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(54) Title: BISPECIFIC ANTI-HER2 ANTIBODY

(57) Abstract: Humanized bispecific anti-HER2 antibodies that comprise one antigen binding site containing variable regions of heavy and light chain of trastuzumab, and another antigen binding site containing variable regions of heavy and light chain of pertuzumab. The bispecific anti-HER2 antibody is effective for treating cancer, such as breast cancer, gastric cancer, or ovarian cancer. Preferred bispecific anti-HER2 antibodies are afucosylated antibodies. Also provided Chinese Hamster ovary(CHO) mutant cell line that has a dysfunctional Slc35C1 gene, which is the only dysfunctional gene in the mutant that affects glycan regulation.

BISPECIFIC ANTI-HER2 ANTIBODY

REFERENCE TO SEQUENCE LISTING, TABLE OR COMPUTER PROGRAM

The Sequence Listing is concurrently submitted herewith with the specification as an ASCII formatted text file via EFS-Web with a file name of Sequence_Listing.txt with a creation date of March 13, 2017, and a size of 30.7 kilobytes. The Sequence Listing filed via EFS-Web is part of the specification and is hereby incorporated in its entirety by reference herein.

TECHNICAL FIELD

The present invention pertains to the fields of oncology therapy and molecular immunology, and relates to an anti-Her2 antibody, and pharmaceutical compositions and uses thereof. In particular, the present invention relates to humanized bispecific anti-Her2 antibodies that comprise one antigen binding site containing variable regions of heavy and light chain of trastuzumab, and another antigen binding site containing variable regions of heavy and light chain of pertuzumab. The present invention also relates to Chinese Hamster ovary (CHO) mutant cell line that has a dysfunctional *Slc35C1* gene; the mutant cell line expresses proteins having reduced fucosylation.

BACKGROUND OF THE INVENTION

Human epidermal growth factor receptor 2 (abbreviated as Her2, ERBB2, HER2/neu or c-erbB2) is a protein encoded by ERBB2 gene. In normal cells, Her2 has a very low expression level; but Her2 is highly expressed during the period of embryonic development, and is very important in the regulation of cell proliferation, differentiation, development, adhesion and migration (Gutierrez, C. and R. Schiff, HER2: biology, detection, and clinical implications. Arch Pathol Lab Med, 2011. 135(1): p. 55-62.).

Her2 belongs to the family of human epidermal growth factor receptor, and this family consists of 4 members: Her1 (EGFR), Her2, Her3 and Her4. Her2 has no specific ligand, and the activation of its downstream pathway depends on formation of homologous or heterologous dimers (Gutierrez et al, Arch Pathol Lab Med, 2011. 135(1): p. 55-62.). Human epidermal growth factors are all locate on cell surface, and have a similar structure: one extracellular domain (ECD) binding to a ligand, one single transmembrane α-helix transmembrane domain and one intracellular region that consists of an intracellular

membrane-proximal domain, a tyrosine kinase catalytic domain and a tyrosine-rich C-terminal tail domain playing a regulatory role (Eccles, Int J Dev Biol, 2011. 55(7-9): p. 685-96). The extracellular domain (ECD) of human epidermal growth factor can further be separated into 4 subdomains, i.e., regions I, II, III and IV, in which regions II and IV are cysteine-rich domains and participate in dimerization and activation of the receptor.

Overexpression of Her2 may results in disorders of cell normal functions, and usually closely relates to tumor genesis and development. The homologous or heterologous polymerization of Her2 may lead to phosphorylation of tyrosine residues of the receptor, and initiate many signal pathways and causes cell proliferation and tumor genesis. As a biomarker for prognosis and prediction, amplification or overexpression of Her2 gene occurs in about 15-30% breast cancer and 10-30% gastric/esophageal cancer. Overexpression of Her2 may also be observed in other tumors such as ovary, endometrium, bladder, lung, colon, and head-neck tumors.

In breast cancer, Her2 is commonly recognized as a predictive factor and a therapeutic target. Since Her2 has no specific ligand, its antibodies usually inhibit tumor cells by blocking dimerization and activation of the receptor and mediating killing effect of immune system.

At present, Trastuzumab and Pertuzumab are the main Her2-targeted therapeutic antibodies commercially available.

In 1998, FDA approved a Her2-targeting humanization monoclonal antibody, trastuzumab (also called as HERCEPTIN®; humanization degree 95%) of Genentech Inc. This antibody recognizes Her2 extracellular domain IV juxtamembrane epitope, and its antigen affinity constant can be up to 0.1 nmol/L. Trastuzumab recognizes the epitope consisting of the 3 loops (557-561,570-573 and 593-603) at the C-terminal of section IV. Because the epitope may be close to or directly interact with the binding domain of its dimerization partner, trastuzumab's binding to the epitope may induce steric hindrance inhibiting the dimerization process. In addition, trastuzumab's binding may also protect the extracellular domain of the Her2 receptor from the attack by proteinase for hydrolysis.

The mechanisms of action of trastuzumab may include: immune-induced bioactivities (antibody dependent cell-mediated cytotoxicity (ADCC) and Natural killer cell activity), inducing the internalization of Her2 receptor, inhibiting DNA repair, breaking PI3K pathway, activating p27kipl induced G1 cycle stoppage, stimulating cancer cell apoptosis and inhibiting the activation of intracellular p95 domain off of the extracellular domain of the receptor[4,5]. Among them, there have been reports about trastuzumab induced immuno-

mediated therapeutic bioactivities. In particular, ADCC plays an important role, as it was shown in a BT474 xenograft mouse model, when the Fc receptor was knocked out, the inhibition rate of cancer growth was reduced from 96% to 29% (Nat Med, 2000, 6:443-6). Kohrt et al (J Clin Invest, 2012. 122(3): 1066-75) report that stimulation of natural killer cells with a CD137-specific antibody enhances trastuzumab efficacy in xenotransplant models of breast cancer.

Trastuzumab is currently used as a first-line drug for treatment of breast cancer, and is effective in treatment of metastatic breast cancer with Her2 overexpression, and its objective reflection rate of single drug first-line treatment is 30-50%; but it has unsatisfied effect in treating metastatic breast cancer with lower Her2 expression, and resistance has been developed in a number of patients for whom the antibody is initially effective within 1 year. This may be related to shielding of antigen epitopes or abnormal activation of receptor signaling pathway caused by changes of some gene expressions in tumor cells. In addition, Her2 together with other members (Her1, Her3 and Her4) of the family can form ligand-dependent or ligand-independent heterologous dimers, thereby activating downstream pathways, and then resulting proliferation of tumor cells, while trastuzumab cannot inhibit formation of heterologous dimers, so this may be one of reasons for the development of resistance.

Pertuzumab (PERJETA®) was approved by FDA for marketing in USA in 2012, and has certain curative effects on advanced prostate cancer, non-small cell lung cancer, ovarian cancer and breast cancer, but its curative effects still depend on Her2 expression level.

Pertuzumab recognizes key sites for heterologous dimerization of Her2 extracellular domain II, and the epitope recognized thereby are located in segment 245-311 of II subregion center, and key residues are H245, V286, S288, L295, H296 and K311. In which, L295, H296 are key sites for mediating heterologous dimerization of Her2 and Her3, and L295A/H296A double mutation can completely block heterologous dimerization of Her2/Her3 (Franklin, M.C., et al., Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. Cancer Cell, 2004. 5(4): p. 317-28.). Hence, Pertuzumab can be used for effectively inhibiting the formation of Her2/Her3 heterologous dimer, but does not show obvious inhibition effects on the formation of EGFR/Her2 heterologous dimer.

At present, there is a need for developing new anti-HER2 antibodies.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the structure of a preferred embodiment of humanized bispecific anti-Her2 antibodies of the present invention.

FIG. 2 shows a flow chart of the method for preparing a CHO mutant CHOK1-AF, in which the *Slc35c1* gene is knocked out.

FIG. 3 shows the fucose expression level of CHO-K1 cells (A) and CHOK1-AF cells (B) determined by FACS.

FIG. 4 shows intact molecular weight spectrum of MBS301.

FIG. 5 shows intact molecular weight spectrum of MBS301 after N-saccharide excision modification.

FIG. 6 shows results of SEC-HPLC analysis of MIL203AF, MIL204AF, and MBS301.

FIG. 7 shows analytic results of N-glycotypes of MIL203/204 and MBS301.

FIG. 8 shows ADCC action to SKBR-3 cells.

FIG. 9 shows ADCC action to BT474 cells.

FIG. 10 shows ADCC action to SW480 cells.

FIG. 11 shows ADCC action to HCC1419.

FIG. 12 shows cell direct killing effects to BT474 cells.

FIG. 13 shows cell direct killing effects to MDA-MB-175 cells.

FIG.14 shows cell direct killing effects to SKBR-3 cells.

FIG. 15 shows cell direct killing effects to HCC1419 cells.

FIG. 16 shows cell direct killing effects to NCI-N87 cells.

FIG. 17 shows CDC action to BT474 cells.

FIG. 18 shows inhibitory effect on *in vivo* tumor growth of human ovary cancer cells SKOV3 in nude mice.

FIG. 19 shows inhibitory effects on *in vivo* tumor growth of human breast cancer cells BT474 in mice.

FIG. 20 shows inhibitory effects on *in vivo* tumor growth of human stomach cancer cells NCI-N87 in mice.

FIG. 21 shows inhibitory effects on *in vivo* tumor volume in a Trastuzumab-resistant stomach cancer GA055 PDX model.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

As used herein, the term "about" refers to ± 10% of the recited value.

As used herein, the term "an effective amount" refers to an amount to obtain or at least partially obtain a desired effect. An effective amount can be determined by a skilled technician in the art. For example, an effective amount for treatment use depends on severity of disease to be treated, general status of immune system of a patient, general status of a patient such as age, body weight and gender, administration method for drugs, and other therapies simultaneously applied.

As used herein, the term "adjuvant" refers to a non-specific immune enhancer, when it is delivered with an antigen, it can enhance immune response of a subject to the antigen or change type of immune response. There are many kinds of adjuvants, including but not being limited to aluminum adjuvants (e.g., aluminum hydroxide), Freund's adjuvants, lipopolysaccharides, and cell factors. Freund's adjuvants are the most popular adjuvants in animal tests at present, while aluminum hydroxide adjuvant is often used in clinical experiments.

As used herein, the term "antibody" refers to an immune globulin usually consisting of two pairs of polypeptide chains (each pair has a light (L) chain and a heavy (H) chain). The antibody light chain can be classified as κ light chain and λ light chain. The heavy chain can be classified as μ, δ, γ, α or ε, and isotypes of antibody are separately defined as IgM, IgD, IgG, IgA and IgE. In light chain and heavy chain, variable region and constant region are linked via "J" region with about 12 or more amino acids, and heavy chain further contains "D" region with about 3 or more amino acids. Each heavy chain consists of a heavy chain variable region (V_H) and heavy constant region (C_H). Heavy chain consists of 3 domains (CH1, CH2, and CH3). Each light chain consists of a light chain variable region (V_L) and a light chain constant region (C_L). The constant regions of antibody can mediate immune globulin to bind to host tissues or factors, including various cells (e.g., effector cells) of immune system and first component (C1q) of classical complement system. V_H and V_L regions can further be classified as high variability regions (called as complementary determining region (CDR)), in which relatively conservative regions called as framework regions (FR) are scattered. These V_H and V_L regions are composed of 3 CDR regions and 4 FR regions in order of: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4, from amino terminal to

carboxyl terminal. Variable regions (V_H and V_L) of each pair of heavy chain/light chain form an antibody binding site.

As used herein, "antibody-dependent cell-mediated cytotoxicity" (ADCC) is a mechanism of cell-mediated immune defense whereby an effector cell of the immune system actively lyses a target cell, whose membrane-surface antigens have been bound by specific antibodies.

As used herein, the term "antigen-binding fragment" of antibody refers to a polypeptide containing a fragment of full-length antibody, which remains ability of specifically binding to the same antigen to which the full-length antibody binds, and/or competes with the full-length antibody to specifically bind to antigen.

As used herein, the term "complement-dependent cytotoxicity" (CDC) is a function of the complement system. It is the processes in the immune system that kill pathogens by damaging their membranes without the involvement of antibodies or cells of the immune system.

As used herein, the term "core fucose" refers to a fucose linked to GlcNAC in connection with asparagine in N-saccharide core pentasaccharides.

As used herein, the term "EC50" refers to concentration for 50% of maximal effect, that is, a concentration that causes 50% of maximal effect.

As used herein, the term "FcγRIIIa" is a 50-70kDa glycoprotein, belonging to Ig superfamily, having two C2 structures, and its gene is located at 1q23-24 of chromosome. FcγRIII binds to human IgG, IgG3, and is a low affinity receptor. FcγRIII comprises 2 allotypes, FcγRIII A and FcγRIII B. FcγRIII A (AAH17865.1, GenBank) has a transmembrane structure and is mainly distributed in macrophages, NK cells and eosinophilic granulocytes, in which macrophages have a high expression level of FcγRIII A, while mononuclear cells have a lower expression level. FcγRIII A relates to disulfide bond-linked CD3ζ or FcεR I γ chain dimer, in which FcγRIII A relates to CD3 complex γ chain on macrophages, while FcγRIIIA relates to ζ chain on NK/LGL.

As used herein, the term "FcRn" is neonate Fc receptor (P61769, UniProtKB/Swiss-Prot), which is a heterologous dimer consisting of a large subunit and a small subunit, the large subunit has a molecular weight of 45-53 kD, called as α chain; the small subunit is β2 microglobulin (β2m), has a molecular weight of 14 kD, called as β chain, the two chains are bound together in a non-covalent bond form. When physiologic pH is 7.4, FcRn does not

bind to IgG, but under condition of endosome acidic pH 6-6.5, affinity of FcRn to IgG Fc ranges from nanomoles to micromoles.

As used herein, the term "Her2" refers to Her2 in full-length (NP_004439.2), or extracellular fragments or domains I, II, III or IV of Her2, or fragments containing at least one of them; or comprises a fusion proteins containing a Her2 extracellular fragment. However, those skilled in the art would understand that the amino acid sequence of Her2 may have a naturally generated or artificially introduced mutation or variation (including but not being limited to replacement, deletion and/or addition) without influencing its biological function. Hence, in the present invention, the term "Her2" should include any one of these sequences.

As used herein, the term "host cell" refers to a cell into which a vector can be introduced, which includes but is not limited to, for example, prokaryotic cells such as *E. coli* or Bacterium subtilis, fungus cells such as yeast cells or Aspergillus, insect cells such as S2 fruit fly cells or Sf9 cells, or animal cells such as fibroblasts, CHO cells, COS cells, NSO cells, Hela cells, BHK cells, HEK293 cells or human cells.

As used herein, the term "K_D" refers to a dissociation equilibrium constant for a specific antibody-antigen interaction, which is used to describe binding affinity between the antibody and the antigen.

As used herein, the term "pharmaceutically acceptable carrier and/or excipient" refers to a carrier and/or excipient pharmacologically and/or physiologically compatible to a subject and an active component, for example, see Remington's Pharmaceutical Sciences. Edited by Gennaro AR, 19th ed. Pennsylvania: Mack Publishing Company, 1995. A pharmaceutically acceptable carrier includes but is not limited to: pH regulators, surfactants, adjuvants, ion strength enhancers. For example, pH regulators include but are not limited to phosphate buffer solutions; surfactants include but are not limited to cationic, anionic or nonionic surfactants, for example, Tween-80; ion strength enhancers include but are not limited to sodium chloride.

As used herein, the term "specifically binding" refers to a non-random binding reaction between two molecules, for example, a reaction between an antibody and its antigen.

As used herein, the term "vector" refers to a nucleic acid vector that can be used for inserting polynucleotide. When a vector enables an inserted polynucleotide to express a protein encoded thereby, the vector is called as expression vector. Vector can be introduced into a host cell by transformation, transduction or transfection, so that a genetic material element carried by the vector is expressed in the host cell. Vectors are well-known by those

skilled in the art, including but not being limited to: plasmids, phasmids, cosmids, artificial chromosomes, for example, yeast artificial chromosomes (YAC), bacterial artificial chromosomes (BAC) or P1-sourced artificial chromosomes (PAC); phages such as λ phages or M13 phages and animal viruses. The animal viruses usable as vectors include but are not limited to retroviruses (including lentiviruses), adenoviruses, adeno-associated viruses, herpes viruses (e.g., herpes simplex virus), poxviruses, baculoviruses, papillomaviruses, papovaviruses (e.g., SV40). A vector can contain a plurality of expression-controlling elements, including but not being limited to promoter sequence, transcription initiation sequence, enhancer sequences, selection element and reporter gene. In addition, vector may further contain replication initiation site.

Description

The present invention is directed to a humanized bispecific anti-Her2 antibody or a bispecific antigen-binding fragment thereof, comprising one antigen binding site containing variable regions of heavy and light chain of trastuzumab, and another antigen binding site containing variable regions of heavy and light chain of pertuzumab. The bispecific antibody recognizes Her2 extracellular domains IV and II.

The anti-Her2 antibody or antigen-binding fragments of the present invention comprises a first heavy chain and a first light chain relating to trastuzumab, and a second heavy chain and a second light chain relating to pertuzumab.

The first heavy chain comprises a V_H having CDRs of which the amino acid sequences are shown in SEQ ID NOs: 1-3, and a C_H having an amino acid sequence as shown in SEQ ID NO: 7. In one embodiment, the non-CDR region is derived from a human antibody.

The second heavy chain comprising a V_H having CDRs of which the amino acid sequences are shown in SEQ ID NOs: 4-6, and a C_H having an amino acid sequence as shown in SEQ ID NO: 8. In one embodiment, the non-CDR region is derived from a human antibody.

In the first heavy chain,

CDR1: GFNIKDTY (SEQ ID NO: 1)

CDR2: IYPTNGYT (SEQ ID NO: 2)

CDR3: SRWGGDGFYAMDY (SEQ ID NO: 3).

In the second heavy chain,

CDR1: GFTFTDYT (SEQ ID NO: 4)

CDR2: VNPNSGGS (SEQ ID NO: 5)

CDR3: ARNLGPSFYFDY (SEQ ID NO: 6).

Constant region of the first heavy chain (SEQ ID NO: 7):

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHT
FPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH
TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV
DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI
EKTISKAKGQPREPQVYTLPPSREEMTKNQVSLSCAVKGFYPSDIAVEWESNGQP
ENNYKTTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSL
SLSPGK

Constant region of the second heavy chain (SEQ ID NO: 8):

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHT
FPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH
TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV
DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI
EKTISKAKGQPREPQVYTLPPSREEMTKNQVSLWCLVKGFYPSDIAVEWESNGQP
ENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSL
SLSPGK

In one embodiment, the first heavy chain V_H has an amino acid sequence as shown below:

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARI
YPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGF
YAMDYWGQGTLVTVSS (SEQ ID NO: 9)

The second heavy chain V_H has an amino acid sequence as shown below:

EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVRQAPGKGLEWVAD
VNPNSGGSIYNQRFKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARNLGPSF
YFDYWGQGTLVTVSS (SEQ ID NO: 10)

The anti-Her2 antibody or antigen-binding fragments thereof further comprises a first light chain and a second light chain.

The first light chain comprising a V_L having CDRs of which the amino acid sequences are shown in SEQ ID NOs: 11-13. In one embodiment, the non-CDR region is derived from a human antibody.

The second light chain comprising a V_L having CDRs of which the amino acid sequences are shown in SEQ ID NOs: 14-16. In one embodiment, the non-CDR region is derived from a human antibody.

In the first light chain,
CDR1: QDVNTA (SEQ ID NO: 11)
CDR2: SASFLYS (SEQ ID NO: 12)
CDR3: QQHYTTPPT (SEQ ID NO: 13).

In the second light chain,
CDR1: QDVSIG (SEQ ID NO: 14)
CDR2: SASYRYT (SEQ ID NO: 15)
CDR3: QQYYIYPYT (SEQ ID NO: 16).

In one embodiment of the present invention, the first light chain V_L has an amino acid sequence as shown below:
DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSAS FLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIK (SEQ ID NO: 17)

The second light chain V_L has an amino acid sequence as shown below:
DIQMTQSPSSLSASVGDRVTITCKASQDVSIGVAWYQQKPGKAPKLLIYSAS YRYTGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYIYPYTFGQGTKVEIK (SEQ ID NO: 18)

The present invention is directed to a humanized bispecific anti-Her2 antibody or an antigen binding fragment thereof, comprising: a first heavy chain comprising a variable region (V_H) having an amino acid sequence of SEQ ID NO: 9, a first light chain comprising a

variable region (V_L) having an amino acid sequence of SEQ ID NO: 17, a second heavy chain comprising a variable region (V_H) having an amino acid sequence of SEQ ID NO: 10, and a second light chain comprising a variable region (V_L) having an amino acid sequence of SEQ ID NO: 18,

wherein the first V_H and the first V_L form a first antigen binding site specific for extracellular domain IV of HER2, and the second V_H and the second V_L form a second antigen binding site specific for extracellular domain II of HER2.

In one embodiment of the present invention, the anti-Her2 antibody or an antigen binding fragment thereof further comprises a first light chain C_H and/or a second light chain C_H having an amino acid sequence of SEQ ID NO: 19:

RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGN SQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGE C (SEQ ID NO: 19)

In one embodiment of the present invention, the anti-Her2 antibody or an antigen binding fragment thereof comprises a first heavy chain having an amino acid sequence as SEQ ID NO: 22, wherein the underlined part is amino acid sequence of heavy chain variable region:

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPT NGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDY WGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCD KTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKT ISKAKGQPREPQVYTLPPSREEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYK TTPPVLDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 22)

In one embodiment of the present invention, the anti-Her2 antibody or an antigen binding fragment thereof comprises a first light chain having an amino acid sequence as SEQ ID NO: 23, wherein the underlined part is amino acid sequence of light chain variable region:

DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFL

YSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRT VAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQES VTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 23)

In one embodiment of the present invention, the anti-Her2 antibody or an antigen binding fragment thereof comprises a second heavy chain having an amino acid sequence SEQ ID NO: 25; wherein the underlined part is amino acid sequence of heavy chain variable region:

EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVRQAPGKGLEWVADVN PNSGGSIYNQRFKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARNLGPSFYFDYW GQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKT HTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISK AKGQPREPQVYTLPPSREEMTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTP PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 25)

In one embodiment of the present invention, the anti-Her2 antibody or an antigen binding fragment thereof comprises a second light chain having an amino acid sequence SEQ ID NO: 26, wherein the underlined part is amino acid sequence of light chain variable region:

DIQMTQSPSSLSASVGDRVTITCKASQDVSIGVAWYQQKPGKAPKLLIYSASYRYTGV PSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYYIYPYTFGQGTKVEIKRTVAAPSVFIF PPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYS LSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 26)

In one embodiment of the present invention, the anti-Her2 antibody or an antigen binding fragment thereof, contains fucose glycotype ≤25%, ≤20%, ≤15%, ≤10%, ≤8%, ≤6%, ≤5%, ≤4%, ≤3%, ≤2%, ≤1.5%, or ≤1.1% of the total saccharides that are attached to the Fc region of the antibody. The content of fucose glycotype is obtain by summing contents of all fucose-containing glycotypes, e.g., determined by N-saccharide determination method.

In one embodiment of the present invention, the anti-Her2 antibody or an antigen binding fragment thereof binds to Her2 protein with an EC50 of less than about 100 nM, for example, less than about 10 nM, 1 nM, 0.9 nM, 0.8 nM, 0.7 nM, 0.6 nM, 0.5 nM, 0.4 nM, 0.3 nM, 0.2 nM, 0.1 nM or less. The EC50 may be determined by Biacore method.

The bispecific antibody of the present invention combines pertuzumab and trastuzumab, and benefits from more complete blocking of the Her2-mediated signal transduction. Trastuzumab inhibits the formation of Her2 homodimerization and prevents the extracellular domain of Her2 undergoing proteolytic cleavage to form constitutively active p95 proteins; Pertuzumab blocks Her2 heterodimer formation and then completely blocks Her2-mediated signal transduction. When used alone, pertuzumab and trastuzumab do not have CDC activity. However, the bispecific antibody of the present invention exhibits strong CDC activity as observed in at least one *in vitro* cell based assay.

The bispecific antibody of the present invention targets different Her2 epitopes, which enhances its tumor suppressive effects and achieves a synergistic effect, and enhances the ADCC function.

In one embodiment, the bispecific antibody of the present invention lacks core fucose residue from the Fc N-glycans, and exhibits strong ADCC at low concentrations. This is because afucosylated antibody enhances its binding affinity with the Fc gamma receptor IIIa (FcγRIIIa) on the natural killer (NK) cells, and hence increases the antibody's ADCC activity. At the same time, afucosylated antibody can suppress the inhibitory effect from human immunoglobulin G (IgG) in serum for binding to the Fc gamma receptor IIIa (FcγRIIIa) on the natural killer (NK) and macrophage cells as the latter's binding affinity with FcγRIIIa is much weaker.

Removal of the core fucosylation to increase the antibody affinity with FcγRIIIa is one of the most effective ways to increase ADCC. Most therapeutic antibodies currently on the market are heavily fucosylated because they are produced by mammalian cell lines such as Chinese Hamster ovary (CHO) with intrinsic enzyme activity responsible for the core-fucosylation of the Fc N-glycans of the products. The present invention provides a CHO mutant that has a dysfunctional *Slc35C1* gene, which encodes the GDP-fucose transporter SLC35C1 that critically regulates the fucosylation of glycans. The CHO mutant of the present invention only contains one dysfunctional gene from CHO that affects the regulation of glycan, i.e., the *Slc35C1* gene is knocked out; the CHO mutant does not contain any other dysfunctional gene that affects the regulation of glycans. For example, the CHO mutant does not contain a dysfunctional gene that affects the production of sialic acids. The SLC35C1-

deficient CHO cells of the present invention produce antibody with fucose content about ≤10%, ≤8%, ≤6%, ≤4%, ≤3%, ≤2%, ≤1.5%, or ≤1.1% of the total saccharides that are attached to the Fc region of the antibody.

The present invention provides a method to produce mutant SLC35C1-deficient CHO cells. The method uses the zinc finger enzyme knock-out technique to knock out the key fucose-modified protein GFT (GDP-fucose transporter) in the host CHO cells, and thus the fucosylated level of the antibody produced is effectively reduced. This method can block both the classical and the compensatory pathways of fucosylation, so the method is effective in reducing fucosylation. The method comprises using zinc-finger nuclease technique to design two GFT zinc-finger nucleases for GFT gene *Slc35c1* sequence (GenBank: BAE16173.1); the two zinc-finger nucleases are designed to bind double-stranded DNA of the target gene separately. The two zinc-finger nuclease sequences for GFT are cloned to construct two expression vectors. The two expression vectors are co-transfected into the target CHO cells by a suitable method known to a skilled person, e.g., by electrotransfection technique. After transfection, the transfected cells are cultivated, performed passage and amplification. The clones without fucosylation modification are selected via multi-turns of negative separation and clonal culture. One specific method is illustrated in FIG. 2.

The present invention provides a method to remove the core fucosylation of the bispecific antibody, which improve the ADCC effect of the antibody. In the present method, the bispecific antibody is produced using a CHO mutant that has a dysfunctional *Slc35C1* gene, e.g. CHOK1-AF, which results in the core-fucose level of less than 1.5%. The MBS301 of the present patent has a 10-fold increase in ADCC activity compared to MIL203/204, which does not remove the core fucose unit.

The bispecific antibody of the present invention, e.g., MBS301 is designed to bind against Her2 extracellular domains IV and II; it has higher cell direct killing activity, ADCC activity, CDC activity and the tumor suppressing ability in mice than using each antibody alone. MBS301 exhibits higher cell direct killing activity, higher ADCC activity, when compared with the combination use of trastuzumab and pertuzumab in *in vitro* cell line activity studies, while CDC activity is similar to the combination use of trastuzumab and pertuzumab.

In one embodiment, the bispecific antibody of the present invention is a "knob-into-hole" antibody, which has modified amino acid sequence in the CH3 region to facilitate the pairing of the heterologous half-antibodies. For example, the constant region of the first heavy chain has 3 mutations from human Fc; the mutations are T369S, L371A, and Y410V in SEQ ID

NO: 7. The constant region of the second heavy chain has 1 mutation from human Fc; the mutation is T368W in SEQ ID NO: 8. The "knob-into-hole" structure antibody maintains the normal antibody structure and size and provide bifunctional activity.

In one embodiment, the present invention relates to isolated nucleic acid molecules which are capable of encoding the first and the second heavy chains and the first and the second light chain of the Her2 antibody of the present invention.

In another aspect, the present invention relates to a vector, which comprises the isolated nucleic acid of the present invention.

In another aspect, the present invention relates to a host cell, which comprises the isolated nucleic acid molecule of the present invention, or the vector of the present invention. Preferably, the host cell is CHOK1-AF cell. Preferably, in the host cell, the gene of GFT (key protein in fucose modification pathway) is site-directly knocked out. Preferably, the knockout is performed by zinc finger nuclease technique. Preferably, the SLC35c1 sequence in the gene of GFT (GenBank accession number: BAE16173.1) is site-directly knocked out. In one embodiment of the present invention, the fucose is core fucose.

In another aspect, the present invention relates to a conjugate, which comprises an anti-Her2 antibody or an antigen binding fragment thereof and a coupling part, wherein, the anti-Her2 antibody is the anti-Her2 antibody or an antigen binding fragment thereof according to any one of items of the present invention, the coupling part is a detectable label; preferably, the coupling part is a radioactive isotope, a fluorescent material, a luminescent material, a colored material or an enzyme.

In another aspect, the present invention relates to a kit, which comprises the anti-Her2 antibody or an antigen binding fragment thereof according to the present invention, or comprises the conjugate of the present invention. The kit may further comprise a second antibody, which specifically recognizes the anti-Her2 antibody or an antigen binding fragment thereof; optionally, the second antibody further comprises a detectable label, such as a radioactive isotope, a fluorescent material, a luminescent material, a colored material or an enzyme.

In another aspect, the present invention relates to a use of the anti-Her2 antibody or an antigen binding fragment thereof according to the present invention or the conjugate of the present invention in manufacturing a kit, wherein the kit is used for detecting the existence of Her2 or the level of Her2 in a sample.

In another aspect, the present invention relates to a pharmaceutical composition, which comprises the anti-Her2 antibody or an antigen binding fragment thereof or the

conjugate of the present invention; optionally, further comprises a pharmaceutically acceptable carrier and/or an excipient; optionally, further comprises one or more chemotherapeutic drugs or cytotoxic drugs. The chemotherapeutic drug or cytotoxic drug may be selected from: (1) drugs acting on DNA chemical structure: alkylating agent such as mechlorethamines, nitroso urines, methylsulfonic acid esters; platinum compounds such as cis-platinum, carboplatin and oxaliplatin, etc.; mitomycin (MMC); (2) drugs affecting synthesis of nucleic acids: dihydrofolate reductase inhibitors such as methotrexate (MTX) and Alimta, etc.; thymidine synthase inhibitor such as fluorouracils (5FU, FT-207, capecitabine), etc.; purine nucleoside synthase inhibitors such as 6-mercaptopurine (6-MP) and 6-TG, etc.; nucleotide reductase inhibitors such as hydroxyurea (HU), etc.; DNA polymerase inhibitors such as cytarabine (Ara-C) and Gemz, etc.; (3) drugs acting on nucleic acid transcription: drugs for inhibiting RNA synthesis by selectively acting on DNA templates, inhibiting DNA-dependent RNA polymerase, such as: actinomycin D, rubidomycin, adriamycin, epirubicin, aclacinomycin, mithramycin, etc.; (4) drugs mainly acting on microtubulin synthesis: paclitaxel, docetaxel, vinblastinum, vinorelbine, podophyllotoxins, homoharringtonine; (5) other cytotoxic drugs: asparaginase mainly inhibiting protein synthesis; hormones: antiestrogens: tamoxifen, droloxifen, exemestane, etc.; aromatase inhibitors: aminoglutethimide, lentaron, letrozole, Arimidex, etc.; antiandrogens: Flutamide RH-LH agonists/antagonists: zoladex, enantone, etc.; biological response regulators: interferons mainly inhibiting tumors via body immune functions; interleukin-2; thymosins; monoclonal antibodies: rituximab (MabThera); Cetuximab (C225); HERCEPTIN® (trastuzumab); Bevacizumab (Avastin); cell differentiation inducers such as Tretinoins; cell apoptosis inducers. The bispecific antibodies and compositions thereof as disclosed by the invention can be used in drug combinations with one or more of the aforesaid anti-tumor drugs.

In another aspect, the present invention relates to a use of the anti-Her2 antibody or an antigen binding fragment thereof of the present invention or the conjugate of the present invention in the manufacture of a medicament for prophylaxis and/or treatment and/or diagnosis of cancer; the cancer is selected from breast cancer, gastric cancer, esophagus cancer, ovarian cancer, endometrial cancer, bladder cancer, lung cancer, colon cancer, head-and-neck cancer and prostate cancer; for example, the prostate cancer is advanced prostate cancer; and the breast cancer is metastatic breast cancer.

The present invention is further directed to a method for treating cancer. The method comprises the step of administering an effective amount of the anti-Her2 antibody or an

antigen binding fragment thereof of the present invention to a subject in need thereof. The cancer includes breast cancer, gastric cancer, ovarian cancer, esophagus cancer, endometrial cancer, bladder cancer, lung cancer, colon cancer, head-and-neck cancer and prostate cancer.

The pharmaceutical composition of the present invention can be applied by systemic administration or local administration. Systemic administration includes oral, parenteral (such as intravenous, intramuscular, subcutaneous, or rectal), and other systemic routes of administration. In systemic administration, the active compound first reaches plasma and then distributes into target tissues.

Dosing of the composition can vary based on the extent of the cancer and each patient's individual response. For systemic administration, plasma concentrations of the active compound delivered can vary; but are generally 1×10^{-10}-1×10^{-4} moles/liter, and preferably $1x10^{-8}$-1×10^{-5} moles/liter.

Those of skill in the art will recognize that a wide variety of delivery mechanisms are also suitable for the present invention.

The present invention is useful in treating a mammal subject, such as humans, horses, and dogs. The present invention is particularly useful in treating humans.

The invention is further illustrated by the following examples.

EXAMPLES

The abbreviations/terms used in the examples are provided as follows.

MIL40: a HERCEPTIN® sample as prepared by the inventors, which is in consistence with HERCEPTIN® amino acid sequence.

MIL41: a PERJETA® sample as prepared by the inventors, which is in consistence with the amino acid sequence of PERJETA®.

MIL203: an incomplete antibody (semi-antibody), in which amino acid sequences of heavy chain and light chain are designed as Example 1.

MIL203AF: an amino acid sequence identical to MIL203, except that it is expressed in fucose-knockout engineering cell line (CHOK1-AF). In its N-saccharide modified glycotypes, the ratio of glycotypes without core fucose is $\geq$ 98.5%, i.e., the core fucose is $<$ 1.5%.

MIL204: an incomplete antibody (semi-antibody), in which amino acid sequences of heavy chain and light chain are designed as Example 1.

MIL204AF: the amino acid sequence of the Fab of MIL204AF is identical to that of MIL204, but MIL204AF is expressed in fucose-knockout engineering cell line (CHOK1-AF). In its N-saccharide modified glycotypes, the ratio of glycotypes without core fucose is ≥ 98.5%, i.e., the core fucose is < 1.5%.

MIL203/204: a bifunctional antibody formed by assembling MIL203 and MIL204.

MBS301: a bifunctional antibody formed by assembling MIL203AF and MIL204AF.

Example 1: Amino acid sequence design and gene sequence optimization of heavy chains and light chains of antibodies MIL203 and MIL204

(1) Amino acid sequences of MIL203 light chain and heavy chain

The heavy chain of MIL203 has the amino acid sequence of SEQ ID NO: 22.

The light chain of MIL203 has the amino acid sequence of SEQ ID NO: 23.

(2) Nucleic Acid sequences of light chain and heavy chain of MIL203

The optimized gene sequences for encoding light chain and heavy chain of MIL203 are as follows.

MIL203 heavy chain base sequence is shown as SEQ ID NO: 20, wherein the underlined part is base sequence of heavy chain variable region.

gaggtgcagctggtggagagcggcggcggcctggtgcagcccggcggcagcctgcgcctgagctgcgccgccagcggc
ttcaacatcaaggatacctacatccactgggtgcgccaggctcccggcaagggcctggagtgggtggcccgcatctaccccaccaac
ggctacacccgctacgccgatagcgtgaagggccgcttcaccatcagcgccgataccagcaagaacaccgcctacctgcagatgaa
cagcctgcgcgccgaggataccgccgtgtactactgcagccgctggggcggcgatggcttctacgccatggattactggggccagg
gcaccctggtcaccgtgagcagcgctagcaccaagggcccatcggtcttcccctggcaccctcctccaagagcacctctgggggc
acagcggccctgggctgcctggtcaaggactacttccccgaaccggtgacggtgtcgtggaactcaggcgccctgaccagcggcgt
gcacaccttcccggctgtcctacagtcctcaggactctactccctcagcagcgtggtgactgtgccctctagcagcttgggcacccaga
cctacatctgcaacgtgaatcacaagcccagcaacaccaaggtggacaagaaagttgagcccaaatcttgtgacaaaactcacacat
gcccaccgtgcccagcacctgaactcctgggggggaccgtcagtcttcctcttcccccaaaacccaaggacaccctcatgatctcccg
gacccctgaggtcacatgcgtggtggtggacgtgagccacgaagaccctgaggtcaagttcaactggtacgtggacggcgtggagg
tgcataatgccaagacaaagccgcgggaggagcagtacaacagcacgtaccgtgtggtcagcgtcctcaccgtcctgcaccaggac
tggctgaatggcaaggagtacaagtgcaaggtctccaacaaagccctcccagcccccatcgagaaaaccatctccaaagccaaagg
gcagccccgagaaccacaggtgtacaccctgcccccatcccgggaagagatgaccaagaaccaggtcagcctgagctgcgcagtc
aaaggcttctatcccagcgacatcgccgtggagtgggagagcaatgggcagccggagaacaactacaagaccacgcctcccgtgct

ggactccgacggctccttcttcctcgtgagcaagctcaccgtggacaagagcaggtggcagcaggggaacgtcttctcatgctccgtg atgcatgaggctctgcacaaccactacacgcagaagagcctctccctgtctccgggtaaa (SEQ ID NO: 20)

MIL203 light chain base sequence is shown as SEQ ID NO: 24, wherein the underlined part is base sequence of light chain variable region:

gatatccagatgacccagagccccagcagcctgagcgccagcgtgggcgatcgcgtgaccatcacctgccgcgccagcca ggatgtgaacaccgccgtggcctggtaccagcagaagcccggcaaggcccccaagctgctgatctacagcgccagcttcctgtaca gcggcgtgcccagccgcttcagcggcagccgcagcggcaccgatttcaccctgaccatcagcagcctgcagcccgaggatttcgcc acctactactgccagcagcactacaccacccccccccaccttcggccagggcaccaaggtggagatcaagcgtacggtggctgcacc atctgtcttcatcttcccgccatctgatgagcagttgaaatctggaactgcctctgttgtgtgcctgctgaataacttctatcccagagaggc caaagtacagtggaaggtggataacgccctccaatcgggtaactcccaggagagtgtcacagagcaggacagcaaggacagcacc tacagcctcagcagcaccctgacgctgagcaaagcagactacgagaaacacaaagtctacgcctgcgaagtcacccatcagggcct gagctcgcccgtcacaaagagcttcaacaggggagagtgt (SEQ ID NO: 24)

(3) Amino acid sequences of light chain and heavy chain of MIL204

The heavy chain of MIL204 has the amino acid sequence of SEQ ID NO: 25.

The light chain of MIL204 has the amino acid sequence of SEQ ID NO: 26.

(4) Nucleic acid sequences of light chain and heavy chain of MIL204

The nucleotide sequence of heavy chain of MIL204 is shown in SEQ ID NO: 21, wherein the underlined part is base sequence of heavy chain variable region.

gaggtgcagctggtggagagcggcggcggcctggtgcagcccggcggcagcctgcgcctgtcctgcgccgccagcggct tcacctttaccgactacaccatggactgggtgcgccaggctcccggcaagggcctggagtgggtggccgacgtgaaccccaacagc ggcggcagcatctacaaccagcgcttcaagggccgcttcaccctgagcgtggaccgcagcaagaacaccctgtacctgcagatgaa cagcctgcgcgccgaggacaccgccgtgtactactgcgcccgcaacctgggccccagcttctacttcgactattgggggcagggca ccctggtcaccgtgagcagcgctagcaccaagggcccatcggtcttccccctggcaccctcctccaagagcacctctgggggcaca gcggccctgggctgcctggtcaaggactacttccccgaaccggtgacggtgtcgtggaactcaggcgccctgaccagcggcgtgca caccttcccggctgtcctacagtcctcaggactctactccctcagcagcgtggtgactgtgccctctagcagcttgggcacccagacct acatctgcaacgtgaatcacaagcccagcaacaccaaggtggacaagaaagttgagcccaaatcttgtgacaaaactcacacatgcc caccgtgcccagcacctgaactcctgggggggaccgtcagtcttcctcttcccccaaaacccaaggacaccctcatgatctcccggac ccctgaggtcacatgcgtggtggtggacgtgagccacgaagaccctgaggtcaagttcaactggtacgtggacggcgtggaggtgc ataatgccaagacaaagccgcgggaggagcagtacaacagcacgtaccgtgtggtcagcgtcctcaccgtcctgcaccaggactgg ctgaatggcaaggagtacaagtgcaaggtctccaacaaagccctcccagcccccatcgagaaaaccatctccaaagccaaagggca gccccgagaaccacaggtgtacaccctgcccccatcccgggaagagatgaccaagaaccaggtcagcctgtggtgcctggtcaaa

ggcttctatcccagcgacatcgccgtggagtgggagagcaatgggcagccggagaacaactacaagaccacgcctcccgtgctgg actccgacggctccttcttcctctacagcaagctcaccgtggacaagagcaggtggcagcaggggaacgtcttctcatgctccgtgat gcatgaggctctgcacaaccactacacgcagaagagcctctccctgtctccgggtaaa (SEQ ID NO: 21)

The nucleotide sequence of light chain of MIL204 is shown in SEQ ID NO: 27, wherein the underlined part is base sequence of heavy chain variable region.

gatatccagatgacccagagcccctccagcctgtccgccagcgtgggcgaccgcgtgaccatcacctgcaaggccagcca ggacgtgagcatcggcgtggcctggtaccagcagaagcccggcaaggcccccaagctgctgatctacagcgcctcctaccgctaca ccggcgtgccctcccgcttcagcggctccggcagcggcaccgactttaccctgaccatctccagcctgcagcccgaggactttgcca cctactactgccagcagtactacatctatccctataccttcggccagggcaccaaggtggagatcaagcgtacggtggctgcaccatct gtcttcatcttcccgccatctgatgagcagttgaaatctggaactgcctctgttgtgtgcctgctgaataacttctatcccagagaggccaa agtacagtggaaggtggataacgccctccaatcgggtaactcccaggagagtgtcacagagcaggacagcaaggacagcacctac agcctcagcagcaccctgacgctgagcaaagcagactacgagaaacacaaagtctacgcctgcgaagtcacccatcagggcctga gctcgcccgtcacaaagagcttcaacaggggagagtgt (SEQ ID NO: 27)

Example 2: Construction of 203 antibody eukaryotic expression vector and 204 antibody eukaryotic expression vector

Expression vector pTGS-FRT-DHFR (Chinese patent ZL200510064335.0) was used, hygromycin selecting label was removed, GS (glutamine synthetase) expression box was added via PshA1 and Xho1 restriction enzyme cutting sites and used as selection markers; wherein GS cDNA could be obtained via RT-PCR from cell line CHO that expressed GS. The vector obtained by modification was named as GS vector.

Based on the GS vector, the completely synthesized light chain constant region (the constant region sequence was SEQ ID NO: 24 or the non-underlined sequence in SEQ ID NO: 27) was inserted via BsiwI and NotI restriction enzyme cutting sites; then, the completely synthesized 203 heavy chain constant region and 204 heavy chain constant region (the constant region sequences were separately non-underlined sequences in SEQ ID NO: 20 and SEQ ID NO:21) were separately inserted via Nhe I and XhoI restriction enzyme cutting sites; and after modification of constant regions, GS-203 vector containing 203 light chain constant region and heavy chain constant region and GS-204 vector containing 204 light chain constant region and heavy chain constant region were separately obtained.

The genes for 203 light chain variable region and heavy chain variable region and 204 light chain variable region and heavy chain variable region (which were separately

underlined sequences in SEQ ID NO: 24, SEQ ID NO: 27, SEQ ID NO: 20 and SEQ ID NO: 21) were completely synthesized, and inserted via construction into pGEM-TEasy vector to obtain vectors separately named as pGEM-TEasy-203/Vκ vector, pGEM-TEasy-204/Vκ vector, pGEM-TEasy-203/V_H vector and pGEM-TEasy-204/V_H vector.

The pGEM-TEasy-203/Vκ and pGEM-TEasy-204/Vκ were separately digested with ClaI and BsiwI, to separately obtain 203 light chain variable region gene and 204 light chain variable region gene.

The GS-203 vector and GS-204 vector as above constructed were separately taken in an amount of 1μg, and separately digested with ClaI and BsiwI.

The GS-203 vector digested with ClaI and BsiwI as above-obtained and 203 light chain variable region were linked with T4 DNA ligase; and the GS-204 vector digested with ClaI and BsiwI as above-obtained and 204 light chain variable region were linked with T4 DNA ligase. The resultant plasmids carrying 203 light chain and 204 light chain were separately named as pTGS-203Vκ vector and pTGS-204Vκ vector.

The pGEM-TEasy-203/V_H and pGEM-TEasy-204/V_H were separately taken and digested with EcoR I and Nhe I, to separately obtain 203 heavy chain variable region gene and 204 heavy chain variable region gene. The pTGS-203Vκ vector and pTGS-204Vκ vector were separately taken in amount of 1μg, and separately digested with EcoR I and Nhe I. The pTGS-203Vκ digested with EcoR I and Nhe I as above obtained and 203 heavy chain variable region gene, as well as the pTGS-204Vκ and 204 heavy chain variable region gene were separately linked with T4 DNA ligase. Based on the pTGS-203Vκ and the pTGS-204Vκ, the plasmids separately carrying antibody 203 heavy chain variable region gene and antibody 204 heavy chain variable region gene were obtained, which were separately named as 203 antibody eukaryotic expression vector and 204 antibody eukaryotic expression vector.

Example 3: Fucose knockout and suspension acclimatization of host cells

CHO-K1 cells (ATCC: 58995535) purchased from ATCC were subjected to gene knockout so that the proteins expressed by themselves nearly or completely did not have fucosylation modification, and the obtained fucose-knockout host cells were named as CHOK1-AF. Specific method comprised: modifying expression system by genetic engineering technique, in which site-specific knockout of key protein GFT for fucosylation modification route was carried out in host cell CHO-K1 for antibody expression to effectively reduce fucose modification level of antibody. This method could simultaneously block typical

fucosylation mechanism and compensation mechanism, so as to achieve complete removal of fucosylation. Specific technical route was shown in FIG. 2, in which by using zinc-finger nuclease technique, two GFT zinc-finger nuclease sequences were designed for GFT gene SLC35c1 sequence (GenBank: BAE16173.1) and separately used to bind double-stranded DNA of target genes. Expression vector plasmids were correspondingly constructed, and the two plasmids were co-transfected into CHO-K1 cells by electrotransfection technique. The transfected cells were static cultivated on 6-well plate for 24h and then transferred in 125mL shake flask and cultured under shaking so as to perform passage and amplification in the shake flask. By using the specific affinity of saccharide-binding agglutinin LCA (Lens culinaris agglutinin) to protein fucosyl, the co-transfected cells were stained with biotin-LCA, negative separation was carried out by using anti-biotin microBeads and MACs LD column in combination, clonal culture was further performed, and fucose knockout level of clonal cells was determined by flow cytometry technique; and clone 1G7 without fucosylation modification was obtained via multi-turns of negative separation and clonal culture.

FIG.3 shows the fucose expression level of CHO-K1 cells (A) and CHOK1-AF cells (B). The dark color-filled peak refers to the control cells which do not express fucose. The black line peak represents the fucose expression level of CHO-K1 (A) or CHOK1-AF cells (B) determined by FCAS using Lens culinaris agglutinin (LCA) reagent, which has high specific binding affinity to the fucose unit. The results show that CHO-K1 cells express high level of fucose and CHOK1-AF cells do not express the fucose.

The CHOK1-AF cell was deposited in China General Microbiological Culture Collection Center (No. 1 West Beichen Road, Chaoyang District, Beijing 100101, China) on June 14, 2017, with a deposit number of CGMCC No.14287.

The total RNA of clone 1G7 without fucosylation modification was extracted, after reverse transcription, the gene encoding GDP transport protein was taken and sequenced to confirm that this gene was mutated successfully, and could not be normally expressed.

Further acclimatization and culture: post-thawed host cell gmt4$^-$-CHO-K1 was subjected to adherent culture in seed culture medium (see: Table 1-1) (containing 10% calf serum), serum was gradually reduced (from 10%, 5%, 2.5%, 1%, 0.5%, to totally free of serum), transferred in a shake flask to perform suspension acclimatization, and passage was performed by about 10 times in total. When host cells were completely suspended and stably increased exponentially, stable host cells capable of growing in seed culture medium were finally obtained.

Example 4: Preparation of supernatant containing MIL203AF and MIL204AF antibodies

By using electrotransfection technique, the 203 antibody eukaryotic expression vector and 204 antibody eukaryotic expression vector obtained in Example 2 were separately transfected into target host cell CHOK1-AF, 50μM MSX (methionine sulfoxmine) was added to seed culture medium, culture was performed at 37°C, CO_2 incubator for 2-4 weeks, the cells survived in this culture medium were picked out, and ELISA method was used to detect cells capable of expressing antibody. Subclone screening was performed by limiting dilution method, and after 6-8 weeks of culture and screening, monoclonal cell lines capable of effectively expressing MIL203AF and MIL204AF antibodies were obtained.

Preparation of specific culture media: the culture media were prepared according to the components as shown in Tables 1-1, 1-2 and 1-3. After being filtered under sterile condition with 0.22μm membrane, they were used for cell culture.

Table 1-1: Seed culture medium

No.	Component	Content
1	water for injection (25±5°C)	0.9L
2	Pluronic F-68	1.0g/L
3	Glucose	8.8g/L
4	Culture medium powder Maxgrow 202	7.44g/L
5	sodium bicarbonate	1.98g/L
6	sodium chloride	3.47g/L
7	1M HEPES	15ml/L
8	5M HCl or 5M NaOH	Regulated to pH = 7.0±0.1
diluted to 1L		

Table 1-2: Production culture medium

No.	Component	Content
1	water for injection (25±5°C)	0.8L
2	Sodium hydroxide	0.8g/L
3	Culture medium powder Maxpro 302	11.5g/L
4	1g/L vitamin B12 stock solution	1-2ml/L
5	10g/L ferrous sulfate stock solution	0.4-0.6ml/L

6	Sodium dihydrogen phosphate monohydrate	0.35g/L
7	Glucose (monohydrate)	8.8g/L
8	L-cysteine hydrochloride monohydrate	0.3-0.375g/L
9	Pluronic F-68	1 g/L
11	sodium chloride	1.55g/L
12	5M HCl	5.6ml/L
13	sodium bicarbonate	1.22g/L
14	1M HEPES	7.5ml/L
15	5M HCl or 5M NaOH	Regulated to pH = 7.0±0.1
diluted to 1L		

Table 1-3: Fed-batch culture medium

No.	Component	Content
1	water for injection (25±5°C)	0.8L
2	5M NaOH	7.325mL
3	Anhydrous disodium hydrogen phosphate	3.09g/L
4	Fed-batch medium powder Maxfeed 402	39.03 g/L
5	50g/L L-tyrosine disodium salt dihydrate	23.8mL
6	50g/L L-cysteine hydrochloride monohydrate	23.2mL
7	Glucose	50.0g/L
8	1.75g/L vitamin B12	0.3mL
9	5g/L ferrous sulfate heptahydrate	0.3mL
10	Pluronic F-68	0.3g
11	sodium chloride	0.24g
12	sodium bicarbonate	0.366g
13	5M HCl or 5M NaOH	Regulated to pH = 7.0±0.1
diluted to 1L		

The cell line was amplified by multi-step culture with culture media, in which seeding density was $0.5\pm0.2\times10^6$ cells/ml, passage was performed once per 2-4 days, when sufficient cells were obtained by amplification, they were transferred to fermentation culture medium

(the medium comprised: production culture medium : seed culturing medium = 1: 1), the culture period in the fermentation culture medium was 12-14 days, and fed-batch culture medium was added in 10% volume on the 3rd, 6th, 9th day, the supernatant was obtained after the end of culture. Thus, MIL203AF and MIL204AF were obtained, respectively.

The method for preparing MIL203 and MIL204 referred to the method for preparing MIL203AF and MIL204AF in the present example, except that the host cells were CHO-K1 cells (ATCC: 58995535) purchased from ATCC, and fucose knockout was not carried out.

Example 5: Assembling MBS301 bispecific antibody

1. Capture of semi-antibody

The supernatant of cell fermentation broth obtained in Example 4 was filtered with 0.2μm membrane, and capture was performed by using Protein A column. Firstly, the column was balanced with low-salt Tris, pH7.5 buffer solution, then the supernatant was loaded, the column was then eluted with low-salt Tris, pH7.5 buffer solution, the column was further eluted with high-salt potassium phosphate, pH6.0 buffer solution, the column was then eluted and balanced with low-salt Tris, pH7.5 buffer solution, and finally eluted with low pH acetate buffer solution to obtain semi-antibody. The semi-antibody solution was regulated with Tris base solution to pH5.5, added with a suitable amount of Arg and preserved.

2. Assembly

The concentration of semi-antibody was determined with 280nm absorbance using spectrophotometer. The semi-antibody was mixed in molar ratio of 1:1, regulated with Tris Base buffer solution to pH8.0, added with an amount of reducing agent GSH, reacted at 25°C and low speed stirring overnight. The reducing agent was removed by desalting column (or ultrafiltration), and the reaction was terminated.

3. Anion (QSFF)

The sample as assembled and replaced was regulated to have pH of 8.0, conductivity of 3.5 mS/cm, filtered with 0.22μm membrane. Firstly, an anionic chromatographic column was balanced with low-salt Tris, pH8.0 buffer solution, then the sample was loaded on the anionic chromatographic column, breakthrough component was collected, low salt Tris, pH8.0 buffer solution was then used for elution until UV280 trended to base line. The collected breakthrough sample was regulated with acetic acid solution to pH5.5.

4. Cation (50HS)

The sample as collected in the anion procedure was filtered with 0.22μm membrane. The sample was loaded on 50HS column, then balanced with low-concentration acetate,

pH5.5 buffer solution, eluted in linear gradient manner with 0-100% high-concentration acetate, pH 5.5, 20 CV, and eluted components were collected.

The obtained MBS301 antibody was used in the following examples.

Example 6: Determining molecular weight by mass spectra

1. Experimental method

Preparation of de-sugared sample: 500μg of MBS301 antibody was desalted with 10kD ultrafiltration tube, added with 10μL of G7 digestion buffer solution, 3μL of PNGase F, diluted with ultrapure water to 100μL, mixed homogeneously and sealed with sealing film, and placed in 37°C water-bath overnight;

LC-MS analysis: the MBS301 or the de-sugared sample was diluted to 2.5mg/ml, desalted with PLRP-S chromatographic column: using 10 min gradient from 95% mobile phase A (0.1%FA water), 5% mobile phase B (0.1%FA acetonitrile) to 95% mobile phase B, and maintaining for 10min; after being desalted with reverse chromatographic column, mass spectrometry was performed with TripleTOF 4600 (AB Sciex), and data was subjected to deconvolution analysis with Analyst TF1.6.

2. Experimental results

The mass spectrometry results of intact protein molecular weight of MBS301 were shown in FIG. 4, MBS301 consisted of a plurality of molecules with different molecular weights, which corresponded to different glycotypes, and fucose was not found in these glycotypes.

After removal of N-saccharide modification, the spectrometry results of MBS301 were shown in FIG. 5, in which its intact protein molecular weight of 145,154 was in conformity with the theoretical molecular weight, which indicated that the assembly of MIL203AF and MIL204AF was successful.

Example 7: Molecular-exclusion chromatography (SEC-HPLC)

1. Experimental method

Mobile phase: 0.2 mol/L potassium phosphate buffer solution, 0.25 mol/L potassium chloride, pH6.2±0.1

Preparation of sample: the sample to be tested was diluted with mobile phase to 2 mg/mL

Chromatographic conditions: sample injector temperature was 6°C, sample size: 25μl, flow rate: 0.5ml/min, signal: 280 nm, column temperature: 30°C, isocratic elution for 30min.

2. Experimental results

The SEC spectra of the MIL203AF, MIL204AF before assembly and the MBS301 after assembly were shown in FIG. 6. It could be seen that before assembly, MIL203AF had many semi-antibody (44.7%) and macromolecules; before assembly, MIL204AF had a broad monomer peak pattern, which indicated that their molecular sizes were not evenly distributed; however, after assembly of MBS301, the molecular size distribution pattern becomes clean, and monomer purity was 99.1%.

Example 8: N-glycotype analysis

1. Experimental method:

500μg of antibody was desalted with 10kD ultrafiltration tube, added with 10μL of G7 digestion buffer solution, 3μL of PNGase F, diluted with ultrapure water to 100μL, mixed evenly and sealed with sealing film, placed in 37°C water-bath overnight. The digested sample was added to 300μL of pre-cooled ethanol, mixed evenly and stood for 30min, centrifuged at 12000rpm for 5 min, the supernatant was taken and concentrated and dried under vacuum. DMSO and acetic acid were mixed in ratio of 350μL:150μL, 5mg of 2-AB, 6mg of Sodium Cyanoborohydride were taken and dissolved in 100μL of the mixture solution of DMSO and acetic acid, 10μL of the mixture solution was taken, placed in 65°C oven, after derivation for 3h, 200μL of a mixture solution of 80% acetonitrile and water was added, centrifuged for 2min, and supernatant was collected.

Chromatographic column: WATERS Acquity UPLC BEH Amide 1.7μm, 2.1×50mm Column;

Column temperature: 40°C;

Excitation wavelength: λex=330nm; λem=420nm;

Sample size: 10μL;

The chromatographic column was balanced with 20% mobile phase A (100mM ammonium formate pH4.5), 80% mobile phase B (100% acetonitrile), after loading sample, the percentage of phase A was increased to 40% after 36min.

2. Experimental results:

The assembled MIL203/204, MBS301 had glycotype spectra as shown in FIG. 7. It could be seen in FIG. 7 and Tables 2 and 3 that, in comparison with MIL203/204, MBS301

had a significantly decreased fucose content, and the percentage of fucose-containing glycotype G0F was only 1.1%.

Table 2: Percentages of glycotypes of MIL203/204 glycotypes

Name	G0F-GN	G0	G0F	MAN5	G1F	G1F'	G2F	G2FS	G2FS2
203-204	1.06	0.62	44.73	0.98	9.27	5.87	8.69	10.53	9.40

Table 3: Percentages of glycotypes of MBS301

Name	G0-GN	G0	G0F	MAN5	G1	G1'	G2	G2S	G2S2
203AF-204AF	3.34	46.50	1.1	1.49	7.73	6.11	4.89	5.65	7.77

Example 9: Analysis of Her2 binding activity for antibody

1. Experimental method:

HBS-EP+ Buffer was used to dilute MIL40, MIL41, mixture of MIL40 and MIL41 (1:1), MIL203/204, and MBS301 samples to 0.1μg/ml, respectively, to form ligands. HER2 (Sino Biological Inc, 10004-H08H) was diluted with HBS-EP+ Buffer to 4μg/ml, 2μg/ml, 1μg/ml, 0.5μg/ml, 0.25μg/ml and 0.125μg/ml, to form analytes. The ligands (antibodies) were fixed by an indirect capture method, in which 25μg/ml of Anti-Human IgG antibody (BR100839, GE) was firstly bound to surface of CM5 chip via amino coupling covalent bond, then ligands and analytes were bound. Under BIACORE® (analyzers for automatically measuring and investigating the interactions of biomolecules) Wizard mode, affinity analysis experiment was performed in multi-cycle mode by separately using MIL40, MIL41, mixture of MIL40 and MIL41, and MBS301 samples as ligands, and using HER2 as analytes. The analysis for each sample comprised 3 start-up samples, 1 zero concentration control sample, 6 gradient concentration samples, and 1 repeat concentration sample, after the end of each cycle, the chip was regenerated with 3M $MgCl_2$ regenerating solution. The capture time for each concentration cycle of analyte was set as 90s, ligand solution flow rate was 10 μl/min; the binding time for ligand and analyte was 180s, analyte solution flow rate was 30 μl/min; dissociation time was 1200s. The original data was introduced in BIACORE® X100 analysis software, zero concentration control was deducted, reference channel was deducted to

eliminate volume effect, and 1:1 binding mode of Kinetics analysis method was used for fitting curves, and data were collated.

2. Experimental results:

Table 4. The Her2 binding dynamic constants determined by Biacore technique

Sample name	ka (1/Ms)	kd (1/s)	K_D(M)
MIL40	3.293 E+5	1.772E-4	5.383E-10
MIL41	1.974E+5	2.117E-4	1.073E-9
MIL40/MIL41	3.172E+5	1.481E-4	4.668E-10
MIL203-204	3.320E+5	1.240E-4	3.735E-10
MBS301	3.465 E+5	1.161E-4	3.350E-10

It could be seen from the table that according to binding dynamic constants, MBS301 and MIL203/204 were superior to MIL41 in Her2 binding activity, and substantially equivalent to MIL40, and the mixture of MIL40 and MIL41 (1:1).

Example 10: Analysis of FcγRIIIa binding activity

1. Experimental method:

FcγRIIIa (Sino Biological Inc, 10389-H08C1) was diluted with HBS-EP Buffer to 0.2 μg/ml, to form a ligand. HBS-EP Buffer was used for separately diluting MIL40, MIL41, mixture of MIL40 and MIL41 (1:1), MIL203/204, and MBS301 samples to 360μg/ml, 120μg/ml, 40μg/ml, 13.3μg/ml, 4.4μg/ml, to form analytes. The ligand FcγRIIIa was fixed by indirect capture method, in which 50μg/ml of Anti-His IgG was firstly bound to surface of CM5 chip via amino coupling covalent bond, then the ligand and analyte were bound. Under Biacore Wizard mode, affinity analysis experiment was performed in multi-cycle mode by using FcγRIIIa as ligand and separately using MIL40, MIL41, mixture of MIL40 and MIL41, and MBS301 samples as analyte, respectively. The analysis for each sample comprised 3 start-up samples, 1 zero concentration control sample, 5 gradient concentration samples, and 1 repeat concentration sample, after the end of each cycle, the chip was regenerated with 10 mM Glycine-HCl, pH 1.5 regenerating solution. The capture time for each concentration cycle of analyte was set as 60s, ligand solution flow rate was 10 μl/min; the binding time for ligand and analyte was 180s, analyte solution flow rate was 30 μl/min; dissociation time was 180s. The CM5 chip coupled with Anti-His IgG was placed in slot, and samples were tested and analyzed. The original data was introduced in BIACORE™ X100 analysis software, zero

concentration control was deducted, reference channel was deducted to eliminate volume effect, and homeostasis model assessment of affinity analysis method was used for fitting curves, and data were collated.

2. Experimental results:

It could be seen from Table 6 that MBS301 showed the lowest K_D value, which indicated that it had the strongest binding activity to FcγRIIIa, obviously stronger than that of MIL40, MIL41, mixture of MIL40 and MIL41, MIL203/204, and this exhibited the superiority of glycosylation-modified MBS301.

Table 5.

	K_D(M) E-7	K_D(M)E-7	K_DMean (M)E-7
MIL41	8.290	8.059	8.175
MIL40	3.194	3.022	3.108
MBS301	1.252	1.096	1.174
MIL203/204	5.886	5.852	5.869
MIL41/MIL40 mixture	4.312	4.297	4.305

Example 11: Analysis of ADCC activity

1. Experimental method:

Target breast cancer cell SKBR-3 (purchased from ATCC, CRL-2326), effector cell NK92MI-CD16a (purchased from Huabo Bio) were centrifuged at 1200 rpm for 4 min, supernatants were discarded, ADCC experimental culture medium was used to resuspend cells, then centrifuged at 1200rpm for 4min, supernatants were discarded, ADCC experimental culture medium was used to resuspend cells, and the cell viability should be ≥90% according to cell counting. SKBR-3 cell density was regulated to 1.25×10^5/ml, NK92MI-CD16a cell density was regulated to 6.25×10^5/ml.

Antibodies of different concentrations were separately added to achieve final concentrations of 0.000001μg/ml, 0.00001μg/ml, 0.0001μg/ml, 0.001μg/ml, 0.01μg/ml, 0.1μg/ml, 1μg/ml, 10μg/ml, respectively, then effector cells and target cells (effector-target ratio was 5:1) were added, incubated at 37°C for 6h, LDH developing solution was added, 100μL/well, stood away from light at room temperature for 20 min. Determination was performed with MD SpectraMax i3.

With regard to target breast cancer cell BT474 (purchased from ATCC, CRL-2326), colon cancer SW480 (purchased from the Cell Bank of Chinese Academy of Sciences,

TCHU172), the ratio of ADCC effector cell to target cell was 10:1, that is, the target cell density was 1.25×10^5/ml, and the effector cell density was 1.25×10^6/ml. Other methods were the same for SKBR-3.

With regard to target breast cancer cell HCC1419 (Trastuzumab resistant, purchased from ATCC, CRL-2326), the ADCC action method was the same for SKBR-3.

Calculation of killing rate:

Background group: culture medium group

Minimum release group: target cell group

Maximum release group: target cell + lysis solution group

Experimental groups: target cell + effector cell

Killing rate (%)= [(experimental group – minimum release group) / (maximum release group – minimum release group)] × 100

2. Experimental results:

FIGs. 8-11 show the results that the ADCC activities of MBS301 to different target cells were significantly superior to MIL40, MIL41, MIL40 and MIL41 administrated in combination (1: 1) and MIL203/204, and killing effects depended on antibody dosage.

Example 12: Analysis for direct cell-killing activity

1. Experimental materials

Human breast cancer BT474 cell (purchased from ATCC, HTB-20).

Human breast cancer MDA-MB-175 cell (purchased from ATCC, HTB-25).

Human breast cancer SKBR-3 cell (purchased from ATCC, HTB-30).

Human breast cancer HCC1419 cell (purchased from ATCC, CRL-2326).

Human gastric cancer NCI-N87 cell (purchased from the Cell Bank of Chinese Academy of Sciences, TCHU130).

Among these cells, BT474 was triple positive cell, Her-2 high expression; MDA-MB-175, SKBR-3 HER-2, positive, lower expression in comparison with BT474; HCC1419 was HERCEPTIN®-resistant strain.

2. Experimental method:

Human breast cancer BT474 cells (purchased from ATCC, HTB-20) in logarithmic phase were counted, viability rate > 90%, regulated to have cell density of 6.7×10^4 cells/ml, mixed evenly, inoculated in an amount of 150μl/well on a cell culture plate. Antibody drugs MIL40, MIL41, MIL40/MIL41 administrated in combination, MIL203/204, MBS301 were

diluted then added in an amount of 50μl/well to 96-well culture plate on which cells were spread in advance, for each antibody drug, 9 concentrations, 2.5μg/ml, 1.25μg/ml, 0.625μg/ml, 0.313μg/ml, 0.156μg/ml, 0.078μg/ml, 0.039μg/ml, 0.020μg/ml, 0.010μg/ml, were set, and repeated wells were set for each concentration; in addition, a drug-free control group and a cell culture medium blank control group were set as well. The culture plate was placed in a cell incubator and incubated for 120 h, then 10μl of CCK-8 solution was added to each well, after shaking, the culture plate was placed in the incubator and incubated for 3-5 h, OD_{450} values were determined with ELISA. Inhibition rates of drugs to cells were calculated by the following formula: inhibition rate = (1-(drug group OD_{450} – blank group OD_{450}) / (control group OD_{450} – blank group OD_{450}))*100%.

Human breast cancer MDA-MB-175 cells (purchased from ATCC, HTB-25) in logarithmic phase were counted, viability rate >90%, regulated to have cell density of 1×10^5 cells/ml, mixed evenly, inoculated in an amount of 100μl/well on a cell culture 96-well plate. For each antibody drug, 10 concentrations, 500 μg/ml, 125 μg/ml, 31.25 μg/ml, 5.208 μg/ml, 0.868 μg/ml, 0.145 μg/ml, 0.0241 μg/ml, 0.00402 μg/ml, 0.000670 μg/ml, 0.000112 μg/ml, were set. The culture plate was placed in a cell culture incubator and incubated for 72h, and other methods were the same for BT474 cells.

Human breast cancer SKBR-3 cells (purchased from ATCC, HTB-30) in logarithmic phase were counted, viability rate >90%, regulated to have cell density of 1×10^5 cells/ml, mixed evenly, inoculated in an amount of 100μl/well on a 96-well plate for cell culture. For each antibody drug, 9 concentrations, 100 μg/ml, 25 μg/ml, 6.25 μg/ml, 1.56 μg/ml, 0.39 μg/ml, 0.098 μg/ml, 0.0244 μg/ml, 0.0061 μg/ml, 0.0015 μg/ml, were set. The culture plate was placed in a cell culture incubator and incubated for 120h, and other methods were the same for BT474 cells.

Human breast cancer HCC1419 cells (purchased from ATCC, CRL-2326) in logarithmic phase were counted, viability rate >90%, regulated to have cell density of 5×10^4 cells/ml, mixed evenly, inoculated in an amount of 100μl/well on a 96-well plate for cell culture. For each antibody drug, 9 concentrations, 100 μg/ml, 25 μg/ml, 6.25 μg/ml, 1.56 μg/ml, 0.39 μg/ml, 0.098 μg/ml, 0.0244 μg/ml, 0.0061 μg/ml, 0.0015 μg/ml, were set. The culture plate was placed in a cell culture incubator and incubated for 120h, and other methods were the same for BT474 cells.

Human gastric cancer NCI-N87 cells (purchased from the Cell Bank of Chinese Academy of Sciences, TCHU130) in logarithmic phase were counted, viability rate >90%, regulated to have cell density of 5×10^4 cells/ml, mixed evenly, inoculated in an amount of

100μl/well on a 96-well plate for cell culture. For each antibody drug, 9 concentrations, 10 μg/ml, 3.33 μg/ml, 1.11 μg/ml, 0.37 μg/ml, 0.123 μg/ml, 0.041 μg/ml, 0.0137 μg/ml, 0.0045 μg/ml, 0.0015 μg/ml, were set. The culture plate was placed in a cell culture incubator and incubated for 7h, and other methods were the same for BT474 cells.

3. Experimental results:

As shown in FIG. 12, MIL203/204, MBS301 had inhibition rates to BT474 cells higher than those of MIL40, MIL40/MIL41 mixture, and MIL41 had the weakest inhibition activity.

As shown in FIG. 13, MIL203/204, MBS301 had inhibition rates to MDA-MB-175 cells significantly higher than that of MIL40, and very close to the inhibition rates of MIL41, MIL40/MIL41 mixture (1:1).

As shown in FIG. 14, MIL203/204, MBS301 had inhibition rates to SKBR-3 cells higher than those of MIL40, MIL41, MIL40/MIL41 mixture.

As shown in FIG.15, MIL40, MIL41 had no significant inhibition effect to breast cancer cell HCC1419, MIL40/MIL41 administrated in combination (1:1) could inhibit cell proliferation, MIL203/204 and MBS301 showed the highest inhibition rate, and their activities were significantly superior to MIL40 and MIL41 administrated in combination (1:1).

As shown in FIG. 16, MIL40 and MIL41administrated in combination (1:1) had inhibition effect to gastric cancer cell NCI-N87 superior to MIL40, MIL41showed no significant inhibition effect; MIL203/204 and MBS301 showed the highest inhibition rate, and their activities were significantly superior to MIL40 and MIL41 administered in combination (1:1).

Example 13: CDC activity

1. Experimental method:

Target cells BT474 was centrifuged at 1200 rpm for 4 min, supernatant was discarded, the cells were resuspended with 1%FBS culture medium, counted, cell viability should be ≥90%. Cell density of BT474 cells was regulated to 2×10^5/ml, 50μl per well.

Antibodies of different concentrations were separately added, and their final concentrations were 100 μg/ml, 25 μg/ml, 6.25 μg/ml, 1.56 μg/ml, 0.39 μg/ml, 0.098 μg/ml, 0.0244 μg/ml, 0.0061 μg/ml, respectively, 50μl of rabbit complement (1:20 dilution) was added, incubated at 37°C for 2h, added with LDH developing solution, 80μL/well, stood away from light at room temperature for 20 min. Determination was performed with MD SpectraMax i3.

Calculation of killing rate:

Background group: culture medium group

Minimum release group: target cell group

Maximum release group: target cell + lysis solution group

Experimental groups: target cell + complement

Killing rate (%)= [(experimental group – minimum release group) / (maximum release group – minimum release group)] × 100

2. Experimental results:

It could be seen from Fig.17 that MIL40, MIL41 separately acted on target cell BT474 did not exhibit CDC activity, but when they were administered in combination, they showed CDC effect, bifunctional antibodies MIL203/204, MBS301 had CDC activities significantly stronger than that of MIL40 and MIL41 administered in combination, and presented antibody dose-dependent CDC killing effect.

Example 14: Analysis for antibody FcRn binding activity

1. Experimental method

FcRn (Sino Biological Inc, CT009-H08H) was diluted with HBS-EP Buffer to 0.2μg/ml, to form a ligand. HBS-EP Buffer was used for separately diluting MIL40, MIL41, mixture of MIL40 and MIL41, MIL203/204, and MBS301 samples to 360μg/ml, 120μg/ml, 40μg/ml, 13.3μg/ml, 4.4μg/ml, to form analytes. The ligand FcRn-His tag was fixed by indirect capture method, in which 50μg/ml of Anti-His IgG was firstly bound to surface of CM5 chip via amino coupling covalent bond, then the ligand and analytes were bound. Under Biacore Wizard mode, affinity analysis experiment was performed in multi-cycle mode by separately using FcRn as ligand, using MIL40, MIL41, mixture of MIL40 and MIL41, and MBS301 samples as analytes. The analysis for each sample comprised 3 startup samples, 1 zero concentration control sample, 5 gradient concentration samples, and 1 repeat concentration sample, after the end of each cycle, the chip was regenerated with 10 mM Glycine-HCl, pH 1.5 regenerating solution. The capture time for each concentration cycle of analyte was set as 60s, ligand solution flow rate was 10 μl/min; the binding time for ligand and analyte was 180s, analyte solution flow rate was 30 μl/min; dissociation time was 180s. The CM5 chip coupled with Anti-His IgG was placed in slot, and samples were tested and analyzed. The original data was introduced in BIACORE^{TM} X100 analysis software, zero concentration control was deducted, reference channel was deducted to eliminate volume

effect, and homeostasis model assessment of affinity analysis method was used for fitting curves, and data were collated.

2. Experimental results:

It could be seen from Table 6 that MBS301 showed the lowest K_D value, which indicated that it had the strongest binding activity to FcRn, significantly superior to MIL40, MIL41, mixture of MIL40 and MIL41, and substantially equivalent to MIL203/204.

Table 6. The FcRn binding dynamic constants determined by Biacore technique

	K_D(M) E-7	K_D(M) E-7	K_D mean (M)E-7
MIL41	5.337	4.495	4.916
MIL40	5.891	5.60	5.746
MBS301	1.930	2.128	2.029
MIL203/204	2.081	2.059	2.07
MIL41/MIL40 MIX	6.050	4.034	5.042

Example 15: Experiment for *in vivo* tumor suppression in nude mice

1. Experimental method

6-8 week Nu/Nu nude mice, bodyweight 17.0-22.0g, 80 female mice/batch, purchased from Beijing Vital River Experimental Animal Technology Co., Ltd., animal certificate: SCXK (Beijing)-2012-0001. The experimental animals were fed sterile IVC cages with independent air supply, 5 mice per cage. Padding material was corncob padding material (size: 4-6 mm) sterilized with ^{60}Co radiation, the mice were fed with sterilized fodder that was specifically formulated for mice, and given purified water to drink freely. In laboratory for animal experiment, room temperature was kept around 25°C, relative humidity was kept at 40-70%, and illuminated 12 h per day.

The nude mice were hypodermically inoculated with SKO-V3. When tumor volumes were grown to be about 1500-2000mm^3, tumor blocks were taken out under aseptic condition, and cut into about 1.0×1.0×1.0mm^3 pieces, which was hypodermically inoculated to nude mice at axilla of right forelimb. After hypodermically inoculated tumors had sizes of 100-300mm^3, they were randomly grouped according to tumor size. SKO-V3 cell culture: the cells were cultured in DMEM cell culture medium containing 10% fetal calf serum (supplemented with penicillin and streptomycin, 100μl/ml for each), placed in a cell incubator at 37°C and 5%CO_2, medium was replaced once per 1-2 days. Passage was

performed by using 0.25% trypsin digestion, after centrifugation at 1000r/min for 5 min, supernatant was discarded, and fresh culture medium was added for passage and culture.

After hypodermic transplantation, tumor-bearing animals that meet standards were selected, and randomly grouped according to tumor size, about 8 animals per group, administration was performed by caudal vein injection, twice per week, for consecutive 2 weeks.

The experimental animals were observed every day in terms of taking food, drinking water and movement, bodyweight and tumor size of each animal were measured every 3 days, and the animals were executed by neck dislocation at the end of experiment, tumors visible to naked eye were stripped and weighed. All tissues obtained by dissection were placed and preserved in 4% formaldehyde for conventional pathological detection.

The data were expressed in X± s; tumor growth inhibition rate = (experimental group tumor volume – administration group tumor volume) / control group tumor volume × 100%; tumor volume = $1/2ab^2$ (a = tumor long diameter; b = tumor short diameter);

2. Experimental results

As shown in FIG. 18, all tumors in tumor-bearing mice grew, in which tumors of control group showed progressive growth, while the growth of tumors of administration groups was slowed down to different degrees or stopped. At the end of observation period, the nude mice of control group were of drooped spirit, asarcia, skin shrinkage, and slow moving.

Tumor growth curves were plotted according to tumor sizes and time. In SKO-V3 cell tumor-bearing mice group, bifunctional antibodies MIL203/204, MBS301, and MIL40/MIL41 administrated in combination could effectively inhibit growth of SKO-V3 tumors, and their tumor inhibition abilities were superior to MIL40 and MIL41 alone.

Example 16. Anti-Her2 bispecific antibody MBS301 treatment for reducing human breast tumor volume in mice

Human Breast Cancer Cell line BT474

This human breast cancer cell line has been established from the ductal carcinoma of a breast cancer patient. BT474 cell line was routinely cultured in DMEM medium (Gibco, America) supplemented with 10 % fetal bovine serum (Gibco, America) at 37°C in a water- saturated atmosphere at 5% $C0_2$.

Mice

Female BALB/c Nude mice; age 5-6 weeks; body weight 15-17 g (Beijing Vital River Laboratory Animal Technology Co., Ltd.); they were maintained under specific-pathogen-

free condition with daily cycles of 12 h light and 12 h darkness. After arrival animals were housed in the quarantine part of the animal facility for one week to get accustomed to new environment and for observation. Food and water were provided ad libitum.

Tumor cell injection

At the day of injection tumor cells were harvested from culture flasks. Cell titer was adjusted to 1 x 10^8/ml. Before injection, 17β-ESTRADIOL pellet (Innovative Research of America) was subcutaneously implanted into the back of BALB/c nude mice. Tumor cell suspension was carefully mixed with MATRIGEL® (biological cell culture substrate) at the ratio of 1:1, then the cell suspension was 5 x 10e7/ml, BT474 cells were injected in a volume of 0.2ml into the right mammary fat pad of each mouse.

Treatment

Mice were randomized for tumor volume of 125mm^3 and subsequently treated twice weekly with a volume of l0ml/kg intravenous injection. For combination treatment MIL40 and MIL41 were given at the same time (see Table 7).

Table 7.

Group	No. of animals	Compound	Dose (mg/kg)	Route/Mode of administration
1	6	vehicle	-	i.v. twice weekly
2	6	MIL40	13.5	i.v. twice weekly
3	6	MIL41	13.5	i.v. twice weekly
4	6	MIL40 plus MIL41	6.75 plus 6.75	i.v. twice weekly
5	6	MIL203/304	13.5	i.v. twice weekly
6	6	MBS301	13.5	i.v. twice weekly

The results are shown in FIG. 19. MBS301 inhibited the growth of BT474 tumors more effectively than MIL40, as effectively as the 1:1 mixture of MIL40 with MIL41.

Example 17. Anti-Her2 bispecific antibody MBS301 treatment for reducing human stomach tumor volume in mice

Human Gastric Cancer Cell Line NCI-N87

This human stomach cancer cell has derived from metastatic site of NCI-N87 cell line was routinely cultured in 1640 medium (Gibco, America) supplemented with 10 % fetal bovine serum (Gibco, America) at 37°C in a water- saturated atmosphere at 5% CO_2.

Mice

Female BALB/c Nude mice; age 6-7 weeks; body weight 18-22 g (Beijing Vital River Laboratory Animal Technology Co., Ltd.); they were maintained under specific-pathogen-free condition with daily cycles of 12 h light and 12 h darkness. After arrival animals were housed in the quarantine part of the animal facility for one week to get accustomed to new environment and for observation. Food and water were provided ad libitum.

Tumor cell injection

At the day of injection tumor cells were harvested from culture flasks. Cell titer was adjusted to 5 x 10e7 / ml. Tumor cell suspension was carefully mixed with Matrigel at the ratio of 1:1, then the cell suspension was 2.5 x 10e7/ml, NCI-N87 cells were subcutaneously injected in a volume of 0.2ml into the right back of each mouse.

Treatment

Mice were randomized for tumor volume of 110mm^3 and subsequently treated o weekly with a volume of 10ml/kg intravenous injection. For combination treatment MIL40 and MIL41 were given at the same time (see Table 8).

Table 8.

Group	**No. of animals**	**Compound**	**Dose (mg/kg)**	**Route/Mode of administration**
1	6	vehicle	-	i.v. once weekly
2	6	MIL40	20	i.v. once weekly
3	6	MIL41	20	i.v. once weekly
4	6	MIL40 plus MIL41	10 plus 10	i.v. once weekly
5	6	MIL203/304	20	i.v. once weekly
6	6	MBS301(=MBS301)	20	i.v. once weekly

The results are shown in FIG. 20. MBS301 inhibited the growth of NCI-N87 tumors more effectively than MIL40, as effectively as MIL40 concomitant with MIL41.

In the *in vivo* tumor growth inhibition studies of Examples 16 and 17, both MIL40/MIL41 combination and MBS301 inhibited tumor growth, and there was no significant difference between the two groups. The additional tumor cell killing activity via ADCC of afucosylated MBS301 was not displayed in the results of Example 16 and 17; this is because humanized antibody cannot activate NK cells and macrophages of BALB/c nude mice. However, in the *in vitro* cell based ADCC assays, MBS301 exhibited significant enhanced ADCC activity in comparison with the mixture of MIL40 with MIL41 (see Examples 11-13, and FIGs. 8-17).

Example 18. Human Gastric cancer GA0055 Patient derived xenograft (PDX) nude mice model

This tumor tissue has been established from the stomach of an Asian female, age 69, its

pathology diagnosis was clear cell adenocarcinoma of anterior wall of gastric antrum, ulcerative type, IHC(immunohistochemistry) results was HER-2(+) with high mRNA expression level.

Mice

Female BALB/c Nude mice were maintained under specific-pathogen-free condition with daily cycles of 12 h light and 12 h darkness. After arrival, animals were housed in the quarantine part of the animal facility for one week to get accustomed to new environment and for observation. Food and water were provided ad libitum.

Tumor Inoculation

Each mouse was inoculated subcutaneously at the right flank with primary human gastric cancer model GA0055 fragment (2-3 mm in diameter) for tumor development. When average tumor size reached 146 mm^3, mice were randomly grouped into 3 groups (see Table 9).

Table 9.

Group	No. of animals	Compound	Dose (mg/kg)	Route/Mode of administration
1	6	vehicle	-	i.v. twice weekly
2	6	HERCEPTIN®	13.5	i.v. twice weekly

3	6	MBS301	13.5	i.v. twice weekly

In this stomach cancer PDX model, MBS301 inhibited the growth of tumors more effectively than HERCEPTIN®, the final tumor growth inhibition ratio of MBS301 is 77.82%, while Herceptin is 50.15%. After treatment for 18 days, there was significant difference in tumor size between MBS301 and Herceptin, as shown in FIG. 21.

It is to be understood that the foregoing describes preferred embodiments of the present invention and that modifications may be made therein without departing from the scope of the present invention as set forth in the claims.

WHAT IS CLAIMED IS:

1. A humanized bispecific anti-Her2 antibody or an antigen binding fragment thereof, comprising:

a first heavy chain comprising a variable region (first V_H) having an amino acid sequence of SEQ ID NO: 9,

a first light chain comprising a variable region (first V_L) having an amino acid sequence of SEQ ID NO: 17,

a second heavy chain comprising a variable region (second V_H) having an amino acid sequence of SEQ ID NO: 10, and

a second light chain comprising a variable region (second V_L) having an amino acid sequence of SEQ ID NO: 18,

wherein the first V_H and the first V_L form a first antigen binding site specific for extracellular domain IV of HER2, and the second V_H and the second V_L form a second antigen binding site specific for extracellular domain II of HER2.

2. The antibody according to Claim 1, wherein the first heavy chain further comprises a constant region having an amino acid sequence of SEQ ID NO: 7.

3. The antibody according to Claim 2, wherein the first heavy chain comprises an amino acid sequence of SEQ ID NO: 22.

4. The antibody according to Claim 1, wherein the first light chain further comprises a constant region having an amino acid sequence of SEQ ID NO: 19.

5. The antibody according to Claim 4, wherein the first light chain comprises an amino acid sequence of SEQ ID NO: 23.

6. The antibody according to Claim 1, wherein the second heavy chain further comprises a constant region having an amino acid sequence of SEQ ID NO: 8.

7. The antibody according to Claim 6, wherein the second heavy chain comprises an amino acid sequence of SEQ ID NO: 25.

8. The antibody according to Claim 1, wherein the second light chain further comprises a constant region having an amino acid sequence of SEQ ID NO: 19.

9. The antibody according to Claim 8, wherein the second light chain comprises an amino acid sequence of SEQ ID NO: 26.

10. The antibody according to Claim 1, comprising fucose in an amount of no more than 10% of the total saccharides that are attached to the Fc region of the antibody.

11. The antibody according to Claim 1, comprising fucose in an amount of no more than 5% of the total saccharides that are attached to the Fc region of the antibody.

12. An isolated nucleic acid molecule encoding the antibody or the antigen binding fragment
of Claim 1.

13. An isolated host cell or a non-human organism transformed or transfected with the nucleic
acid molecule of Claim 12.

14. A pharmaceutical composition comprising the antibody or the antigen binding fragment of
Claim 1 and a pharmaceutically acceptable carrier, diluent and/or adjuvant.

15. A method for treating cancer, comprising administering an effective amount of the antibody or the antigen binding fragment of Claim 1 to a subject in need thereof.

16. The method of Claim 15, wherein the cancer is breast cancer, gastric cancer, ovarian cancer, esophagus cancer, endometrial cancer, bladder cancer, lung cancer, colon cancer,

or head and neck cancer, or prostate cancer.

17. The method of Claim 15, wherein the cancer is breast cancer, gastric cancer, or ovarian Cancer.

18. Chinese Hamster ovary (CHO) mutant cell line comprising a dysfunctional *Slc35C1* gene, wherein the CHO mutant cell line only contains one dysfunctional gene that affects the regulations of glycan.

19. The CHO mutant cell line of Claim 18, which expresses proteins having $\leq 5\%$ fucosylation of the total saccharides that are attached to the proteins.

20. The CHO mutant cell line of Claim 18, having a deposit number of CGMCC No.14287.

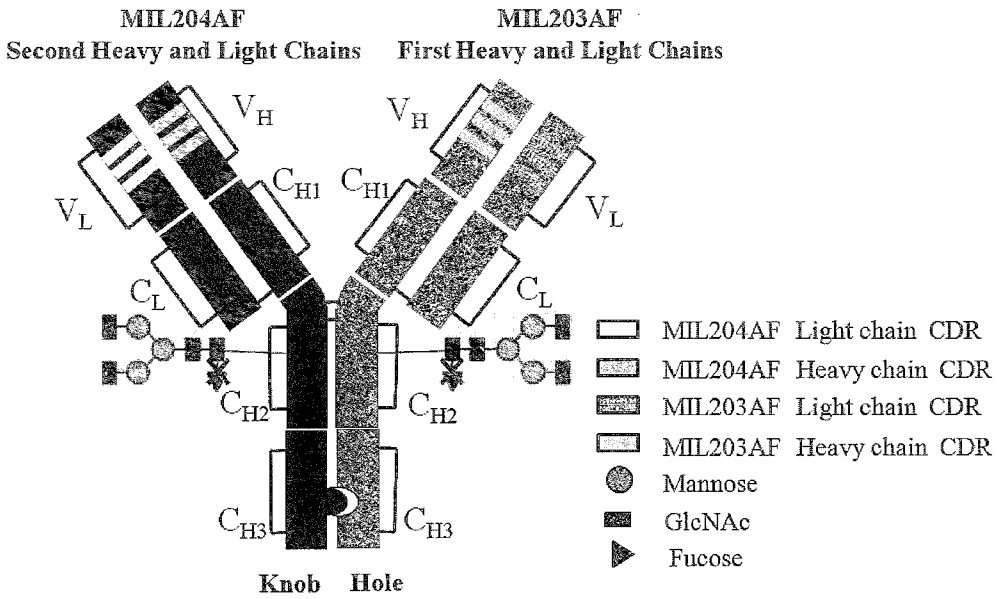
MIL204AF
Second Heavy and Light Chains
MIL203AF
First Heavy and Light Chains
V_H
V_H
C_{H1}
C_{H1}
V_L
V_L
C_L
C_L
C_{H2}
C_{H2}
C_{H3}
C_{H3}
Knob
Hole
MIL204AF Light chain CDR
MIL204AF Heavy chain CDR
MIL203AF Light chain CDR
MIL203AF Heavy chain CDR
Mannose
GlcNAc
Fucose

FIG. 1

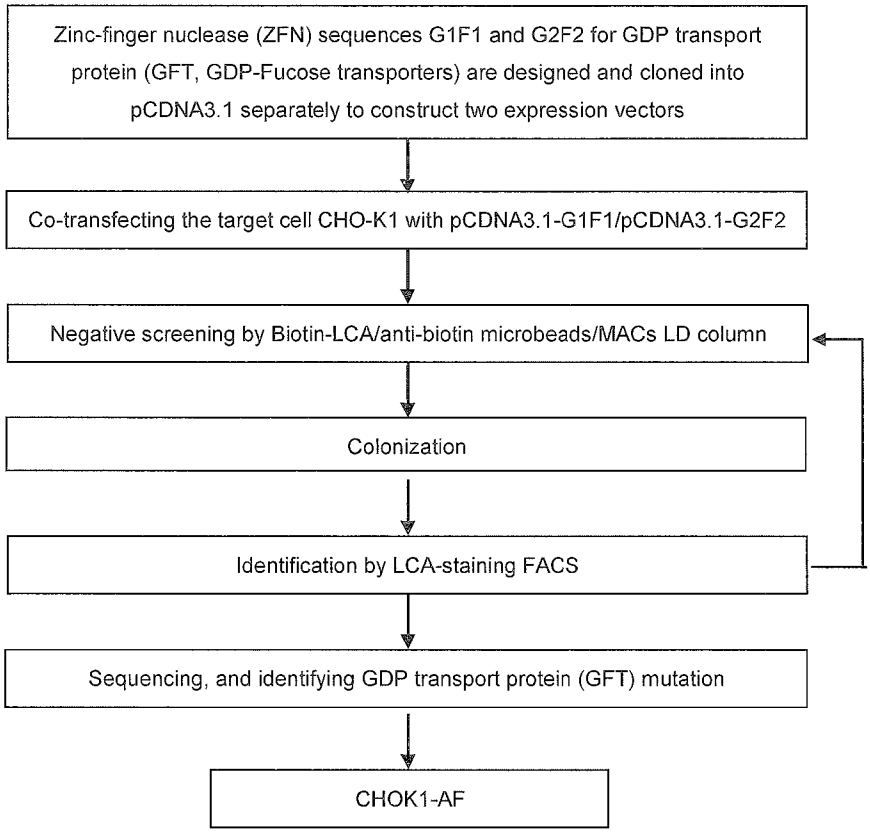
Zinc-finger nuclease (ZFN) sequences G1F1 and G2F2 for GDP transport protein (GFT, GDP-Fucose transporters) are designed and cloned into pCDNA3.1 separately to construct two expression vectors
Co-transfecting the target cell CHO-K1 with pCDNA3.1-G1F1/pCDNA3.1-G2F2
Negative screening by Biotin-LCA/anti-biotin microbeads/MACs LD column
Colonization
Identification by LCA-staining FACS
Sequencing, and identifying GDP transport protein (GFT) mutation
CHOK1-AF
Multi-rounds of negative screening screening

FIG 2

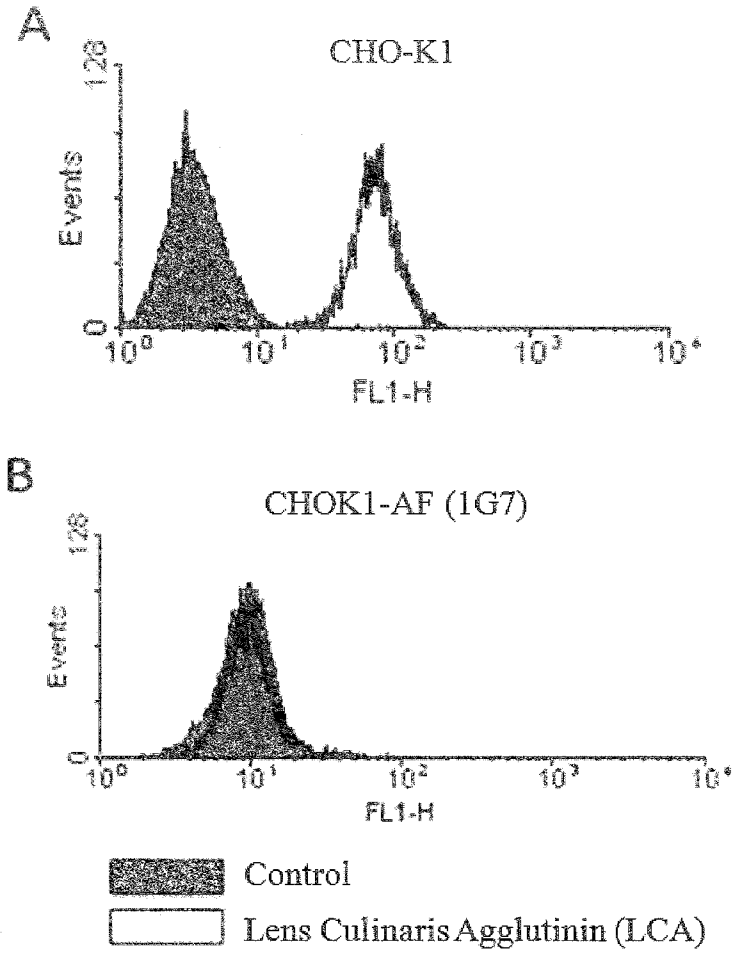
A
CHO-K1
128
Events
0
10^0
10^1
10^2
10^3
10^4
FL1-H
B
CHOK1-AF (1G7)
128
Events
0
10^0
10^1
10^2
10^3
10^4
FL1-H
Control
Lens Culinaris Agglutinin (LCA)

FIG. 3

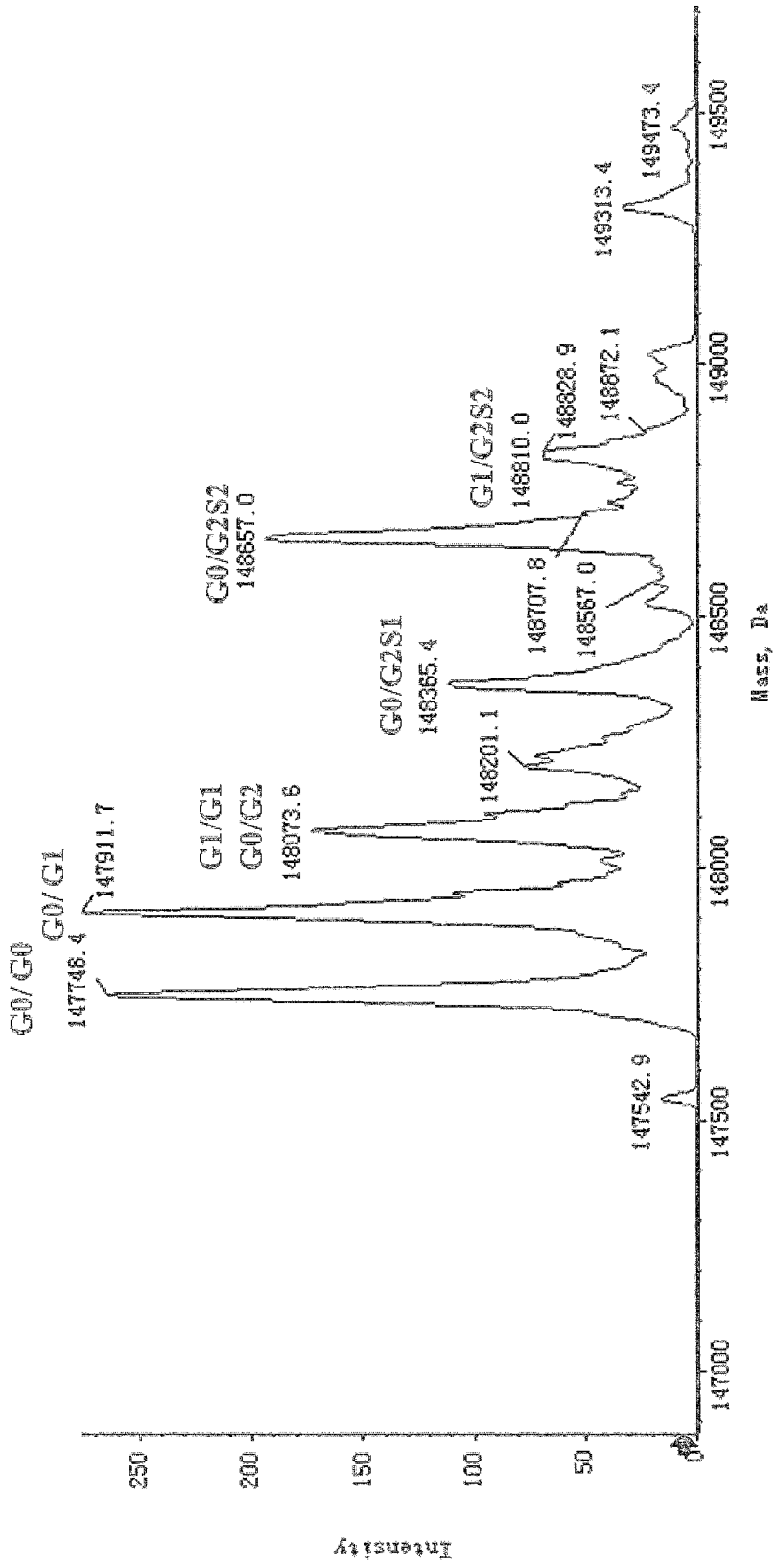
G0/G0
147746.4
G0/G1
147911.7
G1/G1
G0/G2
148073.6
G0/G2S2
148657.0
G0/G2S1
148365.4
148201.1
148707.8
148567.0
G1/G2S2
148810.0
148828.9
148672.1
149313.4
149473.4
147542.9
147000
147500
148000
148500
149000
149500
Mass, Da
0
50
100
150
200
250
Intensity

FIG. 4

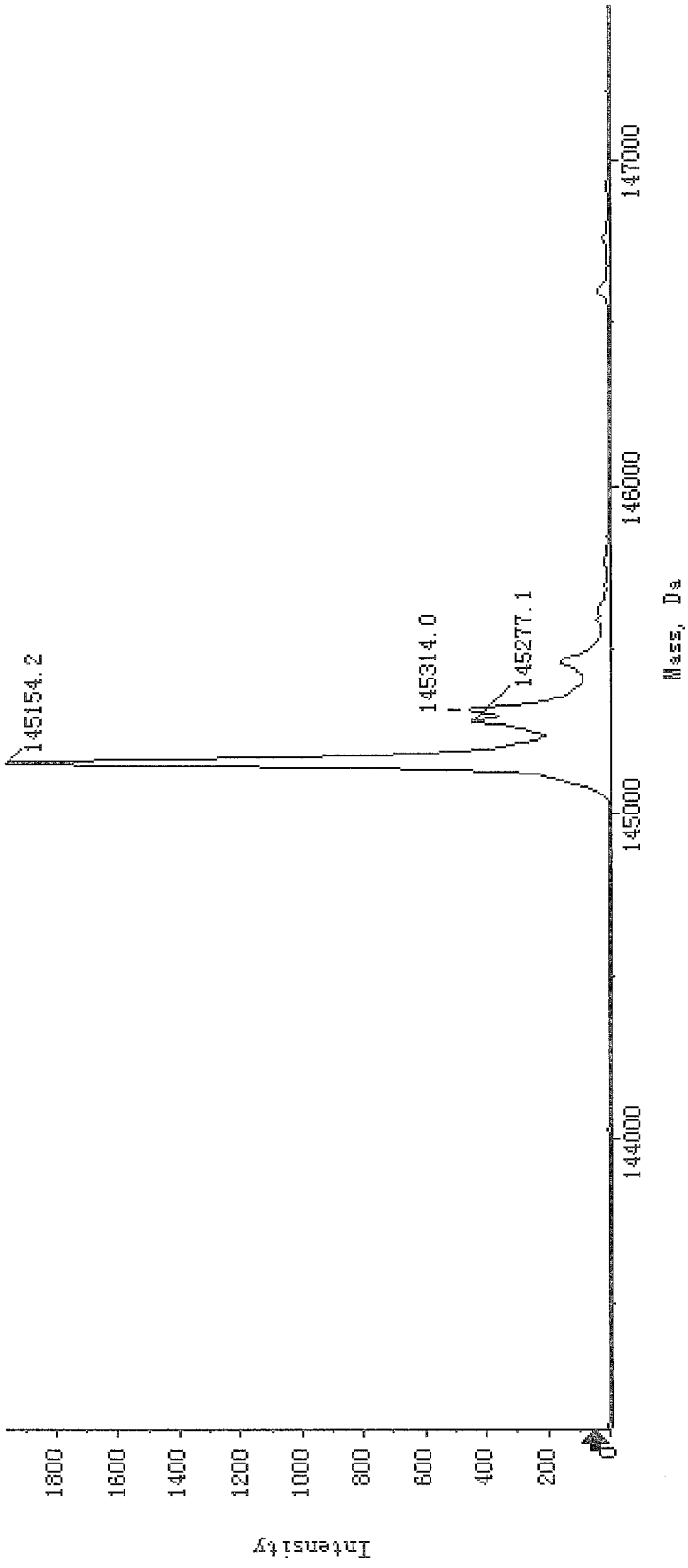
145154.2
145314.0
145277.1
Mass, Da
144000
145000
146000
147000
Intensity
0
200
400
600
800
1000
1200
1400
1600
1800

FIG. 5

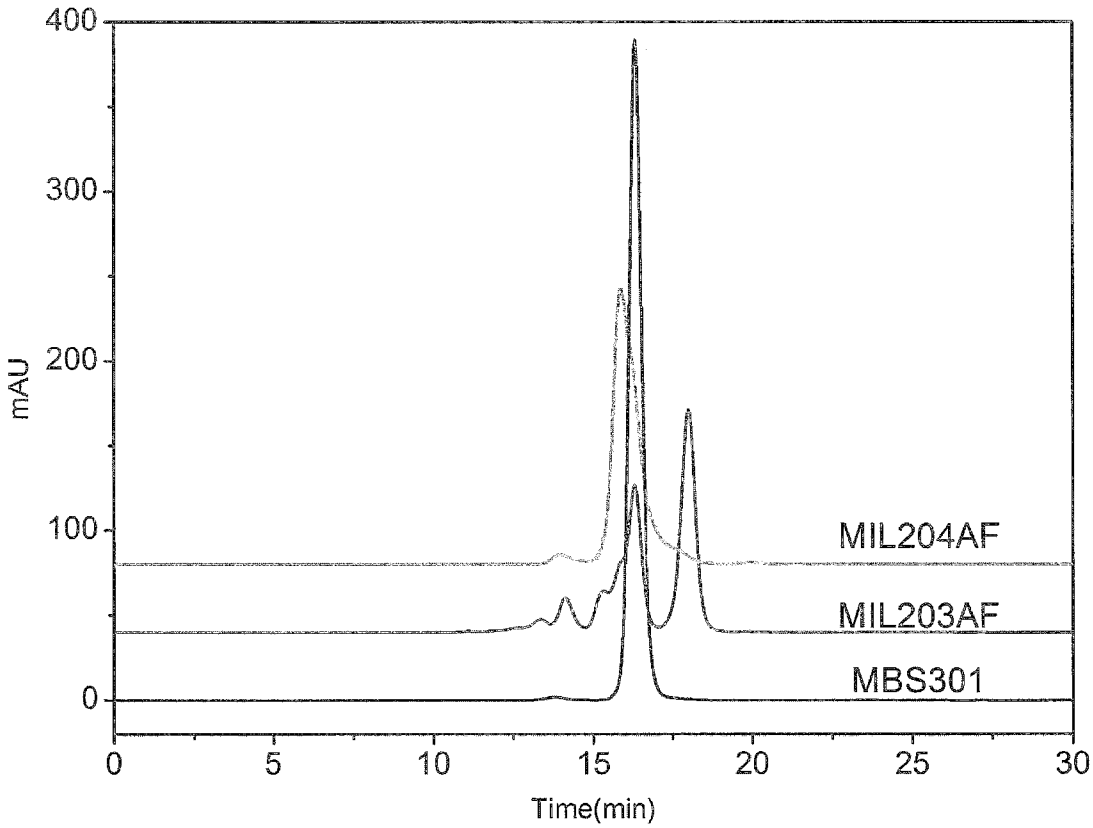
400
300
200
100
0
mAU
0
5
10
15
20
25
30
Time(min)
MIL204AF
MIL203AF
MBS301

FIG. 6

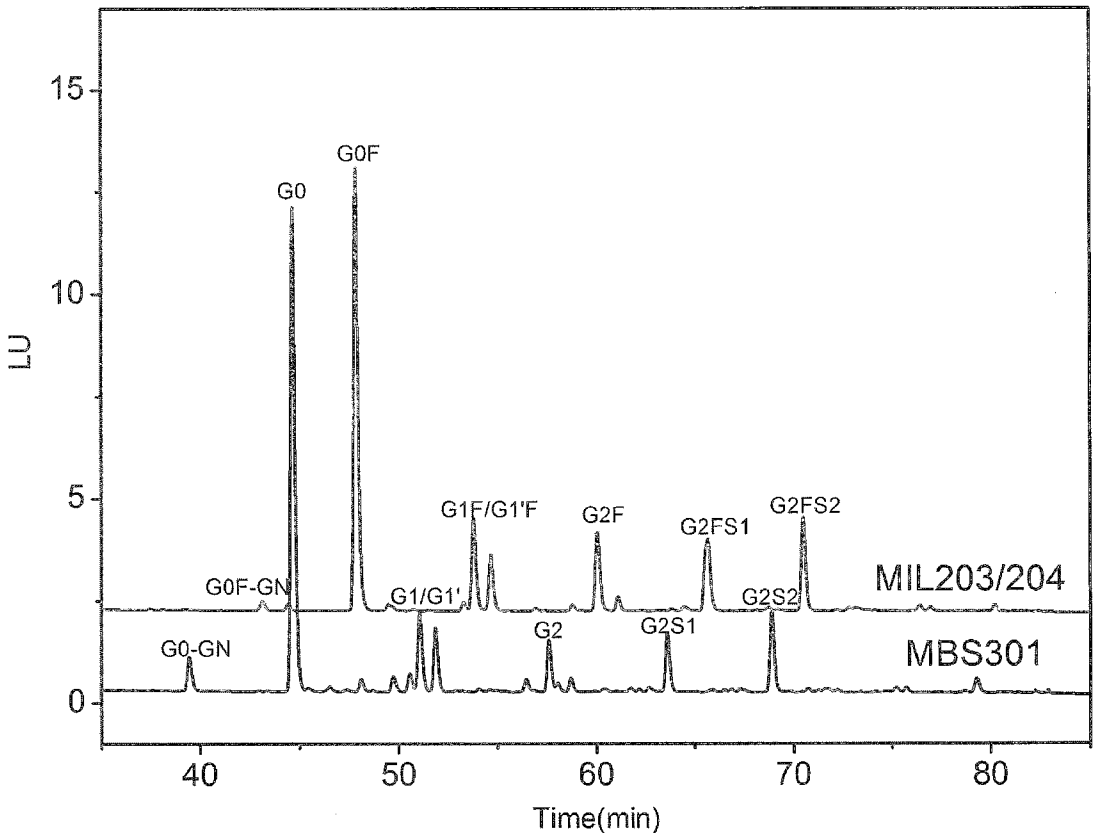
15
10
5
0
LU
G0
G0F
G0F-GN
G0-GN
G1F/G1'F
G1/G1'
G2F
G2
G2FS1
G2S1
G2FS2
G2S2
MIL203/204
MBS301
40
50
60
70
80
Time(min)

FIG. 7

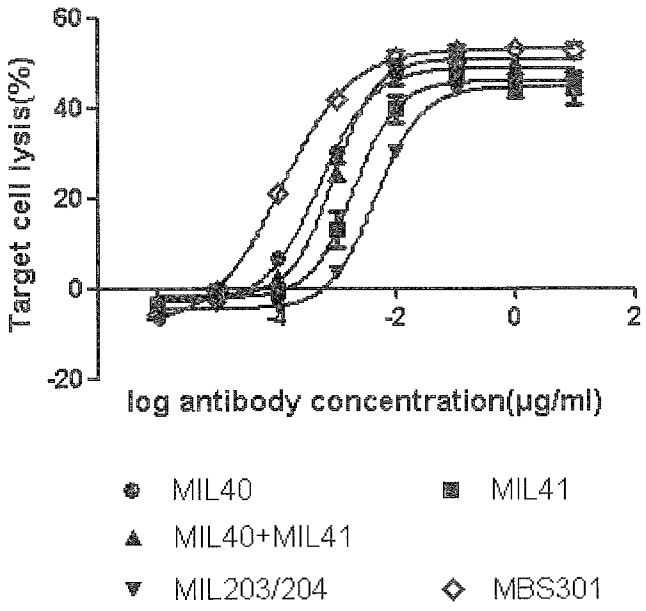
60
40
20
0
-20
Target cell lysis(%)
-2
0
2
log antibody concentration(μg/ml)
MIL40
MIL41
MIL40+MIL41
MIL203/204
MBS301

FIG. 8

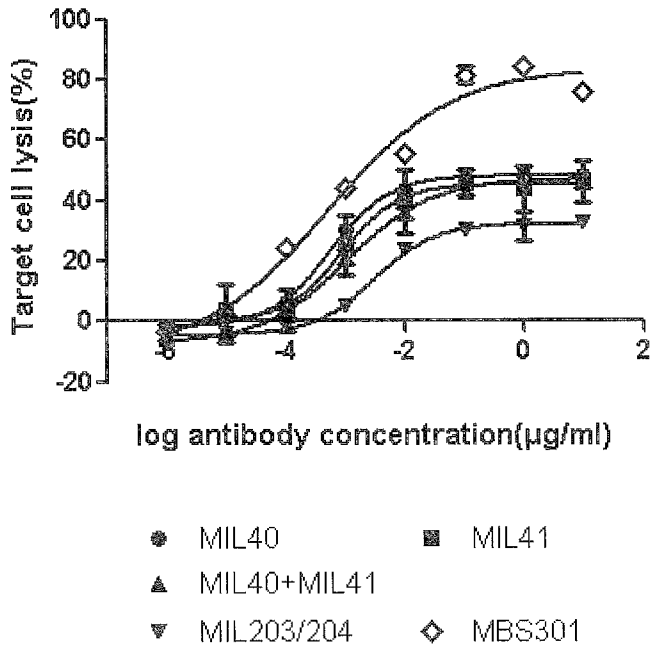
100
80
60
40
20
0
-20
Target cell lysis(%)
-6
-4
-2
0
2
log antibody concentration(μg/ml)
MIL40
MIL41
MIL40+MIL41
MIL203/204
MBS301

FIG. 9

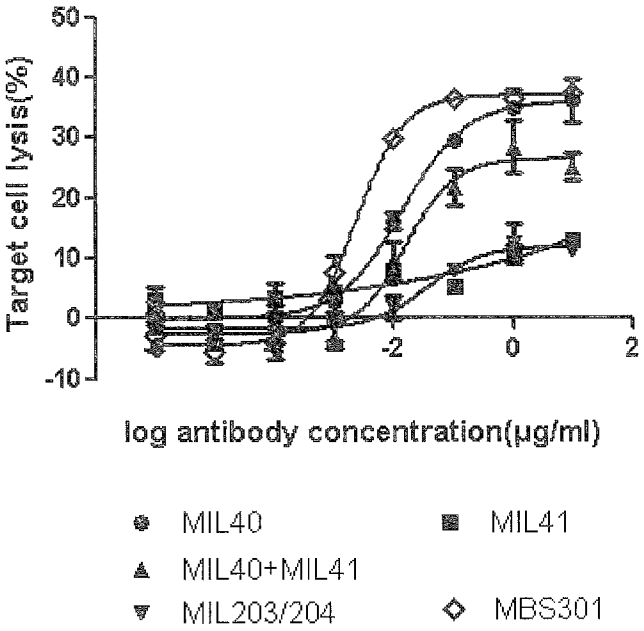
Target cell lysis(%)
50
40
30
20
10
0
-10
-2
0
2
log antibody concentration(μg/ml)
MIL40
MIL41
MIL40+MIL41
MIL203/204
MBS301

FIG. 10

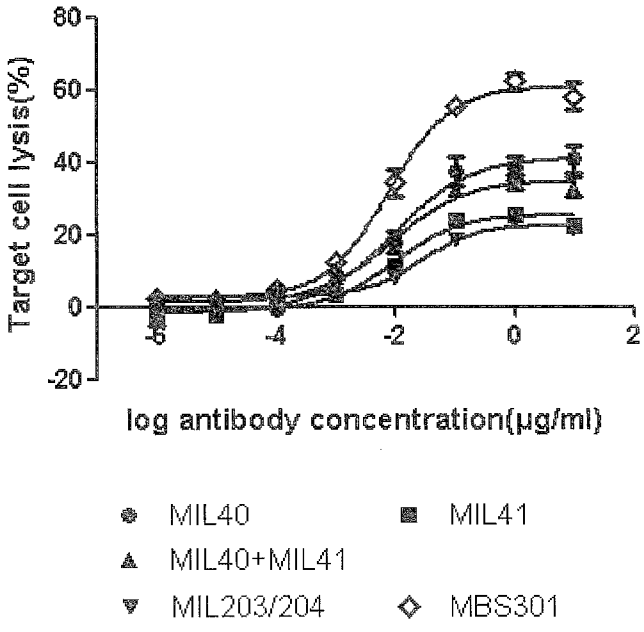
Target cell lysis(%)
80
60
40
20
0
-20
-6
-4
-2
0
2
log antibody concentration(μg/ml)
MIL40
MIL41
MIL40+MIL41
MIL203/204
MBS301

FIG. 11

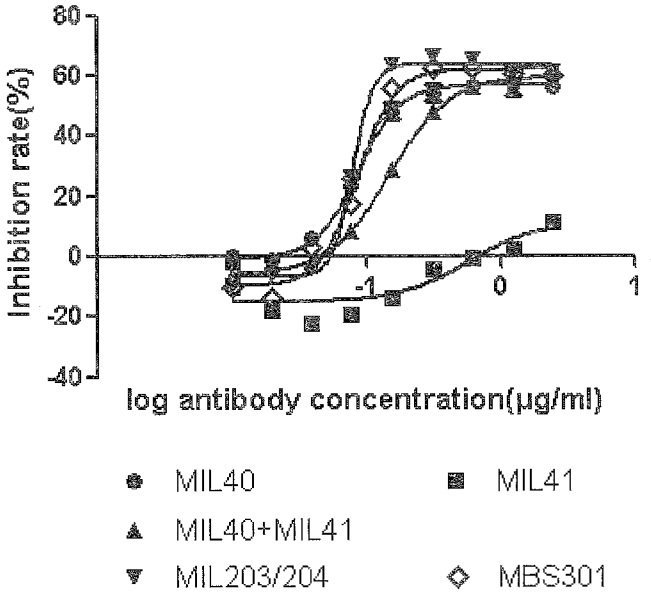
Inhibition rate(%)
80
60
40
20
0
-20
-40
-1
0
1
log antibody concentration(μg/ml)
MIL40
MIL41
MIL40+MIL41
MIL203/204
MBS301

FIG. 12

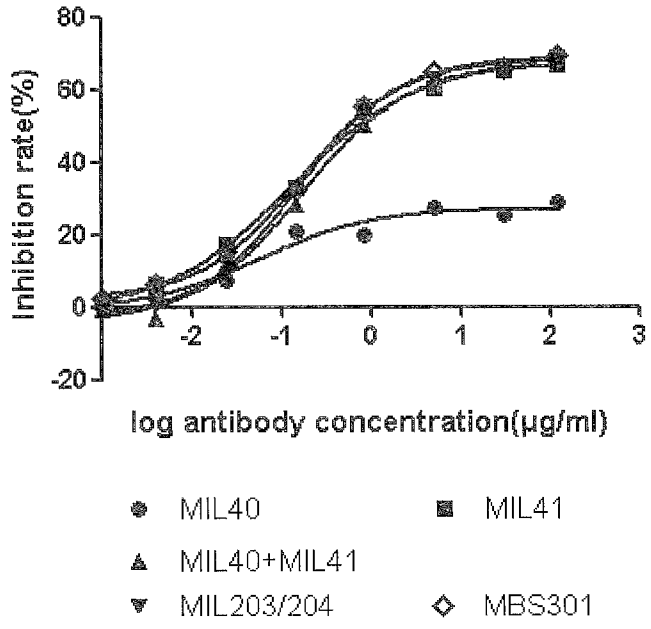
Inhibition rate(%)
80
60
40
20
0
-20
-2
-1
0
1
2
3
log antibody concentration(μg/ml)
MIL40
MIL41
MIL40+MIL41
MIL203/204
MBS301

FIG. 13

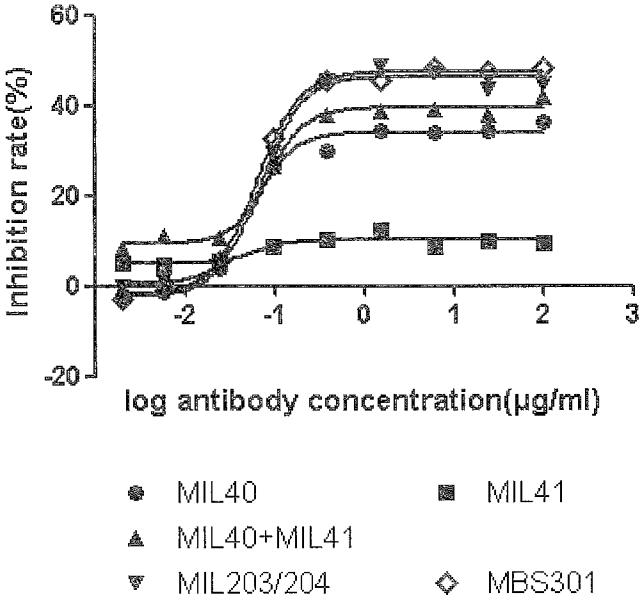
Inhibition rate(%)
60
40
20
0
-20
-2
-1
0
1
2
3
log antibody concentration(μg/ml)
MIL40
MIL41
MIL40+MIL41
MIL203/204
MBS301

FIG. 14

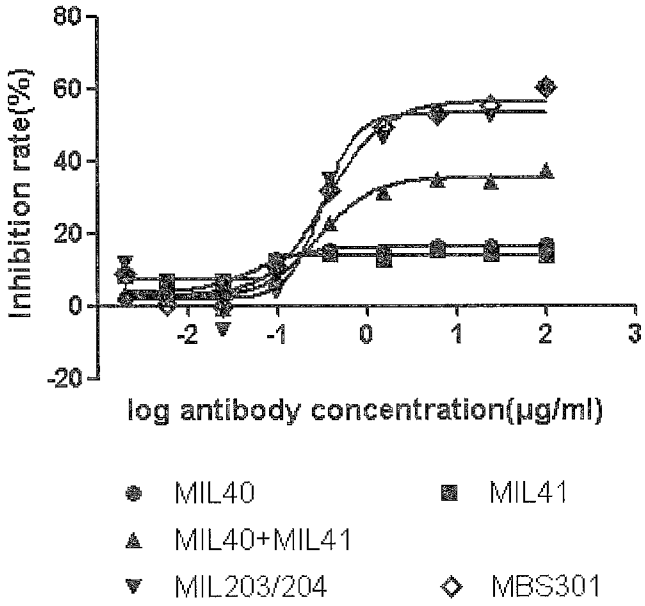
Inhibition rate(%)
80
60
40
20
0
-20
-2
-1
0
1
2
3
log antibody concentration(μg/ml)
MIL40
MIL41
MIL40+MIL41
MIL203/204
MBS301

FIG. 15

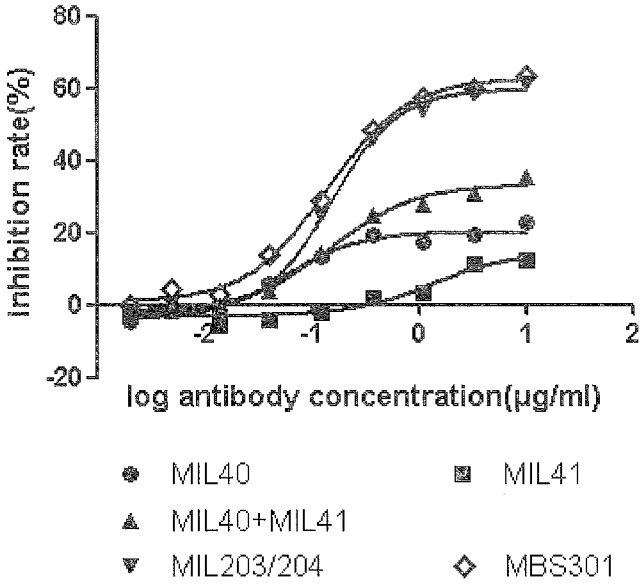
Inhibition rate(%)
80
60
40
20
0
-20
-2
-1
0
1
2
log antibody concentration(μg/ml)
MIL40
MIL41
MIL40+MIL41
MIL203/204
MBS301

FIG. 16

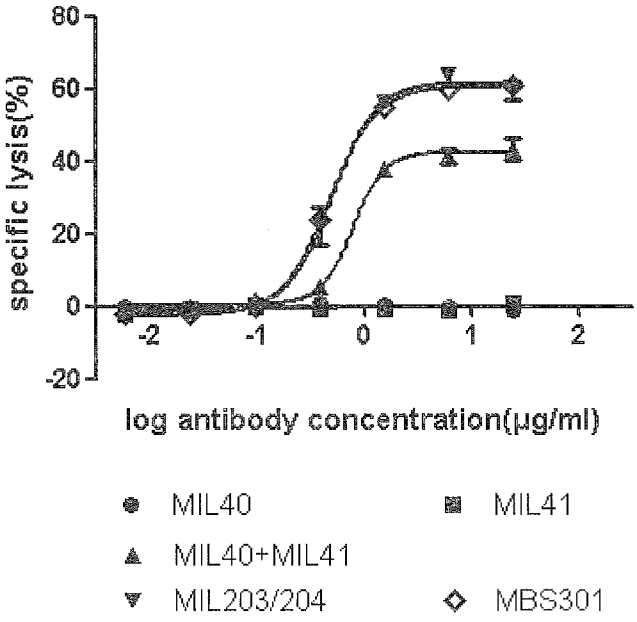
specific lysis(%)
80
60
40
20
0
-20
-2
-1
0
1
2
log antibody concentration(μg/ml)
MIL40
MIL41
MIL40+MIL41
MIL203/204
MBS301

FIG. 17

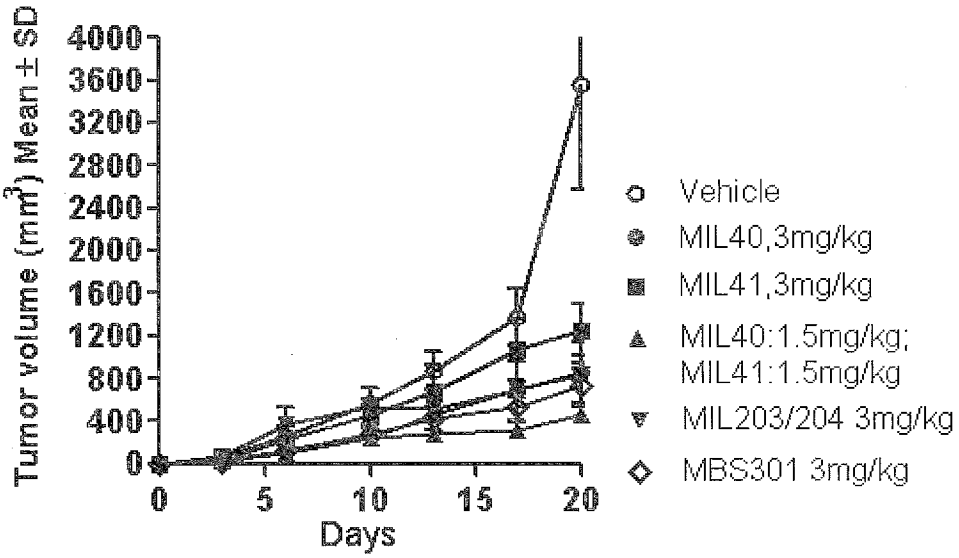
Tumor volume (mm3) Mean ± SD
4000
3600
3200
2800
2400
2000
1600
1200
800
400
0
0
5
10
15
20
Days
Vehicle
MIL40,3mg/kg
MIL41,3mg/kg
MIL40:1.5mg/kg;
MIL41:1.5mg/kg
MIL203/204 3mg/kg
MBS301 3mg/kg

FIG.18

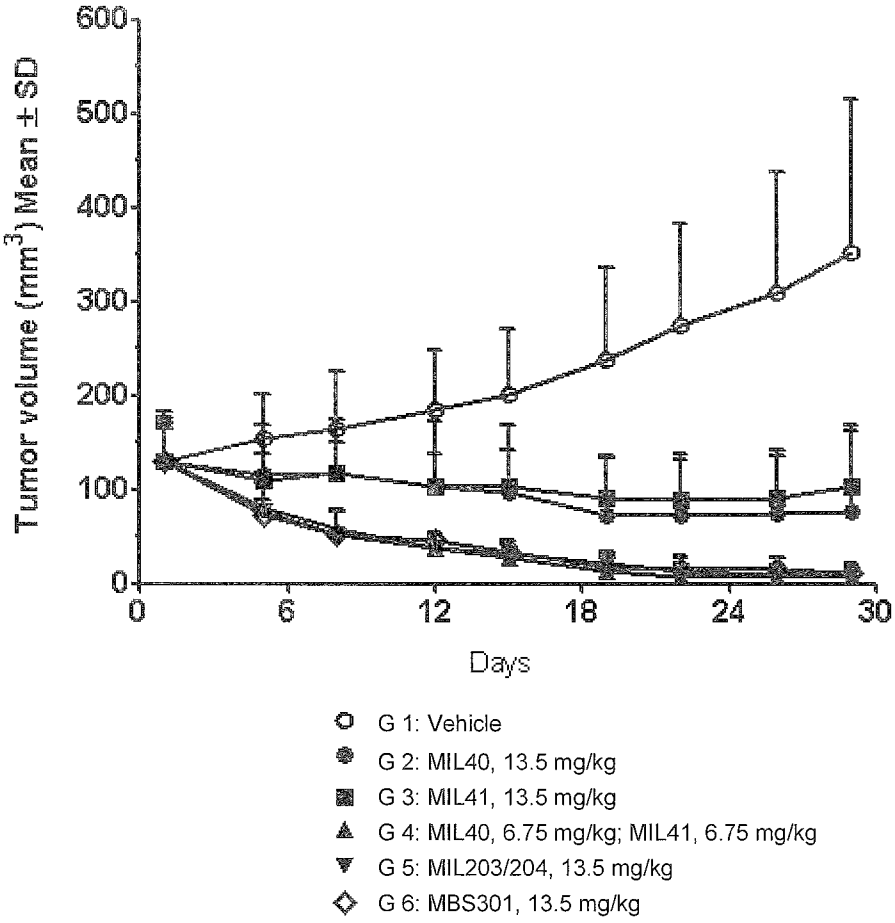
Tumor volume (mm^3) Mean ± SD
600
500
400
300
200
100
0
0
6
12
18
24
30
Days
G 1: Vehicle
G 2: MIL40, 13.5 mg/kg
G 3: MIL41, 13.5 mg/kg
G 4: MIL40, 6.75 mg/kg; MIL41, 6.75 mg/kg
G 5: MIL203/204, 13.5 mg/kg
G 6: MBS301, 13.5 mg/kg

FIG. 19

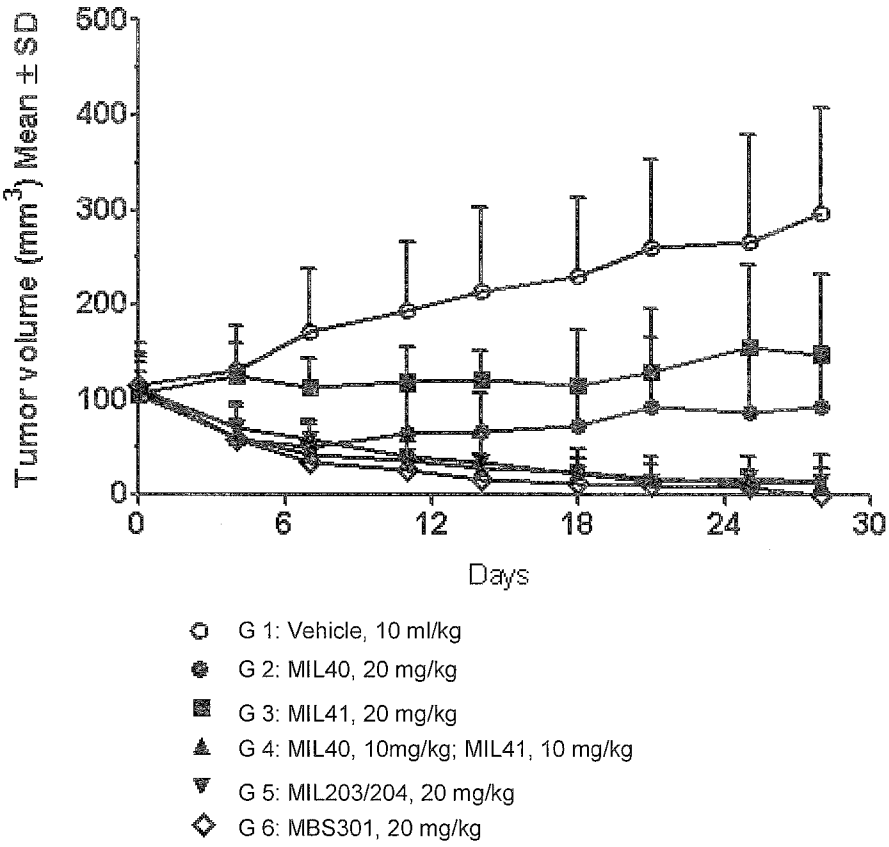
Tumor volume (mm3) Mean ± SD
500
400
300
200
100
0
0
6
12
18
24
30
Days
G 1: Vehicle, 10 ml/kg
G 2: MIL40, 20 mg/kg
G 3: MIL41, 20 mg/kg
G 4: MIL40, 10mg/kg; MIL41, 10 mg/kg
G 5: MIL203/204, 20 mg/kg
G 6: MBS301, 20 mg/kg

FIG. 20

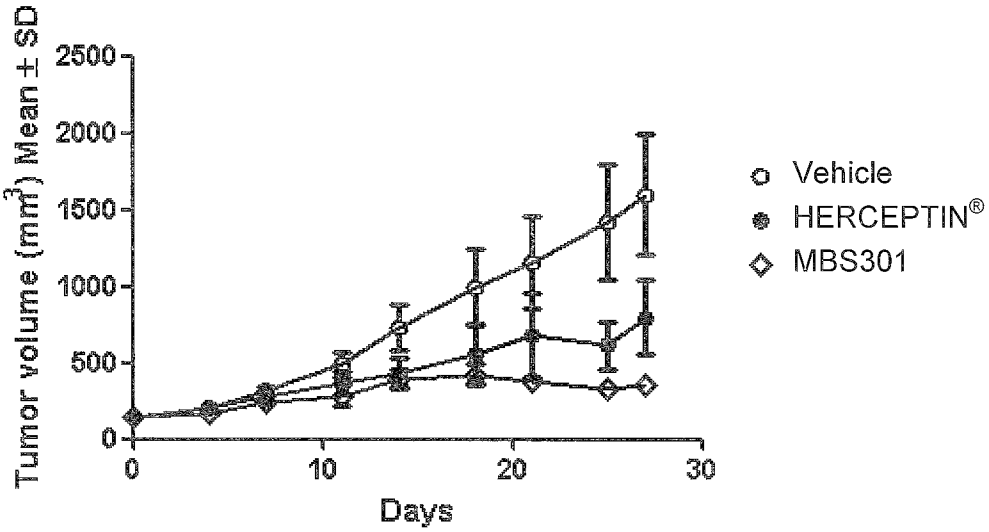
Tumor volume (mm3) Mean ± SD
2500
2000
1500
1000
500
0
0
10
20
30
Days
Vehicle
HERCEPTIN®
MBS301

FIG. 21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2017/093816

A. CLASSIFICATION OF SUBJECT MATTER

C07K 16/46(2006.01)i; C07K 16/30(2006.01)i; C12N 15/13(2006.01)i; A61K 39/395(2006.01)i; A61P 35/00(2006.01)i; C12N 5/16(2006.01)i

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)

C07K; C12N; A61K; A61P

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)

CNABS , VEN , CNTXT , USTXT , EPTXT , SIPOABS , DWPI , CNKI , GOOGLE, PubMed, ISI Web of Knowledge, NCBI GenBank, EBI-EMBL, SEQ ID NOs: 7-10, 17-19, 22-23, 25-26, HER2, antibody, bispecific, trastuzumab, pertuzumab, extracellular domain, saccharide, cancer, tumor, CHO cell, Slc35C1, mutant

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2015077891 A1 (ZYMEWORKS INC.) 04 June 2015 (2015-06-04) description, paragraghs 5, 60, 61, 94, 96, 99, 106, 109-114, 119, 123-128, 130, 132, 134-135, 186, 213, 241, 244, Fig.1A	1-17
X	MENG, Y. C. "Effective suppression of breast tumor growth by a bispecific antibody targeting distinct ErbB2 epitopes." *CHINESE MASTER'S THESES FULL-TEXT DATABASE (MEDICINE AND HEALTH SCIENCES).*, 15 May 2014 (2014-05-15), Article E072-620	1-5, 8-9, 12-17
X	WO 2015091738 A1 (F. HOFFMANN-LA ROCHE AG) 25 June 2015 (2015-06-25) description, page 25, lines 29-33, page 26, lines 1-10, page 49, lines 24, page 58, lines 21-22, page 66, lines 4-6, page 67 lines 17-23, pages 132-133, Table 32	1, 4, 8, 10-17
X	CHAN, K. F. et al. "Inactivation of GDP-fucose transporter gene(Slc35c1) in CHO cells by ZFNs, TALENs and CRISPR-Cas9 for production of fucose-free antibodies." *BIOTECHNOLOGY JOURNAL.*, Vol. 11, 16 October 2015 (2015-10-16), pages 399-414	18-20

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

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 September 2017	**17 October 2017**

Name and mailing address of the ISA/CN	Authorized officer
STATE INTELLECTUAL PROPERTY OFFICE OF THE P.R.CHINA **6, Xitucheng Rd., Jimen Bridge, Haidian District, Beijing 100088** **China**	**GUO,Tingting**
Facsimile No. **(86-10)62019451**	Telephone No. **(86-10)62413879**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2017/093816

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2015164665 A1 (GENENTECH, INC.) 29 October 2015 (2015-10-29) the whole document	1-20
A	TANG, Q. et al. "Development of antibody drugs targeting against HER2 for cancer therapy." *ACTA PHARMACEUTICA SINICA.*, Vol. 47, No. 10, 12 October 2012 (2012-10-12), pages 1297-1305	1-20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2017/093816

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 filed or furnished:

   a. (means)

   ☐ on paper

   ☑ in electronic form

   b. (time)

   ☑ in the international application as filed

   ☐ together with the international application in electronic form

   ☐ subsequently to this Authority for the purposes of search

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 in 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/CN2017/093816

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.: **15-17**
because they relate to subject matter not required to be searched by this Authority, namely:

[1] The subject matter of claims 15-17 relates to the treatment of human body by therapy, therefore does not warrant an international search according to the criteria set out in PCT Rule 39.1(iv). An international search is still carried out on the basis of the use of the antibody or the antigen binding fragment of claim 1 for the manufacturing of a medicament for the treatment of cancer.

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

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

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2017/093816

Patent document cited in search report	Publication date (day/month/year)	Patent family member(s)	Publication date (day/month/year)
WO 2015077891 A1	04 June 2015	KR 20160091961 A	03 August 2016
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		CN 106163558 A	23 November 2016
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		MX 2016014007 A	11 January 2017
		EP 3134440 A1	01 March 2017