab-39SH_aFuc, and Bs4Ab-39SH_aFuc.

- [0092] **FIG. 30B** shows ligand-dependent proliferation assays using the cell line MCF-7 (human invasive breast ductal adenocarcinoma), which showed that all of the ADCC enhanced afucosylated bispecific antibodies retain in vitro anti-proliferative activity. In each experiment, the antibody samples used were: R347 control, 39S plus trastuzumab, Bs2Ab-39SH_aFuc, Bs3Ab-39SH_aFuc, and Bs4Ab-39SH_aFuc.
- [0093] **FIG. 31A** shows ADCC cytotoxicity assays using the MDA-MB-361 cells as the target and engineered NK effector cells (stably expressing CD16), which showed that each of the afucosylated bispecific antibodies has more potent ADCC activity as compared to the same construct having fucosylated glycoforms. The antibody samples used were: (1) R347 control, (2) trastuzumab, (3) trastuzumab_aFuc, (4) Bs2Ab-39SH, and (5) Bs2Ab-39SH_aFuc,
- [0094] **FIG. 31B** shows ADCC cytotoxicity assays using the MDA-MB-361 cells as the target and engineered NK effector cells (stably expressing CD16), which showed that each of the afucosylated bispecific antibodies has more potent ADCC activity as compared to the same construct having fucosylated glycoforms. The antibody samples used were: (1) R347 control, (2) trastuzumab, (3) trastuzumab_aFuc, (4) Bs3Ab-39SH, and (5) Bs3Ab-39SH_aFuc.
- [0095] **FIG. 31C** shows ADCC cytotoxicity assays using the MDA-MB-361 cells as the target and engineered NK effector cells (stably expressing CD16), which showed that each of the afucosylated bispecific antibodies has more potent ADCC activity as compared to the same construct having fucosylated glycoforms. The antibody samples used were: (1) R347 control, (2) trastuzumab, (3) trastuzumab_aFuc, (4) Bs4Ab-39SH, and (5) Bs4Ab-39SH_aFuc.
- [0096] **FIG. 32** shows the structure of the tubulysin 1508 payload. The double bond of the maleimide group at the far right readily reacts with the thiol group found on cysteine to form a stable carbon-sulfur bond.
- [0097] **FIG. 33** shows an exemplary site-specific antibody drug conjugation process, using the Bs2Ab-FCC construct as the derivatizable platform. The process comprises the steps of (a) uncapping the side chains of the derivatizable amino acids (*e.g.*, cysteines), (b) oxidizing, (c) conjugating a payload (*e.g.*, a cytotoxic agent such as tubulysin), and (d) polishing by removing conjugation reagents and non-reacted payload.

DETAILED DESCRIPTION OF THE INVENTION

[0098] The present disclosure provides optimized anti-HER2 antibodies, and bispecific antibodies derived from such optimized anti-HER2 antibodies. Related polynucleotides, vectors, cells, and pharmaceutical compositions comprising such optimized anti-HER2 antibodies or bispecific anti-HER2 antibodies are also provided. Also provided are methods of making such optimized anti-HER2 antibodies or bispecific antibodies. In addition, the disclosure provides are methods of using the optimized anti-HER2 antibodies or bispecific anti-HER2 antibodies, for example, methods of treating cancer in a subject in need thereof.

[0099] The present disclosure also provides antibody-drug conjugates (ADC) derived from optimized anti-HER2 antibodies and bispecific antibodies derived from such optimized anti-HER2 antibodies. Also provided are methods of making ADC derived from such optimized antibodies and bispecific antibodies. Also provided are methods of using the ADC derived from optimized anti-HER2 antibodies and bispecific antibodies, for example, methods of treating cancer in a subject in need thereof. Also provided are methods to treat patients with cancers resistant to chemotherapy (e.g., tumors in T-DM1 non-responder or poor-responder patients); to treat patients that are relapsed, refractory, on non-eligible to be treated with other therapies, in particular mono-specific ADC therapies (e.g. T-DM1); or to treat patients after pretreatment with other therapies (e.g. T-DM1).

[00100] In particular, the instant disclosure provides anti-HER2 binding molecules that are suitable for treating cancer expressing low levels of HER2.

[00101] In order that the present invention can be more readily understood, certain terms are first defined. Additional definitions are set forth throughout the detailed description.

I. Definitions

[00102] Before describing the present invention in detail, it is to be understood that this invention is not limited to specific compositions or process steps, as such can vary. As used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the context clearly dictates otherwise. The terms "a" (or

"an"), as well as the terms "one or more," and "at least one" can be used interchangeably herein.

[00103] Furthermore, "and/or" where used herein is to be taken as specific disclosure of each of the two specified features or components with or without the other. Thus, the term "and/or" as used in a phrase such as "A and/or B" herein is intended to include "A and B," "A or B," "A" (alone), and "B" (alone). Likewise, the term "and/or" as used in a phrase such as "A, B, and/or C" is intended to encompass each of the following aspects: A, B, and C; A, B, or C; A or C; A or B; B or C; A and C; A and B; B and C; A (alone); B (alone); and C (alone).

[00104] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure is related. For example, the Concise Dictionary of Biomedicine and Molecular Biology, Juo, Pei-Show, 2nd ed., 2002, CRC Press; The Dictionary of Cell and Molecular Biology, 3rd ed., 1999, Academic Press; and the Oxford Dictionary Of Biochemistry And Molecular Biology, Revised, 2000, Oxford University Press, provide one of skill with a general dictionary of many of the terms used in this disclosure.

[00105] Units, prefixes, and symbols are denoted in their Système International de Unites (SI) accepted form. Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, amino acid sequences are written left to right in amino to carboxy orientation. The headings provided herein are not limitations of the various aspects, which can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the specification in its entirety.

[00106] It is understood that wherever aspects are described herein with the language "comprising," otherwise analogous aspects described in terms of "consisting of" and/or "consisting essentially of" are also provided.

[00107] Amino acids are referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, are referred to by their commonly accepted single-letter codes.

[00108] The terms "HER2" and "HER2 receptor" are used interchangeably herein, and refer to the ErbB2 protein (also referred to as HER2/neu in the literature). As used herein, the terms are intended to include variants (*e.g.*, splice variants), isoforms, and homologs of HER2 (both orthologs and paralogs). In some aspects, binding of an anti-HER2 binding molecule disclosed herein to HER2 inhibits the growth of cells expressing HER2 (*i.e.* typically tumor cells, and in particular cancer cells expressing low levels of HER2) by inhibiting formation of heteromeric complexes between HER2 and other ErbB family members, *e.g.* inhibiting heterodimerization with EGFR or HER3.

[00109] HER2 is a receptor tyrosine kinase and is composed of an extracellular domain (ECD), which consists of (i) two leucine-rich domains (domain I/L1 and domain III/L2) responsible for ligand binding, and (ii) two cysteine-rich domains (domain II/CR1 and domain IV/CR2) responsible for receptor dimerization; a transmembrane domain; and an intracellular tyrosine kinase domain. Alternative splice variants of HER2 exist. Examples of alternative splice variants of HER2 include p100 and herstatin (two soluble forms), as well as 611-CTF, 687-CTF, 648-CTF, and Δ 16HER2. The soluble forms of HER2 can interact with full-length receptors (p185) and inhibit receptor dimerization; 687-CTF is inactive; 611-CTF and 648-CTF can activate several intracellular signal transduction pathways; and Δ 16HER2 lacks the amino acids 634–649 in domain IV, which induce a conformational change that promotes the formation of constitutively activated HER2 homodimers. The extracellular portion of mature HER2, without the signal sequence, corresponds to positions 23-652 of canonical Isoform 1 (see Uniprot P04626; see also "Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab." Cho *et al.*, Nature 421:756-760 (2003), incorporated herein by reference in its entirety). The pertuzumab antibody binds to an epitope within domain II of HER2. Antibodies that do not bind to the pertuzumab binding epitope within domain II include trastuzumab, which binds to an epitope within domain IV.

[00110] The terms "inhibit," "block," and "suppress" are used interchangeably herein and refer to any statistically significant decrease in biological activity, including full blocking of the activity. For example, "inhibition" can refer to a decrease of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% in biological activity. Accordingly, when the terms "inhibition" or "suppression" are applied to describe, *e.g.*, an effect on

ligand-mediated HER2 phosphorylation, the term refers to the ability of an anti-HER2 antibody or a HER2-binding molecule comprising an antigen binding fragment thereof, to statistically significantly decrease the phosphorylation of HER2 induced by an EGF-like ligand, relative to the phosphorylation in an untreated (control) cell.

[00111] The cell which expresses HER2 can be a naturally occurring cell or cell line (*e.g.*, a cancer cell) or can be recombinantly produced by introducing a nucleic acid encoding HER2 into a host cell. In one aspect, the anti-HER2 binding molecule, *e.g.*, an anti-HER2 antibody or a HER2-binding molecule comprising an antigen binding fragment thereof, inhibits ligand mediated phosphorylation of HER2 by at least 10%, or at least 20%, or at least 30%, or at least 40%, or at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or about 100%, as determined, for example, by Western blotting followed by probing with an anti-phosphotyrosine antibody or by ELISA.

[00112] The terms "growth suppression" or "growth inhibition" of a cell expressing HER2, as used herein, refer to the ability of anti-HER2 binding molecule, *e.g.*, an anti-HER2 antibody or a HER2-binding molecule comprising an antigen-binding fragment thereof, to statistically significantly decrease proliferation of cells expressing HER2 relative to the proliferation in the absence of the anti-HER2 binding molecule. In one aspect, the proliferation of cells expressing HER2 (*e.g.*, cancer cells) can be decreased by at least 10%, or at least 20%, or at least 30%, or at least 40%, or at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or about 100% when cells are contacted with an anti-HER2 binding molecule, *e.g.*, an anti-HER2 antibody or a HER2-binding molecule comprising an antigen-binding fragment thereof, relative to the proliferation measured in the absence of the anti-HER2 binding molecule (control conditions). Cell proliferation can be measured according to various methods known in the art, *e.g.*, by counting the number of viable cells, identifying the presence of markers of growth markers, measuring the incorporation of molecules (*e.g.*, radioactively labelled molecules such as ^3H -thymidine), by measuring the size of a tumor (*e.g.*, by volume or by weight), etc. In certain aspects, growth suppression refers to reduction in the number, size, or distribution of metastases.

[00113] As used throughout the instant specification, the phrase "anti-HER2 binding molecule" refers, for example, to (i) antibodies and antigen-binding fragments thereof

which bind the same epitope as or are derived from the 1.39.1 antibody (see PCT Publ. No. WO 2008/019290) disclosed in the instant application, *e.g.*, the 39S antibody and antigen-binding fragments thereof, and in general molecules comprising such antibodies and antigen-binding fragments thereof; (ii) anti-HER2 antibodies and other HER2-binding molecules which bind the same epitope as or are derived from the 39S antibody which incorporate additional antigen-binding moieties, *e.g.*, bispecific antibodies; (iii) antibody-drug conjugates (ADC) comprising at least one of the molecules according to (i) or (ii) conjugated to a cytotoxic moiety (*e.g.*, a small molecule anticancer agent, a radionuclide, etc.), and (iv) anti-HER2 molecules according to (i) or (ii) having enhanced ADCC. As used herein, the term "39S antibody" refers to a lead optimized monoclonal antibody derived from the 1.39.1 antibody disclosed in PCT Publ. No. WO 2008/019290, wherein said optimized antibody comprises a VH comprising the amino acids of SEQ ID NO:15 and a VL comprising the amino acids of SEQ ID NO:16.

[00114] As used herein, the term "tubulysin" refers both collectively and individually to the naturally occurring tubulysins, and analogs and derivatives of tubulysins. Illustrative examples of tubulysins are disclosed, for example, in WO2004005326A2, WO2012019123A1, WO2009134279A1, WO2009055562A1, WO2004005327A1, US7754885, US20100240701, US7816377, US20110021568, and US20110263650 and in the Examples provided herein. It is to be understood that such derivatives include, for example, tubulysin prodrugs or tubulysins that include one or more protection or protecting groups, one or more linking moieties.

[00115] Cellular proliferation can be assayed using art recognized techniques which measure rate of cell division, and/or the fraction of cells within a cell population undergoing cell division, and/or rate of cell loss from a cell population due to terminal differentiation or cell death (*e.g.*, thymidine incorporation).

[00116] The terms "antibody" or "immunoglobulin," as used interchangeably herein, include whole antibodies and any antigen binding fragment or single chains thereof and combinations thereof (*e.g.*, bispecific antibodies).

[00117] A typical antibody comprises at least two heavy (H) chains and two light (L) chains interconnected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as VH) and a heavy chain constant region

(abbreviated herein as CH). The heavy chain constant region is comprised of three domains, CH1, CH2, and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as VL) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed Complementarity Determining Regions (CDR), interspersed with regions that are more conserved, termed framework regions (FW). Each VH and VL is composed of three CDRs and four FWs, arranged from amino-terminus to carboxy-terminus in the following order: FW1, CDR1, FW2, CDR2, FW3, CDR3, FW4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies can mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (*e.g.*, effector cells) and the first component (C1q) of the classical complement system. Exemplary antibodies of the present disclosure include typical antibodies, scFvs, and combinations thereof where, for example, an scFv is covalently linked (for example, via peptidic bonds or via a chemical linker) to the N-terminus of either the heavy chain and/or the light chain of a typical antibody, or intercalated in the heavy chain and/or the light chain of a typical antibody.

[00118] The term "antibody" means an immunoglobulin molecule that recognizes and specifically binds to a target, such as a protein, polypeptide, peptide, carbohydrate, polynucleotide, lipid, or combinations of the foregoing through at least one antigen recognition site within the variable region of the immunoglobulin molecule. As used herein, the term "antibody" encompasses intact polyclonal antibodies, intact monoclonal antibodies, antibody fragments (such as Fab, Fab', F(ab')₂, and Fv fragments), single chain variable fragment (scFv), disulfide stabilized scFvs, multispecific antibodies such as bispecific antibodies generated from at least two intact antibodies and/or antigen binding fragments thereof, chimeric antibodies, humanized antibodies, human antibodies, fusion proteins comprising an antigen determination portion of an antibody, and any other modified immunoglobulin molecule comprising an antigen recognition site so long as the antibodies exhibit the desired biological activity.

[00119] An antibody can be of any the five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, or subclasses (isotypes) thereof (*e.g.* IgG1, IgG2, IgG3, IgG4, IgA1

and IgA2), based on the identity of their heavy-chain constant domains referred to as alpha, delta, epsilon, gamma, and mu, respectively. The different classes of immunoglobulins have different and well known subunit structures and three-dimensional configurations. Antibodies can be naked or conjugated to other molecules such as toxins, radioisotopes, etc. to form ADCs.

[00120] A "blocking" antibody or an "antagonist" antibody is one which inhibits or reduces biological activity of the antigen it binds, such as HER2. In a certain aspect, blocking antibodies or antagonist antibodies substantially or completely inhibit the biological activity of the antigen. Desirably, the biological activity is reduced by 10%, 20%, 30%, 50%, 70%, 80%, 90%, 95%, or even 100%.

[00121] The terms "anti-HER2 antibody" or "anti-HER2" refers to an antibody that is capable of binding HER2 with sufficient affinity such that the antibody is useful as a therapeutic agent or diagnostic reagent in targeting HER2. The extent of binding of an anti-HER2 antibody to an unrelated, non-HER2 protein is less than about 10% of the binding of the antibody to HER2 as measured, *e.g.*, by a radioimmunoassay (RIA), or BIACORE™ (using recombinant HER2 as the analyte and antibody as the ligand, or *vice versa*), or other binding assays known in the art. In certain aspects, an antibody that binds to HER2 has a dissociation constant (K_D) of $\leq 1 \mu\text{M}$, $\leq 100 \text{ nM}$, $\leq 10 \text{ nM}$, $\leq 1 \text{ nM}$, $\leq 0.1 \text{ nM}$, $\leq 10 \text{ pM}$, $\leq 1 \text{ pM}$, or $\leq 0.1 \text{ pM}$.

[00122] The terms "antigen binding fragment" refers to a portion of an intact antibody and refers to the antigenic determining variable regions of an intact antibody. It is known in the art that the antigen binding function of an antibody can be performed by fragments of a full-length antibody. Examples of antibody fragments include, but are not limited to Fab, Fab', F(ab')₂, and Fv fragments, linear antibodies, single chain antibodies, and multispecific antibodies formed from antibody fragments.

[00123] A "monoclonal antibody" refers to a homogeneous antibody population involved in the highly specific recognition and binding of a single antigenic determinant, or epitope. This is in contrast to polyclonal antibodies that typically include different antibodies directed against different antigenic determinants.

[00124] The term "monoclonal antibody" encompasses both intact and full-length monoclonal antibodies as well as antibody fragments (such as Fab, Fab', F(ab')₂, Fv),

single chain variable fragments (scFv), fusion proteins comprising an antibody portion, and any other modified immunoglobulin molecule comprising an antigen recognition site. Furthermore, "monoclonal antibody" refers to such antibodies made in any number of ways including, but not limited to, by hybridoma, phage selection, recombinant expression, and transgenic animals (*e.g.*, expression of a human antibody in a transgenic mouse).

[00125] The term "humanized antibody" refers to an antibody derived from a non-human (*e.g.*, murine) immunoglobulin, which has been engineered to contain minimal non-human (*e.g.*, murine) sequences. Typically, humanized antibodies are human immunoglobulins in which residues from the CDRs are replaced by residues from the CDRs of a non-human species (*e.g.*, mouse, rat, rabbit, or hamster) that have the desired specificity, affinity, and capability (Jones *et al.*, 1986, *Nature*, 321:522-525; Riechmann *et al.*, 1988, *Nature*, 332:323-327; Verhoeyen *et al.*, 1988, *Science*, 239:1534-1536). In some instances, the FW residues of a human immunoglobulin are replaced with the corresponding residues in an antibody from a non-human species that has the desired specificity, and/or affinity, and/or capability.

[00126] The humanized antibody can be further modified by the substitution of additional residues either in the FW regions and/or within the replaced non-human residues to refine and optimize antibody specificity, and/or affinity, and/or capability. In general, the humanized antibody will comprise substantially all of at least one, and typically two or three, variable domains containing all or substantially all of the CDR regions that correspond to the non-human immunoglobulin whereas all or substantially all of the FW regions are those of a human immunoglobulin consensus sequence. The humanized antibody can also comprise at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin. Examples of methods used to generate humanized antibodies are described in U.S. Pat. Nos. 5,225,539 or 5,639,641.

[00127] A "variable region" of an antibody refers to the variable region of the antibody light chain or the variable region of the antibody heavy chain, either alone or in combination. The variable regions of the heavy and light chain each consist of four FW regions connected by three CDR regions. The CDRs in each chain are held together in close proximity by the FW regions and, with the CDRs from the other chain, contribute

to the formation of the antigen-binding site of antibodies. There are at least two techniques for determining CDRs: (1) an approach based on cross-species sequence variability (*i.e.*, Kabat *et al.* Sequences of Proteins of Immunological Interest, (5th ed., 1991, National Institutes of Health, Bethesda Md.)); and (2) an approach based on crystallographic studies of antigen-antibody complexes (Al-lazikani *et al.* (1997) *J. Molec. Biol.* 273:927-948)). In addition, combinations of these two approaches are sometimes used in the art to determine CDRs. The Kabat numbering system is generally used when referring to a residue in the variable domain (approximately residues 1-107 of the light chain and residues 1-113 of the heavy chain) (*e.g.*, Kabat *et al.*, Sequences of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)).

[00128] The phrases "amino acid position numbering as in Kabat," "Kabat position," and grammatical variants thereof refer to the numbering system used for heavy chain variable domains or light chain variable domains of the compilation of antibodies in Kabat *et al.*, Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991). Using this numbering system, the actual linear amino acid sequence can contain fewer or additional amino acids corresponding to a shortening of, or insertion into, a FW or CDR of the variable domain. For example, a heavy chain variable domain can include a single amino acid insert (residue 52a according to Kabat) after residue 52 of H2 and inserted residues (*e.g.*, residues 82a, 82b, and 82c, *etc.* according to Kabat) after heavy chain FW residue 82.

TABLE 1

Loop	Kabat	AbM	Chothia
L1	L24-L34	L24-L34	L24-L34
L2	L50-L56	L50-L56	L50-L56
L3	L89-L97	L89-L97	L89-L97
H1	H31-H35B	H26-H35B (Kabat Numbering)	H26-H32..34
H1	H31-H35	H26-H35 (Chothia Numbering)	H26-H32
H2	H50-H65	H50-H58	H52-H56
H3	H95-H102	H95-H102	H95-H102

[00129] The Kabat numbering of residues can be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence. Chothia refers instead to the location of the structural loops (Chothia and Lesk, J. Mol. Biol. 196:901-917 (1987)). The end of the Chothia CDR-H1 loop when numbered using the Kabat numbering convention varies between H32 and H34 depending on the length of the loop (this is because the Kabat numbering scheme places the insertions at H35A and H35B; if neither 35A nor 35B is present, the loop ends at 32; if only 35A is present, the loop ends at 33; if both 35A and 35B are present, the loop ends at 34). The AbM hypervariable regions represent a compromise between the Kabat CDRs and Chothia structural loops, and are used by Oxford Molecular's AbM antibody modeling software.

[00130] IMGT (ImMunoGeneTics) also provides a numbering system for the immunoglobulin variable regions, including the CDRs. See *e.g.*, Lefranc, M.P. *et al.*, Dev. Comp. Immunol. 27: 55-77(2003), which is herein incorporated by reference. The IMGT numbering system was based on an alignment of more than 5,000 sequences, structural data, and characterization of hypervariable loops and allows for easy comparison of the variable and CDR regions for all species. According to the IMGT numbering schema VH-CDR1 is at positions 26 to 35, VH-CDR2 is at positions 51 to 57, VH-CDR3 is at positions 93 to 102, VL-CDR1 is at positions 27 to 32, VL-CDR2 is at positions 50 to 52, and VL-CDR3 is at positions 89 to 97.

[00131] For all heavy chain constant region amino acid positions discussed in the present invention, numbering is according to the EU index first described in Edelman et al., 1969, Proc. Natl. Acad. Sci. USA 63(1):78-85, describing the amino acid sequence of myeloma protein Eu, which is the first human IgG1 sequenced. The Eu index of Edelman et al. is also set forth in Kabat et al., 1991, Sequences of Proteins of Immunological Interest, 5th Ed., United States Public Health Service, National Institutes of Health, Bethesda. Thus, the phrases "EU index as set forth in Kabat" or "EU index of Kabat" and "position ... according to the EU index as set forth in Kabat," and grammatical variants thereof refer to the residue numbering system based on the human IgG1 Eu antibody of Edelman et al. as set forth in Kabat 1991.

- [00132] The numbering system used for the variable domains (both heavy chain and light chain) and light chain constant region amino acid sequence is that set forth in Kabat 1991.
- [00133] As used herein the Fc region includes the polypeptides comprising the constant region of an antibody excluding the first constant region immunoglobulin domain. Thus Fc refers to the last two constant region immunoglobulin domains of IgA, IgD, and IgG, and the last three constant region immunoglobulin domains of IgE and IgM, and the flexible hinge N-terminal to these domains. For IgA and IgM Fc can include the J chain. For IgG, Fc comprises immunoglobulin domains Cgamma2 and Cgamma3 (Cγ2 and Cγ3) and the hinge between Cgamma1 (Cγ1) and Cgamma2 (Cγ2).
- [00134] Although the boundaries of the Fc region can vary, the human IgG heavy chain Fc region is usually defined to comprise residues C226 or P230 to its carboxyl-terminus, wherein the numbering is according to the EU index as set forth in Kabat. Fc can refer to this region in isolation, or this region in the context of an antibody, antibody fragment, or Fc fusion protein.
- [00135] Polymorphisms have been observed at a number of different positions within antibody constant regions (e.g., Fc positions, including but not limited to positions 270, 272, 312, 315, 356, and 358 as numbered by the EU index as set forth in Kabat), and thus slight differences between the presented sequence and sequences in the prior art can exist. Polymorphic forms of human immunoglobulins have been well-characterized. At present, 18 Gm allotypes are known: G1m (1, 2, 3, 17) or G1m (a, x, f, z), G2m (23) or G2m (n), G3m (5, 6, 10, 11, 13, 14, 15, 16, 21, 24, 26, 27, 28) or G3m (b1, c3, b3, b0, b3, b4, s, t, g1, c5, u, v, g5) (Lefranc, et al., The human IgG subclasses: molecular analysis of structure, function and regulation. Pergamon, Oxford, pp. 43-78 (1990); Lefranc, G. et al., 1979, Hum. Genet.: 50, 199-211). It is specifically contemplated that the antibodies of the present invention may be incorporate any allotype, isoallotype, or haplotype of any immunoglobulin gene, and are not limited to the allotype, isoallotype or haplotype of the sequences provided herein.
- [00136] The term "human antibody" means an antibody produced by a human or an antibody having an amino acid sequence corresponding to an antibody produced by a human made using any technique known in the art (e.g., recombinant expression in

cultures cells, or expression in transgenic animals). Thus, the term human antibody also encompasses an antibody having an amino acid sequence corresponding to an antibody originally produced by a human (or an engineered variant or derivative thereof) but expressed in a non-human system (e.g., produced by chemical synthesis; recombinantly expressed in microbial, mammal, or insect cells; or expressed in an animal subject). Accordingly, an antibody obtained from a human subject or from human cells (e.g., hybridoma or cell line expressing a recombinant antibody or fragment thereof) and subsequently expressed in an animal, *e.g.*, mice, is considered a human antibody. This definition of a human antibody includes intact or full-length antibodies, fragments thereof, and/or antibodies comprising at least one human heavy and/or light chain polypeptide such as, for example, an antibody comprising murine light chain and human heavy chain polypeptides.

[00137] The term "chimeric antibodies" refers to antibodies wherein the amino acid sequence of the immunoglobulin molecule is derived from two or more animal species. Typically, the variable region of both light and heavy chains corresponds to the variable region of antibodies derived from one species of mammals (*e.g.*, mouse, rat, rabbit, etc.) with the desired specificity, and/or affinity, and/or capability while the constant regions are homologous to the sequences in antibodies derived from another species (usually human) to avoid eliciting an immune response in that species.

[00138] The term "epitope" as used herein refers to an antigenic protein determinant capable of binding to an HER2 antibody or HER2 binding molecule disclosed herein. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. The part of an antibody or binding molecule that recognizes the epitope is called a paratope. The epitopes of protein antigens are divided into two categories, conformational epitopes and linear epitopes, based on their structure and interaction with the paratope. A conformational epitope is composed of discontinuous sections of the antigen's amino acid sequence. These epitopes interact with the paratope based on the 3-D surface features and shape or tertiary structure of the antigen. By contrast, linear epitopes interact with the paratope based on their

primary structure. A linear epitope is formed by a continuous sequence of amino acids from the antigen.

[00139] The term "antibody binding site" refers to a region in the antigen (*e.g.*, HER2) comprising a continuous or discontinuous site (*i.e.*, an epitope) to which a complementary antibody specifically binds. Thus, the antibody binding site can contain additional areas in the antigen which are beyond the epitope and which can determine properties such as binding affinity and/or stability, or affect properties such as antigen enzymatic activity or dimerization. Accordingly, even if two antibodies bind to the same epitope within an antigen, if the antibody molecules establish distinct intermolecular contacts with amino acids outside of the epitope, such antibodies are considered to bind to distinct antibody binding sites.

[00140] "Binding affinity" generally refers to the strength of the sum total of non-covalent interactions between a single binding site of a molecule (*e.g.*, an antibody) and its binding partner (*e.g.*, an antigen). Unless indicated otherwise, as used herein, "binding affinity" refers to intrinsic binding affinity which reflects a 1:1 interaction between members of a binding pair (*e.g.*, antibody and antigen). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (K_D). Affinity can be measured by common methods known in the art, including those described herein. Low-affinity antibodies generally bind antigen slowly and tend to dissociate readily, whereas high-affinity antibodies generally bind antigen faster and tend to remain bound longer. A variety of methods of measuring binding affinity are known in the art, any of which can be used for purposes of the present disclosure.

[00141] "Potency" is normally expressed as an IC_{50} value, in nM unless otherwise stated. IC_{50} is the median inhibitory concentration of an antigen-binding molecule. In functional assays, IC_{50} is the concentration that reduces a biological response by 50% of its maximum. In ligand-binding studies, IC_{50} is the concentration that reduces receptor binding by 50% of maximal specific binding level. IC_{50} can be calculated by any number of means known in the art. Improvement in potency can be determined by measuring, *e.g.*, against the 39S parent antibody.

[00142] The fold improvement in potency for the anti-HER2 binding molecule disclosed herein (for example, as compared to the 39S parent antibody, trastuzumab, or

combinations thereof) can be at least about 2-fold, at least about 4-fold, at least about 6-fold, at least about 8-fold, at least about 10-fold, at least about 20-fold, at least about 30-fold, at least about 40-fold, at least about 50-fold, at least about 60-fold, at least about 70-fold, at least about 80-fold, at least about 90-fold, at least about 100-fold, at least about 110-fold, at least about 120-fold, at least about 130-fold, at least about 140-fold, at least about 150-fold, at least about 160-fold, at least about 170-fold, or at least about 180-fold or more.

[00143] "Antibody-dependent cell-mediated cytotoxicity" or "ADCC" refers to a form of cytotoxicity in which secreted immunoglobulins bound onto Fc receptors (FcRs) present on certain cytotoxic cells (*e.g.*, Natural Killer (NK) cells, neutrophils, and macrophages) enable these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cell with cytotoxins. Specific high-affinity IgG antibodies directed to the surface of target cells "arm" the cytotoxic cells and are absolutely required for such killing. Lysis of the target cell is extracellular, requires direct cell-to-cell contact, and does not involve complement. It is contemplated that, in addition to antibodies, other proteins comprising Fc regions, specifically Fc fusion proteins, having the capacity to bind specifically to an antigen-bearing target cell will be able to effect cell-mediated cytotoxicity. For simplicity, the cell-mediated cytotoxicity resulting from the activity of an Fc fusion protein is also referred to herein as ADCC activity.

[00144] A polypeptide, antibody, polynucleotide, vector, cell, or composition which is "isolated" is a polypeptide, antibody, polynucleotide, vector, cell, or composition which is in a form not found in nature. Isolated polypeptides, antibodies, polynucleotides, vectors, cells or compositions include those which have been purified to a degree that they are no longer in a form in which they are found in nature. In some aspects, an antibody, polynucleotide, vector, cell, or composition which is isolated is substantially pure.

[00145] The term "subject" refers to any animal (*e.g.*, a mammal), including, but not limited to humans, non-human primates, rodents, and the like, which is to be the recipient of a particular treatment. Typically, the terms "subject" and "patient" are used interchangeably herein in reference to a human subject.

- [00146] The term "pharmaceutical composition" refers to a preparation which is in such form as to permit the biological activity of the active ingredient (*e.g.*, an anti-HER2 binding molecule disclosed herein) to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the composition would be administered. Such composition can be sterile.
- [00147] An "effective amount" of an anti-HER2 binding molecule as disclosed herein is an amount sufficient to carry out a specifically stated purpose. An "effective amount" can be determined empirically and in a routine manner, in relation to the stated purpose.
- [00148] The term "therapeutically effective amount" refers to an amount of an anti-HER2 binding molecule disclosed herein or other drug effective to "treat" a disease or disorder in a subject or mammal.
- [00149] The word "label" when used herein refers to a detectable compound or composition which is conjugated directly or indirectly to an anti-HER2 binding molecule disclosed herein so as to generate a "labeled" anti-HER2 binding molecule. The label can be detectable by itself (*e.g.*, radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, can catalyze chemical alteration of a substrate compound or composition which is detectable.
- [00150] Terms such as "derivatizable group" and "derivatizable functional group" are used interchangeably and refer to a functional group that is capable of reacting to permit the formation of a covalent bond between an anti-HER2 binding molecule disclosed herein (*e.g.*, a HER2 antibody) and another substance. In some aspects, such substance is a therapeutic moiety (*e.g.*, a cytotoxin), a detectable label, a polymer (*e.g.*, PEG), etc. Exemplary derivatizable groups include thiol, hydroxyl, amino, carboxy, and amide, as well as modified forms thereof, such as activated or protected forms.
- [00151] Terms such as "treating" or "treatment" or "to treat" or "alleviating" or "to alleviate" refer to both (1) therapeutic measures that cure, slow down, lessen symptoms of, and/or halt progression of a diagnosed pathologic condition or disorder and (2) prophylactic or preventative measures that prevent and/or slow the development of a targeted pathologic condition or disorder. Thus, those in need of treatment include those already with the disorder; those prone to have the disorder; and those in whom the disorder is to be prevented. In certain aspects, a subject is successfully "treated" for

cancer according to the methods of the present disclosure if the patient shows, *e.g.*, total, partial, or transient remission of a certain type of cancer.

[00152] The terms "cancer", "tumor", "cancerous", and "malignant" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancers include but are not limited to, carcinoma including adenocarcinomas, lymphomas, blastomas, melanomas, sarcomas, and leukemias. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, Hodgkin's and non-Hodgkin's lymphoma, pancreatic cancer, glioblastoma, glioma, cervical cancer, ovarian cancer, liver cancer such as hepatic carcinoma and hepatoma, bladder cancer, breast cancer (including hormonally mediated breast cancer, see, *e.g.*, Innes *et al.* (2006) *Br. J. Cancer* 94:1057-1065), colon cancer, colorectal cancer, endometrial carcinoma, myeloma (such as multiple myeloma), salivary gland carcinoma, kidney cancer such as renal cell carcinoma and Wilms' tumors, basal cell carcinoma, melanoma, prostate cancer, vulval cancer, thyroid cancer, testicular cancer, esophageal cancer, various types of head and neck cancer and cancers of mucinous origins, such as, mucinous ovarian cancer, cholangiocarcinoma (liver) and renal papillary carcinoma. In some aspects, the term cancer as used herein specifically refers to cancer expressing HER2. In some specific aspects, the term cancer refers to cancers expression low levels of HER2.

[00153] A "low level of HER2 " as used herein, refers to a cancer cell, subject, or patient exhibiting a score of less than 2+ (*e.g.*, 1+) when using a HERCEPT® (DakoCytomation California Inc., Carpinteria, CA) classification, or a cancer, cancer cell, subject or patient that has been identified as such, for example, by FISH.

[00154] To determine HER2 expression in the cancer, various diagnostic/prognostic assays are available. In one aspect, HER2 overexpression can be analyzed by IHC, *e.g.*, by using HERCEPT® (Dako). Paraffin embedded tissue sections from a tumor biopsy can be subjected to the IHC assay and accorded a HER2 protein staining intensity criteria as follows/ Alternatively, or additionally, FISH assays such as the INFORM™ (sold by Ventana, Ariz.) or PATHVISION™ (Vysis, Ill.) can be carried out on formalin-fixed, paraffin-embedded tumor tissue to determine the extent (if any) of HER2 overexpression in the tumor.

[00155] "Low" is a term that refers to a measure that is less than normal, less than a standard such as a predetermined measure or a subgroup measure that is relatively less than another subgroup measure. For example, low HER2 means a measure of HER2 that is less than a normal HER2 measure in a particular set of samples of patients that is HER2 positive. A normal HER2 measure can be determined according to any method available to one skilled in the art. Low HER2 can also mean a measure of HER2 that is less than a predetermined measure, such as a predetermined cutoff. Low HER2 can also mean a measure wherein a low HER2 subgroup is relatively lower than another subgroup. For example, without limitation, according to the present specification, two distinct patient subgroups can be created by dividing samples around a mathematically determined point, such as, without limitation, a median, thus creating a group whose measure is low (*i.e.*, less than the median) with respect to another group whose measure is high. HER2 can be measured by any method known to one skilled in the art such as, for example, without limitation, using the eTag method or using any standard IHC method such as HERCEPT®. As another example, low level of HER2 refers to a low level of HER2 homodimers, which means a measure of HER2 homodimers that is less than a normal measure of HER2 homodimers in a particular set of samples or patients that is HER2 positive. Low HER2 homodimers can also mean a measure that is less than a predetermined measure, such as a predetermined cutoff. Low HER2 homodimers can also mean a measure wherein a low HER2 homodimer subgroup is relatively less than another subgroup. HER2 homodimers can be measured by any method known in the art such as Fluorescence resonance energy transfer (FRET), Bioluminescent resonance energy transfer (BRET), proximity ligation assay (PLA), dimer-specific antibodies or eTag or any other method that is well known to one skilled in the art.

[00156] As used herein, the term "carcinomas" refers to cancers of epithelial cells, which are cells that cover the surface of the body, produce hormones, and make up glands. Examples of carcinomas are cancers of the skin, lung, colon, stomach, breast, prostate and thyroid gland.

[00157] "Polynucleotide," or "nucleic acid," as used interchangeably herein, refer to polymers of nucleotides of any length, and include DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their

analog, or any substrate that can be incorporated into a polymer by DNA or RNA polymerase. A polynucleotide can comprise modified nucleotides, such as methylated nucleotides and their analogs. The preceding description applies to all polynucleotides referred to herein, including RNA and DNA.

[00158] The term "vector" means a construct, which is capable of delivering, and in some aspects, expressing, one or more gene(s) or sequence(s) of interest in a host cell. Examples of vectors include, but are not limited to, viral vectors, naked DNA or RNA expression vectors, plasmid, cosmid or phage vectors, DNA or RNA expression vectors associated with cationic condensing agents, DNA or RNA expression vectors encapsulated in liposomes, and certain eukaryotic cells, such as producer cells.

[00159] The terms "polypeptide," "peptide," and "protein" are used interchangeably herein to refer to polymers of amino acids of any length. The polymer can be linear or branched, it can comprise modified amino acids, and it can be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component. Also included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), as well as other modifications known in the art. It is understood that, because the polypeptides of the instant disclosure are based upon antibodies, in certain aspects, the polypeptides can occur as single chains or associated chains.

[00160] A "recombinant" polypeptide or protein refers to a polypeptide or protein produced via recombinant DNA technology. Recombinantly produced polypeptides and proteins expressed in engineered host cells are considered isolated for the purpose of the invention, as are native or recombinant polypeptides which have been separated, fractionated, or partially or substantially purified by any suitable technique. The polypeptides disclosed herein can be recombinantly produced using methods known in the art. Alternatively, the proteins and peptides disclosed herein can be chemically synthesized.

[00161] Unless otherwise specified, the term "substituted" as used herein in the context of modifications to the chemical structure of the cytotoxic agents, i.e. tubulysins pertains to

a parent group which bears one or more substituents. The term "substituent" is used herein in the conventional sense and refers to a chemical moiety which is covalently attached to, or if appropriate, fused to, a parent group. A wide variety of substituents are well known, and methods for their formation and introduction into a variety of parent groups are also well known. Examples of chemical substituents are described in more detail below.

[00162] The phrase "optionally substituted" as used herein in the context of modifications to the chemical structure of cytotoxic agents, pertains to a parent group which may be unsubstituted or which may be substituted.

[00163] The terms "substituted," "amino acid substitution" and the like as used herein in the context of polypeptides refers to replacing an amino acid residue present in a parent polypeptide with another amino acid residue. An amino acid can be substituted in a parent polypeptide, for example, via chemical peptide synthesis or through recombinant methods known in the art. Accordingly, references to a "substitution at position X" or "substitution at position X" refer to the substitution of an amino acid present at position X with an alternative amino acid residue. In some aspects, substitution patterns can be described according to the schema AXY, wherein A is the single letter code corresponding to the amino acid naturally present at position X, and Y is the substituting amino acid residue. Accordingly, L234F would refer to the substitution of the leucine amino acid (L) at position 234 with a phenylalanine (F). In other aspects, substitution patterns can be described according to the schema XY, wherein Y is the single letter code corresponding to the amino acid residue substituting the amino acid naturally present at position X. Accordingly, 239C would refer to the substitution of the native amino acid at position 239 with a cysteine (C).

[00164] A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art, including basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine,

valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Thus, if an amino acid in a polypeptide is replaced with another amino acid from the same side chain family, the substitution is considered to be conservative. In another aspect, a string of amino acids can be conservatively replaced with a structurally similar string that differs in order and/or composition of side chain family members.

[00165] Non-conservative amino acid substitutions include those in which (i) a residue having an electropositive side chain (*e.g.*, Arg, His or Lys) is substituted for, or by, an electronegative residue (*e.g.*, Glu or Asp), (ii) a hydrophilic residue (*e.g.*, Ser or Thr) is substituted for, or by, a hydrophobic residue (*e.g.*, Ala, Leu, Ile, Phe or Val), (iii) a cysteine or proline is substituted for, or by, any other residue, or (iv) a residue having a bulky hydrophobic or aromatic side chain (*e.g.*, Val, His, Ile or Trp) is substituted for, or by, one having a smaller side chain (*e.g.*, Ala, Ser) or no side chain (*e.g.*, Gly).

[00166] Other amino acid substitutions can be readily identified by workers of ordinary skill. For example, for the amino acid alanine, a substitution can be taken from any one of D-alanine, glycine, beta-alanine, L-cysteine and D-cysteine. For lysine, a replacement can be any one of D-lysine, arginine, D-arginine, homo-arginine, methionine, D-methionine, omithine, or D- ornithine. Generally, substitutions in functionally important regions that can be expected to induce changes in the properties of isolated polypeptides are those in which (i) a polar residue, *e.g.*, serine or threonine, is substituted for (or by) a hydrophobic residue, *e.g.*, leucine, isoleucine, phenylalanine, or alanine; (ii) a cysteine residue is substituted for (or by) any other residue; (iii) a residue having an electropositive side chain, *e.g.*, lysine, arginine or histidine, is substituted for (or by) a residue having an electronegative side chain, *e.g.*, glutamic acid or aspartic acid; or (iv) a residue having a bulky side chain, *e.g.*, phenylalanine, is substituted for (or by) one not having such a side chain, *e.g.*, glycine. The likelihood that one of the foregoing non-conservative substitutions may alter functional properties of the protein is also correlated to the position of the substitution with respect to functionally important regions of the protein: some non-conservative substitutions may accordingly have little or no effect on biological properties.

[00167] The term "amino acid insertion" refers to introducing a new amino acid residue between two amino acid residues present in the parent sequence. An amino acid can be

inserted in a parent sequence, for example, via chemical peptide synthesis or through recombinant methods known in the art. Accordingly as used herein, the phrases "insertion between positions X and Y" or "insertion between Kabat positions X and Y," wherein X and Y correspond to amino acid positions (*e.g.*, a cysteine amino acid insertion between positions 239 and 240), refers to the insertion of an amino acid between the X and Y positions, and also to the insertion in a nucleic acid sequence of a codon encoding an amino acid between the codons encoding the amino acids at positions X and Y. Insertion patterns can be described according to the schema AXins, wherein A is the single letter code corresponding to the amino acid being inserted, and X is the position preceding the insertion. Accordingly, C239ins would refer to the insertion of a cysteine amino acid (C) after position 239 (*i.e.*, an insertion between position 239 and 240).

[00168] The term "percent sequence identity" between two polypeptide or polynucleotide sequences refers to the number of identical matched positions shared by the sequences over a comparison window, taking into account additions or deletions (*i.e.*, gaps) that must be introduced for optimal alignment of the two sequences. A matched position is any position where an identical nucleotide or amino acid is presented in both the target and reference sequence. Gaps presented in the target sequence are not counted since gaps are not nucleotides or amino acids. Likewise, gaps presented in the reference sequence are not counted since target sequence nucleotides or amino acids are counted, not nucleotides or amino acids from the reference sequence.

[00169] The percentage of sequence identity is calculated by determining the number of positions at which the identical amino-acid residue or nucleic acid base occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. The comparison of sequences and determination of percent sequence identity between two sequences can be accomplished using readily available software both for online use and for download. Suitable software programs are available from various sources, and for alignment of both protein and nucleotide sequences. One suitable program to determine percent sequence identity is *bl2seq*, part of the BLAST suite of program available from the U.S. government's National Center for Biotechnology Information BLAST web site

(blast.ncbi.nlm.nih.gov). BL2seq performs a comparison between two sequences using either the BLASTN or BLASTP algorithm. BLASTN is used to compare nucleic acid sequences, while BLASTP is used to compare amino acid sequences. Other suitable programs are, *e.g.*, Needle, Stretcher, Water, or Matcher, part of the EMBOSS suite of bioinformatics programs and also available from the European Bioinformatics Institute (EBI) at www.ebi.ac.uk/Tools/psa.

[00170] Different regions within a single polynucleotide or polypeptide target sequence that aligns with a polynucleotide or polypeptide reference sequence can each have their own percent sequence identity. It is noted that the percent sequence identity value is rounded to the nearest tenth. For example, 80.11, 80.12, 80.13, and 80.14 are rounded down to 80.1, while 80.15, 80.16, 80.17, 80.18, and 80.19 are rounded up to 80.2. It also is noted that the length value will always be an integer.

[00171] In certain aspects, the percentage identity "X" of a first amino acid sequence to a second sequence amino acid is calculated as $100 \times (Y/Z)$, where Y is the number of amino acid residues scored as identical matches in the alignment of the first and second sequences (as aligned by visual inspection or a particular sequence alignment program) and Z is the total number of residues in the second sequence. If the length of a first sequence is longer than the second sequence, the percent identity of the first sequence to the second sequence will be higher than the percent identity of the second sequence to the first sequence.

[00172] One skilled in the art will appreciate that the generation of a sequence alignment for the calculation of a percent sequence identity is not limited to binary sequence-sequence comparisons exclusively driven by primary sequence data. Sequence alignments can be derived from multiple sequence alignments. One suitable program to generate multiple sequence alignments is ClustalW2, available from www.clustal.org. Another suitable program is MUSCLE, available from www.drive5.com/muscle/. ClustalW2 and MUSCLE are alternatively available, *e.g.*, from the EBI.

[00173] It will also be appreciated that sequence alignments can be generated by integrating sequence data with data from heterogeneous sources such as structural data (*e.g.*, crystallographic protein structures), functional data (*e.g.*, location of mutations), or phylogenetic data. A suitable program that integrates heterogeneous data to generate a

multiple sequence alignment is T-Coffee, available at www.tcoffee.org, and alternatively available, *e.g.*, from the EBI. It will also be appreciated that the final alignment used to calculate percent sequence identity can be curated either automatically or manually.

II. Anti-HER2-binding Molecules

[00174] The present disclosure provides anti-HER2 binding molecules, *e.g.*, anti-HER2 antibodies or molecules comprising HER2-binding fragments thereof, that specifically bind HER2.

[00175] The full-length amino acid (aa) and nucleotide (nt) sequences for HER2 are known in the art (see, *e.g.*, UniProt Acc. No. P04626 for human HER2). See, *e.g.*, Yamamoto *et al.*, *Nature* 319:230-234 (1986); Coussens *et al.*, *Science* 230:1132-1139 (1985); Tal *et al.*, *Mol. Cell. Biol.* 7:2597-2601 (1987); Semba *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 82:6497-6501 (1985); King *et al.*, *Science* 229:974-976 (1985); Sarkar *et al.*, *DNA Cell Biol.* 12:611-615 (1993); Giri *et al.*, *Mol. Cell. Biol.* 25:11005-11018 (2005); Anido *et al.*, *EMBO J.* 25:3234-3244 (2006); Birrane *et al.*, *J. Biol. Chem.* 278:1399-1402 (2003); Ivancic *et al.*, *J. Biomol. NMR* 27:205-219 (2003); Cho *et al.*, *Nature* 421:756-760 (2003); Franklin *et al.*, *Cancer Cell* 5:317-328 (2004); Bostrom *et al.*, *Science* 323:1610-1614 (2009); Eigenbrot *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 107:15039-15044 (2010); Stephens *et al.*, *Nature* 431:525-526 (2004); Greenman *et al.*, *Nature* 446:153-158 (2007); all of which are herein incorporated by reference in their entireties.

[00176] In certain aspects, the anti-HER2 binding molecules are antibodies or antigen-binding fragments thereof. In some aspects, the anti-HER2 binding molecules, *e.g.*, anti-HER2 antibodies or molecules comprising HER2-binding fragments thereof, comprise a Fab, a Fab', a F(ab')₂, a Fd, a single chain Fv, scFv, disulfide stabilized scFv, a disulfide linked Fv, a V-NAR domain, an IgNar, an intrabody, an IgGΔCH2, a minibody, a F(ab')₃, a tetrabody, a triabody, a diabody, a single-domain antibody, DVD-Ig, Fcab, mAb², a (scFv)₂, or a scFv-Fc. In some aspects, the antibody is of the IgG type, for example of the IgG1 type.

[00177] In some aspects, the anti-HER2 binding molecules are monospecific. In other aspects, the anti-HER2 binding molecules are bispecific, trispecific, tetraspecific, etc. In

other aspects, the anti-HER2 binding molecules are multispecific. In some aspects, the anti-HER2 binding molecules are monovalent, bivalent, trivalent, tetravalent, etc. In yet other aspects, the anti-HER2 binding molecules are multivalent. In specific aspects, the anti-HER2 binding molecules are bivalent, *e.g.*, an antibody comprising two HER2 specific antigen binding sites. In specific aspects, the anti-HER2 binding molecules are bispecific, *i.e.*, the molecule can specifically bind to two different antigens (*e.g.*, two different epitopes on the same or different molecules). In some specific aspects, the anti-HER2 binding molecules are bivalent and tetravalent, *e.g.*, an antibody comprising four antigen-binding sites that are capable of binding to two different antigens (*e.g.*, two different epitopes on the same or different molecules).

[00178] In certain aspects, the anti-HER2 binding molecules comprise antibodies or antigen-binding fragments thereof have a binding site that is substantially the same as the binding site of the 1.39.1 antibody (see PCT Publ. No. WO 2008/019290, which is herein incorporated by reference in its entirety). In a specific aspect, the anti-HER2 binding molecules comprise antibodies or antigen-binding fragments thereof bind one or more amino acid residues of SEQ ID NO: 52. In certain aspects, the anti-HER2 binding molecules comprise antibodies or antigen-binding fragments thereof have a binding site that overlaps the binding site of the 1.39.1 antibody.

[00179] In certain aspects, the anti-HER2 binding molecules comprise antibodies or antigen-binding fragments thereof comprising a VH and/or a VL that have been modified compared to the VH (SEQ ID NO:43) and/or the VL (SEQ ID NO:44) of the parent 1.39.1 antibody (see PCT Publ. No. WO 2008/019290). The modifications introduced in the parent antibody can include mutations (*e.g.*, point mutations or replacement of an entire subsequence) in the CDR regions and/or in the FW regions of the VH and VL as compared to the parent 1.39.1 antibody.

[00180] In some aspects, the VH-CDR1 (SEQ ID NO:45) of the parent 1.39.1 antibody has been replaced with a VH-CDR1 comprising the amino acids of SEQ ID NO: 1. In some aspects, VH-CDR1 (SEQ ID NO:45) of the parent 1.39.1 antibody has been replaced with a VH-CDR1 consisting of the amino acids of SEQ ID NO: 1.

[00181] In other aspects, VH-CDR3 (SEQ ID NO:46) of the parent 1.39.1 antibody has been replaced with a VH-CDR3 comprising the amino acids of SEQ ID NO: 3. In other

aspects, VH-CDR3 (SEQ ID NO:46) of the parent 1.39.1 antibody has been replaced with a VH-CDR3 consisting of the amino acids of SEQ ID NO: 3.

[00182] In some aspects, VH-CDR1 (SEQ ID NO:45) and VH-CDR3 (SEQ ID NO:46) of the parent 1.39.1 antibody have been replaced with a VH-CDR1 and a VH-CDR3 comprising or consisting of the amino acids of SEQ ID NO: 1 and the amino acids of SEQ ID NO:3, respectively.

[00183] In some aspects, the VL-CDR1 (SEQ ID NO:47) of the parent 1.39.1 antibody has been replaced with a VL-CDR1 comprising the amino acids of SEQ ID NO:4. In some aspects, VL-CDR1 (SEQ ID NO:3) of the parent 1.39.1 antibody has been replaced with a VH-CDR1 consisting of the amino acids of SEQ ID NO: 4.

[00184] In some aspects, the VL FW1 region (SEQ ID NO:48) of the parent 1.39.1 antibody has been replaced with a VL FW1 comprising the amino acids of SEQ ID NO: 11. In some aspects, the VL FW1 region (SEQ ID NO:48) of the parent 1.39.1 antibody has been replaced with a FW1 consisting of the amino acids of SEQ ID NO: 11.

[00185] In other aspects, the VL FW2 region (SEQ ID NO:49) of the parent 1.39.1 antibody has been replaced with a FW2 comprising the amino acids of SEQ ID NO: 12. In other aspects, the VL FW2 region (SEQ ID NO:49) of the parent 1.39.1 antibody has been replaced with a FW2 consisting of the amino acids of SEQ ID NO: 12.

[00186] In other aspects, the VL FW3 region (SEQ ID NO:50) of the parent 1.39.1 antibody has been replaced with a FW3 comprising the amino acids of SEQ ID NO: 13. In other aspects, the VL FW3 region (SEQ ID NO:50) of the parent 1.39.1 antibody has been replaced with a VL FW2 consisting of the amino acids of SEQ ID NO: 13.

[00187] In some aspects, the VL FW1 region (SEQ ID NO:48) and/or the FW2 region (SEQ ID NO:49) and/or the FW3 region (SEQ ID NO:50) of the parent 1.39.1 antibody have been replaced with a FW1 and/or a FW2 and/or a FW3 independently comprising or consisting of the amino acids of SEQ ID NOS: 11, 12, or 13, respectively.

[00188] In some aspects the present disclosure provides an anti-HER2 binding molecule comprising an immunoglobulin heavy chain variable region (VH) and an immunoglobulin light chain variable region (VL), wherein the VH comprises the amino acids of SEQ ID NO:15. In some aspects, the present disclosure provides an anti-HER2 binding molecule comprising a VH and a VL, wherein the VL comprises the amino acids

of SEQ ID NO:16. In some aspects, the VH comprises the amino acids of SEQ ID NO:15 and the VL comprises the amino acids of SEQ ID NO:16. In some aspect, an anti-HER2 binding molecule disclosed herein comprises an antibody, or a HER2-binding fragment thereof.

[00189] In certain aspects, an anti-HER2 binding molecule of the instant disclosure comprises an immunoglobulin heavy chain (VH) and an immunoglobulin light chain (VL), wherein the binding molecule comprises:

- (i) VH-CDR1 comprising the amino acids of SEQ ID NO: 1;
- (ii) VH-CDR2 comprising the amino acids of SEQ ID NO: 2;
- (iii) VH-CDR3 comprising the amino acids of SEQ ID NO: 3;
- (iv) VL-CDR1 comprising the amino acids of SEQ ID NO: 4;
- (v) VL-CDR2 comprising the amino acids of SEQ ID NO: 5; and,
- (vi) VL-CDR3 comprising the amino acids of SEQ ID NO: 6.

[00190] In certain aspects, an anti-HER2 binding molecule of the instant disclosure (*e.g.*, an anti-HER2 antibody or HER2-binding fragment thereof, or a bispecific anti-HER2 antibody) comprises an antibody VL and an antibody VH, wherein the VL comprises an amino acid sequence at least about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, or about 100% identical to a reference VL comprising or consisting of the amino acids of SEQ ID NO: 16.

[00191] In other aspects, an anti-HER2 binding molecule of the instant disclosure (*e.g.*, an anti-HER2 antibody or HER2-binding fragment thereof, or a bispecific anti-HER2 antibody) comprises an antibody VL and an antibody VH, wherein the VH comprises an amino acid sequence at least about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, or about 100% identical to a reference VH comprising or consisting of the amino acids of SEQ ID NO:15.

[00192] In other aspects, an anti-HER2 binding molecule of the instant disclosure (*e.g.*, an anti-HER2 antibody or HER2-binding fragment thereof, or a bispecific anti-HER2 antibody) comprises a VL comprising a sequence at least about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, or about 100% identical to a reference VL comprising or consisting of the amino acids of SEQ ID NO:16, and further comprises a VH comprising a sequence at least about 80%, about 85%, about

90%, about 95%, about 96%, about 97%, about 98%, about 99%, or about 100% identical to a reference VH comprising or consisting of the amino acids of SEQ ID NO:15.

[00193] In some aspects, the anti-HER2 binding molecule of the instant disclosure (*e.g.*, an anti-HER2 antibody or HER2-binding fragment thereof, or a bispecific anti-HER2 antibody) comprises a heavy chain constant region or fragment thereof. In some specific aspects, the heavy chain constant region is an IgG constant region. The IgG constant region can comprise a light chain constant region selected from the group consisting of a kappa constant region and a lambda constant region.

[00194] In certain aspects, an anti-HER2 binding molecule of the instant disclosure (*e.g.*, an anti-HER2 antibody or HER2-binding fragment thereof, or a bispecific anti-HER2 antibody) can bind HER2 with substantially the same or better affinity as the 1.39.1 parent antibody. Thus, in one aspect, an anti-HER2 binding molecule of the instant disclosure (*e.g.*, an anti-HER2 antibody or HER2-binding fragment thereof, or a bispecific anti-HER2 antibody) specifically binds HER2 and antigenic fragments thereof with a dissociation constant or k_d (k_{off}/k_{on}) of less than 10^{-6} M, or of less than 10^{-7} M, or of less than 10^{-8} M, or of less than 10^{-9} M, or of less than 10^{-10} M, or of less than 10^{-11} M, or of less than 10^{-12} M, or of less than 10^{-13} M.

[00195] In another aspect, an anti-HER2 binding molecule of the instant disclosure (*e.g.*, an anti-HER2 antibody or HER2-binding fragment thereof, or a bispecific anti-HER2 antibody) binds to HER2 and/or antigenic fragments thereof with a k_{off} of less than $1 \times 10^{-3} \text{ s}^{-1}$, or less than $2 \times 10^{-3} \text{ s}^{-1}$. In other aspects, an anti-HER2 binding molecule of the instant disclosure (*e.g.*, an anti-HER2 antibody or HER2-binding fragment thereof, or a bispecific anti-HER2 antibody) binds to HER2 and antigenic fragments thereof with a k_{off} of less than 10^{-3} s^{-1} , less than $5 \times 10^{-3} \text{ s}^{-1}$, less than 10^{-4} s^{-1} , less than $5 \times 10^{-4} \text{ s}^{-1}$, less than 10^{-5} s^{-1} , less than $5 \times 10^{-5} \text{ s}^{-1}$, less than 10^{-6} s^{-1} , less than $5 \times 10^{-6} \text{ s}^{-1}$, less than less than $5 \times 10^{-7} \text{ s}^{-1}$, less than 10^{-8} s^{-1} , less than $5 \times 10^{-8} \text{ s}^{-1}$, less than 10^{-9} s^{-1} , less than $5 \times 10^{-9} \text{ s}^{-1}$, or less than 10^{-10} s^{-1} .

[00196] In another aspect, an anti-HER2 binding molecule of the instant disclosure (*e.g.*, an anti-HER2 antibody or HER2-binding fragment thereof, or a bispecific anti-HER2 antibody) binds to HER2 and/or antigenic fragments thereof with an association rate constant or k_{on} rate of at least $10^5 \text{ M}^{-1} \text{ s}^{-1}$, at least $5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, at least $10^6 \text{ M}^{-1} \text{ s}^{-1}$, at

least $5 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, at least $10^7 \text{ M}^{-1} \text{ s}^{-1}$, at least $5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, or at least $10^8 \text{ M}^{-1} \text{ s}^{-1}$, or at least $10^9 \text{ M}^{-1} \text{ s}^{-1}$.

[00197] In other aspects, the VH and/or VL amino acid sequences can be 85%, 90%, 95%, 96%, 97%, 98% or 99% similar to the sequences set forth above, and comprise 1, 2, 3, 4, 5 or more conservative substitutions. An anti-HER2 binding molecule disclosed herein having VH and VL regions having high (*i.e.*, 80% or greater) similarity to the VH region of SEQ ID NOs:16 and/or VL region of SEQ ID NOs:15, respectively, can be obtained by mutagenesis (*e.g.*, site-directed or PCR-mediated mutagenesis) of their respective encoding nucleic acid molecules, followed by testing of the altered antibody for retained function using the functional assays described herein.

[00198] The affinity and/or avidity of an anti-HER2 binding molecule disclosed herein for an antigen can be determined experimentally using any suitable method well known in the art, *e.g.*, flow cytometry, enzyme-linked immunosorbent assay (ELISA), or radioimmunoassay (RIA), or kinetics (*e.g.*, BIACORE™ analysis). Direct binding assays as well as competitive binding assay formats can also be readily employed. See, for example, Berzofsky *et al.*, "Antibody-Antigen Interactions," In Fundamental Immunology, Paul, W. E., Ed., Raven Press: New York, N.Y. (1984); Kuby, Immunology, W. H. Freeman and Company: New York, N.Y. (1992); and methods described herein.

[00199] The measured affinity of the interaction of a particular anti-HER2 binding molecule disclosed herein with a HER2 antigen can vary if measured under different conditions (*e.g.*, salt concentration, pH, temperature). Thus, measurements of affinity and other antigen-binding parameters (*e.g.*, K_D or K_d , k_{on} , k_{off}) are made with standardized solutions of anti-HER2 binding molecule and antigen, and a standardized buffer, as known in the art and such as the buffer described herein.

[00200] It also known in the art that affinities measured using BIACORE™ analysis can vary depending on which one of the reactants is bound to the chip. In this respect, affinity can be measured using a format in which the targeting anti-HER2 binding molecule is immobilized onto the chip (referred to as an "IgG down" format) or using a format in which the target protein (*e.g.*, HER2) is immobilized onto the chip (referred to as, *e.g.*, a "HER2 down" format).

III. Bispecific Anti-HER2 Binding Molecules

- [00201] The present disclosure also provides bispecific anti-HER2 antibodies comprising a first immunoglobulin antigen-binding domain and a second immunoglobulin antigen-binding domain, wherein:
- (i) the first and second immunoglobulin antigen-binding domains specifically bind to distinct HER2 antibody binding sites;
 - (ii) the first immunoglobulin antigen-binding domain binds to a first HER2 antibody binding site which comprises an epitope within domain II of HER2; and,
 - (iii) the first HER2 antibody binding site is distinct from the antibody binding site of pertuzumab.
- [00202] In some aspects, the first immunoglobulin antigen-binding domain binds to a HER2 antibody binding site which comprises an epitope within domain II of HER2. In some aspects, the first HER2 antibody binding site is identical the HER2 antibody binding site of the 1.39.1 or 39S antibodies. In some aspects, the first HER2 antibody binding site partially overlaps with the HER2 antibody binding site of the 1.39.1 or 39S antibodies. In other aspects, the first HER2 antibody binding site is distinct from the HER antibody binding site of the 1.39.1 or 39S antibodies.
- [00203] In some aspects, the second immunoglobulin antigen-binding domain binds to a second HER2 antibody binding site which comprises an epitope within domain IV of HER2. In some aspects, the second HER2 antibody binding site is identical the HER2 antibody binding site of trastuzumab. In some aspects, the second HER2 antibody binding site partially overlaps with the HER2 antibody binding site of trastuzumab. In other aspects, the second HER2 antibody binding site is distinct from the HER antibody binding site of trastuzumab.
- [00204] In some aspects, the bispecific anti-HER2 antibody comprises a comprising a first immunoglobulin antigen-binding domain and a second immunoglobulin antigen-binding domain, wherein (i) the first and second immunoglobulin antigen-binding domains specifically bind to distinct HER2 antibody binding sites, (ii) the first immunoglobulin antigen-binding domain binds to a first HER2 antibody binding site which comprises an epitope within domain II of HER2 and is distinct from the antibody binding site of

pertuzumab, and (iii) the second immunoglobulin antigen-binding domain competes with HERCEPTIN® for binding to domain IV of HER2.

[00205] The present disclosure also provides bispecific anti-HER2 molecules which bind the same epitope as or are derived from the anti-HER2 binding molecules disclosed above (*i.e.*, lead optimized antibodies derived from the 1.39.1 parent antibody, for example, the 39S antibody). In some aspects, such bispecific anti-HER2 molecules are bispecific anti-HER2 antibodies and molecules derived from such bispecific antibodies. In some aspects, such molecules derived from the bispecific anti-HER2 molecules described herein are antibody-drug conjugates (ADC). In certain aspects, the ADCs provided herein have reduced ADCC activity. In some aspects, such molecules derived from the bispecific anti-HER2 molecules described herein have enhanced ADCC activity.

[00206] The present disclosure also provides a bispecific HER antibody comprising a first immunoglobulin and a second immunoglobulin antigen-binding domain wherein (i) the first and second immunoglobulin antigen-binding domains specifically bind to distinct HER2 epitopes; and (ii) wherein the first immunoglobulin antigen-binding domain binds HER2 to one or more amino acid residues in SEQ ID NO: 52. In some aspects, the second immunoglobulin antigen-binding domain binds HER2 at an epitope within domain IV. In other aspects, the second immunoglobulin antigen-binding domain binds HER2 to one or more amino acid residues in SEQ ID NO: 53.

[00207] Accordingly, in one aspect, the instant disclosure provides bispecific anti-HER2 antibodies comprising a first immunoglobulin antigen-binding domain and a second immunoglobulin antigen-binding domain, wherein the first immunoglobulin antigen-binding domain comprises a heavy chain (HC) variable region (VH), and a light chain (LC) variable region (VL) comprising:

(i) a variable heavy chain CDR-1 (VH-CDR1) sequence identical to SEQ ID NO: 1 or identical to SEQ ID NO: 1 except for up to 1, 2, 3, or 4 amino acid substitutions;

(ii) a variable heavy chain CDR-2 (VH-CDR2) sequence identical to SEQ ID NO: 2 or identical to SEQ ID NO: 2 except for up to 1, 2, 3, or 4 amino acid substitutions;

(iii) a variable heavy chain CDR-3 (VH-CDR3) sequence identical to SEQ ID NO: 3 or identical to SEQ ID NO: 3 except for up to 1, 2, 3, or 4 amino acid substitutions;

(iv) a variable light chain CDR-1 (VL-CDR1) sequence identical to SEQ ID NO: 4 or identical to SEQ ID NO: 4 except for up to 1, 2, 3, or 4 amino acid substitutions;

(v) a variable light chain CDR-2 (VL-CDR2) sequence identical to SEQ ID NO: 5 or identical to SEQ ID NO: 5 except for up to 1, 2, 3, or 4 amino acid substitutions; and,

(vi) a variable light chain CDR-3 (VL-CDR3) sequence identical to SEQ ID NO: 6 or identical to SEQ ID NO: 6 except for up to 1, 2, 3, or 4 amino acid substitutions;

wherein the second immunoglobulin antigen-binding domain comprises an scFv antibody fragment; and, wherein the first and second immunoglobulin antigen-binding domains specifically bind to distinct HER2 epitopes.

[00208] In some aspects, the first immunoglobulin antigen-binding domain of the bispecific anti-HER2 antibody comprises at least one heterologous variable domain framework region (FW) different relative to the FW regions of an immunoglobulin antigen-binding domain comprising a VH comprising the amino acids of SEQ ID NO:43 and a VL comprising the amino acids of SEQ ID NO:44;

[00209] In some aspects, the first immunoglobulin antigen-binding domain of the bispecific anti-HER2 antibody comprises (i) a variable light chain framework 1 (VL-FW1) comprising the amino acids of SEQ ID NO:11; (ii) a variable light chain framework 2 (VL-FW2) comprising the amino acids of SEQ ID NO:12; (iii) a VL-variable light chain framework 3 (VL-FW3) comprising the amino acids of SEQ ID NO:13; (iv) a variable light chain framework 4 (VL-FW4) comprising the amino acids of SEQ ID NO:14; or (v) any combination thereof.

[00210] In some aspects, the first immunoglobulin antigen-binding domain of the bispecific HER2 antibody comprises (i) a VL-FW1 consisting of the amino acids of SEQ ID NO:11; (ii) a VL-FW2 consisting of the amino acids of SEQ ID NO:12; (iii) a VL-FW3 consisting of the amino acids of SEQ ID NO:13; (iv) a VL-FW4 consisting of the amino acids of SEQ ID NO:14; or (v) any combination thereof.

[00211] In other aspects, the bispecific anti-HER2 antibody disclosed herein comprises a first immunoglobulin antigen binding-domain and a second immunoglobulin antigen-binding domain, wherein:

(i) the first immunoglobulin antigen-binding domain comprises a VH and a VL, wherein the VH comprises the amino acids of SEQ ID NO:15 or SEQ ID NO:43;

- (ii) the second immunoglobulin antigen-binding domain comprises an scFv antibody fragment; and,
- (iii) the first and second immunoglobulin antigen-binding domains specifically bind to distinct HER2 epitopes.

[00212] In other aspects, the bispecific anti-HER2 antibody comprises a first immunoglobulin antigen binding-domain and a second immunoglobulin antigen-binding domain, wherein:

- (i) the first immunoglobulin antigen-binding domain comprises a VH and a VL, wherein the VH consists of the amino acids of SEQ ID NO:15 or SEQ ID NO:43;
- (ii) the second immunoglobulin antigen-binding domain comprises an scFv antibody fragment; and,
- (iii) the first and second immunoglobulin antigen-binding domains specifically bind to distinct HER2 epitopes.

[00213] In some aspects, the bispecific anti-HER2 antibody comprises a first immunoglobulin antigen binding-domain and a second immunoglobulin antigen-binding domain, wherein:

- (i) the first immunoglobulin antigen-binding domain comprises a VH and a VL, wherein the VL comprises the amino acids of SEQ ID NO:16 or SEQ ID NO:44;
- (ii) the second immunoglobulin antigen-binding domain comprises an scFv antibody fragment; and,
- (iii) the first and second immunoglobulin antigen-binding domains specifically bind to distinct HER2 epitopes.

[00214] In some aspects, the bispecific anti-HER2 antibody comprises a first immunoglobulin antigen binding-domain and a second immunoglobulin antigen-binding domain, wherein:

- (i) the first immunoglobulin antigen-binding domain comprises a VH and a VL, wherein the VL consists of the amino acids of SEQ ID NO:16 or SEQ ID NO:44;
- (ii) the second immunoglobulin antigen-binding domain comprises an scFv antibody fragment; and,
- (iii) the first and second immunoglobulin antigen-binding domains specifically bind to distinct HER2 epitopes.

[00215] In some aspects, the bispecific anti-HER2 antibody comprises a first immunoglobulin antigen binding-domain and a second immunoglobulin antigen-binding domain, wherein:

- (i) the first immunoglobulin antigen-binding domain comprises a VH and a VL, wherein the VL comprises the amino acids of SEQ ID NO:16 or SEQ ID NO:44, and the VH comprises the amino acids of SEQ ID NO:15 or SEQ ID NO:45;
- (ii) the second immunoglobulin antigen-binding domain comprises an scFv antibody fragment; and,
- (iii) the first and second immunoglobulin antigen-binding domains specifically bind to distinct HER2 epitopes.

[00216] In some aspects, the bispecific anti-HER2 antibody comprises a first immunoglobulin antigen binding-domain and a second immunoglobulin antigen-binding domain, wherein:

- (i) the first immunoglobulin antigen-binding domain comprises a VH and a VL, wherein the VL consists of the amino acids of SEQ ID NO:16 or SEQ ID NO:44, and the VH consists of the amino acids of SEQ ID NO:15 or SEQ ID NO: 43;
- (ii) the second immunoglobulin antigen-binding domain comprises an scFv antibody fragment; and,
- (iii) the first and second immunoglobulin antigen-binding domains specifically bind to distinct HER2 epitopes.

[00217] In some aspects, the first immunoglobulin antigen binding domain of the bispecific anti-HER2 antibody comprises or consists of:

- (a) a VH further comprising a heavy chain constant region or a fragment thereof, and a VL comprising a light chain constant region (LC) or a fragment thereof;
- (b) a single chain Fv ("scFv");
- (c) a diabody;
- (d) a minibody;
- (e) an F(ab')₂; or
- (f) an F(ab).

[00218] In some aspects, the heavy chain constant region or fragment thereof of the bispecific anti-HER2 antibody is an IgG constant region. In some aspects, the IgG

constant region or fragment thereof is an IgG1, IgG2, IgG3, or IgG4 constant region. In specific aspects, the IgG constant region is an IgG1 constant region. In some aspects, the first immunoglobulin antigen binding domain of the bispecific anti-HER2 antibody comprises a VL comprising a light chain constant region (LC), wherein the LC constant region is a kappa constant region. In some aspects, the first immunoglobulin antigen binding domain of the bispecific anti-HER2 antibody comprises a VL comprising a light chain constant region (LC), wherein the LC constant region is a lambda constant region.

[00219] In some aspects, the first immunoglobulin antigen-binding domain of the bispecific anti-HER2 antibody is, for example, a monoclonal antibody, a humanized antibody, a chimeric antibody, or an affinity optimized antibody. In some aspects, the first immunoglobulin antigen-binding domain of the bispecific anti-HER2 antibody is, for example, a human antibody. In some aspects, the human antibody is expressed in a transgenic mouse (see, for example, Bruggemann, "Human antibody expression in transgenic mice, " *Arch. Immunol. Therap. Exper.* 49: 203-208, 2001, which is herein incorporated by reference in its entirety).

[00220] In some aspects, the first immunoglobulin antigen-binding domain of the bispecific anti-HER2 antibody does not compete with trastuzumab or pertuzumab for epitope binding. In some aspects, the first and second immunoglobulin antigen binding domains of the bispecific anti-HER2 antibody specifically bind to distinct HER2 epitopes. In some aspects, the distinct HER2 epitopes are non-overlapping.

[00221] In some aspects, the second immunoglobulin antigen-binding domain of the bispecific anti-HER2 antibody (i) specifically binds to the same HER2 epitope as the trastuzumab antibody; and/or (ii) competitively inhibits HER2 binding by the trastuzumab antibody; and/or (iii) comprises at least one, at least two, at least three, at least four, at least five, or at least six complementarity determining regions (CDRs) of comprising the amino acids of any one of SEQ ID NOs: 54 to 59.

[00222] In some aspects, the second immunoglobulin antigen-binding domain of the bispecific anti-HER2 antibody comprises an scFv. In some specific aspects, the second immunoglobulin antigen-binding domain of the bispecific anti-HER2 antibody comprises a disulfide stabilized scFv (ds-scFv). In some aspects, the disulfide stabilized scFv specifically binds to the same HER2 epitope as the trastuzumab antibody.

[00223] A stabilizing disulfide can be engineered between the VH and VL regions of an scFv by introducing cysteine substitutions at positions selected such that the cysteine residues can form a disulfide bond. In particular, such a disulfide can be introduced in the framework regions such that the VL and VH regions are linked by a disulfide bond. The position of representing but not limiting residues in the VH and VL regions meeting these criteria are provided in TABLE 2.

TABLE 2: VH-VL pairs‡

VH44 + VL100	VH55 + VL101	VH101 + VL46
VH44 + VL105	VH100 + VL50	VH105 + VL43
VH45 + VL87	VH98 + VL 46	VH106 + VL57

‡ Numbering in TABLE 2 is according to the Kabat index as set forth in Kabat.. It will be understood that the wild type amino acid residue at these positions will vary. Regardless of the wild type amino acid residue each position of a given pair will be substituted with a Cysteine.

[00224] The scFv disclosed herein are obtainable from or produced by any suitable source, whether natural or not, or it may be a recombinant scFv, a synthetic scFv, a semi-synthetic scFv, a derivatized scFv, a fermentation optimized scFv, a fusion protein or equivalents, mutants and derivatives thereof as long as it retains the required binding specificity of the scFv's of the present disclosure. These include a scFv with binding specificity which has amino acid substitutions or has sugars or other molecules attached to amino acid functional groups. The term "derivative" or "derivatized" as used herein with respect to an scFv includes chemical modification of an scFv. Illustrative of such modifications would be replacement of hydrogen by an alkyl, acyl, or amino group.

[00225] In some aspects, the second immunoglobulin antigen-binding domain is an scFv comprising:

- (i) a VH-CDR1 comprising the amino acids of SEQ ID NO:54;
- (ii) a VH-CDR2 comprising the amino acids of SEQ ID NO:55;
- (iii) a VH-CDR3 comprising the amino acids of SEQ ID NO:56;

- (iv) a VL-CDR1 comprising the amino acids of SEQ ID NO:57;
- (v) a VL-CDR2 comprising the amino acids of SEQ ID NO:58; and,
- (vi) a VL-CDR3 comprising the amino acids of SEQ ID NO:59.

[00226] In some aspects, the second immunoglobulin antigen-binding domain of the bispecific anti-HER2 antibody consists of a scFv that specifically binds to the same HER2 epitope as the trastuzumab antibody and comprises VH and VL derived from the VH and VL of the trastuzumab antibody (e.g., the native VH and/or VL present in the trastuzumab antibody, or VH and/or VL mutants with stabilizing mutations, e.g., the pairs of mutations shown in TABLE 2). See Goldenger, Clin. Ther. 21:309-18 (1999). Accordingly, in some aspects, the scFv that binds the same epitope as trastuzumab comprises a VH comprising the amino acids of SEQ ID NO:17, and a VL comprising the amino acids of SEQ ID NO:18. In some aspects, the scFv that binds the same epitope as trastuzumab comprises a VH consisting of the amino acids SEQ ID NO:17, and a VL consisting of the amino acids of SEQ ID NO:18. In some specific aspects, the VH and VL of the scFv that binds the same epitope as trastuzumab are covalently linked via a peptide linker. In some aspects, the peptide linker comprises the amino acids of SEQ ID NO:19. In some aspects, the peptide linker consists of the amino acids of SEQ ID NO:19.

[00227] As discussed above, a person skilled in the art will appreciate that scFv's that bind the same epitope as trastuzumab include sequences derived from trastuzumab, comprising for example mutant sequences wherein at least one amino acid has been deleted or substituted with respect to a parent sequence in the trastuzumab antibody, as long as the resulting molecule is capable of specifically binding to the same HER2 epitope as the trastuzumab antibody, e.g., mutations designed to introduce at least one stabilizing disulfide between the VH and VL of the scFv.

[00228] In some aspects, the second immunoglobulin antigen-binding domain of the bispecific anti-HER2 antibody is covalently linked to the carboxy-terminus of the HC of the first immunoglobulin antigen-binding domain. In some aspects, the bispecific anti-HER2 antibody comprises at least one linker interposed between the second immunoglobulin antigen binding domain and the carboxy-terminus of the HC of the first immunoglobulin antigen-binding domain. In some specific aspects, one linker is

interposed between the second immunoglobulin antigen binding domain and the carboxy-terminus of the HC of the first immunoglobulin antigen-binding domain.

[00229] In some aspects, the second immunoglobulin antigen-binding domain is covalently linked to the amino-terminus of the HC of the first immunoglobulin antigen-binding domain. In some aspects, the bispecific anti-HER2 antibody comprises at least one linker interposed between the second immunoglobulin antigen-binding domain and the amino-terminus of the HC of the first immunoglobulin antigen-binding domain. In specific aspects, one linker is interposed between the second immunoglobulin antigen-binding domain and the amino-terminus of the HC of the first immunoglobulin antigen-binding domain.

[00230] In some aspects, the second immunoglobulin antigen binding domain of the bispecific anti-HER2 antibody is covalently intercalated in the sequence of the HC of the first immunoglobulin antigen-binding domain. In some aspects, the second immunoglobulin antigen binding domain of the bispecific anti-HER2 antibody is covalently intercalated between the CH1 region and CH2 region of the HC of the first immunoglobulin antigen-binding domain. In some aspects, one or more linkers connect the second immunoglobulin antigen binding domain of the bispecific anti-HER2 antibody to the CH1 region and/or the CH2 region of the HC of the first immunoglobulin antigen-binding domain.

[00231] In some specific aspects, the bispecific anti-HER2 antibody comprises (i) a linker interposed between the CH1 region of the HC of the first immunoglobulin antigen binding domain and the second immunoglobulin antigen binding domain; and (ii) a second linker interposed between the second immunoglobulin antigen binding domain and the CH2 region of the HC of the of the first immunoglobulin antigen-binding domain. In some aspects, the first linker and the second linker are identical. In some aspects, the first linker and the second linker are different. In some aspects, one or more of the linkers comprise a peptide linker. In some aspects, the peptide linker comprises at least one, at least two, at least three, at least four, at least five, at least 10, at least 15, at least 20, at least 25, or at least 30 amino acids. In some aspects, the peptide linker comprises more than 20 amino acids. In some aspects, the peptide linker comprises a peptide having the formula $\text{Ser}_x[(\text{Gly})_y\text{-Ser}_4]_z$ where x is from 0 to 1, y is from 1 to 4, and

z is from 1 to 10. In some aspects, the peptide linker comprises a sequence selected from SEQ ID NOS:19, 20, 21 or 22.

[00232] In some aspects, the bispecific anti-HER2 antibody comprises a heavy chain comprising a constant region comprising an Fc domain. In some aspects, the Fc domain comprises a CH2 region, and/or a CH3 region, and/or fragments thereof. In some specific aspects, the Fc domain comprises a CH2 region and a CH3 region. In some aspects, the Fc domain consists of a CH2 region and a CH region. In some aspects, the Fc domain is an IgG Fc domain, for example, an Fc domain from an IgG1, IgG2, IgG3, or IgG4. In some aspects, the IgG Fc domain is a human or humanized IgG Fc domain. In some aspects, the Fc domain is an IgG1 Fc domain.

[00233] In some aspects, the IgG Fc domain, for example an IgG1 Fc domain, is a native (wild type) domain. In some aspects, the native IgG1 Fc domain comprises the amino acids of SEQ ID NO: 23. In other aspects, the native IgG1 Fc domain consists of the amino acids of SEQ ID NO: 23. In other aspects, the Fc domain is a mutant IgG domain, for example, a mutant IgG1, IgG2, IgG3, or IgG4 domain. In some specific aspects, the mutant Fc domain is a mutant IgG1 Fc domain.

[00234] In some aspects, the mutant IgG domain, for example, a human or humanized IgG1 Fc domain, comprises at least one mutation capable of reducing the ADCC activity of the bispecific anti-HER2 antibody. In certain aspects, at least one mutation capable of reducing the ADCC activity of the anti-HER2 bispecific antibody is an amino acid substitution. In some aspects, the bispecific anti-HER2 antibody with reduced ADCC activity comprises at least one amino acid substitution selected from L234F, S239C, S239A, a cysteine amino acid insertion between positions 239 and 240, or any combination thereof, wherein the amino acid position numbering is according to the EU index as set forth in Kabat. Numerous mutations capable of reducing the ADCC activity of an antibody are known in the art. For example, see the mutations described in WO2012175751, WO2011149999, WO2011066501, WO2000042072, WO2011120134, which are herein incorporated by reference in their entireties. Antibodies with reduced ADCC effector function also include those with substitution of one or more of Fc region residues 238, 265, 269, 270, 297, 327 and 329 (U.S. Pat. No. 6,737,056), wherein the amino acid position numbering is according to the EU index as set forth in Kabat. Such

Fc mutants also include Fc mutants with substitutions at two or more of amino acid positions 265, 269, 270, 297 and 327, including Fc mutant with substitution of residues 265 and 297 to alanine (U.S. Pat. No. 7,332,581, wherein the amino acid position numbering is according to the EU index as set forth in Kabat).

[00235] In some aspects, the mutant IgG domain, for example, a human or humanized IgG1 Fc domain, comprises at least one mutation capable of enhancing the ADCC activity of the bispecific anti-HER2 antibody. In certain aspects, at least one mutation capable of enhancing the ADCC activity of the anti-HER2 bispecific antibody is an amino acid substitution. In some aspects, the bispecific anti-HER2 antibody with enhanced ADCC activity comprises at least one amino acid substitution selected from S239A, S239D, A330L, I332E, E333A, K334A or any combination thereof, wherein the amino acid position numbering is according to the EU index as set forth in Kabat. Additional mutations which enhance ADCC activity are known to one skilled in the art including but not limited to those exemplified in Tables 2, and 6-10 of US 6,737,056; the tables presented in Figure 41 of US 2006/024298; the tables presented in Figures 5, 12, and 15 of US 2006/235208; the tables presented in Figures 8, 9 and 10 of US 2006/0173170 and the tables presented in Figures 8, 9 and 10 of WO 09/058492 .

[00236] In some aspects, the mutant IgG1 Fc domain can comprise at least one amino acid substitution introducing a derivatizable functional group. In some aspects, the mutant IgG1 Fc domain comprises one to three amino acid substitutions introducing a derivatizable functional group. In some aspects, the derivatizable group is the sulfhydryl side chain of a cysteine amino acid. In particular aspects, the substituted amino acid or amino acids occur at accessible sites of the anti-HER2 binding molecule. By substituting those amino acid residues with cysteine, reactive thiol groups are thereby positioned at accessible sites of the anti-HER2 binding molecule and can be used to conjugate the anti-HER2 binding molecule to other moieties, such as drug moieties or linker-drug moieties, to create an immunoconjugate, as described further herein. Cysteine engineered antibodies can be generated as described, *e.g.*, in U.S. Pat. No. 7,521,541.

[00237] In some aspects, the derivatizable group is introduced at Kabat position 239, 248, 254, 258, 273, 279, 282, 284, 286, 287, 289, 297, 298, 312, 324, 326, 330, 335, 337, 339, 350, 355, 356, 359, 360, 361, 375, 383, 384, 389, 398, 400, 413, 415, 418, 422, 435, 440,

441, 442, 443 or 446, at an amino acid inserted between positions 239 and 240, or any combination thereof, wherein the amino acid position numbering is according to the EU index as set forth in Kabat. In some aspects, the amino acid or amino acid substitution introducing a derivatizable sulfhydryl group is selected from the group consisting of S239C, 248C, 254C, 258C, 273C, 279C, 282C, 284C, 286C, 287C, 289C, 297C, 298C, 312C, 324C, 326C, 330C, 335C, 337C, 339C, 350C, 355C, 356C, 359C, 360C, 361C, 375C, 383C, 384C, 389C, 398C, 400C, 413C, 415C, 418C, 422C, 435C, 440C, 441C, S442C, 443C and 446C, a cysteine amino acid insertion between positions 239 and 240, or any combination thereof, wherein the amino acid position numbering is according to the EU index as set forth in Kabat. In some aspects, the amino acid or amino acid substitution introducing a derivatizable sulfhydryl group is S239C and/or S442C.

[00238] Selectively derivatizable groups are well known in the art, such as an amino group, sulfhydryl group, pendant oxyamino, or other nucleophilic groups. Derivatizable groups can be joined to a polypeptide chain via one or more linkers. Ligands (*e.g.*, therapeutic moieties, detectable labels, half-life extending polymers, etc.) can be attached to the derivatizable groups using the appropriate attachment chemistry. This coupling chemistry can include, for example, amide, urea, thiourea, oxime, aminoacetyl amide, etc.

[00239] In some aspects, the Fc domain has an altered type of glycosylation that enhances ADCC activity. The glycosylation of the Fc region can be modified to increase or decrease effector function (see for examples, Umana et al, 1999, Nat. Biotechnol 17:176-180; Davies et al., 2001, Biotechnol Bioeng 74:288-294; Shields et al, 2002, J Biol Chem 277:26733-26740; Shinkawa et al., 2003, J Biol Chem 278:3466-3473; U.S. Pat. Nos. 6,602,684; 6,946,292; 7,064,191; 7,214,775; 7,393,683; 7,425,446; 7,504,256; U.S. Publication. Nos. 2003/0157108; 2003/0003097; 2009/0010921; POTELEAGENT™ technology (Biowa, Inc. Princeton, N.J.); GLYCOMAB™ glycosylation engineering technology (GLYCART biotechnology AG, Zurich, Switzerland)). In some aspects, the Fc domain is a hypofucosylated antibody Fc domain having reduced amounts of fucosyl residues (see for examples, U.S. Patent Application Publication No.2005/0226867). In one aspect, these antibodies with increased effector function, specifically ADCC, as generated in host cells (*e.g.*, CHO cells, Lemna minor) engineered to produce highly defucosylated antibody with over 100-fold higher ADCC compared to antibody produced

by the parental cells (e.g., Mori et al., 2004, *Biotechnol Bioeng* 88:901–908; Cox et al., 2006, *Nat Biotechnol.*, 24:1591-7). In some aspects, the Fc domain has increased bisecting GlcNAc structures (e.g., Umana et al, 1999, *Nat. Biotechnol* 17:176-180; US2009/0010921).

[00240] In some aspects, the mutant Fc domain comprises the amino acids of SEQ ID NO: 24A, 24C, 25A or 25C.

[00241] In some aspects, the mutant Fc domain comprises the amino acids of SEQ ID NO: 24B or SEQ ID NO:25B.

[00242] Also provided in the instant disclosure are bispecific anti-HER2 antibodies comprising a first and a second polypeptide chain associated with each other, wherein the first polypeptide chain is selected from:

- (1) [TZ_S]-[L₁]-[_BVH]-[_BCH]-[Fc_X]
- (2) [_BVH]-[_BCH]-[Fc_X]-[L₂]-[TZ_S]
- (3) [_BVH]-[_BCH]-[L₃]-[TZ_S]-[L₄]-[Fc_X]

wherein

TZ_S is an scFv that binds the same epitope recognized by the trastuzumab antibody;

L₁, L₂, L₃, and L₄ are peptide linkers;

Fc_X is an Fc domain;

_BVH and _BCH are the VH and CH1 regions, respectively, of an antibody capable of binding to an HER2 epitope distinct from the epitope recognized by the trastuzumab antibody.

[00243] In some aspects, the distinct epitope comprises one or more amino acids within SEQ ID NO: 52.

[00244] In some aspects, the epitope recognized by the trastuzumab antibody comprises one or more amino acid residues in SEQ ID NO: 53.

[00245] In some aspects, the second polypeptide chain comprises [_BVL]-[CL] wherein _BVL is the VL region of an antibody capable of binding to an HER2 epitope distinct from the epitope recognized by the trastuzumab antibody, and CL is an IgG light chain constant region. In some aspects, CL is selected from the group consisting of a human kappa constant region and a human lambda constant region.

[00246] In some aspects, _BVL comprises the amino acids of SEQ ID NO:16. In some aspects, _BVL comprises the amino acids of SEQ ID NO:44. In some aspects, _BVL comprises:

- (i) a variable light chain CDR-1 (VL-CDR1) identical to SEQ ID NO: 4 or identical to SEQ ID NO: 4 except for up to 1, 2, 3, or 4 amino acid substitutions;
- (ii) a variable light chain CDR-2 (VL-CDR2) identical to SEQ ID NO: 5 or identical to SEQ ID NO: 5 except for up to 1, 2, 3, or 4 amino acid substitutions; and,
- (iii) a variable light chain CDR-3 (VL-CDR3) identical to SEQ ID NO: 6 or identical to SEQ ID NO: 6 except for up to 1, 2, 3, or 4 amino acid substitutions.

[00247] In some specific aspects, CL comprises the amino acids of SEQ ID NO:27A.

[00248] In some specific aspects, CL comprises the amino acids of SEQ ID NO:27B.

[00249] In some aspects, [TZ_S] comprises:

- (i) a VH-CDR1 comprising the amino acids of SEQ ID NO:54;
- (ii) a VH-CDR2 comprising the amino acids of SEQ ID NO:55;
- (iii) a VH-CDR3 comprising the amino acids of SEQ ID NO:56;
- (iv) a VL-CDR1 comprising the amino acids of SEQ ID NO:57;
- (v) a VL-CDR2 comprising the amino acids of SEQ ID NO:58; and
- (vi) a VL-CDR3 comprising the amino acids of SEQ ID NO:59.

[00250] In some aspects, [TZ_S] is a disulfide stabilized scFv. In some aspects, [TZ_S] comprises (i) a VH comprising or consisting of the amino acids of SEQ ID NO:17, or a variant thereof, and (ii) a VL comprising or consisting of the amino acids of SEQ ID NO:18, or a variant thereof, covalently linked by a peptide linker. Numerous linkers (for example, peptide linkers) suitable for linking the VH and VL moieties of an scFv are known in the art. In some aspects, the linker is a peptide linker comprising or consisting of the amino acids of SEQ ID NO:19. In other aspects, [TZ_S] comprises or consists of the amino acids of SEQ ID NO:28.

[00251] In some aspects, a hinge polypeptide links the [_BCH] and [Fc_x]. In some aspects, the hinge polypeptide comprises or consists of the amino acids of SEQ ID NO: 26. In some aspects, the [Fc_x] comprises at least one amino acid substitution introducing a derivatizable group. In other aspects, the [Fc_x] comprises one to three amino acid substitution introducing a derivatizable group. In yet other aspects, the [Fc_x] comprises

more than three amino acid substitutions introducing a derivatizable. In some aspects, all the derivatizable groups are identical. In other aspects, at least one derivatizable group is different from the rest. In some aspects, all the derivatizable groups are different. In some aspects, the derivatizable group is a sulfhydryl group (e.g., the sulfhydryl group of a cysteine). In some aspects, the derivatizable group is protected.

[00252] In some aspects, the derivatizable group is introduced at position 239, 248, 254, 258, 273, 279, 282, 284, 286, 287, 289, 297, 298, 312, 324, 326, 330, 335, 337, 339, 350, 355, 356, 359, 360, 361, 375, 383, 384, 389, 398, 400, 413, 415, 418, 422, 435, 440, 441, 442, 443, or 446, or between positions 239 and 240, or any combination thereof, wherein the amino acid position numbering is according to the EU index as set forth in Kabat. In some aspects, the derivatizable group is a sulfhydryl group in at least one cysteine amino acid substitution comprising S239C, 248C, 254C, 258C, 273C, 279C, 282C, 284C, 286C, 287C, 289C, 297C, 298C, 312C, 324C, 326C, 330C, 335C, 337C, 339C, 350C, 355C, 356C, 359C, 360C, 361C, 375C, 383C, 384C, 389C, 398C, 400C, 413C, 415C, 418C, 422C, 435C, 440C, 441C, S442C, 443C and 446C, a cysteine amino acid insertion between positions 239 and 240, or any combination thereof, wherein the amino acid position numbering is according to the EU index as set forth in Kabat. In some aspects, the amino acid or amino acid substitution introducing a derivatizable sulfhydryl group is S239C and/or S442C. In some aspects, the amino acid or amino acid substitution introducing a derivatizable sulfhydryl group is a cysteine amino acid insertion between positions 239 and 240 and/or S442C.

[00253] In other aspects, [Fcx] comprises the amino acids of any one of SEQ ID NOs: 23, 24A, 24C, 25A or 25C.

[00254] In other aspects, [Fcx] comprises the amino acids of any one of SEQ ID NOs: 23, 24B, or 25B.

[00255] In certain aspects, [L₁], [L₂], [L₃], and [L₄] comprise the amino acids of linker sequences independently selected from the group consisting of SEQ ID NOs: 19, 20, 21, and 22. In some aspects, all the linkers are different. In some aspects, at least two of the linkers are identical. One skilled in the art would understand that the linkers can be peptide, non-peptide, or combination of peptide and non-peptide linkers. In some specific aspects, (i) [L₁] comprises or consists of the amino acids of SEQ ID NO:20; (ii) [L₂]

comprises or consists of the amino acids of SEQ ID NO:20; (iii) [L₃] comprises or consists of the amino acids of SEQ ID NO:21; and, (iv) [L₄] comprises or consists of the amino acids of SEQ ID NO:22.

[00256] In some aspects, [B_{VH}] comprises:

- (i) a variable heavy chain CDR-1 (VH-CDR1) identical to SEQ ID NO: 1 or identical to SEQ ID NO: 1 except for up to 1, 2, 3, or 4 amino acid substitutions;
- (ii) a variable heavy chain CDR-2 (VH-CDR2) identical to SEQ ID NO: 2 or identical to SEQ ID NO: 2 except for up to 1, 2, 3, or 4 amino acid substitutions; and
- (iii) a variable heavy chain CDR-3 (VH-CDR3) identical to SEQ ID NO: 3 or identical to SEQ ID NO: 3 except for up to 1, 2, 3, or 4 amino acid substitutions.

[00257] In some aspects, [B_{VH}] comprises or consists of the amino acids of SEQ ID NO:15 or SEQ ID NO: 43.

[00258] In some aspects, [B_{VL}] comprises:

- (i) a variable light chain CDR-1 (VL-CDR1) identical to SEQ ID NO: 4 or identical to SEQ ID NO: 4 except for up to 1, 2, 3, or 4 amino acid substitutions;
- (ii) a variable light chain CDR-2 (VL-CDR2) identical to SEQ ID NO: 5 or identical to SEQ ID NO: 5 except for up to 1, 2, 3, or 4 amino acid substitutions; and,
- (iii) a variable light chain CDR-3 (VL-CDR3) identical to SEQ ID NO: 6 or identical to SEQ ID NO: 6 except for up to 1, 2, 3, or 4 amino acid substitutions.

[00259] In some specific aspects, [B_{VL}] comprises or consists of the amino acids of SEQ ID NO:16 or SEQ ID NO: 44. In some aspects, [B_{CH}] comprises or consists of the amino acids of SEQ ID NO: 29.

[00260] In some specific aspects, the instant disclosure provides bispecific anti-HER2 antibodies comprising a first polypeptide chain and a second polypeptide chain, wherein (i) the first polypeptide chain comprises or consists of an amino acid sequence selected from the group consisting of SEQ ID NOs: 30, 31A, 32A, 32C, 33A, 33C, 34, 34A, 35A, 36A, 36C, 37A, 37C, 38, 38A, 39A, 40A, 40C, 41A and 41C, and (ii) the second polypeptide chain comprises or consists of the sequence of SEQ ID NO:42A or 42B, wherein the bispecific anti-HER2 antibodies are conjugated to a therapeutic moiety.

[00261] In some specific aspects, the instant disclosure provides bispecific anti-HER2 antibodies comprising a first polypeptide chain and a second polypeptide chain, wherein

(i) the first polypeptide chain comprises or consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 30, 31B, 32B, 33B, 34B, 35B, 36B, 37B, 38B, 39B, 40B, and 41B, and (ii) the second polypeptide chain comprises or consists of the sequence of SEQ ID NO: 42A or 42B, wherein bispecific anti-HER2 antibodies have enhanced ADCC activity.

IV. Antibody-Drug Conjugates (ADCs)

[00262] The instant disclosure also provides antibody-drug conjugates (ADCs) comprising at least one of the anti-HER2 binding molecules disclosed herein (*e.g.*, antibodies which bind the same epitope as or are derived from the 39S antibody or HER2-binding fragments thereof, or the bispecific anti-HER2 antibodies disclosed herein) conjugated to at least one therapeutic moiety. Accordingly, in some aspects, the ADC comprises a bispecific anti-HER2 antibody disclosed herein conjugated to at least one therapeutic moiety (*e.g.*, a cytotoxin), wherein said bispecific anti-HER2 antibody comprises (i) a first immunoglobulin antigen-binding domain and (ii) a second immunoglobulin antigen-binding domain, wherein the first immunoglobulin antigen-binding domain comprises a heavy chain (HC) variable region (VH) and a light chain (LC) variable region (VL) comprising:

- (i) a variable heavy chain CDR-1 (VH-CDR1) sequence identical to SEQ ID NO: 1 or identical to SEQ ID NO: 1 except for up to 1, 2, 3, or 4 amino acid substitutions;
 - (ii) a variable heavy chain CDR-2 (VH-CDR2) sequence identical to SEQ ID NO: 2 or identical to SEQ ID NO: 2 except for up to 1, 2, 3, or 4 amino acid substitutions;
 - (iii) a variable heavy chain CDR-3 (VH-CDR3) sequence identical to SEQ ID NO: 3 or identical to SEQ ID NO: 3 except for up to 1, 2, 3, or 4 amino acid substitutions;
 - (iv) a variable light chain CDR-1 (VL-CDR1) sequence identical to SEQ ID NO: 4 or identical to SEQ ID NO: 4 except for up to 1, 2, 3, or 4 amino acid substitutions;
 - (v) a variable light chain CDR-2 (VL-CDR2) sequence identical to SEQ ID NO: 5 or identical to SEQ ID NO: 5 except for up to 1, 2, 3, or 4 amino acid substitutions; and,
 - (vi) a variable light chain CDR-3 (VL-CDR3) sequence identical to SEQ ID NO: 6 or identical to SEQ ID NO: 6 except for up to 1, 2, 3, or 4 amino acid substitutions;
- wherein:

- (a) the second immunoglobulin antigen-binding domain comprises an scFv antibody fragment; and,
- (b) the first and second immunoglobulin antigen-binding domains specifically bind to distinct HER2 epitopes.

[00263] In some aspects, the first immunoglobulin antigen-binding domain of the ADC comprises at least one heterologous variable domain framework region (FW) different relative to the FW regions of an immunoglobulin antigen-binding domain comprising a VH comprising the amino acids of SEQ ID NO:43, and a VL comprising the amino acids of SEQ ID NO:44;

[00264] In some aspects, the ADC further comprises at least one optional spacer which can be intercalated between the side chain of an amino acid in a polypeptide chain of the anti-HER2 binding molecule (for example, an amino in the heavy chain of an anti-HER2 bispecific antibody disclosed herein) and the therapeutic moiety. In some aspects, the at least one spacer is a peptidic spacer. In other aspects, the at least one spacer is a non-peptidic spacer. In some aspects, the spacer is unstable, such an acid labile spacer (*e.g.*, a hydrazine). In other aspects, the spacer is an enzyme cleavable peptide, *e.g.*, a cleavable dipeptide. In some aspects, the spacer is uncleavable (hydrolytically stable), for example, a thioether spacer or a hindered disulfide spacer. In some aspects, the intercalated between the side chain of an amino acid in a polypeptide chain of the anti-HER2 binding molecule (for example, an amino in the heavy chain of an anti-HER2 bispecific antibody disclosed herein) and the additional therapeutic moiety is MCC (N-succinimidyl-4(maleimidomethyl) cyclohexane)).

[00265] Hydrolytically stable spacers are substantially stable in water and do not react with water at useful pH values, including but not limited to, under physiological conditions for an extended period of time. Hydrolytically unstable or degradable spacers are degradable in water or in aqueous solutions, including for example, blood.

[00266] Enzymatically unstable or degradable spacers can be degraded by one or more enzymes. By way of example only, PEG and related polymers can include degradable spacers in the polymer backbone or in the linker group between the polymer backbone and one or more of the terminal functional groups of the polymer molecule. Such degradable spacers include, but are not limited to, ester linkages formed by the reaction

of PEG carboxylic acids or activated PEG carboxylic acids with alcohol groups on a biologically active agent, wherein such ester groups generally hydrolyze under physiological conditions to release the biologically active agent. Other hydrolytically degradable spacers include but are not limited to carbonate linkages; imine linkages resulting from reaction of an amine and an aldehyde; phosphate ester linkages formed by reacting an alcohol with a phosphate group; hydrazone linkages which are a reaction product of a hydrazide and an aldehyde; acetal linkages that are the reaction product of an aldehyde and an alcohol; orthoester linkages that are the reaction product of a formate and an alcohol; peptide linkages formed by an amine group, including but not limited to, at an end of a polymer such as PEG, and a carboxyl group of a peptide; and oligonucleotide linkages formed by a phosphoramidite group, including but not limited to, at the end of a polymer, and a 5' hydroxyl group of an oligonucleotide.

[00267] In some aspects, the ADC comprises two, three, four, five, six, seven, eight, nine or ten therapeutic moieties. In some specific aspects, the ADC comprises two, three, or four therapeutic moieties. In some aspects, all therapeutic moieties are the same. In some aspects, at least one therapeutic moiety is different from the rest. In some aspects, all therapeutic moieties are different. In some aspects, all the spacers (*e.g.*, peptidic and/or non-peptidic spacers) are the same. In some aspects, at least one spacer is different from the rest. In still other aspects, all the spacers are different.

[00268] In some aspects, each therapeutic moiety is chemically conjugated to the side chain of an amino acid at a specific position in the Fc region of the anti-HER2 binding molecule (*e.g.*, a bispecific anti-HER2 antibody disclosed herein).

[00269] In some aspects, the specific positions in the Fc region are selected from the group consisting of 239, 248, 254, 258, 273, 279, 282, 284, 286, 287, 289, 297, 298, 312, 324, 326, 330, 335, 337, 339, 350, 355, 356, 359, 360, 361, 375, 383, 384, 389, 398, 400, 413, 415, 418, 422, 435, 440, 441, 442, 443, 446, an insertion between positions 239 and 240, and combinations thereof, wherein the amino acid position numbering is according to the EU index as set forth in Kabat.

[00270] In some aspects, the specific positions in the Fc region are 239, 442, or both, wherein the amino acid position numbering is according to the EU index as set forth in Kabat. In some aspects, the specific positions in the Fc region consist of 442 and an

amino acid insertion between positions 239 and 240, wherein the amino acid position numbering is according to the EU index as set forth in Kabat.

[00271] In some aspects, the amino acid side chain where the therapeutic moiety is conjugated is a sulfhydryl side chain, for example, the sulfhydryl group of a cysteine amino acid. In some aspects, at least one therapeutic moiety is chemically conjugated to the side chain of an amino acid located at a position outside of the Fc region of the anti-HER2 binding molecule (*e.g.*, a bispecific anti-HER2 antibody disclosed herein). In some aspects, all the therapeutic moieties are chemically conjugated to the side chain of an amino acid located at a position outside of the Fc region of the anti-HER2 binding molecule (*e.g.*, a bispecific anti-HER2 antibody disclosed herein). In some aspects, at least one therapeutic moiety is genetically incorporated into the polypeptide chain of the anti-HER2 binding molecule (*e.g.*, a bispecific anti-HER2 antibody disclosed herein) using recombinant techniques known in the art.

[00272] In some aspects, the therapeutic moiety comprises a cytotoxin, a radioisotope, a radioisotope, an immunomodulator, a cytokine, a lymphokine, a chemokine, a growth factor, a tumor necrosis factor, a hormone, a hormone antagonist, an enzyme, an oligonucleotide, a DNA, an RNA, an siRNA, an RNAi, a microRNA, a photoactive therapeutic agent, an anti-angiogenic agent, a pro-apoptotic agent, a peptide, a lipid, a carbohydrate, a chelating agent or combinations thereof.

[00273] In some specific aspects, the cytotoxin is an auristatin, a tubulysin, a maytansinoid or a pyrrolobenzodiazepine (PBD). In another specific aspect, the cytotoxin is tubulysin 1508.

[00274] In specific aspects, the ADC comprises a bispecific anti-HER2 antibody disclosed herein, wherein said antibody comprises:

(i) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO: 32A and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises a therapeutic moiety (*e.g.*, a tubulysin 1508 molecule) covalently linked to a cysteine amino acid at position 239, wherein the amino acid position numbering is according to the EU index as set forth in Kabat;

- (ii) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:33A and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises two therapeutic moieties (*e.g.*, two tubulysin 1508 molecules) covalently linked to cysteine amino acids respectively located at positions 239 and 442 wherein the amino acid position numbering is according to the EU index as set forth in Kabat;
- (iii) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:36A and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises a therapeutic moiety (*e.g.*, a tubulysin 1508 molecule) covalently linked to a cysteine amino acid at position 239, wherein the amino acid position numbering is according to the EU index as set forth in Kabat;
- (iv) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:37A and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises two therapeutic moieties (*e.g.*, two tubulysin 1508 molecules) covalently linked to cysteine amino acids respectively located at positions 239 and 442, wherein the amino acid position numbering is according to the EU index as set forth in Kabat;
- (v) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:40A and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises a therapeutic moiety (*e.g.*, a tubulysin 1508 molecule) covalently linked to a cysteine amino acid at position 239, wherein the amino acid position numbering is according to the EU index as set forth in Kabat; or,
- (vi) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:41A and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises two therapeutic moieties (*e.g.*, two tubulysin 1508 molecules) covalently linked to cysteine amino acids respectively located at positions 239 and 442, wherein the amino acid position numbering is according to the EU index as set forth in Kabat.

[00275] In specific aspects, the ADC comprises a bispecific anti-HER2 antibody disclosed herein, wherein said antibody comprises:

- (i) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:62 and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises a therapeutic moiety (*e.g.*, a tubulysin 1508 molecule) covalently linked to a cysteine amino acid inserted between positions 239 and 240, wherein the amino acid position numbering is according to the EU index as set forth in Kabat;
- (ii) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:33C and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises two therapeutic moieties (*e.g.*, two tubulysin 1508 molecules) covalently linked to a cysteine amino acid inserted between positions 239 and 240 and a cysteine amino acid located at position 442, wherein the amino acid position numbering is according to the EU index as set forth in Kabat;
- (iii) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:36C and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises a therapeutic moiety (*e.g.*, a tubulysin 1508 molecule) covalently linked to a cysteine amino acid inserted between positions 239 and 240, wherein the amino acid position numbering is according to the EU index as set forth in Kabat;
- (iv) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:37C and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises two therapeutic moieties (*e.g.*, two tubulysin MEDI 1508 molecules) covalently linked to a cysteine amino acid inserted between positions 239 and 240 and a cysteine amino acid located at position 442, wherein the amino acid position numbering is according to the EU index as set forth in Kabat;
- (v) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:40C and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises a therapeutic moiety (*e.g.*, a tubulysin 1508 molecule) covalently linked to a cysteine amino acid inserted

between positions 239 and 240, wherein the amino acid position numbering is according to the EU index as set forth in Kabat; or,

(vi) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:41C and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises two therapeutic moieties (*e.g.*, two tubulysin 1508 molecules) covalently linked to a cysteine amino inserted between positions 239 and 240 and a cysteine amino acid located at position 442, wherein the amino acid position numbering is according to the EU index as set forth in Kabat.

[00276] ADC molecules disclosed herein comprise at least one of the anti-HER2 binding molecules disclosed herein (*e.g.*, antibodies which bind the same epitope as or are derived from the 39S antibody or HER2-binding fragments thereof, or the bispecific anti-HER2 antibodies disclosed herein) which has been derivatized or linked (*e.g.*, chemically or recombinantly) to another molecule (*e.g.*, a peptide, small drug molecule, detectable molecule, etc.). In general, anti-HER2 antibodies or portions thereof are derivatized such that their HER2 binding is not affected adversely by the derivatization or labeling. Accordingly, the anti-HER2 antibodies and antibody portions of the instant disclosure are intended to include both intact and modified forms of the anti-HER2 binding molecules described herein. For example, an anti-HER2 binding molecule disclosed herein or HER2-binding portion thereof can be functionally linked (by chemical coupling, genetic fusion, noncovalent association, or otherwise) to one or more other molecular entities, such as a cytotoxic agent, a pharmaceutical agent, a detection agent, and/or a protein or peptide that can mediate association of the anti-HER2 binding molecule with another molecule (such as a streptavidin core region or a polyhistidine tag).

[00277] One type of derivatized molecule can be produced by crosslinking two or more molecular entities, *e.g.*, an anti-HER2 binding molecule disclosed herein and a therapeutic moiety (*e.g.*, a cytotoxin such as tubulysin 1508). Suitable crosslinkers include those that are heterobifunctional, *i.e.*, having two distinctly reactive groups separated by an appropriate spacer (*e.g.*, m-maleimidobenzoyl-N-hydroxysuccinimide ester); or homobifunctional (*e.g.*, disuccinimidyl suberate). Such crosslinkers are available, for example, from Pierce Chemical Company, Rockford, IL. Additional

bifunctional coupling agents include N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate, iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene).

[00278] Another type of derivatized molecule can be produced by incorporating a detectable label. Useful detection agents include fluorescent compounds (*e.g.*, fluorescein, fluorescein isothiocyanate, rhodamine, 5-dimethylamine-1-naphthalenesulfonyl chloride, phycoerythrin, lanthanide phosphors and the like), enzymes that are useful for detection (*e.g.*, horseradish peroxidase, β -galactosidase, luciferase, alkaline phosphatase, glucose oxidase and the like), epitopes recognized by a secondary reporter (*e.g.*, leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags, etc.). In some aspects, detectable labels can be attached by at least one spacer arm. Spacer arms can be of various lengths to reduce potential steric hindrance.

[00279] An anti-HER2 binding molecule can also be labeled with a radiolabeled amino acid. The radiolabel can be used for both diagnostic and therapeutic purposes. For instance, the radiolabel can be used to detect HER2-expressing cells by X-ray or other diagnostic techniques such as positron emission tomography (PET).

[00280] Further, the radiolabel can be used therapeutically as a toxin for HER2-expressing cells, such as those which cause unwanted immune response. Examples of labels for polypeptides include, but are not limited to, the following radioisotopes or radionuclides: ^3H , ^{14}C , ^{15}N , ^{35}S , ^{90}Y , ^{99}Tc , ^{111}In , ^{125}I and ^{131}I . In some aspects, the anti-HER2 binding molecule can be labeled with a paramagnetic, radioactive, or fluorogenic ion that is detectable upon imaging. In some aspects, the paramagnetic ion is chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) or erbium (III). In other aspects, the radioactive ion is iodine-123,

technetium-99, indium-111, rhenium-188, rhenium-186, copper-67, iodine-131, yttrium-90, iodine-125, astatine-211, and gallium-67. In other aspects, the anti-HER2 binding molecule is labeled with an X-ray imaging agent such as lanthanum (III), gold (III), lead (II), and bismuth (III). An anti-HER2 binding molecule can also be derivatized with a chemical group, for example a polymer such as polyethylene glycol (PEG), a methyl group, an ethyl group, or a carbohydrate group. These groups are useful to improve the biological characteristics of the antibody, *e.g.*, to increase serum half-life or to increase tissue binding.

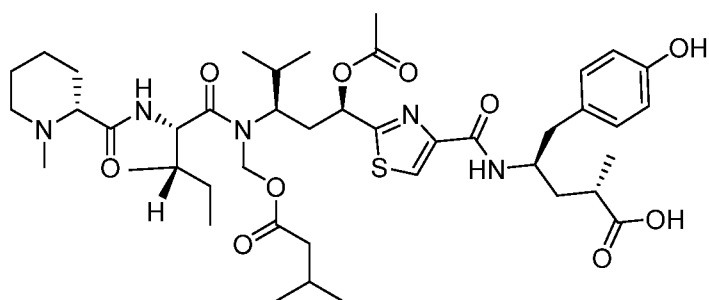
[00281] The term "cytotoxic agent" as used herein is defined broadly and refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells (cell death), and/or exerts anti-neoplastic/anti-proliferative effects. For example, cytotoxic agent prevents directly or indirectly the development, maturation, or spread of neoplastic tumor cells. The term includes also such agents that cause a cytostatic effect only and not a mere cytotoxic effect. The term includes chemotherapeutic agents as specified below, as well as other HER2 antagonists, anti-angiogenic agents, tyrosine kinase inhibitors, protein kinase A inhibitors, members of the cytokine family, radioactive isotopes, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin.

[00282] The term "chemotherapeutic agent" is a subset of the term "cytotoxic agent" comprising natural or synthetic chemical compounds. Examples of chemotherapeutic agents include alkylating agents, for example, nitrogen mustards, ethyleneimine compounds, alkyl sulphonates and other compounds with an alkylating action such as nitrosoureas, cisplatin and dacarbazine; antimetabolites, for example, folic acid, purine or pyrimidine antagonists; mitotic inhibitors, for example, *Vinca* alkaloids and derivatives of podophyllotoxin; cytotoxic antibiotics and camptothecin derivatives. Other chemotherapeutic agents are amifostine (ETHYOL®), cisplatin, dacarbazine (DTIC), dactinomycin, mechlorethamine (nitrogen mustard), streptozocin, cyclophosphamide, carmustine (BCNU), lomustine (CCNU), doxorubicin (ADRIAMYCIN®), doxorubicin lipo (DOXIL®), gemcitabine (GEMZAR®), daunorubicin, daunorubicin lipo (DAUNOXOME®), procarbazine, mitomycin, cytarabine, etoposide, methotrexate, 5-fluorouracil (5-FU), vinblastine, vincristine, bleomycin, paclitaxel (TAXOL®), docetaxel (TAXOTERE®),

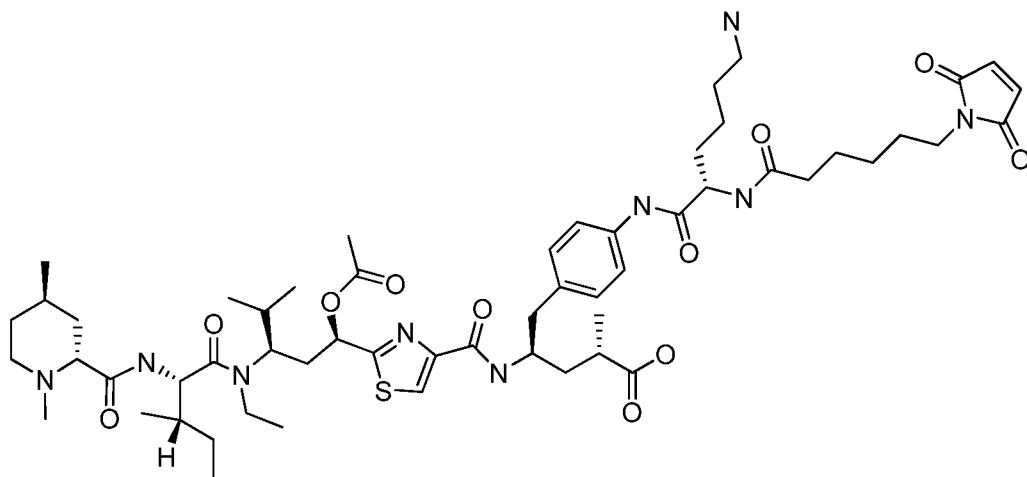
aldesleukin, asparaginase, busulfan, carboplatin, cladribine, camptothecin, CPT-11, 10-hydroxy-7-ethyl-camptothecin (SN38), gefitinib (IRESSA®), dacarbazine, floxuridine, fludarabine, hydroxyurea, ifosfamide, idarubicin, mesna, interferon alpha, interferon beta, irinotecan, mitoxantrone, topotecan, leuprolide, megestrol, melphalan, mercaptopurine, plicamycin, mitotane, pegaspargase, pentostatin, pipobroman, plicamycin, streptozocin, tamoxifen, teniposide, testolactone, thioguanine, thiotepa, uracil mustard, vinorelbine, chlorambucil aromatase inhibitors, and combinations thereof.

IV.A Tubulysins

[00283] In some aspects, the ADC comprises an anti-HER2 binding molecule disclosed herein (*e.g.*, the 39S antibody or a derivative thereof, or one of the bispecific anti-HER2 antibodies disclosed herein) conjugated to one or more tubulysin molecules (see structure of tubulysin A and tubulysin 1508 below).



Tubulysin A



Tubulysin 1508 (*compound T32*)

[00284] Tubulysins are members of a class of natural products isolated from myxobacterial species (Sasse *et al.*, J. Antibiot. 53:879-885 (2000)). As cytoskeleton interacting agents, tubulysins are mitotic poisons that inhibit tubulin polymerization and lead to cell cycle arrest and apoptosis (Steinmetz *et al.*, Chem. Int. Ed. 43:4888-4892 (2004); Khalil *et al.*, ChemBioChem. 7:678-683 (2006); Kaur *et al.*, Biochem. J. 396: 235-242 (2006)). Tubulysins are extremely potent cytotoxic molecules, exceeding the cell growth inhibition of any clinically relevant traditional chemotherapeutic, *e.g.*, epothilones, paclitaxel, and vinblastine. Furthermore, they are potent against multidrug resistant cell lines (Domling *et al.*, Mol. Diversity 9:141-147 (2005)). These compounds show high cytotoxicity tested against a panel of cancer cell lines with IC₅₀ values in the low picomolar range; thus, they are of interest as anticancer therapeutics. See, *e.g.*, WO2012019123, which is herein incorporated by reference in its entirety. Tubulysin conjugates are disclosed, *e.g.*, in U.S. Pat. No. 7,776,814.

[00285] In some aspects, the tubulysin molecule or derivative thereof is a prodrug.

IV.B Maytansine and Maytansinoids

[00286] In some aspects, the ADC comprises an anti-HER2 binding molecule disclosed herein (*e.g.*, the 39S antibody or a derivative thereof, or one of the bispecific anti-HER2 antibodies disclosed herein) conjugated to one or more maytansinoid molecules.

[00287] Maytansinoids are mitotic inhibitors which act by inhibiting tubulin polymerization. Maytansine was first isolated from the east African shrub *Maytenus serrata* (U.S. Pat. No. 3,896,111). Subsequently, it was discovered that certain microbes also produce maytansinoids, such as maytansinol and C-3 maytansinol esters (U.S. Pat. No. 4,151,042). Synthetic maytansinol and derivatives and analogues thereof are disclosed, for example, in U.S. Pat. Nos. 4,137,230; 4,248,870; 4,256,746; 4,260,608; 4,265,814; 4,294,757; 4,307,016; 4,308,268; 4,308,269; 4,309,428; 4,313,946; 4,315,929; 4,317,821; 4,322,348; 4,331,598; 4,361,650; 4,364,866; 4,424,219; 4,450,254; 4,362,663; and 4,371,533.

[00288] Maytansinoid drug moieties are attractive drug moieties in antibody drug conjugates because they are: (i) relatively accessible to prepare by fermentation or chemical modification, derivatization of fermentation products, (ii) amenable to

derivatization with functional groups suitable for conjugation through the non-disulfide linkers to antibodies, (iii) stable in plasma, and (iv) effective against a variety of tumor cell lines. Immunoconjugates containing maytansinoids, methods of making same, and their therapeutic use are disclosed, for example, in U.S. Pat. Nos. 5,208,020, 5,416,064 and European Patent EP0425235B1; Liu *et al.*, Proc. Natl. Acad. Sci. USA 93:8618-8623 (1996) (described immunoconjugates comprising a maytansinoid designated DM1); and Chari *et al.*, Cancer Research 52:127-131 (1992).

[00289] Trastuzumab emtansine (ado-trastuzumab emtansine, T-DM1, trade name KADCYLA®) is an antibody-drug conjugate consisting of the monoclonal antibody trastuzumab (HERCEPTIN®) conjugated to the maytansinoid mertansine (DM1). See, *e.g.*, LoRusso *et al.*, Clin. Cancer Res. 20:6437-47 (2011), which is herein incorporated by reference in its entirety. An engineered thio-Trastuzumab-DM1 ADC has also been described in Junutual *et al.*, Clin. Cancer Res. 16:4769-78 (2010), which is herein incorporated by reference in its entirety.

[00290] Antibody-maytansinoid conjugates are prepared by chemically linking an antibody to a maytansinoid molecule without significantly diminishing the biological activity of either the antibody or the maytansinoid molecule. See, *e.g.*, U.S. Pat. No. 5,208,020. An average of 3-4 maytansinoid molecules conjugated per antibody molecule has shown efficacy in enhancing cytotoxicity of target cells without negatively affecting the function or solubility of the antibody, although even one molecule of toxin/antibody would be expected to enhance cytotoxicity over the use of naked antibody. Maytansinoids are well known in the art and can be synthesized by known techniques or isolated from natural sources. Suitable maytansinoids are disclosed, for example, in U.S. Pat. No. 5,208,020.

[00291] In some aspects, the maytansinoid molecule, variant, or derivative thereof is a prodrug.

IV.C Auristatins and Dolastatins

[00292] In some aspects, the ADC comprises an anti-HER2 binding molecule disclosed herein (*e.g.*, the 39S antibody or a derivative thereof, or one of the bispecific anti-HER2 antibodies disclosed herein) conjugated to dolastatins or dolostatin peptidic analogs and

derivatives, the auristatins (U.S. Pat. Nos. 5,635,483; 5,780,588). Dolastatins and auristatins have been shown to interfere with microtubule dynamics, GTP hydrolysis, and nuclear and cellular division (Woyke *et al.*, Antimicrob. Agents and Chemother. 45:3580-3584 (2001)) and have anticancer activity (U.S. Pat. No. 5,663,149). The dolastatin or auristatin drug moiety can be attached to the antibody through the N (amino) terminus or the C (carboxyl) terminus of the peptidic drug moiety (See, *e.g.*, WO2002088172).

[00293] In some aspects, the auristatine or dolastatine molecule, variant, or derivative thereof is a prodrug.

IV.D Calicheamicin

[00294] In some aspects, the ADC comprises an anti-HER2 binding molecule disclosed herein (*e.g.*, the 39S antibody or a derivative thereof, or one of the bispecific anti-HER2 antibodies disclosed herein) conjugated to one or more calicheamicin molecules. Members of the calicheamicin family of antibiotics are capable of producing double-stranded DNA breaks at sub-picomolar concentrations. The calicheamicins are a class of enediyne antibiotics derived from the bacterium *Micromonospora echinospora*, with calicheamicin γ 1 being the most notable. Other calicheamicins are β 1Br, γ 1Br, α 2I, α 3I, β 1I, γ 1I, and Δ 1I. See Lee *et al.*, Journal of Antibiotics 42(7):1070–87 (1989). For the preparation of conjugates of the calicheamicin family, see U.S. Pat. Nos. 5,712,374, 5,714,586, 5,739,116, 5,767,285, 5,770,701, 5,770,710, 5,773,001, 5,877,296. Structural analogues of calicheamicin which can be used include, but are not limited to, γ 1I, α 2I, α 3I, N-acetyl- γ 1I, PSAG and θ 11 (Hinman *et al.*, Cancer Research 53:3336-3342 (1993), Lode *et al.*, Cancer Research 58:2925-2928 (1998) and the aforementioned U.S. patents to American Cyanamid).

[00295] In some aspects, the calicheamicin molecule, variant, or derivative thereof is a prodrug.

IV.E Duocarmycins

[00296] In some aspects, the ADC comprises an anti-HER2 binding molecule disclosed herein (*e.g.*, the 39S antibody or a derivative thereof, or one of the bispecific anti-HER2

antibodies disclosed herein) conjugated to one or more duocarmycin molecules. Duocarmycins are members of a series of related natural products first isolated from *Streptomyces* bacteria and they are potent antitumor antibiotics. *See* Boger. (1991). Chemtracts: Organic Chemistry 4 (5): 329–349 (1991); Tercel *et al.*, Chem. Int. Ed. Engl. 52(21):5442-6 (2013); Boger & Douglas, Proc. Natl. Acad. Sci. USA 92(9): 3642–3649 (1995); Cacciari *et al.*, Expert Opinion on Therapeutic Patents 10(12):1853-71 (2000).

[00297] Natural duocarmycins include duocarmycin A, duocarmycin B1, duocarmycin B2, duocarmycin C1, duocarmycin C2, duocarmycin D, duocarmycin SA, and CC-1065. Synthetic analogs include adozelesin, bizelesin, and carzelesin (U-80244).

[00298] In some aspects, the duocarmycin molecule, variant, or derivative thereof is a prodrug.

IV.F Pyrrolobenzodiazepine

[00299] In some aspects, the drug is a pyrrolobenzodiazepine (PBD). PBDs are relatively small molecules and some have the ability to recognize and covalently bind to specific sequences in the minor groove of DNA and thus exhibit antibiotic/antitumor activity. A number of PBDs and derivatives thereof are known in the art, for example, PBD dimers (e.g., SJG-136 or SG2000), C2-unsaturated PBD dimers, pyrrolobenzodiazepine dimers bearing C2 aryl substitutions (e.g., SG2285), PBD dimer pro-drug that is activated by hydrolysis (e.g., SG2285), and polypyrrole-PBD (e.g., SG2274). PBDs are further described WO 2000/012507, WO 2007/039752, WO 2005/110423, WO 2005/085251, and WO 2005/040170, and U.S. Pat. No. 7,612,062, each of which is incorporated by reference herein in its entirety.

IV.G Other Cytotoxic Agents

[00300] In some aspects, the ADC comprises an anti-HER2 binding molecule disclosed herein (e.g., the 39S antibody or a derivative thereof, or one of the bispecific anti-HER2 antibodies disclosed herein) conjugated to other antitumor agents, for example, BCNU, anthracyclines (e.g., daunomycin or adriamycin), taxenes (e.g., paclitaxel), streptozocin, *Vinca* alkaloids (e.g., vincristine), 5-fluorouracil, the family of agents known collectively as LL-E33288 complex (see U.S. Pat. Nos. 5,053,394, and 5,770,710), esperamicins (see

U.S. Pat. No. 5,877,296), . The ADC can also comprise an anti-HER2 binding molecule disclosed herein (*e.g.*, the 39S antibody or a derivative thereof, or one of the bispecific anti-HER2 antibodies disclosed herein) conjugated to enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, *Phytolacca americana* proteins (PAPI, PAPII, and PAP-S), *Momordica charantia* inhibitor, curcin, crotin, *Saponaria officinalis* inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. See, for example, WO199321232. In some aspects, the cytotoxic agent is a light activated drug.

V. Anti-HER2 Binding Molecules that Bind to the Same Epitope as the 39S Antibody

[00301] In another aspect, the present disclosure provides anti-HER2 binding molecules that bind to the same epitope(s) as do the 39S antibody (*e.g.*, antibodies derived from the 39S antibody or antigen-binding fragments thereof, bispecific anti-HER2 antibodies, or ADCs).

[00302] Such anti-HER2 binding molecules that bind to the same epitope as the 39S antibody can be identified based on their ability to cross-compete (*e.g.*, to competitively inhibit the binding of, in a statistically significant manner) with the 39S antibody in standard HER2 binding assays. Accordingly, in one aspect, the instant disclosure provides anti-HER2 binding molecules (*e.g.*, antibodies derived from the 39S antibody or antigen-binding fragments thereof, bispecific anti-HER2 antibodies, or ADCs) that compete for binding to HER2 with the 39S antibody antigen-binding fragments thereof. The ability of a test antibody to inhibit the binding of, *e.g.*, the 39S antibody or binding molecules derived from the 39S antibody (*e.g.*, bispecific antibodies or ADCs) demonstrates that the test antibody can compete with the 39S antibody for binding to HER2; such anti-HER2 binding molecule can, according to non-limiting theory, bind to the same or a related (*e.g.*, a structurally similar or spatially proximal) epitope on HER2 as the 39S antibody or antigen-binding fragment thereof with which it competes.

[00303] In one aspect, the anti-HER2 molecule that binds to the same epitope on HER2 as the 39S antibody or binding molecules derived from the 39S antibody is a human

monoclonal antibody or an antigen-binding fragment thereof, a bispecific anti-HER2 antibody (*e.g.*, a bispecific antibody comprising two HER2 binding regions, at least one of which recognizes the same epitope as the 39S antibody), or an ADC (*e.g.*, an ADC comprising at least one antigen-binding moiety recognizing the same epitope as the 39S; or an ADC comprising a bispecific antibody which comprises two HER2 binding regions, at least one of which recognizes the same epitope as the 39S antibody).

VI. Mechanisms of Action of anti-HER2 Binding Molecules

[00304] The present disclosure provides anti-HER2 binding molecules (*e.g.*, bispecific anti-HER2 antibodies, or ADCs) comprising a HER2-binding domain binding the same epitope as or derived from the 1.39.1 or 39S antibodies, wherein such anti-HER2-binding molecules induce internalization upon binding to the HER2 target. Also provided are anti-HER2 binding molecules (*e.g.*, bispecific anti-HER2 antibodies, or ADCs) comprising a HER2-binding domain binding the same epitope as or derived from the 1.39.1 or 39S antibodies, wherein such anti-HER2-binding molecules promote effective lysosomal trafficking following internalization. The present disclosure also provides anti-HER2 binding molecules (*e.g.*, bispecific anti-HER2 antibodies, or ADCs) comprising a HER2-binding domain binding the same epitope as or derived from the 1.39.1 or 39S antibodies, wherein such anti-HER2-binding molecules induce HER2 target internalization and/or degradation.

[00305] In some aspects, an anti-HER2 binding molecule disclosed herein can reduce, block, or suppress HER2 phosphorylation. In other aspects, an anti-HER2 binding molecule disclosed herein can reduce, block, or suppress ligand-induced AKT phosphorylation. In some aspects, the anti-HER2 binding molecule can reduce, block, or suppress ligand-induced AKT phosphorylation in low HER2-expressing cancer cells.

[00306] In still other aspects, an anti-HER2 binding molecule, *e.g.*, an anti-HER2 antibody or antigen-binding fragment thereof disclosed in the instant application can reduce, disrupt, or suppress ligand-induced HER2-HER3 dimerization.

[00307] In some aspects, an ADC comprising an anti-HER2 binding molecule disclosed herein can inhibit cancer stem cell (CSC) sphere formation and/or proliferation. In some aspects, an anti-HER2 binding molecule disclosed herein exhibits a cytotoxic effect on

CSCs. In some aspects, an ADC comprising an anti-HER2 binding molecule disclosed herein can inhibit tumor growth and/or induce tumor regression in tumors expressing low levels of HER2 (e.g., +1 to +2 via HercepTest). In certain aspects, an ADC comprising an anti-HER2 binding molecule disclosed herein can inhibit tumor growth and/or induce tumor regression in tumors resistant to T-DM1.

[00308] In some aspects, an HER2 binding molecule, *e.g.*, an anti-HER2 antibody or antigen-binding fragment thereof lacks ADCC activity. In specific aspects, an anti-HER2 binding molecule, *e.g.*, an anti-HER2 antibody or antigen-binding fragment thereof can reduce or suppress HER2 phosphorylation, AKT phosphorylation, and/or tumor colony formation via a ligand-independent mechanism of action.

VII. Preparation of Anti-HER2 Binding Molecules

[00309] Anti-HER2 binding molecules of the present disclosure (for example, antibodies or antigen-binding fragments thereof which bind the same epitope as or are derived from the 1.39.1 or 39S antibodies, bispecific antibodies and ADCs comprising the same) can be prepared according to methods known in the art. For example, anti-HER2 binding molecules binding to the same epitope as the 39S antibody disclosed herein can be generated using hybridoma methods, such as those described by Kohler & Milstein (1975) Nature 256:495.

[00310] Using the hybridoma method, a mouse, hamster, or other appropriate host animal, is immunized as described above to elicit the production by lymphocytes of antibodies that will specifically bind to an immunizing antigen. Lymphocytes can also be immunized *in vitro*. Following immunization, the lymphocytes are isolated and fused with a suitable myeloma cell line using, for example, polyethylene glycol, to form hybridoma cells that can then be selected away from unfused lymphocytes and myeloma cells. Hybridomas that produce monoclonal antibodies directed specifically against a chosen antigen as determined by immunoprecipitation, immunoblotting, or by an *in vitro* binding assay (*e.g.* radioimmunoassay (RIA); enzyme-linked immunosorbent assay (ELISA)) can then be propagated either in *in vitro* culture using standard methods (Goding, Monoclonal Antibodies: Principles and Practice, Academic Press, 1986) or *in vivo* as ascites tumors in an animal. The monoclonal antibodies can then be purified from the culture medium or ascites fluid as described for polyclonal antibodies above.

[00311] Anti-HER2 binding molecules of the present disclosure (for example, antibodies or antigen-binding fragments thereof which bind the same epitope as or are derived from the 1.39.1 or 39S antibodies, bispecific antibodies and ADCs comprising the same) can also be made using recombinant DNA methods as described in U.S. Pat. No. 4,816,567. The polynucleotides encoding a monoclonal antibody are isolated from mature B-cells or hybridoma cell, such as by RT-PCR using oligonucleotide primers that specifically amplify the genes encoding the heavy and light chains of the antibody, and their sequence is determined using conventional procedures. The isolated polynucleotides encoding the heavy and light chains are then cloned into suitable expression vectors, which when transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, monoclonal antibodies are generated by the host cells. Also, recombinant anti-HER2 monoclonal antibodies or molecules comprising antigen-binding fragments thereof of the desired species can be isolated from phage display libraries expressing CDRs of the desired species as described (McCafferty *et al.*, Nature 348:552-554 (1990); Clarkson *et al.*, Nature 352:624-628 (1991); and Marks *et al.*, J. Mol. Biol. 222:581-597 (1991)).

[00312] The polynucleotide(s) encoding an anti-HER2 binding molecule of the present disclosure (for example, antibodies or antigen-binding fragments thereof which bind the same epitope as or are derived from the 39S antibody, bispecific antibodies and ADCs comprising the same) can further be modified in a number of different manners using recombinant DNA technology to generate alternative anti-HER2 binding molecules. In some aspects, the constant domains of the light and heavy chains of, for example, a mouse monoclonal antibody can be substituted (1) for those regions of, for example, a human antibody to generate a chimeric antibody or (2) for a non-immunoglobulin polypeptide to generate a fusion antibody. In some aspects, the constant regions are truncated or removed to generate the desired antibody fragment of a monoclonal antibody. Site-directed or high-density mutagenesis of the variable region can be used to optimize specificity, affinity, etc. of a monoclonal antibody.

[00313] In certain aspects, the anti-HER2 binding molecule of the present disclosure is a human antibody or antigen-binding fragment thereof. Human antibodies can be directly prepared using various techniques known in the art. Immortalized human B lymphocytes

immunized *in vitro* or isolated from an immunized individual that produce an antibody directed against a target antigen can be generated (See, *e.g.*, Cole *et al.*, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985); Boemer *et al.*, J. Immunol. 147:86-95 (1991); and U.S. Pat. No. 5,750,373). One or more cDNAs encoding the antibody in the immortalized B lymphocyte can then be prepared and inserted into an expression vector and/or a heterologous host cell for expression of a non-naturally-occurring recombinant version of the antibody.

[00314] Also, the anti-HER2 human antibody or antigen-binding fragment thereof can be selected from a phage library, where that phage library expresses human antibodies or fragments thereof as fusion proteins with heterologous phage proteins, as described, for example, in Vaughan *et al.*, Nat. Biotech. 14:309-314 (1996); Sheets *et al.*, Proc. Natl. Acad. Sci. 95:6157-6162 (1998); Hoogenboom and Winter, J. Mol. Biol. 227:381 (1991), and Marks *et al.*, J. Mol. Biol. 222:581 (1991)). Techniques for the generation and use of antibody phage libraries are also described in U.S. Pat. Nos. 5,969,108, 6,172,197, 5,885,793, 6,521,404; 6,544,731; 6,555,313; 6,582,915; 6,593,081; 6,300,064; 6,653,068; 6,706,484; and 7,264,963, each of which is incorporated by reference in its entirety.

[00315] Affinity maturation strategies and chain shuffling strategies (Marks *et al.*, BioTechnology 10:779-783 (1992), incorporated by reference in its entirety) are known in the art and can be employed to generate high affinity human antibodies or antigen-binding fragments thereof.

[00316] In some aspects, an anti-HER2 binding molecule of the present disclosure can be a humanized antibody. Methods for engineering, humanizing or resurfacing non-human or human antibodies can also be used and are well known in the art. A humanized, resurfaced or similarly engineered antibody can have one or more amino acid residues from a source that is non-human, *e.g.*, but not limited to, mouse, rat, rabbit, non-human primate or other mammal. These non-human amino acid residues are replaced by residues that are often referred to as "import" residues, which are typically taken from an "import" variable, constant or other domain of a known human sequence. Such imported sequences can be used to reduce immunogenicity or reduce, enhance or modify binding, affinity, on-rate, off-rate, avidity, specificity, half-life, or any other suitable characteristic, as known in the art. In general, the CDR residues are directly and most substantially involved in

influencing HER2 binding. Accordingly, part or all of the non-human or human CDR sequences are maintained while the non-human sequences of the variable and constant regions can be replaced with human or other amino acids. In certain aspects, human CDRs are inserted into non-human antibody scaffolds to make an antibody with reduced immunogenicity in an animal model system, *e.g.*, a "murinized" antibody.

[00317] Anti-HER2 binding molecules, *e.g.*, antibodies, can optionally be humanized, resurfaced, or engineered with retention of high affinity for the antigen HER2 and other favorable biological properties. To achieve this goal, humanized (or human) or engineered anti-HER2 antibodies and resurfaced antibodies can be optionally prepared by a process of analysis of the parental sequences and various conceptual humanized and engineered products using three-dimensional models of the parental, engineered, and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art.

[00318] Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, *i.e.*, the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen, such as HER2. In this way, framework residues can be selected and combined from the consensus and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved.

[00319] Humanization, resurfacing or engineering of the anti-HER2 binding molecules disclosed herein can be performed using any known method, such as but not limited to those described in, Jones *et al.*, Nature 321:522 (1986); Riechmann *et al.*, Nature 332:323 (1988); Verhoeyen *et al.*, Science 239:1534 (1988)), Sims *et al.*, J. Immunol. 151: 2296 (1993); Chothia and Lesk, J. Mol. Biol. 196:901 (1987), Carter *et al.*, Proc. Natl. Acad. Sci. U.S.A. 89:4285 (1992); Presta *et al.*, J. Immunol. 151:2623 (1993), U.S. Pat. Nos. 5,639,641, 5,723,323; 5,976,862; 5,824,514; 5,817,483; 5,814,476; 5,763,192; 5,723,323; 5,766,886; 5,714,352; 6,204,023; 6,180,370; 5,693,762; 5,530,101; 5,585,089; 5,225,539; 4,816,567, 7,557,189; 7,538,195; and 7,342,110; WO90/14443; WO90/14424;

WO90/14430; and EP229246, each of which is entirely incorporated herein by reference, including the references cited therein.

[00320] In certain aspects an anti-HER2 antibody fragment is provided. Various techniques are known for the production of antibody fragments. Traditionally, these fragments are derived via proteolytic digestion of intact antibodies (for example Morimoto *et al.*, J. Biochem. Biophys. Methods 24:107-117 (1993); Brennan *et al.*, Science, 229:81 (1985)). In certain aspects, anti-HER2 antibody fragments are produced recombinantly. Fab, Fv, and scFv antibody fragments can all be expressed in and secreted from *E. coli* or other host cells, thus allowing the production of large amounts of these fragments. Such anti-HER2 antibody fragments can also be isolated from the antibody phage libraries discussed above. The anti-HER2 antibody fragments can also be linear antibodies as described in U.S. Pat. No. 5,641,870. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner.

[00321] Techniques can be adapted for the production of single-chain antibodies specific to the same HER2 epitope as the 39S antibody (see, *e.g.*, U.S. Pat. No. 4,946,778). In addition, methods can be adapted for the construction of Fab expression libraries (see, *e.g.*, Huse *et al.*, Science 246:1275-1281 (1989)) to allow rapid and effective identification of monoclonal Fab fragments with the desired specificity for HER2, or derivatives, fragments, analogs or homologs thereof. Antibody fragments can be produced by techniques in the art including, but not limited to: (a) a F(ab')₂ fragment produced by pepsin digestion of an antibody molecule; (b) a Fab fragment generated by reducing the disulfide bridges of an F(ab')₂ fragment, (c) a Fab fragment generated by the treatment of the antibody molecule with papain and a reducing agent, and (d) Fv fragments.

[00322] In some aspects, especially in the case of antibody fragments, an anti-HER2 antibody or antigen-binding fragment thereof can be modified in order to increase its serum half-life. This can be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody or antibody fragment by mutation of the appropriate region in the antibody or antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody or antibody fragment at either end or in the middle (*e.g.*, by DNA or peptide synthesis), or by YTE mutation. Other methods to increase the

serum half-life of an antibody or antigen-binding fragment thereof, *e.g.*, conjugation to a heterologous molecule such as PEG are well known in the art.

[00323] Heteroconjugate anti-HER2 binding molecules, *e.g.*, bispecific antibodies which bind the same epitope as or are derived from the 39S antibody disclosed herein or ADCs, can be prepared using recombinant biology technology as well as *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, bispecific antibodies or ADCs can be chemically constructed using a disulfide exchange reaction or by forming a thioether bond. Suitable reagents for this purpose are known in the art, and include iminothiolate and methyl-4-mercaptobutyrimidate.

[00324] It will be noted that in certain aspects, the anti-HER2 binding molecules can be engineered to fuse the CH3 domain directly to the hinge region of the respective modified antibodies or fragments thereof. In other constructs, a peptide spacer can be inserted between the hinge region and the modified CH2 and/or CH3 domains. For example, compatible constructs can be expressed wherein the CH2 domain has been deleted and the remaining CH3 domain (modified or unmodified) is joined to the hinge region with a 5-20 amino acid spacer. Such a spacer can be added, for instance, to ensure that the regulatory elements of the constant domain remain free and accessible or that the hinge region remains flexible. However, it should be noted that amino acid spacers can, in some cases, prove to be immunogenic and elicit an unwanted immune response against the construct. Accordingly, in certain aspects, any spacer added to the construct will be relatively non-immunogenic, or even omitted altogether, so as to maintain the desired biochemical qualities of the modified antibodies.

[00325] Besides the deletion of whole constant region domains, it will be appreciated that the anti-HER2 binding molecule can be provided by the partial deletion or substitution of a few or even a single amino acid. For example, the mutation of a single amino acid in selected areas of the CH2 domain can be enough to substantially reduce Fc binding and thereby increase tumor localization. Moreover, as alluded to above, the constant regions of the disclosed anti-HER2 binding molecule can be modified through the mutation or substitution of one or more amino acids that enhances the profile of the resulting construct. In this respect it is possible to disrupt the activity provided by a conserved

binding site (*e.g.*, Fc binding) while substantially maintaining the configuration and immunogenic profile of the modified antibody or antigen-binding fragment thereof. Certain aspects can comprise the addition of one or more amino acids to the constant region to enhance desirable characteristics such as decreasing or increasing effector function or provide for more cytotoxin or carbohydrate attachment. In such aspects, specific sequences derived from selected constant region domains can be inserted or replicated.

VIII. Polynucleotides Encoding HER2-Binding Molecules

[00326] In certain aspects, the present disclosure provides polynucleotides comprising nucleic acid sequences that encode an anti-HER2 binding molecule disclosed herein that specifically binds HER2. For example, the instant disclosure provides a polynucleotide comprising a nucleic acid sequence that encodes an anti-HER2 binding molecule such as an antibody or a fragment thereof (*e.g.*, a molecule which binds the same epitope as or is derived from the 39S antibody). The polynucleotides of the instant disclosure can be in the form of RNA or in the form of DNA. DNA includes cDNA, genomic DNA, and synthetic DNA; and can be double-stranded or single-stranded, and if single stranded can be the coding strand or non-coding (anti-sense) strand. In certain aspects the DNA is a cDNA that is used to produce a non-naturally-occurring recombinant antibody.

[00327] In certain aspects, the polynucleotides are isolated. In certain aspects, the polynucleotides are substantially pure. In certain aspects the polynucleotides comprise the coding sequence for the mature polypeptide fused in the same reading frame to a polynucleotide (either natural or heterologous) which aids, for example, in expression and secretion of a polypeptide from a host cell (*e.g.*, a leader sequence which functions as a secretory sequence for controlling transport of a polypeptide from the cell). The polypeptide having a leader sequence is a preprotein and can have the leader sequence cleaved by the host cell to form the mature form of the polypeptide. The polynucleotides can also encode for an anti-HER2 binding molecule proprotein which is the mature protein plus additional 5' amino acid residues. In certain aspects, the polynucleotides are altered to optimize codon usage for a certain host cell.

[00328] In certain aspects the polynucleotides comprise the coding sequence for the mature anti-HER2 binding molecule, *e.g.*, an anti-HER2 antibody or an antigen-binding

fragment thereof fused in the same reading frame to a heterologous marker sequence that allows, for example, for purification of the encoded polypeptide. For example, the marker sequence can be a hexa-histidine (His6) tag supplied, for example, by a pQE-9 vector to provide for purification of the mature polypeptide fused to the marker in the case of a bacterial host. In other aspects, the marker sequence can be a hemagglutinin (HA) tag derived, for example, from the influenza hemagglutinin protein, when a mammalian host (*e.g.*, COS-7 cells) is used.

[00329] The present disclosure further relates to variants of the described polynucleotides encoding, for example, HER2-binding fragments, analogs, and derivatives of the anti-HER2 binding molecules of the present disclosure.

[00330] The polynucleotide variants can contain alterations in the coding regions, non-coding regions, or both. In some aspects the polynucleotide variants contain alterations which produce silent substitutions, additions, or deletions, but do not alter the properties or activities of the encoded polypeptide. In some aspects, nucleotide variants are produced by silent substitutions due to the degeneracy of the genetic code. Polynucleotide variants can be produced for a variety of reasons, *e.g.*, to optimize codon expression for a particular host (change codons in the human mRNA to those preferred by a bacterial host such as *E. coli*). Vectors and cells comprising the polynucleotides described herein are also provided.

[00331] In some aspects a DNA sequence encoding an anti-HER2 binding molecule, *e.g.*, an anti-HER2 antibody or an antigen-binding fragment thereof can be constructed by chemical synthesis, for example, using an oligonucleotide synthesizer. Such oligonucleotides can be designed based on the amino acid sequence of the desired polypeptide and selecting those codons that are favored in the host cell in which the recombinant polypeptide of interest will be produced. Standard methods can be applied to synthesize an isolated polynucleotide sequence encoding an isolated polypeptide of interest. For example, a complete amino acid sequence can be used to construct a back-translated gene. Further, a DNA oligomer containing a nucleotide sequence coding for the particular isolated polypeptide can be synthesized. For example, several small oligonucleotides coding for portions of the desired polypeptide can be synthesized and

then ligated. The individual oligonucleotides typically contain 5' or 3' overhangs for complementary assembly.

[00332] Once assembled (by synthesis, site-directed mutagenesis or another method), the polynucleotide sequences encoding a particular isolated polypeptide of interest will be inserted into an expression vector and operatively linked to an expression control sequence appropriate for expression of the protein in a desired host. Proper assembly can be confirmed, for example, by nucleotide sequencing, restriction mapping, and expression of a biologically active polypeptide in a suitable host. As is well known in the art, in order to obtain high expression levels of a transfected gene in a host, the gene must be operatively linked to transcriptional and translational expression control sequences that are functional in the chosen expression host.

[00333] In certain aspects, recombinant expression vectors are used to amplify and express DNA encoding anti-HER2 binding molecules. Recombinant expression vectors are replicable DNA constructs which have synthetic or cDNA-derived DNA fragments encoding, for example, a polypeptide chain of an anti-HER2 antibody or and antigen-binding fragment thereof, operatively linked to suitable transcriptional or translational regulatory elements derived from mammalian, microbial, viral or insect genes. A transcriptional unit generally comprises an assembly of (1) a genetic element or elements having a regulatory role in gene expression, for example, transcriptional promoters or enhancers, (2) a structural or coding sequence which is transcribed into mRNA and translated into protein, and (3) appropriate transcription and translation initiation and termination sequences, as described in detail below. Such regulatory elements can include an operator sequence to control transcription.

[00334] The ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants can additionally be incorporated. DNA regions are operatively linked when they are functionally related to each other. For example, DNA for a signal peptide (secretory leader) is operatively linked to DNA for a polypeptide if it is expressed as a precursor which participates in the secretion of the polypeptide; a promoter is operatively linked to a coding sequence if it controls the transcription of the sequence; or a ribosome binding site is operatively linked to a coding sequence if it is positioned so as to permit translation. Structural elements

intended for use in yeast expression systems include a leader sequence enabling extracellular secretion of translated protein by a host cell. Alternatively, where recombinant protein is expressed without a leader or transport sequence, it can include an N-terminal methionine residue. This residue can optionally be subsequently cleaved from the expressed recombinant protein to provide a final product.

[00335] The choice of expression control sequence and expression vector will depend upon the choice of host. A wide variety of expression host/vector combinations can be employed. Useful expression vectors for eukaryotic hosts, include, for example, vectors comprising expression control sequences from SV40, bovine papilloma virus, adenovirus and cytomegalovirus. Useful expression vectors for bacterial hosts include known bacterial plasmids, such as plasmids from *E. coli*, including pCR 1, pBR322, pMB9 and their derivatives, wider host range plasmids, such as M13 and filamentous single-stranded DNA phages.

[00336] Suitable host cells for expression of anti-HER2 binding molecules, *e.g.*, anti-HER2 antibodies or antigen-binding fragments thereof, include prokaryotes, yeast, insect or higher eukaryotic cells under the control of appropriate promoters. Prokaryotes include gram negative or gram positive organisms, for example *E. coli* or bacilli. Higher eukaryotic cells include established cell lines of mammalian origin as described below. Cell-free translation systems could also be employed. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described by Pouwels *et al.* (Cloning Vectors: A Laboratory Manual, Elsevier, N.Y., 1985), the relevant disclosure of which is hereby incorporated by reference.

[00337] Additional information regarding methods of protein production, including antibody production, can be found, *e.g.*, in U.S. Publ. No. 2008/0187954, U.S. Patent Nos. 6,413,746 and 6,660,501, and Int'l Pat. Publ. No. WO 04009823, each of which is hereby incorporated by reference in its entirety.

[00338] Various mammalian or insect cell culture systems can also be advantageously employed to express recombinant anti-HER2 binding molecules, *e.g.*, anti-HER2 antibodies or antigen-binding fragments thereof. Expression of recombinant proteins in mammalian cells can be performed because such proteins are generally correctly folded, appropriately modified and completely functional. Examples of suitable mammalian host

cell lines include HEK-293 and HEK-293T, the COS-7 lines of monkey kidney cells, described by Gluzman (Cell 23:175, 1981), and other cell lines including, for example, L cells, C127, 3T3, Chinese hamster ovary (CHO), NSO, HeLa, and BHK cell lines. Mammalian expression vectors can comprise nontranscribed elements such as an origin of replication, a suitable promoter and enhancer linked to the gene to be expressed, and other 5' or 3' flanking nontranscribed sequences, and 5' or 3' nontranslated sequences, such as necessary ribosome binding sites, a polyadenylation site, splice donor and acceptor sites, and transcriptional termination sequences. Baculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow & Summers, BioTechnology 6:47 (1988).

[00339] Anti-HER2 binding molecules, *e.g.*, anti-HER2 antibodies or antigen-binding fragments thereof, produced by a transformed host can be purified according to any suitable method. Such standard methods include, for example, chromatography (*e.g.*, ion exchange, affinity and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for protein purification. Affinity tags such as hexahistidine, maltose binding domain, influenza coat sequence, glutathione-S-transferase, etc., can be attached to the protein to allow easy purification by passage over an appropriate affinity column. Isolated proteins can also be physically characterized using, for example, proteolysis, nuclear magnetic resonance or x-ray crystallography.

[00340] For example, supernatants from systems which secrete recombinant protein into culture media can be first concentrated using a commercially available protein concentration filter, for example, an AMICON® or Millipore PELLICON® ultrafiltration unit. Following the concentration step, the concentrate can be applied to a suitable purification matrix. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed.

[00341] Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Finally, one or more reversed-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, *e.g.*, silica gel having pendant methyl or other aliphatic groups, can be employed

to further purify an HER2-binding molecule. Some or all of the foregoing purification steps, in various combinations, can also be employed to provide a homogeneous recombinant protein.

[00342] A recombinant anti-HER2 binding molecule, *e.g.*, an anti-HER2 antibody or antigen-binding fragment thereof, produced in bacterial culture can be isolated, for example, by initial extraction from cell pellets, followed by one or more concentration, salting-out, aqueous ion exchange or size exclusion chromatography steps. High performance liquid chromatography (HPLC) can be employed for final purification steps. Microbial cells employed in expression of a recombinant protein can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

[00343] Methods known in the art for purifying antibodies and other proteins also include, for example, those described in U.S. Pat. Publ. Nos. US20080312425, US20080177048, and US20090187005, each of which is hereby incorporated by reference in its entirety.

IX. Treatment Methods Using Therapeutic Anti-HER2 Binding Molecules

[00344] The present disclosure also provides methods directed to the use of anti-HER2 binding molecules, *e.g.*, antibodies, including antigen-binding fragments, variants, and derivatives thereof, to treat patients having a disease associated with HER2 expression or HER2-expressing cells.

[00345] By "HER2-expressing cell" is meant a cell expressing the HER2 protein. Methods for detecting and/or quantitating HER2 expression in cells are well known in the art and include, but are not limited to, PCR techniques, immunohistochemistry (*e.g.*, HERCEPTTM), flow cytometry, Western blot, ELISA, and the like. In some aspects, the methods disclosed herein are applied to treatment and diagnostic method where the cancer cells are expressing HER2 at low levels.

[00346] The methods for diagnosis and treatment of various diseases and disorders with an anti-HER2 binding molecule disclosed herein, refer to anti-HER2 antibodies (*e.g.*, the 39S antibody, variants, derivatives, and HER2 -binding fragments; bispecific anti-HER2 molecules of the instant disclosure; and ADC molecules of the instant disclosure) that retain the desired properties of the anti-HER2 binding molecules of the instant disclosure, *e.g.*, capable of specifically binding HER2 and neutralizing HER2 activity.

- [00347] In some aspects, the anti-HER2 binding molecules are human or humanized anti-HER2 binding molecules that mediate human ADCC; or comprise known anti-HER2 antibodies that mediate ADCC; or comprise anti-HER2 binding molecules that are engineered such that they mediate ADCC.
- [00348] In some aspects, the anti-HER2 binding molecules are human or humanized anti-HER2 binding molecules that do not mediate human ADCC; or comprise known anti-HER2 antibodies that do not mediate ADCC; or comprise anti-HER2 binding molecules that are engineered such that they do not mediate ADCC.
- [00349] In one aspect, treatment includes the application or administration of an anti-HER2 binding molecule, *e.g.*, an antibody or antigen binding fragment, variant, or derivative thereof of the current disclosure to a subject or patient, or application or administration of the anti-HER2 binding molecule to an isolated tissue or cell line from a subject or patient, where the subject or patient has a disease, a symptom of a disease, or a predisposition toward a disease. In another aspect, treatment is also intended to include the application or administration of a pharmaceutical composition comprising the anti-HER2 binding molecule, *e.g.*, an antibody or antigen binding fragment, variant, or derivative thereof of the current disclosure to a subject or patient, or application or administration of a pharmaceutical composition comprising the anti-HER2 binding molecule to an isolated tissue or cell line from a subject or patient, who has a disease, a symptom of a disease, or a predisposition toward a disease.
- [00350] The anti-HER2 binding molecules, *e.g.*, antibodies or antigen-binding fragments, variants, or derivatives thereof of the present disclosure are useful for the treatment of various cancers. In one aspect, the instant disclosure relates to anti-HER2 binding molecules, *e.g.*, antibodies or antigen-binding fragments, variants, or derivatives thereof for use as a medicament, in particular for use in the treatment or prophylaxis of cancer. Examples of cancer include, but are not limited to breast cancer, colon cancer, lung cancer, gastric cancer, head and neck squamous cells cancer, melanoma, pancreatic cancer, or prostate cancer. In some specific cases, the cancer expresses low levels of HER2 as determined, for example, via HERCEPTEST™.
- [00351] In accordance with the methods of the present disclosure, at least one anti-HER2 binding molecule, *e.g.*, an antibody or antigen binding fragment, variant, or derivative

thereof as defined elsewhere herein is used to promote a positive therapeutic response with respect to cancer. The term "positive therapeutic response" with respect to cancer treatment refers to an improvement in the disease in association with the activity of these anti-HER2 binding molecules, *e.g.*, antibodies or antigen-binding fragments, variants, or derivatives thereof, and/or an improvement in the symptoms associated with the disease.

[00352] For example, an improvement in the disease can be characterized as a complete response. The term "complete response" refers to an absence of clinically detectable disease with normalization of any previously test results. Alternatively, an improvement in the disease can be categorized as being a partial response. A "positive therapeutic response" encompasses a reduction or inhibition of the progression and/or duration of cancer, the reduction or amelioration of the severity of cancer, and/or the amelioration of one or more symptoms thereof resulting from the administration of an anti-HER2 binding molecule of the instant disclosure.

[00353] In specific aspects, such terms refer to one, two or three or more results following the administration of anti-HER2 binding molecules of the instant disclosure:

- (1) a stabilization, reduction or elimination of the cancer cell population;
- (2) a stabilization or reduction in cancer growth;
- (3) an impairment in the formation of cancer;
- (4) eradication, removal, or control of primary, regional and/or metastatic cancer;
- (5) a reduction in mortality;
- (6) an increase in disease-free, relapse-free, progression-free, and/or overall survival, duration, or rate;
- (7) an increase in the response rate, the durability of response, or number of patients who respond or are in remission;
- (8) a decrease in hospitalization rate,
- (9) a decrease in hospitalization lengths,
- (10) the size of the cancer (*e.g.*, in volume) is maintained and does not increase or increases by less than 10%, preferably less than 5%, preferably less than 4%, preferably less than 2%, and
- (11) an increase in the number of patients in remission.

(12) a decrease in the number of adjuvant therapies (*e.g.*, chemotherapy or hormonal therapy) that would otherwise be required to treat the cancer.

[00354] Clinical response can be assessed using screening techniques such as PET, magnetic resonance imaging (MRI) scan, x-radiographic imaging, computed tomographic (CT) scan, flow cytometry or fluorescence-activated cell sorter (FACS) analysis, histology, gross pathology, and blood chemistry, including but not limited to changes detectable by ELISA, RIA, chromatography, and the like. In addition to these positive therapeutic responses, the subject undergoing therapy with the anti-HER2 binding molecule, *e.g.*, an antibody or antigen-binding fragment, variant, or derivative thereof, can experience the beneficial effect of an improvement in the symptoms associated with the disease.

[00355] The anti-HER2 binding molecules, *e.g.*, antibodies or antigen-binding fragments, variants, or derivatives thereof of the instant disclosure can be used in combination with any known therapies for cancer, including any agent or combination of agents that are known to be useful, or which have been used or are currently in use, for treatment of cancer, *e.g.*, colon cancer, lung cancer, gastric cancer, head and neck squamous cells cancer, and breast cancer. The second agent or combination of agents of the pharmaceutical combination formulation or dosing regimen preferably has complementary activities to anti-HER2 binding molecule(s) of the instant disclosure such that they do not adversely affect each other.

[00356] Anticancer agents include drugs used to treat malignancies, such as cancerous growths. Drug therapy can be used alone, or in combination with other treatments such as surgery or radiation therapy. Several classes of drugs can be used in cancer treatment, depending on the nature of the organ involved. For example, breast cancers are commonly stimulated by estrogens, and can be treated with drugs which inactivate the sex hormones. Similarly, prostate cancer can be treated with drugs that inactivate androgens, the male sex hormone.

[00357] Anti-cancer agents for use in certain methods of the present disclosure include, among others, antibodies (*e.g.*, antibodies which bind IGF-1R, antibodies which bind EGFR, antibodies which bind HER2 or HER3), small molecules targeting IGF1R, small molecules targeting EGFR, small molecules targeting HER2, antimetabolites, alkylating

agents, topoisomerase inhibitors, microtubule targeting agents, kinase inhibitors, protein synthesis inhibitors, immunotherapeutic agents, hormonal therapies, glucocorticoids, aromatase inhibitors, mTOR inhibitors, chemotherapeutic agents, protein kinase B inhibitors, phosphatidylinositol 3-kinase (PI3K) inhibitors, cyclin dependent kinase (CDK) inhibitors, RLR9, CD289, enzyme inhibitors, anti-TRAIL, MEK inhibitors, etc.

[00358] In specific aspects, the anti-HER2 binding molecules disclosed herein, *e.g.*, antibodies or antigen-binding fragments thereof, can be administered in combination with other antibodies or antibody fragments targeting epidermal growth factor receptor (EGFR), *e.g.* Erbitux® (cetuximab) or panitumumab (VECTIBIX®).

[00359] In other aspects, the anti-HER2 binding molecules disclosed herein can be administered in combination with kinase inhibitors, *e.g.*, tyrosine kinase inhibitors. In some other specific aspects, the anti-HER2 binding molecules disclosed herein can be administered in combination with inhibitors of the tyrosine kinase activity associated with EGFR and/or HER2/neu, *e.g.*, lapatinib. In some aspects, the anti-HER2 binding molecules of the instant disclosure can be administered in combination with antimitotic agents. In some specific aspects, the anti-HER2 binding molecules of the instant disclosure can be administered in combination with agents that stabilize the mitotic spindle microtubule assembly, *e.g.*, paclitaxel or docetaxel.

[00360] Where the combined therapies comprise administration of an anti-HER2 binding molecule in combination with administration of another therapeutic agent, the methods of the instant disclosure encompass co-administration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order. In some aspects, the anti-HER2 binding molecules described herein are administered in combination with other drugs, wherein the antibody or antigen-binding fragment, variant, or derivative thereof and the therapeutic agent(s) can be administered sequentially, in either order, or simultaneously (*i.e.*, concurrently or within the same time frame).

[00361] The combination therapy can provide "synergy" and prove "synergistic", *i.e.*, the effect achieved when the active ingredients used together is greater than the sum of the effects that results from using the compounds separately. A synergistic effect can be attained when the active ingredients are: (1) co-formulated and administered or delivered simultaneously in a combined, unit dosage formulation; (2) delivered by alternation or in

parallel as separate formulations; or (3) by some other regimen. When delivered in alternation therapy, a synergistic effect can be attained when the compounds are administered or delivered sequentially, *e.g.*, by different injections in separate syringes. In general, during alternation therapy, an effective dosage of each active ingredient is administered sequentially, *i.e.*, serially, whereas in combination therapy, effective dosages of two or more active ingredients are administered together.

[00362] In some aspects, the anti-HER2 binding molecule, *e.g.*, an anti-HER2 antibody or antigen binding fragment thereof of the instant disclosure, can be administered in a synergistic combination with a growth factor receptor (EGFR) inhibitor. In some aspects, the EGFR inhibitor is an antibody. In specific aspects, the EGFR inhibitor antibody is ERBITUX® (cetuximab) or VECTIBIX® (panitumumab). In specific aspects, the anti-HER2 binding molecules of the instant disclosure, *e.g.*, antibodies or antigen-binding fragments thereof, can be administered in a synergistic combination with inhibitors of the tyrosine kinase activity associated with EGFR and/or HER2/neu, *e.g.*, lapatinib. In some aspects, the anti-HER2 binding molecules of the instant disclosure can be administered in a synergistic combination with an antimetabolic agent. In some specific aspects the antimetabolic agent stabilizes the mitotic spindle microtubule assembly. In some specific aspects, the antimetabolic agent is paclitaxel or docetaxel.

[00363] A further aspect is the use of anti-HER2 binding molecules of the instant disclosure, *e.g.*, antibodies or antigen-binding fragments, variants, or derivatives thereof, for diagnostic monitoring of protein levels in tissue as part of a clinical testing procedure, *e.g.*, to determine the efficacy of a given treatment regimen. For example, detection can be facilitated by coupling the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β -galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material

includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin; and examples of suitable radioactive material include ^{125}I , ^{131}I , ^{35}S , or ^3H .

[00364] A further aspect is the use of anti-HER2 binding molecules of the instant disclosure, *e.g.*, antibodies or antigen-binding fragments, variants, or derivatives thereof, to treat a cancer patient resistant to a HER2-targeting therapeutic agent, for example an antibody targeting an epitope within domain IV of HER2 such as trastuzumab. In some aspects, the therapeutic agent comprises a moiety targeting the same epitope as trastuzumab, and a cytotoxic moiety. In some aspects, such cytotoxic moiety is a maytansinoid. In some specific aspects, the maytansinoid is DM-1.

[00365] In some aspects, the methods of treatment disclosed herein comprise the administration of an anti-HER2 binding molecule which is an anti-HER2 antibody comprising a heavy chain (HC) variable region (VH) and a light chain (LC) variable region (VL) comprising:

- (i) a variable heavy chain CDR-1 (VH-CDR1) identical to SEQ ID NO: 1 or identical to SEQ ID NO: 1 except for up to 1, 2, 3, or 4 amino acid substitutions;
- (ii) a variable heavy chain CDR-2 (VH-CDR2) identical to SEQ ID NO: 2 or identical to SEQ ID NO: 2 except for up to 1, 2, 3, or 4 amino acid substitutions;
- (iii) a variable heavy chain CDR-3 (VH-CDR3) identical to SEQ ID NO: 3 or identical to SEQ ID NO: 3 except for up to 1, 2, 3, or 4 amino acid substitutions;
- (iv) a variable light chain CDR-1 (VL-CDR1) identical to SEQ ID NO: 4 or identical to SEQ ID NO: 4 except for up to 1, 2, 3, or 4 amino acid substitutions;
- (v) a variable light chain CDR-2 (VL-CDR2) identical to SEQ ID NO: 5 or identical to SEQ ID NO: 5 except for up to 1, 2, 3, or 4 amino acid substitutions; and,
- (vi) a variable light chain CDR-3 (VL-CDR3) identical to SEQ ID NO: 6 or identical to SEQ ID NO: 6 except for up to 1, 2, 3, or 4 amino acid substitutions.

[00366] In some aspects, the methods of treatment disclosed herein comprise the administration of an anti-HER2 binding molecule which is a bispecific anti-HER2 antibody comprising a first immunoglobulin antigen-binding domain and a second immunoglobulin antigen-binding domain, wherein (i) the first and second immunoglobulin antigen-binding domains specifically bind to distinct HER2 antibody binding sites, (ii) the first immunoglobulin antigen-binding domain binds to a first HER2

antibody binding site which comprises an epitope within domain II of HER2, and (iii) the first HER2 antibody binding site is distinct from the antibody binding site of pertuzumab.

[00367] In some aspects, the methods of treatment disclosed herein comprise the administration of an anti-HER2 binding molecule which is a bispecific anti-HER2 antibody comprising a first immunoglobulin antigen-binding domain and a second immunoglobulin antigen-binding domain, wherein the first immunoglobulin antigen-binding domain comprises a heavy chain (HC) variable region (VH) and a light chain (LC) variable region (VL) comprising:

- (i) a variable heavy chain CDR-1 (VH-CDR1) identical to SEQ ID NO: 1 or identical to SEQ ID NO: 1 except for up to 1, 2, 3, or 4 amino acid substitutions;
- (ii) a variable heavy chain CDR-2 (VH-CDR2) identical to SEQ ID NO: 2 or identical to SEQ ID NO: 2 except for up to 1, 2, 3, or 4 amino acid substitutions;
- (iii) a variable heavy chain CDR-3 (VH-CDR3) identical to SEQ ID NO: 3 or identical to SEQ ID NO: 3 except for up to 1, 2, 3, or 4 amino acid substitutions;
- (iv) a variable light chain CDR-1 (VL-CDR1) identical to SEQ ID NO: 4 or identical to SEQ ID NO: 4 except for up to 1, 2, 3, or 4 amino acid substitutions;
- (v) a variable light chain CDR-2 (VL-CDR2) identical to SEQ ID NO: 5 or identical to SEQ ID NO: 5 except for up to 1, 2, 3, or 4 amino acid substitutions; and,
- (vi) a variable light chain CDR-3 (VL-CDR3) identical to SEQ ID NO: 6 or identical to SEQ ID NO: 6 except for up to 1, 2, 3, or 4 amino acid substitutions.

[00368] In some aspects, the first immunoglobulin antigen-binding domain of such anti-HER2 binding molecule comprises at least one heterologous variable domain framework region (FW) different relative to the FW regions of an immunoglobulin antigen-binding domain comprising a VH comprising the amino acids SEQ ID NO:43 and a VL comprising the amino acids of SEQ ID NO:44. In some aspects, the first immunoglobulin antigen-binding domain comprises:

- (i) a variable light chain framework 1 (VL-FW1) comprising the amino acids of SEQ ID NO:11;
- (ii) a VL-FW2 comprising the amino acids of SEQ ID NO:12;
- (iii) a VL-FW3 comprising the amino acids of SEQ ID NO:13;
- (iv) a VL-FW4 comprising the amino acids of SEQ ID NO:14; or

(vi) any combination thereof.

[00369] In some aspects, the first immunoglobulin antigen-binding domain of such anti-HER2 binding molecule comprises a VH and a VL, wherein the VH comprises the amino acids of SEQ ID NO:15 or 43; and wherein the first and second immunoglobulin antigen-binding domains specifically bind to distinct HER2 epitopes. In some aspects, the first immunoglobulin antigen-binding domain of such anti-HER2 binding molecule a VH and a VL, wherein the VL comprises the amino acids of SEQ ID NO:16 or 44; and wherein the first and second immunoglobulin antigen-binding domains specifically bind to distinct HER2 epitopes. In some aspects, the first immunoglobulin antigen-binding domain of such anti-HER2 binding molecule comprises a VH and a VL, wherein the VH comprises the amino acids of SEQ ID NO:15; and wherein the VL comprises the amino acids of SEQ ID NO: 16.

[00370] In some aspects, the second immunoglobulin antigen-binding domain of such anti-HER2 binding molecule (a) specifically binds to the same HER2 epitope as the trastuzumab antibody; and/or (b) competitively inhibits HER2 binding by the trastuzumab antibody; and/or (c) comprises at least one, at least two, at least three, at least four, at least five, or at least six complementarity determining regions (CDRs) comprising the amino acids of any one of SEQ ID NOs: 54 to 59. In some aspects, the second immunoglobulin antigen-binding domain of such anti-HER2 binding molecule is an scFv comprising (i) a VH-CDR1 comprising the amino acids of SEQ ID NO:54; (ii) a VH-CDR2 comprising the amino acids of SEQ ID NO:55; (iii) a VH-CDR3 comprising the amino acids of SEQ ID NO:56; (iv) a VL-CDR1 comprising the amino acids of SEQ ID NO:57; (v) a VL-CDR2 comprising the amino acids of SEQ ID NO:58; and (vi) a VL-CDR3 comprising the amino acids of SEQ ID NO:59.

[00371] In other aspects, the methods of treatment disclosed herein comprise the administration of an anti-HER2 binding molecule which is an ADC comprising a bispecific anti-HER2 antibody, wherein said antibody comprises:

(i) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO: 32A and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises a therapeutic moiety (*e.g.*, a tubulysin 1508 molecule) covalently linked to a cysteine amino acid at position 239,

wherein the amino acid position numbering is according to the EU index as set forth in Kabat;

(ii) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:33A and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises two therapeutic moieties (*e.g.*, two tubulysin 1508 molecules) covalently linked to cysteine amino acids respectively located at positions 239 and 442 wherein the amino acid position numbering is according to the EU index as set forth in Kabat;

(iii) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:36A and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises a therapeutic moiety (*e.g.*, a tubulysin 1508 molecule) covalently linked to a cysteine amino acid at position 239, wherein the amino acid position numbering is according to the EU index as set forth in Kabat;

(iv) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:37A and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises two therapeutic moieties (*e.g.*, two tubulysin 1508 molecules) covalently linked to cysteine amino acids respectively located at positions 239 and 442, wherein the amino acid position numbering is according to the EU index as set forth in Kabat;

(v) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:40A and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises a therapeutic moiety (*e.g.*, a tubulysin 1508 molecule) covalently linked to a cysteine amino acid at position 239, wherein the amino acid position numbering is according to the EU index as set forth in Kabat; or,

(vi) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:41A and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises two therapeutic moieties (*e.g.*, two tubulysin 1508 molecules) covalently linked to cysteine amino acids

respectively located at positions 239 and 442, wherein the amino acid position numbering is according to the EU index as set forth in Kabat.

[00372] In other aspects, the methods of treatment disclosed herein comprise the administration of an anti-HER2 binding molecule which is an ADC comprising a bispecific anti-HER2 antibody, wherein said antibody comprises:

- (i) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:32C and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises a therapeutic moiety (*e.g.*, a tubulysin 1508 molecule) covalently linked to a cysteine amino acid inserted between positions 239 and 240, wherein the amino acid position numbering is according to the EU index as set forth in Kabat;
- (ii) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:3C3 and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises two therapeutic moieties (*e.g.*, two tubulysin 1508 molecules) covalently linked to a cysteine amino inserted between positions 239 and 240 and a cysteine amino acid located at position 442 wherein the amino acid position numbering is according to the EU index as set forth in Kabat;
- (iii) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:36C and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises a therapeutic moiety (*e.g.*, a tubulysin 1508 molecule) covalently linked to a cysteine amino acid inserted between positions 239 and 240, wherein the amino acid position numbering is according to the EU index as set forth in Kabat;
- (iv) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:37C and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises two therapeutic moieties (*e.g.*, two tubulysin 1508 molecules) covalently linked to a cysteine amino inserted between positions 239 and 240 and a cysteine amino acid located at position 442, wherein the amino acid position numbering is according to the EU index as set forth in Kabat;

(v) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:40C and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises a therapeutic moiety (*e.g.*, a tubulysin 1508 molecule) covalently linked to a cysteine amino acid inserted between positions 239 and 240, wherein the amino acid position numbering is according to the EU index as set forth in Kabat; or,

(vi) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:67 and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises two therapeutic moieties (*e.g.*, two tubulysin 1508 molecules) covalently linked to a cysteine amino acid inserted between positions 239 and 240 and a cysteine amino acid located at position 442, wherein the amino acid position numbering is according to the EU index as set forth in Kabat.

X. Pharmaceutical Compositions and Administration Methods

[00373] Methods of preparing and administering anti-HER2 binding molecules, *e.g.*, antibodies, or antigen-binding fragments, variants, or derivatives thereof (*e.g.*, an ADC such as Bs2-4T), to a subject in need thereof are well known to or are readily determined by those skilled in the art. The route of administration of the anti-HER2 binding molecule, *e.g.*, antibody, or antigen-binding fragment, variant, or derivative thereof (*e.g.*, an ADC such as Bs2-4T), can be, *e.g.*, oral, parenteral, by inhalation or topical. The term parenteral as used herein includes, *e.g.*, intravenous, intraarterial, intraperitoneal, intramuscular, subcutaneous, rectal, or vaginal administration. However, in other methods compatible with the teachings herein, anti-HER2 binding molecules, *e.g.*, antibodies, or antigen-binding fragments, variants, or derivatives thereof (*e.g.*, an ADC such as Bs2-4T), of the instant disclosure can be delivered directly to the site of the adverse cellular population thereby increasing the exposure of the diseased tissue to the therapeutic agent.

[00374] As discussed herein, anti-HER2 binding molecules of the instant disclosure, *e.g.*, antibodies, or antigen-binding fragments, variants, or derivatives thereof (*e.g.*, an ADC such as Bs2-4T), can be administered in a pharmaceutically effective amount for the *in vivo* treatment of HER2-expressing cell-mediated diseases such as certain types of

cancers. The pharmaceutical compositions can comprise pharmaceutically acceptable carriers, including, *e.g.*, water, ion exchangers, proteins, buffer substances, and salts. Preservatives and other additives can also be present. The carrier can be a solvent or dispersion medium. Suitable formulations for use in the therapeutic methods disclosed herein are described in Remington's Pharmaceutical Sciences (Mack Publishing Co.) 16th ed. (1980).

[00375] In any case, sterile injectable solutions can be prepared by incorporating an active compound (*e.g.*, an anti-HER2 antibody, or antigen-binding fragment, variant, or derivative thereof, *e.g.*, an ADC such as Bs2-4T, by itself or in combination with other active agents) in the required amount in an appropriate solvent followed by filtered sterilization. Further, the preparations can be packaged and sold in the form of a kit. Such articles of manufacture can have labels or package inserts indicating that the associated compositions are useful for treating a subject suffering from, or predisposed to a disease or disorder.

[00376] Parenteral formulations can be a single bolus dose, an infusion or a loading bolus dose followed with a maintenance dose. These compositions can be administered at specific fixed or variable intervals, *e.g.*, once a day, or on an "as needed" basis.

[00377] The composition can be administered as a single dose, multiple doses or over an established period of time in an infusion. Dosage regimens also can be adjusted to provide the optimum desired response (*e.g.*, a therapeutic or prophylactic response).

[00378] Therapeutically effective doses of the compositions of the present disclosure, for treatment of HER2-expressing cell-mediated diseases such as certain types of cancers including *e.g.*, colon cancer, lung cancer, gastric cancer, head and neck squamous cells cancer, melanoma, pancreatic cancer, prostate cancer, and breast cancer, vary depending upon many different factors, including means of administration, target site, physiological state of the patient, whether the patient is human or an animal, other medications administered, and whether treatment is prophylactic or therapeutic. In some specific aspects, the cancer expresses low levels of HER2 as determined, for example, using HERCEPTTM. Usually, the patient is a human, but non-human mammals including transgenic mammals can also be treated. Treatment dosages can be titrated using routine methods known to those of skill in the art to optimize safety and efficacy.

[00379] The amount of at least one anti-HER2 binding molecule, *e.g.*, antibody or binding fragment, variant, or derivative thereof (*e.g.*, an ADC such as Bs2-4T) to be administered can be readily determined by one of ordinary skill in the art without undue experimentation. Factors influencing the mode of administration and the respective amount of at least one anti-HER2 binding molecule, *e.g.*, antibody, antigen-binding fragment, variant or derivative thereof (*e.g.*, an ADC such as Bs2-4T), include, but are not limited to, the severity of the disease, the history of the disease, and the age, height, weight, health, and physical condition of the individual undergoing therapy. Similarly, the amount of anti-HER2 binding molecule, *e.g.*, antibody, or fragment, variant, or derivative thereof (*e.g.*, an ADC such as Bs2-4T), to be administered will be dependent upon the mode of administration and whether the subject will undergo a single dose or multiple doses of this agent.

[00380] The present disclosure also provides for the use of an anti-HER2 binding molecule, *e.g.*, an antibody or antigen-binding fragment, variant, or derivative thereof (*e.g.*, an ADC such as Bs2-4T), in the manufacture of a medicament for treating a type of cancer, including, *e.g.*, breast cancer, colon cancer, lung cancer, gastric cancer, head and neck squamous cells cancer, melanoma, pancreatic cancer, and prostate cancer. In some specific aspects, the cancer expresses low levels of HER2 as determined, for example, using HERCEPTEST™.

[00381] The disclosure also provides for the use of an anti-HER2 binding molecule, *e.g.*, antibody of the instant disclosure, or antigen-binding fragment, variant, or derivative thereof, in the manufacture of a medicament for treating a subject for treating a type of cancer. In some specific aspects, the cancer expresses low levels of HER2 as determined, for example, using HERCEPTEST™. In certain aspects, the medicament is used in a subject that has been pretreated with at least one other therapy.

[00382] By "pretreated" or "pretreatment" is intended the subject has received one or more other therapies (*e.g.*, been treated with at least one other anti-cancer therapy) prior to receiving the medicament comprising the anti-HER2 binding molecule, *e.g.*, antibody or antigen-binding fragment, variant, or derivative thereof (*e.g.*, an ADC such as Bs2-4T). It is not necessary that the subject was a responder to pretreatment with the prior therapy or therapies. Thus, the subject that receives the medicament comprising the anti-HER2

binding molecule, *e.g.*, an antibody or antigen-binding fragment, variant, or derivative thereof (*e.g.*, an ADC such as Bs2-4T) could have responded, responded poorly, or could have failed to respond to pretreatment with the prior therapy, or to one or more of the prior therapies where pretreatment comprised multiple therapies. Accordingly, the present disclosure provides methods to treat patients that are poor responders or non-responders to other therapies (*e.g.*, treatment with an antibody or an ADC such as T-DM1) comprising administering an anti-HER2 binding molecule disclosed herein, *e.g.*, an antibody or binding fragment, a variant, or a derivative thereof (*e.g.*, an ADC such as Bs2-4T). Also provided are methods to prevent resistance to cancer therapies (*e.g.*, resistance to treatment with an antibody or an ADC such as T-DM1) comprising administering an anti-HER2 binding molecule disclosed herein, *e.g.*, an antibody or binding fragment, a variant, or a derivative thereof (*e.g.*, an ADC such as Bs2-4T).

[00383] Even if a patient has been previously treated with a HER2 inhibitor, a person skilled in the art can determine whether a person showed no response after the treatment with the HER2 inhibitor. For example, a non-response to an inhibitor may be reflected in an increased suffering from cancer, such as an increased growth of a cancer/tumor and/or increase in the size of a tumor, the (increase in) the formation of metastases or a increase in the number or size of metastases. A non-response may also be the development of a tumor or metastases, for example after resection of a tumor, in the shortening of time to disease progression, or in the increase in the size of (a) tumor(s) and/or (a) metastases, for example in neoadjuvant therapy. Based on these parameters or other parameters known in the art, a patient group can be identified that does not respond to treatment with HER2 inhibitors, like pertuzumab, trastuzumab, or T-DM1. Such group of patients then could be treated with the anti-HER2 binding molecules disclosed herein, *e.g.*, antibody of the disclosure, or antigen-binding fragment, variant, or derivative thereof (*e.g.*, an ADC such as Bs2-4T).

[00384] The present disclosure provides also methods to treat patients that are, for example, poor-responders or non-responders to another therapy. The term "non-responder" as used herein can refer to an individual/patient/subject that is less likely to respond to a treatment using a HER2 inhibitor (*e.g.*, pertuzumab, trastuzumab, or T-DM1). "Less likely to respond" as used herein refers to a decreased likeliness that a

pathological complete response will occur in a patient treated with a HER2 inhibitor. In some aspects, a patient can be initially a good responder, and resistance to HER2 inhibitors can develop during treatment with such HER2 inhibitors (e.g., pertuzumab, trastuzumab, or T-DM1), leading to poor or no-response to the treatment.

[00385] The term "good responder" as used herein refers to an individual whose tumor does not demonstrate growth, metastases, increase in number or size of metastases, etc. during or after treatment using a HER2 inhibitor (like pertuzumab, trastuzumab, or T-DM1), for example based on serial imaging studies, an individual that does not experience tumor growth, metastases, increase in number or size of metastases, etc. over a period of time (e.g., about 1 year following initial diagnosis), and/or an individual that experiences a certain life span (e.g., about 2 years or more following initial diagnosis).

[00386] The term "poor responder" as used herein refers to an individual whose tumor grows or metastasizes during or shortly thereafter standard therapy, for example using a HER2 inhibitor (like pertuzumab, trastuzumab, or T-DM1), or who experiences adverse clinical effects attributable to the tumor.

[00387] In cases where it is assessed that the subject is a "non-responder," a "poor-responder" or is "less likely to respond" (based, for example, on the presence of certain biomarkers in the cancer cells), the subject could be treated with the anti-HER2 binding molecules disclosed herein, e.g., antibody of the disclosure, or antigen-binding fragment, variant, or derivative thereof (e.g., an ADC such as Bs2-4T).

[00388] The instant disclosure also provides for the co-administration of an anti-HER2 binding molecule, e.g., antibody of the disclosure, or antigen-binding fragment, variant, or derivative thereof (e.g., an ADC such as Bs2-4T), and at least one other therapy. The anti-HER2 binding molecule and the at least one other therapy can be co-administered together in a single composition or can be co-administered together at the same time or overlapping times in separate compositions. In some aspects, an anti-HER2 binding molecule, e.g., antibody of the disclosure, or antigen-binding fragment, variant, or derivative thereof (e.g., an ADC such as Bs2-4T), can be used as an adjuvant therapy.

[00389] The instant disclosure also provides for the use of an anti-HER2 binding molecule, e.g., antibody of the disclosure, or antigen-binding fragment, variant, or derivative thereof (e.g., an ADC such as Bs2-4T), in the manufacture of a medicament for

treating a subject for treating cancer, wherein the anti-HER2 binding molecule is administered before a subject has been treated with at least one other therapy.

XI. Diagnostics

[00390] The present disclosure further provides diagnostic methods useful for during diagnosis of HER2-expressing cell-mediated diseases such as certain types of cancer including, *e.g.*, colon cancer, lung cancer, gastric cancer, head and neck squamous cells cancer, melanoma, pancreatic cancer, prostate cancer, and breast cancer, which involves measuring the expression level of HER2 protein or transcript in tissue or other cells or body fluid from an individual and comparing the measured expression level with a standard HER2 expression level in normal tissue or body fluid, whereby an increase in the expression level compared to the standard is indicative of a disorder.

[00391] The anti-HER2 binding molecules of the instant disclosure and antigen-binding fragments, variants, and derivatives thereof, can be used to assay HER2 protein levels in a biological sample using classical immunohistological methods known to those of skill in the art (*e.g.*, see Jalkanen, *et al.*, *J. Cell. Biol.* 101:976-985 (1985); Jalkanen *et al.*, *J. Cell Biol.* 105:3087-3096 (1987)). Other antibody-based methods useful for detecting HER2 protein expression include immunoassays, such as the enzyme linked immunosorbent assay (ELISA), immunoprecipitation, or Western blotting. Suitable assays are described in more detail elsewhere herein.

[00392] By "assaying the expression level of HER2 polypeptide" is intended qualitatively or quantitatively measuring or estimating the level of HER2 polypeptide in a first biological sample either directly (*e.g.*, by determining or estimating absolute protein level) or relatively (*e.g.*, by comparing to the disease associated polypeptide level in a second biological sample). HER2 polypeptide expression level in the first biological sample can be measured or estimated and compared to a standard HER2 polypeptide level, the standard being taken from a second biological sample obtained from an individual not having the disorder or being determined by averaging levels from a population of individuals not having the disorder. As will be appreciated in the art, once the "standard" HER2 polypeptide level is known, it can be used repeatedly as a standard for comparison.

[00393] By "biological sample" is intended any biological sample obtained from an individual, cell line, tissue culture, or other source of cells potentially expressing HER2. Methods for obtaining tissue biopsies and body fluids from mammals are well known in the art.

XII. Kits Comprising Anti-HER2 Binding Molecules

[00394] The present disclosure also provides kits that comprise an anti-HER2 binding molecule disclosed herein, *e.g.*, an anti-HER2 antibody or antigen binding fragment thereof, that can be used to perform the methods described herein. In certain aspects, a kit comprises at least one purified anti-HER2 binding molecule or an antigen-binding fragment thereof in one or more containers. In some aspects, the kits contain all of the components necessary and/or sufficient to perform a detection assay, including all controls, directions for performing assays, and any necessary software for analysis and presentation of results. One skilled in the art will readily recognize that the disclosed anti-HER2 binding molecules disclosed herein, *e.g.*, an anti-HER2 antibody or antigen binding fragment thereof, can be readily incorporated into one of the established kit formats which are well known in the art.

XIII. Immunoassays

[00395] Anti-HER2 binding molecules, *e.g.*, antibodies or antigen-binding fragments thereof, variants, or derivatives thereof of the molecules of the instant disclosure can be assayed for immunospecific binding by any method known in the art. The immunoassays that can be used include but are not limited to competitive and non-competitive assay systems using techniques such as Western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, to name but a few. Such assays are routine and well known in the art (see, *e.g.*, Ausubel *et al.*, eds, (1994) Current Protocols in Molecular Biology (John Wiley & Sons, Inc., NY) Vol. 1, which is incorporated by reference herein in its entirety).

[00396] Anti-HER2 binding molecules disclosed herein, *e.g.*, bispecific anti-HER2 antibodies or antigen-binding fragments thereof, variants, or derivatives thereof of the molecules of the instant disclosure, can be employed histologically, as in immunofluorescence, immunoelectron microscopy or non-immunological assays, for *in situ* detection of HER2 receptors or conserved variants or peptide fragments thereof. *In situ* detection can be accomplished by removing a histological specimen from a patient, and applying thereto a labeled HER2-binding molecule, *e.g.*, a bispecific anti-HER2 antibody or antigen-binding fragment thereof, variant, or derivative thereof, preferably applied by overlaying the labeled HER2-binding molecule (*e.g.*, an antibody or fragment) onto a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of HER2, or conserved variants or peptide fragments, but also its distribution in the examined tissue. Using the present disclosure, those of ordinary skill will readily perceive that any of a wide variety of histological methods (such as staining procedures) can be modified in order to achieve such *in situ* detection.

[00397] The binding activity of a given lot of anti-HER2 binding molecule, *e.g.*, a bispecific anti-HER2 antibody or antigen-binding fragment thereof, variant, or derivative thereof, can be determined according to well-known methods. Those skilled in the art will be able to determine operative and optimal assay conditions for each determination by employing routine experimentation.

[00398] Methods and reagents suitable for determination of binding characteristics of an anti-HER2 binding molecule of the instant disclosure, *e.g.*, a bispecific anti-HER2 antibody or antigen-binding fragment thereof, variant, or an altered/mutant derivative thereof, are known in the art and/or are commercially available. Equipment and software designed for such kinetic analyses are commercially available (*e.g.*, BIAcore, BIAevaluation software, GE Healthcare; KinExa Software, Sapidyne Instruments).

[00399] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, Sambrook *et al.*, ed. (1989) *Molecular Cloning A Laboratory Manual* (2nd ed.; Cold Spring Harbor Laboratory Press); Sambrook *et al.*, ed. (1992) *Molecular Cloning: A Laboratory Manual*,

(Cold Springs Harbor Laboratory, NY); D. N. Glover ed., (1985) DNA Cloning, Volumes I and II; Gait, ed. (1984) Oligonucleotide Synthesis; Mullis *et al.* U.S. Pat. No. 4,683,195; Hames and Higgins, eds. (1984) Nucleic Acid Hybridization; Hames and Higgins, eds. (1984) Transcription And Translation; Freshney (1987) Culture Of Animal Cells (Alan R. Liss, Inc.); Immobilized Cells And Enzymes (IRL Press) (1986); Perbal (1984) A Practical Guide To Molecular Cloning; the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Miller and Calos eds. (1987) Gene Transfer Vectors For Mammalian Cells, (Cold Spring Harbor Laboratory); Wu *et al.*, eds., Methods In Enzymology, Vols. 154 and 155; Mayer and Walker, eds. (1987) Immunochemical Methods In Cell And Molecular Biology (Academic Press, London); Weir and Blackwell, eds., (1986) Handbook Of Experimental Immunology, Volumes I-IV; Manipulating the Mouse Embryo, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (1986); and in Ausubel *et al.* (1989) Current Protocols in Molecular Biology (John Wiley and Sons, Baltimore, Md.).

[00400] General principles of antibody engineering are set forth in Borrebaeck, ed. (1995) Antibody Engineering (2nd ed.; Oxford Univ. Press). General principles of protein engineering are set forth in Rickwood *et al.*, eds. (1995) Protein Engineering, A Practical Approach (IRL Press at Oxford Univ. Press, Oxford, Eng.). General principles of antibodies and antibody-hapten binding are set forth in: Nisonoff (1984) Molecular Immunology (2nd ed.; Sinauer Associates, Sunderland, Mass.); and Steward (1984) Antibodies, Their Structure and Function (Chapman and Hall, New York, N.Y.). Additionally, standard methods in immunology known in the art and not specifically described are generally followed as in Current Protocols in Immunology, John Wiley & Sons, New York; Stites *et al.*, eds. (1994) Basic and Clinical Immunology (8th ed; Appleton & Lange, Norwalk, Conn.) and Mishell and Shiigi (eds) (1980) Selected Methods in Cellular Immunology (W.H. Freeman and Co., NY).

[00401] Standard reference works setting forth general principles of immunology include Current Protocols in Immunology, John Wiley & Sons, New York; Klein (1982) J., Immunology: The Science of Self-Nonself Discrimination (John Wiley & Sons, NY); Kennett *et al.*, eds. (1980) Monoclonal Antibodies, Hybridoma: A New Dimension in Biological Analyses (Plenum Press, NY); Campbell (1984) "Monoclonal Antibody

Technology" in Laboratory Techniques in Biochemistry and Molecular Biology, ed. Burden *et al.*, (Elsevier, Amsterdam); Goldsby *et al.*, eds. (2000) Kuby Immunology (4th ed.; H. Freeman & Co.); Roitt *et al.* (2001) Immunology (6th ed.; London: Mosby); Abbas *et al.* (2005) Cellular and Molecular Immunology (5th ed.; Elsevier Health Sciences Division); Kontermann and Dubel (2001) Antibody Engineering (Springer Verlag); Sambrook and Russell (2001) Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Press); Lewin (2003) Genes VIII (Prentice Hall 2003); Harlow and Lane (1988) Antibodies: A Laboratory Manual (Cold Spring Harbor Press); Dieffenbach and Dveksler (2003) PCR Primer (Cold Spring Harbor Press).

XIV. Embodiments

- [00402] Embodiments are designated according to an "EnXm" schema, where E means "embodiment"; *n* is the embodiment ordinal number; *X* is optional and can be A or B, where A denotes embodiments specifically related to ADC constructs, and B denotes embodiments specifically related to construct with enhanced ADCC; and *m* is an optional sub-ordinal number indicating additional embodiments within a class (e.g., A1, B1, etc.).
- [00403] E1. A bispecific anti-HER2 antibody comprising a first immunoglobulin antigen-binding domain and a second immunoglobulin antigen-binding domain, wherein (i) the first and second immunoglobulin antigen-binding domains specifically bind to distinct HER2 antibody binding sites, (ii) the first immunoglobulin antigen-binding domain binds to a first HER2 antibody binding site which comprises an epitope within domain II of HER2, and (iii) the first HER2 antibody binding site is distinct from the antibody binding site of pertuzumab.
- [00404] E2. The bispecific anti-HER2 antibody of E1 wherein the second immunoglobulin antigen-binding domain binds to a second HER2 antibody binding site which comprises an epitope within domain IV of HER2.
- [00405] E3. The bispecific anti-HER2 antibody of E2 wherein the second HER2 antibody binding site is identical to the HER2 antibody binding site of trastuzumab.
- [00406] E4. The bispecific anti-HER2 antibody of E2 wherein the second HER2 antibody binding site partially overlaps with the HER2 antibody binding site of trastuzumab.
- [00407] E5. The bispecific anti-HER2 antibody of E2 wherein the second HER2 antibody binding site is distinct from the HER antibody binding site of trastuzumab.

- [00408] E6. A bispecific HER2 antibody comprising a first immunoglobulin antigen-binding domain and a second immunoglobulin antigen-binding domain, wherein the first and second immunoglobulin antigen-binding domains specifically bind to distinct HER2 epitopes; and wherein the first immunoglobulin antigen-binding domain binds HER2 to one or more amino acid residues in SEQ ID NO: 52.
- [00409] E7. The bispecific antibody according to E6, wherein the second immunoglobulin antigen-binding domain binds HER2 at an epitope within domain IV.
- [00410] E8. The bispecific antibody according to E6, wherein the second immunoglobulin antigen binding domain binds HER2 to one or more amino acid residues in SEQ ID NO: 53.
- [00411] E9. The bispecific antibody according to E1-E8, wherein the first immunoglobulin antigen-binding domain comprises a heavy chain (HC) variable region (VH) and a light chain (LC) variable region (VL) comprising:
- (i) a variable heavy chain CDR-1 (VH-CDR1) identical to SEQ ID NO: 1 or identical to SEQ ID NO: 1 except for up to 1, 2, 3, or 4 amino acid substitutions;
 - (ii) a variable heavy chain CDR-2 (VH-CDR2) identical to SEQ ID NO: 2 or identical to SEQ ID NO: 2 except for up to 1, 2, 3, or 4 amino acid substitutions;
 - (iii) a variable heavy chain CDR-3 (VH-CDR3) identical to SEQ ID NO: 3 or identical to SEQ ID NO: 3 except for up to 1, 2, 3, or 4 amino acid substitutions;
 - (iv) a variable light chain CDR-1 (VL-CDR1) identical to SEQ ID NO: 4 or identical to SEQ ID NO: 4 except for up to 1, 2, 3, or 4 amino acid substitutions;
 - (v) a variable light chain CDR-2 (VL-CDR2) identical to SEQ ID NO: 5 or identical to SEQ ID NO: 5 except for up to 1, 2, 3, or 4 amino acid substitutions; and,
 - (vi) a variable light chain CDR-3 (VL-CDR3) identical to SEQ ID NO: 6 or identical to SEQ ID NO: 6 except for up to 1, 2, 3, or 4 amino acid substitutions.
- [00412] E10. The bispecific antibody according to E9, wherein the first immunoglobulin antigen-binding domain comprises at least one heterologous variable domain framework region (FW) different relative to the FW regions of an immunoglobulin antigen-binding domain comprising a VH comprising the amino acids SEQ ID NO:43 and a VL comprising the amino acids of SEQ ID NO:44.

- [00413] E11. The bispecific anti-HER2 antibody according to E10, wherein the first immunoglobulin antigen-binding domain comprises:
- (i) a variable light chain framework 1 (VL-FW1) comprising the amino acids of SEQ ID NO:11;
 - (ii) a VL-FW2 comprising the amino acids of SEQ ID NO:12;
 - (iii) a VL-FW3 comprising the amino acids of SEQ ID NO:13;
 - (iv) a VL-FW4 comprising the amino acids of SEQ ID NO:14; or
 - (v) any combination thereof.
- [00414] E12. A bispecific anti-HER2 antibody comprising a first immunoglobulin antigen binding-domain and a second immunoglobulin antigen-binding domain, wherein the first immunoglobulin antigen-binding domain comprises a VH and a VL, wherein the VH comprises the amino acids of SEQ ID NO:15 or 43;and wherein the first and second immunoglobulin antigen-binding domains specifically bind to distinct HER2 epitopes.
- [00415] E13. A bispecific anti-HER2 antibody comprising a first immunoglobulin antigen binding-domain and a second immunoglobulin antigen-binding domain, wherein the first immunoglobulin antigen-binding domain comprises a VH and a VL, wherein the VL comprises the amino acids of SEQ ID NO:16 or 44;and wherein the first and second immunoglobulin antigen-binding domains specifically bind to distinct HER2 epitopes.
- [00416] E14. The bispecific anti-HER2 antibody of E12 or E13, wherein the VH comprises the amino acids of SEQ ID NO:15; and wherein the VL comprises the amino acids of SEQ ID NO: 16.
- [00417] E15. The bispecific anti-HER2 antibody according to any one of E1 to E14, wherein the first immunoglobulin antigen-binding domain comprises an scFv antibody fragment.
- [00418] E16. The bispecific anti-HER2 antibody according to any one of E1 to E15, wherein the second immunoglobulin antigen-binding domain comprises an scFv antibody fragment.

- [00419] E17. The bispecific anti-HER2 antibody according to thereof of any one of E1 to E16, wherein the first immunoglobulin antigen binding domain comprises or consists of:
- (a) a VH further comprising a heavy chain constant region or a fragment thereof and a VL comprising a light chain constant region (LC) or a fragment thereof;
 - (b) a single chain Fv ("scFv");
 - (c) a diabody;
 - (d) a minibody;
 - (e) an F(ab')₂; or
 - (f) an F(ab).
- [00420] E18. The bispecific anti-HER2 antibody according to E17, wherein the heavy chain constant region or fragment thereof is an IgG constant region.
- [00421] E19. The bispecific HER2 antibody according to E18, wherein the IgG constant region or fragment thereof is an IgG1 constant region.
- [00422] E20. The bispecific HER2 antibody according to any one of E17 to E19, wherein the LC constant region is a kappa constant region.
- [00423] E21. The bispecific HER2 antibody according to any one of E17 to E19, wherein the LC constant region is a lambda constant region.
- [00424] E22. The bispecific anti-HER2 antibody according to any one of E1 to E21, wherein the first immunoglobulin antigen-binding domain is a monoclonal antibody.
- [00425] E23. The bispecific anti-HER2 antibody according to any one of E1 to E22, wherein the first immunoglobulin antigen-binding domain is a humanized antibody.
- [00426] E24. The bispecific anti-HER2 antibody according to any one of E1 to E22, wherein the first immunoglobulin antigen-binding domain is a human antibody.
- [00427] E25. The bispecific anti-HER2 antibody according to any one of E1 to E22, wherein the first immunoglobulin antigen-binding domain is a chimeric antibody.
- [00428] E26. The bispecific anti-HER2 antibody according to any one of E1 to E25, wherein the first immunoglobulin antigen-binding domain is an affinity optimized antibody.

- [00429] E27. The bispecific anti-HER2 antibody according to any one of E1 to E26, wherein the first immunoglobulin antigen-binding domain does not compete with trastuzumab or pertuzumab for epitope binding.
- [00430] E28. The bispecific anti-HER2 antibody according to any one of E1 to E27, wherein the first and second immunoglobulin antigen binding domains specifically bind to distinct non-overlapping HER2 epitopes.
- [00431] E29. The bispecific anti-HER2 antibody according to any one of E1 to E28, wherein:
- (a) the second immunoglobulin antigen-binding domain specifically binds to the same HER2 epitope as the trastuzumab antibody;
 - (b) the second immunoglobulin antigen-binding domain competitively inhibits HER2 binding by the trastuzumab antibody; or
 - (c) the second immunoglobulin antigen-binding domain comprises at least one, at least two, at least three, at least four, at least five, or at least six complementarity determining regions (CDRs) comprising the amino acids of any one of SEQ ID NOs: 54 to 59.
- [00432] E30. The bispecific anti-HER2 antibody according to E29, wherein the second immunoglobulin antigen-binding domain is an scFv comprising:
- (i) a VH-CDR1 comprising the amino acids of SEQ ID NO:54;
 - (ii) a VH-CDR2 comprising the amino acids of SEQ ID NO:55;
 - (iii) a VH-CDR3 comprising the amino acids of SEQ ID NO:56;
 - (iv) a VL-CDR1 comprising the amino acids of SEQ ID NO:57;
 - (v) a VL-CDR2 comprising the amino acids of SEQ ID NO:58; and
 - (vi) a VL-CDR3 comprising the amino acids of SEQ ID NO:59.
- [00433] E31. The bispecific anti-HER2 antibody according to E30, wherein the scFv is a disulfide stabilized scFv.
- [00434] E32. The bispecific anti-HER2 antibody according to E30 or E31, wherein said scFv comprises a VH comprising the amino acids of SEQ ID NO:17, and a VL comprising the amino acid of SEQ ID NO:18.
- [00435] E33. The bispecific anti-HER2 antibody according to E32, wherein the VH and VL of the scFv are covalently linked via a peptide linker.

- [00436] E34. The bispecific anti-HER2 antibody according to E33, wherein the peptide linker comprises the amino acids of SEQ ID NO:19.
- [00437] E35. The bispecific anti-HER2 antibody according to any one of E29 to E34, wherein the second immunoglobulin antigen-binding domain is covalently linked to the carboxy-terminus of the HC of the first immunoglobulin antigen-binding domain.
- [00438] E36. The bispecific anti-HER2 antibody according to E35, comprising a linker interposed between the second immunoglobulin antigen binding domain and the carboxy-terminus of the HC of the first immunoglobulin antigen-binding domain.
- [00439] E37. The bispecific anti-HER2 antibody according to any one of E29 to E34, wherein the second immunoglobulin antigen-binding domain is covalently linked to the amino-terminus of the HC of the first immunoglobulin antigen-binding domain.
- [00440] E38. The bispecific anti-HER2 antibody according to E37, comprising a linker interposed between the second immunoglobulin antigen-binding domain and the amino-terminus of the HC of the first immunoglobulin antigen-binding domain.
- [00441] E39. The bispecific anti-HER2 antibody according to E29 to E34, wherein the second immunoglobulin antigen binding domain is covalently intercalated in the polypeptide chain of the HC of the first immunoglobulin antigen-binding domain.
- [00442] E40. The bispecific anti-HER2 antibody according to E39, wherein the second immunoglobulin antigen binding domain is covalently intercalated between the CH1 region and CH2 region of the HC of the first immunoglobulin antigen-binding domain.
- [00443] E41. The bispecific anti-HER2 antibody according to E40, comprising:
(i) a linker interposed between the CH1 region of the HC of the first immunoglobulin antigen binding domain and the second immunoglobulin antigen binding domain; and
(ii) a second linker interposed between the second immunoglobulin antigen binding domain and the CH2 region of the HC of the of the first immunoglobulin antigen-binding domain.
- [00444] E42. The bispecific anti-HER2 antibody according to E41, wherein the first linker and the second linker are identical.
- [00445] E43. The bispecific anti-HER2 antibody according to E41, wherein the first linker and the second linker are different.

- [00446] E44. The bispecific anti-HER2 antibody according to any one of E36, E38, and E41 to E43, wherein one or more of the linkers comprise a peptide linker.
- [00447] E45. The bispecific anti-HER2 antibody according to E44, wherein the peptide linker comprises at least two, at least three, at least four, at least five, at least 10, at least 20, or at least 30 amino acids.
- [00448] E46. The bispecific anti-HER2 antibody according to any one of E44 or E45, wherein the peptide linker comprises a peptide having the formula $\text{Ser}_x[(\text{Gly})_y\text{-Ser}_4]_z$ where x is from 0 to 1, y is from 1 to 4, and z is from 1 to 10.
- [00449] E47. The bispecific anti-HER2 antibody according to E46, wherein the peptide linker comprises SEQ ID NO:19-22.
- [00450] E48. The bispecific anti-HER2 antibody according to any one of E1 to E47, wherein the heavy chain comprises a constant region comprising an Fc domain.
- [00451] E49. The bispecific anti-HER2 antibody according to E48, wherein the Fc domain comprises a CH2 region and a CH3 region.
- [00452] E50. The bispecific anti-HER2 antibody according to E48, wherein the Fc domain is an IgG1 Fc domain.
- [00453] E51. The bispecific anti-HER2 antibody according to E50, wherein the IgG1 Fc domain is a native IgG1 Fc domain.
- [00454] E52. The bispecific anti-HER2 antibody according to E51, wherein the native IgG1 Fc domain comprises the amino acids of SEQ ID NO: 23.
- [00455] E53. The bispecific anti-HER2 antibody according to E48, wherein the Fc domain is a mutant IgG1 Fc domain.
- [00456] E54A. The bispecific anti-HER2 antibody according to E53, wherein the mutant IgG1 Fc domain comprises at least one mutation capable of reducing the ADCC activity of the bispecific antibody.
- [00457] E54B. The bispecific anti-HER2 antibody according to E53, wherein the mutant IgG1 Fc domain comprises at least one mutation capable of enhancing the ADCC activity of the bispecific antibody.
- [00458] E55A. The bispecific anti-HER2 antibody according to E54A, wherein at least one mutation capable of reducing the ADCC activity of the bispecific antibody is an amino acid substitution.

- [00459] E55B. The bispecific anti-HER2 antibody according to E54B, wherein at least one mutation capable of enhancing the ADCC activity of the bispecific antibody is an amino acid substitution.
- [00460] E56A. The bispecific anti-HER2 antibody according to E55A, comprising at least one amino acid substitution comprising L234F, S239A, S239C, a cysteine amino acid insertion between positions 239 and 240 or any combination thereof, wherein the amino acid position numbering is according to the EU index as set forth in Kabat.
- [00461] E56B. The bispecific anti-HER2 antibody according to E55B, comprising at least one amino acid substitution comprising, S239A, S239D, A330L, I332E, E333A, K334A, or any combination thereof, wherein the amino acid position numbering is according to the EU index as set forth in Kabat.
- [00462] E57A. The bispecific anti-HER2 antibody according to E55A or E56A, wherein said mutant IgG1 Fc domain comprises at least one amino acid substitution introducing a derivatizable group.
- [00463] E57B. The bispecific anti-HER2 antibody according to any one of E48 to E53, E54B, E55B or E56B wherein the Fc domain has an altered type of glycosylation that enhances ADCC activity.
- [00464] E58A. The bispecific anti-HER2 antibody according to E57A, wherein said mutant IgG1 Fc domain comprises one to three amino acid substitution introducing a derivatizable group.
- [00465] E58B. The bispecific anti-HER2 antibody according to E57B, wherein the Fc domain is a hypofucosylated antibody having reduced amounts of fucosyl residues.
- [00466] E59A. The bispecific anti-HER2 antibody according to E57A or E58A, wherein the derivatizable group in a sulfhydryl group.
- [00467] E59B. The bispecific anti-HER2 antibody according to E57B or E58B, wherein the Fc domain has increased bisecting GlcNAc structures.
- [00468] E60. The bispecific anti-HER2 antibody according to E59A, wherein the at least one amino acid substitution comprises S239C, 248C, 254C, 258C, 273C, 279C, 282C, 284C, 286C, 287C, 289C, 297C, 298C, 312C, 324C, 326C, 330C, 335C, 337C, 339C, 350C, 355C, 356C, 359C, 360C, 361C, 375C, 383C, 384C, 389C, 398C, 400C, 413C, 415C, 418C, 422C, 435C, 440C, 441C, S442C, 443C, a cysteine amino acid

insertion between positions 239 and 240, and 446C, or any combination thereof, wherein the amino acid position numbering is according to the EU index as set forth in Kabat.

[00469] E61A. The bispecific anti-HER2 antibody according to any one E54A, E55A, E56A, E57A, E58A, E59A, or E60, wherein the mutant Fc domain comprises the amino acids of SEQ ID NO: 24A, SEQ ID NO: 24C, SEQ ID NO: 25A or SEQ ID NO:25C.

[00470] E61B. The bispecific anti-HER2 antibody according to any one E54B, E55B, E56B, E57B, E58B, E59B, or E60, wherein the mutant Fc domain comprises the amino acids of SEQ ID NO: 24B or SEQ ID NO:25B.

[00471] E62. A bispecific anti-HER2 antibody comprising a first and a second polypeptide chain associated with each other, wherein the first polypeptide chain is selected from:

(1) [TZ_S]-[L₁]-[_BVH]-[_BCH]-[Fc_x]

(2) [_BVH]-[_BCH]-[Fc_x]-[L₂]-[TZ_S]

(3) [_BVH]-[_BCH]-[L₃]-[TZ_S]-[L₄]-[Fc_x]

wherein

TZ_S is an scFv that binds the same epitope as trastuzumab;

L₁, L₂, L₃, and L₄ are peptide linkers;

Fc_x is an Fc domain;

_BVH and _BCH are the VH and CH1 regions, respectively, of an antibody capable of binding to an HER2 epitope distinct from the epitope recognized by the trastuzumab antibody.

[00472] E63. The bispecific anti-HER2 antibody according to E62, wherein the distinct epitope comprises one or more amino acids within SEQ ID NO: 52.

[00473] E64. The bispecific anti-HER2 antibody according to E62 or E63, wherein the second chain comprises [_BVL]-[CL] wherein BVL is the VL region of an antibody capable of binding to an HER2 epitope distinct from the epitope recognized by the trastuzumab antibody, and CL is a IgG light chain constant region.

[00474] E65. The bispecific anti-HER2 antibody according to E64, wherein CL is selected from the group consisting of a human kappa constant region and a human lambda constant region.

- [00475] E66. The bispecific anti-HER2 antibody according to E64 or E65, wherein _BVL comprises:
- (i) a variable light chain CDR-1 (VL-CDR1) identical to SEQ ID NO: 4 or identical to SEQ ID NO: 4 except for up to 1, 2, 3, or 4 amino acid substitutions;
 - (ii) a variable light chain CDR-2 (VL-CDR2) identical to SEQ ID NO: 5 or identical to SEQ ID NO: 5 except for up to 1, 2, 3, or 4 amino acid substitutions; and,
 - (iii) a variable light chain CDR-3 (VL-CDR3) identical to SEQ ID NO: 6 or identical to SEQ ID NO: 6 except for up to 1, 2, 3, or 4 amino acid substitutions.
- [00476] E67. The bispecific anti-HER2 antibody according to E64, E65 or E66, wherein _BVL comprises the amino acids of SEQ ID NO:16 or 44.
- [00477] E68. The bispecific anti-HER2 antibody according to E65, wherein CL comprises the amino acids of SEQ ID NO:27A or SEQ ID NO: 27B.
- [00478] E69. The bispecific anti-HER2 antibody according to any one of E62 to E68, wherein [TZ_S] comprises:
- (i) a VH-CDR1 comprising the amino acids of SEQ ID NO:54;
 - (ii) a VH-CDR2 comprising the amino acids of SEQ ID NO:55;
 - (iii) a VH-CDR3 comprising the amino acids of SEQ ID NO:56;
 - (iv) a VL-CDR1 comprising the amino acids of SEQ ID NO:57;
 - (v) a VL-CDR2 comprising the amino acids of SEQ ID NO:58; and
 - (vi) a VL-CDR3 comprising the amino acids of SEQ ID NO:59.
- [00479] E70. The bispecific anti-HER2 antibody according to any one of E62 to E69, wherein [TZ_S] is a disulfide stabilized scFv.
- [00480] E71. The bispecific anti-HER2 antibody according to any one of E62 to E70, wherein [TZ_S] comprises a VH comprising the amino acids of SEQ ID NO:17 and a VL comprising the amino acids of SEQ ID NO:18, covalently linked by a peptide linker.
- [00481] E72. The bispecific anti-HER2 antibody according to E71, wherein the peptide linker comprises the amino acids of SEQ ID NO:19.
- [00482] E73. The bispecific anti-HER2 antibody according to any one of E69 to E72, wherein [TZ_S] comprises of the amino acids of SEQ ID NO:28.
- [00483] E74. The bispecific anti-HER2 antibody according to any one of E62 to E73, wherein a hinge polypeptide links the [_BCH] and [FcX].

- [00484] E75. The bispecific anti-HER2 antibody according to E74, wherein the hinge polypeptide comprises or consists of the amino acids of SEQ ID NO: 26.
- [00485] E76A. The bispecific anti-HER2 antibody according to any one of E62 to E75, wherein the [Fc_x] comprises at least one amino acid substitution introducing a derivatizable group.
- [00486] E76B. The bispecific anti-HER2 antibody according to any one of E62 to E75, wherein [Fc_x] comprises at least one mutation capable of enhancing the ADCC activity of the bispecific antibody.
- [00487] E77A. The bispecific anti-HER2 antibody according to E76A, wherein the [Fc_x] comprises one to three amino acid substitution introducing a derivatizable group.
- [00488] E77B. The bispecific anti-HER2 antibody according to E76B, wherein at least one mutation capable of enhancing the ADCC activity of the bispecific antibody is an amino acid substitution.
- [00489] E78A. The bispecific anti-HER2 antibody according to E76A or E77A, wherein the derivatizable group is a sulfhydryl group.
- [00490] E78B. The bispecific anti-HER2 antibody according to E77B, comprising at least one amino acid substitution comprising, S239A, S239D, A330L, I332E, E333A, K334A, or any combination thereof, wherein the amino acid position numbering is according to the EU index as set forth in Kabat.
- [00491] E78B1. The bispecific anti-HER2 antibody according to any one of E62 to E75, E76B, E77B or E78B wherein the Fc domain has an altered type of glycosylation that enhances ADCC activity.
- [00492] E78B2. The bispecific anti-HER2 antibody according to E78B1, wherein the Fc domain is a hypofucosylated antibody having reduced amounts of fucosyl residues.
- [00493] E78B3. The bispecific anti-HER2 antibody according to E78B1 or E78B2, wherein the Fc domain has increased bisecting GlcNAc structures.
- [00494] E79. The bispecific anti-HER2 antibody according to E78A, wherein the at least one amino acid substitution comprises S239C, 248C, 254C, 258C, 273C, 279C, 282C, 284C, 286C, 287C, 289C, 297C, 298C, 312C, 324C, 326C, 330C, 335C, 337C, 339C, 350C, 355C, 356C, 359C, 360C, 361C, 375C, 383C, 384C, 389C, 398C, 400C, 413C, 415C, 418C, 422C, 435C, 440C, 441C, S442C, 443C, 446C, a cysteine amino acid

insertion between positions 239 and 240, or any combination thereof, wherein the amino acid position numbering is according to the EU index as set forth in Kabat.

[00495] E80A. The bispecific anti-HER2 antibody according to any one of E62 to E75, E76A, E77A, E78A, or E79, wherein [Fcx] comprises the amino acids of any one of SEQ ID NOS: 23, 24A, 24C, 25A and 25C.

[00496] E80B. The bispecific anti-HER2 antibody according to any one of E62 to E75, E76B, E77B, E78B, E78B1, E78B2, E78B3, or E79, wherein [Fcx] comprises the amino acids of any one of SEQ ID NOS: 23, 24B, and 25B.

[00497] E81. The bispecific anti-HER2 antibody according to any one of E62 to E79, wherein [L₁], [L₂], [L₃], and [L₄] comprise amino acids independently selected from the group consisting of SEQ ID NOS: 19, 20, 21, and 22.

[00498] E82. The bispecific anti-HER2 antibody according to any one of E62 to E79, wherein:

- (i) [L₁] comprises the amino acids of SEQ ID NO:19;
- (ii) [L₂] comprises the amino acids of SEQ ID NO:20;
- (iii) [L₃] comprises the amino acids of SEQ ID NO:21; and,
- (iv) [L₄] comprises the amino acids of SEQ ID NO:22.

[00499] E83. The bispecific anti-HER2 antibody according to any one of E62 to E82, wherein [BVH] comprises:

- (i) a variable heavy chain CDR-1 (VH-CDR1) identical to SEQ ID NO: 1 or identical to SEQ ID NO: 1 except for up to 1, 2, 3, or 4 amino acid substitutions;
- (ii) a variable heavy chain CDR-2 (VH-CDR2) identical to SEQ ID NO: 2 or identical to SEQ ID NO: 2 except for up to 1, 2, 3, or 4 amino acid substitutions; and
- (iii) a variable heavy chain CDR-3 (VH-CDR3) identical to SEQ ID NO: 3 or identical to SEQ ID NO: 3 except for up to 1, 2, 3, or 4 amino acid substitutions

[00500] E84. The bispecific anti-HER2 antibody according to any one of E62 to E82, wherein [BVH] comprises SEQ ID NO:15 or 43.

[00501] E85. The bispecific anti-HER2 antibody according to any one of E62 to E84, wherein [BCH] comprises the amino acids of SEQ ID NO: 29.

[00502] E86. The bispecific anti-HER2 antibody according to any one of E64 to E85, wherein [BVL] comprises:

- (i) a variable light chain CDR-1 (VL-CDR1) identical to SEQ ID NO: 4 or identical to SEQ ID NO: 4 except for up to 1, 2, 3, or 4 amino acid substitutions;
- (ii) a variable light chain CDR-2 (VL-CDR2) identical to SEQ ID NO: 5 or identical to SEQ ID NO: 5 except for up to 1, 2, 3, or 4 amino acid substitutions; and
- (iii) a variable light chain CDR-3 (VL-CDR3) identical to SEQ ID NO: 6 or identical to SEQ ID NO: 6 except for up to 1, 2, 3, or 4 amino acid substitutions.

[00503] E87. The bispecific anti-HER2 antibody according to any one of E64 to E85, wherein [BVL] comprises the amino acids of SEQ ID NO:16 or 44.

[00504] E88A. The bispecific HER2 antibody according to claim any one of E62 to E87, wherein first polypeptide chain comprises the amino acids of any one of SEQ ID NOs: 30, 31A, 32A, 32C, 33A, 33C, 34, 35A, 36A, 36C, 37A, 37C, 38, 38A, 39A, 40A, 40C, 41A or 41C, and a second polypeptide chain comprising the amino acids of SEQ ID NO:42A or 42B, wherein the bispecific HER2 antibody is conjugated to a therapeutic moiety.

[00505] E88B. The bispecific HER2 antibody according to claim any one of E62 to E87, wherein first polypeptide chain comprises the amino acids of any one of SEQ ID NOs: 30, 31B, 32B, 33B, 34, 35B, 36B, 37B, 38, 38B, 39B, 40B, or 41B, and a second polypeptide chain comprising the amino acids of SEQ ID NO:42A or 42B, wherein the bispecific HER2 antibody has enhanced ADCC activity.

[00506] E89. The bispecific HER2 antibody according to any one of E1 to E88B, wherein the bispecific HER2 antibody induces internalization upon binding to the HER2 target.

[00507] E90. The bispecific HER2 antibody according to E89, wherein the bispecific HER2 antibody promotes effective lysosomal trafficking following internalization.

[00508] E91. The bispecific HER2 antibody according to any one of E1 to E88B, wherein the bispecific HER2 antibody induces HER2 target degradation.

[00509] E92. The bispecific HER2 antibody according to any one of E1 to E88B, wherein the bispecific HER2 antibody blocks ligand-induced AKT phosphorylation in low HER2-expressing cancer cells.

- [00510] E93. The bispecific HER2 antibody according to any one of E1 to E88B, wherein the bispecific HER2 antibody disrupts ligand-induced HER2:HER3 dimerization.
- [00511] E94. An anti-HER2 binding molecule comprising an immunoglobulin heavy chain (VH) and an immunoglobulin light chain (VL), wherein the binding molecule comprises:
- (i) VH-CDR1 comprising the amino acids of SEQ ID NO: 1;
 - (ii) VH-CDR2 comprising the amino acids of SEQ ID NO: 2;
 - (iii) VH-CDR3 comprising the amino acids of SEQ ID NO: 3;
 - (iv) VL-CDR1 comprising the amino acids of SEQ ID NO: 4;
 - (v) VL-CDR2 comprising the amino acids of SEQ ID NO: 5; and,
 - (vi) VL-CDR3 comprising the amino acids of SEQ ID NO: 6.
- [00512] E95. An anti-HER2 binding molecule comprising an immunoglobulin heavy chain (VH) and an immunoglobulin light chain (VL), wherein the VH comprises the amino acids of SEQ ID NO:15.
- [00513] E96. An anti-HER2 binding molecule comprising a VH and a VL, wherein the VL comprises the amino acids of SEQ ID NO:16.
- [00514] E97. The binding molecule of E94 or E95 or E96, wherein the VH comprises the amino acids of SEQ ID NO:15 and the VL comprises the amino acids of SEQ ID NO:16.
- [00515] E98. The binding molecule of any one of E94 to E97 comprising an antibody, or antigen-binding fragment thereof.
- [00516] E99A. An antibody-drug conjugate (ADC) comprising the bispecific HER2 antibody according to any one E1 to E93 or the anti-HER2 binding molecule according to any of E94 to E98 and at least one therapeutic moiety.
- [00517] E100A. The ADC according to E99A, further comprising at least one optional spacer.
- [00518] E101A. The ADC according to E100A, where at least one spacer is a peptidic spacer.
- [00519] E102A. The ADC according to E100A, wherein at least one spacer is a nonpeptidic spacer.

- [00520] E103A. The ADC according to any one of E99A to E102A, comprising two, three, or four therapeutic moieties.
- [00521] E104A. The ADC according to any one of E99A to E103A, where all therapeutic moieties are the same.
- [00522] E105A. The ADC according to any one of E99A to E104A, wherein each therapeutic moiety is chemically conjugated to the side chain of an amino acid at a specific position in the Fc region of the bispecific antibody.
- [00523] E106A. The ADC according to E105A, where the specific positions are selected from the group consisting of 239, 248, 254, 258, 273, 279, 282, 284, 286, 287, 289, 297, 298, 312, 324, 326, 330, 335, 337, 339, 350, 355, 356, 359, 360, 361, 375, 383, 384, 389, 398, 400, 413, 415, 418, 422, 435, 440, 441, 442, 443, 446, an insertion between positions 239 and 240, and combinations thereof, wherein the amino acid position numbering is according to the EU index as set forth in Kabat.
- [00524] E107A. The ADC according to E105A or 106A, wherein the specific positions are 239, 442, or both, wherein the amino acid position numbering is according to the EU index as set forth in Kabat.
- [00525] E108A. The ADC according to E105A or 106A, wherein the specific positions are 442 and an amino acid insertion between positions 239 and 240, wherein the amino acid position numbering is according to the EU index as set forth in Kabat.
- [00526] E109A. The ADC according to E105A to E108A, wherein the amino acid side chain is a sulfhydryl side chain.
- [00527] E110A. An ADC comprising the bispecific HER2 antibody according to any one of E1 to E98, wherein said antibody comprises:
- (i) a first polypeptide chain comprising the amino acids of SEQ ID NO: 32A and a second polypeptide chain comprising the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises a therapeutic moiety covalently linked to a cysteine amino acid at position 239, wherein the amino acid position numbering is according to the EU index as set forth in Kabat;
 - (ii) a first polypeptide chain comprising the amino acids of SEQ ID NO:33A and a second polypeptide chain comprising the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises two therapeutic moieties covalently linked to cysteine

amino acids respectively located at positions 239 and 442, wherein the amino acid position numbering is according to the EU index as set forth in Kabat;

(iii) a first polypeptide chain comprising the amino acids of SEQ ID NO:36A and a second polypeptide chain comprising the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises a therapeutic moiety covalently linked to a cysteine amino acid at position 239, wherein the amino acid position numbering is according to the EU index as set forth in Kabat;

(iv) a first polypeptide chain comprising the amino acids of SEQ ID NO:37A and a second polypeptide chain comprising the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises two therapeutic moieties covalently linked to cysteine amino acids respectively located at positions 239 and 442, wherein the amino acid position numbering is according to the EU index as set forth in Kabat;

(v) a first polypeptide chain comprising the amino acids of SEQ ID NO:40A and a second polypeptide chain comprising the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises a therapeutic moiety covalently linked to a cysteine amino acid at position 239, wherein the amino acid position numbering is according to the EU index as set forth in Kabat; or

(vi) a first polypeptide chain comprising the amino acids of SEQ ID NO:41A and a second polypeptide chain comprising the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises two therapeutic moieties covalently linked to cysteine amino acids respectively located at positions 239 and 442, wherein the amino acid position numbering is according to the EU index as set forth in Kabat.

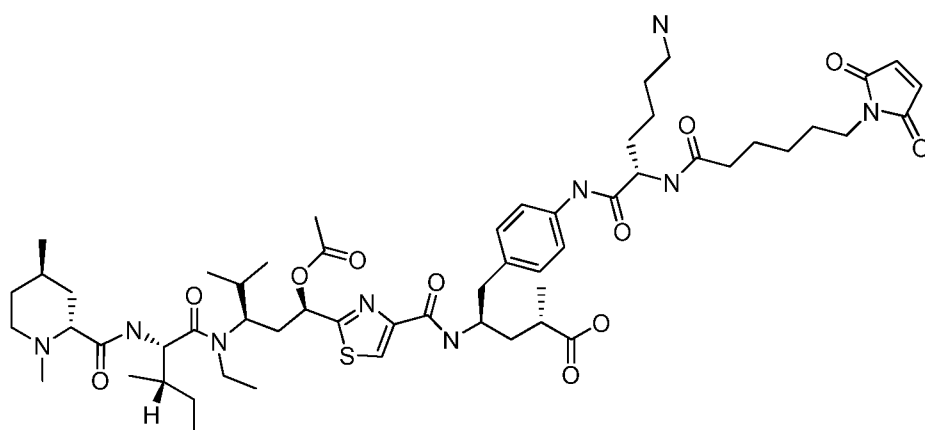
[00528] E111A1. An ADC comprising the bispecific HER2 antibody according to any one of claims 1 to 113, wherein said antibody comprises:

(i) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:32C and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises a therapeutic moiety covalently linked to a cysteine amino acid inserted between positions 239 and 240, wherein the amino acid position numbering is according to the EU index as set forth in Kabat;

- (ii) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:33C and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises two therapeutic moieties covalently linked to a cysteine amino inserted between positions 239 and 240 and a cysteine amino acid located at position 442, wherein the amino acid position numbering is according to the EU index as set forth in Kabat;
- (iii) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:36C and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises a therapeutic moiety covalently linked to a cysteine amino acid inserted between positions 239 and 240, wherein the amino acid position numbering is according to the EU index as set forth in Kabat;
- (iv) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:37C and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises two therapeutic moieties covalently linked to a cysteine amino inserted between positions 239 and 240 and a cysteine amino acid located at position 442, wherein the amino acid position numbering is according to the EU index as set forth in Kabat;
- (v) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:40C and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises a therapeutic moiety covalently linked to a cysteine amino acid inserted between positions 239 and 240, wherein the amino acid position numbering is according to the EU index as set forth in Kabat; or,
- (vi) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:41C and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises two therapeutic moieties covalently linked to a cysteine amino inserted between positions 239 and 240 and a cysteine amino acid located at position 442, wherein the amino acid position numbering is according to the EU index as set forth in Kabat.

- [00529] E112A. The ADC according to any one of E99A to E111A, wherein the therapeutic moiety comprises a cytotoxin, a radioisotope, a radioisotope, an immunomodulator, a cytokine, a lymphokine, a chemokine, a growth factor, a tumor necrosis factor, a hormone, a hormone antagonist, an enzyme, an oligonucleotide, a DNA, an RNA, an siRNA, an RNAi, a microRNA, a photoactive therapeutic agent, an anti-angiogenic agent, a pro-apoptotic agent, a peptide, a lipid, a carbohydrate, a chelating agent, or combinations thereof.
- [00530] E113A. The ADC according to E112A, wherein the cytotoxin is a tubulysin, an auristatin, a maytansinoid or a pyrrolobenzodiazepine (PBD).
- [00531] E114. An isolated nucleic acid molecule or a set of nucleic acid molecules encoding a bispecific HER2 antibody according to any one of E1 to E93 or an anti-HER2 binding molecule according to any one of E94 to E98, or a complement thereof.
- [00532] E115. A vector or a set of vectors comprising the nucleic acid molecule or set of the nucleic acid molecules of E114, or a complement thereof.
- [00533] E116. A host cell comprising an isolated nucleic acid molecule or a set of nucleic acid molecules according to E114, or the vector or set of vectors according to E115.
- [00534] E117. A host cell expressing a bispecific HER2 antibody according to any one of E1 to E93 or an anti-HER2 binding molecule according to any one of E94 to E98.
- [00535] E118. A method for producing a bispecific HER2 antibody according to any one of E1 to E93 or an anti-HER2 binding molecule according to any one of E94 to E98 comprising culturing the host cell according to any one of E116 or E117 and recovering the antibody from the culture medium.
- [00536] E119. A pharmaceutical composition comprising a bispecific HER2 antibody according to any one of E1 to E93, an anti-HER2 binding molecule according to any one of E94 to E98, or an ADC according to any one of E99A to E113A1, and a pharmaceutically acceptable carrier.
- [00537] E120. A method of treating a HER2-expressing cancer comprising administering a bispecific HER2 antibody according to any one of E1 to E93, an anti-HER2 binding molecule according to any one of E94 to E98, an ADC according to any one of E99A to E113A1 or the composition according to E119 to a subject in need thereof.

- [00538] E121. The method according to E120, wherein the cancer is a low HER2-expressing cancer.
- [00539] E122. The method according to any one of E120 or E121, further comprising administering at least one additional therapeutic agent.
- [00540] E123. The method according to E122, wherein the at least one additional therapeutic agent is a radionuclide or a chemotherapeutic agent.
- [00541] E124. A method to target a therapeutic moiety to cells expressing HER2 comprising administering the therapeutic moiety fused or conjugated to a bispecific HER2 antibody according to any one of E1 to E93, an anti-HER2 binding molecule according to any one of E94 to E98, or an ADC according to any one of E99A to E113A1.
- [00542] E125. A method to increase the activity of a therapeutic moiety comprising conjugating the moiety to a bispecific HER2 antibody according to any one of E1 to E93, an anti-HER2 binding molecule according to any one of E94 to E98, or an ADC according to any one of E99A to E113A1.
- [00543] E126. A method to improve the pharmacokinetic properties of a therapeutic moiety comprising conjugating the moiety to a bispecific HER2 antibody according to any one of E1 to E93, an anti-HER2 binding molecule according to any one of E94 to E98, or an ADC according to any one of E99A to E113A1.
- [00544] E127. The method according to any of E122 to E126, wherein the therapeutic moiety is a cytotoxin, a radioisotope, an immunomodulator, a cytokine, a lymphokine, a chemokine, a growth factor, a tumor necrosis factor, a hormone, a hormone antagonist, an enzyme, an oligonucleotide, a DNA, an RNA, an siRNA, an RNAi, a microRNA, a photoactive therapeutic agent, an anti-angiogenic agent, a pro-apoptotic agent, a peptide, a lipid, a carbohydrate, or a chelating agent.
- [00545] E128. The method according to E127, wherein the cytotoxin is a tubulysin, an auristatin, a maytansinoid or a pyrrolobenzodiazepine (PBD).
- [00546] E129. The ADC according to E113A or the method according to E128, wherein the cytotoxin is tubulysin 1508 having the following structure:



[00547] E130. A method to treat resistance to a HER2-targeting therapeutic agent comprising administering a bispecific HER2 antibody according to any one of E1 to E93, an anti-HER2 binding molecule according to any one of E94 to E98, or an ADC according to any one of E99A to E113A to a patient in need thereof.

XV. Sequences

[00548] Table 3 below provides the sequence reference number (SEQ ID NO:), amino acid sequence and comments regarding the sequences.

TABLE 3

SEQ ID NO	SEQUENCE	Comments
1	SYSMS	VH-CDR1 from affinity optimized S39 antibody
2	SISSSSSYIYYADSVKG	VH-CDR2 from affinity optimized S39 antibody
3	GGDAYNYYYFDY	VH-CDR3 from affinity optimized S39 antibody
4	KSSQSVFFRSNNKNILA	VL-CDR1 from affinity optimized S39 antibody
5	WASSRES	VL-CDR2 from affinity optimized S39 antibody
6	QQYFGSPFT	VL-CDR3 from affinity optimized S39 antibody
7	EVQLVESGGGLVKPGGSLRLSCAASGFTFS	VH-FR1 from affinity optimized S39 antibody
8	WVRQAPGKGLEWVS	VH-FR2 from affinity optimized S39 antibody
9	RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR	VH-FR3 from affinity optimized S39 antibody
10	WGQGTLLVTVSS	VH-FR4 from affinity optimized S39 antibody

SEQ ID NO	SEQUENCE	Comments
11	DIVMTQTPLSLSVTPGQPASISC	VL-FR1 from affinity optimized S39 antibody
12	WYLQKPGQPPQLLIY	VL-FR2 from affinity optimized S39 antibody
13	GVPDRFSGSGSGTDFTLKISRVEAEDVGVIYC	VL-FR3 from affinity optimized S39 antibody
14	FGPGTKVDIK	VL-FR4 from affinity optimized S39 antibody
15	EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYSMSWVRQAPGKLEWVSS ISSSSSYIYYADSVKGRFTISRDNKNSLYLQMNSLRAEDTAVYYCARGG DAYNYYYFDYWGGQTLVTVSS	VH2 – VH from clone 39S antibody
16	DIVMTQTPLSLSVTPGQPASISCKSSQSVFFRSNNKNILAWYLQKPGQPP QLLIYWASSRESGVDRFSGSGSGTDFTLKISRVEAEDVGVIYCQYFSGS PFTFGPGTKVDIK	VL2 – VL from clone 39S antibody
17	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGK X LEWVAR IYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWG GDGFYAMDYWGQTLVTVSS	VH1 – VH of the Domain IV scFv X in SEQ ID NO: 17 is K or C
18	DIQMTQSPSSLSASVGVDRVITTCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGSRSGTDFTLTISSLPQEDFATYYCQQHYTTPPTFG X GTKVEIK	VL1 – VL from of the Domain IV scFv X in SEQ ID NO:18 is Q or C
19	GGGGSGGGSGGGSGGGGS	L _x linker, x = 1-4 - scFv Linker – Located between VL and VH in scFv
20	GGGGSGGGSGGGSGGGGS	L _x linker, x = 1-4 - Used in Bs2Ab- FCC and Bs2Ab-FC constructs
21	EPKSC G KTGGGGSGGGGS	L _x linker, x = 1-4 - Used in Bs4Ab- FCC and Bs4Ab-FC constructs
22	SGGGSGGGSGGGGS	L _x linker, x = 1-4 - Hinge
23	CPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKF NWWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN KALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPS DIAVEWESNGQPENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSC SVMHEALHNHYTQKSLSLSPGK	Native IgG1 Fc Fc portion can include EU residues 226 to its carboxyl-terminus. The highlighted positions correspond to sites of known allelic variation.
24A	CPPCPAPE E LG G PSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKF NWWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN KALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPS DIAVEWESNGQPENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSC SVMHEALHNHYTQKSLSLSPGK	Fc IgG1 L234F S239C (“FC”), for ADC constructs. Mutations within boxes. The highlighted positions correspond to sites of known allelic variation.
24B	CPPCPAPELLGGP D VFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKF NWWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN KALPAP E EKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPS DIAVEWESNGQPENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSC SVMHEALHNHYTQKSLSLSPGK	Fc IgG1 S239D I332E, for ADCC constructs. Mutations within boxes. The highlighted positions correspond to sites of known allelic variation.
24C	CPPCPAPELLGGPS C VFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKF FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVS NKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYP SDIAVEWESNGQPENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSC CSVMHEALHNHYTQKSLSLSPGK	Fc IgG1 C239-ins, for ADC constructs. Mutations within boxes. The highlighted positions correspond to sites of known allelic variation.
25A	CPPCPAPE E LG G PSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKF NWWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN KALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPS DIAVEWESNGQPENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSC	Fc IgG1 L234F S239C S442C (“FCC”), for ADC constructs. Mutations within boxes. The highlighted positions correspond to

SEQ ID NO	SEQUENCE	Comments
	SVMHEALHNHYTQKSL <u>C</u> LSPGK	sites of known allelic variation.
25B	CPPCPAPELLGGP <u>D</u> VFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP <u>P</u> <u>E</u> EKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFC	Fc IgG1 S239D A330L I332E, for ADCC constructs. Mutations within boxes. The highlighted positions correspond to sites of known allelic variation.
25C	CPPCPAPELLGGP <u>S</u> VFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFC	Fc IgG1 C239-ins S442C, for ADC constructs. Mutations within boxes. The highlighted positions correspond to sites of known allelic variation.
26	EPKSCDKTHT	IgG1 hinge present in Bs2 and Bs3
27A	RTVAAPSVFIFPPSDEQLKSGTASVCLLNFFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC	CL kappa. The highlighted positions correspond to sites of known allelic variation.
27B	QPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC	CL lambda. The highlighted positions correspond to sites of known allelic variation.
28	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKX ₁ LEWVARITYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGLTVTVSSGGGGSGGGSGGGSGGGSDIQMTQSPSLSASVGDRTVITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFYLSGVP	Trastuzumab scFv sequence. X ₁ = K or C; X ₂ = Q or C. In disulfide-stabilized scFv X ₁ = X ₂ = C.
29	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTWNSGALTSKVHTFPFAVLQSSGLYSLSSVTVTPSSSLGTQTYICNVNHKPSNTKVDKRV	CH1 from 39S antibody – EU residues 118 to 215. The highlighted positions correspond to sites of known allelic variation.
30	DIQMTQSPSSLSASVGDRTVITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFYLSGVPVPSRFGSGRSGTDFTLTITSSLPQEDFATYYCQQHYTTPPTFGCGTKVEIKGGGGSGGGSGGGSGGGSGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKCLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGLTVTVSSGGGGSGGGSGGGSEVQLVESGGGLVKPGGSLRLSCAASGFTFSYSSMSWVRQAPGKLEWVSSISSSSYIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARGGDAYNYYYFDYWGQGLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTWNSGALTSKVHTFPFAVLQSSGLYSLSSVTVTPSSSLGTQTYICNVNHKPSNTKVDKRVPEPKSCDKTHTCPPCPAPELLGGP <u>S</u> VFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP <u>A</u> <u>P</u> <u>I</u> <u>E</u> KTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFC	Heavy chain of Bs2Ab-native Fc construct. The underlined positions indicate where certain substitutions may be made. The highlighted positions correspond to sites of known allelic variation.
31A	DIQMTQSPSSLSASVGDRTVITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFYLSGVPVPSRFGSGRSGTDFTLTITSSLPQEDFATYYCQQHYTTPPTFGCGTKVEIKGGGGSGGGSGGGSGGGSGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKCLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGLTVTVSSGGGGSGGGSGGGSEVQLVESGGGLVKPGGSLRLSCAASGFTFSYSSMSWVRQAPGKLEWVSSISSSSYIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARGGDAYNYYYFDYWGQGLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTWNSGALTSKVHTFPFAVLQSSGLYSLSSVTVTPSSSLGTQTYICNVNHKPSNTKVDKRVPEPKSCDKTHTCPPCPAPELLGGP <u>S</u> VFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP <u>A</u> <u>P</u> <u>I</u> <u>E</u> KTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFC	Heavy chain of Bs2Ab2 with L234F ("F"). Construct for ADC. The highlighted positions correspond to sites of known allelic variation.

SEQ ID NO	SEQUENCE	Comments
	TSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPE <u>F</u> LGGP <u>S</u> VFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP <u>A</u> <u>P</u> <u>I</u> <u>E</u> <u>K</u> TISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVSCSVMHEALHNHYTQKSL <u>S</u> LSPGK	
31B	DIQMTQSPSSLSASVGDRTTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSGSGSGSGSGSGGSEVQLVESGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKCLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGTLLTVSSGGGSGGGGSGGGGSEVQLVESGGLVQPGGSLRLSCAASGFTFSSYSMSWVRQAPGKGLEWVSSISSSSSYIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARGGDAYNYYYFDYWGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPE <u>L</u> LGGP <u>S</u> VFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP <u>A</u> <u>P</u> <u>E</u> <u>E</u> <u>K</u> TISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVSCSVMHEALHNHYTQKSL <u>S</u> LSPGK	Heavy chain of Bs2Ab2 with I332E. Construct with enhanced ADCC. The highlighted positions correspond to sites of known allelic variation.
32A	DIQMTQSPSSLSASVGDRTTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSGSGSGSGSGSGGSEVQLVESGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKCLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGTLLTVSSGGGSGGGGSGGGGSEVQLVESGGLVQPGGSLRLSCAASGFTFSSYSMSWVRQAPGKGLEWVSSISSSSSYIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARGGDAYNYYYFDYWGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPE <u>F</u> LGGP <u>C</u> VFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP <u>A</u> <u>P</u> <u>I</u> <u>E</u> <u>K</u> TISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVSCSVMHEALHNHYTQKSL <u>S</u> LSPGK	Heavy chain of Bs2Ab with L234F, S239C ("FC"). Construct for ADC. The highlighted positions correspond to sites of known allelic variation.
32B	DIQMTQSPSSLSASVGDRTTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSGSGSGSGSGSGGSEVQLVESGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKCLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGTLLTVSSGGGSGGGGSGGGGSEVQLVESGGLVQPGGSLRLSCAASGFTFSSYSMSWVRQAPGKGLEWVSSISSSSSYIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARGGDAYNYYYFDYWGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPE <u>L</u> LGGP <u>D</u> VFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP <u>A</u> <u>P</u> <u>E</u> <u>E</u> <u>K</u> TISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVSCSVMHEALHNHYTQKSL <u>S</u> LSPGK	Heavy chain of Bs2Ab with S239D and I332E. Construct with enhanced ADCC. The highlighted positions correspond to sites of known allelic variation.
32C	DIQMTQSPSSLSASVGDRTTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSGSGSGSGSGSGGSEVQLVESGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKCLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGTLLTVSSGGGSGGGGSGGGGSEVQLVESGGLVQPGGSLRLSCAASGFTFSSYSMSWVRQAPGKGLEWVSSISSSSSYIYYADSVKGRFTISRDNAKNSLYLQMNSLRA	Heavy chain of Bs2Ab with C239-ins ("C"). Construct for ADC. The highlighted positions correspond to sites of known allelic variation.

SEQ ID NO	SEQUENCE	Comments
	EDTAVYYCARGGDAYNYYYFDYWGQGT ^L LVTVSSASTKGPSVF ^L PLAPSSKSTSGGTAALGCLVKDYFPEPVT ^S WNSGALTSGVHTFPAVLQSSGLYSLSSVTV ^P SSSLGTQTYICNVNHKPSNTKVDKRV ^E PKSCDKTHTCPPCPAPE ^L LGGPS ^C VFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAP ^I IEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNV ^F SCSV ^M MHEALH ^N HYTQKSL ^S LSPGK	
33A	DIQMTQSPSSLSASVGDRTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTITSSSLQPEDFATYYCQQHYTTPPTFGCGTKVEIKGGGGSGGGSGGGSGGGSGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKCLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT ^L LVTVSSGGGGSGGGSGGGSGGGSEVQLVESGGGLVQPGGSLRLSCAASGFTFSYSMSWVRQAPGKGLEWVSSISSSSSYIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARGGDAYNYYYFDYWGQGT ^L LVTVSSASTKGPSVF ^L PLAPSSKSTSGGTAALGCLVKDYFPEPVT ^S WNSGALTSGVHTFPAVLQSSGLYSLSSVTV ^P SSSLGTQTYICNVNHKPSNTKVDKRV ^E PKSCDKTHTCPPCPAPE ^F LGGP ^C VFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAP ^I IEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNV ^F SCSV ^M MHEALHNHYTQKSL ^C LSPGK	Heavy chain of Bs2Ab with L234F, S239C and S442C ("FCC"). Construct for ADC. The highlighted positions correspond to sites of known allelic variation.
33B	DIQMTQSPSSLSASVGDRTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTITSSSLQPEDFATYYCQQHYTTPPTFGCGTKVEIKGGGGSGGGSGGGSGGGSGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKCLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT ^L LVTVSSGGGGSGGGSGGGSGGGSEVQLVESGGGLVQPGGSLRLSCAASGFTFSYSMSWVRQAPGKGLEWVSSISSSSSYIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARGGDAYNYYYFDYWGQGT ^L LVTVSSASTKGPSVF ^L PLAPSSKSTSGGTAALGCLVKDYFPEPVT ^S WNSGALTSGVHTFPAVLQSSGLYSLSSVTV ^P SSSLGTQTYICNVNHKPSNTKVDKRV ^E PKSCDKTHTCPPCPAPE ^L LGGP ^D VFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP ^L P ^E EKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNV ^F SCSV ^M MHEALHNHYTQKSL ^S LSPGK	Heavy chain of Bs2Ab with S239D, A330L, and I332E. Construct with enhanced ADCC. The highlighted positions correspond to sites of known allelic variation.
33C	DIQMTQSPSSLSASVGDRTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTITSSSLQPEDFATYYCQQHYTTPPTFGCGTKVEIKGGGGSGGGSGGGSGGGSGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKCLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT ^L LVTVSSGGGGSGGGSGGGSGGGSEVQLVESGGGLVQPGGSLRLSCAASGFTFSYSMSWVRQAPGKGLEWVSSISSSSSYIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARGGDAYNYYYFDYWGQGT ^L LVTVSSASTKGPSVF ^L PLAPSSKSTSGGTAALGCLVKDYFPEPVT ^S WNSGALTSGVHTFPAVLQSSGLYSLSSVTV ^P SSSLGTQTYICNVNHKPSNTKVDKRV ^E PKSCDKTHTCPPCPAPE ^L LGGPS ^C VFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAP ^I IEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNV ^F SCSV ^M MHEALH ^N HYTQKSL ^C LSPGK	Heavy chain of Bs2Ab with C239-ins and S442C ("CC"). Construct for ADC. The highlighted positions correspond to sites of known allelic variation.
34	EVQLVESGGGLVQPGGSLRLSCAASGFTFSYSMSWVRQAPGKGLEWVSSISSSSSYIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARGGDAYNYYYFDYWGQGT ^L LVTVSSASTKGPSVF ^L PLAPSSKSTSGGTAALGCLVKDYFPEPVT ^S WNSGALTSGVHTFPAVLQSSGLYSLSSVTV ^P SSSLGTQTYICNVNHKPSNTKVDKRV ^E PKSCDKTHTCPPCPAPE ^L LGGP ^S VFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY	Heavy chain of Bs3Ab native Fc. The highlighted positions correspond to sites of known allelic variation.

SEQ ID NO	SEQUENCE	Comments
	NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPTEKTIKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFFLYSKLTVDKSRWQQGNVSCFVSVMHEALHNHYTQKSLSLSPGKGGGSGGGGSGGGGSDIQMTQSPSSLSASVGDRTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFVLYSGVPSRFSGSRSGTDFTLTISSSLQPEDFATYYCQQHYTTPPTFGCGTKVEIKGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFTNIDKYIHWRQAPGKCLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRADTAVYYCSRWGGDGFYAMDYWGQGTLLVTVSS	
35A	EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYSMSWVRQAPGKGLEWVSSISSSSSYIYYADSVKGRFTISRDNANKNSLYLQMNSLRADTAVYYCARGGDAYNYYYFDYWGQGTLLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKRVPEPKSCDKTHTCPPCPAPEFLGGP ^S VFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPTEKTIKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFFLYSKLTVDKSRWQQGNVSCFVSVMHEALHNHYTQKSLSLSPGKGGGSGGGGSGGGGSDIQMTQSPSSLSASVGDRTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFVLYSGVPSRFSGSRSGTDFTLTISSSLQPEDFATYYCQQHYTTPPTFGCGTKVEIKGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFTNIDKYIHWRQAPGKCLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRADTAVYYCSRWGGDGFYAMDYWGQGTLLVTVSS	Heavy chain of Bs3Ab with L234F ("F"). Construct for ADC. The highlighted positions correspond to sites of known allelic variation.
35B	EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYSMSWVRQAPGKGLEWVSSISSSSSYIYYADSVKGRFTISRDNANKNSLYLQMNSLRADTAVYYCARGGDAYNYYYFDYWGQGTLLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKRVPEPKSCDKTHTCPPCPAPE ^L LGGP ^S VFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPTEKTIKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFFLYSKLTVDKSRWQQGNVSCFVSVMHEALHNHYTQKSLSLSPGKGGGSGGGGSGGGGSDIQMTQSPSSLSASVGDRTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFVLYSGVPSRFSGSRSGTDFTLTISSSLQPEDFATYYCQQHYTTPPTFGCGTKVEIKGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFTNIDKYIHWRQAPGKCLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRADTAVYYCSRWGGDGFYAMDYWGQGTLLVTVSS	Heavy chain of Bs3Ab with I332E. Construct with enhanced ADCC. The highlighted positions correspond to sites of known allelic variation.
36A	EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYSMSWVRQAPGKGLEWVSSISSSSSYIYYADSVKGRFTISRDNANKNSLYLQMNSLRADTAVYYCARGGDAYNYYYFDYWGQGTLLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKRVPEPKSCDKTHTCPPCPAPEFLGGP ^C VFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPTEKTIKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFFLYSKLTVDKSRWQQGNVSCFVSVMHEALHNHYTQKSLSLSPGKGGGSGGGGSGGGGSDIQMTQSPSSLSASVGDRTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFVLYSGVPSRFSGSRSGTDFTLTISSSLQPEDFATYYCQQHYTTPPTFGCGTKVEIKGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFTNIDKYIHWRQAPGKCLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRADTAVYYCSRWGGDGFYAMDYWGQGTLLVTVSS	Heavy chain of Bs3Ab with L234F and S239C ("FC"). Construct for ADC. The highlighted positions correspond to sites of known allelic variation.
36B	EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYSMSWVRQAPGKGLEWVSSISSSSSYIYYADSVKGRFTISRDNANKNSLYLQMNSLRADTAVYYCARGGDAYNYYYFDYWGQGTLLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKRVPEPKSCDKTHTCPPCPAPE ^L LGGP ^D VFLFPPK	Heavy chain of Bs3Ab with S239D and I332E. Construct with enhanced ADCC. The highlighted positions correspond to sites of known allelic variation.

SEQ ID NO	SEQUENCE	Comments
	PKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP <u>AP</u> EKTISKAKGQPREP QVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP VLDSGDSFFLYSKLTVDKSRWQQGNVSCSVMHEALHNHYTQKSLS <u>L</u> SPG KGGGSGGGGSGGGGSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVA WYQQKPGKAPKLLIYSASF ¹ LYSGVPSRFSGSRSGTDFLTITSSLPEDFA TYYCQQHYHTTPPTFGCGTKVEIKGGGSGGGGSGGGGSGGGGSEVQLVES GGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKCLEWVARIYPTNGY TRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAM DYWGQGT ² LVTVSS	
36C	EVQLVESGGGLVKPGGSLRLSCAASGFTFSSYSMSWVRQAPGKGLEWVSS ISSSSSYIYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARGG DAYNYYYFDYWGQGT ² LVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLV KDYFPEPVTISWNSGALTSGVHTFPAVLQSSGLYSLSSVTVTPSSSLGTQ TYICNVNHKPSNTKVDKRVPEPKSCDKTHTCPPCPAPE <u>L</u> GGPS <u>C</u> VFLFPP PKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP <u>AP</u> EKTISKAKGQPRE PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP PVLDSGDSFFLYSKLTVDKSRWQQGNVSCSVMHEALHNHYTQKSLS <u>L</u> SP GKGGGSGGGGSGGGGSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAV AWYQQKPGKAPKLLIYSASF ¹ LYSGVPSRFSGSRSGTDFLTITSSLPEDF ATYYCQQHYHTTPPTFGCGTKVEIKGGGSGGGGSGGGGSGGGGSEVQLVE SGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKCLEWVARIYPTNG YTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYA MDYWGQGT ² LVTVSS	Heavy chain of Bs3Ab with L234F and C239-ins ("C"). Construct for ADC. The highlighted positions correspond to sites of known allelic variation.
37A	EVQLVESGGGLVKPGGSLRLSCAASGFTFSSYSMSWVRQAPGKGLEWVSS ISSSSSYIYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARGG DAYNYYYFDYWGQGT ² LVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLV KDYFPEPVTISWNSGALTSGVHTFPAVLQSSGLYSLSSVTVTPSSSLGTQ TYICNVNHKPSNTKVDKRVPEPKSCDKTHTCPPCPAPE <u>F</u> LGGP <u>C</u> VFLFPPK PKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP <u>AP</u> EKTISKAKGQPREP QVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP VLDSGDSFFLYSKLTVDKSRWQQGNVSCSVMHEALHNHYTQKSLS <u>C</u> LSPG KGGGSGGGGSGGGGSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVA WYQQKPGKAPKLLIYSASF ¹ LYSGVPSRFSGSRSGTDFLTITSSLPEDFA TYYCQQHYHTTPPTFGCGTKVEIKGGGSGGGGSGGGGSGGGGSEVQLVES GGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKCLEWVARIYPTNGY TRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAM DYWGQGT ² LVTVSS	Heavy chain of Bs3Ab with L234F S239C and S442C ("FC"). Construct for ADC. The highlighted positions correspond to sites of known allelic variation.
37B	EVQLVESGGGLVKPGGSLRLSCAASGFTFSSYSMSWVRQAPGKGLEWVSS ISSSSSYIYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARGG DAYNYYYFDYWGQGT ² LVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLV KDYFPEPVTISWNSGALTSGVHTFPAVLQSSGLYSLSSVTVTPSSSLGTQ TYICNVNHKPSNTKVDKRVPEPKSCDKTHTCPPCPAPE <u>L</u> GGP <u>D</u> VFLFPPK PKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP <u>LP</u> EKTISKAKGQPREP QVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP VLDSGDSFFLYSKLTVDKSRWQQGNVSCSVMHEALHNHYTQKSLS <u>L</u> SPG KGGGSGGGGSGGGGSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVA WYQQKPGKAPKLLIYSASF ¹ LYSGVPSRFSGSRSGTDFLTITSSLPEDFA TYYCQQHYHTTPPTFGCGTKVEIKGGGSGGGGSGGGGSGGGGSEVQLVES GGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKCLEWVARIYPTNGY TRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAM DYWGQGT ² LVTVSS	Heavy chain of Bs3Ab with S239D, A330L, and I332E. Construct with enhanced ADCC. The highlighted positions correspond to sites of known allelic variation.
37C	EVQLVESGGGLVKPGGSLRLSCAASGFTFSSYSMSWVRQAPGKGLEWVSS ISSSSSYIYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARGG DAYNYYYFDYWGQGT ² LVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLV KDYFPEPVTISWNSGALTSGVHTFPAVLQSSGLYSLSSVTVTPSSSLGTQ	Heavy chain of Bs3Ab with C239-ins and S442C ("CC"). Construct for ADC. The highlighted positions correspond to sites of known allelic

SEQ ID NO	SEQUENCE	Comments
	TYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPE <u>L</u> GGPS <u>C</u> VFLFPP KPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ YNSTYRVVSVLTVHLQDWLNGKEYCKKVSNAKALPA <u>P</u> IEKTISKAKGQPRE PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTP PVLDSGDSFFLYSKLTVDKSRWQQGNVSCSVMHEALHNHYTQKSLS <u>C</u> LSP GKGGGGSGGGSGGGGSDIQMTQSPSSLSASVGDRTITCRASQDVNTAV AWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISLQPEDF ATYYCQQHYTTPPTFGCGTKVEIKGGGGSGGGSGGGSGGGSGGGSEVQLVE SGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKCLEWVARIYPTNG YTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYA MDYWGQGTLLTVSS	variation.
38	EVQLVESGGGLVKPGGSLRLSCAASGFTFSYSSMSWVRQAPGKLEWVSS ISSSSSYIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARGG DAYNYYYFDYWGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLV KDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ TYICNVNHKPSNTKVDKRVEPKSCGKTGGGGSGGGGSDIQMTQSPSSLSA SVGDRTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSG SRSGTDFTLTISLQPEDFATYYCQQHYTTPPTFGCGTKVEIKGGGGSG GGGGSGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHW VRQAPGKCLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSL RAEDTAVYYCSRWGGDGFYAMDYWGQGTLLTVSSSGGGSGGGGSCPPCP APE <u>L</u> GGP <u>S</u> VFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVD GVEVHNAKTKPREEQYNSTYRVVSVLTVHLQDWLNGKEYCKKVSNAKALPA P <u>I</u> EKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVSCSVMHE ALHNHYTQKSLS <u>S</u> LSPGK	Heavy chain of Bs4Ab-native Fc. The highlighted positions correspond to sites of known allelic variation.
39A	EVQLVESGGGLVKPGGSLRLSCAASGFTFSYSSMSWVRQAPGKLEWVSS ISSSSSYIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARGG DAYNYYYFDYWGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLV KDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ TYICNVNHKPSNTKVDKRVEPKSCGKTGGGGSGGGGSDIQMTQSPSSLSA SVGDRTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSG SRSGTDFTLTISLQPEDFATYYCQQHYTTPPTFGCGTKVEIKGGGGSG GGGGSGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHW VRQAPGKCLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSL RAEDTAVYYCSRWGGDGFYAMDYWGQGTLLTVSSSGGGSGGGGSCPPCP APE <u>F</u> LGGP <u>S</u> VFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVD GVEVHNAKTKPREEQYNSTYRVVSVLTVHLQDWLNGKEYCKKVSNAKALPA P <u>I</u> EKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVSCSVMHE ALHNHYTQKSLS <u>S</u> LSPGK	Heavy chain of Bs4Ab with L234F ("F"). Construct for ADC. The highlighted positions correspond to sites of known allelic variation.
39B	EVQLVESGGGLVKPGGSLRLSCAASGFTFSYSSMSWVRQAPGKLEWVSS ISSSSSYIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARGG DAYNYYYFDYWGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLV KDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ TYICNVNHKPSNTKVDKRVEPKSCGKTGGGGSGGGGSDIQMTQSPSSLSA SVGDRTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSG SRSGTDFTLTISLQPEDFATYYCQQHYTTPPTFGCGTKVEIKGGGGSG GGGGSGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHW VRQAPGKCLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSL RAEDTAVYYCSRWGGDGFYAMDYWGQGTLLTVSSSGGGSGGGGSCPPCP APE <u>L</u> GGP <u>S</u> VFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVD GVEVHNAKTKPREEQYNSTYRVVSVLTVHLQDWLNGKEYCKKVSNAKALPA P <u>E</u> EKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVSCSVMHE ALHNHYTQKSLS <u>S</u> LSPGK	Heavy chain Bs4Ab with I332E. Construct with enhanced ADCC. The highlighted positions correspond to sites of known allelic variation.
40A	EVQLVESGGGLVKPGGSLRLSCAASGFTFSYSSMSWVRQAPGKLEWVSS ISSSSSYIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARGG DAYNYYYFDYWGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLV	Heavy chain of Bs4Ab with L234F and S239C ("FC"). Construct for ADC. The highlighted positions

SEQ ID NO	SEQUENCE	Comments
	KDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQ TYICNVNHKPSNTKVDKRVKPKSCGKTGGGGSGGGGSDIQMTQSPSSLSA SVGDRVTITCRASQDVNTAVAWYQQKPKGAPKLLIYSASFLYSGVPSRF GSRSGTDFTLTISLQPEDFATYYCQQHYTTPPTFGCGTKVEIKGGGGSG GGGGSGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHW VRQAPGKCLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSL RAEDTAVYYCSRWGGDGFYAMDYWGQGLVTVSSSGGGSGGGGSCPPCP APEFLGGP <u>C</u> VFLLFPKPKDLMISRTPEVTCVVVDVSHEDPEVKFNWYVD GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP <u>A</u> <u>P</u> IEKTIKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHE ALHNHYTQKSLSLSPGK	correspond to sites of known allelic variation.
40B	EVQLVESGGGLVQPGGSLRLSCAASGFTFSYSMSWVRQAPGKLEWVSS ISSSSSYIYYADSVKGRFTISRDNANKNSLYLQMNSLRAEDTAVYYCARGG DAYNYYYFDYWGQGLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLV KDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQ TYICNVNHKPSNTKVDKRVKPKSCGKTGGGGSGGGGSDIQMTQSPSSLSA SVGDRVTITCRASQDVNTAVAWYQQKPKGAPKLLIYSASFLYSGVPSRF GSRSGTDFTLTISLQPEDFATYYCQQHYTTPPTFGCGTKVEIKGGGGSG GGGGSGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHW VRQAPGKCLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSL RAEDTAVYYCSRWGGDGFYAMDYWGQGLVTVSSSGGGSGGGGSCPPCP APEFLGGP <u>D</u> VFLLFPKPKDLMISRTPEVTCVVVDVSHEDPEVKFNWYVD GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP <u>A</u> <u>P</u> IEKTIKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHE ALHNHYTQKSLSLSPGK	Heavy chain of Bs4Ab with S239D and I332E for ADCC. Construct with enhanced ADCC. The highlighted positions correspond to sites of known allelic variation.
40C	EVQLVESGGGLVQPGGSLRLSCAASGFTFSYSMSWVRQAPGKLEWVSS ISSSSSYIYYADSVKGRFTISRDNANKNSLYLQMNSLRAEDTAVYYCARGG DAYNYYYFDYWGQGLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLV KDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQ TYICNVNHKPSNTKVDKRVKPKSCGKTGGGGSGGGGSDIQMTQSPSSLSA SVGDRVTITCRASQDVNTAVAWYQQKPKGAPKLLIYSASFLYSGVPSRF GSRSGTDFTLTISLQPEDFATYYCQQHYTTPPTFGCGTKVEIKGGGGSG GGGGSGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHW VRQAPGKCLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSL RAEDTAVYYCSRWGGDGFYAMDYWGQGLVTVSSSGGGSGGGGSCPPCP APEFLGGP <u>C</u> VFLLFPKPKDLMISRTPEVTCVVVDVSHEDPEVKFNWYVD DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP <u>A</u> PIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAV EWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMH EALHNHYTQKSLSLSPGK	Heavy chain of Bs4Ab with and C239-ins ("C"). Construct for ADC. The highlighted positions correspond to sites of known allelic variation.
41A	EVQLVESGGGLVQPGGSLRLSCAASGFTFSYSMSWVRQAPGKLEWVSS ISSSSSYIYYADSVKGRFTISRDNANKNSLYLQMNSLRAEDTAVYYCARGG DAYNYYYFDYWGQGLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLV KDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQ TYICNVNHKPSNTKVDKRVKPKSCGKTGGGGSGGGGSDIQMTQSPSSLSA SVGDRVTITCRASQDVNTAVAWYQQKPKGAPKLLIYSASFLYSGVPSRF GSRSGTDFTLTISLQPEDFATYYCQQHYTTPPTFGCGTKVEIKGGGGSG GGGGSGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHW VRQAPGKCLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSL RAEDTAVYYCSRWGGDGFYAMDYWGQGLVTVSSSGGGSGGGGSCPPCP APEFLGGP <u>C</u> VFLLFPKPKDLMISRTPEVTCVVVDVSHEDPEVKFNWYVD GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP <u>A</u> <u>P</u> IEKTIKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHE ALHNHYTQKSLSLSPGK	Heavy chain of Bs4Ab with L234F, S239C and S442C ("FCC"). Construct for ADC. The highlighted positions correspond to sites of known allelic variation.
41B	EVQLVESGGGLVQPGGSLRLSCAASGFTFSYSMSWVRQAPGKLEWVSS ISSSSSYIYYADSVKGRFTISRDNANKNSLYLQMNSLRAEDTAVYYCARGG	Heavy chain of Bs4Ab with S239D, A330L, and I332E. Construct with

SEQ ID NO	SEQUENCE	Comments
	DAYNYYYFDYWGGQTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTWNSGALTSGLVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCGKTGGGSGGGGSDIQMTQSPSSLSASVGRDVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFVLYSGVPSRFGSRSGTDFTLTITSSLPEDFATYYCQQHYTTPPTFGCGTKVEIKGGGSGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKCLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQTLVTVSSSGGGGSGGGGSCPPCPAPE <u>L</u> LGGS <u>D</u> VFLFPKPKDTLMISRTPVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP <u>L</u> <u>P</u> EEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSL <u>S</u> LSPGK	enhanced ADCC. The highlighted positions correspond to sites of known allelic variation.
41C	EVQLVESGGGLVQPGGSLRLSCAASGFTFSYSSMSWVRQAPGKLEWVSSISSSSSYIYYADSVKGRFTISRDNANKNSLYLQMNSLRAEDTAVYYCARGGDAYNYYYFDYWGGQTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTWNSGALTSGLVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCGKTGGGSGGGGSDIQMTQSPSSLSASVGRDVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFVLYSGVPSRFGSRSGTDFTLTITSSLPEDFATYYCQQHYTTPPTFGCGTKVEIKGGGSGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKCLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQTLVTVSSSGGGGSGGGGSCPPCPAPE <u>L</u> LGGS <u>C</u> VFLFPKPKDTLMISRTPVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP <u>A</u> <u>P</u> IEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSL <u>C</u> LSPGK	Heavy chain of Bs4Ab with C239-ins and S442C ("CC"). Construct for ADC. The highlighted positions correspond to sites of known allelic variation.
42A	DIVMTQTPLSLSVTPGQPASISCKSSQSVFFRSNNKNILAWYLQKPGQPPQLLIYWASSRESGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCQQYFGSPFTFGPGTKVDIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC	LC construct with kappa chain common to all the antibody constructs. The highlighted positions correspond to sites of known allelic variation.
42B	DIVMTQTPLSLSVTPGQPASISCKSSQSVFFRSNNKNILAWYLQKPGQPPQLLIYWASSRESGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCQQYFGSPFTFGPGTKVDIKQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTTPSKQSNKNYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTEC	LC construct with lambda chain option for all the antibody constructs. The highlighted positions correspond to sites of known allelic variation.
43	EVQLVESGGGLVQPGGSLRLSCAASGFTFSYSSMNWVRQAPGKLEWVSSISSSSSYIYYADSVKGRFTISRDNANKNSLYLQMNSLRAEDTAVYYCARGGDGYNYYYFDYWGGQTLVTVSS	VH from clone 1.39.1 antibody
44	DIVMTQSPDLSAVSLGERATITCKSSQSVFFRSNNKNCLAWYQQRPGQPPNLLIYWASSRESGVPDRFSGSGSGTDFTALTITSLQTEDVAVYYCQQYFGSPFTFGPGTKVDIK	VL from clone 1.39.1 antibody
45	SYSM	VH clone 1.39.1 CDR1 (sequence from non lead optimized antibody)
46	GGDGYNYYYFDY	VH clone 1.39.1 CDR3 (sequence from non lead optimized antibody)
47	KSSQSVFFRSNNKNCLA	VL clone 1.39.1 CDR1 (sequence from non lead optimized antibody)
48	DIVMTQSPDLSAVSLGERATITC	VL clone 1.39.1 FW1 (sequence from non lead optimized antibody)
49	WYQQRPGQPPNLLIY	VL clone 1.39.1 FW2 (sequence from non lead optimized antibody)
50	GVPDRFSGSGSGTDFTALTITSLQTEDVAVYYC	VL clone 1.39.1 FW3 (sequence from non lead optimized antibody)

SEQ ID NO	SEQUENCE	Comments
51	TQVCTGTDMLRLPASPETHLDMRLHLYQGCQVVQGNLELTYPNTASLS FLQDIQEVQGYVLIAHNQVRQVPLQRLRIVRGTLQFEDNYALAVLDNGDP LNNTTPVTGASPGGLRELQLRSLTEILKGGVLIQRNPQLCYQDTILWKDI FHKNNQLALTLIDTNRSRACHPCSPMCKGSRWGESSEDCQSLTRTV CAG GCARCKGPLPTDCCHEQCAAGCTGPKHSDCLACLFHNSGICELHCPALV TYNTDTFESMPNPEGRYTFGASCVTACPYNYLSTDVGSCTLVCPLHNQEV TAEDGTQRCEKCSKPCARVCYGLGMEHLREVRVTSANIQEFAGCKKIFG SLAFLPESFDGDPASNTAPLQPEQLQVFETLEEITGYLYISAWPDSLPLD SVFQNLQVIRGRI LHNGAYSLTLQGLGISWLGLRSLRELGSGLALIHNT HLCFVHTVPWDQLFRNPHQALLHTANRPEDECVEGLACHQLCARGHCWG PGPTQCVNCSQFLRGQECVEECRVLQGLPREYVNAHCLPCHPECQPQNG SVTCFGPEADQCVACAHYKDPPFCVARCP SGVKPDL SYMPIWKFPDEEGA CQPCPINCTHSCVDLDDKGCPAEQRASPLT	Extracellular portion of mature HER2 (positions 23-652 of canonical Isoform 1; without signal sequence). Natural variant: W452C. See Uniprot P04626. See also "Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab." Cho <i>et al.</i> , Nature 421:756-760 (2003), incorporated herein by reference in its entirety.
52	SLTRTV CAGGCARCKGP	HER2 epitope of clone 1.39.1 derived antibodies.
53	LPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVARCP SGVKPDL SY MPIWKFPDEEGACQP	HER2 epitope binding region of trastuzumab (HERCEPTIN®). Reported to span residues 538-603 (or residues 561-625 in HER2 with signal sequence); reported to be a conformational epitope with contacts at three loop regions.
54	DTYIH	Domain IV scFv VH-CDR1
55	RIYPTNGYTRYADSVKG	Domain IV scFv VH-CDR2
56	WGGDGFYAMDY	Domain IV scFv VH-CDR3
57	RASQDVNTAVA	Domain IV scFv VL-CDR1
58	SASFLYS	Domain IV scFv VL-CDR2
59	QQHYTTPPT	Domain IV scFv VL-CDR3

[00549] The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

[00550] Aspects of the present disclosure can be further defined by reference to the following non-limiting examples, which describe in detail preparation of certain antibodies of the present disclosure and methods for using antibodies of the present disclosure. It will be apparent to those skilled in the art that many modifications, both to materials and methods, can be practiced without departing from the scope of the present disclosure.

[00551] A number of HER2 antibodies have been approved for use in the treatment of breast cancer patients whose tumor(s) overexpress HER2 including trastuzumab (HERCEPTIN®; see U.S. Pat. No. 5,821,337), pertuzumab (PERJETA™; Patent Publication WO2001/00245) and T-DM1 (ado-trastuzumab emtansine, KADCYLA™, an antibody-drug conjugate consisting of the monoclonal antibody trastuzumab linked to the cytotoxic agent mertansine (DM1), Niculescu-Duvaz et. al., 2010, Curr. Opin. Mol. Ther. 12:350–60). However, these therapies are not indicated for the majority of patients, who express lower levels of HER2. Additionally, there are patients who do not respond to these therapies or who become resistant. Thus, there is an unmet medical need for superior therapeutics to address these patients.

[00552] As detailed in the specific examples provided below, highly potent bispecific antibodies were generated by combining an optimized fully human anti-HER2 antibody binding a newly described epitope within domain II of HER2 with an scFv binding a known epitope within domain IV of HER2. A number of different bispecific antibody configurations were generated and tested. The unique bispecific antibodies provided exhibit biological activities not seen with any of the mono-specific anti-HER2 antibodies tested. In many assays the bispecific antibodies also demonstrate a synergistic activity over mono-specific anti-HER2 antibodies. A number of the *in vitro* and *in vivo* activities of the bispecific antibodies provided herein are further enhanced by the addition of cytotoxic agents (e.g., tubulysin 1508) in the absence of or severely reduced binding activities to Fc gamma receptors. Additionally, it was found that the *in vitro* activity of the unique bispecific antibodies provided herein may also be enhanced by enhancing ADCC activity, for example by altering glycosylation (e.g., using POTELLEGENT™ technology (Biowa, Inc. Princeton, N.J.) to generated hypofucosylated antibodies having enhanced ADCC activity). These data suggest that the bispecific antibodies may have therapeutic use, particularly as ADCs or as ADCC enhanced antibodies, for the treatment of cancers expressing a wide range of HER2 levels, including patients currently ineligible for treatment with trastuzumab, pertuzumab or T-DM1. In addition, the bispecific antibodies may have therapeutic use for the treatment of cancer patients that have failed existing anti-HER2 therapies.

Example 1

1.1. Lead Optimization

[00553] AZ1.39.1 is a fully human monoclonal antibody against human HER2 that does not compete for binding with either trastuzumab or pertuzumab (Patent Publication WO 2008/019290). 39S is a lead-optimized antibody generated from AZ1.39.1 (Figure 1) as detailed below. Site directed mutagenesis was used to replace an unpaired Cys residue in the CDR1 of light chain with Ile and a potential isomerization site (DG) in the CDR3 of heavy chain was removed by changing the DG to DA. The resulting variant demonstrated the same binding specificity and *in vitro* anti-proliferative activity as AZ1.39.1 (data not shown). To generate higher affinity binders, mutagenesis was applied to the CDR residues of heavy chain and the mutants were expressed as IgG in mammalian cells and screened for their binding activity to the recombinant human HER2 extracellular domain protein by capture ELISA. Clones with significantly higher binding signal than the wild-type control were sequenced to reveal the mutation information. A combinatorial library was constructed from the identified mutations and screened for binding activity to the recombinant human HER2 extracellular domain protein by capture ELISA. 39S was found to have the highest binding activity, and further sequence analysis revealed that 39S carried a single N5S mutation in the CDR1 of heavy chain. The affinity of 39S to human HER2 (K_D) is ~ 1.0 nM, determined by BIAcore, in contrast the reported affinity of the parent 1.39.1 antibody is ~ 2.0 nM. Thus, this single mutation resulted in a 2 fold increase in binding affinity over the parental antibody. To improve the antibody expression level the framework sequence, in particular FR1, FR2 and FR3 of the light chain, was swapped from IGKV4-1 + Jk3.01 to the IGVK2D germline sequence, resulting in an approximately 2-fold increase in IgG expression level measured after 7 days in culture (Figure 2).

1.2. Binding Specificity and Species Cross-reactivity of Lead-optimized Antibody 39S

[00554] To determine whether 39S retains the binding specificity and species cross-reactivity of its parental antibody AZ1.39.1, binding of 39S to the receptors of human ErbB family (EGFR, HER2, HER3, and HER4), the mouse Her2, and the cynomolgus

monkey Her2 was examined by capture ELISA. Briefly, 96-well plates were coated with one of the following recombinant extracellular domain proteins: human EGFR, human HER2, human HER3, human HER4, mouse Her2, or cynomolgus monkey Her2. Antibody to be tested was prepared by diluting in a stepwise 1:3 serial dilution ranging from 50 nM down to 0.28 pM and then added to the wells in duplicate. After one hour of incubation, plates were washed, and goat anti-human IgG Fab HRP-conjugated secondary antibody was added to each well and the plates were incubated for 1 hour. The plates were washed and TMB substrate was added and incubated for 5-20 min to allow color development. The stop solution was added to the wells at the end of reaction and the plates were read at 450 nm. Binding signal (absorbance at 450 nm) was plotted against antibody concentration using Prism software. Results show that 39S, similar to AZ1.39.1, can bind to human HER2 and cynomolgus monkey Her2, but not to Human EGFR, HER3, HER4, or mouse Her2 (data not shown).

1.3. 39S Epitope Mapping and Characterization

[00555] The domain II of human HER2 was identified as the epitope of 39S by swapping domains between human and mouse Her2 molecules. Mouse Her2 was chosen as the chimeric partner because it is not recognized by 39S, but shares 85% sequence identity with human HER2. Chimeric variants targeting each domain of HER2 were constructed (see method below) as listed in Table 4, including four knock-out (KO, loss-of-function) variants replacing domain I, II, III, or IV of human HER2 with the mouse counterpart, and one knock-in (KI, gain-of-function) variant grafting the domain II of human HER2 into mouse Her2 molecule. The chimeric variant nomenclature denotes types of variants (KO/KI) and the number of domains swapped. The binding profiles of 39S to these variants were characterized using a SPR based instrument ProteOn™ by capturing variants on sensor surfaces using anti-human and mouse Her2 polyclonal antibodies (see method below). The expression of the variants was monitored by anti-His polyclonal antibody using ProteOn™. The binding results of 39S to the loss-of-function variants have demonstrated that domain II is the epitope-containing domain. 39S did not bind to the variant of KO_II encoding for mouse domain II (Table 4), while retained binding to the variant of KO_I encoding for mouse domain I. Although the variants knocking out

human domain III and IV (KO_III and KO_IV) did not express, these two domains were excluded as the epitope of 39S basing on the binding results on the gain-of-function variant KI_II. This gain-of-function variant (KI_II), encoding for mouse domain I, III and IV and human domain II, was recognized by 39S with similar binding affinity (168pM) as to human HER2 (84pM). Therefore, the domain II (amino acid 146-310) of human HER2 was identified as the epitope-containing domain of 39S by both loss-of-function and gain-of-function variants.

[00556] We further refined the epitope of 39S and identified the region of amino acid 192-208 in domain II as the critical epitope region. A series of chimeric human/mouse variants were constructed targeting short regions of domain II possessing different amino acid sequences between human and mouse Her2 proteins, as listed in Table 4. Seven knock-out variants were generated by replacing each of the following human HER2 regions with the mouse counterparts, including amino acids 146-208, 159-162, 171-187, 192-208, 250-261, 276-285, and 295-296. In addition, one knock-in variant was constructed by grafting the region of amino acid 192-208 of human HER2 to the mouse molecule. The variant nomenclature denotes types of variants (KO/KI) and the swapped regions with amino acid numbering. All variants had detectable expression levels by anti-His polyclonal antibody (Table 4). 39S did not bind to any of the chimeric variants, in which the region of amino acid 192-208 of human HER2 was replaced by mouse residues (KO_146-208, KO_192-208). The binding of 39S was not impacted when substituting any other regions of human HER2 with mouse amino acids (KO_159-162, KO_171-187, KO_250-261, KO_276-285, and KO_295-296). Furthermore, 39S bind to the KI variant encoding human 192-208 (KI_192-208) with a KD of 72 pM, comparable to the KD (84 pM) of human HER2. Taken together, the region of amino acid 192-208 in domain II of human HER2 was identified as the functional epitope of 39S.

[00557] The variant nomenclature denotes types of variants (KO/KI) and the swapped regions with amino acid numbering. Amino acid positions were denoted basing on the numbering scheme of mature human HER2 sequences without its signal peptide. The expression levels of all variants were monitored by an anti-His polyclonal antibody. The binding profiles of 39S to these variants were characterized using a SPR based instrument ProteOn™ by capturing variants on sensor surfaces using anti-human and mouse Her2

polyclonal antibodies. By capturing Her2 proteins on sensor surfaces, the measured apparent binding affinities of 39S are expected to be higher than the monovalent binding affinity in the format of immobilizing 39S on chip surfaces.

Table 4. Binding profiles of 39S to chimeric human/mouse Her2 variants

	Variants	The swapped human HER2 amino acids	Expression	The binding affinity of 39S (K_D)
Chimeric variants targeting domains	KO_I	1-145	Yes	95 pM
	KO_II	146-310	Yes	No binding
	KO_III	311-433	No expression	-
	KO_IV	434-646	No expression	-
	KI_II	146-310	Yes	163 pM
Chimeric variants targeting short regions of domain II of human HER2, which possess different protein sequences between human and mouse	KO_146-208	146-208	Yes	No binding
	KO_159-162	159-162	Yes	101 pM
	KO_171-187	171-187	Yes	38 pM
	KO_192-208	192-208	Yes	No binding
	KO_250-261	250-261	Yes	187 pM
	KO_276-285	276-285	Yes	106 pM
	KO_295-296	295-296	Yes	84.7 pM
	KI_192-208	192-208	Yes	72 pM
Controls	Mouse Her2	N/A	Yes	No binding
	Human Her2	N/A	Yes	84 pM

[00558] Construction and expression of chimeric human/mouse Her2 variants were constructed as follows. Briefly, DNAs encoding chimeric human/mouse Her2 variants with a His tag were assembled and amplified by overlapping PCR using human and mouse Her2 plasmids as templates (MedImmune). The assembled DNAs were cloned

into the mammalian expression vector pEBNA (MedImmune). HEK293F cells were then transiently transfected with the various constructs using 293fectin and standard protocols according to the manufacturer's instructions (Invitrogen).

[00559] The binding characteristics of 39S to chimeric human/mouse variants were studied using a ProteOn™ XPR36 instrument (BioRad). Standard amine coupling was used to immobilize an anti-human or mouse Her2 polyclonal antibody (R&D System) in 10 mM sodium acetate (pH 5.0) to the surface of a GLC biosensor chip at ~5000 resonance units (RU) for each channel. The chimeric proteins in cell culture supernatant were injected and captured by anti-human or mouse polyclonal antibodies onto the GLC surface with a ~200RU response. Anti-HER2 mAb 39S was diluted in phosphate-buffered saline (PBS) (pH 7.4) with 0.005% Tween-20 from 10 nM to 0.625nM (1:2 dilution), and injected at 100 µL/min for 180 sec with a 600- second dissociation time. Expression levels of chimeric variants were monitored by flowing anti-His polyclonal antibody (MedImmune) under the same conditions as injecting 39S. The surface was regenerated twice by injecting glycine buffer (10mM, pH 1.5) at 100 µL/min for 30 sec. All sensorgram data were processed with the ProteOn™ Manager 3.0.1 software.

[00560] A FACS-based binding competition assay was used to determine whether antibodies competed for binding to the same epitope as trastuzumab, and/or pertuzumab. BT-474 cells were harvested, resuspended in FACS buffer, and 2.5×10^5 cells/well were added to a 96-well U-bottom plate. Antibody to be tested (R347 IgG1 isotype control, trastuzumab, pertuzumab, AZ1.39.1, and 39S) was prepared by diluting in FACS buffer containing 2 µg/mL of Alexa-Fluor 647-labeled 39S antibody in a stepwise 1:4 serial dilution ranging from 500 µg/mL down to 1.9 ng/mL and then added to cells in triplicate. After 1 hour of staining on ice, cells were washed 3 times with ice-cold FACS buffer and then fixed with 2% PFA. Cells were analyzed by a BD LSR II machine with BD FACSDiva™ software. Data were analyzed with FlowJo software and presented as mean MFI ± SEM (n=3).

[00561] Figure 4 shows that 39S binds to the same epitope as its parental antibody AZ1.39.1; and 39S does not compete with trastuzumab or pertuzumab for binding. The binding sites of 39S, pertuzumab and trastuzumab are indicated by arrows on the ribbon structure of HER2 provided in Figure 3.

1.4. In Vitro Activity of 39S

[00562] A panel of human cancer cell lines expressing various levels of HER2 was selected for evaluating the anti-proliferative activity of antibodies or antibody combinations. HER2 expression level in cells was determined by HercepTest[®] and quantitative FACS (Table 5).

TABLE 5: HER2 Expression Levels on Human Cancer Cell Lines

Cell Line	Cancer Type	HercepTest [®] Score	Relative HER2 Density on Cell
BT-474	Breast	3+	1,841,884
SKBR-3	Breast	3+	1,517,135
NCI-N87	Gastric	3+	1,292,978
SKOV-3	Ovarian	3+	349,178
MDA-MB-361	Breast	2+/3+	252,249
JIMT-1	Breast	2+	65,573
MDA-MB-453	Breast	2+	77,314
MDA-MB-175-VII	Breast	2+	29,437
RT-112	Bladder	2+	7,664
MCF7-GTU	Breast	2+	6,058
ZR-75-1	Breast	1+	5,892
T47D	Breast	1+	6,124
MCF-7	Breast	0	3,646
MDA-MB-468	Breast	0	Undetectable

[00563] *Proliferation Inhibition Assay:* Cells were plated in serum-containing culture media at a density of 5,000 to 20,000 per well (depending on the growth kinetics of each cell line) of 96-well plates in a volume of 100 μ L. A 2X concentration of each dose of antibody or antibody combination to be tested was prepared by diluting the test articles in culture medium. One hundred microliters of each test article was added to cells in triplicate such that the final dose curve ranged from 10 μ g/mL down to 0.15 ng/mL in a stepwise 1:4 serial dilution series. For ligand-dependent proliferation inhibition assay, cells were plated in serum-free media and a 4X concentration of each dose of antibody or

antibody combination to be tested was prepared by diluting the test articles in serum-free medium. Fifty microliters of each test article was added to cells in triplicate and then 50 μ L of Heregulin- β 1 at a concentration of 32 ng/mL diluted in serum-free medium was added to cells to achieve the final Heregulin- β 1 concentration of 8 ng/mL and the final antibody dose curve ranged from 100 μ g/mL down to 6.1 ng/mL in a stepwise 1:4 serial dilution series. The treated cells were cultured at 37°C / 5% CO₂ for 4 to 7 days (depending on the growth kinetics of each particular cell line). Cell viability was determined using Cell Titer Glo from Promega according to manufacturer's instructions. Data are analyzed using the GraphPad Prism software and presented as percent growth inhibition relative to the untreated control.

[00564] Figure 5 shows that 39S has a similar activity as pertuzumab in inhibiting ligand-driven proliferation in MCF-7 cells. In combination with trastuzumab or pertuzumab, 39S shows additive or synergistic inhibition of ligand-dependent proliferation with potency comparable to trastuzumab and pertuzumab combination. Similar results were observed in other cells lines such as MDA-MB-361 and RT-112 (data not shown).

[00565] Figure 6 shows that 39S, like pertuzumab, has a limited activity in inhibiting NCI-N87 cell proliferation in serum-containing media. However when combined with trastuzumab or pertuzumab 39S demonstrates a strong synergistic effect in inhibiting cell growth. Synergy between 39S and trastuzumab or pertuzumab is much greater than that seen with the combination of trastuzumab and pertuzumab. Similar results were observed in BT-474 cells (data not shown).

Example 2

2.1. Bispecific Antibody Construction

[00566] *Cloning of Bs2Ab-39SH, Bs3Ab-39SH and Bs4Ab-39SH.* Bispecific expression constructs were generated by cloning the variable domains for the anti-HER2 domain IV antibody (SEQ ID NOs: 17 and 18 where X is C) anti-HER2 antibody 39S (SEQ ID NOs: 15 and 16) into expression vectors comprising the appropriate constant regions. The anti-HER2 domain IV variable binding domain was constructed as single-chain Fv (scFv). Using the amino acid sequences of SEQ ID NOs: 17 and 18 (where X-C) above a codon optimized DNA sequence for maximum mammalian protein expression was designed and

synthesized. The Bs2Ab and Bs3Ab constructs were generated using methods similar to those described in Dimasi et al., (2009) J. Mol. Biol. 393, 672-692. The Bs4Ab construct was generated using method similar to those described in Patent Publication WO2013070565A1. The final synthetic gene contains two cysteine mutations, one in the light-chain at position 100 and one on the heavy chain at position 44, respectively. These cysteines will form an interchain disulfide bond between the VL and VH domains in order to stabilize the scFv. The two VL and VH domains of the scFv were linked using 20 amino acids residues $(G_4S)_4$. The scFv in Bs2Ab was linked to the N-terminus of the heavy chain by using a 10 amino acid residue linker $(G_4S)_2$. The scFv in the Bs3Ab format was linked to the C-terminus of the antibody CH3 domain by using a 10 amino acid residue linker $(G_4S)_2$. Two linkers of sequence $(G_4S)_2$ were used for linking the scFv in the Bs4Ab backbone. Construct identity and fidelity was determined using DNA sequence.

[00567] Figure 7 provides a schematic diagram of each of the Bs2Ab-39SH, Bs3Ab-39SH, Bs4Ab-39SH bispecific antibody formats (Panels A, B and C, respectively) generated for binding to HER2 antigen. The bispecific antibodies have two binding units, each of which binds a different epitope on the same antigen. The binding units are labeled on the figure. The molecule is bilaterally symmetric with respect to the binding units. As depicted, Bs2Ab-39SH, Bs3Ab-39SH, Bs4Ab-39SH formats refers to bispecific antibodies in which a scFv is fused to the amino terminus of the variable region (Bs2Ab-39SH), inserted into a modified hinge region (Bs4Ab-39SH) or the carboxy-terminus of CH3 (Bs3Ab-39SH) of a heavy chain through a linker (e.g., $(G_4S)_2$). The three bispecific constructs shown are comprised of an anti-HER2 domain IV binding scFv fused to an anti-HER2 domain II human IgG1 via a glycine serine linker (e.g., $(G_4S)_2$).

[00568] The amino acid sequences of the Bs2Ab-39SH, Bs3Ab-39SH and Bs4Ab-39SH constructs are provided in Figure 8 (also see SEQ ID NOs: 30, 34, and 38 native Fc region). Figure 8A shows the bispecific antibody heavy chain amino acid sequences for Bs2Ab-39SH and possible substitution sites for enhanced ADCC and or site specific Antibody Drug Conjugation for two and four drug loading. The anti-HER2 domain IV scFv is in the VL- $(G_4S)_4$ linker-VH format and is genetically linked to the amino terminus of the anti-HER2 domain II antibody heavy chain via a $(G_4S)_2$ linker. The

amino acid substitutions and or insertions depicted within can be made in the CH2 and CH3 of the antibody for enhanced ADCC and site specific conjugation. Figure 8B shows the bispecific antibody heavy chain amino acid sequences for Bs3Ab-39SH and possible substitution sites for enhanced ADCC and or site specific Antibody Drug Conjugation for two and four drug loading. The anti-HER2 domain IV scFv is in the VL-(G₄S)₄ linker-VH format and is genetically linked to the carboxy-terminus of the anti-HER2 domain II antibody heavy chain via a (G₄S)₂ linker. The amino acid substitutions and or insertions depicted within can be made in the CH2 and CH3 of the antibody for enhanced ADCC and site specific conjugation. Figure 8C shows the bispecific antibody heavy chain amino acid sequences for Bs4Ab-39SH and possible substitution sites for enhanced ADCC and or site specific Antibody Drug Conjugation for two and four drug loading. The anti-HER2 domain IV scFv is in the VL-(G₄S)₄ linker-VH format and is inserted into a modified hinge region of the anti-HER2 domain II antibody heavy chain via two (G₄S)₂ linkers. The amino acid substitutions and or insertions depicted within can be made in the CH2 and CH3 of the antibody for enhanced ADCC and site specific conjugation.

2.2. Binding Specificity and Species Cross-reactivity of Bispecific Antibody

[00569] Binding specificity and species cross-reactivity of bispecific antibodies to the recombinant extracellular domain protein of human EGFR, human HER2, human HER3, human HER4, mouse Her2, or cynomolgus monkey Her2 were determined by capture ELISA as described in Example 1. Results show that all bispecific antibodies tested, including Bs2Ab-39SH, Bs3Ab-39SH and Bs4Ab-39SH, are able to bind to human HER2 and cynomolgus monkey Her2 at similar potency and none of them shows binding to the Human EGFR, HER3, HER4, or the mouse Her2 (data not shown), suggesting the bispecific antibodies retain the binding specificity of species cross-reactivity of their parental monoclonal antibodies.

[00570] Binding kinetics of the bispecific antibodies to human HER2 and cynomolgus monkey Her2 are determined by BIAcore (data not shown). The affinity to human HER2 (K_D) is 113 pM for Bs2Ab-39SH, and 236 pM for Bs4Ab-39SH.

2.3. In Vitro Activity of Bispecific Antibody

[00571] Activity of bispecific antibody in inhibiting ligand-driven cell proliferation was determined using method described in Example 1. Results show that Bs2Ab-39SH, Bs3Ab-39SH and Bs4Ab-39SH have similar potency in MDA-MB-361 cells (Figure 9A) and MCF-7 cells (Figure 9B), which is also comparable to the activity of parental antibody combination (39S plus trastuzumab). Similar results were observed in other cell lines including NCI-N87 and RT-112 (data not shown).

2.4. Disruption of HER2:HER3 Heterodimerization by Bispecific Antibody

[00572] T47D cells were harvested, washed and resuspended in serum-free media. Cells were seeded at a density of 1×10^6 cells/well in a 6-well plate and then incubated overnight at 37°C/5% CO₂. The next day cells were pre-treated for 1 hour with antibody to be tested (R347 IgG1 isotype control, trastuzumab, pertuzumab, 39S, and Bs2Ab-39SH) at a concentration of 500 nM. After pre-treatment, heregulin- β 1 was added at a final concentration of 8 ng/mL and cells were incubated for 5 min at 37°C/5% CO₂. Cells were then washed twice with ice-cold 1x PBS, lysed and immune-precipitated using mouse anti-human HER2 (clone 44E7) antibody and the Pierce Classic IP Kit from Thermo Scientific according to manufacturer's instructions. Immune-precipitated protein samples were eluted in Laemmli buffer containing 2-mecaptoethanol and analyzed by Western blot using standard protocol. Rabbit anti-human HER2 (clone 29D8) antibody was used to detect HER2 and rabbit anti-human HER3 (C-17) polyclonal antibody was used to detect HER3 in the Western blot analysis.

[00573] Figure 10 shows that Bs2Ab-39SH and 39S, similar to pertuzumab, can disrupt HER2:HER3 heterodimerization induced by ligand stimulation.

2.5. Clustering of HER2 by Bispecific Antibody

[00574] To examine whether the bispecific antibody can cross-link HER2 to form a large complex, the recombinant human HER2 extracellular domain protein was mixed with Bs2Ab-39SH or trastuzumab at various molar ratios and incubated at room temperature for 30 min. The formed immune complexes were separated by HPLC size-exclusion

chromatography; the sizes of which were then analyzed by Multi-Angle Light Scattering (MALS) assay.

[00575] Figure 11 shows the representative data derived from antibody:HER2 molar ratio of 1:1 (data at other molar ratios not shown). Results indicate that Bs2Ab-39SH can cross-link many HER2 molecules to form a protein complex as large as 1716 kDa in size, while trastuzumab can only bind to two HER2 molecules in maximal to form a 320 kDa complex. Similar results were observed with Bs4Ab-39SH (data not shown).

2.6. Enhanced Internalization and Lysosomal Trafficking by Bispecific Antibody

[00576] Antibody internalization was measured by FACS. BT-474 cells were harvested from a T150 flask, resuspended in ice-cold culture media and then added to a 96-well U-bottom plate at 1×10^6 cells/well. Cells were pelleted by centrifugation at 4°C. Media were flicked off, and cell pellets were resuspended in triplicate in 150 μ L of ice-cold culture media containing 10 μ g/mL antibody or antibody combination to be tested (R347 IgG1 isotype control, trastuzumab, pertuzumab, AZ1.39.1, 39S, trastuzumab+39S, trastuzumab+pertuzumab, pertuzumab+39S, trastuzumab+pertuzumab+39S, Bs2Ab-39SH, Bs3Ab-39SH, and Bs4Ab-39SH). Cells were incubated on ice for 1 hour and then washed to remove unbound antibodies. An aliquot of cells was kept on ice; the rest was incubated at 37°C/5% CO₂ for a different period of time (30 min, 1 hour, 2 hours, or 4 hours) and then cooled on ice immediately. Cells were washed twice with ice-cold FACS buffer and then fixed with 4% PFA for 20 min. Following fixation, cells were stained with anti-human IgG Alexa-Fluor 488 and analyzed by a BD LSR II machine and BD FACSDiva™ software. Data were analyzed with FlowJo software. Receptor-antibody complex internalization was calculated as percent mean fluorescent intensity (MFI) loss at 37°C relative to that on ice after subtracted by the background value of MFI derived from the untreated control.

[00577] Figure 12 shows that the bispecific antibody (Bs2Ab-39SH, Bs3Ab-39SH, Bs4Ab-39SH) can induce a much faster and stronger internalization than any single mono-specific antibody or antibody combination. Pertuzumab, AZ1.39.1, trastuzumab+pertuzumab, pertuzumab+39S, and trastuzumab+pertuzumab+39S have similar or lower internalization profiles to that of 39S and are not shown in the graph.

Similar results were observed in cell lines NCI-N87, MDA-MB-361 and RT-112 (data not shown).

[00578] Confocal microscopy method was used to visualize antibody internalization and lysosomal trafficking. BT-474 cells were harvested from a T-150 flask and resuspended in ice-cold culture media and then added to a 96-well U-bottom plate at 2.5×10^5 cells/well. Cells were pelleted by centrifugation at 4°C. Media were flicked off, and cell pellets were resuspended in 150 µL of ice-cold culture media in the presence of 10 µg/mL antibody to be tested, such as R347 IgG1 isotype control, trastuzumab, Bs2Ab-39SH, and Bs4Ab-39SH. Cells were incubated at 37°C/5% CO₂ for a different period of time (30 min, 2 hours, 4 hours, or 6 hours) and then cooled on ice immediately. Cells were washed twice with ice-cold FACS buffer and then fixed and permeabilized using BD Biosciences Cytofix/Cytoperm™ Fixation/Permeabilization Solution according to manufacturer's instructions. Cells were stained in the dark with anti-human IgG Alexa-Fluor 488, and mouse anti-human LAMP-1 (clone H4A3) followed by anti-mouse IgG Alexa-Fluor 647. After staining, cells were cytopspun onto positive charge slides and coverslipped with ProLong® Gold Antifade Reagent containing DAPI. Cells were then visualized by Leica SP5 Confocal Microscope and the Leica Application Suite Advanced Fluorescence software suite.

[00579] Figure 13 shows the antibody internalization and trafficking to the lysosomes. Both Bs2Ab-39SH and Bs4Ab-39SH promote much faster internalization and stronger lysosomal trafficking than trastuzumab, which show little to no internalization (data for Bs4Ab-39SH not shown).

[00580] Western blot analysis was used to monitor lysosomal degradation of HER2. BT-474 cells were harvested, washed and resuspended in culture media. Cells were seeded at a density of 5×10^4 cells/well in a 96-well plate and treated at 37°C/5% CO₂ with antibody to be tested (R347 IgG1 isotype control, trastuzumab, pertuzumab, 39S, trastuzumab+pertuzumab, trastuzumab+39S, pertuzumab+39S, trastuzumab+pertuzumab+39S, Bs2Ab-39SH, Bs3Ab-39SH, and Bs4Ab-39SH) at a concentration of 500 nM for 2 hours, 6 hours, or 24 hours. At the end of treatment, cells were washed twice with ice-cold 1x PBS and then lysed in M-PER Mammalian Protein Extract buffer from Thermo Scientific according to manufacturer's instructions. Protein

concentration of each lysate was measured by BCA assay. Equal amount of protein in each lysate was loaded onto gel in Western blot. Rabbit anti-human HER2 (clone 29D8) antibody and rabbit anti-human GAPDH (clone D16H11) antibody were used to detect HER2 and GAPDH, respectively.

[00581] Figure 14 shows that treatment with the bispecific antibody (Bs2Ab-39SH, Bs3Ab-39SH, or Bs4Ab-39SH) leads to the significant HER2 degradation in BT-474 cells, and mono-specific antibody or antibody combination induces no or limited HER2 degradation.

Example 3

3.1. Cloning of site-specific mutants for site-specific conjugation and for ablating Fc gamma receptor binding activities

[00582] Standard overlapping PCR methods were used to introduce the mutation L234F, S239C and S442C, independently or in combination (L234F-S239C (FC) and L234F-S239C-S442C (FCC)) into the Fc portion of the Bs2Ab-39SH and Bs4Ab-39SH constructs. The Primers were designed to contain desired mutations and flanking primers, containing restriction sites to facilitate directional cloning, were used to amplify the Fc fragments containing the specific mutations. Modified Fc PCR products were cloned into mammalian expression vectors using defined restrictions sites. Identities of Fc mutations were confirmed by DNA sequence analysis.

[00583] Figure. 15 shows illustrations of three anti-HER2 bispecific antibodies, (Bs2Ab-39SH, Bs3Ab-39SH, Bs4Ab-39SH) with amino acids substitutions for site specific conjugation. In addition, the L234F substitution on the CH2 of the antibody heavy chain (not depicted in the figure) is used to minimize Fc gamma receptor binding. Where 2 DAR is desired a cysteine substitution is engineered at site 1 (e.g., S239C) or site 2 (e.g., S442C), and for 4 DAR a cysteine substitution is engineered at both sites. The bispecific-drug constructs are also referred to herein as Bs2-2T/Bs2-4T, Bs3-2T/Bs34T, and Bs4-2T/Bs4-4T.

[00584] The resulting bispecific antibodies were conjugated to the tubulysin 1508 payload essentially as described below (see Example 5).

3.2. Binding Specificity and Species Cross-reactivity of Bispecific ADC

[00585] To determine whether the conjugation of tubulysin 1508 alters antigen binding specificity and species cross-reactivity, binding activity of bispecific ADC to the recombinant extracellular domain protein of human EGFR, human HER2, human HER3, human HER4, mouse Her2, and cynomolgus monkey Her2 were confirmed by capture ELISA as described in Example 1. Results show that conjugation did not change the antigen binding specificity and species cross-reactivity of the bispecific ADC. Both Bs2-4T and Bs4-4T can bind to human HER2 and cynomolgus monkey Her2 at the same potency as their unconjugated version; and none of them shows binding to the human EGFR, HER3, HER4, or the mouse Her2 (data not shown). Binding kinetics of the bispecific ADC to human HER2 are determined by BIAcore and results show that the affinity to human HER2 (K_D) is 120 pM for Bs2-4T, and 271 pM for Bs4-4T.

3.3. Disruption of Intracellular Microtubule Network by Bispecific ADC

[00586] To examine the disruption of intracellular microtubule network by anti-HER2 ADC, three cell lines were selected: SKOV-3, JIMT-1, and RT112, which represent the T-DM1 eligible, the T-DM1 non-responder, and the T-DM1 ineligible, respectively. On day 1, cells were harvested by trypsinization, resuspended in culture media, and then seeded in 8-well chamber slides at a density of 6×10^4 cells/well. Slides were incubated at 37°C/5% CO₂ overnight. On day 2, media were aspirated to remove any unattached cells and then fresh media containing 5 nM ADC to be tested were added to the cells. Slides were incubated at 37°C/5% CO₂ overnight. On day 3, each chamber was washed twice with 1x PBS. Cells were then fixed with 4% PFA for 20 min. At the end of fixation, the chambers were removed and slides were stained following standard immunofluorescence procedures. Briefly, cells were permeabilized using Triton X-100 and washed with 1x PBS containing Tween-20. Rabbit anti-human α -Tubulin Alexa-Fluor 488 (clone 11H10) was diluted by 1:100 in DAKO antibody diluent and added to the slides. After incubation at room temperature for 1 hour, slides were coverslipped with ProLong® Gold Antifade Reagent containing DAPI. Stained cells were visualized by the Leica SP5 Confocal Microscope and the Leica Application Suite Advanced Fluorescence software suite.

[00587] Figure 16A shows in SKOV-3, a cell line representing T-DM1 eligible patients, that both T-DM1 and Bs2-4T are capable of disrupting microtubule networks. Similar results were observed in SKBR-3 cells (data not shown).

[00588] Figure 16B shows in JIMT-1, a cell line representing T-DM1 non-responder patients, that only Bs2-4T is able to disrupt intracellular microtubule networks.

[00589] Figure 16C shows in RT-112, a cell line representing T-DM1 ineligible patients, that Bs2-4T is able to disrupt intracellular microtubule networks, while T-DM1 is inactive.

3.4. In Vitro Activity of Bispecific ADC

[00590] A panel of human cancer cell lines expressing various levels of HER2 was used to evaluate the cytotoxic activity of bispecific ADC (Table 5 in Example 1). Briefly, cells were harvested, resuspended, and plated in serum-containing culture media at a density of 5,000 to 20,000 per well (depending on the growth kinetics of each cell line) of 96-well plates in a volume of 100 μ L. A 2X concentration of each dose of antibody or ADC to be tested was prepared by diluting the test articles in culture medium. One hundred microliters of each test article was added to cells in triplicate such that the final dose curve ranged from 5 nM down to 0.08 pM in a stepwise 1:4 serial dilution series. The treated cells were incubated at 37°C/5% CO₂ for 3 to 4 days, depending on the growth kinetics of each particular cell line. Cell viability was determined using Cell Titer Glo according to manufacturer's instructions. Data are analyzed by the GraphPad Prism software and presented as percent growth inhibition relative to the untreated control. EC₅₀ values were determined using Sigmoidal non-linear regression analysis with GraphPad Prism software and were summarized in Table 6.

Table 6: *In vitro* potency of anti-HER2 ADC in a panel of cancer cell lines (EC₅₀ in pM)

Cell Line	T-DM1	Bs2-2T	Bs4-2T	Bs2-4T	Bs4-4T
SKBR-3	82.6	12.9	10.4	4.0	2.6
NCI-N87	275.1	36.8	25.6	23.7	20.0
SKOV-3	116.1	13.6	8.9	8.7	5.6
MDA-MB-361	266.0	15.1	10.0	4.0	2.7

JIMT-1	Inactive	13.3	7.1	5.1	3.1
MDA-MB-453	344.3	19.0	13.0	10.5	8.6
RT-112	Inactive	4635	2938	36.5	23.5
MCF7-GTU	Inactive	Inactive	Inactive	84.5	61.4
ZR-75-1	Inactive	1543	753.5	18.1	20.9
T47D	Inactive	Inactive	Inactive	Inactive	Inactive
MCF-7	Inactive	Inactive	Inactive	Inactive	Inactive
MDA-MB-468	Inactive	Inactive	Inactive	Inactive	Inactive

[00591] Figure 17A shows the cytotoxic activity of Bs2-2T and Bs2-4T relative to T-DM1 and various controls in SKBR-3, a human breast cancer cell line representing the T-DM1 eligible patients. Data indicate that both Bs2-2T and Bs2-4T are more potent than T-DM1 in SKBR-3 cells.

[00592] Figure 17B shows the cytotoxic activity of Bs4-2T and Bs4-4T relative to T-DM1 and various controls in SKBR-3, a human breast cancer cell line representing the T-DM1 eligible patients. Data indicate that both Bs4-2T and Bs4-4T are more potent than T-DM1 in SKBR-3 cells.

[00593] Figure 18A shows the cytotoxic activity of Bs2-2T and Bs2-4T relative to T-DM1 and various controls in JIMT-1, a human breast cancer cell line representing the T-DM1 eligible but non-responder patients. Data indicate that both Bs2-2T and Bs2-4T are very potent in killing JIMT-1 cells, while T-DM1 shows no activity.

[00594] Figure 18B shows the cytotoxic activity of Bs4-2T and Bs4-4T relative to T-DM1 and various controls in JIMT-1, a human breast cancer cell line representing the T-DM1 eligible but non-responder patients. Data indicate that both Bs4-2T and Bs4-4T are very potent in killing JIMT-1 cells, while T-DM1 shows no activity.

[00595] Figure 19A shows the cytotoxic activity of Bs2-2T and Bs2-4T relative to T-DM1 and various controls in ZR-75-1, a human breast cancer cell line representing the T-DM1 ineligible patients. Data indicate that Bs2-4T is the most active in killing ZR-75-1 cells, while Bs2-2T has a lower level of activity and T-DM1 shows no or limited cytotoxic activity.

[00596] Figure 19B shows the cytotoxic activity of Bs4-2T and Bs4-4T relative to T-DM1 and various controls in ZR-75-1, a human breast cancer cell line representing the T-DM1 ineligible patients. Data indicate that Bs4-4T is the most active in killing ZR-75-1 cells, while Bs4-2T has a lower level of activity and T-DM1 shows no or limited cytotoxic activity.

[00597] Figure 20 shows the cytotoxic activity of Bs2-2T and Bs2-4T relative to T-DM1 and various controls in MDA-MB-468, a human breast cancer cell line with no HER2 expression. Data indicate that neither Bs2-2T nor Bs2-4T is active in MDA-MB-468 cells, indicating that the cytotoxic activity of Bs2-2T and Bs2-4T is target (i.e. HER2)-dependent. Similar results were observed with Bs4-2T and Bs4-4T (data not shown).

3.5. In Vivo Activity of Bispecific ADC Constructs

[00598] *Cell Line-Based Xenograft (CBX) Tumor Models and Patient-Derived Xenograft (PDX) Tumor Models:* All mouse experiments were carried out in compliance with the guidelines published by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and protocols approved by the MedImmune Institutional Animal Care and Use Committee. Athymic nude mice between 4-8 weeks of age were used in the studies. In subcutaneous CBX models, animals were injected unilaterally on the right flank with tumor cells harvested from culture at a specific passage lot. In orthotopic CBX models, xenografts were established by injecting 5×10^6 cells per mouse (suspended in 50% matrigel) into the mammary fat pad on the right flank of animals. In PDX models, animals are implanted unilaterally on the flank with tumor fragments harvested from host animals each implanted from a specific passage lot. Pre-study tumor volumes were recorded for each experiment beginning approximately one week prior to its estimated start date. When tumors reach the appropriate tumor volume initiation (TVI) range (typically 150-250 mm³), animals are randomized into treatment and control groups and dosing initiated. Animals were dosed once weekly unless otherwise specified with test articles via intravenous or intraperitoneal injection. Animals were observed daily and tumor dimensions and body weights were measured and recorded twice weekly. Tumor volumes are calculated using the following formula: tumor volume = $\pi \div 6$ (length x width²). Tumor growth curve was presented as the mean tumor volume (mm³) $\pm$ SEM.

- [00599] Figure 21A shows that even at lower concentrations (1 mg/kg) Bs2-4T has higher *in vivo* activity relative to T-DM1 (3 mg/kg) and various controls (all at 3 mg/kg) in MDA-MB-361 model, a human breast cancer CBX tumor model representing the T-DM1 ineligible patients. Data demonstrate that Bs2-4T induced a dose-dependent tumor growth inhibition, and at 3 mg/kg dose Bs2-4T induced complete tumor regression while T-DM1 showed limited activity. At 3 mg/kg Bs4-4T also exhibited higher *in vivo* activity relative to T-DM1 (Figure 21B).
- [00600] FIG. 22 shows the *in vivo* activity of Bs2-2T relative to Bs2-4T and T-DM1 in ST996 model. ST996 is a primary PDX model derived from a triple-negative breast cancer patient (HER2 IHC: 1+; ER-; PR-). Data demonstrate that Bs2-2T at 3 mg/kg dose induced a robust tumor growth inhibition, although its anti-tumor potency was slightly reduced as compared to Bs2-4T. In contrast, T-DM1 showed no activity in ST996 model. Similar results were observed in additional low HER2-expressing PDX models ST738, ST455B, and ST821 (data not shown).
- [00601] Figure 23 shows the *in vivo* activity of Bs2-4T relative to T-DM1 and various controls in ST225 model, a human breast cancer PDX tumor model representing T-DM1 eligible patients. Data demonstrate that Bs2-4T at 3 mg/kg dose induced complete and durable tumor regression. In contrast, T-DM1 only induced tumor stasis during the treatment phase and the tumor re-grew quickly after the treatment was stopped.
- [00602] Figure 24 shows the *in vivo* activity of Bs2-4T relative to T-DM1 and various controls in JIMT-1 model, a human breast cancer CBX orthotopic tumor model representing T-DM1 eligible but non-responder patients. Data demonstrate that Bs2-4T at 3 mg/kg dose induced complete and durable tumor regression. In contrast, T-DM1 or T-DM1 plus pertuzumab combination showed no activity.
- [00603] Figure 25 shows the *in vivo* activity of Bs2-4T relative to T-DM1 and various controls in ST455B model. ST455B is a primary PDX model derived from a triple-negative breast cancer patient (HER2 IHC: 1+, ER-, PR-). Data demonstrate that Bs2-4T at 3 mg/kg dose induced complete tumor regression while T-DM1 showed no activity. To further expand the finding shown in Figure 25, we have examined Bs2-4T in another 16 PDX models derived from breast cancer patients with relatively low levels of HER2 expression (+1 to +2 via HercepTest). Other criteria were also considered in the selection

of these models, including the degree of heterogeneity in HER2 expression, ER/PR status and histopathologic subclass, to maximize the diversity of tumor subtypes in the study. Table 7 summarizes the *in vivo* activity of Bs2-4T in these 17 different PDX breast cancer models. Bs2-4T demonstrated potent anti-tumor activity regardless of the histopathologic subclass and ER/PR status of the tumor. At the dose of 1 mg/kg, 41% of the tumor models showed tumor regression and 6% showed tumor stasis. At the dose of 3 mg/kg, 71% of the models showed tumor regression and 12% showed tumor stasis.

[00604] These studies demonstrate the superior activity of the Bispecific ADCs of the instant invention as compared to mono-specific ADC therapies (e.g. T-DM1) across a wide range of breast cancer models. In particular, the Bispecific ADCs of the instant invention have *in vivo* activity in cancer models with low levels of HER2 expression and in models of T-DM1 non-responder patients.

[00605] **Table 7:** Summary of *in vivo* efficacy of Bs2-4T in a panel of PDX breast cancer models representing HER2-low/T-DM1 ineligible patients. At the time-point showing maximal response to the Bs2-4T treatment, if the tumor volume is decreased as compared to the tumor volume at dosing initiated (TVI), the *in vivo* efficacy is expressed as percent tumor volume change over the TVI. Otherwise the *in vivo* efficacy is expressed as percent tumor volume change relative to the vehicle control. Responsiveness to the treatment ranked as “regression” if the tumor volume decreased by >20%, “stasis” if the tumor volume changed <20%, and “progression” if the tumor volume increased by >20%.

Efficacy	1 mg/kg	3 mg/kg
Regression	41% (7/17)	70% (12/17)
Stasis	6% (1/17)	12% (2/17)
Progression	53% (9/17)	18% (3/17)

3.6. Activity of Bispecific ADC in Tumor Models with Acquired T-DM1 Resistance

[00606] NCI-N87 cells with acquired resistance to T-DM1 were generated through continuous treatment with gradually increased concentration of T-DM1 up to 5 µg/mL. *In*

vitro cytotoxic activity of Bs2-4T relative to T-DM1 was examined in both parental and resistant cell lines as described in Example 3. Results shown in Figure 26A indicate that both Bs2-4T and T-DM1 are active in the parental NCI-N87 cells although Bs2-4T is more potent than T-DM1 (left panel). In the resistant cell line, T-DM1 has lost the activity, whereas Bs2-4T is still active in killing the resistant cells (right panel). Other resistant cell lines were also generated through continuous treatment with T-DM1, including BT-474, SKOV-3, and MDA-MB-361, and similar cytotoxic activity was observed with Bs2-4T in these resistant cell lines (data not shown).

[00607] To establish an *in vivo* tumor model with acquired T-DM1 resistance, the T-DM1-resistant NCI-N87 cells were injected subcutaneously into the immune-deficient mice. The tumor-bearing mice were treated with 3 mg/kg of T-DM1. It appeared that the *in vitro* T-DM1 resistance was not fully translated into the *in vivo* resistance, reflected by considerable variations in tumor growth among animals. Thus, mice with large refractory tumors ($\sim 1000 \text{ mm}^3$ in volume) were selected and the tumor tissue fragments were passaged to new mice until the tumors grew consistently in the presence of weekly treatment of 3 mg/kg T-DM1. After three passages, stable resistant tumors evolved and these tumors were fragmented and implanted into mice to evaluate the *in vivo* activity of Bs2-4T. As demonstrated in Figure 26B, tumors relapsed from the repeated T-DM1 treatment were not only resistant to T-DM1, but also unresponsive to T-DM1 and pertuzumab combination treatment. In contrast, Bs2-4T induced a robust and sustained tumor regression after treatment, suggesting its potential as an effective therapy for the T-DM1 relapsed/refractory patients.

[00608] Figure 26B shows that Bs2-4T induces tumor regression in the T-DM1 resistant NCI-N87 tumor model. Tumor growth curves, in response to weekly intravenous dosing of Bs2-4T (3 mg/kg), T-DM1 (3 mg/kg) or other control antibody/ADC (3 mg/kg, except for pertuzumab which is 10 mg/kg) for a total of 4 doses, are shown as the mean tumor volume (mm^3) $\pm$ SEM (n=7). *P < 0.001 by Student's t test as compared to the untreated control group.

3.7. *In Vivo* Anti-tumor Activity of Bispecific ADC is not Attenuated by Pretreatment of Trastuzumab

[00609] ST225 PDX tumor fragments were implanted unilaterally on the flank of athymic nude mice. Mice were randomized into treatment and control groups and dosing initiated when tumor volumes reached 200-250 mm³. In the treatment groups, mice were either dosed with vehicle buffer or trastuzumab (3 mg/kg). Three days later, mice received weekly intravenous injection of Bs2-4T for a total 4 doses. Figure 27 shows that pretreatment of trastuzumab does not attenuate the anti-tumor activity of Bs2-4T in the ST225 PDX model, suggesting that a washout period may not be needed if Bs2-4T is used to treat patients that are relapsed/refractory to trastuzumab or T-DM1. ST225 is a primary breast cancer PDX model with HER2 overexpression. Tumor growth curves in response to various treatment are presented in Figure 27 as the mean tumor volume (mm³) $\pm$ SEM (n=10).

3.8. Bystander Effect of Bispecific ADC

[00610] NCI-N87 cells were stably transfected with **green fluorescent protein (GFP)** and MDA-MB-468 cells were stably transfected with **red fluorescent protein (RFP)**. Both cell lines were harvested, washed, resuspended in culture media and then seeded in the same well of a 6-well plate as co-culture. As a control, each cell line was seeded in a different 6-well plate as single culture (Figure 28A). To adjust to different growth kinetics, NCI-N87 cells were seeded at 5×10^5 cells/well and MDA-MB-468 cells were seeded at 2×10^5 cells/well. Cells were incubated for 2 days at 37°C/5% CO₂. Media were aspirated and fresh media containing 5 nM of antibody or ADC to be tested was added to cells and the plates were incubated for 4 days at 37°C/5% CO₂. At the end of treatment, cells in each well were collected, washed, resuspended in 100 μ L of ice-cold FACS buffer and then fixed with 2% PFA. Cells were analyzed by a BD LSR II machine and BD FACSDiva™ software. Data were analyzed with FlowJo software.

[00611] Figure 28B demonstrates that Bs2-4T can kill both HER2-overexpressing and HER2-null cells in a co-culture, suggesting that Bs2-4T has bystander effect. In contrast, T-DM1 cannot kill HER2-null cells in a co-culture, suggesting that it does not have bystander effect. As controls, both Bs2-4T and T-DM1 showed potent killing of NCI-

N87 cells in single cultures and neither Bs2-4T nor T-DM1 showed killing of MDA-MB-468 cells in single cultures (data not shown).

3.9. Activity of Bispecific ADC against Cancer Stem Cells

[00612] *Cancer Stem Cell Sphere Assay:* MDA-MB-361 cells were cultured under standard tissue culture conditions in Leibovitz's L-15 medium supplemented with 20% FBS. Cells were harvested by trypsinization, washed twice with PBS, and resuspended to 30,000 cells per mL in stem cell medium (SCM: DMEM/F12 supplemented with 20 ng/mL EGF, 10ng/mL bFGF, 5 mg/mL insulin, 0.4% BSA and 1% knock-out serum replacement). To form primary spheres, 1 mL of cells were plated into 24-well ultra-low attachment plates and treated with 10 pM of either R347-4T, T-DM1 or BS2-4T and incubated for 4 days at 37°C/5% CO₂. At the end of treatment, the primary spheres were harvested, dissociated using 0.05% trypsin and resuspended at a density of 30,000 cells/mL in SCM containing the same antibody treatment as the primary sphere culture. Cells were then plated in triplicate into 96-well ultra-low attachment plates and incubated for 4 days. Sphere cell viability was determined using Cell Titer Glo according to manufacturer's instructions. Data are presented as fold of CSC sphere formation relative to the untreated control. Figure 29 (left panel) shows that Bs2-4T inhibited CSC sphere formation by 84% and T-DM1 had no activity in inhibiting CSC sphere formation. Similar results were observed in other cancer cell lines including BT-474, JIMT-1 and T47D (data not shown).

[00613] *Assessment of Cancer Stem Cells in Xenograft Tumors Treated with Bs2-4T:* Tumors from MDA-BM-361 xenograft studies evaluating the *in vivo* activity of Bs2-4T were excised, cut into 4 mm pieces and cryopreserved using Cryostor. Frozen tumor pieces were thawed at 37°C, washed twice in Hank's Balanced Salt Solution (HBSS) and further minced using sterile scalpel blades. To obtain single-cell suspensions, the tumor pieces were then mixed with 200 units of ultrapure collagenase III per mL of DMEM/F12 medium. The tumor suspension was incubated at 37°C for approximately 1 hour, with mechanical disruption every 30 minutes. At the end of the incubation, cells were filtered through a 70-µm nylon mesh and washed twice with HBSS. Following the last wash, cells were put through a 40-µm cell strainer and counted using a Vi-Cell XR Cell

Viability Analyzer. Cells were assayed for aldehyde dehydrogenase activity as a measure of CSCs using the Stemcell Technologies Aldefluor kit and following manufacturer's instructions. The cells were run on an LSRII flow cytometer and analyzed with FlowJo. Figure 29 (right panel) shows that the treatment with Bs2-4T resulted in reduction of CSC by 54% in MDA-MB-361 breast cancer xenograft model.

Example 4

4.1. Afucosylated Bispecific Antibody Production and Characterization

[00614] The bispecific antibodies were expressed using POTELEGENT™ technology (Biowa, Inc. Princeton, N.J.) to generated afucoslyated antibodies having enhanced ADCC activity. Table 8 shows the K_D (nM) as measured by BIAcore of Bs2Ab-39SH and Bs4Ab-39SH bispecific antibodies and trastuzumab with fucosylation (_Fuc) and without fucosylation (_aFuc) binding to Fc gamma receptors (FcγRs) and C1q demonstrating that the afucosylated antibodies have enhanced binding to FcγRI, and both alleles of FcγRIIIa.

Table 8: K_D (nM) Measured by BIAcore

Antibody	FcgRI	FcgRIIa	FcgRIIb	FcgRIIIa (158F)	FcgRIIIa (158V)	C1q
Bs2Ab-39SH_Fuc	7.0	1300	2750	1990	267	75.6
Bs2Ab-39SH_aFuc	0.98	1250	2400	216	40	130
Bs4Ab-39SH_Fuc	13.0	1190	2790	1780	228	88.6
Bs4Ab-39SH_aFuc	0.98	1260	2560	223	45	446
Trastuzumab_Fuc	5.1	1270	2650	2119	240	72.7
Trastuzumab_aFuc	0.65	1210	2190	305	60	134

[00615] *Measurement of Equilibrium Binding Constants: Human Fc Gamma Receptors:* The binding constants (K_D) for the binding of anti-HER2 bispecific antibodies to human FcγRs were measured on a ProteOn XPR36 instrument. Briefly, the bispecific antibodies were immobilized at high density on a GLC sensor chip using a standard amino coupling chemistry as outlined by the instrument manufacturer. The final surface density of IgG measured approximately 3000 RU. A reference flow cell was also prepared on this sensor

chip using the identical immobilization protocol minus protein. Stock solutions of each FcγR were prepared at either 4000 nM, 16,000 nM, or 32,000 nM in instrument buffer (phosphate buffered saline [PBS]/Tween/Ethylenediaminetetraaceticacid [EDTA] buffer containing 50 mM phosphate, pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.005% Tween-20), and then serially diluted (1:3) in the same buffer to obtain the desired concentration series for each receptor: 1.82 nM-4,000 nM (FcγRI), 197.5 nM-16,000 nM (FcγRIIA), 395.1 nM-16,000 nM (FcγRIIb), 21.9 nM-16,000 nM (hFcγRIIIA-158V), and 395-32,000 nM (FcγRIIIA-158F). Each concentration of FcγR was injected over both the bispecific antibody and reference cell surfaces at a flow rate of 25 μL/min for 8 min, during which binding data were collected. Between injections, the surfaces were regenerated (i.e., bound FcγR was removed) with a 60-sec pulse of 5 mM HCl. Several buffer injections were also interspersed throughout the injection series. Later, one of these buffer injections along with the reference cell data was used to correct the binding data for any injection artifacts (e.g., nonspecific binding) through a technique commonly referred to as “double-referencing” (Myszka, 1999). After all binding data were collected, individual data sets were averaged for binding (Response at equilibrium [Req]) at each concentration (C), and then fit to a 1:1 binding isotherm (Req vs. C) plot. From this, the equilibrium binding constants, K_D , were derived using the vendor’s evaluation software, version 3.1.0.6.

[00616] *Measurement of Equilibrium Binding Constants: Human FcRn Protein:* The affinity (K_D) for the binding of the bispecific antibodies to human FcRn protein (huFcRn) was measured on a ProteOn XPR36 instrument. Briefly, bispecific antibodies were immobilized at high density on a GLC sensor chip using a standard amino coupling chemistry, as described above. A stock solutions of huFcRn protein was prepared at 3000 nM in instrument buffer (50 mM sodium phosphate buffer, pH 6, containing 150 mM NaCl, and 0.05% Tween-20), and then serially diluted (3:1) to 1.37 nM in the same buffer. Each concentration of huFcRn was sequentially injected over the bispecific antibody and reference cell surfaces, connected in series, at a flow rate of 25 μL/min for 16 min. Binding data were collected, followed by a 60-sec injection of 50 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl, and 0.05% Tween 20 between injections of each receptor or buffer blank to regenerate the IgG surface (i.e., remove

bound huFcRn protein). Several buffer injections were also interspersed throughout the injection series. Later, one of these buffer injections was used along with the reference cell data to correct the raw data sets for injection artifacts (e.g., nonspecific binding) through “double-referencing” (Myszka, 1999). After all binding data was collected, individual data sets were averaged for binding (Req) at each concentration (C), and then fit to a 1:1 binding isotherm (Req vs. C) plot. From this, the equilibrium binding constants, K_D , were derived using the vendor’s BIAevaluation software, v. 4.1. Results show that the bispecific antibodies have similar K_D values which are comparable to conventional IgG1.

4.2. In Vitro Activity of Afucosylated Bispecific Antibody

[00617] Activity of afucosylated bispecific antibody in inhibiting ligand-driven cell proliferation was determined using method described in Example 1. Results show that afucosylated Bs2Ab-39SH, afucosylated Bs3Ab-39SH and afucosylated Bs4Ab-39SH have similar anti-proliferative potency in MDA-MB-361 cells (Figure 30A) and MCF-7 cells (Figure 30B), which is also comparable to the activity of parental antibody combination (39S plus trastuzumab). Similar results were observed in other cell lines including NCI-N87 and RT-112 (data not shown).

4.3. Enhanced ADCC Activity of Afucosylated Bispecific Antibody

[00618] KC1333, a human natural killer (NK) cell line expressing FcγRIIIa, was used as an effector cell and MDA-MB-361 cell line was used as a target cell in the ADCC assay to evaluate the ADCC activity of anti-HER2 antibodies. Both cell lines were harvested, washed and resuspended in assay medium. KC1333 were resuspended at a density of 1×10^6 cells/mL and MDA-MB-361 at 4×10^5 cells/mL. Fifty microliter of each cell line was added to the wells in a 96-well U-bottom plate to achieve target:effector ratio of 1:2.5. A 3X concentration of each dose of antibody was prepared by diluting the test articles in assay medium. Fifty microliter of each test article was added to cells in triplicate such that the final dose curve ranged from 10 μg/mL down to 0.15 ng/mL in a stepwise 1:4 serial dilution series. Plates were centrifuged to pellet cells in each well and then incubated overnight at 37°C/5% CO₂. The next day LDH in the supernatant of each well

was quantified using Promega's CytoTox 96® Non-Radioactive Cytotoxicity Assay according to manufacturer's instructions. Data are analyzed using the GraphPad Prism software and presented as percent cytotoxicity relative to the untreated control.

[00619] Figures 31A through 31C show the ADCC activity of afucosylated bispecific antibodies relative to trastuzumab and afucosylated trastuzumab in MDA-MB-361 cells. Data suggest that the ADCC activity of afucosylated antibody is higher than its fucosylated version and the rank of ADCC potency among afucosylated bispecific antibodies is Bs2Ab-39SH_aFuc > Bs4Ab-39SH_aFuc > Bs3Ab-39SH_aFuc. Similar results were observed in other cell lines including BT-474, NCI-N87, MDA-MB-453, T47D, and JIMT-1 (data not shown).

Example 5

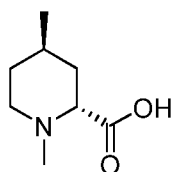
5.1. Tubulysin 1508 Payload Synthesis

[00620] Synthesis of tubulysin 1508 cytotoxin payload as shown in Figure 32 for conjugation is detailed in the following illustrative example in which, unless stated otherwise:

- (i) temperatures are given in degrees Celsius (°C); when operations were carried out at room temperature or ambient temperature, that is, in a range of 18-25 °C, unless otherwise stated;
- (ii) solutions were dried over anhydrous sodium sulphate or magnesium sulphate; evaporation of organic solvent was carried out using a rotary evaporator under reduced pressure (4.5 – 30 mmHg) with a bath temperature of up to 30 °C;
- (iii) chromatography means flash chromatography on silica gel; thin layer chromatography (TLC) was carried out on silica gel plates;
- (iv) in general, the course of reactions was followed by TLC or liquid chromatography/mass spectroscopy (LC/MS) and reaction times are given for illustration only;
- (v) final products have satisfactory proton nuclear magnetic resonance (NMR) spectra and/or mass spectra data;

- (vi) yields are given for illustration only and are not necessarily those which can be obtained by diligent process development; preparations were repeated if more material was required;
- (vii) when given, nuclear magnetic resonance (NMR) data is in the form of delta (δ) values for major diagnostic protons, given in part per million (ppm) relative to tetramethylsilane (TMS) as an internal standard, determined at 300 or 400 MHz in d_6 -DMSO unless otherwise stated;
- (viii) chemical symbols have their usual meanings;
- (ix) solvent ratio is given in volume:volume (v/v) terms; and
- (x) purification of the compounds was carried out using one or more of the following methods:
 - a) flash chromatography on regular silica gel;
 - b) flash chromatography on silica gel using Isco Combiflash® separation system: RediSep normal phase flash column, flow rate, 30-40 ml/min (ISCO MPLC); please add the ISCO reverse phase column
 - c) Gilson semiprep HPLC separation system: YMC pack ODS-AQ column, 100x20mm, S 5 μ m 12 nm, water (0.1% trifluoroacetic acid) and acetonitrile (0.1% trifluoroacetic acid) as solvents, 20 min run;

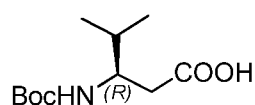
Compound T1



[00621] To a solution of (2R,4R)-4-methylpiperidine-2-carboxylic acid (2 g, 13.97 mmol) in MeOH (40 mL) and water (40.0 mL) was added paraformaldehyde (2.52 g, 27.94 mmol) and Pd/C (10%) (0.8 g, 7.52 mmol). The reaction mixture was stirred under a hydrogen atmosphere at room temperature overnight. From TLC, the reaction was not completed. Another one equivalent of paraformaldehyde (2.52 g, 27.94 mmol) was added and the reaction mixture was stirred another 24 hours. TLC indicated the reaction was completed and reaction mixture was filtered, washed the catalyst with MeOH (2 $\times$ 30

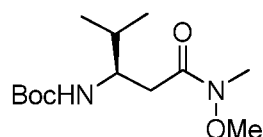
mL). The filtrate was concentrated in vacuo to give crude product as a white solid, which was washed with ether (3×30 mL), dried in high vacuum overnight to yield (2R,4R)-1,4-dimethylpiperidine-2-carboxylic acid (T1) (1.870 g, 85 %) as a white solid. LC-MS: 158 ($M+1$); ^1H NMR (400 MHz, DEUTERIUM OXIDE) δ ppm 0.97 (d, $J=5.52$ Hz, 3 H), 1.54 (br. s, 1 H), 1.71 - 1.87 (m, 3 H), 1.91 - 2.07 (m, 1 H), 2.84 (s, 3 H), 3.13 (td, $J=8.41, 3.76$ Hz, 1 H), 3.35 (m, 1 H), 3.65 (m, 1 H).

Compound T2



[00622] Di-tert-Butyl dicarbonate (243.0 g, 1.1 mol) was added dropwise to a suspension of (R)-3-amino-4-methyl pentanoic acid (commercially available) (133.0 g, 1.0 mol) and Na_2CO_3 (212 g, 2.0 mol) in acetone (1 L) and water (1 L) with stirring at room temperature. The reaction mixture was stirred overnight and the organic solvent was removed under reduced pressure. The residue was diluted with water (1 L) and washed with EtOAc (500 mL $\times 3$). The aqueous phase was acidified with 2N HCl solution to pH=3 and the resulting mixture was extracted with EtOAc (800 mL $\times 3$). The combined extracts were washed with brine (800 mL $\times 1$), dried (anhydrous Na_2SO_4) and concentrated to give compound (T2) (224.0 g, 97% yield) as an oil, which was used in the next step without further purification.

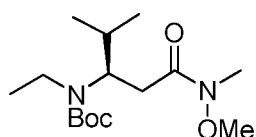
Compound T3



[00623] Triethylamine (67 g, 0.61 mol) was added to a suspension of compound (T2) (140.0 g, 0.61 mol) and N,O-dimethylhydroxylamine hydrochloride (74.1 g, 0.76 mol) in

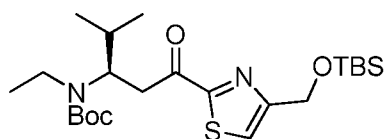
CH₂Cl₂ (1.4 L) with stirring at 0°C. The suspension was stirred for 0.5 hour and EDCI (74 g, 0.61 mol) was added in portions at 0 °C. The reaction mixture was stirred for 2 hours at 0°C and water (800 mL) was added. The organic phase was separated, washed with 5% KHSO₄ solution (800 mL×3), saturated NaHCO₃ solution (800 mL×3) and brine (800 mL×1), was dried (anhydrous Na₂SO₄) and concentrated to dryness. The residue was purified by flash column chromatography on silica gel (EtOAc/Hexane=1:5) to afford compound (**T3**) (141.0 g, 84% yield) as an oil. ¹H NMR (300 MHz, CDCl₃): δ 5.26 (m, 1H), 3.75 (m, 1H), 3.70 (s, 3H), 3.15 (s, 3H), 2.60~2.80 (m, 2H), 1.85 (m, 1H), 1.41 (s, 9H), 0.90 (d, J = 6.6 Hz, 3H), 0.88 (d, J = 6.6 Hz, 3H).

Compound **T4**



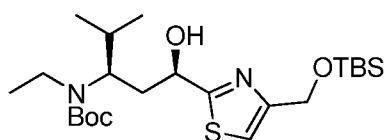
[00624] Iodoethane (250.0 g, 1.6 mol) was added to a solution of compound (**T3**) (55.0 g, 0.2 mol) in DMF (1.1 L) with stirring at 0°C. Then NaH (60% suspension, 24.0 g, 0.60 mol) was added in portions at 0°C and the reaction mixture was allowed to warm to room temperature and stirred for 12 hours. The reaction was quenched with water (2 L) carefully and EtOAc (2 L) was added. The organic phase was separated, washed with 5% KHSO₄ solution (800 mL×3), saturated NaHCO₃ solution (800 mL×3) and brine (800 mL×1), dried (anhydrous Na₂SO₄) and concentrated to dryness. The residue was purified by flash column chromatography on silica gel (EtOAc/Hexane=1:10) to afford compound (**T4**) (35.1 g, 58% yield) as an oil. ¹H NMR (300 MHz, CDCl₃): δ 3.70 (s, 3H), 3.65 (m, 1H), 3.10-3.30 (m, 5H), 2.50-2.95 (m, 2H), 1.90-2.20 (m, 1H), 1.40~1.55 (m, 9H), 1.10 (t, J = 7.2 Hz, 3H), 0.90 (d, J = 6.6 Hz, 3H), 0.88 (d, J = 6.6 Hz, 3H).

Compound **T5**



[00625] A solution of n-BuLi (106 ml, 2.5N in hexane, 0.17 mol) was added dropwise to a solution of 2-bromo-4-((tert-butyldimethylsilyloxy)methyl)thiazole (74 g, 0.24 mol) (prepared as described in Wipf, P et al Org. Lett. 2007, 9(8), p. 1605) in dry THF (500 mL) at -78°C under N₂ with stirring over 1 hour. The suspension was stirred for further 30 min and then a solution of compound (T4) (51.0 g, 0.17 mol) in dry THF (300 mL) was added dropwise over 30 min at -78°C. The reaction mixture was stirred for 1 hour at -78°C and then allowed to warm to room temperature and stirred for 12 hours. The reaction was quenched with 20% aqueous ammonium chloride solution (1 L) and the organic solvent was removed under reduced pressure. The resulting mixture was extracted with EtOAc (800 mL×3). The combined organic phases were washed with 5% KHSO₄ solution (800 mL×3), saturated NaHCO₃ solution (800 mL×3) and brine (800 mL×1), dried (Na₂SO₄) and concentrated to dryness. The crude material was purified by flash column chromatography on silica gel (EtOAc/Hexane=1:10) to afford compound (T5) (58.1 g, 73% yield) as an oil. ¹H NMR (300 MHz, CDCl₃): δ 7.53 (m, 1H), 4.90 (s, 2H), 4.04 (m, 1H), 3.35 (m, 2H), 3.15 (m, 2H), 2.00 (m, 1H), 1.40 (s, 9H), 0.80-1.20 (m, 18H), 0.14 (s, 6H).

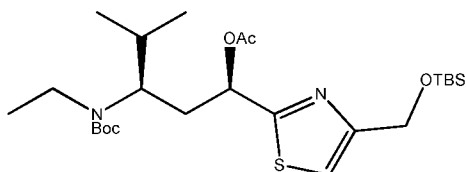
Compound T6



[00626] LiBH₄ (4.8 g, 0.22 mol) was added in portions to a solution of compound (T5) (47.1 g, 0.1 mol) in methanol (500 mL) at room temperature over a period of 0.5 hour with stirring. The suspension was stirred for 2 hours and the solvent was removed under reduced pressure. The residue was dissolved in EtOAc (800 mL) and the resulting solution was washed with saturated NaHCO₃ solution (500 mL×3) and brine (500 mL×1), dried (Na₂SO₄) and concentrated to dryness. The crude material was purified by flash column chromatography (EtOAc/Hexane=1:6) to afford compound (T6) (13.5 g, 28% yield) and its isomer (T6') (21.0 g, 45% yield). ¹H NMR (300 MHz, CHLOROFORM-d)

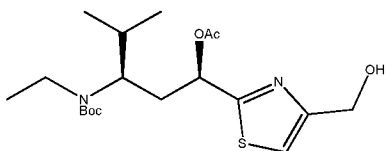
δ ppm -0.06 - 0.05 (m, 6 H) 0.76 - 0.89 (m, 15 H) 1.12 (t, $J=6.97$ Hz, 3 H) 1.39 (s, 9 H) 1.55 - 2.05 (m, 3 H) 2.86 - 3.21 (m, 2 H) 3.76 - 3.96 (m, 1 H) 4.73 (d, $J=1.13$ Hz, 4 H) 7.01 (s, 1 H).

Compound T7



[00627] Acetyl chloride (45.2 g, 0.58 mol) was added dropwise to a solution of compound (T6) (34.0 g, 72 mmol) in pyridine (500 mL) at 0°C with stirring over 10 min. The reaction mixture was allowed to warm to room temperature and stirred for 12 hours. The reaction was quenched with water (200 mL) and the solvent was removed under reduced pressure. The residue was treated with CH₂Cl₂ (800 mL) and the resulting mixture was washed with 5% KHSO₄ solution (800 mL×3), saturated NaHCO₃ solution (800 mL×3) and brine (800 mL×1), dried (Na₂SO₄) and concentrated to dryness. The crude material was purified by flash column chromatography on silica gel (EtOAc/ Hexane=1:10) to afford compound (T7) (25.7 g, 69% yield) as an oil. ¹H NMR (300 MHz, CDCl₃): δ 7.15 (m, 1H), 5.95 (m, 1H), 4.84 (s, 2H), 4.04 (m, 1H), 3.10 (m, 2H), 2.35 (m, 1H), 2.15 (s, 3H), 2.00 (m, 1H), 1.70 (m, 1H), 1.45 (s, 9H), 1.25 (t, $J = 7.2$ Hz, 3H), 0.80~1.10 (m, 15H), 0.08 (s, 6H).

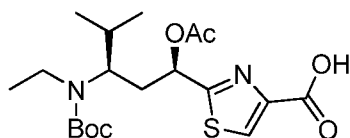
Compound T8



[00628] A solution of tetrabutylammonium fluoride (65.3 g, 0.25 mol) in THF (200 mL) was added dropwise to a solution of compound (7) (25.7 g, 50 mmol) in THF (300 mL) at 0°C with stirring. The reaction mixture was allowed to warm to room temperature and

stirred for 4 hours. Water (800 mL) was added and the organic solvent was removed under reduced pressure. The residue was treated with CH_2Cl_2 (800 mL) and the resulting mixture was washed with 5% KHSO_4 solution (800 mL $\times$ 3), saturated NaHCO_3 solution (800 mL $\times$ 3) and brine (800 mL $\times$ 1), dried (Na_2SO_4) and concentrated to dryness. The crude material was purified by flash column chromatography on silica gel (EtOAc/Hexane=1:4) to afford compound (**T8**) (19.5 g, 98% yield) as an oil. ^1H NMR (300 MHz, CDCl_3): δ 8.26 (m, 1H), 5.95 (m, 1H), 4.83 (m, 2H), 4.10 (m, 1H), 3.17 (m, 2H), 2.40 (m, 1H), 2.20 (s, 3H), 2.18 (m, 1H), 1.75 (m, 1H), 1.56 (s, 9H), 1.10-1.30 (m, 3H), 0.80-1.05 (m, 6H).

Compound T9

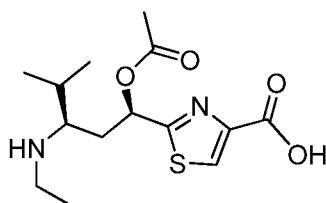


[00629] Dess-Martin reagent (32.7 g, 75 mmol) was added to a solution of compound (**T8**) (20.0 g, 50 mmol) in dichloromethane (300 mL) and the reaction mixture was stirred at room temperature for 12 hours. The mixture was washed with sodium hydroxide solution (1N, 300 mL $\times$ 3), sodium thiosulfate solution (1N, 300 mL $\times$ 3), saturated NaHCO_3 (300 mL $\times$ 3) solution and brine (300 mL $\times$ 1), respectively. The organic layer was dried (Na_2SO_4) and concentrated to dryness to give the corresponding aldehyde.

[00630] This crude aldehyde was dissolved in tert-butyl alcohol (500 mL) and a solution of sodium chlorite (80%, 36.4 g, 320 mmol) and sodium dihydrogenphosphate monohydrate (105 g, 0.77 mol) in water (300 mL) was added dropwise over 1 hour at room temperature. The reaction mixture was stirred for 3 hours and diluted with hydrochloric acid solution (0.1N, 500 mL). The resulting mixture was extracted with EtOAc (500 mL $\times$ 1) and the combined organic layers were washed with 5% KHSO_4 solution (500 mL $\times$ 3) and brine (500 mL $\times$ 1), dried over Na_2SO_4 and concentrated to dryness. The residue was purified by flash column chromatography on silica gel ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ =100:5) to afford compound (**T9**) (15.4 g, 58% yield). ^1H NMR (300

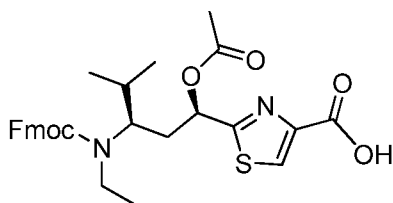
MHz, CDCl₃): δ 9.90 (br s, 1H), 8.27 (s, 1H), 5.96 (m, 1H), 4.07 (m, 1H), 3.15 (m, 1H), 2.35 (m, 1H), 2.20 (s, 3H), 2.18 (m, 1H), 1.75 (m, 1H), 1.45 (s, 9H), 1.20 (t, J = 7.2 Hz, 3H), 0.98 (d, J = 6.6 Hz, 3H), 0.88 (d, J = 6.6 Hz, 3H).

Compound T10



[00631] To a solution of 2-((1R,3R)-1-acetoxy-3-((tert-butoxycarbonyl)(ethyl)amino)-4-methylpentyl)thiazole-4-carboxylic acid (**T9**) (6.5g, 15.68 mmol) in DCM (60 mL) was added TFA (30 mL) in dropwise at 0 °C. The mixture was stirred at 0 °C for 1 hour. The solvent was evaporated *in vacuo* to give crude product (**T10**). The crude product was used to next step reaction without further purification (7.2 grams). LC-MS : 315 (M + 1).

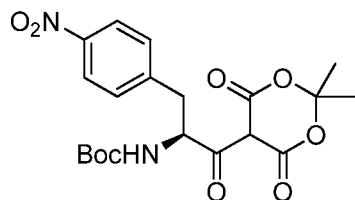
Compound T11



[00632] To the solution of 2-((1R,3R)-1-acetoxy-3-(ethylamino)-4-methylpentyl)thiazole-4-carboxylic acid 4, Trifluoroacetic acid salt (**T10**) (5g, 11.67 mmol) and sodium bicarbonate (9.80 g, 116.71 mmol) in a mixture of acetone (300 mL) and water (150 mL) was added (9H-fluoren-9-yl)methyl (2,5-dioxopyrrolidin-1-yl) carbonate (3.94 g, 11.67 mmol). The mixture was stirred at room temperature overnight. LCMS indicated the reaction was completed. The mixture was acidified to (pH 2) with hydrochloric acid and acetone was evaporated *in vacuo*. The product was extracted with DCM (3 × 300 mL). The combined organic extracts were washed with 0.1% HCl solution (200 mL), brine (200 mL), dried over Na₂SO₄, and evaporated *in vacuo*. The residue was purified by flash

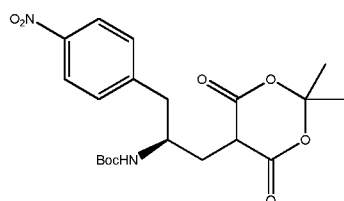
chromatography (silica gel, MeOH/DCM, MeOH from 0% to 5%,) to give 2-(((1R,3R)-3-(((9H-fluoren-9-yl)methoxy)carbonyl)(ethyl)amino)-1-acetoxy-4-methylpentyl)thiazole-4-carboxylic acid (**T11**) (3.53 g, 54.6 %) as a white solid. LC-MS : 537.2 (M+1); ¹H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 0.84 (d, *J*=6.78 Hz, 3 H), 0.92 - 1.05 (m, 5 H), 1.14 (d, *J*=3.01 Hz, 1 H), 1.73 (dt, *J*=10.23, 6.43 Hz, 1 H), 1.92 - 2.05 (m, 1 H), 2.12 - 2.27 (m, 4 H), 2.28 - 2.44 (m, 1 H), 2.90 - 3.33 (m, 2 H), 3.98 (t, *J*=9.29 Hz, 1 H), 4.12 - 4.32 (m, 1 H), 4.47 - 4.82 (m, 2 H), 5.95 (dd, *J*=10.92, 2.89 Hz, 1 H), 7.29 - 7.45 (m, 4 H), 7.55 - 7.69 (m, 2 H), 7.72 - 7.81 (m, 2 H), 8.22 - 8.29 (m, 1 H).

Compound **T12**



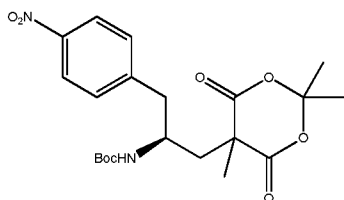
[00633] DMAP (106 g, 0.86 mol) was added to a solution of Boc-L-4-nitro-Phenylalanine (1800 g, 0.58 mol) and Meldrum's acid (92 g, 0.64 mol) in dichloromethane (1.5 L). The resulting solution was cooled at -5°C under N₂ atmosphere, followed by addition of DCC (240 g, 1.16 mol) in dichloromethane (1 L) over 1 h. The mixture was stirred overnight at 0-5°C. Then the precipitated N,N'-dicyclohexylurea was removed by filtration and the filtrate was washed with 5% aqueous HCl (1 L $\times$ 3), and brine (1 L $\times$ 1), and was dried over MgSO₄. After removal of MgSO₄ by filtration, the organic phase was concentrated to dryness. The residue was triturated with EtOAc/hexane (1:1, 500 mL), and was dried to afford compound (**T12**) (130.0 g, 51% yield) as a yellow solid.

Compound **T13**



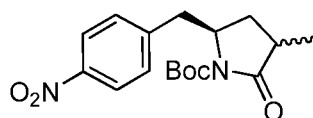
[00634] AcOH (400 mL) was added to a solution of compound (**T12**) (130.0 g, 0.298 mol) in dichloromethane (1.5 L) at -5°C under N₂. Solid NaBH₄ (22.7 g, 0.597 mol) was added in small portions over 2 hours (gas evolution and exothermic). After stirring for additional 3 h at -5 °C, TLC indicated the reaction was complete. The mixture was quenched with brine (1 L). The organic layer was separated, and washed sequentially with water (1 L×2), aqueous saturated NaHCO₃ (1 L×3) and brine (1 L×3), and was dried over MgSO₄. The filtrate was concentrated to dryness and afford compound (**T13**) (70.3 g, 55 % yield) as a yellow solid. ¹H NMR (300 MHz, CDCl₃): δ 8.18 (d, J=8.7 Hz, 2H), 7.41 (d, J=8.7 Hz, 2H), 4.58 (m, 1H), 4.29 (m, 1H), 3.85 (m, 1H), 2.97 (d, J=6.6 Hz, 2H), 2.27 (m, 2H), 1.80 (s, 3H), 1.76 (s, 3H), 1.35 (s, 9H).

Compound **T14**



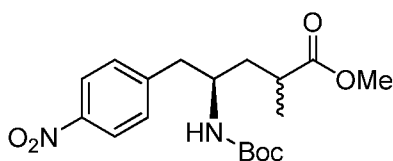
[00635] K₂CO₃ (35 g, 0.25 mol) and MeI (36 g, 0.25 mol) were added to a solution of compound (**T13**) (70.3 g, 0.167 mol) in acetone (400 mL) and DMF (400 mL). The mixture was stirred overnight at room temperature. TLC showed the starting material was consumed. Water (2 L) was added and the mixture was stirred for an additional hour. The precipitated solid was collected by filtration, was washed with water, was dried to afford compound (**T14**) (34.5 g, 47% yield) as a pale yellow solid. ¹H NMR (300 MHz, CDCl₃): δ 8.17 (d, J=8.7 Hz, 2H), 7.34 (d, J=8.7 Hz, 2H), 4.22 (m, 1H), 3.85 (m, 1H), 2.85 (m, 2H), 2.22 (m, 2H), 1.73 (s, 3H), 1.73 (s, 3H), 1.52 (s, 3H), 1.31 (s, 9H).

Compound **T15**



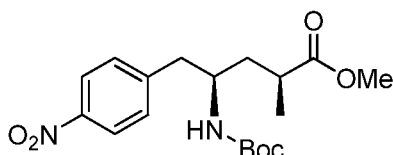
[00636] Compound (**T14**) (34.5 g, 79.1 mmol) was dissolved in toluene (500 mL). The solution was heated under reflux for 40 hours. TLC indicated the reaction complete. The solvent was removed to afford compound (**T15**) (30g), which was used for next step without further purification.

Compound T16



[00637] K_2CO_3 (22 g, 0.16 mol) was added to a solution of compound (**T15**) (30 g, 79 mmol) in MeOH (300 mL). The mixture was stirred for 3 hours at room temperature. TLC showed complete conversion. The solvent was removed, the residue was dissolved in dichloromethane (500 mL), washed with brine (500 mL $\times$ 3), dried over $MgSO_4$. After removal of $MgSO_4$ by filtration, the organic phase was concentrated to dryness. The residue was further purified by silica gel chromatography (EtOAc/Hexane=1:10) and afforded compound (**T16**) (23.5 g, 81 % yield for two steps) as 1:1 diastereomeric mixture. 1H NMR (300 MHz, $CDCl_3$): δ 8.13 (d, $J=8.7$ Hz, 2H), 7.34 (d, $J=8.7$ Hz, 2H), 4.43 (m, 1H), 3.85 (m, 1H), 3.65 (s, 3H), 2.85 (m, 2H), 2.65 (m, 1H), 1.85 (m, 1H), 1.50 (m, 1H), 1.30 (s, 9H), 1.15 (t, $J=6.6$ Hz, 3H).

Compound T17



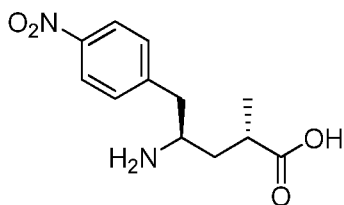
[00638] 50 g of compound (**T16**) was subjected to chiral chromatography using SFC (supercritical fluid chromatography) on a Chiralpak ID 21×250 mm, 5 μ column using mobile phase A 90% carbon dioxide and phase B isopropanol 10% at a 60 ml/min flow rate. The separation was performed at 40 °C and detection at 270 nM. Baseline separation was achieved and two fractions were isolated. Peak B was desired compound (**T17**) and was obtained as a solid 27.4 g (55%).

[00639] >99:1 diastereomeric excess on a Chiralpak IA column 4.6×250 mm, 5 μ , 10% 1:1 Methanol:Isopropanol in hexane with 0.1% diethylamine modifier.

[00640] LC/MS (2 minute, Acid _CV10.olg method 367 (M + 1), 1.16 minutes.

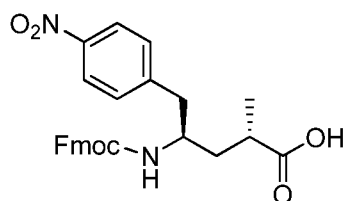
[00641] ^1H NMR (400 MHz, METHANOL- d_4) δ ppm 8.16 (d, $J=8.53$ Hz, 2 H) 7.46 (d, $J=8.53$ Hz, 2 H) 3.79 - 3.93 (m, 1 H) 3.68 (s, 3 H) 2.90 - 2.99 (m, 1H) 2.71 - 2.81 (m, 1 H) 2.47 - 2.59 (m, 1 H) 1.81 - 1.95 (m, 1 H) 1.55 - 1.66 (m, 1 H) 1.32 (s, 9 H) 1.21 - 1.25 (m, 2 H) 1.16 (d, $J=7.03$ Hz, 3 H).

Compound **T18**



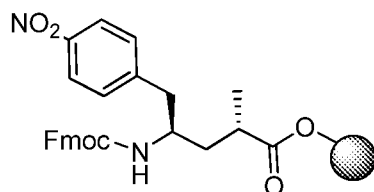
[00642] A solution of (2S,4R)-methyl 4-((tert-butoxycarbonyl)amino)-2-methyl-5-(4-nitrophenyl)pentanoate (**T17**) (3.5g, 9.55 mmol) in 6N HCl aqueous solution (8.0 mL, 263.30 mmol) was heated at 130 °C in microwave for 30 min. The reaction mixture was lyophilized to afford (2S,4R)-4-amino-2-methyl-5-(4-nitrophenyl)pentanoic acid (**T18**) as a solid. The product was used in the next step reaction without further purification. (3.2 g). LC-MS : 253 (M + 1); ^1H NMR (400 MHz, DEUTERIUM OXIDE) δ ppm 1.12 (d, $J=7.28$ Hz, 3 H), 1.62 - 1.76 (m, 1 H), 1.90 - 2.02 (m, 1 H), 2.56 - 2.68 (m, 1 H), 3.02 - 3.11 (m, 2 H), 3.58 - 3.69 (m, 1 H), 7.47 (d, $J=8.53$ Hz, 2 H), 8.18 (d, $J=8.78$ Hz, 2 H).

Compound T19



[00643] To the solution of compound (T18) (0.43 g, 1.49 mmol) and sodium bicarbonate (1.251 g, 14.89 mmol) in a mixture of acetone (30 mL) and water (15 mL) was added (9H-fluoren-9-yl)methyl 2,5-dioxopyrrolidin-1-yl carbonate (0.502 g, 1.49 mmol). The mixture was stirred at room temperature for overnight. LCMS indicated the reaction was completed. The mixture was acidified to pH 2 with hydrochloric acid and acetone was evaporated *in vacuo*. The product was extracted with DCM (3 X 60 mL). The combined organic extracts were washed with 1N HCl solution (40 mL), brine (40 mL), dried over Na₂SO₄, and evaporated *in vacuo*. The residue was purified by silica gel flash chromatography, EtOAc from 0% to 100% in DCM, to give (2S,4R)-4-(((9H-fluoren-9-yl)methoxy)carbonylamino)-2-methyl-5-(4-nitrophenyl)pentanoic acid (0.630 g, 89 %) (T19) as a white solid. LC-MS : 475.5 (M+H); ¹H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 0.81 - 1.06 (m, 1 H), 1.08 - 1.28 (m, 2 H), 1.33 - 1.75 (m, 1 H), 1.77 - 2.11 (m, 1 H), 2.36 - 2.69 (m, 2 H), 2.76 - 3.18 (m, 1 H), 3.43 - 4.08 (m, 1 H), 4.09 - 4.19 (m, 1 H), 4.21 - 4.53 (m, 2 H), 4.54 - 4.80 (m, 1 H), 7.18 - 7.58 (m, 8 H), 7.66 - 7.82 (m, 2 H), 7.95 - 8.17 (m, 2 H), 8.67 (br. s., 1 H).

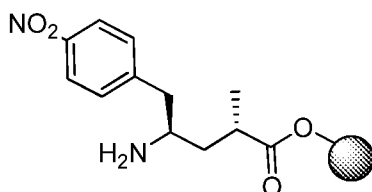
Compound T20



[00644] DIEA (0.419 mL, 2.40 mmol) was added to a solution of (2S,4R)-4-(((9H-fluoren-9-yl)methoxy)carbonylamino)-2-methyl-5-(4-nitrophenyl)pentanoic acid (0.380 g, 0.80 mmol) (T19) in DCM (4.5 mL), and the mixture was stirred at room temperature

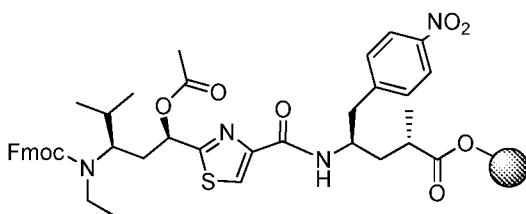
for 5 min, then 2-Chlorotrityl chloride resin (0.5 g, 0.80 mmol) was added to the mixture. The mixture was shaken at room temperature overnight, the resulting resin was washed with DMF (3 X 6 mL), MeOH (3 X 6 mL), and DCM (3 X 6 mL), then treated with DIEA (0.419 mL, 2.40 mmol) and MeOH/DCM (1:1, 5 mL) at room temperature for 30 min. Resulting resin was filtered, washed with DMF (3 X 6 mL), MeOH (3 X 6 mL), and DCM (3 X 6 mL), dried in high vacuum overnight. Small amount of compound was cleaved from resin, and analyzed by LCMS. The resulting resin (**T20**) was used for next step reaction. LC-MS : 475 (M + 1)

Compound **T21**



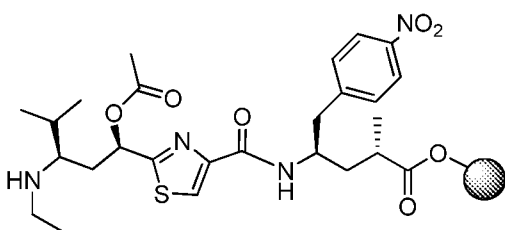
[00645] To the resin (**T20**) (0.5 g, 0.80 mmol) was added 20% piperidine in DMF (5 mL). The mixture was shaken at room temperature for 6 min, the resulting resin was filtered, washed with DMF (3 × 6 mL), MeOH (3 × 6 mL), DCM (3 × 6 mL), dried *in vacuo*. Small amount of the compound was cleaved from resin, analyzed by LCMS, which indicated the reaction was completed. The resulting resin (**T21**) was used for the next step reaction. LC-MS: 253 (M + H).

Compound **T22**



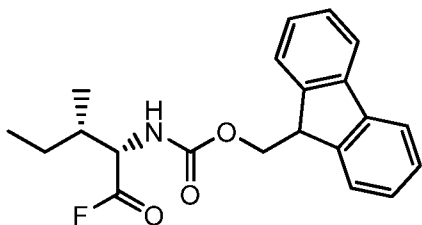
[00646] To the resin (**T21**) (0.5 g, 1.88 mmol) was added a solution of 2-((1R,3R)-3-(((9H-fluoren-9-yl)methoxy)carbonyl)(ethyl)amino)-1-acetoxy-4-methylpentyl)thiazole-4-carboxylic acid (**3**) (1.108 g, 2.07 mmol), HATU (1.428 g, 3.76 mmol), 2,4,6-trimethylpyridine (0.500 mL, 3.76 mmol), and DIEA (0.656 mL, 3.76 mmol) in DMF (5 mL) at room temperature. The mixture was shaken at room temperature for two hours, and the resulting resin was filtered, washed with DMF (3 × 6 mL), MeOH (3 × 6 mL), and DCM (3 × 6 mL), dried *in vacuo*. Small amount of the compound was cleaved from resin, analyzed by LCMS, which indicated the reaction was completed. The resulting resin (**T22**) was used for the next step. LC-MS: 771 (M + H).

Compound **T23**



[00647] To the resin (**T22**) (0.5 g, 0.80 mmol) was added 20% piperidine in DMF (5 mL). The mixture was shaken at room temperature for 6 min, the resulting resin was filtered, washed with DMF (3 × 6 mL), MeOH (3 × 6 mL), DCM (3 × 6 mL), dried *in vacuo*. Small amount of the compound was cleaved from resin, analyzed by LCMS, which indicated the reaction was completed. The resulting resin (**T23**) was used in the next reaction step. LC-MS : 549 (M + 1).

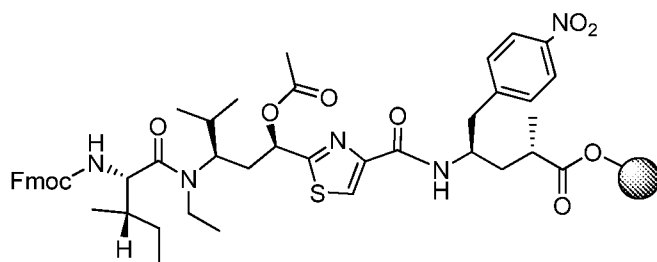
Compound **T24**



[00648] To a solution of (2S,3S)-2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-3-methylpentanoic acid (Fmoc-Isoleucine) (7 g, 19.81 mmol) and pyridine (1.602 mL, 19.81 mmol) in DCM (120 mL) was added via cannula a solution of DAST (3.11 mL, 23.77 mmol) in DCM (20 mL) over 10 min. The reaction mixture was stirred at room temperature for 1 hour, diluted with DCM (80 mL), washed with ice-cold water (2 × 200 mL), the organic layer was dried over MgSO₄, filtered, and evaporated in vacuo to give (9H-fluoren-9-yl)methyl (2S,3S)-1-fluoro-3-methyl-1-oxopentan-2-ylcarbamate (6.65 g, 94 %) as a white solid. An esterification test was performed to assure quantitative acid fluoride formation by dissolving Fmoc-Ile-F (5 mg) in anhydrous MeOH (0.3 mL) and DIEA (0.030 mL) and allowing to react at room temperature for 15 min. The mixture was then evaporated in vacuo and analyzed by LCMS, showed less than 1% of Fmoc-Ile-OH present.

[00649] ¹H NMR (400 MHz, CHLOROFORM-d) δ ppm 0.83 - 1.12 (m, 6 H) 1.18 - 1.37 (m, 1 H) 1.42 - 1.59 (m, 1 H) 2.01 (br. s., 1 H) 4.26 (t, J=6.78 Hz, 1 H) 4.44 - 4.63 (m, 3 H) 5.20 (d, J=8.53 Hz, 1 H) 7.31 - 7.39 (m, 2 H) 7.40 - 7.47 (m, 2 H) 7.61 (d, J=7.28 Hz, 2 H) 7.80 (d, J=7.53 Hz, 2 H).

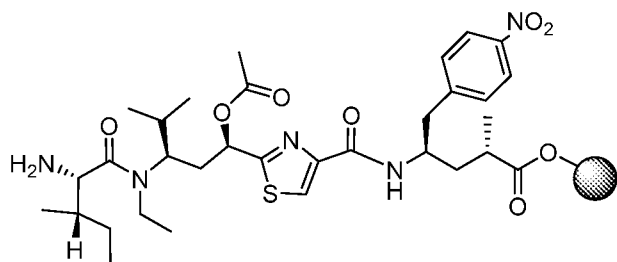
Compound T25



[00650] To the resin (**T23**) (0.5 g, 0.80 mmol) was added a solution of (9H-fluoren-9-yl)methyl (2S,3S)-1-fluoro-3-methyl-1-oxopentan-2-ylcarbamate (**T24**) (0.569 g, 1.60 mmol), DMAP (4.89 mg, 0.04 mmol), and DIEA (0.419 mL, 2.40 mmol) in DCM (5 mL) at room temperature. The mixture was shaken at room temperature for overnight, the resulting resin was filtered, washed with DMF (3 × 6 mL), MeOH (3 × 6 mL), DCM (3 × 6 mL), dried in high vacuum. Small amount of compound was cleaved from resin, and

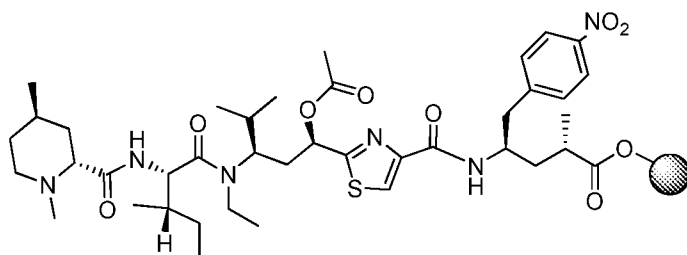
analyzed by LC/MS, LCMS indicated the reaction was completed. The resulting resin (**T25**) was used in the next reaction step. LC-MS : 884 (M + H).

Compound **T26**



[00651] To the resin (**T25**) (0.5 g, 0.80 mmol) was added 20% piperidine in DMF (5 mL). The mixture was shaken at room temperature for 6 min, the resulting resin was filtered, washed with DMF (3 × 6 mL), MeOH (3 × 6 mL), DCM (3 × 6 mL), dried *in vacuo*. Small amount of the compound was cleaved from resin, analyzed by LCMS, which indicated the reaction was completed. The resulting resin (**T26**) was used in the next reaction step. LC-MS: 662 (M + 1).

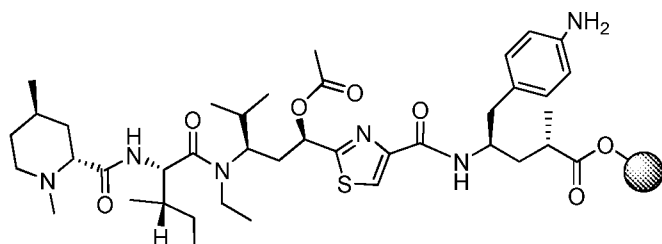
Compound **T27**



[00652] To the resin (**T26**) (0.5 g, 0.80 mmol) was added a solution of (2R,4R)-1,4-dimethylpiperidine-2-carboxylic acid (**1**) (0.252 g, 1.60 mmol), HATU (0.608 g, 1.60 mmol), 2,4,6-trimethylpyridine (0.320 mL, 2.40 mmol), and DIEA (0.419 mL, 2.40 mmol) in DMF (5 mL). The mixture was shaken at room temperature for 2 hours, the resulting resin was filtered, washed with DMF (3 × 6 mL), MeOH (3 × 6 mL), and DCM (3 × 6 mL), dried *in vacuo*. Small amount of the compound was cleaved from resin,

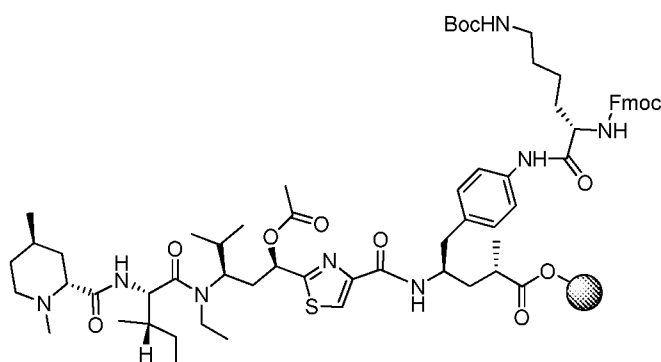
analyzed by LCMS, which indicated the reaction was completed. The resulting resin (**T27**) was used in the next reaction step. LC-MS: 801 (M + 1).

Compound **T28**



[00653] To the resin (**T27**) was added a solution of tin(II) chloride dehydrate (1.805 g, 8.00 mmol), and sodium acetate (0.197 g, 2.40 mmol) in DMF (5 mL). The mixture was shaken at room temperature for 4 hours, the resulting resin was filtered, washed with DMF (3 × 6 mL), MeOH (3 × 6 mL), and DCM (3 × 6 mL), and dried *in vacuo*. Small amount of the compound was cleaved from resin, analyzed by LCMS, which indicated the reaction was completed. The resulting resin (**T28**) was used for the next step. LC-MS: 771 (M + H).

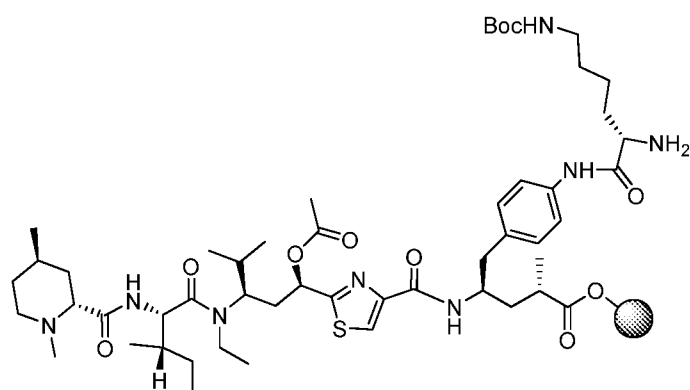
Compound **T29**



[00654] To the resin (**T28**) (0.2 g, 0.32 mmol) was added a solution of (S)-2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-6-(tert-butoxycarbonylamino)hexanoic acid (commercially available) (0.300 g, 0.64 mmol), HATU (0.243 g, 0.64 mmol), 2,4,6-

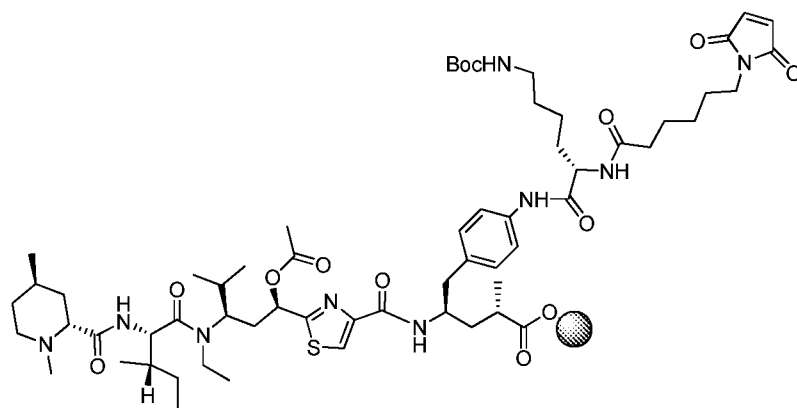
trimethylpyridine (0.128 mL, 0.96 mmol), and DIEA (0.168 mL, 0.96 mmol) in DMF (4 mL). The mixture was shaken at room temperature for 2 hours, the resulting resin was filtered, washed with DMF (3×2 mL), MeOH (3×2 mL), and DCM (3×2 mL), and dried *in vacuo*. Small amount of the compound was cleaved from resin, analyzed by LCMS, which indicated the reaction was completed. The resulting resin (**T29**) was used to next step reaction. LC-MS: 1221 ($M + 1$).

Compound T30



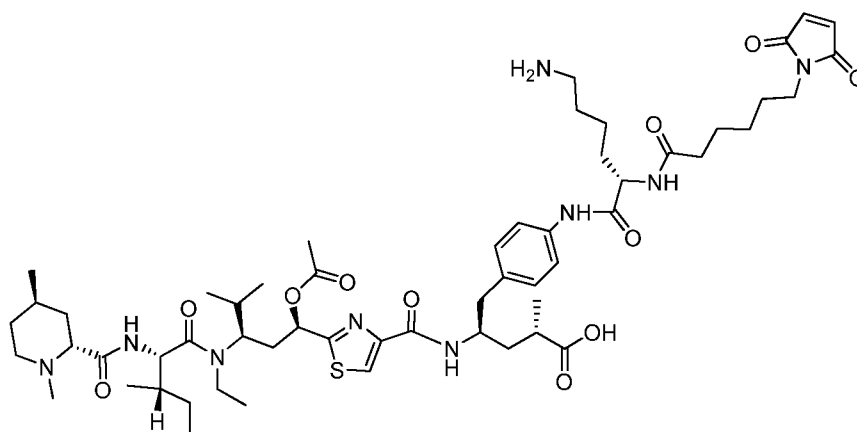
[00655] To the resin (**T29**) (0.2 g, 0.32 mmol) was added 20% piperidine in DMF (2 mL). The mixture was shaken at room temperature for 6 min, the resulting resin was filtered, washed with DMF (3×3 mL), MeOH (3×3 mL), DCM (3×3 mL), dried *in vacuo*. Small amount of the compound was cleaved from resin, analyzed by LCMS, which indicated the reaction was completed. The resulting resin (**T30**) was used to next step reaction. LC/MS : 999 ($M + H$).

Compound T31



[00656] To the resin (**T30**) (0.2 g, 0.32 mmol) was added a solution of 2,5-dioxopyrrolidin-1-yl 6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoate (0.148 g, 0.48 mmol) in DMF (2 mL), followed by N-METHYLMORPHOLINE (0.106 mL, 0.96 mmol) at room temperature. The mixture was shaken at room temperature for 2 hours, the resulting resin was filtered, washed with DMF (3 × 3 mL), DCM (3 × 3 mL), dried *in vacuo*. Small amount of the compound was cleaved from resin, analyzed by LCMS, which indicated the reaction was completed. The resulting resin (**T31**) was used for the next step. LC-MS: 1192 (M + 1).

Compound T32 (tubulysin 1508)



[00657] To the resin (**T31**) (0.2 g, 0.32 mmol) was added DCM (1 mL), and TFA (1 mL) at room temperature. The mixture was shaken at room temperature for 20 min, then filtered. The resin was washed with DCM/TFA (1:1, 3 × 2 mL), the filtrates were evaporated *in vacuo*. The residue was purified by reverse phase HPLC (ACN/H₂O (contained 0.1%TFA), ACN from 5% to 75% in 14 min.) The pure fractions were lyophilized to give (2S,4R)-4-(2-((1R,3R)-1-acetoxy-3-((2S,3S)-2-((2R,4R)-1,4-dimethylpiperidine-2-carboxamido)-N-ethyl-3-methylpentanamido)-4-methylpentyl)thiazole-4-carboxamido)-5-(4-((S)-6-amino-2-(6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanamido)hexanamido)phenyl)-2-methylpentanoic acid (**T32**) (0.095 g, 22.48 %) as a white solid. LC-MS : 1092 [M+1]; ¹H NMR (400 MHz, METHANOL-d₄) δ ppm 7.99 (s, 1 H), 7.34 (d, *J* = 8.53 Hz, 2 H), 7.10 (d, *J* = 8.53 Hz, 2 H), 6.66 (s, 2 H),

5.64 (d, $J=10.79$ Hz, 1 H), 4.50 - 4.61 (m, 1 H), 4.21 - 4.35 (m, 2 H), 3.92 (d, $J=9.29$ Hz, 1 H), 3.69 (br. s., 1 H), 3.37 (t, $J=7.15$ Hz, 2 H), 3.15-3.35 (m, 4H), 3.04 (dt, $J=3.58$, 1.85 Hz, 1 H), 2.84 (t, $J=7.65$ Hz, 2 H), 2.76 (d, $J=7.03$ Hz, 2 H), 2.62 (br. s., 2 H), 2.38 - 2.52 (m, 2 H), 2.25 (t, $J=11.54$ Hz, 1 H), 2.16 (t, $J=7.40$ Hz, 2 H), 2.04 - 2.11 (m, 4 H), 1.70 - 2.00 (m, 7 H) 1.42 - 1.69 (m, 11 H), 1.34 - 1.40 (m, 1 H), 1.27 (t, $J=6.78$ Hz, 3 H), 1.16 - 1.24 (m, 2 H), 1.01 - 1.14 (m, 7 H), 0.90 (d, $J=6.78$ Hz, 3 H), 0.94 (d, $J=6.53$ Hz, 3 H), 0.84 (t, $J=7.40$ Hz, 3 H), 0.79 (d, $J=6.53$ Hz, 3 H).

5.2. Conjugation of Tubulysin 1508

[00658] *Compound* T32 (tubulysin 1508) comprises a linker and a maleimide group that is readily conjugated to a thiol residue of an antibody forming a thiol-maleimide linkage. Cytotoxins comprising a maleimide group (e.g., tubulysin 1508) may be conjugated to specific cysteine residues engineered into the anti-HER2 antibodies provided herein (e.g. Bs2Ab-39SH, Bs3Ab-39SH, or Bs4Ab-39SH) of the invention. Alternatively, or optionally one may use classical conjugation methods to attach a cytotoxic agent the antibodies described. Method for conjugation to native lysine and cysteine residues are well known in the art. Representative methods for site specific (at engineered cysteine residues) and classic conjugation (at native cysteine residues) are provided below.

[00659] A representative site-specific antibody drug conjugation process is outlined in Figure 33 and includes the steps of (a) uncapping the side chains of the derivatizable amino acids (e.g., cysteines), (b) oxidizing, (c) conjugating a payload (e.g., a cytotoxic agent such as tubulysin 1508), and (d) polishing by removing conjugation reagents and non-reacted payload. For example conjugation to an engineered cysteine may be carried out by formulating the antibody in 1X PBS with 1 mM thylenediaminetetraacetic acid. Mild reduction is used generate free thiols by adding forty equivalences of Tris(2-carboxyethyl)phosphine hydrochloride per antibody is incubated at 37°C for three hours. Three successive dialysis in 1X PBS with 1mM Ethylenediaminetetraacetic acid are used to remove the Tris(2-carboxyethyl)phosphine hydrochloride (alternatively desalting columns may be used). The antibody interchain disulphide bonds are allowed to re-form by addition ~20 equivalences of dehydroabiatic acid (dhAA) and incubation ~four hours at room temperature. In preparation for conjugation, dimethyl sulfoxide was add to the

antibody to ten percent v/v and 8 or 12 equivalences of the tubulysin 1508 payload in Dimethyl sulfoxide is added and incubated at room temperature for ~1 hour (alternatively incubate at 4°C for ~16 hours) for 2T and 4T drug loading, respectively. The reaction is quenched by adding ~4 molar equivalence of N-acetyl cysteine (NAC) per payload. The free payload was removed from the conjugated antibody by Ceramic Hydroxyapatite following the manufacture recommendations. The final product may be subjected to buffer-exchange if desired. The conjugated antibodies may be analysed by non-reducing and reducing SDS-PAGE to confirm purity and conjugation to the heavy chain.

[00660] Antibody–drug conjugates with drugs randomly conjugated to native cysteine residues are prepared by partial reduction of the antibody followed by reaction with desired linker–drug. The antibody at a concentration of 5 mg/mL is partially reduced by addition of ~3 molar equivalents of DTT at pH 8.0, followed by incubation at ~37 °C for ~2 h. The reduction reaction is then chilled in ice and the excess DTT removed, for example via diafiltration. The linker–drug is then added to linker–drug/thiol molar ratio of ~1:10. The conjugation reaction is carried out in the presence of ~10% v/v of DMSO. After conjugation, excess free cysteine (~2 fold molar ration over linker–drug) is added to quench unreacted linker–drug to produce the cysteine–linker–drug adduct. The reaction mixture is purified (e.g, by hydrophobic interaction chromatography) and may be subjected to buffer-exchange into PBS. Drug load distribution is determined using standard methods such as hydrophobic interaction chromatography and reduced reverse phase chromatography.

5.3. Chemical Abbreviations

Ac	acetyl
ACN	acetonitrile
Boc	di- <i>tert</i> -butyl dicarbonate
t-Bu	tert-butyl
Bzl	benzyl, where Bzl-OMe is methoxybenzyl and Bzl-Me is methylbenzene
Cbz or Z	benzyloxy-carbonyl, where Z-Cl and Z-Br are chloro- and bromobenzyloxy carbonyl, respectively
DAST	diethylaminosulfur trifluoride
DCM	dichloromethane
DIAD	diisopropyl azodicarboxylate

DIC	N,N'-diisopropylcarbodiimide
DIEA	diethylisopropylamine
DMF	<i>N,N</i> -dimethylformamide
DTT	dithiothreitol
EtOAc	ethyl acetate
Et2O	diethylether
Fmoc	9 <i>H</i> -fluoren-9-ylmethoxycarbonyl
HATU	1-[Bis(dimethylamino)methylene]-1 <i>H</i> -1,2,3-triazolo[4,5- <i>b</i>]pyridinium 3-oxid hexafluorophosphate
HCl	hydrochloric acid
LC-MS	liquid chromatography-mass spectrometry
MeOH	methanol
Na2CO3	sodium bicarbonate
NaHCO3	sodium hydrogen carbonate
PAB	para-aminobenzyloxycarbonyl
RT	room temperature
TEA	triethylamine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography

INCORPORATION BY REFERENCE

[00661] All references cited herein, including patents, patent applications, papers, text books, and the like, and the references cited therein, to the extent that they are not already, are hereby incorporated herein by reference in their entireties for all purposes.

EQUIVALENTS

[00662] The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the embodiments. The foregoing description and Examples detail certain embodiments and describes the best mode contemplated by the inventors. It will be appreciated, however, that no matter how detailed the foregoing may appear in text, the embodiments may be practiced in many ways and the claims include any equivalents thereof.

WHAT IS CLAIMED IS:

1. A bispecific anti-HER2 antibody comprising a first immunoglobulin antigen-binding domain and a second immunoglobulin antigen-binding domain, wherein (i) the first and second immunoglobulin antigen-binding domains specifically bind to distinct HER2 antibody binding sites, (ii) the first immunoglobulin antigen-binding domain binds to a first HER2 antibody binding site which comprises an epitope within domain II of HER2, and (iii) the first HER2 antibody binding site is distinct from the antibody binding site of pertuzumab.

2. The bispecific antibody according to claim 1, wherein the first immunoglobulin antigen-binding domain comprises a heavy chain (HC) variable region (VH) and a light chain (LC) variable region (VL) comprising:

- (i) a variable heavy chain CDR-1 (VH-CDR1) identical to SEQ ID NO: 1 or identical to SEQ ID NO: 1 except for up to 1, 2, 3, or 4 amino acid substitutions;
- (ii) a variable heavy chain CDR-2 (VH-CDR2) identical to SEQ ID NO: 2 or identical to SEQ ID NO: 2 except for up to 1, 2, 3, or 4 amino acid substitutions;
- (iii) a variable heavy chain CDR-3 (VH-CDR3) identical to SEQ ID NO: 3 or identical to SEQ ID NO: 3 except for up to 1, 2, 3, or 4 amino acid substitutions;
- (iv) a variable light chain CDR-1 (VL-CDR1) identical to SEQ ID NO: 4 or identical to SEQ ID NO: 4 except for up to 1, 2, 3, or 4 amino acid substitutions;
- (v) a variable light chain CDR-2 (VL-CDR2) identical to SEQ ID NO: 5 or identical to SEQ ID NO: 5 except for up to 1, 2, 3, or 4 amino acid substitutions; and,
- (vi) a variable light chain CDR-3 (VL-CDR3) identical to SEQ ID NO: 6 or identical to SEQ ID NO: 6 except for up to 1, 2, 3, or 4 amino acid substitutions.

3. A bispecific anti-HER2 antibody comprising a first immunoglobulin antigen binding-domain and a second immunoglobulin antigen-binding domain, wherein the first immunoglobulin antigen-binding domain comprises a VH and a VL, wherein

- (a) the VH comprises the amino acids of SEQ ID NO:15 or 43;
- (b) the VL comprises the amino acids of SEQ ID NO:16 or 44;

and wherein the first and second immunoglobulin antigen-binding domains specifically bind to distinct HER2 epitopes.

4. The bispecific anti-HER2 antibody according to thereof of any one of claims 1 to 3, wherein the first immunoglobulin antigen binding domain and/or the second immunoglobulin antigen-binding domain comprises or consists of (a) a VH further comprising a heavy chain constant region or a fragment thereof and a VL comprising a light chain constant region (LC) or a fragment thereof; (b) a single chain Fv ("scFv"); (c) a diabody; (d) a minibody; (e) an F(ab')₂; or (f) an F(ab).

5. The bispecific anti-HER2 antibody according to claim 4, wherein (a) the heavy chain constant region or fragment thereof is an IgG constant region; and/or (b) the LC constant region is a kappa constant region or a lambda constant region.

6. The bispecific anti-HER2 antibody according to any one of claims 1 to 5, wherein the first immunoglobulin antigen-binding domain is a monoclonal antibody, a humanized antibody, a human antibody, a chimeric antibody, or an affinity optimized antibody.

7. The bispecific anti-HER2 antibody according to any one of claims 1 to 6, wherein:

- (a) the second immunoglobulin antigen-binding domain specifically binds to the same HER2 epitope as the trastuzumab antibody;
- (b) the second immunoglobulin antigen-binding domain competitively inhibits HER2 binding by the trastuzumab antibody; or
- (c) the second immunoglobulin antigen-binding domain comprises at least one, at least two, at least three, at least four, at least five, or at least six complementarity determining regions (CDRs) comprising the amino acids of any one of SEQ ID NOs: 54 to 59.

8. The bispecific anti-HER2 antibody according to claim 7, wherein the second immunoglobulin antigen-binding domain is an scFv comprising:

- (i) a VH-CDR1 comprising the amino acids of SEQ ID NO:54;
- (ii) a VH-CDR2 comprising the amino acids of SEQ ID NO:55;
- (iii) a VH-CDR3 comprising the amino acids of SEQ ID NO:56;
- (iv) a VL-CDR1 comprising the amino acids of SEQ ID NO:57;
- (v) a VL-CDR2 comprising the amino acids of SEQ ID NO:58; and
- (vi) a VL-CDR3 comprising the amino acids of SEQ ID NO:59.

9. The bispecific anti-HER2 antibody according to claim 7 or claim 8, wherein

(a) the second immunoglobulin antigen-binding domain is covalently linked to the carboxy-terminus of the HC of the first immunoglobulin antigen-binding domain;

(b) the second immunoglobulin antigen-binding domain is covalently linked to the amino-terminus of the HC of the first immunoglobulin antigen-binding domain; or,

(c) the second immunoglobulin antigen binding domain is covalently intercalated in the polypeptide chain of the HC of the first immunoglobulin antigen-binding domain.

10. The bispecific anti-HER2 antibody according to any one of claims 1 to 9, wherein the heavy chain comprises a constant region comprising an Fc domain.

11. The bispecific anti-HER2 antibody according to claim 10, wherein the Fc domain comprises at least one mutation capable of reducing or enhancing the ADCC activity of the bispecific antibody.

12. A bispecific anti-HER2 antibody comprising a first and a second polypeptide chain associated with each other, wherein the first polypeptide chain is selected from:

(1) [TZ_S]-[L₁]-[_BVH]-[_BCH]-[Fc_x]

(2) [_BVH]-[_BCH]-[Fc_x]-[L₂]-[TZ_S]

(3) [_BVH]-[_BCH]-[L₃]-[TZ_S]-[L₄]-[Fc_x]

wherein

TZ_S is an scFv that binds the same epitope as trastuzumab;

L₁, L₂, L₃, and L₄ are peptide linkers;

Fc_x is an Fc domain;

_BVH and _BCH are the VH and CH1 regions, respectively, of an antibody capable of binding to an HER2 epitope distinct from the epitope recognized by the trastuzumab antibody;

and wherein the second chain comprises [_BVL]-[CL] wherein _BVL is the VL region of an antibody capable of binding to an HER2 epitope distinct from the epitope recognized by the trastuzumab antibody, and CL is a IgG light chain constant region selected from the group consisting of a human kappa constant region and a human lambda constant region.

13. The bispecific anti-HER2 antibody according to claim 12, wherein _BVL comprises

- (i) a variable light chain CDR-1 (VL-CDR1) identical to SEQ ID NO: 4 or identical to SEQ ID NO: 4 except for up to 1, 2, 3, or 4 amino acid substitutions;

- (ii) a variable light chain CDR-2 (VL-CDR2) identical to SEQ ID NO: 5 or identical to SEQ ID NO: 5 except for up to 1, 2, 3, or 4 amino acid substitutions; and,
- (iii) a variable light chain CDR-3 (VL-CDR3) identical to SEQ ID NO: 6 or identical to SEQ ID NO: 6 except for up to 1, 2, 3, or 4 amino acid substitutions;

wherein [TZ_s] comprises

- (i) a VH-CDR1 comprising the amino acids of SEQ ID NO:54;
- (ii) a VH-CDR2 comprising the amino acids of SEQ ID NO:55;
- (iii) a VH-CDR3 comprising the amino acids of SEQ ID NO:56;
- (iv) a VL-CDR1 comprising the amino acids of SEQ ID NO:57;
- (v) a VL-CDR2 comprising the amino acids of SEQ ID NO:58; and
- (vi) a VL-CDR3 comprising the amino acids of SEQ ID NO:59;

wherein the [Fcx] comprises

- (i) at least one amino acid substitution introducing a derivatizable group; and/or,
- (ii) at least one mutation capable of enhancing the ADCC activity of the bispecific antibody;

and wherein [B^{VH}] comprises

- (i) a variable heavy chain CDR-1 (VH-CDR1) identical to SEQ ID NO: 1 or identical to SEQ ID NO: 1 except for up to 1, 2, 3, or 4 amino acid substitutions;
- (ii) a variable heavy chain CDR-2 (VH-CDR2) identical to SEQ ID NO: 2 or identical to SEQ ID NO: 2 except for up to 1, 2, 3, or 4 amino acid substitutions; and
- (iii) a variable heavy chain CDR-3 (VH-CDR3) identical to SEQ ID NO: 3 or identical to SEQ ID NO: 3 except for up to 1, 2, 3, or 4 amino acid substitutions.

14. The bispecific HER2 antibody according to any one of claims 1 to 13, wherein the bispecific HER2 antibody:

- (a) induces internalization upon binding to the HER2 target;
- (b) promotes effective lysosomal trafficking following internalization;
- (c) induces HER2 target degradation;
- (d) blocks ligand-induced AKT phosphorylation in low HER2-expressing cancer cells;

- (e) disrupts ligand-induced HER2:HER3 dimerization; or
- (f) combinations thereof.

15. An antibody-drug conjugate (ADC) comprising the bispecific HER2 antibody according to any one of claims 1 to 14 and least one, two, three, or four therapeutic moieties, and optionally comprising at least one spacer.

16. The ADC according to claim 15, wherein each therapeutic moiety is chemically conjugated to the side chain of an amino acid at a specific position in the Fc region of the bispecific antibody.

17. The ADC according to claim 16, where the specific positions are selected from the group consisting of 239, 248, 254, 258, 273, 279, 282, 284, 286, 287, 289, 297, 298, 312, 324, 326, 330, 335, 337, 339, 350, 355, 356, 359, 360, 361, 375, 383, 384, 389, 398, 400, 413, 415, 418, 422, 435, 440, 441, 442, 443, 446, an insertion between positions 239 and 240, and combinations thereof, wherein the amino acid position numbering is according to the EU index as set forth in Kabat.

18. The ADC according to claim 17, wherein the specific positions are 239, 442, or both, wherein the amino acid position numbering is according to the EU index as set forth in Kabat.

19. An ADC comprising the bispecific HER2 antibody according to any one of claims 1 to 14, wherein said antibody comprises:

- (i) a first polypeptide chain comprising the amino acids of SEQ ID NO: 32A and a second polypeptide chain comprising the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises a therapeutic moiety covalently linked to a cysteine amino acid at position 239, wherein the amino acid position numbering is according to the EU index as set forth in Kabat;
- (ii) a first polypeptide chain comprising the amino acids of SEQ ID NO:33A and a second polypeptide chain comprising the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises two therapeutic moieties covalently linked to cysteine amino acids respectively located at positions 239 and 442, wherein the amino acid position numbering is according to the EU index as set forth in Kabat;

- (iii) a first polypeptide chain comprising the amino acids of SEQ ID NO:36A and a second polypeptide chain comprising the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises a therapeutic moiety covalently linked to a cysteine amino acid at position 239, wherein the amino acid position numbering is according to the EU index as set forth in Kabat;
- (iv) a first polypeptide chain comprising the amino acids of SEQ ID NO:37A and a second polypeptide chain comprising the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises two therapeutic moieties covalently linked to cysteine amino acids respectively located at positions 239 and 442, wherein the amino acid position numbering is according to the EU index as set forth in Kabat;
- (v) a first polypeptide chain comprising the amino acids of SEQ ID NO:40A and a second polypeptide chain comprising the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises a therapeutic moiety covalently linked to a cysteine amino acid at position 239, wherein the amino acid position numbering is according to the EU index as set forth in Kabat; or,
- (vi) a first polypeptide chain comprising the amino acids of SEQ ID NO:41A and a second polypeptide chain comprising the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises two therapeutic moieties covalently linked to cysteine amino acids respectively located at positions 239 and 442, wherein the amino acid position numbering is according to the EU index as set forth in Kabat.
- (vii) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:32A and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises a therapeutic moiety covalently linked to a cysteine amino acid inserted between positions 239 and 240, wherein the amino acid position numbering is according to the EU index as set forth in Kabat;
- (viii) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:33C and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises two

therapeutic moieties covalently linked to a cysteine amino inserted between positions 239 and 240 and a cysteine amino acid located at position 442, wherein the amino acid position numbering is according to the EU index as set forth in Kabat;

- (ix) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:36C and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises a therapeutic moiety covalently linked to a cysteine amino acid inserted between positions 239 and 240, wherein the amino acid position numbering is according to the EU index as set forth in Kabat;
- (x) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:37C and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises two therapeutic moieties covalently linked to a cysteine amino inserted between positions 239 and 240 and a cysteine amino acid located at position 442, wherein the amino acid position numbering is according to the EU index as set forth in Kabat;
- (xi) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:40C and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises a therapeutic moiety covalently linked to a cysteine amino acid inserted between positions 239 and 240, wherein the amino acid position numbering is according to the EU index as set forth in Kabat; or,
- (xii) a first polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:41C and a second polypeptide chain comprising or consisting of the amino acids of SEQ ID NO:42A, wherein the first polypeptide chain comprises two therapeutic moieties covalently linked to a cysteine amino inserted between positions 239 and 240 and a cysteine amino acid located at position 442, wherein the amino acid position numbering is according to the EU index as set forth in Kabat.

20. The ADC according to any one of claims 15 to 19, wherein the therapeutic moiety comprises a cytotoxin, a radioisotope, an immunomodulator, a cytokine, a lymphokine, a chemokine, a growth factor, a tumor necrosis factor, a hormone, a hormone antagonist, an enzyme, an oligonucleotide, a DNA, an RNA, an siRNA, an RNAi, a microRNA, a photoactive therapeutic agent, an anti-angiogenic agent, a pro-apoptotic agent, a peptide, a lipid, a carbohydrate, a chelating agent, or combinations thereof.

21. The ADC according to claim 20, wherein the cytotoxin is a tubulysin, an auristatin, a maytansinoid or a pyrrolobenzodiazepine (PBD).

22. A pharmaceutical composition comprising a bispecific HER2 antibody according to any one of claims 1 to 14, or an ADC according to any one of claims 15 to 21, and a pharmaceutically acceptable carrier.

23. A method of treating a HER2-expressing cancer comprising administering a bispecific HER2 antibody according to any one of claims 1 to 14, an ADC according to any one of claims 15 to 21, or the pharmaceutical composition according to claim 22 to a subject in need thereof.

24. The method according to claim 23, wherein the cancer is a low HER2-expressing cancer.

25. A method to treat resistance to a HER2-targeting therapeutic agent comprising administering a bispecific HER2 antibody according to any one of claims 1 to 14, an ADC according to any one of claims 15 to 21, or the pharmaceutical composition according to claim 22 to a subject in need thereof.

AZ1.39.1 VL Germline IGKV4-1+JK3.01 → IGKV2D

WT: DIVMTQSPDSLAIVSLGERATITCKSSQSVFFRSNNKNCLAWYQQRPGQPPNLLIYWASSRES
LO: DIVMTQTPLSLSVTPGQPASISCKSSQSVFFRSNNKNILAWYLOKPGQPPOLLIYWASSRES
WT: GVPDRFSGSGGTDFALTISSSLQTEDVAVYYCQQYFGSGPETFGPGTKVDIK
LO: GVPDRFSGSGGTDFTLKISRVEAEDVGYYCQQYFGSGPETFGPGTKVDIK

AZ1.39.1 VH Germline VH3-21+JH4

WT: EVQLVESGGGLVKPGGSLRLSCAASGETFSSYSNNWVRQAPGKGLEWVSSISSSSSYIYYADSVKG
LO: EVQLVESGGGLVKPGGSLRLSCAASGETFSSYSNSWVRQAPGKGLEWVSSISSSSSYIYYADSVKG
WT: RETISRDNAKNSLYLOMNSLRAEDTAVYYCARGGDGYNYEYDWGQGTLTWVSS
LO: RETISRDNAKNSLYLOMNSLRAEDTAVYYCARGGDAINYEYDWGQGTLTWVSS

FIG. 1

Construct ^a	IgG in sups (µg/ml) ^b
After LC-FR swapping	140
Before LC-FR swapping	72

a: All constructs have the same heavy chain as G4A (AZ1.39.1 (WT) heavy chain with DG mutated to DA in CDR3H)

b: Average from two Octet-96 quants after 7 days in culture of 293F cells transfected with corresponding IgG vectors; cv% ~10%

FIG. 2

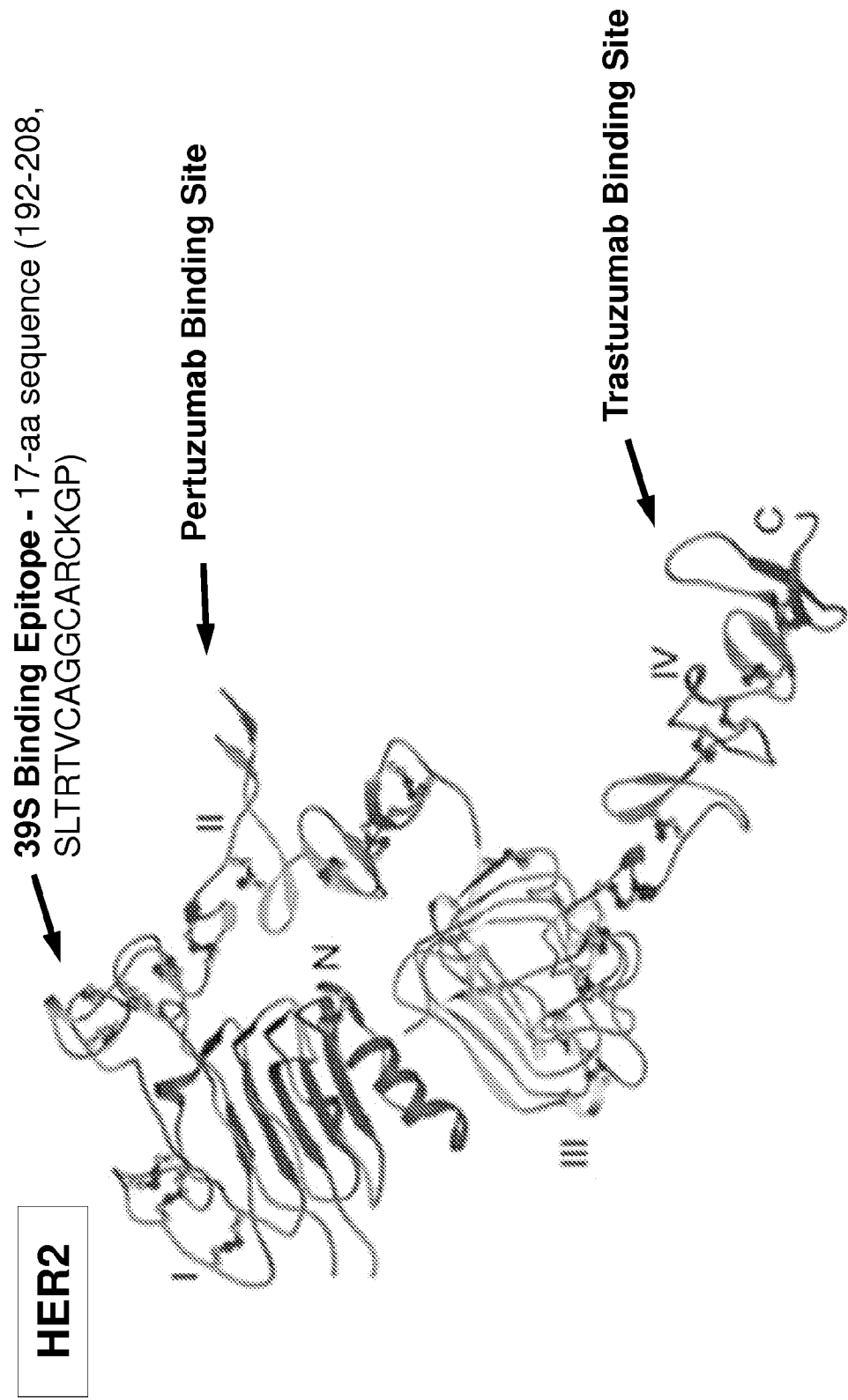


FIG. 3

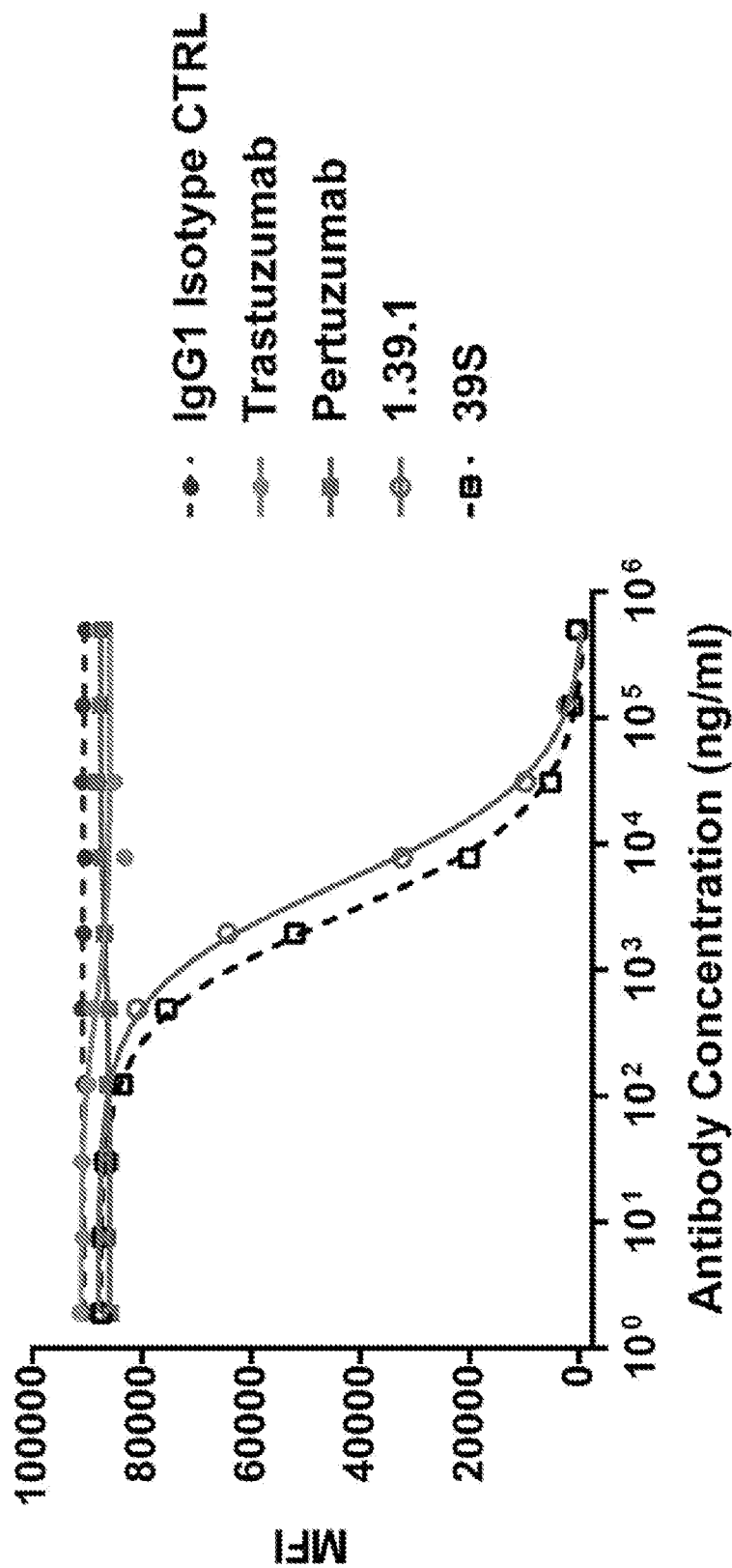


FIG. 4

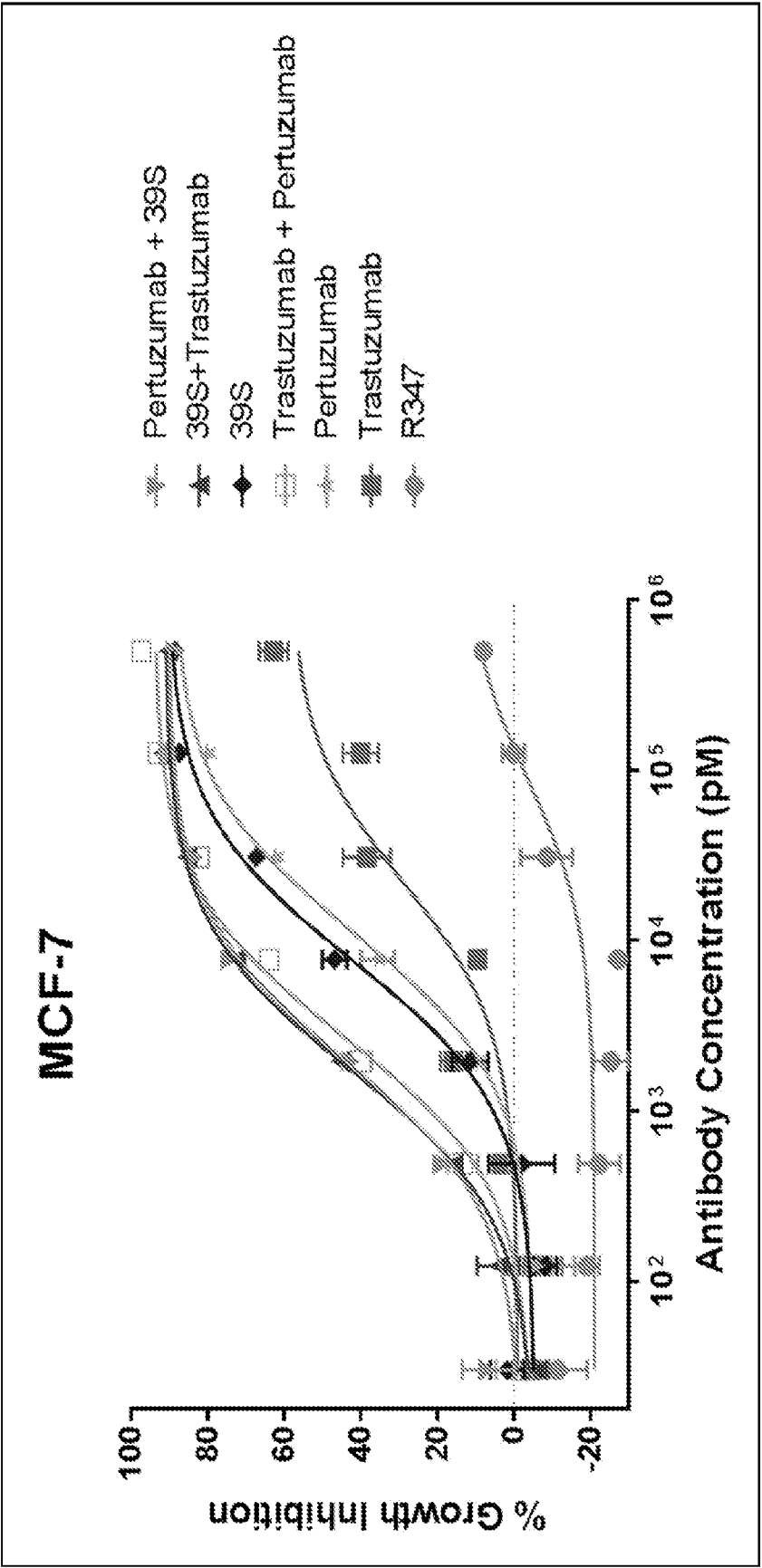
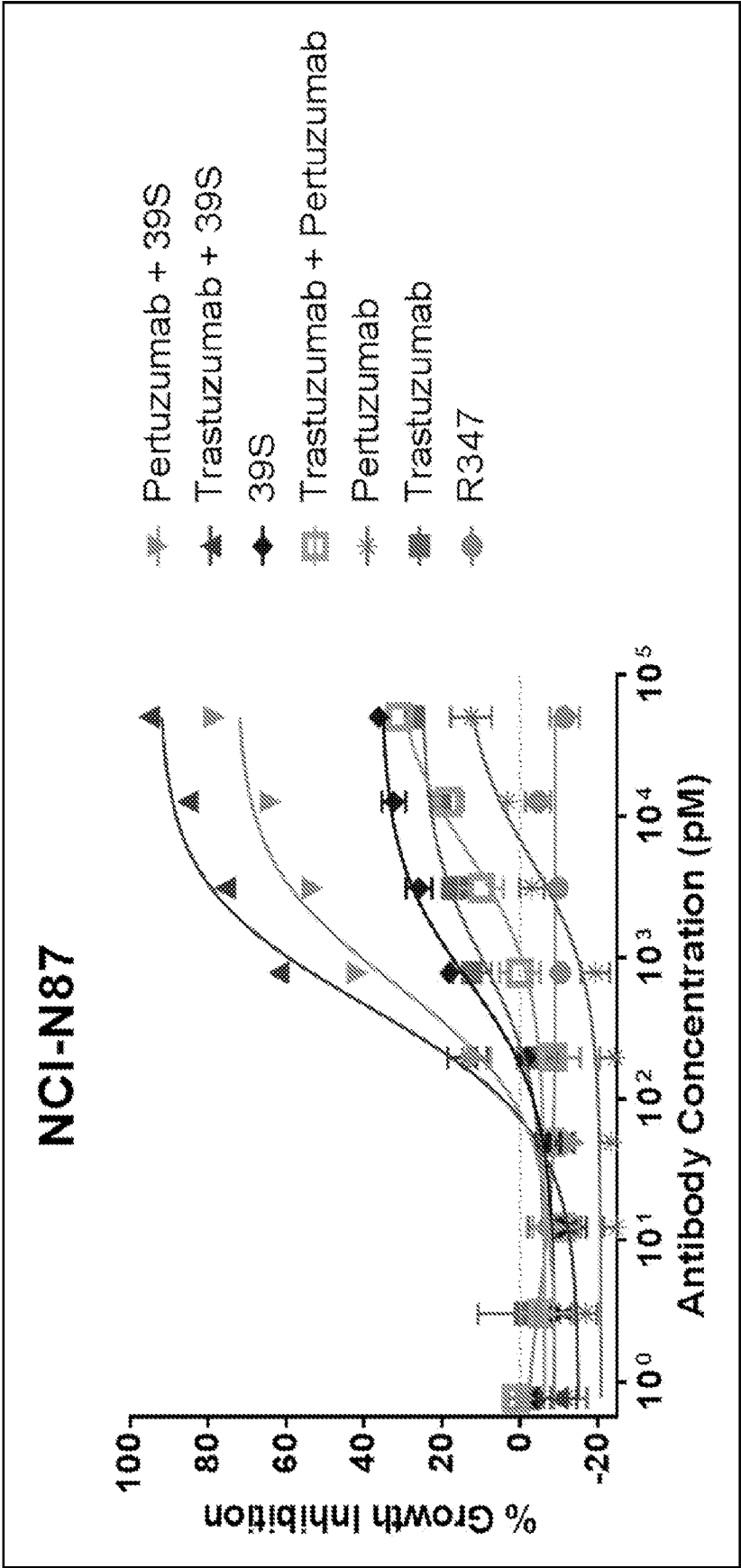


FIG. 5



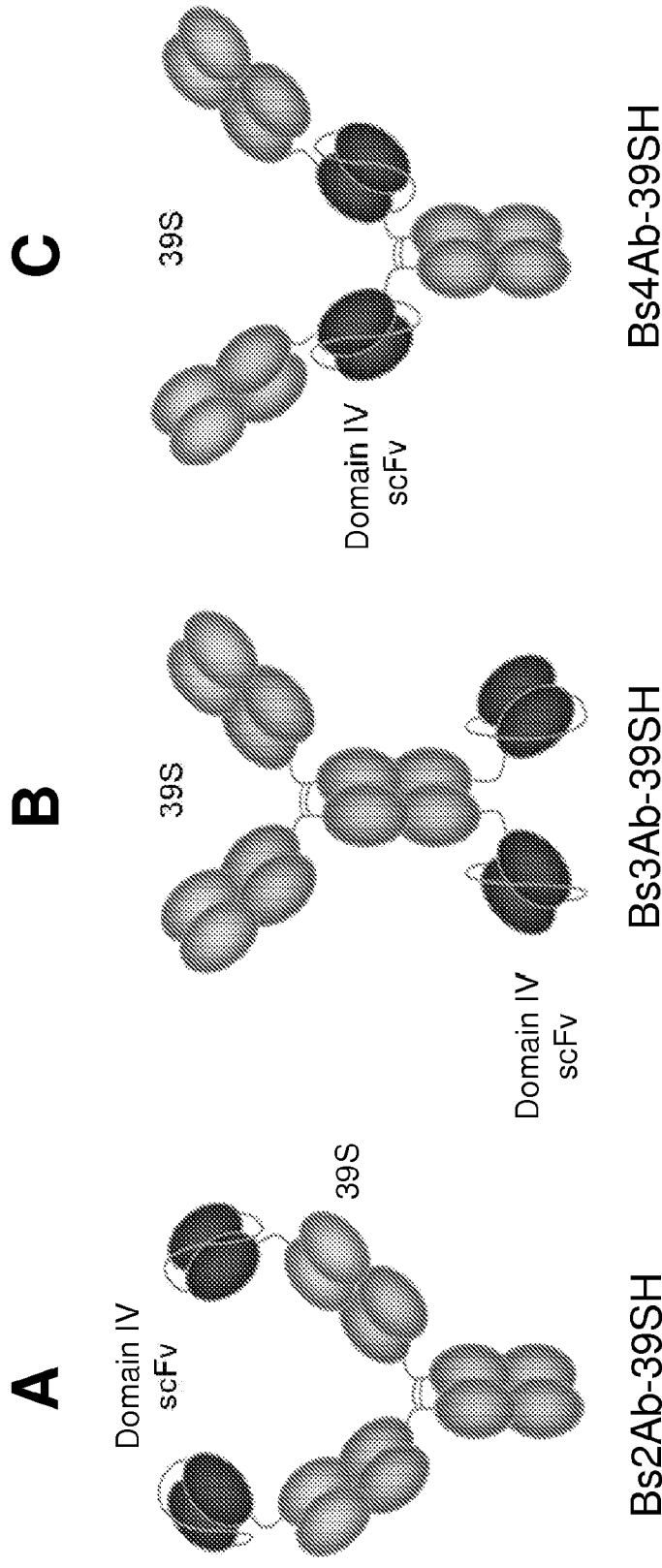


FIG. 7

DIQMTQSPSSLASVGDVRTITCRASQDVNTAVAWYQQKPKGKAPKLLIYSASFVPSRFSGRSGTDFTLTISSLQPEDFA
TYYCQQHYTTPTFGCGTKVEIKGGGGGGGGGGGGGGGGGGVQVPGGSLRLSCAASGPNIKDTYIHVVRQAP
GKCLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYCSRWGGDGFYAMDYWGQGLTVTVSSGGGGS
GGGGGGGGSEVQLVESGGGLVKPAGGSLRLSCAASGFTFSSYSMSWVRQAPGKLEWVSSISSSSYIYYADSVKGRFTISRDN
AKNSLYLQMNSLRAEDTAVYCARGGDAINYFYFDYWGQGLTVTVSSASTKGPSVFP LAPSSKSTSGGTAALGCLVKDYFPEPV
TVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKRVKPKSCDKTHTCPPCPAPEX1LG
PX2*VFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY
KCKVSNKALPX3PX4EKTISKAKGQPREPQVYTLPPSRREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSD
GSFFLYSKLTVDKSRWQQGNVFSVMSVHEALHNHYTQKSLX5LLSPGK

EU#					
234	X1 = L for WT;	L for ADCC;	F for ADC	"F", "FC", "FCC"	
239	X2 = S for WT;	S or D for ADCC;	C for ADC	"FC", "FCC"	
239i	* = absent for WT;	absent for ADCC;	C for ADC	239-ins "C"	
330	X3 = A for WT;	L or A for ADCC;	A for ADC		
332	X4 = I for WT;	E for ADCC;	I for ADC		
442	X5 = S for WT;	S for ADCC;	C for ADC	"FCC" and "CC"	

FIG. 8A

EVQLVESGGGLVKPGGSLRLSCAASGFTFSSYSMSWVRQAPGKGLEWVSSISSSSSYIYYADSVKGRFTISRDNAKNSLYLQMN
SLRAEDTAVYYCARGGDAYNYFYFDYWGGTLVTVSSASTKGPSVFPLAPSSKSTSGTAALGCLVKDYFPEPVTVSWNSGALT
SGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQYICNVNHKPSNTKVDKRVKSCDKTHTCPPCPAPEX1LGGPX2*VFLFPP
KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYCKVSNKALP
X3PX4EKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLT
VDKSRWQQGNVFCSCVMHEALHNHYTQKSLX5LSPGKGGSGGGSGGSDIQMTQSPSSLSASVGDRTITCRASQDVNTA
VAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGRSGTDFLTITISLQPEDFATYYCQQHYTTPPTFGCGTKVEIKGGGSGGGG
SGGGSGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKCLEWVARITYPTNGYTRYADSVKGRFTISAD
TSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQTLVTVSS

EU#					
234	X1 = L for WT;	L for ADCC;	F for ADC	"F", "FC", "FCC"	
239	X2 = S for WT;	S or D for ADCC;	C for ADC	"FC", "FCC"	
239i	* = absent for WT;	absent for ADCC;	C for ADC	239-ins "C"	
330	X3 = A for WT;	L or A for ADCC;	A for ADC		
332	X4 = I for WT;	E for ADCC;	I for ADC		
442	X5 = S for WT;	S for ADCC;	C for ADC	"FCC" and "CC"	

FIG. 8B

EVQLVESGGGLVKPGGSLRLSCAASGFTFSSYSMSWVRQAPGKGLEWVSSISSSSYIYYADSVKGRFTISRDNAKNSLYLQMN
SLRAEDTAVYYCARGGDAYNYFYFDYWGGTLVTVSSASTKGPSVFPLAPSSKSTSGTAALGCLVKDYFPEPVTVSWNSGALT
SGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCGKTGGGGGGGSDIQMTQSPSSLSASV
GDRVITICRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGRSGTDFTLTISLQPEDFATYCYCQHYTTPPTFG
CGTKVEIKGGGGGGGGGGGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKCLEWVARIYPTNG
YTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGLVTVSSSGGGGGGSCPPCPAPEX
1LGGPX2*VFLFPKPKDITLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLN
GKEYCKVSNKALPX3X4EKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTITPPV
LDSDGSFFFLYSKLTVDKSRWQQGNVFSQSVMHENHHTQKSLX5LSPGK

EU#		L for ADCC;	F for ADC	"F", "FC", "FCC"
234	X1 = L for WT;	L for ADCC;	F for ADC	"F", "FC", "FCC"
239	X2 = S for WT;	S or D for ADCC;	C for ADC	"FC", "FCC"
239i	* = absent for WT;	absent for ADCC;	C for ADC	239-ins "C"
330	X3 = A for WT;	L or A for ADCC;	A for ADC	
332	X4 = I for WT;	E for ADCC;	I for ADC	
442	X5 = S for WT;	S for ADCC;	C for ADC	"FCC" and "CC"

FIG. 8C

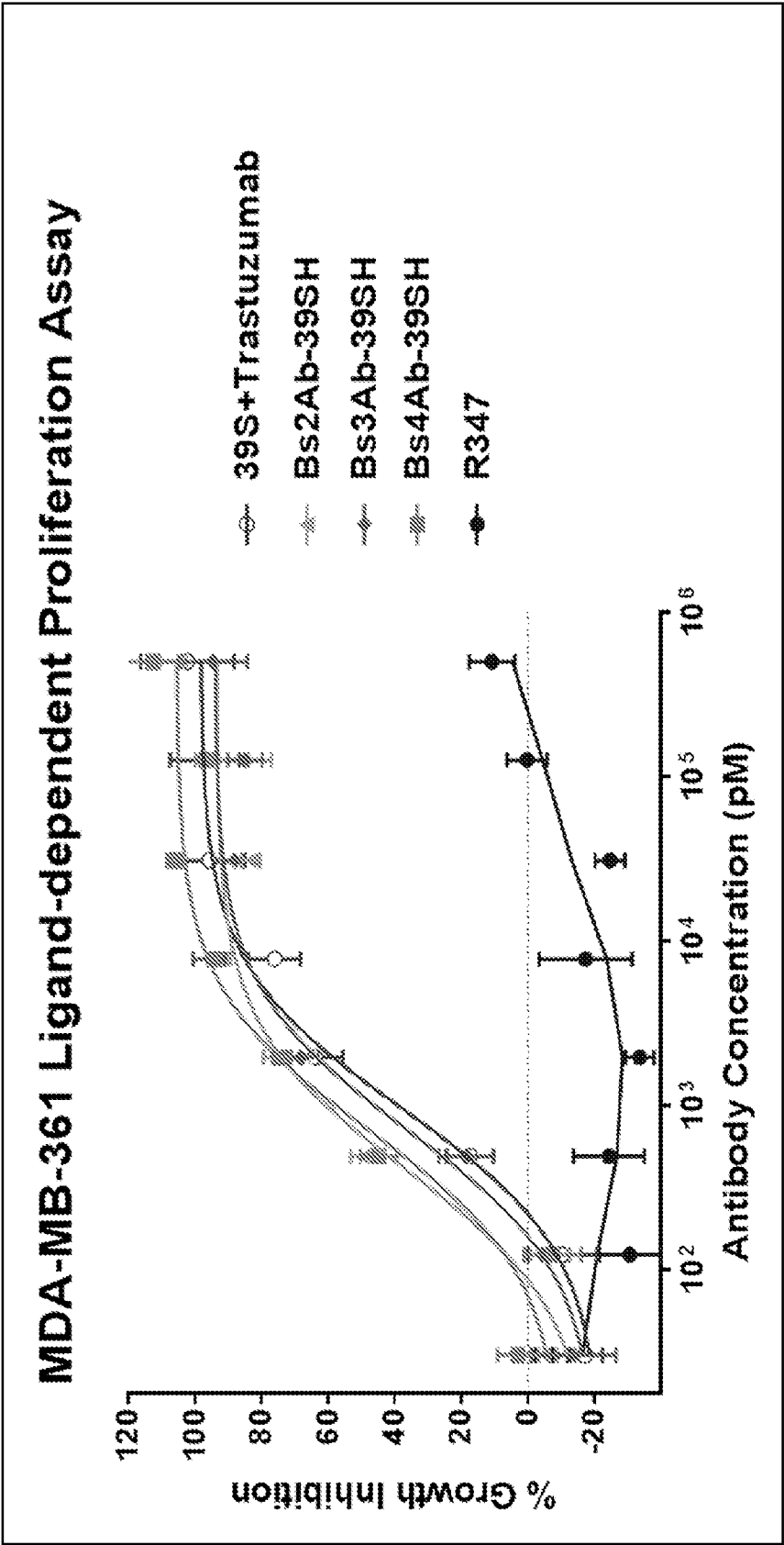


FIG. 9A

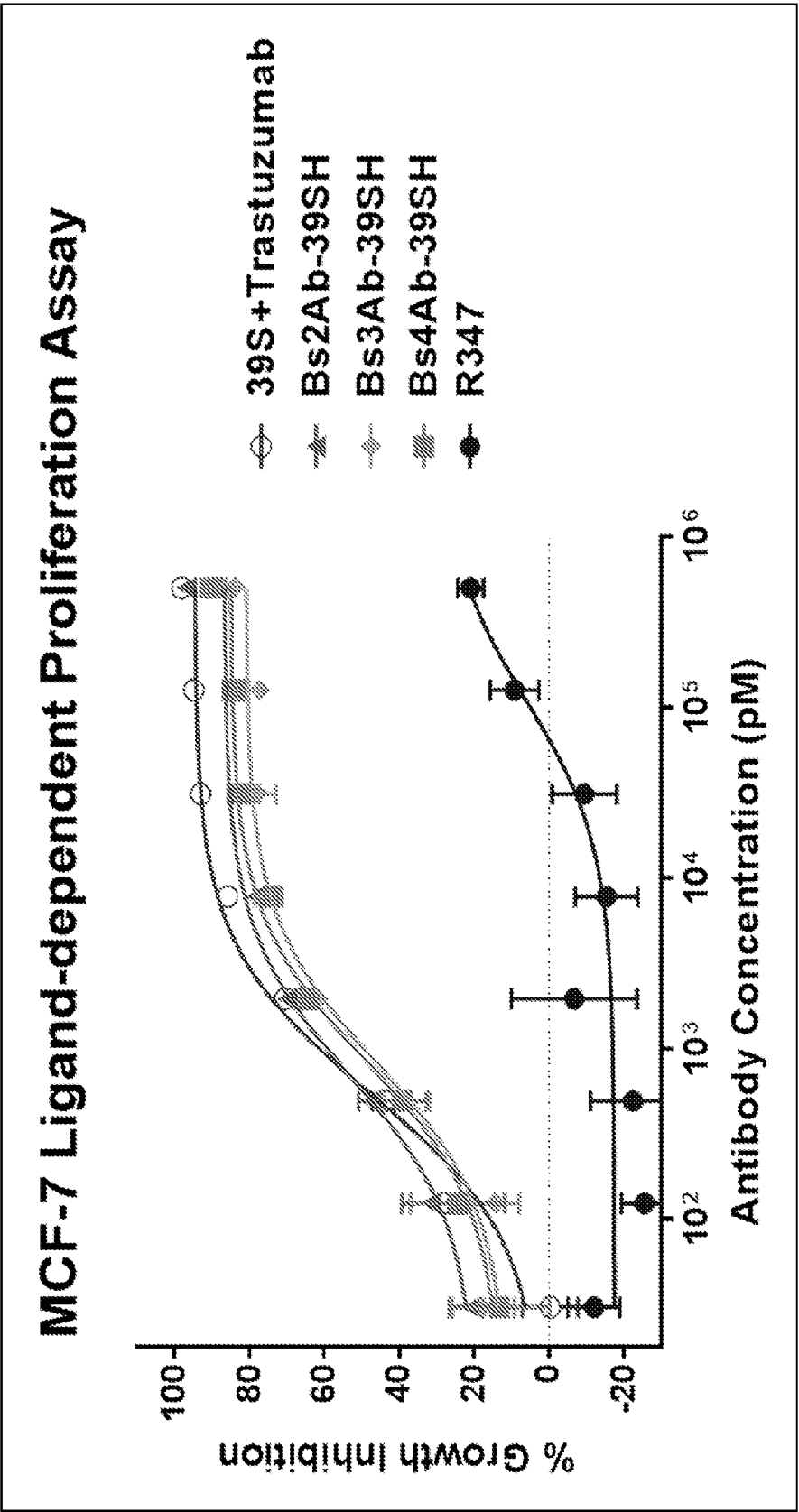


FIG. 9B

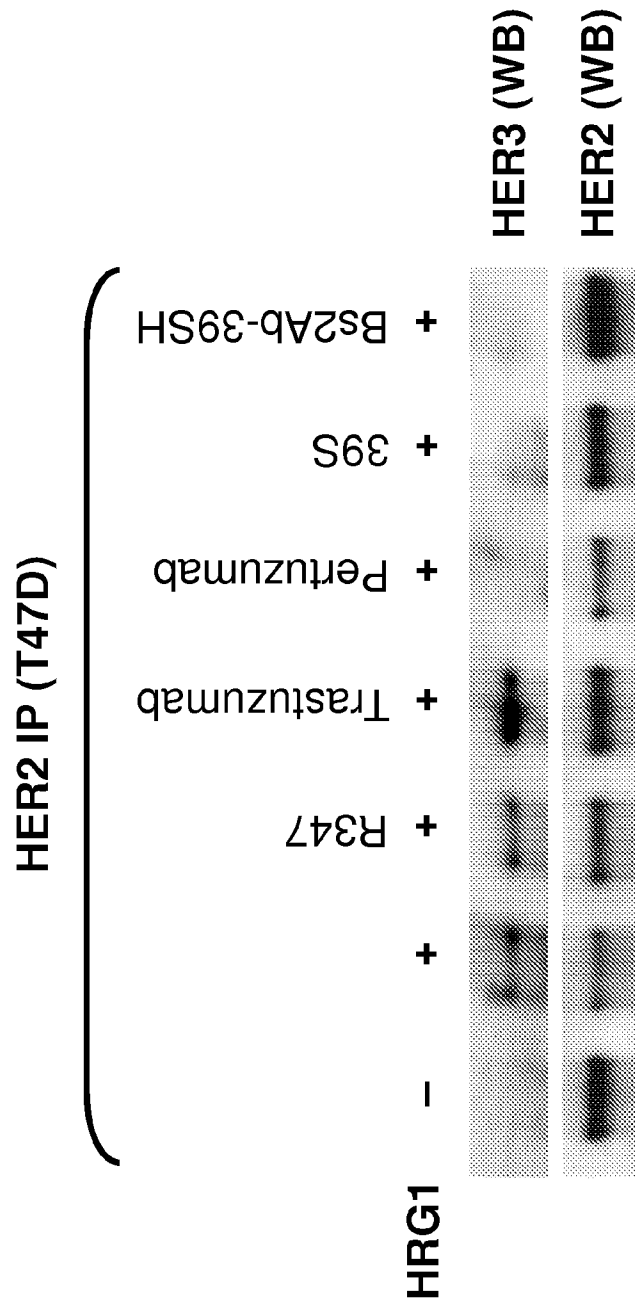


FIG. 10

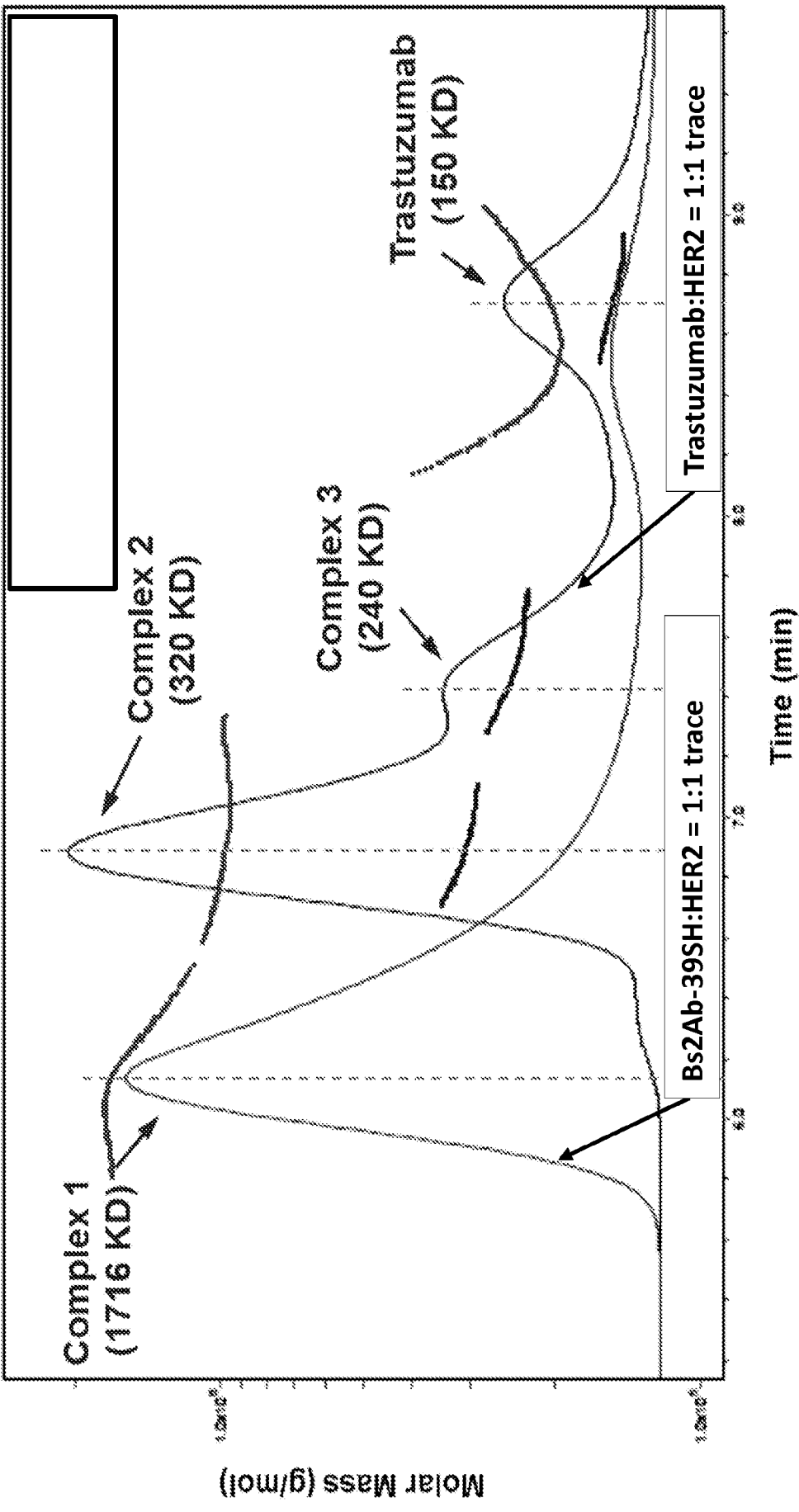


FIG. 11

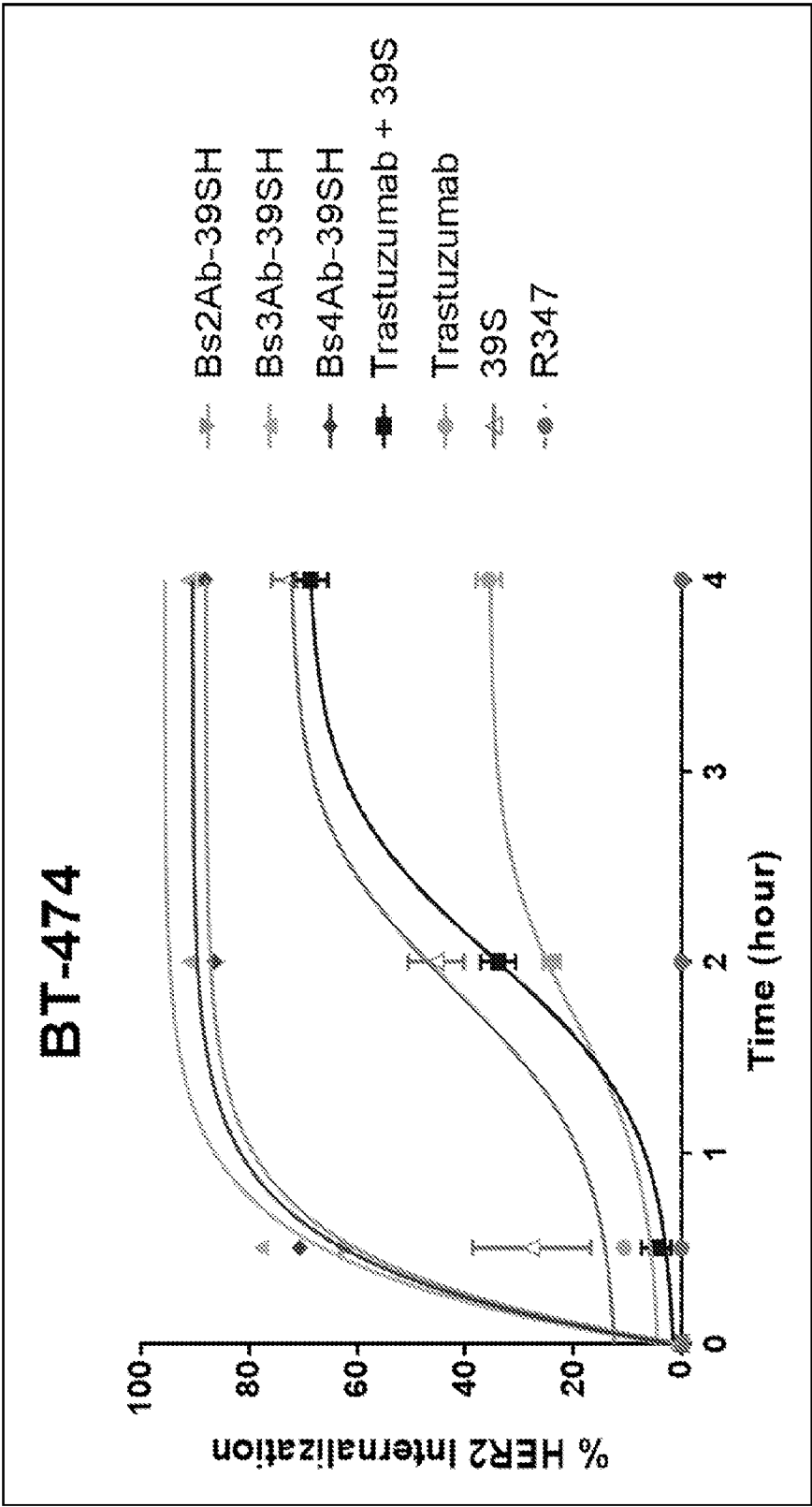


FIG. 12

T = 6 hrs

T = 4 hrs

T = 2 hrs

T = 30 min

T = 0

R347

Trastuzumab

Bs2Ab-39SH
(Unarmed)

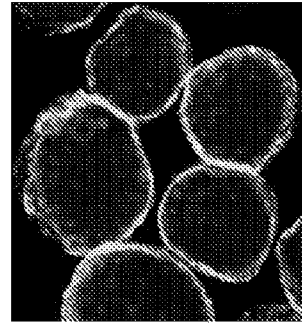
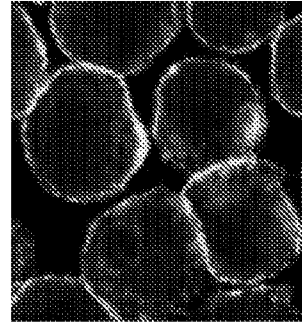
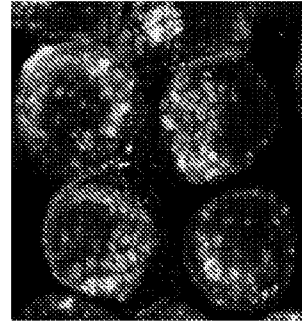
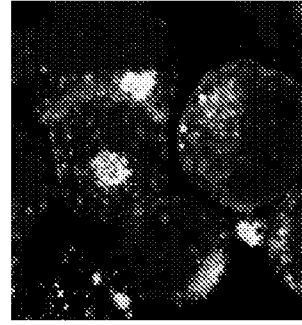
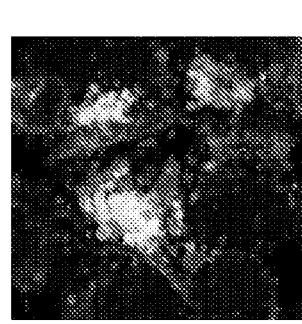
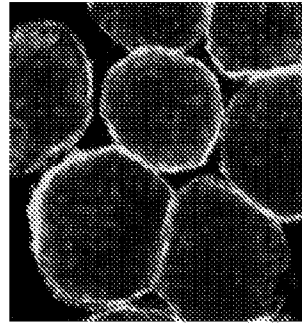
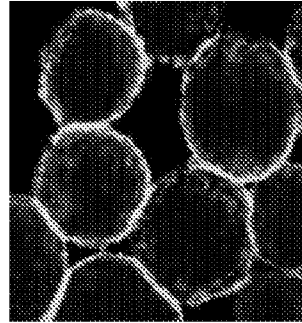
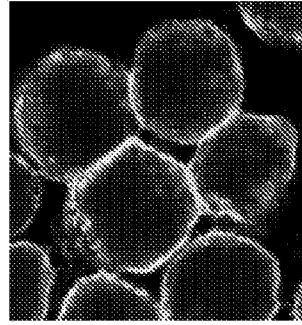
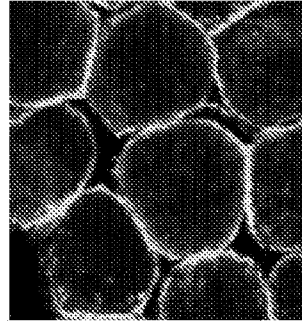
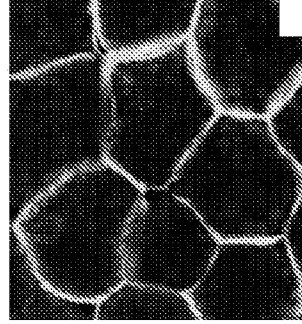
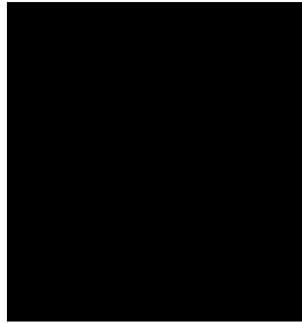
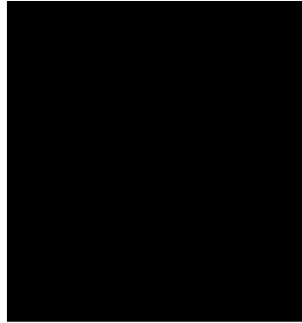
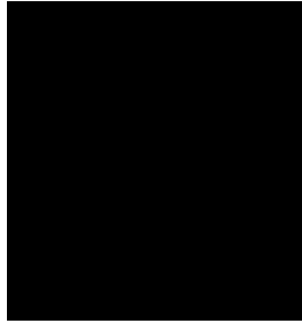
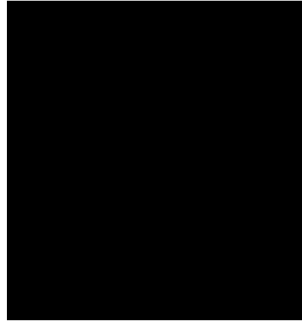
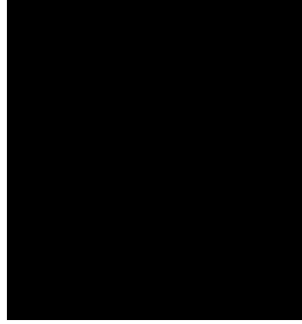


FIG. 13

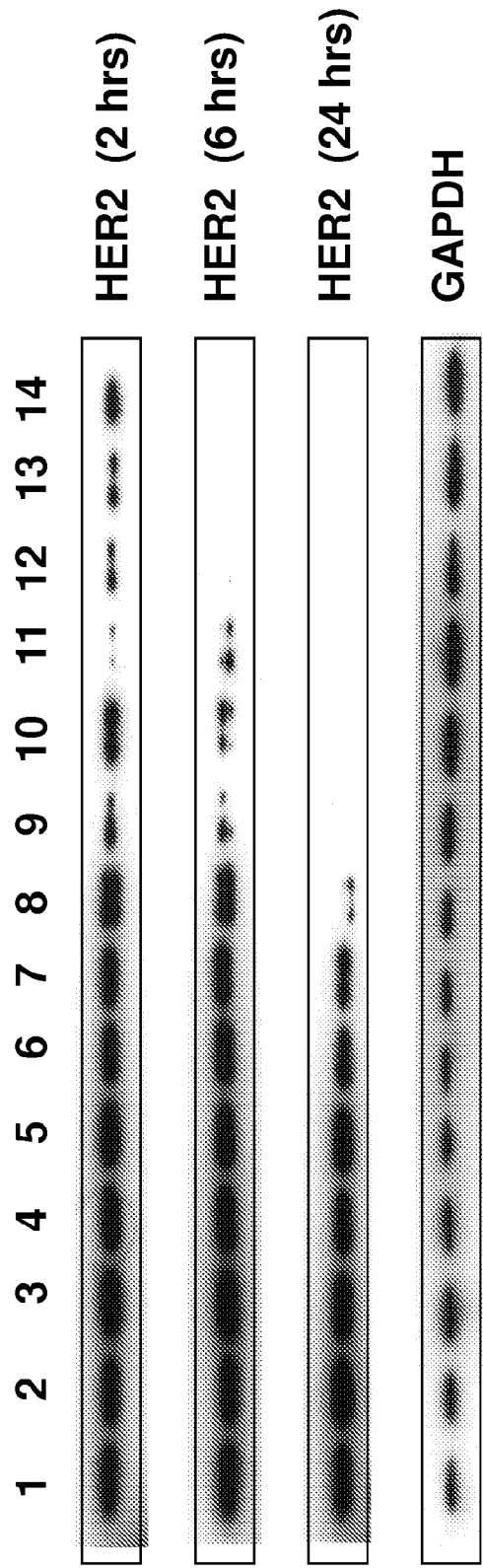


FIG. 14

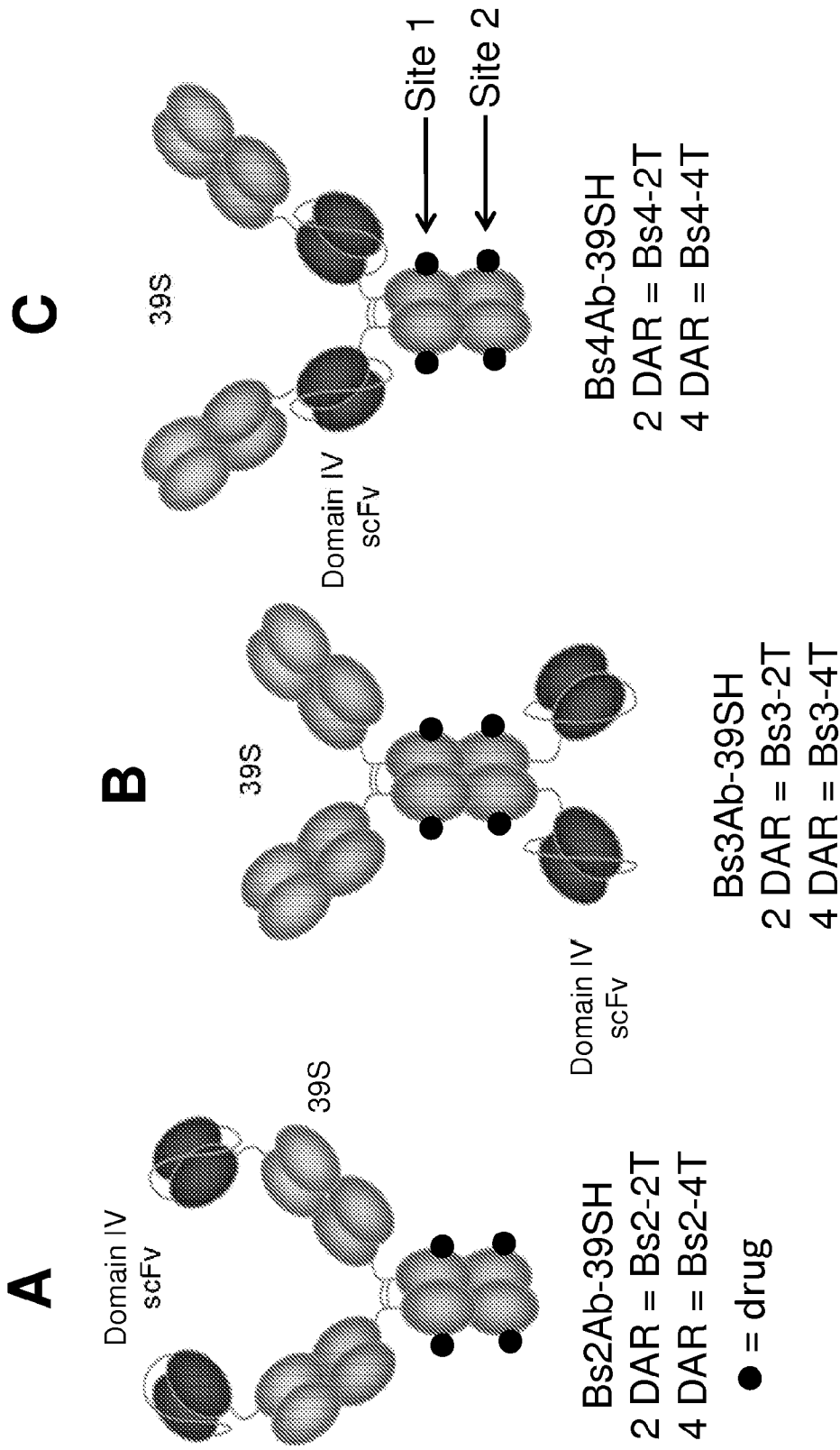


FIG. 15

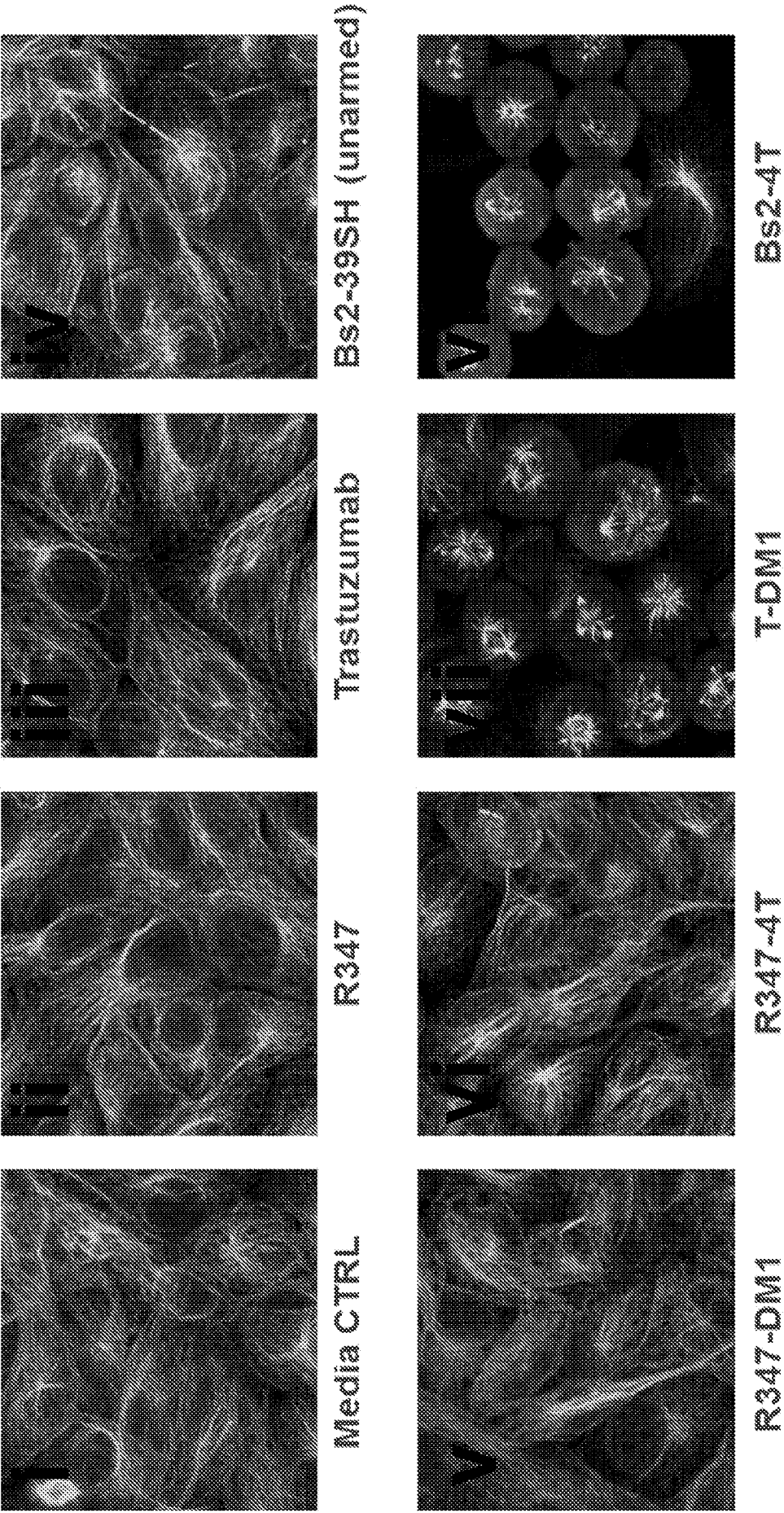


FIG. 16A

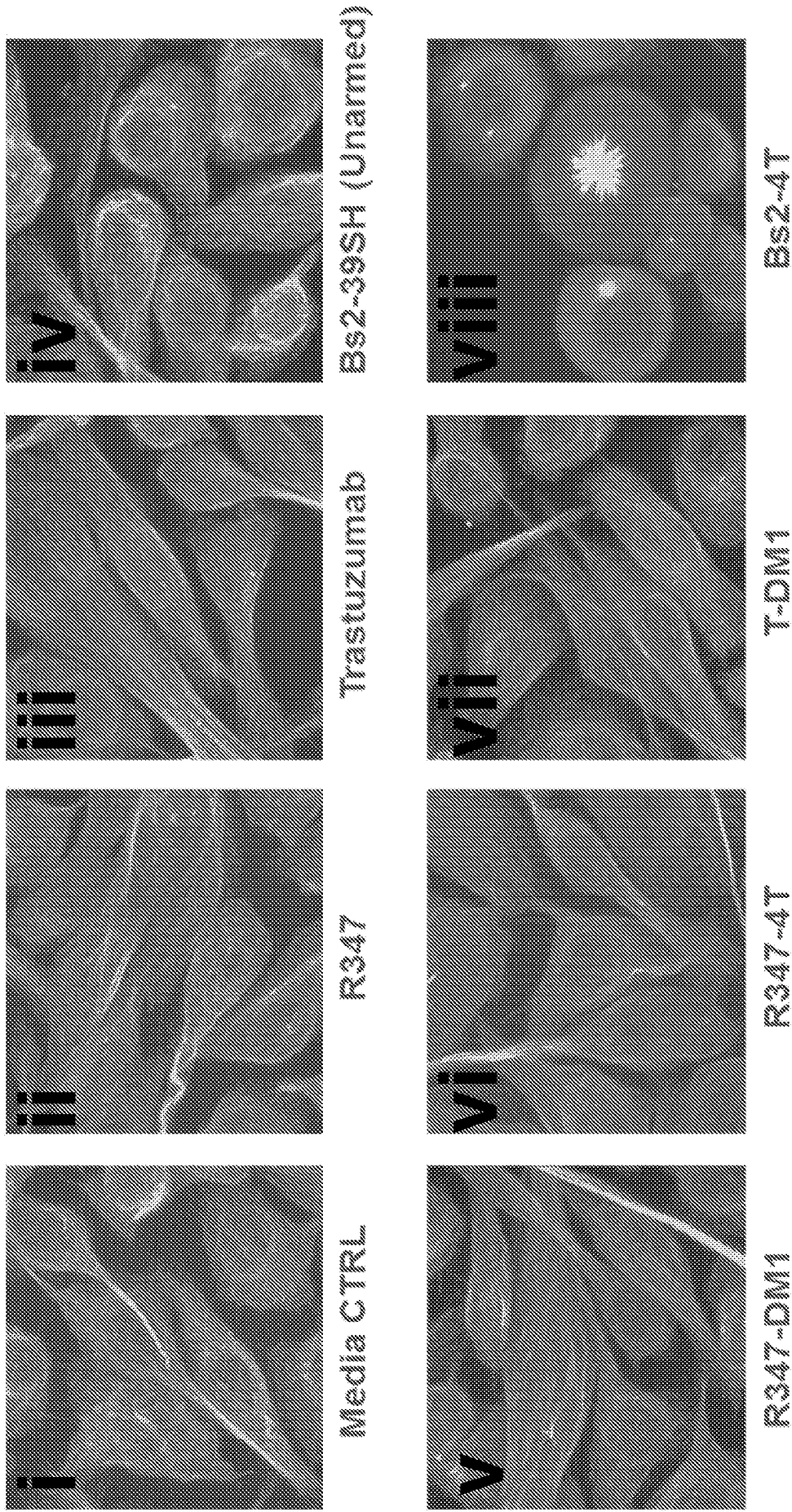


FIG. 16B

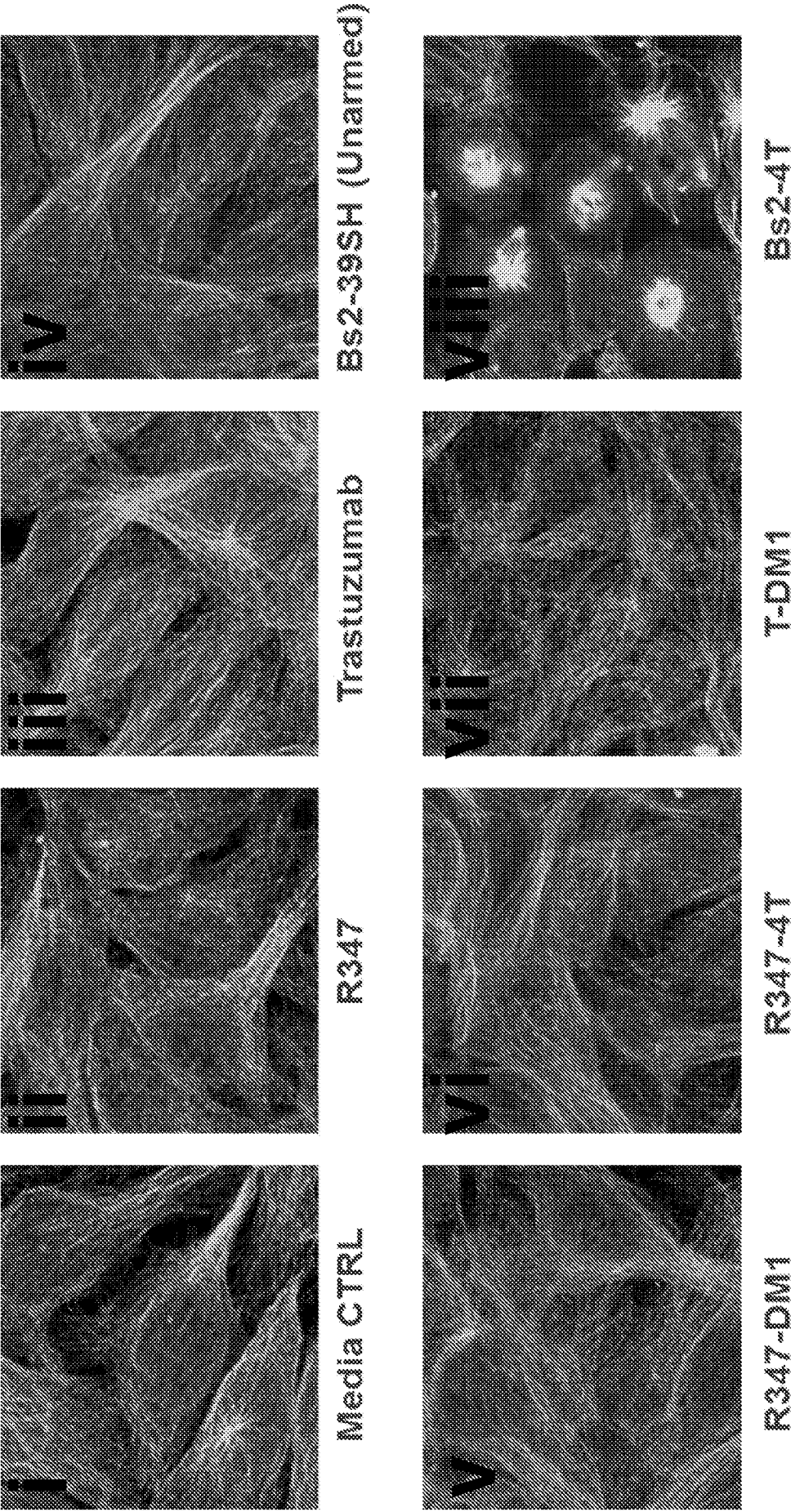


FIG. 16C

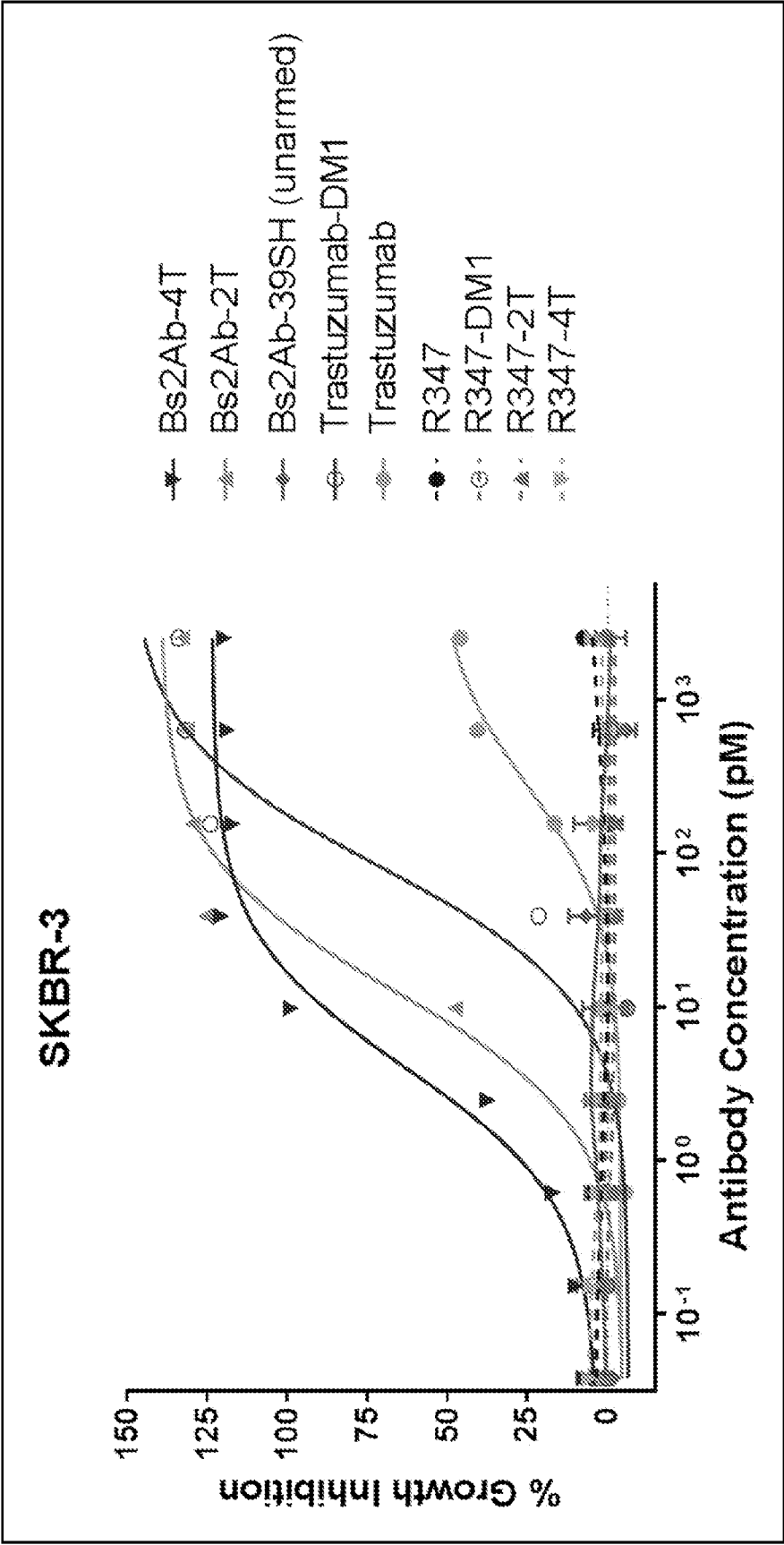


FIG. 17A

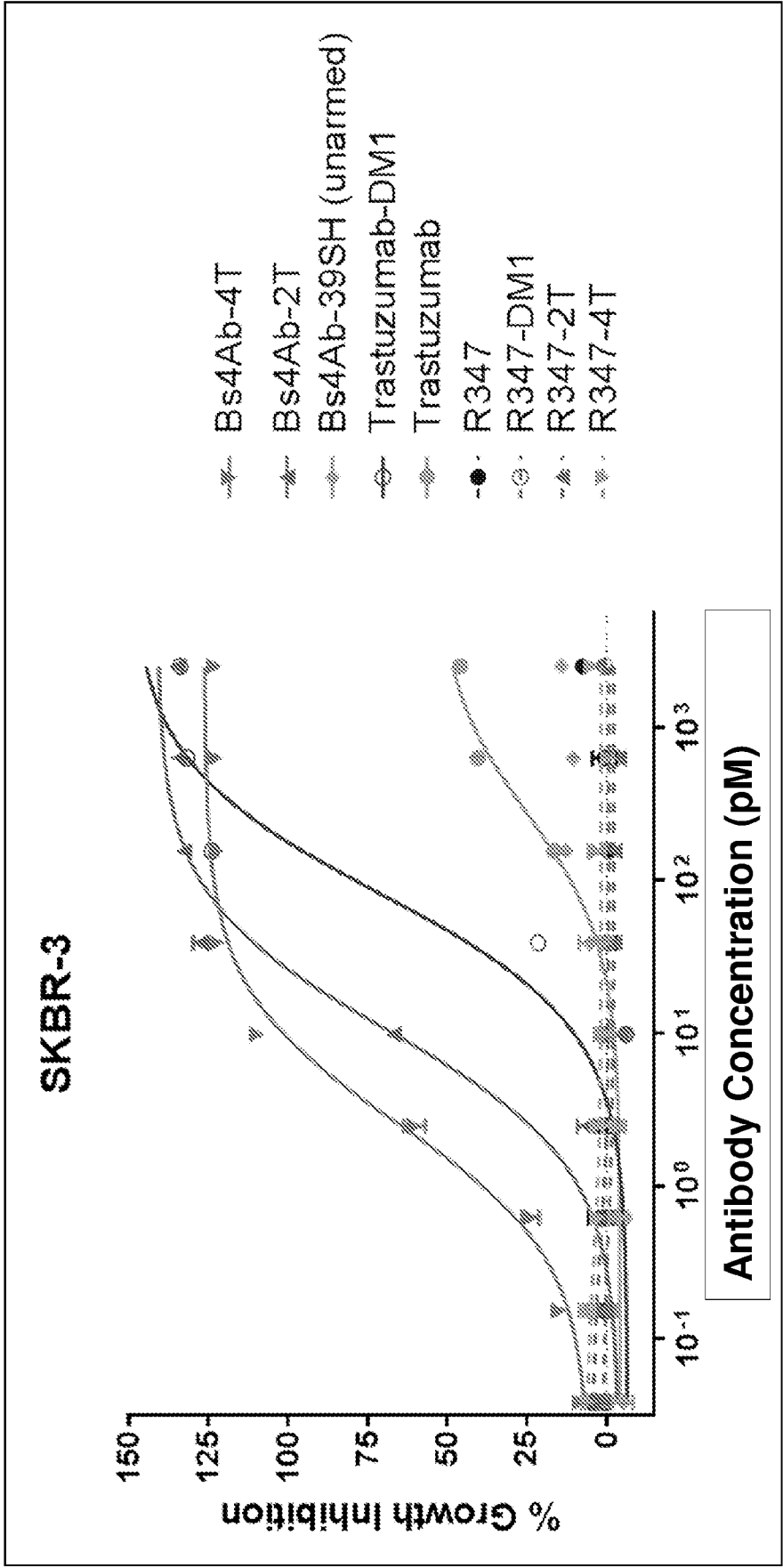


FIG. 17B

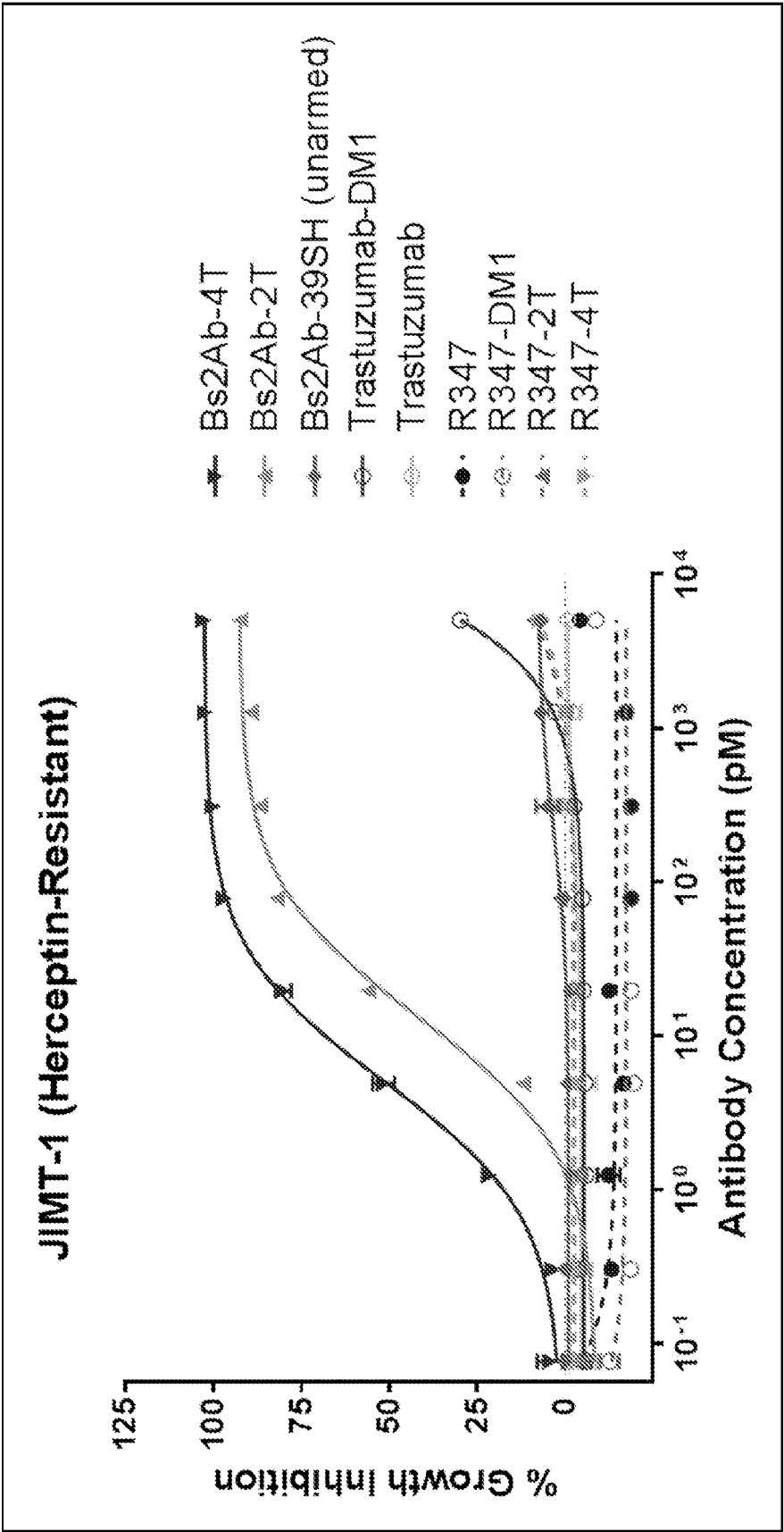


FIG. 18A

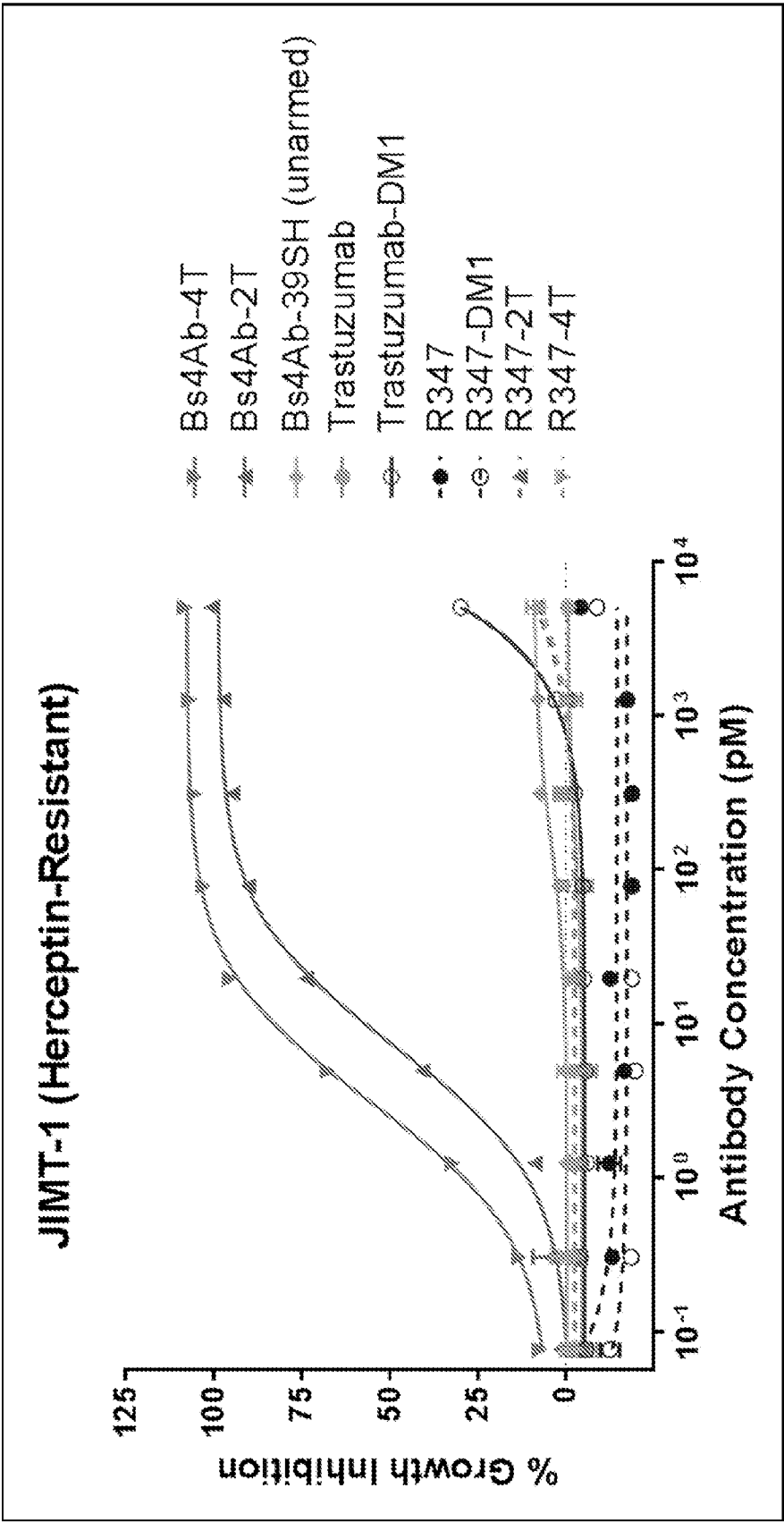


FIG. 18B

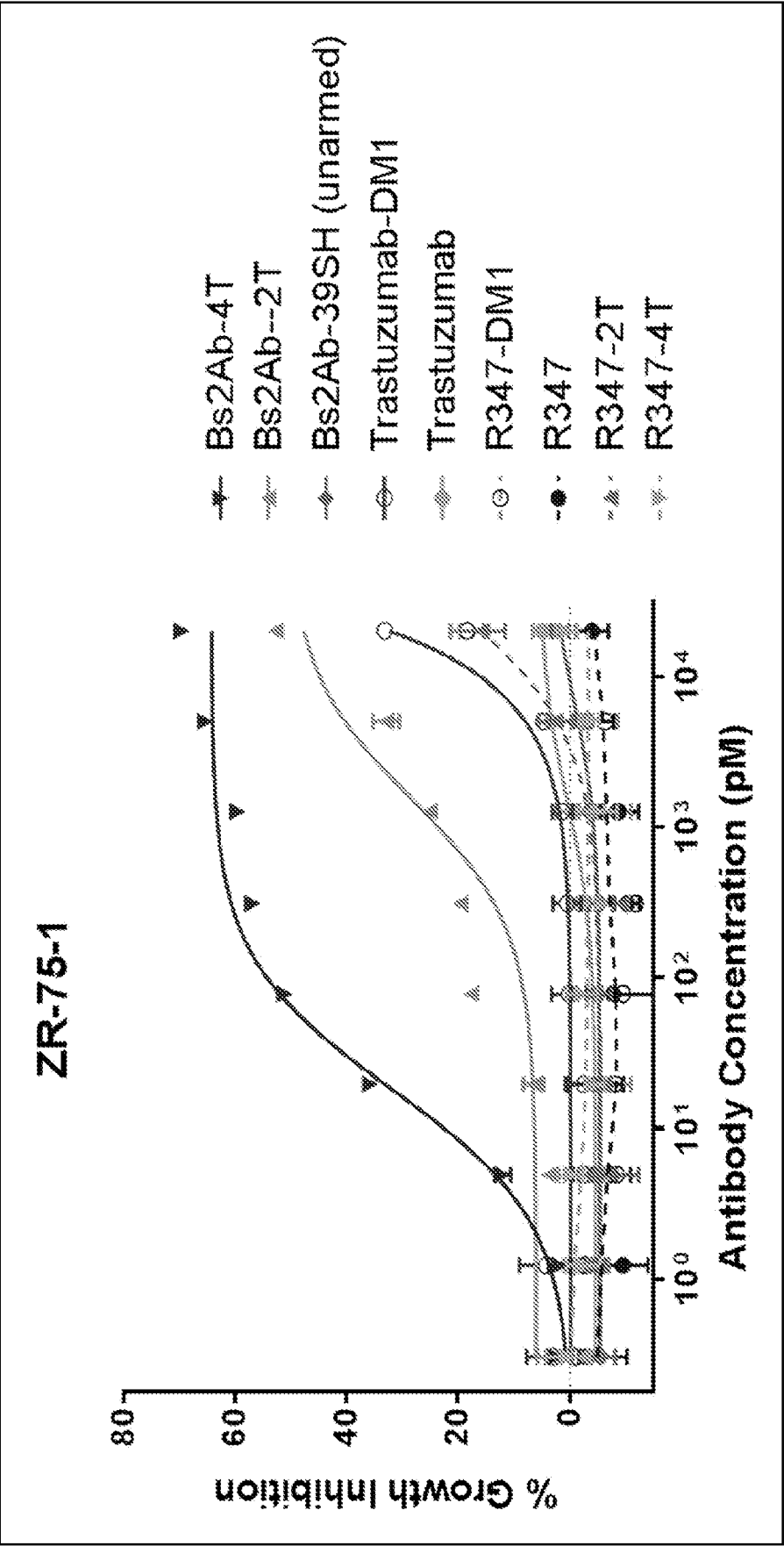


FIG. 19A

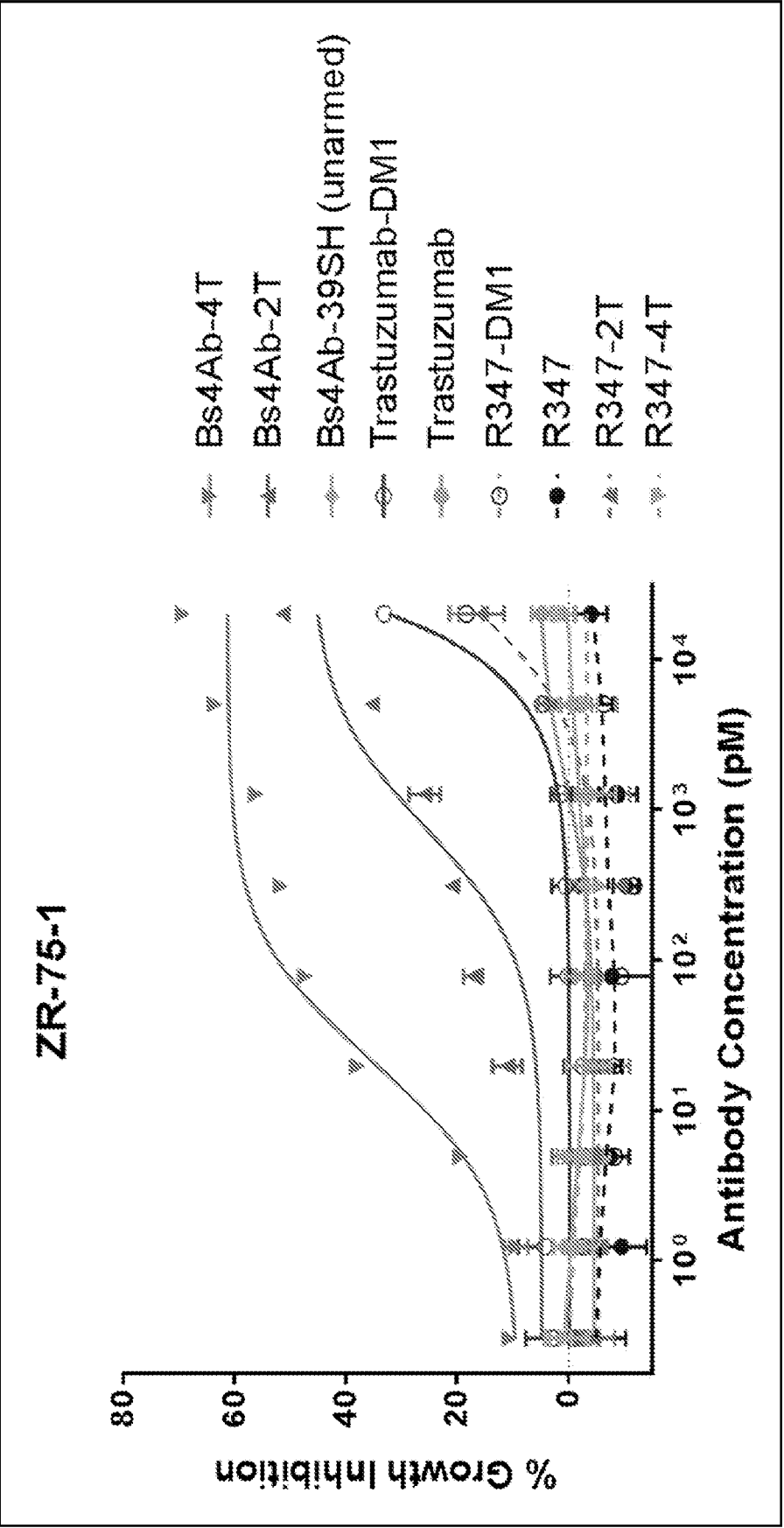


FIG. 19B

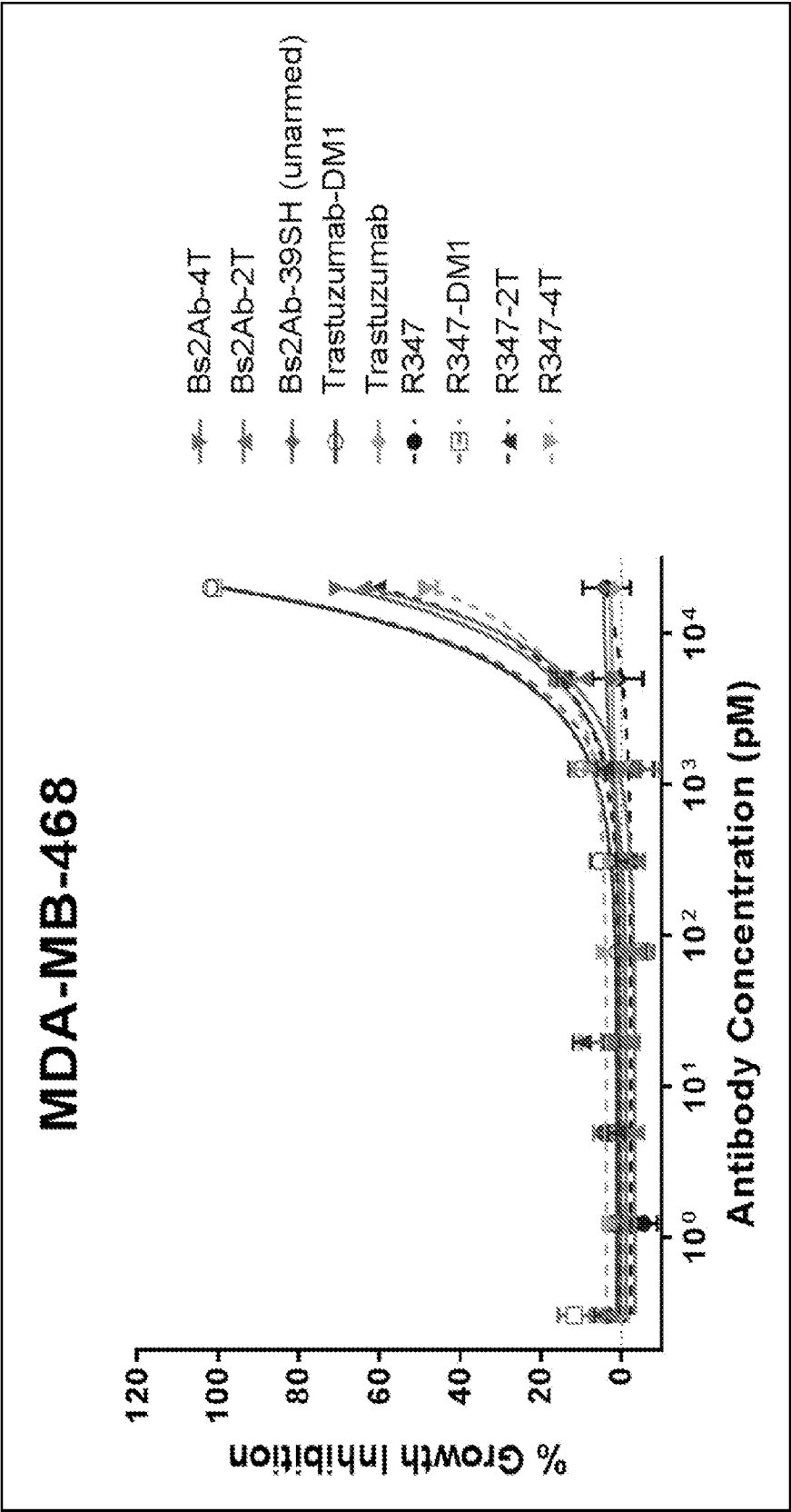


FIG. 20

MBA-MB-361 (sc serial implant, with estrogen treatment)

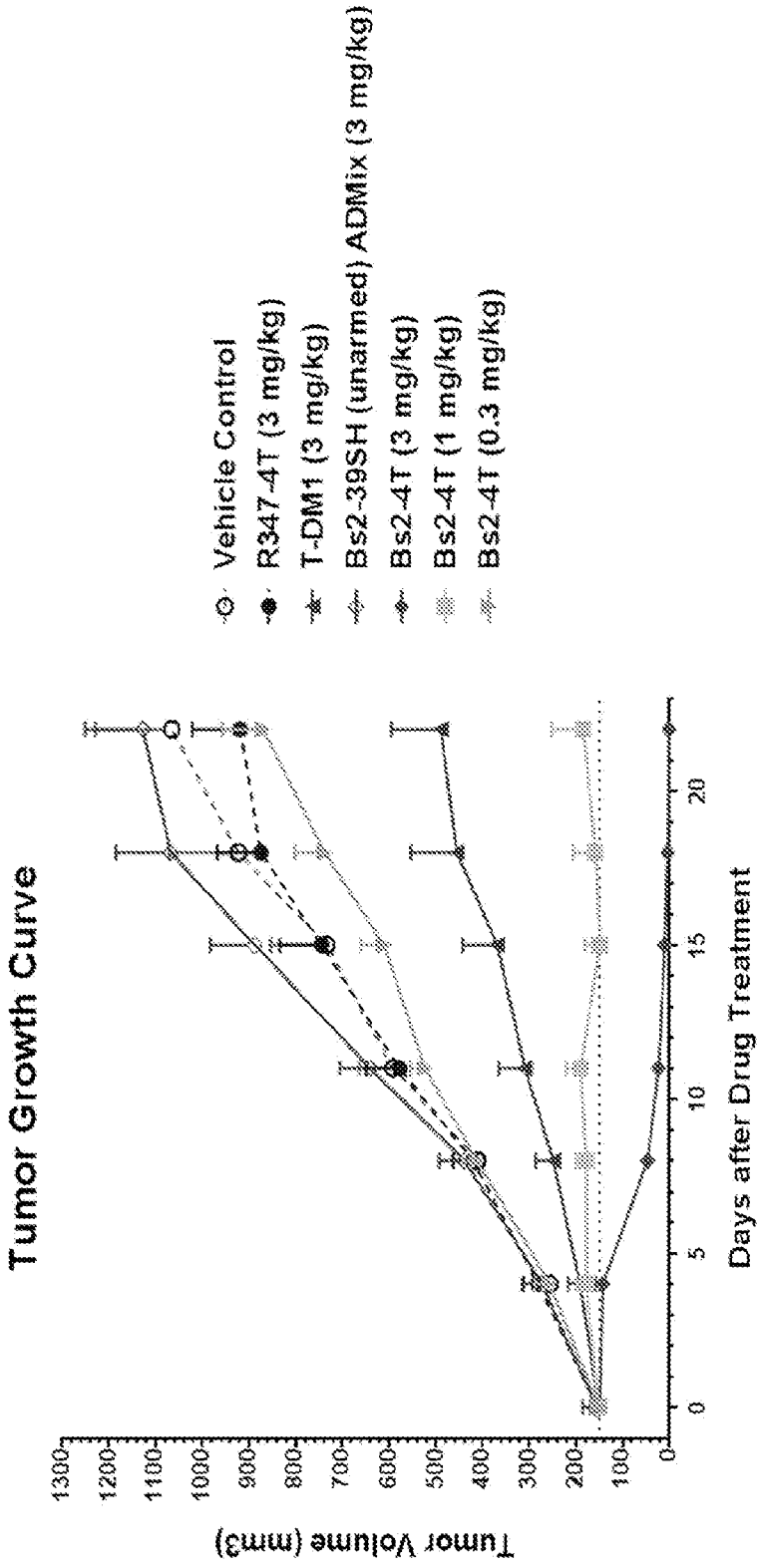


FIG. 21A

MBA-MB-361 (sc serial implant, with estrogen treatment)

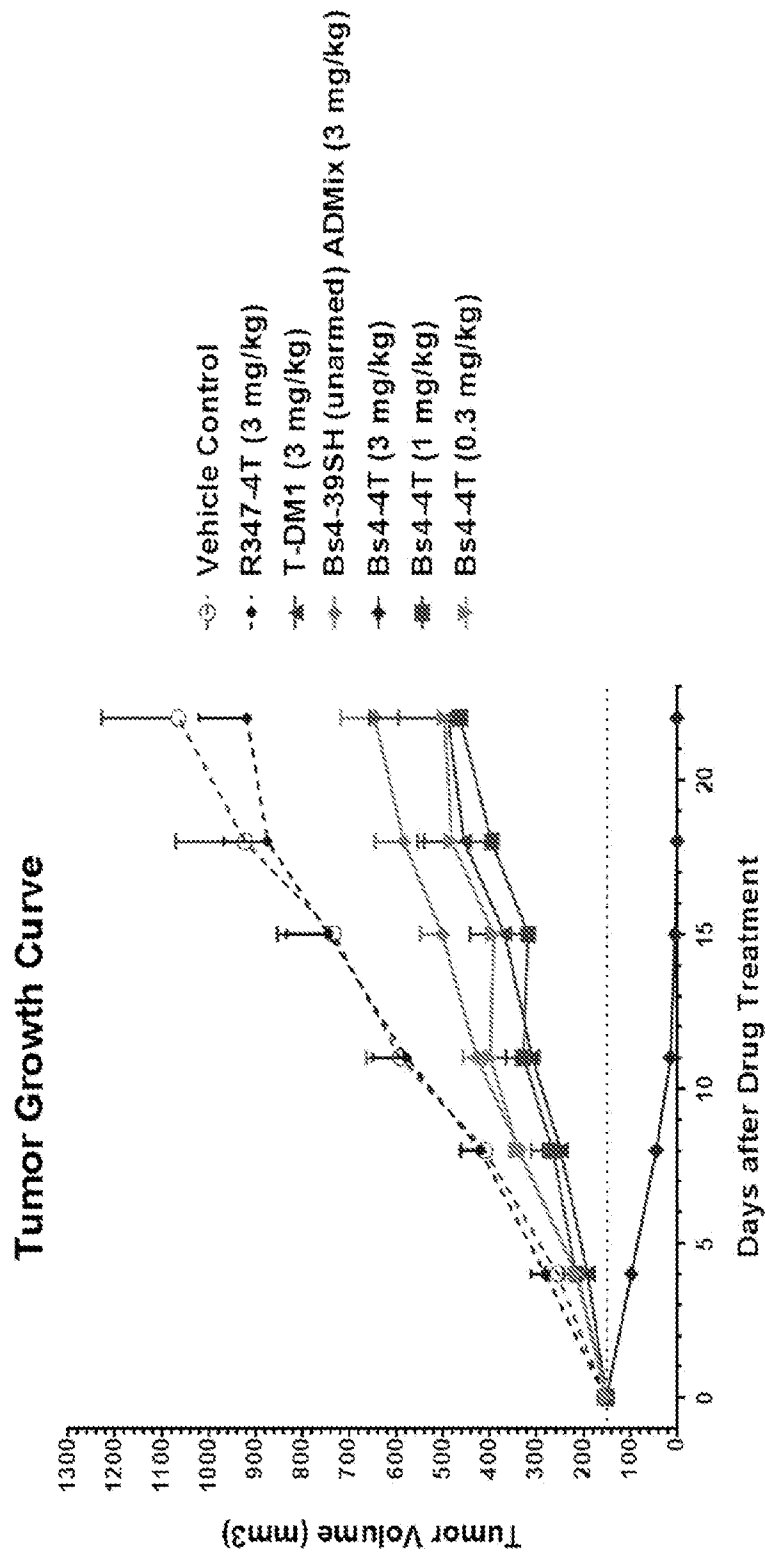


FIG. 21B

ST996 (HER2¹⁺/ER⁻/PR⁻) Tumor Growth Curve

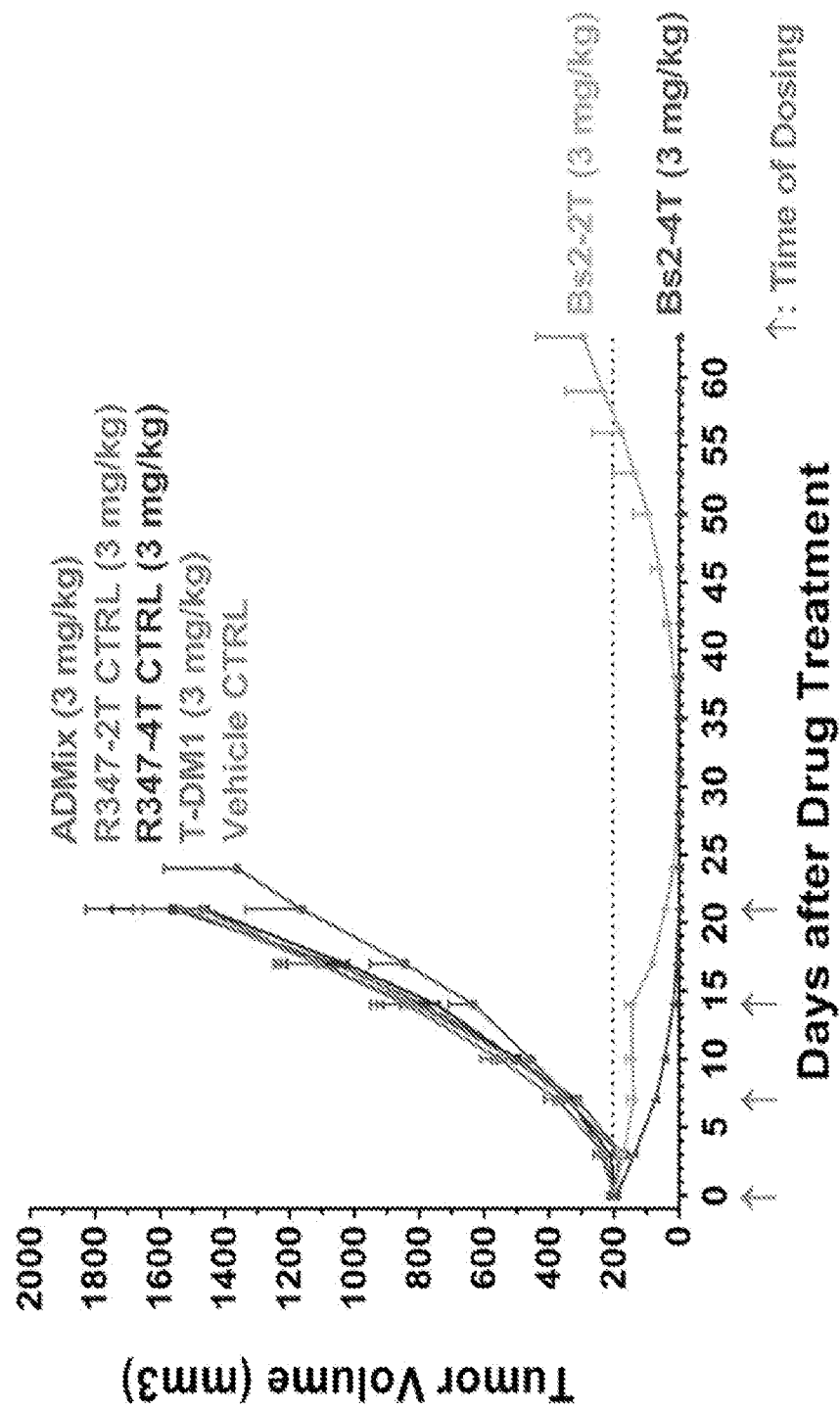


FIG. 22

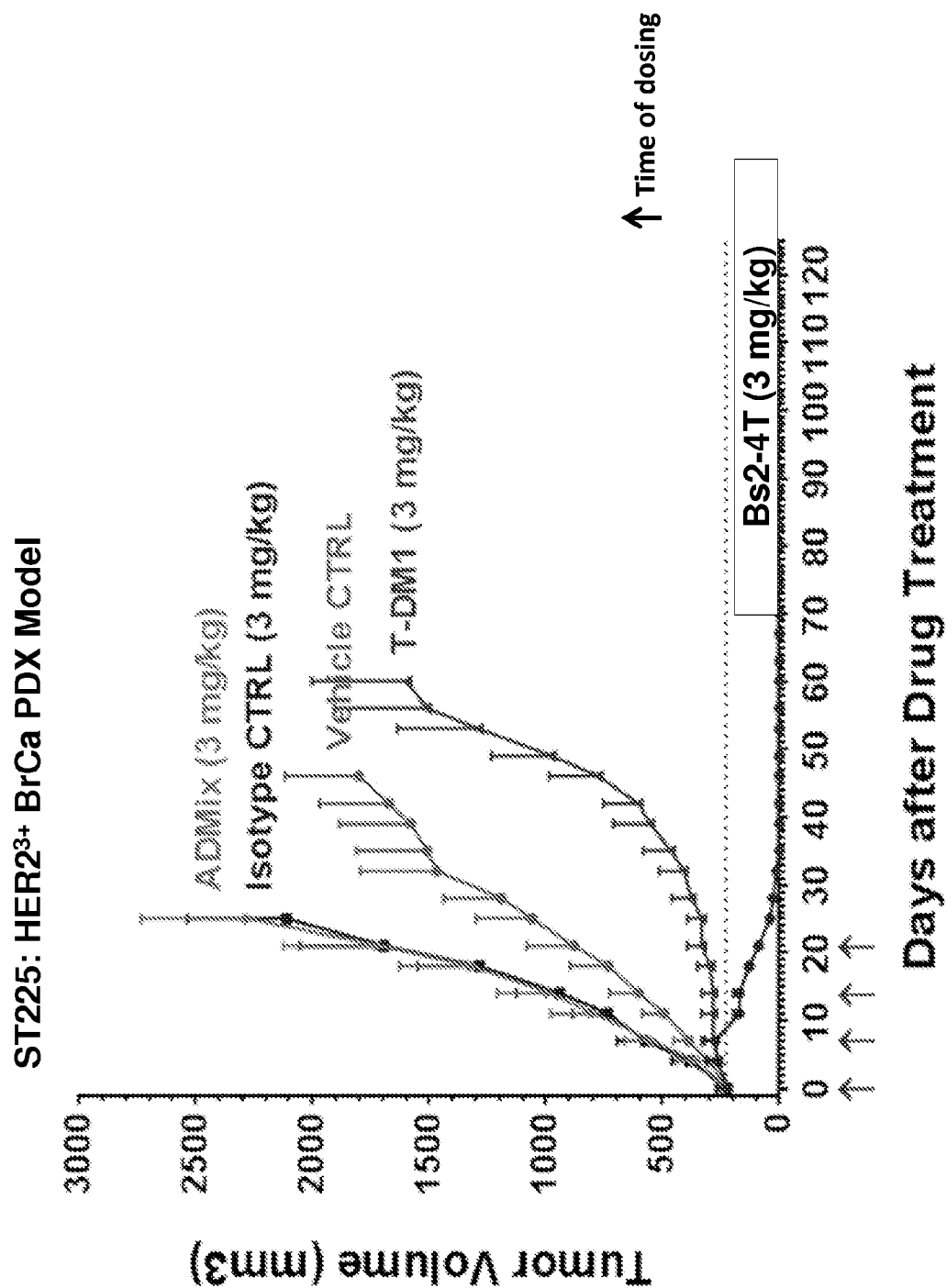


FIG. 23

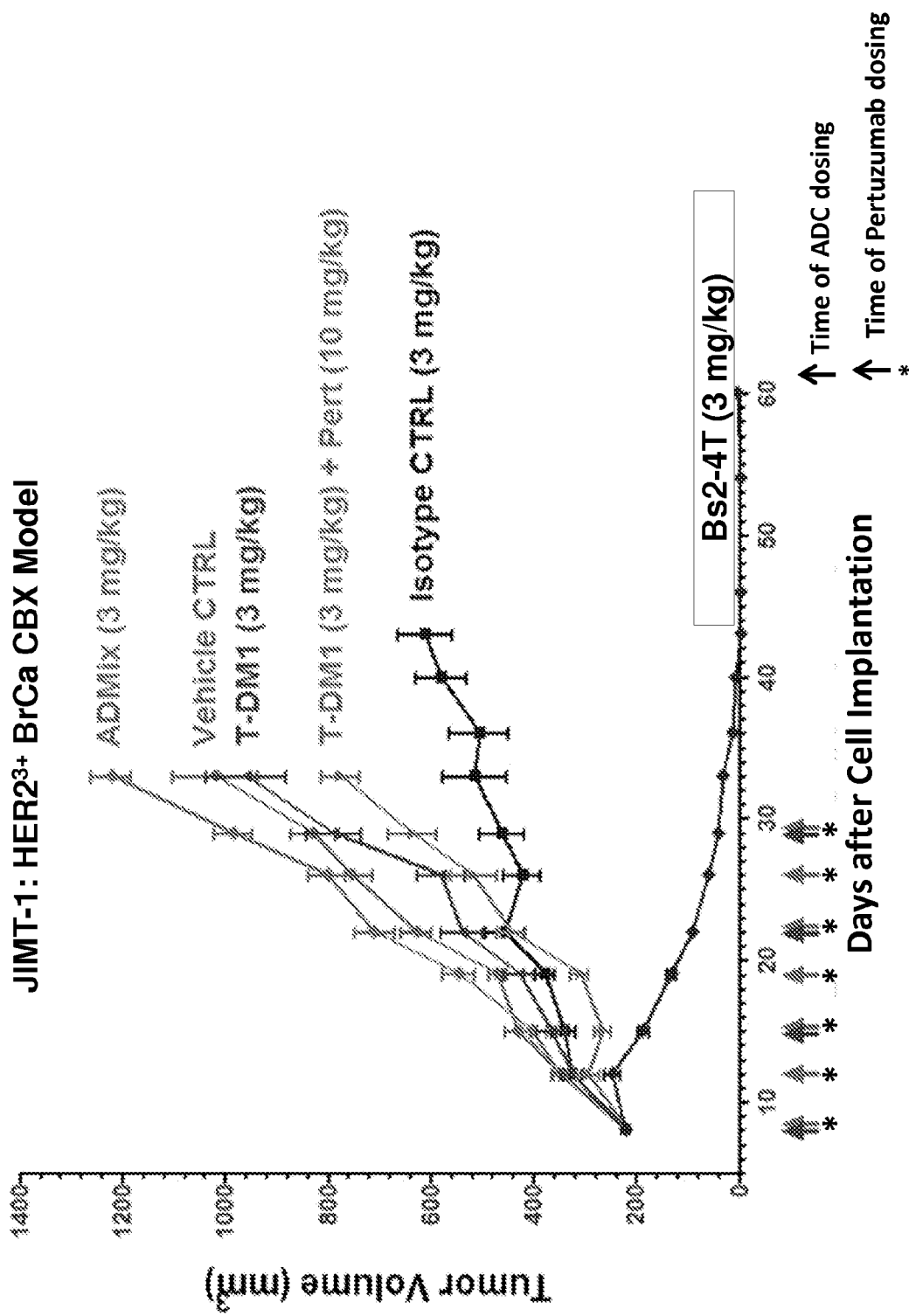


FIG. 24

ST455B: Triple-negative (ER⁻/PR⁻/HER2¹⁺) BrCa PDX Model

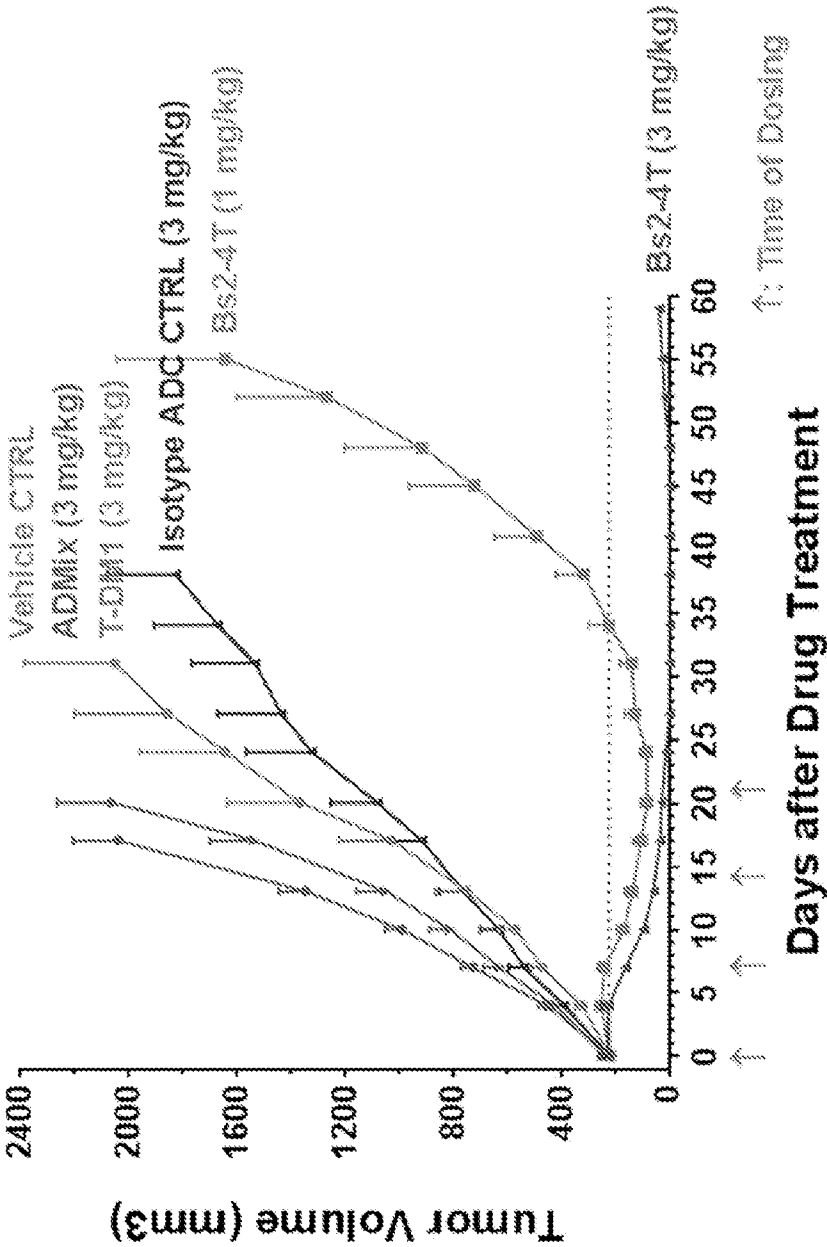


FIG. 25

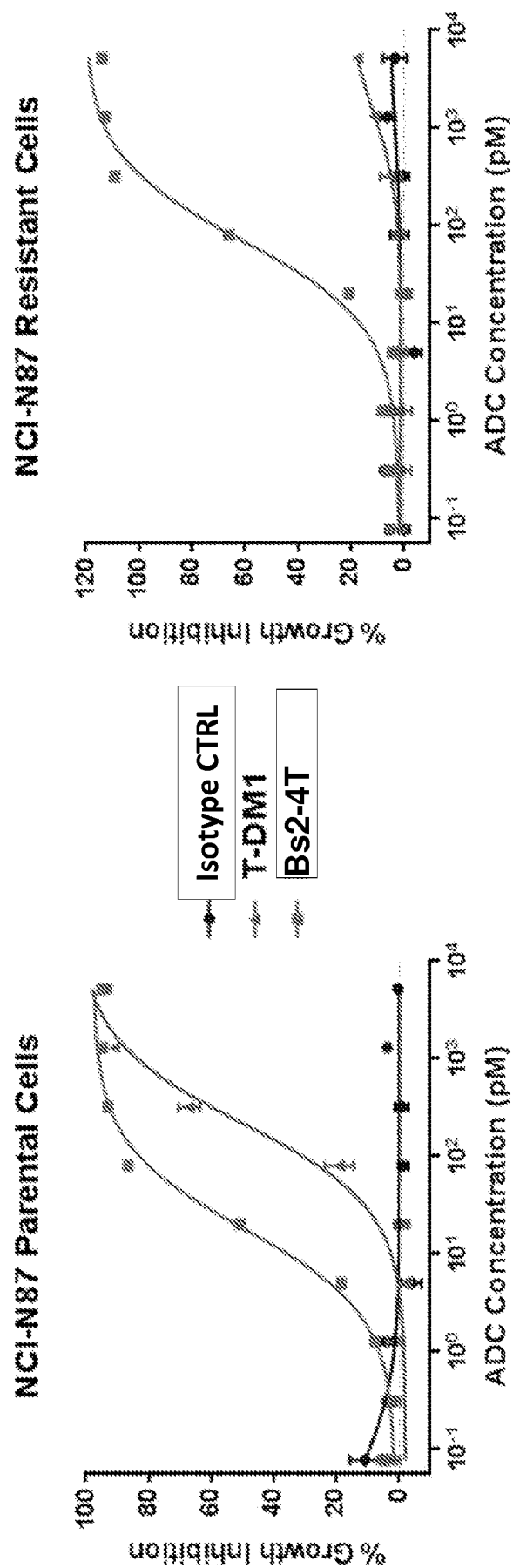


FIG. 26A

T-DM1 Resistant NCI-N87 Model

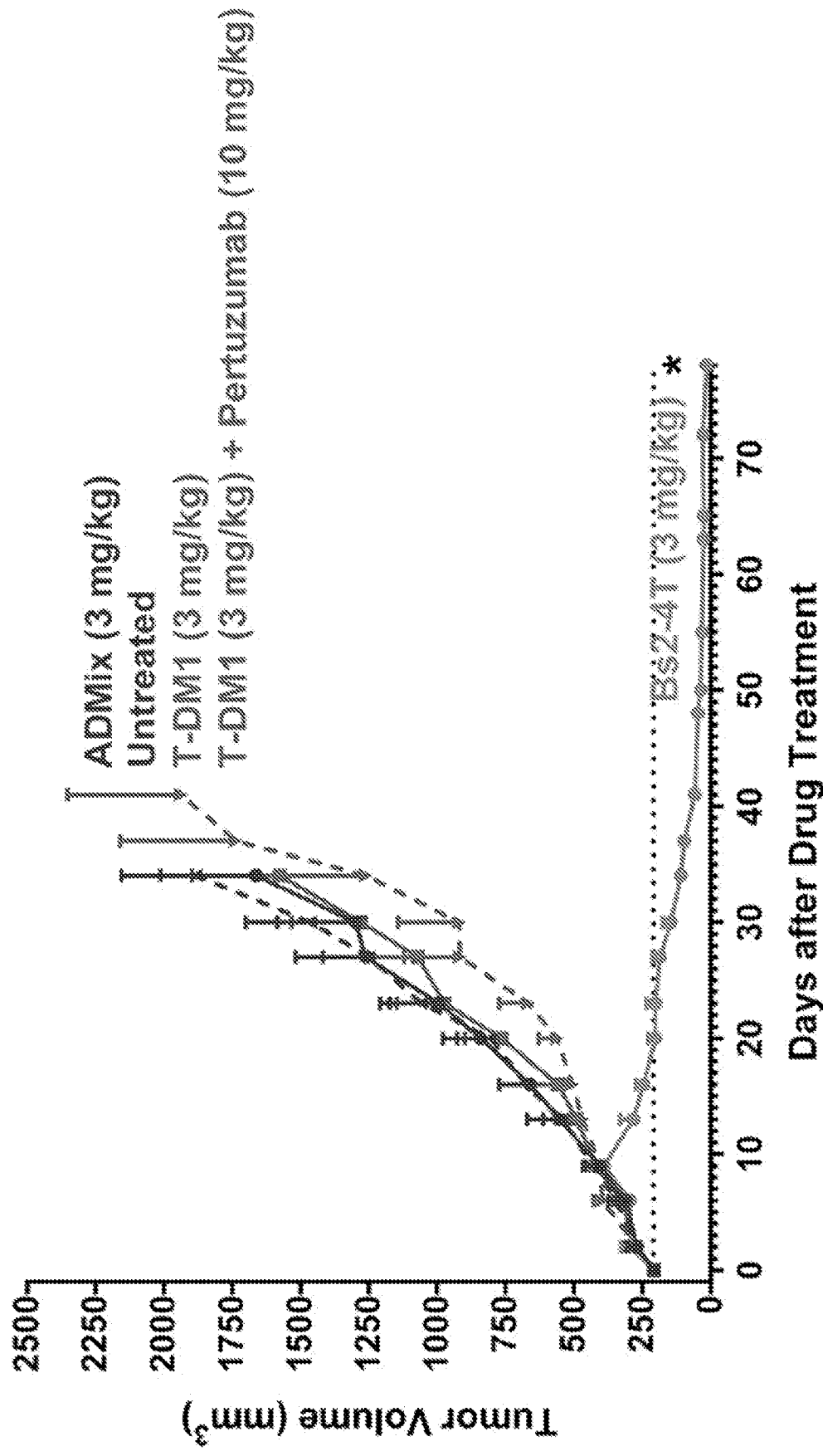


FIG. 26B

ST225 (HER2³⁺) PDX Model

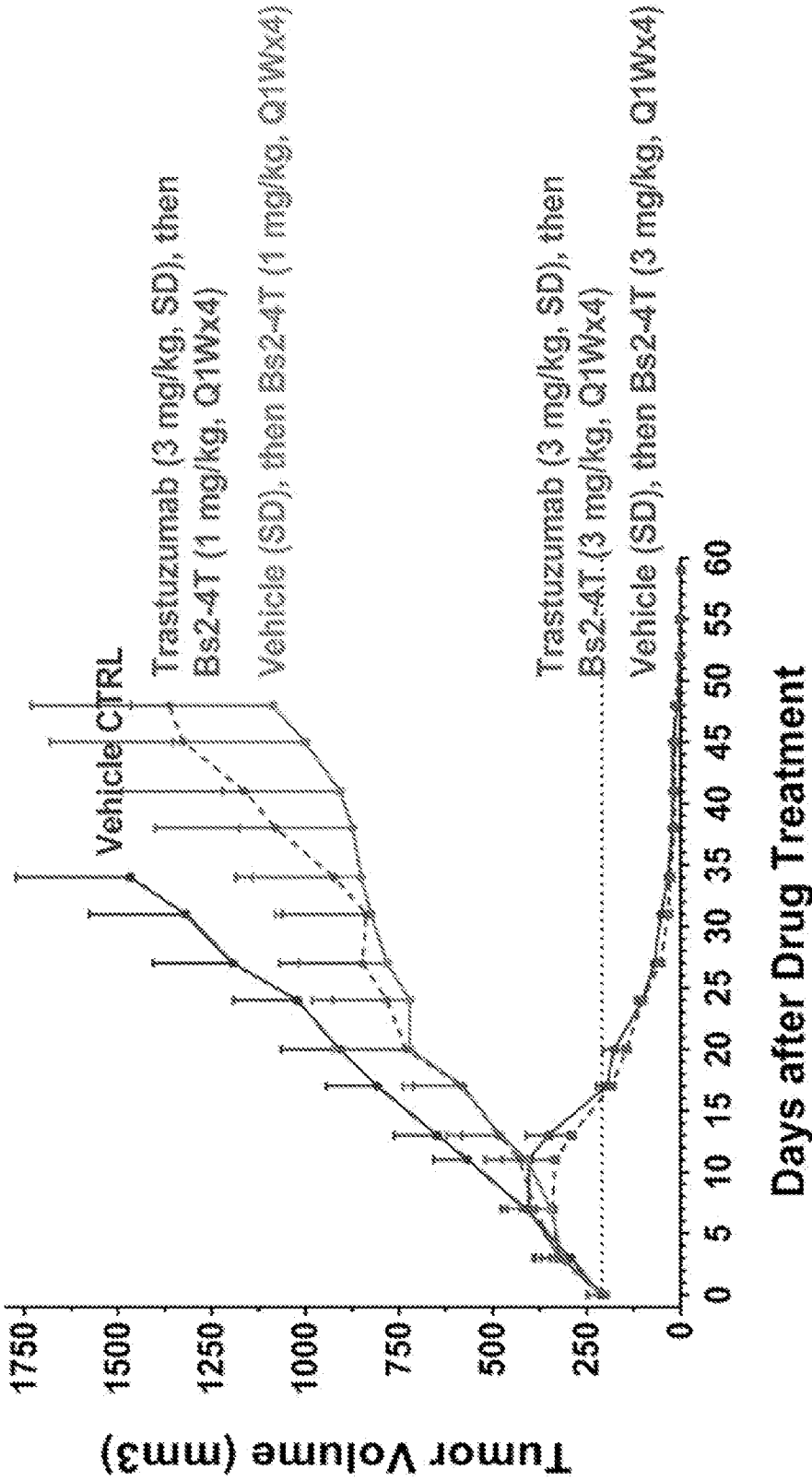


FIG. 27

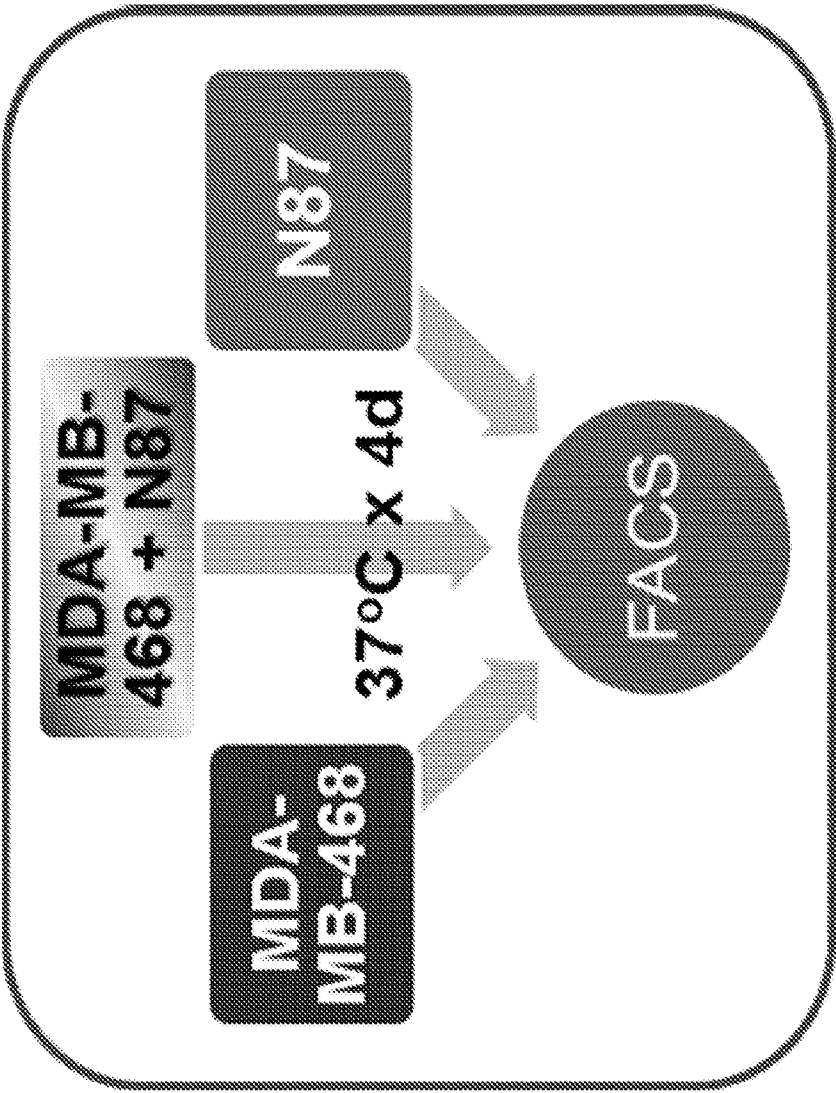


FIG. 28A

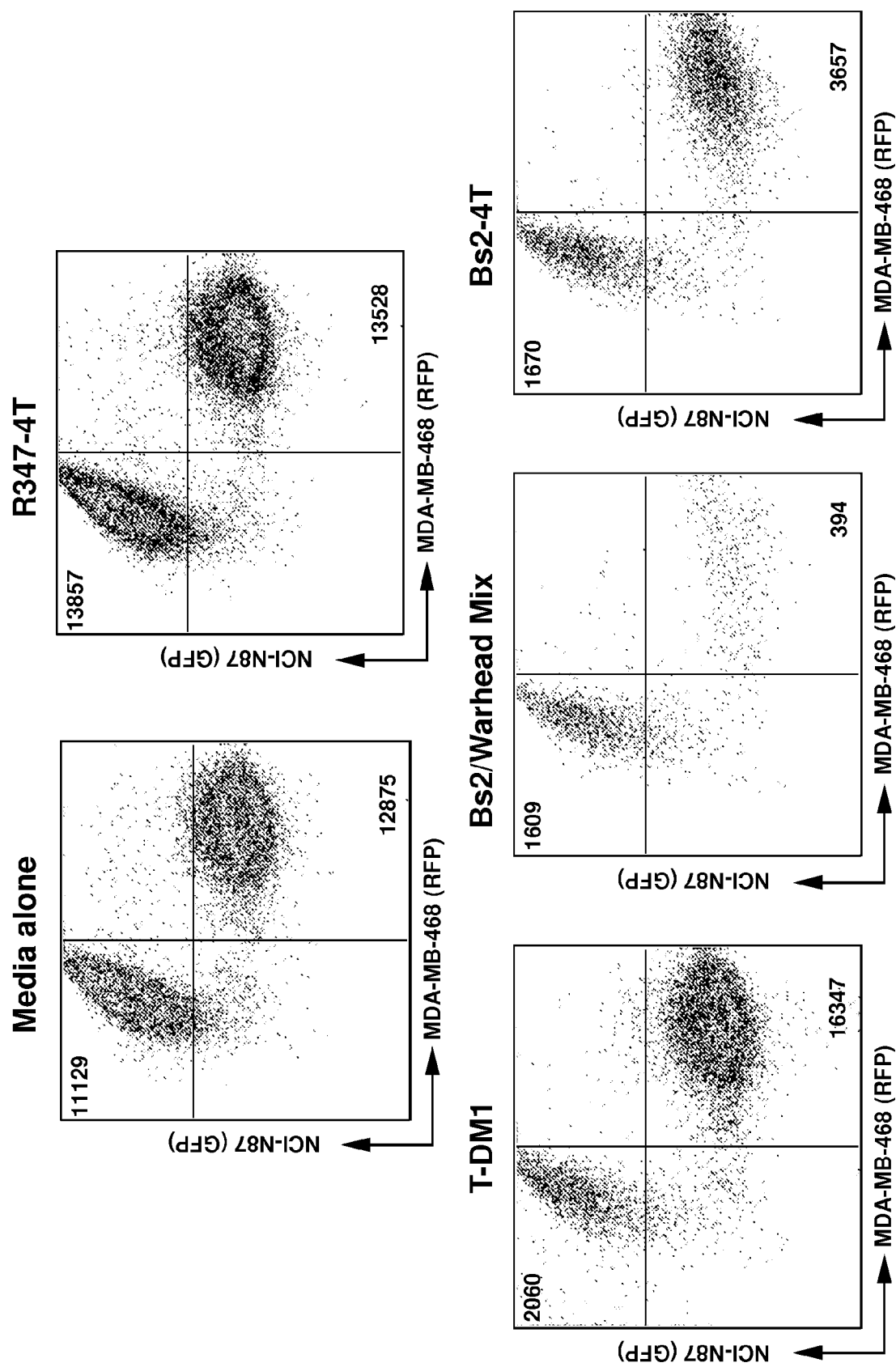


FIG. 28B

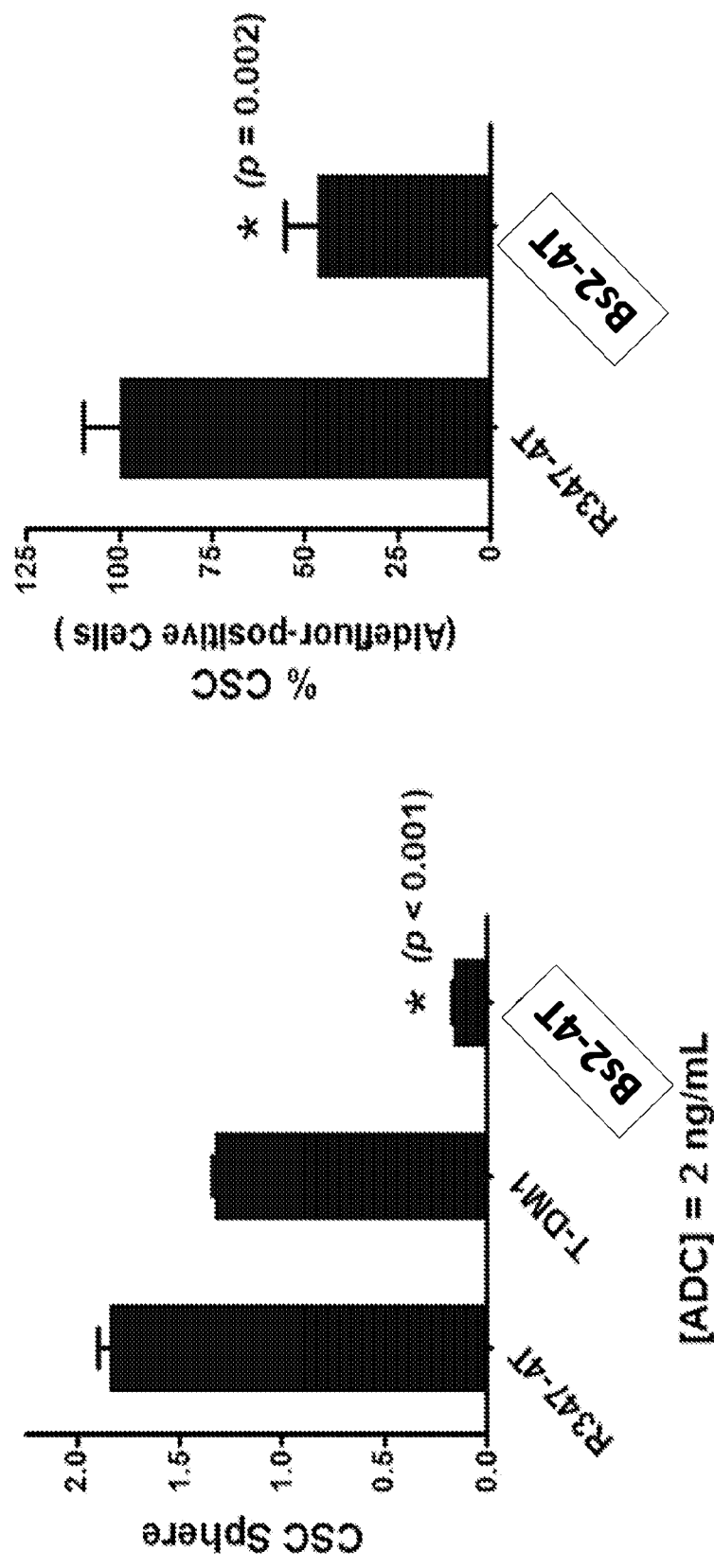


FIG. 29

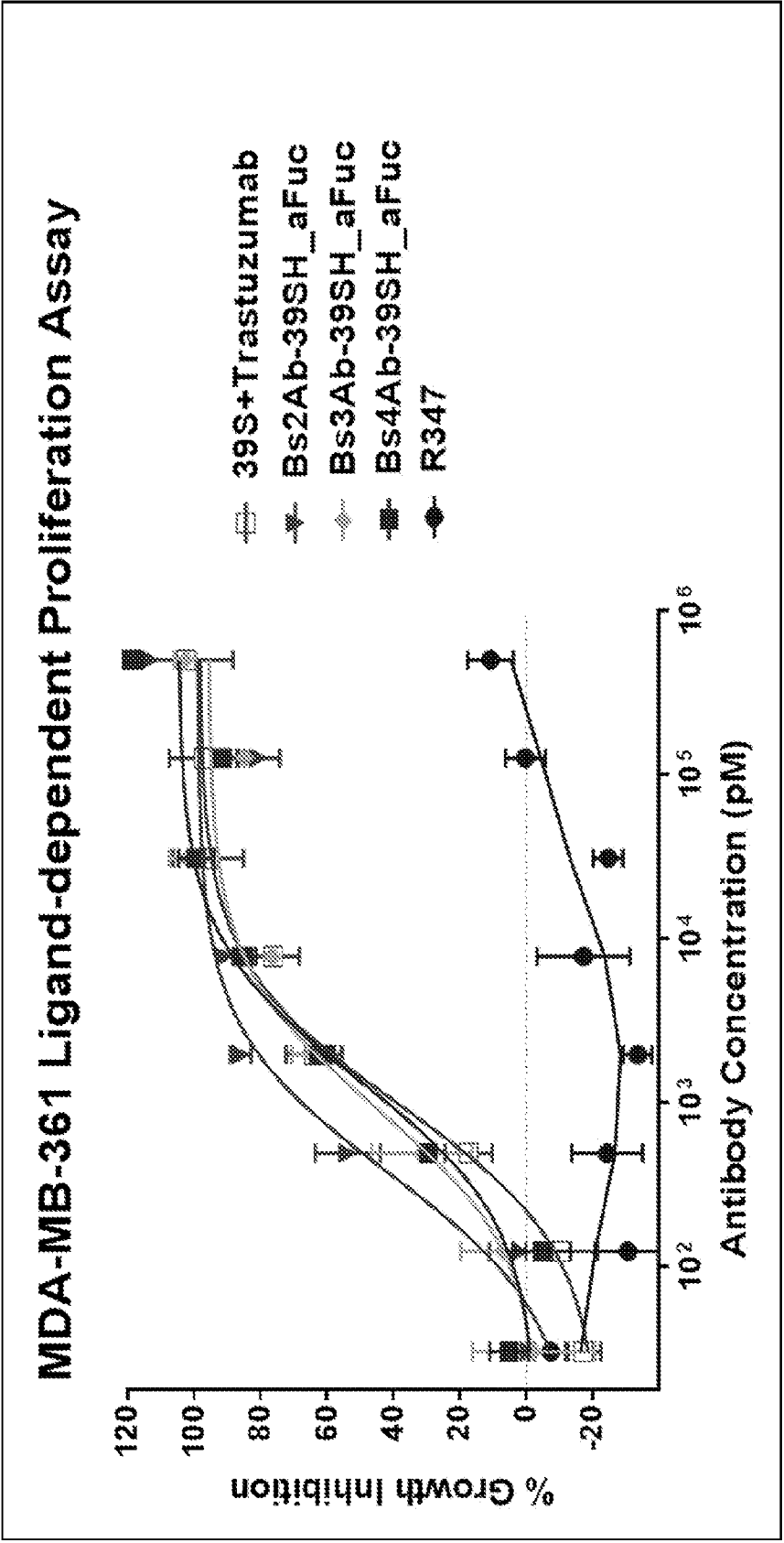


FIG. 30A

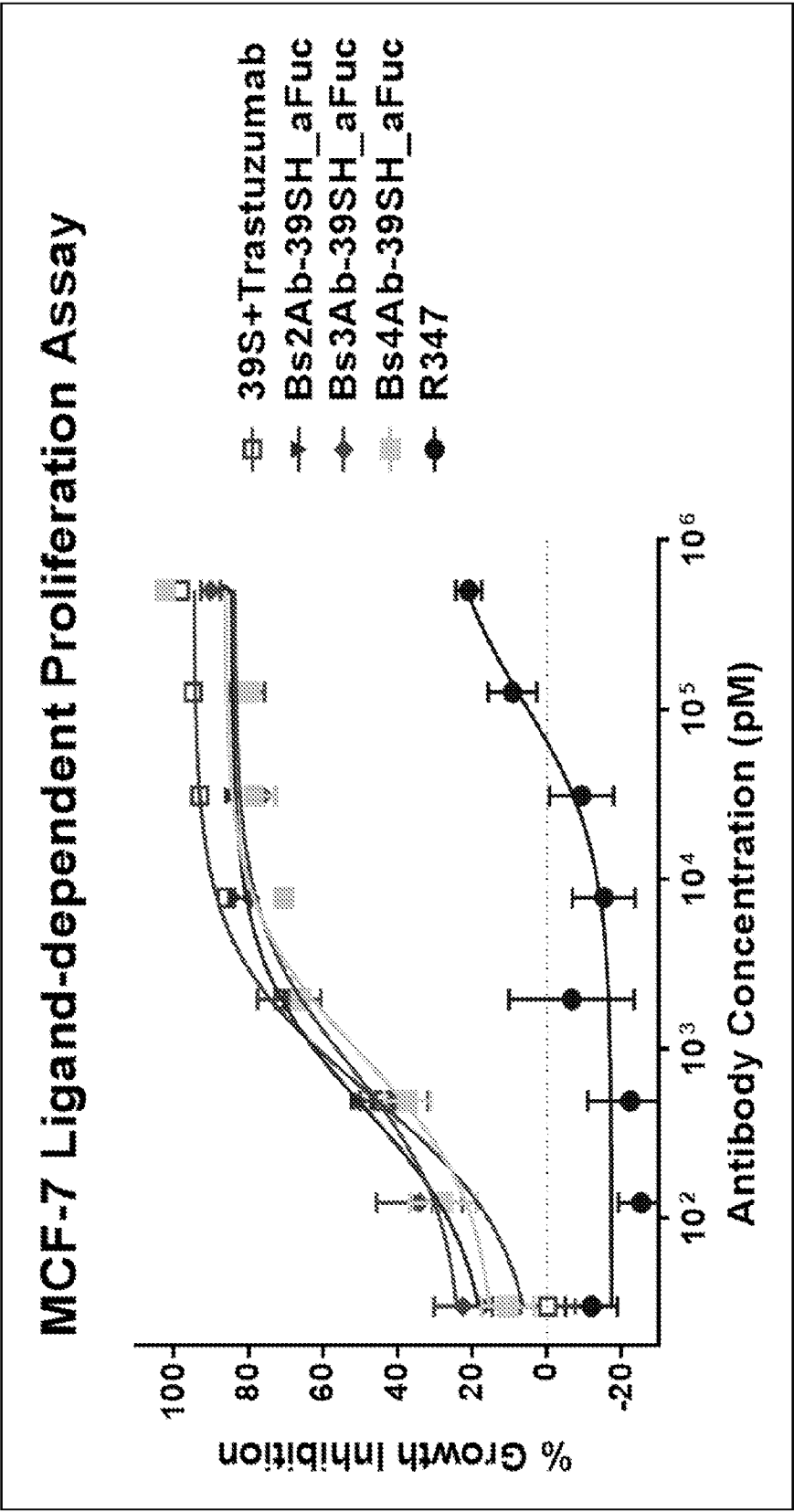


FIG. 30B

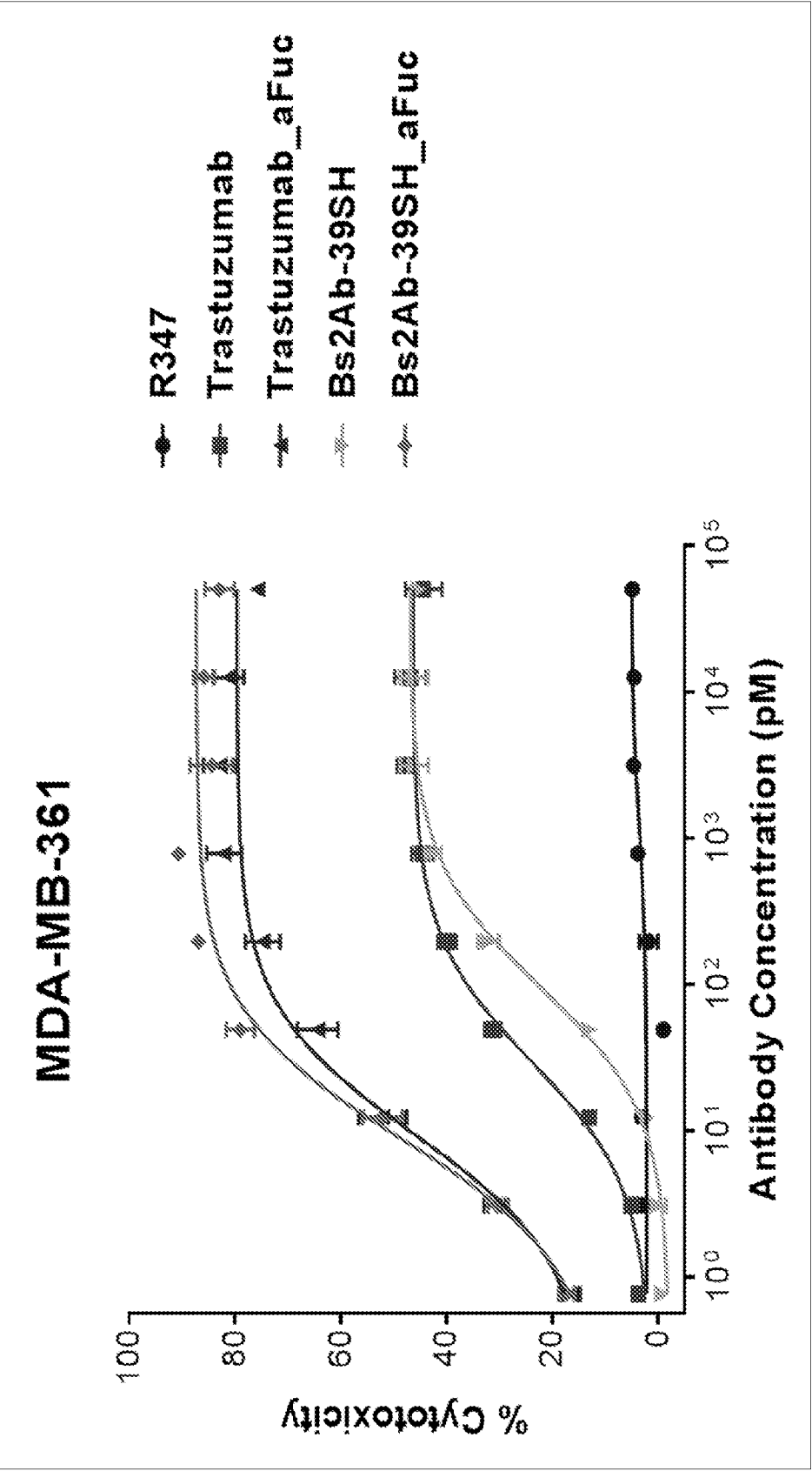


FIG. 31A

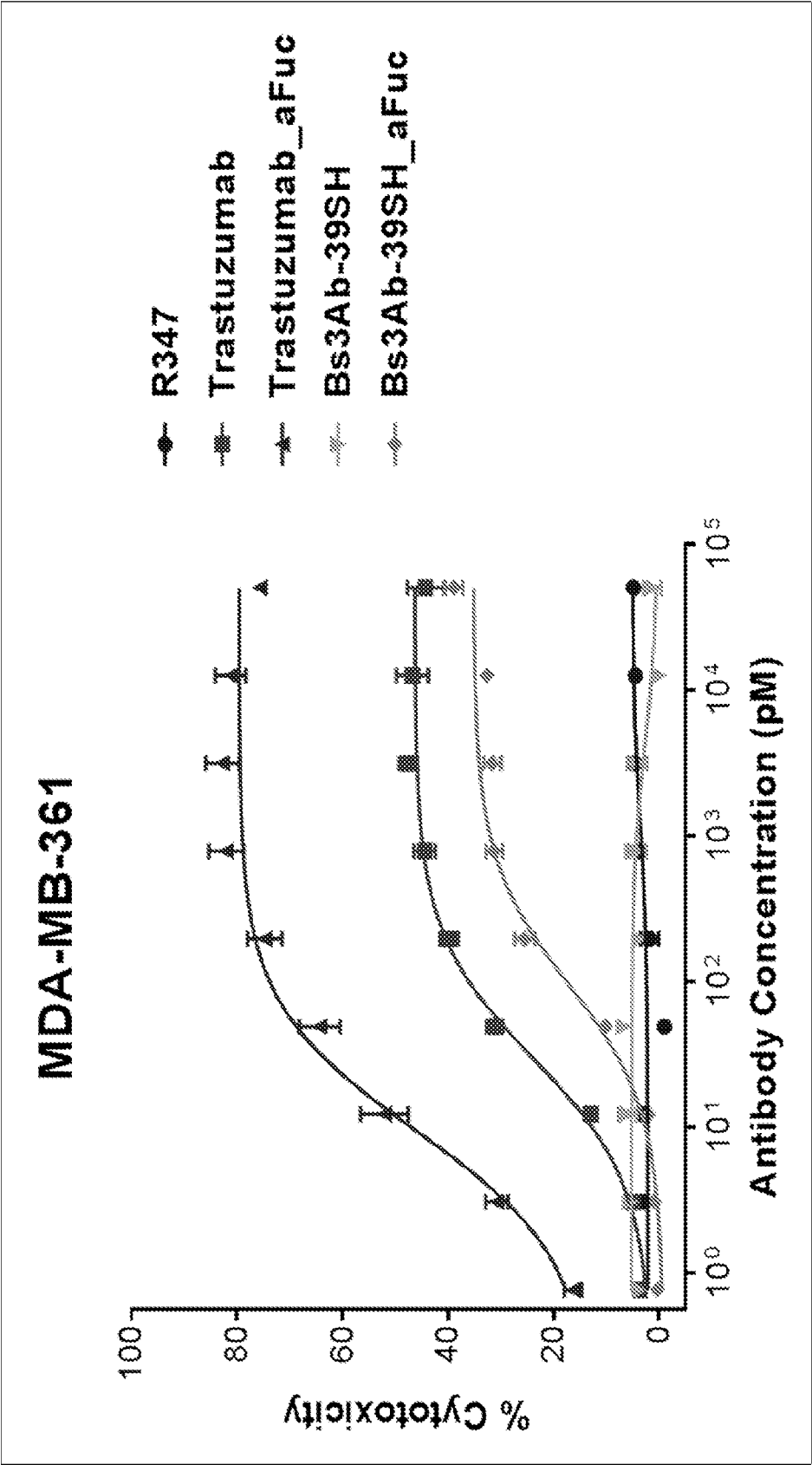


FIG. 31B

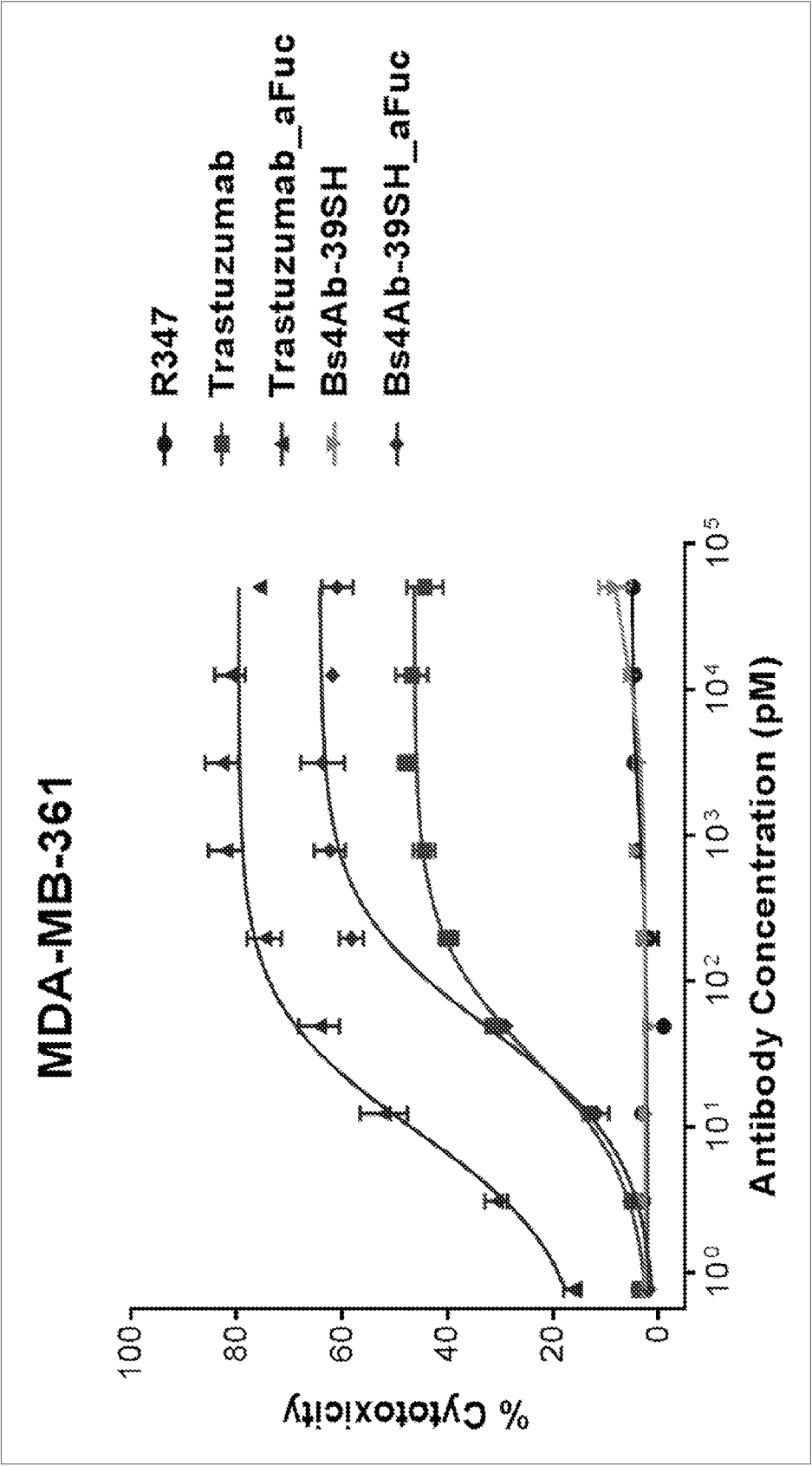


FIG. 31C

Tubulysin 1508 Payload (compound T-32)

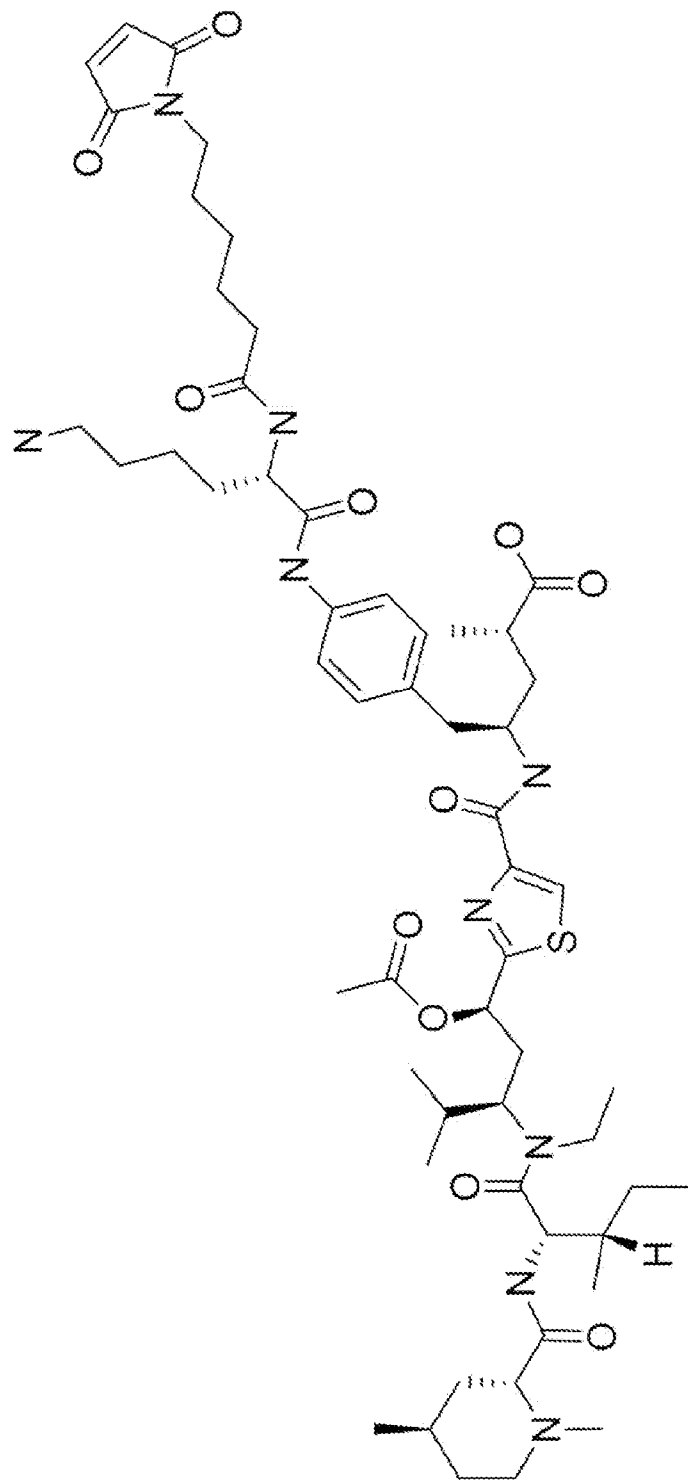


FIG. 32

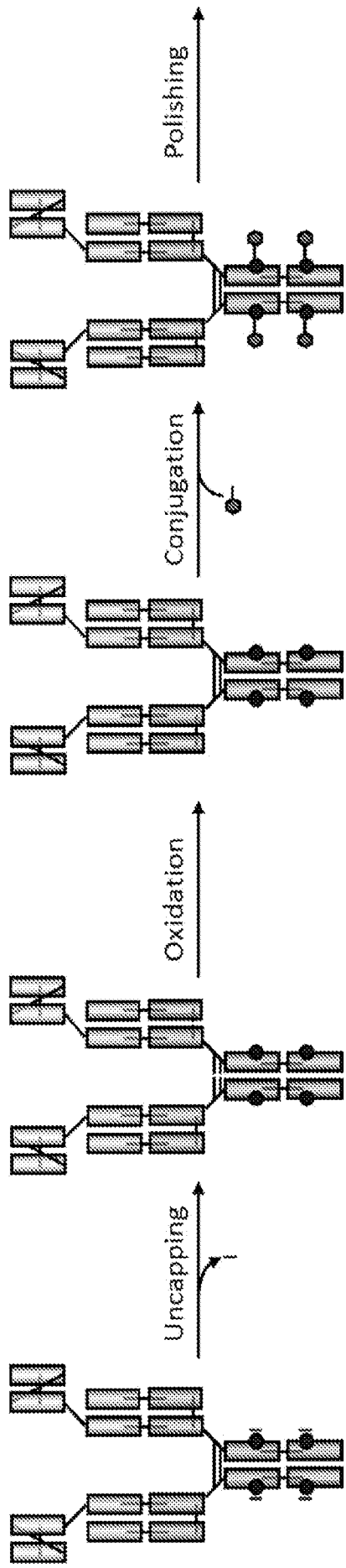


FIG. 33

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US15/25232

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

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☐ forming part of the international application as filed:
 - ☐ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☒ furnished subsequent to the international filing date for the purposes of international search only:
 - ☒ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).
2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US15/25232

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.: 6-11, 14-25
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:

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.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US15/25232

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 39/395, 45/06; C07K 16/40 (2015.01)

CPC - A61K 2039/505; C07K 2317/33, 2317/21

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8): A61K 39/395, 45/06; C07K 16/40

CPC: A61K 2039/505; C07K 2317/33, 2317/21, 2317/73, 2317/76

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

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

PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, INPADOC Data); Google; Google Scholar; NCBI/BLAST/PubMed; Dialog ProQuest; The Lens.org; 'HER2,' 'ErbB2,' antibody, binding, domain, pertuzumab, bispecific, 'Fc domain,' linker, 'scFV,' trastuzumab, peptide

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2013/0089544 A1 (MEDIMMUNE LIMITED); April 11, 2013; paragraphs [0003], [0030]-[0033], [0044], [0146], [0187], [0203], [0233], [0256], [0280]; SEQ ID NOs: 36, 38	1-5, 12, 13
Y	WO 2012/143523 A1 (GENMAB A/S); October 26, 2012; page 4 paragraph 4, page 46 paragraph 2, page 52 paragraph 2	1-5
Y	WO 2012/088290 A2 (ABBOTT LABORATORIES); June 28, 2012; page 4, lines 22-31; page 7, line 16; page 24, lines 12-24; SEQ ID NO: 171	12, 13
Y	US 7776330 B2 (YAZAKI, PJ et al.); August 17, 2010; SEQ ID NO: 30	13

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

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

24 July 2015 (24.07.2015)

Date of mailing of the international search report

07 AUG 2015

Name and mailing address of the ISA/

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P.O. Box 1450, Alexandria, Virginia 22313-1450

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Authorized officer

Shane Thomas

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A61K 45/06(2006.01)

C07K 16/40(2006.01)

权利要求书5页 说明书125页 附图47页

(54)发明名称

双特异性HER2抗体

(57)摘要

本发明涉及抗HER2结合分子(例如,抗体及其抗原结合片段)、衍生的HER2结合分子(例如,双特异性抗HER2抗体)以及结合该HER2受体的细胞外结构域的抗体-药物轭合物(ADC)。还提供包含这些披露的组合物的药物配制品以及用于治疗与HER2介导的信号转导相关联的疾病的方法。

1. 一种双特异性抗HER2抗体, 包含第一免疫球蛋白抗原结合结构域和第二免疫球蛋白抗原结合结构域, 其中(i)该第一免疫球蛋白抗原结合结构域和该第二免疫球蛋白抗原结合结构域特异性结合至不同HER2抗体结合位点, (ii)该第一免疫球蛋白抗原结合结构域结合至第一HER2抗体结合位点, 该第一HER2抗体结合位点包含HER2的结构域II内的表位, 并且(iii)该第一HER2抗体结合位点与帕妥珠单抗的抗体结合位点不同。

2. 根据权利要求1所述的双特异性抗体, 其中该第一免疫球蛋白抗原结合结构域包含重链(HC)可变区(VH)和轻链(LC)可变区(VL), 该重链可变区和该轻链可变区包含:

(i)与SEQ ID NO:1相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:1相同的可变重链CDR-1(VH-CDR1);

(ii)与SEQ ID NO:2相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:2相同的可变重链CDR-2(VH-CDR2);

(iii)与SEQ ID NO:3相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:3相同的可变重链CDR-3(VH-CDR3);

(iv)与SEQ ID NO:4相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:4相同的可变轻链CDR-1(VL-CDR1);

(v)与SEQ ID NO:5相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:5相同的可变轻链CDR-2(VL-CDR2); 以及,

(vi)与SEQ ID NO:6相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:6相同的可变轻链CDR-3(VL-CDR3)。

3. 一种双特异性抗HER2抗体, 包含第一免疫球蛋白抗原结合结构域和第二免疫球蛋白抗原结合结构域, 其中该第一免疫球蛋白抗原结合结构域包含VH和VL, 其中

(a)该VH包含SEQ ID NO:15或43的氨基酸;

(b)该VL包含SEQ ID NO:16或44的氨基酸;

并且其中该第一免疫球蛋白抗原结合结构域和该第二免疫球蛋白抗原结合结构域特异性结合至不同HER2表位。

4. 根据权利要求1至3中任一项所述的双特异性抗HER2抗体, 其中该第一免疫球蛋白抗原结合结构域和/或该第二免疫球蛋白抗原结合结构域包含以下各项或由其组成:(a)VH和VL, 该VH进一步包含重链恒定区或其片段, 并且该VL包含轻链恒定区(LC)或其片段;(b)单链Fv(“scFv”);(c)双体抗体;(d)小抗体;(e)F(ab')₂; 或(f)F(ab)。

5. 根据权利要求4所述的双特异性抗HER2抗体, 其中(a)该重链恒定区或其片段是IgG恒定区; 和/或(b)该LC恒定区是κ恒定区或λ恒定区。

6. 根据权利要求1至5中任一项所述的双特异性抗HER2抗体, 其中该第一免疫球蛋白抗原结合结构域是单克隆抗体、人源化抗体、人抗体、嵌合抗体、或亲和力优化的抗体。

7. 根据权利要求1至6中任一项所述的双特异性抗HER2抗体, 其中:

(a)该第二免疫球蛋白抗原结合结构域特异性结合至与该曲妥珠单抗抗体相同的HER2表位;

(b)该第二免疫球蛋白抗原结合结构域通过该曲妥珠单抗抗体竞争性地抑制HER2结合; 或

(c)该第二免疫球蛋白抗原结合结构域包含至少一个、至少两个、至少三个、至少四个、

至少五个、或至少六个互补决定区(CDR),这些互补决定区包含SEQ ID NO:54至59中的任一个的氨基酸。

8.根据权利要求7所述的双特异性抗HER2抗体,其中该第二免疫球蛋白抗原结合结构域是scFv,所述scFv包含:

- (i)包含SEQ ID NO:54的氨基酸的VH-CDR1;
- (ii)包含SEQ ID NO:55的氨基酸的VH-CDR2;
- (iii)包含SEQ ID NO:56的氨基酸的VH-CDR3;
- (iv)包含SEQ ID NO:57的氨基酸的VL-CDR1;
- (v)包含SEQ ID NO:58的氨基酸的VL-CDR2;以及
- (vi)包含SEQ ID NO:59的氨基酸的VL-CDR3。

9.根据权利要求7或权利要求8所述的双特异性抗HER2抗体,其中

(a)该第二免疫球蛋白抗原结合结构域被共价连接至该第一免疫球蛋白抗原结合结构域的该HC的羧基末端上;

(b)该第二免疫球蛋白抗原结合结构域被共价连接至该第一免疫球蛋白抗原结合结构域的该HC的氨基末端上;或,

(c)该第二免疫球蛋白抗原结合结构域被共价嵌入在该第一免疫球蛋白抗原结合结构域的该HC的多肽链中。

10.根据权利要求1至9中任一项所述的双特异性抗HER2抗体,其中该重链包含恒定区,该恒定区包含Fc结构域。

11.根据权利要求10所述的双特异性抗HER2抗体,其中该Fc结构域包含能够降低或增强该双特异性抗体的ADCC活性的至少一个突变。

12.一种双特异性抗HER2抗体,包含彼此相关联的第一多肽链和第二多肽链,其中该第一多肽链选自以下各项:

- (1)[TZS]-[L₁]-[BVH]-[BCH]-[Fc_x]
- (2)[BVH]-[BCH]-[Fc_x]-[L₂]-[TZS]
- (3)[BVH]-[BCH]-[L₃]-[TZS]-[L₄]-[Fc_x]

其中

TZS是结合与曲妥珠单抗相同的表位的scFv;

L₁、L₂、L₃以及L₄是肽接头;

Fc_x是Fc结构域;

BVH和BCH分别是抗体的VH区和CH1区,该抗体能够结合至与该曲妥珠单抗抗体识别的该表位不同的HER2表位;

并且其中该第二链包含[bVL]-[CL],其中bVL是能够结合至与该曲妥珠单抗抗体识别的该表位不同的HER2表位的抗体的该VL区,并且CL是选自下组的IgG轻链恒定区,该组由以下各项组成:人κ恒定区和人λ恒定区。

13.根据权利要求12所述的双特异性抗HER2抗体,其中bVL包含

(i)与SEQ ID NO:4相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:4相同的可变轻链CDR-1(VL-CDR1);

(ii)与SEQ ID NO:5相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:5相同

的可变轻链CDR-2(VL-CDR2);以及,

(iii)与SEQ ID NO:6相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:6相同的可变轻链CDR-3(VL-CDR3);

其中[TZs]包含

(i)包含SEQ ID NO:54的氨基酸的VH-CDR1;

(ii)包含SEQ ID NO:55的氨基酸的VH-CDR2;

(iii)包含SEQ ID NO:56的氨基酸的VH-CDR3;

(iv)包含SEQ ID NO:57的氨基酸的VL-CDR1;

(v)包含SEQ ID NO:58的氨基酸的VL-CDR2;以及

(vi)包含SEQ ID NO:59的氨基酸的VL-CDR3;

其中该[Fcx]包含

(i)引入可衍生基团的至少一个氨基酸取代;和/或,

(ii)能够增强该双特异性抗体的ADCC活性的至少一个突变;

并且其中[bVH]包含

(i)与SEQ ID NO:1相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:1相同的可变重链CDR-1(VH-CDR1);

(ii)与SEQ ID NO:2相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:2相同的可变重链CDR-2(VH-CDR2);以及

(iii)与SEQ ID NO:3相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:3相同的可变重链CDR-3(VH-CDR3)。

14.根据权利要求1至13中任一项所述的双特异性HER2抗体,其中该双特异性HER2抗体:

(a)在结合至该HER2靶标时诱导内化;

(b)在内化之后促进有效的溶酶体运输;

(c)诱导HER2靶标降解;

(d)阻断低HER2表达癌症细胞中的配体诱导的AKT磷酸化;

(e)破坏配体诱导的HER2:HER3二聚化;或

(f)其组合。

15.一种抗体-药物轭合物(ADC),包含根据权利要求1至14中任一项所述的双特异性HER2抗体以及至少一个、两个、三个、或四个治疗部分,并且任选地包含至少一个间隔物。

16.根据权利要求15所述的ADC,其中每个治疗部分被化学轭合至该双特异性抗体的该Fc区中的特定位置处的氨基酸侧链上。

17.根据权利要求16所述的ADC,其中这些特定位置选自下组,该组由以下各项组成:239、248、254、258、273、279、282、284、286、287、289、297、298、312、324、326、330、335、337、339、350、355、356、359、360、361、375、383、384、389、398、400、413、415、418、422、435、440、441、442、443、446、在位置239与240之间的插入、以及其任何组合,其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引。

18.根据权利要求17所述的ADC,其中这些特定位置是239、442或两者,其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引。

19. 一种ADC, 包含根据权利要求1至14中任一项所述的双特异性HER2抗体, 其中所述抗体包含:

(i) 包含SEQ ID NO:32A的氨基酸的第一多肽链和包含SEQ ID NO:42A的氨基酸的第二多肽链, 其中该第一多肽链包含共价连接至位置239处的半胱氨酸氨基酸上的治疗部分, 其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

(ii) 包含SEQ ID NO:33A的氨基酸的第一多肽链和包含SEQ ID NO:42A的氨基酸的第二多肽链, 其中该第一多肽链包含共价连接至分别位于位置239和442处的半胱氨酸氨基酸上的两个治疗部分, 其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

(iii) 包含SEQ ID NO:36A的氨基酸的第一多肽链和包含SEQ ID NO:42A的氨基酸的第二多肽链, 其中该第一多肽链包含共价连接至位置239处的半胱氨酸氨基酸上的治疗部分, 其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

(iv) 包含SEQ ID NO:37A的氨基酸的第一多肽链和包含SEQ ID NO:42A的氨基酸的第二多肽链, 其中该第一多肽链包含共价连接至分别位于位置239和442处的半胱氨酸氨基酸上的两个治疗部分, 其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

(v) 包含SEQ ID NO:40A的氨基酸的第一多肽链和包含SEQ ID NO:42A的氨基酸的第二多肽链, 其中该第一多肽链包含共价连接至位置239处的半胱氨酸氨基酸上的治疗部分, 其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引; 或,

(vi) 包含SEQ ID NO:41A的氨基酸的第一多肽链和包含SEQ ID NO:42A的氨基酸的第二多肽链, 其中该第一多肽链包含共价连接至分别位于位置239和442处的半胱氨酸氨基酸上的两个治疗部分, 其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引。

(vii) 包含SEQ ID NO:32A的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链, 其中该第一多肽链包含共价连接至位置239与240之间插入的半胱氨酸氨基酸上的治疗部分, 其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

(viii) 包含SEQ ID NO:33C的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链, 其中该第一多肽链包含共价连接至位置239与240之间插入的半胱氨酸氨基和位于位置442处的半胱氨酸氨基酸上的两个治疗部分, 其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

(ix) 包含SEQ ID NO:36C的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链, 其中该第一多肽链包含共价连接至位置239与240之间插入的半胱氨酸氨基酸上的治疗部分, 其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

(x) 包含SEQ ID NO:37C的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链, 其中该第一多肽链包含共价连接至位置239与240之间插入的半胱氨酸氨基和位于位置442处的半胱氨酸氨基酸上的两个治疗部分, 其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

(xi) 包含SEQ ID NO:40C的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链, 其中该第一多肽链包含共价连接至位置239与240之间插入的半胱氨酸氨基酸上的治疗部分, 其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

引;或,

(xii)包含SEQ ID NO:41C的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链,其中该第一多肽链包含共价连接至位置239与240之间插入的半胱氨酸氨基和位于位置442处的半胱氨酸氨基酸上的两个治疗部分,其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引。

20.根据权利要求15至19中任一项所述的ADC,其中该治疗部分包括细胞毒素、放射性同位素、免疫调节剂、细胞因子、淋巴因子、趋化因子、生长因子、肿瘤坏死因子、激素、激素拮抗剂、酶、寡核苷酸、DNA、RNA、siRNA、RNAi、微小RNA、光敏治疗剂、抗血管生成剂、促凋亡剂、肽、脂质、碳水化合物、螯合剂或其组合。

21.根据权利要求20所述的ADC,其中该细胞毒素是微管溶素、奥瑞他汀、美登木素生物碱或吡咯并苯并二氮杂萘(PBD)。

22.一种药物组合物,包含根据权利要求1至14中任一项所述的双特异性HER2抗体、或根据权利要求15至21中任一项所述的ADC、以及药学上可接受的载体。

23.一种治疗HER2表达癌症的方法,该方法包括向有需要的受试者给予根据权利要求1至14中任一项所述的双特异性HER2抗体、根据权利要求15至21中任一项所述的ADC、或根据权利要求22所述的药物组合物。

24.根据权利要求23所述的方法,其中该癌症是低HER2表达癌症。

25.一种治疗对HER2靶向治疗剂的抗性的方法,该方法包括向有需要的受试者给予根据权利要求1至14中任一项所述的双特异性HER2抗体、根据权利要求15至21中任一项所述的ADC、或根据权利要求22所述的药物组合物。

双特异性HER2抗体

[0001] 本申请要求2014年4月11日提交的美国临时专利申请号61/978,516和2015年1月23日提交的美国临时专利申请号62/107,050的权益,这些临时专利申请的披露内容通过引用以其全文结合在此。

背景

[0002] 本发明提供特异性结合至HER2的组合物以及用于使用此类组合物来治疗癌症的方法。

[0003] 受体酪氨酸激酶的HER家族是细胞生长、分化和存活的重要介导体。受体家族包括四个不同成员,这四个不同成员包括表皮生长因子受体(EGFR、ErbB1或HER1)、HER2(ErbB2或p185neu)、HER3(ErbB3)以及HER4(ErbB4或tyro2)。

[0004] HER2最初被鉴定为来自化学治疗的大鼠的成神经细胞瘤的转化基因的产物。在乳腺癌和卵巢癌中观察到人同源物扩增并且与较差预后相关。还在其他癌瘤中观察到HER2的过表达(由于基因扩增而频繁地但非一致地),这些癌瘤包括胃癌、子宫内膜癌、唾液腺癌、肺癌、肾癌、结肠癌、甲状腺癌、胰腺癌以及膀胱癌。HER2还可以在前列腺癌中过表达。

[0005] HER受体通常以不同组合存在于细胞中,并且异源二聚化被认为使对各种HER配体的细胞反应的多样性增加(厄普(Earp)等人,乳癌研究和治疗(Breast Cancer Research and Treatment)35:115-132(1995))。虽然EGF和TGF α 不结合HER2,但EGF刺激EGFR和HER2以形成异源二聚体,该异源二聚体活化EGFR并且引起HER2在该异源二聚体中的转磷酸化。二聚化和/或转磷酸化看起来似乎活化HER2酪氨酸激酶。参见厄普等人,同上。同样,当HER3与HER2共表达时,形成活性信号传导复合体并且针对HER2的抗体能够破坏这种复合体(希利科沃夫斯基(Sliwkowski)等人,生物化学杂志(J.Biol.Chem.),269:14661-14665(1994))。

[0006] 在本领域中描述了靶向HER2的众多抗体(参见,例如布雷克(Hudziak)等人,分子细胞生物学(Mol.Cell.Biol.)9:1165-1172(1989);美国专利号5,677,171;芬迪丽(Fendly)等人,癌症研究(Cancer Research)50:1550-1558(1990);可斯(Kotts)等人,体外(In vitro)26(3):59A(1990);厄普等人,生长调节(Growth Regulation)1:72-82(1991);谢泼德(Shepard)等人,临床免疫学杂志(J.Clin.Immunol.)11:117-127(1991);库玛(Kumar)等人,分子细胞生物学(Mol.Cell.Biol.)1:979-986(1991);路易斯(Lewis)等人,癌症免疫现状(Cancer Immunol.Immunother.)37:255-263(1993);彼得拉斯(Pietras)等人,致癌基因(Oncogene)9:1829-1838(1994);维提塔(Vitetta)等人,癌症研究54:5301-5309(1994);希利科沃夫斯基等人,生物化学杂志269(20):14661-14665(1994);斯科德(Scott)等人,生物化学杂志266:14300-5(1991);迪苏沙(D'souza)等人,美国科学院院刊(Proc.Natl.Acad.Sci.)91:7202-7206(1994);路易斯等人,癌症研究56:1457-1465(1996);以及沙佛(Schaefer)等人,致癌基因15:1385-1394(1997)。

[0007] 具有不同特性的其他HER2抗体描述于塔利亚布埃(Tagliabue)等人,国际癌症杂志(Int.J.Cancer)47:933-937(1991);麦肯齐盖尔(McKenzie)等人,致癌基因4:543-548(1989);梅尔(Maier)等人,癌症研究(Cancer Res.)51:5361-5369(1991);贝卡斯(Bacus)等人,分子癌变(Molecular Carcinogenesis)3:350-362(1990);斯坦克斯克(Stancovski)

等人,PNAS(USA)88:8691-8695(1991);贝卡斯等人,癌症研究52:2580-2589(1992);徐等人,国际癌症杂志53:401-408(1993);W094/00136;卡斯普尔泽克等人,癌症研究52:2771-2776(1992);汉考克(Hancock)等人,癌症研究51:4575-4580(1991);沙文(Shawver)等人,癌症研究54:1367-1373(1994);阿特阿加(Arteaga)等人,癌症研究54:3758-3765(1994);哈韦特(Harwerth)等人,生物化学杂志267:15160-15167(1992);美国专利号5,783,186;以及克拉帕(Klapper)等人,致癌基因14:2099-2109(1997)中。

[0008] 曲妥珠单抗(赫赛汀® (HERCEPTIN®);参见美国专利号5,821,337)(鼠HER2抗体4D5的重组人源化形式)在患有HER2过表达转移性乳癌的患者中是临床活性的,这些患者以往接受过广泛的抗癌疗法(巴塞尔加(Baselga)等人,临床肿瘤学杂志(J.Clin.Oncol.)14:737-744(1996))。为了靶向HER信号传导途径,帕妥珠单抗(Perjeta™;参见专利公开W02001/00245)被开发为抑制HER2与其他HER受体二聚化的鼠抗体2C4的人源化形式,从而抑制配体驱动的磷酸化和活化,以及RAS和AKT途径的下游活化。Ado-曲妥珠单抗emtansine(T-DM1; KADCYLA®)是批准用于对曲妥珠单抗有抗性的患有HER2过表达转移性乳癌的患者、连接至细胞毒性剂美登素上的曲妥珠单抗的抗体药物轭合物。

[0009] 虽然,充分证实了曲妥珠单抗在乳癌中的治疗功效,但它被严格限制并且仅批准用于30%的肿瘤过表达HER2的乳癌患者。70%的乳癌患者不能或不充分地响应于曲妥珠单抗,因为他们单独的肿瘤不能过表达或不能充分地表达HER2。在其他癌症和/或单独的癌症中,HER2以43%至69%范围内的很大比例情况下过表达,然而,通常,在大多数肿瘤中HER2表达水平是基本上低的。此外,HER2受体的过表达经常由编码基因扩增而引起(海因斯(Hynes)等人,自然评论:癌症(Nat Rev Cancer)5:341(2005))。因此,目前的共识是:抗HER2单克隆抗体疗法在具有低HER2表达或缺失过表达的肿瘤中是效率低下的。此外,对这些抗HER2抗体的抗性是重大问题。

[0010] 鉴于在表达低水平HER2和对当前疗法有抗性的特定癌症中缺乏有效的抗HER2疗法,存在对能够有效地结合至表达更高范围水平HER2的癌症细胞并且抑制这些癌症细胞生长的改进抗体的需要,这些能力通过例如以下各项来实现:(i)抗体依赖性细胞介导的细胞毒性(ADCC)和/或(ii)由于作为抗体药物轭合物(ADC)的轭合至抗体上的有效负载引起的细胞毒性作用和/或(iii)抑制介导信号传导的受体(例如通过抑制受体二聚化和/或介导受体内化)。

[0011] 因此,本披露的目的是提供有效抑制HER2介导的细胞信号传导的改进免疫治疗剂,这些改进免疫治疗剂可用于治疗HER2表达癌症,包括其中HER2不以高水平表达的癌症。

概述

[0012] 本披露提供包含免疫球蛋白重链(VH)和免疫球蛋白轻链(VL)的抗HER2结合分子,其中该VH包含SEQ ID NO:15的氨基酸序列。还提供包含VH和VL的抗HER2结合分子,其中该VL包含SEQ ID NO:16的氨基酸序列。在一些方面中,该VH包含SEQ ID NO:15的氨基酸序列并且该VL包含SEQ ID NO:16的氨基酸序列。在一些方面中,该抗HER2结合分子包括抗体或其抗原结合片段。

[0013] 本披露还提供包含第一免疫球蛋白抗原结合结构域和第二免疫球蛋白抗原结合结构域的双特异性抗HER2抗体,其中(i)该第一免疫球蛋白抗原结合结构域和该第二免疫球蛋白抗原结合结构域特异性结合至不同HER2抗体结合位点,(ii)该第一免疫球蛋白抗原

结合结构域结合至第一HER2抗体结合位点,该第一HER2抗体结合位点包含HER2的结构域II内的表位,并且(iii)该第一HER2抗体结合位点与帕妥珠单抗的抗体结合位点不同。在一些方面中,该第二免疫球蛋白抗原结合结构域结合至第二HER2抗体结合位点,该第二HER2抗体结合位点包含HER2的结构域IV内的表位。在一些方面中,该第二HER2抗体结合位点与曲妥珠单抗的HER2抗体结合位点相同。在一些方面中,该第二HER2抗体结合位点与曲妥珠单抗的HER2抗体结合位点部分重叠。在其他方面中,该第二HER2抗体结合位点与曲妥珠单抗的HER抗体结合位点不同。

[0014] 本披露还提供包含第一免疫球蛋白抗原结合结构域和第二免疫球蛋白抗原结合结构域的双特异性抗HER2抗体,其中该第一免疫球蛋白抗原结合结构域和该第二免疫球蛋白抗原结合结构域特异性结合至不同HER2表位;并且其中该第一免疫球蛋白抗原结合结构域在包含SEQ ID NO:52中的一个或多个氨基酸残基的表位上结合HER2。

[0015] 在一些方面中,该第一免疫球蛋白抗原结合结构域在包含SEQ ID NO:52中的一个或多个氨基酸残基的表位上结合HER2,并且该第二免疫球蛋白抗原结合结构域在结构域IV内的表位上特异性结合HER2。在一些方面中,该第二免疫球蛋白抗原结合结构域在包含SEQ ID NO:53中的一个或多个氨基酸残基的表位上结合HER2。

[0016] 在一些方面中,该第一免疫球蛋白抗原结合结构域包含重链可变区(VH)和轻链可变区(VL),该重链可变区和该轻链可变区包含:

(i)与SEQ ID NO:1相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:1相同的可变重链CDR-1(VH-CDR1)序列;

(ii)与SEQ ID NO:2相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:2相同的可变重链CDR-2(VH-CDR2)序列;

(iii)与SEQ ID NO:3相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:3相同的可变重链CDR-3(VH-CDR3)序列;

(iv)与SEQ ID NO:4相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:4相同的可变轻链CDR-1(VL-CDR1)序列;

(v)与SEQ ID NO:5相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:5相同的可变轻链CDR-2(VL-CDR2)序列;以及,

(vi)与SEQ ID NO:6相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:6相同的可变轻链CDR-3(VL-CDR3)序列。

[0017] 在某些方面中,相对于包含VH(包含SEQ ID NO:43的氨基酸序列)和VL(包含SEQ ID NO:44的氨基酸序列)的免疫球蛋白抗原结合结构域,该第一免疫球蛋白抗原结合结构域包含至少一个异源可变结构域构架区(FW)。

[0018] 在某些方面中,该第一免疫球蛋白抗原结合结构域包含scFv抗体片段。

[0019] 在某些方面中,该第二免疫球蛋白抗原结合结构域包含scFv抗体片段。

[0020] 在一些方面中,该双特异性抗HER2抗体的该至少一个异源FW区包含第一免疫球蛋白抗原结合结构域,该第一免疫球蛋白抗原结合结构域进一步包含(i)包含SEQ ID NO:11的可变轻链构架1(VL-FW1)氨基酸序列;(ii)包含SEQ ID NO:12的VH-FW2氨基酸序列;(iii)包含SEQ ID NO:13的VH-FW3氨基酸序列;(iv)包含SEQ ID NO:14的VH-FW4氨基酸序列;或(vi)其任何组合。

[0021] 在一些方面中,该双特异性抗HER2抗体包含第一免疫球蛋白抗原结合结构域和第二免疫球蛋白抗原结合结构域,其中该第一免疫球蛋白抗原结合结构域包含VH和VL,其中该VH氨基酸序列包含SEQ ID NO:15;其中该第一免疫球蛋白抗原结合结构域或该第二免疫球蛋白抗原结合结构域包含scFv抗体片段;并且其中该第一免疫球蛋白抗原结合结构域和该第二免疫球蛋白抗原结合结构域特异性结合至不同HER2表位。

[0022] 在一些方面中,该双特异性抗HER2抗体包含第一免疫球蛋白抗原结合结构域和第二免疫球蛋白抗原结合结构域,其中该第一免疫球蛋白抗原结合结构域包含VH和VL,其中该VL氨基酸序列包含SEQ ID NO:16;其中该第一免疫球蛋白抗原结合结构域或该第二免疫球蛋白抗原结合结构域包含scFv抗体片段;并且其中该第一免疫球蛋白抗原结合结构域和该第二免疫球蛋白抗原结合结构域特异性结合至不同HER2表位。

[0023] 在一些方面中,该双特异性抗HER2抗体包含VH和VL,其中该VH氨基酸序列包含SEQ ID NO:15;并且其中该VL氨基酸序列包含SEQ ID NO:16,其中该第一免疫球蛋白抗原结合结构域或该第二免疫球蛋白抗原结合结构域包含scFv抗体片段;并且其中该第一免疫球蛋白抗原结合结构域和该第二免疫球蛋白抗原结合结构域特异性结合至不同HER2表位。

[0024] 在一些方面中,在此披露的该双特异性抗HER2抗体包含第一免疫球蛋白抗原结合结构域,该第一免疫球蛋白抗原结合结构域包含以下各项或由其组成:(a)VH和VL,该VH进一步包括重链恒定区或其片段并且该VL包含轻链恒定区或其片段;(b)单链Fv(“scFv”);(c)双体抗体;(d)小抗体;(e) $F(ab')_2$;或(f) $F(ab)$ 。在相同的方面中,该重链恒定区或其片段是IgG恒定区。在一些方面中,该IgG恒定区或其片段是IgG1恒定区。在一些方面中,该LC恒定区是 κ 恒定区。

[0025] 在一些方面中,该LC恒定区是 λ 恒定区。在一些方面中,该第一免疫球蛋白抗原结合结构域是单克隆抗体。在一些方面中,该第一免疫球蛋白抗原结合结构域是人源化抗体。在一些方面中,该第一免疫球蛋白抗原结合结构域是人抗体。在一些方面中,该第一免疫球蛋白抗原结合结构域是嵌合抗体。在一些方面中,该第一免疫球蛋白抗原结合结构域是亲和力和优化的抗体。在一些方面中,该第一免疫球蛋白抗原结合结构域不与曲妥珠单抗或帕妥珠单抗竞争表位结合。在一些方面中,该第一免疫球蛋白抗原结合结构域和该第二免疫球蛋白抗原结合结构域特异性结合至不同的非重叠HER2表位。

[0026] 在一些方面中,该双特异性抗HER2抗体包含第一免疫球蛋白抗原结合结构域和第二免疫球蛋白抗原结合结构域,其中(a)该第二免疫球蛋白抗原结合结构域特异性结合至与曲妥珠单抗抗体相同的HER2的结构域IV中的表位;(b)该第二免疫球蛋白抗原结合结构域通过曲妥珠单抗抗体竞争性地抑制HER2结合;和/或(c)该第二免疫球蛋白抗原结合结构域包含至少一个、至少两个、至少三个、至少四个、至少五个、或至少六个互补决定区(CDR),这些互补决定区具有选自下组的氨基酸,该组由SEQ ID NO:54至59组成。

[0027] 在一些方面中,该第二免疫球蛋白抗原结合结构域包含scFv,该scFv包含:(i)包含SEQ ID NO:54的氨基酸的VH-CDR1;(ii)包含SEQ ID NO:55的氨基酸的VH-CDR2;(iii)包含SEQ ID NO:56的氨基酸的VH-CDR3;(iv)包含SEQ ID NO:57的氨基酸的VL-CDR1;(v)包含SEQ ID NO:58的氨基酸的VL-CDR2;以及(vi)包含SEQ ID NO:59的氨基酸的VL-CDR3。在一些方面中,该scFv是二硫化物稳定的scFv。在一些方面中,该scFv包含VH和VL,该VH包含SEQ ID NO:17的氨基酸并且该VL包含SEQ ID NO:18的氨基酸。

[0028] 在一些方面中,该scFv的该VH和该VL通过肽接头共价连接。在一些方面中,该肽接头包含SEQ ID NO:19的氨基酸序列。

[0029] 在一些方面中,该双特异性抗HER2抗体的该第二免疫球蛋白抗原结合结构域被共价连接至该第一免疫球蛋白抗原结合结构域的HC的羧基末端上。在一些方面中,该双特异性抗HER2抗体包含插入在该第二免疫球蛋白抗原结合结构域与该第一免疫球蛋白抗原结合结构域的HC的羧基末端之间的接头。在其他方面中,该双特异性抗HER2抗体的该第二免疫球蛋白抗原结合结构域被共价连接至该第一免疫球蛋白抗原结合结构域的HC的氨基末端上。在一些方面中,该双特异性抗HER2抗体包含插入在该第二免疫球蛋白抗原结合结构域与该第一免疫球蛋白抗原结合结构域的HC的氨基末端之间的接头。

[0030] 在一些方面中,该双特异性抗HER2抗体的该第二免疫球蛋白抗原结合结构域被共价嵌入在该第一免疫球蛋白抗原结合结构域的HC的多肽链中。在一些方面中,该第二免疫球蛋白抗原结合结构域被共价嵌入在该第一免疫球蛋白抗原结合结构域的HC的CH1区与CH2区之间。在一些方面中,该双特异性抗HER2抗体包含插入在该第一免疫球蛋白抗原结合结构域的HC的CH1区与该第二免疫球蛋白抗原结合结构域之间的接头,以及插入在该第二免疫球蛋白抗原结合结构域与该第一免疫球蛋白抗原结合结构域的HC的CH2区之间的第二接头。

[0031] 在一些方面中,该第一接头和该第二接头是相同的。在其他方面中,该第一接头和该第二接头是不同的。在一些方面中,这些接头中的一个或多个包括肽接头。在一些方面中,该肽接头包含至少一个、至少两个、至少三个、至少四个、至少五个、至少10个、至少20个、或至少30个氨基酸。在其他方面中,该肽接头包括具有化学式 $\text{Ser}_x[(\text{Gly})_y-\text{Ser}_4]_z$ 的肽,其中x是从0至1,y是从1至4,并且z是从1至10。在一些方面中,该肽接头包含SEQ ID NO:19、20、21或22。

[0032] 在一些方面中,该双特异性抗HER2抗体包含可包括Fc结构域的重链,该Fc结构域包含CH2和CH3区。在一些方面中,该Fc结构域是IgG1Fc结构域。在其他方面中,该IgG1 Fc结构域是天然IgG1 Fc结构域。在一些方面中,该天然IgG1 Fc结构域包含SEQ ID NO:23的氨基酸序列。

[0033] 在一些方面中,该Fc结构域是突变IgG1 Fc结构域。在其他方面中,该突变IgG1 Fc结构域包含能够降低该双特异性抗体的ADCC活性的至少一个突变。在一些方面中,能够降低该双特异性抗体的ADCC活性的至少一个突变是氨基酸取代。在一些方面中,至少一个氨基酸取代包括L234F、S239A、S239C、或其任何组合。

[0034] 在一些方面中,该突变IgG1 Fc结构域包含引入可衍生基团的至少一个氨基酸取代。在一些方面中,该可衍生基团是氢硫基基团。在一些方面中,该至少一个氨基酸取代包括S239C、248C、254C、273C、279C、282C、284C、286C、287C、289C、297C、298C、312C、324C、326C、330C、335C、337C、339C、350C、355C、356C、359C、360C、361C、375C、383C、384C、389C、398C、400C、413C、415C、418C、422C、440C、441C、S442C、443C以及446C、或其任何组合。在一些方面中,该突变Fc结构域包含SEQ ID NO:24或SEQ ID NO:25的氨基酸。

[0035] 还提供包含彼此相关联的第一多肽链和第二多肽链的双特异性抗HER2抗体,其中该第一多肽链包含选自以下各项的序列:

(1)[TZs]-[L1]-[BVH]-[BCH]-[FCx]

(2)_BVH]-[_BCH]-[Fc_x]-[L₂]-[TZ_s]

(3)_BVH]-[_BCH]-[L₃]-[TZ_s]-[L₄]-[Fc_x]

其中

TZ_s是结合与曲妥珠单抗相同的表位的scFv;

L₁、L₂、L₃以及L₄是肽接头;

Fc_x是Fc结构域;

_BVH和_BCH分别是抗体的VH区和CH1区,该抗体能够结合至与曲妥珠单抗抗体识别的表位不同的HER2表位。在某些方面中,该不同的表位包含SEQ ID NO:52中的一个或多个氨基酸残基。

[0036] 在一些方面中,铰链多肽连接[_BCH]和[Fc_x]。在特定方面中,该铰链多肽包含SEQ ID NO:26的氨基酸或替代地由其组成。

[0037] 在一些方面中,该双特异性抗HER2抗体的该第二链包含序列[_BVL]-[CL],其中_BVL是能够结合至与曲妥珠单抗抗体识别的表位不同的HER2表位的抗体的VL区,并且CL是IgG轻链恒定区。在一些方面中,CL选自下组,该组由以下各项组成:人κ恒定区和人λ恒定区。在一些方面中,该_BVL包含(i)与SEQ ID NO:4相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:4相同的可变轻链CDR-1(VL-CDR1);(ii)与SEQ ID NO:5相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:5相同的可变轻链CDR-2(VL-CDR2);以及(iii)与SEQ ID NO:6相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:6相同的可变轻链CDR-3(VL-CDR3)。在一些方面中,_BVL包含SEQ ID NO:16或SEQ ID NO:44的氨基酸。

[0038] 在其他方面中,CL是包含SEQ ID NO:27A的氨基酸序列的κ轻链。

[0039] 在其他方面中,CL是包含SEQ ID NO:27B的氨基酸序列的λ轻链。

[0040] 在一些方面中,[TZ_s]包含:(i)包含SEQ ID NO:54的氨基酸的VH-CDR1;(ii)包含SEQ ID NO:55的氨基酸的VH-CDR2;(iii)包含SEQ ID NO:56的氨基酸的VH-CDR3;(iv)包含SEQ ID NO:57的氨基酸的VL-CDR1;(v)包含SEQ ID NO:58的氨基酸的VL-CDR2;以及(vi)包含SEQ ID NO:59的氨基酸的VL-CDR3。在一些方面中,[TZ_s]是二硫化物稳定的scFv。在一些方面中,[TZ_s]包含通过肽接头共价连接的VH和VL,该VH包含SEQ ID NO:17的氨基酸序列并且该VL包含SEQ ID NO:18的氨基酸序列。在一些方面中,该肽接头包含SEQ ID NO:19的氨基酸序列。在一些方面中,[TZ_s]包含SEQ ID NO:28的氨基酸序列。

[0041] 在一些方面中,[Fc_x]的氨基酸序列选自下组,该组由以下各项组成:SEQ ID NO:23、24A、24B、24C、25A、25B以及25C。在一些方面中,[L₁]、[L₂]、[L₃]以及[L₄]的氨基酸独立地选自下组,该组由以下各项组成:SEQ ID NO:19、20、21以及22。在其他方面中,(i)[L₁]包含SEQ ID NO:20的氨基酸;(ii)[L₂]包含SEQ ID NO:20的氨基酸;(iii)[L₃]包含SEQ ID NO:21的氨基酸;并且(iv)[L₄]包含SEQ ID NO:22的氨基酸。

[0042] 在一些方面中,[_BVH]包含(i)与SEQ ID NO:1相同或除了高达1、2、3或1个氨基酸取代以外与SEQ ID NO:1相同的可变重链CDR-1(VH-CDR1);(ii)与SEQ ID NO:2相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:2相同的可变重链CDR-2(VH-CDR2);以及(iii)与SEQ ID NO:3相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:3相同的可变重链CDR-3(VH-CDR3)。在一些方面中,[_BVH]包含SEQ ID NO:15或SEQ ID NO:43。在其他方面中,[_BCH]包含SEQ ID NO:29的氨基酸序列。

[0043] 在一些方面中,[_BVL]包含(i)与SEQ ID NO:4相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:4相同的可变轻链CDR-1(VL-CDR1);(ii)与SEQ ID NO:5相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:5相同的可变轻链CDR-2(VL-CDR2);以及(iii)与SEQ ID NO:6相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:6相同的可变轻链CDR-3(VL-CDR3)。在其他方面中,[_BVL]包含SEQ ID NO:16的氨基酸。

[0044] 在一些方面中,该双特异性抗HER2抗体的该第一多肽链包含选自下组的氨基酸序列,该组由以下各项组成:SEQ ID NO:30、31A、31B、32A、32B、32C、33A、33B、33C、34、35A、35B、36A、36B、36C、37A、37B、37C、38、39A、39B、40A、40B、40C、41A、41B以及41C,并且该双特异性抗HER2抗体的该第二多肽链包含SEQ ID NO:42A或42B的氨基酸。

[0045] 在一些方面中,该双特异性抗HER2抗体的该第二免疫球蛋白抗原结合结构域在结合至HER2靶标时诱导内化。在一些方面中,该双特异性HER2抗体的该第二免疫球蛋白抗原结合结构域在内化之后促进有效的溶酶体运输。在一些方面中,该双特异性HER2抗体的该第二免疫球蛋白抗原结合结构域诱导HER2靶标降解。在一些方面中,该双特异性HER2抗体的该第二免疫球蛋白抗原结合结构域在低HER2表达的癌症细胞中阻断配体诱导的AKT磷酸化。在一些方面中,该双特异性HER2抗体的该第一免疫球蛋白抗原结合结构域破坏配体诱导的HER2:HER3二聚化。

[0046] 本披露还提供包含在此披露的抗HER2结合分子或在此披露的抗HER2双特异性抗体的ADC,该ADC进一步包含至少一个治疗部分。在一些方面中,该ADC进一步包含至少一个任意的间隔物。在一些方面中,该至少一个间隔物是肽间隔物。在一些方面中,该至少一个间隔物是非肽间隔物。

[0047] 用于ADC的常规耦联策略依赖于通过赖氨酸或半胱氨酸随机地将有效负载(例如,治疗部分)耦联至抗体上。因此,在一些方面中,该ADC被随机地耦联至治疗部分上。在具体方面中,在特定位置处使用反应性氨基酸残基将治疗部分位点特异性耦联至抗体上可得到具有均匀化学计量的均质ADC制剂。在一些方面中,该ADC包含两个、三个、或四个或更多个治疗部分。在一些方面中,所有治疗部分是相同的。在一些方面中,每个治疗部分被化学耦联至该双特异性抗体的Fc区中的特定卡巴特位置处的氨基酸侧链上。在一些方面中,这些特定卡巴特位置是239、442、或两者。在一些方面中,这些特定位置是卡巴特位置442、在卡巴特位置239与240之间的氨基酸插入、或两者。在一些方面中,该氨基酸侧链是氢硫基侧链。在一些方面中,该治疗部分包括细胞毒素、放射性同位素、奥瑞他汀、美登木素生物碱或吡咯并苯并二氮杂草(PBD)、或其组合。在某些方面中,该细胞毒素是微管溶素。在某些方面中,该微管溶素是化合物T32(在此又称为“微管溶素1508”或简称为“1508”)。

[0048] 本披露还提供包含双特异性抗HER2抗体的ADC,其中所述抗体包含:

(i)包含SEQ ID NO:32的氨基酸的第一多肽链和包含SEQ ID NO:42A的氨基酸的第二多肽链,其中该第一多肽链包含共价连接至卡巴特位置239处的半胱氨酸氨基酸上的微管溶素分子。

(ii)包含SEQ ID NO:33的氨基酸的第一多肽链和包含SEQ ID NO:42A的氨基酸的第二多肽链,其中该第一多肽链包含共价连接至分别位于卡巴特位置239和442处的半胱氨酸氨基酸上的两个微管溶素分子。

(iii)包含SEQ ID NO:40的氨基酸的第一多肽链和包含SEQ ID NO:42A的氨基酸的第

二多肽链,其中该第一多肽链包含共价连接至卡巴特位置239处的半胱氨酸氨基酸上的微管溶素分子。

(iv)包含SEQ ID NO:41的氨基酸的第一多肽链和包含SEQ ID NO:42A的氨基酸的第二多肽链,其中该第一多肽链包含共价连接至分别位于卡巴特位置239和442处的半胱氨酸氨基酸上的两个微管溶素分子。

[0049] 本披露还提供编码抗HER2结合分子(例如,在此披露的双特异性抗HER2抗体)、或其补体的分离的核酸分子或核酸分子组。还提供包含此类核酸分子或核酸分子组、或其补体的载体或载体组。还提供包含该分离的核酸分子或核酸分子组、或该载体或载体组的宿主细胞。还提供表达在此披露的抗HER2结合分子或双特异性抗HER2抗体的宿主细胞。还提供用于产生抗HER2结合分子或双特异性抗HER2抗体的方法,该方法包括培养该宿主细胞并且从培养基中回收该抗体。

[0050] 本披露还提供药物组合物,该药物组合物包含抗HER2结合分子或抗HER2双特异性抗体以及药学上可接受的载体。还提供治疗HER2表达的癌症的方法,该方法包括向有需要的受试者给予在此披露的抗HER2结合分子、在此披露的抗HER2双特异性抗体、在此披露的ADC、或在此披露的药物组合物。在一些方面中,该癌症是低HER2表达的癌症。在一些方面中,该方法进一步包括给予至少一种另外的治疗剂。在一些方面中,该至少一种另外的治疗剂是放射性核素或化疗剂。还提供将治疗剂或预防剂靶向表达HER2的细胞表面的方法,该方法包括将该试剂耦合至在此披露的抗HER2结合分子、或在此披露的抗HER2双特异性抗体上。还提供增加治疗部分的活性的方法,该方法包括将该试剂耦合至在此披露的抗HER2结合分子、或在此披露的抗HER2双特异性抗体上。还提供改进治疗剂或预防剂的药物代谢动力学特性的方法,该方法包括将该试剂耦合至在此披露的抗HER2结合分子、或在此披露的抗HER2双特异性抗体上。在一些方面中,该治疗部分是细胞毒素。在一些方面中,该细胞毒素是微管溶素。在一些方面中,该微管溶素是化合物T32,在此又称为“微管溶素1508”或简称为“1508”。还提供治疗对HER2靶向的治疗剂的抗性的方法,该方法包括向有需要的患者给予在此披露的双特异性HER2抗体、在此披露的抗HER2结合分子、或在此披露的ADC。在一些方面中,该患者对包括曲妥珠单抗和/或美登木素生物碱DM1的HER2靶向的治疗剂有抗性。

附图/图简要说明

[0051] 图1示出相应于前导优化的39S抗体(L0)和亲本AZ 1.39.1抗体(WT)的VL区和VH区的氨基酸序列的序列比对。指示了CDR1、CDR2以及CDR3的位置。强调了相对于亲本抗体不同的氨基酸残基。还强调了VL区中的替换的构架区。

[0052] 图2.示出将轻链构架改变为种系IGKV2D可引起加倍表达。

[0053] 图3.示出HER2的细胞外结构域的结构带状示意图。用箭头指示了39S、帕妥珠单抗以及结构域IV scFv的结合位点。39S的结合位点包括与帕妥珠单抗不同的结构域II内的氨基酸。

[0054] 图4示出使用DL-680标记的39S(2 μ g/ml)和不同浓度的未标记的单克隆抗体(R347对照、曲妥珠单抗、帕妥珠单抗、AZ 1.39.1以及39S)的FACS竞争测定。

[0055] 图5示出使用不同抗体组合和细胞系MCF-7的配体依赖性增殖测定,从而证实在与曲妥珠单抗和/或帕妥珠单抗组合给予时39S协同地抑制所测定的细胞系的生长。对于每个

实验,标绘的抗体样品是:R347对照、曲妥珠单抗、帕妥珠单抗加上曲妥珠单抗、39S、39S加上曲妥珠单抗、以及39S加上帕妥珠单抗。

[0056] 图6示出使用不同抗体组合和细胞系NCI-N87的配体依赖性增殖测定,从而证实与曲妥珠单抗和/或帕妥珠单抗组合给予时39S协同地抑制所测定的细胞系的生长。对于每个实验,标绘的抗体样品是:R347对照、曲妥珠单抗、帕妥珠单抗加上曲妥珠单抗、39S、39S加上曲妥珠单抗、以及39S加上帕妥珠单抗。

[0057] 图7描绘双特异性构建体,其中HER2结合部分(在结构域IV内结合的半胱氨酸稳定的scFv)已被基因融合至39S抗体结构内的不同位置上。scFv被融合至39S抗体重链中的每一个的羧基末端(称为“Bs3Ab-39SH”)上(图7B),融合至39S抗体重链中的每一个的氨基末端(称为“Bs2Ab-39SH”)上(图7A),或嵌入在39S抗体重链中的每一个的CH1区与CH2区之间(称为“Bs4Ab-39SH”)(图7C)。

[0058] 图8A示出Bs2抗体的重链序列,从而指示在野生型重链和可对于ADCC活性或对于产生具有降低ADCC活性和/或位点特异性结合的ADC而优化的重链中的卡巴特位置234、239、239-ins、330、332以及442处的氨基酸。

[0059] 图8B示出Bs3抗体的重链序列,从而指示在野生型重链和可对于ADCC活性或对于产生具有降低ADCC活性和/或位点特异性结合的ADC而优化的重链中的卡巴特位置234、239、239-ins、330、332以及442处的氨基酸。

[0060] 图8C示出Bs4抗体的重链序列,从而指示在野生型重链和可对于ADCC活性或对于产生具有降低ADCC活性和/或位点特异性结合的ADC而优化的重链中的卡巴特位置234、239、239-ins、330、332以及442处的氨基酸。

[0061] 图9A示出使用不同双特异性抗体构建体和MDA-MB-361细胞的配体依赖性增殖测定,从而证实Bs2Ab-39SH、Bs3Ab-39SH以及Bs4Ab-39SH在MDA-MB-361和MCF-7细胞中具有类似的效力(分别为图9A和图9B),这还可与亲本抗体组合(39S加上曲妥珠单抗)的活性比较。对于每个实验,标绘的抗体样品是:R347对照、Bs2Ab-39SH、Bs3Ab-39SH、Bs4Ab-39SH、以及39S加上曲妥珠单抗。

[0062] 图9B示出使用不同双特异性抗体构建体和MCF-7细胞的配体依赖性增殖测定,从而证实Bs2Ab-39SH、Bs3Ab-39SH以及Bs4Ab-39SH在MDA-MB-361和MCF-7细胞中具有类似的效力(分别为图9A和图9B),这还可与亲本抗体组合(39S加上曲妥珠单抗)的活性比较。对于每个实验,标绘的抗体样品是:R347对照、Bs2Ab-39SH、Bs3Ab-39SH、Bs4Ab-39SH、以及39S加上曲妥珠单抗。

[0063] 图10示出从T47D细胞中免疫沉淀(IP)HER2,接着免疫印迹法(WB)检测HER2和HER3以测量在存在或不存在抗HER2抗体情况下由调蛋白(HRG1)诱导的HER2:HER3二聚化的破坏。在对照条件(不具有抗体并且不具有HRG1;不具有抗体但具有HRG1;或具有对照抗体R347并且具有HRG1)下孵育T47D细胞,并且之后使用HRG1加上(i)曲妥珠单抗、(ii)帕妥珠单抗、或(iii)39S抗体、或(iv)Bs2Ab-39SH双特异性构建体孵育。

[0064] 图11示出代表性HPLC大小排阻色谱法分布图(profile),从而示出从1:1摩尔比的抗体:HER2中衍生的免疫复合体的大小分离。Bs2Ab-39SH可交联许多HER2分子以形成大小为1716kDa大的复合体,而曲妥珠单抗最大仅可结合至两个HER2分子以形成320kDa复合体。

[0065] 图12呈现基于FACS的受体内化测定,从而表明在图6中呈现的三种HER2双特异性

抗体形式在细胞系BT-474中快速内化。图上标绘的是：R347对照、曲妥珠单抗、39S、曲妥珠单抗加上39S、Bs2Ab-39SH、Bs3Ab-39SH、以及BsAb-39SH。在MCF-7、T47D、RT-112、MDA-MB-361、以及NCI-N87细胞系中看到类似的结果(数据未示出)。

[0066] 图13示出由BT-474细胞进行的Bs2Ab-39SH内化的动力学。在添加Bs2Ab-39SH构建体、曲妥珠单抗或R347对照之后的0分钟、30分钟、60分钟、120分钟、以及360分钟处取得显微图像。使用CELLTRACKER™绿(美国生命技术公司(Life Technologies Corp.))对BT-474细胞的细胞质进行染色,并且用ALEXAFLUOR®647(美国生命技术公司)标记抗体。

[0067] 图14是示出在使用抗体样品孵育BT-474细胞2小时、6小时、以及24小时之后HER2的降解程度或缺乏该HER2的蛋白质免疫印迹。GAPDH被用作对照。所测试的抗体样品是:(泳道1)R347对照抗体、(泳道2)曲妥珠单抗、(泳道3)帕妥珠单抗、(泳道4)曲妥珠单抗和帕妥珠单抗、(泳道5)39S、(泳道6)曲妥珠单抗和39S、(泳道7)帕妥珠单抗和39S、(泳道8)曲妥珠单抗、帕妥珠单抗以及39S、(泳道9)Bs2Ab-39SH、(泳道10)Bs2Ab-39SH_aFuc(均质无岩藻糖基化的Bs2Ab-39SH)、(泳道11)Bs3Ab-39SH、(泳道12)Bs3Ab-39SH_aFuc(均质无岩藻糖基化的Bs3Ab-39SH_aFuc)、(泳道13)Bs4Ab-39SH、以及(泳道14)Bs4Ab-39SH_aFuc(均质无岩藻糖基化的Bs4Ab-39SH)。

[0068] 图15是从Bs2Ab-39SH(图A)、Bs3Ab-39SH(图B)以及Bs4Ab-39SH(图C)抗HER2构建体衍生的示例性ADC的详细卡通示意图。由圆形指示ADC构建体的CH2和CH3结构域中的两个潜在工程化的细胞毒性剂耦联位点。在希望的药物与抗体比(DAR)是2比1的情况下,可使用或者位点1或者位点2。在希望DAR为4的情况下,使用位点1和位点2两者。可工程化替代的和/或另外的位点。对于具有大约2的DAR的ADC,双特异性抗HER2 ADC在此被缩写为“Bs2Ab-2T”、“Bs3Ab-2T”以及“Bs4Ab-2T”(或简称为“Bs2-2T”、“Bs3-2T”以及“Bs4-2T”),并且对于具有大约4的DAR的ADC,被缩写为“Bs2Ab-4T”、“Bs3Ab-4T”以及“Bs4Ab-4T”(或简称为“Bs2-4T”、“Bs3-4T”以及“Bs4-4T”)。通过在图7中提供的名称引用缺乏任何细胞毒性剂的双特异性构建体并且可被鉴定为“未待用的”。如在此所提供(参见,例如图8),可选择工程化的耦联位点来降低或去除ADCC功能。替代地或另外地,抗体的Fc部分可进一步包含降低或去除ADCC活性的另外的突变。替代地,可使用经典耦联方法来产生ADC,诸如通过抗体的常有多种赖氨酸残基或天然半胱氨酸残基耦联至抗体上,从而产生异质抗体-药物耦合物混合物。

[0069] 图16A呈现使用1μg/ml的在每个图像下指示的抗体处理的SKOV-3细胞的免疫荧光图像,从而示出细胞内微管网络的破坏或缺乏该细胞内微管网络。示出了微管染色。测试了以下样品:不具有任何抗体的培养基对照(图像i)、R347对照抗体(图像ii)、曲妥珠单抗(图像iii)、Bs2Ab-39SH(图像iv)、R347-DM1(耦合至细胞毒性剂美登木素生物碱DM1上的R347对照抗体)(图像v)、R347-4T(耦合至4个细胞毒性剂微管溶素分子上的R347对照抗体)(图像vi)、T-DM1(耦合至美登木素生物碱DM1上的曲妥珠单抗)(图像vii)、以及Bs2-4T(耦合至4个细胞毒性剂微管溶素分子上的Bs2Ab-39SH)(图像viii)。

[0070] 图16B呈现使用1μg/ml的在每个图像下指示的抗体处理的JIMT-1细胞的免疫荧光图像,从而示出细胞内微管网络的破坏或缺乏该细胞内微管网络。示出了微管染色。测试了以下样品:不具有任何抗体的培养基对照(图像i)、R347对照抗体(图像ii)、曲妥珠单抗(图像iii)、Bs2Ab-39SH(图像iv)、R347-DM1(耦合至细胞毒性剂美登木素生物碱DM1上的R347对照抗体)(图像v)、R347-4T(耦合至4个细胞毒性剂微管溶素分子上的R347对照抗体)(图

像vi)、T-DM1(轭合至美登木素生物碱DM1上的曲妥珠单抗)(图像vii)、以及Bs2-4T(轭合至4个细胞毒性剂微管溶素分子上的Bs2Ab-39SH)(图像viii)。

[0071] 图16C呈现使用1 μ g/ml的在每个图像下指示的抗体处理的RT-112细胞的免疫荧光图像,从而示出细胞内微管网络的破坏或缺乏该细胞内微管网络。示出了微管染色。测试了以下样品:不具有任何抗体的培养基对照(图像i)、R347对照抗体(图像ii)、曲妥珠单抗(图像iii)、Bs2Ab-39SH(图像iv)、R347-DM1(轭合至细胞毒性剂美登木素生物碱DM1上的R347对照抗体)(图像v)、R347-4T(轭合至4个细胞毒性剂微管溶素分子上的R347对照抗体)(图像vi)、T-DM1(轭合至美登木素生物碱DM1上的曲妥珠单抗)(图像vii)、以及Bs2-4T(轭合至4个细胞毒性剂微管溶素分子上的Bs2Ab-39SH)(图像viii)。

[0072] 图17A示出相对于SKBR-3人乳癌细胞系上的T-DM1、未轭合(未待用的)Bs2Ab-39SH以及曲妥珠单抗具有2或4的DAR的Bs2Ab形式的细胞毒性活性。还示出对于R347(R347对照抗体)、与2或4个微管溶素轭合的R347(分别为R347-2T和R3474T)、轭合至DM1上的R347的曲线。Bs2-2T和Bs2-4T两者比T-DM1更有效。

[0073] 图17B示出相对于SKBR-3人乳癌细胞系上的T-DM1、未轭合(未待用的)Bs4Ab-39SH以及曲妥珠单抗具有2或4的DAR的Bs4Ab形式的细胞毒性活性(图B)。还示出对于R347(R347对照抗体)、与2或4个微管溶素轭合的R347(分别为R347-2T和R3474T)、轭合至DM1上的R347的曲线。Bs4-2T和Bs4-4T两者比T-DM1更有效。

[0074] 图18A示出相对于JIMT-1人乳癌细胞系上的T-DM1、未轭合(未待用的)Bs2Ab-39SH以及曲妥珠单抗具有2或4的DAR的Bs2Ab形式的细胞毒性活性。还示出对于R347(R347对照抗体)、与2或4个微管溶素轭合的R347(分别为R347-2T和R3474T)、轭合至DM1上的R347的曲线。Bs2-2T和Bs2-4T两者在杀伤JIMT-1细胞上是非常有效的,而T-DM1示出无活性。

[0075] 图18B示出相对于JIMT-1人乳癌细胞系上的T-DM1、未轭合(未待用的)Bs4Ab-39SH以及曲妥珠单抗具有2或4的DAR的Bs4Ab形式的细胞毒性活性(图B)。还示出对于R347(R347对照抗体)、与2或4个微管溶素轭合的R347(分别为R347-2T和R3474T)、轭合至DM1上的R347的曲线。Bs4-2T和Bs4-4T两者在杀伤JIMT-1细胞上是非常有效的,而T-DM1示出无活性。

[0076] 图19A示出相对于ZR-75-1人乳癌细胞系上的T-DM1、未轭合(未待用的)Bs2Ab-39SH以及曲妥珠单抗具有2或4的DAR的Bs2Ab形式的细胞毒性活性。还示出对于R347(R347对照抗体)、与2或4个微管溶素轭合的R347(分别为R347-2T和R3474T)、轭合至DM1上的R347的曲线。Bs2-4T在杀伤ZR-75-1细胞上是最有活性的,而Bs2-2T具有较低水平的活性并且T-DM1示出无细胞毒性活性或限制的细胞毒性活性。

[0077] 图19B示出相对于ZR-75-1人乳癌细胞系上的T-DM1、未轭合(未待用的)Bs4Ab-39SH以及曲妥珠单抗具有2或4的DAR的Bs4Ab形式的细胞毒性活性(图B)。还示出对于R347(R347对照抗体)、与2或4个微管溶素轭合的R347(分别为R347-2T和R3474T)、轭合至DM1上的R347的曲线。Bs4-4T在杀伤ZR-75-1细胞上是最有活性的,而Bs4-2T具有较低水平的活性并且T-DM1示出无细胞毒性活性或限制的细胞毒性活性。

[0078] 图20示出相对于MDA-MB-468人乳癌细胞系上的T-DM1、未轭合(未待用的)Bs2Ab-39SH以及曲妥珠单抗具有2或4的DAR的Bs2Ab形式的细胞毒性活性(图A)。还示出对于R347(R347对照抗体)、与2或4个微管溶素轭合的R347(分别为R347-2T和R3474T)、轭合至DM1上的R347的曲线。数据指示Bs2-2T或Bs2-4T在MDA-MB-468细胞中都不是有效的,从而指示

Bs2-2T和Bs2-4T的细胞毒性活性是靶标(HER2)依赖的。对于Bs4-2T和Bs4-4T观察到类似的结果(数据未示出)。

[0079] 图21A示出抗HER2 ADC在MDA-MB-361肿瘤模型中的活性。示出了相应于使用以下各项处理的小鼠的肿瘤生长曲线:(1)媒介物对照、(2)在3mg/kg下的R347-4T(辄合至4个微管溶素分子上的R347对照抗体)、(3)在3mg/kg下的T-DM1(曲妥珠单抗-DM1 ADC)、(4)在3mg/kg下的Bs2 ADMix(与微管溶素混合的Bs2Ab-39SH-(FCC)构建体)、以及(5)在0.3、1以及3mg/kg下的Bs2-4T(辄合至4个微管溶素上的Bs2Ab-39SH-(FCC)构建体)。在括号之间指示了浓度。

[0080] 图21B示出抗HER2 ADC在MDA-MB-361肿瘤模型中的活性。示出了相应于使用以下各项处理的小鼠的肿瘤生长曲线:(1)媒介物对照、(2)在3mg/kg下的R347-4T(辄合至4个微管溶素分子上的R347对照抗体)、(3)在3mg/kg下的T-DM1(曲妥珠单抗-DM1 ADC)、(4)在3mg/kg下的Bs4ADMix(与微管溶素混合的Bs4Ab-39SH-(FCC)构建体)、以及(5)在0.3、1以及3mg/kg下的Bs4-4T(辄合至4个微管溶素上的Bs4Ab-39SH-(FCC)构建体)。在括号之间指示了浓度。

[0081] 图22示出抗HER2 ADC在ST996三阴性(ER-/PR-/HER2-1+)PDX肿瘤模型中的活性。示出了相应于使用以下各项处理的小鼠的肿瘤生长曲线:(1)媒介物对照(CTRL)、(2)同种型R347-2T CTRL(辄合至2个微管溶素分子上的R347对照抗体)、(3)同种型R347-4T CTRL(辄合至4个微管溶素分子上的R347对照抗体)、(4)T-DM1(曲妥珠单抗-DM1ADC)、(5)ADMix(与微管溶素混合的构建体)、(6)Bs2-2T,即辄合至2个微管溶素上的Bs2Ab-39SH-(FCC)构建体、或(7)Bs2-4T,即辄合至4个微管溶素上的Bs2Ab-39SH-(FCC)构建体。在括号之间指示了浓度。还通过箭头指示了给药时间。响应于不同处理的肿瘤生长曲线呈现为平均肿瘤体积(mm^3) $\pm$ SEM。

[0082] 图23示出抗HER2 ADC在ST225(HER2-3+)BrCa PDX肿瘤模型中的活性。示出了相应于使用以下各项处理的小鼠的肿瘤生长曲线:(1)媒介物对照、(2)R347-4T(辄合至4个微管溶素分子上的R347对照抗体)、(3)T-DM1(曲妥珠单抗-DM1 ADC)、(4)BS2 ADMix(与微管溶素混合的Bs2Ab-39SH-(FCC)构建体)、以及(5)Bs2-4T(辄合至4个微管溶素上的Bs2Ab-39SH-(FCC)构建体)。在括号之间指示了浓度。

[0083] 图24示出抗HER2 ADC在T-DM-1未响应者JIMT-1(HER2-3+)BrCa CBX肿瘤模型中的活性。示出了相应于使用以下各项处理的小鼠的肿瘤生长曲线:(1)媒介物对照、(2)R347-4T(辄合至4个微管溶素分子上的R347对照抗体)、(3)T-DM1(曲妥珠单抗-DM1 ADC)、(4)BS2 ADMix(与微管溶素混合的Bs2Ab-39SH-(FCC)构建体)、以及(5)Bs2-4T(辄合至4个微管溶素上的Bs2Ab-39SH-(FCC)构建体)。在括号之间指示了浓度。

[0084] 图25示出抗HER2 ADC在ST455B三阴性(ER-/PR-/HER2-1+)BrCa PDX肿瘤模型中的活性。示出了相应于使用以下各项处理的小鼠的肿瘤生长曲线:(1)媒介物对照(CTRL)、(2)同种型CTRL(辄合至4个微管溶素分子上的R347对照抗体)、(3)T-DM1(曲妥珠单抗-DM1 ADC)、(4)BS2 ADMix(与微管溶素混合的Bs2Ab-39SH-(FCC)构建体)、以及(5)Bs2-4T(辄合至4个微管溶素上的Bs2Ab-39SH-(FCC)构建体)。在括号之间指示了浓度。

[0085] 图26A示出Bs2-4T和T-DM1对亲本NCI-N87细胞系(左图)和具有对T-DM1的获得性抗性的NCI-N87细胞系(右图)的细胞毒性活性。Bs2-4T在亲本细胞系中具有更有效的活性

并且在杀伤T-DM1抗性细胞上仍有活性。

[0086] 图26B示出抗HER2 ADC在T-DM1抗性NCI-N87肿瘤模型中的活性。示出了相应于未处理的小鼠和使用以下各项处理的小鼠的肿瘤生长曲线：(1)ADMix(与微管溶素混合的构建体)、(2)T-DM1(曲妥珠单抗-DM1ADC)、(3)T-DM1(曲妥珠单抗-DM1 ADC)加上帕妥珠单抗、或(4)Bs2-4T,即融合至4个微管溶素上的Bs2Ab-39SH-(FCC)构建体。在括号之间指示了浓度。响应于不同处理的肿瘤生长曲线呈现为平均肿瘤体积(mm^3) $\pm$ SEM($n=7$)。与未处理的对照组比较,由学生t检验得到 $*P<0.001$ 。

[0087] 图27示出在使用曲妥珠单抗预处理之后的抗HER2 ADC的活性。示出了相应于使用以下各项处理的小鼠的肿瘤生长曲线：(1)媒介物CTRL(对照)、(2)曲妥珠单抗接着是Bs2-4T、(3)媒介物接着是Bs2-4T、(4)曲妥珠单抗接着是Bs2-4T、或(5)媒介物接着是Bs2-4T。在括号之间指示了浓度和剂量方案。响应于不同处理的肿瘤生长曲线呈现为平均肿瘤体积(mm^3) $\pm$ SEM($n=10$)。

[0088] 图28A示出评价双特异性ADC的旁观者效应的测定的示意图。

[0089] 图28B示出使用以下各项处理的细胞的FACS分析结果：(i)单独培养基；(ii)R347-T4对照；(iii)T-DM1；(iv)Bs2Ab-39SH混合物；以及(v)Bs2-4T。在两个象限中的细胞数减少指示：Bs2-4T可杀伤共培养物中的HER2表达细胞和无HER2细胞两者,从而表明Bs2-4T具有旁观者效应。相反,T-DM1不能杀伤共培养物中的无HER2细胞,从而表明它不具有旁观者效应。

[0090] 图29示出相对于T-DM1和R347-4T(融合至4个微管溶素分子上的R347对照抗体),Bs2-4T对癌症干细胞(CSC)球体形成的活性(左图)；并且相对于R34-4T(融合至4个微管溶素分子上的R234对照抗体),对异种移植肿瘤中的CSC的活性(右图)。

[0091] 图30A示出使用细胞系MDA-MB-361(从脑转移衍生的人导管乳腺上皮癌)的配体依赖性增殖测定,这些测定示出所有ADCC增强的无岩藻糖基化的双特异性抗体均保留体外抗增殖性活性。在每个实验中,所使用的抗体样品是：R347对照、39S加上曲妥珠单抗、Bs2Ab-39SH_aFuc、Bs3Ab-39SH_aFuc、以及Bs4Ab-39SH_aFuc。

[0092] 图30B示出使用细胞系MCF-7(人侵入性导管乳腺癌)的配体依赖性增殖测定,这些测定示出所有ADCC增强的无岩藻糖基化的双特异性抗体均保留体外抗增殖性活性。在每个实验中,所使用的抗体样品是：R347对照、39S加上曲妥珠单抗、Bs2Ab-39SH_aFuc、Bs3Ab-39SH_aFuc、以及Bs4Ab-39SH_aFuc。

[0093] 图31A示出使用作为靶标的MDA-MB-361细胞和工程化的NK效应子细胞(稳定表达CD16)的ADCC细胞毒性测定,这些测定示出与具有岩藻糖基化的糖形的相同构建体相比,每种无岩藻糖基化的双特异性抗体具有更有效的ADCC活性。所使用的抗体样品是：(1)R347对照、(2)曲妥珠单抗、(3)曲妥珠单抗_aFuc、(4)Bs2Ab-39SH、以及(5)Bs2Ab-39SH_aFuc。

[0094] 图31B示出使用作为靶标的MDA-MB-361细胞和工程化的NK效应子细胞(稳定表达CD16)的ADCC细胞毒性测定,这些测定示出与具有岩藻糖基化的糖形的相同构建体相比,每种无岩藻糖基化的双特异性抗体具有更有效的ADCC活性。所使用的抗体样品是：(1)R347对照、(2)曲妥珠单抗、(3)曲妥珠单抗_aFuc、(4)Bs3Ab-39SH、以及(5)Bs3Ab-39SH_aFuc。

[0095] 图31C示出使用作为靶标的MDA-MB-361细胞和工程化的NK效应子细胞(稳定表达CD16)的ADCC细胞毒性测定,这些测定示出与具有岩藻糖基化的糖形的相同构建体相比,每

种无岩藻糖基化的双特异性抗体具有更有效的ADCC活性。所使用的抗体样品是：(1)R347对照、(2)曲妥珠单抗、(3)曲妥珠单抗_{aFuc}、(4)Bs4Ab-39SH、以及(5)Bs4Ab-39SH_{aFuc}。

[0096] 图32示出微管溶素1508有效负载的结构。在最右边处的马来酰亚胺基团的双键易于与半胱氨酸上存在的巯基基团反应以形成稳定的碳-硫键。

[0097] 图33示出使用Bs2Ab-FCC构建体作为可衍生平台的示例性位点特异性抗体药物轭合方法。该方法包括以下步骤：(a)打开可衍生氨基酸(例如，半胱氨酸)的侧链，(b)氧化，(c)轭合有效负载(例如，细胞毒性剂诸如微管溶素)并且(d)通过去除轭合试剂和未反应的有效负载来抛光。

发明详细说明

[0098] 本披露提供优化的抗HER2抗体、以及从此类优化的抗HER2抗体中衍生的双特异性抗体。还提供相关的多核苷酸、载体、细胞、以及包含此类优化的抗HER2抗体或双特异性抗HER2抗体的药物组合物。还提供制备此类优化的抗HER2抗体或双特异性抗体的方法。另外，本披露提供使用这些优化的抗HER2抗体或双特异性抗HER2抗体的方法，例如治疗有需要的受试者的癌症的方法。

[0099] 本披露还提供从优化的抗HER2抗体和从此类优化的抗HER2抗体中衍生的双特异性抗体中衍生的抗体-药物轭合物(ADC)。还提供制备从此类优化的抗体和双特异性抗体中衍生的ADC的方法。还提供使用从优化的抗HER2抗体和双特异性抗体中衍生的ADC的方法，例如治疗有需要的受试者的癌症的方法。还提供治疗患有对化学疗法有抗性的癌症(例如，T-DM1未响应者或较差响应者患者中的肿瘤)的患者；治疗使用其他疗法(具体地单特异性ADC疗法(例如T-DM1))治疗会复发、难治疗、不适合的患者；或在使用其他疗法(例如T-DM1)预先治疗之后治疗患者的方法。

[0100] 具体地说，本披露提供适用于治疗表达低水平HER2的癌症的抗HER2结合分子。

[0101] 为了使本发明可更容易理解，首先定义某些术语。另外的定义贯穿详细说明而阐述。

I. 定义

[0102] 在详细描述本发明之前，应当了解的是，本发明并不限于特定的组合物或方法步骤，因为这些组合物或方法步骤可以改变。如在本说明书和随附权利要求书中所使用，除非上下文另外明确说明，否则单数形式“一个/种”和“该”包括复数参考对象。术语“一个”(或“一种”)，以及术语“一个或多个/一种或多种”和“至少一个”在此可以互换地使用。

[0103] 此外，在此使用“和/或”应当理解为在有或没有另一者的情况下，两个指定的特征或组分中的每一者的特定披露。因此，如在此在措词如“A和/或B”中所使用的术语“和/或”旨在包括“A和B”、“A或B”、“A”(单独)、以及“B”(单独)。同样，如在词组如“A、B和/或C”中使用的术语“和/或”旨在包括以下方面：A、B以及C；A、B或C；A或C；A或B；B或C；A和C；A和B；B和C；A(单独)；B(单独)；以及C(单独)。

[0104] 除非另外定义，在此所使用的所有技术和科学术语具有与本披露涉及的领域所属的技术人员通常所理解的相同的意义。例如，简明生物医学与分子生物学词典(the Concise Dictionary of Biomedicine and Molecular Biology)，卓培秀(Juo, Pei-Show)，第2版，2002，CRC出版社；细胞与分子生物学词典(The Dictionary of Cell and Molecular Biology)，第3版，1999，美国学术出版社(Academic Press)；以及牛津生物化学

与分子生物学辞典(the Oxford Dictionary Of Biochemistry And Molecular Biology),修订版,2000,牛津大学出版社(Oxford University Press)为技术人员提供了用于本披露中的许多术语的通用词典。

[0105] 单位、前缀以及符号均以它们的国际单位系统(SI)接受形式表示。数值范围包括定义该范围的数字。除非另外指明,否则氨基酸序列以氨基到羧基的方向从左到右书写。在此提供的小标题不是不同方面的限制,可以通过作为一个整体参考本说明书来获得这些方面。因此,通过以其全文参考说明书,更完全地定义了就在以下定义的术语。

[0106] 应当理解,当用语言“包含”来说明方面时,还提供了关于“由……组成”和/或“主要由……组成”描述的其他类似方面。

[0107] 氨基酸在此通过它们的通常已知的三字母符号或通过由IUPAC-IUB生物化学命名委员会(IUPAC-IUB Biochemical Nomenclature Commission)推荐的单字母符号来提及。同样地,核苷酸通过它们的普遍公认的单字母代码来提及。

[0108] 术语“HER2”和“HER2受体”在此可互换使用,并且是指ErbB2蛋白(在文献中又称为HER2/neu)。如在此所使用,这些术语旨在包括HER2的变体(例如,剪接变体)、同工型、以及同源物(直向同源物和横向同源物两者)。在一些方面中,在此披露的抗HER2结合分子与HER2的结合抑制了表达HER2的细胞(即典型地肿瘤细胞,并且具体地表达低水平HER2的癌症细胞)的生长,该抑制通过抑制HER2与其他ErbB家族成员之间的异聚复合体形成,例如抑制与EGFR或HER3异源二聚化来实现。

[0109] HER2是受体酪氨酸激酶并且包含细胞外结构域(ECD),该HER2由以下各项组成:(i)两个负责配体结合的富含亮氨酸的结构域(结构域I/L1和结构域III/L2)、以及(ii)两个负责受体二聚化的富含半胱氨酸的结构域(结构域II/CR1和结构域IV/CR2);跨膜结构域;以及细胞内酪氨酸激酶结构域。存在替代的HER2的剪接变体。替代的HER2的剪接变体的实例包括p100和赫斯达汀(两种可溶性形式)、以及611-CTF、687-CTF、648-CTF、以及 Δ 16HER2。HER2的可溶性形式可与全长受体(p185)相互作用并且抑制受体二聚化;687-CTF是无活性的;611-CTF和648-CTF可活化若干细胞内信号转导途径;并且 Δ 16HER2缺乏结构域IV中的氨基酸634-649,这诱导促进组成型活化的HER2同源二聚体形成的构象变化。不具有信号序列的成熟HER2的细胞外部分相应于标准同工型1的位置23-652(参见Uniprot P04626;还参见“单独HER2和与赫赛汀Fab复合的HER2的细胞外区的结构(Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab.)”Cho等人,自然(Nature)421:756-760(2003),通过引用以其全文结合在此)。帕妥珠单抗结合至HER2的结构域II内的表位。不结合至结构域II内的帕妥珠单抗结合表位的抗体包括曲妥珠单抗,该曲妥珠单抗结合至结构域IV内的表位。

[0110] 术语“抑制”、“阻断”以及“压制”在此可互换使用并且是指任何统计学显著的生物活性的降低,包括活性的完全阻断。例如,“抑制”可以指生物活性约10%、20%、30%、40%、50%、60%、70%、80%、90%或100%的降低。因此,当术语“抑制”或“压制”被用于描述例如对配体介导的HER2磷酸化的作用时,术语是指相对于未处理(对照)细胞中的磷酸化,抗HER2抗体或包含其抗原结合片段的HER2结合分子统计学显著地降低由EGF样配体诱导的HER2磷酸化的能力。

[0111] 表达HER2的细胞可以是天然存在的细胞或细胞系(例如,癌症细胞)或可以通过将

编码HER2的核酸引入到宿主细胞中来重组产生。在一个方面中,该抗HER2结合分子(例如,抗HER2抗体或包含其抗原结合片段的HER2结合分子)将配体介导的HER2磷酸化抑制至少10%、或至少20%、或至少30%、或至少40%、或至少50%、或至少60%、或至少70%、或至少80%、或至少90%、或约100%,如例如通过蛋白质印迹法接着是使用抗磷酸酪氨酸抗体探测或通过ELISA来确定。

[0112] 如在此所使用的术语表达HER2的细胞的“生长压制”或“生长抑制”是指相对于不存在抗HER2结合分子情况下的增殖,抗HER2结合分子(例如,抗HER2抗体或包含其抗原结合片段的HER2结合分子)统计学显著地降低表达HER2的细胞的增殖的能力。在一个方面中,当细胞与抗HER2结合分子(例如,抗HER2抗体或包含其抗原结合片段的HER2结合分子)接触时,相对于不存在该抗HER2结合分子情况下(对照条件)测量的增殖,表达HER2的细胞(例如,癌症细胞)的增殖可被降低至少10%、或至少20%、或至少30%、或至少40%、或至少50%、或至少60%、或至少70%、或至少80%、或至少90%、或约100%。细胞增殖可根据本领域中已知的不同方法来测量,例如通过计数活细胞数,鉴定生长标记物的标记物存在,测量分子(例如,放射性标记的分子,诸如³H-胸苷)的掺入,通过测量肿瘤的大小(例如,按体积计或按重量计)等。在某些方面中,生长压制是指转移的数量、大小或分布减小。

[0113] 如贯穿本说明书所使用,短语“抗HER2结合分子”是指例如(i)结合与本申请中披露的1.39.1抗体相同的表位或从该1.39.1抗体中衍生的抗体及其抗原结合片段(参见PCT公开号W0 2008/019290),例如39S抗体及其抗原结合片段,并且通常是包含此类抗体及其抗原结合片段的分子;(ii)结合与掺入另外的抗原结合部分的39S抗体相同的表位或从该39S抗体中衍生的抗HER2抗体和其他HER2结合分子,例如双特异性抗体;(iii)包含轭合至细胞毒性部分(例如,小分子抗癌剂、放射性核素等)上的根据(i)或(ii)的分子中的至少一种的抗体-药物轭合物(ADC);以及(iv)具有增强的ADCC的根据(i)或(ii)的抗HER2分子。如在此所使用,术语“39S抗体”是指从PCT公开号W0 2008/019290中披露的1.39.1抗体中衍生的前导优化的单克隆抗体,其中所述优化的抗体包含VH和VL,该VH包含SEQ ID NO:15的氨基酸并且该VL包含SEQ ID NO:16的氨基酸。

[0114] 如在此所使用,术语“微管溶素”是总体地和单独地指天然存在的微管溶素、以及微管溶素的类似物和衍生物。微管溶素的说明性实例披露于例如W02004005326A2、W02012019123A1、W02009134279A1、W02009055562A1、W02004005327A1、US7754885、US20100240701、US7816377、US20110021568以及US20110263650中并且披露于在此提供的实例中。应当理解,此类衍生物包括例如微管溶素前药或包括一种或多种保护或保护基团、一个或多个连接部分的微管溶素。

[0115] 可使用本领域公认的技术来测定细胞增殖,这些技术测量了细胞分裂速率、和/或细胞群内经历细胞分裂的细胞分数、和/或由于终末分化或细胞死亡(例如,胸苷掺入)引起的细胞从细胞群中的损失率。

[0116] 如在此互换使用的术语“抗体”或“免疫球蛋白”包括全抗体和其任何抗原结合片段或其单链以及其组合(例如,双特异性抗体)。

[0117] 典型的抗体包含由二硫键相互连接的至少两个重(H)链和两个轻(L)链。每个重链包含重链可变区(在此缩写为VH)和重链恒定区(在此缩写为CH)。该重链恒定区包含三个结构域(CH1、CH2以及CH3)。每个轻链包含轻链可变区(在此缩写为VL)和轻链恒定区。该轻链

恒定区包含一个结构域CL。这些VH区和VL区可被进一步细分为称为互补决定区(CDR)的高变区,这些高变区与称为构架区(FW)的更保守的区相互间置。每个VH和VL包含以下列顺序从氨基末端至羧基末端安排的三个CDR和四个FW:FW1、CDR1、FW2、CDR2、FW3、CDR3、FW4。重链和轻链的可变区含有与抗原相互作用的结合结构域。抗体的恒定区可介导免疫球蛋白与宿主细胞或因子的结合,包括免疫系统的不同细胞(例如,效应子细胞)和经典补系统的第一组分(C1q)。本披露的示例性抗体包括典型的抗体、scFv以及其组合,其中,例如scFv被共价连接(例如,通过肽键或通过化学接头)至典型抗体的重链和/或轻链的N末端上,或嵌入在典型抗体的重链和/或轻链中。

[0118] 术语“抗体”意指通过位于免疫球蛋白分子的可变区内的至少一个抗原识别位点识别并特异性地结合至靶标诸如蛋白、多肽、肽、碳水化合物、多核苷酸、脂质、或上述物质的组合的免疫球蛋白分子。如在此所使用,术语“抗体”涵盖完整多克隆抗体、完整单克隆抗体、抗体片段(诸如Fab、Fab'、F(ab')₂、以及Fv片段)、单链可变片段(scFv)、二硫化物稳定的scFv、多特异性抗体诸如从至少两个完整抗体和/或其抗原结合片段中产生的双特异性抗体、嵌合抗体、人源化抗体、人抗体、包含抗体的抗原决定部分的融合蛋白以及包含抗原识别位点的任何其他修饰的免疫球蛋白分子,只要这些抗体展现希望的生物活性。

[0119] 抗体可以是以下五大类免疫球蛋白中的任一种:IgA、IgD、IgE、IgG、以及IgM,或其亚类(同种型)(例如IgG1、IgG2、IgG3、IgG4、IgA1以及IgA2),这基于它们的重链恒定结构域的特性分别被称为 α 、 δ 、 ϵ 、 γ 、以及 μ 。不同类的免疫球蛋白具有不同的且熟知的亚基结构和三维构型。抗体可以是裸的或辄合至其他分子诸如毒素、放射性同位素等上以形成ADC。

[0120] “阻断”抗体或“拮抗剂”抗体是抑制或降低其结合的抗原诸如HER2的生物活性的抗体。在某一方面中,阻断抗体或拮抗剂抗体基本上或完全地抑制抗原的生物活性。希望地,该生物活性被降低10%、20%、30%、50%、70%、80%、90%、95%、或甚至100%。

[0121] 术语“抗HER2抗体”或“抗HER2”是指能够以足够的亲和力结合HER2的抗体,这样使得该抗体作为靶向HER2的治疗剂或诊断试剂是有用的。抗HER2抗体与不相关非HER2蛋白的结合程度小于约该抗体与HER2的结合的10%,如例如通过放射免疫测定(RIA)或BIAcore™(使用重组HER2作为分析物并且使用抗体作为配体,或反之亦然)、或其他本领域中已知的结合测定所测量。在某些方面中,结合至HER2的抗体具有 $\leq 1\mu\text{M}$ 、 $\leq 100\text{nM}$ 、 $\leq 10\text{nM}$ 、 $\leq 1\text{nM}$ 、 $\leq 0.1\text{nM}$ 、 $\leq 10\text{pM}$ 、 $\leq 1\text{pM}$ 、或 $\leq 0.1\text{pM}$ 的解离常数(K_D)。

[0122] 术语“抗原结合片段”是指完整抗体的一部分并且是指完整抗体的抗原决定可变区。本领域已知的是,抗体的抗原结合功能可以由全长抗体的片段完成。抗体片段的实例包括但不限于:Fab、Fab'、F(ab')₂、以及Fv片段、线性抗体、单链抗体、以及从抗体片段形成的多特异性抗体。

[0123] “单克隆抗体”是指涉及高度特异性识别和结合单个抗原决定簇或表位的均质抗体群体。这与典型地包括针对不同抗原决定簇的不同抗体的多克隆抗体相反。

[0124] 术语“单克隆抗体”涵盖完整单克隆抗体和全长单克隆抗体两者以及抗体片段(诸如Fab、Fab'、F(ab')₂、Fv)、单链可变片段(scFv)、包含抗体部分的融合蛋白、以及包含抗原识别位点的任何其他修饰的免疫球蛋白分子。此外,“单克隆抗体”是指通过任何数量的方式制备的此类抗体,包括但不限于通过杂交瘤、噬菌体选择、重组表达、以及转基因动物(例如,在转基因小鼠中表达人抗体)。

[0125] 术语“人源化抗体”是指从非人(例如,鼠)免疫球蛋白中衍生的抗体,该抗体被工程化成含有最少的非人(例如,鼠)序列。典型地,人源化抗体是人免疫球蛋白,其中来自CDR的残基被来自具有希望的特异性、亲和力以及能力的非人物种(例如,小鼠、大鼠、兔、或仓鼠)的CDR的残基替换(琼斯(Jones)等人,1986,自然,321:522-525;瑞彻曼(Riechmann)等人,1988,自然,332:323-327;威霍英(Verhoeyen)等人,1988,科学(Science),239:1534-1536)。在一些例子中,人免疫球蛋白的FW残基被来自具有希望的特异性和/或亲和力和/或能力的非人物种的抗体中的相应残基替换。

[0126] 该人源化抗体可通过取代FW区中的另外残基和/或在替换的非人残基内精修并优化抗体特异性和/或亲和力和/或能力来进一步修饰。通常,该人源化抗体将基本上包含所有至少一个、并且典型地两个或三个可变结构域,这些可变结构域含有所有或基本上所有的相应于非人免疫球蛋白的CDR区,而所有或基本上所有的FW区是人免疫球蛋白共有序列的那些。该人源化抗体还可以包含免疫球蛋白恒定区或结构域(Fc)的至少一部分,典型地是人免疫球蛋白的至少一部分。用于产生人源化抗体的方法的实例描述于美国专利号5,225,539或5,639,641中。

[0127] 抗体的“可变区”是指抗体轻链的可变区或抗体重链的可变区,单独地或组合地。重链和轻链的这些可变区各自自由通过三个CDR区连接的四个FW区组成。每个链中的CDR由FW区紧密靠近地保持在一起,并且与来自另一个链的CDR促成了抗体的抗原结合位点的形成。存在用于确定CDR的至少两种技术:(1)基于跨物种序列变异性的方法(即,卡巴特等人,具有免疫学兴趣的蛋白质序列(Sequences of Proteins of Immunological Interest),(第5版,1991,美国国家卫生研究院(National Institutes of Health),马里兰州贝塞斯达(Bethesda Md.));以及(2)基于抗原-抗体复合体的结晶学研究的方法(奥拉卡尼(A1-lazikani)等人(1997)分子生物学杂志(J.Molec.Biol.)273:927-948)。另外,在本领域中有时使用这两种方法的组合来确定CDR。当引用可变结构域中的残基(大约轻链的残基1-107和重链的残基1-113)时,通常使用卡巴特编号系统(例如,卡巴特等人,具有免疫学兴趣的序列,第5版,公共卫生服务(Public Health Service),美国国家卫生研究院,马里兰州贝塞斯达(1991))。

[0128] 短语“如用卡巴特编号的氨基酸位置”、“卡巴特位置”以及其语法变体是指在卡巴特等人,具有免疫学兴趣的蛋白质序列,第5版,公共卫生服务,美国国家卫生研究院,马里兰州贝塞斯达(1991)中用于抗体编译的重链可变结构域或轻链可变结构域的编号系统。使用这个编号系统,实际的线性氨基酸序列可以含有相应于可变结构域的FW或CDR的缩短、或插入的较少的或另外的氨基酸。例如,重链可变结构域可以包括在H2的残基52之后的单个氨基酸插入(根据卡巴特的残基52a)以及在重链FW残基82之后的插入残基(例如,根据卡巴特的残基82a、82b、以及82c等)。

表1

环	卡巴特	AbM	乔西亚
L1	L24-L34	L24-L34	L24-L34
L2	L50-L56	L50-L56	L50-L56
L3	L89-L97	L89-L97	L89-L97
H1	H31-H35B	H26-H35B (卡巴特编号)	H26-H32..34
H1	H31-H35	H26-H35 (乔西亚编号)	H26-H32
H2	H50-H65	H50-H58	H52-H56
H3	H95-H102	H95-H102	H95-H102

[0129] 可以通过抗体序列与“标准”卡巴特编号序列在同源区的比对而针对给定的抗体确定残基的卡巴特编号。替代地,乔西亚是指结构环的位置(乔西亚和莱斯克(Lesk),分子生物学杂志196:901-917(1987))。当使用卡巴特编号惯例进行编号时,乔西亚CDR-H1环的端部取决于该环的长度在H32与H34之间变化(这是因为卡巴特编号方案在H35A和H35B处放置插入;如果35A或35B都不存在,该环在32处结束;如果仅存在35A,该环在33处结束;如果存在35A和35B两者,该环在34处结束)。AbM高变区代表卡巴特CDR与乔西亚结构环之间的折衷,并且通过牛津大学分子AbM抗体建模软件来使用。

[0130] IMGT(ImMunoGeneTics)也提供了用于免疫球蛋白可变区包括CDR的编号系统。参见,例如勒弗朗M.P.(Lefranc,M.P.)等人,发育和比较免疫学(Dev.Comp.Immunol.)27:55-77(2003),该参考文献通过引用结合在此。该IMGT编号系统基于多于5,000个序列的比对、结构数据以及高变环的表征,并且允许对于所有物种进行可变区和CDR区的简单比较。根据该IMGT编号方案,VH-CDR1是在位置26至35处,VH-CDR2是在位置51至57处,VH-CDR3是在位置93至102处,VL-CDR1是在位置27至32处,VL-CDR2是在位置50至52处,并且VL-CDR3是在位置89至97处。

[0131] 对于在本发明中讨论的所有重链恒定区氨基酸位置,根据首次为爱德曼(Edelman)等人,1969,美国科学院院刊63(1):78-85中描述的EU索引进行编号,该参考文献描述了骨髓瘤蛋白Eu的氨基酸序列,该氨基酸序列是首次人IgG1测序的。爱德曼等人的EU索引也阐述于卡巴特等人,1991,具有免疫兴趣的蛋白质序列,第5版,美国公共卫生服务,国家卫生研究院,贝塞斯达中。因此,短语“如在卡巴特中所阐述的EU索引”或“卡巴特的EU索引”和“根据如在卡巴特中所阐述的EU索引的位置……”以及其语法变体是指基于如在卡巴特1991中所阐述的爱德曼等人的人IgG1 Eu抗体的残基编号系统。

[0132] 用于可变结构域(重链和轻链两者)和轻链恒定区氨基酸序列的编号系统是在卡巴特1991中阐述的编号系统。

[0133] 如在此所使用,Fc区包括包含抗体的除第一恒定区免疫球蛋白结构域之外的恒定区的多肽。因此,Fc是指IgA、IgD、以及IgG的最后两个恒定区免疫球蛋白结构域、以及IgE和IgM的最后三个恒定区免疫球蛋白结构域、以及在上述结构域的N末端的柔性铰链。对于IgA和IgM,Fc可以包括J链。对于IgG,Fc包含免疫球蛋白结构域C γ 2和C γ 3(C γ 2和C γ 3)以及C γ 1(C γ 1)与C γ 2(C γ 2)之间的铰链。

[0134] 虽然Fc区的界限可以改变,但人IgG重链Fc区通常被定义成包含其羧基末端的残基C226或P230,其中编号是根据如在卡巴特中阐述的EU索引。Fc可以是指孤立地这个区,或在抗体、抗体片段或Fc融合蛋白的背景下的这个区。

[0135] 已在抗体恒定区的多个不同位置(例如,Fc位置,包括但不限于如通过在卡巴特中所阐述的EU索引进行编号的位置270、272、312、315、356、以及358)处观察到多态性,并且因此所呈现的序列与现有技术序列之间可存在略微的差异。已充分表征了人免疫球蛋白的多态性形式。目前,已知18种Gm同种异型:G1m(1、2、3、17)或G1m(a、x、f、z)、G2m(23)或G2m(n)、G3m(5、6、10、11、13、14、15、16、21、24、26、27、28)或G3m(b1、c3、b3、b0、b3、b4、s、t、g1、c5、u、v、g5)(勒弗朗(Lefranc)等人,人IgG亚类:结构、功能以及调控的分子分析(The human IgG subclasses:molecular analysis of structure,function and regulation).培格曼(Pergamon),牛津,第43-78页(1990);勒弗朗G.等人,1979,美国人类遗传学杂志(Hum.Genet.):50,199-211)。确切地想到,本发明的抗体可掺入任何免疫球蛋白基因的任何同种异型、异种型或单倍型,并且不限于在此提供的序列的同种异型、异种型或单倍型。

[0136] 术语“人抗体”意指由人产生的抗体或具有相应于使用本领域中已知的任何技术(例如,在培养物细胞中重组表达、或在转基因动物中表达)由人制备产生的抗体的氨基酸序列的抗体。因此,术语人抗体还涵盖具有相应于最初由人(或其工程化的变体或衍生物)产生但在非人系统中表达(例如,由化学合成产生;在微生物、哺乳动物或昆虫细胞中重组表达;或在动物受试者中表达)的抗体的氨基酸序列的抗体。因此,从人受试者或从人细胞(例如,表达重组抗体或其片段的杂交瘤或细胞系)中获得并且随后在例如小鼠的动物中表达的抗体被认为是人抗体。人抗体的这种定义包括完整或全长抗体、其片段、和/或包含至少一个人重链和/或轻链多核苷酸的抗体,诸如例如包含鼠轻链和人重链多核苷酸的抗体。

[0137] 术语“嵌合抗体”是指其中免疫球蛋白分子的氨基酸序列从两种或更多种动物物种中衍生的抗体。典型地,轻链和重链两者的可变区相应于从具有希望的特异性和/或亲和力和/或能力的哺乳动物(例如,小鼠、大鼠、兔等)中的一种物种中衍生的抗体的可变区,而恒定区与从另一种物种(通常人)中衍生的抗体中的序列同源以便避免在这些物种中引发免疫应答。

[0138] 如在此所使用的术语“表位”是指能够结合至在此披露的HER2抗体或HER2结合分子的抗原性蛋白质决定簇。表位通常由诸如氨基酸或糖侧链的分子的化学活性表面基团组成,并且通常具有特定的三维结构特征以及特定的电荷特征。抗体或结合分子的识别该表位的部分被称为互补位。蛋白质抗原的表位基于它们的结构与互补位的相互作用而被分成两类(构象表位和线性表位)。构象表位包含抗原的氨基酸序列的不连续区段。这些表位基于抗原的3D表面特征和形状或三级结构而与互补位相互作用。相反,线性表位基于它们的一级结构而与互补位相互作用。线性表位由来自抗原的氨基酸的连续序列形成。

[0139] 术语“抗体结合位点”是指抗原(例如,HER2)中包含互补抗体特异性结合至其上的连续或不连续位点(即,表位)的区域。因此,该抗体结合位点可含有抗原中的另外区域,这些区域超出表位并且可决定诸如结合亲和力和/或稳定性的特性或影响诸如抗原酶促活性或二聚化的特性。因此,即使两个抗体结合至抗原内的相同表位,如果抗体分子建立与表位外部氨基酸的不同分子间接触,则此类抗体被认为结合至不同抗体结合位点。

[0140] “结合亲和力”通常是指在分子(例如抗体)的单一结合位点与其结合配偶体(例

如,抗原)之间的非共价相互作用的总和的强度。除非另有说明,如在此所使用,“结合亲和力”是指反映在结合对的成员(例如,抗体和抗原)之间的1:1相互作用的固有结合亲和力。分子X对于其配偶体Y的亲和力通常可以通过解离常数(K_D)表示。可以通过本领域中已知的常见方法来测量亲和力,包括在此描述的那些。低亲和力抗体通常缓慢地结合抗原并且倾向于容易解离,而高亲和力抗体通常更快地结合抗原并且倾向于更长地保持结合。多种测量结合亲和力的方法在本领域中是已知的,出于本披露的目的而可以使用它们中的任何一种。

[0141] 除非另外说明,否则“效力”通常表达为以nM计的 IC_{50} 值。 IC_{50} 是抗原结合分子的中值抑制浓度。在功能性测定中, IC_{50} 是将生物应答降低其最大值的50%的浓度。在配体结合研究中, IC_{50} 是将受体结合降低最大特异性结合水平的50%的浓度。可以通过本领域中已知的任何数量的手段来计算 IC_{50} 。效力的改进可通过例如针对39S亲本抗体测量来确定。

[0142] 对于在此披露的抗HER2结合分子的效力改进倍数(例如,与39S亲本抗体、曲妥单抗或其组合相比)可以是至少约2倍、至少约4倍、至少约6倍、至少约8倍、至少约10倍、至少约12倍、至少约14倍、至少约16倍、至少约18倍、至少约20倍、至少约30倍、至少约40倍、至少约50倍、至少约60倍、至少约70倍、至少约80倍、至少约90倍、至少约100倍、至少约110倍、至少约120倍、至少约130倍、至少约140倍、至少约150倍、至少约160倍、至少约170倍、或至少约180倍或更多。

[0143] “抗体依赖性细胞介导的细胞毒性”或“ADCC”是指细胞毒性的一种形式,其中结合至存在于某些细胞毒性细胞(例如,天然杀伤(NK)细胞、嗜中性粒细胞、以及巨噬细胞)上的Fc受体(FcR)上的分泌的免疫球蛋白使得这些细胞毒性效应子细胞能够特异性地结合至带有抗原的靶细胞并且随后用细胞毒素杀伤该靶细胞。针对靶细胞表面的特异性高亲和力IgG抗体“武装”细胞毒性细胞并且是这种杀伤所需要的。靶细胞的溶解是细胞外的,要求直接的细胞与细胞接触,并且不涉及补体。应当想到,除了抗体以外,具有特异性结合至带有抗原的靶细胞的能力的包含Fc区的其他蛋白质(确切地Fc融合蛋白)将能够实现细胞介导的细胞毒性。为了简明起见,由Fc融合蛋白的活性产生的细胞介导的细胞毒性在此又称为ADCC活性。

[0144] “分离的”多肽、抗体、多核苷酸、载体、细胞或组合物是以不存在于自然中的形式的多肽、抗体、多核苷酸、载体、细胞或组合物。分离的多肽、抗体、多核苷酸、载体、细胞或组合物包括已被纯化至它们不再以存在于自然中的形式的程度的那些。在一些方面中,分离的抗体、多核苷酸、载体、细胞或组合物是基本上纯的。

[0145] 术语“受试者”是指有待作为具体处理的接受者的任何动物(例如,哺乳动物),包括但不限于人、非人灵长类动物、啮齿动物等。典型地,对于人受试者,在此可互换使用术语“受试者”和“患者”。

[0146] 术语“药物组合物”是指以容许活性成分(例如,在此披露的抗HER2结合分子)的生物活性有效这样的形式的制剂,并且该制剂不含有对向其给予该组合物的受试者有不可接受的毒性的另外组分。此种组合物可以是无菌的。

[0147] 如在此所披露的抗HER2结合分子的“有效量”是足以执行确切说明的目的的量。关于说明的目的,可以根据经验并且以常规方式确定“有效量”。

[0148] 术语“治疗有效量”是指有效于“治疗”受试者或哺乳动物的疾病或病症的在此披露的抗HER2结合分子或其他药物的量。

[0149] 当在此使用时,词语“标记”是指直接或间接结合至在此披露的抗HER2结合分子上以便产生“标记的”抗HER2结合分子的可检测化合物或组合物。该标记本身可以是可检测的(例如,放射性同位素标记或荧光标记),或在酶标记的情况下,可以催化可检测的底物化合物或组合物的化学改变。

[0150] 术语诸如“可衍生基团”和“可衍生官能团”可互换使用并且是指能够反应以容许介于在此披露的抗HER2结合分子(例如,HER2抗体)与另一种物质之间形成共价键的官能团。在一些方面中,此种物质是治疗部分(例如,细胞毒素)、可检测标记、聚合物(例如,PEG)等。示例性可衍生基团包括巯基、羟基、氨基、羧基、以及酰胺、以及其改性的形式,诸如被活化或被保护的形式。

[0151] 术语诸如“治疗(treating)”或“治疗(treatment)”或“治疗(to treat)”或“减轻(alleviating)”或“减轻(to alleviate)”是指(1)治愈、减缓、减轻诊断的病理病状或病症的症状和/或停止诊断的病理病状或病症的进展的治疗性措施以及(2)防止和/或减慢靶向的病理病状或病症的发展的预防性或防御性措施两者。因此,有治疗需要的那些包括已经患有病症的那些;倾向于患有病症的那些;以及其中有待防止病症的那些。在某些方面中,如果患者表现出例如全部、部分或暂时消退某种类型的癌症,则受试者根据本披露的方法被成功地“治疗”癌症。

[0152] 术语“癌症”、“肿瘤”、“癌性”以及“恶性”是指或描述哺乳动物中典型地特征在于不规则细胞生长的生理病状。癌症的实例包括但不限于癌瘤,包括腺癌、淋巴瘤、胚细胞瘤、黑色素瘤、肉瘤、以及白血病。此类癌症的更多具体实例包括鳞状细胞癌、小细胞肺癌、非小细胞肺癌、胃肠癌、霍奇金氏淋巴瘤和非霍奇金氏淋巴瘤、胰腺癌、成胶质细胞瘤、神经胶质瘤、宫颈癌、卵巢癌、肝癌诸如肝细胞癌和肝细胞瘤、膀胱癌、乳癌(包括激素介导的乳癌,参见例如,英尼斯(Innes)等人(2006)英国癌症杂志(Br. J. Cancer)94:1057-1065)、结肠癌、结肠直肠癌、子宫内膜癌、骨髓瘤(诸如多发性骨髓瘤)、唾液腺癌、肾癌诸如肾细胞癌和维尔姆斯氏瘤、基底细胞癌、黑色素瘤、前列腺癌、外阴癌、甲状腺癌、睾丸癌、食管癌、不同类型的头颈部癌和粘液性来源的癌症,诸如粘液性卵巢癌、胆管癌(肝脏)以及肾乳头状癌。在一些方面中,如在此所使用的术语癌症确切地是指表达HER2的癌症。在一些特定方面中,术语癌症是指表达低水平HER2的癌症。

[0153] 如在此所使用的“低水平HER2”是指当使用 HERCEPTEST®(DakoCytomation加利福尼亚公司,卡奔塔利亚(Carpenteria),CA)分类时表现出小于2+(例如,1+)分数的癌症细胞、受试者或患者,或例如通过FISH已鉴定为如此的癌症、癌症细胞、受试者或患者。

[0154] 为了确定癌症中的HER2表达,不同诊断/预后测定是可用的。在一个方面中,HER2过表达可通过IHC,例如通过使用HERCEPTEST®(Dako)来分析。来自肿瘤活组织检查的石蜡包埋的组织切片可受到IHC测定并且根据如下的HER2蛋白质染色强度标准。替代地或另外地,可在福尔马林固定的、石蜡包埋的肿瘤组织上进行FISH测定诸如INFORM™(由塔纳亚利桑那州(Ventana, Ariz.)销售)或PATHVISION™(Vysis, Ill.),以便确定肿瘤中的HER2过表达的程度(如果存在的话)。

[0155] “低”是指小于正常、小于标准(诸如预先确定的量度)的量度,或相对小于另一个亚组量度的亚组量度的术语。例如,低HER2意指小于HER2阳性的一个具体患者样品组中的正常HER2量度的HER2的量度。正常HER2量度可以根据本领域技术人员可用的任何方法来确

定。低HER2还可以意指小于预先确定的量度的HER2的量度,诸如预先确定的截止低HER2还可以意指其中低HER2亚组相对低于另一个亚组的量度。例如,但不限于,根据本说明书,两个不同的患者亚组可通过在数学上确定的点(诸如但不限于中值)周围对样品进行划分来创建,从而创建了其量度相对于量度高的另一个组是较低的(即,小于该中值)的组。HER2可通过本领域技术人员已知的任何方法来测量,诸如例如但不限于,使用eTag方法或使用任何标准IHC方法诸如HERCEPTEST®。作为另一个实例,低水平HER2是指低水平HER2同源二聚体,这意指小于HER2阳性的一个具体样品或患者组中的正常HER2同源二聚体量度的HER2同源二聚体的量度。低HER2同源二聚体还可以意指小于预先确定的量度(诸如预先确定的截止)的量度。低HER2同源二聚体还可以意指其中低HER2同源二聚体亚组相对低于另一个亚组的量度。HER2同源二聚体可通过本领域中已知的任何方法来测量,诸如荧光共振能量转移(FRET)、生物发光共振能量转移(BRET)、邻位连接测定(PLA)、二聚体特异性抗体或eTag或本领域技术人员熟知的任何其他方法。

[0156] 如在此所使用,术语“癌瘤”是指上皮细胞的癌症,这些上皮细胞是覆盖身体表面、产生激素并且构成腺体的细胞。癌瘤的实例是皮肤癌、肺癌、结肠癌、胃癌、乳癌、前列腺癌以及甲状腺癌。

[0157] 如在此可互换使用的“多核苷酸”或“核酸”是指具有任何长度的核苷酸的聚合物,并且包括DNA和RNA。这些核苷酸可以是脱氧核糖核苷酸、核糖核苷酸、修饰的核苷酸或碱基和/或它们的类似物、或可通过DNA或RNA聚合酶掺入到聚合物中的任何底物。多核苷酸可包含修饰的核苷酸,诸如甲基化的核苷酸和它们的类似物。先前描述适用于在此提到的所有多核苷酸,包括RNA和DNA。

[0158] 术语“载体”意指能够递送,并且在一些方面中,在宿主细胞中表达感兴趣的一个或多个基因或一个或多个序列的构建体。载体的实例包括,但不限于病毒载体、裸DNA或RNA表达载体、质粒、粘粒或噬菌体载体、与阳离子缩合剂相连的DNA或RNA表达载体、包封在脂质体中的DNA或RNA表达载体、以及某些真核细胞诸如生产者细胞。

[0159] 术语“多肽”、“肽”以及“蛋白质”在此可互换使用是指具有任何长度的氨基酸的聚合物。该聚合物可以是直链或支链的,它可包含修饰的氨基酸,并且它可被非氨基酸中断。这些术语还涵盖天然地或通过介入而修饰的氨基酸聚合物;例如,二硫键形成、糖基化、脂质化、乙酰化、磷酸化、或任何其他操纵或修饰,诸如与标记组分轭合。还包括在定义内的是例如,含有氨基酸(包括例如,非天然氨基酸等)的一种或多种类似物、以及本领域中已知的其他修饰的多肽。应当理解,因为本披露的多肽是基于抗体的,所以在某些方面中,多肽可作为单链或相连的链存在。

[0160] “重组”多肽或蛋白质是指通过重组DNA技术产生的多肽或蛋白质。如同已经通过任何适合的技术来分离、分级分离或部分地或基本上纯化的天然或重组多肽一样,出于本发明的目的,在工程化的宿主细胞中表达的重组产生的多肽和蛋白质也被认为是分离的。在此披露的多肽可使用本领域中已知的方法来重组产生。替代地,在此披露的蛋白质和肽可被化学合成。

[0161] 除非另外说明,否则在对细胞毒性剂(即微管溶素)的化学结构进行修饰的背景下在此所使用的术语“取代的”涉及带有一个或多个取代基的母体基团。在常规意义上在此使用术语“取代基”,并且是指共价附接至、或如果适当的话融合至母体基团上的化学部分。各

种各样的取代基是熟知的,并且用于这些取代基形成和引入到各种母体基团中的方法也是熟知的。化学取代基的实例在以下更详细地进行了描述。

[0162] 在对细胞毒性剂的化学结构进行修饰的背景下在此所使用的短语“任选取代的”涉及可以是未取代的或可以是取代的母体基团。

[0163] 在多肽背景下在此所使用的术语“取代的”、“氨基酸取代”等是指使用另一个氨基酸残基替换存在于母体多肽中的氨基酸残基。可例如通过化学肽合成或通过本领域中已知的重组方法来在母体多肽中取代氨基酸。因此,提到“位置X处的取代”或“位置X处的取代”是指使用替代的氨基酸残基取代位置X处存在的氨基酸。在一些方面中,可根据模式AXY描述取代方式,其中A是相应于在位置X处天然存在的氨基酸的单字母代码,并且Y是取代的氨基酸残基。因此,L234F将是指使用苯丙氨酸(F)取代位置234处的亮氨酸氨基酸(L)。在其他方面中,可根据模式XY描述取代方式,其中Y是相应于取代在位置X处天然存在的氨基酸的氨基酸残基的单字母代码。因此,239C将是指使用半胱氨酸(C)取代位置239处的天然氨基酸。

[0164] “保守氨基酸取代”是其中氨基酸残基被具有类似侧链的氨基酸残基替换的取代。在本领域中已定义了具有类似侧链的氨基酸残基的家族,这些家族包括碱性侧链(例如,赖氨酸、精氨酸、组氨酸)、酸性侧链(例如,天冬氨酸、谷氨酸)、不带电荷的极性侧链(例如,甘氨酸、天冬酰胺、谷氨酰胺、丝氨酸、苏氨酸、酪氨酸、半胱氨酸)、非极性的侧链(例如,丙氨酸、缬氨酸、亮氨酸、异亮氨酸、脯氨酸、苯丙氨酸、甲硫氨酸、色氨酸)、 β -分支侧链(例如,苏氨酸、缬氨酸、异亮氨酸)以及芳香族侧链(例如,酪氨酸、苯丙氨酸、色氨酸、组氨酸)。因此,如果多肽中的氨基酸被来自相同侧链家族的另一种氨基酸替换,则取代被认为是保守的。在另一个方面中,氨基酸串可被在侧链家族成员的顺序和/或组成上不同、在结构上类似的串保守地替换。

[0165] 非保守氨基酸取代包括以下那些,其中(i)具有正电性侧链的残基(例如,Arg、His或Lys)被取代成负电性残基(例如,Glu或Asp)或被负电性残基取代,(ii)亲水性残基(例如,Ser或Thr)被取代成疏水性残基(例如,Ala、Leu、Ile、Phe或Val)或被疏水性残基取代,(iii)半胱氨酸或脯氨酸被取代成任何其他残基或被任何其他残基取代,或(iv)具有大体积疏水性或芳香族侧链的残基(例如,Val、His、Ile或Trp)被取代成具有更小侧链的残基(例如,Ala、Ser)或无侧链的残基(例如,Gly)或被具有更小侧链的残基或无侧链的残基取代。

[0166] 其他氨基酸取代可通过普通技术人员容易地鉴定。例如,对于氨基酸丙氨酸,可从D-丙氨酸、甘氨酸、 β -丙氨酸、L-半胱氨酸以及D-半胱氨酸中的任一个中得到取代。对于赖氨酸,替代可以是D-赖氨酸、精氨酸、D-精氨酸、同源精氨酸、甲硫氨酸、D-甲硫氨酸、鸟氨酸、或D-鸟氨酸中的任一个。通常,可预期诱导分离多肽的特性变化的功能重要区域中的取代是以下那些,其中(i)极性残基例如丝氨酸或苏氨酸被取代成疏水性残基例如亮氨酸、异亮氨酸、苯丙氨酸、或丙氨酸(或被其取代);(ii)半胱氨酸残基被取代成任何其他残基(或被其取代);(iii)具有正电性侧链的残基例如赖氨酸、精氨酸或组氨酸被取代成具有负电性侧链的残基例如谷氨酸或天冬氨酸(或被其取代);或(iv)具有大体积侧链的残基例如苯丙氨酸被取代成不具有这样侧链的残基例如甘氨酸(或被其取代)。前述非保守取代中的一种可改变蛋白质的功能特性的可能性还与关于蛋白质的功能重要区域的取代位置相关:一

些非保守取代可因此对生物特性具有较小或没有作用。

[0167] 术语“氨基酸插入”是指在亲本序列中存在的两个氨基酸残基之间引入新的氨基酸残基。可例如通过化学肽合成或通过本领域中已知的重组方法来在亲本序列中插入氨基酸。因此,如在此所使用,短语“在位置X与Y之间插入”或“在卡巴特位置X与Y之间插入”是指在X位置与Y位置之间插入氨基酸,而且是指是在编码位置X与Y处的氨基酸的密码子之间在编码氨基酸的密码子的核酸序列中的插入,其中X和Y相应于氨基酸位置(例如,位置239与240之间的半胱氨酸氨基酸插入)。可根据模式AXins描述插入方式,其中A是相应于插入的氨基酸的单字母代码,并且X是该插入之前的位置。因此,C239ins将是指是在位置239之后插入半胱氨酸氨基酸(C)(即,在位置239与240之间插入)。

[0168] 术语两个多肽或多核苷酸序列之间的“序列一致性百分比”是指考虑到为了最佳比对这两个序列而必须引入的添加或缺失(即,空隙),在比较窗口上由这些序列共用的相同匹配位置的数目。匹配位置是其中相同核苷酸或氨基酸呈现在靶标序列与参考序列两者中的任何位置。呈现在靶标序列中的空隙未计数,因为空隙并非核苷酸或氨基酸。同样地,呈现在参考序列中的空隙未计数,因为对靶标序列核苷酸或氨基酸进行计数,而不对来自参考序列的核苷酸或氨基酸进行计数。

[0169] 序列一致性百分比的计算是通过确定相同氨基酸残基或核酸碱基出现在两个序列中的位置的数目以便产生匹配位置数目,将匹配位置数目除以比较窗口中的位置总数并且将结果乘以100,从而产生序列一致性百分比。序列的比较和两个序列之间的序列一致性百分比的确定可以使用供在线使用与下载两者的易于得到的软件来完成。适合的软件程序可从不同来源获得,并且用于比对蛋白质与核苷酸序列两者。确定序列一致性百分比的一种适合程序是bl2seq,该程序是可从美国政府的国家生物技术信息中心(National Center for Biotechnology Information)BLAST网站(blast.ncbi.nlm.nih.gov)获得的BLAST程序套件的一部分。Bl2seq使用BLASTN或BLASTP算法在两个序列之间执行比较。BLASTN用于比较核酸序列,而BLASTP用于比较氨基酸序列。其他适合程序是例如Needle、Stretcher、Water或Matcher,它们是EMBOSS生物信息学程序套件的一部分并且也可在www.ebi.ac.uk/Tools/psa上从欧洲生物信息学研究所(European Bioinformatics Institute;EBI)获得。

[0170] 与多核苷酸或多肽参考序列比对的单一多核苷酸或多肽靶标序列内的不同区域可各自具有其本身的序列一致性百分比。应当指出的是序列一致性百分比值被四舍五入至最近的十分位。例如,80.11、80.12、80.13以及80.14向下舍入至80.1,而80.15、80.16、80.17、80.18以及80.19向上舍入至80.2。还应当指出的是长度值将总是整数。

[0171] 在某些方面中,第一氨基酸序列与第二序列氨基酸的一致性百分比“X”被计算为 $100 \times (Y/Z)$,其中Y是在该第一序列和该第二序列比对中作为相同匹配计分的氨基酸残基数(如通过目测检查或具体序列比对程序所比对)并且Z是该第二序列中的残基总数。如果第一序列的长度长于该第二序列,该第一序列与该第二序列的一致性百分比将高于该第二序列与该第一序列的一致性百分比。

[0172] 本领域技术人员应当了解用于计算序列一致性百分比的序列比对的产生不限于完全由基本序列数据驱动的二元序列-序列比较。序列比对可得自多重序列比对。产生多重序列比对的一种适合程序是可从www.CLUSTAL.org获得的ClustalW2。另一种适合程序是可从www.drive5.com/muscle/获得的MUSCLE。ClustalW2和MUSCLE可替代地从例如EBI获得。

[0173] 还应当了解序列比对可以通过将序列数据与来自异类来源的数据诸如结构数据(例如,结晶学蛋白质结构)、功能数据(例如,突变位置)或系统发生数据集集成来产生。将异类数据集集成以便产生多重序列比对的适合程序是T-Coffee,它可从www.tcoffee.org获得,并且替代地可从例如EBI获得。还应当了解用于计算序列一致性百分比的最终比对可以自动或手动地管理(curate)。

II. 抗HER2结合分子

[0174] 本披露提供特异性结合HER2的抗HER2结合分子,例如包含其HER2结合片段的抗HER2抗体或分子。

[0175] 对于HER2的全长氨基酸(aa)和核苷酸(nt)序列是本领域中已知的(参见,例如对于人HER2的UniProt登录号P04626)。参见,例如耀司(Yamamoto)等人,自然319:230-234(1986);寇森斯(Coussens)等人,科学230:1132-1139(1985);涛(Tal)等人,分子细胞生物学7:2597-2601(1987);萨姆巴(Semba)等人,美国科学院院刊(Proc.Natl.Acad.Sci.U.S.A.)82:6497-6501(1985);金(King)等人,科学229:974-976(1985);萨卡尔(Sarkar)等人,DNA细胞生物学(DNA Cell Biol.)12:611-615(1993);吉里(Giri)等人,分子细胞生物学25:11005-11018(2005);阿尼多(Anido)等人,欧洲分子生物学学会杂志(EMBO J.)25:3234-3244(2006);必然尼(Birrane)等人,生物化学杂志278:1399-1402(2003);伊万茨(Ivancic)等人,生物分子学核磁共振杂志(J.Biomol.NMR)27:205-219(2003);曹(Cho)等人,自然421:756-760(2003);富兰克林(Franklin)等人,癌症细胞(Cancer Cell)5:317-328(2004);博斯特罗姆(Bostrom)等人,科学323:1610-1614(2009);埃金伯特(Eigenbrot)等人,美国科学院院刊107:15039-15044(2010);斯蒂芬斯(Stephens)等人,自然431:525-526(2004);格林曼(Greenman)等人,自然446:153-158(2007);所有这些参考文献通过引用以其全文结合在此。

[0176] 在某些方面中,这些抗HER2结合分子是抗体或其抗原结合片段。在一些方面中,这些抗HER2结合分子(例如,抗HER2抗体或包含其HER2结合片段的分子)包含Fab、Fab'、F(ab')₂、Fd、单链Fv、scFv、双硫化物稳定的scFv、二硫化物连接的Fv、V-NAR结构域、IgNar、内抗体、IgG△CH2、小抗体、F(ab')₃、四体抗体、三体抗体、双体抗体、单一结构域抗体、DVD-Ig、Fcab、mAb²、(scFv)₂、或scFv-Fc。在一些方面中,该抗体具有IgG类型,例如具有IgG1类型。

[0177] 在一些方面中,这些抗HER2结合分子是单特异性的。在其他方面中,这些抗HER2结合分子是双特异性的、三特异性的、四特异性的等。在其他方面中,这些抗HER2结合分子是多特异性的。在一些方面中,这些抗HER2结合分子是单价的、二价的、三价的、四价的等。在又其他方面中,这些抗HER2结合分子是多价的。在特定方面中,这些抗HER2结合分子是二价的,例如包含两个HER2特异性抗原结合位点的抗体。在特定方面中,这些抗HER2结合分子是双特异性的,即,分子可特异性结合至两个不同抗原(例如,相同或不同分子上的两个不同表位)。在一些特定方面中,这些抗HER2结合分子是二价的和四价的,例如,包含能够结合至两个不同抗原(例如,相同或不同分子上的两个不同表位)的四个抗原结合位点的抗体。

[0178] 在特定方面中,这些抗HER2结合分子包括具有与1.39.1抗体的结合位点基本上相同的结合位点的抗体或其抗原结合片段(参见PCT公开号WO 2008/019290,该专利通过引用以其全文结合在此)。在特定方面中,这些抗HER2结合分子包括结合SEQ ID NO:52的一个或

多个氨基酸残基的抗体或其抗原结合片段。在某些方面中,这些抗HER2结合分子包括具有重叠1.39.1抗体的结合位点的抗体或其抗原结合片段。

[0179] 在某些方面中,这些抗HER2结合分子包括包含与亲本1.39.1抗体的VH(SEQ ID NO:43)和/或VL(SEQ ID NO:44)相比已被修饰的VH和/或VL的抗体或其抗原结合片段(参见PCT公开号WO 2008/019290)。在亲本抗体中引入的修饰可以包括与亲本1.39.1抗体相比在VH和VL的CDR区和/或在FW区中的突变(例如,点突变或替换整个子序列)。

[0180] 在一些方面中,亲本1.39.1抗体的VH-CDR1(SEQ ID NO:45)被包含SEQ ID NO:1的氨基酸的VH-CDR1替换。在一些方面中,亲本1.39.1抗体的VH-CDR1(SEQ ID NO:45)被由SEQ ID NO:1的氨基酸组成的VH-CDR1替换。

[0181] 在其他方面中,亲本1.39.1抗体的VH-CDR3(SEQ ID NO:46)被包含SEQ ID NO:3的氨基酸的VH-CDR3替换。在其他方面中,亲本1.39.1抗体的VH-CDR3(SEQ ID NO:46)被由SEQ ID NO:3的氨基酸组成的VH-CDR3替换。

[0182] 在一些方面中,亲本1.39.1抗体的VH-CDR1(SEQ ID NO:45)和VH-CDR3(SEQ ID NO:46)被分别包含SEQ ID NO:1的氨基酸和SEQ ID NO:3的氨基酸或由其组成的VH-CDR1和VH-CDR3替换。

[0183] 在一些方面中,亲本1.39.1抗体的VL-CDR1(SEQ ID NO:47)被包含SEQ ID NO:4的氨基酸的VL-CDR1替换。在一些方面中,亲本1.39.1抗体的VL-CDR1(SEQ ID NO:47)被由SEQ ID NO:4的氨基酸组成的VL-CDR1替换。

[0184] 在一些方面中,亲本1.39.1抗体的VL FW1区(SEQ ID NO:48)被包含SEQ ID NO:11的氨基酸的VL FW1替换。在一些方面中,亲本1.39.1抗体的VL FW1区(SEQ ID NO:48)被由SEQ ID NO:11的氨基酸组成的FW1替换。

[0185] 在其他方面中,亲本1.39.1抗体的VL FW2区(SEQ ID NO:49)被包含SEQ ID NO:12的氨基酸的FW2替换。在其他方面中,亲本1.39.1抗体的VL FW2区(SEQ ID NO:49)被由SEQ ID NO:12的氨基酸组成的FW2替换。

[0186] 在其他方面中,亲本1.39.1抗体的VL FW3区(SEQ ID NO:50)被包含SEQ ID NO:13的氨基酸的FW3替换。在其他方面中,亲本1.39.1抗体的VL FW3区(SEQ ID NO:50)被由SEQ ID NO:13的氨基酸组成的VLFW2替换。

[0187] 在一些方面中,亲本1.39.1抗体的VL FW1区(SEQ ID NO:48)和/或FW2区(SEQ ID NO:49)和/或FW3区(SEQ ID NO:50)被独立地分别包含SEQ ID NO:11、12或13的氨基酸或由其组成的FW1和/或FW2和/或FW3替换。

[0188] 在一些方面中,本披露提供包含免疫球蛋白重链可变区(VH)和免疫球蛋白轻链可变区(VL)的抗HER2结合分子,其中该VH包含SEQ ID NO:15的氨基酸。在一些方面中,本披露提供包含VH和VL的抗HER2结合分子,其中该VL包含SEQ ID NO:16的氨基酸。在一些方面中,该VH包含SEQ ID NO:15的氨基酸并且该VL包含SEQ ID NO:16的氨基酸。在一些方面中,在此披露的抗HER2结合分子包括抗体或其HER2结合片段。

[0189] 在某些方面中,本披露的抗HER2结合分子包含免疫球蛋白重链(VH)和免疫球蛋白轻链(VL),其中该结合分子包含:

- (i)包含SEQ ID NO:1的氨基酸的VH-CDR1;
- (ii)包含SEQ ID NO:2的氨基酸的VH-CDR2;

- (iii)包含SEQ ID NO:3的氨基酸的VH-CDR3;
- (iv)包含SEQ ID NO:4的氨基酸的VL-CDR1;
- (v)包含SEQ ID NO:5的氨基酸的VL-CDR2;以及,
- (vi)包含SEQ ID NO:6的氨基酸的VL-CDR3;

[0190] 在某些方面中,本披露的抗HER2结合分子(例如,抗HER2抗体或其HER2结合片段、或双特异性抗HER2抗体)包含抗体VL和抗体VH,其中该VL包含与参考VL至少约80%、约85%、约90%、约95%、约96%、约97%、约98%、约99%、或约100%一致的氨基酸序列,该参考VL包含SEQ ID NO:16的氨基酸或由其组成。

[0191] 在其他方面中,本披露的抗HER2结合分子(例如,抗HER2抗体或其HER2结合片段、或双特异性抗HER2抗体)包含抗体VL和抗体VH,其中该VH包含与参考VH至少约80%、约85%、约90%、约95%、约96%、约97%、约98%、约99%、或约100%一致的氨基酸序列,该参考VH包含SEQ ID NO:15的氨基酸或由其组成。

[0192] 在其他方面中,本披露的抗HER2结合分子(例如,抗HER2抗体或其HER2结合片段、或双特异性抗HER2抗体)包含VL,该VL包含与参考VL至少约80%、约85%、约90%、约95%、约96%、约97%、约98%、约99%、或约100%一致的氨基酸序列,该参考VL包含SEQ ID NO:16的氨基酸或由其组成,并且进一步包含VH,该VH包含于参考VH至少约80%、约85%、约90%、约95%、约96%、约97%、约98%、约99%、或约100%一致的氨基酸序列,该参考VH包含SEQ ID NO:15的氨基酸或由其组成。

[0193] 在一些方面中,本披露的该抗HER2结合分子(例如,抗HER2抗体或其HER2结合片段、或双特异性抗HER2抗体)包含重链恒定区或其片段。在一些方面中,该重链恒定区是IgG恒定区。该IgG恒定区可包含选自下组的轻链恒定区,该组由以下各项组成: κ 恒定区和 λ 恒定区。

[0194] 在某些方面中,本披露的抗HER2结合分子(例如,抗HER2抗体或其HER2结合片段、或双特异性抗HER2抗体)可以与1.39.1亲本抗体基本上相同或更好的亲和力结合HER2。因此,在一个方面中,本披露的抗HER2结合分子(例如,抗HER2抗体或其HER2结合片段、或双特异性抗HER2抗体)特异性结合HER2及其抗原片段,其中解离常数或 $k_d(k_{off}/k_{on})$ 小于 $10^{-6}M$ 、或小于 $10^{-7}M$ 、或小于 $10^{-8}M$ 、或小于 $10^{-9}M$ 、或小于 $10^{-10}M$ 、或小于 $10^{-11}M$ 、或小于 $10^{-12}M$ 、或小于 $10^{-13}M$ 。

[0195] 在另一个方面中,本披露的抗HER2结合分子(例如,抗HER2抗体或其HER2结合片段、或双特异性抗HER2抗体)以小于 $1 \times 10^{-3}s^{-1}$ 、或小于 $2 \times 10^{-3}s^{-1}$ 的 k_{off} 结合至HER2和/或其抗原片段。在其他方面中,本披露的抗HER2结合分子(例如,抗HER2抗体或其HER2结合片段、或双特异性抗HER2抗体)结合至HER2及其抗原片段,其中 k_{off} 小于 $10^{-3}s^{-1}$ 、小于 $5 \times 10^{-3}s^{-1}$ 、小于 $10^{-4}s^{-1}$ 、小于 $5 \times 10^{-4}s^{-1}$ 、小于 $10^{-5}s^{-1}$ 、小于 $5 \times 10^{-5}s^{-1}$ 、小于 $10^{-6}s^{-1}$ 、小于 $5 \times 10^{-6}s^{-1}$ 、小于 $5 \times 10^{-7}s^{-1}$ 、小于 $10^{-8}s^{-1}$ 、小于 $5 \times 10^{-8}s^{-1}$ 、小于 $10^{-9}s^{-1}$ 、小于 $5 \times 10^{-9}s^{-1}$ 、或小于 $10^{-10}s^{-1}$ 。

[0196] 在另一个方面中,本披露的抗HER2结合分子(例如,抗HER2抗体或其HER2结合片段、或双特异性抗HER2抗体)结合至HER2和/或其抗原片段,其中缔合速率常数或 k_{on} 速率是至少 $10^5M^{-1}s^{-1}$ 至少 $5 \times 10^5M^{-1}s^{-1}$ 、至少 $10^6M^{-1}s^{-1}$ 、至少 $5 \times 10^6M^{-1}s^{-1}$ 至少 $10^8M^{-1}s^{-1}$ 、至少 $5 \times 10^7M^{-1}s^{-1}$ 、或至少 $10^8M^{-1}s^{-1}$ 、或至少 $10^9M^{-1}s^{-1}$ 。

[0197] 在其他方面中,VH和/或VL氨基酸序列可与以上阐述的序列85%、90%、95%、

96%、97%、98%或99%类似,并且包含1、2、3、4、5或更多个保守取代。具有与SEQ ID NO:16的VH区和/或SEQ ID NO:15的VL区分别具有高(即,80%或更大)相似性的VH区和VL区的在此披露的抗HER2结合分子,可通过诱变(例如,定点或PCR介导的诱变)它们各自的编码核酸分子,接着使用在此描述的功能测定对于保留的功能测试改变的抗体来获得。

[0198] 在此披露的抗HER2结合分子对抗原的亲合力和/或亲合力可使用本领域中熟知的任何适合方法来实验确定,例如,流式细胞术、酶联免疫吸附测定(ELISA)、或放射免疫测定(RIA)、或动力学(例如,BIACORE™分析)。也可以容易地采用直接结合测定以及竞争结合测定。参见,例如在基础免疫学(Fundamental Immunology),保罗W.E.(Paul,W.E.)编著,Raven出版社:纽约,N.Y.(1984)中的博卓夫斯基(Berzofsky)等人,“抗体-抗原相互作用”(“Antibody-Antigen Interactions,”);库彼(Kuby),免疫学(Immunology),W.H.弗里曼(W.H.Freeman)和公司:纽约,N.Y.(1992);以及在此描述的方法。

[0199] 如果在不同条件(例如,盐浓度、pH、温度)下测量,在此披露的具体抗HER2结合分子与HER2抗原的相互作用的测量的亲和力可改变。因此,使用标准化的抗HER2结合分子和抗原溶液和如本领域中所知的标准化的缓冲液并且诸如在此描述的缓冲液来进行亲和力和其他抗原结合参数(例如, K_D 或 K_d 、 k_{on} 、 k_{off})的测量。

[0200] 在本领域中还已知的是使用BIACORE™分析测量的亲和力可取决于哪一种反应物被结合至芯片而改变。在这个方面中,可使用其中靶向的抗HER2结合分子被固定到芯片上的形式(称为“IgG向下”形式)或使用其中靶蛋白质(例如,HER2)被固定到芯片上的形式(称为例如“HER2向下”形式)来测量亲和力。

III. 双特异性抗HER2结合分子

[0201] 本披露还提供包含第一免疫球蛋白抗原结合结构域和第二免疫球蛋白抗原结合结构域的双特异性抗HER2抗体,其中:

(i)该第一免疫球蛋白抗原结合结构域和该第二免疫球蛋白抗原结合结构域特异性结合至不同HER2抗体结合位点;

(ii)该第一免疫球蛋白抗原结合结构域结合至第一HER2抗体结合位点,该第一HER2抗体结合位点包含HER2的结构域II内的表位;并且,

(iii)该第一HER2抗体结合位点与帕妥珠单抗的抗体结合位点不同。

[0202] 在一些方面中,该第一免疫球蛋白抗原结合结构域结合至HER2抗体结合位点,该HER2抗体结合位点包含HER2的结构域II内的表位。在一些方面中,该第一HER2抗体结合位点与1.39.1或39S抗体的HER2抗体结合位点相同。在一些方面中,该第一HER2抗体结合位点与1.39.1或39S抗体的HER2抗体结合位点部分重叠。在其他方面中,该第一HER2抗体结合位点与1.39.1或39S抗体的HER抗体结合位点不同。

[0203] 在一些方面中,该第二免疫球蛋白抗原结合结构域结合至第二HER2抗体结合位点,该第二HER2抗体结合位点包含HER2的结构域IV内的表位。在一些方面中,该第二HER2抗体结合位点与曲妥珠单抗的HER2抗体结合位点相同。在一些方面中,该第二HER2抗体结合位点与曲妥珠单抗的HER2抗体结合位点部分重叠。在其他方面中,该第二HER2抗体结合位点与曲妥珠单抗的HER抗体结合位点不同。

[0204] 在一些方面中,该双特异性抗HER2抗体包含第一免疫球蛋白抗原结合结构域和第二免疫球蛋白抗原结合结构域,其中(i)该第一免疫球蛋白抗原结合结构域和该第二免疫

球蛋白抗原结合结构域特异性结合至不同HER2抗体结合位点,(ii)该第一免疫球蛋白抗原结合结构域结合至第一HER2抗体结合位点,该第一HER2抗体结合位点包含HER2的结构域II内的表位并且与帕妥珠单抗的抗体结合位点不同,并且(iii)该第二免疫球蛋白抗原结合结构域与HERCEPTIN®竞争结合至HER2的结构域IV。

[0205] 本披露还提供结合与以上披露的抗HER2结合分子相同的表位或从以上披露的抗HER2结合分子中衍生的双特异性抗HER2分子(即,从1.39.1亲本抗体例如39S抗体中衍生的前导优化的抗体)。在一些方面中,此类双特异性抗HER2分子是双特异性抗HER2抗体和从此类双特异性抗体中衍生的分子。在一些方面中,从在此描述的这些双特异性抗HER2分子中衍生的此类分子是抗体-药物轭合物(ADC)。在某些方面中,在此提供的这些ADC具有降低的ADCC活性。在一些方面中,从在此描述的这些双特异性抗HER2分子中衍生的此类分子具有增强的ADCC活性。

[0206] 本披露还提供包含第一免疫球蛋白和第二免疫球蛋白抗原结合结构域的双特异性HER抗体,其中(i)该第一免疫球蛋白抗原结合结构域和该第二免疫球蛋白抗原结合结构域特异性结合至不同HER2表位;并且(ii)其中该第一免疫球蛋白抗原结合结构域将HER2结合至SEQ ID NO:52中的一个或多个氨基酸残基上。在一些方面中,该第二免疫球蛋白抗原结合结构域在结构域IV内的表位处结合HER2。在一些方面中,该第二免疫球蛋白抗原结合结构域将HER2结合至SEQ ID NO:53中的一个或多个氨基酸残基上。

[0207] 因此,在一个方面中,本披露提供包含第一免疫球蛋白抗原结合结构域和第二免疫球蛋白抗原结合结构域的双特异性抗HER2抗体,其中该第一免疫球蛋白抗原结合结构域包含重链(HC)可变区(VH)和轻链(LC)可变区(VL),该重链可变区和该轻链可变区包含:

(i)与SEQ ID NO:1相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:1相同的可变重链CDR-1(VH-CDR1)序列;

(ii)与SEQ ID NO:2相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:2相同的可变重链CDR-2(VH-CDR2)序列;

(iii)与SEQ ID NO:3相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:3相同的可变重链CDR-3(VH-CDR3)序列;

(iv)与SEQ ID NO:4相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:4相同的可变轻链CDR-1(VL-CDR1)序列;

(v)与SEQ ID NO:5相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:5相同的可变轻链CDR-2(VL-CDR2)序列;以及,

(vi)与SEQ ID NO:6相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:6相同的可变轻链CDR-3(VL-CDR3)序列;

其中该第二免疫球蛋白抗原结合结构域包含scFv抗体片段;并且,其中该第一免疫球蛋白抗原结合结构域和该第二免疫球蛋白抗原结合结构域特异性结合至不同HER2表位。

[0208] 在一些方面中,该双特异性抗HER2抗体的该第一免疫球蛋白抗原结合结构域包含相对于包含VH(包含SEQ ID NO:43的氨基酸)和VL(包含SEQ ID NO:44的氨基酸)的免疫球蛋白抗原结合结构域的FW区不同的至少一个异源可变结构域构架区(FW)。

[0209] 在一些方面中,该双特异性抗HER2抗体的该第一免疫球蛋白抗原结合结构域包含:(i)包含SEQ ID NO:11的氨基酸的可变轻链构架1(VL-FW1);(ii)包含SEQ ID NO:12的

氨基酸的可变轻链构架2(VL-FW2);(iii)包含SEQ ID NO:13的氨基酸的VL-可变轻链构架3(VL-FW3);(iv)包含SEQ ID NO:14的氨基酸的可变轻链构架4(VL-FW4);或(v)其任何组合。

[0210] 在一些方面中,该双特异性HER2抗体的该第一免疫球蛋白抗原结合结构域包含:(i)由SEQ ID NO:11的氨基酸组成的VL-FW1;(ii)由SEQ ID NO:12的氨基酸组成的VL-FW2;(iii)由SEQ ID NO:13的氨基酸组成的VL-FW3;(iv)由SEQ ID NO:14的氨基酸组成的VL-FW4;或(v)其任何组合。

[0211] 在其他方面中,在此披露的该双特异性抗HER2抗体包含第一免疫球蛋白抗原结合结构域和第二免疫球蛋白抗原结合结构域,其中:

(i)该第一免疫球蛋白抗原结合结构域包含VH和VL,其中该VH包含SEQ ID NO:15或SEQ ID NO:43的氨基酸;

(ii)该第二免疫球蛋白抗原结合结构域包含scFv抗体片段;并且,

(iii)该第一免疫球蛋白抗原结合结构域和该第二免疫球蛋白抗原结合结构域特异性结合至不同HER2表位。

[0212] 在其他方面中,该双特异性抗HER2抗体包含第一免疫球蛋白抗原结合结构域和第二免疫球蛋白抗原结合结构域,其中:

(i)该第一免疫球蛋白抗原结合结构域包含VH和VL,其中该VH由SEQ ID NO:15或SEQ ID NO:43的氨基酸组成;

(ii)该第二免疫球蛋白抗原结合结构域包含scFv抗体片段;并且,

(iii)该第一免疫球蛋白抗原结合结构域和该第二免疫球蛋白抗原结合结构域特异性结合至不同HER2表位。

[0213] 在一些方面中,该双特异性抗HER2抗体包含第一免疫球蛋白抗原结合结构域和第二免疫球蛋白抗原结合结构域,其中:

(i)该第一免疫球蛋白抗原结合结构域包含VH和VL,其中该VL包含SEQ ID NO:16或SEQ ID NO:44的氨基酸;

(ii)该第二免疫球蛋白抗原结合结构域包含scFv抗体片段;并且,

(iii)该第一免疫球蛋白抗原结合结构域和该第二免疫球蛋白抗原结合结构域特异性结合至不同HER2表位。

[0214] 在一些方面中,该双特异性抗HER2抗体包含第一免疫球蛋白抗原结合结构域和第二免疫球蛋白抗原结合结构域,其中:

(i)该第一免疫球蛋白抗原结合结构域包含VH和VL,其中该VL由SEQ ID NO:16或SEQ ID NO:44的氨基酸组成;

(ii)该第二免疫球蛋白抗原结合结构域包含scFv抗体片段;并且,

(iii)该第一免疫球蛋白抗原结合结构域和该第二免疫球蛋白抗原结合结构域特异性结合至不同HER2表位。

[0215] 在一些方面中,该双特异性抗HER2抗体包含第一免疫球蛋白抗原结合结构域和第二免疫球蛋白抗原结合结构域,其中:

(i)该第一免疫球蛋白抗原结合结构域包含VH和VL,其中该VL包含SEQ ID NO:16或SEQ ID NO:44的氨基酸,并且该VH包含SEQ ID NO:15或SEQ ID NO:45的氨基酸;

(ii)该第二免疫球蛋白抗原结合结构域包含scFv抗体片段;并且,

(iii)该第一免疫球蛋白抗原结合结构域和该第二免疫球蛋白抗原结合结构域特异性结合至不同HER2表位。

[0216] 在一些方面中,该双特异性抗HER2抗体包含第一免疫球蛋白抗原结合结构域和第二免疫球蛋白抗原结合结构域,其中:

(i)该第一免疫球蛋白抗原结合结构域包含VH和VL,其中该VL由SEQ ID NO:16或SEQ ID NO:44的氨基酸组成,并且该VH由SEQ ID NO:15或SEQ ID NO:43的氨基酸组成;

(ii)该第二免疫球蛋白抗原结合结构域包含scFv抗体片段;并且,

(iii)该第一免疫球蛋白抗原结合结构域和该第二免疫球蛋白抗原结合结构域特异性结合至不同HER2表位。

[0217] 在一些方面中,该双特异性抗HER2抗体的该第一免疫球蛋白抗原结合结构域包含以下各项或由其组成:

(a)VH和VL,该VH进一步包含重链恒定区或其片段,并且该VL包含轻链恒定区(LC)或其片段;

(b)单链Fv(“scFv”);

(c)双体抗体;

(d)小抗体;

(e)F(ab')₂;或

(f)F(ab)。

[0218] 在一些方面中,该双特异性抗HER2抗体的该重链恒定区或其片段是IgG恒定区。在一些方面中,该IgG恒定区或其片段是IgG1、IgG2、IgG3、或IgG4恒定区。在特定方面中,该IgG恒定区是IgG1恒定区。在一些方面中,该双特异性抗HER2抗体的该第一免疫球蛋白抗原结合结构域包含VL,该VL包含轻链恒定区(LC),其中该LC恒定区是κ恒定区。在一些方面中,该双特异性抗HER2抗体的该第一免疫球蛋白抗原结合结构域包含VL,该VL包含轻链恒定区(LC),其中该LC恒定区是λ恒定区。

[0219] 在一些方面中,该双特异性抗HER2抗体的该第一免疫球蛋白抗原结合结构域是例如单克隆抗体、人源化抗体、嵌合抗体、或亲和力优化的抗体。在一些方面中,该双特异性抗HER2抗体的该第一免疫球蛋白抗原结合结构域是例如人抗体。在一些方面中,该人抗体在转基因小鼠中表达(参见,例如布吕格曼(Bruggemann),“在转基因小鼠中的人抗体表达”(“Human antibody expression in transgenic mice”),实验免疫学与治疗学文献(Arch.Immunol.Therap.Exper.)49:203-208,2001,该参考文献通过引用以其全文结合在此)。

[0220] 在一些方面中,该双特异性抗HER2抗体的该第一免疫球蛋白抗原结合结构域不与曲妥珠单抗或帕妥珠单抗竞争表位结合。在一些方面中,该双特异性抗HER2抗体的该第一免疫球蛋白抗原结合结构域和该第二免疫球蛋白抗原结合结构域特异性结合至不同HER2表位。在一些方面中,这些不同HER2表位是非重叠的。

[0221] 在一些方面中,该双特异性抗HER2抗体的该第二免疫球蛋白抗原结合结构域(i)特异性结合至与曲妥珠单抗抗体相同的HER2表位;和/或(ii)通过曲妥珠单抗抗体竞争性地抑制HER2结合;和/或(iii)包含至少一个、至少两个、至少三个、至少四个、至少五个、或至少六个互补决定区(CDR),这些互补决定区包含SEQ ID NO:54至59中的任一个的氨基酸。

[0222] 在一些方面中,该双特异性抗HER2抗体的该第二免疫球蛋白抗原结合结构域包含scFv。在一些特定方面中,该双特异性抗HER2抗体的该第二免疫球蛋白抗原结合结构域包含二硫化物稳定的scFv(ds-scFv)。在一些方面中,该二硫化物稳定的scFv特异性结合至与曲妥珠单抗抗体相同的HER2表位。

[0223] 可通过在选择使得半胱氨酸残基可形成二硫键的位置处引入半胱氨酸取代而在scFv的VH区和VL区之间对稳定的二硫化物进行工程化。具体地说,可在构架区中引入这种二硫化物以使得VL区和VH区通过二硫键来连接。表2中提供了VH区和VL区中满足这些标准的代表性但非限制性的残基的位置。

表2:VH-VL对

VH44+VL100	VH55+VL101	VH101+VL46
VH44+VL105	VH100+VL50	VH105+VL43
VH45+VL87	VH98+VL46	VH106+VL57

表2中的编号根据在卡巴特中所阐述的卡巴特索引。将理解,在这些位置处的野生型氨基酸残基将发生变化。无论是否是野生型氨基酸残基,给定对的每个位置都将由半胱氨酸取代。

[0224] 在此披露的scFv可获自或通过任何适合的来源(无论天然与否)产生,或它可以是重组scFv、合成scFv、半合成scFv、衍生化的scFv、发酵优化的scFv、其融合蛋白或等效物、突变体以及衍生物,只要它保持本披露的scFv要求的结合特异性。这些包括具有氨基酸取代或具有附接至氨基酸官能团上的糖或其他分子的带有结合特异性的scFv。关于scFv如在此所使用的术语“衍生物”或“衍生化”包括scFv的化学改性。此类改性的示例将是通过烷基、酰基、或氨基基团来替换氢。

[0225] 在一些方面中,该第二免疫球蛋白抗原结合结构域是scFv,该scFv包含:

- (i)包含SEQ ID NO:54的氨基酸的VH-CDR1;
- (ii)包含SEQ ID NO:55的氨基酸的VH-CDR2;
- (iii)包含SEQ ID NO:56的氨基酸的VH-CDR3;
- (iv)包含SEQ ID NO:57的氨基酸的VL-CDR1;
- (v)包含SEQ ID NO:58的氨基酸的VL-CDR2;以及,
- (vi)包含SEQ ID NO:59的氨基酸的VL-CDR3。

[0226] 在一些方面中,该双特异性抗HER2抗体的该第二免疫球蛋白抗原结合结构域由特异性结合至与曲妥珠单抗抗体相同的HER2表位的scFv组成,并且包含从曲妥珠单抗抗体的VH和VL中衍生的VH和VL(例如,存在于曲妥珠单抗抗体中的天然VH和/或VL、或具有稳定突变例如在表2中示出的突变对的VH和/或VL突变体)。参见高登格(Goldenger),临床治疗(Clin. Ther.)21:309-18(1999)。因此,在一些方面中,结合与曲妥珠单抗相同表位的该scFv包含VH和VL,该VH包含SEQ ID NO:17的氨基酸并且该VL包含SEQ ID NO:18的氨基酸。在一些方面中,结合与曲妥珠单抗相同表位的该scFv包含VH和VL,该VH由SEQ ID NO:17的氨基酸组成并且该VL由SEQ ID NO:18的氨基酸组成。在一些特定方面中,结合与曲妥珠单抗相同表位的该scFv的该VH和该VL通过肽接头共价连接。在一些方面中,该肽接头包含SEQ ID NO:19的氨基酸。在一些方面中,该肽接头由SEQ ID NO:19的氨基酸组成。

[0227] 如上所讨论,本领域技术人员将了解,结合与曲妥珠单抗相同表位的scFv包括从

曲妥珠单抗衍生的序列,从而包含例如突变序列,其中相对于曲妥珠单抗抗体中的亲本序列,至少一个氨基酸被缺失或取代,只要所得分子能够特异性结合至与曲妥珠单抗抗体相同的HER2表位,例如被设计成在该scFv的VH与VL之间引入至少一个稳定二硫化物的突变。

[0228] 在一些方面中,该双特异性抗HER2抗体的该第二免疫球蛋白抗原结合结构域被共价连接至该第一免疫球蛋白抗原结合结构域的HC的羧基末端上。在一些方面中,该双特异性抗HER2抗体包含插入在该第二免疫球蛋白抗原结合结构域与该第一免疫球蛋白抗原结合结构域的HC的羧基末端之间的至少一个接头。在一些特定方面中,一个接头被插入在该第二免疫球蛋白抗原结合结构域与该第一免疫球蛋白抗原结合结构域的HC的羧基末端之间。

[0229] 在一些方面中,该第二免疫球蛋白抗原结合结构域被共价连接至该第一免疫球蛋白抗原结合结构域的HC的氨基末端上。在一些方面中,该双特异性抗HER2抗体包含插入在该第二免疫球蛋白抗原结合结构域与该第一免疫球蛋白抗原结合结构域的HC的氨基末端之间的至少一个接头。在特定方面中,一个接头被插入在该第二免疫球蛋白抗原结合结构域与该第一免疫球蛋白抗原结合结构域的HC的氨基末端之间。

[0230] 在一些方面中,该双特异性抗HER2抗体的该第二免疫球蛋白抗原结合结构域被共价嵌入在该第一免疫球蛋白抗原结合结构域的HC的序列中。在一些方面中,该双特异性抗HER2抗体的该第二免疫球蛋白抗原结合结构域被共价嵌入在该第一免疫球蛋白抗原结合结构域的HC的CH1区与CH2区之间。在一些方面中,一个或多个接头将该双特异性抗HER2抗体的该第二免疫球蛋白抗原结合结构域连接至该第一免疫球蛋白抗原结合结构域的HC的CH1区和/或CH2区上。

[0231] 在一些特定方面中,该双特异性抗HER2抗体包含(i)插入在该第一免疫球蛋白抗原结合结构域的HC的CH1区与该第二免疫球蛋白抗原结合结构域之间的接头;以及(ii)插入在该第二免疫球蛋白抗原结合结构域与该第一免疫球蛋白抗原结合结构域的HC的CH2区之间的第二接头。在一些方面中,该第一接头和该第二接头是相同的。在一些方面中,该第一接头和该第二接头是不同的。在一些方面中,这些接头中的一个或多个包括肽接头。在一些方面中,该肽接头包含至少一个、至少两个、至少三个、至少四个、至少五个、至少10个、至少15个、至少20个、至少25个、或至少30个氨基酸。在一些方面中,该肽接头包含多于20个氨基酸。在一些方面中,该肽接头包括具有化学式 $\text{Ser}_x[(\text{Gly})_y-\text{Ser}_4]_z$ 的肽,其中x是从0至1,y是从1至4,并且z是从1至10。在一些方面中,该肽接头包含选自SEQ ID NO:19、20、21或22的序列。

[0232] 在一些方面中,该双特异性抗HER2抗体包含重链,该重链包含包括Fc结构域的恒定区。在一些方面中,该Fc结构域包含CH2区、和/或CH3区、和/或其片段。在一些方面中,该Fc结构域包含CH2区和CH3区。在一些方面中,该Fc结构域由CH2区和CH区组成。在一些方面中,该Fc结构域是IgG Fc结构域,例如,来自IgG1、IgG2、IgG3、或IgG4的Fc结构域。在一些方面中,该IgG Fc结构域是人或人源化IgG Fc结构域。在一些方面中,该Fc结构域是IgG1 Fc结构域。

[0233] 在一些方面中,该IgG Fc结构域例如IgG1 Fc结构域是天然(野生型)结构域。在一些方面中,该天然IgG1 Fc结构域包含SEQ ID NO:23的氨基酸。在一些方面中,该天然IgG1 Fc结构域由SEQ ID NO:23的氨基酸组成。在其他方面中,该Fc结构域是突变IgG结构域,例

如,突变IgG1、IgG2、IgG3、或IgG4结构域。在一些特定方面中,该突变Fc结构域是突变IgG1 Fc结构域。

[0234] 在一些方面中,该突变IgG结构域例如人或人源化IgG1 Fc结构域包含能够降低该双特异性抗HER2抗体的ADCC活性的至少一个突变。在某些方面中,能够降低该抗HER2双特异性抗体的ADCC活性的至少一个突变是氨基酸取代。在一些方面中,具有降低的ADCC活性的该双特异性抗HER2抗体包含选自以下各项的至少一个氨基酸取代:L234F、S239C、S239A、位置239与240之间的半胱氨酸氨基酸插入、或其任何组合,其中氨基酸位置编号根据在卡巴特中所阐述的EU索引。能够降低抗体的ADCC活性的众多突变是本领域中已知的。例如,参见描述于W02012175751、W02011149999、W02011066501、W02000042072、W02011120134中的突变,这些专利通过引用以其全文结合在此。具有降低的ADCC效应子功能的抗体还包括具有Fc区残基238、265、269、270、297、327以及329中的一个或多个的取代的那些(美国专利号6,737,056),其中氨基酸位置编号根据在卡巴特中所阐述的EU索引。此类Fc突变体还包括具有在氨基酸位置265、269、270、297以及327中的两个或更多个处的取代的Fc突变体,包括具有残基265和297至丙氨酸的取代的Fc突变体(美国专利号7,332,581,其中氨基酸位置编号根据在卡巴特中所阐述的EU索引)。

[0235] 在一些方面中,该突变IgG结构域例如人或人源化IgG1 Fc结构域包含能够增强该双特异性抗HER2抗体的ADCC活性的至少一个突变。在某些方面中,能够增强该抗HER2双特异性抗体的ADCC活性的至少一个突变是氨基酸取代。在一些方面中,具有增强的ADCC活性的该双特异性抗HER2抗体包含选自以下各项的至少一个氨基酸取代:S239A、S239D、A330L、I332E、E333A、K334A或其任何组合,其中氨基酸位置编号根据在卡巴特中所阐述的EU索引。增强ADCC活性的另外突变是本领域技术人员已知的,包括但不限于以下各项中的那些:在US 6,737,056的表2和表6-10中举例说明的那些;在US 2006/024298的图41中呈现的表格;在US 2006/235208的图5、图12以及图15中呈现的表格;在US 2006/0173170的图8、图9以及图10中呈现的表格以及W0 09/058492的图8、图9以及图10中呈现的表格。

[0236] 在一些方面中,该突变IgG1 Fc结构域可以包含引入可衍生官能团的至少一个氨基酸取代。在一些方面中,该突变IgG1 Fc结构域包含引入可衍生官能团的一至三个氨基酸取代。在一些方面中,该可衍生基团是半胱氨酸氨基酸的氢硫基侧链。在具体方面中,该取代的氨基酸或多个氨基酸发生在该抗HER2结合分子的可接近位点处。通过使用半胱氨酸取代那些氨基酸残基,从而反应性巯基基团被定位在该抗HER2结合分子的可接近位点处并且可以被用来将该抗HER2结合分子辄合至其他部分(诸如药物部分或接头-药物部分)上以便产生如在此进一步所述的免疫辄合物。半胱氨酸工程化的抗体可以如例如在美国专利号7,521,541中所述的来产生。

[0237] 在一些方面中,该可衍生基团被引入在卡巴特位置239、248、254、258、273、279、282、284、286、287、289、297、298、312、324、326、330、335、337、339、350、355、356、359、360、361、375、383、384、389、398、400、413、415、418、422、435、440、441、442、443或446处、在位置239与240之间插入的氨基酸处、或其任何组合,其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引。在一些方面中,引入可衍生氢硫基基团的氨基酸或氨基酸取代选自下组,该组由以下各项组成:S239C、248C、254C、258C、273C、279C、282C、284C、286C、287C、289C、297C、298C、312C、324C、326C、330C、335C、337C、339C、350C、355C、356C、359C、360C、361C、

375C、383C、384C、389C、398C、400C、413C、415C、418C、422C、435C、440C、441C、S442C、443C以及446C、位置239与240之间的半胱氨酸插入、或其任何组合,其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引。在一些方面中,引入可衍生氢硫基基团的氨基酸或氨基酸取代是S239C和/或S442C。

[0238] 选择性可衍生的基团是本领域中熟知的,诸如氨基基团、氢硫基基团、侧链氧氨基、或其他亲核基团。可衍生基团可以通过一个或多个接头连接至多肽链上。配体(例如,治疗部分、可检测标记、半衰期延长聚合物等)可以使用适当的附接化学物附接至可衍生基团上。这种偶联化学物可以包括,例如酰胺、脲、硫脲、脞、氨基乙酰胺等。

[0239] 在一些方面中,该Fc结构域具有增强ADCC活性的改变类型的糖基化。该Fc区的糖基化可以被修饰成增加或降低效应子功能(参见例如,优玛娜(Umana)等人,1999,自然生物技术(Nat. Biotechnol)17:176-180;戴维斯(Davies)等人,2001,生物技术与生物工程(Biotechnol Bioeng)74:288-294;希尔兹(Shields)等人,2002,生物化学杂志(J Biol Chem)277:26733-26740;新川(Shinkawa)等人,2003,生物化学杂志278:3466-3473;美国专利号6,602,684;6,946,292;7,064,191;7,214,775;7,393,683;7,425,446;7,504,256;美国公开号2003/0157108;2003/0003097;2009/0010921;POTELLEAGENT™技术(Biowa公司,新泽西州普林斯顿(Princeton, N.J.));GLYCOMAB™糖基化工程化技术(GLYCART生物技术股份有限公司(GLYCART biotechnology AG),瑞士苏黎世(Zurich, Switzerland)))。在一些方面中,该Fc结构域是具有降低量的岩藻糖基残基的低度岩藻糖基化抗体Fc结构域(参见例如美国专利申请公开号2005/0226867)。在一个方面中,如宿主细胞(例如,CHO细胞,浮萍(Lemna minor))中产生的、具有增加的效应子功能(尤其是ADCC)的这些抗体被工程化来产生ADCC比通过亲本细胞产生的抗体高出100倍以上的高度去岩藻糖基化的抗体(例如,莫里(Mori)等人,2004,生物技术与生物工程88:901-908;考克斯(Cox)等人,2006,自然生物技术24:1591-7)。在一些方面中,该Fc结构域具有增加的等分GlcNAc结构(例如,优玛娜等人,1999,自然生物技术17:176-180;US2009/0010921)。

[0240] 在一些方面中,该突变Fc结构域包含SEQ ID NO:24A、24C、25A或25C的氨基酸。

[0241] 在一些方面中,该突变Fc结构域包含SEQ ID NO:24B或SEQ ID NO:25B的氨基酸。

[0242] 在本披露中还提供包含彼此相关联的第一多肽链和第二多肽链的双特异性抗HER2抗体,其中该第一多肽链选自以下各项:

- (1)[TZ_s]-[L₁]-[BVH]-[BCH]-[Fc_x]
- (2)[BVH]-[BCH]-[Fc_x]-[L₂]-[TZ_s]
- (3)[BVH]-[BCH]-[L₃]-[TZ_s]-[L₄]-[Fc_x]

其中

TZ_s是结合与由曲妥珠单抗抗体识别的相同表位的scFv;

L₁、L₂、L₃以及L₄是肽接头;

Fc_x是Fc结构域;

BVH和BCH分别是抗体的VH区和CH1区,该抗体能够结合至与曲妥珠单抗抗体识别的表位不同的HER2表位。

[0243] 在一些方面中,该不同的表位包含SEQ ID NO:52内的一个或多个氨基酸。

[0244] 在一些方面中,曲妥珠单抗抗体识别的表位包含SEQ ID NO:53中的一个或多个氨

基酸残基。

[0245] 在一些方面中,该第二多肽链包含_BVL-[CL],其中_BVL是能够结合至与曲妥珠单抗抗体识别的表位不同的HER2表位的抗体的VL区,并且CL是IgG轻链恒定区。在一些方面中,CL选自下组,该组由以下各项组成:人κ恒定区和人λ恒定区。

[0246] 在一些方面中,_BVL包含SEQ ID NO:16的氨基酸。在一些方面中,_BVL包含SEQ ID NO:44的氨基酸。在一些方面中,_BVL包含:

(i)与SEQ ID NO:4相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:4相同的可变轻链CDR-1(VL-CDR1);

(ii)与SEQ ID NO:5相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:5相同的可变轻链CDR-2(VL-CDR2);以及,

(iii)与SEQ ID NO:6相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:6相同的可变轻链CDR-3(VL-CDR3)。

[0247] 在一些特定方面中,CL包含SEQ ID NO:27A的氨基酸。

[0248] 在一些特定方面中,CL包含SEQ ID NO:27B的氨基酸。

[0249] 在一些方面中,[TZ_s]包含:

(i)包含SEQ ID NO:54的氨基酸的VH-CDR1;

(ii)包含SEQ ID NO:55的氨基酸的VH-CDR2;

(iii)包含SEQ ID NO:56的氨基酸的VH-CDR3;

(iv)包含SEQ ID NO:57的氨基酸的VL-CDR1;

(v)包含SEQ ID NO:58的氨基酸的VL-CDR2;以及

(vi)包含SEQ ID NO:59的氨基酸的VL-CDR3。

[0250] 在一些方面中,[TZ_s]是二硫化物稳定的scFv。在一些方面中,[TZ_s]包含通过肽接头共价连接的(i)VH,该VH包含SEQ ID NO:17的氨基酸或其变体或由其组成,以及(ii)VL,该VL包含SEQ ID NO:18的氨基酸或其变体或由其组成。适用于连接scFv的VH和VL部分的众多接头(例如,肽接头)是本领域中已知的。在一些方面中,该接头是包含SEQ ID NO:19的氨基酸或由其组成的肽接头。在其他方面中,[TZ_s]包含SEQ ID NO:28的氨基酸或由其组成。

[0251] 在一些方面中,铰链多肽连接_BCH和[Fcx]。在一些方面中,该铰链多肽包含SEQ ID NO:26的氨基酸或由其组成。在一些方面中,该[Fcx]包含引入可衍生基团的至少一个氨基酸取代。在其他方面中,该[Fcx]包含引入可衍生基团的一至三个氨基酸取代。在又其他方面中,该[Fcx]包含引入可衍生基团的多于三个氨基酸取代。在一些方面中,所有可衍生基团是相同的。在其他方面中,至少一个可衍生基团与其余的可衍生基团不同。在一些方面中,所有可衍生基团是不同的。在一些方面中,该可衍生基团是氢硫基基团(例如,半胱氨酸的氢硫基基团)。在一些方面中,该可衍生基团是被保护的。

[0252] 在一些方面中,该可衍生基团被引入在位置239、248、254、258、273、279、282、284、286、287、289、297、298、312、324、326、330、335、337、339、350、355、356、359、360、361、375、383、384、389、398、400、413、415、418、422、435、440、441、442、443或446处、或在位置239与240之间、或其任何组合,其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引。在一些方面中,该可衍生基团是至少一个半胱氨酸氨基酸取代中的氢硫基基团,该氨基酸取代包括S239C、248C、254C、258C、273C、279C、282C、284C、286C、287C、289C、297C、298C、312C、

324C、326C、330C、335C、337C、339C、350C、355C、356C、359C、360C、361C、375C、383C、384C、389C、398C、400C、413C、415C、418C、422C、435C、440C、441C、S442C、443C以及446C、位置239与240之间的半胱氨酸插入、或其任何组合,其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引。在一些方面中,引入可衍生氢硫基基团的氨基酸或氨基酸取代是S239C和/或S442C。在一些方面中,引入可衍生氢硫基基团的氨基酸或氨基酸取代是位置239与240之间的半胱氨酸氨基酸插入和/或S442C。

[0253] 在其他方面中,[Fc_x]包含SEQ ID NO:23、24A、24C、25A或25C中的任一个的氨基酸。

[0254] 在其他方面中,[Fc_x]包含SEQ ID NO:23、24B或25B中的任一个的氨基酸。

[0255] 在某些方面中,[L₁],[L₂],[L₃]以及[L₄]包含独立地选自下组的接头序列的氨基酸,该组由以下各项组成:SEQ ID NO:19、20、21以及22。在一些方面中,所有接头是不同的。在一些方面中,这些接头中的至少两个是相同的。本领域技术人员将理解,这些接头可以是肽接头、非肽接头、或肽接头和非肽接头的组合。在一些特定方面中,(i)[L₁]包含SEQ ID NO:20的氨基酸或由其组成;(ii)[L₂]包含SEQ ID NO:20的氨基酸或由其组成;(iii)[L₃]包含SEQ ID NO:21的氨基酸或由其组成;并且(iv)[L₄]包含SEQ ID NO:22的氨基酸或由其组成。

[0256] 在一些方面中,[B_{VH}]包含:

(i)与SEQ ID NO:1相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:1相同的可变重链CDR-1(VH-CDR1);

(ii)与SEQ ID NO:2相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:2相同的可变重链CDR-2(VH-CDR2);以及

(iii)与SEQ ID NO:3相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:3相同的可变重链CDR-3(VH-CDR3)。

[0257] 在一些方面中,[B_{VH}]包含SEQ ID NO:15或SEQ ID NO:43的氨基酸或由其组成。

[0258] 在一些方面中,[B_{VL}]包含:

(i)与SEQ ID NO:4相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:4相同的可变轻链CDR-1(VL-CDR1);

(ii)与SEQ ID NO:5相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:5相同的可变轻链CDR-2(VL-CDR2);以及,

(iii)与SEQ ID NO:6相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:6相同的可变轻链CDR-3(VL-CDR3)。

[0259] 在一些特定方面中,[B_{VL}]包含SEQ ID NO:16或SEQ ID NO:44的氨基酸或由其组成。在一些方面中,[B_{CH}]包含SEQ ID NO:29的氨基酸或由其组成。

[0260] 在一些特定方面中,本披露提供包含第一多肽链和第二多肽链的双特异性抗HER2抗体,其中(i)该第一多肽链包含选自下组的氨基酸序列或由其组成,该组由以下各项组成:SEQ ID NO:30、31A、32A、32C、33A、33C、34、34A、35A、36A、36C、37A、37C、38、38A、39A、40A、40C、41A以及41C,并且(ii)该第二多肽链包含SEQ ID NO:42A或42B的序列或由其组成,其中该双特异性抗HER2抗体被轭合至治疗部分上。

[0261] 在一些特定方面中,本披露提供包含第一多肽链和第二多肽链的双特异性抗HER2

抗体,其中(i)该第一多肽链包含选自下组的氨基酸序列或由其组成,该组由以下各项组成:SEQ ID NO:30、31B、32B、33B、34B、35B、36B、37B、38B、39B、40B以及41B,并且(ii)该第二多肽链包含SEQ ID NO:42A或42B的序列或由其组成,其中双特异性抗HER2抗体具有增强的ADCC活性。

IV. 抗体-药物轭合物(ADC)

[0262] 本披露还提供抗体-药物轭合物(ADC),该抗体-药物轭合物包含轭合至至少一个治疗部分上的在此披露的抗HER2结合分子(例如,结合与39S抗体相同表位或从39S抗体中衍生的抗体或其HER2结合片段、或在此披露的双特异性抗HER2抗体)中的至少一个。因此,在一些方面中,该ADC包含轭合至至少一个治疗部分(例如,细胞毒素)上的在此披露的双特异性抗HER2抗体,其中所述双特异性抗HER2抗体包含(i)第一免疫球蛋白抗原结合结构域和(ii)第二免疫球蛋白抗原结合结构域,其中该第一免疫球蛋白抗原结合结构域包含重链(HC)可变区(VH)和轻链(LC)可变区(VL),该重链可变区和该轻链可变区包含:

(i)与SEQ ID NO:1相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:1相同的可变重链CDR-1(VH-CDR1)序列;

(ii)与SEQ ID NO:2相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:2相同的可变重链CDR-2(VH-CDR2)序列;

(iii)与SEQ ID NO:3相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:3相同的可变重链CDR-3(VH-CDR3)序列;

(iv)与SEQ ID NO:4相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:4相同的可变轻链CDR-1(VL-CDR1)序列;

(v)与SEQ ID NO:5相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:5相同的可变轻链CDR-2(VL-CDR2)序列;以及,

(vi)与SEQ ID NO:6相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:6相同的可变轻链CDR-3(VL-CDR3)序列;

其中:

(a)该第二免疫球蛋白抗原结合结构域包含scFv抗体片段;并且,

(b)该第一免疫球蛋白抗原结合结构域和该第二免疫球蛋白抗原结合结构域特异性结合至不同HER2表位。

[0263] 在一些方面中,该ADC的该第一免疫球蛋白抗原结合结构域包含相对于包含VH(包含SEQ ID NO:43的氨基酸)和VL(包含SEQ ID NO:44的氨基酸)的免疫球蛋白抗原结合结构域的FW区不同的至少一个异源可变结构域构架区(FW)。

[0264] 在一些方面中,该ADC进一步包含至少一个任选的间隔物,该至少一个任选的间隔物可被嵌入在抗HER2结合分子的多肽链中的氨基酸侧链(例如,在此披露的抗HER2双特异性抗体的重链中的氨基)与治疗部分之间。在一些方面中,该至少一个间隔物是肽间隔物。在其他方面中,该至少一个间隔物是非肽间隔物。在一些方面中,该间隔物是不稳定的,诸如酸不稳定的间隔物(例如,胍)。在其他方面中,该间隔物是酶可裂解的肽,例如可裂解的二肽。在一些方面中,该间隔物是不可裂解的(水解稳定的),例如硫醚间隔物或位阻的二硫化物间隔物。在一些方面中,嵌入在抗HER2结合分子的多肽链中的氨基酸侧链(例如,在此披露的抗HER2双特异性抗体的重链中的氨基)与另外的治疗部分之间的是MCC(N-琥珀酰亚

胺基-4(马来酰亚胺基甲基)环己烷))。

[0265] 水解稳定的间隔物在水中是基本上稳定的并且在有用的pH值下(包括但不限于在生理条件下持续延长的时间周期)不与水反应。水解不稳定或可降解的间隔物在水中或在水溶液(包括例如血液)中可降解。

[0266] 酶促不稳定或可降解的间隔物可被一种或多种酶降解。仅通过举例,PEG和相关的聚合物可以包括聚合物骨架中的可降解间隔物或在聚合物骨架与聚合物分子的末端官能团中的一个或多个之间的接头基团中的可降解间隔物。此类可降解间隔物包括,但不限于由PEG羧酸或活化的PEG羧酸与生物活性剂上的醇基团的反应形成的酯键,其中此类酯基团通常在生理条件下水解以释放该生物活性剂。其他水解可降解的间隔物包括但不限于碳酸酯键;由胺和醛的反应产生的亚胺键;由醇与磷酸盐基团反应形成的磷酸酯键;作为酰肼和醛的反应产物的腙键;作为醛和醇的反应产物的缩醛键;作为甲酸盐和醇的反应产物的原酸酯键;由包括但不限于聚合物诸如PEG的一端处的胺基团和肽的羧基基团形成的肽键;以及由包括但不限于聚合物的一端处的亚磷酰胺基团和寡核苷酸的5'羟基基团形成的寡核苷酸键。

[0267] 在一些方面中,该ADC包含两个、三个、四个、五个、六个、七个、八个、九个或十个治疗部分。在一些特定方面中,该ADC包含两个、三个、或四个治疗部分。在一些方面中,所有治疗部分是相同的。在一些方面中,至少一个治疗部分与其余的治疗部分不同。在一些方面中,所有治疗部分是不同的。在一些方面中,所有间隔物(例如,肽间隔物和/或非肽间隔物)是相同的。在一些方面中,至少一个间隔物与其余的间隔物不同。在还其他方面中,所有间隔物是不同的。

[0268] 在一些方面中,每个治疗部分被化学耦联至该抗HER2结合分子(例如,在此披露的双特异性抗HER2抗体)的Fc区中的特定位置处的氨基酸侧链上。

[0269] 在一些方面中,Fc区中的特定位置选自下组,该组由以下各项组成:239、248、254、258、273、279、282、284、286、287、289、297、298、312、324、326、330、335、337、339、350、355、356、359、360、361、375、383、384、389、398、400、413、415、418、422、435、440、441、442、443、446、在位置239与240之间的插入、以及其任何组合,其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引。

[0270] 在一些方面中,Fc区中的特定位置是239、442或两者,其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引。在一些方面中,Fc区中的特定位置由442和位置239与240之间的氨基酸插入组成,其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引。

[0271] 在一些方面中,其中耦联治疗部分的氨基酸侧链是氢硫基侧链,例如半胱氨酸氨基酸的氢硫基基团。在一些方面中,至少一个治疗部分被化学耦联至位于该抗HER2结合分子(例如,在此披露的双特异性抗HER2抗体)的Fc区外的位置处的氨基酸侧链上。在一些方面中,所有治疗部分被化学耦联至位于该抗HER2结合分子(例如,在此披露的双特异性抗HER2抗体)的Fc区外的位置处的氨基酸侧链上。在一些方面中,使用本领域中已知的重组技术将至少一个治疗部分基因掺入到该抗HER2结合分子(例如,在此披露的双特异性抗HER2抗体)的多肽链中。

[0272] 在一些方面中,该治疗部分包括细胞毒素、放射性同位素、放射性同位素、免疫调节剂、细胞因子、淋巴因子、趋化因子、生长因子、肿瘤坏死因子、激素、激素拮抗剂、酶、寡核

苷酸、DNA、RNA、siRNA、RNAi、微小RNA、光敏治疗剂、抗血管生成剂、促凋亡剂、肽、脂质、碳水化合物、螯合剂或其组合。

[0273] 在一些特定方面中,该细胞毒素是奥瑞他汀、微管溶素、关登木素生物碱或吡咯并苯并二氮杂草(PBD)。在另一个特定方面中,该细胞毒素是微管溶素1508。

[0274] 在特定方面中,该ADC包含在此披露的双特异性抗HER2抗体,其中所述抗体包含:

(i)包含SEQ ID NO:32A的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链,其中该第一多肽链包含共价连接至位置239处的半胱氨酸氨基酸上的治疗部分(例如,微管溶素1508分子),其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

(ii)包含SEQ ID NO:33A的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链,其中该第一多肽链包含共价连接至分别位于位置239和442处的半胱氨酸氨基酸上的两个治疗部分(例如,两个微管溶素1508分子),其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

(iii)包含SEQ ID NO:36A的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链,其中该第一多肽链包含共价连接至位置239处的半胱氨酸氨基酸上的治疗部分(例如,微管溶素1508分子),其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

(iv)包含SEQ ID NO:37A的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链,其中该第一多肽链包含共价连接至分别位于位置239和442处的半胱氨酸氨基酸上的两个治疗部分(例如,两个微管溶素1508分子),其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

(v)包含SEQ ID NO:40A的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链,其中该第一多肽链包含共价连接至位置239处的半胱氨酸氨基酸上的治疗部分(例如,微管溶素1508分子),其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;或,

(vi)包含SEQ ID NO:41A的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链,其中该第一多肽链包含共价连接至分别位于位置239和442处的半胱氨酸氨基酸上的两个治疗部分(例如,两个微管溶素1508分子),其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引。

[0275] 在特定方面中,该ADC包含在此披露的双特异性抗HER2抗体,其中所述抗体包含:

(i)包含SEQ ID NO:62的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链,其中该第一多肽链包含共价连接至位置239与240之间插入的半胱氨酸氨基酸上的治疗部分(例如,微管溶素1508分子),其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

(ii)包含SEQ ID NO:33C的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链,其中该第一多肽链包含共价连接至位置239与240之间插入的半胱氨酸氨基和位于位置442处的半胱氨酸氨基酸上的两个治疗部分(例如,两个微管溶素1508分子),其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

(iii)包含SEQ ID NO:36C的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的

氨基酸或由其组成的第二多肽链,其中该第一多肽链包含共价连接至位置239与240之间插入的半胱氨酸氨基酸上的治疗部分(例如,微管溶素1508分子),其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

(iv)包含SEQ ID NO:37C的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链,其中该第一多肽链包含共价连接至位置239与240之间插入的半胱氨酸氨基和位于位置442处的半胱氨酸氨基酸上的两个治疗部分(例如,两个微管溶素MEDI 1508分子),其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

(v)包含SEQ ID NO:40C的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链,其中该第一多肽链包含共价连接至位置239与240之间插入的半胱氨酸氨基酸上的治疗部分(例如,微管溶素1508分子),其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;或,

(vi)包含SEQ ID NO:41C的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链,其中该第一多肽链包含共价连接至位置239与240之间插入的半胱氨酸氨基和位于位置442处的半胱氨酸氨基酸上的两个治疗部分(例如,两个微管溶素1508分子),其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引。

[0276] 在此披露的ADC分子包含被衍生化或连接(例如,化学地或重组地)至另一个分子(例如,肽、小药物分子、可检测分子等)上的在此披露的抗HER2结合分子(例如,结合与39S抗体相同表位或从39S抗体中衍生的抗体或其HER2结合片段、或在此披露的双特异性抗HER2抗体)中的至少一种。通常,抗HER2抗体或其部分被衍生化使得它们的HER2结合不受衍生化或标记的不利影响。因此,本披露的抗HER2抗体和抗体部分意图包括在此描述的抗HER2结合分子的完整形式和修饰形式两者。例如,在此披露的抗HER2结合分子或其HER2结合部分可以被功能连接(通过化学偶联、基因融合、非共价缔合、或以另外的方式)至一个或多个其他分子实体上,诸如细胞毒性剂、药物试剂、检测剂、和/或可以介导该抗HER2结合分子与另一个分子(诸如抗生物素蛋白链菌素核心区或聚组氨酸标签)的缔合的蛋白质或肽。

[0277] 一种类型的衍生化的分子可以通过交联两个或更多个分子实体例如在此披露的抗HER2结合分子和治疗部分(例如,细胞毒素诸如微管溶素1508)来产生。适合的交联剂包括杂双官能性(即,具有被适当的间隔物分开的两个不同反应性基团)的那些(例如,间-马来酰亚胺苯甲酰基-N-羟基琥珀酰亚胺酯);或同双官能性的那些(例如,二琥珀酰亚胺基辛二酸酯)。此类交联剂可例如购自Pierce化学公司,罗克福德(Rockford),IL。另外的双官能偶联剂包括N-琥珀酰亚胺基-3-(2-吡啶基二巯基)丙酸酯(SPDP)、琥珀酰亚胺基-4-(N-马来酰亚胺甲基)环己烷-1-羧酸酯、亚胺基硫烷(iminothiolane)(IT)、亚胺酸酯的双官能衍生物(诸如盐酸二甲基己二酸亚氨酯)、活性酯类(诸如二琥珀酰亚胺基辛二酸酯)、醛类(诸如戊二醛)、双-叠氮化合物(诸如双(对-叠氮基苯甲酰基)己二胺)、双-重氮衍生物(诸如双-(对-重氮苯甲酰基)-乙二胺)、二异氰酸酯类(诸如亚甲代苯基2,6-二异氰酸酯)、以及双-活性氟化合物(诸如1,5-二氟-2,4-二硝基苯)。

[0278] 另一种类型的衍生化的分子可以通过掺入可检测标记来产生。有用的检测剂包括荧光化合物(例如,荧光素、异硫氰酸荧光素、罗丹明、5-二甲胺-1-萘磺酰氯、藻红蛋白、镧系元素磷光体等)、可用于检测的酶类(例如,辣根过氧化物酶、 β -半乳糖苷酶、荧光素酶、碱性磷酸酶、葡萄糖氧化酶等)、由二级报告分子识别的表位(例如,亮氨酸拉链对序列、二级

抗体的结合位点、金属结合结构域、表位标签等)。在一些方面中,可检测标记可以通过至少一个间隔物臂来附接。间隔物臂可以具有不同的长度以便减少潜在的空间位阻。

[0279] 抗HER2结合分子还可以用放射性标记的氨基酸来标记。放射性标记可以用于诊断目的和治疗目的两者。例如,放射性标记可以用来通过X-射线或其他诊断技术诸如正电子发射断层摄影术(PET)来检测HER2表达的细胞。

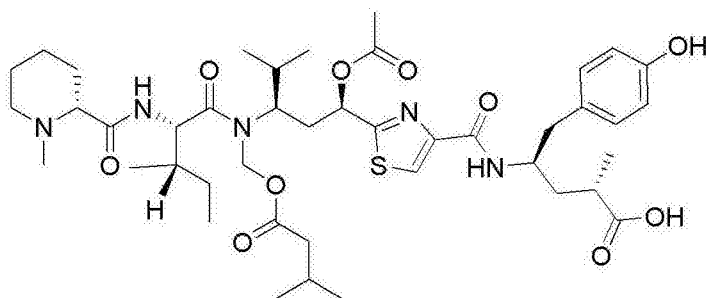
[0280] 此外,放射性标记可以被治疗用作HER2表达的细胞的毒素,诸如引起不希望的免疫应答的那些。多肽的标记的实例包括,但不限于以下放射性同位素或放射性核素: ^3H 、 ^{14}C 、 ^{15}N 、 ^{35}S 、 ^{90}Y 、 ^{99}Tc 、 ^{111}In 、 ^{125}I 以及 ^{131}I 。在一些方面中,该抗HER2结合分子可以用在成像时可检测的顺磁性、放射性或发荧光的离子来标记。在一些方面中,该顺磁性离子是铬(III)、锰(II)、铁(III)、铁(II)、钴(II)、镍(II)、铜(II)、钆(III)、钐(III)、铽(III)、钕(III)、钷(II)、铽(III)、镱(III)、铕(III)或铈(III)。在其他方面中,该放射性离子是碘-123、钨-99、铟-111、铯-188、铯-186、铜-67、碘-131、钇-90、碘-125、碲-211、以及镓-67。在其他方面中,该抗HER2结合分子用X-射线成像剂来标记,诸如镧(III)、金(III)、铅(II)、以及铋(III)。抗HER2结合分子还可以用化学基团例如聚合物诸如聚乙二醇(PEG)、甲基基团、乙基基团、或碳水化合物基团来衍生化。这些基团有助于改进抗体的生物特征,例如增加血清半衰期或增加组织结合。

[0281] 如在此所使用的术语“细胞毒性剂”被广泛地定义并且是指抑制或防止细胞功能和/或引起细胞破坏(细胞死亡)和/或施加抗肿瘤/抗增殖作用的物质。例如,细胞毒性剂直接或间接防止增生性肿瘤细胞的发展、突变、或扩散。该术语还包括仅引起细胞抑制作用而无纯粹的细胞毒性作用的此类试剂。该术语包括如下说明的化疗剂、以及其他HER2拮抗剂、抗血管生成剂、酪氨酸激酶抑制剂、蛋白质激酶A抑制剂、细胞因子家族的成员、放射性同位素、以及毒素诸如细菌、真菌、植物或动物来源的酶促活性毒素。

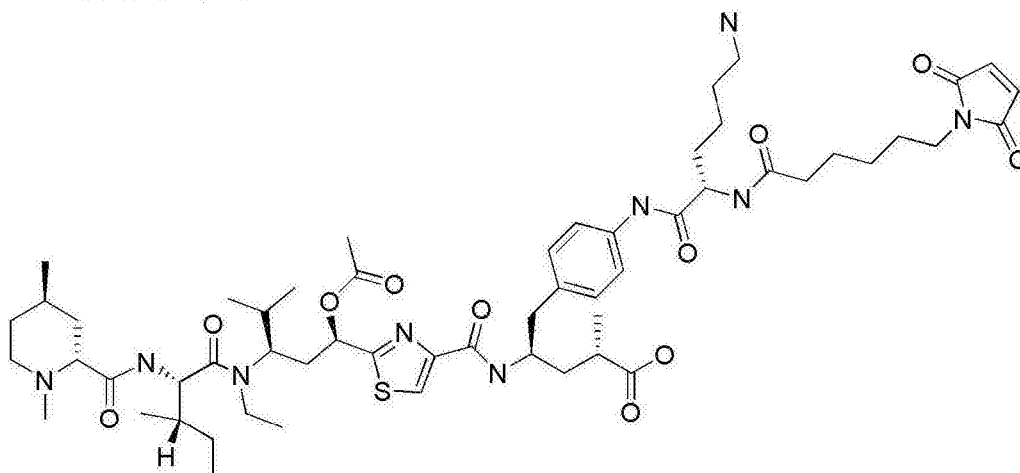
[0282] 术语“化疗剂”是包括天然或合成化学化合物的术语“细胞毒性剂”的子集。化疗剂或试剂的实例包括烷基化剂,例如氮芥、亚乙基亚胺化合物、烷基磺酸盐以及具有烷基化行为的其他化合物,诸如亚硝基脲、顺铂以及达卡巴嗪;抗代谢物,例如叶酸、嘌呤或嘧啶拮抗剂;有丝分裂抑制剂,例如长春花生物碱和鬼臼毒素的衍生物;细胞毒性抗生素和喜树碱衍生物。其他化疗剂是氮磷汀(ETHYOL®)、顺铂、达卡巴嗪(DTIC)、更生霉素、氮芥(mechlorethamine)(氮芥(nitrogen mustard))、链脲霉素、环磷酰胺、卡莫司汀(BCNU)、洛莫司汀(CCNU)、阿霉素(亚德里亚霉素®(ADRIAMYCIN®))、阿霉素脂质体(DOXIL®)、吉西他滨(健择®(GEMZAR®))、柔红霉素、柔红霉素脂质体(DAUNOXOME®)、丙卡巴肼、丝裂霉素、阿糖胞苷、依托泊苷、甲氨喋呤、5-氟尿嘧啶(5-FU)、长春花碱、长春新碱、博来霉素、紫杉醇(特素®(TAXOL®))、多西他赛(TAXOTERE®)、阿地白介素、门冬酰胺酶、白消安、卡铂、克拉屈滨、喜树碱、CPT-11、10-羟基-7-乙基-喜树碱(SN38)、吉非替尼(易瑞沙®(IRESSA®))、达卡巴嗪、氟尿苷、氟达拉滨、羟基脲、异环磷酰胺、伊达比星、关司钠、干扰素 α 、干扰素 β 、伊立替康、米托蒽醌、拓扑替康、利普安、甲地孕酮、关法仑、巯基嘌呤、普卡霉素、米托坦、培加帕酶、喷司他丁、哌泊溴烷、普卡霉素、链脲霉素、他莫昔芬、替尼泊苷、睾内酯、硫代鸟嘌呤、噻替派、尿嘧啶氮芥、长春瑞滨、苯丁酸氮芥芳香化酶抑制剂、以及其组合。

IV.A微管溶素

[0283] 在一些方面中,该ADC包含辄合至一个或多个微管溶素分子上(参见以下微管溶素A和微管溶素1508的结构)的在此披露的抗HER2结合分子(例如,39S抗体或其衍生物、或在此披露的双特异性抗HER2抗体中的一种)。



微管溶素 A



微管溶素 1508 (化合物 T32)

[0284] 微管溶素是从粘细菌物种中分离的一类天然产物的成员(萨斯(Sasse)等人,抗生素杂志(J.Antibiot.)53:879-885(2000))。作为细胞骨架相互作用剂,微管溶素是抑制微管蛋白聚合并且引起细胞周期停止和细胞凋亡的有丝分裂毒剂(施泰因梅茨(Steinmetz)等人,德国应用化学(Chem.Int.Ed.)43:4888-4892(2004);卡里尔(Khalil)等人,生物化学(ChemBioChem.)7:678-683(2006);考尔(Kaur)等人,生物化学杂志(Biochem.J.)396:235-242(2006))。微管溶素是超越任何临床相关的传统化疗剂(例如,埃坡霉素、紫杉醇、以及长春花碱)的细胞生长抑制的极其有效的细胞毒性分子。此外,它们对多药物抗性的细胞系是有效的(多姆灵(Domling)等人,分子多样性(Mol.Diversity)9:141-147(2005))。这些化合物对具有低皮摩尔范围内的 IC_{50} 值的一组癌症细胞系示出所测试的高细胞毒性;因此,它们作为抗癌治疗剂是感兴趣的。参见,例如W02012019123,该专利通过引用以其全文结合在此。微管溶素辄合物被披露于例如美国专利号7,776,814中。

[0285] 在一些方面中,该微管溶素分子或其衍生物是前药。

IV.B美登素和美登木素生物碱

[0286] 在一些方面中,该ADC包含辄合至一个或多个美登木素生物碱分子上的在此披露的抗HER2结合分子(例如,39S抗体或其衍生物、或在此披露的双特异性抗HER2抗体中的一种)。

[0287] 关登木素生物碱是通过抑制微管蛋白聚合起作用的有丝分裂抑制剂。关登素首先是从东非灌木齿叶关登木(*Maytenus serrata*)中分离的(美国专利号3,896,111)。随后,发现某些微生物也产生关登木素生物碱,诸如关登醇和C-3关登醇酯(美国专利号4,151,042)。合成关登醇及其衍生物和类似物被披露于例如美国专利号4,137,230;4,248,870;4,256,746;4,260,608;4,265,814;4,294,757;4,307,016;4,308,268;4,308,269;4,309,428;4,313,946;4,315,929;4,317,821;4,322,348;4,331,598;4,361,650;4,364,866;4,424,219;4,450,254;4,362,663;以及4,371,533中。

[0288] 关登木素生物碱药物部分在抗体药物轭合物中是有吸引力的药物部分,因为它们:(i)通过发酵或化学改性、衍生化发酵产物相对易于制备,(ii)适于使用适合从非二硫化物接头轭合至抗体的官能团进行衍生化,(iii)在血浆中稳定的,并且(iv)对各种肿瘤细胞系有效的。含有关登木素生物碱的免疫轭合物、制备这些免疫轭合物的方法、以及这些免疫轭合物的治疗用途被披露于例如美国专利号5,208,020、5,416,064以及欧洲专利EP0425235B1;刘(Liu)等人,美国科学院院刊93:8618-8623(1996)(描述包含称为DM1的关登木素生物碱的免疫轭合物);以及查丽(Chari)等人,癌症研究52:127-131(1992)中。

[0289] 曲妥珠单抗emtansine(ado-曲妥珠单抗emtansine,T-DM1,商品名Kadcyla®)是由轭合至关登木素生物碱关登素(DM1)上的单克隆抗体曲妥珠单抗(赫赛汀®)组成的抗体-药物轭合物。参见,例如洛鲁索(LoRusso)等人,临床癌症研究(Clin.Cancer Res.)20:6437-47(2011),该参考文献通过引用以其全文结合在此。工程化的硫代-曲妥珠单抗-DM1 ADC还被描述于朱那托尔(Junutual)等人,临床癌症研究16:4769-78(2010)中,该参考文献通过引用以其全文结合在此。

[0290] 抗体-关登木素生物碱轭合物通过将抗体化学连接至关登木素生物碱分子上而不明显减弱该抗体或该关登木素生物碱分子的生物活性来制备。参见,例如美国专利号5,208,020。每个抗体分子轭合平均3-4个关登木素生物碱分子在增强靶细胞的细胞毒性方面显示功效而不负面影响该抗体的功能或溶解性,虽然毒素/抗体的即使一个分子将预期在使用裸抗体过程中增强细胞毒性。关登木素生物碱是本领域中熟知的并且可以通过已知的技术来合成或从天然来源中分离。适合的关登木素生物碱被披露于例如美国专利号5,208,020中。

[0291] 在一些方面中,该关登木素生物碱分子、其变体或衍生物是前药。

IV.C奥瑞他汀和多拉司他汀

[0292] 在一些方面中,该ADC包含轭合至多拉司他汀或多拉司他汀肽类似物和衍生物、奥瑞他汀上(美国专利号5,635,483;5,780,588)的在此披露的抗HER2结合分子(例如,39S抗体或其衍生物、或在此披露的双特异性抗HER2抗体中的一种)。显示多拉司他汀和奥瑞他汀干扰微管动力学、GTP水解以及细胞核和细胞分裂(沃克(Woyke)等人,抗微生物制剂和化学疗法(Antimicrob.Agents and Chemother.)45:3580-3584(2001))并且具有抗癌活性(美国专利号5,663,149)。多拉司他汀或奥瑞他汀药物部分可以通过肽药物部分的N(氨基)末端或C(羧基)末端被附接至抗体上(参见,例如W02002088172)。

[0293] 在一些方面中,该奥瑞他汀或多拉司他汀分子、其变体或衍生物是前药。

IV.D卡奇霉素

[0294] 在一些方面中,该ADC包含轭合至一个或多个卡奇霉素分子上的在此披露的抗

HER2结合分子(例如,39S抗体或其衍生物、或在此披露的双特异性抗HER2抗体中的一种)。抗生素的卡奇霉素家族成员能够在亚皮摩尔浓度下产生双链DNA断裂。卡奇霉素是从细菌棘孢小单孢菌(*Micromonospora echinospora*)中衍生的一类烯二炔抗生素,其中卡奇霉素 γ 1是最著名的。其他卡奇霉素是 β 1Br、 γ 1Br、 α 2I、 α 3I、 β 1I、 γ 1I、以及 Δ 1I。参见,李(Lee)等人,抗生素杂志(*Journal of Antibiotics*)42(7):1070-87(1989)。对于卡奇霉素家族的轭合物的制备,参见美国专利号5,712,374、5,714,586、5,739,116、5,767,285、5,770,701、5,770,710、5,773,001、5,877,296。可以使用的卡奇霉素的结构类似物包括,但不限于 γ 1I、 α 2I、 α 3I、N-乙酰基- γ 1I、PSAG以及 θ 1I(欣曼(Hinman)等人,癌症研究53:3336-3342(1993),洛得(Lode)等人,癌症研究58:2925-2928(1998)以及前面提到的美国氰胺公司(American Cyanamid)的美国专利)。

[0295] 在一些方面中,该卡奇霉素分子、其变体或衍生物是前药。

IV.E多卡米素

[0296] 在一些方面中,该ADC包含轭合至一个或多个多卡米素分子上的在此披露的抗HER2结合分子(例如,39S抗体或其衍生物、或在此披露的双特异性抗HER2抗体中的一种)。多卡米素是首先从链霉菌属中分离的一系列相关天然产物的成员并且它们有效的抗肿瘤抗生素。参见博格(Boger)(1991)*Chemtracts:有机化学*(*Chemtracts:Organic Chemistry*)4(5):329-349(1991);特塞尔(Tercel)等人,英文国际版化学杂志(*Chem.Int.Ed.Engl.*)52(21):5442-6(2013);博格和道格拉斯(Douglas),美国科学院院刊92(9):3642-3649(1995);卡恰里(Cacciari)等人,治疗术专利专家评论(*Expert Opinion on Therapeutic Patents*)10(12):1853-71(2000)。

[0297] 天然多卡米素包括多卡米素A、多卡米素B1、多卡米素B2、多卡米素C1、多卡米素C2、多卡米素D、多卡米素SA、以及CC-1065。合成类似物包括阿多来新、比折来新、以及卡折来新(U-80244)。

[0298] 在一些方面中,该多卡米素分子、其变体或衍生物是前药。

IV.F吡咯并苯并二氮杂草

[0299] 在一些方面,该药物是吡咯并苯并二氮杂草(PBD)。PBD是相对小的分子并且一些具有识别且共价结合至DNA小沟中的特定序列的能力,并且因此展现出抗生素/抗肿瘤活性。许多PBD及其衍生物是本领域中已知的,例如PBD二聚体(例如,SJG-136或SG2000)、C2-不饱和的PBD二聚体、带有C2芳基取代的吡咯并苯并二氮杂草二聚体(例如,SG2285)、通过水解活化的PBD二聚体前药(例如,SG2285)、以及聚吡咯-PBD(例如,SG2274)。WO 2000/012507、WO 2007/039752、WO 2005/110423、WO 2005/085251以及WO 2005/040170以及美国专利号7,612,062PBD进一步描述了PBD,这些专利中的每一个通过引用以其全文结合在此。

IV.G其他细胞毒性剂

[0300] 在一些方面中,该ADC包含轭合至其他抗肿瘤剂例如BCNU、蒽环类(例如,e.g.,道诺霉素或阿霉素)、紫杉烷类(例如,紫杉醇)、链佐星、长春花生物碱(例如,长春新碱)、5-氟尿嘧啶、共同作为LL-E33288复合体已知的试剂的家族(参见美国专利号5,053,394和5,770,710)、埃斯波霉素(参见美国专利号5,877,296)上的在此披露的抗HER2结合分子(例如,39S抗体或其衍生物、或在此披露的双特异性抗HER2抗体中的一种)。该ADC还可以包含轭合至酶促活性毒素及其片段上的在此披露的抗HER2结合分子(例如,39S抗体或其衍生

物、或在此披露的双特异性抗HER2抗体中的一种),可以使用的这些酶促活性毒素及其片段包括白喉A链、白喉毒素的非结合活性片段、外毒素A链(来自铜绿假单胞菌)、蓖麻毒素A链、相思豆毒素A链、蒴莲根毒素A链、 α -八叠球菌素、油桐蛋白、石竹素蛋白、美洲商陆蛋白(PAPI、PAPII、以及PAP-S)、苦瓜抑制剂、麻风树毒蛋白、巴豆毒蛋白、肥皂草抑制剂、白树毒素、米托毒素(mitogellin)、局限曲菌素、酚霉素、伊诺霉素、以及单端孢霉烯类。参见,例如W0199321232。在一些方面中,该细胞毒性剂是光活化药物。

V. 结合至与39S抗体相同表位的抗HER2结合分子

[0301] 在另一个方面中,本披露提供结合至与39S抗体相同的一个或多个表位的抗HER2结合分子(例如,从39S抗体中衍生的抗体或其抗原结合片段、双特异性抗HER2抗体、或ADC)。

[0302] 结合至与39S抗体相同表位的此类抗HER2结合分子可以基于它们在标准HER2结合测定中与39S抗体交叉竞争(例如,以统计学显著的方式竞争性地抑制结合)的能力来进行鉴定。因此,在一个方面中,本披露提供与39S抗体或其抗原结合片段竞争结合至HER2的抗HER2结合分子(例如,从39S抗体中衍生的抗体或其抗原结合片段、双特异性抗HER2抗体、或ADC)。测试抗体抑制例如39S抗体或从39S抗体中衍生的结合分子(例如,双特异性抗体或ADC)的结合的能力证实了该测试抗体可以与39S抗体竞争结合至HER2;此种抗HER2结合分子可以根据非限制性理论结合至与其竞争的与39S抗体或其抗原结合片段相同或相关(例如,结构上类似或空间上接近)的HER2上的表位。

[0303] 在一个方面中,结合至与39S抗体或从39S抗体中衍生的结合分子相同的HER2上的表位的该抗HER2分子是人单克隆抗体或其抗原结合片段、双特异性抗HER2抗体(例如,包含两个HER2结合区的双特异性抗体,这两个HER2结合区中的至少一个识别与39S抗体相同的表位)、或ADC(例如,包含识别与39S相同表位的至少一个抗原结合部分的ADC;或包含包含有两个HER2结合区的双特异性抗体的ADC,这两个HER2结合区中的至少一个识别与39S抗体相同的表位)。

VI. 抗HER2结合分子的作用机制

[0304] 本披露提供包含结合与1.39.1或39S抗体相同表位或从1.39.1或39S抗体中衍生的HER2结合结构域的抗HER2结合分子(例如,双特异性抗HER2抗体、或ADC),其中此类抗HER2结合分子在结合至该HER2靶标时诱导内化。还提供包含结合与1.39.1或39S抗体相同表位或从1.39.1或39S抗体中衍生的HER2结合结构域的抗HER2结合分子(例如,双特异性抗HER2抗体、或ADC),其中此类抗HER2结合分子在内化之后促进有效的溶酶体运输。本披露还提供包含结合与1.39.1或39S抗体相同表位或从1.39.1或39S抗体中衍生的HER2结合结构域的抗HER2结合分子(例如,双特异性抗HER2抗体、或ADC),其中此类抗HER2结合分子诱导HER2靶标内化和/或降解。

[0305] 在一些方面中,在此披露的抗HER2结合分子可以诱导、阻断、或压制HER2磷酸化。在其他方面中,在此披露的抗HER2结合分子可以诱导、阻断、或压制配体诱导的AKT磷酸化。在一些方面中,该抗HER2结合分子可以诱导、阻断、或压制低HER2表达癌症细胞中的配体诱导的AKT磷酸化。

[0306] 在还其他方面中,本披露中所披露的抗HER2结合分子例如抗HER2抗体或其抗原结合片段可以诱导、破坏、或压制配体诱导的HER2-HER3二聚化。

[0307] 在一些方面中,包含在此披露的抗HER2结合分子的ADC可以抑制癌症干细胞(CSC)球体形成和/或增殖。在一些方面中,在此披露的抗HER2结合分子展现出对CSC的细胞毒性作用。在一些方面中,包含在此披露的抗HER2结合分子的ADC可以在表达低水平HER2的肿瘤中抑制肿瘤生长和/或诱导肿瘤消退(例如,通过HercepTest的+1至+2)。在某些方面中,包含在此披露的抗HER2结合分子的ADC可以在对T-DM1有抗性的肿瘤中抑制肿瘤生长和/或诱导肿瘤消退。

[0308] 在一些方面中,HER2结合分子例如抗HER2抗体或其抗原结合片段缺乏ADCC活性。在特定方面中,抗HER2结合分子例如抗HER2抗体或其抗原结合片段可以通过配体非依赖性作用机制降低或压制HER2磷酸化、AKT磷酸化和/或肿瘤集落形成。

VII. 抗HER2结合分子的制备

[0309] 可以根据本领域中已知的方法来制备本披露的抗HER2结合分子(例如,结合与1.39.1或39S抗体相同表位或从1.39.1或39S抗体中衍生的抗体或其抗原结合片段、双特异性抗体以及包含这些抗体的ADC)。例如,使用诸如由科勒(Kohler)和米尔斯坦(Milstein)(1975)自然256:495所描述的那些杂交瘤方法来产生在此披露的结合与39S抗体相同表位的抗HER2结合分子。

[0310] 使用该杂交瘤方法,小鼠、仓鼠、或其他适当的宿主动物被如上所述免疫或诱导通过抗体的淋巴细胞进行的产生,这些淋巴细胞将特异性结合至免疫抗原。淋巴细胞还可以被体外免疫。免疫之后,分离这些淋巴细胞并且使用例如聚乙二醇与适合的骨髓瘤细胞系融合,以便形成随后可以从未融合的淋巴细胞和骨髓瘤细胞中选择离开的杂交瘤细胞。如通过免疫沉淀、免疫印迹、或通过体外结合测定(例如,放射免疫测定(RIA);酶联免疫吸附测定(ELISA))所确定的特异性针对所选择抗原的产生单克隆抗体的杂交瘤随后可以使用标准方法(高定(Goding),单克隆抗体:原理和实践(Monoclonal Antibodies:Principles and Practice),美国学术出版社,1986)在体外培养物中或在体内作为动物中的腹水肿瘤来繁殖。这些单克隆抗体随后可以对于上述的多克隆抗体从所述的培养基或腹水中纯化出来。

[0311] 还可以使用如在美国专利号4,816,567中所述的重组DNA方法来制备本披露的抗HER2结合分子(例如,结合与1.39.1或39S抗体相同表位或从1.39.1或39S抗体中衍生的抗体或其抗原结合片段、双特异性抗体以及包含这些抗体的ADC)。诸如通过使用特异性扩增编码抗体重链和轻链的基因的寡核苷酸引物的RT-PCR,将编码单克隆抗体的多核苷酸从成熟B细胞或杂交瘤细胞中分离出来,并且使用常规程序确定它们的序列。然后将编码重链和轻链的分离的多核苷酸克隆到适合的表达载体中,当转染时这些表达载体进入不另外产生免疫球蛋白的蛋白质的宿主细胞诸如大肠杆菌细胞、猿猴COS细胞、中国仓鼠卵巢(CHO)细胞或骨髓瘤细胞中,通过这些宿主细胞来产生单克隆抗体。同样,如所述的可以将希望物种的重组抗HER2单克隆抗体或其包含抗原结合片段的分子从表达希望物种的CDR的噬菌体展示文库中分离出来(麦卡弗蒂(McCafferty)等人,自然348:552-554(1990);克莱森(Clarkson)等人,自然352:624-628(1991);以及马克斯(Marks)等人,分子生物学杂志(J.Mol.Biol.)222:581-597(1991))。

[0312] 可以使用重组DNA技术以多种不同方式进一步修饰编码本披露的抗HER2结合分子的一种或多种多核苷酸(例如,结合与39S抗体相同表位或从39S抗体中衍生的抗体或其抗

原结合片段、双特异性抗体以及包含这些抗体的ADC),以便产生替代的抗HER2结合分子。在一些方面中,例如小鼠单克隆抗体的轻链和重链的恒定结构域可以被取代成(1)例如人抗体的那些区以便产生嵌合抗体或(2)非免疫球蛋白多肽以便产生融合抗体。在一些方面中,恒定区被截断或去除以产生单克隆抗体的希望的抗体片段。可变区的定点或高密度诱变可以被用来优化单克隆抗体的特异性、亲和力等。

[0313] 在某些方面中,本披露的抗HER2结合分子是人抗体或其抗原结合片段。人抗体可以使用本领域中已知的不同技术来直接制备。可以产生体外免疫或从产生针对靶抗原的抗体的免疫个体中分离的永生化的B淋巴细胞(参见,例如科尔(Cole)等人,单克隆抗体和癌症治疗(Monoclonal Antibodies and Cancer Therapy),阿兰R.丽丝(Alan R.Liss),第77页(1985);波伊默(Boemer)等人,免疫学杂志(J.Immunol.)47:86-95(1991);以及美国专利号5,750,373)。然后可以制备在永生化的B淋巴细胞中编码抗体的一个或多个cDNA并且插入表达载体和/或异源宿主细胞中以便用于表达抗体的非天然存在的重组形式。

[0314] 同样,抗HER2人抗体或其抗原结合片段可以选自噬菌体文库,其中该噬菌体文库将人抗体或其片段表达为具有异源噬菌体蛋白质的融合蛋白,如例如在沃恩(Vaughan)等人,自然-生物技术(Nat.Biotech.)14:309-314(1996);希茨(Sheets)等人,美国科学院院刊95:6157-6162(1998);霍金布姆(Hoogenboom)和温特(Winter),分子生物学杂志227:381(1991)以及马克斯等人,分子生物学杂志222:581(1991))中所述。用于产生和使用抗体噬菌体文库的技术还被描述于美国专利号5,969,108;6,172,197;5,885,793;6,521,404;6,544,731;6,555,313;6,582,915;6,593,081;6,300,064;6,653,068;6,706,484;以及7,264,963中,这些美国专利中的每一个通过引用以其全文结合。

[0315] 亲和力成熟策略和链改组策略(马克思等人,生物技术(BioTechnology)10:779-783(1992),该参考文献通过引用以其全文结合)是本领域中已知的,并且可以被用来产生高亲和力人抗体或其抗原结合片段。

[0316] 在一些方面中,本披露的抗HER2结合分子可以是人源化抗体。也可以使用用于工程化、人源化或表面重修非人抗体或人抗体的方法并且这些方法是本领域中熟知的。人源化、表面重修或类似工程化的抗体可以具有来自非人来源例如但不限于小鼠、大鼠、非人灵长类动物或其他哺乳动物的一个或多个氨基酸残基。这些非人氨基酸残基被经常称为“输入”残基的残基替换,这些输入残基典型地取自已知人序列的“输入”可变、恒定或其他结构域。此类输入的序列可以被用来降低免疫原性或降低、增强或改进结合、亲和力、开(on)速率、关(off)速率、亲合力、特异性、半衰期、或本领域中所知的任何其他适合的特征。通常,CDR残基是直接和最主要地参与影响HER2结合的。因此,非人或人CDR序列的部分或全部被维持而可变区和恒定区的非人序列可以用人或其他氨基酸替换。在某些方面中,人CDR被插入非人抗体支架中以便在动物模型系统中制备具有降低免疫原性的抗体,例如“鼠源化”抗体。

[0317] 抗HER2结合分子例如抗体可以任选地被人源化、表面重修、或工程化,其中保留对抗原HER2的高亲和力和其他有利的生物特性。为了实现这个目标,人源化(或人)或工程化的抗HER2抗体和表面重修的抗体可以任选地通过使用亲本、工程化和人源化序列的三维模型对亲本序列和不同概念人源化和工程化产物进行分析的方法来制备。三维免疫球蛋白模型是普遍可得到的并且是本领域技术人员熟知的。

[0318] 例证和展示所选择的候选免疫球蛋白序列的可能的三维构象结构的计算机程序是可用的。对这些展示的检查容许分析残基在候选免疫球蛋白序列功能中可能的作用,即,分析影响候选免疫球蛋白结合其抗原诸如HER2能力的残基。以这种方式,可以从共有序列和输入序列中选择并合并构架残基以使得实现希望的抗体特征,诸如对一个或多个靶抗原的亲合力增加。

[0319] 对在此披露的抗HER2结合分子的人源化、表面重修或工程化可以使用任何已知的方法来进行,诸如但不限于描述于以下参考文献中的那些:琼斯等人,自然321:522(1986);瑞彻曼等人,自然332:323(1988);威霍英等人,科学239:1534(1988)、斯姆斯(Sims)等人,免疫学杂志151:2296(1993);乔西亚和莱斯克,分子生物学杂志196:901(1987)、卡特等人,美国科学院院刊89:4285(1992);普雷斯塔(Presta)等人,免疫学杂志151:2623(1993)、美国专利号5,639,641;5,723,323;5,976,862;5,824,514;5,817,483;5,814,476;5,763,192;5,723,323;5,766,886;5,714,352;6,204,023;6,180,370;5,693,762;5,530,101;5,585,089;5,225,539;4,816,567;7,557,189;7,538,195;以及7,342,110;W090/14443;W090/14424;W090/14430;以及EP229246,这些参考文献中的每一个通过引用整体结合在此,包括其中引用的参考文献。

[0320] 在某些方面中,提供抗HER2抗体片段。已知不同技术用于产生抗体片段。在传统上,这些片段是通过完整抗体的蛋白水解消化而衍生的(例如森本晃司(Morimoto)等人,生物化学与生物物理杂志方法(J.Biochem.Biophys.Methods)24:107-117(1993);布伦南(Brennan)等人,科学,229:81(1985))。在某些方面中,重组产生抗HER2抗体片段。所有Fab、Fv和scFv抗体片段都可以表达在大肠杆菌或其他宿主细胞中并且从其分泌,因而允许产生大量的这些片段。还可以从以上讨论的抗体噬菌体文库中分离此类抗HER2抗体片段。这些抗HER2抗体片段还可以是如在美国专利号5,641,870中所述的线性抗体。用于产生抗体片段的其他技术对于熟练的从业人员将是清楚的。

[0321] 技术可以适用于产生对与39S抗体相同HER2表位有特异性的单链抗体(参见,例如美国专利号4,946,778)。另外,方法可以适用于构建Fab表达文库(参见,例如休斯(Huse)等人,科学246:1275-1281(1989))以便允许快速和有效的鉴定对HER2、或其衍生物、片段、类似物或同源物具有希望的特异性的单克隆Fab片段。抗体片段可以通过本领域的技术来产生,这些抗体片段包括但不限于:(a)由抗体分子的胃蛋白酶消化产生的F(ab')₂片段;(b)由还原F(ab')₂片段的二硫化物桥键产生的Fab片段;(c)由使用木瓜蛋白酶和还原剂处理抗体分子产生的Fab片段以及(d)Fv片段。

[0322] 在一些方面中,尤其在抗体片段的情况下,抗HER2抗体或其抗原结合片段可以被修饰以便增加它的血清半衰期。这可以例如通过以下方式来实现:通过突变抗体或抗体片段中的适当的区域将补救受体结合表位掺入抗体或抗体片段中或通过表位掺入随后融合至任一端或在中间处的抗体或抗体片段的肽标签中(例如,通过DNA或肽合成)、或通过YTE突变。增加抗体或其抗原结合片段的血清半衰期的其他方法(例如,耦合至异源分子诸如PEG)是本领域中熟知的。

[0323] 杂轭合物抗HER2结合分子例如在此披露的结合与39S抗体相同表位或从39S抗体中衍生的双特异性抗体或ADC可以使用重组生物学技术以及体外使用合成蛋白质化学方面的方法(包括涉及交联剂的那些)来制备。例如,可以使用二硫化物交换反应或通过形成硫

醚键来化学构建双特异性抗体或ADC。出于这个目的的适合试剂是本领域中已知的,并且包括亚氨基硫醇酯和4-巯基丁亚胺酸甲酯。

[0324] 应当指出的是在某些方面中,抗HER2结合分子可以被工程化成直接将CH3结构域融合至对应修饰的抗体或其片段的铰链区。在其他构建体中,肽间隔物可以被插入在铰链区与修饰的CH2和/或CH3结构域之间。例如,可以表达相容的构建体,其中CH2结构域已缺失并且剩余的CH3结构域(修饰或未修饰的)使用5-20个氨基酸的间隔物而连接至铰链区。可以例如添加这样一种间隔物以便确保恒定结构域的调节元件保持游离并且可接近或确保铰链区保持柔性。然而,应当指出氨基酸间隔物可在一些情况下被证明是免疫原性的并且针对构建体引发不希望的免疫应答。因此,在某些方面中,添加至构建体的任何间隔物将是相对非免疫原性的,或甚至总体上省略的,以便维持修饰抗体的希望的生物化学品质。

[0325] 除了缺失整个恒定区结构域之外,应当了解抗HER2结合分子可以通过部分缺失或取代几个或甚至单个氨基酸来提供。例如,对CH2结构域的选择区域中的单个氨基酸进行突变可足以基本上降低Fc结合并且因而增加肿瘤定位。此外,如上所暗示,披露的抗HER2结合分子的恒定区可以通过突变或取代增强所得构建体的特征(profile)的一个或多个氨基酸来修饰。在这个方面,可能破坏由保守结合位点(例如,Fc结合)提供的活性而维持修饰的抗体或其抗原结合片段的构象和免疫原性特征。某些方面可以包括将一个或多个氨基酸添加至恒定区以便增强希望的特征诸如降低或增加效应子功能或提供更多细胞毒素或碳水化合物附接。在此类方面中,从选择的恒定区结构域中衍生的特定序列可以被插入或复制。

VIII. 编码HER2结合分子的多核苷酸

[0326] 在某些方面中,本披露提供包含编码特异性结合HER2的在此披露的抗HER2结合分子的核酸序列的多核苷酸。例如,本披露提供包含编码抗HER2结合分子诸如抗体或其片段(例如,结合与39S抗体相同表位或从39S抗体中衍生的分子)的核酸序列的多核苷酸。本披露的多核苷酸可以呈RNA形式或呈DNA形式。DNA包括cDNA、基因组DNA、以及合成DNA;并且可以是双链或单链的,并且如果是单链的,可以是编码链或非编码(反义)链。在某些方面中,该DNA是用来产生非天然存在的重组抗体的cDNA。

[0327] 在某些方面中,这些多核苷酸是分离的。在某些方面中,这些多核苷酸是基本上纯的。在某些方面中,这些多核苷酸包含被融合在与例如辅助从宿主细胞表达和分泌多肽的多核苷酸(天然或异源的)相同阅读构架中的成熟多肽的编码序列(例如,用作控制多肽从细胞运输的分泌序列的前导序列)。具有前导序列的多肽是前蛋白并且可以具有由宿主细胞裂解的前导序列以便形成多肽的成熟形式。这些多核苷酸还可以编码作为成熟蛋白加上另外的5'氨基酸残基的抗HER2结合分子前蛋白。在某些方面中,这些多核苷酸被改变成针对某一宿主细胞优化密码子使用。

[0328] 在某些方面中,这些多核苷酸包含被融合在与异源标记序列相同阅读构架中的成熟抗HER2结合分子(例如,抗HER2抗体或其抗原结合片段)的编码序列,该异源标记序列允许例如纯化编码的多肽。例如,该标记序列可以是例如由pQE-9载体供应的以便提供在细菌宿主的情况下融合至标记物的成熟多肽的纯化的六组氨酸(His6)标签。在其他方面中,该标记序列可以是在使用哺乳动物宿主(例如,COS-7细胞)时,例如从流感血凝素蛋白中衍生的血凝素(HA)标签。

[0329] 本披露进一步涉及例如编码本披露的抗HER2结合分子的HER2结合片段、类似物、

以及衍生物的所述多核苷酸的变体。

[0330] 这些多核苷酸变体可以在编码区、非编码区、或两者中含有改变。在一些方面中，这些多核苷酸变体含有产生沉默取代、添加、或缺失的改变，但不改变编码的多肽的特性或活性。在一些方面中，通过由于遗传密码的简并性引起的沉默取代来产生核苷酸变体。可以出于各种原因来产生多核苷酸变体，例如为了优化特定宿主的密码子表达(将人mRNA中的密码子改变成细菌宿主诸如大肠杆菌优选的那些)。还提供包含在此描述的多核苷酸的载体和细胞。

[0331] 在一些方面中，编码抗HER2结合分子(例如，抗HER2抗体或其抗原结合片段)的DNA序列可以通过化学合成，例如使用寡核苷酸合成仪来构建。可以基于希望多肽的氨基酸序列并且选择有利于其中将产生感兴趣重组多肽的宿主细胞的那些密码子来设计此类寡核苷酸。标准方法可以适用于合成编码感兴趣的分离多肽的分离多核苷酸序列。例如，可以使用完整的氨基酸序列来构建反向翻译的基因。此外，可以合成含有编码特定分离多肽的核苷酸序列的DNA低聚物。例如，可以合成编码希望多肽的部分的若干小寡核苷酸并且然后进行连接。各个寡核苷酸典型地含有用于互补组装的5'或3'突出端。

[0332] 一旦组装(通过合成、定点诱变或另一种方法)，编码感兴趣的特定分离多肽的多核苷酸序列将被插入表达载体中并且可操作地连接至适用于在希望的宿主中表达蛋白质的表达控制序列上。合适的组装可以例如通过核苷酸测序、限制性图谱以及在适合宿主中表达生物活性多肽来证实。正如本领域中熟知的，为了在宿主中获得转染基因的高表达水平，该基因必须可操作地连接至在选择的表达宿主中有功能性的转录和翻译表达控制序列上。

[0333] 在某些方面中，重组表达载体被用来扩增和表达编码抗HER2结合分子的DNA。重组表达载体是可复制的DNA构建体，这些DNA构建体具有编码例如抗HER2抗体或和其抗原结合片段的多肽链、可操作地连接至从哺乳动物、微生物、病毒或昆虫基因中衍生的适合转录或翻译调节元件上的合成或cDNA衍生的DNA片段。转录单元通常包括对以下各项的组装：(1)在基因表达方面具有调节作用的基因元件或多个元件，例如转录启动子或增强子，(2)被转录到mRNA中并且翻译成蛋白质的结构或编码序列，以及(3)如下详细所述的适当转录和翻译起始序列和终止序列。此类调节元件可以包括控制转录的操纵子序列。

[0334] 可以另外地掺入通常由复制起点赋予的在宿主中复制的能力、以及促进识别转化体的选择基因。当DNA区是功能上彼此相关的时候，它们可操作地连接。例如，如果信号肽(分泌前导)的DNA被表达为参与多肽分泌的前体，则它可操作地连接至多肽的DNA上；如果启动子控制序列的转录，则它可操作地连接至编码序列上；或如果核糖体结合位点为了容许翻译而定位，则它可操作地连接至编码序列上。意图用于酵母表达系统中的结构元件包括能实现通过宿主细胞细胞外分泌翻译的蛋白质的前导序列。可替代地，在不使用前导或运输序列表达重组蛋白的情况下，它可以包括N-末端的甲硫氨酸残基。这个残基可以任选地随后从表达的重组蛋白中裂解，以便提供最终产物。

[0335] 表达控制序列和表达载体的选择将取决于宿主的选择。可以采用各种各样的表达宿主/载体组合。对于真核宿主的有用表达载体包括例如，包含来自SV40、牛乳头瘤病毒、腺病毒以及巨细胞病毒的表达控制序列的载体。对于细菌宿主的有用表达载体包括已知的细菌质粒，诸如来自大肠杆菌的质粒，包括pCR 1、pBR322、pMB9以及它们的衍生物、更宽宿主

范围的质粒,诸如M13和丝状单链DNA噬菌体。

[0336] 用于表达抗HER2结合分子(例如抗HER2抗体或其抗原结合片段)的适合宿主细胞包括在适当启动子控制下的原核细胞、酵母、昆虫或更高等真核细胞。原核细胞包括革兰阴性或革兰阳性生物体,例如大肠杆菌或细菌。更高等真核细胞包括如下所述已确立的哺乳动物来源的细胞系。还可以采用无细胞的翻译系统。用于与细菌、真菌、酵母以及哺乳动物细胞宿主一起使用的适当克隆和表达载体由泊威斯(Pouwels)等人(克隆载体:实验室手册(Cloning Vectors:A Laboratory Manual),纽约,爱思唯尔(Elsevier,N.Y.),1985)所述,该参考文献的相关披露内容特此通过引用结合。

[0337] 关于蛋白质产生(包括抗体产生)的方法的另外信息可见于例如美国公开号2008/0187954、美国专利号6,413,746和6,660,501、以及国际专利公开号WO 04009823中,这些专利中的每一个特此通过引用以其全文结合。

[0338] 还可以有利地采用不同哺乳动物或昆虫细胞培养物系统来表达重组抗HER2结合分子,例如抗HER2抗体或其抗原结合片段。可以进行哺乳动物细胞中的重组蛋白的表达,因为此类蛋白质通常是正确折叠、适当修饰并且完全有功能的。适合的哺乳动物宿主细胞系的实例包括HEK-293和HEK-293T,由格祖曼(Gluzman)(细胞(Cell)23:175,1981)所述的COS-7猴肾脏细胞系;以及其他细胞系,包括例如L细胞、C127、3T3、中国仓鼠卵巢(CHO)、NSO、HeLa、以及BHK细胞系。哺乳动物表达载体可以包含非转录元件,诸如复制起点、连接至有待表达的基因上的适合启动子和增强子、及其他5'或3'侧翼非转录序列、以及5'或3'非翻译序列,诸如必要的核糖体结合位点、聚腺苷酸化位点、剪接供体和受体位点、以及转录终止序列。用于在昆虫细胞中产生异源蛋白的杆状病毒系统由卢科(LuckoW)和萨默斯(Summers),生物技术6:47(1988)进行了综述。

[0339] 可以根据任何适合的方法来纯化由转化的宿主产生的抗HER2结合分子(例如,抗HER2抗体或其抗原结合片段)。此类标准方法包括,例如色谱法(例如,离子交换、亲和力以及定大小柱色谱法)、离心、差别溶解性、或通过蛋白质纯化的任何其他标准技术。亲和力标签诸如六组氨酸、麦芽糖结合结构域、流感外壳序列、谷胱甘肽S-转移酶等可以被附接至蛋白质上,以便允许通过在适当亲和力柱上穿过而简单纯化。还可以使用例如蛋白水解、核磁共振或x-射线结晶学来物理表征分离蛋白质。

[0340] 例如,可以首先使用商业上可获得的蛋白质浓缩过滤器例如AMICON®或Millipore PELLICON®超滤单元来浓缩来自将重组蛋白分泌到培养基中的系统的上清液。在浓缩步骤之后,可以将浓缩物施加至适合的纯化基质中。可替代地,可以采用阴离子交换树脂,例如具有侧链二乙基氨基乙基(DEAE)基团的基质或基底。这些基质可以是丙烯酰胺、琼脂糖、葡聚糖、纤维素或常用于蛋白质纯化的其他类型。可替代地,可以采用阳离子交换步骤。

[0341] 适合的阳离子交换剂包括包含磺基丙基或羧甲基基团的不同不溶性基质。最后,可以采用使用疏水性RP-HPLC介质(例如,具有侧链甲基或其他脂肪族基团的硅胶)的一个或多个反相高效液相色谱法(RP-HPLC)步骤来进一步纯化HER2结合分子。还可以不同的组合采用前述纯化步骤中的一些或全部来提供均质重组蛋白。

[0342] 可以例如通过初始从细胞沉淀物中提取、接着进行一个或多个浓缩、盐析、水性离子交换或大小排阻色谱法步骤来分离在细菌培养物中产生的重组抗HER2结合分子,例如抗

HER2抗体或其抗原结合片段。可以采用高效液相色谱法(HPLC)用于最终的纯化步骤。在重组蛋白的表达中采用的微生物细胞可以通过任何合宜方法来破坏,包括冻融循环、超声、机械破坏、或使用细胞裂解剂。

[0343] 本领域中已知的用于纯化抗体和其他蛋白质的方法还包括例如描述于美国专利公开号US20080312425、US20080177048以及US20090187005中的那些,这些专利中的每一个特此通过引用以其全文结合。

IX. 使用治疗性抗HER2结合分子的治疗方法

[0344] 本披露还提供涉及使用抗HER2结合分子(例如抗体,包括其抗原结合片段、变体以及衍生物)来治疗患有与HER2表达或HER2表达细胞相关联的疾病的患者的方法。

[0345] “HER2表达细胞”意指表达HER2蛋白的细胞。用于检测和/或定量细胞中的HER2表达的方法是本领域中熟知的,并且包括但不限于PCR技术、免疫组织化学(例如,Herceptest™)、流式细胞术、蛋白质印迹、ELISA等。在一些方面中,在此披露的方法适用于其中癌症细胞以低水平表达HER2的治疗和诊断方法。

[0346] 用于使用在此披露的抗HER2结合分子诊断和治疗不同疾病和病症的方法是指保留本披露的抗HER2结合分子的希望特性的抗HER2抗体(例如,39S抗体、变体、衍生物、以及HER2结合片段;本披露的双特异性抗HER2分子;以及本披露的ADC分子)例如能够特异性结合HER2并且中和HER2活性。

[0347] 在一些方面中,抗HER2结合分子是介导人ADCC的人或人源化抗HER2结合分子;或包括介导ADCC的已知抗HER2抗体;或包括工程化成使得它们介导ADCC的抗HER2结合分子。

[0348] 在一些方面中,抗HER2结合分子是不介导人ADCC的人或人源化抗HER2结合分子;或包括不介导ADCC的已知抗HER2抗体;或包括工程化成使得它们不介导ADCC的抗HER2结合分子。

[0349] 在一个方面中,治疗包括向受试者或患者施加或给予本披露的抗HER2结合分子(例如,抗体或其抗原结合片段、变体、或衍生物),或向来自受试者或患者的分离组织或细胞系施加或给予该抗HER2结合分子,其中该受试者或患者具有疾病、疾病的症状、或易患疾病的倾向。在另一个方面中,治疗还意图包括向受试者或患者施加或给予包含本披露的抗HER2结合分子(例如,抗体或其抗原结合片段、变体、或衍生物)的药物组合物,或向来自受试者或患者的分离组织或细胞系施加或给予包含抗HER2结合分子的药物组合物,该受试者或患者具有疾病、疾病的症状、或易患疾病的倾向。

[0350] 本披露的抗HER2结合分子(例如,抗体或其抗原结合片段、变体、或衍生物)有用于治疗不同癌症。在一个方面中,本披露涉及用作药剂、具体地用于治疗或预防癌症的抗HER2结合分子,例如抗体或其抗原结合片段、变体、或衍生物。癌症的实例包括,但不限于乳癌、结肠癌、肺癌、胃癌、头颈部鳞状细胞癌、黑色素瘤、胰腺癌、或前列腺癌。在一些特定情况下,如例如通过HERCEPTEST™所确定,该癌症表达低水平的HER2。

[0351] 根据本披露的方法,如在此其他地方所定义的至少一种抗HER2结合分子(例如抗体或其抗原结合片段、变体、或衍生物)被用来促进关于癌症的阳性治疗应答。关于癌症治疗的术语“阳性治疗应答”是指改进与这些抗HER2结合分子(例如,抗体或其抗原结合片段、变体、或衍生物)的活性相关联的疾病,和/或改进与疾病相关联的症状。

[0352] 例如,疾病的改进可被表征为完全应答。术语“完全应答”是指使用对任何先前测

试结果进行归一化而不存在临床可检测的疾病。可替代地,疾病的改进可被归类为部分应答。“阳性治疗应答”涵盖由给予本披露的抗HER2结合分子产生的降低或抑制癌症的进展和/或持续时间、降低或改善癌症的严重性、和/或改善其一种或多种症状。

[0353] 在特定方面中,此类术语是指在给予本披露的抗HER2结合分子之后的一种、两种或三种或更多种结果:

- (1)稳定、降低或消除癌症细胞群;
- (2)稳定或降低癌症生长;
- (3)损害癌症形成;
- (4)消灭、去除、或控制原发性、局部性和/或转移性癌症;
- (5)降低死亡率;
- (6)增加无疾病、无复发、无进展、和/或总体存活率、持续时间、或速率;
- (7)增加应答速率、应答持久性、或响应或处于缓解的患者数;
- (8)降低住院治疗率;
- (9)减小住院期;
- (10)维持癌症大小(例如,以体积计)并且不增加或增加小于10%、优选地小于5%、优选地小于4%、优选地小于2%,以及
- (11)增加处于缓解的患者数;
- (12)减少将另外地要求治疗癌症的辅助疗法(例如,化疗或激素疗法)数。

[0354] 临床应答可以使用筛选技术来评价,这些筛选技术诸如PET、磁共振成像(MRI)扫描、x-射线照相成像、计算机断层摄影(CT)扫描、流式细胞术或荧光激活细胞分选仪(FACS)分析、组织学、粗略病理学、以及血液化学,包括但不限于通过ELISA、RIA、色谱法等可检测的变化。除了这些阳性治疗应答以外,经受使用抗HER2结合分子(例如,抗体或其抗原结合片段、变体、或衍生物)的疗法的受试者可以经历与疾病相关联的症状改进的有益作用。

[0355] 本披露的抗HER2结合分子(例如,抗体或其抗原结合片段、变体、或衍生物)可与任何已知的癌症疗法(包括已知有用、或已使用或目前正使用的任何试剂或试剂组合)组合使用,以便用于治疗癌症,例如结肠癌、肺癌、胃癌、头颈部鳞状细胞癌、以及乳癌。药物组合配制品或给药方案的第二试剂或试剂组合优选地具有与本披露的一种或多种抗HER2结合分子互补的活性,这样使得它们不会彼此不利地影响。

[0356] 抗癌剂包括用来治疗恶性肿瘤诸如癌性生长的药物。可以单独地、或与其他治疗诸如外科手术或放射疗法组合使用药物疗法。取决于涉及的器官性质,可以在癌症治疗中使用若干种类的药物。例如,乳癌通常通过雌激素来刺激,并且可以使用使性激素失活的药物来治疗。类似地,前列腺癌可以使用使雄激素(雄性激素)失活的药物来治疗。

[0357] 用于本披露的某些方法中的抗癌剂尤其包括抗体(例如,结合IGF-1R的抗体、结合EGFR的抗体、结合HER2或HER3的抗体)、靶向IGF1R的小分子、靶向EGFR的小分子、靶向HER2的小分子、抗代谢物、烷基化剂、拓扑异构酶抑制剂、微管靶向剂、激酶抑制剂、蛋白质合成抑制剂、免疫治疗剂、激素疗法、糖皮质激素、芳香酶抑制剂、mTOR抑制剂、化疗剂、蛋白激酶B抑制剂、磷脂酰肌醇3-激酶(PI3K)抑制剂、细胞周期蛋白依赖性激酶(CDK)抑制剂、RLr9、CD289、酶抑制剂、抗TRAIL、MEK抑制剂等。

[0358] 在特定方面中,在此披露的抗HER2结合分子(例如,抗体或其抗原结合片段)可与

靶向表皮生长因子受体(EGFR)的其他抗体或抗体片段(例如爱必妥® (Erbix®)(西妥昔单抗)或帕尼单抗(VECTIBIX®))组合给予。

[0359] 在其他方面中,在此披露的抗HER2结合分子可与激酶抑制剂(例如,酪氨酸激酶抑制剂)组合给予。在一些其他特定方面中,在此披露的抗HER2结合分子可与具有与EGFR和/或HER2/neu相关联的酪氨酸激酶活性的抑制剂(例如,拉帕替尼)组合给予。在一些方面中,本披露的抗HER2结合分子可与抗有丝分裂剂组合给予。在一些特定方面中,本披露的抗HER2结合分子可与稳定有丝分裂纺锤体微管组件的试剂(例如紫杉醇或多西他赛)组合给予。

[0360] 在组合疗法包括与另一种治疗剂给予组合而给予抗HER2结合分子的情况下,本披露的方法涵盖使用分开的配制品或单一药物配制品、以及以任一顺序的连续给予来进行的共同给予。在一些方面中,与其他药物组合给予在此描述的抗HER2结合分子,其中可依次、以任一顺序、或同时(即,同时地或在相同时间范围内)给予抗体或其抗原结合片段、变体、或衍生物以及一种或多种治疗剂。

[0361] 组合疗法可提供“协同作用”并且被证明是“协同的”,即,当一起使用活性成分时所实现的作用大于由分开使用化合物产生的作用的总和。当活性成分(1)共同配制和同时给予或递送在组合单位剂量配制品中;(2)作为分开的配制品通过交替或平行的方式递送;或(3)通过一些其他方案时,可获得协同作用。当以交替疗法递送时,当例如通过分开的注射器中的不同注射依次给予或递送化合物时,可获得协同作用。通常,在交替疗法的过程中,依次即连续给予每种活性成分的有效剂量,而在组合疗法中,一起给予两种或更多种活性成分的有效剂量。

[0362] 在一些方面中,可与生长因子受体(EGFR)抑制剂协同组合给予本披露的抗HER2结合分子,例如抗HER2抗体或其抗原结合片段。在一些方面中,该EGFR抑制剂是抗体。在特定方面中,该EGFR抑制剂抗体是爱必妥®(西妥昔单抗)或VECTIBIX®(帕尼单抗)。在特定方面中,本披露的抗HER2结合分子(例如,抗体或其抗原结合片段)可与具有与EGFR和/或HER2/neu相关联的酪氨酸激酶活性的抑制剂(例如,拉帕替尼)协同组合给予。在一些方面中,本披露的抗HER2结合分子可与抗有丝分裂剂协同组合给予。在一些特定方面中,该抗有丝分裂剂稳定有丝分裂纺锤体微管组件。在一些特定方面中,该抗有丝分裂剂是紫杉醇或多西他赛。

[0363] 另一方面是本披露的抗HER2结合分子(例如,抗体或其抗原结合片段、变体、或衍生物)用于诊断监测组织中的蛋白质水平的用途,该诊断监测作为临床测试程序中的一部分例如以便确定给定治疗方案的功效。例如,通过使抗体偶联到可检测物质上可促进检测。可检测物质的实例包括不同酶、辅基、荧光材料、发光材料、生物发光材料、以及放射性材料。适合的酶的实例包括辣根过氧化物酶、碱性磷酸酶、 β -半乳糖苷酶或乙酰胆碱酯酶;适合的辅基复合体的实例包括抗生物素蛋白链菌素/生物素和亲和素/生物素;适合的荧光材料的实例包括伞形酮、荧光素、异硫氰酸荧光素、罗丹明、二氯三嗪胺荧光素、丹磺酰氯或藻红蛋白;发光材料的实例包括鲁米诺;生物发光材料的实例包括荧光素酶、荧光素和水母发光蛋白;适合的放射性材料的实例包括 ^{125}I 、 ^{131}I 、 ^{35}S 或 ^3H 。

[0364] 另一方面是本披露的抗HER2结合分子(例如,抗体或其抗原结合片段、变体、或衍生物)治疗对HER2靶向的治疗剂(例如靶向HER2的结构域IV内的表位的抗体诸如曲妥珠单

抗)有抗性的癌症患者的用途。在一些方面中,该治疗剂包含靶向与曲妥珠单抗相同表位的一部分、以及细胞毒性部分。在一些方面中,此种细胞毒性部分是美登木素生物碱。在一些特定方面中,该美登木素生物碱是DM-1。

[0365] 在一些方面中,在此披露的治疗方法包括给予抗HER2结合分子,该抗HER2结合分子是包含重链(HC)可变区(VH)和轻链(LC)可变区(VL)的抗HER2抗体,该重链可变区和轻链可变区包含:

(i)与SEQ ID NO:1相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:1相同的可变重链CDR-1(VH-CDR1);

(ii)与SEQ ID NO:2相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:2相同的可变重链CDR-2(VH-CDR2);

(iii)与SEQ ID NO:3相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:3相同的可变重链CDR-3(VH-CDR3);

(iv)与SEQ ID NO:4相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:4相同的可变轻链CDR-1(VL-CDR1);

(v)与SEQ ID NO:5相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:5相同的可变轻链CDR-2(VL-CDR2);以及,

(vi)与SEQ ID NO:6相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:6相同的可变轻链CDR-3(VL-CDR3)。

[0366] 在一些方面中,在此披露的治疗方法包括给予抗HER2结合分子,该抗HER2结合分子是包含第一免疫球蛋白抗原结合结构域和第二免疫球蛋白抗原结合结构域的双特异性抗HER2抗体,其中(i)该第一免疫球蛋白抗原结合结构域和该第二免疫球蛋白抗原结合结构域特异性结合至不同HER2抗体结合位点,(ii)该第一免疫球蛋白抗原结合结构域结合至第一HER2抗体结合位点,该第一HER2抗体结合位点包含HER2的结构域II内的表位,并且(iii)该第一HER2抗体结合位点与帕妥珠单抗的抗体结合位点不同。

[0367] 在一些方面中,在此披露的治疗方法包括给予抗HER2结合分子,该抗HER2结合分子是包含第一免疫球蛋白抗原结合结构域和第二免疫球蛋白抗原结合结构域的双特异性抗HER2抗体,其中该第一免疫球蛋白抗原结合结构域包含重链(HC)可变区(VH)和轻链(LC)可变区(VL),该重链可变区和该轻链可变区包含:

(i)与SEQ ID NO:1相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:1相同的可变重链CDR-1(VH-CDR1);

(ii)与SEQ ID NO:2相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:2相同的可变重链CDR-2(VH-CDR2);

(iii)与SEQ ID NO:3相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:3相同的可变重链CDR-3(VH-CDR3);

(iv)与SEQ ID NO:4相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:4相同的可变轻链CDR-1(VL-CDR1);

(v)与SEQ ID NO:5相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:5相同的可变轻链CDR-2(VL-CDR2);以及,

(vi)与SEQ ID NO:6相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:6相同

的可变轻链CDR-3(VL-CDR3)。

[0368] 在一些方面中,此种抗HER2结合分子的该第一免疫球蛋白抗原结合结构域包含相对于包含VH(包含SEQ ID NO:43的氨基酸)和VL(包含SEQ ID NO:44的氨基酸)的免疫球蛋白抗原结合结构域的FW区不同的至少一个异源可变结构域构架区(FW)。在一些方面中,该第一免疫球蛋白抗原结合结构域包含:

- (i)包含SEQ ID NO:11的氨基酸的可变轻链构架1(VL-FW1);
- (ii)包含SEQ ID NO:12的氨基酸的VL-FW2;
- (iii)包含SEQ ID NO:13的氨基酸的VL-FW3;
- (iv)包含SEQ ID NO:14的氨基酸的VL-FW4;或
- (vi)其任何组合。

[0369] 在一些方面中,此种抗HER2结合分子的该第一免疫球蛋白抗原结合结构域包含VH和VL,其中该VH包含SEQ ID NO:15或43的氨基酸;并且其中该第一免疫球蛋白抗原结合结构域和该第二免疫球蛋白抗原结合结构域特异性结合至不同HER2表位。在一些方面中,此种抗HER2结合分子的该第一免疫球蛋白抗原结合结构域包含VH和VL,其中该VL包含SEQ ID NO:16或44的氨基酸;并且其中该第一免疫球蛋白抗原结合结构域和该第二免疫球蛋白抗原结合结构域特异性结合至不同HER2表位。在一些方面中,此种抗HER2结合分子的该第一免疫球蛋白抗原结合结构域包含VH和VL,其中该VH包含SEQ ID NO:15的氨基酸;并且其中该VL包含SEQ ID NO:16的氨基酸。

[0370] 在一些方面中,此种抗HER2结合分子的该第二免疫球蛋白抗原结合结构域(a)特异性结合至与曲妥珠单抗抗体相同的HER2表位;和/或(b)通过曲妥珠单抗抗体竞争性地抑制HER2结合;和/或(c)包含至少一个、至少两个、至少三个、至少四个、至少五个、或至少六个互补决定区(CDR),这些互补决定区包含SEQ ID NO:54至59中的任一个的氨基酸。在一些方面中,此种抗HER2结合分子的该第二免疫球蛋白抗原结合结构域是scFv,该scFv包含:
(i)包含SEQ ID NO:54的氨基酸的VH-CDR1;(ii)包含SEQ ID NO:55的氨基酸的VH-CDR2;
(iii)包含SEQ ID NO:56的氨基酸的VH-CDR3;(iv)包含SEQ ID NO:57的氨基酸的VL-CDR1;
(v)包含SEQ ID NO:58的氨基酸的VL-CDR2;以及(vi)包含SEQ ID NO:59的氨基酸的VL-CDR3。

[0371] 在其他方面中,在此披露的治疗方法包括给予抗HER2结合分子,该抗HER2结合分子是包含双特异性抗HER2抗体的ADC,其中所述抗体包含:

(i)包含SEQ ID NO:32A的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链,其中该第一多肽链包含共价连接至位置239处的半胱氨酸氨基酸上的治疗部分(例如,微管溶素1508分子),其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

(ii)包含SEQ ID NO:33A的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链,其中该第一多肽链包含共价连接至分别位于位置239和442处的半胱氨酸氨基酸上的两个治疗部分(例如,两个微管溶素1508分子),其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

(iii)包含SEQ ID NO:36A的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链,其中该第一多肽链包含共价连接至位置239处的半胱氨

酸氨基酸上的治疗部分(例如,微管溶素1508分子),其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

(iv)包含SEQ ID NO:37A的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链,其中该第一多肽链包含共价连接至分别位于位置239和442处的半胱氨酸氨基酸上的两个治疗部分(例如,两个微管溶素1508分子),其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

(v)包含SEQ ID NO:40A的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链,其中该第一多肽链包含共价连接至位置239处的半胱氨酸氨基酸上的治疗部分(例如,微管溶素1508分子),其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;或,

(vi)包含SEQ ID NO:41A的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链,其中该第一多肽链包含共价连接至分别位于位置239和442处的半胱氨酸氨基酸上的两个治疗部分(例如,两个微管溶素1508分子),其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引。

[0372] 在其他方面中,在此披露的治疗方法包括给予抗HER2结合分子,该抗HER2结合分子是包含双特异性抗HER2抗体的ADC,其中所述抗体包含:

(i)包含SEQ ID NO:32C的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链,其中该第一多肽链包含共价连接至位置239与240之间插入的半胱氨酸氨基酸上的治疗部分(例如,微管溶素1508分子),其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

(ii)包含SEQ ID NO:3C3的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链,其中该第一多肽链包含共价连接至位置239与240之间插入的半胱氨酸氨基和位于位置442处的半胱氨酸氨基酸上的两个治疗部分(例如,两个微管溶素1508分子),其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

(iii)包含SEQ ID NO:36C的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链,其中该第一多肽链包含共价连接至位置239与240之间插入的半胱氨酸氨基酸上的治疗部分(例如,微管溶素1508分子),其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

(iv)包含SEQ ID NO:37C的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链,其中该第一多肽链包含共价连接至位置239与240之间插入的半胱氨酸氨基和位于位置442处的半胱氨酸氨基酸上的两个治疗部分(例如,两个微管溶素1508分子),其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

(v)包含SEQ ID NO:40C的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链,其中该第一多肽链包含共价连接至位置239与240之间插入的半胱氨酸氨基酸上的治疗部分(例如,微管溶素1508分子),其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;或,

(vi)包含SEQ ID NO:67的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链,其中该第一多肽链包含共价连接至位置239与240之间插入的半胱氨酸氨基和位于位置442处的半胱氨酸氨基酸上的两个治疗部分(例如,两个微管溶

素1508分子),其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引。

X. 药物组合物和给予方法

[0373] 制备抗HER2结合分子(例如,抗体或其抗原结合片段、变体、或衍生物)(例如,ADC诸如Bs2-4T)并且向有需要的受试者给予这些抗HER2结合分子的方法对于本领域技术人员来说是熟知的或容易由本领域技术人员来确定。抗HER2结合分子(例如,抗体或其抗原结合片段、变体、或衍生物)(例如,ADC诸如Bs2-4T)的给予途径可以是例如口服、肠胃外、吸入或局部。如在此所使用的术语肠胃外包括例如静脉内、动脉内、腹膜内、肌内、皮下、直肠或经阴道给予。然而,在与在此的传授内容相容的其他方法中,本披露的抗HER2结合分子(例如,抗体或其抗原结合片段、变体、或衍生物)(例如,ADC诸如Bs2-4T)可以直接递送至有害细胞群的部位,从而增加患病组织对治疗剂的暴露。

[0374] 如在此所讨论,本披露的抗HER2结合分子(例如,抗体或其抗原结合片段、变体、衍生物)(例如,ADC诸如Bs2-4T)可以用于体内治疗HER2表达细胞介导的疾病诸如某些类型的癌症的药理学上有效的量来给予。药物组合物可以包含药理学上可接受的载体,包括例如水、离子交换剂、蛋白质、缓冲物质、以及盐。还可以存在防腐剂和其他添加剂。该载体可以是溶剂或分散介质。用于在此披露的治疗方法中的适合配制品被描述于《雷明登氏药理学全书》(Remington's Pharmaceutical Sciences)(Mack出版公司),第16版(1980)中。

[0375] 在任何情况下,无菌可注射溶液可以通过以下方式来制备:以要求的量将活性化合物(例如,单独或与其他活性剂组合的抗HER2抗体或其抗原结合片段、变体、或衍生物,例如ADC诸如Bs2-4T)掺入在适当溶剂中,接着进行过滤灭菌。此外,制剂可以被包装并且呈试剂盒的形式销售。此类制造物品可以具有标签或包装说明书(package insert),从而指示相关组合物是有用于治疗遭受、或易患疾病或病症的受试者。

[0376] 肠胃外配制品可以是单次推注剂量、输注剂量或负荷推注剂量随后是维持剂量。可以具体固定的或可变的间隔给予这些组合物,例如,每日一次,或以“在需要时”为基础。

[0377] 可以作为单次剂量、多次剂量或以输注形式在确立的时间周期内给予组合物。也可以调整剂量方案以便提供最佳期望反应(例如治疗性或预防性反应)。

[0378] 用于治疗HER2表达细胞介导的疾病诸如某些类型的癌症(包括例如,结肠癌、肺癌、胃癌、头颈部鳞状细胞癌、黑色素瘤、胰腺癌、前列腺癌、以及乳癌)的本披露的组合物的治疗有效量取决于许多不同因素而改变,这些因素包括给予方式、目标部位、患者的生理状态、患者是否是人或动物、给予的其他药剂、以及治疗是否是预防性或治疗性的。在一些特定方面中,如例如使用HERCEPTTM所确定,该癌症表达低水平的HER2。通常,该患者是人,但是也可以治疗非人哺乳动物(包括转基因哺乳动物)。治疗剂量可以使用本领域技术人员已知的常规方法来滴定,以便最优化安全性和功效。

[0379] 有待给予的至少一种抗HER2结合分子(例如,抗体或其结合片段、变体、或衍生物)(例如,ADC诸如Bs2-4T)的量可以在没有过分实验的情况下通过本领域技术人员容易地确定。影响至少一种抗HER2结合分子(例如,抗体、其抗原结合片段、变体或衍生物)(例如,ADC诸如Bs2-4T)的给予方式和对应的量的因素包括,但不限于经历疗法的个体的疾病严重性、疾病史、以及年龄、身高、体重、健康以及身体状况。类似地,有待给予的抗HER2结合分子(例如,抗体或其片段、变体、或衍生物)(例如,ADC诸如Bs2-4T)的量将取决于给予方式以及该受试者是否将经历这种试剂的单次剂量或多次剂量。

[0380] 本披露还提供抗HER2结合分子(例如,抗体或其抗原结合片段、变体、或衍生物)(例如,ADC诸如Bs2-4T)在制造用于治疗一种类型癌症的药剂中的用途,该癌症包括例如乳腺癌、结肠癌、肺癌、胃癌、头颈部鳞状细胞癌、黑色素瘤、胰腺癌、以及前列腺癌。在一些特定方面中,如例如使用HERCEPTEST™所确定,该癌症表达低水平的HER2。

[0381] 本披露还提供抗HER2结合分子(例如,本披露的抗体或其抗原结合片段、变体、或衍生物)在制造用于治疗受试者以便用于治疗一种类型癌症的药剂中的用途。在一些特定方面中,如例如使用HERCEPTEST™所确定,该癌症表达低水平的HER2。在某些方面中,在已使用至少一种其他疗法预先治疗的受试者中使用该药剂。

[0382] “预先治疗(pretreated)”或“预先治疗(pretreatment)”意图是指受试者在接受包含抗HER2结合分子(例如,抗体或其抗原结合片段、变体、或衍生物)(例如,ADC诸如Bs2-4T)的药剂之前已接受一种或多种其他疗法(例如,使用至少一种其他抗癌疗法进行治疗)。受试者不必是对使用先前的疗法或多个疗法进行的预先治疗的响应者。因此,接受包含抗HER2结合分子(例如,抗体或其抗原结合片段、变体、或衍生物)(例如,ADC诸如Bs2-4T)的药剂的受试者可以响应于、较差响应于、或可以不响应于使用先前疗法进行的预先治疗、或其中包括多个疗法的预先治疗的先前疗法中的一种或多种。因此,本披露提供治疗对其他疗法(例如,使用抗体或ADC诸如T-DM1的治疗)是较差响应者或未响应者的患者的方法,这些方法包括给予在此披露的抗HER2结合分子(例如,抗体或其结合片段、变体、或衍生物)(例如,ADC诸如Bs2-4T)。还提供预防对癌症疗法有抗性(例如,对使用抗体或ADC诸如T-DM1的治疗有抗性)的方法,这些方法包括给予在此披露的抗HER2结合分子(例如,抗体或其结合片段、变体、或衍生物)(例如,ADC诸如Bs2-4T)。

[0383] 即使先前已使用HER2抑制剂对患者进行治疗,但本领域技术人员可以确定人们在使用该HER2抑制剂治疗之后是否显示无应答。例如,对抑制剂的无应答可以反映在患癌增加中,诸如癌症/肿瘤生长增加和/或肿瘤大小增加、转移形成(增加)或转移数目或大小增加。无应答还可以是肿瘤或转移发展(例如在肿瘤切除之后)、疾病进展的时间缩短、或一种或多种肿瘤和/或一种或多种转移大小增加(例如在新辅助疗法中)。基于本领域中已知的这些参数或其他参数,可以鉴定不响应于使用HER2抑制剂(像帕妥珠单抗、曲妥珠单抗、或T-DM1)的治疗的患者组。此种患者组然后可以使用在此披露的抗HER2结合分子(例如,本披露的抗体、或其抗原结合片段、变体、或衍生物)(例如,ADC诸如Bs2-4T)来治疗。

[0384] 本披露还提供治疗例如对另一种疗法是较差响应者或未响应者的患者的方法。如在此所使用的术语“未响应者”可以是指不太可能响应于使用HER2抑制剂(例如,帕妥珠单抗、曲妥珠单抗、或T-DM1)的治疗的个体/患者/受试者。如在此所使用的“不太可能响应”是指在使用HER2抑制剂治疗的患者中将发生病理学完全应答的可能性降低。在一些方面中,患者开始可以是良好响应者,并且对HER2抑制剂的抗性可以在使用此类HER2抑制剂(例如,帕妥珠单抗、曲妥珠单抗、或T-DM1)治疗的过程中发展,从而引起对该治疗的较差应答或无应答。

[0385] 如在此所使用的术语“良好响应者”是指在使用HER2抑制剂(像帕妥珠单抗、曲妥珠单抗、或T-DM1)治疗过程中或之后,其肿瘤不表现出生长、转移、转移数目或大小增加等的个体,例如基于连续成像研究;在时间周期(例如,在初始诊断之后约1年)内不经历肿瘤生长、转移、转移数目和大小增加等的个体;和/或经历某一寿命(例如,在初始诊断之后约2

年或更长)的个体。

[0386] 如在此所使用的术语“较差响应者”是指例如使用HER2抑制剂(像帕妥珠单抗、曲妥珠单抗、或T-DM1)在标准疗法过程中或之后的短期内其肿瘤生长或转移、或经历归因于肿瘤的不良临床作用的个体。

[0387] 在其中评价受试者是“未响应者”、“较差响应者”或“不太可能响应”(例如基于在癌症细胞中存在某些生物标记物)的情况下,受试者可以使用在此披露的抗HER2结合分子(例如,本披露的抗体或其抗原结合片段、变体、或衍生物)(例如,ADC诸如Bs2-4T)来治疗。

[0388] 本披露还提供共同给予抗HER2结合分子(例如,本披露的抗体或其抗原结合片段、变体、或衍生物)(例如,ADC诸如Bs2-4T)以及至少一种其他疗法。该抗HER2结合分子和该至少一种其他疗法可以一起共同给予在单个组合物中,或可以同时或在重叠的时间里一起共同给予在分开的组合物中。在一些方面中,抗HER2结合分子(例如,本披露的抗体或其抗原结合片段、变体、或衍生物)(例如,ADC诸如Bs2-4T)可以用作辅助疗法。

[0389] 本披露还提供抗HER2结合分子(例如,本披露的抗体或其抗原结合片段、变体、或衍生物)(例如,ADC诸如Bs2-4T)在制造用于治疗受试者以便用于治疗癌症的药剂中的用途,其中该抗HER2结合分子在受试者用至少一种其他疗法治疗之前给予。

XI. 诊断学

[0390] 本披露进一步提供有助于在诊断HER2表达细胞介导的疾病诸如某些类型癌症过程中的诊断方法,这些癌症包括例如结肠癌、肺癌、胃癌、头颈部鳞状细胞癌、黑色素瘤、胰腺癌、前列腺癌、以及乳癌,这些诊断方法涉及测量来自个体的组织或其他细胞或体液中的HER2蛋白或转录物的表达水平并且将测量的表达水平与正常组织或体液中的标准HER2表达水平进行比较,因而与标准相比的表达水平增加指示病症。

[0391] 使用本领域技术人员已知的经典免疫组织学方法,本披露的抗HER2结合分子及其抗原结合片段、变体、以及衍生物可以被用来测定生物样品中的HER2蛋白水平(例如,参见加坎能(Jalkanen)等人,细胞生物学杂志(J.Cell.Biol.)101:976-985(1985);加坎能等人,细胞生物学杂志105:3087-3096(1987))。可用于检测HER2蛋白表达的其他基于抗体的方法包括免疫测定,诸如酶联免疫吸附测定(ELISA)、免疫沉淀、或蛋白质印迹。适合的测定在此其他地方更详细地描述。

[0392] “测定HER2多肽的表达水平”意图是指直接(例如,通过确定或评估绝对蛋白质水平)或相对(例如,通过与第二生物样品中的疾病相关联的多肽水平进行比较)定性地或定量地测量或评估第一生物样品中的HER2多肽水平。可以测量或评估该第一生物样品中的HER2多肽表达水平并且与标准HER2多肽水平进行比较,该标准得自从不具有病症的个体获得的第二生物样品或通过来自不具有病症的个体群体的水平进行平均化来确定。正如本领域中将了解的,一旦已知“标准”HER2多肽水平,它可以被重复地使用作为比较的标准。

[0393] “生物样品”意图是指从个体、细胞系、组织培养物、或潜在表达HER2的其他细胞来源获得的任何生物样品。用于从哺乳动物获得组织活检物和体液的方法是本领域中熟知的。

XII. 包括抗HER2结合分子的试剂盒

[0394] 本披露还提供可用来进行在此描述的方法、包括在此披露的抗HER2结合分子(例如,抗HER2抗体或其抗原结合片段)的试剂盒。在某些方面中,试剂盒包括在一个或多个容

器中的至少一种纯化的抗HER2结合分子或其抗原结合片段。在一些方面中,这些试剂盒含有进行检测测定所必需和/或足以进行检测测定的所有组分(包括用于进行检测的所有控制、指导),以及用于分析和呈现结果的任何必需软件。本领域技术人员将易于认识到,在此披露的所披露的抗HER2结合分子(例如,抗HER2抗体或其抗原结合片段)可易于与本领域中熟知的已建立的试剂盒形式相结合。

XIII. 免疫测定

[0395] 抗HER2结合分子(例如抗体或其抗原结合片段、本披露分子的变体或衍生物)可以通过在本领域中已知的任何方法来针对免疫特异性结合进行测定。可使用的免疫测定包括但不限于使用诸如以下技术的竞争性和非竞争性测定系统:蛋白质印迹、放射免疫测定、ELISA(酶联免疫吸附测定)、“夹心”免疫测定、免疫沉淀测定、沉淀素反应、凝胶扩散沉淀素反应、免疫扩散测定、凝集测定、补体固定测定、免疫放射测定、荧光免疫测定、蛋白质A免疫测定,仅举几个例子。此类测定是常规的并且在本领域是熟知的(参见,例如奥苏贝尔(Ausubel)等人编著,(1994),现代分子生物学实验技术(Current Protocols in Molecular Biology)(约翰威利父子公司,纽约(John Wiley&Sons,Inc.,NY))第1卷,该参考文献通过引用以其全文结合在此)。

[0396] 在此披露的抗HER2结合分子(例如双特异性抗HER2抗体或其抗原结合片段、本披露的变体或其衍生物)可以如在免疫荧光、免疫电子显微术或非免疫测定中被组织学地采用,以便用于原位检测HER2受体或其保守变体或肽片段。原位检测可以通过以下方式来实现:从患者中取得组织学样本,并且向其施加标记的HER2结合分子(例如,双特异性抗HER2抗体或其抗原结合片段、其变体或衍生物,优选地通过覆盖该标记的HER2结合分子(例如,抗体或片段)来施加)到生物样品上。通过使用这样一个程序,不仅可能确定HER2、或保守变体或肽片段的存在,而且可能确定它在所检查的组织中的分布。使用本披露,技术人员将容易地认识到可以修改任何各种组织学方法(诸如染色程序)以便实现这样的原位检测。

[0397] 可以根据熟知的方法来确定给定批次抗HER2结合分子(例如,双特异性抗HER2抗体或其抗原结合片段、其变体或衍生物)的结合活性。本领域技术人员将能够通过采用常规实验来确定每种确定的操作和最优测定条件。

[0398] 适用于确定本披露的抗HER2结合分子(例如,双特异性抗体或其抗原结合片段、其变体或改变/突变的衍生物)的结合特征的方法和试剂在本领域中是已知的和/或是商业上可获得的。设计用于此类动力学分析的仪器和软件是商业上可获得的(例如,BIAcore,BIA评估软件,通用健康医疗公司(GE Healthcare);KinExa软件,Sapidyne仪器公司(Sapidyne Instruments))。

[0399] 除非另外指示,否则本发明的实践将采用细胞生物学、细胞培养、分子生物学、转基因生物学、微生物学、重组DNA以及免疫学的常规技术,这些技术在本领域的技能范围内。此类技术在文献中得到充分解释。参见,例如桑布鲁克(Sambrook)等人编著(1989)分子克隆实验室手册(Molecular Cloning A Laboratory Manual)(第2版;冷泉港实验室出版社(Cold Spring Harbor Laboratory Press));桑布鲁克等人编著(1992)分子克隆实验室手册,(冷泉港实验室,纽约);D.N.格洛夫(D.N.Glover)编著,(1985)DNA克隆(DNA Cloning),第I和II卷;盖特(Gait)编著(1984)寡核苷酸合成(Oligonucleotide Synthesis);穆利斯(Mullis)等人美国专利号4,683,195;哈莫斯(Hames)和希金斯(Higgins)编著(1984)核酸

杂交(Nucleic Acid Hybridization);哈莫斯和希金斯编著(1984)转录和翻译(Transcription And Translation);弗雷什尼(Freshney)(1987)动物细胞培养(Culture Of Animal Cells)Alan R.Liss公司(Alan R.Liss,Inc.);固定的细胞和酶(Immobilized Cells And Enzymes)(IRL出版社)(1986);培博(Perbal)(1984)分子克隆的实践指导;酶学论文、方法(A Practical Guide To Molecular Cloning;the treatise,Methods In Enzymology)(美国学术出版社公司,纽约);米勒(Miller)和卡洛(Calos)编著(1987)哺乳动物细胞的基因转移载体(Gene Transfer Vectors For Mammalian Cells),(冷泉港实验室);吴(Wu)等人编著,酶学方法(Methods In Enzymology),第154和155卷;迈尔(Mayer)和沃克(Walker)编著(1987)细胞和分子生物学中的免疫化学方法(美国学术出版社,伦敦);维尔(Weir)和布莱克威尔(Blackwell)编著(1986)实验免疫学手册(Handbook Of Experimental Immunology),第1-IV卷;操纵小鼠胚胎(Manipulating the Mouse Embryo),冷泉港实验室出版社,冷泉港,纽约,(1986);以及奥苏贝尔等人(1989)现代分子生物学实验技术(约翰威利父子公司,马里兰州巴尔的摩)。

[0400] 在博雷贝克(Borrebaeck)编著(1995)抗体工程化(Antibody Engineering)(第2版;牛津大学出版社)中阐述了抗体工程化的普遍原理。在里克伍德(Rickwood)等人编著(1995)蛋白质工程化,实践方法(Protein Engineering,A Practical Approach)(英国牛津,牛津大学出版社的IRL出版社)中阐述了蛋白质工程化的普遍原理。抗体和抗体-半抗原结合的普遍原理被阐述于:尼森夫(Nisonoff)(1984)分子免疫学(Molecular Immunology)(第2版;Sinauer Associates公司,桑德兰,Mass.);以及斯特华(Steward)(1984)抗体、它们的结构和功能(Antibodies,Their Structure and Function)(查普曼(Chapman)和霍尔(Hall),纽约)中。另外,本领域中已知的并且不明确描述的免疫学标准方法通常如下所述:现代免疫学实验技术,约翰威利父子公司,纽约;斯蒂茨(Stites)等人编著(1994)基本和临床免疫学(Basic and Clinical Immunology)(第8版;康涅狄格州阿普尔顿和兰格,诺瓦克(Appleton&Lange,Norwalk,Conn.))以及米歇尔(Mishell)和师伊吉(Shiigi)(编著)(1980)细胞免疫学的选定方法(Selected Methods in Cellular Immunology)(W.H.弗里曼和有限公司,纽约)。

[0401] 阐述免疫学普遍原理的标准参考著作包括现代免疫学实验技术,约翰威利父子公司,纽约;克莱因(Klein)(1982)免疫学杂志:自身-非自身区别科学(J.,Immunology:The Science of Self-Nonself Discrimination)(约翰威利父子公司,纽约);肯尼特(Kennett)等人编著(1980)单克隆抗体,杂交瘤:生物学分析的新局面(Monoclonal Antibodies,Hybridoma:A New Dimension in Biological Analyses)(纽约Plenum出版社);坎贝尔(Campbell)(1984)生物化学和分子生物学实验技术中的“单克隆抗体技术”(“Monoclonal Antibody Technology”in Laboratory Techniques in Biochemistry and Molecular Biology),布登(Burden)等人编著,(Elsevier,Amsterdam);戈尔兹比(Goldsby)等人编著(2000)Kuby免疫学(Kuby Immunology)(第4版;H.弗里曼和有限公司);罗义特(Roitt)等人(2001)免疫学(第6版;伦敦:莫斯比(London:Mosby));阿巴斯(Abbas)等人(2005)细胞和分子免疫学(Cellular and Molecular Immunology)(第5版;Elsevier健康科学部(Elsevier Health Sciences Division));康特曼(Kontermann)和迪贝尔(Dubel)(2001)抗体工程化(Springer Verlag);桑布鲁克和拉塞尔(Russell)(2001)

分子克隆:实验室手册(冷泉港出版社);列文(Lewin)(2003)基因VIII(Genes VIII)(Prentice Hall 2003);哈洛(Harlow)和莱恩(Lane)(1988)抗体:实验室手册(Antibodies: A Laboratory Manual)(冷泉港出版社);迪芬巴赫(Dieffenbach)和维克斯列尔(Dveksler)(2003)PCR引物(PCR Primer)(冷泉港出版社)。

XIV. 实施例

[0402] 根据“EnXm”模式命名实施例,其中E意指“实施例”;n是实施例序数;X是任选的并且可以是A或B,其中A指代确切地与ADC构建体相关的实施例,并且B指代确切地与具有增强的ADCC的构建体相关的实施例;并且m是指示类别内的另外实施例的任选子序数(例如,A1、B1等)。

[0403] E1.一种双特异性抗HER2抗体,包含第一免疫球蛋白抗原结合结构域和第二免疫球蛋白抗原结合结构域,其中(i)该第一免疫球蛋白抗原结合结构域和该第二免疫球蛋白抗原结合结构域特异性结合至不同HER2抗体结合位点,(ii)该第一免疫球蛋白抗原结合结构域结合至第一HER2抗体结合位点,该第一HER2抗体结合位点包含HER2的结构域II内的表位,并且(iii)该第一HER2抗体结合位点与帕妥珠单抗的抗体结合位点不同。

[0404] E2.如E1所述的双特异性抗HER2抗体,其中该第二免疫球蛋白抗原结合结构域结合至第二HER2抗体结合位点,该第二HER2抗体结合位点包含HER2的结构域IV内的表位。

[0405] E3.如E2所述的双特异性抗HER2抗体,其中该第二HER2抗体结合位点与曲妥珠单抗的HER2抗体结合位点相同。

[0406] E4.如E2所述的双特异性抗HER2抗体,其中该第二HER2抗体结合位点与曲妥珠单抗的HER2抗体结合位点部分重叠。

[0407] E5.如E2所述的双特异性抗HER2抗体,其中该第二HER2抗体结合位点与曲妥珠单抗的HER抗体结合位点不同。

[0408] E6.一种双特异性HER2抗体,包含第一免疫球蛋白抗原结合结构域和第二免疫球蛋白抗原结合结构域,其中该第一免疫球蛋白抗原结合结构域和该第二免疫球蛋白抗原结合结构域特异性结合至不同HER2表位;并且其中该第一免疫球蛋白抗原结合结构域将HER2结合至SEQ ID NO:52中的一个或多个氨基酸残基上。

[0409] E7.根据E6所述的双特异性抗体,其中该第二免疫球蛋白抗原结合结构域在结构域IV内的表位处结合HER2。

[0410] E8.根据E6所述的双特异性抗体,其中该第二免疫球蛋白抗原结合结构域将HER2结合至SEQ ID NO:53中的一个或多个氨基酸残基上。

[0411] E9.根据E1-E8所述的双特异性抗体,其中该第一免疫球蛋白抗原结合结构域包含重链(HC)可变区(VH)和轻链(LC)可变区(VL),该重链可变区和该轻链可变区包含:

(i)与SEQ ID NO:1相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:1相同的可变重链CDR-1(VH-CDR1);

(ii)与SEQ ID NO:2相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:2相同的可变重链CDR-2(VH-CDR2);

(iii)与SEQ ID NO:3相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:3相同的可变重链CDR-3(VH-CDR3);

(iv)与SEQ ID NO:4相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:4相同

的可变轻链CDR-1(VL-CDR1);

(v)与SEQ ID NO:5相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:5相同的可变轻链CDR-2(VL-CDR2);以及,

(vi)与SEQ ID NO:6相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:6相同的可变轻链CDR-3(VL-CDR3)。

[0412] E10.根据E9所述的双特异性抗体,其中该第一免疫球蛋白抗原结合结构域包含相对于包含VH(包含SEQ ID NO:43的氨基酸)和VL(包含SEQ ID NO:44的氨基酸)的免疫球蛋白抗原结合结构域的FW区不同的至少一个异源可变结构域构架区(FW)。

[0413] E11.根据E10所述的双特异性抗HER2抗体,其中该第一免疫球蛋白抗原结合结构域包含:

- (i)包含SEQ ID NO:11的氨基酸的可变轻链构架1(VL-FW1);
- (ii)包含SEQ ID NO:12的氨基酸的VL-FW2;
- (iii)包含SEQ ID NO:13的氨基酸的VL-FW3;
- (iv)包含SEQ ID NO:14的氨基酸的VL-FW4;或
- (v)其任何组合。

[0414] E12.一种双特异性抗HER2抗体,包含第一免疫球蛋白抗原结合结构域和第二免疫球蛋白抗原结合结构域,其中该第一免疫球蛋白抗原结合结构域包含VH和VL,

其中该VH包含SEQ ID NO:15或43的氨基酸;并且

其中该第一免疫球蛋白抗原结合结构域和该第二免疫球蛋白抗原结合结构域特异性结合至不同HER2表位。

[0415] E13.一种双特异性抗HER2抗体,包含第一免疫球蛋白抗原结合结构域和第二免疫球蛋白抗原结合结构域,其中该第一免疫球蛋白抗原结合结构域包含VH和VL,

其中该VL包含SEQ ID NO:16或44的氨基酸;并且

其中该第一免疫球蛋白抗原结合结构域和该第二免疫球蛋白抗原结合结构域特异性结合至不同HER2表位。

[0416] E14.如E12或E13所述的双特异性抗HER2抗体,

其中该VH包含SEQ ID NO:15的氨基酸;并且

其中该VL包含SEQ ID NO:16的氨基酸。

[0417] E15.根据E1至E14中任一项所述的双特异性抗HER2抗体,其中该第一免疫球蛋白抗原结合结构域包含scFv抗体片段。

[0418] E16.根据E1至E15中任一项所述的双特异性抗HER2抗体,其中该第二免疫球蛋白抗原结合结构域包含scFv抗体片段。

[0419] E17.根据E1至E16中任一项所述的双特异性抗HER2抗体,其中该第一免疫球蛋白抗原结合结构域包含以下各项或由其组成:

(a)VH和VL,该VH进一步包含重链恒定区或其片段,并且该VL包含轻链恒定区(LC)或其片段;

(b)单链Fv(“scFv”);

(c)双体抗体;

(d)小抗体;

(e)F(ab')₂;或

(f)F(ab)。

[0420] E18.根据E17所述的双特异性抗HER2抗体,其中该重链恒定区或其片段是IgG恒定区。

[0421] E19.根据E18所述的双特异性HER2抗体,其中该IgG恒定区或其片段是IgG1恒定区。

[0422] E20.根据E17至E19中任一项所述的双特异性HER2抗体,其中该LC恒定区是κ恒定区。

[0423] E21.根据E17至E19中任一项所述的双特异性HER2抗体,其中该LC恒定区是λ恒定区。

[0424] E22.根据E1至E21中任一项所述的双特异性抗HER2抗体,其中该第一免疫球蛋白抗原结合结构域是单克隆抗体。

[0425] E23.根据E1至E22中任一项所述的双特异性抗HER2抗体,其中该第一免疫球蛋白抗原结合结构域是人源化抗体。

[0426] E24.根据E1至E22中任一项所述的双特异性抗HER2抗体,其中该第一免疫球蛋白抗原结合结构域是人抗体。

[0427] E25.根据E1至E22中任一项所述的双特异性抗HER2抗体,其中该第一免疫球蛋白抗原结合结构域是嵌合抗体。

[0428] E26.根据E1至E25中任一项所述的双特异性抗HER2抗体,其中该第一免疫球蛋白抗原结合结构域是亲和力优化的抗体。

[0429] E27.根据E1至E26中任一项所述的双特异性抗HER2抗体,其中该第一免疫球蛋白抗原结合结构域不与曲妥珠单抗或帕妥珠单抗竞争表位结合。

[0430] E28.根据E1至E27中任一项所述的双特异性抗HER2抗体,其中该第一免疫球蛋白抗原结合结构域和该第二免疫球蛋白抗原结合结构域特异性结合至不同的非重叠HER2表位。

[0431] E29.根据E1至E28中任一项所述的双特异性抗HER2抗体,其中:

(a)该第二免疫球蛋白抗原结合结构域特异性结合至与该曲妥珠单抗抗体相同的HER2表位;

(b)该第二免疫球蛋白抗原结合结构域通过该曲妥珠单抗抗体竞争性地抑制HER2结合;或

(c)该第二免疫球蛋白抗原结合结构域包含至少一个、至少两个、至少三个、至少四个、至少五个、或至少六个互补决定区(CDR),这些互补决定区包含SEQ ID NO:54至59中的任一个的氨基酸。

[0432] E30.根据E29所述的双特异性抗HER2抗体,其中该第二免疫球蛋白抗原结合结构域是scFv,所述scFv包含:

(i)包含SEQ ID NO:54的氨基酸的VH-CDR1;

(ii)包含SEQ ID NO:55的氨基酸的VH-CDR2;

(iii)包含SEQ ID NO:56的氨基酸的VH-CDR3;

(iv)包含SEQ ID NO:57的氨基酸的VL-CDR1;

(v)包含SEQ ID NO:58的氨基酸的VL-CDR2;以及

(vi)包含SEQ ID NO:59的氨基酸的VL-CDR3。

[0433] E31.根据E30所述的双特异性抗HER2抗体,其中该scFv是二硫化物稳定的scFv。

[0434] E32.根据E30或E31所述的双特异性抗HER2抗体,其中所述scFv包含VH和VL,该VH包含SEQ ID NO:17的氨基酸并且该VL包含SEQ ID NO:18的氨基酸。

[0435] E33.根据E32所述的双特异性抗HER2抗体,其中该scFv的该VH和该VL通过肽接头共价连接。

[0436] E34.根据E33所述的双特异性抗HER2抗体,其中该肽接头包含SEQ ID NO:19的氨基酸。

[0437] E35.根据E29至E34中任一项所述的双特异性抗HER2抗体,其中该第二免疫球蛋白抗原结合结构域被共价连接至该第一免疫球蛋白抗原结合结构域的该HC的羧基末端上。

[0438] E36.根据E35所述的双特异性抗HER2抗体,包含插入在该第二免疫球蛋白抗原结合结构域与该第一免疫球蛋白抗原结合结构域的该HC的羧基末端之间的接头。

[0439] E37.根据E29至E34中任一项所述的双特异性抗HER2抗体,其中该第二免疫球蛋白抗原结合结构域被共价连接至该第一免疫球蛋白抗原结合结构域的该HC的氨基末端上。

[0440] E38.根据E37所述的双特异性抗HER2抗体,包含插入在该第二免疫球蛋白抗原结合结构域与该第一免疫球蛋白抗原结合结构域的该HC的氨基末端之间的接头。

[0441] E39.根据E29至E34所述的双特异性抗HER2抗体,其中该第二免疫球蛋白抗原结合结构域被共价嵌入在该第一免疫球蛋白抗原结合结构域的该HC的多肽链中。

[0442] E40.根据E39所述的双特异性抗HER2抗体,其中该第二免疫球蛋白抗原结合结构域被共价嵌入在该第一免疫球蛋白抗原结合结构域的该HC的CH1区与CH2区之间。

[0443] E41.根据E40所述的双特异性抗HER2抗体,包含:

(i)插入在该第一免疫球蛋白抗原结合结构域的该HC的CH1区与该第二免疫球蛋白抗原结合结构域之间的接头;以及

(ii)插入在该第二免疫球蛋白抗原结合结构域与该第一免疫球蛋白抗原结合结构域的该HC的CH2区之间的第二接头。

[0444] E42.根据E41所述的双特异性抗HER2抗体,其中该第一接头和该第二接头是相同的。

[0445] E43.根据E41所述的双特异性抗HER2抗体,其中该第一接头和该第二接头是不同的。

[0446] E44.根据E36、E38以及E41至E43中任一项所述的双特异性抗HER2抗体,其中这些接头中的一个或多个包括肽接头。

[0447] E45.根据E44所述的双特异性抗HER2抗体,其中该肽接头包含至少两个、至少三个、至少四个、至少五个、至少10个、至少20个、或至少30个氨基酸。

[0448] E46.根据E44或E45中任一项所述的双特异性抗HER2抗体,其中该肽接头包括具有化学式 $\text{Ser}_x[(\text{Gly})_y\text{-Ser}_4]_z$ 的肽,其中x是从0至1,y是从1至4,并且z是从1至10。

[0449] E47.根据E46所述的双特异性抗HER2抗体,其中该肽接头包含SEQ ID NO:19-22。

[0450] E48.根据E1至E47中任一项所述的双特异性抗HER2抗体,其中该重链包含恒定区,该恒定区包含Fc结构域。

- [0451] E49. 根据E48所述的双特异性抗HER2抗体, 其中该Fc结构域包含CH2区和CH3区。
- [0452] E50. 根据E48所述的双特异性抗HER2抗体, 其中该Fc结构域是IgG1 Fc结构域。
- [0453] E51. 根据E50所述的双特异性抗HER2抗体, 其中该IgG1 Fc结构域是天然IgG1 Fc结构域。
- [0454] E52. 根据E51所述的双特异性抗HER2抗体, 其中该天然IgG1 Fc结构域包含SEQ ID NO:23的氨基酸。
- [0455] E53. 根据E48所述的双特异性抗HER2抗体, 其中该Fc结构域是突变IgG1 Fc结构域。
- [0456] E54A. 根据E53所述的双特异性抗HER2抗体, 其中该突变IgG1Fc结构域包含能够降低该双特异性抗体的ADCC活性的至少一个突变。
- [0457] E54B. 根据E53所述的双特异性抗HER2抗体, 其中该突变IgG1Fc结构域包含能够增强该双特异性抗体的ADCC活性的至少一个突变。
- [0458] E55A. 根据E54A所述的双特异性抗HER2抗体, 其中能够降低该双特异性抗体的ADCC活性的至少一个突变是氨基酸取代。
- [0459] E55B. 根据E54B所述的双特异性抗HER2抗体, 其中能够增强该双特异性抗体的ADCC活性的至少一个突变是氨基酸取代。
- [0460] E56A. 根据E55A所述的双特异性抗HER2抗体, 包含至少一个氨基酸取代, 该至少一个氨基酸取代包括L234F、S239A、S239C、位置239与240之间的半胱氨酸氨基酸插入或其任何组合, 其中氨基酸位置编号根据在卡巴特中所阐述的EU索引。
- [0461] E56B. 根据E55B所述的双特异性抗HER2抗体, 包含至少一个氨基酸取代, 该至少一个氨基酸取代包括S239A、S239D、A330L、I332E、E333A、K334A或其任何组合, 其中氨基酸位置编号根据在卡巴特中所阐述的EU索引。
- [0462] E57A. 根据E55A或E56A所述的双特异性抗HER2抗体, 其中所述突变IgG1 Fc结构域包含引入可衍生基团的至少一个氨基酸取代。
- [0463] E57B. 根据E48至E53、E54B、E55B或E56B中任一项所述的双特异性抗HER2抗体, 其中该Fc结构域具有增强ADCC活性的改变类型的糖基化。
- [0464] E58A. 根据E57A所述的双特异性抗HER2抗体, 其中所述突变IgG1 Fc结构域包含引入可衍生基团的一至三个氨基酸取代。
- [0465] E58B. 根据E57B所述的双特异性抗HER2抗体, 其中该Fc结构域是具有减少量的岩藻糖基残基的无岩藻糖基化的抗体。
- [0466] E59A. 根据E57A或E58A所述的双特异性抗HER2抗体, 其中该可衍生基团是氢硫基基团。
- [0467] E59B. 根据E57B或E58B所述的双特异性抗HER2抗体, 其中该Fc结构域具有增加的等分G1cNAc结构。
- [0468] E60. 根据E59A所述的双特异性抗HER2抗体, 其中该至少一个氨基酸取代包括S239C、248C、254C、258C、273C、279C、282C、284C、286C、287C、289C、297C、298C、312C、324C、326C、330C、335C、337C、339C、350C、355C、356C、359C、360C、361C、375C、383C、384C、389C、398C、400C、413C、415C、418C、422C、435C、440C、441C、S442C、443C、位置239与240之间的半胱氨酸插入、以及446C、或其任何组合, 其中氨基酸位置编号根据如在卡巴特中所阐述的EU

索引。

[0469] E61A. 根据E54A、E55A、E56A、E57A、E58A、E59A、或E60中任一项所述的双特异性抗HER2抗体,其中该突变Fc结构域包含SEQ ID NO:24A、SEQ ID NO:24C、SEQ ID NO:25A或SEQ ID NO:25C的氨基酸。

[0470] E61B. 根据E54B、E55B、E56B、E57B、E58B、E59B、或E60中任一项所述的双特异性抗HER2抗体,其中该突变Fc结构域包含SEQ ID NO:24B或SEQ ID NO:25B的氨基酸。

[0471] E62. 一种双特异性抗HER2抗体,包含彼此相关联的第一多肽链和第二多肽链,其中该第一多肽链选自以下各项:

- (1) [TZ_s]-[L₁]-[BVH]-[BCH]-[Fc_x]
- (2) [BVH]-[BCH]-[Fc_x]-[L₂]-[TZ_s]
- (3) [BVH]-[BCH]-[L₃]-[TZ_s]-[L₄]-[Fc_x]

其中

TZ_s是结合与曲妥珠单抗相同的表位的scFv;

L₁、L₂、L₃以及L₄是肽接头;

Fc_x是Fc结构域;

BVH和BCH分别是抗体的VH区和CH1区,该抗体能够结合至与该曲妥珠单抗抗体识别的该表位不同的HER2表位。

[0472] E63. 根据E62所述的双特异性抗HER2抗体,其中该不同表位包含SEQ ID NO:52内的一个或多个氨基酸。

[0473] E64. 根据E62或E63所述的双特异性抗HER2抗体,其中该第二链包含[BVL]-[CL],其中BVL是能够结合至与该曲妥珠单抗抗体识别的该表位不同的HER2表位的抗体的VL区,并且CL是IgG轻链恒定区。

[0474] E65. 根据E64所述的双特异性抗HER2抗体,其中CL选自下组,该组由以下各项组成:人K恒定区和人λ恒定区。

[0475] E66. 根据E64或E65所述的双特异性抗HER2抗体,其中BVL包含:

(i) 与SEQ ID NO:4相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:4相同的可变轻链CDR-1(VL-CDR1);

(ii) 与SEQ ID NO:5相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:5相同的可变轻链CDR-2(VL-CDR2);以及,

(iii) 与SEQ ID NO:6相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:6相同的可变轻链CDR-3(VL-CDR3)。

[0476] E67. 根据E64、E65或E66所述的双特异性抗HER2抗体,其中BVL包含SEQ ID NO:16或44的氨基酸。

[0477] E68. 根据E65所述的双特异性抗HER2抗体,其中CL包含SEQ ID NO:27A或SEQ ID NO:27B的氨基酸。

[0478] E69. 根据E62至E68中任一项所述的双特异性抗HER2抗体,其中[TZ_s]包含:

- (i) 包含SEQ ID NO:54的氨基酸的VH-CDR1;
- (ii) 包含SEQ ID NO:55的氨基酸的VH-CDR2;
- (iii) 包含SEQ ID NO:56的氨基酸的VH-CDR3;

- (iv)包含SEQ ID NO:57的氨基酸的VL-CDR1;
- (v)包含SEQ ID NO:58的氨基酸的VL-CDR2;以及
- (vi)包含SEQ ID NO:59的氨基酸的VL-CDR3。

[0479] E70.根据E62至E69中任一项所述的双特异性抗HER2抗体,其中[TZ_s]是二硫化物稳定的scFv。

[0480] E71.根据E62至E70中任一项所述的双特异性抗HER2抗体,其中[TZ_s]包含通过肽接头共价连接的VH和VL,该VH包含SEQ ID NO:17的氨基酸并且该VL包含SEQ ID NO:18的氨基酸。

[0481] E72.根据E71所述的双特异性抗HER2抗体,其中该肽接头包含SEQ ID NO:19的氨基酸。

[0482] E73.根据E69至E72中任一项所述的双特异性抗HER2抗体,其中[TZ_s]包含SEQ ID NO:28的氨基酸。

[0483] E74.根据E62至E73中任一项所述的双特异性抗HER2抗体,其中铰链多肽连接该[BCH]和[FcX]。

[0484] E75.根据E74所述的双特异性抗HER2抗体,其中该铰链多肽包含SEQ ID NO:26的氨基酸或由其组成。

[0485] E76A.根据E62至E75中任一项所述的双特异性抗HER2抗体,其中该[FcX]包含引入可衍生基团的至少一个氨基酸取代。

[0486] E76B.根据E62至E75中任一项所述的双特异性抗HER2抗体,其中[FcX]包含能够增强该双特异性抗体的ADCC活性的至少一个突变。

[0487] E77A.根据E76A所述的双特异性抗HER2抗体,其中该[FcX]包含引入可衍生基团的一至三个氨基酸取代。

[0488] E77B.根据E76B所述的双特异性抗HER2抗体,其中能够增强该双特异性抗体的ADCC活性的至少一个突变是氨基酸取代。

[0489] E78A.根据E76A或E77A所述的双特异性抗HER2抗体,其中该可衍生基团是氢硫基基团。

[0490] E78B.根据E77B所述的双特异性抗HER2抗体,包含至少一个氨基酸取代,该至少一个氨基酸取代包括S239A、S239D、A330L、I332E、E333A、K334A或其任何组合,其中氨基酸位置编号根据在卡巴特中所阐述的EU索引。

[0491] E78B1.根据E62至E75、E76B、E77B或E78B中任一项所述的双特异性抗HER2抗体,其中该Fc结构域具有增强ADCC活性的改变类型的糖基化。

[0492] E78B2.根据E78B1所述的双特异性抗HER2抗体,其中该Fc结构域是具有减少量的岩藻糖基残基的无岩藻糖基化的抗体。

[0493] E78B3.根据E78B1或E78B2所述的双特异性抗HER2抗体,其中该Fc结构域具有增加的等分GlcNAc结构。

[0494] E79.根据E78A所述的双特异性抗HER2抗体,其中该至少一个氨基酸取代包括S239C、248C、254C、258C、273C、279C、282C、284C、286C、287C、289C、297C、298C、312C、324C、326C、330C、335C、337C、339C、350C、355C、356C、359C、360C、361C、375C、383C、384C、389C、398C、400C、413C、415C、418C、422C、435C、440C、441C、S442C、443C、446C、位置239与240之间

的半胱氨酸插入、或其任何组合,其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引。

[0495] E80A.根据E62至E75、E76A、E77A、E78A、或E79中任一项所述的双特异性抗HER2抗体,其中[Fcx]包含SEQ ID NO:23、24A、24C、25A以及25C中的任一个的氨基酸。

[0496] E80B.根据E62至E75、E76B、E77B、E78B、E78B1、E78B2、E78B3、或E79中任一项所述的双特异性抗HER2抗体,其中[Fcx]包含SEQ ID NO:23、24B、以及25B中的任一个的氨基酸。

[0497] E81.根据E62至E79中任一项所述的双特异性抗HER2抗体,其中[L₁],[L₂],[L₃]以及[L₄]包含独立地选自下组的氨基酸,该组由以下各项组成:SEQ ID NO:19、20、21以及22。

[0498] E82.根据E62至E79中任一项所述的双特异性抗HER2抗体,其中:

(i)[L₁]包含SEQ ID NO:19的氨基酸;

(ii)[L₂]包含SEQ ID NO:20的氨基酸;

(iii)[L₃]包含SEQ ID NO:21的氨基酸;以及,

(iv)[L₄]包含SEQ ID NO:22的氨基酸。

[0499] E83.根据E62至E82中任一项所述的双特异性抗HER2抗体,其中[BVH]包含:

(i)与SEQ ID NO:1相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:1相同的可变重链CDR-1(VH-CDR1);

(ii)与SEQ ID NO:2相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:2相同的可变重链CDR-2(VH-CDR2);以及

(iii)与SEQ ID NO:3相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:3相同的可变重链CDR-3(VH-CDR3)。

[0500] E84.根据E62至E82中任一项所述的双特异性抗HER2抗体,其中[BVH]包含SEQ ID NO:15或43。

[0501] E85.根据E62至E84中任一项所述的双特异性抗HER2抗体,其中[BCH]包含SEQ ID NO:29的氨基酸。

[0502] E86.根据E64至E85中任一项所述的双特异性抗HER2抗体,其中[BVL]包含:

(i)与SEQ ID NO:4相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:4相同的可变轻链CDR-1(VL-CDR1);

(ii)与SEQ ID NO:5相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:5相同的可变轻链CDR-2(VL-CDR2);以及

(iii)与SEQ ID NO:6相同或除了高达1、2、3或4个氨基酸取代以外与SEQ ID NO:6相同的可变轻链CDR-3(VL-CDR3)。

[0503] E87.根据E64至E85中任一项所述的双特异性抗HER2抗体,其中[BVL]包含SEQ ID NO:16或44的氨基酸。

[0504] E88A.根据E62至E87中任一项所述的双特异性HER2抗体,其中第一多肽链包含SEQ ID NO:30、31A、32A、32C、33A、33C、34、35A、36A、36C、37A、37C、38、38A、39A、40A、40C、41A或41C中的任一个的氨基酸,并且第二多肽链包含SEQ ID NO:42A或42B的氨基酸,其中该双特异性HER2抗体被轭合至治疗部分上。

[0505] E88B.根据E62至E87中任一项所述的双特异性HER2抗体,其中第一多肽链包含SEQ ID NO:30、31B、32B、33B、34、35B、36B、37B、38、38B、39B、40B或41B中的任一个的氨基酸,并且第二多肽链包含SEQ ID NO:42A或42B的氨基酸,其中该双特异性HER2抗体具有增强的

ADCC活性。

[0506] E89.根据E1至E88B中任一项所述的双特异性HER2抗体,其中该双特异性HER2抗体在结合至该HER2靶标时诱导内化。

[0507] E90.根据E89所述的双特异性HER2抗体,其中该双特异性HER2抗体在内化之后促进有效的溶酶体运输。

[0508] E91.根据E1至E88B中任一项所述的双特异性HER2抗体,其中该双特异性HER2抗体诱导HER2靶标降解。

[0509] E92.根据E1至E88B中任一项所述的双特异性HER2抗体,其中该双特异性HER2抗体阻断低HER2表达癌症细胞中的配体诱导的AKT磷酸化。

[0510] E93.根据E1至E88B中任一项所述的双特异性HER2抗体,其中该双特异性HER2抗体破坏配体诱导的HER2:HER3二聚化。

[0511] E94.一种抗HER2结合分子,包含免疫球蛋白重链(VH)和免疫球蛋白轻链(VL),其中该结合分子包含:

- (i)包含SEQ ID NO:1的氨基酸的VH-CDR1;
- (ii)包含SEQ ID NO:2的氨基酸的VH-CDR2;
- (iii)包含SEQ ID NO:3的氨基酸的VH-CDR3;
- (iv)包含SEQ ID NO:4的氨基酸的VL-CDR1;
- (v)包含SEQ ID NO:5的氨基酸的VL-CDR2;以及,
- (vi)包含SEQ ID NO:6的氨基酸的VL-CDR3;

[0512] E95.一种抗HER2结合分子,包含免疫球蛋白重链(VH)和免疫球蛋白轻链(VL),其中该VH包含SEQ ID NO:15的氨基酸。

[0513] E96.一种包含VH和VL的抗HER2结合分子,其中该VL包含SEQ ID NO:16的氨基酸。

[0514] E97.如E94或E95或E96所述的结合分子,其中该VH包含SEQ ID NO:15的氨基酸并且该VL包含SEQ ID NO:16的氨基酸。

[0515] E98.如权利要求E94至E97中任一项所述的结合分子,包括抗体或其抗原结合片段。

[0516] E99A.一种抗体-药物缀合物(ADC),包含根据E1至E93中任一项所述的双特异性HER2抗体或根据E94至E98中任一项所述的抗HER2结合分子以及至少一个治疗部分。

[0517] E100A.根据E99A所述的ADC,进一步包含至少一个任意的间隔物。

[0518] E101A.根据E100A所述的ADC,其中至少一个间隔物是肽间隔物。

[0519] E102A.根据E100A所述的ADC,其中至少一个间隔物是非肽间隔物。

[0520] E103A.根据E99A至E102A中任一项所述的ADC,包含两个、三个、或四个治疗部分。

[0521] E104A.根据E99A至E103A中任一项所述的ADC,其中所有治疗部分是相同的。

[0522] E105A.根据E99A至E104A中任一项所述的ADC,其中每个治疗部分被化学缀合至该双特异性抗体的该Fc区中的特定位置处的氨基酸侧链上。

[0523] E106A.根据E105A所述的ADC,其中这些特定位置选自下组,该组由以下各项组成: 239、248、254、258、273、279、282、284、286、287、289、297、298、312、324、326、330、335、337、339、350、355、356、359、360、361、375、383、384、389、398、400、413、415、418、422、435、440、441、442、443、446、在位置239与240之间的插入、以及其任何组合,其中氨基酸位置编号根

据如在卡巴特中所阐述的EU索引。

[0524] E107A. 根据E105A或106A所述的ADC, 其中这些特定位置是239、442或两者, 其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引。

[0525] E108A. 根据E105A或106A所述的ADC, 其中这些特定位置是442和位置239与240之间的氨基酸插入, 其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引。

[0526] E109A. 根据E105A至E108A所述的ADC, 其中该氨基酸侧链是氢硫基侧链。

[0527] E110A. 一种ADC, 包含根据E1至E98中任一项所述的双特异性HER2抗体, 其中所述抗体包含:

(i) 包含SEQ ID NO:32A的氨基酸的第一多肽链和包含SEQ ID NO:42A的氨基酸的第二多肽链, 其中该第一多肽链包含共价连接至位置239处的半胱氨酸氨基酸上的治疗部分, 其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

(ii) 包含SEQ ID NO:33A的氨基酸的第一多肽链和包含SEQ ID NO:42A的氨基酸的第二多肽链, 其中该第一多肽链包含共价连接至分别位于位置239和442处的半胱氨酸氨基酸上的两个治疗部分, 其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

(iii) 包含SEQ ID NO:36A的氨基酸的第一多肽链和包含SEQ ID NO:42A的氨基酸的第二多肽链, 其中该第一多肽链包含共价连接至位置239处的半胱氨酸氨基酸上的治疗部分, 其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

(iv) 包含SEQ ID NO:37A的氨基酸的第一多肽链和包含SEQ ID NO:42A的氨基酸的第二多肽链, 其中该第一多肽链包含共价连接至分别位于位置239和442处的半胱氨酸氨基酸上的两个治疗部分, 其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

(v) 包含SEQ ID NO:40A的氨基酸的第一多肽链和包含SEQ ID NO:42A的氨基酸的第二多肽链, 其中该第一多肽链包含共价连接至位置239处的半胱氨酸氨基酸上的治疗部分, 其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引; 或

(vi) 包含SEQ ID NO:41A的氨基酸的第一多肽链和包含SEQ ID NO:42A的氨基酸的第二多肽链, 其中该第一多肽链包含共价连接至分别位于位置239和442处的半胱氨酸氨基酸上的两个治疗部分, 其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引。

[0528] E111A1. 一种ADC, 包含根据权利要求1至113中任一项所述的双特异性HER2抗体, 其中所述抗体包含:

(i) 包含SEQ ID NO:32C的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链, 其中该第一多肽链包含共价连接至位置239与240之间插入的半胱氨酸氨基酸上的治疗部分, 其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

(ii) 包含SEQ ID NO:33C的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链, 其中该第一多肽链包含共价连接至位置239与240之间插入的半胱氨酸氨基和位于位置442处的半胱氨酸氨基酸上的两个治疗部分, 其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

(iii) 包含SEQ ID NO:36C的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链, 其中该第一多肽链包含共价连接至位置239与240之间插入的半胱氨酸氨基酸上的治疗部分, 其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引;

引；

(iv)包含SEQ ID NO:37C的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链,其中该第一多肽链包含共价连接至位置239与240之间插入的半胱氨酸氨基和位于位置442处的半胱氨酸氨基酸上的两个治疗部分,其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引；

(v)包含SEQ ID NO:40C的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链,其中该第一多肽链包含共价连接至位置239与240之间插入的半胱氨酸氨基酸上的治疗部分,其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引；或,

(vi)包含SEQ ID NO:41C的氨基酸或由其组成的第一多肽链和包含SEQ ID NO:42A的氨基酸或由其组成的第二多肽链,其中该第一多肽链包含共价连接至位置239与240之间插入的半胱氨酸氨基和位于位置442处的半胱氨酸氨基酸上的两个治疗部分,其中氨基酸位置编号根据如在卡巴特中所阐述的EU索引。

[0529] E112A.根据E99A至E111A中任一项所述的ADC,其中该治疗部分包括细胞毒素、放射性同位素、放射性同位素、免疫调节剂、细胞因子、淋巴因子、趋化因子、生长因子、肿瘤坏死因子、激素、激素拮抗剂、酶、寡核苷酸、DNA、RNA、siRNA、RNAi、微小RNA、光敏治疗剂、抗血管生成剂、促凋亡剂、肽、脂质、碳水化合物、螯合剂或其组合。

[0530] E113A.根据E112A所述的ADC,其中该细胞毒素是微管溶素、奥瑞他汀、美登木素生物碱或吡咯并苯并二氮杂萘(PBD)。

[0531] E114.一种分离的核酸分子或核酸分子组,编码根据E1至E93中任一项所述的双特异性HER2抗体或根据E94至E98中任一项所述的抗HER2结合分子、或其补体。

[0532] E115.一种载体或载体组,包含如E114所述的核酸分子或核酸分子组、或其补体。

[0533] E116.一种宿主细胞,包含根据E114所述的分离的核酸分子或核酸分子组、或根据E115所述的载体或载体组。

[0534] E117.一种宿主细胞,表达根据E1至E93中任一项所述的双特异性HER2抗体或根据E94至E98中任一项所述的抗HER2结合分子。

[0535] E118.一种用于产生根据E1至E93中任一项所述的双特异性HER2抗体或根据E94至E98中任一项所述的抗HER2结合分子的方法,该方法包括培养根据E116或E117中任一项所述的宿主细胞并且从培养基中回收该抗体。

[0536] E119.一种药物组合物,包含根据E1至E93中任一项所述的双特异性HER2抗体、根据E94至E98中任一项所述的抗HER2结合分子、或根据E99A至E113A1中任一项所述的ADC、以及药学上可接受的载体。

[0537] E120.一种治疗HER2表达癌症的方法,该方法包括向有需要的受试者给予根据E1至E93中任一项所述的双特异性HER2抗体、根据E94至E98中任一项所述的抗HER2结合分子、根据E99A至E113A1中任一项所述的ADC或根据E119所述的组合物。

[0538] E121.根据E120所述的方法,其中该癌症是低HER2表达癌症。

[0539] E122.如E120或E121中任一项所述的方法,进一步包括给予至少一种另外的治疗剂。

[0540] E123.根据E122所述的方法,其中该至少一种另外的治疗剂是放射性核素或化疗

剂。

[0541] E124. 一种将治疗部分靶向至表达HER2的细胞上的方法, 该方法包括给予融合或轭合至根据E1至E93中任一项所述的双特异性HER2抗体、根据E94至E98中任一项所述的抗HER2结合分子、或根据E99A至E113A1中任一项所述的ADC上的该治疗部分。

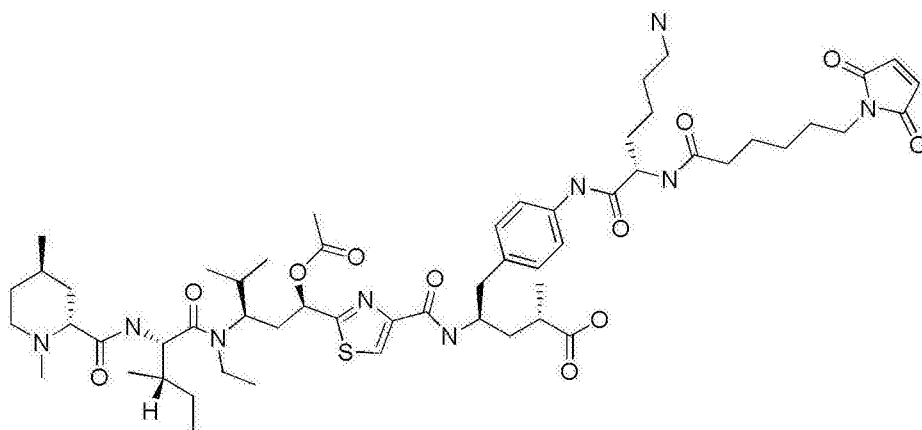
[0542] E125. 一种增加治疗部分的活性的方法, 该方法包括将该部分轭合至根据E1至E93中任一项所述的双特异性HER2抗体、根据E94至E98中任一项所述的抗HER2结合分子、或根据E99A至E113A1中任一项所述的ADC上。

[0543] E126. 一种改进治疗部分的药物代谢动力学特性的方法, 该方法包括将该部分轭合至根据E1至E93中任一项所述的双特异性HER2抗体、根据E94至E98中任一项所述的抗HER2结合分子、或根据E99A至E113A1中任一项所述的ADC上。

[0544] E127. 根据E112至E126中任一项所述的方法, 其中该治疗部分是细胞毒素、放射性同位素、免疫调节剂、细胞因子、淋巴因子、趋化因子、生长因子、肿瘤坏死因子、激素、激素拮抗剂、酶、寡核苷酸、DNA、RNA、siRNA、RNAi、微小RNA、光敏治疗剂、抗血管生成剂、促凋亡剂、肽、脂质、碳水化合物、或螯合剂。

[0545] E128. 根据E127所述的方法, 其中该细胞毒素是微管溶素、奥瑞他汀、美登木素生物碱或吡咯并苯并二氮杂萘(PBD)。

[0546] E129. 根据E113A所述的ADC或根据E128所述的方法, 其中该细胞毒素是具有以下结构的微管溶素1508:



[0547] E130. 一种治疗对HER2靶向治疗剂的抗性的方法, 该方法包括向有需要的患者给予根据E1至E93中任一项所述的双特异性HER2抗体、根据E94至E98中任一项所述的抗HER2结合分子、或根据E99A至E113A中任一项所述的ADC。

XV. 序列

[0548] 以下表3提供了序列参考号(SEQ ID NO:)、氨基酸序列以及关于这些序列的评述。

表3

SEQ ID NO	序列	评述
1	SYSMS	来自亲和力优化的S39抗体的VH-CDR1
2	SISSSSSYIYYADSVKG	来自亲和力优化的S39抗体的VH-CDR2
3	GGDAYNYYYFDY	来自亲和力优化的S39抗体的VH-CDR3
4	KSSQSVFFRSNNKNILA	来自亲和力优化的S39抗体的VL-CDR1
5	WASSRES	来自亲和力优化的S39抗体的VL-CDR2
6	QQYFGSPFT	来自亲和力优化的S39抗体的VL-CDR3
7	EVQLVESGGGLVKPGGSLRLSCAASGFTFS	来自亲和力优化的S39抗体的VH-FR1
8	WVRQAPGKGLEWVS	来自亲和力优化的S39抗体的VH-FR2
9	RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR	来自亲和力优化的S39抗体的VH-FR3
10	WGQGTLVTVSS	来自亲和力优化的S39抗体

SEQ ID NO	序列	评述
		的VH-FR4
11	DIVMTQTPLSLSVTPGQPASISC	来自亲和力优化的S39抗体的VL-FR1
12	WYLQKPGQPPQLLIY	来自亲和力优化的S39抗体的VL-FR2
13	GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC	来自亲和力优化的S39抗体的VL-FR3
14	FGPGTKVDIK	来自亲和力优化的S39抗体的VL-FR4
15	EVQLVESGGGLVKPGGSLRLSCAASGFTFSSYSMSWVRQAPGKGLEWVSSISSSSYIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARGGDAYNYYYFDYWGQGTLVTVSS	来自克隆39S抗体的VH2 - VH
16	DIVMTQTPLSLSVTPGQPASISCKSSQSVFFRSNNKNILAWYLQKPGQPPQLLIYWASSRESGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCQQYFGSPFTFGPGTKVDIK	来自克隆39S抗体的VL2 - VL
17	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKXLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGTLLTVSS	结构域IV scFv的VH1 - VH SEQ ID NO: 17中的X是K或C
18	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISLQPEDFATYYCQQHYTTPPTFGXGTKVEIK	结构域IV scFv的VL1 - VL SEQ ID NO:18中的X是Q或C
19	GGGGSGGGGSGGGGSGGGGS	L _x 接头, x = 1-4 - scFv接头 - 位于scFv中的VL与VH之间
20	GGGGSGGGGSGGGGS	L _x 接头, x = 1-4 - 用于Bs2Ab-FCC和Bs2Ab-FC构建体中
21	EPKSCGKTGGGGSGGGGS	L _x 接头, x = 1-4 - 用于Bs4Ab-FCC和Bs4Ab-FC构建体中
22	SGGGGSGGGGS	L _x 接头, x = 1-4 - 铰链
23	CPPCPAPPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDI	天然IgG1 Fc Fc部分可以包括其羧基末端的EU残基226。强调的位置相应于已知等位基因变异的

SEQ ID NO	序列	评述
	AVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSVSMHEALHNHYTQKSLSLSPGK	位点。
24A	CPPCPAPEFLGGPCVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSVSMHEALHNHYTQKSLSLSPGK	对于ADC构建体, Fc IgG1 L234F S239C (“FC”)。突变在方块内。强调的位置相应于已知等位基因变异的位点。
24B	CPPCPAPELLGGPDVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSVSMHEALHNHYTQKSLSLSPGK	对于ADCC构建体, Fc IgG1 S239D I332E。突变在方块内。强调的位置相应于已知等位基因变异的位点。
24C	CPPCPAPELLGGPSCVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSVSMHEALHNHYTQKSLSLSPGK	对于ADC构建体, Fc IgG1 C239-ins。突变在方块内。强调的位置相应于已知等位基因变异的位点。
25A	CPPCPAPEFLGGPCVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSVSMHEALHNHYTQKSLSLSPGK	对于ADC构建体, Fc IgG1 L234F S239C S442C (“FCC”)。突变在方块内。强调的位置相应于已知等位基因变异的位点。
25B	CPPCPAPELLGGPDVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSVSMHEALHNHYTQKSLSLSPGK	对于ADCC构建体, Fc IgG1 S239D A330L I332E。突变在方块内。强调的位置相应于已知等位基因变异的位点。

SEQ ID NO	序列	评述
	QKSLSLSPGK	
25C	CPPCPAPELLGGPS <u>C</u> VFLFPPKPKDTLMISRT PEVTCVVVDVSHEDPEVKFNWYVDGVEVH NAKTKPREEQYNSTYRVVSVLTVLHQDWL NGKEYKCKVSNKALPAPIEKTISKAKGQPRE PQVYITLPPSREEMTKNQVSLTCLVKGFYPSD IAVEWESNGQPENNYKTTTPVLDSGGSFFLY SKLTVDKSRWQQGNVFSVMSVMEALHNHY TQKSL <u>C</u> LSPGK	对于ADC构建体, Fc IgG1 C239-ins S442C。突变在方块内。强调的位置相应于已知等位基因变异的位点。
26	EPKSCDKTHT	存在于Bs2和Bs3中的IgG1铰链
27A	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNFF YPREAKVQWKVDNALQSGNSQESVTEQDS KDSTYLSSTLTLSKADYEKHKVYACEVTH QGLSSPVTKSFNRGEC	CL κ 。强调的位置相应于已知等位基因变异的位点。
27B	QPKAAPSVTLFPPSSEELQANKATLVCLISDF YPGAVTVAWKADSSPVKAGVETTTPSKQSN NKYAASSYLSLTPEQWKSHRSYSCQVTHEG STVEKTVAPTEC	CL λ 。强调的位置相应于已知等位基因变异的位点。
28	EVQLVESGGGLVQPGGSLRLSCAASGFNID TYIHWRVQAPGK <u>X</u> ₁ LEWVARIYPTNGYTRY ADSVKGRFTISADTSKNTAYLQMNSLR AEDTAVYYCSRWGGDGFYAMDYWGQGT LVTVSSGGGGSGGGGSGGGGSGGGGSDIQMTQSP SSLSASVGDRTITCRASQDVNTAVAWYQQ KPGKAPKLLIYSASFLYSGVPSRFSRSGTD FTLTISLQPEDFATYYCQQHYTTPPTFG <u>X</u> ₂ G TKVEIK	曲妥珠单抗scFv序列。X ₁ =K或C; X ₂ =Q或C。在二硫化物稳定的scFv中, X ₁ =X ₂ =C。
29	ASTKGPSVFPLAPSSKSTSGGTAAALGCLVKD YFPEPVTISWNSGALTSGVHTFPAVLQSSGL YSLSSVVTVPSSSLGTQTYICNVNHKPSNTK VDKRV	来自39S抗体的CH1 - EU残基118至215。强调的位置相应于已知等位基因变异的位点。
30	DIQMTQSPSSLSASVGDRTITCRASQDVNT AVAWYQQKPGKAPKLLIYSASFLYSGVPSRF SGSRSGTDFTLTISLQPEDFATYYCQQHYTT PPTFGCGTKVEIKGGGGSGGGGSGGGGSGG GGSEVQLVESGGGLVQPGGSLRLSCAASGF NIKDTYIHWVRQAPGKCLEWVARIYPTNGY TRYADSVKGRFTISADTSKNTAYLQMNSLR AEDTAVYYCSRWGGDGFYAMDYWGQGT LVTVSSGGGGSGGGGSGGGGSEVQLVESGGG LVKPGGSLRLSCAASGFTFSYMSWVRQAP	Bs2Ab天然Fc构建体的重链。如下划线的位置指示可以进行某些取代的地方。强调的位置相应于已知等位基因变异的位点。

SEQ ID NO	序列	评述
	GKGLEWVSSISSSSSYIYYADSVKGRFTISRDN NAKNSLYLQMNSLRAEDTAVYYCARGGDA YNYYYFDYWGQGTLVTVSSASTKGPSVFPL APSSKSTSGGTAALGCLVKDYFPEPVTVSW NSGALTSGVHTFPAVLQSSGLYSLSSVVTVP SSSLGTQTYICNVNHKPSNTKVDKRVEPKSC DKTHTCPPCPAPE <u>LLGGP</u> <u>S</u> VFLFPPKPKDTL MISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQ DWLNGKEYKCKVSNKALP <u>API</u> EKTISKAKG QPREPQVYTLPPSREEMTKNQVSLTCLVKGF YPSDIAVEWESNGQPENNYKTTTPVLDSDGS FFLYSKLTVDKSRWQQGNVFCFSVMHEALH NHYTQKSL <u>S</u> LSPGK	
31A	DIQMTQSPSSLSASVGDRVTITCRASQDVNT AVAWYQQKPGKAPKLLIYSASFLYSGVPSRF SGSRSGTDFTLTISSLQPEDFATYYCQQHYTT PPTFGCGTKVEIKGGGGSGGGGSGGGGSGG GGSEVQLVESGGGLVQPGGSLRLSCAASGF NIKDTYIHWVRQAPGKCLEWVARIYPTNGY TRYADSVKGRFTISADTSKNTAYLQMNSLR AEDTAVYYCSRWGGDGFYAMDYWGQGT LTVSSGGGGSGGGGSGGGGSEVQLVESGGG LVKPGGSLRLSCAASGFTFSSYSMSWVRQAP GKGLEWVSSISSSSSYIYYADSVKGRFTISRDN NAKNSLYLQMNSLRAEDTAVYYCARGGDA YNYYYFDYWGQGTLVTVSSASTKGPSVFPL APSSKSTSGGTAALGCLVKDYFPEPVTVSW NSGALTSGVHTFPAVLQSSGLYSLSSVVTVP SSSLGTQTYICNVNHKPSNTKVDKRVEPKSC DKTHTCPPCPAPE <u>FLGGP</u> <u>S</u> VFLFPPKPKDTL MISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQ DWLNGKEYKCKVSNKALP <u>API</u> EKTISKAKG QPREPQVYTLPPSREEMTKNQVSLTCLVKGF YPSDIAVEWESNGQPENNYKTTTPVLDSDGS FFLYSKLTVDKSRWQQGNVFCFSVMHEALH NHYTQKSL <u>S</u> LSPGK	具有 L234F (“F”) 的 Bs2Ab2 的重链。对于 ADC 的构建体。强调的位置相应于已知等位基因变异的位点。
31B	DIQMTQSPSSLSASVGDRVTITCRASQDVNT AVAWYQQKPGKAPKLLIYSASFLYSGVPSRF SGSRSGTDFTLTISSLQPEDFATYYCQQHYTT PPTFGCGTKVEIKGGGGSGGGGSGGGGSGG GGSEVQLVESGGGLVQPGGSLRLSCAASGF NIKDTYIHWVRQAPGKCLEWVARIYPTNGY TRYADSVKGRFTISADTSKNTAYLQMNSLR AEDTAVYYCSRWGGDGFYAMDYWGQGT LTVSSGGGGSGGGGSGGGGSEVQLVESGGG	具有 I332E 的 Bs2Ab2 的重链。具有增强的 ADCC 的构建体。强调的位置相应于已知等位基因变异的位点。

SEQ ID NO	序列	评述
	LVKPGGSLRLSCAASGFTFSSYSMSWVRQAP GKGLEWVSSISSSSSSYIYYADSVKGRFTISR NAKNSLYLQMNSLRAEDTAVYYCARGGDA YNYYYFDYWGQGLTVTVSSASTKGPSVFPL APSSKSTSGGTAALGCLVKDYFPEPVTVSW NSGALTSGVHTFPAVLQSSGLYSLSSVTVTP SSSLGTQTYICNVNHKPSNTKVDKRVEPKSC DKTHTCPPCPAPE <u>LLGGP</u> <u>SVFL</u> FPPKPKDTL MISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQ DWLNGKEYKCKVSNKALP <u>AP</u> <u>EE</u> KTISKAKG QPREPQVYTLPPSREEMTKNQVSLTCLVKGF YPSDIAVEWESNGQPENNYKTTTPVLDSGGS FFLYSKLTVDKSRWQQGNVFCFSVMHEALH NHYTQKSL <u>SL</u> SPGK	
32A	DIQMTQSPSSLSASVGDRVTITCRASQDVNT AVAWYQQKPGKAPKLLIYSASF LY SGVPSRF SGSRSGTDFTLTISSLQPEDFATYYCQQHYTT PPTFGCGTKVEIKGGGGSGGGGSGGGGSGG GGSEVQLVESGGGLVQPGGSLRLSCAASGF NIKDTYIHWVRQAPGKCLEWVARIYPTNGY TRYADSVKGRFTISADTSKNTAYLQMNSLR AEDTAVYYCSRWGGDGFYAMDYWGQGLT VTVSSGGGGSGGGGSGGGGSEVQLVESGGG LVKPGGSLRLSCAASGFTFSSYSMSWVRQAP GKGLEWVSSISSSSSSYIYYADSVKGRFTISR NAKNSLYLQMNSLRAEDTAVYYCARGGDA YNYYYFDYWGQGLTVTVSSASTKGPSVFPL APSSKSTSGGTAALGCLVKDYFPEPVTVSW NSGALTSGVHTFPAVLQSSGLYSLSSVTVTP SSSLGTQTYICNVNHKPSNTKVDKRVEPKSC DKTHTCPPCPAPE <u>FLGGP</u> <u>CVFL</u> FPPKPKDTL MISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQ DWLNGKEYKCKVSNKALP <u>API</u> <u>E</u> KTISKAKG QPREPQVYTLPPSREEMTKNQVSLTCLVKGF YPSDIAVEWESNGQPENNYKTTTPVLDSGGS FFLYSKLTVDKSRWQQGNVFCFSVMHEALH NHYTQKSL <u>SL</u> SPGK	具有 L234F 、 S239C （“FC”）的Bs2Ab的重链。 对于ADC的构建体。强调的 位置相应于已知等位基因变 异的位点。
32B	DIQMTQSPSSLSASVGDRVTITCRASQDVNT AVAWYQQKPGKAPKLLIYSASF LY SGVPSRF SGSRSGTDFTLTISSLQPEDFATYYCQQHYTT PPTFGCGTKVEIKGGGGSGGGGSGGGGSGG GGSEVQLVESGGGLVQPGGSLRLSCAASGF NIKDTYIHWVRQAPGKCLEWVARIYPTNGY TRYADSVKGRFTISADTSKNTAYLQMNSLR AEDTAVYYCSRWGGDGFYAMDYWGQGLT	具有 S239D和I332E的Bs2Ab 的重链。具有增强的ADCC 的构建体。强调的位置相应 于已知等位基因变异的位 点。

SEQ ID NO	序列	评述
	VTVSSGGGGSGGGSGGGGSEVQLVESGGG LVKPGGSLRLSCAASGFTFSSYSMSWVRQAP GKGLEWVSSISSSSSSYIYYADSVKGRFTISR NAKNSLYLQMNSLRAEDTAVYYCARGGDA YNYYYFDYWGQGTLLTVSSASTKGPSVFPL APSSKSTSGGTAALGCLVKDYFPEPVTVSW NSGALTSGVHTFPAVLQSSGLYSLSSVVTVP SSSLGTQTYICNVNHKPSNTKVDKRVEPKSC DKHTHTCPPCPAPE <u>L</u> GGP <u>D</u> VFLFPPKPKDTL MISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQ DWLNGKEYKCKVSNKALP <u>A</u> PEIEKTISKAKG QPREPQVYTLPPSREEMTKNQVSLTCLVKGF YPSDIAVEWESNGQPENNYKTTTPVLDSDGS FFLYSKLTVDKSRWQQGNVFCFSVMHEALH NHYTQKSLSLSPGK	
32C	DIQMTQSPSSLSASVGDRVTITCRASQDVNT AVAWYQQKPGKAPKLLIYSASFLYSGVPSRF SGRSGTDFLTIISSLQPEDFATYYCQQHYTT PPTFGCGTKVEIKGGGGSGGGGSGGGGSGG GGSEVQLVESGGGLVQPGGSLRLSCAASGF NIKDTYIHWVRQAPGKCLEWVARIYPTNGY TRYADSVKGRFTISADTSKNTAYLQMNSLR AEDTAVYYCSRWGGDGFYAMDYWGQGTLL VTVSSGGGGSGGGSGGGGSEVQLVESGGG LVKPGGSLRLSCAASGFTFSSYSMSWVRQAP GKGLEWVSSISSSSSSYIYYADSVKGRFTISR NAKNSLYLQMNSLRAEDTAVYYCARGGDA YNYYYFDYWGQGTLLTVSSASTKGPSVFPL APSSKSTSGGTAALGCLVKDYFPEPVTVSW NSGALTSGVHTFPAVLQSSGLYSLSSVVTVP SSSLGTQTYICNVNHKPSNTKVDKRVEPKSC DKHTHTCPPCPAPE <u>L</u> GGP <u>C</u> VFLFPPKPKDTL MISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQ DWLNGKEYKCKVSNKALP <u>A</u> PIEIEKTISKAKG QPREPQVYTLPPSREEMTKNQVSLTCLVKGF YPSDIAVEWESNGQPENNYKTTTPVLDSDGS FFLYSKLTVDKSRWQQGNVFCFSVMHEALH NHYTQKSLSLSPGK	具有 C239-ins (“C”) 的 Bs2Ab 的重链。对于 ADC 的构建体。强调的位置相应于已知等位基因变异的位点。
33A	DIQMTQSPSSLSASVGDRVTITCRASQDVNT AVAWYQQKPGKAPKLLIYSASFLYSGVPSRF SGRSGTDFLTIISSLQPEDFATYYCQQHYTT PPTFGCGTKVEIKGGGGSGGGGSGGGGSGG GGSEVQLVESGGGLVQPGGSLRLSCAASGF NIKDTYIHWVRQAPGKCLEWVARIYPTNGY TRYADSVKGRFTISADTSKNTAYLQMNSLR	具有 L234F、S239C 以及 S442C (“FCC”) 的 Bs2Ab 的重链。对于 ADC 的构建体。强调的位置相应于已知等位基因变异的位点。

SEQ ID NO	序列	评述
	AEDTAVYYCSRWGGDGFYAMDYWGQGT LVTSSGGGGSGGGSGGGGSEVQLVESGGG LVKPGGSLRLSCAASGFTSSYMSWVRQAP GKGLEWVSSISSSSSYIYYADSVKGRFTISR NAKNSLYLQMNSLRAEDTAVYYCARGGDA YNYYYFDYWGQGT LVTSSASTKGPSVFPL APSSKSTSGGTAALGCLVKDYFPEPVT VSW NSGALTSGVHTFPAVLQSSGLYSLSSVVTVP SSSLGTQTYICNVNHKPSNTKVDKRVEPKSC DKTHTCPPCPAPE <u>FLGGP</u> CVFLFPPKPKDTL MISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQ DWLNGKEYKCKVSNKALP <u>API</u> EKTISKAKG QPREPQVYTLPPSREEMTKNQVSLTCLVKGF YPSDIAVEWESNGQPENNYKTTTPVLDSDGS FFLYSKLTVDKSRWQQGNVFSCSVMHEALH NHYTQKSL <u>CL</u> SPGK	
33B	DIQMTQSPSSLSASVGDRVTITCRASQDVNT AVAWYQQKPGKAPKLLIYSASFLYSGVPSRF SGSRSGTDFTLTISSLQPEDFATYYCQQHYTT PPTFGCGTKVEIKGGGGSGGGSGGGSGGGSGG GGSEVQLVESGGGLVQPGGSLRLSCAASGF NIKDTYIHWVRQAPGKCLEWVARIYPTNGY TRYADSVKGRFTISADTSKNTAYLQMNSLR AEDTAVYYCSRWGGDGFYAMDYWGQGT LVTSSGGGGSGGGSGGGGSEVQLVESGGG LVKPGGSLRLSCAASGFTSSYMSWVRQAP GKGLEWVSSISSSSSYIYYADSVKGRFTISR NAKNSLYLQMNSLRAEDTAVYYCARGGDA YNYYYFDYWGQGT LVTSSASTKGPSVFPL APSSKSTSGGTAALGCLVKDYFPEPVT VSW NSGALTSGVHTFPAVLQSSGLYSLSSVVTVP SSSLGTQTYICNVNHKPSNTKVDKRVEPKSC DKTHTCPPCPAPE <u>LLGGP</u> DVFLFPPKPKDTL MISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQ DWLNGKEYKCKVSNKALP <u>LPE</u> EKTISKAKG QPREPQVYTLPPSREEMTKNQVSLTCLVKGF YPSDIAVEWESNGQPENNYKTTTPVLDSDGS FFLYSKLTVDKSRWQQGNVFSCSVMHEALH NHYTQKSL <u>SL</u> SPGK	具有 S239D、A330L 以及 I332E 的 Bs2Ab 的重链。具有增强的 ADCC 的构建体。强调的位置相应于已知等位基因变异的位点。
33C	DIQMTQSPSSLSASVGDRVTITCRASQDVNT AVAWYQQKPGKAPKLLIYSASFLYSGVPSRF SGSRSGTDFTLTISSLQPEDFATYYCQQHYTT PPTFGCGTKVEIKGGGGSGGGSGGGSGGGSGG GGSEVQLVESGGGLVQPGGSLRLSCAASGF NIKDTYIHWVRQAPGKCLEWVARIYPTNGY	具有 C239-ins 和 S442C (“CC”) 的 Bs2Ab 的重链。对于 ADC 的构建体。强调的位置相应于已知等位基因变异的位点。

SEQ ID NO	序列	评述
	TRYADSVKGRFTISADTSKNTAYLQMNSLR AEDTAVYYCSRWGGDGFYAMDYWGQGT LTVSSGGGGSGGGSGGGGSEVQLVESGGG LVKPGGSLRLSCAASGFTFSSYSMSWVRQAP GKGLEWVSSISSSSSYIYYADSVKGRFTISR DAKNSLYLQMNSLRAEDTAVYYCARGGDA YNYYYFDYWGQGT LTVSSASTKGPSVFPL APSSKSTSGGTAALGCLVKDYFPEPVT VSW NSGALTSGVHTFPAVLQSSGLYSLSSVVTVP SSSLGTQTYICNVNHKPSNTKVDKRVEPKSC DKTHTCPPCPAPE <u>L</u> LGGP <u>S</u> CVFLFPPKPKDTL MISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQ DWLNGKEYKCKVSNKALP <u>A</u> PIEKTISKAKG QPREPQVYTLPPSREEMTKNQVSLTCLVKGF YPSDIAVEWESNGQPENNYKTPPVLDSDGS FFLYSKLTVDKSRWQQGNVFSCSVMHEALH NHYTQKSL <u>C</u> LSPGK	
34	EVQLVESGGGLVKPGGSLRLSCAASGFTFSS YMSWVRQAPGKGLEWVSSISSSSSYIYYAD SVKGRFTISRDNKNSLYLQMNSLRAEDTA VYYCARGGDAYNYYYFDYWGQGT LTVSS ASTKGPSVFPLAPSSKSTSGGTAALGCLVKD YFPEPVT VSWNSGALTSGVHTFPAVLQSSGL YSLSSVVTVPSSSLGTQTYICNVNHKPSNTK VDKRVEPKSCDKTHTCPPCPAPE <u>L</u> LGGP <u>S</u> VF LFPPKPKDTLMISRTPEVTCVVVDVSHEDPE VKFNWYVDGVEVHNAKTKPREEQYNSTYR VVSVLTVLHQDWLNGKEYKCKVSNKALP <u>A</u> <u>P</u> IEKTISKAKGQPREPQVYTLPPSREEMTKN QVSLTCLVKGFYPSDIAVEWESNGQPENNY KTPPVLDSDGSFFLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSL <u>S</u> LSPGKGGGG SGGGGSGGGGSDIQMTQSPSSLSASVGDRV TITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGSRSGTDFLTITSSLPEDF ATYYCQQHYTTPPTFGCGTKVEIKGGGGSG GGGSGGGSGGGGSEVQLVESGGGLVQPG GSLRLSCAASGFNIKDTYIHWVRQAPGKCLE WVARITYPTNGYTRYADSVKGRFTISADTSK NTAYLQMNSLRAEDTAVYYCSRWGGDGFY AMDYWGQGT LTVSS	Bs3Ab天然Fc的重链。强调的位置相应于已知等位基因变异的位点。
35A	EVQLVESGGGLVKPGGSLRLSCAASGFTFSS YMSWVRQAPGKGLEWVSSISSSSSYIYYAD SVKGRFTISRDNKNSLYLQMNSLRAEDTA VYYCARGGDAYNYYYFDYWGQGT LTVSS ASTKGPSVFPLAPSSKSTSGGTAALGCLVKD	具有L234F (“F”) 的Bs3Ab的重链。对于ADC的构建体。强调的位置相应于已知等位基因变异的位点。

SEQ ID NO	序列	评述
	YFPEPVTVSWNSGALTSGVHTFPAVLQSSGL YSLSSVVTVPSSSLGTQTYICNVNHKPSNTK VDKRVEPKSCDKTHTCPPCPAPE <u>FLGGP</u> <u>S</u> VF LFPPKPKDTLMISRTPEVTCVVVDVSHEDPE VKFNWYVDGVEVHNAKTKPREEQYNSTYR VVSVLTVLHQDWLNGKEYKCKVSNKALP <u>A</u> <u>P</u> IEKTISKAKGQPREPQVYTLPPSREEMTKN QVSLTCLVKGFYPSDIAVEWESNGQPENNY KTTTPVLDSGDSFFLYSKLTVDKSRWQQGN VFSCSVMEALHNHYTQKSLS <u>L</u> SPGKGGGG SGGGGSGGGGSDIQMTQSPSSLSASVGDRVT ITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGSRSGTDFLTISLQPEDF ATYYCQQHYTTPPTFGCGTKVEIKGGGGSG GGGSGGGGSGGGGSEVQLVESGGGLVQPG GSLRLSCAASGFNIKDTYIHWVRQAPGKCLE WVARIYPTNGYTRYADSVKGRFTISADTSK NTAYLQMNSLRAEDTAVYYCSRWGGDGFY AMDYWGQGTLVTVSS	
35B	EVQLVESGGGLVKPGGSLRLSCAASGFTFSS YSMSWVRQAPGKGLEWVSSISSSSSYIYYAD SVKGRFTISRDNANKSLYLQMNSLRAEDTA VYYCARGGDAYNYYFFDYWGQGTLVTVSS ASTKGPSVFPLAPSSKSTSGGTAALGCLVKD YFPEPVTVSWNSGALTSGVHTFPAVLQSSGL YSLSSVVTVPSSSLGTQTYICNVNHKPSNTK VDKRVEPKSCDKTHTCPPCPAPE <u>L</u> GGP <u>S</u> VF LFPPKPKDTLMISRTPEVTCVVVDVSHEDPE VKFNWYVDGVEVHNAKTKPREEQYNSTYR VVSVLTVLHQDWLNGKEYKCKVSNKALP <u>A</u> <u>P</u> EEKTISKAKGQPREPQVYTLPPSREEMTKN QVSLTCLVKGFYPSDIAVEWESNGQPENNY KTTTPVLDSGDSFFLYSKLTVDKSRWQQGN VFSCSVMEALHNHYTQKSLS <u>L</u> SPGKGGGG SGGGGSGGGGSDIQMTQSPSSLSASVGDRVT ITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGSRSGTDFLTISLQPEDF ATYYCQQHYTTPPTFGCGTKVEIKGGGGSG GGGSGGGGSGGGGSEVQLVESGGGLVQPG GSLRLSCAASGFNIKDTYIHWVRQAPGKCLE WVARIYPTNGYTRYADSVKGRFTISADTSK NTAYLQMNSLRAEDTAVYYCSRWGGDGFY AMDYWGQGTLVTVSS	具有I332E的Bs3Ab的重链。 具有增强的ADCC的构建体。强调的位置相应于已知等位基因变异的位点。
36A	EVQLVESGGGLVKPGGSLRLSCAASGFTFSS YSMSWVRQAPGKGLEWVSSISSSSSYIYYAD SVKGRFTISRDNANKSLYLQMNSLRAEDTA VYYCARGGDAYNYYFFDYWGQGTLVTVSS	具有 L234F 和 S239C (“FC”) 的Bs3Ab的重链。对于ADC的构建体。强调的

SEQ ID NO	序列	评述
	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKD YFPEPVTVSWNSGALTSGVHTFPAVLQSSGL YSLSSVVTVPSSSLGTQTYICNVNHKPSNTK VDKRVEPKSCDKTHTCPPCPAPE <u>FLGGP</u> CVF LFPPKPKDTLMISRTPEVTCVVVDVSHEDPE VKFNWYVDGVEVHNAKTKPREEQYNSTYR VVSVLTVLHODWLNGKEYKCKVSNKALP <u>A</u> <u>P</u> IEKTISKAKGQPREPQVYTLPPSREEMTKN QVSLTCLVKGFYPSDIAVEWESNGQPENNY KTTTPVLDSGDSFFLYSKLTVDKSRWQQGN VFSCSVMEALHNHYTQKSLSLSPGKGGGG SGGGGSGGGGSDIQMTQSPSSLSASVGDRV ITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGSRSGTDFLTISLQPEDF ATYYCQQHYTTPPTFGCGTKVEIKGGGGSG GGGSGGGGSGGGGSEVQLVESGGGLVQPG GSLRLSCAASGFNIKDTYIHWVRQAPGKCLE WVARIYPTNGYTRYADSVKGRFTISADTSK NTAYLQMNSLRAEDTAVYYCSRWGGDGFY AMDYWGQGTLLTVSS	位置相应于已知等位基因变异的位点。
36B	EVQLVESGGGLVKPGGSLRLSCAASGFTFSS YSMSWVRQAPGKGLEWVSSISSSSYIYYAD SVKGRFTISRDNKNSLYLQMNSLRAEDTA VYYCARGGDAYNYYFYDWGQGTLLTVSS ASTKGPSVFPLAPSSKSTSGGTAALGCLVKD YFPEPVTVSWNSGALTSGVHTFPAVLQSSGL YSLSSVVTVPSSSLGTQTYICNVNHKPSNTK VDKRVEPKSCDKTHTCPPCPAPE <u>LLGGP</u> DVF LFPPKPKDTLMISRTPEVTCVVVDVSHEDPE VKFNWYVDGVEVHNAKTKPREEQYNSTYR VVSVLTVLHODWLNGKEYKCKVSNKALP <u>A</u> <u>P</u> IEKTISKAKGQPREPQVYTLPPSREEMTKN QVSLTCLVKGFYPSDIAVEWESNGQPENNY KTTTPVLDSGDSFFLYSKLTVDKSRWQQGN VFSCSVMEALHNHYTQKSLSLSPGKGGGG SGGGGSGGGGSDIQMTQSPSSLSASVGDRV ITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGSRSGTDFLTISLQPEDF ATYYCQQHYTTPPTFGCGTKVEIKGGGGSG GGGSGGGGSGGGGSEVQLVESGGGLVQPG GSLRLSCAASGFNIKDTYIHWVRQAPGKCLE WVARIYPTNGYTRYADSVKGRFTISADTSK NTAYLQMNSLRAEDTAVYYCSRWGGDGFY AMDYWGQGTLLTVSS	具有 S239D 和 I332E 的 Bs3Ab 的重链。具有增强的 ADCC 的构建体。强调的位置相应于已知等位基因变异的位点。
36C	EVQLVESGGGLVKPGGSLRLSCAASGFTFSS YSMSWVRQAPGKGLEWVSSISSSSYIYYAD SVKGRFTISRDNKNSLYLQMNSLRAEDTA	具有 L234F 和 C239-ins (“C”) 的 Bs3Ab 的重链。对于 ADC 的构建体。强调的位

SEQ ID NO	序列	评述
	VYYCARGGDAYNYYYFDYWGGTGLVTVSS ASTKGPSVFPLAPSSKSTSGGTAALGCLVKD YFPEPVTVSWNSGALTSGVHTFPAVLQSSGL YSLSSVVTVPSSSLGTQTYICNVNHKPSNTK VDKRVEPKSCDKTHTCPPCPAPE <u>L</u> GGP <u>S</u> CV FLPPKPKDTLMISRTPEVTCVVVDVSHEDP EVKFNWYVDGVEVHNAKTKPREEQYNSTY RVVSVLTVLHQDWLNGKEYKCKVSNKALP <u>A</u> PIEKTISKAKGQPREPQVYTLPPSREEMTK NQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTTPVLDSDGSFFLYSKLTVDKSRWQQG NVFSCSVMHREALHNHYTQKSL <u>S</u> LSPGKGGG SGGGGSGGGGSDIQMTQSPSSLSASVGDR VTITCRASQDVNTAVAWYQQKPGKAPKLLI YSASFLYSGVPSRFSGRSGTDFTLTISLQPE DFATYYCQQHYTTPPTFGCGTKVEIKGGGG SGGGGSGGGGSGGGGSEVQLVESGGGLVQP GGSLRLSCAASGFNIKDTYIHWVRQAPGKCL EWVARIYPTNGYTRYADSVKGRFTISADTSK NTAYLQMNSLRAEDTAVYYCSRWGGDGFY AMDYWGQGTGLVTVSS	置相应于已知等位基因变异的位点。
37A	EVQLVESGGGLVKPGGSLRLSCAASGFTFSS YSMSWVRQAPGKGLEWVSSISSSSSYIYYAD SVKGRFTISRDNKNSLYLQMNSLRAEDTA VYYCARGGDAYNYYYFDYWGGTGLVTVSS ASTKGPSVFPLAPSSKSTSGGTAALGCLVKD YFPEPVTVSWNSGALTSGVHTFPAVLQSSGL YSLSSVVTVPSSSLGTQTYICNVNHKPSNTK VDKRVEPKSCDKTHTCPPCPAPE <u>F</u> LGGP <u>C</u> VF LFPPKPKDTLMISRTPEVTCVVVDVSHEDPE VKFNWYVDGVEVHNAKTKPREEQYNSTYR VVSVLTVLHQDWLNGKEYKCKVSNKALP <u>A</u> <u>P</u> IEKTISKAKGQPREPQVYTLPPSREEMTKN QVSLTCLVKGFYPSDIAVEWESNGQPENNY KTTTPVLDSDGSFFLYSKLTVDKSRWQQGN VFSCSVMHREALHNHYTQKSL <u>C</u> LSPGKGGGG SGGGGSGGGGSDIQMTQSPSSLSASVGDRVT ITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGRSGTDFTLTISLQPEDF ATYYCQQHYTTPPTFGCGTKVEIKGGGGSG GGGSGGGGSGGGGSEVQLVESGGGLVQPG GSLRLSCAASGFNIKDTYIHWVRQAPGKCLE WVARIYPTNGYTRYADSVKGRFTISADTSK NTAYLQMNSLRAEDTAVYYCSRWGGDGFY AMDYWGQGTGLVTVSS	具有 L234F、S239C 以及 S442C (“FC”) 的 Bs3Ab 的重链。对于 ADC 的构建体。强调的位置相应于已知等位基因变异的位点。
37B	EVQLVESGGGLVKPGGSLRLSCAASGFTFSS YSMSWVRQAPGKGLEWVSSISSSSSYIYYAD	具有 S239D、A330L 以及 I332E 的 Bs3Ab 的重链。具有

SEQ ID NO	序列	评述
	SVKGRFTISRDNANKNSLYLQMNSLRAEDTA VYYCARGGDAYNYYYFDYWGQGTLLTVSS ASTKGPSVFPLAPSSKSTSGGTAALGCLVKD YFPEPVTVSWNSGALTSGVHTFPAVLQSSGL YSLSSVTVTPSSSLGTQTYICNVNHKPSNTK VDKRVEPKSCDKTHTCPPCPAPE <u>LLGGP</u> <u>D</u> VF LFPPPKPDTLMISRTPEVTCVVVDVSHEDPE VKFNWYVDGVEVHNAKTKPREEQYNSTYR VVSVLTVLHQDWLNGKEYKCKVSNKALP <u>L</u> <u>P</u> EEKTISKAKGQPREPQVYTLPPSREEMTKN QVSLTCLVKGFYPSDIAVEWESNGQPENNY KTTTPVLDSDGSFFLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKS <u>L</u> SLSPGKGGGG SGGGGSGGGGSDIQMTQSPSSLSASVGDRVT ITCRASQDVNTAVAWYQQKPGKAPKLLIYS ASFLYSGVPSRFSGRSGTDFTLTISLQPEDF ATYYCQQHYTTPPTFGCGTKVEIKGGGGSG GGGSGGGGSGGGGSEVQLVESGGGLVQPG GSLRLSCAASGFNIKDTYIHWVRQAPGKCLE WVARIYPTNGYTRYADSVKGRFTISADTSK NTAYLQMNSLRAEDTAVYYCSRWGGDGFY AMDYWGQGTLLTVSS	增强的ADCC的构建体。强调的位置相应于已知等位基因变异的位点。
37C	EVQLVESGGGLVKPGGSLRLSCAASGFTFSS YSMSWVRQAPGKGLEWVSSISSSSSYIYYAD SVKGRFTISRDNANKNSLYLQMNSLRAEDTA VYYCARGGDAYNYYYFDYWGQGTLLTVSS ASTKGPSVFPLAPSSKSTSGGTAALGCLVKD YFPEPVTVSWNSGALTSGVHTFPAVLQSSGL YSLSSVTVTPSSSLGTQTYICNVNHKPSNTK VDKRVEPKSCDKTHTCPPCPAPE <u>LLGGP</u> <u>S</u> <u>C</u> V FLPPPKPDTLMISRTPEVTCVVVDVSHEDP EVKFNWYVDGVEVHNAKTKPREEQYNSTY RVVSVLTVLHQDWLNGKEYKCKVSNKALP <u>A</u> <u>P</u> IEKTISKAKGQPREPQVYTLPPSREEMTK NQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTTPVLDSDGSFFLYSKLTVDKSRWQQG NVFSCSVMHEALHNHYTQKS <u>L</u> <u>C</u> LSPGKGGG GSGGGGSGGGGSDIQMTQSPSSLSASVGDR VTITCRASQDVNTAVAWYQQKPGKAPKLLI YSASFLYSGVPSRFSGRSGTDFTLTISLQPE DFATYYCQQHYTTPPTFGCGTKVEIKGGGG SGGGGSGGGGSGGGGSEVQLVESGGGLVQP GGSLRLSCAASGFNIKDTYIHWVRQAPGKCL EWVARIYPTNGYTRYADSVKGRFTISADTSK NTAYLQMNSLRAEDTAVYYCSRWGGDGFY AMDYWGQGTLLTVSS	具有 C239-ins 和 S442C (“CC”) 的Bs3Ab的重链。对于ADC的构建体。强调的位置相应于已知等位基因变异的位点。
38	EVQLVESGGGLVKPGGSLRLSCAASGFTFSS	Bs4Ab天然Fc的重链。强调

SEQ ID NO	序列	评述
	YMSWVRQAPGKGLEWVSSISSSSSYIYYAD SVKGRFTISRDNANKNSLYLQMNSLRAEDTA VYYCARGGDAYNYYYFDYWGQGTLLTVSS ASTKGPSVFPLAPSSKSTSGGTAALGCLVKD YFPEPVTVSWNSGALTSGVHTFPAVLQSSGL YSLSSVVTVPSSSLGTQTYICNVNHKPSNTK VDKRVEPKSCGKTGGGSGGGGSDIQMTQS PSSLSASVGDRVTITCRASQDVNTAVAWYQ QKPGKAPKLLIYSASFLYSGVPSRFSGRSGT DFTLTISSLQPEDFATYYCQQHYTTPPTFGCG TKVEIKGGGGSGGGSGGGSGGGGSEVQL VESGGGLVQPGGSLRLSCAASGFNIKDTYIH WVRQAPGKCLEWVARIYPTNGYTRYADSV KGRFTISADTSKNTAYLQMNSLRAEDTAVY YCSRWGGDGFYAMDYWGQGTLLTVSSSGG GSGGGGSCPPCPAPE <u>L</u> GGP <u>S</u> VFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTV LHQDWLNGKEYKCKVSNKALP <u>A</u> PIEKTISK AKGQPREPQVYTLPPSREEMTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNYKTTPPV L DSDGSFFLYSKLTVDKSRWQQGNVFCFSVM HEALHNHYTQKSLSLSPGK	的位置相应于已知等位基因 变异的位点。
39A	EVQLVESGGGLVKPGGSLRLSCAASGFTFSS YMSWVRQAPGKGLEWVSSISSSSSYIYYAD SVKGRFTISRDNANKNSLYLQMNSLRAEDTA VYYCARGGDAYNYYYFDYWGQGTLLTVSS ASTKGPSVFPLAPSSKSTSGGTAALGCLVKD YFPEPVTVSWNSGALTSGVHTFPAVLQSSGL YSLSSVVTVPSSSLGTQTYICNVNHKPSNTK VDKRVEPKSCGKTGGGSGGGGSDIQMTQS PSSLSASVGDRVTITCRASQDVNTAVAWYQ QKPGKAPKLLIYSASFLYSGVPSRFSGRSGT DFTLTISSLQPEDFATYYCQQHYTTPPTFGCG TKVEIKGGGGSGGGSGGGSGGGGSEVQL VESGGGLVQPGGSLRLSCAASGFNIKDTYIH WVRQAPGKCLEWVARIYPTNGYTRYADSV KGRFTISADTSKNTAYLQMNSLRAEDTAVY YCSRWGGDGFYAMDYWGQGTLLTVSSSGG GSGGGGSCPPCPAPE <u>F</u> LGGP <u>S</u> VFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTV LHQDWLNGKEYKCKVSNKALP <u>A</u> PIEKTISK AKGQPREPQVYTLPPSREEMTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNYKTTPPV L DSDGSFFLYSKLTVDKSRWQQGNVFCFSVM HEALHNHYTQKSLSLSPGK	具有L234F (“F”) 的Bs4Ab 的重链。对于ADC的构建 体。强调的位置相应于已知 等位基因变异的位点。

SEQ ID NO	序列	评述
39B	EVQLVESGGGLVKPGGSLRLSCAASGFTFSS YSMSWVRQAPGKGLEWVSSISSSSSYIYYAD SVKGRFTISRDNKNSLYLQMNSLRAEDTA VYYCARGGDAYNYYYFDYWGQGTLLTVSS ASTKGPSVFPLAPSSKSTSGGTAALGCLVKD YFPEPVTISWNSGALTSGVHTFPAVLQSSGL YSLSSVTVTPSSSLGTQTYICNVNHKPSNTK VDKRVEPKSCGKTGGGSGGGGSDIQTQS PSSLSASVGDRVTITCRASQDVNTAVAWYQ QKPGKAPKLLIYSASFLYSGVPSRFSGRSGT DFTLTISLQPEDFATYYCQGHYTPPTFGCG TKVEIKGGGSGGGGSGGGGSGGGGSEVQL VESGGGLVQPGGSLRLSCAASGFNIKDTYIH WVRQAPGKCLEWVARIYPTNGYTRYADSV KGRFTISADTSKNTAYLQMNSLRAEDTAVY YCSRWGGDGFYAMDYWGQGTLLTVSSSGG GGSGGGGSCPPCPAPE <u>L</u> GGP <u>S</u> VFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTV LHQDWLNGKEYKCKVSNKALP <u>A</u> EEKTISK AKGQPREPQVYTLPPSREEMTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNYKTTTPVL DSDGSFFLYSKLTVDKSRWQQGNVFCFSVM HEALHNHYTQKSLSLSPGK	具有I332E的Bs4Ab的重链。 具有增强的ADCC的构建体。强调的位置相应于已知等位基因变异的位点。
40A	EVQLVESGGGLVKPGGSLRLSCAASGFTFSS YSMSWVRQAPGKGLEWVSSISSSSSYIYYAD SVKGRFTISRDNKNSLYLQMNSLRAEDTA VYYCARGGDAYNYYYFDYWGQGTLLTVSS ASTKGPSVFPLAPSSKSTSGGTAALGCLVKD YFPEPVTISWNSGALTSGVHTFPAVLQSSGL YSLSSVTVTPSSSLGTQTYICNVNHKPSNTK VDKRVEPKSCGKTGGGSGGGGSDIQTQS PSSLSASVGDRVTITCRASQDVNTAVAWYQ QKPGKAPKLLIYSASFLYSGVPSRFSGRSGT DFTLTISLQPEDFATYYCQGHYTPPTFGCG TKVEIKGGGSGGGGSGGGGSGGGGSEVQL VESGGGLVQPGGSLRLSCAASGFNIKDTYIH WVRQAPGKCLEWVARIYPTNGYTRYADSV KGRFTISADTSKNTAYLQMNSLRAEDTAVY YCSRWGGDGFYAMDYWGQGTLLTVSSSGG GGSGGGGSCPPCPAPE <u>F</u> LGGP <u>C</u> VFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTV LHQDWLNGKEYKCKVSNKALP <u>A</u> PIEKTISK AKGQPREPQVYTLPPSREEMTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNYKTTTPVL DSDGSFFLYSKLTVDKSRWQQGNVFCFSVM HEALHNHYTQKSLSLSPGK	具有 L234F 和 S239C (“FC”) 的Bs4Ab的重链。 对于ADC的构建体。强调的位置相应于已知等位基因变异的位点。

SEQ ID NO	序列	评述
40B	EVQLVESGGGLVKPGGSLRLSCAASGFTFSS YSMSWVRQAPGKGLEWVSSISSSSSSYIYYAD SVKGRFTISRDNKNSLYLQMNSLRAEDTA VYYCARGGDAYNYYYFDYWGGQGLTVTVSS ASTKGPSVFPLAPSSKSTSGGTAALGCLVKD YFPEPVTVSWNSGALTSGVHTFPAVLQSSGL YSLSSVTVTPSSSLGTQTYICNVNHKPSNTK VDKRVEPKSCGKTGGGSGGGGSDIQTQS PSSLSASVGDRVTITCRASQDVNTAVAWYQ QKPGKAPKLLIYSASFLYSGVPSRFSGRSGT DFTLTISLQPEDFATYYCQGHYTTPTFGCG TKVEIKGGGSGGGGSGGGGSGGGGSEVQL VESGGGLVQPGGSLRLSCAASGFNIKDTYIH WVRQAPGKCLEWVARIYPTNGYTRYADSV KGRFTISADTSKNTAYLQMNSLRAEDTAVY YCSRWGGDGFYAMDYWGQGLTVTVSSSGG GGSGGGGSCPPCPAPE <u>L</u> GGP <u>D</u> VFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTV LHQDWLNGKEYKCKVSNKALP <u>A</u> PEKTISK AKGQPREPQVYTLPPSREEMTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNYKTTPPV DSDGSFFLYSKLTVDKSRWQQGNVFCFSVM HEALHNHYTQKSLSLSPGK	对于ADCC，具有S239D和I332E的Bs4Ab的重链。具有增强的ADCC的构建体。强调的位置相应于已知等位基因变异的位点。
40C	EVQLVESGGGLVKPGGSLRLSCAASGFTFSS YSMSWVRQAPGKGLEWVSSISSSSSSYIYYAD SVKGRFTISRDNKNSLYLQMNSLRAEDTA VYYCARGGDAYNYYYFDYWGGQGLTVTVSS ASTKGPSVFPLAPSSKSTSGGTAALGCLVKD YFPEPVTVSWNSGALTSGVHTFPAVLQSSGL YSLSSVTVTPSSSLGTQTYICNVNHKPSNTK VDKRVEPKSCGKTGGGSGGGGSDIQTQS PSSLSASVGDRVTITCRASQDVNTAVAWYQ QKPGKAPKLLIYSASFLYSGVPSRFSGRSGT DFTLTISLQPEDFATYYCQGHYTTPTFGCG TKVEIKGGGSGGGGSGGGGSGGGGSEVQL VESGGGLVQPGGSLRLSCAASGFNIKDTYIH WVRQAPGKCLEWVARIYPTNGYTRYADSV KGRFTISADTSKNTAYLQMNSLRAEDTAVY YCSRWGGDGFYAMDYWGQGLTVTVSSSGG GGSGGGGSCPPCPAPE <u>L</u> GGP <u>S</u> VFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTV LHQDWLNGKEYKCKVSNKALP <u>A</u> PIEKTISK AKGQPREPQVYTLPPSREEMTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNYKTTPPV DSDGSFFLYSKLTVDKSRWQQGNVFCFSVM HEALHNHYTQKSLSLSPGK	具有和C239-ins (“C”) 的Bs4Ab的重链。对于ADC的构建体。强调的位置相应于已知等位基因变异的位点。

SEQ ID NO	序列	评述
41A	EVQLVESGGGLVKPGGSLRLSCAASGFTFSS YSMSWVRQAPGKGLEWVSSISSSSSSYIYYAD SVKGRFTISRDNANKNSLYLQMNSLRAEDTA VYYCARGGDAYNYYYFDYWGQGTLLTVSS ASTKGPSVFPLAPSSKSTSGGTAALGCLVKD YFPEPVTVSWNSGALTSGVHTFPAVLQSSGL YSLSSVTVTPSSSLGTQTYICNVNHKPSNTK VDKRVEPKSCGKTGGGSGGGGSDIQMTQS PSSLSASVGDRVTITCRASQDVNTAVAWYQ QKPGKAPKLLIYSASF LY SGVPSRFSGRSGT DFTLTIS SL QPEDFATYYCQQH Y TTPTFGCG TKVEIKGGGGSGGGSGGGSGGGGSEVQL VESGGGLVQPGGSLRLSCAASGFNIKDTYIH WVRQAPGKCLEWVARIYPTNGYTRYADSV KGRFTISADTSKNTAYLQMNSLRAEDTAVY YCSRWGGDGFYAMDYWGQGTLLTVSSSGG GGSGGGGSCPPCPAPE <u>FLGGP</u> CVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTV LHQDWLNGKEYKCKVSNKALP <u>API</u> EKTISK AKGQPREPQVYTLPPSREEMTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNYKTTPPV LDS DS GSFFLYSKLTVDKSRWQQGNVFS CS SVM HEALHNHYTQKSL <u>CL</u> SPGK	具有 L234F、S239C 以及 S442C (“FCC”) 的 Bs4Ab 的重链。对于 ADC 的构建体。强调的位置相应于已知等位基因变异的位点。
41B	EVQLVESGGGLVKPGGSLRLSCAASGFTFSS YSMSWVRQAPGKGLEWVSSISSSSSSYIYYAD SVKGRFTISRDNANKNSLYLQMNSLRAEDTA VYYCARGGDAYNYYYFDYWGQGTLLTVSS ASTKGPSVFPLAPSSKSTSGGTAALGCLVKD YFPEPVTVSWNSGALTSGVHTFPAVLQSSGL YSLSSVTVTPSSSLGTQTYICNVNHKPSNTK VDKRVEPKSCGKTGGGSGGGGSDIQMTQS PSSLSASVGDRVTITCRASQDVNTAVAWYQ QKPGKAPKLLIYSASF LY SGVPSRFSGRSGT DFTLTIS SL QPEDFATYYCQQH Y TTPTFGCG TKVEIKGGGGSGGGSGGGSGGGGSEVQL VESGGGLVQPGGSLRLSCAASGFNIKDTYIH WVRQAPGKCLEWVARIYPTNGYTRYADSV KGRFTISADTSKNTAYLQMNSLRAEDTAVY YCSRWGGDGFYAMDYWGQGTLLTVSSSGG GGSGGGGSCPPCPAPE <u>LLGGP</u> DVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTV LHQDWLNGKEYKCKVSNKALP <u>LPE</u> EKTISK AKGQPREPQVYTLPPSREEMTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNYKTTPPV LDS DS GSFFLYSKLTVDKSRWQQGNVFS CS SVM HEALHNHYTQKSL <u>SL</u> SPGK	具有 S239D、A330L 以及 I332E 的 Bs4Ab 的重链。具有增强的 ADCC 的构建体。强调的位置相应于已知等位基因变异的位点。

SEQ ID NO	序列	评述
41C	EVQLVESGGGLVKPGGSLRLSCAASGFTFSS YSMSWVRQAPGKGLEWVSSISSSSSYIYYAD SVKGRFTISRDNKNSLYLQMNSLRAEDTA VYYCARGGDAYNYYYFDYWGQGLTVTVSS ASTKGPSVFPLAPSSKSTSGGTAAALGCLVKD YFPEPVTVSWNSGALTSGVHTFPAVLQSSGL YSLSSVTVTPSSSLGTQTYICNVNHKPSNTK VDKRVEPKSCGKTGGGSGGGGSDIQMTQS PSSLSASVGDRVTITCRASQDVNTAVAWYQ QKPGKAPKLLIYSASFLYSGVPSRFSGRSGT DFTLTISLQPEDFATYYCQQHYTTPPTFGCG TKVEIKGGGSGGGSGGGSGGGSGGGSEVQL VESGGGLVQPGGSLRLSCAASGFNIKDTYIH WVRQAPGKCLEWVARIYPTNGYTRYADSV KGRFTISADTSKNTAYLQMNSLRAEDTAVY YCSRWGGDGFYAMDYWGQGLTVTVSSSGG GGSGGGGSCPPCPAPE <u>L</u> GGP <u>S</u> CVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWY VDGVEVHNAKTKPREEQYNSTYRVVSVLTV LHQDWLNGKEYKCKVSNKALP <u>A</u> PIEKTISK AKGQPREPQVYTLPPSREEMTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNYKTTPPV LDSGDSFFLYSKLTVDKSRWQQGNVFCFSVM HEALHNHYTQKSL <u>C</u> LSPGK	具有 C239-ins 和 S442C (“CC”) 的Bs4Ab的重链。对于ADC的构建体。强调的位置相应于已知等位基因变异的位点。
42A	DIVMTQTPLSLSVTPGPASISCKSSQSVFFR SNNKNILAWYLQKPGQPPQLLIYWASSRESG VPDRFSGSGSGTDFTLKISRVEAEDVGVYYC QQYFGSPFTFGPGTKVDIKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKV DNALQSGNSQESVTEQDSKSTYSLSSLTTL SKADYEKHKVYACEVTHQGLSSPVTKSFNR GEC	具有与所有抗体构建体共有的κ链的LC构建体。强调的位置相应于已知等位基因变异的位点。
42B	DIVMTQTPLSLSVTPGPASISCKSSQSVFFR SNNKNILAWYLQKPGQPPQLLIYWASSRESG VPDRFSGSGSGTDFTLKISRVEAEDVGVYYC QQYFGSPFTFGPGTKVDIKQPKAAPSVTLFPP SSEELQANKATLVCLISDFYPGAVTVAWKA DSSPVKAGVETTPSKQSNKYAASSYLSLT PEQWKS SYSCQVTHEGSTVEKTVAPTEC	具有对于所有抗体构建体的λ链选项的LC构建体。强调的位置相应于已知等位基因变异的位点。
43	EVQLVESGGGLVKPGGSLRLSCAASGFTFSS YSMNWVRQAPGKGLEWVSSISSSSSYIYYA DSVKGRFTISRDNKNSLYLQMNSLRAEDT AVYYCARGGDGYNYYYFDYWGQGLTVTV SS	来自克隆1.39.1抗体的VH
44	DIVMTQSPDSLAVSLGERATITCKSSQSVFFR SNNKNCLAWYQQRPGQPPNLLIYWASSRES GVPDRFSGSGSGTDFTALTISLQTEDVAVYY	来自克隆1.39.1抗体的VL

SEQ ID NO	序列	评述
	CQQYFGSPFTFGPGTKVDIK	
45	SYSM	VH克隆1.39.1 CDR1 (来自非前导优化的抗体的序列)
46	GGDGYNYYYFDY	VH克隆1.39.1 CDR3 (来自非前导优化的抗体的序列)
47	KSSQSVFFRSNNKNCLA	VL克隆1.39.1 CDR1 (来自非前导优化的抗体的序列)
48	DIVMTQSPDSLAVSLGERATITC	VL克隆1.39.1 FW1 (来自非前导优化的抗体的序列)
49	WYQQRPGQPPNLLIY	VL克隆1.39.1 FW2 (来自非前导优化的抗体的序列)
50	GVPDRFSGSGSGTDFALTISSLQTEDVAVYYC	VL克隆1.39.1 FW3 (来自非前导优化的抗体的序列)
51	TQVCTGTDMKLRLPASPETHLDMRLRHL YQG CQVVQGNLELTYLPTNASLSFLQDIQEVQGY VLIAHNQVRQVPLQRLRIVRGTQLFEDNYAL AVLDNGDPLNNTTPVTGASPGGLRELQLRSL TEILKGGVLIQRNPQLCYQDTILWKDIFHKN NQLALTLIDTNRSRACHPCSPMCKGSR CWG ESSEDCQSLTRTV CAGGCARCKGPLPTDCCH EQCAAGCTGPKHSDCLACLFHFNHSGICELHC PALVTYNTDTFESMPNPEGRYTFGASCVTAC PYNYLSTDVGSC TLV CPLHNQEV TAEDGTQRCEKCSKPCARVCYGLGMEHLRE VRAVTSANIQEFAGCKKIFGSLAFLPESFDGD PASNTAPLQPEQLQVFETLEEITGYLYISAWP DSL PDL SVFQNLQVIRGRILHNGAYSLTLQG LGISWLGLRSLRELGSGGLALIHNTHL CFVH TVPWDQLFRNPHQALLHTANRPEDEC V GEG LACHQLCARGHCWGPGPTQCVNCSQFLRG QECVEECRVLQGLPREYVNARHCLPCHPEC QPQNGSVTCFGPEADQCVACAHYKDPPFCV ARCP SGVKPDLSYMPIWKFPDEEGA CQPCPINCTHSCVDLDDKGCPAEQRASPLT	成熟HER2的细胞外部分 (标准同工型1的位置23-652; 不具有信号序列)。天然变体: W452C。参见Uniprot P04626。还参见“单独HER2和与赫赛汀Fab复合的HER2的细胞外区的结构”("Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab.") 曹等人, 自然421:756-760 (2003), 该参考文献通过引用以其全文结合在此。
52	SLTRTV CAGGCARCKGP	克隆1.39.1衍生的抗体的HER2表位。
53	LPCHPECQPQNGSVTCFGPEADQCVACAHY KDPPFCVARCP SGVKPDLSYMPIWKFPDEEG ACQP	曲妥珠单抗(赫赛汀®)的HER2表位结合区。报道了跨越残基538-603 (或具有信号序列的HER2中的残基561-625); 报道了在三个环区处接触的构象表位。

SEQ ID NO	序列	评述
54	DTYIH	结构域IV scFv VH-CDR1
55	RIYPTNGYTRYADSVKG	结构域IV scFv VH-CDR2
56	WGGDGFYAMDY	结构域IV scFv VH-CDR3
57	RASQDVNTAVA	结构域IV scFv VL-CDR1
58	SASFLYS	结构域IV scFv VL-CDR2
59	QQHYTTPPT	结构域IV scFv VL-CDR3

[0549] 通过说明而不是通过限制的方式,提供以下实例。

实例

[0550] 本披露的多方面可以进一步通过参考以下非限制性实例进行定义,这些非限制性实例详细描述了本披露的某些抗体的制备以及使用本披露抗体的方法。对于本领域技术人员来说将会清楚的是,在不背离本披露的范围的情况下,可以对材料和方法两者做出许多修改。

[0551] 许多HER2抗体被批准用于治疗其一种或多种肿瘤过表达HER2的乳癌患者,包括曲妥珠单抗(赫赛汀®;参见美国专利号5,821,337)、帕妥珠单抗(PERJETA™;专利公开号W02001/00245)和T-DM1(ado-曲妥珠单抗emtansine,KADCYLA™,由连接至细胞毒性剂美登素(DM1)上的单克隆抗体曲妥珠单抗组成的抗体-药物轭合物,尼古莱斯库(Niculescu)-杜凡斯(Duvaz)等人,2010,分子治疗新进展(Curr.Opin.Mol.Ther.)12:350-60)。然而,这些疗法并没有被指出供表达低水平HER2的大部分患者使用。另外地,存在不响应于这些疗法或变得抗性的患者。因此,存在对解决这些患者的卓越治疗学的未能满足的医疗需求。

[0552] 如在以下提供的具体实例中详细说明,通过将结合HER2的结构域II内的新描述的表位的优化全人抗HER2抗体与结合HER2的结构域IV内的已知表位的scFv组合来产生高度有效的双特异性抗体。产生并且测试了许多不同双特异性抗体构象。所提供的独特双特异性抗体展现出对于任何所测试的单特异性抗HER2抗体未看出的生物活性。在许多测定中,双特异性抗体还证实了优于单特异性抗HER2抗体的协同活性。在此提供的双特异性抗体的许多体外和体内活性通过在不存在或对Fcγ受体的结合活性严重减少的情况下添加细胞毒性剂(例如,微管溶素1508)来进一步增强。另外,发现在此提供的独特双特异性抗体的体外活性还可以通过增强ADCC活性,例如通过改变糖基化(例如,使用POTELLEGENT™技术(新泽西州普林斯顿Biowa公司(Biowa, Inc.Princeton, N.J.))以产生具有增强ADCC活性的无岩藻糖基化的抗体来增强。这些数据表明双特异性抗体可以具有治疗用途,具体地作为ADC或作为ADCC增强的抗体,以便用于治疗表达宽范围HER2水平的癌症,包括当前不适合使用曲妥珠单抗、帕妥珠单抗或T-DM1治疗的患者。另外,双特异性抗体可以具有用于治疗对现有抗HER2疗法以失败告终的癌症患者的治疗用途。

实例1

1.1.前导优化

[0553] AZ1.39.1是不与曲妥珠单抗或帕妥珠单抗竞争结合的针对人HER2的全人单克隆抗体(专利公开号WO 2008/019290)。39S是如下详细说明书的从AZ1.39.1中产生的前导优化抗体(图1)。定点诱变被用来使用Ile替换轻链CDR1中的未配对Cys残基,并且重链CDR3中的潜在异构化位点(DG)通过将该DG改变成DA来去除。所得变体证实了与AZ1.39.1相同的结合特异性和体外抗增殖活性(数据未示出)。为了产生更高亲和力结合剂,对重链的CDR残基施加诱变,并且突变体被表达为哺乳动物细胞中的IgG并且通过捕获ELISA对它们与重组人HER2细胞外结构域蛋白的结合活性进行筛选。对具有比野生型对照显著更高的结合信号的克隆进行测序以揭示突变信息。从鉴定的突变中构建组合文库并且通过捕获ELISA对与重组人HER2细胞外结构域蛋白的结合活性进行筛选。发现39S具有最高结合活性,并且进一步的序列分析揭示39S携带重链CDR1中的单个N5S突变。通过BIAcore所确定,39S与人HER2的亲和力(K_D)是大约1.0nM,相反所报道的亲本1.39.1抗体的亲和力是大约2.0nM。因此,这单个突变引起优于亲本抗体的2倍结合亲和力增加。为了改进抗体表达水平,构架序列(具体地轻链FR1、FR2以及FR3)从IGKV4-1+Jk3.01换至IGVK2D种系序列,从而引起培养7天后测量的IgG表达水平增加大约2倍(图2)。

1.2. 前导优化抗体39S的结合特异性和物种交叉反应性

[0554] 为了确定39S是否保留其亲本抗体AZ1.39.1的结合特异性和物种交叉反应性,通过捕获ELISA来检查39S与人ErbB家族的受体(EGFR、HER2、HER3以及HER4)、小鼠Her2、以及食蟹猴Her2的结合。简单地说,使用以下重组细胞外结构域蛋白中的一种涂布96孔板:人EGFR、人HER2、人HER3、人HER4、小鼠Her2、或食蟹猴Her2。通过在从50nM降至0.28pM范围内以逐步1:3系列稀释进行稀释并且然后一式二份添加至孔中来制备有待测试的抗体。在孵育1小时之后,对板进行洗涤,并且将山羊抗人IgG Fab HRP-耦联的二级抗体添加至每个孔中并且将板孵育1小时。对板进行洗涤并且添加TMB底物且孵育5-20min以便允许显色。在反应结束时将停止溶液添加至孔中并且在450nm处对板进行读数。使用Prism软件,针对抗体浓度对结合信号(450nm处的吸光度)进行绘图。结果显示类似于AZ1.39.1的39S可以结合至人HER2和食蟹猴Her2,但不能结合至人EGFR、HER3、HER4、或小鼠Her2(数据未示出)。

1.3. 39S表位作图和表征

[0555] 通过在人Her2分子与小鼠Her2分子之间交换结构域,将人HER2的结构域II鉴定为39S的表位。将小鼠Her2选择为嵌合配偶体,因为它不能被39S识别但与人HER2共享85%的序列一致性。如在表4中所列,构建靶向HER2的每个结构域的嵌合变体(参见以下方法),包括使用小鼠对应部分替换人HER2的结构域I、II、III或IV的四个敲除(KO,丧失功能)变体,以及将人HER2的结构域II移植到小鼠Her2分子中的一个敲入(KI,获得功能)变体。嵌合变体命名指代变体的类型(KO/KI)和所交换的结构域数。使用基于SPR的仪器ProteOn™,通过使用抗人和小鼠Her2多克隆抗体捕获传感器表面上的变体来表征39S与这些变体的结合特征(参见以下方法)。通过使用ProteOn™的抗His多克隆抗体来监测变体的表达。39S与丧失功能的变体的结合结果证实了结构域II是含有表位的结构域。39S不结合至编码小鼠结构域II的KO_II的变体(表4),而保留结合至编码小鼠结构域I的KO_I的变体。虽然敲除人结构域III和IV(KO_III和KO_IV)的变体不表达,但基于获得功能的变体KI_II的结合结果,这两个结构域被排除作为39S的表位。这种编码小鼠结构域I、III以及IV和人结构域II的获得功能的变体(KI_II)被具有与人HER2(84pM)类似的结合亲和力(168pM)的39S识别。因此,通过

丧失功能和获得功能的变体,人HER2的结构域II(氨基酸146-310)被鉴定为39S的含有表位的结构域。

[0556] 我们进一步细化了39S的表位并且将结构域II中的氨基酸192-208的区鉴定为关键表位区。构建一系列靶向结构域II的短区的嵌合人/小鼠变体,这些结构域II的短区在人Her2蛋白与小鼠Her2蛋白之间具有不同的氨基酸序列,如在表4中所列。通过使用小鼠对应部分替换以下人HER2区中的每一个来产生七个敲除变体,包括氨基酸146-208、159-162、171-187、192-208、250-261、276-285、以及295-296。另外,通过将人HER2的氨基酸192-208的区移植至小鼠分子中来构建一个敲入变体。变体命名指代变体的类型(KO/KI)和具有氨基酸编号的交换区。所有变体具有通过抗His多克隆抗体得到的可检测表达水平(表4)。39S不结合至任何嵌合变体,其中人HER2的氨基酸192-208的区被小鼠残基(KO_146-208、KO_192-208)替换。当用小鼠氨基酸(KO_159-162、KO_171-187、KO_250-261、KO_276-285、以及KO_295-296)取代人HER2的任何其他区时,39S的结合不受影响。此外,与人HER2的KD(84pM)相比,39S以72pM的KD结合至编码人192-208的KI变体(KI_192-208)。总的来说,人HER2结构域II中的氨基酸192-208的区被鉴定为39S的功能表位。

[0557] 变体命名指代变体的类型(KO/KI)和具有氨基酸编号的交换区。基于不具有其信号肽的成熟人HER2序列的编号方案来指代氨基酸位置。通过抗His多克隆抗体来监测所有变体的表达水平。使用基于SPR的仪器ProteOn™,通过使用抗人和小鼠Her2多克隆抗体捕获传感器表面上的变体来表征39S与这些变体的结合特征。通过捕获传感器表面上的Her2蛋白,预期39S的测量的表观结合亲和力高于以将39S固定在芯片表面上的形式的单价结合亲和力。

表4. 39S与嵌合人/小鼠Her2变体的结合特征

	变体	交换的人 HER2氨基酸	表达	39S的结合亲 和力 (K_D)
靶向结构域的嵌合 变体	KO_I	1-145	是	95 pM
	KO_II	146-310	是	无结合
	KO_III	311-433	无表 达	-
	KO_IV	434-646	无表 达	-
	KI_II	146-310	是	163 pM
靶向人HER2的结构 域II的短区的嵌合变 体, 这些嵌合变体 具有人与小鼠之间 不同的蛋白质序列	KO_146-208	146-208	是	无结合
	KO_159-162	159-162	是	101 pM
	KO_171-187	171-187	是	38 pM
	KO_192-208	192-208	是	无结合
	KO_250-261	250-261	是	187 pM
	KO_276-285	276-285	是	106 pM
	KO_295-296	295-296	是	84.7 pM
	KI_192-208	192-208	是	72 pM
对照	小鼠Her2	不适用	是	无结合
	人Her2	不适用	是	84 pM

[0558] 如下构建嵌合人/小鼠Her2变体的构建和表达。简单地说, 组装编码具有His标签的嵌合人/小鼠Her2变体的DNA, 并且使用人和小鼠Her2质粒作为模板(MedImmune)通过重叠PCR来扩增。将组装的DNA克隆到哺乳动物表达载体pEBNA(MedImmune)中。然后根据制造商说明书(英杰公司(Invitrogen)), 使用293fectin和标准方案用不同构建体瞬时转染HEK293F细胞。

[0559] 使用ProteOn™ XPR36仪器(BioRad)研究39S与嵌合人/小鼠变体的结合特征。使用标准胺偶联将在10mM乙酸钠(pH 5.0)中的抗人或小鼠Her2多克隆抗体(R&D系统公司(R&D System))以对于每个通道大约5000个共振单位(RU)固定至GLC生物传感器芯片的表面上。注射细胞培养上清液中的嵌合蛋白并且通过抗人或小鼠多克隆抗体捕获到GLC表面上, 其中具有大约200RU反应。将抗HER2 mAb 39S稀释在具有0.005%吐温-20的从10nM至0.625nM (1:2稀释)的磷酸盐缓冲盐水(PBS)(pH 7.4)中, 并且以100μL/min注射持续180秒, 其中具有600秒解离时间。通过在与注射39S相同的条件下使抗His多克隆抗体(MedImmune)流动来监测嵌合变体的表达水平。通过以100μL/min注射甘氨酸缓冲液(10mM, pH 1.5)持续30秒来

使表面再生两次。使用ProteOn™ Manager 3.0.1软件处理所有传感图数据。

[0560] 使用基于FACS的结合竞争测定来确定抗体是否竞争结合至与曲妥珠单抗和/或帕妥珠单抗相同的表位。收获BT-474细胞,重新悬浮于FACS缓冲液中,并且将 2.5×10^5 个细胞/孔添加至96孔U型底板中。通过在从500 μ g/mL降至1.9ng/mL范围内以逐步1:4系列稀释来稀释在含有2 μ g/mL Alexa-Fluor 647-标记的39S抗体的FACS缓冲液中制备有待测试的抗体(R347 IgG1同种型对照、曲妥珠单抗、帕妥珠单抗、AZ1.39.1以及39S),并且然后一式三份添加至细胞中。在冰上染色1小时之后,用冰冷FACS缓冲液洗涤细胞3次并且然后用2% PFA固定。通过具有BD FACSDiva™软件的BD LSR II机器来分析细胞。使用FlowJo软件分析数据并且作为平均MFI $\pm$ SEM(n=3)呈现。

[0561] 图4示出39S结合至与其亲本抗体AZ1.39.1相同的表位;并且39S不与曲妥珠单抗或帕妥珠单抗竞争结合。在图3中提供的HER2的带状结构上通过箭头指示39S、帕妥珠单抗以及曲妥珠单抗的结合位点。

1.4. 39S的体外活性

[0562] 选择一组表达不同水平HER2的人癌症细胞系以用于评估抗体或抗体组合的抗增殖活性。通过HerceptTest®和定量FACS来确定细胞中的HER2表达水平(表5)。

表5:人癌症细胞系上的HER2表达水平

细胞系	癌症类型	HerceptTest® 得分	细胞上的相对HER2密度
BT-474	乳房	3+	1,841,884
SKBR-3	乳房	3+	1,517,135
NCI-N87	胃	3+	1,292,978
SKOV-3	卵巢	3+	349,178
MDA-MB-361	乳房	2+/3+	252,249
JIMT-1	乳房	2+	65,573
MDA-MB-453	乳房	2+	77,314
MDA-MB-175-VII	乳房	2+	29,437
RT-112	膀胱	2+	7,664
MCF7-GTU	乳房	2+	6,058
ZR-75-1	乳房	1+	5,892
T47D	乳房	1+	6,124
MCF-7	乳房	0	3,646
MDA-MB-468	乳房	0	不可检测

[0563] 增殖抑制测定：以100 μ L的体积，以96孔板的每个孔5,000至20,000的密度（取决于每种细胞系的生长动力学）将细胞铺放在含有血清的培养基中。通过将测试物品稀释在培养基中制备有待测试的每种剂量抗体或抗体组合的2X浓缩物。一式三份将100微升每种测试物品添加至细胞中，这样使得最终剂量曲线在以逐步1:4系列稀释从10 μ g/mL降至0.15ng/mL范围内。对于配体依赖性增殖抑制测定，将细胞铺放在无血清的培养基中，并且通过将测试物品稀释在无血清培养基中制备有待测试的每种剂量抗体或抗体组合的4X浓缩物。一式三份将50微升每种测试物品添加至细胞中，并且然后将以32ng/mL浓度稀释在无血清培养基中的50 μ L调蛋白- β 1添加至细胞中以便实现8ng/mL的最终调蛋白- β 1浓度，并且最终抗体剂量曲线以逐步1:4系列稀释从100 μ g/mL降至6.1ng/mL范围内。在37 $^{\circ}$ C/5%CO₂下培养经过处理的细胞持续4至7天（取决于每种具体细胞系的生长动力学）。根据制造商说明书，使用来自普洛麦格公司（Promega）的细胞滴度Glo来确定细胞活力。使用GraphPad Prism软件分析数据，并且作为相对于未处理的对照的生长抑制百分比呈现。

[0564] 图5示出39S具有与帕妥珠单抗类似的在抑制MCF-7细胞中的配体驱动的增殖方面的活性。与曲妥珠单抗或帕妥珠单抗组合，39S示出与曲妥珠单抗和帕妥珠单抗组合相比具有功效的对配体依赖性增殖的加和或协同抑制。在其他细胞系诸如MDA-MB-361和RT-112中观察到类似结果（数据未示出）。

[0565] 图6示出39S（像帕妥珠单抗）在含有血清的培养基中抑制NCI-N87细胞增殖方面具有限制的活性。然而，当与曲妥珠单抗或帕妥珠单抗组合时，39S证实了在抑制细胞生长方面的强协同作用。39S与曲妥珠单抗或帕妥珠单抗之间的协同性比对于曲妥珠单抗和帕妥珠单抗的组合看出的协同性要大得多。在BT-474细胞中观察到类似的结果（数据未示出）。

实例2

2.1. 双特异性抗体构建

[0566] 对Bs2Ab-39SH、Bs3Ab-39SH以及Bs4Ab-39SH进行克隆。通过将抗HER2结构域IV抗体（SEQ ID NO:17和18，其中X是C）、抗HER2抗体39S（SEQ ID NO:15和16）的可变结构域克隆到包含适当恒定区的表达载体中来产生双特异性表达构建体。将抗HER2结构域IV可变结合结构域构建为单链Fv（scFv）。使用以上SEQ ID NO:17和18（其中X-C）的氨基酸序列，设计并且合成用于最大哺乳动物蛋白质表达的密码子优化的DNA序列。使用类似于描述于迪马斯（Dimasi）等人，（2009）分子生物学杂志393,672-692中的那些方法来产生Bs2Ab和Bs3Ab构建体。使用类似于描述于专利公开号W02013070565A1中的那些方法来产生Bs4Ab构建体。最终合成的基因含有两个半胱氨酸突变，分别是在位置100处的轻链中的一个突变和在位置44处的重链上的一个突变。这些半胱氨酸将在VL结构域与VH结构域之间形成链间二硫键以便稳定scFv。使用20个氨基酸残基（G₄S）₄来连接scFv的两个VL结构域和VH结构域。通过使用10个氨基酸残基接头（G₄S）₂来将Bs2Ab中的scFv连接至重链N-末端上。通过使用10个氨基酸残基接头（G₄S）₂来将Bs3Ab形式中的scFv连接至抗体CH3结构域的C-末端上。具有序列（G₄S）₂的两个接头用于连接Bs4Ab骨架中的scFv。使用DNA序列来确定构建一致性和保真度。

[0567] 图7提供产生用于结合至HER2抗原的Bs2Ab-39SH、Bs3Ab-39SH、Bs4Ab-39SH双特异性抗体形式中的每一种的示意图（分别是图A、图B以及图C）。双特异性抗体具有两个结合单元，这两个结合单元中的每一个结合相同抗原上的不同表位。在图中标记了这些结合单元。关于这些结合单元，分子是双侧对称的。如所描绘，Bs2Ab-39SH、Bs3Ab-39SH、Bs4Ab-39SH形

式是指其中scFv通过接头(例如(G₄S)₂)被融合至可变区的氨基末端(Bs2Ab-39SH)、插入修饰的铰链区(Bs4Ab-39SH)或重链的CH3的羧基-末端(Bs3Ab-39SH)的双特异性抗体。所示的三种双特异性构建体包含通过甘氨酸丝氨酸接头(例如(G₄S)₂)融合至抗HER2结构域II人IgG1的抗HER2结构域IV结合scFv。

[0568] Bs2Ab-39SH、Bs3Ab-39SH以及Bs4Ab-39SH构建体的氨基酸序列提供于图8中(还参见SEQ ID NO:30、34以及38天然Fc区)。图8A示出Bs2Ab-39SH的双特异性抗体重链氨基酸序列和用于增强的ADCC的可能取代位点和或对于两种和四种药物负载的位点特异性抗体药物轭合。抗HER2结构域IV scFv是以VL-(G₄S)₄接头-VH形式并且通过(G₄S)₂接头基因连接至抗HER2结构域II抗体重链的氨基末端上。可在抗体的CH2和CH3中进行所描绘的氨基酸取代和或插入,以便用于增强的ADCC和位点特异性轭合。图8B示出Bs3Ab-39SH的双特异性抗体重链氨基酸序列和用于增强的ADCC的可能取代位点和或对于两种和四种药物负载的位点特异性抗体药物轭合。抗HER2结构域IV scFv是以VL-(G₄S)₄接头-VH形式并且通过(G₄S)₂接头基因连接至抗HER2结构域II抗体重链的羧基末端上。可在抗体的CH2和CH3中进行所描绘的氨基酸取代和或插入,以便用于增强的ADCC和位点特异性轭合。图8C示出Bs4Ab-39SH的双特异性抗体重链氨基酸序列和用于增强的ADCC的可能取代位点和或对于两种和四种药物负载的位点特异性抗体药物轭合。抗HER2结构域IV scFv是以VL-(G₄S)₄接头-VH形式并且通过两个(G₄S)₂接头插入抗HER2结构域II抗体重链的修饰的铰链区中。可在抗体的CH2和CH3中进行所描绘的氨基酸取代和或插入,以便用于增强的ADCC和位点特异性轭合。

2.2. 双特异性抗体的结合特异性和物种交叉反应性

[0569] 通过如在实例1中所述的捕获ELISA来确定双特异性抗体与人EGFR、人HER2、人HER3、人HER4、小鼠Her2、或食蟹猴Her2的重组细胞外结构域蛋白的结合特异性和物种交叉反应性。结果显示所测试的所有双特异性抗体(包括Bs2Ab-39SH、Bs3Ab-39SH以及Bs4Ab-39SH)能够以类似功效结合至人HER2和食蟹猴Her2并且它们中没有一个显示结合至人EGFR、HER3、HER4或小鼠Her2(数据未示出),从而表明双特异性抗体保留其亲本单克隆抗体的物种交叉反应性的结合特异性。

[0570] 通过BIAcore来确定双特异性抗体与人HER2和食蟹猴Her2的结合动力学(数据未示出)。针对Bs2Ab-39SH对人HER2的亲和力(K_D)是113pM,并且针对Bs4Ab-39SH是236pM。

2.3. 双特异性抗体的体外活性

[0571] 使用在实例1中描述的方法来确定双特异性抗体在抑制配体驱动的细胞增殖方面的活性。结果显示Bs2Ab-39SH、Bs3Ab-39SH以及Bs4Ab-39SH在MDA-MB-361细胞(图9A)和MCF-7细胞(图9B)中具有类似的功效,这还与亲本抗体组合(39S加上曲妥珠单抗)的活性可比较。在其他细胞系(包括NCI-N87和RT-112)中观察到类似结果(数据未示出)。

2.4. 通过双特异性抗体来破坏HER2:HER3异源二聚化

[0572] 收获T47D细胞,洗涤并且重新悬浮于无血清培养基中。以 1×10^6 个细胞/孔的密度将细胞种于6孔板中,并且然后在37°C/5%CO₂下孵育过夜。第二天,使用500nM浓度下的有待测试的抗体(R347 IgG1同种型对照、曲妥珠单抗、帕妥珠单抗、39S以及Bs2Ab-39SH)对细胞进行预处理1小时。在预处理后,以最终浓度8ng/mL添加调蛋白-β1并且在37°C/5%CO₂下孵育细胞5min。然后使用冰冷1x PBS洗涤细胞两次,溶解并且根据制造商说明书使用小鼠抗人HER2(克隆44E7)抗体和来自赛默飞科技公司(Thermo Scientific)的Pierce经典IP试

剂盒来进行免疫沉淀。用含有2-巯基乙醇的Laemmli缓冲液洗脱免疫沉淀的蛋白质样品,并且使用标准方案通过蛋白质印迹来进行分析。在蛋白质印迹分析中,兔抗人HER2(克隆29D8)抗体被用来检测HER2并且兔抗人HER3(C-17)多克隆抗体被用来检测HER3。

[0573] 图10示出Bs2Ab-39SH和39S(类似于帕妥珠单抗)可以破坏由配体刺激诱导的HER2:HER3异源二聚化。

2.5. 通过双特异性抗体进行的HER2聚集

[0574] 为了检查双特异性抗体是否可以交联HER2以形成大型复合体,以不同摩尔比将重组人HER2细胞外结构域蛋白与Bs2Ab-39SH或曲妥珠单抗混合,并且在室温下孵育30min。通过HPLC大小排阻色谱法来分离所形成的免疫复合体;然后通过多角度光散射(MALS)测定来分析这些免疫复合体的大小。

[0575] 图11示出来源于1:1摩尔比的抗体:HER2的代表性数据(以其他摩尔比的数据未示出)。结果指示Bs2Ab-39SH可交联许多HER2分子以形成大小为1716 kDa大的蛋白质复合体,而曲妥珠单抗最大仅可结合至两个HER2分子以形成320 kDa复合体。对于Bs4Ab-39SH观察到类似的结果(数据未示出)。

2.6. 通过双特异性抗体进行的增强的内化和溶酶体运输

[0576] 通过FACS来测量抗体内化。从T150烧瓶中收获BT-474细胞,重新悬浮于冰冷培养基中并且然后以 1×10^6 个细胞/孔添加至96孔U型底板中。在4℃下通过离心来沉淀细胞。弹去培养基,并且一式三份将细胞沉淀物重新悬浮于150μL的含有10μg/mL有待测试的抗体或抗体组合(R347 IgG1同种型对照、曲妥珠单抗、帕妥珠单抗、AZ1.39.1、39S、曲妥珠单抗+39S、曲妥珠单抗+帕妥珠单抗、帕妥珠单抗+39S、曲妥珠单抗+帕妥珠单抗+39S、Bs2Ab-39SH、Bs3Ab-39SH以及Bs4Ab-39SH)的冰冷培养基中。在冰上孵育细胞1小时并且然后洗涤以去除未结合抗体。将等分试样的细胞保持在冰上;剩余细胞在37℃/5%CO₂下孵育不同的时间周期(30min、1小时、2小时或4小时),并且然后立即在冰上冷却。使用冰冷FACS缓冲液洗涤细胞两次并且然后使用4%PFA固定20min。固定之后,使用抗人IgG Alexa-Fluor 488对细胞进行染色,并且通过BD LSR II机器和BD FACSDiva™软件来进行分析。使用FlowJo软件分析数据。受体-抗体复合体内化被计算为相对于在扣除来源于未处理对照的MFI背景值之后在冰上的荧光强度的在37℃下的平均荧光强度(MFI)损失百分比。

[0577] 图12示出双特异性抗体(Bs2Ab-39SH、Bs3Ab-39SH、Bs4Ab-39SH)可以诱导比任何单一单特异性抗体或抗体组合更快和更强的内化。帕妥珠单抗、AZ1.39.1、曲妥珠单抗+帕妥珠单抗、帕妥珠单抗+39S以及曲妥珠单抗+帕妥珠单抗+39S具有类似于或低于39S的内化特征并且在图形中未示出。在细胞系NCI-N87、MDA-MB-361以及RT-112中观察到类似结果(数据未示出)。

[0578] 共聚焦显微镜方法被用来可视化抗体内化和溶酶体运输。从T-150烧瓶中收获BT-474细胞并且重新悬浮于冰冷培养基中,并且然后以 2.5×10^5 个细胞/孔添加至96孔U型底板中。在4℃下通过离心来沉淀细胞。弹去培养基,并且将细胞沉淀物重新悬浮于150μL的存在10μg/mL有待测试的抗体(诸如R347 IgG1同种型对照、曲妥珠单抗、Bs2Ab-39SH以及Bs4Ab-39SH)的冰冷培养基中。将细胞在37℃/5%CO₂下孵育不同的时间周期(30min、2小时、4小时或6小时),并且然后立即在冰上冷却。使用冰冷FACS缓冲液洗涤细胞两次并且然后后根据制造商说明书使用BD生物科技公司(BD Biosciences)Cytofix/Cytoperm™固定/透

化溶液进行固定和透化。使用抗人IgG Alexa-Fluor 488和小鼠抗人LAMP-1(克隆H4A3)接着使用抗小鼠IgG Alexa-Fluor 647在黑暗中对细胞进行染色。染色后,将细胞旋转涂片到正电荷载玻片上并且使用含有DAPI的ProLong®Gold Antifade试剂用盖玻片盖好。然后通过Leica SP5共聚焦显微镜和Leica应用套件高级荧光软件套件来可视化细胞。

[0579] 图13示出抗体内化和对溶酶体的运输。Bs2Ab-39SH和Bs4Ab-39SH两者促进比曲妥单抗更快的内化和更强的溶酶体运输,这显示很少甚至没有内化(针对Bs4Ab-39SH的数据未示出)。

[0580] 蛋白质印迹分析被用来监测HER2的溶酶体降解。收获BT-474细胞,洗涤并且重新悬浮于培养基中。以 5×10^4 个细胞/孔的密度将细胞种于96孔板中并且使用500nM浓度下的有待测试的抗体(R347 IgG1同种型对照、曲妥单抗、帕妥单抗、39S、曲妥单抗+帕妥单抗、曲妥单抗+39S、帕妥单抗+39S、曲妥单抗+帕妥单抗+39S、Bs2Ab-39SH、Bs3Ab-39SH以及Bs4Ab-39SH)在37℃/5%CO₂下进行处理,持续2小时、6小时或24小时。在处理结束时,使用冰冷1x PBS洗涤细胞两次并且然后根据制造商说明书溶解于来自赛默飞科技公司的M-PER哺乳动物蛋白质提取缓冲液中。通过BCA测定来测量每种溶解产物的蛋白质浓度。将等量每种溶解产物中的蛋白质负载到蛋白质印迹中的凝胶上。兔抗人HER2(克隆29D8)抗体和兔抗人GAPDH(克隆D16H11)抗体被用来分别检测HER2和GAPDH。

[0581] 图14示出使用双特异性抗体(Bs2Ab-39SH、Bs3Ab-39SH或Bs4Ab-39SH)的处理引起BT-474细胞中的显著HER2降解,并且单特异性抗体或抗体组合诱导没有或有限的HER2降解。

实例3

3.1. 对用于位点特异性铰合和用于消融Fc γ 受体结合活性的位点特异性突变体进行克隆

[0582] 标准重叠PCR方法被用来独立地或组合地(L234F-S239C(FC)和L234F-S239C-S442C(FCC))将突变L234F、S239C以及S442C引入Bs2Ab-39SH和Bs4Ab-39SH构建体的Fc部分中。引物被设计成含有希望的突变,并且含有限制性位点以促进定向克隆的侧翼引物被用来扩增含有特定突变的Fc片段。使用限定的限制性位点将修饰的Fc PCR产物克隆到哺乳动物表达载体中。通过DNA序列分析来证实Fc突变的身份。

[0583] 图15示出具有用于位点特异性铰合的氨基酸取代的三种抗HER2双特异性抗体(Bs2Ab-39SH、Bs3Ab-39SH、Bs4Ab-39SH)的说明。另外,抗体重链CH₂上的L234F取代(图中未描绘)被用来使Fc γ 受体结合最小化。在希望2DAR的情况下,在位点1(例如,S239C)或位点2(例如,S442C)处工程化半胱氨酸取代,并且对于4DAR,在这两个位点处工程化半胱氨酸取代。双特异性药物构建体在此又称为Bs2-2T/Bs2-4T、Bs3-2T/Bs34T以及Bs4-2T/Bs4-4T。

[0584] 所得双特异性抗体被铰合至主要如下所述的微管溶素1508有效负载上(参见实例5)。

3.2. 双特异性ADC的结合特异性和物种交叉反应性

[0585] 为了确定铰合微管溶素1508是否改变抗原结合特异性和物种交叉反应性,通过如在实例1中所述的捕获ELISA来证实双特异性ADC与人EGFR、人HER2、人HER3、人HER4、小鼠Her2以及食蟹猴Her2的重组细胞外结构域蛋白的结合活性。结果显示铰合不改变双特异性ADC的抗原结合特异性和物种交叉反应性。Bs2-4T和Bs4-4T两者可以与其未铰合形式相同

的功效结合至人HER2和食蟹猴Her2;并且它们中没有一个显示结合至人EGFR、HER3、HER4或小鼠Her2(数据未示出)。通过BIAcore来确定双特异性ADC与人HER2的结合动力学,并且结果显示针对Bs2-4T对人HER2的亲和力(K_D)是120pM,并且针对Bs4-4T是271pM。

3.3. 通过双特异性ADC来破坏细胞内微管网络

[0586] 为了检查通过抗HER2ADC对细胞内微管网络的破坏,选择了三种细胞系:SKOV-3、JIMT-1以及RT112,这些分别代表T-DM1适合、T-DM1未响应者以及T-DM1不适合。在第1天,通过胰蛋白酶消化来收获细胞,将其重新悬浮于培养基中,并且然后以 6×10^4 个细胞/孔的密度种于8孔腔室载玻片中。在37℃/5%CO₂下孵育载玻片过夜。在第2天,吸出培养基以去除任何未附着的细胞并且然后将含有5nM有待测试的ADC的新鲜培养基添加至细胞中。在37℃/5%CO₂下孵育载玻片过夜。在第3天,使用1x PBS洗涤每个腔室两次。然后使用4%PFA固定细胞,持续20min。在固定结束时,去除腔室并且根据标准免疫荧光程序对载玻片进行染色。简单地说,使用Triton X-100透化细胞并且使用含有吐温-20的1x PBS洗涤。以1:100将兔抗人 α -微管蛋白Alexa-Fluor 488(克隆11H10)稀释在DAKO抗体稀释物中并且添加至载玻片中。在室温下孵育1小时之后,使用含有DAPI的ProLong®Gold Antifade试剂用盖玻片盖好。通过Leica SP5共聚焦显微镜和Leica应用套件高级荧光软件套件来可视化染色的细胞。

[0587] 图16A示出,在SKOV-3(代表T-DM1适合的患者的细胞系)中,T-DM1和Bs2-4T两者能够破坏微管网络。在SKBR-3细胞中观察到类似的结果(数据未示出)。

[0588] 图16B示出,在JIMT-1(代表T-DM1未响应者患者的细胞系)中,仅Bs2-4T能够破坏细胞内微管网络。

[0589] 图16C示出,在RT-112(代表T-DM1不适合的患者的细胞系)中,Bs2-4T能够破坏细胞内微管网络,而T-DM1是无效的。

3.4. 双特异性ADC的体外活性

[0590] 表达不同水平HER2的一组人癌症细胞系被用来评估双特异性ADC的细胞毒性活性(实例1中的表5)。简单地说,收获细胞,将其重新悬浮并且以100 μ L的体积,以96孔板的每个孔5,000至20,000的密度(取决于每种细胞系的生长动力学)将细胞铺放在含有血清的培养基中。通过将测试物品稀释在培养基中制备有待测试的每种剂量抗体或ADC的2X浓缩物。一式三份将100微升每种测试物品添加至细胞中,这样使得最终剂量曲线在以逐步1:4系列稀释从5nM降至0.08pM范围内。在37℃/5%CO₂下孵育经过处理的细胞持续3至4天,取决于每种具体细胞系的生长动力学。根据制造商说明书,使用细胞滴度Glo来确定细胞活力。通过GraphPad Prism软件分析数据,并且作为相对于未处理的对照的生长抑制百分比呈现。使用具有GraphPad Prism软件的S形非线性回归分析来确定EC₅₀值并且概括于表6中。

表6:一组癌症细胞系中抗HER2ADC的体外功效(以pM计的EC₅₀)

细胞系	T-DM1	Bs2-2T	Bs4-2T	Bs2-4T	Bs4-4T
SKBR-3	82.6	12.9	10.4	4.0	2.6
NCI-N87	275.1	36.8	25.6	23.7	20.0
SKOV-3	116.1	13.6	8.9	8.7	5.6
MDA-MB-361	266.0	15.1	10.0	4.0	2.7
JIMT-1	无活性的	13.3	7.1	5.1	3.1

MDA-MB-453	344.3	19.0	13.0	10.5	8.6
RT-112	无活性的	4635	2938	36.5	23.5
MCF7-GTU	无活性的	无活性的	无活性的	84.5	61.4
ZR-75-1	无活性的	1543	753.5	18.1	20.9
T47D	无活性的	无活性的	无活性的	无活性的	无活性的
MCF-7	无活性的	无活性的	无活性的	无活性的	无活性的
MDA-MB-468	无活性的	无活性的	无活性的	无活性的	无活性的

[0591] 图17A示出在SKBR-3(代表T-DM1适合的患者的人乳腺癌细胞系)中,Bs2-2T和Bs2-4T相对于T-DM1和不同对照的细胞毒性活性。数据指示在SKBR-3细胞中,Bs2-2T和Bs2-4T两者比T-DM1更有效。

[0592] 图17B示出在SKBR-3(代表T-DM1适合的患者的人乳腺癌细胞系)中,Bs4-2T和Bs4-4T相对于T-DM1和不同对照的细胞毒性活性。数据指示在SKBR-3细胞中,Bs4-2T和Bs4-4T两者比T-DM1更有效。

[0593] 图18A示出在JIMT-1(代表T-DM1适合但未响应者的患者的人乳腺癌细胞系)中,Bs2-2T和Bs2-4T相对于T-DM1和不同对照的细胞毒性活性。数据指示Bs2-2T和Bs2-4T两者在杀伤JIMT-1细胞上是非常有效的,而T-DM1示出无活性。

[0594] 图18B示出在JIMT-1(代表T-DM1适合但未响应者的患者的人乳腺癌细胞系)中,Bs4-2T和Bs4-4T相对于T-DM1和不同对照的细胞毒性活性。数据指示Bs4-2T和Bs4-4T两者在杀伤JIMT-1细胞上是非常有效的,而T-DM1示出无活性。

[0595] 图19A示出在ZR-75-1(代表T-DM1不适合的患者的人乳腺癌细胞系)中,Bs2-2T和Bs2-4T相对于T-DM1和不同对照的细胞毒性活性。数据指示Bs2-4T在杀伤ZR-75-1细胞上是最有活性的,而Bs2-2T具有较低水平的活性并且T-DM1示出无细胞毒性活性或限制的细胞毒性活性。

[0596] 图19B示出在ZR-75-1(代表T-DM1不适合的患者的人乳腺癌细胞系)中,Bs4-2T和Bs4-4T相对于T-DM1和不同对照的细胞毒性活性。数据指示Bs4-4T在杀伤ZR-75-1细胞上是最有活性的,而Bs4-2T具有较低水平的活性并且T-DM1示出无细胞毒性活性或限制的细胞毒性活性。

[0597] 图20示出在MDA-MB-468(不具有HER2表达的人乳腺癌细胞系)中,Bs2-2T和Bs2-4T相对于T-DM1和不同对照的细胞毒性活性。数据指示Bs2-2T或Bs2-4T在MDA-MB-468细胞中都不是有效的,从而指示Bs2-2T和Bs2-4T的细胞毒性活性是靶标(即HER2)依赖的。对于Bs4-2T和Bs4-4T观察到类似的结果(数据未示出)。

3.5. 双特异性ADC构建体的体内活性

[0598] 基于细胞系的异种移植(CBX)肿瘤模型和患者来源的异种移植(PDX)肿瘤模型:依从实验室动物护理评估和鉴定协会(AAALAC)公开的准则和MedImmune公司机构动物保护和委员会批准的方案进行所有小鼠实验。在研究中使用4-8周龄之间的无胸腺裸鼠。在皮下CBX模型中,使用从特定传代批下的培养物中收获的肿瘤细胞在右翼上对动物进行单侧注射。在正位CBX模型中,通过将每个小鼠 5×10^6 个细胞(悬浮于50%基质胶中)注入动物右翼上的乳房脂垫中来建立异种移植。在PDX模型中,使用从宿主动物中收获的肿瘤片段在侧翼上对动物进行单侧植入,每种宿主动物从特定传代批中植入。在其评估开始日

期之前大约一周开始对每个实验记录研究前肿瘤体积。当肿瘤达到适当的肿瘤体积开始(TVI)范围(典型地150-250mm³)时,将动物随机化成处理组和对照组并且开始给药。除非另外指出,否则每周一次对动物进行给药,其中测试物品通过静脉内或腹膜内注射。每日观察动物并且测量肿瘤尺寸和体重,并且每周两次进行记录。使用以下公式计算肿瘤体积:肿瘤体积= $\pi \div 6$ (长度 $\times$ 宽度²)。肿瘤生长曲线呈现为平均肿瘤体积(mm³) $\pm$ SEM。

[0599] 图21A示出甚至在更低浓度(1mg/kg)下,Bs2-4T在MDA-MB-361模型(代表T-DM1不适合的患者的人乳癌CBX肿瘤模型)中相对于T-DM1(3mg/kg)和不同对照(所有都在3mg/kg下)具有更高的体内活性。数据证实了Bs2-4T诱导剂量依赖性肿瘤生长抑制,并且在3mg/kg剂量下,Bs2-4T诱导完全肿瘤消退而T-DM1示出限制的活性。在3mg/kg下,Bs4-4T还展现出相对于T-DM1的更高体内活性(图21B)。

[0600] 图22示出在ST996模型中,Bs2-2T相对于Bs2-4T和T-DM1的体内活性。ST996是来源于三阴性乳癌患者(HER2IHC:1+;ER-;PR-)的原代PDX模型。数据证实了在3mg/kg剂量下的Bs2-2T诱导有力的肿瘤生长抑制,虽然与Bs2-4T相比它的抗肿瘤功效略微降低。相反,T-DM1在ST996模型中示出无活性。在另外的低HER2表达PDX模型ST738、ST455B以及ST821中观察到类似的结果(数据未示出)。

[0601] 图23示出在ST225模型(代表T-DM1适合的患者的乳癌PDX肿瘤模型)中,Bs2-4T相对于T-DM1和不同对照的体内活性。数据证实了在3mg/kg剂量下的Bs2-4T诱导完全和持久的肿瘤消退。相反,T-DM1仅在处理期过程中诱导肿瘤停滞并且停止在处理之后迅速地肿瘤重新生长。

[0602] 图24示出在JIMT-1模型(代表T-DM1适合但未响应者的患者的乳癌CBX正位肿瘤模型)中,Bs2-4T相对于T-DM1和不同对照的体内活性。数据证实了在3mg/kg剂量下的Bs2-4T诱导完全和持久的肿瘤消退。相反,T-DM1或T-DM1加上帕妥珠单抗组合示出无活性。

[0603] 图25示出在ST455B模型中,Bs2-4T相对于T-DM1和不同对照的体内活性。ST455B是来源于三阴性乳癌患者(HER2IHC:1+、ER-、PR-)的原代PDX模型。数据证实了在3mg/kg剂量下的Bs2-4T诱导完全肿瘤消退而T-DM1示出无活性。为了进一步扩大在图25中示出的发现,我们检查了在另外16个PDX模型中的Bs2-4T,这些模型来源于具有相对低水平HER2表达(通过HerceptTest得到的+1至+2)的乳癌患者。在这些模型的选择中还考虑到其他标准(包括HER2表达的异质性程度、ER/PR状态以及病理组织学子类)来使研究中的肿瘤亚型多样性最大化。表7概括了在这些17个不同PDX乳癌模型中的Bs2-4T体内活性。Bs2-4T证实了与肿瘤的病理组织学子类和ER/PR状态无关的有效抗肿瘤活性。在1mg/kg的剂量下,41%的肿瘤模型示出肿瘤消退并且6%示出肿瘤停滞。在3mg/kg的剂量下,71%的模型示出肿瘤消退并且12%示出肿瘤停滞。

[0604] 这些研究证实了与单特异性ADC疗法(例如T-DM1)相比,本发明的双特异性ADC在宽范围乳癌模型上的卓越活性。具体地说,本发明的双特异性ADC在具有低水平HER2表达的癌症模型和在T-DM1未响应者患者的模型中具有体内活性。

[0605] 表7:Bs2-4T在一组代表低HER2/T-DM1不适合的患者的PDX乳癌模型中的体内功效的概述。在示出对Bs2-4T处理的最大应答的时间点处,如果与开始给药下的肿瘤体积(TVI)相比肿瘤体积减小,则体内功效表达为随TVI的肿瘤体积变化百分比。另外,体内功效表达为相对于媒介物对照的肿瘤体积变化百分比。如果肿瘤体积减小 $>20\%$,对处理的响应性

分类为“消退”，如果肿瘤体积变化 $<20\%$ ，则分类为“停滞”并且如果肿瘤体积增加 $>20\%$ ，则分类为“进展”。

功效	1 mg/kg	3 mg/kg
消退	41% (7/17)	70% (12/17)
停滞	6% (1/17)	12% (2/17)
进展	53% (9/17)	18% (3/17)

3.6. 双特异性ADC在具有获得性T-DM1抗性的肿瘤模型中的活性

[0606] 通过使用逐渐增加浓度高达 $5\mu\text{g/mL}$ 的T-DM1的连续处理来产生具有获得性对T-DM1的抗性的NCI-N87细胞。如在实例3中所述，在亲本和抗性细胞系两者中检查Bs2-4T相对于T-DM1的体外细胞毒性活性。图26A中示出的结果指示Bs2-4T和T-DM1两者在亲本NCI-N87细胞中是有效的，虽然Bs2-4T比T-DM1更有效(左图)。在抗性细胞系中，T-DM1失去活性，而Bs2-4T仍在杀伤抗性细胞上有效的(右图)。还通过使用T-DM1(包括BT-474、SKOV-3以及MDA-MB-361)的连续处理来产生其他抗性细胞系，并且在这些抗性细胞系中针对Bs2-4T观察到类似的细胞毒性活性(数据未示出)。

[0607] 为了建立具有获得性T-DM1抗性的体内肿瘤模型，将T-DM1抗性的NCI-N87细胞皮下注入免疫缺陷的小鼠中。使用 3mg/kg 的T-DM1来处理荷瘤小鼠。可以看出，体外T-DM1抗性不能完全翻译成体内抗性，通过动物之中的肿瘤生长大量的变化来反映。因此，选择具有大型顽固性肿瘤(以体积计大约 1000mm^3)的小鼠，并且将肿瘤组织片段传代至新小鼠直到在 3mg/kg T-DM1的每周处理存在下肿瘤一致生长。在三代之后，稳定的抗性肿瘤演变并且对这些肿瘤进行片段化并且植入小鼠中，以便评估Bs2-4T的体内活性。如在图26B中所证实，由重复的T-DM1处理复发的肿瘤不仅对T-DM1有抗性，而且对T-DM1和曲妥珠单抗组合处理无反应。相反，Bs2-4T在处理之后诱导有力和持续的肿瘤消退，从而表明其作为用于T-DM1复发/顽固性患者的有效疗法的潜力。

[0608] 图26B示出Bs2-4T在T-DM1抗性NCI-N87肿瘤模型中诱导肿瘤消退。响应于每周静脉内给药Bs2-4T(3mg/kg)、T-DM1(3mg/kg)或其他对照抗体/ADC(3mg/kg ，除了对于帕妥珠单抗是 10mg/kg)总共4次剂量的肿瘤生长曲线作为平均肿瘤体积(mm^3) $\pm$ SEM($n=7$)示出。与未处理的对照组比较，由学生t检验得到 $*P<0.001$ 。

3.7. 双特异性ADC的体内抗肿瘤活性不会被曲妥珠单抗的预处理所衰减

[0609] 在无胸腺裸鼠的侧翼上单侧地植入ST225PDX肿瘤片段。将小鼠随机化成处理组和对照组并且当肿瘤体积达到 $200\text{--}250\text{mm}^3$ 时开始给药。在处理组中，使用媒介物缓冲液或曲妥珠单抗(3mg/kg)对小鼠进行给药。三天后，小鼠接受每周静脉内注射Bs2-4T总共4次剂量。图27示出曲妥珠单抗的预处理不会衰减Bs2-4T在ST225 PDX模型中的抗肿瘤活性，从而表明如果使用Bs2-4T来治疗对曲妥珠单抗或T-DM1复发/顽固性的患者的话，可以不需要清洗期。ST225是具有HER2过表达的原代乳癌PDX模型。响应于不同处理的肿瘤生长曲线在图27中作为平均肿瘤体积(mm^3) $\pm$ SEM($n=10$)呈现。

3.8. 双特异性ADC的旁观者效应

[0610] 使用绿色荧光蛋白(GFP)稳定转染NCI-N87细胞,并且使用红色荧光蛋白(RFP)稳定转染MDA-MB-468细胞。收获这两种细胞系,洗涤,重新悬浮于培养基中,并且然后种于6孔板的相同孔中作为共培养物。作为对照,将每个细胞系种于不同6孔板中作为单一培养物(图28A)。为了调节至不同生长动力学,以 5×10^5 个细胞/孔种下NCI-N87细胞并且以 2×10^5 个细胞/孔种下MDA-MB-468细胞。在 $37^\circ\text{C}/5\%\text{CO}_2$ 下孵育细胞2天。吸出培养基并且将含有5nM有待测试的抗体或ADC的新鲜培养基添加至细胞中,并且在 $37^\circ\text{C}/5\%\text{CO}_2$ 下孵育板4天。在处理结果时,收集每个孔中的细胞,洗涤,重新悬浮于100 μL 的冰冷FACS缓冲液中并且然后使用2%PFA来固定。通过BD LSR II机器和BD FACSDiva™软件来分析细胞。使用FlowJo软件分析数据。

[0611] 图28证实了Bs2-4T可杀伤共培养物中的HER2过表达细胞和无HER2细胞两者,从而表明Bs2-4T具有旁观者效应。相反,T-DM1不能杀伤共培养物中的无HER2细胞,从而表明它不具有旁观者效应。作为对照,Bs2-4T和T-DM1两者示出对单一培养物中的NCI-N87细胞的有效杀伤,并且Bs2-4T或T-DM1都没有示出对单一培养物中的MDA-MB-468细胞的杀伤。

3.9. 双特异性ADC对抗癌症干细胞的活性

[0612] 癌症干细胞球体测定:在标准组织培养条件下,在补充有20%FBS的莱博维茨(Leibovitz)的L-15培养基中培养MDA-MB-361细胞。通过胰蛋白酶消化收获细胞,使用PBS洗涤两次,并且重新悬浮于干细胞培养基(补充有20ng/mL EGF、10ng/mL bFGF、5mg/mL胰岛素、0.4%BSA以及1%敲除血清替代品的SCM:DMEM/F12)中达到30,000个细胞/mL。为了形成原代球体,将1mL细胞铺放到24孔超低附着板中并且使用10pM R347-4T、T-DM1或BS2-4T处理,并且在 $37^\circ\text{C}/5\%\text{CO}_2$ 下孵育4天。在处理结束时,收获原代球体,使用0.05%胰蛋白酶解离并且以30,000个细胞/mL的密度重新悬浮于含有与原代球体培养相同的抗体处理的SCM中。然后一式三份将细胞铺放到96孔超低附着板中并且孵育4天。根据制造商说明书,使用细胞滴度Glo来确定球体细胞活力。数据呈现为相对于未处理的对照的CSC球体形成倍数。图29(左图)示出Bs2-4T使CSC球体形成抑制84%并且T-DM1对抑制CSC球体形成没有活性。在其他癌症细胞系(包括BT-474、JIMT-1以及T47D)中观察到类似结果(数据未示出)。

[0613] 在使用Bs2-4T处理的异种移植瘤中评价癌症干细胞:切除来自评估Bs2-4T体内活性的MDA-BM-361异种移植瘤研究的肿瘤,切成4mm小片并且使用Cryostor进行冻存。在 37°C 下融化冷冻的肿瘤小片,用汉克斯平衡盐溶液(HBSS)洗涤两次并且使用无菌手术刀刀片进一步切碎。为了获得单细胞悬浮液,然后将肿瘤小片与200单位的超纯胶原酶III/mL DMEM/F12培养基混合。在 37°C 下孵育肿瘤悬浮液持续大约1小时,其中每30分钟进行机械破坏。在孵育结束时,通过70- μm 尼龙筛过滤细胞并且使用HBSS洗涤两次。在最后一次洗涤之后,使细胞放入穿过40- μm 细胞滤器并且使用Vi-CellXR细胞活力分析仪进行计数。使用干细胞技术公司(Stemcell Technologies)Aldefluor试剂盒并且根据制造商说明书测定细胞的醛脱氢酶活性作为CSC量度。在LSRII流式细胞仪上运行细胞并且使用FlowJo进行分析。图29(右图)示出在MDA-MB-361乳癌异种移植瘤模型中,使用Bs2-4T的处理引起CSC降低54%。

实例4

4.1. 无岩藻糖基化的双特异性抗体产生和表征

[0614] 使用POTELLEGENT™技术(新泽西州普林斯顿Biowa公司)表达双特异性抗体以便产

生具有增强的ADCC活性的无岩藻糖基化的抗体。表8示出如通过BIAcore所测量的Bs2Ab-39SH和Bs4Ab-39SH双特异性抗体以及结合至Fc γ 受体(Fc γ R)和C1q的具有岩藻糖基化(Fuc)和不具有岩藻糖基化(_aFuc)的曲妥珠单抗的K_D(nM),从而证实了无岩藻糖基化的抗体具有增强的与Fc γ RI以及Fc γ RIIIa的这两个等位基因的结合。

表8:由BIAcore测量的K_D(nM)

抗体	FcgRI	FcgRIIa	FcgRIIb	FcgRIIIa (158F)	FcgRIIIa (158V)	C1q
Bs2Ab-39SH_Fuc	7.0	1300	2750	1990	267	75.6
Bs2Ab-39SH_aFuc	0.98	1250	2400	216	40	130
Bs4Ab-39SH_Fuc	13.0	1190	2790	1780	228	88.6
Bs4Ab-39SH_aFuc	0.98	1260	2560	223	45	446
曲妥珠单抗_Fuc	5.1	1270	2650	2119	240	72.7
曲妥珠单抗_aFuc	0.65	1210	2190	305	60	134

[0615] 平衡结合常数的测量:人Fc γ 受体:在ProteOn XPR36仪器上测量对于抗HER2双特异性抗体与人Fc γ R结合的结合常数(K_D)。简单地说,使用如仪器制造商所述的标准氨基偶联化学性质,在高密度下将双特异性抗体固定在GLC传感器芯片上。IgG的最终表面密度测量为大约3000RU。还使用除去蛋白质的相同固定方案而在此传感器芯片上制备了参考流细胞。在仪器缓冲液(含有50mM磷酸盐,pH 7.4,0.15 M NaCl,3mM EDTA以及0.005%吐温-20的磷酸盐缓冲盐水[PBS]/吐温/乙二胺四乙酸[EDTA]缓冲液)中制备4000nM、16,000nM或32,000nM的每种Fc γ R的储备溶液,并且然后在相同缓冲液中连续稀释(1:3)以便获得每种受体的希望的浓度系列:1.82nM-4,000nM(Fc γ RI)、197.5nM-16,000nM(Fc γ RIIA)、395.1nM-16,000nM(Fc γ RIIb)、21.9nM-16,000nM(hFc γ RIIIA-158V)以及395-32,000mM(Fc γ RIIIA-158F)。在双特异性抗体和参考细胞两者表面上以25 μ L/min的流速注射每种浓度的Fc γ R,持续8min,在此过程中收集结合数据。在注射之间,使用60秒脉冲5mM HCl来再生表面(即,去除结合的Fc γ R)。还贯穿注射系列,散布若干缓冲液注射。后来,这些缓冲液注射中的一种连同参考细胞数据一起被用来通过普遍称为“双重参考”(Myszka,1999)的技术校正任何注射伪像(例如,非特异性结合)的结合数据。在收集所有结合数据之后,对各个浓度(C)下的结合(在平衡状态[Req]下的反应)的个别数据集合取平均值,并且然后将其拟合至1:1结合等温(在平衡状态下的反应对比浓度)图。由此,使用供应商的评估软件3.1.0.6版本来衍生平衡结合常数K_D。

[0616] 平衡结合常数的测量:人FcRn蛋白:在ProteOn XPR36仪器上测量对于双特异性抗体与人FcRn蛋白(huFcRn)结合的亲和力(K_D)。简单地说,使用如上所述的标准氨基偶联化学性质,在高密度下将双特异性抗体固定在GLC传感器芯片上。在仪器缓冲液(含有150mM NaCl以及0.05%吐温-20的50mM磷酸钠缓冲液,pH 6)中制备3000nM的huFcRn蛋白的储备溶液,并且然后在相同缓冲液中连续稀释(3:1)至1.37nM。在串联连接的双特异性抗体和参考细胞表面上,以25 μ L/min的流速依次注射每种浓度的huFcRn持续16min。收集结合数据,接着在注射每种受体或缓冲液空白之间60秒注射含有150mM NaCl和0.05%吐温20的50mM磷

酸钠缓冲液, pH7.4以便再生IgG表面(即, 去除结合的huFcRn蛋白)。还贯穿注射系列, 散布若干缓冲液注射。后来, 使用这些缓冲液注射中的一种连同参考细胞数据来通过“双重参考”(Myszka, 1999)校正注射伪像(例如, 非特异性结合)的原数据集合。在收集所有结合数据之后, 对各个浓度(C)下的结合(R_{eq})的个别数据集合取平均值, 并且然后将其拟合至1:1结合等温(在平衡状态下的反应对比浓度)图。由此, 使用供应商的BIA评估软件4.1版本来衍生平衡结合常数 K_D 。结果显示双特异性抗体具有与常规IgG1可比较的类似 K_D 值。

4.2. 无岩藻糖基化的双特异性抗体的体外活性

[0617] 使用在实例1中描述的方法来确定无岩藻糖基化的双特异性抗体在抑制配体驱动的细胞增殖方面的活性。结果显示无岩藻糖基化的Bs2Ab-39SH、无岩藻糖基化的Bs3Ab-39SH以及无岩藻糖基化的Bs4Ab-39SH在MDA-MB-361细胞(图30A)和MCF-7细胞(图30B)中具有类似的抗增殖功效, 这还与亲本抗体组合(39S加上曲妥珠单抗)的活性可比较。在其他细胞系(包括NCI-N87和RT-112)中观察到类似结果(数据未示出)。

4.3. 无岩藻糖基化的双特异性抗体的增强的ADCC活性

[0618] 使用KC1333(表达Fc γ RIIIa的人自然杀伤(NK)细胞系)作为效应子细胞并且使用MDA-MB-361细胞系作为ADCC测定中的靶细胞以便评估抗HER2抗体的ADCC活性。收获这两种细胞系, 洗涤并且重新悬浮于测定培养基中。以 1×10^6 个细胞/mL的密度重新悬浮KC1333并且以 4×10^5 个细胞/mL的密度重新悬浮MDA-MB-361。将50微升每种细胞系添加至96孔U形底板中的孔中, 以便达到1:2.5的靶标:效应子。通过将测试物品稀释在测定培养基中制备每种剂量抗体的3X浓缩物。一式三份将50微升每种测试物品添加至细胞中, 这样使得最终剂量曲线在以逐步1:4系列稀释从10 μ g/mL降至0.15ng/mL范围内。对板进行离心以使每个孔中的细胞沉淀并且然后在37 $^{\circ}$ C/5%CO $_2$ 下孵育过夜。第二天, 根据制造商说明书使用普洛麦格公司的CytoTox 96 $\oplus$ 非放射性细胞毒性测定来定量每个孔的上清液中的LDH。使用GraphPad Prism软件分析数据, 并且作为相对于未处理的对照的细胞毒性百分比呈现。

[0619] 图31A至31C示出无岩藻糖基化的双特异性抗体相对于曲妥珠单抗和无岩藻糖基化的曲妥珠单抗在MDA-MB-361细胞中的ADCC活性。数据表明无岩藻糖基化的抗体的ADCC活性高于其岩藻糖基化的形式, 并且无岩藻糖基化的双特异性抗体之间的ADCC功效的范围是Bs2Ab-39SH_aFuc>Bs4Ab-39SH_aFuc>Bs3Ab-39SH_aFuc。在其他细胞系包括BT-474、NCI-N87、MDA-MB-453、T47D以及JIMT-1中观察到类似结果(数据未示出)。

实例5

5.1. 微管溶素1508有效负载合成

[0620] 除非另外说明, 否则用于耦合的如在图32中所示的微管溶素1508细胞毒素有效负载的合成在以下说明性实例中详细说明, 其中:

(i)以摄氏度($^{\circ}$ C)给出温度; 当在室温或环境温度下进行操作时, 即, 在18 $^{\circ}$ C-25 $^{\circ}$ C的范围内, 除非另外说明;

(ii)经过无水硫酸钠或硫酸镁干燥溶液; 在减压(4.5-30mmHg)下使用旋转蒸发器进行有机溶剂的蒸发, 其中浴温高达至30 $^{\circ}$ C;

(iii)色谱法意指硅胶上的快速色谱法; 在硅胶板上进行薄层色谱法(TLC);

(iv)通常, 反应期之后是TLC或液相色谱法/质谱法(LC/MS), 并且仅出于说明给出反应时间;

(v)最终产物具有满意的质子核磁共振(NMR)谱和/或质谱数据;

(vi)仅出于说明给出产率并且不必是可通过酌情工艺开发所获得的那些;如果要求更多材料,重复制备;

(vii)当给定的核磁共振(NMR)数据是以主要诊断质子的 $\delta(\text{delta})(\delta)$ 值形式时,给出在300或400MHz下在 d_6 -DMSO中确定的相对于作为内标物的四甲基硅烷(TMS)的一百万分之一(ppm),除非另外说明;

(viii)化学符号具有它们通常的意义;

(ix)以体积:体积(v/v)方式给出溶剂比;并且

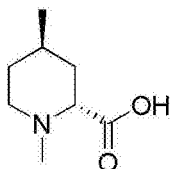
(x)使用以下方法中的一种或多种进行化合物的纯化:

a)在常规硅胶上进行的快速色谱法;

b)使用Isco**Combiflash®**分离系统:RediSep正相快速柱,流速30-40ml/min(ISCO MPLC),在硅胶上进行的快速色谱法;请添加ISCO反相柱

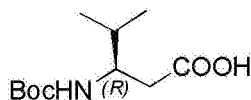
c)Gilson semiprep HPLC分离系统:YMC pack ODS-AQ柱子,100×20mm,S 5 μ m 12nm,水(0.1%三氟乙酸)和乙腈(0.1%三氟乙酸)作为溶剂,运行20min;

化合物T1



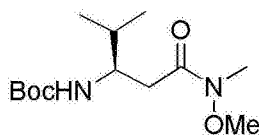
[0621] 向(2R,4R)-4-甲基哌啶-2-甲酸(2g,13.97mmol)在MeOH(40mL)和水(40.0mL)中的溶液中添加多聚甲醛(2.52g,27.94mmol)和Pd/C(10%)(0.8g,7.52mmol)。在室温下,在氢气气氛下搅拌反应混合物过夜。通过TLC,反应未完成。添加另一个当量的多聚甲醛(2.52g,27.94mmol)并且将反应混合物再搅拌24小时。TLC指示反应完成并且过滤反应混合物,使用MeOH(2×30mL)洗涤催化剂。在真空中浓缩滤液以得到呈白色固体的粗产物,使用乙醚(3×30mL)洗涤该粗产物,在高真空中干燥过夜以得到呈白色固体的(2R,4R)-1,4-二甲基哌啶-2-甲酸(T1)(1.870g,85%)。LC-MS:158(M+1);¹H NMR(400MHz,重水) δ ppm 0.97(d,J=5.52Hz,3H),1.54(br.s,1H),1.71-1.87(m,3H),1.91-2.07(m,1H),2.84(s,3H),3.13(td,J=8.41,3.76Hz,1H),3.35(m,1H),3.65(m,1H)。

化合物T2



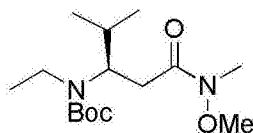
[0622] 将二碳酸二叔丁酯(243.0g,1.1mol)逐滴添加至(R)-3-氨基-4-甲基戊酸(商业上可获得)(133.0g,1.0mol)和Na₂CO₃(212g,2.0mol)在丙酮(1L)和水(1L)中的悬浮液中,同时在室温下搅拌。搅拌反应混合物过夜并且在减压下去除有机溶剂。使用水(1L)稀释残余物并且使用EtOAc(500mL×3)洗涤。使用2N HCl溶液对水相进行酸化达到pH=3,并且使用EtOAc(800mL×3)萃取所得混合物。使用盐水(800mL×1)洗涤合并的萃取物,干燥(无水Na₂SO₄)并且浓缩以便得到呈油状物的化合物(T2)(224.0g,97%产率),该化合物无需进一步纯化而用于下一步中。

化合物T3



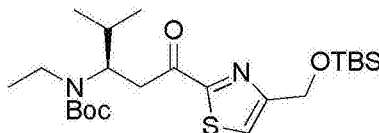
[0623] 将三乙胺(67g, 0.61mol)添加至化合物(T2)(140.0g, 0.61mol)和N, O-二甲基羟胺盐酸盐(74.1g, 0.76mol)在CH₂Cl₂(1.4L)中的悬浮液中, 同时在0℃下搅拌。搅拌悬浮液0.5小时并且在0℃下按份添加EDCI(74g, 0.61mol)。在0℃下搅拌反应混合物2小时并且添加水(800mL)。分离有机相, 使用5%KHSO₄溶液(800mL×3)、饱和NaHCO₃溶液(800mL×3)以及盐水(800mL×1)洗涤, 干燥(无水Na₂SO₄)并且浓缩至干燥。通过快速柱色谱法在硅胶(EtOAc/己烷=1:5)上纯化残余物, 得到呈油状物的化合物(T3)(141.0g, 84%产率)。¹H NMR(300MHz, CDCl₃): δ5.26(m, 1H), 3.75(m, 1H), 3.70(s, 3H), 3.15(s, 3H), 2.60~2.80(m, 2H), 1.85(m, 1H), 1.41(s, 9H), 0.90(d, J=6.6Hz, 3H), 0.88(d, J=6.6Hz, 3H)。

化合物T4



[0624] 将碘乙烷(250.0g, 1.6mol)添加至化合物(T3)(55.0g, 0.2mol)在DMF(1.1L)中的溶液中, 同时在0℃下搅拌。然后在0℃下按份添加NaH(60%悬浮液, 24.0g, 0.60mol), 并且允许反应混合物加温至室温且搅拌12小时。使用水(2L)小心地猝灭反应并且添加EtOAc(2L)。分离有机相, 使用5%KHSO₄溶液(800mL×3)、饱和NaHCO₃溶液(800mL×3)以及盐水(800mL×1)洗涤, 干燥(无水Na₂SO₄)并且浓缩至干燥。通过快速柱色谱法在硅胶(EtOAc/己烷=1:10)上纯化残余物, 得到呈油状物的化合物(T4)(35.1g, 58%产率)。¹H NMR(300MHz, CDCl₃): δ3.70(s, 3H), 3.65(m, 1H), 3.10~3.30(m, 5H), 2.50~2.95(m, 2H), 1.90~2.20(m, 1H), 1.40~1.55(m, 9H), 1.10(t, J=7.2Hz, 3H), 0.90(d, J=6.6Hz, 3H), 0.88(d, J=6.6Hz, 3H)。

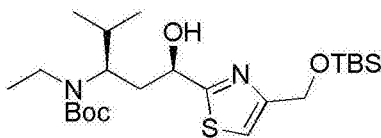
化合物T5



[0625] 在-78℃下, 在N₂下将正丁基锂(106ml, 2.5N己烷溶液, 0.17mol)逐滴添加至2-溴-4-((叔丁基二甲基甲硅烷基氧基)甲基)噻唑(74g, 0.24mol)(如在微泊夫P(Wipf, P)等人, 有机通讯(Org. Lett.)2007, 9(8), 第1605页中所述的制备)在干燥THF(500mL)中的溶液中, 同时经过1小时搅拌。进一步搅拌悬浮液30min并且然后在-78℃下经过30min逐滴添加化合物(T4)(51.0g, 0.17mol)在干燥THF(300mL)中的溶液。在-78℃下搅拌反应混合物1小时并且然后允许加温至室温且搅拌12小时。使用20%水性氯化铵溶液(1L)猝灭反应, 并且在减压下去除有机溶剂。用EtOAc(800mL×3)萃取所得混合物。使用5%KHSO₄溶液(800mL×3)、饱和NaHCO₃溶液(800mL×3)以及盐水(800mL×1)洗涤合并的有机相, 干燥(Na₂SO₄)并且浓缩至干燥。通过快速柱色谱法在硅胶(EtOAc/己烷=1:10)上纯化粗材料, 得到呈油状物的化合物(T54)(58.1g, 73%产率)。¹H NMR(300MHz, CDCl₃): δ7.53(m, 1H), 4.90(s, 2H), 4.04(m, 1H), 3.35(m, 2H), 3.15(m, 2H), 2.00(m, 1H), 1.40(s, 9H), 0.80~1.20(m, 18H), 0.14(s,

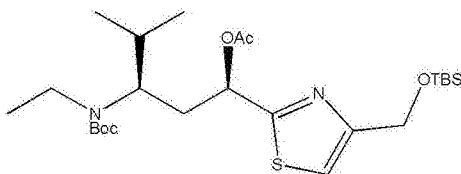
6H)。

化合物T6



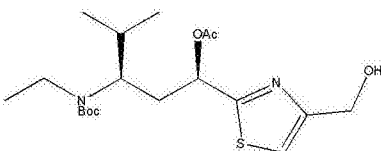
[0626] 在室温下,经过0.5小时周期将 LiBH_4 (4.8g,0.22mol)按份添加至化合物(T5)(47.1g,0.1mol)在甲醇(500mL)中的溶液中,同时进行搅拌。搅拌悬浮液2小时并且在减压下去除溶剂。将残余物溶解于 EtOAc (800mL)中,并且使用饱和 NaHCO_3 溶液(500mL $\times$ 3)和盐水(500mL $\times$ 1)洗涤所得溶液,干燥(Na_2SO_4)并且浓缩至干燥。通过快速柱色谱法(EtOAc /己烷=1:6)来纯化粗材料,得到化合物(T6)(13.5g,28%产率)和它的异构体(T6')(21.0g,45%产率)。 ^1H NMR(300MHz,氯仿-d) δ ppm-0.06-0.05(m,6H)0.76-0.89(m,15H)1.12(t,J=6.97Hz,3H)1.39(s,9H)1.55-2.05(m,3H)2.86-3.21(m,2H)3.76-3.96(m,1H)4.73(d,J=1.13Hz,4H)7.01(s,1H)。

化合物T7



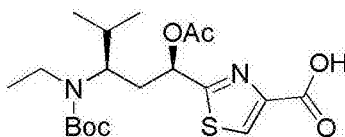
[0627] 在0℃下,将乙酰氯(45.2g,0.58mol)逐滴添加至化合物(T6)(34.0g,72mmol)在吡啶(500mL)中的溶液中,同时经过10min搅拌。允许反应物加温至室温并且搅拌12小时。使用水(200mL)猝灭反应并且在减压下去除溶剂。使用 CH_2Cl_2 (800mL)处理残余物,并且使用5% KHSO_4 溶液(800mL $\times$ 3)、饱和 NaHCO_3 溶液(800mL $\times$ 3)以及盐水(800mL $\times$ 1)洗涤所得混合物,干燥(Na_2SO_4)并且浓缩至干燥。通过快速柱色谱法在硅胶(EtOAc /己烷=1:10)上纯化粗材料,得到呈油状物的化合物(T7)(25.7g,69%产率)。 ^1H NMR(300MHz, CDCl_3): δ 7.15(m,1H),5.95(m,1H),4.84(s,2H),4.04(m,1H),3.10(m,2H),2.35(m,1H),2.15(s,3H),2.00(m,1H),1.70(m,1H),1.45(s,9H),1.25(t,J=7.2Hz,3H),0.80~1.10(m,15H),0.08(s,6H)。

化合物T8



[0628] 在0℃下,将四丁基氟化铵(65.3g,0.25mol)在THF(200mL)中的溶液逐滴添加至化合物(7)(25.7g,50mmol)在THF(300mL)中的溶液中,同时搅拌。允许反应物加温至室温并且搅拌4小时。添加水(800mL)并且在减压下去除有机溶剂。使用 CH_2Cl_2 (800mL)处理残余物,并且使用5% KHSO_4 溶液(800mL $\times$ 3)、饱和 NaHCO_3 溶液(800mL $\times$ 3)以及盐水(800mL $\times$ 1)洗涤所得混合物,干燥(Na_2SO_4)并且浓缩至干燥。通过快速柱色谱法在硅胶(EtOAc /己烷=1:4)上纯化粗材料,得到呈油状物的化合物(T8)(19.5g,98%产率)。 ^1H NMR(300MHz, CDCl_3): δ 8.26(m,1H),5.95(m,1H),4.83(m,2H),4.10(m,1H),3.17(m,2H),2.40(m,1H),2.20(s,3H),2.18(m,1H),1.75(m,1H),1.56(s,9H),1.10-1.30(m,3H),0.80-1.05(m,6H)。

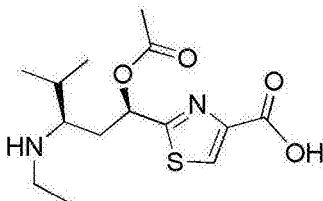
化合物T9



[0629] 将戴斯-马丁试剂(32.7g, 75mmol)添加至化合物(T8)(20.0g, 50mmol)在二氯甲烷(300mL)中的溶液中并且在室温下搅拌反应混合物12小时。分别使用氢氧化钠溶液(1N, 300mL×3)、硫代硫酸钠溶液(1N, 300mL×3)、饱和NaHCO₃(300mL×3)溶液以及盐水(300mL×1)洗涤混合物。干燥(Na₂SO₄)有机层并且浓缩至干燥以得到相应的醛。

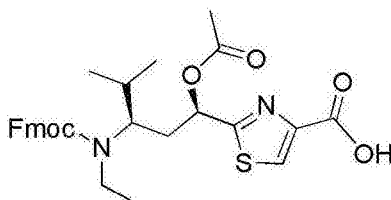
[0630] 将此粗醛溶解于叔丁醇(500mL)中, 并且在室温下经过1小时逐滴添加亚氯酸钠(80%, 36.4g, 320mmol)和磷酸二氢钠一水合物(105g, 0.77mol)在水(300mL)中的溶液。搅拌反应混合物3小时并且使用盐酸溶液(0.1N, 500mL)稀释。用EtOAc(500mL×1)萃取所得混合物, 并且使用5%KHSO₄溶液(500mL×3)和盐水(500mL×1)洗涤合并的有机层, 经过Na₂SO₄干燥并且浓缩至干燥。通过快速柱色谱法在硅胶(CH₂Cl₂/MeOH=100:5)上纯化残余物, 得到化合物(T9)(15.4g, 58%产率)。¹H NMR(300MHz, CDCl₃): δ9.90(br s, 1H), 8.27(s, 1H), 5.96(m, 1H), 4.07(m, 1H), 3.15(m, 1H), 2.35(m, 1H), 2.20(s, 3H), 2.18(m, 1H), 1.75(m, 1H), 1.45(s, 9H), 1.20(t, J=7.2Hz, 3H), 0.98(d, J=6.6Hz, 3H), 0.88(d, J=6.6Hz, 3H)。

化合物T10



[0631] 在0℃下, 向2-((1R,3R)-1-乙酰氧基-3-((叔丁氧基羰基)(乙基)氨基)-4-甲基戊基)噻唑-4-甲酸(T9)(6.5g, 15.68mmol)在DCM(60mL)中的溶液中添加TFA(30mL)。在0℃下搅拌混合物1小时。真空蒸发溶剂以得到粗产物(T10)。该粗产物无需进一步纯化而用于下一步反应中(7.2克)。LC-MS: 315(M+1)。

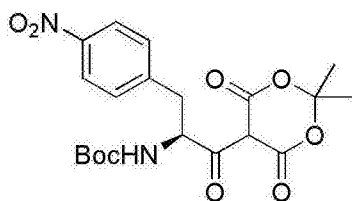
化合物T11



[0632] 向2-((1R,3R)-1-乙酰氧基-3-((乙基氨基)-4-甲基戊基)噻唑-4-甲酸4、三氟乙酸盐(T10)(5g, 11.67mmol)和碳酸氢钠(9.80g, 116.71mmol)在丙酮(300mL)和水(150mL)的混合物中的溶液中添加(9H-芴-9-基)甲基(2,5-二氧化吡咯烷-1-基)碳酸酯(3.94g, 11.67mmol)。在室温下搅拌混合物过夜。LCMS指示反应完成。使用盐酸对混合物进行酸化达到(pH 2)并且在真空中蒸发丙酮。用DCM(3×300mL)萃取产物。使用0.1% HCl溶液(200mL)、盐水(200mL)洗涤合并的有机萃取物, 经过Na₂SO₄干燥, 并且在真空中蒸发。通过快速色谱法(硅胶, MeOH/DCM, 从0%至5%的MeOH)纯化残余物, 得到呈白色固体的2-((1R,3R)-3-(((9H-芴-9-基)甲氧基)羰基)(乙基)氨基)-1-乙酰氧基-4-甲基戊基)噻唑-4-甲酸(T11)(3.53g, 54.6%)。LC-MS: 537.2(M+1); ¹H NMR(400MHz, 氯仿-d) δppm 0.84(d, J=6.78Hz,

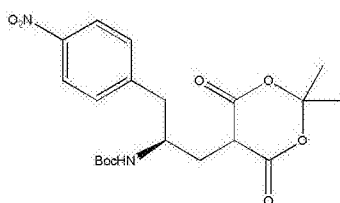
3H), 0.92-1.05(m, 5H), 1.14(d, $J=3.01\text{Hz}$, 1H), 1.73(dt, $J=10.23, 6.43\text{Hz}$, 1H), 1.92-2.05(m, 1H), 2.12-2.27(m, 4H), 2.28-2.44(m, 1H), 2.90-3.33(m, 2H), 3.98(t, $J=9.29\text{Hz}$, 1H), 4.12-4.32(m, 1H), 4.47-4.82(m, 2H), 5.95(dd, $J=10.92, 2.89\text{Hz}$, 1H), 7.29-7.45(m, 4H), 7.55-7.69(m, 2H), 7.72-7.81(m, 2H), 8.22-8.29(m, 1H)。

化合物T12



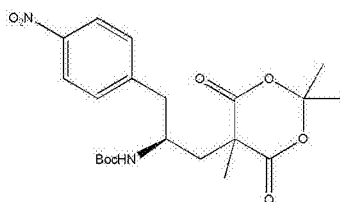
[0633] 将DMAP(106g, 0.86mol)添加至Boc-L-4-硝基-苯基丙氨酸(1800g, 0.58mol)和麦氏酸(92g, 0.64mol)在二氯甲烷(1.5L)中的溶液中。在 -5°C 下,在 N_2 气氛下冷却所得溶液,接着经过1h添加二氯甲烷(1L)中的DCC(240g, 1.16mol)。在 0°C - 5°C 下搅拌混合物过夜。然后通过过滤去除沉淀的 $\text{N,N}'$ -二氯己基脒,并且使用5%水性HCl(1L $\times$ 3)和盐水(1L $\times$ 1)洗涤滤液,并且经过 MgSO_4 干燥。在通过过滤去除 MgSO_4 之后,浓缩有机相至干燥。使用EtOAc/己烷(1:1, 500mL)研磨残余物,并且干燥以得到呈黄色固体的化合物(T12)(130.0g, 51%产率)。

化合物T13



[0634] 在 -5°C 下,在 N_2 下将AcOH(400mL)添加至化合物(T12)(130.0g, 0.298mol)在二氯甲烷(1.5L)中的溶液中。经过2小时按小份添加固体 NaBH_4 (22.7g, 0.597mol)(气体逸出和放热)。在 -5°C 下再搅拌3h之后,TLC指示反应完全。使用盐水(1L)猝灭混合物。分离有机层,并且使用水(1L $\times$ 2)、水性饱和 NaHCO_3 (1L $\times$ 3)以及盐水(1L $\times$ 3)依次洗涤,并且经过 MgSO_4 干燥。浓缩滤液至干燥并且得到呈黄色固体的化合物(T13)(70.3g, 55%产率)。 ^1H NMR(300MHz, CDCl_3): δ 8.18(d, $J=8.7\text{Hz}$, 2H), 7.41(d, $J=8.7\text{Hz}$, 2H), 4.58(m, 1H), 4.29(m, 1H), 3.85(m, 1H), 2.97(d, $J=6.6\text{Hz}$, 2H), 2.27(m, 2H), 1.80(s, 3H), 1.76(s, 3H), 1.35(s, 9H)。

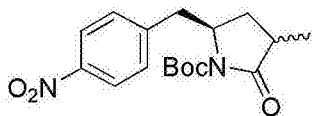
化合物T14



[0635] 将 K_2CO_3 (35g, 0.25mol)和MeI(36g, 0.25mol)添加至化合物(T13)(70.3g, 0.167mol)在丙酮(400mL)和DMF(400mL)中的溶液中。在室温下搅拌混合物过夜。TLC显示起始材料被消耗。添加水(2L)并且再搅拌混合物1小时。通过过滤收集沉淀的固体,使用水洗涤,干燥,得到呈浅黄色固体的化合物(T14)(34.5g, 47%产率)。 ^1H NMR(300MHz, CDCl_3): δ

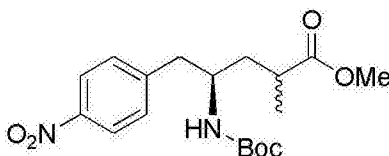
8.17(d, $J=8.7\text{Hz}$, 2H), 7.34(d, $J=8.7\text{Hz}$, 2H), 4.22(m, 1H), 3.85(m, 1H), 2.85(m, 2H), 2.22(m, 2H), 1.73(s, 3H), 1.73(s, 3H), 1.52(s, 3H), 1.31(s, 9H)。

化合物T15



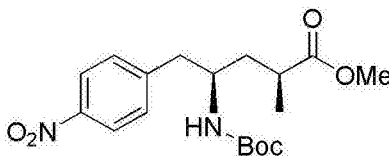
[0636] 将化合物(T14)(34.5g, 79.1mmol)溶解于甲苯(500mL)中。在回流下加热溶液40小时。TLC指示反应完成。去除溶剂以得到化合物(T15)(30g), 该化合物无需进一步纯化而用于下一步中。

化合物T16



[0637] 将 K_2CO_3 (22g, 0.16mol)添加至化合物(T15)(30g, 79mmol)在MeOH(300mL)中的溶液中。在室温下搅拌混合物3小时。TLC显示完全转化。去除溶剂, 将残余物溶解于二氯甲烷(500mL)中, 使用盐水(500mL $\times$ 3)洗涤, 经过 MgSO_4 干燥。在通过过滤去除 MgSO_4 之后, 浓缩有机相至干燥。通过硅胶色谱法(EtOAc/己烷=1:10)来进一步纯化残余物并且得到呈1:1非对映异构混合物的化合物(T16)(23.5g, 对于两步的产率为81%)。 ^1H NMR(300MHz, CDCl_3): δ 8.13(d, $J=8.7\text{Hz}$, 2H), 7.34(d, $J=8.7\text{Hz}$, 2H), 4.43(m, 1H), 3.85(m, 1H), 3.65(s, 3H), 2.85(m, 2H), 2.65(m, 1H), 1.85(m, 1H), 1.50(m, 1H), 1.30(s, 9H), 1.15(t, $J=6.6\text{Hz}$, 3H)。

化合物T17



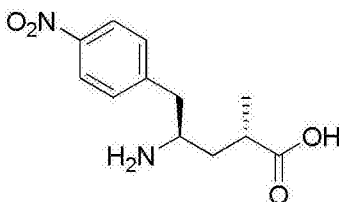
[0638] 使用SFC(超临界流体色谱法), 在Chiralpak ID 21 $\times$ 250mm, 5 μ 柱上, 使用流动相A 90%二氧化碳和流动相B异丙醇10%, 在60ml/min流速下使50g的化合物(T16)受到手性色谱法。在40 $^\circ\text{C}$ 下进行分离并且在270nm下检测。达到基线分离并且分离两种级分。峰B是希望的化合物(T17)并且呈固体获得27.4g(55%)。

[0639] 在Chiralpak IA柱4.6 $\times$ 250mm, 5 μ , 10%1:1甲醇:异丙醇的己烷溶液, 具有0.1%二乙胺改性剂上的非对应异构体过量 $>99:1$ 。

[0640] LC/MS(2分钟, 酸_CV10.0lp方法367(M+1), 1.16分钟。

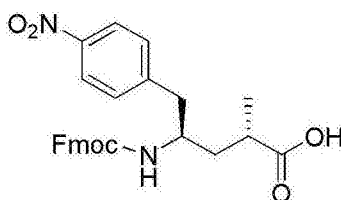
[0641] ^1H NMR(400MHz, 甲醇- d_4) δ ppm 8.16(d, $J=8.53\text{Hz}$, 2H) 7.46(d, $J=8.53\text{Hz}$, 2H) 3.79-3.93(m, 1H) 3.68(s, 3H) 2.90-2.99(m, 1H) 2.71-2.81(m, 1H) 2.47-2.59(m, 1H) 1.81-1.95(m, 1H) 1.55-1.66(m, 1H) 1.32(s, 9H) 1.21-1.25(m, 2H) 1.16(d, $J=7.03\text{Hz}$, 3H)。

化合物T18



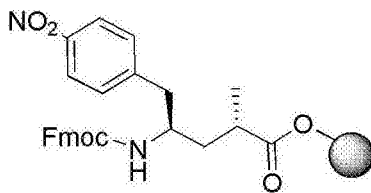
[0642] 在130℃下,在微波中加热(2S,4R)-甲基4-((叔丁氧基羰基)氨基)-2-甲基-5-(4-硝基苯基)戊酸酯(T17)(3.5g,9.55mmol)在6N HCl水溶液(8.0mL,263.30mmol)中的溶液,持续30min。冻干反应混合物,得到呈固体的(2S,4R)-4-氨基-2-甲基-5-(4-硝基苯基)戊酸(T18)。产物无需进一步纯化而用于下一步反应中。(3.2g)。LC-MS:253(M+1);¹H NMR(400MHz,重水)δppm 1.12(d,J=7.28Hz,3H),1.62-1.76(m,1H),1.90-2.02(m,1H),2.56-2.68(m,1H),3.02-3.11(m,2H),3.58-3.69(m,1H),7.47(d,J=8.53Hz,2H),8.18(d,J=8.78Hz,2H)。

化合物T19



[0643] 向化合物(T18)(0.43g,1.49mmol)和碳酸氢钠(1.251g,14.89mmol)在丙酮(30mL)和水(15mL)的混合物中的溶液中添加(9H-芴-9-基)甲基2,5-二氧化吡咯烷-1-基碳酸酯(0.502g,1.49mmol)。在室温下搅拌混合物过夜。LCMS指示反应完成。使用盐酸对混合物进行酸化达到pH 2并且在真空中蒸发丙酮。用DCM(3×60mL)萃取产物。使用1N HCl溶液(40mL)、盐水(40mL)洗涤合并的有机萃取物,经过Na₂SO₄干燥,并且在真空中蒸发。通过硅胶快速色谱法(从0%至100%EtOAc的DCM溶液)纯化残余物,得到呈白色固体的(2S,4R)-4-(((9H-芴-9-基)甲氧基)羰基氨基)-2-甲基-5-(4-硝基苯基)戊酸(0.630g,,89%)(T19)。LC-MS:475.5(M+H);¹H NMR(400MHz,氯仿-d)δppm 0.81-1.06(m,1H),1.08-1.28(m,2H),1.33-1.75(m,1H),1.77-2.11(m,1H),2.36-2.69(m,2H),2.76-3.18(m,1H),3.43-4.08(m,1H),4.09-4.19(m,1H),4.21-4.53(m,2H),4.54-4.80(m,1H),7.18-7.58(m,8H),7.66-7.82(m,2H),7.95-8.17(m,2H),8.67(br.s.,1H)。

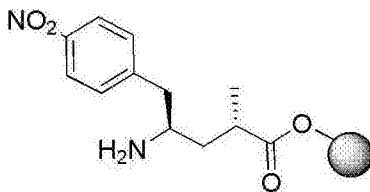
化合物T20



[0644] 将DIEA(0.419mL,2.40mmol)添加至(2S,4R)-4-(((9H-芴-9-基)甲氧基)羰基氨基)-2-甲基-5-(4-硝基苯基)戊酸(0.380g,0.80mmol)(T19)在DCM(4.5mL)中的溶液中,并且在室温下搅拌混合物5min,然后将2-氯三苯甲基氯树脂(0.5g,0.80mmol)添加至混合物中。在室温下摇晃混合物过夜,使用DMF(3×6mL)、MeOH(3×6mL)以及DCM(3×6mL)洗涤所得树脂,然后在室温下用DIEA(0.419mL,2.40mmol)和MeOH/DCM(1:1,5mL)处理30min。过滤所得树脂,使用DMF(3×6mL)、MeOH(3×6mL)以及DCM(3×6mL)洗涤,在高真空中干燥过夜。从

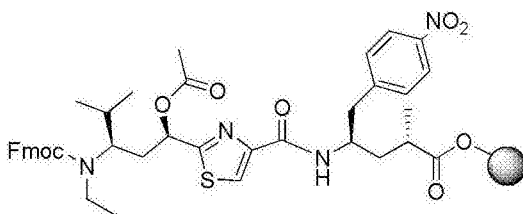
树脂上裂解少量化合物,并且通过LCMS来分析。所得树脂(T20)用于下一步反应。LC-MS:475(M+1)

化合物T21



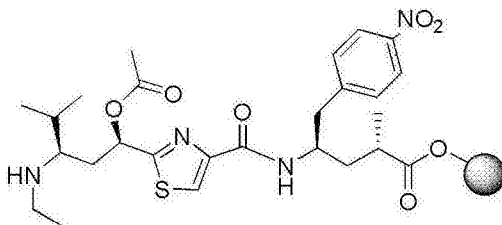
[0645] 向树脂(T20)(0.5g,0.80mmol)中添加DMF(5mL)中的20%哌啶。在室温下摇晃混合物6min,过滤所得树脂,使用DMF(3×6mL)、MeOH(3×6mL)、DCM(3×6mL)洗涤,在真空中干燥。从树脂上裂解少量化合物,通过LCMS来分析,该LCMS指示反应完成。所得树脂(T21)用于下一步反应。LC-MS:253(M+H)。

化合物T22



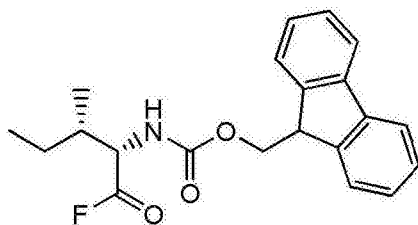
[0646] 在室温下,向树脂(T21)(0.5g,1.88mmol)中添加2-(((1R,3R)-3-((((9H-芴-9-基)甲氧基)羰基)(乙基)氨基)-1-乙酰氧基-4-甲基戊基)噻唑-4-甲酸(3)(1.108g,2.07mmol)、HATU(1.428g,3.76mmol)、2,4,6-三甲基吡啶(0.500mL,3.76mmol)以及DIEA(0.656mL,3.76mmol)在DMF(5mL)中的溶液。在室温下摇晃混合物两小时,并且过滤所得树脂,使用DMF(3×6mL)、MeOH(3×6mL)以及DCM(3×6mL)洗涤,在真空中干燥。从树脂上裂解少量化合物,通过LCMS来分析,该LCMS指示反应完成。所得树脂(T22)用于下一步骤。LC-MS:771(M+H)。

化合物T23



[0647] 向树脂(T22)(0.5g,0.80mmol)中添加DMF(5mL)中的20%哌啶。在室温下摇晃混合物6min,过滤所得树脂,使用DMF(3×6mL)、MeOH(3×6mL)、DCM(3×6mL)洗涤,在真空中干燥。从树脂上裂解少量化合物,通过LCMS来分析,该LCMS指示反应完成。所得树脂(T23)用于下一步反应。LC-MS:549(M+1)。

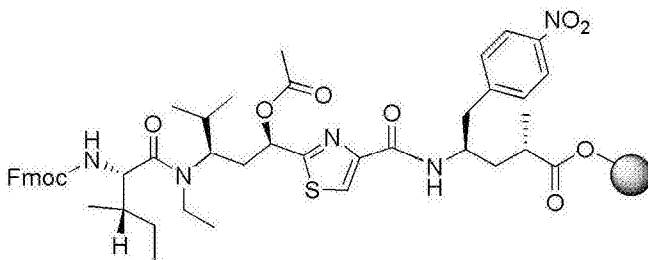
化合物T24



[0648] 向(2S,3S)-2-(((9H-芴-9-基)甲氧基)羰基氨基)-3-甲基戊酸(Fmoc-异亮氨酸)(7g,19.81mmol)和吡啶(1.602mL,19.81mmol)在DCM(120mL)中的溶液中通过插管经过10min添加DAST(3.11mL,23.77mmol)在DCM(20mL)中的溶液。在室温下搅拌反应混合物1小时,使用DCM(80mL)稀释,使用冰冷水(2×200mL)洗涤,经过MgSO₄干燥有机层,过滤并且在真空中蒸发,得到呈白色固体的(9H-芴-9-基)甲基(2S,3S)-1-氟代-3-甲基-1-氧代戊-2-基氨基甲酸酯(6.65g,94%)。进行酯化测试以确保通过将Fmoc-Ile-F(5mg)溶解于无水MeOH(0.3mL)和DIEA(0.030mL)中并且允许在室温下反应15min来进行定量酰氟形成。然后在真空中蒸发混合物并且通过LCMS来分析,示出存在小于1%的Fmoc-Ile-OH。

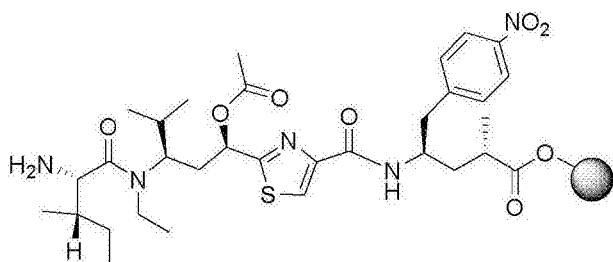
[0649] ¹H NMR(400MHz,氯仿-d) δ ppm 0.83-1.12(m,6H)1.18-1.37(m,1H)1.42-1.59(m,1H)2.01(br.s.,1H)4.26(t,J=6.78Hz,1H)4.44-4.63(m,3H)5.20(d,J=8.53Hz,1H)7.31-7.39(m,2H)7.40-7.47(m,2H)7.61(d,J=7.28Hz,2H)7.80(d,J=7.53Hz,2H)。

化合物T25



[0650] 在室温下,向树脂(T23)(0.5g,0.80mmol)中添加(9H-芴-9-基)甲基(2S,3S)-1-氟代-3-甲基-1-氧代戊-2-基氨基甲酸酯(T24)(0.569g,1.60mmol)、DMAP(4.89mg,0.04mmol)以及DIEA(0.419mL,2.40mmol)在DCM(5mL)中的溶液。在室温下摇晃混合物过夜,过滤所得树脂,使用DMF(3×6mL)、MeOH(3×6mL)、DCM(3×6mL)洗涤,在高真空中干燥。从树脂上裂解少量化合物,并且通过LC/MS来分析,LCMS指示反应完成。所得树脂(T25)用于下一反应步骤中。LC-MS:884(M+H)。

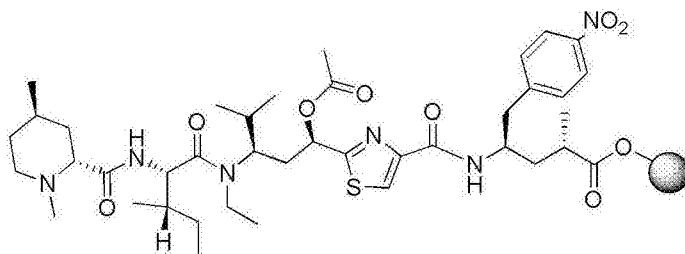
化合物T26



[0651] 向树脂(T25)(0.5g,0.80mmol)中添加DMF(5mL)中的20%哌啶。在室温下摇晃混合物6min,过滤所得树脂,使用DMF(3×6mL)、MeOH(3×6mL)、DCM(3×6mL)洗涤,在真空中干燥。从树脂上裂解少量化合物,通过LCMS来分析,该LCMS指示反应完成。所得树脂(T26)用于

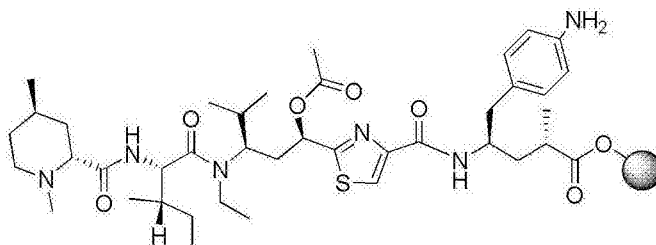
下一反应步骤中。LC-MS:662(M+1)。

化合物T27



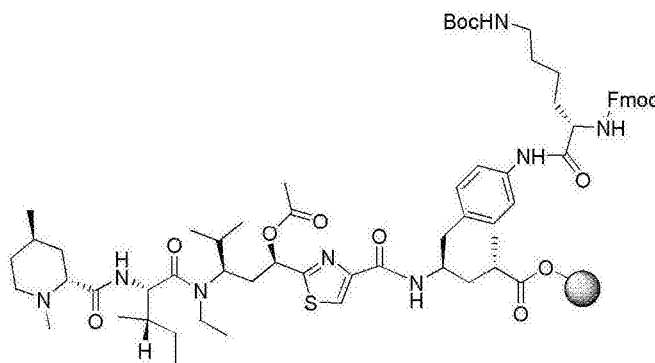
[0652] 向树脂(T26)(0.5g,0.80mmol)中添加(2R,4R)-1,4-二甲基哌啶-2-甲酸(1)(0.252g,1.60mmol)、HATU(0.608g,1.60mmol)、2,4,6-三甲基吡啶(0.320mL,2.40mmol)以及DIEA(0.419mL,2.40mmol)在DMF(5mL)中的溶液。在室温下摇晃混合物2小时,过滤所得树脂,使用DMF(3×6mL)、MeOH(3×6mL)以及DCM(3×6mL)洗涤,在真空中干燥。从树脂上裂解少量化合物,通过LCMS来分析,该LCMS指示反应完成。所得树脂(T27)用于下一反应步骤中。LC-MS:801(M+1)。

化合物T28



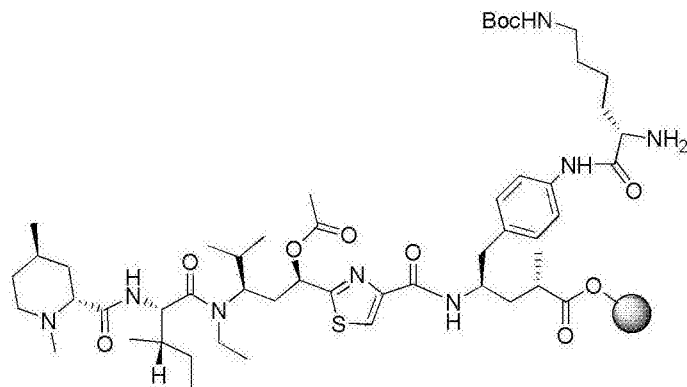
[0653] 向树脂(T27)中添加二氯化锡(II)二水(1.805g,8.00mmol)和乙酸钠(0.197g,2.40mmol)在DMF(5mL)中的溶液。在室温下摇晃混合物4小时,过滤所得树脂,使用DMF(3×6mL)、MeOH(3×6mL)以及DCM(3×6mL)洗涤,并且在真空中干燥。从树脂上裂解少量化合物,通过LCMS来分析,该LCMS指示反应完成。所得树脂(T28)用于下一步骤。LC-MS:771(M+H)。

化合物T29



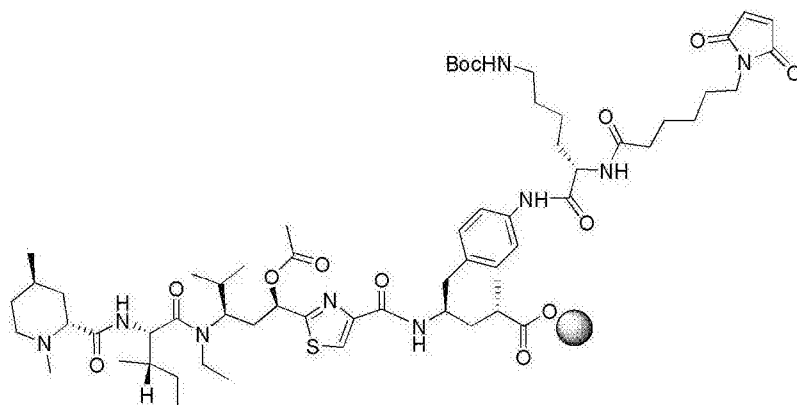
[0654] 向树脂(T28)(0.2g,0.32mmol)中添加(S)-2-(((9H-芴-9-基)甲氧基)羰基氨基)-6-(叔丁氧基羰基氨基)己酸(商业上可获得)(0.300g,0.64mmol)、HATU(0.243g,0.64mmol)、2,4,6-三甲基吡啶(0.128mL,0.96mmol)以及DIEA(0.168mL,0.96mmol)在DMF(4mL)中的溶液。在室温下摇晃混合物2小时,过滤所得树脂,使用DMF(3×2mL)、MeOH(3×2mL)以及DCM(3×2mL)洗涤,并且在真空中干燥。从树脂上裂解少量化合物,通过LCMS来分析,该LCMS指示反应完成。所得树脂(T29)用于下一步反应。LC-MS:1221(M+1)。

化合物T30



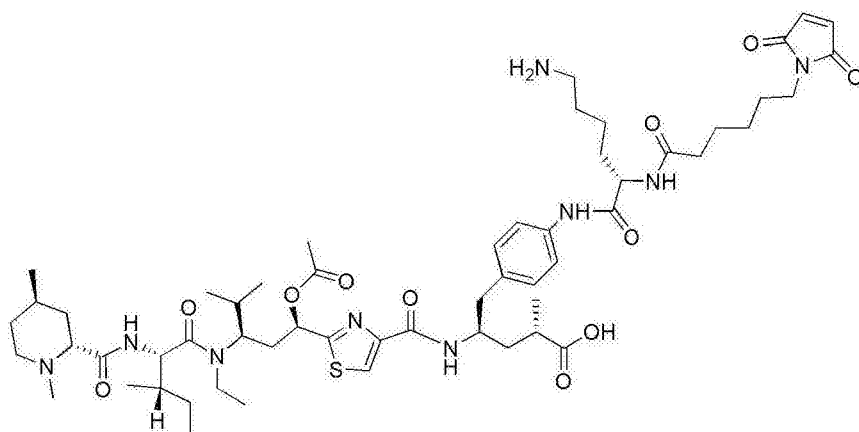
[0655] 向树脂(T29)(0.2g,0.32mmol)中添加DMF(2mL)中的20%哌啶。在室温下摇晃混合物6min,过滤所得树脂,使用DMF(3×3mL)、MeOH(3×3mL)、DCM(3×3mL)洗涤,在真空中干燥。从树脂上裂解少量化合物,通过LCMS来分析,该LCMS指示反应完成。所得树脂(T30)用于下一步反应。LC/MS:999(M+H)。

化合物T31



[0656] 在室温下,向树脂(T30)(0.2g,0.32mmol)中添加2,5-二氧代吡咯烷-1-基6-(2,5-二氧代-2,5-二氢-1H-吡咯-1-基)己酸酯(0.148g,0.48mmol)在DMF(2mL)中的溶液,接着添加N-甲基吗啉(0.106mL,0.96mmol)。在室温下摇晃混合物2小时,过滤所得树脂,使用DMF(3×3mL)、DCM(3×3mL)洗涤,在真空中干燥。从树脂上裂解少量化合物,通过LCMS来分析,该LCMS指示反应完成。所得树脂(T31)用于下一步骤。LC-MS:1192(M+1)。

化合物T32(微管溶素1508)



[0657] 在室温下,向树脂(T31)(0.2g,0.32mmol)中添加DCM(1mL)和TFA(1mL)。在室温下摇晃混合物20min,然后过滤。使用DCM/TFA(1:1,3×2mL)洗涤树脂,在真空中蒸发滤液。通过反相HPLC(ACN/H₂O(含有0.1%TFA),在14min中ACN从5%至75%)。冻干纯级分,得到呈白色固体的(2S,4R)-4-(2-((1R,3R)-1-乙酰氧基-3-((2S,3S)-2-((2R,4R)-1,4-二甲基哌啶-2-甲酰氨基)-N-乙基-3-甲基戊酰氨基)-4-甲基戊基)噻唑-4-甲酰氨基)-5-(4-((S)-6-氨基-2-(6-(2,5-二氧化-2,5-二氢-1H-吡咯-1-基)己酰氨基)己酰氨基)苯基)-2-甲基戊酸(T32)(0.095g,22.48%)。LC-MS:1092[M+1];¹H NMR(400MHz,甲醇-d₄)δppm 7.99(s,1H),7.34(d,J=8.53Hz,2H),7.10(d,J=8.53Hz,2H),6.66(s,2H),5.64(d,J=10.79Hz,1H),4.50-4.61(m,1H),4.21-4.35(m,2H),3.92(d,J=9.29Hz,1H),3.69(br.s.,1H),3.37(t,J=7.15Hz,2H),3.15-3.35(m,4H),3.04(dt,J=3.58,1.85Hz,1H),2.84(t,J=7.65Hz,2H),2.76(d,J=7.03Hz,2H),2.62(br.s.,2H),2.38-2.52(m,2H),2.25(t,J=11.54Hz,1H),2.16(t,J=7.40Hz,2H),2.04-2.11(m,4H),1.70-2.00(m,7H)1.42-1.69(m,11H),1.34-1.40(m,1H),1.27(t,J=6.78Hz,3H),1.16-1.24(m,2H),1.01-1.14(m,7H),0.90(d,J=6.78Hz,3H),0.94(d,J=6.53Hz,3H),0.84(t,J=7.40Hz,3H),0.79(d,J=6.53Hz,3H)。

5.2.微管溶素1508的轭合

[0658] 化合物T32(微管溶素1508)包含接头和容易轭合至抗体的巯基残基上从而形成巯基-马来酰亚胺连接的马来酰亚胺基团。包含马来酰亚胺基团的细胞毒素(例如,微管溶素1508)可被轭合至特定半胱氨酸残基上,被工程化成本发明的在此提供的抗HER2抗体(例如Bs2Ab-39SH、Bs3Ab-39SH或Bs4Ab-39SH)。可替代地或任选地,人们可以使用经典轭合方法来将细胞毒性剂附接至所描述的抗体上。用于轭合至天然赖氨酸和半胱氨酸残基上的方法是本领域中熟知的。以下提供了用于位点特异性(在工程化的半胱氨酸残基处)和经典的轭合(在天然半胱氨酸残基处)的代表性方法。

[0659] 代表性位点特异性抗体药物轭合方法在图33中所述,并且包括以下步骤:(a)打开可衍生氨基酸(例如,半胱氨酸)的侧链,(b)氧化,(c)轭合有效负载(例如,细胞毒性剂诸如微管溶素1508)并且(d)通过去除轭合试剂和未反应的有效负载来抛光。例如,轭合至工程化的半胱氨酸上可以通过在具有1mM乙二胺四乙酸的1X PBS中配制抗体来进行。通过添加每个抗体40当量的三(2-羧乙基)膦盐酸盐,在37℃下孵育3小时,使用温和还原来产生游离巯基。使用在具有1mM乙二胺四乙酸的1X PBS中的三次连续透析来去除三(2-羧乙基)膦盐酸盐(可替代地可以使用脱盐柱)。通过添加大约20当量的脱氢枞酸(dhAA)并且在室温下孵育大约4小时来允许抗体链间二硫键重新形成。在准备轭合中,将二甲亚砷添加至抗体中达到10%v/v,并且分别对于2T和4T药物负载,添加8或12当量的二甲亚砷中的微管溶素1508有效负载并且在室温下孵育大约1小时(可替代地在4℃下孵育大约16小时)。通过添加每个有效负载大约4摩尔当量的N-乙酰基半胱氨酸(NAC)来猝灭反应。根据制造商建议通过陶瓷羟基磷灰石来从轭合的抗体中去除游离的有效负载。如果需要,可以使最终产物受到缓冲液交换。可以通过非还原性和还原性SDS-PAGE来分析轭合的抗体,以便确认纯度和与重链的轭合。

[0660] 具有随机轭合至天然半胱氨酸残基上的药物的抗体-药物轭合物通过部分还原抗体接着与希望的接头-药物反应来制备。通过在pH 8.0下添加大约3摩尔当量的DTT,接着在大约37℃下孵育大约2h来部分还原5mg/mL浓度的抗体。然后在冰中冷却还原反应并且例如

通过渗滤来去除过量DTT。然后添加接头-药物至大约1:10摩尔比的接头-药物/巯基。在大约10% v/v的DMSO存在下进行轭合反应。在轭合之后,添加过量游离的半胱氨酸(接头-药物的大约2倍摩尔比)来猝灭未反应的接头-药物,以便产生半胱氨酸-接头-药物加合物。纯化反应混合物(例如,通过疏水相互作用色谱法)并且使其受到缓冲交换进入PBS中。使用标准方法诸如疏水相互作用色谱法和还原性反相色谱法来确定药物负载分布。

5.3. 化学缩写

Ac	乙酰基
ACN	乙腈
Boc	二碳酸二叔丁酯
t-Bu	叔丁基
Bzl	苄基,其中Bzl-OMe是甲氧基苄基并且Bzl-Me是甲苯
Cbz或Z	苄基氧基-羰基,其中Z-Cl和Z-Br分别是氯代-苄基氧基羰基和溴代苄基氧基羰基
DAST	三氟化二乙基氨基硫
DCM	二氯甲烷
DIAD	偶氮二甲酸二异丙酯
DIC	N,N'-二异丙基碳二亚胺
DIEA	二乙基异丙胺
DMF	N,N-二甲基甲酰胺
DTT	二硫苏糖醇
EtOAc	乙酸乙酯
Et2O	二乙醚
Fmoc	9H-芴-9-基甲氧基羰基
HATU	1-[二(二甲基氨基)亚甲基]-1H-1,2,3-三唑并[4,5-b]吡啶3-氧化六氟磷酸盐
HCl	盐酸
LC-MS	液相色谱法-质谱
MeOH	甲醇
Na2CO3	碳酸钠
NaHCO3	碳酸氢钠
PAB	对氨基苯甲基氧羰基
RT	室温
TEA	三乙胺
TFA	三氟乙酸
THF	四氢呋喃
TLC	薄层色谱法

通过引用结合

[0661] 出于所有目的,在此引用的所有参考文献,包括专利、专利申请、论文、教科书等以及其中引用的参考文献(如同它们还未曾引用过的程度)通过引用以其全文结合在此。

等效物

[0662] 前述书面说明书被认为足以使本领域的技术人员能够实践这些实施例。前述说明和实例详述了某些实施例,并且描述了诸位发明人所期望的最佳模式。然而,将了解到,无论前述内容以文本形式表现的如何详细,这些实施例仍可以许多方式实践,并且权利要求书包括其任何等效物。

AZ1.39.1.VL 种系 IGKV4-1-Jk3.01 → IGKV2D

WT: DYNITQSPDGLAVSLGDSRATISCHSSQSTFEFSKNNKNOIAWYQDRFQQRNIIKKWASCRRES
 LO: **GIYVSGTPPSISLWTRPGOPASISQKSSQSSWZFSNNENILAPFLDKPFGSPDPIHISWASPEPS**

WT: GVPDRSSGSGAEEALLIGSSQTEEDVAVTYCCYFGSDPTKFSFPTVDLK
 LO: **GVPRFSGSGSOTLFLFISFVERENVVANGCCYFGSDPTKFSFPTVDLK**

AZ1.39.1.VH 种系 VH3-21+JH4

WT: EYDLVSGGSLVKFGSSIFLSZAAAGPSESLSANNVHQAFGELEHWSSLSMSSTLYVADSVK
 LO: **SYDQVERSSGFLVKFGSLNHSITWASGEPSESLSMSVYEQAPDPSGLHWSSLSMSSTLYVADSVK**

WT: RFTLEFNKNSIYLCMMISLASEDAVFIQAFSDENNDYFGVWQGLITVSS
 LO: **RTTIFEDNKNISLYECINISLASEDAVYVYFASGSDATYNTFDYKQGSTLVTVSS**

图1

构建体 ^a	上清液中的IgG (μg/ml) ^b
在LC-FR交换之后	140
在LG-FR交换之前	72

a: 所有构建体具有与G4A (具有突变成CDR3H中的DA的DG的AZ1.39.1 (WT) 重链) 相同的重链

b: 在对使用相应IgG载体转染的293F细胞进行培养7天之后来自两个Octet-96显示数量的平均值; cv%大约10%

图2

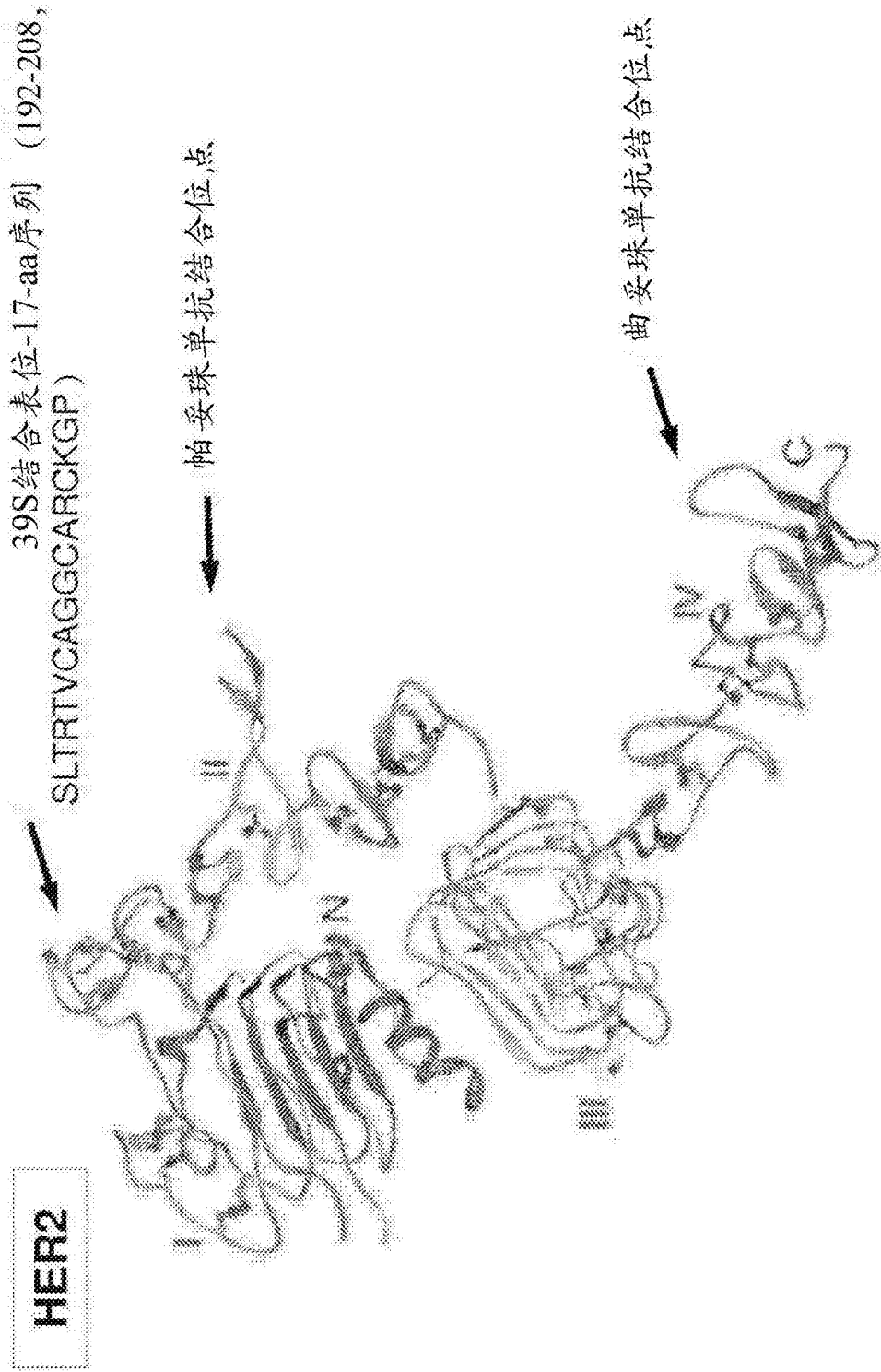


图3

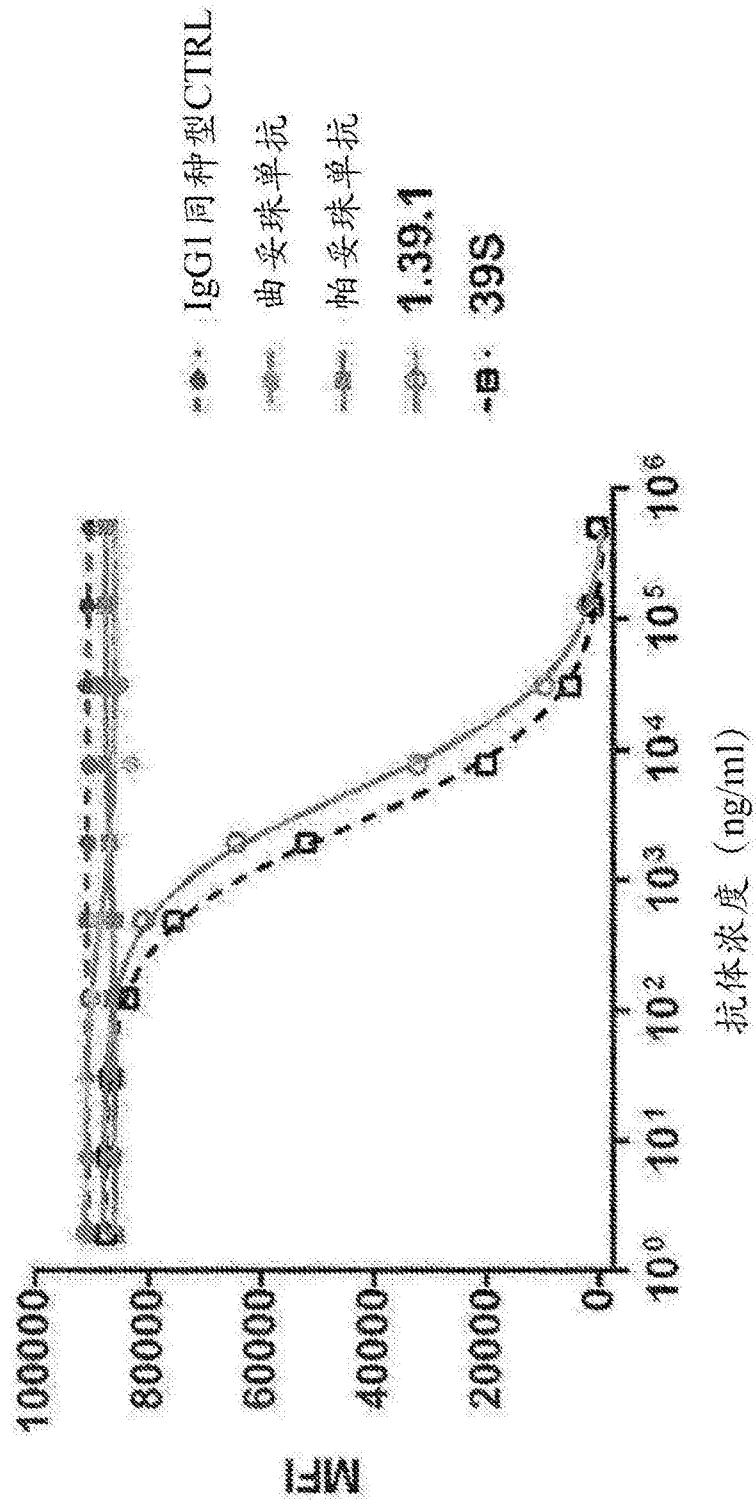


图4

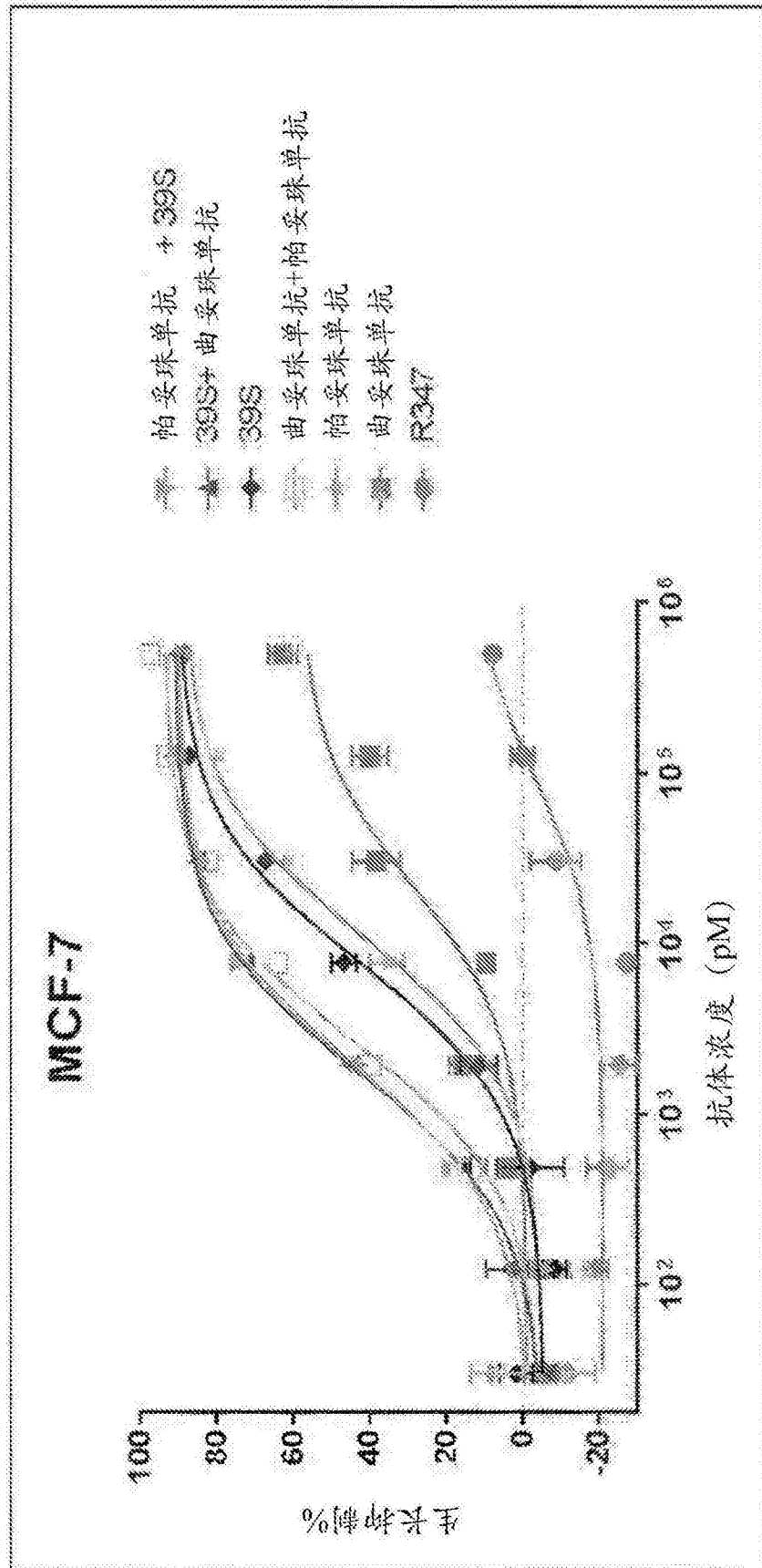


图5

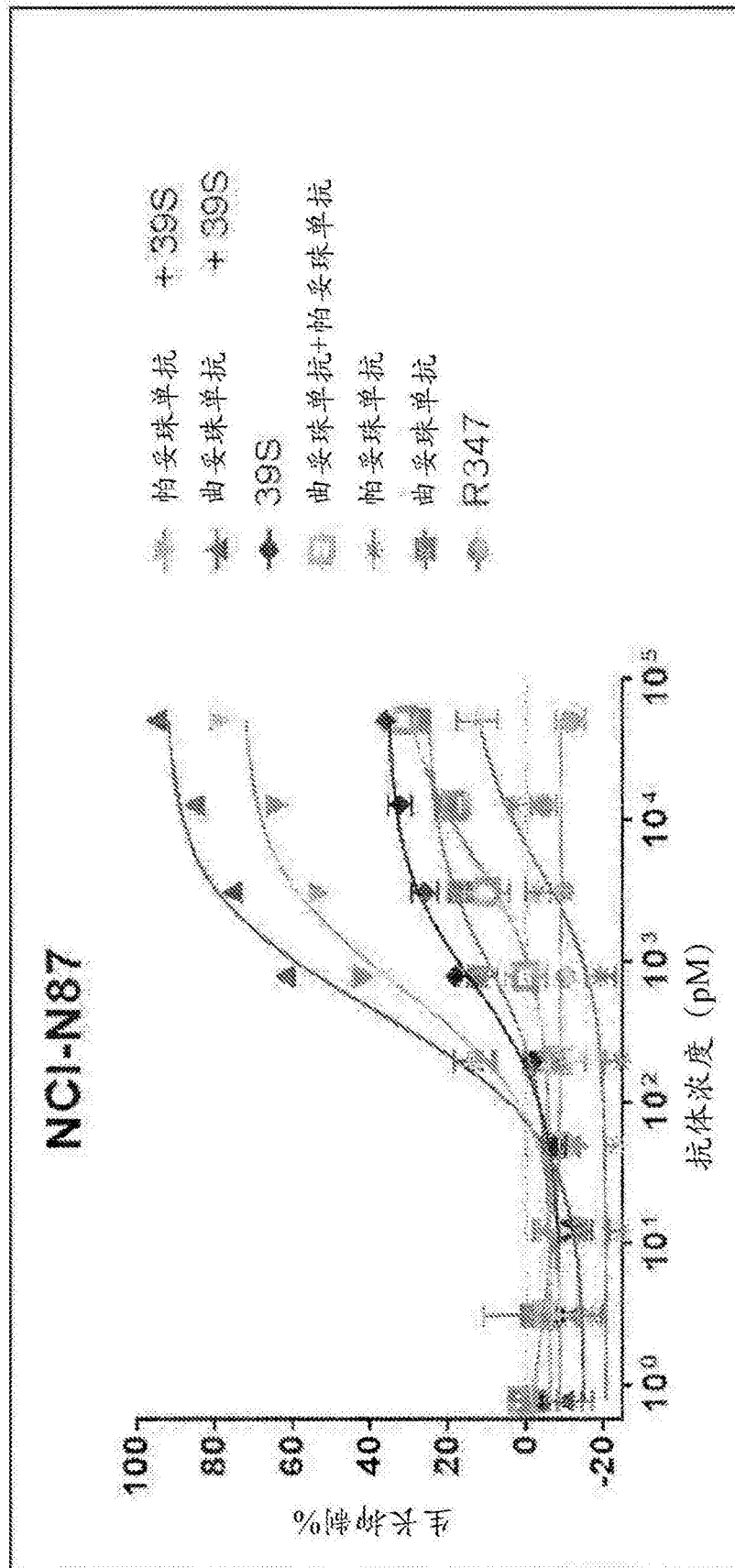


图6

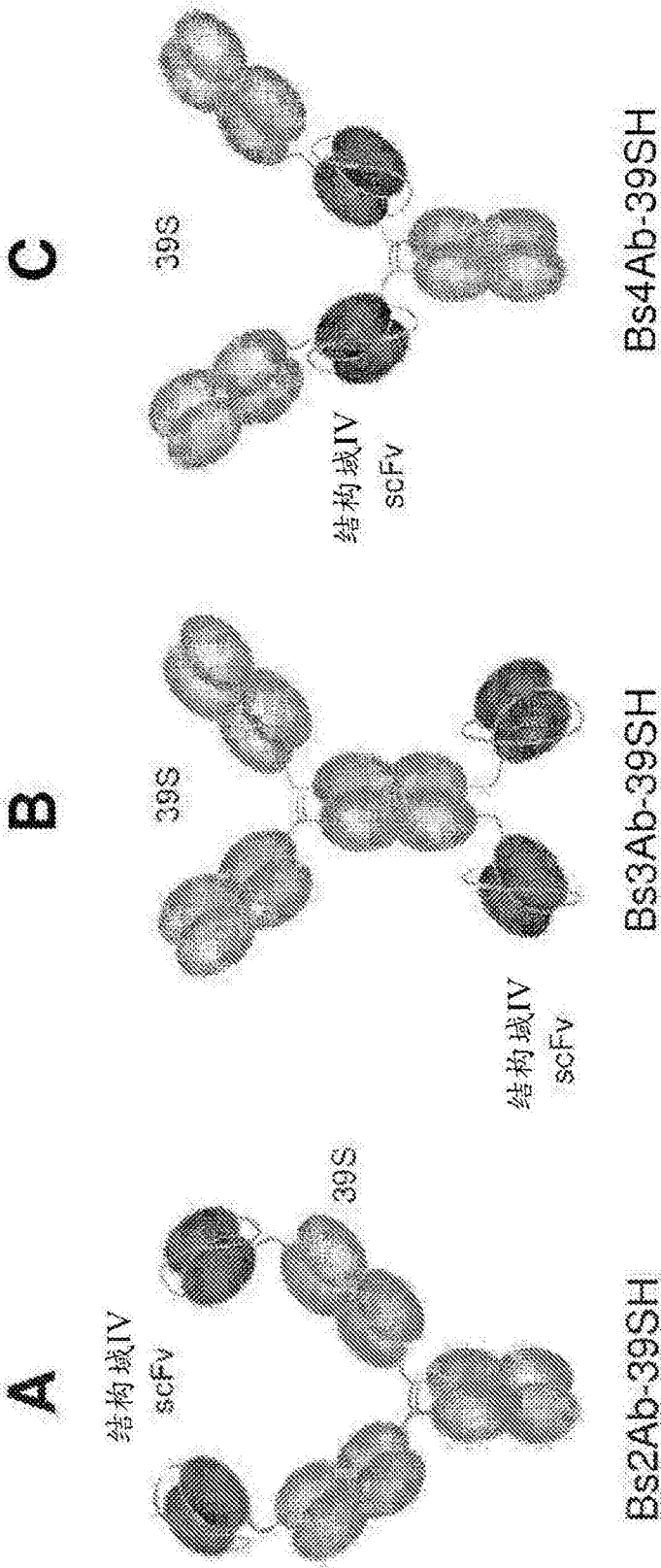


图7

[illegible]

234	X1	对于野生型是L;	对于ADCC是L;	对于ADC“F”、“FC”、“FCC”是F
239	X2	对于野生型是S;	对于ADCC是S或D;	对于ADC“FC”、“FCC”是C
239.1	*	对于野生型不存在;	对于ADCC不存在;	对于ADC 239-ins“C”是C
330	X3	对于野生型是A;	对于ADCC是L或A;	对于ADC是A
332	X4	对于野生型是I;	对于ADCC是E;	对于ADC是I
442	X5	对于野生型是S;	对于ADCC是S;	对于ADC“FCC”和“CC”是C

图 8A

EVQLVESGGGLVPGGSLRLSCAASGFTTESSYSMSNVRQAPGKGLEWVSSISSSSYIYADSVKGFETISRDNAKNSLYLQMR				
SLRAEDTAVNYCARGGDAYNYFYFDYWGQGTLVTVSSASTKGPSVEFLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT				
SGVHTFPANLQSSGLYSLSNVVTVPSSSLGIQIYICNVNHKPSNIVDKRVEPKSCDKTHICPPCFAPAE <u>X1</u> LGGP <u>X2</u> *VFLPFP				
KPRDTLMISRTTEVTGVVDVSHEDDEVKFNWYDGVGVHNAKTKPREEQYNSTYEVVSVLTVLHQDNLNGKEYKCKVSNKALP				
<u>X3</u> <u>X4</u> EKTISKAKGQPREPQVYTLPPSRREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKITTPFVLDSDGGSTFLYSKLT				
VUKSRWQQGNVFSCSVMHEALHNHYTQKSL <u>X5</u> LSFGKGGGSGGSGGSDIQMTQSPSSLSASVGRVTITCRASQDVNTA				
VANYQQKPKAKPKLLIYSASELYSGVPSRFSGSRSGIDEFLIISLQPEDEFAIYYCQOHYITPFTGCCGKVEIKGGGSGGGG				
SGGGSGGGSGSEVQLVESGGGLVQPGGSLRLSCAASGCFNIKDTYIHNVROAPGKCLEWVARIYPTNGYTRYADSVKGRFTISAD				
TSKNTAYLQNNSLRAEDTAVNYCSEWGGDGEYAMDYWGQGTILVTVSS				
EU#				
234	X1 =	对于野生型是L;	对于ADCC是L;	对于ADC“F”、“FC”、“FCC”是F
239	X2 =	对于野生型是S;	对于ADCC是S或D;	对于ADC“FC”、“FCC”是C
239i	*	对于野生型不存在;	对于ADCC不存在;	对于ADC 239-ins“C”是C
330	X3 =	对于野生型不存在;	对于ADCC是L或A;	对于ADC是A
332	X4 =	对于野生型是A;	对于ADCC是L或A;	对于ADC是I
442	X5 =	对于野生型是I;	对于ADCC是E;	对于ADC“FCC”和“CC”是C
		对于野生型是S;	对于ADCC是S;	

图8B

EVQVLVESGGGLVKPGGSLRLSCAASGFTESYSSMSNVRQAPGKGLEWVSSLSSSSSYLYYADSVKGRFTISRDNAKNSLYLQMR
SLRAEDTAVNYCARGDAYNYFYFDYWGQGTLLVTVSSASTKGPSVEPLAPSSKSTSCGTAAAGCLVKDYFPEPVTVSWNSGALT
SGVHTFPAYLQSSGLYSLSVTVPSSSLGIQIYICNVNHRKPSNIKVDRKVEPRSCCKTGGGSGGGGSDIQMTQSPSSLSASV
GDRVYITCRASQDVNIATAVANYQQPKGKAPKLLIYSASELYSGVPSPRFGSSPSGTDFTLTISLQPEDFAITYYCCQHYTIPDFG
CGTKVEIKGGSGGGCGGGGSEVOLVESGGELVQPGGSLRLSCAASGFTFNIDYIHWVRQAPGKCLEWVARITYPING
YIYRYADSVKGRFTISADTSKNIAYLQMNSLRAEDTAVNYCSRWGDDGEYAMDYWGQGTLLVTVSSSGGGSGGGGSCPPAPAEF**X**
1LGSP**x2***VELFPFKPKDILMISRIPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTRKREEQYNSTYRVVSVLTVLHQDWLNL
GKEYKCKVSNKALP**x3****x4**ENTISKARGQPREPQVYTLPPSRHEMTKNQVSLTCLVKGYTSPDIAVEWESNGQPENNYKTTIPVY
LQSDGSGSTFYSKLTVERSGRWQQGNVFTCSVMHEALHNHYTQCKSL**x5**LSFGK

2234	X1	对于野生型是L;	对于ADCC是L;	对于ADC“F”、“FC”、“FCC”是F
2239	X2	对于野生型是S;	对于ADCC是S或D;	对于ADC“FC”、“FCC”是C
2391	*	对于野生型不存在;	对于ADCC不存在;	对于ADC 239-ins“C”是C
330	X3	对于野生型是A;	对于ADCC是L或A;	对于ADC是A
332	X4	对于野生型是I;	对于ADCC是E;	对于ADC是I
442	X5	对于野生型是S;	对于ADCC是S;	对于ADC“FCC”和“CC”是C

图 8C

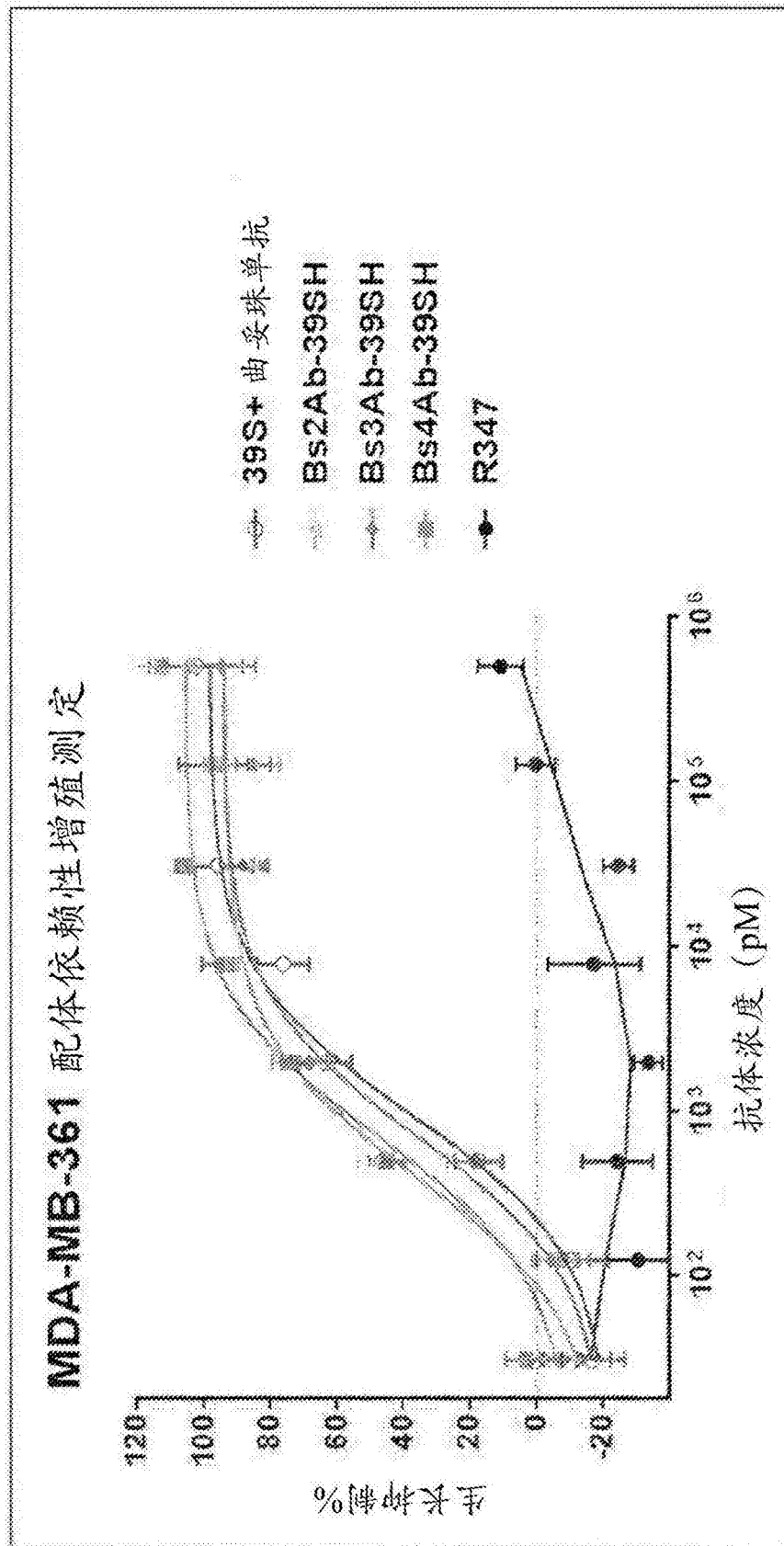


图9A

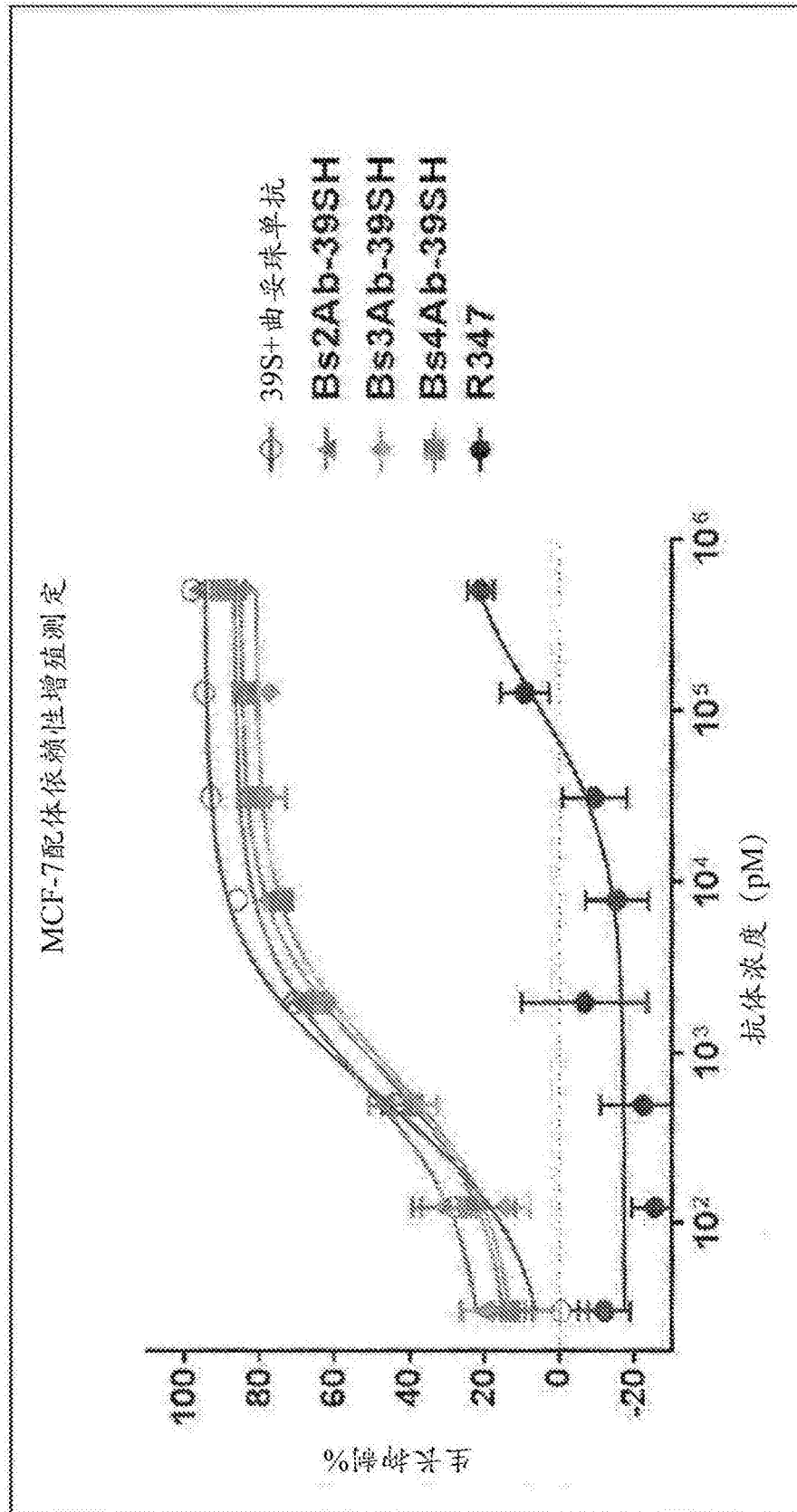


图9B

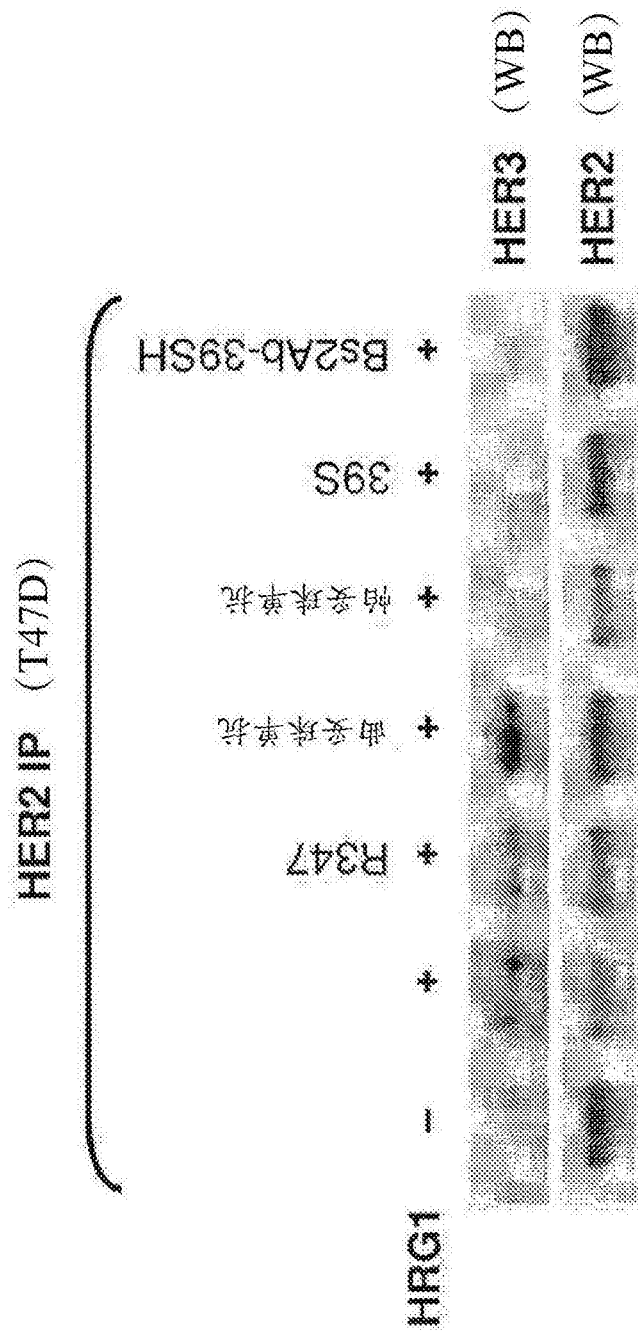


图10

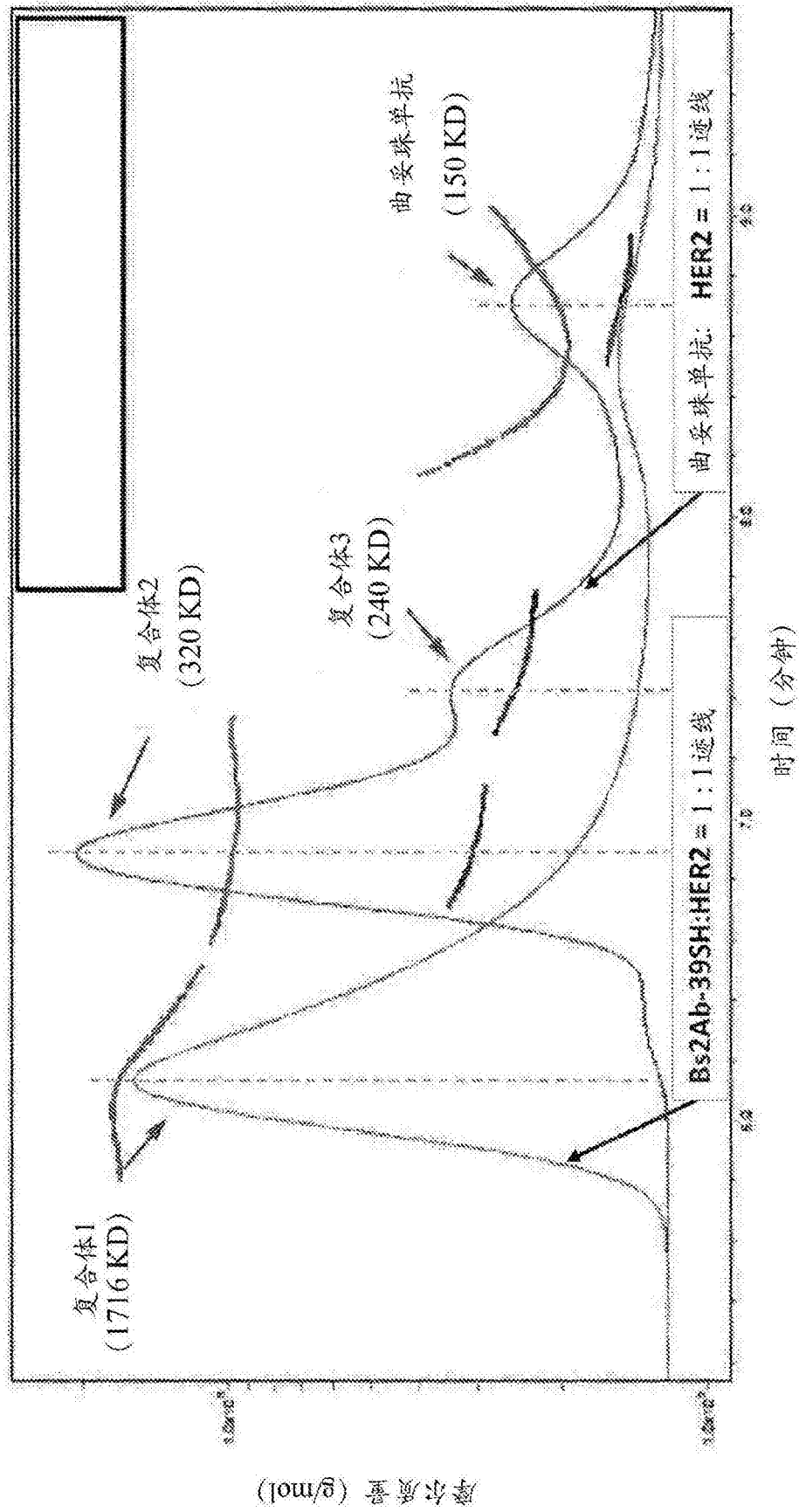


图11

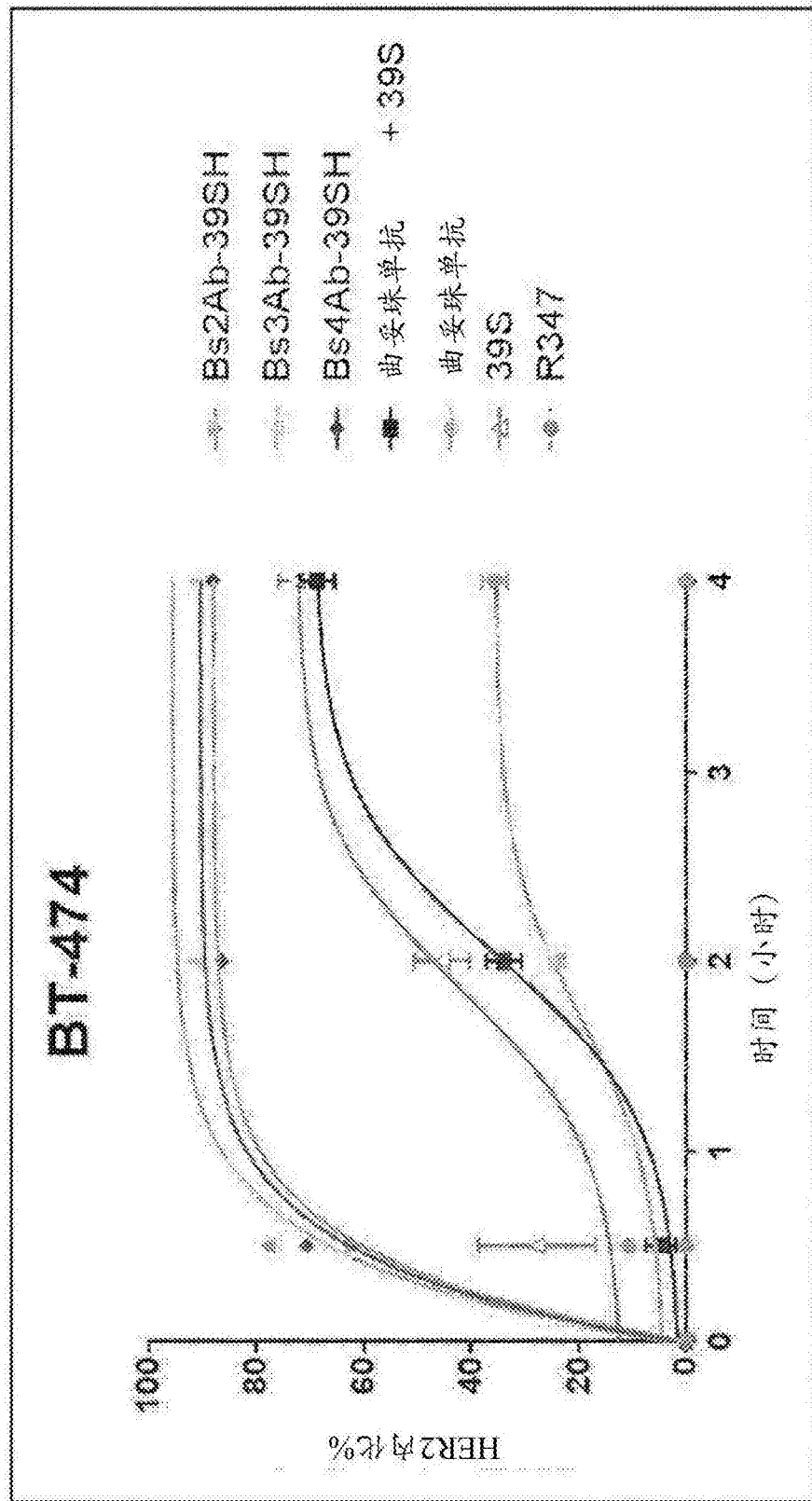


图12

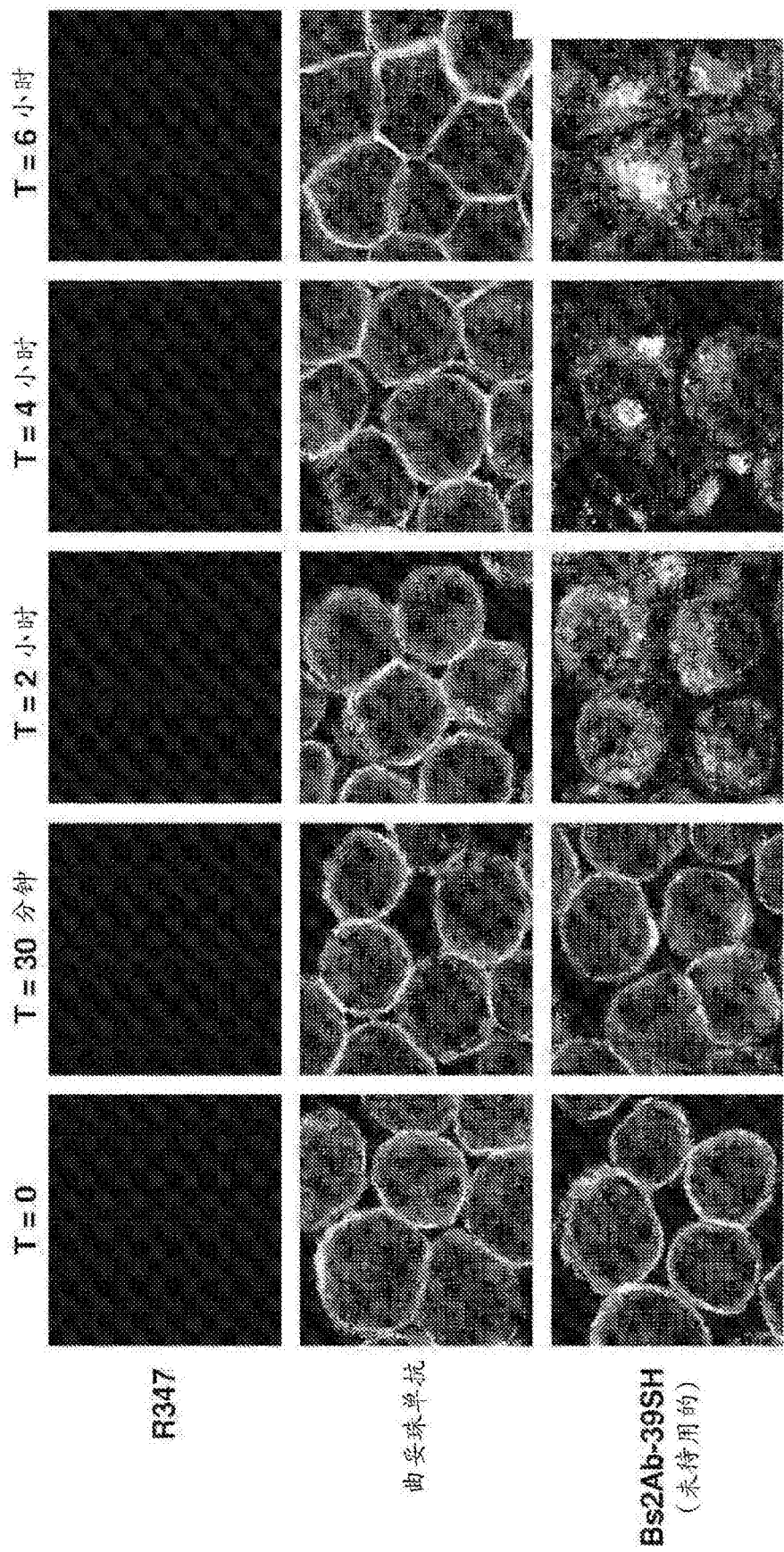


图13

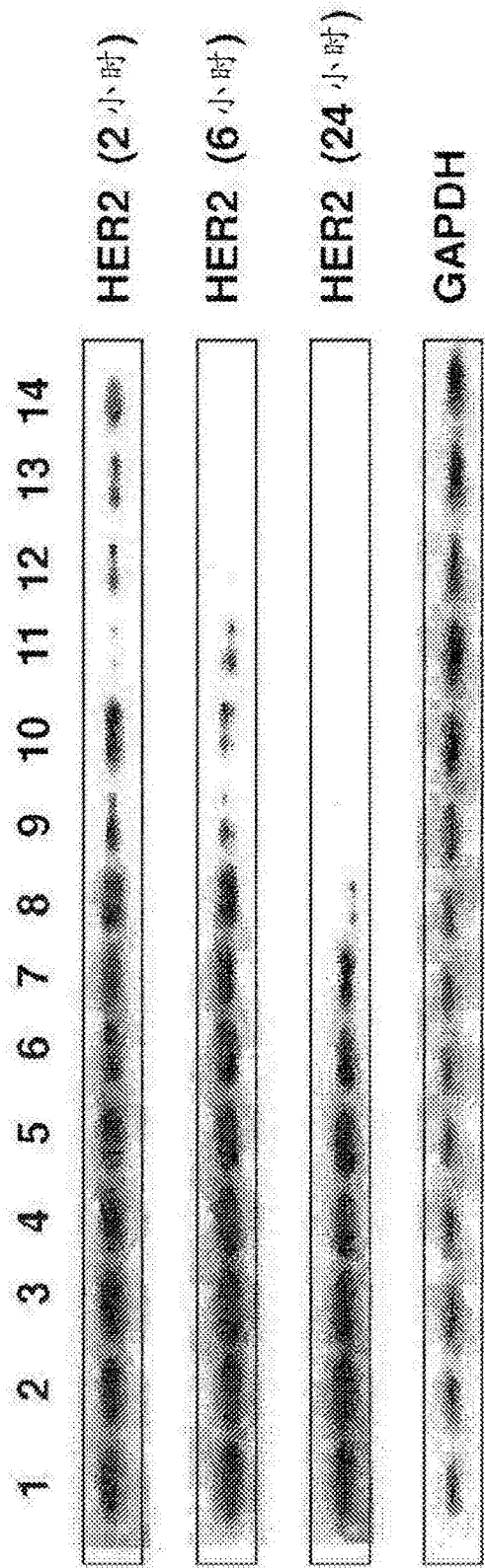


图14

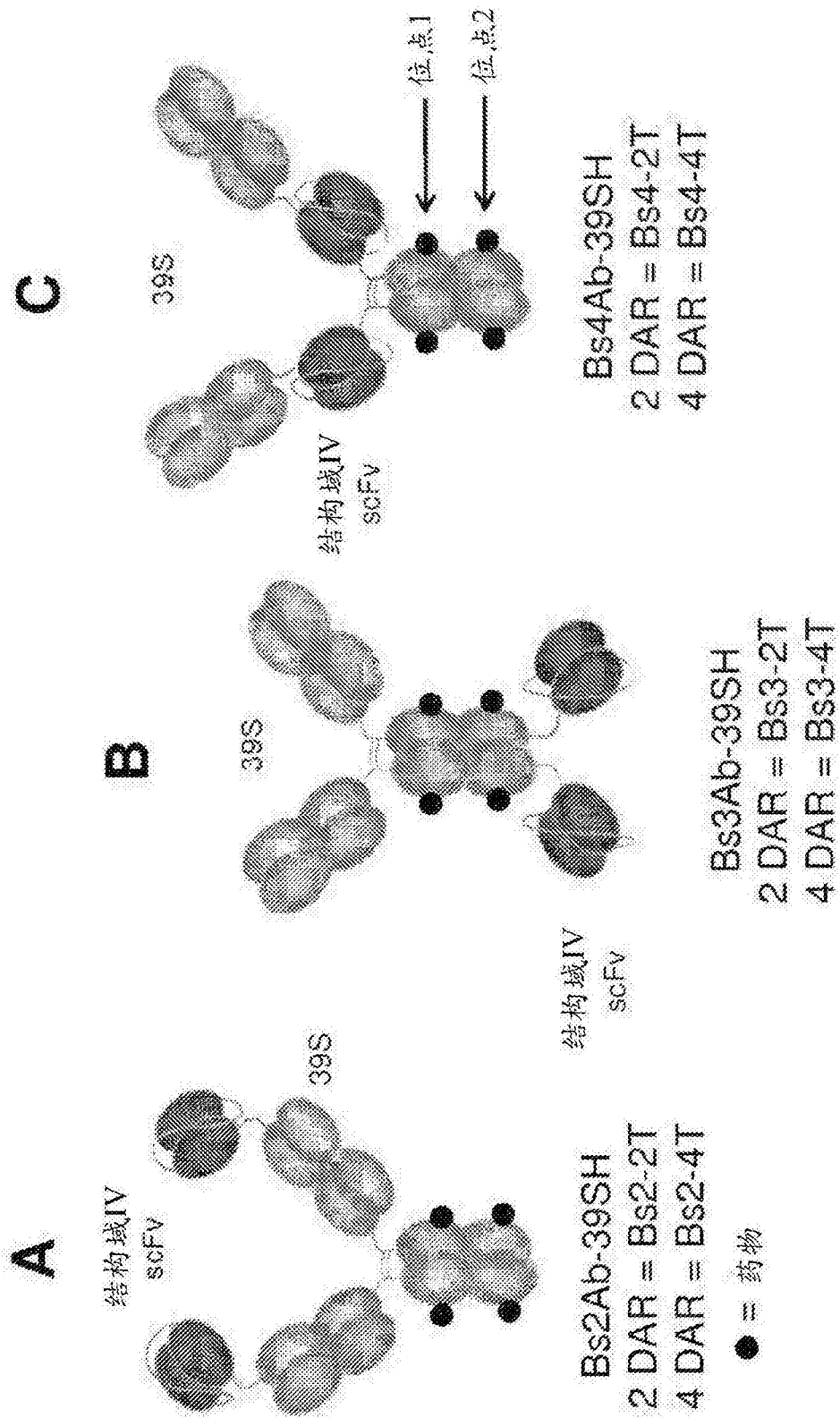


图15

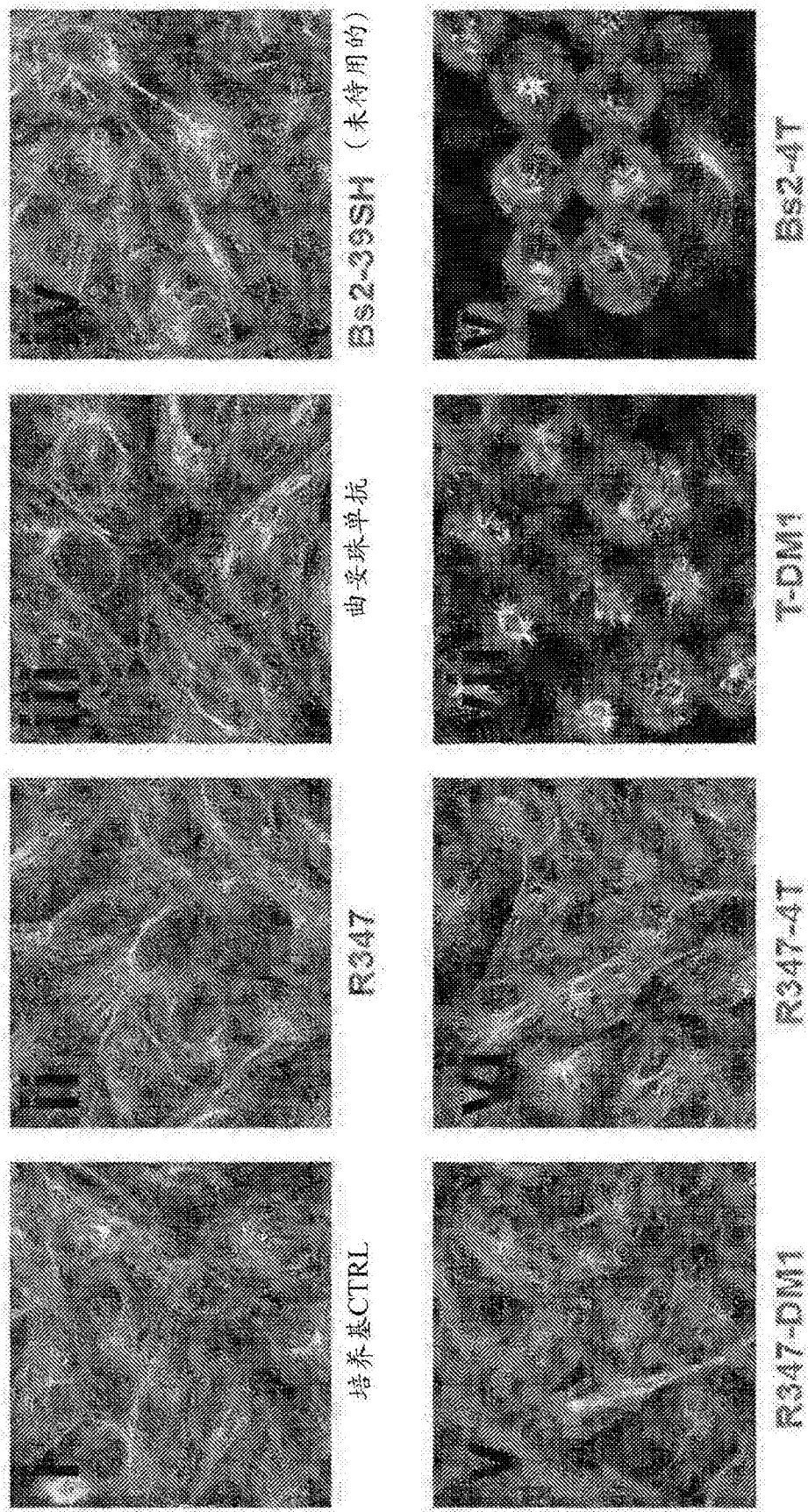


图16A

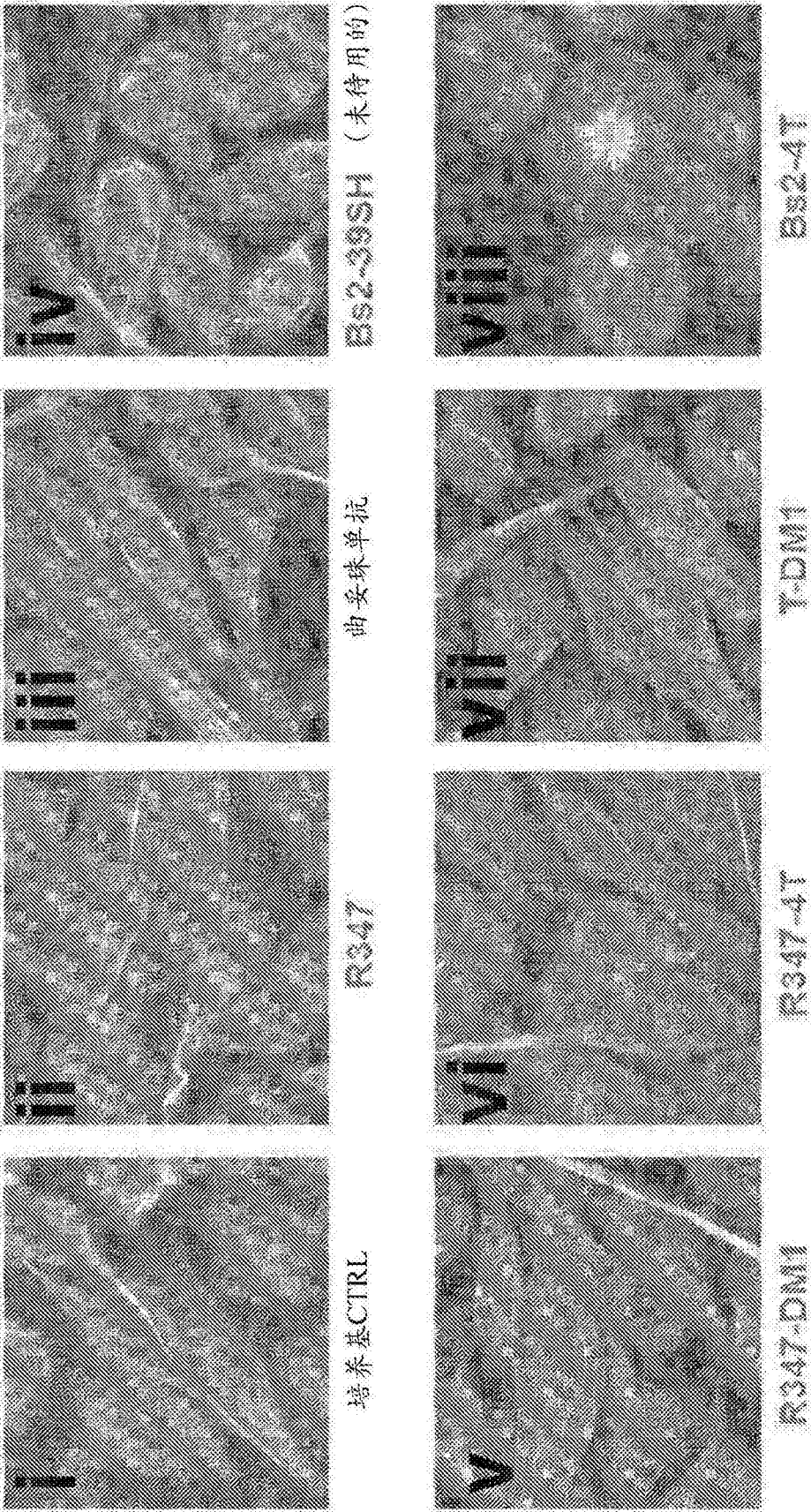


图16B

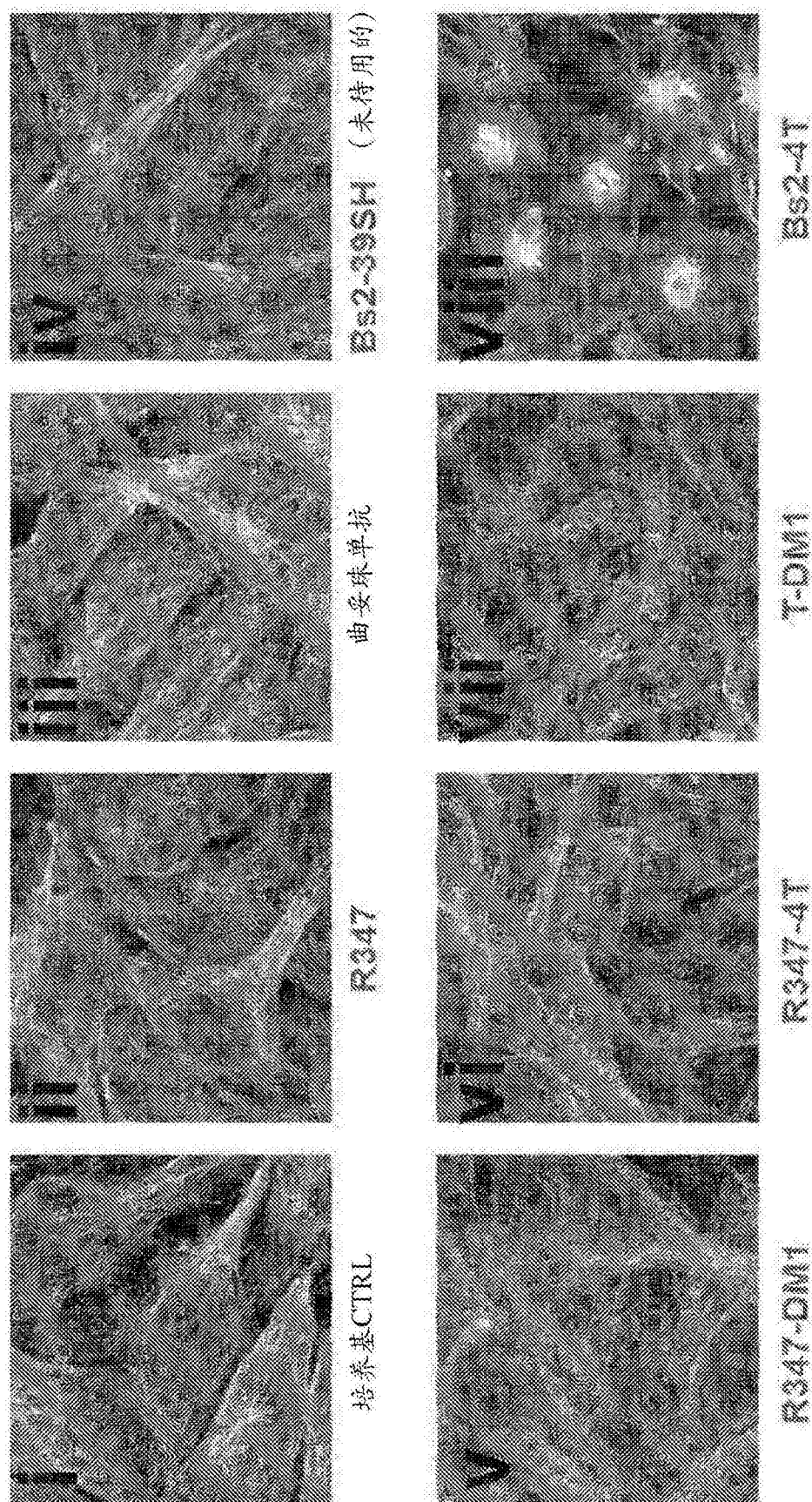


图16C

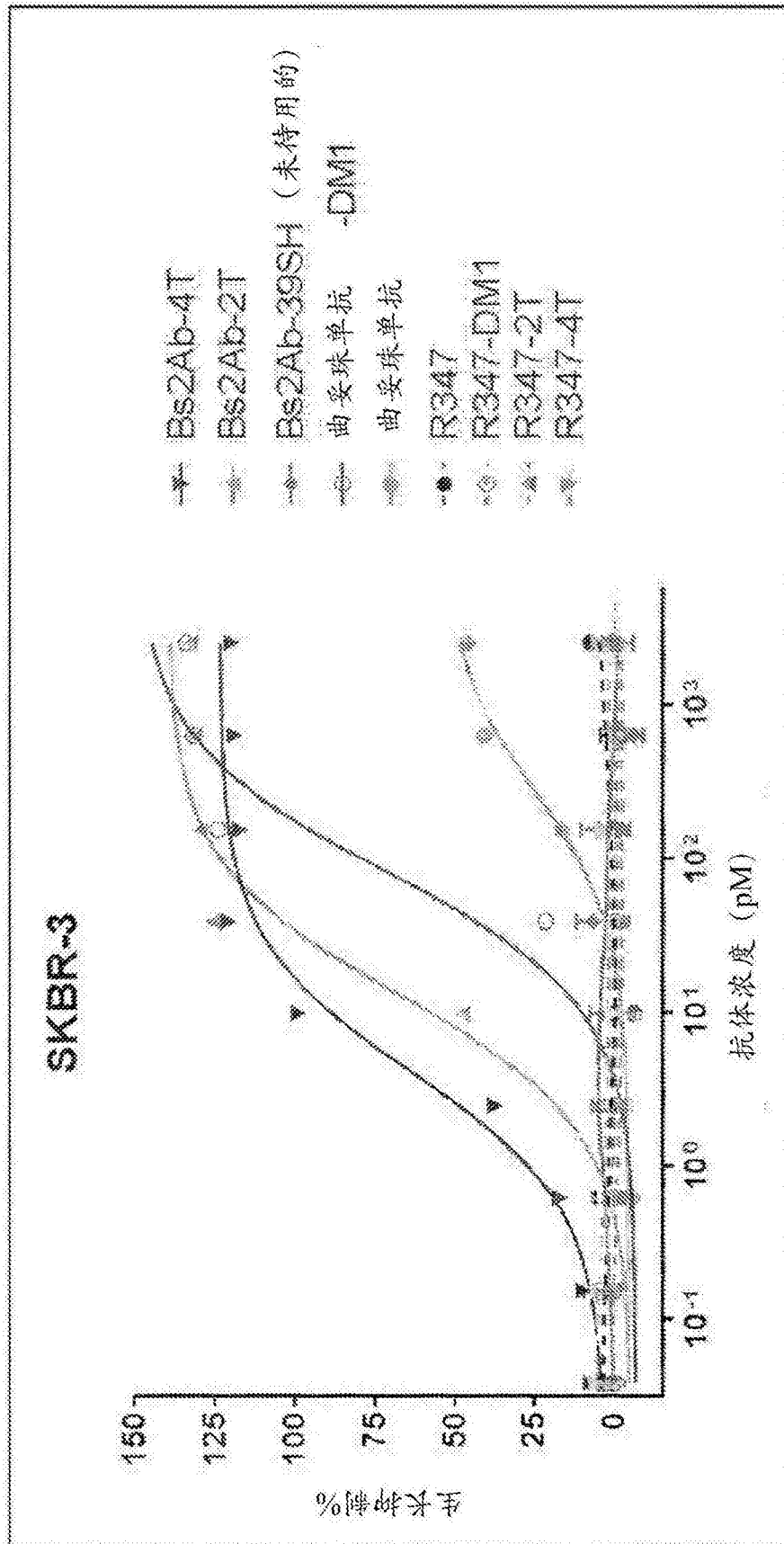


图17A

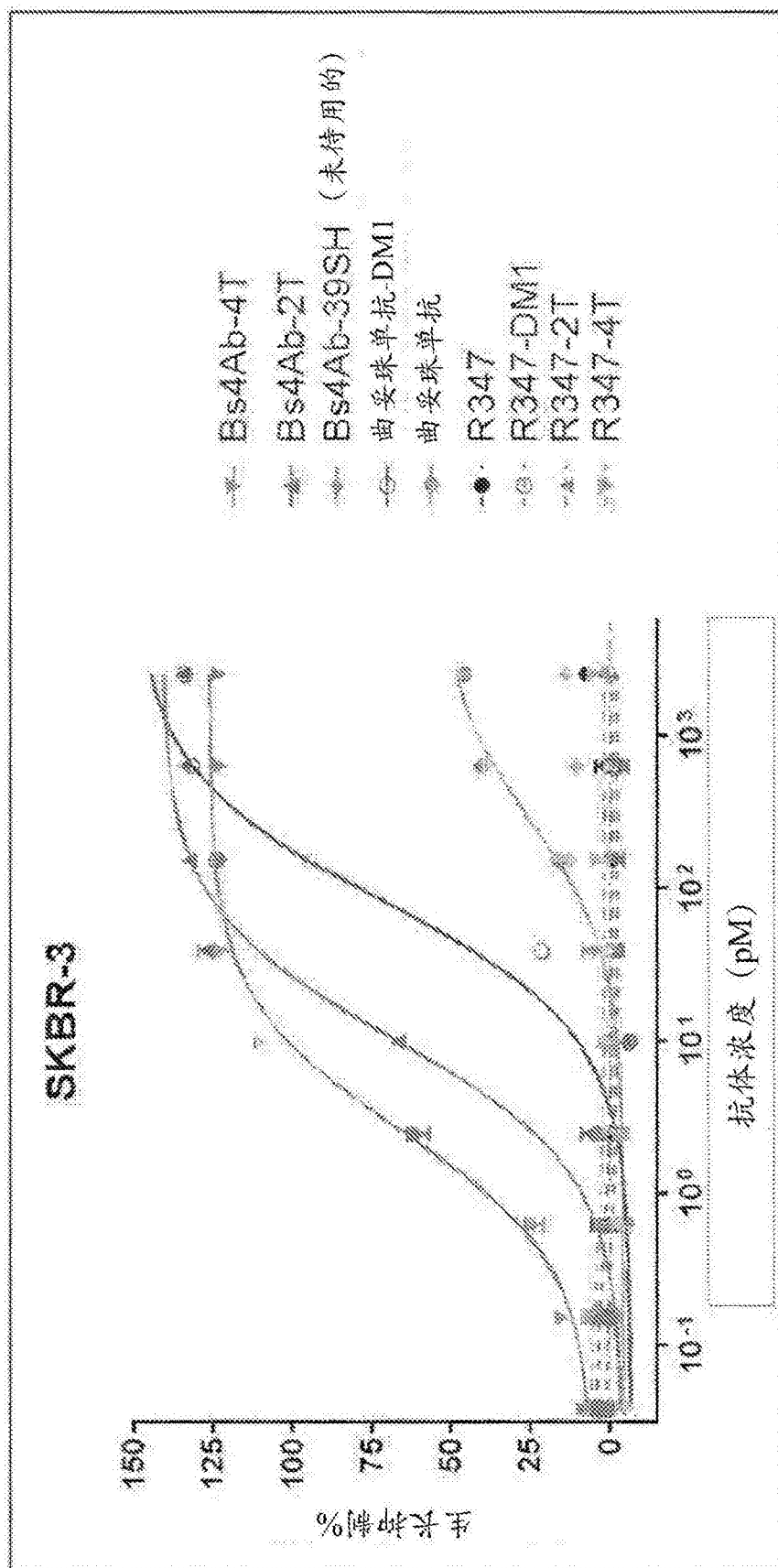


图17B

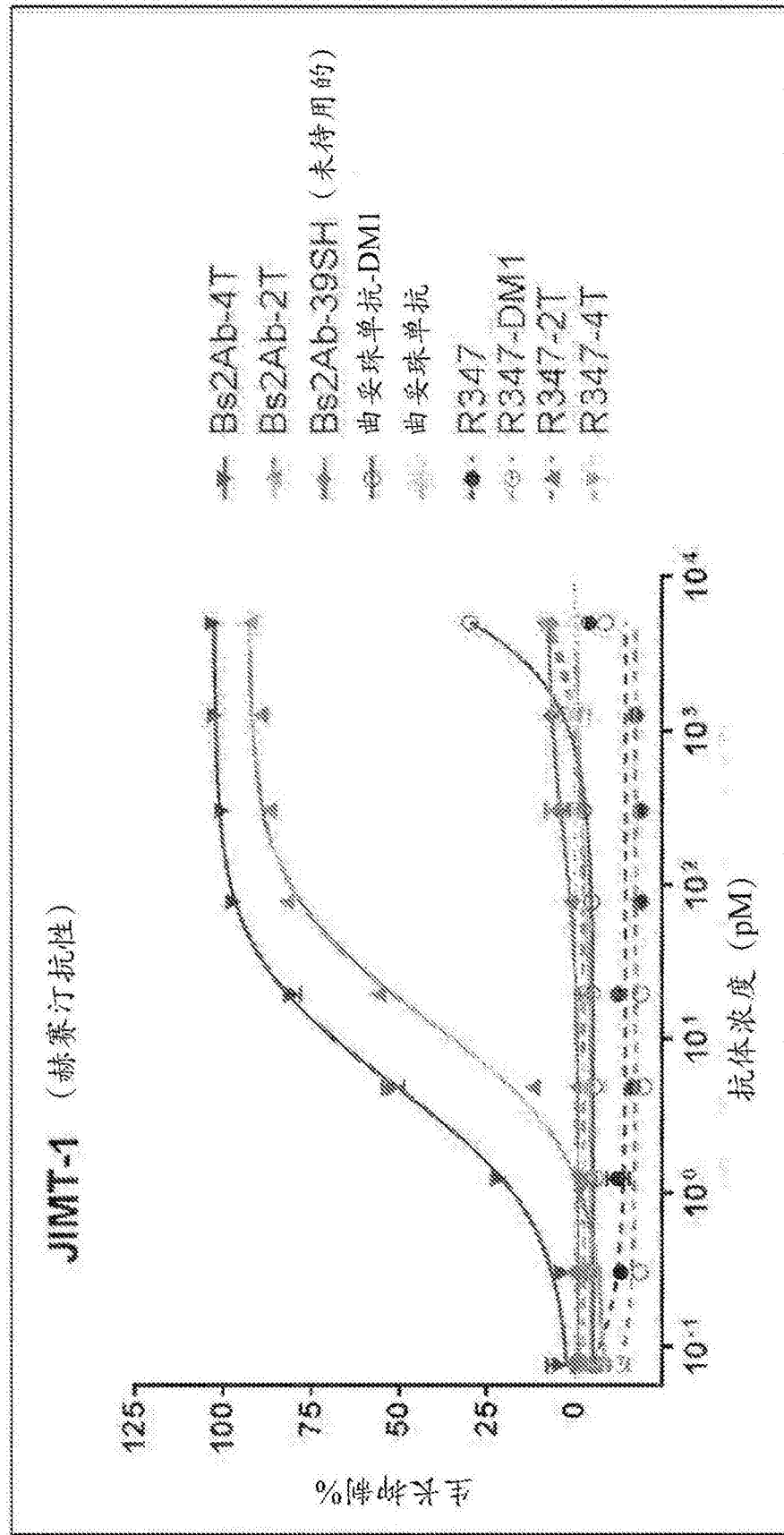


图18A

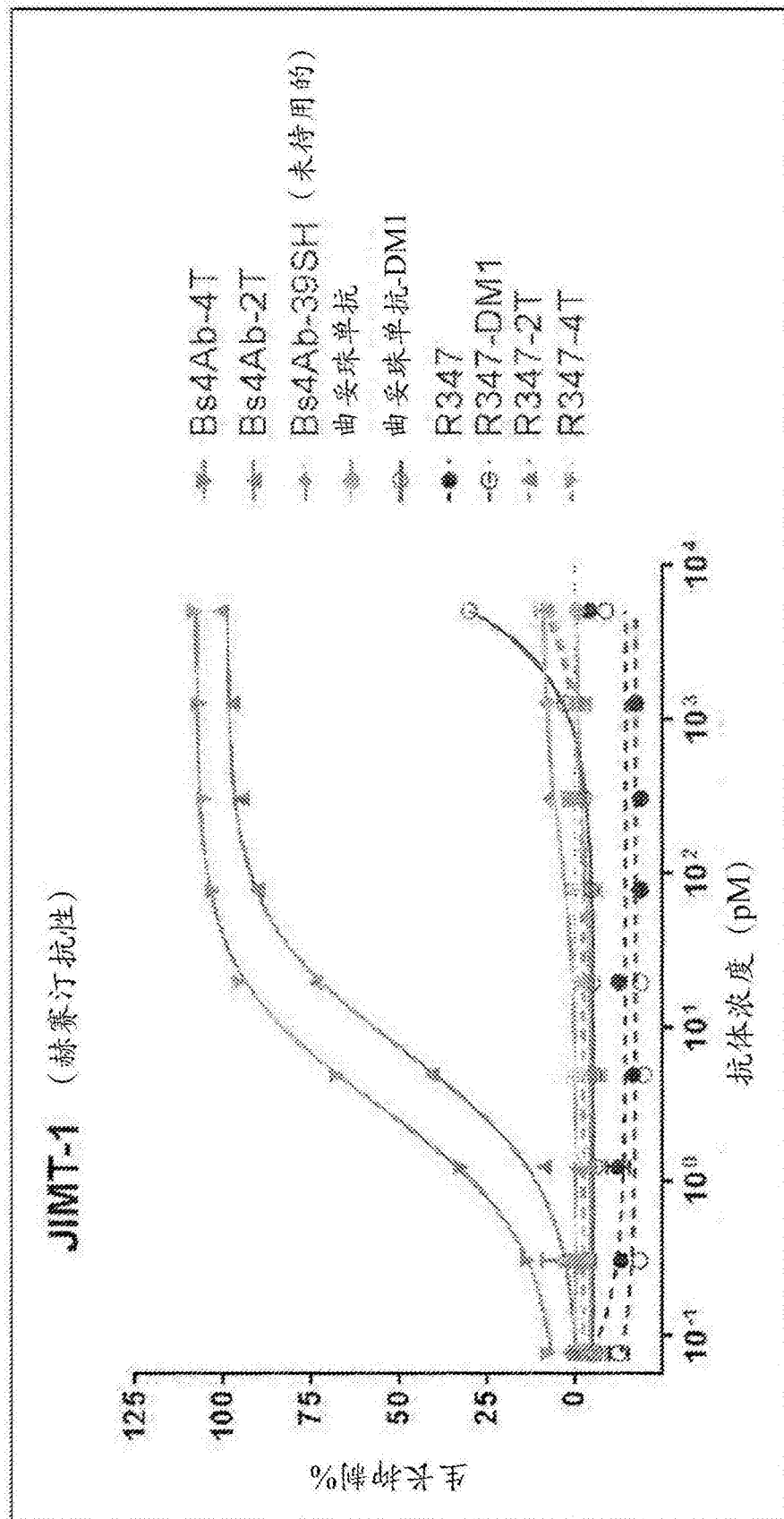


图18B

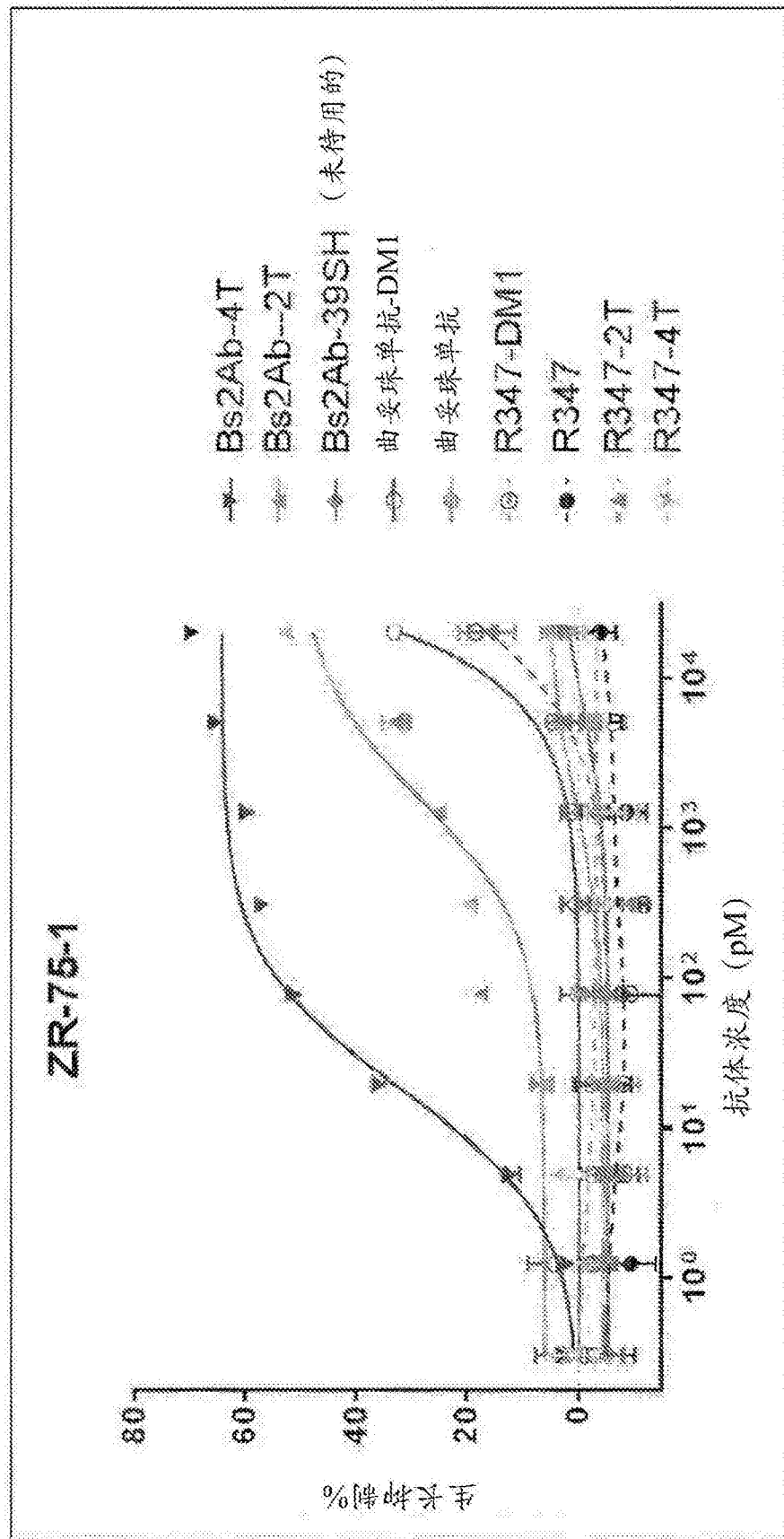


图19A

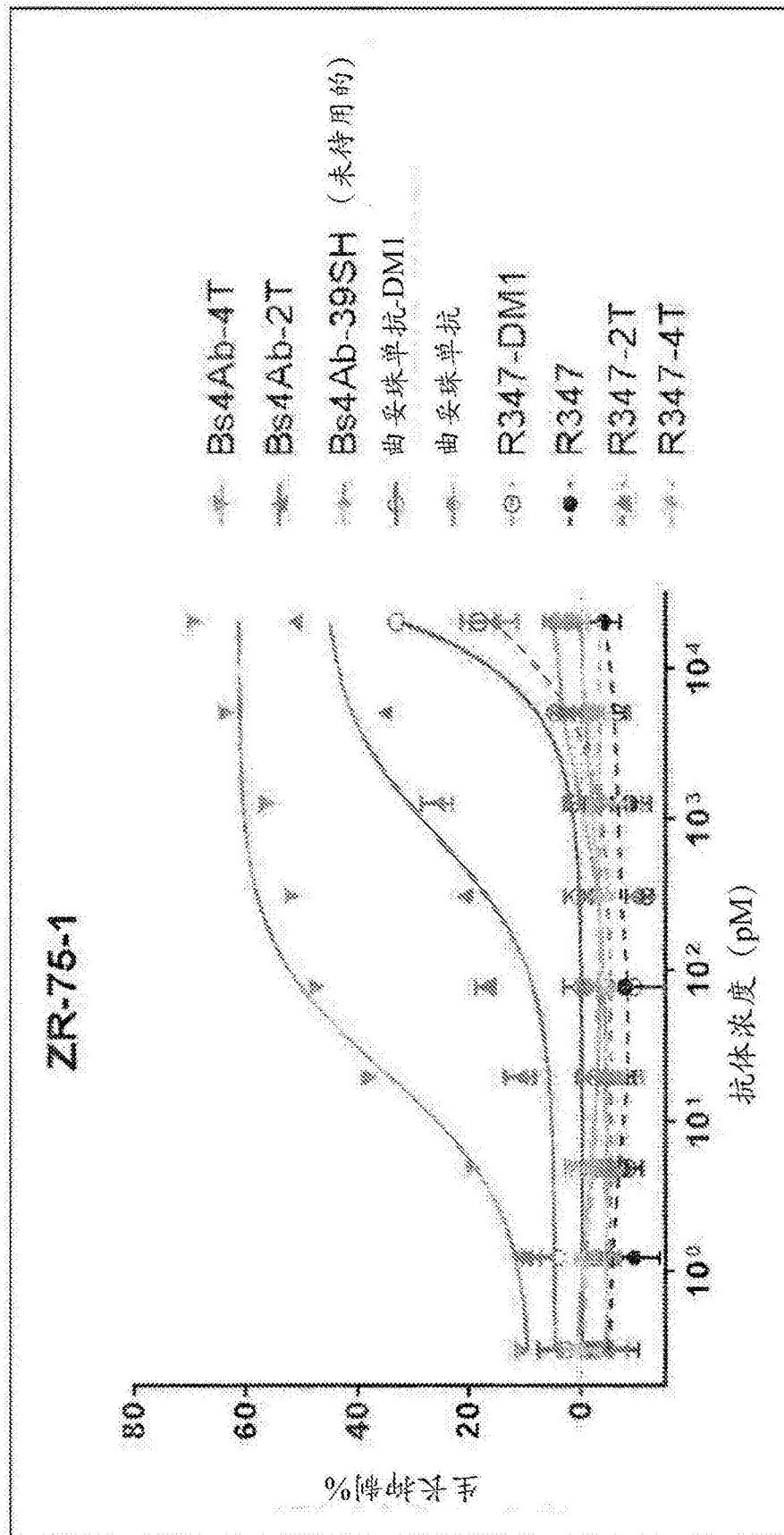


图19B

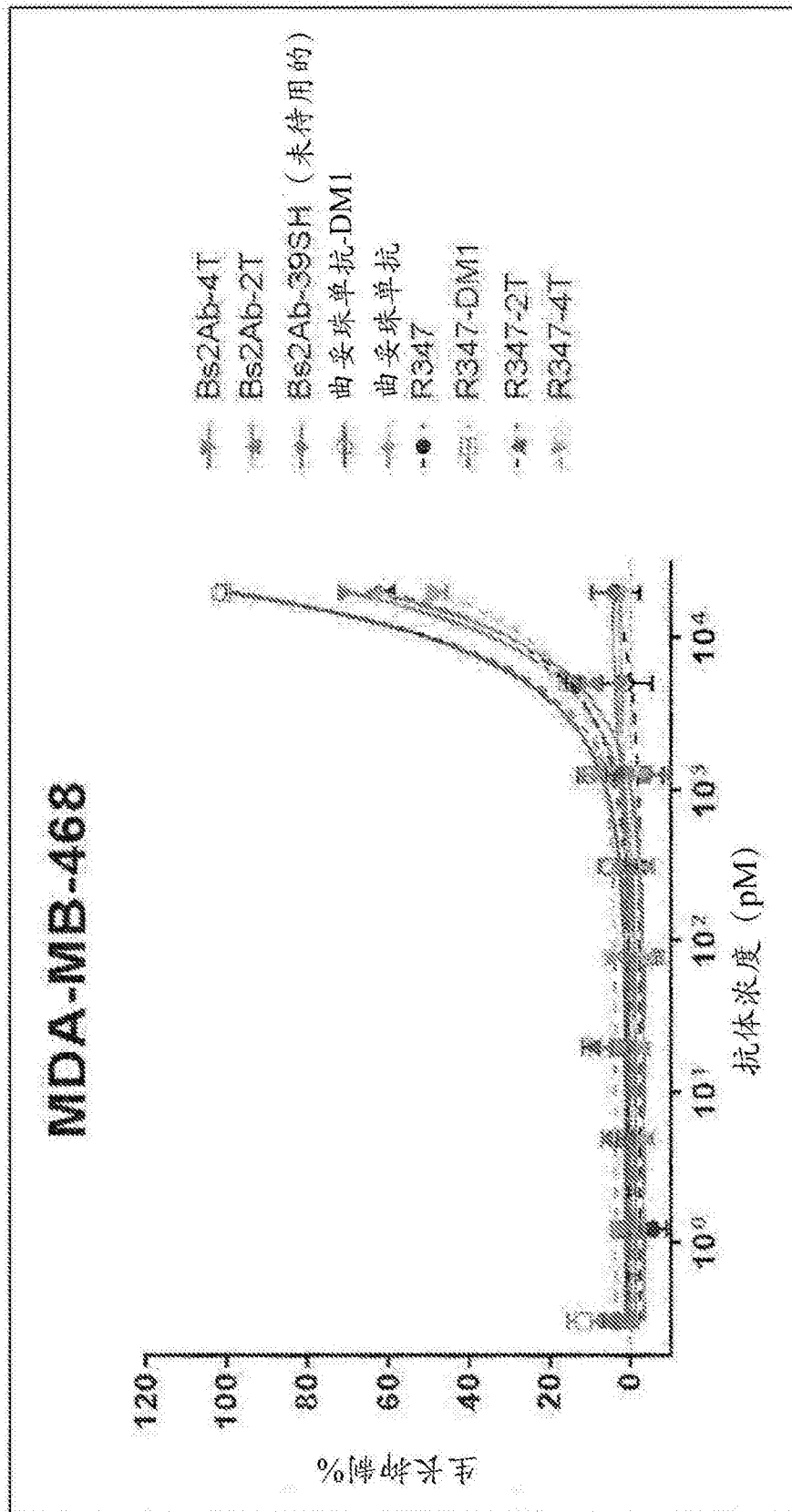


图20

MBA-MB-361 (使用雌激素处理的sc系列植入物)

肿瘤生长曲线

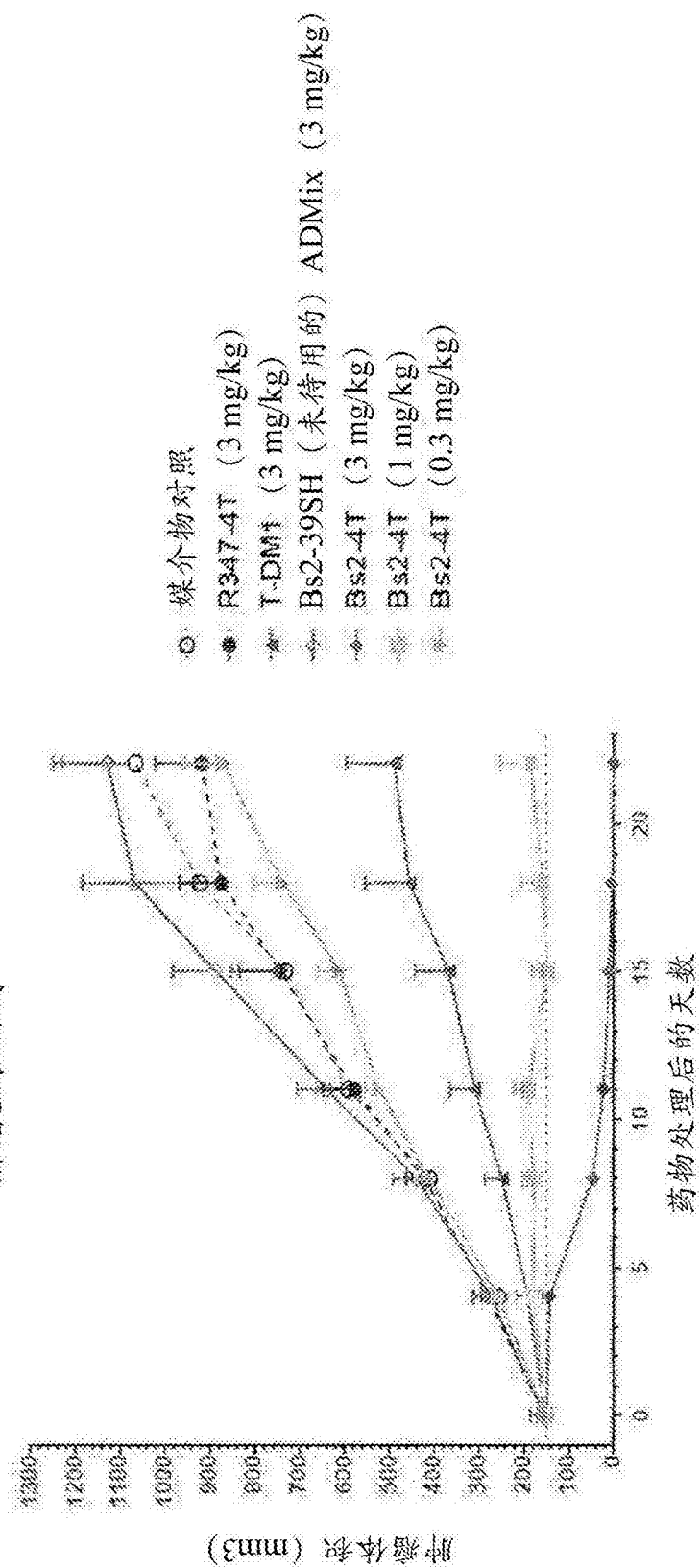


图21A

MBA-MB-361 (使用雌激素处理的sc系列植入物)

肿瘤生长曲线

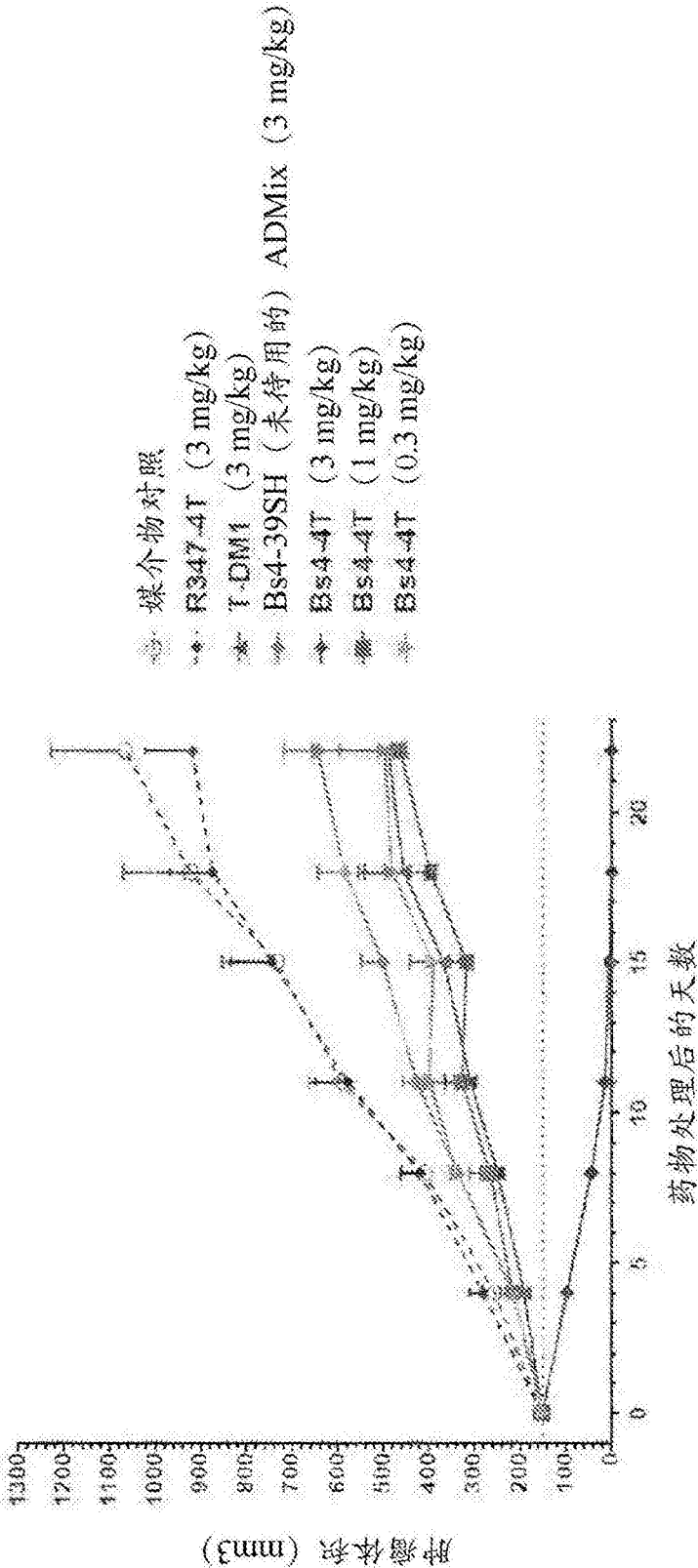


图21B

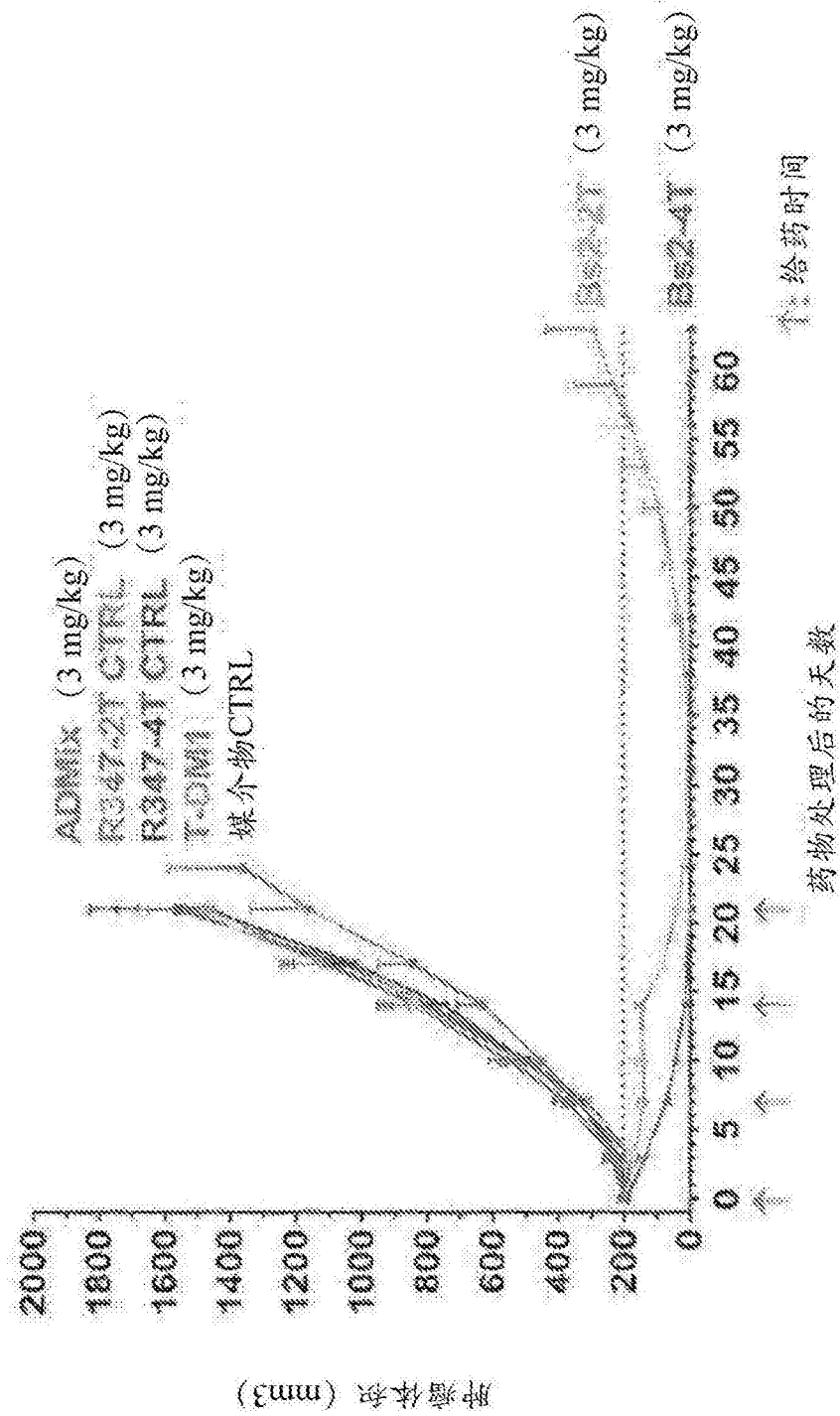
ST996 (HER2⁺/ER-/PR-) 肿瘤生长曲线

图22

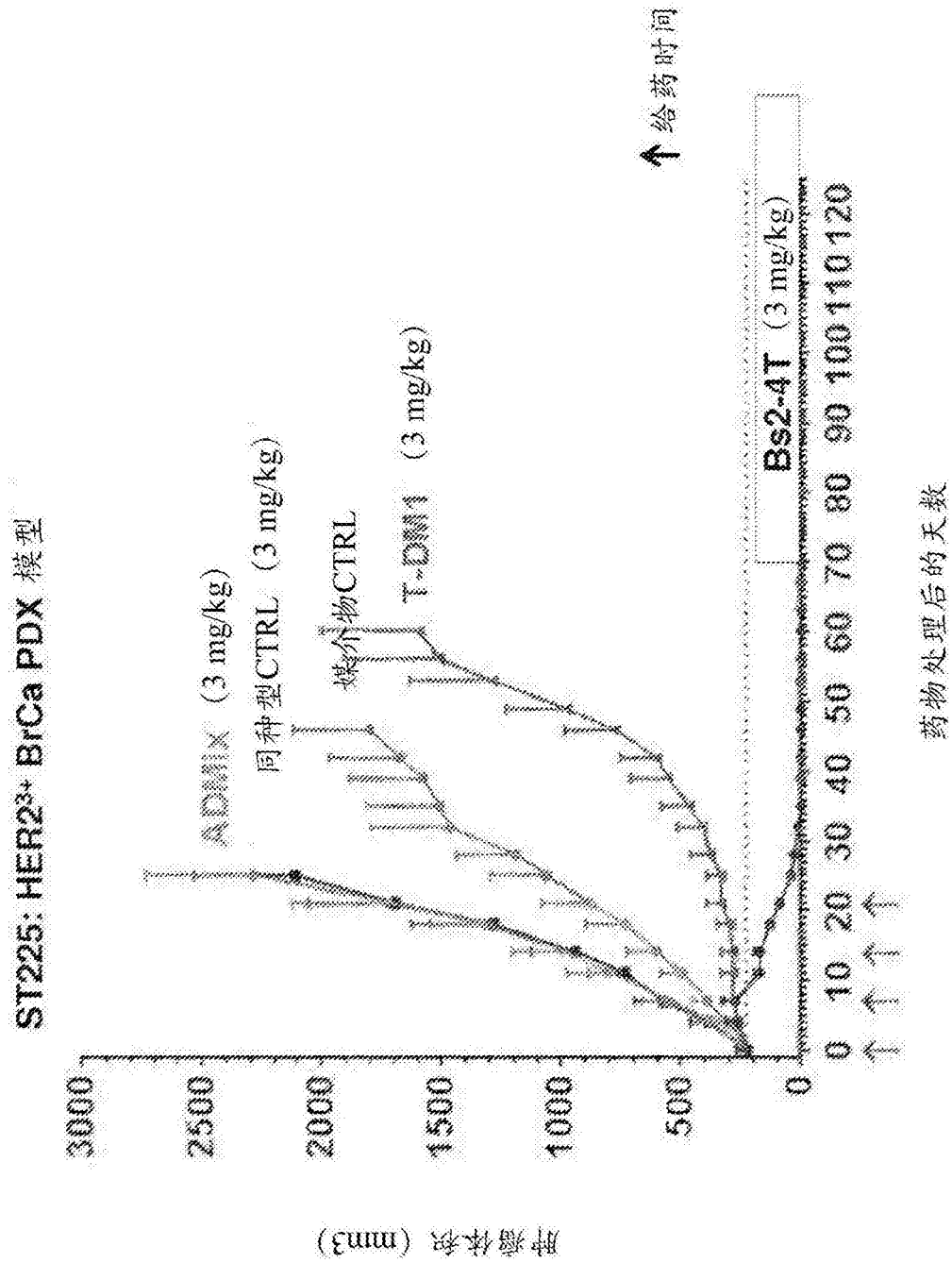


图23

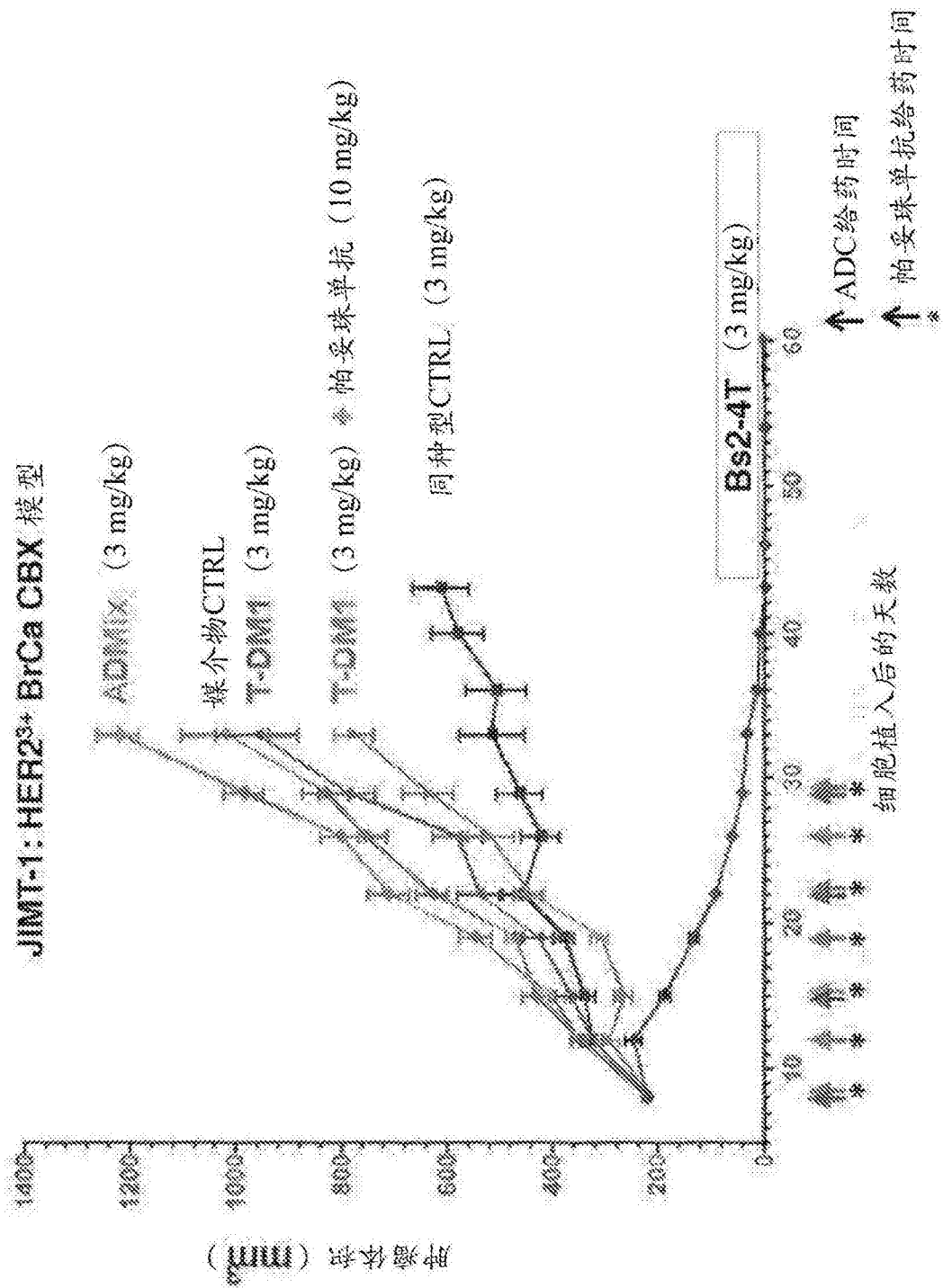


图24

ST455B:三阴性 (ER/PR/HER2⁺) BrCa PDX模型

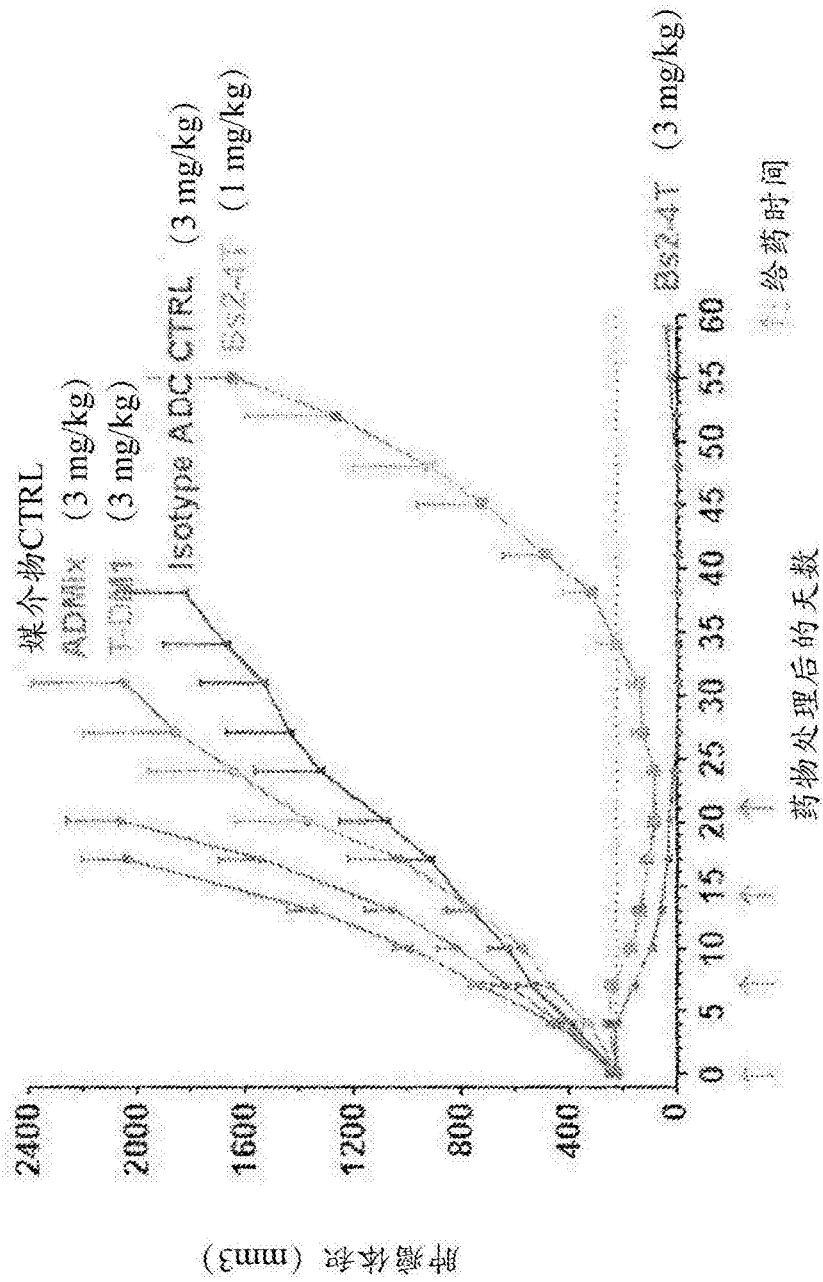


图25

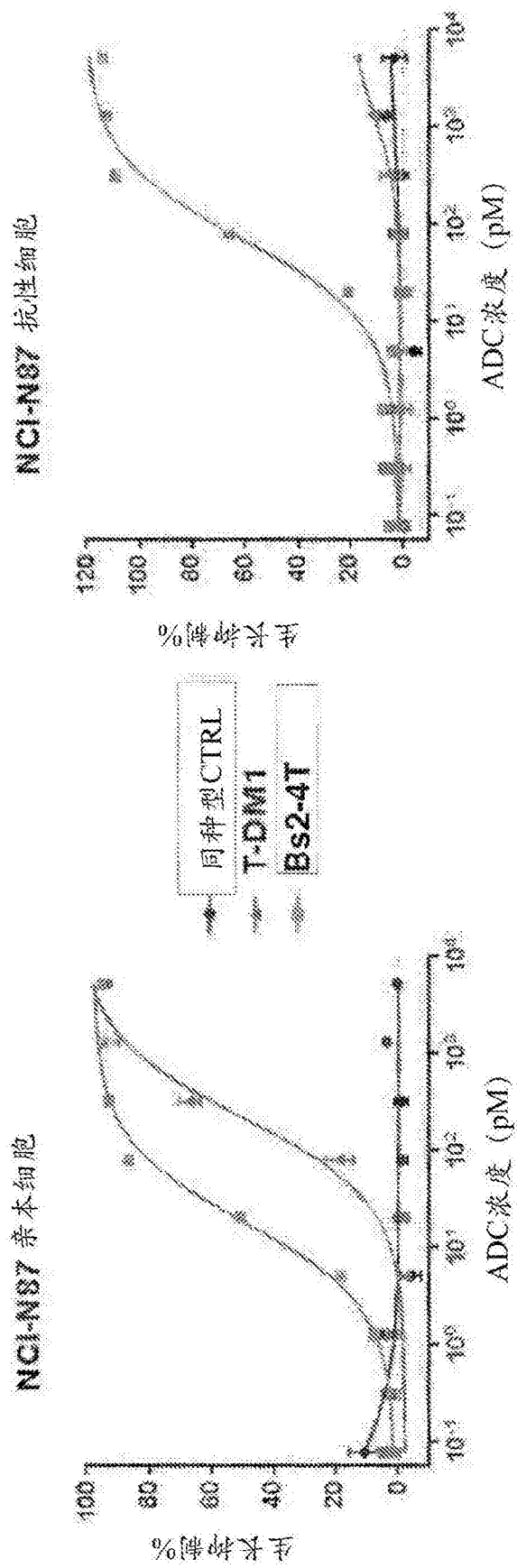


图26A

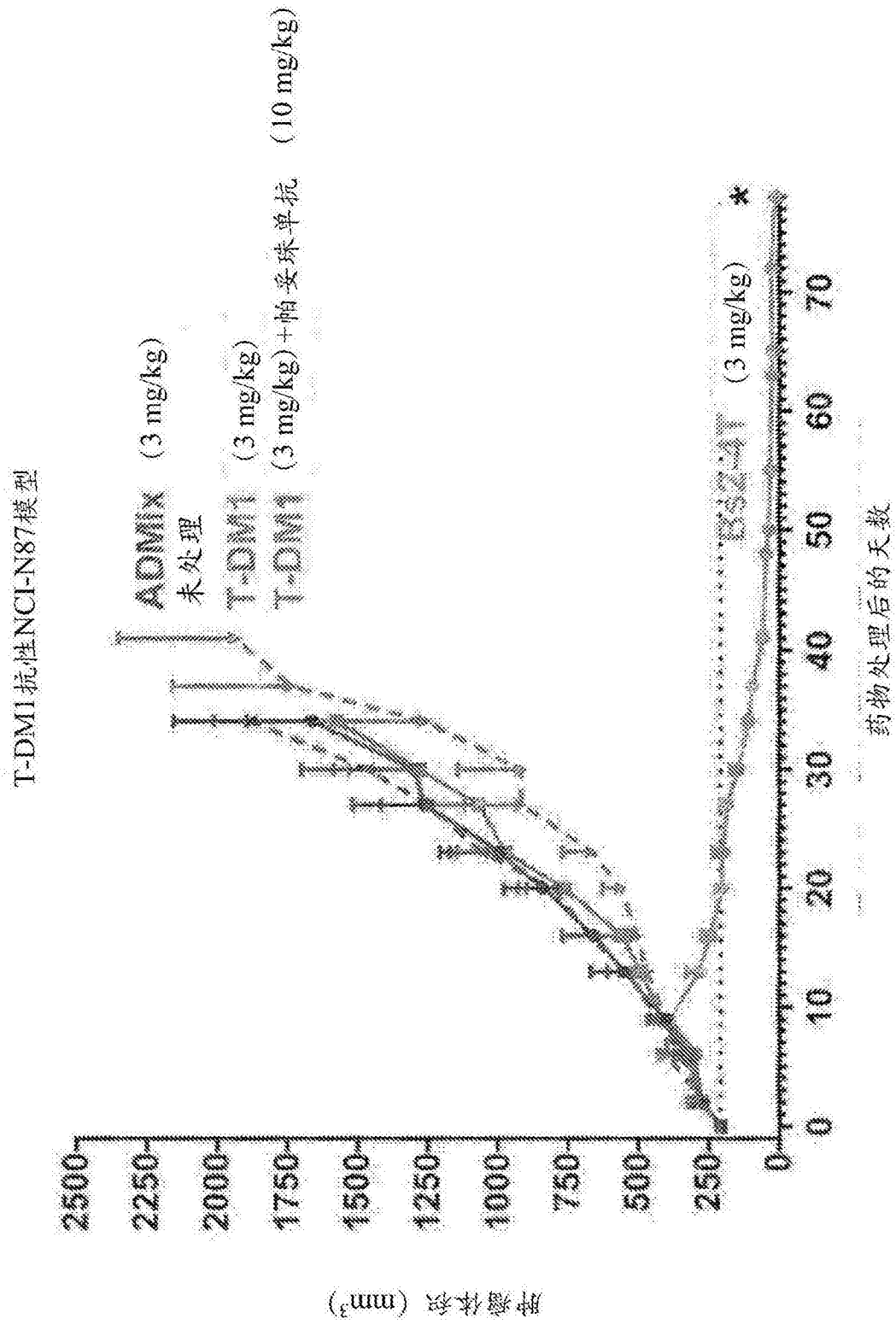


图26B

ST225 (HER2³⁺) PDX模型

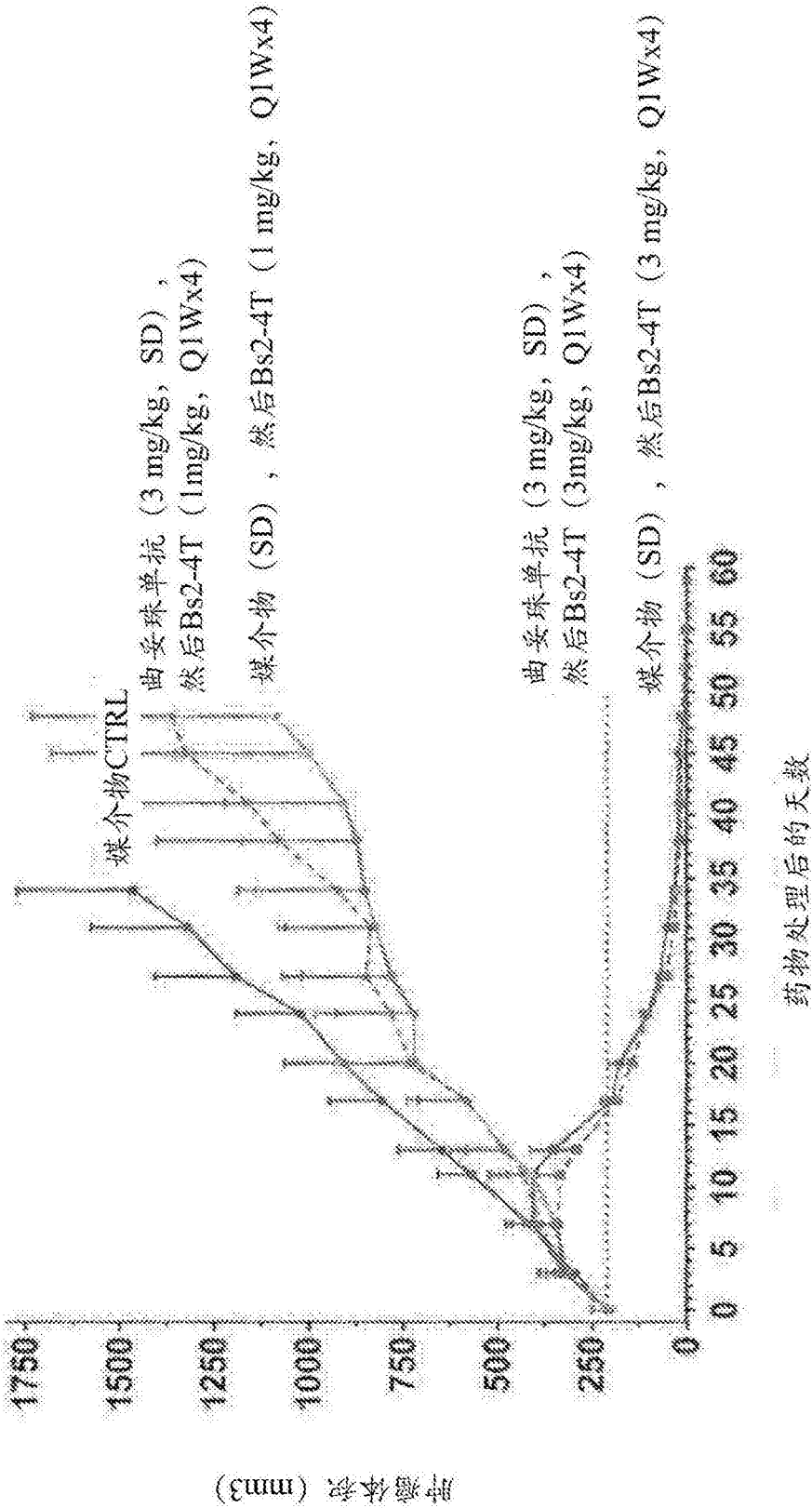


图27

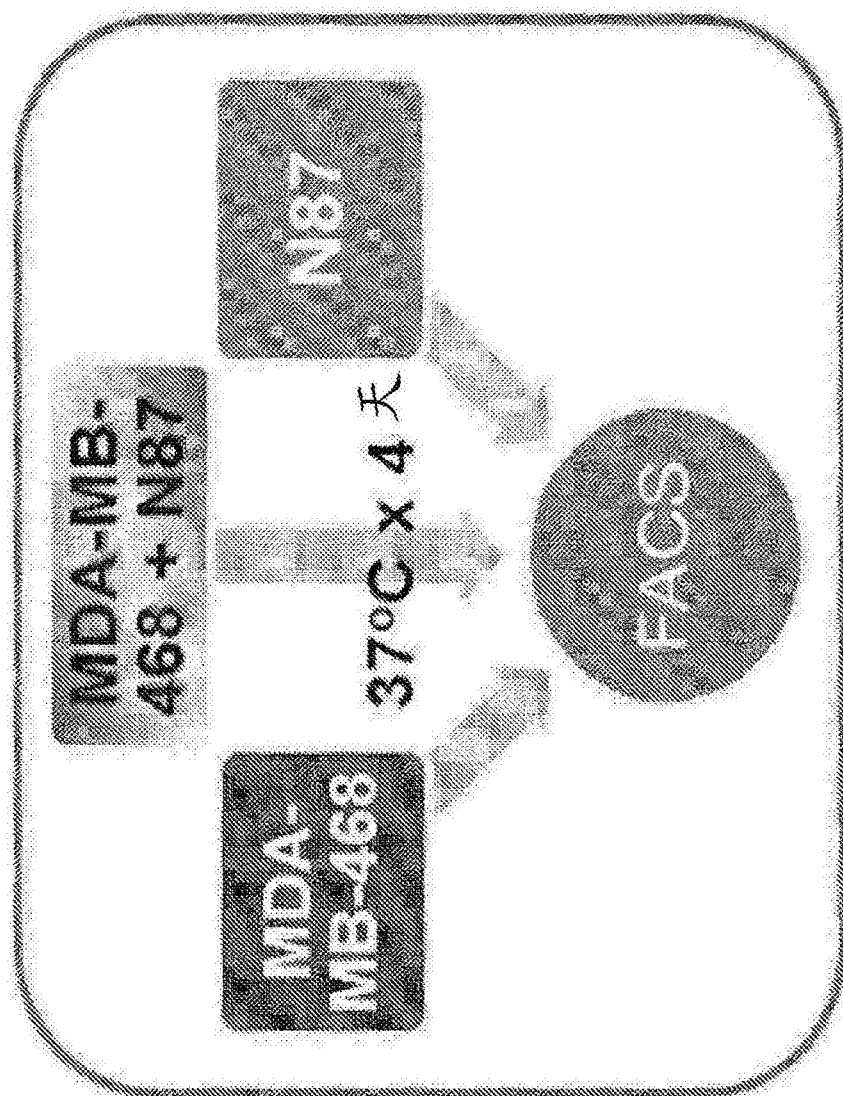


图28A

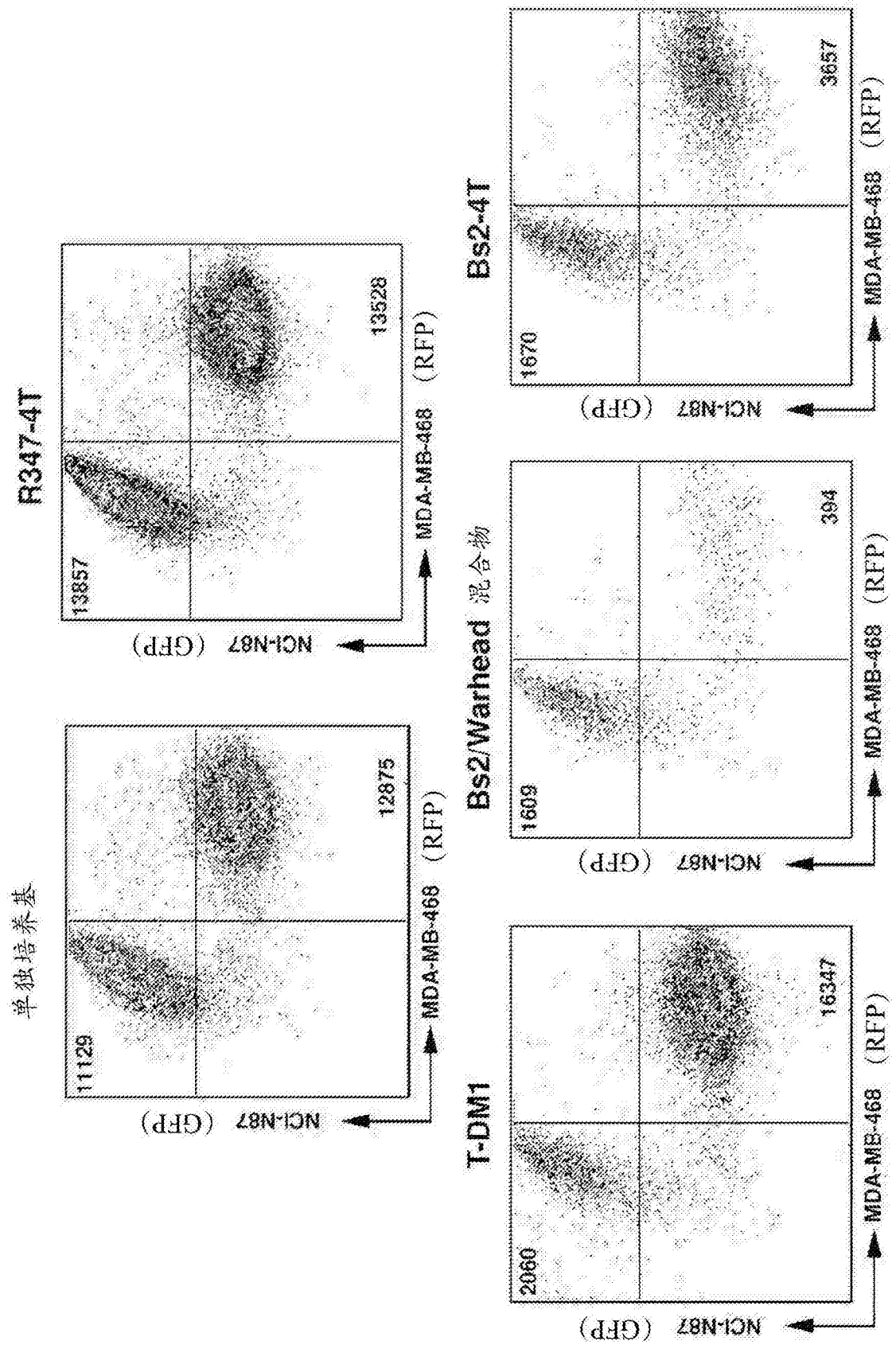


图28B

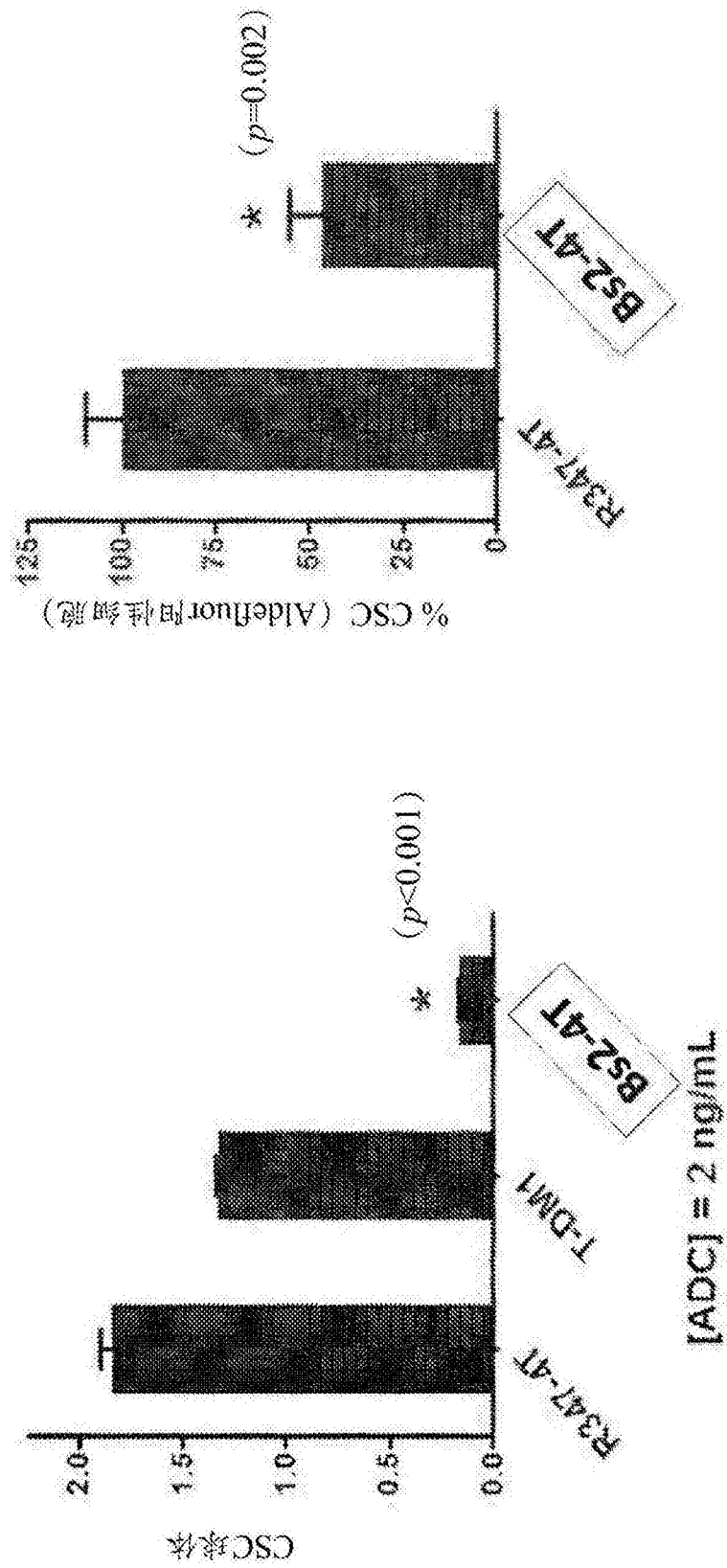


图29

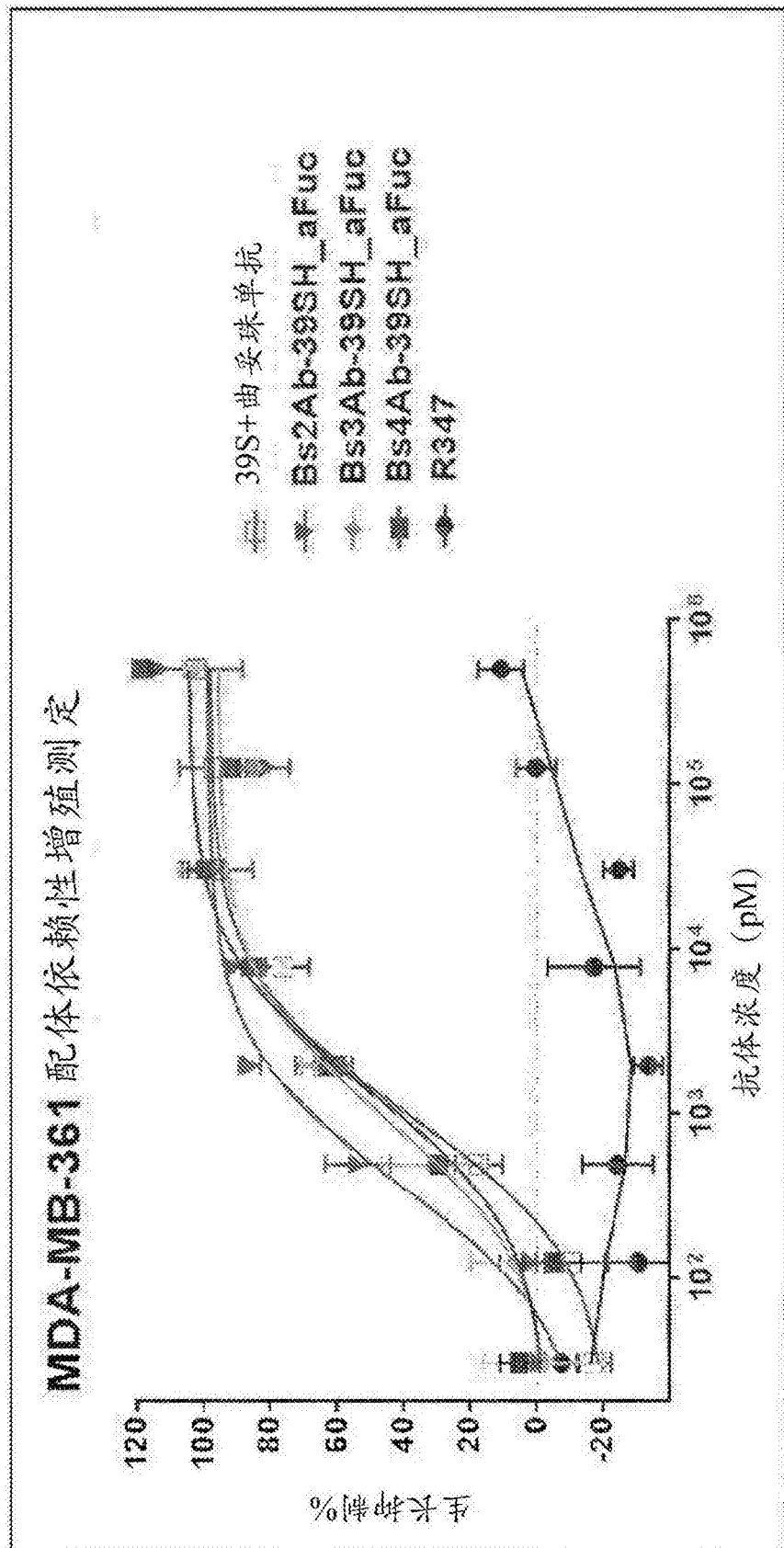


图30A

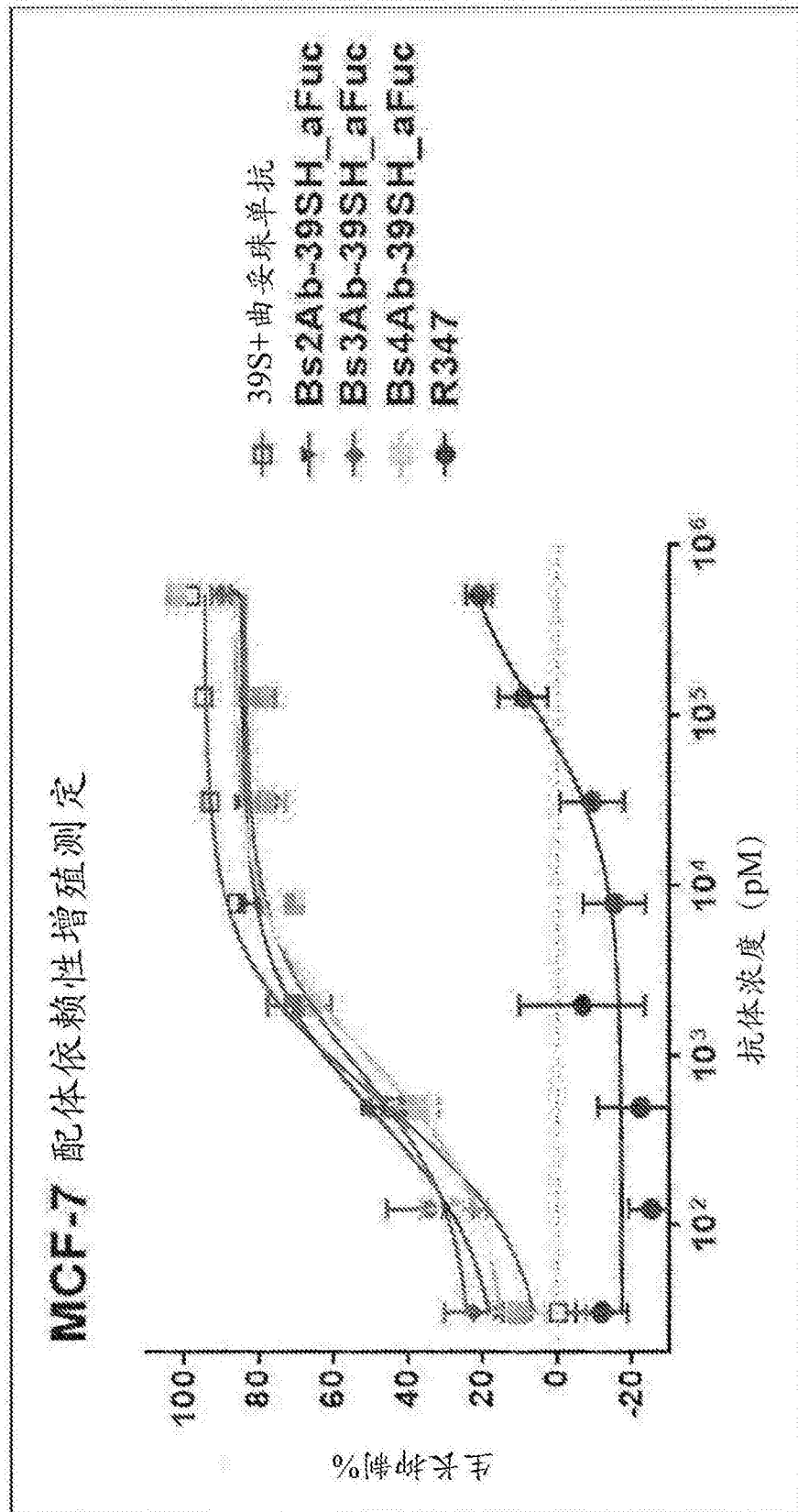


图30B

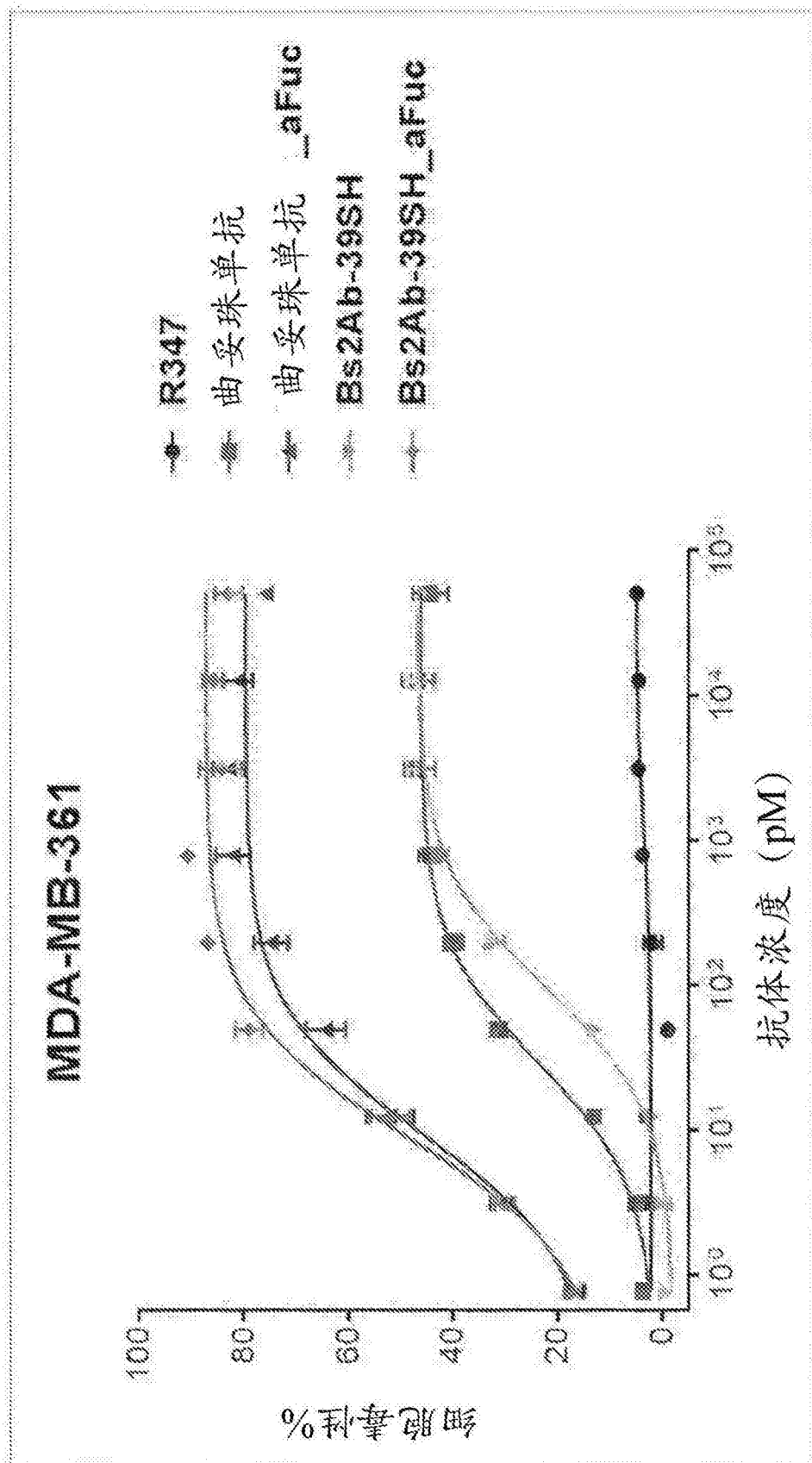


图31A

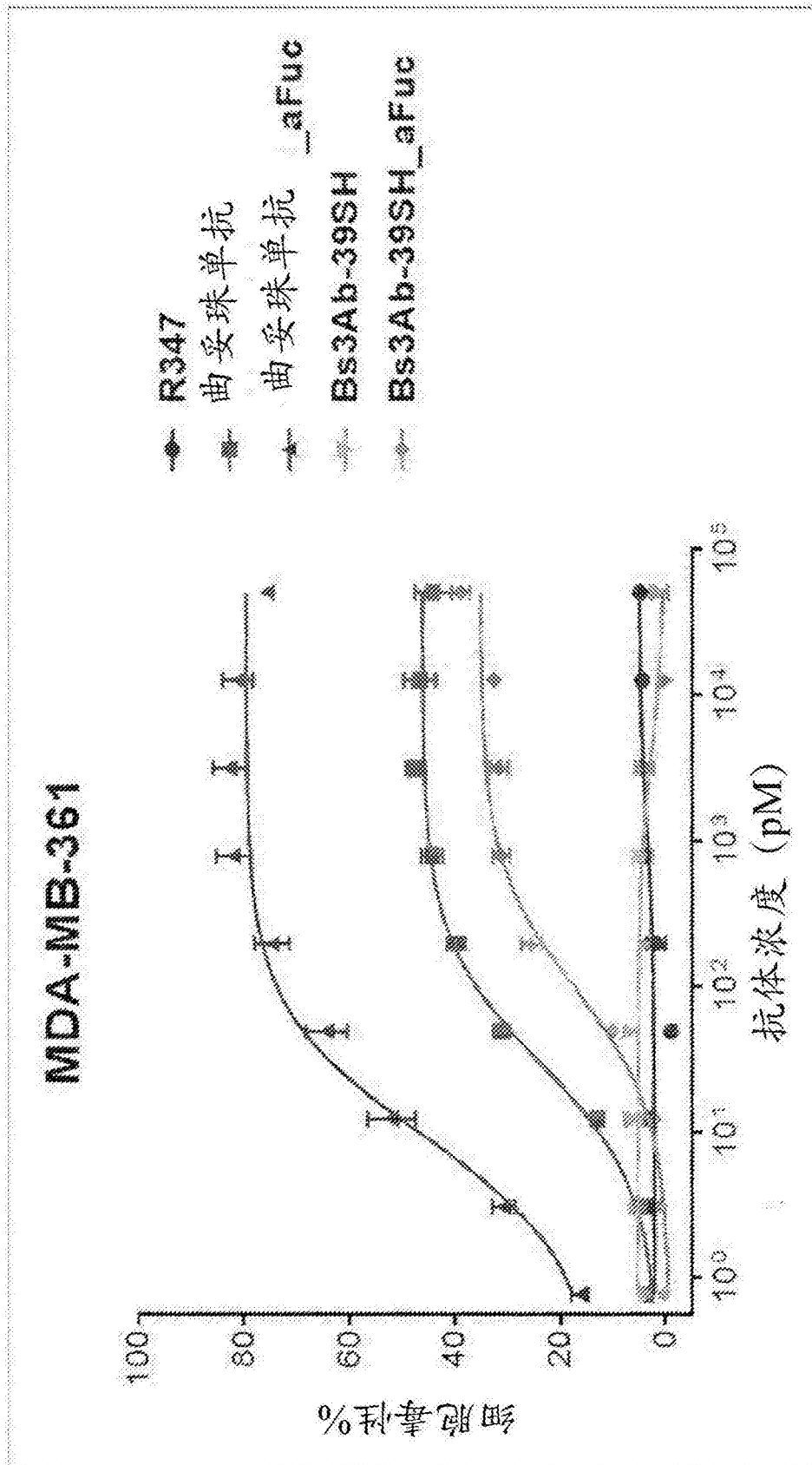


图31B

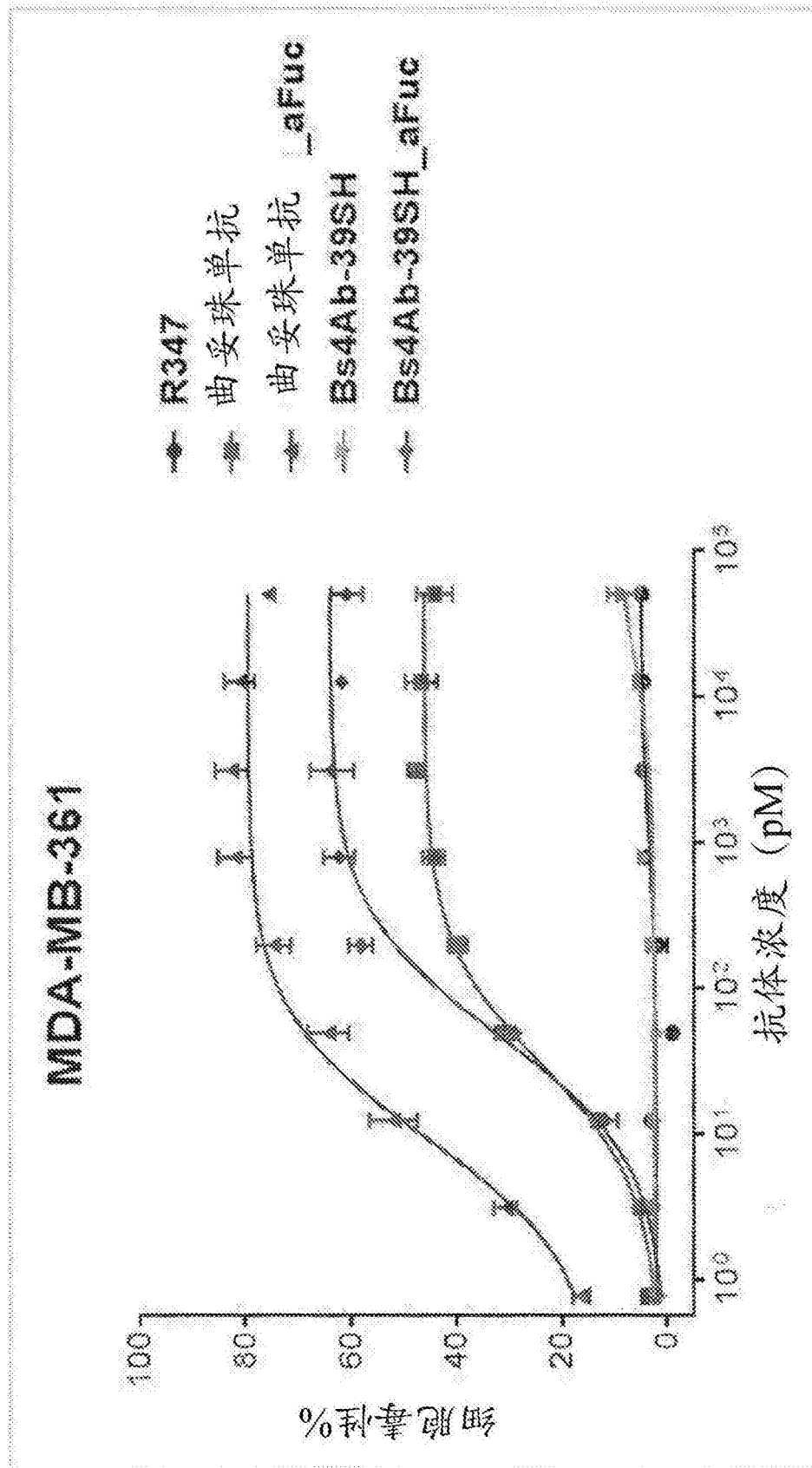


图31C

微管溶素1508有效负载 (化合物T-32)

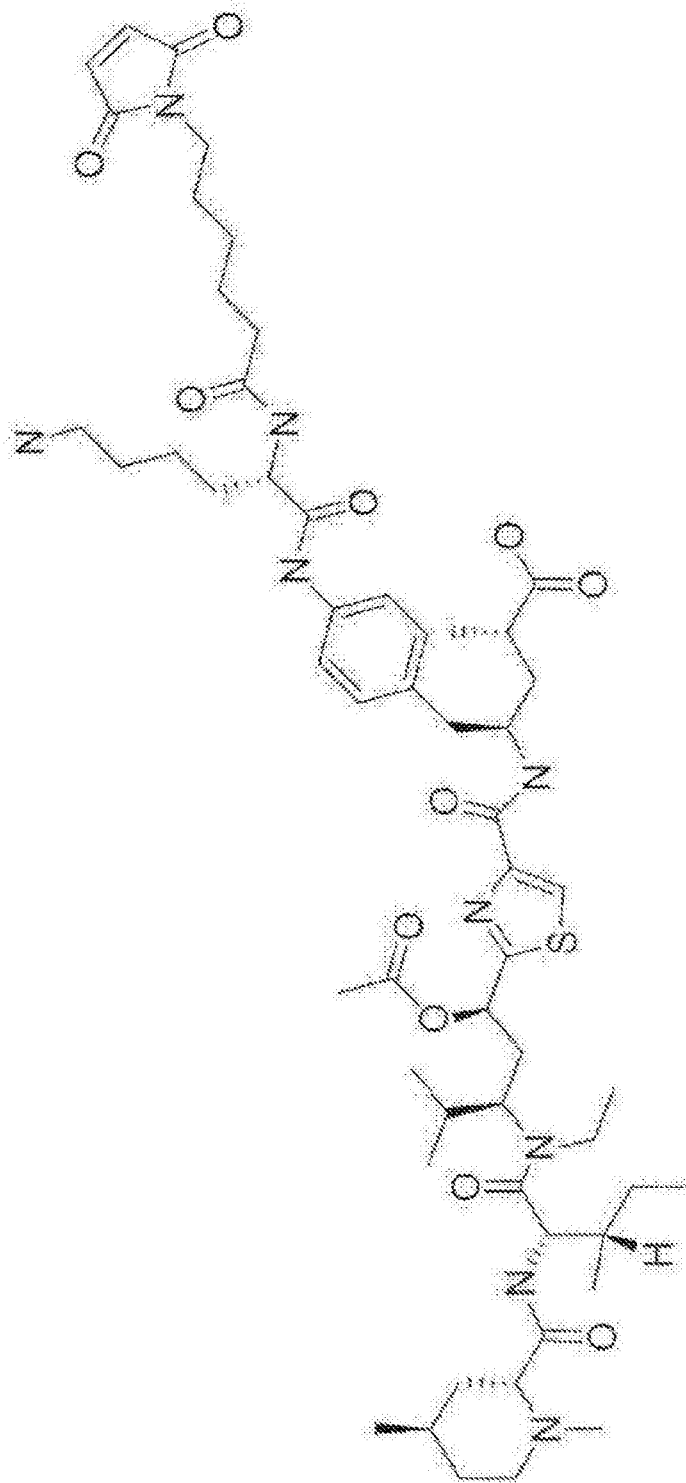


图32

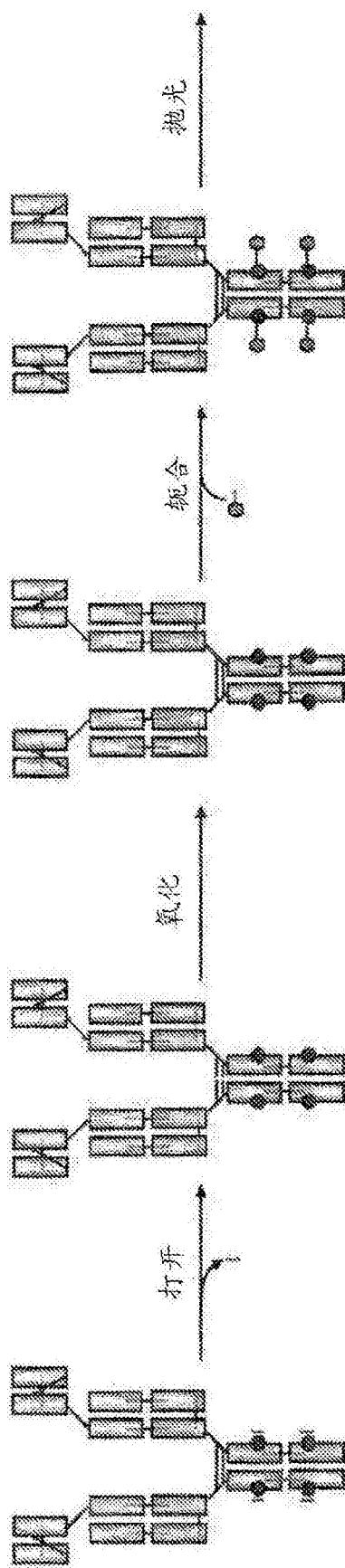


图33