ANTI-VEGF/DLL4 DUAL VARIABLE DOMAIN IMMUNOGLOBULINS AND USES THEREOF

[001] This application claims the benefit of priority under 35 U.S.C. § 119 of U.S. Provisional Application No. 61/721,072, filed November 1, 2012, and U.S. Provisional Application No. 61/787,927, filed March 15, 2013, both of which are incorporated herein by reference in their entirety.

[002] Disclosed herein are multivalent and multispecific binding proteins, methods of making the binding proteins, and their uses in the diagnosis, inhibition, prevention and/or treatment of cancers, tumors, and/or other angiogenesis-dependent diseases.

[003] Engineered proteins, such as multispecific binding proteins capable of binding two or more antigens, are known in the art. Such multispecific binding proteins can be generated using cell fusion, chemical conjugation, or recombinant DNA techniques. There are a variety of multispecific binding protein structures known in the art; however many such structures and methods have distinct disadvantages.

[004] Bispecific antibodies have been produced using quadroma technology. However, the presence of mis-paired by-products and significantly reduced production yields with this technology means that sophisticated purification procedures are required. Bispecific antibodies can also be produced by chemical conjugation of two different mAbs. However, this approach does not yield homogeneous preparations.

[005] Other approaches used previously include coupling of two parental antibodies with a hetero-bifunctional crosslinker, production of tandem single-chain Fv molecules, diabodies, bispecific diabodies, single-chain diabodies, and di-diabodies. However, each of these approaches have disadvantages. In addition, a multivalent antibody construct comprising two Fab repeats in the heavy chain of an IgG and capable of binding four antigen molecules has been described (see PCT Publication No. WO 0177342 and Miller et al. (2003) J. Immunol. 170(9): 4854-61).

[006] Ligand-receptor systems have co-evolved to maintain specificity. Their interactions activate specific signaling for a particular biological activity. However, non-ligand-receptor binding proteins such as mono-specific antibodies, bi- or multi-specific binding proteins, noncompetitive antibody combinations or other receptor binding proteins to an extracellular domain (ECD) of a receptor may recognize epitopes distinct from a receptor ligand-binding site. Binding to such a distinct epitope(s) on the ECD of

a receptor may transduce conformational changes to the intracellular domain, which may result in a novel unexpected signaling cascade.

[007] US Patent No. 7,612,181 (incorporated herein by reference in its entirety) provides a novel family of binding proteins capable of binding two or more antigens with high affinity, which are called dual variable domain binding proteins (DVD binding protein) or dual variable domain immunoglobulins (DVD-Ig™). DVDs molecules are tetravalent dual-specific Ig-like proteins capable of binding two distinct epitopes on the same molecule or two different molecules simultaneously. DVDs are unique binding proteins comprised of two variable domains fused to the N-terminus of a bivalent antibody. The variable domains may be directly fused to one another or connected via synthetic peptide linkers of assorted length and amino acid composition. DVDs can be engineered with intact and functional Fc domains, allowing then to mediate appropriate effector functions. DVD format, due to its flexibility of choice of antibody pair, orientation of two antigen-binding domains and the length of the linker that joins them, may provide for novel therapeutic modalities.

[008] While a variety of structures are provided in the art, some with advantages and disadvantages, specific constructs are required for preparing multivalent binding proteins with specific properties and which bind to specific targets. Additionally, new variable domain sequences can further improve the properties of the binding proteins. Specifically, improved DVDs that bind to DLL4 and VEGF could prove beneficial. Accordingly, disclosed herein are dual variable domain immunoglobulins using the binding protein framework disclosed in US Patent No. 7,612,181 (incorporated herein by reference in its entirety) and containing particular first and second polypeptide chains, each comprising first and second variable domain sequences (e.g., those listed in Table 2) that form functional binding sites for VEGF and DLL4. In some embodiments, the first and second polypeptide chains comprise first and second variable domain sequences that each contain the three CDRs from one of the sequences listed in Table 2 and form functional binding sites for VEGF and DLL4.

[009] DLL4 is a ligand involved in cell-to-cell signaling through the Notch receptor pathway. Such cell-to-cell communication is required for many biological processes such as differentiation, proliferation, and homeostasis. The Notch-signaling pathway is one system that is utilized by a wide range of eukaryotes. This pathway, especially the Notch receptor, is also critical for functional tumor angiogenesis. Thus, inhibition of Notch receptor function, blockage of the Notch receptor, and/or blockage of

the Notch-signaling pathway are potential strategies for anticancer compositions and therapies. Small molecule inhibitors of the Notch receptor have often proven to be toxic because they suppress wild type (normal) tissue expression of Notch receptors throughout the body. Thus, different members of the Notch-signaling pathway should be considered as potential targets for therapeutics. A vasculature ligand for the Notch receptor is Delta 4 or Delta-like 4 (DLL4). Largely expressed in the vasculature, DLL4 is critical for vascular development (Yan et al., *Clin. Cancer Res.*, 13(24): 7243-7246 (2007); Shutter et al., *Genes Dev.*, 14(11): 1313-1318 (2000); Gale et al., *Proc. Natl. Acad. Sci. USA*, 101(45): 15949-15954 (2004); Krebs et al., *Genes Dev.*, 14(11): 1343-1352 (2000)). Mice heterozygous for DLL4 are embryonically lethal due to major defects in vascular development (Gale et al., *Proc. Natl. Acad. Sci. USA*, 101(45): 15949-15954 (2004); Duarte et al., *Genes Dev.*, 18(20): 2474-2478 (2004); Krebs et al., *Genes Dev.*, 18(20): 2469-2473 (2004)).

[010] The expression of DLL4 can be induced by VEGF (Liu et al., *Mol. Cell Biol.*, 23(1): 14- 25 (2003); Lobov et al., *Proc. Natl. Acad. Sci. USA*, 104(9): 3219-3224 (2007)). VEGF is a signal protein produced by cells involved in angiogenesis. Additionally, DLL4 can negatively regulate VEGF signaling, in part through repressing VEGFR2 and inducing VEGR1 (Harrington et al., *Microvasc. Res.*, 75(2): 144-154 (2008); Suchting et al., *Proc. Natl. Acad. Sci. USA*, 104(9): 3225-3230 (2007)). Exquisite coordination between DLL4 and VEGF is essential for functional angiogenesis, making both DLL4 and VEGF potential targets for therapeutic intervention.

[011] In addition to their physiological role, DLL4 and VEGF are also up-regulated in tumor blood vessels (Gale et al., *Proc. Natl. Acad. Sci. USA*, 101(45): 15949-15954 (2004); Mailhos et al., *Differentiation*, 69(2-3): 135-144 (2001); Patel et al., *Cancer Res.*, 65(19): 8690-8697 (2005); Patel et al., *Clin. Cancer Res.*, 12(16): 4836-4844 (2006); Noguera-Troise et al., *Nature*, 444(7122): 1032-1037 (2006)). Blockade of DLL4 has been shown to inhibit primary tumor growth in multiple models (Noguera-Troise et al., *Nature*, 444(7122): 1032-1037 (2006); Ridgway et al., *Nature*, 444(7122): 1083-1087 (2006); Scehnet et al., *Blood*, 109(11): 4753-4760 (2007)). The inhibition of DLL4 is even effective against tumors that are resistant to anti-VEGF therapy. Thus, the combinatorial inhibition of both DLL4 and VEGF could provide an enhanced anti-tumor therapy. Interestingly, unlike VEGF inhibition that reduces tumor vessel formation, DLL4 blockade leads to an increase in tumor vasculature density wherein the vessels are abnormal, cannot support efficient blood transport, and are

effectively nonfunctional. Thus, disruption of both VEGF and DLL4 provides for different methods of action for potential anti-cancer treatment.

[012] While antibodies and various binding constructs are known in the art, there remains a need for better targeting and efficiency of binding to VEGF and DLL4, e.g., to treat cancer and tumorogenesis. There is thus a need in the art for improved multivalent binding proteins capable of binding DLL4 and VEGF. Accordingly, novel binding proteins are provided, wherein the binding proteins are capable of binding DLL4 and VEGF. In some embodiments, the binding proteins are capable of, e.g., binding to DLL4 and VEGF with improved binding affinity and/or neutralization potency.

SUMMARY

[012a] In one aspect, there is provided a binding protein comprising first and second polypeptide chains, each independently comprising VD1-(X1)n-VD2-C-(X2)n, wherein

VD1 is a first variable domain;

VD2 is a second variable domain;

C is a constant domain;

X1 is a linker;

X2 is an Fc region;

n is 0 or 1,

wherein the VD1 domains on the first and second polypeptide chains form a first functional target binding site and the VD2 domains on the first and second polypeptide chains form a second functional target binding site, and wherein the binding protein is capable of binding DLL4 and VEGF, wherein:

(i) the variable domains that form a functional target binding site for DLL4 comprise:

three CDRs from SEQ ID NO: 39 and three CDRs from SEQ ID NO: 40, and

(ii) the variable domains that form a functional target binding site for VEGF comprise:

three CDRs from SEQ ID NO: 41 and three CDRs from SEQ ID NO: 42.

[012b] In one aspect, there is provided a binding protein conjugate comprising a binding protein as disclosed herein, wherein the binding protein conjugate further comprising an agent, wherein the agent is an immunoadhesion molecule, an imaging agent, a therapeutic agent, or a cytotoxic agent.

[012c] In one aspect, there is provided an isolated nucleic acid encoding the binding protein as disclosed herein.

[012d] In one aspect, there is provided a vector comprising the isolated nucleic acid as disclosed herein.

[012e] In one aspect, there is provided a host cell comprising the vector as disclosed herein.

[012f] In one aspect, there is provided a method of producing a binding protein comprising culturing the host cell as disclosed herein in culture medium under conditions sufficient to produce the binding protein.

[012g] In one aspect, there is provided a protein produced by the method as disclosed herein.

[012h] In one aspect, there is provided a composition comprising the binding protein as disclosed herein and at least one additional agent.

[012i] In one aspect, there is provided a use of the binding protein as disclosed herein, or the pharmaceutical composition as disclosed herein, in the manufacture of a medicament for treating a disease characterized by vascular overgrowth, edema, or aberrant DLL4 and/or VEGF expression or activity in a subject.

[012j] In one aspect, there is provided a method of detecting the presence of at least one of VEGF or DLL4, or a fragment thereof, in a test sample by an immunoassay, wherein the immunoassay comprises contacting the test sample with at least one binding protein and at least one detectable label, wherein the at least one binding protein comprises the binding protein as disclosed herein.

[012k] In one aspect, there is provided kit for use in assaying a test sample for the presence, amount, or concentration of DLL4, VEGF, or both, the kit comprising
(a) instructions for assaying the test sample for VEGF and/or DLL4 or fragment(s) thereof; and
(b) at least one binding protein comprising the binding protein as disclosed herein.

[013] Binding proteins capable of targeting two epitopes are provided, wherein the binding proteins are capable of binding DLL4 and VEGF. In an embodiment, binding proteins capable of binding epitopes of DLL4 and VEGF with high affinity are provided. In an embodiment, the binding proteins comprise a dual variable domain binding protein framework that contains the CDR and variable domain sequences listed in Table 2. In an embodiment, the dual variable domain binding protein framework comprises the framework disclosed in US Patent No. 7,612,181 (incorporated herein by reference in its entirety).

[014] In one embodiment, binding proteins comprising a polypeptide chain that can bind two epitopes of two different proteins (VEGF and DLL4) are provided, wherein the polypeptide chain comprises VD1-(X1)n-VD2-C-(X2)n, wherein VD1 is a first variable domain, VD2 is a second variable domain, C is a constant domain, X1

represents an amino acid or polypeptide, X2 represents an Fc region and n is 0 or 1, are provided. In some embodiments, the VD1 and VD2 in the binding protein are heavy chain variable domains. In certain embodiments, VD1 and VD2 are capable of binding an epitope of DLL4 and an epitope of VEGF. In some embodiments, C is a heavy chain constant domain, such as CH1. In certain embodiments, X1 is a linker with the proviso that X1 is not CH1.

[015] In various embodiments, the binding protein disclosed herein comprises a polypeptide chain that binds an epitope of DLL4 and an epitope of VEGF, wherein the polypeptide chain comprises VD1-(X1)n-VD2-C-(X2)n, wherein VD1 comprises a first heavy chain variable domain, VD2 comprises a second heavy chain variable domain, C comprises a heavy chain constant domain, X1 comprises a linker, and X2 comprises an Fc region. In an embodiment, X1 is a linker with the proviso that it is not CH1. In an embodiment, the VD1 and VD2 heavy chain variable domains each comprise three CDRs chosen from the CDRs in SEQ ID NO: 39, 41, 43, 45, 47, 49, 51,

or 53 (i.e., CDRs 1-3 from one of those SEQ ID NOs), wherein at least one of the VD1 and/or VD2 heavy chain variable domains comprises the three CDRs in SEQ ID NO: 39. In another embodiment, the binding protein is capable of binding DLL4 and VEGF. In an embodiment, the VD1 and VD2 heavy chain variable domains comprise SEQ ID NO: 39, 41, 43, 45, 47, 49, 51, or 53, wherein at least one of the VD1 and/or VD2 heavy chain variable domains comprises SEQ ID NO: 39.

[016] In various embodiments, the binding protein disclosed herein comprises a polypeptide chain that binds an epitope of DLL4 and an epitope of VEGF, wherein the polypeptide chain comprises VD1-(X1)n-VD2-C-(X2)n, wherein VD1 comprises a first light chain variable domain, VD2 comprises a second light chain variable domain, C comprises a light chain constant domain, X1 comprises a linker, and X2 does not comprise an Fc region. In an embodiment, X1 is a linker with the proviso that it is not a CH1 or a CL. In an embodiment, the VD1 and VD2 light chain variable domains each comprise three CDRs chosen from the CDRs in SEQ ID NO: 40, 42, 44, 46, 48, 50, 52, or 54 (i.e., CDRs 1-3 from one of those SEQ ID NOs), wherein at least one of the VD1 and/or VD2 light chain variable domains comprises the three CDRs in SEQ ID NO: 40. In another embodiment, the binding protein is capable of binding DLL4 and VEGF. In an embodiment, the VD1 and VD2 light chain variable domains each comprise SEQ ID NO: 40, 42, 44, 46, 48, 50, 52, or 54, wherein at least one of the VD1 and/or VD2 light chain variable domains comprises SEQ ID NO: 40.

[017] In another embodiment, a binding protein that binds an epitope of DLL4 and an epitope of VEGF is disclosed. In some embodiments, the binding protein comprises first and second polypeptide chains, wherein each of the first and second polypeptide chains independently comprises VD1-(X1)n-VD2-C-(X2)n, wherein VD1 is a first variable domain, VD2 is a second variable domain, C is a constant domain, X1 is a linker, X2 is an Fc region, and n is 0 or 1, wherein the VD1 domains on the first and second polypeptide chains form a first functional target binding site and the VD2 domains on the first and second polypeptide chains form a second functional target binding site. In an embodiment, X2 comprises an Fc region when n=1, and X2 does not comprise an Fc region when n=0. In some embodiments, the X1 sequences on the first and second polypeptide chains are the same. In other embodiments, the X1 sequences on the first and second polypeptide chains are different. In some embodiments X1 on at least one of the polypeptide chains is not a CH1 domain and/or a CL domain. In one embodiment, the X1 sequence is a short (e.g., 6, 5, 4, 3, or 2 amino acid) linker. In another embodiment, the the X1 sequence is a long (e.g., 6, 7, 8,

9, 10, 11, 12, 15, 20, 25, 30, or greater amino acid) linker. In another embodiment, the X1 sequence on one of the two polypeptide chains is a short linker and the X1 sequence on the other polypeptide chain is a long linker. In an embodiment, the VD1 and VD2 heavy chain variable domains each comprise three CDRs from SEQ ID NO: 39, 41, 43, 45, 47, 49, 51, or 53 (i.e., CDRs 1-3 from one of those SEQ ID NOs), wherein at least one of the VD1 and/or VD2 heavy chain variable domains comprises the three CDRs in SEQ ID NO: 39; and the VD1 and VD2 light chain variable domains comprise three CDRs from SEQ ID NO: 40, 42, 44, 46, 48, 50, 52, or 54 (i.e., CDRs 1-3 from one of those SEQ ID NOs), wherein at least one of the VD1 and/or VD2 light chain variable domains comprises the three CDRs in SEQ ID NO: 40. In another embodiment, the binding protein is capable of binding DLL4 and VEGF. In an embodiment, the VD1 and VD2 heavy chain variable domains comprise SEQ ID NO: 39, 41, 43, 45, 47, 49, 51, or 53, wherein at least one of the VD1 and/or VD2 heavy chain variable domains comprises SEQ ID NO: 39, and the VD1 and VD2 light chain variable domains comprise SEQ ID NO: 40, 42, 44, 46, 48, 50, 52, or 54, wherein at least one of the VD1 and/or VD2 light chain variable domains comprises SEQ ID NO: 40.

[018] In various embodiments, a binding protein is disclosed that is capable of binding VEGF and DLL4. In some embodiments, the binding protein comprises first and second polypeptide chains, wherein each of the first and second polypeptide chains independently comprises VD1-(X1)n-VD2-C-(X2)n, wherein VD1 is a first variable domain, VD2 is a second variable domain, C is a constant domain, X1 is a linker, X2 is an Fc region, and n is 0 or 1, wherein the VD1 domains on the first and second polypeptide chains form a first functional target binding site and the VD2 domains on the first and second polypeptide chains form a second functional target binding site. In an embodiment, the variable domains that form a functional target binding site for VEGF comprise three CDRs from SEQ ID NO: 41 and three CDRs from SEQ ID NO: 42 (e.g., CDRs 1-3 from SEQ ID NO: 41 and CDRs 1-3 from SEQ ID NO: 42 present on separate chains, with the CDRs on each chain arranged in the specified order and separated by suitable framework sequences to form a functional binding site). In an embodiment, the variable domains that form a functional target binding site for DLL4 comprise three CDRs from SEQ ID NO: 39 and three CDRs from SEQ ID NO: 40 (e.g., CDRs 1-3 from SEQ ID NO: 39 and CDRs 1-3 from SEQ ID NO: 40 present on separate chains, with the CDRs on each chain arranged in the specified order and separated by suitable framework sequences to form a functional binding site). In an embodiment, the binding protein comprises a functional target binding site for VEGF

comprising three CDRs from SEQ ID NO: 41 and three CDRs from SEQ ID NO: 42 (e.g., CDRs 1-3 from SEQ ID NO: 41 and CDRs 1-3 from SEQ ID NO: 42), and a functional target binding site for DLL4 comprising three CDRs from SEQ ID NO: 39 and three CDRs from SEQ ID NO: 40 (e.g., CDRs 1-3 from SEQ ID NO: 39 and CDRs 1-3 from SEQ ID NO: 40). In an embodiment, the binding protein comprises a functional target binding site for VEGF comprising SEQ ID NO: 41 and SEQ ID NO: 42, and a functional target binding site for DLL4 comprising SEQ ID NO: 39 and SEQ ID NO: 40. In an embodiment, the binding protein comprises a first polypeptide chain comprising SEQ ID NO: 56 and a second polypeptide chain comprising SEQ ID NO: 64. In an embodiment, the binding protein comprises a first polypeptide chain comprising SEQ ID NO: 73 and a second polypeptide chain comprising SEQ ID NO: 74. In some embodiments, the variable domains that form a binding site for DLL4 comprise those from US application publication no. 20110217237, and/or the variable domains that form a binding site for VEGF comprise those from US application publication no 20100076178, which are incorporated herein by reference in their entirety. In an embodiment, the binding protein comprises h1A11.1-SL-Av. In an embodiment, the h1A11.1-SL-Av binding protein comprises an Fc region from a human IgG1 LALA mutant.

[019] The development and production of a binding protein suitable for use as a human therapeutic agent, e.g., as an anticancer/antitumor agent, can require more than the identification of a binding protein capable of binding to a desired target or targets. For instance, a candidate may bind its target(s) but exhibit a reduced ability to inhibit or neutralize its desired target, may prove difficult to stably formulate, may exhibit undesirable pharmacokinetic properties, or may prove difficult to produce in a suitable expression system (e.g., expression in a host cell such as CHO). Thus, factors to consider in developing a suitable therapeutic agent include, but are not limited to, (a) the binding kinetics (on-rate, off-rate and affinity) for both the inner and outer antigen-binding domains, (b) potencies in various biochemical and cellular bioassays, (c) in vivo efficacies in relevant tumor models, (d) pharmacokinetic and pharmacodynamics properties, (e) manufacturability, including protein expression level in selected cell lines, scalability, post-translational modification, physicochemical properties such as monomer percentage, solubility, and stability (intrinsic, freeze/thaw, storage stability, etc.), (f) formulation properties, (g) potential immunogenicity risk, and (h) toxicological properties of a molecule. Binding mode and valency may also be evaluated, as these can affect binding properties and cellular potencies of a molecule. For some binding proteins, even small changes in the amino acid sequences of the

variable domains, constant domains, and/or linkers can potentially impact (positively or negatively) one or more of these factors, and so the combination of factors can be evaluated to select a lead candidate. Once a lead candidate is identified, further in vivo evaluation of therapeutic properties are conducted, included evaluation of safety, efficacy, and potency in animal and human subjects.

[020] It has been found, unexpectedly, that a binding protein comprising SEQ ID NO: 56 and SEQ ID NO: 64, or comprising SEQ ID NO: 73 and SEQ ID NO: 74, exhibited a superior combination of properties in this regard, such as a binding kinetic (i.e., a dissociation constant) surpassing a therapeutically useful threshold, improved neutralization ability, enhanced in vivo efficacy, superior formulatability, a desirable glycosylation pattern, a favorable pharmacokinetic profile, and efficient expression in host cells, as compared to other evaluated binding proteins comprising different variable domains and/or linker sequences. The compared binding proteins can include those having the same variable domain sequences and orientations but with different linkers, as well as those having altered binding domain orientations and/or other variable domain sequences (e.g., affinity matured sequences, fully human sequences, etc.). In some embodiments, these superior properties are dependent on the selection of particular variable domain sequences (e.g., SEQ ID NOs: 39-42), particular orientations of the VEGF and DLL4 binding domains in the inner and outer positions (e.g., the orientations provided in SEQ ID NO: 56 and SEQ ID NO: 64), particular linker sequences (e.g., the heavy chain variable domains and short linker sequence used in SEQ ID NO: 56 and the light chain variable domains and long linker sequence used in SEQ ID NO: 64), and/or particular constant domain sequences (e.g., the constant domain sequences used in SEQ ID NOs: 73 and 74). In some embodiments, merely changing the linker sequence can have a significant impact on functional properties. For example, selecting the linker sequence used in SEQ ID NO: 73 and 74 (along with the variable and constant domains included in those SEQ ID NOs) can provide unexpected improvement in the human therapeutic properties of the binding protein. For instance, Table 9 demonstrates the effects of different linker sequences on in vivo anti-tumor efficacy, as measured in colorectal adenocarcinoma and glioblastoma xenograft models. Thus, for example, using the sequences in SEQ ID NOs: 56 and 64, or in SEQ ID NOs: 73 and 74 (including the linker sequences included therein), can produce an unexpected gain in anti-tumor efficacy in vivo.

[021] In various embodiments, a binding protein disclosed herein exhibits a desired binding affinity, blocking ability, and/or neutralization potency for VEGF and/or

DLL4, for instance levels roughly comparable to those observed for antibodies against VEGF (e.g., AVASTIN®) or DLL4 (e.g., antibody h1A11.1). In some embodiments, the binding protein exhibits increased affinity, blocking ability, and/or neutralization potency for VEGF and/or DLL4, as compared to DVD-Ig binding proteins comprising other variable domain sequences or linkers. In some embodiments, the binding protein *comprises a functional target binding site for VEGF comprising three CDRs from* SEQ ID NO: 41 and three CDRs from SEQ ID NO: 42, and a functional target binding site for DLL4 comprising three CDRs from SEQ ID NO: 39 and three CDRs from SEQ ID NO: 40; or comprises a functional target binding site for VEGF comprising SEQ ID NO: 41 and SEQ ID NO: 42, and a functional target binding site for DLL4 comprising SEQ ID NO: 39 and SEQ ID NO: 40; or comprises SEQ ID NO: 56 and SEQ ID NO: 64; or comprises SEQ ID NO: 73 and SEQ ID NO: 74. For instance, the binding protein (e.g., the binding protein comprising SEQ ID NO: 56 and SEQ ID NO: 64, or comprising SEQ ID NO: 73 and SEQ ID NO: 74) can be capable of binding to VEGF with a dissociation constant (K_D) of at most about 7.0×10^{-10} M, as measured by surface plasmon resonance, and/or blocking VEGF activity with an IC50 of at most about 3.8 nM, as measured in a VEGFR1 Competition ELISA; and/or capable of binding to DLL4 with a dissociation constant (K_D) of at most about 1.0×10^{-8} M, as measured by surface plasmon resonance, and/or blocking DLL4 activity with an IC50 of at most about 1.09 nM, as measured in a Notch Competition ELISA.

[022] In some embodiments, the binding protein (e.g., the binding protein comprising SEQ ID NO: 56 and SEQ ID NO: 64, or comprising SEQ ID NO: 73 and SEQ ID NO: 74) can exhibit increased neutralization potency for DLL4 when also in the presence of VEGF, as compared to a mixture of VEGF and DLL4 antibodies (e.g., the parental antibodies used to provide variable domains for the binding protein). In some embodiments, the binding protein can exhibit an order of magnitude increase in the neutralization potency for DLL4 when also in the presence of VEGF, as compared to a mixture of VEGF and DLL4 antibodies (e.g., the parental antibodies used to provide variable domains for the binding protein). For instance, Table 24 demonstrates that a binding protein comprising SEQ ID NO: 73 and SEQ ID NO: 74 can exhibit an order of magnitude increase in the neutralization potency for DLL4 when also in the presence of at least about 1.2 nM VEGF (e.g., at least about 1.2, 1.5, 2, 2.5, 5, 10, 50, 150, or more), as compared to a mixture of VEGF and DLL4 antibodies (the parental antibodies used to provide variable domains for the binding protein). This DLL4 neutralization property may be beneficial because, in the in vivo situation of treatment for a tumor, VEGF levels are usually higher in the vicinity of a tumor than in the

circulation generally, allowing for both improved targeting and enhanced functional DLL4 neutralization acitivity at the tumor site.

[023] In various embodiments, a binding protein (e.g., the binding protein comprising SEQ ID NO: 56 and SEQ ID NO: 64, or comprising SEQ ID NO: 73 and SEQ ID NO: 74) exhibits improved properties, e.g., improved safety, increased stability, greater potency, reduced inflammation or immune response, or other beneficial in vivo human therapeutic properties, as compared to other treatments for cancers and/or vascularized tumors. Treatments suitable for comparison can include administration of a small molecule anti-cancer agent, or an antibody against VEGF (e.g., AVASTIN®) and/or DLL4 (e.g., antibody h1A11.1), or a DVD-Ig binding protein comprising other variable domain sequences and/or linkers. In some embodiments, the binding protein exhibits improved properties over a current standard of care treatment for cancer and/or a vascularized tumor. For instance, the binding protein can exhibit improved binding kinetics, superior in vivo therapeutic efficacy, enhanced formulatability (including reduced aggregation and improved storage stability), improved pharmacokinetics, reduced inflammation or immune response, and/or enhanced host cell expression levels.

[024] In some embodiments, a binding protein (e.g., the binding protein comprising SEQ ID NO: 56 and SEQ ID NO: 64, or comprising SEQ ID NO: 73 and SEQ ID NO: 74) exhibits superior (e.g., additive and/or superadditive) effects in combination with one or more anti-cancer agents, as compared to an anti-VEGF antibody or an anti-DLL4 antibody in combination with one or more anti-cancer agent, or as compared to a DVD-Ig binding protein comprising other variable domain sequences and/or linkers in combination with one or more anti-cancer agent. For instance, the superior binding properties can be those identified in Tables 27-30 and 34. For instance, the binding protein can exhibit at least about a 50% or greater (e.g., 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 125, 150% or more) inhibition of tumor groth or delay in tumor growth after administration alone or in combination with one or more anti-cancer agent, as compared to an untreated tumor. The anti-cancer agent can be, for instance, one or more of Irinotecan, FOLFIRI, Temozolomide, Gemcitabine, Paclitaxel, 5-FU, and Capecitabine, or any other small molecule or biologic agent used in the treatment of a particular cancer. The binding protein alone or in combination with the one or more anti-cancer agent can be used as a medicament to treat, e.g., colon cancer, gliobastoma, pancreatic cancer, or breast cancer.

[025] In an embodiment, a Dual Variable Domain (DVD-Ig) binding protein comprises two first and two second polypeptide chains as described in the previous paragraph (i.e., comprising four polypeptide chains), wherein each of the polypeptide chains independently comprises VD1-(X1)n-VD2-C-(X2)n, wherein VD1 is a first variable domain, VD2 is a second variable domain, C is a constant domain, X1 is a linker, X2 is an Fc region, and n is 0 or 1. In some embodiments, the first chain is a heavy chain and is paired with a second chain that is a light chain. Such a DVD-Ig binding protein comprises four functional target binding sites. In some embodiments, the X1 linker on the first and second polypeptide chains are the same or different. In some embodiments, the DVD-Ig binding proteins comprise at least two variable domain sequences (e.g., VD1 and VD2) capable of binding two or more epitopes (e.g., two, three, or four) of the same or different proteins, in any orientation. In some embodiments, VD1 and VD2 are independently chosen. In an embodiment, the VD1 and VD2 heavy chain variable domains each comprise three CDRs from SEQ ID NO: 39, 41, 43, 45, 47, 49, 51, or 53, wherein at least one of the VD1 and/or VD2 heavy chain variable domains comprises the three CDRs in SEQ ID NO: 39, and the VD1 and VD2 light chain variable domains comprise three CDRs from SEQ ID NO: 40, 42, 44, 46, 48, 50, 52, or 54, wherein at least one of the VD1 and/or VD2 light chain variable domains comprises the three CDRs in SEQ ID NO: 40. In another embodiment, the binding protein is capable of binding DLL4 and VEGF. In an embodiment, the VD1 and VD2 heavy chain variable domains each comprise SEQ ID NO: 39, 41, 43, 45, 47, 49, 51, or 53, wherein at least one of the VD1 and/or VD2 heavy chain variable domains comprises SEQ ID NO: 39, and the VD1 and VD2 light chain variable domains each comprise SEQ ID NO: 40, 42, 44, 46, 48, 50, 52, or 54, wherein at least one of the VD1 and/or VD2 light chain variable domains comprises SEQ ID NO: 40.

[026] In another embodiment, the Dual Variable Domain binding protein comprises a heavy chain and a light chain sequence as shown in Table 2, wherein at least one of the VD1 and/or VD2 heavy chain variable domains comprises SEQ ID NO: 39 and/or at least one of the VD1 and/or VD2 light chain variable domains comprises SEQ ID NO: 40.

[027] In a further embodiment, any of the heavy chain, light chain, two chain, or four chain embodiments includes at least one X1 linker comprising the linkers selected from SEQ ID NO: 1-38. In an embodiment, X2 is an Fc region. In another embodiment, X2 is a variant Fc region.

[028] In still another embodiment, the Fc region, if present in the first polypeptide, is a native sequence Fc region or a variant sequence Fc region. In yet another embodiment, the Fc region is an Fc region from an IgG1, an Fc region from an IgG2, an Fc region from an IgG3, an Fc region from an IgG4, an Fc region from an IgA, an Fc region from an IgM, an Fc region from an IgE, or an Fc region from an IgD. In certain embodiments, the Fc region is an Fc region from a human IgG1 LALA mutant, which is a mutant of the b12 antibody that provides protection against the HIV virus.

[029] A method of making a binding protein that binds two different target proteins is provided. In an embodiment, the method of making a binding protein comprises the steps of a) obtaining a first parent antibody, or antigen binding portion thereof, that binds a first epitope; b) obtaining a second parent antibody, or antigen binding portion thereof, that binds a second epitope; c) preparing construct(s) encoding any of the binding proteins described herein; and d) expressing the polypeptide chains, such that a binding protein that binds the first and the second epitope is generated.

[030] In any of the embodiments herein, the VD1 heavy chain variable domain, if present, and light chain variable domain, if present, can be from a first parent antibody or antigen binding portion thereof; the VD2 heavy chain variable domain, if present, and light chain variable domain, if present, can be from a second parent antibody or antigen binding portion thereof. The first and second parent antibodies can be the same or different.

[031] In one embodiment, the first parent antibody or antigen binding portion thereof, binds a first antigen, and the second parent antibody or antigen binding portion thereof, binds a second antigen. In an embodiment, the first and second antigens are different antigens. In another embodiment, the first parent antibody or antigen binding portion thereof binds the first antigen with a potency different from the potency with which the second parent antibody or antigen binding portion thereof binds the second antigen. In yet another embodiment, the first parent antibody or antigen binding portion thereof binds the first antigen with an affinity different from the affinity with which the second parent antibody or antigen binding portion thereof binds the second antigen.

[032] In another embodiment, the first parent antibody or antigen binding portion thereof, and the second parent antibody or antigen binding portion thereof are a human antibody, CDR grafted antibody, humanized antibody, and/or affinity matured antibody.

[033] In another embodiment, the binding protein possesses at least one desired property exhibited by the first parent antibody or antigen binding portion thereof, or by the second parent antibody or antigen binding portion thereof. Alternatively, the first parent antibody or antigen binding portion thereof and the second parent antibody or antigen binding portion thereof possess at least one desired property exhibited by the binding protein. In an embodiment, the desired property is one or more antibody parameters. In another embodiment, the antibody parameters are antigen specificity, affinity to antigen, potency, biological function, epitope recognition, stability, solubility, production efficiency, immunogenicity, pharmacokinetics, bioavailability, tissue cross reactivity, or orthologous antigen binding. In an embodiment, the binding protein is multivalent. In another embodiment, the binding protein is multispecific. The multivalent and or multispecific binding proteins described herein have desirable properties particularly from a therapeutic standpoint. For instance, the multivalent and or multispecific binding protein may (1) be internalized (and/or catabolized) faster than a bivalent antibody by a cell expressing an antigen to which the antibodies bind; (2) be an agonist binding protein; and/or (3) induce cell death and/or apoptosis of a cell expressing an antigen to which the multivalent binding protein is capable of binding. The "parent antibody", which provides at least one antigen binding specificity of the multivalent and or multispecific binding protein, may be one that is internalized (and/or catabolized) by a cell expressing an antigen to which the antibody binds; and/or may be an agonist, cell death-inducing, and/or apoptosis-inducing antibody, and the multivalent and or multispecific binding protein as described herein may display improvement(s) in one or more of these properties. Moreover, the parent antibody may lack any one or more of these properties, but may acquire one or more of them when constructed as a multivalent binding protein as described herein.

[034] In another embodiment, the binding protein has an on rate constant (K_{on}) to one or more targets of at least about $10^2\,M^{-1}s^{-1}$; at least about $10^3\,M^{-1}s^{-1}$; at least about $10^4\,M^{-1}s^{-1}$; at least about $10^5\,M^{-1}s^{-1}$; or at least about $10^6\,M^{-1}s^{-1}$, as measured by surface plasmon resonance. In an embodiment, the binding protein has an on rate constant (K_{on}) to one or more targets from about $10^2\,M^{-1}s^{-1}$ to about $10^3\,M^{-1}s^{-1}$; from about $10^3\,M^{-1}s^{-1}$ to about $10^4\,M^{-1}s^{-1}$; from about $10^4\,M^{-1}s^{-1}$ to about $10^5\,M^{-1}s^{-1}$; or from about $10^5\,M^{-1}s^{-1}$ to about $10^6\,M^{-1}s^{-1}$, as measured by surface plasmon resonance.

[035] In another embodiment, the binding protein has an off rate constant (K_{off}) for one or more targets of at most about $10^{-2}\,s^{-1}$; at most about $10^{-3}\,s^{-1}$; at most about $10^{-4}\,s^{-1}$; at most about $10^{-5}\,s^{-1}$; or at most about $10^{-6}\,s^{-1}$, as measured by surface

plasmon resonance. In an embodiment, the binding protein has an off rate constant (K_{off}) to one or more targets of about 10^{-2} s^{-1} to about $10^{-3}s^{-1}$; of about 10^{-3} s^{-1} to about 10^{-4} s^{-1}; of about 10^{-4} s^{-1} to about 10^{-5} s^{-1}; or of about 10^{-5} s^{-1} to about 10^{-6} s^{-1}, as measured by surface plasmon resonance.

[036] In another embodiment, the binding protein has an equilibrium dissociation constant (K_D) to one or more targets of at most about 10^{-7} M; at most about 10^{-8} M; at most about 10^{-9} M; at most about 10^{-10} M; at most about 10^{-11} M; or at most about 10^{-12} M. In an embodiment, the binding protein has an equilibrium dissociation constant (K_D) to its targets of about 10^{-7} M to about 10^{-8} M; of about 10^{-8} M to about 10^{-9} M; of about 10^{-9} M to about 10^{-10} M; of about 10^{-10} M to about 10^{-11} M; or of about 10^{-11} M to about 10^{-12} M.

[037] In some embodiments, an anti-DLL4/anti-VEGF binding protein exhibits increased potency (e.g., increased ability to interfere with, inhibit and/or neutralize DLL4 and/or VEGF activity) as compared to an anti-DLL4 or anti-VEGF antibody. In some embodiments, the potency of the binding protein can be evaluated in any assay for evaluating VEGF and/or DLL4 activity, e.g., a VEGF and/or DLL4 binding ELISA assay, a BIACORE™ assay, a DLL4-Notch reporter assay, a VEGF-stimulated Endothelial Cell Proliferation/Survival assay, or any other assay known to one of skill in the art. In some embodiments, the binding protein exhibits increased DLL4 potency in the presence of VEGF.

[038] In another embodiment, a conjugate is provided, comprising any of the binding proteins described herein and further comprising an agent. In an embodiment, the agent is an immunoadhesion molecule, an imaging agent, a therapeutic agent, or a cytotoxic agent. In an embodiment, the imaging agent is a radiolabel, an enzyme, a fluorescent label, a luminescent label, a bioluminescent label, a magnetic label, or biotin. In another embodiment, the radiolabel is ^{3}H, ^{14}C, ^{35}S, ^{90}Y, ^{99}Tc, ^{111}In, ^{125}I, ^{131}I, ^{177}Lu, ^{166}Ho, or ^{153}Sm. In yet another embodiment, the therapeutic or cytotoxic agent is an anti-metabolite, an alkylating agent, an antibiotic, a growth factor, a cytokine, an anti-angiogenic agent, an anti-mitotic agent, an anthracycline, toxin, or an apoptotic agent. In some embodiments, the agent is one or more of: irinotecan, leucovorin, 5-FU, temozolomide, gemcitabine, and paclitaxel. In an embodiment, the agent is irinotecan. In an embodiment, the agent is leucovorin. In an embodiment, the agent is 5-FU. In an embodiment, the agent is irinotecan, leucovorin, and 5-FU. In an embodiment, the agent is temozolomide. In an embodiment, the agent is gemcitabine. In an embodiment, the agent is paclitaxel.

[039] In another embodiment, the conjugate comprises a binding protein and a drug. In an embodiment, the binding protein in the conjugate comprises first and second polypeptide chains, wherein each of the first and second polypeptide chains independently comprises VD1-(X1)n-VD2-C-(X2)n, wherein VD1 is a first variable domain, VD2 is a second variable domain, C is a constant domain, X1 is a linker, and X2 is an Fc region, wherein the VD1 domains on the first and second polypeptide chains form a first functional target binding site and the VD2 domains on the first and second polypeptide chains form a second functional target binding site, and wherein the binding protein is capable of binding VEGF and DLL4. In some embodiments, the VD1 and VD2 domains comprise CDRs or variable domain sequences from any of the sequences disclosed in Table 2, paired and arranged to form functional binding sites for VEGF and DLL4. In an embodiment, the drug in the conjugate is selected from the group consisting of a mitotic inhibitor, an antitumor antibiotic, an immunomodulating agent, a vector for gene therapy, an alkylating agent, an antiangiogenic agent, an antimetabolite, a boron-containing agent, a chemoprotective agent, a hormone, an antihormone agent, a corticosteroid, a photoactive therapeutic agent, an oligonucleotide, a radionuclide agent, a topoisomerase inhibitor, a tyrosine kinase inhibitor, and a radiosensitizer. In another embodiment, the drug is selected from the group consisting of Ixempra, dolastatin 10, dolatstin 15, auristatin E, auristatin PE, monomethyl auristatin D (MMAD or auristatin D derivative), monomethyl auristatin E (MMAE or auristatin E derivative), monomethyl auristatin F (MMAF or auristatin F derivative), auristatin F phenylenediamine (AFP), auristatin EB (AEB), auristatin EFP (AEFP), 5-benzoylvaleric acid-AE ester (AEVB), methotrexate, daunorubicin, vincristine, maytansine, maytansinol, C-3 esters of maytansinol, ansamitocin P1, ansamitocin P2, ansamitocin P3, ansamitocin P4, docetaxel, paclitaxel, nanoparticle paclitaxel, vindesine sulfate, vincristine, vinblastine, vinorelbine, actinomycines, actinomycin D, anthramycin, chicamycin A, DC-18, mazethramycin, neothramycin A, neothramycin B, prothracarcin B, SG2285, sibanomicin, sibiromycin, anthracyclines, daunorubicin, doxorubicin, epirubicin, idarubicin, calicheamicins, γ_1^I, α_2^I, α_3^I, N-acetyl-γ_1^I, PSAG, θ^I_1, duocarmycins, adozelesin, bizelesin, and carzelesin, bleomycin, mitomycin, plicamycin, bacillus calmette-guerin (BCG), levamisole, cancer vaccines, recombinant bivalent human papillomavirus (HPV) vaccine types 16 and 18 vaccine, recombinant quadrivalent human papillomavirus (HPV) types 6, 11, 16, and 18 vaccine, sipuleucel-T, cytokines, parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH), hepatic growth factor;

fibroblast growth factor, prolactin, placental lactogen, tumor necrosis factor, mullerian-inhibiting substance, mouse gonadotropin-associated peptide, inhibin, activin, vascular endothelial growth factor, integrin, thrombopoietin (TPO), nerve growth factors such as NGF, platelet-growth factor, transforming growth factors (TGFs), insulin-like growth factor-I and -II, erythropoietin (EPO), osteoinductive factors, interferons such as interferon α, β, and γ, colony stimulating factors (CSFs), granulocyte-macrophage-C-SF (GM-CSF), and granulocyte-CSF (G-CSF), interleukins (ILs) such as IL-1, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12, tumor necrosis factor and other polypeptide factors including LIF and kit ligand (KL), colony-stimulating factors, erythropoietin (epoetin), filgrastim, sargramostim, promegapoietin, Oprelvekin, immunomodulating gene therapeutics, nucleic acid encoding a functional, therapeutic gene that is used to replace a mutated or otherwise dysfuntional (*e.g.* truncated) gene associated with cancer, nucleic acid that encodes for or otherwise provides for the production of a therapeutic protein to treat cancer, alkyl sulfonates, busulfan, nitrogen mustards, chlorambucil, cyclophosphamide, estramustine, ifosfamide, mechlorethamine, and melphalan, nitrosoureas, carmustine, fotemustine, lomustine, nimustine, streptozocin, triazines and hydrazines, dacarbazine, procarbazine, temozolomide, ethylenimimes, thiopeta, diaziquone, mitomycin C, methylamine derivatives, epoxides, altretamine, dianhydrogalactitol, dibromodulcitol, angiostatin, ABX EFG, C1-1033, PKI-166, EGF vaccine, EKB-569, GW2016, ICR-62, EMD 55900, CP358, PD153035, AG1478, IMC-C225, OSI-774, Erlotinib, angiostatin, arrestin, endostatin, BAY 12-9566 and w/fluorouracil or doxorubicin, canstatin, carboxyamidotriozole and with paclitaxel, EMD121974, S-24, vitaxin, dimethylxanthenone acetic acid, IM862, Interleukin-12, Interleukin-2, NM-3, HuMV833, PTK787, RhuMab, angiozyme, IMC-1C11, Neovastat, marimstat, prinomastat, BMS-275291, COL-3, MM1270, SU101, SU6668, SU11248, SU5416, with paclitaxel, with gemcitabine and cisplatin, and with irinotecan and cisplatin and with radiation, tecogalan, temozolomide and PEG interferon α2b, tetrathiomolybdate, TNP-470, thalidomide, CC-5013 and with taxotere, tumstatin, 2-methoxyestradiol, VEGF trap, mTOR inhibitors (deforolimus, everolimus, and temsiroiimus), tyrosine kinase inhibitors (*e.g.*, imatinib, gefitinib, dasatinib, sunitinib, nilotinib, lapatinib, sorafenib, phosphoinositide 3-kinases (PI3K), folic acid antagonists, methotrexate, 4-amino-folic acid, lometrexol, pemetrexed, trimetrexate, a pyrimidine antagonists, azacitidine, capecitabine, cytarabine, decitabine, 5-fluorouracil, 5-fluoro-2'-deoxyuridine 5'-phosphate, 5-fluorouridine triphosphate, gemcitabine, foxuridine, a purine antagonist azathioprine, cladribine, mercaptopurine, fludarabine, pentostatin, 6-thioguanine,

adenosine deaminase inhibitors, Cladribine, Fludarabine, Nelarabine, Pentostatin, borophycin, bortezomib, chemoprotective agents, amifostine, dexrazoxane, mesna, androgens, estrogens, medroxyprogesterone acetate, progestins, aminoglutethimide, anastrozole, bicalutamide, chlorotrianises, cyproterone acetate, degarelix, exemestane, flutamide, fulvestrant, goserelin, letrozole, leuprolide, lupron, medroxyprogesterone acetate, Megestrol acetate, tamoxifen, triptorelin, asparaginase, dacarbazine, hydroxyurea, levamisole, mitotane, procarbazane, tretinoin, glucocorticoids, prednisone, chromagens, dyes, antisense oligonucleotides whether naturally occurring or synthesized using standard and/or non-standard nucleotides (including RNA interference (RNAi)), double-stranded RNA (dsRNA), small interfering RNA (siRNA), microRNA (miRNA), aptamers, CpG oligonucleotides, ribozymes, angiozyme, ^{111}In, ^{177}Lu, ^{212}Bi, ^{213}Bi, ^{211}At, ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{90}Y, ^{125}I, ^{131}I, ^{32}P, ^{33}P, ^{47}Sc, ^{111}Ag, ^{67}Ga, ^{142}Pr, ^{153}Sm, ^{161}Tb, ^{166}Dy, ^{166}Ho, ^{186}Re, ^{188}Re, ^{189}Re, ^{212}Pb, ^{223}Ra, ^{225}Ac, ^{59}Fe, ^{75}Se, ^{77}As, ^{89}Sr, ^{99}Mo, ^{105}Rh, ^{109}Pd, ^{143}Pr, ^{149}Pm, ^{169}Er, ^{194}Ir, ^{198}Au, ^{199}Au, ^{211}Pb, Co-58, Ga-67, Br-80m, Tc-99m, Rh-103m, Pt-109, In-111 1, Sb-119, I-125, Ho-161 , Os-189m, Ir-192, Dy-152, At-211 , Bi-212, Ra-223, Rn-219, Po-215, Bi-21 1, Ac-225, Fr-221, At-217, Bi-213, Fm-255, ^{11}C, ^{13}N, ^{15}0, ^{75}Br, ^{198}Au, ^{224}Ac, ^{126}I, ^{133}I, ^{77}Br, ^{113m}In, ^{95}Ru, ^{97}Ru, ^{103}Ru, ^{105}Ru, ^{107}Hg, ^{203}Hg, ^{121m}Te, ^{122m}Te, ^{125m}Te, ^{165}Tm, ^{167}Tm, ^{168}Tm, ^{197}Pt, ^{109}Pd, ^{105}Rh, ^{142}Pr, ^{143}Pr, ^{161}Tb, ^{166}Ho, ^{199}Au, ^{57}Co, ^{58}Co, ^{51}Cr, ^{59}Fe, ^{75}Se, ^{201}Tl, ^{225}Ac, ^{76}Br, ^{169}Yb, taxane, cisplatin, metronidazole, misonidazole, desmethylmisonidazole, pimonidazole, etanidazole, nimorazole, mitomycin C, RSU 1069, SR 4233, E09, RB 6145, nicotinamide, 5-bromodeoxyuridine (BUdR), 5-iododeoxyuridine (IUdR), bromodeoxycytidine, fluorodeoxyuridine (FUdR), hydroxyurea, hematoporphyrin derivatives, Photofrin(r), benzoporphyrin derivatives, NPe6, tin etioporphyrin (SnET2), pheoborbide a, bacteriochlorophyll a, naphthalocyanines, phthalocyanines, zinc phthalocyanine, camptothecins, irinotecan, topotecan, amsacrine, daunorubicin, doxotrubicin, epipodophyllotoxins, ellipticines, epirubicin, etoposide, razoxane, teniposide, Axitinib, Bosutinib, Cediranib, Dasatinib, Erlotinib, Gefitinib, Imatinib, Lapatinib, Lestaurtinib, Nilotinib, Semaxanib, Sunitinib, Vandetanib, abrin, abrin A chain, alpha toxin, Aleurites fordii proteins, amatoxin, crotin, curcin, dianthin proteins, diptheria toxin, diphtheria A chain, nonbinding active fragments of diphtheria toxin, deoxyribonuclease (Dnase), gelonin, mitogellin, modeccin A chain, momordica charantia inhibitor, neomycin, onconase, phenomycin, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), pokeweed antiviral protein, Pseudomonas endotoxin, Pseudomonas exotoxin, exotoxin A chain from Pseudomonas aeruginosa, restrictocin, ricin, ricin A chain, ribonuclease (Rnase), sapaonaria officinalis inhibitor, saporin, alpha-sarcin, Staphylcoccal

enterotoxin-A, tetanus toxin, cisplatin, carboplatin, and oxaliplatin (Eloxatin, Sanofi Aventis), proteasome inhibitors, PS-341, HDAC inhibitors, vorinostat, belinostat, entinostat, mocetinostat, panobinostat, COX-2 inhibitors, substituted ureas, heat shock protein inhibitors, Geldanamycin, adrenocortical suppressants, tricothecenes, A12, 19D12, Cp751-871, H7C10, alphaIR3, ScFV/FC, EM/164, Matuzumab, Erbitux, Vectibix, mAb 806, Nimotuxumab, AVEO, AMG102, 5D5 (OA-5d5), H244G11, Ab #14 (MM 121-14), Herceptin, 1B4C3; 2D1D12, NVP-AEW541-A, BMS-536,924 (1H-benzoimidazol-2-yl)-1H-pyridin-2-one), BMS-554,417, Cycloligan, TAE226, PQ401 , Iressa, CI-1033 (PD 183805), Lapatinib (GW-572016), Tykerb, Tarceva, PKI-166, PD-158780, EKB-569, Tyrphostin AG 1478 (4-(3-Chloroanillino)-6,7-dimethoxyquinazoline), PHA665752, ARQ 197, Capecitabine, 5-Trifluoromethyl-2'-deoxyuridine, Methotrexate sodium, Raltitrexed, Pemetrexed, Tegafur, Cytosine Arabinoside (Cytarabine), 5-azacytidine, 6-mercaptopurine (Mercaptopurine, 6-MP), Azathioprine, 6-thioguanine, Pentostatin, Fludarabine phosphate, Cladribine (2-CdA, 2-chlorodeoxyadenosine), Ribonucleotide Reductase Inhibitor, Cyclophosphamide, Neosar, ifosfamide, Thiotepa, BCNU→ 1,3-bis(2-chloroethyl)-1-nitosourea, CCNU→ 1, -(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (methyl CCNU), Hexamethylmelamine, busulfan, Procarbazine HCL, Dacarbazine (DTIC), chlorambucil, melphalan, carboplatin, oxaliplatin, doxorubicin HCL, daunorubicin citrate, mitoxantrone HCL, actinomycin D, etoposide, topotecan HCl, teniposide, irinotecan HCL(CPT-II), vincristine, vinblastine sulfate, vinorelbine tartrate, vindesine sulphate, pac1itaxel, docetaxel, abraxane, ixabepilone, imatinib mesylate, sunitinib malate, sorafenib toslate, nilotinib hydrochloride monohydrate, L-asparaginase, alpha interferon, Avastin, IL-2, Aldesleukin, Proleukin, IL-12, Toremifene citrate, Fulvestrant, raloxifene HCL, anastrazole, letrozole, Fadrozole (CGS 16949A), exemestane, leuprolide acetate, Lupron, goserelin acetate, triptorelin pamoate, buserelin, Nafarelin, cetrorelix, bicalutamide, nilutamide, megestrol acetate, somatostatin Analogs, prendinsolone, dexamethasone, ketoconazole, sirolimus, temsirolimus (CCI-779), deforolimus (AP23573), Irinotecan; Leucovorin; Folfiri; 5-FU; Enalapril; Nifedipine; Clonidine; Temozolomide; Gemcitabine; Capecitabine; Paclitaxel; Regorafenib; Pertuzumab and everolimus (RAD001).

[040] In some embodiments, a composition is disclosed comprising one or more binding protein as disclosed herein and one or more additional agent, e.g., a chemotherapeutic agent. For example, the composition can comprise one or more binding proteins in solution with one or more additional agents. In some embodiments, the agent is one or more of: irinotecan, leucovorin, 5-FU, temozolomide, gemcitabine,

and paclitaxel. In an embodiment, the agent is irinotecan. In an embodiment, the agent is leucovorin. In an embodiment, the agent is 5-FU. In an embodiment, the agent is irinotecan, leucovorin, and 5-FU. In an embodiment, the agent is temozolomide. In an embodiment, the agent is gemcitabine. In an embodiment, the agent is paclitaxel.

[041] In another embodiment, the binding protein is a crystallized binding protein and exists as a crystal. In an embodiment, the crystal is a carrier-free pharmaceutical controlled release crystal. In another embodiment, the crystallized binding protein has a greater half life in vivo than the soluble counterpart of the binding protein. In yet another embodiment, the crystallized binding protein retains biological activity.

[042] In another embodiment, the binding protein described herein is glycosylated. For example, the glycosylation pattern is a human glycosylation pattern.

[043] An isolated nucleic acid encoding any one of the binding proteins disclosed herein is also provided. A further embodiment provides a vector comprising the isolated nucleic acid disclosed herein wherein the vector is pcDNA; pTT (Durocher et al. (2002) Nucleic Acids Res. 30(2); pTT3 (pTT with additional multiple cloning site; pEFBOS (Mizushima and Nagata (1990) Nucleic Acids Res. 18(17); pBV; pJV; pcDNA3.1 TOPO; pEF6 TOPO; pBOS; pHybE; or pBJ. In an embodiment, the vector is a vector disclosed in US Patent Publication No. 20090239259.

[044] In another aspect, a host cell is transformed with the vector disclosed herein. In an embodiment, the host cell is a prokaryotic cell, for example, E. Coli. In another embodiment, the host cell is a eukaryotic cell, for example, a protist cell, an animal cell, a plant cell, or a fungal cell. In an embodiment, the host cell is a mammalian cell including, but not limited to, CHO, COS, NS0, SP2, PER.C6, or a fungal cell, such as *Saccharomyces cerevisiae*, or an insect cell, such as Sf9. In an embodiment, two or more binding proteins, e.g., with different specificities, are produced in a single recombinant host cell. For example, the expression of a mixture of antibodies has been called Oligoclonics™ (Merus.B.V., The Netherlands) US Patent Nos. 7,262,028 and 7,429,486.

[045] A method of producing a binding protein disclosed herein comprising culturing any one of the host cells disclosed herein in a culture medium under conditions sufficient to produce the binding protein is provided. In an embodiment, 50%-75% of the binding protein produced by this method is a dual specific tetravalent

binding protein. In another embodiment, 75%-90% of the binding protein produced by this method is a dual specific tetravalent binding protein. In another embodiment, 90%-95% of the binding protein produced is a dual specific tetravalent binding protein.

[046] One embodiment provides a composition for the release of a binding protein wherein the composition comprises a crystallized binding protein, an ingredient, and at least one polymeric carrier. In an embodiment, the polymeric carrier is poly (acrylic acid), a poly (cyanoacrylate), a poly (amino acid), a poly (anhydride), a poly (depsipeptide), a poly (ester), poly (lactic acid), poly (lactic-co-glycolic acid) or PLGA, poly (b-hydroxybutryate), poly (caprolactone), poly (dioxanone), poly (ethylene glycol), poly ((hydroxypropyl) methacrylamide, poly [(organo)phosphazene], a poly (ortho ester), poly (vinyl alcohol), poly (vinylpyrrolidone), a maleic anhydride- alkyl vinyl ether copolymer, a pluronic polyol, albumin, alginate, cellulose, a cellulose derivative, collagen, fibrin, gelatin, hyaluronic acid, an oligosaccharide, a glycaminoglycan, a sulfated polysaccharide, or blends and copolymers thereof. In an embodiment, the ingredient is albumin, sucrose, trehalose, lactitol, gelatin, hydroxypropyl-β- cyclodextrin, methoxypolyethylene glycol, or polyethylene glycol.

[047] Another embodiment provides a method for treating a mammal comprising the step of administering to the mammal an effective amount of a *composition disclosed herein.*

[048] A pharmaceutical composition comprising a binding protein disclosed herein and a pharmaceutically acceptable carrier is provided. In some embodiments, the pharmaceutical composition comprises at least one additional therapeutic agent for treating a disorder. For example, the additional agent may be a therapeutic agent, a chemotherapeutic agent; an imaging agent, a cytotoxic agent, an angiogenesis inhibitor, a kinase inhibitor (including but not limited to a KDR and a TIE-2 inhibitor), a co-stimulation molecule modulator (including but not limited to anti-B7.1, anti-B7.2, CTLA4-Ig, anti-CD20), an adhesion molecule blocker (including but not limited to an anti-LFA-1 antibody, an anti-E/L selectin antibody, a small molecule inhibitor), an anti-cytokine antibody or functional fragment thereof (including but not limited to an anti-IL-18, an anti-TNF, or an anti-IL-6/cytokine receptor antibody), an anti-VEGF mAb; an anti-DLL4 mAb; methotrexate, cyclosporin, rapamycin, FK506, a detectable label or reporter, a TNF antagonist, an antirheumatic, a muscle relaxant, a narcotic, a non-steroid anti-inflammatory drug (NSAID), an analgesic, an anesthetic, a sedative, a local anesthetic, a neuromuscular blocker, an antimicrobial, an antipsoriatic, a corticosteriod, an anabolic steroid, an erythropoietin, an immunization, an immunoglobulin, an

immunosuppressive, a growth hormone, a hormone replacement drug, a radiopharmaceutical, an antidepressant, an antipsychotic, a stimulant, an asthma medication, a beta agonist, an inhaled steroid, an epinephrine or analog, a cytokine, or a cytokine antagonist. In some embodiments, the additional therapeutic agent is a chemotherapeutic agent. In some embodiments, the additional agent is one or more of: irinotecan, leucovorin, 5-FU, temozolomide, gemcitabine, and paclitaxel. In an embodiment, the agent is irinotecan. In an embodiment, the agent is leucovorin. In an embodiment, the agent is 5-FU. In an embodiment, the agent is irinotecan, leucovorin, and 5-FU. In an embodiment, the agent is temozolomide. In an embodiment, the agent is gemcitabine. In an embodiment, the agent is paclitaxel.

[049] In various embodiments, a method is provided for diagnosing and/or treating a human subject suffering from a disorder which can be diagnosed and/or treated by targeting VEGF and/or DLL4 (e.g., any angiogenesis disorder or any other disorder associated with aberrant expression of VEGF and/or DLL4), comprising administering to the human subject a binding protein disclosed herein such that the activity of the target, or targets, in the human subject is inhibited and one or more symptoms is alleviated or treatment is achieved is provided. The binding proteins provided herein can be used to diagnose and/or treat humans suffering from primary and metastatic cancers, including carcinomas of breast, colon, rectum, lung, oropharynx, hypopharynx, esophagus, stomach, pancreas, liver, gallbladder and bile ducts, small intestine, urinary tract (including kidney, bladder and urothelium), female genital tract (including cervix, uterus, and ovaries as well as choriocarcinoma and gestational trophoblastic disease), male genital tract (including prostate, seminal vesicles, testes and germ cell tumors), endocrine glands (including the thyroid, adrenal, and pituitary glands), and skin, as well as hemangiomas, melanomas, sarcomas (including those arising from bone and soft tissues as well as Kaposi's sarcoma), tumors of the brain, nerves, eyes, and meninges (including astrocytomas, gliomas, glioblastomas, retinoblastomas, neuromas, neuroblastomas, Schwannomas, and meningiomas), tumors arising from hematopoietic malignancies, acute leukemia, acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), B cell lymphoma, Burkitt's lymphoma, chronic myelocytic leukemia (CML), chronic lymphocytic leukemia (CLL), hairy cell leukemia, Hodgkin's and non-Hodgkin's lymphomas, hematopoietic malignancies, Kaposi's sarcoma, malignamt lymphoma, malignant histiocytosis, malignant melanoma, multiple myeloma, paraneoplastic syndrome/hypercalcemia of malignancy, or solid tumors.

[050] In some embodiments, a method of treating cancer in a patient comprises administering one or more of the binding proteins disclosed herein or a pharmaceutical composition thereof. In an embodiment, the cancer is colon cancer. In an embodiment, the cancer is glioblastoma. In an embodiment, the cancer is pancreatic cancer. In an embodiment, the cancer is breast cancer. In some embodiments, the methods of treating cancer, comprising administering one or more of the binding proteins disclosed herein or a pharmaceutical composition thereof, produce a reduction in tumor growth or a delay in tumor growth that is at least about equivalent to the expected additive effects of a combination of an anti-VEGF antibody and an anti-DLL4 antibody. In some embodiments, the methods produce a reduction in tumor growth or a delay in tumor growth that is more than additive (e.g., a larger reduction than that expected from adding the predicted effects of an anti-VEGF antibody and an anti-DLL4 antibody).

[051] In some embodiments, a method of treating a cancer comprises administering one or more of the binding proteins disclosed herein or a pharmaceutical composition thereof, in combination with one or more additional agents, e.g., a chemotherapeutic or biological agent. In some embodiments, the agent is one or more of: regorafenib (STIVAGRA™), pertuzumab (PERJECTA™), irinotecan, leucovorin, 5-FU, temozolomide, gemcitabine, and paclitaxel. In an embodiment, the agent is irinotecan. In an embodiment, the agent is leucovorin. In an embodiment, the agent is 5-FU. In an embodiment, the agent is irinotecan, leucovorin, and 5-FU. In an embodiment, the agent is temozolomide. In an embodiment, the agent is gemcitabine. In an embodiment, the agent is paclitaxel. In some embodiments, the methods of treating cancer, comprising administering one or more of the binding proteins disclosed herein or a pharmaceutical composition thereof, in combination with one or more additional agents, produce a reduction in tumor growth or a delay in tumor growth that is at least equivalent to the expected additive effects of a combination of the binding protein and the additional agent. In some embodiments, the methods produce a reduction in tumor growth or a delay in tumor growth that is more than additive (e.g., a larger reduction than that expected from adding the predicted effects of the binding protein and the additional agent).

[052] In some embodiments, a method of treating colon cancer comprises administering one or more of the binding proteins disclosed herein or a pharmaceutical composition thereof, optionally in combination with one or more of irinotecan, leucovorin, and 5-FU. In some embodiments, a method of treating glioblastoma

comprises administering one or more of the binding proteins disclosed herein or a pharmaceutical composition thereof, optionally in combination with temozolomide. In some embodiments, a method of treating pancreatic cancer comprises administering one or more of the binding proteins disclosed herein or a pharmaceutical composition thereof, optionally in combination with gemcitabine. In some embodiments, a method of treating breast cancer comprises administering one or more of the binding proteins disclosed herein or a pharmaceutical composition thereof, optionally in combination with paclitaxel.

[053] In various embodiments, the binding proteins provided herein can be administered in combination with one or more anti-hypertensive agent. The one or more anti-hypertensive agent can be selected from the group consisting of a diuretic, an adrenergic receptor antagonist, a calcium channel blocker, renin inhibitors, ACE inhibitors, angiotensin II receptor antagonists, vasodilators, and alpha-2 agonists. For example, the agent can be one or more of clonidine, enalapril; nifedipine; methyldopa, hydralazine, prazosin, reserpine, moxonidine, guanfacine, perindopril/indapamide, lofexidine, and metirosine. In some embodiments, the binding proteins provided herein can be administered in combination with one or more anticoagulant. For example, the anticoagulant can be one or more of warfarin, heparin, low molecular weight heparin, dalteparin sodium, argatroban, bivalirudin, lepirudin, and dextrose. In some embodiment, the binding proteins provided herein can be administered in combination with one or more anti-hypertensive agent and one or more anticoagulant.

[054] In various embodiments, the binding proteins provided herein can be used to diagnose and/or treat humans suffering from macular degeneration (including the wet form), diabetic retinopathy, and/or any other disease or disorder characterized by vascular overgrowth or edema.

[055] In an embodiment, the binding proteins, or antigen-binding portions thereof, are used to treat cancer or in the prevention or inhibition of metastases from the tumors described herein, either when used alone or in combination with radiotherapy and/or chemotherapeutic agents.

[056] In an embodiment, the chemotherapeutic or biological agents with which binding proteins provided herein can be combined include the following:13-cis-Retinoic Acid; 2-CdA; 2-Chlorodeoxyadenosine; 5-Azacitidine; 5-Fluorouracil; 5-FU; 6-Mercaptopurine; 6-MP; 6-TG; 6-Thioguanine; Abraxane; Accutane®; Actinomycin-D; Adriamycin®; Adrucil®; Afinitor®; Agrylin®; Ala-Cort®; Aldesleukin; Alemtuzumab; ALIMTA; Alitretinoin; Alkaban-AQ®; Alkeran®; All-transretinoic Acid; Alpha Interferon;

Altretamine; Amethopterin; Amifostine; Aminoglutethimide; Anagrelide; Anandron®; Anastrozole; Arabinosylcytosine; Ara-C Aranesp®; Aredia®; Arimidex®; Aromasin®; Arranon®; Arsenic Trioxide; Arzerra™; Asparaginase; ATRA; Avastin®; Azacitidine; BCG; BCNU; Bendamustine; Bevacizumab; Bexarotene; BEXXAR®; Bicalutamide; BiCNU; Blenoxane®; Bleomycin; Bortezomib; Busulfan; Busulfex®; C225; Calcium Leucovorin; Campath®; Camptosar®; Camptothecin-11; Capecitabine Carac™; Carboplatin; Carmustine; Carmustine Wafer; Casodex®; CC-5013; CCI-779; CCNU; CDDP; CeeNU; Cerubidine®; Cetuximab; Chlorambucil; Cisplatin; Citrovorum Factor; Cladribine; Cortisone; Cosmegen®; CPT-11; Cyclophosphamide; Cytadren®; Cytarabine; Cytarabine Liposomal; Cytosar-U®; Cytoxan®; Dacarbazine; Dacogen; Dactinomycin; Darbepoetin Alfa; Dasatinib; Daunomycin; Daunorubicin; Daunorubicin Hydrochloride; Daunorubicin Liposomal; DaunoXome®; Decadron; Decitabine; Delta-Cortef®; Deltasone®; Denileukin; Diftitox; DepoCyt™; Dexamethasone; Dexamethasone Acetate; Dexamethasone Sodium Phosphate; Dexasone; Dexrazoxane; DHAD; DIC; Diodex; Docetaxel; Doxil®; Doxorubicin; Doxorubicin Liposomal; Droxia™; DTIC; DTIC-Dome®; Duralone®; Efudex®; Eligard™; Ellence™; Eloxatin™; Elspar®; Emcyt®; Epirubicin; Epoetin Alfa; Erbitux; Erlotinib; Erwinia L-asparaginase; Estramustine; Ethyol Etopophos®; Etoposide; Etoposide Phosphate; Eulexin®; Everolimus; Evista®; Exemestane; Fareston®; Faslodex®; Femara®; Filgrastim; Floxuridine; Fludara®; Fludarabine; Fluoroplex®; Fluorouracil; Fluorouracil (cream); Fluoxymesterone; Flutamide; Folinic Acid; FUDR®; Fulvestrant; Gefitinib; Gemcitabine; Gemtuzumab ozogamicin; Gemzar; Gleevec™; Gliadel® Wafer; GM-CSF; Goserelin; Granulocyte-Colony Stimulating Factor (G-CSF); Granulocyte Macrophage Colony Stimulating Factor (G-MCSF); Halotestin®; Herceptin®; Hexadrol; Hexalen®; Hexamethylmelamine; HMM; Hycamtin®; Hydrea®; Hydrocort Acetate®; Hydrocortisone; Hydrocortisone Sodium Phosphate; Hydrocortisone Sodium Succinate; Hydrocortone Phosphate; Hydroxyurea; Ibritumomab; Ibritumomab Tiuxetan; Idamycin®; Idarubicin Ifex®; Interferon-alpha; Interferon-alpha-2b (PEG Conjugate); Ifosfamide; Interleukin-11 (IL-11); Interleukin-2 (IL-2); Imatinib mesylate; Imidazole Carboxamide; Intron A®; Iressa®; Irinotecan; Isotretinoin; Ixabepilone; Ixempra™; KADCYCLA®; Kidrolase (t) Lanacort®; Lapatinib; L-asparaginase; LCR; Lenalidomide; Letrozole; Leucovorin; Leukeran; Leukine™; Leuprolide; Leurocristine; Leustatin™; Liposomal Ara-C; Liquid Pred®; Lomustine; L-PAM; L-Sarcolysin; Lupron®; Lupron Depot®; Matulane®; Maxidex; Mechlorethamine; Mechlorethamine Hydrochloride; Medralone®; Medrol®; Megace®; Megestrol; Megestrol Acetate; Melphalan; Mercaptopurine; Mesna; Mesnex™; Methotrexate; Methotrexate Sodium;

Methylprednisolone; Meticorten®; Mitomycin; Mitomycin-C; Mitoxantrone M-Prednisol®; MTC; MTX; Mustargen®; Mustine; Mutamycin®; Myleran®; Mylocel™; Mylotarg®; Navelbine®; Nelarabine; Neosar®; Neulasta™; Neumega®; Neupogen®; Nexavar®; Nilandron®; Nilotinib; Nilutamide; Nipent®; Nitrogen Mustard Novaldex®; Novantrone®; Nplate; Octreotide; Octreotide acetate; Ofatumumab; Oncospar®; Oncovin®; Ontak®; Onxal™; Oprelvekin; Orapred®; Orasone®; Oxaliplatin; Paclitaxel; Paclitaxel Protein-bound; Pamidronate; Panitumumab; Panretin®; Paraplatin®; Pazopanib; Pediapred®; PEG Interferon; Pegaspargase; Pegfilgrastim; PEG-INTRON™; PEG-L-asparaginase; PEMETREXED; Pentostatin; Phenylalanine Mustard; Platinol®; Platinol-AQ®; Prednisolone; Prednisone; Prelone®; Procarbazine; PROCRIT®; Proleukin®; Prolifeprospan 20 with Carmustine Implant; Purinethol®; Raloxifene; Revlimid®; Rheumatrex®; Rituxan®; Rituximab; Roferon-A®; Romiplostim; Rubex®; Rubidomycin hydrochloride; Sandostatin®; Sandostatin LAR®; Sargramostim; Solu-Cortef®; Solu-Medrol®; Sorafenib; SPRYCEL™; STI-571; Streptozocin; SU11248; Sunitinib; Sutent®; Tamoxifen Tarceva®; Targretin®; Tasigna®; Taxol®; Taxotere®; Temodar®; Temozolomide Temsirolimus; Teniposide; TESPA; Thalidomide; Thalomid®; TheraCys®; Thioguanine; Thioguanine Tabloid®; Thiophosphoamide; Thioplex®; Thiotepa; TICE®; Toposar®; Topotecan; Toremifene; Torisel®; Tositumomab; Trastuzumab; Treanda®; Tretinoin; Trexall™; Trisenox®; TSPA; TYKERB®; VCR; Vectibix™; Velban®; Velcade®; VePesid®; Vesanoid®; Viadur™; Vidaza®; Vinblastine; Vinblastine Sulfate; Vincasar Pfs®; Vincristine; Vinorelbine; Vinorelbine tartrate; VLB; VM-26; Vorinostat; Votrient; VP-16; Vumon®; Xeloda®; Zanosar®; Zevalin™; Zinecard®; Zoladex®; Zoledronic acid; Zolinza; or Zometa®, and/or any other agent not specifically listed here that target similar pathways.

[057] In another embodiment, methods of treating a patient suffering from a disorder comprise the step of administering one or more of the binding proteins disclosed herein alone or administering the binding protein(s) before, concurrently, or after the administration of a second agent. In a particular embodiment, the pharmaceutical compositions disclosed herein are administered to a patient by oral, parenteral, subcutaneous, intramuscular, intravenous, intrarticular, intrabronchial, intraabdominal, intracapsular, intracartilaginous, intracavitary, intracelial, intracerebellar, intracerebroventricular, intracolic, intracervical, intragastric, intrahepatic, intramyocardial, intraosteal, intrapelvic, intrapericardiac, intraperitoneal, intrapleural, intraprostatic, intrapulmonary, intrarectal, intrarenal, intraretinal,

intraspinal, intrasynovial, intrathoracic, intrauterine, intravesical, bolus, vaginal, rectal, buccal, sublingual, intranasal, or transdermal administration.

[058] In various embodiments, methods of determining the presence, amount or concentration of one or more antigens, or fragments thereof, in a test sample are provided, wherein the one or more antigens or fragments thereof are DLL4 and/or VEGF. The method comprises assaying the test sample for the antigen, or fragment thereof, by an immunoassay. The immunoassay (i) employs at least one binding protein and at least one detectable label and (ii) comprises comparing a signal generated by the detectable label as a direct or indirect indication of the presence, amount or concentration of the antigen, or fragment thereof, in the test sample to a signal generated as a direct or indirect indication of the presence, amount or concentration of the antigen, or fragment thereof, in a control or a calibrator. The calibrator is optionally part of a series of calibrators in which each of the calibrators differs from the other calibrators in the series by the concentration of the antigen, or fragment thereof. The method can comprise (i) contacting the test sample with at least one capture agent, which binds to an epitope on the antigen, or fragment thereof, so as to form a complex comprising the capture agent and the antigen or fragment thereof (ii) contacting the complex comprising the capture agent and the antigen or fragment thereof with at least one detection agent, which comprises a detectable label and binds to an epitope on the antigen, or fragment thereof, that is not bound by the capture agent, to form a detection complex, and (iii) determining the presence, amount or concentration of the antigen, or fragment thereof, in the test sample based on the signal generated by the detectable label in the detection complex formed in (ii), wherein at least one capture agent and/or at least one detection agent is the at least one binding protein.

[059] Alternatively, in some embodiments the method of determining the presence, amount or concentration of one or more antigens, or fragments thereof, in a test sample can comprise (i) contacting the test sample with at least one capture agent, which binds to an epitope on the antigen, or fragment thereof, so as to form a complex comprising the capture agent and the antigen or fragment thereof and simultaneously or sequentially, in either order, contacting the test sample with detectably labeled antigen, or fragment thereof, which can compete with any antigen, or fragment thereof, in the test sample for binding to the at least one capture agent, wherein any antigen, or fragment thereof, present in the test sample and the detectably labeled antigen compete with each other to form a detection complex and (ii) determining the presence,

amount or concentration of the antigen, or fragment thereof, in the test sample based on the signal generated by the detectable label in the detection complex formed in (i), wherein at least one capture agent is the at least one binding protein and wherein the signal generated by the detectable label in the capture detection complex is inversely proportional to the amount or concentration of antigen, or fragment thereof, in the test sample.

[060] In various embodiments, the test sample can be from a patient, in which case the method can further comprise diagnosing, prognosticating, or assessing the efficacy of therapeutic/prophylactic treatment of the patient. If the method further comprises assessing the efficacy of therapeutic/prophylactic treatment of the patient, the method optionally further comprises modifying the therapeutic/prophylactic treatment of the patient as needed to improve efficacy. The method can be adapted for use in an automated system or a semi-automated system. Accordingly, the methods described herein also can be used to determine whether or not a subject has or is at risk of developing a given disease, disorder or condition. Specifically, such a method can comprise the steps of:

[061] (a) determining the concentration or amount of one or more analytes, or fragments thereof, in a test sample from a subject (e.g., using the methods described herein, or methods known in the art); and

[062] (b) comparing the concentration or amount of the analyte(s), or fragment(s) thereof, as determined in step (a) with a predetermined level, wherein, if the concentration or amount of analyte(s) determined in step (a) is favorable with respect to a predetermined level, then the subject is determined not to have or be at risk for a given disease, disorder or condition. However, if the concentration or amount of analyte(s) determined in step (a) is unfavorable with respect to the predetermined level, then the subject is determined to have or be at risk for a given disease, disorder or condition.

[063] Additionally, provided herein are methods of monitoring the progression of a disease in a subject. In some embodiments, the methods comprise the steps of:

[064] (a) determining the concentration or amount in a test sample from a subject of one or more analyte(s);

[065] (b) determining the concentration or amount of analyte(s) in a later test sample from the same subject; and

[066] (c) comparing the concentration or amount of analyte(s) as determined in step (b) with the concentration or amount of analyte(s) determined in step (a), wherein if the concentration or amount determined in step (b) is unchanged or is unfavorable when compared to the concentration or amount determined in step (a), then the disease in the subject is determined to have continued, progressed or worsened. By comparison, if the concentration or amount as determined in step (b) is favorable when compared to the concentration or amount as determined in step (a), then the disease in the subject is determined to have discontinued, regressed or improved.

[067] Optionally, the methods of monitoring the progression of a disease further comprises comparing the concentration or amount of analyte(s) as determined in step (b), for example, with a predetermined level. Further, optionally the methods comprise treating the subject with one or more pharmaceutical compositions for a period of time if the comparison shows that the concentration or amount of analyte(s) as determined in step (b), for example, is unfavorably altered with respect to the predetermined level.

[068] Also provided is a kit for assaying a test sample for the presence or concentration of one or more antigens, or fragments thereof, wherein the one or more antigens are DLL4 and/or VEGF. The kit comprises at least one binding protein, as described herein, for assaying the test sample for an antigen, or fragment thereof, and instructions for assaying the test sample for an antigen, or fragment thereof. In an embodiment, the at least one binding protein is optionally detectably labeled.

Brief Description of the Drawings

[069] Figure 1 is a schematic representation of Dual Variable Domain (DVD) binding protein constructs and shows the strategy for generation of a DVD binding protein from two parent antibodies.

Detailed Description

[070] Multivalent and/or multispecific binding proteins capable of binding epitopes on two different proteins are provided. Dual variable domain binding proteins (also referred to as DVDs, DVD binding proteins, or dual variable domain immunoglobulins (DVD-Ig^{TM})), and pharmaceutical compositions thereof, as well as nucleic acids, recombinant expression vectors and host cells for making such DVD binding proteins are also provided. Methods of using the DVD binding proteins to detect specific antigens, either in vitro or in vivo are also provided.

[071] Unless otherwise defined herein, scientific and technical terms used herein have the meanings that are commonly understood by those of ordinary skill in the art. In the event of any latent ambiguity, definitions provided herein take precedent over any dictionary or extrinsic definition. Unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. The use of "or" means "and/or" unless stated otherwise. The use of the term "including", as well as other forms, such as "includes" and "included", is not limiting. Any range described here will be understood to include the endpoints and all values between the endpoints.

[072] The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described. All documents, or portions of documents, cited in this application, including but not limited to patents, patent applications, articles, books, and treatises, are hereby expressly incorporated by reference in their entirety for any purpose. To the extent documents incorporated by reference contradict the disclosure contained in the specification, the specification will supersede any contradictory material.

[073] Generally, nomenclatures used in connection with cell and tissue culture, molecular biology, immunology, microbiology, genetics and protein and nucleic acid chemistry and hybridization described herein are those well known and commonly used in the art. The methods and techniques provided herein are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated. Enzymatic reactions and purification techniques are performed according to manufacturer's specifications, as commonly accomplished in the art or as described herein unless otherwise indicated. The nomenclatures used in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well known and commonly used in the art unless otherwise indicated. Standard techniques are used for chemical syntheses, chemical analyses, pharmaceutical preparation, formulation, and delivery, and treatment of patients.

[074] So that the disclosure may be more readily understood, select terms are defined below.

[075] The term "antibody" refers to an immunoglobulin (Ig) molecule, which is generally comprised of four polypeptide chains, two heavy (H) chains and two light

(L) chains, or a functional fragment, mutant, variant, or derivative thereof, that retains the epitope binding features of an Ig molecule. Such fragment, mutant, variant, or derivative antibody formats are known in the art. In an embodiment of a full-length antibody, each heavy chain is comprised of a heavy chain variable region (VH) and a heavy chain constant region (CH). The CH is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (VL) and a light chain constant region (CL). The CL is comprised of a single CL domain. The VH and VL can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDRs), interspersed with regions that are more conserved, termed framework regions (FRs). Generally, each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. Immunoglobulin molecules can be of any type (e.g., IgG, IgE, IgM, IgD, IgA and IgY), class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2), or subclass.

[076] The term "bispecific antibody" refers to an antibody that binds one antigen (or epitope) on one of its two binding arms (one pair of HC/LC), and binds a different antigen (or epitope) on its second binding arm (a different pair of HC/LC). A bispecific antibody has two distinct antigen binding arms (in both specificity and CDR sequences), and is monovalent for each antigen to which it binds. Bispecific antibodies include those generated by quadroma technology (Milstein and Cuello (1983) Nature 305(5934): 537-40), by chemical conjugation of two different monoclonal antibodies (Staerz et al. (1985) Nature 314(6012): 628-31), or by knob-into-hole or similar approaches which introduces mutations in the Fc region (Holliger et al. (1993) Proc. Natl. Acad. Sci. USA 90(14): 6444-6448).

[077] An "affinity matured" antibody is an antibody with one or more alterations in one or more CDRs thereof which result an improvement in the affinity of the antibody for antigen, compared to a parent antibody which does not possess those alteration(s). Exemplary affinity matured antibodies will have nanomolar or even picomolar affinities for the target antigen. Affinity matured antibodies are produced by procedures known in the art. Marks et al. (1992) BioTechnology 10:779-783 describes affinity maturation by VH and VL domain shuffling. Random mutagenesis of CDR and/or framework residues is described by Barbas et al. (1994) Proc. Nat. Acad. Sci. USA 91:3809-3813; Schier et al. (1995) Gene 169:147-155; Yelton et al. (1995) J. Immunol. 155:1994-2004; Jackson et al. (1995) J. Immunol. 154(7):3310-9; Hawkins et al. (1992) J. Mol. Biol. 226:889-896 and mutation at selective mutagenesis positions,

contact or hypermutation positions with an activity enhancing amino acid residue as described in US Patent No. 6,914,128.

[078] The term "CDR-grafted antibody" refers to an antibody that comprises heavy and light chain variable region sequences in which the sequences of one or more of the CDR regions of VH and/or VL are replaced with CDR sequences of another antibody. For example, the two antibodies can be from different species, such as antibodies having murine heavy and light chain variable regions in which one or more of the murine CDRs has been replaced with human CDR sequences.

[079] The term "humanized antibody" refers to an antibody from a non-human species that has been altered to be more "human-like", i.e., more similar to human germline sequences. One type of humanized antibody is a CDR-grafted antibody, in which non-human CDR sequences are introduced into human VH and VL sequences to replace the corresponding human CDR sequences. A "humanized antibody" is also an antibody or a variant, derivative, analog or fragment thereof that comprises framework region (FR) sequences having substantially identity (e.g., at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% identity) to the amino acid sequence of a human antibody FR sequences and at least one CDR having substantial identity (e.g., at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% identity) to the amino acid sequence of a non-human CDR. A humanized antibody may comprise substantially all of at least one, and typically two, variable domains (Fab, Fab', F(ab') 2, FabC, Fv) in which the sequence of all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin (i.e., donor antibody) and the sequence of all or substantially all of the FR regions are those of a human immunoglobulin. The humanized antibody can also include the CH1, hinge, CH2, CH3, and CH4 regions of the heavy chain from a human antibody. In an embodiment, a humanized antibody also comprises at least a portion of a human immunoglobulin Fc region. In some embodiments, a humanized antibody only contains a humanized light chain. In some embodiments, a humanized antibody only contains a humanized heavy chain. In some embodiments, a humanized antibody only contains a humanized variable domain of a light chain and/or humanized variable domain of a heavy chain. In some embodiments, a humanized antibody contains a light chain as well as at least the variable domain of a heavy chain. In some embodiments, a humanized antibody contains a heavy chain as well as at least the variable domain of a light chain.

[080] The terms "dual variable domain binding protein" and "dual variable domain immunoglobulin" refer to a binding protein that has two variable domains in each of its two binding arms (e.g., a pair of HC/LC) (see PCT Publication No. WO 02/02773), each of which is able to bind to an antigen. In an embodiment, each variable domain binds different antigens or epitopes. In another embodiment, each variable domain binds the same antigen or epitope. In another embodiment, a dual variable domain binding protein has two identical antigen binding arms, with identical specificity and identical CDR sequences, and is bivalent for each antigen to which it binds. In an embodiment, the DVD binding proteins may be monospecific, i.e., capable of binding one antigen or multispecific, i.e., capable of binding two or more antigens. DVD binding proteins comprising two heavy chain DVD polypeptides and two light chain DVD polypeptides are referred to as a DVD-Ig™. In an embodiment, each half of a four chain DVD binding protein comprises a heavy chain DVD polypeptide, and a light chain DVD polypeptide, and two antigen binding sites. In an embodiment, each binding site comprises a heavy chain variable domain and a light chain variable domain with a total of 6 CDRs involved in antigen binding per antigen binding site.

[081] The term "antiidiotypic antibody" refers to an antibody raised against the amino acid sequence of the antigen combining site of another antibody. Antiidiotypic antibodies may be administered to enhance an immune response against an antigen.

[082] The term "biological activity" refers to any one or more biological properties of a molecule (whether present naturally as found in vivo, or provided or enabled by recombinant means). Biological properties include, but are not limited to, binding a receptor, inducing cell proliferation, inhibiting cell growth, inducing other cytokines, inducing apoptosis, and enzymatic activity.

[083] The term "neutralizing" refers to counteracting the biological activity of an antigen when a binding protein specifically binds to the antigen. In an embodiment, a neutralizing binding protein binds to an antigen (e.g., a cytokine) and reduces its biologically activity by at least about 20%, 40%, 60%, 80%, 85% or more.

[084] "Specificity" refers to the ability of a binding protein to selectively bind an antigen.

[085] "Affinity" is the strength of the interaction between a binding protein and an antigen, and is determined by the sequence of the CDRs of the binding protein as well as by the nature of the antigen, such as its size, shape, and/or charge. Binding

proteins may be selected for affinities that provide desired therapeutic end-points while minimizing negative side-effects. Affinity may be measured using methods known to one skilled in the art (US 20090311253).

[086] The term "potency" refers to the ability of a binding protein to achieve a desired effect, and is a measurement of its therapeutic efficacy. Potency may be assessed using methods known to one skilled in the art (US 20090311253).

[087] The term "cross-reactivity" refers to the ability of a binding protein to bind a target other than that against which it was raised. Generally, a binding protein will bind its target tissue(s)/antigen(s) with an appropriately high affinity, but will display an appropriately low affinity for non-target normal tissues/antigens. Individual binding proteins are generally selected to meet two criteria: (1) antibody binding, as visualized using staining methods known in the art, to tissue appropriate for the known expression of the antibody target and (2) a similar staining pattern between human and tox species (e.g., mouse and cynomolgus monkey) tissues from the same organ. These and other methods of assessing cross-reactivity are known to one skilled in the art (US 20090311253).

[088] The term "biological function" refers the specific *in vitro* or *in vivo* actions of a binding protein. Binding proteins may target several classes of antigens and achieve desired therapeutic outcomes through multiple mechanisms of action. Binding proteins may target soluble proteins, cell surface antigens, and/or extracellular protein deposits. Binding proteins may agonize, antagonize, or neutralize the activity of their targets. Binding proteins may assist in the clearance of the targets to which they bind, or may result in cytotoxicity when bound to cells. Portions of two or more antibodies may be incorporated into a multivalent format to achieve more than one distinct function in a single binding protein molecule. *in vitro* assays and *in vivo* models used to assess biological function are known to one skilled in the art (US 20090311253).

[089] A "stable" binding protein is one in which the binding protein essentially retains its physical stability, chemical stability and/or biological activity upon storage. A multivalent binding protein that is stable *in vitro* at various temperatures for an extended period of time is desirable. Methods of stabilizing binding proteins and assessing their stability at various temperatures are known to one skilled in the art (US 20090311253).

[090] The term "solubility" refers to the ability of a protein to remain dispersed within an aqueous solution. The solubility of a protein in an aqueous formulation depends upon the proper distribution of hydrophobic and hydrophilic amino acid residues, and therefore, solubility can correlate with the production of correctly folded proteins. A person skilled in the art will be able to detect an increase or decrease in solubility of a binding protein using routine HPLC techniques and methods known to one skilled in the art (US 20090311253).

[091] Binding proteins may be produced using a variety of host cells or may be produced *in vitro*, and the relative yield per effort determines the "production efficiency." Factors influencing production efficiency include, but are not limited to, host cell type (prokaryotic or eukaryotic), choice of expression vector, choice of nucleotide sequence, and methods employed. The materials and methods used in binding protein production, as well as the measurement of production efficiency, are known to one skilled in the art (US 20090311253).

[092] The term "immunogenicity" means the ability of a substance to induce an immune response. Administration of a therapeutic binding protein may result in a certain incidence of an immune response. Potential elements that might induce immunogenicity in a multivalent format may be analyzed during selection of the parental antibodies, and steps to reduce such risk can be taken to optimize the parental antibodies prior to incorporating their sequences into a multivalent binding protein format. Methods of reducing the immunogenicity of antibodies and binding proteins are known to one skilled in the art (US 20090311253).

[093] The terms "label" and "detectable label" mean a moiety attached to a member of a specific binding pair, such as an antibody or its analyte to render a reaction (e.g., a binding) between the members of the specific binding pair, detectable. The labeled member of the specific binding pair is referred to as "detectably labeled." Thus, the term "labeled binding protein" refers to a protein with a label incorporated that provides for the identification of the binding protein. In an embodiment, the label is a detectable marker that can produce a signal that is detectable by visual or instrumental means, e.g., incorporation of a radiolabeled amino acid or attachment to a polypeptide of biotinyl moieties that can be detected by marked avidin (e.g., streptavidin containing a fluorescent marker, or enzymatic activity that can be detected by optical or colorimetric methods). Examples of labels for polypeptides include, but are not limited to, the following: radioisotopes or radionuclides (e.g., ^{3}H, ^{14}C, ^{35}S, ^{90}Y, ^{99}Tc, ^{111}In, ^{125}I, ^{131}I, ^{177}Lu, ^{166}Ho, or ^{153}Sm); chromogens, fluorescent labels (e.g., FITC, rhodamine,

lanthanide phosphors), enzymatic labels (e.g., horseradish peroxidase, luciferase, alkaline phosphatase); chemiluminescent markers; biotinyl groups; predetermined polypeptide epitopes recognized by a secondary reporter (e.g., leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags); and magnetic agents, such as gadolinium chelates. Representative examples of labels commonly employed for immunoassays include moieties that produce light, e.g., acridinium compounds, and moieties that produce fluorescence, e.g., fluorescein. In this regard, the moiety itself may not be detectably labeled but may become detectable upon reaction with yet another moiety.

[094] The term "conjugate" refers to a binding protein, such as an antibody or DVD-Ig, that is chemically linked to a second chemical moiety, such as a therapeutic or cytotoxic agent. The term "agent" includes a chemical compound, a mixture of chemical compounds, a biological macromolecule, or an extract made from biological materials. Examples of therapeutic or cytotoxic agents include, but are not limited to, taxol, cytochalasin B, gramicidin D, emetine, mitomycin, etoposide, teniposide, vincristine, vinblastine, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, metoprolol, atenolol, bisoprolol, and puromycin and analogs or homologs thereof. When employed in the context of an immunoassay, the conjugate may be a detectably labeled antibody or DVD-Ig used as the detection agent.

[095] The terms "crystal" and "crystallized" refer to a binding protein (e.g., an antibody), or antigen binding portion thereof, that exists in the form of a crystal. Crystals are one form of the solid state of matter, which is distinct from other forms such as the amorphous solid state or the liquid crystalline state. Crystals are composed of regular, repeating, three-dimensional arrays of atoms, ions, molecules (e.g., proteins such as antibodies), or molecular assemblies (e.g., antigen/antibody complexes). These three-dimensional arrays are arranged according to specific mathematical relationships that are well-understood in the field. The fundamental unit, or building block, that is repeated in a crystal is called the asymmetric unit. Repetition of the asymmetric unit in an arrangement that conforms to a given, well-defined crystallographic symmetry provides the "unit cell" of the crystal. Repetition of the unit cell by regular translations in all three dimensions provides the crystal. See Giege, R. and Ducruix, A. Barrett, CRYSTALLIZATION OF NUCLEIC ACIDS AND PROTEINS, A

PRACTICAL APPROACH, 2nd ea., pp. 20 1-16, Oxford University Press, New York, New York, (1999).

[096] The term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Other vectors include RNA vectors. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply, "expression vectors"). In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, other forms of expression vectors are also included, such as viral vectors (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions. A group of pHybE vectors (US Patent Application Serial No. 61/021,282) can be used for parental antibody and DVD binding protein cloning. V1, derived from pJP183; pHybE-hCg1,z,non-a V2, can be used for cloning of antibody and DVD heavy chains with a wildtype constant region. V2, derived from pJP191; pHybE-hCk V3, can be used for cloning of antibody and DVD light chains with a kappa constant region. V3, derived from pJP192; pHybE-hCl V2, can be used for cloning of antibody and DVD light chains with a lambda constant region. V4, built with a lambda signal peptide and a kappa constant region, can be used for cloning of DVD light chains with a lambda-kappa hybrid V domain. V5, built with a kappa signal peptide and a lambda constant region, can be used for cloning of DVD light chains with a kappa-lambda hybrid V domain. V7, derived from pJP183; pHybE-hCg1,z,non-a V2, can be used for cloning of antibody and DVD heavy chains with a (234,235 AA) mutant constant region.

[097] The terms "recombinant host cell" or "host cell" refer to a cell into which exogenous DNA has been introduced. Such terms refer not only to the particular subject cell, but to the progeny of such a cell. Because certain modifications may occur

in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term "host cell" as used herein. In an embodiment, host cells include prokaryotic and eukaryotic cells. In an embodiment, eukaryotic cells include protist, fungal, plant and animal cells. In another embodiment, host cells include, but are not limited to, the prokaryotic cell line *E.Coli*; mammalian cell lines CHO, HEK 293, COS, NS0, SP2 and PER.C6; the insect cell line Sf9; and the fungal cell *Saccharomyces cerevisiae*.

[098] The term "transfection" encompasses a variety of techniques commonly used for the introduction of exogenous nucleic acid (e.g., DNA) into a host cell, e.g., electroporation, calcium-phosphate precipitation, DEAE-dextran transfection and the like.

[099] The term "cytokine" refers to a protein released by one cell population that acts on another cell population as an intercellular mediator. The term "cytokine" includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

[0100] The term "biological sample" means a quantity of a substance from a living thing or formerly living thing. Such substances include, but are not limited to, blood, (e.g., whole blood), plasma, serum, urine, amniotic fluid, synovial fluid, *endothelial cells*, *leukocytes*, *monocytes*, *other cells*, *organs*, *tissues*, *bone marrow*, lymph nodes and spleen.

[0101] The term "component" refers to an element of a composition. In relation to a diagnostic kit, for example, a component may be a capture antibody, a detection or conjugate antibody, a control, a calibrator, a series of calibrators, a sensitivity panel, a container, a buffer, a diluent, a salt, an enzyme, a co-factor for an enzyme, a detection reagent, a pretreatment reagent/solution, a substrate (e.g., as a solution), a stop solution, and the like that can be included in a kit for assay of a test sample. Thus, a "component" can include, in some embodiments, a polypeptide or other analyte as above, that is immobilized on a solid support, such as by binding to an anti-analyte (e.g., anti-polypeptide) antibody. In some embodiments, one or more components can be in solution or lyophilized.

[0102] "Control" refers to a composition that does not comprise an analyte ("negative control") or does comprise the analyte ("positive control"). A positive control can comprise a known concentration of analyte. "Control," "positive control," and

"calibrator" may be used interchangeably herein to refer to a composition comprising a known concentration of analyte. A "positive control" can be used to establish assay performance characteristics and is a useful indicator of the integrity of reagents (e.g., analytes).

[0103] "Predetermined cutoff" and "predetermined level" refer generally to an assay cutoff value that is used to assess diagnostic/prognostic/therapeutic efficacy results by comparing the assay results against the predetermined cutoff/level, where the predetermined cutoff/level already has been linked or associated with various clinical parameters (e.g., severity of disease, progression/nonprogression/improvement, etc.). While the present disclosure may provide exemplary predetermined levels, it is well-known that cutoff values may vary depending on the nature of the immunoassay (e.g., antibodies employed, etc.). It further is well within the ordinary skill of one in the art to adapt the disclosure herein for other immunoassays to obtain immunoassay-specific cutoff values for those other immunoassays based on this disclosure. Whereas the precise value of the predetermined cutoff/level may vary between assays, correlations as described herein (if any) may be generally applicable.

[0104] A "Pretreatment reagent," e.g., a lysis, precipitation and/or solubilization reagent, as used in a diagnostic assay as described herein, is one that lyses any cells and/or solubilizes any analyte that is/are present in a test sample. Pretreatment is not necessary for all samples, as described further herein. Among other things, solubilizing the analyte (e.g., polypeptide of interest) may entail release of the analyte from an endogenous binding proteins present in the sample. A pretreatment reagent may be homogeneous (not requiring a separation step) or heterogeneous (requiring a separation step). In some embodiments, when using a heterogeneous pretreatment reagent, precipitated analyte-binding proteins are removed from the test sample prior to proceeding to the next step of the assay.

[0105] "Quality control reagents" in the context of immunoassays and kits described herein, include, but are not limited to, calibrators, controls, and sensitivity panels. One or more "calibrator(s)" or "standard(s)" are typically used in order to establish calibration (standard) curves for interpolation of the concentration of a target molecule, such as an antibody or an analyte. In some embodiments, a single calibrator, which is near a predetermined positive/negative cutoff, can be used. Alternatively, in other embodiments multiple calibrators (i.e., more than one calibrator or a varying

amount of calibrator(s)) can be used to establish a "sensitivity panel" or a "sensitivity gradient"

[0106] The term "specific binding partner" refers to a member of a specific binding pair. A specific binding pair comprises two different molecules that specifically bind to each other through chemical or physical means. In various embodiments, in addition to antigen and antibody specific binding, other specific binding pairs can include biotin and avidin (or streptavidin), carbohydrates and lectins, complementary nucleotide sequences, effector and receptor molecules, cofactors and enzymes, enzyme inhibitors and enzymes, and the like. Furthermore, specific binding pairs can include, in some embodiments, members that are analogs of the original specific binding members, for example, an analyte-analog. Immunoreactive specific binding members include antigens, antigen fragments, and antibodies, including monoclonal and polyclonal antibodies as well as complexes, fragments, and variants (including fragments of variants) thereof, whether isolated or recombinantly produced.

[0107] The term "Fc region" defines the C-terminal region of an immunoglobulin heavy chain, which may be generated by papain digestion of an intact antibody. The Fc region may be a native sequence Fc region or a variant Fc region. The Fc region of an immunoglobulin generally comprises two constant domains, a CH2 domain and a CH3 domain, and optionally comprises a CH4 domain. Replacements of amino acid residues in the Fc portion to alter antibody effector function are known in the art (e.g., US Patent Nos. 5,648,260 and 5,624,821). The Fc region mediates several important effector functions, e.g., cytokine induction, antibody dependent cell mediated cytotoxicity (ADCC), phagocytosis, complement dependent cytotoxicity (CDC), and the half-life/clearance rate of antibody and antigen-antibody complexes. In some cases these effector functions are desirable for a therapeutic immunoglobulin but in other cases might be unnecessary or even deleterious, depending on the therapeutic objectives.

[0108] The term "antigen-binding portion" of a binding protein means one or more fragments of a binding protein (e.g., an antibody) that retain the ability to specifically bind to an antigen. The antigen-binding function of a binding protein can be performed by fragments of a full-length antibody, as well as bispecific, dual specific, or multi-specific formats. Examples of binding fragments encompassed within the term "antigen-binding portion" of an binding protein include (i) an Fab fragment, a monovalent fragment consisting of the *VL*, *VH*, *CL* and *CH1* domains; (ii) an $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide

bridge at the hinge region; (iii) an Fd fragment consisting of the VH and CH1 domains; (iv) an Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment, which comprises a single variable domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are encoded by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. Other forms of single chain antibodies, such as diabodies are also encompassed. In addition, single chain antibodies also include "linear antibodies" comprising a pair of tandem Fv segments (VH-CH1-VH-CH1) which, together with complementary light chain polypeptides, form a pair of antigen binding regions.

[0109] The term "multivalent binding protein" means a binding protein comprising two or more antigen binding sites. In an embodiment, the multivalent binding protein is engineered to have three or more antigen binding sites, and is not a naturally occurring antibody. The term "multispecific binding protein" refers to a binding protein capable of binding two or more related or unrelated targets. In an embodiment, the DVD binding proteins provided herein comprise two or more antigen binding sites and are tetravalent or multivalent binding proteins.

[0110] The term "linker" means an amino acid residue or a polypeptide comprising two or more amino acid residues joined by peptide bonds that are used to link two polypeptides (e.g., two VH or two VL domains). Examples of such linker polypeptides are well known in the art (see, e.g., Holliger et al. (1993) Proc. Natl. Acad. Sci. USA 90:6444-6448; Poljak et al. (1994) Structure 2:1121-1123).

[0111] The terms "Kabat numbering", "Kabat definitions" and "Kabat labeling" are used interchangeably herein. These terms, which are recognized in the art, refer to a system of numbering amino acid residues which are more variable (i.e., hypervariable) than other amino acid residues in the heavy and light chain variable regions of an antibody, or an antigen binding portion thereof (Kabat et al. (1971) Ann. NY Acad. Sci. 190:382-391 and, Kabat et al. (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242). For the heavy chain variable region, the hypervariable region ranges from amino acid positions 31 to 35 for CDR1, amino acid positions 50 to 65 for CDR2, and amino acid positions 95 to 102 for CDR3. For the light chain variable

region, the hypervariable region ranges from amino acid positions 24 to 34 for CDR1, amino acid positions 50 to 56 for CDR2, and amino acid positions 89 to 97 for CDR3.

[0112] The term "CDR" means a complementarity determining region within an immunoglobulin variable region sequence. There are three CDRs in each of the variable regions of the heavy chain and the light chain, which are designated CDR1, CDR2 and CDR3, for each of the heavy and light chain variable regions. The term "CDR set" refers to a group of three CDRs that occur in a single variable region capable of binding the antigen. The exact boundaries of these CDRs have been defined differently according to different systems. The system described by Kabat (Kabat et al. (1987) and (1991)) not only provides an unambiguous residue numbering system applicable to any variable region of an antibody, but also provides precise residue boundaries defining the three CDRs. These CDRs may be referred to as Kabat CDRs. Chothia and colleagues (Chothia and Lesk (1987) J. Mol. Biol. 196:901-917; Chothia et al. (1989) Nature 342:877-883) found that certain sub- portions within Kabat CDRs adopt nearly identical peptide backbone conformations, despite having great diversity at the level of amino acid sequence. These sub-portions were designated as L1, L2 and L3 or H1, H2 and H3 where the "L" and the "H" designates the light chain and the heavy chain regions, respectively. These regions may be referred to as Chothia CDRs, which have boundaries that overlap with Kabat CDRs. Other boundaries defining CDRs overlapping with the Kabat CDRs have been described by Padlan (1995) FASEB J. 9:133-139 and MacCallum (1996) J. Mol. Biol. 262(5):732-45). Still other CDR boundary definitions may not strictly follow one of the herein systems, but will nonetheless overlap with the Kabat CDRs, although they may be shortened or lengthened in light of prediction or experimental findings that particular residues or groups of residues or even entire CDRs do not significantly impact antigen binding. The methods used herein may utilize CDRs defined according to any of these systems, although certain embodiments use Kabat or Chothia defined CDRs.

[0113] The term "epitope" means a region of an antigen that is bound by a binding protein, e.g., a region capable of specificly binding to an immunoglobulin or T-cell receptor. In certain embodiments, epitope determinants include chemically active surface groupings of molecules such as amino acids, sugar side chains, phosphoryl, or sulfonyl, and, in certain embodiments, may have specific three dimensional structural characteristics, and/or specific charge characteristics. In an embodiment, an epitope comprises the amino acid residues of a region of an antigen (or fragment thereof) known to bind to the complementary site on the specific binding partner. An antigenic

fragment can contain more than one epitope. In certain embodiments, a binding protein specifically binds an antigen when it recognizes its target antigen in a complex mixture of proteins and/or macromolecules. Binding proteins "bind to the same epitope" if the antibodies cross-compete (e.g., one prevents the other from binding to the binding protein, or inhibits the modulating effect on the other of binding to the binding protein). The methods of visualizing and modeling epitope recognition are known to one skilled in the art (US 20090311253).

[0114] "Pharmacokinetics" refers to the process by which a drug is absorbed, distributed, metabolized, and excreted by an organism. In some embodiments, to generate a multivalent binding protein molecule with a desired pharmacokinetic profile, parent monoclonal antibodies with similarly desired pharmacokinetic profiles are selected. PK profiles of the selected parental monoclonal antibodies can be easily determined, for example using rodents in methods known to one skilled in the art (US 20090311253).

[0115] "Bioavailability" refers to the amount of active drug that reaches its target following administration. Bioavailability is function of several of the previously described properties, including stability, solubility, immunogenicity and pharmacokinetics, and can be assessed using methods known to one skilled in the art (US 20090311253).

[0116] The term "surface plasmon resonance" means an optical phenomenon that allows for the analysis of real-time biospecific interactions by detection of alterations in protein concentrations within a biosensor matrix, for example using the BIAcore® system (BIAcore International AB, a GE Healthcare Co., Uppsala, Sweden and Piscataway, NJ). For further descriptions, see Jönsson et al. (1993) Ann. Biol. Clin. 51:19-26. The term "K_{on}" means the on rate constant for association of a binding protein (e.g., an antibody or DVD) to the antigen to form a bound complex (e.g., a DVD/antigen complex). The term "K_{on}" also means "association rate constant", or "ka", as is used interchangeably herein. This value indicating the binding rate of a binding protein to its target antigen or the rate of complex formation between a binding protein, (e.g., an antibody) and antigen. This is also shown by the equation below:

Antibody ("Ab") + Antigen ("Ag")→Ab-Ag

[0117] The term "K_{off}" means the off rate constant for dissociation, or "dissociation rate constant", of a binding protein (e.g., an antibody or DVD) froma bound complex (e.g., a DVD/antigen complex), as is known in the art. This value

indicates the dissociation rate of a binding protein (e.g., an antibody) from its target antigen or the separation of an Ab-Ag complex over time into free antibody and antigen, as shown by the equation below:

$$Ab + Ag \leftarrow Ab\text{-}Ag$$

[0118] The terms "K_D" and "equilibrium dissociation constant" means the value obtained in a titration measurement at equilibrium, or by dividing the dissociation rate constant (K_{off}) by the association rate constant (K_{on}). The association rate constant, the dissociation rate constant and the equilibrium dissociation constant, are used to represent the binding affinity of a binding protein (e.g., an antibody or DVD) to an antigen. Methods for determining association and dissociation rate constants are well known in the art. Using fluorescence–based techniques offers high sensitivity and the ability to examine samples in physiological buffers at equilibrium. Other experimental approaches and instruments such as a BIAcore® (biomolecular interaction analysis) assay, can be used (e.g., instrument available from BIAcore International AB, a GE Healthcare company, Uppsala, Sweden). Additionally, a KinExA® (Kinetic Exclusion Assay) assay, available from Sapidyne Instruments (Boise, Idaho), can also be used.

[0119] The term "variant" means a polypeptide that differs from a given polypeptide in amino acid sequence by the addition (e.g., insertion), deletion, or conservative substitution of amino acids, but that retains the biological activity of the given polypeptide (e.g., a variant VEGF antibody can compete with anti-VEGF antibody for binding to VEGF). A conservative substitution of an amino acid, i.e., replacing an amino acid with a different amino acid of similar properties (e.g., hydrophilicity and/or degree or distribution of charged regions) is recognized in the art as typically involving a minor change. These minor changes can be identified, in part, by considering the hydropathic index of amino acids, as understood in the art (see, e.g., Kyte et al. (1982) J. Mol. Biol. 157: 105-132). In one aspect, amino acids having hydropathic indexes of ± 2 are substituted. The hydrophilicity of amino acids can also be used to reveal substitutions that would result in proteins that retain biological function. A consideration of the hydrophilicity of amino acids in the context of a peptide permits calculation of the greatest local average hydrophilicity of that peptide, a useful measure that has been reported to correlate well with antigenicity and immunogenicity (see, e.g., US Patent No. 4,554,101). Substitution of amino acids having similar hydrophilicity values can result in peptides retaining biological activity, for example immunogenicity, as is understood in the art. In one aspect, substitutions are performed with amino acids having hydrophilicity values within ± 2 of each other. Both the hydrophobicity index and

the hydrophilicity value of amino acids are influenced by the particular side chain of that amino acid. Consistent with that observation, amino acid substitutions that are compatible with biological function are understood to depend on the relative similarity of the amino acids, and particularly the side chains of those amino acids, as revealed by the hydrophobicity, hydrophilicity, charge, size, and other properties. The term "variant" also includes polypeptides or fragments thereof that have been differentially processed, such as by proteolysis, phosphorylation, or other post-translational modification, yet retain biological activity and/or antigen reactivity, e.g., the ability to bind to VEGF and/or DLL4. The term "variant" encompasses fragments of a variant unless otherwise defined. A variant may be 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%,85%, 84%, 83%, 82%, 81%, 80%, 79%, 78%, 77%, 76%, or 75% identical to the wild type sequence.

I. Generation of binding proteins

[0120] Binding proteins capable of binding two different antigens, and methods of making the same are provided. The binding protein can be generated using various techniques. Expression vectors, host cell and methods of generating the binding protein are also provided.

A. Generation of parent monoclonal antibodies

[0121] The variable domains of the DVD binding protein can be obtained from parent antibodies (Abs), including polyclonal Abs and monoclonal Abs (mAbs) capable of binding antigens of interest. These antibodies may be naturally occurring or may be generated by recombinant technology. The person of ordinary skill in the art is well familiar with many methods for producing antibodies, including, but not limited to using hybridoma techniques, selected lymphocyte antibody method (SLAM), use of a phage, yeast, or RNA-protein fusion display or other library, immunizing a non-human animal comprising at least some of the human immunoglobulin locus, and preparation of chimeric, CDR-grafted, and humanized antibodies. See, e.g., US Patent Publication No. 20090311253 A1. Variable domains may also be prepared using affinity maturation techniques.

B. Criteria for selecting parent monoclonal antibodies

[0122] An embodiment is provided comprising selecting parent antibodies with at least one or more properties desired in the DVD binding protein molecule. In an embodiment, the desired property is one or more antibody parameters, such as, for example, antigen specificity, affinity to antigen, potency, biological function, epitope

recognition, stability, solubility, production efficiency, immunogenicity, pharmacokinetics, bioavailability, tissue cross reactivity, or orthologous antigen binding. See, e.g., US Patent Publication No. 20090311253.

C. Binding protein molecules

[0123] In various embodiments, the binding protein may be designed such that two different light chain variable domains (VL) from the two different parent monoclonal antibodies are linked in tandem directly or via a linker by recombinant DNA techniques, followed by the light chain constant domain CL. Similarly, the heavy chain comprises two different heavy chain variable domains (VH) linked in tandem, directly or via a linker, followed by the constant domain CH1 and Fc region (**Figure 1**).

[0124] In various embodiments , the variable domains can be obtained using recombinant DNA techniques from parent antibodies generated by any one of the methods described herein. In an embodiment, the variable domain is a murine heavy or light chain variable domain. In another embodiment, the variable domain is a CDR grafted or a humanized variable heavy or light chain domain. In an embodiment, the variable domain is a human heavy or light chain variable domain.

[0125] In various embodiments, the linker sequence may be a single amino acid or a polypeptide sequence. In an embodiment, the choice of linker sequences is based on crystal structure analysis of several Fab molecules. There is a natural flexible linkage between the variable domain and the CH1/CL constant domain in Fab or antibody molecular structure. This natural linkage generally comprises approximately 10-12 amino acid residues, contributed by 4-6 residues from the C-terminus of a V domain and 4-6 residues from the N-terminus of a CL/CH1 domain. In some embodiments, DVD binding proteins are generated using N-terminal 5-6 amino acid residues, or 11-12 amino acid residues, of a CL or CH1 as a linker in the light chain and heavy chains, respectively. The N-terminal residues of a CL or CH1 domains, particularly the first 5-6 amino acid residues, can adopt a loop conformation without strong secondary structures, and therefore can act as flexible linkers between the two variable domains. The N-terminal residues of a CL or CH1 domains are natural extension of the variable domains, as they are part of the Ig sequences, and therefore their use minimizes to a large extent any immunogenicity potentially arising from the linkers and junctions.

[0126] In various embodiments, the binding proteins disclosed herein include at least one linker comprising one or more of SEQ ID NO: 1-38 (Table 1). In an embodiment, X2 is an Fc region. In another embodiment, X2 is a variant Fc region.

Table 1: List of Linker Sequences

SEQ ID NO:	Sequence	SEQ ID NO:	Sequence
1	ASTKGPSVFPLAP	20	RADAAAAGGPGS
2	ASTKGP	21	RADAAAA
3	GGGGSG	22	SAKTTPKLEEGEFSEARV
4	GGGGSGGGGS	23	ADAAP
5	GGGGSGGGGSGGGG	24	ADAAPTVSIFPP
6	TVAAPSVFIFPP	25	TVAAP
7	TVAAP	26	TVAAPSVFIFPP
8	GGGGSG	27	QPKAAP
9	GGSGGGGSG	28	QPKAAPSVTLFPP
10	GGSGGGGSGGGGS	29	AKTTPP
11	GGSGG	30	AKTTPPSVTPLAP
12	GGSGGGGSGGGS	31	AKTTAP
13	AKTTPKLEEGEFSEAR	32	AKTTAPSVYPLAP
14	AKTTPKLEEGEFSEARV	33	GGGGSGGGGSGGGGS
15	AKTTPKLGG	34	GENKVEYAPALMALS
16	SAKTTPKLGG	35	GPAKELTPLKEAKVS
17	SAKTTP	36	GHEAAAVMQVQYPAS
18	RADAAP	37	TVAAPSVFIFPPTVAAPSVFIFPP
19	RADAAPTVS	38	ASTKGPSVFPLAPASTKGPSVFPLAP

[0127] Other linker sequences may include any sequence of any length derived from a CL/CH1 domain but not all residues of a CL/CH1 domain; for example the first 5-12 amino acid residues of a CL/CH1 domain. In another example, the light chain linkers can be selected from Cκ or Cλ; and the heavy chain linkers can be derived from CH1 of any isotype, including Cγ1, Cγ2, Cγ3, Cγ4, Cα1, Cα2, Cδ, Cε, and Cμ. Linker sequences may also be derived from other proteins such as Ig-like proteins (e.g., TCR, FcR, KIR); G/S based sequences (e.g., G4S repeats); hinge region-derived sequences; and other natural sequences from other proteins. Other linker sequences

may include any sequence of any length comprising G/S repeats (e.g., a sequence comprising repeats of a GGGS motif), or any other peptide linkers.

[0128] In an embodiment, a constant domain is linked to the two linked variable domains using recombinant DNA techniques. In an embodiment, a sequence comprising linked heavy chain variable domains is linked to a heavy chain constant domain and a sequence comprising linked light chain variable domains is linked to a light chain constant domain. In an embodiment, the constant domains are human heavy chain constant domains and human light chain constant domains respectively. In an embodiment, the DVD heavy chain is further linked to an Fc region. The Fc region may be a native sequence Fc region or a variant Fc region. In another embodiment, the Fc region is a human Fc region. In another embodiment, the Fc region includes Fc region from IgG1, IgG2, IgG3, IgG4, IgA, IgM, IgE, or IgD.

[0129] In an embodiment, an antibody or functional antigen binding fragment thereof is disclosed, comprising an antibody having a functional binding site for one of the target antigens listed in Table 2 and comprising a paired VH and VL region selected from the pairs listed in Table 2, or comprising the CDR regions of those VH and VL regions. For example, the antibody can comprise VH and VL regions from Table 2 that form a functional binding site for DLL4. In an embodiment, a functional antigen binding fragment retains variable domain regions (e.g., the CDR regions taken from the paired VH and VL sequences in Table 2) sufficient to bind the target antigen. A functional antigen binding fragment can include a humanized, fully human, camelized, single-chain, chimeric, synthetic, recombinant, hybrid, mutated, back-mutated, or CDR-grafted antibody, or a Fab, F(ab')2, Fv, scFv, Fd, dAb, VHH (also referred to as a nanobody), or other antibody fragment that retains antigen-binding function, including bi-specific or multi-specific antibodies.

[0130] In an embodiment, a binding protein is disclosed comprising variable domains selected from those in Table 2. In an embodiment, a binding protein comprises a first chain and a second chain and two functional binding sites, as described above, and wherein the first and second chains of the binding protein form two functional binding sites for one or more of the target antigens listed in Table 2. In an embodiment, each functional binding site comprises paired VH and VL sequences selected from the pairs listed in Table 2 (e.g., the paired VH and VL sequences forming a binding site for DLL4), or comprising the CDR regions of those VH and VL regions. In some embodiments, the first chain comprises a first VH sequence and a second VH sequence selected from Table 2, or contains the CDR sequences from those VH

sequences, and a second chain comprising a first VL sequence and a second VL sequence selected from Table 2, or contains the CDR sequences from those VL sequences. In other embodiments, the VH-VL arrangement of first or second binding sites is flipped across the two chains, such that each chain comprises a VH domain from one binding site and a VL domain from the other binding site, while retaining two functional binding sites.

[0131] In an embodiment, two heavy chain DVD polypeptides and two light chain DVD polypeptides are combined to form a DVD binding protein. Table 2 lists amino acid sequences of VH and VL regions of exemplary antibodies useful for treating disease. In an embodiment, a DVD comprising at least two of the VH and/or VL regions listed in Table 2, in any orientation, is provided, wherein at least one of the VH and/or VL sequences is SEQ ID NO: 39 or SEQ ID NO: 40. In some embodiments, VD1 and VD2 are independently chosen. The VH and VL domain sequences provided below comprise complementarity determining regions (CDRs) and framework sequences. In some embodiments, one or more of these CDRs and/or framework sequences are replaced, without loss of function, by other CDRs and/or framework sequences from binding proteins that are known in the art to bind to the same antigen.

Table 2: List of Amino Acid Sequences of VH and VL Regions of Anti-DLL4 and Anti-VEGF Antibodies for Generating Binding Proteins, Including Multivalent Binding Proteins
(CDR sequences in bold)

SEQ ID No.	ABT Unique ID	Protein region	Sequence 12345678901234567890123456789012345678901234567890
39	h1A11.1	DLL4 VH	EVQLVESGGGLVQPGGSLRLSCAAS**GFTFSNFPMA**WVRQA PGKGLEWVAT**ISSSDGTTYYRDSVKG**RFTISRDNAKNSLY LQMNSLRAEDTAVYYCAR**GYYNSPFAY**WGQGTLVTVSS
40	h1A11.1	DLL4 VL	DIQMTQSPSSLSASVGDRVTITC**RASEDIYSNLA**WYQQKP GKAPKLLIY**DTNNLAD**GVPSRFSGSGSGTDFTLTISSLQP EDFATYYC**QQYNNYPPT**FGQGTKLEIKR
41	Av	VEGF VH (seq 1)	EVQLVESGGGLVQPGGSLRLSCAA**SGYTFTNYGMN**WVRQAP GKGLEWVGW**INTYTGEPTYAADFKR**RFTFSLDTSKSTAYLQ MNSLRAEDTAVYYCAK**YPHYYGSSHWYFDV**WGQGTLVTVSS
42	Av	VEGF VL (seq 1)	DIQMTQSPSSLSASVGDRVTITC**SASQDISNYLN**WYQQKPG KAPKVLIY**FTSSLHS**GVPSRFSGSGSGTDFTLTISSLQPED FATYYC**QQYSTVPW**TFGQGTKVEIKR
43	AB285VH	VEGF VH (seq 2)	EVTLRESGPALVKPTQTLTLTCTASGYTFTNYGMNWVRQPP GKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSQAVLT MTNMDPVDTATYYCAKYPHYYGSSHWYFDVWGQGTTVTVSS
44	AB285VL	VEGF VL (seq 2)	DIVMTQSPDSLAVSLGERATINCSASQDISNYLNWYQQKPG QAPKVLIYFTSSLHSGVPDRFSGSGSGTDFTLTISSLQAED VAVYYCQQYSTVPWTFGGGTKVEIKR
45	AB288VH	VEGF VH (seq 3)	EVQLVQSGTEVKKPGESLKISCKASGYTFTNYGMNWVRQMP GKGLEWVGWINTYTGEPTYAADFKRQFTFSLDTSFSTAFLQ WSSLKASDTAMYYCAKYPHYYGSSHWYFDVWGQGTMVTVSS
46	AB288VL	VEGF VL (seq 3)	EIVMTQSPATLSVSPGERATLSCSASQDISNYLNWYQQKPG QAPRVLIYFTSSLHSDVPARFSGSGSGTEFTLTISSLQSED FAVYYCQQYSTVPWTFGQGTRLEIKR
47	AB305VH	VEGF VH (seq 4)	EVQLLESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAP GKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTAYLQ MNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTLVTVSS
48	AB305VL	VEGF VL (seq 4)	EIVMTQSPGTLSLSPGERATLSCSASQDISNYLNWYQQKPG QAPRVLIYFTSSLHSGVPDRFSGSGSGTDFTLTISRLEPED FAVFYCQQYSTVPWTFGQGTKVEIKR
49	AB308VH	VEGF VH (seq 5)	EVQLVESGGGLVQPGRSLRLSCAASGYTFTNYGMNWVRQAP GKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTAKSSAYLQ MNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTLVTVSS
50	AB308VL	VEGF VL (seq 5)	DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPG KAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTISSLQPED VATYYCQQYSTVPWTFGQGTKVEIKR

SEQ ID No.	ABT Unique ID	Protein region	Sequence 12345678901234567890123456789012345678901234567890
51	AB318VH	VEGF VH (seq 6)	EVQLVESGGGLVQPANSLKLSCAASGYTFTNYGMNWVRQSP KKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTAKSTAYLQ MDSLRSEDTATYYCAKYPHYYGSSHWYFDVWGQGVLVTVSS
52	AB318VL	VEGF VL (seq 6)	DIRMTQSPASLSASLGETVNIECSASQDISNYLNWYQQKPG KAPQVLIYFTSSLHSGVPSRFSGSGSGTQFSLKINSLQSED VATYYCQQYSTVPWTFGGGTKLELKR
53	AB333VH	VEGF VH (seq 7)	QVQLQQSGAELMKPGASVKLSCKATGYTFTNYGMNWVKQRP GHGLEWVGWINTYTGEPTYAADFKRKFTFTLDTSSSTAYIQ LISLTTEDSAIYYCAKYPHYYGSSHWYFDVWGQGTLLTVSA
54	AB333VL	VEGF VL (seq 7)	DILMTQSPAILSVSPGERVSFSCSASQDISNYLNWYQQRTN GAPRVLIYFTSSLHSGVPSRFSGGGSGTDFTLSINSVESED IADYYCQQYSTVPWTFGAGTKLELKR

[0132] In some embodiments, DVD binding proteins are provided, comprising a VH region selected from SEQ ID NO: 55-63. In certain embodiments, a DVD binding protein comprises a VL region selected from SEQ ID NO: 64-73. In some embodiments, a DVD binding protein comprises a VH region selected from SEQ ID NO: 55-63 and 74 and a VL region selected from SEQ ID NO: 64-73. The amino acid sequences for these VH and VL domains are shown below in Table 3.

Table 3: DVD binding proteins directed against epitopes of DLL4 and VEGF
(Linker sequence underlined; CDR sequences in bold)

SEQ ID No.	ABT Unique ID	Protein region	Sequence 12345678901234567890123456789012345678901234567890
55	h1A11.1-L-Av VH	DLL4 VH and VEGF VH	EVQLVESGGGLVQPGGSLRLSCAAS**GFTFSNFPMA**WVRQA PGKGLEWVAT**ISSSDGTTYYRDSVKG**RFTISRDNAKNSLY LQMNSLRAEDTAVYYCAR**GYYNSPFAY**WGQGTLVTVSS*AS TKGPSVFPLAP*EVQLVESGGGLVQPGGSLRLSCAA**SGYTF TNYGMN**WVRQAPGKGLEWVG**WINTYTGEPTYAADFKR**RFT FSLDTSKSTAYLQMNSLRAEDTAVYYCAK**YPHYYGSSHWY FDV**WGQGTLVTVSS
56	h1A11.1-S-Av VH	DLL4 VH and VEGF VH	EVQLVESGGGLVQPGGSLRLSCAAS**GFTFSNFPMA**WVRQA PGKGLEWVAT**ISSSDGTTYYRDSVKG**RFTISRDNAKNSLY LQMNSLRAEDTAVYYCAR**GYYNSPFAY**WGQGTLVTVSS*AS TKGP*EVQLVESGGGLVQPGGSLRLSCAA**SGYTFTNYGMN**W VRQAPGKGLEWVG**WINTYTGEPTYAADFKR**RFTFSLDTSK STAYLQMNSLRAEDTAVYYCAK**YPHYYGSSHWYFDV**WGQG TLVTVSS

SEQ ID No.	ABT Unique ID	Protein region	Sequence 12345678901234567890123456789012345678901234567890
57	h1A11.1-GS10-Av VH	DLL4 VH and VEGF VH	EVQLVESGGGLVQPGGSLRLSCAAS**GFTFSNFPMA**WVRQA PGKGLEWVAT**ISSSDGTTYYRDSVKG**RFTISRDNAKNSLY LQMNSLRAEDTAVYYCAR**GYYNSPFAY**WGQGTLVTVSS*GG GGSGGGGS*EVQLVESGGGLVQPGGSLRLSCAA**SGYTFTNY GMN**WVRQAPGKGLEWVGW**INTYTGEPTYAADFKR**RFTFSL DTSKSTAYLQMNSLRAEDTAVYYCAK**YPHYYGSSHWYFDV** WGQGTLVTVSS
58	h1A11.1-GS14-Av VH	DLL4 VH and VEGF VH	EVQLVESGGGLVQPGGSLRLSCAAS**GFTFSNFPMA**WVRQA PGKGLEWVAT**ISSSDGTTYYRDSVKG**RFTISRDNAKNSLY LQMNSLRAEDTAVYYCAR**GYYNSPFAY**WGQGTLVTVSS*GG GGSGGGGSGGGG*EVQLVESGGGLVQPGGSLRLSCAA**SGYT FTNYGMN**WVRQAPGKGLEWVGW**INTYTGEPTYAADFKR**RF TFSLDTSKSTAYLQMNSLRAEDTAVYYCAK**YPHYYGSSHW YFDV**WGQGTLVTVSS
59	Av-L-h1A11.1 VH	VEGF VH and DLL4 VH	EVQLVESGGGLVQPGGSLRLSCAA**SGYTFTNYGMN**WVRQA PGKGLEWVGW**INTYTGEPTYAADFKR**RFTFSLDTSKSTAY LQMNSLRAEDTAVYYCAK**YPHYYGSSHWYFDV**WGQGTLVT VSS*ASTKGPSVFPLAP*EVQLVESGGGLVQPGGSLRLSCAA S**GFTFSNFPMA**WVRQAPGKGLEWVAT**ISSSDGTTYYRDSV KG**RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR**GYYNSP FAY**WGQGTLVTVSS
60	Av-S-h1A11.1 VH	VEGF VH and DLL4 VH	EVQLVESGGGLVQPGGSLRLSCAA**SGYTFTNYGMN**WVRQA PGKGLEWVGW**INTYTGEPTYAADFKR**RFTFSLDTSKSTAY LQMNSLRAEDTAVYYCAK**YPHYYGSSHWYFDV**WGQGTLVT VSS*ASTKGP*EVQLVESGGGLVQPGGSLRLSCAAS**GFTFSN FPMA**WVRQAPGKGLEWVAT**ISSSDGTTYYRDSVKG**RFTIS RDNAKNSLYLQMNSLRAEDTAVYYCAR**GYYNSPFAY**WGQG TLVTVSS
61	Av-GS6-h1A11.1 VH	VEGF VH and DLL4 VH	EVQLVESGGGLVQPGGSLRLSCAA**SGYTFTNYGMN**WVRQA PGKGLEWVGW**INTYTGEPTYAADFKR**RFTFSLDTSKSTAY LQMNSLRAEDTAVYYCAK**YPHYYGSSHWYFDV**WGQGTLVT VSS*GGGGSG*EVQLVESGGGLVQPGGSLRLSCAAS**GFTFSN FPMA**WVRQAPGKGLEWVAT**ISSSDGTTYYRDSVKG**RFTIS RDNAKNSLYLQMNSLRAEDTAVYYCAR**GYYNSPFAY**WGQG TLVTVSS
62	Av-GS10-h1A11.1 VH	VEGF VH and DLL4 VH	EVQLVESGGGLVQPGGSLRLSCAA**SGYTFTNYGMN**WVRQA PGKGLEWVGW**INTYTGEPTYAADFKR**RFTFSLDTSKSTAY LQMNSLRAEDTAVYYCAK**YPHYYGSSHWYFDV**WGQGTLVT VSS*GGGGSGGGGS*EVQLVESGGGLVQPGGSLRLSCAAS**GF TFSNFPMA**WVRQAPGKGLEWVAT**ISSSDGTTYYRDSVKG**R FTISRDNAKNSLYLQMNSLRAEDTAVYYCAR**GYYNSPFAY** WGQGTLVTVSS
63	Av-GS14-h1A11.1 VH	VEGF VH and DLL4 VH	EVQLVESGGGLVQPGGSLRLSCAA**SGYTFTNYGMN**WVRQA PGKGLEWVGW**INTYTGEPTYAADFKR**RFTFSLDTSKSTAY LQMNSLRAEDTAVYYCAK**YPHYYGSSHWYFDV**WGQGTLVT VSS*GGGGSGGGGSGGGG*EVQLVESGGGLVQPGGSLRLSCA AS**GFTFSNFPMA**WVRQAPGKGLEWVAT**ISSSDGTTYYRDS VKG**RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR**GYYNS PFAY**WGQGTLVTVSS

SEQ ID No.	ABT Unique ID	Protein region	Sequence 12345678901234567890123456789012345678901234567890
64	h1A11.1-L-Av VL	DLL4 VL and VEGF VL	DIQMTQSPSSLSASVGDRVTITC**RASEDIYSNLA**WYQQKP GKAPKLLIY**DTNNLAD**GVPSRFSGSGSGTDFTLTISSLQP EDFATYYC**QQYNNYPPT**FGQGTKLEIKR*TVAAPSVFIFPP* DIQMTQSPSSLSASVGDRVTITC**SASQDISNYLN**WYQQKP GKAPKVLIY**FTSSLHS**GVPSRFSGSGSGTDFTLTISSLQP EDFATYYC**QQYSTVPW**TFGQGTKVEIKR
65	h1A11.1-S-Av VL	DLL4 VL and VEGF VL	DIQMTQSPSSLSASVGDRVTITC**RASEDIYSNLA**WYQQKP GKAPKLLIY**DTNNLAD**GVPSRFSGSGSGTDFTLTISSLQP EDFATYYC**QQYNNYPPT**FGQGTKLEIKR*TVAAP*DIQMTQS PSSLSASVGDRVTITC**SASQDISNYLN**WYQQKPGKAPKVL IY**FTSSLHS**GVPSRFSGSGSGTDFTLTISSLQPEDFATYY C**QQYSTVPW**TFGQGTKVEIKR
66	h1A11.1-GS10-Av VL	DLL4 VL and VEGF VL	DIQMTQSPSSLSASVGDRVTITC**RASEDIYSNLA**WYQQKP GKAPKLLIY**DTNNLAD**GVPSRFSGSGSGTDFTLTISSLQP EDFATYYC**QQYNNYPPT**FGQGTKLEIKR*GGSGGGGSG*DIQ MTQSPSSLSASVGDRVTITC**SASQDISNYLN**WYQQKPGKA PKVLIY**FTSSLHS**GVPSRFSGSGSGTDFTLTISSLQPEDF ATYYC**QQYSTVPW**TFGQGTKVEIKR
67	h1A11.1-GS14-Av VL	DLL4 VL and VEGF VL	DIQMTQSPSSLSASVGDRVTITC**RASEDIYSNLA**WYQQKP *GKAPKLLIY**DTNNLAD**GVPSRFSGSGSGTDFTLTISSLQP* EDFATYYC**QQYNNYPPT**FGQGTKLEIKR*GGSGGGGSGGGG* *S*DIQMTQSPSSLSASVGDRVTITC**SASQDISNYLN**WYQQK PGKAPKVLIY**FTSSLHS**GVPSRFSGSGSGTDFTLTISSLQ PEDFATYYC**QQYSTVPW**TFGQGTKVEIKR
68	Av-L-h1A11.1 VL	VEGF VL and DLL4 VL	DIQMTQSPSSLSASVGDRVTITC**SASQDISNYLN**WYQQKP GKAPKVLIY**FTSSLHS**GVPSRFSGSGSGTDFTLTISSLQPE DFATYYC**QQYSTVPW**TFGQGTKVEIKR*TVAAPSVFIFPP*D IQMTQSPSSLSASVGDRVTITC**RASEDIYSNLA**WYQQKPG KAPKLLIY**DTNNLAD**GVPSRFSGSGSGTDFTLTISSLQPE DFATYYC**QQYNNYPPT**FGQGTKLEIKR
69	Av-S-h1A11.1 VL	VEGF VL and DLL4 VL	DIQMTQSPSSLSASVGDRVTITC**SASQDISNYLN**WYQQKP GKAPKVLIY**FTSSLHS**GVPSRFSGSGSGTDFTLTISSLQPE DFATYYC**QQYSTVPW**TFGQGTKVEIKR*TVAAP*DIQMTQSP SSLSASVGDRVTITC**RASEDIYSNLA**WYQQKPGKAPKLLI Y**DTNNLAD**GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC **QQYNNYPPT**FGQGTKLEIKR
70	Av-GS6-h1A11.1 VL	VEGF VL and DLL4 VL	DIQMTQSPSSLSASVGDRVTITC**SASQDISNYLN**WYQQKP GKAPKVLIY**FTSSLHS**GVPSRFSGSGSGTDFTLTISSLQPE DFATYYC**QQYSTVPW**TFGQGTKVEIKR*GGSGG*DIQMTQSP SSLSASVGDRVTITC**RASEDIYSNLA**WYQQKPGKAPKLLI Y**DTNNLAD**GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC **QQYNNYPPT**FGQGTKLEIKR
71	Av-GS10-h1A11.1 VL	VEGF VL and DLL4 VL	DIQMTQSPSSLSASVGDRVTITC**SASQDISNYLN**WYQQKP GKAPKVLIY**FTSSLHS**GVPSRFSGSGSGTDFTLTISSLQPE DFATYYC**QQYSTVPW**TFGQGTKVEIKR*GGSGGGGSG*DIQM TQSPSSLSASVGDRVTITC**RASEDIYSNLA**WYQQKPGKAP KLLIY**DTNNLAD**GVPSRFSGSGSGTDFTLTISSLQPEDFA TYYC**QQYNNYPPT**FGQGTKLEIKR

SEQ ID No.	ABT Unique ID	Protein region	Sequence 12345678901234567890123456789012345678901234567890
72	Av-GS14-h1A11.1 VL	VEGF VL and DLL4 VL	DIQMTQSPSSLSASVGDRVTITC**SASQDISNYLN**WYQQKP GKAPKVLIY**FTSSLHS**GVPSRFSGSGSGTDFTLTISSLQPE DFATYYC**QQYSTVPW**TFGQGTKVEIKR*GGSGGGGSGGGS*D IQMTQSPSSLSASVGDRVTITC**RASEDIYSNLA**WYQQKPG KAPKLLIY**DTNNLAD**GVPSRFSGSGSGTDFTLTISSLQPE DFATYYC**QQYNNYPPT**FGQGTKLEIKR

Table 3a: Full length binding proteins directed against epitopes of DLL4 and VEGF

73	h1A11.1-SL-Av light chain	DIQMTQSPSSLSASVGDRVTITC**RASEDIYSNLA**WYQQKPGKAP KLLIY**DTNNLAD**GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC **QQYNNYPPT**FGQGTKLEIKRTVAAPSVFIFPPDIQMTQSPSSLS ASVGDRVTITC**SASQDISNYLN**WYQQKPGKAPKVLIY**FTSSLHS** GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC**QQYSTVPW**TFGQ GTKVEIKR**TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREA** **KVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEK** **HKVYACEVTHQGLSSPVTKSFNRGEC**
74	h1A11.1-SL-Av heavy chain	EVQLVESGGGLVQPGGSLRLSCAAS**GFTFSNFPMA**WVRQAPGKG LEWVAT**ISSSDGTTYYRDSVKG**RFTISRDNAKNSLYLQMNSLRA EDTAVYYCAR**GYYNSPFAY**WGQGTLVTVSSASTKGPEVQLVESG GGLVQPGGSLRLSCAA**SGYTFTNYGMN**WVRQAPGKGLEWVGW**IN** **TYTGEPTYAADFKR**RFTFSLDTSKSTAYLQMNSLRAEDTAVYYC AK**YPHYYGSSHWYFDV**WGQGTLVTVSS**ASTKGPSVFPLAPSSKS** **TSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG** **LYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDK** **THTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDV** **SHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLH** **QDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPS** **REEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVL** **DSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLS** **LSPGK**

[0133] Table 3a provides the full-length heavy and light chan sequences for binding proteins directed against VEGF and DLL4. Linker sequences are underlined, while CDRs and constant region sequences are in bold.

[0134] Detailed descriptions of specific DVD binding proteins capable of binding specific targets, and methods of making the same, are provided in the Examples section below.

D. Production of binding proteins

[0135] The binding proteins provided herein may be produced by any of a number of techniques known in the art. For example, the binding proteins can be expressed in host cells, wherein expression vector(s) encoding the DVD heavy and DVD light chains is (are) transfected into a host cell by standard techniques. In some embodiments, the DVD binding proteins provided herein are expressed in prokaryotic host cells. In other embodiments, the DVD binding proteins are expressed in eukaryotic cells, for example, mammalian host cells.

[0136] In an exemplary system for recombinant expression of DVD proteins, a recombinant expression vector encoding both the DVD heavy chain and the DVD light chain is introduced into DHFR-CHO cells by calcium phosphate-mediated transfection. Within the recombinant expression vector, the DVD heavy and light chain genes are each operatively linked to CMV enhancer/AdMLP promoter regulatory elements to drive high levels of transcription of the genes. The recombinant expression vector also carries a DHFR gene, which allows for selection of CHO cells that have been transfected with the vector using methotrexate selection/amplification. The selected host cells are cultured to allow for expression of the DVD heavy and light chains and intact DVD protein is recovered from the culture medium. Standard molecular biology techniques are used to prepare the recombinant expression vector, transfect the host cells, select for transformants, culture the host cells and recover the DVD protein from the culture medium. In various embodiments, a method of synthesizing a DVD protein by culturing a host cell in a suitable culture medium until a DVD protein is synthesized is also provided herein. In some embodiments, the method can further comprise isolating the DVD protein from the culture medium.

[0137] A feature of a DVD binding protein is that it can be produced and purified in a similar way to a conventional antibody. In some embodiments, the production of a DVD binding protein results in a homogeneous, single major product with desired dual-specific activity, without the need for sequence modification of the constant region or chemical modifications. Other previously described methods to generate "bi-specific", "multi-specific", and "multi-specific multivalent" full length binding proteins can lead to the intracellular or secreted production of a mixture of assembled inactive, mono-specific, multi-specific, multivalent, full length binding proteins, and multivalent full length binding proteins with a combination of different binding sites.

[0138] In some embodiments, the design of the DVD proteins provided herein leads to a dual variable domain light chain and a dual variable domain heavy chain that

assemble primarily to the desired "dual-specific multivalent full length binding proteins" after expression in host cells.

[0139] In some embodiments, at least 50%, at least 75% or at least 90% (or any percentage in between) of the expressed and assembled dual variable domain immunoglobulin molecules are the desired dual-specific tetravalent protein, and therefore possess enhanced commercial utility. Thus, in certain embodiments, a method to express a dual variable domain light chain and a dual variable domain heavy chain in a single cell leading to a single primary product of a "dual-specific tetravalent full length binding protein" is provided.

[0140] In various embodiments, methods of expressing a dual variable domain light chain and a dual variable domain heavy chain in a single cell leading to a "primary product" of a "dual-specific tetravalent full length binding protein", where the "primary product" is more than 50%, such as more than 75% or more than 90% (or any *percentage in between*), *of all assembled protein, comprising a dual variable domain* light chain and a dual variable domain heavy chain are provided.

II. Uses of binding proteins

[0141] Given their ability to bind to one, two, or more antigens, the binding proteins provided herein can be used, in certain embodiments, to detect the antigen(s) in a sample (e.g., a biological sample, such as serum or plasma), using a conventional immunoassay, such as an enzyme linked immunosorbent assays (ELISA), a radioimmunoassay (RIA), or tissue immunohistochemistry. In some embodiments, the binding protein is directly or indirectly labeled with a detectable substance to facilitate detection of the bound or unbound antibody. Suitable detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin. An example of a luminescent material is luminol and examples of suitable radioactive materials include ^{3}H, ^{14}C, ^{35}S, ^{90}Y, ^{99}Tc, ^{111}In, ^{125}I, ^{131}I, ^{177}Lu, ^{166}Ho, and ^{153}Sm.

[0142] In various embodiments, the binding proteins provided herein are capable of neutralizing the activity of their antigen targets *in vitro* and/or *in vivo*.

Accordingly, in certain embodiments the binding proteins can be used to inhibit antigen activity, e.g., in a cell culture containing the antigens, in human subjects, or in other mammalian subjects who have the antigens with which a binding protein cross-reacts. In another embodiment, a method for reducing antigen activity in a human or non-human animal subject suffering from a disease or disorder in which the antigen is detrimental is provided. In various embodiments, a binding protein provided herein can be administered to a human or non-human animal subject for diagnostic or therapeutic purposes (e.g., to detect or treat a disease, such as a disease characterized by abherrant VEGF and/or DLL4 expression).

[0143] As used herein, the term "a disorder in which antigen activity is detrimental" is intended to include diseases and/or other disorders in which the presence of the antigen in a subject suffering from the disorder has been shown to be or is suspected of being responsible for the pathophysiology of the disorder and/or a factor that contributes to a worsening of the disorder. Accordingly, a disorder in which antigen activity is detrimental is a disorder in which reduction of antigen activity is expected to alleviate the symptoms and/or progression of the disorder. Such disorders may be evidenced, for example, by an increase in the concentration of the antigen in a biological fluid of a subject suffering from the disorder (e.g., an increase in the concentration of antigen in serum, plasma, synovial fluid, etc., of the subject). Non-limiting examples of disorders that can be treated with the binding proteins provided herein include those disorders discussed below and in the section pertaining to pharmaceutical compositions comprising the binding proteins.

[0144] In various embodiments, DVD binding proteins are useful as therapeutic agents to increase the binding to a detrimental antigens and/or to simultaneously block two different antigen targets (DLL4 and/or VEGF) to enhance efficacy/safety and/or increase patient coverage.

[0145] Additionally, in some embodiments, DVD binding proteins provided herein can be employed for tissue-specific delivery (e.g., to target a tissue marker and/or a disease mediator for enhanced local PK, thus providing higher efficacy and/or lower toxicity), including intracellular delivery (e.g., targeting a DVD to an intracellular molecule). In some embodiments, DVD binding proteins can also serve as carrier proteins to deliver antigens to a specific location via binding to a non-neutralizing epitope of that antigen and also to increase the half-life of the antigen. Furthermore, a DVD binding protein can be designed, in certain embodiments, to either be physically linked to medical devices implanted into patients or to target these medical devices

(see Burke et al. (2006) Advanced Drug Deliv. Rev. 58(3): 437-446; Hildebrand et al. (2006) Surface and Coatings Technol. 200(22-23): 6318-6324; Drug/ device combinations for local drug therapies and infection prophylaxis, Wu (2006) Biomaterials 27(11):2450-2467; Mediation of the cytokine network in the implantation of orthopedic devices, Marques (2005) Biodegradable Systems in Tissue Engineer. Regen. Med. 377-397).

A. Use of binding proteins in various diseases

[0146] In various embodiments, the binding proteins provided herein are useful as therapeutic molecules to treat various diseases or disorders, e.g., diseases or disorders associated with detrimental expression or expression levels of DLL4 and/or VEGF. In some embodiments, one or more binding proteins can be administered to diagnose, treat or enhance anti-tumor therapies and/or may be beneficial in the treatment of primary and/or metastic cancers. In various embodiments, one or more binding proteins can be administered to diagnose, treat or enhance treatment an oncologic condition. In other embodiments, one or more binding proteins can be administered to diagnose, treat or enhance treatment of any other disease or disorder characterized by abherrant angiogenesis (e.g., general autoimmune and inflammatory disorders, wound healing). In various embodiments, administration of one or more binding proteins leads to binding to VEGF and/or DLL4, which may neutralize or otherwise reduce the levels of VEGF and/or DLL4 in a patient suffering from a condition characterized by excessive VEGF and/or DLL4 levels.

[0147] Without limiting the disclosure, further information on certain disease conditions is provided.

1. Oncological disorders

[0148] Monoclonal antibody therapy has emerged as an important therapeutic modality for cancer (von Mehren et al. (2003) Annu. Rev. Med. 54:343-69). The use of a dual-specific antibody, as disclosed herein, that targets two separate oncongenic mediators, will likely provide additional benefit compared to a mono-specific therapy.

[0149] In various embodiments, oncologic diseases that can be diagnosed and/or treated with the compositions and methods provided herein include, but are not limited to, primary or metastatic cancer, breast cancer, colon cancer, rectum cancer, lung cancer, non-small cell lung cancer, adenocarcinoma, oropharynx cancer, hypopharynx cancer, esophageal cancer, stomach cancer, pancreatic cancer, liver cancer, gallbladder cancer, bile duct cancer, small intestine cancer, urinary tract cancer

(including kidney, bladder and urothelium), female genital tract cancer (including cervix, uterus, and ovaries as well as choriocarcinoma and gestational trophoblastic disease), male genital tract cancer (including prostate, seminal vesicles, testes and germ cell tumors), endocrine gland cancer (including the thyroid, adrenal, and pituitary glands), skin cancer, hemangiomas, melanomas, sarcomas (including those arising from bone and soft tissues as well as Kaposi's sarcoma), tumors of the brain, nerves, eyes, and meninges (including astrocytomas, gliomas, glioblastomas, retinoblastomas, neuromas, neuroblastomas, Schwannomas, and meningiomas), solid tumors arising from hematopoietic malignancies such as leukemias, and lymphomas (both Hodgkin's and non-Hodgkin's lymphomas), stomach cancer, bladder cancer, prostate cancer, rectal cancer, hematopoietic malignancies, leukemia, lymphoma, Abetalipoprotemia, acrocyanosis, acute and chronic parasitic or infectious processes, acute leukemia, acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), B cell lymphoma, Burkitt's lymphoma, chronic myelocytic leukemia (CML), chronic lymphocytic leukemia (CLL), colorectal carcinoma, hairy cell leukemia, Hodgkin's disease, Kaposi's sarcoma, malignamt lymphoma, malignant histiocytosis, malignant melanoma, multiple myeloma, non-hodgkins lymphoma, pancreatic carcinoma, paraneoplastic syndrome/hypercalcemia of malignancy, sarcomas, solid tumors, or any other angiogenesis independent or dependent diseases characterized by aberrant DLL4 or VEGF activity.

[0150] In various embodiments, DVD binding proteins that bind DLL4 and/or VEG, or antigen-binding portions thereof, are used to diagnose and/or to treat cancer and/or to prevent metastasis, either when used alone or in combination with radiotherapy and/or other chemotherapeutic agents.

2. Macular degeneration

[0151] In various embodiments, the compositions and methods provided herein can be used to treat macular degeneration, including neovascular (wet) macular degeneration. Macular degeneration is a medical condition that results in a loss of vision in the center of the visual field due to retina damage. Neovascular (wet) macular degeneration results from abnormal blood vessel growth, ultimately leading to blood and protein leakage below the macula that can cause irreversible damage to the photoreceptors.

[0152] In various embodiments, DVDs that bind DLL4 and/or VEGF, or antigen-binding portions thereof, are used to diagnose and/or to treat macular degeneration, either when used alone or in combination with other therapeutic agents.

3. Diabetes and Diabetic Retinopathy

[0153] Diabetes mellitus type 1 is a form of diabetes mellitus that results from autoimmune destruction of insulin-producing beta cells in the pancreas. The subsequent lack of insulin leads to increased blood and urine glucose. Diabetic retinopathy involves damage to the retina as a complication of diabetes.

[0154] Diabetic retinopathy is the result of small changes in the vascular system of the retina that result from hyperglycemia-induced pericyte death and weakening of the vascular walls. As the disease progresses, severe nonproliferative diabetic retinopathy enters an advanced stage where blood vessels proliferate. Without treatment, the new blood vessels can bleed, cloud vision, and further damage the retina. Fibrovascular proliferation can also cause retinal detachment. The proliferating blood vessels can also grow into the anterior chamber of the eye and cause neovascular glaucoma.

[0155] VEGF and DLL4 signaling are believed to play important roles in mediating diabetic endothelial dysfunction and vasculopathy, including involvement in the pathogenesis of both diabetic nephropathy and retinopathy. *J. Exp. Med.* 209(5): 1011-28 (2012); *Nephrol. Dial. Transplant.* 18 (8): 1427-1430 (2003); *PNAS* 109(27): E1868-77 (2012); US Patent Application No. 20110189176 (Skokos et al). Thus, VEGF and DLL4 may represent potential targets for diagnosis and/or therapy for diabetes and/or diabetic retinopathy using a DVD of the present disclosure (e.g., to identify serum levels and/or to alter levels of VEGF and/or DLL4 in a patient). In various embodiments, DVDs that bind DLL4 and/or VEGF, or antigen-binding portions thereof, are used to diagnose and/or to treat diabetes mellitus type 1 and/or diabetic retinopathy, either when used alone or in combination with other therapeutic agents.

4. Atherosclerosis

[0156] VEGF and DLL4 are believed to be involved in the progression of atherosclerosis. *Circulation* 98(20):2108-16 (1998); *PNAS* 109(27): E1868-77 (2012). In particular, VEGF expression has been shown in atherosclerotic lesions in human coronary arteries, suggesting a role for VEGF in the progression of human coronary atherosclerosis, as well as in recanalization processes in obstructive coronary diseases. Similarly, inhibition of DLL4 signaling using a neutralizing anti-DLL4 antibody has been shown to attenuate the development of atherosclerosis and diminishe plaque calcification. Thus, VEGF and DLL4 levels may represent potential targets for diagnosis and/or therapy for atherosclerosis using a DVD of the present disclosure

(e.g., to identify serum levels and/or to alter levels of VEGF and/or DLL4 in a patient). in various embodiments, DVDs that bind DLL4 and/or VEGF, or antigen-binding portions thereof, are used to diagnose and/or to treat atherosclerosis, either when used alone or in combination with other therapeutic agents.

III. Pharmaceutical Compositions

[0157] In various embodiments, pharmaceutical compositions comprising one or more binding proteins, either alone or in combination with prophylactic agents, therapeutic agents, and/or pharmaceutically acceptable carriers are provided herein. In various embodiments, nonlimiting examples of the uses of the pharmaceutical compositions disclosed herein include diagnosing, detecting, and/or monitoring a disorder, preventing, treating, managing, and/or ameliorating a disorder or one or more symptoms thereof, and/or in research. The formulation of pharmaceutical compositions, either alone or in combination with prophylactic agents, therapeutic agents, and/or pharmaceutically acceptable carriers, are known to one skilled in the art (US Patent Publication No. 20090311253 A1).

[0158] In various embodiments, a pharmaceutical formulation comprises one or more amino acid, one or more polysaccharide and/or polysorbate, and a binding protein present at a concentration of between about 0.1 and 100 mg/ml, inclusive of endpoints (e.g., 0.1-10, 1-10, .01-50, 1-50, 1-100, 10-100, 25-100, 25-50, or 50-100 mg/ml), where the formulation is at a pH between about 5.0 and 7.0, inclusive of endpoints (e.g., a pH of about 5.0-6.0, 5.5-6.0, 5.0-6.5, 5.5-6.5, or 6.0-7.0). In some embodiments, the binding protein comprises comprises first and second polypeptide chains, wherein each of the first and second polypeptide chains independently comprises VD1-(X1)n-VD2-C-(X2)n, wherein VD1 is a first variable domain, VD2 is a second variable domain, C is a constant domain, X1 is a linker, X2 is an Fc region, and n is 0 or 1, wherein the VD1 domains on the first and second polypeptide chains form a first functional target binding site and the VD2 domains on the first and second polypeptide chains form a second functional target binding site. In an embodiment, the binding protein comprises a first polypeptide chain comprising SEQ ID NO: 56 and a second polypeptide chain comprising SEQ ID NO: 64. In an embodiment, the binding protein comprises a first polypeptide chain comprising SEQ ID NO: 73 and a second polypeptide chain comprising SEQ ID NO: 74. In an embodiment, at least one amino acid in the formulation is histidine and is present at a concentration of about 10-20 mM, 10-15mM, 15-20mM, or about 15mM. In an embodiment, at least one polysaccharide in the formulation is sucrose and is present at a concentration of about 0-8.0%

weight/volume (w/v). In an embodiment, the polysorbate in the formulation is polysorbate 80 and is at a concentration of about 0-0.06% w/v. In an embodiment, at least one amino acid in the formulation is arginine and is present at a concentration of about 0-1.5% w/v (e.g., 0.5-1.5, 1.0-1.5, or 0.5-1.0 w/v). In an embodiment, the binding protein is present in the formulation at a concentration of about 0.1-100 mg/ml, (e.g., about 1-100 mg/ml, or about 1-15 mg/ml, or about 1-7.5 mg/ml, or about 2.5-7.5 mg/ml, or about 5-7.5 mg/ml, or about 25-100 mg/ml, or about 20-60 mg/ml, or about 25-50 mg/ml, or about 25 mg/ml, or about 50 mg/ml, or about 0.1-60 mg/ml, , or about about 0.1-25 mg/ml, or about 1.0-60 mg/ml, or about 0.5-60 mg/ml, or about 0.1-2.0 mg/ml, or about 0.5-2.0 mg/ml, or about 1-5 mg/ml, or about 1-7.5 mg/ml, or about 1-15 mg/ml, or about 0.5 mg/ml, or about 1.0 mg/m).

[0159] In various embodiments, the pharmaceutical formulation is an aqueous formulation, a lyophilized formulation, or a lyophilized and rehydrated formulation. In an embodiment, the hydrating solution is dextrose and/or saline (e.g., dextrose at a concentration of about 5% w/v and/or the saline at a concentration of about 0.9% w/v). In an embodiment, the pharmaceutical formulation comprises about 15 mM histidine, about 0.03% (w/v) polysorbate 80, about 4% (w/v) sucrose, and about 0.1-25 mg/ml of the binding protein, or about 1-15 mg/ml of the binding protein, and is at a pH of about 6, wherein the binding protein comprises SEQ ID NO: 56 and SEQ ID NO: 64 or SEQ ID NO: 73 and SEQ ID NO: 74. In an embodiment, the binding protein comprises h1A11.1-SL-Av. In an embodiment, the h1A11.1-SL-Av binding protein comprises an Fc region from a human IgG1 LALA mutant. In an embodiment, the formulation further comprises at least one additional agent.

[0160] In various embodiments, a formulation is used containing about 25 mg/ml binding protein (e.g., a binding protein comprising SEQ ID NO: 56 and SEQ ID NO: 64 or SEQ ID NO: 73 and SEQ ID NO: 74), about 15 mM histidine, 0.03% polysorbate 80 (weight/volume, w/v), 4.0% sucrose (w/v), and a pH of about 6.0. In some embodiments, the formulation does not comprise arginine. In some embodiments, the formulation exhibits unexpectedly improved freeze-thaw stability, liquid formulation stability, and/or lyophilized formulation stability, as compared to other formulations comprising other components or concentrations.

[0161] In some embodiments, the formulations discussed above, and containing a binding protein comprising SEQ ID NO: 56 and SEQ ID NO: 64, or comprising SEQ ID NO: 73 and SEQ ID NO: 74, exhibit unexpected improvements in formulatability. For instance, the formulations avoid undesirable gelling or the

formation of high levels of aggregates when stored at 5°C. In some embodiments, the formulations are stable under both storage (5°C) and accelerated (40°C) conditions.

[0162] Methods of administering a prophylactic or therapeutic agent provided herein include, but are not limited to, oral administration, parenteral administration (e.g., intradermal, intramuscular, intraperitoneal, intravenous and subcutaneous), epidural administration, intratumoral administration, mucosal administration (e.g., intranasal and oral routes) and pulmonary administration (e.g., aerosolized compounds administered with an inhaler or nebulizer). The formulation of pharmaceutical compositions for specific routes of administration, and the materials and techniques necessary for the various methods of administration are available and known to one skilled in the art (US Patent Publication No. 20090311253 A1).

[0163] In various embodiments, dosage regimens may be adjusted to provide *for an optimum desired response (e.g., a therapeutic or prophylactic response). For* example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. In some embodiments, parenteral compositions are formulated in dosage unit form for ease of administration and uniformity of dosage. The term "dosage unit form" refers to physically discrete units *suited as unitary dosages for the mammalian subjects to be treated; each unit* containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier.

[0164] An exemplary, non-limiting range for a therapeutically or prophylactically effective amount of a binding protein provided herein is about 0.1-100 mg/kg, (e.g., about 0.1-0.5, 0.1-1, 0.1-10, 0.1-20, 0.1-50, 0.1-75, 1-10, 1-15, 1-7.5, 1.25-15, 1.25-7.5, 2.5-7.5, 2.5-15, 5-15, 5-7.5,1-20, 1-50, 7-75, 1-100, 5-10, 5-15, 5-20, 5-25, 5-50, 5-75, 10-20, 10-50, 10-75, or 10-100 mg/kg, or any concentration in between). In some embodiments, the binding protein is present in a pharmaceutical composition at a therapeutically effective concentration, e.g., a concentration of about 0.1-100 mg/ml (e.g., about 0.1-0.5, 0.1-1, 0.1-10, 0.1-20, 0.1-50, 0.1-75, 1-10, 1-20, 1-50, 1-75, 1-100, 5-10, 5-15, 5-20, 5-25, 5-50, 5-75, 10-20, 10-50, 10-75, or 10-100 mg/ml, or any concentration in between). Note that dosage values may vary with the type and/or severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens may be adjusted over time according to the individual need and/or the professional judgment of the person administering or supervising the administration of the compositions, and that dosage

ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition.

IV. Combination Therapy

[0165] In various embodiments, a binding protein provided herein can also be administered alone or in combination with one or more additional therapeutic agents used to treat a diseass or disorder, e.g., a disease or disorder associated with detrimental expression or expression levels of DLL4 and/or VEGF. In some embodiments, the one or more binding proteins can be administered in combination with one or more therapeutic agents to diagnose, treat or enhance anti-tumor therapies and/or to treat primary and/or metastic cancers. In various embodiments, one or more binding proteins can be administered in combination with one or more therapeutic agents to diagnose, treat or enhance treatment an oncologic condition. In other embodiments, one or more binding proteins can be administered in combination with one or more therapeutic agents to diagnose, treat or enhance treatment of any other disease or disorder characterized by abherrant angiogenesis (e.g., general autoimmune and inflammatory disorders, wound healing). In various embodiments, administration of one or more binding proteins in combination with one or more therapeutic agents leads to a neutralization or other reduction in the levels of VEGF and/or DLL4 in a patient suffering from a condition characterized by excessive VEGF and/or DLL4 levels, as well as other therapeutic changes resulting from administration of the binding proteins and/or one or more therapeutic agents.

[0166] In various embodiments, the one or more additional agents is selected by the skilled artisan for its intended purpose. For example, the additional agent can be a therapeutic agent recognized in the art as being useful to treat cancer. The combination therapy can also include more than one additional agent, e.g., two, three, four, five, or more additional agents.

[0167] In various embodiments, combination therapy agents include, but are not limited to, antiangiogenic agents, antineoplastic agents, radiotherapy, chemotherapy such as DNA alkylating agents, cisplatin, carboplatin, anti-tubulin agents, paclitaxel, docetaxel, taxol, doxorubicin, gemcitabine, gemzar, anthracyclines, adriamycin, topoisomerase I inhibitors, topoisomerase II inhibitors, 5-fluorouracil (5-FU), leucovorin, irinotecan, receptor tyrosine kinase inhibitors (e.g., erlotinib, gefitinib), and siRNAs.

[0168] Non-limiting examples of chemotherapeutic agents with which binding proteins provided herein can be combined include the following:13-cis-Retinoic Acid; 2-CdA; 2-Chlorodeoxyadenosine; 5-Azacitidine; 5-Fluorouracil; 5-FU; 6-Mercaptopurine; 6-MP; 6-TG; 6-Thioguanine; Abraxane; Accutane®; Actinomycin-D; Adriamycin®; Adrucil®; Afinitor®; Agrylin®; Ala-Cort®; Aldesleukin; Alemtuzumab; ALIMTA; Alitretinoin; Alkaban-AQ®; Alkeran®; All-transretinoic Acid; Alpha Interferon; Altretamine; Amethopterin; Amifostine; Aminoglutethimide; Anagrelide; Anandron®; Anastrozole; Arabinosylcytosine; Ara-C Aranesp®; Aredia®; Arimidex®; Aromasin®; Arranon®; Arsenic Trioxide; Arzerra™; Asparaginase; ATRA; Avastin®; Azacitidine; BCG; BCNU; Bendamustine; Bevacizumab; Bexarotene; BEXXAR®; Bicalutamide; BiCNU; Blenoxane®; Bleomycin; Bortezomib; Busulfan; Busulfex®; C225; Calcium Leucovorin; Campath®; Camptosar®; Camptothecin-11; Capecitabine; Carac™; Carboplatin; Carmustine; Carmustine Wafer; Casodex®; CC-5013; CCI-779; CCNU; CDDP; CeeNU; Cerubidine®; Cetuximab; Chlorambucil; Cisplatin; Citrovorum Factor; Cladribine; Cortisone; Cosmegen®; CPT-11; Cyclophosphamide; Cytadren®; Cytarabine; Cytarabine Liposomal; Cytosar-U®; Cytoxan®; Dacarbazine; Dacogen; Dactinomycin; Darbepoetin Alfa; Dasatinib; Daunomycin; Daunorubicin; Daunorubicin Hydrochloride; Daunorubicin Liposomal; DaunoXome®; Decadron; Decitabine; Delta-Cortef®; Deltasone®; Denileukin; Diftitox; DepoCyt™; Dexamethasone; Dexamethasone Acetate; Dexamethasone Sodium Phosphate; Dexasone; Dexrazoxane; DHAD; DIC; Diodex; Docetaxel; Doxil®; Doxorubicin; Doxorubicin Liposomal; Droxia™; DTIC; DTIC-Dome®; Duralone®; Efudex®; Eligard™; Ellence™; Eloxatin™; Elspar®; Emcyt®; Epirubicin; Epoetin Alfa; Erbitux; Erlotinib; Erwinia L-asparaginase; Estramustine; Ethyo;l Etopophos®; Etoposide; Etoposide Phosphate; Eulexin®; Everolimus; Evista®; Exemestane; Fareston®; Faslodex®; Femara®; Filgrastim; Floxuridine; Fludara®; Fludarabine; Fluoroplex®; Fluorouracil; Fluorouracil (cream); Fluoxymesterone; Flutamide; Folinic Acid; FUDR®; Fulvestrant; Gefitinib; Gemcitabine; Gemtuzumab ozogamicin; Gemzar; Gleevec™; Gliadel® Wafer; GM-CSF; Goserelin; Granulocyte-Colony Stimulating Factor (G-CSF); Granulocyte Macrophage Colony Stimulating Factor (G-MCSF); Halotestin®; Herceptin®; Hexadrol; Hexalen®; Hexamethylmelamine; HMM; Hycamtin®; Hydrea®; Hydrocort Acetate®; Hydrocortisone; Hydrocortisone Sodium Phosphate; Hydrocortisone Sodium Succinate; Hydrocortone Phosphate; Hydroxyurea; Ibritumomab; Ibritumomab Tiuxetan; Idamycin®; Idarubicin Ifex®; Interferon-alpha; Interferon-alpha-2b (PEG Conjugate); Ifosfamide; Interleukin-11 (IL-11); Interleukin-2 (IL-2); Imatinib mesylate; Imidazole Carboxamide; Intron A®; Iressa®; Irinotecan; Isotretinoin; Ixabepilone; Ixempra™;

KADCYCLA®; Kidrolase (t) Lanacort®; Lapatinib; L-asparaginase; LCR; Lenalidomide; Letrozole; Leucovorin; Leukeran; Leukine™; Leuprolide; Leurocristine; Leustatin™; Liposomal Ara-C; Liquid Pred®; Lomustine; L-PAM; L-Sarcolysin; Lupron®; Lupron Depot®; Matulane®; Maxidex; Mechlorethamine; Mechlorethamine Hydrochloride; Medralone®; Medrol®; Megace®; Megestrol; Megestrol Acetate; Melphalan; Mercaptopurine; Mesna; Mesnex™; Methotrexate; Methotrexate Sodium; Methylprednisolone; Meticorten®; Mitomycin; Mitomycin-C; Mitoxantrone M-Prednisol®; MTC; MTX; Mustargen®; Mustine; Mutamycin®; Myleran®; Mylocel™; Mylotarg®; Navelbine®; Nelarabine; Neosar®; Neulasta™; Neumega®; Neupogen®; Nexavar®; Nilandron®; Nilotinib; Nilutamide; Nipent®; Nitrogen Mustard Novaldex®; Novantrone®; Nplate; Octreotide; Octreotide acetate; Ofatumumab; Oncospar®; Oncovin®; Ontak®; Onxal™; Oprelvekin; Orapred®; Orasone®; Oxaliplatin; Paclitaxel; Paclitaxel Protein-bound; Pamidronate; Panitumumab; Panretin®; Paraplatin®; Pazopanib; Pediapred®; PEG Interferon; Pegaspargase; Pegfilgrastim; PEG-INTRON™; PEG-L-asparaginase; PEMETREXED; Pentostatin; Phenylalanine Mustard; Platinol®; Platinol-AQ®; Prednisolone; Prednisone; Prelone®; Procarbazine; PROCRIT®; Proleukin®; Prolifeprospan 20 with Carmustine Implant; Purinethol®; Raloxifene; Revlimid®; Rheumatrex®; Rituxan®; Rituximab; Roferon-A®; Romiplostim; Rubex®; Rubidomycin hydrochloride; Sandostatin®; Sandostatin LAR®; Sargramostim; Solu-Cortef®; Solu-Medrol®; Sorafenib; SPRYCEL™; STI-571; Streptozocin; SU11248; Sunitinib; Sutent®; Tamoxifen Tarceva®; Targretin®; Tasigna®; Taxol®; Taxotere®; Temodar®; Temozolomide Temsirolimus; Teniposide; TESPA; Thalidomide; Thalomid®; TheraCys®; Thioguanine; Thioguanine Tabloid®; Thiophosphoamide; Thioplex®; Thiotepa; TICE®; Toposar®; Topotecan; Toremifene; Torisel®; Tositumomab; Trastuzumab; Treanda®; Tretinoin; Trexall™; Trisenox®; TSPA; TYKERB®; VCR; Vectibix™; Velban®; Velcade®; VePesid®; Vesanoid®; Viadur™; Vidaza®; Vinblastine; Vinblastine Sulfate; Vincasar Pfs®; Vincristine; Vinorelbine; Vinorelbine tartrate; VLB; VM-26; Vorinostat; Votrient; VP-16; Vumon®; Xeloda®; Zanosar®; Zevalin™; Zinecard®; Zoladex®; Zoledronic acid; Zolinza; or Zometa®.

[0169] In an embodiment, the binding proteins provided herein are used in combination with one or more of: Temozolomide®, irinotecan, leucovorin, 5-FU, gemcitabine, and paclitaxel. In an embodiment, binding protein h1A11.1-SL-Av is used in combination with one or more of: Temozolomide®, irinotecan, leucovorin, 5-FU, gemcitabine, and paclitaxel.

[0170] In various embodiments, the binding proteins provided herein may also be combined with an agent, such as methotrexate, 6-MP, azathioprine sulphasalazine, mesalazine, olsalazine chloroquinine/hydroxychloroquine, pencillamine, aurothiomalate (intramuscular and oral), azathioprine, cochicine, a corticosteroid (oral, inhaled and local injection), a beta-2 adrenoreceptor agonist (salbutamol, terbutaline, salmeteral), a xanthine (theophylline, aminophylline), cromoglycate, nedocromil, ketotifen, ipratropium, oxitropium, cyclosporin, FK506, rapamycin, mycophenolate mofetil, leflunomide, an NSAID, for example, ibuprofen, a corticosteroid such as prednisolone, a phosphodiesterase inhibitor, an adensosine agonist, an antithrombotic agent, a complement inhibitor, an adrenergic agent, an agent which interferes with signalling by proinflammatory cytokines such as TNF-α or IL-1 (e.g., IRAK, NIK, IKK , p38 or a MAP kinase inhibitor), an IL-1β converting enzyme inhibitor, a TNFα converting enzyme (TACE) inhibitor, a T-cell signalling inhibitor such as a kinase inhibitor, a metalloproteinase inhibitor, sulfasalazine, azathioprine, a 6-mercaptopurine, an angiotensin converting enzyme inhibitor, a soluble cytokine receptor or derivative thereof (e.g., a soluble p55 or p75 TNF receptor or the derivative p75TNFRIgG (Enbrel™) or p55TNFRIgG (Lenercept), sIL-1RI, sIL-1RII, sIL-6R), an antiinflammatory cytokine (e.g., IL-4, IL-10, IL-11, IL-13 and TGFβ), celecoxib, folic acid, hydroxychloroquine sulfate, rofecoxib, etanercept, infliximab, naproxen, valdecoxib, sulfasalazine, methylprednisolone, meloxicam, methylprednisolone acetate, gold sodium thiomalate, aspirin, triamcinolone acetonide, propoxyphene napsylate/apap, folate, nabumetone, diclofenac, piroxicam, etodolac, diclofenac sodium, oxaprozin, oxycodone hcl, hydrocodone bitartrate/apap, diclofenac sodium/misoprostol, fentanyl, anakinra, human recombinant, tramadol hcl, salsalate, sulindac, cyanocobalamin/fa/pyridoxine, acetaminophen, alendronate sodium, prednisolone, morphine sulfate, lidocaine hydrochloride, indomethacin, glucosamine sulf/chondroitin, amitriptyline hcl, sulfadiazine, oxycodone hcl/acetaminophen, olopatadine hcl, misoprostol, naproxen sodium, omeprazole, cyclophosphamide, rituximab, IL-1 TRAP, MRA, CTLA4-IG, IL-18 BP, anti-IL-18, Anti-IL15, BIRB-796, SCIO-469, VX-702, AMG-548, VX-740, Roflumilast, IC-485, CDC-801, or Mesopram. In some embodiments, combinations can include methotrexate or leflunomide and cyclosporine.

[0171] In some embodiments, the pharmaceutical compositions provided herein may include a "therapeutically effective amount" or a "prophylactically effective amount" of a binding protein provided herein. A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result. A therapeutically effective amount of the binding protein

may be determined by a person skilled in the art and may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the binding protein to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the antibody, or antibody binding portion, are outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount will be less than the therapeutically effective amount.

V. Diagnostics

[0172] The disclosure herein also provides diagnostic applications, including, but not limited to, diagnostic assay methods using one or more binding proteins, diagnostic kits containing one or more binding proteins, and methods and kits for use in automated and/or semi-automated systems. In some embodiments, the methods and kits can be employed in the detection, monitoring, and/or treatment of a disease or disorder in an individual.

A. Assay Method

[0173] In various embodiments, methods are provided for determining the presence, amount and/or concentration of at least one analyte, or fragment thereof, in a test sample using at least one binding protein. Exemplary assays include, but are not limited to, immunoassays and/or methods employing mass spectrometry.

[0174] For example, immunoassays provided by the present disclosure may include sandwich immunoassays, radioimmunoassays (RIA), enzyme immunoassays (EIA), enzyme-linked immunosorbent assays (ELISA), competitive-inhibition immunoassays, fluorescence polarization immunoassays (FPIA), enzyme multiplied immunoassay techniques (EMIT), bioluminescence resonance energy transfer (BRET), and homogenous chemiluminescent assays, among others.

[0175] In some embodiments, a method of determining the presence, amount or concentration of one or more antigens, or fragments thereof, in a test sample is provided, wherein the one or more antigens or fragments thereof are DLL4 and/or VEGF. The method comprises assaying the test sample for the antigen, or fragment thereof, by an immunoassay. The immunoassay (i) employs at least one binding protein and at least one detectable label and (ii) comprises comparing a signal generated by the detectable label as a direct or indirect indication of the presence,

amount or concentration of the antigen, or fragment thereof, in the test sample to a signal generated as a direct or indirect indication of the presence, amount or concentration of the antigen, or fragment thereof, in a control or a calibrator. The calibrator is optionally part of a series of calibrators in which each of the calibrators differs from the other calibrators in the series by the concentration of the antigen, or fragment thereof. The method can comprise (i) contacting the test sample with at least one capture agent, which binds to an epitope on the antigen, or fragment thereof, so as to form a complex comprising the capture agent and the antigen or fragment thereof (ii) contacting the complex comprising the capture agent and the antigen or fragment thereof with at least one detection agent, which comprises a detectable label and binds to an epitope on the antigen, or fragment thereof, that is not bound by the capture agent, to form a detection complex, and (iii) determining the presence, amount or concentration of the antigen, or fragment thereof, in the test sample based on the signal generated by the detectable label in the detection complex formed in (ii), wherein at least one capture agent and/or at least one detection agent is the at least one binding protein.

[0176] Alternatively, in some embodiments, the method of determining the presence, amount or concentration of one or more antigens, or fragments thereof, in a test sample can comprise (i) contacting the test sample with at least one capture agent, which binds to an epitope on the antigen, or fragment thereof, so as to form a complex comprising the capture agent and the antigen or fragment thereof and simultaneously or sequentially, in either order, contacting the test sample with detectably labeled antigen, or fragment thereof, which can compete with any antigen, or fragment thereof, in the test sample for binding to the at least one capture agent, wherein any antigen, or fragment thereof, present in the test sample and the detectably labeled antigen compete with each other to form a detection complex and (ii) determining the presence, amount or concentration of the antigen, or fragment thereof, in the test sample based on the signal generated by the detectable label in the detection complex formed in (i), wherein at least one capture agent is the at least one binding protein and wherein the signal generated by the detectable label in the capture detection complex is inversely proportional to the amount or concentration of antigen, or fragment thereof, in the test sample.

[0177] In various embodiments, the test sample can be from a patient, in which case the method can further comprise diagnosing, prognosticating, or assessing the efficacy of therapeutic/prophylactic treatment of the patient. If the method further

comprises assessing the efficacy of therapeutic/prophylactic treatment of the patient, the method optionally further comprises modifying the therapeutic/prophylactic treatment of the patient as needed to improve efficacy. The method can be adapted for use in an automated system or a semi-automated system. Accordingly, the methods described herein also can be used to determine whether or not a subject has or is at risk of developing a given disease, disorder or condition. Specifically, such a method can comprise the steps of:

[0178] (a) determining the concentration or amount of one or more analytes, or fragments thereof, in a test sample from a subject (e.g., using the methods described herein, or methods known in the art); and (b) comparing the concentration or amount of the analyte(s), or fragment(s) thereof, as determined in step (a) with a predetermined level, wherein, if the concentration or amount of analyte(s) determined in step (a) is favorable with respect to a predetermined level, then the subject is determined not to have or be at risk for a given disease, disorder or condition. However, if the concentration or amount of analyte(s) determined in step (a) is unfavorable with respect to the predetermined level, then the subject is determined to have or be at risk for a given disease, disorder or condition.

[0179] Additionally, provided herein are methods of monitoring the progression of a disease in a subject. In some embodiments, the methods comprise the steps of:

(a) determining the concentration or amount in a test sample from a subject of one or more analyte(s);

(b) determining the concentration or amount of analyte(s) in a later test sample from the same subject; and

(c) comparing the concentration or amount of analyte(s) as determined in step (b) with the concentration or amount of analyte(s) determined in step (a), wherein if the concentration or amount determined in step (b) is unchanged or is unfavorable when compared to the concentration or amount determined in step (a), then the disease in the subject is determined to have continued, progressed or worsened. By comparison, if the concentration or amount as determined in step (b) is favorable when compared to the concentration or amount as determined in step (a), then the disease in the subject is determined to have discontinued, regressed or improved.

[0180] Optionally, the methods of monitoring the progression of a disease further comprises comparing the concentration or amount of analyte(s) as determined in step (b), for example, with a predetermined level. Further, optionally the methods comprise treating the subject with one or more pharmaceutical compositions for a period of time if the comparison shows that the concentration or amount of analyte(s) as determined in step (b), for example, is unfavorably altered with respect to the predetermined level.

[0181] In some embodiments, the presence, amount, or concentration of an analyte or fragment thereof is detected in a sample using a detectable label such as a chemiluminescent label (acridinium compound). In some embodiments, the chemiluminescent signal that is generated can be detected using routine techniques known to those skilled in the art. Based on the intensity of the signal generated, the amount or concentration of analyte in the sample can be quantified. Specifically, in some embodiments, the amount of analyte in the sample may be proportional to the intensity of the signal generated. In certain embodiments, the amount of analyte present can be quantified by comparing the amount of light generated to a standard curve for analyte or by comparison to a reference standard or calibrator. The standard curve can be generated using serial dilutions or solutions of known concentrations of analyte by mass spectroscopy, gravimetric methods, and other techniques known in the art.

[0182] Analyte immunoassays generally can be conducted using any format known in the art, such as, but not limited to, a sandwich format. For example, in the immunoassays one or more binding proteins can be used to capture the analyte (or a fragment thereof) in the test sample (these binding proteins are frequently referred to as a "capture" binding proteins) and one or more binding proteins can be used to bind a detectable (namely, quantifiable) label to the sandwich (these binding proteins are frequently referred to as the "detection" binding proteins, the "conjugate," or the "conjugates"). Thus, in the context of an exemplary sandwich immunoassay format, a DVD (or a fragment, a variant, or a fragment of a variant thereof) as described herein can be used as a capture binding protein, a detection binding protein, or both. For example, a DVD having a first domain that can bind an epitope on a first analyte (or a fragment thereof) and a second domain that can bind an epitope on a second analyte (or a fragment thereof) can be used as a capture and/or detection binding protein to detect, and optionally quantify, one or more analytes (e.g., DLL4 and/or VEGF). In a further example, employing DVD having differential affinities within a sandwich assay

can provide an avidity advantage. In the context of immunoassays as described herein, it generally may be helpful or desired to incorporate one or more linkers within the structure of a DVD. When present, optimally the linker should be of sufficient length and structural flexibility to enable binding of an epitope by the inner domains as well as binding of another epitope by the outer domains. In this regard, if a DVD can bind two different analytes and one analyte is larger than the other, desirably the larger analyte is bound by the outer domains.

[0183] In various embodiments, a sample being tested (e.g., a sample suspected of containing analyte or a fragment thereof) can be contacted with at least one capture binding protein and at least one detection binding protein either simultaneously or sequentially and in any order. For example, the test sample can first be contacted with at least one capture binding protein and then (sequentially) with at least one detection binding protein. Alternatively, the test sample can be first contacted with at least one detection binding protein and then (sequentially) with at least one capture binding protein. In yet another alternative, the test sample can be contacted simultaneously with a capture binding protein and a detection binding protein. In various embodiments, competitive inhibition immunoassays comprising one or more DVD disclosed herein can be used to detect the presence, amount, or concentration of one or more analytes or fragments thereof (e.g., DLL4 and/or VEGF).

[0184] In various embodiments, the detectable label can be bound to the binding proteins either directly or through a coupling agent. An example of a coupling agent that can be used is EDAC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, hydrochloride), which is commercially available from Sigma-Aldrich, St. Louis, MO. Other coupling agents that can be used are known in the art. Methods for binding a detectable label to a binding protein are known in the art.

[0185] In various embodiments, the presence or amount of label bound to a complex comprising analyte and DVD in a detection assay can be quantified using techniques known in the art. For example, if an enzymatic label is used, the labeled complex can be reacted with a substrate for the label that gives a quantifiable reaction such as the development of color. If the label is a radioactive label, the label can be quantified using appropriate means, such as a scintillation counter. If the label is a fluorescent label, the label can be quantified by stimulating the label and detecting a fluorescent signal. If the label is a chemiluminescent label, the label can be quantified by detecting the light emitted either visually or by using luminometers, x-ray film, high speed photographic film, a CCD camera, etc. In some embodiments, once the amount

of the label in the complex has been quantified, the concentration of analyte or a fragment thereof in the test sample can determined by appropriate means, such as by use of a standard curve that has been generated using serial dilutions of analyte or a fragment thereof of known concentration or by any other calibrator.

[0186] In an embodiment, a chemiluminescent microparticle immunoassay, in particular one employing the ARCHITECT® automated analyzer (Abbott Laboratories, Abbott Park, IL), is used.

[0187] In some embodiments, methods employing mass spectrometry are provided by the present disclosure and include, but are not limited to MALDI (matrix-assisted laser desorption/ionization) and SELDI (surface-enhanced laser desorption/ionization).

[0188] Methods for collecting, handling, processing, and analyzing biological test samples using immunoassays and mass spectrometry are well-known to one skilled in the art and are provided for in the practice of the present disclosure (US 2009-0311253 A1).

B. Kit

[0189] In various embodiments, a kit for assaying a test sample for the presence, amount and/or concentration of at least one analyte, or fragment thereof, in a test sample is also provided. In some embodiments, the kit comprises at least one component for assaying the test sample for the analyte, or fragment thereof, and instructions for assaying the test sample for the analyte, or fragment thereof. The at least one component for assaying the test sample for the analyte, or fragment thereof, can include a composition comprising a binding protein, as disclosed herein, and/or a fragment, a variant, or a fragment of a variant thereof. In some embodiments, the component is optionally immobilized on a solid phase.

[0190] Optionally, in some embodiments, the kit may comprise a calibrator or control, which may comprise isolated or purified analyte. In certain embodiments, the kit can comprise at least one component for assaying the test sample for an analyte by immunoassay and/or mass spectrometry. The kit components, including the analyte, binding protein, and/or anti-analyte binding protein, or fragments thereof, may be optionally labeled using any art-known detectable label (US 2009-0311253 A1).

C. Automation

[0191] In various embodiments, the kits (or components thereof) and the methods of determining the presence, amount and/or concentration of at least one analyte in a test sample can be adapted for use in a variety of automated and semi-automated systems, as described, for example, in US Patent Nos. 5,089,424 and 5,006,309, and as commercially marketed, for example, by Abbott Laboratories (Abbott Park, IL) as ARCHITECT®.

[0192] Other automated or semiautomated platforms that could be used with the binding proteins include, but are not limited to, AxSYM®, IMx® (see, for example, US Patent No. 5,294,404, PRISM®, EIA (bead), and Quantum™ II (all from Abbott Laboratories), as well as other platforms known in the art. Additionally, the assays, kits and kit components can be employed in other formats, for example, on electrochemical and/or other hand-held or point-of-care assay systems. The present disclosure is, for example, applicable to the commercial Abbott Point of Care (i-STAT®, Abbott Laboratories) electrochemical immunoassay system that performs sandwich immunoassays. Immunosensors and their methods of manufacture and operation in single-use test devices are described, for example in, US Patent No. 5,063,081, 7,419,821, and 7,682,833; and US Publication Nos. 20040018577, 20060160164 and US 20090311253.

[0193] It will be readily apparent to those skilled in the art that other suitable modifications and adaptations of the methods described herein are obvious and may be made using suitable equivalents without departing from the scope of the embodiments disclosed herein. Having now described certain embodiments in detail, the same will be more clearly understood by reference to the following examples, which are included for purposes of illustration only and are not intended to be limiting.

EXAMPLES

Example 1: Construction of Anti-DLL4/Anti-VEGF DVD Molecules

[0194] The variable domain sequences from a humanized anti-DLL4 mAb (h1A11.1) and anti-VEGF mAb (Av) were used to design the VH and VL domains of anti-DLL4/anti-VEGF DVD molecules. Variable regions were synthesized using two-step PCR. Primers were designed with homologous flanking regions to the cloning vector and the linker region between each DVD variable pair. Bacterial transformation was performed to identify positive clones and constructs were harvested and purified for use in mammalian transformation using standard protocols known in the art.

[0195] The variable domains of the heavy and light chain were cloned in-frame into mutant human IgG1 (L234, 235A) heavy-chain and kappa light-chain constant regions, respectively, to generate anti-DLL4/anti-VEGF DVD molecules (Table 4).

Table 4. Anti-DLL4/Anti-VEGF DVD Constructs

DVD Name	Heavy Chain (HC)	Light Chain (LC)
h1A11.1-LL-Av	h1A11.1-L-Av HC	h1A11.1-L-Av LC
h1A11.1-LS-Av	h1A11.1-L-Av HC	h1A11.1-S-Av LC
h1A11.1-SL-Av	h1A11.1-S-Av HC	h1A11.1-L-Av LC
h1A11.1-SS-Av	h1A11.1-S-Av HC	h1A11.1-S-Av LC
h1A11.1-GS10-Av	h1A11.1-GS10-Av HC	h1A11.1-GS10-Av LC
h1A11.1-GS14-Av	h1A11.1-GS14-Av HC	h1A11.1-GS14-Av LC
Av-LL-h1A11.1	Av-L-h1A11.1 HC	Av-L-h1A11.1 LC
Av-LS-h1A11.1	Av-L-h1A11.1 HC	Av-S-h1A11.1 LC
Av-SL-h1A11.1	Av-S-h1A11.1 HC	Av-L-h1A11.1 LC
Av-SS-h1A11.1	Av-S-h1A11.1 HC	Av-S-h1A11.1 LC
Av-GS6-h1A11.1	Av-GS6-h1A11.1 HC	Av-GS6-h1A11.1 LC
Av-GS10-h1A11.1	Av-GS10-h1A11.1 HC	Av-GS10-h1A11.1 LC
Av-GS14-h1A11.1	Av-GS14-h1A11.1 HC	Av-GS14-h1A11.1 LC

Example 2: Affinity Determination of Anti-DLL4/Anti-VEGF DVD Constructs

[0196] A BIACORE assay (Biacore, Inc, Piscataway, NJ) was used to evaluate the binding of DVDs to a purified recombinant DLL4 extracellular domain

(ECD) or to $VEGF_{165}$, as determined by surface plasmon resonance-based measurements made on a Biacore 2000, Biacore 3000 or Biacore T100 (GE Healthcare, Piscataway, NJ) at 25°C. For DLL4 binding kinetic measurements the assay buffer was HBS-EPB: 10mM Hepes, pH7.5, 150mM NaCl, 3mM EDTA, 0.005% Tween 20, 0.1mg/ml BSA (Sigma A7906). For VEGF binding kinetic measurements the assay buffer was HBS-EP+(3N01B): 10mM Hepes, pH7.5, 300mM NaCl, 3mM EDTA, 0.05% Tween 20, 0.1mg/ml BSA (Sigma A7906). For example, approximately 9000 RU of goat anti-human Fc specific polyclonal antibody (Thermo Fisher Scientific Inc., Rockford, IL) diluted in 10 mM sodium acetate (pH 4.5) is directly immobilized across a CM5 research grade biosensor chip using a standard amine coupling kit according to manufacturer's instructions and procedures at 25 ug/ml. Unreacted moieties on the biosensor surface are blocked with ethanolamine. For kinetic analysis, rate equations derived from the 1:1 Langmuir binding model are fitted simultaneously to multiple antigen injections (using global fit analysis) with the use of Scrubber 2 (BioLogic Software), Biacore Biaevaluation 4.0.1 software or Biacore T100 Evaluation software. Purified antibodies are diluted in running buffer for capture across goat anti-human Fc reaction surfaces. Antibodies to be captured as a ligand (1 ug/ml) are injected over reaction matrices at a flow rate of 10 ul/min. During the assay, all measurements are referenced against the capture surface alone (i.e. with no captured antibody). The association and dissociation rate constants, K_{on} ($M^{-1}s^{-1}$) and K_{off} (s^{-1}) are determined under a continuous flow rate of 80 ul/min. Rate constants are derived by making kinetic binding measurements at different antigen concentrations ranging from 1.23 – 900 nM, as a 3-fold dilution series, and included buffer-only injections (to be used for double referencing). The equilibrium dissociation constant K_D (M) of the reaction between antibodies and the target antigen is then calculated from the kinetic rate constants by the following formula: $K_D = K_{off}/K_{on}$. Binding is recorded as a function of time and kinetic rate constants are calculated. In this assay, on-rates as fast as 10^6 $M^{-1}s^{-1}$ and off-rates as slow as 10^{-6} s^{-1} can be measured. The antigen binding affinities of the anti-DLL4/anti-VEGF DVDs are summarized in Table 5 and 6.

Table 5. Biacore Kinetics of Anti-DLL4/Anti-VEGF DVD Binding Proteins

DVD Name	BIAcore human $DLL4_{529}$			BIAcore human $VEGF_{165}$		
	Kon ($M^{-1}s^{-1}$)	Koff (s^{-1})	K_D (M)	Kon ($M^{-1}s^{-1}$)	Koff (s^{-1})	K_D (M)
Av	N/A	N/A	N/A	1.19E+05	3.47E-05	2.9E-10
h1A11.1	1.60E+05	1.93E-03	1.2E-08	N/A	N/A	N/A

DVD Name	BIAcore human $DLL4_{529}$			BIAcore human $VEGF_{165}$		
h1A11.1-LL-Av	2.05E+05	2.63E-03	1.2E-08	5.19E+04	2.44E-05	4.7E-10
h1A11.1-LS-Av	2.15E+05	2.36E-03	1.1E-08	2.26E+04	2.83E-05	1.3E-09
h1A11.1-SL-Av	2.17E+05	2.24E-03	1.0E-08	4.57E+04	3.20E-05	7.0E-10
h1A11.1-SS-Av	1.92E+05	2.25E-03	1.2E-08	7.32E+03	5.42E-05	7.4E-09
h1A11.GS10-Av	2.52E+05	2.33E-03	9.3E-09	1.92E+04	4.26E-05	2.2E-09
h1A11.1-GS14-Av	2.40E+05	2.33E-03	9.7E-09	2.87E+04	3.72E-05	1.3E-09
Av-GS6-h1A11.1	1.41E+04	7.03E-04	5.0E-08	1.94E+05	3.72E-05	1.9E-10
Av-GS10-h1A11.1	3.45E+04	1.01E-03	2.9E-08	1.84E+05	3.59E-05	1.9E-10
Av-GS14-h1A11.1	3.97E+04	1.34E-03	3.4E-08	1.82E+05	3.08E-05	1.7E-10

N/A: not applicable

Table 6. Additional Biacore Kinetics of h1A11.1-SL-Av DVD

DVD Name	BIAcore cynomolgus monkey $DLL4_{529}$			BIAcore mouse $DLL4_{530}$		
	Kon ($M^{-1}s^{-1}$)	Koff (s^{-1})	K_D (M)	Kon ($M^{-1}s^{-1}$)	Koff (s^{-1})	K_D (M)
h1A11.1-SL-Av	4.43E+05	2.49E-03	5.6E-09	3.22E+05	7.74E-03	2.4E-08

Example 3: *In vitro* Characterization of the Anti-DLL4/Anti-VEGF DVD Molecules

Example 3.1: DLL4 Binding Activity as Determined by Flow Cytometry (FACS)

[0197] Stable HEK293G cell lines overexpressing full-length DLL4 were harvested from tissue culture flasks, washed four times and resuspended in phosphate buffered saline (PBS) containing 1% bovine serum albumin and 1 mM $CaCl_2$ (FACS buffer). 1.5 $x10^5$ cells were incubated with DVD binding proteins at various concentrations in FACS buffer for 60 minutes on ice. Cells were washed twice and 50 uL of R-phycoerythrin-conjugated anti-rat IgG $F(ab')_2$ fragment (1:200 dilution in FACS buffer) (Jackson ImmunoResearch, West Grove, PA, Cat.#112-116-072) was added. Following an incubation on ice (4°C, 60 minutes), cells were washed three times and resuspended in FACS buffer. Fluorescence was measured using a Becton Dickinson FACSCalibur-HTS (Becton Dickinson, San Jose, CA). Data was analyzed using Graphpad Prism software and EC_{50} values were reported as the concentration of antibody to achieve 50% of maximal antibodies binding to DLL4 expressing cells.

Example 3.2: DLL4-blocking Activity of the Anti-DLL4/Anti-VEGF DVD Proteins as Determined by Inhibition of Notch-1 Interaction with Soluble DLL4 Extracellular Domain

[0198] 96-well Nunc-Immuno plates (#439454 for huDLL4 ELISA) and 96-well Costar plates (#9018 for muDLL4 ELISA) were coated with 16 nM human Notch-1 (R&D Systems #3647-TK, 100 µl/well in D-PBS) and incubated overnight at 4°C. Plates were then washed 3X with wash buffer (PBS, 0.05% Tween-20) and blocked with 200 µl/well blocking buffer (D-PBS, 1% BSA, 1 mM $CaCl_2$, 0.05% Tween-20) for 1 hour at 25°C. While blocking, biotin labeled DLL4 extracellular domain (14 nM) was mixed with antibody (30 pM-66 nM, 3-fold serial dilution in blocking buffer) for 1 hour at 25°C with shaking. Assay plates were washed after blocking, and incubated with DLL4/ antibody mixtures (100 µl/well, 1 hour at 25°C with shaking). Plates were washed again and 100 µl/well of streptavidin conjugated with HRP (Fitzgerald #65R-S104PHRPx, diluted 1:5,000 in blocking buffer) was added for 1 hour at 25°C with shaking. After a final wash, plates were developed using 100 µl/well substrate (TMB Sigma #T8665), and the reaction was stopped using 100 µl/well 1N HCl, and the absorbance was read at 450 nm. Data was analyzed using Graphpad Prism software and IC_{50} values were reported as the concentration of antibody required to achieve 50% reduction of DLL4 binding to Notch1.

Example 3.3: DLL4-blocking Activity of the Anti-DLL4/Anti-VEGF DVD Proteins as Determined by Inhibition of DLL4-Dependent Notch Activation Using a Notch reporter assay

[0199] 96-well black clear-bottom tissue culture plates were seeded overnight with engineered EA.hy926 cells expressing luciferase driven by a Notch-responsive promoter (7,000 cells/well). Antibodies serially diluted from 200 nM were mixed for 15 minutes with an equal volumn of solution containing HEK293G cells expressing full-length DLL4 (5,000 cells/well). The 293G/DLL4 cells were co-cultured with EA.hy926 Notch reporter cells for 24 hrs in the presence of testing antibodies. Luciferase activity was analyzed using Promega's substrate (Promega # E2940). Data was analyzed using Graphpad Prism software and IC_{50} values were reported as the concentration of antibody required to achieve a 50% reduction of DLL4-induced Notch activation.

Example 3.4: VEGF Binding Activity of Anti-DLL4/Anti-VEGF DVD Proteins as Determined by Capture ELISA

[0200] ELISA plates (Nunc, MaxiSorp, Rochester, NY) were incubated overnight at 4°C with anti-human Fc antibody (5 µg/ml in PBS, Jackson Immunoresearch, West Grove, PA). Plates were washed three times in wash buffer (PBS containing 0.05% Tween 20), and blocked for 1 hour at 25°C in blocking buffer (PBS containing 1% BSA). Wells were washed three times, and each antibody or DVD was serially diluted in PBS containing 0.1% BSA before incubating at 25°C for 1 hour. The wells were washed three times, and biotinylated VEGF (2 nM) was added to the plates and incubated for 1 hour at 25°C. The wells were washed three times, and then incubated for 1 hour at 25°C with streptavidin-HRP (KPL #474-3000, Gaithersburg, MD). The wells were washed three times, and 100 µl of ULTRA-TMB ELISA (Pierce, Rockford, IL) was added per well. Following color development, the reaction was stopped with 1M HCl and absorbance at 450 nM was measured.

Example 3.5: VEGF-blocking Activity of Anti-DLL4/Anti-VEGF DVD Proteins as Determined by Inhibition of VEGF Interaction with VEGFR1

[0201] ELISA plates (Nunc, MaxiSorp, Rochester, NY) were incubated overnight at 4°C with 100 µl of PBS containing recombinant VEGFR1 extra-cellular domain-Fc fusion protein (5 µg/ml, R&D systems, Minneapolis, MN). Plates were washed three times in washing buffer (PBS containing 0.05% Tween 20), and blocked for 1 hour at 25°C in blocking buffer (PBS containing 1% BSA). Each antibody and DVD was serially diluted in PBS containing 0.1% BSA and incubated with 50 µl of 2 nM biotinylated VEGF for 1 hour at 25°C. The mixtures of antibody and biotinylated VEGF or DVD and biotinylated VEGF (100 µl) were then added to the VEGFR1-Fc coated wells and incubated at 25°C for 10 minutes. The wells were washed three times, and then incubated for 1 hour at 25°C with 100 µl of streptavidin-HRP (KPL #474-3000, Gaithersburg, MD). The wells were washed three times, and 100 µl of ULTRA-TMB ELISA (Pierce, Rockford, IL) was added per well. Following color development, the reaction was stopped with 1M HCl and absorbance at 450 nM was measured.

Example 3.6: VEGF-blocking Activity of Anti-DLL4/Anti-VEGF DVD Protein as Determined by Inhibition of VEGF-stimulated Endothelial Cell Proliferation/Survival

[0202] Prior to plating for the assay, TIME (ATCC) or HUVEC (passage 2-6) endothelial cells were maintained in EBM-2 (Lonza-Clonetics, Walkersville, MD) supplemented with EGM-2 SingleQuots (Lonza-Clonetics, Walkersville, MD, #CC-4176). Cells were plated at 10,000 cells/well on collagen-coated black 96-well plates in 100 µl EMB-2 with 0.1% FBS in the absence of growth factors. The following day the

media was replaced with 0.1% FBS in the absence of growth factors. The following day the media was replaced with 100 µl of EMB-2 (without growth factors or serum) and incubated for four hours prior to the addition of VEGF and antibodies or DVDs. Anti-VEGF monoclonal antibodies or DVDs were serially diluted in EMB-2 with 0.1% BSA and were pre-incubated with recombinant human $VEGF_{165}$ (50 ng/ml) for 1 hour at 25°C in 50 µl. Mixtures of antibody and VEGF or DVD and VEGF were then added to the cells (50 µl), and the plates were incubated at 37°C in a humidified, 5% CO_2 atmosphere for 72 hours. Cell survival/proliferation was measured indirectly by assessing ATP levels using an ATPlite kit (Perkin Elmer, Waltham, MA) according to the manufacturer's instructions.

[0203] The *in vitro* activities of the anti-DLL4/anti-VEGF DVDs, as characterized by the above-mentioned assays, are summarized in Table 7.

Table 7. *In Vitro* Characterization of Anti-DLL4/Anti-VEGF DVDs

DVD Name	Human DLL4			Human VEGF		
	Binding	Functional Blockade		Binding	Functional Blockade	
	FACS EC_{50} (nM)	Notch Competition ELISA IC_{50} (nM)	Notch Activation IC_{50} (nM)	ELISA EC_{50} (nM)	VEGFR1 Competition ELISA IC_{50} (nM)	Endothelial Cell Proliferation IC_{50} (nM)
Av-LL-h1A11.1		2.43				
Av-LS-h1A11.1		2.77				
Av-SL-h1A11.1		7.38				
Av-SS-h1A11.1		3503				
h1A11.1-LL-Av	5.04	0.79	4.56	0.12	3.8	0.42
h1A11.1-LS-Av	5	0.76	4.59	0.16	7.7	0.57
h1A11.1-SL-Av	4.35	1.09	5.34	0.55	3.8	0.61
h1A11.1-SS-Av	3.75	0.91	7.47	2.5	26	4.2
h1A11.1-GS10-Av		0.65		0.99	37.2	1.21
h1A11.1-GS14-Av		0.68		0.41	20.2	0.84
Av-GS6-h1A11.1		3.41		0.25	7.44	4.14
Av-GS10-h1A11.1		1.5		0.12	2.01	0.57
Av-GS14-h1A11.1		1.54		0.17	4.69	0.48

Example 4: *In vivo* Pharmacokinetic Results of Anti-DLL4/Anti-VEGF DVD

[0204] The pharmacokinetics properties of h1A11.1-SL-Av DVD were assessed in cynomolgus monkeys (n=2 for each dose group) and CD1 mice (n=6 for each dose group) following bolus intravenous administration. The h1A11.1-SL-Av DVD

pharmacokinetic profile in both CD1 mice and cynomolgus monkeys was characteristic of a traditional monoclonal antibody (Table 8).

Table 8: Mean PK Parameters of h1A11.1-SL-Av DVD after Bolus Intravenous Administration

Species	Dose	AUC	Cmax	Vss	CL	T1/2	MRT
Mouse	1	30.4	16.6	56.6	33.6	1.4	1.8
	3	203.1	68.9	46.2	14.9	2.3	3.1
	10	570.3	187.2	102.8	18.3	4.7	5.9
	30	4488.1	496.2	94.4	6.8	9.8	13.7
Monkey	1	109.7	30.3	35.9	10.5	3.1	3.9
	3	403.9	92.8	33.9	7.5	4.3	4.6
	10	1957.1	395.1	35.9	5.1	5.0	7.1
	30	8626.9	1344.4	27.0	3.7	5.5	7.8

Dose: mg/kg; AUC: Area under the concentration curve from 0 to time infinity (d*ug/mL); Cmax: First observed conc. post dosing (ug/mL); Vss: volume of distribution (mL/kg); CL: clearance (mL/day/kg); T1/2: terminal half-life (days); MRT: Mean residence time from 0 to infinity (days).

Example 5: *In vivo* Anti-Tumor Efficacy of Anti-DLL4/Anti-VEGF DVDs

[0205] The effect of anti-DLL4/anti-VEGF DVDs on tumor growth was initially evaluated on HT-29 human colorectal adenocarcinoma xenograft tumors in female athmyic nude mice. Briefly, $2x10^6$ cells were inoculated subcutaneously into the right hind flank. Tumors were allowed to establish for 25 days, at which point tumor volume was determined using electronic caliper measurements using the formula: $L \times W^2/2$. Mice were allocated into treatment groups (n=10 per group) so that each cohort had equivalent mean tumor volume of 214 mm^3 prior to initiation of therapy. Animals were dosed intraperitoneally weekly for four weeks, with tumor volume measured twice a week for the duration of the experiment. Results are shown in Table 9.

[0206] The effect of anti-DLL4/anti-VEGF DVDs on tumor growth was subsequently evaluated on U87-MG human glioblastoma xenograft tumors in female SCID mice. Briefly, $3x10^6$ cells were inoculated subcutaneously into the right hind flank. Tumors were allowed to establish for 17 days, at which point tumor volume was determined using electronic caliper measurements using the formula: $L \times W^2/2$. Mice were allocated into treatment groups (n=10 per group) so that each cohort had equivalent mean tumor volume of 221 mm^3 prior to initiation of therapy. Animals were

dosed intraperitoneally weekly for four weeks, with tumor volume measured twice a week for the duration of the experiment. Results are shown in Table 9.

Table 9. Efficacy of Anti-DLL4/Anti-VEGF DVDs in the HT-29 Colorectal Adenocarcinoma and U87-MG Glioblastoma Xenograft Models

		HT-29		U87-MG	
Treatment	**Dose Route, Regimen**	**%TGI[a]**	**%TGD [b]**	**%TGI[c]**	**%TGD [b]**
h1A11.1-LL-Av	6.7 mg/kg IP, q7dX4	59**	42***	74***	100***
h1A11.1-LS-Av	6.7 mg/kg IP, q7dX4	59**	42***	77***	124***
h1A11.1-SL-Av	6.7 mg/kg IP, q7dX4	68***	61***	81***	100***
h1A11.1-SS-Av	6.7 mg/kg IP, q7dX4	47*	49*	64***	48***

Table 9 key: a. %TGI = Percent tumor growth inhibition = 100 – (T/C x 100), where T = mean tumor volume of treatment group and C = mean tumor volume of treatment control group. Based on day 29 post size match measurements. P values (as indicated by asterisks) are derived from Student's T test comparison of treatment group vs. treatment control group. b. %TGD = Percent tumor growth delay = (T – C)/C x 100, where T = median time to endpoint of treatment group and C=median time to endpoint of treatment control group. Based on an endpoint of 1000 mm^3. P values (as indicated by asterisks) derived from Kaplan Meier log-rank comparison of treatment group vs. treatment control group. c. %TGI = Percent tumor growth inhibition = 100 – (T/C x 100), where T = mean tumor volume of treatment group and C = mean tumor volume of treatment control group. Based on day 24 post size match measurements. P values (as indicated by asterisks) are derived from Student's T test comparison of treatment group vs. treatment control group (* $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$)

Example 6: *In vivo* Combination Efficacy of Anti-DLL4/Anti-VEGF DVDs

[0207] The effect of anti-DLL4/anti-VEGF DVDs in combination with chemotherapy on tumor growth was evaluated on U87-MG human glioblastoma

xenograft tumors in female SCID mice. Briefly, $3x10^6$ cells were inoculated subcutaneously into the right hind flank. Tumors were allowed to establish for 22 days, at which point tumor volume was determined using electronic caliper measurements using the formula: $L \times W^2/2$. Mice were allocated into treatment groups (n=10 per group) so that each cohort had equivalent mean tumor volume of 207 mm^3 prior to initiation of therapy. Animals were dosed intraperitoneally with a single dose of temozolomide® and/or four weekly doses of anti-DLL4 / anti-VEGF DVD, with tumor volume measured twice a week for the duration of the experiment. Results are shown in Table 10.

Table 10. Combination Efficacy of Anti-DLL4/Anti-VEGF DVD and Temozolomide in the U87-MG Glioblastoma Xenograft Model

Treatment	Dose Route, Regimen	%TGI[a]	%TGD [b]
Temozolomide	5 mg/kg IP, qdX1	65***	45***
h1A11.1-SL-Av	6.7 mg/kg IP, q7dX4	69***	100***
Temozolomide + h1A11.1-SL-Av	5 mg/kg IP, qdX1 + 6.7 mg/kg IP, q7dX4	78***	147***

Table 10 key: a. %TGI = Percent tumor growth inhibition = 100 – (T/C x 100), where T = mean tumor volume of treatment group and C = mean tumor volume of treatment control group. Based on day 19 post size match measurements. P values (as indicated by asterisks) are derived from Student's T test comparison of treatment group vs. treatment control group. b. %TGD = Percent tumor growth delay = (T – C)/C x 100, where T = median time to endpoint of treatment group and C=median time to endpoint of treatment control group. Based on an endpoint of 1000 mm^3. P values (as indicated by asterisks) derived from Kaplan Meier log-rank comparison of treatment group vs. treatment control group (* $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$).

Example 7: Preformulation characterization of Anti-DLL4/Anti-VEGF DVDs

[0208] The storage stability (5°C) and accelerated stability (40°C) of an anti-DLL4/anti-VEGF DVD (h1A11.1-SL-Av) was evaluated in the formulations and protein

concentrations listed below. Stability was evaluated by size exclusion chromatography (SE-HPLC) and % aggregrate, % monomer, % fragment, and total species recovered were quantitated. Overall, the formulations cover a pH range of 5 to 7 and a protein concentration range of 1.0 to 118 mg/ml.

[0209] At 5°C and 40°C temperatures and at protein concentrations of 50, 30, and 10 mg/ml, formulations were: 15 mM acetate pH 5; 15 mM phosphate pH 7; 30 mM acetate, 80 mg/ml sucrose, 0.02% Tween 80 at pH 5; 30 mM histidine, 80 mg/ml sucrose, 0.02% Tween 80 at pH 6; PBS (phosphate buffered saline). All formulations contained 0.02% sodium azide to prevent microbial growth during storage. At 5°C and 40°C temperatures and at protein concentrations of 60, 50, 30, and 10 mg/ml, the formulation was 15 mM histidine pH 6 (also containing 0.02% sodium azide to prevent microbial growth during storage). At 5°C and at a protein concentration of 118 mg/ml, the formulation was 15 mM histidine pH 6 (also containing 0.02% sodium azide to prevent microbial growth during storage). At 40°C and at a protein concentration of 1.0 mg/ml, the formulations were 10 mM citrate + 10 mM phosphate at pHs 5, 6, 7. Formulations with protein were filtered to remove possible microbes.

[0210] Freeze-thaw stability was performed by subjecting the protein in formulation to four cycles of freezing at -80°C for at least 20 hours and thawing in a 30°C water bath. The formulations that were tested for freeze-thaw stability are listed below. Stability was evaluated by SE-HPLC and % aggregrate, % monomer, % fragment, and total species recovered were quantitated. The formulations were 15 mM histidine pH 6 at 60 mg/ml protein (also containing 0.02% sodium azide to prevent microbial growth) and 10 mM citrate + 10 mM phosphate at pHs 5, 6, 7 and 1.0 mg/ml protein (filtered to remove possible microbes).

[0211] Finally, differential scanning calorimetry to measure thermal stability was performed on the protein in 10 mM citrate + 10 mM phosphate buffer at pHs 5, 6, 7 and 1.0 mg/ml protein. The onset temperature of unfolding and the midpoint temperatures of unfolding (Tm) of each protein domain were quantitated.

[0212] Based on the data in Tables 11 and 12, h1A11.1-SL-Av satisfied the preformulation criteria for DVD-Ig stability.

Table 11: Accelerated stability at 40°C of h1A11.1-SL-Av at different concentrations and in different buffers, excipients, and pHs

Protein conc (mg/ml)	time	temp (°C)	buffer	pH	% Aggregrate	% Monomer	% Fragment	Total Area
---	pre-	---	---	---	2.71	96.31	0.98	53058

	dialysis							
50, 30, 10	T0	---	ace	5	2.89	96.08	1.03	48033
50, 30, 10	T0	---	his	6	2.81	96.23	0.96	46995
50, 30, 10	T0	---	phos	7	2.91	96.09	1.00	52571
50, 30, 10	T0	---	ace-suc-tw	5	2.54	96.50	0.96	50185
50, 30, 10	T0	---	his-suc-tw	6	2.37	96.62	1.01	50771
50, 30, 10	T0	---	PBS	7	2.90	96.08	1.01	49170
50	T7d	40	ace	5	5.19	93.32	1.49	49028
30	T7d	40	ace	5	3.86	94.68	1.47	48171
10	T7d	40	ace	5	2.60	95.97	1.43	48379
50	T7d	40	his	6	5.25	93.46	1.29	47731
30	T7d	40	his	6	4.13	94.58	1.29	46684
10	T7d	40	his	6	2.73	95.84	1.42	46877
50	T7d	40	phos	7	9.02	89.52	1.46	53429
30	T7d	40	phos	7	6.11	92.40	1.49	51923
10	T7d	40	phos	7	3.94	94.57	1.49	53098
50	T7d	40	ace-suc-tw	5	5.42	92.85	1.73	50373
30	T7d	40	ace-suc-tw	5	4.07	94.06	1.87	48768
10	T7d	40	ace-suc-tw	5	2.66	95.20	2.14	49396
50	T7d	40	his-suc-tw	6	3.44	95.02	1.54	50040
30	T7d	40	his-suc-tw	6	4.16	94.14	1.70	48715
10	T7d	40	his-suc-tw	6	2.86	95.24	1.90	49871
50	T7d	40	PBS	7	8.13	90.28	1.60	49207
30	T7d	40	PBS	7	5.82	92.55	1.63	48853
10	T7d	40	PBS	7	3.62	94.82	1.56	48166
50	T21d	40	ace	5	6.65	90.83	2.51	48536
30	T21d	40	ace	5	4.55	92.91	2.54	48520
10	T21d	40	ace	5	2.71	94.70	2.59	48395
50	T21d	40	his	6	7.01	90.71	2.27	46729
30	T21d	40	his	6	4.69	93.10	2.21	46687
10	T21d	40	his	6	2.77	94.93	2.30	46866
50	T21d	40	phos	7	13.39	83.83	2.78	52244
30	T21d	40	phos	7	9.38	87.76	2.86	53556
10	T21d	40	phos	7	4.77	92.32	2.91	52536
50	T21d	40	ace-suc-tw	5	6.37	90.34	3.30	48268
30	T21d	40	ace-suc-tw	5	4.27	91.91	3.82	47211
10	T21d	40	ace-suc-tw	5	2.26	93.02	4.72	46322
50	T21d	40	his-suc-tw	6	6.84	89.82	3.34	47140
30	T21d	40	his-suc-tw	6	4.60	91.90	3.50	47416
10	T21d	40	his-suc-tw	6	2.67	93.66	3.67	48166
50	T21d	40	PBS	7	12.13	84.81	3.06	49845
30	T21d	40	PBS	7	8.09	88.78	3.13	48108
10	T21d	40	PBS	7	4.20	92.63	3.17	48803

Buffer key (all buffers contain 0.02% sodium azide to prevent microbial growth):
ace = 15 mM acetate pH 5; **his** = 15 mM histidine pH 6; **phos** = 15 mM phosphate pH 7
ace-suc-tw = 30 mM acetate, 80 mg/ml sucrose, 0.02% Tw80
his-suc-tw = 30 mM histidine, 80 mg/ml sucrose, 0.02% Tw80
PBS = phosphate buffered saline

Table 12. Storage stability at 5°C of h1A11.1-SL-Av at different concentrations and in different buffers, excipients, and pHs (buffer key same as in Table 11)

Protein conc (mg/ml)	time	temp (°C)	buffer	pH	% Aggregrate	% Monomer	% Fragment	Total Area
---	pre-dialysis	---	---	---	2.71	96.31	0.98	53058
50, 30, 10	T0	---	ace	5	2.89	96.08	1.03	48033
50, 30, 10	T0	---	his	6	2.81	96.23	0.96	46995
50, 30, 10	T0	---	phos	7	2.91	96.09	1.00	52571
50, 30, 10	T0	---	ace-suc-tw	5	2.54	96.50	0.96	50185
50, 30, 10	T0	---	his-suc-tw	6	2.37	96.62	1.01	50771
50, 30, 10	T0	---	PBS	7	2.90	96.08	1.01	49170
50	T7d	5	ace	5	2.96	95.99	1.05	49118
30	T7d	5	ace	5	2.74	96.21	1.06	48434
10	T7d	5	ace	5	2.62	96.23	1.15	48915
50	T7d	5	his	6	2.93	95.87	1.20	47967
30	T7d	5	his	6	2.75	96.06	1.19	47182
10	T7d	5	his	6	2.55	96.31	1.13	47395
50	T7d	5	phos	7	3.15	95.64	1.21	53843
30	T7d	5	phos	7	3.10	95.76	1.14	53372
10	T7d	5	phos	7	2.91	95.96	1.13	53269
50	T7d	5	ace-suc-tw	5	2.75	96.13	1.12	50236
30	T7d	5	ace-suc-tw	5	2.62	96.11	1.27	50026
10	T7d	5	ace-suc-tw	5	2.56	96.18	1.26	49290
50	T7d	5	his-suc-tw	6	2.84	96.10	1.07	50129
30	T7d	5	his-suc-tw	6	2.58	96.19	1.23	49272
10	T7d	5	his-suc-tw	6	2.64	96.08	1.28	50926
50	T7d	5	PBS	7	3.26	95.59	1.15	49502
30	T7d	5	PBS	7	3.07	95.64	1.29	49724
10	T7d	5	PBS	7	2.83	95.87	1.29	49563
50	T21d	5	ace	5	2.57	95.76	1.67	49722
30	T21d	5	ace	5	2.37	96.03	1.60	48882
10	T21d	5	ace	5	2.22	96.09	1.69	49255
50	T21d	5	his	6	2.63	95.63	1.74	44884
30	T21d	5	his	6	2.42	95.95	1.62	47510
10	T21d	5	his	6	2.19	96.08	1.73	47015
50	T21d	5	phos	7	3.06	94.96	1.98	53449
30	T21d	5	phos	7	2.69	95.46	1.85	52938
10	T21d	5	phos	7	2.35	95.84	1.81	52703
50	T21d	5	ace-suc-tw	5	2.25	95.76	1.99	50960
30	T21d	5	ace-suc-tw	5	2.08	95.90	2.02	49042
10	T21d	5	ace-suc-tw	5	1.97	95.84	2.19	49851
50	T21d	5	his-suc-tw	6	2.24	95.62	2.14	49983
30	T21d	5	his-suc-tw	6	2.09	95.86	2.05	48813
10	T21d	5	his-suc-tw	6	1.97	95.83	2.19	49984
50	T21d	5	PBS	7	2.84	95.07	2.09	50641
30	T21d	5	PBS	7	2.27	95.62	2.12	48441
10	T21d	5	PBS	7	1.99	95.94	2.07	48978
50	T10mo	5	his	6	8.05	91.04	0.91	45552
30	T10mo	5	his	6	5.81	93.29	0.90	46607

Protein conc (mg/ml)	time	temp (°C)	buffer	pH	% Aggregrate	% Monomer	% Fragment	Total Area
10	T10mo	5	his	6	3.62	95.46	0.92	46207
50	T10mo	5	his-suc-tw	6	8.08	90.26	1.67	45430
30	T10mo	5	his-suc-tw	6	5.98	92.43	1.58	42967
10	T10mo	5	his-suc-tw	6	3.95	94.25	1.80	42567

Table 13. Storage stability at 5°C, accelerated stability at 40°C, and freeze-thaw stability of h1A11.1-SL-Av at different concentrations and in different buffers and pHs

Protein conc (mg/ml)	time/FT	temp (°C)	buffer	pH	% Aggregrate	% Monomer	% Fragment	Total Area
1	T0	---	cit-phos	5	7.07	92.14	0.80	46824
1	T8d	40	cit-phos	5	2.23	96.39	1.38	47090
1	T22d	40	cit-phos	5	7.10	89.62	3.28	47956
1	FT2	---	cit-phos	5	7.91	90.75	1.34	46502
1	FT4	---	cit-phos	5	7.41	92.18	0.41	52181
1	T0	---	cit-phos	6	7.17	92.33	0.50	45809
1	T8d	40	cit-phos	6	2.56	96.03	1.42	46783
1	T22d	40	cit-phos	6	5.79	91.73	2.48	47401
1	FT2	---	cit-phos	6	7.14	91.48	1.38	45256
1	FT4	---	cit-phos	6	7.09	92.56	0.34	45004
1	T0	---	cit-phos	7	6.82	92.67	0.51	47025
1	T8d	40	cit-phos	7	2.52	95.95	1.53	48080
1	T22d	40	cit-phos	7	5.52	91.58	2.90	48706
1	FT2	---	cit-phos	7	7.23	91.52	1.25	46732
1	FT4	---	cit-phos	7	7.15	92.49	0.36	46561
60 and 118	T0	---	his	6	8.03	91.15	0.82	43528
60	T7d	40	his	6	7.17	91.76	1.07	45333
60	T21d	40	his	6	15.77	82.13	2.10	44729
60	T7d	5	his	6	3.83	95.32	0.86	46774
60	T26d	5	his	6	7.14	92.56	0.30	63982
118	T5mo	5	his	6	12.82	86.65	0.53	55869
60	T5mo	5	his	6	9.46	90.03	0.51	64573
60	FT2	---	his	6	6.71	92.59	0.70	42259
60	FT4	---	his	6	6.33	93.62	0.05	41054

Key:

FT = freeze thaw

FT2 = analysis after two cycles of freeze and thaw; freezing at -80°C and thawing in a 30°C water bath

FT4 = analysis after four cycles of freeze and thaw; freezing at -80°C and thawing in a 30°C water bath

cit-phos = 10 mM citrate + 10 mM phosphate

his = 15 mM histidine + 0.02% sodium azide (azide for preventing microbial growth)

Table 14. Differential scanning calorimetry data of h1A11.1-SL-Av at 1 mg/ml in 10 mM citrate + 10 mM phosphate at different pHs

pH	Onset (°C)	Tm1 (°C)	Tm2 (°C)	Tm3 (°C)	Tm4 (°C)
5	55	68.2	68.86	75.56	81.18
6	58	69.04	70.47	75.24	82.04
7	59	69.52	70.94	74.44	82.06

Example 8: Formulation selection for Anti-DLL4/Anti-VEGF DVDs

[0213] Materials and Methods. The stability of anti-DLL4/anti-VEGF DVD-Ig h1A11.1-SL-Av protein was evaluated in the six formulations listed in Table 15. All formulations were prepared in 15 mM histidine buffer. Formulations F1 to F4 were prepared at 50 mg/mL protein concentration. In these formulations, the pH ranged from 5.5 to 6.0, polysorbate 80 concentration ranged from 0 to 0.05% w/v, sucrose concentration ranged from 0 to 7.5% w/v, and arginine concentration ranged from 0 to 1% w/v. Formulation F4 was prepared in 15 mM histidine buffer at pH 6.0 without any stabilizers and served as a study control for the 50 mg/mL liquid formulation stability assessment. In addition, two formulations were prepared at 25 mg/mL protein concentration at pH 6.0 (Formulations F5 and F6). The composition of polysorbate 80 and sucrose was slightly different in these two formulations; the concentration of polysorbate 80 ranged from 0.025% w/v to 0.03% w/v and the concentration of sucrose ranged from 3.8% w/v to 4% w/v. The Formulations F1 to F5 used material from an early preparation process while the F6 formulation was formulated with material from a more optimized process. The compositions of formulations F5 and F6 are very similar, but stability differences were observed between the two. As the compositions were prepared from different processes, this may be the cause of the observed stability differences.

Table 15. Formulation Composition Description

Formulation Identifier	anti-DLL4 / anti-VEGF DVD Concentration (mg/mL)	Buffer	pH	Polysorbate 80 (Tween 80) (% w/v)	Sucrose (% w/v)	Arginine (% w/v)
F1	50	15 mM Histidine	6.0	0.05	7.5	0
F2	50	15 mM Histidine	5.5	0.05	7.5	0
F3	50	15 mM Histidine	6.0	0.05	7.5	1

F4	50	15 mM Histidine	6.0	0	0	0
F5	25	15 mM Histidine	6.0	0.025	3.8	0
F6	25	15 mM Histidine	6.0	0.03	4.0	0

[0214] In the above formulations, 15 mM histidine buffer was selected because it provides adequate buffering capacity to maintain the target formulation pH. Sucrose was evaluated as a stabilizer against freeze-thaw stress (cryoprotectant) and lyophilization process-induced stress (lyoprotectant). Polysorbate 80 (surfactant) and arginine were added to potentially stabilize the formulation against aggregates and particulates formation.

[0215] The stability of liquid formulations was assessed during Freeze/thaw, and at -80, 5, 25 and 40°C by a broad panel of analytical assays including Visual appearance, % Aggregates by Size Exclusion Chromatography (SE-HPLC), Charge heterogeneity by Cation Exchange Chromatography (CEX-HPLC), Fragmentation by reduced SDS-Capillary Electrophoresis (CE-SDS), and Sub-visible particles by Micro Flow Imaging (MFI) or Light Obscuration (HIAC). These results are provided in Tables 16-19.

Table 16. Freeze-Thaw and Liquid Formulation Stability Results at -80°C

Formulation Identifier	Time (month)	Visual Appearance	% Aggregate by SE-HPLC	CEX-HPLC			% Purity (CE-SDS Reduced)	Sub-visible Particle Counts by MFI/HIAC			Binding Potency by ELISA	
				% Acidic region	% Main peak	% Basic region		≥2μm/mL	≥10μm/mL	≥25μm/mL	% DLL4	% VEGF
F1	0	EFVP	1.0	21.6	61.7	16.7	97.7	3333	5	0	93	113
	3FT	EFVP	1.1	21.5	61.8	16.6	97.7	2388	50	5	NP	NP
	1	EFVP	1.1	21.0	62.1	16.8	97.8	1364	15	5	NP	NP
	3	EFVP	1.1	21.0	62.3	16.6	97.6	714	20	0	NP	NP
F2	0	EFVP	1.3	21.4	61.8	16.8	97.5	1589	15	0	93	113
	3FT	EFVP	1.3	21.4	61.8	16.9	97.6	435	5	5	NP	NP
	1	EFVP	1.4	21.0	62.0	17.0	97.8	315	0	0	NP	NP
	3	EFVP	1.5	20.9	61.9	17.2	97.7	699	5	0	NP	NP
F3	0	EFVP	1.1	21.5	61.7	16.7	97.6	784	0	0	93	113
	3FT	EFVP	1.1	21.3	61.8	16.9	97.5	490	10	0	NP	NP
	1	EFVP	1.1	21.0	61.9	17.1	97.9	250	0	0	NP	NP
	3	EFVP	1.2	21.0	61.8	17.2	97.7	1219	35	0	NP	NP
F4	0	EFVP	1.2	21.6	61.8	16.6	97.4	23707	370	5	93	113
	3FT	TMTC	1.5	21.4	61.8	16.8	97.4	105467	5906	30	NP	NP
	1	TMTC	1.2	21.1	62.0	16.9	97.8	42024	1329	60	NP	NP
	3	TMTC	1.5	21.1	61.9	17.0	97.8	40065	3203	625	NP	NP
F5	0	EFVP	0.9	21.6	61.6	16.7	97.7	2808	5	5	93	113
	3FT	EFVP	1.2	21.5	61.8	16.8	97.6	1949	0	0	NP	NP
	1	EFVP	1.1	21.0	62.2	16.8	97.8	270	5	0	NP	NP
	3	EFVP	1.1	21.0	62.2	16.8	97.6	759	0	0	NP	NP
F6	0	EFVP	1.0	21.6	56.4	22.0	98.1	426	58	1	109	97

	3FT	EFVP	1.1	21.7	55.9	22.4	98.2	193	24	0	115	100
	1	EFVP	1.0	21.6	55.8	22.7	98.2	50	3	0	NP	NP
	3	EFVP	1.1	22.0	55.9	22.1	98.3	254	31	0	89	96

Key: EFVP: Essentially Free of Visible Particles, TMTC: Too Many To Count, NP: Not Performed

Table 17. Liquid Formulation Stability Results at 5°C

Formulation Identifier	Time (month)	Visual Appearance	% Aggregates by SE-HPLC	CEX-HPLC			% Purity (CE- SDS Reduced)	Sub-visible Particle Counts by MFI/HIAC			Binding Potency by *ELISA*	
				% Acidic region	% Main peak	% Basic region		≥2 µm/mL	≥10 µm/mL	≥25 µm/mL	% DLL4	% VEGF
F1	0	EFVP	1.0	21.6	61.7	16.7	97.7	3333	5	0	93	113
	1	EFVP	2.0	21.2	62.4	16.3	97.8	1064	0	0	NP	NP
	3	EFVP	3.0	21.6	62.5	15.9	97.6	3452	15	0	NP	NP
F2	0	EFVP	1.3	21.4	61.8	16.8	97.5	1589	15	0	93	113
	1	EFVP	2.1	20.9	62.3	16.8	97.8	230	5	5	NP	NP
	3	EFVP	3.0	21.1	62.5	16.3	97.8	1454	0	0	NP	NP
F3	0	EFVP	1.1	21.5	61.7	16.7	97.6	784	0	0	93	113
	1	EFVP	2.0	20.8	62.0	17.2	97.8	225	5	0	NP	NP
	3	EFVP	3.2	20.8	61.1	18.1	97.6	1369	5	0	NP	NP
F4	0	EFVP	1.2	21.6	61.8	16.6	97.4	23707	370	5	93	113
	1	EFVP	2.0	21.3	62.3	16.5	97.8	1189	0	0	NP	NP
	3	EFVP	3.3	21.6	62.6	15.8	97.8	6046	145	0	NP	NP
F5	0	EFVP	0.9	21.6	61.6	16.7	97.7	2808	5	5	93	113
	1	EFVP	1.5	21.2	61.9	16.8	97.8	709	10	0	NP	NP
	3	EFVP	2.2	21.4	62.4	16.1	97.6	3203	50	0	NP	NP
F6*	0	EFVP	1.0	21.6	57.0	21.5	98.1	426	58	1	109	97
	1	EFVP	1.1	21.6	55.9	22.5	98.1	2458	164	1	116	99
	3	EFVP	1.2	22.4	55.9	21.7	98.0	34	1	0	101	100

Key: EFVP: Essentially Free of Visible Particles, NP: Not Performed

Table 18. Liquid Formulation Stability Results at 25°C

Formulation Identifier	Time (month)	Visual Appearance	% Aggregates by SE-HPLC	CEX-HPLC			% Purity (CE- SDS Reduced)	Sub-visible Particle Counts by MFI/HIAC			Binding Potency by ELISA	
				% Acidic region	% Main peak	% Basic region		≥2μm/mL	≥10μm/mL	≥25μm/mL	% DLL4	% VEGF
F1	0	EFVP	1.0	21.6	61.7	16.7	97.7	3333	5	0	93	113
	1	EFVP	4.3	23.5	62.2	14.2	97.4	1559	5	5	NP	NP
	3	EFVP	6.7	29.2	57.0	13.9	96.3	9358	964	150	NP	NP
F2	0	EFVP	1.3	21.4	61.8	16.8	97.5	1589	15	0	93	113
	1	EFVP	4.4	22.8	61.6	15.6	97.4	1149	5	0	NP	NP
	3	EFVP	7.1	27.2	56.6	16.2	95.4	6170	95	0	NP	NP
F3	0	EFVP	1.1	21.5	61.7	16.7	97.6	784	0	0	93	113
	1	EFVP	4.9	21.5	58.3	20.2	97.3	834	10	0	NP	NP
	3	EFVP	9.3	24.7	52.2	23.0	96.4	3677	150	10	NP	NP
F4	0	EFVP	1.2	21.6	61.8	16.6	97.4	23707	370	5	93	113
	1	EFVP	4.5	23.5	61.8	14.7	97.2	89299	3053	165	NP	NP
	3	EFVP	7.5	28.6	57.4	14.1	96.6	10527	1279	275	NP	NP
F5	0	EFVP	0.9	21.6	61.6	16.7	97.7	2808	5	5	93	113
	1	EFVP	2.6	23.5	62.0	14.6	97.2	944	15	0	NP	NP
	3	EFVP	3.9	29.4	57.7	12.9	96.2	13575	1259	225	NP	NP
F6	0	EFVP	1.0	21.6	57.0	21.5	98.1	426	58	1	109	97
	1	EFVP	1.2	23.5	54.8	21.7	97.7	386	50	0	100	96

	3	EFVP	1.6	28.8	52.5	18.7	96.2	40	1	0	94	100

Key: EFVP: Essentially Free of Visible Particles, NP: Not Performed

Table 19. Liquid Formulation Stability Results at 40°C

Formulation Identifier	Time (month)	Visual Appearance	% Aggregates by SE-HPLC	CEX-HPLC			% Purity (CE- SDS Reduced)	Sub-visible Particle Counts by MFI/HIAC			Binding Potency by ELISA	
				% Acidic region	% Main peak	% Basic region		≥2µm/mL	≥10µm/mL	≥25µm/mL	% DLL4	% VEGF
F1	0	EFVP	1.0	21.6	61.7	16.7	97.7	3333	5	0	93	113
	1	EFVP	7.2	35.8	43.0	21.2	95.0	1219	15	0	94	104
	3	EFVP	12.8	57.0	23.0	20.0	85.9	21464	635	30	73	72
F2	0	EFVP	1.3	21.4	61.8	16.8	97.5	1589	15	0	93	113
	1	EFVP	7.9	33.8	40.9	25.3	95.1	655	5	0	95	97
	3	EFVP	13.3	52.8	22.7	24.5	86.7	6041	90	0	68	73
F3	0	EFVP	1.1	21.5	61.7	16.7	97.6	784	0	0	93	113
	1	EFVP	11.3	32.1	42.3	25.6	95.0	1464	5	0	97	101
	3	EFVP	18.1	48.6	25.2	26.2	86.3	9103	165	15	81	72
F4	0	EFVP	1.2	21.6	61.8	16.6	97.4	23707	370	5	93	113
	1	EFVP	7.7	34.9	44.2	20.9	94.8	61754	5051	670	101	97
	3	EFVP	13.5	52.8	25.9	21.4	86.3	14000	1729	480	73	76
F5	0	EFVP	0.9	21.6	61.6	16.7	97.7	2808	5	5	93	113

	1	EFVP	3.9	37.0	44.4	18.6	95.0	974	20	0	92	95
	3	EFVP	7.4	59.6	24.5	15.9	87.0	11836	610	55	68	69
F6	0	EFVP	1.0	21.6	56.4	22.0	98.1	426	58	1	109	97
	1	EFVP	1.9	35.0	42.8	22.2	94.5	60	0	0	96	95

Key: EFVP: Essentially Free of Visible Particles

[0216] Lyophilized Formulation Stability Testing. The stability of select formulations containing binding protein h1A11.1-SL-Av was also evaluated after the formulations were lyophilized. The lyophilized drug product stability was assessed for all sucrose-containing formulations (F1, F2, F3, F5 and F6). Stability was assessed after 2 weeks storage at 55°C. Stability was tested by a broad panel of analytical assays including Visual appearance (before and after reconstitution), Reconstitution time, % Aggregates by Size Exclusion Chromatography (SE-HPLC), Charge heterogeneity by Cation Exchange Chromatography (CEX-HPLC), Fragmentation by reduced SDS-Capillary Electrophoresis (CE-SDS), Sub-visible particles by Micro Flow Imaging (MFI) or light obscuration (HIAC), and Water Content by Karl Fischer titration.

[0217] The lyophilized formulation stability testing results are provided in Table 20. Reconstitution time for all evaluated formulations was approximately 1 to 2 minutes. A slight increase in aggregation by SEC and % basic region by CEX was observed for all formulations under the stressed storage condition of 55°C. Minimal changes were observed in all other measured product stability attributes.

Table 20. Lyophilized Formulation Stability Results at 55°C

Formulation Identifier	Time (month)	Visual Appearance		% Aggregates by SE-HPLC	CEX-HPLC			% Purity (CE-SDS Reduced)	Sub-visible Particle Counts by MFI/HIAC		
		Before Recon	After Recon		% Acidic region	% Main peak	% Basic region		≥2 µm/mL	≥10 µm/mL	≥25 µm/mL
F1	0	WTOWC	EFVP	1.1	21.3	61.9	16.8	97.6	749	20	10
	2weeks at 55°C	WTOWC	EFVP	1.6	20.6	58.1	21.3	97.5	1639	15	0
F2	0	WTOWC	EFVP	1.3	21.2	61.8	17.0	97.6	1254	15	0
	2weeks at 55°C	WTOWC	EFVP	2.0	20.4	57.8	21.8	97.6	1609	10	0
F3	0	WTOWC	EFVP	1.1	21.2	61.9	16.9	97.6	719	5	0
	2weeks at 55°C	WTOWC	EFVP	1.4	20.8	59.5	19.8	97.5	475	10	5
F5	0	WTOWC	EFVP	1.0	21.3	61.8	16.9	97.5	844	35	5
	2weeks at 55°C	WTOWC	EFVP	1.5	20.5	58.0	21.5	97.7	270	5	0
F6	0	WTOWC	EFVP	1.0	21.5	56.5	22.0	98.2	205	7	0
	2weeks at 55°C	WTOWC	EFVP	1.5	21.1	53.4	25.5	98.2	126	5	1

Key: WTOWC: White to off-white cake, EFVP: Essentially Free of Visible Particle

[0218] Stability of formulation F6 (lyophilized, 200 binding protein h1A11.1-SL-Av per vial) formulation F6 was also evaluated over a 12 month period. The formulation was stable over the testing period.

[0219] Dose Solution Stability Testing. The anti-DLL4/anti-VEGF binding protein is tested for intravenous administration (IV). Prior to administration, the lyophilized drug product is reconstituted with sterile water for injection (SWFI). Subsequently, the reconstituted product is diluted in a solution that is suitable for IV infusion. The final concentration to which the dose solution is diluted is determined based on the clinical dose administered.

[0220] A study was conducted to evaluate the stability of the diluted anti-DLL4/anti-VEGF binding protein h1A11.1-SL-Av when in dose solutions containing one of two commonly used IV diluents, 0.9% saline and 5% dextrose (D5W). The protein concentrations evaluated were 0.5 and 1 mg/mL. To assess the dose solution stability and compatibility with the infusion components, the dose solutions per test condition were prepared and stored in IV bags (n=2) for 6 hours at RT/RL, and subsequently, a mock infusion study was performed using the commonly used infusion administration components, including an in-line filter. Test samples were pulled directly from the bag after preparing dose solutions in IV bags (T0). In addition, samples collected at the end of the 30 minutes infusion process were tested. The samples were tested by a panel of analytical assays including Visual appearance, % Aggregates by Size Exclusion Chromatography (SE-HPLC) and Sub-visible particles by Light obscuration (HIAC).

[0221] The dose solution stability studies results are provided in Table 21. The aggregate level in the starting material was 0.7%. After the dose solutions were prepared in saline, a clear trend indicating increase in aggregate level upon dilution in saline (T0) and at the end of infusion was observed. In addition, sub-visible particle counts in these samples were high. In comparison, the dose solutions prepared in 5% dextrose (D5W) showed consistently lower % aggregate levels with acceptable stability trends with respect to particulates.

Table 21. Clinical In-Use Stability Results

Diluent	Dose Solution Conc. (mg/mL)	Assays→ / Time Point↓	Visual Appearance	% Aggregates by SE-HPLC	Sub-visible Particle Counts by HIAC		
					≥ 2 µm/mL	≥ 10 µm/mL	≥ 25 µm/mL
0.9% Saline	0.5 mg/mL (Bag #1)	T0	EFVP	2.7	2139	86	8
		Post pump infusion	EFVP	4.7	4398	63	1
	0.5 mg/mL (Bag #2)	T0	EFVP	2.7	2729	189	12
		Post pump infusion	EFVP	3.5	1879	31	2
	1.0 mg/mL (Bag #1)	T0	EFVP	2.4	1774	85	3
		Post pump infusion	EFVP	3.0	2300	24	0
	1.0 mg/mL (Bag #2)	T0	EFVP	1.8	914	23	0
		Post pump infusion	EFVP	2.5	3056	42	2
5% Dextrose	0.5 mg/mL (Bag #1)	T0	EFVP	0.6	1679	31	0
		Post pump infusion	EFVP	0.6	7	0	0
	0.5 mg/mL (Bag #2)	T0	EFVP	0.6	2944	135	0
		Post pump infusion	EFVP	0.6	3	0	0
	1 mg/mL (Bag #1)	T0	EFVP	0.6	1652	23	0
		Post pump infusion	EFVP	0.6	2	0	0
	1 mg/mL (Bag #2)	T0	EFVP	0.6	2105	38	0
		Post pump infusion	EFVP	0.6	6	0	0

EFVP: Essentially free of visible particles

Example 9: Extended preformulation characterization

[0222] Extended preformulation characterization on anti-DLL4/-antiVEGF DVDs was performed to explore how different formulations conditions impact the stability of the DVDs. Data for h1A11.1-LS-Av is presented in Tables 22 and 23. The storage stability (5°C) and accelerated stability (40°C) of the DVD was evaluated in the formulations and protein concentrations listed below. Stability was evaluated by size exclusion chromatography (SE-HPLC) and % aggregrate, % monomer, % fragment, and total species recovered were quantitated. Overall, the formulations cover a pH range of 5 to 7 and a protein concentration range of 10 to 50 mg/ml.

[0223] At 5°C and 40°C temperatures and at concentrations of 50, 30, and 10 mg/ml the following formulations were evaluated: 15 mM acetate pH 5, 15 mM histidine

pH 6, 15 mM phosphate pH 7, 30 mM acetate, 80 mg/ml sucrose, 0.02% Tween 80 at pH 5, 30 mM histidine, 80 mg/ml sucrose, 0.02% Tween 80 at pH 6, and PBS (phosphate buffered saline). All formulations contained 0.02% sodium azide to prevent microbial growth during storage.

[0224] Based on the data in Tables 22 and 23, h1A11.1-LS-Av satisfied the preformulation criteria for DVD-Ig stability.

Table 22. Accelerated stability at 40°C of h1A11.1-LS-Av

Protein conc (mg/ml)	time	temp (°C)	buffer	pH	% Aggregrate	% Monomer	% Fragment	Total Area
---	pre-dialysis	---	---	---	0.21	98.42	1.36	56054
50, 30, 10	T0	---	ace	5	0.28	98.41	1.31	56381
50, 30, 10	T0	---	his	6	0.46	98.23	1.31	54316
50, 30, 10	T0	---	phos	7	0.74	97.86	1.40	53212
50, 30, 10	T0	---	ace-suc-tw	5	0.24	98.16	1.60	56244
50, 30, 10	T0	---	his-suc-tw	6	0.30	98.11	1.59	54076
50, 30, 10	T0	---	PBS	7	0.52	98.05	1.43	50085
50	T7d	40	ace	5	1.63	96.74	1.63	55563
30	T7d	40	ace	5	1.13	97.24	1.62	55194
10	T7d	40	ace	5	0.84	97.49	1.67	55029
50	T7d	40	his	6	2.00	96.62	1.38	53566
30	T7d	40	his	6	1.17	97.46	1.38	52443
10	T7d	40	his	6	0.60	98.00	1.40	53812
50	T7d	40	phos	7	4.31	94.02	1.67	52934
30	T7d	40	phos	7	2.85	95.46	1.69	52663
10	T7d	40	phos	7	1.20	97.11	1.69	52411
50	T7d	40	ace-suc-tw	5	1.10	96.23	2.66	54837
30	T7d	40	ace-suc-tw	5	0.77	96.40	2.83	52474
10	T7d	40	ace-suc-tw	5	0.43	96.39	3.17	50855
50	T7d	40	his-suc-tw	6	1.69	96.27	2.05	53017
30	T7d	40	his-suc-tw	6	1.14	96.84	2.02	52153
10	T7d	40	his-suc-tw	6	0.59	97.30	2.11	52208
50	T7d	40	PBS	7	2.77	95.30	1.93	51623
30	T7d	40	PBS	7	1.73	96.28	1.99	49973
10	T7d	40	PBS	7	0.78	97.25	1.97	50851
50	T21d	40	ace	5	3.66	94.30	2.04	55920
30	T21d	40	ace	5	2.56	95.33	2.10	54188
10	T21d	40	ace	5	1.85	96.00	2.15	55213
50	T21d	40	his	6	4.14	94.28	1.58	54807
30	T21d	40	his	6	2.67	95.79	1.54	53071
10	T21d	40	his	6	1.59	96.82	1.58	54053
50	T21d	40	phos	7	8.52	89.32	2.16	53273
30	T21d	40	phos	7	5.58	92.54	1.89	53162
10	T21d	40	phos	7	3.01	94.89	2.10	52747
50	T21d	40	ace-suc-tw	5	4.12	93.78	2.10	56278

30	T21d	40	ace-suc-tw	5	2.93	94.94	2.13	55481
10	T21d	40	ace-suc-tw	5	1.99	95.75	2.26	54696
50	T21d	40	his-suc-tw	6	4.94	93.21	1.85	54034
30	T21d	40	his-suc-tw	6	*n/a*	*n/a*	*n/a*	*n/a*
10	T21d	40	his-suc-tw	6	2.00	96.30	1.70	52686
50	T21d	40	PBS	7	8.44	89.65	1.90	51697
30	T21d	40	PBS	7	5.54	92.43	2.03	50282
10	T21d	40	PBS	7	2.89	95.05	2.06	51580

Buffer key (all buffers contain 0.02% sodium azide to prevent microbial growth):**ace** = 15 mM acetate pH 5; **his** = 15 mM histidine pH 6; **phos** = 15 mM phosphate pH 7; **ace-suc-tw** = 30 mM acetate, 80 mg/ml sucrose, 0.02% Tween80; **his-suc-tw** = 30 mM histidine, 80 mg/ml sucrose, 0.02% Tween80; **PBS** = phosphate buffered saline

Table 23. Storage stability at 5°C of h1A11.1-LS-Av

Protein conc (mg/ml)	time	temp (°C)	buffer	pH	% Aggregrate	% Monomer	% Fragment	Total Area
---	pre-dialysis	---	---	---	0.21	98.42	1.36	56054
50, 30, 10	T0	---	ace	5	0.28	98.41	1.31	56381
50, 30, 10	T0	---	his	6	0.46	98.23	1.31	54316
50, 30, 10	T0	---	phos	7	0.74	97.86	1.40	53212
50, 30, 10	T0	---	ace-suc-tw	5	0.24	98.16	1.60	56244
50, 30, 10	T0	---	his-suc-tw	6	0.30	98.11	1.59	54076
50, 30, 10	T0	---	PBS	7	0.52	98.05	1.43	50085
50	T7d	5	ace	5	0.18	98.17	1.64	57599
30	T7d	5	ace	5	0.16	98.21	1.64	55889
10	T7d	5	ace	5	0.13	98.17	1.70	53289
50	T7d	5	his	6	0.18	98.14	1.68	55742
30	T7d	5	his	6	0.12	98.06	1.82	53603
10	T7d	5	his	6	0.13	98.07	1.80	53505
50	T7d	5	phos	7	0.23	97.72	2.05	54355
30	T7d	5	phos	7	0.18	97.77	2.04	53561
10	T7d	5	phos	7	0.13	97.72	2.15	53151
50	T7d	5	ace-suc-tw	5	0.09	97.40	2.51	57158
30	T7d	5	ace-suc-tw	5	0.08	97.43	2.49	55025
10	T7d	5	ace-suc-tw	5	0.08	97.34	2.58	53882
50	T7d	5	his-suc-tw	6	0.10	97.48	2.43	55272
30	T7d	5	his-suc-tw	6	0.08	97.63	2.29	52763
10	T7d	5	his-suc-tw	6	0.05	97.41	2.53	52903
50	T7d	5	PBS	7	0.12	97.31	2.58	51698
30	T7d	5	PBS	7	0.09	97.24	2.67	50144
10	T7d	5	PBS	7	0.08	97.28	2.64	50428
50	T21d	5	ace	5	0.87	98.45	0.68	57706
30	T21d	5	ace	5	0.80	98.55	0.65	56566
10	T21d	5	ace	5	0.83	98.47	0.70	54226
50	T21d	5	his	6	1.05	98.29	0.66	55911

30	T21d	5	his	6	0.92	98.40	0.68	54225
10	T21d	5	his	6	0.90	98.41	0.70	54128
50	T21d	5	phos	7	1.25	98.09	0.66	54980
30	T21d	5	phos	7	1.20	98.11	0.69	53903
10	T21d	5	phos	7	1.01	98.29	0.69	53271
50	T21d	5	ace-suc-tw	5	0.92	98.36	0.72	61574
30	T21d	5	ace-suc-tw	5	0.89	98.39	0.72	55532
10	T21d	5	ace-suc-tw	5	0.83	98.46	0.71	55841
50	T21d	5	his-suc-tw	6	1.00	98.27	0.73	55484
30	T21d	5	his-suc-tw	6	0.92	98.37	0.70	53335
10	T21d	5	his-suc-tw	6	0.82	98.49	0.69	53736
50	T21d	5	PBS	7	1.49	97.79	0.71	52405
30	T21d	5	PBS	7	1.29	98.02	0.70	51284
10	T21d	5	PBS	7	1.12	98.18	0.70	51377

The buffer key for Table 23 is the same as in Table 22.

Example 10: Effect of VEGF on the Neutralization Activity of Anti-DLL4/Anti-VEGF DVD in DLL4 Cellular Assay

[0225] To evaluate whether VEGF binding will affect the DLL4 neutralization potency of anti-DLL4/anti-VEGF DVDs, VEGF was included in the DLL4-Notch reporter assay as described in Example 3.3. Briefly, the HEK293G cells expressing human DLL4 were co-cultured with EA.hy926 Notch reporter cells for 24 hrs in the presence of h1A11.1-SL-Av DVD or the mixture of anti-DLL4 mAb (h1A11.1) and anti-VEGF mAb (Av) serially diluted from 300 nM. Recombinant human $VEGF_{165}$ (a physiologically relevant human splice isoform of VEGF) or a negative control protein (BSG2) was also included. DLL4 neutralization potency was determined by evaluating IC_{50} values, the concentration of antibody needed to achieve 50% reduction of DLL4-induced Notch activation. As shown in Table 24, the presence of 6 or 150 nM VEGF greatly increased the DLL4 neutralization potency of h1A11.1-SL-Av DVD. This increased potency is unique to the anti-DLL4/anti-VEGF DVD as the parental mAb mixture exhibited similar potency with or without VEGF included.

Table 24. VEGF Enhances the DLL4 Potency of Anti-DLL4/Anti-VEGF DVD but not the Anti-DLL4/Anti-VEGF Mixture

	IC50 (nM)		
	0nM VEGF	*6 nM VEGF*	*6 nM BSG2*
h1A11.1-SL-Av	12.50	0.61	16.76
h1A11.1 + Av mixture	8.64	9.97	9.55
	IC_{50} (nM)		

	0nM VEGF	150 nM VEGF	150 nM BSG2
h1A11.1-SL-Av	12.69	0.40	14.11
h1A11.1 + Av mixture	9.17	10.32	10.93

[0226] In another experiment, the monovalent Fab fragment of h1A11.1-SL-Av DVD was also evaluated in the DLL4 neutralization cellular assay. In contrast to the DVD Ig, the monovalent DVD Fab has weaker DLL4 neutralization potency. The presence of VEGF improved the potency of the DVD-Fab, but not to the degree as seen with the DVD-Ig (Table 25).

Table 25. Effect of VEGF on the DLL4 Neutralization Potency of Anti-DLL4/Anti-VEGF DVD Ig and DVD Fab

	IC_{50} (nM)		
	0nM VEGF	150 nM VEGF	150 nM BSG2
h1A11.1-SL-Av	13.46	0.41	16.55
h1A11.1-SL-Av Fab	> 40*	4.56	> 40*

* *Precise IC_{50} could not be determined due to inability to fully neutralize DLL4*

[0227] In another experiment, VEGF concentrations were serially titrated down and applied to the DLL4-Notch reporter assay described above. As shown in Table 26, VEGF can enhance the DLL4 neutralization activity of h1A11.1-SL-Av DVD at a concentration as low as 1.2 nM.

Table 26. VEGF Enhances the DLL4 Neutralization Potency of Anti-DLL4/Anti-VEGF DVD

		h1A11.1-SL-Av
	nM	IC_{50} *(nM)*
BSG2	150	11.0
VEGF	150	0.6
	30	0.7
	6	0.6
	1.2	1.1
	0.24	12.7
	0.048	12.3
	0.0096	11.5
	0	11.8

Example 11: *In vivo* combination efficacy of DLL4-VEGF DVD-Igs

[0228] The effect of anti-DLL4-VEGF DVD-Igs in combination with chemotherapy on tumor growth was evaluated on SW-48 human colon xenograft tumors in female SCID mice. Briefly, $5x10^6$ cells were inoculated subcutaneously into the right hind flank. Tumors were allowed to establish for 13 days, at which point tumor volume was determined using electronic caliper measurements using the formula: $L \times W^2/2$. Mice were allocated into treatment groups (n=10 per group) so that each cohort had equivalent mean tumor volume of 211 mm^3 prior to initiation of therapy. Animals were dosed with irinotecan, anti-VEGF mAb, and/or anti-DLL4-VEGF DVD-Ig at the dose and schedule in Table 27. Tumor volume was measured twice a week for the duration of the experiment. Results are shown in Table 27.

Table 27. Combination efficacy of anti-DLL4-VEGF DVD-Ig and irinotecan in the SW-48 colon xenograft model

Treatment	Dose Route, Regimen	%TGI[a]	%TGD[b]
Irinotecan	60 mg/kg IP, q3dX4	77***	106***
Anti-VEGF mAb	10 mg/kg IP, q7dX4	39*	50*
h1A11.1-SL-Av	13.3 mg/kg IP, q7dX4	72***	150***
Anti-VEGF mAb + Irinotecan	10 mg/kg IP, q7dX4 + 60 mg/kg IP, q3dX4	78***	150***
h1A11.1-SL-Av + Irinotecan	13.3 mg/kg IP, q7dX4 + 60 mg/kg IP, q3dX4	90***	228***

Table 27 key: a. %TGI = Percent tumor growth inhibition = 100 – (T/C x 100), where T = mean tumor volume of treatment group and C = mean tumor volume of treatment control group. Based on day 18 post size match measurements. P values (as indicated by asterisks) are derived from Student's T test comparison of treatment group vs. treatment control group. b. %TGD = Percent tumor growth delay = (T – C)/C x 100, where T = median time to endpoint of treatment group and C = median time to endpoint of treatment control group. Based on an endpoint of 1000 mm^3. *P* values (as indicated by asterisks) derived from Kaplan Meier log-rank comparison of treatment group vs. treatment control group: * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$. "q3dX4"

indicates administration every three days for four cycles (i.e., 4 doses), while "q7dX4" indicates administration every seven days for four cycles.

[0229] The effect of anti-DLL4-VEGF DVD-Igs in combination with chemotherapy on tumor growth was also evaluated on HCT-116 human colon xenograft tumors in female SCID mice. Briefly, $5x10^6$ cells were inoculated subcutaneously into the right hind flank. Tumors were allowed to establish for 14 days, at which point tumor volume was determined using electronic caliper measurements using the formula: $L \times W^2/2$. Mice were allocated into treatment groups (n=9 per group) so that each cohort had equivalent mean tumor volume of 192 mm^3 prior to initiation of therapy. Animals were dosed with 5-FU, leucovorin, irinotecan, anti-VEGF mAb, and/or anti-DLL4-VEGF DVD-Ig at the dose and schedule in Table 28. Tumor volume was measured twice a week for the duration of the experiment. Results are shown in Table 28.

Table 28. Combination efficacy of anti-DLL4-VEGF DVD-Ig and FOLFIRI in the HCT-116 colon xenograft model

Treatment	Dose Route, Regimen	%TGI[a]
5-FU Leucovorin Irinotecan (FOLFIRI)	50 mg/kg IV, q7dX3 25 mg/kg PO, q7dX3 30 mg/kg IV, q7dX3	63***
Anti-VEGF mAb	5 mg/kg IP, q7dX4	49***
hIA11.1-SL-Av	6.7 mg/kg IP, q7dX4	67***
Anti-VEGF mAb + FOLFIRI	5 mg/kg IP, q7dX4 + (above) q7dX3	81***
hIA11.1-SL-Av + FOLFIRI	6.7 mg/kg IP, q7dX4 + (above) q7dX3	90***

Table 28 key: a. %TGI = Percent tumor growth inhibition = 100 – (T/C x 100), where T = mean tumor volume of treatment group and C = mean tumor volume of treatment control group. Based on day 26 post size match measurements. P values (as indicated by asterisks) are derived from Student's T test comparison of treatment group vs. treatment control group: * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$. "q7dX3" indicates administration every seven days for three cycles (i.e., 3 doses), while "q7dX4" indicates administration every seven days for four cycles.

[0230] The effect of anti-DLL4-VEGF DVD-Igs in combination with chemotherapy on tumor growth was evaluated on HT-29 human colon xenograft

tumors in female SCID mice. Briefly, $2x10^6$ cells were inoculated subcutaneously into the right hind flank. Tumors were allowed to establish for 25 days, at which point tumor volume was determined using electronic caliper measurements using the formula: $L \times W^2/2$. Mice were allocated into treatment groups (n=10 per group) so that each cohort had equivalent mean tumor volume of 209 mm^3 prior to initiation of therapy. Animals were dosed with irinotecan and/or anti-DLL4-VEGF DVD-Ig, at the dose and schedule in Table 29. Tumor volume was measured twice a week for the duration of the experiment. Results are shown in Table 29.

Table 29. Combination efficacy of anti-DLL4-VEGF DVD-Ig and irinotecan in the HT-29 colon xenograft model

Treatment	Dose Route, Regimen	%TGI[a]	%TGD[b]
Irinotecan	60 mg/kg IP, q3dX4	47*	60*
h1A11.1-SL-Av	6.7 mg/kg IP, q7dX4	42*	45*
h1A11.1-SL-Av + Irinotecan	6.7 mg/kg IP, q7dX4 + 60 mg/kg IP, q3dX4	74**	76*

Table 29 key. a. %TGI = Percent tumor growth inhibition = 100 – (T/C x 100), where T = mean tumor volume of treatment group and C = mean tumor volume of treatment control group. Based on day 20 post size match measurements. P values (as indicated by asterisks) are derived from Student's T test comparison of treatment group vs. treatment control group. b. %TGD = Percent tumor growth delay = (T – C)/C x 100, where T = median time to endpoint of treatment group and C = median time to endpoint of treatment control group. Based on an endpoint of 1000 mm^3. P values (as indicated by asterisks) derived from Kaplan Meier log-rank comparison of treatment group vs. treatment control group: * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$. "q3dX4" indicates administration every three days for four cycles (i.e., 4 doses), while "q7dX4" indicates administration every seven days for four cycles.

[0231] The effect of anti-DLL4-VEGF DVD-Igs in combination with chemotherapy on tumor growth was evaluated on U87-MG human glioblastoma xenograft tumors in female SCID mice. Briefly, $3x10^6$ cells were inoculated subcutaneously into the right hind flank. Tumors were allowed to establish for 13 days, at which point tumor volume was determined using electronic caliper measurements

using the formula: L x W^2/2. Mice were allocated into treatment groups (n=10 per group) so that each cohort had equivalent mean tumor volume of 207 mm^3 prior to initiation of therapy. Animals were dosed with temozolomide, anti-VEGF mAb, and/or anti-DLL4-VEGF DVD-Ig at the dose and schedule in Table 30. Tumor volume was measured twice a week for the duration of the experiment. Results are shown in Table 30.

Table 30. Combination efficacy of anti-DLL4-VEGF DVD-Ig and temozolomide in the U87-MG glioblastoma xenograft model

Treatment	Dose Route, Regimen	%TGI[a]	%TGD[b]
Temozolomide	5 mg/kg IP, qdX1	65***	45***
Anti-VEGF mAb	5 mg/kg IP, q7dX4	47**	45**
h1A11.1-SL-Av	6.7 mg/kg IP, q7dX4	69***	100***
Anti-VEGF mAb + Temozolomide	5 mg/kg IP, q7dX4 + 5 mg/kg IP, qdX1	68***	89***
h1A11.1-SL-Av + Temozolomide	6.7 mg/kg IP, q7dX4 + 5 mg/kg IP, qdX1	78***	155***

Table 30 key. a. %TGI = Percent tumor growth inhibition = 100 – (T/C x 100), where T = mean tumor volume of treatment group and C = mean tumor volume of treatment control group. Based on day 19 post size match measurements. P values (as indicated by asterisks) are derived from Student's T test comparison of treatment group vs. treatment control group. b. %TGD = Percent tumor growth delay = (T – C)/C x 100, where T = median time to endpoint of treatment group and C = median time to endpoint of treatment control group. Based on an endpoint of 1000 mm^3. P values (as indicated by asterisks) derived from Kaplan Meier log-rank comparison of treatment group vs. treatment control group: * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$.

[0232] The effect of anti-DLL4-VEGF DVD-Igs in combination with chemotherapy on tumor growth was evaluated on PA0123 patient-derived human pancreatic xenograft tumors in female NSG mice. Briefly, frozen tumor fragments were implanted subcutaneously into the right hind flank. Tumors were allowed to establish for 28 days, at which point tumor volume was determined using electronic caliper measurements using the formula: L x W^2/2. Mice were allocated into treatment groups (n=7 per group) so that each cohort had equivalent mean tumor volume of 193 mm^3

prior to initiation of therapy. Animals were dosed with gemcitabine and/or anti-DLL4-VEGF DVD-Ig, at the dose and schedule in Table 31. Tumor volume was measured twice a week for the duration of the experiment. Results are shown in Table 31.

Table 31. Combination efficacy of anti-DLL4-VEGF DVD-Ig and gemcitabine in the PA0123 patient-derived pancreatic xenograft model

Treatment	Dose Route, Regimen	%TGI[a]	%TGD[b]
Gemcitabine	100 mg/kg IP, [q3dX4]X2	48**	43**
hIA11.1-SL-Av	13.3 mg/kg IP, q7dX5	54**	75**
hIA11.1-SL-Av + Gemcitabine	13.3 mg/kg IP, q7dX5 + 100 mg/kg IP, [q3dX4]X2	75***	114***

Table 31 key. a. %TGI = Percent tumor growth inhibition = 100 – (T/C x 100), where T = mean tumor volume of treatment group and C = mean tumor volume of treatment control group. Based on day 38 post size match measurements. *P values (as indicated by asterisks) are derived from Student's T test* comparison of treatment group vs. treatment control group. b. %TGD = Percent tumor growth delay = (T – C)/C x 100, where T = median time to endpoint of treatment group and C = median time to endpoint of treatment control group. Based on an endpoint of 1000 mm^3. P values (as indicated by asterisks) derived from Kaplan Meier log-rank comparison of treatment group vs. treatment control group: * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$.

[0233] The effect of anti-DLL4-VEGF DVD-Igs in combination with chemotherapy on tumor growth was evaluated on MDA-MB-231-luc human breast xenograft tumors in female SCID mice. Briefly, $2x10^6$ cells were implanted in the mammary fat pad. Tumors were allowed to establish for 13 days, at which point tumor volume was determined using electronic caliper measurements using the formula: $L \times W^2/2$. Mice were allocated into treatment groups (n=10 per group) so that each cohort had equivalent mean tumor volume of 150 mm^3 prior to initiation of therapy. Animals were dosed with paclitaxel and/or anti-DLL4-VEGF DVD-Ig, at the dose and schedule in Table 32. Tumor volume was measured twice a week for the duration of the experiment. Additionally, bioluminescent images were acquired to monitor and track spontaneous metastasis of cancer cell to the lung and/or lymph nodes Results are shown in Table 32.

Table 32. Combination efficacy of anti-DLL4-VEGF DVD-Ig and paclitaxel in the MDA-MB-231-luc breast xenograft model

Treatment	Dose Route, Regimen	%TGI[a]	%TGD[b]	%met incidence[c]
Paclitaxel	25 mg/kg IP, q4dX3	78***	106***	40*
h1A11.1-SL-Av	6.7 mg/kg IP, q7dX4	56***	85***	50*
h1A11.1-SL-Av + Paclitaxel	6.7 mg/kg IP, q7dX4 + 25 mg/kg IP, q4dX3	92***	179***	0***

Table 32 key. a. %TGI = Percent tumor growth inhibition = 100 – (T/C x 100), where T = mean tumor volume of treatment group and C = mean tumor volume of treatment control group. Based on day 15 post size match measurements. P values (as indicated by asterisks) are derived from Student's T test comparison of treatment group vs. treatment control group. b. %TGD = Percent tumor growth delay = (T – C)/C x 100, where T = median time to endpoint of treatment group and C = median time to endpoint of treatment control group. Based on an endpoint of 1000 mm^3. P values (as indicated by asterisks) derived from Kaplan Meier log-rank comparison of treatment group vs. treatment control group. c. % metastasis incidence = Percent of animals with detectable signal in the lung and/or lymph nodes based on bioluminescent imaging. Treatment control group had 100%. Based on day 22 post size match measurements. P values (as indicated by asterisks) are derived from Student's T test comparison of treatment group vs. treatment control group: * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$.

[0234] The effect of anti-DLL4-VEGF DVD-Igs in combination with chemotherapy on tumor growth was evaluated on SUM149PT human breast xenograft tumors in female SCID mice. Briefly, $1x10^6$ cells were inoculated subcutaneously into the right hind flank. Tumors were allowed to establish for 28 days, at which point tumor volume was determined using electronic caliper measurements using the formula: $L \times W^2/2$. Mice were allocated into treatment groups (n=9 per group) so that each cohort had equivalent mean tumor volume of 183 mm^3 prior to initiation of therapy. Animals were dosed with paclitaxel and/or anti-DLL4-VEGF DVD-Ig, at the dose and schedule in Table 33. Tumor volume was measured twice a week for the duration of the experiment. Results are shown in Table 33.

Table 33. Combination efficacy of anti-DLL4-VEGF DVD-Ig and paclitaxel in the SUM149PT breast xenograft model

Treatment	Dose Route, Regimen	%TGI[a]	%TGD[b]
Paclitaxel	25 mg/kg IP, [q4dX3]X4	60***	173***
h1A11.1-SL-Av	6.7 mg/kg IP, q7dX8	88***	282***
h1A11.1-SL-Av + Paclitaxel	6.7 mg/kg IP, q7dX8 + 25 mg/kg IP, [q4dX3]X4	93***	459***

Table 33 key. a. %TGI = Percent tumor growth inhibition = 100 – (T/C x 100), where T = mean tumor volume of treatment group and C = mean tumor volume of treatment control group. Based on day 22 post size match measurements. P values (as indicated by asterisks) are derived from Student's T test comparison of treatment group vs. treatment control group. b. %TGD = Percent tumor growth delay = (T – C)/C x 100, where T = median time to endpoint of treatment group and C = median time to endpoint of treatment control group. Based on an endpoint of 1000 mm^3. P values (as indicated by asterisks) derived from Kaplan Meier log-rank comparison of treatment group vs. treatment control group: * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$.

[0235] The effect of anti-DLL4-VEGF DVD-Igs in combination with chemotherapy on tumor growth was evaluated on SUM149PT human breast xenograft tumors in female SCID mice. Briefly, $1x10^6$ cells were inoculated subcutaneously into the right hind flank. Tumors were allowed to establish for 25 days, at which point tumor volume was determined using electronic caliper measurements using the formula: $L \times W^2/2$. Mice were allocated into treatment groups (n=10 per group) so that each cohort *had equivalent mean tumor volume of 228 mm^3 prior to initiation of therapy*. Animals were dosed with paclitaxel, anti-VEGF mAb and/or anti-DLL4-VEGF DVD-Ig, at the dose and schedule in **Table 34**. Tumor volume was measured twice a week for the duration of the experiment. Results are shown in **Table 34**.

Table 34. Combination efficacy of anti-DLL4-VEGF DVD-Ig and paclitaxel in the SUM149PT breast xenograft model

Treatment	Dose Route, Regimen	%TGI[a]
Paclitaxel	25 mg/kg IP, [q4dX3]X4	50*
Anti-VEGF mAb	5 mg/kg IP, q7dX8	54*
h1A11.1-SL-Av	6.7 mg/kg IP, q7dX8	78**
Anti-VEGF mAb + Paclitaxel	5 mg/kg IP, q7dX8 + 25 mg/kg IP, [q4dX3]X4	81**
h1A11.1-SL-Av + Paclitaxel	6.7 mg/kg IP, q7dX8 + 25 mg/kg IP, [q4dX3]X4	87**

Table 34 key: a. %TGI = Percent tumor growth inhibition = 100 – (T/C x 100), where T = mean tumor volume of treatment group and C = mean tumor volume of treatment control group. *Based on day 18 post size match measurements.* P values (as indicated by asterisks) are derived from Student's T test comparison of treatment group vs. treatment control group: * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$.

[0236] The effect of anti-DLL4-VEGF DVD-Igs in combination with chemotherapy on tumor growth was further evaluated on HCT-116 human colon xenograft tumors in female SCID mice. Briefly, $5x10^6$ cells were inoculated subcutaneously into the right hind flank. Tumors were allowed to establish for 16 days, at which point tumor volume was determined using electronic caliper measurements using the formula: $L \times W^2/2$. Mice were allocated into treatment groups (n=9 per group) so that each cohort had equivalent mean tumor volume of 198 mm^3 prior to initiation of therapy. Animals were dosed with 5-FU, capecitabine and/or anti-DLL4-VEGF DVD-Ig at the dose and schedule in **Table 35**. Tumor volume measured twice a week for the duration of the experiment. Results are shown in **Table 35**.

Table 35. Combination efficacy of anti-DLL4-VEGF DVD-Ig and 5-FU or anti-DLL4-VEGF DVD-Ig and capecitabine in the HCT-116 colon xenograft model.

Treatment	Dose Route, Regimen	%TGI[a]
5-FU	75 mg/kg IV, q7dX4	50**
Capecitabine	350 mg/kg PO, qdX14	69***
h1A11.1-SL-Av	6.7 mg/kg IP, q7dX4	66***
h1A11.1-SL-Av + 5-FU	6.7 mg/kg IP, q7dX4 + 75 mg/kg IV, q7dX4	84***
h1A11.1-SL-Av + Capecitabine	6.7 mg/kg IP, q7dX4 + 350 mg/kg PO, qdX14	92***

Table 35 key: a. %TGI = Percent tumor growth inhibition = 100 – (T/C x 100), where T = mean tumor volume of treatment group and C = mean tumor volume of treatment control group. Based on day 21 post size match measurements. P values (as indicated by asterisks) are derived from Student's T test comparison of treatment group vs. treatment control group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Example 12: Cross-Species Cellular Potency of h1A11.1-SL-Av DVD

[0237] The cross-species neutralizing potency of h1A11.1-SL-Av DVD against DLL4 was compared in a cell-based assay using immobilized recombinant DLL4 extracellular domain (ECD) across species. This assay was utilized to compare the activity of h1A11.1-SL-Av DVD against the same concentration of DLL4 derived from human, rat, mouse, and cynomolgus monkey species.

[0238] Black 96-well tissue culture plates with clear bottom wells (Costar, #3904) were pre-coated with either 100 µL/well of DLL4-ECD (11 nM) diluted in PBS (Invitrogen, #14190), or 100 µL/well of 11 nM BSA as control (Sigma, #A9576). The assay plates were sealed and incubated at 4°C for 18 hours. On the following day, h1A11.1-SL-AV DVD was serially diluted in assay media DMEM (Invitrogen, #11995) containing 10% FBS. The pre-coated assay plates were rinsed once with assay media before adding 50 µL/well of the serially diluted h1A11.1-SL-Av DVD solution. The assay plates were then incubated at 25°C for 30 minutes. During this incubation, EA.hy926 cells expressing a control renilla luciferase and a firefly luciferase driven by a Notch-responsive promoter were detached from the culture flask using 0.25% Trypsin-EDTA (Invitrogen, #25200), resuspended to $3x10^5$ cells/mL in assay media, and then

added directly to the assay plates at 25 µL cells/well. The assay plates were then incubated at 37°C with 5% CO_2 for 24 hours. Firefly and renilla luciferase assays were performed according to the vendor's protocol (Dual-Glo Luciferase Assay System, Promega, #E2940). Luminescence was read on a plate reader (Victor, Perkin Elmer, #1420-051), and data generated was normalized to the renilla luciferase luminescence signal.

[0239] As shown in Table 36, h1A11.1-SL-Av DVD inhibits human and cynomolgus monkey DLL4 activity with comparable potencies. h1A11.1-SL-Av DVD also inhibits mouse DLL4, albeit with about 3-fold less activity, compared to human DLL4. h1A11.1-SL-Av DVD does not inhibit rat DLL4 activity in the cellular assays.

[0240] The cross-species neutralizing potency of h1A11.1-SL-AV DVD against VEGF was evaluated in a VEGF-stimulated NIH3T3/VEGFR-2 cell proliferation and viability assay.

[0241] NIH3T3 cells stably transfected with the cDNA for full-length human VEGFR-2 was used for the VEGF-stimulated proliferation and survival assays. A confluent culture of NIH3T3/VEGFR-2 stable cell line was detached from the culture flask with 0.25% Trypsin-EDTA (Invitrogen, #25200), and resuspended to $1.33x10^5$ cells/mL with assay media: DMEM (Invitrogen, #11995) containing 0.1% BSA (Sigma, #A9576). Cells were seeded at 10,000 cells/well in 75 µL total volume into black 96-well tissue culture assay plates with clear bottoms (Costar, #3904), and incubated for 24 hours at 37°C with 5% CO_2. The next day, recombinant VEGF protein and h1A11.1-SL-AV DVD were diluted with assay media, mixed in a 96-well polypropylene plate and incubated at 25°C for 30 minutes. The VEGF and h1A11.1-SL-AV DVD cocktails (4X) were then added to cells on an assay plate at 25 µL/well. Assay plates containing treated cells were then incubated at 37°C with 5% CO_2. Seventy-two hours later, a viability assay was performed according to the vendor's protocol (ATPlite 1step, Perkin Elmer, #6016739). Luminescence was read on a plate reader (Victor, Perkin Elmer, #1420-051).

[0242] As shown in Table 36, h1A11.1-SL-AV DVD neutralized cynomolgus monkey VEGF activity with similar potency compared to human VEGF. h1A11.1-SL-AV DVD does not inhibit mouse or rat VEGF in cellular assay.

Table 36: Cross-Species Cellular Potency of h1A11.1-SL-AV DVD

	Potency Assay, IC_{50} (nM)[a]
Human DLL4	0.22 ± 0.14
Cynomolgus monkey DLL4	0.12 ± 0.08
Mouse DLL4	0.65 ± 0.23
Rat DLL4	Undetectable
Human VEGF	0.9 ± 0.1
Cynomolgus monkey VEGF	1.1 ± 0.3
Mouse VEGF	Undetectable
Rat VEGF	Undetectable

Table 36 key: a. Values from DLL4 cellular assay are averages ± standard deviations from four independent experiments. Values from VEGF cellular assay are averages ± standard deviations from three independent experiments.

Example 13: Screening Funnel and Lead Candidate Selection

[0243] Selection of a lead DVD-Ig candidate can require several cycles of *molecular engineering, generating hundreds of potential candidate molecules, followed* by testing in a screening funnel that can include a battery of assays for identifying a DVD-Ig molecule with the properties required for a therapeutic agent, all without guidance regarding the variable domain sequences or other structures that will provide optimal functional properties. Typically these properties include, but are not limited to, an evaluation of: (a) the binding kinetics (on-rate, off-rate and affinity) for both the inner and outer antigen-binding domains, (b) potencies in various biochemical and cellular bioassays, (c) in vivo efficacies in relevant tumor models, (d) pharmacokinetic and pharmacodynamics properties, (e) manufacturability, including protein expression level in selected cell lines, scalability, post-translational modification, physicochemical properties such as monomer percentage, solubility, and stability (intrinsic, freeze/thaw, storage stability, etc.), (f) formulation properties, (g) potential immunogenicity risk, and (h) toxicological properties of a molecule. Binding mode and valency may also be evaluated, as these can affect binding properties and cellular potencies of a molecule. Threshold levels are set for each evaluated parameter, and the binding proteins exhibiting superior levels for each parameter are noted. From the initial screening of hundreds of molecules, often only one or none emerge with properties that satisfy all of the listed parameters, among other factors taken into consideration when identifying a lead candidate for further evaluation. Once a lead candidate is identified, further in vivo

evaluation of therapeutic properties are examined, included safety, efficacy, and potency in animal and human subjects.

Example 14: Anti-DLL4 / Anti-VEGF DVD-Ig Lead Candidate Selection

[0244] The inventors have constructed and evaluated approximately 100 anti-DLL4/anti-VEGF DVD-Ig molecules, including constructs using the VEGF and DLL4 sequences listed in Table 2, as well as CDR-grafted and affinity matured versions of those variable domains and additional humanized and fully human variable domain sequences. Fully human parental anti-DLL4 mAbs were derived by PROfusion mRNA display technology followed by affinity-maturation using yeast display technology. CDR-regrafted parental antibodies were based on anti-DLL4 mAb E9.71, several humanized anti-DLL4 mAbs were prepared based on h1A11.1 and h38H12.11, and several affinity-matured h1A11 antibodies were also produced. The inventors also evaluated numerous different linker sequences in the DVD-Ig binding proteins. The use of different linkers resulted in widely differing properties, even between binding proteins having the same variable domain sequences.

[0245] Out of the approximately 100 DVD-Ig molecules generated, 10 of them were excluded for not achieving a desired level of expression in HEK293 cells by transient transfection, 22 did not exhibit a desired threshold level of binding to DLL4, 19 did not exhibit a desired threshold level of binding to VEGF, several exhibited suboptimal posttranslational modification, including O-linked glycosylation. Several DVD-Igs were excluded for exhibiting binding affinity below a defined threshold. Antigen-binding domains at the inner position of a DVD-Ig may exhibit a reduced level of antigen-binding affinity and potency. For instance, all the DVD-Ig molecules of the h1A11/Av series where the VEGF binding domain is placed at the outer position and the DLL4 binding domain at the inner position exhibited reduced DLL4 binding affinity. These DVD-Igs were therefore not selected for further study. In the opposite orientation (i.e., VEGF binding domain at the inner position and DLL4 binding domain at the outer position), the VEGF binding affinity of h1A11.1-SL-Av was found to be unexpectedly superior, even to binding proteins using the same variable domains and orientations but with other linker sequences, such as h1A11.1-SS-Av (short linkers between the VD1 and VD2 domains on both the heavy and light chains).

[0246] Additionally, affinity maturation was conducted on h1A11 to generate anti-DLL4 parental antibodies with very high affinities and stabilities when in monoclonal antibody form, but in DVD-Ig form these constructs did not exhibit the level of stability observed for h1A11.1-SL-Av, regardless of the linkers used or orientations

of the binding domains. Similarly, affinity maturation of Av (anti-VEGF) variable domains again generated stable parental monoclonal antibodies exhibiting reduced levels of stability regardless linker and orientation manipulation. In addition, affinity maturation, even where it produces superior binding kinetics for monoclonal antibodies, may not predict the activity of the subsequent DVD-Ig. For example, when h1A11.1 was replaced by its affinity-matured variants, these new DVD-Ig molecules displayed reduced stability, shorter in vivo half-life, and decreased anti-tumor activities in tumor models. In addition, DVD-Ig molecules containing affinity-matured h1A11 and/or Av variable domains also exhibited an increased tendency to gel or form high levels of aggregates during storage at 5°C. This undesirable gelling and aggregation was not observed for DVD-Ig molecules comprising the original parental antibody variable domains (i.e., variable domains from antibodies that were not affinity matured) when stored under similar conditions, particularly for DVD-Ig h1A11.1-SL-Av.

[0247] This indicates that antigen binding affinity alone is not the only factor to consider when selecting a clinical candidate and indeed binding affinity may not predict other binding protein properties, such as stability, formulatability, physicochemical properties, in vivo anti-tumor potency and/or pharmacokinetic properties, which are sensitive to even slight structural modifications. For instance, the VEGF and DLL4 neutralization potencies of h1A11.1-LS-Av, h1A11.1-LL-Av and h1A11.1-SL-Av DVD-Ig molecules were broadly comparable (Table 7), but h1A11.1-SL-Av exhibited the highest level of tumor growth inhibitory potency (Table 9). In addition, for the DVD-Ig molecules that retained binding affinities and cellular potencies, some of them displayed less favorable physicochemical properties. For example, although h1A11.1-LL-Av is very similar to the therapeutic lead h1A11.1-SL-Av, the difference between the linkers which connects the two variable domains in the two constructs results in less favorable physicochemical properties for h1A11.1-LL-Av. This demonstrates that even small changes, e.g., to the linker between the two variable domains in a DVD-Ig can have a significant impact on the evaluated therapeutic properties.

[0248] Based on the presence of certain desirable features, as measured in the assays described above, 17 of the approximately 100 or more DVD-Ig molecules were further tested in mouse models for anti-tumor activity and pharmacokinetic properties. DVD-Ig h1A11.1-SL-Av exhibited unexpectedly superior tumor-inhibitory potency and pharmacokinetic properties compared to the other tested binding proteins. Thus, evaluating the exclusion criteria used in the screening funnel, DVD-Ig h1A11.1-SL-Av eventually emerged as a binding protein exhibiting favorable (i.e.,

above threshold) properties in each of the measured parameters. The binding protein was therefore selected for further evaluation and study.

Example 15: Comparison to Earlier VEGF/DLL4 DVD-Igs

[0249] Although h1A11.1-SL-Av was not directly compared to the 96 prior VEGF/DLL4 DVD-Ig molecules published in US Patent Application Publication No. 20100076178 (see Table 5 in that application), an indirect comparison can be made. For instance, h1A11.1-SL-Av exhibited VEGF-binding activity comparable to that of the reference anti-VEGF mAb Av (see Table 5). In contrast, at least two thirds of the 96 VEGF/DLL4 DVD-Ig molecules tested in US Patent Application Publication No. 20100076178 had weaker VEGF-binding activity than the reference anti-VEGF mAb AB014. (AB014 contains the same variable domain sequences as in antibody Av). Accordingly, h1A11.1-SL-Av appears to exhibit improved binding kinetics over a majority of the DVD-Ig constructs tested in the earlier application.

[0250] The preceding examples are intended to illustrate and in no way limit the present disclosure. Other embodiments of the disclosed devices and methods will be apparent to those skilled in the art from consideration of the specification and practice of the devices and methods disclosed herein

Incorporation by Reference

[0251] The contents of all cited references (including literature references, patents, patent applications, and websites) that maybe cited throughout this application are hereby expressly incorporated by reference in their entirety for any purpose, as are the references cited therein. The disclosure will employ, unless otherwise indicated, conventional techniques of immunology, molecular biology and cell biology, which are well known in the art.

[0252] The present disclosure also incorporates by reference in their entirety techniques well known in the field of molecular biology and drug delivery. These techniques include, but are not limited to, techniques described in the following publications:

Ausubel et al. (eds.), CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley &Sons, NY (1993);
Ausubel, F.M. et al. eds., SHORT PROTOCOLS IN MOLECULAR BIOLOGY (4th Ed. 1999) John Wiley & Sons, NY. (ISBN 0-471-32938-X);
CONTROLLED DRUG BIOAVAILABILITY, DRUG PRODUCT DESIGN AND PERFORMANCE, Smolen and Ball (eds.), Wiley, New York (1984);

Giege, R. and Ducruix, A. Barrett, CRYSTALLIZATION OF NUCLEIC ACIDS AND PROTEINS, a Practical Approach, 2nd ea., pp. 20 1-16, Oxford University Press, New York, New York, (1999);

Goodson, in MEDICAL APPLICATIONS OF CONTROLLED RELEASE, vol. 2, pp. 115-138 (1984);

Hammerling, et al., in: MONOCLONAL ANTIBODIES AND T-CELL HYBRIDOMAS 563-681 (Elsevier, N.Y., 1981;

Harlow et al. , ANTIBODIES: A LABORATORY MANUAL, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988);

Kabat et al., SEQUENCES OF PROTEINS OF IMMUNOLOGICAL INTEREST (National Institutes of Health, Bethesda, Md. (1987) and (1991);

Kabat, E.A., *et al.* (1991) SEQUENCES OF PROTEINS OF IMMUNOLOGICAL INTEREST, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242;

Kontermann and Dubel eds., ANTIBODY ENGINEERING (2001) Springer-Verlag. New York. 790 pp. (ISBN 3-540-41354-5).

Kriegler, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY (1990);

Lu and Weiner eds., CLONING AND EXPRESSION VECTORS FOR GENE FUNCTION ANALYSIS (2001) BioTechniques Press. Westborough, MA. 298 pp. (ISBN 1-881299-21-X).

MEDICAL APPLICATIONS OF CONTROLLED RELEASE, Langer and Wise (eds.), CRC Pres., Boca Raton, Fla. (1974);

Old, R.W. & S.B. Primrose, PRINCIPLES OF GENE MANIPULATION: AN INTRODUCTION TO GENETIC ENGINEERING (3d Ed. 1985) Blackwell Scientific Publications, Boston. Studies in Microbiology; V.2:409 pp. (ISBN 0-632-01318-4).

Sambrook, J. et al. eds., MOLECULAR CLONING: A LABORATORY MANUAL (2d Ed. 1989) Cold Spring Harbor Laboratory Press, NY. Vols. 1-3. (ISBN 0-87969-309-6).

SUSTAINED AND CONTROLLED RELEASE DRUG DELIVERY SYSTEMS, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978

Winnacker, E.L. FROM GENES TO CLONES: INTRODUCTION TO GENE TECHNOLOGY (1987) VCH Publishers, NY (translated by Horst Ibelgaufts). 634 pp. (ISBN 0-89573-614-4).

Equivalents

[0253] The disclosure may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The foregoing embodiments are therefore to be considered in all respects illustrative rather than limiting of the disclosure. Scope of the disclosure is thus indicated by the appended

claims rather than by the foregoing description, and all changes that come within the meaning and range of equivalency of the claims are therefore intended to be embraced herein.

CLAIMS

1. A binding protein comprising first and second polypeptide chains, each independently comprising VD1-(X1)n-VD2-C-(X2)n, wherein

VD1 is a first variable domain;

VD2 is a second variable domain;

C is a constant domain;

X1 is a linker;

X2 is an Fc region;

n is 0 or 1,

wherein the VD1 domains on the first and second polypeptide chains form a first functional target binding site and the VD2 domains on the first and second polypeptide chains form a second functional target binding site, and wherein the binding protein is capable of binding DLL4 and VEGF, wherein the first polypeptide chain of the binding protein comprises SEQ ID NO: 56 and the second polypeptide chain of the binding protein comprises SEQ ID NO: 64.

2. The binding protein of claim 1, wherein the binding protein comprises a first polypeptide chain comprising a first VD1-(X1)n-VD2-C-(X2)n, wherein

VD1 is a first heavy chain variable domain;

VD2 is a second heavy chain variable domain;

C is a heavy chain constant domain;

X1 is a linker;

X2 is an Fc region;

n is 0 or 1, and

wherein the binding protein comprises a second polypeptide chain comprising a second VD1-(X1)n-VD2-C-(X2)n, wherein

VD1 is a first light chain variable domain;

VD2 is a second light chain variable domain;

C is a light chain constant domain;

X1 is a linker;

n is 0 or 1 for (X1)n;

n is 0 for (X2)n,

wherein the VD1 domains on the first and second polypeptide chains form a first functional target binding site and the VD2 domains on the first and second polypeptide chains form a second functional target binding site.

3. The binding protein of claim 1 or 2, wherein the binding protein comprises two first and two second polypeptide chains and four functional target binding sites.

4. The binding protein of any one of claims 1-3, wherein the Fc region of the binding protein is an Fc region from an IgG1, IgG2, IgG3, IgG4, IgA, IgM, IgE, or IgD, or a variant thereof.

5. The binding protein of any one of claims 1-3, wherein the Fc region of the binding protein is a variant sequence Fc region.

6. The binding protein of any one of claims 1-3, wherein the binding protein comprises the constant region sequences of SEQ ID NO: 74 and/or SEQ ID NO: 73.

7. The binding protein of any one of claims 1-3, wherein the first and second polypeptide chains of the binding protein comprise SEQ ID NOs: 74 and 73.

8. The binding protein of any one of claims 1-7, wherein the binding protein is capable of:

(a) binding to VEGF with a dissociation constant (K_D) of at most about 7.0×10^{-10} M, as measured by surface plasmon resonance, and/or blocking VEGF activity with an IC50 of at most about 3.8 nM, as measured in a VEGFR1 Competition ELISA; and/or

(b) binding to DLL4 with a dissociation constant (K_D) of at most about 1.0×10^{-8} M, as measured by surface plasmon resonance, and/or blocking DLL4 activity with an IC50 of at most about 1.09 nM, as measured in a Notch Competition ELISA.

9. A binding protein conjugate comprising a binding protein of any one of claims 1-8, the binding protein conjugate further comprising an agent, wherein the agent is an immunoadhension molecule, an imaging agent, a therapeutic agent, or a cytotoxic agent.

10. The binding protein conjugate according to claim 9, wherein the imaging agent is a radiolabel, an enzyme, a fluorescent label, a luminescent label, a bioluminescent label, a magnetic label, or biotin.

11. The binding protein according to any one of claims 1-8, wherein the binding protein is a crystallized binding protein.

12. An isolated nucleic acid encoding the binding protein of any one of claims 1-8.

13. A vector comprising the isolated nucleic acid of claim 12.

14. The vector of claim 13 wherein said vector is selected from the group consisting of: pcDNA, pTT, pTT3, pEFBOS, pBV, pJV, pcDNA3.1 TOPO, pEF6, pHybE, TOPO, and pBJ.

15. A host cell comprising the vector according to claim 13.

16. The host cell according to claim 15, wherein said host cell is a prokaryotic cell.

17. The host cell according to claim 16, wherein said host cell is *Escherichia coli*.

18. The host cell according to claim 15, wherein said host cell is a eukaryotic cell.

19. The host cell according to claim 18, wherein said eukaryotic cell is selected from the group consisting of: a protist cell, an animal cell, a plant cell, and a fungal cell.

20. The host cell according to claim 18, wherein said eukaryotic cell is an animal cell selected from the group consisting of: a mammalian cell, an avian cell, and an insect cell.

21. The host cell according to claim 20, wherein said mammalian cell is a CHO cell.

22. The host cell according to claim 20, wherein said mammalian cell is a COS cell.

23. The host cell according to claim 19, wherein said fungal cell is *Saccharomyces cerevisiae*.

24. The host cell according to claim 20, wherein said insect cell is an Sf9 cell.

25. A method of producing a binding protein comprising culturing the host cell according to any one of claims 15-24 in culture medium under conditions sufficient to produce the binding protein.

26. A protein produced by the method of claim 25.

27. A pharmaceutical composition comprising the binding protein of any one of claims 1-8 and at least one additional agent.

28. The pharmaceutical composition of claim 27, wherein the additional agent comprises at least one of an immunoadhesion molecule, an imaging agent, a therapeutic agent, a cytotoxic agent, a radiolabel, an enzyme, a fluorescent label, a luminescent label, a bioluminescent label, a magnetic label, biotin, an anti-metabolite, an alkylating agent, an antibiotic, a growth factor, a cytokine, an anti-angiogenic agent, an anti-mitotic agent, an anthracycline, toxin, or an apoptotic

agent, a chemotherapeutic agent; an imaging agent, an angiogenesis inhibitor, a kinase inhibitor (including but not limited to a KDR and a TIE-2 inhibitor), a co-stimulation molecule modulator (including but not limited to anti-B7.1, anti-B7.2, CTLA4-Ig, anti-CD20), an adhesion molecule blocker (including but not limited to an anti-LFA-1 antibody, an anti-E/L selectin antibody, an anti-VEGF mAb; an anti-DLL4 mAb; a small molecule inhibitor), an anti-cytokine antibody or functional fragment thereof (including but not limited to an anti-IL-18, an anti-TNF, or an anti-IL-6/cytokine receptor antibody), methotrexate, cyclosporin, rapamycin, FK506, a detectable label or reporter, a TNF antagonist, an antirheumatic, a muscle relaxant, a narcotic, a non-steroid anti-inflammatory drug (NSAID), an analgesic, an anesthetic, a sedative, a local anesthetic, a neuromuscular blocker, an antimicrobial, an antipsoriatic, a corticosteriod, an anabolic steroid, an erythropoietin, an immunization, an immunoglobulin, an immunosuppressive, a growth hormone, a hormone replacement drug, a radiopharmaceutical, an antidepressant, an antipsychotic, a stimulant, an asthma medication, a beta agonist, an inhaled steroid, an epinephrine or analog, a cytokine, a cytokine antagonist, an anti-hypertensive agent, a diuretic, an adrenergic receptor antagonist, a calcium channel blocker, a renin inhibitor, an ACE inhibitor, an angiotensin II receptor antagonist, a vasodilator, an alpha-2 agonist, clonidine, methyldopa, hydralazine, prazosin, reserpine, moxonidine, guanfacine, perindopril/indapamide, lofexidine, metirosine, an anticoagulant, warfarin, heparin, low molecular weight heparin, dalteparin, argatroban, bivalirudin, lepirudin, and dextrose.

29. The pharmaceutical composition of claim 27, wherein the additional agent comprises one or more of a cytotoxic agent, a chemotherapeutic agent, an anti-angiogenic agent, an anti-CTLA4 antibody, a calcium channel blocker, an ACE inhibitor, FOLFIRI, paclitaxel, carboplatin, doxil, topotecan, and cisplatin.

30. Use of the binding protein of any one of claims 1-8, or the pharmaceutical composition of any one of claims 27-29, in the manufacture of a medicament for

treating a disease characterized by vascular overgrowth, edema, or aberrant DLL4 and/or VEGF expression or activity in a subject.

31. The use of claim 30, wherein the medicament further comprises at least one additional agent for treating a disease characterized by vascular overgrowth, edema, or aberrant DLL4 and/or VEGF expression or activity.

32. The use of claim 31, wherein the at least one additional agent comprises one or more of: irinotecan, leucovorin, 5-FU, temozolomide, capecitabine, gemcitabine, and paclitaxel.

33. The use of claim 31, wherein the at least one additional agent comprises one or more of: a cytotoxic agent, a chemotherapeutic agent, an anti-angiogenic agent, an anti-CTLA4 antibody, a calcium channel blocker, an ACE inhibitor, FOLFIRI, paclitaxel, carboplatin, doxil, topotecan, and cisplatin.

34. The use of any one of claims 30-33, wherein the disease is colorectal carcinoma, ovarian cancer, cervical cancer, breast cancer, lung cancer, or pancreatic cancer.

35. The use of any one of claims 30-33, wherein the disease is primary or metastatic cancer, breast cancer, colon cancer, rectum cancer, lung cancer, non-small cell lung cancer, adenocarcinoma, oropharynx cancer, hypopharynx cancer, esophageal cancer, stomach cancer, pancreatic cancer, liver cancer, gallbladder cancer, bile duct cancer, small intestine cancer, urinary tract cancer, kidney cancer, bladder cancer, urothelium cancer, female genital tract cancer, cervical cancer, uterus cancer, ovarian cancer, choriocarcinoma, gestational trophoblastic disease, male genital tract cancer, prostate cancer, seminal vesicles cancer, testical cancer, germ cell tumors, endocrine gland cancer, thyroid cancer, adrenal gland cancer, pituitary gland cancer, skin cancer, hemangiomas, melanomas, sarcomas, bone sarcoma, soft tissue sarcoma, Kaposi's sarcoma, tumors of the brain, tumors of the nerves, tumors of the eyes, tumors of the meninges, astrocytomas, glioma, gliobiastomas,

retinoblastomas, neuromas, neuroblastomas, Schwannomas, and meningiomas, solid tumors arising from hematopoietic malignancies, leukemia, lymphoma, Hodgkin's lymphoma, non-Hodgkin's lymphoma, stomach cancer, bladder cancer, prostate cancer, rectal cancer, hematopoietic malignancies, Abetalipoprotemia, acrocyanosis, acute leukemia, acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), B cell lymphoma, Burkitt's lymphoma, chronic myelocytic leukemia (CML), chronic lymphocytic leukemia (CLL), colorectal carcinoma, hairy cell leukemia, malignant lymphoma, malignant histiocytosis, malignant melanoma, multiple myeloma, pancreatic carcinoma, paraneoplastic syndrome, hypercalcemia of malignancy, sarcomas, solid tumors, macular degeneration, diabetes mellitus type 1, diabetic retinopathy, or atherosclerosis.

36. The use of any one of claims 30-33, wherein the disease is colon cancer and/or the medicament further comprises one or more of irinotecan, leucovorin, temozolomide, gemcitabine, paclitaxel, capecitabine, and 5-FU.

37. The use of any one of claims 30-33, wherein the disease is glioblastoma and/or the medicament further comprises temozolomide.

38. The use of any one of claims 30-33, wherein the disease is pancreatic cancer and/or the medicament further comprises gemcitabine.

39. The use of any one of claims 30-33, wherein the disease is breast cancer and/or the medicament further comprises paclitaxel.

40. The use of any one of claims 30-39, wherein the medicament further comprises:

one or more amino acid,

one or more polysaccharide and/or polysorbate, and

the binding protein at a concentration of 0.1-100 mg/ml,

wherein the pharmaceutical composition is at a pH of 5.0-7.0.

41. The use of claim 40, wherein the one or more amino acid comprises histidine and is present at a concentration of 10-20 mM.

42. The use of claim 40 or 41, wherein the one or more polysaccharide comprises sucrose and is present at a concentration of 0-8.0% weight/volume (w/v).

43. The use of any one of claims 40-42, wherein the polysorbate is polysorbate 80 and is at a concentration of 0-0.06% w/v.

44. The use of any one of claims 40-43, wherein the one or more amino acid comprises arginine and is present at a concentration of 0-1.5% w/v.

45. The use of any one of claims 40-44, wherein the binding protein is present at a concentration of 0.1- 25 mg/ml.

46. The use of claim 40, wherein the composition comprises 15 mM histidine, 0.03% (w/v) polysorbate 80, 4% (w/v) sucrose, and 1-25 mg/ml of the binding protein, and is at a pH of 6.

47. A method of detecting the presence of at least one of VEGF or DLL4, or a fragment thereof, in a test sample by an immunoassay,

wherein the immunoassay comprises contacting the test sample with at least one binding protein and at least one detectable label, wherein the at least one binding protein comprises the binding protein of any one of claims 1-8.

48. The method according to claim 47, wherein the at least one detectable label comprises a radiolabel, an enzyme, a fluorescent label, a luminescent label, a bioluminescent label, a magnetic label, or biotin.

49. The method according to claim 47, wherein the at least one detectable label is on the at least one binding protein.

50. The method according to claim 47, further comprising:

(i) contacting the test sample with the at least one binding protein, wherein the binding protein binds to an epitope on VEGF or DLL4 or fragment thereof in the test sample so as to form a first complex;

(ii) contacting the first complex with at least one detectable label to form a second complex, wherein the detectable label binds to the binding protein or to an epitope on VEGF or DLL4 or fragment thereof in the test sample that is not bound by the binding protein; and

(iii) detecting the presence of VEGF or DLL4 or fragment thereof in the test sample based on the signal generated by the detectable label in the second complex, wherein the presence of VEGF or DLL4 or fragment thereof is directly correlated with the signal generated by the detectable label.

51. The method according to any one of claims 47-50, wherein the test sample is from a patient and the method further comprises diagnosing, prognosticating, or assessing the efficacy of therapeutic or prophylactic treatment of the patient, and wherein if the method further comprises assessing the efficacy of therapeutic or prophylactic treatment of the patient, the method optionally further comprises modifying the therapeutic or prophylactic treatment of the patient as needed to improve efficacy.

52. The method according to any one of claims 47-51, wherein the method is adapted for use in an automated system or a semi-automated system.

53. The method according to any one of claims 47-52, wherein the method determines the amount or concentration of DLL4 and VEGF in the sample.

54. A kit for use in assaying a test sample for the presence, amount, or concentration of DLL4, VEGF, or both, the kit comprising

(a) instructions for assaying the test sample for VEGF and/or DLL4 or fragment(s) thereof; and

(b) at least one binding protein comprising the binding protein of any one of claims 1-8.

55. A pharmaceutical composition comprising the binding protein of any one of claims 1-8, further comprising:

one or more amino acid,

one or more polysaccharide and/or polysorbate, and

the binding protein at a concentration of 0.1-100 mg/ml,

wherein the pharmaceutical composition is at a pH of 5.0-7.0.

56. The pharmaceutical composition of claim 55, wherein the one or more amino acid comprises histidine and is present at a concentration of 10-20 mM.

57. The pharmaceutical composition of claim 55 or 56, wherein the one or more polysaccharide comprises sucrose and is present at a concentration of 0-8.0% weight/volume (w/v).

58. The pharmaceutical composition of any one of claims 55-57, wherein the polysorbate is polysorbate 80 and is at a concentration of 0-0.06% w/v.

59. The pharmaceutical composition of any one of claims 55-58, wherein the one or more amino acid comprises arginine and is present at a concentration of 0-1.5% w/v.

60. The pharmaceutical composition of any one of claims 55-59, wherein the binding protein is present at a concentration of 0.1- 25 mg/ml.

61. The pharmaceutical composition of claim 55, wherein the composition comprises 15 mM histidine, 0.03% (w/v) polysorbate 80, 4% (w/v) sucrose, and 1-25 mg/ml of the binding protein, and is at a pH of 6.