



US012384853B2

(12) **United States Patent**  
**Zhu et al.**

(10) **Patent No.:** **US 12,384,853 B2**

(45) **Date of Patent:** **Aug. 12, 2025**

(54) **ANTI-HER2/PD1 BISPECIFIC ANTIBODY**

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(\*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 653 days.

(21) Appl. No.: **17/295,391**

(22) PCT Filed: **Oct. 22, 2019**

(86) PCT No.: **PCT/CN2019/112467**

§ 371 (c)(1),

(2) Date: **May 19, 2021**

(87) PCT Pub. No.: **WO2020/103629**

PCT Pub. Date: **May 28, 2020**

(65) **Prior Publication Data**

US 2023/0287139 A1 Sep. 14, 2023

(30) **Foreign Application Priority Data**

Nov. 19, 2018 (CN) ..... 201811376950.9

(51) **Int. Cl.**

**C07K 16/00** (2006.01)

**A61P 35/00** (2006.01)

**C07K 16/28** (2006.01)

**C07K 16/32** (2006.01)

**A61K 39/00** (2006.01)

(52) **U.S. Cl.**

CPC ..... **C07K 16/32** (2013.01); **A61P 35/00** (2018.01); **C07K 16/2818** (2013.01); **A61K 2039/505** (2013.01); **C07K 2317/31** (2013.01); **C07K 2317/565** (2013.01); **C07K 2317/622** (2013.01); **C07K 2317/732** (2013.01); **C07K 2317/76** (2013.01); **C07K 2317/92** (2013.01)

(58) **Field of Classification Search**

None

See application file for complete search history.

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(57) **ABSTRACT**

Provided are an anti-HER2/PD1 bispecific antibody, preparation method, and antitumor application. Specifically, a single-chain variable fragment scFv and an immunoglobulin antibody IgG are connected by means of a peptide linker to obtain a bispecific antibody; the bispecific antibody can simultaneously target the tumor cell surface molecule HER2 antigen and T lymphocyte surface molecule PD-1. The results of experimentation show that the bispecific antibody provided is capable of inhibiting the proliferation of HER2-positive tumor cells while also blocking the binding of PD-1/PD-L1, releasing the suppressive state of T cells and performing an antitumor function.

**10 Claims, 13 Drawing Sheets**

**Specification includes a Sequence Listing.**

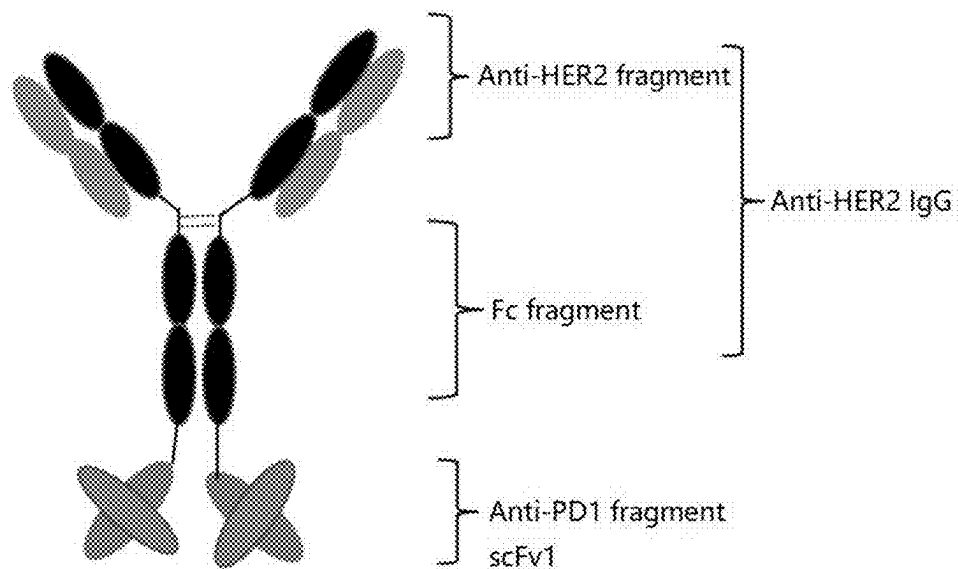


Fig. 1A

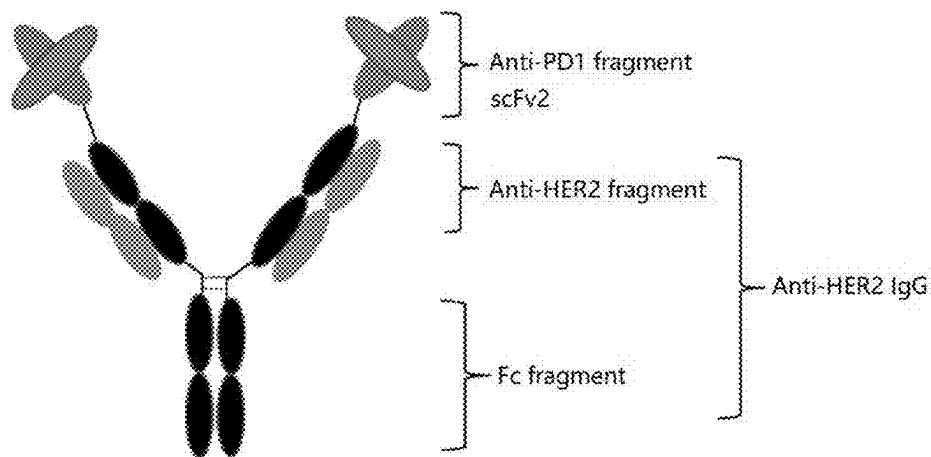


Fig. 1B

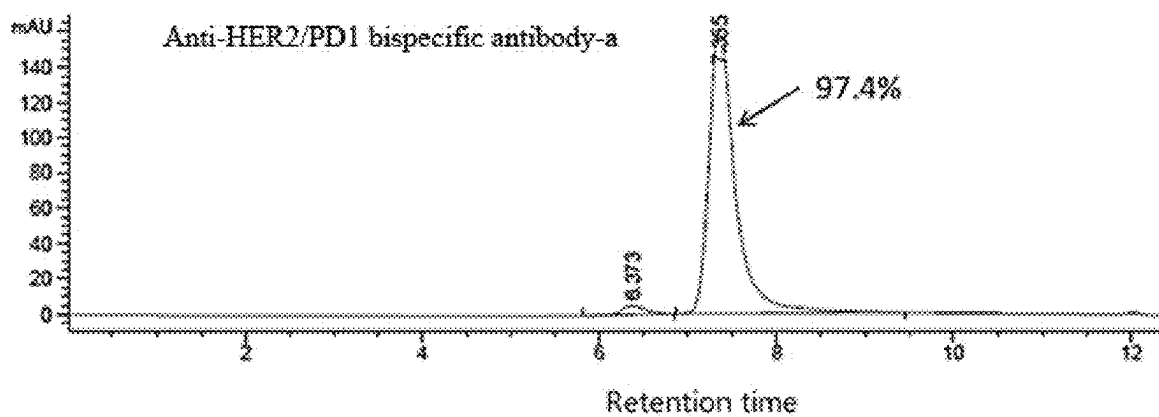


Fig. 2A

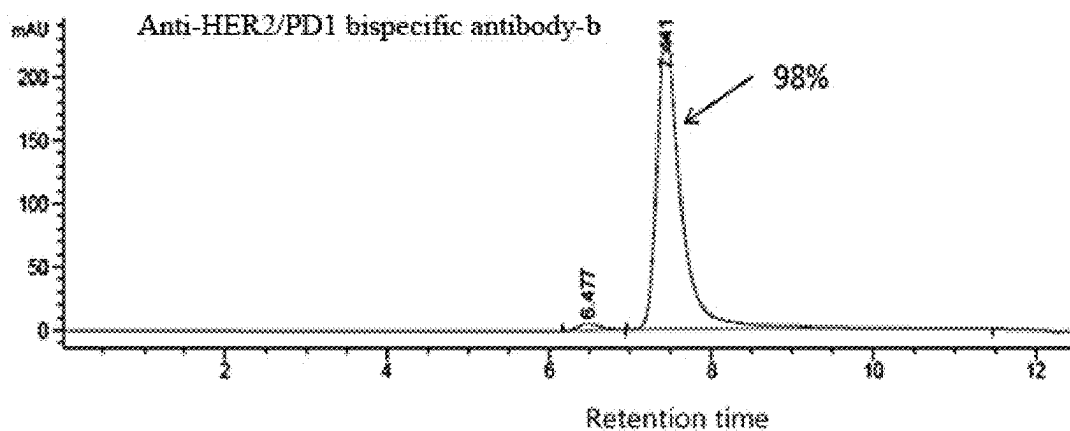


Fig. 2B

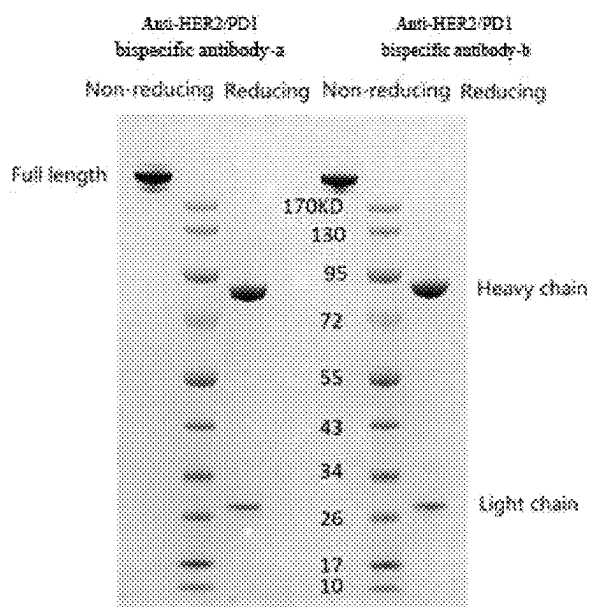


Fig.2C

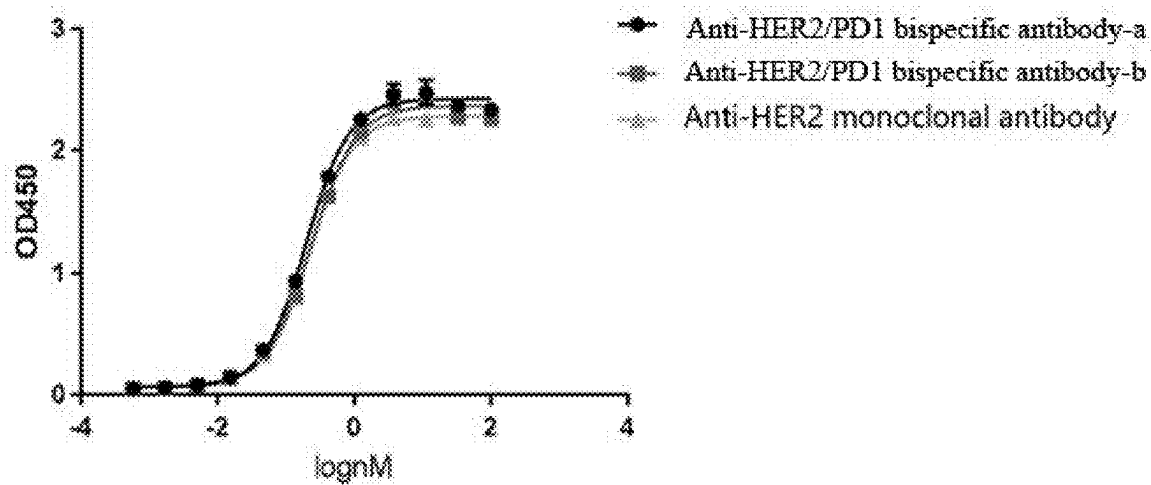


Fig. 3A

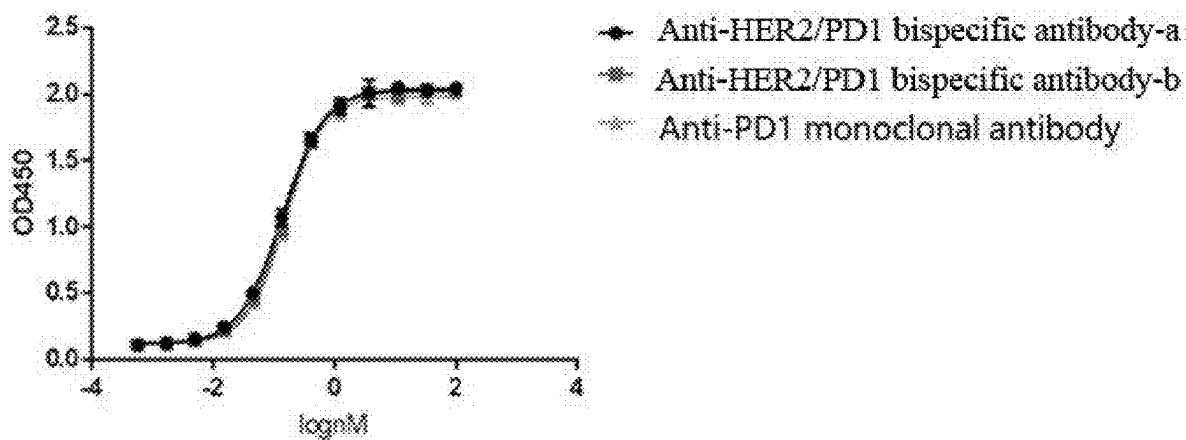


Fig. 3B

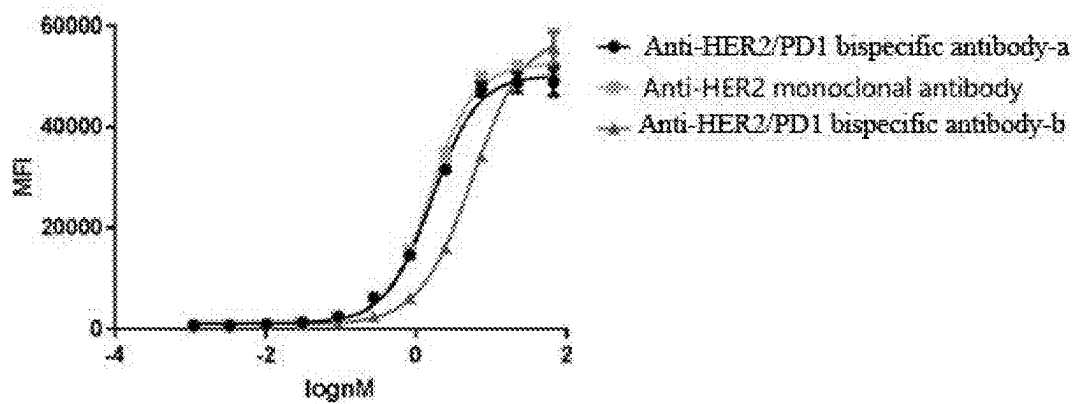


Fig. 4A

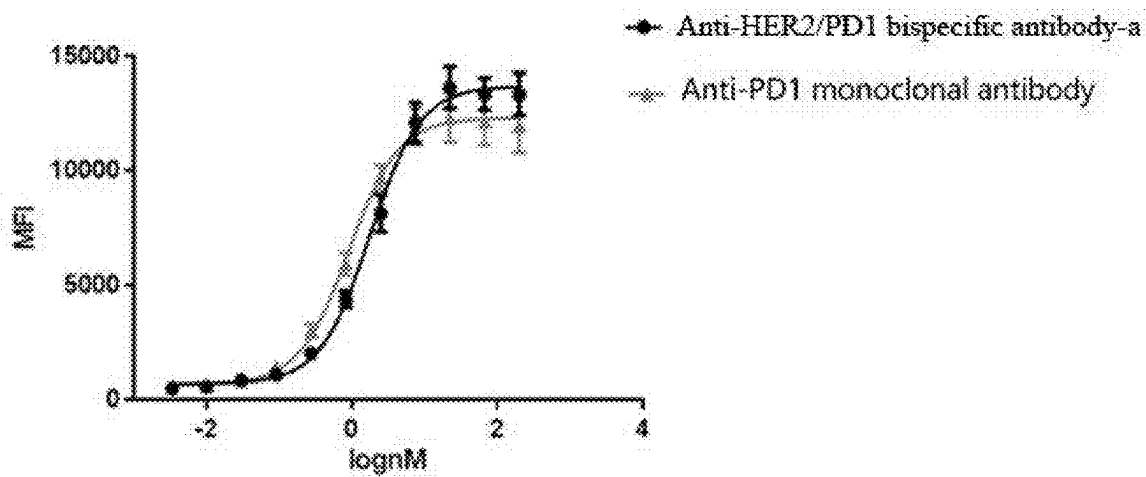


Fig. 4B

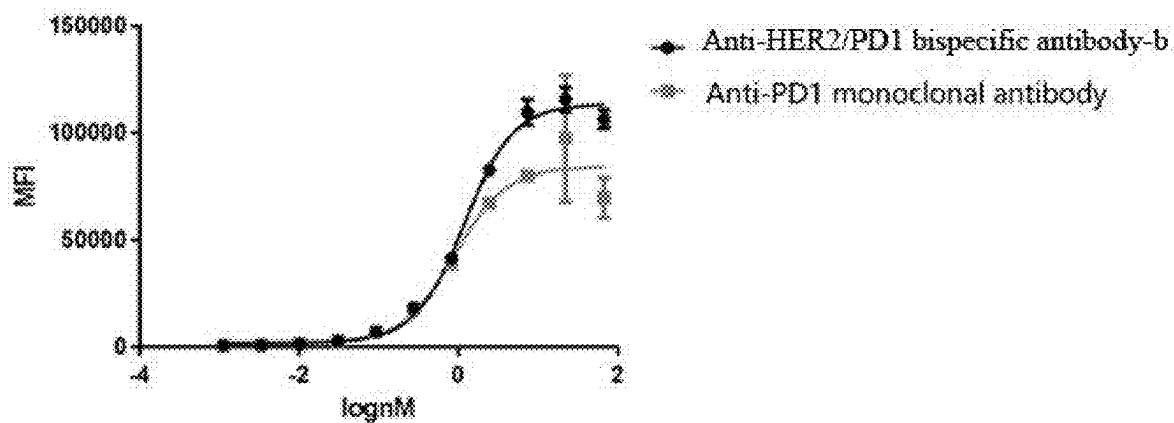


Fig. 4C

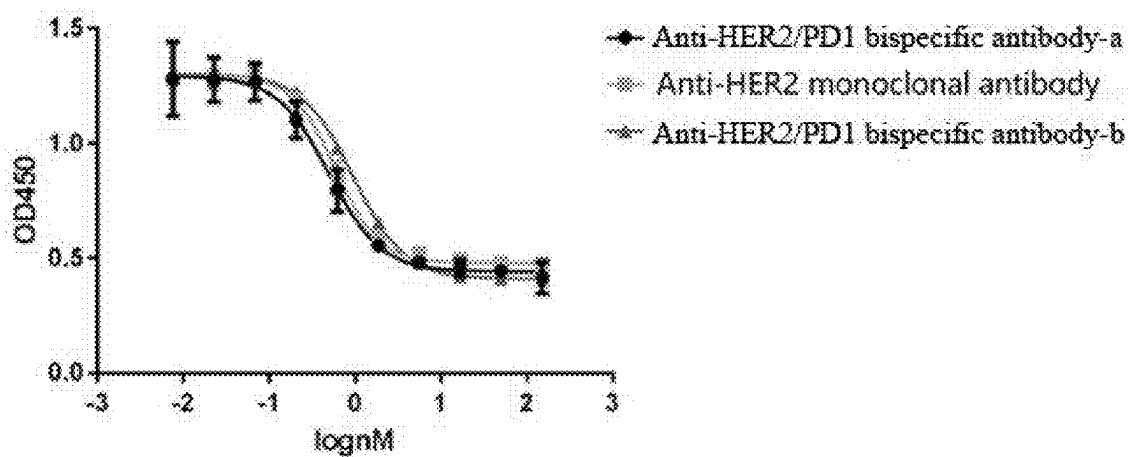


Fig. 5

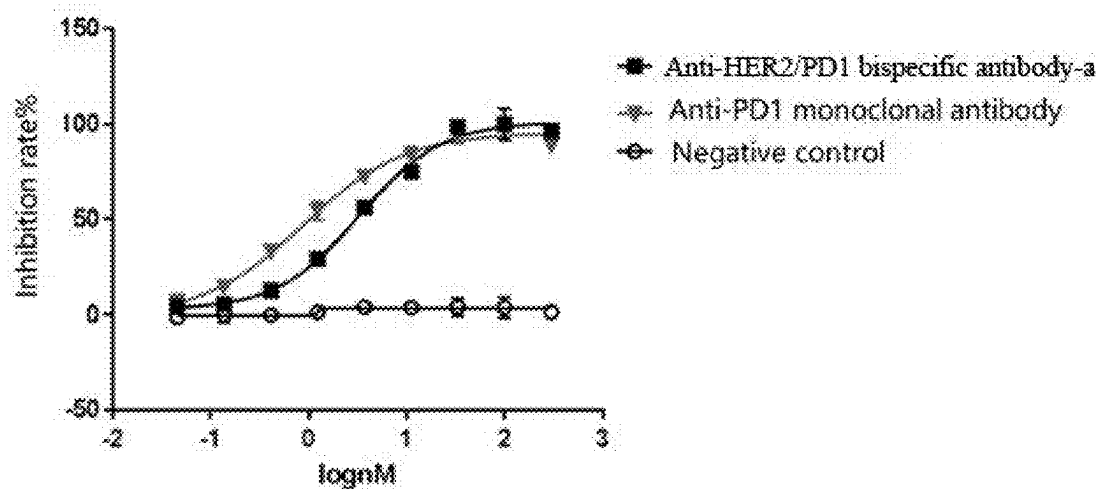


Fig. 6A

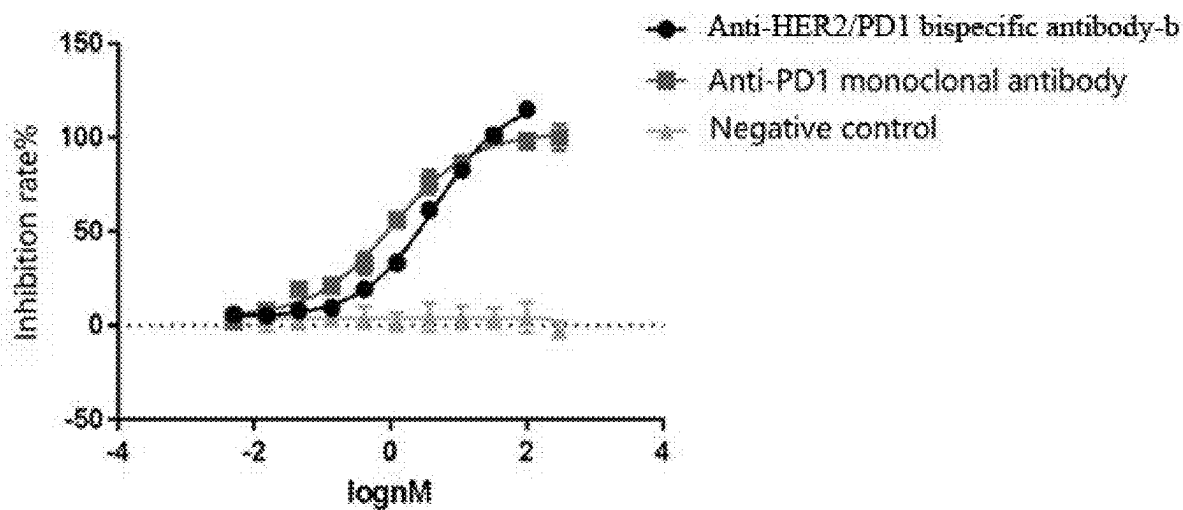


Fig. 6B

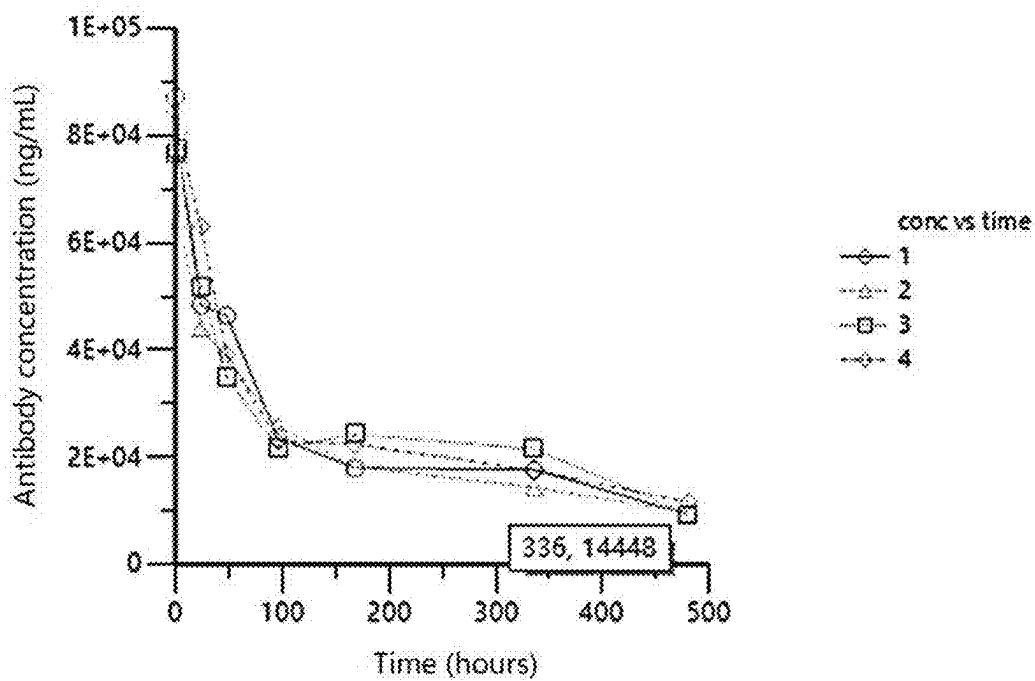


Fig. 7A

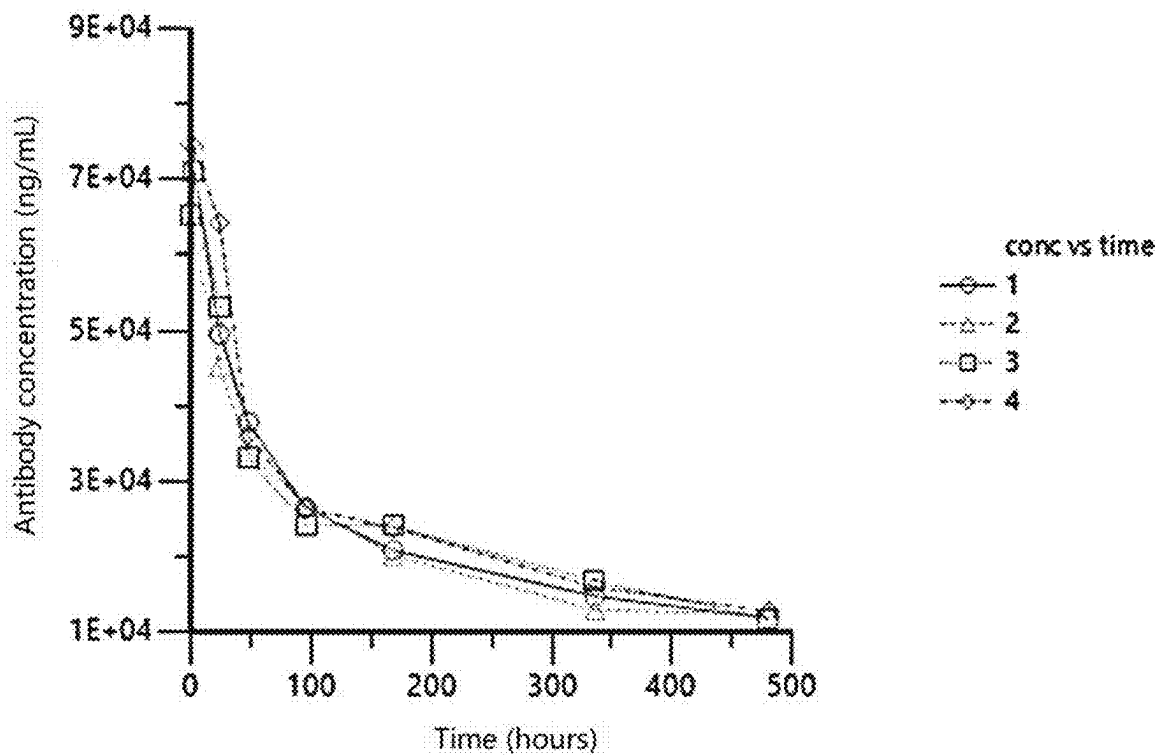


Fig. 7B

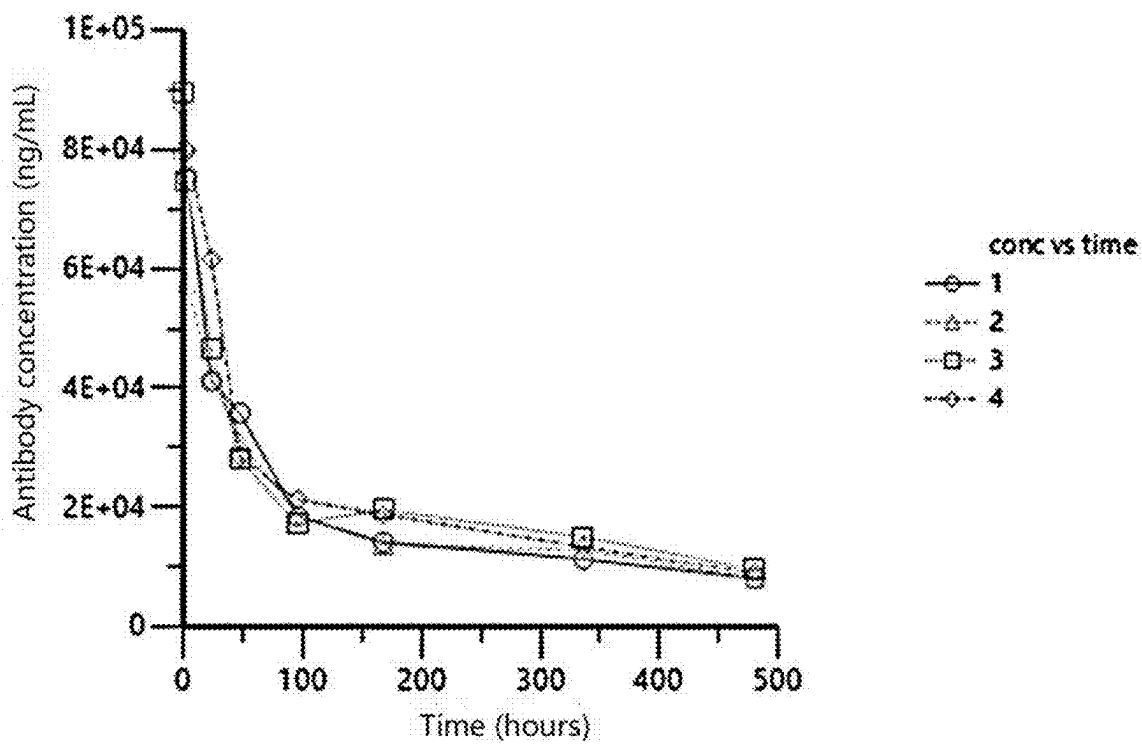


Fig. 7C

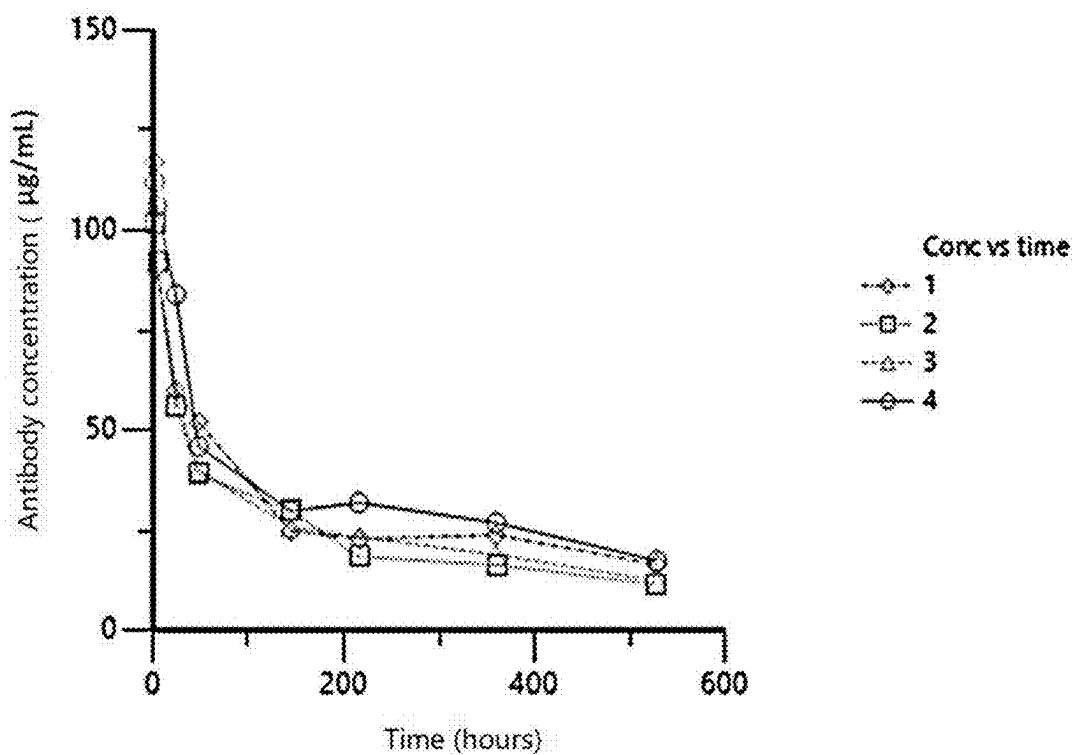


Fig. 7D



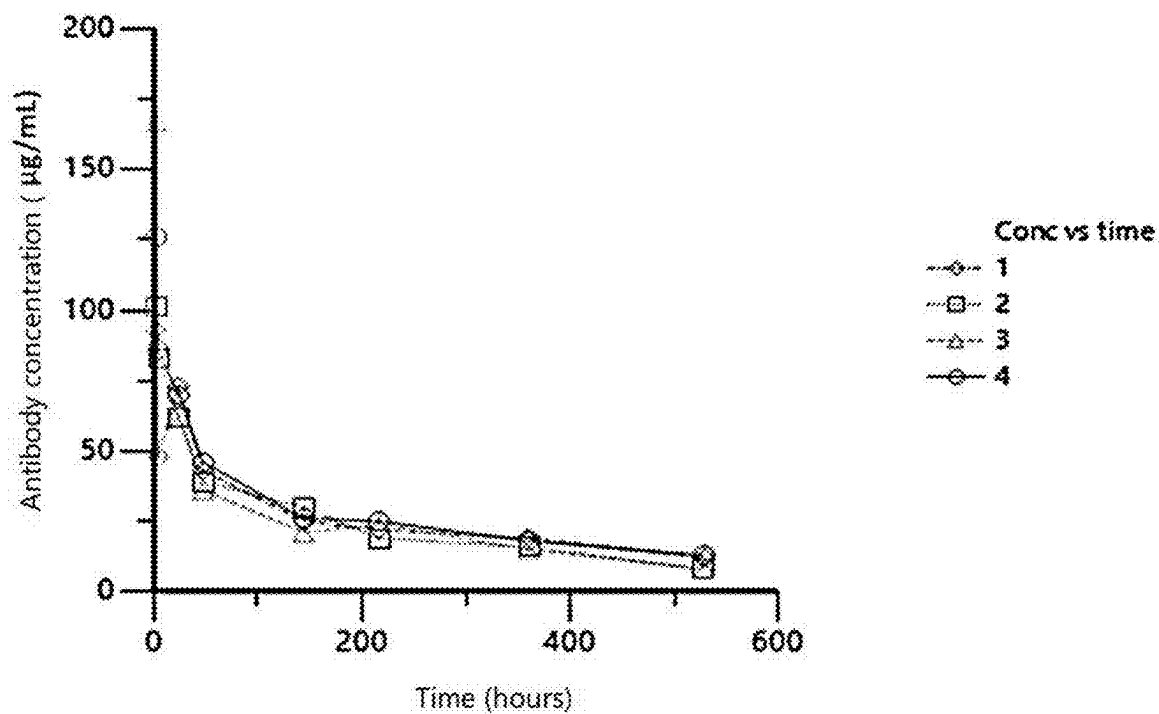


Fig. 7E

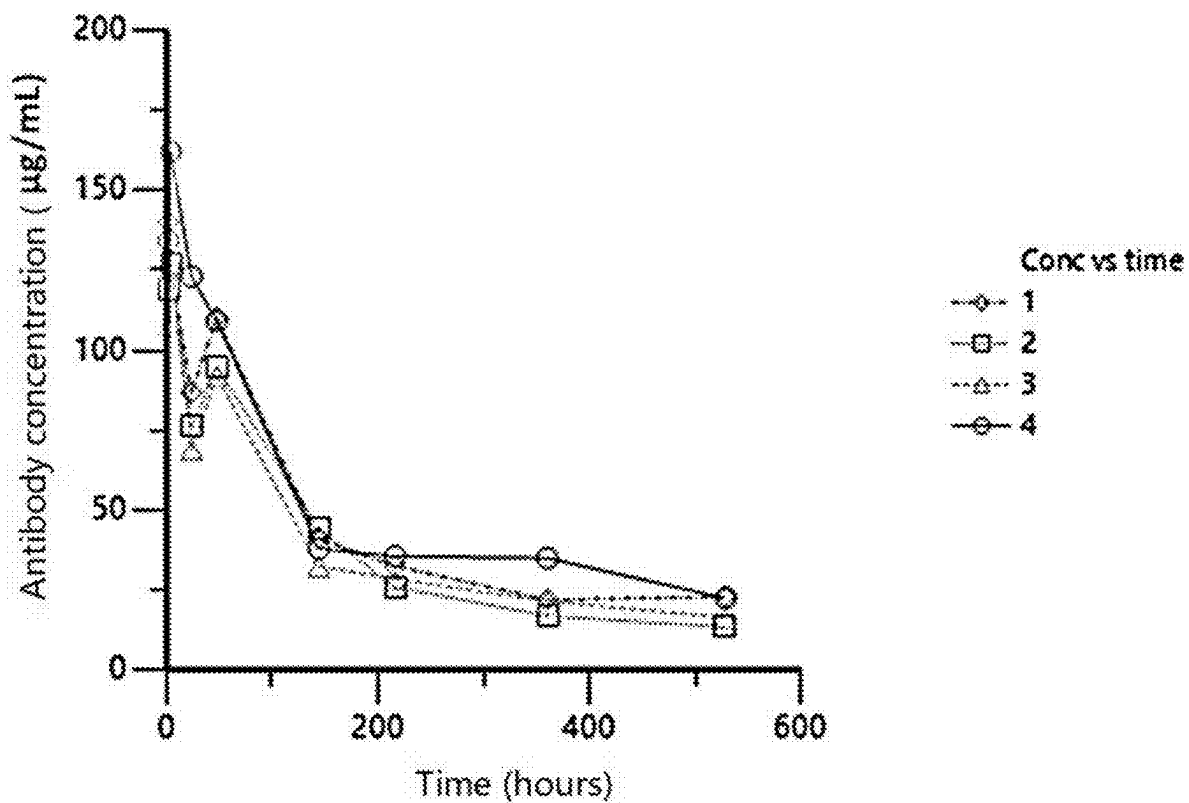


Fig. 7F

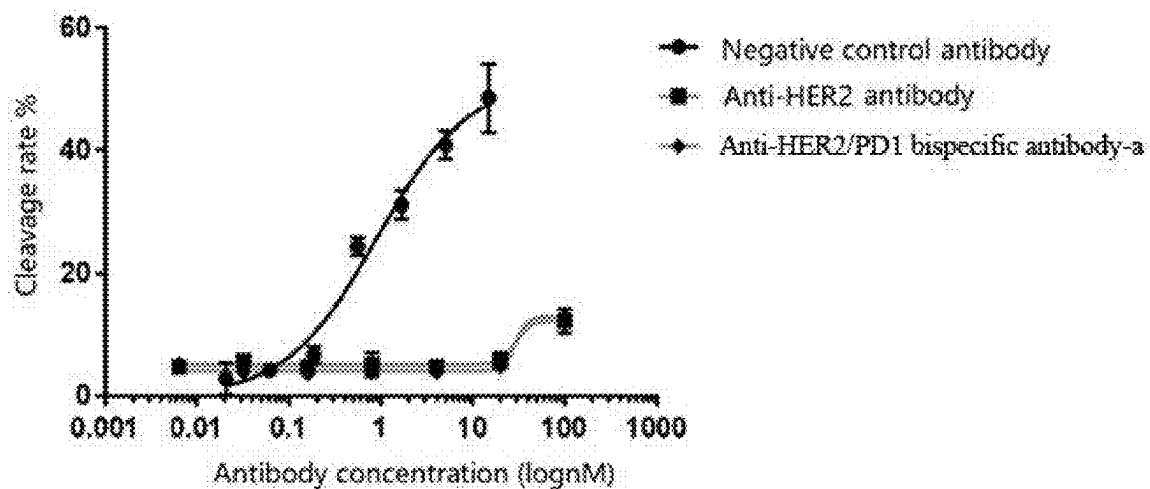


Fig. 8A

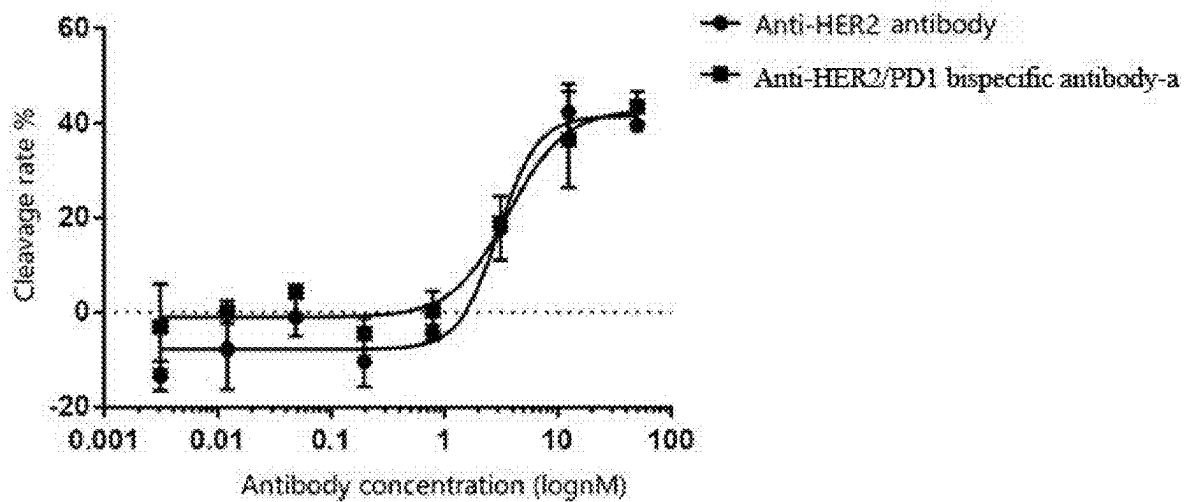


Fig. 8B

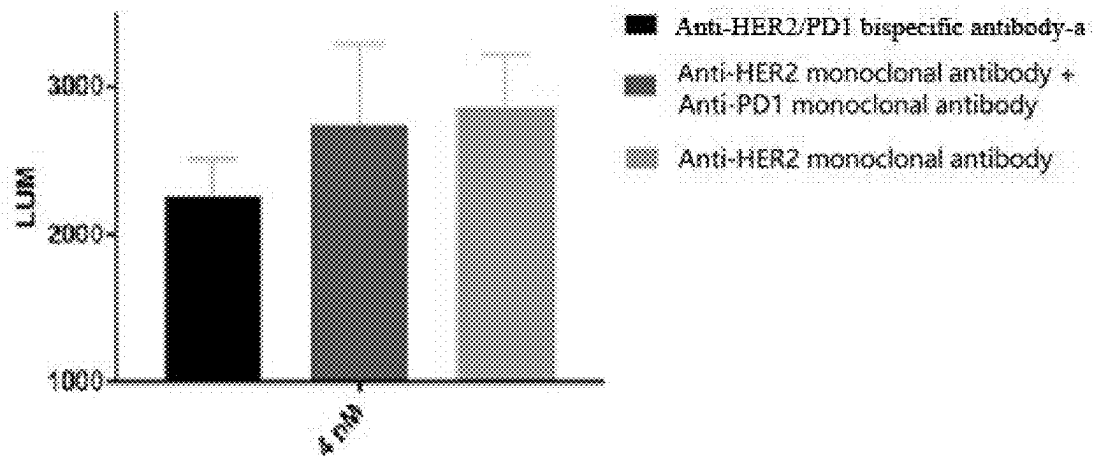


Fig. 9A

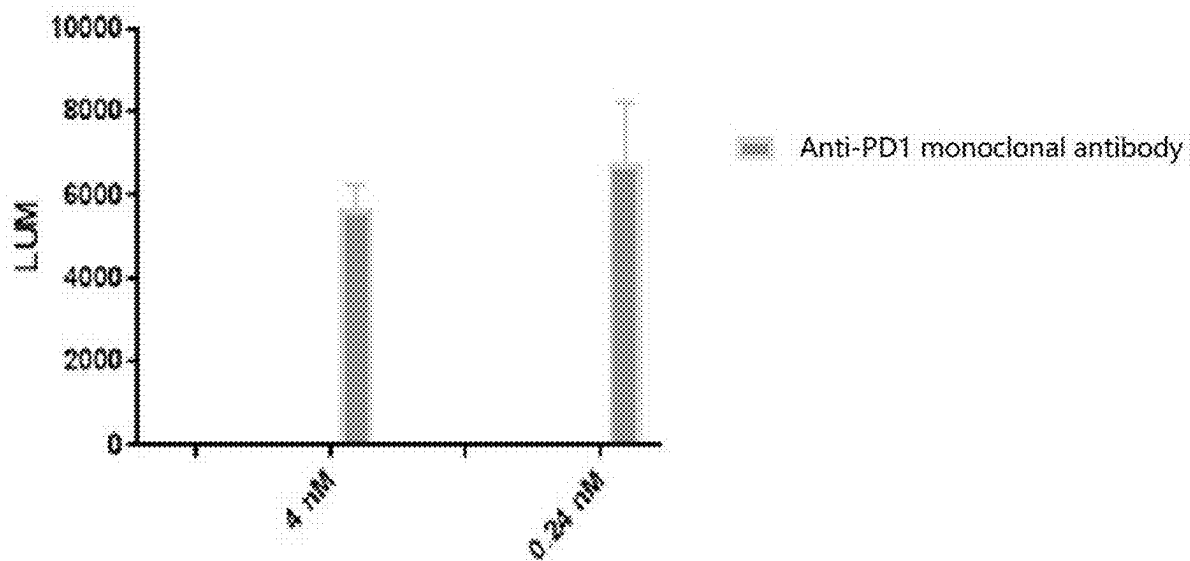


Fig. 9B

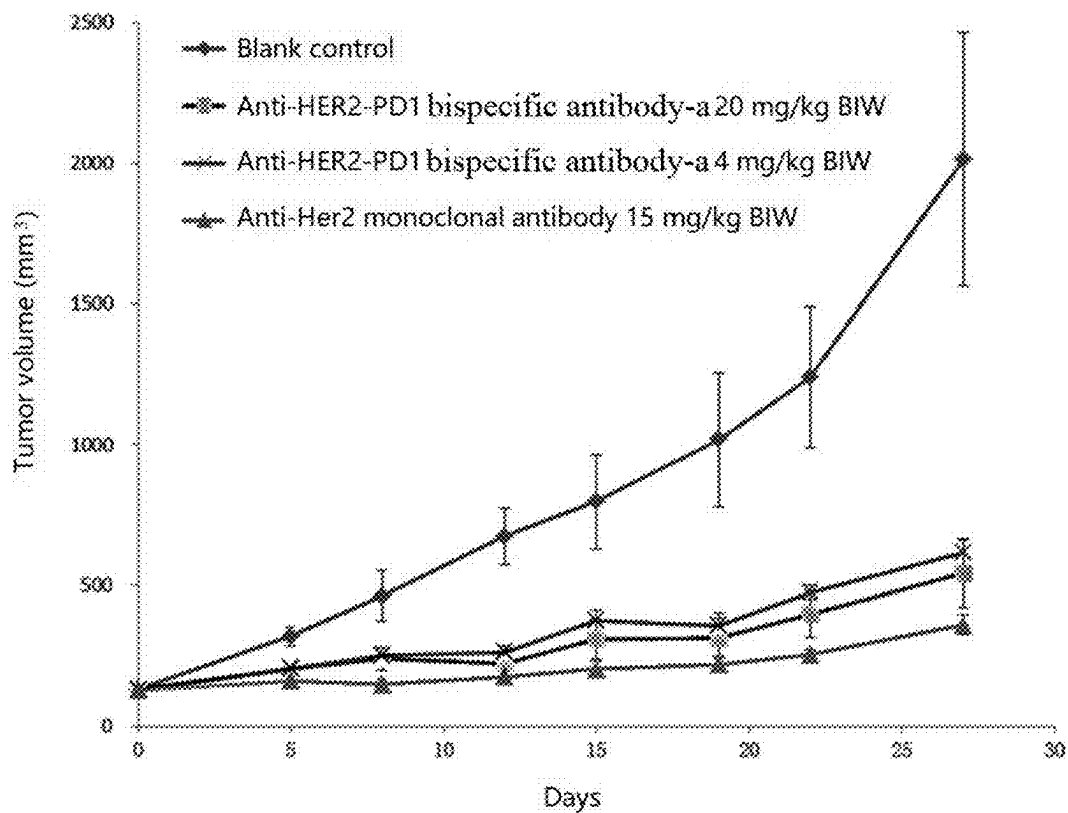


Fig. 10

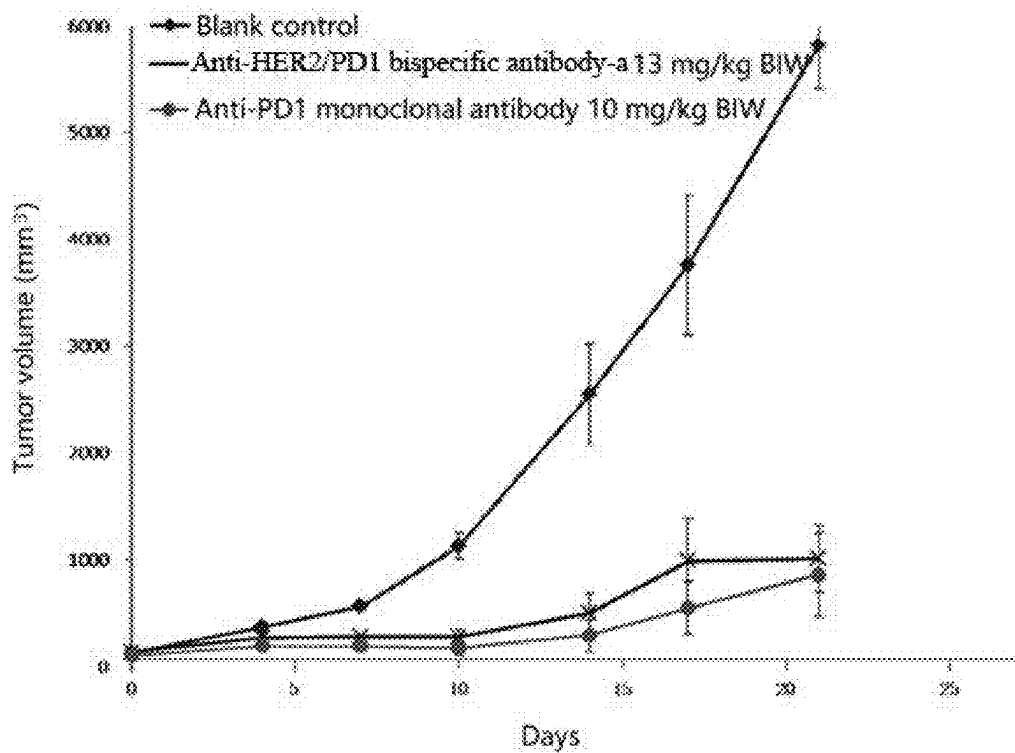


Fig. 11

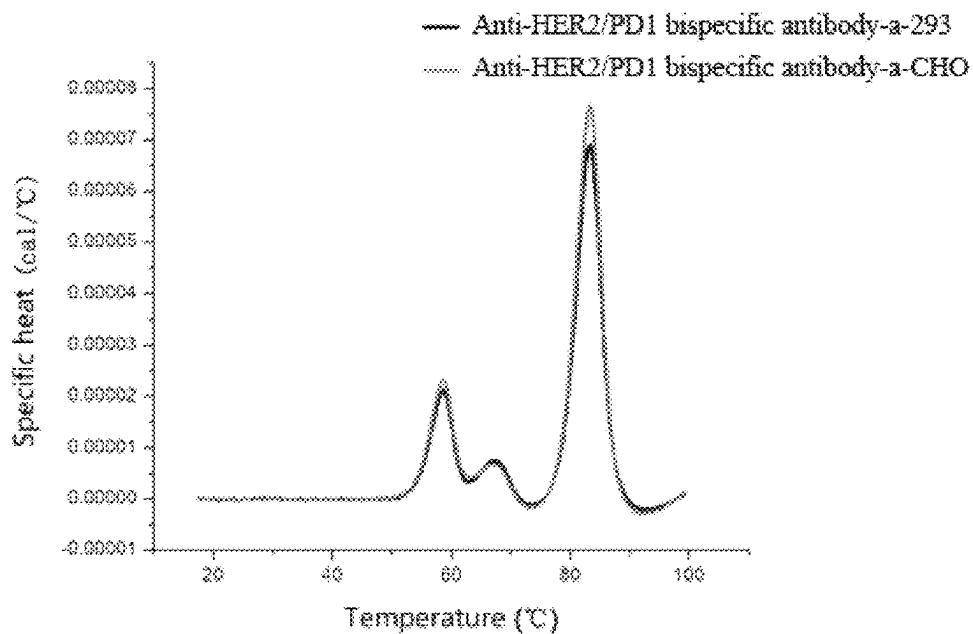


Fig. 12A

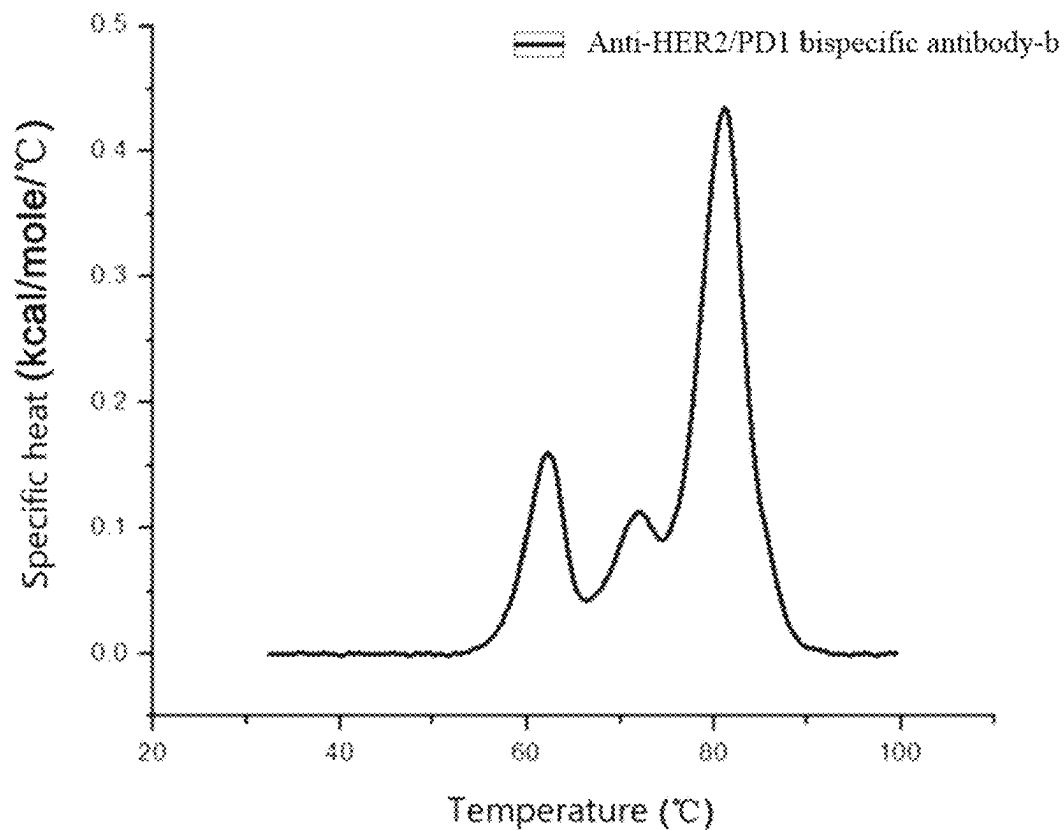


Fig. 12B

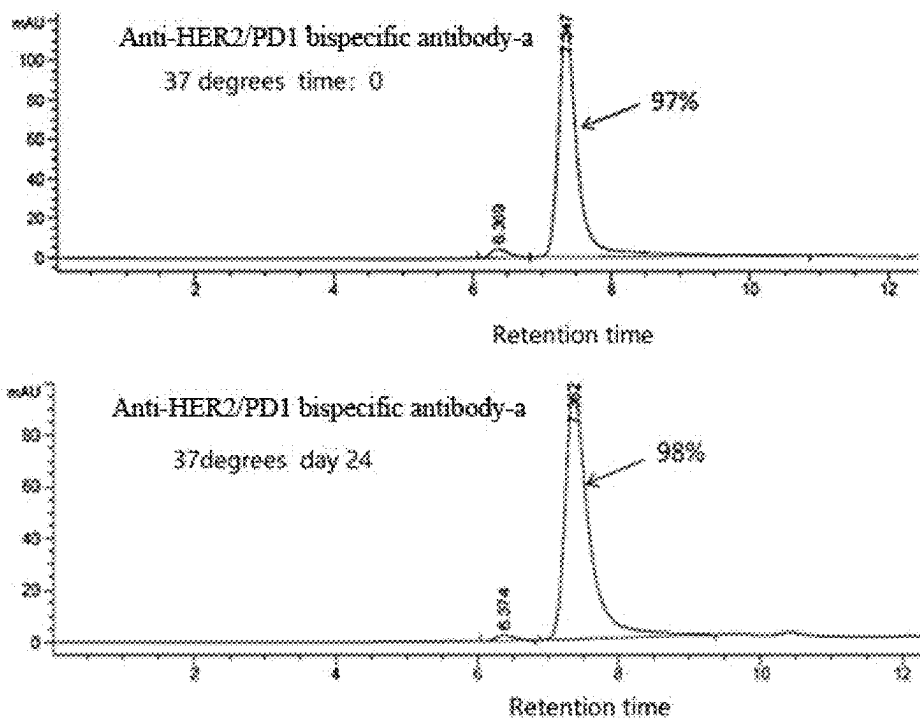


Fig. 12C

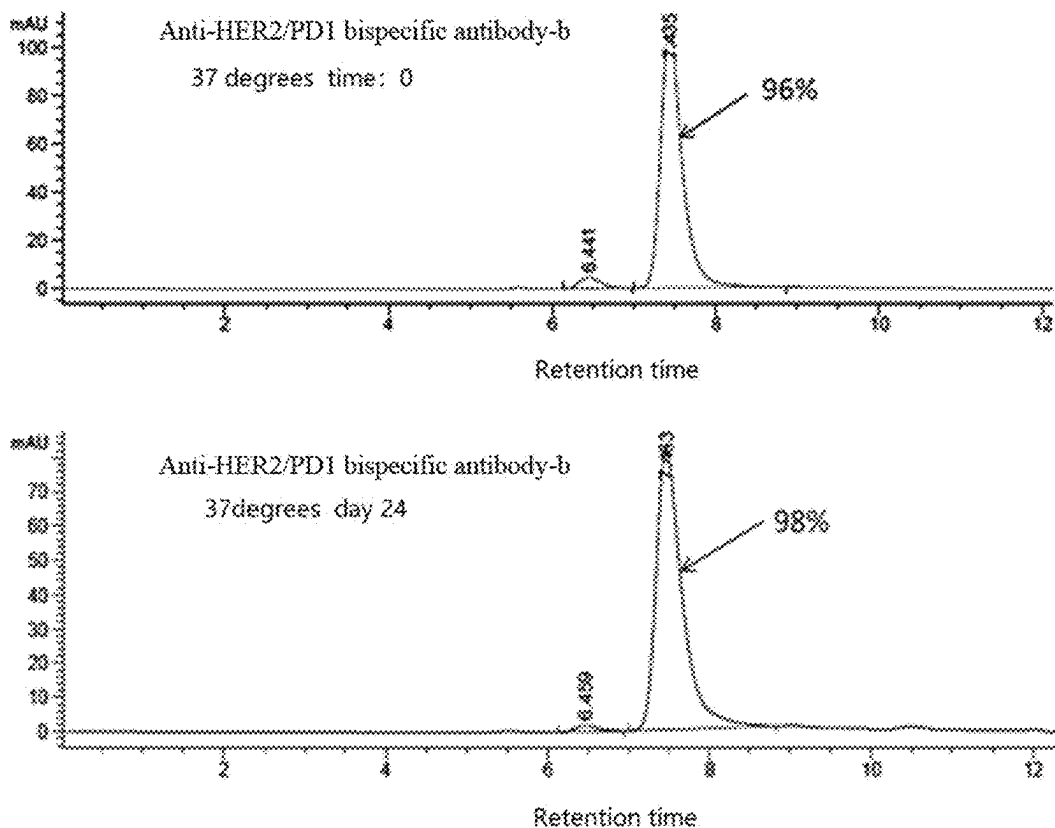


Fig. 12D

**ANTI-HER2/PD1 BISPECIFIC ANTIBODY****RELATED APPLICATIONS**

This application is a national phase application claiming benefit of priority under 35 U.S.C. § 371 to International (PCT) Patent Application serial number PCT/CN2019/112467, filed Oct. 22, 2019, which claims benefit of priority to Chinese Patent Application No. CN 201811376950.9, filed Nov. 19, 2018. The aforementioned applications are expressly incorporated herein by reference in their entirety and for all purposes.

**FIELD OF THE INVENTION**

The invention belongs to the field of tumor treatment and biotechnology, and relates to an anti-HER2 and PD1 bispecific antibody molecule, preparation method and application thereof.

**BACKGROUND OF THE INVENTION**

HER2 (human epidermal growth factor receptor 2) has tyrosine protein kinase activity, which is a member of the human epidermal growth factor receptor family. It is expressed at low levels in only a few normal tissues in adults. However, studies have shown that HER2 is overexpressed in a variety of tumors, for example, such overexpression is present in about 30% of breast cancer patients and 16% of gastric cancer patients. The overexpression of HER2 in tumors can significantly promote tumor angiogenesis, tumor growth, and enhance tumor invasion and metastasis, which is an important indicator of poor prognosis for such patients. Thus, as early as 1998, Herceptin (Genentech/Roche), the first HER2-targeting monoclonal antibody drug, was approved by the FDA for the treatment of HER2-overexpressing breast cancer and gastric cancer.

Human programmed cell death protein 1 (PD-1) is a type I membrane protein consisting of 288 amino acids, the extracellular segment is the Ig variable (V-type) domain responsible for binding ligands, and the intracellular segment is the cytoplasmic tail region responsible for binding signal transduction molecules. The cytoplasmic tail region of PD1 contains two tyrosine-based signal transduction motifs, namely ITIM (Immune Receptor Tyrosine Inhibition Motif) and ITSM (Immune Receptor Tyrosine Transduction Motif). PD-1 is expressed on the surface of activated T lymphocytes, binds to the ligands PD-L1 (programmed cell death-Ligand 1) and PD-L2 (programmed death receptor-Ligand 2) to inhibit the activity of T lymphocytes and related cellular immune responses in vivo. Numerous studies have shown that the interaction of PD-1 and PD-L1 not only maintains the balance of the immune system in the body, but is also the main mechanism that causes tumor cells that express PD-L1 to evade immune surveillance. By blocking the PD1/PD-L1 signaling pathway, the immune system can be activated and the immune killing function of T cells can be restored.

KEYTRUDA® (pembrolizumab) is the first humanized monoclonal antibody against PD-1 on the market, which was approved by the FDA in September 2014 for the treatment of melanoma, and the indications approved until 2018 include: melanoma, non-small cell lung cancer, Hodgkin's lymphoma, head and neck squamous cell carcinoma, bladder cancer, gastric cancer, and solid tumors with MSI-H or dMMR. OPDIVO® (nivolumab) is a anti-PD1 monoclonal antibody of Bristol-Myers Squibb, which was approved

for marketing by the FDA in December 2014, and the indications include: melanoma, non-small cell lung cancer, renal cell carcinoma, classic Hodgkin's lymphoma tumor, head and neck squamous cell carcinoma, bladder cancer, colorectal cancer and hepatocellular carcinoma. The anti-PD1 monoclonal antibody independently developed by Sunshine Guojian is a new humanized anti-PD1 monoclonal antibody. In vivo and in vitro biological activity and anti-tumor activity studies have shown that the biological activity of anti-PD1 is between the positive control drugs Opdivo and Keytruda, and is slightly better than the positive control drug Opdivo in some aspects.

Bispecific antibody (BsAb) refers to an antibody molecule that can bind two (or more) different epitopes at the same time. Compared with traditional monoclonal antibodies, bispecific antibodies have unique mechanisms of action: 1) Bispecific antibodies can bind to two or more different antigen molecules or different epitopes of the same molecule at the same time, but combination therapy often do not have this effect. 2) Bispecific antibodies mediate the interaction between cells, they can bind to two antigens on effector cells and target cells, respectively, build a bridge between effector cells and target cells, and promote the interaction between the cells, for example, mediate killing of tumor cells by immune cells. Therefore, bispecific antibodies have unique advantages that traditional monoclonal antibodies do not possess.

**SUMMARY OF THE INVENTION**

The present invention provides a new bispecific antibody that can specifically bind to HER2 and PD1, and also provides the preparation method and application of the bispecific antibody.

Therefore, the object of the present invention is to provide a bispecific antibody that can specifically bind to HER2 and PD1; provide a nucleotide molecule encoding the bispecific antibody; provide an expression vector comprising the nucleotide molecule; provide a host cell comprising the expression vector; provides a method of preparing the bispecific antibody; provides a pharmaceutical composition comprising the bispecific antibody; provides an application of the bispecific antibody in the preparation of a medicine.

In order to achieve the above objects, the present invention adopts the following technical solutions:

One aspect of the present invention provides a bispecific antibody capable of specifically binding to HER2 and PD1, which comprises an immunoglobulin antibody IgG and two identical single-chain variable region fragments scFv, wherein each single-chain variable fragment scFv comprises a variable region VH and a variable region VL, VH and VL are connected by a peptide linker L1, and each single-chain variable fragment scFv is connected in series with the immunoglobulin antibody IgG by a linker peptide L2.

The "bispecific antibody" in the present invention refers to a bispecific antibody that has two different antigen-binding sites and can simultaneously bind to HER2 and PD1, which comprises two single-chain variable fragments scFv and an immunoglobulin antibody IgG conjugated to it. Each scFv is connected to each heavy chain of immunoglobulin antibody IgG by a peptide linker L2 to form a heavy chain fusion protein of the bispecific antibody, wherein each scFv contains a variable region VH and a variable region VL, and VH and VL are connected by a peptide linker L1.

The "single-chain variable region fragment scFv" in the present invention refers to a fusion protein comprising the variable regions of the immunoglobulin heavy chain VH and

light chain VL. VH and VL are connected by a peptide linker, wherein the fusion protein retains the same antigen specificity as the intact immunoglobulin.

The "immunoglobulin antibody IgG" in the present invention is a molecule of about 150 kDa, which is composed of four peptide chains, containing two identical heavy chains of about 50 kDa and two identical light chains of about 25 kDa, thus having a tetrameric quaternary structure. The two heavy chains are connected to each other by disulfide bonds, and each of them is connected to a light chain. The resulting tetramer has the same two halves, which form a fork or Y-like shape, and each end of the fork contains the same antigen-binding site. IgG antibodies can be divided into multiple subclasses (for example, IgG1, 2, 3, 4) based on small differences in the amino acid sequence of the constant region of the heavy chain.

As a preferred embodiment, VH comprises complementarity determining regions HCDR1-3, wherein HCDR1 has the amino acid sequence as shown in SEQ ID NO: 1, HCDR2 has the amino acid sequence as shown in SEQ ID NO: 2, and HCDR3 has the amino acid sequence as shown in SEQ ID NO: 3;

the VL comprises complementarity determining regions LCDR1-3, wherein LCDR1 has the amino acid sequence as shown in SEQ ID NO: 4, LCDR2 has the amino acid sequence as shown in SEQ ID NO: 5, and LCDR3 has the amino acid sequence as shown in SEQ ID NO: 6;

the heavy chain of the immunoglobulin antibody IgG comprises complementarity determining regions HCDR4-6, wherein HCDR4 has the amino acid sequence as shown in SEQ ID NO: 7, wherein HCDR5 has the amino acid sequence as shown in SEQ ID NO: 8, wherein HCDR6 has the amino acid sequence as shown in SEQ ID NO: 9;

the light chain of the immunoglobulin antibody IgG comprises complementarity determining regions LCDR4-6, wherein LCDR4 has the amino acid sequence as shown in SEQ ID NO: 10, wherein LCDR5 has the amino acid sequence as shown in SEQ ID NO: 11, wherein LCDR6 has the amino acid sequence as shown in SEQ ID NO: 12;

In the art, the binding region of an antibody usually comprises one light chain variable region and one heavy chain variable region, and each variable region comprises 3 CDR domains. The CDR domains of heavy chain and light chain of an antibody are called HCDR and LCDR, respectively. Therefore, a conventional antibody antigen binding site comprises six CDRs, including a set of CDRs from the heavy and light chain V regions, respectively.

As a preferred embodiment, the scFv has the amino acid sequence of VH as shown in SEQ ID NO: 13, the amino acid sequence of VL as shown in SEQ ID NO: 14; the immunoglobulin antibody IgG has the amino acid sequence of the heavy chain variable region as shown in SEQ ID NO: 15, and the amino acid sequence of the light chain variable region is shown in SEQ ID NO: 16.

As a preferred embodiment, the peptide linker L1 has the amino acid sequence as shown in SEQ ID NO: 17.

As a preferred embodiment, the peptide linker L2 has the amino acid sequence as shown in SEQ ID NO: 18.

As a preferred embodiment, the single-chain variable fragment scFv1 has the molecular structure of VL-L1-VH, and the N-terminus of each scFv is connected to the C-terminus of the heavy chain of immunoglobulin antibody IgG by a peptide linker L2.

As a preferred embodiment, the single-chain variable fragment scFv1 has the amino acid sequence as shown in SEQ ID NO: 19.

As a preferred embodiment, the bispecific antibody has the amino acid sequence of the heavy chain as shown in SEQ ID NO: 20, and the amino acid sequence of the light chain as shown in SEQ ID NO: 21.

As a preferred embodiment, the single-chain variable fragment scFv2 has the molecular structure of VH-L1-VL, and the C-terminus of each scFv is connected to the N-terminus of the heavy chain of immunoglobulin antibody IgG by a peptide linker L2.

As a preferred embodiment, the single-chain variable fragment scFv2 has the amino acid sequence as shown in SEQ ID NO: 24.

As a preferred embodiment, the bispecific antibody has the amino acid sequence of the heavy chain as shown in SEQ ID NO: 25, and the amino acid sequence of the light chain as shown in SEQ ID NO: 21.

When constructing the bispecific antibody of the present invention, the problems related to the chemical and physical stability of the bispecific antibody are also solved, such as expressing physically stable molecules, increasing heat and salt-dependent stability, reducing aggregation, increasing solubility at high concentrations, and maintaining affinity for the two antigens HER2 and PD1, etc.

Another aspect of the present invention provides a nucleotide molecule, which encodes the above-mentioned bispecific antibody.

As a preferred embodiment, the nucleotide molecule has the nucleotide sequence encoding the heavy chain of the bispecific antibody capable of specifically binding to HER2 and PD1 as shown in SEQ ID NO: 22, and the nucleotide sequence encoding the light chain thereof as shown in SEQ ID NO: 23; or the nucleotide molecule has the nucleotide sequence encoding the heavy chain of the bispecific antibody capable of specifically binding to HER2 and PD1 as shown in SEQ ID NO: 26, and the nucleotide sequence encoding the light chain thereof as shown in SEQ ID NO: 23.

The preparation method of the nucleotide molecule of the present invention is a conventional preparation method in the art, and preferably includes the following preparation methods: the nucleotide molecule encoding the above-mentioned monoclonal antibody is obtained by gene cloning technology such as PCR method, or the nucleotide molecule encoding the above-mentioned monoclonal antibody is obtained by artificial full-sequence synthesis.

Those skilled in the art know that the nucleotide sequence encoding the amino acid sequence of the bispecific antibody can be replaced, deleted, altered, inserted or added as appropriate to provide a polynucleotide homologue. The polynucleotide homologue of the present invention can be prepared by replacing, deleting or adding one or more bases of the genes encoding the bispecific antibody within the scope of maintaining the activity of the antibody.

Another aspect of the present invention provides an expression vector, which comprises the above-mentioned nucleotide molecule.

The expression vector is a conventional expression vector in the art, which refers to an expression vector containing appropriate regulatory sequences, such as promoter sequences, terminator sequences, polyadenylation sequences, enhancer sequences, marker genes and/or sequences, and other appropriate sequences. The expression vector can be a virus or a plasmid, such as an appropriate phage or phagemid. For more technical details, please refer



to, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, 1989. For many known techniques and protocols for nucleic acid manipulation, please refer to Current Protocols in Molecular Biology, Second Edition, edited by Ausubel et al. The expression vector of the present invention is preferably pDR1, pcDNA3.1(+), pcDNA3.1/ZEO (+), pDHFR, pTT5, pDHFF, pGM-CSF or pCHO 1.0, more preferably pTT5.

In addition, the present invention provides a host cell, which comprises the above expression vector.

The host cell of the present invention may be a variety of conventional host cells in the field, as long as it can make the above-mentioned recombinant expression vector stably replicate itself and the nucleotides carried by it can be effectively expressed. Wherein the host cell includes prokaryotic expression cells and eukaryotic expression cells, and the expression vector preferably includes: COS, CHO (Chinese Hamster Ovary), NS0, sf9, sf21, DH5 $\alpha$ , BL21 (DE3) or TG1, more preferably *E. coli* TG1, BL21(DE3) cells (expressing single-chain antibodies or Fab antibodies) or CHO-K1 cells (expressing full-length IgG antibodies). The above expression vector may be transformed into a host cell to obtain the preferred recombinant expression transformant of the present invention. The transformation method may be a conventional transformation method in the art, preferably a chemical transformation method, a heat shock method or an electrotransformation method.

As a preferred embodiment, the host cell is a eukaryotic cell. Preferred are CHO cells or 293E cells.

Another aspect of the present invention provides a method of preparing the above bispecific antibody capable of specifically binding to HER2 and PD1, and the preparation method comprises the following steps of:

- a) under expression conditions, cultivating the above host cell, to express the bispecific antibody capable of specifically binding to HER2 and PD1;
- b) isolating and purifying the bispecific antibody of step a).

The method of culturing the host cell and the method of isolating and purifying the antibody of the present invention may be a conventional method in the art. For the specific operation method, please refer to the corresponding cell culture technical manual and antibody isolation and purification technical manual. The preparation method of the anti-HER2/PD1 bispecific antibody disclosed in the present invention comprises: culturing the above-mentioned host cell under expression conditions so as to express the bispecific antibody capable of specifically binding to HER2 and PD1; and isolating and purifying the anti-HER2/PD1 bispecific antibody. Using the above method, the recombinant protein can be purified into a substantially homogeneous substance, for example, showing a single band on SDS-PAGE electrophoresis.

The anti-HER2/PD1 bispecific antibody disclosed in the present invention can be isolated and purified by affinity chromatography. According to the characteristics of the affinity column used, conventional methods such as high-salt buffer, pH change, etc. may be used to elute the anti-HER2/PD1 bispecific antibody bound on the affinity column. The inventors of the present invention conducted detection experiments on the obtained anti-HER2/PD1 bispecific antibody, and the experimental results show that the anti-HER2/PD1 bispecific antibody can bind to target cells and antigens well and has a high affinity.

Another aspect of the present invention provides a composition, which comprises the above bispecific antibody

capable of specifically binding to HER2 and PD1 and one or more pharmaceutically acceptable carriers, diluents or excipients.

The bispecific antibody of the present invention can be combined with a pharmaceutically acceptable carrier to form a pharmaceutical preparation composition, so as to exert a therapeutic effect more stably. These preparations can ensure the conformational integrity of the amino acid core sequences of the bispecific antibody of the present invention, and meanwhile, protect the multifunctional groups of the protein from degradation (including but not limited to aggregation, deamination or oxidation). Generally, for liquid formulations, it can be stored at 2° C.-8° C. for at least one year, and for freeze-dried formulations, it can be stored at 30° C. for at least six months. The bispecific antibody preparation may be suspension, water injection, freeze-dried and other preparations commonly used in the pharmaceutical field.

For the water injection or freeze-dried preparation of the bispecific antibody of the present invention, pharmaceutically acceptable carriers preferably include, but are not limited to: one of surfactants, solution stabilizers, isotonic regulators, and buffers or a combination thereof. The surfactants preferably include but are not limited to: nonionic surfactants such as polyoxyethylene sorbitan fatty acid ester (Tween 20 or 80); poloxamer (e.g. poloxamer 188); Triton; sodium dodecyl sulfate (SDS); sodium lauryl sulfate; tetradecyl, linoleyl or octadecylsarcosine; Pluronic; MONA-QUAT<sup>TM</sup>, etc., which should be added in an amount such that the granulation tendency of the anti-HER2/PD1 bispecific antibody is minimized. The solution stabilizers preferably include but are not limited to one or a combination of the following: sugars, for example, reducing sugars and non-reducing sugars; amino acids, for example, monosodium glutamate or histidine; alcohols, for example, triols, higher sugar alcohols, propylene glycol, polyethylene glycol, etc. The solution stabilizer should be added in an amount such that the final formed preparation remains stable for a period of time that is considered stable by those skilled in the art. Isotonicity adjusting agents preferably include but are not limited to, one of sodium chloride, and mannitol, or a combination thereof. The buffers preferably include but are not limited to, one of Tris, histidine buffer, and phosphate buffer, or a combination thereof.

Another aspect of the present invention provides the use of the above bispecific antibody capable of specifically binding to HER2 and PD1, or the above pharmaceutical composition in the preparation of a medicine, the medicine is used for the treatment of cancers or tumors.

The medicine used for the treatment of cancers or tumors of the present invention refers to a medicine that inhibits and/or treats tumors, which may include delay in the development of tumor-related symptoms and/or reduction in the severity of these symptoms, and further include reduction of symptoms associated with pre-existing tumors and prevention of other symptoms, and also include reduction or prevention of tumor metastasis.

The tumors targeted by the medicine of the present invention preferably include, but are not limited to: lung cancer, bone cancer, stomach cancer, pancreatic cancer, skin cancer, head and neck cancer, uterine cancer, ovarian cancer, testicular cancer, uterine cancer, fallopian tube cancer, uterus endometrial cancer, cervical cancer, vaginal cancer, vulvar cancer, rectal cancer, colon cancer, anal cancer, breast cancer, esophageal cancer, small intestine cancer, endocrine system cancer, thyroid cancer, parathyroid cancer, adrenal cancer, urethral cancer, penile cancer, prostate cancer, pan-

creatic cancer, brain cancer, testicular cancer, lymphoma, transitional cell cancer, bladder cancer, kidney cancer or ureteral cancer, renal cell cancer, renal pelvis cancer, Hodgkin's disease, non-Hodgkin's lymphoma, soft tissue sarcoma, solid tumors in children, lymphocytic lymphoma, central nervous system (CNS) tumor, primary central nervous system lymphoma, tumor angiogenesis, spinal tumor, brainstem glioma, pituitary adenoma, melanoma, Kaposi's sarcoma, epidermoid carcinoma, squamous cell carcinoma, T-cell lymphoma, chronic or acute leukemia, and combinations of said cancers.

When the bispecific antibody and its composition of the present invention are administered to animals including humans, the dose may vary depending on the age and weight of the subject, the characteristics and severity of the disease, and the route of administration. The results of animal experiments and various situations may be referred, the total dose cannot exceed a certain range. Specifically, the dose of intravenous injection is 1-1800 mg/day.

The bispecific antibody and its composition of the present invention can also be administered in combination with other anti-tumor drugs to achieve the purpose of more effective treatment of tumors. Such anti-tumor drugs include but are not limited to: 1. cytotoxic drugs: 1) drugs that act on the chemical structure of nucleic acids: alkylating agents such as nitrogen mustards, nitrosoureas, and methyl-sulfonates; platinum compounds such as Cisplatin, Carboplatin and Oxaliplatin, etc.; antibiotics such as Adriamycin/Doxorubicin, Dactinomycin D, Daunorubicin, Epirubicin, Mithramycin, etc.; 2) drugs that affect nucleic acid metabolism: dihydrofolate reductase inhibitors such as Methotrexate (MTX) and Pemetrexed, etc.; thymidine synthase inhibitors such as fluorouracil (5-fluorouracil, Capecitabine), etc.; purine nucleoside synthase inhibitors such as 6-mercaptopurine, etc.; nucleotide reductase inhibitors such as Hydroxycarbamide, etc.; DNA polymerase inhibitors such as Cytosine arabinoside and Gemcitabine, etc.; 3) drugs that act on tubulin: Docetaxel, Vincristine, Vinorelbine, podophyllin, homoharringtonine, etc.; 2. hormonal drugs: anti-estrogens such as Tamoxifen, Droloxifene, Exemestane, etc.; aromatase inhibitors such as Aminoglutethimide, Formestane, Letrozole, Anastrozole, etc.; anti-androgens: flutamine RH-LH agonist/antagonist: Norride, Enanton, etc.; 3. biological response modifier drugs: these drugs mainly regulate the immune function of the body to achieve anti-tumor effects, such as Interferon; Interleukin-2; Thymosins, etc.; 4. monoclonal antibody drugs: Trastuzumab, Rituximab, Cetuximab, Bevacizumab, etc.; 5. other anti-tumor drugs: including some drugs whose mechanisms are not yet clear and need to be further studied. The bispecific antibody and its composition disclosed in the present invention can be used in combination with one or a combination of the above anti-tumor drugs.

The present invention provides a bispecific antibody that combines traditional tumor targeted therapies with immune checkpoint blockades (PD1/PDL1). Anti-HER2/PD1 bispecific antibody simultaneously bound to and bridged the two targets in vitro as well as on the cell surface without loss of affinities in comparison with its respective parental mAb. Further, the BsAb retained the biological activities of each of its parental mAb: it was as effective as the parental mAb in inhibiting proliferation of HER2-expressing tumor cells and in activating T cells via blocking PD1/PDL1 interaction. In animal studies, the BsAb demonstrated equal potency to trastuzumab in a nude mouse xenografted tumor model (a system without human T cell presence), and to the

anti-PD1 mAb (609 A) in the human PD1-transgenic mouse syngeneic tumor model (where the tumor cells does not express human HER2).

The positive and progressive effect of the present invention is that the HER2/PD1 bispecific antibody can simultaneously exert three effects and play a synergistic role in killing tumors. First, it blockaded the PD-1/PD-L1 signaling pathway. PD-L1 is expressed on tumor cells and some immune regulatory cells, while PD-1 is expressed on T cells. The binding of PD-1 and PD-L1 can inhibit the proliferation and activation of T cells. Blockading this pathway can restore the immune killing function of T cells. Second, the Fc segment of anti-HER2 antibody of this bispecific antibody can bind to the Fc receptors of NK and other cells, allowing the immune effector cells of the Fc receptor to show ADCC effects, killing of tumor cells, but no obvious killing of T cells. Third, the anti-HER2 activity of the bispecific antibody can bind to tumor cells that over-express HER2 antigen and inhibit tumor proliferation. In summary, the HER2/PD1 bispecific antibody can simultaneously bind to PD1 and HER2 antigens, blockade signal pathways, and activate immune effector cells, which work together to inhibit and kill tumor cells. Anti-HER2/PD1 bispecific antibody—also has good stability.

#### DESCRIPTION OF THE DRAWINGS

FIG. 1A: Schematic diagram of the structure of anti-HER2/PD1 bispecific antibody-a

FIG. 1B: Schematic diagram of the structure of anti-HER2/PD1 bispecific antibody-b

FIG. 2A: HPLC detection pattern of anti-HER2/PD1 bispecific antibody-a

FIG. 2B: HPLC detection pattern of anti-HER2/PD1 bispecific antibody-b

FIG. 2C: SDS-PAGE detection results of anti-HER2/PD1 bispecific antibody-a and b

FIG. 3A: Binding of anti-HER2/PD1 bispecific antibody-a, -b to HER2 detected by ELISA

FIG. 3B: Binding of anti-HER2/PD1 bispecific antibody-a, -b to PD1-ECD detected by ELISA

FIG. 4A: Binding of anti-HER2/PD1 bispecific antibody-a and anti-HER2/PD1 bispecific antibody-b to BT474 cells detected by FACS

FIG. 4B: Binding of anti-HER2/PD1 bispecific antibody-a to PD1/CHO cells detected by FACS

FIG. 4C: Binding of anti-HER2/PD1 bispecific antibody-b to PD1/CHO cells detected by FACS

FIG. 5: Inhibition of anti-HER2/PD1 bispecific antibody-a and b on the proliferation of BT474 cells in vitro

FIG. 6A: Activity of anti-HER2/PD1 bispecific antibody-a to blockade PD1/PD-L1 binding at cellular level

FIG. 6B: Activity of anti-HER2/PD1 bispecific antibody-b to blockade PD1/PD-L1 binding at cellular level

FIG. 7A: Detection of the half-life of anti-HER2 antibody in anti-HER2/PD1 bispecific antibody-a

FIG. 7B: Detection of the half-life of anti-HER2/PD1 bispecific antibody-a with biotinylated PD1

FIG. 7C: Detection of the half-life of anti-HER2/PD1 bispecific antibody-a with proteinA

FIG. 7D: Detection of the half-life of anti-HER2 antibody in anti-HER2/PD1 bispecific antibody-b

FIG. 7E: Detection of the half-life of anti-PD1 antibody in anti-HER2/PD1 bispecific antibody-b

FIG. 7F: Detection of the half-life of anti-HER2/PD1 bispecific antibody-a with proteinA

FIG. 8A: ADCC towards CD4+ T cells  
 FIG. 8B: ADCC towards BT474 tumor cells  
 FIG. 9A: Synergistic killing effect of anti-HER2/PD1 bispecific antibody-a on N87-PDL1 cells  
 FIG. 9B: Effect of PD1 control monoclonal antibody on N87-PDL1 cells  
 FIG. 10: Anti-tumor effect of anti-HER2/PD1 bispecific antibody-a on NCI-N87 xenograft model  
 FIG. 11: Anti-tumor effect of anti-HER2/PD1 bispecific antibody-a on humanized PD1 mouse MC38 xenograft model  
 FIG. 12A: DSC pattern of anti-HER2/PD1 bispecific antibody-a  
 FIG. 12B: DSC pattern of anti-HER2/PD1 bispecific antibody-b  
 FIG. 12C: Stability at 37° C., SEC-HPLC at day 0 and day 24 of anti-HER2/PD1 bispecific antibody-a  
 FIG. 12D: Stability at 37° C., SEC-HPLC at day 0 and day 24 of anti-HER2/PD1 bispecific antibody-b

#### DETAILED DESCRIPTION OF THE INVENTION

The following examples and experimental examples are intended to further illustrate the present invention, and should not be construed as limiting the present invention. The examples do not include detailed descriptions of traditional methods, such as those methods of constructing vectors and plasmids, methods of inserting genes encoding proteins into such vectors and plasmids or methods of introducing plasmids into host cells. Such methods are well known to those of ordinary skill in the art, and are described in many publications, including Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edition, Cold spring Harbor Laboratory Press.

The experimental materials and sources and the preparation methods of the experimental reagents used in the following examples are specifically described as follows.

#### Experimental Materials:

CHO cells: purchased from Thermo fisher company, catalog number A29133.  
 293E cells: from NRC biotechnology Research Institute.  
 Human breast cancer cell BT474: from the Cell Bank of Chinese Academy of Sciences, catalog number TCHu143.  
 PD-L1aAPC/CHO-K1 cells: purchased from Promega, catalog number J1252.  
 CD4+ T cells: purchased from Allcells, catalog number LP180329.  
 NK92 cells: purchased from ATCC, product number pta8837.  
 Protein A chip: label No: 29139131-AA; lot: 10261132.  
 SD rats: purchased from Zhejiang Weitong Lihua Laboratory Animal Technology Co., Ltd., production license SCXK (Zhejiang) 2018-0001.  
 Human gastric cancer cell line NCI-N87: purchased from the American Type Culture Collection (ATCC).  
 BALB/c nude mice: purchased from Shanghai Lingchang Biological Technology Co., Ltd.  
 MC38 mouse colon cancer cell line: Heyuan Biotechnology (Shanghai) Co., Ltd.  
 Humanized PD1 mouse strain C57BJ/6J-PDCD1eml (Hpdcd1)/Smoc: product number: NM-K1-00015, purchased from Shanghai Southern Model Biotechnology Co., Ltd.  
 PBMC: purchased from Sailybio, catalog number SLB-HP040A.

#### Experimental Reagents:

HRP-labeled mouse anti-human Fab antibody: purchased from sigma, catalog number A0293.  
 Streptavidin HRP: purchased from BD Biosciences, catalog number 554066.  
 Goat anti-human IgG-FITC: purchased from sigma, catalog number F4143.  
 Anti-CD28 antibody: purchased from Abcam, catalog number ab213043.  
 IL-2: purchased from R&D, catalog number 202-IL.  
 PBS: purchased from Sangon Biotech (Shanghai) Co., Ltd., catalog number B548117.  
 PBST: PBS+0.05% Tween 20.  
 BSA: purchased from Sangon Biotech (Shanghai) Co., Ltd., catalog number A60332.  
 TMB: purchased from BD company, catalog number 555214.  
 Bio-Glo: purchased from Promega, catalog number G7940.  
 FBS: purchased from Gibco, catalog number 10099.  
 HBS-EP working solution: purchased from Life science, BR-1006-69.  
 CellTiter-Glo: purchased from promega, catalog number G775B.

#### Laboratory Apparatus:

HiTrap MabSelectSuRe column: purchased from GE company.  
 Beckman Coulter CytoFLEX flow cytometer: purchased from Beckman company.  
 SpectraMax i3x microplate reader: purchased from Molecular Devices company.  
 SpectraMaxM5 microplate reader: purchased from Molecular Devices company.  
 Micro-calorimeter scanning calorimeter: MicroCal VP-Capillary DSC

The HER2 monoclonal antibody in the examples of the present invention refers to a human-mouse chimeric monoclonal antibody obtained by Sunshine Guojian Pharmaceutical company according to the amino acid sequence of Herceptin, according to the same expression and purification method as that of the double antibody in Example 2. The PD1 monoclonal antibody in the examples of the present invention refers to a brand-new anti-PD1 humanized monoclonal antibody disclosed in Chinese patent application CN201710054783.5, which is independently developed by Sunshine Guojian Pharmaceutical company.

#### Example 1. Construction of Anti-HER2/PD1 Bispecific Antibody Molecule

In the present invention, the anti-HER2/PD1 bispecific antibody-a was constructed by connecting anti-HER2 monoclonal antibody IgG and the scFv of anti-PD1 monoclonal antibody in series.

The light chain variable region VL (SEQ ID NO: 14) and heavy chain variable region VH (SEQ ID NO: 13) of the anti-PD1 monoclonal antibody were connected by peptide linker L1 (SEQ ID NO: 17), to obtain the anti-PD1 single-chain antibody fragment VL-L1-VH, i.e., the anti-PD1 fragment scFv1 (SEQ ID NO: 19). The single-chain antibody fragment and the heavy chain of the anti-HER2 monoclonal antibody were connected by L2 (SEQ ID NO: 18), to obtain the heavy chain of the bispecific antibody molecule anti-HER2/PD1 bispecific antibody-a (SEQ ID NO: 20), and the light chain of the anti-HER2 monoclonal antibody (SEQ ID NO: 21) remains unchanged. In order to improve the expression efficiency of antibody molecule in CHO cells, Genewiz

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company was entrusted to perform codon optimization of the nucleic acid sequence of anti-HER2/PD1 bispecific antibody-a molecule. During the optimization, factors such as codon preference, GC content, mRNA secondary structure, repetitive sequence, etc. were mainly considered. Then Genewiz company was entrusted to synthesize the genes. Anti-HER2/PD1 bispecific antibody-a has the nucleic acid sequence of the heavy chain as shown in SEQ ID NO: 22, and the nucleic acid sequence of the light chain as shown in SEQ ID NO: 23. The structure of anti-HER2/PD1 bispecific antibody-a is shown in FIG. 1A, and the sequence is shown in the attached sequence listing.

The molecular construction of anti-HER2/PD1 bispecific antibody-b is as follows:

The light chain variable region VL (SEQ ID NO: 14) and heavy chain variable region VH (SEQ ID NO: 13) of the anti-PD1 monoclonal antibody were connected by peptide linker L1 (SEQ ID NO: 17), to obtain the anti-PD1 single-chain antibody fragment VH-L1-VL, i.e., the anti-PD1 fragment scFv2 (SEQ ID NO: 24). The single-chain antibody fragment and the heavy chain of the anti-HER2 monoclonal antibody were connected by L2 (SEQ ID NO: 18), to obtain the heavy chain of the bispecific antibody molecule anti-HER2/PD1 bispecific antibody-b (SEQ ID NO: 25), and the light chain of the anti-HER2 monoclonal antibody (SEQ ID NO: 21) remains unchanged. In order to improve the expression efficiency of antibody molecule in CHO cells, Genewiz company was entrusted to perform codon optimization of the nucleic acid sequence of anti-HER2/PD1 bispecific antibody-b molecule. During the optimization, factors such as codon preference, GC content, mRNA secondary structure, repetitive sequence, etc. were mainly considered. Then Genewiz company was entrusted to synthesize the genes. Anti-HER2/PD1 bispecific antibody-b has the nucleic acid sequence of the heavy chain as shown in SEQ ID NO: 26, and the nucleic acid sequence of the light chain as shown in SEQ ID NO: 23. The structure of anti-HER2/PD1 bispecific antibody-b is shown in FIG. 1B, and the sequence is shown in the attached sequence listing.

#### Example 2. Expression and Purification of Bispecific Antibody

The DNA fragments of the heavy and light chains of the bispecific antibodies were subcloned into pTT5 vector, and the recombinant plasmids were extracted and co-transfected into CHO cells and/or 293E cells. After the cells were cultured for 5-7 days, the culture solutions were centrifuged at high speed, vacuum filtered with a microporous membrane, and loaded onto HiTrap MabSelectSuRe column. The proteins were eluted in one step with an eluent containing 100 mM citric acid, pH 3.5, and the target samples were recovered and dialyzed against PBS (pH 7.4). The purified proteins were detected by HPLC. The HPLC detection patterns of anti-HER2/PD1 bispecific antibodies-a and b are shown in FIGS. 2A and 2B, respectively. The antibody molecules are in uniform state and the monomer purity is over 97%. The purified anti-HER2/PD1 bispecific antibody-a and b were added with non-reducing electrophoresis buffer and detected by SDS-polyacrylamide gel electrophoresis; the purified anti-HER2/PD1 bispecific antibody-a and -b were added with reducing electrophoresis buffer and boiled, and detected by SDS-polyacrylamide gel electrophoresis. The electropherogram is shown in FIG. 2C. The theoretical molecular weight of the full-length bispecific antibodies is 199 KD.

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#### Example 3. Determination of the Affinity of Bispecific Antibody to Antigen by Enzyme-Linked Immunosorbent Assay (ELISA)

In order to detect the binding affinity of anti-HER2/PD1 bispecific antibody-a and -b to the HER2 antigen, the HER2-ECD-His protein (prepared by Sunshine Guojian) was diluted to 250 ng/ml with PBS buffer (pH7.4), and then added into an ELISA plate at 100  $\mu$ l/well; incubated overnight at 4° C.; the next day, the plate was washed twice with PBST; each well was blocked by adding PBST+1% BSA at 37° C. for 1 h; the plate was washed twice with PBST; then the antibody to be tested that was gradiently diluted with PBS+1% BSA was added, using HER2 monoclonal antibody as positive control, with an initial concentration of 100 nM, gradually diluted 3-fold for a total of 12 gradients, incubated at 37° C. for 1 h. The plate was washed twice with PBST; HRP-labeled mouse anti-human Fab antibody was added and incubated at 37° C. for 40 min; the plate was washed three times with PBST and pat dry; 100  $\mu$ l of TMB was added into each well, and placed in the dark at room temperature (20 $\pm$ 5° C.) for 5 min; 50  $\mu$ l of 2M H<sub>2</sub>SO<sub>4</sub> stop solution was added to each well to stop the substrate reaction. OD value at 450 nm was read using a microplate reader. GraphPad Prism6 was used for data analysis, graphing and calculation of EC<sub>50</sub>. The experimental results are shown in FIG. 3A. The EC<sub>50</sub>s of anti-HER2/PD1 bispecific antibody-a, -b and positive control anti-HER2 monoclonal antibody binding to HER2 were 0.1975 nM, 0.2294 nM and 0.221 nM, respectively. The three have an equivalent affinity.

In order to detect the binding ability of anti-HER2/PD1 bispecific antibody-a and -b to PD1, the recombinant PD1-ECD-hFc protein (prepared by Sunshine Guojian) was diluted to 200 ng/ml with PBS (pH7.4), added into an ELISA plate at 100  $\mu$ l/well; incubated overnight at 4° C. The plate was washed twice with PBST; blocking solution (PBS+2% BSA) was added at 200  $\mu$ l/well, placed at 37° C. for 1 hour, and then the plate was washed once for later use. The antibody to be tested was gradiently diluted with PBS+1% BSA, using anti-PD1 monoclonal antibody as positive control, with an initial concentration of 100 nM, gradually diluted 3-fold for a total of 12 gradients. They were added into the ELISA plate at 100  $\mu$ l/well and placed at 37° C. for 1 h. The plate was washed twice with PBST; HRP-labeled mouse anti-human Fab antibody was added and incubated at 37° C. for 30 min; the plate was washed three times with PBST and pat dry, 100  $\mu$ l of TMB was added into each well, and placed in the dark at room temperature (20 $\pm$ 5° C.) for 5 min; 50  $\mu$ l of 2M H<sub>2</sub>SO<sub>4</sub> stop solution was added into each well to stop the substrate reaction. OD value at 450 nm was read using a microplate reader. GraphPad Prism6 was used for data analysis, graphing and calculation of EC<sub>50</sub>. The experimental results are shown in FIG. 3B. The EC<sub>50</sub>s of anti-HER2/PD1 bispecific antibody-a, -b and positive control anti-PD1 monoclonal antibody binding to PD1 were 0.1384 nM, 0.1525 nM and 0.1557 nM, respectively. The three have an equivalent affinity.

#### Example 4. Detection of the Binding Affinity of Bispecific Antibody to Target Cells

In this experiment, the human breast cancer cell BT474 with high expression of HER2 on the cell surface was used as the target cell, washed three times with PBS containing 0.5% BSA and centrifuged at 300 g for 5 min each time to

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discard the supernatant. The cells were resuspended in PBS containing 0.5% BSA to make the cell concentration  $1 \times 10^6$  cells/mL, and added into a 96-well plate at 100  $\mu$ L/well. Anti-HER2/PD1 bispecific antibody-a, -b and positive control anti-HER2 monoclonal antibody were diluted to 400 nM, and then gradually diluted for a total of 11 gradients, and added into a 96-well plate at 100  $\mu$ L/well, mixed well with BT474 cells, and incubated at 4° C. for 1 hour. The cells were washed twice with PBS to remove unbound antibodies to be tested, and then incubated with 100  $\mu$ L of 10  $\mu$ g/ml goat anti-human IgG-FITC at 4° C. for 30 min, centrifuged at 300 g for 5 min, and washed with PBS twice to remove unbound secondary antibodies. Finally, the cells were resuspended in 200  $\mu$ L PBS, and the binding affinity of the bispecific antibodies to the cells was determined by a Beckman Coulter CytoFLEX flow cytometer. The data obtained was fitted and analyzed by GraphPad Prism6 software. The experimental results are shown in FIG. 4A. Anti-HER2/PD1 bispecific antibody-a and anti-HER2/PD1 bispecific antibody-b can specifically bind to HER2 expressed on the cell surface. The experimental results are shown in FIG. 4A. The  $EC_{50}$ s of anti-HER2/PD1 bispecific antibody-a, -b and positive control anti-HER2 monoclonal antibody binding to BT474 cells were 1.64 nM, 5.669 nM, and 1.556 nM, respectively. Among them, anti-HER2/PD1 bispecific antibody-a and positive control HER2 monoclonal antibody have an equivalent affinity, while anti-HER2/PD1 bispecific antibody-b has a slightly weaker affinity compared to positive control anti-HER2 monoclonal antibody.

Similarly, using CHO stable transfected cells expressing PD1 on the cell surface as target cells, the binding affinities of anti-HER2/PD1 bispecific antibody-a and anti-HER2/PD1 bispecific antibody-b to the cell were determined by flow cytometry. According to the same method as described above, the data obtained was fitted and analyzed by GraphPad Prism 6 software. The experimental results are shown in FIGS. 4B and 4C. Both anti-HER2/PD1 bispecific antibody-a and anti-HER2/PD1 bispecific antibody-b can specifically bind to PD1 expressed on the cell surface. The  $EC_{50}$ s of anti-HER2/PD1 bispecific antibody-a and positive control anti-PD1 monoclonal antibody were 1.777 nM and 0.8981 nM, respectively; the  $EC_{50}$ s of anti-HER2/PD1 bispecific antibody-b and positive control anti-PD1 monoclonal antibody were 1.192 nM and 0.8891 nM, respectively. The three have an equivalent affinity.

#### Example 5. Inhibition of Bispecific Antibody on the Proliferation of BT474 Cells In Vitro

The human breast cancer cell line BT474 expresses the HER2 antigen molecule on its cell surface. Since BT474 cells, when cultured in vitro, their normal proliferation is partly dependent on the growth signal transmitted by HER2 receptor. Adding anti-HER2 antibody to the culture medium can inhibit the cell proliferation. Within a certain range, the concentration of antibody has a dose-effect relationship with the degree of cell proliferation inhibition. The degree of cell proliferation can be detected by CCK-8 (Cell Counting Kit-8) cell proliferation toxicity reagent. The dose-effect relationship curve is an inverse "S" curve.

BT474 cells were digested with trypsin, resuspended and then counted. According to the density of living cells, the cells were adjusted to a density of  $5 \times 10^4$  cells/mL with complete medium, and added into Rows B~G of a 96-well cell culture plate at 100  $\mu$ L/well. 200  $\mu$ L/well of culture medium or PBS was added into the two Rows A and H to seal the edges. The cells were cultured adherently in a 37°

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C., 5% CO<sub>2</sub> incubator for 3 to 5 hours. Anti-HER2/PD1 bispecific antibodies-a and b and positive control anti-HER2 monoclonal antibody samples were prepared to be 300 nM solution using complete medium as diluent, and then gradually diluted 3-fold for a total of 11 gradients. The diluted samples were added to the corresponding cells in the 96-well plate and cultured in a 37° C., 5% CO<sub>2</sub> incubator for 7 days. Chromogenic solution was added into the cell culture plate at a ratio of 1:10 (sample dilution: CCK-8), and incubated in the CO<sub>2</sub> incubator for another 3-5 hours. OD value was measured using 650 nm as the reference wavelength and 450 nm as the detection wavelength. The data obtained was analyzed by GraphPad Prism 6 software. The experimental results are shown in FIG. 5. The  $IC_{50}$  of anti-HER2/PD1 bispecific antibody-a, anti-HER2/PD1 bispecific antibody-b and positive control anti-HER2 monoclonal antibody were 0.4967 nM, 0.9427 nM, 0.5914 nM, respectively. The three have an equivalent inhibition rate.

#### Example 6. Activity of Bispecific Antibody to Blockade the Binding of PD1/PD-L1 at the Cellular Level

The PD-L1 aAPC/CHO-K1 in logarithmic growth phase were digested with trypsin into single cells, transferred to a white, clear-bottom 96-well plate, 100  $\mu$ L/well, 40,000 cells/well, incubated at 37° C., 5% CO<sub>2</sub> overnight. Anti-HER2/PD1 bispecific antibody-a, -b, anti-PD1 monoclonal antibody, and isotype negative control sample were gradually diluted 3-fold to 2 $\times$  working solution concentration from an initial concentration of 600 nM. The PD1 effector cells with a density of  $1.4 \times 10^6$  cells/mL and a cell viability above 95% were diluted with assay buffer to produce a single cell suspension of  $1.25 \times 10^6$  cells/mL.

The supernatant of PD-L1 aAPC/CHO-K1 cells that had been seeded the day before was discarded, and 40  $\mu$ L of gradually diluted bispecific antibody/PD1 monoclonal antibody working solution was added; then an equal volume of PD1 effector cells was added, incubated at 37° C., 5% CO<sub>2</sub> for 6 hours. 80  $\mu$ L of Bio-Glo detection reagent was added into each well, incubated at room temperature for 10 min, and luminescence was read with spectramax i3.

All data were from two replicate wells. The obtained signal values were averaged and then fitted with 4-parameter method to draw the curve, as shown in FIGS. 6A, 6B, and to obtain the data of  $IC_{50}$ , top, bottom, hillslope and etc. of anti-HER2/PD1 bispecific antibody-a, as shown in Table 1:

TABLE 1

	Anti-HER2/PD1 bispecific antibody-a	Anti-PD1 monoclonal antibody
Bottom	2.707	-1.048
Top	101.1	95.61
IgIC <sub>50</sub>	0.5161	-0.05017
Hill Slope	1.048	0.8425
IC <sub>50</sub>	3.282	0.8909

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The data of IC<sub>50</sub>, top, bottom, hillslope and etc. of anti-HER2/PD1 bispecific antibody-b are shown in table 2.

TABLE 2

	Anti-HER2/PD1 bispecific antibody-b	Anti-PD1 monoclonal antibody
Bottom	4.435	4.39
Top	121.3	102.8
IgIC <sub>50</sub>	0.6467	0.03983
Hill Slope	0.8171	0.7548
IC <sub>50</sub>	4.433	1.096

#### Example 7. Affinity of Bispecific Antibody to Antigen Determined by Biacore™ 8K

The kinetic parameters of the binding between the bispecific antibody—and the antigen HER2-ECD-his were determined by the proteinA capture method. The bispecific antibody with a concentration of 1 µg/ml was bound to the Protein A chip, and the antigen HER2-ECD-his was serially diluted 2-fold from 50 nM with 1×HBS-EP working solution, and 6 concentration gradients were provided to bind to the antibody, and dissociated in HBS-EP working solution.

The kinetic parameters of the binding between the bispecific antibody—and the antigen PD1-ECD-his were determined by the proteinA capture method. The bispecific antibody with a concentration of 1 µg/ml was bound to the Protein A chip, and the antigen PD1-ECD-his was serially diluted 2-fold from 250 nM with 1×HBS-EP working solution, and 5 concentration gradients were provided to bind to the antibody, and dissociated in HBS-EP working solution.

The kinetic parameters of the binding of anti-HER2/PD1 bispecific antibody-a to HER2-ECD-His and PD1-ECD-his are shown in Table 3. The results show that anti-HER2/PD1 bispecific antibody-a has good affinity for antigens PD1 and HER2.

TABLE 3

Analyte Solution	ka (1/Ms)	kd (1/s)	KD (M)
PD1-ECD-his	2.43E+04	8.57E-05	3.53E-09
HER2-ECD-his	5.88E+04	1.92E-04	3.27E-09

KD is the affinity constant; ka is the antigen-antibody binding rate; kd is the antigen-antibody dissociation rate; KD=kd/ka.

The kinetic parameters of the binding of anti-HER2/PD1 bispecific antibody-b to HER2-ECD-His and PD1-ECD-his are shown in Table 4. The results show that anti-HER2/PD1 bispecific antibody-b has good affinity for antigens PD1 and HER2.

TABLE 4

Kinetic parameters of anti-HER2/PD1 bispecific antibody-b			
Analyte Solution	ka (1/Ms)	kd (1/s)	KD (M)
PD1-ECD-his	3.85E+04	1.86E-04	4.83E-09
HER2-ECD-his	2.16E+05	1.71E-04	7.92E-10

KD is the affinity constant; ka is the antigen-antibody binding rate; kd is the antigen-antibody dissociation rate; KD=kd/ka.

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#### Example 8. Pharmacokinetic Study of Anti-HER2/PD1 Bispecific Antibody-a and Anti-HER2/PD1 Bispecific Antibody-b

Four SD rats from each group, weighing about 200 g were injected with a dose of 2 mg of antibody by the tail vein, respectively. The blood was collected from the orbit at a specific time after treatment, and after natural coagulation, the blood was centrifuged at 8000 rpm/min to collect the serum.

The serum drug concentration of anti-HER2/PD1 bispecific antibody-a was detected by the following method:

1) Two ELISA plates were coated with 50 ng/well of HER2-His overnight at 4° C. The next day, the plates were washed twice with PBST, and then blocked with PBS+2% BSA at 37° C. for 2 hours. The standard of anti-HER2/PD1 bispecific antibody-a was diluted two-fold from an initial concentration of 0.5 µg/mL into 12 gradients. Each serum sample was diluted 2000 times. The above samples were added into the blocked ELISA plates, incubated at 37° C. for one hour. Then, the plates were washed twice with PBST.

Detection of anti-HER2 antibody: HRP-labeled mouse anti-human Fab antibody was added to one of the plates, diluted 1:3000, 100 µL/well, incubated at 37° C. for 40 min. The plate was washed 4 times with PBST and pat dry. 100 µL of TMB was added into each well, placed in the dark at room temperature (20±5° C.) for 5 min; 50 µL of 2M H<sub>2</sub>SO<sub>4</sub> stop solution was added into each well to stop the substrate reaction. OD value at 450 nm was read using a microplate reader.

Detection of anti-PD1 antibody: Biotinylated PD1-hFc was added into another plate, 7.5 ng/well, incubated for 1 hour. The plate was washed, added with Streptavidin-HRP, diluted 1:1000, placed at 37° C. for 30 min. The plate was washed 4 times with PBST and pat dry. 100 µL of TMB was added into each well, placed in the dark at room temperature (20±5° C.) for 5 min; 50 µL of 2M H<sub>2</sub>SO<sub>4</sub> stop solution was added into each well to stop the substrate reaction. OD value at 450 nm was read using a microplate reader.

2) An ELISA plate was coated with ProteinA to detect the Fab fragment of the antibody, 100 ng/well, overnight at 4° C.; the next day, the plate was washed twice with PBST, and then blocked with PBS+2% BSA at 37° C. for 2 hours. The plate was washed twice with PBST. The standard of anti-HER2/PD1 bispecific antibody-a was diluted two-fold from an initial concentration of 1000 ng/mL into 12 gradients. The rat serum samples were diluted 2000 times. The above two groups of samples were added into the blocked ELISA plate and incubated for 1 hour; the plate was washed twice with PBST; HRP-labeled mouse anti-human Fab antibody was added, and placed at 37° C. for 30 min; the plate was washed three times with PBST, pat dry; 100 µL of TMB was added into each well, and placed in the dark at room temperature (20±5° C.) for 5 min; 50 µL of 2M H<sub>2</sub>SO<sub>4</sub> stop solution was added into each well to stop the substrate reaction. OD value at 450 nm was read using a microplate reader.

Phoenix software was used to calculate the half-life of the antibody drug in rats, and GraphPad Prism6 was used for data analysis and graphing. The pharmacokinetic parameters are shown in Tables 5, 6, and 7, and the experimental results are shown in FIGS. 7A, 7B, and 7C. The half-lives in rats detected by the two methods were: 273 hours for the anti-HER2 antibody, 333 hours for the anti-PD1 antibody according to the first method; the half-life was 333 hours according to the second method. The three sets of data were similar, and it can be inferred that the half-life of anti-HER2/PD1 bispecific antibody-a is about 300 hours.

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The half-life of the anti-HER2 antibody is shown in Table 5:

TABLE 5

Group	HL_Lambda_z (hr)
1	217.1994
2	296.66456
3	245.3652
4	333.21473
Average	273

The half-life of anti-HER2/PD1 bispecific antibody-a detected with biotinylated PD1 is shown in Table 6:

TABLE 6

Group	HL_Lambda_z(hr)
1	382.77325
2	294.69571
3	302.13064
4	353.0152
Average	333

The half-life of anti-HER2/PD1 bispecific antibody-a detected with proteinA is shown in Table 7:

TABLE 7

Group	HL_Lambda_z(hr)
1	346.75496
2	369.60234
3	306.45773
4	310.91707
Average	333

The serum drug concentration of anti-HER2/PD1 bispecific antibody-b was detected by the following method:

1) Detection of anti-HER2 antibody: An ELISA plate was coated with 50 ng/well of HER2-His overnight at 4° C. The next day, the plate was washed twice with PBST, and then blocked with PBS+2% BSA at 37° C. for 2 hours. The standard of anti-HER2/PD1 bispecific antibody-b was diluted two-fold from an initial concentration of 0.5 µg/mL into 12 gradients. Each serum sample was diluted 2000 times, added into the blocked ELISA plate, incubated at 37° C. for one hour. Then, the plate was washed twice with PBST. HRP-labeled mouse anti-human Fab antibody was added, diluted 1:3000, 100 µL/well, incubated at 37° C. for 40 min. The plate was washed 4 times with PBST and pat dry. 100 µl of TMB was added into each well, placed in the dark at room temperature (20±5° C.) for 5 min; 50 µl of 2M H<sub>2</sub>SO<sub>4</sub> stop solution was added into each well to stop the substrate reaction. OD value at 450 nm was read using a microplate reader.

2) Detection of anti-PD1 antibody: An ELISA plate was coated with PD1-ECD-hFc, 20 ng/well. The methods of coating, washing plate, and diluting the standard were the same as above. The serum samples were diluted 1000-2000 times and added into the blocked ELISA plate, incubated at 37° C. for 1 hour. Then the plate was washed twice with PBST. HRP-labeled mouse anti-human Fab antibody was added, diluted 1:3000, 100 µL/well, incubated at 37° C. for 40 min. The plate was washed 4 times with PBST and pat dry. 100 µl of TMB was added into each well, placed in the dark at room temperature (20±5° C.) for 5 min; 50 µl of 2M

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H<sub>2</sub>SO<sub>4</sub> stop solution was added into each well to stop the substrate reaction. OD value at 450 nm was read using a microplate reader.

3) An ELISA plate was coated with ProteinA, 100 ng/well, overnight at 4° C., to detect the Fab fragment of the antibody; the next day, the plate was washed twice with PBST, and then blocked with PBS+2% BSA at 37° C. for 2 hours. The plate was washed twice with PBST. The standard of anti-HER-2/PD1 bispecific antibody-b was diluted two-fold from an initial concentration of 1000 ng/mL into 12 gradients. The rat serum samples were diluted 500-1000 times, added into the blocked ELISA plate and incubated for 1 hour; the plate was washed twice with PBST; HRP-labeled mouse anti-human Fab antibody was added, and placed at 37° C. for 30 min; the plate was washed 4 times, and pat dry. 100 µl of TMB was added into each well, and placed in the dark at room temperature (20±5° C.) for 5 min; 50 µl of 2M H<sub>2</sub>SO<sub>4</sub> stop solution was added into each well to stop the substrate reaction. OD value at 450 nm was read using a microplate reader.

Phoenix software was used to calculate the half-life of the antibody drug in rats, and GraphPad Prism6 was used for data analysis and graphing. The pharmacokinetic parameters are shown in Tables 8, 9, and 10, and the experimental results are shown in FIGS. 7D, 7E, and 7F. The half-lives in rats detected by the three methods were: 312 hours according to the first method; 280 hours according to the second method, and 277 hours according to the third hours. The three sets of data were similar, and it can be inferred that the half-life of anti-HER2/PD1 bispecific antibody-b is about 280 hours.

The detected half-life of the anti-HER2 antibody is shown in Table 8:

TABLE 8

Group	HL_Lambda_z(hr)
1	249.10194
2	279.51118
3	366.19333
4	355.05384
Average	312

The detected half-life of the anti-PD1 antibody is shown in Table 9:

TABLE 9

Group	HL_Lambda_z(hr)
1	375.61812
2	216.88057
3	196.51091
4	331.40524
Average	280

The half-life of anti-HER2/PD1 bispecific antibody-b detected by protein A is shown in Table 10:

TABLE 10

Group	HL_Lambda_z(hr)
1	189.38172
2	333.14994
3	389.77667
4	196.6228
Average	277

### Example 9. ADCC Effect of Anti-HER2/PD1 Bispecific Antibody-a

Because the HER2/PD1 bispecific antibody can not only bind to tumor cells expressing HER2, and bind to T cells expressing PD-1, the Fc segment of the antibody can also bind to NK cells.

On the one hand, this experiment detects whether NK92a cells can kill CD4+ T cells bound to the antibody; on the other hand, it detects whether NK92a cells kill BT474 tumor cells bound to the antibody.

1) Whether NK92a cells have a killing effect on CD4+ T cells: Activated T cells express PD1, which can be bound by the anti-HER2/PD1 bispecific antibody-a. Fc segment of anti-HER2/PD1 bispecific antibody-a can bind to Fc receptor of the effector cells NK, and the addition of NK cells can detect whether T cells are killed.

The experimental methods were as follows:

Activation of CD4+ T cells: Anti-CD3 antibody was prepared with D-PBS into a concentration of 5  $\mu\text{g/mL}$ , and used to coat a 24-well cell culture plate overnight at 4° C. The next day, 5 $\times 10^5$  CD4+ T cells were added into each well, and 2  $\mu\text{g/mL}$  of anti-CD28 antibody and 100 U/mL of IL2 were added at the same time, placed in a 37° C., CO<sub>2</sub> incubator, and activated for 72 h.

The activated T cells were collected, and the expression of PD1 was detected by FACS. Then, the T cells with high expression of PD1 were prepared with 1640 medium containing 5% FBS into 2 $\times 10^5$  cells/mL, and added into a 96-well plate, 50  $\mu\text{L/well}$ .

Dilution of anti-HER2/PD1 bispecific antibody-a and the negative control sample HER2 monoclonal antibody: anti-HER2/PD1 bispecific antibody-a and anti-HER2 monoclonal antibody were prepared into an initial concentration of 400 nM, and subjected to serial 5-fold dilution, and then added into a 96 well plate paved with T cells, incubated in a 37° C., CO<sub>2</sub> incubator for 15 min. During this period, NK92a cells were adjusted to 5 $\times 10^5$  cells/mL with 1640 medium containing 5% FBS, and added into the above 96-well plate at 100  $\mu\text{L/well}$ , incubated in a 37° C., CO<sub>2</sub> incubator for 3 hours.

The 96-well plate was centrifuged at 300 g for 5 min, the supernatant was transferred and centrifuged once again. 100  $\mu\text{L}$  of the supernatant was transferred to another 96-well plate. LDH substrate was added at 50  $\mu\text{L/well}$  and incubated for 15 min. SpectraMaxM5 microplate reader was used to read the values using 450 nm as the detection wavelength and 650 nm as the reference wavelength, and GraphPad Prism6 was used for data analysis and graphing. As shown in FIG. 8A, NK92a cells have no obvious killing effect on CD4+ T cells, and have only a weak killing effect at high concentrations of anti-HER2/PD1 bispecific antibody-a.

#### 2) ADCC Towards BT474 Tumor Cells

The HER2 antigen is expressed on the surface of BT474 cells, which can be combined by the added anti-HER2/PD1 bispecific antibody-a. The Fc segment of anti-HER2/PD1 bispecific antibody-a binds to the Fc receptor of effector cells NK, and the addition of NK cells can detect whether BT474 cells are killed.

The experimental methods were as follows:

BT474 cells were diluted with 1640 medium containing 5% FBS to 2 $\times 10^5$  cells/mL, added into a 96-well flat bottom plate, 50  $\mu\text{L/well}$ , and placed in a 37° C., 5% CO<sub>2</sub> incubator overnight.

Dilution of anti-HER2/PD1 bispecific antibody-a and the negative control sample HER2 monoclonal antibody: anti-HER2/PD1 bispecific antibody-a and HER2 monoclonal

antibody were prepared into an initial concentration of 200 nM, and subjected to gradual 4-fold dilution, and then added into a 96 well plate paved with BT474 cells, incubated in a 37° C., CO<sub>2</sub> incubator for 15 min. During this period, NK92a cells were adjusted to 5 $\times 10^5$  cells/mL with 1640 medium containing 5% FBS, and added into the above 96-well plate at 100  $\mu\text{L/well}$ , incubated in a 37° C., CO<sub>2</sub> incubator for 3 hours.

The 96-well plate was centrifuged at 300 g for 5 min, the supernatant was transferred and centrifuged once again. 100  $\mu\text{L}$  of the supernatant was transferred to another 96-well plate. LDH substrate was added at 50  $\mu\text{L/well}$  and incubated for 15 min. SpectraMaxM5 microplate reader was used to read the values using 450 nm as the detection wavelength and 650 nm as the reference wavelength, and GraphPad Prism6 was used for data analysis and graphing. As shown in FIG. 8B, NK92a cells have significant killing effect on BT474 tumor cells, which is similar to the anti-HER2 monoclonal antibody.

### Example 10. Detection of the Synergistic Effect of Anti-HER2/PD1 Bispecific Antibody-a at the Cellular Level

To detect the synergistic effect of the two targets of anti-HER2/PD1 bispecific antibody—at the cellular level, the following conditions must be met: HER2 antigen is expressed on tumor cells, and the proliferation of tumor cells can be inhibited by HER2 antibody; meanwhile, PD-L1 is expressed, which can bind to PD-1 on T cells, so the addition of anti-PD1 antibody blockade the PD-1/PD-L1 binding, releases the suppression of T cells, and plays a role in killing tumors. Since no cell strain meeting these conditions was screened, a lentiviral transfection method was used to recombine the PD-L1 gene into the human gastric cancer cell strain NCI-N87. PD-L1 was highly expressed on the cell surface of the constructed N87-PDL1 cells, detected by FACS.

N87-PDL1 cells in the logarithmic growth phase were digested with trypsin, diluted to 1 $\times 10^5$ /mL in 1640 medium supplemented with 1% FBS, and transferred to a white, clear-bottom 96-well plate, incubated at 37° C., 5% CO<sub>2</sub> overnight. The next day, the antibody to be tested and fresh PBMC cells were added, 50  $\mu\text{L/well}$ . The antibodies were antiHER2/PD1 bispecific antibody-a, anti-HER2 monoclonal antibody, anti-HER2 monoclonal antibody plus anti-PD1 monoclonal antibody, and anti-PD1 monoclonal antibody, at a concentration of 4 nM. PBMC was diluted in 1640 medium supplemented with 1% FBS, at 10<sup>5</sup>/well, incubated at 37° C. and 5% CO<sub>2</sub> for another 6 days. Then the plate was washed three times with PBS, CellTiter-Glo was diluted 1:1 with medium, and added into the 96-well plate at 100  $\mu\text{L/well}$ . Luminescence was read using spectramax i3. GraphPad Prism was used for data analysis and graphing, see FIGS. 9A and 9B. The data show that anti-HER2/PD1 bispecific antibody-a is more effective than anti-HER2 monoclonal antibody in killing tumors, and better than anti-HER2 monoclonal antibody plus anti-PD1 monoclonal antibody, indicating that the bispecific antibody has a synergistic anti-tumor effect.

### Example 11. Anti-Tumor Effect of Anti-HER2/PD1 Bispecific-Antibody-a on NCI-N87 Xenograft Model

Human gastric cancer cell strain NCI-N87 cells cultured in vitro were collected, and adjusted to a cell concentration



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of  $5 \times 10^7$  cells/mL, resuspended in serum-free medium. Under aseptic conditions, 100  $\mu$ L of cell suspension was inoculated subcutaneously into the back of nude mice. The length and width of the xenografts were measured with a vernier caliper, to calculate the tumor volume. The animals were randomly divided into groups after the tumor volume grew to 100-200 mm<sup>3</sup>.

The dose of the tested sample anti-HER2/PD1 bispecific antibody-a was divided into two groups, 20 mg/kg, 4 mg/kg, i.e., 0.4 mg/mouse, 0.08 mg/mouse, and the dose of positive control drug anti-HER2 monoclonal antibody was 15 mg/kg, i.e., 0.3 mg/mouse. The control group was given the same volume of PBS. The administration route was intraperitoneal administration, with an administration volume of 0.2 mL/mouse (20 g), twice a week for three consecutive weeks.

The xenograft volume was measured twice a week, and the mice were weighed and recorded. Tumor volume (TV) was calculated by the formula:  $TV = \frac{1}{2} \times \text{length} \times \text{width}^2$ . Relative tumor volume (RTV) was calculated according to the measurement results by the calculation formula:  $RTV = V_t / V_0$ . Wherein  $V_0$  is the tumor volume measured at the beginning of the administration (i.e., d0), and  $V_t$  is the tumor volume measured at each time. The evaluation index of anti-tumor activity is the relative tumor proliferation rate T/C (%), which is calculated by the formula:  $T/C (\%) = (TRTV/CRTV) \times 100$  (TRTV: treatment group RTV; CRTV: negative control group RTV); Tumor inhibition rate =  $1 - T/C (\%)$ . Evaluation standard for efficacy: it is ineffective if  $T/C (\%) > 40\%$ ; it is effective if  $T/C (\%) \leq 40\%$  and  $p \leq 0.05$  by statistics analysis. The results of the experiment are shown in FIG. 10. Anti-HER2/PD1 bispecific antibody-a is similar to positive control HER2 monoclonal antibody.

Example 12. Anti-Tumor Effect of Anti-HER2/PD1 Bispecific Antibody-a on Humanized PD1 Mouse MC38 Xenograft Model

The dose of the tested sample anti-HER2/PD1 bispecific antibody-a was 13 mg/kg, the dose of positive control anti-PD1 monoclonal antibody was set to 10 mg/kg, and the control group was given the same volume of normal saline. MC38 mouse colon cancer cells cultured in vitro were collected, and the cell suspension concentration was adjusted to  $1 \times 10^7$  cells/mL. Under aseptic conditions, 100  $\mu$ L of the cell suspension was inoculated subcutaneously into the right rib of humanized PD1 mice. The diameters of subcutaneous xenografts in the humanized PD1 mice were measured with a vernier caliper, and the animals were randomly divided into groups after the average tumor volume grew to 100-200 mm<sup>3</sup>. Anti-PD1 monoclonal antibody and anti-HER2/PD1 bispecific antibody-a were administered according to the dose, and the control group was given the same amount of normal saline, injected intraperitoneally twice a week for 3 consecutive weeks. During the experiment, the diameters of the xenografts were measured twice a week, and the mice were weighed at the same time. Tumor volume (TV) was calculated by the formula:  $TV = \frac{1}{2} \times \text{length} \times \text{width}^2$ . Relative tumor volume (RTV) was calculated according to the measurement results by the calculation

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formula:  $RTV = V_t / V_0$ . Wherein  $V_0$  is the tumor volume measured at the beginning of the administration (i.e., d0), and  $V_t$  is the tumor volume measured at each time. The evaluation index of anti-tumor activity is the relative tumor proliferation rate T/C (%), which is calculated by the formula as follows:  $T/C (\%) = (TRTV/CRTV) \times 100$  (TRTV: treatment group RTV; CRTV: negative control group RTV). Evaluation standard for efficacy: it is ineffective if  $T/C (\%) > 40\%$ ; it is effective if  $T/C (\%) \leq 40\%$  and  $p \leq 0.05$  by statistics analysis. The experiment was repeated twice. The experimental results are shown in FIG. 11. The results indicate that in the humanized PD1 mouse MC38 xenograft model, anti-HER2/PD1 bispecific antibody-a can inhibit tumor growth by blocking PD1, and the tumor inhibitory effect is similar to that of positive control anti-PD1 monoclonal antibody.

Example 13. Study on the Stability of Anti-HER2/PD1 Bispecific Antibody-a and Anti-HER2/PD1 Bispecific Antibody-b

This experiment can be used to evaluate the thermodynamic parameters related to the interaction, such as the unfolding of the protein when the excipients are added, so as to reveal the important mechanism information needed to develop the optimal preparation.

MicroCal VP-Capillary DSC was used in the experiment. The sample and its buffer were filtered with a 0.22  $\mu$ m filter membrane. 400  $\mu$ L of the sample and its matching buffer were placed in a 96-well plate, respectively. The sample was scanned at 25° C.-100° C., scanning rate was 120° C. per hour.

Anti-HER2/PD1 bispecific antibody-a and anti-HER2/PD1 bispecific antibody-b were stored in PBS at pH 7.4. The  $T_m$  values of the bispecific antibodies detected by DSC are shown in Table 11. The patterns are shown in FIGS. 12A and 12B. It can be seen that the bispecific antibodies are relatively stable. The results of subsequent long-term stability experiments at 37° C. also verified this. The HPLC-SEC results are shown in FIGS. 12C and 12D.

TABLE 11

Sample No.	$T_m$ Onset	$T_m1$	$T_m2$
Anti-HER2/PD1 bispecific antibody-a-CHO	50	58	83
Anti-HER2/PD1 bispecific antibody-a-293E	51	58	83
Anti-HER2/PD1 bispecific antibody-b-293E	53	62	81

It can be seen from the above experiments that the bispecific antibodies provided by the present invention have a stable structure. They can bind both HER2 and PD1 antigens at the same time; block the HER2 signaling pathway and can inhibit the proliferation of tumor cells expressing HER2 antigen; can also blockade the PD-1/PD-L1 pathway and restore the immune killing function of T cells and play a role in killing tumor cells. Meanwhile, the anti-HER2/PD1 exhibited strong ADCC towards BT474 tumor cells, comparable to that of anti-HER2 antibody. By contrast, no ADCC towards T cells could be detected.

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           50                  55                  60  
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 Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln His Tyr Thr Thr Pro Pro  
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<210> SEQ ID NO 17  
 <211> LENGTH: 20  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: L1

<400> SEQUENCE: 17

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Ser Gly  
 1                  5                  10                  15  
 Gly Gly Gly Ser  
           20

<210> SEQ ID NO 18  
 <211> LENGTH: 15  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: L2

<400> SEQUENCE: 18

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Ser  
 1                  5                  10                  15

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<210> SEQ ID NO 19
<211> LENGTH: 244
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: scFv1

<400> SEQUENCE: 19

Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser Leu Ser Pro Gly
1             5             10            15

Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Ile Ser Asn Phe
                20            25            30

Leu His Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile
            35            40            45

Lys Tyr Ala Ser Gln Ser Ile Ser Gly Ile Pro Ala Arg Phe Ser Gly
            50            55            60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu Pro
65            70            75            80

Glu Asp Phe Ala Val Tyr Phe Cys Gln Gln Ser Asn Ser Trp Pro His
            85            90            95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Gly Gly Gly Gly Ser
            100           105           110

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Glu
            115           120           125

Val Lys Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly Ser
            130           135           140

Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Ala Phe Ser Ser Tyr Asp
145           150           155           160

Met Ser Trp Val Arg Gln Ala Pro Gly Lys Arg Leu Glu Trp Val Ala
            165           170           175

Thr Ile Ser Gly Gly Gly Arg Tyr Thr Tyr Tyr Pro Asp Thr Val Lys
            180           185           190

Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Ser His Tyr Leu
            195           200           205

Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Phe Cys Ala
            210           215           220

Ser Pro Tyr Gly Gly Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val
225           230           235           240

Thr Val Ser Ser

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<210> SEQ ID NO 20
<211> LENGTH: 709
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: heavy chain

<400> SEQUENCE: 20

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1             5             10            15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Asn Ile Lys Asp Thr
            20            25            30

Tyr Ile His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
            35            40            45

Ala Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr Ala Asp Ser Val
            50            55            60

Lys Gly Arg Phe Thr Ile Ser Ala Asp Thr Ser Lys Asn Thr Ala Tyr

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65					70					75					80
Leu	Gln	Met	Asn	Ser	Leu	Arg	Ala	Glu	Asp	Thr	Ala	Val	Tyr	Tyr	Cys
				85					90					95	
Ser	Arg	Trp	Gly	Gly	Asp	Gly	Phe	Tyr	Ala	Met	Asp	Tyr	Trp	Gly	Gln
			100					105					110		
Gly	Thr	Leu	Val	Thr	Val	Ser	Ser	Ala	Ser	Thr	Lys	Gly	Pro	Ser	Val
		115					120					125			
Phe	Pro	Leu	Ala	Pro	Ser	Ser	Lys	Ser	Thr	Ser	Gly	Gly	Thr	Ala	Ala
	130					135					140				
Leu	Gly	Cys	Leu	Val	Lys	Asp	Tyr	Phe	Pro	Glu	Pro	Val	Thr	Val	Ser
145					150					155					160
Trp	Asn	Ser	Gly	Ala	Leu	Thr	Ser	Gly	Val	His	Thr	Phe	Pro	Ala	Val
				165					170					175	
Leu	Gln	Ser	Ser	Gly	Leu	Tyr	Ser	Leu	Ser	Ser	Val	Val	Thr	Val	Pro
		180						185					190		
Ser	Ser	Ser	Leu	Gly	Thr	Gln	Thr	Tyr	Ile	Cys	Asn	Val	Asn	His	Lys
	195						200					205			
Pro	Ser	Asn	Thr	Lys	Val	Asp	Lys	Lys	Val	Glu	Pro	Lys	Ser	Cys	Asp
	210					215					220				
Lys	Thr	His	Thr	Cys	Pro	Pro	Cys	Pro	Ala	Pro	Glu	Leu	Leu	Gly	Gly
225					230					235					240
Pro	Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	Leu	Met	Ile
			245						250					255	
Ser	Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	Asp	Val	Ser	His	Glu
		260						265					270		
Asp	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	Glu	Val	His
	275						280					285			
Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	Asn	Ser	Thr	Tyr	Arg
	290					295					300				
Val	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	Trp	Leu	Asn	Gly	Lys
305				310						315					320
Glu	Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu	Pro	Ala	Pro	Ile	Glu
			325						330					335	
Lys	Thr	Ile	Ser	Lys	Ala	Lys	Gly	Gln	Pro	Arg	Glu	Pro	Gln	Val	Tyr
		340						345					350		
Thr	Leu	Pro	Pro	Ser	Arg	Glu	Glu	Met	Thr	Lys	Asn	Gln	Val	Ser	Leu
	355					360						365			
Thr	Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp	Ile	Ala	Val	Glu	Trp
	370				375						380				
Glu	Ser	Asn	Gly	Gln	Pro	Glu	Asn	Asn	Tyr	Lys	Thr	Thr	Pro	Pro	Val
385				390						395					400
Leu	Asp	Ser	Asp	Gly	Ser	Phe	Phe	Leu	Tyr	Ser	Lys	Leu	Thr	Val	Asp
			405						410					415	
Lys	Ser	Arg	Trp	Gln	Gln	Gly	Asn	Val	Phe	Ser	Cys	Ser	Val	Met	His
		420						425					430		
Glu	Ala	Leu	His	Asn	His	Tyr	Thr	Gln	Lys	Ser	Leu	Ser	Leu	Ser	Pro
	435						440					445			
Gly	Lys	Gly	Gly	Gly	Gly	Ser	Gly	Gly	Gly	Gly	Ser	Gly	Gly	Gly	Gly
	450					455					460				
Ser	Glu	Ile	Val	Leu	Thr	Gln	Ser	Pro	Ala	Thr	Leu	Ser	Leu	Ser	Pro
465					470					475					480
Gly	Glu	Arg	Ala	Thr	Leu	Ser	Cys	Arg	Ala	Ser	Gln	Ser	Ile	Ser	Asn
			485						490					495	

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Phe Leu His Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu  
                   500                                  505                                  510  
 Ile Lys Tyr Ala Ser Gln Ser Ile Ser Gly Ile Pro Ala Arg Phe Ser  
                   515                                  520                                  525  
 Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu  
                   530                                  535                                  540  
 Pro Glu Asp Phe Ala Val Tyr Phe Cys Gln Gln Ser Asn Ser Trp Pro  
                   545                                  550                                  555                                  560  
 His Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Gly Gly Gly Gly  
                                   565                                  570                                  575  
 Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser  
                                   580                                  585                                  590  
 Glu Val Lys Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly  
                                   595                                  600                                  605  
 Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Ala Phe Ser Ser Tyr  
                                   610                                  615                                  620  
 Asp Met Ser Trp Val Arg Gln Ala Pro Gly Lys Arg Leu Glu Trp Val  
                                   625                                  630                                  635                                  640  
 Ala Thr Ile Ser Gly Gly Gly Arg Tyr Thr Tyr Tyr Pro Asp Thr Val  
                                   645                                  650                                  655  
 Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Ser His Tyr  
                                   660                                  665                                  670  
 Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Phe Cys  
                                   675                                  680                                  685  
 Ala Ser Pro Tyr Gly Gly Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu  
                                   690                                  695                                  700  
 Val Thr Val Ser Ser  
 705

<210> SEQ ID NO 21  
 <211> LENGTH: 214  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: light chain

<400> SEQUENCE: 21

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly  
 1                  5                                  10                                  15  
 Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val Asn Thr Ala  
                   20                                  25                                  30  
 Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile  
                   35                                  40                                  45  
 Tyr Ser Ala Ser Phe Leu Tyr Ser Gly Val Pro Ser Arg Phe Ser Gly  
                   50                                  55                                  60  
 Ser Arg Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro  
                   65                                  70                                  75                                  80  
 Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln His Tyr Thr Thr Pro Pro  
                   85                                  90                                  95  
 Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala  
                   100                                  105                                  110  
 Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly  
                   115                                  120                                  125  
 Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala  
                   130                                  135                                  140



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Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln  
145 150 155 160

Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser  
165 170 175

Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr  
180 185 190

Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser  
195 200 205

Phe Asn Arg Gly Glu Cys  
210

<210> SEQ ID NO 22

<211> LENGTH: 2127

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: heavy chain

<400> SEQUENCE: 22

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gaggccaac tggaggagtc cggaggagga ctggtgcaac ccggcggatc cctccggctg      60
tcttgtgctg ctacgggctt taacatcaag gatacctata tccattgggt taggcaagct      120
cccgtaagg gcttagaatg ggtcgctagg atctacccca ccaatggcta tactcgttac      180
gccgacagcg tgaagggtcg gttcaccatc tccgctgaca cctccaagaa cacagcttac      240
ctccaaatga actctttacg ggccgaggac acagccgtgt actactgttc ccggtgggga      300
ggcgacggct tctatgctat ggattactgg ggccaaggta ctttagtgac agtgtccagc      360
gccagcacia aaggaccttc cgtcttcctt ctggctccct cctccaagag caccagcggc      420
ggaacagctg ctctggctg tctggtgaag gactacttcc ccgaaccgtt taccgtgtct      480
tggaattccg gcgctttaac ctccggcggt cacaccttcc ccgctgtttt acagagcagc      540
ggcctctatt ctttaagctc cgtgggcaca gtgcctagca gctctttagg caccagacc      600
tacatctgca acgtgaacca taagcccagc aataccaagg tcgacaagaa ggtggagccc      660
aagagctgcg ataagaccca cacatgtcct ccttgtcccg ctcccgaaact gctgggagga      720
cccagcgtgt ttttattccc ccccaaaccc aaagacaccc tcatgatcag ccggaccccc      780
gaggtcacat gcgtggtggt ggacgtctcc cacgaagacc ccgaagtcaa gttcaactgg      840
tacgtggacg gagtcgaggt gcataacgcc aagacaaaac cccgggagga gcaatacaac      900
tccacctatc gtgtggtgag cgtgtctacc gtgtgcatc aagattggct gaatggcaag      960
gagtacaagt gcaaggctcg caacaaggct ctgcccgtcc ctatcgagaa gacaatcagc     1020
aaagccaaag gccaaccccc ggagccccc aaagtatactt taccacctcc ccgggaagag     1080
atgaccaaga accaagtctt ttaacatgt ttagtgaaag gcttttacc ctcgacatc     1140
gccgtcgagt gggagtccaa tggccagccc gaaaataact acaagacaac ccccccggtg     1200
ctcgattccg atggatcctt ctttttatac agcaagctca cagtggataa gtcccgggtg     1260
cagcaaggaa acgtgttttc ttgtccgtc atgcaagagg ccctccataa ccactacacc     1320
cagaaatccc tctctctcag ccccggtaaa ggccgaggag gatccggcgg cggcggaagc     1380
ggaggaggcg gctccgagat cgtgtgtgac cagtcccccg ctacactgtc cctctctccc     1440
ggtgaacggg ctactttaag ctgtctgtcc agccaaagca tcagcaactt ttactactgg     1500
taccaacaga aaccgggcca agtcccccg ctgtgatca agtacgcttc ccagagcatc     1560
agcggcattc ccgctaggtt ctccggcagc ggcagcggaa ccgatttcac ttaaccatc     1620

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agctcttttag agcccgagga cttcgccgtc tactttctgcc aacagagcaa tagctggcct 1680
cacacatttcg gccaaagtac aaaggctcag atcaaagggtg gaggtggcag cgggtggcggc 1740
ggcagcgggtg gtggtggaag cggaggcgga ggctccgagg tgaagctcgt ggaatctggc 1800
ggaggtttag tgcagcccg tggtctttta aggtgagct gtgctgccag cggttttgcc 1860
tttagctcct acgacatgag ctgggtgcgt caagctccc gaaagaggct ggagtgggtg 1920
gccacaatct cggcggtgg tcgttatacc tattacccc acacgtcaa aggccggttt 1980
accatcagcc gggacaacgc taagaacagc cattacctcc agatgaattc ttacgggct 2040
gaggacaccg ctgtgtatct ctgtgctagc ccctacggcg gctactttga tgtctgggga 2100
caaggtaccc tcgtgaccgt gagctcc 2127

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<210> SEQ ID NO 23
<211> LENGTH: 642
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: light chain

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<400> SEQUENCE: 23

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gacatccaga tgacacagag cccctcctct ttatccgctt ccgtgggaga tcgtgtcacc 60
atcacttgct gtgcctccca agatgtgaac accgctgtgg cttggtacca gcagaagccc 120
ggtaaggctc ccaagctgct gatctactcc gccagctttt tatactccgg cgtgccctct 180
cgtttctccg gatctcgttc cggcaccgac ttcactttaa ccatcagctc ttacagccc 240
gaggacttcg ccacctacta ctgccagcag cactacacca cccccctac ctteggacaa 300
ggtaccaagg tggagatcaa gaggaccgtg gccgccccct ccgtcttcat ctttccccct 360
tccgacgagc agctgaagtc cggcacagcc tccgtgggtg gtttactgaa taacttctac 420
ccccgggagg ccaaggtgca gtggaagggt gataacgctt tacagtccgg caacagccaa 480
gaaagcgtga ccgaacaaga tagcaaggac tccacctact ctttatcctc cactttaact 540
ttaagcaagg ccgactacga gaagcataag gtgtacgctt gtgaggtgac ccatcaagg 600
ttaagcagcc ccgtgaccaa gtccttcaac cggggcgaat gc 642

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<210> SEQ ID NO 24
<211> LENGTH: 244
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: scFv2

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<400> SEQUENCE: 24

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Glu Val Lys Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1           5           10          15
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Ala Phe Ser Ser Tyr
20          25          30
Asp Met Ser Trp Val Arg Gln Ala Pro Gly Lys Arg Leu Glu Trp Val
35          40          45
Ala Thr Ile Ser Gly Gly Gly Arg Tyr Thr Tyr Tyr Pro Asp Thr Val
50          55          60
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Ser His Tyr
65          70          75          80
Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Phe Cys
85          90          95

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Ala	Ser	Pro	Tyr	Gly	Gly	Tyr	Phe	Asp	Val	Trp	Gly	Gln	Gly	Thr	Leu
			100					105					110		
Val	Thr	Val	Ser	Ser	Gly	Gly	Gly	Gly	Ser	Gly	Gly	Gly	Gly	Ser	Gly
		115					120					125			
Gly	Gly	Gly	Ser	Gly	Gly	Gly	Gly	Ser	Glu	Ile	Val	Leu	Thr	Gln	Ser
		130					135					140			
Pro	Ala	Thr	Leu	Ser	Leu	Ser	Pro	Gly	Glu	Arg	Ala	Thr	Leu	Ser	Cys
					150					155					160
Arg	Ala	Ser	Gln	Ser	Ile	Ser	Asn	Phe	Leu	His	Trp	Tyr	Gln	Gln	Lys
				165					170					175	
Pro	Gly	Gln	Ala	Pro	Arg	Leu	Leu	Ile	Lys	Tyr	Ala	Ser	Gln	Ser	Ile
			180					185					190		
Ser	Gly	Ile	Pro	Ala	Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Asp	Phe
		195					200					205			
Thr	Leu	Thr	Ile	Ser	Ser	Leu	Glu	Pro	Glu	Asp	Phe	Ala	Val	Tyr	Phe
		210				215					220				
Cys	Gln	Gln	Ser	Asn	Ser	Trp	Pro	His	Thr	Phe	Gly	Gln	Gly	Thr	Lys
		225			230					235					240
Val	Glu	Ile	Lys												

<210> SEQ ID NO 25  
 <211> LENGTH: 709  
 <212> TYPE: PRT  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: heavy chain

<400> SEQUENCE: 25

Glu	Val	Lys	Leu	Val	Glu	Ser	Gly	Gly	Gly	Leu	Val	Gln	Pro	Gly	Gly
1				5					10					15	
Ser	Leu	Arg	Leu	Ser	Cys	Ala	Ala	Ser	Gly	Phe	Ala	Phe	Ser	Ser	Tyr
			20					25					30		
Asp	Met	Ser	Trp	Val	Arg	Gln	Ala	Pro	Gly	Lys	Arg	Leu	Glu	Trp	Val
		35					40					45			
Ala	Thr	Ile	Ser	Gly	Gly	Gly	Arg	Tyr	Thr	Tyr	Tyr	Pro	Asp	Thr	Val
		50				55					60				
Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ala	Lys	Asn	Ser	His	Tyr
		65			70					75					80
Leu	Gln	Met	Asn	Ser	Leu	Arg	Ala	Glu	Asp	Thr	Ala	Val	Tyr	Phe	Cys
			85						90					95	
Ala	Ser	Pro	Tyr	Gly	Gly	Tyr	Phe	Asp	Val	Trp	Gly	Gln	Gly	Thr	Leu
			100					105					110		
Val	Thr	Val	Ser	Ser	Gly	Gly	Gly	Gly	Ser	Gly	Gly	Gly	Gly	Ser	Gly
		115					120					125			
Gly	Gly	Gly	Ser	Gly	Gly	Gly	Gly	Ser	Glu	Ile	Val	Leu	Thr	Gln	Ser
		130					135					140			
Pro	Ala	Thr	Leu	Ser	Leu	Ser	Pro	Gly	Glu	Arg	Ala	Thr	Leu	Ser	Cys
					150					155					160
Arg	Ala	Ser	Gln	Ser	Ile	Ser	Asn	Phe	Leu	His	Trp	Tyr	Gln	Gln	Lys
				165					170					175	
Pro	Gly	Gln	Ala	Pro	Arg	Leu	Leu	Ile	Lys	Tyr	Ala	Ser	Gln	Ser	Ile
			180					185					190		
Ser	Gly	Ile	Pro	Ala	Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Asp	Phe
		195					200					205			
Thr	Leu	Thr	Ile	Ser	Ser	Leu	Glu	Pro	Glu	Asp	Phe	Ala	Val	Tyr	Phe

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210	215	220
Cys Gln Gln Ser Asn Ser Trp Pro His Thr Phe Gly Gln Gly Thr Lys 225 230 235 240		
Val Glu Ile Lys Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly 245 250 255		
Gly Gly Ser Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln 260 265 270		
Pro Gly Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Asn Ile 275 280 285		
Lys Asp Thr Tyr Ile His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu 290 295 300		
Glu Trp Val Ala Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr Ala 305 310 315 320		
Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Ala Asp Thr Ser Lys Asn 325 330 335		
Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val 340 345 350		
Tyr Tyr Cys Ser Arg Trp Gly Gly Asp Gly Phe Tyr Ala Met Asp Tyr 355 360 365		
Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly 370 375 380		
Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly 385 390 395 400		
Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val 405 410 415		
Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe 420 425 430		
Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val 435 440 445		
Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val 450 455 460		
Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys 465 470 475 480		
Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu 485 490 495		
Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr 500 505 510		
Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val 515 520 525		
Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val 530 535 540		
Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser 545 550 555 560		
Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu 565 570 575		
Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala 580 585 590		
Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro 595 600 605		
Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln 610 615 620		
Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala 625 630 635 640		

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Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr  
645 650 655

Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu  
660 665 670

Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser  
675 680 685

Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser  
690 695 700

Leu Ser Pro Gly Lys  
705

<210> SEQ ID NO 26

<211> LENGTH: 2127

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: heavy chain

<400> SEQUENCE: 26

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gaggatgaagc tgggtgagtc cggcggcggc ctggtgcagc ctggcggctc cctgaggctg      60
tctctgcgcg cctcgggett cgccttctcc tctacgaca tgtctgggt gaggcaggcc      120
cctggcaaga ggctggagt ggtggccacc atctcggcg gcggcaggta cactactac      180
cctgacaccg tgaagggcag gtccaccatc tccagggaca acgccaagaa ctcccactac      240
ctgcagatga actcctgag ggccgaggac accgcggtgt acttctgcgc ctccccttac      300
ggcggtactc tcgacgtgtg gggccagggc accctggtga ccgtgtcctc cggcgcgggc      360
ggctcggcg gcggcggtc cggcgcgggc ggctcggcg gcggcggtc cgagatcgtg      420
ctgacccagt cccctgccac cctgtcctg tccctggcg agagggccac cctgtcctgc      480
agggcctccc agtccatctc caacttctg cactggtacc agcagaagcc tggccaggcc      540
cctaggctgc tgatcaagta cgcctcccag tccatctccg gcacccctgc cagggtctcc      600
ggctcgggt cgggcacga cttcacctg accatctcct ccctggagcc tgaggacttc      660
gccgtgtact tctgccagca gtccaactcc tggcctcaca ccttcggcca gggcaccaag      720
gtggagatca agggcgcgcg cggtcggc ggcgcggtc cggcgcgcg cggtcggcg      780
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tccgtgttcc tgttccctcc taagcctaag gacaccctga tgatctccag gaccctgag     1560
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aagtcctctg cctctgcccc tggcaag	2127

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What is claimed is:

1. A bispecific antibody capable of specifically binding to HER2 and PD1, comprising:

(a) an immunoglobulin antibody IgG comprising an IgG heavy chain and an IgG light chain, each comprising an N-terminus and a C-terminus; and

(b) two identical single-chain variable fragments scFv each comprising an N-terminus and a C-terminus, wherein each single-chain variable fragment scFv comprises a heavy chain variable region (VH) and a light chain variable region (VL),

wherein the VH and the VL are connected by a peptide linker L1, and each of the two identical single-chain variable fragment scFv independently is connected in series to the immunoglobulin antibody IgG by a peptide linker L2,

wherein the VH of the scFv of the bispecific antibody comprises: a complementarity determining region HCDR1, a complementarity determining region HCDR2, and a complementarity determining region HCDR3,

wherein the HCDR1 has an amino acid sequence comprising SEQ ID NO:1, the HCDR2 has an amino acid sequence comprising SEQ ID NO: 2, and the HCDR3 has an amino acid sequence comprising SEQ ID NO: 3;

wherein the VL of the scFv of the bispecific antibody comprises: a complementarity determining region LCDR1, a complementarity determining region LCDR2, and a complementarity determining region LCDR3,

wherein the LCDR1 has an amino acid sequence comprising SEQ ID NO: 4, the LCDR2 has an amino acid sequence comprising SEQ ID NO: 5, and the LCDR3 has an amino acid sequence comprising SEQ ID NO: 6;

wherein the IgG heavy chain of the immunoglobulin antibody IgG comprises: a complementarity determining region HCDR4, a complementarity determining region HCDR5, and a complementarity determining region HCDR6,

wherein the HCDR4 has an amino acid sequence comprising SEQ ID NO: 7, the HCDR5 has an amino acid sequence comprising SEQ ID NO: 8, the HCDR6 has an amino acid sequence comprising SEQ ID NO: 9;

wherein the IgG light chain of the immunoglobulin antibody IgG comprises: a complementarity determining region LCDR4, a complementarity determining region LCDR5, and a complementarity determining region LCDR6,

wherein LCDR4 has an amino acid sequence comprising SEQ ID NO: 10 LCDR5 has an amino acid sequence comprising SEQ ID NO: 11, LCDR6 has an amino acid sequence comprising SEQ ID NO: 12.

2. The bispecific antibody of claim 1, wherein:

(a) (i) the VH of the single-chain variable fragment scFv has the amino acid sequence comprising SEQ ID NO: 13, and

(ii) the VL of the single-chain variable fragment scFv has an amino acid sequence comprising SEQ ID NO:14; and

(b) (i) the IgG heavy chain has a heavy chain variable region comprising an amino acid sequence comprising SEQ ID NO:15, and

(ii) the IgG light chain has a light chain variable region comprising an amino acid sequence comprising SEQ ID NO:16.

3. The bispecific antibody of claim 1, wherein the peptide linker L1 has an amino acid sequence comprising SEQ ID NO: 17, and/or the peptide linker L2 has an amino acid comprising SEQ ID NO: 18.

4. The bispecific antibody of claim 1, wherein each of the two identical single-chain variable fragments scFv has a molecular structure of VL-L1-VH, and the N-terminus of each of the two identical single-chain variable fragments scFv is independently connected to the C-terminus of the IgG heavy chain of the immunoglobulin antibody IgG by a peptide linker L2.

5. The bispecific antibody of claim 1, wherein each of the two identical single-chain variable fragments scFv has an amino acid sequence comprising SEQ ID NO: 19.

6. The bispecific antibody of claim 1, wherein:

(a) the heavy chain of the bispecific antibody has an amino acid sequence comprising SEQ ID NO:20, and

(b) the light chain of the bispecific antibody has an amino acid sequence of the light chain comprising as shown in SEQ ID NO:21.

7. The bispecific antibody of claim 1, wherein:

(a) each of the two identical single-chain variable fragments scFv has an molecular structure of VH-L1-VL, and

(b) the C-terminus of each of the two identical single-chain variable fragments scFv is independently connected to the N-terminus of the IgG heavy chain of the immunoglobulin antibody IgG by a peptide linker L2.

8. The bispecific antibody of claim 1, wherein each of the two identical single-chain variable fragments scFv has an amino acid sequence comprising SEQ ID NO: 24.

9. The bispecific antibody of claim 1, wherein:

- (a) the heavy chain of the bispecific antibody has an amino acid sequence comprising SEQ ID NO:25, and
- (b) the light chain of the bispecific antibody has an amino acid sequence comprising SEQ ID NO:21.

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10. A composition comprising: the bispecific of claim 1; and, one or more pharmaceutically acceptable carriers, diluents or excipients.

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