IMMUNO IMAGING AGENT FOR USE WITH ANTIBODY-DRUG CONJUGATE THERAPY

FIELD OF THE INVENTION

The invention relates to a companion diagnostic antibody-like binding protein and methods of use.

BACKGROUND OF THE INVENTION

There are a number of challenges in developing drugs for therapeutic use in a patient population. Many initial agents do not reach the market for any number of reasons (*e.g.*, lack of clinical benefit or lack of proven safety in a patient population). Some critical issues for the development of effective and safe drugs are: (1) establishing that a drug interacts with its intended molecular target in a patient population; (2) establishing that the specific target is relevant to treating the particular disease in the same patient population; and (3) identifying a biologically optimal dose based on the drug's effect on the target in the same patient population. The development of an appropriate companion diagnostic for a particular drug helps significantly in developing a drug for use in a clinical patient population.

A companion diagnostic coupled with molecular imaging provides a powerful, non-invasive 4-dimensional assessment of a molecular target and its interaction with drug molecules *in vivo*. Some examples of imaging platforms includes: magnetic resonance imaging (MRI); positron emission tomography (PET); single photon emission tomography (SPECT); optical imaging, computed tomography (CT); ultrasound, X-ray or photoacoustic imaging. Selection of an imaging contrast takes into account the specific molecular probe and intrinsic tissue characteristics. The coupling of a companion diagnostic with molecular imaging allows for a better understanding of a particular drug's interaction with its molecular target in patients, which can help in identifying potential responders to the specific drug and aid in the understanding and determination of the mechanisms of sensitivity and resistance of the drug (and its optimal dosing).

SUMMARY OF THE INVENTION

A companion diagnostic antibody-like binding protein based on the humanized monoclonal antibody, DS6, was developed for use as a diagnostic tool for *in vivo* detection and quantification of the tumor-associated MUC1-sialoglycotope, CA6, by an imaging technique, such as positron emission tomography (PET) bio-imaging. The antibody-like binding protein facilitates patient stratification and early evaluation of the therapeutic efficacy of the humanized DS6 antibody-drug immunoconjugate, huDS6-DM4, which consists of a humanized monoclonal antibody against the tumor-associated MUC1-sialoglycotope, CA6, conjugated to the cytotoxic maytansinoid, DM4.

The companion diagnostic antibody-like binding protein can be used to identify patients and patient subpopulations with high CA6 expression who may preferentially respond to treatment with a drug that targets tissues (*e.g.*, tumor tissues) having high CA6 expression, such as an antibody drug conjugate (ADC) that targets the CA6 epitope. The ADC can be, for example, an huDS6 molecule attached to a cytotoxic agent, such as a maytansine derivative, such as DM1 or DM4, or another agent such as described in the International Publication No. WO 2005/009369. The companion diagnostic antibody-like binding protein can be administered prior to the administration of the ADC, such as to determine whether the subject is likely to respond to treatment with the ADC.

The companion diagnostic antibody-like binding protein can be used for CA6 detection *in vivo* by a bio-imaging technique, such as positron emission tomography (PET), and allows for short exposure times and fast imaging, such as within 48 to 24 hours or less, the companion diagnostic is a highly specific binder with appropriate clearance kinetics.

The invention as disclosed herein provides an antibody-like binding protein that specifically binds CA6, wherein the antibody-like binding protein further comprises a chelator. The antibody-like binding protein can be used as a diagnostic tool for *in vivo* detection and quantification of the human CA6 tumor antigen by a bio-imaging technique, such as PET, wherein the antibody-like binding protein is conjugated to a suitable chelator for coupling to the bio-imaging tracer.

The invention as disclosed herein further provides an antibody-like binding protein that specifically binds CA6, wherein the antibody-like binding protein further

comprises a chelator. The antibody-like binding protein can be used as an imaging or detection agent for tissue expressing the CA6 tumor antigen, wherein the antibody-like binding protein is conjugated to a suitable chelator for coupling to an agent.

The invention as disclosed herein further provides a method of making a DS6 antibody-like binding protein comprising respective V_L and V_H DNA sequences from humanized DS6 antibody arranged in tandem and separated by a linker sequence. The DS6 V_L-Linker-DS6 V_L and DS6 V_H-Linker-DS6 V_H constructs are fused on DNA level at the 3´end to human constant domain sequences, according to formulas [I] and [II] below:

$$V_{L1}\text{-}L_1\text{-}V_{L2}\text{-}C_L \qquad [I]$$

$$V_{H1}\text{-}L_2\text{-}V_{H2}\text{-}C_{H1} \qquad [II]$$

wherein:

V_{L1} is a DS6-based immunoglobulin light chain variable domain;

V_{L2} is a DS6-based immunoglobulin light chain variable domain;

V_{H1} is a DS6-based immunoglobulin heavy chain variable domain;

V_{H2} is a DS6-based immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain, such as a human IGKC immunoglobulin light chain constant domain;

C_{H1} is an immunoglobulin C_{H1} heavy chain constant domain, such as a human immunoglobulin C_{H1} heavy chain constant domain;

L_1 and L_2 are amino acid linkers; and

wherein the polypeptides of V_{L1} and V_{L2} are the same or different DS6-based immunoglobulin light chain variable domain, and the polypeptides of V_{H1} and V_{H2} are the same or different DS6-based immunoglobulin heavy chain variable domain; and wherein the polypeptides of formula I and the polypeptides of formula II form a bivalent monospecific tandem immunoglobulin antibody-like binding protein. The C_L and C_{H1} domains may be joined, such as by a disulfide bond. Furthermore, the polypeptides of formula I and formula II may comprise an optional tag (*e.g.*, polyhistidine or 6x-His tag).

The invention as disclosed herein further provides a method of treating a disorder, such as a cancer in a patient, comprising the steps of obtaining the expression level of CA6 in the patient wherein an antibody-like binding protein that specifically binds CA6, wherein the antibody-like binding protein further comprises a

chelator, is administered to the patient, and making a determination as to whether tissues are expressing CA6; and administering huDS6-DM4 to the patient if the patient has a CA6 expression level that is at least 10% greater than a CA6 expression level in a reference tissue, *e.g.*, 10%, 20%, 30%, 40% or greater than CA6 expression in a reference tissue. In some embodiments, the antibody-like binding protein featured in the invention is used to determine the level of CA6 expression in a tumor tissue such as in a serous ovarian carcinoma, endometriod ovarian carcinoma, neoplasm of the uterine cervix, neoplasm of the endometrius, neoplasm of the vulva, breast carcinoma, pancreatic tumor, and tumor of the urothelium.

The invention as disclosed herein further provides a method of determining whether a cancer patient is a candidate for treatment with huDS6-DM4, the method comprising the steps of obtaining the expression level of CA6 in the patient wherein an antibody-like binding protein that specifically binds CA6, wherein the antibody-like binding protein further comprises a chelator is administered to the patient, and making a determination as to whether the CA6 expression level is at least 10% greater than a CA6 expression level in a reference tissue, *e.g.*, 10%, 20%, 30%, 40% or greater than CA6 expression in a reference tissue; and providing an indication that the cancer patient is a candidate for treatment with huDS6-DM4 when the determination is made in the affirmative.

The invention as disclosed herein further provides a method of monitoring a cancer patient's response to treatment with huDS6-DM4, the method comprising the steps of obtaining the expression level of CA6 in the patient, administering to the patient an antibody-like binding protein that specifically binds CA6, wherein the antibody-like binding protein further comprises a chelator, before and during treatment with huDS6-DM4, making a determination as to whether the CA6 expression level is increased, the same, or decreased as a result of treatment with huDS6-DM4; and providing an indication that treatment should be continued if the level of CA6 expression in the patient has decreased as compared to the level of CA6 expression before treatment with the huDS6-DM4.

The invention provides an antibody-like binding protein that specifically binds CA6, wherein the antibody-like binding protein comprises two polypeptides having structures represented by the formulas [I] and [II] below:

V_{L1}-L_1-V_{L2}-C_L [I]

V_{H1}-L_2-V_{H2}-C_{H1} [II]

wherein:

V_{L1} is a DS6-based immunoglobulin light chain variable domain;

V_{L2} is a DS6-based immunoglobulin light chain variable domain;

V_{H1} is a DS6-based immunoglobulin heavy chain variable domain;

V_{H2} is a DS6-based immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain, such as a human IGKC immunoglobulin light chain constant domain;

C_{H1} is an immunoglobulin C_{H1} heavy chain constant domain, such as a human immunoglobulin C_{H1} heavy chain constant domain;

L_1 and L_2 are amino acid linkers;

wherein the polypeptides of formula I and the polypeptides of formula II form a bivalent monospecific tandem immunoglobulin antibody-like binding protein; and

wherein the antibody-like protein further comprises a chelator.

The invention also provides a method for making an antibody-like binding protein that specifically binds CA6, wherein the antibody-like binding protein further comprises a chelator, comprising:

(a) expressing in a cell one or more nucleic acid molecules encoding polypeptides having structures represented by the formulas [I] and [II] below:

V_{L1}-L_1-V_{L2}-C_L [I]

V_{H1}-L_2-V_{H2}-C_{H1} [II]

wherein:

V_{L1} is a DS6-based immunoglobulin light chain variable domain;

V_{L2} is a DS6-based immunoglobulin light chain variable domain;

V_{H1} is a DS6-based immunoglobulin heavy chain variable domain;

V_{H2} is a DS6-based immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain, such as a human IGKC immunoglobulin light chain constant domain;

C_{H1} is an immunoglobulin C_{H1} heavy chain constant domain, such as a human immunoglobulin C_{H1} heavy chain constant domain;

L_1 and L_2 are amino acid linkers; and

wherein the polypeptides of formula I and the polypeptides of formula II form a bivalent monospecific tandem immunoglobulin antibody-like binding protein; and

(b) attaching the antibody-like binding protein to the chelator.

The invention further provides a method for treating diseased tissue expressing CA6 in a patient comprising administering an antibody-like binding protein that specifically binds CA6 and further comprises a chelator and an imaging agent to the patient; identifying the diseased tissue expressing CA6 in the patient by positron emission tomography (PET) imaging; and determining that the patient is a candidate for treatment with huDS6-DM4 if the CA6 expression in the diseased tissue is greater than or equal to 10% of CA6 expression in a reference standard.

The invention further provides a method of treating CA6 positive cancer in a patient, comprising the steps of acquiring information regarding expression level of CA6 in the patient; and administering huDS6-DM4 to the patient if the expression level of CA6 in the patient is greater than or equal to 10% of CA6 expression in a reference standard.

The invention further provides a method of determining whether a cancer patient is a candidate for treatment with huDS6-DM4, comprising the steps of acquiring information regarding expression level of CA6 in the patient; and determining that the patient is a candidate for treatment with huDS6-DM4 if the expression level of CA6 in the patient is greater than or equal to 10% of CA6 expression in a reference standard.

The invention further provides a method of monitoring a cancer patient's response to treatment with huDS6-DM4, comprising the steps of acquiring information regarding expression level of CA6 in the patient following administration of the huDS6-DM4; and providing an indication that treatment should be continued if

the level of CA6 expression in the patient has decreased as compared to the level of CA6 expression before treatment with the huDS6-DM4.

The invention provides an antibody-like binding protein that specifically binds CA6, wherein the antibody-like binding protein comprises two polypeptides having structures represented by the formulas [I] and [II] below:

$$V_{L1}\text{-}L_1\text{-}V_{L2}\text{-}C_L \qquad [I]$$

$$V_{H1}\text{-}L_2\text{-}V_{H2}\text{-}C_{H1} \qquad [II]$$

wherein:

V_{L1} is a DS6-based immunoglobulin light chain variable domain;

V_{L2} is a DS6-based immunoglobulin light chain variable domain;

V_{H1} is a DS6-based immunoglobulin heavy chain variable domain;

V_{H2} is a DS6-based immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain, such as a human IGKC immunoglobulin light chain constant domain;

C_{H1} is an immunoglobulin C_{H1} heavy chain constant domain, such as a human immunoglobulin C_{H1} heavy chain constant domain;

L_1 and L_2 are amino acid linkers; and

wherein the polypeptides of formula I and the polypeptides of formula II form a bivalent monospecific tandem immunoglobulin antibody-like binding protein.

The invention also provides a method for making an antibody-like binding protein that specifically binds CA6, comprising expressing in a cell one or more nucleic acid molecules encoding polypeptides having structures represented by the formulas [I] and [II] below:

$$V_{L1}\text{-}L_1\text{-}V_{L2}\text{-}C_L \qquad [I]$$

$$V_{H1}\text{-}L_2\text{-}V_{H2}\text{-}C_{H1} \qquad [II]$$

wherein:

V_{L1} is a DS6-based immunoglobulin light chain variable domain;

V_{L2} is a DS6-based immunoglobulin light chain variable domain;

V_{H1} is a DS6-based immunoglobulin heavy chain variable domain;

V_{H2} is a DS6-based immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain, such as a human IGKC immunoglobulin light chain constant domain;

C_{H1} is an immunoglobulin C_{H1} heavy chain constant domain, such as a human immunoglobulin C_{H1} heavy chain constant domain;

L_1 and L_2 are amino acid linkers; and

wherein the polypeptides of formula I and the polypeptides of formula II form a bivalent monospecific tandem immunoglobulin antibody-like binding protein.

Specific embodiments of the invention will become evident from the following more detailed description of certain embodiments and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Schematic representation of the bivalent engineered antibody-like binding protein, a bivalent monospecific tandem immunoglobulin fragment (B-Fab). Examples of B-Fab proteins include: B-Fab with G4SG4S linker (SEQ ID NO: 3) and C-terminal 6x-His tag, B-Fab with G4SG4S linker (SEQ ID NO: 3) without C-terminal 6x-His tag, and B-Fab with G4S linker and C-terminal 6x-His tag.

Figure 2. Assessment of the B-Fab DS6 based engineered antibody with GGGGSGGGGS ($(G4S)_2$) linker (SEQ ID NO: 3) and C-terminal 6x-His tag via gel electrophoresis (Figure 2A), size-exclusion chromatography (SEC) (Figure 2B), and Fluorescence Activated Cell Sorting (FACS) (Figure 2C and Figure 2D).

Figure 3. Assessment of the B-Fab DS6 based engineered antibody with $(G4S)_2$ linker via gel electrophoresis (Figure 3A), SEC (Figure 3B), and FACS (Figure 3C and Figure 3D).

Figure 4. Assessment of the B-Fab DS6 based engineered antibody with GGGGS (G4S) linker and C-terminal 6x-His tag via gel electrophoresis (Figure 4A), SEC (Figure 4B), and FACS (Figure 4C and Figure 4D).

Figure 5. Assessment of the B-Fab DS6 based engineered antibody via FACS using A2780 cells without DOTA (Figure 5A), WISH cells without DOTA (Figure 5B), and

WISH cells with DOTA (Figure 5C), demonstrates that DOTA conjugation does not alter DS6 binding.

Figure 6. Assessment of the ^{64}Cu-DOTA-DS6 B-Fab antibody-like binding protein demonstrates that radiolabelling with ^{64}Cu and DOTA conjugation allows for ideal radiochemistry (Figure 6A) and serum stability (Figure 6B).

Figure 7. Comparison between antibody-like binding proteins having a diabody scaffold (Figure 7A) and B-Fab scaffold (Figure 7B).

DETAILED DESCRIPTION OF THE INVENTION

The invention as disclosed herein relates to a companion diagnostic antibody-like binding protein based on the humanized monoclonal antibody, DS6, to be used as diagnostic tool for *in vivo* detection and quantification of the human CA6 tumor antigen by PET bio-imaging. The companion diagnostic antibody-like binding protein will facilitate patient stratification and early evaluation of the therapeutic efficacy of the DS6 antibody-drug immunoconjugate huDS6-DM4 consisting of a humanized monoclonal antibody against the tumor-associated MUC1-sialoglycotope, CA6, conjugated to the cytotoxic maytansinoid, DM4. The antibody-drug immunoconjugate, huDS6-DM4, is described, for example, in International Publication No. WO 2005/009369, incorporated herein by reference. The DS6 antibody and the CA6 tumor antigen are described, for example, in Kearse *et al.* (Int J Cancer. 2000 Dec 15;88(6):866-72), incorporated herein by reference.

HuDS6-DM4 is a first in class antibody-drug immunoconjugate consisting of a humanized monoclonal antibody (huDS6) against the tumor-associated MUC1-sialoglycotope, CA6, conjugated to the cytotoxic maytansinoid, DM4. Mucin proteins play an essential role in forming protective mucous barriers on epithelial surfaces, and are expressed on the apical surface of epithelial cells that line the mucosal surfaces of many different tissues including lung, breast, ovarian stomach and pancreas. MUC1 (mucin-1) is known to be overexpressed in a number of epithelial cancers, and CA6 overexpression is linked to aggressive behavior in human cancers and poor patient outcome. CA6 has limited distribution among normal adult tissues, thus making the DS6 antibody a promising agent for the selective treatment

CA6 positive tumors (*e.g.*, tumors of the ovaries, uterus, pancreas, breast or lung). Upon antibody/antigen binding and internalization, the huDS6-DM4 immunoconjugate releases DM4, which binds to tubulin and disrupts microtubule assembly/disassembly dynamics, resulting in mitotic arrest of CA6-expressing tumor cells. HuDS6-DM4 is currently undergoing a phase I clinical trial in patients diagnosed with CA6 antigen positive solid tumors.

To identify patients who show expression of the CA6 in tumors and therefore may respond to the treatment with huDS6-DM4, a companion diagnostic tool that binds CA6 with high affinity was developed. Furthermore, this companion diagnostic antibody-like binding protein acts as an early efficacy marker providing an adjunct tool to non-invasively monitor a patient's response to huDS6-DM4 therapy thereby facilitating patient stratification in clinical trials or clinical treatment (*i.e.,* a decrease in CA6 signal correlated to tumor shrinkage or target down-regulation coupled to insufficient efficacy). Such a companion diagnostic can reduce attrition and help deliver effective medicine to patients faster. The companion diagnostic antibody-like binding protein as disclosed herein can also be used for CA6 detection *in vivo* by PET bio-imaging. To allow for short exposure times and fast imaging, *e.g.*, within 48 to 24 hours or less, with high tumor to blood signals, a highly specific binder with appropriate clearance kinetics was needed.

Different novel antibody-like binding proteins derived from the DS6 antibody sequence were developed along with a suitable chelator and radiolabel in order to meet the required specifications for PET imaging (*e.g.*, better tumor penetration, faster clearance kinetics and excellent tumor to blood ratio). Based on pre-determined imaging figures of merit (IFOM), a ~70 kDa antibody fragment (a bivalent monospecific tandem immunoglobulin, termed B-Fab) was chosen for specificity evaluation and eventual clinical translation. The B-Fab format provided the advantages of: (1) high stability to heat stress (incubation at 42°C for one week); (2) high stability in human serum (incubation at 37°C for 24h); and (3) reasonable productivity in transient cell culture (~2mg/L). Compared to full-length antibodies (~150 kDa), antibody fragments (~50-75 kDa) show faster renal elimination rates and blood clearance, which results in higher signal to noise ratios during PET bio-imaging.

The B-Fab DS6 antibody-like binding protein contains two CA6 antigen

recognition sites based on the CA6 antigen recognition sites of the DS6 humanized monoclonal antibody and shows high binding affinity to the CA6 antigen. After conjugation with a chelator (*e.g.*, DOTA, NOTA, DTPA, TETA, or Df) the DS6 B-Fab was radiolabeled and used for *in vivo* PET bio-imaging experiments.

Standard recombinant DNA methodologies were used to construct the polynucleotides that encode the polypeptides which form the companion diagnostic antibody (based on the DS6 antibody) of the invention, incorporate these polynucleotides into recombinant expression vectors, and introduce such vectors into host cells. *See e.g.*, Sambrook *et al.*, 2001, MOLECULAR CLONING: A LABORATORY MANUAL (Cold Spring Harbor Laboratory Press, 3rd ed.). Enzymatic reactions and purification techniques may be performed according to manufacturer's specifications, as commonly accomplished in the art, or as described herein. Unless specific definitions are provided, the nomenclature utilized 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. Similarly, conventional techniques may be used for chemical syntheses, chemical analyses, pharmaceutical preparation, formulation, delivery, and treatment of patients.

1. General Definitions

As utilized in accordance with the present disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings. Unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

The term "polynucleotide" as used herein refers to single-stranded or double-stranded nucleic acid polymers of at least 10 nucleotides in length. In certain embodiments, the nucleotides comprising the polynucleotide can be ribonucleotides or deoxyribonucleotides or a modified form of either type of nucleotide. Such modifications include base modifications such as bromuridine, ribose modifications such as arabinoside and 2',3'-dideoxyribose, and internucleotide linkage modifications such as phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phoshoraniladate and phosphoroamidate. The term "polynucleotide" specifically includes single-stranded

and double-stranded forms of DNA.

An "isolated polynucleotide" is a polynucleotide of genomic, cDNA, or synthetic origin or some combination thereof, which by virtue of its origin the isolated polynucleotide: (1) is not associated with all or a portion of a polynucleotide in which the isolated polynucleotide is found in nature, (2) is linked to a polynucleotide to which it is not linked in nature, or (3) does not occur in nature as part of a larger sequence.

An "isolated polypeptide" is one that: (1) is free of at least some other polypeptides with which it would normally be found, (2) is essentially free of other polypeptides from the same source, *e.g.*, from the same species, (3) is expressed by a cell from a different species, (4) has been separated from at least about 50 percent of polynucleotides, lipids, carbohydrates, or other materials with which it is associated in nature, (5) is not associated (by covalent or noncovalent interaction) with portions of a polypeptide with which the "isolated polypeptide" is associated in nature, (6) is operably associated (by covalent or noncovalent interaction) with a polypeptide with which it is not associated in nature, or (7) does not occur in nature. Such an isolated polypeptide can be encoded by genomic DNA, cDNA, mRNA or other RNA, of synthetic origin, or any combination thereof. Preferably, the isolated polypeptide is substantially free from polypeptides or other contaminants that are found in its natural environment that would interfere with its use (therapeutic, diagnostic, prophylactic, research or otherwise).

As used herein, the term “antibody” encompasses chimeric, humanized, human and murine antibodies.

The term "human antibody" as used herein includes antibodies having variable and constant regions substantially corresponding to human germline immunoglobulin sequences. In some embodiments, human antibodies are produced in non-human mammals, including, but not limited to, rodents, such as mice and rats, and lagomorphs, such as rabbits. In other embodiments, human antibodies are produced in hybridoma cells. In still other embodiments, human antibodies are produced recombinantly.

Naturally occurring antibodies typically comprise a tetramer. Each such tetramer is typically composed of two identical pairs of polypeptide chains, each pair having one full-length "light" chain (typically having a molecular weight of about 25

kDa) and one full-length "heavy" chain (typically having a molecular weight of about 50-70 kDa). The terms "heavy chain" and "light chain" as used herein refer to any immunoglobulin polypeptide having sufficient variable domain sequence to confer specificity for a target antigen. The amino-terminal portion of each light and heavy chain typically includes a variable domain of about 100 to 110 or more amino acids that typically is responsible for antigen recognition. The carboxy-terminal portion of each chain typically defines a constant domain responsible for effector function. Thus, in a naturally occurring antibody, a full-length heavy chain immunoglobulin polypeptide includes a variable domain (V_H) and three constant domains (C_{H1}, C_{H2}, and C_{H3}), wherein the V_H domain is at the amino-terminus of the polypeptide and the C_{H3} domain is at the carboxyl-terminus, and a full-length light chain immunoglobulin polypeptide includes a variable domain (V_L) and a constant domain (C_L), wherein the V_L domain is at the amino-terminus of the polypeptide and the C_L domain is at the carboxyl-terminus.

Mammalian light chains, in particular human light chains, are typically classified as kappa and lambda light chains, and human heavy chains are typically classified as mu, delta, gamma, alpha, or epsilon, and define the antibody's isotype as IgM, IgD, IgG, IgA, and IgE, respectively. IgG has several subclasses, including, but not limited to IgG1, IgG2, IgG3, and IgG4. IgM has subclasses including, but not limited to, IgM1 and IgM2. IgA is similarly subdivided into subclasses including, but not limited to, IgA1 and IgA2. Within full-length light and heavy chains, the variable and constant domains typically are joined by a "J" region of about 12 or more amino acids, with the heavy chain also including a "D" region of about 10 more amino acids. *See, e.g.*, FUNDAMENTAL IMMUNOLOGY (Paul, W., ed., Raven Press, 2nd ed., 1989), which is incorporated by reference in its entirety for all purposes. The variable regions of each light/heavy chain pair typically form an antigen binding site. The variable domains of naturally occurring antibodies typically exhibit the same general structure of relatively conserved framework regions (FR) joined by three hypervariable regions, also called complementarity determining regions or CDRs. The CDRs from the two chains of each pair typically are aligned by the framework regions, which may enable binding to a specific epitope. From the amino-terminus to the carboxyl-terminus, both light and heavy chain variable domains typically comprise the domains FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4.

The term "native Fc" as used herein refers to a molecule comprising the sequence of a non-antigen-binding fragment resulting from digestion of an antibody or produced by other means, whether in monomeric or multimeric form, and can contain the hinge region. The original immunoglobulin source of the native Fc is preferably of human origin and can be any of the immunoglobulins, although IgG1 and IgG2 are preferred. Native Fc molecules are made up of monomeric polypeptides that can be linked into dimeric or multimeric forms by covalent (*i.e.*, disulfide bonds) and non-covalent association. The number of intermolecular disulfide bonds between monomeric subunits of native Fc molecules ranges from 1 to 4 depending on class (*e.g.*, IgG, IgA, and IgE) or subclass (*e.g.*, IgG1, IgG2, IgG3, IgA1, and IgGA2). One example of a native Fc is a disulfide-bonded dimer resulting from papain digestion of an IgG. The term "native Fc" as used herein is generic to the monomeric, dimeric, and multimeric forms.

The term "Fc variant" as used herein refers to a molecule or sequence that is modified from a native Fc but still comprises a binding site for the salvage receptor, FcRn (neonatal Fc receptor). Exemplary Fc variants, and their interaction with the salvage receptor, are known in the art. Thus, the term "Fc variant" can comprise a molecule or sequence that is humanized from a non-human native Fc. Furthermore, a native Fc comprises regions that can be removed because they provide structural features or biological activity that are not required for the antibody-like binding proteins of the invention. Thus, the term "Fc variant" comprises a molecule or sequence that lacks one or more native Fc sites or residues, or in which one or more Fc sites or residues has be modified, that affect or are involved in: (1) disulfide bond formation, (2) incompatibility with a selected host cell, (3) N-terminal heterogeneity upon expression in a selected host cell, (4) glycosylation, (5) interaction with complement, (6) binding to an Fc receptor other than a salvage receptor, or (7) antibody-dependent cellular cytotoxicity (ADCC).

The term "Fc domain" as used herein encompasses native Fc and Fc variants and sequences as defined above. As with Fc variants and native Fc molecules, the term "Fc domain" includes molecules in monomeric or multimeric form, whether digested from whole antibody or produced by other means.

The term "antibody-like binding protein" as used herein refers to a non-naturally occurring (or recombinant) engineered molecule that specifically binds to at

least one target antigen. It encompasses, e.g., F(ab) fragment, F(ab') fragments and diabodies.

In certain aspects, the "antibody-like binding protein" comprises the respective V_L and V_H DNA sequences from an antibody arranged in tandem and separated by a short amino acid linker sequence, according to formulas [I] and [II] below:

$$V_{L1}\text{-}L_1\text{-}V_{L2}\text{-}C_L \qquad [I]$$

$$V_{H1}\text{-}L_2\text{-}V_{H2}\text{-}C_{H1} \qquad [II]$$

wherein:

V_{L1} is an immunoglobulin light chain variable domain;

V_{L2} is an immunoglobulin light chain variable domain;

V_{H1} is an immunoglobulin heavy chain variable domain;

V_{H2} is an immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_{H1} is an immunoglobulin C_{H1} heavy chain constant domain;

L_1 and L_2 are amino acid linkers; and

wherein the polypeptides of formula I and the polypeptides of formula II form a Fab fragment (bivalent monospecific tandem immunoglobulin fragment).

In certain aspects, V_{L1} and V_{L2} are identical polypeptides, and in certain aspects V_{H1} and V_{H2} are identical polypeptides.

In certain aspects, each of L_1 and L_2 has a length of at least 5, 6, 7, 8, 9 or 10 amino acids.

In certain aspects, or typically, the C_L and C_{H1} domains are linked by a disulfide bond.

In certain aspects, each of L_1 and L_2 is either GGGGS or GGGGSGGGGS (SEQ ID NO: 3).

In certain aspects, the polypeptide of formula [I] comprises a sequence at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 1.

In certain aspects, the polypeptide of formula [II] comprises a sequence at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 7.

In certain aspects, said antibody-like binding protein further comprises at least one radiolabel, imaging agent, therapeutic agent, or diagnostic agent.

In certain aspects, said antibody-like binding protein has a specific activity of

at least 1, 1.1, 1.2, 1.3, 1.4 or 1.5 Ci/μMole.

In certain aspects, said antibody-like binding protein can have an apparent Kd of at most 8, 7, 6, 5 or 4 nM when the binding is measured by FACS, e.g. with a free antibody-like binding protein.

In certain aspects, said antibody-like binding protein presents a high stability to heat stress. More specifically, less than 25, 20, 15, 10 or 5% of soluble antibody-like binding protein can be lost after incubation at 42°C for one week.

In certain aspects, said antibody-like binding protein presents a high stability in human serum. More specifically, at least 70, 75, 80, 85, 90 or 95 % of the antibody-like binding protein can be monomeric after incubation at 37°C for 24 hours in human serum.

An "isolated" antibody-like binding protein is one that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would interfere with diagnostic or therapeutic uses for the antibody-like binding protein, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the antibody-like binding protein will be purified: (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody-like binding proteins include the antibody-like binding protein *in situ* within recombinant cells since at least one component of the antibody-like binding protein's natural environment will not be present.

The terms "substantially pure" or "substantially purified" as used herein refer to a compound or species that is the predominant species present (*i.e.*, on a molar basis it is more abundant than any other individual species in the composition). In some embodiments, a substantially purified fraction is a composition wherein the species comprises at least about 50% (on a molar basis) of all macromolecular species present. In other embodiments, a substantially pure composition will comprise more than about 80%, 85%, 90%, 95%, or 99% of all macromolar species present in the composition. In still other embodiments, the species is purified to essential

homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species.

The term "antigen" or "target antigen" as used herein refers to a molecule or a portion of a molecule that is capable of being bound by an antibody or antibody-like binding protein, and additionally is capable of being used in an animal to produce antibodies capable of binding to an epitope of that antigen. A target antigen may have one or more epitopes. With respect to each target antigen recognized by an antibody-like binding protein, the antibody-like binding protein is capable of competing with an intact antibody that recognizes the target antigen. A "bivalent" antibody-like binding protein, other than a "multispecific" or "multifunctional" antibody-like binding protein, is understood to comprise antigen binding sites having identical antigenic specificity.

A bispecific or bifunctional antibody typically is an artificial hybrid antibody having two different heavy chain/light chain pairs and two different binding sites or epitopes. Bispecific antibodies may be produced by a variety of methods including, but not limited to, fusion of hybridomas or linking of F(ab') fragments.

An F(ab) fragment, also referred to as Fab, typically includes one light chain and the V_H and C_{H1} domains of one heavy chain, wherein the V_H-C_{H1} heavy chain portion of the F(ab) fragment cannot form a disulfide bond with another heavy chain polypeptide. As used herein, an F(ab) fragment can also include one light chain containing two variable domains separated by an amino acid linker and one heavy chain containing two variable domains separated by an amino acid linker and a C_{H1} domain. An F(ab) fragment can also include disulfide bond between the C_L and C_{H1}.

An F(ab') fragment typically includes one light chain and a portion of one heavy chain that contains more of the constant region (between the C_{H1} and C_{H2} domains), such that an interchain disulfide bond can be formed between two heavy chains to form an $F(ab')_2$ molecule.

A “diabody” typically is a dimer of two single-chain variable fragments (scFv) that each consist of the heavy chain variable (V_H) and light chain variable (V_L) regions connected by a small peptide linker that is too short for the two variable regions to fold together, forcing scFvs to dimerize.

The phrases "biological property," "biological characteristic," and the term

"activity" in reference to an antibody-like binding protein of the invention are used interchangeably herein and include, but are not limited to, epitope affinity and specificity, ability to antagonize the activity of the antigen target (or targeted polypeptide), the *in vivo* stability of the antibody-like binding protein, and the immunogenic properties of the antibody-like binding protein. Other identifiable biological properties or characteristics of an antibody-like binding protein include, for example, cross-reactivity, (*i.e.*, with non-human homologs of the antigen target, or with other antigen targets or tissues, generally), and ability to preserve high expression levels of protein in mammalian cells. The aforementioned properties or characteristics can be observed or measured using art-recognized techniques including, but not limited to ELISA, competitive ELISA, surface plasmon resonance analysis, *in vitro* and *in vivo* neutralization assays, and immunohistochemistry with tissue sections from different sources including human, primate, or any other source as the need may be.

The term "immunologically functional immunoglobulin fragment" as used herein refers to a polypeptide fragment that contains at least the CDRs of the immunoglobulin heavy or light chains from which the polypeptide fragment was derived. An immunologically functional immunoglobulin fragment is capable of binding to a target antigen.

A "neutralizing" antibody-like binding protein as used herein refers to a molecule that is able to block or substantially reduce an effector function of a target antigen to which it binds. As used herein, "substantially reduce" means at least about 60%, preferably at least about 70%, more preferably at least about 75%, even more preferably at least about 80%, still more preferably at least about 85%, most preferably at least about 90% reduction of an effector function of the target antigen.

The term "epitope" includes any determinant, preferably a polypeptide determinant, capable of specifically 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 groups, or sulfonyl groups, and, in certain embodiments, may have specific three-dimensional structural characteristics and/or specific charge characteristics. An epitope is a region of an antigen that is bound by an antibody or antibody-like binding protein. In certain embodiments, an antibody-like binding protein is said to specifically bind an antigen

when it preferentially recognizes its target antigen in a complex mixture of proteins and/or macromolecules. In preferred embodiments, an antibody-like binding protein is said to specifically bind an antigen when the equilibrium dissociation constant is $\leq 10^{-8}$ M, more preferably when the equilibrium dissociation constant is $\leq 10^{-9}$ M, and most preferably when the dissociation constant is $\leq 10^{-10}$ M.

The dissociation constant (K_D) of an antibody-like binding protein can be determined, for example, by surface plasmon resonance. Generally, surface plasmon resonance analysis measures real-time binding interactions between ligand (a target antigen on a biosensor matrix) and analyte (an antibody-like binding protein in solution) by surface plasmon resonance (SPR) using the BIAcore system (Pharmacia Biosensor; Piscataway, NJ). Surface plasmon analysis can also be performed by immobilizing the analyte (antibody-like binding protein on a biosensor matrix) and presenting the ligand (target antigen). The term "K_D," as used herein refers to the dissociation constant of the interaction between a particular antibody-like binding protein and a target antigen.

The term "specifically binds" as used herein refers to the ability of an antibody-like binding protein or an antigen-binding fragment thereof to bind to an antigen containing an epitope with an Kd of at least about 1×10^{-6} M, 1×10^{-7} M, 1×10^{-8} M, 1×10^{-9} M, 1×10^{-10} M, 1×10^{-11} M, 1×10^{-12} M, or more, and/or to bind to an epitope with an affinity that is at least two-fold greater than its affinity for a nonspecific antigen.

The term "linker" as used herein refers to one or more amino acid residues inserted between immunoglobulin domains to provide sufficient mobility for the domains of the light and heavy chains to fold into cross over dual variable region immunoglobulins. Non-limiting examples include glycine/serine linkers, such as GGGGS or GGGGSGGGGS (SEQ ID NO: 3), and glycine/alanine linkers. A linker is inserted at the transition between variable domains or between variable and constant domains, respectively, at the sequence level. The transition between domains can be identified because the approximate sizes of the immunoglobulin domains are well understood. The precise location of a domain transition can be determined by locating peptide stretches that do not form secondary structural elements such as beta-sheets or alpha-helices as demonstrated by experimental data or as can be assumed by techniques of modeling or secondary structure prediction. The

linkers are independent, but they may in some cases have the same sequence and/or length. Exemplary amino acid linkers are known in the art and are suitable for the uses described herein.

The term “tag” as used herein refers to any linked molecule or affinity based sequence fused, usually in-frame, at any position (typically the N-terminal or C-terminal end) of a polypeptide or antibody-like binding protein. The presence of a suitable tag may serve to improve detection, purification or other characteristics of the antibody-like protein. Suitable affinity based tags include any sequence that may be specifically bound to another moiety (non-limiting examples include poly-histidine, 6x-histidine, FLAG, V5, biotin, HA, GST or MBP). In some instances, a linked molecule may be a light emitting reporter that may include any domain that can report the presence of a polypeptide. Suitable light emitting reporter domains include luciferase, fluorescent proteins or light emitting variants thereof.

The term "vector" as used herein refers to any molecule (*e.g.*, nucleic acid, plasmid, or virus) that is used to transfer coding information to a host cell. The term "vector" includes a nucleic acid molecule that is 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 molecule into which additional DNA segments may be inserted. Another type of vector is a viral vector, wherein additional DNA segments may be inserted into the viral genome. 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. In addition, 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. The terms "plasmid" and "vector" may be used interchangeably herein, as a plasmid is the most commonly used form of vector. However, the invention is intended to include other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses, and adeno-associated viruses), which serve equivalent functions.

The term "operably linked" is used herein to refer to an arrangement of flanking sequences wherein the flanking sequences so described are configured or assembled so as to perform their usual function. Thus, a flanking sequence operably linked to a coding sequence may be capable of effecting the replication, transcription, and/or translation of the coding sequence. For example, a coding sequence is operably linked to a promoter when the promoter is capable of directing transcription of that coding sequence. A flanking sequence need not be contiguous with the coding sequence, so long as it functions correctly. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence and the promoter sequence can still be considered "operably linked" to the coding sequence.

The phrase "recombinant host cell" (or "host cell") as used herein refers to a cell into which a recombinant expression vector has been introduced. A recombinant host cell or host cell is intended to refer not only to the particular subject cell, but also 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 such cells are still included within the scope of the term "host cell" as used herein. A wide variety of host cell expression systems can be used to express the antibody-like binding proteins of the invention, including bacterial, yeast, baculoviral, and mammalian expression systems (as well as phage display expression systems). An example of a suitable bacterial expression vector is pUC19. To express an antibody-like binding protein recombinantly, a host cell is transformed or transfected with one or more recombinant expression vectors carrying DNA fragments encoding the polypeptide chains of the antibody-like binding protein such that the polypeptide chains are expressed in the host cell and, preferably, secreted into the medium in which the host cells are cultured, from which medium the antibody-like binding protein can be recovered.

The term "transformation" as used herein refers to a change in a cell's genetic characteristics, and a cell has been transformed when it has been modified to contain a new DNA. For example, a cell is transformed where it is genetically modified from its native state. Following transformation, the transforming DNA may recombine with that of the cell by physically integrating into a chromosome of the cell, or may be maintained transiently as an episomal element without being replicated, or may

replicate independently as a plasmid. A cell is considered to have been stably transformed when the DNA is replicated with the division of the cell. The term "transfection" as used herein refers to the uptake of foreign or exogenous DNA by a cell, and a cell has been "transfected" when the exogenous DNA has been introduced inside the cell membrane. A number of transfection techniques are well known in the art. Such techniques can be used to introduce one or more exogenous DNA molecules into suitable host cells.

The term "naturally occurring" as used herein and applied to an object refers to the fact that the object can be found in nature and has not been manipulated by man. For example, a polynucleotide or polypeptide that is present in an organism (including viruses) that can be isolated from a source in nature and that has not been intentionally modified by man is naturally-occurring. Similarly, "non-naturally occurring" as used herein refers to an object that is not found in nature or that has been structurally modified or synthesized by man.

As used herein, the twenty conventional amino acids and their abbreviations follow conventional usage. Stereoisomers (*e.g.*, D-amino acids) of the twenty conventional amino acids; unnatural amino acids such as α-, α-disubstituted amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids may also be suitable components for the polypeptide chains of the antibody-like binding proteins of the invention. Examples of unconventional amino acids include: 4-hydroxyproline, γ-carboxyglutamate, ε-N,N,N-trimethyllysine, ε-N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, σ-N-methylarginine, and other similar amino acids and imino acids (*e.g.*, 4-hydroxyproline). In the polypeptide notation used herein, the left-hand direction is the amino terminal direction and the right-hand direction is the carboxyl-terminal direction, in accordance with standard usage and convention.

Naturally occurring residues may be divided into classes based on common side chain properties:

(1) hydrophobic: Met, Ala, Val, Leu, Ile, Phe, Trp, Tyr, Pro;

(2) polar hydrophilic: Arg, Asn, Asp, Gln, Glu, His, Lys, Ser, Thr ;

(3) aliphatic: Ala, Gly, Ile, Leu, Val, Pro;

(4) aliphatic hydrophobic: Ala, Ile, Leu, Val, Pro;

(5) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;

(6) acidic: Asp, Glu;

(7) basic: His, Lys, Arg;

(8) residues that influence chain orientation: Gly, Pro;

(9) aromatic: His, Trp, Tyr, Phe; and

(10) aromatic hydrophobic: Phe, Trp, Tyr.

Conservative amino acid substitutions may involve exchange of a member of one of these classes with another member of the same class. Conservative amino acid substitutions may encompass non-naturally occurring amino acid residues, which are typically incorporated by chemical peptide synthesis rather than by synthesis in biological systems. These include peptidomimetics and other reversed or inverted forms of amino acid residues. Non-conservative substitutions may involve the exchange of a member of one of these classes for a member from another class.

A skilled artisan will be able to determine suitable variants of the polypeptide chains of the antibody-like binding proteins of the invention using well-known techniques. For example, one skilled in the art may identify suitable areas of a polypeptide chain that may be changed without destroying activity by targeting regions not believed to be important for activity. Alternatively, one skilled in the art can identify residues and portions of the molecules that are conserved among similar polypeptides. In addition, even areas that may be important for biological activity or for structure may be subject to conservative amino acid substitutions without destroying the biological activity or without adversely affecting the polypeptide structure.

The term "chelator" is chemical compound in the form of a heterocyclic ring that can contain a metal coordinated to at least two nonmetal ions. The presence of hard acid cation chelate moieties on the antibody-like binding protein helps to ensure rapid *in vivo* clearance of the companion diagnostic.

Chelators are covalently bound to the antibody or antibody-like binding protein using standard methods of bioconjugation. Amine containing residues (*e.g.*, lysine or N-terminus) on the antibody undergo amide bond formation with a chelator containing an activated ester (*e.g.*, an *N*-hydroxysuccinimidyl ester). Sulfur containing residues (*e.g.*, cysteine) undergo conjugation with chelators containing an activated ester or maleimide moiety. Alternatively, bioconjugates are formed when activated carboxylate residues on the antibody undergo amide or thoiester formation

with amine or thiol groups, respectively, on the chelator. Bifunctional linkers, such as, for example, PEG–maleimide (PEG–Mal), succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) or *N*-succinimidyl 3-(2-pyridylthio)propionate (SPDP) are used where necessary.

Chelators are chosen for their metal-binding properties, and may be changed at will. Chelators such as NOTA (1, 4, 7-triaza-cyclononane-N,N',N"-triacetic acid), DOTA (1, 4, 7, 10-tetraazacyclododecane-1, 4, 7,10-tetraacetic acid), DTPA (1, 1, 4, 7, 7-Diethylenetriaminepentaacetic acid), TETA (p-bromoacetamido-benzyl-tetraethylaminetetraacetic acid), and Df (desferrioxamine B) are of use with a variety of radiolabels, radionuclides, radioisotopes, metals and radiometals. DOTA-type chelators, where the ligand includes hard base chelating functions such as carboxylate or amine groups, are most effective for chelating hard acid cations, especially Group IIa and Group IIIa metal cations. Such metal-chelate complexes can be made very stable by tailoring the ring size to the metal of interest. Also, more than one type of chelator may be conjugated to the targetable construct to bind multiple metal ions, *e.g.*, diagnostic radionuclides and/or therapeutic radionuclides.

Particularly useful diagnostic radiolabels, radionuclides or radioisotopes that can be bound to the chelating agents of the antibody or engineered antibody-like binding protein include, but are not limited to, ^{110}In, ^{111}In, ^{177}Lu, ^{18}F, ^{52}Fe, ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{67}Ga, ^{68}Ga, ^{86}Y, ^{90}Y, ^{89}Zr, ^{94}Tc, ^{94}Tc, ^{99m}Tc, ^{120}I, ^{123}I, ^{124}I, ^{125}I, ^{131}I, $^{154\text{-}158}$Gd, ^{32}P, ^{11}C, ^{13}N, ^{15}O, ^{186}Re, ^{188}Re, ^{51}Mn, ^{52m}Mn, ^{55}Co, ^{72}As, ^{75}Br, ^{76}Br, ^{82m}Rb, ^{83}Sr, or other gamma-, beta-, or positron-emitters. The diagnostic radiolabels include a decay energy in the range of 25 to 10,000 keV, more preferably in the range of 25 to 4,000 keV, and even more preferably in the range of 20 to 1,000 keV, and still more preferably in the range of 70 to 700 keV. Total decay energies of useful positron-emitting radionuclides are preferably <2,000 keV, more preferably under 1,000 keV, and most preferably <700 keV. Radiolabels useful as diagnostic agents utilizing gamma-ray detection include, but are not limited to: Cr-51, Co-57, Co-58, Fe-59, Cu-67, Ga-67, Se-75, Ru-97, Tc-99m, In-111, In-114m, I-123, I-125, I-131, Yb-169, Hg-197, and T1-201. Decay energies of useful gamma-ray emitting radiolabels are preferably 20 to 2000 keV, more preferably 60 to 600 keV, and most preferably 100 to 300 keV.

The term "patient" as used herein includes, but is not limited to human and

animal subjects.

A "disorder" is any condition that would benefit from treatment using the antibody or engineered antibody-like binding protein of the invention. "Disorder" and "condition" are used interchangeably herein and include chronic and acute disorders or diseases, including those pathological conditions that predispose a patient to the disorder in question. In certain aspects, the disorder is cancer, such as a solid tumor cancer. In certain specific aspects, the cancer is breast cancer, colon cancer, ovarian carcinoma, endometrial cancer, osteosarcoma, cervical cancer, prostate cancer, lung cancer, synovial carcinoma, pancreatic cancer, kidney cancer, a cancer of the lymphatic system, a sarcoma or a carcinoma in which CA6 is expressed or other cancer yet to be determined in which CA6 glycotope is expressed predominantly. The cancer can be, for example, serous ovarian carcinoma, endometriod ovarian carcinoma, neoplasm of the uterine cervix, neoplasm of the endometrius, neoplasm of the vulva, breast carcinoma, pancreatic tumor, and tumor of the urothelium.

Any disorder that can be treated by the killing of selected cell populations is suitable for use with the antibody-like binding proteins described herein, such as a cancer, an autoimmune disease, such as systemic lupus, rheumatoid arthritis, and multiple sclerosis; a graft rejection, such as renal transplant rejection, liver transplant rejection, lung transplant rejection, cardiac transplant rejection, and bone marrow transplant rejection; graft versus host disease; viral infection, such as mV infection, HIV infection, AIDS, etc.; and parasite infection, such as giardiasis, amoebiasis, schistosomiasis, and others as determined by one of ordinary skill in the art.

A reference expression level can be, for example, the level of CA6 expression in undiseased tissue of the patient (*e.g.*, from the same tissue-type as that containing the tumor), or the reference standard can be, for example, determined from an art-accepted standard, such as derived from expression levels in undiseased tissues from a population of individuals.

The terms "treatment" or "treat" as used herein refer to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those having the disorder as well as those prone to have the disorder or those in which the disorder is to be prevented.

The terms "pharmaceutical composition" or "therapeutic composition" as used herein refer to a compound or composition capable of inducing a desired therapeutic

effect when properly administered to a patient.

The term "pharmaceutically acceptable carrier" or "physiologically acceptable carrier" as used herein refers to one or more formulation materials suitable for accomplishing or enhancing the delivery of an antibody or engineered antibody-like binding protein.

The terms "effective amount" and "therapeutically effective amount" when used in reference to a pharmaceutical composition comprising one or more antibodies or engineered antibody-like binding proteins refers to an amount or dosage sufficient to produce a desired therapeutic result. More specifically, a therapeutically effective amount is an amount of an antibody or engineered antibody-like binding protein sufficient to inhibit, for some period of time, one or more of the clinically defined pathological processes associated with the condition being treated. As used herein, "therapeutically effective amount" also refers to an amount of antibody-like protein effective to visualize a CA6-expressing tissue, *e.g.*, tumor tissue. The effective amount may vary depending on the specific antibody or engineered antibody-like binding protein that is being used, and also depends on a variety of factors and conditions related to the patient being treated and the severity of the disorder. For example, if the antibody or engineered antibody-like binding protein is to be administered *in vivo*, factors such as the age, weight, and health of the patient as well as dose response curves and toxicity data obtained in preclinical animal work would be among those factors considered. The determination of an effective amount or therapeutically effective amount of a given pharmaceutical composition is well within the ability of those skilled in the art.

The terms "acquire" or "acquiring" as used herein, refer to obtaining possession of a physical entity, or a value, *e.g.,* a numerical value, by "directly acquiring" or "indirectly acquiring" the physical entity or value. "Directly acquiring" means performing a process (*e.g.*, performing a synthetic or analytical method) to obtain the physical entity or value. "Indirectly acquiring" refers to receiving the physical entity or value from another party or source (*e.g.*, a third party laboratory that directly acquired the physical entity or value). Directly acquiring a physical entity includes performing a process that includes a physical change in a physical substance, *e.g.*, a starting material. Exemplary changes include making a physical entity from two or more starting materials, shearing or fragmenting a substance, separating or

purifying a substance, combining two or more separate entities into a mixture, performing a chemical reaction that includes breaking or forming a covalent or non-covalent bond. Directly acquiring a value includes performing a process that includes a physical change in a sample or another substance, *e.g.*, performing an analytical process which includes a physical change in a substance, *e.g.*, a sample, analyte, or reagent (sometimes referred to herein as "physical analysis").

Information that is acquired indirectly can be provided in the form of a report, *e.g.*, supplied in paper or electronic form, such as from an online database or application (an "App"). The report or information can be provided by, for example, a healthcare institution, such as a hospital or clinic; or a healthcare provider, such as a doctor or nurse.

One embodiment of the invention provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a therapeutically effective amount of an antibody or engineered antibody-like binding protein.

2. Uses for Antibody-like Binding Proteins

The antibody or engineered antibody-like binding proteins of the invention can be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays for the detection and quantitation of one or more target antigens. The antibody or engineered antibody-like binding proteins will bind the one or more target antigens with an affinity that is appropriate for the assay method being employed.

For diagnostic applications, in certain embodiments, engineered antibody-like binding proteins can be labeled with a detectable moiety. The detectable moiety can be any one that is capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety can be a radiolabel, radioisotope, radionuclide, imaging agent, therapeutic agent or diagnostic agent, such as ^{3}H, ^{14}C, ^{32}P, ^{35}S, ^{125}I, ^{99}Tc, ^{111}In, ^{18}F, ^{11}C, ^{68}Ga, ^{64}Cu, ^{89}Zr, ^{124}I or ^{67}Ga; a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin; or an enzyme, such as alkaline phosphatase, β-galactosidase, or horseradish peroxidase.

A proper reporter moiety can be detected by a particular imaging technique (*e.g.*, $^{18}F/^{11}C/^{68}Ga/^{64}Cu/^{89}Zr/^{124}I$ radiolabeled reporter for PET imaging, $^{99m}Tc/^{111}In$

labeled reporter for SPECT imaging, a fluorescent dye labeled reporter for optical imaging or paramagnetic material labeled reporter for MRI). An appropriate match is recommended between the biologic half-life of an antibody or antibody-like binding protein and the physical half-life of the radiolabel (*see* Table 1).

Table 1. Examples of radiolabels and their half-life

Radiolabel	Half-life
^{68}Ga	68 min
^{18}F	109 min
^{64}Cu	12.7 hr
^{89}Zr	78.4 hr
^{124}I	100.2 hr

The development of an imaging companion diagnostic probe or antibody-like binding protein essentially requires a process similar to drug discovery (*e.g.*, starting with target probe, radiation dosimetry, good laboratory practices toxicity studies, good manufacturing practice production, preclinical assessment, preclinical validation of target probe and clinical assessment). Additional parameters to be evaluated are: optimal timing of imaging relative to tracer administration, correlation with *ex vivo* analysis, tracer biodistribution and target specificity (tested with a radiolabeled non relevant fragment). Once fully developed, a molecular imaging probe can be used as a molecular imaging companion diagnostic for a particular drug or agent.

The engineered antibody-like binding proteins of the invention are also useful for *in vivo* imaging. An antibody-like binding protein labeled with a detectable moiety can be administered to an animal, preferably into the bloodstream, and the presence and location of the labeled antibody in the host assayed. The antibody-like binding protein can be labeled with any moiety that is detectable in an animal, whether by nuclear magnetic resonance, positron emission tomography or other detection means known in the art.

The *in vivo* imaging technique can be used to image a tissue, such as tumor tissue, to assess the level of CA6 glycotope expression in the tissue. Determining the level of CA6 expression in the tissue is useful, for example, to determine whether a patient, such as a cancer patient, will be a good candidate for treatment with a drug that targets a CA6 glycotope. For example, the antibody-like binding protein is useful as a companion diagnostic for a huDM6-DM4 cytotoxic agent.

In one embodiment, a patient is evaluated for treatment with a CA6 targeting

molecule such as huDM6-DM4. The patient is evaluated, for example, by acquiring information regarding the level of CA6 expression in a diseased tissue, such as a tumor tissue (*e.g.*, a tissue biopsy), and if the level of CA6 expression is determined to be greater than 10% of a reference expression level (*e.g.*, greater than or equal to 10%, 20%, 30%, 40% or more than a reference level), then the patient is determined to be a good candidate for treatment with the CA6-targeting molecule. A patient who is a good candidate for treatment with the CA6-targeting molecule is predicted to respond positively to treatment with the molecule. A patient who does not have a suitable level of CA6 expression, *e.g.*, less than 10% CA6 expression as compared to a reference standard, is determined not to be a good candidate for treatment with a CA6-targeting molecule.

A reference expression level can be, for example, the level of CA6 expression in undiseased tissue of the patient (*e.g.*, from the same tissue-type as that containing the tumor), or the reference standard can be, for example, determined from an art-accepted standard, such as derived from expression levels in undiseased tissues from a population of individuals.

In one embodiment, the level of CA6 expression in a tissue of a subject is acquired indirectly, such as from a service-provider, such as from a medical institution, such as a hospital or clinic or doctor's office, or from a healthcare professional, such as a doctor or nurse, or from an insurance provider, or from a database. In another embodiment, the level of CA6 expression is determined directly, such as be administering the antibody-like binding protein featured in the invention to the subject, and determining the level of CA6 expression by a bioimaging technique.

A patient determined to be a good candidate for treatment with a CA6-targeting molecule can optionally be further administered the CA6-targeting molecule. In some embodiments, a patient treated with a CA6 targeting molecule will be monitored at intervals (*e.g.*, weekly, biweekly, monthly, bimonthly) following administration of the CA6 targeting molecule to determine if the CA6 expression level is changing (*e.g.*, decreasing) following treatment with the CA6 targeting molecule. If after a certain amount of time, or after the patient presents with adverse events, if the CA6 expression level in the diseased tissue remains unchanged, a decision can be made to stop or continue treatment with the CA6-targeting molecule.

In one embodiment, a patient is monitored during treatment with a CA6

targeting molecule such as huDM6-DM4. The patient is monitored, for example, by acquiring information regarding the level of CA6 expression in a diseased tissue, such as a tumor tissue, following administration of a CA6 targeting molecule or treatment with a CA6 targeting molecule such as huDM6-DM4, and if the level of CA6 expression is determined to be greater than 10% of a reference expression level (*e.g.*, greater than or equal to 10%, 20%, 30%, 40% or more than a reference level), then the patient is determined to be a good candidate for continued treatment with the CA6-targeting molecule. If the level of CA6 expression is determined to have decreased during treatment with a CA6 targeting molecule such as huDM6-DM4, then the patient is determined to have responded positively to treatment with the molecule and treatment with huDS6-DM4 should continue. A patient who does not have a suitable level of CA6 expression, *e.g.*, less than 10% CA6 expression as compared to a reference standard or who has an increased level of CA6 expression, is determined not to be a good candidate for continued treatment with a CA6-targeting molecule.

The invention as disclosed herein also relates to a kit comprising an antibody-like binding protein and other reagents useful for detecting target antigen levels in biological samples. Such reagents can include a detectable label, blocking serum, positive and negative control samples, and detection reagents.

4. Engineered Antibody-like Binding Protein Compositions and Administration Thereof

Compositions comprising engineered antibody-like binding proteins are within the scope of the invention. Such diagnostic, therapeutic or pharmaceutical compositions can comprise a diagnostic or therapeutically effective amount of an antibody-like binding protein, or antibody-like binding protein-drug conjugate, in admixture with a pharmaceutically or physiologically acceptable formulation agent selected for suitability with the mode of administration.

Acceptable formulation materials preferably are nontoxic to recipients at the dosages and concentrations employed.

The diagnostic or pharmaceutical composition can contain formulation materials for modifying, maintaining, or preserving, for example, the pH, osmolarity, viscosity, clarity, color, isotonicity, odor, sterility, stability, rate of dissolution or

release, adsorption, or penetration of the composition. Suitable formulation materials include, but are not limited to, amino acids (such as glycine, glutamine, asparagine, arginine, or lysine), antimicrobials, antioxidants (such as ascorbic acid, sodium sulfite, or sodium hydrogen-sulfite), buffers (such as borate, bicarbonate, Tris-HCl, citrates, phosphates, or other organic acids), bulking agents (such as mannitol or glycine), chelating agents (such as ethylenediamine tetraacetic acid (EDTA)), complexing agents (such as caffeine, polyvinylpyrrolidone, beta-cyclodextrin, or hydroxypropyl-beta-cyclodextrin), fillers, monosaccharides, disaccharides, and other carbohydrates (such as glucose, mannose, or dextrins), proteins (such as serum albumin, gelatin, or immunoglobulins), coloring, flavoring and diluting agents, emulsifying agents, hydrophilic polymers (such as polyvinylpyrrolidone), low molecular weight polypeptides, salt-forming counterions (such as sodium), preservatives (such as benzalkonium chloride, benzoic acid, salicylic acid, thimerosal, phenethyl alcohol, methylparaben, propylparaben, chlorhexidine, sorbic acid, or hydrogen peroxide), solvents (such as glycerin, propylene glycol, or polyethylene glycol), sugar alcohols (such as mannitol or sorbitol), suspending agents, surfactants or wetting agents (such as pluronics; PEG; sorbitan esters; polysorbates such as polysorbate 20 or polysorbate 80; triton; tromethamine; lecithin; cholesterol or tyloxapal), stability enhancing agents (such as sucrose or sorbitol), tonicity enhancing agents (such as alkali metal halides – preferably sodium or potassium chloride – or mannitol sorbitol), delivery vehicles, diluents, excipients and/or pharmaceutical adjuvants (*see, e.g.,* REMINGTON'S PHARMACEUTICAL SCIENCES (18th Ed., A.R. Gennaro, ed., Mack Publishing Company 1990), and subsequent editions of the same, incorporated herein by reference for any purpose).

The optimal diagnostic or pharmaceutical composition will be determined by a skilled artisan depending upon, for example, the intended route of administration, delivery format, and desired dosage. Such compositions can influence the physical state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the antibody-like binding protein.

The primary vehicle or carrier in a diagnostic or pharmaceutical composition can be either aqueous or non-aqueous in nature. For example, a suitable vehicle or carrier for injection can be water, physiological saline solution, or artificial cerebrospinal fluid, possibly supplemented with other materials common in

compositions for parenteral administration. Neutral buffered saline or saline mixed with serum albumin are further exemplary vehicles. Other exemplary pharmaceutical compositions comprise Tris buffer of about pH 7.0-8.5, or acetate buffer of about pH 4.0-5.5, which can further include sorbitol or a suitable substitute. In one embodiment of the invention, engineered antibody-like binding protein compositions can be prepared for storage by mixing the selected composition having the desired degree of purity with optional formulation agents in the form of a lyophilized cake or an aqueous solution. Further, the antibody-like binding proteins can be formulated as a lyophilizate using appropriate excipients such as sucrose.

The diagnostic or pharmaceutical compositions of the invention can be selected for parenteral delivery. Alternatively, the compositions can be selected for inhalation or for delivery through the digestive tract, such as orally. The preparation of such pharmaceutically acceptable compositions is within the skill of the art.

The formulation components are present in concentrations that are acceptable to the site of administration. For example, buffers are used to maintain the composition at physiological pH or at a slightly lower pH, typically within a pH range of from about 5 to about 8.

When parenteral administration is contemplated, the diagnostic or therapeutic compositions for use in this invention can be in the form of a pyrogen-free, parenterally acceptable, aqueous solution comprising the desired antibody-like binding protein in a pharmaceutically acceptable vehicle. A particularly suitable vehicle for parenteral injection is sterile distilled water in which an antibody-like binding protein is formulated as a sterile, isotonic solution, properly preserved. Yet another preparation can involve the formulation of the desired molecule with an agent, such as injectable microspheres, bio-erodible particles, polymeric compounds (such as polylactic acid or polyglycolic acid), beads, or liposomes, that provides for the controlled or sustained release of the product which can then be delivered via a depot injection. Hyaluronic acid can also be used, and this can have the effect of promoting sustained duration in the circulation. Other suitable means for the introduction of the desired molecule include implantable drug delivery devices.

In one embodiment, a diagnostic or pharmaceutical composition can be formulated for inhalation. For example, an antibody or engineered antibody-like binding protein can be formulated as a dry powder for inhalation. Antibody-like

binding proteins inhalation solutions can also be formulated with a propellant for aerosol delivery. In yet another embodiment, solutions can be nebulized.

It is also contemplated that certain formulations can be administered orally. In one embodiment of the invention, antibody-like binding proteins that are administered in this fashion can be formulated with or without those carriers customarily used in the compounding of solid dosage forms such as tablets and capsules. For example, a capsule can be designed to release the active portion of the formulation at the point in the gastrointestinal tract when bioavailability is maximized and pre-systemic degradation is minimized. Additional agents can be included to facilitate absorption of the antibody-like binding protein. Diluents, flavorings, low melting point waxes, vegetable oils, lubricants, suspending agents, tablet disintegrating agents, and binders can also be employed.

Another diagnostic or pharmaceutical composition can involve an effective quantity of antibody-like binding protein in a mixture with non-toxic excipients that are suitable for the manufacture of tablets. By dissolving the tablets in sterile water, or another appropriate vehicle, solutions can be prepared in unit-dose form. Suitable excipients include, but are not limited to, inert diluents, such as calcium carbonate, sodium carbonate or bicarbonate, lactose, or calcium phosphate; or binding agents, such as starch, gelatin, or acacia; or lubricating agents such as magnesium stearate, stearic acid, or talc.

Additional diagnostic or pharmaceutical compositions of the invention will be evident to those skilled in the art, including formulations involving antibody-like binding protein in sustained- or controlled-delivery formulations. Techniques for formulating a variety of other sustained- or controlled-delivery means, such as liposome carriers, bio-erodible microparticles or porous beads and depot injections, are also known to those skilled in the art. Additional examples of sustained-release preparations include semipermeable polymer matrices in the form of shaped articles, *e.g.* films, or microcapsules. Sustained release matrices can include polyesters, hydrogels, polylactides, copolymers of L-glutamic acid and gamma ethyl-L-glutamate, poly (2-hydroxyethyl-methacrylate), ethylene vinyl acetate, or poly-D(-)-3-hydroxybutyric acid. Sustained-release compositions can also include liposomes, which can be prepared by any of several methods known in the art.

Diagnostic or pharmaceutical compositions of the invention to be used for *in vivo* administration typically must be sterile. This can be accomplished by filtration through sterile filtration membranes. Where the composition is lyophilized, sterilization using this method can be conducted either prior to, or following, lyophilization and reconstitution. The composition for parenteral administration can be stored in lyophilized form or in a solution. In addition, parenteral compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

Once the diagnostic or pharmaceutical composition has been formulated, it can be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or as a dehydrated or lyophilized powder. Such formulations can be stored either in a ready-to-use form or in a form (*e.g.*, lyophilized) requiring reconstitution prior to administration.

The invention also encompasses kits for producing a single-dose administration unit. The kits can each contain both a first container having a dried protein and a second container having an aqueous formulation. Also included within the scope of this invention are kits containing single and multi-chambered pre-filled syringes (*e.g.*, liquid syringes and lyosyringes).

The composition can also be administered locally via implantation of a membrane, sponge, or other appropriate material onto which the desired molecule has been absorbed or encapsulated. Where an implantation device is used, the device can be implanted into any suitable tissue or organ, and delivery of the desired molecule can be via diffusion, timed-release bolus, or continuous administration.

EXAMPLES

The Examples that follow are illustrative of specific embodiments of the invention, and various uses thereof. They are set forth for explanatory purposes only, and should not be construed as limiting the scope of the invention in any way.

Example 1: Radiolabeling and *in vitro* characterization of humanized DS6 monoclonal antibody and DS6-DM4

A clinically useful molecular imaging agent produces high contrast images

within a reasonable amount of time following administration, demonstrates tumor penetration, has faster clearance kinetics and exhibits an excellent tumor to blood ratio. A caveat of using intact native antibodies in bioimaging is inherently slow kinetics of tumor uptake and blood clearance; they also tend to have high background and require a long time (hours to days) to obtain clear images of the tumor.

It has been previously shown that a radiolabeled tracer combined with bio-imaging can translate from a preclinical tumor model to a clinical setting (Williams *et al.*, 2008, *Cancer Biother and Radiopharm* 23(6):797; Liu *et al.*, (2009) *Mol Imaging Biol* 12:530). We further demonstrated that a radiolabeled DS6 antibody and DS6-DM4 effectively targeted WISH xenograft tumors in mice. Based on these results, three engineered antibody-like binding proteins were developed for optimized pharmacokinetics as an imaging based companion diagnostic.

Example 2: Expression of DS6 engineered B-Fab

The expression plasmids encoding the heavy and light chains of the corresponding constructs were propagated in *E. coli* DH5α cells. Plasmids used for transfection were prepared from *E. coli* using the Qiagen EndoFree Plasmid Mega Kit (polypeptide and DNA sequences for the B-Fab antibody-like binding protein are in Table 2).

The DS6 antibody-like binding protein B-Fab was produced by transient transfection of HEK293 cells in FreeStyle F17 medium (Invitrogen). After 7 days after transfection, supernatant was harvested, and subjected to centrifugation and 0.2μm filtration to remove particles. Purification was carried out by capture on KappaSelect (GE Healthcare). After binding the KappaSelect column, the column was washed with PBS or HEPES buffer (50mM HEPES, 150mM NaCl, pH 7.2, FP12016) followed by elution with 0.1M Glycin (pH 2.5) and neutralization with 1M Tris/HCl pH 9. The purification polishing step consisted of size exclusion chromatography using Superdex 200 (GE Healthcare) with PBS or HEPES buffer (50mM HEPES, 150mM NaCl, pH 7.2, FP12016). Following the polishing step, the purification product was concentrated by ultrafiltration, and finally the DS6 B-Fab was sterile filtered using a 0.22 μm membrane.

Protein concentration was determined by measurement of absorbance at 280 nm. Each batch was analyzed by SDS-PAGE under reducing and non-reducing

conditions to determine the purity and molecular weight of each subunit and of the monomer. The quality of purified DS6 B-Fab was checked by the analytical methods described below.

Table 2. Polypeptide and DNA sequences for DS6 based B-Fab

Amino acid sequence for: V_{L1}-L_1-V_{L2}-C_L (SEQ ID NO: 1)	EIVLTQSPATMSASPGERVTITCSAHSSVSFMHWFQQKPGTSPKLWIY STSSLASGVPARFGGSGSGTSYSLTISSMEAEDAATYYCQQRSSFPLT FGAGTKLELK***GGGGSGGGGS***EIVLTQSPATMSASPGERVTITCSAHSS VSFMHWFQQKPGTSPKLWIYSTSSLASGVPARFGGSGSGTSYSLTISS MEAEDAATYYCQQRSSFPLTFGAGTKLELK**RTVAAPSVFIFPPSDEQL KSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTY SLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC**
Amino acid sequence for: Anti-CA6 V_{L1} (SEQ ID NO: 2)	EIVLTQSPATMSASPGERVTITCSAHSSVSFMHWFQQKPGTSPKLWIY STSSLASGVPARFGGSGSGTSYSLTISSMEAEDAATYYCQQRSSFPLT FGAGTKLELK
Amino acid sequence for: L_1 Linker (SEQ ID NO: 3)	GGGGSGGGGS
Amino acid sequence for: Anti-CA6 V_{L2} (SEQ ID NO: 2)	EIVLTQSPATMSASPGERVTITCSAHSSVSFMHWFQQKPGTSPKLWIY STSSLASGVPARFGGSGSGTSYSLTISSMEAEDAATYYCQQRSSFPLT FGAGTKLELK
Amino acid sequence for: C_L Human IGKC (SEQ ID NO: 4)	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQ SGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSS PVTKSFNRGEC
DNA coding sequence for: V_{L1}-L_1-V_{L2}-C_L (SEQ ID NO: 5)	gaaatcgtgctgacccagagccccgccaccatgtctgccagccctggc gagagagtcaccatcacctgtagcgcccacagcagcgtcagtttcatg cactggttccagcagaagcccggcaccagcccaaagctgtggatctac agcaccagcagcctcgccagcggcgtcccagctcgctttggcggcagc ggctctggcaccagctacagcctgaccatcagcagcatggaagccgag gacgccgccacctactactgccagcagcggagcagctttcccctgacc ttcggcgctggcaccaagctggaactgaagggcggaggcggatccggc ggcggaggctccgagattgtgctgacacagtctccagccaccatgagc gcctccccaggcgagcgcgtgacaatcacatgctccgcccactcctcc gtgtcttttatgcattggtttcagcagaaacctgggacatcccctaaa ctctggatctactccacctcctccctggcctccggggtgcccgctaga tttggaggctctggcagcggcacctcctactccctgaccatctcctct atggaagctgaagatgctgcaacatattattgccagcagagaagctcc ttcccactgacatttggggccggaacaaagctcgagctgaagcgtacg gtggccgctccttccgtgttcatcttccctccctccgacgagcagctg aagtccggcaccgcctccgtggtgtgtctgctgaacaacttctaccct cgggaggccaaggtgcagtggaaggtggacaacgccctgcagtccggc aactcccaggagtccgtcaccgagcaggactccaaggacagcacctac tccctgtcctccaccctgaccctgtccaaggccgactacgagaagcac aaggtgtacgcctgtgaggtgacccaccagggcctgtccagccctgtg accaagtccttcaaccggggcgagtgc
DNA coding sequence for: V_{L1}-L_1-V_{L2}-C_L (SEQ ID NO: 6) With signal sequence	atgggctggtcctgcatcatcctgtttctggtggccacagccaccggc gtgcacagcgaaatcgtgctgacccagagccccgccaccatgtctgcc agccctggcgagagagtcaccatcacctgtagcgcccacagcagcgtc agtttcatgcactggttccagcagaagcccggcaccagcccaaagctg tggatctacagcaccagcagcctcgccagcggcgtcccagctcgcttt ggcggcagcggctctggcaccagctacagcctgaccatcagcagcatg gaagccgaggacgccgccacctactactgccagcagcggagcagcttt cccctgaccttcggcgctggcaccaagctggaactgaagggcggaggc ggatccggcggcggaggctccgagattgtgctgacacagtctccagcc

	accatgagcgcctccccaggcgagcgcgtgacaatcacatgctccgcc cactcctccgtgtcttttatgcattggtttcagcagaaacctgggaca tcccctaaactctggatctactccacctcctccctggcctccggggtg cccgctagatttggaggctctggcagcggcacctcctactccctgacc atctcctctatggaagctgaagatgctgcaacatattattgccagcag agaagctccttcccactgacatttggggccggaacaaagctcgagctg aagcgtacggtggccgctccttccgtgttcatcttccctccctccgac gagcagctgaagtccggcaccgcctccgtggtgtgtctgctgaacaac ttctaccctcgggaggccaaggtgcagtggaaggtggacaacgccctg cagtccggcaactcccaggagtccgtcaccgagcaggactccaaggac agcacctactccctgtcctccaccctgaccctgtccaaggccgactac gagaagcacaaggtgtacgcctgtgaggtgacccaccagggcctgtcc agccctgtgaccaagtccttcaaccggggcgagtgc
Amino acid sequence for: V_{H1}-L_2-V_{H2}-C_{H1} (SEQ ID NO: 7)	EAQLVQSGAEVVKPGASVKMSCKASGYTFTSYNMHWVKQTPGQGLEWI GYIYPGNGATNYNQKFQGKATLTADPSSSTAYMQISSLTSEDSAVYFC ARGDSVPFAYWGQGTLVTVSA***GGGGSGGGGS***EAQLVQSGAEVVKPGAS VKMSCKASGYTFTSYNMHWVKQTPGQGLEWIGYIYPGNGATNYNQKFQ GKATLTADPSSSTAYMQISSLTSEDSAVYFCARGDSVPFAYWGQGTLV TVSA**ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSG ALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNT KVDKKVEPKSCDKTHT**
Amino acid sequence for: Anti-CA6 V_{H1} (SEQ ID NO: 8)	EAQLVQSGAEVVKPGASVKMSCKASGYTFTSYNMHWVKQTPGQGLEWI GYIYPGNGATNYNQKFQGKATLTADPSSSTAYMQISSLTSEDSAVYFC ARGDSVPFAYWGQGTLVTVSA
Amino acid sequence for: L_2 Linker (SEQ ID NO: 3)	GGGGSGGGGS
Amino acid sequence for: Anti-CA6 V_{H2} (SEQ ID NO: 8)	EAQLVQSGAEVVKPGASVKMSCKASGYTFTSYNMHWVKQTPGQGLEWI GYIYPGNGATNYNQKFQGKATLTADPSSSTAYMQISSLTSEDSAVYFC ARGDSVPFAYWGQGTLVTVSA
Amino acid sequence for: C_{H1} Human constant heavy (SEQ ID NO: 9)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK KVEPKSCDKTHT
DNA coding sequence for V_{H1}-L_2-V_{H2}-C_{H1} (SEQ ID NO: 10)	gaggcccagctggtgcagtctggcgctgaggtggtcaagcctggggcc agcgtgaagatgagctgcaaggccagcggctacaccttcaccagctac aacatgcactgggtcaagcagaccccagggcagggcctggaatggatt ggctacatctaccccggcaacggcgccaccaactacaaccagaagttc cagggcaaggctaccctgaccgccgaccctagcagcagcaccgcctac atgcagatcagcagcctgaccagcgaggacagcgccgtgtacttctgc gccagaggcgacagcgtgcccttcgcctattggggccagggcaccctg gtcacagtgtctgctggtggcggaggatccggcggaggcggaagcgaa gcccagctcgtccagagcggagccgaggtcgtgaaaccaggcgcctct gtgaagatgtcttgcaaggcctctggctatacctttacctcctataat atgcattgggtcaaacagacacctggacagggactcgagtggatcgga tatatctatcctggaaatggggccacaaattacaatcagaaatttcag gggaaagccacactgacagccgatcccagctcctccacagcctatatg cagattagctctctgacctccgaggactccgccgtgtatttttgtgcc cggggagactccgtgccttttgcttactggggacagggcacactcgtg acagtgtccgccgcttccaccaagggcccctccgtgtttcctctggcc cccagcagcaagagcacctctggcggaacagccgccctgggctgcctg gtcaaggactacttccccgagcccgtgaccgtgtcttggaactctggc gccctgacctccggcgtccacaccttccagccgtgctgcagagcagc ggcctgtactctctgagcagcgtcgtgaccgtgcccagcagcagcctg gggacccagacctacatctgcaacgtgaaccacaagcccagcaacacc aaggtggacaagaaggtggaacccaagagctgcgacaagacccacacc
DNA coding sequence for V_{H1}-L_2-V_{H2}-C_{H1} (SEQ ID NO: 11)	atgggctggtcctgcatcatcctgtttctggtggccacagccaccggc gtgcactctgaggcccagctggtgcagtctggcgctgaggtggtcaag cctggggccagcgtgaagatgagctgcaaggccagcggctacaccttc accagctacaacatgcactgggtcaagcagaccccagggcagggcctg

With signal sequence.	gaatggattggctacatctaccccggcaacggcgccaccaactacaac cagaagttccagggcaaggctaccctgaccgccgaccctagcagcagc accgcctacatgcagatcagcagcctgaccagcgaggacagcgccgtg tacttctgcgccagaggcgacagcgtgcccttcgcctattggggccag ggcaccctggtcacagtgtctgctggtggcggaggatccggcggaggc ggaagcgaagcccagctcgtccagagcggagccgaggtcgtgaaacca ggcgcctctgtgaagatgtcttgcaaggcctctggctatacctttacc tcctataatatgcattgggtcaaacagacacctggacagggactcgag tggatcggatatatctatcctggaaatggggccacaaattacaatcag aaatttcaggggaaagccacactgacagccgatcccagctcctccaca gcctatatgcagattagctctctgacctccgaggactccgccgtgtat ttttgtgcccggggagactccgtgccttttgcttactggggacagggc acactcgtgacagtgtccgccgcttccaccaagggcccctccgtgttt cctctggccccagcagcaagagcacctctggcggaacagccgccctg ggctgcctggtcaaggactacttccccgagcccgtgaccgtgtcttgg aactctggcgccctgacctccggcgtccacacctttccagccgtgctg cagagcagcggcctgtactctctgagcagcgtcgtgaccgtgcccagc agcagcctggggacccagacctacatctgcaacgtgaaccacaagccc agcaacaccaaggtggacaagaaggtggaacccaagagctgcgacaag acccacacc

Example 3: Development of engineered antibody-like binding proteins based on the DS6 antibody

In order to develop an imaging based companion diagnostic antibody-like binding protein for huDS6-DM4, three engineered antibody-like binding proteins (B-Fab, *see* Figure 1) based on CA6 binding sequences from the humanized DS6 monoclonal antibody were created. Each of the three engineered antibody-like binding proteins was purified and tested as described below; the results are shown in Table 3. Desirable characteristics include >95% purity by HPLC or SDS-PAGE, ~10mg/mL concentration, a stable peptide, binding similar to the DS6 antibody, and to determine whether the presence of a poly-histidine (6x-His) tag affects PET tracers. Additional selection criteria includes: a tumor signal of >5 %ID/g; a tumor to muscle ratio of 3:1; efficient renal clearance; and a statistically significant difference ($p<0.05$) between unblocked and blocked uptake (*see* Table 4 for summary of quality criteria for radiolabeled chelated B-Fab).

Table 3. Summary of DS6 engineered antibody-like binding proteins

Format	Description	Purity (SEC)	ID (MS)	Stability (Tmelt)	K_d (nM)
B-Fab 1151	$(G4S)_2$ linker with C-terminal 6xHis tag	>95%	OK	58°C	3.4
B-Fab 1152	G4S linker with C-terminal 6xHis tag	>95%	OK	59°C	88
B-Fab 1153	$(G4S)_2$ linker without C-terminal 6xHis tag	>95%	OK	57°C	2.3
DS6 mAb	Control DS6 monoclonal antibody				2.0

Table 4. Summary of quality criteria for radiolabeled chelated antibody-like binding protein

Imaging Companion Diagnostic Test Specifications	Acceptance Criteria/Limit
Purity (HPLC)	>95-98% monomeric
In vitro cell binding assay	>90% for CA6+ cell lines <5% for CA6- cell lines – low nM
Stability	>80% after 24 hours at 37°C in serum
Imaging parameters	Tumor %IC/g ≥ 5 Tumor to normal tissue ratio 3:1 Specific Activity (Ci/µmole) 2-5

It was determined that the B-Fab antibody containing a $(G4S)_2$ linker, and with or without a 6x-His tag, maintained binding efficiencies similar to the control DS6 monoclonal antibody from which the CA6 binding sequences were based. The

quality of each of the purified antibody-like binding protein was checked by the following analytical methods:

a) Analytical size-exclusion chromatography (SEC)

Analytical SEC was performed using an ÄKTA explorer 10 (GE Healthcare) equipped with a TSKgel G3000SWXL column (7.8mm x 30cm) and TSKgel SWXL guard column (Tosoh Bioscience). The analysis was run at 1mL/min using 250 mM NaCl, 100 mM Na-phosphate pH 6.7 with detection at 280 nm. Thirty microliters of protein sample (at 0.5-1 mg/mL) were applied onto the column. For estimation of the molecular size, the column is calibrated using a gel filtration standard mixture (MWGF-1000, SIGMA Aldrich). Data evaluation was performed using UNICORN software v5.11.

b) Intact mass analysis by LC-MS

Each sample was diluted to a concentration of 0.01mg/mL and then reduced by adding DTT (final 10mM). Prior to separation, the sample was trapped for 20 minutes and desalted with 20 µL/min on a monolithic trap column with 2% Acetonitrile / 0.1% TFA (v/v) prior to elution with a gradient ranging from 15% Eluent A (H_2O / 0.05 %TFA) to 50 % Eluent B (Acetonitrile / 0.05% TFA).

Each sample was separated operating in nanoflow (300 nL/min) on a monolithic column (PSDVB; 100µm I.D. x 5 cm) at 37°C. Introduction of each sample was carried out using electrospray needles from new objective with an outer diameter of 365µm, inner diameter of 75µm and an end tip diameter of 15 µm plus sheath gas. After acquisition on a QStar XL, the spectra were summed over the corresponding time range and deconvoluted using the protein reconstruction tool delivered with BioAnalyst from Applied Biosystems/MDS Sciex.

c) Protein identification by peptide mass fingerprint analysis using LC-MS

The lane of interest was punched out, carbamidomethylated and digested with endoproteinase trypsin. Subsequently the sample was analyzed by LC-MS/MS using an OrbiTrap XL mass spectrometer. The obtained spectra were compared with the user defined database Swiss-Prot PP to which the given construct was added, as well as to the database Swiss-Prot all species.

Each of three engineered DS6-based antibody-like binding proteins were chemically conjugated to the hard acid cation chelator DOTA (1, 4, 7, 10-

tetraazacyclododecane-1, 4, 7, 10-tetraacetic acid), radiolabeled with ^{64}Cu, and used for *in vivo* PET bio-imaging experiments in preclinical tumor models and *in vitro* cell binding assays in order to characterize each of the three engineered DS6-based antibody-like binding proteins. A summary of the data from the activities and experiments for each engineered antibody can be seen in Table 5 and Figures 2-4.

Table 5. Summary of activities of DS6 engineered antibody-like binding proteins

	B-Fabs
Purity (SEC)	>95%
In vitro stability: Freeze/thaw stress (3x -80°C/RT) Heat stress (1 week at 42°C)	 Well tolerated Well tolerated
Serum stability (24 hours at 37°C)	>90% monomeric
FACS binding affinities (WISH cell line, CA6+)	2nM
Radiochemistry yield	60-80%
Imaging parameters: Tumor (WISH, CA6+) Tumor (A2780, CA6-)	 7.5-10.4% ID/g 4-5.6% ID/g
Biodistribution Liver Kidney Blood Muscle	 7-10% ID/g 57-73% ID/g 2-3% ID/g ~1% ID/g
WISH tumor to normal tissue ratio: Tumor/Blood Tumor/Muscle	 4-6 9-11
WISH (CA6+) tumor to A2780 (CA6-) tumor ratio	 1.7-1.9

WISH is a cell line positive for the CA6 antigen.
A2780 is a cell line negative for the CA6 antigen.

All the above antibody-like binding proteins and their corresponding DOTA conjugates (1.5-2.5 DOTA/fragment) had high affinity (K_d = 4-20 nM) to CA6 positive cells (WISH cell line), indicating that DOTA derivatization does not adversely affect the affinity towards the antigen. The fragments had low affinity towards CA6 negative cells (A2780 cell line). The ^{64}Cu labeled agents were evaluated by human serum stability studies and *in vivo* imaging and 24-hour biodistribution studies in nude mice bearing either WISH or A2780 subcutaneous tumors. The ^{64}Cu-DOTA-B-Fab was synthesized in high yield (RCY - 80%, SA - 55 GBq/μmole, >99% purity) and gave good results in 24 hour serum stability (94±5%,

n=3) tests. *In-vivo* biodistribution experiments (*see* Table 6) in xenograft tumor-bearing mice showed relatively high WISH tumor uptake (7.6±0.87 %ID/g, n=4, 20-206 mg) and low A2780 tumor uptake (5.1±0.92 %ID/g, n=3, 50-270 mg) combined with high tumor/muscle (8.7:1), tumor/blood (3.6:1) and positive/negative tumor (1.8, $p<0.05$, n=7) ratios. Tumor uptake was surpassed only by the kidneys (62±4 %ID/g) and liver (10±0.75 %ID/g).

Table 6. Summary of biodistribution studies in xenograft tumor bearing mice

	WISH tumor (%ID/g)	A2780 tumor (%ID/g)	WISH/A2780 ratio	Liver (%ID/g)	Kidneys (%ID/g)
^{64}Cu-DOTA-B-Fab	7.6±0.87	5.1±0.92	1.5	10±0.75	62±4
^{64}Cu-DOTA-anti lyzosyme-B-Fab	4.84±1.02	3.84±0.49	1.26	15.2±1.3	70±9.6
^{64}Cu-DOTA-anti-DM4-B-Fab	4.04±0.43	3.03±0.24	1.33	10.4±1.6	52±8.3

^{64}Cu-DOTA-B-Fab was further evaluated in WISH bearing tumor animals for specificity via blocking studies *in vivo*. Blocking was performed either by administration of B-Fab (2 mg, n=5) or DS6 (1 mg, n=4) at 2.5 or 25 hours respectively prior to the administration of the radiolabeled agent. B-Fab blocking afforded a 23% ($p<0.05$, n=8) decrease in WISH tumor uptake while DS6 blocking achieved a 26% ($p<0.05$, n=7) decrease. Control tumors (n=3) were not blocked in this study. These preclinical studies suggest that ^{64}Cu-DOTA-B-Fab is a suitable companion diagnostic for huDS6-DM4 in patients.

Example 4: *In vivo* PET studies of developed engineered antibodies in preclinical tumor models

a) B-Fab $(G4S)_2$ linker and C-terminal 6x-His tag

The B-Fab 1151 is a DS6-based engineered antibody like binding protein containing both L1 and L2 linkers as GGGGSGGGGS ($(G4S)_2$) (SEQ ID NO: 3) and containing a C-terminal 6x-His tag expressed. The protein was purified by IMAC and SEC and *in vitro* binding solution was prepared at 1.8 mg/ml in PBS and binding affinity was characterized using flow cytometry in the WISH CA6+ cell line (Figure 2C and Figure 2D).

b) B-Fab $(G4S)_2$ linker

The B-Fab 1153 is a DS6-based engineered antibody-like binding protein

containing a both L1 and L2 linkers as $(G4S)_2$ and not containing a C-terminal 6x-His tag. Since this protein was lacking a His tag the protein was purified by IMAC, Kappa-select and SEC. The *in vitro* binding solution was prepared at 1.6 mg/ml in PBS and binding affinity was characterized using flow cytometry in the WISH CA6+ cell line (Figure 3C and Figure 3D).

c) B-Fab G4S linker and C-terminal 6x-His tag

The B-Fab 1152 is a DS6-based engineered antibody-like binding protein containing a both L1 and L2 linkers as a single copy of G4S and containing a C-terminal 6x-His tag. The protein was purified by IMAC and SEC and the *in vitro* binding solution was prepared at 1.8 mg/ml in PBS and binding affinity was characterized using flow cytometry in the WISH CA6+ cell line (Figure 4C and Figure 4D).

Example 5: *In vitro* binding results for DS6 with and without DOTA

In order to show that DOTA conjugation does not alter DS6 activity, binding of the DS6 antibody-like binding protein and the DOTA-DS6 conjugated antibody was tested *in vitro* on CA6 positive WISH cell line and the A2780 CA6 negative cell line. Fluorescent activated cell sorting experiments determined that DOTA conjugation does not alter DS6 binding in the CA6 positive WISH cells (*see* Figure 5).

Example 6: Radiolabeling and Stability of ^{64}Cu-DOTA-DS6 B-Fab antibody-like binding protein

Human serum stability studies and 24-hour biodistribution studies using DOTA conjugated DS6 B-Fab labeled with copper-64 were performed in nude mice bearing either CA6 positive (WISH) or CA6 negative (A2780) subcutaneous tumors were. Radiochemistry studies show that the ^{64}Cu-DOTA-DS6 B-Fab engineered antibody-like binding protein has a radiochemical yield of ~30%, a radiochemical purity of >95%, and a specific activity of 1.9 Ci/μmole (*see* Figure 6A). Serum stability experiments of the ^{64}Cu-DOTA-DS6 B-Fab antibody-like binding protein were performed for 24 hours in human serum at 37°C, and show 97.2% activity at the 0 hour time point and 96.3% activity at the 24 hour time point, demonstrating no significant loss in activity after 24 hours (*see* Figure 6B).

Example 7: Comparison between antibody-like binding proteins having different scaffolds

The properties of the B-Fab antibody-like binding protein described above were compared with those of a diabody that specifically binds CA6, in order to assess which scaffold would be the most suitable for use as an imaging companion diagnostic. The amino acid sequence of that anti-CA6 diabody, which forms a homodimer, is shown as SEQ ID NO: 12 (Table 7). In some experiments, that homodimeric diabody further comprised a C-terminal tag selected from the group consisting of a GGC tag, a tag of SEQ ID NO: 13, and a tag of SEQ ID NO: 14.

As shown in Figure 7, the diabody has a scaffold that is different from the scaffold of the B-Fab antibody-like binding protein. For instance, it does not comprise any C_L or any C_{H1} domain.

Table 7. Polypeptide sequence for the diabody that specifically binds CA6

Amino acid sequence for the diabody (SEQ ID NO: 12)	EIVLTQSPATMSASPGERVTITCSAHSSVSFMHWFQQKPGTSPKLWIY STSSLASGVPARFGGSGSGTSYSLTISSMEAEDAATYYCQQRSSFPLT FGAGTKLELKGGGSGGGGEAQLVQSGAEVVKPGASVKMSCKASGYTFT SYNMHWVKQTPGQGLEWIGYIYPGNGATNYNQKFQGKATLTADPSSST AYMQISSLTSEDSAVYFCARGDSVPFAYWGQGTLVTVSA
C-terminal tag (SEQ ID NO: 13)	GGHHHHHH
C-terminal tag (SEQ ID NO: 14)	GGCGGHHHHHH

The results are shown in tables 8A to 8C below. These results were obtained by comparing a diabody of SEQ ID NO: 12 fused to a tag of SEQ ID NO: 14, with the B-Fab 1153 (which contains a (G4S)2 linker but no His-tag).

Table 8A. Comparison between the B-Fab and the diabody

Scaffold	Aggregation (SEC)	In Vitro Stability: Freeze/thaw stress (3X - 80°C-->RT)	In Vitro Stability: Heat Stress (1 week at 42°C)	FACS binding assay (Free) Kd app (nM)	FACS binding assay (DOTA Conjugate) Kd app (nM)
B-Fab	>95% monomeric	Well tolerated	Well tolerated	4	7.4
Diabody	>95% monomeric	Well tolerated	Significant loss (~25%) of soluble protein	10	7.5

Table 8B. Comparison between the B-Fab and the diabody

Scaffold	Radio chemical yield	Radio chemical purity	Specific acitivity (Ci/μMole)	Serum stability of radiolabeled scaffolds: 24 h at 37°C
B-Fab	40 – 80%	>99%	1.5	>90% monomeric
Diabody	10 – 15%	>99%	0.3	~40% monomeric

Table 8C. Comparison between the B-Fab and the diabody

Scaffold	Tumor signal in antigen positive tumor model (WISH) (%ID/g) 24 hrs P.I.	Tumor signal in antigen negative tumor model (A2780) (%ID/g) 24 hrs P.I.	Tumor to muscle ratio in antigen positive tumor model (WISH) 24 hrs P.I.	Tumor to blood ratio in antigen positive tumor model (A2780) 24 hrs P.I.	Primary route of clearance	Biodistribution Profile 24 hrs Post Injection (P.I.)

B-Fab	7.5 – 10.4 [Three independent expts]	4 – 5.6 [Three independent expts]	9 – 11 [Three independent expts]	4 – 6 [Three independent expts]	Renal	Yes Kidney: 57-73%ID/g Liver: 7-10%ID/g
Diabody	4.3	3.2	~4	~2	Renal	Yes Kidney: 104%ID/g Liver: 10%IDg

From these results it can be concluded that the B-Fab is better than the diabody for use as a companion diagnostic. For example, the diabody presented degradation in the plasma stability assay and in the temperature stress assay, whereas the B-Fab was stable in both assays.

While the invention has been described in terms of various embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the appended claims cover all such equivalent variations that come within the scope of the invention as claimed. In addition, the section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

Each embodiment herein described may be combined with any other embodiment or embodiments unless clearly indicated to the contrary. In particular, any feature or embodiment indicated as being preferred or advantageous may be combined with any other feature or features or embodiment or embodiments indicated as being preferred or advantageous, unless clearly indicated to the contrary.

All references cited in this application are expressly incorporated by reference herein.

WHAT IS CLAIMED IS:

1. An antibody-like binding protein that specifically binds CA6, wherein the antibody-like binding protein comprises two polypeptides having structures represented by the formulas [I] and [II] below:

$$V_{L1}\text{-}L_1\text{-}V_{L2}\text{-}C_L \qquad [I]$$

$$V_{H1}\text{-}L_2\text{-}V_{H2}\text{-}C_{H1} \qquad [II]$$

wherein:

V_{L1} is a DS6-based immunoglobulin light chain variable domain;

V_{L2} is a DS6-based immunoglobulin light chain variable domain;

V_{H1} is a DS6-based immunoglobulin heavy chain variable domain;

V_{H2} is a DS6-based immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain, such as a human IGKC immunoglobulin light chain constant domain;

C_{H1} is an immunoglobulin C_{H1} heavy chain constant domain, such as a human immunoglobulin C_{H1} heavy chain constant domain;

L_1 and L_2 are amino acid linkers;

wherein the polypeptides of formula I and the polypeptides of formula II form a bivalent monospecific tandem immunoglobulin antibody-like binding protein; and

wherein the antibody-like protein further comprises a chelator.

2. The antibody-like binding protein of claim 1, wherein L_1 is GGGGS or GGGGSGGGGS (SEQ ID NO: 3).

3. The antibody-like binding protein of claim 1 or 2, wherein L_2 is GGGGS or GGGGSGGGGS (SEQ ID NO: 3).

4. The antibody-like binding protein of any one of claims 1 to 3, wherein L_1 and L_2 are identical.

5. The antibody-like binding protein of any one of claims 1 to 4, wherein V_{L1} and

V_{L2} are identical.

6. The antibody-like binding protein of any one of claims 1 to 5, wherein V_{H1} and V_{H2} are identical.

7. The antibody-like binding protein of any one of claims 1 to 6, wherein the polypeptide of formula [I] comprises SEQ ID NO: 1.

8. The antibody-like binding protein of any one of claims 1 to 7, wherein the polypeptide of formula [II] comprises SEQ ID NO: 7.

9. The antibody-like binding protein of any one of claims 1 to 8, wherein the chelator is a hard acid cation chelator.

10. The antibody-like binding protein of claim 9, wherein the hard acid cation chelator is DOTA (1, 4, 7, 10-tetraazacyclododecane-1, 4, 7, 10-tetraacetic acid), NOTA (1, 4, 7-triaza-cyclononane-N,N',N''-triacetic acid), DTPA (1, 1, 4, 7, 7-Diethylenetriaminepentaacetic acid), or TETA (p-bromoacetamido-benzyl-tetraethylaminetetraacetic acid).

11. The antibody-like binding protein of claim 10, wherein the hard acid cation chelator is DOTA.

12. The antibody-like protein of any one of claims 9 to 11, wherein the hard acid chelator is covalently attached.

13. The antibody-like protein of claim 12, wherein between 1 and 5 copies of the hard acid chelator are covalently attached.

14. The antibody-like binding protein of any one of claims 1 to 13, further comprising a tag.

15. The antibody-like binding protein of claim 14, wherein the tag is 6x-Histidine, biotin, MYC, V5, FLAG, MBP, or glutathione-S-transferase or HA.

16. The antibody-like binding protein of claim 15, wherein the tag is a 6x-Histidine tag.

17. The antibody-like binding protein of any one of claims 1 to 16, further comprising at least one radiolabel, imaging agent, therapeutic agent, or diagnostic agent.

18. The antibody-like binding protein of claim 17, wherein the radiolabel, imaging agent, therapeutic agent, or diagnostic agent is ^{110}In, ^{111}In, ^{177}Lu, ^{18}F, ^{52}Fe, ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{67}Ga, ^{68}Ga, ^{86}Y, ^{90}Y, ^{89}Zr, ^{94}Tc, ^{94}Tc, ^{99m}Tc, ^{120}I, ^{123}I, ^{124}I, ^{125}I, ^{131}I, $^{154\text{-}158}$Gd, ^{32}P, ^{11}C, ^{13}N, ^{15}O, ^{186}Re, ^{188}Re, ^{51}Mn, ^{52m}Mn, ^{55}Co, ^{72}As, ^{75}Br, ^{76}Br, ^{82m}Rb, or ^{83}Sr.

19. The antibody-like binding protein of claim 18, wherein the radiolabel, imaging agent, therapeutic agent, or diagnostic agent is ^{64}Cu.

20. The antibody-like binding protein of claim 17, wherein the radiolabel, imaging agent, therapeutic agent, or diagnostic agent comprises one or more agents for magnetic resonance imaging (MRI); positron emission tomography (PET); single photon emission tomography (SPECT); optical imaging, computed tomography (CT); ultrasound, X-ray or photoacoustic imaging.

21. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and an amount of the antibody-like binding protein of any one of claims 1 to 20 effective to image a CA6 expressing tissue.

22. A method for making an antibody-like binding protein that specifically binds CA6, wherein the antibody-like binding protein further comprises a chelator, comprising:

(a) expressing in a cell one or more nucleic acid molecules encoding polypeptides having structures represented by the formulas [I] and [II] below:

$$V_{L1}\text{-}L_1\text{-}V_{L2}\text{-}C_L \qquad [I]$$

$$V_{H1}\text{-}L_2\text{-}V_{H2}\text{-}C_{H1} \qquad [II]$$

wherein:

V_{L1} is a DS6-based immunoglobulin light chain variable domain;

V_{L2} is a DS6-based immunoglobulin light chain variable domain;

V_{H1} is a DS6-based immunoglobulin heavy chain variable domain;

V_{H2} is a DS6-based immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain, such as a human IGKC immunoglobulin light chain constant domain;

C_{H1} is an immunoglobulin C_{H1} heavy chain constant domain, such as a human immunoglobulin C_{H1} heavy chain constant domain;

L_1 and L_2 are amino acid linkers; and

wherein the polypeptides of formula I and the polypeptides of formula II form a bivalent monospecific tandem immunoglobulin antibody-like binding protein; and

(b) attaching the antibody-like binding protein to the chelator.

23. The method of claim 22, wherein L_1 is GGGGS or GGGGSGGGGS (SEQ ID NO: 3).

24. The method of claim 22 or 23, wherein L_2 is GGGGS or GGGGSGGGGS (SEQ ID NO: 3).

25. The method of any one of claims 22 to 24, wherein L_1 and L_2 are identical.

26. The method of any one of claims 22 to 25, wherein V_{L1} and V_{L2} are identical.

27. The method of any one of claims 22 to 26, wherein V_{H1} and V_{H2} are identical.

28. The method of any one of claims 22 to 27, wherein the polypeptide of formula [I] comprises SEQ ID NO: 1.

29. The method of any one of claims 22 to 28, wherein the polypeptide of formula [II] comprises SEQ ID NO: 7.

30. The method of any one of claims 22 to 29, wherein the chelator is a hard acid cation chelator.

31. The method of claim 30, wherein the hard acid cation chelator is DOTA (1, 4, 7, 10-tetraazacyclododecane-1, 4, 7, 10-tetraacetic acid), NOTA (1, 4, 7-triaza-cyclononane-N,N',N''-triacetic acid), DTPA (1, 1, 4, 7, 7-Diethylenetriaminepentaacetic acid) or TETA (p-bromoacetamido-benzyl-tetraethylaminetetraacetic acid).

32. The method of claim 31, wherein the hard acid cation chelator is DOTA.

33. The method of any one of claims 22 to 32, wherein the hard acid chelator is covalently attached.

34. The method of any one of claims 22 to 33, wherein between 1 and 5 copies of the hard acid chelator are covalently attached.

35. A method for treating diseased tissue expressing CA6 in a patient comprising:

(a) administering an antibody-like binding protein that specifically binds CA6 and further comprises a chelator and an imaging agent to the patient;

(b) identifying the diseased tissue expressing CA6 in the patient by positron emission tomography (PET) imaging; and

(c) determining that the patient is a candidate for treatment with huDS6-DM4 if the CA6 expression in the diseased tissue is greater than or equal to 10% of CA6 expression in a reference standard.

36. The method of claim 35, further comprising administering huDS6-DM4 if the patient is determined to be a candidate for treatment with huDS6-DM4.

37. The method of claim 35 or 36, further comprising monitoring the subject's response to huDS6-DM4 using the antibody-like binding protein of step (a).

38. The method of any one of claims 35 to 37, wherein said antibody-like binding protein is the antibody-like binding protein of any one of claims 1 to 20.

39. A method of treating CA6 positive cancer in a patient, comprising the steps of:

(a) acquiring information regarding expression level of CA6 in the patient; and

(b) administering huDS6-DM4 to the patient if the expression level of CA6 in the patient is greater than or equal to 10% of CA6 expression in a reference standard.

40. The method of claim 39 wherein the information regarding expression level of CA6 in the patient is acquired by administering an antibody-like protein that specifically binds CA6 and comprises a chelator and an imaging agent, and detecting the localization of the imaging agent in the patient using a bioimaging technique.

41. The method of claim 40, wherein said antibody-like binding protein is the antibody-like binding protein of any one of claims 1 to 20.

42. The method of any one of claims 39 to 41, wherein the CA6 positive cancer is serous ovarian carcinoma, endometriod ovarian carcinoma, neoplasm of the uterine cervix, neoplasm of the endometrius, neoplasm of the vulva, breast carcinoma, pancreatic tumor, and tumor of the urothelium.

43. The method of any one of claims 39 to 42, wherein the information regarding expression level of CA6 in the patient is acquired by magnetic resonance imaging (MRI); positron emission tomography (PET); single photon emission tomography (SPECT);

optical imaging, computed tomography (CT); ultrasound, X-ray, or photoacoustic imaging.

44. The method of any one of claims 40 to 42, wherein the chelator is a hard acid cation chelator.

45. The method of claim 44, wherein the hard acid cation chelator is DOTA (1, 4, 7, 10-tetraazacyclododecane-1, 4, 7, 10-tetraacetic acid), NOTA (1, 4, 7-triaza-cyclononane-N,N',N''-triacetic acid), DTPA (1, 1, 4, 7, 7-Diethylenetriaminepentaacetic acid), or TETA (p-bromoacetamido-benzyl-tetraethylaminetetraacetic acid).

46. The method of claim 45, wherein the hard acid cation chelator is DOTA.

47. The method of any one of claims 40 to 46, wherein the antibody-like binding protein further comprises a tag.

48. The method of claim 47, wherein the tag is a 6x-Histidine tag.

49. The method of any one of claims 40 to 48, wherein the antibody-like binding protein further comprises at least one radiolabel, imaging agent, therapeutic agent, or diagnostic agent.

50. The method of claim 49, wherein the radiolabel, imaging agent, therapeutic agent, or diagnostic agent is ^{110}In, ^{111}In, ^{177}Lu, ^{18}F, ^{52}Fe, ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{67}Ga, ^{68}Ga, ^{86}Y, ^{90}Y, ^{89}Zr, ^{94}Tc, ^{94}Tc, ^{99m}Tc, ^{120}I, ^{123}I, ^{124}I, ^{125}I, ^{131}I, $^{154\text{-}158}$Gd, ^{32}P, ^{11}C, ^{13}N, ^{15}O, ^{186}Re, ^{188}Re, ^{51}Mn, ^{52m}Mn, ^{55}Co, ^{72}As, ^{75}Br, ^{76}Br, ^{82m}Rb, or ^{83}Sr.

51. The method of claim 50, wherein the radiolabel, imaging agent, therapeutic agent, or diagnostic agent is ^{64}Cu.

52. The method of any one of claims 49 to 51, wherein the radiolabel, imaging agent,

therapeutic agent, or diagnostic agent comprises one or more agents for magnetic resonance imaging (MRI); positron emission tomography (PET); single photon emission tomography (SPECT); optical imaging, computed tomography (CT); ultrasound, X-ray, or photoacoustic imaging.

53. A method of determining whether a cancer patient is a candidate for treatment with huDS6-DM4, comprising the steps of:

(a) acquiring information regarding expression level of CA6 in the patient; and

(b) determining that the patient is a candidate for treatment with huDS6-DM4 if the expression level of CA6 in the patient is greater than or equal to 10% of CA6 expression in a reference standard.

54. The method of claim 53, wherein the information regarding expression level of CA6 in the patient is acquired by administering an antibody-like protein that specifically binds CA6 and comprises a chelator and an imaging agent, and detecting the localization of the imaging agent in the patient using a bioimaging technique.

55. The method of claim 54, wherein said antibody-like binding protein is the antibody-like binding protein of any one of claims 1 to 20.

56. The method of any one of claims 53 to 55, wherein the information regarding expression level of CA6 in the patient is acquired by magnetic resonance imaging (MRI); positron emission tomography (PET); single photon emission tomography (SPECT); optical imaging, computed tomography (CT); ultrasound, X-ray or photoacoustic imaging.

57. The method of any one of claims 54 to 56, wherein the chelator is a hard acid cation chelator.

58. The method of claim 57, wherein the hard acid cation chelator is DOTA (1, 4, 7,

10-tetraazacyclododecane-1, 4, 7, 10-tetraacetic acid), NOTA (1, 4, 7-triaza-cyclononane-N,N',N''-triacetic acid), DTPA (1, 1, 4, 7, 7-Diethylenetriaminepentaacetic acid), or TETA (p-bromoacetamido-benzyl-tetraethylaminetetraacetic acid).

59. The method of claim 58, wherein the hard acid cation chelator is DOTA.

60. The method of claim 54, wherein the antibody-like binding protein further comprises tag.

61. The method of claim 60, wherein the tag is a 6x-Histidine tag.

62. The method of claim 54, wherein the antibody-like binding protein further comprises at least one radiolabel, imaging agent, therapeutic agent, or diagnostic agent.

63. The method of claim 62, wherein the radiolabel, imaging agent, therapeutic agent, or diagnostic agent is ^{110}In, ^{111}In, ^{177}Lu, ^{18}F, ^{52}Fe, ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{67}Ga, ^{68}Ga, ^{86}Y, ^{90}Y, ^{89}Zr, ^{94}Tc, ^{94}Tc, ^{99m}Tc, ^{120}I, ^{123}I, ^{124}I, ^{125}I, ^{131}I, $^{154\text{-}158}$Gd, ^{32}P, ^{11}C, ^{13}N, ^{15}O, ^{186}Re, ^{188}Re, ^{51}Mn, ^{52m}Mn, ^{55}Co, ^{72}As, ^{75}Br, ^{76}Br, ^{82m}Rb, or ^{83}Sr.

64. The method of claim 63, wherein the radiolabel, imaging agent, therapeutic agent, or diagnostic agent is ^{64}Cu.

65. The method of any one of claims 62 to 64, wherein the radiolabel, imaging agent, therapeutic agent, or diagnostic agent comprises one or more agents for magnetic resonance imaging (MRI); positron emission tomography (PET); single photon emission tomography (SPECT); optical imaging, computed tomography (CT); ultrasound, X-ray or photoacoustic imaging.

66. A method of monitoring a cancer patient's response to treatment with huDS6-DM4, comprising the steps of:

(a) acquiring information regarding expression level of CA6 in the patient following administration of the huDS6-DM4; and

(b) providing an indication that treatment should be continued if the level of CA6 expression in the patient has decreased as compared to the level of CA6 expression before treatment with the huDS6-DM4.

67. The method of claim 66, wherein the information regarding expression level of CA6 in the patient is acquired by:

treating the patient with huDS6-DM4 for a prescribed period of time;

administering an antibody-like protein that specifically binds CA6 and comprises a chelator and an imaging agent; and

detecting the localization of the imaging agent in the patient using a bioimaging technique.

68. The method of claim 67, wherein said antibody-like binding protein that specifically binds CA6 is the antibody-like binding protein of any one of claims 1 to 20.

69. The method of any one of claims 66 to 68, the information regarding expression level of CA6 in the patient is acquired by magnetic resonance imaging (MRI); positron emission tomography (PET); single photon emission tomography (SPECT); optical imaging, computed tomography (CT); ultrasound, X-ray or photoacoustic imaging.

70. The method of any one of claims 67 to 68, wherein the chelator is a hard acid cation chelator.

71. The method of claim 70, wherein the hard acid cation chelator is DOTA (1, 4, 7, 10-tetraazacyclododecane-1, 4, 7, 10-tetraacetic acid), NOTA (1, 4, 7-triaza-cyclononane-N,N',N''-triacetic acid), DTPA (1, 1, 4, 7, 7-Diethylenetriaminepentaacetic acid), or TETA (p-bromoacetamido-benzyl-tetraethylaminetetraacetic acid).

72. The method of claim 71, wherein the hard acid cation chelator is DOTA.

73. The method of any one of claims 67 to 72, wherein the antibody-like binding protein further comprises a tag.

74. The method of claim 73, wherein the tag is a 6x-Histidine tag.

75. The method of any one of claims 67 to 74, wherein the antibody-like binding protein further comprises at least one radiolabel, imaging agent, therapeutic agent, or diagnostic agent.

76. The method of claim 75, wherein the radiolabel, imaging agent, therapeutic agent, or diagnostic agent is ^{110}In, ^{111}In, ^{177}Lu, ^{18}F, ^{52}Fe, ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{67}Ga, ^{68}Ga, ^{86}Y, ^{90}Y, ^{89}Zr, ^{94}Tc, ^{94}Tc, ^{99m}Tc, ^{120}I, ^{123}I, ^{124}I, ^{125}I, ^{131}I, $^{154\text{-}158}$Gd, ^{32}P, ^{11}C, ^{13}N, ^{15}O, ^{186}Re, ^{188}Re, ^{51}Mn, ^{52m}Mn, ^{55}Co, ^{72}As, ^{75}Br, ^{76}Br, ^{82m}Rb, or ^{83}Sr.

77. The method of claim 76, wherein the radiolabel, imaging agent, therapeutic agent, or diagnostic agent is ^{64}Cu.

78. The method of any one of claims 75 to 77, wherein the wherein the radiolabel, imaging agent, therapeutic agent, or diagnostic agent comprises one or more agents for magnetic resonance imaging (MRI); positron emission tomography (PET); single photon emission tomography (SPECT); optical imaging, computed tomography (CT); ultrasound, X-ray, or photoacoustic imaging.