ANTI-VEGF AND PD-1 BISPECIFIC ANTIBODIES AND USES THEREOF

FIELD OF THE INVENTION

[0001] The present invention relates bispecific antibodies containing an anti-VEGF binding region conjugated to an anti-PD-1 binding region and uses thereof for treating cancer.

BACKGROUND OF THE INVENTION

[0002] Angiogenesis generates new blood vessels by budding or dividing them from existing ones (capillaries, small arteries and veins). This mechanism is both beneficial and essential for maintaining many normal physiological processes, such as embryonic development, wound healing, and repair. On the other hand, excessive blood vessel proliferation or angiogenesis is also associated with pathological processes, such as tumor growth, metastasis, and inflammation. The key reason for *in vivo* proliferation of blood vessels is the ability of endothelial cells to excessively divide and proliferate and to insert into the existing vascular wall. Vascular endothelial growth factor ("VEGF") is the most important and potent angiogenic factor in vascular endothelial cell growth. The importance of VEGF in angiogenesis has been well demonstrated by studies in VEGF knock-out mice. Mouse embryos carrying one wild-type copy of VEGF gene while the other copy is knocked-out died at 11 to 12 days of development due to a decrease and abnormalities in vascular angiogenesis (Carmeliet P et. al., Nature 1996, 380:435; Ferrara N et. al., Nature 1996, 380:439).

[0003] Overexpression of VEGF has been observed in a variety of malignancies, such as colorectal, stomach, ovarian, breast cancers, hepatocellular carcinoma and multiple myeloma. The level of VEGF expression is highly correlated with tumor growth, relapse and metastasis (Dvorak H F et. al: J Exp Med 1991; 174:1275-8; Brown L F et. al., Cancer Res 1993; 53:4727-35; Weidner N, Semple J P, Welch W R and Folkman J: N Engl J Med 1991; 324:1-8). In recent years, accumulating data from a series of animal experiments have shown that blocking angiogenesis by inhibiting the interaction of VEGF and VEGF-R via gene manipulation or administration of drugs leads to tumor ischemia and necrosis, which in turn results in inhibition of tumor growth, metastasis and, ultimately, prolongation of overall survival. Therefore, drug development targeting VEGF mediated angiogenesis has become a hot area of research worldwide.

[0004] There are currently two major approaches in developing anti-angiogenesis drugs that target the VEGF and VEGF-R pathway. The first involves an inhibitor that antagonizes the tyrosine kinase located in the intracellular domain of the VEGF receptor ("VEGF-R"). Such antagonistic inhibitors are generally small-molecule chemical drugs, with the prototypical drugs being SUTENT® (Sunitinib), which was developed and marketed by Pfizer, and NEXAVAR® (Sorafenib), which was developed and marketed by Bayer (Germany) and Onyx Pharmaceuticals.

[0005] The second approach involves large protein molecules, either an antibody or a fusion protein employing the Fc receptor of an antibody, which can directly block the binding of VEGF to VEGF-R. One drug developed via this approach is AVASTIN® (Bevacizumab), a humanized anti-VEGF monoclonal antibody drug which was developed and produced by Roche/Genentech and obtained FDA approval in February 2004. AVASTIN® (Bevacizumab or its biosimilars) is so far the only anti-VEGF monoclonal antibody available on the global market. AVASTIN®, by highly specific binding to VEGF, prevents VEGF from binding to VEGF-R, and thus blocks angiogenesis and inhibits tumor proliferation (Presta L G et. al., Cancer Res, 1997, 57: 4593; Hurwitz H et. al., N Engl J Med, 2004; 350:2335). AVASTIN® (Bevacizumab) has been approved by the FDA for treatment of metastatic colorectal cancer ("mCRC"), advanced non-squamous non-small cell lung cancer ("NSCLC"), glioblastoma, metastatic renal cell carcinoma ("mRCC"), and various other solid tumors. AVASTIN® (Bevacizumab) also received approval from China SFDA in February 2010 for the treatment of colon cancer.

[0006] However, AVASTIN® (Bevacizumab) still has the following shortcomings: i) similar to most other monoclonal antibodies, it can only bind to a part of VEGF region or epitope but cannot bind to other epitopes or cover other areas of VEGF antigen; and ii) previous animal experiments and recent clinic research have shown that administering AVASTIN® (Bevacizumab) alone was not able to neutralize VEGF entirely, or completely inhibit VEGF mediated angiogenesis *in vivo*. Therefore, there is a need in the art for new monoclonal antibodies or other therapeutic agents that can bind to new binding sites on VEGF and at the same time inhibit the binding of VEGF to VEGF-R.

[0007] In addition, it has long been believed that tumor formation and development are closely related to the functional status of the host immune system. Normally, the host immune system plays the role of immune surveillance by surveilling the growth of mutated tumor cells and

inhibiting tumor metastasis and reoccurrence. However, in case of the loss of function or suppression of the host immune system, tumor metastasis and recurrence accelerate and may be life-threatening in severe cases. Therefore, tumor immunotherapy, which attacks or kills tumor directly by mobilizing the host immune system, is one of the goals being pursued in the clinic. Since 2011, a string of major breakthroughs in immunotherapy have revolutionized cancer treatment (Topalian S L et al, "Cancer immunotherapy comes of age," JCO 2011; 29:4828-4836; Pardoll D & Drake C, "Immunotherapy earns its spot in the ranks of cancer therapy," J Exp Med 2012; 209: 201-209; Page D B, Postow M A, Callahan M K, Allison J P and Wolchok J D, "Immune modulation in cancer with antibodies," Annu Rev Med 2014; 65:185-202). These revolutionary breakthroughs mainly result from progress in basic immunological research, as well as the advent and development of modern biotechnology represented by hybridomas, genetically engineered antibodies, and the like.

[0008] Basic immunological research shows that cellular immunity mediated by T lymphocytes is extremely crucial in surveilling and/or directly attacking and killing off cancer cells. T lymphocytes can be broadly separated into two categories: T helper cells, which mainly regulate and control immune function, and cytotoxic T cells ("CTL"), which engage in recognizing target antigens and directly attack and kill target cells.

[0009] Full activation and proliferation of helper T cells or CTLs generally require a synergy of two signal pathways. The first signal is antigen specific and is mediated by the interaction between T-cell receptor ("TcR") expressed on T cells and antigen peptide-MHC (major histocompatibility complex) expressed on target cells or antigen-presenting cells ("APC"). The second signal is antigen non-specific and is mediated by the interaction between co-stimulatory molecules or co-inhibitory molecules expressed on T-cells and their corresponding ligands expressed on target cells or APCs.

[0010] Co-stimulatory molecules, which up-regulate immune response mainly include CD28 and its ligand B7-1 ("CD80") or B7-2 ("CD86"), CD40 and its ligand CD40L, CD137 (also called 4-1BB) and its ligand CD137-L, and CD278 ("ICOS", Inducible T-cell costimulatory) and its ligand ICOS-L. These co-inhibitory molecules, which are also called immune checkpoint inhibitors, down-regulate immune response and mainly include CTLA-4 (Cytotoxic T-lymphocyte Antigen-4) and its ligand B7-1 (CD80) or B7-2 (CD86), PD-1 (programmed death-1) and its ligand PD-L1 or PD-L2, LAG-3 (Lymphocyte activation gene-3) and its ligand, TIM-3

(T-cell Immunoglobulin domain and Mucin domain 3) and its ligand, BTLA (B and T Lymphocyte Attenuator). These co-stimulatory/co-inhibitory molecules are very similar in structure and most of them are the members of immunoglobulin superfamily (Chen L P: Co-inhibitory molecules of the B7-CD28 family in the control of T cell immunity. Nature Immunol 2004, 336-347).

[0011] In principle, there are at least two different approaches to up-regulate the immune response. The first approach is to directly up-regulate T-cell function by increasing the expression or function of co-stimulatory molecules such as CD28 on T cells. The second approach is to indirectly up-regulate T cell function by releasing immunosuppression mediated by co-inhibitory molecules such as CTLA-4, PD-1/PD-L1, TIM-3, LAG-3, BTLA or other factors. Up-regulation of the immune response by the first approach can be achieved by using agonistic antibodies that bind to CD28 or other co-stimulatory molecules.

[0012] However, it is widely believed in the medical field that the agonist antibodies such as anti-CD28 antibodies have a high safety risk. This was seen in a Phase 1 study of an anti-CD28 monoclonal antibody drug (code: TGN1412) in England in 2006 in which six healthy subjects had extremely severe adverse reactions to the drug on the day of infusion. (Suntharalingam G, et al, N Engl J Med 2006; 355: 1018-1028). In contrast, antagonistic monoclonal antibody drugs that eliminate or reduce the immunosuppression mediated by CTLA-4 or PD-1/PD-L1 and other factors have been the most successful anti-tumor drugs world-wide, owing to the distinctive anti-tumor curative effect and acceptable safety shown in multiple international clinic studies. The development of anti-tumor drugs that target PD-1/PD-L1 has been particularly remarkable (Quezada S A and Peggs K S: British Journal of Cancer 2013; 108: 1560-1565; Flemming A: Nat Rev Drug Discov. 2012, 11:601).

[0013] The PD-1 gene was first discovered and cloned by Tasuku Honjo and his colleagues in 1992, and it has one IgV-like domain in the extracellular region thereof with 23% homology to CTLA-4 (Ishida, Y., Agata, Y, Shibahara, K. and Honjo, T.: EMBO J. 1992; 11:3887).

[0014] PD-1 is mainly expressed on activated T lymphocytes, B lymphocytes, mononuclear and other immune cells (Yasutoshi Agata et al, International Immunology 1996; 8: 675). There are two receptors or ligands of PD-1: PD-L1 (Freeman G J et al, JEM 2000; 192: 1027-1034), also known as B7-H1 (Dong H et al, Nature Medicine 1999; 5: 1365-1369), and PD-L2 (Latchman Y et al, Nat. Immunol. 2001; 2: 261-268), also known as B7-DC (Tseng S Y et al, JEM 2001; 193:

839-845). PD-L1 and PD-L2 are mainly expressed on target cells such as tumor cells or APCs (Thompson R H et al, Cancer Res 2006; 66: 3881-3885).

[0015] The phenomenon of PD-1 participating in the down-regulation of *in vivo* immunologic function was first observed in PD-1 knockout mice. It was found that knocking out PD-1 in C57BL/6 mice led to the development of lupoid glomerulonephritis and arthritis (Nishimura H et al: Immunity 1999; 11:141), while knocking it out in Balb/c mice caused the formation of high titer, anti-cardiac muscle tissue antibodies, thereby causing severe autoimmune cardiomyopathy (Nishimura H. et al: Science 2001; 291:319). Expressing PD-L1 or PD-L2 in normal tissues or cells *in vivo* prevent the cells from being attacked, killed and rejected by peripheral lymphocytes (Keir M. E. et al: J Exp Med 2006; 203: 883-895; Keir M E, Butte M J, Freeman G J, Sharpe A H: Annu Rev Immunol 2008; 26: 677-704).

[0016] Unfortunately, mutated tumor cells can also up-regulate the expression of PD-L1 or PD-L2, which bind to PD-1 on lymphocytes and inhibit their function, allowing them to keep growing by evading immune attack, killing, and rejection (Dong H et al, Nature Medicine 2002; 8: 793-800; Azuma T et al, Blood 2008; 111: 3635-3643). Blocking the binding of PD-1 on lymphocytes to PD-L1/PD-L2 on tumor cells can restore the function of lymphocytes to immunologically recognize and kill mutated tumor cells, thus suppressing tumor growth and potentially eradicating or rejecting tumor cells (Iwai Y et al, PNAS 2002; 99: 1229; Hirano F et al, Cancer Res 2005; 65: 1089-1096).

[0017] Because the binding of PD-1 to its ligand (PD-L1 or PD-L2) is characterized by the extensive binding domain involved, with more than dozens of amino acid sites engaging in the binding, and because the human PD-1 protein only shares 60% sequence identity with its mouse homolog, it is speculated that novel anti-PD-1 monoclonal antibodies could be developed with specific binding domains/different epitopes by using traditional mouse immunization and hybridoma technology. These new anti-PD-1 monoclonal antibodies, with their unique antigen binding domains or epitopes, are expected to have stronger *in vitro* and *in vivo* biological activity, and a safer and more superior curative effect than the currently marketed PD-1 monoclonal antibodies, OPDIVO® (Nivolumab) or KEYTRUDA® (Pembrolizumab). On one hand, these new anti-PD-1 antibodies can be used as pharmaceutical ingredients, either in combination or sequence with other marketed PD-1 monoclonal antibody drugs or PD-L1 monoclonal antibody drugs, to further enhance host immunologic function and anti-tumor

effects. On the other hand, these new anti-PD-1 antibodies are expected to be developed into novel immune function enhancers or used alone as anti-tumor pharmaceutical preparations.

[0018] Accordingly, there is a need in the art for improved ways of identifying and treating cancers mediated by both VEGF and PD-1 utilizing a bispecific antibody containing both anti-VEGF and anti-PD-1 binding regions.

SUMMARY OF THE INVENTION

[0019] Provided herein is a bispecific binding protein, which may comprise one or more anti-VEGF binding regions and one or more anti-PD-1 binding regions. The bispecific binding protein may be in a Coloma and Morrison-type IgG-single chain fragment variable (scFv) tetravalent format. The anti-VEGF binding region may comprise (a) an anti-VEGF light chain comprising an anti-VEGF light chain variable region and an anti-VEGF light chain constant region; and (b) an anti-VEGF heavy chain comprising an anti-VEGF heavy chain variable region and an anti-VEGF heavy chain constant region.

[0020] The anti-VEGF light chain variable region may comprise a complementarity determining region (CDR1) comprising the sequence set forth in SEQ ID NO: 8, a CDR2 comprising the sequence set forth in SEQ ID NO: 9, and a CDR3 comprising the sequence set forth in SEQ ID NO: 10. The anti-VEGF light chain variable region may comprise the sequence set forth in SEQ ID NO: 2. The anti-VEGF light chain may comprise a human Kappa light chain constant region. The human Kappa light chain constant region may comprise the sequence set forth in SEQ ID NO: 52.

[0021] The anti-VEGF heavy chain variable region may comprise a CDR1 comprising the sequence set forth in SEQ ID NO: 5, a CDR2 comprising the sequence set forth in SEQ ID NO: 6, and a CDR3 comprising the sequence set forth in SEQ ID NO: 7. The anti-VEGF heavy chain variable region may comprise the sequence set forth in SEQ ID NO: 1. The anti-VEGF heavy chain constant region may comprise a human IgG1 heavy chain constant region. The human IgG1 heavy chain constant region may comprise the sequence set forth in SEQ ID NO: 51. The human IgG1 heavy chain constant region may comprise Fc-silent mutations, which may comprise L234A, L235A, and G237A substitutions. The human IgG1 heavy chain constant region may comprise the sequence set forth in SEQ ID NO: 69. The human IgG1 heavy chain constant region may further comprise FcRn binding enhancing mutations. The FcRn binding

enhancing mutations may comprise M428L and N434S substitutions. The substitutions may have positions in the human IgG1 heavy chain constant region relative to the EU index. The human IgG1 heavy chain constant region may comprise the sequences set forth in SEQ ID NO: 70.

[0022] The anti-PD-1 binding region may comprise an anti-PD-1 heavy chain variable region and an anti-PD-1 light chain variable region. A scFv may comprise the anti-PD-1 binding region. The anti-PD-1 heavy chain variable region may comprise a CDR1 comprising the sequence set forth in SEQ ID NO: 21, a CDR2 comprising the sequence set forth in SEQ ID NO: 22, and a CDR3 comprising the sequence set forth in SEQ ID NO: 23. The anti-PD-1 heavy chain variable region may comprise the sequence set forth in SEQ ID NO: 17. The anti-PD-1 light chain variable region may comprise a CDR1 comprising the sequence set forth in SEQ ID NO: 24, a CDR2 comprising the sequence set forth in SEQ ID NO: 25, and a CDR3 comprising the sequence set forth in SEQ ID NO: 26. The anti-PD-1 light chain variable region may comprise the sequence set forth in SEQ ID NO: 18.

[0023] The scFv may comprise a first linker linking the anti-PD-1 heavy chain variable region to the anti-PD-1 light chain variable region. The first linker may comprise the sequence set forth in one of SEQ ID NOs: 55-58. The scFv may comprise, from N-terminus to C-terminus, the anti-PD-1 heavy chain variable region, the first linker comprising the sequence set forth in SEQ ID NO: 57, and the anti-PD-1 light chain variable region. The scFv may comprise, from N-terminus to C-terminus, the anti-PD-1 light chain variable region, the first linker comprising the sequence set forth in SEQ ID NO: 57, and the anti-PD-1 heavy chain variable region.

[0024] The scFv may further comprise one of the one or more anti-VEGF binding regions. The anti-VEGF binding region may comprise an anti-VEGF heavy chain comprising an anti-VEGF heavy chain variable region comprising the sequence set forth in SEQ ID NO: 1 and a heavy chain constant region comprising the sequence set forth in SEQ ID NO: 51, 69, or 70. The C-terminus of the anti-VEGF heavy chain may be linked to the N-terminus of the anti-PD-1 binding region via a second linker. The second linker may comprise the sequence set forth in SEQ ID NO: 58. The anti-PD-1 heavy chain variable region may comprise the sequence set forth in SEQ ID NO: 53 and the anti-PD-1 light chain variable region may comprise the sequence set forth in SEQ ID NO: 54. The scFv may comprise a first linker linking the anti-PD-1 heavy chain variable region to the anti-PD-1 light chain variable region. The first linker may comprise the sequence set forth in one of SEQ ID NOs: 55-58.

[0025] The scFv may comprise, from N-terminus to C-terminus, the anti-PD-1 heavy chain variable region, the first linker comprising the sequence set forth in SEQ ID NO: 57, and the anti-PD-1 light chain variable region. The scFv may comprise, from N-terminus to C-terminus, the anti-PD-1 light chain variable region, the first linker comprising the sequence set forth in SEQ ID NO: 57, and the anti-PD-1 heavy chain variable region. The scFv may further comprise one of the one or more anti-VEGF binding regions. The anti-VEGF binding region may comprise an anti-VEGF heavy chain comprising an anti-VEGF heavy chain variable region comprising the sequence set forth in SEQ ID NO: 1 and a heavy chain constant region comprising the sequence set forth in SEQ ID NO: 51, 69, or 70. The C-terminus of the anti-VEGF heavy chain may be linked to the N-terminus of the anti-PD-1 binding region via a second linker. The second linker may comprise the sequence set forth in SEQ ID NO: 58. The scFv may comprise the sequence set forth in one of SEQ ID NOs: 59, 60, 71, 72, or 73. The bispecific binding protein may comprise an anti-VEGF light chain comprising an anti-VEGF variable light chain region comprising the sequence set forth in SEQ ID NO: 2 fused to a light chain constant region comprising the sequence set forth in SEQ ID NO: 52. The anti-VEGF light chain may complex with the scFv, which may be via the anti-VEGF heavy chain in the scFv.

[0026] Provided herein is a bispecific binding protein comprising one or more anti-VEGF binding regions and one or more anti-PD-1 binding regions. The bispecific binding protein may comprise a single chain variable fragment (scFv) comprising an anti-VEGF binding region and an anti-PD-1 binding region, and an anti-VEGF light chain. The anti-VEGF light chain may complex with the scFv. The scFv may comprise an anti-VEGF heavy chain region, a first linker, an anti-PD-1 heavy chain variable region, a second linker, and an anti-PD-1 light chain region.

[0027] The anti-VEGF heavy chain region may comprise an anti-VEGF heavy chain variable region comprising the sequence set forth in SEQ ID NO: 3 linked via its C-terminus to a heavy chain constant region comprising the sequence set forth in SEQ ID NO: 51. The first linker may comprise the sequence set forth in SEQ ID NO: 57, wherein the first linker connects a C-terminus of the anti-VEGF heavy chain region to the anti-PD-1 heavy chain variable region. The anti-PD-1 heavy chain variable region may comprise the sequence set forth in SEQ ID NO: 54. The second linker may comprise the sequence set forth in SEQ ID NO: 58. The second linker may connect a C-terminus of the anti-PD-1 heavy chain variable region to the anti-PD-1 light chain region. The anti-PD-1 light chain region may comprise the sequence set forth in SEQ ID

NO: 53. The scFv may comprise the sequence set forth in SEQ ID NO: 61. The anti-VEGF light chain may comprise an anti-VEGF light chain variable region comprising the sequence set forth in SEQ ID NO: 4 connected via its C-terminus to a light chain constant region comprising the sequence set forth in SEQ ID NO: 52. The anti-VEGF light chain may complex with the scFv, which may be via the anti-VEGF heavy chain in the scFv.

[0028] Provided herein is a pharmaceutical composition comprising the bispecific binding protein and a pharmaceutically acceptable excipient. The pharmaceutical composition may be suitable for intravenous or subcutaneous injection. The pharmaceutical composition may comprise 10-30 mg/mL or 20 mg/mL of the bispecific binding protein. The pharmaceutical composition may comprise one or more of histidine buffer, trehalose dihydrate, and polysorbate 80. The pharmaceutical composition may comprise 10-30 mM histidine buffer, 6-12% (w/v) trehalose dihydrate, and 0.02-0.06% (w/v) polysorbate 80. The pharmaceutical composition may comprise about 20 mM histidine buffer, about 9% (w/v) trehalose dihydrate, and about 0.04% (w/v) polysorbate 80. The pharmaceutical composition may have a pH of 5-6 or about 5.5.

[0029] Provided herein is a method of treating a cancer in a subject in need thereof. The method may comprise administering the bispecific binding protein or pharmaceutical composition to the subject. A therapeutically effective amount of the bispecific binding protein may be administered, which may be about 0.3-25 mg/kg, 0.3-20 mg/kg, 0.3 mg/kg, 1 mg/kg, 3 mg/kg, 10 mg/kg, or 20 mg/kg. Also provided are the bispecific binding protein for use in treating cancer; the pharmaceutical composition comprising the bispecific binding protein for treating cancer; and use of the bispecific binding protein or pharmaceutical composition in the manufacture of a medicament for treating cancer. The cancer may be lung cancer, ovarian cancer, breast cancer, liver cancer, brain cancer, cervical cancer, pancreatic cancer, renal cancer, testicular cancer, prostate cancer, neuroblastoma, mantle cell lymphoma, or a hematological malignancy.

[0030] The cancer may be a solid tumor, which may be colorectal cancer or a lung cancer. The colorectal cancer may be one or more of micro-satellite stable and proficient mismatch repair. The lung cancer may be a non-small cell lung cancer (NSCLC) or a small cell lung cancer (SCLC). The NSLC may be PD-L1+. The SCLC may be extensive-stage.

[0031] The bispecific binding protein or pharmaceutical composition may be administered intravenously or subcutaneously. The bispecific binding protein, pharmaceutical composition, or medicament may be for intravenous or subcutaneous administration. The bispecific binding

protein or pharmaceutical composition may be administered intravenously at a dose of 0.3-25 mg/kg, 0.3-20 mg/kg, 0.3 mg/kg, 1 mg/kg, 3 mg/kg, 10 mg/kg, or 20 mg/kg. The intravenous administration may be performed over a minimum of 60 or 120 minutes, such as a minimum of 60 minutes when the bispecific binding protein dose is no more than 10 mg/kg and a minimum of 120 minutes when the bispecific binding protein dose is at least 10 mg/kg, such as 20 mg/kg. The bispecific binding protein or pharmaceutical composition may be administered every 1, 2, 3, or 4 weeks.

DESCRIPTION OF THE DRAWINGS

[0032] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office by request and payment of the necessary fee.

[0033] FIG. 1A-C show the basic design and schematic structure of three bispecific antibodies: hPV19-derived VPD1-HL (FIG. 1A), hPV19-derived VPD1-LH (FIG. 1B), and Bevacizumab-derived VPD2-HL (FIG. 1C), all in the Coloma and Morrison type of IgG-single chain fragment variable (scFv) tetravalent format.

[0034] FIG. 2A-B show the DNA constructs and molecules of bispecific antibodies: VPD1-HL and VPD1-LH (FIG. 2A), and VPD2-HL (FIG. 2B). The structures of these, including the order and number of GGGGS (SEQ ID NO: 55) units in the flexible linkers used in the scFv and bispecific antibody-heavy chain are described in the table below.

Table 1

Molecule Name	**Source of Light chain**	**Source of Heavy chain (IgG1-backbone)**	**No. of G4S units (connector)**	**Source of anti-PD-1 (scFv)***	**Orientation of scFv**	**No. of G4S units (linker)**
VPD1-HL	hPV19	hPV19	3	AI-025	VH-G4S-VL	4
VPD1-LH	hPV19	hPV19	3	AI-025	VL-G4S-VH	4
VPD2-HL	Bevacizumab	Bevacizumab	3	AI-025	VH-G4S-VL	4
VPD2-LH	Bevacizumab	Bevacizumab	3	AI-025	VL-G4S-VH	4
*All scFv have disulfide modifications VH44cys and VL100cys as described herein.						

[0035] FIG. 3A and 3B show alignments of the amino acid sequences of the heavy chain variable region (VH) and light chain variable region (VL), respectively, of hPV19 (VH: SEQ ID NO: 1; VL: SEQ ID NO: 2) with those of Bevacizumab (VH: SEQ ID NO: 3; VL: SEQ ID NO: 4).

[0036] FIG. 4 shows an alignment of the VH sequence of hAB21 with other major anti-PD-1 antibodies.

[0037] FIG. 5 shows an alignment of the VL sequence of hAB21 with other major anti-PD-1 antibodies.

[0038] FIG. 6A and 6B show an alignment of the amino acid sequences of the VH and VL, respectively, of hAB21 (VH: SEQ ID NO: 17; VL: SEQ ID NO: 18) with that of Penpulimab (VH: SEQ ID NO: 39; VL: SEQ ID NO: 48).

[0039] FIG. 7A-C show SEC-HPLC analysis of VPD1-HL (FIG. 7A), VPD2-LH (FIG. 7B), and VPD2-HL (FIG. 7C).

[0040] FIG. 8 shows the binding of VPD1 or VPD2 to human VEGF165 antigen in an indirect ELISA test. The data are shown in the table below.

Table 2

Concentration (μg/ml)	**VPD2-HL**	**VPD1-HL**	**VPD1-LH**	**Avastin**	**hPV19**	**ONC392**
10	1.449	1.533	1.475	1.636	1.606	0.046
5	1.492	1.449	1.461	1.655	1.754	0.045
2.5	1.444	1.257	1.226	1.557	1.684	0.046
1.25	0.969	0.908	0.863	1.52	1.418	0.044
0.625	0.661	0.567	0.523	1.413	1.104	0.044
0.315	0.429	0.371	0.358	1.032	0.742	0.045
0.156	0.281	0.203	0.178	0.782	0.532	0.04
0.078	0.174	0.116	0.118	0.500	0.300	0.042
0.039	0.119	0.079	0.085	0.313	0.171	0.043
0.019	0.077	0.063	0.06	0.189	0.108	0.047
0.009	0.059	0.049	0.053	0.119	0.077	0.043
0.005	0.049	0.047	0.047	0.079	0.057	0.042
EC50 (μg/mL)	0.803	1.073	1.108	0.190	0.398	NA
EC50 (nmol/mL)	4.02	5.37	5.54	1.27	2.65	NA

[0041] FIG. 9 shows the binding of VPD1 or VPD2 to human PD-1-his antigen in an indirect ELISA test. The data are shown in the table below.

Table 3

Concentration (µg/ml)	VPD2-HL	VPD1-HL	VPD1-LH	hAB21	Nivolumab	Pembro	ONC392
10	1.133	1.218	1.057	1.161	0.753	1.032	0.043
5	1.026	1.039	1.058	1.363	1.011	1.089	0.046
2.5	0.910	0.904	0.946	1.283	0.916	1.102	0.046
1.25	0.730	0.755	0.853	1.149	0.900	0.965	0.049
0.625	0.538	0.588	0.637	1.035	0.789	0.947	0.045
0.312	0.358	0.332	0.413	0.819	0.737	0.759	0.044
0.156	0.196	0.195	0.244	0.560	0.506	0.555	0.045
0.078	0.126	0.146	0.157	0.368	0.324	0.343	0.045
0.039	0.092	0.094	0.095	0.203	0.202	0.199	0.049
0.019	0.065	0.066	0.073	0.118	0.122	0.119	0.044
0.009	0.052	0.053	0.054	0.077	0.080	0.082	0.042
0.005	0.047	0.049	0.048	0.060	0.059	0.059	0.042
EC50 (µg/mL)	0.869	1.018	0.507	0.204	0.135	0.162	NA
EC50 (nmol/mL)	4.35	5.09	2.54	1.36	0.90	1.08	NA

[0042] FIG. 10A shows a diagram of sandwich ELISA with either VPD1 or VPD2 simultaneously binding to human VEGF and PD-1 antigens with a plate pre-coated with the human PD-1 antigen.

[0043] FIG. 10B shows the results of VPD1 or VPD2 binding to the human VEGF and PD-1 antigens tested using the sandwich ELISA diagrammed in FIG. 10A. The data are shown in the table below.

Table 4

Antibody Concentration	hPV19	hAB21	ONC392	VPD1 HL	VPD1 LH	VPD2 HL
10	0.077	0.055	0.059	1.662	1.694	1.651
5	0.074	0.056	0.057	1.550	1.636	1.566
2.5	0.069	0.053	0.059	1.378	1.579	1.513
1.25	0.068	0.055	0.058	1.115	1.376	1.328
0.625	0.069	0.052	0.055	0.817	1.065	1.124
0.312	0.064	0.063	0.055	0.592	0.808	0.810
0.156	0.065	0.054	0.052	0.393	0.554	0.554
0.078	0.062	0.058	0.054	0.266	0.369	0.391
0.039	0.061	0.056	0.055	0.176	0.238	0.274
0.019	0.063	0.055	0.052	0.120	0.161	0.163
0.009	0.007	0.058	0.057	0.087	0.112	0.116
0	0.087	0.064	0.063	0.058	0.059	0.006

[0044] FIG. 11A shows a diagram of sandwich ELISA with either VPD1 or VPD2 simultaneously binding to human VEGF and PD-1 antigens with a 96-well plate pre-coated with the human VEGF165 antigen.

[0045] FIG. 11B shows the results of VPD1 or VPD2 binding to the human VEGF and PD-1 antigens using the sandwich ELISA diagrammed in FIG. 11A. The data are shown in the table below.

Table 5

Antibody concentration	VPD1-HL	VPD1-LH	VPD2-HL	Avastin	hAB21	hPV19	ONC392
10	2.150	2.674	2.689	0.275	0.302	0.116	0.167
5	2.492	2.553	2.618	0.226	0.240	0.112	0.144
2.5	2.265	2.257	2.463	0.214	0.234	0.124	0.154
1.25	1.565	1.771	2.188	0.233	0.255	0.161	0.154
0.625	1.395	1.261	1.654	0.226	0.207	0.151	0.135
0.312	0.435	0.458	0.711	0.148	0.165	0.135	0.139
0.156	0.417	0.383	0.502	0.158	0.142	0.121	0.109
0.078	0.261	0.276	0.356	0.155	0.134	0.109	0.109
0.039	0.212	0.219	0.257	0.151	0.130	0.107	0.113
0.019	0.175	0.167	0.202	0.141	0.116	0.103	0.109
0.009	0.153	0.150	0.159	0.137	0.114	0.105	0.117
0	0.084	0.116	0.122	0.138	0.112	0.11	0.099

[0046]

[0047] FIG. 12 shows binding curves and affinity of VPD1-HL, VPD2-HL and Avastin to human VEGF165 antigen in a Fortebio assay. The data are shown in the table below.

Table 6

Sample ID	ka (1/Ms)	kd (1/s)	KD (M)	KD ratio
VPD1-HL	7.25E+5	6.54E-5	9.03E-11	-
VPD2-HL	3.47E+5	4.87E-4	1.40E-9	15.53
Avastin	2.69E+5	3.40E-4	1.26E-9	13.99

[0048] FIG. 13 shows binding curves and affinity of VPD1-HL, VPD2-HL and hAB21 to human PD-1 antigen in a Fortebio assay. The data are shown below.

Table 7

Sample ID	ka (1/Ms)	kd (1/s)	KD (M)	KD ratio
VPD1-HL	1.75E+5	2.76E-4	1.58E-9	-
VPD2-HL	2.04E+5	1.08E-3	5.28E-9	3.35
hAB21	2.49E+5	1.16E-3	4.67E-9	2.97

[0049] FIG 14 shows the activity of VPD1-HL or VPD1-LH in blocking PD-1/PD-L1 interaction in a competitive ELISA test. The data are shown in the table below.

Table 8

Concent.	OD at 492nm					
(μg/mL)	VPD1-HL	VPD1-LH	hAB21	Pembro	Opdivo	ONC392
5	0.080	0.081	0.043	0.046	0.043	0.368
2.5	0.142	0.148	0.043	0.049	0.046	0.343
1.25	0.234	0.224	0.057	0.052	0.063	0.350
0.625	0.301	0.288	0.112	0.115	0.142	0.351
0.312	0.327	0.331	0.205	0.200	0.218	0.358
0.156	0.346	0.360	0.264	0.256	0.293	0.367
0.078	0.343	0.343	0.288	0.283	0.300	0.330
0.039	0.346	0.345	0.293	0.303	0.308	0.336
0.019	0.347	0.351	0.321	0.307	0.312	0.325
0.009	0.360	0.345	0.325	0.311	0.319	0.331
0.004	0.349	0.358	0.319	0.309	0.320	0.343
0.002	0.365	0.359	0.299	0.304	0.322	0.337
IC50 (μg/mL)	1.73	1.64	0.37	0.37	0.45	NA
IC50 (nM)	8.65	8.20	2.47	2.47	3.00	NA

[0050] FIG. 15 shows the activity of VPD1-HL or VPD1-LH in blocking VEGF/VEGFR1 interaction in a competitive ELISA test. The data are shown in the table below.

Table 9

Concentration								
(μg/mL)	Avastin	hPV19	ONC-392	VPD1-HL	VPD1-LH	VPD2-HL	Aflibercept	VPD3
100	0.086	0.205	1.429	0.048	0.050	0.065	0.049	0.154
50	0.083	0.072	1.410	0.053	0.065	0.088	0.050	0.345
25	0.086	0.074	1.358	0.066	0.066	0.116	0.055	0.571
12.5	0.101	0.088	1.270	0.088	0.092	0.226	0.071	0.951
6.25	0.164	0.101	1.299	0.165	0.171	0.454	0.080	1.120
3.125	0.422	0.199	1.359	0.435	0.460	0.785	0.112	1.255
1.562	0.714	0.487	1.318	0.876	0.909	1.047	0.173	1.241
0.781	0.842	0.965	1.282	1.139	1.134	1.173	0.504	1.237
0.390	0.872	1.124	1.241	1.207	1.188	1.137	0.976	1.217
0.195	1.079	1.217	1.254	1.238	1.251	1.169	1.118	1.206
0.097	1.132	1.278	1.210	1.234	1.214	1.177	1.152	1.209
0.048	1.146	1.300	1.278	1.219	1.209	1.235	1.166	1.301
IC50 (μg/mL)	1.78	1.16	NA	2.26	2.38	4.36	0.68	21.49
IC50 (nM)	11.87	7.77	NA	11.30	11.91	21.82	6.80	107.45

[0051] FIG. 16 shows the activity of VPD1-HL or VPD1-LH in blocking VEGF/VEGFR2 interaction in a competitive ELISA test. The data are shown in the table below.

Table 10

Abs Concent.	Avastin	hPV19	ONC392	VPD1-HL	VPD1-LH	VPD2-HL	Aflibercept	VPD3
100	0.057	0.047	1.079	0.046	0.043	0.046	0.043	0.073
50	0.045	0.070	1.124	0.046	0.044	0.05	0.042	0.091
25	0.047	0.051	1.040	0.045	0.044	0.049	0.047	0.246
12.5	0.051	0.043	1.032	0.046	0.047	0.065	0.044	0.513
6.25	0.062	0.044	0.998	0.056	0.059	0.164	0.044	0.816
3.125	0.161	0.063	1.129	0.176	0.188	0.385	0.049	0.896
1.565	0.417	0.206	1.013	0.611	0.633	0.718	0.061	0.855
0.781	0.695	0.699	1.018	0.867	0.871	0.866	0.279	0.873
0.390	0.810	0.847	0.972	0.935	0.915	0.885	0.752	0.832
0.195	0.956	0.924	0.930	0.982	0.100	0.887	0.837	0.832
0.097	1.035	0.944	0.907	0.922	0.857	0.820	0.867	0.797
IC50 (μg/mL)	1.15	1.04	NA	1.89	1.95	2.81	0.63	14.60
IC50 (nM)	7.69	6.90	NA	9.50	9.80	14.00	6.30	73.00

[0052] FIG. 17A-B show schematic structures of VPD1-3A-HL and VPD1-3A-LH, respectively. Descriptions of the structures are provided in the table below.

Table 11

Molecule Name	**Anti-VEGF backbone (IgG1)**	**Mutations in IgG1-Fc region**	**No. of G4S unit connector**	**Source of anti-PD-1 (scFv)***	**Orientation of scFv**	**No. of G4S units (linker)**
VPD1-3A-LH	hPV19	L234A/L235A/G237A	3	AI-025	VL-G4S-VH	4
VPD1-3A-HL	hPV19	L234A/L235A/G237A	3	AI-025	VH-G4S-VL	4
*All scFv have disulfide modifications VH44cys and VL100cys as described herein.						

[0053] FIG. 18 shows the binding of VPD1-3A mutant (VPD1-3A-HL and VPD1-3A-LH) to human CD64 (FcγRI) in an ELISA test. The data are shown in the table below.

Table 12

Ab Conc.	**ONC-392**	**hAB21**	**VPD1-HL**	**VPD1-3A-HL**	**VPD1-3A-LH**	**VPD3**	**Avastin**	**hPV19**
30	0.415	0.062	0.471	0.106	0.073	0.072		0.457
10		0.061	0.444	0.082	0.063	0.059	0.455	0.433
3.333	0.37	0.057	0.441	0.067	0.064	0.055	0.429	0.466
1.111	0.362	0.055	0.383	0.063	0.061	0.053	0.384	0.469
0.370	0.312	0.05	0.314	0.06	0.059	0.048	0.294	0.376
0.123	0.242	0.047	0.212	0.066	0.055	0.044	0.201	0.281
0.041	0.149	0.044	0.131	0.065	0.05	0.043	0.127	0.186
0.013	0.094	0.043	0.079	0.047	0.047	0.045	0.083	0.106
0.004	0.079	0.044	0.057	0.045	0.059	0.046	0.057	0.068
0.001	0.081	0.043	0.047	0.044	0.045	0.043	0.05	0.061

[0054] FIG. 19 shows the binding of VPD1-HL, VPD1-3A-HL, VPD1-3A-LH, and VPD3 to human PD1 antigen in an ELISA test. The data are shown in the table below.

Table 13

Concentration of antibody(μg/ml)	hAB21	ONC-392	VPD3	VPD1-3A-HL	VPD1-3A-LH	VPD1-HL
10	0.936	0.088	0.747	0.656	0.748	0.797
5	0.866	0.062	0.685	0.746	0.718	0.781
2.5	0.837	0.052	0.581	0.739	0.719	0.801
1.25	0.733	0.045	0.54	0.584	0.671	0.606
0.625	0.72	0.043	0.389	0.535	0.632	0.542
0.3125	0.649	0.041	0.238	0.41	0.528	0.381
0.156	0.659	0.041	0.162	0.269	0.328	0.245
0.078	0.526	0.041	0.102	0.148	0.21	0.137
0.039	0.367	0.04	0.072	0.098	0.127	0.095
0.019	0.256	0.04	0.055	0.072	0.089	0.068
0.009	0.162	0.05	0.05	0.077	0.067	0.064
0.004	0.114	0.051	0.051	0.056	0.059	0.051
EC50 (μg/ml)	**0.021**	**NA**	**0.724**	**0.294**	**0.192**	**0.413**

[0055] FIG. 20 shows the bio-activity of VPD1-HL, VPD1-3A-HL, and VPD1-3A-LH in a cell-based VEGFR2-Luc reporter assay. The data are shown in the tables below.

Table 14

	VPD1-3A-HL	VPD1-3A-LH	VPD1-HL
IC50	10.86	9.847	11.72

Table 15

Avastin	VPD3	hPV19
66.21	47.55	9.803

[0056] FIG. 21 shows the bio-activity of VPD1-HL, VPD1-3A-HL, and VPD1-3A-LH in a cell-based PD-1-NFAT-Luc reporter assay. The data are shown in the table below.

Table 16

	hAB21	VPD1-3A-HL	VPD1-3A-LH	VPD3	VPD1-HL	Penpulimab	VPD1-LH	ONC392
60	26227	22362	27197	23524	25651	26075	26825	6752
20	27259	24354	25215	23212	26745	25641	26877	7776
6.666	26333	23018	23838	20378	24677	25383	25283	8378
2.222	24715	21076	22156	18847	23364	23370	23468	8630
0.740	23070	17681	18559	15145	18149	20386	19567	8378
0.246	22098	14479	15293	12870	14407	18949	16427	8670
0.082	17093	11226	11518	10788	11546	14217	12526	9068
0.027	11252	9138	9564	8398	9214	10764	10084	8478
0.009	9280	8020	8832	8662	8564	9484	9318	8650
0.003	9228	8504	8716	8290	8824	8724	8774	8716
EC50 (μg/mL)	**0.094**	**0.385**	**0.554**	**0.982**	**0.545**	**0.185**	**0.412**	**NA**

[0057] FIG. 22 shows the schematic structure of bispecific antibody VPD1-3A&LS-HL. Its structure is summarized in the table below.

Table 17

Molecule Name	**Anti-VEGF backbone (IgG1)**	**Mutations in IgG1-Fc region**	**No. of G4S unit connector**	**Source of anti-PD-1 (scFv)***	**Orientation of scFv**	**No. of G4S units (linker)**
VPD1-3A&LS-HL	hPV19	L234A/L235A/G237A; M428L/N434S	3	AI-025	VH-G4S-VL	4
*scFv has disulfide modifications VH44cys and VL100cys as described herein.						

[0058] FIG. 23 shows bio-activity of VPD1-3A-HL and VPD1-3A&LS-HL in a PD-1 reporter assay. The data are shown in the table below.

Table 18

Conc. (μg/mL)	hAB21	VPD1-3A	VPD1-LS	VPD3	ONC-392
60	69283	64712	69886	51817	19059
20	73867	73563	83016	72792	19835
6.667	73388	73557	71414	62175	21726
2.222	69987	63127	5619	58213	21760
0.741	66354	46181	47688	42914	20390
0.247	51432	36413	33282	31400	23826
0.083	37285	25719	28016	27002	23456
0.027	28024	24468	27151	26860	22048
0.009	25851	27411	27765	27077	25235
0.003	26358	26225	28535	25741	26689
EC50 (μg/mL)	0.199	0.755	0.929	0.791	NA
EC50 (nM)	1.33	3.78	4.64	3.96	NA

[0059] FIG. 24 shows activity of VPD1-3A-HL and VPD1-3A&LS-HL in blocking VEGF/VEGFR2 interaction in a blocking ELISA test. The data are shown in the table below.

Table 19

Block the binding of VEGF165 to VEGF-R2 (OD492nm)			
Conc. (μg/mL)	VPD1-3A	VPD3	VPD1-3A&LS
100	0.125	0.129	0.124
33.3	0.149	0.149	0.132
11.1	0.140	0.261	0.127
3.700	0.144	0.508	0.136
1.230	0.173	0.640	0.142
0.410	0.239	0.762	0.249
0.130	0.482	0.732	0.575
0.040	0.736	0.713	0.739
0.015	0.785	0.731	0.752
0.005	0.742	0.741	0.751
IC50 (μg/mL)	**0.15**	**5.14**	**0.20**
IC50 (nM)	**0.73**	**25.73**	**1.02**

[0060] FIG. 25A-B show the binding of VPD1-3A-HL and VPD1-3A&LS-HL to ELISA plate co-coated with human VEGF165 and human /PD-1 antigens, where FIG. 25A shows a schematic of the ELISA and FIG. 25B shows the results. The data in FIG. 25B are shown in the table below.

Table 20

Conc. (μg/mL)	ONC392	VPD1-3A	VPD3	VPD1-3A-LS	hAB21	hPV19
2	0.059	4.084	3.348	3.975	3.573	4.264
0.666	0.043	3.704	2.841	3.575	3.452	4.120
0.222	0.048	2.899	1.802	2.513	3.368	3.835
0.074	0.047	1.591	0.843	1.234	2.825	2.984
0.024	0.042	0.672	0.360	0.517	1.846	1.601
0.008	0.044	0.287	0.168	0.223	0.880	0.674
0.0027	0.043	0.135	0.090	0.110	0.401	0.288
0.0009	0.044	0.077	0.059	0.066	0.180	0.136
EC50 (μg/mL)	NA	0.118	0.231	0.161	0.024	0.039
EC50 (nM)	NA	0.59	1.15	0.80	0.16	0.26

[0061] FIG. 26A-C show the bio-activity of VPD1-3A-HL (FIG. 26A), VPD1-3A&LS-HL (FIG. 26B), and VPD3 (FIG. 26C) in a PD-1 cell reporter assay in the presence of human VEGF165.

[0062] FIG 27 shows VPD1-3A-HL *in vivo* anti-tumor activity in comparison to VPD2-HL in human A673 tumor/nude model mice. The dosing schedule is shown in the table below.

Table 21

Group	Antibodies	Dosage
1 (n=9)	PBS	_
2 (n=10)	VPD1-3A-HL	5 mg/kg, twice weekly
3 (n=10)	VPD2-HL	5 mg/kg, twice weekly

[0063] FIG. 28 shows additional VPD1-3A-HL *in vivo* anti-tumor activity in comparison to VPD2-HL in human A673 tumor/nude model mice.

[0064] FIG. 29 shows VPD1-3A-HL *in vivo* anti-tumor activity in comparison to hPV19, AI-025 or combination of hPV19 plus AI-025 in human A375 tumor/NCG-hPBMC model mice. The experiment groups are tabulated below.

Table 22

Group	Antibodies	Dosage
1 (n=10)	PBS	_
2 (n=10)	hPV19	5 mg/kg, twice weekly
3 (n=10)	AI-025	5 mg/kg, twice weekly
4 (n=10)	hPV19+AI-025	5 mg/kg each, twice weekly
5 (n=10)	VPD1-3A-HL	6.6 mg/kg, twice weekly

[0065] FIG. 30A shows a comparison of the *in vivo* anti-tumor activity of a 1.6 mg/kg dose of VPD1-3A-HL and VPD3 in human A375 tumor/NCG-hPBMC model mice.

[0066] FIG. 30B shows a comparison of the *in vivo* anti-tumor activity of a 6.6 mg/kg dose of VPD1-3A-HL and VPD3 in human A375 tumor / NCG-hPBMC model mice.

[0067] FIG. 31 shows a comparison of *in vivo* anti-tumor activity VPD1-3A-HL, VPD1-3A-LH, and VPD3 in MC38-hVEGF tumor in human PD-1 knock-in model mice.

[0068] FIG. 32 shows additional VPD1-3A-HL, VPD1-3A-LH, and VPD3 *in vivo* anti-tumor activity in MC38-hVEGF tumor in human PD-1 knock-in mice model. The dotted lines indicate tumor volume at 500 mm^3.

[0069] FIG. 33A-B illustrate a cell-based bio-potency assay with (FIG. 33A) and without (FIG. 33B) anti-PD-1 mAb.

[0070] FIG. 34 is a study diagram of AI-081 monotherapy dose escalation.

[0071] FIG. 35 is a study diagram of Phase 2 Part B Cohort B1 dose optimization for AI-081 monotherapy in 2L colorectal cancer.

[0072] FIG. 36 is a study diagram of Phase 2 Part B Cohort B2 dose optimization for AI-081 monotherapy in PD-L1+ NSCLC.

[0073] FIG. 37 is a study diagram of Phase 2 Part B Cohort B3 dose optimization for AI-081 plus chemotherapy combination therapy in advanced SCLC.

DETAILED DESCRIPTION

[0074] The present inventors have now discovered anti-VEGF/anti-PD-1 binding proteins in a bispecific antibody format that exhibit surprisingly improved activity.

1. Definitions

[0075] The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting. As used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. The word "about" in association with a numeric value denotes a reasonable approximation of that value. In certain cases, "about" may be construed as being within as much as 10% of the specific value with which it is associated. For example, the phrase "about 100" would encompass any value between 90 and 110.

[0076] For recitation of numeric ranges herein, each intervening number there between with the same degree of precision is explicitly contemplated. For example, for the range of 6-9, the numbers 7 and 8 are contemplated in addition to 6 and 9, and for the range 6.0-7.0, the numbers 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, and 7.0 are explicitly contemplated.

[0077] "Treatment" or "treating," when referring to protection of an animal from a disease, means preventing, suppressing, repressing, or completely eliminating the disease. Preventing the disease involves administering a composition of the disclosure to an animal prior to onset of the disease. Suppressing the disease involves administering a composition of the disclosure to an animal after induction of the disease but before its clinical appearance. Repressing the disease

involves administering a composition of the disclosure to an animal after clinical appearance of the disease.

[0078] As used herein, the term "antibody" is intended to denote an immunoglobulin molecule that possesses a "variable region" antigen recognition site. The term "variable region" is intended to distinguish such domain of the immunoglobulin from domains that are broadly shared by antibodies (such as an antibody Fc domain). The term antibody includes monoclonal antibodies, multi-specific antibodies, human antibodies, humanized antibodies, synthetic antibodies, chimeric antibodies, camelid antibodies, single chain antibodies, disulfide-linked Fvs (sdFv), Fabs-in-tandem (FIT) molecules, intrabodies, and anti-idiotypic (anti-Id) antibodies (including, e.g., anti-Id and anti-anti-Id antibodies to antibodies of the invention). In particular, such antibodies include immunoglobulin molecules of any type (e.g., IgG, IgE, IgM, IgD, IgA and IgY), class (e.g., IgG_1, IgG_2, IgG_3, IgG_4, IgA_1 and IgA_2) or subclass.

[0079] As used herein, the term "antigen binding fragment" of an antibody refers to one or more portions of an antibody that contain the antibody's complementarity determining region (CDR) that comprise the antibody's "variable region" antigen recognition site, and exhibit an ability to specifically bind antigen. Such fragments include Fab', $F(ab')_2$, Fv, scFv, and mutants thereof, naturally occurring variants, and fusion proteins comprising the antibody's "variable region" antigen recognition site and a heterologous protein (e.g., a toxin, an antigen recognition site for a different antigen, an enzyme, a receptor or receptor ligand, etc.). As used herein, the term "fragment" refers to a peptide or polypeptide comprising an amino acid sequence of at least 5 contiguous amino acid residues, at least 10 contiguous amino acid residues, at least 15 contiguous amino acid residues, at least 20 contiguous amino acid residues, at least 25 contiguous amino acid residues, at least 40 contiguous amino acid residues, at least 50 contiguous amino acid residues, at least 60 contiguous amino residues, at least 70 contiguous amino acid residues, at least 80 contiguous amino acid residues, at least 90 contiguous amino acid residues, at least 100 contiguous amino acid residues, at least 125 contiguous amino acid residues, at least 150 contiguous amino acid residues, at least 175 contiguous amino acid residues, at least 200 contiguous amino acid residues, or at least 250 contiguous amino acid residues.

[0080] Human, chimeric or humanized antibodies are particularly preferred for *in vivo* use in humans, however, murine antibodies or antibodies of other species may be advantageously employed for many uses (for example, *in vitro* or *in situ* detection assays, acute *in vivo* use, etc.).

[0081] A "chimeric antibody" is a molecule in which different portions of the antibody are derived from different immunoglobulin molecules such as antibodies having a variable region derived from a non-human antibody and a human immunoglobulin constant region. Chimeric antibodies comprising one or more CDRs from a non-human species and framework regions from a human immunoglobulin molecule can be produced using a variety of techniques known in the art including, for example, CDR-grafting (EP 239,400; International Publication No. WO 91/09967; and U.S. Pat. Nos. 5,225,539, 5,530,101, and 5,585,089, the contents of each of which are incorporated herein in their entirety), veneering or resurfacing (EP 592,106; EP 519,596, the contents of each of which are incorporated herein by reference), and chain shuffling (U.S. Pat. No. 5,565,332, the contents of which are incorporated herein by reference.

[0082] As used herein, the term "humanized antibody" refers to an immunoglobulin comprising a human framework region and one or more CDRs from a non-human (usually a mouse or rat) immunoglobulin. The non-human immunoglobulin providing the CDRs is called the "donor" and the human immunoglobulin providing the framework is called the "acceptor." Constant regions need not be present, but if they are, they must be substantially identical to human immunoglobulin constant regions, i.e., at least about 85-90%, preferably about 95% or more identical. Hence, all parts of a humanized immunoglobulin, except possibly the CDRs, are substantially identical to corresponding parts of natural human immunoglobulin sequences. A humanized antibody is an antibody comprising a humanized light chain and a humanized heavy chain immunoglobulin. For example, a humanized antibody would not encompass a typical chimeric antibody, because, e.g., the entire variable region of a chimeric antibody is non-human. The donor antibody may be referred to as having been "humanized," by the process of "humanization," because the resultant humanized antibody is expected to bind to the same antigen as the donor antibody that provides the CDRs. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which hypervariable region residues of the recipient are replaced by hypervariable region residues from a non-human species (donor antibody) such as mouse, rat, rabbit or a non-human primate having the desired specificity, affinity, and capacity. In some instances, Framework Region (FR) residues of the human

immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable regions correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin that immunospecifically binds to an FcγRIIB polypeptide, that has been altered by the introduction of amino acid residue substitutions, deletions or additions (i.e., mutations).

2. Bispecific Anti-PD-1/Anti-VEGF Binding Protein Compositions

[0083] Provided herein is a bispecific binding protein, which binds to VEGF and PD-1. The bispecific binding protein comprises at least one anti-VEGF binding region and at least one anti-PD-1 binding region. The bispecific binding protein may specifically target tumor cells expressing VEGF while simultaneously binding to T-cells expressing PD-1. Without being bound by theory, the bispecific binding protein may attract T cells to a tumor site, where the T cells may infiltrate the tumor and lead to tumor cytotoxicity.

a. Anti-VEGF Binding Region

[0084] The anti-VEGF binding region may specifically target VEGF, which may be human. The anti-VEGF binding region may comprise at least one region of an anti-VEGF antibody or an antigen binding fragment thereof. The anti-VEGF binding region may be a scFv.

[0085] The anti-VEGF antibody may be hPV19. The anti-VEGF binding region may comprise at least one light chain variable region comprising a complementarity determining region (CDR) 1 of hPV19 comprising the sequence set forth in SEQ ID NO: 8, a CDR2 of hPV19 comprising the sequence set forth in SEQ ID NO: 9, and a CDR3 of hPV19 comprising the sequence set forth in SEQ ID NO: 10. The anti-VEGF binding region may comprise at least one light chain variable region of hPV19 comprising the following sequence:
DIVMTQSPDSLAVSLGERATINCKSSQSLLNSRTRKNFLAWYQQKPGQSPKLLIYWAST RESGVPDRFTGSGSGTDFTLTISSVQAEDLAVYYCKQSYNLYTFGGGTNLEIKR (SEQ ID NO: 2)

[0086] The anti-VEGF binding region may comprise at least one heavy chain variable region comprising a CDR1 of hPV19 comprising the sequence set forth in SEQ ID NO: 5, a CDR2 of hPV19 comprising the sequence set forth in SEQ ID NO: 6, and a CDR3 of hPV19 comprising the sequence set forth in SEQ ID NO: 7. The anti-VEGF binding region may comprise at least one heavy chain variable region of hPV19 comprising the following sequence: DVQLVQSGVEVKNPGASVKVSCRASGYSFTNSGINWVKQAPGKGLKWMGWINTYTGE PTYADDFKGRFAFSLETSASSAYLQINNLKNEDTATYFCARFGDGYYWFFDVWGAGTT VTVSS (SEQ ID NO: 1)

[0087] The sequences of hPV19 may be as disclosed in the U.S. Patent No. 9,580,498, the contents of which are hereby incorporated by reference in their entirety.

[0088] In another example, the anti-VEGF antibody is Bevacizumab. The anti-VEGF binding region may comprise a light chain variable region comprising a CDR1 of Bevacizumab comprising the sequence set forth in SEQ ID NO: 14, a CDR2 of Bevacizumab comprising the sequence set forth in SEQ ID NO: 15, and a CDR3 of Bevacizumab comprising the sequence set forth in SEQ ID NO: 16. The anti-VEGF binding region may comprise a light chain variable region of Bevacizumab comprising the following sequence: DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIYFTSSLHSGVPS RFSGSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQGTKVEIKR (SEQ ID NO: 4)

[0089] The anti-VEGF binding region may comprise a heavy chain variable region comprising a CDR1 of Bevacizumab comprising the sequence set forth in SEQ ID NO: 11, a CDR2 of Bevacizumab comprising the sequence set forth in SEQ ID NO: 12, and a CDR3 of Bevacizumab comprising the sequence set forth in SEQ ID NO: 13. The anti-VEGF binding region may comprise a heavy chain variable region of Bevacizumab comprising the following sequence: EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVGWINTYTGE PTYAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWG QGTLVTVSS (SEQ ID NO: 3)

[0090] The sequence of Bevacizumab antibody may be as disclosed in DrugBank (go.drugbank.com/drugs/DB00112, the contents of which are incorporated herein in their entirety.

b. Anti-PD-1 Binding Region

[0091] The anti-PD-1 binding region may specifically target PD-1, which may be human. The anti-PD-1 binding region may comprise at least one region of an anti-PD-1 antibody or an antigen binding fragment thereof. The anti-PD-1 antibody may be hAB21 (AI-025). The anti-PD-1 binding region may comprise at least one light chain variable region comprising a CDR1 of hAB21 comprising the sequence set forth in SEQ ID NO: 24, a CDR2 of hAB21 comprising the sequence set forth in SEQ ID NO: 25, and a CDR3 of hAB21 comprising the sequence set forth in SEQ ID NO: 26. The anti-PD-1 binding region may comprise at least one light chain variable region of hAB21 comprising the following sequence:
DIQLTQSPSFLSASVGDRVTITCKASQDAGSAVAWYQQKPGKAPKLLIYWASTRHTGVP
SRFSGSGSGTEFTLTISSLQPEDFATYYCQQYSSYPWTFGGGTKLEIKR (SEQ ID NO: 18)

[0092] The anti-PD-1 binding region may comprise at least one heavy chain variable region comprising a CDR1 of hAB21 comprising the sequence set forth in SEQ ID NO: 21, a CDR2 of hAB21 comprising the sequence set forth in SEQ ID NO: 22, and a CDR3 of hAB21 comprising the sequence set forth in SEQ ID NO: 23. The anti-PD-1 binding region may comprise at least one heavy chain variable region of hAB21 comprising the following sequence:
EVQLVESGGGLVQPGGSLRLSCAASGFTFSRYDMSWVRQAPGKGLEWVSTISGGGRYT
YYPDSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCTSPYGNYGMDYWGQGTSVT
VSS (SEQ ID NO: 17)

The hAB21 antibody may be as disclosed in U.S. Patent No. 11,345,754, the contents of which are incorporated herein in their entirety.

[0093] The anti-PD-1 antibody may be mAB7. The anti-PD-1 binding region may comprise at least one light chain variable region comprising a CDR1 of mAB7 comprising the sequence set forth in SEQ ID NO: 30, a CDR2 of mAB7 comprising the sequence set forth in SEQ ID NO: 31, and a CDR3 of mAB7 comprising the sequence set forth in SEQ ID NO: 32. The anti-PD-1 binding region may comprise at least one light chain variable region of mAB7 comprising the following sequence:
QIVLTQSATIMSASLGERVTMTCTASSSVSSSYLHWYQQKPGSSPKLWIYSTSNLASGVP
ARFSGSGSGTSYSLTISSMEAEDAATYYCHQYHRSPLTFGAGTKLEIK (SEQ ID NO: 20).

[0094] The anti-PD-1 binding region may comprise at least one heavy chain variable region comprising a CDR1 of mAB7 comprising the sequence set forth in SEQ ID NO: 27, a CDR2 of mAB7 comprising the sequence set forth in SEQ ID NO: 28, and a CDR3 of mAB7 comprising the sequence set forth in SEQ ID NO: 29. The anti-PD-1 binding region may comprise at least one heavy chain variable region of mAB7 comprising the following sequence: QIQLVQSGPELKKPGETVKISCKASGYTFTDYSMHWVKQAPGKGLKWMGWIKVETGG PTYAEDFKGRFAFSLETSARTAYLQINNLKNEDTATYFCARDYYGNYYYAMDYWGQG TSVTVSS (SEQ ID NO: 19)

[0095] The anti-PD-1 binding region may be a scFv. The scFv may comprise a disulfide-bond modification made by changing a glycine at position 44 in the VH into a cysteine ("VH44cys") and a glycine at position 100 in the VL into a cysteine ("VL100cys"). The modification may make the scFv structurally more stable and may result in a significant improvement in the structure stability and potency of the scFv (Jennifer S. Michaelson et al., mAbs, Vol 1 (2):128-141 (2009)).

[0096] The VH44cys-VH100cys modifications may be made to a binding region of hAB21. The anti-PD-1 binding region may comprise at least one modified light chain variable region of hAB21 comprising the VL100cys mutation, and may comprise the following sequence: DIQLTQSPSFLSASVGDRVTITCKASQDAGSAVAWYQQKPGKAPKLLIYWASTRHTGVP SRFSGSGSGTEFTLTISSLQPEDFATYYCQQYSSYPWTFG**C**GTKLEIKR (SEQ ID NO: 54)

[0097] The anti-PD-1 binding region may comprise at least one modified heavy chain variable region of hAB21 comprising the VH44cys mutation, and may comprise the following sequence: EVQLVESGGGLVQPGGSLRLSCAASGFTFSRYDMSWVRQAPGK**C**LEWVSTISGGGRYT YYPDSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCTSPYGNYGMDYWGQGTSVT VSS (SEQ ID NO: 53)

a. Heavy Chain Constant Regions

[0098] Anti-PD-1 and anti-VEGF binding regions disclosed herein may comprise one or more antibody heavy chain constant regions or portions thereof. Heavy chain regions disclosed herein may comprise a heavy chain constant region or a portion thereof. The heavy chain constant region may comprise a wild-type heavy chain constant region or a fragment thereof. Heavy chain

constant regions are known in the art. The wild-type heavy chain constant region may be an IgG1 or IgG4 constant region. The IgG1 constant region may comprise the following sequence: ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSS GLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGG PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR DELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDK SRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 51).

[0099] The IgG4 constant region may comprise the following sequence: ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSS GLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTKVDKRVESKYGPPCPSCPAPEFLGGPSV FLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNST YRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEM TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRW QEGNVFSCSVMHEALHNHYTQKSLSLSLGK (SEQ ID NO: 62).

[0100] The heavy chain constant region may be modified with one or more amino acid substitutions to avoid or reduce one or more of antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), or antibody-dependent cell-mediated phagocytosis (ADCP) activity. The position of each amino acid substitution may be numbered according to the EU numbering system, also called the EU index, as described in Kabat, et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991). The heavy chain IgG1 constant region may comprise L234A, L235, and G237A substitutions, and may comprise the following sequence. ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSS GLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPEAAGA PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR DELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDK SRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 69)

[0101] The heavy chain constant region may also L234A, L235, G237A, M428L and N434S substitutions, and may comprise the following sequence.
ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSS
GLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPEAAGA
PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY
NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR
DELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDK
SRWQQGNVFSCSVLHEALHSHYTQKSLSLSPGK (SEQ ID NO: 70)

b. Light Chain Constant Regions

[0102] Anti-PD-1 and anti-VEGF binding regions disclosed herein may comprise one or more antibody light chain constant regions or portions thereof. Light chain antibody regions disclosed herein may comprise a light chain constant region or a portion thereof. The light chain constant region may comprise a wild-type light chain constant region or a fragment thereof. Light chain constant regions are known in the art. The light chain constant region may be a human Kappa (κ) or Lambda (λ) chain constant region. The human κ constant region may comprise the following sequence:
TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQD
SKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 52)

c. Combinations of Anti-VEGF and Anti-PD-1 Binding Regions

[0103] Each anti-VEGF binding region of the bispecific antibody may comprise at least one region of an anti-VEGF antibody or an antigen binding fragment thereof, and each anti-PD-1 binding region of the bispecific antibody may comprise at least one region of an anti-PD-1 antibody or an antigen binding fragment thereof. The bispecific binding protein may comprise a bridge region that bridges one or more anti-VEGF binding regions to one or more anti-PD-1 binding regions. Each anti-VEGF binding region and each anti-PD-1 binding region may comprise a single chain antibody region or a Fabs-in-tandem (FIT) molecule. Each single chain antibody region may comprise a scFv. The single chain antibody region or FIT molecule may comprise a combination of anti-PD-1 and anti-VEGF binding regions.

[0104] The bispecific binding protein may comprise one or more heavy chain variable regions described herein, one or more light chain variable regions described herein, or a combination thereof. Each variable region may independently bind to VEGF or PD-1. The variable regions

may be arranged in tandem. The bispecific binding protein may comprise a single chain antibody region, which may be an scFv. Each variable region may be connected to another variable region via a flexible linker. The flexible linker may comprise the amino acid sequence $(GGGGS)_n$ (SEQ ID NO: 55), where n is 1, 2, 3, or 4. The flexible linker may comprise the sequence set forth in one of SEQ ID NOs: 56-58.

[0105] In one example, the scFv comprises an anti-PD-1 binding region and an anti-VEGF binding region. The scFV may comprise an anti-PD-1 heavy chain variable region connected to an anti-PD-1 light chain variable region via a flexible linker. The scFv may have an anti-PD-1 binding region comprising a VH-linker-VL ("HL") structure or an anti-PD-1 VL-linker-VH ("LH") structure, wherein the N-terminus of the anti-PD-1 binding region is connected to the C-terminal end of a heavy chain of the anti-VEGF binding region comprising at least a portion of an anti-VEGF antibody via a flexible linker.

[0106] In one example, the bispecific binding protein is in a Coloma and Morrison type of IgG-scFv tetravalent format (M. Josefina Coloma & Sherie L. Morrison: Design and production of novel tetravalent bispecific antibodies. Nature Biotechnology 1997, 15:159-163). The anti-VEGF antibody, which may be a scFv, may be fused at its N-terminus to the C-terminus of a heavy chain CH3 domain of the anti-PD-1 antibody or portion thereof, or to the C-terminal end of a hinge region of the anti-PD-1 antibody or portion thereof. The anti-PD-1 antibody portion may be the heavy chain of an anti-PD-1 antibody. Alternatively, the anti-PD-1 antibody, which may be a scFv, may be fused at its N-terminus to the C-terminus of a heavy chain CH3 domain of the anti-VEGF antibody region or to the C-terminal end of a hinge region of the anti-VEGF antibody region.

[0107] A flexible linker may connect the C-terminus of the heavy chain domain to the N-terminus of the VH (e.g., for a HL structure) or the VL (e.g., for a LH structure) of the scFv. The flexible linker may comprise $(GGGGS)_n$ (SEQ ID NO: 55), where n = 1, 2, 3, or 4. Co-expression of an anti-VEGF antibody light chain or anti-PD-1 light chain corresponding to the heavy chain portion fused to the scFv may result in an IgG-heavy chain-scFv (CH3-scFv) or $F(ab')_2$-$scFv_2$ (Hinge-scFv) fusion protein comprising four binding sites, two for each antigen. That is, the anti-VEGF or anti-PD-1 antibody light chain may complex with the respective anti-VEGF or anti-PD-1 antibody heavy chain, respectively, fused to the anti-PD-1 or anti-VEGF scFv, respectively. The anti-VEGF antibody may be a source of the IgG backbone, and the scFv

may be from a PD-1 antibody. The scFv may be created by linking the heavy chain variable region with the light chain variable region by the flexible linker.

[0108] The VEGF binding region of the bispecific binding protein may be derived from hPV19 and referred to herein as "VPD-1" (i.e., either VPD1-HL or VPD1-LH). The VEGF binding region may comprise a heavy chain constant region, and may comprise the heavy chain variable region of hPV19 linked to the heavy chain constant region. The C-terminus of the heavy chain constant region, which may be that of hPV19, may be connected via the flexible linker comprising the sequence set forth in SEQ ID NO: 57 to the N-terminus of a hAB21-derived scFv in the HL format or the LH format. The hAB21-based scFv may comprise the VH of hAB21 connected via its C-terminus to the N-terminus of the VL of hAB21 via the flexible linker comprising the sequence set forth in SEQ ID NO: 58 ("HL" format); or may comprise the VL of hAB21 connected via its C-terminus to the N-terminus of the VH of hAB21 via the flexible linker comprising the sequence set forth in SEQ ID NO: 58 ("LH"). VPD-1 may also comprise the light chain of hPV19. The light chain of hPV19 may complex with the VH of hPV19 linked to the hAB21-based scFv. Examples of VPD1-HL and VPD1-LH are shown in FIG. 1A-B, respectively, and in FIG. 2A. The heavy chain portion of VPD-1 may comprise the sequence set forth in SEQ ID NO: 59 or 60. The light chain portion of VPD-1 may comprise the sequence set forth in SEQ ID NO: 2 fused to a light chain constant region, which may comprise the sequence set forth in SEQ ID NO: 52.

[0109] The bispecific binding protein may be derived from hPV19 and may be referred to herein as VPD1-3A-HL or VPD1-3A-LH. The structure of VPD1-3A-HL may be identical to that of VPD1-HL and the structure of VPD1-3A-LH may be identical to that of VPD1-LH, except that heavy chain constant region in the heavy chain of VPD1-HL or VPD1-LH is replaced with a constant region comprising L234A/L235A/G237A substitutions, which may comprise the sequence set forth in SEQ ID NO: 69. The heavy chain of VPD1-3A-HL may comprise the sequence set forth in SEQ ID NO: 71. The heavy chain of VPD1-3A-LH may comprise the sequence set forth in SEQ ID NO: 72. The light chain portion of VPD1-3A-HL and VPD1-3A-LH may be the same as for VPD-1.

[0110] The bispecific binding protein may be derived from hPV19 and may be referred to herein as VPD1-3A&LS-HL. The structure of VPD1-3A&LS-HL may be identical to that of VPD1-HL, except that heavy chain constant region in the heavy chain of VPD1-HL is replaced with a

constant region comprising L234A/L235A/G237A/M428L/N434S substitutions, which may comprise the sequence set forth in SEQ ID NO: 70. The heavy chain of VPD1-3A&LS-HL may comprise the sequence set forth in SEQ ID NO: 73. The light chain portion of VPD1-3A&LS-HL and VPD1-3A&LS-LH may be the same as for VPD-1.

The bispecific antibody may be derived from Bevacizumab and referred to herein as "VPD-2" (i.e., either VPD2-HL or VPD2-LH). VPD-2 may be a comparator molecule. The C-terminus of the heavy chain of Bevacizumab may be connected via the flexible linker comprising the sequence set forth in SEQ ID NO: 57 to the N-terminus of the hAB21-based anti-VEGF scFv in the HL format or the LH format. The hAB21-based scFv may comprise the VH of hAB21 connected via its C-terminus to the N-terminus of the VL of hAB21 via the flexible linker comprising the sequence set forth in SEQ ID NO: 58 ("HL" format); or may comprise the VL of hAB21 connected via its C-terminus to the N-terminus of the VH of hAB21 via the flexible linker comprising the sequence set forth in SEQ ID NO: 58 ("LH"). VPD-2 may also comprise the light chain of Bevacizumab. The light chain of Bevacizumab may complex with the heavy chain of Bevacizumab linked to the hAB21-based scFv. Examples of VPD-2 are shown in FIG. 1C and FIG. 2B. The heavy chain portion of VPD-2 may comprise the sequence set forth in SEQ ID NO: 61. The light chain portion of VPD-2 may comprise the sequence set forth in SEQ ID NO: 4 fused to a light chain constant region, which may comprise the sequence set forth in SEQ ID NO: 52.

[0111] Provided herein is a further comparator molecule, which may be a Ivonescimab ("AK112") analog molecule, referred to herein as "VPD3-HL" or "VPD3". The sequence of VPD3 may be based on the disclosed sequences of Ivonescimab in WHO-International Nonproprietary Names for Pharmaceutical Substances (INN). VPD-3 may comprise a heavy chain and a light chain. The heavy chain of VPD-3 may comprise the sequence set forth in SEQ ID NO: 62. The light chain of VPD-3 may the sequence set forth in SEQ ID NO: 4 (Bevacizumab-light chain).

3. Methods of Treatment

[0112] Provided herein are uses of bispecific binding proteins disclosed herein in a method of treating cancer, which may be in a subject in need thereof. The method may comprise administering the bispecific binding protein, or a pharmaceutical composition comprising the foregoing, to the subject. A therapeutically effective amount of the bispecific binding protein

may be administered to the subject. The bispecific binding protein or pharmaceutical composition thereof may also be used in the manufacture of a medicament for treating or preventing cancer or another abnormal proliferative disease. As used herein, the term "cancer" refers to a neoplasm or tumor resulting from abnormal uncontrolled growth of cells. As used herein, cancer explicitly includes leukemia and lymphomas. The term refers to a disease involving cells that have the potential to metastasize to distal sites. The subject may be a human.

[0113] The cancer or other abnormal proliferative disease may be (but is not limited to) one or more of the following: carcinoma, including that of the bladder, breast, colon, kidney, liver, lung, ovary, pancreas, stomach, cervix, thyroid and skin; including squamous cell carcinoma; hematopoietic tumors of lymphoid lineage, including leukemia, acute lymphocytic leukemia, acute lymphoblastic leukemia, B-cell lymphoma, T-cell lymphoma, Burkett's lymphoma; hematopoietic tumors of myeloid lineage, including acute and chronic myelogenous leukemias and promyelocytic leukemia; tumors of mesenchymal origin, including fibrosarcoma and rhabdomyosarcoma; other tumors, including melanoma, seminoma, tetratocarcinoma, neuroblastoma and glioma; tumors of the central and peripheral nervous system, including astrocytoma, neuroblastoma, glioma, and schwannomas; tumors of mesenchymal origin, including fibrosarcoma, rhabdomyosarcoma, and osteosarcoma; and other tumors, including melanoma, xenoderma pigmentosum, keratoactanthoma, seminoma, thyroid follicular cancer and teratocarcinoma. It is also contemplated that cancers caused by aberrations in apoptosis would also be treated by the methods and compositions of the invention. Such cancers may include, but are not be limited to, follicular lymphomas, carcinomas with p53 mutations, hormone dependent tumors of the breast, prostate and ovary, and precancerous lesions such as familial adenomatous polyposis, and myelodysplastic syndromes. In specific embodiments, malignancy or dysproliferative changes (such as metaplasias and dysplasias), or hyperproliferative disorders, are treated or prevented by the methods and compositions of the invention in the ovary, bladder, breast, colon, lung, skin, pancreas, or uterus. The cancer may also be sarcoma, melanoma, or leukemia.

[0114] The cancer may be a solid tumor. The solid tumor may be unresectable. The solid tumor may be a colorectal cancer, which may be micro-satellite stable (MSS) or proficient mismatch repair (pMMR), or both. The solid tumor may be a lung cancer, such as a non-small cell lung

cancer (NSCLC) or a small cell lung carcinoma (SCLC), which may be extensive-stage SCLC. The NSCLC may be PD-L1+. The solid tumor may be endometrial cancer.

[0115] The bispecific binding protein may be used in combination with one or more other anti-tumor therapies, including but not limited to, current standard and experimental chemotherapies, hormonal therapies, biological therapies, immunotherapies, radiation therapies, or surgery. In some embodiments, the bispecific antibody may be administered in combination with a therapeutically or prophylactically effective amount of one or more agents, therapeutic antibodies or other agents known to those skilled in the art for the treatment and/or prevention of cancer, autoimmune disease, infectious disease, or intoxication. Such agents include for example, any biological response modifiers, cytotoxins, antimetabolites, alkylating agents, antibiotics, or anti-mitotic agents, or immunotherapeutics disclosed herein.

[0116] The bispecific binding protein may be used in combination with one or more anti-tumor immunotherapies. The anti-tumor immunotherapy may involve molecules that disrupt or enhance alternative immunomodulatory pathways (such as CTLA-4, TIM3, TIM4, OX40, CD40, GITR, 4-1-BB, B7-H1, PD-1, B7-H3, B7-H4, LIGHT, BTLA, ICOS, CD27 or LAG3) or modulate the activity of effecter molecules such as cytokines (e.g., IL-4, IL-7, IL-10, IL-12, IL-15, IL-17, GF-beta, IFNg, Flt3, BLys) and chemokines (e.g., CCL21) in order to enhance the immunomodulatory effects. The bispecific binding protein may be administered in combination with molecules that activate different stages or aspects of the immune response to achieve a broader immune response. The bispecific binding protein may be combined with anti-4-1BB antibodies, without exacerbating autoimmune side effects.

[0117] The bispecific binding protein may be used in combination with one or more standard of care (SOC) anti-cancer therapies. The SOC anti-cancer therapy may be carboplatin, which may be used when the cancer is an extensive small cell lung cancer. The carboplatin may be administered via an intravenous infusion at an area under the curve of 5 mg/mL over a period of 15-60 minutes every 3 weeks for 4 cycles. The SOC anti-cancer therapy may be cisplatin, which may be used when the cancer is an extensive small cell lung cancer. The cisplatin may be administered via an intravenous infusion at 75-80 mg/m^2 over a period of about 30 minutes for 4 cycles. The SOC anti-cancer therapy may be etoposide, which may be used when the cancer is an extensive small cell lung cancer. The etoposide may be administered via an intravenous infusion at 80-100 mg/m^2 over a period of about 60 minutes for 4 cycles.

4. Production

[0118] The bispecific binding protein may be prepared using a eukaryotic expression system. The expression system may entail expression from a vector in mammalian cells, such as Chinese Hamster Ovary (CHO) cells. The system may also be a viral vector, such as a replication-defective retroviral vector that may be used to infect eukaryotic cells. The bispecific binding protein may also be produced from a stable cell line that expresses the antibody from a vector or a portion of a vector that has been integrated into the cellular genome. The stable cell line may express the antibody from an integrated replication-defective retroviral vector.

[0119] The bispecific binding protein may be purified using, for example, chromatographic methods such as affinity chromatography, ion exchange chromatography, hydrophobic interaction chromatography, DEAE ion exchange, gel filtration, and hydroxyapatite chromatography. In some embodiments, fusion proteins can be engineered to contain an additional domain containing amino acid sequence that allows the polypeptides to be captured onto an affinity matrix. For example, the antibodies described herein comprising the Fc region of an immunoglobulin domain can be isolated from cell culture supernatant or a cytoplasmic extract using a protein A column. In addition, a tag such as c-myc, hemagglutinin, polyhistidine, or Flag™ (Kodak) can be used to aid polypeptide purification. Such tags can be inserted anywhere within the polypeptide, including at either the carboxyl or amino terminus. Other fusions that can be useful include enzymes that aid in the detection of the polypeptide, such as alkaline phosphatase. Immunoaffinity chromatography also can be used to purify polypeptides.

5. Pharmaceutical Compositions

[0120] Provided herein is a pharmaceutical composition comprising a bispecific binding protein disclosed herein, and a physiologically acceptable carrier or excipient. The pharmaceutical composition may comprise a prophylactically or therapeutically effective amount of the bispecific binding protein, and a pharmaceutically acceptable carrier.

[0121] In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant (e.g., Freund's adjuvant (complete and incomplete), excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers may be sterile liquids, such as water and oils, including those of

petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The pharmaceutical composition, if desired, may also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions may take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like.

[0122] Generally, the ingredients of the pharmaceutical composition may be supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the pharmaceutical composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the pharmaceutical composition is administered by injection, an ampoule of sterile water for injection or saline may be provided so that the ingredients may be mixed prior to administration.

[0123] The pharmaceutical composition may be formulated as neutral or salt forms. Pharmaceutically acceptable salts include, but are not limited to, those formed with anions such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with cations such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

[0124] In one example, the pharmaceutical composition comprises one or more of histidine buffer, trehalose dihydrate, and polysorbate 80. The pharmaceutical composition may comprise 10-30 mM histidine buffer. The pharmaceutical composition may comprise 6-12% (w/v) trehalose dihydrate. The pharmaceutical composition may comprise 0.02-0.06% (w/v) polysorbate 80. In one example, the pharmaceutical composition comprises 20 mM histidine buffer, 9% (w/v) trehalose dihydrate, and 0.04% (w/v) polysorbate 80. The pharmaceutical composition may have a pH of 5-6 or 5.5. The pharmaceutical composition may comprise 10-30

mg/mL of the bispecific binding protein and may comprise 20 mg/mL of the bispecific binding protein.

[0125] The pharmaceutical composition may be in a vial. The vial may contain 10 mL of the pharmaceutical composition.

6. Methods of Administration

[0126] Methods of administering the compositions and the pharmaceutical compositions thereof include, but are not limited to, parenteral administration (e.g., intradermal, intramuscular, intraperitoneal, intravenous and subcutaneous), epidural, and mucosal (e.g., intranasal and oral routes). In a specific embodiment, the composition is administered intramuscularly, intravenously, or subcutaneously. The composition may be administered by any convenient route, for example, by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with one or more other biologically active agents. Administration can be systemic or local. In one example, the composition is administered intravenously.

[0127] A dose of 0.3-25 mg/kg of the bispecific antibody may be administered. The dose may be 0.3-20 mg/kg. The dose may be 0.3, 1, 3, 10, or 20 mg/kg. The bispecific antibody may be administered intravenously over a period of at least 60 minutes or 120 minutes. When the bispecific antibody is administered at a dose of 10 mg/kg or less, the infusion may be performed over a minimum of 60 minutes. When the bispecific antibody is administered at a dose of over 10 mg/kg, such as a dose of 20 mg/kg, the infusion may be performed over a minimum of 120 minutes. The bispecific antibody may be administered every 1, 2, 3, 4, or 5 weeks. In one example, the bispecific antibody is administered every 3 weeks.

EXAMPLES

[0128] The disclosure has multiple aspects, illustrated by the following non-limiting examples.

Example 1

Constructs

Expression and Purification of Bispecific Antibodies

[0129] A high-yield transient expression system based on suspension-adapted Chinese Hamster Ovary (CHO) cells ("CHO-S") was used to produce bispecific antibodies. The table below shows the plasmid DNA pairings used in transient transfection.

Table 23 List of plasmid DNA pairing for transit cell transfection

Antibody name	Heavy-chain plasmid	Light-chain plasmid
VPD1-LH	VPD1-heavy chain (scFv-LH)	hPV19- light chain (LC)
VPD1-HL	VPD1-heavy chain (scFv-HL)	hPV19-light chain (LC)
VPD2-HL	VPD2-heavy chain (scFv-HL)	Bevacizumab-light chain (LC)-
VPD3-HL	AK112-heavy chain (scFv-HL)	Bevacizumab-light chain (LC)-

[0130] The above plasmid DNAs were paired and mixed with transfection reagent Polyethylenimine (PEI) and then added into the CHO-cell suspension. On Day 1 and Day 5 after the transfection, MetaCell Titer Enhancer (Cellplus Bio, 0.7% volume) and MetaCell CHO TransFeed (Cellplus Bio, 10% volume) were added respectively. Cell culture supernatants were collected on Day 9 after the transfection and antibody protein in the collected supernatants was purified by one-step Protein-A affinity chromatography method using Mab Select Sure resin (Cytiva) with an elution buffer (50 mM NaAc, pH 3.4). The eluted protein samples were immediately neutralized by adding 1/10 volume of 1M Tris-HCl, pH=8.0.

[0131] The concentration of the purified antibody samples was determined by a UV based method using NanoPhotometer measurement (Implen GmbH, München, Germany). The quality of the purified antibodies was analyzed by SDS-PAGE and/or size exclusion-high-performance liquid chromatography (SEC-HPLC) analysis.

Example 2

Bispecific Antibody Constructs

Construction of VPD1 (VPD1-HL or VPD1-LH) Bispecific Antibody

[0132] VPD-1 was created by pairing hPV19-light chain with hPV19-heavy chain appended with a disulfide-bound stabilized anti-PD-1 scFv from hAB21. VPD2 was created by pairing Bevacizumab-light chain with Bevacizumab-heavy chain appended with a disulfide-bound stabilized anti-PD-1 scFv from hAB21. The disulfide-bound stabilized anti-PD-1 scFv, either in VH-$(GGGGS)_4$ (SEQ ID NO: 58)-VL orientation or in VL-$(GGGGS)_4$ (SEQ ID NO: 58)-VH orientation was connected to the C-terminal end of the anti-VEGF IgG heavy chain by a $(GGGGS)_3$ (SEQ ID NO: 57) linker.

[0133] The heavy chain of the VPD-1 bispecific molecule (VPD-1-LH or VPD1-HL) was generated by fusing the DNAs encoding the heavy chain variable region of the anti-VEGF antibody hPV19 (SEQ ID NO: 1) with the DNAs encoding the human IgG1 heavy chain constant region (SEQ ID NO: 51), followed by the DNAs encoding the disulfide-bound stabilized scFv from anti-PD-1 antibody hAB21. The light chain was generated by fusing the DNAs encoding the light chain variable region of the anti-VEGF antibody hPV19 (SEQ ID NO: 2) with the DNAs encoding the human Kappa light chain constant region (SEQ ID NO: 52).

[0134] For the construction of VPD-LH, a disulfide-bound stabilized scFv was created in VH (SEQ ID NO: 53)-$(GGGGS)_4$ (SEQ ID NO: 58)-VL (SEQ ID NO: 54) orientation. For the construction of VPD-HL, a disulfide-bound stabilized scFV was created in VL (SEQ ID NO: 46)-$(GGGGS)_4$ (SEQ ID NO: 58)-VH (SEQ ID NO: 45) orientation. The whole heavy chain amino acid sequences of VPD1-LH and VPD1-HL are shown the table below.

Table 24

SEQ ID NO: 59 (Heavy chain of VPD1-LH)	**DVQLVQSGVEVKNPGASVKVSCRASGYSFTNSGINWV KQAPGKGLKWMGWINTYTGEPTYADDFKGRFAFSLE TSASSAYLQINNLKNEDTATYFCARFGDGYYWFFDVW GAGTTVTVSS**ASTKGPSVFPLAPSSKSTSGGTAALGCLV KDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSV VTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVD VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVV SVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQ PREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWE SNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPGK*GGGGSGGGGSGG GGS*DIQLTQSPSFLSASVGDRVTITCKASQDAGSAVAWY QQKPGKAPKLLIYWASTRHTGVPSRFSGSGSGTEFTLTISS LQPEDFATYYCQQYSSYPWTFGCGTKLEIKR*GGGGSGGG GSGGGGSGGGGS*EVQLVESGGGLVQPGGSLRLSCAASGF TFSRYDMSWVRQAPGKCLEWVSTISGGGRYTYYPDSVK GRFTISRDNSKNTLYLQMNSLRAEDTAVYYCTSPYGNYG MDYWGQGTSVTVSS
SEQ ID NO: 60 (Heavy chain of VPD1-HL)	**DVQLVQSGVEVKNPGASVKVSCRASGYSFTNSGINWV KQAPGKGLKWMGWINTYTGEPTYADDFKGRFAFSLE TSASSAYLQINNLKNEDTATYFCARFGDGYYWFFDVW GAGTTVTVSS**ASTKGPSVFPLAPSSKSTSGGTAALGCLV KDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSV VTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVD VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVV

	SVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQ PREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWE SNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPGK*GGGGSGGGGSGG GGS*EVQLVESGGGLVQPGGSLRLSCAASGFTFSRYDMSW VRQAPGKCLEWVSTISGGGRYTYYPDSVKGRFTISRDNS KNTLYLQMNSLRAEDTAVYYCTSPYGNYGMDYWGQGT SVTVSS*GGGGSGGGGSGGGGSGGGGS*DIQLTQSPSFLSAS VGDRVTITCKASQDAGSAVAWYQQKPGKAPKLLIYWAS TRHTGVPSRFSGSGSGTEFTLTISSLQPEDFATYYCQQYSS YPWTFGCGTKLEIKR

Construction of VPD2 (VPD2-HL) Bispecific Antibody

[0135] The heavy chain of VPD2 bispecific molecules (Bevacizumab-derived) was generated by fusing the DNAs encoding the heavy chain variable region of the anti-VEGF antibody Bevacizumab (SEQ ID NO: 3) with the DNAs encoding the human IgG1 heavy chain constant region (SEQ ID NO: 51), followed by the DNAs encoding the disulfide-bound stabilized scFv from anti-PD-1 antibody hAB21 as described above. The light chain of VPD2 was created by fusing the DNAs encoding the Bevacizumab light chain variable region (SEQ ID NO: 4) with the DNAs encoding the human Kappa light chain constant region (SEQ ID NO: 52). The whole heavy chain amino acid sequences of the VPD2- HL are shown in the table below.

Table 25 Amino acid sequences of heavy chain variable region of VPD2-HL

SEQ ID NO: 61 (Heavy chain of VPD2-HL)	**EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNW VRQAPGKGLEWVGWINTYTGEPTYAADFKRRFTFSL DTSKSTAYLQMNSLRAEDTAVYYCAKYPHYYGSSHW YFDVWGQGTLVTVSS**ASTKGPSVFPLAPSSKSTSGGTAA LGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLY SLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSC DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTC VVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNST YRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISK AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIA VEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW QQGNVFSCSVMHEALHNHYTQKSLSLSPGK*GGGGSGGG GSGGGGS*EVQLVESGGGLVQPGGSLRLSCAASGFTFSRY DMSWVRQAPGKCLEWVSTISGGGRYTYYPDSVKGRFTIS RDNSKNTLYLQMNSLRAEDTAVYYCTSPYGNYGMDYW GQGTSVTVSS*GGGGSGGGGSGGGGSGGGGS*DIQLTQSPSF LSASVGDRVTITCKASQDAGSAVAWYQQKPGKAPKLLIY

	WASTRHTGVPSRFSGSGSGTEFTLTISSLQPEDFATYYCQ QYSSYPWTFGCGTKLEIKR

Construction of VPD3-HL Bispecific Antibody

[0136] An Ivonescimab ("AK112") analog molecule (defined here as "VPD3-HL" or "VPD3") was also generated based on the disclosed sequences of Ivonescimab (AK112) in WHO-International Nonproprietary Names for Pharmaceutical Substances (INN).

Generation of Bispecific Molecules with Reduced Binding to FcRs

[0137] To avoid or reduce ADCC/CDC/ADCP activity, Fc-silent variants of the bispecific molecules were generated by introducing L234A/L235A/G237A (3A mutations) into the IgG1-heavy chain. Based on the overall performance in antigen binding affinity, receptor blocking, SEC-HPLC profile, hPV19 derived VPD1-HL and VPD1-LH were selected as the parental bispecific antibodies for constructing the Fc-silent molecules. **Figure 17** shows the schematic structure of the two bispecific antibodies with 3A-mutations: VPD1-3A-HL derived from VPD1-HL, and VPD1-3A-LH derived VPD1-LH. **Table 8** shows the amino acid sequence of the heavy chain for these two bispecific antibodies with 3A mutations (Fc-silent): VPD1-3A-HL (SEQ ID NO: 71) and VPD1-3A-LH (SEQ ID NO: 72).

Table 26 Amino acid sequences of heavy chain of VPD1-3A-HL and VPD1-3A-LH

SEQ ID NO: 69 (IgG1-heavy chain-constant region with L234A/L235A/ G237A mutations)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVS WNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQT YICNVNHKPSNTKVDKRV**EPKSCDKTHTCPPCPAPEAA** GAPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTL PPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN YKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMH EALHNHYTQKSLSLSPGK
SEQ ID NO: 71 (heavy chain of VPD1-3A-HL)	**DVQLVQSGVEVKNPGASVKVSCRASGYSFTNSGINWV KQAPGKGLKWMGWINTYTGEPTYADDFKGRFAFSLE TSASSAYLQINNLKNEDTATYFCARFGDGYYWFFDVW GAGTTVTVSS**ASTKGPSVFPLAPSSKSTSGGTAALGCLV KDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSV VTVPSSSLGTQTYICNVNHKPSNTKVDKRV**EPKSCDKTH TCPPCPAPEAAGAPSVFLFPPKPKDTLMISRTPEVTCVVV** DVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRV VSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKG QPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEW ESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQG

	NVFSCSVMHEALHNHYTQKSLSLSPGK*GGGGSGGGGSG GGGS*EVQLVESGGGLVQPGGSLRLSCAASGFTFSRYDMS WVRQAPGKCLEWVSTISGGGRYTYYPDSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCTSPYGNYGMDYWGQG TSVTVSS*GGGGSGGGGSGGGGSGGGGS*DIQLTQSPSFLSA SVGDRVTITCKASQDAGSAVAWYQQKPGKAPKLLIYWA STRHTGVPSRFSGSGSGTEFTLTISSLQPEDFATYYCQQYS SYPWTFGTKLEIKR
SEQ ID NO: 72 (heavy chain of VPD1-3A-LH)	**DVQLVQSGVEVKNPGASVKVSCRASGYSFTNSGINWV KQAPGKGLKWMGWINTYTGEPTYADDFKGRFAFSLE TSASSAYLQINNLKNEDTATYFCARFGDGYYWFFDVW GAGTTVTVSS**ASTKGPSVFPLAPSSKSTSGGTAALGCLV KDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSV VTVPSSSLGTQTYICNVNHKPSNTKVDKRV**EPKSCDKTH TCPPCPAPEAAGAPSVFLFPPKPKDTLMISRTPEVTCVVV** DVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRV VSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKG QPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEW ESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQG NVFSCSVMHEALHNHYTQKSLSLSPGK*GGGGSGGGGSG GGGS*DIQLTQSPSFLSASVGDRVTITCKASQDAGSAVAW YQQKPGKAPKLLIYWASTRHTGVPSRFSGSGSGTEFTLTI SSLQPEDFATYYCQQYSSYPWTFGCGTKLEIKR*GGGGSG GGGSGGGGSGGGGS*EVQLVESGGGLVQPGGSLRLSCAAS GFTFSRYDMSWVRQAPGKCLEWVSTISGGGRYTYYPDS VKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCTSPYGN YGMDYWGQGTSVTVSS

[0138] To construct the VPD1-3A-HL and VPD1-3A-LH bispecific molecules, the heavy chain was generated by synthesizing DNA encoding SEQ ID NO: 71 and 72, respectively, and cloned into expression vectors. And the light chain used for pairing the VPD1-3A-HL or VPD1-3A-LH heavy chain was generated by synthesizing DNA encoding the light chain variable region of the anti-VEGF antibody hPV19 (SEQ ID NO: 2) and the DNA encoding the human Kappa light chain constant region (SEQ ID NO: 52) and cloned into expression vectors. These DNA were synthesized and cloned into mammalian expression plasmid vectors by Genescript Co. Plasmids containing these DNA inserts were introduced into bacteria by transformation and plasmid DNA were obtained by the method of mini-or mid-preparation.

[0139] The obtained plasmid DNA was mixed with transfection reagent polyethylenimine (PEI) and added into the CHO-cell suspension. Transfected cell culture supernatants were collected and antibody protein in the collected supernatants was purified by one-step Protein-A affinity

chromatography method using Mab Select Sure resin (Cytiva) with the elution buffer (50mM NaAc, pH3.4). Eluted protein samples were immediately neutralized by adding 1/10 volume of 1M Trs-HCl, pH=8.0. The concentration of the purified antibody samples was determined by a UV based method using NanoPhotometer measurement (Implen GmbH, München, Germany). The quality of the purified antibodies was analyzed by SDS-PAGE and/or size exclusion-high-performance liquid chromatography (SEC-HPLC) analysis. SEC-HPLC analysis showed that the purified sample of VPD1-3A-HL and VPD1-3A-LH all has a monomer peak of over 95%.

Generation of Bispecific Molecules with Enhanced FcRn Binding

[0140] A bispecific anti-VEGF/PD-1 variant with enhanced binding to the neonatal Fc receptor (FcRn) was also generated. Specifically, in this example, the bispecific anti-VEGF/PD-1 variant, VPD1-3A&LS-HL, was generated by introducing two additional amino acid mutants (M428L and N434S: named as LS mutation) into the CH3 domain of VPD1-3A-HL molecule. Figure 22 shows the schematic structure of bispecific antibody VPD1-3A&LS-HL.

[0141] To construct the VPD1-3A&LS-HL bispecific antibody, the heavy chain was generated by synthesizing DNA encoding SEQ ID NO: 73 and cloned into expression vectors. The light chain used for pairing the heavy chain was generated by synthesizing DNA encoding the light chain variable region of the anti-VEGF antibody hPV19 (SEQ ID NO: 2) and the DNA encoding the human Kappa light chain constant region (SEQ ID NO: 52) and cloned into expression vectors.

[0142] These DNA were synthesized and cloned into mammalian expression plasmid vectors by Genescript Co. Plasmids containing these DNA inserts were introduced into bacteria by transformation and plasmid DNA were obtained by the method of mini-or mid-preparation.

[0143] The obtained plasmid DNA was mixed with transfection reagent polyethylenimine (PEI) and added into the CHO-cell suspension. Transfected cell culture supernatants were collected and antibody protein in the collected supernatants was purified by one-step Protein-A affinity chromatography method using Mab Select Sure resin (Cytiva) with the elution buffer (50mM NaAc, pH3.4). Eluted protein samples were immediately neutralized by adding 1/10 volume of 1M Tris-HCl, pH=8.0. The concentration of the purified antibody samples was determined by a UV based method using NanoPhotometer measurement (Implen GmbH, München, Germany). The quality of the purified antibodies was analyzed by SDS-PAGE and/or size exclusion-high-

performance liquid chromatography (SEC-HPLC) analysis. SEC-HPLC analysis showed that the purified antibody sample of VPD1-3A&LS-HL has a monomer peak of over 95%.

[0144] The table below shows the amino acid sequence of the heavy chain (SEQ ID NO: 73) for the bispecific antibody VPD1-3A&LS-HL with both the 3A mutations (Fc-silent mutant) and the LS mutations (FcRn binding enhanced mutant).

Table 27 Amino acid sequences of the heavy chain of VPD1-3A&LS-HL

SEQ ID NO: 73 (heavy chain of VPD1-3A&LS-HL)	**DVQLVQSGVEVKNPGASVKVSCRASGYSFTNSGINWV KQAPGKGLKWMGWINTYTGEPTYADDFKGRFAFSLE TSASSAYLQINNLKNEDTATYFCARFGDGYYWFFDVW GAGTTVTVSS**ASTKGPSVFPLAPSSKSTSGGTAALGCLV KDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSV VTVPSSSLGTQTYICNVNHKPSNTKVDKRV**EPKSCDKTH TCPPCPAPEAAGAPSVFLFPPKPKDTLMISRTPEVTCVVV** DVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRV VSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKG QPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEW ESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQG NVFSCSVLHEALHSHYTQKSLSLSPGK*GGGGSGGGGSGG GGS*EVQLVESGGGLVQPGGSLRLSCAASGFTFSRYDMSW VRQAPGKCLEWVSTISGGGRYTYYPDSVKGRFTISRDNS KNTLYLQMNSLRAEDTAVYYCTSPYGNYGMDYWGQGT SVTVSS*GGGGSGGGGSGGGGSGGGGS*DIQLTQSPSFLSAS VGDRVTITCKASQDAGSAVAWYQQKPGKAPKLLIYWAS TRHTGVPSRFSGSGSGTEFTLTISSLQPEDFATYYCQQYSS YPWTFGTKLEIKR

Example 3

SEC-HPLC Analysis of the Purified Bispecific Antibodies

[0145] Approximately 10μg of the purified antibodies (VPD1-LH, VPD1-HL and VPD2-HL) were loaded into a MabPac-SEC-1 column (7.8x300mm) (Thermo Fisher Scientific) and analyzed using Waters Alliance e2695 HPLC system (Waters). **Figure 7** shows one representative of the SEC-HPLC analysis results. VPD1-HL, VPD1-LH and VPD2-HL all showed a main monomer peak with a retention time (RT) at about 8.5 min. The percentage of the monomer in VPD1-HL, VPD1-LH and VPD2-HL was at 99.33, 92.29 and 89.46%, respectively.

Example 4

Determination of the Binding of Bispecific Antibodies to VEGF and PD-1

ELISA Assay for the Binding to Human VEGF165

[0146] An indirect ELISA method was used to detect the binding of the bi-specific antibodies (VPD1-HL, VPD1-LH and VPD2-HL and etc.) to human VEGF protein. The 96-well plates were coated with 2μg/mL (in 1x PBS solution, 50μL per well) of human VEGF165 protein (AcroBiosystems, Cat #VE5-H4210, expressed from HEK293 cells) at 37°C for 2 hours or at 4°C overnight. After blocking with PBST-1% skim milk at 37°C for 1 hour and washing with PBS-0.1% Tween 20 buffer (PBST) 3 times, 2-fold serial dilutions of VPD1-HL, VPD1-LH, VPD2-HL or control samples (all starting at 10μg/mL, diluted in PBST-0.5% skim milk) were added (50 μL per well) and the plates were incubated at 37°C for 1 hour. After washing with PBST for 3 times, peroxidase conjugated goat anti-human IgG (Fc specific)-antibody (Sigma, Cat# A0170), diluted with PBST-0.5% skim milk at 1:2000, was added (50μL per well) and the plates were then incubated at 37°C for 1 hour. After washing again with PBST for 3 times, o-Phenylenediamine (OPD)/0.3% H_2O_2 substrates was added into each well (50μL per well) for color development. After setting at room temp for 5-10 min, 1M HCl solution was added into each well (50μL per well) to stop the reaction and then the plates were inserted into a plate reader (Multiskan FC, Thermo) and the OD values at wavelength of 492nm (OD492nm) in each well was recorded. The subsequent OD value vs antibody concentration curves and the EC50 values were generated/calculated by the GraphPad Prism 10.1.1.

[0147] In some experiments, one or two anti-VEGF antibodies hPV19 (humanized IgG1) or Bevacizumab (humanized IgG1) were included as the positive control, and irrelevant antibodies anti-CTLA4 antibody ONC-392 (humanized IgG1) or Ipilimumab (IgG1) served as the negative control. **Figure 8** shows one of the representative VEGF165 antigen binding ELISA results. VPD1-HL, VPD1-LH, VPD2-HL all show a similar binding to human VEGF in this assay.

ELISA Assay for Binding to Human PD-1

[0148] An indirect ELISA method was also used to detect the binding of bispecific antibodies (VPD1-HL, VPD1-LH, VPD2-HL and etc.) to human PD-1. The procedures are the same as those described above except that the antigen used for coating the plates was human PD-1 instead of human VEGF.

[0149] In this example, the antigen used for coating the plates was a histidine tagged human PD-1 (Human PD-1 protein, his tag, AcroBiosystems, Cat # H5221, expressed from HEK293 cells) and the final concentration for the coating was 1-2μg/mL.

[0150] In some experiments, one or more anti-PD-1 antibodies such as hAB21(AI-025, IgG4), Pembrolizumab (IgG4) or Nivolumab (IgG4) were included as the positive control, and irrelevant antibodies anti-CTLA4 antibody ONC-392 (humanized IgG1) or Ipilimumab (IgG1) included as the negative control. **Figure 9** shows one of the representative PD-1 antigen binding ELISA results. VPD1-HL, VPD1-LH, VPD2-HL all show a similar binding to human PD-1 in this assay.

Example 5

Determination of the Simultaneous Binding of Bispecific Antibodies to Both Human VEGF and PD-1 Antigens

[0151] A sandwich ELISA method was developed to detect the simultaneous binding of bispecific antibodies (VPD1-HL, VPD1-LH and VPD2-HL) to both human VEGF and PD-1 antigens. 96-well plates were coated with 2μg/mL (in 1x PBS solution, 50μL per well) of HEK293 cell derived recombinant human VEGF165 protein (AcroBiosystems, Cat # VE5-H4210) at 37°C for 2 hours or at 4°C overnight. After blocking with PBST-1 % skim milk at 37°C for 1 hour and washing with PBS-0.1% Tween 20 buffer (PBST) 3 times, 2-fold serial dilutions of VPD1-HL, VPD1-LH and VPD2-HL or control samples (all starting at 10 μg/mL, diluted in PBST-0.5 % skim milk) were added (50 μL per well) and the plates were then incubated at 37°C for 1 hour. After washing with PBST for 3 times, biotinylated human PD-1-his protein (Biotinylated Human PD-1/PDCD1 Protein, AVITAG™, His Tag from AcroBiosystems, Cat. No. PD1-H82E4, expressed from HEK293 cells) was added (diluted with PBST-0.5 % skim milk to 10 μg/mL, 50 μL per well) and the plates were then incubated at 37°C for 1 hour. After washing with PBST for 3 times, horse radish peroxidase (HRP)-conjugated Avidin (Sigma, Cat #A7419, diluted with PBST-0.5 % skim milk to 1:2000) was added (50μL per well) and the plates were incubated at 37°C for 1 hour. After washing again with PBST 3 times, o-Phenylenediamine (OPD)/0.3% H_2O_2 substrates were added into each well (50μL per well) for color development. After setting at room temp for 5-10 min, 1M HCl solution was added into each well (50μL per well) to stop the reaction and then the plates were inserted into a plate

reader (Multiskan FC, Thermo) and the OD values at wavelength of 492 nm (OD492nm) in each well were recorded. The OD value vs antibody concentration curves were generated by the GraphPad Prism 10.1.1.

[0152] Figure 10 shows VPD1 or VPD2 simultaneously binding to human VEGF and PD-1 antigens in the sandwich ELISA with plate pre-coated with human PD-1 antigen. As shown in the figure, only the bi-specific antibodies (VPD1-HL, VPD1-LH and VPD2-HL), but not the parental mAbs (hPV19, Avastin or hAB21), can simultaneously bind to both human VEGF and PD-1 antigens, VPD1-HL and VPD1-LH showed similar binding as VPD2 -HL in this assay.

[0153] Figure 11 shows VPD1 or VPD2 simultaneously binding to human VEGF and PD-1 antigens in in the sandwich ELISA with 96-well plates pre-coated with human VEGF165 antigen. Again, as shown in the figure, only the bi-specific antibodies (VPD1-HL, VPD1-LH and VPD2-HL), but not the parental mAbs (hPV19, Avastin or hAB21), can simultaneously bind to both human VEGF and PD-1 antigens. VPD1-HLHand VPD1-LH showed similar binding as VPD2-HL in this assay.

Example 6

Binding kinetics of VPD1 or VPD2 to VEGF and PD-1

Binding Affinity to Human VEGF and PD-1 Measured by Biolayer Interferometry Assay

[0154] A biolayer interferometry assay (BLI) based Fortebio (Fortbio Blitz) assay was used to analyze the binding kinetics of VPD1 or VPD2 to VEGF and PD-1. Bio-Layer Interferometry (BLI) is a label-free optical technique to study the interactions between an immobilized receptor/ligand and analytes in solution. The BLI approach has a similar concept to Surface Plasmon Resonance technique (SPR, Biacore) in the sense that the receptor/ligand is immobilized on a surface, and analytes are in solution.

Binding Kinetics and Affinity to Human VEGF

To measure the binding kinetics and affinity to VEGF, the bispecific antibodies VPD1-HL, VPD2-HL or Avastin were dissolved in PBST pH 7.4 buffer at 5μg/mL and captured by a biosensor AHC (Octet® anti-Human IgG Fc capture, Fortebio). The captured biosensor was then inserted into the tube with a binding solution containing different concentrations (0, 11, 33 and 100nM) of human VEGF165 (Acro biosystems, Cat#VE5-H4210). The association time in the

tube was set for 5 min and the dissociation was monitored for 20 min. The association/dissociation curves were recorded, and the kinetics parameters, such as, association (Ka), dissociation (Kd) constants and binding affinity (KD) were determined using 1:1 binding model. **Figure 12** shows the association/dissociation curves of VEGF binding.

Table 28 Binding affinity to human VEGF165

Sample ID	ka (1/Ms)	kd (1/s)	KD (M)	KD ratio
VPD1-HL	7.25E+5	6.54E-5	9.03E-11	-
VPD2-HL	3.47E+5	4.87E-4	1.40E-9	15.53
Avastin	2.69E+5	3.40E-4	1.26E-9	13.99

[0155] As shown in the above table, VPD1-HL has 14- to 15-fold higher VEGF binding affinity than Avastin or Avastin derived VPD2-HL in this assay, due to its faster binding and lower dissociation rate.

Binding Kinetics and Affinity to Human PD-1

[0156] To measure the binding kinetics and affinity of the bispecific antibodies to PD-1, samples of VPD1-HL, VPD2-HL or hAB21 were also dissolved at 5μg/mL in PBST pH 7.4 buffer and each captured by a biosensor AHC (OCTET® anti-Human IgG Fc capture, Fortebio). The captured biosensor was then inserted into the binding solution containing different concentrations (0, 11, 33 and 100nM) of human PD-1-his (Acro biosystems, Cat# H5221). The association time was in the tube was set for 5 min and the dissociation was monitored for 20 min. The association/dissociation curves were recorded, and the kinetics parameters, such as, association (Ka), dissociation (Kd) constants and binding affinity (KD) were determined using 1:1 binding model. Binding affinities (KD) were recorded and determined using a 1:1 binding model. **Figure 13** shows the association/dissociation curves of PD-1 binding.

Table 29 Binding affinity to human PD1-his

Test Sample	ka (1/Ms)	kd (1/s)	KD (M)	KD ratio
VPD1-HL	1.75E5	2.76E-4	1.58E-9	-
VPD2-HL	2.04E5	1.08E-3	5.28E-9	3.35
hAB21	2.49E5	1.16E-3	4.67E-9	2.97

[0157] As shown in the above table, VPD1-HL has about a 3-fold higher PD-1 binding affinity than VPD2-HL or hAB21, mainly due to its lower dissociation rate.

Example 7

VPD1-HL or VPD-LH Blocks PD-1/PD-L1 Interaction in Competitive ELISA Assay

[0158] A competitive ELISA assay was developed and used for testing the potency of the bispecific antibodies in blocking the interaction of PD-1 with its receptors such as PD-L1. 96-well plates were coated with 10μg/mL (50 μL per well) of recombinant human PD-1-hFc protein (Sino Biologicals, Cat #10377-H02H) at 37°C for 2 hours or at 4°C overnight. After blocking with PBST-1% milk at 37°C for 1 hour and washing with PBST 3 times, a mixture of 2-fold serial dilutions of bispecific antibodies (VPD1-HL, VPD1-LH), or control samples (starting at 10μg/mL) and a fixed concentration (1μg/mL) of Biotinylated human PD-1 Protein, AVITAG™, His Tag (Acrobiosystems, Cat # H8214, expressed from HEK293 cells) was added (50 μL per well) and the plates were then incubated at 37°C for 1 hour. After washing with PBST 3 times, horse radish peroxidase (HRP)-conjugated Avidin (Sigma, Cat #A7419, diluted with PBST-0.5 % skim milk to 1:2000) was added (50μL per well) and the plates were incubated at 37°C for 1 hour. After washing again with PBST 3 times, o-Phenylenediamine (OPD)/0.3% H_2O_2 substrates were added into each well (50μL per well) for color development. After setting at room temp for 5-10 min, 1M HCl solution was added into each well (50μL per well) to stop the reaction and then the plates were inserted into the plate reader (Multiskan FC, Thermo) and the OD values at wavelength of 492 nm (OD492 nm) in each well were recorded. The OD value vs antibody concentration curves and IC_{50} values were generated/calculated by the GraphPad Prism 10.1.1. **Figure 14** shows that VPD1-HL or VPD1-LH has a similar PD-1/PD-L1 blocking activity in this competitive ELISA assay.

Example 8

Bispecific Antibodies Block the Binding of Human VEGF to Human VEGF-R (VEGF-R1, VEGFR-2) in Function Blocking ELISA Assay

[0159] A functional blocking ELISA assay was developed and used for probing the bio-potency of the bispecific antibodies in blocking the interaction of VEGF with VEGF-R (VEGF-R1, VEGF-R2).

Generation of Human VEGFR1(D2D3)-Fc and VEGFR2 (D2D3)-Fc Fusion Proteins

[0160] To develop the blocking assay, we created two Fc fusion proteins: VEGFR1(D2D3)-Fc, and VEGFR2(D2D3)-Fc. VEGFR1(D2D3)-Fc fusion protein was created by fusing the extracellular Ig-like domain 2 (D2) and domain 3 (D3) of human VEGF-R1 with the Fc (hinge-CH2-CH3) domain of human IgG1-heavy chain. VEGFR2(D2D3)-Fc fusion protein was created by fusing the extracellular Ig-like domain 2 (D2) and domain 3 (D3) of human VEGF-R2 with the Fc (hinge-CH2-CH3) of human IgG1-heavy chain. For both VEGF-R1 and VEGF-R2, it was known that the Ig-like D2 and D3 are the main units responsible for mediating their high affinity binding to VEGF. The amino acid sequences of human VEGF-R1 extracellular domain 2 and domain 3 (VEGFR1-D2D3) had the sequence set forth in SEQ ID NO: 63, and human VEGF-R2 extracellular domain 2 and domain 3 (VEGFR2-D2D3) had the sequence set forth in SEQ ID NO: 64.

[0161] VEGFR1-D2D3-Fc fusion protein was generated by fusing DNA encoding human VEGFR1-D2D3 (SEQ ID NO: 63) with DNA encoding human IgG1-Hinge-CH2-CH3 (SEQ ID NO: 66). The signal peptide (SEQ ID NO: 65) from the human CD24 gene was used for generating this fusion protein.

[0162] Similarly, VEGFR2-D2D3-Fc fusion protein was generated by fusing DNA encoding human VEGFR2-D2D3 (SEQ ID NO: 64) with DNA encoding human IgG1-Hinge-CH2-CH3 ((SEQ ID NO: 66). The signal peptide (SEQ ID NO: 65) from human CD24 gene was used for generating this fusion protein. Complete amino acid sequences of VEGFR1-D2D3-Fc and VEGFR2-D2D3-Fc had the sequences set forth in SEQ ID NO: 67 and 68, respectively.

Table 30 Fusion protein sequences

SEQ ID NO: 63 (VEGFR1-D2D3)	**TGRPFVEMYSEIPEIIHMTEGRELVIPCRVTSPNITVT** **LKKFPLDTLIPDGKRIIWDSRKGFIISNATYKEIGLLT** **CEATVNGHLYKTNYLT**HRQTNTIIDVQISTPRPVKLLR GHTLVLNCTATTPLNTRVQMTWSYPDEKNKRASVRRRI DQSNSHANIFYSVLTIDKMQNKDKGLYTCRVRSGPSFK SVNTSVHIYDK
SEQ ID NO: 64 (VEGFR2-D2D3)	***SD*YRSPFIASVSDQHGVVYITENKNKTVVIPCLGSISN** **LNVSLCARYPEKRFVPDGNRISWDSKKGFTIPSYMIS**

	YAGMVFCEAKINDESYQSIMYIVVVVGYRIYDVVLSP SHGIELSVGEKLVLNCTARTELNVGIDFNWEYPSSKHQH KKLVNRDLKTQSGSEMKKFLSTLTIDGVTRSDQGLYTC AASSGLMTKKNSTFVRVH
SEQ ID NO: 66 (Hinge-CH2-CH3)	EPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISR TPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPR EEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLY SKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS PG*
SEQ ID NO: 65 (Signal peptide from human CD24 gene)	MGRAMVARLGLGLLLLALLLPTQIYS
SEQ ID NO: 67 (VEGFR1-D2D3-Fc)	*SD*TGRPFVEMYSEIPEIIHMTEGRELVIPCRVTSPNITVTL KKFPLDTLIPDGKRIIWDSRKGFIISNATYKEIGLLTCEAT VNGHLYKTNYLTHRQTNTIIDVQISTPRPVKLLRGHTLV LNCTATTPLNTRVQMTWSYPDEKNKRASVRRRIDQSNS HANIFYSVLTIDKMQNKDKGLYTCRVRSGPSFKSVNTS VHIYDKRVEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKP KDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV HNAKT KPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK ALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSL TCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSF FLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSL SLSPG*

SEQ ID NO: 68 (VEGFR2-D2D3-Fc)	*SD*YRSPFIASVSDQHGVVYITENKNKTVVIPCLGSISNLN VSLCARYPEKRFVPDGNRISWDSKKGFTIPSYMISYAGM VFCEAKINDESYQSIMYIVVVVGYRIYDVVLSPSHGIELS VGEKLVLNCTARTELNVGIDFNWEYPSSKHQHKKLVN RDLKTQSGSEMKKFLSTLTIDGVTRSDQGLYTCAASSGL MTKKNSTFVRVHEPKSSDKTHTCPPCPAPELLGGPSVFL FPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK EYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRE EMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTT PPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEAL HNHYTQKSLSLSPG*

[0163] The plasmid DNA was transiently transfected into CHO-S cells by the PEI method and cell-culture supernatants were then collected for purification of the Fc-fusion proteins by the standard one step Protein A affinity column method. ELISA assay results showed that the purified VEGFR1-Fc and VEGFR2-Fc both specifically bind to human VEGF165 protein coated on the 96-well plates, and therefore, can be used as the reagents for the subsequent VEGF/VEGFR1 or VEGF/VEGFR2 receptor blocking assay.

Bispecific Antibodies Block the Interaction of VEGF with VEGF-R in Function Blocking ELISA Assay

[0164] A function blocking ELISA assay was developed and used to detect the ability and potency of the bispecific antibodies in blocking the interaction of human VEGF165 to VEGF-R1 or VEGF-R2. In this assay, Aflibercept (VEGF-Trap, Regeneron pharmaceuticals/Bayer) a 115 kDa Ig-Fc fusion protein consisting of the Ig-like domain 2 of VEGF-R1, the Ig like domain D3 of VEGF-R2 and the hinge-CH2-CH3 region of human IgG1 was included as a positive control sample, and an anti-CTLA4 antibody ONC392 as a negative control. 96-well plates were coated with 1μg/mL (50μL per well) of the recombinant human VEGFR1(D2D3)-Fc or VEGFR2(D2D3)-Fc protein (derived from the above Example 10.1) at 37°C for 2 hours or at 4°C overnight. After blocking with PBST-1% milk at 37°C for 1 hour and washing with PBST 3

times, a mixture of 2-fold serial dilutions of bispecific antibodies (VPD1-HL, VPD1-LH, VPD2-HL and VPD3), or control samples (starting at 100μg/mL) and a fixed concentration of biotin-labeled VEGF165 (0.03μg/mL, biotinylated Human VEGF165-His (from Acrobiosystms, Cat # VE5-H82Q0, expressed from human HEK293 cells) was added (50μL per well) and the plates were then incubated at 37°C for 1 hour.

[0165] After washing with PBST 3 times, horse radish peroxidase (HRP)-conjugated Avidin (Sigma, Cat #A7419, diluted with PBST-0.5% skim milk to 1:2000) was added (50μL per well) and the plates were incubated at 37°C for 1 hour. After washing again with PBST 3 times, o-Phenylenediamine (OPD)/0.3% H_2O2 substrates were added into each well (50μL per well) for color development. After setting at room temp for 5-10 min, 1M HCl solution was added into each well (50μL per well) to stop the reaction and then the plates were inserted into the plate reader (Multiskan FC, Thermo) and the OD values at wavelength of 492 nm (OD492 nm) in each well were recorded.

[0166] The OD value vs antibody concentration curves and IC_{50} values were generated and calculated by the GraphPad Prism 10.1.1. **Figure 15** shows that hPV19 derived bi-specific antibodies (VPD1-HL and VPD1-LH) have a nearly 2-fold higher potency than bevacizumab-derived VPD2-HL and nearly 10-fold higher than VPD3 in blocking VEGF binding to VEGFR1. The IC50 of VPD1-HL, VPD1-LH, VPD2-HL and VPD3 are at 11.30, 11.91, 21.82 and 107.45nM, respectively. As expected, the positive control sample of Aflibercept (VEGF-Trap) showed a very high blocking activity (IC50 at 6.80 nM), whereas the negative control sample of ONC392 showed no blocking activity.

[0167] **Figure 16** shows one of the representative VEGF/VEGF-R2 blocking ELISA assay results. Again, as shown in the figure and underneath table, hPV19 derived bi-specific antibodies (VPD1-HL and VPD1-LH) also have a nearly 50% higher potency than bevacizumab derived VPD2-HL and 7.5-fold higher than VPD3 in blocking VEGF binding to VEGFR2. The IC50 of VPD1-HL, VPD1-LH, VPD2-HL and VPD3 are at 9.50, 9.80, 14.0 and 73.00nM, respectively.

Example 9

ELISA Assay for Probing the Binding of VPD1-3A-HL or VPD1-3A-LH Bispecific Antibodies to Human FcγRI (CD64)

[0168] An indirect ELISA method was used for probing the binding of bispecific antibodies VPD1-3A-HL or VPD1-3A-LH to human FcγRI (CD64). The ELISA procedures were the same as described above except that human FcγRI protein (CD64) was used instead of human VEGF to coat the plates. A 96-well plate was coated with recombinant human FcγRI protein (CD64-His, from Acrobiosystems, Cat #, FCA-H52H1) at 5μg/mL in 1x PBS. After washing away of unbound protein and blocked with 1x PBST-1% skim milk in, 2-fold serial dilutions of VPD-1, VPD1-3A mutants (VPD1-3A-HL and VPD1-3A-LH), hAB21 (IgG4 isotype mAb) or IgG1 isotype mAbs (Avastin, hPV19 and ONC392), all starting at 10μg/mL, were added into the plate. The bound antibodies were detected by adding HRP-conjugated goat anti-human IgG (Fab-specific) antibodies (Sigma, A0293) followed by the substrate OPD. **Figure 18** shows VPD1-3A mutants (VPD1-3A-HL and VPD1-3A-LH) as well as IgG4 isotype of anti-PD-1 antibody hAB21 (AI-025), have no binding to human FcγRI (CD64), whereas the parental VPD1 (wild-type IgG1) and the three IgG1 antibodies (Avastin, hPV19 and ONC392) all showed a high-affinity binding to the human FcγRI receptor (CD64) protein.

Example 10

Binding Kinetics and Affinity of VPD1-3A-HL and VPD3 to Human VEGF

[0169] To measure the binding kinetics and affinity of VEGF, samples of the bispecific antibodies VPD1-3A-HL or VPD3 were dissolved in PBST pH 7.4 buffer at 5μg/mL and captured by a biosensor AHC (OCTET® anti-Human IgG Fc capture, Fortebio). The captured biosensor was then inserted into the tube with a binding solution containing different concentrations (0, 3.7, 11, 33 and 100nM) of human VEGF165 (Acro biosystems, Cat#VE5-H4210). The association time in the tube was set for 5 min and the dissociation was monitored for 60 min. The association/dissociation curves were recorded, and the kinetics parameters, such as, association (Ka), dissociation (Kd) constants and binding affinity (KD) were determined using 1:1 binding model. As shown in the table below, VPD1-3A-HL had over a 200-fold higher

VEGF binding affinity than VPD3-HL, mainly due to its extremely very slow dissociation rate (＜1E-7 in VPD1-3A vs. 6.98E-4 in VPD3).

Table 31 Binding affinity to human VEGF165

Sample ID	ka (1/Ms)	kd (1/s)	KD (M)	KD ratio
VPD1-3A-HL	3.79E+5	＜1E-7	＜1E-12	-
VPD3-HL	3.21E+5	6.98E-4	2.17E-10	>200

Example 11

VPD1-3A Mutants (VPD1-3A-HL and VPD1-3A-LH) Maintain the High Binding Activity to VEGF and PD-1 as that of VPD1

[0170] The binding of VPD1-3A mutants (VPD1-3A-HL and VPD1-3A-LH) to VEGF and PD-1 was examined in an ELISA assay. The ELISA assay described above was used to test the binding of VPD1-3A mutants (VPD1-3A-HL and VPD1-3A-LH) to human PD-1-his antigen.

[0171] As shown in **Figure 19,** similar to the parental molecule VPD1-HL, VPD1-3A-HL and VPD1-3A-LH also have higher PD-1 binding activity than Avastin/Panpulimab-derived VPD3 (AK112) in this assay. The EC50 of VPD1-HL, VPD1-3A-HL and VPD1-3A-LH in this assay was at 0.294, 0.413 and 0.192μg/mL, respectively, about 1.75- to 3.78-fold lower than that of VPD3 (0.724μg/mL).

Example 12

Determination of the Biopotency of Bispecific Antibodies in Cell-Based Luciferase-Reporter Assay

Determination of the Biopotency of Bispecific Antibodies in Cell-Based Luciferase-VEGFR2 Reporter Assay

[0172] A luciferase-VEGFR2 reporter cell-based luminescence assay was developed and used for determination of the bio-potency of the bispecific antibodies. The reporter cell line (Cell line name: H_VEGF Reporter 293 Cell Lin, Cat # GM-C09057) was purchased from Genomeditech (Shanghai) Co., Ltd. This cell line expressed both a human VEGF-R2 gene (cDNA) and a Luciferase reporter gene that is under the control of the nuclear factor of activated T cells

(NFAT) signaling response element. When these cells are stimulated with human VEGF protein, the VEGF/VEGF-R2 interaction leads to the downstream signal transduction events and NFAT driven luciferase reporter gene expression. Inhibition of VEGF binding to VEGF-R2 by either anti-VEGF or anti-VEGFR2 antibodies results in a decrease in luminescence. The luminescence signal intensity (Lum) is reversely correlated with the bio-potency of anti-VEGF or anti-VEGFR2 antibody: i.e., the lower of the luminescence signal, the higher of the potency.

[0173] To measure the bio-potency of the variants, H_VEGF Reporter 293 cells (culture medium: DMEM+10% FBS+1% P.S+4 μg/mL Blasticidin+400μg/mL G418) were first seeded into a 96-well plate (2×10^5 cells/mL, 100μL per well) and incubated at 37°C, 5% CO_2 for 16 hours. The next day, 3-fold serial dilution samples of different antibodies or the negative control antibody ONC392, with human VEGF165 protein (Acro biosystems, Cat. #VE5-H4210) at the final concentration of 10 ng/mL, were added into the plate and incubated at 37°C, 5% CO_2 for 2 days. At the end of the incubation, Bio-Glo solution was added into the 96-well plate (100μL/well) and the luminescence signal in the plate was detected by a Multimode microplate reader (PerkinElmer Victor X3).

[0174] As shown in **Figure 20,** VPD-1, VPD1-3A-HL and VPD1-3A-LH all had a higher biopotency than Avastin/Panpulimab-derived VPD3 (AK112). The IC50 of VPD-1, VPD1-3A-HL and VPD1-3A-LH in this assay were measured at 11.72, 10.86 and 9.85ng/mL, respectively, approximately 4.5-fold lower than that of VPD3 (AK112), which was measured at 47.55ng/mL. The IC50 of the parental anti-VEGF mAb hPV19 was measured at 9.80ng/mL, and 6.75-fold lower than that of Avastin, which was measured at 66.21 ng/mL.

Determination of the Biopotency of Bispecific Antibodies in Cell-Based Luciferase-PD-1/PD-L1 Reporter Assay

[0175] A luciferase PD-1/PD-L1 reporter cell-based luminescence assay was also used for determination of the bio-potency of the bispecific antibodies. The principle and procedure of this cell-based assay are illustrated in Figure 39. This assay relies on the co-culture of two types of engineered cells: one is CHO-PD-L1-CD3scFv cell stably expressing human PD-L1 and the membrane-anchored anti-CD3 single chain antibody fragment (CD3 scFv) on the cell surface; the other is a Jurkat-NFAT-PD-1 cell stably expressing human PD-1 on the cell surface and the luciferase reporter gene under the control of NFAT (nuclear factor of activated T cells) response elements. When these two types of cells are incubated together, an activation signal cascade is

initiated via the interaction between the anti-CD3scFv on CHO cells and TcR/CD3 complex on Jurkat cells, leading to the downstream signal transduction events and NFAT driven luciferase reporter gene expression; but a co-engagement of PD-L1 on CHO cells with PD-1 on Jurkat T cells would block the TcR/CD3 mediated signal transduction and inhibits the activation of NFAT pathway and its associated luciferase reporter gene expression. When a neutralizing anti-PD-1 or anti-PDL1 antibody is added into the co-culture, it releases the PD-L1/PD-1 blockage and restores the TcR/CD3 mediated signal cascade in Jurkat cells and NFAT driven luciferase reporter gene expression, thus generating a luminescence signal upon the addition of Bio-Glo substrates into the culture. The luminescence signal intensity is correlated with the bioactivity of anti-PD-1 or anti-PDL1 antibody, i.e., the higher of a luminescence signal, the higher the potency.

[0176] To measure the potency of the bispecific antibodies, CHO-PD-L-CD3scFv cells (2×10^6 cells/mL) were first seeded into a 96-well plate (0.1mL/well) and incubated at 37±3°C, 5%±1% CO_2 for 16 hours. The next day, three-fold serial dilution samples of different bispecific antibodies, or the parental anti-PD-1 antibodies such as hAB21 or the negative control antibody ONC392, all starting at 60μg/mL, were added into the plate and co-incubated with Jurkat-PD-1-NFAT cells (2×10^6 cells/mL, add 0.1mL/well) at 37°C, 5% for 6±2 hours. At the end of the co-incubation, Bio-Glo solution was added into the 96-well plate (100μL/well) and the luminescence signal in the plate was detected by a Multimode microplate reader (PerkinElmer Victor X3). As shown in **Figure 21**, VPD-1 and its 3A mutants VPD1-3A-HL or VPD1-3A-LH all had a higher bio-potency than Avastin/Panpulimab-derived VPD3 (AK112). The EC50 of VPD1-HL, VPD1-LH, VPD1-3A-HL and VPD1-3A-LH in this assay was measured at 0.545, 0.412, 0.385, an 0.554μg/mL, respectively, approximately 2-fold lower than that of VPD3 (AK112), which was measured at 0.982μg/mL. The EC50 of the parental anti-PD-1 mAb hAB21 was measured at 0.094μg/mL, also about 2-fold lower than that of Penpulimab, which was measured at 0.185μg/mL.

Example 13

Determination of the Binding of VPD1-3A-HL or VPD1-3A&LS-HL to Human FcRn by BLI-based Fortebio Assay

[0177] A BLI-based Fortebio assay was used for the determination of the binding of VPD1-3A-HL or VPD1-3A&LS-HL to human FcRn at different pH (pH 6.0, 6.5 and 7.4). Table 14 shows the summary of the binding parameters. Compared with VPD1-3A-HL, VPD1-3A&LS-HL mutant showed about 7.0-, 5.8-, and 7.3-fold higher binding to human FcRn at pH of 7.4, 6.5 and 6.0, respectively.

Table 32 FcRn binding parameters

	Loading Sample	ka (1/Ms)	kd (1/s)	KD (M)
pH7.4				
	VPD1-3A-HL	5.91E+4	1.63E-2	2.76E-7
	VPD1-3A&LS-HL	2.45E+5	9.61E-3	3.93E-8
pH6.5	VPD1-3A-HL	1.71E+5	5.83E-3	3.14E-8
	VPD1-3A&LS-HL	4.51E+5	2.46E-3	5.45E-9
pH6.0	VPD1-3A-HL	3.24E+5	4.20E-3	1.29E-8
	VPD1-3A&LS-HL	3.56E+5	6.31E-4	1.77E-9

Example 14

Determination of the Binding of VPD1-HL, VPD1-3A-HL and VPD1-3A&LS-HL to Fcγ Receptors I-III (CD16, CD32 and CD64)

[0178] A BLI-based Fortebio assay was also used to further probe the binding of VPD1-HL, VPD1-3A-HL and VPD1-3A&LS-HL to human and mouse Fcγ receptors including FcγRI receptor (CD64), FcγRIIA (CD32A), FcγRIIB (CD32B) and FcγRIII (CD16).

[0179] Recombinant human or mouse Fc receptors (all from AcroBiosystesm) were dissolved at 5μg/mL in PBST and captured on individual Octet® SA StreptaAvidin Biosensors (Fortibio). The sensors were inserted into tubes containing PBST pH7.4 buffer with VPD1-HL, VPD1-3A-HL and VPD1-3A&LS-HL at concentrations of 0, 11, 33 and100 nM. The association time was set for 2 min and the dissociation was monitored for 5 min. The binding curves were recorded and kinetics parameters, such as, association (Ka), dissociation (Kd) constants and binding affinity (KD) and determined using 1:1 binding model. Compared with VPD1-HL, both VPD1-

3A-HL and VPD1-3A&LS-HL respectively had 240- and 16-fold reduction in binding to the human FcγRI (CD64).

[0180] In addition, VPD1-3A-HL also had almost no binding to the high affinity variant of human FcγRIII (CD16A, V176), whereas VPD1-3A&LS-HL had detectable but 2.5-fold lower binding. Interestingly, the impact on low-affinity CD16A variant (F176) was less clear. VPD1-3A&LS-HL also showed reduced binding to the human FcγRIIA (CD32A). VPD1-3A-HL and VPD1-3A&LS-HL also showed a 3-fold reduction binding to the FcγRIIB (CD32B).

Table 33 Binding Parameters to Human FcγRs

Captured human FcγR	**Loading Sample**	**ka (1/Ms)**	**kd (1/s)**	**KD (M)**
Biotinylated CD64	VPD1-HL VPD1-3A-HL VPD1-3A&LS-HL	1.568E5 1.694E4 1.819E5	3.147E-4 8.124E-3 5.996E-3	2.007E-9 4.795E-7 3.297E-8
Biotinylated CD32A, H167his	VPD1-HL VPD1-3A-HL VPD1-3A&LS-HL	1.094E5 6.958E4 2.902E4	1.627E-2 1.357E-2 1.250E-2	1.487E-7 1.951E-7 4.307E-7
Biotinylated CD32A, R167his	VPD1-HL VPD1-3A-HL VPD1-3A&LS-HL	1.491E5 5.330E4 4.161E4	8.613E-3 3.088E-3 4.826E-3	5.778E-8 5.795E-8 1.160E-7
Biotinylated CD32B/C his	VPD1-HL VPD1-3A-HL VPD1-3A&LS-HL	1.148E5 4.463E4 3.166E4	6.834E-3 8.464E-3 5.701E-3	5.953E-8 1.827E-7 1.801E-7
Biotinylated CD16A, F176his	VPD1-HL VPD1-3A-HL VPD1-3A&LS-HL	3.091E4 2.114E4 3.066E4	1.314E-2 1.126E-2 1.057E-2	4.251E-7 5.325E-7 3.446E-7
Biotinylated CD16A, V176his	VPD1-HL VPD1-3A-HL VPD1-3A&LS-HL	3.648E4 3.900E3 1.722E4	7.711E-3 5.734 9.016E-3	2.114E-7 3.900E-3 5.235E-7

[0181] As shown in Table 16, VPD1-3A-HL and VPD1-3A&LS-HL had essentially lost binding to mouse FcgRI (CD64). However, both mutants showed normal binding to mouse FcgRIII (CD16). Modest reduction was observed to mouse FcgRIIB (CD32B).

Table 34 Binding parameters to mouse FcγRs

Loading Sample	ka (1/Ms)	kd (1/s)	KD (M)
Biotinylated mouse CD64, his (5μg/mL)			
VPD1-HL	1.620E5	5.872E-3	3.624E-8
VPD1-3A-HL	1.122E2	2.324E-2	**2.071E-4**
VPD1-3A&LS-HL	1.473E3	7.461E-2	**5.065E-5**
Biotinylated mouse CD32b, his (5μg/mL)			
VPD1-HL	3.950E4	7.249E-3	1.835E-7
VPD1-3A-HL	3.426E4	3.502E-3	1.022E-7
VPD1-3A&LS-HL	2.837E4	1.218E-3	4.292E-8
Biotinylated mouse CD16, his (5μg/mL)			
VPD1-HL	2.030E4	1.310E-2	6.455E-7
VPD1-3A-HL	1.358E4	9.824E-3	7.235E-7
VPD1-3A&LS-HL	8.534E3	5.163E-3	6.050E-7

Example 15

Determination of the Bio-Potency of VPD1-3A&LS-HL in PD-1/PDL1 Reporter Assay

[0182] The bio-potency of VPD1-3A&LS-HL was examined in the PD-1/PDL1 reporter assay. As shown in **Figure 23**, VPD1-3A&LS-HL, VPD1-3A-HL and VPD3 had similar activity in this assay. The EC50 of VPD1-3A, VPD1-3A&LS-HL and VPD3 were measured at 3.78, 4.64 and 3.96 nM, respectively.

Example 16

Determination of the Bio-Potency of VPD1-3A&LS-HL in VEGF-VEGFR2 Blocking ELISA Assay

[0183] The bio-potency of VPD1-3A&LS-HL was also examined in the VEGF-VEGFR2 blocking ELISA assay. As shown in **Figure 24**, VPD1-3A&LS-HL had a similar high bio-potency as that of VPD1-3A-HL in blocking the binding of VEGF165 to VEGFR2. The IC50 of

VPD1-3A&LS-HL and VPD1-3A-HL were measured at 1.02 and 0.73nM, respectively, about 25- to 35-fold higher than that of VPD3-HL, which was measured at 25.73nM.

Example 17

Determination the Binding of Bispecific Antibodies VPD1-3A-HL or VPD1-3A&LS-HL to Plate Co-Coating with Both Human VEGF and PD-1 Antigens

[0184] Binding of bispecific antibodies VPD1-3A-HL or VPD1-3A&LS-HL to plate co-coated with both human VEGF and PD-1 antigens was determined by an ELISA assay. The ELISA method used in this example is similar to one described above, except that a mixture sample of two antigens (VEGF and PD-1, at 1:10 ration) was coated in the 96-well plates. As shown in **Figure 25,** VPD1-3A-HL showed about 2-fold higher binding to the mixture of VEGF165 and PD-1-his antigen (antigen coating ratio at 1:10) than Avastin/Panpulimab-derived VPD3 (AK112), while VPD1-3A&LS-HL had modestly better binding than VPD3.

Example 18

Presence of VEGF Increases the Potency of VPD1-3A-HL or VPD1-3A&LS-HL in PD-1 Cell Reporter Assay

[0185] The same Luciferase-PD-1/PD-L1 reporter cell-based assay was used here except that in the cell co-culture, human VEGF (VEGF165 from AcroBiosystems, used at final concentration at 0, 10, 100 and 100ng/mL) was added. **Figure 26** showed that the presence of human VEGF165 protein increased the potency of VPD1-3A-HL or VPD-1-3A&LS-HL in a dose-response manner, suggesting the co-operative effect of the two ligands for releasing PD-1-mediated immune suppression. In contrast, the co-operative effect was not observed when VPD3 is tested under the same condition.

Examples 19-22

Summary of *In Vivo* Anti-Tumor Activity

[0186] A series of *in vivo* experiments have been conducted to study the efficacy and safety of the various bispecific antibodies. The tables below describe the animal models, tumor cell lines used in these studies and the findings of each study. In most of the models, VPD1 derived bispecific antibodies such as VPD1-3A-HL, VPD1-3A-LH or VPD-3A&LS-HL generally

showed a better in-vivo anti-tumor efficacy than co-administration of parental anti-VEGF mAb (hPV19) or anti-PD-1 mAb (AI-025). Moreover, VPD1-3A-HL, VPD1-3A-LH or VPD-3A&LS-HL also showed a better efficacy than Bevacizumab/Penpulimab-derived bispecific antibody VPD3 (AK112) and VPD2-HL, another bispecific mAb with anti-VEGF mAb Bevacizumab as the IgG backbone.

Table 35 List of *in vivo* animal models and tested results

No.	**Tumor Model**	**Major finding and results**	**Example No. and Figures**
001	Human A673 in nude mice	VPD1-3A has a better efficacy than VPD2	Example 19 Figs. 27, 28
002	Human A375, NCG/human PBMC mice	VPD1-3A or VPD1-3A-LS has a better efficacy than combo of hPV19 and AI-025	Example 20 Figs. 29
003	Human A375, NCG/human PBMC mice, dose titration	VPD1-3A has a better efficacy than VPD3 (AK112)	Example 21 Figs. 30
004	MC38-hVEGF in human PD-1 gene knock-in C57BL/6 mice	VPD1-3A (HL) or VPD1-3A (LH) has a better efficacy than VPD3 (AK112)	Example 22 Figs. 31, 32

Example 19

Efficacy of Bispecific Antibodies in Nude Mice with Human A673 Xenograft Tumor

[0187] The in vivo anti-tumor activity of bispecific antibodies (VPD1-3A-HL and VPD2-HL) was tested in nude mice subcutaneously inoculated with human A673 tumor cells. A673 is a human rhabdomyosarcoma (Ewing tumor) cell line that grows in culture and forms tumors in immunodeficient mice. The A673 xenograft tumor model has been widely used in the study of

angiogenesis and response to anti-angiogenic agents such as Avastin (A4.6.1) or VEGF-trap (*Nature 1993;362:841-4, Cancer Res 1997, 57:4593, Toxicol Pathol 1999;27:14-21*).

[0188] In this example, A673 tumor cells (purchased from Shanghai Institute of Biochemistry and Cell Biology, SIBCB, www.cellbank.org.cn) were inoculated into 4-weeks old female BALB/c-Nude mice (BALB/cNj-Foxn1nu/Gpt, purchased from GemPharmatech, Naijing, China).

Table 36 Summary of mouse tumor model, treatment group and dosage

Group	**Dosage (mg/kg)**	**Number of mice**
Group 1: PBS (Vehicle control)	0	9
Group 2: VPD1-3A-HL	5 mg/kg, twice weekly	10
Group 3: VPD2-HL	5 mg/kg, twice weekly	10
Tumor model	Human A673 tumor cells subcutaneously inoculated into Bable/c nude mice, when tumor reached approximately 60-70mm^3, mice were randomly divided into 4 groups (n=9 or 10) and then received different treatment	
Treatment Schedule	Intraperitoneal injection (i.p.) once every 3-4 days, for a total of 5 injections; Mice were euthanized when tumor growing volume reached at 3000mm^3	

[0189] The A673 cells were cultured in T75 flasks in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FCS and 100U/mL penicillin and 100μg/mL streptomycin in a CO2 incubator (37°c, 5% CO_2). The nude mice were housed and maintained in the animal facility of Genpharma Co. Ltd. (Suzhou, China) under pathogen-free conditions.

[0190] To establish tumors in nude mice, A673 cells were collected from cultured flasks by trypsin treatment (TRYPSIN-0.25% EDTA, from Beyotime). After centrifugation, cell pellets were re-suspended at 1x10^7 cells/mL in 1xPBS, and 0.1 mL of the suspended cells was then immediately injected subcutaneously (s.c.) into the flank of each individual mouse (1x10^6 cells per mouse). When tumors grow to approximately 60-70mm^3 (6 to 7 days after tumor cell inoculation), mice were randomly divided into 4 different groups (n=9 or 10) and received the administration of PBS (the vehicle control), VPD1-3A-HL or VPD2-HL at a dose of 5mg/kg by intraperitoneal (i.p) injection twice weekly (once every 3-4 days) for a total of 5 doses.

[0191] Mice were observed for survival and signs of toxicity (mouse body weight was used as a reference index for indirect determination of drug toxicity), and tumor volume (V) was measured every 3-4 days (length × width) with a caliper. The tumor volume was calculated with the formula, $V= 0.5a \times b^2$, wherein "a" is the long diameter (length) and "b" is the short diameter (width) of tumor.

[0192] **Figure 27** shows the growth curve of human A673 tumors in the nude mice from different treatment groups (Data shown in the figure are mean±SEM), and **Table 19** shows the actual data (expressed as mean±SEM). In summary, the A673 tumors in the PBS vehicle control treatment group grew very rapidly, reaching an average of 2333mm^3 by Day 15 after the start of treatment. In the VPD1-3A-HL or VPD2-HL treatment groups, tumor growth was very significantly suppressed ($P<0.001$, Two-way ANNOVE analysis) with the mean tumor volume measured at Day 15 after the start of treatment only at 281 and 665mm^3, respectively. At the same dose of 5 mg/kg, VPD1-3A-HL showed a better efficacy than bevacizumab derived bispecific antibody VPD2-HL ($p=0.093$, Mann-Whitney test). Also as further shown in the **Figure 28**, the VPD1-3A-HL treatment group showed improved anti-tumor activity over the other two groups, with no tumor volume larger than 1000mm^3 observed during the whole period (up day 21 after tumor inoculation). Tumor Growth Volume in the Different Nude Mice with Human A673 Xenograft Tumor Treatment Groups (Pls remove HP19, as the data are confusing and it is meaningless point to make in a model where there is no human PD1.

Table 37 Tumor growth volume in different group after the treatment (Mean±SEM)

Days	Tumor volume (mm^3)		
	Vehicle (n=9)	VPD1-3A-HL 5 mg/kg (n=10)	VPD2-HL 5 mg/kg (n=10)
0[a]	41.22±11.95	44.70±11.65	45.00±10.93
4	276.00±95.99	94.10±37.42	207.30±81.62
8	772.44±170.80	412.50±117.71	331.90±131.14
12	1468.44±392.18	410.40±118.86	586.40±166.01
15	2333.56±28.07	281.17±136.75#	665.83±123.74

a: days after the start of the treatment

#: compared with VPD2-HL group, p=0.093 (Mann-Whitney test)

Example 20

Efficacy of VPD1-3A-HL or VPD1-3A&LS-HL in /NCG-hPBMC Mice with Human A375 Melanoma Xenograft Model: comparison with co-administration of anti-PD1 and anti-VEGF antibodies

[0193] To further test the anti-tumor effect of VPD1-3A-HL or VPD1-3A&LS-HL, human A375 tumor melanoma xenograft model was developed in immunodeficient NCG mice reconstituted with human peripheral blood mononuclear cells. A375 is a human melanoma cell line that grows in culture and forms a xenograft in immunodeficient mice. The A375 model has been widely used to study immune-checkpoint agents such as anti-PD-1/PD-L1 mAbs or bispecific antibodies such as anti-PDL1/VEGF-trap (Cell 2015, 162: 1242-1256;Nature Comm 2021;12:346; Front. Immunol. 2021, 12:778978).

[0194] A375 cells (purchased from Shanghai Institute of Biochemistry and Cell Biology SIBCB, www.cellbank.org.cn) were maintained and cultured in T75 flasks in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FCS and penicillin-streptomycin in a CO_2 incubator (37°C, 5% CO_2). The NCG mice (NOD/ShiLtJGpt-Prkdcem26Cd52Il2rgem26Cd22/Gpt, purchased from GemPharmatech, Naijing, China) were housed and maintained in the animal facility of Beigene Ltd. (GuangZhou, China) under pathogen-free conditions. The table below summarizes the tumor model and treatment schedule for each Group. **Figure 29** shows the growth curve of human A375 tumors in NCG/hPBMC mice treated with different antibodies (Data shown in the figure are mean±SD). As shown in **Figure 29**, the VPD1-3A-HL treatment group showed statistically (Two-way ANNOVE analysis) significant better anti-tumor activity than hPV19 ($P<0.05$,), AI-025 ($P<0.01$), and more importantly, also statistically very significant better than the combination of hPV19 and AI-025 ($P<0.01$). These data clearly demonstrate that VPD1-3A-HL has more potent anti-tumor activity than co-administration of anti-VEGF and anti-PD-1 antibodies.

Table 38 Summary of mouse tumor model, treatment group and schedule

Group	Dosage (mg/kg)	Number of mice
Group 1: PBS (Vehicle control)	0	10
Group 2: hPV19	5 mg/kg, twice weekly	10

Group 3: AI-025	5 mg/kg, twice weekly	10
Group 4: hPV19+AI-025	5 mg/kg each, twice weekly	10
Group 5: VPD1-3A-HL	6.6 mg/kg, twice weekly	10
Group 6: VPD3-3A&LS-HL	6.6 mg/kg, twice weekly	10
Tumor model	Human A375 melanoma cells subcutaneously inoculated into NCG mice reconstituted with human PBMCs, when tumors reached approximately 70 mm^3, mice were randomly divided into 6 groups (n= 10) and then received different treatment	
Treatment Schedule	Intraperitoneal injection (i.p.) once every 3-4 days, for a total of 6 injections	

Example 21

Dose-Titration Study in NCG-hPBMC Mice with Human A375 Melanoma Xenograft Model: Comparing the Efficacy of VPD1-3A-HL, VPD1-3A&LS-HL and VPD3 (AK112)

[0195] The same NCG-hPBMC mice with human A375 melanoma Xenograft was used in a dose-titration study for comparing the efficacy of VPD1-3A-HL andVPD3 (AK112). The design of the dose-titration study is shown the table below. As shown in **Figure 30**, VPD1-3A-HL has a statistically (Two-way ANNOVE analysis) significant better in-vivo anti-tumor efficacy than VPD3 at both 1.6 mg/kg ($P<0.01$) and 6.6 mg/kg ($P<0.05$).

Table 39 Dose-titration study: treatment group and dosage

Group	**Dosage (mg/kg)**	**Number of mice**
Group 1: PBS (Vehicle control)	0	10
Group 2: VPD1-3A-HL	1.6 mg/kg, twice weekly	11
Group 3: VPD1-3A-HL	6.6 mg/kg, twice weekly	11
Group 4: VPD3 (AK112)	1.6 mg/kg, twice weekly	11
Group 5: VPD3 (AK112)	6.6 mg/kg, twice weekly	11
Tumor model	Human A375 melanoma cells subcutaneously inoculated into NCG mice reconstituted with human PBMCs, when tumors reached approximately 70 mm3, mice were randomly divided into 5 groups (n=10 or 11) and then received different treatment	
Treatment Schedule	Intraperitoneal injection (i.p.) once every 3-4 days, for a total of 6 injections	

Example 22

Efficacy Study of VPD1-3A-HL, VPD1-3A-LH and VPD3 in Human PD-1 Knock-in C57BJ/B6 Mice transplanted with syngeneic MC38 Colon cancer cell line expressing human VEGF

[0196] MC38 is a mouse tumor cell line grows in culture and in immune-competent C57BJ/B6 mice. The MC38 model has been widely used to study immune-checkpoint inhibitors such as anti-PD-1 antibodies or anti-CTLA4 antibodies. In order to evaluate the anti-tumor activity of anti-human PD1 and anti-VEGF bispecific antibodies in a syngeneic mice with intact immune system, we used tumor cell MC38-hVEGF (cell line name: MC-38-188), which has been stably transfected with human a cDNA encoding VEGF165 having the sequence set forth in SEQ ID NO: 74 and a gene encoding GFP-Luc having the sequence set forth in SEQ ID NO: 75 (luciferase protein linked with EGFP protein by a 22 amino acid long-P2A peptide). This cell line consistently secreted high levels of human VEGF165 into the culture (up to 500 ng/mL) and rapidly formed tumor in C57BJ/B6 mice.

[0197] To evaluate anti-PD-1 activities, mouse MC38-hVEGF tumor cells (MC-38-188) were subcutaneously inoculated into human PD-1 knock-in C57J/B6 mice ($1x10^6$ cells per a mouse) and mice with tumors were randomly divided into different groups and received the treatment as shown in the table below. As shown in **Figure 31**, VPD1-3A-HL and VPD1-3A-LH all have statistically (Two-way ANNOVE analysis) significantly ($P<0.05$) better anti-tumor activity than that of VPD3 (AK112). Alas as shown in **Figure 32,** in either VPD1-3A-HL, VPD1-3A-LH, 5 of 6 mice showed tumor growth suppression or shanking; whereas in the VPD3 (AK112) treatment group only 3 of 6 mice showed the tumor growth suppression or shanking.

Table 40 Summary of mouse tumor model, treatment group and schedule

Group		**Dosage (mg/kg)**	**Number of mice**
Group 1: PBS (Vehicle control)		0	6
Group 2: VPD1-3A-HL		6.6 mg/kg, 3 times weekly	6
Group 3: VPD1-3A-LH		6.6 mg/kg, 3 times weekly	6
Group 4: VPD3 (AK112)		6.6 mg/kg, 3 times weekly	6
Tumor model	Mouse MC38-hVEGF tumor cells (MC-38-188) subcutaneously inoculated into human PD-1 knock-in C57J/B6 mice ($1x10^6$ cells per a mouse), when tumor reached approximately 60-70 mm^3, mice		

	were randomly divided into 4 groups (n=6) and then received treatment
Treatment Schedule	Intraperitoneal injection (i.p.) 3 times weekly, for a total of 4 injections

Example 23

Clinical study of a bi-specific anti-VEGF/PD-1 antibody

[0198] This example describes a clinical study on a bi-specific anti-VEGF/PD-1 antibody, which may be VPD1-3A-HL or VPD1-3A-LH, in patients with advanced solid tumors.

Indication

Monotherapy dose-escalation cohorts (Part A)

[0199] The study will include patients with a histologically or cytologically confirmed diagnosis of solid tumors who have locally advanced or metastatic disease and have failed or are intolerant to standard therapy, standard therapy does not confer survival benefit, or standard therapy is not available.

Dose expansion in colorectal and lung cancers (Part B)

[0200] Cancer patients will be recruited into three cohorts based on indications:

[0201] Cohort B1: Monotherapy dose expansion in patients with microsatellite stable (MSS) colorectal cancer (CRC) and progressed on first-line systemic therapy.

[0202] Cohort B2: Monotherapy dose expansion in PD-L1+ non-small cell lung carcinoma (NSCLC) (PD-L1 TPS $\geq$1%).

[0203] Cohort B3: Combination with chemotherapy: Dose expansion in extensive stage small cell lung carcinoma (ES-SCLC).

Summary of Rationale

[0204] PD(L)-1 and VEGF(R) are clinically validated targets for treatment of multiple cancer types. Combination therapies using agents targeting one of the two pathways have been approved for therapy of multiple cancers, including lung cancer, breast cancer, liver cancer and renal cancer. More recent studies have suggested that monotherapy using bispecific monoclonal antibodies targeting both PD(L)-1 and VEGF appeared to offer multiple benefit to cancer

patients, including convenience of administration, high tolerability-relating to better cancer-selective action and strong clinical activities attributed to co-operative interaction to targets in multiple cancer indications, particularly colorectal cancer and lung cancer.

[0205] The anti-VEGF/PD-1 antibody to be studied, referred to as AI-081, is a humanized monoclonal antibody with a functionally silenced Fc and two $F(ab)_2$ targeting PD-1 and VEGF. Due to the higher affinity of anti-PD-1 and anti-VEGF domains and better cooperative interaction to the two ligands, AI-081 exhibits 4-6-folder higher potency in blocking the PD-1 and VEGF receptors than other clinical stage PD(L)-1/VEGF bispecific antibodies. Preclinical toxicity studies in non-human primates have demonstrated that AI-081 is well tolerated at the highest dose tested (200 mg/kg).

[0206] This will be a Phase 1-2 study for evaluating the safety, pharmacokinetics (PK), and efficacy of AI-081 and it is consisting of two integrated parts: Part A is the first-in-human dose escalation study to determine the recommended Phase 2 dose (RP2D) of AI-081 monotherapy, while Part B are dose optimization trials comparing the safety and clinical activities of AI-081 at RP2D and one dose level lower than RP2D (RP2D-1), either as monotherapy or in combination therapy with standard of care (SOC) in selected indications.

[0207] In Part A, patients with solid tumors that have progressed on SOC will be enrolled for determining the safety, PK and clinical activities of AI-081 monotherapy in cancer patients. The RP2D of AI-081 will be identified through this part of the study.

[0208] To further evaluating the optimal dose of AI-081, patients with colorectal cancer or lung cancers will receive AI-081 at RP2D and RP2D-1 either as monotherapy or in combination with SOC chemotherapy in Part B. These indications are chosen for dose optimization as it has been reported that other agents of the same class have demonstrated promising efficacy signals in patients with microsatellite stable (MSS)/ proficient mismatch repair (pMMR) colorectal cancer, NSCLC and endometrial cancer with manageable toxicities. Thus, this suggests that these patients may benefit from a bispecific monoclonal antibody targeting both PD(L)1 and VEGF.

[0209] In Part B cohort B1, patients with MSS colorectal cancer and progressed on first-line systemic therapy, will be enrolled for monotherapy with AI-081. It has been reported that clinical response has been observed in patients with MSS/pMMR colorectal cancer treated with other agents of the same class.

[0210] In Part B cohort B2, patients with unresectable PD-L1+ NSCLC and are naïve to systemic therapy will be randomized to receive AI-081 monotherapy at either RP2D or RP2D-1 dose levels. A primary analysis of an agent of the same class suggests that these patients may benefit from AI-081 monotherapy in NSCLC patients with PD-L1 TPS ≥1%.

[0211] In Part B cohort B3, patients with unresectable SCLC and are naïve to systemic therapy will be randomized to receive SOC chemotherapy in addition to AI-081 at either RP2D or RP2D-1 dose levels. Clinical response has been reported in SCLC patients using agents of the same class, suggests that these patients have higher potential to benefit from AI-081 when used in combination with chemotherapy.

Summary of Study Design

Part A: First-in-human dose escalation

[0212] This is a Phase I open label, dose-escalation study of AI-081 as a single agent in patients with advanced/metastatic solid tumors. The study will evaluate five dose levels of AI-081 starting from subtherapeutic dose of 0.3 mg/kg and 1.0 mg/kg in single patient dose escalation, and 3.0 mg/kg, 10 mg/kg and 20 mg/kg using traditional 3+3 study design. Single patient dose escalation is planned for the first 2 dose levels. Intra-patient dose escalation (IPDE) should occur only if ≤ Grade 2 toxicity was observed during the previous treatment cycle and could potentially be escalated in each cycle with different dose level up to the dose level of 3 mg/kg. The decision to allow for IPDE should be made after considering the potential safety profile of the investigational drug including cumulative toxicity. The study will be changed to conventional 3+3 study design when grade 2 or greater AE, unless it is unequivocally due to the underlying disease or an extraneous cause, occurred or reached 3.0 mg/kg dose level, whichever comes first. The maximal tolerant dose (MTD) is defined as the highest dose level at which DLT is observed in 1 out of 6 subjects or in more than 1 but <30% of subjects if more than 6 patients are enrolled at that dose level. If MTD is defined, the recommended Phase II dose (RP2D) is defined as the same dose level as that of MTD. If the MTD has not been reached in the five dose levels tested, the highest dose level with no DLT among at least 6 patients will be declared as the RP2D.

Part B: Dose optimization: monotherapy or combination with chemotherapy in colorectal and lung cancer indications

[0213] Part B is a Phase 2 dose optimization study that will categorize patients into three cohorts based on cancer indications. Cohort B1 will randomize second-line (2L) CRC patients who progressed on prior systemic therapy and will receive monotherapy of AI-081. Treatment-naïve PD-L1+ NSCLC patients in Cohort B2 and treatment-naïve ES-SCLC patients in Cohort B3 will receive AI-081 as monotherapy and in combination with SOC chemotherapy, respectively. Each cohort will consist of 58 patients (29 patients per arm) who will be randomized into RP2D or RP2D-1 dose levels at 1:1 ratio.

[0214] An interim safety review will be performed when the 10th patients in each arm in the three cohorts have received at least 2 doses of AI-081. If the number of DLT in an arm has reached the threshold specified in the stopping rule, the enrollment to that arm will be stopped but enrollment will continue in the other arm. If the number of DLT in both arms has reached the threshold specified in the stopping rule, the enrollment of both arms will be stopped and dose reduction to two dose level below RP2D (RP2D-2) and three dose level below RP2D (RP2D-3) will be executed.

Key Study Eligibility Criteria

[0215] The following describe the study eligibility criteria.

Table 41 Eligibility Criteria for Part A

Inclusion Criteria	**Exclusion Criteria**
1. Age ≥ 18 years old. 2. Male or Female 3. Must have ECOG score ≤ 1. 4. A histological or cytological diagnosis of solid tumors and metastatic disease or locally advanced disease 5. Must have measurable target lesion according to RECIST V1.1. 6. Adequate organ function as determined by laboratory tests. 7. Voluntary agreement to participate as evidenced by written informed consent. 8. Female patient: negative pregnancy test and agreement on contraceptive methods. 9. Male patient: agreement on contraceptive methods.	1. Patients who have not recovered to NCI CTCAE grade ≤ 1 from an adverse event (AE) due to cancer therapeutics except the chemotherapy-associated peripheral neuropathy (motor or sensory) or alopecia. Patients with ongoing and adequately controlled endocrine immune-related AEs are considered stable and eligible for enrollment. The washout period for treatment regimen containing monoclonal antibodies is 28 days. Palliative radiotherapy for painful metastases or metastases in potentially sensitive locations (e.g., epidural space) ≥ 7 days prior to the first dose of study drug. Best supportive care, such as thyroxine, insulin, steroid replacement treatment, blood transfusion and therapy for non-cancer condition are allowed. 2. Patients who are currently enrolled in any other clinical trial testing an investigational agent or device, or with concurrent anticancer treatment (except palliative bone-directed radiotherapy), immune therapy, or cytokine therapy or

10. Agree to give archival or other diagnostic tissue recut slides or an optional new tumor biopsy.	anticipated to require another antineoplastic therapy during the study. 3. Patients who are on chronic systemic steroid therapy at doses higher than 10 mg/day prednisone or equivalent within 7 days before first treatment. 4. Patients who have brain metastases or leptomeningeal metastases. 5. Patient with a different cancer other than the one treated under this protocol, which requires systemic treatments within 24 months prior to C1D1. 6. Patient has history of grade ≥3 allergic or hypersensitivity to IV infusion medications, or severe allergic reactions to food, pollen, oral medications, or atopic dermatitis or asthmatic episodes that required hospitalization. 7. Within past 6 months with history of significant cardiovascular acute myocardial infarction, acute coronary syndrome, ischemic or hemorrhagic stroke, revascularization procedures, acute pulmonary embolism or any disorders resulted in LVEF < 40% at the time of screening or colitis, small bowel obstruction, hepatitis or pancreatitis adrenal insufficiency, or severe immunotherapy related AE (irAE≥ grade 3). 8. Patients who have acute infections which require systemic treatments within 14 days prior to C1D1. 9. Patients who, in the opinion of the treating Investigator, have a history or current evidence of any condition, therapy, or laboratory abnormality that might confound the results of the study, interfere with the patient's participation for the full duration of the study, or make study participation not in the best interest of the patient, in the opinion of the treating Investigator. Investigators should discuss the case with the Sponsor and/or study leaders. 10. Patients with known psychiatric or substance abuse disorders may interfere with cooperation with the requirements of the trial. 11. Patients who are pregnant or breastfeeding or plan pregnancy or fathering the child during the study or within 6 months after the last dosing of study drug 12. Patients with tumor surrounds important blood vessels or has obvious necrosis, cavitation, or invades surrounding important organs and blood vessels or otherwise with high risk of fatal hemorrhage 13. Uncontrolled hypertension: systolic pressure ≥ 150 millimeters of mercury (mmHg) or diastolic pressure ≥ 90 mmHg on repeated measurements that cannot be managed by standard antihypertension medications ≤ 28 days before the first dose of study drug(s). 14. Medical history of cardiovascular diseases, gastrointestinal perforation or gastrointestinal fistula within 6 months prior to the first dose. 15. Patients with clinically symptomatic pleural effusion, pericardial effusion, or ascites requiring frequent drainage. 16. With a history of interstitial lung disease, non-infectious pneumonitis, or uncontrolled systemic diseases, including diabetes, hypertension, pulmonary fibrosis, acute lung diseases, etc.

Table 42 Eligibility Criteria for Part B

Inclusion Criteria	**Exclusion Criteria**
1. Age ≥ 18 years old. 2. Male or Female 3. Must have ECOG score ≤ 1. 4. Patients should meet the following criteria defined in each cohort: a) Cohort B1: A histologically confirmed diagnosis of unresectable and metastatic colorectal cancer (CRC). b) Cohort B2: A histological or cytological diagnosis of PD-L1+ NSCLC with unresectable metastatic disease or locally advanced disease. c) Cohort B3: Histologically or cytologically confirmed extensive-stage small cell lung cancer (ES-SCLC) 5. Must have measurable target lesion according to RECIST V1.1. 6. Adequate organ function as determined by laboratory. 7. Voluntary agreement to participate as evidenced by written informed consent. 8. Female patient: negative pregnancy test and agreement on contraceptive methods. 9. Male patient: agreement on contraceptive methods. 10. Agree to give archival or other diagnostic tissue recut slides or an optional new tumor biopsy.	1. Patients who have the following conditions based on the cohorts: a. Cohort B1: Tumor is MSI-H/dMMR per a standard local testing method. b. Cohort B2 and B3: i. Cancer tissue is positive for actional genomic alterations in EGFR, HER2, ROS, and ALK. ii. Patient with prior systemic therapy. 2. Patients who are currently enrolled in any other clinical trial testing an investigational agent or device, or with concurrent anticancer treatment (except palliative bone-directed radiotherapy), immune therapy, or cytokine therapy or anticipated to require another antineoplastic therapy during the study. 3. Patients who are on chronic systemic steroid therapy at doses higher than 10 mg/day prednisone or equivalent within 7 days before first treatment. 4. Patients who have brain metastases or leptomeningeal metastases. 5. Patient with a different cancer other than the one treated under this protocol, which requires systemic treatments within 24 months prior to C1D1. 6. Patient has history of grade ≥3 allergic or hypersensitivity to IV infusion medications, or severe allergic reactions to food, pollen, oral medications, or atopic dermatitis or asthmatic episodes that required hospitalization. 7. Within past 6 months with history of significant cardiovascular acute myocardial infarction, acute coronary syndrome, ischemic or hemorrhagic stroke, revascularization procedures, acute pulmonary embolism or any disorders resulted in LVEF < 40% at the time of screening or colitis, small bowel obstruction, hepatitis or pancreatitis adrenal insufficiency, or severe immunotherapy related AE (irAE≥ grade 3). 8. Patients who have acute infections which require systemic treatments within 14 days prior to C1D1. 9. Patients who, in the opinion of the treating Investigator, have a history or current evidence of any condition, therapy, or laboratory abnormality that might confound the results of the study, interfere with the patient's participation for the full duration of the study, or make study participation not in the best interest of the patient, in the opinion of the treating Investigator. Investigators should discuss the case with the Sponsor and/or study leaders. 10. Patients with known psychiatric or substance abuse disorders may interfere with cooperation with the requirements of the trial. 11. Patients who are pregnant or breastfeeding or plan pregnancy or fathering the child during the study or within 6 months after the last dosing of study drug 12. Patients with tumor surrounds important blood vessels or has obvious necrosis, cavitation, or invades surrounding important organs and blood vessels or otherwise with high risk of fatal hemorrhage

	13. Uncontrolled hypertension: systolic pressure ≥ 150 millimeters of mercury (mmHg) or diastolic pressure ≥ 90 mmHg on repeated measurements that cannot be managed by standard antihypertension medications ≤ 28 days before the first dose of study drug(s). 14. Medical history of gastrointestinal perforation or gastrointestinal fistula within 6 months prior to the first dose. 15. Patients with clinically symptomatic pleural effusion, pericardial effusion, or ascites requiring frequent drainage. 16. With a history of interstitial lung disease, non-infectious pneumonitis, or uncontrolled systemic diseases, including diabetes, hypertension, pulmonary fibrosis, acute lung diseases, etc.

Dosage/Dosage Form, Route, and Dose Regimen

[0216] In Part A, 5 dose levels of AI-081 will be evaluated: 0.3 mg/kg, 1.0 mg/kg, 3.0 mg/kg, 10 mg/kg, and 20 mg/kg.

[0217] AI-081 will be administered via IV infusion over approximately 30 minutes for dose levels of 0.3 mg/kg and 1.0 mg/kg, a minimum of 60 minutes for 3.0 mg/kg and 10 mg/kg, and a minimum of 120 minutes for 20 mg/kg dose level. The AI-081 dosing interval will be 21 days ± 3 days (every 3 weeks [Q3W]).

[0218] The study will be carried out by using an accelerated titration design. Single patient will be dosed at lower dose levels 1 and 2 (0.3 mg/kg and 1.0 mg/kg). Intra-patient dose escalation should occur only if ≤ Grade 2 toxicity was observed during the previous treatment cycle and could potentially be escalated in each cycle with different dose level up to the dose level of 3 mg/kg. The decision to allow for IPDE should be made after considering the potential safety profile of the investigational drug including cumulative toxicity. There will be up to a total of 17 cycles in 12 months. The pre-dose ADA will be tested at all cycles.

[0219] (1) The first subject will be dosed at dose level 1 (0.3 mg/kg, Q3W). With a minimal of 21 days observation without any ≥ grade 2 adverse event (AE), unless it is unequivocally due to the underlying disease or an extraneous cause, the same subject may be dosed at dose level 2 (1.0 mg/kg, Q3W) at C2D1. If no ≥ grade 2 AE in cycle 2, the same subject may be dosed at dose level 3 (3.0 mg/kg, Q3W) at C3D1. The first subject may continue the dose escalation if there is no ≥ grade 2 AE. The study will proceed to dose level 3 with three subjects at 3 mg/kg, Q3W with a minimal of 1 day apart in each first dose. The study will continue as conventional 3+3 design and subjects at each dose level will complete at least 21 days DLT observation period

before the next dose escalation. For 3 subjects at dose level 3, if there is no ≥ grade 2 AE, intra-patient dose escalation is allowed.

[0220] (2) If the first subject at the 2 lower dose levels has any Grade 2 or greater AE, unless it is unequivocally due to the underlying disease or an extraneous cause, during the DLT period (Cycle 1, 21 days), the study will be converted to 3+3 study design. Two additional subjects will be dosed at the same dose level as the first subject who had Grade 2 or greater AE.

[0221] (3) After converted to 3+3 design, for each of the 3 subjects, the subject without ≥ grade 2 AE in cycle 1 will be allowed to escalate to the next dose level at cycle 2. Such subject may continue the intra-patient dose escalation in subsequent cycles till one dose level lower than the final dose level. The subject that has any grade 2 AE or non-DLT grade 3 AE will continue the treatment at the prior dose level if the AE is resolved to grade 1 or 0 within 14 days. The subject that has any DLT will discontinue the treatment and exit the study. Additional subjects will be enrolled to maintain the minimal of 3 subjects in each dose level in the 3+3 design. The final dose level will have 6 subjects.

[0222] (4) If there is one DLT event in the 3 subjects at any dose level, additional 3 subjects will be enrolled and dosed at the same dose level. If no more than 1 in 6 subjects has DLT, the study will be escalated to the next dose level. The highest dose with 1/6 DLT will be defined as MTD.

[0223] (5) If there are 2 or more subjects out of 6 subjects who have DLT at any dose level, dose de-escalation will be initiated.

[0224] (6) Dose de-escalation will be conducted by selecting intermediate dose level at 13 mg/kg if 2 or more DLT was observed at 20 mg/kg dose level. An intermediate dose level at 6 mg/kg may be selected if 2 or more DLT were observed at 10 mg/kg dose level. After dose de-escalation, 6 subjects will be enrolled at that intermediate dose level.

[0225] (7) The RP2D is defined as the MTD if MTD is reached. If MTD is not reached, the maximum dose level with no DLT among at least 6 subjects will be defined as the RP2D.

[0226] There will be up to a total of 17 cycles in 12 months.

[0227] In Part B, patients will receive AI-081 at RP2D or RP2D-1 through intravenous infusion. The duration of infusion is the same as described for Part A.

[0228] Study treatment should be stopped for unacceptable toxicity, confirmed disease progression, voluntary withdrawal by the patient, or at 1 year (17 cycles), whichever occurs first. Dose reduction is allowed in Part B if the number of DLT reached pre-specified stopping rules.

Patient Inclusion Criteria

[0229] 1) Patient is ≥ 18 years of age on the day of signing informed consent.

[0230] 2) Male or female, female patient of childbearing potential must have negative pregnancy test.

[0231] 3) Patient must have a performance status of ≤ 1 on the ECOG Performance Scale.

[0232] 4) Patients must have a histological or cytological diagnosis of solid tumors and have metastatic disease or locally advanced disease.

[0233] (a) In the Part A dose escalation study of AI-081 monotherapy, patients with advanced/metastatic solid tumors, with measurable disease as determined by RECIST 1.1, who have progressed or are intolerant to standard therapy, standard therapy does not confer survival benefit, or standard therapy is not available, are eligible for participation.

[0234] (b) In the Part B dose optimization study, patients should meet the following criteria defined in each cohort:

[0235] i. Cohort B1: Locally advanced and metastatic colorectal cancer (CRC).

[0236] a. Patients must have received one line of prior systemic therapy in recurrent or metastatic setting. Patients who received adjuvant chemotherapy and had recurrence during or within 6 months of completion of the adjuvant chemotherapy can count this as a line of therapy.

[0237] b. Patients must be treatment naïve from VEGF-targeting therapy.

[0238] c. The tumor must have been assessed as microsatellite stable (MSS) status per a standard local testing method.

[0239] d. No growth factor support, transfusions, or albumin administration within 14 days of randomization of study treatment.

[0240] ii. Cohort B2: Locally advanced or metastatic NSCLC.

[0241] a. Patients must have a PD-L1 tumor score of at least 1% (PD-L1 TPS ≥ 1%).

[0242] iii. Cohort B3: Extensive-stage small cell lung cancer (ES-SCLC).

[0243] a. This stage of SCLC is defined as Stage IV [Tany, Nany, M1a/b] or T3-4 due to multiple lung nodules that were too extensive or had tumor/nodal volume that was too large to be

encompassed in a tolerable radiation plan by the American Joint Committee on Cancer, Seventh Edition.

[0244] 5) Measurable disease as determined by RECIST 1.1 (either a or b or both):

[0245] a. Tumor mass: Must be accurately measurable in at least 1 dimension (longest diameter to be recorded) with a minimum size of:

[0246] 1. 10 mm by computed tomography (CT) scan (CT scan slide thickness must be <5 mm),

[0247] Or:

[0248] 2. 20 mm by chest X-ray (if clearly defined and surrounded by aerated lung).

[0249] b. Malignant lymph nodes: ≥15 mm in short axis when assessed by CT scan (CT scan slice thickness must be <5 mm). The measurement should be two dimensions at axial plane. The short axis should be in perpendicular to long diameter.

6) Patient must have adequate organ function as indicated by the following laboratory values:

Table 43 Laboratory values indicative of adequate organ function

System	**Laboratory Value**
Hematological	
Absolute neutrophil count (ANC)	≥ 1,500 /μL
Platelets[a]	≥ 100,000 / μL
Hemoglobin[a]	≥ 9 g/dL or ≥5.6 mmol/L
Renal	
eGFR (with 2021 CKD-EPI Creatinine equation)	≥ 30 mL/minute
Hepatic	
Serum total bilirubin	≤ 1.5 X ULN OR Direct bilirubin ≤ ULN for patients with total bilirubin levels > 1.5 ULN
Aspartate transaminase (AST) and alanine transaminase (ALT)	≤ 3 X ULN OR ≤ 5 X ULN for patients with active liver metastases
General	
LDH	≤ ULN
[a]Criteria must be met without packed red blood cell (pRBC) transfusion within last 2 weeks. Patients can be on stable dose of erythropoietin (≥ approximately 3 months). pRBC or platelet transfusion as best supportive care in the study is allowed and there is no waiting period for next dosing.	

[0250] 7) Patient has voluntarily agreed to participate by giving written informed consent.

[0251] 8) Female patients enrolled in the study, if having childbearing potential (WOCBP) and sexually active, must agree to use adequate and effective birth control starting with the first dose of study drug through 90 days after the last dose of study therapy.

[0252] 9) Male patients, if sexually active, must agree to use adequate and effective methods of contraception starting with the first dose of study drug through 90 days after the last dose of study therapy.

Patient Exclusion Criteria

[0253] A patient meeting any of the following criteria is not eligible to participate in this study:

[0254] 1) Patients who have the following conditions based on the cohorts:

[0255] (a) In Part A:

[0256] i. Patients who have not recovered to NCI CTCAE Grade 1 or better from AEs due to cancer therapeutics except chemotherapy associated peripheral neuropathy (motor or sensory), or alopecia, or endocrine related AE, in which recovery to ≤ Grade 2 is allowed. The washout period for cancer therapeutic drugs (such as chemotherapy, radiation, or targeted therapy) is 21 days. The washout period for treatment regimen containing monoclonal antibodies is 28 days. Palliative radiotherapy for painful metastases or metastases in potentially sensitive locations (e.g., epidural space) ≥ 7 days prior to the first dose of study drug. Best supportive care, such as thyroxine, insulin, steroid replacement treatment, blood transfusion and therapy for non-cancer condition are allowed.

[0257] (b) In Part B Cohort B1:

[0258] i. Tumors is MSI-H/dMMR per a standard local testing method.

[0259] ii. Received any immune checkpoint inhibitor or experimental immunologic agents.

[0260] iii. Received regorafenib or trifluridine/tipiracil as prior therapy(ies).

[0261] iv. Partial or complete bowel obstruction within the last 3 months, signs/symptoms of bowel obstruction, or known radiologic evidence of impending obstruction.

[0262] v. Refractory ascites.

[0263] vi. Liver metastases by computed tomography or magnetic resonance imaging. Note: Patients with definitively treated liver metastases (this includes surgical resection, including microwave or radiofrequency ablation, or stereotactic body radiation therapy, but not yttrium-90

or chemotherapy alone) may be eligible if they were treated at least 6 months prior to enrollment with no evidence of metastatic disease in the liver on subsequent imaging.

[0264] vii. Patients who require treatment with strong cytochrome P450 3A4 inducers or inhibitors.

[0265] (c) In Part B Cohort B2 and B3:

[0266] i. Cancer tissue is positive for actional genomic alterations in EGFR, HER2, ROS, and ALK.

[0267] ii. Patients who had received prior systemic therapy.

[0268] 2) Patients who are currently enrolled in any other clinical trial testing an investigational agent or device, or with concurrent anticancer treatment (except palliative bone-directed radiotherapy), chemotherapy, immunotherapy, or cytokine therapy or anticipated to require another antineoplastic therapy during the study.

[0269] 3) Patients who are on chronic systemic steroid therapy at doses higher than 10 mg/day prednisone or equivalent within 7 days before first treatment.

[0270] 4) Patients who have brain metastases or leptomeningeal metastases.

[0271] 5) Patient with a different cancer other than the one treated under this protocol, which requires systemic treatments within 24 months prior to C1D1.

[0272] 6) Patient has history of grade $\geq$3 allergic or hypersensitivity to IV infusion medications, or severe allergic reactions to food, pollen, oral medications, or atopic dermatitis or asthmatic episodes that required hospitalization.

[0273] 7) Patients with tumor surrounds important blood vessels or has obvious necrosis, cavitation, or invades surrounding important organs and blood vessels or otherwise with high risk of fatal hemorrhage.

[0274] 8) Medical history of cardiovascular diseases, gastrointestinal perforation or gastrointestinal fistula within 6 months prior to the first dose.

[0275] 9) Patients with clinically symptomatic pleural effusion, pericardial effusion, or ascites requiring frequent drainage.

[0276] 10) Within past 6 months with history of significant cardiovascular acute myocardial infarction, acute coronary syndrome, ischemic or hemorrhagic stroke, revascularization procedures, acute pulmonary embolism or any disorders resulted in LVEF < 40% at the time of screening.

[0277] 11) Patients who have acute infections which require systemic treatments within 14 days prior to C1D1.

[0278] 12) With a history of interstitial lung disease, non-infectious pneumonitis, or uncontrolled systemic diseases, including diabetes, hypertension, pulmonary fibrosis, acute lung diseases, etc.

[0279] 13) Patients with known psychiatric or substance abuse disorders may interfere with cooperation with the requirements of the trial.

[0280] 14) Patients who, in the opinion of the treating Investigator, have a history or current evidence of any condition, therapy, or laboratory abnormality that might confound the results of the study, interfere with the patient's participation for the full duration of the study, or make study participation not in the best interest of the patient, in the opinion of the treating Investigator.

[0281] 15) Patients who are pregnant or breastfeeding or plan pregnancy or fathering the child during the study or within 6 months after the last dosing of study drug.

[0282] 16) Uncontrolled hypertension: systolic pressure $\geq$ 150 millimeters of mercury (mmHg) or diastolic pressure $\geq$ 90 mmHg on repeated measurements that cannot be managed by standard antihypertension medications $\leq$ 28 days before the first dose of study drug(s).

TREATMENT PLAN AND TIMING OF PROCEDURES

Summary of the Study Design

[0283] This will be a first-in-human open-label dose escalation and dose optimization study of AI-081 in patients with advanced/metastatic solid tumors.

[0284] This study consists of two parts:

[0285] (1) Part A (FIG. 34) is a phase I dose escalation study of AI-081 monotherapy in patients with advanced/metastatic solid tumors. The objective of Part A is to define the RP2D of AI-081 monotherapy.

[0286] (2) Part B is a phase II dose optimization study of AI-081 that consists of three, parallel, dose expansion cohorts to compare the efficacy and safety of AI-081 as monotherapy or in combination with chemotherapy at RP2D and one dose level lower than RP2D (RP2D-1).

[0287] a. Cohort B1 (FIG. 35) will enroll metastatic CRC patients who had progressed after one line of systemic therapy.

[0288] b. Cohort B2 (FIG. 36) will enroll metastatic PD-L1+ NSCLC patients who have not received any systemic therapy.

[0289] c. Cohort B3 (FIG. 37) will enroll extensive-stage SCLC patients who have not received any systemic therapy.

[0290] Study treatment (both monotherapy and combination therapy) may be continued for additional cycles (optional) after a patient has confirmed progressive disease (PD) based on iRECIST if the patient tolerates the treatment and study clinical investigator considers there is potential clinical benefit.

[0291] Study treatment (both monotherapy and combination therapy) should be stopped for unacceptable toxicity, voluntary withdrawal by the patient, or at 1 year (17 cycles), whichever occurs first.

Part A: Phase I Dose Escalation of AI-081 Monotherapy

[0292] In Part A, patients with solid tumors that have progressed on SOC will be enrolled for determining the safety, PK and clinical activities of AI-081 monotherapy in cancer patients. Five dose levels of AI-081 will be evaluated: 0.3 mg/kg, 1.0 mg/kg, 3.0 mg/kg, 10 mg/kg and 20 mg/kg.

[0293] AI-081 will be administered via IV infusion over approximately 30 minutes for dose levels of 0.3 mg/kg and 1.0 mg/kg, a minimum of 60 minutes for 3.0 mg/kg and 10 mg/kg, and a minimum of 120 minutes for 20 mg/kg dose level. The AI-081 dosing interval will be 21 days ± 3 days (every 3 weeks [Q3W]).

[0294] The study will be carried out by using an accelerated titration design. Single patient will be dosed at lower dose level 1 and 2 (0.3 mg/kg and 1.0 mg/kg). Intra-patient dose escalation should occur only if ≤ Grade 2 toxicity was observed during the previous treatment cycle and could potentially be escalated in each cycle with different dose level up to the dose level of 3 mg/kg. The decision to allow for IPDE should be made after considering the potential safety profile of the investigational drug including cumulative toxicity. The pre-dosing plasma ADA will be tested at all cycles.

[0295] (1) The first subject will be dosed at dose level 1 (0.3 mg/kg, Q3W). With a minimal of 21 days observation without any ≥ grade 2 adverse event (AE), unless it is unequivocally due to the underlying disease or an extraneous cause, the same subject may be dosed at dose level 2 (1.0 mg/kg, Q3W) at C2D1. If no ≥ grade 2 AE in cycle 2, the same subject may be dosed at

dose level 3 (3.0 mg/kg, Q3W) at C3D1. The first subject may continue the dose escalation if there is no ≥ grade 2 AE. The study will proceed to dose level 3 with three subjects at 3 mg/kg, Q3W with a minimal of 1 day apart in each first dose. The study will continue as conventional 3+3 design and subjects at each dose level will complete at least 21 days DLT observation period before the next dose escalation. For 3 subjects at dose level 3, if there is no ≥ grade 2 AE, intra-patient dose escalation is allowed.

[0296] (2) If the first subject at the 2 lower dose levels has any Grade 2 or greater AE, unless it is unequivocally due to the underlying disease or an extraneous cause, during the DLT period (Cycle 1, 21 days), the study will be converted to 3+3 study design. Two additional subjects will be dosed at the same dose level as the first subject who had Grade 2 or greater AE.

[0297] (3) After converted to 3+3 design, for each of the 3 subjects, the subject without ≥ grade 2 AE in cycle 1 will be allowed to escalate to the next dose level at cycle 2. Such subject may continue the intra-patient dose escalation in subsequent cycles till one dose level lower than the final dose level. The subject that has any grade 2 AE or non-DLT grade 3 AE will continue the treatment at the prior dose level if the AE is resolved to grade 1 or 0 within 14 days. The subject that has any DLT will discontinue the treatment and exit the study. Additional subjects will be enrolled to maintain the minimal of 3 subjects in each dose level in the 3+3 design. The final dose level will have 6 subjects.

[0298] (4) If there is one DLT event in the 3 subjects at any dose level, additional 3 subjects will be enrolled and dosed at the same dose level. If no more than 1 in 6 subjects has DLT, the study will be escalated to the next dose level. The highest dose with 1/6 DLT will be defined as MTD.

[0299] (5) If there are 2 or more subjects out of 6 subjects who have DLT at any dose level, dose de-escalation will be initiated.

[0300] (6) Dose de-escalation will be conducted by selecting intermediate dose level at 13 mg/kg if 2 or more DLT was observed at 20 mg/kg dose level. An intermediate dose level at 6 mg/kg may be selected if 2 or more DLT were observed at 10 mg/kg dose level. After dose de-escalation, 6 subjects will be enrolled at that intermediate dose level.

[0301] (7) The RP2D is defined as the MTD if MTD is reached. If MTD is not reached, the maximum dose level with no DLT among at least 6 subjects will be defined as the RP2D.

[0302] There will be up to a total of 17 cycles in 12 months.

[0303] The maximal tolerant dose (MTD) is defined as the highest dose level at which DLT is observed in 1 out of 6 subjects or in more than 1 but <30% of subjects if more than 6 patients are enrolled at that dose level. If MTD is defined, the recommended Phase II dose (RP2D) is defined as the same dose level as that of MTD. If the MTD has not been reached in the five dose levels tested, the highest dose level with no DLT among at least 6 patients will be declared as the RP2D.

[0304] At the end of Part A, SRC meeting will be held for safety review and to determine the RP2D of AI-081.

[0305] 5.1.2 Part B: Phase II Dose Optimization of AI-081 Monotherapy and Combination therapy with Chemotherapy

[0306] In Part B, patients will be categorized into three cohorts based on the specified cancer indications to determine the efficacy and safety of AI-081 as monotherapy or in combination with chemotherapy, by using the RP2D determined from Part A. Part B of this study will only commence when RP2D is determined from Part A.

[0307] Cohort B1 will randomize 2L CRC patients who progressed on prior systemic therapy and will receive monotherapy of AI-081. Cohort B2 will randomize PD-L1+ NSCLC; while Cohort B3 will enroll ES-SCLC. Treatment-naïve patients who enrolled in Cohort B2 and B3 will receive AI-081 at either RP2D or RP2D-1 levels in combination with SOC chemotherapy. Each cohort will consist of 58 patients who will be randomized into RP2D or RP2D-1 dose levels at 1:1 ratio.

[0308] An interim safety review will be performed when the 10th patients in each arm in the three cohorts have received at least 2 doses of AI-081. If the number of DLT in an arm has reached the threshold specified in the stopping rule, the enrollment to that arm will be stopped but enrollment will continue in the other arm. If the number of DLT in both arm has reached the threshold specified, the enrollment of both arms will be stopped and dose reduction to two dose level below RP2D (RP2D-2) and three dose level below RP2D (RP2D-3) will be executed.

AI-081 Information and Administration

Description of AI-081

[0309] AI-081 is supplied as a sterile, clear, colorless to slight brown, preservative-free aqueous solution for parenteral administration.

Physical Form

[0310] AI-081 is supplied as a sterile, clear, colorless to slight brown, preservative-free aqueous solution for parenteral administration.

Dosage Formulation

[0311] AI-081 has been formulated as single dose injection solution, at a protein concentration of 20 mg/mL. Each AI-081 vial contains 200 mg of AI-081 in an extractable volume of 10 mL formulated in 20 mM histidine buffer, 9% (w/v) trehalose dihydrate, and 0.04% (w/v) polysorbate 80, at pH 5.5.

Route of Administration

[0312] AI-081 will be administrated by intravenous infusion over a minimum of 30-120 minutes depending on dose levels (30 minutes for 0.3 mg/kg and 1 mg/kg, and a minimum of 60 minutes for 3 mg/kg and 10 mg/kg and a minimum of 120 minutes for 20 mg/kg), Q3W.

Packaging, Storage and Stability

[0313] AI-081 is supplied in clear borosilicate glass vials with chlorobutyl rubber stoppers and aluminum flip off seals. AI-081 is stored refrigerated at 2-8° C until use. Stability studies of AI-061 at long term storage temperature of 5 ± 3 °C and under accelerated stability conditions (25 ± 2 °C / 60 ± 5% RH are ongoing.

Drug Preparation for Clinical Use

[0314] The dose of AI-081 for administration must be prepared using aseptic technique. +

AI-081 IV Infusion

[0315] All patients must have pre-medication in first treatment of AI-081 to prevent infusion reaction. AI-081 IV infusion will be given approximately 30 minutes for dose levels of 0.3 mg/kg and 1.0 mg/kg, a minimum of 60 minutes for 3.0 mg/kg and 10 mg/kg, and a minimum of 120 minutes for 20 mg/kg dose level.

[0316] The drug product of AI-081 has the concentration of 20 mg/mL. The drug product should be diluted with normal saline solution to a final concentration.

AI-081 Dose Adjustment

[0317] If a patient has AEs possibly related to the study drug that may potentially lead to severe AEs in the opinion of the Investigator, the dose may be adjusted to a lower level for future treatment. Alternatively, the Investigator may wish to withhold the treatment until the initial TRAEs are resolved before having the patient restart the treatment at a reduced dose level. If the SRC deems that one dose level cohort has excessive delayed toxicity beyond the DLT period, the SRC may recommend the remaining patients in the cohort be treated at lower doses or less frequent dosing. Dose adjustment refers to dose reduction from 20 to 10 to 6 to 3 mg/kg and more than 1 level of dose reduction, i.e., from 20 mg/kg to 3 mg/kg is permitted. After the dose adjusted to lower levels, the Investigator may re-evaluate the patient and adjust the dose to a higher level up to the original dose level in the study design.

Local Cancer Treatment

[0318] During the treatment period, patients may become eligible for such local curative treatment. The study will allow patients to have optional local therapies to achieve the best outcome or to receive palliative treatment as part of the best supportive care. Patients may continue the AI-081 treatment after the local therapy. For palliative radiation therapy that is not overlapping with study scheduled treatment, patients may continue to receive study treatment as scheduled without a wash-out period. Any local therapies or procedures, whether curative or palliative, should be documented.

Drug Preparation for Clinical Use

[0319] The dose of AI-081 for administration must be prepared using aseptic technique following the instructions in AI-081 Pharmacy Manual. The diluted drug is stable for up to 4 hours at room temperature. The time from needle puncture of the product vial to start of administration should not exceed 4 hours at room temperature. The drug should be removed from its long-term storage temperature and prepared for administration on the same day as treatment. The prepared drug product should not be stored at room temperature for more than 4 hours prior to administration. If in-use storage time exceeds 4 hours, a new dose must be prepared from new vials. Any unused portion in the product vial should be discarded according to local institutional guidelines.

CLAIMS

1. A bispecific binding protein comprising one or more anti-VEGF binding regions and one or more anti-PD-1 binding regions.

2. The bispecific binding protein of claim 1, wherein the bispecific binding protein has a Coloma and Morrison-type IgG-single chain fragment variable (scFv) tetravalent format.

3. The bispecific binding protein of claim 1, wherein the anti-VEGF binding region comprises (a) an anti-VEGF light chain comprising an anti-VEGF light chain variable region and an anti-VEGF light chain constant region; and (b) an anti-VEGF heavy chain comprising an anti-VEGF heavy chain variable region and an anti-VEGF heavy chain constant region.

4. The bispecific binding protein of claim 3, wherein the anti-VEGF light chain variable region comprises a complementarity determining region (CDR1) comprising the sequence set forth in SEQ ID NO: 8, a CDR2 comprising the sequence set forth in SEQ ID NO: 9, and a CDR3 comprising the sequence set forth in SEQ ID NO: 10; optionally wherein the anti-VEGF light chain variable region comprises the sequence set forth in SEQ ID NO: 2.

5. The bispecific binding protein of claim 4, wherein the anti-VEGF light chain comprises a human Kappa light chain constant region.

6. The bispecific binding protein of claim 5, wherein the human Kappa light chain constant region comprises the sequence set forth in SEQ ID NO: 52.

7. The bispecific binding protein of claim 3, wherein the anti-VEGF heavy chain variable region comprise a CDR1 comprising the sequence set forth in SEQ ID NO: 5, a CDR2 comprising the sequence set forth in SEQ ID NO: 6, and a CDR3 comprising the sequence set forth in SEQ ID NO: 7; optionally wherein the anti-VEGF heavy chain variable region comprises the sequence set forth in SEQ ID NO: 1.

8. The bispecific binding protein of claim 3, wherein the anti-VEGF heavy chain constant region comprises a human IgG1 heavy chain constant region.

9. The bispecific binding protein of claim 8, wherein the human IgG1 heavy chain constant region comprises the sequence set forth in SEQ ID NO: 51.

10. The bispecific binding protein of claim 8, wherein the human IgG1 heavy chain constant region comprises Fc-silent mutations.

11. The bispecific binding protein of claim 10, wherein the Fc-silent mutations comprise L234A, L235A, and G237A substitutions, wherein the substitutions have positions in the human IgG1 heavy chain constant region relative to the EU index.

12. The bispecific binding protein of claim 11, wherein the human IgG1 heavy chain constant region comprises the sequence set forth in SEQ ID NO: 69.

13. The bispecific binding protein of claim 11, wherein the human IgG1 heavy chain constant region further comprises FcRn binding enhancing mutations.

14. The bispecific binding protein of claim 12, wherein the FcRn binding enhancing mutations comprise M428L and N434S substitutions, wherein the substitutions have positions in the human IgG1 heavy chain constant region relative to the EU index.

15. The bispecific binding protein of claim 14, wherein the human IgG1 heavy chain constant region comprises the sequences set forth in SEQ ID NO: 70.

16. The bispecific binding protein of claim 1, wherein the anti-PD-1 binding region comprises an anti-PD-1 heavy chain variable region and an anti-PD-1 light chain variable region.

17. The bispecific binding protein of claim 1, wherein a scFv comprises the anti-PD-1 binding region.

18. The bispecific binding protein of claim 17, wherein the anti-PD-1 heavy chain variable region comprises a CDR1 comprising the sequence set forth in SEQ ID NO: 21, a CDR2 comprising the sequence set forth in SEQ ID NO: 22, and a CDR3 comprising the sequence set forth in SEQ ID NO: 23; optionally wherein the anti-PD-1 heavy chain variable region comprises the sequence set forth in SEQ ID NO: 17; and wherein the anti-PD-1 light chain variable region comprises a CDR1 comprising the sequence set forth in SEQ ID NO: 24, a CDR2 comprising the

sequence set forth in SEQ ID NO: 25, and a CDR3 comprising the sequence set forth in SEQ ID NO: 26; optionally wherein the anti-PD-1 light chain variable region comprises the sequence set forth in SEQ ID NO: 18.

19. The bispecific binding protein of claim 18, wherein the scFv comprises a first linker linking the anti-PD-1 heavy chain variable region to the anti-PD-1 light chain variable region.

20. The bispecific binding protein of claim 19, wherein the first linker comprises the sequence set forth in one of SEQ ID NOs: 55-58.

21. The bispecific binding protein of claim 20, wherein the scFv comprises, from N-terminus to C-terminus: (a) the anti-PD-1 heavy chain variable region, the first linker comprising the sequence set forth in SEQ ID NO: 57, and the anti-PD-1 light chain variable region; or (b) the anti-PD-1 light chain variable region, the first linker comprising the sequence set forth in SEQ ID NO: 57, and the anti-PD-1 heavy chain variable region.

22. The bispecific binding protein of claim 21, wherein the scFv further comprises one of the one or more anti-VEGF binding regions, wherein the anti-VEGF binding region comprises an anti-VEGF heavy chain comprising an anti-VEGF heavy chain variable region comprising the sequence set forth in SEQ ID NO: 1 and a heavy chain constant region comprising the sequence set forth in SEQ ID NO: 51, 69, or 70, and wherein the C-terminus of the anti-VEGF heavy chain is linked to the N-terminus of the anti-PD-1 binding region via a second linker.

23. The bispecific binding protein of claim 22, wherein the second linker comprises the sequence set forth in SEQ ID NO: 58.

24. The bispecific binding protein of claim 17, wherein the anti-PD-1 heavy chain variable region comprises the sequence set forth in SEQ ID NO: 53 and the anti-PD-1 light chain variable region comprises the sequence set forth in SEQ ID NO: 54.

25. The bispecific binding protein of claim 17, wherein the scFv comprises a first linker linking the anti-PD-1 heavy chain variable region to the anti-PD-1 light chain variable region.

26. The bispecific binding protein of claim 2519, wherein the first linker comprises the sequence set forth in one of SEQ ID NOs: 55-58.

27. The bispecific binding protein of claim 26, wherein the scFv comprises, from N-terminus to C-terminus: (a) the anti-PD-1 heavy chain variable region, the first linker comprising the sequence set forth in SEQ ID NO: 57, and the anti-PD-1 light chain variable region; or (b) the anti-PD-1 light chain variable region, the first linker comprising the sequence set forth in SEQ ID NO: 57, and the anti-PD-1 heavy chain variable region.

28. The bispecific binding protein of claim 27, wherein the scFv further comprises one of the one or more anti-VEGF binding regions, wherein the anti-VEGF binding region comprises an anti-VEGF heavy chain comprising an anti-VEGF heavy chain variable region comprising the sequence set forth in SEQ ID NO: 1 and a heavy chain constant region comprising the sequence set forth in SEQ ID NO: 51, 69, or 70, and wherein the C-terminus of the anti-VEGF heavy chain is linked to the N-terminus of the anti-PD-1 binding region via a second linker.

29. The bispecific binding protein of claim 28, wherein the second linker comprises the sequence set forth in SEQ ID NO: 58.

30. The bispecific binding protein of claim 29, wherein the scFv comprises the sequence set forth in one of SEQ ID NOs: 59, 60, 71, 72, or 73.

31. The bispecific binding protein of claim 30, comprising an anti-VEGF light chain comprising an anti-VEGF variable light chain region comprising the sequence set forth in SEQ ID NO: 2 fused to a light chain constant region comprising the sequence set forth in SEQ ID NO: 52, wherein the anti-VEGF light chain complexes with the scFv.

32. A pharmaceutical composition comprising the bispecific binding protein of claim 1 and a pharmaceutically acceptable excipient.

33. The pharmaceutical composition of claim 32, suitable for intravenous or subcutaneous injection.

34. The pharmaceutical composition of claim 33, wherein the pharmaceutical composition comprises 10-30 mg/mL of the bispecific binding protein.

35. The pharmaceutical composition of claim 34, wherein the pharmaceutical composition comprises 20 mg/mL of the bispecific binding protein.

36. The pharmaceutical composition of claim 32, comprising one or more of histidine buffer, trehalose dihydrate, and polysorbate 80.

37. The pharmaceutical composition of claim 36, comprising 10-30 mM histidine buffer, 6-12% (w/v) trehalose dihydrate, and 0.02-0.06% (w/v) polysorbate 80.

38. The pharmaceutical composition of claim 37, wherein the pharmaceutical composition comprises about 20 mM histidine buffer, about 9% (w/v) trehalose dihydrate, and about 0.04% (w/v) polysorbate 80.

39. The pharmaceutical composition of claim 32 having a pH of 5-6.

40. The pharmaceutical composition of claim 39 having a pH of about 5.5.

41. A method of treating cancer in a subject in need thereof, comprising administering the bispecific binding protein of claim 1 to the subject.

42. The method of claim 41, wherein the cancer is lung cancer, ovarian cancer, breast cancer, liver cancer, brain cancer, cervical cancer, pancreatic cancer, renal cancer, testicular cancer, prostate cancer, neuroblastoma, mantle cell lymphoma, or a hematological malignancy.

43. The method of claim 42, wherein the cancer is a solid tumor.

44. The method of claim 43, wherein the solid tumor is a colorectal cancer or a lung cancer.

45. The method of claim 44, wherein the solid tumor is a colorectal cancer that is one or more of micro-satellite stable and proficient mismatch repair.

46. The method of claim 44, wherein the solid tumor is a lung cancer that is a non-small cell lung cancer (NSCLC) or a small cell lung cancer (SCLC).

47. The method of claim 46, wherein the solid tumor is a PD-L1+ NSCLC.

48. The method of claim 46, wherein the solid tumor is an extensive-stage SCLC.

49. The method of claim 41, wherein the bispecific binding protein is administered intravenously at a dose of 0.3-25 mg/kg or 0.3-20 mg/kg.

50. The method of claim 49, wherein the bispecific binding protein is administered at a dose of 0.3, 1, 3, 10, or 20 mg/kg.

51. The method of claim 41, wherein the bispecific binding protein is administered intravenously or subcutaneously.

52. The method of claim 41, where the bispecific binding protein is administered once every 1-4 weeks.

53. The method of claim 52, where the bispecific binding protein is administered once every two weeks

54. The method of claim 52, where the bispecific binding protein is administered once every three weeks.

55. The method of claim 52, where the bispecific binding protein is administered once every 4 weeks.

56. The method of claim 52, where the bispecific binding protein is administered once every week.

57. The bispecific binding protein of claim 1 for use in treating cancer.

58. The bispecific binding protein of claim 57, wherein the cancer is lung cancer, ovarian cancer, breast cancer, liver cancer, brain cancer, cervical cancer, pancreatic cancer, renal cancer, testicular cancer, prostate cancer, neuroblastoma, mantle cell lymphoma, or a hematological malignancy.

59. The bispecific binding protein of claim 57, wherein the cancer is a solid tumor.

60. The bispecific binding protein of claim 59, wherein the solid tumor is a colorectal cancer or a lung cancer.

61. The bispecific binding protein of claim 60, wherein the solid tumor is a colorectal cancer that is one or more of micro-satellite stable and proficient mismatch repair.

62. The bispecific binding protein of claim 60, wherein the solid tumor is a lung cancer that is a non-small cell lung cancer (NSCLC) or a small cell lung cancer (SCLC).

63. The bispecific binding protein of claim 62, wherein the solid tumor is a PD-L1+ NSCLC.

64. The bispecific binding protein of claim 62, wherein the solid tumor is an extensive-stage SCLC.

65. The bispecific binding protein of claim 57, wherein the bispecific binding protein is for intravenous or subcutaneous administration.

66. Use of the bispecific binding protein of claim 1 in the manufacture of a medicament for treating cancer.

67. The use of claim 66, wherein the cancer is lung cancer, ovarian cancer, breast cancer, liver cancer, brain cancer, cervical cancer, pancreatic cancer, renal cancer, testicular cancer, prostate cancer, neuroblastoma, mantle cell lymphoma, or a hematological malignancy.

68. The use of claim 66, wherein the cancer is a solid tumor.

69. The use of claim 68, wherein the solid tumor is a colorectal cancer or a lung cancer.

70. The use of claim 69, wherein the solid tumor is a colorectal cancer that is one or more of micro-satellite stable and proficient mismatch repair.

71. The use of claim 6970, wherein the solid tumor is a lung cancer that is a non-small cell lung cancer (NSCLC) or a small cell lung cancer (SCLC).

72. The use of claim 71, wherein the solid tumor is a PD-L1+ NSCLC.

73. The use of claim 71, wherein the solid tumor is an extensive-stage SCLC.

74. The use of claim 66, wherein the bispecific binding protein is for intravenous or subcutaneous administration.

75. A bispecific binding protein comprising one or more anti-VEGF binding regions and one or more anti-PD-1 binding regions, wherein the bispecific binding protein comprises a single chain variable fragment (scFv) comprising an anti-VEGF binding region and an anti-PD-1 binding region, and an anti-VEGF light chain, wherein the anti-VEGF light chain complexes with the scFv, wherein:

(a) the scFv comprises an anti-VEGF heavy chain region, a first linker, an anti-PD-1 heavy chain variable region, a second linker, and an anti-PD-1 light chain region, wherein:

(i) the anti-VEGF heavy chain region comprises an anti-VEGF heavy chain variable region comprising the sequence set forth in SEQ ID NO: 3 linked via its C-terminus to a heavy chain constant region comprising the sequence set forth in SEQ ID NO: 51;

(ii) the first linker comprises the sequence set forth in SEQ ID NO: 57, wherein the first linker connects a C-terminus of the anti-VEGF heavy chain region to the anti-PD-1 heavy chain variable region;

(iii) the anti-PD-1 heavy chain variable region comprises the sequence set forth in SEQ ID NO: 54;

(iv) the second linker comprise the sequence set forth in SEQ ID NO: 58, wherein the second linker connects a C-terminus of the anti-PD-1 heavy chain variable region to the anti-PD-1 light chain region; and

(v) the anti-PD-1 light chain region comprises the sequence set forth in SEQ ID NO: 53

optionally wherein the scFv comprises the sequence set forth in SEQ ID NO: 61; and

(b) the anti-VEGF light chain comprises an anti-VEGF light chain variable region comprising the sequence set forth in SEQ ID NO: 4 connected via its C-terminus to a light chain constant region comprising the sequence set forth in SEQ ID NO: 52.