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Anti-PD-1 and anti-VEGFA bifunctional antibody, pharmaceutical composition thereof and use thereof

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ABSTRACT

The present invention relates to the fields of tumor treatment and molecular immunology, and specifically, to an anti-VEGFA/PD-1 bifunctional antibody, a pharmaceutical composition thereof and use thereof. Specifically, the anti-VEGFA/PD-1 bifunctional antibody comprises a first protein functional region targeting VEGFA and a second protein functional region targeting PD-1. The bifunctional antibody of the present invention can specifically bind to VEGFA and PD-1, specifically relieve immunosuppression of VEGFA and PD-1 in an organism, and inhibit tumor-induced angiogenesis, thus having good application prospect.

SPECIFICATION

ANTI-PD-1/VEGFA BIFUNCTIONAL ANTIBODY, PHARMACEUTICAL COMPOSITION THEREOF AND USE THEREOF

CROSS REFERENCE

This application is a divisional of Australian Patent Application No. 2019332708, which is the Australian National Phase Application of PCT/CN2019/103618, and claims priority to 20181102548.4 dated 30 August 2018. The entire content of each of these applications is hereby incorporated herein by reference.

REFERENCE TO A SEQUENCE LISTING

Preceding applications contained a Sequence Listing which was originally submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on 1 March 2020, is named “IEC190067PCT-SEQUENCELISTING.txt” and is 24.3 kilobytes in size. The present application contains a sequence listing which has been submitted electronically as an XML document in the ST.26 format and is hereby incorporated by reference in its entirety. Said XML copy, created on 11 February 2026, is named “P0030509AUD1 Sequence Listing.xml” and is 37.3 kilobytes in size.

TECHNICAL FIELD

The present invention relates to the fields of tumor treatment and immunobiology, particularly to an anti-PD-1/VEGFA bifunctional antibody, a pharmaceutical composition thereof and use thereof. Specifically, the present invention relates to an anti-human PD-1/human

VEGFA bifunctional antibody, a pharmaceutical composition thereof and use thereof.

BACKGROUND

Tumor, especially a malignant tumor, is a serious health-threatening disease in the world today, and it is the second leading cause of death among various diseases. In recent years, the incidence of the disease has been increasing remarkably. Malignant tumor is characterized by poor treatment response, high late metastasis rate and poor prognosis. Although conventional treatment methods (such as radiotherapy, chemotherapy and surgical treatment) adopted clinically at present alleviate the pain to a great extent and prolong the survival time, the methods have great limitations, and it is difficult to further improve their efficacy.

There are two distinct stages of tumor growth, namely, from a slow growth stage without blood vessels to a rapid proliferation stage with blood vessels. The angiogenesis enables the tumor to acquire enough

[Text continues on page 2]

nutrition to complete the blood vessel switching stage, and if there is no angiogenesis, the primary tumor will be no more than 1-2 mm, and thus the metastasis cannot be realized.

Vascular Endothelial Growth Factor (VEGF) is a growth factor which can promote division and proliferation of endothelial cells, promote formation of new blood vessels and improve blood vessel permeability, and it binds to vascular endothelial growth factor receptors on the cell surface and plays a role by activating tyrosine kinase signal transduction pathways. In tumor tissues, tumor cells, and macrophages and mast cells invading into tumors can secrete high-level VEGF, stimulate tumor vascular endothelial cells in a paracrine form, promote proliferation and migration of endothelial cells, induce angiogenesis, promote continuous growth of tumor, improve vascular permeability, cause fibrin deposition in surrounding tissues, and promote infiltration of mononuclear cells, fibroblast and endothelial cells, which facilitates formation of tumor stroma and entry of tumor cells into new blood vessels, and promote tumor metastasis. Therefore, inhibiting tumor angiogenesis is considered to be one of the most promising tumor treatment methods at present. The VEGF family includes: VEGFA, VEGFB, VEGFC, VEGFD and PIGF. Vascular Endothelial Growth Factor Receptors (VEGFRs) include VEGFR1 (also known as Flt1), VEGFR2 (also known as KDR or Flk1), VEGFR3 (also known as Flt4), and Neuropilin-1 (NRP-1). The first three receptors are similar in structure, belong to a tyrosine kinase superfamily, and are composed of an extramembrane region, a transmembrane segment and an intramembrane region, where the extramembrane region is composed of an immunoglobulin-like domain, and the intramembrane region is a tyrosine kinase region. VEGFR1 and VEGFR2 are located primarily on the surface of vascular endothelial

cells, and VEGFR3 is located primarily on the surface of lymphatic endothelial cells.

Molecules of the VEGF family have different affinities for these receptors. VEGFA mainly acts in combination with VEGFR1, VEGFR2 and NRP-1. VEGFR1 is the earliest found receptor and has a higher affinity for VEGFA than VEGFR2 under normal physiological conditions, but it has a lower tyrosinase activity in intracellular segment than VEGFR2 (Ma Li, J. Chinese *Journal of Birth Health and Heredity*, 24 (5): 146-148 (2016)).

VEGFR2 is the primary regulator of angiogenesis and vascular engineering, and has a much higher tyrosine kinase activity than VEGFR1. VEGFR2, after binding to ligand VEGFA, mediates the proliferation, differentiation and the like of vascular endothelial cells, as well as the formation process of blood vessels and the permeability of blood vessels (Roskoski R Jr. *et al.*, *Crit Rev Oncol Hematol*, 62(3): 179-213 (2007)). VEGFA, after binding to VEGFR2, mediates the transcriptional expression of intracellular related protein genes through the downstream PLC-γ-PKC-Raf-MEK-MAPK signaling pathway, and thus promotes the proliferation of vascular endothelial cells (Takahashi T *et al.*, *Oncogene*, 18(13): 2221-2230 (1999)).

VEGFR3 is one of the tyrosine kinase family members, and mainly expresses embryonic vascular endothelial cell and adult lymphatic endothelial cells, and VEGFC and VEGFD bind to VEGFR3 to stimulate proliferation and migration of lymphatic endothelial cells and promote neogenesis of lymphatic vessels; NRP-1 is a non-tyrosine kinase transmembrane protein and is incapable of independently transducing biological signals, and it is able to mediate signaling only after forming a complex with a VEGF tyrosine kinase receptor. (Ma Li, *Chinese Journal*

***of Birth Health and Heredity*, 24(5): 146-148 (2016)).**

VEGFA and VEGFR2 are mainly involved in regulation of angiogenesis, where before and after the binding of VEGFA to VEGFR2, a cascade reaction of numerous intermediate signals in upstream and downstream pathways is formed, and finally the physiological functions are changed by proliferation, survival, migration, permeability increase and infiltration to peripheral tissues, *etc*. of endothelial cells (Dong Hongchao *et al.*, Sep. 2014, *Journal of Modern Oncology*, 22(9): 2231-3).

Currently, there are several humanized monoclonal antibodies targeting human VEGF, particularly VEGFA, such as bevacizumab, which has been approved by the U.S. Food and Drug Administration for the treatment of various tumors such as non-small cell lung cancer, renal cell carcinoma, cervical cancer, and metastatic colorectal cancer in succession during 2004.

The programmed cell death receptor-1 (PD-1), also known as CD279, is a type I transmembrane glycoprotein membrane surface receptor, belongs to the CD28 immunoglobulin superfamily, and is commonly expressed in T cells, B cells, and myeloid cells. PD-1 has two natural ligands, PD-L1 and PD-L2. Both PD-L1 and PD-L2 belong to the B7 superfamily and are expressed constitutively or inducibly on the membrane surface a variety of cells, including nonhematopoietic cells and a variety of tumor cells. PD-L1 is mainly expressed on T cells, B cells, DC and microvascular endothelial cells and a variety of tumor cells, while PD-L2 is expressed only on antigen presenting cells such as dendritic cells and macrophages. The interaction between PD-1 and its ligands can inhibit the activation of lymph, the proliferation of T cells, and the secretion of cytokines such as IL-2 and IFN-γ.

A large number of researches show that a tumor microenvironment can protect tumor cells from being damaged by immune cells, expression of PD-1 in lymphocytes infiltrated in the tumor microenvironment is up-regulated, and various primary tumor tissues are PD-L1 positive in immunohistochemical analysis, such as lung cancer, liver cancer, ovarian cancer, skin cancer, colon cancer and glioma. Meanwhile, the expression of PD-L1 in the tumor is significantly correlated with poor prognosis of cancer patients. Blocking the interaction between PD-1 and its ligands can promote the tumor-specific T cell immunity and enhance the immune elimination efficiency of tumor cells. A large number of clinical trials show that antibodies targeting PD-1 or PD-L1 can promote infiltration of CD8$^+$ T cells into tumor tissues and up-regulate anti-tumor immune effector factors such as IL-2, IFN-γ, granzyme B and perforin, thereby effectively inhibiting the growth of tumors.

In addition, anti-PD-1 antibodies may also be used in the treatment of viral chronic infections. Viral chronic infections are often accompanied by a loss of function of virus-specific effector T cells and a reduction in its number. The interaction between PD-1 and PD-L1 can be blocked by injecting a PD-1 antibody, thereby effectively inhibiting the exhaustion of effector T cells in viral chronic infection.

Due to the broad anti-tumor prospect and surprising efficacy of PD-1 antibodies, it is widely accepted in the industry that antibodies targeting the PD-1 pathway will bring about breakthroughs in the treatment of a variety of tumors: for the treatment of non-small cell lung cancer, renal cell carcinoma, ovarian cancer and melanoma (Homet M. B., Parisi G., *et al.*, Anti-PD-1 therapy in melanoma. *Semin Oncol.* 2015 Jun; 42(3): 466-473), and lymphoma and anemia (Held SA, Heine A, *et al.*, Advances in immunotherapy of chronic myeloid leukemia CML. *Curr Cancer Drug*

Targets 2013 Sep; 13(7): 768-74).

The bifunctional antibody, also known as bispecific antibody, is a specific medicament that targets two different antigens simultaneously, and can be produced by immunoselection purification. In addition, the bispecific antibody can also be produced by genetic engineering, which has certain advantages due to corresponding flexibility in aspects such as the optimization of binding sites, consideration of synthetic form, and yield. Currently, the bispecific antibody has been demonstrated to exist in over 45 forms (Müller D, Kontermann RE. Bispecific antibodies for cancer immunotherapy: current perspectives. *BioDrugs* 2010; 24: 89-98). A number of bispecific antibodies have been developed in the form of IgG-ScFv, namely the Morrison form (Coloma M. J., Morrison S. L. Design and production of novel tetravalent bispecific antibodies. *Nat Biotechnol.*, 1997; 15: 159-163), which has been demonstrated to be one of the ideal forms of the bispecific antibodies because of its similarity to the naturally existing IgG form and advantages in antibody engineering, expression and purification (Miller B. R., Demarest S. J., *et al.*, Stability engineering of scFvs for the development of bispecific and multivalent antibodies. *Protein Eng Des Sel* 2010; 23: 549-57; Fitzgerald J, Lugovskoy A. Rational engineering of antibody therapeutics targeting multiple oncogene pathways. *MAbs* 2011; 3: 299-309).

Currently, there is a need to develop a bifunctional antibody medicament targeting both PD-1 and VEGF (*e.g.*, VEGFA).

SUMMARY

Through in-depth research and creative efforts, and based on commercially available VEGFA monoclonal antibody Avastin (bevacizumab) and 14C12H1L1 acquired before (see Chinese patent publication No. CN106977602A), the inventors has acquired a humanized bifunctional antibody named VP101, which is capable of simultaneously binding to VEGFA and PD-1, and blocking the binding of VEGFA to VEGFR2 and that of PD-1 to PD-L1.

The inventors have surprisingly found that VP101 is capable of:

effectively binding to PD-1 on the surface of human immune cells, relieving immunosuppression mediated by PD-L1 and PD-1, and promoting secretion of IFN-γ and IL-2 by human immune cells;

effectively inhibiting VEGFA-induced proliferation of vascular endothelial cells, and thereby inhibiting tumor-induced angiogenesis; and/or

having the potential of being used for preparing medicaments for preventing and treating malignant tumors such as liver cancer, lung cancer, melanoma, renal tumor, ovarian cancer and lymphoma.

The present invention is detailed below.

One aspect of the present invention relates to a bispecific antibody, which comprises:

a first protein functional region targeting VEGFA, and

a second protein functional region targeting PD-1;

preferably,

the first protein functional region is an anti-VEGFA antibody or an antigen-binding fragment thereof, a heavy chain variable region of the anti-VEGFA antibody comprising HCDR1-HCDR3 with amino acid sequences set forth in SEQ ID NOs: 15-17 respectively, and a light chain variable region of the anti-VEGFA antibody comprising LCDR1-LCDR3

with amino acid sequences set forth in SEQ ID NOs: 18-20 respectively; and

the second protein functional region is an anti-PD-1 antibody or an antigen-binding fragment thereof, a heavy chain variable region of the anti-PD-1 antibody comprising HCDR1-HCDR3 with amino acid sequences set forth in SEQ ID NOs: 21-23 respectively, and a light chain variable region of the anti-PD-1 antibody comprising LCDR1-LCDR3 with amino acid sequences set forth in SEQ ID NOs: 24-26 respectively.

In some embodiments of the present invention, the bispecific antibody is provided, wherein,

the anti-VEGFA antibody or the antigen-binding fragment thereof is selected from Fab, Fab', F(ab')$_2$, Fd, Fv, dAb, a complementarity determining region fragment, a single chain antibody, a humanized antibody, a chimeric antibody, and a diabody;

and/or,

the anti-PD-1 antibody or the antigen-binding fragment thereof is selected from Fab, Fab', F(ab')$_2$, Fd, Fv, dAb, a complementarity determining region fragment, a single chain antibody, a humanized antibody, a chimeric antibody, and a diabody.

In some embodiments of the present invention, the bispecific antibody is in IgG-scFv form.

In some embodiments of the present invention, the first protein functional region is an immunoglobulin, a heavy chain variable region of the immunoglobulin comprising HCDR1-HCDR3 with amino acid sequences set forth in SEQ ID NOs: 15-17 respectively, and a light chain variable region of the immunoglobulin comprising LCDR1-LCDR3 with

amino acid sequences set forth in SEQ ID NOs: 18-20 respectively; and the second protein functional region is a single chain antibody, a heavy chain variable region of the single chain antibody comprising HCDR1-HCDR3 with amino acid sequences set forth in SEQ ID NOs: 21-23 respectively, and a light chain variable region of the single chain antibody comprising LCDR1-LCDR3 with amino acid sequences set forth in SEQ ID NOs: 24-26 respectively;

or,

the first protein functional region is a single chain antibody, a heavy chain variable region of the single chain antibody comprising HCDR1-HCDR3 with amino acid sequences set forth in SEQ ID NOs: 21-23 respectively, and a light chain variable region of the single chain antibody comprising LCDR1-LCDR3 with amino acid sequences set forth in SEQ ID NOs: 24-26 respectively; and the second protein functional region is an immunoglobulin, a heavy chain variable region of the immunoglobulin comprising HCDR1-HCDR3 with amino acid sequences set forth in SEQ ID NOs: 15-17 respectively, and a light chain variable region of the immunoglobulin comprising LCDR1-LCDR3 with amino acid sequences set forth in SEQ ID NOs: 18-20 respectively.

In a specific embodiment of the present invention, a bispecific antibody is provided, which comprises:

a first protein functional region targeting VEGFA, and

a second protein functional region targeting PD-1;

wherein,

the first protein functional region is an immunoglobulin, a heavy chain variable region of the immunoglobulin comprising HCDR1-HCDR3 with

amino acid sequences set forth in SEQ ID NOs: 15-17 respectively, and a light chain variable region of the immunoglobulin comprising LCDR1-LCDR3 with amino acid sequences set forth in SEQ ID NOs: 18-20 respectively; and the second protein functional region is a single chain antibody, a heavy chain variable region of the single chain antibody comprising HCDR1-HCDR3 with amino acid sequences set forth in SEQ ID NOs: 21-23 respectively, and a light chain variable region of the single chain antibody comprising LCDR1-LCDR3 with amino acid sequences set forth in SEQ ID NOs: 24-26 respectively;

or,

the first protein functional region is a single chain antibody, a heavy chain variable region of the single chain antibody comprising HCDR1-HCDR3 with amino acid sequences set forth in SEQ ID NOs: 21-23 respectively, and a light chain variable region of the single chain antibody comprising LCDR1-LCDR3 with amino acid sequences set forth in SEQ ID NOs: 24-26 respectively; and the second protein functional region is an immunoglobulin, a heavy chain variable region of the immunoglobulin comprising HCDR1-HCDR3 with amino acid sequences set forth in SEQ ID NOs: 15-17 respectively, and a light chain variable region of the immunoglobulin comprising LCDR1-LCDR3 with amino acid sequences set forth in SEQ ID NOs: 18-20 respectively.

In some embodiments of the present invention, the bispecific antibody is provided, wherein,

the amino acid sequence of the heavy chain variable region of the immunoglobulin is set forth in SEQ ID NO: 5, and the amino acid sequence of the light chain variable region of the immunoglobulin is set forth in SEQ ID NO: 7; and the amino acid sequence of the heavy chain variable region of the single chain antibody is set forth in SEQ ID NO: 9, and the amino acid sequence of the light chain variable region of the single chain antibody is set forth in SEQ ID NO: 11;

or,

the amino acid sequence of the heavy chain variable region of the single chain antibody is set forth in SEQ ID NO: 9, and the amino acid sequence of the light chain variable region of the single chain antibody is set forth in SEQ ID NO: 11; and the amino acid sequence of the heavy chain variable region of the immunoglobulin is set forth in SEQ ID NO: 5, and the amino acid sequence of the light chain variable region of the immunoglobulin is set forth in SEQ ID NO: 7.

In some embodiments of the present invention, the bispecific antibody is provided, wherein,

the immunoglobulin comprises a non-CDR region derived from a species other than murine, such as from a human antibody.

In some embodiments of the present invention, the bispecific antibody is provided, wherein,

the immunoglobulin comprises constant regions derived from a human antibody;

preferably, the constant regions of the immunoglobulin are selected from constant regions of human IgG1, IgG2, IgG3, and IgG4.

In some embodiments of the present invention, the bispecific antibody is provided, wherein,

the heavy chain constant region of the immunoglobulin is human Ig gamma-1 chain C region or human Ig gamma-4 chain C region, and its

light chain constant region is human Ig kappa chain C region.

In some embodiments of the present invention, the constant regions of the immunoglobulin are humanized. For example, each heavy chain constant region is Ig gamma-1 chain C region, ACCESSION: P01857, and each light chain constant region is Ig kappa chain C region, ACCESSION: P01834.

In some embodiments of the present invention, the bispecific antibody is provided, wherein the first protein functional region and the second protein functional region are linked directly or via a linker fragment;

preferably, the linker fragment is (GGGGS)m, wherein m is a positive integer such as 1, 2, 3, 4, 5, or 6, and GGGGS (SEQ ID NO: 14) is a constituent unit of the linker.

In some embodiments of the present invention, the bispecific antibody is provided, wherein the numbers of the first protein functional region and the second protein functional region are each independently 1, 2 or more.

In some embodiments of the present invention, the bispecific antibody is provided, wherein 1 immunoglobulin and 2 single chain antibodies, preferably two identical single chain antibodies, are present.

In some embodiments of the present invention, the bispecific antibody is provided, wherein the immunoglobulin is an IgG, IgA, IgD, IgE, or IgM, preferably an IgG, such as an IgG1, IgG2, IgG3 or IgG4.

In some embodiments of the present invention, the bispecific antibody is provided, wherein the single chain antibody is linked to the C-terminus of the heavy chain of the immunoglobulin. Since an immunoglobulin has two heavy chains, two single chain antibody molecules are linked to one immunoglobulin molecule. Preferably, the two single chain antibody

molecules are identical.

In some embodiments of the present invention, the bispecific antibody is provided, wherein two single chain antibodies are present, and one terminus of each single chain antibody is linked to the C-terminus or the N-terminus of one of the two heavy chains of the immunoglobulin.

In some embodiments of the present invention, a disulfide bond is present between the V_H and the V_L of the single chain antibody. Methods for introducing a disulfide bond between the VH and VL of an antibody are well known in the art, see, for example, US 5,747,654; Rajagopal *et al., Prot. Engin.* 10(1997)1453-1459; Reiter *et al., Nat. Biotechnol.* 14(1996)1239-1245; Reiter *et al., Protein Engineering* 8(1995)1323-1331; Webber *et al., Molecular Immunology* 32(1995)249-258; Reiter *et al., Immunity* 2(1995)281-287; Reiter *et al., JBC* 269(1994)18327-18331; Reiter *et al., Inter. J. of Cancer* 58(1994)142-149; or Reiter *et al., Cancer Res*. 54(1994)2714-2718, which are incorporated herein by reference.

In some embodiments of the present invention, the bispecific antibody is provided, wherein the bispecific antibody binds to a VEGFA protein and/or a PD-1 protein with a K_D of less than 10^{-5} M, such as less than 10^{-6} M, 10^{-7} M, 10^{-8} M, 10^{-9} M or 10^{-10} M or less; preferably, the K_D is measured by a Fortebio molecular interaction instrument.

In some embodiments of the present invention, the bispecific antibody is provided, wherein,

the bispecific antibody binds to the VEGFA protein with an EC_{50} of less than 1 nM, less than 0.5 nM, less than 0.2 nM, less than 0.15 nM, or less than 0.14 nM; preferably, the EC_{50} is detected by indirect ELISA;

and/or,

the bispecific antibody binds to the PD-1 protein with an EC_{50} of less than 1 nM, less than 0.5 nM, less than 0.2 nM, less than 0.17 nM, less than 0.16 nM, or less than 0.15 nM; preferably, the EC_{50} is detected by indirect ELISA.

Another aspect of the present invention relates to an isolated nucleic acid molecule encoding the bispecific antibody according to any embodiment of the present invention.

The present invention also relates to a vector comprising the isolated nucleic acid molecule of the present invention.

The present invention also relates to a host cell comprising the isolated nucleic acid molecule of the present invention or comprising the vector of the present invention.

Another aspect of the present invention relates to a method for preparing the bispecific antibody according to any embodiment of the present invention, which comprises culturing the host cell of the present invention in a suitable condition and isolating the bispecific antibody from the cell cultures.

Another aspect of the present invention relates to a conjugate, comprising a bispecific antibody and a conjugated moiety, wherein the bispecific antibody is the bispecific antibody according to any embodiment of the present invention, and the conjugated moiety is a detectable label; preferably, the conjugated moiety is a radioisotope, a fluorescent substance, a luminescent substance, a colored substance, or an enzyme.

Another aspect of the present invention relates to a kit comprising the bispecific antibody according to any embodiment of the present invention or comprising the conjugate of the present invention;

preferably, the kit further comprises a second antibody capable of specifically binding to the bispecific antibody; optionally, the second antibody further comprises a detectable label, such as a radioisotope, a fluorescent substance, a luminescent substance, a colored substance, or an enzyme.

Another aspect of the present invention relates to use of the bispecific antibody according to any embodiment of the present invention in preparing a kit for detecting the presence or level of VEGFA and/or PD-1 in a sample.

Another aspect of the present invention relates to a pharmaceutical composition comprising the bispecific antibody according to any embodiment of the present invention or comprising the conjugate of the present invention; optionally, it further comprises a pharmaceutically acceptable excipient.

The bispecific antibody of the present invention or the pharmaceutical composition of the present invention may be formulated into any dosage form known in the pharmaceutical field, such as tablet, pill, suspension, emulsion, solution, gel, capsule, powder, granule, elixir, troche, suppository, injection (including injection solution, sterile powder for injection and concentrated solution for injection), inhalant, and spray. The preferred dosage form depends on the intended mode of administration and therapeutic use. The pharmaceutical composition of the present invention should be sterile and stable under the conditions of manufacture and storage. One preferred dosage form is an injection. Such injections may be sterile injection solutions. For example, sterile injection solutions can be prepared by the following method: a necessary amount of the bispecific antibody of the present invention is added in an appropriate solvent, and optionally, other desired ingredients (including,

but not limited to, pH regulators, surfactants, adjuvants, ionic strength enhancers, isotonic agents, preservatives, diluents, or any combination thereof) are added at the same time, followed by filtration and sterilization. In addition, sterile injection solutions can be prepared as sterile lyophilized powders (*e.g.*, by vacuum drying or lyophilizing) for convenient storage and use. Such sterile lyophilized powders may be dispersed in a suitable carrier (*e.g.*, sterile pyrogen-free water) prior to use.

In addition, the bispecific antibody of the present invention may be present in a pharmaceutical composition in unit dose form for ease of administration. In some embodiments, the unit dose is at least 1 mg, at least 5 mg, at least 10 mg, at least 15 mg, at least 20 mg, at least 25 mg, at least 30 mg, at least 45 mg, at least 50 mg, at least 75 mg, or at least 100 mg. Where the pharmaceutical composition is in a liquid (*e.g.*, injection) dosage form, it may comprise the bispecific antibody of the present invention at a concentration of at least 0.1 mg/mL, such as at least 0.25 mg/mL, at least 0.5 mg/mL, at least 1 mg/mL, at least 2.5 mg/mL, at least 5 mg/mL, at least 8 mg/mL, at least 10 mg/mL, at least 15 mg/mL, at least 25 mg/mL, at least 50 mg/mL, at least 75 mg/mL, or at least 100 mg/mL.

The bispecific antibody or the pharmaceutical composition of the present invention may be administered by any suitable method known in the art, including, but not limited to, oral, buccal, sublingual, ocular, topical, parenteral, rectal, intrathecal, intracisternal, inguinal, intravesical, topical (*e.g.*, powder, ointment, or drop), or nasal route. However, for many therapeutic uses, the preferred route/mode of administration is parenteral (such as intravenous injection, subcutaneous injection, intraperitoneal injection, and intramuscular injection). Those skilled in the art will appreciate that the route and/or mode of administration will

vary depending on the intended purpose. In a preferred embodiment, the bispecific antibody or the pharmaceutical composition of the present invention is administered by intravenous infusion or injection.

The bispecific antibody or the pharmaceutical composition provided herein can be used alone or in combination, or used in combination with additional pharmaceutically active agents (*e.g.*, a tumor chemotherapeutic drug). Such an additional pharmaceutically active agent may be administered prior to, concurrently with, or subsequent to the administration of the bispecific antibody of the present invention or the pharmaceutical composition of the present invention.

In the present invention, the administration regimen may be adjusted to achieve the optimal desired response (*e.g.*, a therapeutic or prophylactic response). For example, it may be a single administration, may be multiple administrations over a period of time, or may be characterized by reducing or increasing the dose proportionally with the emergency degree of the treatment.

Another aspect of the present invention relates to use of the bispecific antibody according to any embodiment of the present invention or the conjugate of the present invention in preparing a medicament for preventing and/or treating a malignant tumor, wherein preferably, the malignant tumor is selected from colon cancer, rectal cancer, lung cancer such as non-small cell lung cancer, liver cancer, ovarian cancer, skin cancer, glioma, melanoma, renal tumor, prostate cancer, bladder cancer, gastrointestinal cancer, breast cancer, brain cancer and leukemia.

Another aspect of the present invention relates to use of the bispecific antibody according to any embodiment of the present invention or the conjugate of the present invention in preparing:

(1)

a medicament or an agent for detecting the level of VEGFA in a sample,

a medicament or an agent for blocking binding of VEGFA to VEGFR2,

a medicament or an agent for down-regulating the activity or level of VEGFA,

a medicament or an agent for relieving the stimulation of VEGFA on vascular endothelial cell proliferation,

a medicament or an agent for inhibiting vascular endothelial cell proliferation, or

a medicament or an agent for blocking tumor angiogenesis;

and/or

(2)

a medicament or an agent for blocking the binding of PD-1 to PD-L1,

a medicament or an agent for down-regulating the activity or level of PD-1,

a medicament or an agent for relieving the immunosuppression of PD-1 in an organism,

a medicament or an agent for promoting IFN-γsecretion in T lymphocytes, or

a medicament or an agent for promoting IL-2 secretion in T lymphocytes.

In one embodiment of the present invention, the use is non-therapeutic and/or non-diagnostic.

Another aspect of the present invention relates to an *in vivo* or *in vitro* method comprising administering to a cell an effective amount of the bispecific antibody according to any embodiment of the present invention or the conjugate of the present invention, and the method is selected from:

(1)

a method for detecting the level of VEGFA in a sample,

a method for blocking the binding of VEGFA to VEGFR2,

a method for down-regulating the activity or level of VEGFA,

a method for relieving the stimulation of VEGFA on vascular endothelial cell proliferation,

a method for inhibiting vascular endothelial cell proliferation, or

a method for blocking tumor angiogenesis;

and/or

(2)

a method for blocking the binding of PD-1 to PD-L1,

a method for down-regulating the activity or level of PD-1,

a method for relieving the immunosuppression of PD-1 in an organism,

a method for promoting IFN-γ secretion in T lymphocytes, or

a method for promoting IL-2 secretion in T lymphocytes.

In one embodiment of the present invention, the *in vitro* method is non-therapeutic and/or non-diagnostic.

In the *in vitro* experiment of the present invention, the anti-VEGFA antibody and the anti-VEGFA/PD-1 bifunctional antibody both can inhibit HUVEC cell proliferation, and the anti-PD-1 antibody and the anti-VEGFA/PD-1 bifunctional antibody both can promote the secretion of IFN-γ and/or IL-2 and activate immune reaction.

Another aspect of the present invention relates to a method for preventing and/or treating a malignant tumor, comprising administering to a subject in need an effective amount of the bispecific antibody according to any embodiment of the present invention or the conjugate of the present invention, wherein preferably, the malignant tumor is selected from colon cancer, rectal cancer, lung cancer such as non-small cell lung cancer, liver cancer, ovarian cancer, skin cancer, glioma, melanoma, renal tumor, prostate cancer, bladder cancer, gastrointestinal cancer, breast cancer, brain cancer and leukemia.

A typical non-limiting range of a therapeutically or prophylactically effective amount of the bispecific antibody of the present invention is 0.02-50 mg/kg, such as 0.1-50 mg/kg, 0.1-25 mg/kg, or 1-10 mg/kg. It should be noted that the dose may vary with the type and severity of the symptom to be treated. Furthermore, those skilled in the art will appreciate that for any particular patient, the particular administration regimen will be adjusted over time according to the needs of the patient and the professional judgment of the physician; the dose ranges given herein are for illustrative purpose only and do not limit the use or scope of the pharmaceutical composition of the present invention.

In the present invention, the subject may be a mammal, such as a human.

Provided is the bispecific antibody or the conjugate according to any embodiment of the present invention for use in preventing and/or

treating a malignant tumor, wherein preferably, the malignant tumor is selected from colon cancer, rectal cancer, lung cancer such as non-small cell lung cancer, liver cancer, ovarian cancer, skin cancer, glioma, melanoma, renal tumor, prostate cancer, bladder cancer, gastrointestinal cancer, breast cancer, brain cancer and leukemia.

Provided is the bispecific antibody or conjugate according to any embodiment of the present invention for use in:

(1)

detecting the level of VEGFA in a sample,

blocking the binding of VEGFA to VEGFR2,

down-regulating the activity or level of VEGFA,

relieving the stimulation of VEGFA on vascular endothelial cell proliferation,

inhibiting vascular endothelial cell proliferation, or

blocking tumor angiogenesis;

and/or

(2)

blocking the binding of PD-1 to PD-L1,

down-regulating the activity or level of PD-1,

relieving the immunosuppression of PD-1 in an organism,

promoting IFN-γ secretion in T lymphocytes, or

promoting IL-2 secretion in T lymphocytes.

Antibody drugs, especially monoclonal antibodies, have achieved good efficacy in the treatment of various diseases. Traditional experimental methods for acquiring these therapeutic antibodies are to immunize animals with the antigen and acquire antibodies targeting the antigen in the immunized animals, or to improve those antibodies with lower affinity for the antigen by affinity maturation.

The variable regions of the light chain and the heavy chain determine the binding of the antigen; the variable region of each chain contains three hypervariable regions called Complementarity Determining Regions (CDRs) (CDRs of the heavy chain (H Chain) comprise HCDR1, HCDR2, and HCDR3, and CDRs of the light chain (L Chain) comprise LCDR1, LCDR2, and LCDR3, which are named by Kabat *et al.*, see Bethesda M.d., Sequences of Proteins of Immunological Interest, Fifth Edition, NIH Publication (1-3) 1991: 91-3242).

Preferably, CDRs may also be defined by the IMGT numbering system, see Ehrenmann, Francois, Quentin Kaas, and Marie-Paule Lefranc. "IMGT/3Dstructure-DB and IMGT/DomainGapAlign: a database and a tool for immunoglobulins or antibodies, T cell receptors, MHC, IgSF and MhcSF." *Nucleic acids research* 38.suppl_1 (2009): D301-D307.

The amino acid sequences of the CDR regions of the monoclonal antibody sequences in (1) to (13) below were analyzed by technical means well known to those skilled in the art, for example by VBASE2 database and according to the IMGT definition, and the results are as follows:

(1) <u>Bevacizumab</u>

The amino acid sequence of the heavy chain variable region is set forth in SEQ ID NO: 5, and the amino acid sequence of the light chain variable region is set forth in SEQ ID NO: 7.

The amino acid sequences of the 3 CDR regions of its heavy chain variable region are as follows:

HCDR1: GYTFTNYG (SEQ ID NO: 15)

HCDR2: INTYTGEP (SEQ ID NO: 16)

HCDR3: AKYPHYYGSSHWYFDV (SEQ ID NO: 17)

The amino acid sequences of the 3 CDR regions of its light chain variable region are as follows:

LCDR1: QDISNY (SEQ ID NO: 18)

LCDR2: FTS (SEQ ID NO: 19)

LCDR3: QQYSTVPWT (SEQ ID NO: 20)

(2) 14C12H1L1

The amino acid sequence of the heavy chain variable region is set forth in SEQ ID NO: 9, and the amino acid sequence of the light chain variable region is set forth in SEQ ID NO: 11.

The amino acid sequences of the 3 CDR regions of its heavy chain variable region are as follows:

HCDR1: GFAFSSYD (SEQ ID NO: 21)

HCDR2: ISGGGRYT (SEQ ID NO: 22)

HCDR3: ANRYGEAWFAY (SEQ ID NO: 23)

The amino acid sequences of the 3 CDR regions of its light chain variable region are as follows:

LCDR1: QDINTY (SEQ ID NO: 24)

LCDR2: RAN (SEQ ID NO: 25)

LCDR3: LQYDEFPLT (SEQ ID NO: 26)

(3) <u>VP101</u>

The amino acid sequences of the 9 CDR regions of its heavy chains are as follows:

HCDR1: GYTFTNYG (SEQ ID NO: 15)

HCDR2: INTYTGEP (SEQ ID NO: 16)

HCDR3: AKYPHYYGSSHWYFDV (SEQ ID NO: 17)

HCDR4: GFAFSSYD (SEQ ID NO: 21)

HCDR5: ISGGGRYT (SEQ ID NO: 22)

HCDR6: ANRYGEAWFAY (SEQ ID NO: 23)

HCDR7: QDINTY (SEQ ID NO: 24)

HCDR8: RAN (SEQ ID NO: 25)

HCDR9: LQYDEFPLT (SEQ ID NO: 26)

The amino acid sequences of the 3 CDR regions of its light chain variable region are as follows:

LCDR1: QDISNY (SEQ ID NO: 18)

LCDR2: FTS (SEQ ID NO: 19)

LCDR3: QQYSTVPWT (SEQ ID NO: 20)

In the present invention, unless otherwise defined, the scientific and technical terms used herein have the meanings generally understood by those skilled in the art. In addition, the laboratory operations of cell culture, molecular genetics, nucleic acid chemistry and immunology used herein are the routine procedures widely used in the corresponding

fields. Meanwhile, in order to better understand the present invention, the definitions and explanations of the relevant terms are provided below.

As used herein, when referring to the amino acid sequence of VEGFA protein (GenBank ID: NP_001165097.1), it includes the full length of the VEGFA protein, as well as a fusion protein of VEGFA, such as a fragment fused to an Fc protein fragment of mouse or human IgG (mFc or hFc). However, those skilled in the art will appreciate that in the amino acid sequence of the VEGFA protein, mutations or variations (including but not limited to, substitutions, deletions and/or additions) can be naturally generated or artificially introduced without affecting biological functions thereof. Therefore, in the present invention, the term "VEGFA protein" should include all such sequences, including their natural or artificial variants. In addition, when describing the sequence fragment of the VEGFA protein, it also includes the corresponding sequence fragments in its natural or artificial variants. In one embodiment of the present invention, the amino acid sequence of the VEGFA protein is shown as the underlined part of SEQ ID NO: 1 (without the last 6 His, a total of 302 amino acids).

As used herein, when referring to the amino acid sequence of VEGFR2 protein (also known as KDR, GenBank ID: NP_002244), it includes the full length of the VEGFR2 protein, or the extracellular fragment VEGFR2-ECD of VEGFR2, or a fragment comprising VEGFR2-ECD, and it also includes a fusion protein of VEGFR2-ECD, such as a fragment fused to an Fc protein fragment of mouse or human IgG (mFc or hFc). However, those skilled in the art will appreciate that in the amino acid sequence of the VEGFR2 protein, mutations or variations (including but not limited to, substitutions, deletions and/or additions)

can be naturally generated or artificially introduced without affecting biological functions thereof. Therefore, in the present invention, the term "VEGFR2 protein" should include all such sequences, including their natural or artificial variants. In addition, when describing the sequence fragment of the VEGFR2 protein, it also includes the corresponding sequence fragments in its natural or artificial variants. In one embodiment of the present invention, the amino acid sequence of the extracellular fragment VEGFR2-ECD of VEGFR2 is shown as the wavy-underlined part of SEQ ID NO: 4 (766 amino acids).

As used herein, unless otherwise specified, the VEGFR is VEGFR1 and/or VEGFR2; specific protein sequence thereof is a sequence known in the prior art, and reference may be made to the sequence disclosed in the existing literature or GenBank. For example, VEGFR1 (VEGFR1, NCBI Gene ID: 2321); VEGFR2 (VEGFR2, NCBI Gene ID: 3791).

As used herein, when referring to the amino acid sequence of PD-1 protein (Programmed cell death protein 1, NCBI GenBank: NM_005018), it includes the full length of the PD-1 protein, or the extracellular fragment PD-1ECD of PD-1 or a fragment comprising PD-1ECD, and it also includes a fusion protein of PD-1ECD, such as a fragment fused to an Fc protein fragment of a mouse or human IgG (mFc or hFc). However, it will be appreciated by those skilled in the art that in the amino acid sequence of PD-1 protein, mutations or variations (including but not limited to substitutions, deletions and/or additions) can be naturally generated or artificially introduced without affecting biological functions thereof. Therefore, in the present invention, the term "PD-1 protein" should include all such sequences, including their natural or artificial variants. In addition, when describing the sequence fragment of the PD-1 protein, it also includes the corresponding sequence fragments in its natural or artificial variants.

As used herein, the term EC_{50} refers to the concentration for 50% of maximal effect, *i.e.* the concentration that can cause 50% of the maximal effect.

As used herein, the term "antibody" refers to an immunoglobulin molecule that generally consists of two pairs of polypeptide chains (each pair with one "light" (L) chain and one "heavy" (H) chain). In a general sense, the heavy chain can be interpreted as a polypeptide chain with a larger molecular weight in an antibody, and the light chain refers to a polypeptide chain with a smaller molecular weight in an antibody. Light chains are classified as κ and λ light chains. Heavy chains are generally classified as μ, δ, γ, α, or ε, and isotypes of antibodies are defined as IgM, IgD, IgG, IgA, and IgE, respectively. In light chains and heavy chains, the variable region and constant region are linked by a "J" region of about 12 or more amino acids, and the heavy chain also comprises a "D" region of about 3 or more amino acids. Each heavy chain consists of a heavy chain variable region (V_H) and a heavy chain constant region (C_H). The heavy chain constant region consists of 3 domains (C_{H1}, C_{H2}, and C_{H3}). Each light chain consists of a light chain variable region (V_L) and a light chain constant region (C_L). The light chain constant region consists of one domain C_L. The constant region of the antibody can mediate the binding of immunoglobulins to host tissues or factors, including the binding of various cells of the immune system (*e.g.*, effector cells) to the first component (C1q) of classical complement system. The V_H and V_L regions can be further subdivided into highly variable regions (called Complementarity Determining Regions (CDRs)), between which conservative regions called framework regions (FRs) are distributed. Each V_H and V_L consists of 3 CDRs and 4 FRs arranged from amino terminus to carboxyl terminus in the following order: FR1, CDR1, FR2,

CDR2, FR3, CDR3, FR4. The variable regions (V_H and V_L) of each heavy chain/light chain pair form antibody binding sites, respectively. The assignment of amino acids to the regions or domains may be based on Kabat Sequences of Proteins of Immunological Interest (National Institutes of Health, Bethesda, Md. (1987 and 1991)), or Chothia & Lesk *J. Mol. Biol.* 196(1987): 901-917; Chothia *et al. Nature* 342(1989): 878-883 or the definition of IMGT numbering system, see Ehrenmann, Francois, Quentin Kaas, and Marie-Paule Lefranc. "IMGT/3Dstructure-DB and IMGT/DomainGapAlign: a database and a tool for immunoglobulins or antibodies, T cell receptors, MHC, IgSF and MhcSF." *Nucleic acids research* 38.suppl_1 (2009): D301-D307. In particular, the heavy chain may also comprise more than 3 CDRs, such as 6, 9, or 12. For example, in the bispecific antibody of the present invention, the heavy chain may be a ScFv with the C-terminus of the heavy chain of IgG antibody linked to another antibody, and in this case, the heavy chain comprises 9 CDRs. The term "antibody" is not limited by any specific method for producing antibody. For example, the antibody includes, in particular, a recombinant antibody, a monoclonal antibody, and a polyclonal antibody. Antibodies can be different isotypes, such as antibody IgG (*e.g.*, subtype IgG1, IgG2, IgG3 or IgG4), IgA1, IgA2, IgD, IgE or IgM.

As used herein, the term "antigen binding fragment", also known as the "antigen binding portion", refers to a polypeptide comprising the fragment of a full-length antibody, which maintains the ability to specifically bind to the same antigen to which the full-length antibody binds, and/or competes with the full-length antibody for the specific binding to an antigen. See generally, Fundamental Immunology, Ch. 7 (Paul, W., ed., 2nd edition, Raven Press, N.Y. (1989), which is incorporated by reference herein in its entirety for all purposes. An antigen-binding fragment of an antibody can be produced by recombinant DNA technique or by enzymatic or chemical cleavage of an intact antibody. In some cases, the antigen binding fragment includes Fab, Fab', F $(ab')_2$, Fd, Fv, dAb, and complementarity determining region (CDR) fragment, single chain antibody fragment (*e.g.*, scFv), chimeric antibody, diabody and polypeptide that comprises at least a portion of an antibody sufficient to impart specific antigen binding ability to a polypeptide.

As used herein, the term "Fd fragment" refers to an antibody fragment consisting of V_H and C_{H1} domains; the term "Fv fragment" refers to an antibody fragment consisting of the V_L and V_H domains of a single arm of an antibody; the term "dAb fragment" refers to an antibody fragment consisting of a V_H domain (Ward *et al.*, *Nature* 341 (1989):544-546); the term "Fab fragment" refers to an antibody fragment consisting of V_L, V_H, C_L and C_{H1} domains; and the term "$F(ab')_2$ fragment" refers to an antibody fragment comprising two Fab fragments linked by the disulfide bridge on a hinge region.

In some cases, the antigen binding fragment of the antibody is a single chain antibody (*e.g.*, scFv) in which the V_L and V_H domains are paired to form a monovalent molecule via a linker that enables them to produce a single polypeptide chain (see, *e.g.*, Bird *et al.*, *Science* 242 (1988):423-426 and Huston *et al.*, *Proc. Natl. Acad. Sci.* USA 85 (1988):5879-5883). Such scFv molecules may have a general structure: NH_2-V_L-linker-V_H-COOH or NH_2-V_H-linker-V_L-COOH. An appropriate linker in prior art consists of a repeating GGGGS amino acid sequence or a variant thereof. For example, a linker having the amino acid sequence $(GGGGS)_4$ can be used, and variants thereof can also be used (Holliger *et al.*, *Proc. Natl. Acad. Sci. USA* 90 (1993): 6444-6448). Other linkers useful in the present

invention are described by Alfthan *et al., Protein Eng.* 8 (1995): 725-731, *Choi et al., Eur. J. Immunol.* 31 (2001): 94-106, Hu *et al., Cancer Res.* 56 (1996): 3055-3061, Kipriyanov *et al., J. Mol. Biol.* 293 (1999): 41-56, and Roovers *et al., Cancer Immunol.* (2001).

In some cases, the antigen binding fragment of the antibody is a diabody, that is, a bivalent antibody, in which the V_H and V_L domains are expressed on a single polypeptide chain. However, the linker used is too short to allow the pairing of the two domains on the same chain, thereby the domains are forced to pair with the complementary domains on the other chain and two antigen binding sites are generated (see, *e.g.*, Holliger P. *et al., Proc. Natl. Acad. Sci. USA* 90 (1993):6444-6448, and Poljak RJ *et al., Structure* 2 (1994):1121-1123).

Antigen binding fragments (*e.g.*, the above mentioned antibody fragments) of antibodies can be obtained from given antibodies by using conventional techniques known to those skilled in the art *(e.g.*, recombinant DNA technique or enzymatic or chemical cleavage), and the antigen binding fragments of the antibodies are screened for specificity in the same way as for intact antibodies.

As used herein, unless otherwise clearly defined in the context, when referring to the term "antibody", it includes not only intact antibodies but also antigen binding fragments of antibodies.

As used herein, the terms "mAb" and "monoclonal antibody" refer to an antibody or a fragment thereof that is derived from a group of highly homologous antibodies, *i.e.* from a group of identical antibody molecules, except for natural mutations that may occur spontaneously. The monoclonal antibody has a high specificity for a single epitope on an antigen. The polyclonal antibody, relative to the monoclonal antibody,

generally comprises at least two or more different antibodies which generally recognize different epitopes on an antigen. Monoclonal antibodies can generally be obtained by hybridoma technique first reported by Kohler *et al.* (*Nature*, 256:495, 1975), and can also be obtained by recombinant DNA technique (for example, see U.S. Patent 4,816,567).

As used herein, the term "chimeric antibody" refers to an antibody of which a part of the light or/and heavy chains is derived from an antibody (which may be derived from a specific species or belong to a specific antibody class or subclass), and the other part of the light or/and heavy chains are derived from another antibody (which may be derived from the same or different species or belong to the same or different antibody class or subclass). But in any case, it retains the binding activity for the target antigen (U.S. Patent 4,816,567 to Cabilly *et al.*; Morrison *et al., Proc. Natl. Acad. Sci. USA,* 81 (1984):6851-6855).

As used herein, the term "humanized antibody" refers to an antibody or antibody fragment obtained when all or a part of CDR regions of a human immunoglobulin (receptor antibody) are replaced by the CDR regions of a non-human antibody (donor antibody), wherein the donor antibody may be a non-human (*e.g.*, mouse, rat or rabbit) antibody having expected specificity, affinity or reactivity. In addition, some amino acid residues in the framework regions (FRs) of the receptor antibody can also be replaced by the amino acid residues of corresponding non-human antibodies or by the amino acid residues of other antibodies to further improve or optimize the performance of the antibody. For more details on humanized antibodies, see, *e.g.*, Jones *et al., Nature,* 321 (1986): 522-525; Reichmann *et al., Nature,* 332:323 329 (1988); Presta, *Curr. Op. Struct. Biol.,* 2 (1992): 593-596, and Clark, *Immunol. Today* 21 (2000): 397-402.

As used herein, the term "epitope" refers to a site on the antigen that an immunoglobulin or antibody specifically binds to. "Epitope" is also called in the art as an "antigenic determinant". The epitope or antigenic determinant generally consists of chemically active surface groups of a molecule such as amino acids or carbohydrates or sugar side chains, and usually has specific three-dimensional structural characteristics and specific charge characteristics. For example, the epitope generally includes at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 consecutive or non-consecutive amino acids in a unique spatial conformation, which can be "linear" or "conformational". See, for example, Epitope Mapping Protocols in Methods in Molecular Biology, Vol. 66, G. E. Morris, Ed. (1996). In a linear epitope, all interacting sites between a protein and an interacting molecule (*e.g.*, an antibody) exist linearly along the primary amino acid sequence of the protein. In a conformational epitope, the interacting sites exist across the protein amino acid residues that are separated from each other.

As used herein, the term "isolated" refers to obtained by artificial means from natural state. If a certain "isolated" substance or component appears in nature, it may be that change occurs in its natural environment, or that it is isolated from the natural environment, or both. For example, a certain non-isolated polynucleotide or polypeptide naturally exists in a certain living animal, and the same polynucleotide or polypeptide with a high purity isolated from such a natural state is called isolated polynucleotide or polypeptide. The term "isolated" does not exclude the existence of artificial or synthetic substances or other impurities that do not affect the activity of the substance.

As used herein, the term "vector" refers to a nucleic acid vehicle into which a polynucleotide can be inserted. When a vector allows for the

expression of the protein encoded by the inserted polynucleotide, the vector is called an expression vector. A vector can be introduced into a host cell by transformation, transduction, or transfection so that the genetic substance elements carried by the vector can be expressed in the host cell. Vectors are well known to those skilled in the art, including but not limited to: plasmids; phagemids; cosmids; artificial chromosomes, such as yeast artificial chromosome (YAC), bacterial artificial chromosome (BAC), or P1-derived artificial chromosome (PAC); phages such as lambda phages or M13 phages, and animal viruses. Animal viruses that can be used as vectors include, but are not limited to, retroviruses (including lentiviruses), adenoviruses, adeno-associated viruses, herpes viruses (such as herpes simplex virus), poxviruses, baculoviruses, papillomaviruses, and papovaviruses (such as SV40). A vector can contain a variety of elements that control expression, including, but not limited to, promoter sequences, transcription initiation sequences, enhancer sequences, selection elements, and reporter genes. In addition, the vector may further contain a replication initiation site.

As used herein, the term "host cell" refers to cells to which the vector can be introduced, including but not limited to prokaryotic cells such as *E. coli* or *bacillus subtilis*, fungal cells such as yeast cells or *aspergillus*, insect cells such as S2 drosophila cells or Sf9, or animal cells such as fibroblast, CHO cells, COS cells, NSO cells, HeLa cells, BHK cells, HEK 293 cells, or human cells.

As used herein, the term "specifically bind" refers to a non-random binding reaction between two molecules, such as a reaction between an antibody and an antigen it targets. In some embodiments, an antibody that specifically binds to an antigen (or an antibody that is specific for an antigen) means that the antibody binds to the antigen with an affinity

(K_D) of less than about 10^{-5} M, such as less than about 10^{-6} M, 10^{-7} M, 10^{-8} M, 10^{-9} M or 10^{-10} M or less. In some embodiments of the present invention, the term "target" refers to specific binding.

As used herein, the term "K_D" refers to a dissociation equilibrium constant for a specific antibody-antigen interaction, which is used to describe the binding affinity between the antibody and the antigen. The smaller the equilibrium dissociation constant, the tighter the antibody-antigen binding, and the higher the affinity between the antibody and the antigen. Generally, antibodies bind to antigens with a dissociation equilibrium constant (K_D) of less than about 10^{-5} M, such as less than about 10^{-6} M, 10^{-7} M, 10^{-8} M, 10^{-9} M or 10^{-10} M or less, for example, as determined in a BIACORE instrument using Surface Plasmon Resonance (SPR).

As used herein, the terms "monoclonal antibody" and "mAb" have the same meaning and can be used interchangeably; the terms "polyclonal antibody" and "PcAb" have the same meaning and can be used interchangeably; the terms "polypeptide" and "protein" have the same meaning and can be used interchangeably. Besides, in the present invention, amino acids are generally represented by single-letter and three-letter abbreviations known in the art. For example, alanine can be represented by A or Ala.

As used herein, the term "pharmaceutically acceptable excipient" refers to a carrier and/or vehicle that is pharmacologically and/or physiologically compatible with the subject and the active ingredient, which is well known in the art (see, *e.g.*, Remington's Pharmaceutical Sciences. Edited by Gennaro AR, 19th ed. Pennsylvania: Mack Publishing Company, 1995) and includes, but is not limited to, pH regulators, surfactants, adjuvants, and ionic strength enhancers. For

example, the pH regulators include, but are not limited to, phosphate buffer; the surfactants include, but are not limited to, cationic, anionic, or non-ionic surfactants, such as Tween-80; the ionic strength enhancers include, but are not limited to, sodium chloride.

As used herein, the term "adjuvant" refers to a non-specific immune enhancer, which can enhance the immune response of an organism to antigens or change the type of immune response when delivered into the organism together with the antigens or delivered into the organism in advance. There are various adjuvants, including but not limited to aluminum adjuvant (such as aluminum hydroxide), Freund's adjuvant (such as complete Freund's adjuvant and incomplete Freund's adjuvant), corynebacterium parvum, lipopolysaccharide, cytokine, *etc*. The Freund's adjuvant is the most commonly used adjuvant in animal experiments. The aluminum hydroxide adjuvant is used more in clinical trials.

As used herein, the term "effective amount" refers to an amount sufficient to obtain or at least partially obtain desired effect. For example, a prophylactically effective amount (*e.g.*, for a disease associated with PD-1 binding to PD-L1 or overexpression of VEGF, such as a tumor) is an amount sufficient to prevent, stop, or delay the onset of the disease (*e.g.*, a disease associated with PD-L1 binding to PD-L1 or overexpression of VEGF, such as a tumor); a therapeutically effective amount is an amount sufficient to cure or at least partially stop the disease and its complications in a patient suffering from the disease. It is undoubtedly within the ability of those skilled in the art to determine such an effective amount. For example, the amount effective for therapeutic use will depend on the severity of the disease to be treated, the overall state of the immune system of the patient, the general

condition of the patient such as age, weight and sex, the mode of drug administration, and other treatments administered concurrently, *etc*.

Advantages of the present invention:

The bispecific antibody VP101 can specifically bind to VEGFA well, effectively block the binding of VEGFA to VEGFR2, and specifically relieve the immunosuppression of VEGFA in an organism and the promoting effect of VEGFA on angiogenesis; VP101 can specifically bind to PD-1 well, effectively block the binding of PD-1 to PD-L1, and specifically relieve the immunosuppression of PD-1 in an organism, and activate the immune response.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the SDS-PAGE detection results of bifunctional antibody VP101. The samples of the four lanes from left to right and their respective loading amounts are: antibody in non-reduced protein electrophoresis loading buffer, 1 μg; antibody in reduced protein electrophoresis loading buffer, 1 μg; Marker, 5 μL; BSA, 1 μg.

FIG. 2 shows the detection results of kinetic characteristic parameters of the binding of antibody VP101 to PD-1.

FIG. 3 shows the detection results of kinetic characteristic parameters of the binding of antibody BsAbB7 to PD-1.

FIG. 4 shows the detection results of kinetic characteristic parameters of the binding of antibody BsAbB8 to PD-1.

FIG. 5 shows the detection results of kinetic characteristic parameters of the binding of antibody 14C12H1L1 to PD-1.

FIG. 6 shows the detection results of kinetic characteristic parameters of the binding of antibody nivolumab to PD-1.

FIG. 7 shows the detection results of kinetic characteristic parameters of the binding of antibody VP101 to VEGF.

FIG. 8 shows the detection results of kinetic characteristic parameters of the binding of antibody BsAbB7 to VEGF.

FIG. 9 shows the detection results of kinetic characteristic parameters of the binding of antibody BsAbB8 to VEGF.

FIG. 10 shows the detection results of kinetic characteristic parameters of the binding of antibody bevacizumab to VEGF.

FIG. 11 shows the binding activity of antibody VP101 to VEGFA detected by indirect ELISA.

FIG. 12 shows the respective binding activities of antibodies VP101, BsAbB7, BsAbB8 and bevacizumab to VEGFA-his detected by indirect ELISA.

FIG. 13 shows the binding activity of antibody VP101 to PD-1 detected by indirect ELISA.

FIG. 14 shows the respective binding activities of antibodies VP101, BsAbB7, BsAbB8, 14C12H1L1 and nivolumab to PD-1 detected by indirect ELISA.

FIG. 15 shows the activity of antibody VP101 in competing with VEGFR2 for binding to VEGFA detected by competitive ELISA.

FIG. 16 shows the activity of antibody VP101 in competing with PD-L1 for binding to PD-1 detected by competitive ELISA.

FIG. 17 shows the binding EC_{50} of antibodies 14C12H1L1 and VP101 to 293T-PD-1 cell surface protein PD-1 detected by FACS.

FIG. 18 shows the binding EC_{50} of antibodies VP101, BsAbB7 and BsAbB8 to 293T-PD-1 cell surface protein PD-1 detected by FACS.

FIG. 19 shows the activity of antibodies VP101 and 14C12H1L1 in competing with PD-L1 for binding to 293T-PD-1 cell surface protein PD-1 detected by FACS.

FIG. 20 shows the activity of antibodies 14C12H1L1, VP101, BsAbB7, BsAbB8, 14C12H1L and nivolumab in competing with PD-L1 for binding to 293T-PD-1 cell surface protein PD-1 detected by FACS.

FIG. 21 shows the neutralization bioactivity of antibodies VP101, BsAbB7 and BsAbB8 in blocking VEGF to activate NFAT signaling pathway.

FIG. 22 shows the effect of bevacizumab and VP101 on HUVEC cell proliferation.

FIG. 23 shows the effect of VP101 on secretion of IFN-γ in mixed culture system of DC and PBMC cells.

FIG. 24 shows the effect of VP101, BsAbB7 and BsAbB8 on secretion of IFN-γ in mixed culture system of DC and PBMC cells.

FIG. 25 shows the effect of VP101, BsAbB7 and BsAbB8 on secretion of IL-2 in mixed culture system of DC and PBMC cells.

FIG. 26 shows the effect of antibodies 14C12H1L1 and VP101 on secretion of the cytokine IL-2 induced by mixed culture of PBMC and Raji-PD-L1 cells detected by ELISA.

FIG. 27 shows effect of antibodies 14C12H1L1 and VP101 on secretion of the cytokine IFN-γ induced by mixed culture of PBMC and Raji-PD-L1 cells detected by ELISA.

FIG. 28 shows effect of antibodies 14C12H1L1, VP101, BsAbB7 and BsAbB8 on secretion of the cytokine IFN-γ induced by mixed culture of PBMC and Raji-PD-L1 cells detected by ELISA.

FIG. 29 shows effect of antibodies 14C12H1L1, VP101, BsAbB7 and BsAbB8 on secretion of the cytokine IL-2 induced by mixed culture of PBMC and Raji-PD-L1 cells detected by ELISA.

FIG. 30 shows the inhibition of tumor growth by VP101 at different concentrations.

FIG. 31 shows effect of VP101 at different concentrations on body weight of mouse.

DETAILED DESCRIPTION

The embodiments of the present invention will be described in detail below with reference to the examples. Those skilled in the art will understand that the following examples are only used to illustrate the present invention, and should not be regarded as limiting the scope of the present invention. The cases without the specific descriptions of techniques or conditions were carried out according to the technologies or conditions described in the literature in the art (*e.g.*, see, Guide to Molecular Cloning Experiments, authored by J. Sambrook *et al.*, and translated by Huang Peitang *et al.*, third edition, Science Press) or according to the product manual. Reagents or instruments used are all commercially available conventional products if the manufacturers thereof are not specified.

In the following examples of the present invention, the marketed antibody bevacizumab (trade name Avastin®) for the same target was purchased from Roche as a control antibody, or was prepared according to Preparation Example 4.

In the following examples of the present invention, the marketed antibody nivolumab for the same target (trade name Opdivo®) was purchased from BMS as a control antibody.

In the following examples of the present invention, the amino acid sequences of the control antibodies BsAbB7 and BsAbB8 were identical to the amino acid sequences of BsAbB7 and BsAbB8 respectively in Chinese Patent Publication CN105175545A.

Preparation Example 1: Preparation of Fusion Proteins PD-1-mFc, PD-1-hFc and PD-L1-hFc

The preparation of fusion proteins PD-1-mFc, PD-1-hFc and PD-L1-hFc and the SDS-PAGE electrophoresis detection are carried out by fully referring to Preparation Example 1 of Chinese Patent Publication CN106632674A.

The amino acid sequences and the encoding nucleotide sequences of the fusion proteins PD-1-mFc, PD-1-hFc and PD-L1-hFc in this preparation example are the same as those of PD-1-mFc, PD-1-hFc and PDL-1-hFc respectively in the Preparation Example 1 of Chinese Patent Publication CN106632674A.

Fusion proteins PD-1-mFc, PD-1-hFc and PD-L1-hFc were thus obtained.

Preparation Example 2: Expression and Purification of Fusion Protein VEGFA-His

1. Construction of plasmid VEGFA-His

PCR amplification was performed using VEGFA human cDNA (purchased from Origene) as a template and the hVEGFA-His fragment was purified and isolated using an ordinary DNA product purification kit. The isolated hVEGFA-His fragment and an expression vector pcDNA3.1 were enzyme-digested with XbaI&HindIII-HF, and a target gene fragment was isolated by gel extraction and ligated with a linear expression vector by T4 ligase. Then all the ligation products were transformed into DH5a chemically competent cells and coated on an Agar plate with Amp. Well separated single colonies were selected for colony PCR identification, PCR positive clones were inoculated to an LB culture medium for culture, and a bacteria solution was taken and sent to Guangzhou Invitrogen Biotechnology for sequencing verification. The alignment of the sequencing results showed that the insertion sequence of the positive recon was completely correct.

2. Expression and purification of fusion protein VEGFA-His

After the recombinant plasmid VEGFA-his was transfected into 293F cells (purchased from Invitrogen) for 7 days according to the manual in lipofectamin transfection kit (purchased from Invitrogen), the culture medium was subjected to high-speed centrifugation, supernatant concentration and buffer exchange into Binding Buffer A, and then loaded onto a HisTrap column, and proteins were linearly eluted with Elution Buffer A. The primary pure sample was subjected to buffer exchange into Binding Buffer B with a HiTrap Desalting column and loaded onto a HiTrap Q column, proteins were linearly eluted with Elution Buffer B, and the target sample was isolated and buffer exchanged into PBS. The purified sample was added to a reduced protein electrophoresis loading buffer for SDS-PAGE electrophoresis detection.

The fusion protein VEGFA-His was thus obtained.

The amino acid sequence of VEGFA-His is as follows (171 aa):

APMAEGGGQNHHEVVKFMDVYQRSYCHPIETLVDIFQEYPDEIEYIFKPSCVPLMRCGGCCNDEGLECVPTEESNITMQIMRIKPHQGQHIGEMSFLQHNKCECRPKKDRARQENPCGPCSERRKHLFVQDPQTCKCSCKNTDSRCKARQLELNERTCRCDKPRRHHHHHH（SEQ ID NO: 1）

wherein, the underlined part is the amino acid sequence of VEGFA.

Nucleotide sequence encoding VEGFA-His (513 bp)

GCACCCATGGCCGAGGGCGGCGGCCAGAACCACCACGAGGTGGTGAAGTTCATGGACGTGTACCAGAGAAGCTACTGCCACCCCATCGAGACCCTGGTGGACATCTTCCAGGAGTACCCCGACGAGATCGAGTACATCTTCAAGCCCAGCTGCGTGCCCCTGATGAGATGCGGCGGCTGCTGCAACGACGAGGGCCTGGAGTGCGTGCCCACCGAGGAGAGCAACATCACCATGCAGATCATGAGAATCAAGCCCCACCAGGGCCAGCACATCGGCGAGATGAGCTTCCTGCAGCACAACAAGTGCGAGTGCAGACCCAAGAAGGACAGAGCCAGACAGGAGAACCCCTGCGGCCCCTGCAGCGAGAGAAGAAAGCACCTGTTCGTGCAGGACCCCCAGACCTGCAAGTGCAGCTGCAAGAACACCGACAGCAGATGCAAGGCCAGACAGCTGGAGCTGAACGAGAGAACCTGCAGATGCGACAAGCCCAGAAGACATCATCACCATCACCAC（SEQ ID NO: 2）

Preparation Example 3: Expression and Purification of Fusion Protein VEGFR2-hFc

1. Synthesis of gene VEGFR2-hFc:

The amino acids corresponding to the extracellular fragment VEGFR2 ECD of gene VEGFR2 (Vascular Endothelial Growth Factor Receptor 2, NCBI GenBank: NP_002244) were fused with TEV and the Fc protein fragment of human IgG (hFc) respectively (SEQ ID NO: 3). Genscript was entrusted to synthesize corresponding encoding nucleotide sequence (SEQ ID NO: 4).

VEGFR2, Vascular Endothelial Growth Factor Receptor 2, NCBI GenBank NP_002244;

hFc: Ig gamma-1 chain C region, ACCESSION: P01857, 106-330;

Amino acid sequence of fusion protein VEGFR2-hFc: (998 aa)

**MQSKVLLAVALWLCVETRAASVGLPSVSLDLPRLSIQKDILTIKANTTL
QITCRGQRDLDWLWPNNQSGSEQRVEVTECSDGLFCKTLTIPKVIGND
TGAYKCFYRETDLASVIYVYVQDYRSPFIASVSDQHGVVYITENKNKTV
VIPCLGSISNLNVSLCARYPEKRFVPDGNRISWDSKKGFTIPSYMISYAG
MVFCEAKINDESYQSIMYIVVVVGYRIYDVVLSPSHGIELSVGEKLVLN
CTARTELNVGIDFNWEYPSSKHQHKKLVNRDLKTQSGSEMKKFLSTL
TIDGVTRSDQGLYTCAASSGLMTKKNSTFVRVHEKPFVAFGSGMESLV
EATVGERVRIPAKYLGYPPPEIKWYKNGIPLESNHTIKAGHVLTIMEVS
ERDTGNYTVILTNPISKEKQSHVVSLVVYVPPQIGEKSLISPVDSYQYGT
TQTLTCTVYAIPPPHHIHWYWQLEEECANEPSQAVSVTNPYPCEEWRS
VEDFQGGNKIEVNKNQFALIEGKNKTVSTLVIQAANVSALYKCEAVNK
VGRGERVISFHVTRGPEITLQPDMQPTEQESVSLWCTADRSTFENLTW
YKLGPQPLPIHVGELPTPVCKNLDTLWKLNATMFSNSTNDILIMELKN
ASLQDQGDYVCLAQDRKTKKRHCVVRQLTVLERVAPTITGNLENQTT
SIGESIEVSCTASGNPPPQIMWFKDNETLVEDSGIVLKDGNRNLTIRRVR
KEDEGLYTCQACSVLGCAKVEAFFIIEGAQEKTNLESRENLYFQGTHT
CPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVK
FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK
CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLV
KGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW
QQGNVFSCSVMHEALHNHYTQKSLSLSPGK（SEQ ID NO: 3）**

wherein, the wavy-underlined part is the ECD part of VEGFR2, the framed part is TEV enzyme digestion site, and the solid-underlined part is hFc part.

Nucleotide sequence encoding fusion protein VEGFR2-hFc: (2997 bp)

ATGCAGAGCAAGGTGCTGCTGGCCGTCGCCCTGTGGCTCTGCGTG
GAGACCCGGGCCGCCTCTGTGGGTTTGCCTAGTGTTTCTCTTGATC
TGCCCAGGCTCAGCATACAAAAAGACATACTTACAATTAAGGCTAA
TACAACTCTTCAAATTACTTGCAGGGGACAGAGGGACTTGGACTGG
CTTTGGCCCAATAATCAGAGTGGCAGTGAGCAAAGGGTGGAGGTG
ACTGAGTGCAGCGATGGCCTCTTCTGTAAGACACTCACAATTCCAA
AAGTGATCGGAAATGACACTGGAGCCTACAAGTGCTTCTACCGGGA
AACTGACTTGGCCTCGGTCATTTATGTCTATGTTCAAGATTACAGAT
CTCCATTTATTGCTTCTGTTAGTGACCAACATGGAGTCGTGTACATT
ACTGAGAACAAAAACAAAACTGTGGTGATTCCATGTCTCGGGTCCA
TTTCAAATCTCAACGTGTCACTTTGTGCAAGATACCCAGAAAAGAG
ATTTGTTCCTGATGGTAACAGAATTTCCTGGGACAGCAAGAAGGGC
TTTACTATTCCCAGCTACATGATCAGCTATGCTGGCATGGTCTTCTG
TGAAGCAAAAATTAATGATGAAAGTTACCAGTCTATTATGTACATA
GTTGTCGTTGTAGGGTATAGGATTTATGATGTGGTTCTGAGTCCGT
CTCATGGAATTGAACTATCTGTTGGAGAAAAGCTTGTCTTAAATTGT
ACAGCAAGAACTGAACTAAATGTGGGGATTGACTTCAACTGGGAAT
ACCCTTCTTCGAAGCATCAGCATAAGAAACTTGTAAACCGAGACCT
AAAAACCCAGTCTGGGAGTGAGATGAAGAAATTTTTGAGCACCTTA
ACTATAGATGGTGTAACCCGGAGTGACCAAGGATTGTACACCTGTG
CAGCATCCAGTGGGCTGATGACCAAGAAGAACAGCACATTTGTCAG
GGTCCATGAAAAACCTTTTGTTGCTTTTGGAAGTGGCATGGAATCT
CTGGTGGAAGCCACGGTGGGGGAGCGTGTCAGAATCCCTGCGAAG
TACCTTGGTTACCCACCCCCAGAAATAAAATGGTATAAAAATGGAA
TACCCCTTGAGTCCAATCACACAATTAAAGCGGGGCATGTACTGAC
GATTATGGAAGTGAGTGAAAGAGACACAGGAAATTACACTGTCATC
CTTACCAATCCCATTTCAAAGGAGAAGCAGAGCCATGTGGTCTCTC

TGGTTGTGTATGTCCCACCCCAGATTGGTGAGAAATCTCTAATCTC
TCCTGTGGATTCCTACCAGTACGGCACCACTCAAACGCTGACATGT
ACGGTCTATGCCATTCCTCCCCCGCATCACATCCACTGGTATTGGC
AGTTGGAGGAAGAGTGCGCCAACGAGCCCAGCCAAGCTGTCTCAG
TGACAAACCCATACCCTTGTGAAGAATGGAGAAGTGTGGAGGACTT
CCAGGGAGGAAATAAAATTGAAGTTAATAAAAATCAATTTGCTCTA
ATTGAAGGAAAAAACAAAACTGTAAGTACCCTTGTTATCCAAGCGG
CAAATGTGTCAGCTTTGTACAAATGTGAAGCGGTCAACAAAGTCGG
GAGAGGAGAGAGGGTGATCTCCTTCCACGTGACCAGGGGTCCTGA
AATTACTTTGCAACCTGACATGCAGCCCACTGAGCAGGAGAGCGTG
TCTTTGTGGTGCACTGCAGACAGATCTACGTTTGAGAACCTCACAT
GGTACAAGCTTGGCCCACAGCCTCTGCCAATCCATGTGGGAGAGTT
GCCCACACCTGTTTGCAAGAACTTGGATACTCTTTGGAAATTGAAT
GCCACCATGTTCTCTAATAGCACAAATGACATTTTGATCATGGAGC
TTAAGAATGCATCCTTGCAGGACCAAGGAGACTATGTCTGCCTTGC
TCAAGACAGGAAGACCAAGAAAAGACATTGCGTGGTCAGGCAGCT
CACAGTCCTAGAGCGTGTGGCACCCACGATCACAGGAAACCTGGA
GAATCAGACGACAAGTATTGGGGAAAGCATCGAAGTCTCATGCACG
GCATCTGGGAATCCCCCTCCACAGATCATGTGGTTTAAAGATAATG
AGACCCTTGTAGAAGACTCAGGCATTGTATTGAAGGATGGGAACCG
GAACCTCACTATCCGCAGAGTGAGGAAGGAGGACGAAGGCCTCTA
CACCTGCCAGGCATGCAGTGTTCTTGGCTGTGCAAAAGTGGAGGCA
TTTTTCATAATAGAAGGTGCCCAGGAAAAGACGAACTTGGAATCTA
GAGAAAACCTGTATTTTCAGGGCACTCACACATGCCCACCGTGCCC
AGCACCTGAACTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCA
AAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACAT
GCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCA
ACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGC
CGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCC

TCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGTTGGACTCCGACGGCTCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCCGGGAAATGA （SEQ ID NO: 4）

wherein, the wavy-underlined part is the ECD part of VEGFR2, the framed part is TEV enzyme digestion site, and the solid-underlined part is hFc part.

2. Construction of plasmid pUC57simple-VEGFR2-hFc:

The VEGFR2-hFc encoding gene synthesized by Genscript was cloned into an expression vector pUC57simple (provided by Genscript), and a pUC57simple-VEGFR2-hFc plasmid was obtained.

3. Construction of recombinant plasmid pcDNA3.1-VEGFR2-hFc:

The plasmid pUC57simple-VEGFR2-hFc was enzyme-digested (Xba I and BamH I), and the fusion gene fragment VEGFR2-hFc isolated by electrophoresis was ligated with expression vector pcDNA3.1 (purchased from Invitrogen) to give pcDNA3.1-VEGFR2-hFc, which was transfected into competent *E. coli* cell DH5a (purchased from TIANGEN); the transfection and culture were performed according to the manual. The positive pcDNA3.1-VEGFR2-hFc colonies were screened, *E. coli* was amplified according to a conventional method, and a kit (purchased from Tiangen Biotech (Beijing) Co., Ltd., DP103-03) was then used and a recombinant plasmid pcDNA3.1-VEGFR2-hFc was extracted according to the manual of the kit.

4. Transfection of recombinant plasmid pcDNA3.1-VEGFR2-hFc into 293F cells

The recombinant plasmid pcDNA3.1-VEGFR2-hFc was transfected into 293F cells (purchased from Invitrogen) according to the lipofectamin transfection kit (purchased from Invitrogen).

5. SDS-PAGE electrophoresis detection of VEGFR2-hFc protein

After transfecting the recombinant plasmid pcDNA3.1-VEGFR2-hFc into 293F cells for 7 days, the culture medium was subjected to high-speed centrifugation, microporous membrane vacuum filtration and purification in a Mabselect SuRe column to obtain a VEGFR2-hFc fusion protein sample, and a part of the sample was added into a reduced protein electrophoresis loading buffer for SDS-PAGE electrophoresis detection.

The fusion protein VEGFR2-hFc was thus obtained.

Preparation Example 4: Preparation of Anti-VEGFA Antibody Bevacizumab

Chinese Patent Publication CN1259962A is referred to for the amino acid sequences of the heavy chain variable region and the light chain variable region of the marketed VEGFA monoclonal antibody Avastin (bevacizumab). Genscript was entrusted to synthesize nucleotide sequences encoding the heavy chain variable region and the light chain variable region.

Amino acid sequence of the heavy chain variable region of bevacizumab: (123 aa)

EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTLVTVSS（SEQ ID NO: 5）

Nucleotide sequence encoding the heavy chain variable region of bevacizumab: (369 bp)

GAGGTGCAGCTGGTCGAGTCCGGGGGGGGGCTGGTGCAGCCAGGCGGGTCTCTGAGGCTGAGTTGCGCCGCTTCAGGGTACACCTTCACAAACTATGGAATGAATTGGGTGCGCCAGGCACCAGGAAAGGGACTGGAGTGGGTCGGCTGGATCAACACTTACACCGGGGAACCTACCTATGCAGCCGACTTTAAGCGGCGGTTCACCTTCAGCCTGGATACAAGCAAATCCACTGCCTACCTGCAGATGAACAGCCTGCGAGCTGAGGACACCGCAGTCTACTATTGTGCTAAATATCCCCACTACTATGGGAGCAGCCATTGGTATTTTGACGTGTGGGGGCAGGGGACTCTGGTGACAGTGAGCAGC（SEQ ID NO: 6）

Amino acid sequence of the light chain variable region of bevacizumab: (107 aa)

DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQGTKVEIK（SEQ ID NO: 7）

Nucleotide sequence encoding the light chain variable region of bevacizumab: (321 bp)

GATATTCAGATGACTCAGAGCCCCTCCTCCCTGTCCGCCTCTGTGGGCGACAGGGTCACCATCACATGCAGTGCTTCACAGGATATTTCCAACTACCTGAATTGGTATCAGCAGAAGCCAGGAAAAGCACCCAAGGTGCTGATCTACTTCACTAGCTCCCTGCACTCAGGAGTGCCAAGCCGGTTCAGCGGATCCGGATCTGGAACCGACTTTACTCTGACCATTTCTAGTCTGCAGCCTGAGGATTTCGCTACATACTATTGCCAGCAGTATTCT

ACCGTGCCATGGACATTTGGCCAGGGGACTAAAGTCGAGATCAAG（SEQ ID NO: 8）

The heavy chain constant regions were all Ig gamma-1 chain C region, ACCESSION: P01857; the light chain constant regions were all Ig kappa chain C region, ACCESSION: P01834.

The heavy chain cDNA and the light chain cDNA of bevacizumab were cloned into vector pcDNA3.1, and the recombinant expression plasmid of the antibody bevacizumab was obtained. The recombinant plasmid was transfected into 293F cells. The 293F cell culture medium was purified and then detected.

The anti-VEGFA monoclonal antibody Avastin (bevacizumab) was thus obtained.

Preparation Example 5: Preparation and Detection of Anti-PD-1 Humanized Antibody 14C12H1L1

The preparation was carried out according to the Examples 3-4 described in Chinese Patent Publication CN106977602A.

The amino acid sequences of the heavy chain variable region and the light chain variable region of humanized antibody 14C12H1L1, and the nucleotide sequence encoding the same are also the same as those described in Examples 3-4 of Chinese Patent Publication CN106977602A, and are also provided herein as follows:

Amino acid sequence of the heavy chain variable region of humanized antibody 14C12H1L1: (118 aa)

EVQLVESGGGLVQPGGSLRLSCAASGFAFSSYDMSWVRQAPGKGLDWVATISGGGRYTYYPDSVKGRFTISRDNSKNNLYLQMNSLRAEDTALYYCANRYGEAWFAYWGQGTLVTVSS（SEQ ID NO: 9）

Nucleotide sequence encoding the heavy chain variable region of humanized antibody 14C12H1L1: (354 bp)

GAAGTGCAGCTGGTCGAGTCTGGGGGAGGGCTGGTGCAGCCCGGCGGGTCACTGCGACTGAGCTGCGCAGCTTCCGGATTCGCCTTTAGCTCCTACGACATGTCCTGGGTGCGACAGGCACCAGGAAAGGGACTGGATTGGGTCGCTACTATCTCAGGAGGCGGGAGATACACCTACTATCCTGACAGCGTCAAGGGCCGGTTCACAATCTCTAGAGATAACAGTAAGAACAATCTGTATCTGCAGATGAACAGCCTGAGGGCTGAGGACACCGCACTGTACTATTGTGCCAACCGCTACGGGGAAGCATGGTTTGCCTATTGGGGGCAGGGAACCCTGGTGACAGTCTCTAGT (SEQ ID NO: 10)

Amino acid sequence of the light chain variable region of humanized antibody 14C12H1L1: (107 aa)

DIQMTQSPSSMSASVGDRVTFTCRASQDINTYLSWFQQKPGKSPKTLIYRANRLVSGVPSRFSGSGSGQDYTLTISSLQPEDMATYYCLQYDEFPLTFGAGTKLELK（SEQ ID NO: 11）

Nucleotide sequence encoding the light chain variable region of humanized antibody 14C12H1L1: (321 bp)

GACATTCAGATGACTCAGAGCCCCTCCTCCATGTCCGCCTCTGTGGGCGACAGGGTCACCTTCACATGCCGCGCTAGTCAGGATATCAACACCTACCTGAGCTGGTTTCAGCAGAAGCCAGGGAAAAGCCCCAAGACACTGATCTACCGGGCTAATAGACTGGTGTCTGGAGTCCCAAGTCGGTTCAGTGGCTCAGGGAGCGGACAGGACTACACTCTGACCATCAGCTCCCTGCAGCCTGAGGACATGGCAACCTACTATTGCCTGCAGTATGATGAGTTCCCACTGACCTTTGGCGCCGGGACAAAACTGGAGCTGAAG（SEQ ID NO: 12）

The anti-PD-1 humanized antibody 14C12H1L1 was thus obtained.

Preparation Example 6: Preparation and Identification of hIgG

The sequence of Human Anti-Hen Egg Lysozyme IgG (anti-HEL, *i.e.*, human IgG, abbreviated as hIgG) is derived from a variable region sequence of the Fab F10.6.6 sequence in the research published by Acierno *et al.*, which is entitled "Affinity maturation increases the stability and plasticity of the Fv domain of anti-protein antibodies" (Acierno *et al.*, *J Mol Biol.* 2007; 374(1): 130-46). The preparation method is as follows:

Nanjing Genscript Biology was entrusted to carry out codon optimization of amino acids and gene synthesis on heavy and light chain (complete sequence or variable region) genes of human IgG antibody, and by referring to the standard technologies introduced in the "Guide to Molecular Cloning Experiments (Third Edition)" and using standard molecular cloning technologies such as PCR, enzyme digestion, DNA gel extraction, ligation transformation, colony PCR or enzyme digestion identification, the heavy and light chain genes were respectively subcloned into the antibody heavy chain expression vector and antibody light chain expression vector of the mammalian expression system, and the heavy and light chain genes of the recombinant expression vector were further sequenced and analyzed. After the sequence was verified to be correct, endotoxin-free expression plasmids were prepared in a large scale, and the heavy and light chain plasmids were transiently co-transfected into HEK293 cells for expression of recombinant antibody. After 7 days of culture, the cell culture medium was collected and affinity purified using an rProtein A column (GE), and the quality of the resulting antibody sample was determined using SDS-PAGE and SEC-HPLC standard analysis techniques.

The hIgG was thus obtained, and used in Examples 8-9 below.

Example 1: Sequence Design, Preparation and Detection of Heavy and Light Chains of Bifunctional Antibody VP101

1. Sequence design

The structure of the bifunctional antibody VP101 of the present invention is in the Morrison form (IgG-scFv), *i.e.* C-termini of two heavy chains of an IgG antibody are each linked to a scFv fragment of another antibody, and the main composition design of the heavy and light chains is as shown in Table 1 below.

Table 1: Composition design of the heavy and light chains of VP101

Bifunctional antibody No.	Heavy chain			Light chain
	IgG part	Linker fragment	scFv part	
VP101	Bevacizumab-H	Linker1	14C12H1$_V$-Linker1-14C12L1$_V$	Bevacizumab-L

In the Table 1 above:

(1) Those with "V" labeled at lower right corner refer to the variable region of corresponding heavy chain or the variable region of corresponding light chain. For those without "V" label, the corresponding heavy or light chain is the full length comprising the constant region. The corresponding sequences described in the above preparation examples are referred to for the amino acid sequences of these variable regions or the full length and the nucleotide sequences encoding them.

(2) The amino acid sequence of linker 1 is GGGGSGGGGSGGGGSGGGGS (SEQ ID NO: 13)

2. Expression and purification of antibody VP101

The heavy chain cDNA sequence and the light chain cDNA sequence of VP101 were each cloned into vector pUC57simple (provided by Genscript) to obtain plasmids pUC57simple-VP101H and pUC57simple-VP101L, respectively.

Plasmids pUC57simple-VP101H and pUC57simple-VP101L were enzyme-digested (HindIII&EcoRI), and heavy and light chains isolated by electrophoresis were subcloned into vector pcDNA3.1, and recombinant plasmids were extracted to co-transfect 293F cells. After 7 days of cell culture, the culture medium was centrifuged at high speed, and the supernatant was concentrated and loaded onto a HiTrap MabSelect SuRe column. The protein was further eluted in one step with Elution Buffer, and the target sample antibody VP101 was isolated and buffer exchanged into PBS.

2. Detection of antibody VP101

The purified sample was added to both a reduced protein electrophoresis loading buffer and a non-reduced protein electrophoresis loading buffer, and then boiled for SDS-PAGE electrophoresis detection. The electropherogram of VP101 is shown in FIG. 1. The target protein of the reduced protein sample is at 75 kD and 25 kD, and the target protein of the non-reduced protein sample (single antibody) is at 200 kD.

Unless otherwise specified, the humanized antibody VP101 used in the following experiments was prepared by the method of this example.

Example 2: Detection of Kinetic Parameters of Humanized Antibody VP101

1. Detection of kinetic parameters of the binding of humanized antibody VP101 to PD-1-mFc

The sample dilution buffer was PBS (0.02% Tween-20, 0.1% BSA, pH7.4). 5 μg/mL antibody was immobilized to an AHC sensor with the immobilization height being about 0.4 nM. The sensor was equilibrated in a buffer for 60 s, and the antibody immobilized to the sensor bound to PD-1-mFc at a concentration of 0.62-50 nM (three-fold gradient dilution) for 120 s, and then the antigen and antibody dissociated in the buffer for 300 s. The data were analyzed by 1:1 model fitting to obtain affinity constants. The data acquisition software was Fortebio Data Acquisition 7.0, and the data analysis software was Fortebio Data Analysis 7.0. Kinetic parameters of the binding of antibodies VP101, BsAbB7, BsAbB8, 14C12H1L1 and the control antibody nivolumab to PD-1-mFc are shown in Table 2, and the detection results of the kinetic characteristic parameters are shown in FIG. 2, FIG. 3, FIG. 4, FIG. 5 and FIG. 6, respectively.

Table 2: Kinetic parameters of the binding of humanized antibody VP101, BsAbB7, BsAbB8, 14C12H1L1 and the control antibody nivolumab to PD-1-mFc

Sample ID	K_D (M)	Kon (1/Ms)	S E (kon)	Kdis (1/s)	S E (kdis)	Rmax (nm)
VP101	1.68E-10	3.22E+05	1.44E+04	5.40E-05	3.16E-05	0.14-0.28
BsAbB7	1.62E-10	3.27E+05	2.60E+04	5.30E-05	6.24E-05	0.01-0.11
BsAbB8	4.06E-10	3.39E+05	2.04E+04	1.37E-04	4.61E-05	0.01-0.13
14C12H1L1	1.64E-10	4.55E+05	1.61E+04	7.47E-05	2.98E-05	0.24-0.28
Nivolumab	2.32E-10	5.85E+05	2.03E+04	1.36E-04	3.47E-05	0.02-0.14

K_D is affinity constant; kon is binding rate of antigen and antibody; kdis is dissociation rate of antigen and antibody; K_D = kdis/kon.

The results show that the antibodies VP101 and BsAbB7 are equivalent in terms of affinity for PD-1-mFc; the affinity constant of VP101 for

PD-1-mFc is significantly smaller than that of BsAbB8, suggesting that VP101 has better binding activity; the dissociation rate constant for VP101 and PD-1-mFc was significantly smaller than BsAbB8 and 14C12H1L1, suggesting that VP101 binds to antigen more stably with a dissociation rate slower than that of 14C12H1L1 and BsAbB8.

2. Detection of kinetic parameters of the binding of humanized antibody VP101 to VEGF-His

The sample dilution buffer was PBS (0.02% Tween-20, 0.1% BSA, pH7.4). 1 μg/mL VEGF-His was immobilized to the HIS1K sensor for 20 s, then the sensor was equilibrated in a buffer for 60 s, and the VEGF immobilized on the sensor bound to the antibody at a concentration of 12.34-1000 nM (three-fold gradient dilution) for 120 s, and then the antigen and antibody dissociated in the buffer for 300 s. The data were analyzed by 1:1 model fitting to obtain affinity constants. The data acquisition software was Fortebio Data Acquisition 7.0, and the data analysis software was Fortebio Data Analysis 7.0.

Kinetic parameters of the binding of antibodies VP101, BsAbB7, BsAbB8 and the control antibody bevacizumab to VEGF-His are shown in Table 3, and the detection results of kinetic characteristic parameters are shown in FIG. 7, FIG. 8, FIG. 9 and FIG. 10 respectively.

Table 3: Kinetic parameters of the binding of antibodies VP101, BsAbB7, BsAbB8 and the control antibody bevacizumab to VEGF-His

Sample ID	K_D (M)	Kon (1/Ms)	S E (kon)	Kdis (1/s)	S E (kdis)	Rmax (nm)
VP101	5.21E-10	1.55E+05	9.67E+03	8.05E-05	4.66E-05	0.39-0.60
BsAbB7	5.14E-10	1.57E+05	9.67E+03	8.05E-05	4.83E-05	0.36-0.53
BsAbB8	6.33E-10	1.71E+05	1.07E+04	1.08E-04	4.64E-05	0.39-0.56
Bevacizumab	7.24E-10	1.23E+05	7.09E+03	8.90E-05	4.53E-05	0.29-0.41

The results show that the antibodies VP101 and BsAbB7 are equivalent in terms of affinity for the antigen, and the affinity constant of VP101 is significantly smaller than that of BsAbB8 and the control antibody bevacizumab, suggesting that VP101 has better binding activity; the dissociation rate constant of VP101 for VEGF-His is significantly smaller than that of BsAbB8, suggesting that VP101 binds to antigen more stably with a slower dissociation rate than that of BsAbB8.

Example 3: Detection of Binding Activity of Antibody VP101 to Antigen by ELISA

1. Detection of binding activity of antibody VP101 to antigen VEGFA-his by indirect ELISA

The method is specified as follows:

The microplate was coated with VEGFA-His and incubated at 37 °C for 2 hours. After being washed, the microplate was blocked with 1% BSA for 2 hours. After being washed, the microplate was added with the gradiently diluted antibody and incubated at 37 °C for 30 minutes. After being washed, the microplate was added with the enzyme-labeled goat anti-human IgG secondary antibody working solution and incubated for 30 minutes at 37 °C. After being washed, the microplate was added with TMB chromogenic solution for color developing for 5 minutes in the absence of light, and then stop solution was added to terminate the chromogenic reaction. Then the microplate was put into a microplate reader immediately, and the OD value of each well in the microplate was read at 450 nm. SoftMax Pro 6.2.1 was used to analyze and process the data.

The detection result of the binding of antibody VP101 to antigen VEGFA-His is shown in FIG. 11. The absorbance intensities at each dose are shown in Table 4. The binding EC_{50} of antibody was calculated by curve fitting using antibody concentration as the abscissa and absorbance value as the ordinate, and the results are shown in Table 4 below.

Table 4: Binding of bifunctional antibody to VEGFA-his (Indirect ELISA)

Antibody concentration (µg/mL)	Coating: VEGFA-His (1 µg/mL)			
	VP101		Bevacizumab	
1.0000	3.045	2.943	2.798	2.974
0.3333	3.037	2.861	2.816	2.993
0.1111	2.901	2.689	2.653	2.700
0.0370	2.597	2.460	2.445	2.555
0.0123	2.013	1.914	1.998	2.074
0.0041	1.115	1.086	1.446	1.363
0.0014	0.524	0.496	0.640	0.729
0.0000	0.099	0.091	0.094	0.083
Secondary antibody	Goat anti-human IgG (H+L), HRP (1:5000)			
EC_{50}(nM)	0.036		0.035	

The results show that antibody VP101 is able to bind to VEGFA protein efficiently and its binding efficiency is dose-dependent, and the two antibodies are equivalent in terms of binding activity to human VEGFA.

2. Detection of respective binding activities of antibodies VP101, BsAbB7 and BsAbB8 to antigen VEGFA-His by indirect ELISA

The method is specified as follows:

The microplate was coated with VEGFA-His and incubated overnight at 4 °C. After being washed, the microplate was blocked with 1% BSA (dissolved in PBS) for 2 hours. After being washed, the microplate was added with the gradiently diluted antibody and incubated at 37 °C for 30 minutes. After being washed, the microplate was added with the horseradish peroxidase-labeled goat anti-human IgG Fc (Jackson, 109-035-098) working solution and incubated for 30 minutes at 37 °C.

After being washed, the microplate was added with TMB (Neogen, 308177) for color developing for 5 minutes in the absence of light, and then stop solution was added to terminate the chromogenic reaction. Then the microplate was put into a microplate reader immediately, and the OD value of each well in the microplate was read at 450 nm. SoftMax Pro 6.2.1 was used to analyze and process the data.

The result of the binding of antibody VP101 to antigen VEGFA-His is shown in FIG. 12. The absorbance intensities at each dose are shown in Table 5. The binding EC_{50} of antibody was calculated by curve fitting using antibody concentration as the abscissa and absorbance value as the ordinate, and the results are shown in Table 5 below.

Table 5: Respective binding activities of VP101, BsAbB7, BsAbB8 and bevacizumab to VEGFA-His (Indirect ELISA)

Antibody concentration (µg/mL)	Antibody coating: VEGFA-His 1 µg/mL							
	VP101		BsAbB7		BsAbB8		Bevacizumab	
1.000	3.112	3.090	3.074	3.081	3.070	3.093	3.137	3.138
0.333	3.026	2.961	2.954	2.941	2.946	2.968	3.075	3.086
0.111	2.802	2.684	2.575	2.621	2.631	2.618	2.965	2.999
0.037	1.972	1.876	1.656	1.668	1.756	1.709	2.504	2.503
0.012	0.994	0.915	0.754	0.764	0.809	0.814	1.476	1.454
0.004	0.436	0.391	0.317	0.332	0.347	0.339	0.711	0.700
0.001	0.197	0.177	0.151	0.155	0.159	0.155	0.318	0.311
0	0.083	0.063	0.086	0.076	0.095	0.072	0.066	0.064
Secondary antibody	Horseradish peroxidase-labeled goat anti-human IgG Fc, HRP (1:5000)							
EC_{50}(nM)	0.130		0.171		0.159		0.092	

The results show that the antibodies VP101, BsAbB7, BsAbB8 and bevacizumab all can bind to the VEGF protein efficiently and their binding efficiency is dose-dependent, and antibody VP101 has a higher binding activity to human VEGF than BsAbB7 and BsAbB8.

3. Detection of binding activity of antibody VP101 to antigen PD-1 by indirect ELISA

The method is specified as follows:

The microplate was coated with human PD-1-mFc and incubated overnight at 4 °C. After being blocked with 1% BSA at 37 °C for 2 hours, the microplate was added with antibody, and then incubated at 37 °C for 30 minutes. After the microplate was washed and patted dry, the HRP-labeled goat anti-human IgG (H+L) secondary antibody (Jackson, 109-035-088) was added, and the microplate was incubated at 37 °C for 30 minutes. After the microplate was washed and patted dry, TMB (Neogen, 308177) was added for color developing for 5 minutes, and then stop solution was added to terminate the color development. Then the microplate was put into a microplate reader immediately, and the OD value of each well in the microplate was read at 450 nm. SoftMax Pro 6.2.1 was used to analyze and process the data.

The detection result of the binding of antibody VP101 to antigen PD-1 is shown in FIG. 13. The absorbance intensities at each dose are shown in Table 6. By quantitative analysis of the bound antibody VP101, the curve simulation was performed to obtain the binding efficiency EC$_{50}$ of the antibody, which is shown in Table 6 below.

Table 6: Binding of bifunctional antibody to PD-1 (Indirect ELISA)

Antibody dilution gradient	Antibody coating: PD-1-mFc 0.5 μg/mL					
	VP101		Nivolumab		14C12H1L1	
0.333μg/ml	3.109	3.063	3.137	3.130	3.298	3.278
1:3	3.016	2.926	3.139	3.140	3.245	3.352
1:9	2.461	2.513	2.802	2.758	3.104	3.155
1:27	1.638	1.675	1.949	1.810	2.352	2.549
1:81	0.787	0.791	0.933	0.990	1.382	1.421
1:243	0.301	0.656	0.348	0.375	0.612	0.596

Antibody dilution gradient	Antibody coating: PD-1-mFc 0.5 μg/mL					
	VP101		Nivolumab		14C12H1L1	
1:729	0.136	0.145	0.159	0.162	0.253	0.247
0	0.068	0.056	0.053	0.053	0.053	0.053
EC_{50}(nM)	0.06		0.061		0.037	

The results show that antibody VP101 is able to bind to PD-1 protein efficiently and its binding efficiency is dose-dependent.

4. Detection of respective binding activities of antibodies VP101, BsAbB7 and BsAbB8 to antigen PD-1 by indirect ELISA

The method is specified as follows:

The microplate was coated with human PD-1-mFc and incubated overnight at 4 °C. After being blocked with 1% BSA at 37 °C for 2 hours, the microplate was added with antibody, and then incubated at 37 °C for 30 minutes. After the microplate was washed and patted dry, the horseradish peroxidase-labeled goat anti-human IgG Fc (Jackson, 109-035-098) was added, and the microplate was incubated at 37 °C for 30 minutes. After the microplate was washed and patted dry, TMB (Neogen, 308177) was added for color developing for 5 minutes, and then stop solution was added to terminate the color development. Then the microplate was put into a microplate reader immediately, and the OD value of each well in the microplate was read at 450 nm. SoftMax Pro 6.2.1 was used to analyze and process the data.

The detection result of the binding of antibody VP101 to antigen PD-1 is shown in FIG. 14. The absorbance intensities at each dose are shown in Table 7. By quantitative analysis of the bound antibody VP101, the curve simulation was performed to give the binding efficiency EC_{50} of the antibody, which is shown in Table 7 below.

Table 7: Respective binding activities of antibodies VP101, BsAbB7, BsAbB8, 14C12H1L1 and nivolumab to PD-1 (Indirect ELISA)

Antibody concentration (µg/mL)	Antigen coating: PD-1-mFc 0.5 µg/mL									
	VP101		BsAbB7		BsAbB8		14C12 H1L1		Nivolumab	
0.333	2.717	2.709	2.732	2.755	2.716	2.715	2.947	2.966	2.823	2.824
0.111	2.507	2.381	2.318	2.321	2.377	2.409	2.923	2.967	2.747	2.758
0.037	1.709	1.616	1.491	1.457	1.522	1.549	2.656	2.694	2.208	2.293
0.012	0.916	0.822	0.732	0.711	0.797	0.775	2.049	2.060	1.348	1.389
0.004	0.413	0.394	0.333	0.321	0.368	0.351	1.139	1.132	0.629	0.638
0.001	0.195	0.191	0.167	0.174	0.181	0.174	0.552	0.541	0.295	0.295
0.000	0.140	0.123	0.110	0.103	0.117	0.118	0.254	0.248	0.152	0.157
0.000	0.099	0.095	0.089	0.074	0.100	0.081	0.083	0.075	0.078	0.084
Secondary antibody	Horseradish peroxidase-labeled goat anti-human IgG Fc, HRP (1:5000)									
EC_{50}(nM)	0.146		0.199		0.173		0.045		0.095	

The results show that the antibody VP101 can bind to the PD-1 protein efficiently and its binding efficiency is dose-dependent, and antibody VP101 has a higher binding activity to human PD-1 than BsAbB7 and BsAbB8.

5. Detection of activity of antibody VP101 in competing with VEGFR2 for binding to antigen VEGFA by competitive ELISA

The method is specifically as follows:

The microplate was coated with VEGF-His and incubated at 37 °C for 2 hours. After being washed, the microplate was blocked with 1% BSA for 1 hour at 37 °C. After being washed, the microplate was added with the gradiently diluted antibodies and human VEGFR2 ECD-mFc-bio (final concentration: 0.02 µg/mL) and incubated at room temperature for 2 hours. After being washed, the microplate was added with HRP-labeled streptavidin SA-HRP (1:4000) working solution and incubated at 37 °C for 30 minutes. After being washed, the microplate was added with TMB chromogenic solution for color developing for 5 minutes in the absence of

light, and then stop solution was added to terminate the chromogenic reaction. Then the microplate was put into a microplate reader immediately, and the OD value of each well in the microplate was read at 450 nm. SoftMax Pro 6.2.1 was used to analyze and process the data.

The detection results are shown in FIG 15. The absorbance intensities at each dose are shown in Table 8. By quantitative analysis of the absorbance intensities of the bound antibodies, the curve simulation was performed to give the binding efficiency EC_{50} of the antibodies (Table 8).

Table 8: Detection of antibody in competing with VEGFR2-mFc for binding to the antigen VEGFA-His by competitive ELISA

Coating: VEGF-His (2 μg/mL)				
Antibody concentration (μg/mL)	VP101		Bevacizumab	
10.000	0.133	0.133	0.103	0.104
3.333	0.161	0.149	0.114	0.109
1.111	0.624	0.563	0.374	0.351
0.370	1.055	1.051	0.905	0.982
0.123	1.059	1.075	0.964	1.049
0.041	1.137	1.068	1.062	1.141
0.014	1.106	1.138	1.010	1.169
0.000	1.155	1.131	1.173	1.153
Receptor	VEGFR2 ECD-mFc-bio, 0.02μg/ml			
Secondary antibody	SA-HRP（1:4000)			
EC_{50} (nM)	5.324		5.086	

The results show that the antibody VP101 can effectively bind to the antigen VEGFA and inhibit the binding of VEGFR2 to VEGFA, and its efficiency in inhibiting the binding of VEGFR2 to VEGFA is dose-dependent.

6. Detection of antibody VP101 in competing with PD-L1 for binding to antigen PD-1 by competitive ELISA

The method is specifically as follows:

The microplate was coated with PD-1-hFc and incubated overnight at 4 °C. After the microplate was blocked with 1% BSA for 2 hours, antibodies at different concentrations were each mixed with PD-L1-hFc for 10 minutes (see Table 10 for the dilution concentrations). After incubation at 37 °C for 30 minutes, the microplate was washed and patted dry. Then enzyme-labeled secondary antibody was added, and the microplate was incubated at 37 °C for 30 minutes. After the microplate was washed and patted dry, TMB was added for color developing for 5 minutes, and then stop solution was added to terminate the color development. Then the microplate was put into a microplate reader immediately, and the OD value of each well in the microplate was read at 450 nm (see Table 10). SoftMax Pro 6.2.1 was used to analyze and process the data.

The detection results are shown in FIG 16. The absorbance intensities at each dose are shown in Table 9. By quantitative analysis of the bound antibody VP101, the curve simulation was performed to give the binding efficiency EC_{50} of the antibody (Table 9).

Table 9: Detection of bifunctional antibody competing with PD-L1 for binding to PD-1 by competitive ELISA

Antibody dilution gradient	Antigen coating: PD-1-hFc 0.5 µg/mL					
	VP101		Nivolumab		14C12H1L1	
5µg/ml	0.096	0.063	0.058	0.058	0.062	0.063
1:3	0.064	0.077	0.059	0.059	0.061	0.064
1:9	0.166	0.160	0.061	0.062	0.066	0.071
1:27	0.867	0.848	0.284	0.335	0.262	0.193
1:81	1.217	1.149	0.973	1.007	0.968	0.882
1:243	1.196	1.949	1.139	1.144	1.122	1.051
1:729	1.183	1.250	1.127	1.185	1.052	1.059
0	1.153	1.276	0.960	1.071	1.027	1.024

Antibody dilution gradient	**Antigen coating: PD-1-hFc 0.5 μg/mL**		
	VP101	**Nivolumab**	**14C12H1L1**
Receptor	**PD-L1-mFc 0.3μg/ml**		
Secondary antibody	**Goat anti-mouse IgG (H+L), HRP conjugated (1:5000)**		
EC_{50}(nM)	**1.216**	**0.842**	**0.745**

The results show that antibody VP101 can effectively bind to antigen PD-1 and inhibit the binding of ligand PD-L1 to PD-1, and its efficiency in inhibiting the binding of PD-L1 to PD-1 is dose-dependent.

<u>Example 4: Binding of antibody VP101 to cell membrane surface antigen</u>

Firstly, 293T cells expressing PD-1 antigen was constructed, and then the specific binding capacity of the antibody to the cell membrane surface antigen was analyzed and verified by flow cytometry.

1. Construction of 293T cells expressing PD-1 antigen

The vector pLenti6.3-PD-1 of PD-1 (the vector pLenti6.3 was purchased from Invitrogen) was transfected into 293T cells, and clone group 293T-PD-1 cells which stably express PD-1 were obtained by screening.

2. Detection of binding of antibody to cell surface antigen

The 293T-PD-1 expressing antigen obtained in the previous step was digested with pancreatin by a conventional pancreatin digestion method, and the number of cells in each collection tube was made to be 2×10^5. Antibody diluting solutions with concentration gradiently diluted with PBSA (1% BSA) were each incubated with 293T-PD-1 cells on ice for 2 hours, and then each tube was added with 100 μL of FITC goat anti-human IgG (1:500) and incubated on ice for 1 hour. Then PBS was used for washing, and 300 μL of PBSA was used to resuspend the cells, and fluorescence signals (MFI) were detected with FITC channel on a flow cytometer.

The results are shown in FIG. 17, and the MFI values at each concentration are shown in Table 10. By fluorescence quantification analysis and curve fitting of the bound 14C12H1L1 antibody, the binding EC_{50} of the VP101 antibody was calculated to be 3.5 nM.

Table 10: Analysis of fluorescence intensity of the binding of VP101 to 293T-PD-1 surface antigen detected by FACS

Antibody (nM)	**0.14**	**0.41**	**1.23**	**3.70**	**11.11**	**33.33**	**100**	**EC_{50}**
Bevacizumab	**3.2**	**2.2**	**2.0**	**2.3**	**2.7**	**3.8**	**5.7**	**-**
Nivolumab	**33.3**	**74.9**	**171.9**	**357.9**	**481.9**	**498.3**	**478.4**	**2.1**
14C12H1L1	**48.1**	**99.7**	**201.5**	**409.0**	**600.2**	**655.4**	**670.8**	**2.9**
VP101	**30.8**	**61.8**	**135.7**	**286.9**	**487.7**	**534.0**	**528.6**	**3.5**

The results show that the VP101 antibody can effectively bind to the PD-1 antigen on the 293T-PD-1 host cell surface, and its binding efficiency is dose-dependent, and bevacizumab has no binding activity to 293T-PD-1, which indicates that the binding of VP101 to 293T-PD-1 is specific.

3. The binding of antibodies VP101, BsAbB7 and BsAbB8 to the cell surface antigen was detected by referring to the experimental procedure described in step 2 of this example.

The results are shown in FIG. 18, and the MFI values at each concentration are shown in Table 11. By fluorescence quantification analysis and curve fitting of the bound antibody, the binding EC_{50} values of nivolumab, 14C12H1L1, VP101, BsAbB7 and BsAbB8 were calculated to be 7.853 nM, 3.607 nM, 7.896 nM, 9.943 nM and 10.610 nM, respectively.

Table 11: Analysis of fluorescence intensities of the binding of VP101, BsAbB7 and BsAbB8 to 293T-PD-1 surface antigen detected by FACS

Antibody (nM)	0.014	0.14	0.41	1.23	3.7	11	30	100	EC50(nM)
Bevacizumab	1.89	1.90	2.20	1.92	2.04	2.48	2.80	2.43	-
Nivolumab	3.91	15.30	34.69	94.04	234.34	533.63	640.15	804.69	7.853
14C12H1L1	7.40	29.55	69.16	175.54	422.53	868.45	831.27	813.58	3.607
VP101	3.47	16.16	38.75	93.08	216.76	509.23	810.37	783.58	7.896
BsAbB7	3.85	14.86	37.45	83.78	202.40	465.10	837.61	846.80	9.943
BsAbB8	4.41	16.77	36.86	89.89	210.40	457.91	804.43	863.35	10.610

The results show that the VP101 antibody can bind to the membrane surface PD-1 of 293T-PD1 in a dose-dependent manner. Bevacizumab has no binding activity to 293T-PD-1, which indicates that the binding of VP101 to 293T-PD-1 is specific.

Example 5: Competitive binding of antibody VP101 to cell membrane surface antigen

1. A competitive flow cytometry method was adopted to detect the EC_{50} of the VP101 in competing with PD-L1 for binding to the cell membrane surface antigen PD-1, and the method is specified as follows:

The 293T-PD-1 cells was digested in a conventional way, and divided into several samples with 300,000 cells for each, which were then subjected to centrifugation and washing. Then each tube was added with 100 µL of corresponding gradiently diluted antibody and incubated on ice for 30 minutes; 100 µL of PD-L1-mFc was then added to each tube, and the mixture was mixed well to reach a final concentration of 20 nM, and then incubated on ice for 1 hour. Then 500 µL of 1% PBSA was added, and the mixture was centrifuged at 5600 rpm for 5 minutes to remove the supernatant. 100 µL of FITC coat anti mouse antibody diluted at a ratio of 1:500 was then added into each tube, and the mixture was incubated

on ice for 40 minutes in the absence of light after being mixed well. Then the mixture was centrifuged, washed and resuspended, and then transferred to a loading tube for testing.

The results are shown in FIG. 19, and the MFI values at each concentration are shown in Table 12. By fluorescence quantification analysis and curve fitting, the binding EC_{50} values of the antibodies VP101 and 14C12H1L1 were calculated to be 8.33 nM and 4.37 nM, respectively.

Table 12: Analysis of fluorescence intensities of 14C12H1L1 and VP101 in competing for binding to 293T-PD-1 surface antigen detected by FACS

Antibody (nM)	0.05	0.14	0.41	1.23	3.70	11.11	33.33	100.00	EC_{50}	R^2
14C12H1L1	288.17	287.29	277.09	237.22	177.80	12.04	10.32	9.87	4.37	0.988
VP101	272.66	264.39	279.11	272.26	239.18	99.29	17.05	14.91	8.33	0.999

The results show that the VP101 antibody can effectively block the binding of PDL-1 to PD-1 on the surface of 293T-PD-1 host cells in a dose-dependent manner.

2. EC_{50} values of VP101, BsAbB7, BsAbB8, 14C12H1L1 and nivolumab in competing with PD-L1 for binding to the cell membrane surface antigen PD-1 were detected by using competitive flow cytometry and referring to the experimental procedure described in step 1 of this example.

The results are shown in FIG. 20, and the MFI values at each concentration are shown in Table 13. By fluorescence quantification analysis and curve fitting, the competitive binding EC50 values of antibodies VP101, BsAbB7, BsAbB8, 14C12H1L1 and nivolumab were calculated to be 15.04 nM, 22.25 nM, 19.25 nM, 9.21 nM and 9.72 nM, respectively.

Table 13: Analysis of fluorescence intensities of VP101, BsAbB7,

BsAbB8, 14C12H1L1 and nivolumab in competing for binding to 293T-PD-1 surface antigen detected by FACS

Antibody (nM)	0.14	0.41	1.23	3.7	11.11	33.33	100	300	EC_{50}	R^2
VP101	1441.94	1380.62	1368.15	1288.34	982.69	112.90	8.49	8.21	15.04	0.9971
BsAbB7	1412.62	1377.27	1339.60	1341.27	1094.35	417.70	9.23	9.18	22.25	0.9985
BsAbB8	1578.36	1521.50	1427.20	1429.85	1137.74	359.69	9.73	9.68	19.25	0.9962
14C12H1L1	1384.08	1551.05	1462.85	1296.64	580.45	12.93	13.37	14.99	9.21	0.9950
Nivolumab	1539.58	1552.37	1483.84	1300.81	713.56	70.92	60.77	56.92	9.72	0.9969

The results show that the activity of the antibody 14C12H1L1 is equivalent to that of the marketed antibody nivolumab targeting PD-1, and is superior to that of the bifunctional antibody VP101. The activity of the antibody VP101 is superior that of to BsAbB7 and BsAbB8.

Example 6: Detection of Neutralization Bioactivity of Antibodies VP101, BsAbB7 and BsAbB8 in Blocking VEGF to Activate NFAT Signaling Pathway

1. Construction of 293T-NFAT-(opv)KDR(C7) cells

KDR (VEGFR2) vector pCDH-KDRFL(OPV)-GFP-Puro (Vector pCDH-GFP-Puro is purchased from Youbio) and NFAT vector pNFAT-luc-hygro (vector pGL4-luc2P-hygro is purchased from Promega) were transfected into 293T cells, and a clone group 293T-NFAT-(opv) KDR(C7) cells stably expressing KDR and NFAT luciferase reporter genes were obtained by screening.

2. 293T-NFAT-(opv)KDR(C7) cells were collected and centrifuged for 5 minutes to remove the supernatant; DMEM+10%FBS medium was used to resuspend the cells, and the cell number was counted and the cell viability was detected; then the cell concentration was adjusted to be in a

proper range, and 50000 cells/50 µL cell suspension was added into each well of a black 96-well plate;

Corresponding antibodies (final concentrations being 300, 100, 10, 2, 0.2, 0.02, 0.002 nM) and VEGF (final concentration being 30 ng/mL) were diluted according to the experimental design, and the antibodies targeting VEGF were preincubated with VEGF for 1 hour at room temperature before being added into the cells. Blank and isotype controls (final volume of each well being 100 µL) were designed and incubated in a carbon dioxide incubator at 37 °C, 5% CO_2 for 4 hours; 50 µL of Luciferase Assay System was added to each well, and Relative Fluorescence Units (RLUs) were detected by a multi-label microplate tester within 5 minutes.

The experimental results are shown in FIG. 21, and the EC_{50} values for each antibody are shown in Table 14.

Table 14: Detection of neutralization bioactivity of antibodies VP101, BsAbB7 and BsAbB8 in blocking VEGF to activate NFAT signaling pathway by reporter assay

Sample	VP101	BsAbB7	BsAbB8	Bevacizumab
EC_{50}(nM)	1.2400	1.2170	1.7280	0.7730

The results show that the EC_{50} of VP101 is 1.240 nM, the EC_{50} of BsAbB7 is 1.217 nM, the EC_{50} of BsAbB8 is 1.728 nM, and the EC_{50} of bevacizumab is 0.773 nM, and the experimental results show that the activity of VP101 and BsAbB7 in blocking VEGF to activate NFAT signaling pathway is better than that of BsAbB8.

Example 7: Experiment of VP101 Antibody Inhibiting VEGFA-Induced HUVEC Cell Proliferation

HUVEC cells (purchased from Allcell) in a good growth state, after the

cell concentration was adjusted to be 1.5×10^4/mL, were inoculated into a 96-well plate at 200 µL/well, and then incubated in an incubator at 37 °C, 5% CO_2 for 24 hours. Then it was observed that the cells adhered well, and then culture medium was discarded. 20 nM VEGFA prepared by using 1640 containing 2% FBS was then added into the 96-well plate at 200 µL/well, and antibodies at different concentrations were added, followed by incubation for 72 hours. 72 hours later, the culture medium was discarded and MTT was added. 4 hours later, the MTT was discarded and DMSO was added, and then a microplate reader was used to measure the OD value at 490 nm.

The results are shown in FIG. 22. The results show that the humanized antibodies VP101 and bevacizumab both can effectively inhibit VEGFA-induced HUVEC cell proliferation in a dose-dependent manner, and the pharmacological activity of VP101 in inhibiting VEGFA-induced HUVEC cell proliferation is higher than that of bevacizumab at the same dose.

Example 8: Promotion of Secretion of Cytokines IFN-γ and IL-2 in Mixed Lymphocyte Reaction

1. Promotion of secretion of IFN-γ by VP101, 14C12H1L1 and nivolumab in mixed culture system of DC and PBMC cells

PBMCs were isolated by Ficoll-Paque Plus (GE Healthcare) and added to IL-4 (Peprotech 200-04, 1000 U/mL) and GM-CSF (Peprotech 300-03, 1000 U/mL) for 6 days of induction, and then TNF-α (Peprotech 300-01A, 200 U/mL) was additionally added for 3 days of induction to obtain mature DC cells.

On the day of co-culture, fresh PBMCs were isolated from peripheral blood of another donor, and the obtained mature DC cells were mixed with the freshly isolated PBMCs of another donor at a ratio of 1:10, and meanwhile antibodies at different concentrations (hIgG as a control) were added. After co-culture for 5-6 days, cell supernatant was collected and assayed for IFN-γ content using an ELISA kit (purchased from Dakewe).

The effect of VP101 on secretion of IFN-γ in mixed culture system of DC and PBMC cells is shown in FIG. 23. As can be seen in FIG. 23, VP101 can effectively promote secretion of IFN-γ in a dose-dependent manner. In addition, at doses of 30 nM and 300 nM, VP101 has greater activity in promoting secretion of IFN-γ than equivalent 14C12H1L1, and at dose level of 30 nM, it has greater activity in promoting secretion of IFN-γ than equivalent nivolumab.

2. Promotion of secretion of IL-2 and IFN-γ by VP101, BsAbB7 and BsAbB8b in mixed culture system of DC and PBMC cells

Step 1 in this example was referred to for the experimental method, namely PBMCs were isolated by Ficoll-Paque Plus (GE Healthcare) and added to IL-4 (Peprotech 200-04, 1000 U/mL) and GM-CSF (Peprotech 300-03, 1000 U/mL) for 6 days of induction, and then TNF-α (Peprotech 300-01A, 200 U/mL) was additionally added for 3 days of induction to obtain mature DC cell.

On the day of co-culture, fresh PBMCs were isolated from peripheral blood of another donor, and the obtained mature DC cells were mixed with the freshly isolated PBMCs of another donor at a ratio of 1:10, and meanwhile antibodies at different concentrations (hIgG as a control) were added; after co-culture for 5-6 days, cell supernatant was collected

and assayed for IL-2 and IFN-γ content using an ELISA kit (purchased from Dakewe).

The effect of VP101 on secretion of IFN-γ in mixed culture system of DC and PBMC cells is shown in FIG. 24. As can be seen from FIG. 24, VP101 can effectively promote secretion of IFN-γ in a dose-dependent manner. The pharmacological activity of VP101 in promoting secretion of IFN-γ is significantly better than that of BsAbB7 and BsAbB8.

The effect of VP101 on secretion of IL-2 in mixed culture system of DC and PBMC cells is shown in FIG. 25. As can be seen from FIG. 25, VP101 can effectively promote secretion of IL-2 in a dose-dependent manner, and the pharmacological activity of VP101 in promoting secretion of IL-2 is better than that of BsAbB7 and BsAbB8.

3. Promotion of secretion of IL-2 and IFN-γ by VP101, 14C12H1L1 and nivolumab in mixed culture system of PBMC and Raji-PD-L1 cells

PD-L1 was stably transfected into Raji cells through lentivirus infection, and Raji-PD-L1 cells stably expressing PD-L1 were obtained after dosing and screening; PBMCs, after two days of stimulation by SEB, were cultured in together with mitomycin C-treated Raji-PD-L1.

The results are shown in FIGs. 26 and 27. The results show that VP101 can effectively promote secretion of IL-2 and IFN-γ, and at dose level of 300 nM, the activity of VP101 in promoting secretion of IL-2 is significantly better than that of equivalent 14C12H1L1 and nivolumab.

The isotype control antibody to be studied was Human Anti-Hen Lysozyme (anti-HEL, *i.e.*, human IgG, abbreviated as hIgG), and it was prepared as described in Preparation Example 6 above.

4. The promotion of secretion of IL-2 and IFN-γ by VP101, BsAbB7 and BsAbB8 in mixed culture system of PBMC and Raji-PD-L1 cells was studied by referring to the experimental method described in step 3 of this example.

The results of secretion of IFN-γ are shown in FIG. 28. The results show that VP101 can effectively promote secretion of IFN-γ in a dose-dependent manner. At the same time, VP101 is significantly better than BsAbB7 at the same dose, while the pharmacological activity of VP101 is significantly better than that of BsAbB8 at dose levels of 3 nM and 30 nM.

The results of secretion of IL-2 are shown in FIG. 29. The results show that VP101 can effectively promote secretion of IL-2 in a dose-dependent manner. At the same time, the pharmacological activity of VP101 is significantly better than that of BsAbB7 at doses of 3 nM and 300 nM, while VP101 is equivalent to BsAbB8 at the same dose.

Example 9: Experiment of Inhibition of Tumor Growth *In Vivo* by VP101

To detect the *in vivo* tumor-inhibiting activity of VP101, U87MG cells (human glioma cells, purchased from ATCC) were first inoculated subcutaneously into 5-7 week old female Scid Beige mice (purchased from Vital River), and the modeling and specific mode of administration were shown in Table 15. After the administration, the length and width of each group of tumors were measured, and the tumor volume was calculated.

Table 15: Dosing regimen of treating U87MG tumor xenograft Scid Beige mouse model with VP101

Grouping	n	Tumor xenograft	Condition of administration
Isotype control 40 mg/kg	7	U-87MG, 5 million cells/mouse subcutaneously	Isotype control antibody, hIgG, 40 mg/kg, injected intravenously on days 0, 7 and 13
Bevacizumab 30mg/kg	8		Bevacizumab 30 mg/kg, injected intravenously on days 0, 7 and 13
VP101 40mg/kg	7		VP101 40 mg/kg, injected intravenously on days 0, 7 and 13
VP101 4mg/kg	7		VP101 4 mg/kg, injected intravenously on days 0, 7 and 13

The results are shown in FIG. 30. The results show that compared with an isotype control antibody hIgG (the preparation method is the same as that of the Preparation Example 6), bevacizumab and VP101 at different doses can effectively inhibit the growth of mouse tumors, and the high-dose VP101 is better than that of low-dose VP 101 in inhibiting tumors.

Furthermore, as shown in FIG. 31, VP101 does not affect the body weight of tumor-bearing mouse.

While the content of the present invention has provided complete and clear description of its disclosed embodiments, it is not limited thereto. For those skilled in the art, modifications and replacements to the present invention are possible with the guidance of these descriptions, and such modifications and replacements are included within the scope of the present invention. The full scope of the present invention is given by the appended claims and any equivalent thereof.

CLAIMS

1. A bispecific antibody, comprising:

a first protein functional region targeting VEGFA, and

a second protein functional region targeting PD-1;

wherein:

the first protein functional region is an anti-VEGFA antibody or an antigen-binding fragment thereof, a heavy chain variable region of the anti-VEGFA antibody comprising HCDR1-HCDR3 with amino acid sequences set forth in SEQ ID NOs: 15-17 respectively, and a light chain variable region of the anti-VEGFA antibody comprising LCDR1-LCDR3 with amino acid sequences set forth in SEQ ID NOs: 18-20 respectively; and

the second protein functional region is an anti-PD-1 antibody or an antigen-binding fragment thereof, a heavy chain variable region of the anti-PD-1 antibody comprising HCDR1-HCDR3 with amino acid sequences set forth in SEQ ID NOs: 21-23 respectively, and a light chain variable region of the anti-PD-1 antibody comprising LCDR1-LCDR3 with amino acid sequences set forth in SEQ ID NOs: 24-26 respectively.

2. The bispecific antibody according to claim 1, wherein,

the anti-VEGFA antibody or the antigen-binding fragment thereof is selected from Fab, Fab', F(ab')$_2$, Fd, Fv, dAb, a complementarity determining region fragment, a single chain antibody, a humanized antibody, a chimeric antibody, and a diabody;

and/or,

the anti-PD-1 antibody or the antigen-binding fragment thereof is selected from Fab, Fab', F(ab')$_2$, Fd, Fv, dAb, a complementarity determining region fragment, a single chain antibody, a humanized antibody, a chimeric antibody, and a diabody.

3. The bispecific antibody according to claim 1, wherein,

the first protein functional region is an immunoglobulin, a heavy chain variable region of the immunoglobulin comprising HCDR1-HCDR3 with amino acid sequences set forth in SEQ ID NOs: 15-17 respectively, and a light chain variable region of the immunoglobulin comprising LCDR1-LCDR3 with amino acid sequences set forth in SEQ ID NOs: 18-20 respectively; and the second protein functional region is a single chain antibody, a heavy chain variable region of the single chain antibody comprising HCDR1-HCDR3 with amino acid sequences set forth in SEQ ID NOs: 21-23 respectively, and a light chain variable region of the single chain antibody comprising LCDR1-LCDR3 with amino acid sequences set forth in SEQ ID NOs: 24-26 respectively;

or,

the first protein functional region is a single chain antibody, a heavy chain variable region of the single chain antibody comprising HCDR1-HCDR3 with amino acid sequences set forth in SEQ ID NOs: 21-23 respectively, and a light chain variable region of the single chain antibody comprising LCDR1-LCDR3 with amino acid sequences set forth in SEQ ID NOs: 24-26 respectively; and the second protein functional region is an immunoglobulin, a heavy chain variable region of the immunoglobulin comprising HCDR1-HCDR3 with amino acid sequences set forth in SEQ ID NOs: 15-17 respectively, and a light chain variable region of the immunoglobulin comprising LCDR1-LCDR3 with amino acid sequences set forth in SEQ ID NOs: 18-20 respectively.

4. The bispecific antibody according to claim 3, wherein,

the amino acid sequence of the heavy chain variable region of the immunoglobulin is set forth in SEQ ID NO: 5, and the amino acid sequence of the light chain variable region of the immunoglobulin is set forth in SEQ ID NO: 7; and the amino acid sequence of the heavy chain variable region of the single chain antibody is set forth in SEQ ID NO: 9, and the amino acid sequence of the light chain variable region of the single chain antibody is set forth in SEQ ID NO: 11;

or,

the amino acid sequence of the heavy chain variable region of the single chain antibody is set forth in SEQ ID NO: 9, and the amino acid sequence of the light chain variable region of the single chain antibody is set forth in SEQ ID NO: 11; and the amino acid sequence of the heavy chain variable region of the immunoglobulin is set forth in SEQ ID NO: 5, and the amino acid sequence of the light chain variable region of the immunoglobulin is set forth in SEQ ID NO: 7.

5. The bispecific antibody according to claim 3 or 4, wherein the immunoglobulin is an IgG, IgA, IgD, IgE, or IgM; preferably, the immunoglobulin is an IgG.

6. The bispecific antibody according to claim 3 or 4, wherein two single chain antibodies are present, and one terminus of each single chain antibody is linked to the C-terminus or the N-terminus of one of the two heavy chains of the immunoglobulin.

7. The bispecific antibody according to claim 3 or 4, wherein,

the immunoglobulin comprises a non-CDR region derived from a species other than murine, such as from a human antibody.

8. The bispecific antibody according to claim 3 or 4, wherein,

the immunoglobulin comprises constant regions derived from a human antibody;

preferably, the constant regions of the immunoglobulin are selected from constant regions of human IgG1, IgG2, IgG3, and IgG4.

9. The bispecific antibody according to claim 3 or 4, wherein,

the heavy chain constant region of the immunoglobulin is human Ig gamma-1 chain C region or human Ig gamma-4 chain C region, and its light chain constant region is human Ig kappa chain C region.

10. The bispecific antibody according to claim 1 or 2, wherein the first and second protein functional regions are linked directly or via a linker fragment;

preferably, the linker fragment is (GGGGS)m, wherein m is a positive integer such as 1, 2, 3, 4, 5, or 6.

11. The bispecific antibody according to claim 1 or 2, wherein the numbers of the first and second protein functional regions are each independently 1, 2 or more.

12. The bispecific antibody according to claim 1 or 2, wherein,

the bispecific antibody binds to the VEGFA protein with an EC_{50} of less than 1 nM, less than 0.5 nM, less than 0.2 nM, less than 0.15 nM, or less than 0.14 nM; preferably, the EC_{50} is detected by indirect ELISA;

and/or,

the bispecific antibody binds to the PD-1 protein with an EC_{50} of less than 1 nM, less than 0.5 nM, less than 0.2 nM, less than 0.17 nM, less than 0.16 nM, or less than 0.15 nM; preferably, the EC_{50} is detected by indirect ELISA.

13. An isolated nucleic acid molecule, encoding the bispecific antibody according to any one of claims 1-12.

14. A vector, comprising the isolated nucleic acid molecule according to claim 13.

15. A host cell, comprising the isolated nucleic acid molecule according to claim 13 or the vector according to claim 14.

16. A method for preparing the bispecific antibody according to any one of claims 1-12, comprising: culturing the host cell according to claim 15 in a suitable condition, and isolating the bispecific antibody from the cell cultures.

17. A conjugate, comprising a bispecific antibody and a conjugated moiety, wherein the bispecific antibody is the bispecific antibody according to any one of claims 1-12, and the conjugated moiety is a detectable label; preferably, the conjugated moiety is a radioisotope, a fluorescent substance, a luminescent substance, a colored substance, or an enzyme.

18. A kit, comprising the bispecific antibody according to any one of claims 1-12 or the conjugate according to claim 17;

wherein preferably, the kit further comprises a second antibody capable of specifically binding to the bispecific antibody; optionally, the second antibody further comprises a detectable label, such as a radioisotope, a fluorescent substance, a luminescent substance, a colored substance, or an enzyme.

19. Use of the bispecific antibody according to any one of claims 1-12 in preparing a kit for detecting the presence or level of VEGFA and/or PD-1 in a sample.

20. A pharmaceutical composition, comprising the bispecific antibody according to any one of claims 1-12 or the conjugate according to claim 17, and optionally, a pharmaceutically acceptable excipient.

21. Use of the bispecific antibody according to any one of claims 1-12 or the conjugate according to claim 17 in preparing a medicament for preventing and/or treating a malignant tumor, wherein preferably, the malignant tumor is selected from colon cancer, rectal cancer, lung cancer such as non-small cell lung cancer, liver cancer, ovarian cancer, skin cancer, glioma, melanoma, renal tumor, prostate cancer, bladder cancer, gastrointestinal cancer, breast cancer, brain cancer and leukemia.

22. Use of the bispecific antibody according to any one of claims 1-12 or the conjugate according to claim 17 in preparing:

(1)

a medicament or an agent for detecting the level of VEGFA in a sample,

a medicament or an agent for blocking the binding of VEGFA to VEGFR2,

a medicament or an agent for down-regulating the activity or level of VEGFA,

a medicament or an agent for relieving the stimulation of VEGFA on vascular endothelial cell proliferation,

a medicament or an agent for inhibiting vascular endothelial cell proliferation, or

a medicament or an agent for blocking tumor angiogenesis;

and/or

(2)

a medicament or an agent for blocking the binding of PD-1 to PD-L1,

a medicament or an agent for down-regulating the activity or level of PD-1,

a medicament or an agent for relieving the immunosuppression of PD-1 in an organism,

a medicament or an agent for promoting IFN-γsecretion in T lymphocytes, or

a medicament or an agent for promoting IL-2 secretion in T lymphocytes.

23. An *in vivo* or *in vitro* method, comprising administering to a cell an effective amount of the bispecific antibody according to any one of claims 1-12 or the conjugate according to claim 17, wherein the method is selected from:

(1)

a method for detecting the level of VEGFA in a sample,

a method for blocking the binding of VEGFA to VEGFR2,

a method for down-regulating the activity or level of VEGFA,

a method for relieving the stimulation of VEGFA on vascular endothelial cell proliferation,

a method for inhibiting vascular endothelial cell proliferation, or

a method for blocking tumor angiogenesis;

and/or

(2)

a method for blocking the binding of PD-1 to PD-L1,

a method for down-regulating the activity or level of PD-1,

a method for relieving the immunosuppression of PD-1 in an organism,

a method for promoting IFN-γ secretion in T lymphocytes, or

a method for promoting IL-2 secretion in T lymphocytes.

24. A method for preventing and/or treating a malignant tumor, comprising administering to a subject in need an effective amount of the bispecific antibody according to any one of claims 1-12 or the conjugate according to claim 17, wherein preferably, the malignant tumor is selected from colon cancer, rectal cancer, lung cancer such as non-small cell lung cancer, liver cancer, ovarian cancer, skin cancer, glioma, melanoma, renal tumor, prostate cancer, bladder cancer, gastrointestinal cancer, breast cancer, brain cancer and leukemia.

25. The bispecific antibody according to any one of claims 1-12 or the conjugate according to claim 17 for use in preventing and/or treating a malignant tumor, wherein preferably, the malignant tumor is selected from colon cancer, rectal cancer, lung cancer such as non-small cell lung cancer, liver cancer, ovarian cancer, skin cancer, glioma, melanoma, renal tumor, prostate cancer, bladder cancer, gastrointestinal cancer, breast cancer, brain cancer and leukemia.

26. The bispecific antibody according to any one of claims 1-12 or the conjugate according to claim 17 for use in:

(1)

detecting the level of VEGFA in a sample,

blocking the binding of VEGFA to VEGFR2,

down-regulating the activity or level of VEGFA,

relieving the stimulation of VEGFA on vascular endothelial cell proliferation,

inhibiting vascular endothelial cell proliferation, or

blocking tumor angiogenesis;

and/or

(2)

blocking the binding of PD-1 to PD-L1,

down-regulating the activity or level of PD-1,

relieving the immunosuppression of PD-1 in an organism,

promoting IFN-γ secretion in T lymphocytes, or

promoting IL-2 secretion in T lymphocytes.

2026201467 26 Feb 2026

DRAWINGS OF SPECIFICATION

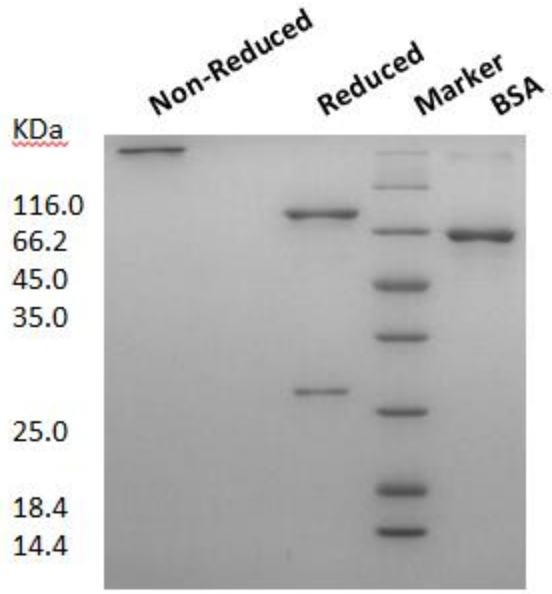


FIG. 1

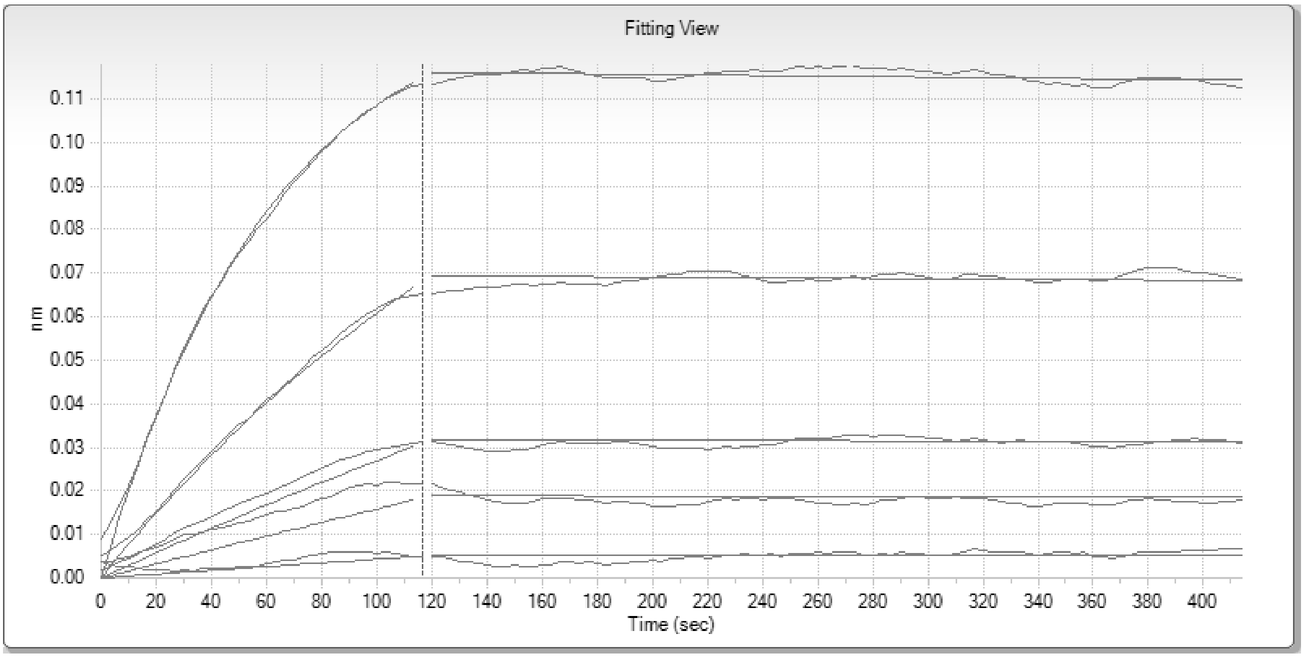
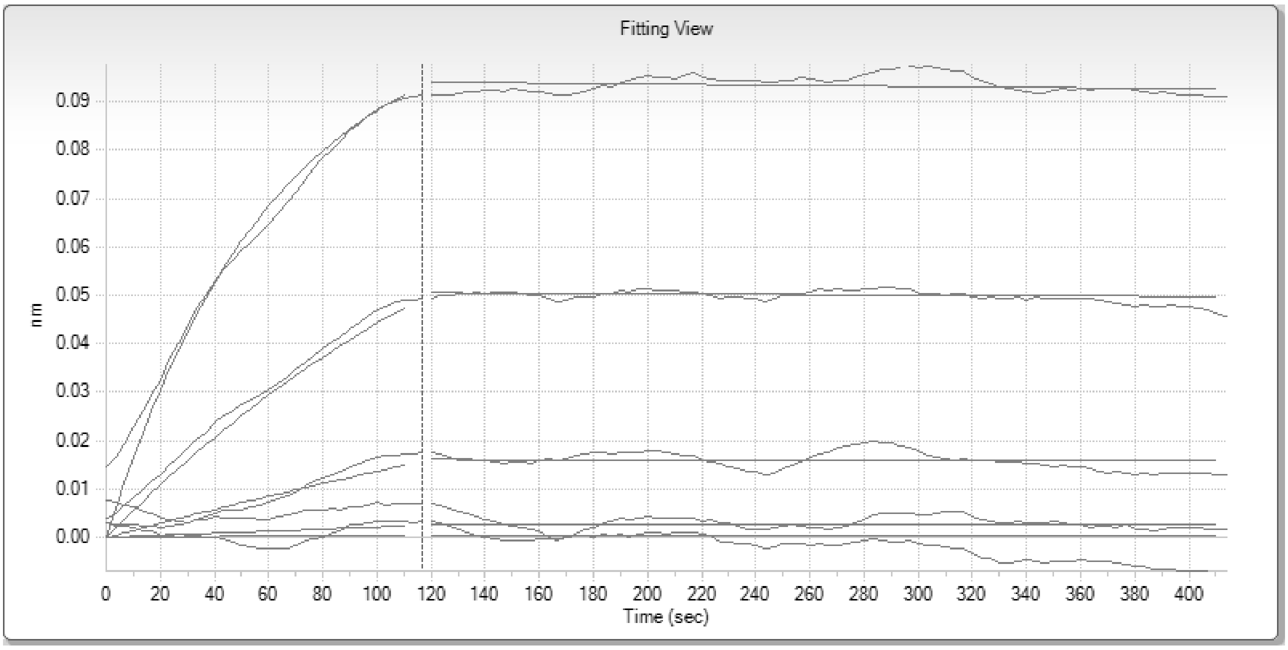


FIG. 2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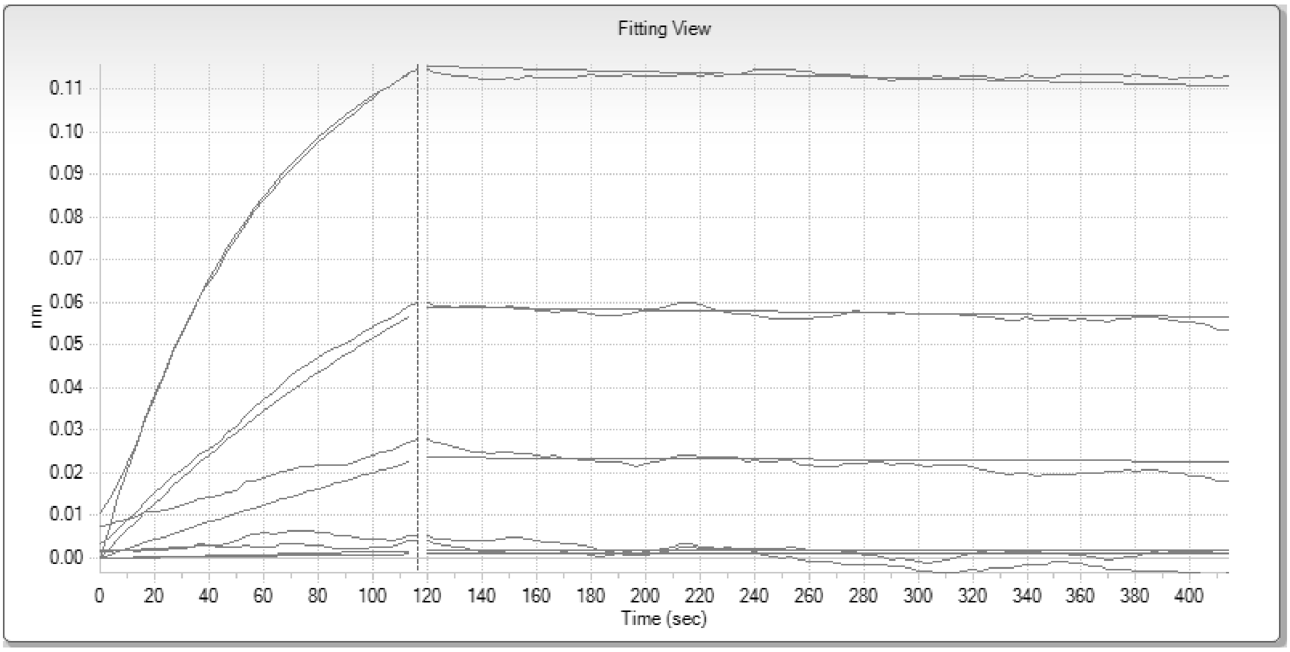
400
Time (sec)

FIG. 3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0
20
40
60
80
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200
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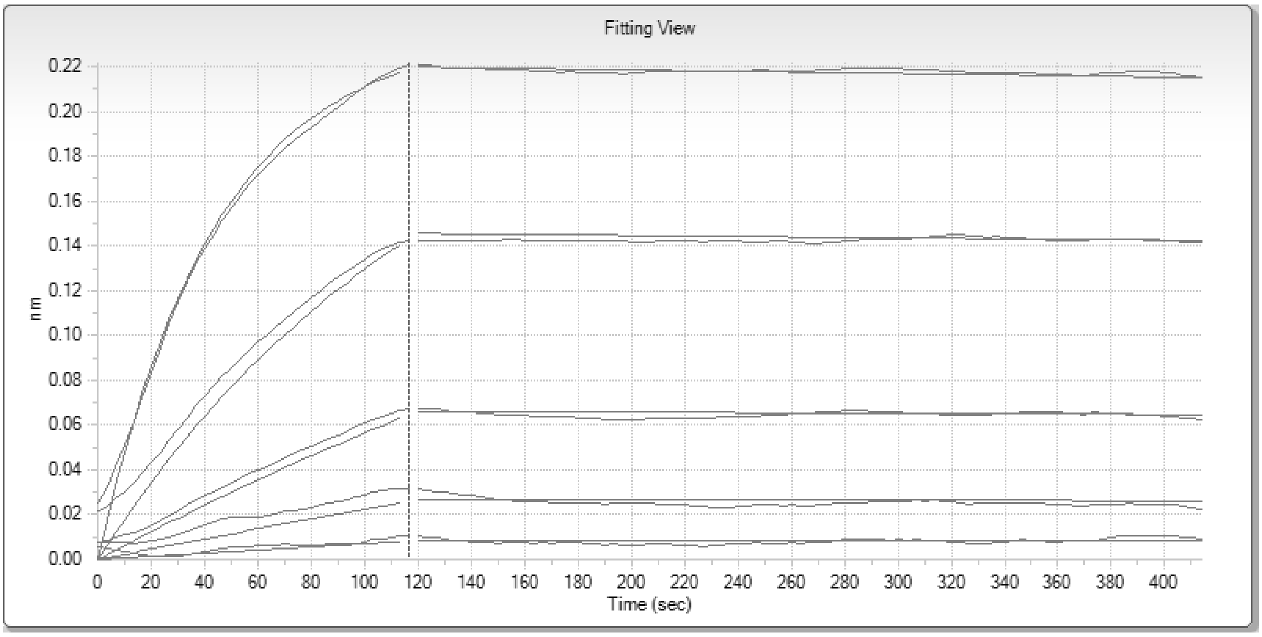
360
380
400
Time (sec)

FIG. 4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nm
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380
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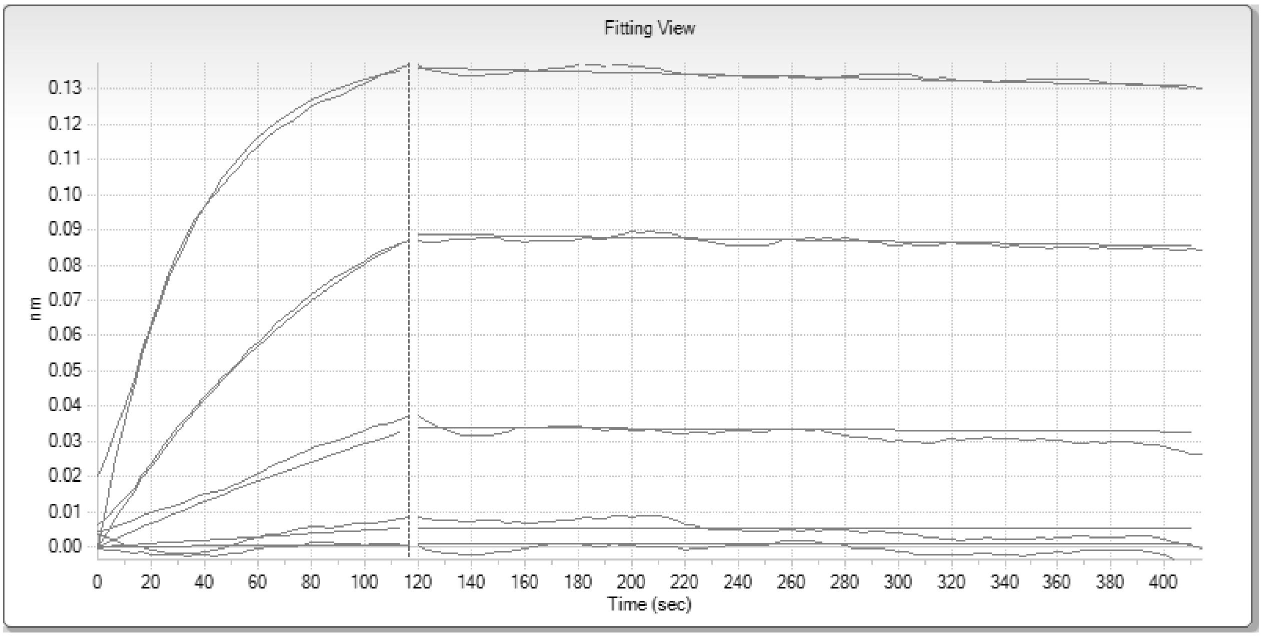
Time (sec)

FIG. 5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nm
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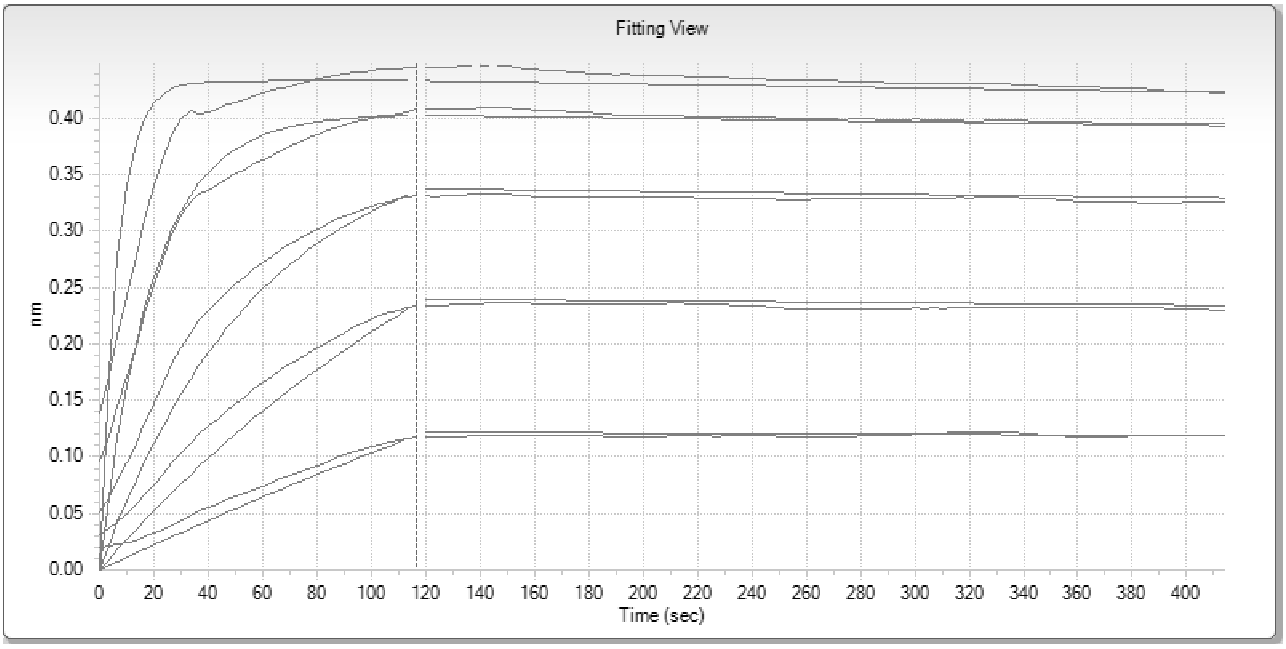
Time (sec)

FIG. 6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180
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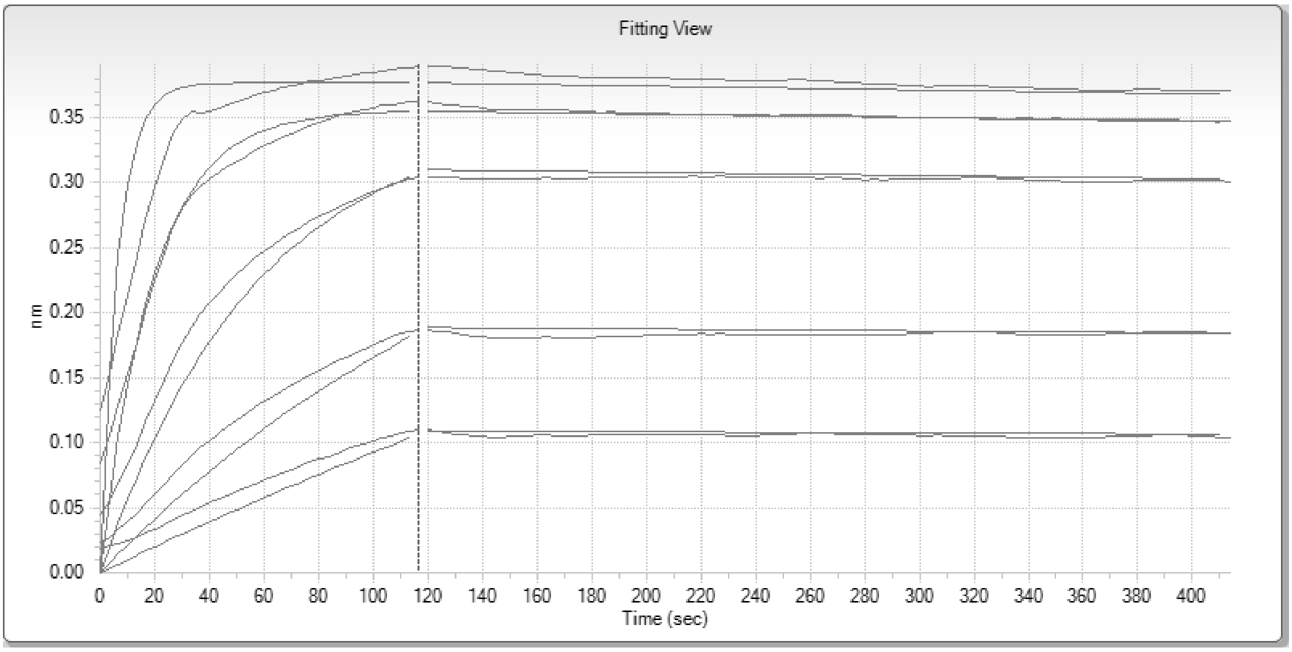
360
380
400
Time (sec)

FIG. 7


Fitting View
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0
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180
200
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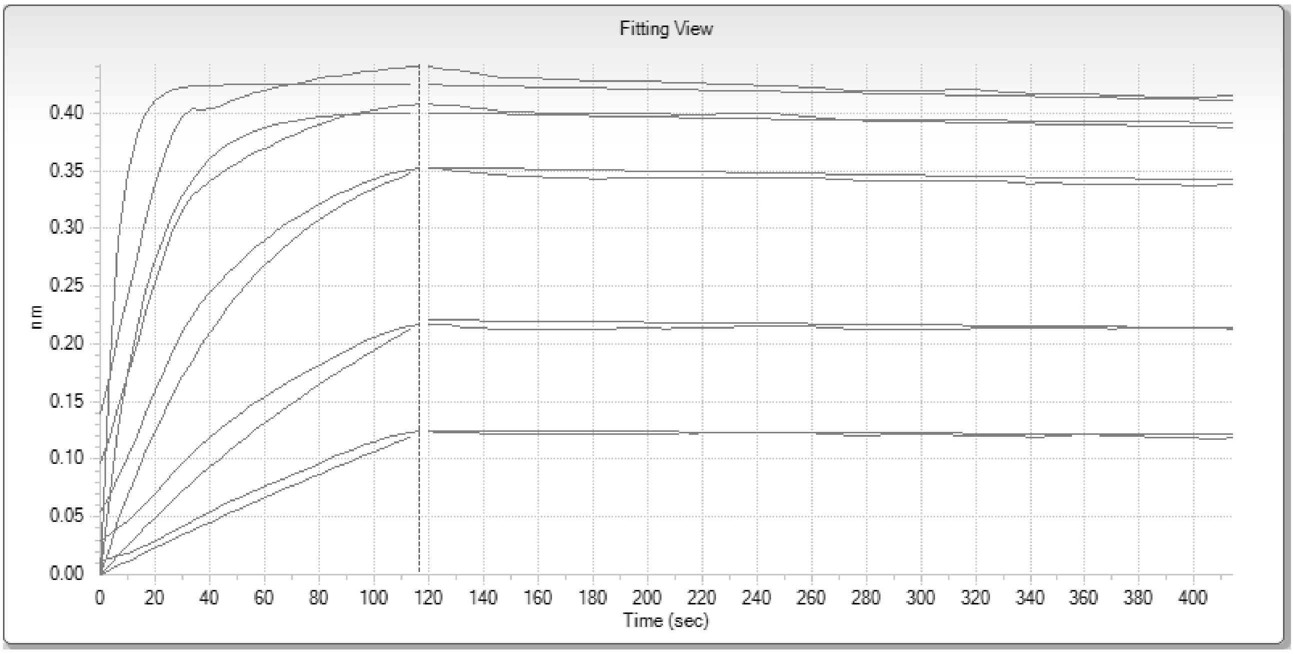
240
260
280
300
320
340
360
380
400
Time (sec)

FIG. 8


Fitting View
nm
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0.10
0.15
0.20
0.25
0.30
0.35
0.40
0
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40
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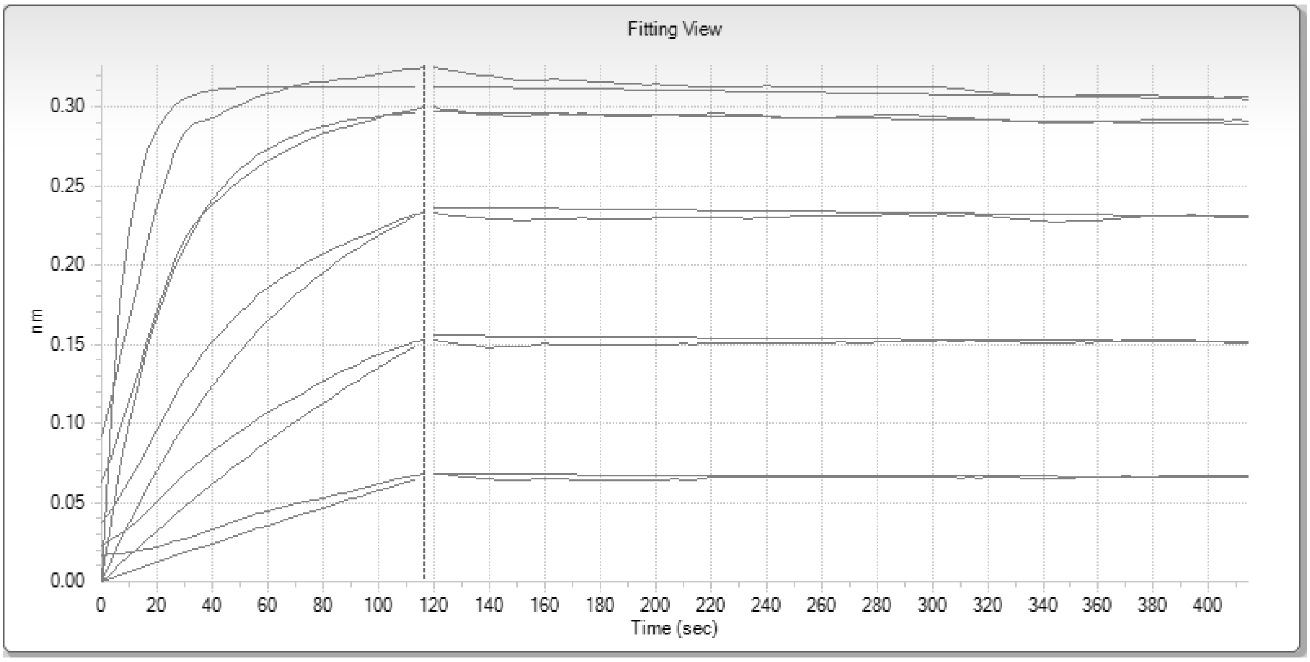
340
360
380
400
Time (sec)

FIG. 9


Fitting View
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40
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240
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340
360
380
400
Time (sec)

FIG. 10

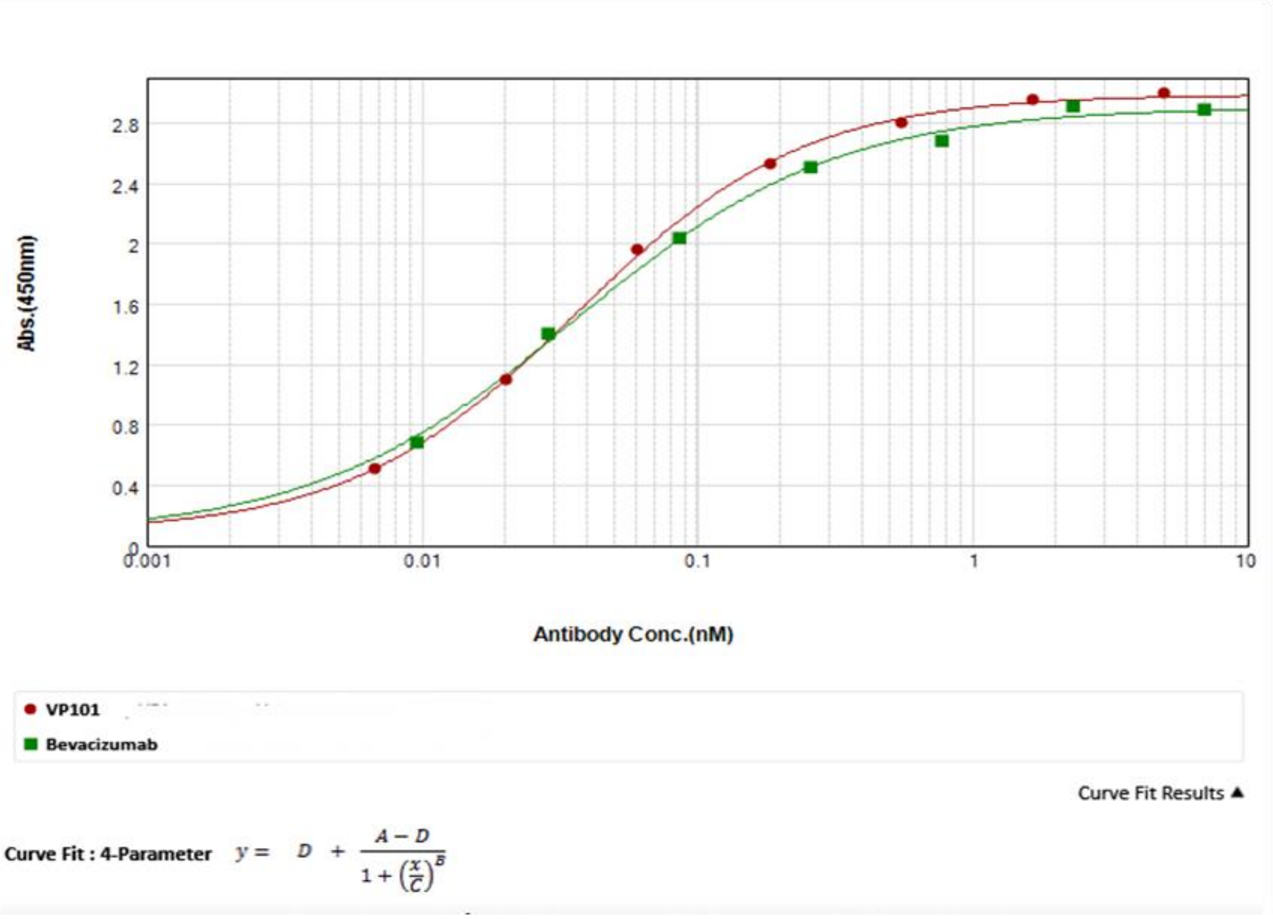
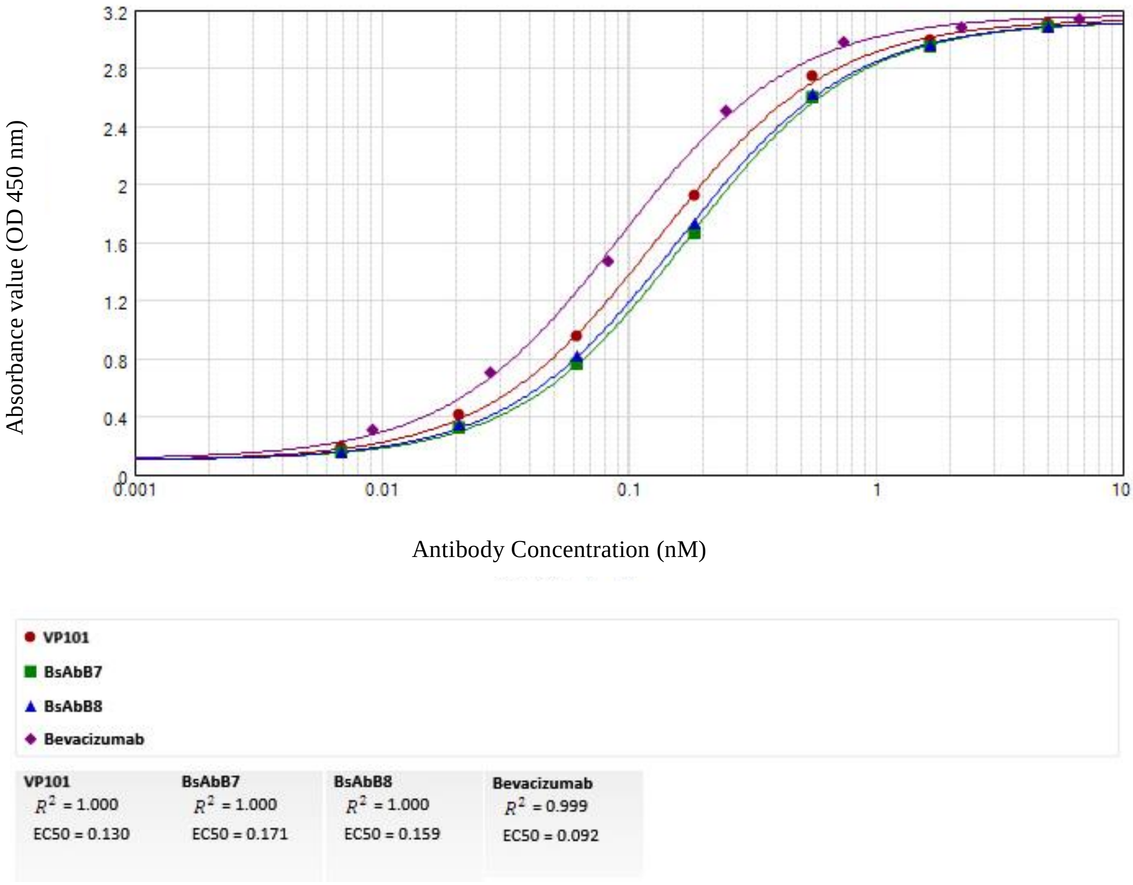

Abs.(450nm)
2.8
2.4
2
1.6
1.2
0.8
0.4
0
0.001
0.01
0.1
1
10
Antibody Conc.(nM)
● VP101
🟩 Bevacizumab
Curve Fit Results ▲
Curve Fit : 4-Parameter
$y = D + \frac{A - D}{1 + \left(\frac{x}{C}\right)^B}$

FIG. 11



VP101	BsAbB7	BsAbB8	Bevacizumab
R^2 = 1.000	R^2 = 1.000	R^2 = 1.000	R^2 = 0.999
EC50 = 0.130	EC50 = 0.171	EC50 = 0.159	EC50 = 0.092

FIG. 12

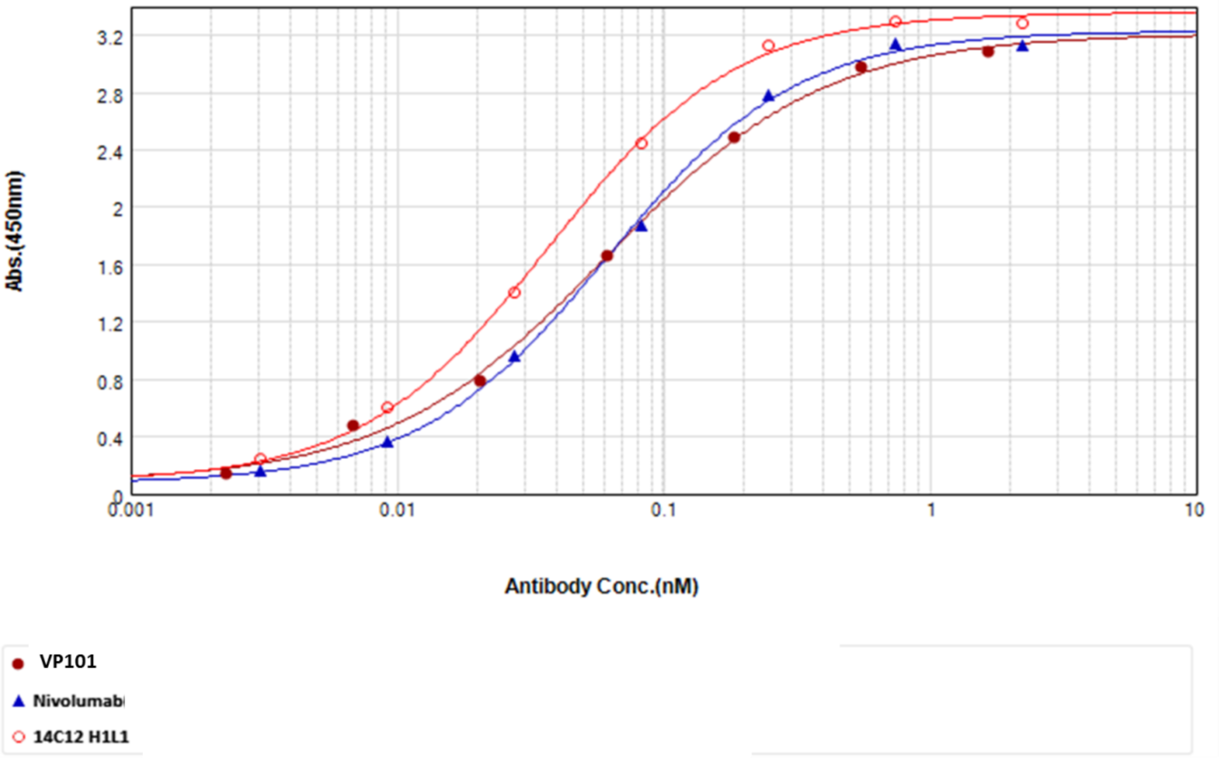
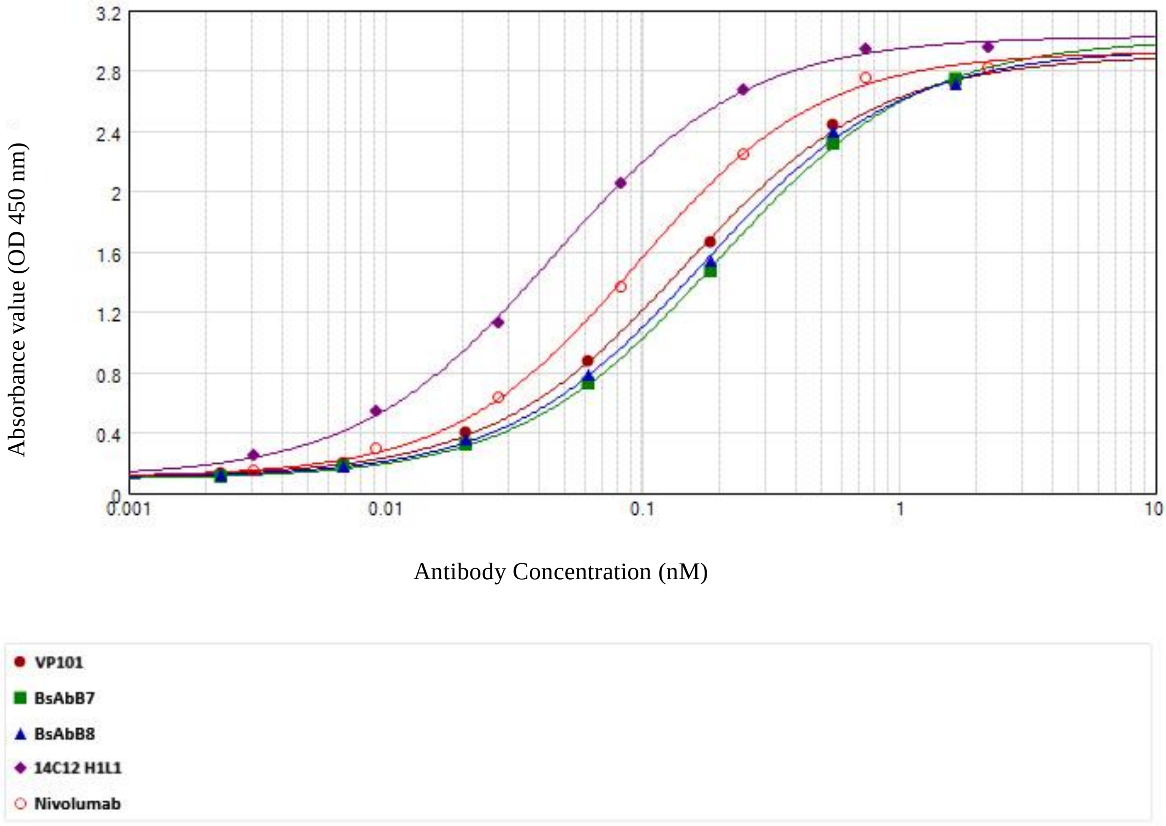
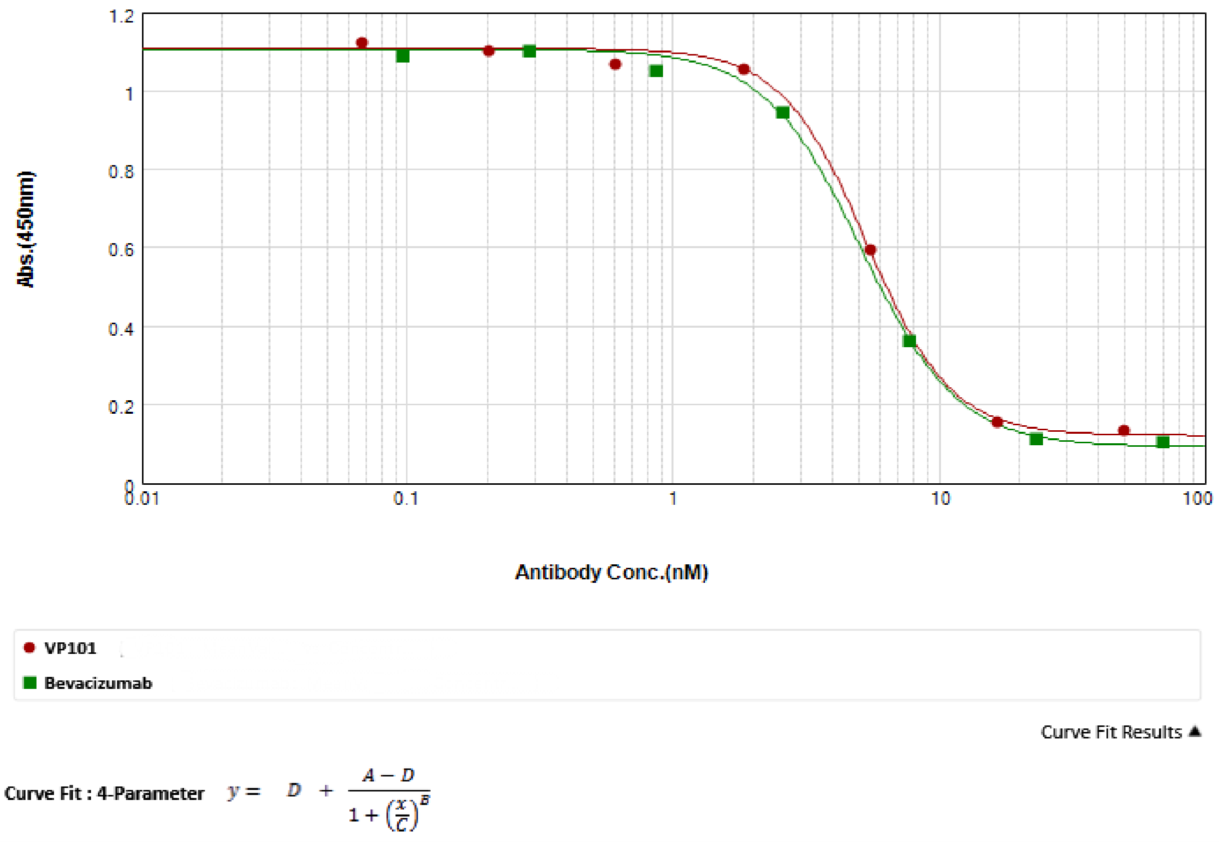


FIG. 13



VP101	BsAbB7	BsAbB8	14C12 H1L1	Nivolumab
R^2 = 0.999	R^2 = 1.000	R^2 = 0.999	R^2 = 0.999	R^2 = 0.999
EC50 = 0.146	EC50 = 0.199	EC50 = 0.173	EC50 = 0.045	EC50 = 0.095

FIG. 14



$$y = D + \frac{A - D}{1 + \left(\frac{x}{C}\right)^{B}}$$

FIG. 15

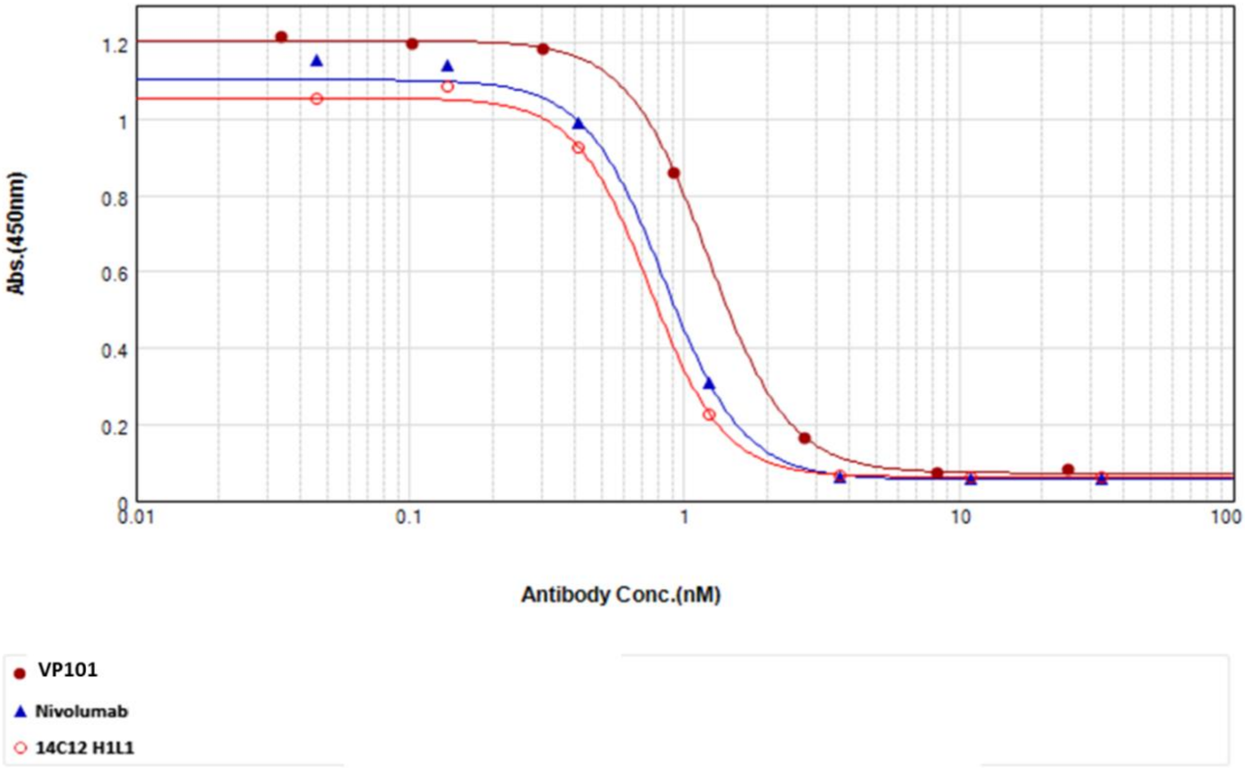


FIG. 16

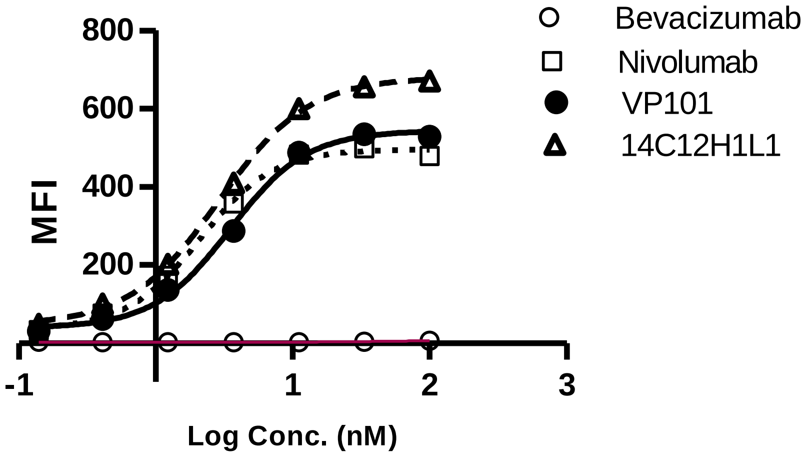

800
600
400
200
MFI
-1
1
2
3
Log Conc. (nM)
○ Bevacizumab
□ Nivolumab
● VP101
△ 14C12H1L1

FIG. 17

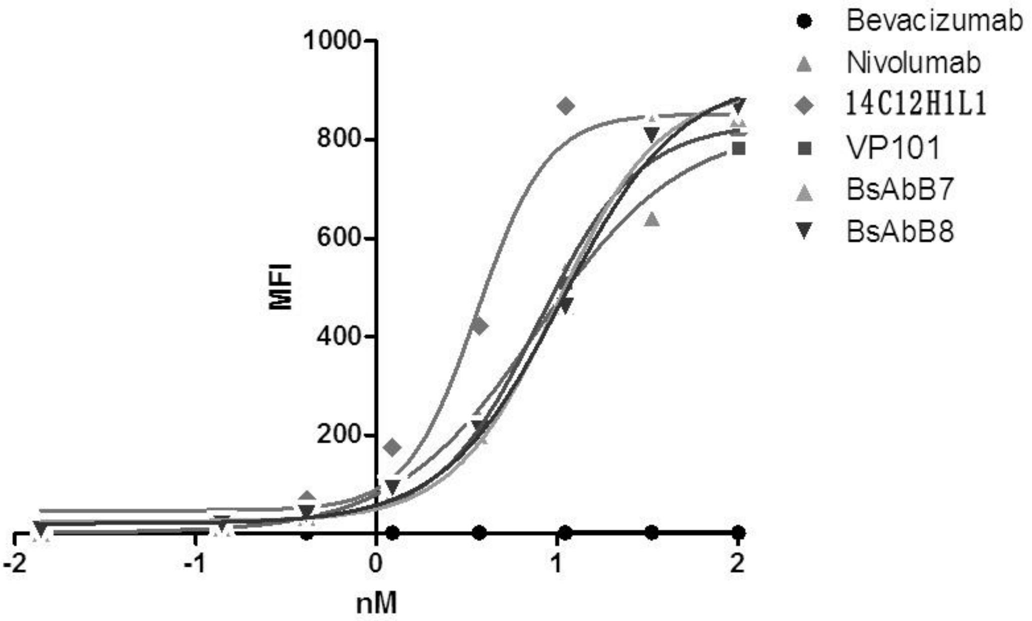
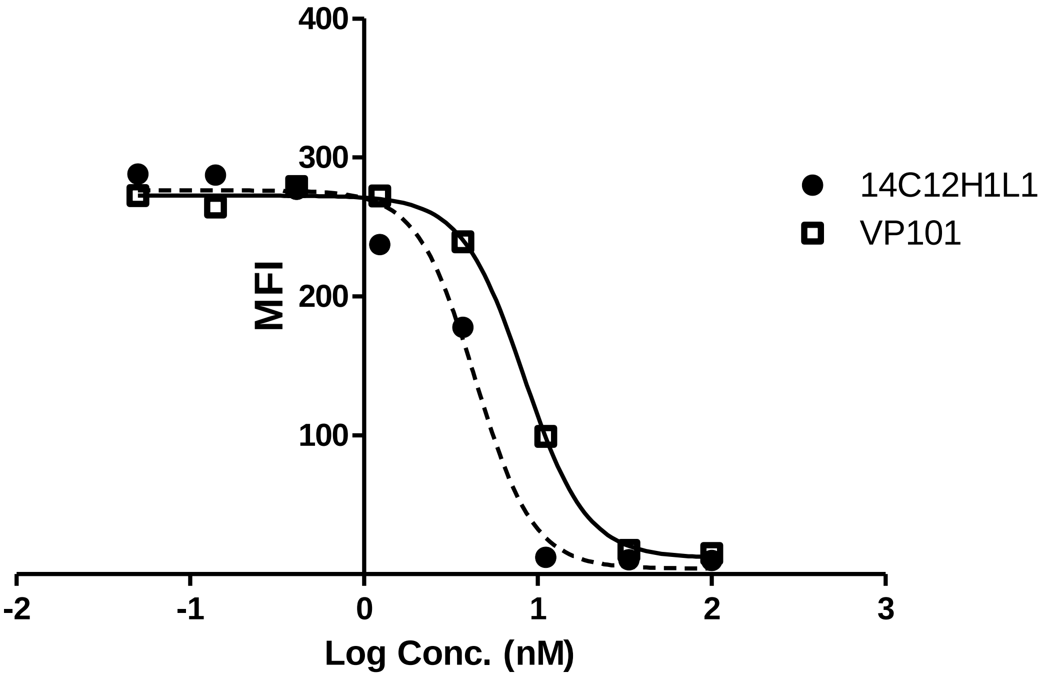

1000
800
600
400
200
MFI
-2
-1
0
1
2
nM
● Bevacizumab
▲ Nivolumab
◆ 14C12H1L1
■ VP101
▲ BsAbB7
▼ BsAbB8

FIG. 18


MFI
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0
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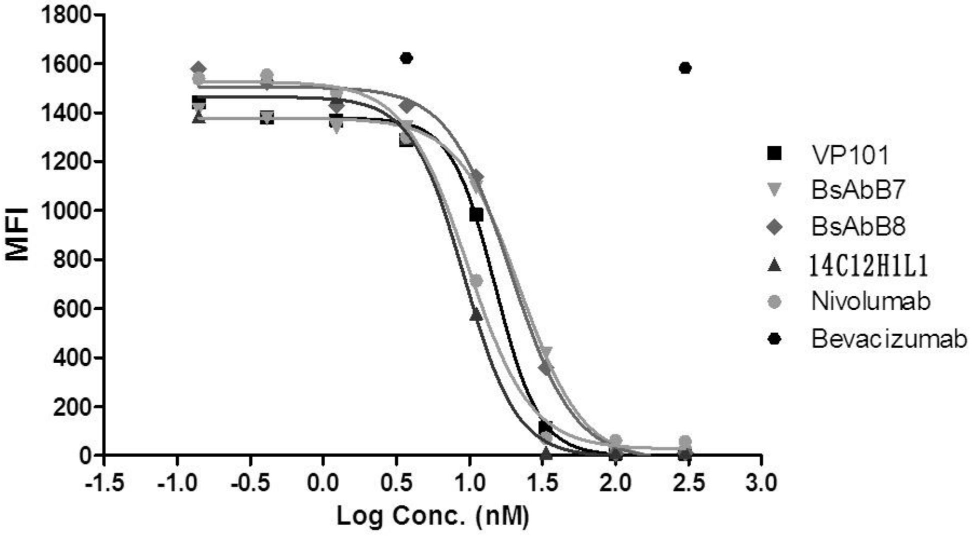
3
Log Conc. (nM)
● 14C12H1L1
□ VP101

FIG. 19


MFI
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1000
800
600
400
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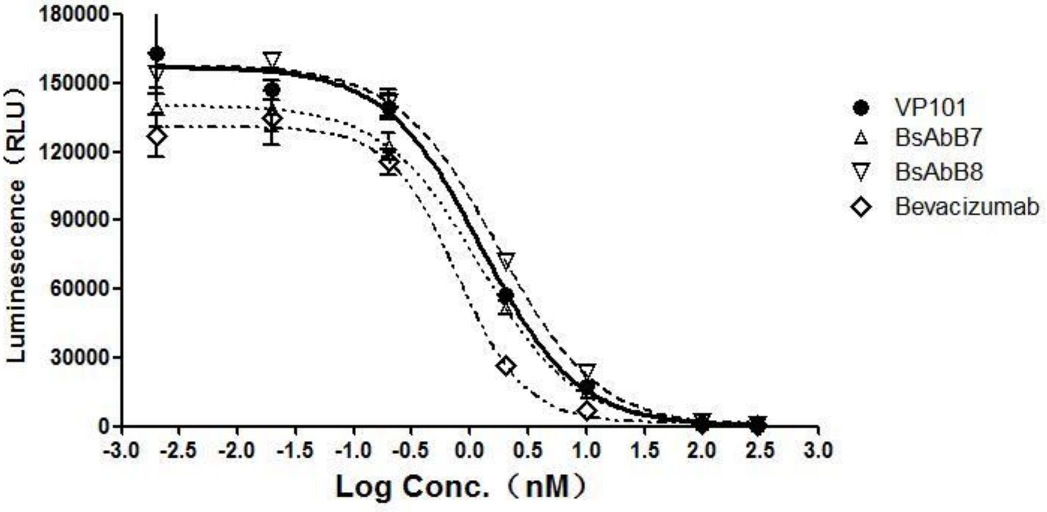
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Log Conc. (nM)
■ VP101
▼ BsAbB7
◆ BsAbB8
▲ 14C12H1L1
● Nivolumab
● Bevacizumab

FIG. 20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120000
90000
60000
30000
0
Luminesecence（RLU）
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-2.5
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Log Conc. （nM）
● VP101
△ BsAbB7
▽ BsAbB8
◇ Bevacizumab

FIG. 21

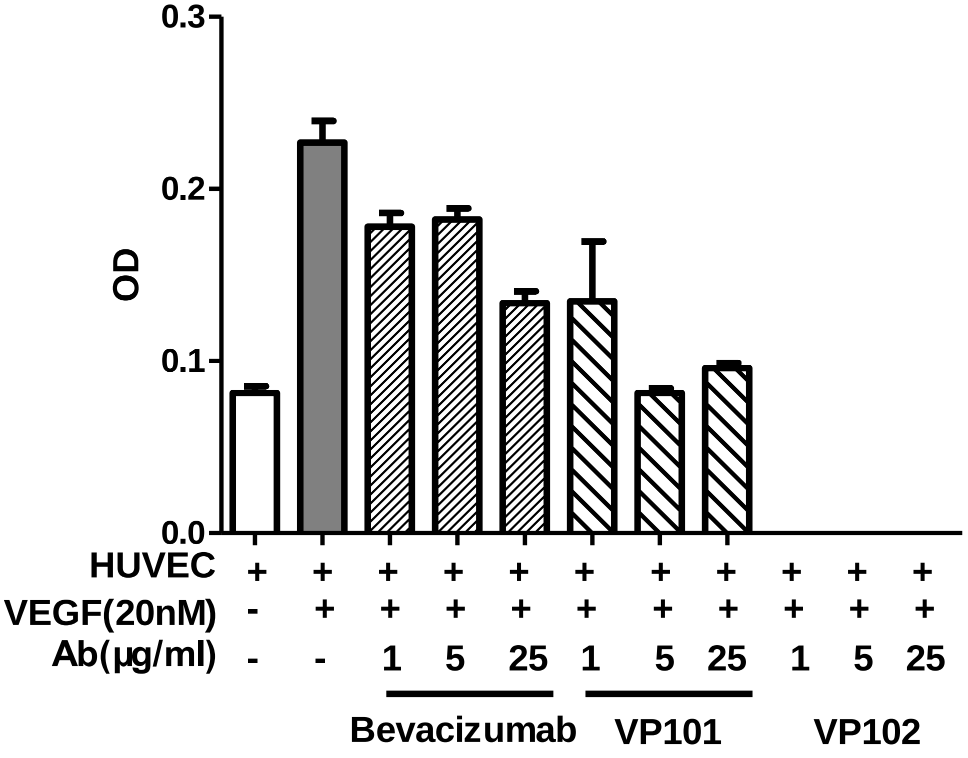
0.3
0.2
0.1
0.0
OD
HUVEC + + + + + + + + + + +
VEGF(20nM) - + + + + + + + + + +
Ab(μg/ml) - - 1 5 25 1 5 25 1 5 25
Bevacizumab
VP101
VP102

FIG. 22

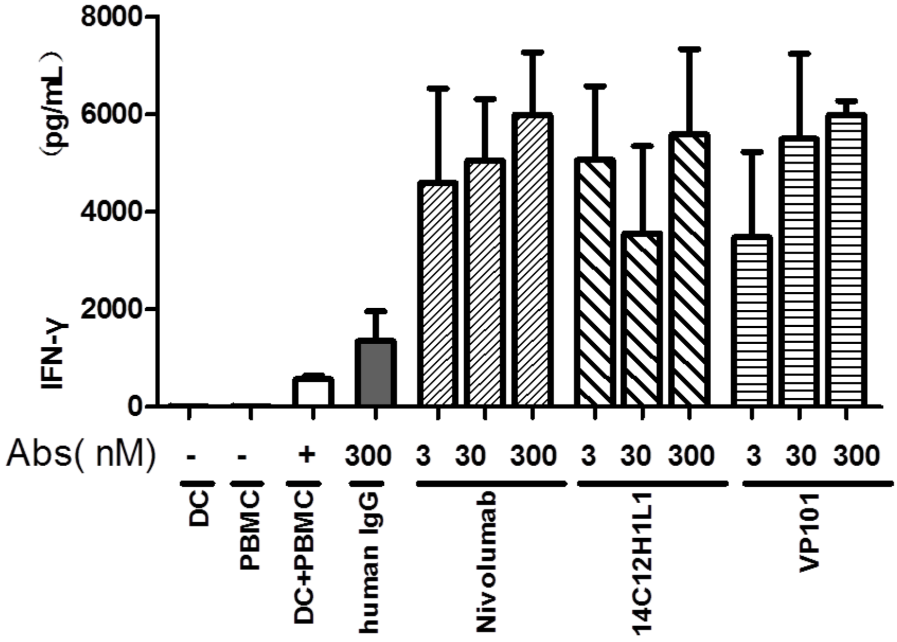
8000
6000
4000
2000
0
IFN-γ（pg/mL）
Abs(nM)
-
-
+
300
3 30 300
3 30 300
3 30 300
DC
PBMC
DC+PBMC
human IgG
Nivolumab
14C12H1L1
VP101

FIG. 23

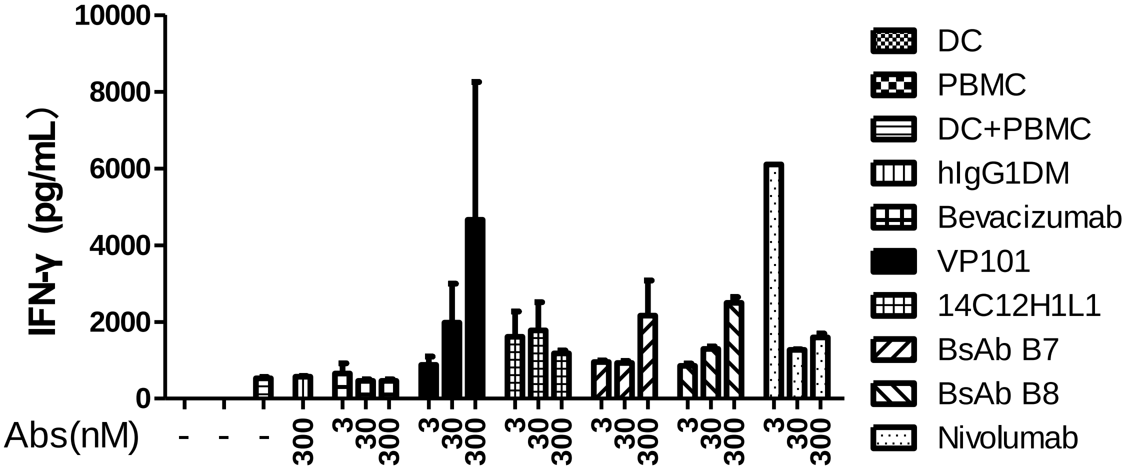
10000
8000
6000
4000
2000
0
IFN-γ (pg/mL)
Abs(nM)
- - -
300
3 30 300
3 30 300
3 30 300
3 30 300
3 30 300
3 30 300
DC
PBMC
DC+PBMC
hIgG1DM
Bevacizumab
VP101
14C12H1L1
BsAb B7
BsAb B8
Nivolumab

FIG. 24

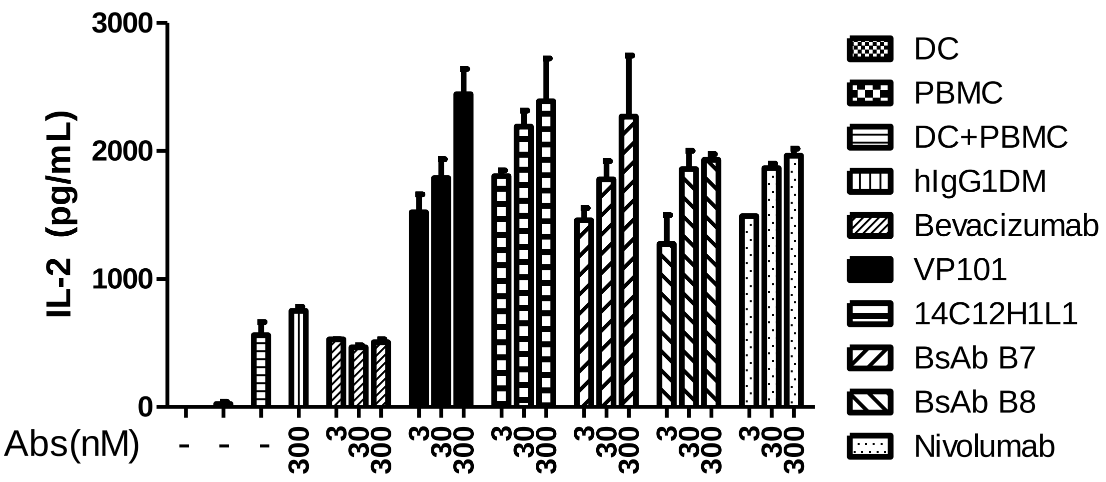
3000
2000
1000
0
IL-2 (pg/mL)
Abs(nM)
-
-
-
300
3
30
300
3
30
300
3
30
300
3
30
300
3
30
300
3
30
300
DC
PBMC
DC+PBMC
hIgG1DM
Bevacizumab
VP101
14C12H1L1
BsAb B7
BsAb B8
Nivolumab

FIG. 25

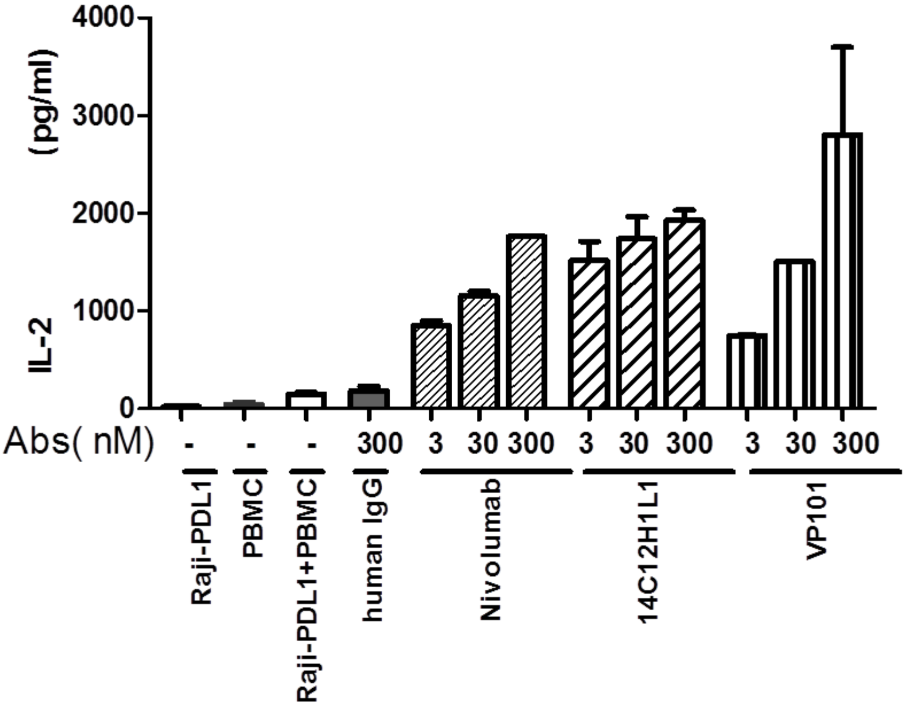
4000
3000
2000
1000
0
IL-2 (pg/ml)
Abs(nM)
-
-
-
300
3
30
300
3
30
300
3
30
300
Raji-PDL1
PBMC
Raji-PDL1+PBMC
human IgG
Nivolumab
14C12H1L1
VP101

FIG. 26

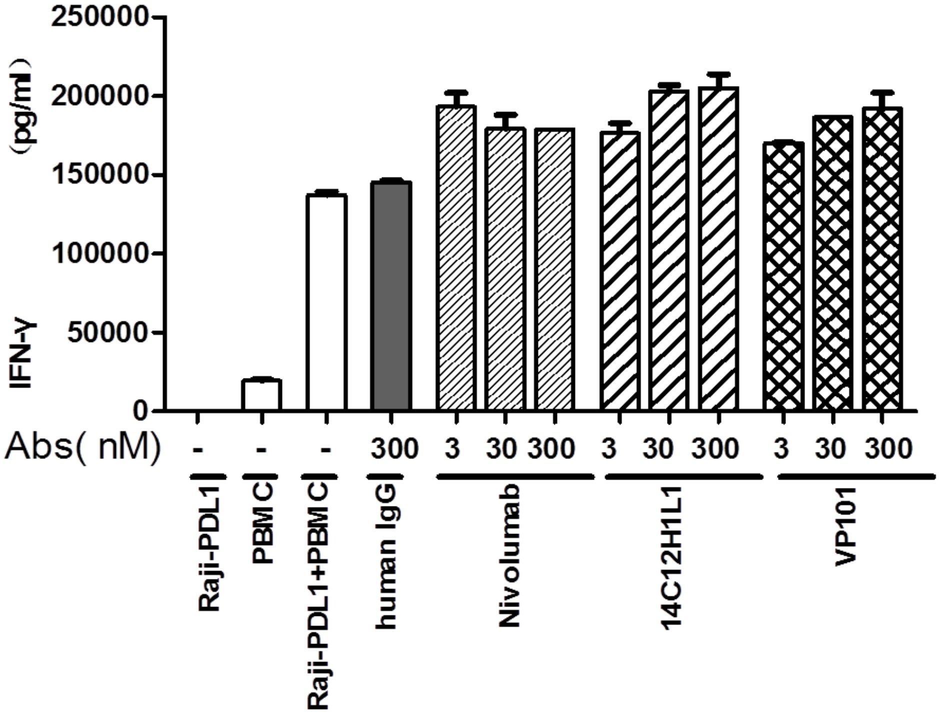
250000
200000
150000
100000
50000
0
IFN-γ （pg/ml）
Abs(nM)
-
-
-
300
3
30
300
3
30
300
3
30
300
Raji-PDL1
PBMC
Raji-PDL1+PBMC
human IgG
Nivolumab
14C12H1L1
VP101

FIG. 27

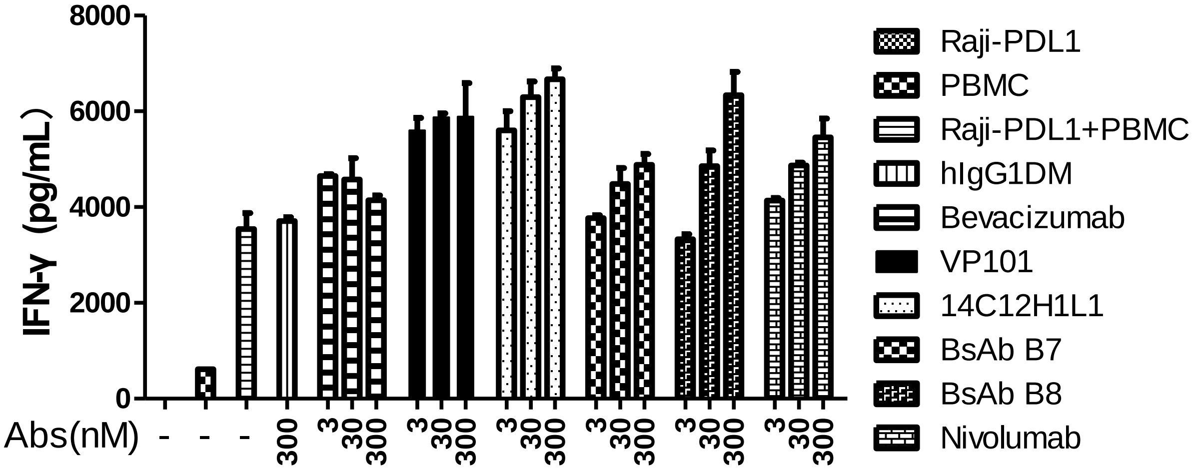
8000
6000
4000
2000
0
IFN-γ (pg/mL)
Abs(nM)
-
-
-
300
3
30
300
3
30
300
3
30
300
3
30
300
3
30
300
3
30
300
Raji-PDL1
PBMC
Raji-PDL1+PBMC
hIgG1DM
Bevacizumab
VP101
14C12H1L1
BsAb B7
BsAb B8
Nivolumab

FIG. 28

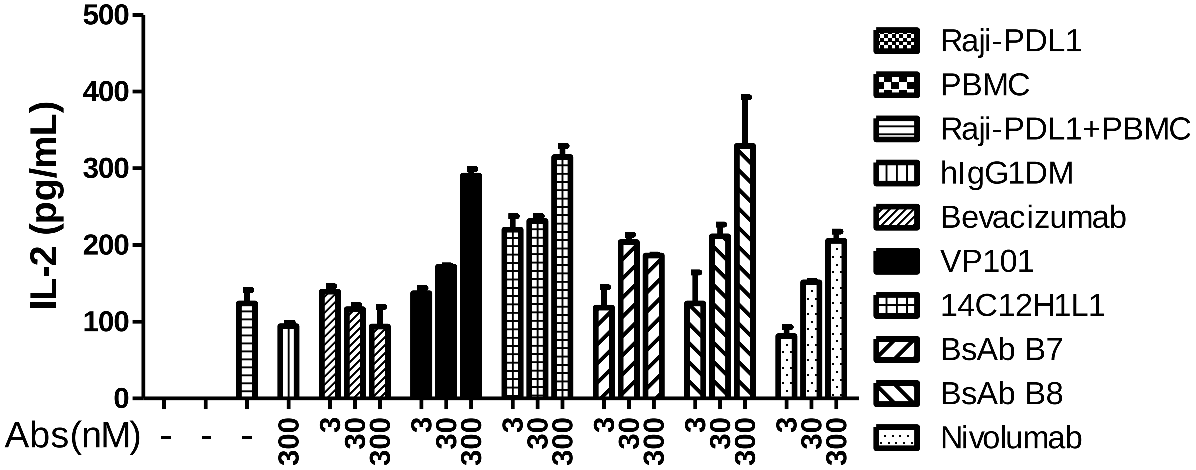
IL-2 (pg/mL)
500
400
300
200
100
0
Abs(nM)
-
-
-
300
3
30
300
3
30
300
3
30
300
3
30
300
3
30
300
3
30
300
Raji-PDL1
PBMC
Raji-PDL1+PBMC
hIgG1DM
Bevacizumab
VP101
14C12H1L1
BsAb B7
BsAb B8
Nivolumab

FIG. 29

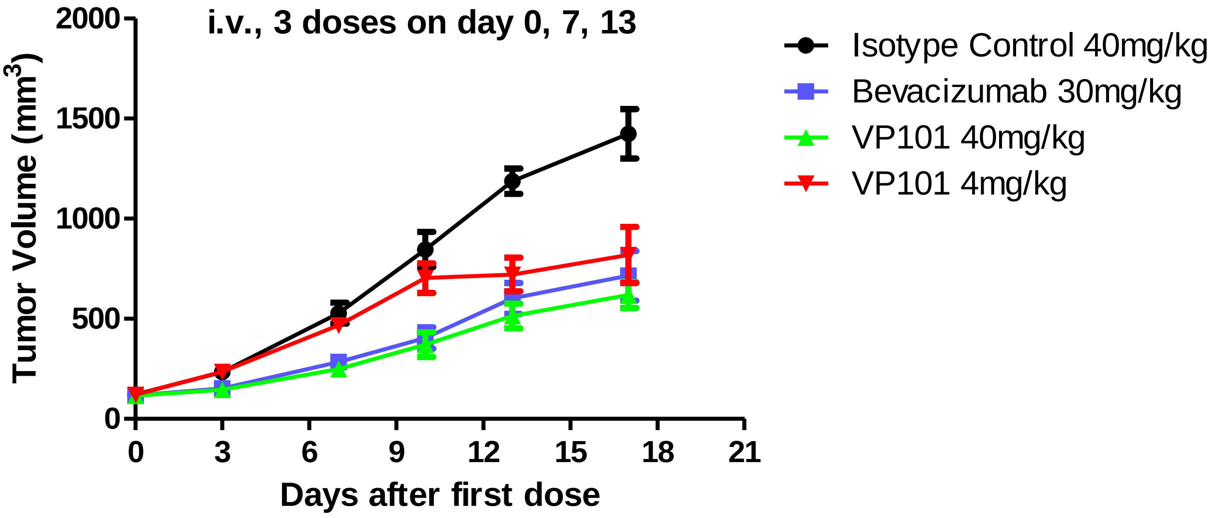
i.v., 3 doses on day 0, 7, 13
Tumor Volume (mm^3)
2000
1500
1000
500
0
0
3
6
9
12
15
18
21
Days after first dose
Isotype Control 40mg/kg
Bevacizumab 30mg/kg
VP101 40mg/kg
VP101 4mg/kg

FIG. 30

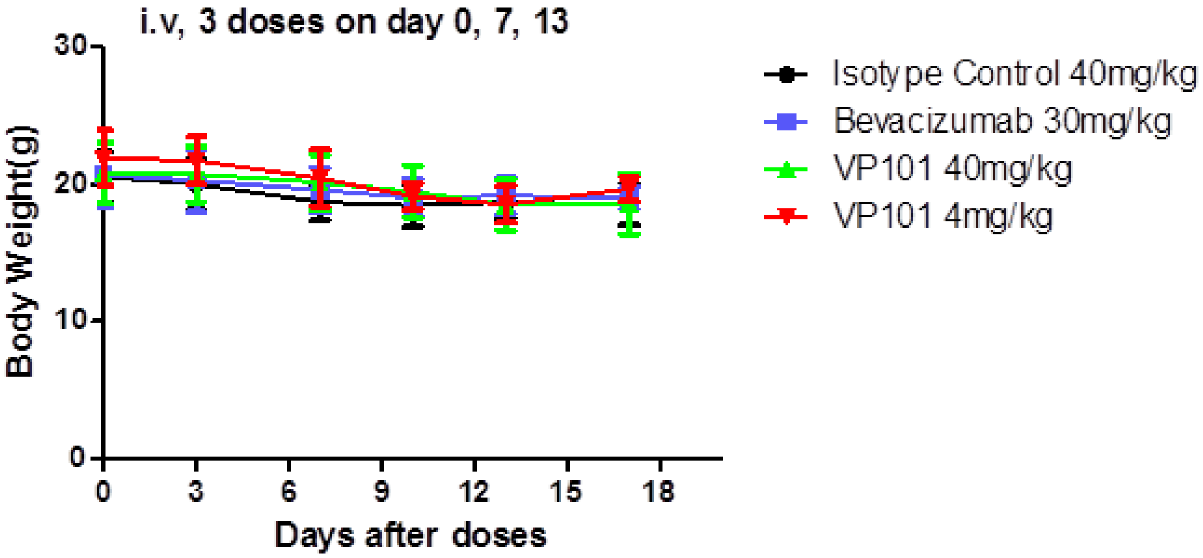
i.v, 3 doses on day 0, 7, 13
Body Weight(g)
30
20
10
0
0
3
6
9
12
15
18
Days after doses
Isotype Control 40mg/kg
Bevacizumab 30mg/kg
VP101 40mg/kg
VP101 4mg/kg

FIG. 31

Sequence Listing

1	**Sequence Listing Information**	
1-1	File Name	P0030509AUD1 - Sequence Listing.xml
1-2	DTD Version	V1_3
1-3	Software Name	WIPO Sequence
1-4	Software Version	2.3.0
1-5	Production Date	2026-02-11
1-6	Original free text language code	
1-7	Non English free text language code	
2	**General Information**	
2-1	Current application: IP Office	
2-2	Current application: Application number	
2-3	Current application: Filing date	
2-4	Current application: Applicant file reference	IEC190067PCT
2-5	Earliest priority application: IP Office	CN
2-6	Earliest priority application: Application number	201811002548.4
2-7	Earliest priority application: Filing date	2018-08-30
2-8en	Applicant name	AKESO BIOPHARMA, INC
2-8	Applicant name: Name Latin	
2-9en	Inventor name	
2-9	Inventor name: Name Latin	
2-10en	Invention title	ANTI-PD-1/VEGFA BIFUNCTIONAL ANTIBODY, PHARMACEUTICAL COMPOSITION THEREOF AND USE THEREOF
2-11	Sequence Total Quantity	26

3-1	**Sequences**	
3-1-1	Sequence Number [ID]	1
3-1-2	Molecule Type	AA
3-1-3	Length	171
3-1-4	Features	**REGION 1..171**
	Location/Qualifiers	note=The amino acid sequence of VEGFA-His
		source 1..171
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-1-5	Residues	APMAEGGGQN HHEVVKFMDV YQRSYCHPIE TLVDIFQEYP DEIEYIFKPS CVPLMRCGGC 60 CNDEGLECVP TEESNITMQI MRIKPHQGQH IGEMSFLQHN KCECRPKKDR ARQENPCGPC 120 SERRKHLFVQ DPQTCKCSCK NTDSRCKARQ LELNERTCRC DKPRRHHHHH H 171
3-2	**Sequences**	
3-2-1	Sequence Number [ID]	2
3-2-2	Molecule Type	DNA
3-2-3	Length	513
3-2-4	Features	**misc_feature 1..513**
	Location/Qualifiers	note=Nucleotide sequence of VEGFA-His
		source 1..513
		mol_type=other DNA
		organism=synthetic construct
	NonEnglishQualifier Value	
3-2-5	Residues	gcacccatgg ccgagggcgg cggccagaac caccacgagg tggtgaagtt catggacgtg 60 taccagagaa gctactgcca ccccatcgag accctggtgg acatcttcca ggagtacccc 120 gacgagatcg agtacatctt caagcccagc tgcgtgcccc tgatgagatg cggcggctgc 180 tgcaacgacg agggcctgga gtgcgtgccc accgaggaga gcaacatcac catgcagatc 240 atgagaatca agccccacca gggccagcac atcggcgaga tgagcttcct gcagcacaac 300 aagtgcgagt gcagacccaa gaaggacaga gccagacagg agaacccctg cggcccctgc 360 agcgagagaa gaaagcacct gttcgtgcag gacccccaga cctgcaagtg cagctgcaag 420 aacaccgaca gcagatgcaa ggccagacag ctggagctga acgagagaac ctgcagatgc 480 gacaagccca gaagacatca tcaccatcac cac 513
3-3	**Sequences**	
3-3-1	Sequence Number [ID]	3
3-3-2	Molecule Type	AA
3-3-3	Length	998
3-3-4	Features	**REGION 1..998**
	Location/Qualifiers	note=The amino acid sequence of Fusion protein VEGFR2- hFc
		source 1..998
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-3-5	Residues	MQSKVLLAVA LWLCVETRAA SVGLPSVSLD LPRLSIQKDI LTIKANTTLQ ITCRGQRDLD 60 WLWPNNQSGS EQRVEVTECS DGLFCKTLTI PKVIGNDTGA YKCFYRETDL ASVIYVYVQD 120 YRSPFIASVS DQHGVVYITE NKNKTVVIPC LGSISNLNVS LCARYPEKRF VPDGNRISWD 180 SKKGFTIPSY MISYAGMVFC EAKINDESYQ SIMYIVVVVG YRIYDVVLSP SHGIELSVGE 240 KLVLNCTART ELNVGIDFNW EYPSSKHQHK KLVNRDLKTQ SGSEMKKFLS TLTIDGVTRS 300 DQGLYTCAAS SGLMTKKNST FVRVHEKPFV AFGSGMESLV EATVGERVRI PAKYLGYPPP 360 EIKWYKNGIP LESNHTIKAG HVLTIMEVSE RDTGNYTVIL TNPISKEKQS HVVSLVVYVP 420 PQIGEKSLIS PVDSYQYGTT QTLTCTVYAI PPPHHIHWYW QLEEECANEP SQAVSVTNPY 480 PCEEWRSVED FQGGNKIEVN KNQFALIEGK NKTVSTLVIQ AANVSALYKC EAVNKVGRGE 540 RVISFHVTRG PEITLQPDMQ PTEQESVSLW CTADRSTFEN LTWYKLGPQP LPIHVGELPT 600 PVCKNLDTLW KLNATMFSNS TNDILIMELK NASLQDQGDY VCLAQDRKTK KRHCVVRQLT 660 VLERVAPTIT GNLENQTTSI GESIEVSCTA SGNPPPQIMW FKDNETLVED SGIVLKDGNR 720 NLTIRRVRKE DEGLYTCQAC SVLGCAKVEA FFIIEGAQEK TNLESRENLY FQGTHTCPPC 780 PAPELLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSHE DPEVKFNWYV DGVEVHNAKT 840 KPREEQYNST YRVVSVLTVL HQDWLNGKEY KCKVSNKALP APIEKTISKA KGQPREPQVY 900 TLPPSRDELT KNQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTPPVLD SDGSFFLYSK 960 LTVDKSRWQQ GNVFSCSVMH EALHNHYTQK SLSLSPGK 998
3-4	**Sequences**	
3-4-1	Sequence Number [ID]	4
3-4-2	Molecule Type	DNA
3-4-3	Length	2997
3-4-4	Features	**misc_feature 1..2997**
	Location/Qualifiers	note=Nucleotide sequence of Fusion protein VEGFR2- hFc
		source 1..2997
		mol_type=other DNA
		organism=synthetic construct
	NonEnglishQualifier Value	
3-4-5	Residues	atgcagagca aggtgctgct ggccgtcgcc ctgtggctct gcgtggagac ccgggccgcc 60 tctgtgggtt tgcctagtgt ttctcttgat ctgcccaggc tcagcataca aaaagacata 120

		cttacaatta aggctaatac aactcttcaa attacttgca ggggacagag ggacttggac 180 tggctttggc ccaataatca gagtggcagt gagcaaaggg tggaggtgac tgagtgcagc 240 gatggcctct tctgtaagac actcacaatt ccaaaagtga tcggaaatga cactggagcc 300 tacaagtgct tctaccggga aactgacttg gcctcggtca tttatgtcta tgttcaagat 360 tacagatctc catttattgc ttctgttagt gaccaacatg gagtcgtgta cattactgag 420 aacaaaaaca aaactgtggt gattccatgt ctcgggtcca tttcaaatct caacgtgtca 480 ctttgtgcaa gatacccaga aaagagattt gttcctgatg gtaacagaat ttcctgggac 540 agcaagaagg gctttactat tcccagctac atgatcagct atgctggcat ggtcttctgt 600 gaagcaaaaa ttaatgatga aagttaccag tctattatgt acatagttgt cgttgtaggg 660 tataggattt atgatgtggt tctgagtccg tctcatggaa ttgaactatc tgttggagaa 720 aagcttgtct taaattgtac agcaagaact gaactaaatg tggggattga cttcaactgg 780 gaataccctt cttcgaagca tcagcataag aaacttgtaa accgagacct aaaaacccag 840 tctgggagtg agatgaagaa atttttgagc accttaacta tagatggtgt aacccggagt 900 gaccaaggat tgtacacctg tgcagcatcc agtgggctga tgaccaagaa gaacagcaca 960 tttgtcaggg tccatgaaaa acctttgtt gcttttggaa gtggcatgga atctctggtg 1020 gaagccacgg tgggggagcg tgtcagaatc cctgcgaagt accttggtta cccaccccca 1080 gaaataaaat ggtataaaaa tggaataccc cttgagtcca atcacacaat taaagcgggg 1140 catgtactga cgattatgga agtgagtgaa agagacacag gaaattacac tgtcatcctt 1200 accaatccca tttcaaagga gaagcagagc catgtggtct ctctggttgt gtatgtccca 1260 ccccagattg gtgagaaatc tctaatctct cctgtggatt cctaccagta cggcaccact 1320 caaacgctga catgtacggt ctatgccatt cctcccccgc atcacatcca ctggtattgg 1380 cagttggagg aagagtgcgc caacgagccc agccaagctg tctcagtgac aaacccatac 1440 ccttgtgaag aatggagaag tgtggaggac ttccagggag gaaataaaat tgaagttaat 1500 aaaaatcaat ttgctctaat tgaaggaaaa aacaaaactg taagtaccct tgttatccaa 1560 gcggcaaatg tgtcagcttt gtacaaatgt gaagcggtca acaaagtcgg gagaggagag 1620 agggtgatct ccttccacgt gaccaggggt cctgaaatta ctttgcaacc tgacatgcag 1680 cccactgagc aggagagcgt gtctttgtgg tgcactgcag acagatctac gtttgagaac 1740 ctcacatggt acaagcttgg cccacagcct ctgccaatcc atgtgggaga gttgcccaca 1800 cctgtttgca agaacttgga tactctttgg aaattgaatg ccaccatgtt ctctaatagc 1860 acaaatgaca ttttgatcat ggagcttaag aatgcatcct tgcaggacca aggagactat 1920 gtctgccttg ctcaagacag gaagaccaag aaaagacatt gcgtggtcag gcagctcaca 1980 gtcctagagc gtgtggcacc cacgatcaca ggaaacctgg agaatcagac gacaagtatt 2040 ggggaaagca tcgaagtctc atgcacggca tctgggaatc cccctccaca gatcatgtgg 2100 tttaaagata atgagaccct tgtagaagac tcaggcattg tattgaagga tgggaaccgg 2160 aacctcacta tccgcagagt gaggaaggag gacgaaggcc tctacacctg ccaggcatgc 2220 agtgttcttg gctgtgcaaa agtggaggca tttttcataa tagaaggtgc ccaggaaaag 2280 acgaacttgg aatctagaga aaacctgtat tttcagggca ctcacacatg cccaccgtgc 2340 ccagcacctg aactcctggg gggaccgtca gtcttcctct tccccccaaa acccaaggac 2400 accctcatga tctcccggac ccctgaggtc acatgcgtgg tggtggacgt gagccacgaa 2460 gaccctgagg tcaagttcaa ctggtacgtg gacggcgtgg aggtgcataa tgccaagaca 2520 aagccgcggg aggagcagta caacagcacg taccgtgtgg tcagcgtcct caccgtcctg 2580 caccaggact ggctgaatgg caaggagtac aagtgcaagg tctccaacaa agccctccca 2640 gcccccatcg agaaaaccat ctccaaagcc aaagggcagc cccgagaacc acaggtgtac 2700 accctgcccc catcccggga tgagctgacc aagaaccagg tcagcctgac ctgcctggtc 2760 aaaggcttct atcccagcga catcgccgtg gagtgggaga gcaatgggca gccggagaac 2820 aactacaaga ccacgcctcc cgtgttggac tccgacggct ccttcttcct ctacagcaag 2880 ctcaccgtgg acaagagcag gtggcagcag gggaacgtct tctcatgctc cgtgatgcat 2940 gaggctctgc acaaccacta cacgcagaag agcctctccc tgtctcccgg gaaatga 2997
3-5	**Sequences**	
3-5-1	Sequence Number [ID]	5
3-5-2	Molecule Type	AA
3-5-3	Length	123
3-5-4	Features Location/Qualifiers	**REGION 1..123** note=The amino acid sequence of Bevacizumab heavy chain variable region **source 1..123** mol_type=protein organism=synthetic construct
	NonEnglishQualifier Value	
3-5-5	Residues	EVQLVESGGG LVQPGGSLRL SCAASGYTFT NYGMNWVRQA PGKGLEWVGW INTYTGEPTY 60 AADFKRRFTF SLDTSKSTAY LQMNSLRAED TAVYYCAKYP HYYGSSHWYF DVWGQGTLVT 120 VSS 123
3-6	**Sequences**	
3-6-1	Sequence Number [ID]	6
3-6-2	Molecule Type	DNA
3-6-3	Length	369
3-6-4	Features Location/Qualifiers	**misc_feature 1..369** note=Nucleotide sequence of Bevacizumab heavy chain variable region **source 1..369** mol_type=other DNA organism=synthetic construct
	NonEnglishQualifier Value	
3-6-5	Residues	gaggtgcagc tggtcgagtc cgggggggg ctggtgcagc caggcgggtc tctgaggctg 60 agttgcgccg cttcagggta caccttcaca aactatggaa tgaattgggt gcgccaggca 120

		ccaggaaagg gactggagtg ggtcggctgg atcaacactt acaccgggga acctacctat 180
		gcagccgact ttaagcggcg gttcaccttc agcctggata caagcaaatc cactgcctac 240
		ctgcagatga acagcctgcg agctgaggac accgcagtct actattgtgc taaatatccc 300
		cactactatg ggagcagcca ttggtatttt gacgtgtggg ggcaggggac tctggtgaca 360
		gtgagcagc 369
3-7	**Sequences**	
3-7-1	Sequence Number [ID]	7
3-7-2	Molecule Type	AA
3-7-3	Length	107
3-7-4	Features	**REGION 1..107**
	Location/Qualifiers	note=The amino acid sequence of Bevacizumab light chain variable regi on
		source 1..107
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-7-5	Residues	DIQMTQSPSS LSASVGDRVT ITCSASQDIS NYLNWYQQKP GKAPKVLIYF TSSLHSGVPS 60
		RFSGSGSGTD FTLTISSLQP EDFATYYCQQ YSTVPWTFGQ GTKVEIK 107
3-8	**Sequences**	
3-8-1	Sequence Number [ID]	8
3-8-2	Molecule Type	DNA
3-8-3	Length	321
3-8-4	Features	**misc_feature 1..321**
	Location/Qualifiers	note=Nucleotide sequence of Bevacizumab light chain variable region
		source 1..321
		mol_type=other DNA
		organism=synthetic construct
	NonEnglishQualifier Value	
3-8-5	Residues	gatattcaga tgactcagag cccctcctcc ctgtccgcct ctgtgggcga cagggtcacc 60
		atcacatgca gtgcttcaca ggatatttcc aactacctga attggtatca gcagaagcca 120
		ggaaaagcac ccaaggtgct gatctacttc actagctccc tgcactcagg agtgccaagc 180
		cggttcagcg gatccggatc tggaaccgac tttactctga ccatttctag tctgcagcct 240
		gaggatttcg ctacatacta ttgccagcag tattctaccg tgccatggac atttggccag 300
		gggactaaag tcgagatcaa g 321
3-9	**Sequences**	
3-9-1	Sequence Number [ID]	9
3-9-2	Molecule Type	AA
3-9-3	Length	118
3-9-4	Features	**REGION 1..118**
	Location/Qualifiers	note=The amino acid sequence of humanized antibody 14C12H1L1 heavy ch ain variable region
		source 1..118
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-9-5	Residues	EVQLVESGGG LVQPGGSLRL SCAASGFAFS SYDMSWVRQA PGKGLDWVAT ISGGGRYTYY 60
		PDSVKGRFTI SRDNSKNNLY LQMNSLRAED TALYYCANRY GEAWFAYWGQ GTLVTVSS 118
3-10	**Sequences**	
3-10-1	Sequence Number [ID]	10
3-10-2	Molecule Type	DNA
3-10-3	Length	354
3-10-4	Features	**misc_feature 1..354**
	Location/Qualifiers	note=Nucleotide sequence of humanized antibody 14C12H1L1 heavy chain variable region
		source 1..354
		mol_type=other DNA
		organism=synthetic construct
	NonEnglishQualifier Value	
3-10-5	Residues	gaagtgcagc tggtcgagtc tgggggaggg ctggtgcagc ccggcgggtc actgcgactg 60
		agctgcgcag cttccggatt cgcctttagc tcctacgaca tgtcctgggt gcgacaggca 120
		ccaggaaagg gactggattg ggtcgctact atctcaggag gcgggagata cacctactat 180
		cctgacagcg tcaagggccg gttcacaatc tctagagata acagtaagaa caatctgtat 240
		ctgcagatga acagcctgag ggctgaggac accgcactgt actattgtgc caaccgctac 300
		ggggaagcat ggtttgccta ttgggggcag ggaaccctgg tgacagtctc tagt 354
3-11	**Sequences**	
3-11-1	Sequence Number [ID]	11
3-11-2	Molecule Type	AA
3-11-3	Length	107
3-11-4	Features	**REGION 1..107**
	Location/Qualifiers	note=The amino acid sequence of humanized antibody 14C12H1L1 light ch ain variable region
		source 1..107
		mol_type=protein
		organism=synthetic construct

	NonEnglishQualifier Value	
3-11-5	Residues	DIQMTQSPSS MSASVGDRVT FTCRASQDIN TYLSWFQQKP GKSPKTLIYR ANRLVSGVPS 60 RFSGSGSGQD YTLTISSLQP EDMATYYCLQ YDEFPLTFGA GTKLELK 107
3-12	**Sequences**	
3-12-1	Sequence Number [ID]	12
3-12-2	Molecule Type	DNA
3-12-3	Length	321
3-12-4	Features Location/Qualifiers	**misc_feature 1..321** note=Nucleotide sequence of humanized antibody 14C12H1L1 light chain variable region **source 1..321** mol_type=other DNA organism=synthetic construct
	NonEnglishQualifier Value	
3-12-5	Residues	gacattcaga tgactcagag cccctcctcc atgtccgcct ctgtgggcga cagggtcacc 60 ttcacatgcc gcgctagtca ggatatcaac acctacctga gctggtttca gcagaagcca 120 gggaaaagcc ccaagacact gatctaccgg gctaatagac tggtgtctgg agtcccaagt 180 cggttcagtg gctcagggag cggacaggac tacactctga ccatcagctc cctgcagcct 240 gaggacatgg caacctacta ttgcctgcag tatgatgagt tcccactgac ctttggcgcc 300 gggacaaaac tggagctgaa g 321
3-13	**Sequences**	
3-13-1	Sequence Number [ID]	13
3-13-2	Molecule Type	AA
3-13-3	Length	20
3-13-4	Features Location/Qualifiers	**REGION 1..20** note=The amino acid sequence of Linker 1 **source 1..20** mol_type=protein organism=synthetic construct
	NonEnglishQualifier Value	
3-13-5	Residues	GGGGSGGGGS GGGGSGGGGS 20
3-14	**Sequences**	
3-14-1	Sequence Number [ID]	14
3-14-2	Molecule Type	AA
3-14-3	Length	5
3-14-4	Features Location/Qualifiers	**REGION 1..5** note=linker fragment **source 1..5** mol_type=protein organism=synthetic construct
	NonEnglishQualifier Value	
3-14-5	Residues	GGGGS 5
3-15	**Sequences**	
3-15-1	Sequence Number [ID]	15
3-15-2	Molecule Type	AA
3-15-3	Length	8
3-15-4	Features Location/Qualifiers	**REGION 1..8** note=HCDR1 of Antibody Bevacizumab **source 1..8** mol_type=protein organism=synthetic construct
	NonEnglishQualifier Value	
3-15-5	Residues	GYTFTNYG 8
3-16	**Sequences**	
3-16-1	Sequence Number [ID]	16
3-16-2	Molecule Type	AA
3-16-3	Length	8
3-16-4	Features Location/Qualifiers	**REGION 1..8** note=HCDR2 of Antibody Bevacizumab **source 1..8** mol_type=protein organism=synthetic construct
	NonEnglishQualifier Value	
3-16-5	Residues	INTYTGEP 8
3-17	**Sequences**	
3-17-1	Sequence Number [ID]	17
3-17-2	Molecule Type	AA
3-17-3	Length	16
3-17-4	Features Location/Qualifiers	**REGION 1..16** note=HCDR3 of Antibody Bevacizumab

		source 1..16	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-17-5	Residues	AKYPHYYGSS HWYFDV	16
3-18	**Sequences**		
3-18-1	Sequence Number [ID]	18	
3-18-2	Molecule Type	AA	
3-18-3	Length	6	
3-18-4	Features	**REGION 1..6**	
	Location/Qualifiers	note=LCDR1 of Antibody Bevacizumab	
		source 1..6	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-18-5	Residues	QDISNY	6
3-19	**Sequences**		
3-19-1	Sequence Number [ID]	19	
3-19-2	Molecule Type		
3-19-3	Length		
3-19-4	Features		
	Location/Qualifiers		
	NonEnglishQualifier Value		
3-19-5	Residues	000	3
3-20	**Sequences**		
3-20-1	Sequence Number [ID]	20	
3-20-2	Molecule Type	AA	
3-20-3	Length	9	
3-20-4	Features	**REGION 1..9**	
	Location/Qualifiers	note=LCDR3 of Antibody Bevacizumab	
		source 1..9	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-20-5	Residues	QQYSTVPWT	9
3-21	**Sequences**		
3-21-1	Sequence Number [ID]	21	
3-21-2	Molecule Type	AA	
3-21-3	Length	8	
3-21-4	Features	**REGION 1..8**	
	Location/Qualifiers	note=HCDR1 of Antibody 14C12H1L1	
		source 1..8	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-21-5	Residues	GFAFSSYD	8
3-22	**Sequences**		
3-22-1	Sequence Number [ID]	22	
3-22-2	Molecule Type	AA	
3-22-3	Length	8	
3-22-4	Features	**REGION 1..8**	
	Location/Qualifiers	note=HCDR2 of Antibody 14C12H1L1	
		source 1..8	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-22-5	Residues	ISGGGRYT	8
3-23	**Sequences**		
3-23-1	Sequence Number [ID]	23	
3-23-2	Molecule Type	AA	
3-23-3	Length	11	
3-23-4	Features	**REGION 1..11**	
	Location/Qualifiers	note=HCDR3 of Antibody 14C12H1L1	
		source 1..11	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-23-5	Residues	ANRYGEAWFA Y	11

3-24	**Sequences**		
3-24-1	Sequence Number [ID]	24	
3-24-2	Molecule Type	AA	
3-24-3	Length	6	
3-24-4	Features Location/Qualifiers	**REGION 1..6** note=LCDR1 of Antibody 14C12H1L1 **source 1..6** mol_type=protein organism=synthetic construct	
	NonEnglishQualifier Value		
3-24-5	Residues	QDINTY	6
3-25	**Sequences**		
3-25-1	Sequence Number [ID]	25	
3-25-2	Molecule Type		
3-25-3	Length		
3-25-4	Features Location/Qualifiers		
	NonEnglishQualifier Value		
3-25-5	Residues	000	3
3-26	**Sequences**		
3-26-1	Sequence Number [ID]	26	
3-26-2	Molecule Type	AA	
3-26-3	Length	9	
3-26-4	Features Location/Qualifiers	**REGION 1..9** note=LCDR3 of Antibody 14C12H1L1 **source 1..9** mol_type=protein organism=synthetic construct	
	NonEnglishQualifier Value		
3-26-5	Residues	LQYDEFPLT	9