absence of exogenously added HER3 ligand. In some embodiments, the inhibitory activity can be 20%, 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95%, or even 100%.

[0072] The term "inhibition of HER3 ligand-dependent growth of tumor cells" refers to the ability of a molecule to inhibit the growth of tumor cells in media containing exogenously added HER3 ligand. In some embodiments, the inhibitory activity can be 20%, 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95%, or even 100%.

[0073] The term "anti-HER3 antibody" or "an antibody that binds to HER3" refers to an antibody that is capable of binding HER3 with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting HER3. The extent of binding of an anti-HER3 antibody to an unrelated, non-HER3 protein can be less than about 10% (e.g., about 9%, about 8%, about 7%, about 6%, about 5%, about 4%, about 3%, about 2%, or about 1%) of the binding of the antibody to HER3 as measured, e.g., by a radioimmunoassay (RIA). In certain embodiments, an antibody that binds to HER3 has a dissociation constant (Kd) of ≤1 μM, ≤100 nM, ≤10 nM, ≤5 nM, ≤1 nM, ≤0.9 nM, ≤0.8 nM, ≤0.7 nM, ≤0.6 nM, ≤0.5 nM, ≤0.4 nM, ≤0.3 nM, ≤0.2 nM, or ≤0.1 nM.

[0074] The term "antibody fragment" refers to a portion of an intact antibody and refers to the antigenic determining variable regions of an intact antibody. Examples of antibody fragments include, but are not limited to Fab, Fab', F(ab')2, and Fv fragments, linear antibodies, single chain antibodies, and multispecific antibodies formed from antibody fragments.

[0075] A "monoclonal antibody" refers to a homogeneous antibody population involved in the highly specific recognition and binding of a single antigenic determinant, or epitope. This is in contrast to polyclonal antibodies that typically include different antibodies directed against different antigenic determinants. The term "monoclonal antibody" encompasses both intact and full-length monoclonal antibodies as well as antibody fragments (such as Fab, Fab', F(ab')2, Fv), single chain (scFv) mutants, fusion proteins comprising an antibody portion, and any other modified immunoglobulin molecule comprising an antigen recognition site. Furthermore, "monoclonal antibody" refers to such antibodies made in any number of manners including but not limited to by hybridoma, phage selection, recombinant expression, and transgenic animals.

[0076] The term "humanized antibody" refers to forms of non-human (e.g., murine) antibodies that are specific immunoglobulin chains, chimeric immunoglobulins, or fragments thereof that contain minimal non-human (e.g., murine) sequences. Typically, humanized

antibodies are human immunoglobulins in which residues from the complementary determining region (CDR) are replaced by residues from the CDR of a non-human species (e.g., mouse, rat, rabbit, hamster) that have the desired specificity, affinity, and capability (Jones et al., 1986, *Nature*, 321:522-525; Riechmann et al., 1988, *Nature*, 332:323-327; Verhoeyen et al., 1988, *Science*, 239:1534-1536). In some instances, the Fv framework region (FR) residues of a human immunoglobulin are replaced with the corresponding residues in an antibody from a non-human species that has the desired specificity, affinity, and capability. The humanized antibody can be further modified by the substitution of additional residues either in the Fv framework region and/or within the replaced non-human residues to refine and optimize antibody specificity, affinity, and/or capability. In general, the humanized antibody will comprise substantially all of at least one, and typically two or three, variable domains containing all or substantially all of the CDR regions that correspond to the non-human immunoglobulin whereas all or substantially all of the FR residues are those of a human immunoglobulin consensus sequence. The humanized antibody can also comprise at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin. Examples of methods used to generate humanized antibodies are described in U.S. Pat. 5,225,539.

[0077] A "variable region" of an antibody refers to the variable region of the antibody light chain or the variable region of the antibody heavy chain, either alone or in combination. The variable regions of the heavy and light chain each consist of four framework regions (FR) connected by three complementarity determining regions (CDRs) also known as hypervariable regions. The CDRs in each chain are held together in close proximity by the FRs and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies. There are at least two techniques for determining CDRs: (1) an approach based on cross-species sequence variability (see Kabat et al. Sequences of Proteins of Immunological Interest, (5th ed., 1991, National Institutes of Health, Bethesda Md.)); and (2) an approach based on crystallographic studies of antigen-antibody complexes (see Al-lazikani et al (1997) J. *Molec. Biol.* 273:927-948)). In addition, combinations of these two approaches are sometimes used in the art to determine CDRs.

[0078] The Kabat numbering system is generally used when referring to a residue in the variable domain (approximately residues 1-107 of the light chain and approximately residues 1-113 of the heavy chain) (see Kabat et al., Sequences of Immunological Interest. 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)).

[0079] The amino acid position numbering as in Kabat, refers to the numbering system used for heavy chain variable domains or light chain variable domains of the compilation of antibodies in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991). Using this numbering system, the actual linear amino acid sequence can contain fewer or additional amino acids corresponding to a shortening of, or insertion into, a FR or CDR of the variable domain. For example, a heavy chain variable domain can include a single amino acid insert (residue 52a according to Kabat) after residue 52 of H2 and inserted residues (e.g., residues 82a, 82b, and 82c, etc., according to Kabat) after heavy chain FR residue 82. The Kabat numbering of residues can be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence. The Chothia numbering system also can be used when referring to a residue in the variable domain (see Chothia and Lesk J. Mol. Biol. 196:901-917 (1987)). The Chothia numbering system refers instead to the location of the structural loops (Chothia and Lesk J. Mol. Biol. 196:901-917 (1987)). The end of the Chothia CDR-H1 loop when numbered using the Kabat numbering convention varies between H32 and H34 depending on the length of the loop (this is because the Kabat numbering scheme places the insertions at H35A and H35B; if neither 35A nor 35B is present, the loop ends at 32; if only 35A is present, the loop ends at 33; if both 35A and 35B are present, the loop ends at 34). The AbM hypervariable regions represent a compromise between the Kabat CDRs and Chothia structural loops, and are used by Oxford Molecular's AbM antibody modeling software.

Loop	Kabat	AbM	Chothia
L1	L24-L34	L24-L34	L24-L34
L2	L50-L56	L50-L56	L50-L56
L3	L89-L97	L89-L97	L89-L97
H1	H31-H35B	H26-H35B (Kabat Numbering)	H26-H32..34
H1	H31-H35	H26-H35 (Chothia Numbering)	H26-H32
H2	H50-H65	H50-H58	H52-H56
H3	H95-H102	H95-H102	H95-H102

[0080] The term "human antibody" means an antibody produced by a human or an antibody having an amino acid sequence corresponding to an antibody produced by a human

made using any technique known in the art. This definition of a human antibody includes intact or full-length antibodies, fragments thereof, and/or antibodies comprising at least one human heavy and/or light chain polypeptide such as, for example, an antibody comprising murine light chain and human heavy chain polypeptides.

[0081] The term "chimeric antibodies" refers to antibodies wherein the amino acid sequence of the immunoglobulin molecule is derived from two or more species. Typically, the variable region of both light and heavy chains corresponds to the variable region of antibodies derived from one species of mammals (e.g., mouse, rat, rabbit, etc.) with the desired specificity, affinity, and capability, while the constant regions are homologous to the sequences in antibodies derived from another (usually human) to avoid eliciting an immune response in that species.

[0082] The term "epitope" or "antigenic determinant" are used interchangeably herein and refer to that portion of an antigen capable of being recognized and specifically bound by a particular antibody. When the antigen is a polypeptide, epitopes can be formed both from contiguous amino acids and noncontiguous amino acids juxtaposed by tertiary folding of a protein. Epitopes formed from contiguous amino acids are typically retained upon protein denaturing, whereas epitopes formed by tertiary folding are typically lost upon protein denaturing. An epitope typically includes at least 3, and more usually, at least 5 or 8-10 amino acids in a unique spatial conformation.

[0083] "Binding affinity" generally refers to the strength of the sum total of noncovalent interactions between a single binding site of a molecule (e.g., an antibody) and its binding partner (e.g., an antigen). Unless indicated otherwise, as used herein, "binding affinity" refers to intrinsic binding affinity which reflects a 1:1 interaction between members of a binding pair (e.g., antibody and antigen). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (Kd). Affinity can be measured by common methods known in the art, including those described herein. Low-affinity antibodies generally bind antigen slowly and tend to dissociate readily, whereas high-affinity antibodies generally bind antigen faster and tend to remain bound longer. A variety of methods of measuring binding affinity are known in the art, any of which can be used for purposes of the present invention. Specific illustrative embodiments are described in the following.

[0084] "Or better" when used herein to refer to binding affinity refers to a stronger binding between a molecule and its binding partner. "Or better" when used herein refers to a stronger binding, represented by a smaller numerical Kd value. For example, an antibody

which has an affinity for an antigen of "0.6 nM or better," the antibody's affinity for the antigen is <0.6 nM, e.g., 0.59 nM, 0.58 nM, 0.57 nM, etc., or any value less than 0.6 nM.

[0085] By "specifically binds," it is generally meant that an antibody binds to an epitope via its antigen binding domain, and that the binding entails some complementarity between the antigen binding domain and the epitope. According to this definition, an antibody is said to "specifically bind" to an epitope when it binds to that epitope, via its antigen binding domain more readily than it would bind to a random, unrelated epitope. The term "specificity" is used herein to qualify the relative affinity by which a certain antibody binds to a certain epitope. For example, antibody "A" may be deemed to have a higher specificity for a given epitope than antibody "B," or antibody "A" may be said to bind to epitope "C" with a higher specificity than it has for related epitope "D."

[0086] By "preferentially binds," it is meant that the antibody specifically binds to an epitope more readily than it would bind to a related, similar, homologous, or analogous epitope. Thus, an antibody which "preferentially binds" to a given epitope would more likely bind to that epitope than to a related epitope, even though such an antibody may cross-react with the related epitope.

[0087] An antibody is said to "competitively inhibit" binding of a reference antibody to a given epitope if it preferentially binds to that epitope to the extent that it blocks, to some degree, binding of the reference antibody to the epitope. Competitive inhibition may be determined by any method known in the art, for example, competition ELISA assays. An antibody may be said to competitively inhibit binding of the reference antibody to a given epitope by at least 95%, at least 90%, at least 85%, at least 80%, at least 70%, at least 60%, or at least 50%.

[0088] The phrase "substantially similar," or "substantially the same," as used herein, denotes a sufficiently high degree of similarity between two numeric values (generally one associated with an antibody of the invention and the other associated with a reference/comparator antibody), such that one of skill in the art would consider the difference between the two values to be of little or no biological and/or statistical significance within the context of the biological characteristic measured by said values (e.g., Kd values). The difference between said two values can be less than about 50%, less than about 40%, less than about 30%, less than about 20%, or less than about 10% as a function of the value for the reference/comparator antibody.

[0089] A polypeptide, antibody, polynucleotide, vector, cell, or composition which is "isolated" is a polypeptide, antibody, polynucleotide, vector, cell, or composition which is in a form not found in nature. Isolated polypeptides, antibodies, polynucleotides, vectors, cell or compositions include those which have been purified to a degree that they are no longer in a form in which they are found in nature. In some embodiments, an antibody, polynucleotide, vector, cell, or composition which is isolated is substantially pure.

[0090] As used herein, "substantially pure" refers to material which is at least 50% pure (i.e., free from contaminants), at lest 60% pure, at least 70% pure, at least 80% pure, at least 90% pure, at least 95% pure, at least 96% pure, at least 97% pure, at least 98% pure, or at least 99% pure.

[0091] The term "immunoconjugate" or "conjugate" as used herein refers to a compound or a derivative thereof that is linked to a cell binding agent (i.e., an anti-HER3 antibody or fragment thereof) and is defined by a generic formula: C-L-A, wherein C = cytotoxin, L = linker, and A = cell binding agent or anti-HER3 antibody or antibody fragment. Immunoconjugates can also be defined by the generic formula in reverse order: A-L-C.

[0092] A "linker" is any chemical moiety that is capable of linking a compound, usually a drug, such as a maytansinoid, to a cell-binding agent such as an anti HER3 antibody or a fragment thereof in a stable, covalent manner. Linkers can be susceptible to or be substantially resistant to acid-induced cleavage, light-induced cleavage, peptidase-induced cleavage, esterase-induced cleavage, and disulfide bond cleavage, at conditions under which the compound or the antibody remains active. Suitable linkers are well known in the art and include, for example, disulfide groups, thioether groups, acid labile groups, photolabile groups, peptidase labile groups and esterase labile groups. Linkers also include charged linkers, and hydrophilic forms thereof as described herein and known in the art.

[0093] The terms of "tumor" and "neoplasm" refer to a solid lesion formed by an abnormal growth of cells (termed neoplastic) which can be benign, pre-malignant or malignant. The term of "cancer" refer to malignant tumor/neoplasm that describes a class of diseases in which a population of cells display uncontrolled growth (division beyond the normal limits), invasion (intrusion on and destruction of adjacent tissues), and sometimes metastasis (spread to other locations in the body via lymph or blood). Examples of cancer include, but are not limited to carcinoma, sarcoma, lymphoma, leukemia, blastoma, and germ cell tumor. Examples of "cancer" or "tumorigenic" diseases which can be treated and/or prevented include acute lymphoblastic leukemia, acute myeloid leukemia, adrenocortical

carcinoma, anal cancer, basal cell carcinoma, bile duct cancer, bladder cancer, bone cancer, brain tumors (including astrocytoma, brain stem glioma, craniopharyngioma, ependymoblastoma, ependymoma, medulloblastoma, medulloepithelioma, pineal parenchymal tumors of intermediate differentiation, supratentorial primitive neuroectodermal tumors, and pineoblastoma), breast cancer, bronchial tumors, Burkitt lymphoma, carcinoid tumors, central nervous system atypical teratoid/rhabdoid tumors, cervical cancer, chordoma, chronic lymphocytic leukemia, chronic myelogenous leukemia, chronic myeloproliferative disorders, colon cancer, colorectal cancer, cutaneous T-Cell lymphoma, embryonal tumors, endometrial cancer, esophageal cancer, Ewing sarcoma, gallbladder cancer, gastric cancer, germ cell tumors, hairy cell leukemia, head and neck cancer, hepatocellular cancer, Hodgkin lymphoma, hypopharyngeal cancer, intraocular melanoma, islet cell tumors, Kaposi sarcoma, kidney cancer, Langerhans cell histiocytosis, laryngeal cancer, liver cancer, malignant fibrous histiocytoma of bone and osteosarcoma, melanoma, Merkel cell carcinoma, mesothelioma, mouth cancer, multiple myeloma/plasma cell neoplasm, mycosis fungoides, myelodysplastic/myeloproliferative neoplasms, nasal cavity and paranasal sinus cancer, nasopharyngeal cancer, neuroblastoma, non-Hodgkin lymphoma, non-small cell lung cancer, oral cancer, oropharyngeal cancer, ovarian cancer, pancreatic cancer, parathyroid cancer, penile cancer, pharyngeal cancer, pituitary tumor, prostate cancer, rectal cancer, renal cell cancer, retinoblastoma, rhabdomyosarcoma, salivary gland cancer, Sézary syndrome, skin cancer (nonmelanoma), small cell lung cancer, small intestine cancer, soft tissue sarcoma, squamous cell carcinoma, squamous neck cancer, stomach cancer, T-cell lymphoma, testicular cancer, throat cancer, thymoma and thymic carcinoma, thyroid cancer, urethral cancer, uterine cancer, vaginal cancer, vulvar cancer, Waldenström macroglobulinemia, and Wilms tumor.

[0094] The terms “cancer cell,” “tumor cell,” and grammatical equivalents refer to the total population of cells derived from a tumor or a pre-cancerous lesion, including both non-tumorigenic cells, which comprise the bulk of the tumor cell population, and tumorigenic cells, including cancer stem cells. As used herein, the term “tumor cell” will be modified by the term “non-tumorigenic” when referring solely to those tumor cells lacking the capacity to renew and differentiate to distinguish those tumor cells from cancer stem cells.

[0095] The term “subject” refers to any animal (e.g., a mammal), including, but not limited to humans, non-human primates, rodents, and the like, which is to be the recipient of

a particular treatment. Typically, the terms "subject" and "patient" are used interchangeably herein in reference to a human subject.

[0096] Administration "in combination with" one or more further therapeutic agents includes simultaneous (concurrent) and consecutive administration in any order.

[0097] The term "pharmaceutical formulation" refers to a preparation which is in such form as to permit the biological activity of the active ingredient to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered. The formulation can be sterile.

[0098] An "effective amount" of an antibody as disclosed herein is an amount sufficient to carry out a specifically stated purpose. An "effective amount" can be determined empirically and in a routine manner, in relation to the stated purpose.

[0099] The term "therapeutically effective amount" refers to an amount of an antibody or other drug effective to "treat" a disease or disorder in a subject or mammal. In the case of cancer, the therapeutically effective amount of the drug can reduce the number of cancer cells; reduce the tumor size; inhibit (i.e., slow to some extent or stop) cancer cell infiltration into peripheral organs; inhibit (i.e., slow to some extent or stop) tumor metastasis; inhibit, to some extent, tumor growth; and/or relieve to some extent one or more of the symptoms associated with the cancer. See the definition herein of "treating". To the extent the drug can prevent growth and/or kill existing cancer cells, it can be cytostatic and/or cytotoxic. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically but not necessarily, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount will be less than the therapeutically effective amount.

[0100] The word "label" when used herein refers to a detectable compound or composition which is conjugated directly or indirectly to the antibody so as to generate a "labeled" antibody. The label can be detectable by itself (e.g., radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, can catalyze chemical alteration of a substrate compound or composition which is detectable.

[0101] A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer, regardless of mechanism of action. Chemotherapeutic agents include, for example, cisplatin, paclitaxel, gemcitabine, cyclophosphamide, doxorubicin, vincristine, prednisone, fludarabine, etoposide, methotrexate, lenalidomide, chlorambucil, bentamustine and/or modified versions of such chemotherapeutics.

[0102] Terms such as "treating" or "treatment" or "to treat" or "alleviating" or "to alleviate" refer to both 1) therapeutic measures that cure, slow down, lessen symptoms of, and/or halt progression of a diagnosed pathologic condition or disorder and 2) prophylactic or preventative measures that prevent and/or slow the development of a targeted pathologic condition or disorder. Thus, those in need of treatment include those already having the disorder; those prone to have the disorder; and those in whom the disorder is to be prevented. In certain embodiments, a subject is successfully "treated" for cancer according to the methods of the present invention if the patient shows one or more of the following: a reduction in the number of or complete absence of cancer cells; a reduction in the tumor size; inhibition of or an absence of cancer cell infiltration into peripheral organs including, for example, the spread of cancer into soft tissue and bone; inhibition of or an absence of tumor metastasis; inhibition or an absence of tumor growth; relief of one or more symptoms associated with the specific cancer; reduced morbidity and mortality; improvement in quality of life; reduction in tumorigenicity, tumorgenic frequency, or tumorgenic capacity, of a tumor; reduction in the number or frequency of cancer stem cells in a tumor; differentiation of tumorigenic cells to a non-tumorigenic state; or some combination of effects.

[0103] "Polynucleotide," or "nucleic acid," as used interchangeably herein, refer to polymers of nucleotides of any length, and include DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a polymer by DNA or RNA polymerase. A polynucleotide can comprise modified nucleotides, such as methylated nucleotides and their analogs. If present, modification to the nucleotide structure can be imparted before or after assembly of the polymer. The sequence of nucleotides can be interrupted by non-nucleotide components. A polynucleotide can be further modified after polymerization, such as by conjugation with a labeling component. Other types of modifications include, for example, "caps," substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, cabamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example, proteins (e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as

unmodified forms of the polynucleotide(s). Further, any of the hydroxyl groups ordinarily present in the sugars can be replaced, for example, by phosphonate groups, phosphate groups, protected by standard protecting groups, or activated to prepare additional linkages to additional nucleotides, or can be conjugated to solid supports. The 5' and 3' terminal OH can be phosphorylated or substituted with amines or organic capping group moieties of from 1 to 20 carbon atoms. Other hydroxyls can also be derivatized to standard protecting groups. Polynucleotides can also contain analogous forms of ribose or deoxyribose sugars that are generally known in the art, including, for example, 2'-O-methyl-, 2'-O-allyl, 2'-fluoro- or 2'-azido-ribose, carbocyclic sugar analogs, .alpha.-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs and abasic nucleoside analogs such as methyl riboside. One or more phosphodiester linkages can be replaced by alternative linking groups. These alternative linking groups include, but are not limited to, embodiments wherein phosphate is replaced by P(O)S ("thioate"), P(S)S ("dithioate"), "(O)NR_2 ("amidate"), P(O)R, P(O)OR', CO or CH_2 ("formacetal"), in which each R or R' is independently H or substituted or unsubstituted alkyl (1-20 C) optionally containing an ether (--O--) linkage, aryl, alkenyl, cycloalkyl, cycloalkenyl or araldyl. Not all linkages in a polynucleotide need be identical. The preceding description applies to all polynucleotides referred to herein, including RNA and DNA.

[0104] The term "vector" means a construct, which is capable of delivering, and optionally expressing, one or more gene(s) or sequence(s) of interest in a host cell. Examples of vectors include, but are not limited to, viral vectors, naked DNA or RNA expression vectors, plasmid, cosmid or phage vectors, DNA or RNA expression vectors associated with cationic condensing agents, DNA or RNA expression vectors encapsulated in liposomes, and certain eukaryotic cells, such as producer cells.

[0105] The terms "polypeptide," "peptide," and "protein" are used interchangeably herein to refer to polymers of amino acids of any length. The polymer can be linear or branched, it can comprise modified amino acids, and it can be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component. Also included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), as well as other modifications known in the art. It is understood that, because the polypeptides of this

invention are based upon antibodies, in certain embodiments, the polypeptides can occur as single chains or associated chains.

[0106] The terms "identical" or percent "identity" in the context of two or more nucleic acids or polypeptides, refer to two or more sequences or subsequences that are the same or have a specified percentage of nucleotides or amino acid residues that are the same, when compared and aligned (introducing gaps, if necessary) for maximum correspondence, not considering any conservative amino acid substitutions as part of the sequence identity. The percent identity can be measured using sequence comparison software or algorithms or by visual inspection. Various algorithms and software are known in the art that can be used to obtain alignments of amino acid or nucleotide sequences. One such non-limiting example of a sequence alignment algorithm is the algorithm described in Karlin et al, 1990, *Proc. Natl. Acad. Sci.*, 87:2264-2268, as modified in Karlin et al., 1993, *Proc. Natl. Acad. Sci.*, 90:5873-5877, and incorporated into the NBLAST and XBLAST programs (Altschul et al., 1991, *Nucleic Acids Res.*, 25:3389-3402). In certain embodiments, Gapped BLAST can be used as described in Altschul et al., 1997, *Nucleic Acids Res.* 25:3389-3402. BLAST-2, WU-BLAST-2 (Altschul et al., 1996, *Methods in Enzymology*, 266:460-480), ALIGN, ALIGN-2 (Genentech, South San Francisco, California) or Megalign (DNASTAR) are additional publicly available software programs that can be used to align sequences. In certain embodiments, the percent identity between two nucleotide sequences is determined using the GAP program in GCG software (e.g., using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 90 and a length weight of 1, 2, 3, 4, 5, or 6). In certain alternative embodiments, the GAP program in the GCG software package, which incorporates the algorithm of Needleman and Wunsch (*J. Mol. Biol.* (48):444-453 (1970)) can be used to determine the percent identity between two amino acid sequences (e.g., using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5). Alternatively, in certain embodiments, the percent identity between nucleotide or amino acid sequences is determined using the algorithm of Myers and Miller (CABIOS, 4:11-17 (1989)). For example, the percent identity can be determined using the ALIGN program (version 2.0) and using a PAM120 with residue table, a gap length penalty of 12 and a gap penalty of 4. Appropriate parameters for maximal alignment by particular alignment software can be determined by one skilled in the art. In certain embodiments, the default parameters of the alignment software are used. In certain embodiments, the percentage identity "X" of a first amino acid sequence to a second

sequence amino acid is calculated as 100 x (Y/Z), where Y is the number of amino acid residues scored as identical matches in the alignment of the first and second sequences (as aligned by visual inspection or a particular sequence alignment program) and Z is the total number of residues in the second sequence. If the length of a first sequence is longer than the second sequence, the percent identity of the first sequence to the second sequence will be longer than the percent identity of the second sequence to the first sequence.

[0107] As a non-limiting example, whether any particular polynucleotide sequence has a certain percentage sequence identity (e.g., is at least 80% identical, at least 85% identical, at least 90% identical, and in some embodiments, at least 95%, 96%, 97%, 98%, or 99% identical) to a reference sequence can, in certain embodiments, be determined using the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). Bestfit uses the local homology algorithm of Smith and Waterman, *Advances in Applied Mathematics* 2: 482 489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

[0108] In some embodiments, two nucleic acids or polypeptides of the invention are substantially identical, meaning they have at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, and in some embodiments at least 95%, 96%, 97%, 98%, 99% nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using a sequence comparison algorithm or by visual inspection. Identity can exist over a region of the sequences that is at least about 10, about 20, about 40-60 residues in length or any integral value therebetween, and can be over a longer region than 60-80 residues, for example, at least about 90-100 residues, and in some embodiments, the sequences are substantially identical over the full length of the sequences being compared, such as the coding region of a nucleotide sequence for example.

[0109] A "conservative amino acid substitution" is one in which one amino acid residue is replaced with another amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art, including basic side

chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). For example, substitution of a phenylalanine for a tyrosine is a conservative substitution. In some embodiments, conservative substitutions in the sequences of the polypeptides and antibodies of the invention do not abrogate the binding of the polypeptide or antibody containing the amino acid sequence, to the antigen(s), i.e., the HER3, to which the polypeptide or antibody binds. Methods of identifying nucleotide and amino acid conservative substitutions which do not eliminate antigen binding are well- known in the art (see, e.g., Brummell et al., *Biochem.* 32: 1180-1 187 (1993); Kobayashi et al. *Protein Eng.* 12(10):879-884 (1999); and Burks et al. *Proc. Natl. Acad. Sci. USA* 94:.412-417 (1997)).

[0110] As used in the present disclosure and claims, the singular forms "a," "an," and "the" include both singular and plural forms unless the context clearly dictates otherwise.

[0111] It is understood that wherever embodiments are described herein with the language "comprising," otherwise analogous embodiments described in terms of "consisting of" and/or "consisting essentially of" are also provided.

[0112] The term "and/or" as used in a phrase such as "A and/or B" herein is intended to include both "A and B," "A or B," "A," and "B." Likewise, the term "and/or" as used in a phrase such as "A, B, and/or C" is intended to encompass each of the following embodiments: A, B, and C; A, B, or C; A or C; A or B; B or C; A and C; A and B; B and C; A (alone); B (alone); and C (alone).

II. HER3 binding agents

[0113] The present invention provides agents that specifically bind HER3. These agents are referred to herein as "HER3 binding agents." The full-length amino acid sequences for human HER3 (Genbank Accession Number NP_001973.2), macaca HER3 (Genbank Accession Number XP_001113953.1), and murine HER3 (NP_034283.1) are known in the art and also provided herein as represented by SEQ ID NOs:1-3, respectively.

[0114] In certain embodiments, the HER3 binding agents are antibodies, immunoconjugates or polypeptides. In some embodiments, the HER3 binding agents are humanized antibodies.

[0115] In certain embodiments, the HER3-binding agents have one or more of the following effects: inhibit proliferation of tumor cells, reduce the tumorigenicity of a tumor by reducing the frequency of cancer stem cells in the tumor, inhibit tumor growth, increase survival, trigger cell death of tumor cells, differentiate tumorigenic cells to a non-tumorigenic state, or prevent metastasis of tumor cells.

[0116] In certain embodiments, the HER3-binding agents are capable of inducing antibody dependent cell mediated cytotoxicity (ADCC). For example, treatment of cells with the HER3-binding agents can result in ADCC activity that produces at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or even 100% cell lysis. Treatment of cells with the HER3-binding agents can result in ADCC activity that produces about 10-20%, about 20-30%, about 30-40%, about 40-50%, about 50-60%, about 60-70%, about 70-80%, about 80-90%, or about 90-100% cell lysis. Treatment of cells with the HER3-binding agents can also result in ADCC activity that produces about 10-50%, about 20-50%, about 30-50%, about 30-60%, about 40-60%, about 40-70%, about 50-80%, about 60-90%, or about 70-100% cell lysis. In some particular embodiments, the HER3-binding agents are capable of inducing ADCC in SKBR3 and MCF7 (Figure 24).

[0117] In some embodiments, the HER3-binding agents are capable of inhibiting ligand-independent tumor cell growth. For example, treatment of cells with the HER3-binding agents can inhibit at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or even 100% of ligand-independent tumor cell growth. In some particular embodiments, the HER3-binding agents are capable of inhibiting cell growth in SKBR3, BT474, MDA-MB453 and ZR75-30 cell lines in a ligand-independent manner (Figures 1 and 17-20).

[0118] In certain embodiments, immunoconjugates or other agents that specifically bind human HER3 trigger cell death via a cytotoxic agent. For example, in certain embodiments, an antibody to human HER3 is conjugated to a maytansinoid that is activated in tumor cells

expressing HER3 antigen by internalization and intracellular processing. In certain alternative embodiments, the HER3-binding agent or antibody is not conjugated.

[0119] The HER3-binding agents include HER3 antibodies HER3-3, HER3-8, HER3-10, as described herein, and fragments, variants and derivatives thereof. The HER3-binding agents also include HER3-binding agents that specifically bind to the same HER3 epitope as an antibody selected from the group consisting of HER3-3, HER3-8, and HER3-10. In one embodiment, the HER3-binding agents specifically bind to to amino acid residues 20-342 of SEQ ID NO: 1 (human HER3). In a specific embodiment, the HER3-binding agents of the invention include an antibody or fragment thereof that specifically binds to amino acid residues 20-342 of SEQ ID NO: 1 (human HER3). In another specific embodiment, the HER3 antibody or fragment thereof binds to an epitope within amino acid residues 20-342 of SEQ ID NO: 1. The HER3-binding agents also include HER3-binding agents that competitively inhibit an antibody selected from the group consisting of HER3-3, HER3-8, and HER3-10.

[0120] The HER3-binding agents also include HER3-binding agents that comprise the heavy and light chain CDR sequences of HER3-3, HER3-8, or HER3-10 and their related sequences. The CDR sequences of HER3-3, HER3-8 and HER3-10 are described in Tables 1 and 2 below.

Table 1: Variable heavy chain CDR amino acid sequences

Antibody	VH-CDR1	VH-CDR2	VH-CDR3
HER3-3	TDYWIH (SEQ ID NO:4)	AIDTDTY (SEQ ID NO:5)	SLLRLLYFDY (SEQ ID NO:6)
		Kabat HC (murine) AIDTDTYTSYNQQFKG (SEQ ID NO:7)	
		Kabat HC (humanized) AIDTDTYTSYNQKFQG (SEQ ID NO:8)	
HER3-8	TSDYAWN (SEQ ID NO:9)	FISYSGS (SEQ ID NO:10)	Murine and humanized v1.01 KLGRLYYFDY (SEQ ID NO:11)
		Kabat HC (murine and humanized) FISYSGSTSYNPSLKS (SEQ ID NO:12)	Humanized v1.00 RLGRLYYFDY (SEQ ID NO: 13)
HER3-10	TDYTMN (SEQ ID NO:14)	LINPYNDI (SEQ ID NO:15)	RGPRGAWFAY (SEQ ID NO:16)
		Kabat HC CDR2 (murine) LINPYNDITAYNQKFKG (SEQ ID NO:17)	
		Kabat HC CDR2 (humanized) LINPYNDITAYNQKFQG (SEQ ID NO:18)	

Table 2: Variable light chain CDR amino acid sequences

Antibody	VL-CDR1	VL-CDR2	VL-CDR3
HER3-3	RASQSITDYLH (SEQ ID NO:19)	FASQSIS (SEQ ID NO:20)	QNGHSFPLT (SEQ ID NO:21)
HER3-8	RASQSINDYLH (SEQ ID NO:22)	FASHSIS (SEQ ID NO:23)	QNGHSFPWT (SEQ ID NO:24)
HER3-10	RASTSVYNYGNSFMH (SEQ ID NO:25)	LASNLES (SEQ ID NO:26)	QQNSEDPWT (SEQ ID NO:27)

[0121] The present invention also provides HER3 binding agents that comprise one or more of the CDRs of HER3-3, HER3-8, or HER3-10, as shown in Table 1 and Table 2 (i.e., one or more of SEQ ID NOs:4-27). In one embodiment, the invention provides antibodies (including molecules comprising, or alternatively consisting of, antibody fragments or variants) that specifically bind to a polypeptide or polypeptide fragment of HER3, wherein

said antibodies comprise, or alternatively consist of, a polypeptide comprising the amino acid sequence of one, two, three, or more variable heavy (VH) chain CDRs selected from the group consisting of SEQ ID NOs:4-18 (as shown in Table 1), and/or one, two, three or more variable light (VL) chain CDRs selected from the group consisting of SEQ ID NOs:19-27 (as shown in Table 2). In particular, the invention provides for antibodies that comprise, or alternatively consist of, a polypeptide comprising the amino acid sequence of a VH CDR1 and a VL CDR1, a VH CDR1 and a VL CDR2, a VH CDR1 and a VL CDR3, a VH CDR2 and a VL CDR1, VH CDR2 and VL CDR2, a VH CDR2 and a VL CDR3, a VH CDR3 and a VH CDR1, a VH CDR3 and a VL CDR2, a VH CDR3 and a VL CDR3, or any combination thereof, of the VH CDRs referred to in Table 1 (SEQ ID NOs:4-18) and the VL CDRs referred to in Table 2 (SEQ ID NOs:19-27). In a preferred embodiment, one or more of these combinations are from the VL and VH chains of the same antibody (e.g., from the VL and VH chains of HER3-3, from the VL and VH chains of HER3-8, or from the VL and VH chains of HER3-10).

[0122] In one embodiment, the invention encompasses an antibody or fragment thereof wherein the polypeptide sequence of the VH chain comprises a VH CDR1, a VH CDR2, and a VH CDR3 referred to in Table 1. In another one embodiment, the invention encompasses an antibody or fragment thereof wherein the polypeptide sequence of the VL chain comprises a VL CDR1, a VL CDR2, and a VL CDR3 referred to in Table 2. For example, the invention encompasses an antibody or fragment thereof wherein the polypeptide sequence of the VH chain comprises SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6, and/or wherein the polypeptide sequence of the VL chain comprises SEQ ID NO:19, SEQ ID NO:20, and SEQ ID NO:21. The invention also encompasses an antibody or fragment thereof wherein the polypeptide sequence of the VH chain comprises SEQ ID NO:9, SEQ ID NO:10, and SEQ ID NO:11, and/or wherein the polypeptide sequence of the VL chain comprises SEQ ID NO:22, SEQ ID NO:23, and SEQ ID NO:24. In another embodiment, the invention encompasses an antibody or fragment thereof wherein the polypeptide sequence of the VH chain comprises SEQ ID NO:9, SEQ ID NO:10, and SEQ ID NO:13, and/or wherein the polypeptide sequence of the VL chain comprises SEQ ID NO:22, SEQ ID NO:23, and SEQ ID NO:24. In yet another embodiment, the invention encompasses an antibody or fragment thereof wherein the polypeptide sequence of the VH chain comprises SEQ ID NO:14, SEQ ID NO:15, and SEQ ID NO:16, and/or wherein the polypeptide sequence of the VL chain comprises SEQ ID NO:25, SEQ ID NO:26, and SEQ ID NO:27.

[0123] In another embodiment, the HER3 binding molecules can be antibodies or antigen binding fragments that specifically bind to HER3 that comprise one or more of the CDRs of HER3-3, HER3-8, or HER3-10 as shown in Table 1 and Table 2 (i.e., one or more of SEQ ID NOs:4-27) with up to four (i.e., 0, 1, 2, 3, or 4) conservative amino acid substitutions per CDR.

[0124] In one embodiment, polypeptides of the invention comprise one of the individual variable light chains or variable heavy chains described herein. Antibodies and polypeptides of the invention can also comprise both a variable light chain and a variable heavy chain. The variable heavy chain and variable light chain sequences of murine and humanized HER3-3, HER3-8, and HER3-10 antibodies are provided in Tables 3 and 4 below.

Table 3: Variable heavy chain amino acid sequences

Antibody	Variable Heavy Chain Amino Acid Sequence (SEQ ID NO)
muHER3-3	QVQLQQPGAELVMPGASVKMSCKASGYTFTDYWIHWVKQRPGQGL EWIGAIDTDTYTSYNQQFKGKATLTVDESSSTAYMQLSSLTSEDSAV YYCARSLLRLLYFDYWGQGTTLTVSS (SEQ ID NO: 28)
huHER3-3	QVQLVQPGAEVVKPGASVKMSCKASGYTFTDYWIHWVKQRPGQGL EWIGAIDTDTYTSYNQKFQGKATLTVDESSSTAYMQLSSLTSEDSAV YYCARSLLRLLYFDYWGQGTTLTVSS (SEQ ID NO: 29)
muHER3-8	DVQLQESGPGLVTPSQSLSLTCTVTGSSITSDYAWNWIRQFPGNKLE WMGFISYSGSTSYNPSLKSRISITRDTSKNQFFLHLSSVTSEDTATYYC SRKLGRLYYFDYWGQGTTLTVSS (SEQ ID NO: 30)
huHER3.8v.1.00	QVQLQESGPGLVKPSQSLSLTCTVTGSSITSDYAWNWIRQHPGNKLE WMGFISYSGSTSYNPSLKSRISITRDTSKNQFFLHLSSVTAADTATYYC SRRLGRLYYFDYWGQGTLLTVSS (SEQ ID NO: 31)
huHER3.8v.1.01	QVQLQESGPGLVKPSQSLSLTCTVTGSSITSDYAWNWIRQHPGNKLE WMGFISYSGSTSYNPSLKSRISITRDTSKNQFFLHLSSVTAADTATYYC SRKLGRLYYFDYWGQGTLLTVSS (SEQ ID NO: 32)
muHER3-10	EVQLQQSGPELVKPGVSMKISCKASGYSFTDYTMNWVKQSHGKNLE WIGLINPYNDITAYNQKFKGKATLSVNKSSSTAYMELLSLTSEDSAVY YCARRGPRGAWFAYWGQGTLVTVSA (SEQ ID NO: 33)
huHER3-10	QVQLVQSGPEVVKPGVSMKISCKASGYTFTDYTMNWVKQSPGQNLE WIGLINPYNDITAYNQKFQGKATLSVDKSSSTAYMELLSLTSEDSAVY YCARRGPRGAWFAYWGQGTLVTVSA (SEQ ID NO: 34)

Table 4: Variable light chain amino acid sequences

Antibody	Variable Light Chain Amino Acid Sequence (SEQ ID NO)
muHER3-3	DIVMTQSPATLSVTPGDRVSLSCRASQSITDYLHWYQQKSHESPRLLI KFASQSISGIPSRFSGSGSGSDFTLSINSVEPEDVGVYYCQNGHSFPLTF

	GAGTKLELKR (SEQ ID NO: 35)
huHER3-3	EIVMTQSPATLSVTPGDRVTLSCRASQSITDYLHWYQQKPGQSPRLLI KFASQSISGIPDRFSGSGSGSDFTLSISSVEPEDVGVYYCQNGHSFPLTF GQGTKLELKR (SEQ ID NO: 36)
muHER3-8	DIVMTQSPATLSVTPGDRVSLSCRASQSINDYLHWYQQKSHESPRLLI KFASHSISGIPSRFSGSGSGSDFTLTINSVEPEDVGVYYCQNGHSFPWT FGGGTKLEIKR (SEQ ID NO: 37)
huHER3.8v.1.00 and huHER3.8v.1.01	EIVMTQSPATLSVTPGDRVSLSCRASQSINDYLHWYQQKPGQSPRLLI KFASHSISGIPDRFSGSGSGSDFTLTISSVEPEDVGVYYCQNGHSFPWT FGGGTKLEIKR (SEQ ID NO: 38)
muHER3-10	NIVLTQSPASLAVSLGQRATISCRASTSVYNYGNSFMHWYQQMPGQP PKLLIYLASNLESGVPARFSGSGSRTDFTLTIDPVEADDAATYYCQQN SEDPWTFGGGTKLEIKR (SEQ ID NO: 39)
huHER3-10	DIVLTQSPASLAVSPGQRATISCRASTSVYNYGNSFMHWYQQMPGQP PKLLIYLASNLESGVPARFSGSGSRTDFTLTIDPVEANDAATYYCQQN SEDPWTFGQGTKLEIKR (SEQ ID NO: 40)

[0125] In one embodiment, the HER3-binding agent comprises a variable heavy chain comprising or consisting of a polypeptide sequence selected from the group consisting of SEQ ID NOs:28-34 and a variable light chain comprising or consisting of a polypeptide sequence selected from the group consisting of SEQ ID NOs:35-40. Also provided are polypeptides that comprise: (a) a polypeptide having at least about 90% sequence identity to a sequence selected from the group consisting of SEQ ID NOs:28-34; and/or (b) a polypeptide having at least about 90% sequence identity to a sequence selected from the group consisting of SEQ ID NOs:35-40. In certain embodiments, the polypeptide comprises a polypeptide having at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity to a sequence selected from the group consisting of SEQ ID NOs:28-40. Thus, in certain embodiments, the polypeptide comprises (a) a polypeptide having at least about 95% sequence identity to a sequence selected from the group consisting of SEQ ID NOs:28-34, and/or (b) a polypeptide having at least about 95% sequence identity to a sequence selected from the group consisting of SEQ ID NOs:35-40. In certain embodiments, the polypeptide comprises (a) a polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NOs:28-34; and/or (b) a polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NOs:35-40. In certain embodiments, the polypeptide is an antibody and/or the polypeptide specifically binds HER3. In certain embodiments, the polypeptide is a murine, chimeric, or humanized antibody that specifically binds HER3. In certain embodiments, the

polypeptide having a certain percentage of sequence identity to any one of SEQ ID NOs:28-40 differs from SEQ ID NOs:28-40 by conservative amino acid substitutions only.

[0126] In one embodiment, polypeptides of the invention comprise one of the individual light chains or heavy chains described herein. Antibodies and polypeptides can also comprise both a light chain and a heavy chain. The full length heavy and light chain sequences of murine and humanized HER3-3, HER3-8, and HER3-10 antibodies are provided in Tables 5 and 6 below.

Table 5: Full length heavy chain amino acid sequences

Antibody	Full Length Heavy Chain Amino Acid Sequence (SEQ ID NO)
muHER3-3	QVQLQQPGAELVMPGASVKMSCKASGYTFTDYWIHWVKQRPGQGL EWIGAIDTDTYTSYNQQFKGKATLTVDESSSTAYMQLSSLTSEDSAV YYCARSLLRLLYFDYWGQGTTLTVSSAKTTPPSVYPLAPGSAAQTNS MVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLESDLYTLSSSV TVPSSMRPSETVTCNVAHPASSTKVDKKIVPRDCGCKPCICTVPEVSS VFIFPPKPKDVLTITLTPKVTCVVVDISKDDPEVQFSWFVDDVEVHTA QTQPREEQFNSTFRSVSELPIMHQDWLNGKEFKCRVNSAAFPAPIEKT ISKTKGRPKAPQVYTIPPPKEQMAKDKVSLTCMITDFFPEDITVEWQW NGQPAENYKNTQPIMNTNGSYFVYSKLNVQKSNWEAGNTFTCSVLH EGLHNHHTEKSLSHSPGK (SEQ ID NO: 41)
huHER3-3	DTYTSYNQKFQGKATLTVDESSSTAYMQLSSLTSEDSAVYYCARSLL RLLYFDYWGQGTTLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLV KDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLG TQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFL FPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK TKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK TISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWE SNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVM HEALHNHYTQKSLSLSPG (SEQ ID NO: 42)
muHER3-8	DVQLQESGPGLVTPSQSLSLTCTVTGSSITSDYAWNWIRQFPGNKLE WMGFISYSGSTSYNPSLKSRISITRDTSKNQFFLHLSSVTSEDTATYYC SRKLGRLYYFDYWGQGTTLTVSSAKTTPPSVYPLAPGSAAQTNSMVT LGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLESDLYTLSSSVTVPS SMRPSETVTCNVAHPASSTKVDKKIVPRDCGCKPCICTVPEVSSVFIFP PKPKDVLTITLTPKVTCVVVDISKDDPEVQFSWFVDDVEVHTAQTQP REEQFNSTFRSVSELPIMHQDWLNGKEFKCRVNSAAFPAPIEKTISKT KGRPKAPQVYTIPPPKEQMAKDKVSLTCMITDFFPEDITVEWQWNGQ PAENYKNTQPIMNTNGSYFVYSKLNVQKSNWEAGNTFTCSVLHEGL HNHHTEKSLSHSPGK (SEQ ID NO: 43)
huHER3.8v.1.00	QVQLQESGPGLVKPSQSLSLTCTVTGSSITSDYAWNWIRQHPGNKLE WMGFISYSGSTSYNPSLKSRISITRDTSKNQFFLHLSSVTAADTATYYC SRRLGRLYYFDYWGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAA LGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTV

	PSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLG GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVE VHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL PAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDI AVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVF SCSVMHEALHNHYTQKSLSLSPG (SEQ ID NO: 44)
huHER3.8v.1.01	QVQLQESGPGLVKPSQSLSLTCTVTGSSITSDYAWNWIRQHPGNKLE WMGFISYSGSTSYNPSLKSRISITRDTSKNQFFLHLSSVTAADTATYYC SRKLGRLYYFDYWGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAA LGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTV PSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLG GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVE VHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL PAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDI AVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVF SCSVMHEALHNHYTQKSLSLSPG (SEQ ID NO: 45)
muHER3-10	EVQLQQSGPELVKPGVSMKISCKASGYSFTDYTMNWVKQSHGKNLE WIGLINPYNDITAYNQKFKGKATLSVNKSSSTAYMELLSLTSEDSAVY YCARRGPRGAWFAYWGQGTLVTVSAAKTTPPSVYPLAPGSAAQTNS MVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLESDLYTLSSSV TVPSSMRPSETVTCNVAHPASSTKVDKKIVPRDCGCKPCICTVPEVSS VFIFPPKPKDVLTITLTPKVTCVVVDISKDDPEVQFSWFVDDVEVHTA QTQPREEQFNSTFRSVSELPIMHQDWLNGKEFKCRVNSAAFPAPIEKT ISKTKGRPKAPQVYTIPPPKEQMAKDKVSLTCMITDFFPEDITVEWQW NGQPAENYKNTQPIMNTNGSYFVYSKLNVQKSNWEAGNTFTCSVLH EGLHNHHTEKSLSHSPGK (SEQ ID NO: 46)
huHER3-10	QVQLVQSGPEVVKPGVSMKISCKASGYTFTDYTMNWVKQSPGQNLE WIGLINPYNDITAYNQKFQGKATLSVDKSSSTAYMELLSLTSEDSAVY YCARRGPRGAWFAYWGQGTLVTVSAASTKGPSVFPLAPSSKSTSGG TAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSV VTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPE LLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN KALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFY PSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQG NVFSCSVMHEALHNHYTQKSLSLSPG (SEQ ID NO: 47)

Table 6: Full length light chain amino acid sequences

Antibody	Full Length Light Chain Amino Acid Sequence (SEQ ID NO)
muHER3-3	DIVMTQSPATLSVTPGDRVSLSCRASQSITDYLHWYQQKSHESPRLLI KFASQSISGIPSRFSGSGSGSDFTLSINSVEPEDVGVYYCQNGHSFPLTF GAGTKLELKRADAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINV KWKIDGSERQNGVLNSWTDQDSKDSTYSMSSTLTLTKDEYERHNSY TCEATHKTSTSPIVKSFNRNEC (SEQ ID NO: 48)
huHER3-3	EIVMTQSPATLSVTPGDRVTLSCRASQSITDYLHWYQQKPGQSPRLLI KFASQSISGIPDRFSGSGSGSDFTLSISSVEPEDVGVYYCQNGHSFPLTF

	GQGTKLELKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKV QWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVY ACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 49)
muHER3-8	DIVMTQSPATLSVTPGDRVSLSCRASQSINDYLHWYQQKSHESPRLLI KFASHSISGIPSRFSGSGSGSDFTLTINSVEPEDVGVYYCQNGHSFPWT FGGGTKLEIKRADAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINV KWKIDGSERQNGVLNSWTDQDSKDSTYSMSSTLTLTKDEYERHNSY TCEATHKTSTSPIVKSFNRNEC (SEQ ID NO: 50)
huHER3.8v.1.00 and huHER3.8v.1.01	EIVMTQSPATLSVTPGDRVSLSCRASQSINDYLHWYQQKPGQSPRLLI KFASHSISGIPDRFSGSGSGSDFTLTISSVEPEDVGVYYCQNGHSFPWT FGGGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKV YACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 51)
muHER3-10	NIVLTQSPASLAVSLGQRATISCRASTSVYNYGNSFMHWYQQMPGQP PKLLIYLASNLESGVPARFSGSGSRTDFTLTIDPVEADDAATYYCQQN SEDPWTFGGGTKLEIKRADAAPTVSIFPPSSEQLTSGGASVVCFLNNF YPKDINVKWKIDGSERQNGVLNSWTDQDSKDSTYSMSSTLTLTKDE YERHNSYTCEATHKTSTSPIVKSFNRNEC (SEQ ID NO: 52)
huHER3-10	DIVLTQSPASLAVSPGQRATISCRASTSVYNYGNSFMHWYQQMPGQP PKLLIYLASNLESGVPARFSGSGSRTDFTLTIDPVEANDAATYYCQQN SEDPWTFGQGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNF YPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKAD YEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 53)

[0127] In one embodiment, the HER3-binding agent comprises a heavy chain comprising or consisting of a polypeptide sequence selected from the group consisting of SEQ ID NOs:41-47 and a light chain comprising or consisting of a polypeptide sequence selected from the group consisting of SEQ ID NOs:48-53. Also provided are polypeptides that comprise: (a) a polypeptide having at least about 90% sequence identity to a sequence selected from the group consisting of SEQ ID NOs:41-47; and/or (b) a polypeptide having at least about 90% sequence identity to a sequence selected from the group consisting of SEQ ID NOs:48-53. In certain embodiments, the polypeptide comprises a polypeptide having at least about 85%, at least about 90%, at least aobut 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity to a sequence selected from the group consisting of SEQ ID NOs:41-53. Thus, in certain embodiments, the polypeptide comprises (a) a polypeptide having at least about 95% sequence identity to a sequence selected from the group consisting of SEQ ID NOs:41-47, and/or (b) a polypeptide having at least about 95% sequence identity to a sequence selected from the group consisting of SEQ ID NOs:48-53. In certain embodiments, the polypeptide comprises (a) a polypeptide having an amino acid sequence a sequence selected from the group consisting of SEQ ID NOs:41-

47; and/or (b) a polypeptide having an amino acid sequence a sequence selected from the group consisting of SEQ ID NOs:48-53. In certain embodiments, the polypeptide is an antibody and/or the polypeptide specifically binds HER3. In certain embodiments, the polypeptide is a murine, chimeric, or humanized antibody that specifically binds HER3. In certain embodiments, the polypeptide having a certain percentage of sequence identity to any onr of SEQ ID NOs:41-53 differs from SEQ ID NOs:41-53 by conservative amino acid substitutions only.

[0128] In certain embodiments, the HER3 antibody can be the antibody produced from a hybridoma selected from the group consisting of ATCC Accession No. PTA-11145, deposited on July 1, 2010, ATCC Accession No. PTA-11146, deposited on July 1, 2010, and ATCC Accession No. PTA-11147, deposited on July 1, 2010. In one embodiment, the HER3 antibody binds to the same epitope as an antibody produced by a hybridoma selected from the group consisting of ATCC Accession No. PTA-11145, deposited on July 1, 2010, ATCC Accession No. PTA-11146, deposited on July 1, 2010, and ATCC Accession No. PTA-11147, deposited on July 1, 2010. The antibody produced by the hybridoma can be a purified antibody.

[0129] Monoclonal antibodies can be prepared using hybridoma methods, such as those described by Kohler and Milstein (1975) Nature 256:495. Using the hybridoma method, a mouse, hamster, or other appropriate host animal, is immunized as described above to elicit the production of antibodies by B lymphocytes that will specifically bind to an immunizing antigen. Lymphocytes can also be immunized *in vitro*. Following immunization, the lymphocytes are isolated and fused with a suitable myeloma cell line using, for example, polyethylene glycol and electrofusion, to form hybridoma cells that can then be selected away from unfused lymphocytes and myeloma cells in the HAT media. Hybridomas that produce monoclonal antibodies directed specifically against a chosen antigen as determined by FACS binding assay, immunoprecipitation, immunoblotting, or by an *in vitro* binding assay (e.g., radioimmunoassay (RIA); enzyme-linked immunosorbent assay (ELISA)) can then be propagated in either *in vitro* culture using standard methods (Goding, Monoclonal Antibodies: Principles and Practice, Academic Press, 1986) or *in vivo* as ascites tumors in an animal. The monoclonal antibodies can then be purified from the culture medium or ascites fluid as described for polyclonal antibodies above.

[0130] Alternatively, monoclonal antibodies can also be made using recombinant DNA methods as described in U.S. Patent 4,816,567. The polynucleotides encoding a monoclonal

antibody are isolated from mature B-cells or hybridoma cell, such as by RT-PCR using oligonucleotide primers that specifically amplify the genes encoding the heavy and light chains of the antibody, and their sequence is determined using conventional procedures. The isolated polynucleotides encoding the heavy and light chains are then cloned into suitable expression vectors, which when transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, monoclonal antibodies are generated by the host cells. Also, recombinant monoclonal antibodies or fragments thereof of the desired species can be isolated from phage display libraries expressing CDRs of the desired species as described (McCafferty et al., 1990, Nature, 348:552-554; Clackson et al., 1991, Nature, 352:624-628; and Marks et al., 1991, J. Mol. Biol., 222:581-597).

[0131] The polynucleotide(s) encoding a monoclonal antibody can further be modified in a number of different manners using recombinant DNA technology to generate alternative antibodies. In some embodiments, the constant domains of the light and heavy chains of, for example, a mouse monoclonal antibody can be substituted 1) for those regions of, for example, a human antibody to generate a chimeric antibody or 2) for a non-immunoglobulin polypeptide to generate a fusion antibody. In some embodiments, the constant regions are truncated or removed to generate the desired antibody fragment of a monoclonal antibody. Site-directed or high-density mutagenesis of the variable region can be used to optimize specificity, affinity, etc. of a monoclonal antibody.

[0132] In some embodiments, the monoclonal antibody against the human HER3 is a humanized antibody. In certain embodiments, such antibodies are used therapeutically to reduce antigenicity and HAMA (human anti-mouse antibody) responses when administered to a human subject. Humanized antibodies can be produced using various techniques known in the art. In certain alternative embodiments, the antibody to HER3 is a human antibody.

[0133] In one embodiment, the humanized HER3 antibody is an antibody encoded by plasmid phHER3-8v1.00 (ATCC Accession No. PTA-11144, deposited on July 1, 2010). In another embodiment, the humanized HER3 antibody is an antibody produced by a host cell transformed or transfected with plasmid phHER3-8v1.00 (ATCC Accession No. PTA-11144, deposited on July 1, 2010).

[0134] Human antibodies can be directly prepared using various techniques known in the art. Immortalized human B lymphocytes immunized *in vitro* or isolated from an immunized individual that produces an antibody directed against a target antigen can be generated (see,

e.g., Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985); Boemer et al., 1991, J. Immunol., 147 (1):86-95; and U.S. Patent 5,750,373). Also, the human antibody can be selected from a phage library, where that phage library expresses human antibodies, as described, for example, in Vaughan et al., 1996, Nat. Biotech., 14:309-314, Sheets et al., 1998, Proc. Nat'l. Acad. Sci., 95:6157-6162, Hoogenboom and Winter, 1991, J. Mol. Biol., 227:381, and Marks et al., 1991, J. Mol. Biol., 222:581. Techniques for the generation and use of antibody phage libraries are also described in U.S. Patent Nos. 5,969,108, 6,172,197, 5,885,793, 6,521,404; 6,544,731; 6,555,313; 6,582,915; 6,593,081; 6,300,064; 6,653,068; 6,706,484; and 7,264,963; and Rothe et al., 2007, J. Mol. Bio., doi:10.1016/j.jmb.2007.12.018 (each of which is incorporated by reference in its entirety). Affinity maturation strategies and chain shuffling strategies (Marks et al., 1992, Bio/Technology 10:779-783, incorporated by reference in its entirety) are known in the art and can be employed to generate high affinity human antibodies.

[0135] Humanized antibodies can also be made in transgenic mice containing human immunoglobulin loci that are capable upon immunization of producing the full repertoire of human antibodies in the absence of endogenous immunoglobulin production. This approach is described in U.S. Patent Nos 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; and 5,661,016.

[0136] This invention also encompasses bispecific antibodies that specifically recognize a HER3. Bispecific antibodies are antibodies that are capable of specifically recognizing and binding at least two different epitopes. The different epitopes can either be within the same molecule or on different molecules. For example, the antibodies can specifically recognize and bind HER3 as well as, for example, 1) other tumor antigens, 2) an effector molecule on a leukocyte such as a T-cell receptor (e.g., CD3) or Fc receptor (e.g., CD64, CD32, or CD16) or 3) a cytotoxic agent as described in detail below.

[0137] Exemplary bispecific antibodies can bind to two different epitopes, at least one of which originates in a polypeptide of the invention. Alternatively, an anti-antigenic arm of an immunoglobulin molecule can be combined with an arm which binds to a triggering molecule on a leukocyte such as a T cell receptor molecule (e.g., CD2, CD3, CD28, or B7), or Fc receptors for IgG (e.g., CD64, CD32, or CD16) so as to focus cellular defense mechanisms to the cell expressing the particular antigen. Bispecific antibodies can also be used to direct cytotoxic agents to cells which express a particular antigen. These antibodies possess an antigen-binding arm and an arm which binds a cytotoxic agent or a radionuclide chelator,

such as EOTUBE, DPTA, DOTA, or TETA. Techniques for making bispecific antibodies are common in the art (Millstein et al., 1983, Nature 305:537-539; Brennan et al., 1985, Science 229:81; Suresh et al, 1986, Methods in Enzymol. 121:120; Traunecker et al., 1991, EMBO J. 10:3655-3659; Shalaby et al., 1992, J. Exp. Med. 175:217-225; Kostelny et al., 1992, J. Immunol. 148:1547-1553; Gruber et al., 1994, J. Immunol. 152:5368; and U.S. Patent 5,731,168). Antibodies with more than two valencies are also contemplated. For example, trispecific antibodies can be prepared (Tutt et al., J. Immunol. 147:60 (1991)). Thus, in certain embodiments the antibodies to HER3 are multispecific.

[0138] In certain embodiments are provided an antibody fragment to, for example, increase tumor penetration. Various techniques are known for the production of antibody fragments. Traditionally, these fragments are derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., 1993, Journal of Biochemical and Biophysical Methods 24:107-117; Brennan et al., 1985, Science, 229:81). In certain embodiments, antibody fragments are produced recombinantly. Fab, Fv, and scFv antibody fragments can all be expressed in and secreted from *E. coli* or other host cells, thus allowing the production of large amounts of these fragments. Such antibody fragments can also be isolated from the antibody phage libraries discussed above. The antibody fragment can also be linear antibodies as described in U.S. Patent No 5,641,870, for example, and can be monospecific or bispecific. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner.

[0139] According to the present invention, techniques can be adapted for the production of single-chain antibodies specific to HER3 (see U.S. Pat. No. 4,946,778). In addition, methods can be adapted for the construction of Fab expression libraries (Huse, et al., Science 246:1275-1281 (1989)) to allow rapid and effective identification of monoclonal Fab fragments with the desired specificity for HER3, or derivatives, fragments, analogs or homologs thereof. Antibody fragments can be produced by techniques in the art including, but not limited to: (a) a F(ab')2 fragment produced by pepsin digestion of an antibody molecule; (b) a Fab fragment generated by reducing the disulfide bridges of an F(ab')2 fragment, (c) a Fab fragment generated by the treatment of the antibody molecule with papain and a reducing agent, and (d) Fv fragments.

[0140] It can further be desirable, especially in the case of antibody fragments, to modify an antibody in order to increase its serum half-life. This can be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody fragment by mutation of

the appropriate region in the antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody fragment at either end or in the middle (e.g., by DNA or peptide synthesis).

[0141] Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune cells to unwanted cells (U.S. Pat. No. 4,676,980). It is contemplated that the antibodies can be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins can be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate.

[0142] For the purposes of the present invention, it should be appreciated that modified antibodies can comprise any type of variable region that provides for the association of the antibody with the polypeptides of a human HER3. In this regard, the variable region can comprise or be derived from any type of mammal that can be induced to mount a humoral response and generate immunoglobulins against the desired tumor associated antigen. As such, the variable region of the modified antibodies can be, for example, of human, murine, or non-human primate (e.g., cynomolgus monkeys, macaques, etc.) origin. In some embodiments both the variable and constant regions of the modified immunoglobulins are human. In other embodiments the variable regions of compatible antibodies (usually derived from a non-human source) can be engineered or specifically tailored to improve the binding properties or reduce the immunogenicity of the molecule. In this respect, variable regions useful in the present invention can be humanized or otherwise altered through the inclusion of imported amino acid sequences.

[0143] In certain embodiments, the variable domains in both the heavy and light chains are altered by at least partial replacement of one or more CDRs and, if necessary, by partial framework region replacement and sequence changing. Although the CDRs can be derived from an antibody of the same class or even subclass as the antibody from which the framework regions are derived, it is envisaged that the CDRs will be derived from an antibody of a different class and possibly from an antibody from a different species. It is not alway necessary to replace all of the CDRs with the complete CDRs from the donor variable region to transfer the antigen binding capacity of one variable domain to another. Rather, in some cases it is only necessary to transfer those residues that are necessary to maintain the

activity of the antigen binding site. Given the explanations set forth in U.S. Patent Nos. 5,585,089, 5,693,761 and 5,693,762, it will be well within the competence of those skilled in the art, either by carrying out routine experimentation or by trial and error testing to obtain a functional antibody with reduced immunogenicity.

[0144] Alterations to the variable region notwithstanding, those skilled in the art will appreciate that the modified antibodies of this invention will comprise antibodies (e.g., full-length antibodies or immunoreactive fragments thereof) in which at least a fraction of one or more of the constant region domains has been deleted or otherwise altered so as to provide desired biochemical characteristics, such as increased tumor localization or reduced serum half-life when compared with an antibody of approximately the same immunogenicity comprising a native or unaltered constant region. In some embodiments, the constant region of the modified antibodies will comprise a human constant region. Modifications to the constant region compatible with this invention comprise additions, deletions or substitutions of one or more amino acids in one or more domains. That is, the modified antibodies disclosed herein can comprise alterations or modifications to one or more of the three heavy chain constant domains (CH1, CH2 or CH3) and/or to the light chain constant domain (CL). In some embodiments, modified constant regions wherein one or more domains are partially or entirely deleted are contemplated. In some embodiments, the modified antibodies will comprise domain deleted constructs or variants wherein the entire CH2 domain has been removed (ΔCH2 constructs). In some embodiments, the omitted constant region domain will be replaced by a short amino acid spacer (e.g., 10 residues) that provides some of the molecular flexibility typically imparted by the absent constant region.

[0145] Besides their configuration, it is known in the art that the constant region mediates several effector functions. For example, binding of the C1 component of complement to antibodies activates the complement system. Activation of complement is important in the opsonisation and lysis of cell pathogens. The activation of complement also stimulates the inflammatory response and can also be involved in autoimmune hypersensitivity. Further, antibodies bind to immune effector cells via interaction of antibody's Fc region with a Fc receptor (FcR) on an immune effector cell. There are a number of Fc receptors which are specific for different classes of antibody, including IgG (immunoglobulin gamma receptors), IgE (immunoglobulin eta receptors), IgA (immunoglobulin alpha receptors) and IgM (immunoglobulin mu receptors). Binding of antibody to Fc receptors on cell surfaces triggers a number of important and diverse biological responses including engulfment and destruction

of antibody-coated particles, clearance of immune complexes, lysis of antibody-coated target cells by killer cells (called antibody-dependent cell-mediated cytotoxicity, or ADCC), release of inflammatory mediators, placental transfer, and control of immunoglobulin production.

[0146] In certain embodiments, the HER3-binding antibodies provide for altered effector functions that, in turn, affect the biological profile of the administered antibody. For example, the deletion or inactivation (through point mutations or other means) of a constant region domain can reduce Fc receptor binding of the circulating modified antibody thereby increasing tumor localization. In other cases, it can be that constant region modifications, consistent with this invention, moderate complement binding and thus reduce the serum half life and nonspecific association of a conjugated cytotoxin. Yet other modifications of the constant region can be used to eliminate disulfide linkages or oligosaccharide moieties that allow for enhanced localization due to increased antigen specificity or antibody flexibility. Similarly, modifications to the constant region in accordance with this invention can easily be made using well known biochemical or molecular engineering techniques well within the purview of the skilled artisan.

[0147] In certain embodiments, a HER3-binding agent that is an antibody does not have one or more effector functions. For instance, in some embodiments, the antibody has no antibody-dependent cellular cytotoxicity (ADCC) activity and/or no complement-dependent cytotoxicity (CDC) activity. In certain embodiments, the antibody does not bind to an Fc receptor and/or complement factors. In certain embodiments, the antibody has no effector function.

[0148] It will be noted that in certain embodiments, the modified antibodies can be engineered to fuse the CH3 domain directly to the hinge region of the respective modified antibodies. In other constructs it can be desirable to provide a peptide spacer between the hinge region and the modified CH2 and/or CH3 domains. For example, compatible constructs could be expressed wherein the CH2 domain has been deleted and the remaining CH3 domain (modified or unmodified) is joined to the hinge region with a 5-20 amino acid spacer. Such a spacer can be added, for instance, to ensure that the regulatory elements of the constant domain remain free and accessible or that the hinge region remains flexible. However, it should be noted that amino acid spacers can, in some cases, prove to be immunogenic and elicit an unwanted immune response against the construct. Accordingly, in certain embodiments, any spacer added to the construct will be relatively non-immunogenic,

or even omitted altogether, so as to maintain the desired biochemical qualities of the modified antibodies.

[0149] Besides the deletion of whole constant region domains, it will be appreciated that the antibodies of the present invention can be provided by the partial deletion or substitution of a few or even a single amino acid. For example, the mutation of a single amino acid in selected areas of the CH2 domain can be enough to substantially reduce Fc binding and thereby increase tumor localization. Similarly, it can be desirable to simply delete that part of one or more constant region domains that control the effector function (e.g., complement CLQ binding) to be modulated. Such partial deletions of the constant regions can improve selected characteristics of the antibody (serum half-life) while leaving other desirable functions associated with the subject constant region domain intact. Moreover, as alluded to above, the constant regions of the disclosed antibodies can be modified through the mutation or substitution of one or more amino acids that enhances the profile of the resulting construct. In this respect it can be possible to disrupt the activity provided by a conserved binding site (e.g., Fc binding) while substantially maintaining the configuration and immunogenic profile of the modified antibody. Certain embodiments can comprise the addition of one or more amino acids to the constant region to enhance desirable characteristics, such as decreasing or increasing effector function or provide for more cytotoxin or carbohydrate attachment. In such embodiments it can be desirable to insert or replicate specific sequences derived from selected constant region domains.

[0150] The present invention further embraces variants and equivalents which are substantially homologous to the chimeric, humanized and human antibodies, or antibody fragments thereof, set forth herein. These can contain, for example, conservative substitution mutations, i.e., the substitution of one or more amino acids by similar amino acids. For example, conservative substitution refers to the substitution of an amino acid with another amino acid within the same general class such as, for example, one acidic amino acid with another acidic amino acid, one basic amino acid with another basic amino acid or one neutral amino acid with another neutral amino acid. What is intended by a conservative amino acid substitution is well known in the art.

[0151] The polypeptides of the present invention can be recombinant polypeptides, natural polypeptides, or synthetic polypeptides comprising an antibody, or fragment thereof, against a human HER3. It will be recognized in the art that some amino acid sequences of the invention can be varied without significant effect of the structure or function of the

protein. Thus, the invention further includes variations of the polypeptides which show substantial activity or which include regions of an antibody, or fragment thereof, against HER3 protein. Such mutants include deletions, insertions, inversions, repeats, and type substitutions.

[0152] The polypeptides and analogs can be further modified to contain additional chemical moieties not normally part of the protein. Those derivatized moieties can improve the solubility, the biological half life or absorption of the protein. The moieties can also reduce or eliminate any desirable side effects of the proteins and the like. An overview for those moieties can be found in Remington's Pharmaceutical Sciences, 20th ed., Mack Publishing Co., Easton, PA (2000).

[0153] The isolated polypeptides described herein can be produced by any suitable method known in the art. Such methods range from direct protein synthesis methods to constructing a DNA sequence encoding isolated polypeptide sequences and expressing those sequences in a suitable transformed host. In some embodiments, a DNA sequence is constructed using recombinant technology by isolating or synthesizing a DNA sequence encoding a wild-type protein of interest. Optionally, the sequence can be mutagenized by site-specific mutagenesis to provide functional analogs thereof. See, e.g. Zoeller et al., Proc. Nat'l. Acad. Sci. USA 81:5662-5066 (1984) and U.S. Patent No. 4,588,585.

[0154] In some embodiments a DNA sequence encoding a polypeptide of interest is constructed by chemical synthesis using an oligonucleotide synthesizer. Such oligonucleotides can be designed based on the amino acid sequence of the desired polypeptide and selecting those codons that are favored in the host cell in which the recombinant polypeptide of interest will be produced. Standard methods can be applied to synthesize an isolated polynucleotide sequence encoding an isolated polypeptide of interest. For example, a complete amino acid sequence can be used to construct a back-translated gene. Further, a DNA oligomer containing a nucleotide sequence coding for the particular isolated polypeptide can be synthesized. For example, several small oligonucleotides coding for portions of the desired polypeptide can be synthesized and then ligated. The individual oligonucleotides typically contain 5' or 3' overhangs for complementary assembly.

[0155] Once assembled (by synthesis, site-directed mutagenesis or another method), the polynucleotide sequences encoding a particular isolated polypeptide of interest will be inserted into an expression vector and operatively linked to an expression control sequence appropriate for expression of the protein in a desired host. Proper assembly can be confirmed

by nucleotide sequencing, restriction mapping, and expression of a biologically active polypeptide in a suitable host. As is well known in the art, in order to obtain high expression levels of a transfected gene in a host, the gene must be operatively linked to transcriptional and translational expression control sequences that are functional in the chosen expression host.

[0156] In certain embodiments, recombinant expression vectors are used to amplify and express DNA encoding antibodies, or fragments thereof, against human HER3. Recombinant expression vectors are replicable DNA constructs which have synthetic or cDNA-derived DNA fragments encoding a polypeptide chain of an anti-HER3 antibody, or fragment thereof, operatively linked to suitable transcriptional or translational regulatory elements derived from mammalian, microbial, viral or insect genes. A transcriptional unit generally comprises an assembly of (1) a genetic element or elements having a regulatory role in gene expression, for example, transcriptional promoters or enhancers, (2) a structural or coding sequence which is transcribed into mRNA and translated into protein, and (3) appropriate transcription and translation initiation and termination sequences, as described in detail below. Such regulatory elements can include an operator sequence to control transcription. The ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants can additionally be incorporated. DNA regions are operatively linked when they are functionally related to each other. For example, DNA for a signal peptide (secretory leader) is operatively linked to DNA for a polypeptide if it is expressed as a precursor which participates in the secretion of the polypeptide; a promoter is operatively linked to a coding sequence if it controls the transcription of the sequence; or a ribosome binding site is operatively linked to a coding sequence if it is positioned so as to permit translation. Structural elements intended for use in yeast expression systems include a leader sequence enabling extracellular secretion of translated protein by a host cell. Alternatively, where recombinant protein is expressed without a leader or transport sequence, it can include an N-terminal methionine residue. This residue can optionally be subsequently cleaved from the expressed recombinant protein to provide a final product.

[0157] The choice of expression control sequence and expression vector will depend upon the choice of host. A wide variety of expression host/vector combinations can be employed. Useful expression vectors for eukaryotic hosts, include, for example, vectors comprising expression control sequences from SV40, bovine papilloma virus, adenovirus and cytomegalovirus. Useful expression vectors for bacterial hosts include known bacterial

plasmids, such as plasmids from *Esherichia coli*, including pCR 1, pBR322, pMB9 and their derivatives, wider host range plasmids, such as M13 and filamentous single-stranded DNA phages.

[0158] Suitable host cells for expression of a HER3-binding polypeptide or antibody (or a HER3 protein to use as an antigen) include prokaryotes, yeast, insect or higher eukaryotic cells under the control of appropriate promoters. Prokaryotes include gram negative or gram positive organisms, for example *E. coli* or bacilli. Higher eukaryotic cells include established cell lines of mammalian origin as described below. Cell-free translation systems could also be employed. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described by Pouwels et al. (Cloning Vectors: A Laboratory Manual, Elsevier, N.Y., 1985), the relevant disclosure of which is hereby incorporated by reference. Additional information regarding methods of protein production, including antibody production, can be found, e.g., in U.S. Patent Publication No. 2008/0187954, U.S. Patent Nos. 6,413,746 and 6,660,501, and International Patent Publication No. WO 04009823, each of which is hereby incorporated by reference herein in its entirety.

[0159] Various mammalian or insect cell culture systems are also advantageously employed to express recombinant protein. Expression of recombinant proteins in mammalian cells can be performed because such proteins are generally correctly folded, appropriately modified and completely functional. Examples of suitable mammalian host cell lines include the COS-7 lines of monkey kidney cells, described by Gluzman (Cell 23:175, 1981), and other cell lines capable of expressing an appropriate vector including, for example, L cells, C127, 3T3, Chinese hamster ovary (CHO), HeLa and BHK cell lines. Mammalian expression vectors can comprise nontranscribed elements such as an origin of replication, a suitable promoter and enhancer linked to the gene to be expressed, and other 5' or 3' flanking nontranscribed sequences, and 5' or 3' nontranslated sequences, such as necessary ribosome binding sites, a polyadenylation site, splice donor and acceptor sites, and transcriptional termination sequences. Baculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow and Summers, Biotechnology 6:47 (1988).

[0160] The proteins produced by a transformed host can be purified according to any suitable method. Such standard methods include chromatography (e.g., ion exchange, affinity and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for protein purification. Affinity tags such as hexahistidine, maltose

binding domain, influenza coat sequence and glutathione-S-transferase can be attached to the protein to allow easy purification by passage over an appropriate affinity column. Isolated proteins can also be physically characterized using such techniques as proteolysis, nuclear magnetic resonance and x-ray crystallography.

[0161] For example, supernatants from systems which secrete recombinant protein into culture media can be first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a suitable purification matrix. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Finally, one or more reversed-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, e.g., silica gel having pendant methyl or other aliphatic groups, can be employed to further purify a HER3-binding agent. Some or all of the foregoing purification steps, in various combinations, can also be employed to provide a homogeneous recombinant protein.

[0162] Recombinant protein produced in bacterial culture can be isolated, for example, by initial extraction from cell pellets, followed by one or more concentration, salting-out, aqueous ion exchange or size exclusion chromatography steps. High performance liquid chromatography (HPLC) can be employed for final purification steps. Microbial cells employed in expression of a recombinant protein can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

[0163] Methods known in the art for purifying antibodies and other proteins also include, for example, those described in U.S. Patent Publication Nos. 2008/0312425, 2008/0177048, and 2009/0187005, each of which is hereby incorporated by reference herein in its entirety.

[0164] In certain embodiments, the HER3-binding agent is a polypeptide that is not an antibody. A variety of methods for identifying and producing non-antibody polypeptides that bind with high affinity to a protein target are known in the art. See, e.g., Skerra, Curr. Opin. Biotechnol., 18:295-304 (2007), Hosse et al., Protein Science, 15:14-27 (2006), Gill et al., Curr. Opin. Biotechnol., 17:653-658 (2006), Nygren, FEBS J., 275:2668-76 (2008), and Skerra, FEBS J., 275:2677-83 (2008), each of which is incorporated by reference herein in its

entirety. In certain embodiments, phage display technology has been used to identify/produce the HER3-binding polypeptide. In certain embodiments, the polypeptide comprises a protein scaffold of a type selected from the group consisting of, but not limited to, protein A, a lipocalin, a fibronectin domain, an ankyrin consensus repeat domain, and thioredoxin.

[0165] In some embodiments, the HER3-binding agent is a non-protein molecule. In certain embodiments, the HER3-binding agent is a small molecule. Combinatorial chemistry libraries and techniques useful in the identification of non-protein HER3-binding agents are known to those skilled in the art. See, e.g., Kennedy et al., J. Comb. Chem, 10:345-354 (2008), Dolle et al, J. Comb. Chem., 9:855-902 (2007), and Bhattacharyya, Curr. Med. Chem., 8:1383-404 (2001), each of which is incorporated by reference herein in its entirety. In certain further embodiments, the HER3-binding agent is a carbohydrate, a glycosaminoglycan, a glycoprotein, or a proteoglycan.

[0166] In certain embodiments, the HER3-binding agent is a nucleic acid aptamer. Aptamers are polynucleotide molecules that have been selected (e.g., from random or mutagenized pools) on the basis of their ability to bind to another molecule. In some embodiments, the aptamer comprises a DNA polynucleotide. In certain alternative embodiments, the aptamer comprises an RNA polynucleotide. In certain embodiments, the aptamer comprises one or more modified nucleic acid residues. Methods of generating and screening nucleic acid aptamers for binding to proteins are well known in the art. See, e.g., U.S. Patent Nos. 5,270,163, 5,683,867, 5,763,595, 6,344,321, 7,368,236, 5,582,981, 5,756,291, 5,840,867, 7,312,325, 7,329,742, International Patent Publication Nos. WO 02/077262, WO 03/070984, U.S. Patent Application Publication Nos. 2005/0239134, 2005/0124565, and 2008/0227735, each of which is incorporated by reference herein in its entirety.

III. Immunoconjugates

[0167] The present invention is also directed to conjugates (also referred to herein as immunoconjugates), comprising the anti-HER3 antibodies, antibody fragments, and their functional equivalents as disclosed herein, linked or conjugated to a drug or prodrug. Suitable drugs or prodrugs are known in the art. The drugs or prodrugs can be cytotoxic agents. The cytotoxic agent used in the cytotoxic conjugate of the present invention can be any compound that results in the death of a cell, or induces cell death, or in some manner

decreases cell viability, and includes, for example, maytansinoids and maytansinoid analogs. Other suitable cytotoxic agents are, for example, benzodiazepines, taxoids, CC-1065 and CC-1065 analogs, duocarmycins and duocarmycin analogs, enediynes, such as calicheamicins, dolastatin and dolastatin analogs including auristatins, tomaymycin derivaties, leptomycin derivaties, methotrexate, cisplatin, carboplatin, daunorubicin, doxorubicin, vincristine, vinblastine, melphalan, mitomycin C, chlorambucil and morpholino doxorubicin.

[0168] Such conjugates can be prepared by using a linking group in order to link a drug or prodrug to the antibody or functional equivalent. Suitable linking groups are well known in the art and include, for example, disulfide groups, thioether groups, acid labile groups, photolabile groups, peptidase labile groups and esterase labile groups.

[0169] The drug or prodrug can, for example, be linked to the anti-HER3 antibody or fragment thereof through a disulfide bond. The linker molecule or crosslinking agent comprises a reactive chemical group that can react with the anti-HER3 antibody or fragment thereof. The reactive chemical groups for reaction with the cell-binding agent can be *N*-succinimidyl esters and *N*-sulfosuccinimidyl esters. Additionally, the linker molecule comprises a reactive chemical group, which can be a dithiopyridyl group that can react with the drug to form a disulfide bond. Linker molecules include, for example, *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) (see, e.g., Carlsson et al., *Biochem. J.*, *173:* 723-737 (1978)), *N*-succinimidyl 4-(2-pyridyldithio)butanoate (SPDB) (see, e.g., U.S. Patent No. 4,563,304), *N*-succinimidyl 4-(2-pyridyldithio)2-sulfobutanoate (sulfo-SPDB) (see U.S. Publication No. 20090274713) , *N*-succinimidyl 4-(2-pyridyldithio) pentanoate (SPP) (see, e.g., CAS Registry number 341498-08-6), 2-iminothiolane, and acetylsuccinic anhydride. For example, the antibody or cell binding agent can be modified with crosslinking reagents and the antibody or cell binding agent containing free or protected thiol groups thus derived is then reacted with a disulfide- or thiol-containing maytansinoid to produce conjugates. The conjugates can be purified by chromatography, including but not limited to HPLC, size-exclusion, adsorption, ion exchange and affinity capture, dialysis or tangential flow filtration.

[0170] In another aspect of the present invention, the anti-HER3 antibody is linked to cytotoxic drugs via disulfide bonds and a polyethylene glycol spacer to enhance the potency, solubility, or efficacy of the immunoconjugate. Such cleavable hydrophilic linkers are described in WO2009/0134976. The additional benefit of this linker design is the desired high monomer ratio and the minimal aggregation of the antibody-drug conjugate. Specifically, conjugates of cell-binding agents and drugs linked via disulfide group (-S-S-)

bearing polyethylene glycol spacers ($(CH_2CH_2O)_{n=1-14}$) with a narrow range of drug load of 2-8 are described that show relatively high potent biological activity toward cancer cells and have the desired biochemical properties of high conjugation yield and high monomer ratio with minimal protein aggregation.

[0171] Specifically contemplated in this aspect is an anti-HER3 antibody drug conjugate of formula (I) or a conjugate of formula (I'):

$$CB–[X_l–(–CH_2–CH_2O–)_n–Y–D]_m \quad (I)$$

$$[D\text{-}Y\text{-}(–CH_2–CH_2O–)_n–X_l]_m\text{-}CB \quad (I')$$

wherein: CB represents an anti-HER3 antibody or fragment thereof; D represents a drug; X represents an aliphatic, an aromatic or a heterocyclic unit attached to the cell-binding agent via a thioether bond, an amide bond, a carbamate bond, or an ether bond; Y represents an aliphatic, an aromatic or a heterocyclic unit attached to the drug via a disulfide bond; l is 0 or 1; m is an integer from 2 to 8; and n is an integer from 1 to 24.

[0172] In some embodiments, m is an integer from 2 to 6.

[0173] In some embodiments, m is an integer from 3 to 5.

[0174] In some embodiments, n is an integer form 2 to 8. Alternatively, as disclosed in, for example, U.S. Patent Nos. 6,441,163 and 7,368,565, the drug can be first modified to introduce a reactive ester suitable to react with a cell-binding agent. Reaction of these drugs containing an activated linker moiety with a cell-binding agent provides another method of producing a cell-binding agent drug conjugate. Maytansinoids can also be linked to anti-HER3 antibody or fragment using PEG linking groups, as set forth, for example, in U.S. Patent No. 6,716,821. These PEG non-cleavable linking groups are soluble both in water and in non-aqueous solvents, and can be used to join one or more cytotoxic agents to a cell binding agent. Exemplary PEG linking groups include heterobifunctional PEG linkers that react with cytotoxic agents and cell binding agents at opposite ends of the linkers through a functional sulfhydryl or disulfide group at one end, and an active ester at the other end. As a general example of the synthesis of a cytotoxic conjugate using a PEG linking group, reference is again made to U.S. Patent No. 6,716,821, which is incorporated entirely by reference herein. Synthesis begins with the reaction of one or more cytotoxic agents bearing a reactive PEG moiety with a cell-binding agent, resulting in displacement of the terminal active ester of each reactive PEG moiety by an amino acid residue of the cell binding agent, to yield a cytotoxic conjugate comprising one or more cytotoxic agents covalently bonded to

a cell binding agent through a PEG linking group. Alternatively, the cell binding agent can be modified with the bifunctional PEG crosslinker to introduce a reactive disulfide moiety (such as a pyridyldisulfide), which can then be treated with a thiol-containing maytansinoid to provide a conjugate. In another method, the cell binding agent can be modified with the bifunctional PEG crosslinker to introduce a thiol moiety which then can be treated with a reactive disulfide-containing maytansinoid (such as a pyridyldisulfide), to provide a conjugate.

[0175] Antibody-maytansinoid conjugates with non-cleavable linkers can also be prepared. Such crosslinkers are described in the art (see, e.g., U.S. Publication No. 20050169933) and include but are not limited to, *N*-succinimidyl 4-(maleimidomethyl) cyclohexanecarboxylate (SMCC). In some embodiments, the antibody is modified with crosslinking reagents such as succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC), sulfo-SMCC, maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), sulfo-MBS or succinimidyl-iodoacetate, as described in the literature, to introduce 1-10 reactive groups (Yoshitake et al, Eur. J. Biochem., 101:395-399 (1979); Hashida et al, J. Applied Biochem., 56-63 (1984); and Liu et al, Biochem., 18:690-697 (1979)). The modified antibody is then reacted with the thiol-containing maytansinoid derivative to produce a conjugate. The conjugate can be purified by gel filtration through a Sephadex G25 column or by dialysis or tangential flow filtration. The modified antibodies are treated with the thiol-containing maytansinoid (1 to 2 molar equivalent/maleimido group) and antibody-maytansinoid conjugates are purified by gel filtration through a Sephadex G-25 column, chromatography on a ceramic hydroxyapatite column, dialysis or tangential flow filtration, or a combination of methods thereof. Typically, an average of 1-10 maytansinoids per antibody is linked. One method is to modify antibodies with succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC) to introduce maleimido groups followed by reaction of the modified antibody with a thiol-containing maytansinoid to give a thioether-linked conjugate. Again, conjugates with 1 to 10 drug molecules per antibody molecule result. Maytansinoid conjugates of antibodies, antibody fragments, and other proteins are made in the same way.

[0176] In another aspect of the invention, the HER3 antibody is linked to the drug via a non-cleavable bond through the intermediacy of a PEG spacer. Suitable crosslinking reagents comprising hydrophilic PEG chains that form linkers between a drug and the anti-HER3 antibody or fragment are also well known in the art, or are commercially available

(for example from Quanta Biodesign, Powell, Ohio). Suitable PEG-containing crosslinkers can also be synthesized from commercially available PEGs themselves using standard synthetic chemistry techniques known to one skilled in the art. The drugs can be reacted with bifunctional PEG-containing cross linkers to give compounds of the following formula, Z – X_l–(–CH_2–CH_2–O–)$_n$–Y_p–D, by methods described in detail in U.S. Patent Publication No. 20090274713 and in International Patent Publication No. WO2009/0134976, which can then react with the cell binding agent to provide a conjugate. Alternatively, the cell binding agent can be modified with the bifunctional PEG crosslinker to introduce a thiol-reactive group (such as a maleimide or haloacetamide) which can then be treated with a thiol-containing maytansinoid to provide a conjugate. In another method, the cell binding agent can be modified with the bifunctional PEG crosslinker to introduce a thiol moiety which can then be treated with a thiol-reactive maytansinoid (such as a maytansinoid bearing a maleimide or haloacetamide), to provide a conjugate.

[0177] Accordingly, another aspect of the present invention is an anti-HER3 antibody drug conjugate of formula (II) or of formula (II'):

$$CB–[X_l–(–CH_2–CH_2–O–)_n–Y_p–D]_m \quad (II)$$

$$[D\text{-}Y_p–(–CH_2–CH_2–O–)_n–X_l]_m\text{-}CB \quad (II')$$

wherein: CB represents an anti-HER3 antibody or fragment thereof; D represents a drug; X represents an aliphatic, an aromatic or a heterocyclic unit bonded to the cell-binding agent via a thioether bond, an amide bond, a carbamate bond, or an ether bond; Y represents an aliphatic, an aromatic, or a heterocyclic unit bonded to the drug via a covalent bond selected from the group consisting of a thioether bond, an amide bond, a carbamate bond, an ether bond, an amine bond, a carbon-carbon bond and a hydrazone bond; l is 0 or 1; p is 0 or 1; m is an integer from 2 to 15; and n is an integer from 1 to 2000.

[0178] In some embodiments, m is an integer from 2 to 8; and

[0179] In some embodiments, n is an integer from 1 to 24.

[0180] In some embodiments, m is an integer from 2 to 6.

[0181] In some embodiments, m is an integer from 3 to 5.

[0182] In some embodiments, n is an integer from 2 to 8. Examples of suitable PEG-containing linkers include linkers having an *N*-succinimidyl ester or *N*-sulfosuccinimidyl ester moiety for reaction with the anti-HER3 antibody or fragment thereof, as well as a

maleimido- or haloacetyl-based moiety for reaction with the compound. A PEG spacer can be incorporated into any crosslinker known in the art by the methods described herein.

[0183] Many of the linkers disclosed herein are described in detail in U.S. Patent Publication Nos. 20050169933 and 20090274713, and in International Patent Publication No. WO2009/0134976; the contents of which are entirely incorporated herein by reference.

[0184] The present invention includes aspects wherein about 2 to about 8 drug molecules ("drug load"), for example, maytansinoid, are linked to an anti-HER3 antibody or fragment thereof. The anti-tumor effect of a conjugate comprising about 2 to about 8 drug molecules is much more efficacious as compared to a drug load of a lesser or higher number of drugs linked to the same cell binding agent. "Drug load," as used herein, refers to the number of drug molecules (e.g., a maytansinoid) that can be attached to a cell binding agent (e.g., an anti-HER3 antibody or fragment thereof). In one aspect the number of drug molecules that can be attached to a cell binding agent can average from about 2 to about 8 (e.g., 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8.0, 8.1). In one embodiment, the drug is $N^{2'}$-deacetyl-$N^{2'}$-(3-mercapto-1-oxopropyl)-maytansine (DM1) or $N^{2'}$-deacetyl-$N^{2'}$-(4-mercapto-4-methyl-1-oxopentyl) maytansine (DM4).

[0185] The anti-HER3 antibody or fragment thereof can be modified by reacting a bifunctional crosslinking reagent with the anti-HER3 antibody or fragment thereof, thereby resulting in the covalent attachment of a linker molecule to the anti-HER3 antibody or fragment thereof. As used herein, a "bifunctional crosslinking reagent" is any chemical moiety that covalently links a cell-binding agent to a drug, such as the drugs described herein. In another method, a portion of the linking moiety is provided by the drug. In this respect, the drug comprises a linking moiety that is part of a larger linker molecule that is used to join the cell-binding agent to the drug. For example, to form the maytansinoid DM1, the side chain at the C-3 hydroxyl group of maytansine is modified to have a free sulfhydryl group (SH). This thiolated form of maytansine can react with a modified cell-binding agent to form a conjugate. Therefore, the final linker is assembled from two components, one of which is provided by the crosslinking reagent, while the other is provided by the side chain from DM1.

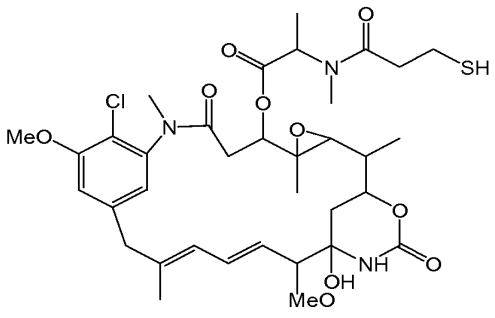
[0186] The drug molecules can also be linked to the antibody molecules through an intermediary carrier molecule such as serum albumin.

[0187] As used herein, the expression "linked to a cell-binding agent" or "linked to an anti-HER3 antibody or fragment" refers to the conjugate molecule comprising at least one drug derivative bound to a cell-binding agent, or bound to an anti-HER3 antibody or fragment thereof via a suitable linking group, or a precursor thereof. One exemplary linking group is SMCC.

[0188] In certain embodiments, cytotoxic agents useful in the present invention are maytansinoids and maytansinoid analogs. Examples of suitable maytansinoids include esters of maytansinol and maytansinol analogs. Included are any drugs that inhibit microtubule formation and that are highly toxic to mammalian cells, as are maytansinol and maytansinol analogs.

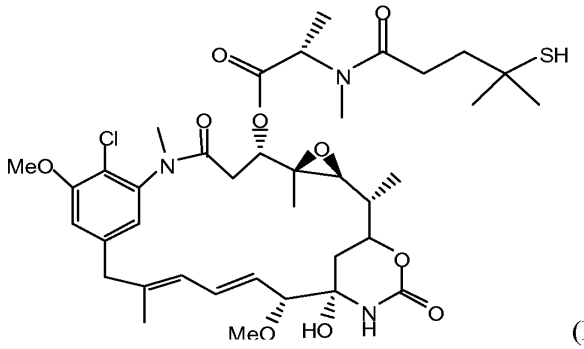
[0189] Examples of suitable maytansinol esters include those having a modified aromatic ring and those having modifications at other positions. Such suitable maytansinoids are disclosed in U.S. Patent Nos. 4,424,219; 4,256,746; 4,294,757; 4,307,016; 4,313,946; 4,315,929; 4,331,598; 4,361,650; 4,362,663; 4,364,866; 4,450,254; 4,322,348; 4,371,533; 5,208,020; 5,416,064; 5,475,092; 5,585,499; 5,846,545; 6,333,410; 7,276,497 and 7,473,796.

[0190] In a certain embodiment, the immunoconjugates of the invention utilize the thiol-containing maytansinoid (DM1), formally termed $N^{2'}$-deacetyl-$N^{2'}$-(3-mercapto-1-oxopropy1)-maytansine, as the cytotoxic agent. DM1 is represented by the following structural formula (III):



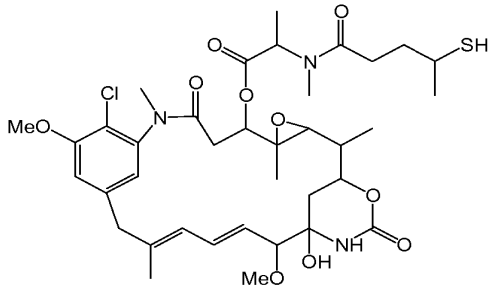
(III)

[0191] In another embodiment, the conjugates of the present invention utilize the thiol-containing maytansinoid $N^{2'}$-deacetyl-$N^{2'}$(4-methy1-4-mercapto-1- oxopentyl)-maytansine (e.g., DM4) as the cytotoxic agent. DM4 is represented by the following structural formula (IV):



(IV)

[0192] Another maytansinoid comprising a side chain that contains a sterically hindered thiol bond is $N^{2'}$-deacetyl-N-$^{2'}$(4-mercapto-1-oxopentyl)-maytansine (termed DM3), represented by the following structural formula (V):

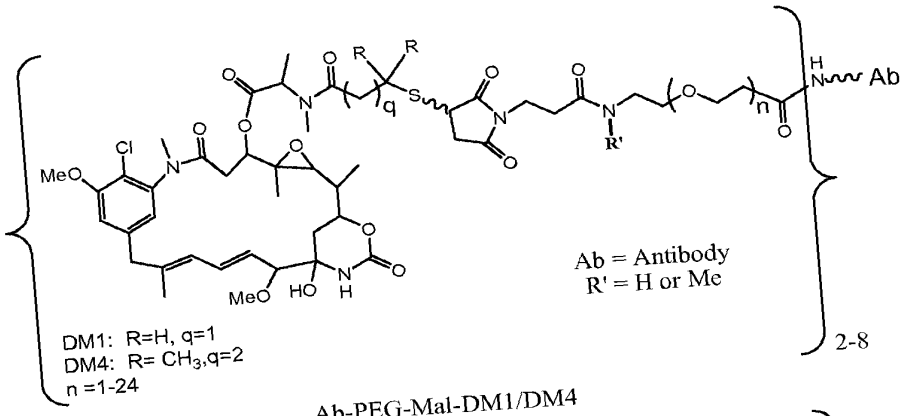


(V)

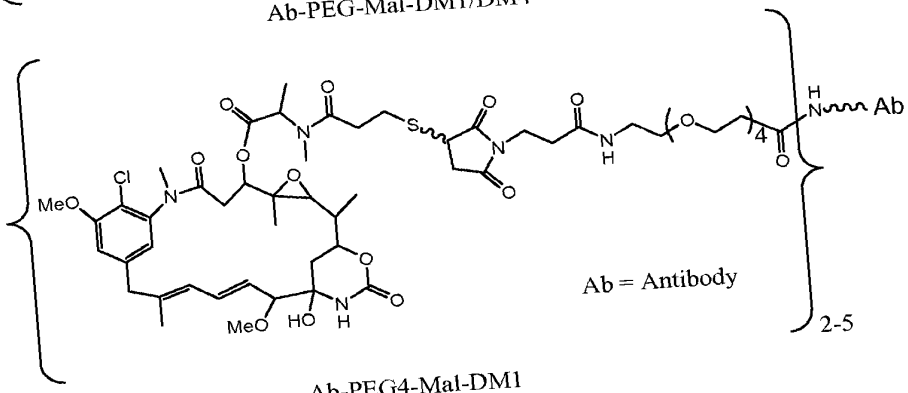
[0193] Each of the maytansinoids taught in U.S. Patent Nos. 5,208,020 and 7,276,497 can also be used in the conjugate of the present invention. In this regard, the entire disclosure of U.S. Patent Nos. 5,208,020 and 7,276,697 is incorporated herein by reference.

[0194] Many positions on maytansinoids can serve as the position to chemically link the linking moiety. For example, the C-3 position having a hydroxyl group, the C-14 position modified with hydroxymethyl, the C-15 position modified with hydroxyl, and the C-20 position having a hydroxy group are all expected to be useful. In some embodiments, the C-3 position serves as the position to chemically link the linking moiety, and in some particular embodiments, the C-3 position of maytansinol serves as the position to chemically link the linking moiety.

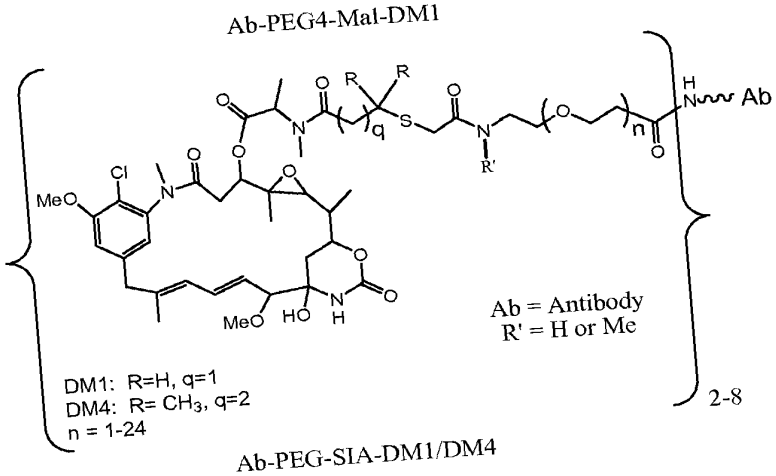
[0195] Structural representations of some conjugates are shown below:



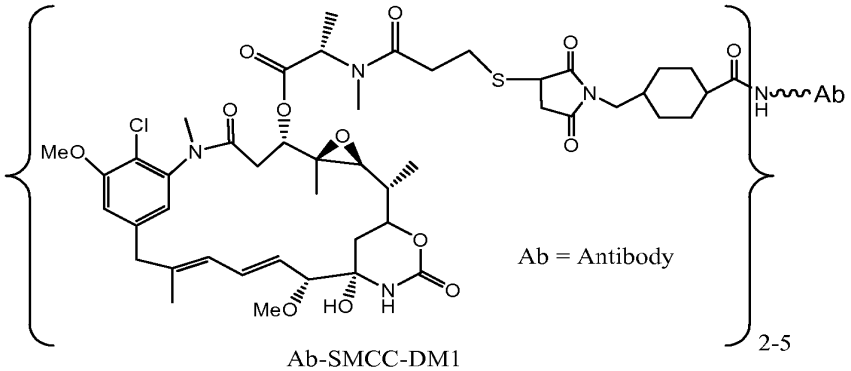
Ab-PEG-Mal-DM1/DM4 (VI)



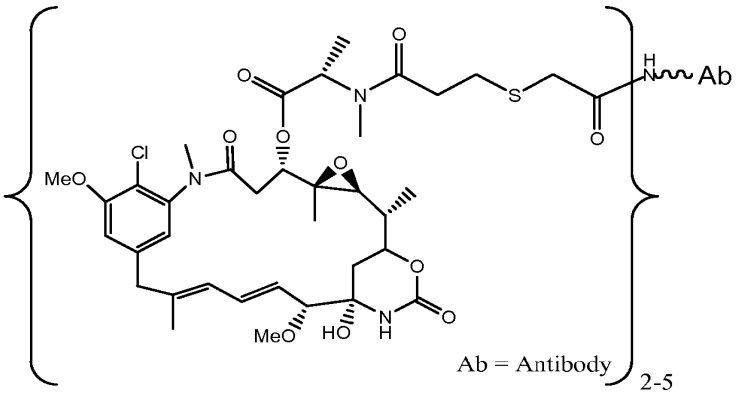
Ab-PEG4-Mal-DM1 (VII)



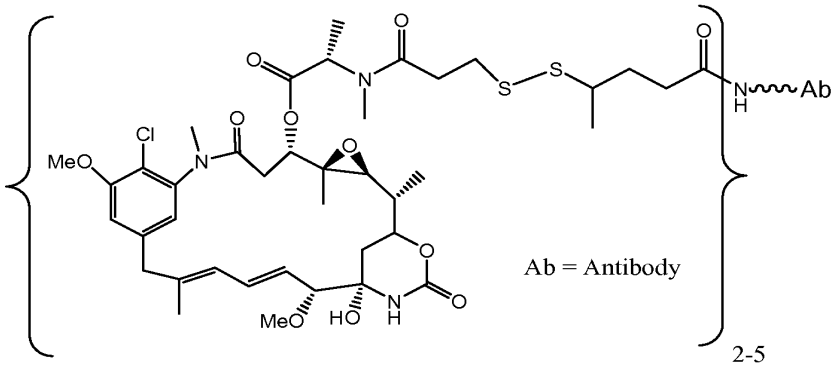
Ab-PEG-SIA-DM1/DM4 (VIII)



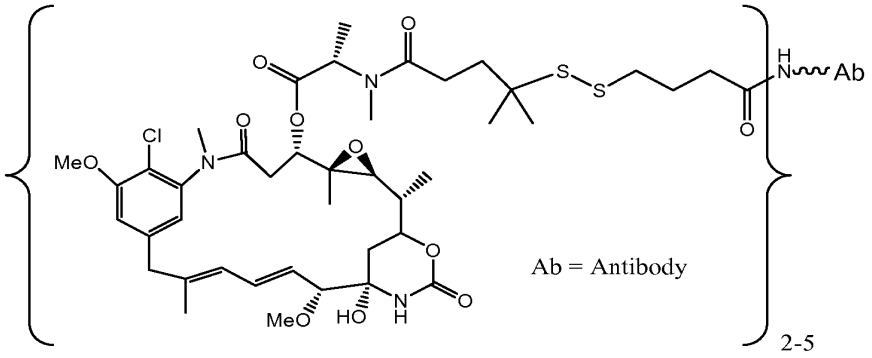
Ab-SMCC-DM1 (IX)



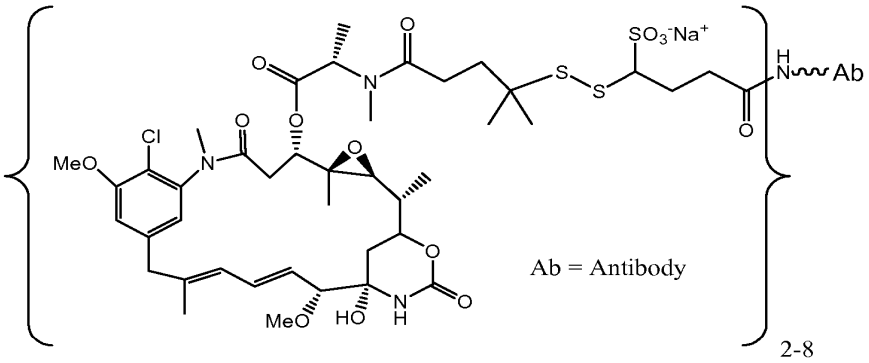
Ab-SIA-DM1 (X)



Ab-SPP-DM1 (XI)



Ab-SPDB-DM4 (XII)



Ab-sulfo-SPDB-DM4 (XIII)

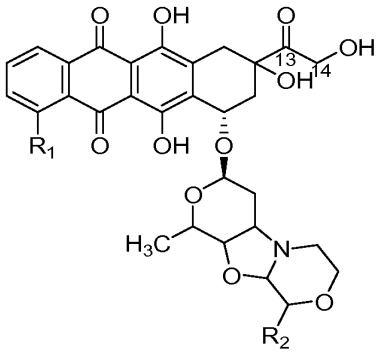
[0196] Several descriptions for producing such antibody-maytansinoid conjugates are provided in U.S. Patent Nos. 6,333,410, 6,441,163, 6,716,821, and 7,368,565, each of which is incorporated herein in its entirety.

[0197] In general, a solution of an antibody in aqueous buffer can be incubated with a molar excess of maytansinoids having a disulfide moiety that bears a reactive group. The reaction mixture can be quenched by addition of excess amine (such as ethanolamine, taurine, etc.). The maytansinoid-antibody conjugate can then be purified by gel filtration.

[0198] The number of maytansinoid molecules bound per antibody molecule can be determined by measuring spectrophotometrically the ratio of the absorbance at 252 nm and 280 nm. The average number of maytansinoid molecules/antibody can be, for example, 1-10, 2-9, 2-8, 2-7, 2-6, or 2-5.

[0199] Anthracycline compounds, as well as derivatives, intermediates and modified versions thereof, can also be used to prepare anti-HER3 immunoconjugates. For example, doxorubicin, doxorubicin derivatives, doxorubicin intermediates, and modified doxorubicins can be used in anti-HER3 conjugates. Exemplary compounds are described in International

Patent Publication WO 2010/009124, which is herein incorporated by reference in its entirety. Such compounds include, for example, compounds of the following formula:



wherein R_1 is a hydrogen atom, hydroxy or methoxy group and R_2 is a C_1-C_8 alkoxy group, or a pharmaceutically acceptable salt thereof.

[0200] Conjugates of antibodies with maytansinoid or other drugs can be evaluated for their ability to suppress proliferation of various unwanted cell lines *in vitro*. For example, cell lines such as the 300-19 cell lines that overexpress human HER3 or macaque HER3 and breast cancer cell line SKBR3, can easily be used for the assessment of cytotoxicity of these compounds. Cells to be evaluated can be exposed to the compounds for 4 to 5 days and the surviving fractions of cells measured in direct assays by known methods. IC_{50} values can then be calculated from the results of the assays.

[0201] The immunoconjugates can, according to some embodiments described herein, be internalized into the cells and processed intracellularly. The immunoconjugate, therefore, can exert a therapeutic effect when it is taken up by, or internalized by, a HER3-expressing cell. In some particular embodiments, the immunoconjugate comprises an antibody, antibody fragment, or polypeptide linked to a cytotoxic agent by a cleavable linker, and the cytotoxic agent is cleaved from the antibody, antibody fragment, or polypeptide, wherein it is internalized by a HER3-expressing cell.

[0202] In another aspect of the invention siRNA molecules can be linked to the antibodies of the present invention instead of a drug. siRNAs can be linked to the antibodies of the present invention by methods commonly used for the modification of oligonucleotides (see, for example, U.S. Patent Publication Nos. 2005/0107325 and 2007/0213292). Thus, the siRNA in its 3' or 5'-phosphoromidite form can be reacted with one end of the crosslinker bearing a hydroxyl functionality to give an ester bond between the siRNA and the crosslinker. Similarly, reaction of the siRNA phosphoramidite with a crosslinker bearing a

terminal amino group results in linkage of the crosslinker to the siRNA through an amine. Alternatively, the siRNA can be derivatized by standard chemical methods to introduce a thiol group. This thiol-containing siRNA can be reacted with an antibody that has been modified to introduce an active disulfide or maleimide moiety, to produce a cleavable or non cleavable conjugate. Between 1 - 20 siRNA molecules can be linked to an antibody by this method.

IV. Polynucleotides

[0203] In certain embodiments, the invention encompasses polynucleotides comprising polynucleotides that encode a polypeptide that specifically binds HER3 or a fragment of such a polypeptide. For example, the invention provides a polynucleotide comprising a nucleic acid sequence that encodes an antibody to a human HER3 or encodes a fragment of such an antibody. The polynucleotides of the invention can be in the form of RNA or in the form of DNA. DNA includes cDNA, genomic DNA, and synthetic DNA; and can be double-stranded or single-stranded, and if single stranded can be the coding strand or non-coding (anti-sense) strand.

[0204] In certain embodiments, the polynucleotides are isolated. In certain embodiments, the polynucleotides are substantially pure.

[0205] The invention provides a polynucleotide comprising a polynucleotide encoding a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs:4-53. The invention also provides a polynucleotide comprising a polynucleotide encoding a polypeptide comprising an amino acid sequence that is at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% identical to an amino acid sequence selected from the group consisting of SEQ ID NOs:4-53.

[0206] The invention further provides a polynucleotide comprising a sequence selected from those shown in Tables 7-10 below.

Table 7: Variable heavy chain polynucleotide sequences

Antibody	VH Polynucleotide Sequence (SEQ ID NO)
muHER3-3	gacattgtgatgactcagtctccagccaccctgtctgtgactccaggagatagagtctctctttcctgcagggcc agccagagtattaacgactacttacactggtatcaacaaaaatcacatgagtctccaaggcttctcatcaaatttg cttcccactccatctctgggatcccctccaggttcagtggcagtggatcagggtcagatttcactctcactatcaa cagtgtggaacctgaagatgttggagtgtattactgtcaaaatggtcacagctttccgtggacgttcggtggag

	gcaccaagctggaaatcaaacgg (SEQ ID NO: 54)
huHER3-3	aagcttgccaccatgggttggtcctgtatcatactgttcctggttgcaactgccaccggcgtacactcccaggtg cagctggtgcagccaggagcagaagtagtgaagcccggagcaagcgtcaaaatgtcctgcaaggctagcg gctacacattcaccgactattggatacactgggtgaagcagcggcctggccaaggactggagtggatcggtg ccattgacaccgacacttacacatcctataaccagaaattccagggcaaagctactctgactgttgatgagtcaa gcagcacagcttacatgcaactctccagcctcactagtgaggacagtgccgtctattactgcgcacgatctttg ctgaggctcctgtactttgactattggggccagggaaccaccctgacagtatcttctgcctcaacaaagggccc (SEQ ID NO: 55)
muHER3-8	gatgtgcagcttcaggagtcgggacctggcctggtgacaccttctcagtctctgtccctcacctgcactgtcact ggctcctcaatcaccagtgattatgcctggaactggatccggcagtttccaggaaacaaactggagtggatgg gcttcatcagttacagtggtagcactagctacaacccatctctcaaaagtcgaatctctatcactcgagacacat ccaagaaccagttcttcctgcacttgagttctgtgacttctgaggacacagccacatattactgttcaaggaaact gggacgtctttactactttgactactggggccaaggcaccactctcacagtctcctca (SEQ ID NO: 56)
huHER3-8v.1.00	aagcttgccaccatgggatggtcctgtattattttgttcctggtcgccactgcaactggtgtgcatagtcaggtgc agctgcaagagtcaggccctggtttggtgaaacccagccagagtctgtctctgacctgcaccgttaccggctct tccatcacttccgattacgcatggaattggatcaggcaacatccaggaaataaactggagtggatgggcttcat ctcttatagtggttctacctcctacaaccccagtttgaaaagtcgaatcagtataactcgcgatacttccaaaaac cagttctttctccatctgagcagtgtgaccgctgcagacaccgctacctattactgttcccggaagctgggccga ctctattattttgactactgggggcaggggactctgttgactgtcagctctgcctccacaaagggccc (SEQ ID NO: 57)
huHER3-8v.1.01	aagcttgccaccatgggatggtcctgtattattttgttcctggtcgccactgcaactggtgtgcatagtc aggtgcagctgcaagagtcaggccctggtttggtgaaacccagccagagtctgtctctgacctgcaccgt taccggctcttccatcacttccgattacgcatggaattggatcaggcaacatccaggaaataaactggag tggatgggcttcatctcttatagtggttctacctcctacaaccccagtttgaaaagtcgaatcagtataa ctcgcgatacttccaaaaaccagttctttctccatctgagcagtgtgaccgctgcagacaccgctaccta ttactgttctagaaagctgggccgactctattattttgactactgggggcaggggactctgttgactgtc Agctctgcctccacaaagggccc (SEQ ID NO: 58)
muHER3-10	actgactacaccatgaactgggtgaagcagagccatggaaagaaccttgagtggattggacttattaatcctta caatgatattactgcctacaaccagaagttcaagggcaaggccacattatctgtaaacaagtcatccagcacag cctacatggagctcctcagtctgacatctgaagactctgcagtctattattgtgcaagacggggcccaagggg ggcctggtttgcttactggggccaagggactctggtcactgtctctgca (SEQ ID NO: 59)
huHER3-10	aagcttgccaccatgggctggagttgtatcattctgttcctcgttgcaaccgccaccggtgtgcacagccaggtt cagctggtacagtctggacctgaagtggttaagccaggcgtttccatgaaaattagctgtaaggcctccggcta tacattcaccgattataccatgaattgggtgaagcaaagtcctggacagaacctggaatggattggcttgatcaa tccatacaacgacattacagcatacaaccagaagtttcaagggaaggctaccttgtctgtggacaaatctagct ctaccgcctacatggagctgttgagcttgacctccgaggacagtgccgtttattactgtgctaggcgcgggcca aggggcgcatggtttgcctattggggccagggtacactggtaacagtgagtgctgcatctacaaagggccc (SEQ ID NO: 60)

Table 8: Variable light chain polynucleotide sequences

Antibody	VL Polynucleotide Sequence (SEQ ID NO)
muHER3-3	gacattgtgatgactcagtctccagccaccctgtctgtgactccaggagatagagtctctctttcctgcagggcc agccagagtattaccgactacttacactggtatcaacaaaaatcacatgagtctccaaggcttctcatcaaatttg cttcccaatccatctctgggatcccctccaggttcagtggcagtggatcagggtcagatttcactctcagtatcaa cagtgtggaacctgaagatgttggagtgtattactgtcaaaatggtcacagctttcctctcacgttcggtgctggg

	accaagctggagctgaaacgg (SEQ ID NO: 61)
huHER3-3	gaattcgccaccatgggttggtcctgtatcatccttttctggtcgctacagccacaggggtgcacagtgagata gtgatgacccagtctccagctactctctccgtcacccccggcgatcgagttacccttcctgccgggcttccca gtctattaccgattaccttcactggtatcagcagaagcctggtcagagcccccgcctgctgattaaattcgcctc acagtccatcagtggtattccagatcgttttagcggttccggctccggctcagactttactctttccatcagttcag tggagcctgaagacgtgggggtgtattactgtcagaatggccactccttcccttgacttttggacagggcacc aagttggagctcaagcgtacg (SEQ ID NO: 62)
muHER3-8	gacattgtgatgactcagtctccagccaccctgtctgtgactccaggagatagagtctctctttcctgcagggcc agccagagtattaacgactacttacactggtatcaacaaaaatcacatgagtctccaaggcttctcatcaaatttg cttcccactccatctctgggatcccctccaggttcagtggcagtggatcagggtcagatttcactctcactatcaa cagtgtggaacctgaagatgttggagtgtattactgtcaaaatggtcacagctttccgtggacgttcggtggag gcaccaagctggaaatcaaacgg (SEQ ID NO: 63)
huHER3-8v.1.00 and huHER3-8v.1.01	gaattcgccaccatgggatggagttgcatcatcctgttcctggtagccaccgccacaggggtgcatagtgaga tagtaatgacacagagccctgcaaccctgtccgttactcctggtgatagggtatcactgtcatgtagggccagt caatccattaatgactacctgcattggtatcagcagaagcctggacaatccccacgattgctgatcaaatttgcta gccacagcatctcaggaatccccgacagattttcaggctccggcagtggatcagacttcacactgacaatctct agtgtcgagcctgaagatgtcggcgtgtattactgccagaacggccatagtttcccatggacatttgggggggg gtacaaaattggagatcaaacgtacg (SEQ ID NO: 64)
muHER3-10	actgactacaccatgaactgggtgaagcagagccatggaaagaaccttgagtggattggacttattaatcctta caatgatattactgcctacaaccagaagttcaagggcaaggccacattatctgtaaacaagtcatccagcacag cctacatggagctcctcagtctgacatctgaagactctgcagtctattattgtgcaagacggggcccaagggg ggcctggtttgcttactggggccaagggactctggtcactgtctctgca (SEQ ID NO: 65)
huHER3-10	gaattcgccaccatgggatggtcatgtatcattctgtttctggtggctacagcaactggggtacacagtgatatc gtgctgacccaaagtcctgcttcactggccgtgtcccctggccagcgcgcaactatcagttgtagggcttctac ctccgtatacaattacggtaactccttcatgcactggtaccagcagatgcccggtcagccccccaaactgttgat ctaccttgcttccaacctggaatcaggcgtcccagcccgttttagcggttccggctcacgaacagatttcaccct tactattgaccccgtcgaggccaacgatgctgcaacatactattgccagcagaatagtgaagatccttggacat ttggccagggaactaagttggaaattaaacgtacg (SEQ ID NO: 66)

Table 9: Full-length heavy chain polynucleotide sequences

Antibody	Full-Length Heavy Chain Polynucleotide Sequence (SEQ ID NO)
huHER3-3	aagcttgccaccatgggttggtcctgtatcatactgttcctggttgcaactgccaccggcgtacactcccaggtg cagctggtgcagccaggagcagaagtagtgaagcccggagcaagcgtcaaaatgtcctgcaaggctagcg gctacacattcaccgactattggatacactgggtgaagcagcggcctggccaaggactggagtggatcggtg ccattgacaccgacacttacacatcctataaccagaaattccagggcaaagctactctgactgttgatgagtcaa gcagcacagcttacatgcaactctccagcctcactagtgaggacagtgccgtctattactgcgcacgatctttg ctgaggctcctgtactttgactattggggccagggaaccaccctgacagtatcttctgcctcaacaaagggccc atcagttttccccttggctccaagttctaaatccacaagcggtggaacagctgcactgggatgcctcgttaaaga ttatttccctgagcctgtgacagtgagctggaatagcggagcattgacttcaggtgtgcacacttttcccgctgtg ttgcagtcctccggtctgtactcactgtccagtgtcgtaaccgtcccttctagcagcttgggaacccagacctac atctgtaacgtcaaccataaaccatccaacacaaaggtggataagaaggttgaaccaaagagctgtgataaga cacatacatgccctccttgtcctgcaccagagctcctcggaggtccatctgtgttcctgtttcccccaaaccca aggacactcttatgatctctcgtactccagaggtcacctgtgttgttgtcgacgtgagccatgaagatcccgagg ttaaattcaactggtacgtggatggagtcgaggttcacaatgccaagaccaagcccagggaggagcaatata attctacatatcgggtagtgagcgttctgaccgtgctccaccaagattggctcaatggaaaagagtacaagtgc aaggtgtccaacaaggctcttcccgctcccattgagaaaactatctccaaagccaaggggcagccacgggaa

	ccccaggtgtatacattgccccccatctagagacgagctgaccaagaaccaggtgagtctcacttgtctggtcaa ggggttttacccttctgacattgctgtagagtgggagtctaacggacagccagaaaacaactacaagacaact ccccccagtgctggacagcgacgggagcttcttcctctactccaagttgactgtagacaagtctagatggcagc aaggaaacgttttctcctgctcagtaatgcatgaggctctgcacaatcactatacccagaaatcactgtcccttag cccagggtgactcgag (SEQ ID NO: 67)
huHER3-8v.1.00	aagcttgccaccatgggatggtcctgtattattttgttcctggtcgccactgcaactggtgtgcatagtcaggtgc agctgcaagagtcaggccctggtttggtgaaacccagccagagtctgtctctgacctgcaccgttaccggctct tccatcacttccgattacgcatggaattggatcaggcaacatccaggaaataaactggagtggatgggcttcat ctcttatagtggttctacctcctacaaccccagtttgaaaagtcgaatcagtataactcgcgatacttccaaaaac cagttctttctccatctgagcagtgtgaccgctgcagacaccgctacctattactgttcccggaagctgggccga ctctattattttgactactgggggcaggggactctgttgactgtcagctctgcctccacaaagggcccatcagttt tcccccttggctccaagttctaaatccacaagcggtggaacagctgcactgggatgcctcgttaaagattatttcc ctgagcctgtgacagtgagctggaatagcggagcattgacttcaggtgtgcacacttttcccgctgtgttgcagt cctccggtctgtactcactgtccagtgtcgtaaccgtcccttctagcagcttgggaacccagacctacatctgta acgtcaaccataaaccatccaacacaaaggtggataagaaggttgaaccaaagagctgtgataagacacata catgccctccttgtcctgcaccagagctcctcggaggtccatctgtgttcctgtttcccccaaacccaaggaca ctcttatgatctctcgtactccagaggtcacctgtgttgttgtcgacgtgagccatgaagatcccgaggttaaatt caactggtacgtggatggagtcgaggttcacaatgccaagaccaagcccagggaggagcaatataattctac atatcgggtagtgagcgttctgaccgtgctccaccaagattggctcaatggaaaagagtacaagtgcaaggtg tccaacaaggctcttcccgctcccattgagaaaactatctccaaagccaaggggcagccacgggaaccccag gtgtatacattgccccccatctagagacgagctgaccaagaaccaggtgagtctcacttgtctggtcaaggggtt ttacccttctgacattgctgtagagtgggagtctaacggacagccagaaaacaactacaagacaactcccccca gtgctggacagcgacgggagcttcttcctctactccaagttgactgtagacaagtctagatggcagcaaggaa acgttttctcctgctcagtaatgcatgaggctctgcacaatcactatacccagaaatcactgtcccttagcccagg gtgactcgag (SEQ ID NO: 68)
huHER3-8v.1.01	aagcttgccaccatgggatggtcctgtattattttgttcctggtcgccactgcaactggtgtgcatagtcaggtgc agctgcaagagtcaggccctggtttggtgaaacccagccagagtctgtctctgacctgcaccgttaccggctct tccatcacttccgattacgcatggaattggatcaggcaacatccaggaaataaactggagtggatgggcttcat ctcttatagtggttctacctcctacaaccccagtttgaaaagtcgaatcagtataactcgcgatacttccaaaaac cagttctttctccatctgagcagtgtgaccgctgcagacaccgctacctattactgttctagaaagctgggccga ctctattattttgactactgggggcaggggactctgttgactgtcagctctgcctccacaaagggcccatcagttt tcccccttggctccaagttctaaatccacaagcggtggaacagctgcactgggatgcctcgttaaagattatttcc ctgagcctgtgacagtgagctggaatagcggagcattgacttcaggtgtgcacacttttcccgctgtgttgcagt cctccggtctgtactcactgtccagtgtcgtaaccgtcccttctagcagcttgggaacccagacctacatctgta acgtcaaccataaaccatccaacacaaaggtggataagaaggttgaaccaaagagctgtgataagacacata catgccctccttgtcctgcaccagagctcctcggaggtccatctgtgttcctgtttcccccaaacccaaggaca ctcttatgatctctcgtactccagaggtcacctgtgttgttgtcgacgtgagccatgaagatcccgaggttaaatt caactggtacgtggatggagtcgaggttcacaatgccaagaccaagcccagggaggagcaatataattctac atatcgggtagtgagcgttctgaccgtgctccaccaagattggctcaatggaaaagagtacaagtgcaaggtg tccaacaaggctcttcccgctcccattgagaaaactatctccaaagccaaggggcagccacgggaaccccag gtgtatacattgccccccatctagagacgagctgaccaagaaccaggtgagtctcacttgtctggtcaaggggtt ttacccttctgacattgctgtagagtgggagtctaacggacagccagaaaacaactacaagacaactcccccca gtgctggacagcgacgggagcttcttcctctactccaagttgactgtagacaagtctagatggcagcaaggaa acgttttctcctgctcagtaatgcatgaggctctgcacaatcactatacccagaaatcactgtcccttagcccagg gtgactcgag (SEQ ID NO:69)
huHER3-10	aagcttgccaccatgggctggagttgtatcattctgttcctcgttgcaaccgccaccggtgtgcacagccaggtt cagctggtacagtctggacctgaagtggttaagccaggcgtttccatgaaaattagctgtaaggcctccggcta tacattcaccgattataccatgaattgggtgaagcaaagtcctggacagaacctggaatggattggcttgatcaa tccatacaacgacattacagcatacaaccagaagtttcaagggaaggctaccttgtctgtggacaaatctagct

	ctaccgcctacatggagctgttgagcttgacctccgaggacagtgccgtttattactgtgctaggcgcgggcca aggggcgcatggtttgcctattggggccagggtacactggtaacagtgagtgctgcatctacaaagggcccat cagttttccccttggctccaagttctaaatccacaagcggtggaacagctgcactgggatgcctcgttaaagatt atttccctgagcctgtgacagtgagctggaatagcggagcattgacttcaggtgtgcacacttttcccgctgtgtt gcagtcctccggtctgtactcactgtccagtgtcgtaaccgtcccttctagcagcttgggaacccagacctacat ctgtaacgtcaaccataaaccatccaacacaaaggtggataagaaggttgaaccaaagagctgtgataagac acatacatgccctccttgtcctgcaccagagctcctcggaggtccatctgtgttcctgtttccccccaaacccaa ggacactcttatgatctctcgtactccagaggtcacctgtgttgttgtcgacgtgagccatgaagatcccgaggt taaattcaactggtacgtggatggagtcgaggttcacaatgccaagaccaagcccagggaggagcaatataa ttctacatatcgggtagtgagcgttctgaccgtgctccaccaagattggctcaatggaaaagagtacaagtgca aggtgtccaacaaggctcttcccgctcccattgagaaaactatctccaaagccaaggggcagccacgggaac cccaggtgtatacattgcccccatctagagacgagctgaccaagaaccaggtgagtctcacttgtctggtcaa ggggttttaccttctgacattgctgtagagtgggagtctaacggacagccagaaaacaactacaagacaact cccccagtgctggacagcgacgggagcttcttcctctactccaagttgactgtagacaagtctagatggcagc aaggaaacgttttctcctgctcagtaatgcatgaggctctgcacaatcactatacccagaaatcactgtcccttag cccagggtgactcgag (SEQ ID NO: 70)

Table 10: Full-length light chain polynucleotide sequences

Antibody	Full-length Light Chain Polynucleotide Sequence (SEQ ID NO)
huHER3-3	Gaattcgccaccatgggttggtcctgtatcatccttttctggtcgctacagccacaggggtgcacagtgagata gtgatgacccagtctccagctactctctccgtcacccccggcgatcgagttacccttcctgccgggcttccca gtctattaccgattaccttcactggtatcagcagaagcctggtcagagcccccgcctgctgattaaattcgcctc acagtccatcagtggtattccagatcgttttagcggttccggctccggctcagactttactctttccatcagttcag tggagcctgaagacgtgggggtgtattactgtcagaatggccactccttcccccttgacttttggacagggcacc aagttggagctcaagcgtacggtggctgcaccatctgtcttcatcttcccgccatctgatgagcagttgaaatct ggaactgcctctgttgtgtgcctgctgaataacttctatcccagagaggccaaagtacagtggaaggtggataa cgccctccaatcgggtaactcccaggagagtgtcacagagcaggacagcaaggacagcacctacagcctc agcagcaccctgacgctgagcaaagcagactacgagaaacacaaagtctacgcctgcgaagtcacccatca gggcctgagctcgcccgtcacaaagagcttcaacaggggagagtgttag (SEQ ID NO: 71)
huHER3-8v.1.00 and huHER3-8v.1.01	gaattcgccaccatgggatggagttgcatcatcctgttcctggtagccaccgccacaggggtgcatagtgaga tagtaatgacacagagccctgcaaccctgtccgttactcctggtgatagggtatcactgtcatgtagggccagt caatccattaatgactacctgcattggtatcagcagaagcctggacaatccccacgattgctgatcaaatttgcta gccacagcatctcaggaatccccgacagattttcaggctccggcagtggatcagacttcacactgacaatctct agtgtcgagcctgaagatgtcggcgtgtattactgccagaacggccatagtttcccatggacatttgggggggg gtacaaaattggagatcaaacgtacggtggctgcaccatctgtcttcatcttcccgccatctgatgagcagttga aatctggaactgcctctgttgtgtgcctgctgaataacttctatcccagagaggccaaagtacagtggaaggtg gataacgccctccaatcgggtaactcccaggagagtgtcacagagcaggacagcaaggacagcacctaca gcctcagcagcaccctgacgctgagcaaagcagactacgagaaacacaaagtctacgcctgcgaagtcacc catcagggcctgagctcgcccgtcacaaagagcttcaacaggggagagtgttag (SEQ ID NO: 72)
huHER3-10	gaattcgccaccatgggatggtcatgtatcattctgtttctggtggctacagcaactggggtacacagtgatatc gtgctgacccaaagtcctgcttcactggccgtgtcccctggccagcgcgcaactatcagttgtagggcttctac ctccgtatacaattacggtaactccttcatgcactggtaccagcagatgcccggtcagccccccaaactgttgat ctaccttgcttccaacctggaatcaggcgtcccagcccgttttagcggttccggctcacgaacagatttcaccct tactattgaccccgtcgaggccaacgatgctgcaacatactattgccagcagaatagtgaagatccttggacat ttggccagggaactaagttggaaattaaacgtacggtggctgcaccatctgtcttcatcttcccgccatctgatg agcagttgaaatctggaactgcctctgttgtgtgcctgctgaataacttctatcccagagaggccaaagtacagt

	ggaaggtggataacgccctccaatcgggtaactcccaggagagtgtcacagagcaggacagcaaggacag cacctacagcctcagcagcaccctgacgctgagcaaagcagactacgagaaacacaaagtctacgcctgcg aagtcacccatcagggcctgagctcgcccgtcacaaagagcttcaacaggggagagtgttag (SEQ ID NO: 73)

[0207] Also provided is a polynucleotide having at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity to SEQ ID NOs:54-73. In one embodiment, the invention encompasses a polynucleotide having at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity to SEQ ID NOs:54-73 wherein the polynucleotide encodes a polypeptide that binds HER3.

[0208] In certain embodiments the polynucleotides comprise the coding sequence for the mature polypeptide fused in the same reading frame to a polynucleotide which aids, for example, in expression and secretion of a polypeptide from a host cell (e.g. a leader sequence which functions as a secretory sequence for controlling transport of a polypeptide from the cell). The polypeptide having a leader sequence is a preprotein and can have the leader sequence cleaved by the host cell to form the mature form of the polypeptide. The polynucleotides can also encode for a proprotein which is the mature protein plus additional 5' amino acid residues. A mature protein having a prosequence is a proprotein and is an inactive form of the protein. Once the prosequence is cleaved an active mature protein remains.

[0209] In certain embodiments the polynucleotides comprise the coding sequence for the mature polypeptide fused in the same reading frame to a marker sequence that allows, for example, for purification of the encoded polypeptide. For example, the marker sequence can be a hexa-histidine tag supplied by a pQE-9 vector to provide for purification of the mature polypeptide fused to the marker in the case of a bacterial host, or the marker sequence can be a hemagglutinin (HA) tag derived from the influenza hemagglutinin protein when a mammalian host (e.g., COS-7 cells) is used.

[0210] The present invention further relates to variants of the above described polynucleotides encoding, for example, fragments, analogs, and derivatives.

[0211] The polynucleotide variants can contain alterations in the coding regions, non-coding regions, or both. In some embodiments the polynucleotide variants contain alterations which produce silent substitutions, additions, or deletions, but do not alter the properties or

activities of the encoded polypeptide. In some embodiments, nucleotide variants are produced by silent substitutions due to the degeneracy of the genetic code. Polynucleotide variants can be produced for a variety of reasons, e.g., to optimize codon expression for a particular host (change codons in the human mRNA to those preferred by a bacterial host such as *E. coli*).

[0212] Vectors and cells comprising the polynucleotides described herein are also provided.

V. Methods of use and pharmaceutical compositions

[0213] The HER3-binding agents (including antibodies, immunoconjugates, and polypeptides) of the invention are useful in a variety of applications including, but not limited to, therapeutic treatment methods, such as the treatment of cancer, such as solid cancers. In certain embodiments, the HER3-binding agents are useful for inhibiting tumor growth, inducing differentiation, reducing tumor volume, and/or reducing the tumorigenicity of a tumor. The methods of use can be *in vitro, ex vivo*, or *in vivo* methods. In certain embodiments, the HER3-binding agent or antibody or immunoconjugate, or polypeptide is an antagonist of the human HER3 to which it binds.

[0214] In one aspect, anti-HER3 antibodies and immunoconjugates of the invention are useful for detecting the presence of HER3 in a biological sample. The term "detecting" as used herein encompasses quantitative or qualitative detection. In certain embodiments, a biological sample comprises a cell or tissue. In certain embodiments, such tissues include normal and/or cancerous tissues that express HER3 at higher levels relative to other tissues.

[0215] In one aspect, the invention provides a method of detecting the presence of HER3 in a biological sample. In certain embodiments, the method comprises contacting the biological sample with an anti-HER3 antibody under conditions permissive for binding of the anti-HER3 antibody to HER3 antigen, and detecting whether a complex is formed between the anti-HER3 antibody and HER3 antigen.

[0216] In one aspect, the invention provides a method of diagnosing a disorder associated with increased expression of HER3 antigen. In certain embodiments, the method comprises contacting a test cell with an anti-HER3 antibody; determining the level of expression (either quantitatively or qualitatively) of HER3 antigen by the test cell by detecting binding of the anti-HER3 antibody to HER3 antigen; and comparing the level of expression of HER3 antigen by the test cell with the level of expression of HER3 antigen by a control cell (e.g., a

normal cell of the same tissue origin as the test cell or a cell that expresses HER3 at levels comparable to such a normal cell), wherein a higher level of expression of HER3 by the test cell as compared to the control cell indicates the presence of a disorder associated with increased expression of HER3. In certain embodiments, the test cell is obtained from an individual suspected of having a disorder associated with increased expression of HER3. In certain embodiments, the disorder is a cell proliferative disorder, such as a cancer or a tumor.

[0217] In certain embodiments, a method of diagnosis or detection, such as those described above, comprises detecting binding of an anti-HER3 antibody to HER3 antigen expressed on the surface of a cell or in a membrane preparation obtained from a cell expressing HER3 on its surface. In certain embodiments, the method comprises contacting a cell with an anti-HER3 antibody under conditions permissive for binding of the anti-HER3 antibody to HER3, and detecting whether a complex is formed between the anti-HER3 antibody and HER3 on the cell surface. An exemplary assay for detecting binding of an anti-HER3 antibody to HER3 expressed on the surface of a cell is a "FACS" assay.

[0218] Certain other methods can be used to detect binding of anti-HER3 antibodies to HER3 antigen. Such methods include, but are not limited to, antigen-binding assays that are well known in the art, such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, fluorescent immunoassays, protein A immunoassays, and immunohistochemistry (IHC).

[0219] In certain embodiments, anti-HER3 antibodies are labeled. Labels include, but are not limited to, labels or moieties that are detected directly (such as fluorescent, chromophoric, electron-dense, chemiluminescent, and radioactive labels), as well as moieties, such as enzymes or ligands, that are detected indirectly, e.g., through an enzymatic reaction or molecular interaction.

[0220] In certain embodiments, anti-HER3 antibodies are immobilized on an insoluble matrix. Immobilization entails separating the anti-HER3 antibody from any HER3 that remains free in solution. This conventionally is accomplished by either insolubilizing the anti-HER3 antibody before the assay procedure, as by adsorption to a water-insoluble matrix or surface (Bennich et al., U.S. Patent No. 3,720,760), or by covalent coupling (for example, using glutaraldehyde cross-linking), or by insolubilizing the anti-HER3 antibody after formation of a complex between the anti-HER3 antibody and HER3 antigen, e.g., by immunoprecipitation.

[0221] Any of the above embodiments of diagnosis or detection can be carried out using an immunoconjugate of the invention in place of or in addition to an anti-HER3 antibody.

[0222] In one embodiment, the invention provides a method of treating, preventing or ameliorating a disease comprising administering a HER3-binding agent or antagonist (e.g., an anti-HER3 antibody) to a patient, thereby treating the disease. In certain embodiments, the disease treated with the HER3-binding agent or antagonist (e.g., an anti-HER3 antibody) is a cancer. Examples of diseases which can be treated and/or prevented include acute lymphoblastic leukemia, acute myeloid leukemia, adrenocortical carcinoma, anal cancer, basal cell carcinoma, bile duct cancer, bladder cancer, bone cancer, brain tumors (including astrocytoma, brain stem glioma, craniopharyngioma, ependymoblastoma, ependymoma, medulloblastoma, medulloepithelioma, pineal parenchymal tumors of intermediate differentiation, supratentorial primitive neuroectodermal tumors, and pineoblastoma), breast cancer, bronchial tumors, Burkitt lymphoma, carcinoid tumors, central nervous system atypical teratoid/rhabdoid tumors, cervical cancer, chordoma, chronic lymphocytic leukemia, chronic myelogenous leukemia, chronic myeloproliferative disorders, colon cancer, colorectal cancer, cutaneous T-Cell lymphoma, embryonal tumors, endometrial cancer, esophageal cancer, Ewing sarcoma, gallbladder cancer, gastric cancer, germ cell tumors, hairy cell leukemia, head and neck cancer, hepatocellular cancer, Hodgkin lymphoma, hypopharyngeal cancer, intraocular melanoma, islet cell tumors, Kaposi sarcoma, kidney cancer, Langerhans cell histiocytosis, laryngeal cancer, liver cancer, malignant fibrous histiocytoma of bone and osteosarcoma, melanoma, Merkel cell carcinoma, mesothelioma, mouth cancer, multiple myeloma/plasma cell neoplasm, mycosis fungoides, myelodysplastic/myeloproliferative neoplasms, nasal cavity and paranasal sinus cancer, nasopharyngeal cancer, neuroblastoma, non-Hodgkin lymphoma, non-small cell lung cancer, oral cancer, oropharyngeal cancer, ovarian cancer, pancreatic cancer, parathyroid cancer, penile cancer, pharyngeal cancer, pituitary tumor, prostate cancer, rectal cancer, renal cell cancer, retinoblastoma, rhabdomyosarcoma, salivary gland cancer, Sézary syndrome, skin cancer (nonmelanoma), small cell lung cancer, small intestine cancer, soft tissue sarcoma, squamous cell carcinoma, squamous neck cancer, stomach cancer, T-cell lymphoma, testicular cancer, throat cancer, thymoma and thymic carcinoma, thyroid cancer, urethral cancer, uterine cancer, vaginal cancer, vulvar cancer, Waldenström macroglobulinemia, and Wilms tumor. In certain embodiments, the cancer is characterized by HER3 expressing cells to which the HER3-binding agent (e.g., antibody) binds.

[0223] The present invention provides for methods of treating cancer comprising administering a therapeutically effective amount of a HER3-binding agent to a subject (e.g., a subject in need of treatment). In certain embodiments, the cancer is a solid cancer. In certain embodiments, the cancer is selected from the group consisting of B cell lymphomas, NHL, precursor B cell lymphoblastic leukemia/lymphoma and mature B cell neoplasms, B cell chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma (SLL), B cell prolymphocytic leukemia, lymphoplasmacytic lymphoma, mantle cell lymphoma (MCL), follicular lymphoma (FL), low grade, intermediate-grade and high-grade (FL), cutaneous follicle center lymphoma, marginal zone B cell lymphoma, MALT type marginal zone B cell lymphoma, nodal marginal zone B cell lymphoma, splenic type marginal zone B cell lymphoma, hairy cell leukemia, diffuse large B cell lymphoma, Burkitt's lymphoma, plasmacytoma, plasma cell myeloma, post-transplant lymphoproliferative disorder, Waldenstrom's macroglobulinemia, and anaplastic large-cell lymphoma (ALCL). In certain embodiments, the subject is a human.

[0224] The present invention further provides methods for inhibiting tumor growth using the antibodies or other agents described herein. In certain embodiments, the method of inhibiting tumor growth comprises contacting a cell with a HER3-binding agent (e.g., antibody*) in vitro*. For example, an immortalized cell line or a cancer cell line that expresses HER3 is cultured in medium to which is added the antibody or other agent to inhibit tumor growth. In some embodiments, tumor cells are isolated from a patient sample such as, for example, a tissue biopsy, pleural effusion, or blood sample and cultured in medium to which is added a HER3-binding agent to inhibit tumor growth.

[0225] In some embodiments, the method of inhibiting tumor growth comprises contacting the tumor or tumor cells with the HER3-binding agent (e.g., antibody) *in vivo*. In certain embodiments, contacting a tumor or tumor cell with a HER3-binding agent is undertaken in an animal model. For example, HER3-binding agents can be administered to xenografts expressing HER3 that have been grown in immunocompromised mice (e.g., NOD/SCID mice) to inhibit tumor growth. In some embodiments, cancer stem cells are isolated from a patient sample such as, for example, a tissue biopsy, pleural effusion, or blood sample and injected into immunocompromised mice that are then administered a HER3-binding agent to inhibit tumor cell growth. In some embodiments, the HER3-binding agent is administered at the same time or shortly after introduction of tumorigenic cells into the

animal to prevent tumor growth. In some embodiments, the HER3-binding agent is administered as a therapeutic after the tumorigenic cells have grown to a specified size.

[0226] In certain embodiments, the method of inhibiting tumor growth comprises administering to a subject a therapeutically effective amount of a HER3-binding agent. In certain embodiments, the subject is a human. In certain embodiments, the subject has a tumor or has had a tumor removed.

[0227] In certain embodiments, the tumor expresses the HER3 to which the HER3-binding agent or antibody binds. In certain embodiments, the tumor overexpresses the human HER3.

[0228] In addition, the invention provides a method of reducing the tumorigenicity of a tumor in a subject, comprising administering a therapeutically effective amount of a HER3-binding agent to the subject. In certain embodiments, the tumor comprises cancer stem cells. In certain embodiments, the frequency of cancer stem cells in the tumor is reduced by administration of the HER3-binding agent.

[0229] The invention further provides methods of differentiating tumorigenic cells into non-tumorigenic cells comprising contacting the tumorigenic cells with a HER3-binding agent, for example, by administering the HER3-binding agent to a subject that has a tumor comprising the tumorigenic cells or that has had such a tumor removed.

[0230] The use of the HER3-binding agents, polypeptides, or antibodies described herein to induce the differentiation of cells, including, but not limited to tumor cells, is also provided. For example, methods of inducing cells to differentiate comprising contacting the cells with an effective amount of a HER3-binding agent (e.g., an anti-HER3 antibody) described herein are envisioned. Methods of inducing cells in a tumor in a subject to differentiate comprising administering a therapeutically effective amount of a HER3-binding agent, polypeptide, or antibody to the subject are also provided. In certain embodiments, the tumor is a pancreatic tumor. In certain other embodiments, the tumor is a lung tumor. In some embodiments, the treatment methods comprise administering a therapeutically effective amount of the HER3-binding agent, polypeptide, or antibody to the subject.

[0231] The present invention further provides pharmaceutical compositions comprising one or more of the HER3-binding agents described herein. In certain embodiments, the pharmaceutical compositions further comprise a pharmaceutically acceptable vehicle. These pharmaceutical compositions find use in inhibiting tumor growth and treating cancer in human patients.

[0232] In certain embodiments, formulations are prepared for storage and use by combining a purified antibody or agent of the present invention with a pharmaceutically acceptable vehicle (e.g., carrier, excipient) (Remington, The Science and Practice of Pharmacy 20th Edition Mack Publishing, 2000). Suitable pharmaceutically acceptable vehicles include, but are not limited to, nontoxic buffers such as phosphate, citrate, and other organic acids; salts such as sodium chloride; antioxidants including ascorbic acid and methionine; preservatives (e.g., octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride; benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens, such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight polypeptides (e.g., less than about 10 amino acid residues); proteins such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; carbohydrates such as monosacchandes, disaccharides, glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g., Zn-protein complexes); and non-ionic surfactants such as TWEEN or polyethylene glycol (PEG).

[0233] The pharmaceutical compositions of the present invention can be administered in any number of ways for either local or systemic treatment. Administration can be topical (such as to mucous membranes, including vaginal and rectal delivery) such as transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders; pulmonary (e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer, intratracheal, intranasal, epidermal and transdermal); oral; or parenteral including intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial (e.g., intrathecal or intraventricular) administration.

[0234] An antibody or immunoconjugate of the invention can be combined in a pharmaceutical combination formulation, or dosing regimen as combination therapy, with a second compound having anti-cancer properties that differs from the antibody or immunoconjugate. The second compound of the pharmaceutical combination formulation or dosing regimen can have complementary activities to the antibody or immunoconjugate of the combination such that they do not adversely affect each other. Pharmaceutical compositions comprising the HER3-binding agent and the second anti-cancer agent are also provided. For example, HER3-binding agents can be administered in combination with, but

not limited to, a chemotherapeutic agent, HER2 antagonists, such as trastuzumab and lapatinib, and/or EGFR antagonists, such as cetuximab, panitumumab, erlotinib and gefitinib.

[0235] For the treatment of the disease, the appropriate dosage of an antibody or agent of the present invention depends on the type of disease to be treated, the severity and course of the disease, the responsiveness of the disease, whether the antibody or agent is administered for therapeutic or preventative purposes, previous therapy, patient's clinical history, and so on, all at the discretion of the treating physician. The antibody or agent can be administered one time or over a series of treatments lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved (e.g., reduction in tumor size). Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient and will vary depending on the relative potency of an individual antibody or agent. The administering physician can easily determine optimum dosages, dosing methodologies and repetition rates. In certain embodiments, dosage is from 0.01μg to 100mg (e.g., 0.01μg, 0.1μg, 1.0μg, 10μg, 50μg, 0.1mg, 0.5mg, 1mg, 5mg, 10mg, 20mg, 30mg, 40mg, 50mg, or 100mg) per kg of body weight, and can be given once or more daily, weekly, monthly or yearly. In certain embodiments, the antibody or other HER3-binding agent is given once every two weeks or once every three weeks. In certain embodiments, the dosage of the antibody or other HER3-binding agent is from about 0.1mg to about 20mg (e.g., 0.1mg, 0.5mg, 1mg, 5mg, 10mg, 15mg, or 20mg) per kg of body weight. The treating physician can estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues.

[0236] The combination therapy can provide "synergy" and prove "synergistic", i.e., the effect achieved when the active ingredients used together is greater than the sum of the effects that results from using the compounds separately. A synergistic effect can be attained when the active ingredients are: (1) co-formulated and administered or delivered simultaneously in a combined, unit dosage formulation; (2) delivered by alternation or in parallel as separate formulations; or (3) by some other regimen. When delivered in alternation therapy, a synergistic effect can be attained when the compounds are administered or delivered sequentially, e.g., by different injections in separate syringes. In general, during alternation therapy, an effective dosage of each active ingredient is administered sequentially, i.e., serially, whereas in combination therapy, effective dosages of two or more active ingredients are administered together.

VI. Kits comprising HER3 binding agents

[0237] The present invention provides kits that comprise the antibodies, immunoconjugates or other agents described herein and that can be used to perform the methods described herein. In certain embodiments, a kit comprises at least one purified antibody against HER3 in one or more containers. In some embodiments, the kits contain all of the components necessary and/or sufficient to perform a detection assay, including all controls, directions for performing assays, and any necessary software for analysis and presentation of results. One skilled in the art will readily recognize that the disclosed antibodies, immunoconjugates or other agents of the present invention can be readily incorporated into one of the established kit formats which are well known in the art.

[0238] Further provided are kits comprising a HER3-binding agent (e.g., a HER3-binding antibody), as well as a second anti-cancer agent that differs from the HER3-binding agent. In certain embodiments, the second anti-cancer agent is a chemotherapeutic agent (e.g., cisplatin).

[0239] Embodiments of the present disclosure can be further defined by reference to the following non-limiting examples, which describe in detail preparation of certain antibodies of the present disclosure and methods for using antibodies of the present disclosure. It will be apparent to those skilled in the art that many modifications, both to materials and methods, can be practiced without departing from the scope of the present disclosure.

EXAMPLES

[0240] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application

[0241] Cell lines

Cell line	Origin	Source
MCF7	Breast adenocarcinoma	ATCC-HTB-22
SKBR3	Breast adenocarcinoma	ATCC-HTB-30
BT474	Breast ductal carcinoma	ATCC-HTB-20
MDA-MB453	Breast metastatic carcinoma	ATCC-HTB-131
ZR75-30	Breast ductal carcinoma	ATCC-CRL-1504

[0242] All cell lines were grown in media suggested by the suppliers at 37°C. Cells were passaged by diluting into fresh media twice per week and maintained between 0.2 to $1x10^6$ cells/ml.

EXAMPLE 1

[0243] Production of murine HER3 antibodies

[0244] Human HER3 (huHER3)-overexpressing 300-19 cells, a pre-B cell line derived from a Balb/c mouse (Reth MG et al. 1985, Nature, 317: 353-355), were used as immunogen for mouse immunization to produce murine anti HER3 antibodies. To generate huHER3 overexpressing 300-19 cell lines, the human HER3 amino acid sequence (Genbank accession NP_001973) was codon-optimized and the cDNA was synthesized by Blue Heron Biotechnology (Bothell, WA). An expression plasmid (pSRα-huHER3) that contained the entire huHER3 coding sequence (CDS) flanked by XbaI and BamHI restriction sites was constructed and transfected into the 300-19 cells to stably express high levels of huHER3 on the cell surface. Immunization was done in Balb/c mice (Charles River Laboratory, Wilmington, MA) by subcutaneous injection of $5x10^6$ HER3-expressing 300-19 cells per mouse every 2 weeks for 5 times. The immunized mice were boosted with another dose of antigen three days before being sacrificed for hybridoma generation. The spleen from the mouse was collected according to standard animal protocols and was ground between two sterile, frosted microscopic slides to obtain a single cell suspension in RPMI-1640 medium. After the red blood cells were lysed with ACK lysing buffer, the spleen cells were then mixed with murine myeloma P3X63Ag8.653 cells (P3 cells) (J. F. Kearney et al. 1979, *J Immunol, 123*: 1548-1550) at a ratio of 1 P3 cell for every 3 spleen cells. The mixture of spleen cells and P3 cells was washed and treated with pronase in fusion media (0.3M mannitol/D-sorbitol, 0.1mM $CaCl_2$, 0.5mM $MgCl_2$ and 1mg/ml BSA) at room temperature for 3 min. The reaction was stopped by addition of FBS; cells were then washed, resuspended in 2 ml cold fusion media and fused with BTX ECM 2001 electrofusion machine (Harvard Apparatus). The fused cells were added gently to RPMI-1640 selection medium containing hypoxanthine-aminopterin-thymidine (HAT) (Sigma H-0262), incubated for 20min at 37°C, and then seeded into flat bottom 96-well plates at 200μl/well. The plates were then incubated in a 5% CO_2 incubator at 37ºC. Incubation was continued until hydridoma clones were ready for antibody screening. Other techniques of immunization and hybridoma production can also be

used, including those described in J. Langone and H. Vunakis (Eds., Methods in Enzymology, Vol. 121, "Immunochemical Techniques, Part I"; Academic Press, Florida) and E. Harlow and D. Lane ("Antibodies: A Laboratory Manual";1988; Cold Spring Harbor Laboratory Press, New York, NY).

[0245] Hybridoma screening and selection

[0246] Culture supernatants from the hybridoma were screened by flow cytometry for mouse monoclonal antibodies that bind to the human HER3 positive MCF7 cells. 50µl of hybridoma supernatants was incubated for 1h with MCF7 cells ($1x10^5$ cells per sample). Then, the cells were washed twice and incubated for 1h at 4°C with 100µL of PE-conjugated goat anti-mouse IgG-antibody (Jackson Immunoresearch, West Grove, PA; 6µg/mL in FACS buffer (PBS supplemented with 0.5% BSA)). The cells were pelleted again, washed with FACS buffer and resuspended in 200µL of PBS containing 1% formaldehyde. Samples were acquired using a FACSCalibur flow cytometer with the HTS multiwell sampler or a FACS array flow cytometer and analyzed using CellQuest Pro (all from BD Biosciences, San Diego, CA).

[0247] The hybridoma clones that tested positive were subcloned by limiting dilution. One subclone from each hybridoma, which showed the same reactivity against HER3 as the parental cells by flow cytometry, was chosen for subsequent analysis. Stable subclones were cultured and the antibody was isotyped using commercial isotyping reagents (Roche 1493027).

[0248] A total of 11 independent fusions were conducted over the course of this investigation. A single fusion experiment routinely yielded approximately between 400 and 900 hybridoma clones. All the resulting hybridoma clones were screened for HER3 binding by flow cytometry and a total of 31 hybridoma clones showed specific binding to HER3.

[0249] Antibody purification

[0250] Antibodies were purified from hybridoma subclone supernatants using standard methods, such as, for example Protein A or G chromatography (HiTrap Protein A or G HP, 1mL, Amersham Biosciences). Briefly, supernatant was prepared for chromatography by the addition of 1/10 volume of 1M Tris/HCl buffer, pH 8.0. The pH-adjusted supernatant was filtered through a 0.22µm filter membrane and loaded onto column equilibrated with binding buffer (PBS, pH 7.3). The column was washed with binding buffer until a stable baseline was obtained with no absorbance at 280nm. Antibody was eluted with 0.1M acetic acid buffer containing 0.15M NaCl, pH 2.8, using a flow rate of 0.5mL/min. Fractions of

approximately 0.25mL were collected and neutralized by the addition of 1/10 volume of 1M Tris/HCl, pH 8.0. The peak fraction(s) was dialyzed overnight twice against 1x PBS and sterilized by filtering through a 0.2 μm filter membrane. Purified antibody was quantified by absorbance at A280.

[0251] Protein A purified fractions were further polished using ion exchange chromatography (IEX) with quaternary ammonium (Q) chromatography for murine antibodies. Briefly, samples from protein A purification were buffer exchanged into binding buffer (10mM Tris, 10mM sodium chloride, pH 8.0) and filtered through a 0.22μm filter membrane. The prepared sample was then loaded onto a Q fast flow resin (GE Lifesciences) that was equilibrated with binding buffer at a flow rate of 120cm/hr. Column size was chosen to have sufficient capacity to bind all the monoclonal antibody in the sample. The column was then washed with binding buffer until a stable baseline was obtained with no absorbance at 280nm. Antibody was eluted by initiating a gradient from 10mM to 500mM sodium chloride in 20 column volume (CV). Peak fractions were collected based on absorbance measurement at 280nm (A280). The percentage of monomer was assessed with size exclusion chromatography (SEC) on a TSK gel G3000SWXL, 7.8 × 300 mm with a SWXL guard column, 6.0 × 40 mm (Tosoh Bioscience, Montgomeryville, PA) using an Agilent HPLC 1100 system (Agilent, Santa Clara, CA). Fractions with monomer content above 95% were pooled, buffer exchanged to PBS (pH 7.4) using a TFF system, and sterilized by filtering through a 0.2μm filter membrane. The IgG concentration of purified antibody was determined by A280 using an extinction coefficient of 1.47. Alternative methods such as ceramic hydroxyapatite (CHT) were also used to polish antibodies with good selectivity. Type II CHT resin with 40μm particle size (Bio-Rad Laboratories) were used with a similar protocol as described for IEX chromatography. The binding buffer for CHT corresponds to 20mM sodium phosphate, pH 7.0 and antibody was eluted with a gradient of 20-160mM sodium phosphate over 20 CV.

EXAMPLE 2

[0252] Inhibition of HER3 ligand independent growth of SKBR3 cells

[0253] The present invention focuses on a novel class of HER3 antibodies that inhibit the HER3 ligand independent growth of cancer cells. During antibody selection, the ability of HER3 antibodies to inhibit cancer cell growth was tested using *in vitro* cell proliferation assays with SKBR3 breast cancer cell line. The SKBR3 cells were chosen because this cell

line constitutively activates HER3 signaling in the presence of serum *in vitro* (Kraus MH. et al. Proc. Natl. Acad. Sci. USA, 90, 2900-2904 (1993)). In this experiment (Figure 1A), SKBR3 target cells were plated at 2,000 cells per well in normal growth media containing 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA). 10μg/ml purified anti-HER3 antibodies were added to the cells and the cultures were further incubated at 37°C in a humidified 5% CO_2 incubator for 4 to 5 days. Level of cell proliferation was determined using colorimetric WST-8 assay (Dojindo Molecular Technologies, Rockville, MD). WST-8 is reduced by dehydrogenases in the living cells to an orange formazan product that is soluble in tissue culture medium, and the amount of formazan produced is directly proportional to the number of living cells. 10% of the final volume of WST-8 was added to each well and plates were incubated at 37°C in a humidified 5% CO_2 incubator for an additional 2-4h. Plates were then analyzed by measuring the absorbance at 450 nm (A450) in the Spectra Max M2 plate reader (Molecular Devices, Sunnyvale, CA). Background A450 absorbance of wells with media and WST-8 only was subtracted from all values. The percent cell proliferation was calculated by dividing each treated sample value by the average value of wells with untreated cell (percent cell proliferation = 100* (A450 treated sample – A450 background)/ (A450 untreated sample – A450 background)).

[0254] A representative result of an SKBR3 cell proliferation assay is presented in Figure 1A. The result showed that muHER3-3, muHER3-8 and muHER3-10 were able to inhibit proliferation of SKBR3 cells as effective as trastuzumab (Tmab) which was shown to inhibit HER2-overexpressing breast cancer cells in ligand-independent manner (Junttila TT et al. Cancer Cell 15, 429-440 (2009). Other muHER3 antibodies were found to be less effective. The fact that there are only 3 out of 31 anti HER3 antibodies that are as effective as trastuzumab in inhibiting SKBR3 cell proliferation suggests that this functional property is unique and rare for anti-HER3 antibodies.

[0255] The WST-8 cell proliferation assay result was confirmed using clonogenic assay (Figure 1B). In this experiment, 2,000 SKBR3 cells/well were seeded into 6-well plates in 2ml normal growth media containing 10% FBS. 12μg/ml antibodies were added to the cells and the culture was continued for 14 days at 37ºC until colonies appeared. After washing once with PBS, the cell colonies were stained with crystal violet and counted using COLCOUNT colony counter (Oxford Optronix, Oxford, OX, UK). The experiment was done in duplicate, the colony counts were added and normalized so that 100% represents the colony count of untreated cells and 0% represents zero colonies. As shown in Figure 1B,

Tmab reduced colony count to ~30% while the muHER3-3, muHER3-8 and muHER3-10 antibodies were able to reduce the colony count to ≤20%. This result confirmed the unique properties of anti-HER3 antibodies of the invention in inhibiting the HER3 ligand-independent growth of SKBR3 cell line. This activity was further confirmed later using humanized HER3 antibodies in three additional cell lines (see Example 12).

EXAMPLE 3

[0256] Binding specificity and affinity of muHER3 antibodies

[0257] Binding specificity of the anti-HER3 antibodies of the invention was tested by flow cytometric assay. FACS histograms demonstrating the binding of muHER3-3, muHER3-8, and muHER3-10 antibodies to the human HER3 expressing 300-19 cells but not to the parental 300-19 cells are shown in Figure 2. All murine antibodies were incubated for 1h at 4°C with either huHER3-expressing 300-19 cells or the non-transfected 300-19 cells ($1x10^5$ cells per sample) in 100μL FACS buffer. Then, the cells were pelleted, washed twice, and incubated for 1h with 100μL of PE-conjugated goat anti-mouse IgG-antibody (Jackson Immunoresearch, West Grove, PA). The cells were pelleted again, washed with FACS buffer and resuspended in 200μL of PBS containing 1% formaldehyde. Samples were acquired using a FACSCalibur flow cytometer with the HTS multiwell sampler or a FACS array flow cytometer and analyzed using CellQuest Pro (all from BD Biosciences, San Diego, CA) or FlowJo (Tree Star, Ashland, OR). The FACS histograms of huHER3-expressing 300-19 cells incubated with muHER3-3, muHER3-8 or muHER3-10 antibodies showed a fluorescence shift, while the parental 300-19 cells did not, demonstrating HER3 specific reactivity of these muHER3 antibodies (Figure 2).

[0258] To obtain the binding affinity of these antibodies, varying concentration of murine antibodies were incubated with huHER3-expressing 300-19 cells and processed as described above for flow cytometric analysis. Data analysis was performed using FlowJo (Tree Star, Ashland, OR) and for each sample the geometric mean fluorescence intensity (GM) was plotted against the antibody concentration in a semi-log plot (Figure 3). A dose-response curve was generated by non-linear regression and the EC50 value of each curve, which corresponds to the apparent dissociation constant (Kd) of each antibody, was calculated using GraphPad Prism v4 (GraphPad software, San Diego, CA). A strong shift in fluorescence was observed for all antibodies tested and the Kd values correspond to 0.30nM, 0.23nM and 0.068nM for muHER3-3, muHER3-8 and muHER3-10 antibodies, respectively.

[0259] To verify that these antibodies can also bind to HER3 endogenously expressed by human cancer cells, binding experiments were performed with HER3-positive SKBR3 breast cancer cells using varying concentrations of muHER3 antibodies. A dose-response curve was generated by non-linear regression and the Kd of each antibody was calculated using GraphPad Prism v4. A strong shift in fluorescence was observed for all antibodies tested and the Kd values correspond to 0.21nM, 0.31nM and 0.052nM for muHER3-3, muHER3-8 and muHER3-10 antibodies, respectively. These Kd values closely matched the Kd values generated with huHER3-overexpressing 300-19 cells.

[0260] Crossreactivity with macaque HER3 antigen

[0261] Macaque HER3 (maHER3) amino acid sequence (Genbank accession XP_001113953) shows 98.7% identity with the huHER3 sequence (Genbank accession NP_001973) with only 5 amino acid differences in the extracellular domain that spans 624 amino acids. To characterize anti-HER3 antibody binding to the maHER3, 300-19 cell line expressing maHER3 extracellular domain was generated. The maHER3 extracellular domain sequence was codon-optimized, the cDNA was ligated to the huHER3 transmembrane and intracellular domain, and an expression plasmid, pSRα-chiHER3, that expresses the maHER3 extracellular domain was constructed. The plasmid was transfected to the 300-19 cells to stably express high levels of maHER3 extracellular domain on the cell surface.

[0262] The binding of muHER3-3, muHER3-8 and muHER3-10 antibodies to the maHER3 antigen was tested in flow cytometric assay as described above. As shown in Figure 4, muHER3-3, muHER3-8 and muHER3-10 antibodies bound to the 300-19 cells expressing maHER3 extracellular domain in a dose dependent manner (Figure 4A-C, respectively). Table 11 summarizes the Kd value for each muHER3 antibody. As shown in Table 11, muHER3-3, muHER3-8 and muHER3-10 antibodies bind well to both huHER3 and maHER3 antigens with very similar Kd, whereas some muHER3 antibodies, such as muHER3-19, muHER3-21, muHER3-22, muHER3-23 and muHER3-26, antibodies failed to recognize the maHER3 antigen although these antibodies bind to huHER3 antigen at high affinity. This result demonstrates that a 5 amino acid difference in the HER3 extracellular domain can alter the binding affinity of some muHER3 antibodies, but it does not change the binding property of muHER3-3, muHER3-8 and muHER3-10 antibodies.

Table 11. muHER3 antibody binding affinity to huHER3 and maHER3 antigens.

Clone #	Isotype	huHER3 Kd (nM)	maHER3 Kd (nM)
muHER3-3	IgG1	0.30	0.14
muHER3-8	IgG1	0.23	0.24
muHER3-10	IgG1	0.068	0.042
muHER3-17	IgG2a	0.26	0.12
muHER3-18	IgG1	0.26	2.9
muHER3-19	IgG1	0.41	100
muHER3-21	IgG1	0.16	19
muHER3-22	IgG1	0.092	14
muHER3-23	IgG1	0.19	21
muHER3-24	IgG1	0.42	0.15
muHER3-25	IgG2a	0.05	0.046
muHER3-26	IgG1	4.3	650
muHER3-27	IgG1	0.064	0.054

EXAMPLE 4

[0263] Inhibition of ligand-induced HER3 activation

[0264] Ligand interaction with HER3 receptor induces phosphorylation of HER3 as well as activation of downstream signaling pathway as indicated by phosphorylation of AKT. To investigate the capacity of muHER3 antibodies to inhibit ligand-induced HER3 activation, whole cell ELISA were performed. In this experiment, $2x10^5$ MCF7 cells/well were seeded in collagen-coated 96 well plate (Genetix, Boston, MA) in presence of normal growth media containing 10% FBS. After overnight incubation at 37ºC, cells were then washed and starved in serum free media for 24h at 37ºC. Cells were preincubated with 10μg/ml anti-HER3 antibodies for 1h at 37ºC and then treated with 30ng/ml HRG1β1 ligand (#377-HB/CF; R&D systems, Minneapolis, MN) for 10min at RT. Medium was flicked out and cells were fixed with 4% formaldehyde solution in PBS for 1h at RT. Formaldehyde solution was removed and cells were washed with wash buffer (PBS/0.1% Tween 20). Cells were quenched with 1% H_2O_2, 0.1% NaN_3 in wash buffer for 20 min at RT, then blocked with 5% BSA/PBS for 2 hr at RT. Primary antibody phospho-HER3 (Tyr1289) rabbit monoclonal antibody (#4791; Cell Signaling, Boston, MA; 1:300 dilution) or anti phospho-AKT (Ser473) rabbit monoclonal antibody (#4060; Cell Signaling, Boston, MA; 1:300 dilution) was added and incubated overnight at 4ºC. The plate was washed 4 times with wash buffer, and then incubated with goat anti-rabbit IgG-HRP (Jackson Immunoresearch, West Grove, PA; 1:7000 dilution) for 1h at RT. The plate was washed 4 times with wash buffer and Tetramethylbenzidine (TMBW; Bio-FX, Owings Mills, MD) was added for about 10min.

The reaction was stopped by addition of 100µl stop reagent (STPR; Bio-FX, Owings Mills, MD) and the absorbance was read at 450 nm with a reference wavelength of 570 nm using the Spectra Max M2 plate reader (Molecular Devices, Sunnyvale, CA).

[0265] The OD results were normalized so that 0% indicated the level of phosphorylation in the absence of HRG1β1 ligand and 100% indicated the level of phosphorylation in the presence of HRG1β1 ligand without any anti-HER3 antibody treatment. Figures 5 and 6 show representative results of phospho-HER3 (pHER3) and phospho-AKT (pAKT) inhibition, respectively. These results demonstrate that anti-HER3 antibodies of the invention were able to reduce ligand-induced HER3 activation as indicated by decreased HER3 and AKT phosphorylation. The muHER3-3, muHER3-8 and muHER3-10 antibodies reduced pHER3 level to ≤ 20% (Figure 5) and pAKT level to ≤ 50% (Figure 6).

EXAMPLE 5

[0266] Inhibition of ligand-induced MCF7 cell proliferation

[0267] HRG1β1 ligand binding to HER3 receptor can induce proliferation of HER3-positive cancer cells, such as MCF7 breast cancer cells. To determine the capacity of anti-HER3 antibodies of the invention to inhibit ligand-induced cell proliferation, MCF7 cells were plated at 2,000 cells per well and incubated in serum free media overnight at 37°C. Cells were pre-treated with 10µg/ml of anti-HER3 antibodies for 1h at 37°C. 20ng/ml HRG1β1 ligand was then added to the cells and the cells were further incubated at 37°C for 3 to 4 days. The level of cell proliferation was determined using colorimetric WST-8 assay (Dojindo Molecular Technologies, Rockville, MD) as described in Example 2. The results were normalized so that 0% indicated the level of cell proliferation in the absence of HRG1β1 ligand and 100% indicated the level of cell proliferation in the presence of HRG1β1 ligand without any anti-HER3 antibody treatment.

[0268] A representative result of ligand-induced MCF7 proliferation assay is presented in Figure 7, demonstrating that anti-HER3 antibodies of the invention were able to reduce ligand-induced MCF7 cell proliferation. The muHER3-8 antibody performed the best with the ability to reduce cell proliferation up to 40%, while muHER3-10 and muHER3-3 antibodies were less active in this assay.

EXAMPLE 6

[0269] Inhibition of ligand-induced HER2/HER3 dimerization

[0270] HRG1β1 ligand binding to HER3 receptor can induce formation of HER2/HER3 dimers in cells that express both RTKs. To determine the capacity of anti-HER3 antibodies of the invention to inhibit ligand-induced HER2/HER3 dimer formation, the following experiments were performed. MCF7 cells were first grown overnight in normal growth media containing 10% FBS. Cells were washed and starved in serum free media for 6h at 37°C. 10μg/ml anti-HER3 antibodies were added and the cells were further incubated overnight at 37ºC. Media was removed and cells were incubated with 30ng/ml HRG1β1 ligand for 15min at RT. Cells were then washed with PBS and lysed in M-PER lysis buffer (Thermo Fisher, Rockford, IL) for 20min on ice. Cell lysates were spun down and the supernatant was incubated with biotinylated mouse monoclonal antibody against HER2 (clone 3B5, #ab79205; Abcam, Cambridge, MA) for 4h on ice. Streptavidin Agarose Resins (#20349; Thermo Scientific, Rockford, IL) were added and rotated at 4ºC overnight. The resins were washed five times with lysis buffer and resuspended in β-mercaptoethanol containing NuPAGE LDS sample buffer (Invitrogen). The samples were heated at 80ºC for 10min and separated by SDS-PAGE using NuPAGE Novex 4-12% Bis-Tris gels with MES running buffer (Invitrogen). The proteins were then transferred to a nitrocellulose membrane using Xcell SureLock Mini-Cell system (Invitrogen). The membrane was blocked with ECL Advanced Blocking Reagent (#CPK1075; GE Healthcare, Piscataway, NJ) and incubated with anti-HER3 antibody (clone C-17 (#sc-285); Santa Cruz Biotechnology, Santa Cruz, CA; 1:300 dilution) or anti-HER2 antibody (clone 29D8 (#2165); Cell Signaling, Boston, MA; 1:1000 dilution) at 4ºC overnight. The membrane was washed in Tris-buffered saline (TBS) and incubated with goat anti-rabbit IgG HRP (Jackson Immunoresearch; 1:4000 dilution). After washing, the signal was detected using an ECL system (#RPN2109; GE Healthcare).

[0271] As shown in Figure 8, upon treatment with HRG1β1 ligand, there was an increase of HER2/HER3 dimerization as indicated by increased HER3 antigen that was co-precipitated with HER2 (lane 2). Treatment of cells with muHER3 antibodies, particularly muHER3-8 antibody, significantly inhibited the HER2/HER3 dimer formation and reduced the formation of HER2/HER3 dimers to the basal level or even lower.

EXAMPLE 7

[0272] Ligand binding competition

[0273] One mechanism of action of HER3 signaling inhibition is the blockade of ligand binding. To determine whether anti-HER3 antibodies of the invention can inhibit ligand binding to the HER3 receptor, binding of biotinylated HRG1β1 ligand to the SKBR3 cells was measured by flow cytometry in the presence of anti-HER3 antibodies. Biotinylation of HRG1β1 ligand was done using EZ-link Micro Sulfo-NHS-LC-biotinylation kit (Pierce, Rockland, IL) according to the manufacturer's instruction. $5x10^4$ SKBR3 cells/well were first incubated with varying concentration of anti-HER3 antibodies for 1h on ice. 10nM biotinylated HRG1β1 ligand was added to each well and incubation was continued for 1h on ice. Cells were then washed twice with FACS buffer and incubated with streptavidin-PE conjugate (Jackson Immunoresearch, West Grove, PA; 1:200 dilution) for 1h on ice. Cells were washed twice with FACS buffer and analyzed in FACSarray using FlowJo program. The geometric mean fluorescence intensities were plotted against the antibody concentration in a semi-log plot.

[0274] Representative results of the ligand competition assay were shown in Figure 9. The muHER3-3 and muHER3-8 antibodies were found to compete with HRG1β1 ligand binding, while muHER3-18 and muHER3-26 antibodies only had partial inhibition, and some anti-HER3 antibodies, such as muHER3-10, muHER3-27 and muHER3-28 antibodies, failed to compete with HRG1β1 ligand binding (Figure 9A). Figure 9B compared the capacity of muHER3-3, muHER3-8 and U3-Pharma/Amgen's U1-59 antibodies in blocking HRG1β1 ligand binding. (The recombinant U1-59 antibody was generated based on sequence data published in U.S. Patent Application Publication number 2008/012434 and the antibody characteristics, such as binding affinity and inhibitory activity of the ligand-induced HER3 and AKT phosphorylation, have been confirmed). A dose-response curve was generated by non-linear regression and the EC50 value of each curve was calculated using GraphPad Prism v4 (GraphPad software, San Diego, CA). The EC50 values correspond to 0.32nM, 0.29nM and 2.2nM for muHER3-3, muHER3-8 and U3-Pharma's U1-59 antibodies, respectively. This data demonstrates that the muHER3-3 and muHER3-8 antibodies are better than the U1-59 antibody in blocking the ligand binding.

EXAMPLE 8

[0275] Anti-HER3 antibody binding competition

[0276] To distinguish binding epitopes of anti-HER3 antibodies, antibody binding competition assays were done using flow cytometry. In this experiment, binding of biotinylated 'reference' antibody to the HER3 positive SKBR3 cells was measured in the presence of varying concentrations of 'competing' naked antibodies. In the experiments shown in Figure 10, U3-pharma's U1-59 antibody (Figure 10A) and muHER3-8 antibody (Figure 10B) were used as reference antibodies. Biotinylation of the reference antibodies was done using EZ-link Micro Sulfo-NHS-LC-biotinylation kit (Pierce, Rockland, IL) according to the manufacturer's instructions. The biotinylated reference antibody with concentration at Kd (1nM for U1-59 and 0.2nM for muHER3-8 antibody) was first mixed with varying concentration of competing antibody. The antibody mixture was incubated with $5x10^4$ MCF7 cells/well for 1h on ice. Cells were then washed twice with FACS buffer and incubated with streptavidin-PE conjugate (Jackson Immunoresearch, West Grove, PA; 1:200 dilution) for 1h on ice. Cells were washed twice with FACS buffer and analyzed in FACSarray using FlowJo program. The geometric mean fluorescence intensities were plotted against the competing antibody concentration in a semi-log plot.

[0277] As shown in Figure 10A, none of the anti-HER3 antibodies of the invention competed with U1-59 antibody, while the positive control antibody (the naked U1-59 antibody) inhibited the binding of the biotinylated U1-59 antibody in dose dependent manner. Corroborating this result, U1-59 antibody failed to compete with biotinylated muHER3-8 antibody (Figure 10B). In addition, muHER3-8 antibody was found to compete with muHER3-2, muHER3-3 and muHER3-10 antibodies, but not with muHER3-12 and muHER3-14 (Figure 10B). These results indicate that muHER3-3, muHER3-8 and muHER3-10 antibodies may recognize the same epitope or adjacent epitopes on HER3 antigen that is distinct from the U1-59 antibody's epitope.

EXAMPLE 9

[0278] Cloning and sequencing of the VL and VH regions of the muHER3-8 antibody

[0279] Total cellular RNA was prepared from $5x10^6$ cells of the muHER3-8 hybridoma using an RNeasy kit (QIAgen) according to the manufacturer's protocol. cDNA was

subsequently synthesized from total RNA using the SuperScript II cDNA synthesis kit (Invitrogen).

[0280] The procedure for the first round degenerate PCR reaction on the cDNA derived from hybridoma cells was based on methods described in Wang et al. (J Immunol Methods. 233: 167-77 (2000)) and Co et al. (J Immunol. 148: 1149-54 (1992)). VH sequences were amplified by PCR using the following degenerate primers: EcoMH1 CTTCCGGAATTCSARGTNMAGCTGSAGSAGTC (SEQ ID NO:74), EcoMH2 CTTCCGGAATTCSARGTNMAGCTGSAGSAGTCWGG (SEQ ID NO:75) and BamIgG1 GGAGGATCCATAGACAGATGGGGGTGTCGTTTTGGC (SEQ ID NO:76). VL sequences were amplified by PCR using the following degenerate primers: SacIMK GGAGCTCGAYATTGTGMTSACMCARWCTMCA (SEQ ID NO:77) and HindKL TATAGAGCTCAAGCTTGGATGGTGGGAAGATGGATACAGTTGGTGC (SEQ ID NO:78). (Mixed bases are defined as follows: N=G+A+T+C, S=G+C, Y=C+T, M=A+C, R=A+G, W=A+T). The PCR reaction mixtures were then run on a 1% low melt agarose gel, the 300 to 400 bp bands were excised, purified using Zymo DNA mini columns, and sent to Agencourt Biosciences for sequencing. The respective 5' and 3' PCR primers were used as sequencing primers to generate the variable region cDNAs from both directions. The amino acid sequences of VH and VL regions were predicted from the DNA sequencing results.

[0281] Since the degenerate primers used to clone the VL and VH cDNA sequences alter the 5' end sequences, additional sequencing efforts were needed to verify the complete sequences. The preliminary cDNA sequences were used to search the NCBI IgBlast site (http://www.ncbi.nlm.nih.gov/igblast/) for the murine germline sequences from which the antibody sequences are derived. PCR primers were then designed to anneal to the germline linked leader sequence of the murine antibody so that this new PCR reaction would yield a complete variable region cDNA sequence, unaltered by the PCR primers. The PCR reactions, band purifications, and sequencing were performed as described above.

[0282] Mass determination for sequence confirmation

[0283] The cDNA sequence information for the variable region was combined with the germline constant region sequence to obtain full length antibody cDNA sequences. The molecular weights of the heavy chain and light chain were then calculated and compared with the molecular weights obtained by LC/MS analyses of the muHER3-8 antibody. The molecular weight measurements are consistent with the cDNA sequences for both the muHER3-8 light and heavy chain.

EXAMPLE 10

[0284] Antibody humanization

[0285] The HER3-3, HER3-8, and HER3-10 antibodies were humanized following resurfacing methods previously described, such as, for example in Roguska et al., Proc. Natl. Acad. Sci., USA, 91(3):969-973 (1994) and Roguska et al., Protein Eng. 9(10):895-904 (1996), which are incorporated in their entirety herein by reference. Resurfacing generally involves identification of the variable region framework surface residues in both the light and heavy chains and replacing them with human equivalents. The murine CDR's are preserved in the resurfaced antibody. Exemplary CDRs of HER3-3, HER3-8, and HER3-10 are defined as indicated in Table 12. To minimize concerns about the impact of conjugating lysines that fall within the murine CDR's, lysine 95 in murine HER3-8 heavy chain CDR3 was replaced with arginine for humanized version 1.00 (shown in italic), so both versions of the heavy chain CDR3 are given. In addition to the heavy chain CDR2 definition employed for resurfacing, the table provides exemplary Kabat defined heavy chain CDR2's for both the murine and human versions of HER3-3, HER3-8, and HER3-10. The underlined sequence indicates the portion of the Kabat heavy chain CDR2 that was not considered a CDR for resurfacing.

[0286] Surface residue positions were defined as any position with a relative accessibility of 30% or greater (Pedersen J.T. et. al, J. Mol. Biol. 1994; 235: 959-973). The calculated surface residues were then aligned with human germline surface sequences to identify the most homologous human surface sequence. The human germline sequence used as the replacement surface for the light chain variable domains of both HER3-3 and HER3-8 was IGKV3-NL5*01 while IGKV7-3*01 was used for the light chain variable domain of HER3-10. The human germline sequences used as the replacement surfaces for the heavy chain variable domains of HER3-3, HER3-8, and HER3-10 were IGHV1-69*12, IGHV4-31*03, and IGHV1-69*10, respectively. The specific framework surface residue changes for HER3-3, HER3-8, and HER3-10 are shown in Figures 11, 12, and 13 respectively. Since the resurfaced HER3-8 heavy chain included the CDR3 lysine 95 to arginine 95 substitution for preferred version 1.00, a second resurfaced version (v1.01) was also generated with murine lysine 95 retained. Figure 14 shows the alignment of the resurfaced sequences for HER3-3, HER3-8, and HER3-10 variable domain of both light chain and heavy chain with their murine counterparts.

Table 12

HER3-3 CDR's	**HER3-8 CDR's**	**HER3-10 CDR's**
Light Chain	**Light Chain**	**Light Chain**
CDR1: RASQSITDYLH (SEQ ID NO:19) CDR2: FASQSIS (SEQ ID NO:20) CDR3: QNGHSFPLT (SEQ ID NO:21)	CDR1: RASQSINDYLH (SEQ ID NO:22) CDR2: FASHSIS (SEQ ID NO:23) CDR3: QNGHSFPWT QNGHSFPWT	CDR1: RASTSVYNYGNSFMH (SEQ ID NO:25) CDR2: LASNLES (SEQ ID NO:26) CDR3: QQNSEDPWT (SEQ ID NO:27)
Heavy Chain	**Heavy Chain**	**Heavy Chain**
CDR1: TDYWIH (SEQ ID NO:4) CDR2: AIDTDTY (SEQ ID NO:5) CDR3: SLLRLLYFDY (SEQ ID NO:6)	CDR1: TSDYAWN (SEQ ID NO:9) CDR2: FISYSGS (SEQ ID NO:10) Murine & human v1.01 CDR3: KLGRLYYFDY (SEQ ID NO:11) Human v1.00 CDR3: *R*LGRLYYFDY (SEQ ID NO: 13)	CDR1: TDYTMN (SEQ ID NO:14) CDR2: LINPYNDI (SEQ ID NO:15) CDR3: RGPRGAWFAY (SEQ ID NO:16)
Kabat HER3-3 HC CDR2	**Kabat HER3-8 HC CDR2**	**Kabat HER3-10 HC CDR2**
Murine HC CDR2: AIDTDTY<u>TSYNQQFKG</u> (SEQ ID NO:7) Humanized HC CDR2: AIDTDTY<u>TSYNQKFQG</u> (SEQ ID NO:8)	Murine & human HC CDR2: FISYSGS<u>TSYNPSLKS</u> (SEQ ID NO:12)	Murine HC CDR2: LINPYNDI<u>TAYNQKFKG</u> (SEQ ID NO:17) Human HC CDR2: LINPYNDI<u>TAYNQKFQG</u> (SEQ ID NO:18)

[0287] Recombinant expression of huHER3 antibodies

[0288] The variable region sequences for huHER3-3, huHER3-8 and huHER3-10 were codon-optimized and synthesized by Blue Heron Biotechnology. The sequences are flanked

by restriction enzyme sites for cloning in-frame with the respective constant sequences in single chain mammalian expression plasmids. The light chain variable region is cloned into EcoRI and BsiWI sites in the pAbKZeo plasmid. The heavy chain variable region is cloned into the HindIII and Apa1 sites in the pAbG1Neo plasmid. These plasmids can be used to express the recombinant antibodies in either transient or stable mammalian cell transfections. Transient transfections to express recombinant antibodies in HEK 293T cells were performed using a modified PEI procedure (Durocher, Y. et al., Nucleic Acids Res. 30:E9 (2002)). Supernatant was purified by Protein A as describe in Example 1. Polishing chromatography steps were performed using either carboxymethyl (CM) fast flow ion exchange (IEX) resin (GE Lifesciences) and 10 mM potassium phosphate, 10 mM sodium chloride binding buffer (pH 7.5) or the alternative CHT methods described above.

EXAMPLE 11

[0289] Binding affinity of humanized antibodies

[0290] Binding affinity of the humanized antibodies of huHER3-3, huHER3-8 and huHER3-10 were compared with their murine counterparts in flow cytometric assay using huHER3-expressing 300-19 cells. The binding assays were performed as described in Example 3. Figure 15 depicts the dose-response curves generated by non-linear regression for each antibody. The value for the apparent dissociation constant (Kd) of each antibody was calculated using GraphPad Prism v4 (GraphPad software, San Diego, CA). The results demonstrate that humanization did not change the binding affinity of muHER3 antibodies of the invention. In the case of HER3-8 antibody, humanization even increased the binding affinity by almost two fold; Kd of muHER3-8 and huHER3-8v.1.00 antibodies was 0.23nM and 0.12nM, respectively (Figure 12C).

[0291] Antibody competition assay was also performed with biotinylated muHER3-8 antibody as the reference antibody, and muHER3-8, huHER3-8v.1.00 and huHER3-8v.1.01 antibodies as competing antibodies. The assay was performed as described in Example 8. As shown in Figure 16, huHER3-8v.1.01 antibody competed with the biotinylated muHER3-8 antibody in the exact fashion as the murine counterpart, while huHER3-8v.1.00 antibody was better than the murine counterpart. The EC50 of the huHER3-8v.1.00 antibody and the muHER3-8 antibody curve were 0.24nM and 0.76nM, respectively. Because of this, huHER3-8v.1.00 antibody was chosen for further characterization and it is referred to as the huHER3-8 antibody.

EXAMPLE 12

[0292] Inhibition of HER3 ligand-independent growth of SKBR3, BT474, MDA-MB453 and ZR75-30 cells

[0293] To confirm the unique capacity of anti-HER3 antibodies of the invention to inhibit the HER3 ligand-independent growth of cancer cells, *in vitro* cell proliferation assays were performed using SKBR3, BT474, MDA-MB453 and ZR75-30 breast cancer cell lines. These cell lines were chosen because they constitutively activate HER3 signaling in the absence of exogenous ligands (Kraus MH. et al. Proc. Natl. Acad. Sci. USA, 90, 2900-2904 (1993)).

[0294] The experiments were carried out as described in Example 2. In brief, target cells were plated at 2,000 cells per well in normal growth media containing 10% FBS. The cells were further incubated at 37°C for 4 to 6 days in presence of 10 μg/ml anti-HER3 antibodies. The level of cell proliferation was determined using colorimetric WST-8 assay. The OD results were normalized so that 100% represents cells grown in normal growth media and in absence of anti-HER3 antibodies, and 0% represents cells grown in the media containing 0.5% FBS. In these experiments, huHER3-3, huHER3-8 and huHER3-10 antibodies were compared with U3 Pharma's U1-59 (human IgG1) and Merrimack's antibody #6 (referred as 'M-6'; humanized IgG2). The recombinant U1-59 and M-6 antibodies were generated based on sequence data published in U.S. Patent Application Publication number 2008/0124345 and International application number PCT/US2008/002119, respectively. The characteristics of both antibodies, such as binding affinity and inhibitory activity of the ligand-induced HER3 and AKT phosphorylation, have been confirmed.

[0295] As shown in Figures 17-20, huHER3 antibodies of the invention performed significantly better ($p \leq 0.05$) than U1-59 and M-6 antibodies in inhibiting cell proliferation of SKBR3, BT474, MDA-MB453 and ZR75-30 cell lines. In particular, the huHER3-8 antibody performed the best among these anti-HER3 antibodies.

EXAMPLE 13

[0296] Inhibition of ligand-induced MCF7 cell proliferation by huHER3 antibodies

[0297] To confirm the capacity of the humanized anti-HER3 antibodies to inhibit ligand-induced cell proliferation, experiments were performed as described in Example 5. In brief, after starvation in serum free media, MCF7 cells were pre-treated with 10μg/ml of anti-HER3 antibodies for 1h at 37°C. 30ng/ml HRG1β1 ligand was added to the cells and the cultures

were further incubated at 37°C for 3 to 4 days. The level of cell proliferation was determined using colorimetric WST-8 assay.

[0298] As shown in Figure 21A, the huHER3-8 and huHER3-10 antibodies significantly reduced MCF7 cell proliferation to 40-45%. In contrast, the huHER3-3 antibody, in spite of the strong capacity to inhibit ligand binding (see Figure 9), had little effect in blocking ligand-induced cell proliferation. This result corroborates the finding described in Figure 7 with the murine antibody counterparts. The M-6 antibody, at 10μg/ml antibody concentration, had the least activity in this experiment. Figure 21B depicts the dose dependent inhibition of MCF7 cell proliferation by the huHER3-8 antibody.

EXAMPLE 14

[0299] Inhibition of ligand-induced HER3 activation by huHER3 antibodies

[0300] To confirm the capacity of the humanized HER3 antibodies in inhibiting HER3 activation, whole cell ELISA was performed as described in Example 4. In brief, MCF7 cells were plated in collagen-coated plates (Genetix), serum starved, pre-treated with 10μg/ml anti-HER3 antibodies for 1h at 37ºC, and then incubated with 30ng/ml HRG1β1 ligand (R&D systems) for 10-15min at RT. After washing, cells were fixed with formaldehyde, quenched, and blocked with 5% BSA/PBS. Cells were then incubated with anti phospho-HER3 (Tyr1289) antibody (Cell Signaling) or anti phospho-AKT (Ser473) antibody (Cell Signaling) for overnight at 4ºC. After washing, goat anti-rabbit IgG-HRP (Jackson Immunoresearch) was added. The signal was detected by adding Tetramethylbenzidine (TMBW; Bio-FX) and the absorbance was read at 450 nm with using the Spectra Max M2 plate reader (Molecular Devices).

[0301] The OD results were normalized so that 0% indicated the level of phosphorylation in the absence of HRG1β1 ligand and 100% indicated the level of phosphorylation in the presence of HRG1β1 ligand without any anti-HER3 antibody treatment. Figures 22A and 22B show representative results of phospho-HER3 (pHER3) and phospho-AKT (pAKT) inhibition, respectively. In these experiments, Merrimack's M-6 antibody was included for comparison. As shown, the huHER3-8 and huHER3-10 antibodies were better than the M-6 antibody in inhibiting ligand-induced HER3 phsophorylation (Figure 22A); and the huHER3-3, huHER3-8 and huHER3-10 antibodies were better than or as active as the M-6 antibody in inhibiting ligand-induced AKT phosphorylation (Figure 22B).

EXAMPLE 15

[0302] Inhibition of ligand-induced HER2/HER3 dimerization by huHER3 antibodies

[0303] To confirm the capacity of the huHER3 antibodies in inhibiting ligand-induced HER2/HER3 dimerization, experiments were performed as described in Example 6. In brief, after serum starvation, MCF7 cells were pre-treated with 10μg/ml anti-HER3 antibodies and cells were activated by addition of 30ng/ml HRG1β1 ligand for 15min at RT. Protein cell lysates were prepared using M-PER lysis buffer and HER2 antigen was immunoprecipitated from the cell lysates using biotinylated anti-HER2 antibody (clone 3B5, Abcam) and Streptavidin Agarose Resins (Thermo Scientific). The immunoprecipitated proteins were separated in an SDS-PAGE gel, transferred to nitrocellulose membrane, and stained with anti-HER2 antibody (clone 29D8, Cell Signaling) or anti-HER3 antibody (clone C-17, Santa Cruz).

[0304] As shown in Figure 23, HRG1β1 ligand treatment increased HER2/HER3 dimerization as indicated by increased HER3 antigen that was co-precipitated with HER2 (lane 2). Pre-treatment of cells with muHER3 antibodies significantly reduced the formation of HER2/HER3 dimers to the basal level or even lower.

EXAMPLE 16

[0305] ADCC activity of huHER3 antibodies

[0306] A lactate dehydrogenase (LDH) release assay was used to measure antibody-dependent cell mediated cytotoxicity (ADCC) of tumor cell lines using freshly isolated human natural killer (NK) cells as effector cells (Shields RL, *J Biol Chem*. 2001 *276*(9):6591-604). The NK cells were first isolated from human peripheral blood from a normal donor (Research Blood Components, Inc., Brighton, MA) using a modified protocol for the NK cell Isolation Kit II (#130-091-152; Miltenyi Biotec, Auburn, CA). Peripheral blood was diluted 2-fold with 1x PBS. 25mL of diluted blood was carefully layered over 25mL of Ficoll Paque in a 50mL conical tube and centrifuged at 400g for 45 min at RT. The peripheral blood mononuclear cells (PBMC) were collected from the interface, transferred into a new conical 50mL tube, and washed once with 1x PBS. The PBMC were counted and resuspended at a concentration of $2.5x10^7$cells/100μl with MACS buffer (1x PBS, 0.5% BSA, 2mM EDTA), and then 1/4x volume of NK cell Biotin-Antibody Cocktail were added to the cell suspension. The NK cell Biotin-Antibody Cocktail contains biotinylated antibodies that bind to the

lymphocytes, except for NK cells, resulting in a negative selection of NK cells. The mixture was incubated at 4°C for 10 min, and then 3/5x volume of MACS buffer and 2/5x volume of NK cell MicroBead cocktail that would bind to the biotinylated antibodies were added. The cell-antibody mixture was incubated for another 15 min at 4°C. Next, cells were washed once with 50mL of MACS buffer and resuspended in 3mL of MACS buffer. NK cells were separated as negative fraction using autoMACS separator (Miltenyi Biotec). The resulting NK cells were plated into 30mL of complete RPMI media (RPMI-1640 supplemented with 5% fetal bovine serum, 1% penicillin-streptomycin, 1mM HEPES, 1mM Sodium Pyruvate, 1% 100X MEM non-essential Amino Acid Solution) overnight. The subsequent assay and all dilutions were carried out in RHBP medium (RPMI-1640 medium supplemented with 20mM HEPES, pH 7.4, 0.1% BSA and 1% penicillin-streptomycin).

[0307] Various concentrations of antibodies in RHBP medium were aliquoted in duplicate at 50μL/well into a round bottom 96-well plate. The target cells were resuspended at 10^6 cells/mL in RHBP medium and added at 100μL/well to each well containing antibody dilutions. The plate containing target cells and antibody dilutions was incubated for 30 min at RT. NK cells were then added to the wells containing the target cells at 50μL/well. The typical ratio was 1 target cell to 3-4 NK cells. The following controls were set up for each experiment: NK cells alone, target cells alone (spontaneous LDH release), target cells with NK cells (antibody independent LDH release), target cells with 10% Triton X-100 (maximum LDH release). The mixtures were incubated at 37°C for 4h to allow for cell lysis. Plates were centrifuged for 10 min at 1200 rpm, and 100μL of the supernatant was carefully transferred to a new flat-bottom 96-well plate. LDH reaction mixture (100μL/well) from the Cytotoxicity Detection Kit (Roche 1 644 793) was added to each well and incubated at room temperature for 5 to 30 min. The optical density (OD) of samples was measured at 490 nm (OD490). The percent specific lysis of each sample was determined using the following formula: percent specific lysis = (sample value - spontaneous release)/ (maximum release - spontaneous release) *100.

[0308] Figures 24A and 24B show ADCC activity of the huHER3 antibodies in BT474 and MCF7, respectively. The huHER3-10 antibody showed the best ADCC activity with maximal cell lysis at 55% and 17% for BT474 and MCF7 cells, respectively. The huHER3-3 and huHER3-8 antibodies showed similar maximal cell lysis at 35% in BT474 cells. In contrast, M-6 antibody did not induce a significant cell lysis in both BT474 and MCF7 cells.

EXAMPLE 17

[0309] Preparation of huHER3 antibody-SMCC-DM1

[0310] The (Succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC, Pierce Biotechnology, Inc) linker was dissolved in dimethylacetamide (DMA). The huHER3 antibody was modified with SMCC to introduce maleimides into the antibody by incubating the antibody at 5mg/mL in 50mM potassium phosphate, 50mM NaCl, 2mM EDTA, pH 6.5 with a 10 molar excess of SMCC. After approximately 100 minutes at ambient temperature, the reaction mixture was purified using a SEPHADEX™ G25 column equilibrated with the same potassium phosphate buffer. Antibody containing fractions were pooled and used for subsequent steps.

[0311] The SMCC-modified antibody was reacted with a 10mM solution of DM1 at a 1.7 molar excess relative to the maleimide linker. The reaction was stirred at ambient temperature for approximately 18 hours. The conjugation reaction mixture was filtered through a SEPHADEX™ G25 gel filtration column equilibrated with 1×PBS at pH 6.5. The huHER3 antibody-SMCC-DM1 conjugate was then dialyzed into buffer containing 10mM histidine, 250mM glycine, 1% sucrose, at pH 5.5. The number of DM1 molecules linked per antibody molecule was determined using the previously reported extinction coefficients for antibody and DM1 (Liu et al., *Proc. Natl. Acad. Sci.* USA, *93*, 8618-8623 (1996)). The percentage of free maytansinoid present after the conjugation reaction was determined by injecting 20-50μg conjugate onto a HiSep column equilibrated in 25% acetonitrile in 100mM ammonium acetate buffer, pH 7.0, and eluting in acetonitrile. The peak area of total free maytansinoid species (eluted in the gradient and identified by comparison of elution time with known standards) was measured using an absorbance detector set to a wavelength of 252 nm and compared with the peak area relative to bound maytansinoid (eluted in the conjugate peak in the column flow-through fractions) to calculate the percentage of total free maytansinoid species. Conjugates with 3.5-4 DM1 molecules per huHER3 antibody were obtained with <1% present as unconjugated maytansinoid.

[0312] Preparation of huHER3-8 antibody-SPDB-DM4

[0313] The exemplary N-succinimidyl 4-(2-pyridyldithio) butanoate (SPDB) linker was dissolved in ethanol. The huHER3 antibody was incubated at 8mg/mL with a 5.5-5 fold molar excess of SPDB linker for approximately 2 hours at room temperature in 50mM potassium phosphate buffer (pH 6.5) containing 50mM NaCl, 2mM EDTA, and 3% ethanol.

The SPDB modified antibody was diluted 2-fold in PBS, pH 6.5 and modified with a 1.5 fold molar excess of the maytansinoid DM4 by the addition of a concentrated solution (15-30mM) of DM4 in dimethylacetamide (DMA). After overnight incubation at room temperature, the conjugated antibody was purified by chromatography on SEPHADEX™ G25F equilibrated with 10mM histidine, 250mM glycine, 1% sucrose, at pH 5.5. The number of DM4 molecules linked per antibody molecule was determined using the previously reported extinction coefficients for antibody and maytansinoid (Widdison WC et al. J Med Chem, 49:4392-4408 (2006)). The percentage of total free maytansinoid species were determined as described above. Conjugates with 3.5-4 DM4 molecules per huHER3 antibody were obtained with <1% present as unconjugated maytansinoid.

[0314] Preparation of huHER3-8 antibody-PEG4-mal-DM1

[0315] The N-hydroxysuccinimidyl-(polyethylene glycol) 4-(N-maleimidomethyl)-DM1 (NHS-PEG4-mal-DM1) reagent was dissolved in DMA. The huHER3 antibody was incubated at 5mg/mL in 50mM potassium phosphate, 150mM NaCl, 2mM EDTA, pH 7.5 with a 7 fold molar excess of NHS-PEG4-mal-DM1 (10% DMA total). After approximately 2 hours at ambient temperature, the reaction mixture was purified using a SEPHADEX™ G25 column equilibrated in 1x PBS, pH 7.4. Antibody containing fractions were pooled and dialyzed into buffer containing 10mM histidine, 250mM glycine, 1% sucrose, at pH 5.5. The number of DM1 molecules linked per antibody and the percentage of total free maytansinoid species were determined as described above. Conjugates with 3.5-4 DM4 molecules per huCD37-3 antibody were obtained with <1% present as unconjugated maytansinoid.

EXAMPLE 18

[0316] Binding affinity of maytansinoid conjugates

[0317] After conjugation to SMCC-DM1, SPDB-DM4 or PEG4-mal-DM1, binding affinity of the exemplary huHER3-8 antibody to huHER3 positive SKBR3 cells and maHER3-overexpressing 300-19 cells was assayed by flow cytometry as described in Example 3. Figure 25A shows the binding curve of huHER3-8 naked antibody and maytansinoid conjugates to SKBR3 cells. The Kds calculated from the binding curves were 0.14nM for huHER3-8 antibody, 0.49nM for huHER3-8-SMCC-DM1, 0.29nM for huHER3-8-SPDB-DM4, and 0.27nM for huHER3-8-PEG-mal-DM1 conjugates. Figure 25B shows the binding curve of huHER3-8 naked antibody and maytansinoid conjugates to maHER3-overexpressing 300-19 cells. The Kds calculated from the binding curves were 0.19nM for

huHER3-8 antibody, 0.29nM for huHER3-8-SMCC-DM1, 0.23nM for huHER3-8-SPDB-DM4, and 0.28nM for huHER3-8-PEG-mal-DM1 conjugates. Altogether these data demonstrate that SMCC-DM1, SPDB-DM4 or PEG4-mal-DM1 conjugation does not notably alter the binding affinity of the exemplary huHER3-8 antibody to both huHER3 and maHER3 antigens.

EXAMPLE 19

[0318] *In vitro* cytotoxicity assays

[0319] The ability of anti-HER3-Ab-maytansinoid conjugates to inhibit cell growth was measured using *in vitro* cytotoxicity assays as described in Example 2. Briefly, target cells were plated at 2,000 cells per well in 100μL in complete RPMI media containing 10% FBS. Conjugates were diluted into complete RPMI media using 3-fold dilution series and 100μL were added per well. The final concentration typically ranged from $1x10^{-8}$ M to $1.5x10^{-12}$ M. Cells were incubated at 37°C in a humidified 5% CO2 incubator for 3-4 days. Viability of the remaining cells was determined by colorimetric WST-8 assay and the absorbance at 450 nm (A450) was measured in a multiwell plate reader. The surviving fraction was calculated by dividing each treated sample value by the average value of wells with untreated cells. The surviving fraction value was plotted against the antibody-conjugate concentration in a semi-log plot for each treatment.

[0320] The *in vitro* cytotoxicity of huHER3-3-SMCC-DM1, huHER3-8 SMCC-DM1, huHER3-8-SPDB-DM4 and huHER3-8-PEG4-Mal-DM1 conjugates was compared to the activity of a non-specific maytansionid conjugates such as chKTI-SMCC-DM1, chKTI-SPDB-DM4, anti-EpCAM chB38.1-PEG4-mal-DM1 and anti-CanAg huC242-SMCC-DM1 conjugates. The results from a typical cytotoxicity assay are shown in Figure 26A and 26B for huHER3-overexpressing 300-19 cells and maHER3-overexpressing 300-19 cells, respectively. The huHER3-3 Ab and huHER3-8 Ab conjugates resulted in high specific cell killing as compared to the control conjugate on both huHER3 and maHER3-overexpressing 300-19 cells. As shown in Figure 26A with the huHER3-overexpressing 300-19 cells, the EC50 values correspond to 0.19nM for huHER3-3-SMCC-DM1, 0.17nM for huHER3-8-SMCC-DM1, and 0.18nM for huHER3-8-SPDB-DM4 whereas only 53nM for chKTI-SMCC-DM1 and 4.4nM for chKTI-SPDB-DM4 conjugates. In the maHER3-overexpressing cells (Figure 26B), the EC50 values correspond to 0.059nM for huHER3-8-SMCC-DM1, 0.099nM for huHER3-8-SPDB-DM4, and 0.045nM for huHER3-8-PEG-mal-DM1

conjugates. In contrast, the non-binding conjugates only kill less than 0.3 fraction of the cells at the highest concentration tested.

EXAMPLE 20

[0321] Epitope Mapping

[0322] The HER-3 extracellular domain (ECD) of about 620 amino acids is made up of four sub-domains consisting of L1 (amino acids 20-184), the amino terminal domain (amino acids, 1-19); S1 (amino acids 185-327) and S2 (amino acids 500-632), which are the two Cysteine-rich domains; and the L2 domain (amino acids 328-499), which is flanked by the two Cysteine-rich domains. The epitopes of the humanized anti-HER-3 antibodies of the invention were mapped to defined domains or combination of domains in the HER-3 extracellular region by engineering various chimeric human/murine HER-3 ECD molecules.

[0323] HER-3 variants cloning and expression

[0324] The entire human HER-3 protein sequence of 1342 amino acids was codon optimized, synthesized, and cloned into the pSRa mammalian expression vector by Blue Heron Biotechnologies. An Fc fusion of human HER-3 was built by cloning HER-3 ECD (amino acids 1-632) in frame with a murine IgG2A hinge, CH2, and CH3 region in the pmuFc2ANL mammalian expression vector. In addition, a murine HER-3 ECD (amino acids 1-632) Fc fusion was similarly constructed including unique internal restriction sites taken from conservative sequences within the human HER-3 Fc construct, providing convenient domain swap cloning sites for each of the four domains. The restriction sites, as well as domain divisions, are illustrated in Figure 27. By taking advantage of the matching unique restriction sites in both the murine and human HER-3 Fc fusion constructs, a couple of chimeric human/murine HER-3 ECD Fc fusion proteins were built, including chL1-S1 (human residues 1-342 with murine residues 343-632) and chL2-S2 (human residues 343-632 with murine residues 1-342). Both forms of HER-3 ECD Fc tagged proteins were expressed via transient transfection of HEK 293T cells and purified from the supernatant of the transfected cells using protein A affinity chromatography.

[0325] Antibody binding to various HER-3 ECD-Fc constructs

[0326] The humanized anti-HER-3 antibodies huHER3-3, huHER3-8, and huHER3-10 were tested in ELISA for binding to the various HER-3 ECD Fc constructs described above. As can been seen in Figure 28, all three of these antibodies bound to human HER-3 ECD Fc protein with sub-nanomolar affinity. In contrast, huHER3-3 and huHER3-8 antibodies only

marginally bound to murine HER-3 ECD while huHER3-10 antibody did not bind to murine HER-3 at all (Figure 29). U1-59 antibody previously shown to cross-react with murine HER-3 was included as a positive control. All of the antibodies tested bound to the chL1-S1 (human residues 1-342 with murine residues 343-632) Fc construct (Figure 30); but the huHER3-3, huHER3-8, and huHER3-10 antibodies either did not, or weakly bound to chL2-S2-Fc protein (Figure 31) similar to their respective observed murine HER-3-Fc binding (Figure 29). These results suggest that the epitopes of the three antibodies of the invention, huHER3-3, huHER3-8, and huHer-10, can be localized within residues 20-342 in the extracellular domain of human HER-3. The epitope mapping results were consistent with the results of antibody binding competition assay (Example 8) where the three antibodies of the invention competed with each other for binding to HER-3 but they do not compete with U1-59 antibody binding (Figure 10).

EXAMPLE 21

[0327] Inhibition of basal level of pHER3 in SKBR3, MDA-MB453 and ZR75-30 cells

[0328] As shown in Example 12, anti-HER3 antibodies of the invention are unique because of their capacity in inhibiting the basal proliferation of breast cancer cell lines such as SKBR3 (Figure 17), BT474 (Figure 18), MDA-MB453 (Figure 19) and ZR75-30 (Figure 20). These four cell lines constitutively activate HER3 signaling in the absence of exogenous ligands (Kraus M.H. et al., Proc. Natl. Acad. Sci. USA, 90: 2900-2904 (1993)). In order to assess the anti-proliferative activity of anti-HER3 antibodies of the invention, the capacity of huHER3-8 antibody to inhibit the basal level of phospho-HER3 (pHER3) in SKBR3, MDA-MB453 and ZR75-30 cells was evaluated. Merrimack's M-6 antibody was included as a control. In brief, tumor cells were plated in 6-well plates at 5 x 10^5 cells/well. The next day, antibodies were added at the indicated concentration, and the cells were further incubated for 20-24 hours. Cells were then washed with HBSS and lysed. The level of pHER3 was measured by ELISA using pErbB3 ELISA kit (catalog number DYC1769-2, R & D Systems). The level of pHER3 detected in each cell line is set forth in Figures 32A-C.

[0329] As shown in Figures 32A-C, huHER3-8 antibody was more potent than Merrimack's M-6 antibody in inhibiting the pHER3 level in the absence of exogenous HER3 ligand (pHER3 basal level) in all three cell lines evaluated in this study. These data demonstrate the superior activity of anti-HER3 antibodies of the invention to inhibit the basal proliferation of tumor cell lines in which HER3 signaling is constitutively activated.

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[0330] It is to be appreciated that the Detailed Description section, and not the Summary and Abstract sections, is intended to be used to interpret the claims. The Summary and Abstract sections sets forth one or more, but not all, exemplary embodiments of the present invention as contemplated by the inventor(s), and thus, are not intended to limit the present invention and the appended claims in any way.

[0331] The present invention has been described above with the aid of functional building blocks illustrating the implementation of specified functions and relationships thereof. The boundaries of these functional building blocks have been arbitrarily defined herein for the convenience of the description. Alternate boundaries can be defined so long as the specified functions and relationships thereof are appropriately performed.

[0332] The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art, readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance.

[0333] The breadth and scope of the present invention should not be limited by any of the above-described exemplary embodiments, but should be defined only in accordance with the following claims and their equivalents.

[0334] All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

[0335] The terms “comprising,” “having,” “including,” and “containing” are to be construed as open-ended terms (i.e., meaning “including, but not limited to,”) unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the

specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0336] Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

WHAT IS CLAIMED IS:

1. An isolated antibody or antigen binding fragment thereof that binds to HER3 protein, wherein the antibody or antigen binding fragment thereof binds to the same HER3 epitope as an antibody purified from the cell line of ATCC Accession No. PTA-11145, ATCC Accession No. PTA-11146, or ATCC Accession No. PTA-11147.

2. An isolated antibody or antigen binding fragment thereof that binds to HER3 protein, wherein the antibody or antigen binding fragment thereof binds to the same HER3 epitope as an antibody selected from the group consisting of:

(a) an antibody comprising a first amino acid sequence that is at least 90% identical to SEQ ID NO: 28 and a second amino acid sequence that is at least 90% identical to SEQ ID NO: 35;

(b) an antibody comprising a first amino acid sequence that is at least 90% identical to SEQ ID NO: 29 and a second amino acid sequence that is at least 90% identical to SEQ ID NO: 36;

(c) an antibody comprising a first amino acid sequence that is at least 90% identical to SEQ ID NO: 30 and a second amino acid sequence that is at least 90% identical to SEQ ID NO: 37;

(d) an antibody comprising a first amino acid sequence that is at least 90% identical to SEQ ID NO: 31 and a second amino acid sequence that is at least 90% identical to SEQ ID NO: 38;

(e) an antibody comprising a first amino acid sequence that is at least 90% identical to SEQ ID NO: 32 and a second amino acid sequence that is at least 90% identical to SEQ ID NO: 38;

(f) an antibody comprising a first amino acid sequence that is at least 90% identical to SEQ ID NO: 33 and a second amino acid sequence that is at least 90% identical to SEQ ID NO: 39; and

(g) an antibody comprising a first amino acid sequence that is at least 90% identical to SEQ ID NO: 34 and a second amino acid sequence that is at least 90% identical to SEQ ID NO: 40.

3. The antibody or antigen binding fragment thereof of claim 2, wherein the antibody or antigen binding fragment thereof binds to the same HER3 epitope as an antibody selected from the group consisting of:

(a) an antibody comprising a first amino acid sequence that is at least 95% identical to SEQ ID NO: 28 and a second amino acid sequence that is at least 95% identical to SEQ ID NO: 35;

(b) an antibody comprising a first amino acid sequence that is at least 95% identical to SEQ ID NO: 29 and a second amino acid sequence that is at least 95% identical to SEQ ID NO: 36;

(c) an antibody comprising a first amino acid sequence that is at least 95% identical to SEQ ID NO: 30 and a second amino acid sequence that is at least 95% identical to SEQ ID NO: 37;

(d) an antibody comprising a first amino acid sequence that is at least 95% identical to SEQ ID NO: 31 and a second amino acid sequence that is at least 95% identical to SEQ ID NO: 38;

(e) an antibody comprising a first amino acid sequence that is at least 95% identical to SEQ ID NO: 32 and a second amino acid sequence that is at least 95% identical to SEQ ID NO: 38;

(f) an antibody comprising a first amino acid sequence that is at least 95% identical to SEQ ID NO: 33 and a second amino acid sequence that is at least 95% identical to SEQ ID NO: 39; and

(g) an antibody comprising a first amino acid sequence that is at least 95% identical to SEQ ID NO: 34 and a second amino acid sequence that is at least 95% identical to SEQ ID NO: 40.

4. An isolated antibody or antigen binding fragment thereof that binds to HER3 protein, wherein the antibody or antigen binding fragment thereof competitively inhibits the binding of an antibody selected from the group consisting of:

(a) an antibody purified from the cell line of ATCC Accession No. PTA-11145;

(b) an antibody purified from the cell line of ATCC Accession No. PTA-11146; and

(c) an antibody purified from the cell line of ATCC Accession No. PTA-11147.

5. An isolated antibody or antigen binding fragment thereof that binds to HER3 protein, wherein the antibody or antigen binding fragment thereof competitively inhibits the binding of an antibody selected from the group consisting of:

(a) an antibody comprising a first amino acid sequence that is at least 90% identical to SEQ ID NO: 28 and a second amino acid sequence that is at least 90% identical to SEQ ID NO: 35;

(b) an antibody comprising a first amino acid sequence that is at least 90% identical to SEQ ID NO: 29 and a second amino acid sequence that is at least 90% identical to SEQ ID NO: 36;

(c) an antibody comprising a first amino acid sequence that is at least 90% identical to SEQ ID NO: 30 and a second amino acid sequence that is at least 90% identical to SEQ ID NO: 37;

(d) an antibody comprising a first amino acid sequence that is at least 90% identical to SEQ ID NO: 31 and a second amino acid sequence that is at least 90% identical to SEQ ID NO: 38;

(e) an antibody comprising a first amino acid sequence that is at least 90% identical to SEQ ID NO: 32 and a second amino acid sequence that is at least 90% identical to SEQ ID NO: 38;

(f) an antibody comprising a first amino acid sequence that is at least 90% identical to SEQ ID NO: 33 and a second amino acid sequence that is at least 90% identical to SEQ ID NO: 39; and

(g) an antibody comprising a first amino acid sequence that is at least 90% identical to SEQ ID NO: 34 and a second amino acid sequence that is at least 90% identical to SEQ ID NO: 40.

6. The antibody or antigen binding fragment thereof of claim 5, wherein the antibody or antigen binding fragment thereof competitively inhibits the binding of an antibody selected from the group consisting of:

(a) an antibody comprising a first amino acid sequence that is at least 95% identical to SEQ ID NO: 28 and a second amino acid sequence that is at least 95% identical to SEQ ID NO: 35;

(b) an antibody comprising a first amino acid sequence that is at least 95% identical to SEQ ID NO: 29 and a second amino acid sequence that is at least 95% identical to SEQ ID NO: 36;

(c) an antibody comprising a first amino acid sequence that is at least 95% identical to SEQ ID NO: 30 and a second amino acid sequence that is at least 95% identical to SEQ ID NO: 37;

(d) an antibody comprising a first amino acid sequence that is at least 95% identical to SEQ ID NO: 31 and a second amino acid sequence that is at least 95% identical to SEQ ID NO: 38;

(e) an antibody comprising a first amino acid sequence that is at least 95% identical to SEQ ID NO: 32 and a second amino acid sequence that is at least 95% identical to SEQ ID NO: 38;

(f) an antibody comprising a first amino acid sequence that is at least 95% identical to SEQ ID NO: 33 and a second amino acid sequence that is at least 95% identical to SEQ ID NO: 39; and

(g) an antibody comprising a first amino acid sequence that is at least 95% identical to SEQ ID NO: 34 and a second amino acid sequence that is at least 95% identical to SEQ ID NO: 40.

7. An isolated antibody or antigen binding fragment thereof that binds to HER3 protein, wherein the antibody or antigen binding fragment thereof comprises:

(i) a first amino acid sequence comprising

(a) an amino acid sequence selected from the group consisting of SEQ ID NOs: 4, 9, 14, and variants thereof with 1 conservative amino acid substitution;

(b) an amino acid sequence selected from the group consisting of SEQ ID NOs: 5, 7, 8, 10, 12, 15, 17, 18, and variants thereof with 1 conservative amino acid substitution; and

(c) an amino acid sequence selected from the group consisting of SEQ ID NOs: 6, 11, 13, 16, and variants thereof with 1 conservative amino acid substitution; and

(ii) a second amino acid sequence comprising

(d) an amino acid sequence selected from the group consisting of SEQ ID NOs: 19, 22, 25, and variants thereof with 1 conservative amino acid substitution;

(e) an amino acid sequence selected from the group consisting of SEQ ID NOs; 20, 23, 26, and variants thereof with 1 conservative amino acid substitution; and

(f) an amino acid sequence selected from the group consisting of SEQ ID NOs: 21, 24, 27, and variants thereof with 1 conservative amino acid substitution.

8. An isolated antibody or antigen binding fragment thereof that binds to HER3 protein, wherein the antibody or antigen binding fragment thereof is selected from the group consisting of:

(a) an antibody or antigen binding fragment thereof comprising a first amino acid sequence comprising SEQ ID NOs: 4, 5, and 6 and a second amino acid sequence comprising SEQ ID NOs: 19, 20, and 21;

(b) an antibody or antigen binding fragment thereof comprising a first amino acid sequence comprising SEQ ID NOs: 9, 10, and 11 and a second amino acid sequence comprising SEQ ID NOs: 22, 23, and 24;

(c) an antibody or antigen binding fragment thereof comprising a first amino acid sequence comprising SEQ ID NOs: 9, 10, and 13 and a second amino acid sequence comprising SEQ ID NOs: 22, 23, and 24;

(d) an antibody or antigen binding fragment thereof comprising a first amino acid sequence comprising SEQ ID NOs: 14, 15, and 16 and a second amino acid sequence comprising SEQ ID NOs: 25, 26, and 27; and

(e) variants of (a) to (d) with 1, 2, 3, or 4 conservative amino acid substitutions.

9. The antibody or antigen binding fragment thereof of claim 8, wherein the antibody or antigen binding fragment thereof is selected from the group consisting of:

(a) an antibody or antigen binding fragment thereof comprising a first amino acid sequence that is at least 90% identical to SEQ ID NO: 28 and a second amino acid sequence that is at least 90% identical to SEQ ID NO: 35;

(b) an antibody or antigen binding fragment thereof comprising a first amino acid sequence that is at least 90% identical to SEQ ID NO: 29 and a second amino acid sequence that is at least 90% identical to SEQ ID NO: 36;

(c) an antibody or antigen binding fragment thereof comprising a first amino acid sequence that is at least 90% identical to SEQ ID NO: 30 and a second amino acid sequence that is at least 90% identical to SEQ ID NO: 37;

(d) an antibody or antigen binding fragment thereof comprising a first amino acid sequence that is at least 90% identical to SEQ ID NO: 31 and a second amino acid sequence that is at least 90% identical to SEQ ID NO: 38;

(e) an antibody or antigen binding fragment thereof comprising a first amino acid sequence that is at least 90% identical to SEQ ID NO: 32 and a second amino acid sequence that is at least 90% identical to SEQ ID NO: 38;

(f) an antibody or antigen binding fragment thereof comprising a first amino acid sequence that is at least 90% identical to SEQ ID NO: 33 and a second amino acid sequence that is at least 90% identical to SEQ ID NO: 39; and

(g) an antibody or antigen binding fragment thereof comprising a first amino acid sequence that is at least 90% identical to SEQ ID NO: 34 and a second amino acid sequence that is at least 90% identical to SEQ ID NO: 40.

10. The antibody or antigen binding fragment thereof of claim 8, wherein the antibody or antigen binding fragment thereof is selected from the group consisting of:

(a) an antibody or antigen binding fragment thereof comprising a first amino acid sequence that is at least 95% identical to SEQ ID NO: 28 and a second amino acid sequence that is at least 95% identical to SEQ ID NO: 35;

(b) an antibody or antigen binding fragment thereof comprising a first amino acid sequence that is at least 95% identical to SEQ ID NO: 29 and a second amino acid sequence that is at least 95% identical to SEQ ID NO: 36;

(c) an antibody or antigen binding fragment thereof comprising a first amino acid sequence that is at least 95% identical to SEQ ID NO: 30 and a second amino acid sequence that is at least 95% identical to SEQ ID NO: 37;

(d) an antibody or antigen binding fragment thereof comprising a first amino acid sequence that is at least 95% identical to SEQ ID NO: 31 and a second amino acid sequence that is at least 95% identical to SEQ ID NO: 38;

(e) an antibody or antigen binding fragment thereof comprising a first amino acid sequence that is at least 95% identical to SEQ ID NO: 32 and a second amino acid sequence that is at least 95% identical to SEQ ID NO: 38;

(f) an antibody or antigen binding fragment thereof comprising a first amino acid sequence that is at least 95% identical to SEQ ID NO: 33 and a second amino acid sequence that is at least 95% identical to SEQ ID NO: 39; and

(g) an antibody or antigen binding fragment thereof comprising a first amino acid sequence that is at least 95% identical to SEQ ID NO: 34 and a second amino acid sequence that is at least 95% identical to SEQ ID NO: 40.

11. The antibody or antigen binding fragment thereof of claim 8, wherein the antibody or antigen binding fragment thereof is selected from the group consisting of:

(a) an antibody or antigen binding fragment thereof comprising a first amino acid sequence that is at least 99% identical to SEQ ID NO: 28 and a second amino acid sequence that is at least 99% identical to SEQ ID NO: 35;

(b) an antibody or antigen binding fragment thereof comprising a first amino acid sequence that is at least 99% identical to SEQ ID NO: 29 and a second amino acid sequence that is at least 99% identical to SEQ ID NO: 36;

(c) an antibody or antigen binding fragment thereof comprising a first amino acid sequence that is at least 99% identical to SEQ ID NO: 30 and a second amino acid sequence that is at least 99% identical to SEQ ID NO: 37;

(d) an antibody or antigen binding fragment thereof comprising a first amino acid sequence that is at least 99% identical to SEQ ID NO: 31 and a second amino acid sequence that is at least 99% identical to SEQ ID NO: 38;

(e) an antibody or antigen binding fragment thereof comprising a first amino acid sequence that is at least 99% identical to SEQ ID NO: 32 and a second amino acid sequence that is at least 99% identical to SEQ ID NO: 38;

(f) an antibody or antigen binding fragment thereof comprising a first amino acid sequence that is at least 99% identical to SEQ ID NO: 33 and a second amino acid sequence that is at least 99% identical to SEQ ID NO: 39; and

(g) an antibody or antigen binding fragment thereof comprising a first amino acid sequence that is at least 99% identical to SEQ ID NO: 34 and a second amino acid sequence that is at least 99% identical to SEQ ID NO: 40.

12. The antibody or antigen binding fragment thereof of any of claims 1-11, wherein the antibody or antigen binding fragment thereof binds to human HER3 protein.

13. The antibody or antigen binding fragment thereof of claim 12, wherein the human HER3 protein comprises SEQ ID NO: 1.

14. The antibody or antigen binding fragment thereof of any of claims 1-11, wherein the antibody or antigen binding fragment thereof binds to an epitope within amino acid residues 20-342 of SEQ ID NO: 1.

15. The antibody or antigen binding fragment thereof of any of claims 1-14, wherein the antibody or antigen binding fragment thereof is murine, non-human, humanized, chimeric, resurfaced, or human.

16. The antibody or antigen binding fragment thereof of any of claims 1-15, wherein the antibody or antigen binding fragment thereof is capable of inhibiting basal proliferation of tumor cells in which HER3 is constitutively activated.

17. The antibody or antigen binding fragment thereof of any of claims 1-16, wherein the antibody or antigen binding fragment thereof is capable of inhibiting HER3 ligand-dependent growth of tumor cells expressing HER3.

18. The antibody or antigen binding fragment thereof of any of claims 1-17, wherein the antibody or antigen binding fragment thereof is capable of inhibiting HER3 ligand-induced HER3 signaling.

19. The antibody or antigen binding fragment thereof of any of claims 1-18, wherein the antibody or antigen binding fragment thereof is capable of inhibiting basal HER3 signaling in the absence of exogenous HER3 ligand.

20. The antibody or antigen binding fragment thereof of any of claims 1-19, wherein the antibody or antigen binding fragment thereof is capable of inhibiting HER3 ligand binding to HER3 receptor.

21. The antibody or antigen binding fragment thereof of any of claims 1-20, wherein the antibody or antigen binding fragment thereof is capable of inhibiting HER3 ligand-induced HER2 and HER3 dimerization.

22. The antibody or antigen binding fragment thereof of any of claims 1-21, wherein the antibody or antigen binding fragment thereof is capable of inducing antibody dependent cell mediated cytotoxicity (ADCC).

23. The antibody or antigen binding fragment thereof of any one of claims 1-22, wherein the antibody or antigen binding fragment thereof has similar binding affinity for human HER3 and macaque HER3.

24. The antibody or antigen binding fragment thereof of any one of claims 1-23, wherein the antibody or antigen binding fragment thereof binds to human HER3 and macaque HER3 with a Kd of 0.3 nM or better.

25. The antibody or antigen binding fragment thereof of any one of claims 1-24, which is a full length antibody.

26. The antibody or antigen binding fragment thereof of any one of claims 1-24, which is an antigen binding fragment.

27. The antibody or antigen binding fragment thereof of any one of claims 1-24, wherein the antibody or antigen binding fragment thereof comprises a Fab, Fab', F(ab')2, Fd, single chain Fv or scFv, disulfide linked Fv, V-NAR domain, IgNar, intrabody, IgGΔCH2, minibody, F(ab')3, tetrabody, triabody, diabody, single-domain antibody, DVD-Ig, Fcab, mAb2, (scFv)2, or scFv-Fc.

28. An isolated antibody that binds to HER3 protein, wherein the antibody is selected from the group consisting of:

(a) an antibody purified from the cell line of ATCC Accession No. PTA-11145;

(b) an antibody purified from the cell line of ATCC Accession No. PTA-11146; and

(c) an antibody purified from the cell line of ATCC Accession No. PTA-11147.

29. An isolated polypeptide that binds to HER3 protein, wherein the polypeptide is selected from the group consisting of:

(a) a polypeptide comprising a first amino acid sequence comprising SEQ ID NOs: 4, 5, and 6 and a second amino acid sequence comprising SEQ ID NOs: 19, 20, and 21;

(b) a polypeptide comprising a first amino acid sequence comprising SEQ ID NOs: 9, 10, and 11 and a second amino acid sequence comprising SEQ ID NOs: 22, 23, and 24;

(c) a polypeptide comprising a first amino acid sequence comprising SEQ ID NOs: 9, 10, and 13 and a second amino acid sequence comprising SEQ ID NOs: 22, 23, and 24;

(d) a polypeptide comprising a first amino acid sequence comprising SEQ ID NOs: 14, 15, and 16 and a second amino acid sequence comprising SEQ ID NOs: 25, 26, and 27; and

(e) variants of (a) to (d) comprising 1, 2, 3, or 4 conservative amino acid substitutions.

30. The polypeptide of claim 29, wherein the polypeptide is selected from the group consisting of:

(a) a polypeptide comprising a first amino acid sequence that is at least 90% identical to SEQ ID NO: 28 and a second amino acid sequence that is at least 90% identical to SEQ ID NO: 35;

(b) a polypeptide comprising a first amino acid sequence that is at least 90% identical to SEQ ID NO: 29 and a second amino acid sequence that is at least 90% identical to SEQ ID NO: 36;

(c) a polypeptide comprising a first amino acid sequence that is at least 90% identical to SEQ ID NO: 30 and a second amino acid sequence that is at least 90% identical to SEQ ID NO: 37;

(d) a polypeptide comprising a first amino acid sequence that is at least 90% identical to SEQ ID NO: 31 and a second amino acid sequence that is at least 90% identical to SEQ ID NO: 38;

(e) a polypeptide comprising a first amino acid sequence that is at least 90% identical to SEQ ID NO: 32 and a second amino acid sequence that is at least 90% identical to SEQ ID NO: 38;

(f) a polypeptide comprising a first amino acid sequence that is at least 90% identical to SEQ ID NO: 33 and a second amino acid sequence that is at least 90% identical to SEQ ID NO: 39; and

(g) a polypeptide comprising a first amino acid sequence that is at least 90% identical to SEQ ID NO: 34 and a second amino acid sequence that is at least 90% identical to SEQ ID NO: 40.

31. The polypeptide claim 29, wherein the polypeptide is selected from the group consisting of:

(a) a polypeptide comprising a first amino acid sequence that is at least 95% identical to SEQ ID NO: 28 and a second amino acid sequence that is at least 95% identical to SEQ ID NO: 35;

(b) a polypeptide comprising a first amino acid sequence that is at least 95% identical to SEQ ID NO: 29 and a second amino acid sequence that is at least 95% identical to SEQ ID NO: 36;

(c) a polypeptide comprising a first amino acid sequence that is at least 95% identical to SEQ ID NO: 30 and a second amino acid sequence that is at least 95% identical to SEQ ID NO: 37;

(d) a polypeptide comprising a first amino acid sequence that is at least 95% identical to SEQ ID NO: 31 and a second amino acid sequence that is at least 95% identical to SEQ ID NO: 38;

(e) a polypeptide comprising a first amino acid sequence that is at least 95% identical to SEQ ID NO: 32 and a second amino acid sequence that is at least 95% identical to SEQ ID NO: 38;

(f) a polypeptide comprising a first amino acid sequence that is at least 95% identical to SEQ ID NO: 33 and a second amino acid sequence that is at least 95% identical to SEQ ID NO: 39; and

(g) a polypeptide comprising a first amino acid sequence that is at least 95% identical to SEQ ID NO: 34 and a second amino acid sequence that is at least 95% identical to SEQ ID NO: 40.

32. The polypeptide of claim 29, wherein the polypeptide is selected from the group consisting of:

(a) a polypeptide comprising a first amino acid sequence that is at least 99% identical to SEQ ID NO: 28 and a second amino acid sequence that is at least 99% identical to SEQ ID NO: 35;

(b) a polypeptide comprising a first amino acid sequence that is at least 99% identical to SEQ ID NO: 29 and a second amino acid sequence that is at least 99% identical to SEQ ID NO: 36;

(c) a polypeptide comprising a first amino acid sequence that is at least 99% identical to SEQ ID NO: 30 and a second amino acid sequence that is at least 99% identical to SEQ ID NO: 37;

(d) a polypeptide comprising a first amino acid sequence that is at least 99% identical to SEQ ID NO: 31 and a second amino acid sequence that is at least 99% identical to SEQ ID NO: 38;

(e) a polypeptide comprising a first amino acid sequence that is at least 99% identical to SEQ ID NO: 32 and a second amino acid sequence that is at least 99% identical to SEQ ID NO: 38;

(f) a polypeptide comprising a first amino acid sequence that is at least 99% identical to SEQ ID NO: 33 and a second amino acid sequence that is at least 99% identical to SEQ ID NO: 39; and

(g) a polypeptide comprising a first amino acid sequence that is at least 99% identical to SEQ ID NO: 34 and a second amino acid sequence that is at least 99% identical to SEQ ID NO: 40.

33. An isolated cell producing the antibody or antigen binding fragment thereof of any one of claims 1-28 or the polypeptide of any one of claims 29-32.

34. A method of making the antibody or antigen-binding fragment thereof of any one of claims 1-28 or the polypeptide of any one of claims 29-32, comprising:

(a) culturing the cell of claim 32 so as to produce the antibody, antigen-binding fragment thereof, or polypeptide; and

(b) isolating the antibody, antigen-binding fragment thereof, or polypeptide from the cultured cell.

35. An immunoconjugate having the formula (A) - (L) - (C), wherein

(A) is an antibody or antigen binding fragment thereof of any one of claims 1-28 or a polypeptide of any one of claims 29-32;

(L) is a linker; and

(C) is a cytotoxic agent; and

wherein the linker (L) links (A) to (C).

36. An immunoconjugate having the formula (A) - (L) - (C), wherein

(A) is an antibody or antigen binding fragment that specifically binds to HER3;

(L) is a non-cleavable linker; and

(C) is a cytotoxic agent; and

wherein said linker (L) links (A) to (C).

37. An immunoconjugate having the formula (A) - (L) - (C), wherein

(A) is an antibody or antigen binding fragment that specifically binds to HER3;

(L) is a linker; and

(C) is a maytansinoid; and

wherein said linker (L) links (A) to (C).

38. The immunoconjugate of any one of claims 35-37, wherein the linker is selected from the group consisting of a cleavable linker, a non-cleavable linker, a hydrophilic linker, and a dicarboxylic acid based linker.

39. The immunoconjugate of claim 38, wherein the linker is selected from the group consisting of N-succinimidyl 4-(2-pyridyldithio)pentanoate (SPP); N-succinimidyl 4-(2-pyridyldithio)butanoate (SPDB) or N-succinimidyl 4-(2-pyridyldithio)-2-sulfobutanoate (sulfo-SPDB); N-succinimidyl 4-(maleimidomethyl) cyclohexanecarboxylate (SMCC); N-sulfosuccinimidyl 4-(maleimidomethyl) cyclohexanecarboxylate (sulfoSMCC); N-succinimidyl-4-(iodoacetyl)-aminobenzoate (SIAB); and N-succinimidyl-[(N-maleimidopropionamido)-tetraethyleneglycol] ester (NHS-PEG4-maleimide).

40. The immunoconjugate of any one of claims 35-39, wherein the cytotoxic agent is selected from the group consisting of a maytansinoid, maytansinoid analog, doxorubicin, a modified doxorubicin, benzodiazepine, taxoid, CC-1065, CC-1065 analog, duocarmycin, duocarmycin analog, calicheamicin, dolastatin, dolastatin analog, aristatin, tomaymycin derivative, and leptomycin derivative or a prodrug of the agent.

41. The immunoconjugate of claim 40, wherein the cytotoxic agent is a maytansinoid.

42. The immunoconjugate of claim 41, wherein the maytansinoid is N(2')-deacetyl-N(2')-(3-mercapto-1-oxopropyl)-maytansine (DM1) or N(2')-deacetyl-N2-(4-mercapto-4-methyl-1-oxopentyl)-maytansine (DM4).

43. A pharmaceutical composition comprising the antibody or antigen binding fragment thereof of any one of claims 1-28, the polypeptide of any one of claims 29-32, or the immunoconjugate of any one of claims 35-42 and a pharmaceutically acceptable carrier.

44. The pharmaceutical composition of claim 43, wherein the composition further comprises an anti-cancer agent that differs from the antibody, antigen binding fragment thereof, polypeptide, or immunoconjugate.

45. The pharmaceutical composition of claim 44, wherein the anti-cancer agent is a chemotherapeutic agent.

46. A diagnostic reagent comprising the antibody or antigen binding fragment thereof of any one of claims 1-28, the polypeptide of any one of claims 29-32, or the immunoconjugate of any one of claims 35-42 which is labeled.

47. The diagnostic reagent of claim 46, wherein the label is selected from the group consisting of a radiolabel, a fluorophore, a chromophore, an imaging agent, and a metal ion.

48. A kit comprising the antibody or antigen binding fragment thereof of any one of claims 1-28, the polypeptide of any one of claims 29-32, or the immunoconjugate of any one of claims 35-42.

49. A method for inhibiting the growth of a cell expressing HER3 comprising contacting the cell with the antibody or antigen binding fragment thereof of any one of claims 1-28, the polypeptide of any one of claims 29-32, the immunoconjugate of any one of claims 35-42, or the pharmaceutical composition of any one of claims 43-44 to thereby inhibit the growth of the cell.

50. A method for treating a patient having cancer comprising administering to the patient a therapeutically effective amount of the antibody or antigen binding fragment thereof of any one of claims 1-28, the polypeptide of any one of claims 29-32, the immunoconjugate of any one of claims 35-42, or the pharmaceutical composition of any one of claims 43-44 to thereby treat the patient.

51. The method of claim 50, further comprising administering an anti-cancer agent that differs from the antibody, antigen binding fragment thereof, polypeptide, or immunoconjugate to the patient.

52. The method of claim 51, wherein the anti-cancer agent is a chemotherapeutic agent.

53. The method of any one of claims 50-52, wherein the cancer is selected from the group consisting of breast cancer, lung cancer, ovarian cancer, colon cancer, and other solid tumors.

54. An isolated polynucleotide comprising a nucleic acid sequence that encodes a polypeptide that is at least 90% identical to a sequence selected from the group consisting of SEQ ID NOs: 28-40.

55. The polynucleotide of claim 54, wherein the polynucleotide comprises a nucleic acid sequence that encodes a polypeptide that is at least 95% identical to a sequence selected from the group consisting of SEQ ID NOs: 28-40.

56. The polynucleotide of claim 54, wherein the polynucleotide comprises a nucleic acid sequence that encodes a polypeptide that is at least 99% identical to a sequence selected from the group consisting of SEQ ID NOs: 28-40.

57. The polynucleotide of any one of claims 54-56, wherein the polynucleotide comprises a nucleic acid sequence that is at least 90% identical to a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 54-73.

58. The polynucleotide of claim 57, wherein the polynucleotide comprises a nucleic acid sequence that is at least 95% identical to a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 54-73.

59. The isolated polynucleotide of claim 57, wherein the polynucleotide comprises a nucleic acid sequence that is at least 99% identical to a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 54-73.

60. A vector comprising the polynucleotide of any one of claims 54-59.

61. A host cell comprising the vector of claim 60.

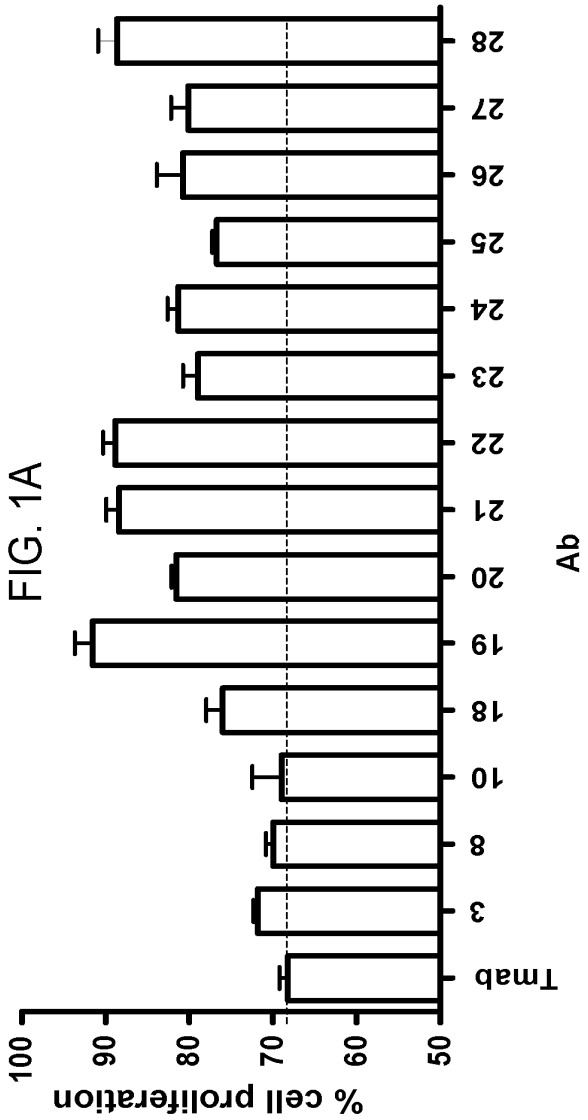

% cell proliferation
100
90
80
70
60
50
Tmab
3
8
10
18
19
20
21
22
23
24
25
26
27
28
Ab

FIG. 1A

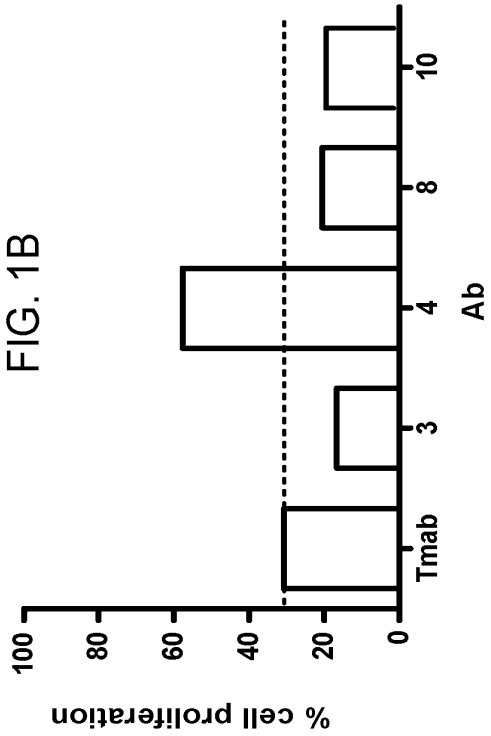

% cell proliferation
100
80
60
40
20
0
Tmab
3
4
8
10
Ab

FIG. 1B

FIG. 2

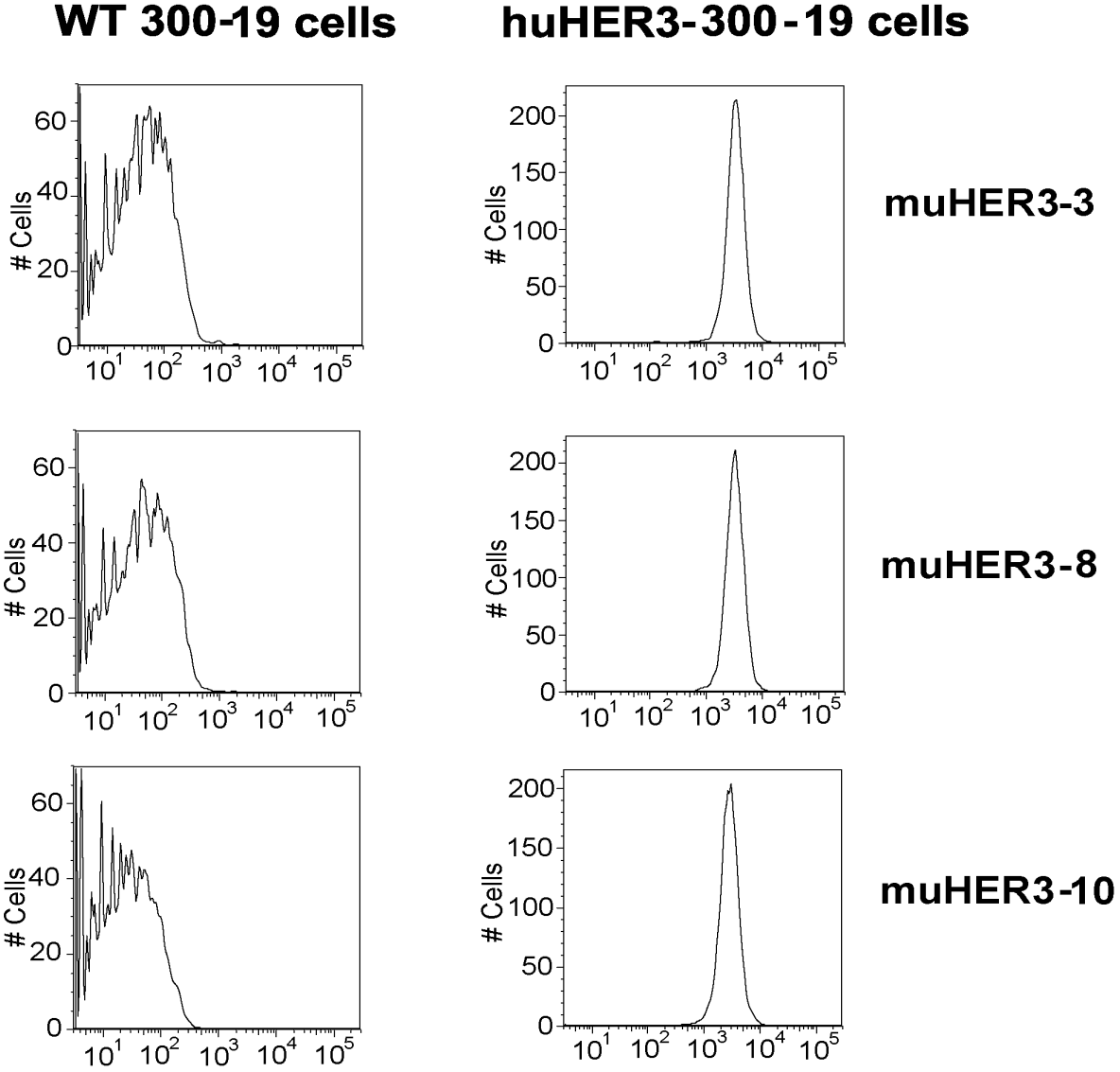
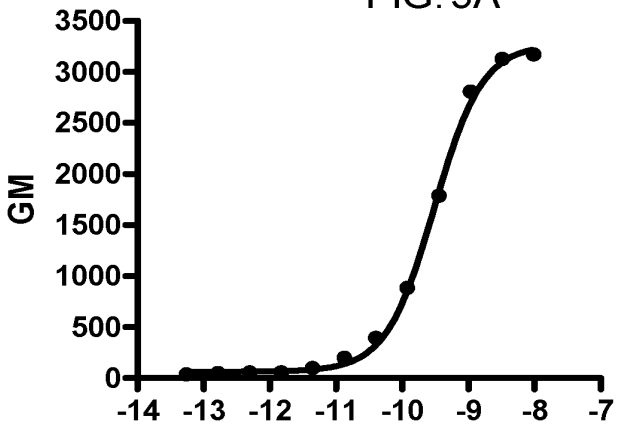
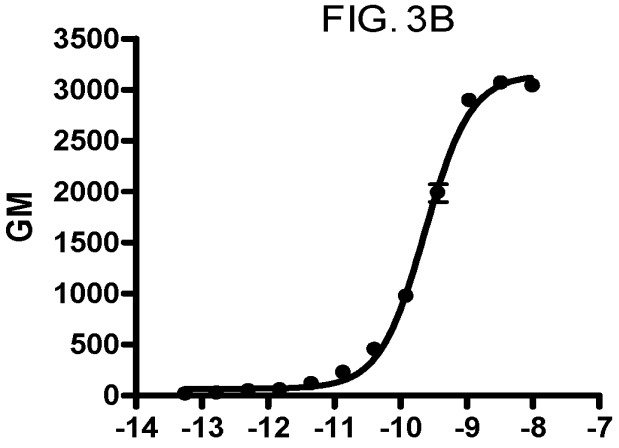

WT 300-19 cells
huHER3-300-19 cells
Cells
muHER3-3
muHER3-8
muHER3-10

FIG. 3A



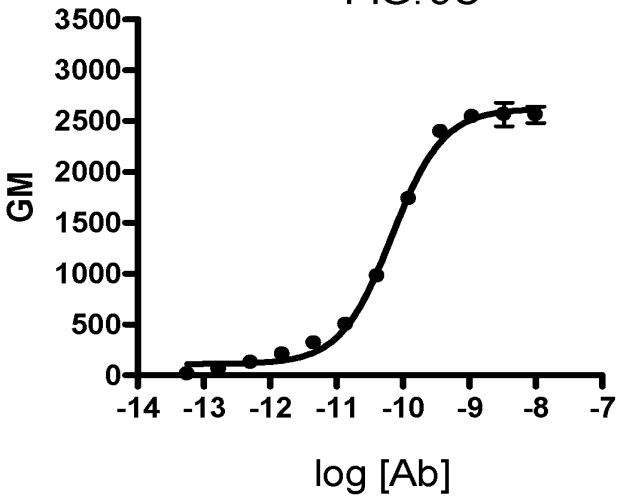
muHER3-3

FIG. 3B



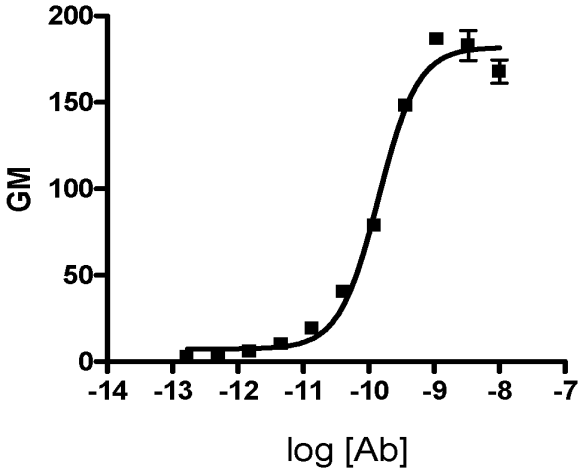
muHER3-8

FIG. 3C



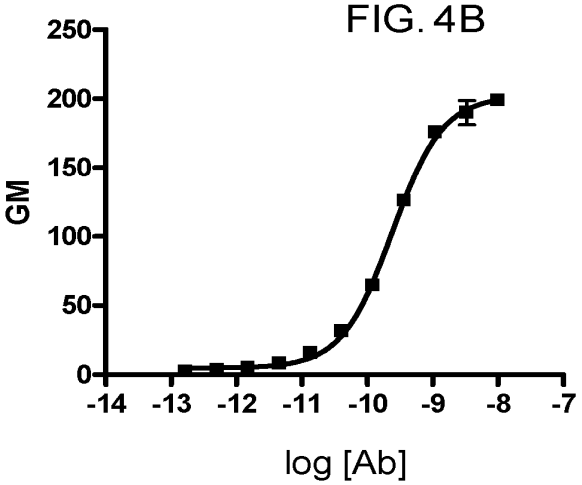
muHER3-10

FIG. 4A



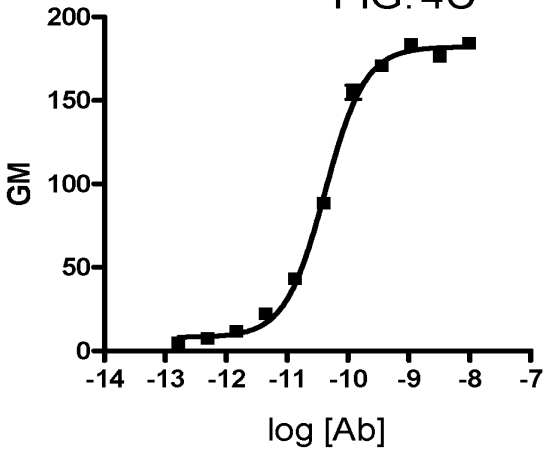
muHER3-3

FIG. 4B



muHER3-8

FIG. 4C



muHER3-10

FIG. 5

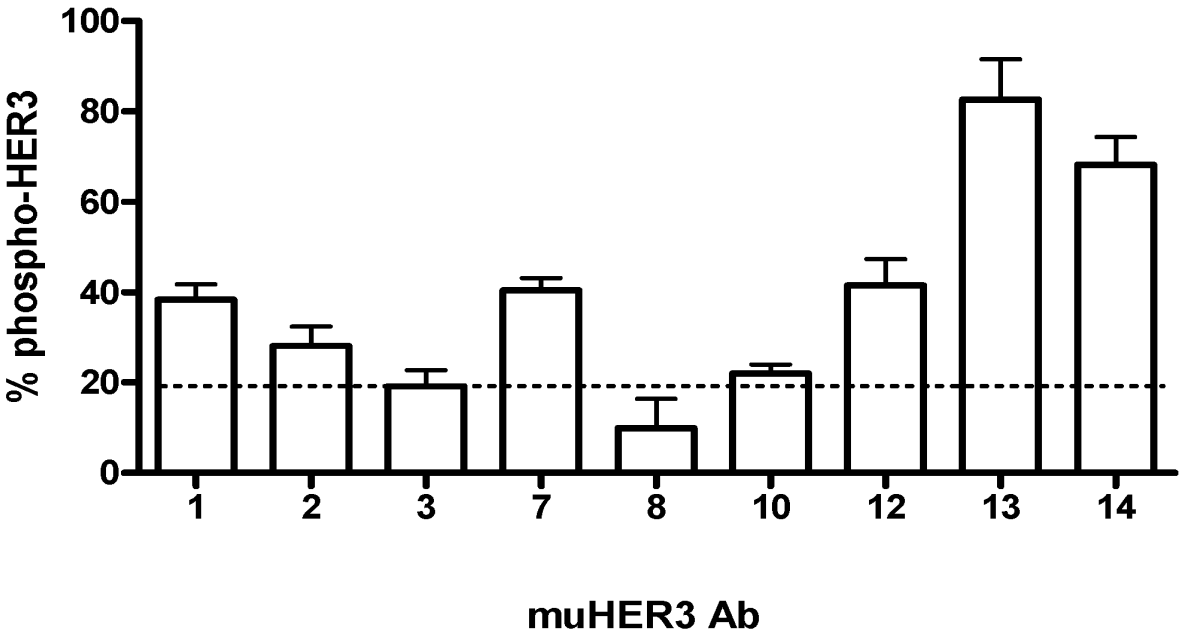


FIG. 6

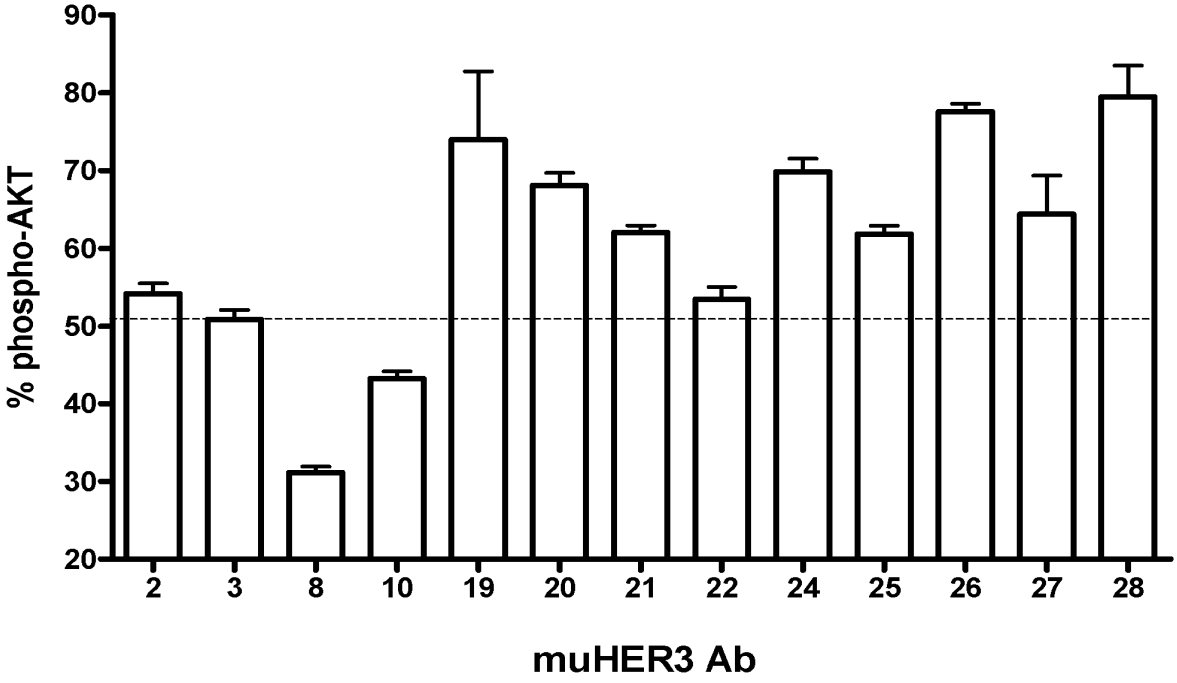

90
80
70
60
50
40
30
20
% phospho-AKT
2
3
8
10
19
20
21
22
24
25
26
27
28
muHER3 Ab

FIG. 7

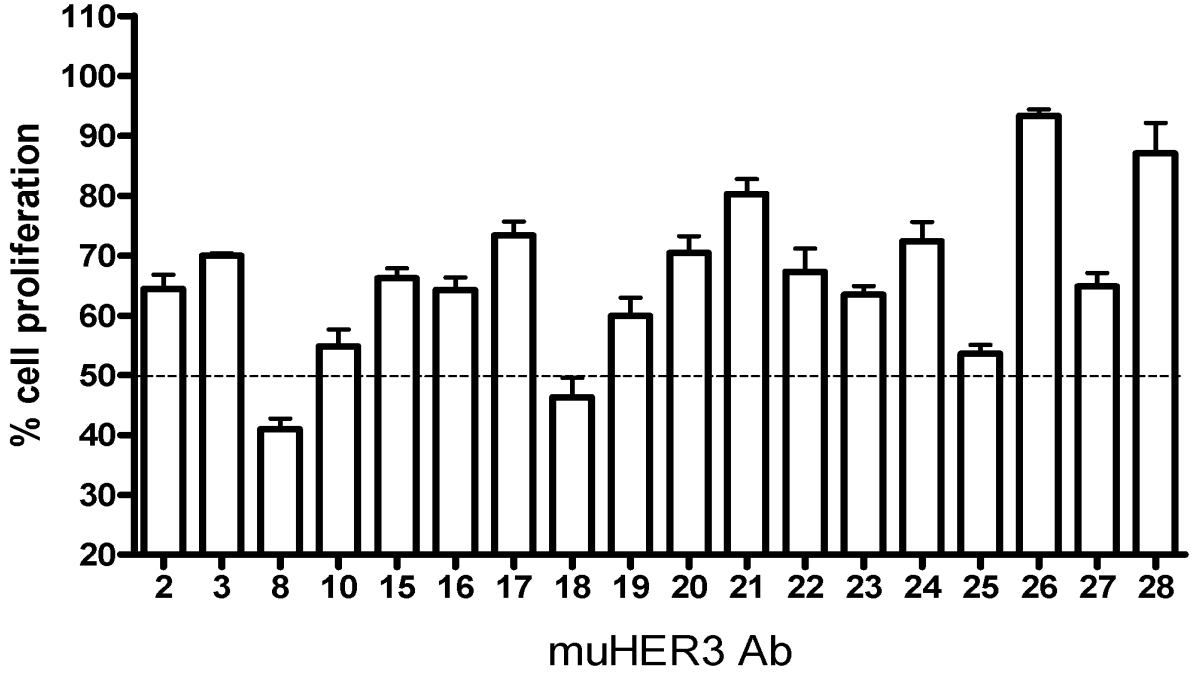

% cell proliferation
110
100
90
80
70
60
50
40
30
20
2
3
8
10
15
16
17
18
19
20
21
22
23
24
25
26
27
28
muHER3 Ab

FIG. 8

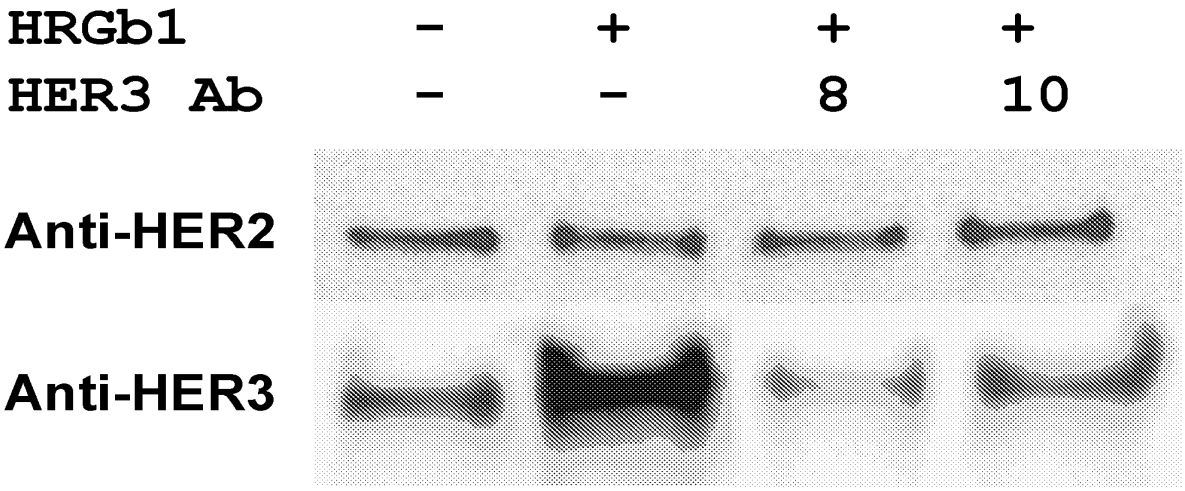


FIG. 9A

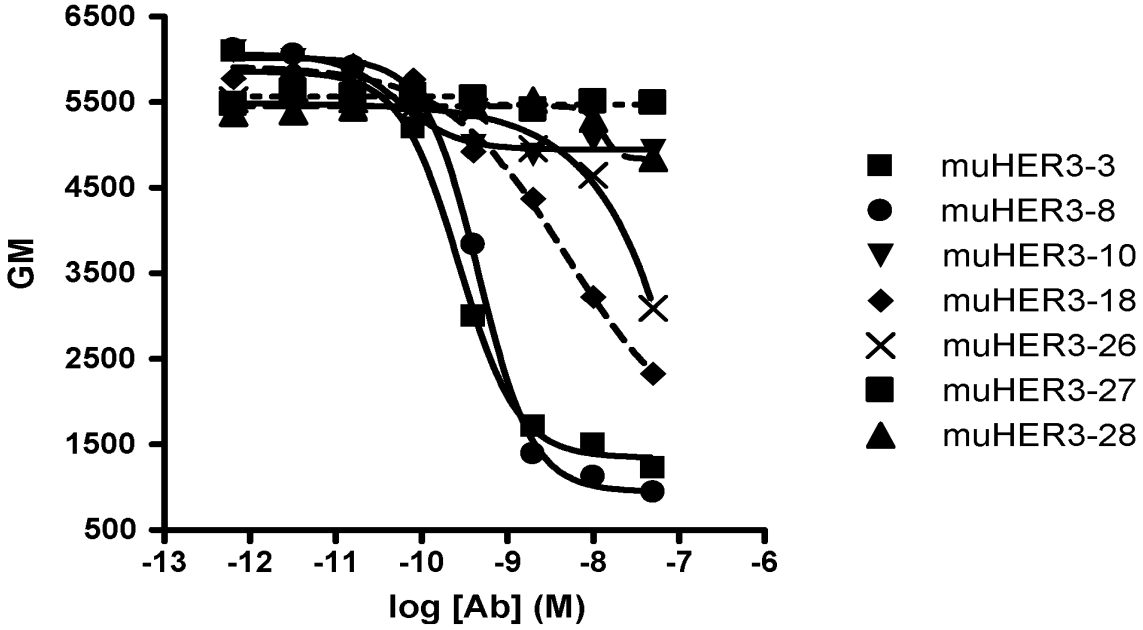

6500
5500
4500
3500
2500
1500
500
GM
-13
-12
-11
-10
-9
-8
-7
-6
log [Ab] (M)
muHER3-3
muHER3-8
muHER3-10
muHER3-18
muHER3-26
muHER3-27
muHER3-28

FIG. 9B

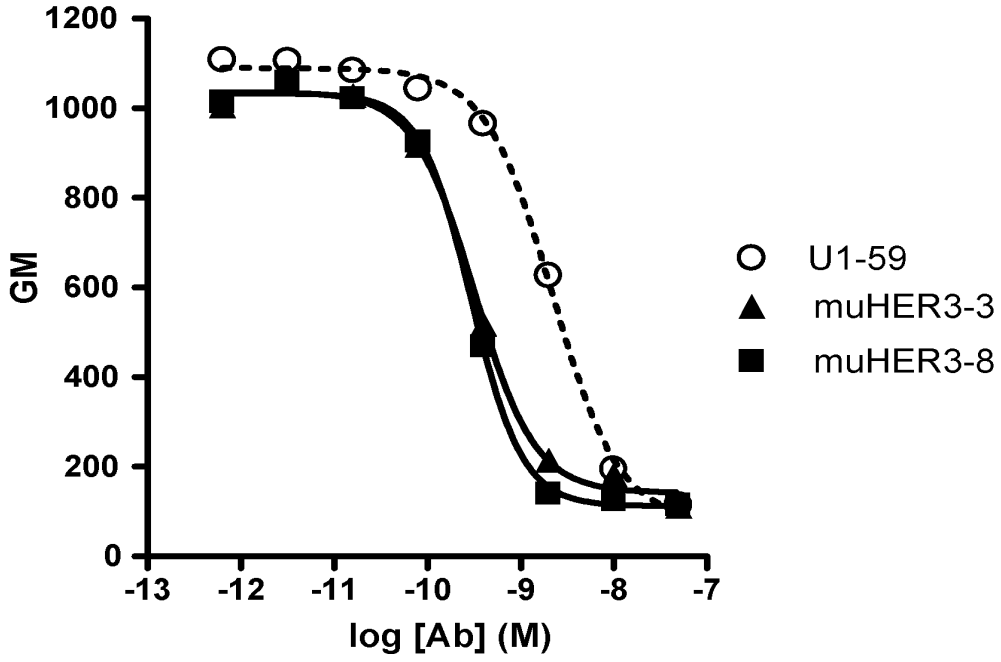

1200
1000
800
600
400
200
0
GM
-13
-12
-11
-10
-9
-8
-7
log [Ab] (M)
U1-59
muHER3-3
muHER3-8

FIG. 10A

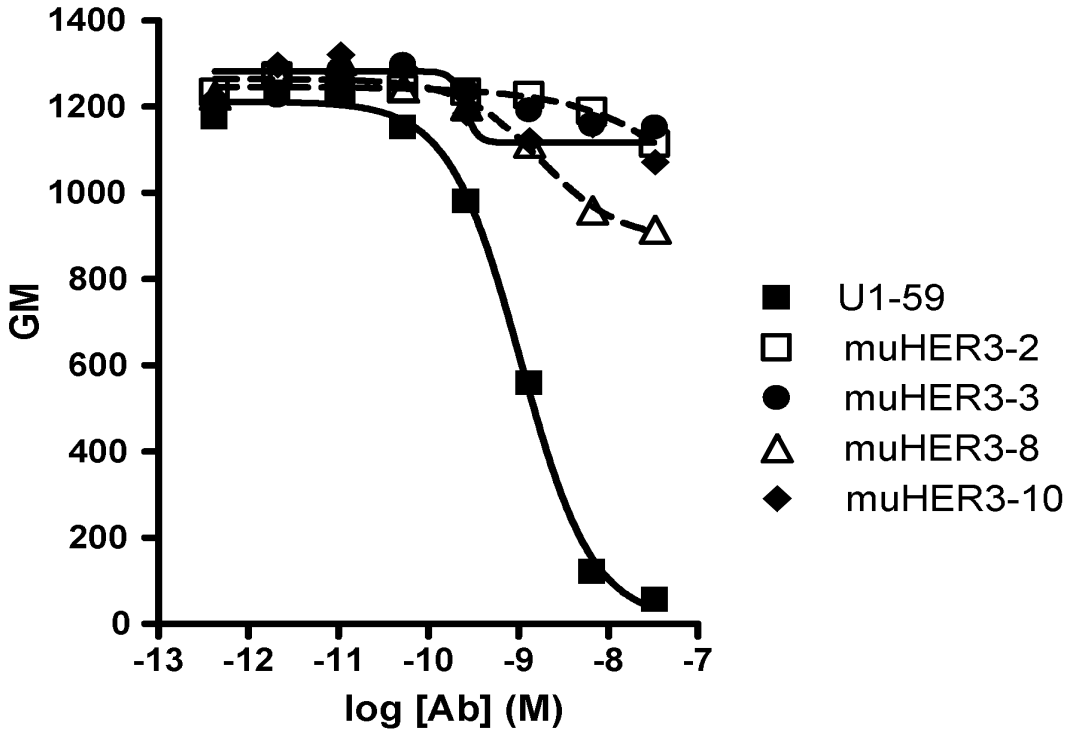


FIG. 10B

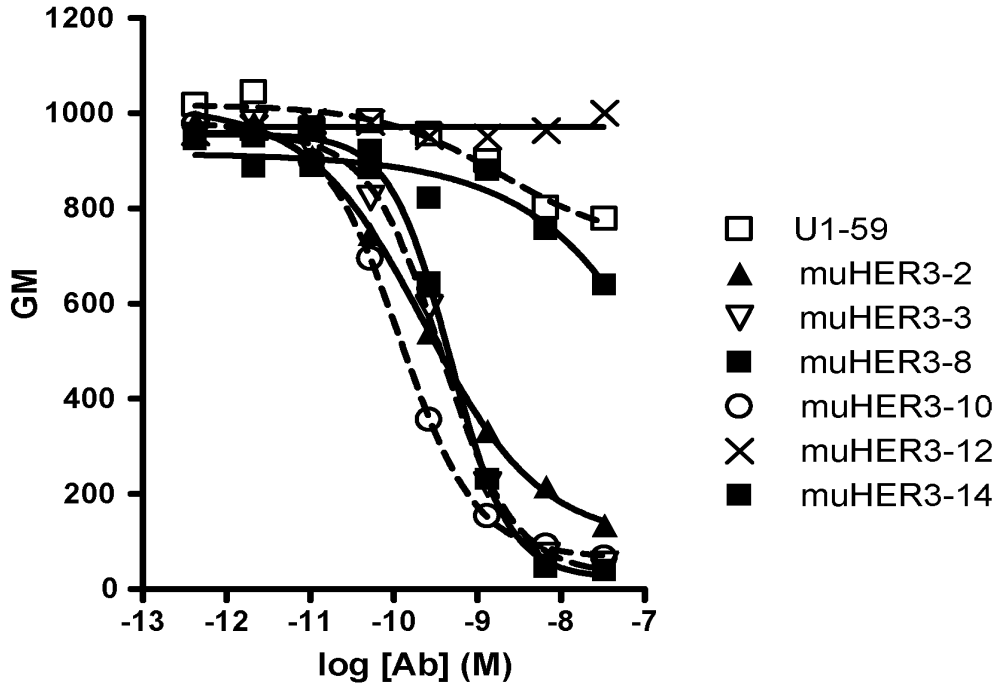


FIG. 11A

HER3-3 V_L		
Kabat position	Murine residue	Human residue
1	**D**	***E***
3	V	V
5	T	T
9	A	A
10	T	T
18	R	R
20	**S**	***T***
40	**S**	***P***
41	**H**	***G***
42	**E**	***Q***
45	R	R
57	G	G
60	**S**	***D***
67	S	S
70	D	D
76	**N**	***S***
79	E	E
80	P	P
81	E	E
100	**A**	***Q***
103	K	K
107	K	K
108	R	R

FIG. 11B

HER3-3 V_H		
Kabat position	Murine residue	Human residue
1	Q	Q
3	Q	Q
5	**Q**	***V***
9	A	A
11	**L**	***V***
13	**M**	***K***
14	P	P
19	K	K
23	K	K
28	T	T
41	P	P
42	G	G
43	Q	Q
61	Q	Q
62	**Q**	***K***
64	**K**	***Q***
65	G	G
73	E	E
74	S	S
82B	S	S
84	S	S
85	E	E
105	Q	Q
112	S	S

FIG. 12A

HER3-8 V_L		
Kabat position	Murine residue	Human residue
1	**D**	***E***
3	V	V
9	A	A
10	T	T
18	R	R
40	**S**	***P***
41	**H**	***G***
42	**E**	***Q***
45	R	R
57	G	G
60	**S**	***D***
67	S	S
70	D	D
76	**N**	***S***
80	P	P
81	E	E
103	K	K
107	K	K
108	R	R

FIG. 12B

HER3-8 V_H			
Kabat position	Murine residue	Human v1.00 residue	Human v1.01 residue
1	**D**	***Q***	***Q***
3	Q	Q	Q
5	Q	Q	Q
9	P	P	P
11	L	L	L
13	**T**	***K***	***K***
15	S	S	S
16	Q	Q	Q
26	G	G	G
41	Q	Q	Q
42	**F**	***H***	***H***
43	P	P	P
61	P	P	P
62	S	S	S
64	K	K	K
65	S	S	S
74	S	S	S
75	K	K	K
82B	S	S	S
84	**S**	***A***	***A***
85	**E**	***A***	***A***
95	**K**	***R***	**K**
105	Q	Q	Q
108	**T**	***L***	***L***
112	S	S	S

FIG. 13A

HER3-10 V_L		
Kabat position	Murine residue	Human residue
1	**N**	***D***
3	V	V
5	T	T
9	A	A
15	**L**	***P***
17	Q	Q
18	R	R
40	P	P
41	G	G
42	Q	Q
45	K	K
57	G	G
60	A	A
67	S	S
70	D	D
80	A	A
81	**D**	***N***
100	**G**	***Q***
103	K	K
105	E	E
107	K	K
108	R	R

FIG. 13B

HER3-10 V_H		
Kabat position	Murine residue	Human residue
1	**E**	***Q***
3	Q	Q
5	**Q**	***V***
11	**L**	***V***
13	K	K
14	P	P
19	K	K
23	K	K
28	**S**	***T***
41	**H**	***P***
42	G	G
43	**K**	***Q***
61	Q	Q
62	K	K
64	**K**	***Q***
65	G	G
72	**N**	***D***
73	K	K
74	S	S
82B	S	S
84	S	S
85	E	E
105	Q	Q
108	L	L
112	S	S

14/32

FIG. 14

A

```
                1                                                                 60
muHer3-3 VL     DIVMTQSPATLSVTPGDRVSLSCRASQSITDYLHWYQQKSHESPRLLIKFASQSISGIPS
huHer3-3 VL     E--------------------T--------------------PGQ------------------D

                61                                                       108
muHer3-3 VL     RFSGSGSGSDFTLSINSVEPEDVGVYYCQNGHSFPLTFGAGTKLELKR
huHer3-3 VL     ----------------S-----------------------Q---------
```

B

```
                1                                                                 60
muHer3-3 VH     QVQLQQPGAELVMPGASVKMSCKASGYTFTDYWIHWVKQRPGQGLEWIGAIDTDTYTSYN
huHer3-3 VH     ----V-----V-K-------------------------------------------------

                61                                                            118
muHer3-3 VH     QQFKGKATLTVDESSSTAYMQLSSLTSEDSAVYYCARSLLRLLYFDYWGQGTTLTVSS
huHer3-3 VH     -K-Q-----------------------------------------------------------
```

C

```
                1                                                                 60
muHer3-8 VL     DIVMTQSPATLSVTPGDRVSLSCRASQSINDYLHWYQQKSHESPRLLIKFASHSISGIPS
huHer3-8 VL     E-----------------------------------PGQ------------------D

                61                                                       108
muHer3-8 VL     RFSGSGSGSDFTLTINSVEPEDVGVYYCQNGHSFPWTFGGGTKLEIKR
huHer3-8 VL     ----------------S-----------------------------------
```

D

```
                1                                                                 60
      muHer3-8 VH     DVQLQESGPGLVTPSQSLSLTCTVTGSSITSDYAWNWIRQFPGNKLEWMGFISYSGSTSY
huHer3-8 VHv1.00      Q-----------K-------------------------------H----------------
huHer3-8 VHv1.01      Q-----------K-------------------------------H----------------

                61                                                            119
      muHer3-8 VH     NPSLKSRISITRDTSKNQFFLMLSSVTSEDTATYYCSRKLGRLYYFDYWGQGTTLTVSS
huHer3-8 VHv1.00      ---------------------------RA---------R--------------L-----
huHer3-8 VHv1.01      ---------------------------RA-----------------------L-----
```

E

```
                1                                                                 60
muHer3-10 VL    NIVLTQSPASLAVSLGQRATISCRASTSVYNYGNSFMHWYQQKPGQPPKLLIYLASNLES
huHer3-10 VL    D--------------P-----------------------------------------------

                61                                                       112
muHer3-10 VL    GVPARFSGSGSRTDFTLTIDPVEADDRATYYCQQNSEDPWTFGGGTKLEIKR
huHer3-10 VL    -------------------------N-------------------Q---------
```

F

```
                1                                                                 60
muHer3-10 VH    EVQLQQSGPELVKPGVSMKISCKASGYSFTDYTMNWVKQSNGKNLEWIGLINPYNGITAY
huHer3-10 VH    Q---V-----V----------------T-------------P-Q-----------------

                61                                                            119
muHer3-10 VH    NQKFKGKATLSVNKSSSTAYMELLSLTSEDSAVYYCARRGPRGAWFAYWGQGTLVTVSA
huHer3-10 VH    ----Q--------D-------------------------------------------------
```

FIG. 15A

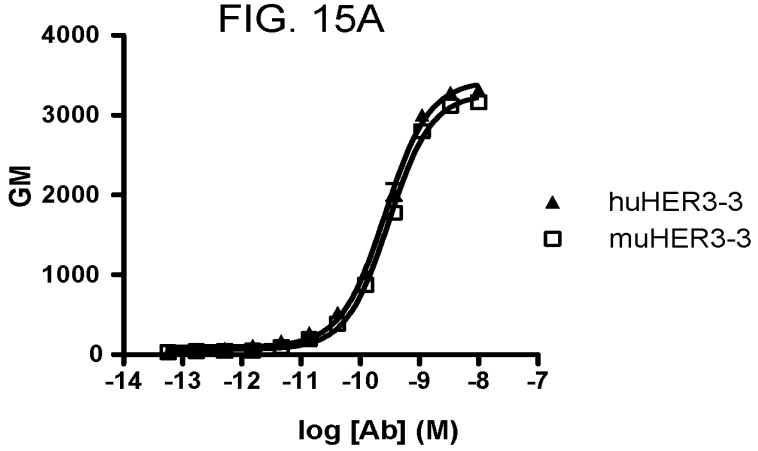


FIG. 15B

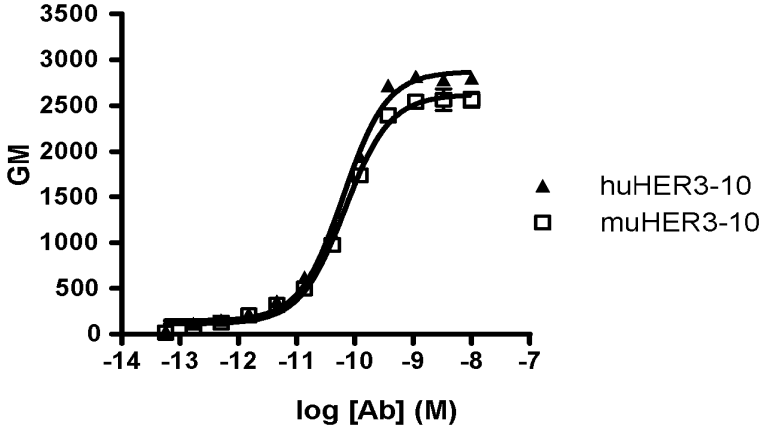


FIG. 15C

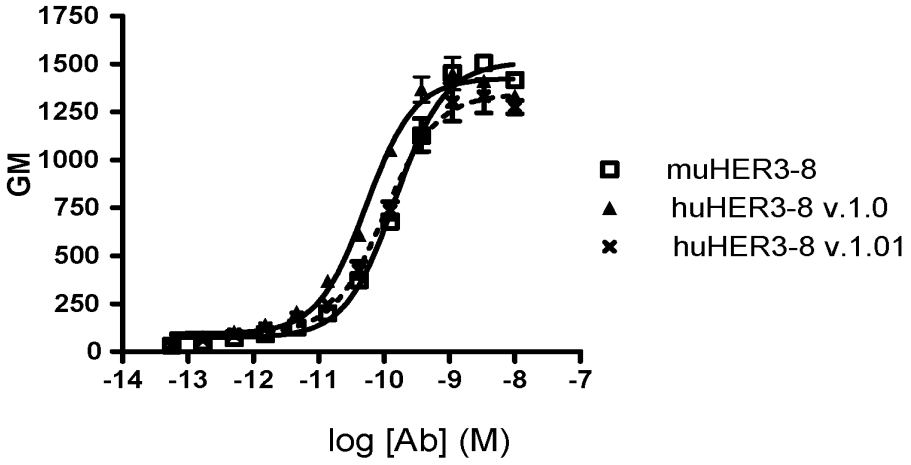


FIG. 16

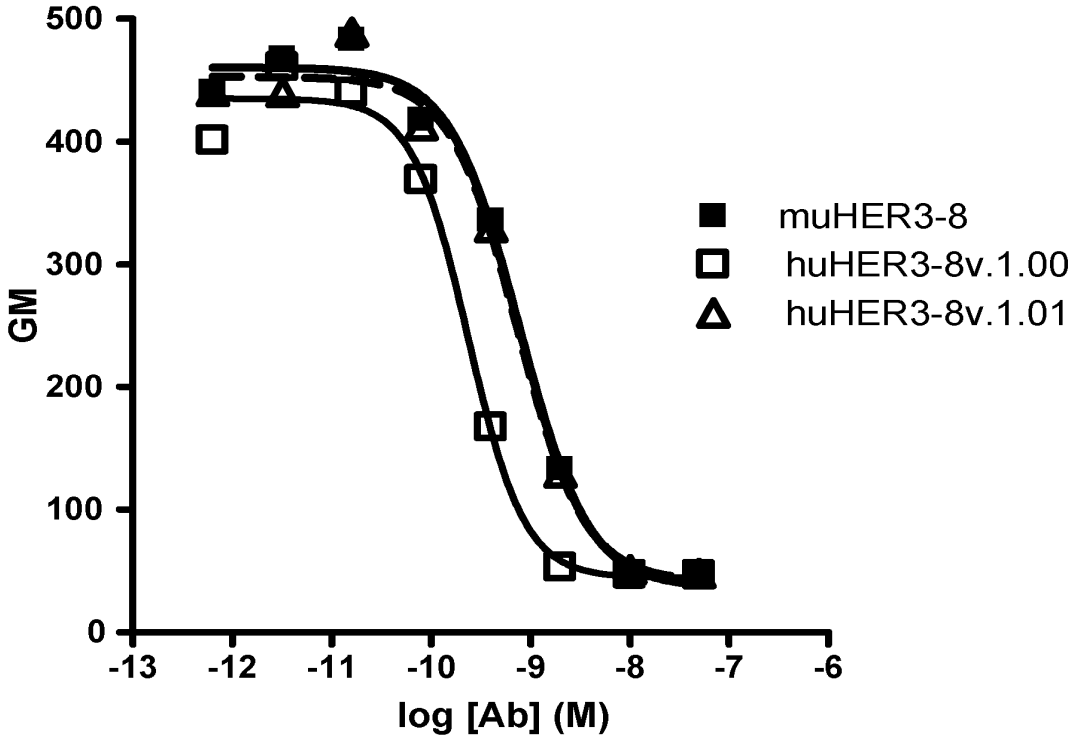

500
400
300
200
100
0
GM
-13
-12
-11
-10
-9
-8
-7
-6
log [Ab] (M)
muHER3-8
huHER3-8v.1.00
huHER3-8v.1.01

FIG. 17

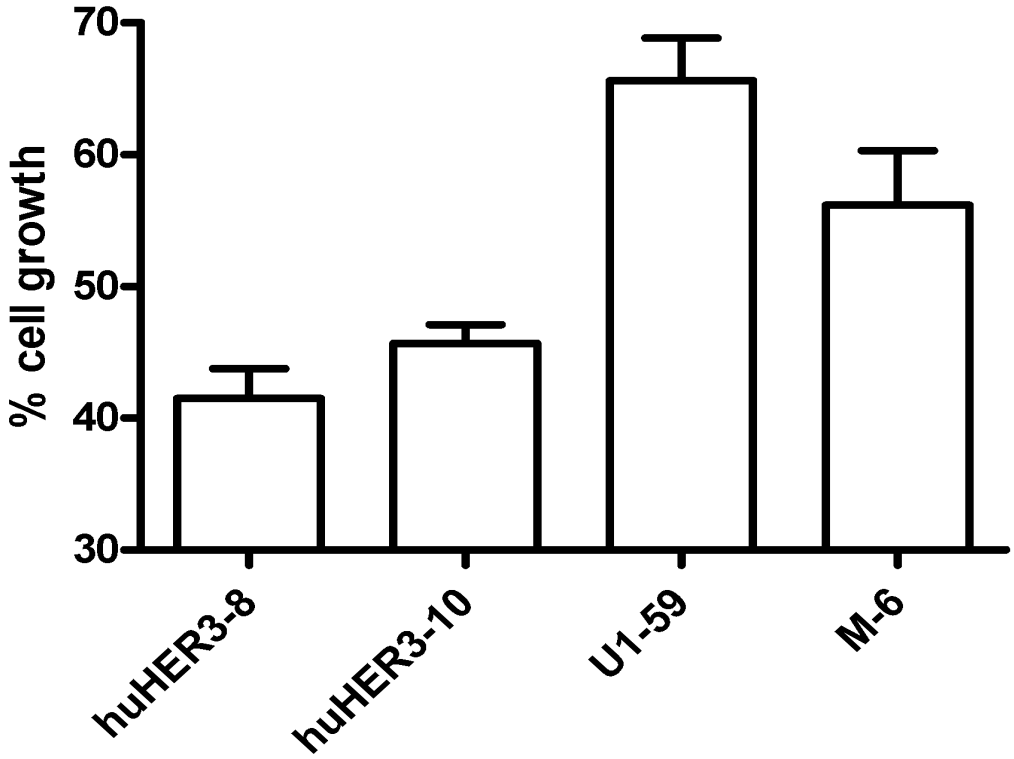


FIG. 18

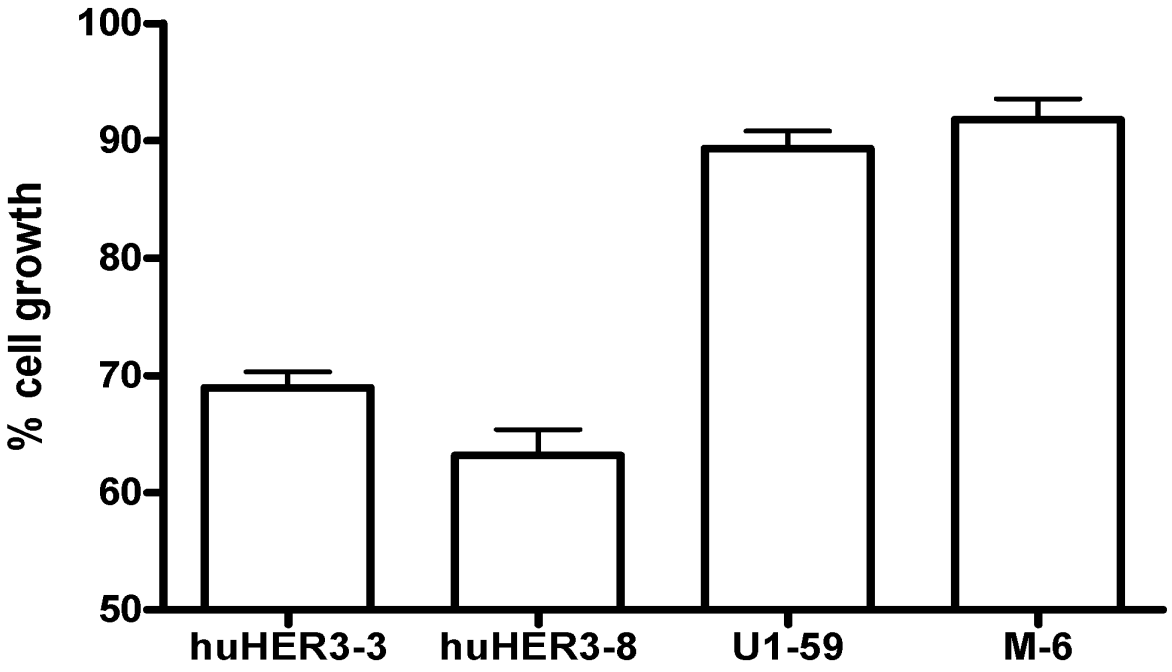


FIG. 19

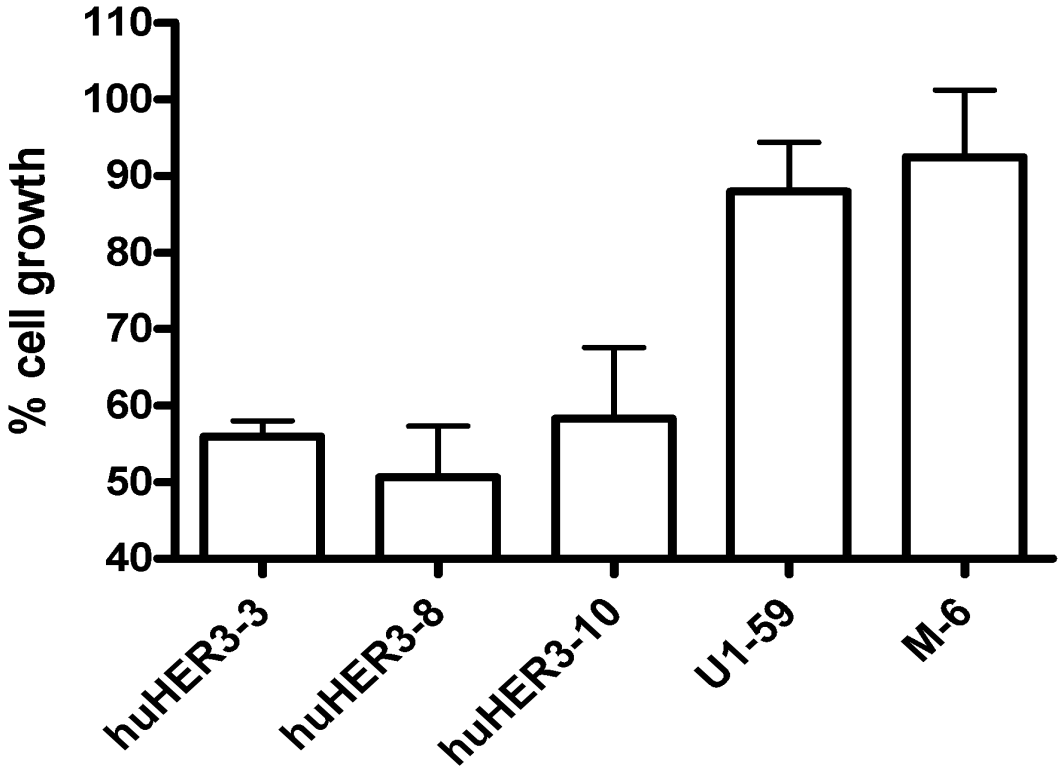

110
100
90
80
70
60
50
40
% cell growth
huHER3-3
huHER3-8
huHER3-10
U1-59
M-6

FIG. 20

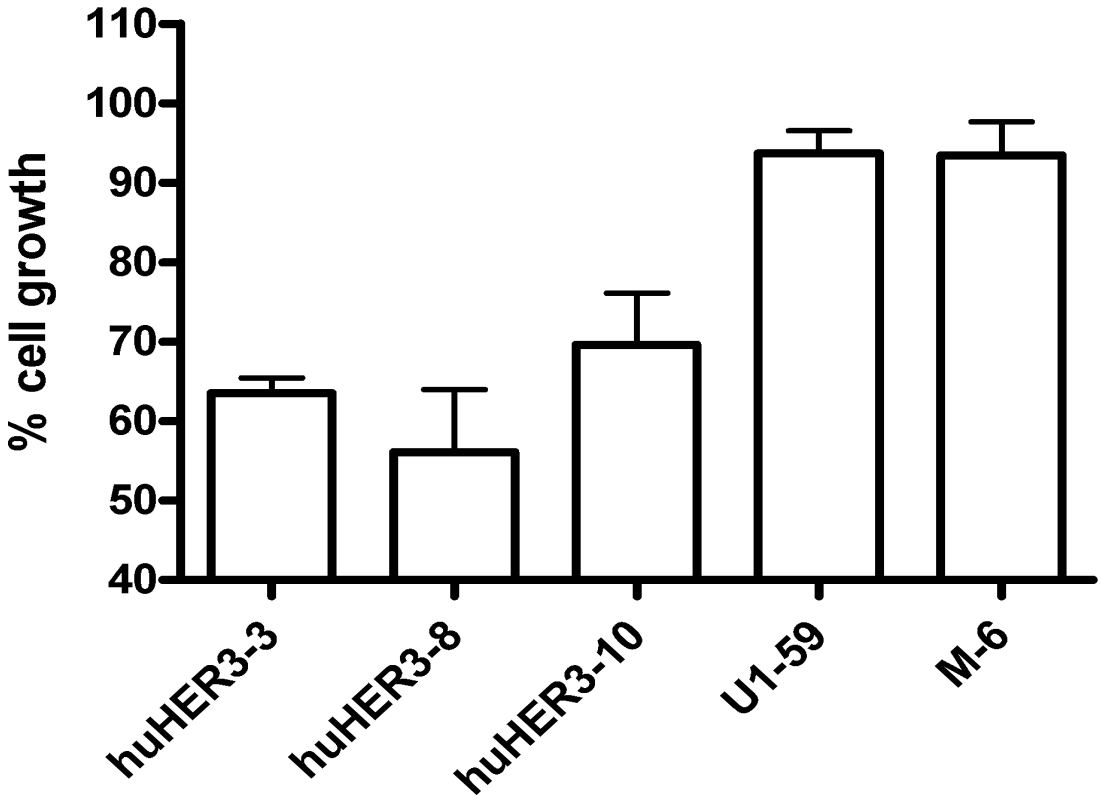

% cell growth
110
100
90
80
70
60
50
40
huHER3-3
huHER3-8
huHER3-10
U1-59
M-6

FIG. 21A

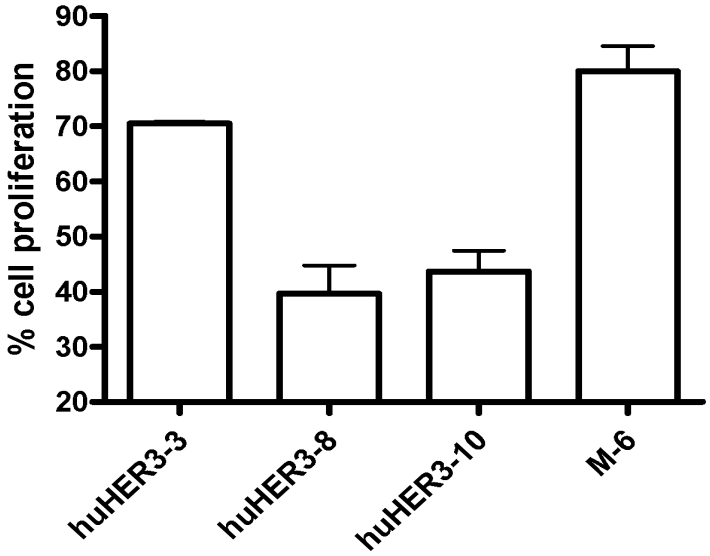


FIG. 21B

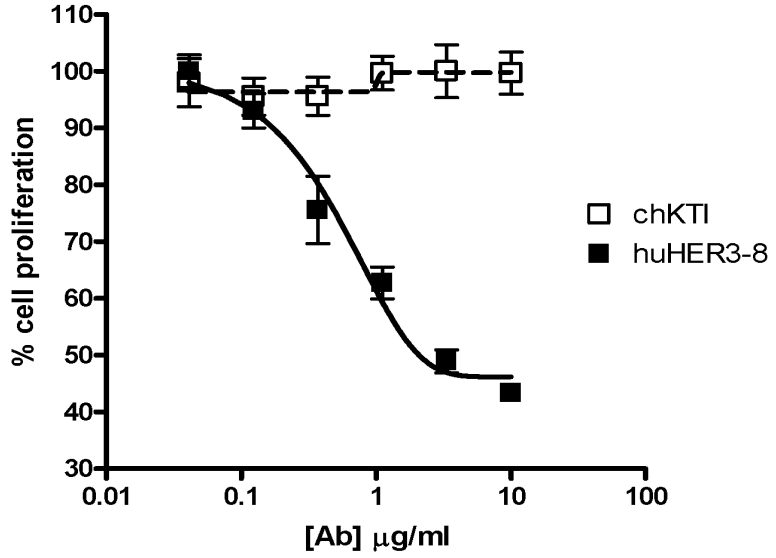


FIG. 22A

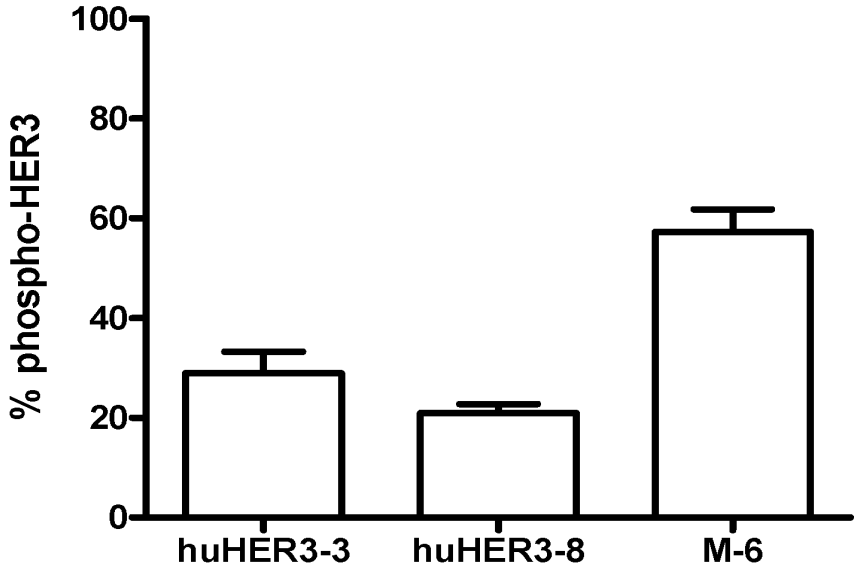


FIG. 22B

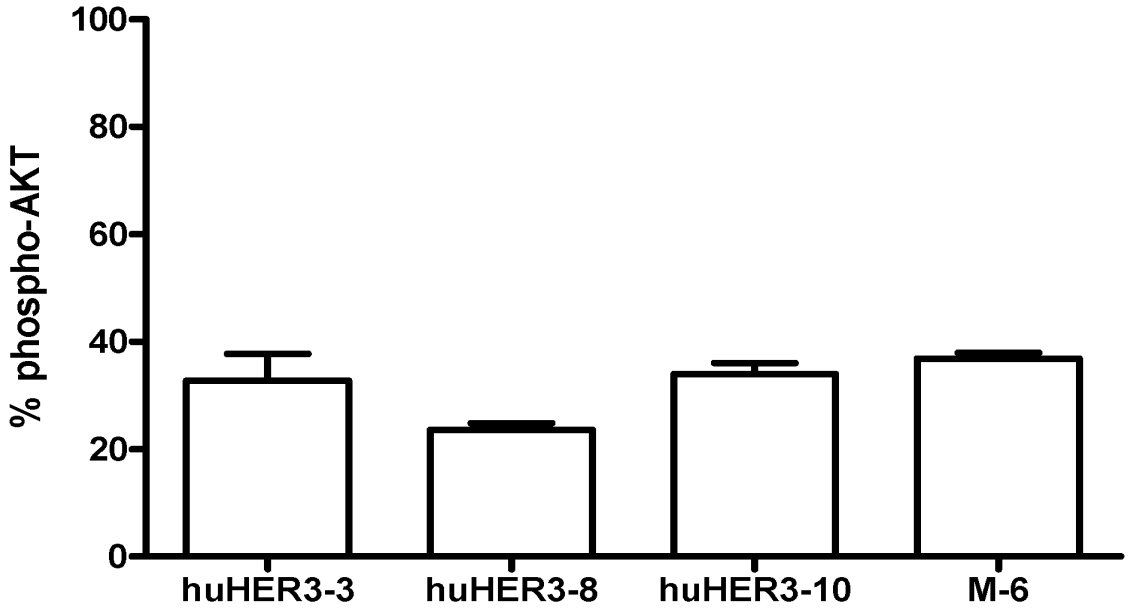
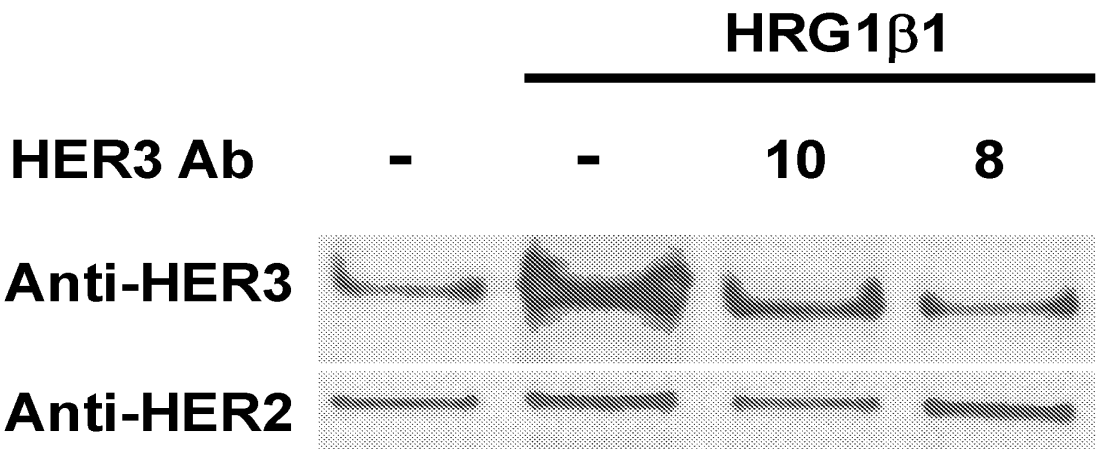


FIG. 23



24/32

FIG.24A

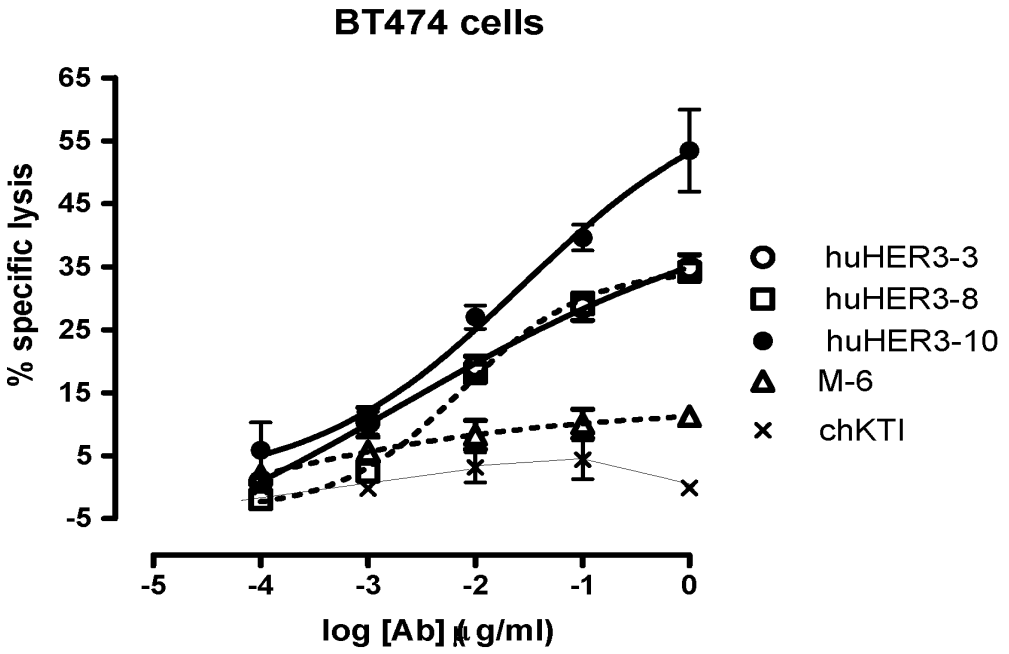
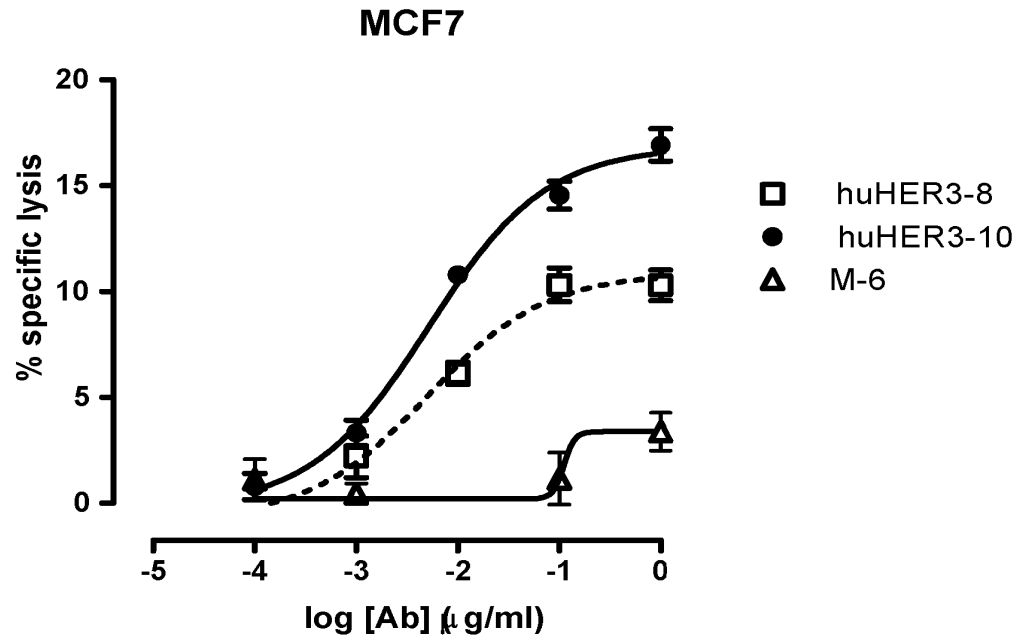


FIG.24B



25/32

FIG. 25A

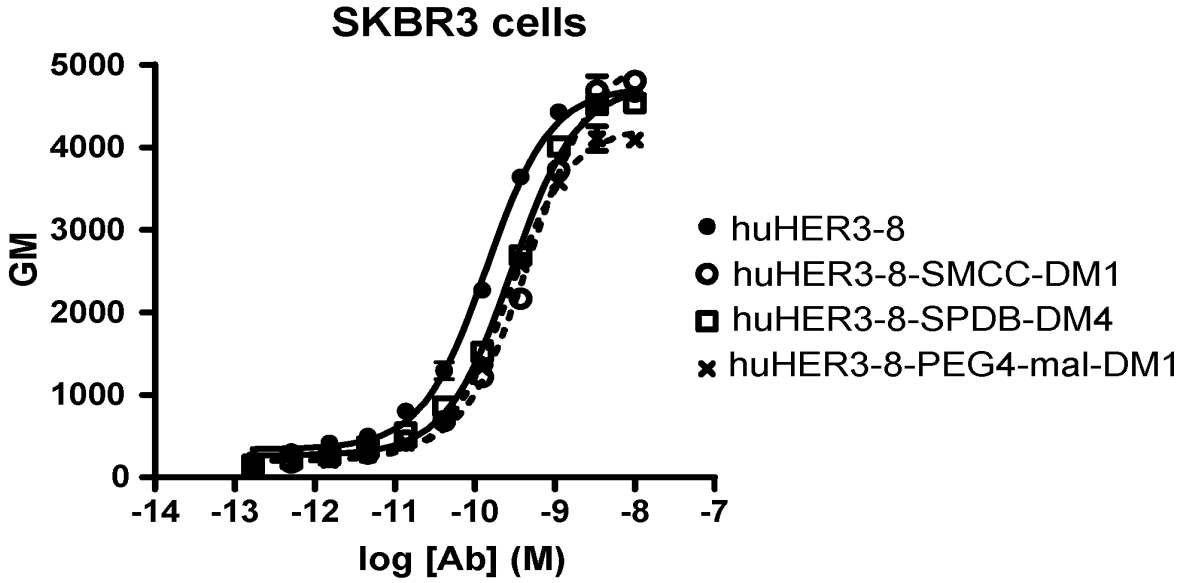


FIG. 25B

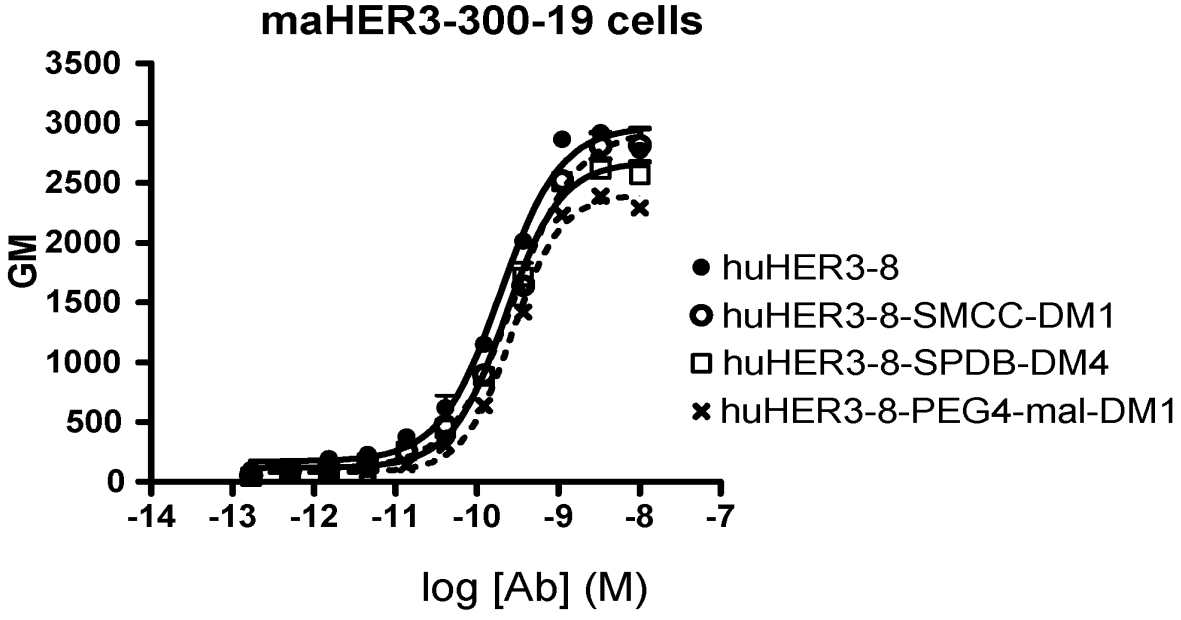


FIG. 26A

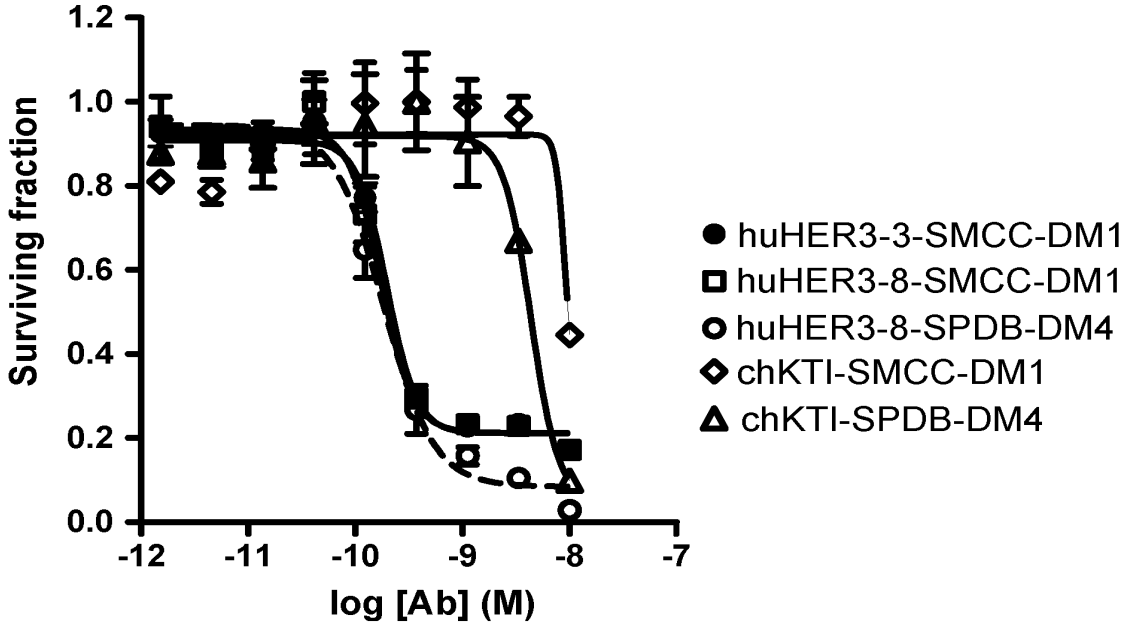
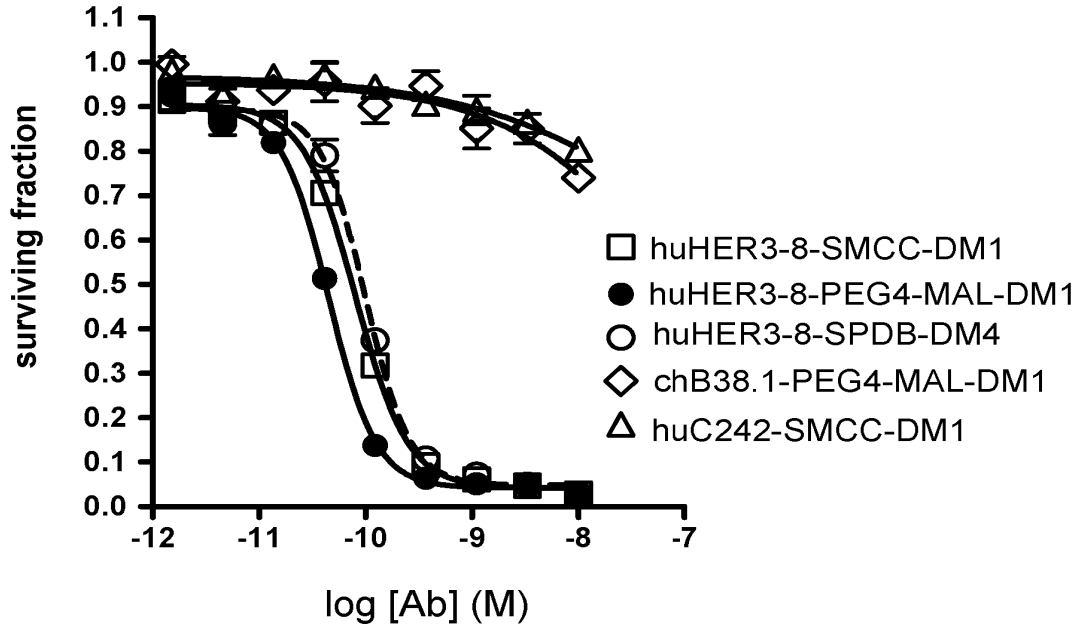


FIG. 26B



27/32

FIG. 27

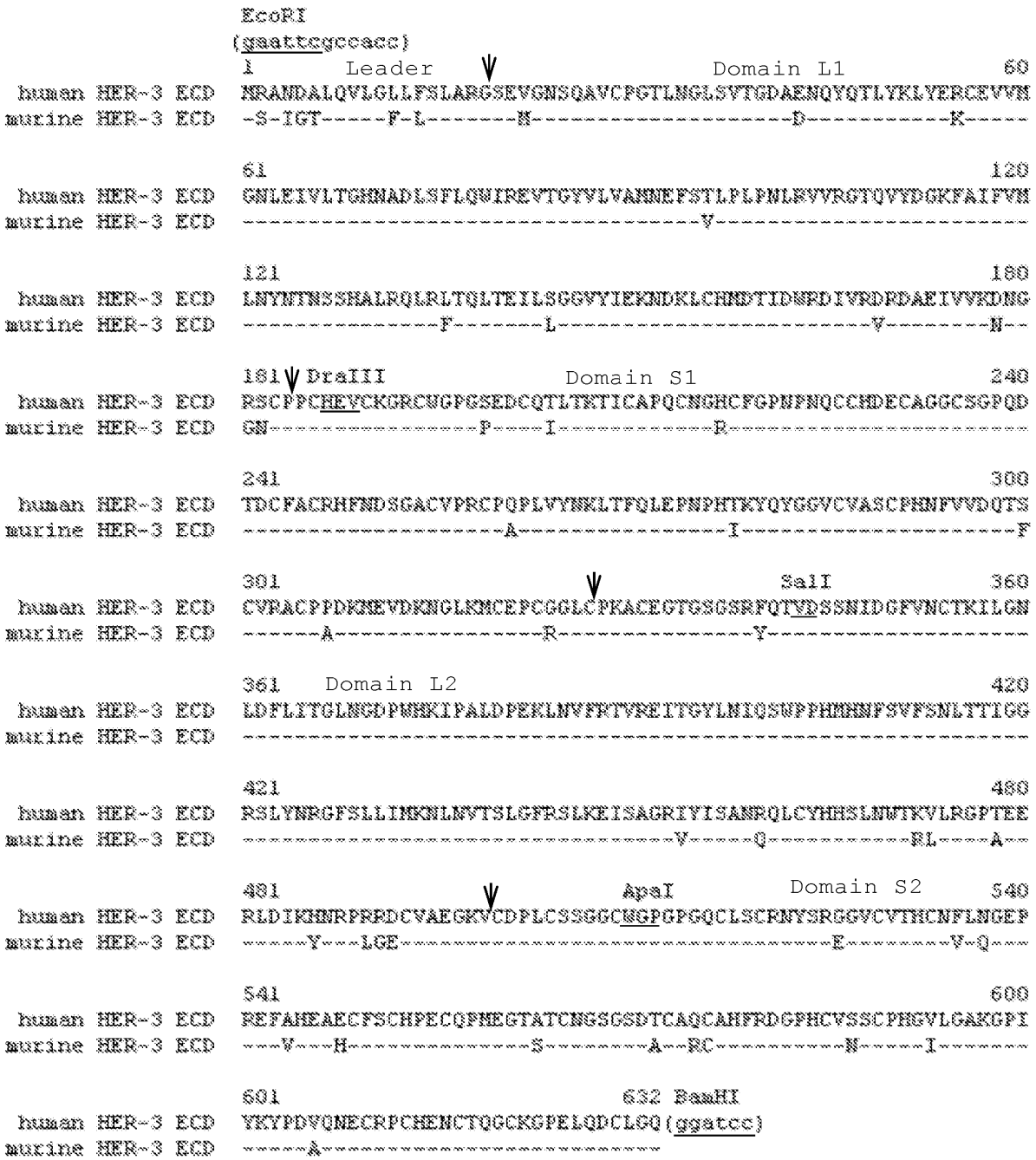
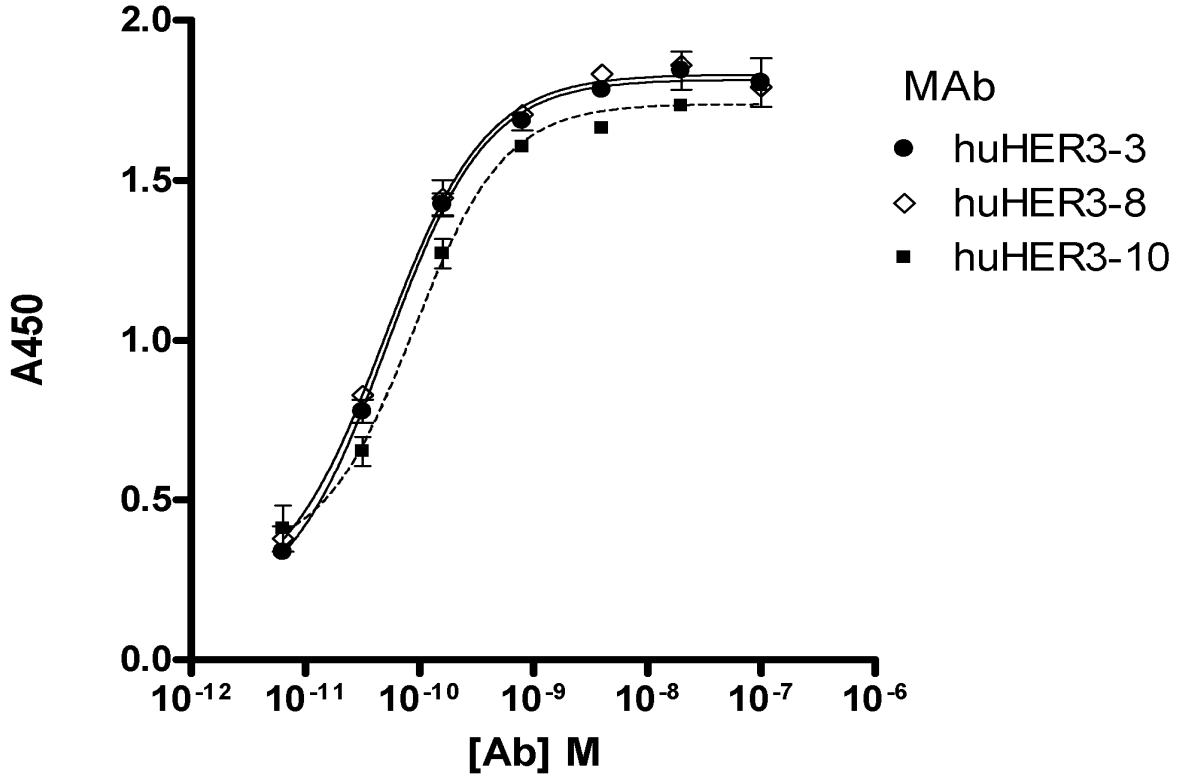
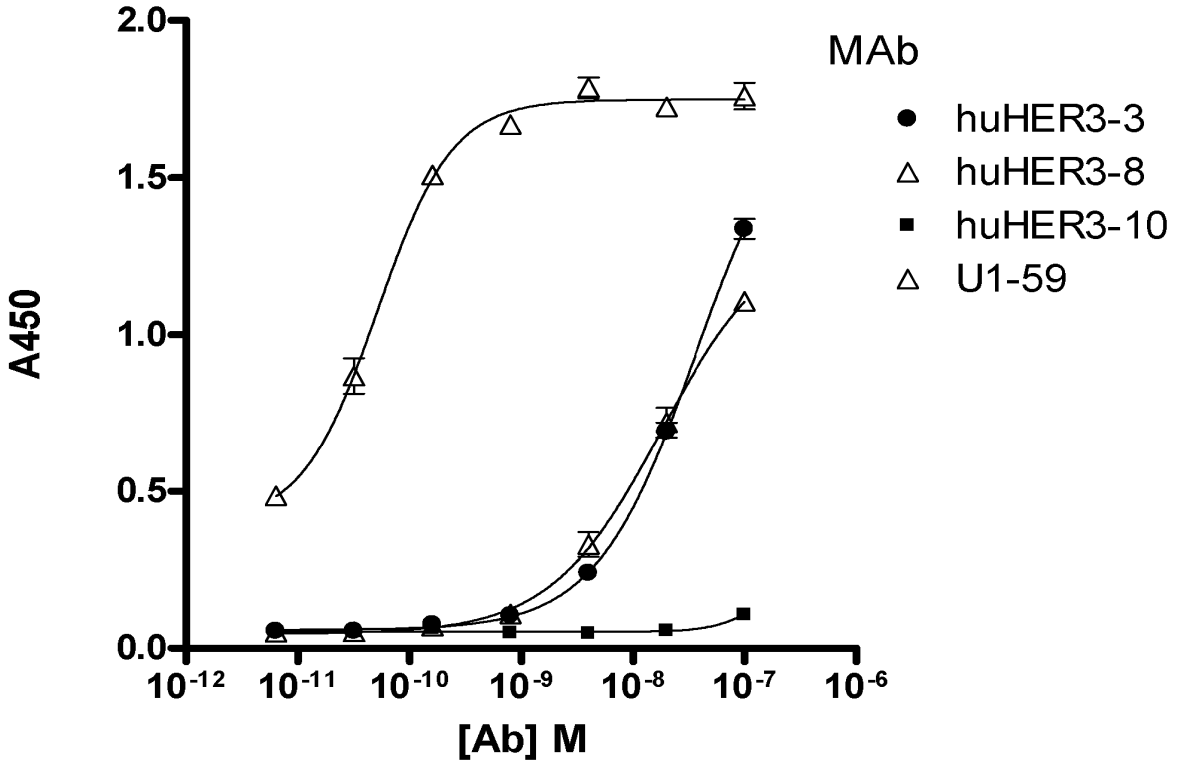

EcoRI
(gaattcgccacc)
1 Leader Domain L1 60
human HER-3 ECD MRANDALQVLGLLFSLARGSEVGNSQAVCPGTLNGLSVTGDAENQYQTLYKLYERCEVVM
murine HER-3 ECD -S-IGT-----F-L-------N----------D----------K-----
61 120
human HER-3 ECD GNLEIVLTGHNADLSFLQWIREVTGYVLVAMNEFSTLPLPNLRVVRGTQVYDGKFAIFVM
murine HER-3 ECD ----------V----------
121 180
human HER-3 ECD LNYNTNSSHALRQLRLTQLTEILSGGVYIEKNDKLCHMDTIDWRDIVRDRDAEIVVKDNG
murine HER-3 ECD ---------------F-------L----------V--------N--
181 DraIII Domain S1 240
human HER-3 ECD RSCPPCHEVCKGRCWGPGSEDCQTLTKTICAPQCNGHCFGPNPNQCCHDECAGGCSGPQD
murine HER-3 ECD GN----------P----I----------R----------
241 300
human HER-3 ECD TDCFACRHFNDSGACVPRCPQPLVYNKLTFQLEPNPHTKYQYGGVCVASCPHNFVVDQTS
murine HER-3 ECD ----------A----------I----------F
301 SalI 360
human HER-3 ECD CVRACPPDKMEVDKNGLKMCEPCGGLCPKACEGTGSGSRFQTVDSSNIDGFVNCTKILGN
murine HER-3 ECD ------A----------R----------Y----------
361 Domain L2 420
human HER-3 ECD LDFLITGLNGDPWHKIPALDPEKLNVFRTVREITGYLNIQSWPPHMHNFSVFSNLTTIGG
murine HER-3 ECD ----------
421 480
human HER-3 ECD RSLYNRGFSLLIMKNLNVTSLGFRSLKEISAGRIYISANRQLCYHHSLNWTKVLRGPTEE
murine HER-3 ECD ----------V-----Q----------RL----A--
481 ApaI Domain S2 540
human HER-3 ECD RLDIKHNRPRRDCVAEGKVCDPLCSSGGCWGPGPGQCLSCRNYSRGGVCVTHCNFLNGEP
murine HER-3 ECD -----Y---LGE----------E--------V-Q---
541 600
human HER-3 ECD REFAHEAECFSCHPECQPMEGTATCNGSGSDTCAQCAHFRDGPHCVSSCPHGVLGAKGPI
murine HER-3 ECD ---V---H----------S--------A--RC----------N-----I--------
601 632 BamHI
human HER-3 ECD YKYPDVQNECRPCHENCTQGCKGPELQDCLGQ(ggatcc)
murine HER-3 ECD -----A----------

FIG. 28



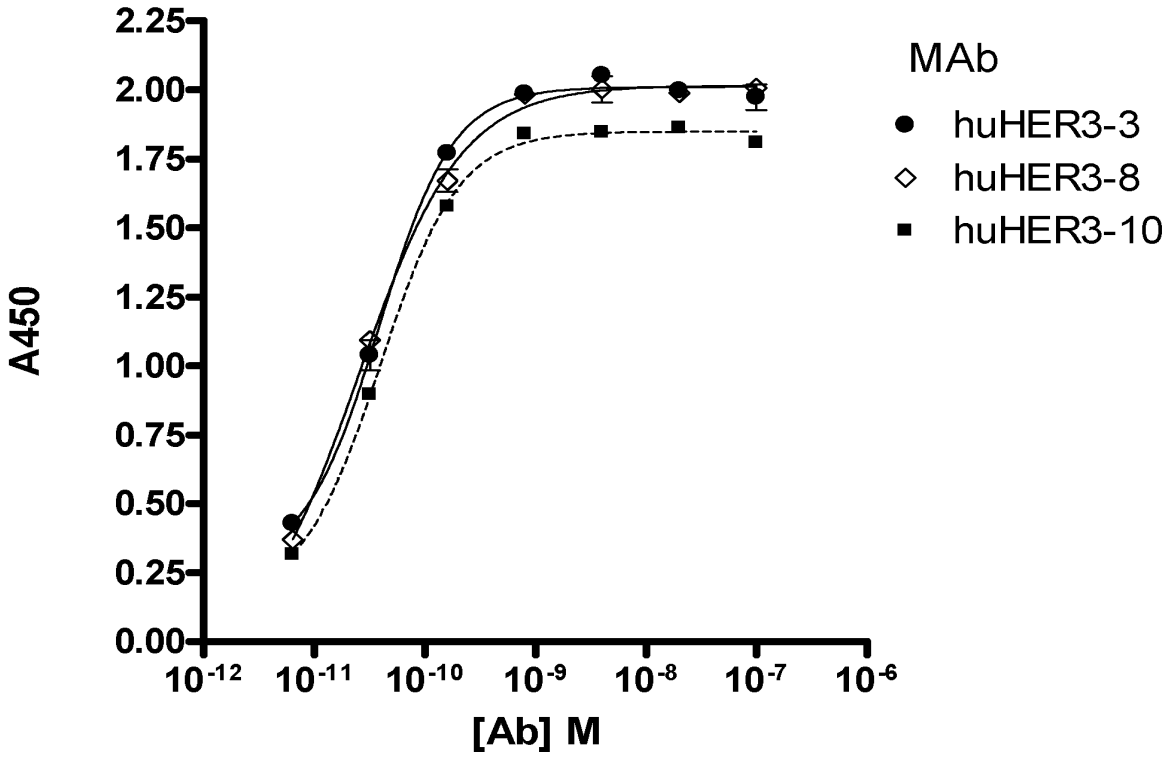
MAb	EC50 (M)
huHER3-3	5.16×10^{-11}
huHER3-8	4.94×10^{-11}
huHER3-10	8.93×10^{-11}

FIG. 29



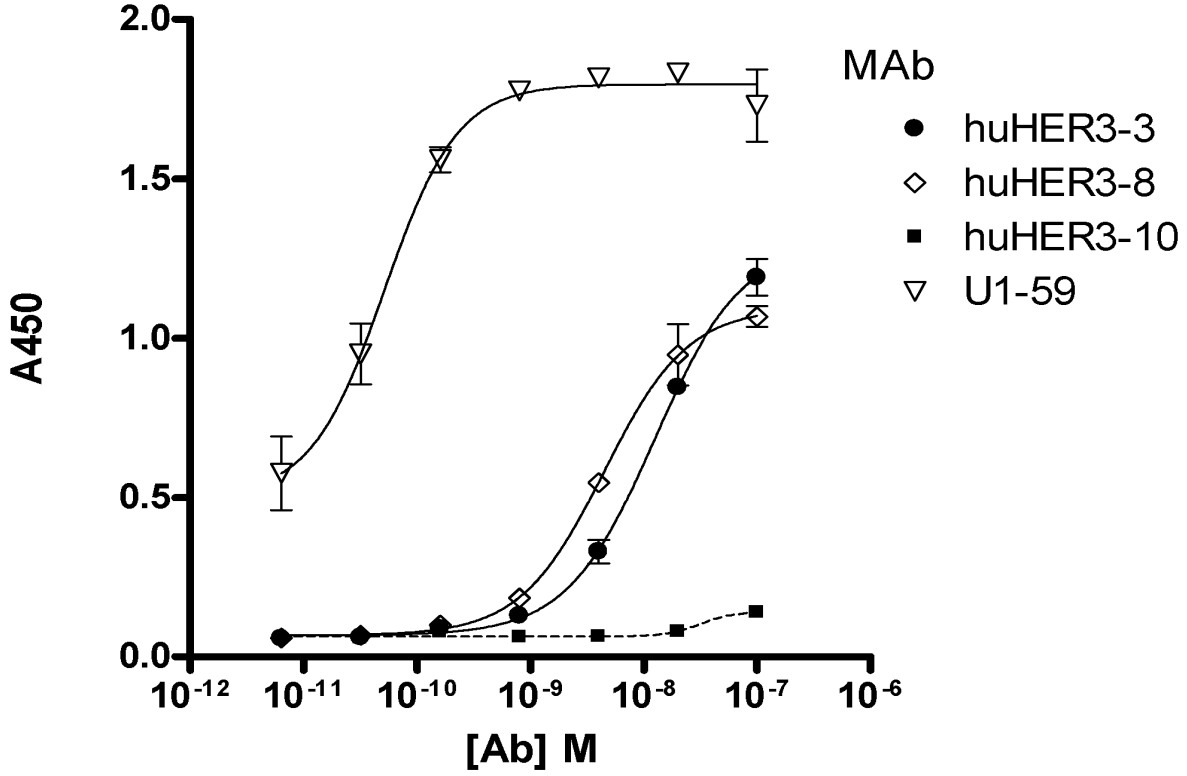
MAb	EC50 (M)
huHER3-3	3.63×10^{-8}
huHER3-8	1.78×10^{-8}
huHER3-10	1.14×10^{-7}
U1-59 Ab	5.14×10^{-11}

FIG. 30



MAb	EC50 (M)
huHER3-3	3.88×10^{-11}
huHER3-8	2.50×10^{-11}
huHER3-10	8.93×10^{-11}

FIG. 31



MAb	EC50 (M)
huHER3-3	3.63×10^{-8}
huHER3-8	1.78×10^{-8}
huHER3-10	1.14×10^{-7}
U1-59 Ab	5.14×10^{-11}

FIG. 32A

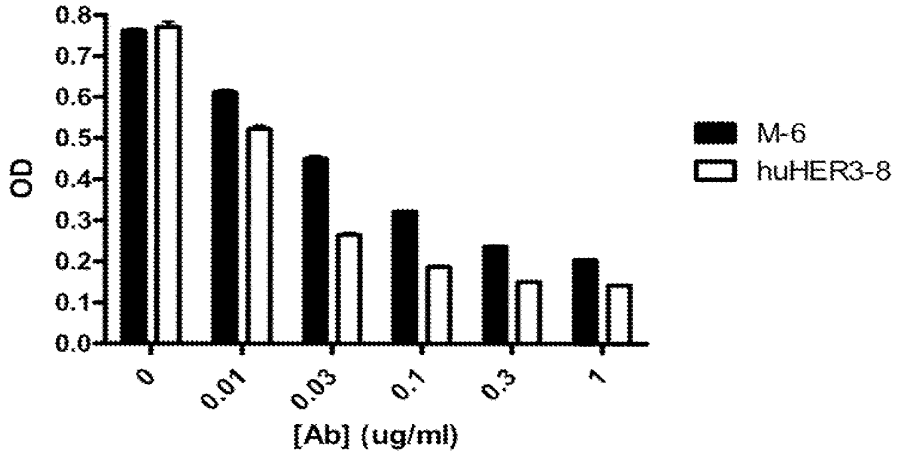


FIG. 32B

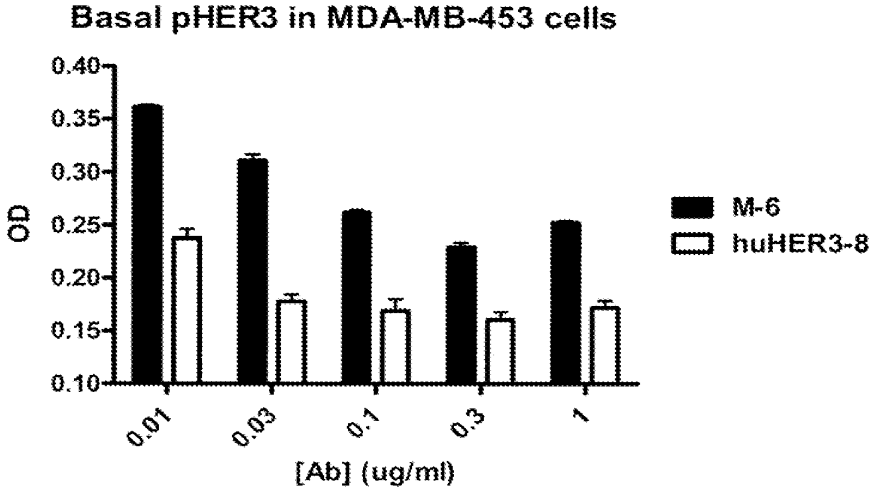
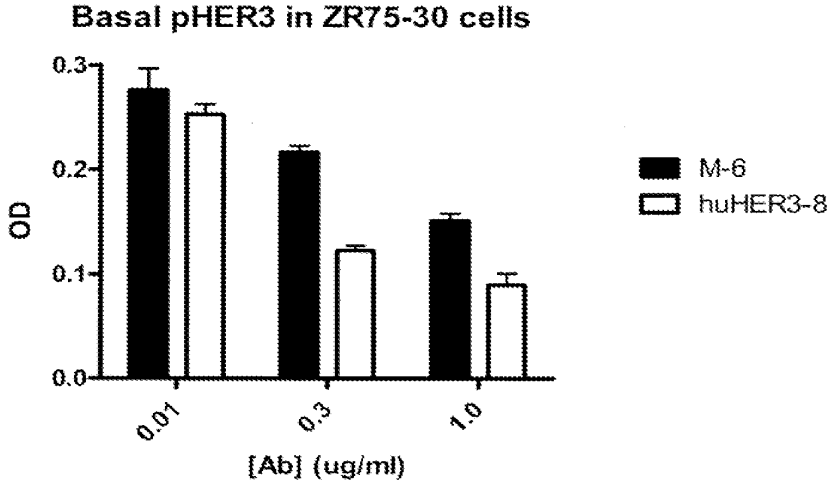


FIG. 32C



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(75) Inventor/Applicant *(for US only)*: **DIMASI, Nazzareno** [IT/US]; C/o Medimmune, LLC, One Medimmune Way, Gaithersburg, MD 20878 (US).

(74) Agents: **KASTENMAYER, James** et al.; Medimmune, LLC, One Medimmune Way, Gaithersburg, MD 20878 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

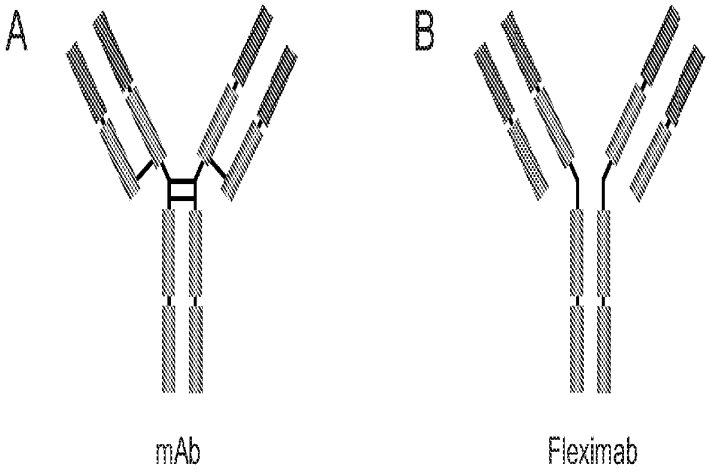
Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: ANTIBODY SCAFFOLD FOR HOMOGENOUS CONJUGATION

FIGURE 1



(57) Abstract: Provided in some embodiments are antibodies comprising a heavy chain having no native interchain cysteine amino acids, a light chain having no native interchain cysteine amino acids, and having no native interchain disulphide linkages between the heavy chain and the light chain. Also provided in certain embodiments are antibodies comprising a heavy chain having no native interchain cysteine amino acids, a light chain having no native interchain cysteine amino acids, and having no native interchain disulphide linkages between the heavy chain and the light chain where the native interchain cysteine amino acids have been replaced by amino acids having no thiol moiety.

WO 2012/064733 A2

OnCusp
Ex. 1002

ANTIBODY SCAFFOLD FOR HOMOGENOUS CONJUGATION

Sequence Listing

The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on November 7, 2011, is named MED0595P.txt and is 34,826 bytes in size.

Field

The technology relates in part to engineered antibodies and antibody conjugates. Such antibodies and conjugates can be utilized for diagnostic and therapeutic applications in some embodiments.

Background

Antibodies, which also are referred to as immunoglobulins (Ig) are proteins that naturally occur in blood or other bodily fluids of vertebrates. Antibodies are immune system agents that bind to and neutralize foreign objects, such as bacteria and viruses.

Naturally occurring antibodies generally include two larger heavy chains and two smaller light chains. In the case of native full-length antibodies, these chains join together to form a "Y-shaped" protein (e.g., FIG. 1A). Heavy chains and light chains include cysteine amino acids that can be joined to one another via disulfide linkages. Heavy chains are joined to one another in an antibody by disulfide linkages between cysteine amino acids in each chain. Light chains are joined to heavy chains also by disulfide linkages between cysteine amino acids in the chains. Such disulfide linkages generally are formed between thiol side chain moieties of the free cysteine amino acids. Three particular cysteine amino acids in each heavy chain and one particular cysteine in each light chain sometimes are referred to as "interchain cysteines" as they generally participate in disulfide linkages between antibody chains.

An antibody may be conjugated to another molecule, which can be useful for diagnostic applications (e.g., the conjugated molecule can be a detectable label) or therapeutic applications (e.g., the conjugated molecule can be a toxin). The molecule can be conjugated to the antibody by a linkage with a cysteine amino acid of the antibody in certain instances.

Summary

Provided herein, in some embodiments, is an antibody that includes a heavy chain having no native interchain cysteine amino acids, a light chain having no native interchain cysteine amino acids, and no native interchain disulphide linkages between the heavy chain and the light chain. In certain embodiments the antibody comprises no interchain disulphide linkages between the heavy chain and the light chain under conditions that native antibodies could form interchain disulphide linkages between the chains. In some embodiments, provided is an antibody, comprising a heavy chain having no interchain cysteine amino acids; a light chain having no interchain cysteine amino acids; and no interchain disulphide linkages between the heavy chain and the light chain.

In various embodiments the antibody comprises two heavy chains and two light chains. The antibody is sometimes a full-length antibody. In some embodiments the heavy chain is about 400 to about 500 amino acids in length (e.g., about 420, 440, 460, 480 amino acids; about 446 amino acids in length) and the light chain is about 200 to about 300 amino acids in length (e.g., about 220, 240, 260, 280 amino acids; about 214 amino acids in length). In certain embodiments the antibody is a human antibody. In various embodiments the antibody is a humanized antibody.

In some embodiments, provided is an antibody that comprises: (i) a IgG1, IgG2, IgG3 or IgG4 isotype heavy chain constant region lacking interchain cysteines, and (ii) a kappa or lambda light chain constant region lacking an interchain cysteine. In certain embodiments, interchain cysteine amino acids are substituted by a non-cysteine amino acid (e.g., an amino acid that does not contain a thiol moiety) in antibodies that lack interchain cysteines. In some embodiments, provided is a fragment of a full-length antibody that comprises (i) a portion of an IgG1, IgG2, IgG3 and IgG4 isotype heavy chain constant region lacking interchain cysteines, and optionally (ii) a kappa or lambda light chain constant region, or portion thereof, lacking an interchain cysteine. In certain embodiments, an antibody comprises an Fc region or fragment thereof. Examples of IgG1, IgG2, IgG3 or IgG4 isotype heavy chain constant region amino acid sequences are shown in SEQ ID NOs: 5, 6, 7 and 8, respectively, and examples of kappa and lambda light chain constant region amino acid sequences are shown in SEQ ID NOs: 9 and 10, respectively, hereafter.

>IgG1 (SEQ ID NO: 5)
ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSL
SSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPK

DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD
WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIA
VEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSL
SLSPGK

>IgG2 (SEQ ID NO: 6)
ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSL
SSVVTVPSSNFGTQTYTCNVDHKPSNTKVDKTVERKCCVECPPCPAPPVAGPSVFLFPPKPKDTL
MISRTPEVTCVVVDVSHEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTFRVVSVLTVVHQDWLN
GKEYKCKVSNKGLPAPIEKTISKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEW
ESNGQPENNYKTTPPMLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
GK

>IgG3 (SEQ ID NO: 7)

ASTKGPSVFPLAPCSRSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSL
SSVVTVPSSSLGTQTYTCNVNHKPSNTKVDKRVELKTPLGDTTHTCPRCPEPKSCDTPPPCPRCP
EPKSCDTPPPCPRCPEPKSCDTPPPCPRCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVD
VSHEDPEVQFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL
PAPIEKTISKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESSGQPENNYKTT
PPVLDSDGSFFLYSKLTVDKSRWQQGNIFSCSVMHEALHNHFTQKSLSLSPGK

>IgG4 (SEQ ID NO: 8)
ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSL
SSVVTVPSSSLGTKTYTCNVDHKPSNTKVDKRVESKYGPPCPSCPAPEFLGGPSVFLFPPKPKDT
LMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWL
NGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVE
WESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLS
LGK

>kappa (SEQ ID NO: 9)
VAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYS
LSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

>lambda (SEQ ID NO: 10)
KAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAAS
SYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS

Antibodies can be engineered to lack interchain cysteines in a variety of manners. In certain antibodies, one or more or all interchain cysteines independently can be substituted with an amino acid that is not cysteine. An amino acid substituted for an interchain cysteine often does not include a thiol moiety, and sometimes is a serine, threonine, valine, alanine, glycine, leucine or isoleucine, other naturally occurring amino acid, or non-naturally occurring amino acid. Interchain cysteines that replaced by another non-cysteine amino acid sometimes are replaced by the same amino acid or by one or more different amino acids. In some embodiments, one or more or all interchain cysteines can be deleted and not replaced by another amino acid.

The following Table 1 illustrates positions of interchain cysteines in the heavy chain constant region and light chain constant region of particular antibody isotypes with reference to Kabat EU numbering and with reference to the sequences disclosed herein. Each or the interchain cysteine positions present in an antibody or antibody fragment often are deleted or substituted with an amino acid that is not a cysteine.

Table 1: Native Interchain Cysteine Positions in Antibody Constant Regions

Antibody Isotype	Kabat EU/ SEQ ID NO	Position of Cysteine					
Heavy chain	Kabat EU position	131	220	N/A	N/A	226	229
IgG1	Corresponding position in SEQ ID NO: 5	N/A	103	N/A	N/A	109	112
IgG2	Corresponding position in SEQ ID NO: 6	14	103	N/A	N/A	106	109
IgG3	Corresponding position in SEQ ID NO: 7	14	N/A	110	113	118	121
IgG4	Corresponding position in SEQ ID NO: 8	14	N/A	N/A	N/A	106	109
Light chain							
Kappa	Kabat EU Position	214					
	Corresponding position in SEQ ID NO: 9	105					
Lambda	Kabat EU Position	213					
	Corresponding position in SEQ ID NO: 10	102					

The location of corresponding interchain cystein positions in the IgG isotypes may also be identified by reference to Figure 22.

Thus, in some embodiments, provided is an antibody comprising a heavy chain comprising the amino acid sequence of SEQ ID NO: 5, and a light chain comprising the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, where each of the cysteines at positions 103, 109, and 112 in SEQ ID NO: 5, and the cysteine at position 105 in SEQ ID NO: 9 or the cysteine at position 102 in SEQ ID NO: 10, are substituted by an amino acid that is not cysteine. The antibody often comprises no interchain cysteine amino acids and often comprises no interchain disulfide linkages.

Provided also in certain embodiments is an antibody comprising a heavy chain comprising the amino acid sequence of SEQ ID NO: 5, where each of the cysteines at positions 103, 109, and 112 in SEQ ID NO: 5 is substituted by an amino acid that is not cysteine, and where the antibody often comprises no interchain cysteine amino acids and often includes no interchain disulfide linkages.

Also provided in some embodiments is an antibody comprising a heavy chain fragment comprising a portion of the amino acid sequence of SEQ ID NO: 5, provided that should the fragment include an amino acid at position(s) 103, 109, and/or 112 in SEQ ID NO: 5, the amino acid at each of the positions is not a cysteine, and where the antibody often comprises no interchain cysteine amino acids and often includes no interchain disulfide linkages. In some embodiments, such antibodies comprise a light chain comprising the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, where the cysteine at position 105 in SEQ ID NO: 9 or the cysteine at position 102 in SEQ ID NO: 10 is substituted by an amino acid that is not cysteine. In certain embodiments, such antibodies comprise a light chain fragment comprising a portion of the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, provided that should the fragment include position 105 of SEQ ID NO: 9 or position 102 of SEQ ID NO: 10, the amino acid at that position is not a cysteine. In some embodiments, an antibody comprises an amino acid sequence comprising 80% or more amino acid sequence identity (e.g., about 85% or more, 86% or more, 87% or more, 88% or more, 89% or more, 90% or more, 91% or more, 92% or more, 93% or more, 94% or more, 95% or more, 96% or more, 97% or more, 98% or more, 99% or more sequence identity) to an antibody described in this paragraph or the foregoing paragraph, where the antibody often comprises no interchain cysteine amino acids and often comprises no interchain disulfide linkages.

In certain embodiments, provided is an antibody comprising a heavy chain comprising the amino acid sequence of SEQ ID NO: 6, and a light chain comprising the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, where each of the cysteines at positions 14, 103, 106, and 109 in SEQ ID NO: 6, and the cysteine at position 105 in SEQ ID NO: 9 or the cysteine at position 102 in SEQ ID NO: 10, are substituted by an amino acid that is not cysteine. The antibody often comprises no interchain cysteine amino acids and often comprises no interchain disulfide linkages.

Also provided in some embodiments is an antibody comprising a heavy chain comprising the amino acid sequence of SEQ ID NO: 6, where each of the cysteines at positions 14, 103, 106, and 109 in SEQ ID NO: 6 is substituted by an amino acid that is not cysteine, and where the antibody often comprises no interchain cysteine amino acids and often includes no interchain disulfide linkages. Provided also in certain embodiments is an antibody comprising a heavy chain fragment comprising a portion of the amino acid sequence of SEQ ID NO: 6, provided that should the fragment include an amino acid at position(s) 14, 103, 106, and/or 109 in SEQ ID NO: 6, the amino acid at each of the positions is not a cysteine, and where the antibody often comprises no interchain cysteine amino acids and often includes no interchain disulfide linkages. In some

embodiments, such antibodies comprise a light chain comprising the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, where the cysteine at position 105 in SEQ ID NO: 9 or the cysteine at position 102 in SEQ ID NO: 10 is substituted by an amino acid that is not cysteine. Such antibodies, in certain embodiments, can comprise a light chain fragment comprising a portion of the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, provided that should the fragment include position 105 of SEQ ID NO: 9 or position 102 of SEQ ID NO: 10, the amino acid at that position is not a cysteine. In some embodiments, an antibody comprises an amino acid sequence comprising 80% or more amino acid sequence identity (e.g., about 85% or more, 86% or more, 87% or more, 88% or more, 89% or more, 90% or more, 91% or more, 92% or more, 93% or more, 94% or more, 95% or more, 96% or more, 97% or more, 98% or more, 99% or more sequence identity) to an antibody described in this paragraph or the foregoing paragraph, where the antibody often comprises no interchain cysteine amino acids and often comprises no interchain disulfide linkages.

In some embodiments, provided is an antibody comprising a heavy chain comprising the amino acid sequence of SEQ ID NO: 7, and a light chain comprising the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, where each of the cysteines at positions 14, 110, 113, 118 and 121 in SEQ ID NO: 7, and the cysteine at position 105 in SEQ ID NO: 9 or the cysteine at position 102 in SEQ ID NO: 10, are substituted by an amino acid that is not cysteine. The antibody often comprises no interchain cysteine amino acids and often comprises no interchain disulfide linkages.

Also provided in certain embodiments is an antibody comprising a heavy chain comprising the amino acid sequence of SEQ ID NO: 7, where each of the cysteines at positions 14, 110, 113, 118 and 121 in SEQ ID NO: 7 is substituted by an amino acid that is not cysteine, and where the antibody often comprises no interchain cysteine amino acids and often includes no interchain disulfide linkages. In some embodiments, provided is an antibody comprising a heavy chain fragment comprising a portion of the amino acid sequence of SEQ ID NO: 7, provided that should the fragment include an amino acid at position(s) 14, 110, 113, 118 and/or 121 in SEQ ID NO: 7, the amino acid at each of the positions is not a cysteine, and where the antibody often comprises no interchain cysteine amino acids and often includes no interchain disulfide linkages. Such antibodies, in some embodiments, comprise a light chain comprising the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, where the cysteine at position 105 in SEQ ID NO: 9 or the cysteine at position 102 in SEQ ID NO: 10 is substituted by an amino acid that is not cysteine. Such antibodies, in certain embodiments, comprise a light chain fragment comprising a portion of

the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, provided that should the fragment include position 105 of SEQ ID NO: 9 or position 102 of SEQ ID NO: 10, the amino acid at that position is not a cysteine. In some embodiments, an antibody comprises an amino acid sequence comprising 80% or more amino acid sequence identity (e.g., about 85% or more, 86% or more, 87% or more, 88% or more, 89% or more, 90% or more, 91% or more, 92% or more, 93% or more, 94% or more, 95% or more, 96% or more, 97% or more, 98% or more, 99% or more sequence identity) to an antibody described in this paragraph or the foregoing paragraph, where the antibody often comprises no interchain cysteine amino acids and often comprises no interchain disulfide linkages.

In some embodiments, provided is an antibody comprising a heavy chain comprising the amino acid sequence of SEQ ID NO: 8, and a light chain comprising the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, where each of the cysteines at positions 14, 106 and 109 in SEQ ID NO: 8, and the cysteine at position 105 in SEQ ID NO: 9 or the cysteine at position 102 in SEQ ID NO: 10, are substituted by an amino acid that is not cysteine. The antibody often comprises no interchain cysteine amino acids and often comprises no interchain disulfide linkages.

Provided in certain embodiments is an antibody comprising a heavy chain comprising the amino acid sequence of SEQ ID NO: 8, where each of the cysteines at positions 14, 106 and 109 in SEQ ID NO: 8 is substituted by an amino acid that is not cysteine, and where the antibody often comprises no interchain cysteine amino acids and often comprises no interchain disulfide linkages. Provided also in some embodiments is an antibody comprising a heavy chain fragment comprising a portion of the amino acid sequence of SEQ ID NO: 8, provided that should the fragment include an amino acid at position(s) 14, 106 and/or 109 in SEQ ID NO: 8, the amino acid at each of the positions is not a cysteine, and where the antibody often comprises no interchain cysteine amino acids and often includes no interchain disulfide linkages. Such antibodies, in some embodiments, comprise a light chain comprising the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, where the cysteine at position 105 in SEQ ID NO: 9 or the cysteine at position 102 in SEQ ID NO: 10 is substituted by an amino acid that is not cysteine. Such antibodies, in certain embodiments, comprise a light chain fragment comprising a portion of the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, provided that should the fragment include position 105 of SEQ ID NO: 9 or position 102 of SEQ ID NO: 10, the amino acid at that position is not a cysteine. In some embodiments, an antibody comprises an amino acid sequence comprising 80% or more amino acid sequence identity (e.g., about 85% or more, 86% or more, 87% or more, 88% or more, 89% or

more, 90% or more, 91% or more, 92% or more, 93% or more, 94% or more, 95% or more, 96% or more, 97% or more, 98% or more, 99% or more sequence identity) to an antibody described in this paragraph or the foregoing paragraph, where the antibody often comprises no interchain cysteine amino acids and often comprises no interchain disulfide linkages.

In certain embodiments, the amino acid sequence of the light chain in an antibody lacking interchain cysteines is about 80% or more identical to the amino acid sequence of SEQ ID NO: 3 (e.g., about 85% or more, 86% or more, 87% or more, 88% or more, 89% or more, 90% or more, 91% or more, 92% or more, 93% or more, 94% or more, 95% or more, 96% or more, 97% or more, 98% or more, 99% or more or 100% identical to the amino acid sequence of SEQ ID NO: 3). In some embodiments, the amino acid sequence of the light chain is identical to the amino acid sequence in SEQ ID NO: 3 except that it includes 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acid modifications (e.g., substitutions, insertions and/or deletions) relative to the amino acid sequence in SEQ ID NO: 3. In some embodiments, the amino acid sequence of the heavy chain is about 80% or more identical to the amino acid sequence of SEQ ID NO: 4 (e.g., about 85% or more, 86% or more, 87% or more, 88% or more, 89% or more, 90% or more, 91% or more, 92% or more, 93% or more, 94% or more, 95% or more, 96% or more, 97% or more, 98% or more, 99% or more or 100% identical to the amino acid sequence of SEQ ID NO: 4). In some embodiments, the amino acid sequence of the heavy chain is identical to the amino acid sequence in SEQ ID NO: 4 except that it includes 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acid modifications (e.g., substitutions, insertions and/or deletions) relative to the amino acid sequence in SEQ ID NO: 4.

Provided also, in some embodiments, is an antibody lacking interchain cysteines that includes a glycosylation site not present in an antibody counterpart having a native interchain cysteine amino acid. In some embodiments, an antibody lacking native interchain cysteines lacks one or more glycosylation moieties present in a counterpart antibody having interchain cysteine moieties, and in some embodiments, an antibody is aglycosylated. In certain embodiments, an antibody lacking native interchain cysteine amino acids includes one or more engineered glycosylation sites (e.g., one or more non-native glycosylation sites can be engineered into an antibody herein).

In various embodiments, an antibody lacking native interchain cysteines comprises one or more cysteine replacements of non-cysteine surface amino acids in the CH1 domain, CH2 domain, or CH3 domain, or combination thereof, of the antibody, when the CH1 domain, CH2 domain and/or CH3 domain, or portion thereof, is present in the antibody. The antibody sometimes comprises

about 2 to about 40 of the cysteine replacements of the non-cysteine surface amino acids. In some embodiments the antibody comprises about 2 to about 40 free thiols. The antibody is a human antibody in certain embodiments.

The following Table 2 shows certain amino acids located in a loop of the CH1 domain of a heavy chain constant region of an antibody, one or more of which can be substituted by a cysteine amino acid in an antibody lacking native interchain cysteines.

Table 2: CH1 Loop Amino Acids

Antibody Isotype	Kabat EU Position and Corresponding Amino Acid									SEQ ID NO:
	131	132	133	134	135	136	137	138	139	
IgG1	Ser	Ser	Lys	Ser	Thr	Ser	Gly	Gly	Thr	5
IgG2	Cys	Ser	Arg	Ser	Thr	Ser	Glu	Ser	Thr	6
IgG3	Cys	Ser	Arg	Ser	Thr	Ser	Gly	Gly	Thr	7
IgG4	Cys	Ser	Arg	Ser	Thr	Ser	Glu	Ser	Thr	8

In some embodiments, 1, 2, 3, 4, 5, 6, 7, 8 or 9 of the amino acids shown in Table 2 independently are substituted by a cysteine amino acid. Thus, in some embodiments, one or more non-cysteine amino acids at positions 131, 132, 133, 134, 135, 136, 137, 138 and 139 in the loop shown in Table 2 independently may be substituted with a cysteine amino acid in an IgG1, IgG2, IgG3, or IgG4 heavy chain or fragment thereof. In certain embodiments, Ser 131 of an IgG1 molecule or fragment is substituted with cysteine in an IgG1 antibody or counterpart position in an IgG2, IgG3 or IgG4 antibody. The one or more cysteine replacements sometimes independently are at one or more of positions 135 and 139 of an IgG1 antibody or counterpart position in an IgG2, IgG3 or IgG4 antibody, or fragment of an antibody. In some embodiments, one or more non-cysteine amino acids at positions 131, 132, 134, 135, 136, and 139 in the loop shown in Table 2 independently may be substituted with a cysteine amino acid in an IgG1, IgG2, IgG3, or IgG4 heavy chain or fragment thereof.

The following Table 3 shows certain surface amino acids located in CH2 and CH3 domains of a heavy chain constant region of an antibody, one or more of which may be substituted by a cysteine amino acid in an antibody lacking native interchain cysteines.

Table 3: CH2 and CH3 Positions that may be Substituted with Cysteine

Antibody Isotype	Position (Kabat EU) and Corresponding Amino Acid																				SEQ ID NO:
	239	282	289	297	312	324	330	335	337	339	356	359	361	383	384	398	400	422	440	442	
IgG1	Ser	Val	Thr	Asn	Asp	Ser	Ala	Thr	Ser	Ala	Glu	Thr	Asn	Ser	Asn	Leu	Ser	Val	Ser	Ser	5
IgG2	Ser	Val	Thr	Asn	Asp	Ser	Ala	Thr	Ser	Thr	Glu	Thr	Asn	Ser	Asn	Leu	Ser	Val	Ser	Ser	6
IgG3	Ser	Val	Thr	Asn	Asp	Ser	Ala	Thr	Ser	Thr	Glu	Thr	Asn	Ser	Ser	Leu	Ser	Ile	Ser	Ser	7
IgG4	Ser	Val	Thr	Asn	Asp	Ser	Ser	Thr	Ser	Ala	Glu	Thr	Asn	Ser	Asn	Leu	Ser	Val	Ser	Ser	8

In some embodiments of an antibody lacking native interchain cysteines, one or more of the following positions independently may be replaced with a cysteine amino acid: 239, 282, 289, 297, 312, 324, 330, 335, 337, 339, 356, 359, 361, 383, 384, 398, 400, 422, 440, and 442 of an IgG1 antibody or counterpart position in an IgG2, IgG3 or IgG4 antibody. Thus, in some embodiments, one or more non-cysteine amino acids at positions 239, 282, 289, 297, 312, 324, 330, 335, 337, 339, 356, 359, 361, 383, 384, 398, 400, 422, 440, shown in Table 3 independently may be substituted with a cysteine amino acid in an IgG1, IgG2, IgG3 or IgG4 heavy chain or fragment thereof.

In certain embodiments, an antibody lacking interchain cysteines has a stability of about 70% or more compared to an antibody counterpart containing all native interchain cysteines (e.g., about 75% or more, 80% or more, 85% or more, 90% or more, 95% or more of the stability). In some embodiments the stability is in vitro stability, and sometimes, the stability is in serum at about 37 degrees Celsius for 5 days or more. The stability is determined by calorimetry in certain embodiments. In some embodiments, the stability is in vivo stability, and sometimes, the stability is in an animal for 14 days or more. In some embodiments, an antibody lacking native interchain cysteines can include a YTE modification, as described herein.

An antibody lacking interchain cysteines sometimes has a specific binding activity of about 70% or more compared to an antibody counterpart containing all native interchain cysteines (e.g., about 75% or more, 80% or more, 85% or more, 90% or more, 95% or more of the binding activity). In certain embodiments, the specific binding activity is in vitro. The specific binding activity sometimes is quantified by an in vitro homogeneous assay or an in vitro heterogeneous assay. In some embodiments the specific binding activity is in vivo, and sometimes, the specific binding activity is determined in situ.

Certain antibodies can inhibit cell proliferation activity. In certain embodiments, an antibody lacking interchain cysteines has a cell proliferation inhibition activity of about 70% or more compared to an antibody counterpart containing all native interchain cysteines (e.g., about 75% or more, 80% or more, 85% or more, 90% or more, 95% or more of the cell proliferation inhibition). In some embodiments, the cell proliferation inhibition activity is inhibition of cancer cell proliferation. In various embodiments, the activity is in vitro, and sometimes, the activity is in vivo.

In some embodiments, the antibody of any one of the foregoing embodiments is about 90% or more monomeric (e.g., about 95% or more monomeric). The antibody of may be 90% or more monomeric in vitro.

Provided also, in various embodiments, is the antibody of any one of the foregoing embodiments which does not bind detectably to a human leukocyte receptor. The human leukocyte receptor is sometimes a Fc gamma RIII receptor.

Also provided, in some embodiments, is the antibody of any one of the foregoing embodiments, which binds to a neonatal Fc receptor with about 80% or more of the binding affinity compared to an antibody counterpart containing all native interchain cysteines (e.g., about 85% or more, 90% or more, 95% or more of the binding affinity). An antibody as provided sometimes specifically binds to a cell surface molecule. In certain embodiments, the cell surface molecule is internalized in a cell. In some embodiments, the cell surface molecule is a cell surface receptor. In various embodiments, the cell surface receptor comprises a protein kinase domain. The cell surface receptor may be epidermal growth factor receptor (EGFR) protein tyrosine kinase. The cell surface receptor is sometimes a HER3 protein tyrosine kinase.

An antibody lacking native interchain cysteine amino acids may be conjugated to another molecule, in certain embodiments. In some embodiments, provided is an antibody conjugate in association with one or more heterologous molecules. In certain embodiments the antibody conjugate is about 80% or more of antibody products in a conjugation reaction product mixture (e.g., about 85% or more, 90% or more, 95% or more of the antibody products). In some embodiments the foregoing antibody comprises one or more cysteine replacements of non-cysteine surface amino acids in the CH1 domain, CH2 domain, or CH3 domain (to the extent such domains are present in the antibody), or combination thereof, of the antibody, where the one or more heterologous molecules are linked to the one or more cysteine replacements. In various embodiments the one or more heterologous molecules comprise a therapeutic agent. The therapeutic agent sometimes comprises a toxin. In some embodiments the one or more heterologous molecules comprise a diagnostic agent. In certain embodiments the diagnostic agent comprises an imaging agent, and sometimes, the diagnostic agent comprises a detectable label. In some embodiments, the one or more heterologous molecules are linked to the antibody via a linker.

In some embodiments, an antibody lacking interchain cysteines is part of an antibody homomultimer conjugate. Also provided, in certain embodiments, is the antibody comprising one or more cysteine replacements of non-cysteine surface amino acids in the CH1 domain, CH2 domain, or CH3 domain (to the extent such domains are present in the antibody), or combination thereof, of the antibody, where antibodies in the antibody homomultimer conjugate include a disulfide linkage between the one or more cysteine replacements.

Provided also, in some embodiments, is a nucleic acid comprising a nucleotide sequence that encodes an antibody herein. Also provided, in certain embodiments, is a cell comprising a nucleic acid that includes a nucleotide sequence encoding an antibody herein. In various embodiments, provided is an expression system that comprises a nucleic acid that includes a nucleotide sequence encoding an antibody herein. Also provided in certain embodiments is an organism that comprises a nucleic acid that includes a nucleotide sequence encoding an antibody herein. Provided also, in some embodiments, is an organism comprising an expression system that comprises a nucleic acid that includes a nucleotide sequence encoding an antibody herein.

Also provided, in certain embodiments, is a process that comprises expressing an antibody described herein in an expression system. In certain embodiments, such a method includes isolating the antibody, thereby producing an isolated antibody.

In some embodiments a process comprises conjugating an antibody described herein, often where the antibody is isolated, with a heterologous molecule, thereby preparing an antibody conjugate. In certain embodiments, provided is a process that comprises conjugating an antibody described herein, often where the antibody is in isolated form, with other isolated antibody, thereby preparing an antibody multimer.

Provided also, in some embodiments, is a method comprising contacting an antibody described herein with a biological sample, and detecting the presence, absence or amount of antibody specifically bound to a component in the biological sample. The method sometimes comprises linking the antibody to a solid support.

Also provided, in certain embodiments, is a method comprising administering an antibody described herein to cells and detecting the presence, absence or amount of antibody in a location

of the cells. In some embodiments, provided is a method that comprises administering an antibody herein to a subject and detecting the presence, absence or amount of antibody in a tissue of the subject. A method sometimes comprises administering an antibody herein to cells, and detecting the presence, absence or amount of a biological effect associated with the administration of the antibody to the cells. In various embodiments, provided is a method that comprises administering an antibody herein to a subject, and detecting the presence, absence or amount of a biological effect in the subject associated with the administration of the antibody. In certain embodiments, the biological effect is cell proliferation inhibition. Provided also, in some embodiments, is a method that comprises administering an antibody herein to a subject, and monitoring the condition of the subject.

Certain embodiments are described further in the following description, examples, claims and drawings.

Brief Description of the Drawings

The drawings illustrate embodiments of the technology and are not limiting. For clarity and ease of illustration, the drawings are not made to scale and, in some instances, various aspects may be shown exaggerated or enlarged to facilitate an understanding of particular embodiments. Antibodies lacking native interchain cysteines are referred to as "FlexiMab" herein, and particularly as "mAb-Val" when all of the native interchain cysteines are substituted by the amino acid valine.

Figure 1. Panel A is a schematic representation of a human antibody in its classic homodimeric format. In this format, the light and heavy chains are covalently linked by the formation of interchain disulphide bonds (one between the light and heavy chains, and two between the heavy chains at the hinge region). In addition to the interchain disulphide bonds, other non-covalent intermolecular interactions at the light and heavy chains and at the CH3 domains are necessary for the IgG homodimer formation and stabilization. Panel B shows a schematic representation of an antibody lacking interchain cysteines. The FlexiMab antibody is an antibody of the human IgG1 isotype with no interchain disulphide bonds. In mAb-Val, the cysteines at the light and heavy chain involved in the formation of the interchain disulphide bonds are mutated to Valine. mAb-Val does not form interchain disulphide bonds, but is able to assemble as a homodimer similar to a classic antibody, likely due to non-covalent intermolecular interactions at the light and heavy chains and at the CH3 domains.

Figure 2. Panel A is the amino acid sequence of a light chain kappa isotype of a classic anti-EGFR antibody format. The cysteine involved in the formation of the interchain disulphide bond with the heavy chain is represented in larger, bold and underlined text. Panel B is the amino acid sequence of a heavy chain gamma 1 isotype of a classic anti-EGFR antibody format. The cysteine involved in the formation of the interchain disulphide bond with the light chain, and the cysteines at the hinge region involved in the formation of the heavy interchain disulphide bonds are represented in larger, bold and underlined text. Panel C is the amino acid sequence of a light chain kappa isotype of a mAb-Val anti-EGFR antibody. The valine-to-cysteine substitution is represented in larger, bold and underlined text. Panel D is the amino acid sequence of a heavy chain gamma 1 isotype of a mAb-Val anti-EGFR antibody. The valine-to-cysteine substitutions are in larger, bold and underlined text.

Figure 3. This table compares the expression and the monomeric content after protein A purification and overnight dialysis in standard antibody formulation buffer of an anti-EGFR antibody in its classic format (mAb) and a FlexiMab antibody in the mAb-Val format (mAb-Val). These data show that mAb and mAb-Val have similar expression levels when transiently expressed in mammalian cells and have similar monomeric content after traditional protein A purification and after overnight dialysis in classic antibody formulation buffer.

Figure 4. This figure shows SDS-PAGE analysis in non-reducing and reducing condition for an anti-EGFR antibody in classic format (mAb) and a FlexiMab antibody in its mAb-Val format (mAb-Val). The mAb is a covalently linked antibody (in non-reducing condition run with the expected size of about 150 KDa); whereas the mAb-Val is a non-covalently linked homodimer and in non-reducing condition the heavy and light chain run as separate entities with the expected molecular weight (~ 50 KDa for the heavy chain and ~ 25 KDa for the light chain). In reducing conditions, both antibody formats run as independent chains with their predicted molecular weight sizes.

Figure 5. This experiment compares the ELISA binding to coated EGFR of an anti-EGFR antibody in its classic format (mAb, filled circle) and a FlexiMab in its mAb-Val format (mAb-Val, open square). As shown in this figure, mAb and mAb-Val have similar binding signal to EGFR.

Figure 6. This experiment compares the FACS binding to surface expressed EGFR on A431 cells, of an anti-EGFR antibody in its classic format (mAb) and a FlexiMab in its mAb-Val format (mAb-

Val). As shown in this figure, mAb and mAb-Val exhibit similar binding signals to cell-surface expressed EGFR (they have similar mean-fluorescence -intensity values -MFIR-).

Figure 7. This experiment compares ELISA binding to coated EGFR of an anti-EGFR antibody in its classic format (mAb, filled circle) and a FlexiMab in its mAb-Val format (mAb-Val, open square) after 5 days incubation at 37 ºC in PBS. As shown in this figure, mAb and mAb-Val have similar binding signal to EGFR after 5 days incubation at 37 ºC in PBS. This experiment suggests that mAb-Val is about as stable as mAb.

Figure 8. This experiment compares ELISA binding to coated EGFR of an anti-EGFR antibody in its classic format (mAb, filled circle) and a FlexiMab in its mAb-Val format (mAb-Val, open square) after 5 days incubation at 37 ºC in human serum. As shown in this figure, mAb and mAb-Val have similar binding signal to EGFR after 5 days incubation at 37 ºC in human serum. This experiment suggest that mAb-Val is about as stable as mAb in an ex vivo experimental set up. The ELISA was carried out with 5 % final human serum concentration.

Figure 9. This table compares kinetic parameters (K_{on}, K_{off} and K_d) of anti-EGFR (mAb format) and anti-EGFR (a FlexiMab in mAb-Val format) for EGFR using a BIAcore assay. mAb and mAb-Val have similar binding affinity (in the pM range) for EGFR. EGFR was immobilized on the BIAcore surface chip.

Figure 10. This experiment compares the inhibition of cancer cell survival (A431, BxPC3 and H358) of an anti-EGFR antibody in its classic format (mAb, open circle) and in a FlexiMab in its mAb-Val format (mAb-Val, open square). As a negative control a non-binding mAb is used (filled circle). As shown in this figure, mAb and mAb-Val exhibit similar potency in inhibiting the survival of human cancer cells.

Figure 11. This experiment compares the rate and efficiency of internalization and therefore receptor degradation with subsequent cell killing of an anti-Her3 antibody in the classic mAb format (mAb) and in a FlexiMab in its mAb-Val format. In this experiment, cell killing is due to Saporin conjugated to an anti-human IgG1. SKBr3 cells expressing Her3 were used in this experiment. As shown in this experiment, anti-Her3 (mAb) and anti-Her3 (mAb-Val) have similar killing potency of SKBr3 cells. These results suggests that the mAb and mAb-Val have similar internalization

kinetics. This assay was carried out by pre-incubating the two antibodies (mAb and mAb-Val) with an anti-human-IgG-Saporin-conjugated).

Figure 12. This experiment compares the steady-state affinities (K_D) of anti-EGFR (mAb format) and anti-EGFR (FlexiMab in its mAb-Val format) for Fc-gamma-RIIIa (variant 158V and 158F) and for FcRn. The mAb-Val antibody exhibited no detectable binding to Fc-gamma-RIII. However, the mAb and mAb-Val exhibited similar binding affinity for FcRn. These results indicate that ADCC or CDC function is negatively impacted in the mAb-Val variant. The mAb and antibodies lacking interchain cysteines, however, are expected to have similar in vivo half-lives.

Figure 13. This experiment compares the PK of anti-EGFR (mAb format, at 1 mg/kg filled circle and dashed line, and at 10 mg/kg filled square dashed line) and anti-EGFR (mAb-Val format, at 1 mg/kg filled triangle upside straight line, and at 0 mg/kg filled triangle downside straight line) in mice. mAb and mAb-Val have similar in vivo half-lifeves at both high dose (10 mg/kg) and low dose (1 mg/kg).

Figure 14. This experiment compares the transition temperatures (raw data baseline subtracted) analyzed using differential scanning calorimetry for an anti-EGFR antibody in the mAb format (straight-line thermogram) and for FlexiMab in the mAb-Val format (dashed-line thermogram). This experiment shows the presence of two transition peaks for both antibody formats: one peak with a transition temperature of about 82 °C (similar for both antibody formats), and another peak with a transition temperature of 69 °C for the mAb and 65 °C for the mAb-Val. These results indicate that (as expected) the mAb-Val is slightly less stable than the mAb.

Figure 15. This size-exclusion chromatography experiment shows that an anti-EGFR antibody in the FlexiMab mAb-Val format is mostly monomeric (~ 98 %) after protein A purification at 11 mg/ml in 25 mM Histidine-HCl pH 6.

Figure 16. This experiment compares in vivo efficacy (left panel) and body weight (right panel) for an anti-EGFR antibody in classic format (mAb, filled circle) and in FlexiMab in its mAb-Val format (open square). Negative controls include untreated animals (filled rhomb) and animals treated with a mAb isotype control (filled square). The animals were dosed 2 times per week for the entire duration of the experiment. The efficacy study (left panel) shows that the mAb and mAb-Val exhibit similar in vivo efficacy. In addition (right panel), mAb and mAb-Val have similar toxicity (i.e., there

is not a significant difference in total body weight lost for animal treated with mAb and mAb-Val), which compare with control vehicles. The tumor model used in this study is A431 (human epidermoid carcinomas).

Figure 17. This figure shows a schematic ribbon representation of mAb-Val (right panel) with the Valine-to-Cysteine substitutions indicated with filled dots (right panel). The Ser-131 to Thr-139 CH1 loop containing the Cysteine-substitutions is shown in black (right panel). The left panel is a zoom view of the Ser-131 to Thr-139 CH1 loop that shows the structural location of the Ser-131, Ser-132, Ser-134, Thr-135, Ser-136 and Thr-139 (EU numbering) positions. See also, Table 2.

Figure 18. This table shows transient expression in mammalian cells after 7 days post-transfection and the monomeric content after protein A purification in PBS for selected mAb-Val Cysteine mutants. These mutants have been designed to be used for site-specific conjugation. The mutants are single (Ser131Cys, Ser132Cys, Ser134Cys, Thr135Cys, Ser136Cys andThr139Cys), double (Ser131Cys-Thr139Cys) and triple mutants (Ser131Cys-Thr135Cys-Thr139Cys). This combination of mutations can allow the site-specific conjugation of two (single mutant), four (double mutants) and six (triple mutants) druglike entities per antibody. As shown in the table, all mutants have expression comparable to mAb (compare with values reported in Figure 3) and are ~ 96 % monomeric after protein A purification and overnight dialysis in PBS. Antibody preparation for site-specific conjugation, site-specific conjugations and analyses were carried out as described in International Application Publication No. WO 2009/092011 entitled “Cysteine engineered antibodies for site-specific conjugation."

Figure 19. This figure shows show the peptide mapping results of anti-EGFR mAb-Val Cysteine-engineered constructs that have been conjugated using Maleimide-PEG2-Biotin as described in U.S. Patent Application Publication No. 2009092011. The figure shows the mAb-Val technology can afford a nearly homogeneous site-specific drug conjugation for two, four and six drug-like entities per antibody.

Figure 20. This figure shows the amino acid sequence at the hinge region for a mAb and a FlexiMab in its mAb-Val format (SEQ ID NOS 20 and 21, respectively). The Valine and Cysteine position are underlined. In mAb-Val the Threonine shown with the arrow is O-glycosylated, as determined experimentally by intact mass and peptide mapping on an anti-EGFR mAb-Val

construct. This hinge O-glycosylation could make the hinge region in mAb-Val less susceptible to in vivo protease cleavage.

Figure 21. This figure shows constant region amino acid sequences for IgG1, IgG2, IgG3 and IgG4 antibody isotypes and interchain cysteine amino acids that are modified in FlexiMab antibodies described herein. The figure also designates CH1, CH2, CH3 and hinge regions in the heavy chain constant region. Amino acid sequences for IgG1, IgG2, IgG3 and IgG4 heavy chain constant regions, and kappa and lambda light chain constant regions, are designated by SEQ ID NOs: 5, 6, 7, 8, 9 and 10, respectively, and are reproduced herein.

Detailed Description

Provided herein, as more fully discussed below, are engineered antibodies in which all of the naturally occurring interchain cysteine amino acids are substituted with a non-thiol amino acid. Such antibodies are referred to as "antibodies lacking interchain cysteines." Monoclonal antibodies in which native interchain cysteines are substituted are referred to as "FlexiMab" and antibodies in which native interchain cysteines are substituted by the amino acid valine are referred to herein as "mAb-Val." Also provided herein, as more fully discussed below, are antibodies lacking interchain cysteines in which selected intra-chain amino acids are substituted with cysteine.

Antibodies are large, complex and structurally diverse biomolecules, often with many reactive functional groups. Their reactivities with linker reagents and druglinker intermediates are dependent on factors such as pH, concentration, salt concentration, and co-solvents. Furthermore, the multistep conjugation process may be non-reproducible due to difficulties in controlling the reaction conditions and characterizing reactants and intermediates.

Methods are known for attaching a heterologous molecule to an antibody. The molecule sometimes is a detectable label and sometimes is a drug. Conventional means of attaching, i.e. linking through covalent bonds, a heterologous moiety to an antibody generally leads to a heterogeneous mixture of molecules where the heterologous moieties are attached at a number of sites on the antibody. For example, cytotoxic drugs have typically been conjugated to antibodies through the often-numerous lysine or cysteine residues of an antibody, generating a heterogeneous antibody-drug conjugate mixture.

Depending on reaction conditions, the mixture resulting from a conjugation reaction typically contains a distribution of antibodies with from 0 to about 8, or more, attached heterologous moieties. In addition, within each subgroup of conjugates with a particular integer ratio of drug moieties to antibody, is a potentially heterogeneous mixture where the heterologous moiety is attached at various sites on the antibody. Antibodies described herein, which lack all naturally occurring interchain cysteine amino acids, reduce the number of sites to which the heterologous moiety can bind, thereby reducing the heterogeneity of the product mixture. Reducing heterogeneity among reaction products can advantageously result in a more homogeneous and useable product for diagnostic and therapeutic applications. More homogeneity can lead to more certainty in the structure of a therapeutic or diagnostic agent.

Cysteine thiols are reactive at neutral pH, unlike most amines which are protonated and less nucleophilic near pH 7. Since free thiol (R-SH, sulfhydryl) groups are relatively reactive, proteins with cysteine residues often exist in their oxidized form as disulfide-linked oligomers or have internally bridged disulfide groups. The amount of free thiol in a protein may be estimated by the standard Ellman's assay. IgM is an example of a disulfide-linked pentamer, while IgG is an example of a protein with internal disulfide bridges bonding the subunits together. In proteins such as this, reduction of the disulfide bonds with a reagent such as dithiothreitol (DTT) or selenol is required to generate the reactive free thiol. This approach may result in loss of antibody tertiary structure and antigen binding specificity. In certain embodiments, an antibody lacking interchain cysteine amino acids can include one or more engineered cysteines, the latter of which can be conjugated to a heterologous moiety.

Terminology

Methods provided herein often are not limited to specific compositions or process steps, as such may vary. Also, as used herein, the singular form “a”, “an” and “the” include plural referents unless the context clearly dictates otherwise.

Amino acids often are referred to herein by commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, often are referred to by commonly accepted single-letter codes.

The numbering of amino acids in the variable domain, complementarity determining region (CDRs) and framework regions (FR), of an antibody follow, unless otherwise indicated, the Kabat definition as set forth in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991). Using this numbering system, the actual linear amino acid sequence may contain fewer or additional amino acids corresponding to a shortening of, or insertion into, a FR or CDR of the variable domain. For example, a heavy chain variable domain may include a single amino acid insertion (residue 52a according to Kabat) after residue 52 of H2 and inserted residues (e.g. residues 82a, 82b, and 82c, etc according to Kabat) after heavy chain FR residue 82. The Kabat numbering of residues may be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence. Maximal alignment of framework residues frequently requires the insertion of “spacer” residues in the numbering system, to be used for the Fv region. In addition, the identity of certain individual residues at any given Kabat site number may vary from antibody chain to antibody chain due to interspecies or allelic divergence.

Antibodies

Antibodies are immunological proteins that bind a specific antigen. In most mammals, including humans and mice, antibodies are constructed from paired heavy and light polypeptide chains. Each chain is made up of two distinct regions, referred to as the variable (Fv) and constant (Fc) regions. The light and heavy chain Fv regions contain the antigen binding determinants of the molecule and are responsible for binding the target antigen. The Fc regions define the class (or isotype) of antibody (IgG for example) and are responsible for binding a number of natural proteins to elicit important biochemical events.

Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies between the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced interchain disulfide bridges. The two light chain-heavy chain dimers are linked via disulfide bridges between the heavy chains, forming a Y-shaped molecule. The region in which the arms of the Y meet the stem is called the hinge region, and exhibits some flexibility.

Each chain includes constant regions that are representative of the antibody class and variable regions specific to each antibody. The constant region determines the mechanism used to destroy

antigen. Antibodies are divided into five major classes, IgM, IgG, IgA, IgD, and IgE, based on their constant region structure and immune function. The variable and constant regions of both the light and the heavy chains are structurally folded into functional units called domains. Each light chain consists of one variable domain (VL) at one end and one constant domain (CL) at its other end. Each heavy chain has at one end a variable domain (VH) followed by three or four constant domains (CH1, CH2, CH3, CH4).

The arms of the Y contain the site that bind antigen and is called the Fab (fragment, antigen binding) region. It is composed of one constant and one variable domain from each heavy and light chain of the antibody. The constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Light chains are classified as lambda chains or kappa chains based on the amino acid sequence of the light chain constant region. The variable domain of a kappa light chain may also be denoted herein as VK.

The Fc region of an antibody interacts with a number of ligands including Fc receptors and other ligands, imparting an array of important functional capabilities referred to as effector functions. An important family of Fc receptors for the IgG class are the Fc gamma receptors (FcγRs). These receptors mediate communication between antibodies and the cellular arm of the immune system In humans this protein family includes FcγRI (CID64), including isoforms FcγRIA, FcγRIB, and FcγRIC; FcγRII (CD32), including isoforms FcγRIIA, FcγRIIB, and FcγRIIC; and FcγRIII (CD16), including isoforms FcγRIIIA and FcγRIIB. These receptors typically have an extracellular domain that mediates binding to Fc, a membrane spanning region, and an intracellular domain that may mediate some signaling event within the cell. These different FcγR subtypes are expressed on different cell types. For example, in humans, FcγRIIIB is found only on neutrophils, whereas FcγRIIIA is found on macrophages, monocytes, natural killer (NK) cells, and a subpopulation of T-cells.

Formation of the Fc/FcγR complex recruits effector cells to sites of bound antigen, typically resulting in signaling events within the cells and important subsequent immune responses such as release of inflammation mediators, B cell activation, endocytosis, phagocytosis, and cytotoxic attack. The ability to mediate cytotoxic and phagocytic effector functions is a potential mechanism by which antibodies destroy targeted cells. The cell mediated reaction where nonspecific cytotoxic cells that express FcγRs recognize bound antibody on a target cell and subsequently cause lysis of

the target cell is referred to as antibody dependent cell-mediated cytotoxicity (ADCC). The primary cells for mediating ADCC, NK cells, express only FcγRIIIA, whereas monocytes express FcγRI, FcγRII and FcγRIII.

Another important Fc ligand is the complement protein C1q. Fc binding to C1q mediates a process called complement dependent cytotoxicity (CDC). C1q is capable of binding six antibodies, although binding to two IgGs is sufficient to activate the complement cascade. C1q forms a complex with the C1r and C1s serine proteases to form the C1 complex of the complement pathway.

Several key features of antibodies including but not limited to, specificity for target, ability to mediate immune effector mechanisms, and long half-life in serum, make antibodies and related immunoglobulin molecules powerful therapeutics. There are a number of possible mechanisms by which antibodies destroy tumor cells, including anti-proliferation via blockage of needed growth pathways, intracellular signaling leading to apoptosis, enhanced down regulation and/or turnover of receptors, ADCC, CDC, and promotion of an adaptive immune response.

As used herein, the terms “antibody” and “antibodies”, also known as immunoglobulins, encompass monoclonal antibodies (including full-length monoclonal antibodies), polyclonal antibodies, multispecific antibodies formed from at least two different epitope binding fragments (e.g., bispecific antibodies), human antibodies, humanized antibodies, camelised antibodies, chimeric antibodies, single-chain Fvs (scFv), single-chain antibodies, single domain antibodies, domain antibodies. Fragments of antibodies include Fab fragments, F(ab')2 fragments, antibody fragments that exhibit the desired biological activity (e.g. the antigen binding portion), disulfide-linked Fvs (dsFv), and anti-idiotypic (anti-Id) antibodies (including, e.g., anti-Id antibodies to antibodies herein provided), intrabodies, and epitope-binding fragments of any of the above. In particular, antibodies include immunoglobulin molecules and immunologically active fragments of immunoglobulin molecules, i.e., molecules that contain at least one antigen-binding site. Immunoglobulin molecules can be of any isotype (e.g., IgG, IgE, IgM, IgD, IgA and IgY), subisotype (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or allotype (e.g., Gm, e.g., G1m(f, z, a or x), G2m(n), G3m(g, b, or c), Am, Em, and Km(1, 2 or 3)). Antibodies may be derived from any mammal, including, but not limited to, humans, monkeys, pigs, horses, rabbits, dogs, cats, mice, and the like, or other animals such as birds (e.g. chickens). Where a particular antibody or

antibody fragment lacks the residues that are substituted in a FexiMab (e.g., a scFV), that antibody or antibody fragment may be fused to an Fc or other portion of an antibody in the FexiMab format.

Antibodies provided herein include full length or intact antibody, antibody fragments, native sequence antibody or amino acid variants, human, humanized, post-translationally modified, chimeric or fusion antibodies, immunoconjugates, and functional fragments thereof. The antibodies can be modified in the Fc region, and certain modifications can provide desired effector functions or serum half-life. As discussed in more detail in the sections below, with the appropriate Fc regions, a naked antibody bound on the cell surface can induce cytotoxicity, e.g., via antibody-dependent cellular cytotoxicity (ADCC) or by recruiting complement in complement dependent cytotoxicity (CDC), or by recruiting nonspecific cytotoxic cells that express one or more effector ligands that recognize bound antibody on a target cell and subsequently cause phagocytosis of the target cell in antibody dependent cell-mediated phagocytosis (ADCP), or some other mechanism. Where it is desirable to eliminate or reduce effector function, so as to minimize side effects or therapeutic complications, certain other Fc regions may be used. The Fc region of antibodies can be modified to increase the binding affinity for FcRn and thus increase serum half-life. Alternatively, the Fc region can be conjugated to PEG or albumin to increase the serum half-life, or some other conjugation that results in a desired effect.

In certain embodiments, an antibody herein is isolated and/or purified and/or pyrogen free antibodies. The term “purified” as used herein, refers to a molecule of interest that has been identified and separated and/or recovered from a component of its natural environment. Thus, in some embodiments, an antibody provided is a purified antibody where it has been separated from one or more components of its natural environment. The term "isolated antibody" as used herein refers to an antibody which is substantially free of other antibody molecules having different structure or antigenic specificities. A bi- or multi-specific antibody molecule is an isolated antibody when substantially free of other antibody molecules. Thus, in some embodiments, antibodies provided are isolated antibodies which have been separated from antibodies with a different specificity. An isolated antibody may be a monoclonal antibody. An isolated antibody that specifically binds to an epitope, isoform or variant of a target may, however, have cross-reactivity to other related antigens, e.g., from other species (e.g., species homologs). An isolated antibody as provided may be substantially free of one or more other cellular materials. In some embodiments, a combination of "isolated" monoclonal antibodies is provided, and pertains to

antibodies having different specificities and combined in a defined composition. Methods of production and purification/isolation of an antibody are described elsewhere herein.

Isolated antibodies presented comprise antibody amino acid sequences disclosed herein, which can be encoded by any suitable polynucleotide. Isolated antibodies sometimes are provided in formulated form. In some embodiments, an antibody binds to a protein and, thereby partially or substantially alters at least one biological activity of the protein, for example, cellular proliferation activity.

Humanized antibodies

A humanized antibody is an antibody or its variant or fragment thereof which is capable of binding to a predetermined antigen and which comprises a framework region having substantially the amino acid sequence of a human immunoglobulin and a CDR having substantially the amino acid sequence of a non-human immunoglobulin. A humanized antibody comprises substantially all of at least one, and typically two, variable domains in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin (i.e., donor antibody) and all or substantially all of the framework regions are those of a human immunoglobulin consensus sequence. A humanized antibody may also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. The antibody may contain both the light chain as well as at least the variable domain of a heavy chain. The antibody also may include the CH1, hinge, CH2, CH3, and CH4 regions of the heavy chain.

The humanized antibody can be selected from any class of immunoglobulins, including IgM, IgG, IgD, IgA and IgE, and any isotype, including IgG1, IgG2, IgG3 and IgG4. Usually the constant domain is a complement fixing constant domain where it is desired that the humanized antibody exhibit cytotoxic activity, and the class is typically IgG1. Where such cytotoxic activity is not desirable, the constant domain may be of the IgG2 class. The humanized antibody may comprise sequences from more than one class or isotype, and selecting particular constant domains to optimize desired effector functions is within the ordinary skill in the art. The framework and CDR regions of a humanized antibody need not correspond precisely to the parental sequences, e.g., the donor CDR or the consensus framework may be mutagenized by substitution, insertion or deletion of at least one residue so that the CDR or framework residue at that site does not correspond to either the consensus or the import antibody. Such

mutations, however, may not be extensive. At least 75% of the humanized antibody residues may correspond to those of the parental framework region (FR) and CDR sequences, with the correspondence sometimes being 90% or greater or 95% or greater, for example.

Humanization can be essentially performed following methods known in the art, by substituting hypervariable region sequences for the corresponding sequences of a human antibody. Specifically, humanized antibodies may be prepared by methods known in the art including CDR grafting approaches, veneering or resurfacing, chain shuffling strategies, molecular modeling strategies, and the like. These general approaches may be combined with standard mutagenesis and recombinant synthesis techniques to produce antibodies herein with desired properties.

CDR grafting is performed by replacing one or more CDRs of an acceptor antibody (e.g., a human antibody) with one or more CDRs of a donor antibody (e.g., a non-human antibody). Acceptor antibodies may be selected based on similarity of framework residues between a candidate acceptor antibody and a donor antibody and may be further modified to introduce similar residues. Following CDR grafting, additional changes may be made in the donor and/or acceptor sequences to optimize antibody binding and functionality.

Grafting of abbreviated CDR regions is a related approach. Abbreviated CDR regions include the specificity-determining residues and adjacent amino acids, including those at positions 27d-34, 50-55 and 89-96 in the light chain, and at positions 31-35b, 50-58, and 95-101 in the heavy chain. Grafting of specificity-determining residues (SDRs) is premised on the understanding that the binding specificity and affinity of an antibody combining site is determined by the most highly variable residues within each of the CDR regions. Analysis of the three-dimensional structures of antibody-antigen complexes, combined with analysis of the available amino acid sequence data was used to model sequence variability based on structural dissimilarity of amino acid residues that occur at each position within the CDR. Minimally immunogenic polypeptide sequences consisting of contact residues, which are referred to as SDRs, are identified and grafted onto human framework regions.

Veneering or resurfacing is based on the concept of reducing potentially immunogenic amino acid sequences in a rodent or other non-human antibody by resurfacing the solvent accessible exterior of the antibody with human amino acid sequences. Thus, veneered antibodies appear less foreign to human cells. A non-human antibody is veneered by (1) identifying exposed exterior framework

region residues in the non-human antibody, which are different from those at the same positions in framework regions of a human antibody, and (2) replacing the identified residues with amino acids that typically occupy these same positions in human antibodies.

By definition, humanized antibodies are chimeric antibodies. Chimeric antibodies are antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while another portion of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity. Chimeric antibodies of interest herein include "primatized" antibodies comprising variable domain antigen-binding sequences derived from a nonhuman primate (e.g., Old World Monkey, such as baboon, rhesus or cynomolgus monkey) and human constant region sequences.

Provided herein are engineered antibodies that can be utilized to generate anti-idiotype antibodies using techniques known in the art. Also provided herein are methods employing the use of polynucleotides comprising a nucleotide sequence encoding an antibody herein or a fragment thereof. Additionally, various methods are known in the art for obtaining physiologically active molecules whose half-lives are modified either by introducing an FcRn-binding polypeptide into the molecules or by fusing the molecules with antibodies whose FcRn-binding affinities are preserved but affinities for other Fc receptors have been greatly reduced or fusing with FcRn binding domains of antibodies. Specific techniques and methods of increasing half-life of physiologically active molecules are additionally known in the art. Specifically, it is contemplated that the antibodies herein comprise an Fc region comprising amino acid residue mutations (as numbered by the EU index in Kabat): M252Y/S254T/T256E or H433K/N434F/Y436H.

Human antibodies

For some uses, including in vivo use of antibodies in humans and in vitro detection assays, it may be appropriate to use human or chimeric antibodies. Completely human antibodies may be desirable for therapeutic treatment of human subjects. Human antibodies can be made by a variety of methods known in the art including phage display methods described below using antibody libraries derived from human immunoglobulin sequences.

Human antibodies can also be produced using transgenic mice which are incapable of expressing functional endogenous immunoglobulins, but which can express human immunoglobulin genes. For example, the human heavy and light chain immunoglobulin gene complexes may be introduced randomly or by homologous recombination into mouse embryonic stem cells. Alternatively, the human variable region, constant region, and diversity region may be introduced into mouse embryonic stem cells in addition to the human heavy and light chain genes. The mouse heavy and light chain immunoglobulin genes may be rendered non-functional separately or simultaneously with the introduction of human immunoglobulin loci by homologous recombination. In particular, homozygous deletion of the JH region prevents endogenous antibody production. The modified embryonic stem cells are expanded and microinjected into blastocysts to produce chimeric mice. The chimeric mice are then bred to produce homozygous offspring which express human antibodies. The transgenic mice are immunized in the normal fashion with a selected antigen, e.g., all or a portion of a polypeptide of an antibody herein.

Monoclonal antibodies directed against the antigen can be obtained from the immunized, transgenic mice using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies. In addition, companies such as Medarex (Princeton, NJ) provide human antibodies directed against a selected antigen.

Chimeric antibodies comprising one or more CDRs from a non-human species and framework regions from a human immunoglobulin molecule can be produced using a variety of techniques known in the art including, for example, CDR-grafting veneering or resurfacing.

Framework residues in the framework regions may be substituted with the corresponding residue from the CDR donor antibody to alter, and potentially improve, antigen binding. These framework substitutions are identified by methods known in the art, e.g., by modeling of the interactions of the CDR and framework residues to identify framework residues important for antigen binding and sequence comparison to identify unusual framework residues at particular positions.
Also known in the art is a “minilocus” approach. In the minilocus approach, an exogenous Ig locus is mimicked through the inclusion of pieces (individual genes) from the Ig locus. Thus, one or more VH genes, one or more DH genes, one or more JH genes, a mu constant region, and usually a second constant region (sometimes a gamma constant region) are formed into a construct for insertion into an animal.

The generation of human antibodies from mice in which, through microcell fusion, large pieces of chromosomes, or entire chromosomes, have been introduced, is also known in the art. For example, cross-breeding of Kirin's Tc mice with Medarex's minilocus (Humab) mice has generated mice possessing the human IgH transchromosome of the Kirin mice and the kappa chain transgene of the Genpharm mice.

Human antibodies can also be derived by in vitro methods. Suitable examples include but are not limited to phage display (MedImmune (formerly CAT), Morphosys, Dyax, Biosite/Medarex, Xoma, Symphogen, Alexion (formerly Proliferon), Affimed) ribosome display (MedImmune (formerly CAT)), yeast display, and the like. The phage display technology can be used to produce human antibodies and antibody fragments in vitro, from immunoglobulin variable (V) domain gene repertoires from unimmunized donors. According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, such as M13 or fd, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus, the phage mimics some of the properties of the B-cell. Phage display can be performed in a variety of formats as known in the art. A diverse array of anti-oxazolone antibodies has been isolated from a small random combinatorial library of V genes derived from the spleens of immunized mice. A repertoire of V genes from unimmunized human donors can be constructed and antibodies to a diverse array of antigens (including self-antigens) can be isolated essentially following the techniques known in the art. Human antibodies may also be generated by in vitro activated B cells.

Multivalent antibodies

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Thus, the antibodies herein presented can be multivalent antibodies (which are other than of the IgM class) with three or more antigen binding sites (e.g. tetravalent antibodies), which can be readily produced by recombinant expression of nucleic acid encoding the polypeptide chains of the antibody. The multivalent antibody can comprise a dimerization domain and three or more antigen binding sites. In an embodiment a dimerization domain comprises (or consists of) an Fc region or a hinge region. In this scenario, the antibody may comprise an Fc region and three or more antigen binding sites amino-terminal to the Fc region. In certain embodiments the multivalent antibody herein comprises (or consists of) three to about eight antigen binding sites. The multivalent antibody comprises at least one polypeptide chain where the polypeptide chain(s) comprise two or more variable domains. For instance, the polypeptide chain(s) may comprise VD1-(X1)n-VD2-(X2)n-Fc, where VD1 is a first variable domain, VD2 is a second variable domain, Fc is one polypeptide chain of an Fc region, X1 and X2 represent an amino acid or polypeptide, and n is 0 or 1. For instance, the polypeptide chain(s) may comprise: VH-CH1-flexible linker-VH-CH1-Fc region chain; or VH-CH1-VH-CH1-Fc region chain. The multivalent antibody herein may further comprise at least two light chain variable domain polypeptides. The multivalent antibody herein may, for instance, comprise from about two to about eight light chain variable domain polypeptides. The light chain variable domain polypeptides contemplated here comprise a light chain variable domain and, optionally, further comprise a CL domain.

Bi-specific antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of a target protein, or may bind two polypeptides. Other such antibodies may combine a site with a binding site for another protein. An bi-specific antibody arm may sometimes be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (e.g. CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16), so as to focus and localize cellular defense mechanisms to the target protein-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express the antigen. Such antibodies may possess a target -binding arm and an arm which binds the cytotoxic agent (e.g.

saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. F(ab′)2 bispecific antibodies). Methods for making bispecific antibodies are known in the art.

Traditional production of full length bispecific antibodies is based on the co-expression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low.

In another approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. A fusion sometimes is with an Ig heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. The first heavy-chain constant region (CH1) containing the site necessary for light chain bonding may be present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host cell. This provides for greater flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yield of the desired bispecific antibody. It is, however, possible to insert the coding sequences for two or all three polypeptide chains into a single expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios have no significant affect on the yield of the desired chain combination.

In some embodiments of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. This asymmetric structure may facilitate the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation.

The interface between a pair of antibody molecules may be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. An appropriate

interface comprises at least a part of the CH3 domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory “cavities” of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or “heteroconjugate” antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are known in the art.

Bispecific antibodies may be generated from antibody fragments. For example, bispecific antibodies can be prepared using chemical linkage. In one procedure intact antibodies are proteolytically cleaved to generate F(ab′)2 fragments. These fragments are reduced in the presence of the dithiol complexing agent, sodium arsenite, to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab′ fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab′-TNB derivatives is then reconverted to the Fab′-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab′-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Fab′-SH fragments can be directly recovered from E. coli, which can be chemically coupled to form bispecific antibodies. A fully humanized bispecific antibody F(ab′)2 molecule may be created by secreting each Fab′ fragment separately from E. coli and subjecting to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed is able to bind to cells over expressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Bispecific antibodies may also be produced using leucine zippers. The leucine zipper peptides from the Fos and Jun proteins are linked to the Fab′ portions of two different antibodies by gene fusion. The antibody homodimers are reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The “diabody” technology described has provided an additional mechanism

for making bispecific antibody fragments. The fragments comprise a VH connected to a VL by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the VH and VL domains of one fragment are forced to pair with the complementary VL and VH domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers is also known in the art.

Antibody receptors

Cells may display specialized antigens called antibody receptors to which an antibody specifically binds as discussed above. Some receptors are configured to be internalized into the cell. An internalizing receptor may carry a bound antibody and conjugated molecules with it. This property makes antibodies potentially valuable for therapy, since pathogenic cells, including cancer cells, may display unique antigens. Cellular receptors may display functional regions, including protein kinase.

The kinase family is one of the largest target families in the human genome. It is estimated that the human genome includes more than 500 members of the major classes of protein serine/threonine, tyrosine, and dual specificity kinases. Protein phosphorylation is a significant signal transduction mechanisms by which intercellular signals regulate crucial intracellular processes such as ion transport, cellular proliferation, and hormone responses. Growth factor receptors EGFR and HER3 and protein tyrosine kinases. The protein kinase family's key function in signal transduction for all organisms makes it an attractive target class for therapeutic interventions in a number of disease states such as cancer, diabetes, inflammation, and arthritis.

Epidermal growth factor (EGF)-enhanced protein kinase activity in plasma membrane preparations of A-431 human epidermoid carcinoma cells has been shown to involve the phosphorylation of tyrosine residues. The epidermal growth factor receptor (EGFR, ErbB-1, HER1, HER3 in humans) is the cell-surface receptor for members of the EGF-family of extracellular protein ligands. The epidermal growth factor receptor is a member of the ErbB family of receptors, a subfamily of four closely related receptor tyrosine kinases: EGFR (ErbB-1), HER2/c-neu (ErbB-2), Her 3 (ErbB-3) and Her 4 (ErbB-4). All four members of the human epidermal growth factor (EGF) receptor (HER) family are implicated in human cancers.

Provided herein, in some embodiments, are antibodies lacking interchain cysteines that bind to EGFR and HER3. Also provided, in certain embodiments, are antibody lacking interchain cysteines conjugates that bind to EGFR and HER3. The antibody conjugates are sometimes internalized.

Antibody function

Antibodies can effect several functions, such as antigen binding and inducing an immune response, for example.

Antigen binding

The term "antigen" as used herein refers to a molecule that causes an immune response when introduced into an organism and that is capable of binding with specific antibodies. Antibody-antigen binding is mediated by the sum of many weak interactions between the antigen and antibody including, for example, hydrogen bonds, van der Waals forces, and ionic and/or hydrophobic interactions.

An antigen binds to the complementarity regions on an antibody. The corresponding region(s) of the antigen is referred to as an antigenic determinant. Most antigens have multiple determinants; if 2 or more are identical, the antigen is multivalent.

The affinity of an antibody reflects the fit between the antigenic determinant and the antibody binding site and is independent of the number of binding sites. The avidity of the binding reflects the overall stability of the antibody-antigen complex. Avidity is defined as the total binding strength of all binding sites. Thus, both affinity of the antibody for its antigen and the valencies of both the antibody and the antigen influence avidity. Engagement of both, rather than only one, multivalent binding sites may strengthen binding by a factor of as much as 10,000 in a typical IgG molecule.

The multivalent nature of many antibodies and antigens may give rise to secondary reactions such as precipitation, cell clumping, and complement fixation in an organism. Such reactions can be useful in techniques such as western blotting, ELISA, immunoprecipitation, and the like.

Immune function

Antibodies bind and inactivate pathogens, can stimulate removal of pathogens by macrophages and other cells by coating the pathogen, and trigger destruction of pathogens by stimulating other immune responses such as the complement pathway. Antibodies activate the complement pathway by binding to surface antigens on, for example, a bacterium or cancer cell. The Fc region of the antibody then interacts with the complement cascade. The binding of the antibody and of complement cascade molecules attracts phagocytes and marks the microbe or cell for ingestion. Complement system components may form a membrane attack complex to assist antibodies to kill the bacterium or cell directly.

Antibody binding may cause pathogens to agglutinate. Antibody coated pathogens stimulate effector functions in cells that recognize the antibody Fc region. The effector function ultimately results in destruction of the invading microbe or pathogenic cell, e.g. phagocytes will phagocytose, mast cells and neutrophils will degranulate, natural killer cells will release cytokines and cytotoxic molecules.

Transformed tumor cells express abnormal antigens from several sources, including oncogenic viruses, abnormally high levels of the organism's own proteins, and cancer inducing oncogenes. Tumor antigens are presented on major histo-compatiblity (MHC) class I molecules in a manner similar to viral antigens. Antigens activate killer T-Cells and also generate antibodies that trigger the complement system.

An antibody herein may bind to a tumor or other pathogenic cell antigen and trigger cell destruction through an antibody function. In certain embodiments, an antibody herein may be conjugated to a therapeutic molecule, including a diagnostic molecule or toxin, and carry the conjugated molecule to selected site by means of antibody-antigen affinity.

Epitopes

The term "epitope" as used herein refers to a protein determinant capable of binding to an antibody. Epitopes generally include chemically active surface groupings of molecules such as amino acids and/or sugar side chains and generally have specific three dimensional structural

characteristics, as well as specific chemical characteristics (e.g., charge, polarity, basic, acidic, hydrophobicity and the like). Conformational and non-conformational epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents.

In certain embodiments, an epitope is comprised of at least one extracellular, soluble, hydrophilic, external or cytoplasmic portion of a target protein. A specified epitope can comprise any combination of at least one amino acid sequence from of at least 3 amino acid residues to the entire specified portion of contiguous amino acids of the target protein. In some embodiments, the epitope is at least 4 amino acid residues, at least 5 amino acid residues, at least 6 amino acid residues, at least 7 amino acid residues, at least 8 amino acid residues or at least 9 amino acid residues to the entire specified portion of contiguous amino acids of the target protein.

An antibody herein may immunospecifically bind to one or more epitopes specific to the target protein, peptide, subunit, fragment, portion or any combination thereof and generally does not specifically bind to other polypeptides. An epitope may comprise at least one antibody binding region that comprises at least one portion of the target protein.

Antibody fragments

In certain embodiments, the present antibodies are antibody fragments or antibodies comprising these fragments. The antibody fragment comprises a portion of the full length antibody, which generally is the antigen binding or variable region thereof. Examples of antibody fragments include Fab, Fab', F(ab')2, Fd and Fv fragments. Diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies are antibodies formed from these antibody fragments.

Traditionally, these fragments were derived via proteolytic digestion of intact antibodies using techniques known in the art. However, these fragments can now be produced directly by recombinant host cells. Fab, Fv and scFv antibody fragments can all be expressed in and secreted from E. coli, thus allowing the facile production of large amounts of these fragments. In an embodiment, the antibody fragments can be isolated from the antibody phage libraries discussed elsewhere herein. Fab'-SH fragments can also be directly recovered from E. coli and chemically coupled to form F(ab')2 fragments. F(ab')2 fragments can also be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments are known in the art. In various embodiments, the antibody of choice is a single-chain Fv fragment

(scFv). In certain embodiments, the antibody is not a Fab fragment. Fv and scFv are the only species with intact combining sites that are devoid of constant regions; thus, they are suitable for reduced nonspecific binding during in vivo use. scFv fusion proteins may be constructed to yield fusion of an effector protein at either the amino or the carboxy terminus of an scFv.

In certain embodiments, the present antibodies are domain antibodies, e.g., antibodies containing the small functional binding units of antibodies, corresponding to the variable regions of the heavy (VH) or light (VL) chains of human antibodies. Examples of domain antibodies include, but are not limited to, those available from Domantis that are specific to therapeutic targets. Commercially available libraries of domain antibodies can be used to identify antigen domain antibodies. In certain embodiments, antibodies herein comprise a functional binding unit and an Fc gamma receptor functional binding unit.

In certain embodiments, the present antibodies are vaccibodies. Vaccibodies are dimeric polypeptides. Each monomer of a vaccibody consists of a scFv with specificity for a surface molecule on APC connected through a hinge region and a Cγ3 domain to a second scFv. In some embodiments, vaccibodies containing as one of the scFv's an antibody lacking interchain cysteines fragment may be used to juxtapose those cells to be destroyed and an effector cell that mediates ADCC.

In certain embodiments herein, the present antibodies are linear antibodies. Linear antibodies comprise a pair of tandem Fd segments (VH-CH1-VH-CH1) which form a pair of antigen-binding regions. Linear antibodies may be bispecific or monospecific.

Antibody synthesis

An antibody lacking interchain cysteines may be produced by any method known in the art for the synthesis of antibodies, in particular, by chemical synthesis or by recombinant expression techniques.

Any antigen can be used to synthesize an antibody. Examples of antigens, or "targets," are described herein. Cells expressing the desired antigen at their cell surface or membranes prepared from such cells can also be used to generate antibodies. Antibodies herein can be produced recombinantly in an isolated from in bacterial or eukaryotic cells using standard

recombinant DNA methodology. Antigen can be expressed as a tagged (e.g., epitope tag) or other fusion protein to facilitate isolation as well as identification in various assays. Antibodies or binding proteins that bind to various tags and fusion sequences are available as elaborated below. Other forms of antigens useful for generating antibodies herein will be apparent to those skilled in the art.

Various tag polypeptides and their respective antibodies are known in the art. Examples include: poly-histidine (poly-his) or poly-histidine-glycine (poly-his-gly) tags; the flu HA tag polypeptide and its antibody 12CA5; the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody. The FLAG-peptide is recognized by an anti-FLAG M2 monoclonal antibody. Purification of a protein containing the FLAG peptide can be performed by immunoaffinity chromatography using an affinity matrix comprising the anti-FLAG M2 monoclonal antibody covalently attached to agarose. Other tag polypeptides include the KT3 epitope peptide, an α-tubulin epitope peptide, and the T7 gene 10 protein peptide tag.

Polyclonal antibodies, which bind to more than one epitope on an antigen, are raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen (especially when synthetic peptides are used) to a protein that is immunogenic in the species to be immunized. For example, the antigen can be conjugated to keyhole limpet hemocyanin (KLH), serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor, using a bifunctional or derivatizing agent (reactive group), e.g., activated ester (conjugation through cysteine or lysine residues), glutaraldehyde, succinic anhydride, SOCl2, or R1N=C=NR, where R and R1 are different alkyl groups. Conjugates also can be made in recombinant cell culture as fusion proteins.

Polyclonal antibodies to an antigen-of-interest can be produced by various procedures known in the art. For example, an antigenic polypeptide or immunogenic fragment thereof can be administered to various host animals including, but not limited to, rabbits, mice, rats, etc. to induce the production of sera containing polyclonal antibodies specific for the antigen. Various adjuvants may be used to increase the immunological response, depending on the host species, and include but are not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants

such as BCG (bacille Calmette-Guerin) and Corynebacterium parvum. Such adjuvants are also known in the art.

Animals may be immunized against the antigen, immunogenic conjugates, or derivatives by combining an appropriate concentration of antigen or conjugate with adjuvant and injecting the solution at multiple sites. One month later, the animals are boosted with 1/5 to 1/10 the original amount of antigen or conjugate in adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later, the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. In addition, aggregating agents such as alum are suitably used to enhance the immune response.

Monoclonal antibodies are highly specific, being directed against a single antigenic site or multiple antigenic sites in the case of multispecific engineered antibodies. Furthermore, in contrast to polyclonal antibody preparations which include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against the same determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they may be synthesized uncontaminated by other antibodies.

Monoclonal antibodies can be prepared using a wide variety of techniques known in the art including the use of hybridoma, recombinant, and phage display technologies, or a combination thereof. For example, monoclonal antibodies can be produced using hybridoma techniques including those known in the art. The term “monoclonal antibody” as used herein is not limited to antibodies produced through hybridoma technology. The term “monoclonal antibody” refers to an antibody that is derived from a single clone, including any eukaryotic, prokaryotic, or phage clone, and not the method by which it is produced. In some embodiments, an antibody herein is 90% or more monoclonal. Following is a description of representative methods for producing monoclonal antibodies which is not intended to be limiting and may be used to produce, for example, monoclonal mammalian, chimeric, humanized, human, domain, diabodies, vaccibodies, linear and multispecific antibodies.

Methods for producing and screening for specific antibodies using hybridoma technology are known in the art. Briefly, mice can be immunized with a target antigen (either the full length protein or a domain thereof, e.g., the extracellular domain or the ligand binding domain) and once an immune response is detected, e.g., antibodies specific for the target antigen are detected in the

mouse serum, the mouse spleen is harvested and splenocytes isolated. The splenocytes are then fused by known techniques to any suitable myeloma cells, for example cells from cell line SP20 available from the ATCC. Hybridomas are selected and cloned by limited dilution. Hybridoma clones are then assayed by methods known in the art for cells that secrete antibodies capable of binding a polypeptide of an antibody herein. Ascites fluid, which generally contains high levels of antibodies, can be generated by immunizing mice with positive hybridoma clones.
Accordingly, monoclonal antibodies can be generated by culturing a hybridoma cell secreting an antibody herein, where the hybridoma is generated by fusing splenocytes isolated from a mouse immunized with a target antigen with myeloma cells and then screening the hybridomas resulting from the fusion for hybridoma clones that secrete an antibody able to bind to a specific target antigen.

Additionally, lymphocytes may be immunized in vitro. After immunization, lymphocytes are isolated and then fused with a myeloma cell line using a suitable fusing agent or fusion partner, such as polyethylene glycol, to form a hybridoma cell. In certain embodiments, the selected myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a selective medium that selects against the unfused parental cells. In one aspect, the myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 and derivatives e.g., X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines for the production of human monoclonal antibodies are also known in the art.

Once hybridoma cells that produce antibodies of the desired specificity, affinity, and/or activity are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods. Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal, e.g, by i.p. injection of the cells into mice.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional antibody purification procedures such as, for example, affinity chromatography (e.g., using protein A or protein G-Sepharose) or ion-exchange

chromatography, affinity tags, hydroxylapatite chromatography, gel electrophoresis, dialysis, etc. Exemplary purification methods are described in more detail below.

Antibody fragments which recognize specific target antigen epitopes may be generated by any technique known to those of skill in the art. For example, Fab and F(ab')2 fragments herein may be produced by proteolytic cleavage of immunoglobulin molecules, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')2 fragments). F(ab')2 fragments contain the variable region, the light chain constant region and the CH1 domain of the heavy chain. Further, the antibodies herein can also be generated using various phage display methods known in the art and more fully discussed below, including using antibody libraries derived from human immunoglobulin sequences.

Antibody mutation

Antibody mutation techniques are known in the art. In order to create a humanized antibody, for example, a vector for the production of a chimeric anti-murine antibodies lacking interchain cysteines may be constructed. Based, for example, on the sequence of anti-murine mAb, overlapping oligonucleotides encoding variable heavy chain and variable light chain domains (about 69-75 bases in length) may be synthesized and purified. The variable heavy and light domains may be synthesized separately by combining 25 pmol of each of the overlapping oligonucleotides with Pfu DNA polymerase (Stratagene) in a 50 .mu.l PCR reaction consisting of 5 cycles of: denaturing at 94.degree. C. for 20 sec, annealing at 50.degree. C. for 30 sec, ramping to 72.degree. C. over 1 min, and maintaining at 72.degree. C. for 30 sec. Subsequently, the annealing temperature can be increased to 55.degree. C. for 25 cycles. A reverse primer and a biotinylated forward primer may be used to further amplify 1 .mu.l of the fusion product in a 100 .mu.l PCR reaction using the same program. The products may be purified by agarose gel electrophoresis, electroeluted, and phosphorylated by T4 polynucleotide kinase (Boehringer Mannheim) and incubated with streptavidin magnetic beads (Boehringer Mannheim) in 5 mM Tris-Cl, pH 7.5, 0.5 mM EDTA, 1 M NaCl, and 0.05% Tween 20 for 15 min at 25.degree. C. The beads may be washed and the non-biotinylated, minus strand DNA eluted by incubating with 0.15 M NaOH at 25.degree. C. for 10 min. Chimeric anti-murine Fab may be synthesized, for example, in a modified M131X10^4 phage vector, termed M131X10^{4}CS, by hybridization mutagenesis using the variable heavy chain and variable light chain oligonucleotides in 3-fold molar excess of the uridinylated vector template. The M131X10^4 vector may be modified by replacing cysteine residues

at the end of the kappa and .gamma.1 constant regions with serine. The reaction may be electroporated into DH10B cells and titered onto a lawn of XL-1 Blue.

The anti-murine mAb variable region framework sequences are sometimes used to identify the most homologous human germline sequences. The heavy chain and light chain framework residues may be, for example, about 70% or more identical to the corresponding human germline sequences.

Specific residues may be substituted using site directed mutagenesis. PCR primer oligonucleotides may be designed to incorporate nucleotide changes to the coding sequence of the subject antibody that result in substitution of, for example, a cysteine residue for an amino acid at a specific position within the protein. A cysteine substitution mutation may be constructed by designing a primer to change, for example, the codon ACT for threonine at amino acid residue 135 to a TGT codon encoding cysteine. PCR may be performed in a 50 µl reaction in 1.times.PCR buffer (Perkin-Elmer buffer containing 1.5 mM MgCl.sub.2), 200 micromolar concentration of each of the four nucleotides dA, dC, dG and dT, with each oligonucleotide primer present at 0.5 .mu.M, 5 pg of template and 1.25 units of Amplitac DNA Polymerase (Perkin-Elmer) and 0.125 units of PFU DNA Polymerase (Stratagene). Reactions may be performed, for example, in a Robocycler Gradient 96 thermal cycler (Stratagene). The program may entailed: 95 deg C for 3 minutes followed by 25 cycles of 95 deg C for 60 seconds, 45 deg C. or 50 deg C or 55 deg C for 75 seconds, 72 deg C for 60 seconds followed by a hold at 6 deg C. The PCR reactions may be analyzed by agarose gel electrophoresis to identify annealing temperatures that gave significant product of the expected size. The 45-deg C reaction may be "cleaned up" using the QIAquick PCR Purification Kit (Qiagen), digested with appropriate enzymes. The resulting fragment, including the putative T135C mutation, may be gel-purified and ligated into an appropriate plasmid.

Cysteine substituted antibodies

Antibodies herein include, in some embodiments, no interchain cysteines (e.g., they are replaced by non-thiol amino acids), certain amino acids substituted by cysteine, and combinations thereof.

Cysteines substituted by non-thiol amino acids

Interchain cysteines are replaced by non-thiol amino acids in some embodiments. In certain embodiments, interchain cysteine amino acids at positions shown in Table 1 independently are removed or substituted with an amino acid that is not cysteine. Such amino acids include, in some embodiments, naturally occurring and non-classical amino acid that lack a thiol moiety. Naturally occurring amino acids lacking a thiol moiety include glycine, alanine, valine, leucine, isoleucine, proline, serine, threonine, methionine, histidine, lysine, arginine, glutamate, aspartate, glutamine, asparagine, phenylalanine, tyrosine and tryptophan. Examples of non-classical amino acids include ornithine, diaminobutyric acid, norleucine, pyrylalanine, thienylalanine, naphthylalanine and phenylglycine. Other examples of non-classical amino acids are alpha and alpha-disubstituted amino acids, N-alkyl amino acids, lactic acid*, halide derivatives of natural amino acids such as trifluorotyrosine*, p-X-phenylalanine (where X is a halide such as F, Cl, Br, or I)*, allylglycine*, 7-aminoheptanoic acid*, methionine sulfone*, norleucine*, norvaline*, p-nitrophenylalanine*, hydroxyproline#, thioproline*, methyl derivatives of phenylalanine (Phe) such as 4-methyl-Phe*, pentamethyl-Phe*, Phe (4-amino)#, Tyr (methyl)*, Phe (4-isopropyl)*, Tic (1,2,3,4-tetrahydroisoquinoline-3-carboxyl acid)*, diaminopropionic acid, Phe (4-benzyl)*, 4-aminobutyric acid (gamma-Abu)*, 2-aminobutyric acid (alpha-Abu)*, 6-aminohexanoic acid (epsilon-Ahx)*, 2-aminoisobutyric acid (Aib)*, 3-aminopropionic acid*, norvaline*, hydroxyproline, sarcosine, citrulline, homocitrulline, cysteic acid, t-butylglycine*, t-butylalanine*, phenylglycine*, cyclohexylalanine*, fluoroamino acids, designer amino acids such as beta-methyl amino acids, and the like. The notation * indicates a derivative having hydrophobic characteristics and # indicates a derivative having hydrophilic characteristics.

In certain embodiments, 1 to 8 interchain cysteines are replaced by valine (e.g., 2, 3, 4, 5, 6, 7 cysteines are replaced by valine. In some embodiments, amino acids at positions shown in Table 1 all are replaced by valine.

Antibody amino acids substituted by cysteine

In some embodiments, an antibody includes no native interchain cysteines and includes one or more non-cysteine amino acids substituted by cysteine. In some embodiments, 1, 2, 3, 4, 5, 6, 7, 8 or more amino acids of positions in the CH1 domain show in Table 2 are substituted with a

cysteine amino acid, where the numbering system of the constant region is that of the EU index as set forth in Kabat et al. (1991, NTH Publication 91-3242, National Technical Information Service, Springfield, VA). One or more or all of non-cysteine amino acids at positions shown in Table 2 may be replaced by cysteine. In some embodiments, such an antibody is a parent or wild-type antibody. A single substitution of a cysteine residue often results in the display of two cysteine residues in the resultant antibody due to the homodimeric nature of IgG molecules. The resultant antibodies lacking interchain cysteines herein may display at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 or more free thiols for the purpose of conjugation to a heterologous molecule.

In some embodiments, antibodies herein comprise a serine shown at a position in Table 2 substituted to cysteine. In various embodiments, an antibody lacking interchain cysteines comprises a threonine shown at a position in Table 2 substituted to cysteine. In some embodiments, a cysteine engineered antibody herein comprises one or more serines and one or more threonines shown at positions in Table 2 substituted by cysteine. Such antibodies may include an IgG1, IgG2, IgG3 or IgG4 isotype heavy chain or portion thereof.

In some embodiments, an antibody lacking interchain cysteines comprises a cysteine substitution at one or more positions selected from positions 131, 132, 133, 134, 135, 136, 137, 138 and 139 shown in Table 2, where the numbering system of the constant region is that of the EU index as set forth in Kabat et al. (supra). In certain embodiments, a cysteine engineered antibody comprises a cysteine substitution at two or more positions selected from positions131, 132, 133, 134, 135, 136, 137, 138 and 139 shown in Table 2. In certain embodiments, an antibody lacking interchain cysteines comprises cysteine substitutions at three or more positions selected from positions 131, 132, 133, 134, 135, 136, 137, 138 and 139 shown in Table 2. In various embodiments, an antibody lacking interchain cysteines comprises a cysteine substitution at four or more positions selected from positions 131, 132, 133, 134, 135, 136, 137, 138 and 139 shown in Table 2. In various embodiments, an antibody lacking interchain cysteines comprises a cysteine substitution at five or more positions selected from positions 131, 132, 133, 134, 135, 136, 137, 138 and 139 shown in Table 2. In some embodiments, an antibody lacking interchain cysteines comprises a cysteine substitution at six or more positions selected from positions 131, 132, 133, 134, 135, 136, 137, 138 and 139 shown in Table 2. In various embodiments, an antibody lacking interchain cysteines comprises a cysteine substitution at seven or more positions selected from positions131, 132, 133, 134, 135, 136, 137, 138 and 139 shown in Table 2. In certain embodiments, an antibody

lacking interchain cysteines comprises a cysteine substitution at eight or more positions selected from positions 131, 132, 133, 134, 135, 136, 137, 138 and 139 shown in Table 2. In various embodiments, an antibody lacking interchain cysteines comprises a cysteine substitution at nine or more positions selected from positions 131, 132, 133, 134, 135, 136, 137, 138 and 139 shown in Table 2. Positions in SEQ ID NOs: 5 to 10 corresponding to positions 131, 132, 133, 134, 135, 136, 137, 138 and 139 shown in Table 2 are determined by inspection.

A fragment of a full-length antibody may not include a full-length CH1 domain in certain embodiments. An antibody fragment may include one or more of the positions shown in Table 2, and one or more of the non-cysteine amino acids at such positions may be substituted by cysteine.

In some embodiments, an antibody lacking interchain cysteines does not comprise a substitution to at cystein positions 132 and/or 138 in Table 2. In some embodiments, an antibody lacking interchain cysteines positions 132 and/or 138 in Table 2 comprise a substitution that is not cysteine. In certain certain embodiments, an antibody lacking interchain cysteines comprises one or more substitutions at only threonine and/or serine amino acids naturally occurring at positions shown in Table 2, or equivalents thereof.

In an embodiment, an antibody lacking interchain cysteines includes an IgG1 having a serine and/or a threonine substituted for a cysteine at a position shown in Table 2. In various embodiments, antibodies lacking interchain cysteines herein are derived from an IgGI , IgG2, IgG3 or an IgG4 isotype. In certain embodiments, an antibody lacking interchain cysteines is derived from non-IgG formats such as FgAI , Ig A2, IgM, IgD, or IgE. In various embodiments, antibodies herein comprise one or more cysteine engineering of residues corresponding to one or more of positions 131-139 of IgG1 shown in Table 2. In some embodiments, antibodies herein comprise cysteine engineering of residues outlined in the various antibody formats. In some embodiments, antibodies herein comprise antibody fragments including, but not limited to Fab and Fab2 molecule formats.

The 131-139 region of the CH1 domain of the IgG1 molecule is solvent exposed as illustrated in Figure 17. As such, it is envisaged that the 131-139 loop may be expanded (in other words, inclusion of additional amino acids) to facilitate a surface for site-specific conjugation of various agents. In some embodiments, a loop shown in Table 2 of an antibody lacking native interchain cysteines is expanded by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, at least 1, at least 2, at

least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, or at least 15 amino acids. In some embodiments, an expansion of a loop shown in Table 2 occurs after one or more of the 131, 132, 133, 134, 135, 136, 137, 138, or 139 residue in an IgG1 isotype antibody or counterpart position in an IgG2, 3 or 4 isotype antibody. In various embodiments, an expansion of a loop shown in Table 2 occurs after the 131, 132, 133, 134, 135, 136, 137, 138, and 139 residue in an IgG1 isotype antibody or counterpart position in an IgG2, 3 or 4 isotype antibody.

In various embodiments, an expansion of a loop shown in Table 2 may comprise any amino acid, and in various embodiments, the expansion comprises at least one non-naturally occurring cysteine amino acid. In some embodiments, the expansion comprises threonine and/or serine residues, and in various embodiments, the expansion is also coupled with the substitution of non-naturally occurring cysteine residues for non-cysteine residues.

In certain embodiments, antibodies lacking interchain cysteines comprise the formation of at least one non-naturally occurring disulfide bond. The non-naturally occurring disulfide bond may be intrachain or interchain bond. The non-naturally occurring disulfide bond may link two separate antibody molecules together. The formation of a non- naturally occurring disulfide bond may liberate at least one free thiol group previously linked to another cysteine residue.

The engineering of cysteine residues to display free thiol groups may lead to a mixture of antibody species, displaying a high degree of variability of positions of disulfide bonds. For example, the naturally occurring "canonical" disulfide bond may only be represented in some of the antibodies present in a sample. It is understood that the engineering of other non-naturally occurring cysteines may lead to the formation of disulfide bonds other than the "canonical" disulfide bond. In some embodiments, a disulfide bond is formed between the light chain and any non-naturally occurring cysteine residue present in a loop shown in the Table 2. In certain embodiments, a disulfide bond is formed between the light chain and any non- naturally occurring cysteine residue present in a loop shown in the Table 2.

Also presented herein are antibodies lacking interchain cysteines where 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more amino acids at positions selected from Table 3 are substituted by a cysteine amino acid. Thus, 1 to 20 amino acids at positions 239, 282, 289, 297, 312, 324, 330, 335, 337, 339, 356, 359, 361, 383, 384, 398, 400, 422, 440 in Table 3 of an

antibody independently may be replaced with a cysteine amino acid, where the numbering system of the constant region is that of the EU index as set forth in Kabat et al. (1991, NIH Publication 91-3242, National Technical Information Service, Springfield, VA) of a parent or wild type antibody are substituted with a cysteine amino acid. It should be noted that a single substitution of a cysteine residue results in the display of two cysteine residues in the resultant antibody due to the homodimeric nature of IgG molecules. The resultant antibodies lacking interchain cysteines herein may display at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, or more free thiols for the purpose of conjugation to a drug or compound. Such antibodies may include an IgG1, IgG2, IgG3 or IgG4 isotype heavy chain or portion thereof. Positions in SEQ ID NOs: 5 to 10 that correspond to positions 239, 282, 289, 297, 312, 324, 330, 335, 337, 339, 356, 359, 361, 383, 384, 398, 400, 422, 440 in Table 3 are determined by inspection.

In some embodiments, an antibody lacking interchain cysteines comprises a cysteine substitution at one or more positions selected from positions 239, 282, 289, 297, 312, 324, 330, 335, 337, 339, 356, 359, 361, 383, 384, 398, 400, 422, 440 in Table 3, where the numbering system of the constant region is that of the EU index as set forth in Kabat et al. (supra). In certain embodiments, an antibody lacking interchain cysteines comprises at least two substitutions selected from the positions 239, 282, 289, 297, 312, 324, 330, 335, 337, 339, 356, 359, 361, 383, 384, 398, 400, 422, 440 in Table 3. In various embodiments, an antibody lacking interchain cysteines comprises at least three substitutions selected from the positions 239, 282, 289, 297, 312, 324, 330, 335, 337, 339, 356, 359, 361, 383, 384, 398, 400, 422, 440 in Table 3. In some embodiments, an antibody lacking interchain cysteines comprises at least four substitutions selected from the positions 239, 282, 289, 297, 312, 324, 330, 335, 337, 339, 356, 359, 361, 383, 384, 398, 400, 422, 440 in Table 3. In certain embodiments, an antibody lacking interchain cysteines comprises at least five substitutions selected from the positions 239, 282, 289, 297, 312, 324, 330, 335, 337, 339, 356, 359, 361, 383, 384, 398, 400, 422, 440 in Table 3. In various embodiments, an antibody lacking interchain cysteines comprises at least six substitutions selected from the positions 239, 282, 289, 297, 312, 324, 330, 335, 337, 339, 356, 359, 361, 383, 384, 398, 400, 422, 440 in Table 3 of the heavy chain of an antibody. An antibody lacking interchain cysteines sometimes comprises at least seven substitutions selected from the positions 239, 282, 289, 297, 312, 324, 330, 335, 337, 339, 356, 359, 361, 383, 384, 398, 400, 422, 440 in Table 3. In some embodiments, an antibody lacking interchain cysteines comprises at least eight substitutions selected from the positions 239, 282, 289, 297, 312, 324, 330, 335, 337, 339, 356, 359, 361, 383, 384, 398, 400, 422, 440 in Table 3. In

certain embodiments, an antibody lacking interchain cysteines comprises at least nine substitutions selected from the positions 239, 282, 289, 297, 312, 324, 330, 335, 337, 339, 356, 359, 361, 383, 384, 398, 400, 422, 440 in Table 3. In various embodiments, an antibody lacking interchain cysteines comprises at least ten substitutions selected from the positions 239, 282, 289, 297, 312, 324, 330, 335, 337, 339, 356, 359, 361, 383, 384, 398, 400, 422, 440 in Table 3. An antibody lacking interchain cysteines sometimes comprises at least ten substitutions selected from the positions 239, 282, 289, 297, 312, 324, 330, 335, 337, 339, 356, 359, 361, 383, 384, 398, 400, 422, 440 in Table 3. In some embodiments, an antibody lacking interchain cysteines comprises at least eleven substitutions selected from the positions 239, 282, 289, 297, 312, 324, 330, 335, 337, 339, 356, 359, 361, 383, 384, 398, 400, 422, 440 in Table 3. In certain embodiments, an antibody lacking interchain cysteines comprises at least twelve substitutions selected from the positions 239, 282, 289, 297, 312, 324, 330, 335, 337, 339, 356, 359, 361, 383, 384, 398, 400, 422, 440 in Table 3. In various embodiments, an antibody lacking interchain cysteines comprises at least thirteen substitutions selected from the positions 239, 282, 289, 297, 312, 324, 330, 335, 337, 339, 356, 359, 361, 383, 384, 398, 400, 422, 440 in Table 3. The cysteine antibodies lacking interchain cysteines herein sometimes comprise at least fourteen substitutions selected from the positions 239, 282, 289, 297, 312, 324, 330, 335, 337, 339, 356, 359, 361, 383, 384, 398, 400, 422, 440 in Table 3. In some embodiments, an antibody lacking interchain cysteines comprises at least fifteen substitutions selected from the positions 239, 282, 289, 297, 312, 324, 330, 335, 337, 339, 356, 359, 361, 383, 384, 398, 400, 422, 440 in Table 3. In certain embodiments, the antibodies lacking interchain cysteines antibodies herein comprise at least sixteen substitutions selected from the positions 239, 282, 289, 297, 312, 324, 330, 335, 337, 339, 356, 359, 361, 383, 384, 398, 400, 422, 440 in Table 3. In various embodiments, an antibody lacking interchain cysteines comprises at least seventeen substitutions selected from the positions 239, 282, 289, 297, 312, 324, 330, 335, 337, 339, 356, 359, 361, 383, 384, 398, 400, 422, 440 in Table 3. An antibody lacking interchain cysteines sometimes comprises at least eighteen substitutions selected from the positions 239, 282, 289, 297, 312, 324, 330, 335, 337, 339, 356, 359, 361, 383, 384, 398, 400, 422, 440 in Table 3. In some embodiments, an antibody lacking interchain cysteines comprises at least nineteen substitutions selected from the positions 239, 282, 289, 297, 312, 324, 330, 335, 337, 339, 356, 359, 361, 383, 384, 398, 400, 422, 440 in Table 3. In certain embodiments, an antibody lacking interchain cysteines comprises substitutions of the positions 239, 282, 289, 297, 312, 324, 330, 335, 337, 339, 356, 359, 361, 383, 384, 398, 400, 422, 440 in Table 3.

In some embodiments, an antibody lacking interchain cysteines comprises a substitution of at least one naturally occurring amino acid selected from the group consisting of: Ser239, Val282, Thr289, Asn297, Asp312, Ser324, Ala330, Thr335, Ser337, Ala339, Glu356, Thr359, Asn361, Ser383, Asn384, Leu398, Ser400, Ser440, Val422, and Ser442 of the heavy chain of an antibody based on IgG1, or counterpart position in an IgG2, IgG3 or IgG4 antibody (see Table 3). In certain embodiments, an antibody lacking interchain cysteines does not comprise a substitution at position or positions selected from: Ser239, Val282, Thr289, Asn297, Asp312, Ser324, Ala330, Thr335, Ser337, Ala339, Glu356, Thr359, Asn361, Ser383, Asn384, Leu398, Ser400, Ser440, Val422, and Ser442 of the heavy chain of an antibody based on IgG1, or counterpart position in an IgG2, IgG3 or IgG4 antibody (see Table 3).

In various embodiments, an antibody lacking interchain cysteines includes an IgG1 having a naturally occurring amino acid substituted for a cysteine at a position selected from the group consisting of: 239, 282, 289, 297, 312, 324, 330, 335, 337, 339, 356, 359, 361, 383, 384, 398, 400, 440, 422, and 442 of the heavy chain of an IgG1 antibody, or a counterpart position in an IgG2, IgG3 or IgG4 antibody. An antibody lacking interchain cysteines sometimes is derived from an IgG1, IgG2, IgG3 or an IgG4 format. In some embodiments, an antibody lacking interchain cysteines is derived from non-IgG formats such as IgA1, IgA2 IgM, IgD, or IgE. In certain embodiments, antibodies herein comprise cysteine engineering of surface residues of the CH2 and/or CH3 region of an IgG1 molecule or equivalents thereof.

A fragment of a full-length antibody may not include a full-length CH2 domain and/or full-length CH3 domain, in certain embodiments. An antibody fragment may include one or more of the positions shown in Table 3, and one or more of the amino acids at such positions may be substituted by cysteine.

In various embodiments, an antibody herein comprises the expression of an isolated Fc region comprising residues of antibody lacking interchain cysteines. Such isolated Fc regions may be useful as scaffolds for display purposes.

In certain embodiments, provided herein are fusion proteins comprising Fc regions that contain at least one or more substitutions at positions selected from positions 239, 282, 289, 297, 312, 324, 330, 335, 337, 339, 356, 359, 361, 383, 384, 398, 400, 422, 440 in Table 3.

In some embodiments, engineered antibodies herein may comprise one or more non-naturally occurring cysteine amino acids in the loop shown in Table 2 and one or more non-naturally occurring cysteine amino acids at position shown in Table 3.

Fc region

Provided herein, in some embodiments, are antibodies lacking native interchain cysteines with modifications to Fc regions described above (e.g., substitution of non-cysteine positions shown in Table 3 by cysteine amino acids). Also provided are antibodies lacking native interchain cysteines with one or more other Fc modifications described hereafter. Thus, in some embodiments an antibody lacking interchain cysteines may have an amino acid sequence having at least 25%, 35%, 45%, 55%, 65%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% amino acid sequence identity or similarity with the amino acid sequence of the Fc region of a parent antibody.

Certain modifications to the Fc region (e.g., amino acid substitutions and/or additions and/or deletions) can enhance or diminish effector function. In certain embodiments, variant Fc regions of antibodies exhibit a similar level of inducing effector function as compared to native Fc. In various embodiments, a variant Fc region exhibits a higher induction of effector function as compared to the native Fc. A variant Fc region sometimes exhibits lower induction of effector function as compared to the native Fc. In some embodiments, a variant Fc region exhibits higher induction of ADCC as compared to the native Fc. In certain embodiments, a variant Fc region exhibits lower induction of ADCC as compared to the native Fc. In some embodiments, a variant Fc region exhibits higher induction of CDC as compared to the native Fc. In some embodiments, a variant Fc region exhibits lower induction of CDC as compared to the native Fc.

In addition, glycosylation of a Fc region can be modified to increase or decrease effector function. In some embodiments the cysteine engineering creates a glycosylation site not present in an antibody counterpart having a native interchain cysteine amino acid. Accordingly, in some embodiments, the Fc regions of antibodies herein comprise altered glycosylation of amino acid residues. In certain embodiments, the altered glycosylation of the amino acid residues results in lowered effector function. In various embodiments, the altered glycosylation of the amino acid residues results in increased effector function. In various embodiments, the Fc region has reduced glycosylation. In some embodiments, the Fc region is glycosylated.

The addition of sialic acid to the oligosaccharides on IgG molecules may enhances their anti-inflammatory activity and alter their cytotoxicity. Thus, the efficacy of antibody therapeutics may be optimized by selection of a glycoform that is best suited to the intended application. The two oligosaccharide chains interposed between the two CH2 domains of antibodies are involved in the binding of the Fc region to its receptors. IgG molecules with increased sialylation exhibit anti-inflammatory properties whereas IgG molecules with reduced sialylation show increased immunostimulatory properties. Therefore, an antibody therapeutic can be "tailor-made" with an appropriate sialylation profile for a particular application. Methods for modulating the sialylation state of antibodies are known in the art.

In some embodiments, the Fc regions of antibodies herein comprise an altered sialylation profile compared to a reference unaltered Fc region. In certain embodiments, the Fe regions of antibodies herein comprises an increased sialylation profile compared to a reference unaltered Fc region. In various embodiments the Fc regions of antibodies herein comprise an increase in sialylation of about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 60%, about 65%, about 70%, about 80%, about 85%, about 90%, about 95%, about 100%, about 125%, about 150% or more as compared to a reference unaltered Fc region. In some embodiments the Fc regions of antibodies herein comprise an increase in sialylation of about 2 fold, about 3 fold, about 4 fold, about 5 fold, about 10 fold, about 20 fold, about 50 fold or more as compared to an unaltered reference Fc region.

In some embodiments, the Fc regions of antibodies herein comprise a decreased sialylation profile compared to a reference unaltered Fc region. In some embodiments, the Fc regions of antibodies herein comprise a decrease in sialylation of about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 60%, about 65%, about 70%, about 80%, about 85%, about 90%, about 95%, about 100%, about 125%, about 150% or more as compared to a reference unaltered Fc region. In some embodiments the Fc regions of antibodies herein comprise a decrease in sialylation of about 2 fold, about 3 fold, about 4 fold, about 5 fold, about 10 fold, about 20 fold, about 50 fold or more as compared to an unaltered reference Fc region.

The Fc region can also be modified to increase the half-lives of proteins. The increase in half-life allows for the reduction in amount of drug given to a patient as well as reducing the frequency of administration. Accordingly, antibodies herein with increased half- lives may be generated by

modifying (for example, substituting, deleting, or adding) amino acid residues identified as involved in the interaction between the Fc and the FcRn receptor. In certain embodiments, a methionine at position 252, a serine at position 254 and a threonine at position 256 of an IgG1 isotype antibody can be changed to tyrosine, threonine and glutamate, respectively, such that the resulting antibody includes tyrosine-252, threonine-254 and glutamate-256. Such an Fc region of an IgG1 antibody includes a YTE modification and counterpart positions in can be similarly modified in IgG2, IgG3 and IgG4 antibodies. In addition, the half-life of antibodies herein may be increase by conjugation to PEG or Albumin by techniques known in the art. In some embodiments the Fc regions of antibodies herein comprise an increase in half-life of about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 60%, about 65%, about 70%, about 80%, about 85% , about 90%, about 95%, about 100%, about 125%s, about 150% or more as compared to a reference unaltered Fc region. In certain embodiments the Fc regions of antibodies herein comprise an increase in half-life of about 2 fold, about 3 fold, about 4 fold, about 5 fold, about 10 fold, about 20 fold, about 50 fold or more as compared to an unaltered reference Fc region.

In various embodiments, the Fc regions of antibodies herein comprise a decrease in half-life. In some embodiments the Fc regions of antibodies herein comprise a decrease in half-life of about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 60%, about 65%, about 70%, about 80%, about 85%, about 90%, about 95%, about 100%, about 125%, about 150% or more as compared to a reference unaltered Fc region. In some embodiments the Fc regions of antibodies herein comprise a decrease in half-life of about 2 fold, about 3 fold, about 4 fold, about 5 fold, about 10 fold, about 20 fold, about 50 fold or more as compared to an unaltered reference Fc region.

Also presented herein, in certain embodiments, are Fc variant proteins which have altered binding properties for an Fc ligand (e.g., an Fc receptor, Clq) relative to a comparable molecule (e.g., a protein having the same amino acid sequence except having a wild type Fc region). Examples of binding properties include but are not limited to, binding specificity, equilibrium dissociation constant (K_D), dissociation and association rates (k_{off} and k_{on} respectively), binding affinity and/or avidity, It is generally understood that a binding molecule (e.g., a Fc variant protein such as an antibody) with a low K_{on} may be preferable to a binding molecule with a high k_{off}. However, in some instances the value of the k_{on} or k_{off} may be more relevant than the value of the K_D. One

skilled in the art can determine which kinetic parameter is most important for a given antibody application.

The affinities and binding properties of an Fc region for its ligand may be determined by a variety of in vitro assay methods (biochemical or immunological based assays) known in the art for determining Fc-FcγR interactions, i.e., specific binding of an Fc region to an FcR including but not limited to, equilibrium methods (e.g., enzyme-linked immunoabsorbent assay (ELISA), or radioimmunoassay (RIA)), or kinetics (e.g., BIACORE® analysis), and other methods such as indirect binding assays, competitive inhibition assays, fluorescence resonance energy transfer (FRET), gel electrophoresis and chromatography (e.g., gel filtration). These and other methods may utilize a label on one or more of the components being examined and/or employ a variety of detection methods including but not limited to colorimetric, spectrometric, spectrophotometic, fluorescent, luminescent, or isotopic labels.

In some embodiments, the Fc variant protein has enhanced binding to one or more Fc ligand relative to a comparable molecule. In certain embodiments, the Fc variant protein has an affinity for an Fc ligand that is at least 2 fold, at least 3 fold, at least 5 fold, at least 7 fold, at least 10 fold, at least 20 fold, at least 30 fold, at least 40 fold, at least 50 fold, at least 60 fold, at least 70 fold, at least 80 fold, at least 90 fold, at least 100 fold, or at least 200 fold greater than that of a comparable molecule. In various embodiments, the Fc variant protein has enhanced binding to an Fc receptor. In some embodiments, the Fc variant protein has enhanced binding to the Fc receptor FcγRIIIA. In certain embodiments, the Fc variant protein has enhanced biding to the Fc receptor FcγR ITB, In various embodiments, the Fc variant protein has enhanced binding to the Fc receptor FcRn. The Fc variant protein sometimes has enhanced binding to Clq relative to a comparable molecule.

The ability of any particular Fc variant protein to mediate lysis of the target cell by ADCC can be assayed. To assess ADCC activity an Fc variant protein of interest is added to target cells in combination with immune effector cells, which may be activated by the antigen antibody complexes resulting in cytolysis of the target cell. Cytolysis is generally detected by the release of label (e.g. radioactive substrates, fluorescent dyes or natural intracellular proteins) from the lysed cells. Effector cells for such assays may include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Specific examples of in vitro ADCC assays are known in the art.

In some embodiments, an Fc variant protein has enhanced ADCC activity relative to a comparable molecule. In specific embodiments, an Fc variant protein has ADCC activity that is at least 2 fold, or at least 3 fold, at least 5 fold, at least 10 fold, at least 50 fold, or at least 100 fold greater than that of a comparable molecule. In certain embodiments, an Fc variant protein has enhanced binding to the Fc receptor FcγRIIIA and has enhanced ADCC activity relative to a comparable molecule. In some embodiments, the Fc variant protein has both enhanced ADCC activity and enhanced serum half life relative to a comparable molecule.

In certain embodiments, an Fc variant protein has reduced ADCC activity relative to a comparable molecule. In some embodiments, an Fc variant protein has ADCC activity that is at least 2 fold, at least 3 fold, at least 5 fold, at least 10 fold, at least 50 fold, or at least 100 fold lower than that of a comparable molecule. In various embodiments, an Fc variant protein has reduced binding to the Fe receptor FcγRIIIA and has reduced ADCC activity relative to a comparable molecule. The Fc variant protein sometimes has both reduced ADCC activity and enhanced serum half life relative to a comparable molecule.

In an embodiment, an Fc variant protein has enhanced CDC activity relative to a comparable molecule. In some embodiments, an Fc variant protein has CDC activity that is at least 2 fold, at least 3 fold, at least 5 fold, at least 10 fold, at least 50 fold, or at least 100 fold greater than that of a comparable molecule. In certain embodiments, the Fc variant protein has both enhanced CDC activity and enhanced serum half life relative to a comparable molecule. In an embodiment, the Fc variant protein has reduced binding to one or more Fc ligand relative to a comparable molecule. In some embodiments, the Fc variant protein has an affinity for an Fc ligand that is at least 2 fold, at least 3 fold, at least 5 fold, at least 7 fold, at least 10 fold, at least 20 fold, at least 30 fold, at least 40 fold, at least 50 fold, at least 60 fold, at least 70 fold, at least 80 fold, at least 90 fold, at least 100 fold, or at least 200 fold lower than that of a comparable molecule.

In some embodiments, the Fc variant protein has reduced binding to an Fc receptor. In certain embodiments, the Fc variant protein has reduced binding to the Fc receptor FcγRIIIA. In various embodiments, an Fc variant described herein has an affinity for the Fc receptor FcγRIIIA that is at least about 5 fold lower than that of a comparable molecule, where said Fc variant has an affinity for the be receptor FcγRIIB that is within about 2 fold of that of a comparable molecule. The Fc variant protein sometimes has reduced binding to the Fc receptor FcRn. In some embodiments, the Fc variant protein has reduced binding to Clq relative to a comparable molecule.

In some embodiments of Fc variants the Fc region comprises a non naturally occurring amino acid residue at one or more positions selected from the group consisting of 234, 235, 236, 237, 238, 239, 240, 241, 243, 244, 245,247, 251, 252, 254, 255, 256, 262, 263, 264, 265, 266, 267, 268, 269, 279, 280, 284, 292, 296, 297, 298, 299, 305, 313, 316, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 339, 341, 343, 370, 373, 378, 392, 416, 419, 421, 440 and 443 as numbered by the EU index as set forth in Kabat.

Optionally, the Fc region may comprise a non-naturally occurring amino acid residue at additional and/or alternative positions known to one skilled in the art. Provided, in some embodiments, is an Fc variant, where the Fc region comprises at least one non naturally occurring amino acid residue selected from the group consisting of 234D, 234E, 234N, 234Q, 234T, 234H, 234Y, 234I, 234V, 234F, 235A, 235D, 235R, 235W, 235P, 235S, 235N, 235Q, 235T, 235H, 235Y, 235I, 235V, 235F, 236E, 239D, 239E, 239N, 239Q, 239F, 239T, 239H, 239Y, 240I, 240A, 240T, 240M, 241W, 241 L, 241Y, 241E, 241 R. 243W, 243L 243Y, 243R, 243Q, 244H, 245A, 247L, 247V, 247G, 251F, 252Y, 254T, 255L, 256E, 256M, 262I, 262A, 262T, 262E, 263I, 263A, 263T, 263M, 264L, 264I, 264W, 264T, 264R, 264F, 264M, 264Y, 264E, 265G, 265N, 265Q, 265Y, 265F, 265V, 265I, 265L, 265H, 265T, 266I, 266A, 266T, 266M, 267Q, 267L, 268E, 269H, 269Y, 269F, 269R, 270E, 280A, 284M, 292P, 292L, 296E, 296Q, 296D, 296N, 296S, 296T, 296L, 296I, 296H, 269G, 297S, 297D, 297E, 298H, 298I, 298T, 298F, 299I, 299L, 299A, 299S, 299V, 299H, 299F, 299E, 305I, 313F, 316D, 325Q, 325L, 325I, 325D, 325E, 325A, 325T, 325V, 325H, 327G, 327W, 327N, 327L, 328S, 328M, 328D, 328E, 328N, 328Q, 328F, 328I, 328V, 328T, 328H, 328A, 329F, 329H, 329Q, 330K, 330G, 330T, 330C, 330L, 330Y, 330V, 330I, 330F, 330R, 330H, 331G, 331A, 331L, 331M, 331F, 331W, 331K, 331Q, 331E, 331S, 331V, 331I, 331C, 331Y, 331H, 331R, 331N, 331D, 331T, 332D,332S, 332W, 332F, 332E, 332N, 332Q, 332T, 332H, 332Y, 332A, 339T, 370E, 370N, 378D, 392T, 396L, 416G, 419H, 421K, 440Yand 443W as numbered by the EU index as set forth in Kabat. Optionally, the Fc region may comprise additional and/or alternative non naturally occurring amino acid residues known in the art.

Certain amino acid sequence modifications

In certain embodiments, the amino acid sequence of a light chain in an antibody lacking interchain cysteines is about 80% or more identical to the amino acid sequence of SEQ ID NO: 9 or 10 (e.g., about 85% or more, 86% or more, 87% or more, 88% or more, 89% or more, 90% or more, 91% or

more, 92% or more, 93% or more, 94% or more, 95% or more, 96% or more, 97% or more, 98% or more, 99% or more identical to the amino acid sequence of SEQ ID NO: 9 or 10). In some embodiments, the amino acid sequence of a light chain is identical to the amino acid sequence in SEQ ID NO: 9 or 10 except that it includes 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acid modifications (e.g., substitutions, insertions and/or deletions) relative to the amino acid sequence in SEQ ID NO: 9 or 10. In some embodiments, the amino acid sequence of a heavy chain is about 80% or more identical to the amino acid sequence of SEQ ID NO: 5, 6, 7 or 8 (e.g., about 85% or more, 86% or more, 87% or more, 88% or more, 89% or more, 90% or more, 91% or more, 92% or more, 93% or more, 94% or more, 95% or more, 96% or more, 97% or more, 98% or more, 99% or more identical to the amino acid sequence of SEQ ID NO: 5, 6, 7 or 8). In some embodiments, the amino acid sequence of a heavy chain is identical to the amino acid sequence in SEQ ID NO: 5, 6, 7 or 8 except that it includes 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acid modifications (e.g., substitutions, insertions and/or deletions) relative to the amino acid sequence in SEQ ID NO: 5, 6, 7 or 8. As described herein, in some embodiments, a light chain and/or a variable chain sequence can include 1 to 10 engineered cysteine substitutions of non-cysteine amino acids.

In addition to the sequences of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10, the present technology also encompasses further modifications and, the variants and fragments thereof, comprising one or more amino acid residues and/or polypeptide substitutions, additions and/or deletions in the variable light (VL) domain and/or variable heavy (VH) domain and/or Fc region and post translational modifications. Included in these modifications are antibody conjugates where an antibody has been covalently attached to a moiety. Moieties suitable for attachment to the antibodies include but are not limited to, proteins, peptides, drugs, labels, and cytotoxins. These changes to the antibodies may be made to alter or fine tune the characteristics (biochemical, binding and/or functional) of the antibodies as is appropriate for treatment and/or diagnosis of antigen mediated diseases. Methods for forming conjugates, making amino acid and/or polypeptide changes and post-translational modifications are known in the art, some of which are detailed below. The following description is not intended to be limiting, but instead a non-limiting description of some embodiments, more of which will be understood by one of skill in the art. It is also understood that some of the following methods were used to develop the human, humanized and/or chimeric antibody sequences described above. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics.

Amino acid changes to the antibodies necessarily results in sequences that are less than 100% identical to the above identified antibody sequences or parent antibody sequence. In certain embodiments, in this context, the antibodies herein many have about 25% to about 95% sequence identity to the amino acid sequence of either the heavy or light chain variable domain of a parent antibody. Thus, in some embodiments an antibody lacking interchain cysteines may have an amino acid sequence having at least 25%, 35%, 45%, 55%, 65%, 75%, 80%, 85%, 90%, or 95% amino acid sequence identity or similarity with the amino acid sequence of either the heavy or light chain variable domain of a parent antibody. In certain embodiments, an antibody lacking interchain cysteines has an amino acid sequence having at least 25%, 35%, 45%, 55%, 65%, 75%, 80%, 85%, 90%, or 95% amino acid sequence identity or similarity with the amino acid sequence of the heavy or light chain CDR1, CDR2, or CDR3 of a parent antibody. An antibody lacking interchain cysteines may sometimes have an amino acid sequence having at least 25%, 35%, 45%, 55%, 65%, 75%, 80%, 85%, 90%, or 95% amino acid sequence identity or similarity with the amino acid sequence of the heavy or light chain FR1, FR2, FR3 or FR4 of a parent antibody.

In certain embodiments, altered antibodies are generated by one or more amino acid alterations (e.g., substitutions, deletion and/or additions) introduced in one or more of the variable regions of the antibody. In various embodiments, the amino acid alterations are introduced in the framework regions. One or more alterations of framework region residues may result in an improvement in the binding affinity of the antibody for the antigen. This may be especially true when these changes are made to humanized antibodies where the framework region may be from a different species than the CDR regions. Examples of framework region residues to modify include those which non-covalently bind antigen directly, interact with/effect the conformation of a CDR, and/or participate in the VL-VH interface. In some embodiments, from about one to about five framework residues may be altered. Sometimes, this may be sufficient to yield an antibody mutant suitable for use in preclinical trials, even where none of the hypervariable region residues have been altered. Normally, however, an altered antibody may comprise additional hypervariable region alteration(s). In certain embodiments, the hypervariable region residues may be changed randomly, especially where the starting binding affinity of an antibody lacking interchain cysteines for the antigen from the second mammalian species is such that such randomly produced antibodies can be readily screened.

Specific binding activity

Antibodies lacking interchain cysteines herein retain the antigen binding capability of their wild type counterpart. In an embodiment, an antibody lacking interchain cysteines exhibits essentially the same affinity as compared to an antibody prior to cysteine engineering. In some embodiments, antibodies lacking interchain cysteines herein exhibit a reduced affinity as compared to an antibody prior to cysteine engineering. In some embodiments, antibodies lacking interchain cysteines herein exhibit an enhanced affinity as compared to an antibody prior to cysteine engineering.

An antibody herein may have a high binding affinity to one or more of its cognate antigens. For example, an antibody described herein may have an association rate constant or k_{on} rate (antibody (Ab) + antigen->Ab-Ag) of at least 2 X 10^5 $M^{-1}s^{-1}$, at least 5 X10^5 $M^{-1}s^{-1}$, at least 10^6 $M^{-1}s^{-1}$, at least 5 X 10^6 $M^{-1}s^{-1}$, at least 10^7 $M^{-1}s^{-1}$, at least 5 X 10^7 $M^{-1}s^{-1}$,or at least 10^8 $M^{-1}s^{-1}$.

In some embodiments, an antibody may have a k_{off} rate (Ab-Ag -> Ab + Ag) of less than 5x10^{-1} s^{-1}, less than 10^{-1} s^{-1}, less than 5x10^{-2} s^{-1}, less than10^{-2} s^{-1}, less than 5X10^{-3} s^{-1}, less than 10^{-3} s^{-1}, less than 5x10^{-4} s^{-1}, or less than10^{-4} s^{-1}. In a some embodiments, an antibody herein has a k_{off} of less than 5x10^{-5} s^{-1}, less than 10^{-5} s^{-1}, less than 5x10^{-6} s^{-1}, less than10^{-6} s^{-1}, less than 5x10^{-7} s^{-1}, less than10^{-7} s^{-1}, less than 5x10^{-8} s^{-1}, less than10^{-8} s^{-1}, less than 5x10^{-9} s^{-1}, less than10^{-9} s^{-1}, or less than 10^{-10} s^{-1}.

In some embodiments, an antibody may have an affinity constant or K_a (k_{on}/k_{off}) of at least 10^2 M^{-1}, at least 5 X10^2 M^{-1}, at least10^3 M^{-1}, at least 5 X10^3 M^{-1}, at least 10^4 M^{-1}, at least 5 X10^4 M^{-1}, at least 10^5 M^{-1}, at least 5 X10^5 M^{-1}, at least 10^6 M^{-1}, at least 5X10^6 M^{-1}, at least 10^7 M^{-1}, at least 5 X 10^7 M^{-1}, at least 10^8 M^{-1}, at least 5 X 10^8 M^{-1}, at least 10^9 M^{-1}, at least 5 X 10^9 M^{-1}, at least 10^{10} M^{-1}, at least 5 X 10^{10} M^{-1}, at least 10^{11} M^{-1}, at least 5X 10^{11} M^{-1}, at least 10^{12} M^{-1}, at least 5 X 10^{12} M^{-1}, at least 10^{13} M^{-1}, at least 5 X 10^{13} M^{-1}, at least 10^{14} M^{-1}, at least 5 X 10^{14} M^{-1}, at least 10^{15} M^{-1}, or at least 5 X 10^{15} M^{-1}.

In certain embodiments, an antibody may have a dissociation constant or K_d (k_{off}/k_{on}) of less than 5x10^{-2} M, less than 10^{-2} M, less than 5x10^{-3} M, less than 10^{-3} M, less than 5x10^{-4} M, less than 10^{-4} M, less than 5x10^{-5} M, less than 10^{-5} M, less than 5x10^{-6} M, less than 10^{-6} M, less than 5x10^{-7} M, less than 10^{-7} M, less than 5x10^{-8} M, less than 10^{-8} M, less than 5x10^{-9} M, less than 10^{-9} M, less than 5x10^{-10} M, less than 10^{-10} M, less than 5x10^{-11} M, less than 10^{-11} M, less than 5x10^{-12} M,

less than 10^{-12} M, less than $5x10^{-13}$ M, less than 10^{-13} M, less than $5x10^{-14}$ M, less than 10^{-14} M, less than $5x10^{-15}$ M, or less than 10^{-15} M.

An antibody used in accordance with a method described herein may have a dissociation constant (K_d) of less than 3000 pM, less than 2500 pM, less than 2000 pM, less than 1500 pM, less than 1000 pM, less than 750 pM, less than 500 pM, less than 250 pM, less than 200 pM, less than 150 pM, less than 100 pM, less than 75 pM as assessed using a method described herein or known to one of skill in the art (e.g., a BIAcore assay, ELISA) (Biacore International AB, Uppsala, Sweden).

In some embodiments, antibodies herein comprise an epitope binding domain that competes for binding with another antibody. In certain embodiments, antibodies lacking interchain cysteines herein comprise an epitope binding domain that specifically binds to an antigen selected from: PDGFRalpha, PDGFRbeta, PDGF, VEGF, VEGF-A, VEGF-B, VEGF-C. VEGF-D, VEGFE, VEGF-F, VEGFR-1, VEGFR-2, VEGFR-3, FGF, FGF2, HGF, KDR, flt-1, FLK-1 Ang-2, Ang-1, PLGF, CEA, CXCL13, Baff, IL-21, CCL21, TNF-alpha, CXCL12, SDF-1, bFGF, MAC-1, IL23p19, FPR, IGFBP4, CXCR3, TLR4, CXCR2, EphA2, EphA4, EphrinB2, EGFR(ErbB1), HER2(ErbB2 or p185neu), HER3(ErbB3), HER4 ErbB4 or tyro2), SC1, LRP5, LRP6, RAGE, Nav1.7, GLP1, RSV, RSV F protein, Influenza HA protein, Influenza NA protein, HMGB1, CD16, CD19, CD20, CD21, CD28, CD32, CD32b, CD64, CD79, CD22, ICAM-1, FGFR1, FGFR2, HDGF, EphB4, GITR, β-amyloid, hMPV, PIV-1, PIV-2, OX40L, IGFBP3, cMet, PD-1, PLGF, Neprolysin, CTD, IL-18, IL-6, CXCL-13, IL-1R1, IL-15, IL-4R, IgE, PAI-1, NGF, EphA2, CEA, uPARt, DLL-4, αvβ6, α5β1, interferon receptor type I and type II. CD19, ICOS, IL-17, Factor II, Hsp90, IGF, CD19, GM-CSFR, PIV-3, CMV, IL-13, IL-9, and EBV.

In various embodiments, an antibody lacking interchain cysteines comprises at least one epitope binding domain that specifically binds to a member (receptor or ligand) of the TNF superfamily. Various molecules include, but are not limited to Tumor Necrosis Factor-alpha ("TNF-alpha"), Tumor Necrosis Factor-beta ("TNF-beta"), Lymphotoxin-alpha ("LT-alpha"), CD30 ligand, CD27 ligand, CD40 ligand, 4-1 BB ligand, Apo-1 ligand (also referred to as Fas ligand or CD95 ligand), Apo-2 ligand (also referred to as TRAIL), Apo-3 ligand (also referred to as TWEAK), osteoprotegerin (OPG), APRIL, RANK ligand (also referred to as TRANCE), TALL-1 (also referred to as BlyS, BAFF or THANK), DR4, DR5 (also known as Apo-2, TRAIL-R2, TR6, Tango-63, hAPO8, TRICK2, or KILLER), DR6, DcR1,DcR2, DcR3 (also known as TR6 or M68), CAR1, HVEM (also known as ATAR or TR2), GITR, ZTNFR-5, NTR-1, TNFL1, CD30, LTBr, 4-1BB receptor

and TR9.

In some embodiments the antibodies lacking interchain cysteines herein are capable of binding one or more targets selected from the group consisting of 5T4, ABL, ABCF1, ACVR1, ACVR1B, ACVR2, ACVR2B, ACVRL1, ADORA2A, Aggrecan, AGR2, AICDA, AIF1, AIGl, AKAP1, AKAP2, AMH, AMHR2, ANGPT1, ANGPT2, ANGPTL3, ANGPTL4, ANPEP, APC, APOC1, AR, aromatase, ATX, AX1, AZGP1 (zinc-a-glycoprotein),B7.1, B7.2, B7-H1, BAD, BAFF, BAG1, BAI1, BCR, BCL2, BCL6, BDNF, BLNK, BLR1 (MDR15), BIyS, BMP1, BMP2, BMP3B (GDFIO), BMP4, BMP6, BMP8, BMPR1A, BMPR1B, BMPR2, BPAG1 (plectin), BRCA1, C19orflO (IL27w), C3, C4A, C5, C5R1, CANT1, CASP1, CASP4, CAV1, CCBP2 (D6 / JAB61), CCL1 (1-309), CCLI1 (eotaxin), CCL13 (MCP-4), CCL15 (MIP-Id), CCL16 (HCC-4), CCL17 (TARC), CCL18 (PARC), CCL19 (MIP-3b), CCL2 (MCP -1), MCAF, CCL20 (MIP-3a), CCL21 (MEP-2), SLC, exodus-2, CCL22(MDC / STC-I), CCL23 (MPIF-I), CCL24 (MPIF-2 / eotaxin-2), CCL25 (TECK), CCL26(eotaxin-3), CCL27 (CTACK / ILC), CCL28, CCL3 (MIP-Ia), CCL4 (MIPIb), CCL5(RANTES), CCL7 (MCP-3), CCL8 (mcp-2), CCNA1, CCNA2, CCND1, CCNE1, CCNE2, CCRl (CKR1 / HM145), CCR2 (mcp-IRB / RA),CCR3 (CKR3 / CMKBR3), CCR4, CCR5(CMKBR5 / ChemR13), CCR6 (CMKBR6 / CKR-L3 / STRL22 / DRY6), CCR7 (CKR7 / EBI1),CCR8 (CMKBR8 / TERI / CKR-L1), CCR9 (GPR-9-6), CCRL1 (VSHK1), CCRL2 (L-CCR),CD164, CD19, CDIC, CD20, CD200, CD-22, CD24, CD28, CD3, CD33, CD35, CD37, CD38, CD3E, CD3G,CD3Z, CD4, CD40, CD40L, CD44, CD45RB, CD52, CD69, CD72, CD74, CD79A, CD79B, CD8, CD80, CD81, CD83, CD86, CD137, CDH1 (Ecadherin), CDH1O, CDH12, CDH13, CDH18,CDH19, CDH20, CDH5, CDH7, CDH8, CDH9, CDK2, CDK3, CDK4, CDK5, CDK6, CDK7,CDK9, CDKN1A (p21Wap1/Cip1),CDKN1B (p27Kip1), CDKN1C, CDKN2A (p16INK4a),CDKN2B, CDKN2C, CDKN3, CEBPB, CERl, CHGA, CHGB, Chitinase, CHST1O, CKLFSF2,CKLFSF3, CKLFSF4, CKLFSF5, CKLFSF6, CKLFSF7, CKLFSF8, CLDN3, CLDN7 (claudin- 7), CLN3, CLU (clusterin), CMKLR1, CMKOR1 (RDC1), CNR1, COL18A1, COLIA1, COL4A3, COL6A1, CR2, Cripto, CRP, CSF1 (M-CSF), CSF2 (GM-CSF), CSF3 (GCSF), CTLA4, CTL8, CTNNB1 (b-catenin), CTSB (cathepsin B), CX3CL1 (SCYD1), CX3CR1 (V28), CXCL1 (GRO1), CXCL1O (IP-IO), CXCLI1 (I-TAC / IP-9), CXCL12 (SDF1), CXCL13, CXCL14, CXCL16, CXCL2 (GRO2), CXCL3 (GRO3), CXCL5 (ENA-78 / LIX), CXCL6 (GCP-2), CXCL9 (MIG), CXCR3 (GPR9/CKR-L2), CXCR4, CXCR6 (TYMSTR /STRL33 /Bonzo),CYB5, CYC1, CYSLTR1, DAB2IP, DES, DKFZp451J0118, DNCL1, DPP4, E2F1, Engel, Edge, Fennel, EFNA3, EFNB2, EGF, EGFR, ELAC2, ENG, Enola, ENO2, ENO3, EPHA1, EPHA2, EPHA3, EPHA4, EPHA5, EPHA6, EPHA7, EPHA8, EPHA9, EPHA10, EPHB1, EPHB2, EPHB3, EPHB4, EPHB5, EPHB6, EPHRIN-A1, EPHRIN-A2, EPHRINA3, EPHRIN-A4, EPHRIN-A5,

EPHRIN-A6, EPHRIN-B1, EPHRIN-B2, EPHRIN-B3, EPHB4,EPG, ERBB2 (Her-2), EREG, ERK8, Estrogen receptor, Earl, ESR2, F3 (TF), FADD, farnesyltransferase, FasL, FASNf, FCER1A, FCER2, FCGR3A, FGF, FGF1 (aFGF), FGF1O, FGF1 1, FGF12, FGF12B, FGF13, FGF14, FGF16, FGF17, FGF18, FGF19, FGF2 (bFGF), FGF20, FGF21 , FGF22, FGF23, FGF3 (int-2),FGF4 (HST), FGF5, FGF6 (HST-2), FGF7 (KGF), FGF8, FGF9, FGFR3, FIGF (VEGFD), FIL1(EPSILON), FBL1 (ZETA), FLJ12584, FLJ25530, FLRT1 (fibronectin), FLT1, FLT-3, FOS, FOSL1(FRA-1), FY (DARC), GABRP (GABAa), GAGEB1, GAGEC1, GALNAC4S-6ST, GATA3, GD2, GDF5, GFI1, GGT1, GM-CSF, GNAS1, GNRH1, GPR2 (CCR1O), GPR31, GPR44, GPR81 (FKSG80), GRCC1O (C1O), GRP, GSN (Gelsolin), GSTP1, HAVCR2, HDAC, HDAC4, HDAC5, HDAC7A, HDAC9, Hedgehog, HGF, HIF1A, HIP1, histamine and histamine receptors, HLA-A, HLA-DRA, HM74, HMOXI, HSP90, HUMCYT2A, ICEBERG, ICOSL, ID2, IFN-a, IFNA1, IFNA2, IFNA4,IFNA5, EFNA6, BFNA7, IFNB1, IFNgamma, IFNW1, IGBP1, IGF1, IGFIR, IGF2, IGFBP2,IGFBP3, IGFBP6, DL-1, ILIO, ILIORA, ILIORB, IL- 1, IL1R1 (CD121a), IL1R2(CD121b), IL-IRA, IL-2, IL2RA (CD25), IL2RB(CD122), IL2RG(CD132), IL-4, IL-4R(CD123), IL-5, IL5RA(CD125), IL3RB(CD131), IL-6, IL6RA, (CD126), IR6RB(CD130), IL-7, IL7RA(CD127), IL-8, CXCR1 (IL8RA), CXCR2, (IL8RB/CD128), IL-9, IL9R (CD129), IL-10, IL10RA(CD210), IL10RB(CDW210B), IL-11, IL11RA, IL-12, IL-12A, IL-12B, IL-12RB1, IL-12RB2, IL-13, IL13RA1, IL13RA2, IL14, IL15, IL15RA, 1L16, IL17, IL17A, IL17B, IL17C, IL17R, IL18, IL18BP, IL18R1, IL18RAP, IL19, ILIA, ILIB, IL1F10, IL1F5, IL1F6, IL1F7, IL1F8, DL1F9, ILIHYI, ILIR1, IL1R2, ILIRAP, ILIRAPLI, IL1RAPL2, IL1RL1, IL1RL2, ILIRN, IL2, IL20, IL20RA, IL21R, IL22, IL22R, IL22RA2, IL23,DL24, IL25, IL26, IL27, IL28A, IL28B, IL29, IL2RA, IL2RB, IL2RG, IL3, IL30, IL3RA, IL4,IL4R, IL6ST (glycoprotein 130), ILK, INHA, INHBA, INSL3, INSL4, IRAK1, IRAK2, ITGA1, ITGA2,ITGA3, ITGA6 (α6 integrin), ITGAV, ITGB3, ITGB4 (β4 integrin), JAG1, JAK1, JAK3, JTB, JUN, K6HF, KAI1, KDR, KITLG, KLF5 (GC Box BP), KLF6, KLK1O, KLK12, KLK13, KLK14, KLK15, KLK3, KLK4, KLK5, KLK6, KLK9, KRT1, KRT19 (Keratin 19), KRT2A, KRTHB6(hair-specific type II keratin), LAMA5, LEP (leptin), Lingo-p75, Lingo-Troy, LPS, LTA (TNF- b), LTB, LTB4R (GPR16), LTB4R2, LTBR, MACMARCKS, MAG or Omgp , MAP2K7 (c-Jun), MCP-1, MDK, MIB1, midkine, MIF, MISRII, MJP-2,MK, MKI67 (Ki-67), MMP2, MMP9, MS4A1, MSMB, MT3 (metallothionectin-UI), mTOR, MTSS1, MUC1 (mucin), MYC,MYD88, NCK2, neurocan,NFKBI, NFKB2, NGFB (NGF), NGFR, NgR-Lingo, NgRNogo66, (Nogo), NgR-p75, NgR-Troy, NMEI (NM23A), NOTCH, NOTCH1, NOX5, NPPB, NROB1, NR0B2, NRID1, NR1D2, NR1H2, NR1H3, NR1H4, NR1I2, NR1I3, NR2C1, NR2C2, NR2E1, NR2E3, NR2F1, NR2F2, NR2F6, NR3C1, NR3C2, NR4A1, NR4A2, NR4A3, NR5A1, NR5A2, NR6A1, NRP1, NRP2, NT5E, NTN4,ODZI, OPRDI, P2RX7, PAP, PART1, PATE, PAWR, PCA3, PCDGF, PCNA, PDGFA, PDGFB, PDGFRA, PDGFRB, PECAMI, peg-asparaginase, PF4

(CXCL4), PGF, PGR, phosphacan, PIAS2, PI3 Kinase, PIK3CG, PLAU (uPA), PLG,PLXDCl, PKC, PKC-beta, PPBP (CXCL7), PPID, PR1, PRKCQ, PRKD1, PRL, PROC, PROK2, PSAP, PSCA, PTAFR, PTEN, PTGS2 (COX-2), PTN, RAC2 (P21Rac2), RANK, RANK ligand, RARB, RGS1, RGS13, RGS3, RNFI1O (ZNF144), Ron, ROBO2, RXR, S100A2, SCGB 1D2 (lípophilin B), SCGB2A1 (mammaglobin 2), SCGB2A2 (mammaglobin 1), SCYE1 (endothelial Monocyte-activating cytokine), SDF2,SERPENA1, SERPINA3, SERPINB5 (maspin), SERPINEl (PAI-l), SERPINFl, SHIP-1, SHIP-2, SHB1, SHB2, SHBG, SfcAZ, SLC2A2, SLC33A1, SLC43A1, SLIT2, SPP1, SPRR1B (Spr1), ST6GAL1, STAB1, STAT6, STEAP, STEAP2, TB4R2, TBX21, TCP1O, TDGF1, TEK, TGFA, TGFB1, TGFB1I1, TGFB2, TGFB3, TGFBI, TGFBR1, TGFBR2, TGFBR3, THIL, THBS1 (thrombospondin-1), THBS2, THBS4, THPO, TIE (Tie-1), TIMP3, tissue factor, TLR1O, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TNF, TNF-a, TNFAIP2 (B94), TNFAIP3, TNFRSFI1A, TNFRSF1A, TNFRSF1B, TNFRSF21, TNFRSF5, TNFRSF6 (Fas), TNFRSF7, TNFRSF8, TNFRSF9, TNFSF1O (TRAIL), TNFSF1 1 (TRANCE), TNFSF12 (APO3L), TNFSF13 (April), TNFSF13B, TNFSF14 (HVEM-L), TNFSF15 (VEGI), TNFSF18, TNFSF4 (OX40 ligand), TNFSF5 (CD40 ligand), TNFSF6 (FasL), TNFSF7 (CD27 ligand), TNFSF8 (CD30 ligand), TNFSF9 (4-1BB ligand), TOLLIP, Toll-like receptors, TOP2A (topoisomerase lia), TP53, TPM1, TPM2,TRADD, TRAF1, TRAF2, TRAF3, TRAF4, TRAF5, TRAF6, TRKA, TREM1, TREM2, TRPC6, TSLP, TWEAK, Tyrosinase, uPAR, VEGF, VEGFB, VEGFC, versican, VHL C5, VLA-4, Wnt-1, XCL1 (lymphotactin),XCL2 (SCM-lb), XCRl (GPR5 / CCXCR1), YY1, and ZFPM2.

In some embodiments, antibodies lacking interchain cysteines herein comprise an epitope binding domain selected from the group consisting of: abagovomab, abatacept (also know as ORENCIA®), abciximab (also known as REOPRO®, c7E3 Fab), adalimumab (also known as HUMIRA®), adecatumumab, alemtuzumab (also known as CAMPATH®, MabCampath or Campath-1H), altumomab, afelimomab, anatumomab mafenatox, anetumumab, anrukizumab, apolizumab, arcitumomab, aselizumab, atlizumab, atorolimumab, bapineuzumab, basiliximab (also known as SIMULECT®), bavituximab, bectumomab (also known as LYMPHOSCAN®), belimumab (also known as LYMPHO-STAT-B®), bertilimumab, besilesomab, bevacizumab (also known as AVASTIN®), biciromab brallobarbital, bivatuzumab mertansine, campath, canakinumab (also known as ACZ885), cantuzumab mertansine, capromab (also known as PROSTASCINT®), catumaxomab (also known as REMOVAB®), cedelizumab (also known as CIMZIA®), certolizumab pegol, cetuximab (also known as ERBITUX®), clenoliximab, dacetuzumab, dacliximab, daclizumab (also known as ZENAPAX®), denosumab (also known as AMG 162), detumomab, dorlimomab

aritox, dorlixizumab, duntumumab, durimulumab, durmulumab, ecromeximab, eculizumab (also known as SOLIRIS®), edobacomab, edrecolomab (also known as Mab17-1A, PANOREX®), efalizumab (also known as RAPTIVA®), efungumab (also known as MYCOGRAB®), elsilimomab, enlimomab pegol, epitumomab cituxetan, efalizumab, epitumomab, epratuzumab, erlizumab, ertumaxomab (also known as REXOMUN®), etanercept (also known as ENBREL®), etaracizumab (also known as etaratuzumab, VITAXIN®, ABEGRINTM), exbivirumab, fanolesomab (also known as NEUTROSPEC®), faralimomab, felvizumab, fontolizumab (also known as HUZAF®), galiximab, gantenerumab, gavilimomab (also known as ABXCBL®), gemtuzumab ozogamicin (also known as MYLOTARG®), golimumab (also known as CNTO 148), gomiliximab, ibalizumab (also known as TNX-355), ibritumomab tiuxetan (also known as ZEVALIN®), igovomab, imciromab, infliximab (also known as REMICADE®), inolimomab, inotuzumab ozogamicin, ipilimumab (also known as MDX-010, MDX-101), iratumumab, keliximab, labetuzumab, lemalesomab, lebrilizumab, lerdelimumab, lexatumumab (also known as, HGS-ETR2, ETR2-ST01), lexitumumab, libivirumab, lintuzumab, lucatumumab, lumiliximab, mapatumumab (also known as HGSETR1,TRM-1), maslimomab, matuzumab (also known as EMD72000), mepolizumab (also known as BOSATRIA®), metelimumab, milatuzumab, minretumomab, mitumomab, morolimumab, motavizumab (also known as NUMAXTM), muromonab (also known as OKT3), nacolomab tafenatox, naptumomab estafenatox, natalizumab (also known as TYSABRI®, ANTEGREN®), nebacumab, nerelimomab, nimotuzumab (also known as THERACIM hR3®, THERA-CIM-hR3®, THERALOC®) , nofetumomab merpentan (also known as VERLUMA®), ocrelizumab, odulimomab, ofatumumab, omalizumab (also known as XOLAIR®), oregovomab (also known as OVAREX®), otelixizumab, pagibaximab, palivizumab (also known as SYNAGIS®), panitumumab (also known as ABX-EGF, VECTIBIX®), pascolizumab, pemtumomab (also known as THERAGYN®), pertuzumab (also known as 2C4, OMNITARG®), pexelizumab, pintumomab, priliximab, pritumumab, ranibizumab (also known as LUCENTIS®), raxibacumab, regavirumab, reslizumab, rituximab (also known as RITUXAN®, MabTHERA®), rovelizumab, ruplizumab, satumomab, sevirumab, sibrotuzumab, siplizumab (also known as MEDI-507), sontuzumab, stamulumab (also known as MYO-029), sulesomab (also known as LEUKOSCAN®), tacatuzumab tetraxetan, tadocizumab, talizumab, taplitumomab paptox, tefibazumab (also known as AUREXIS®), telimomab aritox, teneliximab, teplizumab, ticilimumab, tocilizumab (also known as ACTEMRA®), toralizumab, tositumomab, trastuzumab (also known as HERCEPTIN®), tremelimumab (also known as CP-675,206), tucotuzumab celmoleukin, tuvirumab, urtoxazumab, ustekinumab (also known as CNTO 1275), vapaliximab, veltuzumab, vepalimomab, visilizumab (also known as NUVION®), volociximab (also known as M200), votumumab (also known as

HUMASPECT®), zalutumumab, zanolimumab (also known as HuMAX-CD4), ziralimumab, or zolimomab aritox.

In certain embodiments, antibodies lacking interchain cysteines herein comprise an epitope binding domain that binds to the same antigen as the antibodies listed above. In some embodiments, antibodies herein comprise an epitope binding domain that competes for binding with an antibody selected from selected from the antibodies listed above.

In vitro binding of an antibody herein may be quantified by means known in the art, for example microarrays, immobilization on a BIAcore® chip, enzyme linked immunoabsorbant assay (ELISA). In certain embodiments, chromogenic reporters and substrates produce an observable color change to indicate the presence of antigen or analyte. In some embodiments assay techniques utilize fluorogenic, electrochemiluminescent, real-time PCR reporters, and electroimmunoassays to create quantifiable signals.

In vivo binding of an antibody herein may be assayed in biological samples using in situ determination. A biological sample may be any tissue, substance or fluid as described below. In situ binding determination techniques include without limitation, staining, dyes, fluorescent labels, radiographic assays, tapping mode atomic force microscopy, and micro-radioisotopic antiglobulin assay.

The modifications inherent in cysteine engineering may affect binding specificity. For example, in some embodiments an antibody lacking interchain cysteines herein does not bind to a human leucocyte receptor. In certain embodiments an antibody herein does not bind to a FcγRIII receptor. The engineered antibody, however, closely mimics the binding of native antibody to the protein kinase domain of certain cell surface receptor molecules including human neonatal Fc leucocyte receptor (FcLR), epidermal growth factor receptor (EGFR) and HER3. Fluorescence indicates binding of an anti-EGFR antibody lacking interchain cysteines EGFR expressed on a cell surface. Saporin-conjugated anti-Her3 (mAb) and anti-Her3 (antibody lacking interchain cysteines) show similar killing potency on SKBr3 cells, suggesting that the mAb and antibody lacking interchain cysteines have similar binding and internalization kinetics.

Expression systems

Recombinant expression of an antibody herein, derivative, analog or fragment thereof, requires construction of an expression vector containing a polynucleotide that encodes the antibody. Once a polynucleotide encoding an antibody or a heavy or light chain of an antibody, or portion thereof, herein has been obtained, the vector for the production of the antibody may be produced by recombinant DNA technology using techniques known in the art. Thus, methods for preparing a protein by expressing a polynucleotide containing an antibody encoding nucleotide sequence are described herein. Methods which are known in the art can be used to construct expression vectors containing antibody coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, in vitro recombinant DNA techniques, synthetic techniques, and in vivo genetic recombination.

Herein provided, thus, are replicable vectors comprising a nucleotide sequence encoding an antibody herein, a heavy or light chain of an antibody, a heavy or light chain variable domain of an antibody or a portion thereof, or a heavy or light chain CDR, operably linked to a promoter. Such vectors may include the nucleotide sequence encoding the constant region of the antibody and the variable domain of the antibody may be cloned into such a vector for expression of the entire heavy chain, the entire light chain, or both the entire heavy and light chains.

The expression vector is transferred to a host cell by conventional techniques and the transfected cells are then cultured by conventional techniques to produce an antibody herein. Thus, provided herein are host cells containing a polynucleotide encoding an antibody herein or fragments thereof, or a heavy or light chain thereof, or portion thereof, or a single chain antibody herein, operably linked to a heterologous promoter. In certain embodiments for the expression of double-chained antibodies, vectors encoding both the heavy and light chains may be co-expressed in the host cell for expression of the entire immunoglobulin molecule, as detailed below.

A variety of host-expression vector systems may be utilized to express the antibodies herein. Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, express an antibody herein in situ. These include but are not limited to microorganisms such as bacteria (e.g., E. coli and B. subtilis) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression

vectors containing antibody coding sequences; yeast (e.g., Saccharomyces; Pichia) transformed with recombinant yeast expression vectors containing antibody coding sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing antibody coding sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing antibody coding sequences; or mammalian cell systems (e.g., COS, CHO, BHK, 293, NS0, and 3T3 cells) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter).

Bacterial cells such as Escherichia coli, and eukaryotic cells, may be used for the expression of a recombinant antibody. For non-limiting example, mammalian cells such as Chinese hamster ovary cells (CHO), in conjunction with a vector such as the major intermediate early gene promoter element from human cytomegalovirus can be an effective expression system for antibodies.

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the antibody being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions of an antibody, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited to, the E. coli expression vector pUR278, in which the antibody coding sequence may be ligated individually into the vector in frame with the lac Z coding region so that a fusion protein is produced; pIN vectors and the like. PGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione 5-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption and binding to matrix glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In an insect system, Autographa californica nuclear polyhedrosis In an insect system, Autographa californica nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in Spodoptera frugiperda cells. The antibody coding sequence may be cloned

individually into nonessential regions of the virus and placed under control of an AcNPV promoter.

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the antibody coding sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a nonessential region of the viral genome (e.g., region El or E3) may result in a recombinant virus that is viable and capable of expressing the antibody in infected hosts. Specific initiation signals may also be required for efficient translation of inserted antibody coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc.

In some embodiments, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, W138, BT483, Hs578T, HTB2, BT2O, NS1 and T47D, NS0 (a murine myeloma cell line that does not endogenously produce any immunoglobulin chains), CRL7O3O and HsS78Bst cells.

For long-term, high-yield production of recombinant proteins, stable expression is appropriate. For example, cell lines which stably express the antibody may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the

introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may be used to engineer cell lines which express the antibody. Such engineered cell lines may be useful in screening and evaluation of compositions that interact directly or indirectly with the antibody.

In certain embodiments, antibodies presented herein are expressed in a cell line with transient expression of the antibody. Transient transfection is a process in which the nucleic acid introduced into a cell does not integrate into the genome or chromosomal DNA of that cell but is maintained as an extrachromosomal element, e.g. as an episome, in the cell. Transcription processes of the nucleic acid of the episome are not affected and a protein encoded by the nucleic acid of the episome is produced.

The cell line, either stable or transiently transfected, is maintained in cell culture medium and conditions known in the art resulting in the expression and production of monoclonal antibodies. In certain embodiments, the mammalian cell culture media is based on commercially available media formulations, including, for example, DMEM or Ham's F12. In some embodiments, the cell culture media is modified to support increases in both cell growth and biologic protein expression. As used herein, the terms “cell culture medium,” “culture medium,” and “medium formulation” refer to a nutritive solution for the maintenance, growth, propagation, or expansion of cells in an artificial in vitro environment outside of a multicellular organism or tissue. Cell culture medium may be optimized for a specific cell culture use, including, for example, cell culture growth medium which is formulated to promote cellular growth, or cell culture production medium which is formulated to promote recombinant protein production. The terms nutrient, ingredient, and component may be used interchangeably to refer to the constituents that make up a cell culture medium.

In various embodiments, the cell lines are maintained using a fed batch method. As used herein, “fed batch method,” refers to a method by which a fed batch cell culture is supplied with additional nutrients after first being incubated with a basal medium. For example, a fed batch method may comprise adding supplemental media according to a determined feeding schedule within a given time period. Thus, a “fed batch cell culture” refers to a cell culture where the cells, typically mammalian, and culture medium are supplied to the culturing vessel initially and additional culture

nutrients are fed, continuously or in discrete increments, to the culture during culturing, with or without periodic cell and/or product harvest before termination of culture.

The cell culture medium used and the nutrients contained therein are known to one of skill in the art. In some embodiments, the cell culture medium comprises a basal medium and at least one hydrolysate, e.g., soy-based, hydrolysate, a yeast-based hydrolysate, or a combination of the two types of hydrolysates resulting in a modified basal medium. The additional nutrients may sometimes include only a basal medium, such as a concentrated basal medium, or may include only hydrolysates, or concentrated hydrolysates. Suitable basal media include, but are not limited to Dulbecco's Modified Eagle's Medium (DMEM), DME/F12, Minimal Essential Medium (MEM), Basal Medium Eagle (BME), RPMI 1640, F-10, F-12, α-Minimal Essential Medium (α-MEM), Glasgow's Minimal Essential Medium (G-MEM), PF CHO (see, e.g., CHO protein free medium (Sigma) or EX-CELL™ 325 PF CHO Serum-Free Medium for CHO Cells Protein-Free (SAFC Bioscience), and Iscove's Modified Dulbecco's Medium. Other examples of basal media which may be used in the technology herein include BME Basal Medium (Gibco-Invitrogen; Dulbecco's Modified Eagle Medium (DMEM, powder) (Gibco-Invitrogen (# 31600)).

In certain embodiments, the basal medium may be is serum-free, meaning that the medium contains no serum (e.g., fetal bovine serum (FBS), horse serum, goat serum, or any other animal-derived serum known to one skilled in the art) or animal protein free media or chemically defined media.

The basal medium may be modified in order to remove certain non-nutritional components found in standard basal medium, such as various inorganic and organic buffers, surfactant(s), and sodium chloride. Removing such components from basal cell medium allows an increased concentration of the remaining nutritional components, and may improve overall cell growth and protein expression. In addition, omitted components may be added back into the cell culture medium containing the modified basal cell medium according to the requirements of the cell culture conditions. In certain embodiments, the cell culture medium contains a modified basal cell medium, and at least one of the following nutrients, an iron source, a recombinant growth factor; a buffer; a surfactant; an osmolarity regulator; an energy source; and non-animal hydrolysates. In addition, the modified basal cell medium may optionally contain amino acids, vitamins, or a combination of both amino acids and vitamins. In some embodiments, the modified basal medium further contains glutamine, e.g, L-glutamine, and/or methotrexate.

In some embodiments, antibody production is conducted in large quantity by a bioreactor process using fed-batch, batch, perfusion or continuous feed bioreactor methods known in the art. Large-scale bioreactors have at least 1000 liters of capacity, sometimes about 1,000 to 100,000 liters of capacity. These bioreactors may use agitator impellers to distribute oxygen and nutrients. Small scale bioreactors refers generally to cell culturing in no more than approximately 100 liters in volumetric capacity, and can range from about 1 liter to about 100 liters. Alternatively, single-use bioreactors (SUB) may be used for either large-scale or small scale culturing.

Temperature, pH, agitation, aeration and inoculum density may vary depending upon the host cells used and the recombinant protein to be expressed. For example, a recombinant protein cell culture may be maintained at a temperature between 30 and 45 degrees Celsius. The pH of the culture medium may be monitored during the culture process such that the pH stays at an optimum level, which may be for certain host cells, within a pH range of 6.0 to 8.0. An impellor driven mixing may be used for such culture methods for agitation. The rotational speed of the impellor may be approximately 50 to 200 cm/sec tip speed, but other airlift or other mixing/aeration systems known in the art may be used, depending on the type of host cell being cultured. Sufficient aeration is provided to maintain a dissolved oxygen concentration of approximately 20% to 80% air saturation in the culture, again, depending upon the selected host cell being cultured. Alternatively, a bioreactor may sparge air or oxygen directly into the culture medium. Other methods of oxygen supply exist, including bubble-free aeration systems employing hollow fiber membrane aerators. A number of selection systems may be used, including but not limited to, the herpes simplex virus thymidine kinase, glutamine synthetase, hypoxanthine guanine phosphoribosyltransferase, and adenine phosphoribosyltransferase genes can be employed in tk-, gs-, hgprt- or aprt- cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate gpt, which confers resistance to mycophenolic acid, neo, which confers resistance to the aminoglycoside G-418, and hygro, which confers resistance to hygromycin.

Methods known in the art of recombinant DNA technology may be applied to select the desired recombinant clone. including but not limited to, the herpes simplex virus thymidine kinase, glutamine synthetase, hypoxanthine guanine phosphoribosyltransferase, and adenine phosphoribosyltransferase genes can be employed in tk-, gs-, hgprt- or aprt- cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes:

dhfr, which confers resistance to methotrexate; gpt, which confers resistance to mycophenolic acid; neo, which confers resistance to the aminoglycoside G-418; and hygro, which confers resistance to hygromycin. Methods known in the art of recombinant DNA technology may be applied to select the desired recombinant clone.

The expression levels of an antibody can be increased by vector amplification. When a marker in the vector system expressing antibody is amplifiable, increase in the level of inhibitor present in culture of host cell may increase the number of copies of the marker gene. Since the amplified region is associated with the antibody gene, production of the antibody may also increase.

The host cell may be co-transfected with two expression vectors of the antibodies herein; the first vector encoding a heavy chain derived polypeptide and the second vector encoding a light chain derived polypeptide. The two vectors may contain identical selectable markers which enable equal expression of heavy and light chain polypeptides. Alternatively, a single vector may be used which encodes, and is capable of expressing, both heavy and light chain polypeptides. In such situations, the light chain should be placed before the heavy chain to avoid an excess of toxic free heavy chain. The coding sequences for the heavy and light chains may comprise cDNA or genomic DNA.

Once an antibody lacking interchain cysteines has been produced by recombinant expression, it may be purified by any method known in the art for purification of an immunoglobulin molecule, for example, by chromatography (e.g., ion exchange, affinity, particularly by affinity for the specific antigen after Protein A, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. Further, the antibodies herein or fragments thereof may be fused to heterologous polypeptide sequences described herein or otherwise known in the art to facilitate purification.

Phage display techniques

In phage display methods, functional antibody domains are displayed on the surface of phage particles which carry the polynucleotide sequences encoding them. In particular, DNA sequences encoding VH and VL domains are amplified from animal cDNA libraries (e.g., human or murine cDNA libraries of lymphoid tissues). The DNA encoding the VH and VL domains are recombined together with an scFv linker by PCR and cloned into a phagemid vector (e.g., p CANTAB 6 or pComb 3 HSS). The vector is electroporated in E. coli and the E. coli is infected with helper phage.

Phage used in these methods are typically filamentous phage including fd and M13 and the VH and VL domains are usually recombinantly fused to either the phage gene III or gene VIII. Phage expressing an antigen binding domain that binds to an epitope of interest can be selected or identified with antigen, e.g., using labeled antigen or antigen bound or captured to a solid surface or bead. Phage display methods are known in the art.

After phage selection, the antibody coding regions from the phage can be isolated and used to generate whole antibodies, including human antibodies, or any other desired antigen binding fragment, and expressed in any desired host, including mammalian cells, insect cells, plant cells, yeast, and bacteria, e.g., as described below. Techniques to recombinantly produce Fab, Fab' and F(ab') 2 fragments can also be employed using methods known in the art.

To generate whole antibodies, PCR primers including VH or VL nucleotide sequences, a restriction site, and a flanking sequence to protect the restriction site can be used to amplify the VH or VL sequences in scFv clones. Utilizing cloning techniques known to those of skill in the art, the PCR amplified VH domains can be cloned into vectors expressing a VH constant region, e.g., the human gamma 4 constant region, and the PCR amplified VL domains can be cloned into vectors expressing a VL constant region, e.g., human kappa or lambda constant regions. The vectors for expressing the VH or VL domains sometimes comprise an EF-1α promoter, a secretion signal, a cloning site for the variable domain, constant domains, and a selection marker such as neomycin. The VH and VL domains may also be cloned into one vector expressing the necessary constant regions. The heavy chain conversion vectors and light chain conversion vectors are then co-transfected into cell lines to generate stable or transient cell lines that express full-length antibodies, e.g., IgG, using techniques known in the art.

Nucleic acids

A polynucleotide may be obtained, and the nucleotide sequence of the polynucleotide determined, by any method known in the art. Since the amino acid sequences of antibodies are known, nucleotide sequences encoding these antibodies can be determined using methods known in the art, e.g., nucleotide codons known to encode particular amino acids are assembled in such a way to generate a nucleic acid that encodes the antibody or fragment thereof herein. Such a polynucleotide encoding the antibody may be assembled from chemically synthesized oligonucleotides which, briefly, involves the synthesis of overlapping oligonucleotides containing

portions of the sequence encoding the antibody, annealing and ligating of those oligonucleotides, and then amplification of the ligated oligonucleotides by PCR.

In some embodiments, a polynucleotide encoding an antibody may be generated from nucleic acid from a suitable source. If a clone containing a nucleic acid encoding a particular antibody is not available, but the sequence of the antibody is known, a nucleic acid encoding the immunoglobulin may be chemically synthesized or obtained from a suitable source (e.g., an antibody cDNA library, or a cDNA library generated from, or nucleic acid, sometimes poly A+ RNA, isolated from, any tissue or cells expressing the antibody by PCR amplification using synthetic primers hybridizable to the 3' and 5' ends of the sequence or by cloning using an oligonucleotide probe specific for the particular gene sequence to identify, e.g., a cDNA clone from a cDNA library that encodes the antibody. Amplified nucleic acids generated by PCR may then be cloned into replicable cloning vectors using any method known in the art.

Once the nucleotide sequence of the antibody is determined, the nucleotide sequence of the antibody may be manipulated using methods known in the art for the manipulation of nucleotide sequences, e.g., recombinant DNA techniques, site directed mutagenesis, PCR, etc. to generate antibodies having a different amino acid sequence, for example to create amino acid substitutions, deletions, and/or insertions.

In certain embodiments, one or more of the CDRs is inserted within framework regions using recombinant DNA techniques. The framework regions may be naturally occurring or consensus framework regions, and sometimes human framework regions. The polynucleotide generated by the combination of the framework regions and CDRs may encode an antibody that specifically binds to EGFR, HER3, or other selected antigen. In some embodiments, as discussed supra, one or more amino acid substitutions may be made within the framework regions, and the amino acid substitutions may improve binding of the antibody to its antigen. Additionally, such methods may be used to make amino acid substitutions or deletions of one or more variable region cysteine residues participating in an intrachain disulfide bond to generate antibodies lacking one or more intrachain disulfide bonds. Other alterations to the polynucleotide are encompassed by the present disclosure and within the skill of the art.

Nucleic acid sequences for exemplarly antibodies of the disclosure are provided below. The codons that are altered are indicated in bold and underline text.

>mAb-VL-CL-kappa-anti-EGFR (SEQ ID NO: 15)

GACATCCAGATGACCCAGAGCCCCAGCAGCCTGAGCGCCAGCGTGGGCGACAGAGTGACCA
TCACCTGCCAGGCCAGCCAGGACATCAGCAACTACCTGAACTGGTATCAGCAGAAGCCCGGC
AAGGCCCCCAAGCTGCTGATCTACGACGCCAGCAACCTGGAGACAGGCGTGCCCAGCAGAT
TCAGCGGCAGCGGCTCCGGCACCGACTTCACCTTCACCATCAGCAGCCTCCAGCCCGAGGA
TATCGCCACCTACTTTTGCCAGCACTTCGACCACCTGCCCCTGGCCTTTGGCGGCGGAACAA
AGGTGGAGATCAAGCGTACGGTGGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGATGAG
CAGTTGAAATCTGGAACTGCCTCTGTTGTGTGCCTGCTGAATAACTTCTATCCCAGAGAGGCC
AAAGTACAGTGGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTCACAGA
GCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCTGACGCTGAGCAAAGCAGAC
TACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCAC
AAAGAGCTTCAACAGGGGAGAG**TGT**

>mAb-VH-CH1gamma1-CH2gamma2-CH3gamma3-anti-EGFR (SEQ ID NO: 16)

CAGGTGCAGCTCCAGGAGAGCGGCCCTGGCCTGGTGAAGCCCAGCGAGACACTGAGCCTGA
CCTGCACCGTGTCCGGCGGCAGCGTGTCCAGCGGCGACTACTACTGGACCTGGATCAGACA
GAGCCCCGGCAAGGGCCTGGAGTGGATCGGCCACATCTACTACAGCGGCAACACCAACTAC
AACCCCAGCCTGAAGTCCAGACTGACCATCAGCATCGACACCAGCAAGACCCAGTTCAGCCT
GAAGCTGTCCAGCGTGACAGCCGCCGACACCGCCATCTACTACTGCGTGAGAGACAGAGTG
ACCGGCGCTTTCGACATCTGGGGCCAGGGCACCATGGTGACCGTGTCCAGCGCGTCGACCA
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TGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGA
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GCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCAT
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>mAb-Val-VL-CL-kappa-anti-EGFR (SEQ ID NO: 17)
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TATCGCCACCTACTTTTGCCAGCACTTCGACCACCTGCCCCTGGCCTTTGGCGGCGGAACAA
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CAGTTGAAATCTGGAACTGCCTCTGTTGTGTGCCTGCTGAATAACTTCTATCCCAGAGAGGCC
AAAGTACAGTGGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTCACAGA
GCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCTGACGCTGAGCAAAGCAGAC
TACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCAC
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>mAb-Val-VH-CH1gamma1-CH2gamma2-CH3gamma3-anti-EGFR (SEQ ID NO: 18)
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GCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACA
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TGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGA
GAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTATAGCAA
GCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCAT
GAGGCTCTGCACAACCACTACACGCAGAAGAGCTTAAGCCTGTCTCCGGGTAAA

Scalable production of antibodies lacking interchain cysteines

An antibody lacking interchain cysteines may be produced by a scalable process. In some embodiments, antibodies lacking interchain cysteines may be produced by a scalable process in the research laboratory that may be scaled up to produce the proteins in analytical scale bioreactors (for example, but not limited to 5L, 10L, 15L, 30L, or 50L bioreactors) while maintaining the functional activity of the proteins. For instance, in an embodiment, proteins produced by scalable processes exhibit low to undetectable levels of aggregation as measured by HPSEC or rCGE, that is, no more than 5%, no more than 4%, no more than 3%, no more than 2%, no more than 1%, or no more than 0.5% aggregate by weight protein, and/or low to undetectable levels of fragmentation, that is, 80% or higher, 85% or higher, 90% or higher, 95% or higher, 98% or higher, or 99% or higher, or 99.5% or higher of the total peak area in the peak(s) representing intact antibodies lacking interchain cysteines.

In certain embodiments, the antibodies lacking interchain cysteines may be produced by a scalable process in the research laboratory that may be scaled up to produce the proteins in production scale bioreactors (for example, but not limited to 75L, 100L, 150L, 300L, or 500L). In some embodiments, the scalable process results in little or no reduction in production efficiency as compared to the production process performed in the research laboratory. In some embodiments, the scalable process produces antibodies lacking interchain cysteines at production efficiency of about 10 mg/L, about 20 m/L, about 30 mg/L, about 50 mg/L, about 75 mg/L, about 100 mg/L, about 125 mg/L, about 150 mg/L, about 175 mg/L, about 200 mg/L, about 250 mg/L, about 300 mg/L or higher.

In various embodiments, the scalable process produces antibodies lacking interchain cysteines at production efficiency of at least about 10 mg/L, at least about 20 mg/L, at least about 30 mg/L, at

least about 50 mg/L, at least about 75 mg/L, at least about 100 mg/L, at least about 125 mg/L, at least about 150 mg/L, at least about 175 mg/L, at least about 200 mg/L, at least about 250 mg/L, at least about 300 mg/L or higher.

In some embodiments, the scalable process produces antibodies lacking interchain cysteines at production efficiency from about 10 mg/L to about 300 mg/L, from about 10 mg/L to about 250 mg/L, from about 10 mg/L to about 200 mg/L, from about 10 mg/L to about 175 mg/L, from about 10 mg/L to about 150 mg/L, from about 10 mg/L to about 100 mg/L, from about 20 mg/L to about 300 mg/L, from about 20 mg/L to about 250 mg/L, from about 20 mg/L to about 200 mg/L, from 20 mg/L to about 175 mg/L, from about 20 mg/L to about 150 mg/L, from about 20 mg/L to about 125 mg/L, from about 20 mg/L to about 100 mg/L, from about 30 mg/L to about 300 mg/L, from about 30 mg/L to about 250 mg/L, from about 30 mg/L to about 200 mg/L, from about 30 mg/L to about 175 mg/L, from about 30 mg/L to about 150 mg/L, from about 30 mg/L to about 125 mg/L, from about 30 mg/L to about 100 mg/L, from about 50 mg/L to about 300 mg/L, from about 50 mg/L to about 250 mg/L, from about 50 mg/L to about 200 mg/L, from 50 mg/L to about 175 mg/L, from about 50 mg/L to about 150 mg/L, from about 50 mg/L to about 125 mg/L, from about 50 mg/L to about 100 mg/L.

Antibody purification and isolation

Once an antibody molecule has been produced by recombinant or hybridoma expression, it may be purified by any method known in the art for purification of an immunoglobulin molecule, for example, by chromatography (e.g., ion exchange, affinity, particularly by affinity for the specific antigens Protein A or Protein G, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. Further, the antibodies of the present technology or fragments thereof may be fused to heterologous polypeptide sequences (referred to herein as “tags”) described above or otherwise known in the art to facilitate purification.

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, are removed, for example, by centrifugation or ultrafiltration. For example, procedures for isolating antibodies which are secreted into the periplasmic space of E. coli are known in the art. Where the antibody is

secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, hydrophobic interaction chromatography, ion exchange chromatography, gel electrophoresis, dialysis, and/or affinity chromatography either alone or in combination with other purification steps. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody and will be understood by one of skill in the art. The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a CH3 domain, the Bakerbond ABX resin (J.T. Baker, Phillipsburg, NJ) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin, SEPHAROSE chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, and performed at low salt concentrations (e.g., from about 0-0.25 M salt).

Thus, in certain embodiments, antibodies as provided herein are substantially purified/isolated. In an embodiment, these isolated/purified recombinantly expressed antibodies may be administered to a patient to mediate a prophylactic or therapeutic effect. In some embodiments these isolated/purified antibodies may be used to diagnose a disease.

Stability

Suitable stability assays are available in the art. In an embodiment, assays pertaining to the stability of proteins known in the art are applied to antibodies described herein to determine their stability. In certain embodiments, the stability of antibodies described herein can be assessed by aggregation and/or fragmentation rate or profile. To determine the level of aggregation or fragmentation, many techniques may be used. In some embodiments, the aggregation and/or fragmentation profile may be assessed by the use of analytical ultracentrifugation (AUC), size-exclusion chromatography (SEC), high performance size-exclusion chromatography (HPSEC), melting temperature (Tm), polyacrylamide gel electrophoresis (PAGE), capillary gel electrophoresis (CGE), light scattering (SLS), Fourier Transform Infrared Spectroscopy (FTIR), circular dichroism (CD), urea-induced protein unfolding techniques, intrinsic tryptophan fluorescence, differential scanning calorimetry, or 1-anilino-8-naphthalenesulfonic acid (ANS) protein binding techniques. In another embodiment, the stability of proteins herein is characterized by polyacrylamide gel electrophoresis (PAGE) analysis. In another embodiment, the stability of the proteins herein is characterized by size exclusion chromatography (SEC) profile analysis.

The thermal melting temperatures (Tm) of the Fab domain of an antibody can be a good indicator of the thermal stability of an antibody and may further provide an indication of the antibody shelf-life. A lower Tm indicates more aggregation and less stability, whereas a higher Tm indicates less aggregation and more stability. Thus, in certain embodiments antibodies having higher Tm are selected and utilized. Tm of a protein or protein fragment (e.g., a Fab domain) can be measured using any standard method known in the art, for example, by differential scanning calorimetry. Stability may be determined in vitro or in vivo. Stability may also be determined in an animal.

Antibodies, like all polypeptides, have an isoelectric point (pI), which is generally defined as the pH at which a polypeptide carries no net charge. Protein solubility is typically lowest when the pH of the solution is equal to the isoelectric point (pI) of the protein. As used herein the pI value is defined as the pI of the predominant charge form. The pI of a protein may be determined by a variety of methods including, but not limited to, isoelectric focusing and various computer algorithms.

The formation of at least one non-naturally occurring disulfide bond may influence the stability of an antibody lacking interchain cysteines herein in comparison to the antibody prior to modification. In some embodiments, the non-naturally occurring disulfide bond may increase stability of the antibody lacking interchain cysteines as compared to the same antibody prior to cysteine

engineering. In various embodiments, the non-naturally occurring disulfide bond may decrease stability of an antibody lacking interchain cysteines as compared to the same antibody prior to cysteine engineering. In certain embodiments an antibody herein has a stability of about 70% or more compared to an antibody counterpart containing all native interchain cysteines. In some embodiments the stability is in vitro stability. In certain embodiments an antibody lacking interchain cysteines herein may be monomeric. In some embodiments an antibody herein may be dimeric.

In certain embodiments, an antibody lacking interchain cysteines has certain biochemical characteristics such as a particular isoelectric point (pI) or melting temperature (Tm). In some embodiments, an antibody lacking interchain cysteines has a pI ranging from 5.5 to 9.5. In certain embodiments, an antibody lacking interchain cysteines has a pI that ranges from about 5.5 to about 6.0, or about 6.0 to about 6.5, or about 6.5 to about 7.0, or about 7.0 to about 7.5, or about 7.5 to about 8.0, or about 8.0 to about 8.5, or about 8.5 to about 9.0, or about 9.0 to about 9.5. In various embodiments, an antibody lacking interchain cysteines has a pI that ranges from 5.5-6.0, or 6.0 to 6.5, or 6.5 to 7.0, or 7.0-7.5, or 7.5-8.0, or 8.0-8.5, or 8.5-9.0, or 9.0-9.5. An antibody lacking interchain cysteines sometimes has a pI of at least 5.5, or at least 6.0, or at least 6.3, or at least 6.5, or at least 6.7, or at least 6.9, or at least 7.1, or at least 7.3, or at least 7.5, or at least 7.7, or at least 7.9, or at least 8.1, or at least 8.3, or at least 8.5, or at least 8.7, or at least 8.9, or at least 9.1, or at least 9.3, or at least 9.5. In some embodiments, an antibody lacking interchain cysteines has a pI of at least about 5.5, or at least about 6.0, or at least about 6.3, or at least about 6.5, or at least about 6.7, or at least about 6.9, or at least about 7.1, or at least about 7.3, or at least about 7.5, or at least about 7.7, or at least about 7.9, or at least about 8.1, or at least about 8.3, or at least about 8.5, or at least about 8.7, or at least about 8.9, or at least about 9.1, or at least about 9.3, or at least about 9.5.

It is possible to optimize solubility by altering the number and location of ionizable residues in the antibody to adjust the pI. For example the pI of a polypeptide can be manipulated by making appropriate amino acid substitutions (e.g., by substituting a charged amino acid such as a lysine, for an uncharged residue such as alanine). Without wishing to be bound by any particular theory, amino acid substitutions of an antibody that result in changes of the pI of the antibody may improve solubility and/or the stability of the antibody. Appropriate amino acid substitutions can be selected for a particular antibody to achieve a desired pI. In some embodiments, a substitution is generated in an antibody to alter the pI. It is contemplated that the substitution(s) of the Fc region that result

in altered binding to FcR (described herein) may also result in a change in the pI. In certain embodiments, substitution(s) of the Fc region are specifically chosen to effect both the desired alteration in FcR binding and any desired change in pI.

In some embodiments, an antibody lacking interchain cysteines has a Tm ranging from 65°C to 120°C. In certain embodiments, an antibody lacking interchain cysteines has a Tm ranging from about 75°C to about 120°C, or about 75°C to about 85°C, or about 85°C to about 95°C, or about 95°C to about 105°C, or about 105°C to about 115°C, or about 115°C to about 120°C. In various embodiments, an antibody lacking interchain cysteines has a Tm ranging from 75°C to 120°C, or 75°C to 85°C, or 85°C to 95°C, or 95°C to 105°C, or 105°C to 115°C, or 115°C to 120°C. An antibody lacking interchain cysteines sometimes has a Tm of at least about 65°C, or at least about 70°C, or at least about 75°C, or at least about 80°C, or at least about 85°C, or at least about 90°C, or at least about 95°C, or at least about 100°C, or at least about 105°C, or at least about 110°C, or at least about 115°C, or at least about 120°C. In various embodiments, an antibody lacking interchain cysteines has a Tm of at least 65°C, or at least 70°C, or at least 75°C, or at least 80°C, or at least 85°C, or at least 90°C, or at least 95°C, or at least 100°C, or at least 105°C, or at least 110°C, or at least 115°C, or at least 120°C.

In various embodiments an antibody lacking interchain cysteines herein is stable at about 37º for five days or more. In some embodiments an antibody herein is stable in an animal for about 14 days or more.

Antibody conjugates

The use of antibody conjugates, i.e. immunoconjugates, for the local delivery of cytotoxic or cytostatic agents, i.e. drugs to kill or inhibit tumor cells in the treatment of cancer theoretically allows targeted delivery of the drug moiety to tumors, and intracellular accumulation therein, where systemic administration of these unconjugated drug agents may result in unacceptable levels of toxicity to normal cells as well as the tumor cells sought to be eliminated Efforts to design and refine antibody conjugates have focused on the selectivity of monoclonal antibodies (mAbs) as well as drug-linking and drug-releasing properties. Both polyclonal antibodies and monoclonal antibodies have been reported as useful in these strategies. Drugs used in these methods include daunomycin, doxorubicin, methotrexate, and vindesine.

Toxins used in antibody-toxin conjugates include bacterial toxins such as diphtheria toxin, plant toxins such as ricin, and gelonin, small molecule toxins such as geldanamycin, maytansinoids, and calicheamicin. The toxins may effect their cytotoxic and cytostatic effects by mechanisms including tubulin binding, DNA binding, or topoisomerase inhibition. Some cytotoxic drugs tend to be inactive or less active when conjugated to large antibodies or protein receptor ligands.

Several antibody conjugates have been approved by the FDA or are in clinical trials. For instance, ZEVALIN® (ibritumomab tiuxetan, Biogen/Idec) is composed of amurine IgG1 kappa monoclonal antibody directed against the CD20 antigen found on the surface of normal and malignant B lymphocytes and 111In or 90Y radioisotope bound by athiourea linker-chelator Although ZEVALIN® has activity against B cell non-Hodgkin's Lymphoma (NHL), administration results in severe and prolonged cytopenias in most patients. MYLOTARG® (gemtuzumab ozogamicin, Wyeth Pharmaceuticals), an antibody-drug conjugate composed of a human CD33 antibody linked to calicheamicin, was also approved in 2000 for the treatment of acute myeloid leukemia by injection. Cantuzumab mertansine (Immunogen, Inc.), an antibody-drug conjugate composed of the human C242 antibody linked via the disulfide linker SPP to the maytansinoid drug moiety, DM1, is advancing in clinical trials for the treatment of cancers that express CanAg, such as colon, pancreatic, gastric, and others. MLN-2704, an antibody-drug conjugate composed of composed of the anti-prostate specific membrane antigen (PSMA) monoclonal antibody linked to the maytansinoid drug moiety, DM1, is under development for the potential treatment of prostate tumors.

The auristatin peptides, auristatin E (AE) and monomethylauristatin (MMAE), synthetic analogs of dolastatin have been conjugated to: (i) chimeric monoclonal antibodies cBR96 (specific to Lewis Y on carcinomas); (ii) cAC10 which is specific to CD30 on hematological malignancies; (iii) anti-CD20 antibodies such as RITUXAN® for the treatment of CD20-expressing cancers and immune disorders; (iv) anti-EphB2R antibodies 2H9 and anti-IL-8 for treatment of colorectal cancer; (v) E-selectin antibody; and (vi) other anti-CD30 antibodies. Variants of auristatin E are disclosed in U.S. Pat. No. 5,767,237 and U.S. Pat. No. 6,124,431. Monomethyl auristatin E conjugated to monoclonal antibodies are disclosed in Senter et al, Proceedings of the American Association for Cancer Research, Volume 45, Abstract Number 623, presented Mar. 28, 2004. Auristatin analogs MMAE and MMAF have been conjugated to various antibodies (WO 2005/081711).

Provided herein, in some embodiments, is a method comprising the use of antibodies lacking interchain cysteines recombinantly fused or chemically conjugated (including both covalent and non-covalent conjugations) to a heterologous agent to generate a fusion protein as targeting moieties (hereinafter referred to as "antibody conjugates"). The heterologous agent may be linked to various regions of an antibody herein, including but not limited to the CH1, CH2, and CH3 domains. In some embodiments, a conjugated antibody herein comprises one or more hererologous agents. In certain embodiments, a conjugated antibody comprises an antibody homomultiplier conjugate.

The heterologous agent may be a polypeptide (or portion thereof, sometimes a polypeptide of at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90 or at least 100 amino acids), nucleic acid, small molecule (less than 1000 daltons), or inorganic or organic compound. The fusion does not necessarily need to be direct, but may occur through linker sequences. Antibodies fused or conjugated to heterologous agents may be used in vivo to detect, treat, manage, or monitor the progression of a disorder using methods known in the art. In some embodiments, the disorder to be detected, treated, managed, or monitored is an autoimmune, inflammatory, infectious disease or cancer related disorder. Methods for fusing or conjugating polypeptides to antibody portions are known in the art.

Additional fusion proteins may be generated through the techniques of gene shuffling, motif-shuffling, exon-shuffling, and/or codon-shuffling (collectively referred to as “DNA shuffling”). DNA shuffling may be employed to alter the activities of antibodies lacking interchain cysteines herein (e.g., antibodies with higher affinities and lower dissociation rates). Antibodies or fragments thereof, or the encoded antibodies or fragments thereof, may be altered by being subjected to random mutagenesis by error-prone PCR, random nucleotide insertion or other methods prior to recombination. One or more portions of a polynucleotide encoding an antibody or antibody fragment may be recombined with one or more components, motifs, sections, parts, domains, fragments, etc. of one or more heterologous agents.

In an embodiment, antibodies lacking interchain cysteines herein or fragments or variants thereof are conjugated to a marker sequence, such as a peptide, to facilitate purification. In certain embodiments, the marker amino acid sequence is a hexahistidine peptide (SEQ ID NO: 19), such as the tag provided in a pQE vector (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, CA, 91311), among others, many of which are commercially available. Hexa-histidine (SEQ ID NO: 19), for

example, provides for convenient purification of the fusion protein. Other peptide tags useful for purification include, but are not limited to, the hemagglutinin "HA" tag, which corresponds to an epitope derived from the influenza hemagglutinin protein and the "flag" tag.

In some embodiments, antibodies herein are conjugated to a diagnostic or detectable agent. In some embodiments the agent is a detectable label. In certain embodiments the agent is an imaging agent. Such antibodies can be useful for monitoring or prognosing the development or progression of a disorder (such as, but not limited to cancer) as part of a clinical testing procedure, such as determining the efficacy of a particular therapy.

Such diagnosis and detection may accomplished by coupling the antibody to detectable substances including, but not limited to various enzymes, such as but not limited to horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase; prosthetic groups, such as but not limited to streptavidin/biotin and avidin/biotin; fluorescent materials, such as but not limited to, umbelliferone, fluorescein, fluorescein isothiocynate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; luminescent materials, such as but not limited to, bioluminescent materials, such as but not limited to, luciferase, luciferin, and aequorin; radioactive materials, such as but not limited to, bismuth (^{213}Bi), carbon (^{14}C), chromium (^{51}Cr), cobalt (^{57}Co), fluorine (^{18}F), gadolinium (^{153}Gd, ^{159}Gd), gallium (^{68}Ga, ^{67}Ga), germanium (^{68}Ge), holmium (^{166}Ho), indium (^{115}In, ^{113}In, ^{112}In, ^{111}In), iodine (^{131}I, ^{123}I, ^{123}I, ^{121}I), lanthanium (^{140}La), lutetium (^{177}Lu), manganese (^{54}Mn), molybdenum (^{99}Mo), palladium (^{103}Pd), phosphorous (^{32}P), praseodymium (^{142}Pr), promethium (^{149}Pm), rhenium (^{186}Re, ^{188}Re), rhodium (^{105}Rh), ruthemium (^{97}Ru), samarium (^{153}Sm), scandium (^{47}Sc), selenium (^{75}Se), strontium (^{85}Sr), sulfur (^{35}S), technetium (^{99}Tc), thallium (^{201}Ti), tin (^{113}Sn, ^{117}Sn), tritium (^{3}H), xenon (^{133}Xe), ytterbium (^{169}Yb, ^{175}Yb), yttrium (^{90}Y), zinc (^{65}Zn); positron emitting metals using various positron emission tomographies, and nonradioactive paramagnetic metal ions.

In certain embodiments, antibodies lacking interchain cysteines herein are conjugated to a therapeutic agent such as a cytotoxin, e.g., a cytostatic or cytocidal agent, a therapeutic agent or a radioactive metal ion, e.g., alpha-emitters. A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Examples include paclitaxel, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, puromycin,

epirubicin, and cyclophosphamide and analogs or homologs thereof. Therapeutic agents include, but are not limited to, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BCNU) and lomustine (CCNU), cyclothosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cisdichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g., vincristine and vinblastine).

In an embodiment, the cytotoxic agent is selected from the group consisting of an enediyne, a lexitropsin, a duocarmycin, a taxane, a puromycin, a dolastatin, a maytansinoid, and a vinca alkaloid. In some embodiments, the cytotoxic agent is paclitaxel, docetaxel, CC-1065, SN-38, topotecan, morpholino-doxorubicin, rhizoxin, cyanomorpholinodoxorubicin,dolastatin-10, echinomycin, combretastatin, calicheamicin, maytansine, DM-1,an auristatin or other dolastatin derivatives, such as auristatin E or auristatin F, AEB, AEVB,AEFP, MMAE (monomethylauristatin E), MMAF (monomethylauristatin F), eleutherobin ornetropsin. The synthesis and structure of auristatin E (dolastatin-10), and its derivatives are known in the art.

In some embodiments, the cytotoxic agent of an antibody conjugate herein is an anti-tubulin agent. Anti-tubulin agents are a well established class of cancer therapy compounds. Examples of anti-tubulin agents include, but are not limited to, taxanes (e.g., Taxol (paclitaxel), docetaxel), T67 (Tularik), vincas, and auristatins (e.g., auristatin E, AEB, AEVB, MMAE, MMAF, AEFP). Antitubulin agents included in this class are also: vinca alkaloids, including vincristine and vinblastine, vindesine and vinorelbine; taxanes such as paclitaxel and docetaxel and baccatin derivatives, epithilone A and B, nocodazole, 5-Fluorouracil and colcimid, estramustine, cryptophysins, cemadotin, maytansinoids, combretastatins, dolastatins, discodermolide and eleutherobin In more specific embodiments, the cytotoxic agent is selected from the group consisting of a vinca alkaloid, a podophyllotoxin, a taxane, a baccatin derivative, a cryptophysin, a maytansinoid, a combretastatin, and a dolastatin. In certain embodiments, the cytotoxic agent is vincristine, vinblastine, vindesine, vinorelbine, VP-16, camptothecin, paclitaxel, docetaxel, epithilone A, epithilone B, nocodazole, coichicine, colcimid, estramustine, cemadotin, discodermolide, maytansine, DM-1, an auristatin or other dolastatin derivatives, such as auristatin E or auristatin F, AEB, AEVB, AEFP, MMAE (monomethylauristatin E), MMAF (monomethylauristatin F),

eleutherobin or netropsin.

In specific embodiments, the drug is a maytansinoid, a group of anti-tubulin agents. The drug is sometimes maytansine. The cytotoxic or cytostatic agent may be DM-1. Maytansine, a natural product, inhibits tubulin polymerization resulting in a mitotic block and cell death. Thus, the mechanism of action of maytansine appears to be similar to that of vincristine and vinblastine. Maytansine, however, is about 200 to 1,000-fold more cytotoxic in vitro than these vinca alkaloids. In an embodiment, the drug is an AEFP.

In some embodiments, the antibodies may be conjugated to other small molecule or protein toxins, such as, but not limited to abrin, brucine, cicutoxin, diphtheria toxin, batrachotoxin, botulism toxin, shiga toxin, endotoxin, tetanus toxin, pertussis toxin, anthrax toxin, cholera toxin falcarinol, fumonisin B1, fumonisin B2, afla toxin, maurotoxin, agitoxin, charybdotoxin, margatoxin, slotoxin, scyllatoxin, hefutoxin, calciseptine, taicatoxin, calcicludine, geldanamycin, gelonin, lotaustralin, ocratoxin A, patulin, ricin, strychnine, trichothecene, zearlenone, and tetradotoxin. Further examples of toxins, spacers, linkers, stretchers and the like, and their structures are known in the art.

As discussed herein, the compounds used for conjugation to the antibody conjugates herein can include conventional chemotherapeutics, such as doxorubicin, paclitaxel, carboplatin, melphalan, vinca alkaloids, methotrexate, mitomycin C, etoposide, and others. In addition, potent agents such CC-1065 analogues, calichiamicin, maytansine, analogues of dolastatin 10, rhizoxin, and palytoxin can be linked to the antibodies using the conditionally stable linkers to form potent immunoconjugates.

In certain embodiments, the cytotoxic or cytostatic agent is a dolastatin. In specific embodiments, the dolastatin is of the auristatin class. In some embodiments, the cytotoxic or cytostatic agent is MMAE. In various embodiments, the cytotoxic or cytostatic agent is AEFP. In some embodiments , the cytotoxic or cytostatic agent is MMAF.

In certain embodiments, antibodies herein are conjugated to a therapeutic agent or drug moiety that modifies a given biological response. Therapeutic agents or drug moieties are not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for

example, a toxin such as abrin, ricin A, pseudomonas exotoxin, cholera toxin, or diphtheria toxin; a protein such as tumor necrosis factor, alpha-interferon, beta-interferon, nerve growth factor, platelet derived growth factor, tissue plasminogen activator, an apoptotic agent, e.g., TNF-alpha, TNF-beta, AIM I, a thrombotic agent or an anti-angiogenic agent, e.g., angiostatin or endostatin; or, a biological response modifier such as, for example, a lymphokine (e.g., interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-7 (IL-7), interleukin-9 (IL-9), interleukin-15 (IL-15), interleukin-12 (IL-12), granulocyte macrophage colony stimulating factor (GMCSF), and granulocyte colony stimulating factor (G-CSF)), or a growth factor (e.g., growth hormone (GH)).

In some embodiments, antibodies herein are conjugated to a polypeptide that comprises poly arginine or poly-lysine residues. In certain embodiments, said polypeptide comprises 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or more amino acid residues. In some embodiments, the poly-arginine polypeptide may comprise at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or more arginine residues. In some embodiments, the poly-lysine polypeptide may comprise at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or more lysine residues. In various embodiments, the polypeptide may comprise any combination of arginine and lysine residues.

In some embodiments, antibodies herein are conjugated to a therapeutic agent such as radioactive materials or macrocyclic chelators useful for conjugating radiometal ions (see above for examples of radioactive materials). In certain embodiments, the macrocyclic chelator is 1,4,7,10-tetraazacyclododecane-N,N',N'',N''-tetraacetic acid (DOTA) which can be attached to the antibody via a linker molecule. Such linker molecules, further discussed herein below, are known in the art. In some embodiments, antibodies herein are conjugated to a nucleic acid. The nucleic acid may be selected from the group consisting of DNA, RNA, short interfering RNA (siRNA), microRNA, hairpin or nucleic acid mimetics such as peptide nucleic acid. In certain embodiments the conjugated nucleic acid is at least 10, at least 20, at least 30, at least 40, at least 50 , at least 60 at least 100, at least 200, at least 500, at least 1000, at least 5000 or more base pairs. The conjugated nucleic acid is sometimes single stranded. In various embodiments, the conjugated nucleic acid is double stranded.

In some embodiments, the conjugated nucleic acid encodes an open reading frame. In some embodiments, the open reading frame encoded by the conjugated nucleic acid corresponds to an

apoptosis inducing protein, a viral protein, an enzyme, or a tumor suppressor protein. Techniques for delivery of such nucleic acids to cells are known in the art.

Techniques for conjugating therapeutic moieties to antibodies are known in the art. Moieties may be conjugated to antibodies by any method known in the art, including, but not limited to aldehyde/Schiff linkage, sulphydryl linkage, acid-labile linkage, cis-aconityl linkage, hydrazone linkage, and enzymatically degradable linkage. Additional techniques for conjugating therapeutic moieties to antibodies are also known. Methods for fusing or conjugating antibodies to polypeptide moieties are also known in the art. The fusion of an antibody to a moiety does not necessarily need to be direct, but may occur through linker sequences. Such linker molecules are known in the art.

Two approaches may be taken to minimize drug activity outside the cells that are targeted by the antibody conjugates herein: first, an antibody that binds to cell membrane receptor but not soluble receptor may be used, so that the drug, including drug produced by the actions of the prodrug converting enzyme, is concentrated at the cell surface of the activated lymphocyte. Another approach for minimizing the activity of drugs bound to the antibodies herein is to conjugate the drugs in a manner that would reduce their activity unless they are hydrolyzed or cleaved off the antibody. Such methods would employ attaching the drug to the antibodies with linkers that are sensitive to the environment at the cell surface of the activated lymphocyte (e.g., the activity of a protease that is present at the cell surface of the activated lymphocyte) or to the environment inside the activated lymphocyte the conjugate encounters when it is taken up by the activated lymphocyte (e.g., in the endosomal or, for example by virtue of pH sensitivity or protease sensitivity, in the lysosomal environment). Examples of linkers that can be used for conjugation of the antibodies herein are known in the art.

In an embodiment, the linker is an acid-labile hydrazone or hydrazide group that is hydrolyzed in the lysosome. In certain embodiments, drugs can be appended to antibodies through other acid-labile linkers, such as cis-aconitic amides, orthoesters, acetals and ketals. Such linkers are relatively stable under neutral pH conditions, such as those in the blood, but are unstable at below pH 5, the approximate pH of the lysosome.

In some embodiments, drugs are attached to the antibodies herein using peptide spacers that are cleaved by intracellular proteases. Target enzymes include cathepsins B and D and plasmin, all of which are known to hydrolyze dipeptide drug derivatives resulting in the release of active drug

inside target cells. In intracellular proteolytic drug release the drug may be highly attenuated when conjugated and the serum stabilities of the conjugates may also be high. In some embodiments, the linker is a malonate linker, a maleimidobeiizoyl linker, or a 3'-N-amide analog.

As discussed above, antibody conjugates may be made by conjugating a compound or a drug to an antibody through a linker. Any linker that is known in the art may be used in the conjugates herein, e.g., bifunctional agents (such as dialdehydes or imidoesters) or branched hydrazone linkers.

In certain, non-limiting, embodiments herein, the linker region between the conjugate moiety and the antibody moiety is cleavable under certain conditions, where cleavage or hydrolysis of the linker releases the drug moiety from the antibody moiety. In some embodiments, the linker is sensitive to cleavage or hydrolysis under intracellular conditions.

In an embodiment, the linker region between the conjugate moiety and the antibody moiety is cleavable if the pH changes by a certain value or exceeds a certain value. In some embodiments herein, the linker is cleavable in the milieu of the lysosome, e.g., under acidic conditions (i.e., a pH of around 5-5.5 or less). In certain embodiments, the linker is a peptidyl linker that is cleaved by a peptidase or protease enzyme, including but not limited to a lysosomal protease enzyme, a membrane-associated protease, an intracellular protease, or an endosomal protease. The linker is sometimes at least two amino acids long, and may be at least three amino acids long. For example, a peptidyl linker that is cleavable by cathepsin-B (e.g., a Gly-Phe-Leu-Gly linker), a thiol-dependent protease that is highly expressed in cancerous tissue, can be used. Other such linkers are known in the art.

In other, non-mutually exclusive embodiments herein, the linker by which the antibody and compound of an antibody conjugate herein are conjugated promotes cellular internalization. In certain embodiments, the linker-drug moiety promotes cellular internalization. In certain embodiments, the linker is chosen such that the structure of the entire antibody conjugate promotes cellular internalization. In various embodiments, the linker is a thioether linker. In some embodiments, the linker is a hydrazone linker.

In certain embodiments, the linker is a disulfide linker. A variety of disulfide linkers are known in the art, including but not limited to those that can be formed using SATA (N-succinimidyl-S-

acetylthioacetate), SPDP (N-succinimidyl-3-(2-pyridyldithio) propionate), SPDB (N-succinimidyl-3-(2-pyridyldithio)butyrate) and SMPT (Nsuccinimidyl- oxycarbonyl-alpha-methyl-alpha-(2-pyridyl-dithio)tol- uene). SPDB and SMPT. A variety of linkers that can be used with the compositions and methods herein are known in the art.

In some embodiments herein, the linker unit of an antibody conjugate links the cytotoxic or cytostatic agent (drug unit; -D) and the antibody unit (-A). In certain embodiments, the linker unit has the general formula:

i. -Ta-Ww--Yy-- where:
ii. -T- is a stretcher unit;
iii. a is 0 or 1;
iv. each --W--is independently an amino acid unit;
v. w is independently an integer ranging from 2 to 12;
vi. --Y-- is a spacer unit; and
vii. y is 0, 1 or 2.

The stretcher unit (-T-), when present, links the antibody unit to an amino acid unit (--W--). Useful functional groups that can be present on an antibody, either naturally or via chemical manipulation include, but are not limited to, sulfhydryl, amino, hydroxyl, the anomeric hydroxyl group of a carbohydrate, and carboxyl. Antibodies lacking interchain cysteines herein present at least one free sulfhydryl groups for conjugation. Other methods of introducing free sulfhydryl groups may involve the reduction of the intramolecular disulfide bonds of an antibody. Sulfhydryl groups can also be generated by reaction of an amino group of a lysine moiety of an antibody with 2-iminothiolane (Traut's reagent) or other sulfhydryl generating reagents.

The amino acid unit (--W--) links the stretcher unit (-T-) to the Spacer unit (--Y--) if the Spacer unit is present, and links the stretcher unit to the cytotoxic or cytostatic agent (Drug unit; D) if the spacer unit is absent.

In some embodiments, --Ww-- is a dipeptide, tripeptide, tetrapeptide, pentapeptide, hexapeptide, heptapeptide, octapeptide, nonapeptide, decapeptide, undecapeptide or dodecapeptide unit. The amino acid unit of the linker unit can be enzymatically cleaved by an enzyme including, but not limited to, a tumor-associated protease to liberate the drug unit (-D) which is protonated in vivo

upon release to provide a cytotoxic drug (D).

In a an embodiment, the amino acid unit is a phenylalanine-lysine dipeptide (phe-lys or FK linker). In some embodiments, the amino acid unit is a valine-citrulline dipeptide (val-cit or VC linker).

The spacer unit (--Y--), when present, links an amino acid unit to the drug unit. Spacer units are of two general types: self-immolative and non self-immolative. A non self-immolative spacer unit is one in which part or all of the spacer unit remains bound to the drug unit after enzymatic cleavage of an amino acid unit from the antibody-linker-drug conjugate or the drug-linker compound. Examples of a non self-immolative spacer unit include, but are not limited to a (glycine-glycine) spacer unit and a glycine spacer unit. When an antibody-linker-drug conjugate herein containing a glycine-glycine spacer unit or a glycine spacer unit undergoes enzymatic cleavage via a tumor-cell associated-protease, a cancer-cell-associated protease or a lymphocyte-associated protease, a glycine-glycine-drug moiety or a glycine-drug moiety is cleaved from A-T-Ww--. To liberate the drug, an independent hydrolysis reaction may take place within the target cell to cleave the glycine drug unit bond.

Additional examples of self-immolative spacers include, but are not limited to aromatic compounds that are electronically equivalent to the PAB group such a 2-aminoimidazol-5-methanol derivatives. Spacers can be used that undergo facile cyclization upon amide bond hydrolysis, such as substituted and unsubstituted 4-aminobutyric acid amides, appropriately substituted ring systems, and 2-aminophenylpropionic acid amides. Elimination of amine-containing drugs that are substituted at the alpha-position of glycine are also examples of self-immolative spacer strategies that can be applied to the antibody-linker-drug conjugates herein.

Methods for conjugating a heterologous molecule to an antibody

Heterologous molecules, such as those described herein may be efficiently conjugated to antibodies herein through the free thiol groups the engineered cysteine residues provide. In one aspect, the method provides for efficiently conjugating heterologus molecules to antibodies lacking interchain cysteines. In some embodiments the conjugation of a heterologus molecule may occur at a free thiol group provided by at least one engineered cysteine residue selected from one or more positions shown in Table 2. In certain embodiments, the conjugation of a heterologus molecule may occur at a free thiol group provided by at least one engineered cysteine residue

selected from one or more positions shown in Table 3.

The engineering of non-naturally occurring cysteine residues into antibodies may alter the disulfide pairing of the heavy and light chains such that a naturally occurring cysteine residue which was part of a disulfide bond is liberated and presents a free thiol group capable of conjugation. In certain embodiments, the method comprises the efficient conjugation of a heterologus molecule to an antibody lacking interchain cysteines at a free thiol group not provided by at least one engineered cysteine residue selected from one or more positions shown in Table 2. In various embodiments, the method comprises the efficient conjugation of a heterologus molecule to an antibody lacking interchain cysteines at a free thiol group not provided by at least one engineered cysteine residue selected from one or more positions shown in the Table 3.

The presence of free thiol groups in antibodies may be determined by various art accepted techniques. The efficiency of conjugation of a heterologus molecule to an antibody may be determined by assessing the presence of free thiols remaining after the conjugation reaction. In certain embodiments, the method herein provides for efficiently conjugating a heterologus molecule to an antibody lacking interchain cysteines. In some embodiments, the conjugation efficiency is at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% , at least 98% or more as measured by the level of free thiol groups remaining after the conjugation reaction.

In some embodiments, the method herein provides for conjugating a heterologus molecule to an antibody where the antibody comprises at least one amino acid substitution, such that 2 or more free thiol groups are formed. In certain embodiments, the method comprises an antibody where the antibody comprises at least one amino acid substitution, such that at least 2, at least 4, at least 6, at least 8, at least 10, at least 12, at least 14, at least 16, at least 18, at least 20, at least 22, at least 24, at least 26, at least 28, at least 30, at least 32, at least 34, at least 36, at least 38, at least 40, or more free thiol groups are formed.

Antibodies herein capable of conjugation may contain free cysteine residues that comprise sulfhydryl groups that are blocked or capped. Such caps include proteins, peptides, ions and other materials that interact with the sulfhydryl group and prevent or inhibit conjugate formation. In some embodiments, antibodies herein may require uncapping prior to a conjugation reaction. In specific

embodiments, antibodies herein are uncapped and display a free sulfhydryl group capable of conjugation. In specific embodiments, antibodies herein are subjected to an uncapping reaction that does not disturb or rearrange the naturally occurring disulfide bonds. In some embodiments, antibodies herein are subjected to an uncapping reaction as presented in PCT application PCT/US09/31294, filed Jan 16, 2009.

In some embodiments, antibodies herein may be subjected to conjugation reactions where the antibody to be conjugated is present at a concentration of at least 1mg/ml, at least 2 mg/ml, at least 3 mg/ml, at least 4 mg/ml, at least 5 mg/ml or higher.

Methods of using antibody conjugates

It is contemplated that the antibody conjugates herein may be used to treat various diseases or disorders, e.g. characterized by the over expression of a tumor antigen. In some embodiments an antibody conjugate herein inhibits tumor proliferation. In certain embodiments an antibody conjugate acts upon a subject in vivo. In certain embodiments an antibody conjugate acts in vitro.

In some embodiments an antibody conjugate is administered to a biological sample. A conjugate may contact a biological a sample, for example, by pipette, decantation, perfusion, injection, wash, bath, rotation, chromatography, or osmosis. In certain embodiments an antibody conjugate herein is cross-linked to a solid support, including but not limited to beads, and exposed to the sample. Bound antigen may be detected as known in the art, including but not limited to enzyme linked labels, secondary reactions, chromatographic, dye, fluorescence, and radiographic detection.

In certain embodiments, an antibody lacking interchain cysteines is conjugated to labels for purposes of diagnostics and other assays where the antibody and/or its associated ligand may be detected. A label conjugated to an antibody and used in the present methods and compositions described herein, is any chemical moiety, organic or inorganic, that exhibits an absorption maximum at wavelengths greater than 280 nm, and retains its spectral properties when covalently attached to an antibody. Labels include, without limitation, a chromophore, a fluorophore, a fluorescent protein, a phosphorescent dye, a tandem dye, a particle, a hapten, an enzyme and a radioisotope.

In certain embodiments, an antibody lacking interchain cysteines is conjugated to a fluorophore. As such, fluorophores used to label antibodies herein presented include, without limitation; a pyrene (including any of the corresponding derivative compounds) , an anthracene, a naphthalene, an acridine, a stilbene, an indole or benzindole, an oxazole or benzoxazole, a thiazole or benzothiazole, a 4-amino-7-nitrobenz-2-oxa-1, 3-diazole (NBD), a cyanine (including any corresponding compounds), a carbocyanine (including any corresponding compounds), a carbostyryl, a porphyrin, a salicylate, an anthranilate, an azulene, a perylene, a pyridine, a quinoline, a borapolyazaindacene (including any corresponding compounds), a xanthene (including any corresponding compounds), an oxazine (including any corresponding compounds) or a benzoxazine, a carbazine (including any corresponding compounds), a phenalenone, a coumarin (including an corresponding compounds disclosed), a benzofuran (including an corresponding compounds) and benzphenalenone (including any corresponding compounds) and derivatives thereof. As used herein, oxazines include resorufins (including any corresponding compounds), aminooxazinones, diaminooxazines, and their benzo-substituted analogs.

In certain embodiments, the fluorophores conjugated to antibodies lacking interchain cysteines include xanthene (rhodol, rhodamine, fluorescein and derivatives thereof) coumarin, cyanine, pyrene, oxazine and borapolyazaindacene. In some embodiments, such fluorophores are sulfonated xanthenes, fluorinated xanthenes, sulfonated coumarins, fluorinated coumarins and sulfonated cyanines. Also included are dyes sold under the tradenames, and generally known as, Alexa Fluor, DyLight, Cy Dyes, BODIPY, Oregon Green, Pacific Blue, IRDyes, FAM, FITC, and ROX.

The choice of the fluorophore attached to an antibody lacking interchain cysteines will determine the absorption and fluorescence emission properties of the conjugated antibody. Physical properties of a fluorophore label that can be used for antibody and antibody bound ligands include, but are not limited to, spectral characteristics (absorption, emission and stokes shift), fluorescence intensity, lifetime, polarization and photo-bleaching rate, or combination thereof. All of these physical properties can be used to distinguish one fluorophore from another, and thereby allow for multiplexed analysis. In certain embodiments, the fluorophore has an absorption maximum at wavelengths greater than 480 nm. In some embodiments, the fluorophore absorbs at or near 488 nm to 514 nm (particularly suitable for excitation by the output of the argon-ion laser excitation source) or near 546 nm (particularly suitable for excitation by a mercury arc lamp). In some embodiments a fluorophore can emit in the NIR (near infra red region) for tissue or whole organism

applications. Other desirable properties of the fluorescent label may include cell permeability and low toxicity, for example if labeling of the antibody is to be performed in a cell or an organism (e.g., a living animal).

In various embodiments, an enzyme is a label and is conjugated to a antibody lacking interchain cysteines. Enzymes are effective labels because amplification of the detectable signal can be obtained resulting in increased assay sensitivity. The enzyme itself often does not produce a detectable response but functions to break down a substrate when it is contacted by an appropriate substrate such that the converted substrate produces a fluorescent, colorimetric or luminescent signal. Enzymes amplify the detectable signal because one enzyme on a labeling reagent can result in multiple substrates being converted to a detectable signal. The enzyme substrate is selected to yield the measurable product, e.g. colorimetric, fluorescent or chemiluminescence. Such substrates are extensively used in the art and are known in the art.

In some embodiments, colorimetric or fluorogenic substrate and enzyme combination uses oxidoreductases such as horseradish peroxidase and a substrate such as 3,3'-diaminobenzidine (DAB) and 3-amino-9-ethylcarbazole (AEC), which yield a distinguishing color (brown and red, respectively). Other colorimetric oxidoreductase substrates that yield detectable products include, but are not limited to: 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), o-phenylenediamine (OPD), 3,3',5,5'-tetramethylbenzidine (TMB), o-dianisidine, 5-aminosalicylic acid, 4-chloro-1-naphthol. Fluorogenic substrates include, but are not limited to, homovanillic acid or 4-hydroxy-3-methoxyphenylacetic acid, reduced phenoxazines and reduced benzothiazines, including Amplex® Red reagent and its variants and reduced dihydroxanthenes, including dihydrofluoresceins and dihydrorhodamines including dihydrorhodamine 123. Peroxidase substrates that are tyramides represent a unique class of peroxidase substrates in that they can be intrinsically detectable before action of the enzyme but are "fixed in place" by the action of a peroxidase in the process described as tyramide signal amplification (TSA). These substrates are extensively utilized to label targets in samples that are cells, tissues or arrays for their subsequent detection by microscopy, flow cytometry, optical scanning and fluorometry.

A colorimetric (and in some cases fluorogenic) substrate and enzyme combination sometimes uses a phosphatase enzyme such as an acid phosphatase, an alkaline phosphatase or a recombinant version of such a phosphatase in combination with a colorimetric substrate such as 5-bromo-6-chloro-3-indolyl phosphate (BCIP), 6-chloro-3-indolyl phosphate, 5-bromo-6-chloro-3-indolyl

phosphate, p-nitrophenyl phosphate, or o-nitrophenyl phosphate or with a fluorogenic substrate such as 4-methylumbelliferyl phosphate, 6,8-difluoro-7-hydroxy-4-methylcoumarinyl phosphate (DiFMUP, U.S. Pat. No. 5,830,912) fluorescein diphosphate, 3-O-methylfluorescein phosphate, resorufin phosphate, 9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) phosphate (DDAO phosphate), or ELF 97, ELF 39 or related phosphates.

Glycosidases, in particular beta-galactosidase, beta-glucuronidase and beta-glucosidase, are additional suitable enzymes. Appropriate colorimetric substrates include, but are not limited to, 5-bromo-4-chloro-3-indolyl beta-D-galactopyranoside (X-gal) and similar indolyl galactosides, glucosides, and glucuronides, o-nitrophenyl beta-D-galactopyranoside (ONPG) and p-nitrophenyl beta-D-galactopyranoside. In some embodiments, fluorogenic substrates include resorufin beta-D-galactopyranoside, fluorescein digalactoside (FDG), fluorescein diglucuronide and their structural variants, 4-methylumbelliferyl beta-D-galactopyranoside, carboxyumbelliferyl beta-D-galactopyranoside and fluorinated coumarin beta-D-galactopyranosides.

Additional enzymes include, but are not limited to, hydrolases such as cholinesterases and peptidases, oxidases such as glucose oxidase and cytochrome oxidases, and reductases for which suitable substrates are known.

Enzymes and their appropriate substrates that produce chemiluminescence are useful for some assays. These include, but are not limited to, natural and recombinant forms of luciferases and aequorins. Chemiluminescence-producing substrates for phosphatases, glycosidases and oxidases such as those containing stable dioxetanes, luminol, isoluminol and acridinium esters are additionally productive.

In some embodiments, haptens such as biotin are also utilized as labels. Biotin is useful because it can function in an enzyme system to further amplify the detectable signal, and it can function as a tag to be used in affinity chromatography for isolation purposes. For detection purposes, an enzyme conjugate that has affinity for biotin is used, such as avidin-HRP. Subsequently a peroxidase substrate is added to produce a detectable signal.

Haptens also include hormones, naturally occurring and synthetic drugs, pollutants, allergens, affector molecules, growth factors, chemokines, cytokines, lymphokines, amino acids, peptides, chemical intermediates, nucleotides and the like.

In certain embodiments, fluorescent proteins are conjugated to the antibodies as a label. Examples of fluorescent proteins include green fluorescent protein (GFP) and the phycobiliproteins and the derivatives thereof. The fluorescent proteins, especially phycobiliprotein, are useful for creating tandem dye labeled labeling reagents. These tandem dyes comprise a fluorescent protein and a fluorophore for the purposes of obtaining a larger stokes shift where the emission spectra is farther shifted from the wavelength of the fluorescent protein's absorption spectra. This may be effective for detecting a low quantity of a target in a sample where the emitted fluorescent light is maximally optimized, in other words little to none of the emitted light is reabsorbed by the fluorescent protein. For this to work, the fluorescent protein and fluorophore function as an energy transfer pair where the fluorescent protein emits at the wavelength that the fluorophore absorbs at and the fluorphore then emits at a wavelength farther from the fluorescent proteins than could have been obtained with only the fluorescent protein. A functional combination may be phycobiliproteins and sulforhodamine fluorophores or sulfonated cyanine fluorophores as known in the art. The fluorophore sometimes functions as the energy donor and the fluorescent protein is the energy acceptor.

In certain embodiments, the label is a radioactive isotope. Examples of suitable radioactive materials include, but are not limited to, iodine (.sup.121I, .sup.123I, .sup.125I, .sup.131I), carbon (.sup.14C), sulfur (.sup.35S), tritium (.sup.3H), indium (.sup.111In, .sup.112In, .sup.113mIn, .sup.115mIn), technetium (.sup.99Tc, .sup.99mTc), thallium (.sup.201Ti), gallium (.sup.68Ga, .sup.67Ga), palladium (.sup.103Pd), molybdenum (.sup.99Mo), xenon (.sup.135Xe), fluorine (.sup.18F), .sup.153Sm, .sup.177Lu, .sup.159Gd, .sup.149Pm, .sup.140La, .sup.175Yb, .sup.166Ho, .sup.90Y, .sup.47Sc, .sup.186Re, .sup.188Re, .sup.142Pr, .sup.105Rh, and .sup.97Ru.

Diagnostic methods of use

In certain embodiments, antibodies lacking interchain cysteines, conjugates and compositions herein presented may be used in vivo and/or in vitro for diagnosing diseases associated with the FlexiMab antibody or the conjugated molecule. This can be achieved, for example, by contacting a sample to be tested, optionally along with a control sample, with the antibody under conditions that allow for formation of a complex between the antibody or conjugate herein and the molecule of interest. Complex formation is then detected (e.g., using an ELISA). When using a control sample

along with the test sample, complex is detected in both samples and any statistically significant difference in the formation of complexes between the samples is indicative of the presence of the molecule of interest in the test sample.

In some embodiments, the technology herein provides a method of determining the presence of a molecule of interest in a sample suspected of containing such a molecule, the method comprising exposing the sample to an antibody lacking interchain cysteines or conjugate, and determining binding of the antibody or conjugate to the molecule of interest in the sample where binding of the antibody or conjugate to the molecule of interest in the sample is indicative of the presence of the molecule of interest in the sample. In some embodiments, the sample is a biological sample. In certain embodiments, the biological sample is from a mammal experiencing or suspected of experiencing disease or disorder associated with the molecule of interest.

In certain embodiments, an antibody lacking interchain cysteines or conjugate may be used to detect the overexpression or amplification of a molecule of interest using an in vivo diagnostic assay. In some embodiments, an antibody lacking interchain cysteines or conjugate is added to a sample where the antibody or conjugate binds the molecule of interest to be detected and is tagged with a detectable label (e.g. a radioactive isotope or a fluorescent label) and externally scanning the patient for localization of the label.

FISH assays such as the INFORM™ (sold by Ventana, Ariz.) or PATHVISION™ (Vysis, Ill.) may be carried out on formalin-fixed, paraffin-embedded tissue to determine the extent (if any) of overexpression of a molecule of interest in the tumor.

In certain embodiments, an antibody lacking interchain cysteines or conjugate may be used in a method of diagnosing a cell proliferative disorder associated with an increase in cells expressing a molecule of interest. In some embodiments, the method comprises contacting test cells in a biological sample with an antibody lacking interchain cysteines or conjugate; determining the level of a molecule of interest in test cells in the sample by detecting binding of an antibody lacking interchain cysteines or conjugate; and comparing the level of antibody bound to cells in a control sample, where the level of antibody bound is normalized to the number molecule of interest expressing cells in the test and control samples, and where a higher level of antibody bound in the test sample as compared to the control sample indicates the presence of a cell proliferative disorder associated with cells expressing the molecule of interest.

In certain embodiments, an antibody lacking interchain cysteines or conjugate may be used in a method of detecting soluble molecule of interest in blood or serum. In some embodiments, the method comprises contacting a test sample of blood or serum from a mammal suspected of experiencing a disorder associated with a molecule of interest with an antibody lacking interchain cysteines of conjugate herein and detecting an increase in soluble molecule of interest in the test sample relative to a control sample of blood or serum from a normal mammal. In some embodiments, the method of detecting is useful as a method of diagnosing a disorder associated with an increase in soluble molecule of interest in blood or serum of a mammal.

Treatment methods of use

In various embodiments the antibody conjugate is administered to cells, for example cancer cells. The biological effect of the antibody conjugate may be observed, including but not limited to cell, death, cell proliferation inhibition, lack of effect, changes in cell morphology, and changes in cellar growth pattern. In some embodiments the antibody conjugate comprises a detectable label as described above. In certain embodiments the label indicates the location of the tumor antigen within the cell.

In certain embodiments an antibody conjugate is administered to a subject in need of treatment. In various embodiments a conjugate carries a drug or toxin targeted to a tumor antigen. The conjugate sometimes carries a detectable label by which the antigen may be identified or localized. Some embodiments comprise detection of the biological effect of the antibody conjugate. In certain embodiments the condition of the subject may be monitored. The medical dose may be adjusted in response to monitoring.

Exemplary conditions or hyperproliferative disorders include benign or malignant tumors, leukemia and lymphoid malignancies. Others include neuronal, glial, astrocytal, hypothalamic, glandular, macrophagal, epithelial, endothelial, and stromal malignancies. Other cancers or hyperproliferative disorders include: cancers of the head, neck, eye, mouth, throat, esophagus, chest, skin, bone, lung, colon, rectum, colorectal, stomach, spleen, kidney, skeletal muscle, subcutaneous tissue, metastatic melanoma, endometrial, prostate, breast, ovaries, testicles, thyroid, blood, lymph nodes, kidney, liver, pancreas, brain, or central nervous system. Examples of cancers that can be prevented, managed, treated or ameliorated in accordance with the methods herein include, but are not limited to, cancer of the head, neck, eye, mouth, throat, esophagus, chest, bone, lung,

colon, rectum, stomach, prostate, breast, ovaries, kidney, liver, pancreas, and brain. Additional cancers include, but are not limited to, the following: leukemias such as but not limited to, acute leukemia, acute lymphocytic leukemia, acute myelocytic leukemias such as myeloblastic, promyelocytic, myelomonocytic, monocytic, erythroleukemia leukemias and myelodysplastic syndrome, chronic leukemias such as but not limited to, chronic myelocytic (granulocytic) leukemia, chronic lymphocytic leukemia, hairy cell leukemia; polycythemia vera; lymphomas such as but not limited to Hodgkin's disease, non-Hodgkin's disease; multiple myelomas such as but not limited to smoldering multiple mycloma, nonsecretory myeloma, osteosclerotic myeloma, plasma cell leukemia, solitary plasmacytoma and extramedullary plasmacytoma, Waldenstrom's macroglobulinemia; monoclonal gammopathy of undetermined significance; benign monoclonal gammopathy; heavy chain disease; bone cancer and connective tissue sarcomas such as but not limited to bone sarcoma, myeloma bone disease, multiple myeloma, cholesteatoma-induced bone osteosarcoma, Paget's disease of bone, osteosarcoma, chondrosarcoma, Ewing's sarcoma, malignant giant cell tumor, fibrosarcoma of bone, chordoma, periosteal sarcoma, soft-tissue sarcomas, angiosarcoma (hemangiosarcoma), fibrosarcoma, Kaposi's sarcoma, leiomyosarcoma, liposarcoma, lymphangiosarcoma, neurilemmoma, rhabdomyosarcoma, and synovial sarcoma; brain tumors such as but not limited to, glioma, astrocytoma, brain stem glioma, ependymoma, oligodendroglioma, nonglial tumor, acoustic neurinoma, craniopharyngioma, medulloblastoma, meningioma, pineocytoma, pineoblastoma, and primary brain lymphoma; breast cancer including but not limited to adenocarcinoma, lobular (small cell) carcinoma, intraductal carcinoma, medullary breast cancer, mucinous breast cancer, tubular breast cancer, papillary breast cancer, Paget's disease (including juvenile Paget's disease) and inflammatory breast cancer; adrenal cancer such as but not limited to pheochromocytom and adrenocortical carcinoma; thyroid cancer such as but not limited to papillary or follicular thyroid cancer, medullary thyroid cancer and anaplastic thyroid cancer; pancreatic cancer such as but not limited to, insulinoma, gastrinoma, glucagonoma, vipoma, somatostatin-secreting tumor, and carcinoid or islet cell tumor; pituitary cancers such as but limited to Cushing's disease, prolactin-secreting tumor, acromegaly, and diabetes insipius; eye cancers such as but not limited to ocular melanoma such as iris melanoma, choroidal melanoma, and cilliary body melanoma, and retinoblastoma; vaginal cancers such as squamous cell carcinoma, adenocarcinoma, and melanoma; vulvar cancer such as squamous cell carcinoma, melanoma, adenocarcinoma, basal cell carcinoma, sarcoma, and Paget's disease; cervical cancers such as but not limited to, squamous cell carcinoma, and adenocarcinoma; uterine cancers such as but not limited to endometrial carcinoma and uterine sarcoma; ovarian cancers such as but not limited to, ovarian epithelial carcinoma, borderline tumor, germ cell tumor, and

stromal tumor; esophageal cancers such as but not limited to, squamous cancer, adenocarcinoma, adenoid cyctic carcinoma, mucoepidermoid carcinoma, adenosquamous carcinoma, sarcoma, melanoma, plasmacytoma, verrucous carcinoma, and oat cell (small cell) carcinoma; stomach cancers such as but not limited to, adenocarcinoma, fungating (polypoid), ulcerating, superficial spreading, diffusely spreading, malignant lymphoma, liposarcoma, fibrosarcoma, and carcinosarcoma; colon cancers; rectal cancers; liver cancers such as but not limited to hepatocellular carcinoma and hepatoblastoma, gallbladder cancers such as adenocarcinoma; cholangiocarcinomas such as but not limited to pappillary, nodular, and diffuse; lung cancers such as non-small cell lung cancer, squamous cell carcinoma (epidermoid carcinoma), adenocarcinoma, large-cell carcinoma and small-cell lung cancer; testicular cancers such as but not limited to germinal tumor, seminoma, anaplastic, classic (typical), spermatocytic, nonseminoma, embryonal carcinoma, teratoma carcinoma, choriocarcinoma (yolk-sac tumor), prostate cancers such as but not limited to, adenocarcinoma, leiomyosarcoma, and rhabdomyosarcoma; penal cancers; oral cancers such as but not limited to squamous cell carcinoma; basal cancers; salivary gland cancers such as but not limited to adenocarcinoma, mucoepidermoid carcinoma, and adenoidcystic carcinoma; pharynx cancers such as but not limited to squamous cell cancer, and verrucous; skin cancers such as but not limited to, basal cell carcinoma, squamous cell carcinoma and melanoma, superficial spreading melanoma, nodular melanoma, lentigo malignant melanoma, acral lentiginous melanoma; kidney cancers such as but not limited to renal cell cancer, adenocarcinoma, hypernephroma, fibrosarcoma, transitional cell cancer (renal pelvis and/or ureter); Wilms' tumor; bladder cancers such as but not limited to transitional cell carcinoma, squamous cell cancer, adenocarcinoma, carcinosarcoma. In addition, cancers include myxosarcoma, osteogenic sarcoma, endotheliosarcoma, lymphangioendotheliosarcoma, mesotheliorna, synovioma, hemangioblastoma, epithelial carcinoma, cystadenocarcinoma, bronchogenic carcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma and papillary adenocarcinomas.

It is also contemplated that cancers caused by aberrations in apoptosis can also be treated by the methods and compositions herein. Such cancers may include, but not be limited to, follicular lymphomas, carcinomas with p53 mutations, hormone dependent tumors of the breast, prostate and ovary, and precancerous lesions such as familial adenomatous polyposis, and myelodysplastic syndromes.

The proteins herein and compositions comprising the same are useful for many purposes, for example, as therapeutics against a wide range of chronic and acute diseases and disorders including, but not limited to, autoimmune and/or inflammatory disorders, which include Sjogren's syndrome, rheumatoid arthritis, lupus psoriasis, atherosclerosis, diabetic and other retinopathies, retrolental fibroplasia, age-related macular degeneration, neovascular glaucoma, hemangiomas, thyroid hyperplasias (including Grave's disease), corneal and other tissue transplantation, and chronic inflammation, sepsis, rheumatoid arthritis, peritonitis, Crohn's disease, reperfusion injury, septicemia, endotoxic shock, cystic fibrosis, endocarditis, psoriasis, arthritis (e.g., psoriatic arthritis), anaphylactic shock, organ ischemia, reperfusion injury, spinal cord injury and allograft rejection.

Examples of autoimmune and/or inflammatory disorders include, but are not limited to, alopecia areata, ankylosing spondylitis, antiphospholipid syndrome, autoimmune Addison's disease, autoimmune diseases of the adrenal gland, autoimmune hemolytic anemia, autoimmune hepatitis, autoimmune oophoritis and orchitis, Sjogren's syndrome, psoriasis, atherosclerosis, diabetic and other retinopathies, retrolental fibroplasia, age-related macular degeneration, neovascular glaucoma, hemangiomas, thyroid hyperplasias (including Grave's disease), corneal and other tissue transplantation, and chronic inflammation, sepsis, rheumatoid arthritis, peritonitis, Crohn's disease, reperfusion injury, septicemia, endotoxic shock, cystic fibrosis, endocarditis, psoriasis, arthritis (e.g., psoriatic arthritis), anaphylactic shock, organ ischemia, reperfusion injury, spinal cord injury and allograft rejection. autoimmune thrombocytopenia, Behcet's disease, bullous pemphigoid, cardiomyopathy, celiac sprue-dermatitis, chronic fatigue immune dysfunction syndrome (CFIDS), chronic inflammatory demyelinating polyneuropathy, Churg-Strauss syndrome, cicatrical pemphigoid, CREST syndrome, cold agglutinin disease, discoid lupus, essential mixed cryoglobulinemia, fibromyalgia-fibromyositis, glomerulonephritis, Guillain-Barre, Hashimoto's thyroiditis, idiopathic pulmonary fibrosis, idiopathic thrombocytopenia purpura (ITP), IgA neuropathy, juvenile arthritis, lichen planus, lupus erythematosus, Meniere's disease, mixed connective tissue disease, multiple sclerosis, type 1 or immune-mediated diabetes mellitus, myasthenia gravis, pemphigus vulgaris, pernicious anemia, polyarteritis nodosa, polychrondritis, polyglandular syndromes, polymyalgia rheumatica, polymyositis and dermatomyositis, primary agammaglobulinemia, primary biliary cirrhosis, psoriasis, psoriatic arthritis, Raynauld's phenomenon, Reiter's syndrome, Rheumatoid arthritis, sarcoidosis, scleroderma, Sjogren's syndrome, stiff-man syndrome, systemic lupus erythematosus, lupus erythematosus, takayasu arteritis, temporal arteristis/giant cell arteritis, ulcerative colitis,

uveitis, vasculitides such as dermatitisherpetiformis vasculitis, vitiligo, and Wegener's granulomatosis.

Examples of inflammatory disorders include, but are not limited to, asthma, encephilitis, inflammatory bowel disease, chronic obstructive pulmonary disease (COPD), allergic disorders, septic shock, pulmonary fibrosis, undifferentitated spondyloarthropathy, undifferentiated arthropathy, arthritis, inflammatory osteolysis, and chronic inflammation resulting from chronic viral or bacteria infections.

The compositions and methods herein can be used with one or more conventional therapies that are used to prevent, manage or treat the above diseases. Also provided, in some embodiments are methods of using antibodies and/or antibody conjugates to inactivate various infectious agents such as viruses, fungi, eukaryotic microbes, and bacteria. In some embodiments the antibodies or antibody conjugates herein may be used to inactivate RSV, hMPV, PIV, or influenza viruses. In some embodiments, the antibodies and/or antibody conjugates herein may be used to inactivate fungal pathogens, such as, but not limited to members of Naegleria, Aspergillus, Blastomyces, Histoplasma, Candida or Tinea genera. In some embodiments, the antibodies and/or antibody conjugates herein may be used to inactivate eukaryotic microbes, such as, but not limited to members of Giardia, Toxoplasma, Plasmodium, Trypanosoma, and Entamoeba genera. In some embodiments, the antibodies and/or antibody conjugates herein may be used to inactivate bacterial pathogens, such as but not limited to members of Staphylococcus, Streptococcus, Pseudomonas, Clostridium, Borrelia, Vibro and Neiserria genera.

The antibodies and/or antibody conjugates herein and compositions comprising the same are useful for many purposes, for example, as therapeutics against a wide range of chronic and acute diseases and disorders including, but not limited to, infectious disease, including viral, bacterial and fungal diseases. Examples of viral pathogens include but are not limited to: adenovirdiae (e.g., mastadenovirus and aviadenovirus), herpesviridae (e.g., herpes simplex virus 1, herpes simplex virus 2, herpes simplex virus 5, and herpes simplex virus 6), leviviridae (e.g., levivirus, enterobacteria phase MS2, allolevirus), poxviridae (e.g., chordopoxvirinae, parapoxvirus, avipoxvirus, capripoxvirus, leporiipoxvirus, suipoxvirus, molluscipoxvirus, and entomopoxvirinae), papovaviridae (e.g., polyomavirus and papillomavirus), paramyxoviridae (e.g.,mparamyxovirus, parainfluenza virus 1, mobillivirus (e.g., measles virus), rubulavirus (e.g.,mumps virus), pneumonovirinae (e.g., pneumovirus, human respiratory synctial virus), and metapneumovirus

(e.g., avian pneumovirus and human metapneumovirus)), picornaviridae (e.g., enterovirus, rhinovirus, hepatovirus (e.g., human hepatitis A virus), cardiovirus, and apthovirus), reoviridae (e.g., orthoreovirus, orbivirus, rotavirus, cypovirus, fijivirus, phytoreovirus, and oryzavirus), retroviridae (e.g., mammalian type B retroviruses, mammalian type C retroviruses, avian type C retroviruses, type D retrovirus group, BLVHTLV retroviruses, lentivirus (e.g. human immunodeficiency virus 1 and human immunodeficiency virus 2), spumavirus), flaviviridae (e.g., hepatitis C virus), hepadnaviridae (e.g., hepatitis B virus), togaviridae (e.g., alphavirus (e.g., sindbis virus) and rubivirus (e.g., rubella virus)), rhabdoviridae (e.g., vesiculovirus, lyssavirus, ephemerovirus, cytorhabdovirus, and necleorhabdovirus), arenaviridae (e.g., arenavirus, lymphocytic choriomeningitis virus, Ippy virus, and lassa virus), and coronaviridae (e.g., coronavirus and torovirus).

Examples of bacterial pathogens include but are not limited to: the Aquaspirillum family, Azospirillum family, Azotobacteraceae family, Bacteroidaceae family, Bartonella species, Bdellovibrio family, Campylobacter species, Chlamydia species (e.g., Chlamydia pneumoniae), clostridium, Enterobacteriaceae family (e.g., Citrobacter species, Edwardsiella, Enterobacter aerogenes, Erwinia species, Escherichia coli, Hafnia species, Klebsiella species, Morganella species, Proteus vulgaris, Providencia, Salmonella species, Serratia marcescens, and Shigella flexneri), Gardinella family, Haemophilus influenzae, Halobacteriaceae family, Helicobacter family, Legionallaceae family, Listeria species, Methylococcaceae family, mycobacteria (e.g., Mycobacterium tuberculosis), Neisseriaceae family, Oceanospirillum family, Pasteurellaceae family, Pneumococcus species, Pseudomonas species, Rhizobiaceae family, Spirillum family, Spirosomaceae family, Staphylococcuss (e.g., methicillin resistant Staphylococcus aureus and Staphylococcus pyrogenes), Streptococcus (e.g., Streptococcus enteritidis, Streptococcus fasciae, and Streptococcus pneumoniae), Vampirovibr Helicobacter family, and Vampirovibrio family.

Examples of fungal pathogens include, but are not limited to: Absidia species (e.g., Absidia corymbifera and Absidia ramosa), Aspergillus species, (e.g., Aspergillus flavus, Aspergillus fumigatus, Aspergillus nidulans, Aspergillus niger, and Aspergillus terreus), Basidiobolus ranarum, Blastomyces dermatitidis, Candida species (e.g.,Candida albicans, Candida glabrata, Candida kerr, Candida krusei, Candida parapsilosis, Candida pseudotropicalis, Candida quillermondii, Candida rugosa, Candida stellatoidea, and Candida tropicalis), Coccidioides immitis, Conidiobolus species, Cryptococcus neoforms, Cunninghamella species, dermatophytes, Histoplasma capsulatum, Microsporum gypseum, Mucor pusillus, Paracoccidioides brasiliensis,

Pseudallescheria boydii, Rhinosporidium seeberi, Pneumocystis carinii, Rhizopus species (e.g., Rhizopus arrhizus, Rhizopus oryzae, and Rhizopus microsporus), Saccharomyces species, Sporothrix schenckii, zygomycetes, and classes such as Zygomycetes, Ascomycetes, the Basidiomycetes, Deuteromycetes, and Oomycetes.

Provided also, in some embodiments, are methods of using antibodies to deplete a cell population. In an embodiment, methods herein may be used in the depletion of the following cell types: eosinophil, basophil, neutrophil, T cell, B cell, mast cell, monocytes, endothelial cell and tumor cell.

In certain embodiments, the antibodies herein and conjugates thereof may also be useful in the diagnosis and detection of diseases of symptoms thereof. In some embodiments, the compositions herein may be useful in the monitoring of disease progression. In various embodiments, the compositions herein may be useful in the monitoring of treatment regimens. In certain embodiments, the compositions herein are useful for diagnosis in an ex vivo application, such as a diagnostic kit.

The compositions herein may be useful in the visualization of target antigens. In some embodiments, the target antigens are cell surface receptors that internalize. In certain embodiments, the target antigen is an intracellular antigen. In some embodiments the target is an intranuclear antigen.

In some embodiments, the antibodies or antibody-drug conjugates herein once bound, internalize into cells where internalization is at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, or at least about 90%, at least about 100%, at least about 110%, at least about 130%, at least about 140%, at least about 150%, at least about 160%, or at least about 170% more than control antibodies as described herein.

In certain embodiments, the antibodies herein once bound, internalize into cells where internalization is 1-10%, 10-20%, 20 - 30%, 30- 40%,40- 50%, 50-60%, 60-70%, 70-80%, 80-90%, 90-100%, 100-110%, 110-120%, 120-130%, 130-140%, 140-150%, 150-160%, 160-170% more than control antibodies as described herein.

Various embodiments, the antibodies herein once bound, internalize into cells where internalization is 1-10%, 10-20%, 20 - 30%, 30- 40%,40- 50%, 50-60%, 60-70%, 70-80%, 80-90%, 90-100%, 100-110%, 110-120%, 120-130%, 130-140%, 140-150%, 150-160%, 160-170% more than control antibodies as determined by the internalization assay using a secondary antibody.

Antibody therapeutics

Pharmaceutical compositions

Provided herein, in some embodiments, is a composition, for non-limiting example, a pharmaceutical composition, containing one or a combination of antibodies, or antibody conjugates herein, formulated together with a pharmaceutically acceptable carrier. Such compositions may include one or a combination of, for example, but not limited to two or more different antibodies herein. For example, a pharmaceutical composition herein may comprise a combination of antibodies that bind to different epitopes on the target antigen or that have complementary activities.

To prepare pharmaceutical or sterile compositions including an antibody or antibody conjugate herein, the antibody/antibody conjugate can be mixed with a pharmaceutically acceptable carrier or excipient. Formulations of therapeutic and diagnostic agents can be prepared by mixing with physiologically acceptable carriers, excipients, or stabilizers in the form of, e.g., lyophilized powders, slurries, aqueous solutions, lotions, or suspensions.

Pharmaceutical compositions herein also can be administered in combination therapy, such as, combined with other agents. For example, the combination therapy can include an antibody herein combined with at least one other therapy where the therapy may be surgery, immunotherapy, chemotherapy, radiation treatment, or drug therapy.

The pharmaceutical compounds herein may include one or more pharmaceutically acceptable salt. Examples of such salts include acid addition salts and base addition salts. Acid addition salts include those derived from nontoxic inorganic acids, such as hydrochloric, nitric, phosphoric, sulfuric, hydrobromic, hydroiodic, phosphorous and the like, as well as from nontoxic organic acids such as aliphatic mono- and dicarboxylic acids, phenyl-substituted alkanoic acids, hydroxy alkanoic acids, aromatic acids, aliphatic and aromatic sulfonic acids and the like. Base addition salts include

those derived from alkaline earth metals, such as sodium, potassium, magnesium, calcium and the like, as well as from nontoxic organic amines, such as N,N'-dibenzylethylenediamine, N methylglucamine, chloroprocaine, choline, diethanolamine, ethylenediamine, procaine and the like.

A pharmaceutical composition herein also may include a pharmaceutically acceptable anti-oxidant. Examples of pharmaceutically acceptable antioxidants include: (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; (2) oilsoluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

Examples of suitable aqueous and non-aqueous carriers that may be employed in the pharmaceutical compositions herein include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of presence of microorganisms may be ensured both by sterilization procedures and by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

Pharmaceutical compositions may be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, liposome, or other ordered structure suitable to high drug concentration. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. In many cases, it may

be suitable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by sterilization microfiltration. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, appropriate methods of preparation include vacuum drying and freeze-drying (lyophilization) that yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

In one embodiment the compositions herein are pyrogen-free formulations which are substantially free of endotoxins and/or related pyrogenic substances. Endotoxins include toxins that are confined inside a microorganism and are released when the microorganisms are broken down or die. Pyrogenic substances also include fever inducing, thermostable substances (glycoproteins) from the outer membrane of bacteria and other microorganisms. Both of these substances can cause fever, hypotension and shock if administered to humans. Due to the potential harmful effects, even low amounts of endotoxins may be appropriately removed from intravenously administered pharmaceutical drug solutions. The Food & Drug Administration ("FDA") has set an upper limit of 5 endotoxin units (EU) per dose per kilogram body weight in a single one hour period for intravenous drug applications. When therapeutic proteins are administered in amounts of several hundred or thousand milligrams per kilogram body weight even trace amounts of endotoxin may appropriately be removed. In an embodiment, endotoxin and pyrogen levels in the composition are less then 10 EU/mg, less then 5 EU/mg, less then 1 EU/mg, less then 0.1 EU/mg, less then 0.01 EU/mg, or less then 0.001 EU/mg. In certain embodiments, endotoxin and pyrogen levels in the composition are less then about 10 EU/mg, less then about 5 EU/mg, less then about 1 EU/mg, or less then about 0.1 EU/mg, less then about 0.01 EU/mg, or less then about 0.001 EU/mg.

In some embodiments, a method comprises administering a composition where said administration is oral, parenteral, intramuscular, intranasal, vaginal, rectal, lingual, sublingual,

buccal, intrabuccal, intravenous, cutaneous, subcutaneous or transdermal.

In certain embodiments, a method further comprises administering a composition in combination with other therapies, such as surgery, chemotherapy, hormonal therapy, biological therapy, immunotherapy or radiation therapy.

Dosing and administration

To prepare pharmaceutical or sterile compositions including an antibody or antibody conjugate herein, the antibody/antibody conjugate is mixed with a pharmaceutically acceptable carrier or excipient. Formulations of therapeutic and diagnostic agents can be prepared by mixing with physiologically acceptable carriers, excipients, or stabilizers in the form of, e.g., lyophilized powders, slurries, aqueous solutions, lotions, or suspensions.

Selecting an administration regimen for a therapeutic depends on several factors, including the serum or tissue turnover rate of the entity, the level of symptoms, the immunogenicity of the entity, and the accessibility of the target cells in the biological matrix. In certain embodiments, an administration regimen maximizes the amount of therapeutic delivered to the patient consistent with an acceptable level of side effects. Accordingly, the amount of biologic delivered depends in part on the particular entity and the severity of the condition being treated. Guidance in selecting appropriate doses of antibodies, cytokines, and small molecules is available in the art.

Determination of the appropriate dose may be made by the clinician, e.g., using parameters or factors known or suspected in the art to affect treatment or predicted to affect treatment. Generally, the dose begins with an amount somewhat less than the optimum dose and it is increased by small increments thereafter until the desired or optimum effect is achieved relative to any negative side effects. Important diagnostic measures include those of symptoms of, e.g., the inflammation or level of inflammatory cytokines produced.

Actual dosage levels of the active ingredients in the pharmaceutical compositions herein may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient. The selected dosage level may depend upon a variety of pharmacokinetic factors including the activity of the particular compositions herein employed, or

the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors known in the medical arts.

Compositions comprising antibodies or antibody conjugates herein can be provided by continuous infusion, or by doses at intervals of, e.g., one day, one week, or 1-7 times per week. Doses may be provided intravenously, subcutaneously, topically, orally, nasally, rectally, intramuscular, intracerebrally, or by inhalation. A specific dose protocol is one involving the maximal dose or dose frequency that avoids significant undesirable side effects. A total weekly dose may be at least 0.05 μg/kg body weight, at least 0.2 μg/kg, at least 0.5 μg/kg, at least 1 μg/kg, at least 10 μg/kg, at least 100 μg/kg, at least 0.2 mg/kg, at least 1.0 mg/kg, at least 2.0 mg/kg, at least 10 mg/kg, at least 25 mg/kg, or at least 50 mg/kg. The dose may be at least 15 μg, at least 20 μg, at least 25 μg, at least 30 μg, at least 35 μg, at least 40 μg, at least 45 μg, at least 50 μg, at least 55 μg, at least 60 μg, at least 65 μg, at least 70 μg, at least 75 μg, at least 80 μg, at least 85 μg, at least 90 μg, at least 95 μg, or at least 100 μg. The doses administered to a subject may number at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12, or more.

For antibodies or antibody conjugates herein, the dosage administered to a patient may be 0.0001 mg/kg to 100 mg/kg of the patient's body weight. The dosage may be between 0.0001 mg/kg and 20 mg/kg, 0.0001 mg/kg and 10 mg/kg, 0.0001 mg/kg and 5 mg/kg, 0.0001 and 2 mg/kg, 0.0001 and 1 mg/kg, 0.0001 mg/kg and 0.75 mg/kg, 0.0001 mg/kg and 0.5 mg/kg, 0.0001 mg/kg to 0.25 mg/kg, 0.0001 to 0.15 mg/kg, 0.0001 to 0.10 mg/kg, 0.001 to 0.5 mg/kg, 0.01 to 0.25 mg/kg or 0.01 to 0.10 mg/kg of the patient's body weight.

The dosage of the antibodies or antibody conjugates herein may be calculated using the patient's weight in kilograms (kg) multiplied by the dose to be administered in mg/kg. The dosage of the antibodies herein may be 150 μg/kg or less, 125 μg/kg or less, 100 μg/kg or less, 95 μg/kg or less, 90 μg/kg or less, 85 μg/kg or less, 80 μg/kg or less, 75 μg/kg or less, 70 μg/kg or less, 65 μg/kg or less, 60 μg/kg or less, 55 μg/kg or less, 50 μg/kg or less, 45 μg/kg or less, 40 μg/kg or less, 35 μg/kg or less, 30 μg/kg or less, 25 μg/kg or less, 20 μg/kg or less, 15 μg/kg or less, 10 μg/kg or less, 5 μg/kg or less, 2.5 μg/kg or less, 2 μg/kg or less, 1.5 μg/kg or less, 1 μg/kg or less, 0.5 μg/kg

or less, or 0.5 μg/kg or less of a patient's body weight.

Unit dose of the antibodies or antibody conjugates herein may be 0.1mg to 20 mg, 0.1 mg to 15 mg, 0.1 mg to 12 mg, 0.1 mg to 10 mg, 0.1 mg to 8 mg, 0.1 mg to 7 mg, 0.1 mg to 5 mg, 0.1 to 2.5 mg, 0.25 mg to 20 mg, 0.25 to 15 mg, 0.25 to 12 mg, 0.25 to 10 mg, 0.25 to 8 mg, 0.25 mg to 7 mg, 0.25 mg to 5 mg, 0.5 mg to 2.5 mg, 1 mg to 20 mg, 1 mg to 15 mg, 1 mg to 12 mg, 1 mg to 10 mg, 1 mg to 8 mg, 1 mg to 7 mg, 1 mg to 5 mg, or 1 mg to 2.5 mg.

The dosage of the antibodies or antibody conjugates herein may achieve a serum titer of at least 0.1 μg/ml, at least 0.5 μg/ml, at least 1 μg/ml, at least 2 μg/ml, at least 5 μg/ml, at least 6 μg/ml, at least 10 μg/ml, at least 15 μg/ml, at least 20 μg/ml, at least 25 μg/ml, at least 50 μg/ml, at least 100 μg/ml, at least 125 μg/ml, at least 150 μg/ml, at least 175 μg/ml, at least 200 μg/ml, at least 225 μg/ml, at least 250 μg/ml, at least 275 μg/ml, at least 300 μg/ml, at least 325 μg/ml, at least 350 μg/ml, at least 375 μg/ml, or at least 400 μg/ml in a subject. Alternatively, the dosage of the antibodies herein may achieve a serum titer of at least 0.1 μg/ml, at least 0.5 μg/ml, at least 1 μg/ml, at least, 2 μg/ml, at least 5 μg/ml, at least 6 μg/ml, at least 10 μg/ml, at least 15 μg/ml, at least 20 μg/ml, at least 25 μg/ml, at least 50 μg/ml, at least 100 μg/ml, at least 125 μg/ml, at least 150 μg/ml, at least 175 μg/ml, at least 200 μg/ml, at least 225 μg/ml, at least 250 μg/ml, at least 275 μg/ml, at least 300 μg/ml, at least 325 μg/ml, at least 350 μg/ml, at least 375 μg/ml, or at least 400 μg/ml in the subject.

Doses of antibodies or antibody conjugates herein may be repeated and the administrations may be separated by at least 1 day, 2 days, 3 days, 5 days, 10 days, 15 days, 30 days, 45 days, 2 months, 75 days, 3 months, or at least 6 months.

An effective amount for a particular patient may vary depending on factors such as the condition being treated, the overall health of the patient, the method route and dose of administration and the severity of side affects.

The route of administration may be by, e.g., topical or cutaneous application, injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial, intracerebrospinal, intralesional, or by sustained release systems or an implant. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lidocaine to ease pain at the site of the injection. In addition, pulmonary administration can also be employed,

e.g., by use of an inhaler or nebulizer, and formulation with an aerosolizing agent. In an embodiment, an antibody, combination therapy, or a composition herein is administered using Alkermes AIR® pulmonary drug delivery technology (Alkermes, Inc., Cambridge, Mass.).

A composition herein may also be administered via one or more routes of administration using one or more of a variety of methods known in the art. As will be appreciated by the skilled artisan, the route and/or mode of administration may vary depending upon the desired results. Selected routes of administration for antibodies herein include intravenous, intramuscular, intradermal, intraperitoneal, subcutaneous, spinal or other parenteral routes of administration, for example by injection or infusion. Parenteral administration may represent modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion. Alternatively, a composition herein can be administered via a non-parenteral route, such as a topical, epidermal or mucosal route of administration, for example, intranasally, orally, vaginally, rectally, sublingually or topically.

If the antibodies herein or conjugates thereof are administered in a controlled release or sustained release system, a pump may be used to achieve controlled or sustained release. Polymeric materials can be used to achieve controlled or sustained release of the therapies herein. Examples of polymers used in sustained release formulations include, but are not limited to, poly(2-hydroxy ethyl methacrylate), poly(methyl methacrylate), poly(acrylic acid), poly(ethylene-co-vinyl acetate), poly(methacrylic acid), polyglycolides (PLG), polyanhydrides, poly(N-vinyl pyrrolidone), poly(vinyl alcohol), polyacrylamide, poly(ethylene glycol), polylactides (PLA), poly(lactide-co-glycolides) (PLGA), and polyorthoesters. In an embodiment, the polymer used in a sustained release formulation is inert, free of leachable impurities, stable on storage, sterile, and biodegradable.

A controlled or sustained release system can be placed in proximity of the prophylactic or therapeutic target, thus requiring only a fraction of the systemic dose. Any technique known in the art can be used to produce sustained release formulations comprising one or more antibodies herein or conjugates thereof.

If the antibody or antibody conjugate herein is administered topically, it can be formulated in the form of an ointment, cream, transdermal patch, lotion, gel, shampoo, spray, aerosol, solution, emulsion, or other form known in the art. For non-sprayable topical dosage forms, viscous to semi-solid or solid forms comprising a carrier or one or more excipients compatible with topical application and having a dynamic viscosity, in some instances, greater than water are typically employed. Suitable formulations include, without limitation, solutions, suspensions, emulsions, creams, ointments, powders, liniments, salves, and the like, which are, if desired, sterilized or mixed with auxiliary agents (e.g., preservatives, stabilizers, wetting agents, buffers, or salts) for influencing various properties, such as, for example, osmotic pressure. Other suitable topical dosage forms include sprayable aerosol preparations where the active ingredient, in some instances, in combination with a solid or liquid inert carrier, is packaged in a mixture with a pressurized volatile (e.g., a gaseous propellant, such as freon) or in a squeeze bottle. Moisturizers or humectants can also be added to pharmaceutical compositions and dosage forms if desired. Examples of such additional ingredients are known in the art.

If the compositions comprising antibodies or antibody conjugates are administered intranasally, it can be formulated in an aerosol form, spray, mist or in the form of drops. In particular, prophylactic or therapeutic agents for use as provided herein can be conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant (e.g.,dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas). In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges (composed of, e.g., gelatin) for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

Methods for co-administration or treatment with a second therapeutic agent, e.g., a cytokine, steroid, chemotherapeutic agent, antibiotic, or radiation, are known in the art. An effective amount of therapeutic may decrease the symptoms by at least 10%; by at least 20%; at least about 30%; at least 40%, or at least 50%.

Additional therapies (e.g., prophylactic or therapeutic agents), which can be administered in combination with the antibodies herein or conjugates thereof, may be administered less than 5 minutes apart, less than 30 minutes apart, 1 hour apart, at about 1 hour apart, at about 1 to about 2 hours apart, at about 2 hours to about 3 hours apart, at about 3 hours to about 4 hours apart, at

about 4 hours to about 5 hours apart, at about 5 hours to about 6 hours apart, at about 6 hours to about 7 hours apart, at about 7 hours to about 8 hours apart, at about 8 hours to about 9 hours apart, at about 9 hours to about 10 hours apart, at about 10 hours to about 11 hours apart, at about 11 hours to about 12 hours apart, at about 12 hours to 18 hours apart, 18 hours to 24 hours apart, 24 hours to 36 hours apart, 36 hours to 48 hours apart, 48 hours to 52 hours apart, 52 hours to 60 hours apart, 60 hours to 72 hours apart, 72 hours to 84 hours apart, 84 hours to 96 hours apart, or 96 hours to 120 hours apart from the antibodies herein. The two or more therapies may be administered within one patient visit or on separate visits.

The antibodies or antibody conjugates herein and the other therapies may be cyclically administered. Cycling therapy involves the administration of a first therapy (e.g., a first prophylactic or therapeutic agent) for a period of time, followed by the administration of a second therapy (e.g., a second prophylactic or therapeutic agent) for a period of time, optionally, followed by the administration of a third therapy (e.g., prophylactic or therapeutic agent) for a period of time and so forth, and repeating this sequential administration, i.e., the cycle in order to reduce the development of resistance to one of the therapies, to avoid or reduce the side effects of one of the therapies, and/or to improve the efficacy of the therapies.

In certain embodiments, the antibodies and antibody conjugates herein can be formulated to ensure proper distribution in vivo. For example, the blood brain barrier (BBB) excludes many highly hydrophilic compounds. To ensure that the therapeutic compounds herein cross the BBB (if desired), they can be formulated, for example, in liposomes. Methods of manufacturing liposomes are known in the art. The liposomes may comprise one or more moieties which are selectively transported into specific cells or organs, thus enhance targeted drug delivery. Exemplary targeting moieties include folate or biotin, mannosides, antibodies, surfactant, and protein A receptor.

Also provided, in some embodiments, are protocols for the administration of pharmaceutical composition comprising antibodies or antibody conjugates herein alone or in combination with other therapies to a subject in need thereof. The therapies (e.g., prophylactic or therapeutic agents) of the combination therapies herein can be administered concomitantly or sequentially to a subject. The therapy (e.g., prophylactic or therapeutic agents) of the combination therapies herein can also be cyclically administered. Cycling therapy involves the administration of a first therapy (e.g., a first prophylactic or therapeutic agent) for a period of time, followed by the administration of a second therapy (e.g., a second prophylactic or therapeutic agent) for a period of time and

repeating this sequential administration, i.e., the cycle, in order to reduce the development of resistance to one of the therapies (e.g., agents) to avoid or reduce the side effects of one of the therapies (e.g., agents), and/or to improve, the efficacy of the therapies.

The therapies (e.g., prophylactic or therapeutic agents) of the combination therapies herein can be administered to a subject concurrently. The term "concurrently" is not limited to the administration of therapies (e.g., prophylactic or therapeutic agents) at exactly the same time, but rather it is meant that a pharmaceutical composition comprising antibodies or antibody conjugates herein are administered to a subject in a sequence and within a time interval such that the antibodies herein or conjugates thereof can act together with the other therapy(ies) to provide an increased benefit than if they were administered otherwise. For example, each therapy may be administered to a subject at the same time or sequentially in any order at different points in time; however, if not administered at the same time, they should be administered sufficiently close in time so as to provide the desired therapeutic or prophylactic effect. Each therapy can be administered to a subject separately, in any appropriate form and by any suitable route. In various embodiments, the therapies (e.g., prophylactic or therapeutic agents) are administered to a subject less than 15 minutes, less than 30 minutes, less than 1 hour apart, at about 1 hour apart, at about 1 hour to about 2 hours apart, at about 2 hours to about 3 hours apart, at about 3 hours to about 4 hours apart, at about 4 hours to about 5 hours apart, at about 5 hours to about 6 hours apart, at about 6 hours to about 7 hours apart, at about 7 hours to about 8 hours apart, at about 8 hours to about 9 hours apart, at about 9 hours to about 10 hours apart, at about 10 hours to about 11 hours apart, at about 11 hours to about 12 hours apart, 24 hours apart, 48 hours apart, 72 hours apart, or 1 week apart. In certain embodiments, two or more therapies (e.g., prophylactic or therapeutic agents) are administered to a within the same patient visit.

The prophylactic or therapeutic agents of the combination therapies may be administered to a subject in the same pharmaceutical composition. In some embodiments, the prophylactic or therapeutic agents of the combination therapies can be administered concurrently to a subject in separate pharmaceutical compositions. The prophylactic or therapeutic agents may be administered to a subject by the same or different routes of administration.

Examples

The examples set forth below illustrate certain embodiments and do not limit the technology.

Example 1 - General cloning procedures.

DNA manipulations were carried out according to standard protocols with reagents purchased from Invitrogen (Carlsbad, CA), New England Biolabs (Ipswich, MA), Qiagen (Valentia, CA) and Fermentas (Glen Burnie, MD). All polymerase chain reactions (PCRs) were carried out using Platinum® Taq DNA Polymerase High Fidelity (Invitrogen). The amplified PCR fragments, analyzed using E-gels (Invitrogen), were digested with the appropriate restriction enzymes and purified using preparative agarose gel (Sigma Aldrich, St. Louis, MO). The purified DNA fragments were ligated into a similarly prepared mammalian expression vector pOE (MedImmune) using reagents and protocols supplied by Invitrogen and New England Biolabs. In this expression vector both light and heavy chains are under the control of respective CMV immediate/early enhancer/promoter with a multiple cloning site and a SV40 poly(A) signal. The ligation mixtures were chemically transformed into *Escherichia coli* Stbl3™ (Invitrogen). Recombinant clones were identified by either colony PCR, using primers complementary to the 5' and 3' ends of the recombinant gene inserts, or by restriction digestion analysis using restriction enzymes that specifically cleave correct clones. All recombinant clones were further verified by DNA sequence analysis using Dye Terminator Cycle Sequencing Kits with AmpliTap (Applied Biosystems, Foster City, CA).

Example 2 - Expression, purification and monomeric content of mAb in a conventional format and FlexiMab.

DNA encoding for mAb and FlexiMab were transfected and proteins were expressed in HEK293F cells cultivated in Invitrogen's Freestyle™ media. The FexiMab used in these examples is a "mab-Val," which is a FlexiMab antibody in which interchain cysteines have been substituted with valine (e.g., **Figure 2**, panels C and D). The term "mAb" refers to the counterpart antibody that does not include such cysteine substitutions (e.g., **Figure 2**, panels A and B).

The culture medium was collected 6 days post-transfection and the two antibodies were purified by standard protein A affinity chromatography in accordance with the manufacturer's protocol (GE Healthcare, Piscataway, NJ). The expression level as shown in **Figure 3** was 145 mg/L and 151 mg/L at day 6 post-transfection for mAb and FlexiMab, respectively.

Total IgG expression was determined using a protein A binding assay. The protein A quantification method is as follow. The culture media was automatically loaded onto a protein A column using an HPLC system (Agilent 1100 Capillary LC System, Foster City, CA). Unbound material was washed with a solution of 100 mM sodium phosphate buffer at pH 6.8, and antibodies were eluted with 0.1% phosphoric acid, pH 1.8. The area corresponding to the eluted peak was integrated and the total antibody concentration was determined by comparing to an IgG standard. The concentrations of the purified antibodies were also determined by reading the absorbance at 280 nm using theoretically determined extinction coefficients. Analytical size-exclusion HPLC chromatography (SEC-HPLC, Agilent 1100 Capillary LC System) was used to determine the monomeric content of constructs. The wavelength was set to 280 nm and the experiments were carried out at 25ºC. SEC-HPLC was carried out using TSK-GEL G3000SWXL column (Tosoh Bioscience LLC, Montgomeryville, PA), which separates globular proteins with molecular weight (MW) that range from approximately 10 to 500 KDa, with a buffer containing 100 mM sodium phosphate, pH 6.8, and at a flow rate of 1 mL/min. A low molecular weight gel filtration calibration kit from Bio-Rad (Hercules, CA) containing vitamin B12 (11,350 Da), equine myoglobin (17,000 Da), chicken ovalbumin (44,000 Da), bovine gamma-globulin (158,000 Da) and thyroglobulin (670,000 Da) was used as molecular mass standards. In addition, a highly purified 99% monomeric IgG was used as immunoglobulin molecular weight standard. As shown in **Figure 3**, the monomeric content after protein A purification for mAb and FlexiMab was 98% and 98%, respectively. Subsequent to protein A purification the proteins were buffer exchanged into 25 mM Histidine-HCl, pH 6.0. The purity of the constructs was analyzed (1) by using analytical SEC-HPLC and as shown in **Figure 3**, and by using (2) standard sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and non-reducing conditions (**Figure 4)**. As shown in **Figure 4**, in non-reducing conditions, **lane 2**, the conventional mAb runs as intact interchain disulphide-linked mAb, whereas in non-reducing conditions FlexiMab runs as two impendent chains (similarly to what expected for a reduced sample) heavy (**upper band in Figure 4 lane 3**) and light chain (**lower band in Figure 4 lane 3**). **Figure 4 lane 5 and line 6** show the heavy and light chains for mAb and FlexiMab in reducing conditions. As expected in reducing conditions there are two distinct protein bands, corresponding to the heavy and light chains, respectively. The molecular weight standards used in the SDS-PAGE analysis are schematically shown on the left of the SDS-PAGE image.

Example 3 - ELISA assays for determining functional binding of the FlexiMab to its antigen (EGFR) and comparison with the conventional mAb.

As shown in **Figure 5**, FlexiMab is able to bind its antigen EGFR in an ELISA analysis comparable with its parental mAb. For the ELISA binding analysis, 2 ug/mL of antigen in 30 µL of PBS, pH 7.4 were coated on microtiter ELISA wells for 1 hour at room temperature. Antigen-coated wells were washed 3 times with PBS containing 0.1% (v/v) Tween-20 and blocked for 1 hour at room temperature with 3% BSA. Antibodies were serially diluted in 30 uL of blocking solution and were incubated for 2 hour at 37ºC, followed by extensive washes with PBS containing 0.1% (v/v) Tween-20. Bound antibodies were detected by HRP-conjugated anti-human-kappa-antibody and visualized with 30 uL of 3,3´,5,5´-tetramethylbenzidine substrate (Pierce). The reaction was stopped by adding 30 uL of 0.18 M sulfuric acid (Pierce). The absorbance at 450 nm was measured using a microtiter ELISA plate reader. The resulting data were analyzed and plotted using Prism 5 software (GraphPad, San Diego, CA).

Example 4 - FACS assays for determining functional binding of the FlexiMab to its native antigen expressed on the surface of A431 (human epithelial carcinoma cell line) cells and comparison with the conventional mAb.

As shown in **Figure 6**, FlexiMab is functionally able to bind its ligand EGFR expressed on the cell surface of A431 cells. As indicated in **Figure 6**, the binding signal for FlexiMab and the conventional mAb is comparable. The mean fluorescence intensity values (MFIR) are schematically shown in the figure for FlexiMab, mAb and controls. MFRI values are comparable for FlexiMab and the conventional mAb. A431 cells were grown in F12-K Medium with 10% FBS and detached using Trypsin (0.25%) (Invitrogen, location?, USA). Cells were washed and resuspended in 3% BSA, in PBS. A total of 100 uL of cells at 1.5 x 10^6 cells/mL were dispensed into a 96 well micro-plate. Cells were stained with primary antibody for 30 minutes, 4°C. Cells were then washed three times and stained with 2 ug/mL anti-human IgG-FITC for 30 min, 4°C, for detecting antibody. After washing with 3% BSA in PBS, cells were resuspended in wash buffer containing 2ug/mL Propidium Iodide. Cell-associated fluorescence was analyzed using the LSR II flow cytometer (Bectin, Dickonson) and plotted using the program FlowJo.

Example 5 - Functional stability of FlexiMab and mAb after incubation at 37°C in PBS for 5 days.

Assays, results for which are shown in **Figure 7,** were carried out to determine if the FlexiMab retains binding activity to its antigen upon temperature induced experimental stress. The antibodies were incubated for 5 days in PBS at 37ºC. Upon incubation, functional activity was determined

using ELISA analysis as described in **Example 4**. As shown in **Figure 7**, ELISA analysis shows that FlexiMab and mAb have comparable binding signals for EGFR, and indicates that FlexiMab is a stable molecule.

Example 6 - Functional stability of FlexiMab and mAb after incubation at 37°C in human serum for 5 days.

Results shown in **Figure 8** were obtained to determine if the FlexiMab retains binding activity to its antigen upon incubation for 5 days in total human serum. The antibodies were incubated for 5 days in total human serum at 37ºC. Upon incubation, functional activity was determined using ELISA analysis as described in **Example 4**, except that the ELISA analysis was carried out with 5% final human serum concentration. As shown in **Figure 8**, this analysis shows that FlexiMab and mAb have comparable binding signals for EGFR, and indicates that FlexiMab is a stable molecule and is not susceptible to protease degradation by serum proteases.

Example 6 - Kinetic parameters of FlexiMab and mAb analyzed using BIAcore.

The results shown in **Figure 9** are BIAcore data carried out to determine the Kon, Koff and Kd of the FlexiMab and mAb. As shown in **Figure 9**, FlexiMab and mAb exhibit similar kinetic parameters for EGFR ligand immobilized on the BIAcore sensor chip. BIAcore experiments were carried out using BIAcore 3000 instrument (Biacore International) and using standard protocols as supplied by the manufacturer. EGFR was coupled to a dextran matrix of a CM5 sensor chip (Pharmacia Biosensor) using a standard amine coupling kit. Excess reactive esters were quenched by injection of 70 µL of 1.0 M ethanolamine hydrochloride (pH 8.5). The antibodies were injected at a flow rate of 5 uL/min. Responses were analyzed using BIOEVALUATION software and Kd is shown in pico-molar units.

Example 7 - Inhibition of cell survival mediated by FlexiMab and mAb.

Results in **Figure 10** show inhibition of cancer cell survival by an anti-EGFR antibody (mAb) in its classic format and of the anti-EGFR FlexiMab antibody variant. A non-binding isotype control antibody was used in these experiments. Three different cell lines were used: A431 (human epidermal carcinoma cells), BxPC3 (human pancreatic cancer cells) and H358 (human non-small lung adenocarcinoma cells). All of these cell lines express EGFR on their cell surface. As shown in

Figure 10, FlexiMab inhibits cancer cell survival and its inhibitory activity compared well with its parental mAb control antibody. The cells were cultured in standard medium and plated to non-tissue culture treated plates to a density of 5K. Antibodies in serial dilution were added to the cells and incubated in standard cell culture incubators at 37ºC for 72 hours. A luminescent cell titer assay (CellTiter-Glo) was used, according to manufacturer's instructions, to determine the number of viable cells by quantization of the ATP present in cell lysates, which is an indicator of metabolically active cells.

Example 8 - Killing of cancer cells by FlexiMab and mAb conjugated with an anti-human-Saporin antibody.

Results in **Figure 11** show killing of cancer cells SKBr3 (human mammary carcinoma cells) over-expressing Her3 on their cell surface. Specific FlexiMab and mAb used in this experiment are anti-Her3 specific antibodies. FlexiMab and mAb show similar in vitro cytotoxicity, indicating that both antibodies bind and internalize similarly to the Her3 receptor. These two antibodies were pre-complexed with an anti-human IgG-Saporin conjugate (Advanced Targeting Systems; San Diego). Upon receptor binding and internalization, Saporin will be released inside the cells. The release of Saporin into the cell will result in protein synthesis inhibition and cell death after approximately 72 hours. In these killing experiments negative control used were un-treated cells and cell-treated with anti-human-IgG-Saporin-conjugate only. As shown in **Figure 11**, the FlexiMab-Saporin conjugated shows a dose-dependence killing of SKBr3 comparable to mAb-Saporin conjugated. SKBr3 cells were plated at 3000 cells/90 uL/well and incubated overnight. Antibodies-Saporin conjugated and controls dilutions as indicated in **Figure 11**, were made in cell culture medium RPMI1640 supplemented with 10% FBS, and 10 uL of each dilution was added to each well in triplicate. The plates were incubated for 72 hours. Cell viability was determined by using CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison WI). As instructed by manufacture, 100 μl of CellTiter-Glo reagent was added into each well and incubated at room temperature for 10 minutes with gentle shaking. The luminescence of each sample in the plates was measured in a plate-reading luminometer and the cytotoxicity was calculated by using the luminescence signal of untreated cells as control with 100% of cell survival. Data analysis was done with Prism software (Graphpad, San Diego).

Example 9 - Steady-state kinetic affinities (Kd) of FlexiMab and mAb for Fcgamma receptors, FcgammaRIIIa genotype variant 158V and genotype variant 158F and FcRn, analyzed using BIAcore.

The example shown in **Figure 12** is BIAcore determined steady-state Kd in Molar units for FlexiMab and mAb. As shown in **Figure 12**, FlexiMab and mAb exhibit similar Kd values for FcRn binding, implying comparable *in vivo* half-life for these two antibodies. A Kd for FcgammaRIIIa could not be measured for FlexiMab (158V and 158F genotypes). These results indicate that Fc-mediated antibody effector functions could be potentially abrogated in the FlexiMab. BIAcore experiments were carried out using BIAcore 3000 instrument (Biacore International) and using standard protocols as supplied by the manufacturer. FcgammaRIIIA (158V and 158F genotypes) were captured on the BIAcore sensor chip using anti-histidine chips (Pharmacia Biosensor) and using a standard protocol as supplied by the manufacturer. For the FcRn measurements, FcRn was directly immobilized onto the BIAcore sensor chip using standard amino coupling chemistry as described in **Example 6**. FlexiMab and mAb were flowed at a flow rate of 5 uL/min over the captured/immobilized receptors. Responses were analyzed using the BIOEVALUATION software.

Example 10 - Pharmacokinetics analysis of FlexiMab and mAb.

Results in **Figure 13** show antibody concentration, in a logrithimic representation, versus time of circulation in mouse for mAb and FlexiMab dosed at 1 mg/kg and 10 mg/kg. As shown in **Figure 13**, mAb and FlexiMab have comparable *in vivo* half-life at high dose (10 mg/kg) and low dose (1 mg/kg). Pharmacokinetic analysis was carried out by intraperitoneal administration of 1 mg/kg and 10 mg/kg of mAb and FlexiMab antibodies into nude mice. The plasma concentrations of the antibodies were measured at 0, 1, 4, 24, 48, 90, 168, 216 and 336 hours post-dose and analyzed using ELISA methods as described in **Examples 3, 5 and 6**. An anti-human-kappa antibody was used for detection and standard curves for quantification were generated using known concentrations of mAb and FlexiMab.

Example 11 - Differential scanning calorimetry (DSC) analysis of FlexiMab and mAb.

The example shown in **Figure 14** is a differential scanning calorimetry analysis of FlexiMab and mAb. Differential scanning calorimetry (DSC) experiments, as depicted in **Figure 14**, at a heating rate of 1 °C/min, were carried out using a Microcal VP-DSC ultrasensitive scanning

microcalorimeter (Microcal, Northampton, MA). The thermograms showed in **Figure 14** are raw data baseline subtracted. DSC experiments were carried out in 25 mM Histidine-HCl, pH 6. All solutions and samples used for DSC were filtered using a 0.22 micron-filter and degassed prior to loading into the calorimeter. The two antibodies used for the DSC studies were >98% monomer as judged by analytical gel filtration chromatography (SEC-HPLC). For each set of measurements, at least four buffer-versus-baseline runs were first obtained. Immediately after, the buffer solution was removed from the sample cell and loaded with approximately 0.75 mL of sample at concentration of 1 mg/mL. For each measurement the reference cell was filled with matched sample buffer. In each sample-versus-buffer experiment, the corresponding buffer-versus-buffer baseline run was subtracted. The raw data were normalized for concentration and scan rate. Data analysis and deconvolution was carried out using the Origin™ DSC software provided by Microcal. **Figure 12** shows that the FlexiMab antibody showed two transition temperature peaks at 65°C and at 82°C, respectively. Whereas, mAb antibody showed two transition temperature peaks at 69°C and at 82°C, respectively. The DSC analysis showed that FlexiMab has a first transition temperature less than 4°C when compared to mAb (first transition peak in **Figure 14**), and has a comparable denaturation transition with the mAb for the second transition peak.

Example 12 - Monomeric content of FlexiMab at 11 mg/mL analyzed by analytical size-exclusion chromatography (SEC-HPLC).

Figure 15 shows results of an SEC-HPLC analysis of FlexiMab at 11 mg/mL in 25 mM Histidine-HCl pH 6. This example shows that FlexiMab is >98% monomeric at high concentration. The SEC-HPLC method performed is described in **Example 2**.

Example 13 - In vivo efficacy and body weight of FlexiMab and mAb using A431 xenograft.

Figure 16 shows tumor growth curves (**left panel**) and body weight curves (**right panel**) of A431 (human epithelial carcinoma cells) xenograft tumor in nude mice. In this example there were four mice groups, untreated control, and irrelevant isotype antibody control dosed at 10 mg/kg, mAb and FlexiMab groups dosed at 3 mg/kg. Nine nude mice were used in each group. The reported points (obtained at day 9, 12, 16, 19, and 23 post-inoculation) are average of tumor volume (**left panel**) and average of body weight (**right panel**) versus time of start of treatment. Mice were dosed with the antibodies when tumor reached an average volume size of 150~200 cubic millimeters. The tumor growth inhibition (DeltaTGI) is schematically reported (**legend at left**

panel). The tumor growth inhibition is 63% and 75% for mAb and FlexiMab, respectively. These results indicate that FlexiMab is efficacious *in vivo* comparable (if not better) than its parental mAb. As also shown (**left panel**) there is no drastic difference in total body weight lost for mAb and FlexiMab.

Example 14 - Design of cysteine mutants in the antibody CH1 region 131-139 (EU nomenclature) using FlexiMab for site-specific drug conjugation.

The example shown in **Figure 17** is a ribbon representation (**left panel**) of a FlexiMab with Fc and Fab domains schematically labeled. The black dots at the hinge and at the heavy and light chains represent valine substitutions of interchain cysteine amino acids (8 in total). Amino acids in the CH1 loop **(expanded view, left panel**) that were targeted for cysteine substitutions are labeled by amino acid and position in the ribbon representation. These residues are serine 131, serine 132, serine 134, threonine 135, serine 136, and threonine 139, and are in a CH1 structure region common to all four human immunoglobulin isotypes (IgG1, IgG2, IgG3 and IgG4; see Table 2). The 131-139 CH1 region is shown below in the sequence alignment as underlined bold text. The first underlined and embolded amino acid in the sequence alignment below corresponds to position 131 (serine in IgG1 and cysteine in IgG2, IgG3 and IgG4. The last underlined and embolded amino acid corresponds to position 139 (threonine in all four isotypes). Standard molecular biology techniques were used to generate single, double or triple FlexiMab cysteine variants.

Subtype	Subequence	SEQ ID NO:
IgG1	ASTKGPSVFPLAP**SSKSTSGGT**AALGCLVKDYFPEPVTVSWNSG ALTSGVHTFPAVLQSS	11
IgG2	ASTKGPSVFPLAP**CSRSTSGGT**AALGCLVKDYFPEPVTVSWNSG ALTSGVHTFPAVLQSS	12
IgG3	ASTKGPSVFPLAP**CSRSTSEST**AALGCLVKDYFPEPVTVSWNSGA LTSGVHTFPAVLQSS	13
IgG4	ASTKGPSVFPLAP**CSRSTSEST**AALGCLVKDYFPEPVTVSWNSGA LTSGVHTFPAVLQSS	14

Example 15 - Expression, purification and monomeric content of FlexiMab cysteine variants.

As shown in **Figure 18**, eight (8) FlexiMab Cysteines variants were generated. Of these eight variants, six (6) are single cysteine variants (Ser131Cys, Ser132Cys, Ser134Cys, Thr135Cys,

Ser136Cys and Thr139Cys), one is a double Cysteine variant (Ser131Cys-Thr139Cys) and one is a triple cysteine variant (Ser131Cys-Thr135Cys-Thr139Cys). The expression level as shown in **Figure 18** was ranging from 108 mg/L to 126 mg/L at day 7 post-transfection for all cysteine variants. This expression level is similar to the expected expression level of the FlexiMab antibody (**Figure 3, Example 2**). Total IgG expression was determined using a protein A binding assay as described in **Example 2**. Analytical size-exclusion HPLC chromatography (SEC-HPLC, Agilent 1100 Capillary LC System) was used, as detailed in **Example 2**, to determine the monomeric content of the cysteine constructs in PBS buffer. As shown in **Figure 18**, the monomeric content for FlexiMab cysteine mutants ranged from 96% to 99% monomer.

Example 16 - Site-specific conjugation, using a Maleimide-PEG(2)-Biotin, of FlexiMab cysteine variants.

Figure 19 shows the efficiency of site-specific conjugation for single, double and triple FlexiMab Cysteine variants. As shown in **Figure 19**, high efficiency of site-specific conjugation is achieved using cysteine variants engineered using the FlexiMab backbone. Because FlexiMab does not have a native interchain cysteine at the hinge or at the heavy and light chains, the site-specific conjugation is efficient and there is no scrambling on interchain disulfide bonds between the native and the engineered cysteines. The antibody cysteine variants used for conjugation were dialyzed overnight in 4 liters of 0.1 M Na-phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.4. The antibodies were removed from the dialysis apparatus and filtered through a 0.2 µM syringe filter. Using sterile Eppendorf tubes, 1 mg of the respective antibody variant was mixed with 1.87 uL of 50 mM TCEP solution [Tris-(2-carboxyethyl)-phosphine); Pierce] and 10 µL of DTPA [Diethylenetriaminepentaacetic acid; Sigma-Aldrich] and incubated at 37ºC for 2 hours under constant rotation. After the incubation, the antibodies were incubated with a 3 molar excess of Maleimide-PEG(2)-Biotin (Pierce). This incubation was done by adding 13.14 uL of a 3.8 uM Maleimide-PEG(2)-Biotin (MW 525.62 Da; Pierce) solution and 78 uL of DMSO (dimethyl sulfoxide; Sigma-Aldrich) to the TCEP-reduced antibody cysteine variants. The mixture was incubated for 30 minutes at 4ºC. The reaction was stopped by adding 2.5 uL of NAC [N-acetyl-L-cysteine; Sigma-Aldrich] and by mixing gently for 5 minutes. The conjugated antibodies were dialyzed overnight in 1X PBS pH 7.4 at 4ºC to remove the non-reacted Maleimide-PEG(2)-Biotin. Efficiency of conjugation was measured using standard mass spectrometry and peptide mapping as described in U.S. Patent Application Publication No. 2009092011 entitled “Cysteine engineered antibodies for site-specific conjugation."

Example 17 - FlexiMab is O-glycosylated at Threonine in position 225 (EU numbering) at upper region of human IgG1 hinge.

Shown in **Figure 20** is a sequence alignment of the upper human IgG1 hinge region for mAb and FlexiMab. In this sequence alignment the cysteines in mAb and the valine substitutions in FlexiMab are underlined. Intact mass and peptide mapping have shown that the threonine at position 225 (EU nomenclature), shown in **Figure 20** with an arrow, is modified post-translationally by a potential O-glycosylation. Standard mass spectrometry and peptide mapping techniques were used.

Example 18 - Examples of embodiments

Provided hereafter are non-limiting examples of some embodiments.

A1. An antibody, comprising:
a heavy chain having no native interchain cysteine amino acids;
a light chain having no native interchain cysteine amino acids; and
no native interchain disulphide linkages between the heavy chain and the light chain.

A1.1. The antibody of embodiment A1, comprising no interchain disulphide linkages between the heavy chain and the light chain.

A1.2. An antibody, comprising:
a heavy chain having no interchain cysteine amino acids;
a light chain having no interchain cysteine amino acids; and
no interchain disulphide linkages between the heavy chain and the light chain.

A2. The antibody of any one of embodiments A1 to A1.2, comprising two heavy chains and two light chains.

A3. The antibody of any one of embodiments A1 to A2, which is a full-length antibody.

A4. The antibody of any one of embodiments A1 to A3, wherein the heavy chain is about 446 amino acids in length and the light chain is about 214 amino acids in length.

A4.1. An antibody comprising a heavy chain comprising the amino acid sequence of SEQ ID NO: 5, and a light chain comprising the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, wherein each of the cysteines at positions 103, 109, and 112 in SEQ ID NO: 5, and the cysteine at position 105 in SEQ ID NO: 9 or the cysteine at position 102 in SEQ ID NO: 10, are substituted by an amino acid that is not cysteine, and wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

A4.2. An antibody comprising a heavy chain comprising the amino acid sequence of SEQ ID NO: 5, wherein each of the cysteines at positions 103, 109, and 112 in SEQ ID NO: 5 are substituted by an amino acid that is not cysteine, and wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

A4.3. An antibody comprising a heavy chain fragment comprising a portion of the amino acid sequence of SEQ ID NO: 5, provided that should the fragment include an amino acid at position(s) 103, 109, and/or 112 in SEQ ID NO: 5, the amino acid at each of the positions is not a cysteine, and wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

A4.4. The antibody of embodiment A4.2 or A4.3, which comprises a light chain comprising the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, wherein the cysteine at position 105 in SEQ ID NO: 9 or the cysteine at position 102 in SEQ ID NO: 10 is substituted by an amino acid that is not cysteine.

A4.5. The antibody of embodiment A4.2 or A4.3, which comprises a light chain fragment comprising a portion of the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, provided that should the fragment include position 105 of SEQ ID NO: 9 or position 102 of SEQ ID NO: 10, the amino acid at that position is not a cysteine.

A4.6. An antibody comprising an amino acid sequence comprising 80% or more amino acid sequence identitiy to an antibody of any one of emboidments A4.1 to A4.5, wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

A4.7. An antibody comprising a heavy chain comprising the amino acid sequence of SEQ ID NO: 6, and a light chain comprising the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, wherein each of the cysteines at positions 14, 103, 106, and 109 in SEQ ID NO: 6, and the cysteine at position 105 in SEQ ID NO: 9 or the cysteine at position 102 in SEQ ID NO: 10, are substituted by an amino acid that is not cysteine, and wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

A4.8. An antibody comprising a heavy chain comprising the amino acid sequence of SEQ ID NO: 6, wherein each of the cysteines at positions 14, 103, 106, and 109 in SEQ ID NO: 6 are substituted by an amino acid that is not cysteine, and wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

A4.9. An antibody comprising a heavy chain fragment comprising a portion of the amino acid sequence of SEQ ID NO: 6, provided that should the fragment include an amino acid at position(s) 14, 103, 106, and/or 109 in SEQ ID NO: 6, the amino acid at each of the positions is not a cysteine, and wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

A4.10. The antibody of embodiment A4.8 or A4.9, which comprises a light chain comprising the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, wherein the cysteine at position 105 in SEQ ID NO: 9 or the cysteine at position 102 in SEQ ID NO: 10 is substituted by an amino acid that is not cysteine.

A4.11. The antibody of embodiment A4.8 or A4.9, which comprises a light chain fragment comprising a portion of the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, provided that should the fragment include position 105 of SEQ ID NO: 9 or position 102 of SEQ ID NO: 10, the amino acid at that position is not a cysteine.

A4.12. An antibody comprising an amino acid sequence comprising 80% or more amino acid sequence identitiy to an antibody of any one of emboidments A4.7 to A4.11, wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

A4.13. An antibody comprising a heavy chain comprising the amino acid sequence of SEQ ID NO: 7, and a light chain comprising the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, wherein each of the cysteines at positions 14, 110, 113, 118 and 121 in SEQ ID NO: 7, and the cysteine at position 105 in SEQ ID NO: 9 or the cysteine at position 102 in SEQ ID NO: 10, are substituted by an amino acid that is not cysteine, and wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

A4.14. An antibody comprising a heavy chain comprising the amino acid sequence of SEQ ID NO: 7, wherein each of the cysteines at positions 14, 110, 113, 118 and 121 in SEQ ID NO: 7 are substituted by an amino acid that is not cysteine, and wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

A4.15. An antibody comprising a heavy chain fragment comprising a portion of the amino acid sequence of SEQ ID NO: 7, provided that should the fragment include an amino acid at position(s) 14, 110, 113, 118 and/or 121 in SEQ ID NO: 7, the amino acid at each of the positions is not a cysteine, and wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

A4.16. The antibody of embodiment A4.14 or A4.15, which comprises a light chain comprising the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, wherein the cysteine at position 105 in SEQ ID NO: 9 or the cysteine at position 102 in SEQ ID NO: 10 is substituted by an amino acid that is not cysteine.

A4.17. The antibody of embodiment A4.14 or A4.15, which comprises a light chain fragment comprising a portion of the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, provided that should the fragment include position 105 of SEQ ID NO: 9 or position 102 of SEQ ID NO: 10, the amino acid at that position is not a cysteine.

A4.18. An antibody comprising an amino acid sequence comprising 80% or more amino acid sequence identitiy to an antibody of any one of emboidments A4.13 to A4.17, wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

A4.19. An antibody comprising a heavy chain comprising the amino acid sequence of SEQ ID NO: 8, and a light chain comprising the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10,

wherein each of the cysteines at positions 14, 106 and 109 in SEQ ID NO: 8, and the cysteine at position 105 in SEQ ID NO: 9 or the cysteine at position 102 in SEQ ID NO: 10, are substituted by an amino acid that is not cysteine, and wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

A4.20. An antibody comprising a heavy chain comprising the amino acid sequence of SEQ ID NO: 8, wherein each of the cysteines at positions 14, 106 and 109 in SEQ ID NO: 8 are substituted by an amino acid that is not cysteine, and wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

A4.21. An antibody comprising a heavy chain fragment comprising a portion of the amino acid sequence of SEQ ID NO: 8, provided that should the fragment include an amino acid at position(s) 14, 106 and/or 109 in SEQ ID NO: 8, the amino acid at each of the positions is not a cysteine, and wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

A4.22. The antibody of embodiment A4.20 or A4.21, which comprises a light chain comprising the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, wherein the cysteine at position 105 in SEQ ID NO: 9 or the cysteine at position 102 in SEQ ID NO: 10 is substituted by an amino acid that is not cysteine.

A4.23. The antibody of embodiment A4.20 or A4.21, which comprises a light chain fragment comprising a portion of the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, provided that should the fragment include position 105 of SEQ ID NO: 9 or position 102 of SEQ ID NO: 10, the amino acid at that position is not a cysteine.

A4.24. An antibody comprising an amino acid sequence comprising 80% or more amino acid sequence identitiy to an antibody of any one of emboidments A4.19 to A4.23, wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

A5. The antibody of any one of embodiments A1 to A4.24, which is a human antibody.

A6. The antibody of any one of embodiments A1 to A5, which is a humanized antibody.

A7. An antibody comprising a heavy chain and a light chain, wherein the amino acid sequence of the light chain is about 80% or more identical to SEQ ID NO: 3 and the amino acid sequence of the heavy chain is about 80% or more identical to SEQ ID NO: 4, and wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

A8. The antibody of any one of embodiments A1 to A7, wherein the native interchain cysteine amino acids are replaced by amino acids having no thiol moiety.

A9. The antibody of embodiment A8, wherein an amino acid at positions in Table 1 each are replaced by an amino acid having no thiol moiety.

A10. The antibody of embodiment A8 or A9, wherein one or more of the native interchain cysteine amino acids are replaced by valine.

A11. The antibody of embodiment A10, wherein all of the native interchain cysteine amino acids are replaced by valine.

A12. The antibody of any one of embodiments A1 to A11, comprising a glycosylation site not present in an antibody counterpart having a native interchain cysteine amino acid.

A13. The antibody of any one of embodiments A1 to A12, which comprises an Fc region or fragment thereof.

A14. The antibody of embodiment A13, comprising one or more cysteine replacements of non-cysteine surface amino acids in the CH1 domain, CH2 domain, or CH3 domain, or combination thereof, of the antibody.

A15. The antibody of embodiment A14, comprising about 2 to about 40 of the cysteine replacements of the non-cysteine surface amino acids.

A16. The antibody of embodiment A14 or A15, which comprises about 2 to about 40 free thiols.

A17. The antibody of any one of embodiments A14 to A16, which is a human or humanized antibody.

A18. The antibody of embodiment A17, wherein the one or more cysteine replacements are at one or more of positions shown in Table 2.

A19. The antibody of embodiment A18, wherein the one or more cysteine replacements are at one or more of serine 131, serine 132, serine 134, threonine 135, serine 136 and threonine 139 of an IgG1 antibody, or counterpart position in an IgG2, IgG3 or IgG4 antibody.

A20. The antibody of embodiment A19, wherein the one or more cysteine replacements are at one or more of serine 131, threonine 135 and threonine 139 of IgG1 antibody, or counterpart position in an IgG2, IgG3 or IgG4 antibody.

A21. The antibody of any one of embodiments A14 to A20, wherein the one or more cysteine replacements are at one or more of positions shown in Table 3.

A22. The antibody of any one of embodiments A1 to A21, which has a stability of about 70% or more compared to an antibody counterpart containing all native interchain cysteines.

A23. The antibody of embodiment A22, wherein the stability is in vitro stability.

A24. The antibody of embodiment A23, wherein the stability is in serum at about 37 degrees Celsius for 5 days or more.

A25. The antibody of embodiment A23, wherein the stability is determined by calorimetry.

A26. The antibody of embodiment A22, wherein the stability is in vivo stability.

A27. The antibody of embodiment A25, wherein the stability is in an animal for 14 days or more.

A28. The antibody of any one of embodiments A1 to A27, which has a specific binding activity of about 70% or more compared to an antibody counterpart containing all native interchain cysteines.

A29. The antibody of embodiment A28, wherein the specific binding activity is in vitro.

A30. The antibody of embodiment A29, wherein the specific binding activity is quantified by an in vitro homogeneous assay or an in vitro heterogeneous assay.

A31. The antibody of embodiment A28, wherein the specific binding activity is in vivo.

A32. The antibody of embodiment A31, wherein the specific binding activity is determined in situ.

A33. The antibody of any one of embodiments A1 to A32, which has a cell proliferation inhibition activity of about 70% or more compared to an antibody counterpart containing all native interchain cysteines.

A34. The antibody of embodiment A33, wherein the cell proliferation inhibition activity is inhibition of proliferation of cancer cells.

A35. The antibody of embodiment A33 or A34, wherein the activity is in vitro.

A36. The antibody of embodiment A33 or A34, wherein the activity is in vivo.

A37. The antibody of any one of embodiments A1 to A36, which is about 90% or more monomeric.

A38. The antibody of embodiment A37, which is 90% or more monomeric in vitro.

A39. The antibody of any one of embodiments A1 to A38, which does not bind detectably to a human leukocyte receptor.

A40. The antibody of embodiment A39, wherein the human leukocyte receptor is a Fc gamma RIII receptor.

A41. The antibody of any one of embodiments A1 to A40, which binds to a neonatal Fc receptor with about 80% or more of the binding affinity compared to an antibody counterpart containing all native interchain cysteines.

A42. The antibody of any one of embodiments A1 to A32, which specifically binds to a cell surface molecule.

A43. The antibody of embodiment A42, wherein the cell surface molecule is internalized in a cell.

A44. The antibody of embodiment A43, wherein the cell surface molecule is a cell surface receptor.

A45. The antibody of embodiment A44, wherein the cell surface receptor comprises a protein kinase domain.

A46. The antibody of embodiment A45, wherein the cell surface receptor is an epidermal growth factor receptor (EGFR) protein tyrosine kinase.

A47. The antibody of embodiment A46, wherein the cell surface receptor is a HER3 protein tyrosine kinase.

B1. The antibody of any one of embodiments A1 to A41, which is an antibody conjugate in association with one or more heterologous molecules.

B2. The antibody of embodiment B1, wherein the antibody conjugate is about 80% or more of antibody products in a conjugation reaction product mixture.

B3. The antibody of embodiment B1 or B2, comprising one or more cysteine replacements of non-cysteine surface amino acids in the CH1 domain, CH2 domain, or CH3 domain, or combination thereof, of the antibody, wherein the one or more heterologous molecules are linked to the one or more cysteine replacements.

B4. The antibody of any one of embodiments B1 to B3, wherein the one or more heterologous molecules comprise a therapeutic agent.

B5. The antibody of embodiment B4, wherein the therapeutic agent comprises a toxin.

B6. The antibody of any one of embodiments B1 to B3, wherein the one or more heterologous molecules comprise a diagnostic agent.

B7. The antibody of embodiment B6, wherein the diagnostic agent comprises an imaging agent.

B8. The antibody of embodiment B7, wherein the diagnostic agent comprises a detectable label.

B9. The antibody of any one of embodiments B1 to B8, wherein the one or more heterologous molecules are linked to the antibody via a linker.

C1. The antibody of any one of embodiments A1 to A47, which is part of an antibody homomultimer conjugate.

C2. The antibody of embodiment C1, comprising one or more cysteine replacements of non-cysteine surface amino acids in the CH1 domain, CH2 domain, or CH3 domain, or combination thereof, of the antibody, wherein antibodies in the antibody homomultimer conjugate include a disulfide linkage between the one or more cysteine replacements.

D1. A nucleic acid comprising a nucleotide sequence that encodes an antibody of any one of embodiments A1 to A47.

D2. A cell comprising a nucleic acid of embodiment D1.

D3. An expression system comprising a nucleic acid of embodiment D1.

D4. An organism comprising a nucleic acid of embodiment D1.

D5. An organism comprising an expression system of embodiment D3.

E1. A process, comprising:

expressing an antibody in an expression system of embodiment D3, and

isolating the antibody, thereby producing an isolated antibody.

E2. The process of embodiment E1, comprising conjugating the isolated antibody with a heterologous molecule, thereby preparing an antibody conjugate.

E3. The process of embodiment E1, comprising conjugating the isolated antibody with other isolated antibody, thereby preparing an antibody multimer.

F1. A method, comprising:

contacting an antibody of any one of embodiments A1 to C2 with a biological sample; and

detecting the presence, absence or amount of antibody specifically bound to a molecule of interest in the biological sample.

F2. The method of embodiment F1, comprising linking the antibody to a solid support.

F3. A method, comprising:

administering an antibody of any one of embodiments A1 to C2 to cells; and

detecting the presence, absence or amount of antibody in a location of the cells.

F4. A method, comprising:

administering an antibody of any one of embodiments A1 to C2 to a subject; and

detecting the presence, absence or amount of antibody in a tissue of the subject.

F5. A method, comprising:

administering an antibody of any one of embodiments A1 to C2 to cells; and

detecting the presence, absence or amount of a biological effect associated with the administration of the antibody to the cells.

F6. A method, comprising:

administering an antibody of any one of embodiments A1 to C2 to a subject; and

detecting the presence, absence or amount of a biological effect in the subject associated with the administration of the antibody.

F7. The method of embodiment F5 or F6, wherein the biological effect is cell proliferation inhibition.

F8. A method, comprising:

administering an antibody of any one of embodiments A1 to C2 to a subject; and

monitoring the condition of the subject.

The entirety of each patent, patent application, publication and document referenced herein hereby is incorporated by reference. Citation of the above patents, patent applications, publications and documents is not an admission that any of the foregoing is pertinent prior art, nor does it constitute any admission as to the contents or date of these publications or documents.

Modifications may be made to the foregoing without departing from the basic aspects of the technology. Although the technology has been described in substantial detail with reference to one or more specific embodiments, those of ordinary skill in the art will recognize that changes may be made to the embodiments specifically disclosed in this application, these modifications and improvements are within the scope and spirit of the technology.

The technology illustratively described herein suitably may be practiced in the absence of any element(s) not specifically disclosed herein. Thus, for example, the term “comprising” in each instance encompasses the terms “consisting essentially of” or “consisting of." The terms and expressions which have been employed are used as terms of description and not of limitation, and use of such terms and expressions do not exclude any equivalents of the features shown and described or portions thereof, and various modifications are possible within the scope of the technology claimed. The term “a” or “an” can refer to one of or a plurality of the elements it modifies (e.g., “a reagent” can mean one or more reagents) unless it is contextually clear either one of the elements or more than one of the elements is described. Use of the term “about” at the beginning of a string of values modifies each of the values (i.e., “about 1, 2 and 3” refers to about 1, about 2 and about 3). In certain instances units and formatting are expressed in HyperText Markup Language (HTML) format, which can be translated to another conventional format by those skilled in the art (e.g., ".sup." refers to superscript formatting). Thus, it should be understood that although the present technology has been specifically disclosed by representative embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and such modifications and variations are considered within the scope of this technology.

Certain embodiments of the technology are set forth in the claim(s) that follow(s).

What is claimed is:

1. An antibody, comprising:
a heavy chain having no native interchain cysteine amino acids;
a light chain having no native interchain cysteine amino acids; and
no native interchain disulphide linkages between the heavy chain and the light chain.

2. The antibody of claim 1, comprising no interchain disulphide linkages between the heavy chain and the light chain.

3. An antibody, comprising:
a heavy chain having no interchain cysteine amino acids;
a light chain having no interchain cysteine amino acids; and
no interchain disulphide linkages between the heavy chain and the light chain.

4. The antibody of any one of claims 1 to 3, comprising two heavy chains and two light chains.

5. The antibody of any one of claims 1 to 4, which is a full-length antibody.

6. The antibody of any one of claims 1 to 5, wherein the heavy chain is about 446 amino acids in length and the light chain is about 214 amino acids in length.

7. An antibody comprising a heavy chain comprising the amino acid sequence of SEQ ID NO: 5, and a light chain comprising the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, wherein each of the cysteines at positions 103, 109, and 112 in SEQ ID NO: 5, and the cysteine at position 105 in SEQ ID NO: 9 or the cysteine at position 102 in SEQ ID NO: 10, are substituted by an amino acid that is not cysteine, and wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

8. An antibody comprising a heavy chain comprising the amino acid sequence of SEQ ID NO: 5, wherein each of the cysteines at positions 103, 109, and 112 in SEQ ID NO: 5

are substituted by an amino acid that is not cysteine, and wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

9. An antibody comprising a heavy chain fragment comprising a portion of the amino acid sequence of SEQ ID NO: 5, provided that should the fragment include an amino acid at position(s) 103, 109, and/or 112 in SEQ ID NO: 5, the amino acid at each of the positions is not a cysteine, and wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

10. The antibody of claim 8 or 9, which comprises a light chain comprising the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, wherein the cysteine at position 105 in SEQ ID NO: 9 or the cysteine at position 102 in SEQ ID NO: 10 is substituted by an amino acid that is not cysteine.

11. The antibody of claim 8 or 9, which comprises a light chain fragment comprising a portion of the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, provided that should the fragment include position 105 of SEQ ID NO: 9 or position 102 of SEQ ID NO: 10, the amino acid at that position is not a cysteine.

12. An antibody comprising an amino acid sequence comprising 80% or more amino acid sequence identity to an antibody of any one of claims 7 to 11, wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

13. An antibody comprising a heavy chain comprising the amino acid sequence of SEQ ID NO: 6, and a light chain comprising the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, wherein each of the cysteines at positions 14, 103, 106, and 109 in SEQ ID NO: 6, and the cysteine at position 105 in SEQ ID NO: 9 or the cysteine at position 102 in SEQ ID NO: 10, are substituted by an amino acid that is not cysteine, and wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

14. An antibody comprising a heavy chain comprising the amino acid sequence of SEQ ID NO: 6, wherein each of the cysteines at positions 14, 103, 106, and 109 in SEQ ID

NO: 6 are substituted by an amino acid that is not cysteine, and wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

15. An antibody comprising a heavy chain fragment comprising a portion of the amino acid sequence of SEQ ID NO: 6, provided that should the fragment include an amino acid at position(s) 14, 103, 106, and/or 109 in SEQ ID NO: 6, the amino acid at each of the positions is not a cysteine, and wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

16. The antibody of claim 14 or 15, which comprises a light chain comprising the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, wherein the cysteine at position 105 in SEQ ID NO: 9 or the cysteine at position 102 in SEQ ID NO: 10 is substituted by an amino acid that is not cysteine.

17. The antibody of claim 14 or 15, which comprises a light chain fragment comprising a portion of the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, provided that should the fragment include position 105 of SEQ ID NO: 9 or position 102 of SEQ ID NO: 10, the amino acid at that position is not a cysteine.

18. An antibody comprising an amino acid sequence comprising 80% or more amino acid sequence identity to an antibody of any one of claims 13 to 17, wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

19. An antibody comprising a heavy chain comprising the amino acid sequence of SEQ ID NO: 7, and a light chain comprising the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, wherein each of the cysteines at positions 14, 110, 113, 118 and 121 in SEQ ID NO: 7, and the cysteine at position 105 in SEQ ID NO: 9 or the cysteine at position 102 in SEQ ID NO: 10, are substituted by an amino acid that is not cysteine, and wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

20. An antibody comprising a heavy chain comprising the amino acid sequence of SEQ ID NO: 7, wherein each of the cysteines at positions 14, 110, 113, 118 and 121 in SEQ

ID NO: 7 are substituted by an amino acid that is not cysteine, and wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

21. An antibody comprising a heavy chain fragment comprising a portion of the amino acid sequence of SEQ ID NO: 7, provided that should the fragment include an amino acid at position(s) 14, 110, 113, 118 and/or 121 in SEQ ID NO: 7, the amino acid at each of the positions is not a cysteine, and wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

22. The antibody of claim 20 or 21, which comprises a light chain comprising the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, wherein the cysteine at position 105 in SEQ ID NO: 9 or the cysteine at position 102 in SEQ ID NO: 10 is substituted by an amino acid that is not cysteine.

23. The antibody of claim 20 or 21, which comprises a light chain fragment comprising a portion of the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, provided that should the fragment include position 105 of SEQ ID NO: 9 or position 102 of SEQ ID NO: 10, the amino acid at that position is not a cysteine.

24. An antibody comprising an amino acid sequence comprising 80% or more amino acid sequence identity to an antibody of any one of claims 19 to 23, wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

25. An antibody comprising a heavy chain comprising the amino acid sequence of SEQ ID NO: 8, and a light chain comprising the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, wherein each of the cysteines at positions 14, 106 and 109 in SEQ ID NO: 8, and the cysteine at position 105 in SEQ ID NO: 9 or the cysteine at position 102 in SEQ ID NO: 10, are substituted by an amino acid that is not cysteine, and wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

26. An antibody comprising a heavy chain comprising the amino acid sequence of SEQ ID NO: 8, wherein each of the cysteines at positions 14, 106 and 109 in SEQ ID NO: 8

are substituted by an amino acid that is not cysteine, and wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

27. An antibody comprising a heavy chain fragment comprising a portion of the amino acid sequence of SEQ ID NO: 8, provided that should the fragment include an amino acid at position(s) 14, 106 and/or 109 in SEQ ID NO: 8, the amino acid at each of the positions is not a cysteine, and wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

28. The antibody of claim 26 or 27, which comprises a light chain comprising the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, wherein the cysteine at position 105 in SEQ ID NO: 9 or the cysteine at position 102 in SEQ ID NO: 10 is substituted by an amino acid that is not cysteine.

29. The antibody of claim 26 or 27, which comprises a light chain fragment comprising a portion of the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10, provided that should the fragment include position 105 of SEQ ID NO: 9 or position 102 of SEQ ID NO: 10, the amino acid at that position is not a cysteine.

30. An antibody comprising an amino acid sequence comprising 80% or more amino acid sequence identity to an antibody of any one of claims 25 to 29, wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

31. The antibody of any one of claims 1 to 30, which is a human antibody.

32. The antibody of any one of claims 1 to 31, which is a humanized antibody.

33. An antibody comprising a heavy chain and a light chain, wherein the amino acid sequence of the light chain is about 80% or more identical to SEQ ID NO: 3 and the amino acid sequence of the heavy chain is about 80% or more identical to SEQ ID NO: 4, and wherein the antibody comprises no interchain cysteine amino acids and no interchain disulfide linkages.

34. The antibody of any one of claims 1 to 33, wherein the native interchain cysteine amino acids are replaced by amino acids having no thiol moiety.

35. The antibody of claim 34, wherein an amino acid at positions in Table 1 each are replaced by an amino acid having no thiol moiety.

36. The antibody of claim 34 or 35, wherein one or more of the native interchain cysteine amino acids are replaced by valine.

37. The antibody of claim 36, wherein all of the native interchain cysteine amino acids are replaced by valine.

38. The antibody of any one of claims 1 to 37, comprising a glycosylation site not present in an antibody counterpart having a native interchain cysteine amino acid.

39. The antibody of any one of claims 1 to 38, which comprises an Fc region or fragment thereof.

40. The antibody of claim 39, comprising one or more cysteine replacements of non-cysteine surface amino acids in the CH1 domain, CH2 domain, or CH3 domain, or combination thereof, of the antibody.

41. The antibody of claim 40, comprising about 2 to about 40 of the cysteine replacements of the non-cysteine surface amino acids.

42. The antibody of claim 40 or 41, which comprises about 2 to about 40 free thiols.

43. The antibody of any one of claims 40 to 42, which is a human or humanized antibody.

44. The antibody of claim 43, wherein the one or more cysteine replacements are at one or more of positions shown in Table 2.

45. The antibody of claim 44, wherein the one or more cysteine replacements are at one or more of serine 131, serine 132, serine 134, threonine 135, serine 136 and threonine 139 of an IgG1 antibody, or counterpart position in an IgG2, IgG3 or IgG4 antibody.

46. The antibody of claim 45, wherein the one or more cysteine replacements are at one or more of serine 131, threonine 135 and threonine 139 of IgG1 antibody, or counterpart position in an IgG2, IgG3 or IgG4 antibody.

47. The antibody of any one of claims 40 to 46, wherein the one or more cysteine replacements are at one or more of positions shown in Table 3.

48. The antibody of any one of claims 1 to 47, which has a stability of about 70% or more compared to an antibody counterpart containing all native interchain cysteines.

49. The antibody of claim 48, wherein the stability is in vitro stability.

50. The antibody of claim 49, wherein the stability is in serum at about 37 degrees Celsius for 5 days or more.

51. The antibody of claim 49, wherein the stability is determined by calorimetry.

52. The antibody of claim 48, wherein the stability is in vivo stability.

53. The antibody of claim 51, wherein the stability is in an animal for 14 days or more.

54. The antibody of any one of claims 1 to 53, which has a specific binding activity of about 70% or more compared to an antibody counterpart containing all native interchain cysteines.

55. The antibody of claim 54, wherein the specific binding activity is in vitro.

56. The antibody of claim 55, wherein the specific binding activity is quantified by an in vitro homogeneous assay or an in vitro heterogeneous assay.

57. The antibody of claim 54, wherein the specific binding activity is in vivo.

58. The antibody of claim 57, wherein the specific binding activity is determined in situ.

59. The antibody of any one of claims 1 to 58, which has a cell proliferation inhibition activity of about 70% or more compared to an antibody counterpart containing all native interchain cysteines.

60. The antibody of claim 59, wherein the cell proliferation inhibition activity is inhibition of proliferation of cancer cells.

61. The antibody of claim 59 or 60, wherein the activity is in vitro.

62. The antibody of claim 59 or 60, wherein the activity is in vivo.

63. The antibody of any one of claims 1 to 62, which is about 90% or more monomeric.

64. The antibody of claim 63, which is 90% or more monomeric in vitro.

65. The antibody of any one of claims 1 to 64, which does not bind detectably to a human leukocyte receptor.

66. The antibody of claim 65, wherein the human leukocyte receptor is a Fc gamma RIII receptor.

67. The antibody of any one of claims 1 to 66, which binds to a neonatal Fc receptor with about 80% or more of the binding affinity compared to an antibody counterpart containing all native interchain cysteines.

68. The antibody of any one of claims 1 to 58, which specifically binds to a cell surface molecule.

69. The antibody of claim 68, wherein the cell surface molecule is internalized in a cell.

70. The antibody of claim 69, wherein the cell surface molecule is a cell surface receptor.

71. The antibody of claim 70, wherein the cell surface receptor comprises a protein kinase domain.

72. The antibody of claim 71, wherein the cell surface receptor is an epidermal growth factor receptor (EGFR) protein tyrosine kinase.

73. The antibody of claim 72, wherein the cell surface receptor is a HER3 protein tyrosine kinase.

74. The antibody of any one of claims 1 to 73, which is an antibody conjugate in association with one or more heterologous molecules.

75. The antibody of claim 74, wherein the antibody conjugate is about 80% or more of antibody products in a conjugation reaction product mixture.

76. The antibody of claim 74 or 75, comprising one or more cysteine replacements of non-cysteine surface amino acids in the CH1 domain, CH2 domain, or CH3 domain, or combination thereof, of the antibody, wherein the one or more heterologous molecules are linked to the one or more cysteine replacements.

77. The antibody of any one of claims 74 to 76, wherein the one or more heterologous molecules comprise a therapeutic agent.

78. The antibody of claim 77, wherein the therapeutic agent comprises a toxin.

79. The antibody of any one of claims 74 to 76, wherein the one or more heterologous molecules comprise a diagnostic agent.

80. The antibody of claim 79, wherein the diagnostic agent comprises an imaging agent.

81. The antibody of claim 80, wherein the diagnostic agent comprises a detectable label.

82. The antibody of any one of claims 74 to 81, wherein the one or more heterologous molecules are linked to the antibody via a linker.

83. The antibody of any one of claims 1 to 73, which is part of an antibody homomultimer conjugate.

84. The antibody of claim 83, comprising one or more cysteine replacements of non-cysteine surface amino acids in the CH1 domain, CH2 domain, or CH3 domain, or combination thereof, of the antibody, wherein antibodies in the antibody homomultimer conjugate include a disulfide linkage between the one or more cysteine replacements.

85. A nucleic acid comprising a nucleotide sequence that encodes an antibody of any one of claims 1 to 73.

86. A cell comprising a nucleic acid of claim 85.

87. An expression system comprising a nucleic acid of claim 85.

88. An organism comprising a nucleic acid of claim 85.

89. An organism comprising an expression system of claim 87.

90. A process, comprising:
expressing an antibody in an expression system of claim 87, and
isolating the antibody, thereby producing an isolated antibody.

91. The process of claim 90, comprising conjugating the isolated antibody with a heterologous molecule, thereby preparing an antibody conjugate.

92. The process of claim 90, comprising conjugating the isolated antibody with other isolated antibody, thereby preparing an antibody multimer.

93. A method, comprising:

contacting an antibody of any one of claims 1 to 84 with a biological sample; and

detecting the presence, absence or amount of antibody specifically bound to a molecule of interest in the biological sample.

94. The method of claim 93, comprising linking the antibody to a solid support.

95. A method, comprising:

administering an antibody of any one of claims 1 to 84 to cells; and

detecting the presence, absence or amount of antibody in a location of the cells.

96. A method, comprising:

administering an antibody of any one of claims 1 to 84 to a subject; and

detecting the presence, absence or amount of antibody in a tissue of the subject.

97. A method, comprising:

administering an antibody of any one of claims 1 to 84 to cells; and

detecting the presence, absence or amount of a biological effect associated with the administration of the antibody to the cells.

98. A method, comprising:

administering an antibody of any one of claims 1 to 84 to a subject; and

detecting the presence, absence or amount of a biological effect in the subject associated with the administration of the antibody.

99. The method of claim 97 or 98, wherein the biological effect is cell proliferation inhibition.

100. A method, comprising:

administering an antibody of any one of claims 1 to 84 to a subject; and

monitoring the condition of the subject.

FIGURE 1

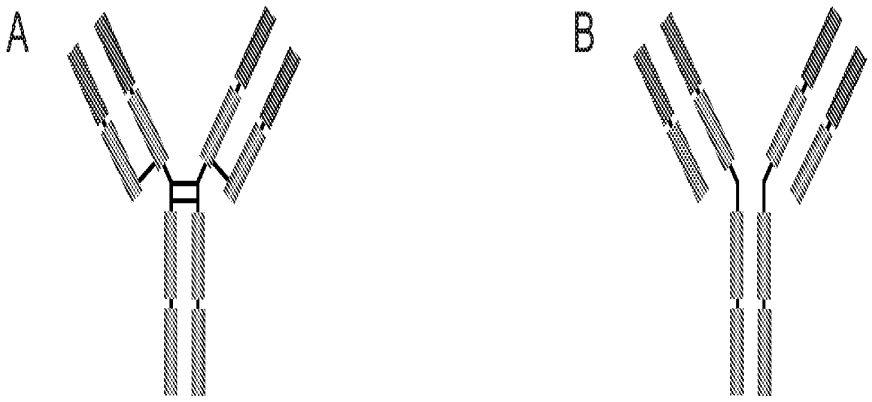


FIGURE 2

A >mAb-VL-CL-kappa-anti-EGFR (SEQ ID NO: 1)

DIQMTQSPSSLSASVGDRVTITCQASQDISNYLNWYQQKPGKAPKLLIYDASNLETGVPSRFSGSGSGTDFTFTISSLQPEDIATYFCQH
FDHLPLAFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLT
LSKADYEKHKVYACEVTHQGLSSPVTKSFNRGE**C**

B >mAb-VH-CH1gamma1-CH2gamma2-CH3gamma3-anti-EGFR (SEQ ID NO: 2)

QVQLQESGPGLVKPSETLSLTCTVSGGSVSSGDYYWTWIRQSPGKGLEWIGHIYYSGNTNYNPSLKSRLTISIDTSKTQFSLKLSSVTAA
DTAIYYCVRDRVTGAFDIWGQGTMVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG
LYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKS**C**DKTHT**C**PP**C**PAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS
HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREE
MTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

C >mAb-Val-VL-CL-kappa-anti-EGFR (SEQ ID NO: 3)

DIQMTQSPSSLSASVGDRVTITCQASQDISNYLNWYQQKPGKAPKLLIYDASNLETGVPSRFSGSGSGTDFTFTISSLQPEDIATYFCQH
FDHLPLAFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLT
LSKADYEKHKVYACEVTHQGLSSPVTKSFNRGE**V**

D >mAb-Val-VH-CH1gamma1-CH2gamma2-CH3gamma3-anti-EGFR (SEQ ID NO: 4)

QVQLQESGPGLVKPSETLSLTCTVSGGSVSSGDYYWTWIRQSPGKGLEWIGHIYYSGNTNYNPSLKSRLTISIDTSKTQFSLKLSSVTAA
DTAIYYCVRDRVTGAFDIWGQGTMVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG
LYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKS**V**DKTHT**V**PP**V**PAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS
HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREE
MTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

FIGURE 3

Format name	Transient expression mg/l at day 6 post-transfection	% monomer after protein A purification	% monomeric after dialysis in 25 mM His-HCl pH 6
mAb	145	98	98
mAb-Val	151	98	98

FIGURE 4

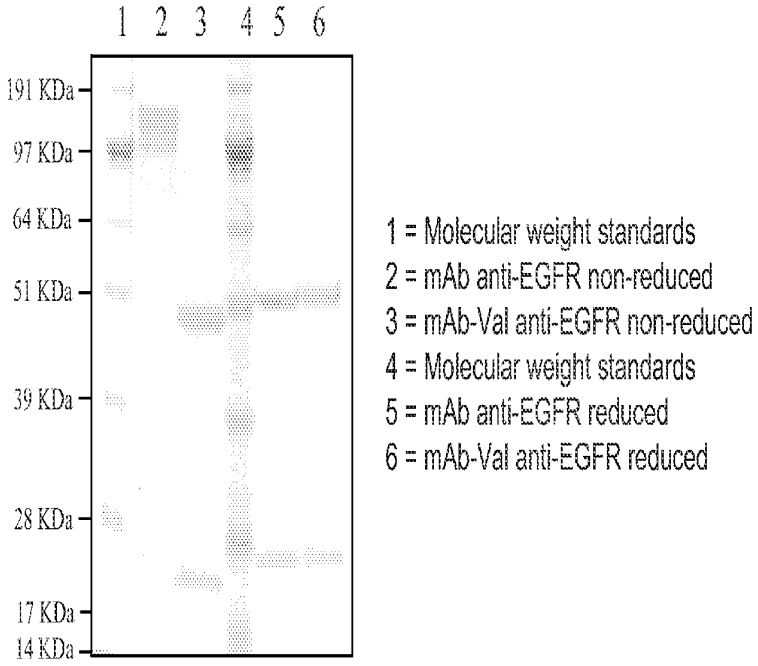

1 2 3 4 5 6
191 KDa
97 KDa
64 KDa
51 KDa
39 KDa
28 KDa
17 KDa
14 KDa
1 = Molecular weight standards
2 = mAb anti-EGFR non-reduced
3 = mAb-Val anti-EGFR non-reduced
4 = Molecular weight standards
5 = mAb anti-EGFR reduced
6 = mAb-Val anti-EGFR reduced

FIGURE 5

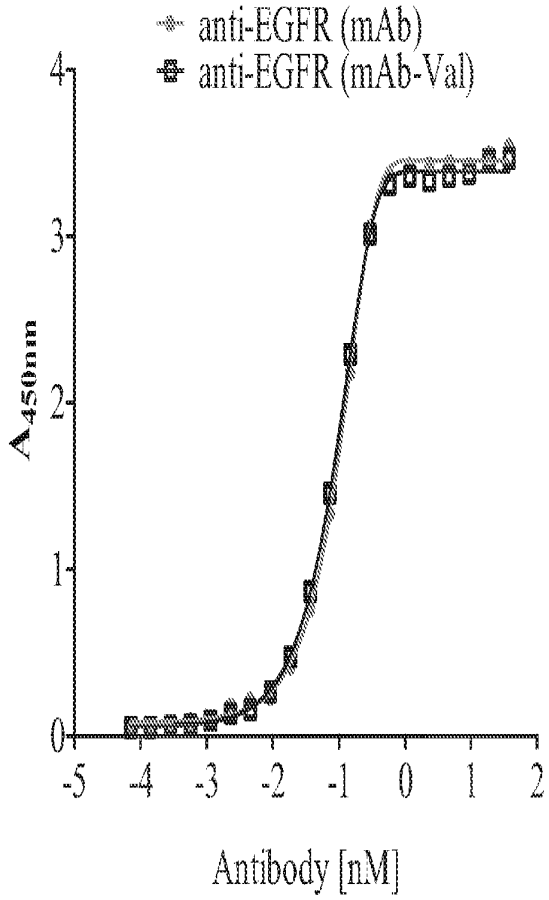

anti-EGFR (mAb)
anti-EGFR (mAb-Val)
A450nm
0
1
2
3
4
-5
-4
-3
-2
-1
0
1
2
Antibody [nM]

FIGURE 6

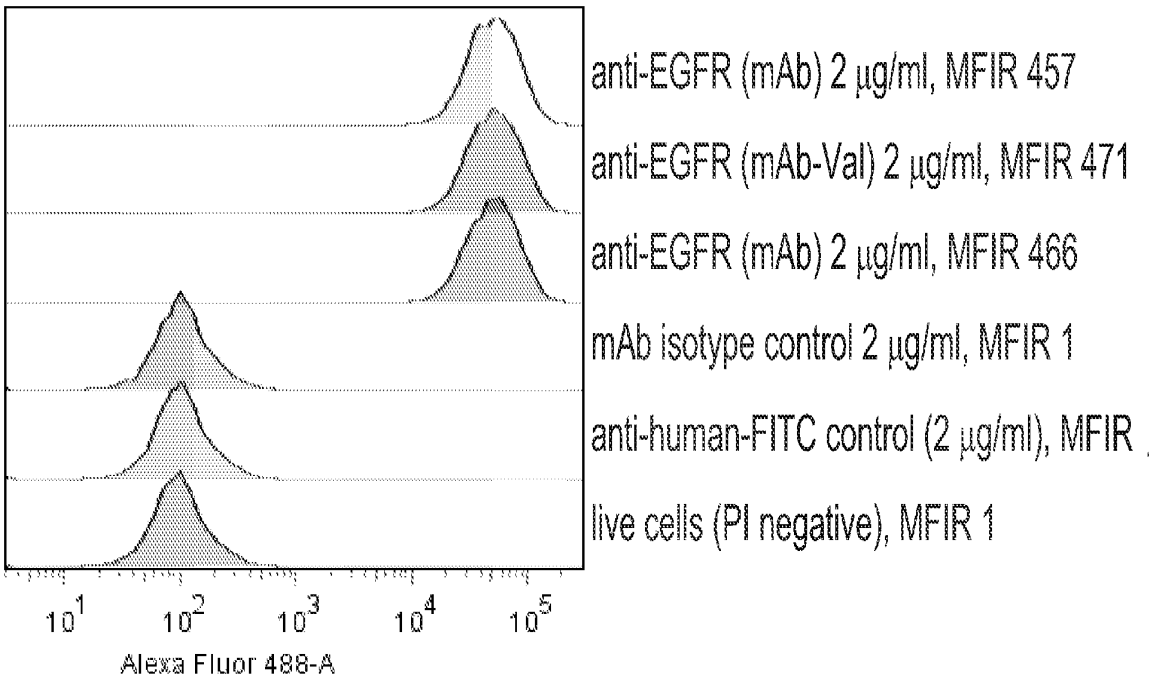

anti-EGFR (mAb) 2 µg/ml, MFIR 457
anti-EGFR (mAb-Val) 2 µg/ml, MFIR 471
anti-EGFR (mAb) 2 µg/ml, MFIR 466
mAb isotype control 2 µg/ml, MFIR 1
anti-human-FITC control (2 µg/ml), MFIR
live cells (PI negative), MFIR 1
10^1
10^2
10^3
10^4
10^5
Alexa Fluor 488-A

FIGURE 7

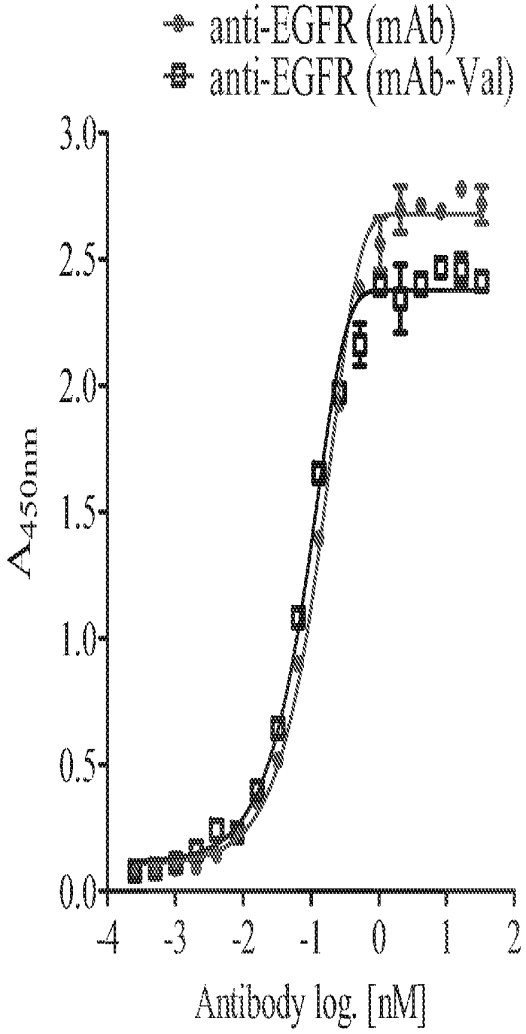

anti-EGFR (mAb)
anti-EGFR (mAb-Val)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
A450nm
-4
-3
-2
-1
0
1
2
Antibody log. [nM]

FIGURE 8

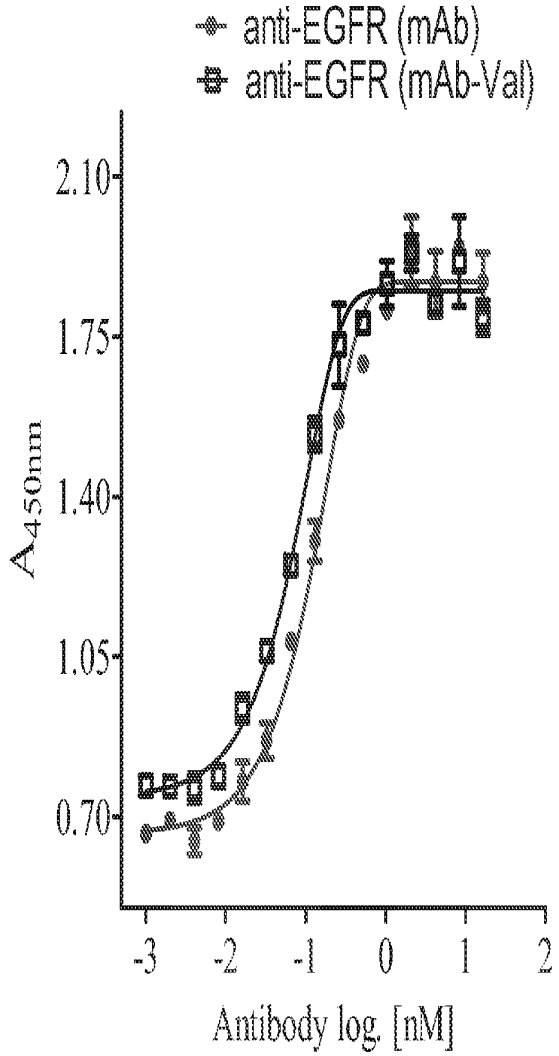

anti-EGFR (mAb)
anti-EGFR (mAb-Val)
2.10
1.75
1.40
1.05
0.70
A450nm
-3
-2
-1
0
1
2
Antibody log. [nM]

FIGURE 9

Construct name	K_{on} (x10^{-5}), M^{-1} s^{-1}	K_{off} (x10^{-4}), s^{-1}	K_d (pM)
anti-EGFR (mAb)	3.38	0.88	261
anti-EGFR (mAb-Val)	3.04	0.96	317

FIGURE 10

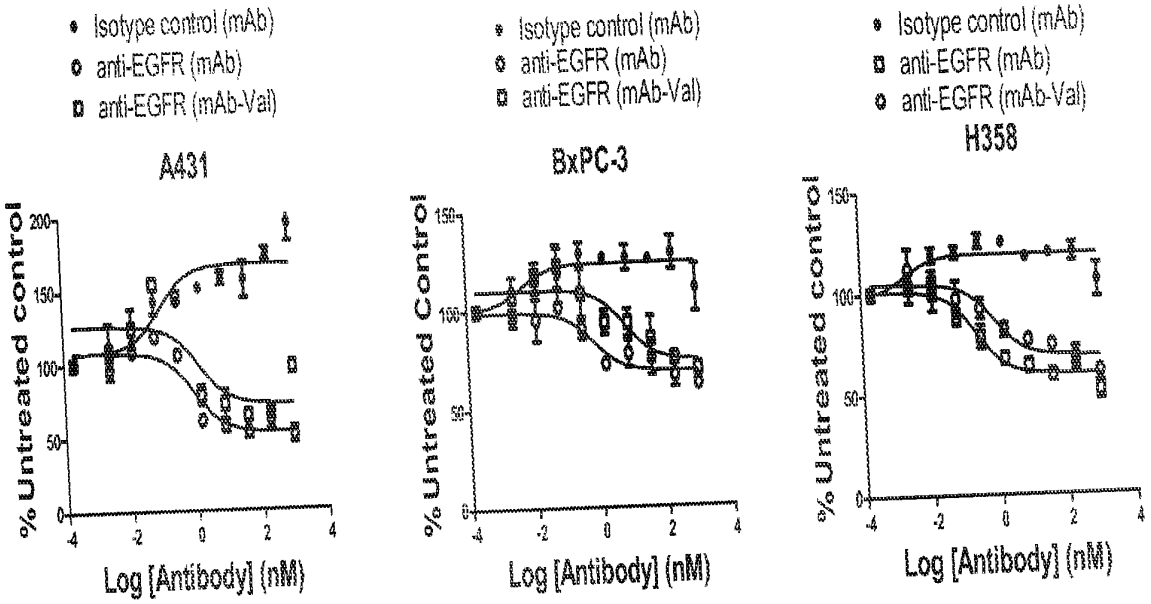

Isotype control (mAb)
anti-EGFR (mAb)
anti-EGFR (mAb-Val)
A431
% Untreated control
Log [Antibody] (nM)
Isotype control (mAb)
anti-EGFR (mAb)
anti-EGFR (mAb-Val)
BxPC-3
% Untreated Control
Log [Antibody] (nM)
Isotype control (mAb)
anti-EGFR (mAb)
anti-EGFR (mAb-Val)
H358
% Untreated control
Log [Antibody] (nM)

FIGURE 11

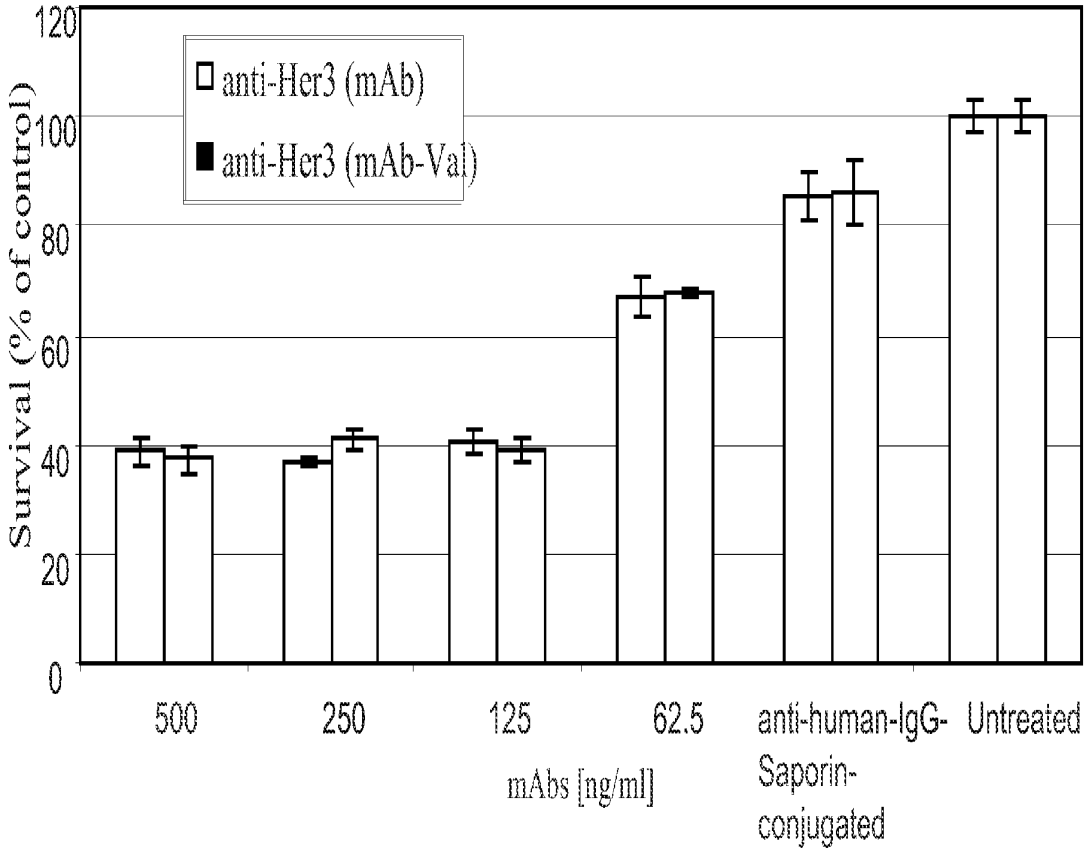

120
100
80
60
40
20
0
Survival (% of control)
anti-Her3 (mAb)
anti-Her3 (mAb-Val)
500
250
125
62.5
anti-human-IgG-Saporin-conjugated
Untreated
mAbs [ng/ml]

FIGURE 12

Construct name	K_D FcγRIIIa 158V (M)	K_D FcγRIIIa 158F (M)	K_D FcRn (M)
anti-EGFR (mAb)	6.69×10^{-07}	2.33×10^{-07}	2.40×10^{-08}
anti-EGFR (mAb-Val)	Not measurable	Not measurable	3.31×10^{-08}

FIGURE 13

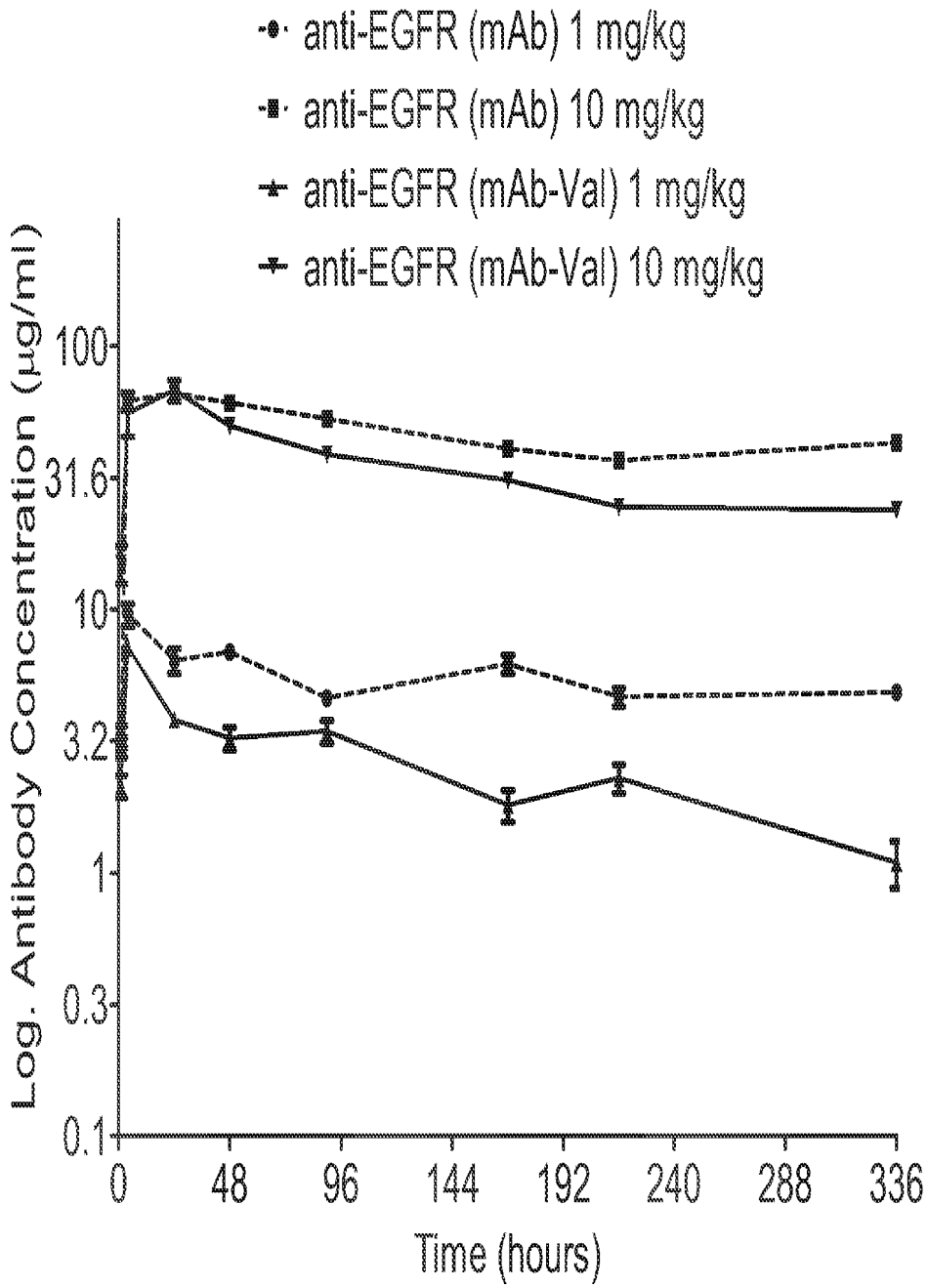

anti-EGFR (mAb) 1 mg/kg
anti-EGFR (mAb) 10 mg/kg
anti-EGFR (mAb-Val) 1 mg/kg
anti-EGFR (mAb-Val) 10 mg/kg
Log. Antibody Concentration (µg/ml)
100
31.6
10
3.2
1
0.3
0.1
0
48
96
144
192
240
288
336
Time (hours)

FIGURE 14

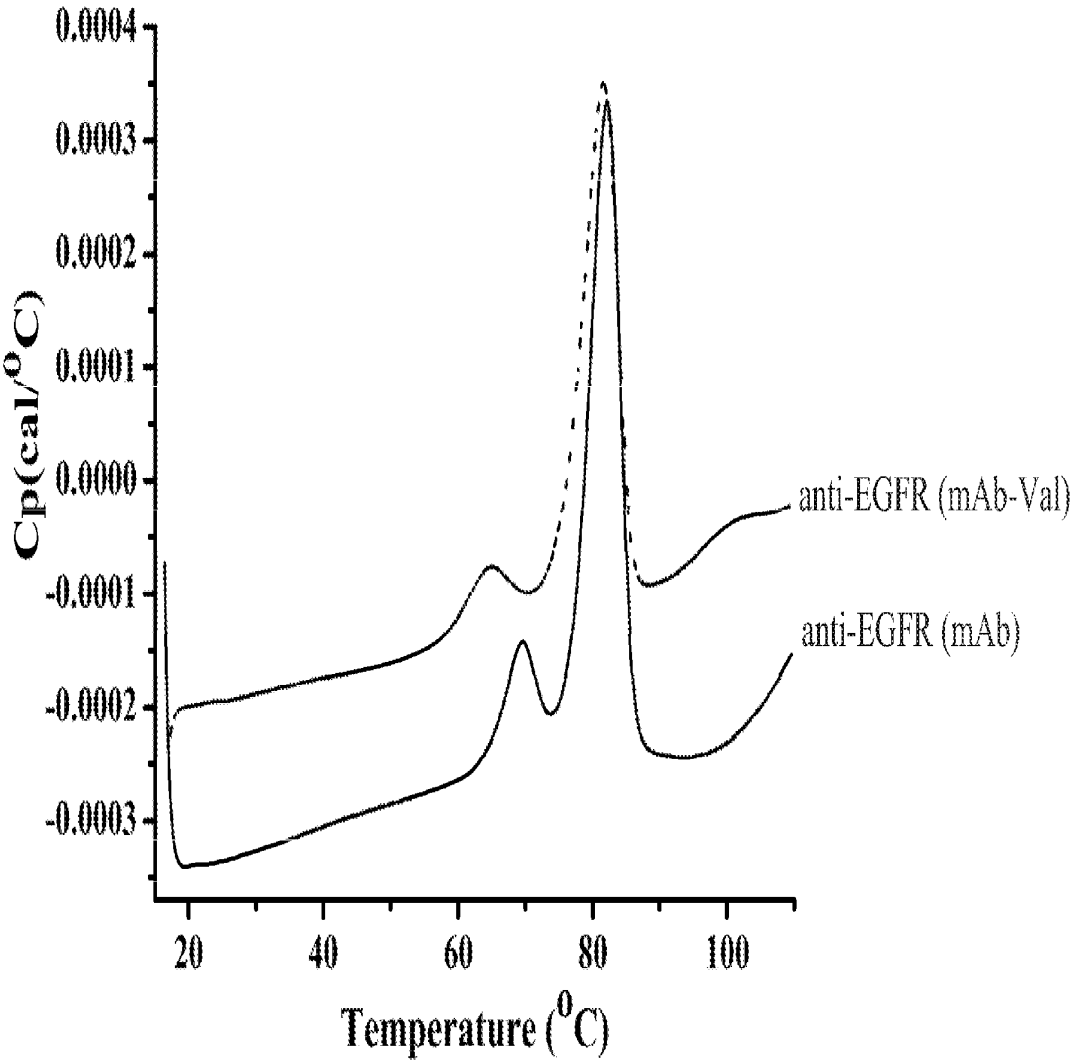
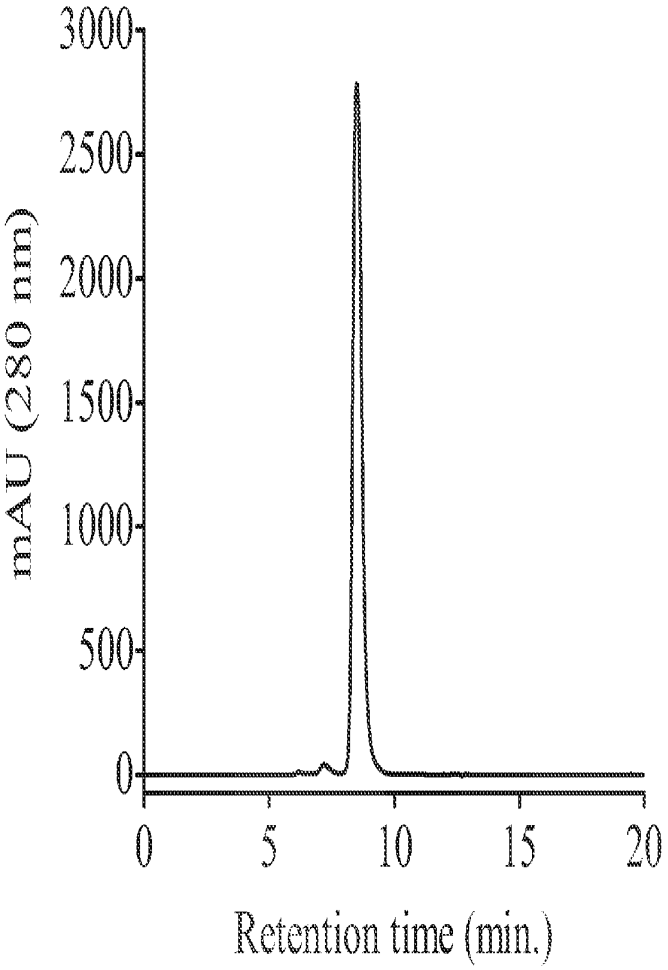

0.0004
0.0003
0.0002
0.0001
0.0000
-0.0001
-0.0002
-0.0003
Cp(cal/°C)
20
40
60
80
100
Temperature (°C)
anti-EGFR (mAb-Val)
anti-EGFR (mAb)

FIGURE 15


3000
2500
2000
1500
1000
500
0
mAU (280 nm)
0
5
10
15
20
Retention time (min.)

WO 2012/064733
PCT/US2011/059775

FIGURE 16

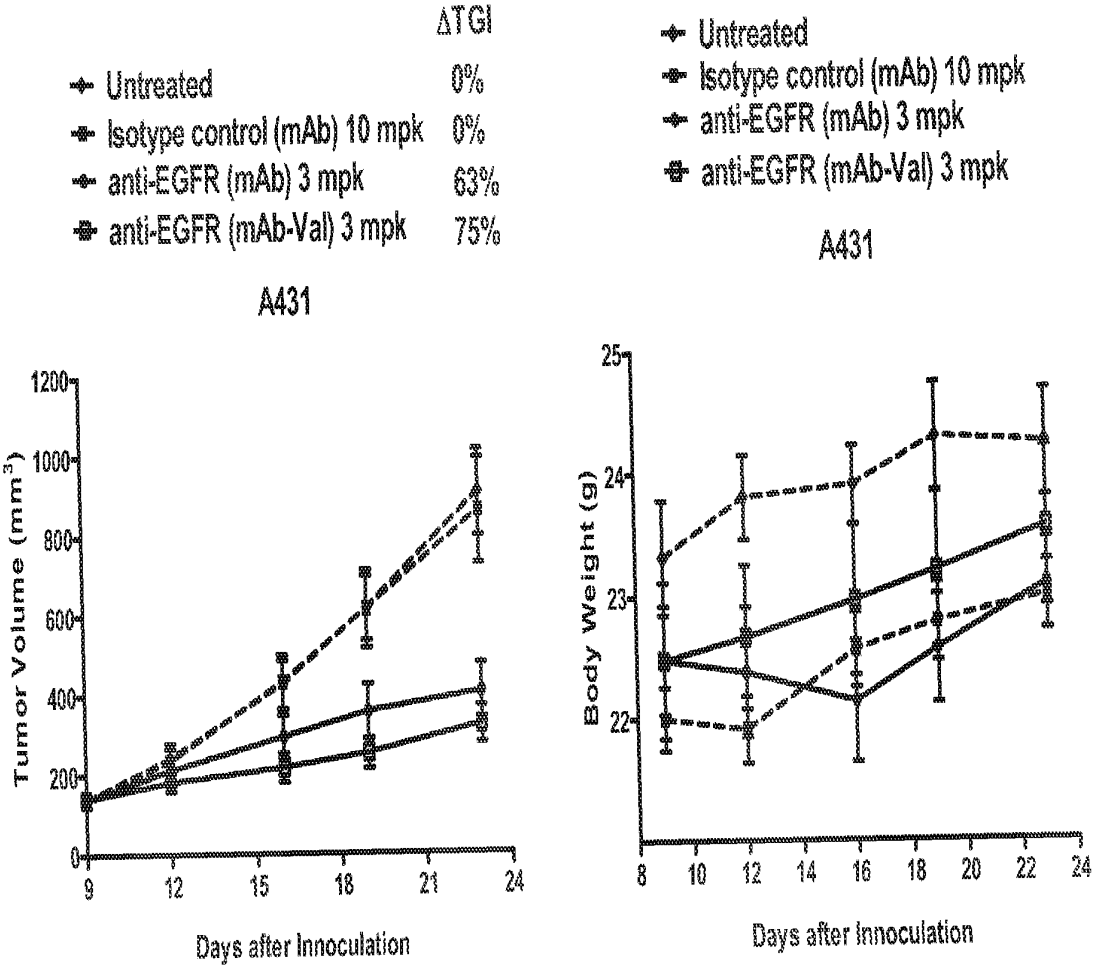


FIGURE 17

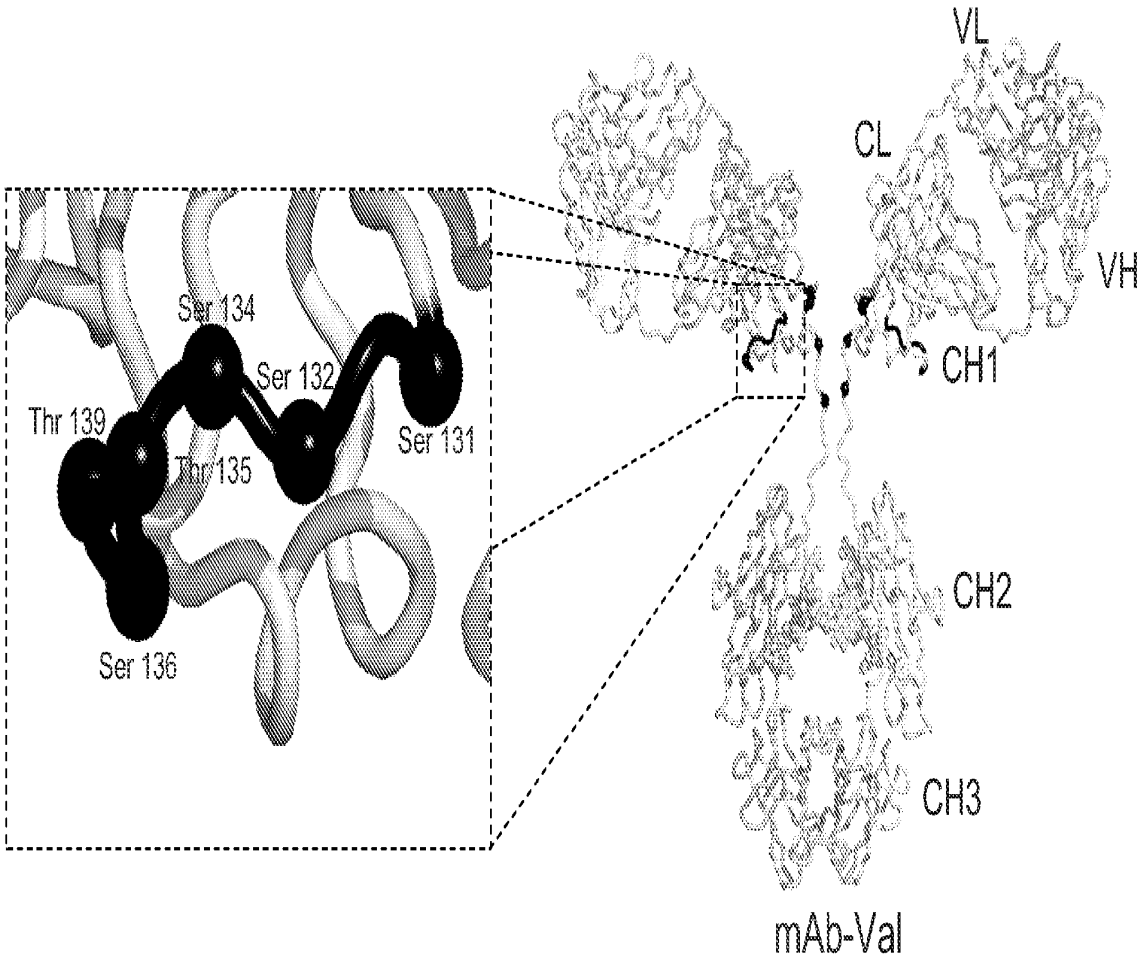

VL
CL
VH
CH1
CH2
CH3
Ser 134
Ser 132
Thr 139
Ser 131
Thr 135
Ser 136
mAb-Val

FIGURE 18

anti-EGFR (mAb-Val)	Day 7 expression (mg/l)	% Monomer in PBS
Ser131Cys	122	99
Ser132Cys	116	98
Ser134Cys	123	98
Thr135Cys	126	98
Ser136Cys	115	99
Thr139Cys	114	98
Ser131Cys-Thr139Cys	111	99
Ser131Cys-Thr135Cys-Thr139Cys	108	96

FIGURE 19

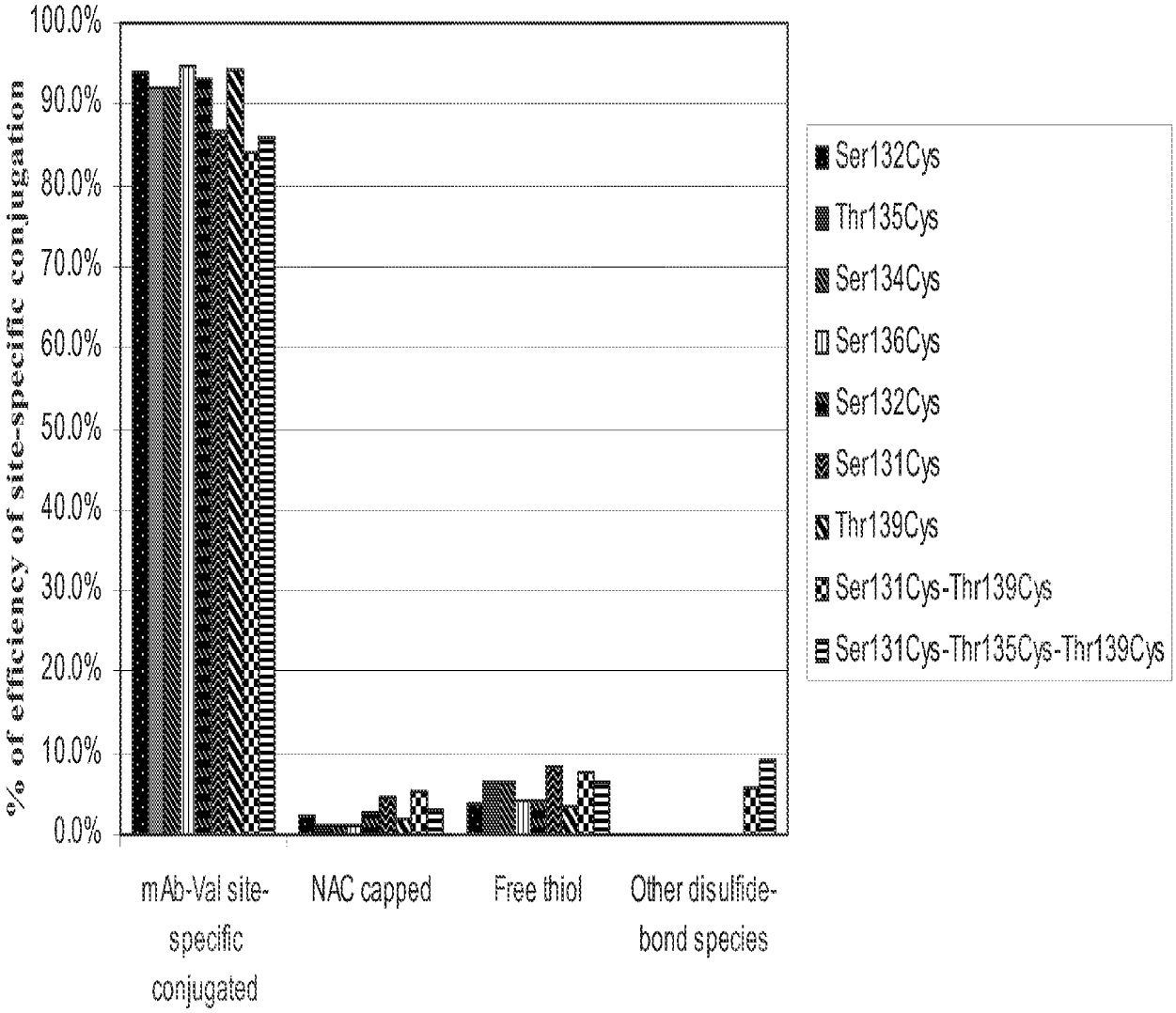
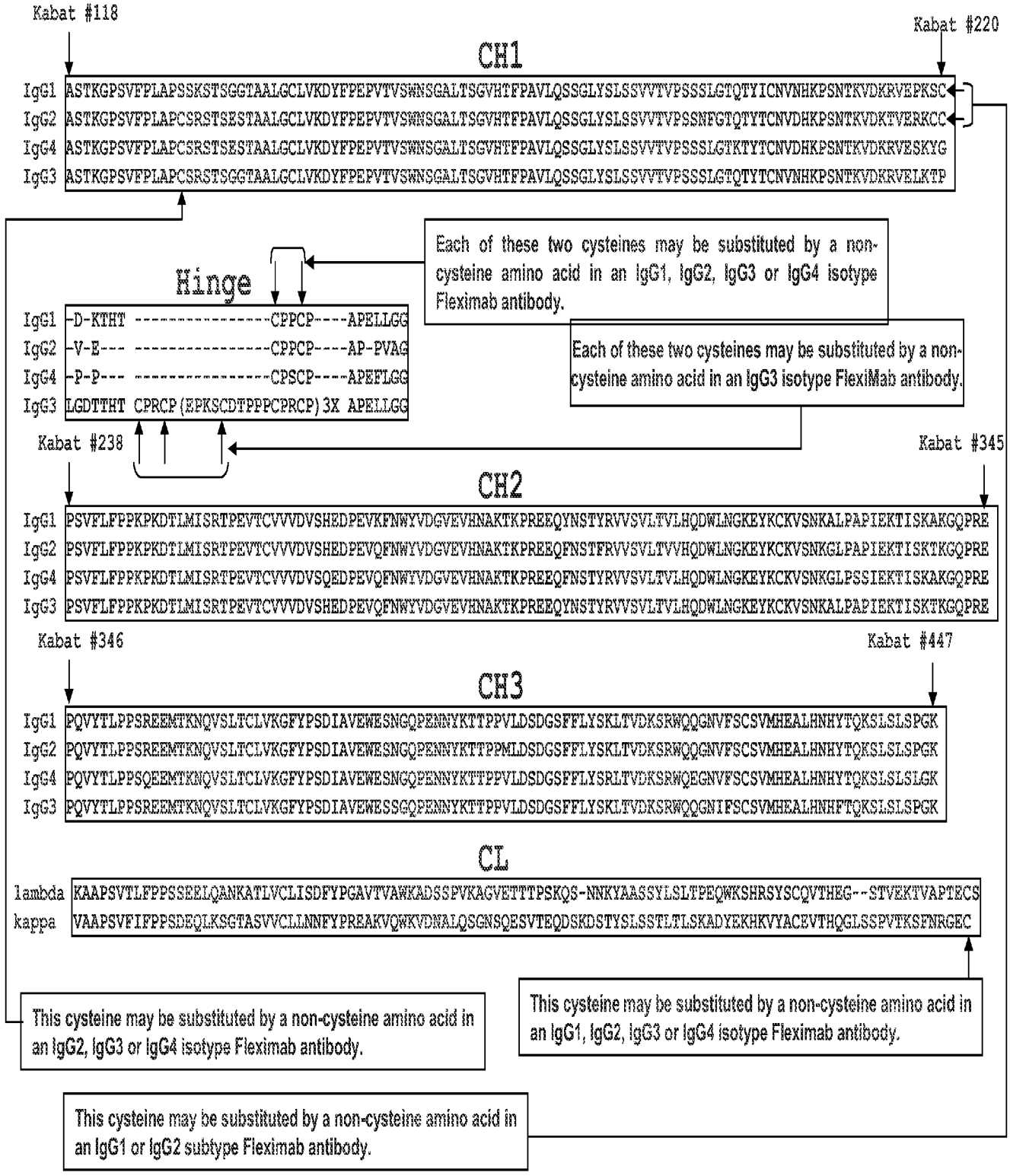

% of efficiency of site-specific conjugation
100.0%
90.0%
80.0%
70.0%
60.0%
50.0%
40.0%
30.0%
20.0%
10.0%
0.0%
mAb-Val site-specific conjugated
NAC capped
Free thiol
Other disulfide-bond species
Ser132Cys
Thr135Cys
Ser134Cys
Ser136Cys
Ser132Cys
Ser131Cys
Thr139Cys
Ser131Cys-Thr139Cys
Ser131Cys-Thr135Cys-Thr139Cys

FIGURE 20

mAb hinge, PKSCDKTHTCPPCPAPE (SEQ ID NO: 20)
mAb-Val hinge, PKSVDKTHTVPPVPAPE (SEQ ID NO: 21)

O-glycosylated in mAb-Val

FIGURE 21


Kabat #118
Kabat #220
CH1
IgG1 ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSC
IgG2 ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVDKTVERKCC
IgG4 ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTKVDKRVESKYG
IgG3 ASTKGPSVFPLAPCSRSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYTCNVNHKPSNTKVDKRVELKTP
Each of these two cysteines may be substituted by a non-cysteine amino acid in an IgG1, IgG2, IgG3 or IgG4 isotype Fleximab antibody.
Hinge
IgG1 -D-KTHT ----------CPPCP----APELLGG
IgG2 -V-E--- ----------CPPCP----AP-PVAG
IgG4 -P-P--- ----------CPSCP----APEFLGG
IgG3 LGDTTHT CPRCP(EPKSCDTPPPCPRCP)3X APELLGG
Each of these two cysteines may be substituted by a non-cysteine amino acid in an IgG3 isotype FlexiMab antibody.
Kabat #238
Kabat #345
CH2
IgG1 PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE
IgG2 PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTFRVVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTKGQPRE
IgG4 PSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPRE
IgG3 PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKTKGQPRE
Kabat #346
Kabat #447
CH3
IgG1 PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
IgG2 PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPMLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
IgG4 PQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK
IgG3 PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESSGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNIFSCSVMHEALHNHFTQKSLSLSPGK
CL
lambda KAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQS-NNKYAASSYLSLTPEQWKSHRSYSCQVTHEG--STVEKTVAPTECS
kappa VAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
This cysteine may be substituted by a non-cysteine amino acid in an IgG2, IgG3 or IgG4 isotype Fleximab antibody.
This cysteine may be substituted by a non-cysteine amino acid in an IgG1, IgG2, IgG3 or IgG4 isotype Fleximab antibody.
This cysteine may be substituted by a non-cysteine amino acid in an IgG1 or IgG2 subtype Fleximab antibody.

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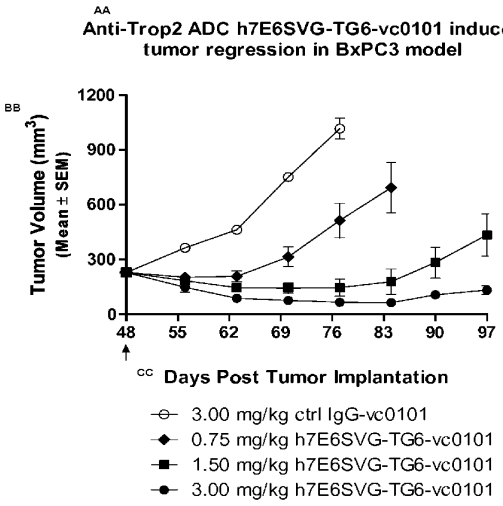
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[Continued on next page]

(54) Title: ANTIBODIES SPECIFIC FOR TROP-2 AND THEIR USES

Figure 9A



(57) Abstract: The present invention provides antibodies that specifically bind to trophoblast cell-surface antigen-2 (Trop-2). The invention further provides antibody conjugates comprising such antibodies, antibody encoding nucleic acids, and methods of obtaining such antibodies. The invention further relates to therapeutic methods for use of these antibodies and Trop-2 antibody conjugates for the treatment of a condition associated with Trop-2 expression (e.g., cancer), such as colon, esophageal, gastric, head and neck, lung, ovarian, or pancreatic cancer.

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ANTIBODIES SPECIFIC FOR TROP-2 AND THEIR USES

Related Applications

This application claims the benefits of U.S. Provisional Application No. 61/559,015 filed November 11, 2011, U.S. Provisional Application No. 61/640,641 filed April 30, 2012, and U.S. Provisional Application No. 61/717,288 filed October 23, 2012 which are hereby incorporated by reference in their entireties.

Field

The present invention relates to antibodies, e.g., full length antibodies or antigen binding fragments thereof, that specifically bind to trophoblast cell-surface antigen (Trop-2). The invention further relates to antibody conjugates (e.g., antibody-drug-conjugates) comprising the Trop-2 antibodies, compositions comprising the Trop-2 antibodies, and methods of using the Trop-2 antibodies and their conjugates for treating conditions associated with Trop-2 expression (e.g., cancer).

Background

Trophoblast cell-surface antigen (Trop-2), also referred to as M1S1, GA733-1 (gastric antigen 733-1), EGP-1 (epithelial glycoprotein-1), or TACSTD2 (tumor-associated calcium signal transducer), is a cell surface glycoprotein originally identified in human placental trophoblast and subsequently found to be highly expressed in most human carcinomas, but showed only restricted or limited expression in normal adult tissues. See, e.g., Varughese et al., Gynecologic Oncology, 122:171-177, 2011. Trop-2 is highly conserved among species. For example, human Trop-2 protein shares 79% identity with murine Trop-2 protein. See Cubas et al., Molecular Cancer, 9:253, 2010. Although the biological role of Trop-2 is still unclear, various studies have shown that overexpression of Trop-2 correlates with increased tumor aggressiveness, metastasis, and poor prognosis in various human carcinomas, such as colon cancer, ovarian cancer, and pancreatic cancer. See, e.g., Fang et al., Int. J. Colorectal Dis, 24:875-884, 2009; Bignotti et al., Eur. J. Cancer, 46:944-953, 2010; and Fong et al., Br J Cancer, 99:1290-1295, 2008. Studies have also shown that Trop-2 contributes to tumor pathogenesis at least in part by activating the ERK1/2 MAPK pathway which has

important implications in cancer cell proliferation, migration, invasion, and survival. See Cubas et al., 2010, supra.

Overexpression of Trop-2 by epithelial tumor cells and its transmembrane location render Trop-2 an attractive target for cancer immunotherapy. Accordingly, a high affinity antibody to Trop-2, particularly to human Trop-2, would make a superior therapeutic agent for cancer treatment in human patients. Although various Trop-2 antibodies have been disclosed (see, e.g., U.S. Pat. No. 7,420,041 (antibody AR47A6.4.2), U.S. Pat. No. 5,850,854 (antibody BR110), U.S. Pat. No. 6,653,104 (antibody RS7), U.S. Pat. No. 7,517,964 (antibody RS7), US2012/0237518), it has been exceedingly difficult to identify monoclonal antibodies having high affinity, high specificity, and potent cytotoxic or tumor killing/inhibition/regression activity. There remains a need for antibodies and other immunotherapeutic agents (such as antibody-drug conjugates) directed against Trop-2 having improved efficacy and safety profile, and which are suitable for use with human patients.

Summary

The invention disclosed herein is directed to antibodies and antibody conjugates (e.g., antibody-drug conjugates) that bind to Trop-2. In one aspect, the invention provides an antibody or antibody conjugate that specifically binds to domain 3 (e.g., amino acid residues 152-206) and domain 4 (e.g., amino acid residues 209-274) of human Trop-2 (SEQ ID NO:27) with a monovalent antibody binding affinity (K_D) of about 6.5 nM or less as measured by surface plasmon resonance.

In another aspect, the invention provides an isolated antibody, or an antigen binding fragment thereof, which specifically binds to Trop-2, wherein the antibody comprises a) a heavy chain variable (VH) region complementary determining regions comprising (i) a VH complementary determining region one (CDR1) comprising the sequence SYGVH (SEQ ID NO: 30), GGSISSY (SEQ ID NO: 36), or GGSISSYGVH (SEQ ID NO: 37); (ii) a VH CDR2 comprising the sequence VIWTX$_1$GX$_2$TDYNSALMX$_3$, wherein X$_1$ is G or S; X$_2$ is S or V; X$_3$ is S or G (SEQ ID NO: 49), or WTX$_1$GX$_2$ wherein X$_1$ is G or S, X$_2$ is S or V (SEQ ID NO: 50); and iii) a VH CDR3 comprising the sequence DGDYDRYTMDY (SEQ ID NO: 35); DYDRYTX$_1$DY, wherein X$_1$ is E or M (SEQ ID NO: 82); or DYDRYTX$_1$DY, wherein X$_1$ is any naturally occurring amino acid,

e.g., A, R, N, D, C, Q, E, G, H, I, L, K, M, F, P, S, T, W, Y, or V (SEQ ID NO: 83) and/or b) a light chain variable region (VL) region complementary determining regions comprising (i) a VL CDR1 comprising the sequence RASKSVSTSX$_1$YSYMH, wherein X$_1$ is G, L, or N (SEQ ID NO: 63); (ii) a VL CDR2 comprising the sequence LASNLES (SEQ ID NO: 55); and (iii) a VL CDR3 comprising the sequence VLQHSRELPYT (SEQ ID NO: 56). In some embodiments, the antibody does not comprise a heavy chain variable region of the sequence QVQLKESGPGLVAPSQSLSITCTVSGFSLTSYGVHWVRQPPGKGLEWLGVIWTGGST DYNSALMSRLSINKDNSKSQVFLKMNSLQTDDTAMYYCARDGDYDRYTMDYWGQGT SVTVSS (SEQ ID NO: 2) and a light chain variable region of the sequence DIVLTQSPASLAVSLGQRATISCRASKSVSTSGYSYMHWYQQKPGQPPKLLIYLASNLE SGVPARFSGSGSGTDFTLNIHPVEEEDAATYYCQHSRELPYTFGGGTKLEIK (SEQ ID NO: 1).

In another aspect, the invention provides an isolated antibody, or an antigen binding fragment thereof, which specifically binds to Trop-2, wherein the antibody comprises: a VH region comprising a VH CDR1, VH CDR2, and VH CDR3 of the VH sequence shown in SEQ ID NO: 5, 84, or 85; and/or a VL region comprising VL CDR1, VL CDR2, and VL CDR3 of the VL sequence shown in SEQ ID NO:3 or 6. In some embodiments, the VH region comprises (i) a VH CDR1 comprising the sequence SYGVH (SEQ ID NO: 30), GGSISSY (SEQ ID NO: 36), or GGSISSYGVH (SEQ ID NO: 37); (ii) a VH CDR2 comprising the sequence VIWTSGVTDYNSALMG (SEQ ID NO: 38) or WTSGV (SEQ ID NO: 39); and (iii) a VH CDR3 comprising the sequence DGDYDRYTMDY (SEQ ID NO: 35) or DYDRYTX$_1$DY, wherein X$_1$ is E or M (SEQ ID NO: 82). In some embodiments, the VL region comprises (i) a VL CDR1 comprising the sequence RASKSVSTSGYSYMH (SEQ ID NO: 54) or RASKSVSTSLYSYMH (SEQ ID NO: 57); (ii) a VL CDR2 comprising the sequence LASNLES (SEQ ID NO: 55); and (iii) a VL CDR3 comprising the sequence QHSRELPYT (SEQ ID NO: 56). In some embodiments, the antibody comprises (a) heavy chain CDRs comprising: (i) a CDR1 comprising the sequence SYGVH (SEQ ID NO: 30), GGSISSY (SEQ ID NO: 36), or GGSISSYGVH (SEQ ID NO: 37); (ii) a CDR2 comprising the sequence VIWTSGVTDYNSALMG (SEQ ID NO: 38) or WTSGV (SEQ ID NO: 39); and (iii) a CDR3 comprising the sequence DGDYDRYTMDY (SEQ ID NO: 35), DYDRYTMDY

(SEQ ID NO: 99), or DYDRYTEDY (SEQ ID NO: 100); and b) light chain CDRs comprising (i) a CDR1 comprising the sequence RASKSVSTSGYSYMH (SEQ ID NO: 54) or RASKSVSTSLYSYMH (SEQ ID NO: 57); (ii) a CDR2 comprising the sequence LASNLES (SEQ ID NO: 55); and (iii) a CDR3 comprising the sequence QHSRELPYT (SEQ ID NO: 56). In some embodiments, the VH region comprises the sequence shown in SEQ ID NO: 5, 84, or 85 or a variant with one or several conservative amino acid substitutions in residues that are not within a CDR and/or the VL region comprises the amino acid sequence shown in SEQ ID NO: 3, 6, or a variant thereof with one or several amino acid substitutions in amino acids that are not within a CDR. In some embodiments, the antibody comprises a light chain comprising the sequence shown in SEQ ID NO: 66 and/or a heavy chain comprising the sequence shown in SEQ ID NO: 65. In some embodiments, the antibody comprises a VH region produced by the expression vector with ATCC Accession No. PTA-12872. In some embodiments, the antibody comprises a VL region produced by the expression vector with ATCC Accession No. PTA-12871.

In another aspect, the invention provides an isolated antibody, or an antigen binding fragment thereof, which specifically binds to domain 1 (e.g., amino acid residues 31-71) of human Trop-2 (SEQ ID NO:27) with a binding affinity (K_D) of about 35 nM or less as measured by surface plasmon resonance.

In another aspect, the invention provides an isolated antibody, or an antigen binding fragment thereof, which specifically binds to Trop-2, wherein the antibody comprises a) a heavy chain variable (VH) region complementary determining regions comprising (i) a VH CDR1 comprising the sequence SYWIN (SEQ ID NO: 40), GYTFTSY (SEQ ID NO: 41), or GYTFTSYWIN (SEQ ID NO: 42); (ii) a VH CDR2 comprising the sequence $NIX_1PSDSYSNYNX_2KFKD$ wherein X_1 is Y or F; X_2 is Q or K (SEQ ID NO: 51), or X_1PSDSY wherein X_1 is Y or F (SEQ ID NO:52); and iii) a VH CDR3 comprising the sequence GSX_1FDY wherein X_1 is S or G (SEQ ID NO: 53); and/or b) a VL region complementary determining regions comprising (i) a VL CDR1 comprising the sequence RASQTIGTSIH (SEQ ID NO: 59); (ii) a VL CDR2 comprising the sequence YASESIS (SEQ ID NO: 60); and (iii) a VL CDR3 comprising the sequence X_1QSX_2SWPFT wherein X_1 is Q or S; X_2 is N or F (SEQ ID NO: 64).

In another aspect, the invention provides an isolated antibody, or an antigen binding fragment thereof, which specifically binds to Trop-2, wherein the antibody comprises: a VH region comprising a VH CDR1, VH CDR2, and VH CDR3 of the VH sequence shown in SEQ ID NO: 13; and/or a VL region comprising VL CDR1, VL CDR2, and VL CDR3 of the VL sequence shown in SEQ ID NO: 12. In some embodiments, the VH region comprises (i) a VH CDR1 comprising the sequence SYWIN (SEQ ID NO: 40), GYTFTSY (SEQ ID NO: 41), or GYTFTSYWIN (SEQ ID NO: 42); (ii) a VH CDR2 comprising the sequence NIFPSDSYSNYNKKFKD (SEQ ID NO: 46) or FPSDSY (SEQ ID NO: 47); and (iii) a VH CDR3 comprising the sequence GSGFDY (SEQ ID NO: 48). In some embodiments, the VL region comprises (i) a VL CDR1 comprising the sequence RASQTIGTSIH (SEQ ID NO: 59); (ii) a VL CDR2 comprising the sequence YASESIS (SEQ ID NO: 60); and (iii) a VL CDR3 comprising the sequence SQSFSWPFT (SEQ ID NO: 62). In some embodiments, the antibody comprises (a) heavy chain CDRs comprising: (i) a CDR1 comprising the sequence SYWIN (SEQ ID NO: 40), GYTFTSY (SEQ ID NO: 41), or GYTFTSYWIN (SEQ ID NO: 42); (ii) a CDR2 comprising the sequence NIFPSDSYSNYNKKFKD (SEQ ID NO: 46) or FPSDSY (SEQ ID NO: 47); and (iii) a VH CDR3 comprising the sequence GSGFDY (SEQ ID NO: 48); and b) light chain CDRs comprising: (i) a CDR1 comprising the sequence RASQTIGTSIH (SEQ ID NO: 59); (ii) a CDR2 comprising the sequence YASESIS (SEQ ID NO: 60); and (iii) a CDR3 comprising the sequence SQSFSWPFT (SEQ ID NO: 62). In some embodiments, the VH region comprises the sequence shown in SEQ ID NO: 13 or a variant with one or several conservative amino acid substitutions in residues that are not within a CDR and/or the VL region comprises the amino acid sequence shown in SEQ ID NO: 12 or a variant thereof with one or several amino acid substitutions in amino acids that are not within a CDR. In some embodiments, the antibody comprises a light chain comprising the sequence shown in SEQ ID NO: 68 and a heavy chain comprising the sequence shown in SEQ ID NO: 67.

In some embodiments, the antibody can be a human antibody, a humanized antibody, or a chimeric antibody. In some embodiments, the antibody is a monoclonal antibody.

In some embodiments, the antibody comprises a constant region. In some embodiments, the antibody is of the human IgG1, IgG2 or IgG2Δa, IgG3, or IgG4

subclass. In some embodiments, the antibody comprises a glycosylated constant region. In some embodiments, the antibody comprises a constant region having increased binding affinity to one or more human Fc gamma receptor(s).

In another aspect, the invention provides an isolated antibody which specifically binds to Trop-2 and competes with the antibodies as described herein (e.g., m7E6, h7E6_SVG, h7E6_SVG4, h7E6_SVG19, h7E6_SVG6, h7E6_SVG20, h7E6_SVG22, h7E6_SVG28, h7E6_SVG30, h7E6_SVGL, h7E6_SVGL1, h7E6_SVGL2, h7E6_SVGL3, h7E6_SVGL4, h7E6_SVGL5, h7E6_SVGN, m6G11, h6G11, or h6G11_FKG_SF). In some embodiments, the antibody competes with h7E6_SVG and has a monovalent antibody binding affinity (K_D) of about 6.5 nM or less as measured by surface plasmon resonance.

In another aspect, the invention provides a conjugate of the antibody or the antigen binding fragment as described herein, wherein the antibody or the antigen binding fragment is conjugated to an agent, wherein the agent is selected from the group consisting of a cytotoxic agent, an immunomodulating agent, an imaging agent, a therapeutic protein, a biopolymer, and an oligonucleotide. In some embodiments, the agent is a cytotoxic agent (e.g., monomethyl auristatin D (MMAD) or other auristatins).

In another aspect, the invention provides an isolated antibody comprising an acyl donor glutamine-containing tag engineered at a specific site of the antibody. In some embodiments, the tag comprises amino acid glutamine (Q) or an amino acid sequence GGLLQGG (SEQ ID NO:78), LLQGA (SEQ ID NO:79), GGLLQGA (SEQ ID NO:81), LLQ, or $LLQX_1X_2X_3X_4X_5$, wherein X_1 is G or P, wherein X_2 is A, G, P, or absent, wherein X_3 is A, G, K, P, or absent, wherein X_4 is G, K, or absent, and wherein X_5 is K or absent (SEQ ID NO: 88). In some embodiments, the Trop-2 antibody or the conjugate as described herein comprises an acyl donor glutamine-containing tag engineered at a specific site, such as G, GGLLQGG (SEQ ID NO:78), LLQGA (SEQ ID NO:79), GGLLQGA (SEQ ID NO:81), LLQ, or $LLQX_1X_2X_3X_4X_5$, wherein X_1 is G or P, wherein X_2 is A, G, P, or absent, wherein X_3 is A, G, K, P, or absent, wherein X_4 is G, K, or absent, and wherein X_5 is K or absent (SEQ ID NO: 88).

In one variation, the invention provides an isolated antibody comprising an acyl donor glutamine-containing tag and an amino acid modification at position 222, 340, or 370 of the antibody (Kabat numbering scheme), wherein the modification is an amino

acid deletion, insertion, substitution, mutation, or any combination thereof. In some embodiments, the Trop-2 antibody or the conjugate as described herein comprises the acyl donor glutamine-containing tag (e.g., Q, GGLLQGG (SEQ ID NO:78), LLQGA (SEQ ID NO:79), GGLLQGA (SEQ ID NO:81), LLQ, or LLQ$X_1X_2X_3X_4X_5$, wherein X_1 is G or P, wherein X_2 is A, G, P, or absent, wherein X_3 is A, G, K, P, or absent, wherein X_4 is G, K, or absent, and wherein X_5 is K or absent (SEQ ID NO: 88)) engineered at a specific site (e.g., at a carboxyl terminus of the heavy or light chain or at an another site) of the Trop-2 antibody and an amino acid modification at position 222, 340, or 370 of the antibody (Kabat numbering scheme). In some embodiments, the amino acid modification is a substitution from lysine to arginine.

In another aspect, the invention provides a conjugate of the antibody or the antigen binding fragment as described herein, wherein the conjugate comprises the formula: antibody-(acyl donor glutamine-containing tag)-(linker)-(cytotoxic agent), wherein the acyl donor glutamine-containing tag is engineered at a specific site of the antibody or the antigen binding fragment (e.g., at a carboxyl terminus of the heavy or light chain or at an another site), wherein the tag is conjugated to a linker (e.g., a linker containing one or more reactive amines (e.g., primary amine NH_2)), and wherein the linker is conjugated to a cytotoxic agent (e.g., MMAD or other auristatins).

In some embodiments, the conjugate is selected from the group consisting of 1) antibody-LLQGA-(acetyl-lysine-valine-citrulline-*p*-aminobenzyloxycarbonyl)-0101, wherein acetyl-lysine-valine-citrulline-*p*-aminobenzyloxycarbonyl is AcLys-VC-PABC, and wherein 0101 is 2-methylalanyl-*N*-[(3*R*,4*S*,5*S*)-3-methoxy-1-{(2*S*)-2-[(1*R*,2*R*)-1-methoxy-2-methyl-3-oxo-3-{[(1*S*)-2-phenyl-1-(1,3-thiazol-2-yl)ethyl]amino}propyl]pyrrolidin-1-yl}-5-methyl-1-oxoheptan-4-yl]-*N*-methyl-L-valinamide; 2) antibody-LLQGA- AcLys-VC-PABC-MMAD; 3) antibody-LLQ$X_1X_2X_3X_4X_5$ (SEQ ID NO: 88)-AcLys-VC-PABC-0101; 4) antibody-LLQ$X_1X_2X_3X_4X_5$ (SEQ ID NO: 88)-AcLys-VC-PABC-MMAD; 5) antibody –GGLLQGG (SEQ ID NO: 78)-AcLys-VC-PABC-0101; and 6) antibody–GGLLQGG (SEQ ID NO: 78)-AcLys-VC-PABC-MMAD. In some embodiments, the conjugate comprises an amino acid substitution from lysine to arginine at position 222. In some embodiments, the conjugate comprises an amino acid lysine (K) deletion at the C-terminus of the heavy chain of the antibody.

In another aspect, the invention provides a conjugate of the antibody or the antigen binding fragment as described herein, the conjugate comprises amino acid substitutions at positions N297Q and K222R, a linker comprising amino-PEG6-propionyl, and a cytotoxic agent (e.g., MMAD or other auristatins).

In another aspect, the invention provides a pharmaceutical composition comprising a therapeutically effective amount of the Trop-2 antibody or the conjugate as described herein and a pharmaceutically acceptable carrier.

In another aspect, the invention provides an isolated polynucleotide comprising a nucleotide sequence encoding the Trop-2 antibody as described herein. In some embodiments, provided is a vector comprising the polynucleotide.

In another aspect, the invention provides an isolated host cell that recombinantly produces the Trop-2 antibody as described herein.

In another aspect, the invention provides a method for treating a condition associated with Trop-2 expression in a subject comprising administering to the subject in need thereof an effective amount of the pharmaceutical composition as described herein. In some embodiments, the condition is a cancer. In some embodiments, the cancer is selected from the group consisting of bladder, breast, cervical, choriocarcinoma, colon, esophageal, gastric, glioblastoma, head and neck, kidney, lung, oral, ovarian, pancreatic, prostate cancer, and skin cancer.

In another aspect, the invention provides a method of inhibiting tumor growth or progression in a subject who has a Trop-2 expressing tumor, comprising administering to the subject in need thereof an effective amount of the pharmaceutical composition as described herein.

In another aspect, the invention provides a method of inhibiting metastasis of Trop-2 expressing cancer cells in a subject, comprising administering to the subject in need thereof an effective amount of the pharmaceutical composition as described herein.

In another aspect, the invention provides a method of inducing tumor regression in a subject who has a Trop-2 expressing tumor, comprising administering to the subject in need thereof an effective amount of the pharmaceutical composition as described herein.

In some embodiments, the antibody or the conjugate as described herein can be administered parenterally in an individual. In some embodiments, the individual is a human.

In some embodiments, the antibody described herein does not comprise a heavy chain variable region of the sequence QVQLKESGPGLVAPSQSLSITCTVSGFSLTSYGVHWVRQPPGKGLEWLGVIWTGGST DYNSALMSRLSINKDNSKSQVFLKMNSLQTDDTAMYYCARDGDYDRYTMDYWGQGT SVTVSS (SEQ ID NO: 2) and a light chain variable region of the sequence DIVLTQSPASLAVSLGQRATISCRASKSVSTSGYSYMHWYQQKPGQPPKLLIYLASNLE SGVPARFSGSGSGTDFTLNIHPVEEEDAATYYCQHSRELPYTFGGGTKLEIK (SEQ ID NO: 1).

Brief Description of the Figures/Drawings

Figure 1 depicts *in vivo* efficacy studies of various Trop-2 mouse antibodies (7E6, 15E2, and 18B1) in target-expressing Colo205 xenograft model.

Figure 2A depicts *in vivo* efficacy studies of various Trop-2 mouse antibodies (7E6, 15E2, and 18B1) in target-expressing A431 xenograft model.

Figure 2B depicts inhibition of A431 Xenograft tumor growth by Trop-2 antibody 7E6 in a dose-response study.

Figure 2C depicts inhibition of A431 Xenograft tumor growth by Trop-2 antibodies 6G11, 7E6, and 18B1 in A431 xenograft model.

Figure 3 depicts the ADCC activity of chimeric and humanized Trop-2 7E6 antibodies in A431 cells. 7E6 corresponds to the chimeric Trop-2 7E6 antibody (e.g., antibody comprising SEQ ID NOs: 2 and 3), h7E6-WT corresponds to an antibody comprising SEQ ID NOs: 4 and 3, h7E6-SVG corresponds to an antibody comprising SEQ ID NOs: 5 and 3, h7E6-L corresponds to an antibody comprising SEQ ID NOs: 4 and 6, h7E6-SVGL corresponds to an antibody comprising SEQ ID NOs: 5 and 6, and anti-EGFR (Epidermal Growth Factor Receptor) corresponds to a positive control antibody.

Figure 4 depicts the amino acid sequence alignment between human Trop-1 (SEQ ID NO: 29) and human Trop-2 (SEQ ID NO: 27), and the consensus sequence (SEQ ID NO: 69).

Figure 5 depicts the amino acid sequence alignment between human Trop-2 (SEQ ID NO: 27) and mouse Trop-2 (SEQ ID NO: 28), and the consensus sequence (SEQ ID NO: 70).

Figure 6A depicts the amino acid sequence alignment of the heavy chain variable regions of Trop-2 antibodies h7E6 (SEQ ID NO: 4), h7E6_SVG (SEQ ID NO: 5), and m7E6 (SEQ ID NO: 2), and their consensus sequence (SEQ ID NO: 71).

Figure 6B depicts the amino acid sequence alignment of the light chain variable regions of Trop-2 antibodies h7E6_VL (SEQ ID NO: 3), h7E6_VL_L (SEQ ID NO: 6), h7E6_VL_N (SEQ ID NO: 7), and m7E6_VL (SEQ ID NO: 1), and their consensus sequence (SEQ ID NO: 72).

Figure 7A depicts the amino acid sequence alignment of the heavy chain variable regions of Trop-2 antibodies h6G11 (SEQ ID NO: 11), h6G11_FKG_SF (SEQ ID NO: 13), and m6G11 (SEQ ID NO: 9), and their consensus sequence (SEQ ID NO: 73).

Figure 7B depicts the amino acid sequence alignment of the light chain variable regions of Trop-2 antibodies h6G11 (SEQ ID NO: 10), h6G11_FKG_SF (SEQ ID NO: 12), and m6G11 (SEQ ID NO: 8), and their consensus sequence (SEQ ID NO: 74).

Figure 8 depicts that chimeric (7E6) Trop-2 antibody conjugated to AcLys-vc-PABC-MMAD induced long term tumor regression in BxPC3 Xenograft model. AcLys-vc-PABC-MMAD corresponds to Acetyl-Lysine-Valine-Citrulline-*p*-aminobenzyloxycarbonyl- Monomethyl Auristatin D. LCQ03 and TG6 correspond to glutamine-containing transglutaminase tags SEQ ID NOs:78 and 79, respectively. NNC-TG1-vcMMAD represents control antibody conjugated to glutamine-containing transglutaminase tag (SEQ ID NO: 75) and vcMMAD.

Figure 9A depicts that humanized (h7E6SVG) Trop-2 antibody conjugated to vc0101 induced tumor regression in pancreatic tumor BxPC3 xenograft model. Vc0101 corresponds to AcLys-vc-PABC-0101 (Acetyl-Lysine-valine-citrulline-*p*-aminobenzyloxycarbonyl-(2-methylalanyl-*N*-[(3*R*,4*S*,5*S*)-3-methoxy-1-{(2*S*)-2-[(1*R*,2*R*)-1-methoxy-2-methyl-3-oxo-3-{[(1*S*)-2-phenyl-1-(1,3-thiazol-2-yl)ethyl]amino}propyl]pyrrolidin-1-yl}-5-methyl-1-oxoheptan-4-yl]-*N*-methyl-L-valinamide). TG6 corresponds to glutamine-containing transglutaminase tag SEQ ID NO:79.

Figure 9B depicts that humanized (h7E6SVG) Trop-2 antibody having amino acid substitutions at positions 297 and 222 and conjugated to PEG6-MMAD induced tumor

regression in pancreatic tumor BxPC3 xenograft model. PEG6-MMAD corresponds to (Propylene Glycol)$_6$-propionyl-MMAD.

Figure 9C depicts that humanized (h7E6SVG) Trop-2 antibody having amino acid substitution at position 222 and conjugated to vc0101 induced tumor regression in pancreatic tumor BxPC3 xenograft model. LCQ04 corresponds to glutamine-containing transglutaminase tag SEQ ID NO: 79. vc0101 corresponds to AcLys-vc-PABC-0101.

Figure 10 depicts that humanized (h7E6SVG) Trop-2 antibody conjugated to vc0101 induced tumor regression in colorectal tumor Colo205 xenograft model. TG6 and LCQ03 correspond to glutamine-containing transglutaminase tags SEQ ID NOs: 79 and 78, respectively.

Figure 11 depicts that humanized (h7E6SVG) Trop-2 antibody conjugated to vc0101 induced tumor regression in Ovarian PDX Ova196756 xenograft model. TG6 corresponds to glutamine-containing transglutaminase tag SEQ ID NO: 79.

Figure 12A shows that humanized (h7E6SVG) Trop-2 antibody conjugated to vc0101 has superior efficacy than gemcitabine to induce tumor regression in Pan0146 pancreatic PDX model. TG6 corresponds to glutamine-containing transglutaminase tag SEQ ID NO: 79 (same as in Figure 12B).

Figure 12B shows that continuous dosing of humanized (h7E6SVG) Trop-2 antibody conjugated to vc0101 resulted in sustained tumor regression in pancreatic PDX Pan0146 xenograft model.

Figure 13 shows that that a single dose of the humanized anti-Trop2 antibody conjugated with PEG6-MMAD induced tumor regression in pancreatic Pan144607 PDX model.

Figure 14 shows that a single dose of the humanized anti-Trop2 antibody conjugated with PEG6-MMAD induced tumor regression in pancreatic Pan0135 PDX model.

Detailed Description

The invention disclosed herein provides antibodies and antibody conjugates (e.g., antibody-drug conjugates) that specifically bind to Trop-2 (e.g., human Trop-2). The invention also provides polynucleotides encoding these antibodies, compositions comprising these antibodies, and methods of making and using these antibodies. The

invention also provides methods for treating a condition associated with Trop-2 expression in a subject, such as cancer (e.g., colon, gastric, head and neck, lung, ovarian, and pancreatic cancer).

General Techniques

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, Molecular Cloning: A Laboratory Manual, second edition (Sambrook et al., 1989) Cold Spring Harbor Press; Oligonucleotide Synthesis (M.J. Gait, ed., 1984); Methods in Molecular Biology, Humana Press; Cell Biology: A Laboratory Notebook (J.E. Cellis, ed., 1998) Academic Press; Animal Cell Culture (R.I. Freshney, ed., 1987); Introduction to Cell and Tissue Culture (J.P. Mather and P.E. Roberts, 1998) Plenum Press; Cell and Tissue Culture: Laboratory Procedures (A. Doyle, J.B. Griffiths, and D.G. Newell, eds., 1993-1998) J. Wiley and Sons; Methods in Enzymology (Academic Press, Inc.); Handbook of Experimental Immunology (D.M. Weir and C.C. Blackwell, eds.); Gene Transfer Vectors for Mammalian Cells (J.M. Miller and M.P. Calos, eds., 1987); Current Protocols in Molecular Biology (F.M. Ausubel et al., eds., 1987); PCR: The Polymerase Chain Reaction, (Mullis et al., eds., 1994); Current Protocols in Immunology (J.E. Coligan et al., eds., 1991); Short Protocols in Molecular Biology (Wiley and Sons, 1999); Immunobiology (C.A. Janeway and P. Travers, 1997); Antibodies (P. Finch, 1997); Antibodies: a practical approach (D. Catty., ed., IRL Press, 1988-1989); Monoclonal antibodies: a practical approach (P. Shepherd and C. Dean, eds., Oxford University Press, 2000); Using antibodies: a laboratory manual (E. Harlow and D. Lane (Cold Spring Harbor Laboratory Press, 1999); The Antibodies (M. Zanetti and J.D. Capra, eds., Harwood Academic Publishers, 1995).

Definitions

An “antibody” is an immunoglobulin molecule capable of specific binding to a target, such as a carbohydrate, polynucleotide, lipid, polypeptide, etc., through at least one antigen recognition site, located in the variable region of the immunoglobulin

molecule. As used herein, the term encompasses not only intact polyclonal or monoclonal antibodies, but also fragments thereof (such as Fab, Fab', $F(ab')_2$, Fv), single chain (ScFv) and domain antibodies (including, for example, shark and camelid antibodies), and fusion proteins comprising an antibody, and any other modified configuration of the immunoglobulin molecule that comprises an antigen recognition site. An antibody includes an antibody of any class, such as IgG, IgA, or IgM (or sub-class thereof), and the antibody need not be of any particular class. Depending on the antibody amino acid sequence of the constant region of its heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2. The heavy-chain constant regions that correspond to the different classes of immunoglobulins are called alpha, delta, epsilon, gamma, and mu, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term "antigen binding fragment" or “antigen binding portion” of an antibody, as used herein, refers to one or more fragments of an intact antibody that retain the ability to specifically bind to a given antigen (e.g., Trop-2). Antigen binding functions of an antibody can be performed by fragments of an intact antibody. Examples of binding fragments encompassed within the term "antigen binding fragment" of an antibody include Fab; Fab'; $F(ab')_2$; an Fd fragment consisting of the VH and CH1 domains; an Fv fragment consisting of the VL and VH domains of a single arm of an antibody; a single domain antibody (dAb) fragment (Ward et al., Nature 341:544-546, 1989), and an isolated complementarity determining region (CDR).

An antibody, an antibody conjugate, or a polypeptide that “preferentially binds” or “specifically binds” (used interchangeably herein) to a target (e.g., Trop-2 protein) is a term well understood in the art, and methods to determine such specific or preferential binding are also well known in the art. A molecule is said to exhibit “specific binding” or “preferential binding” if it reacts or associates more frequently, more rapidly, with greater duration and/or with greater affinity with a particular cell or substance than it does with alternative cells or substances. An antibody “specifically binds” or “preferentially binds” to a target if it binds with greater affinity, avidity, more readily, and/or with greater

duration than it binds to other substances. For example, an antibody that specifically or preferentially binds to a Trop-2 epitope is an antibody that binds this epitope with greater affinity, avidity, more readily, and/or with greater duration than it binds to other Trop-2 epitopes or non-Trop-2 epitopes. It is also understood that by reading this definition, for example, an antibody (or moiety or epitope) that specifically or preferentially binds to a first target may or may not specifically or preferentially bind to a second target. As such, "specific binding" or "preferential binding" does not necessarily require (although it can include) exclusive binding. Generally, but not necessarily, reference to binding means preferential binding.

A "variable region" of an antibody refers to the variable region of the antibody light chain or the variable region of the antibody heavy chain, either alone or in combination. As known in the art, the variable regions of the heavy and light chain each consist of four framework regions (FR) connected by three complementarity determining regions (CDRs) also known as hypervariable regions. The CDRs in each chain are held together in close proximity by the FRs and, with the CDRs from the other chain, contribute to the formation of the antigen binding site of antibodies. There are at least two techniques for determining CDRs: (1) an approach based on cross-species sequence variability (i.e., Kabat et al. Sequences of Proteins of Immunological Interest, (5th ed., 1991, National Institutes of Health, Bethesda MD)); and (2) an approach based on crystallographic studies of antigen-antibody complexes (Al-lazikani et al., 1997, J. Molec. Biol. 273:927-948). As used herein, a CDR may refer to CDRs defined by either approach or by a combination of both approaches.

A "CDR" of a variable domain are amino acid residues within the variable region that are identified in accordance with the definitions of the Kabat, Chothia, the accumulation of both Kabat and Chothia, AbM, contact, and/or conformational definitions or any method of CDR determination well known in the art. Antibody CDRs may be identified as the hypervariable regions originally defined by Kabat et al. See, e.g., Kabat et al., 1992, Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, NIH, Washington D.C. The positions of the CDRs may also be identified as the structural loop structures originally described by Chothia and others. See, e.g., Chothia et al., Nature 342:877-883, 1989. Other approaches to CDR identification include the "AbM definition," which is a compromise between Kabat and Chothia and is

derived using Oxford Molecular's AbM antibody modeling software (now Accelrys®), or the "contact definition" of CDRs based on observed antigen contacts, set forth in MacCallum et al., J. Mol. Biol., 262:732-745, 1996. In another approach, referred to herein as the "conformational definition" of CDRs, the positions of the CDRs may be identified as the residues that make enthalpic contributions to antigen binding. See, e.g., Makabe et al., Journal of Biological Chemistry, 283:1156-1166, 2008. Still other CDR boundary definitions may not strictly follow one of the above approaches, but will nonetheless overlap with at least a portion of the Kabat CDRs, although they may be shortened or lengthened in light of prediction or experimental findings that particular residues or groups of residues or even entire CDRs do not significantly impact antigen binding. As used herein, a CDR may refer to CDRs defined by any approach known in the art, including combinations of approaches. The methods used herein may utilize CDRs defined according to any of these approaches. For any given embodiment containing more than one CDR, the CDRs may be defined in accordance with any of Kabat, Chothia, extended, AbM, contact, and/or conformational definitions.

As used herein, "monoclonal antibody" refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to polyclonal antibody preparations, which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler and Milstein, Nature 256:495, 1975, or may be made by recombinant DNA methods such as described in U.S. Pat. No. 4,816,567. The monoclonal antibodies may also be isolated from phage libraries generated using the techniques described in McCafferty et al., Nature 348:552-554, 1990, for example.

As used herein, "humanized" antibody refers to forms of non-human (*e.g.* murine) antibodies that are chimeric immunoglobulins, immunoglobulin chains, or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen binding subsequences of antibodies) that contain minimal sequence derived from non-human immunoglobulin. Preferably, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, the humanized antibody may comprise residues that are found neither in the recipient antibody nor in the imported CDR or framework sequences, but are included to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin. Preferred are antibodies having Fc regions modified as described in WO 99/58572. Other forms of humanized antibodies have one or more CDRs (CDR L1, CDR L2, CDR L3, CDR H1, CDR H2, or CDR H3) which are altered with respect to the original antibody, which are also termed one or more CDRs “derived from” one or more CDRs from the original antibody.

As used herein, “human antibody” means an antibody having an amino acid sequence corresponding to that of an antibody produced by a human and/or which has been made using any of the techniques for making human antibodies known to those skilled in the art or disclosed herein. This definition of a human antibody includes antibodies comprising at least one human heavy chain polypeptide or at least one human light chain polypeptide. One such example is an antibody comprising murine light chain and human heavy chain polypeptides. Human antibodies can be produced using various techniques known in the art. In one embodiment, the human antibody is selected from a phage library, where that phage library expresses human antibodies

(Vaughan et al., Nature Biotechnology, 14:309-314, 1996; Sheets et al., Proc. Natl. Acad. Sci. (USA) 95:6157-6162, 1998; Hoogenboom and Winter, J. Mol. Biol., 227:381, 1991; Marks et al., J. Mol. Biol., 222:581, 1991). Human antibodies can also be made by immunization of animals into which human immunoglobulin loci have been transgenically introduced in place of the endogenous loci, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. This approach is described in U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; and 5,661,016. Alternatively, the human antibody may be prepared by immortalizing human B lymphocytes that produce an antibody directed against a target antigen (such B lymphocytes may be recovered from an individual or from single cell cloning of the cDNA, or may have been immunized *in vitro).* See, e.g., Cole et al. Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77, 1985; Boerner et al., J. Immunol., 147 (1):86-95, 1991; and U.S. Pat. No. 5,750,373.

The term “chimeric antibody” is intended to refer to antibodies in which the variable region sequences are derived from one species and the constant region sequences are derived from another species, such as an antibody in which the variable region sequences are derived from a mouse antibody and the constant region sequences are derived from a human antibody.

The terms “polypeptide”, “oligopeptide”, “peptide” and “protein” are used interchangeably herein to refer to chains of amino acids of any length, preferably, relatively short (e.g., 10-100 amino acids). The chain may be linear or branched, it may comprise modified amino acids, and/or may be interrupted by non-amino acids. The terms also encompass an amino acid chain that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component. Also included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), as well as other modifications known in the art. It is understood that the polypeptides can occur as single chains or associated chains.

A “monovalent antibody” comprises one antigen binding site per molecule (e.g., IgG or Fab). In some instances, a monovalent antibody can have more than one antigen binding sites, but the binding sites are from different antigens.

A "bivalent antibody" comprises two antigen binding sites per molecule (e.g., IgG). In some instances, the two binding sites have the same antigen specificities. However, bivalent antibodies may be bispecific.

A "bispecific," "dual-specific" or "bifunctional" antibody is a hybrid antibody having two different antigen binding sites. The two antigen binding sites of a bispecific antibody bind to two different epitopes, which may reside on the same or different protein targets.

Antibodies of the invention can be produced using techniques well known in the art, *e.g.*, recombinant technologies, phage display technologies, synthetic technologies or combinations of such technologies or other technologies readily known in the art (see, for example, Jayasena, S.D., Clin. Chem., 45: 1628-50, 1999 and Fellouse, F.A., et al, J. Mol. Biol., 373(4):924-40, 2007).

As known in the art, "polynucleotide," or "nucleic acid," as used interchangeably herein, refer to chains of nucleotides of any length, and include DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a chain by DNA or RNA polymerase. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and their analogs. If present, modification to the nucleotide structure may be imparted before or after assembly of the chain. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. Other types of modifications include, for example, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example, proteins (e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide(s). Further, any of the hydroxyl groups ordinarily present in the sugars may be replaced, for example, by phosphonate groups, phosphate groups, protected by standard protecting groups, or

activated to prepare additional linkages to additional nucleotides, or may be conjugated to solid supports. The 5' and 3' terminal OH can be phosphorylated or substituted with amines or organic capping group moieties of from 1 to 20 carbon atoms. Other hydroxyls may also be derivatized to standard protecting groups. Polynucleotides can also contain analogous forms of ribose or deoxyribose sugars that are generally known in the art, including, for example, 2'-O-methyl-, 2'-O-allyl, 2'-fluoro- or 2'-azido-ribose, carbocyclic sugar analogs, alpha- or beta-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs and abasic nucleoside analogs such as methyl riboside. One or more phosphodiester linkages may be replaced by alternative linking groups. These alternative linking groups include, but are not limited to, embodiments wherein phosphate is replaced by P(O)S("thioate"), P(S)S ("dithioate"), (O)NR_2 ("amidate"), P(O)R, P(O)OR', CO or CH_2 ("formacetal"), in which each R or R' is independently H or substituted or unsubstituted alkyl (1-20 C) optionally containing an ether (-O-) linkage, aryl, alkenyl, cycloalkyl, cycloalkenyl or araldyl. Not all linkages in a polynucleotide need be identical. The preceding description applies to all polynucleotides referred to herein, including RNA and DNA.

As known in the art a "constant region" of an antibody refers to the constant region of the antibody light chain or the constant region of the antibody heavy chain, either alone or in combination.

As used herein, "substantially pure" refers to material which is at least 50% pure (i.e., free from contaminants), more preferably, at least 90% pure, more preferably, at least 95% pure, yet more preferably, at least 98% pure, and most preferably, at least 99% pure.

A "host cell" includes an individual cell or cell culture that can be or has been a recipient for vector(s) for incorporation of polynucleotide inserts. Host cells include progeny of a single host cell, and the progeny may not necessarily be completely identical (in morphology or in genomic DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation. A host cell includes cells transfected in vivo with a polynucleotide(s) of this invention.

As known in the art, the term "Fc region" is used to define a C-terminal region of an immunoglobulin heavy chain. The "Fc region" may be a native sequence Fc region

or a variant Fc region. Although the boundaries of the Fc region of an immunoglobulin heavy chain might vary, the human IgG heavy chain Fc region is usually defined to stretch from an amino acid residue at position Cys226, or from Pro230, to the carboxyl-terminus thereof. The numbering of the residues in the Fc region is that of the EU index as in Kabat. Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md., 1991. The Fc region of an immunoglobulin generally comprises two constant regions, CH2 and CH3.

As used in the art, "Fc receptor" and “FcR” describe a receptor that binds to the Fc region of an antibody. The preferred FcR is a native sequence human FcR. Moreover, a preferred FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and FcγRIII subclasses, including allelic variants and alternatively spliced forms of these receptors. FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. FcRs are reviewed in Ravetch and Kinet, Ann. Rev. Immunol., 9:457-92, 1991; Capel et al., Immunomethods, 4:25-34, 1994; and de Haas et al., J. Lab. Clin. Med., 126:330-41, 1995. “FcR” also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., J. Immunol., 117:587, 1976; and Kim et al., J. Immunol., 24:249, 1994).

The term “compete”, as used herein with regard to an antibody, means that a first antibody, or an antigen binding fragment (or portion) thereof, binds to an epitope in a manner sufficiently similar to the binding of a second antibody, or an antigen binding portion thereof, such that the result of binding of the first antibody with its cognate epitope is detectably decreased in the presence of the second antibody compared to the binding of the first antibody in the absence of the second antibody. The alternative, where the binding of the second antibody to its epitope is also detectably decreased in the presence of the first antibody, can, but need not be the case. That is, a first antibody can inhibit the binding of a second antibody to its epitope without that second antibody inhibiting the binding of the first antibody to its respective epitope. However, where each antibody detectably inhibits the binding of the other antibody with its cognate epitope or ligand, whether to the same, greater, or lesser extent, the antibodies are said to “cross-compete” with each other for binding of their respective epitope(s).

Both competing and cross-competing antibodies are encompassed by the present invention. Regardless of the mechanism by which such competition or cross-competition occurs (e.g., steric hindrance, conformational change, or binding to a common epitope, or portion thereof), the skilled artisan would appreciate, based upon the teachings provided herein, that such competing and/or cross-competing antibodies are encompassed and can be useful for the methods disclosed herein.

A "functional Fc region" possesses at least one effector function of a native sequence Fc region. Exemplary "effector functions" include C1q binding; complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity; phagocytosis; down-regulation of cell surface receptors (e.g. B cell receptor), etc. Such effector functions generally require the Fc region to be combined with a binding domain (e.g. an antibody variable domain) and can be assessed using various assays known in the art for evaluating such antibody effector functions.

A "native sequence Fc region" comprises an amino acid sequence identical to the amino acid sequence of an Fc region found in nature. A "variant Fc region" comprises an amino acid sequence which differs from that of a native sequence Fc region by virtue of at least one amino acid modification, yet retains at least one effector function of the native sequence Fc region. In some embodiments, the variant Fc region has at least one amino acid substitution compared to a native sequence Fc region or to the Fc region of a parent polypeptide, e.g. from about one to about ten amino acid substitutions, and preferably, from about one to about five amino acid substitutions in a native sequence Fc region or in the Fc region of the parent polypeptide. The variant Fc region herein will preferably possess at least about 80% sequence identity with a native sequence Fc region and/or with an Fc region of a parent polypeptide, and most preferably, at least about 90% sequence identity therewith, more preferably, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99% sequence identity therewith.

The term "effector function" refers to the biological activities attributable to the Fc region of an antibody. Examples of antibody effector functions include, but are not limited to, antibody-dependent cell-mediated cytotoxicity (ADCC), Fc receptor binding, complement dependent cytotoxicity (CDC), phagocytosis, C1q binding, and down regulation of cell surface receptors (e.g., B cell receptor; BCR). See, e.g., U.S. Pat No.

6,737,056. Such effector functions generally require the Fc region to be combined with a binding domain (e.g., an antibody variable domain) and can be assessed using various assays known in the art for evaluating such antibody effector functions. An exemplary measurement of effector function is through Fcγ3 and/or C1q binding.

As used herein "antibody-dependent cell-mediated cytotoxicity" or "ADCC" refers to a cell-mediated reaction in which nonspecific cytotoxic cells that express Fc receptors (FcRs) (e.g. natural killer (NK) cells, neutrophils, and macrophages) recognize bound antibody on a target cell and subsequently cause lysis of the target cell. ADCC activity of a molecule of interest can be assessed using an *in vitro* ADCC assay, such as that described in U.S. Patent No. 5,500,362 or 5,821,337. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and NK cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed in vivo, e.g., in an animal model such as that disclosed in Clynes et al., 1998, *PNAS (USA)*, 95:652-656.

"Complement dependent cytotoxicity" or "CDC" refers to the lysing of a target in the presence of complement. The complement activation pathway is initiated by the binding of the first component of the complement system (C1q) to a molecule (e.g. an antibody) complexed with a cognate antigen. To assess complement activation, a CDC assay, e.g. as described in Gazzano-Santoro et al., *J. Immunol. Methods*, 202: 163 (1996), may be performed.

As used herein, "treatment" is an approach for obtaining beneficial or desired clinical results. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, one or more of the following: reducing the proliferation of (or destroying) neoplastic or cancerous cells, inhibiting metastasis of neoplastic cells, shrinking or decreasing the size of Trop-2 expressing tumor, remission of a Trop-2 associated disease (e.g., cancer), decreasing symptoms resulting from a Trop-2 associated disease (e.g., cancer), increasing the quality of life of those suffering from a Trop-2 associated disease (e.g., cancer), decreasing the dose of other medications required to treat a Trop-2 associated disease (e.g., cancer), delaying the progression of a Trop-2 associated disease (e..g, cancer), curing a Trop-2 associated disease (e..g, cancer), and/or prolong survival of patients having a Trop-2 associated disease (e.g., cancer).

"Ameliorating" means a lessening or improvement of one or more symptoms as compared to not administering a Trop-2 antibody or a Trop-2 antibody conjugate. "Ameliorating" also includes shortening or reduction in duration of a symptom.

As used herein, an "effective dosage" or "effective amount" of drug, compound, or pharmaceutical composition is an amount sufficient to effect any one or more beneficial or desired results. For prophylactic use, beneficial or desired results include eliminating or reducing the risk, lessening the severity, or delaying the outset of the disease, including biochemical, histological and/or behavioral symptoms of the disease, its complications and intermediate pathological phenotypes presenting during development of the disease. For therapeutic use, beneficial or desired results include clinical results such as reducing incidence or amelioration of one or more symptoms of various Trop-2 associated diseases or conditions (such as gastric, head and neck, lung, ovarian, and pancreatic cancers), decreasing the dose of other medications required to treat the disease, enhancing the effect of another medication, and/or delaying the progression of the Trop-2 associated disease of patients. An effective dosage can be administered in one or more administrations. For purposes of this invention, an effective dosage of drug, compound, or pharmaceutical composition is an amount sufficient to accomplish prophylactic or therapeutic treatment either directly or indirectly. As is understood in the clinical context, an effective dosage of a drug, compound, or pharmaceutical composition may or may not be achieved in conjunction with another drug, compound, or pharmaceutical composition. Thus, an "effective dosage" may be considered in the context of administering one or more therapeutic agents, and a single agent may be considered to be given in an effective amount if, in conjunction with one or more other agents, a desirable result may be or is achieved.

An "individual" or a "subject" is a mammal, more preferably, a human. Mammals also include, but are not limited to, farm animals, sport animals, pets, primates, horses, dogs, cats, mice and rats.

As used herein, "vector" means a construct, which is capable of delivering, and, preferably, expressing, one or more gene(s) or sequence(s) of interest in a host cell. Examples of vectors include, but are not limited to, viral vectors, naked DNA or RNA expression vectors, plasmid, cosmid or phage vectors, DNA or RNA expression vectors

associated with cationic condensing agents, DNA or RNA expression vectors encapsulated in liposomes, and certain eukaryotic cells, such as producer cells.

As used herein, "expression control sequence" means a nucleic acid sequence that directs transcription of a nucleic acid. An expression control sequence can be a promoter, such as a constitutive or an inducible promoter, or an enhancer. The expression control sequence is operably linked to the nucleic acid sequence to be transcribed.

As used herein, "pharmaceutically acceptable carrier" or "pharmaceutical acceptable excipient" includes any material which, when combined with an active ingredient, allows the ingredient to retain biological activity and is non-reactive with the subject's immune system. Examples include, but are not limited to, any of the standard pharmaceutical carriers such as a phosphate buffered saline solution, water, emulsions such as oil/water emulsion, and various types of wetting agents. Preferred diluents for aerosol or parenteral administration are phosphate buffered saline (PBS) or normal (0.9%) saline. Compositions comprising such carriers are formulated by well known conventional methods (see, for example, Remington's Pharmaceutical Sciences, 18th edition, A. Gennaro, ed., Mack Publishing Co., Easton, PA, 1990; and Remington, The Science and Practice of Pharmacy 21st Ed. Mack Publishing, 2005).

The term “acyl donor glutamine-containing tag” or “glutamine tag” as used herein refers to a polypeptide or a protein containing one or more Gln residue(s) that acts as a transglutaminase amine acceptor. See, e.g., WO2012059882.

The term "k_{on}", as used herein, refers to the rate constant for association of an antibody to an antigen. Specifically, the rate constants (k_{on} and k_{off}) and equilibrium dissociation constants are measured using Fab antibody fragments (i.e. monovalent) and Trop-2 proteins (e.g., Trop-2-Fc fusion protein).

The term "k_{off} ", as used herein, refers to the rate constant for dissociation of an antibody from the antibody/antigen complex.

The term "K_D", as used herein, refers to the equilibrium dissociation constant of an antibody-antigen interaction.

Reference to “about” a value or parameter herein includes (and describes) embodiments that are directed to that value or parameter per se. For example,

description referring to "about X" includes description of "X." Numeric ranges are inclusive of the numbers defining the range.

It is understood that wherever embodiments are described herein with the language "comprising," otherwise analogous embodiments described in terms of "consisting of" and/or "consisting essentially of" are also provided.

Where aspects or embodiments of the invention are described in terms of a Markush group or other grouping of alternatives, the present invention encompasses not only the entire group listed as a whole, but each member of the group individually and all possible subgroups of the main group, but also the main group absent one or more of the group members. The present invention also envisages the explicit exclusion of one or more of any of the group members in the claimed invention.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. In case of conflict, the present specification, including definitions, will control. Throughout this specification and claims, the word "comprise," or variations such as "comprises" or "comprising" will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers. Unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

Exemplary methods and materials are described herein, although methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention. The materials, methods, and examples are illustrative only and not intended to be limiting.

Trop-2 Antibodies and Methods of Making Thereof

The present invention provides an antibody that binds to Trop-2. In one aspect, the invention provides an isolated antibody, or an antigen binding fragment thereof, which specifically binds to domain 3 (e.g., amino acid residues 153-206) and domain 4 (e.g., amino acid residues 209-273) of human Trop-2 (e.g., SEQ ID NO:27) with a monovalent antibody binding affinity (K_D) of 6.5 nM or less as measured by surface plasmon resonance. In another aspect, the invention provides an isolated antibody, or an antigen binding fragment thereof, which specifically binds to domain 1 (e.g., amino

acid residues 27-70) of human Trop-2 (e.g., SEQ ID NO:27) with a binding affinity (K_D) of about 35 nM or less as measured by surface plasmon resonance.

The antibodies and antibody conjugates of the invention are characterized by any one or more of the following characteristics: (a) bind to Trop-2; (b) decrease or downregulate the protein expression of Trop-2; (c) treat, prevent, ameliorate one or more symptoms of a condition associated with Trop-2 expression in a subject (e.g., cancer, such as gastric, head and neck, lung, ovarian, or pancreatic cancer); (d) inhibit tumor growth or progression in a subject (who has a Trop-2 expressing tumor); (e) inhibit metastasis of Trop-2 expressing cancer cells in a subject (who has one or more Trop-2 expressing cancer cells); (f) induce regression (e.g., long-term regression) of a Trop-2 expressing tumor; (g) exert cytotoxic activity in Trop-2 expressing cells; (h) deactivate or downregulate the ERK1/2 MAPK pathway; and (i) block Trop-2 interaction with other yet to be identified factors.

The antibodies useful in the present invention can encompass monoclonal antibodies, polyclonal antibodies, antibody fragments (e.g., Fab, Fab', F(ab')2, Fv, Fc, etc.), chimeric antibodies, bispecific antibodies, heteroconjugate antibodies, single chain (ScFv), mutants thereof, fusion proteins comprising an antibody portion (e.g., a domain antibody), humanized antibodies, and any other modified configuration of the immunoglobulin molecule that comprises an antigen recognition site of the required specificity, including glycosylation variants of antibodies, amino acid sequence variants of antibodies, and covalently modified antibodies. The antibodies may be murine, rat, human, or any other origin (including chimeric or humanized antibodies).

In some embodiments, the Trop-2 antibody as described herein is a monoclonal antibody. For example, the Trop-2 antibody is a humanized monoclonal antibody or a chimeric monoclonal antibody.

In some embodiments, the antibody comprises a modified constant region, such as, for example without limitation, a constant region that has increased potential for provoking an immune response. For example, the constant region may be modified to have increased affinity to an Fc gamma receptor such as, e.g., FcγRI, FcγRIIA, or FcγIII.

In some embodiments, the antibody comprises a modified constant region, such as a constant region that is immunologically inert, that is, having a reduced potential for

provoking an immune response. In some embodiments, the constant region is modified as described in Eur. J. Immunol., 29:2613-2624, 1999; PCT Application No. PCT/GB99/01441; and/or UK Patent Application No. 98099518. The Fc can be human IgG1, human IgG2, human IgG3, or human IgG4. The Fc can be human IgG2 containing the mutation A330P331 to S330S331 (IgG2Δa), in which the amino acid residues are numbered with reference to the wild type IgG2 sequence. Eur. J. Immunol., 29:2613-2624, 1999. In some embodiments, the antibody comprises a constant region of IgG_4 comprising the following mutations (Armour et al., Molecular Immunology 40 585-593, 2003): E233F234L235 to P233V234A235 (IgG4Δc), in which the numbering is with reference to wild type IgG4. In yet another embodiment, the Fc is human IgG4 E233F234L235 to P233V234A235 with deletion G236 (IgG4Δb). In another embodiment, the Fc is any human IgG4 Fc (IgG4, IgG4Δb or IgG4Δc) containing hinge stabilizing mutation S228 to P228 (Aalberse et al., Immunology 105, 9-19, 2002). In another embodiment, the Fc can be aglycosylated Fc.

In some embodiments, the constant region is aglycosylated by mutating the oligosaccharide attachment residue (such as Asn297) and/or flanking residues that are part of the glycosylation recognition sequence in the constant region. In some embodiments, the constant region is aglycosylated for N-linked glycosylation enzymatically. The constant region may be aglycosylated for N-linked glycosylation enzymatically or by expression in a glycosylation deficient host cell.

One way of determining binding affinity of antibodies to Trop-2 is by measuring binding affinity of monofunctional Fab fragments of the antibody. To obtain monofunctional Fab fragments, an antibody (for example, IgG) can be cleaved with papain or expressed recombinantly. The affinity of a Trop-2 Fab fragment of an antibody can be determined by surface plasmon resonance (Biacore™3000™ surface plasmon resonance (SPR) system, Biacore™, INC, Piscataway NJ) equipped with pre-immobilized streptavidin sensor chips (SA) or anti-mouse Fc or anti-human Fc using HBS-EP running buffer (0.01M HEPES, pH 7.4, 0.15 NaCl, 3 mM EDTA, 0.005% v/v Surfactant P20). Biotinylated or Fc fusion human Trop-2 can be diluted into HBS-EP buffer to a concentration of less than 0.5 μg/mL and injected across the individual chip channels using variable contact times, to achieve two ranges of antigen density, either 50-200 response units (RU) for detailed kinetic studies or 800-1,000 RU for screening

assays. Regeneration studies have shown that 25 mM NaOH in 25% v/v ethanol effectively removes the bound Fab while keeping the activity of Trop-2 on the chip for over 200 injections. Typically, serial dilutions (spanning concentrations of 0.1-10x estimated K_D) of purified Fab samples are injected for 1 min at 100 μL/minute and dissociation times of up to 2 hours are allowed. The concentrations of the Fab proteins are determined by ELISA and/or SDS-PAGE electrophoresis using a Fab of known concentration (as determined by amino acid analysis) as a standard. Kinetic association rates (k_{on}) and dissociation rates (k_{off}) are obtained simultaneously by fitting the data globally to a 1:1 Langmuir binding model (Karlsson, R. Roos, H. Fagerstam, L. Petersson, B. (1994). Methods Enzymology 6. 99-110) using the BIAevaluation program. Equilibrium dissociation constant (K_D) values are calculated as k_{off}/k_{on}. This protocol is suitable for use in determining binding affinity of an antibody to any Trop-2, including human Trop-2, Trop-2 of another mammal (such as mouse Trop-2, rat Trop-2, or primate Trop-2), as well as different forms of Trop-2 (e.g., glycosylated Trop-2). Binding affinity of an antibody is generally measured at 25°C, but can also be measured at 37°C.

The Trop-2 antibodies as described herein may be made by any method known in the art. For the production of hybridoma cell lines, the route and schedule of immunization of the host animal are generally in keeping with established and conventional techniques for antibody stimulation and production, as further described herein. General techniques for production of human and mouse antibodies are known in the art and/or are described herein.

It is contemplated that any mammalian subject including humans or antibody producing cells therefrom can be manipulated to serve as the basis for production of mammalian, including human and hybridoma cell lines. Typically, the host animal is inoculated intraperitoneally, intramuscularly, orally, subcutaneously, intraplantar, and/or intradermally with an amount of immunogen, including as described herein.

Hybridomas can be prepared from the lymphocytes and immortalized myeloma cells using the general somatic cell hybridization technique of Kohler, B. and Milstein, C., Nature 256:495-497, 1975 or as modified by Buck, D. W., et al., In Vitro, 18:377-381, 1982. Available myeloma lines, including but not limited to X63-Ag8.653 and those from the Salk Institute, Cell Distribution Center, San Diego, Calif., USA, may be used in the

hybridization. Generally, the technique involves fusing myeloma cells and lymphoid cells using a fusogen such as polyethylene glycol, or by electrical means well known to those skilled in the art. After the fusion, the cells are separated from the fusion medium and grown in a selective growth medium, such as hypoxanthine-aminopterin-thymidine (HAT) medium, to eliminate unhybridized parent cells. Any of the media described herein, supplemented with or without serum, can be used for culturing hybridomas that secrete monoclonal antibodies. As another alternative to the cell fusion technique, EBV immortalized B cells may be used to produce the Trop-2 monoclonal antibodies of the subject invention. The hybridomas are expanded and subcloned, if desired, and supernatants are assayed for anti-immunogen activity by conventional immunoassay procedures (e.g., radioimmunoassay, enzyme immunoassay, or fluorescence immunoassay).

Hybridomas that may be used as source of antibodies encompass all derivatives, progeny cells of the parent hybridomas that produce monoclonal antibodies specific for Trop-2, or a portion thereof.

Hybridomas that produce such antibodies may be grown *in vitro* or *in vivo* using known procedures. The monoclonal antibodies may be isolated from the culture media or body fluids, by conventional immunoglobulin purification procedures such as ammonium sulfate precipitation, gel electrophoresis, dialysis, chromatography, and ultrafiltration, if desired. Undesired activity, if present, can be removed, for example, by running the preparation over adsorbents made of the immunogen attached to a solid phase and eluting or releasing the desired antibodies off the immunogen. Immunization of a host animal with a human Trop-2, or a fragment containing the target amino acid sequence conjugated to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N=C=NR$, where R and R^1 are different alkyl groups, can yield a population of antibodies (e.g., monoclonal antibodies).

If desired, the Trop-2 antibody (monoclonal or polyclonal) of interest may be sequenced and the polynucleotide sequence may then be cloned into a vector for

expression or propagation. The sequence encoding the antibody of interest may be maintained in vector in a host cell and the host cell can then be expanded and frozen for future use. Production of recombinant monoclonal antibodies in cell culture can be carried out through cloning of antibody genes from B cells by means known in the art. See, e.g. Tiller et al., J. Immunol. Methods 329, 112, 2008; U.S. Pat. No. 7,314,622.

In an alternative, the polynucleotide sequence may be used for genetic manipulation to "humanize" the antibody or to improve the affinity, or other characteristics of the antibody. For example, the constant region may be engineered to more nearly resemble human constant regions to avoid immune response if the antibody is used in clinical trials and treatments in humans. It may be desirable to genetically manipulate the antibody sequence to obtain greater affinity to Trop-2 and greater efficacy in inhibiting Trop-2.

There are four general steps to humanize a monoclonal antibody. These are: (1) determining the nucleotide and predicted amino acid sequence of the starting antibody light and heavy variable domains (2) designing the humanized antibody, i.e., deciding which antibody framework region to use during the humanizing process (3) the actual humanizing methodologies/techniques and (4) the transfection and expression of the humanized antibody. See, for example, U.S. Pat. Nos. 4,816,567; 5,807,715; 5,866,692; 6,331,415; 5,530,101; 5,693,761; 5,693,762; 5,585,089; and 6,180,370.

A number of "humanized" antibody molecules comprising an antigen binding site derived from a non-human immunoglobulin have been described, including chimeric antibodies having rodent or modified rodent V regions and their associated CDRs fused to human constant regions. See, for example, Winter et al. Nature 349:293-299, 1991, Lobuglio et al. Proc. Nat. Acad. Sci. USA 86:4220-4224, 1989, Shaw et al. J Immunol. 138:4534-4538, 1987, and Brown et al. Cancer Res. 47:3577-3583, 1987. Other references describe rodent CDRs grafted into a human supporting framework region (FR) prior to fusion with an appropriate human antibody constant region. See, for example, Riechmann et al. Nature 332:323-327, 1988, Verhoeyen et al. Science 239:1534-1536, 1988, and Jones et al. Nature 321:522-525, 1986. Another reference describes rodent CDRs supported by recombinantly engineered rodent framework regions. See, for example, European Patent Publication No. 0519596. These "humanized" molecules are designed to minimize unwanted immunological response

toward rodent anti-human antibody molecules which limits the duration and effectiveness of therapeutic applications of those moieties in human recipients. For example, the antibody constant region can be engineered such that it is immunologically inert (e.g., does not trigger complement lysis). See, e.g. PCT Publication No. PCT/GB99/01441; UK Patent Application No. 9809951.8. Other methods of humanizing antibodies that may also be utilized are disclosed by Daugherty et al., Nucl. Acids Res. 19:2471-2476, 1991, and in U.S. Pat. Nos. 6,180,377; 6,054,297; 5,997,867; 5,866,692; 6,210,671; and 6,350,861; and in PCT Publication No. WO 01/27160.

The general principles related to humanized antibodies discussed above are also applicable to customizing antibodies for use, for example, in dogs, cats, primate, equines and bovines. Further, one or more aspects of humanizing an antibody described herein may be combined, e.g., CDR grafting, framework mutation and CDR mutation.

In one variation, fully human antibodies may be obtained by using commercially available mice that have been engineered to express specific human immunoglobulin proteins. Transgenic animals that are designed to produce a more desirable (e.g., fully human antibodies) or more robust immune response may also be used for generation of humanized or human antibodies. Examples of such technology are Xenomouse™ from Abgenix, Inc. (Fremont, CA) and HuMAb-Mouse® and TC Mouse™ from Medarex, Inc. (Princeton, NJ).

In an alternative, antibodies may be made recombinantly and expressed using any method known in the art. In another alternative, antibodies may be made recombinantly by phage display technology. See, for example, U.S. Pat. Nos. 5,565,332; 5,580,717; 5,733,743; and 6,265,150; and Winter et al., Annu. Rev. Immunol. 12:433-455, 1994. Alternatively, the phage display technology (McCafferty et al., Nature 348:552-553, 1990) can be used to produce human antibodies and antibody fragments *in vitro*, from immunoglobulin variable (V) domain gene repertoires from unimmunized donors. According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, such as M13 or fd, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties

of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus, the phage mimics some of the properties of the B cell. Phage display can be performed in a variety of formats; for review see, e.g., Johnson, Kevin S. and Chiswell, David J., Current Opinion in Structural Biology 3:564-571, 1993. Several sources of V-gene segments can be used for phage display. Clackson et al., Nature 352:624-628, 1991, isolated a diverse array of anti-oxazolone antibodies from a small random combinatorial library of V genes derived from the spleens of immunized mice. A repertoire of V genes from unimmunized human donors can be constructed and antibodies to a diverse array of antigens (including self-antigens) can be isolated essentially following the techniques described by Mark et al., J. Mol. Biol. 222:581-597, 1991, or Griffith et al., EMBO J. 12:725-734, 1993. In a natural immune response, antibody genes accumulate mutations at a high rate (somatic hypermutation). Some of the changes introduced will confer higher affinity, and B cells displaying high-affinity surface immunoglobulin are preferentially replicated and differentiated during subsequent antigen challenge. This natural process can be mimicked by employing the technique known as “chain shuffling.” (Marks et al., Bio/Technol. 10:779-783, 1992). In this method, the affinity of “primary” human antibodies obtained by phage display can be improved by sequentially replacing the heavy and light chain V region genes with repertoires of naturally occurring variants (repertoires) of V domain genes obtained from unimmunized donors. This technique allows the production of antibodies and antibody fragments with affinities in the pM-nM range. A strategy for making very large phage antibody repertoires (also known as “the mother-of-all libraries”) has been described by Waterhouse et al., Nucl. Acids Res. 21:2265-2266, 1993. Gene shuffling can also be used to derive human antibodies from rodent antibodies, where the human antibody has similar affinities and specificities to the starting rodent antibody. According to this method, which is also referred to as “epitope imprinting”, the heavy or light chain V domain gene of rodent antibodies obtained by phage display technique is replaced with a repertoire of human V domain genes, creating rodent-human chimeras. Selection on antigen results in isolation of human variable regions capable of restoring a functional antigen binding site, i.e., the epitope governs (imprints) the choice of partner. When the process is repeated in order to replace the remaining rodent V domain, a human antibody is obtained (see PCT Publication No. WO 93/06213). Unlike traditional

humanization of rodent antibodies by CDR grafting, this technique provides completely human antibodies, which have no framework or CDR residues of rodent origin.

Antibodies may be made recombinantly by first isolating the antibodies and antibody producing cells from host animals, obtaining the gene sequence, and using the gene sequence to express the antibody recombinantly in host cells (e.g., CHO cells). Another method which may be employed is to express the antibody sequence in plants (e.g., tobacco) or transgenic milk. Methods for expressing antibodies recombinantly in plants or milk have been disclosed. See, for example, Peeters, et al. Vaccine 19:2756, 2001; Lonberg, N. and D. Huszar Int. Rev. Immunol 13:65, 1995; and Pollock, et al., J Immunol Methods 231:147, 1999. Methods for making derivatives of antibodies, e.g., humanized, single chain, etc. are known in the art.

Immunoassays and flow cytometry sorting techniques such as fluorescence activated cell sorting (FACS) can also be employed to isolate antibodies that are specific for Trop-2.

The antibodies as described herein can be bound to many different carriers. Carriers can be active and/or inert. Examples of well-known carriers include polypropylene, polystyrene, polyethylene, dextran, nylon, amylases, glass, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble or insoluble for purposes of the invention. Those skilled in the art will know of other suitable carriers for binding antibodies, or will be able to ascertain such, using routine experimentation. In some embodiments, the carrier comprises a moiety that targets the myocardium.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors (such as expression vectors disclosed in PCT Publication No. WO 87/04462), which are then transfected into host cells such as E. coli cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. See, e.g., PCT Publication No. WO 87/04462. The DNA also may be modified, for example, by

substituting the coding sequence for human heavy and light chain constant regions in place of the homologous murine sequences, Morrison et al., Proc. Nat. Acad. Sci. 81:6851, 1984, or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. In that manner, "chimeric" or "hybrid" antibodies are prepared that have the binding specificity of a Trop-2 monoclonal antibody herein.

The Trop-2 antibodies as described herein can be identified or characterized using methods known in the art, whereby reduction of Trop-2 expression levels is detected and/or measured. In some embodiments, a Trop-2 antibody is identified by incubating a candidate agent with Trop-2 and monitoring binding and/or attendant reduction of Trop-2 expression levels. The binding assay may be performed with purified Trop-2 polypeptide(s), or with cells naturally expressing, or transfected to express, Trop-2 polypeptide(s). In one embodiment, the binding assay is a competitive binding assay, where the ability of a candidate antibody to compete with a known Trop-2 antibody for Trop-2 binding is evaluated. The assay may be performed in various formats, including the ELISA format.

Following initial identification, the activity of a candidate Trop-2 antibody can be further confirmed and refined by bioassays, known to test the targeted biological activities. Alternatively, bioassays can be used to screen candidates directly. Some of the methods for identifying and characterizing Trop-2 antibodies are described in detail in the Examples.

Trop-2 antibodies may be characterized using methods well known in the art. For example, one method is to identify the epitope to which it binds, or "epitope mapping." There are many methods known in the art for mapping and characterizing the location of epitopes on proteins, including solving the crystal structure of an antibody-antigen complex, competition assays, gene fragment expression assays, and synthetic peptide-based assays, as described, for example, in Chapter 11 of Harlow and Lane, Using Antibodies, a Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1999. In an additional example, epitope mapping can be used to determine the sequence to which a Trop-2 antibody binds. Epitope mapping is commercially available from various sources, for example, Pepscan Systems (Edelhertweg 15, 8219 PH Lelystad, The Netherlands). The epitope can be a linear

epitope, i.e., contained in a single stretch of amino acids, or a conformational epitope formed by a three-dimensional interaction of amino acids that may not necessarily be contained in a single stretch. Peptides of varying lengths (e.g., at least 4-6 amino acids long) can be isolated or synthesized (e.g., recombinantly) and used for binding assays with a Trop-2 antibody. In another example, the epitope to which the Trop-2 antibody binds can be determined in a systematic screening by using overlapping peptides derived from the Trop-2 sequence and determining binding by the Trop-2 antibody. According to the gene fragment expression assays, the open reading frame encoding Trop-2 is fragmented either randomly or by specific genetic constructions and the reactivity of the expressed fragments of Trop-2 with the antibody to be tested is determined. The gene fragments may, for example, be produced by PCR and then transcribed and translated into protein in vitro, in the presence of radioactive amino acids. The binding of the antibody to the radioactively labeled Trop-2 fragments is then determined by immunoprecipitation and gel electrophoresis. Certain epitopes can also be identified by using large libraries of random peptide sequences displayed on the surface of phage particles (phage libraries). Alternatively, a defined library of overlapping peptide fragments can be tested for binding to the test antibody in simple binding assays. In an additional example, mutagenesis of an antigen binding domain, domain swapping experiments and alanine scanning mutagenesis can be performed to identify residues required, sufficient, and/or necessary for epitope binding. For example, domain swapping experiments can be performed using a mutant Trop-2 in which various fragments of the Trop-2 protein have been replaced (swapped) with sequences from Trop-2 from another species (e.g., mouse), or a closely related, but antigenically distinct protein (e.g., Trop-1). By assessing binding of the antibody to the mutant Trop-2, the importance of the particular Trop-2 fragment to antibody binding can be assessed.

Yet another method which can be used to characterize a Trop-2 antibody is to use competition assays with other antibodies known to bind to the same antigen, i.e., various fragments on Trop-2, to determine if the Trop-2 antibody binds to the same epitope as other antibodies. Competition assays are well known to those of skill in the art.

An expression vector can be used to direct expression of a Trop-2 antibody. One skilled in the art is familiar with administration of expression vectors to obtain expression

of an exogenous protein in vivo. See, e.g., U.S. Pat. Nos. 6,436,908; 6,413,942; and 6,376,471. Administration of expression vectors includes local or systemic administration, including injection, oral administration, particle gun or catheterized administration, and topical administration. In another embodiment, the expression vector is administered directly to the sympathetic trunk or ganglion, or into a coronary artery, atrium, ventrical, or pericardium.

Targeted delivery of therapeutic compositions containing an expression vector, or subgenomic polynucleotides can also be used. Receptor-mediated DNA delivery techniques are described in, for example, Findeis et al., Trends Biotechnol., 1993, 11:202; Chiou et al., Gene Therapeutics: Methods And Applications Of Direct Gene Transfer, J.A. Wolff, ed., 1994; Wu et al., J. Biol. Chem., 263:621, 1988; Wu et al., J. Biol. Chem., 269:542, 1994; Zenke et al., Proc. Natl. Acad. Sci. USA, 87:3655, 1990; and Wu et al., J. Biol. Chem., 266:338, 1991. Therapeutic compositions containing a polynucleotide are administered in a range of about 100 ng to about 200 mg of DNA for local administration in a gene therapy protocol. Concentration ranges of about 500 ng to about 50 mg, about 1 μg to about 2 mg, about 5 μg to about 500 μg, and about 20 μg to about 100 μg of DNA can also be used during a gene therapy protocol. The therapeutic polynucleotides and polypeptides can be delivered using gene delivery vehicles. The gene delivery vehicle can be of viral or non-viral origin (see generally, Jolly, Cancer Gene Therapy,1:51, 1994; Kimura, Human Gene Therapy, 5:845, 1994; Connelly, Human Gene Therapy, 1995, 1:185; and Kaplitt, Nature Genetics, 6:148, 1994). Expression of such coding sequences can be induced using endogenous mammalian or heterologous promoters. Expression of the coding sequence can be either constitutive or regulated.

Viral-based vectors for delivery of a desired polynucleotide and expression in a desired cell are well known in the art. Exemplary viral-based vehicles include, but are not limited to, recombinant retroviruses (see, e.g., PCT Publication Nos. WO 90/07936; WO 94/03622; WO 93/25698; WO 93/25234; WO 93/11230; WO 93/10218; WO 91/02805; U.S. Pat. Nos. 5, 219,740 and 4,777,127; GB Pat. No. 2,200,651; and EP Pat. No. 0 345 242), alphavirus-based vectors (e.g., Sindbis virus vectors, Semliki forest virus (ATCC VR-67; ATCC VR-1247), Ross River virus (ATCC VR-373; ATCC VR-1246) and Venezuelan equine encephalitis virus (ATCC VR-923; ATCC VR-1250; ATCC VR

1249; ATCC VR-532)), and adeno-associated virus (AAV) vectors (see, e.g., PCT Publication Nos. WO 94/12649, WO 93/03769; WO 93/19191; WO 94/28938; WO 95/11984 and WO 95/00655). Administration of DNA linked to killed adenovirus as described in Curiel, Hum. Gene Ther., 1992, 3:147 can also be employed.

Non-viral delivery vehicles and methods can also be employed, including, but not limited to, polycationic condensed DNA linked or unlinked to killed adenovirus alone (see, e.g., Curiel, Hum. Gene Ther., 3:147, 1992); ligand-linked DNA (see, e.g., Wu, J. Biol. Chem., 264:16985, 1989); eukaryotic cell delivery vehicles cells (see, e.g., U.S. Pat. No. 5,814,482; PCT Publication Nos. WO 95/07994; WO 96/17072; WO 95/30763; and WO 97/42338) and nucleic charge neutralization or fusion with cell membranes. Naked DNA can also be employed. Exemplary naked DNA introduction methods are described in PCT Publication No. WO 90/11092 and U.S. Pat. No. 5,580,859. Liposomes that can act as gene delivery vehicles are described in U.S. Pat. No. 5,422,120; PCT Publication Nos. WO 95/13796; WO 94/23697; WO 91/14445; and EP 0524968. Additional approaches are described in Philip, Mol. Cell Biol., 14:2411, 1994 and in Woffendin, Proc. Natl. Acad. Sci., 91:1581, 1994.

In some embodiments, the invention encompasses compositions, including pharmaceutical compositions, comprising antibodies described herein or made by the methods and having the characteristics described herein. As used herein, compositions comprise one or more antibodies that bind to Trop-2, and/or one or more polynucleotides comprising sequences encoding one or more these antibodies. These compositions may further comprise suitable excipients, such as pharmaceutically acceptable excipients including buffers, which are well known in the art.

Accordingly, the invention provides any of the following, or compositions (including pharmaceutical compositions) comprising any of the following: (a) an antibody having a partial light chain sequence of

a)

DIVLTQSPASLAVSLGQRATISCRASKSVSTSGYSYMHWYQQKPGQPPKLLIYLASNLE SGVPARFSGSGSGTDFTLNIHPVEEEDAATYYCQHSRELPYTFGGGTKLEIK (SEQ ID NO: 1),

DIVMTQSPDSLAVSLGERATINCRASKSVSTSGYSYMHWYQQKPGQPPKLLIYLASNL ESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQHSRELPYTFGQGTKLEIK (SEQ ID NO: 3),

DIVMTQSPDSLAVSLGERATINCRASKSVSTSLYSYMHWYQQKPGQPPKLLIYLASNLE SGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQHSRELPYTFGQGTKLEI K (SEQ ID NO:6),

DIVMTQSPDSLAVSLGERATINCRASKSVSTSNYSYMHWYQQKPGQPPKLLIYLASNL ESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQHSRELPYTFGQGTKLEIK (SEQ ID NO: 7),

DILLTQSPAILSVSPGERVSFSCRASQTIGTSIHWYQQRTNGSPRLLIKYASESISGIPSR FSGSGSGTDFTLSINSVESEDIADYYCQQSNSWPFTFGSGTKLEIK (SEQ ID NO: 8),

GVHSEIVLTQSPATLSLSPGERATLSCRASQTIGTSIHWYQQKPGQAPRLLIYYASESIS GIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQQSNSWPFTFGQGTKLEIK (SEQ ID NO: 10), or

EIVLTQSPATLSLSPGERATLSCRASQTIGTSIHWYQQKPGQAPRLLIYYASESISGIPAR FSGSGSGTDFTLTISSLEPEDFAVYYCSQSFSWPFTFGQGTKLEIK (SEQ ID NO: 12)

and/or (b) an antibody having a partial heavy chain sequence of

QVQLKESGPGLVAPSQSLSITCTVSGFSLTSYGVHWVRQPPGKGLEWLGVIWTGGST DYNSALMSRLSINKDNSKSQVFLKMNSLQTDDTAMYYCARDGDYDRYTMDYWGQGT SVTVSS (SEQ ID NO: 2),

QVQLQESGPGLVKPSETLSLTCTVSGGSISSYGVHWIRQPPGKGLEWIGVIWTGGSTD YNSALMSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARDGDYDRYTMDYWGQGTLV TVSS (SEQ ID NO: 4),

QVQLQESGPGLVKPSETLSLTCTVSGGSISSYGVHWIRQPPGKGLEWIGVIWTSGVTD YNSALMGRVTISVDTSKNQFSLKLSSVTAADTAVYYCARDGDYDRYTMDYWGQGTLV TVSS (SEQ ID NO: 5),

QVQLQQPGAELVRPGASVKLSCKASGYTFTSYWINWVKQRPGHGLEWIGNIYPSDSY SNYNQKFKDKATLTVDKSSSTAYMQVSSPTSEDSAVYYCTYGSSFDYWGQGTTVTVS S (SEQ ID NO: 9),

QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYWINWVRQAPGQGLEWMGNIYPSDS YSNYNQKFKDRVTMTRDTSTSTVYMELSSLRSEDTAVYYCARGSSFDYWGQGTLVTV SS (SEQ ID NO: 11), or

QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYWINWVRQAPGQGLEWMGNIFPSDS YSNYNKKFKDRVTMTRDTSTSTVYMELSSLRSEDTAVYYCARGSGFDYWGQGTLVTV SS (SEQ ID NO: 13).

Table 1

mAb	Light Chain	Heavy Chain
m7E6	DIVLTQSPASLAVSLGQRATISC**R ASKSVSTSGYSYMH**WYQQKPG QPPKLLIY**LASNLES**GVPARFSG SGSGTDFTLNIHPVEEEDAATYY C**QHSRELPYT**FGGGTKLEIK (SEQ ID NO: 1)	QVQLKESGPGLVAPSQSLSITCTV S**GFSLTSY**GVHWVRQPPGKGLE WLGV**IWTGGS**TDYNSALMSRLSIN KDNSKSQVFLKMNSLQTDDTAMY YCAR**DGDYDRYTMDY**WGQGTSV TVSS (SEQ ID NO: 2)
h7E6	DIVMTQSPDSLAVSLGERATINC **RASKSVSTSGYSYMH**WYQQKP GQPPKLLIY**LASNLES**GVPDRFS GSGSGTDFTLTISSLQAEDVAVY YC**QHSRELPYT**FGQGTKLEIK (SEQ ID NO: 3)	QVQLQESGPGLVKPSETLSLTCTV S**GGSISSY**GVHWIRQPPGKGLEWI GV**IWTGGS**TDYNSALMSRVTISVD TSKNQFSLKLSSVTAADTAVYYCA R**DGDYDRYTMDY**WGQGTLVTVS S (SEQ ID NO: 4)
h7E6_ SVG	DIVMTQSPDSLAVSLGERATINC **RASKSVSTSGYSYMH**WYQQKP GQPPKLLIY**LASNLES**GVPDRFS GSGSGTDFTLTISSLQAEDVAVY YC**QHSRELPYT**FGQGTKLEIK (SEQ ID NO: 3)	QVQLQESGPGLVKPSETLSLTCTV S**GGSISSY**GVHWIRQPPGKGLEWI GV**IWTSGV**TDYNSALMGRVTISVD TSKNQFSLKLSSVTAADTAVYYCA R**DGDYDRYTMDY**WGQGTLVTVS S (SEQ ID NO: 5)
h7E6_ SVG1	DIVMTQSPDSLAVSLGERATINC **RASKSVSTSGYSYMH**WYQQKP GQPPKLLIY**LASNLES**GVPDRFS GSGSGTDFTLTISSLQAEDVAVY YC**QHSRELPYT**FGQGTKLEIK (SEQ ID NO: 3)	QVQLQESGPGLVKPSETLSLTCTV S**GGSISSY**GVHWIRQPPGKGLEWI GV**IWTSGV**TDYNSALMGRVTISVD TSKNQFSLKLSSVTAADTAVYYCA R**X_1X_2DYDRYTX_3DY**WGQGTLVTV SS wherein X_1, X_2, and X_3 are any naturally occurring amino acids

mAb	Light Chain	Heavy Chain
		(SEQ ID NO: 84)
h7E6_SVG2	DIVMTQSPDSLAVSLGERATINC **RASKSVSTSGYSYMH**WYQQKPGQPPKLLIY**LASNLES**GVPDRFSGSGSGTDFTLTISSLQAEDVAVYYC**QHSRELPYT**FGQGTKLEIK (SEQ ID NO: 3)	QVQLQESGPGLVKPSETLSLTCTVSGGSISSYGVHWIRQPPGKGLEWIGVI**WTSGV**TDYNSALMGRVTISVDTSKNQFSLKLSSVTAADTAVYYCAR**X_1X_2DYDRYTX$_3$DY**WGQGTLVTVSS wherein X_1 and X_2 are any naturally occurring amino acids; X_3 is E or M (SEQ ID NO: 85)
h7E6_SVGL	DIVMTQSPDSLAVSLGERATINC **RASKSVSTSLYSYMH**WYQQKPGQPPKLLIY**LASNLES**GVPDRFSGSGSGTDFTLTISSLQAEDVAVYYC**QHSRELPYT**FGQGTKLEIK (SEQ ID NO: 6)	QVQLQESGPGLVKPSETLSLTCTVSGGSISSYGVHWIRQPPGKGLEWIGVI**WTSGV**TDYNSALMGRVTISVDTSKNQFSLKLSSVTAADTAVYYCAR**DGDYDRYTMDY**WGQGTLVTVSS (SEQ ID NO: 5)
h7E6_SVGN	DIVMTQSPDSLAVSLGERATINC **RASKSVSTSNYSYMH**WYQQKPGQPPKLLIY**LASNLES**GVPDRFSGSGSGTDFTLTISSLQAEDVAVYYC**QHSRELPYT**FGQGTKLEIK (SEQ ID NO: 7)	QVQLQESGPGLVKPSETLSLTCTVSGGSISSYGVHWIRQPPGKGLEWIGVI**WTSGV**TDYNSALMGRVTISVDTSKNQFSLKLSSVTAADTAVYYCAR**DGDYDRYTMDY**WGQGTLVTVSS (SEQ ID NO: 5)
m6G11	DILLTQSPAILSVSPGERVSFSC**RASQTIGTSIH**WYQQRTNGSPRLLIK**YASESIS**GIPSRFSGSGSGTDFTLSINSVESEDIADYYC**QQSNSWPFT**FGSGTKLEIK (SEQ ID NO: 8)	QVQLQQPGAELVRPGASVKLSCKASGYTFT**SY**WINWVKQRPGHGLEWIGNI**YPSDSY**SNYNQKFKDKATLTVDKSSSTAYMQVSSPTSEDSAVYYCTY**GSSFDY**WGQGTTVTVSS (SEQ ID NO: 9)
h6G11	EIVLTQSPATLSLSPGERATLSC**R**	QVQLVQSGAEVKKPGASVKVSCK

mAb	Light Chain	Heavy Chain
	ASQTIGTSIHWYQQKPGQAPRLL IY**YASESIS**GIPARFSGSGSGTDF TLTISSLEPEDFAVYYC**QQSNSW PFT**FGQGTKLEIK (SEQ ID NO: 10)	AS**GYTFTSY**WINWVRQAPGQGLE WMGNI**YPSDSY**SNYNQKFKDRVT MTRDTSTSTVYMELSSLRSEDTAV YYCAR**GSSFDY**WGQGTLVTVSS (SEQ ID NO: 11)
H6G11 _FKG_ SF	EIVLTQSPATLSLSPGERATLSC**R ASQTIGTSIH**WYQQKPGQAPRLL IY**YASESIS**GIPARFSGSGSGTDF TLTISSLEPEDFAVYYC**SQSFSW PFT**FGQGTKLEIK (SEQ ID NO: 12)	QVQLVQSGAEVKKPGASVKVSCK AS**GYTFTSY**WINWVRQAPGQGLE WMGNI**FPSDSY**SNYNKKFKDRVT MTRDTSTSTVYMELSSLRSEDTAV YYCAR**GSGFDY**WGQGTLVTVSS (SEQ ID NO: 13)

In Table 1, the underlined sequences are CDR sequences according to Kabat and in bold according to Chothia.

The invention also provides CDR portions of antibodies to Trop-2 (including Chothia, Kabat CDRs, and CDR contact regions). Determination of CDR regions is well within the skill of the art. It is understood that in some embodiments, CDRs can be a combination of the Kabat and Chothia CDR (also termed "combined CRs" or "extended CDRs"). In some embodiments, the CDRs are the Kabat CDRs. In other embodiments, the CDRs are the Chothia CDRs. In other words, in embodiments with more than one CDR, the CDRs may be any of Kabat, Chothia, combination CDRs, or combinations thereof. Table 2 provides examples of CDR sequences provided herein.

Table 2

Heavy Chain			
mAb	**CDRH1**	**CDRH2**	**CDRH3**
m7E6	SYGVH (SEQ ID NO: 30) (Kabat); GFSLTSY (SEQ ID NO: 31) (Chothia); GFSLTSYGVH (SEQ	VIWTGGSTDYNSALMS (SEQ ID NO: 33) (Kabat) WTGGS (SEQ ID NO:34) (Chothia)	DGDYDRYTMDY (SEQ ID NO: 35)

	ID NO: 32) (extended)		
h7E6	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTGGSTDYNSALMS (SEQ ID NO: 33) (Kabat) WTGGS (SEQ ID NO:34) (Chothia)	DGDYDRYTMDY (SEQ ID NO: 35)
h7E6_ SVG	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	DGDYDRYTMDY (SEQ ID NO: 35)
h7E6_ SVG1	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	X_1X_2DYDRYTX_3D Y wherein X_1, X_2, and X_3 are any naturally occurring amino acids (SEQ ID NO: 86)
h7E6_ SVG2	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	X_1X_2DYDRYTX_3D Y wherein X_1 and X_2, are any naturally occurring amino acids; wherein X_3 is E or M (SEQ ID NO: 87)
h7E6_	SYGVH (SEQ ID NO:	VIWTSGVTDYNSALMG	DYDRYTX_1DY

SVG3	30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	(SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	wherein X_1 is any naturally occurring amino acid; (SEQ ID NO: 83)
h7E6_ SVG4	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	DYDRYTX_1DY wherein X_1 is E or M (SEQ ID NO: 82)
h7E6_ SVG5	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	LGDYDRYTMDY (SEQ ID NO: 103)
h7E6_ SVG6	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	YGDYDRYTMDY (SEQ ID NO: 104)
h7E6_ SVG7	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	FGDYDRYTMDY (SEQ ID NO: 105)

	ID NO: 37) (extended)		
h7E6_SVG8	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	HGDYDRYTMDY (SEQ ID NO: 106)
h7E6_SVG9	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	AGDYDRYTMDY (SEQ ID NO: 107)
h7E6_SVG10	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	TGDYDRYTMDY (SEQ ID NO: 108)
h7E6_SVG11	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	SGDYDRYTMDY (SEQ ID NO: 109)
h7E6_SVG12	SYGVH (SEQ ID NO: 30) (Kabat);	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat)	IGDYDRYTMDY (SEQ ID NO: 110)

	GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	WTSGV (SEQ ID NO:39) (Chothia)	
h7E6_ SVG13	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	RGDYDRYTMDY (SEQ ID NO: 111)
h7E6_ SVG14	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	VGDYDRYTMDY (SEQ ID NO: 112)
h7E6_ SVG15	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	WGDYDRYTMDY (SEQ ID NO: 113)
h7E6_ SVG16	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	QGDYDRYTMDY (SEQ ID NO: 114)

	(extended)		
h7E6_SVG17	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	GGDYDRYTMDY (SEQ ID NO: 115)
h7E6_SVG18	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	KGDYDRYTMDY (SEQ ID NO: 116)
h7E6_SVG19	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	DSDYDRYTMDY (SEQ ID NO: 117)
h7E6_SVG20	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	DKDYDRYTMDY (SEQ ID NO: 118)
h7E6_SVG21	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39)	DHDYDRYTMDY (SEQ ID NO: 119)

	NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	(Chothia)	
h7E6_ SVG22	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	DADYDRYTMDY (SEQ ID NO: 120)
h7E6_ SVG23	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	DFDYDRYTMDY (SEQ ID NO: 121)
h7E6_ SVG24	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	DTDYDRYTMDY (SEQ ID NO: 122)
h7E6_ SVG25	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	DRDYDRYTMDY (SEQ ID NO: 123)

h7E6_SVG26	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	DVDYDRYTMDY (SEQ ID NO: 124)
h7E6_SVG27	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	DQDYDRYTMDY (SEQ ID NO: 125)
h7E6_SVG28	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	DLDYDRYTMDY (SEQ ID NO: 126)
h7E6_SVG29	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	DYDYDRYTMDY (SEQ ID NO: 127)
h7E6_SVG30	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia);	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	DEDYDRYTMDY (SEQ ID NO: 128)

	GGSISSYGVH (SEQ ID NO: 37) (extended)		
h7E6_ SVG31	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	DNDYDRYTMDY (SEQ ID NO: 129)
h7E6_ SVG32	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	DWDYDRYTMDY (SEQ ID NO: 130)
h7E6_ SVGL	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	DGDYDRYTMDY (SEQ ID NO: 35)
h7E6_ SVGL1	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	DSDYDRYTMDY (SEQ ID NO: 117)
h7E6_	SYGVH (SEQ ID NO:	VIWTSGVTDYNSALMG	DKDYDRYTMDY

SVGL2	30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	(SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	(SEQ ID NO: 118)
h7E6_ SVGL3	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	DADYDRYTMDY (SEQ ID NO: 120)
h7E6_ SVGL4	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	DLDYDRYTMDY (SEQ ID NO: 126)
h7E6_ SVGL5	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ ID NO: 37) (extended)	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	DEDYDRYTMDY (SEQ ID NO: 128)
h7E6_ SVGN	SYGVH (SEQ ID NO: 30) (Kabat); GGSISSY (SEQ ID NO: 36) (Chothia); GGSISSYGVH (SEQ	VIWTSGVTDYNSALMG (SEQ ID NO: 38) (Kabat) WTSGV (SEQ ID NO:39) (Chothia)	DGDYDRYTMDY (SEQ ID NO: 35)

	ID NO: 37) (extended)		
h7E6 Heavy Chain Consensus	$GX_1SX_2X_3SY$ wherein X_1 is F or G; X_2 is L or I; X_3 is T or S (SEQ ID NO: 76) (Chothia); $GX_1SX_2X_3SYGVH$ wherein X_1 is F or G; X_2 is L or I; X_3 is T or S (SEQ ID NO: 77)	$VIWTX_1GX_2TDYNSALMX_3$ wherein X_1 is G or S; X_2 is S or V; X_3 is S or G (SEQ ID NO: 49) (Kabat) WTX_1GX_2 wherein X_1 is G or S; X_2 is S or V (SEQ ID NO: 50) (Chothia)	$X_1X_2DYDRYTX_3DY$ wherein X_1, X_2, and X_3 are any naturally occurring amino acids (SEQ ID NO: 86) $X_1X_2DYDRYTX_3DY$ wherein X_1 and X_2, are any naturally occurring amino acids; wherein X_3 is E or M (SEQ ID NO: 87) $DYDRYTX_1DY$ wherein X_1 is any naturally occurring amino acid; (SEQ ID NO: 83) $DYDRYTX_1DY$ wherein X_1 is E or M (SEQ ID NO: 82)
m6G11	SYWIN (SEQ ID NO: 40) (Kabat); GYTFTSY (SEQ ID NO: 41) (Chothia); GYTFTSYWIN (SEQ ID NO: 42) (extended)	NIYPSDSYSNYNQKFKD (SEQ ID NO: 43) (Kabat) YPSDSY (SEQ ID NO: 44) (Chothia)	GSSFDY (SEQ ID NO: 45)
h6G11	SYWIN (SEQ ID NO:	NIYPSDSYSNYNQKFKD	GSSFDY (SEQ ID

	40) (Kabat); GYTFTSY (SEQ ID NO: 41) (Chothia); GYTFTSYWIN (SEQ ID NO: 42) (extended)	(SEQ ID NO: 43) (Kabat) YPSDSY (SEQ ID NO: 44) (Chothia)	NO: 45)
h6G11_FKG_SF	SYWIN (SEQ ID NO: 40) (Kabat); GYTFTSY (SEQ ID NO: 41) (Chothia); GYTFTSYWIN (SEQ ID NO: 42) (extended)	NIFPSDSYSNYNKKFKD (SEQ ID NO: 46) (Kabat) FPSDSY (SEQ ID NO: 47) (Chothia)	GSGFDY (SEQ ID NO: 48)
h6G11 Heavy Chain consensus		NIX_1PSDSYSNYNX_2KFKD wherein X_1 is Y or F; X_2 is Q or K (SEQ ID NO: 51) (Kabat) X_1PSDSY wherein X_1 is Y or F (SEQ ID NO:52) (Chothia)	GSX_1FDY wherein X_1 is S or G (SEQ ID NO: 53)
Light Chain			
mAb	**CDRL1**	**CDRL2**	**CDRL3**
m7E6	RASKSVSTSGYSYMH (SEQ ID NO: 54)	LASNLES (SEQ ID NO: 55)	QHSRELPYT (SEQ ID NO: 56)
h7E6	RASKSVSTSGYSYMH (SEQ ID NO: 54)	LASNLES (SEQ ID NO: 55)	QHSRELPYT (SEQ ID NO: 56)
h7E6_SVG (including h7E6_SVG1 through h	RASKSVSTSGYSYMH (SEQ ID NO: 54)	LASNLES (SEQ ID NO: 55)	QHSRELPYT (SEQ ID NO: 56)

h7E6_SVG32			
h7E6_SVGL (including h7E6_SVGL1 through h7E6_SVGL5	RASKSVSTSLYSYMH (SEQ ID NO: 57)	LASNLES (SEQ ID NO: 55)	QHSRELPYT (SEQ ID NO: 56)
h7E6_SVGN	RASKSVSTSNYSYMH (SEQ ID NO: 58)	LASNLES (SEQ ID NO: 55)	QHSRELPYT (SEQ ID NO: 56)
h7E6 Light Chain Consensus	RASKSVSTSX$_1$YSYMH wherein X$_1$ is G, L, or N (SEQ ID NO: 63)		
m6G11	RASQTIGTSIH (SEQ ID NO: 59)	YASESIS (SEQ ID NO: 60)	QQSNSWPFT (SEQ ID NO: 61)
h6G11	RASQTIGTSIH (SEQ ID NO: 59)	YASESIS (SEQ ID NO: 60)	QQSNSWPFT (SEQ ID NO: 61)
h6G11_FKG_SF	RASQTIGTSIH (SEQ ID NO: 59)	YASESIS (SEQ ID NO: 60)	SQSFSWPFT (SEQ ID NO: 62)
h6G11 Light Chain consensus			X$_1$QSX$_2$SWPFT wherein X$_1$ is Q or S; X$_2$ is N or F (SEQ ID NO:64)

In some embodiments, the present invention provides an antibody that binds to Trop-2 and competes with the antibody as described herein, such as m7E6, h7E6, h7E6_SVG, h7E6_SVG1, h7E6_SVG2, h7E6_SVG3, h7E6_SVG4, h7E6_SVG5, h7E6_SVG6, h7E6_SVG7, h7E6_SVG8, h7E6_SVG9, h7E6_SVG10, h7E6_SVG11, h7E6_SVG12, h7E6_SVG13, h7E6_SVG14, h7E6_SVG15, h7E6_SVG16, h7E6_SVG17, h7E6_SVG18, h7E6_SVG19, h7E6_SVG20, h7E6_SVG21, h7E6_SVG22, h7E6_SVG23, h7E6_SVG24, h7E6_SVG25, h7E6_SVG26, h7E6_SVG27, h7E6_SVG28, h7E6_SVG29, h7E6_SVG30, h7E6_SVG31, h7E6_SVG32, h7E6_SVGL, h7E6_SVGL1, h7E6_SVGL2, h7E6_SVGL3, h7E6_SVGL4, h7E6_SVGL5, h7E6_SVGN, m6G11, h6G11, or h6G11_FKG_SF. In some embodiments, the antibody competes the binding of Trop-2 with antibody h7E6_SVG, h7E6_SVG4, h7E6_SVG19, h7E6_SVG20, h7E6_SVG22, h7E6_SVG28, h7E6_SVG30, h7E6_SVGL, h7E6_SVGL1, h7E6_SVGL2, h7E6_SVGL3, h7E6_SVGL4, h7E6_SVGL5, h7E6_SVGN, or h6G11_FKG_SF. In some embodiments, the antibody competes the binding of Trop-2 with antibody h7E6_SVG and has a monovalent antibody binding affinity (K_D) of about any of or less than about any of 6.5 nM, 6.0 nM, 5.5 nM, 5.0 nM, 4.5 nM, 4.0 nM, 3.5 nM, 3.0 nM, 2.5 nM, 2.0 nM, 1.5 nM, 1.0 nM, 0.5 nM, or 0.25 nM as measured by surface plasmon resonance. In some embodiments, the antibody competes with the binding of Trop-2 with antibody h7E6 and has a monovalent antibody binding affinity (K_D) of about any of or less than about any of 30 nM, 25 nM, 22 nM, 20 nM, 15 nM, or 10 nM. In some embodiments, the competing antibody does not comprise a heavy chain variable region of the sequence QVQLKESGPGLVAPSQSLSITCTVSGFSLTSYGVHWVRQPPGKGLEWLGVIWTGGST DYNSALMSRLSINKDNSKSQVFLKMNSLQTDDTAMYYCARDGDYDRYTMDYWGQGT SVTVSS (SEQ ID NO: 2) and a light chain variable region of the sequence DIVLTQSPASLAVSLGQRATISCRASKSVSTSGYSYMHWYQQKPGQPPKLLIYLASNLE SGVPARFSGSGSGTDFTLNIHPVEEEDAATYYCQHSRELPYTFGGGTKLEIK (SEQ ID NO: 1). In some embodiments, the competing antibody is not antibody AR47A6.4.2, AR52A301.5, AR36A36.11.1, BR110 or RS7.

In some embodiments, the present invention provides an antibody or an antigen binding fragment, which specifically bind to Trop-2, wherein the antibody comprises a VH region comprising a sequence shown in SEQ ID NO: 5, 84, or 85; and/or a VL region

comprising a sequence shown in SEQ ID NO: 3. In some embodiments, the antibody comprises a light chain comprising the sequence DIVMTQSPDSLAVSLGERATINCRASKSVSTSGYSYMHWYQQKPGQPPKLLIYLASNL ESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQHSRELPYTFGQGTKLEIKRTVAAP SVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKD STYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 66) and a heavy chain comprising the sequence QVQLQESGPGLVKPSETLSLTCTVSGGSISSYGVHWIRQPPGKGLEWIGVIWTSGVTD YNSALMGRVTISVDTSKNQFSLKLSSVTAADTAVYYCARDGDYDRYTMDYWGQGTLV TVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFP AVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPC PAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNA KTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGS FFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 65). In some embodiments, the antibody comprises a light chain comprising the sequence SEQ ID NO: 66 and a heavy chain comprising the sequence SEQ ID NO: 101 or 102.

In some embodiments, the present invention provides an antibody or an antigen binding fragment, which specifically bind to Trop-2, wherein the antibody comprises a VH region comprising a sequence shown in SEQ ID NO: 13; and/or a VL region comprising a sequence shown in SEQ ID NO: 12. In some embodiments, the antibody comprises a light chain comprising the sequence EIVLTQSPATLSLSPGERATLSCRASQTIGTSIHWYQQKPGQAPRLLIYYASESISGIPAR FSGSGSGTDFTLTISSLEPEDFAVYYCSQSFSWPFTFGQGTKLEIKRTVAAPSVFIFPPS DEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 68) and a heavy chain comprising the sequence QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYWINWVRQAPGQGLEWMGNIFPSDS YSNYNKKFKDRVTMTRDTSTSTVYMELSSLRSEDTAVYYCARGSGFDYWGQGTLVTV SSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV LQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPA PELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKT

KPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ VYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFF LYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 67).

In some embodiments, the invention also provides CDR portions of antibodies to Trop-2 antibodies based on CDR contact regions. CDR contact regions are regions of an antibody that imbue specificity to the antibody for an antigen. In general, CDR contact regions include the residue positions in the CDRs and Vernier zones which are constrained in order to maintain proper loop structure for the antibody to bind a specific antigen. See, e.g., Makabe et al., J. Biol. Chem., 283:1156-1166, 2007. Determination of CDR contact regions is well within the skill of the art.

The binding affinity (K_D) of the Trop-2 antibody as described herein to Trop-2 (such as human Trop-2) can be about 0.002 to about 200 nM. In some embodiments, the binding affinity is any of about 200 nM, about 100 nM, about 50 nM, about 45 nM, about 40 nM, about 35 nM, about 30 nM, about 25 nM, about 20 nM, about 15 nM, about 10 nM, about 8 nM, about 7.5 nM, about 7 nM, about 6.5 nM, about 6 nM, about 5.5 nM, about 5 nM, about 4 nM, about 3 nM, about 2 nM, about 1 nM, about 500 pM, about 100 pM, about 60 pM, about 50 pM, about 20 pM, about 15 pM, about 10 pM, about 5 pM, or about 2 pM. In some embodiments, the binding affinity is less than any of about 250 nM, about 200 nM, about 100 nM, about 50 nM, about 30 nM, about 20 nM, about 10 nM, about 7.5 nM, about 7 nM, about 6.5 nM, about 6 nM, about 5 nM, about 4.5 nM, about 4 nM, about 3.5 nM, about 3 nM, about 2.5 nM, about 2 nM, about 1.5 nM, about 1 nM, about 500 pM, about 100 pM, about 50 pM, about 20 pM, about 10 pM, about 5 pM, or about 2 pM.

In some embodiments, the binding affinity (e.g., monovalent antibody binding) of the antibodies as described herein is about 35 nM or less as measured by surface plasmon resonance. In some embodiments, the binding affinity (e.g., monovalent antibody binding) of the antibodies as described herein is about 6.5 nM or less as measured by surface plasmon resonance.

The invention also provides methods of making any of these antibodies. The antibodies of this invention can be made by procedures known in the art. The polypeptides can be produced by proteolytic or other degradation of the antibodies, by recombinant methods (i.e., single or fusion polypeptides) as described above or by

chemical synthesis. Polypeptides of the antibodies, especially shorter polypeptides up to about 50 amino acids, are conveniently made by chemical synthesis. Methods of chemical synthesis are known in the art and are commercially available. For example, an antibody could be produced by an automated polypeptide synthesizer employing the solid phase method. See also, U.S. Pat. Nos. 5,807,715; 4,816,567; and 6,331,415.

In another alternative, the antibodies can be made recombinantly using procedures that are well known in the art. In one embodiment, a polynucleotide comprises a sequence encoding the heavy chain and/or the light chain variable regions of antibody m7E6, h7E6, h7E6_SVG, h7E6_SVG1, h7E6_SVG2, h7E6_SVG3, h7E6_SVG4, h7E6_SVG5, h7E6_SVG6, h7E6_SVG7, h7E6_SVG8, h7E6_SVG9, h7E6_SVG10, h7E6_SVG11, h7E6_SVG12, h7E6_SVG13, h7E6_SVG14, h7E6_SVG15, h7E6_SVG16, h7E6_SVG17, h7E6_SVG18, h7E6_SVG19, h7E6_SVG20, h7E6_SVG21, h7E6_SVG22, h7E6_SVG23, h7E6_SVG24, h7E6_SVG25, h7E6_SVG26, h7E6_SVG27, h7E6_SVG28, h7E6_SVG29, h7E6_SVG30, h7E6_SVG31, h7E6_SVG32, h7E6_SVGL, h7E6_SVGL1, h7E6_SVGL2, h7E6_SVGL3, h7E6_SVGL4, h7E6_SVGL5, h7E6_SVGN, m6G11, h6G11, or h6G11_FKG_SF. The sequence encoding the antibody of interest may be maintained in a vector in a host cell and the host cell can then be expanded and frozen for future use. Vectors (including expression vectors) and host cells are further described herein.

The invention also encompasses scFv of antibodies of this invention. Single chain variable region fragments are made by linking light and/or heavy chain variable regions by using a short linking peptide (Bird et al., Science 242:423-426, 1988). An example of a linking peptide is $(GGGGS)_3$ (SEQ ID NO: 80), which bridges approximately 3.5 nm between the carboxy terminus of one variable region and the amino terminus of the other variable region. Linkers of other sequences have been designed and used (Bird et al., 1988, supra). Linkers should be short, flexible polypeptides and preferably comprised of less than about 20 amino acid residues. Linkers can in turn be modified for additional functions, such as attachment of drugs or attachment to solid supports. The single chain variants can be produced either recombinantly or synthetically. For synthetic production of scFv, an automated synthesizer can be used. For recombinant production of scFv, a suitable plasmid

containing polynucleotide that encodes the scFv can be introduced into a suitable host cell, either eukaryotic, such as yeast, plant, insect or mammalian cells, or prokaryotic, such as E. coli. Polynucleotides encoding the scFv of interest can be made by routine manipulations such as ligation of polynucleotides. The resultant scFv can be isolated using standard protein purification techniques known in the art.

Other forms of single chain antibodies, such as diabodies or minibodies are also encompassed. Diabodies are bivalent, bispecific antibodies in which heavy chain variable (VH) and light chain variable (VL) domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (see e.g., Holliger, P., et al., Proc. Natl. Acad Sci. USA 90:6444-6448, 1993; Poljak, R. J., et al., Structure 2:1121-1123, 1994). Minibody includes the VL and VH domains of a native antibody fused to the hinge region and CH3 domain of the immunoglobulin molecule. See, e.g., US5,837,821.

For example, bispecific antibodies, monoclonal antibodies that have binding specificities for at least two different antigens, can be prepared using the antibodies disclosed herein. Methods for making bispecific antibodies are known in the art (see, e.g., Suresh et al., Methods in Enzymology 121:210, 1986). Traditionally, the recombinant production of bispecific antibodies was based on the coexpression of two immunoglobulin heavy chain-light chain pairs, with the two heavy chains having different specificities (Millstein and Cuello, Nature 305, 537-539, 1983).

According to one approach to making bispecific antibodies, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant region sequences. The fusion preferably is with an immunoglobulin heavy chain constant region, comprising at least part of the hinge, CH2 and CH3 regions. It is preferred to have the first heavy chain constant region (CH1), containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are cotransfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments

when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In one approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. This asymmetric structure, with an immunoglobulin light chain in only one half of the bispecific molecule, facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations. This approach is described in PCT Publication No. WO 94/04690.

In another approach, the bispecific antibodies are composed of amino acid modification in the first hinge region in one arm, and the substituted/replaced amino acid in the first hinge region has an opposite charge to the corresponding amino acid in the second hinge region in another arm. This approach is described in International Patent Application No. PCT/US2011/036419 (WO2011/143545).

In another approach, the bispecific antibodies can be generated using a glutamine-containing peptide tag engineered to the antibody directed to an epitope (e.g., Trop-2) in one arm and another peptide tag (e.g., a Lys-containing peptide tag or a reactive endogenous Lys) engineered to a second antibody directed to a second epitope in another arm in the presence of transglutaminase. This approach is described in International Patent Application No. PCT/IB2011/054899 (WO2012/059882).

Heteroconjugate antibodies, comprising two covalently joined antibodies, are also within the scope of the invention. Such antibodies have been used to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (PCT Publication Nos. WO 91/00360 and WO 92/200373; EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents and techniques are well known in the art, and are described in U.S. Pat. No. 4,676,980.

Chimeric or hybrid antibodies also may be prepared *in vitro* using known methods of synthetic protein chemistry, including those involving cross-linking agents. For

example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate.

In the recombinant humanized antibodies, the Fcγ portion can be modified to avoid interaction with Fcγ receptor and the complement and immune systems. The techniques for preparation of such antibodies are described in WO 99/58572. For example, the constant region may be engineered to more resemble human constant regions to avoid immune response if the antibody is used in clinical trials and treatments in humans. See, for example, U.S. Pat. Nos. 5,997,867 and 5,866,692.

The invention encompasses modifications to the antibodies and polypeptides of the invention variants shown in Table 1, including functionally equivalent antibodies which do not significantly affect their properties and variants which have enhanced or decreased activity and/or affinity. For example, the amino acid sequence may be mutated to obtain an antibody with the desired binding affinity to Trop-2. Modification of polypeptides is routine practice in the art and need not be described in detail herein. Examples of modified polypeptides include polypeptides with conservative substitutions of amino acid residues, one or more deletions or additions of amino acids which do not significantly deleteriously change the functional activity, or which mature (enhance) the affinity of the polypeptide for its ligand, or use of chemical analogs.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an antibody with an N-terminal methionyl residue or the antibody fused to an epitope tag. Other insertional variants of the antibody molecule include the fusion to the N- or C-terminus of the antibody of an enzyme or a polypeptide which increases the half-life of the antibody in the blood circulation.

Substitution variants have at least one amino acid residue in the antibody molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include the hypervariable regions, but FR alterations are also contemplated. Conservative substitutions are shown in Table 3 under the heading of "conservative substitutions." If such substitutions result in a

change in biological activity, then more substantial changes, denominated "exemplary substitutions" in Table 3, or as further described below in reference to amino acid classes, may be introduced and the products screened.

Table 3: Amino Acid Substitutions

Original Residue (naturally occurring amino acid)	Conservative Substitutions	Exemplary Substitutions
Ala (A)	Val	Val; Leu; Ile
Arg (R)	Lys	Lys; Gln; Asn
Asn (N)	Gln	Gln; His; Asp, Lys; Arg
Asp (D)	Glu	Glu; Asn
Cys (C)	Ser	Ser; Ala
Gln (Q)	Asn	Asn; Glu
Glu (E)	Asp	Asp; Gln
Gly (G)	Ala	Ala
His (H)	Arg	Asn; Gln; Lys; Arg
Ile (I)	Leu	Leu; Val; Met; Ala; Phe; Norleucine
Leu (L)	Ile	Norleucine; Ile; Val; Met; Ala; Phe
Lys (K)	Arg	Arg; Gln; Asn
Met (M)	Leu	Leu; Phe; Ile
Phe (F)	Tyr	Leu; Val; Ile; Ala; Tyr
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Ser	Ser
Trp (W)	Tyr	Tyr; Phe
Tyr (Y)	Phe	Trp; Phe; Thr; Ser
Val (V)	Leu	Ile; Leu; Met; Phe; Ala; Norleucine

Substantial modifications in the biological properties of the antibody are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the

molecule at the target site, or (c) the bulk of the side chain. Naturally occurring amino acid residues are divided into groups based on common side-chain properties:

(1) Non-polar: Norleucine, Met, Ala, Val, Leu, Ile;

(2) Polar without charge: Cys, Ser, Thr, Asn, Gln;

(3) Acidic (negatively charged): Asp, Glu;

(4) Basic (positively charged): Lys, Arg;

(5) Residues that influence chain orientation: Gly, Pro; and

(6) Aromatic: Trp, Tyr, Phe, His.

Non-conservative substitutions are made by exchanging a member of one of these classes for another class.

Any cysteine residue not involved in maintaining the proper conformation of the antibody also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant cross-linking. Conversely, cysteine bond(s) may be added to the antibody to improve its stability, particularly where the antibody is an antibody fragment such as an Fv fragment.

Amino acid modifications can range from changing or modifying one or more amino acids to complete redesign of a region, such as the variable region. Changes in the variable region can alter binding affinity and/or specificity. In some embodiments, no more than one to five conservative amino acid substitutions are made within a CDR domain. In other embodiments, no more than one to three conservative amino acid substitutions are made within a CDR domain. In still other embodiments, the CDR domain is CDR H3 and/or CDR L3.

Modifications also include glycosylated and nonglycosylated polypeptides, as well as polypeptides with other post-translational modifications, such as, for example, glycosylation with different sugars, acetylation, and phosphorylation. Antibodies are glycosylated at conserved positions in their constant regions (Jefferis and Lund, Chem. Immunol. 65:111-128, 1997; Wright and Morrison, TibTECH 15:26-32, 1997). The oligosaccharide side chains of the immunoglobulins affect the protein's function (Boyd et al., Mol. Immunol. 32:1311-1318, 1996; Wittwe and Howard, Biochem. 29:4175-4180, 1990) and the intramolecular interaction between portions of the glycoprotein, which can affect the conformation and presented three-dimensional surface of the glycoprotein (Jefferis and Lund, supra; Wyss and Wagner, Current Opin. Biotech. 7:409-416, 1996).

Oligosaccharides may also serve to target a given glycoprotein to certain molecules based upon specific recognition structures. Glycosylation of antibodies has also been reported to affect antibody-dependent cellular cytotoxicity (ADCC). In particular, CHO cells with tetracycline-regulated expression of β(1,4)-N-acetylglucosaminyltransferase III (GnTIII), a glycosyltransferase catalyzing formation of bisecting GlcNAc, was reported to have improved ADCC activity (Umana et al., Mature Biotech. 17:176-180, 1999).

Glycosylation of antibodies is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine, asparagine-X-threonine, and asparagine-X-cysteine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-acetylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the original antibody (for O-linked glycosylation sites).

The glycosylation pattern of antibodies may also be altered without altering the underlying nucleotide sequence. Glycosylation largely depends on the host cell used to express the antibody. Since the cell type used for expression of recombinant glycoproteins, e.g. antibodies, as potential therapeutics is rarely the native cell, variations in the glycosylation pattern of the antibodies can be expected (see, e.g. Hse et al., J. Biol. Chem. 272:9062-9070, 1997).

In addition to the choice of host cells, factors that affect glycosylation during recombinant production of antibodies include growth mode, media formulation, culture density, oxygenation, pH, purification schemes and the like. Various methods have been proposed to alter the glycosylation pattern achieved in a particular host organism including introducing or overexpressing certain enzymes involved in oligosaccharide

production (U.S. Pat. Nos. 5,047,335; 5,510,261 and 5,278,299). Glycosylation, or certain types of glycosylation, can be enzymatically removed from the glycoprotein, for example, using endoglycosidase H (Endo H), N-glycosidase F, endoglycosidase F1, endoglycosidase F2, endoglycosidase F3. In addition, the recombinant host cell can be genetically engineered to be defective in processing certain types of polysaccharides. These and similar techniques are well known in the art.

Other methods of modification include using coupling techniques known in the art, including, but not limited to, enzymatic means, oxidative substitution and chelation. Modifications can be used, for example, for attachment of labels for immunoassay. Modified polypeptides are made using established procedures in the art and can be screened using standard assays known in the art, some of which are described below and in the Examples.

In some embodiments of the invention, the antibody comprises a modified constant region, such as a constant region that has increased affinity to a human Fc gamma receptor, is immunologically inert or partially inert, e.g., does not trigger complement mediated lysis, does not stimulate antibody-dependent cell mediated cytotoxicity (ADCC), or does not activate macrophages; or has reduced activities (compared to the unmodified antibody) in any one or more of the following: triggering complement mediated lysis, stimulating antibody-dependent cell mediated cytotoxicity (ADCC), or activating microglia. Different modifications of the constant region may be used to achieve optimal level and/or combination of effector functions. See, for example, Morgan et al., Immunology 86:319-324, 1995; Lund et al., J. Immunology 157:4963-9 157:4963-4969, 1996; Idusogie et al., J. Immunology 164:4178-4184, 2000; Tao et al., J. Immunology 143: 2595-2601, 1989; and Jefferis et al., Immunological Reviews 163:59-76, 1998. In some embodiments, the constant region is modified as described in Eur. J. Immunol., 1999, 29:2613-2624; PCT Application No. PCT/GB99/01441; and/or UK Patent Application No. 9809951.8. In other embodiments, the antibody comprises a human heavy chain IgG2 constant region comprising the following mutations: A330P331 to S330S331 (amino acid numbering with reference to the wild type IgG2 sequence). Eur. J. Immunol., 1999, 29:2613-2624. In still other embodiments, the constant region is aglycosylated for N-linked glycosylation. In some embodiments, the constant region is aglycosylated for N-linked glycosylation by

mutating the glycosylated amino acid residue or flanking residues that are part of the N-glycosylation recognition sequence in the constant region. For example, N-glycosylation site N297 may be mutated to A, Q, K, or H. See, Tao et al., J. Immunology 143: 2595-2601, 1989; and Jefferis et al., Immunological Reviews 163:59-76, 1998. In some embodiments, the constant region is aglycosylated for N-linked glycosylation. The constant region may be aglycosylated for N-linked glycosylation enzymatically (such as removing carbohydrate by enzyme PNGase), or by expression in a glycosylation deficient host cell.

Other antibody modifications include antibodies that have been modified as described in PCT Publication No. WO 99/58572. These antibodies comprise, in addition to a binding domain directed at the target molecule, an effector domain having an amino acid sequence substantially homologous to all or part of a constant region of a human immunoglobulin heavy chain. These antibodies are capable of binding the target molecule without triggering significant complement dependent lysis, or cell-mediated destruction of the target. In some embodiments, the effector domain is capable of specifically binding FcRn and/or FcγRIIb. These are typically based on chimeric domains derived from two or more human immunoglobulin heavy chain C_H2 domains. Antibodies modified in this manner are particularly suitable for use in chronic antibody therapy, to avoid inflammatory and other adverse reactions to conventional antibody therapy.

The invention includes affinity matured embodiments. For example, affinity matured antibodies can be produced by procedures known in the art (Marks et al., Bio/Technology, 10:779-783, 1992; Barbas et al., Proc Nat. Acad. Sci, USA 91:3809-3813, 1994; Schier et al., Gene, 169:147-155, 1995; Yelton et al., J. Immunol., 155:1994-2004, 1995; Jackson et al., J. Immunol., 154(7):3310-9, 1995, Hawkins et al., J. Mol. Biol., 226:889-896, 1992; and PCT Publication No. WO2004/058184).

The following methods may be used for adjusting the affinity of an antibody and for characterizing a CDR. One way of characterizing a CDR of an antibody and/or altering (such as improving) the binding affinity of a polypeptide, such as an antibody, termed "library scanning mutagenesis". Generally, library scanning mutagenesis works as follows. One or more amino acid positions in the CDR are replaced with two or more (such as 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) amino acids

using art recognized methods. This generates small libraries of clones (in some embodiments, one for every amino acid position that is analyzed), each with a complexity of two or more members (if two or more amino acids are substituted at every position). Generally, the library also includes a clone comprising the native (unsubstituted) amino acid. A small number of clones, e.g., about 20-80 clones (depending on the complexity of the library), from each library are screened for binding affinity to the target polypeptide (or other binding target), and candidates with increased, the same, decreased, or no binding are identified. Methods for determining binding affinity are well-known in the art. Binding affinity may be determined using Biacore™ surface plasmon resonance analysis, which detects differences in binding affinity of about 2-fold or greater. Biacore™ is particularly useful when the starting antibody already binds with a relatively high affinity, for example a K_D of about 10 nM or lower. Screening using Biacore™ surface plasmon resonance is described in the Examples, herein.

Binding affinity may be determined using Kinexa Biocensor, scintillation proximity assays, ELISA, ORIGEN immunoassay (IGEN), fluorescence quenching, fluorescence transfer, and/or yeast display. Binding affinity may also be screened using a suitable bioassay.

In some embodiments, every amino acid position in a CDR is replaced (in some embodiments, one at a time) with all 20 natural amino acids using art recognized mutagenesis methods (some of which are described herein). This generates small libraries of clones (in some embodiments, one for every amino acid position that is analyzed), each with a complexity of 20 members (if all 20 amino acids are substituted at every position).

In some embodiments, the library to be screened comprises substitutions in two or more positions, which may be in the same CDR or in two or more CDRs. Thus, the library may comprise substitutions in two or more positions in one CDR. The library may comprise substitution in two or more positions in two or more CDRs. The library may comprise substitution in 3, 4, 5, or more positions, said positions found in two, three, four, five or six CDRs. The substitution may be prepared using low redundancy codons. See, e.g., Table 2 of Balint et al., Gene 137(1):109-18, 1993.

The CDR may be CDRH3 and/or CDRL3. The CDR may be one or more of CDRL1, CDRL2, CDRL3, CDRH1, CDRH2, and/or CDRH3. The CDR may be a Kabat CDR, a Chothia CDR, or an extended CDR.

Candidates with improved binding may be sequenced, thereby identifying a CDR substitution mutant which results in improved affinity (also termed an "improved" substitution). Candidates that bind may also be sequenced, thereby identifying a CDR substitution which retains binding.

Multiple rounds of screening may be conducted. For example, candidates (each comprising an amino acid substitution at one or more position of one or more CDR) with improved binding are also useful for the design of a second library containing at least the original and substituted amino acid at each improved CDR position (i.e., amino acid position in the CDR at which a substitution mutant showed improved binding). Preparation, and screening or selection of this library is discussed further below.

Library scanning mutagenesis also provides a means for characterizing a CDR, in so far as the frequency of clones with improved binding, the same binding, decreased binding or no binding also provide information relating to the importance of each amino acid position for the stability of the antibody-antigen complex. For example, if a position of the CDR retains binding when changed to all 20 amino acids, that position is identified as a position that is unlikely to be required for antigen binding. Conversely, if a position of CDR retains binding in only a small percentage of substitutions, that position is identified as a position that is important to CDR function. Thus, the library scanning mutagenesis methods generate information regarding positions in the CDRs that can be changed to many different amino acids (including all 20 amino acids), and positions in the CDRs which cannot be changed or which can only be changed to a few amino acids.

Candidates with improved affinity may be combined in a second library, which includes the improved amino acid, the original amino acid at that position, and may further include additional substitutions at that position, depending on the complexity of the library that is desired, or permitted using the desired screening or selection method. In addition, if desired, adjacent amino acid position can be randomized to at least two or more amino acids. Randomization of adjacent amino acids may permit additional conformational flexibility in the mutant CDR, which may in turn, permit or facilitate the

introduction of a larger number of improving mutations. The library may also comprise substitution at positions that did not show improved affinity in the first round of screening.

The second library is screened or selected for library members with improved and/or altered binding affinity using any method known in the art, including screening using Biacore™ surface plasmon resonance analysis, and selection using any method known in the art for selection, including phage display, yeast display, and ribosome display.

The invention also encompasses fusion proteins comprising one or more fragments or regions from the antibodies of this invention. In one embodiment, a fusion polypeptide is provided that comprises at least 10 contiguous amino acids of the variable light chain region shown in SEQ ID NOs: 1, 3, 6, 7, 8, 10, and 12 and/or at least 10 amino acids of the variable heavy chain region shown in SEQ ID NOs: 2, 4, 5, 9, 11, and 13. In other embodiments, a fusion polypeptide is provided that comprises at least about 10, at least about 15, at least about 20, at least about 25, or at least about 30 contiguous amino acids of the variable light chain region and/or at least about 10, at least about 15, at least about 20, at least about 25, or at least about 30 contiguous amino acids of the variable heavy chain region. In another embodiment, the fusion polypeptide comprises a light chain variable region and/or a heavy chain variable region, as shown in any of the sequence pairs selected from among SEQ ID NOs: 1 and 2, 3 and 4, 3 and 5, 6 and 5, 7 and 5, 8 and 9, 10 and 11, and 12 and 13. In another embodiment, the fusion polypeptide comprises one or more CDR(s). In still other embodiments, the fusion polypeptide comprises CDR H3 (VH CDR3) and/or CDR L3 (VL CDR3). For purposes of this invention, a fusion protein contains one or more antibodies and another amino acid sequence to which it is not attached in the native molecule, for example, a heterologous sequence or a homologous sequence from another region. Exemplary heterologous sequences include, but are not limited to a "tag" such as a FLAG tag or a 6His tag. Tags are well known in the art.

A fusion polypeptide can be created by methods known in the art, for example, synthetically or recombinantly. Typically, the fusion proteins of this invention are made by preparing an expressing a polynucleotide encoding them using recombinant methods

described herein, although they may also be prepared by other means known in the art, including, for example, chemical synthesis.

This invention also provides compositions comprising antibodies conjugated (for example, linked) to an agent that facilitate coupling to a solid support (such as biotin or avidin). For simplicity, reference will be made generally to antibodies with the understanding that these methods apply to any of the Trop-2 antibody embodiments described herein. Conjugation generally refers to linking these components as described herein. The linking (which is generally fixing these components in proximate association at least for administration) can be achieved in any number of ways. For example, a direct reaction between an agent and an antibody is possible when each possesses a substituent capable of reacting with the other. For example, a nucleophilic group, such as an amino or sulfhydryl group, on one may be capable of reacting with a carbonyl-containing group, such as an anhydride or an acid halide, or with an alkyl group containing a good leaving group (e.g., a halide) on the other.

The invention also provides isolated polynucleotides encoding the antibodies of the invention, and vectors and host cells comprising the polynucleotide.

Accordingly, the invention provides polynucleotides (or compositions, including pharmaceutical compositions), comprising polynucleotides encoding any of the following: the m7E6, h7E6, h7E6_SVG, h7E6_SVG1, h7E6_SVG2, h7E6_SVG3, h7E6_SVG4, h7E6_SVG5, h7E6_SVG6, h7E6_SVG7, h7E6_SVG8, h7E6_SVG9, h7E6_SVG10, h7E6_SVG11, h7E6_SVG12, h7E6_SVG13, h7E6_SVG14, h7E6_SVG15, h7E6_SVG16, h7E6_SVG17, h7E6_SVG18, h7E6_SVG19, h7E6_SVG20, h7E6_SVG21, h7E6_SVG22, h7E6_SVG23, h7E6_SVG24, h7E6_SVG25, h7E6_SVG26, h7E6_SVG27, h7E6_SVG28, h7E6_SVG29, h7E6_SVG30, h7E6_SVG31, h7E6_SVG32, h7E6_SVGL, h7E6_SVGL1, h7E6_SVGL2, h7E6_SVGL3, h7E6_SVGL4, h7E6_SVGL5, h7E6_SVGN, m6G11, h6G11, h6G11_FKG_SF, or any fragment or part thereof having the ability to bind Trop-2.

In another aspect, the invention provides polynucleotides encoding any of the antibodies (including antibody fragments) and polypeptides described herein, such as antibodies and polypeptides having impaired effector function. Polynucleotides can be made and expressed by procedures known in the art.

In another aspect, the invention provides compositions (such as a pharmaceutical compositions) comprising any of the polynucleotides of the invention. In some embodiments, the composition comprises an expression vector comprising a polynucleotide encoding any of the antibodies described herein. In still other embodiments, the composition comprises either or both of the polynucleotides shown in SEQ ID NO: 15 and SEQ ID NO: 14 below:

m7E6 heavy chain variable region

CAGGTCCAACTGCAGGAATCAGGTCCAGGCCTGGTGAAACCGTCTGAAACCCTGA
GCCTGACATGCACCGTGAGCGGTGGTAGTATTAGCTCTTACGGCGTCCATTGGAT
CCGTCAACCGCCTGGTAAAGGTCTGGAATGGATTGGCGTGATCTGGACCGGTGGT
AGCACCGACTATAACAGCGCACTGATGAGCCGCGTGACCATCTCGGTAGACACGT
CGAAAAACCAGTTCAGCCTGAAACTGAGCAGCGTGACCGCCGCGGATACCGCTGT
TTATTACTGCGCACGCGACGGGGATTATGATCGCTACACCATGGATTATTGGGGCC
AGGGTACCCTGGTCACCGTCTCCTCA (SEQ ID NO:15)

m7E6 light chain variable region

GACATTGTGCTGACACAGTCTCCTGCTTCCTTAGCTGTATCTCTGGGGCAGAGGGC
CACCATCTCATGCAGGGCCAGCAAAAGTGTCAGTACATCTGGCTATAGTTATATGC
ACTGGTACCAACAGAAACCAGGACAGCCACCCAAACTCCTCATCTATCTTGCATCC
AACCTAGAATCTGGGGTCCCTGCCAGGTTCAGTGGCAGTGGGTCTGGGACAGACT
TCACCCTCAACATCCATCCTGTGGAGGAGGAGGATGCTGCAACCTATTACTGTCAG
CACAGTAGGGAGCTTCCGTACACGTTCGGAGGGGGGACCAAGCTGGAGATCAAA
(SEQ ID NO:14)

In other embodiments, the composition comprises either or both of the polynucleotides shown in SEQ ID NO: 17 and SEQ ID NO: 16 below:

h7E6 heavy chain variable region

CAGGTCCAACTGCAGGAATCAGGTCCAGGCCTGGTGAAACCGTCTGAAACCCTGA
GCCTGACATGCACCGTGAGCGGTGGTAGTATTAGCTCTTACGGCGTCCATTGGAT
CCGTCAACCGCCTGGTAAAGGTCTGGAATGGATTGGCGTGATCTGGACCGGTGGT
AGCACCGACTATAACAGCGCACTGATGAGCCGCGTGACCATCTCGGTAGACACGT
CGAAAAACCAGTTCAGCCTGAAACTGAGCAGCGTGACCGCCGCGGATACCGCTGT
TTATTACTGCGCACGCGACGGGGATTATGATCGCTACACCATGGATTATTGGGGCC
AGGGTACCCTGGTCACCGTCTCCTCA (SEQ ID NO:17)

h7E6 light chain variable region

GATATCGTAATGACCCAATCTCCGGATTCGCTGGCGGTATCACTGGGCGAACGTG
CCACGATTAACTGCCGTGCAAGCAAATCAGTGTCGACCTCCGGCTACAGCTATATG
CACTGGTATCAACAGAAACCGGGCCAGCCGCCGAAACTGCTGATCTATCTGGCTA
GCAACCTGGAGAGCGGTGTGCCTGATCGCTTTAGTGGCTCCGGTAGCGGTACCGA
TTTCACGCTGACCATCAGCTCCCTGCAGGCAGAAGACGTGGCCGTGTATTATTGTC
AGCACAGCCGTGAGCTGCCGTATACTTTTGGCCAGGGGACAAAACTGGAAATCAA
A (SEQ ID NO:16)

In still other embodiments, the composition comprises either or both of the polynucleotides shown in SEQ ID NO: 18 and SEQ ID NO: 16 below:

h7E6_SVG heavy chain variable region

CAGGTCCAACTGCAGGAATCAGGTCCAGGCCTGGTGAAACCGTCTGAAACCCTGA
GCCTGACATGCACCGTGAGCGGTGGTAGTATTAGCTCTTACGCGTCCATTGGATCC
GTCAACCGCCTGGTAAAGGTCTGGAATGGATTGGCGTGATCTGGACCAGTGGTGT
GACCGACTATAACAGCGCACTGATGGGCCGCGTGACCATCTCGGTAGACACGTCG
AAAAACCAGTTCAGCCTGAAACTGAGCAGCGTGACCGCCGCGGATACCGCTGTTT
ATTACTGCGCACGCGACGGGGATTATGATCGCTACACCATGGATTATTGGGGCCA
GGGTACCCTGGTCACCGTCTCCTCA (SEQ ID NO:18)

h7E6_SVG light chain variable region

GATATCGTAATGACCCAATCTCCGGATTCGCTGGCGGTATCACTGGGCGAACGTG
CCACGATTAACTGCCGTGCAAGCAAATCAGTGTCGACCTCCGGCTACAGCTATATG
CACTGGTATCAACAGAAACCGGGCCAGCCGCCGAAACTGCTGATCTATCTGGCTA
GCAACCTGGAGAGCGGTGTGCCTGATCGCTTTAGTGGCTCCGGTAGCGGTACCGA
TTTCACGCTGACCATCAGCTCCCTGCAGGCAGAAGACGTGGCCGTGTATTATTGTC
AGCACAGCCGTGAGCTGCCGTATACTTTTGGCCAGGGGACAAAACTGGAAATCAA
A (SEQ ID NO:16)

In other embodiments, the composition comprises either or both of the polynucleotides shown in SEQ ID NO: 18 and SEQ ID NO: 19 below:

h7E6_SVGL heavy chain variable region

CAGGTCCAACTGCAGGAATCAGGTCCAGGCCTGGTGAAACCGTCTGAAACCCTGA
GCCTGACATGCACCGTGAGCGGTGGTAGTATTAGCTCTTACGCGTCCATTGGATCC
GTCAACCGCCTGGTAAAGGTCTGGAATGGATTGGCGTGATCTGGACCAGTGGTGT

GACCGACTATAACAGCGCACTGATGGGCCGCGTGACCATCTCGGTAGACACGTCG
AAAAACCAGTTCAGCCTGAAACTGAGCAGCGTGACCGCCGCGGATACCGCTGTTT
ATTACTGCGCACGCGACGGGGATTATGATCGCTACACCATGGATTATTGGGGCCA
GGGTACCCTGGTCACCGTCTCCTCA (SEQ ID NO:18)

h7E6_SVGL light chain variable region

GATATCGTAATGACCCAATCTCCGGATTCGCTGGCGGTATCACTGGGCGAACGTG
CCACGATTAACTGCCGTGCAAGCAAATCAGTGTCGACCTCCTTGTACAGCTATATG
CACTGGTATCAACAGAAACCGGGCCAGCCGCCGAAACTGCTGATCTATCTGGCTA
GCAACCTGGAGAGCGGTGTGCCTGATCGCTTTAGTGGCTCCGGTAGCGGTACCGA
TTTCACGCTGACCATCAGCTCCCTGCAGGCAGAAGACGTGGCCGTGTATTATTGTC
AGCACAGCCGTGAGCTGCCGTATACTTTTGGCCAGGGGACAAAACTGGAAATCAA
A (SEQ ID NO:19) (SEQ ID NO: 19).

In still other embodiments, the composition comprises either or both of the polynucleotides shown in SEQ ID NO: 18 and SEQ ID NO: 20 below:

h7E6_SVGN heavy chain variable region

CAGGTCCAACTGCAGGAATCAGGTCCAGGCCTGGTGAAACCGTCTGAAACCCTGA
GCCTGACATGCACCGTGAGCGGTGGTAGTATTAGCTCTTACGCGTCCATTGGATCC
GTCAACCGCCTGGTAAAGGTCTGGAATGGATTGGCGTGATCTGGACCAGTGGTGT
GACCGACTATAACAGCGCACTGATGGGCCGCGTGACCATCTCGGTAGACACGTCG
AAAAACCAGTTCAGCCTGAAACTGAGCAGCGTGACCGCCGCGGATACCGCTGTTT
ATTACTGCGCACGCGACGGGGATTATGATCGCTACACCATGGATTATTGGGGCCA
GGGTACCCTGGTCACCGTCTCCTCA (SEQ ID NO: 18)

h7E6_SVGN light chain variable region

GATATCGTAATGACCCAATCTCCGGATTCGCTGGCGGTATCACTGGGCGAACGTG
CCACGATTAACTGCCGTGCAAGCAAATCAGTGTCGACCTCCAATTACAGCTATATG
CACTGGTATCAACAGAAACCGGGCCAGCCGCCGAAACTGCTGATCTATCTGGCTA
GCAACCTGGAGAGCGGTGTGCCTGATCGCTTTAGTGGCTCCGGTAGCGGTACCGA
TTTCACGCTGACCATCAGCTCCCTGCAGGCAGAAGACGTGGCCGTGTATTATTGTC
AGCACAGCCGTGAGCTGCCGTATACTTTTGGCCAGGGGACAAAACTGGAAATCAA
A (SEQ ID NO: 20)

In other embodiments, the composition comprises either or both of the polynucleotides shown in SEQ ID NO: 25 and SEQ ID NO: 20 below:

h6G11_FKG_SF heavy chain variable region

CAGGTGCAGTTGGTTCAGAGCGGCGCGGAAGTCAAGAAACCCGGCGCCTCCGTG
AAAGTGAGCTGCAAAGCGAGCGGCTACACCTTCACCAGTTATTGGATTAACTGGGT
GCGCCAGGCCCCAGGCCAGGGGCTGGAGTGGATGGGAAACATCTTCCCATCTGA
CTCTTACAGCAACTATAATAAGAAATTTAAGGATCGCGTAACAATGACCCGTGACAC
CAGCACCAGCACTGTTTACATGGAGCTGAGTTCTCTGCGTTCTGAAGATACCGCCG
TGTACTACTGCGCACGCGGTTCCGGGTTCGATTACTGGGGCCAGGGGACCCTGGT
CACCGTCTCCTCA (SEQ ID NO:25)

h6G11_FKG_SF light chain variable region

GAGATCGTGCTGACCCAAAGTCCAGCCACCCTTTCCCTGTCTCCAGGCGAACGCG
CAACCCTGAGCTGCCGCGCTTCTCAGACCATTGGTACCTCCATTCATTGGTATCAG
CAGAAGCCCGGCCAAGCCCCGCGTCTGCTGATCTATTACGCCTCAGAAAGTATTTC
AGGCATCCCCGCTCGCTTCTCCGGCTCCGGCAGCGGAACCGACTTCACACTTACA
ATCTCTAGTTTGGAGCCAGAAGACTTCGCCGTTTACTACTGTTCGCAGTCTTTTAGC
TGGCCATTTACCTTTGGCCAGGGCACGAAGCTGGAAATCAAG (SEQ ID NO:26)

In other embodiments, the composition comprises either or both of the polynucleotides shown in SEQ ID NO: 22 and SEQ ID NO: 21 below:

m6G11 heavy chain variable region

CAGGTCCAACTGCAGCAGCCTGGGGCTGAGCTGGTGAGGCCTGGGGCTTCAGTG
AAGCTGTCCTGCAAGGCTTCTGGCTACACCTTCACCAGCTACTGGATAAACTGGGT
GAAGCAGAGGCCTGGACATGGCCTTGAGTGGATCGGAAATATTTATCCTTCTGATA
GTTATTCTAACTACAATCAAAAGTTCAAGGACAAGGCCACATTGACTGTAGACAAAT
CCTCCAGCACAGCCTACATGCAGGTCAGCAGCCCGACATCTGAGGACTCTGCGGT
CTATTACTGTACGTACGGTAGTAGCTTTGACTACTGGGGCCAAGGCACCACGGTCA
CCGTCTCCTCA (SEQ ID NO:22)

m6G11 light chain variable region

GACATCTTGCTGACTCAGTCTCCAGCCATCCTGTCTGTGAGTCCAGGAGAAAGAGT
CAGTTTCTCCTGCAGGGCCAGTCAGACCATTGGCACAAGCATACACTGGTATCAGC
AAAGAACAAATGGTTCTCCAAGGCTTCTCATAAAGTATGCTTCTGAGTCTATCTCTG
GGATCCCTTCCAGGTTTAGTGGCAGTGGATCAGGGACAGATTTTACTCTTAGCATC
AACAGTGTGGAGTCTGAAGATATTGCAGATTATTACTGTCAACAAAGTAATAGCTGG
CCATTCACGTTCGGCTCGGGGACCAAGCTGGAAATAAAA (SEQ ID NO:21)

In other embodiments, the composition comprises either or both of the polynucleotides shown in SEQ ID NO: 24 and SEQ ID NO: 23 below:

h6G11 heavy chain variable region

CAGGTGCAGTTGGTTCAGAGCGGCGCGGAAGTCAAGAAACCCGGCGCCTCCGTG
AAAGTGAGCTGCAAAGCGAGCGGCTACACCTTCACCAGTTATTGGATTAACTGGGT
GCGCCAGGCCCCAGGCCAGGGGCTGGAGTGGATGGGAAACATCTACCCATCTGA
CTCTTACAGCAACTATAATCAGAAATTTAAGGATCGCGTAACAATGACCCGTGACAC
CAGCACCAGCACTGTTTACATGGAGCTGAGTTCTCTGCGTTCTGAAGATACCGCCG
TGTACTACTGCGCACGCGGTTCCAGTTTCGATTACTGGGGCCAGGGGACCCTGGT
CACCGTCTCCTCA (SEQ ID NO: 24)

h6G11 light chain variable region

GAGATCGTGCTGACCCAAAGTCCAGCCACCCTTTCCCTGTCTCCAGGCGAACGCG
CAACCCTGAGCTGCCGCGCTTCTCAGACCATTGGTACCTCCATTCATTGGTATCAG
CAGAAGCCCGGCCAAGCCCCGCGTCTGCTGATCTATTACGCCTCAGAAAGTATTTC
AGGCATCCCCGCTCGCTTCTCCGGCTCCGGCAGCGGAACCGACTTCACACTTACA
ATCTCTAGTTTGGAGCCAGAAGACTTCGCCGTTTACTACTGTCAGCAGTCTAACAG
CTGGCCATTTACCTTTGGCCAGGGCACGAAGCTGGAAATCAAG (SEQ ID NO: 23)

Expression vectors, and administration of polynucleotide compositions are further described herein.

In another aspect, the invention provides a method of making any of the polynucleotides described herein.

Polynucleotides complementary to any such sequences are also encompassed by the present invention. Polynucleotides may be single-stranded (coding or antisense) or double-stranded, and may be DNA (genomic, cDNA or synthetic) or RNA molecules. RNA molecules include HnRNA molecules, which contain introns and correspond to a DNA molecule in a one-to-one manner, and mRNA molecules, which do not contain introns. Additional coding or non-coding sequences may, but need not, be present within a polynucleotide of the present invention, and a polynucleotide may, but need not, be linked to other molecules and/or support materials.

Polynucleotides may comprise a native sequence (i.e., an endogenous sequence that encodes an antibody or a portion thereof) or may comprise a variant of such a sequence. Polynucleotide variants contain one or more substitutions, additions,

deletions and/or insertions such that the immunoreactivity of the encoded polypeptide is not diminished, relative to a native immunoreactive molecule. The effect on the immunoreactivity of the encoded polypeptide may generally be assessed as described herein. Variants preferably exhibit at least about 70% identity, more preferably, at least about 80% identity, yet more preferably, at least about 90% identity, and most preferably, at least about 95% identity to a polynucleotide sequence that encodes a native antibody or a portion thereof.

Two polynucleotide or polypeptide sequences are said to be "identical" if the sequence of nucleotides or amino acids in the two sequences is the same when aligned for maximum correspondence as described below. Comparisons between two sequences are typically performed by comparing the sequences over a comparison window to identify and compare local regions of sequence similarity. A "comparison window" as used herein, refers to a segment of at least about 20 contiguous positions, usually 30 to about 75, or 40 to about 50, in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned.

Optimal alignment of sequences for comparison may be conducted using the Megalign program in the Lasergene suite of bioinformatics software (DNASTAR, Inc., Madison, WI), using default parameters. This program embodies several alignment schemes described in the following references: Dayhoff, M.O., 1978, A model of evolutionary change in proteins - Matrices for detecting distant relationships. In Dayhoff, M.O. (ed.) Atlas of Protein Sequence and Structure, National Biomedical Research Foundation, Washington DC Vol. 5, Suppl. 3, pp. 345-358; Hein J., 1990, Unified Approach to Alignment and Phylogenes pp. 626-645 Methods in Enzymology vol. 183, Academic Press, Inc., San Diego, CA; Higgins, D.G. and Sharp, P.M., 1989, CABIOS 5:151-153; Myers, E.W. and Muller W., 1988, CABIOS 4:11-17; Robinson, E.D., 1971, Comb. Theor. 11:105; Santou, N., Nes, M., 1987, Mol. Biol. Evol. 4:406-425; Sneath, P.H.A. and Sokal, R.R., 1973, Numerical Taxonomy the Principles and Practice of Numerical Taxonomy, Freeman Press, San Francisco, CA; Wilbur, W.J. and Lipman, D.J., 1983, Proc. Natl. Acad. Sci. USA 80:726-730.

Preferably, the "percentage of sequence identity" is determined by comparing two optimally aligned sequences over a window of comparison of at least 20 positions,

wherein the portion of the polynucleotide or polypeptide sequence in the comparison window may comprise additions or deletions (i.e., gaps) of 20 percent or less, usually 5 to 15 percent, or 10 to 12 percent, as compared to the reference sequences (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid bases or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the reference sequence (i.e. the window size) and multiplying the results by 100 to yield the percentage of sequence identity.

Variants may also, or alternatively, be substantially homologous to a native gene, or a portion or complement thereof. Such polynucleotide variants are capable of hybridizing under moderately stringent conditions to a naturally occurring DNA sequence encoding a native antibody (or a complementary sequence).

Suitable “moderately stringent conditions” include prewashing in a solution of 5 X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0); hybridizing at 50°C-65°C, 5 X SSC, overnight; followed by washing twice at 65°C for 20 minutes with each of 2X, 0.5X and 0.2X SSC containing 0.1 % SDS.

As used herein, "highly stringent conditions" or "high stringency conditions" are those that: (1) employ low ionic strength and high temperature for washing, for example 0.015 M sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50°C; (2) employ during hybridization a denaturing agent, such as formamide, for example, 50% (v/v) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42°C; or (3) employ 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt’s solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC (sodium chloride/sodium citrate) and 50% formamide at 55°C, followed by a high-stringency wash consisting of 0.1 x SSC containing EDTA at 55°C. The skilled artisan will recognize how to adjust the temperature, ionic strength, etc. as necessary to accommodate factors such as probe length and the like.

It will be appreciated by those of ordinary skill in the art that, as a result of the degeneracy of the genetic code, there are many nucleotide sequences that encode a polypeptide as described herein. Some of these polynucleotides bear minimal homology to the nucleotide sequence of any native gene. Nonetheless, polynucleotides that vary due to differences in codon usage are specifically contemplated by the present invention. Further, alleles of the genes comprising the polynucleotide sequences provided herein are within the scope of the present invention. Alleles are endogenous genes that are altered as a result of one or more mutations, such as deletions, additions and/or substitutions of nucleotides. The resulting mRNA and protein may, but need not, have an altered structure or function. Alleles may be identified using standard techniques (such as hybridization, amplification and/or database sequence comparison).

The polynucleotides of this invention can be obtained using chemical synthesis, recombinant methods, or PCR. Methods of chemical polynucleotide synthesis are well known in the art and need not be described in detail herein. One of skill in the art can use the sequences provided herein and a commercial DNA synthesizer to produce a desired DNA sequence.

For preparing polynucleotides using recombinant methods, a polynucleotide comprising a desired sequence can be inserted into a suitable vector, and the vector in turn can be introduced into a suitable host cell for replication and amplification, as further discussed herein. Polynucleotides may be inserted into host cells by any means known in the art. Cells are transformed by introducing an exogenous polynucleotide by direct uptake, endocytosis, transfection, F-mating or electroporation. Once introduced, the exogenous polynucleotide can be maintained within the cell as a non-integrated vector (such as a plasmid) or integrated into the host cell genome. The polynucleotide so amplified can be isolated from the host cell by methods well known within the art. See, e.g., Sambrook et al., 1989.

Alternatively, PCR allows reproduction of DNA sequences. PCR technology is well known in the art and is described in U.S. Patent Nos. 4,683,195, 4,800,159, 4,754,065 and 4,683,202, as well as PCR: The Polymerase Chain Reaction, Mullis et al. eds., Birkauswer Press, Boston, 1994.

RNA can be obtained by using the isolated DNA in an appropriate vector and inserting it into a suitable host cell. When the cell replicates and the DNA is transcribed

into RNA, the RNA can then be isolated using methods well known to those of skill in the art, as set forth in Sambrook et al., 1989, supra, for example.

Suitable cloning vectors may be constructed according to standard techniques, or may be selected from a large number of cloning vectors available in the art. While the cloning vector selected may vary according to the host cell intended to be used, useful cloning vectors will generally have the ability to self-replicate, may possess a single target for a particular restriction endonuclease, and/or may carry genes for a marker that can be used in selecting clones containing the vector. Suitable examples include plasmids and bacterial viruses, e.g., pUC18, pUC19, Bluescript (e.g., pBS SK+) and its derivatives, mp18, mp19, pBR322, pMB9, ColE1, pCR1, RP4, phage DNAs, and shuttle vectors such as pSA3 and pAT28. These and many other cloning vectors are available from commercial vendors such as BioRad, Strategene, and Invitrogen.

Expression vectors generally are replicable polynucleotide constructs that contain a polynucleotide according to the invention. It is implied that an expression vector must be replicable in the host cells either as episomes or as an integral part of the chromosomal DNA. Suitable expression vectors include but are not limited to plasmids, viral vectors, including adenoviruses, adeno-associated viruses, retroviruses, cosmids, and expression vector(s) disclosed in PCT Publication No. WO 87/04462. Vector components may generally include, but are not limited to, one or more of the following: a signal sequence; an origin of replication; one or more marker genes; suitable transcriptional controlling elements (such as promoters, enhancers and terminator). For expression (i.e., translation), one or more translational controlling elements are also usually required, such as ribosome binding sites, translation initiation sites, and stop codons.

The vectors containing the polynucleotides of interest can be introduced into the host cell by any of a number of appropriate means, including electroporation, transfection employing calcium chloride, rubidium chloride, calcium phosphate, DEAE-dextran, or other substances; microprojectile bombardment; lipofection; and infection (e.g., where the vector is an infectious agent such as vaccinia virus). The choice of introducing vectors or polynucleotides will often depend on features of the host cell.

The invention also provides host cells comprising any of the polynucleotides described herein. Any host cells capable of over-expressing heterologous DNAs can be

used for the purpose of isolating the genes encoding the antibody, polypeptide or protein of interest. Non-limiting examples of mammalian host cells include but not limited to COS, HeLa, and CHO cells. See also PCT Publication No. WO 87/04462. Suitable non-mammalian host cells include prokaryotes (such as *E. coli* or *B. subtillis*) and yeast (such as *S. cerevisae*, *S. pombe*; or *K. lactis*). Preferably, the host cells express the cDNAs at a level of about 5 fold higher, more preferably, 10 fold higher, even more preferably, 20 fold higher than that of the corresponding endogenous antibody or protein of interest, if present, in the host cells. Screening the host cells for a specific binding to Trop-2 or an Trop-2 domain (e.g., domains 1-4) is effected by an immunoassay or FACS. A cell overexpressing the antibody or protein of interest can be identified.

Trop-2 Antibody Conjugates

The present invention also provides a conjugate (or immunoconjugate) of the Trop-2 antibody as described herein, or of the antigen binding fragment thereof, wherein the antibody or the antigen binding fragment is conjugated to an agent (e.g., a cytotoxic agent) for targeted immunotherapy (e.g., antibody-drug conjugates) either directly or indirectly via a linker. For example, a cytotoxic agent can be linked or conjugated to the Trop-2 antibody or the antigen binding fragment thereof as described herein for targeted local delivery of the cytotoxic agent moiety to tumors (e.g., Trop-2 expressing tumor).

Methods for conjugating cytotoxic agent or other therapeutic agents to antibodies have been described in various publications. For example, chemical modification can be made in the antibodies either through lysine side chain amines or through cysteine sulfhydryl groups activated by reducing interchain disulfide bonds for the conjugation reaction to occur. See, e.g., Tanaka et al., FEBS Letters 579:2092-2096, 2005, and Gentle et al., Bioconjugate Chem. 15:658-663, 2004. Reactive cysteine residues engineered at specific sites of antibodies for specific drug conjugation with defined stoichiometry have also been described. See, e.g., Junutula et al., Nature Biotechnology, 26:925-932, 2008. Conjugation using an acyl donor glutamine-containing tag or an endogenous glutamine made reactive (i.e., the ability to form a covalent bond as an acyl donor) by polypeptide engineering in the presence of transglutaminase and an amine (e.g., a cytotoxic agent comprising or attached to a

reactive amine) is also described in International Patent Application Serial No. PCT/IB2011/054899 (WO2012/059882).

In some embodiments, the Trop-2 antibody or the conjugate as described herein comprises an acyl donor glutamine-containing tag engineered at a specific site of the antibody (e.g., a carboxyl terminus, an amino terminus, or at another site in the Trop-2 antibody). In some embodiments, the tag comprises an amino acid glutamine (Q) or an amino acid sequence GGLLQGG (SEQ ID NO:78), LLQGA (SEQ ID NO:79), GGLLQGA (SEQ ID NO:81), LLQ, LLQGPGK (SEQ ID NO: 90), LLQGPG (SEQ ID NO: 91), LLQGPA (SEQ ID NO: 92), LLQGP (SEQ ID NO: 93), LLQP (SEQ ID NO: 94), LLQPGK (SEQ ID NO: 95), LLQGAPGK (SEQ ID NO: 96), LLQGAPG (SEQ ID NO: 97), LLQGAP (SEQ ID NO: 98), $LLQX_1X_2X_3X_4X_5$, wherein X_1 is G or P, wherein X_2 is A, G, P, or absent, wherein X_3 is A, G, K, P, or absent, wherein X_4 is K, G or absent, and wherein X_5 is K or absent (SEQ ID NO: 88), or $LLQX_1X_2X_3X_4X_5$, wherein X_1 is any naturally occurring amino acid and wherein X_2, X_3, X_4, and X_5 are any naturally occurring amino acids or absent (SEQ ID NO: 89). In some embodiments, the Trop-2 antibody or the conjugate as described herein comprises an acyl donor glutamine-containing tag engineered at a specific site of the antibody, wherein the tag comprises an amino acid sequence GGLLQGG (SEQ ID NO:78) engineered at the light chain carboxyl terminus of the Trop-2 antibody. In some embodiments, the Trop-2 antibody or the conjugate as described herein comprises an acyl donor glutamine-containing tag engineered at a specific site of the antibody, wherein the tag comprises an amino acid sequence GGLLQGA (SEQ ID NO:81) engineered at the light chain carboxyl terminus of the Trop-2 antibody. In other embodiments, the Trop-2 antibody or the conjugate as described herein comprises an acyl donor glutamine-containing tag engineered at a specific site of the antibody, wherein the tag comprises an amino acid sequence LLQGA (SEQ ID NO:79) engineered at the heavy chain carboxyl terminus of the Trop-2 antibody and wherein the lysine residue at the heavy chain carboxyl terminus is deleted. In other embodiments, the Trop-2 antibody or the conjugate as described herein comprises an acyl donor glutamine-containing tag engineered at a specific site of the antibody, wherein the tag comprises an amino acid sequence LLQ engineered at the heavy chain carboxyl terminus of the Trop-2 antibody and wherein the lysine residue at the heavy chain carboxyl terminus is deleted. In some embodiments, the Trop-2 antibody or the

conjugate as described herein comprises an amino acid substitution from asparagine (N) to glutamine (Q) at position 297 of the Trop-2 antibody.

Also provided is an isolated antibody comprising an acyl donor glutamine-containing tag and an amino acid modification at position 222, 340, or 370 of the antibody (Kabat numbering scheme), wherein the modification is an amino acid deletion, insertion, substitution, mutation, or any combination thereof. Accordingly, in some embodiments, provided is the Trop-2 antibody or the conjugate as described herein comprising the acyl donor glutamine-containing tag (e.g., Q, GGLLQGG (SEQ ID NO:78), LLQGA (SEQ ID NO:79), GGLLQGA (SEQ ID NO:81), LLQ, LLQGPGK (SEQ ID NO: 90), LLQGPG (SEQ ID NO: 91), LLQGPA (SEQ ID NO: 92), LLQGP (SEQ ID NO: 93), LLQP (SEQ ID NO: 94), LLQPGK (SEQ ID NO: 95), LLQGAPGK (SEQ ID NO: 96), LLQGAPG (SEQ ID NO: 97), LLQGAP (SEQ ID NO: 98), $LLQX_1X_2X_3X_4X_5$, wherein X_1 is G or P, wherein X_2 is A, G, P, or absent, wherein X_3 is A, G, K, P, or absent, wherein X_4 is K, G or absent, and wherein X_5 is K or absent (SEQ ID NO: 88), or $LLQX_1X_2X_3X_4X_5$, wherein X_1 is any naturally occurring amino acid and wherein X_2, X_3, X_4, and X_5 are any naturally occurring amino acids or absent (SEQ ID NO: 89) conjugated at a specific site (e.g., at a carboxyl terminus of the heavy or light chain or at another site) of the Trop-2 antibody and an amino acid modification at position 222, 340, or 370 of the antibody (Kabat numbering scheme). In some embodiments, the amino acid modification is a substitution from lysine to arginine (e.g., K222R, K340R, or K370R). In some embodiments, the Trop-2 antibody or the conjugate as described herein comprises an acyl donor glutamine-containing tag comprising the sequence GGLLQGG (SEQ ID NO:78) engineered at the C-terminus of the Trop-2 antibody light chain and an amino acid substitution from lysine to arginine at position 222 of the antibody (Kabat numbering scheme). In some embodiments, the Trop-2 antibody or the conjugate as described herein comprises an acyl donor glutamine-containing tag comprising the sequence GGLLQGA (SEQ ID NO:81) engineered at the C-terminus of the Trop-2 antibody light chain and an amino acid substitution from lysine to arginine at position 222 of the antibody (Kabat numbering scheme). In some embodiments, the Trop-2 antibody or the conjugate as described herein comprises an acyl donor glutamine-containing tag comprising the sequence LLQGA (SEQ ID NO:79) engineered at the C-terminus of the Trop-2 antibody heavy chain and an amino acid substitution

from lysine to arginine at position 222 of the antibody (Kabat numbering scheme), wherein the lysine residue at the heavy chain carboxyl terminus is deleted. In some embodiments, the Trop-2 antibody or the conjugate as described herein comprises an acyl donor glutamine-containing tag comprising the sequence LLQ engineered at the C-terminus of the Trop-2 antibody heavy chain and an amino acid substitution from lysine to arginine at position 222 of the antibody (Kabat numbering scheme), wherein the lysine residue at the heavy chain carboxyl terminus is deleted. In some embodiments, the Trop-2 antibody or the conjugate as described herein comprises an acyl donor glutamine-containing tag comprising a glutamine engineered at position 297 of the Trop-2 antibody and an amino acid substitution from lysine to arginine at position 222 of the antibody (Kabat numbering scheme).

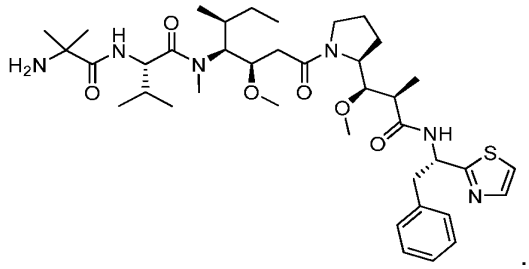
The agents that can be conjugated to the Trop-2 antibodies or the antigen binding fragments of the present invention include, but are not limited to, cytotoxic agents, immunomodulating agents, imaging agents, therapeutic proteins, biopolymers, or oligonucleotides.

Examples of a cytotoxic agent include, but are not limited to, an anthracycline, an auristatin, a dolastatin, CC-1065, a duocarmycin, an enediyne, a geldanamycin, a maytansine, a puromycin, a taxane, a vinca alkaloid, SN-38, tubulysin, hemiasterlin, and stereoisomers, isosteres, analogs or derivatives thereof.

The anthracyclines are derived from bacteria *Strepomyces* and have been used to treat a wide range of cancers, such as leukemias, lymphomas, breast, uterine, ovarian, and lung cancers. Exemplary anthracyclines include, but are not limited to, daunorubicin, doxorubicin (i.e., adriamycin), epirubicin, idarubicin, valrubicin, and mitoxantrone.

Dolastatins and their peptidic analogs and derivatives, auristatins, are highly potent antimitotic agents that have been shown to have anticancer and antifungal activity. See, e.g., U.S. Pat. No. 5,663,149 and Pettit et al., *Antimicrob. Agents Chemother.* 42:2961-2965, 1998. Exemplary dolastatins and auristatins include, but are not limited to, dolastatin 10, auristatin E, auristatin EB (AEB), auristatin EFP (AEFP), MMAD (Monomethyl Auristatin D or monomethyl dolastatin 10), MMAF (Monomethyl Auristatin F or N-methylvaline-valine-dolaisoleuine-dolaproine-phenylalanine), MMAE (Monomethyl Auristatin E or N-methylvaline-valine-dolaisoleuine-dolaproine-

norephedrine}, 5-benzoylvaleric acid-AE ester (AEVB), and other novel auristatins (such as the ones described in U.S. Application No. 61/561,255 and 61/676,423). In some embodiments, the auristatin is 0101 (2-methylalanyl-*N*-[(3*R*,4*S*,5*S*)-3-methoxy-1-{(2*S*)-2-[(1*R*,2*R*)-1-methoxy-2-methyl-3-oxo-3-{[(1*S*)-2-phenyl-1-(1,3-thiazol-2-yl)ethyl]amino}propyl]pyrrolidin-1-yl}-5-methyl-1-oxoheptan-4-yl]-*N*-methyl-L-valinamide) having the following structure:



Duocarmycin and CC-1065 are DNA alkylating agents with cytotoxic potency. See Boger and Johnson, *PNAS* 92:3642-3649, 1995. Exemplary dolastatins and auristatins include, but are not limited to, (+)-docarmycin A and (+)-duocarmycin SA, and (+)-CC-1065.

Enediynes are a class of anti-tumor bacterial products characterized by either nine- and ten-membered rings or the presence of a cyclic system of conjugated triple-double-triple bonds. Exemplary enediynes include, but are not limited to, calicheamicin, esperamicin, and dynemicin.

Geldanamycins are benzoquinone ansamycin antibiotic that bind to Hsp90 (Heat Shock Protein 90) and have been used antitumor drugs. Exemplary geldanamycins include, but are not limited to, 17-AAG (17-N-Allylamino-17-Demethoxygeldanamycin) and 17-DMAG (17-Dimethylaminoethylamino-17-demethoxygeldanamycin).

Maytansines or their derivatives maytansinoids inhibit cell proliferation by inhibiting the microtubules formation during mitosis through inhibition of polymerization of tubulin. See Remillard et al., Science 189:1002-1005, 1975. Exemplary maytansines and maytansinoids include, but are not limited to, mertansine (DM1) and its derivatives as well as ansamitocin.

Taxanes are diterpenes that act as anti-tubulin agents or mitotic inhibitors. Exemplary taxanes include, but are not limited to, paclitaxel (e.g., TAXOL®) and docetaxel (TAXOTERE®).

Vinca alkyloids are also anti-tubulin agents. Exemplary vinca alkyloids include, but are not limited to, vincristine, vinblastine, vindesine, and vinorelbine.

In some embodiments, the agent is an immunomodulating agent. Examples of an immunomodulating agent include, but are not limited to, gancyclovier, etanercept, tacrolimus, sirolimus, voclosporin, cyclosporine, rapamycin, cyclophosphamide, azathioprine, mycophenolgate mofetil, methotrextrate, glucocorticoid and its analogs, cytokines, stem cell growth factors, lymphotoxins, tumor necrosis factor (TNF), hematopoietic factors, interleukins (e.g., interleukin-1 (IL-1), IL-2, IL-3, IL-6, IL-10, IL-12, IL-18, and IL-21), colony stimulating factors (e.g., granulocyte-colony stimulating factor (G-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF)), interferons (e.g., interferons-α, -β and -γ), the stem cell growth factor designated "S 1 factor," erythropoietin and thrombopoietin, or a combination thereof.

In some embodiments, the agent is an imaging agent (e.g., a fluorophore or a PET (Positron Emission Tomography) label, SPECT (Single-Photon Emission Computed Tomorgraphy) label), or MRI (Magnetic Resonance Imaging) label.

Examples of fluorophores include, but are not limited to, fluorescein isothiocyanate (FITC) (e.g., 5-FITC), fluorescein amidite (FAM) (e.g., 5-FAM), eosin, carboxyfluorescein, erythrosine, Alexa Fluor® (e.g., Alexa 350, 405, 430, 488, 500, 514, 532, 546, 555, 568, 594, 610, 633, 647, 660, 680, 700, or 750), carboxytetramethylrhodamine (TAMRA) (e.g., 5,-TAMRA), tetramethylrhodamine (TMR), and sulforhodamine (SR) (e.g., SR101).

In some embodiments, therapeutic or diagnostic radioisotopes or other labels (e.g., PET or SPECT labels) can be incorporated in the agent for conjugation to the Trop-2 antibodies or the antigen binding fragments as described herein. Examples of a radioisotope or other labels include, but are not limited to, ^{3}H, ^{11}C, ^{13}N, ^{14}C, ^{15}N, ^{15}O, ^{35}S, ^{18}F, ^{32}P, ^{33}P, ^{47}Sc, ^{51}Cr, ^{57}Co, ^{58}Co, ^{59}Fe, ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{67}Ga, ^{68}Ga, ^{75}Se, ^{76}Br, ^{77}Br, ^{86}Y, ^{89}Zr, ^{90}Y, ^{94}Tc, ^{95}Ru, ^{97}Ru, ^{99}Tc, ^{103}Ru, ^{105}Rh, ^{105}Ru, ^{107}Hg, ^{109}Pd, ^{111}Ag, ^{111}In, ^{113}In, ^{121}Te, ^{122}Te, ^{123}I, ^{124}I, ^{125}I, ^{125}Te, ^{126}I, ^{131}I, ^{131}In, ^{133}I, ^{142}Pr, ^{143}Pr, ^{153}Pb, ^{153}Sm, ^{161}Tb, ^{165}Tm, ^{166}Dy, ^{166}H, ^{167}Tm, ^{168}Tm, ^{169}Yb, ^{177}Lu, ^{186}Re, ^{188}Re, ^{189}Re, ^{197}Pt, ^{198}Au, ^{199}Au, ^{201}Tl, ^{203}Hg, ^{211}At, ^{212}Bi, ^{212}Pb, ^{213}Bi, ^{223}Ra, ^{224}Ac, and ^{225}Ac.

In some embodiments, the agent is a therapeutic protein including, but is not limited to, a toxin, a hormone, an enzyme, and a growth factor.

Examples of a toxin protein (or polypeptide) include, but are not limited to, dipththeria (e.g., diphtheria A chain), *Pseudomonas* exotoxin and endotoxin, ricin (e.g., ricin A chain), abrin (e.g., abrin A chain), modeccin (e.g., modeccin A chain), alpha-sarcin, Aleurites fordii proteins, dianthin proteins, ribonuclease (RNase), DNase I, *Staphylococcal* enterotoxin-A, pokeweed antiviral protein, gelonin, diphtherin toxin, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, mitogellin, restrictocin, phenomycin, enomycin, tricothecenes, inhibitor cystine knot (ICK) peptides (e.g., ceratotoxins), and conotoxin (e.g., KIIIA or SmIIIa).

In some embodiments, the agent is a biocompatible polymer. The Trop-2 antibodies or the antigen binding fragments as described herein can be conjugated to the biocompatible polymer to increase serum half-life and bioactivity, and/or to extend *in vivo* half-lives. Examples of biocompatible polymers include water-soluble polymer, such as polyethylene glycol (PEG) or its derivatives thereof and zwitterion-containing biocompatible polymers (e.g., a phosphorylcholine containing polymer).

In some embodiments, the agent is an oligonucleotide, such as anti-sense oligonucleotides.

In another aspect, the invention provides a conjugate of the antibody or the antigen binding fragment as described herein, wherein the conjugate comprises the formula: antibody-(acyl donor glutamine-containing tag)-(linker)-(cytotoxic agent), wherein the acyl donor glutamine-containing tag is engineered at a specific site of the antibody or the antigen binding fragment (e.g., at a carboxyl terminus of the heavy or light chain or at an another site), wherein the tag is conjugated to a linker (e.g., a linker containing one or more reactive amines (e.g., primary amine NH_2)), and wherein the linker is conjugated to a cytotoxic agent (e.g., MMAD or other auristatins such as 0101).

Examples of a linker containing one or more reactive amines include, but are not limited to, acetyl-lysine-valine-citrulline-*p*-aminobenzyloxycarbonyl (AcLys-VC-PABC) or amino PEG6-propionyl. See, e.g. WO2012/059882.

In some embodiments, the acyl donor glutamine-containing tag comprises GGLLQGG (SEQ ID NO:78), LLQGA (SEQ ID NO:79), GGLLQGA (SEQ ID NO:81), LLQ, LLQGPGK (SEQ ID NO: 90), LLQGPG (SEQ ID NO: 91), LLQGPA (SEQ ID NO: 92), LLQGP (SEQ ID NO: 93), LLQP (SEQ ID NO: 94), LLQPGK (SEQ ID NO: 95),

LLQGAPGK (SEQ ID NO: 96), LLQGAPG (SEQ ID NO: 97), LLQGAP (SEQ ID NO: 98), $LLQX_1X_2X_3X_4X_5$, wherein X_1 is G or P, wherein X_2 is A, G, P, or absent, wherein X_3 is A, G, K, P, or absent, wherein X_4 is K, G or absent, and wherein X_5 is K or absent (SEQ ID NO: 88), or $LLQX_1X_2X_3X_4X_5$, wherein X_1 is any naturally occurring amino acid and wherein X_2, X_3, X_4, and X_5 are any naturally occurring amino acids or absent (SEQ ID NO: 89).

In some embodiments, the conjugate is 1) antibody-LLQGA (SEQ ID NO: 79)-AcLys-VC-PABC-0101; 2) antibody-LLQGA (SEQ ID NO: 79)-AcLys-VC-PABC-MMAD; 3) antibody-$LLQX_1X_2X_3X_4X_5$ (SEQ ID NO: 88)-AcLys-VC-PABC-0101; 4) antibody-$LLQX_1X_2X_3X_4X_5$ (SEQ ID NO: 88)-AcLys-VC-PABC-MMAD; 5) antibody–GGLLQGG (SEQ ID NO: 78)-AcLys-VC-PABC-0101; and 6) antibody–GGLLQGG (SEQ ID NO: 78)-AcLys-VC-PABC-MMAD. In some embodiments, the acyl donor glutamine-containing tag comprising, e.g., LLQ, SEQ ID NO: 79, 90, 91, 92, 93, 94, 95, 96, 97, or 98, is engineered at the C-terminus of the heavy chain of the antibody, wherein the lysine residue at the C-terminus is deleted. In other embodiments, the acyl donor glutamine-containing tag (e.g., GGLLQGG (SEQ ID NO: 78)) is engineered at the C-terminus of the light chain of the antibody. Examples of the antibody include, but are not limited to, h7E6_SVG, h7E6_SVG1, h7E6_SVG2, h7E6_SVG3, and h7E6_SVG4, h7E6_SVG5, h7E6_SVG6, h7E6_SVG7, h7E6_SVG8, h7E6_SVG9, h7E6_SVG10, h7E6_SVG11, h7E6_SVG12, h7E6_SVG13, h7E6_SVG14, h7E6_SVG15, h7E6_SVG16, h7E6_SVG17, h7E6_SVG18, h7E6_SVG19, h7E6_SVG20, h7E6_SVG21, h7E6_SVG22, h7E6_SVG23, h7E6_SVG24, h7E6_SVG25, h7E6_SVG26, h7E6_SVG27, h7E6_SVG28, h7E6_SVG29, h7E6_SVG30, h7E6_SVG31, h7E6_SVG32, h7E6_SVGL, h7E6_SVGL1, h7E6_SVGL2, h7E6_SVGL3, h7E6_SVGL4, h7E6_SVGL5, h7E6SVGN, h6G11, or h6G11_FKG_SF.

In one variation, the conjugate further comprises an amino acid substitution from lysine to arginine at position 222. Accordingly, for example, the conjugate is 1) antibody–GGLLQGG (SEQ ID NO: 78)-AcLys-VC-PABC-MMAD and comprises K222R; 2) antibody–GGLLQGG (SEQ ID NO: 78)-AcLys-VC-PABC-0101 and comprises K222R; 3) antibody-LLQGA (SEQ ID NO: 79)-AcLys-VC-PABC-0101 and comprises K222R; 4) antibody-LLQGA (SEQ ID NO: 79)-AcLys-VC-PABC-MMAD and comprises K222R; and 5) antibody-$LLQX_1X_2X_3X_4X_5$ (SEQ ID NO: 88)-AcLys-VC-PABC-MMAD and comprises

K222R. In some embodiments, the acyl donor glutamine-containing tag comprising, e.g., LLQ, SEQ ID NO: 79, 90, 91, 92, 93, 94, 95, 96, 97, or 98, is engineered at the C-terminus of the heavy chain of the antibody, wherein the lysine residue at the C-terminus is deleted. In other embodiments, the acyl donor glutamine-containing tag (e.g., GGLLQGG (SEQ ID NO: 78)) is engineered at the C-terminus of the light chain of the antibody. Examples of the antibody include, but are not limited to, h7E6_SVG, h7E6_SVG1, h7E6_SVG2, h7E6_SVG3, and h7E6_SVG4, h7E6_SVG5, h7E6_SVG6, h7E6_SVG7, h7E6_SVG8, h7E6_SVG9, h7E6_SVG10, h7E6_SVG11, h7E6_SVG12, h7E6_SVG13, h7E6_SVG14, h7E6_SVG15, h7E6_SVG16, h7E6_SVG17, h7E6_SVG18, h7E6_SVG19, h7E6_SVG20, h7E6_SVG21, h7E6_SVG22, h7E6_SVG23, h7E6_SVG24, h7E6_SVG25, h7E6_SVG26, h7E6_SVG27, h7E6_SVG28, h7E6_SVG29, h7E6_SVG30, h7E6_SVG31, h7E6_SVG32, h7E6_SVGL, h7E6_SVGL1, h7E6_SVGL2, h7E6_SVGL3, h7E6_SVGL4, h7E6_SVGL5, h6G11, or h6G11_FKG_SF.

In another aspect, the invention provides a conjugate of the antibody or the antigen binding fragment as described herein, the conjugate comprises amino acid substitutions at positions N297Q and K222R, a linker comprising amino-PEG6-propionyl, and a cytotoxic agent (e.g., MMAD or other auristatins). For example, the conjugate is h7E6_SVG, h7E6_SVG1, h7E6_SVG2, h7E6_SVG3, h7E6_SVG4, h7E6_SVG5, h7E6_SVG6, h7E6_SVG7, h7E6_SVG8, h7E6_SVG9, h7E6_SVG10, h7E6_SVG11, h7E6_SVG12, h7E6_SVG13, h7E6_SVG14, h7E6_SVG15, h7E6_SVG16, h7E6_SVG17, h7E6_SVG18, h7E6_SVG19, h7E6_SVG20, h7E6_SVG21, h7E6_SVG22, h7E6_SVG23, h7E6_SVG24, h7E6_SVG25, h7E6_SVG26, h7E6_SVG27, h7E6_SVG28, h7E6_SVG29, h7E6_SVG30, h7E6_SVG31, h7E6_SVG32, h7E6_SVGL, h7E6_SVGL1, h7E6_SVGL2, h7E6_SVGL3, h7E6_SVGL4, h7E6_SVGL5, h6G11, or h6G11_FKG_SF conjugating to amino-PEG6-propionyl and MMAD or h7E6_SVG, h7E6_SVG1, h7E6_SVG2, h7E6_SVG3, h7E6_SVG4, h7E6_SVG5, h7E6_SVG6, h7E6_SVG7, h7E6_SVG8, h7E6_SVG9, h7E6_SVG10, h7E6_SVG11, h7E6_SVG12, h7E6_SVG13, h7E6_SVG14, h7E6_SVG15, h7E6_SVG16, h7E6_SVG17, h7E6_SVG18, h7E6_SVG19, h7E6_SVG20, h7E6_SVG21, h7E6_SVG22, h7E6_SVG23, h7E6_SVG24, h7E6_SVG25, h7E6_SVG26, h7E6_SVG27, h7E6_SVG28,

h7E6_SVG29, h7E6_SVG30, h7E6_SVG31, h7E6_SVG32, h7E6_SVGL, h7E6_SVGL1, h7E6_SVGL2, h7E6_SVGL3, h7E6_SVGL4, h7E6_SVGL5, h6G11, or h6G11_FKG_SF conjugating to amino-PEG6-propionyl and 0101.

Methods of Using the Trop-2 Antibodies and the Antibody Conjugates Thereof

The antibodies and the antibody conjugates of the present invention are useful in various applications including, but are not limited to, therapeutic treatment methods and diagnostic treatment methods.

In one aspect, the invention provides a method for treating a condition associated with Trop-2 expression in a subject. In some embodiments, the method of treating a condition associated with Trop-2 expression in a subject comprises administering to the subject in need thereof an effective amount of a composition (e.g., pharmaceutical composition) comprising the Trop-2 antibodies or the Trop-2 antibody conjugates as described herein. The conditions associated with Trop-2 expression include, but are not limited to, abnormal Trop-2 expression, altered or aberrant Trop-2 expression, Trop-2 overexpression, and a proliferative disorder (e.g., cancer).

Accordingly, in some embodiments, provided is a method of treating a cancer in a subject comprising administering to the subject in need thereof an effective amount of a composition comprising the Trop-2 antibodies or the Trop-2 antibody conjugates as described herein. As used herein, cancers include, but are not limited to bladder, breast, cervical, choriocarcinoma, colon, esophageal, gastric, glioblastoma, head and neck, kidney, lung, oral, ovarian, pancreatic, prostate, and skin cancer. In some embodiments, provided is a method of inhibiting tumor growth or progression in a subject who has a Trop-2 expressing tumor, comprising administering to the subject in need thereof an effective amount of a composition comprising the Trop-2 antibodies or the Trop-2 antibody conjugates as described herein. In other embodiments, provided is a method of inhibiting metastasis of Trop-2 expressing cancer cells in a subject, comprising administering to the subject in need thereof an effective amount of a composition comprising the Trop-2 antibodies or the Trop-2 antibody conjugates as described herein. In other embodiments, provided is a method of inducing regression of a Trop-2 expressing tumor regression in a subject, comprising administering to the

subject in need thereof an effective amount of a composition comprising the Trop-2 antibodies or the Trop-2 antibody conjugates as described herein.

In another aspect, provided is a method of detecting, diagnosing, and/or monitoring a condition associated with Trop-2 expression. For example, the Trop-2 antibodies as described herein can be labeled with a detectable moiety such as an imaging agent and an enzyme-substrate label. The antibodies as described herein can also be used for *in vivo* diagnostic assays, such as *in vivo* imaging (e.g., PET or SPECT), or a staining reagent.

In some embodiments, the methods described herein further comprise a step of treating a subject with an additional form of therapy. In some embodiments, the additional form of therapy is an additional anti-cancer therapy including, but not limited to, chemotherapy, radiation, surgery, hormone therapy, and/or additional immunotherapy.

In some embodiments, the additional form of therapy comprises administering one or more therapeutic agent in addition to the Trop-2 antibodies or the Trop-2 antibody conjugates as described herein. The therapeutic agents include, but are not limited to, a second antibody (e.g., an anti-VEGF antibody, an anti-HER2 antibody, anti-CD25 antibody, and/or an anti-CD20 antibody), an angiogenesis inhibitor, a cytotoxic agent, an anti-inflammatory agent (e.g., paclitaxel, docetaxel, cisplatin, doxorubicin, prednisone, mitomycin, progesterone, tamoxifen, or fluorouracil)

The Trop-2 antibody or the Trop-2 antibody conjugates can be administered to an individual via any suitable route. It should be understood by persons skilled in the art that the examples described herein are not intended to be limiting but to be illustrative of the techniques available. Accordingly, in some embodiments, the Trop-2 antibody or the Trop-2 antibody conjugate is administered to an individual in accord with known methods, such as intravenous administration, e.g., as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerebrospinal, intracranial, transdermal, subcutaneous, intra-articular, sublingually, intrasynovial, via insufflation, intrathecal, oral, inhalation or topical routes. Administration can be systemic, e.g., intravenous administration, or localized. Commercially available nebulizers for liquid formulations, including jet nebulizers and ultrasonic nebulizers are useful for administration. Liquid formulations can be directly nebulized and lyophilized powder can

be nebulized after reconstitution. Alternatively, the Trop-2 antibody or the Trop-2 antibody conjugate can be aerosolized using a fluorocarbon formulation and a metered dose inhaler, or inhaled as a lyophilized and milled powder.

In one embodiment, the Trop-2 antibody or the Trop-2 antibody conjugate is administered via site-specific or targeted local delivery techniques. Examples of site-specific or targeted local delivery techniques include various implantable depot sources of the Trop antibody or the Trop-2 antibody conjugate or local delivery catheters, such as infusion catheters, indwelling catheters, or needle catheters, synthetic grafts, adventitial wraps, shunts and stents or other implantable devices, site specific carriers, direct injection, or direct application. See, e.g., PCT Publication No. WO 00/53211 and U.S. Pat. No. 5,981,568.

Various formulations of the Trop-2 antibody or the Trop-2 antibody conjugate may be used for administration. In some embodiments, the Trop-2 antibody or the Trop-2 antibody conjugate may be administered neat. In some embodiments, of the Trop-2 antibody (or the Trop-2 antibody conjugate) and a pharmaceutically acceptable excipient may be in various formulations. Pharmaceutically acceptable excipients are known in the art, and are relatively inert substances that facilitate administration of a pharmacologically effective substance. For example, an excipient can give form or consistency, or act as a diluent. Suitable excipients include but are not limited to stabilizing agents, wetting and emulsifying agents, salts for varying osmolarity, encapsulating agents, buffers, and skin penetration enhancers. Excipients as well as formulations for parenteral and nonparenteral drug delivery are set forth in Remington, The Science and Practice of Pharmacy 20th Ed. Mack Publishing, 2000.

In some embodiments, these agents are formulated for administration by injection (e.g., intraperitoneally, intravenously, subcutaneously, intramuscularly, etc.). Accordingly, these agents can be combined with pharmaceutically acceptable vehicles such as saline, Ringer's solution, dextrose solution, and the like. The particular dosage regimen, i.e., dose, timing and repetition, will depend on the particular individual and that individual's medical history.

Trop-2 antibodies or the Trop-2 antibody conjugates as described herein can be administered using any suitable method, including by injection (e.g., intraperitoneally, intravenously, subcutaneously, intramuscularly, etc.). The Trop-2 antibody or the Trop-2

antibody conjugate can also be administered via inhalation, as described herein. Generally, for administration of a Trop-2 antibody and a Trop-2 antibody conjugate, an initial candidate dosage can be about 2 mg/kg. For the purpose of the present invention, a typical daily dosage might range from about any of 3 µg/kg to 30 µg/kg to 300 µg/kg to 3 mg/kg, to 30 mg/kg, to 100 mg/kg or more, depending on the factors mentioned above. For example, dosage of about 1 mg/kg, about 2.5 mg/kg, about 5 mg/kg, about 10 mg/kg, and about 25 mg/kg may be used. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of symptoms occurs or until sufficient therapeutic levels are achieved, for example, to inhibit or delay tumor growth/progression or metatstasis of cancer cells. An exemplary dosing regimen comprises administering an initial dose of about 2 mg/kg, followed by a weekly maintenance dose of about 1 mg/kg of the Trop-2 antibody or Trop-2 antibody conjugate, or followed by a maintenance dose of about 1 mg/kg every other week. Other exemplary dosing regimen comprises administering increasing doses (e.g., initial dose of 1 mg/kg and gradual increase to one or more higher doses every week or longer time period). Other dosage regimens may also be useful, depending on the pattern of pharmacokinetic decay that the practitioner wishes to achieve. For example, in some embodiments, dosing from one to four times a week is contemplated. In other embodiments dosing once a month or once every other month or every three months is contemplated. The progress of this therapy is easily monitored by conventional techniques and assays. The dosing regimen (including the Trop-2 antibody or the Trop-2 antibody conjugate used) can vary over time.

For the purpose of the present invention, the appropriate dosage of a Trop-2 antibody or a Trop-2 antibody conjugate will depend on the Trop-2 antibody or the Trop-2 antibody conjugate (or compositions thereof) employed, the type and severity of symptoms to be treated, whether the agent is administered for therapeutic purposes, previous therapy, the patient's clinical history and response to the agent, the patient's clearance rate for the administered agent, and the discretion of the attending physician. Typically the clinician will administer a Trop-2 antibody or a Trop-2 antibody conjugate until a dosage is reached that achieves the desired result. Dose and/or frequency can vary over course of treatment. Empirical considerations, such as the half-life, generally will contribute to the determination of the dosage. For example, antibodies that are

compatible with the human immune system, such as humanized antibodies or fully human antibodies, may be used to prolong half-life of the antibody and to prevent the antibody being attacked by the host's immune system. Frequency of administration may be determined and adjusted over the course of therapy, and is generally, but not necessarily, based on treatment and/or suppression and/or amelioration and/or delay of symptoms, e.g., tumor growth inhibition or delay, etc. Alternatively, sustained continuous release formulations of Trop-2 antibodies or Trop-2 antibody conjugates may be appropriate. Various formulations and devices for achieving sustained release are known in the art.

In one embodiment, dosages for a Trop-2 antibody or a trop-2 antibody conjugate may be determined empirically in individuals who have been given one or more administration(s) of the Trop-2 antibody or its Trop-2 antibody conjugate. Individuals are given incremental dosages of a Trop-2 antibody or a Trop-2 antagonist. To assess efficacy, an indicator of the disease can be followed.

Administration of an Trop-2 antibody or an Trop-2 antibody conjugate in accordance with the method in the present invention can be continuous or intermittent, depending, for example, upon the recipient's physiological condition, whether the purpose of the administration is therapeutic or prophylactic, and other factors known to skilled practitioners. The administration of a Trop-2 antibody or a Trop-2 antibody conjugate may be essentially continuous over a preselected period of time or may be in a series of spaced doses.

In some embodiments, more than one Trop-2 antibody or Trop-2 antibody conjugate may be present. At least one, at least two, at least three, at least four, at least five different or more Trop-2 antibody or Trop-2 antibody conjugate can be present. Generally, those Trop-2 antibodies or Trop-2 antibody conjugates may have complementary activities that do not adversely affect each other. For example, one or more of the following Trop-2 antibody may be used: a first Trop-2 antibody directed to one epitope on Trop-2 and a second Trop-2 antibody directed to a different epitope on Trop-2.

Therapeutic formulations of the Trop-2 antibody or the Trop-2 antibody conjugate used in accordance with the present invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable

carriers, excipients or stabilizers (Remington, The Science and Practice of Pharmacy 21st Ed. Mack Publishing, 2005), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and may comprise buffers such as phosphate, citrate, and other organic acids; salts such as sodium chloride; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens, such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

Liposomes containing the Trop-2 antibody or the Trop-2 antibody conjugate are prepared by methods known in the art, such as described in Epstein, et al., Proc. Natl. Acad. Sci. USA 82:3688, 1985; Hwang, et al., Proc. Natl Acad. Sci. USA 77:4030, 1980; and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Pat. No. 5,013,556. Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in

macroemulsions. Such techniques are disclosed in Remington, The Science and Practice of Pharmacy 21st Ed. Mack Publishing, 2005.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or 'poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and 7 ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT ™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), sucrose acetate isobutyrate, and poly-D-(-)-3-hydroxybutyric acid.

The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by, for example, filtration through sterile filtration membranes. Therapeutic Trop-2 antibody or Trop-2 antibody conjugate compositions are generally 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.

The compositions according to the present invention may be in unit dosage forms such as tablets, pills, capsules, powders, granules, solutions or suspensions, or suppositories, for oral, parenteral or rectal administration, or administration by inhalation or insufflation.

For preparing solid compositions such as tablets, the principal active ingredient is mixed with a pharmaceutical carrier, e.g. conventional tableting ingredients such as corn starch, lactose, sucrose, sorbitol, talc, stearic acid, magnesium stearate, dicalcium phosphate or gums, and other pharmaceutical diluents, e.g. water, to form a solid preformulation composition containing a homogeneous mixture of a compound of the present invention, or a non-toxic pharmaceutically acceptable salt thereof. When referring to these preformulation compositions as homogeneous, it is meant that the active ingredient is dispersed evenly throughout the composition so that the composition may be readily subdivided into equally effective unit dosage forms such as tablets, pills and capsules. This solid preformulation composition is then subdivided into unit dosage forms of the type described above containing from 0.1 to about 500 mg of the active

ingredient of the present invention. The tablets or pills of the novel composition can be coated or otherwise compounded to provide a dosage form affording the advantage of prolonged action. For example, the tablet or pill can comprise an inner dosage and an outer dosage component, the latter being in the form of an envelope over the former. The two components can be separated by an enteric layer that serves to resist disintegration in the stomach and permits the inner component to pass intact into the duodenum or to be delayed in release. A variety of materials can be used for such enteric layers or coatings, such materials including a number of polymeric acids and mixtures of polymeric acids with such materials as shellac, cetyl alcohol and cellulose acetate.

Suitable surface-active agents include, in particular, non-ionic agents, such as polyoxyethylenesorbitans (e.g. Tween™ 20, 40, 60, 80 or 85) and other sorbitans (e.g. Span™ 20, 40, 60, 80 or 85). Compositions with a surface-active agent will conveniently comprise between 0.05 and 5% surface-active agent, and can be between 0.1 and 2.5%. It will be appreciated that other ingredients may be added, for example mannitol or other pharmaceutically acceptable vehicles, if necessary.

Suitable emulsions may be prepared using commercially available fat emulsions, such as Intralipid™, Liposyn™, Infonutrol™, Lipofundin™ and Lipiphysan™. The active ingredient may be either dissolved in a pre-mixed emulsion composition or alternatively it may be dissolved in an oil (e.g. soybean oil, safflower oil, cottonseed oil, sesame oil, corn oil or almond oil) and an emulsion formed upon mixing with a phospholipid (e.g. egg phospholipids, soybean phospholipids or soybean lecithin) and water. It will be appreciated that other ingredients may be added, for example glycerol or glucose, to adjust the tonicity of the emulsion. Suitable emulsions will typically contain up to 20% oil, for example, between 5 and 20%. The fat emulsion can comprise fat droplets between 0.1 and 1.0 μm, particularly 0.1 and 0.5 μm, and have a pH in the range of 5.5 to 8.0.

The emulsion compositions can be those prepared by mixing a Trop-2 antibody or a Trop-2 antibody conjugate with Intralipid™ or the components thereof (soybean oil, egg phospholipids, glycerol and water).

Compositions for inhalation or insufflation include solutions and suspensions in pharmaceutically acceptable, aqueous or organic solvents, or mixtures thereof, and

powders. The liquid or solid compositions may contain suitable pharmaceutically acceptable excipients as set out above. In some embodiments, the compositions are administered by the oral or nasal respiratory route for local or systemic effect. Compositions in preferably sterile pharmaceutically acceptable solvents may be nebulised by use of gases. Nebulised solutions may be breathed directly from the nebulising device or the nebulising device may be attached to a face mask, tent or intermittent positive pressure breathing machine. Solution, suspension or powder compositions may be administered, preferably orally or nasally, from devices which deliver the formulation in an appropriate manner.

Compositions

The compositions used in the methods of the invention comprise an effective amount of a Trop-2 antibody or a Trop-2 antibody conjugate as described herein. Examples of such compositions, as well as how to formulate, are also described in an earlier section and below. In some embodiments, the composition comprises one or more Trop-2 antibodies or Trop-2 antibody conjugates. For example, Trop-2 antibody recognizes human Trop-2. In some embodiments, the Trop-2 antibody is a human antibody, a humanized antibody, or a chimeric antibody. In some embodiments, the Trop-2 antibody comprises a constant region that is capable of triggering a desired immune response, such as antibody-mediated lysis or ADCC. In other embodiments, the Trop-2 antibody comprises a constant region that does not trigger an unwanted or undesirable immune response, such as antibody-mediated lysis or ADCC. In other embodiments, the Trop-2 antibody comprises one or more CDR(s) of the antibody (such as one, two, three, four, five, or, in some embodiments, all six CDRs).

It is understood that the compositions can comprise more than one Trop-2 antibody or Trop-2 antibody conjugate (e.g., a mixture of Trop-2 antibodies that recognize different epitopes of Trop-2). Other exemplary compositions comprise more than one Trop-2 antibody or Trop-2 antibody conjugate that recognize the same epitope(s), or different species of Trop-2 antibodies or Trop-2 antibody conjugate that bind to different epitopes of Trop-2 (e.g., human Trop-2).

The composition used in the present invention can further comprise pharmaceutically acceptable carriers, excipients, or stabilizers (Remington: The

Science and practice of Pharmacy 21st Ed., 2005, Lippincott Williams and Wilkins, Ed. K. E. Hoover), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations, and may comprise buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG). Pharmaceutically acceptable excipients are further described herein.

Kits

The invention also provides kits for use in the instant methods. Kits of the invention include one or more containers comprising the Trop-2 antibody or the Trop-2 antibody conjugate as described herein and instructions for use in accordance with any of the methods of the invention described herein. Generally, these instructions comprise a description of administration of the Trop-2 antibody or the Trop-2 antibody conjugate for the above described therapeutic treatments.

The instructions relating to the use of the Trop-2 antibodies or the Trop-2 antibody conjugates as described herein generally include information as to dosage, dosing schedule, and route of administration for the intended treatment. The containers may be unit doses, bulk packages (e.g., multi-dose packages) or sub-unit doses. Instructions supplied in the kits of the invention are typically written instructions on a label or package insert (e.g., a paper sheet included in the kit), but machine-readable

instructions (e.g., instructions carried on a magnetic or optical storage disk) are also acceptable.

The kits of this invention are in suitable packaging. Suitable packaging includes, but is not limited to, vials, bottles, jars, flexible packaging (e.g., sealed Mylar or plastic bags), and the like. Also contemplated are packages for use in combination with a specific device, such as an inhaler, nasal administration device (e.g., an atomizer) or an infusion device such as a minipump. A kit may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). The container may also have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is a Trop-2 antibody. The container may further comprise a second pharmaceutically active agent.

Kits may optionally provide additional components such as buffers and interpretive information. Normally, the kit comprises a container and a label or package insert(s) on or associated with the container.

Mutations and Modifications

To express the Trop-2 antibodies of the present invention, DNA fragments encoding VH and VL regions can first be obtained using any of the methods described above. Various modifications, e.g. mutations, substitutions, deletions, and/or additions can also be introduced into the DNA sequences using standard methods known to those of skill in the art. For example, mutagenesis can be carried out using standard methods, such as PCR-mediated mutagenesis, in which the mutated nucleotides are incorporated into the PCR primers such that the PCR product contains the desired mutations or site-directed mutagenesis.

One type of substitution, for example, that may be made is to change one or more cysteines in the antibody, which may be chemically reactive, to another residue, such as, without limitation, alanine or serine. For example, there can be a substitution of a non-canonical cysteine. The substitution can be made in a CDR or framework region of a variable domain or in the constant region of an antibody. In some embodiments, the cysteine is canonical.

The antibodies may also be modified, e.g. in the variable domains of the heavy and/or light chains, e.g., to alter a binding property of the antibody. For example, a mutation may be made in one or more of the CDR regions to increase or decrease the K_D of the antibody for Trop-2, to increase or decrease k_{off}, or to alter the binding specificity of the antibody. Techniques in site-directed mutagenesis are well-known in the art. See, e.g., Sambrook et al. and Ausubel et al., supra.

A modification or mutation may also be made in a framework region or constant region to increase the half-life of an Trop-2 antibody. See, e.g., PCT Publication No. WO 00/09560. A mutation in a framework region or constant region can also be made to alter the immunogenicity of the antibody, to provide a site for covalent or non-covalent binding to another molecule, or to alter such properties as complement fixation, FcR binding and antibody-dependent cell-mediated cytotoxicity. According to the invention, a single antibody may have mutations in any one or more of the CDRs or framework regions of the variable domain or in the constant region.

In a process known as “germlining”, certain amino acids in the VH and VL sequences can be mutated to match those found naturally in germline VH and V_L sequences. In particular, the amino acid sequences of the framework regions in the V_H and VL sequences can be mutated to match the germline sequences to reduce the risk of immunogenicity when the antibody is administered. Germline DNA sequences for human VH and VL genes are known in the art (see e.g., the "Vbase" human germline sequence database; see also Kabat, E. A., et al., 1991, Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242; Tomlinson et al., J. Mol. Biol. 227:776-798, 1992; and Cox et al., Eur. J. Immunol. 24:827-836, 1994.

Another type of amino acid substitution that may be made is to remove potential proteolytic sites in the antibody. Such sites may occur in a CDR or framework region of a variable domain or in the constant region of an antibody. Substitution of cysteine residues and removal of proteolytic sites may decrease the risk of heterogeneity in the antibody product and thus increase its homogeneity. Another type of amino acid substitution is to eliminate asparagine-glycine pairs, which form potential deamidation sites, by altering one or both of the residues. In another example, the C-terminal lysine of the heavy chain of an Trop-2 antibody of the invention can be cleaved. In various

embodiments of the invention, the heavy and light chains of the Trop-2 antibodies may optionally include a signal sequence.

Once DNA fragments encoding the VH and VL segments of the present invention are obtained, these DNA fragments can be further manipulated by standard recombinant DNA techniques, for example to convert the variable region genes to full-length antibody chain genes, to Fab fragment genes, or to a scFv gene. In these manipulations, a VL- or VH-encoding DNA fragment is operatively linked to another DNA fragment encoding another protein, such as an antibody constant region or a flexible linker. The term "operatively linked", as used in this context, is intended to mean that the two DNA fragments are joined such that the amino acid sequences encoded by the two DNA fragments remain in-frame.

The isolated DNA encoding the VH region can be converted to a full-length heavy chain gene by operatively linking the VH-encoding DNA to another DNA molecule encoding heavy chain constant regions (CH1, CH2 and CH3). The sequences of human heavy chain constant region genes are known in the art (see e.g., Kabat, E. A., et al., 1991, Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242) and DNA fragments encompassing these regions can be obtained by standard PCR amplification. The heavy chain constant region can be an IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgM or IgD constant region, but most preferably is an IgG1 or IgG2 constant region. The IgG constant region sequence can be any of the various alleles or allotypes known to occur among different individuals, such as Gm(1), Gm(2), Gm(3), and Gm(17). These allotypes represent naturally occurring amino acid substitution in the IgG1 constant regions. For a Fab fragment heavy chain gene, the V_H-encoding DNA can be operatively linked to another DNA molecule encoding only the heavy chain CH1 constant region. The CH1 heavy chain constant region may be derived from any of the heavy chain genes.

The isolated DNA encoding the VL region can be converted to a full-length light chain gene (as well as a Fab light chain gene) by operatively linking the VL-encoding DNA to another DNA molecule encoding the light chain constant region, CL. The sequences of human light chain constant region genes are known in the art (see e.g., Kabat, E. A., et al., 1991, Sequences of Proteins of Immunological Interest, Fifth Edition,

U.S. Department of Health and Human Services, NIH Publication No. 91-3242) and DNA fragments encompassing these regions can be obtained by standard PCR amplification. The light chain constant region can be a kappa or lambda constant region. The kappa constant region may be any of the various alleles known to occur among different individuals, such as Inv(1), Inv(2), and Inv(3). The lambda constant region may be derived from any of the three lambda genes.

To create a scFv gene, the VH- and VL-encoding DNA fragments are operatively linked to another fragment encoding a flexible linker, e.g., encoding the amino acid sequence $(Gly_4\text{-}Ser)_3$, (SEQ ID NO: 80) such that the VH and VL sequences can be expressed as a contiguous single-chain protein, with the VL and VH regions joined by the flexible linker (See e.g., Bird et al., 1988, Science 242:423-426; Huston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; McCafferty et al., 1990, Nature 348:552-554. The single chain antibody may be monovalent, if only a single VH and VL are used, bivalent, if two VH and VL are used, or polyvalent, if more than two VH and VL are used. Bispecific or polyvalent antibodies may be generated that bind specifically to Trop-2 and to another molecule.

In another embodiment, a fusion antibody or immunoadhesin may be made that comprises all or a portion of an Trop-2 antibody of the invention linked to another polypeptide. In another embodiment, only the variable domains of the Trop-2 antibody are linked to the polypeptide. In another embodiment, the VH domain of an Trop-2 antibody is linked to a first polypeptide, while the VL domain of an Trop-2 antibody is linked to a second polypeptide that associates with the first polypeptide in a manner such that the VH and VL domains can interact with one another to form an antigen binding site. In another preferred embodiment, the VH domain is separated from the VL domain by a linker such that the VH and VL domains can interact with one another. The VH-linker- VL antibody is then linked to the polypeptide of interest. In addition, fusion antibodies can be created in which two (or more) single-chain antibodies are linked to one another. This is useful if one wants to create a divalent or polyvalent antibody on a single polypeptide chain, or if one wants to create a bispecific antibody.

In other embodiments, other modified antibodies may be prepared using Trop-2 antibody encoding nucleic acid molecules. For instance, “Kappa bodies” (Ill et al., Protein Eng. 10:949-57, 1997), “Minibodies” (Martin et al., EMBO J., 13:5303-9, 1994),

"Diabodies" (Holliger et al., Proc. Natl. Acad. Sci. USA 90:6444-6448, 1993), or "Janusins" (Traunecker et al., EMBO J. 10:3655-3659, 1991 and Traunecker et al., Int. J. Cancer (Suppl.) 7:51-52, 1992) may be prepared using standard molecular biological techniques following the teachings of the specification.

Bispecific antibodies or antigen binding fragments can be produced by a variety of methods including fusion of hybridomas or linking of Fab' fragments. See, e.g., Songsivilai & Lachmann, Clin. Exp. Immunol. 79:315-321, 1990, Kostelny et al., J. Immunol. 148:1547-1553, 1992. In addition, bispecific antibodies may be formed as "diabodies" or "Janusins." In some embodiments, the bispecific antibody binds to two different epitopes of Trop-2. In some embodiments, the modified antibodies described above are prepared using one or more of the variable domains or CDR regions from the Trop-2 antibodies provided herein.

Representative materials of the present invention were deposited in the American Type Culture Collection (ATCC) on April 26, 2012. Vector having ATCC Accession No. PTA-12872 is a polynucleotide encoding a humanized Trop-2 antibody heavy chain variable region, and vector having ATCC Accession No. PTA-12871 is a polynucleotide encoding a humanized Trop-2 antibody light chain variable region. The deposits were made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture of the deposit for 30 years from the date of deposit. The deposit will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Pfizer, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture of the deposit to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 U.S.C. Section 122 and the Commissioner's rules pursuant thereto (including 37 C.F.R. Section 1.14 with particular reference to 886 OG 638).

The assignee of the present application has agreed that if a culture of the materials on deposit should die or be lost or destroyed when cultivated under suitable conditions, the materials will be promptly replaced on notification with another of the

same. Availability of the deposited material is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

Examples

Example 1: Antibody Binding Affinity Determination for Recombinant Anti-Trop-2 Mouse Antibodies

The affinities of anti-Trop-2 mouse antibodies generated from hybridomas were measured on a surface plasmon resonance Biacore™ 2000 or 3000 biosensor equipped with a research-grade CM5 sensor chip (Biacore™ AB, Uppsala, Sweden – now GE Healthcare). Anti-mouse IgG was first amine coupled to the CM5 sensor surface. Various anti-Trop-2 mouse IgGs were then captured by anti-mouse IgG. Monomeric Trop-2 extracellular domain prepared from papain digestion of Trop-2-Fc fusion protein was then injected as the analyte at 3 fold dilution series. Affinity of anti-Trop-2 mouse antibodies ranges from 7.5 to 31.8 nM. Table 4.

Table 4

Antibody	ka(1/Ms)	kd(1/s)	KD(nM) to huTrop-2
3E9	1.83E+05	2.38E-03	13.0
6G11	2.70E+05	8.60E-03	31.8
7E6	1.60E+05	1.19E-03	7.5
15E2	1.61E+05	4.07E-03	25.3
18B1	4.37E+05	1.04E-02	23.8

Example 2: Domain Mapping of Recombinant Anti-Trop-2 Mouse Antibodies

Domain mapping was done by swapping the Trop-2 extracellular domains with either Trop-1 (EpCAM) or mouse Trop-2 equivalent regions. See Figures 4-5. Anti-Trop-2 antibodies 3E9, 6G11, 7E6, and 18B1 (expressed as recombinant mouse IgG2a) do not bind either human Trop-1 or mouse Trop-2 while 15E2 binds mouse Trop-2 but

not human Trop-1. These hybrid proteins were expressed as human Fc fusion proteins in 293F cells. Binding of anti-Trop-2 antibodies to these domain hybrids were determined by Biacore. Recognition of certain human Trop-2 domains by anti-Trop-2 antibodies were defined when domain swapping results in loss or reduction in binding. Anti-Trop-2 antibody clones 3E9, 7E6, and 15E2 bind to domains 3 and 4 while clones 6G11 and 18B1 bind to domain 1. Table 5. Definition of different domains of Trop-2 can be found, e.g., in Chong et al., J. Biol. Chem. 276(8):5804-13, 2001.

Table 5

Binding of anti-Trop-2 antibodies to different Trop-2 domains					
	domain replacement	domain 1	domain 2	domain 3	domain 4
3E9	hTrop1	-	-	+	+
	mTrop-2	-	-	+/-	+
6G11	hTrop1	+	-	-	-
	mTrop-2	+	-	-	-
7E6	hTrop1	-	-	+	+
	mTrop-2	-	-	-	+
15E2	hTrop1	-	-	+	+/-
	mTrop-2	-	-	+/-	-
18B1	hTrop1	+	-	-	-
	mTrop-2	+	-	-	-

\+ indicates domain replacement results in loss of binding

+/- indicates domain replacement results in decrease of binding

\- indicates domain replacement dose not affect binding

Example 3: *In Vivo* Efficacy Studies With Colo205 Xenograft Model

In vivo efficacy studies of Anti-Trop-2 mouse IgGs were performed with target-expressing colo205 xenograft model. One million colo205 colon cancer cells were implanted subcutaneously into 5-8 weeks old nu/nu mice (day 0). Animals were randomized by body weight the next day (day 1) into different treatment cohorts (control IgG, 7E6, 15E2, or 18B1 group; n=10/group). 20mg/kg of anti-Trop-2 antibodies from different treatment cohorts and control mIgG were administered through bolus tail vein injection three times a week for total 12 doses. All experimental animals were monitored for body weight changes daily. Tumor volume was measured twice a week by a caliper device and calculated with the following formula: Tumor volume = (length x

width^2) / 2. Studies were terminated before tumor volumes reached 2000 mm^3. TGI (% tumor growth inhibition) was determined as [1-tumor sizes (treatment group)/tumor size (control group)] x 100. Table 6 and Figure 1 show that anti-Trop-2 antibodies 7E6, 15E2, and 18B1 inhibit Colo205 xenograft tumor growth *in vivo*.

Table 6

antibody treatment	Mean tumor volume (mm3) on day 43	TGI (Tumor growth inhibition)
control IgG	673.0	0%
7E6	163.8	76%
15E2	582.0	14%
18B1	423.5	37%

Example 4: *In Vivo* Efficacy Studies With A431 Xenograft Model

a) Inhibition of A431 Xenograft Tumor Growth by Anti-Trop-2 antibodies 7E6, 15E2, and 18B1

In vivo efficacy studies of anti-Trop-2 mouse IgGs were performed with target-expressing A431 xenograft model. Two million A431 epidermoid cancer cells were implanted subcutaneously into 5-8 weeks old nu/nu mice until the tumor sizes reached around 100 mm^3. Animals were randomized by tumor sizes and dosing was done through bolus tail vein injection. Anti-Trop-2 antibodies were expressed as recombinant mouse IgG2a antibodies from 293F transient expression. 20mg/kg of anti-Trop-2 antibodies (18B1, 15E2, and 7E6 recombinant mIgG2a) or control IgG were administered through bolus tail vein injection twice a week for total 6 doses. Tumor volume was measured twice a week by a caliper device and calculated with the following formula: Tumor volume = (length x width^2) / 2. Studies were terminated before tumor volumes reached 2000 mm^3. TGI (% tumor growth inhibition) was determined as [1-tumor sizes (treatment group)/tumor size (control group)] x 100. Table 7A and Figure 2A show that anti-Trop 2 antibodies 7E6, 15E2, and 18B1 inhibit A431 xenograft tumor growth *in vivo*.

Table 7A

antibody treatment	Mean tumor volume (mm3) on day 27	TGI (Tumor growth inhibition)
control IgG	1814.8	0%
7E6	770.7	58%
15E2	953.6	47%
18B1	875.5	52%

b) *Inhibition of A431 Xenograft Tumor Growth by Anti-Trop-2 antibody 7E6 in Dose-Response Study*

Using the same xenograft model as described in Example 4a above, various doses of anti-Trop-2 7E6 antibody (5, 10, and 20 mg/kg) or 20 mg/kg control IgG were administered through bolus tail vein injection twice a week for total 6 doses. Table 7B and Figure 2B show inhibition of A431 xenograft tumor growth *in vivo* by various doses of 7E6.

Table 7B

antibody treatment	Mean tumor volume (mm3) on day 27	TGI (Tumor growth inhibition)
control IgG	1814.8	0%
7E6-5mg/kg	913.9	50%
7E6-10mg/kg	897.7	51%
7E6-20mg/kg	875.5	52%

c) *Inhibition of A431 Xenograft Tumor Growth by Anti-Trop-2 antibodies 6G11, 7E6, and 18B1*

Similarly, 20 mg/kg of anti-Trop-2 antibodies (6G11, 7E6, and 18B1 and control IgG), all expressed recombinantly as mIgG2s, were administered through bolus tail vein injection once a week for a total of 3 doses in A431 xenograft model as described in

Example 4a). Table 7C and Figure 2C show that anti-Trop 2 antibodies 6G11, 7E6, and 18B1 inhibit A431 xenograft tumor growth *in vivo*.

Table 7C

antibody treatment	Mean tumor volume (mm3) on day 27	TGI (Tumor growth inhibition)
control IgG	1044.0	0%
6G11	444.8	57%
7E6	561.0	46%
18B1	477.6	54%

Example 5: Antibody Binding Affinity Determination for Humanized Anti-Trop-2 Antibodies – 7E6

The affinities of chimeric and humanized anti-Trop-2 7E6 antibodies were measured on a surface plasmon resonance Biacore™ T200 biosensor equipped with a research-grade CM4 sensor chip (Biacore™ AB, Uppsala, Sweden – now GE Healthcare). The human Trop-2-hFc or cynomolgus monkey Trop-2-hFc proteins were captured on the CM5 EDA chip 37 sensor surface coupled with anti-human Fc. Chimeric and humanized 7E6 recombinant Fab fragments were injected as 3 fold dilution series. Tables 8A and 8B show affinity measurement of humanized anti-Trop-2 7E6 antibodies to human Trop-2 protein and cynomolgus monkey, respectively.

Table 8A

Antibody	ka(1/Ms)	kd(1/s)	KD(nM) to huTrop-2
mouse7E6	1.66E+05	1.34E-03	8.08
h7E6-WT	1.39E+05	2.94E-03	21.10
h7E6_SVG	9.76E+04	4.35E-04	4.46
h7E6_L	1.30E+05	1.50E-04	1.15
h7E6_SVGL	1.31E+05	3.27E-05	0.25

Table 8B

Antibody	ka(1/Ms)	kd(1/s)	KD(nM) to cynoTrop-2
mouse7E6	1.45E+05	2.40E-03	16.50
h7E6-WT	1.44E+05	6.35E-03	44.00
h7E6_SVG	2.65E+05	1.89E-03	7.00
h7E6_L	1.26E+05	3.35E-04	2.66
h7E6_SVGL	1.30E+05	7.42E-05	0.57

Example 6: Antibody Binding Affinity Determination for Humanized Anti-Trop-2 Antibodies – 6G11

The affinities of humanized 6G11 antibodies were measured on a surface plasmon resonance Biacore™ 2000 biosensor equipped with a research-grade CM5 sensor chip (Biacore™ AB, Uppsala, Sweden – now GE Healthcare). Human Trop-2-mFc protein was captured on the CM5 chip coupled with anti-mouse Fc. Human 6G11_WT or Human 6G11_FKG_SF chimeric Fab fragments were injected as 3 fold dilution series. Table 9 shows affinity measurement of humanized anti-Trop-2 6G11 antibodies to human Trop-2 protein.

Table 9

Antibody	ka(1/Ms)	kd(1/s)	KD(nM) to huTrop-2
h6G11_WT	3.60E+05	9.60E-03	27.0
h6G11_FKG_SF	6.40E+05	3.80E-04	0.6

Example 7: Flow Cytometry of Mouse and Humanized Anti-Trop-2 Antibodies on Trop-2 Positive and Negative Tumor Cells

a) 7E6

Binding of mouse chimeric and humanized anti-Trop-2 7E6 antibodies (expressed in human IgG1 subtype) were assessed on Trop-2 expressing (A431 and Colo205) and non-expressing (SW620) cells by flow cytometry. For A431 cell staining, 200,000 cells were incubated with 0.5 ug antibody in 100uL binding buffer (PBS (Phosphate Buffered Saline) + 0.5% BSA (Bovine Serum Albumin)), followed by incubation with Dylight488-conjugated goat anti-human (Fab')2-specific secondary antibody from Jackson immunoresearch Laboratories (West Grove, PA). For Colo205

and SW620 cells, 300,000 cells were used with 1 ug of primary antibody, followed by AlexaFluor 647 anti-human (Fab')2-specific secondary antibody from Jackson immunoresearch Laboratories (West Grove, PA). Table 10 shows at least about 93% binding on Trop-2 positive tumor cells by mouse 7E6 and humanized 7E6 antibodies.

Table 10

	A431 (Trop-2+++)		Colo205 (Trop-2 +)		SW620 (Trop-2 -)	
Antibody	MFI	% positive	MFI	% positive	MFI	% positive
2nd Ab only	1049	3.4	183	3.9	154	2.0
mouse 7E6	103000	94.0	2258	99.0	149	2.2
h7E6_WT	102000	94.8	1761	99.0	143	2.1
h7E6_SVG	121000	96.7	2425	100.0	139	2.4
h7E6_L	110000	95.1	2469	100.0	158	2.5
h7E6_N	149000	98.6	2698	100.0	151	2.3
h7E6_SVGL	107000	93.1	2425	100.0	135	2.6
h7E6_SVGN	128000	98.1	2734	100.0	140	2.5

b) 6G11

Similar to Example 7a), binding of chimeric mouse and humanized anti-Trop-2 6G11 antibodies (expressed in human IgG1 subtype) were assessed on Trop-2 expressing (A431 and Colo205) and non-expressing (SW620) cells by flow cytometry. 300,000 cells were used with 1 ug of primary antibody, followed by AlexaFluor 647 anti-human (Fab')2-specific secondary antibody. Table 11 shows at least about 99% binding on Trop-2 positive tumor cells by mouse 6G11 and humanized 6G11 antibodies.

Table 11

	A431 (Trop-2+++)		Colo205 (Trop-2 +)		SW620 (Trop-2 -)	
Antibody	MFI	% positive	MFI	% positive	MFI	% positive
2nd Ab only	718	4.9	183	3.9	154	2.0
mouse 6G11	16204	99.9	1667	100.0	134	2.5
h6G11_WT	19573	99.9	1716	99.0	154	3.9
h6G11_FKG_SF	20254	99.9	2064	100.0	157	2.5

Example 8: ADCC Activity of Chimeric and Humanized Anti-Trop-2 7E6 hIgG1 Antibodies on A431 Cells

The ADCC (antibody-dependent cytotoxicity) activity of chimeric mouse (h7E6-WT) and humanized anti-Trop-2 7E6 human IgG1antibodies (h7E6_SVG (VH region), h7E6_L (VL region), and h7E6-SVGL (both VH and VL regions)) were determined with the cytoTox 96 non-radioactive cytotoxicity assay kit (Promega, Madison WI). Target expressing A431 cells were seeded at 10,000 cells/well the day before the assay. Donor PBMCs (Cryopreserved Peripheral Blood Mononuclear Cells) were isolated through Ficoll gradient and cultured overnight at 37^0C in X-VIVO medium (Lonza, Wakersville, MO). The antibodies were added to the wells the following day at concentrations indicated in Figure 3, followed by the addition of 500,000 PBMC cells (E:T=50:1) in RPMI+5% FBS (Fetal Bovine Serum). Anti-EGFR (Epidermal Growth Factor Receptor) antibody (ERBITUX®, Imclone, Bridgewater, NJ) was used as a positive control for the assay. The plates were then incubated at 37^0C for 4 hours. At 3 and half hours time point, 20 uL of the lysis solution was added to the target cells alone wells. After spinning the plate at 8000 rpm for 3 minutes, 50uL of supernatants were transferred to another plate. 50ul of substrate was then added to each well and the plate was incubated at room temperature for 30 minutes in the dark. The reaction was stopped by adding 50 uL of stop solution from Promega (Madison, WI) to each well. The plates were then read at 490 nm with a spectrophotometer (Molecular Devices, Sunnyvale, CA). Percentages (%) of specific lysis were calculated with the following formula:

% specific lysis = (treatment LDH (Lactate Dehydrogenase) release - target cell spontaneous LDH release - effector cell spontaneous LDH release) / (target cell maximum LDH release - target cell spontaneous LDH release) x 100

Figure 3 shows that both chimeric mouse and humanized anti-Trop-2 7E6 IgG1 antibodies induced ADCC killing in A431 cells.

Example 9: Cytotoxicity of Anti-Trop-2-ADCs in Trop-2 Positive Cells

Chimeric mouse (6G11 and 7E6) and humanized anti-Trop-2 (h7E6-SVG, h7E6-SVGL, and h7E6-SVGN) antibodies were expressed as human IgG1 subtypes engineered with glutamine-containing transglutaminase ("Q") tags (e.g., TG1, LCQ03, and TG6 correspond to SEQ ID NOs:75, (LLQGG); 78 (GGLLQGA), and 79 (LLQGA),

respectively) and conjugated with AcLys-vcMMAD (Acetyl-Lysine-Valine-Citrulline-MMAD), aminocaproyl-vc-PABC-MMAD (aminocaproyl-Valine-Citrulline-*p*-aminobenzyloxycarbonyl-MMAD), or AcLys-vc-PABC-MMAD (Acetyl-Lysine-Valine-Citrulline-*p*-aminobenzyloxycarbonyl-MMAD) as indicated in Table 12. In one instance, the transglutaminase tags can be engineered at the light chain or heavy chain C-terminus of the antibody; in other instance, the transglutaminase tag (e.g., Q) is engineered at another site of the antibody, such as at position 297 of the human IgG (Kabat numbering scheme). For example, the wild-type amino acid asparagine (N) is substituted with glutamine at position 297 of the Trop-2 antibody (N297Q). Anti-Trop-2 antibody conjugation to MMAD was then achieved via microbial transglutaminase-catalyzed transamidation reaction between the anti-Trop-2 antibody carrying a glutamine-containing tag at the specific site (e.g., carboxyl terminus or amino terminus of the heavy chain or light chain, position 297, or at another site of the antibody) and an amine-containing derivative of the payload (e.g., MMAD). In some instances, the wild-type amino acid lysine at the carboxyl terminus (position 447 in accordance with Kabat numbering scheme) was deleted and replaced with the Q-tag. In other instances, the wild-type amino acid lysine at position 222, 340, or 370 (in accordance with Kabat numbering scheme) was replaced with amino acid arginine ("K222R", "K340R", or "K370R"). For example, the K222R substitution was found to have the surprising effect of resulting in more homogenous antibody and payload conjugate, better intermolecular crosslinking between the antibody and the payload, and/or significant decrease in interchain crosslinking with the glutamine tag on the C terminus of the antibody light chain. In the transamidation reaction, the glutamine on the antibody acted as an acyl donor, and the amine-containing compound acted as an acyl acceptor (amine donor). Purified anti-Trop-2 antibody in the concentration of 1.67 – 4.04 μM was incubated with a 20 – 100 M excess acyl acceptor, ranging between 167 – 404 μM, in the presence of 0.225 – 0.545% (w/v) *Streptoverticillium mobaraense* transglutaminase (ACTIVA™, Ajinomoto, Japan) in 150 – 900 mM NaCl, and 25 mM MES, HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] or Tris HCl buffer at pH range 6.2 – 8.8. The reaction conditions were adjusted for individual acyl acceptor derivatives, and the optimal efficiency and specificity were typically observed for 2.87 μM antibody, 287 μM derivative, and 0.378% (w/v) transglutaminase in 150 mM NaCl, 25 mM Tris HCl, pH

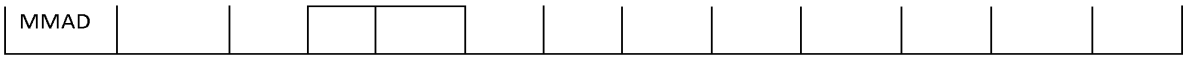
8.8. Following incubation at room temperature for 2.5 hours, the antibody was purified on MabSelect resin (GE Healthcare, Waukesha, WI) using standard affinity chromatography methods known to persons skilled in the art, such as commercial affinity chromatography from GE Healthcare.

Target expressing (A431, BxPC3, CAPAN-2 and Colo205) or non-expressing (SW620) cells were seeded on white walled clear bottom plates at 2000 cells/well for 24 hours before treatment. Cells were treated with 4 fold serially diluted antibody-drug conjugates in triplicates. Cell viability was determined by CellTiter-Glo® Luminescent Cell Viability Assay 96 (Promega, Madison WI) 96 hours after treatment. Relative cell viability was determined as percentage of untreated control. IC50 was calculated by Prism software. Table 12 shows that chimeric mouse and humanized anti-Trop-2 7E6 antibodies conjugated to MMAD through transglutaminase tag exert potent cell killing activity in Trop-2 expressing cells.

Table 12

			A431(Trop-2+++)		BxPC3(Trop-2++)		CAPAN-2(Trop-2+/++)		Colo205 (Trop-2+)		SW620 (Trop-2-)	
Name	conjugate	Loading	Ab-IC50 ug/mL	Ab-IC50 (nM)	Ab-IC50 ug/mL	Ab-IC50 (nM)	Ab-IC50 ug/mL	Ab-IC50 (nM)	Ab-IC50 (ug/mL)	Ab-IC50 (nM)	Ab-IC50 ug/mL	Ab-IC50 (nM)
6G11-TG1-vcMMAD	AcLys-vcMMAD	1.86	0.030	0.197					0.379	2.525		
7E6-TG1-vcMMAD	AcLys-vcMMAD	1.87	0.016	0.109	0.027	0.179	2.320	15.467	0.152	1.011	14.180	94.533
h7E6-WT-TG1-vcMMAD	AcLys-vcMMAD	1.79	0.022	0.144	0.025	0.169	1.956	13.040	0.445	2.969	10.330	68.867
h7E6-L-TG1-vcMMAD	AcLys-vcMMAD	1.85	0.020	0.132	0.035	0.232	2.027	13.513	0.082	0.549	8.535	56.900
h7E6-N-TG1-vcMMAD	AcLys-vcMMAD	1.87	0.024	0.163			2.021	13.473	0.100	0.668	11.150	74.333

h7E6-SVG-TG1-vcMMAD	AcLys-vcMMAD	1.88	0.016	0.105	0.031	0.206	1.659	11.060	0.118	0.787	10.680	71.200
h7E6-SVGL-TG1-vcMMAD	AcLys-vcMMAD	1.85	0.018	0.120			2.440	16.267	0.052	0.345	10.800	72.000
h7E6-SVGN-TG1-vcMMAD	AcLys-vcMMAD	1.87	0.023	0.153	0.051	0.337	2.009	13.393	0.083	0.552	10.410	69.400
NNC-TG1-vcMMAD	Aminocapryl-vcMMAD	1.83			7.260	48.400			22.790	151.933	22.990	153.267
7E6-TG1-vcMMAD	Aminocapryl-vcMMAD	1.87			0.030	0.197					24.180	161.200
h7E6-SVG-TG1-vcMMAD	Aminocapryl-vcMMAD	1.91			0.034	0.229					25.310	168.733
7E6-TG6-AcLys-vc-PABC-MMAD	AcLys-vc-PABC-MMAD	1.87			0.008	0.053						
7E6-K222R-LCQ3-AcLys-vc-PABC-MMAD	AcLys-vc-PABC-MMAD	0.74			0.008	0.054						
h7E6SVG-TG6-AcLys-vc-PABC-MMAD	AcLys-vc-PABC-MMAD	1.93	0.011	0.072	0.032	0.214			0.209	1.393		
h7E6SVG-K222R-LCQ3-AcLys-vc-PABC-	AcLys-vc-PABC-MMAD	1.90	0.01	0.07	0.014	0.092			2.681	17.873		



Example 10: Anti-Trop-2 7E6 Auristatin Conjugate Induced Long Term Tumor Regression in BxPC3 Xenograft Model

In vivo efficacy studies of control antibody hIgG1-TG1-vcMMAD ("NNCTG1-vcMMAD") and chimeric anti-Trop-2 antibody 7E6 conjugated with 1) LCQ03-AcLys-vc-PABC-MMAD K222R variant or 2) TG6-AcLys-vc-PABC-MMAD via transglutaminase were performed with target-expressing BxPC3 xenograft model. For h7E6-K222R-LCQ03-AcLys-vc-PABC-MMAD variant conjugate, the wild-type amino acid lysine in the anti-Trop-2 antibody is substituted with amino acid arginine (i.e., K222R) at position 222 in accordance with Kabat numbering scheme. LCQ03 corresponds to SEQ ID NO:78 (GGLLQGG), and TG6 corresponds to SEQ ID NO:79 (LLQGA). General method of conjugating Trop-2 antibodies using transglutaminase tags LCQ03 and TG6 is described in Example 9. Two million BxPC3 cancer cells were implanted subcutaneously into 5-8 weeks old CB17 SCID mice until the tumor sizes reached around 250 mm^3. Animals were randomized by tumor sizes, and dosing was done through bolus tail vein injection. 3 mg/kg of control hIgG1, chimeric h7E6-K222R-LCQ03-AcLys-vc-PABC-MMAD variant, or chimeric 7E6-TG6-AcLys-vc-PABC-MMAD were administered through bolus tail vein injection once for total of 1 dose. Tumor volume was measured once a week by a Caliper device and calculated with the following formula: Tumor volume = (length x width2) / 2. Studies were terminated before their tumor volumes reached 2000 mm^3. Single dose of the chimeric anti-Trop-2 antibody 7E6 conjugated with AcLys-vc-PABC-MMAD resulted in long term tumor regression. See Figure 8.

Example 11: Cytotoxicity of Anti-Trop-2-ADCs in Trop-2 Positive Cells

Negative control (NNC) and humanized anti-Trop-2 (h7E6SVG) antibodies were expressed as human IgG1 subtypes engineered with glutamine-containing transglutaminase tags at the C-terminus of heavy chain (TG6 (SEQ ID NO: 79)), light chain (LCQ03 (SEQ ID NO: 78) and LCQ04 (SEQ ID NO: 79)) or with N297Q/K222R mutations in the CH2/Hinge domains of heavy chain and conjugated with AcLys-vc-PABC-0101 ("vc0101" or Acetyl-lysine-valine-citrulline-*p*-aminobenzyloxycarbonyl-(2-

methylalanyl-*N*-[(3*R*,4*S*,5*S*)-3-methoxy-1-{(2*S*)-2-[(1*R*,2*R*)-1-methoxy-2-methyl-3-oxo-3-{[(1*S*)-2-phenyl-1-(1,3-thiazol-2-yl)ethyl]amino}propyl]pyrrolidin-1-yl}-5-methyl-1-oxoheptan-4-yl]-*N*-methyl-L-valinamide)) or aminocaproyl-PEG6 (Propylene Glycol)$_6$-propionyl)-MMAD as indicated. 2-methylalanyl-*N*-[(3*R*,4*S*,5*S*)-3-methoxy-1-{(2*S*)-2-[(1*R*,2*R*)-1-methoxy-2-methyl-3-oxo-3-{[(1*S*)-2-phenyl-1-(1,3-thiazol-2-yl)ethyl]amino}propyl]pyrrolidin-1-yl}-5-methyl-1-oxoheptan-4-yl]-*N*-methyl-L-valinamide is a novel auristatin, as described in U.S. Application Nos: 61/561,255 and 61/676,423. Method of conjugating Trop-2 antibodies using transglutaminase tags is described in Example 9. Target expressing or non-expressing (SW620) cells were seeded on white walled clear bottom plates at 2000 (A431, BxPC3, NCI-H292, NCI-H1650, MDA-MB-468 and SW620), 2500 (Calu-3) or 3000 (OVCAR3 and SKBR3) cells per well for 24 hours before treatment. Cells were treated with 4 fold serially diluted antibody-drug conjugates in triplicates. Cell viability was determined by CellTiter-Glo® Luminescent Cell Viability Assay 96 (Promega, Madison, WI) 96 hours after treatment. Relative cell viability was determined as percentage of untreated control. IC50 was calculated by GraphPad Prism 5 software and expressed as concentration (nM) of total Ab. Table 13 shows that humanized anti-Trop-2 7E6 antibodies conjugated to vc0101 or PEG6-MMAD through transglutaminase tag exert potent cell killing activity in Trop-2 expressing cells.

Table 13

Cell name	Cell type	Trop2 expression	h7E6SVG-TG6-vc0101 Ab IC50 (nM)	h7E6SVG-N297Q/K222R-PEG6MMAD Ab IC50 (nM)	h7E6SVG-LCQ03/K222R-vc0101 Ab IC50 (nM)	h7E6SVG-LCQ04/K222R-vc0101 Ab IC50 (nM)	NNC-TG6-vc0101 Ab IC50 (nM)	NNC-N297Q/K222R-PEG6MMAD Ab IC50 (nM)
A431	Epidermoid carcinoma	+++	0.12	0.07	0.03			>267
BxPC3	Pancreas adenocarcinoma	++	0.32	0.03	0.13	0.11		
NCI-H292	Mucoepidermoid pulmonary carcinoma	++	0.63	0.03		1.84		

Calu-3	Lung adenocarcinoma	++	0.53	0.50		1.51		
NCI-H1650	Lung bronchoalveolar carcinoma	++	1.92	0.38			>267	>267
OVCAR3	Ovary adenocarcinoma	+++	0.56	0.98			168.5	>267
MDA-MB-468	Mammary adenocarcinoma	++	0.77	0.19			>267	>267
SKBR3	Mammary adenocarcinoma	+/++	0.42	0.08			200.5	>267
Colo205	Colorectal adenocarcinoma	+	32.89	21.15	174.50			
SW620	Colorectal adenocarcinoma	-	156.20	>267	>267	>267	>267	>267

Example 12: Anti-Trop-2 7E6 Auristatin Conjugate Induced Tumor Regression in Pancreatic Tumor BxPC3 Xenograft Model

In vivo efficacy studies of Trop-2 ADCs were performed with target-expressing BxPC3 xenograft model. Two million BxPC3 pancreatic cancer cells were implanted subcutaneously into 5-8 weeks old CB17/SCID mice until the tumor sizes reached between 300-400 mm^3. Animals were randomized by tumor sizes, and single dose of humanized anti-Trop2 antibody conjugated with 1) TG6-AcLys-vc-PABC-0101 (equivalent to TG6-AcLys-vc-0101 as depicted in Figure 9A); 2) N297Q/K222R-PEG6-MMAD ((Propylene $Glycol)_6$-propionyl-MMAD); and 3) LCQ04-K222R-vc-PABC0101 (equivalent to LCQ04/K222R-vc0101 as depicted in Figure 9C); LCQ04 corresponds to SEQ ID NO: 79 (LLQGA)) and control conjugates were administered through bolus tail vein injection. Tumor volume was measured once a week by a Caliper device and calculated with the following formula: Tumor volume = (length x $width^2$) / 2. Studies were terminated before their tumor volumes reached 2000 mm^3. Figures 9A-9C show that a single dose of the humanized anti-Trop2 antibody conjugated with 1) TG6-AcLys-vc-PABC-0101; 2) N297Q/K222R-PEG6-MMAD; and 3) LCQ04-K222R-vc-PABC0101 resulted in tumor regression in pancreatic tumor BxPC3 xenograft model.

Example 13: Anti-Trop-2 7E6 Auristatin Conjugate Induced Tumor Regression in Colorectal Tumor Colo205 Xenograft Model

In vivo efficacy study of Trop-2 ADCs was performed with target-expressing colo205 xenograft model. Three millions of colo205 colon cancer cells were implanted subcutaneously into 5-8 weeks old nu/nu mice until the tumor sizes reached ~300 mm^3. Animals were randomized by tumor sizes, and single dose of 6mg/kg humanized anti-Trop2 antibody conjugated with 1) TG6-AcLys-vc-PABC-0101 (equivalent to TG6-vc-0101 as depicted in Figure 10); and 2) LCQ03-vc-PABC-0101 having K222R substitution in the hinge/CH2 domains of heavy chain (equivalent to LCQ03/K222R-vc0101 as depicted in Figure10; LCQ03 corresponds to SEQ ID NO: 78 (GGLLQGG)); and control conjugate (IgG-vc-PABC-0101) were administered through bolus tail vein injection. Tumor volume is measured once a week by a Caliper device and calculated with the following formula: Tumor volume = (length x $width^2$) / 2. Studies were terminated before their tumor volumes reached 2000 mm^3. Figure 10 shows that a single dose of the humanized anti-Trop2 antibody conjugated with 1) TG6-AcLys-vc-PABC-0101; and 2) LCQ03-vc-PABC-0101 having K222R substitution in the hinge/CH2 domains of heavy chain resulted in tumor regression in colorectal tumor Colo205 xenograft model.

Example 14: Anti-Trop-2 7E6 Auristatin Conjugate Induced Tumor Regression in Ovarian PDX Ova196756 Xenograft Model

In vivo efficacy study of Trop-2 ADC using humanized anti-Trop2 antibody (h7E6SVG) conjugated with TG6-vc-0101 was performed with target-expressing ovarian cancer patient derived xenograft model (PDX Ova196756). This tumor sample was derived from surgical specimen and propagated in NSG mice (Jackson Laboratories, Bar Harbor, Maine). For efficacy studies, approximately 1-2 mm^3 of tumor fragments were implanted subcutaneously into the lateral flanks of CB17/SCID mice. Animals were randomized by tumor sizes once they reached ~400 mm^3, and a single dose of h7E6SVG-TG6-AcLys-vc-PABC-0101 (0.75 mg/kg and 1.5 mg/kg; equivalent to TG6-vc0101 as depicted in Figure 11) and control conjugate (1.5 mg/kg; IgG-vc-PABC-0101 or IgG-vc0101 as depicted in Figure 11) were administered through bolus tail vein injection. Tumor volume was measured once a week by a caliper device and calculated

with the following formula: Tumor volume = (length x width^2) / 2. Studies were terminated before their tumor volumes reached 2000 mm^3. Figures 11 shows that a single dose of the humanized anti-Trop2 antibody conjugated with TG6-AcLys-vc-PABC-0101 resulted in tumor regression in ovarian PDX Ova196756 xenograft model.

Example 15: Anti-Trop-2 7E6 Auristatin Conjugate Shows Superior Efficacy Than Gemcitabine to Induce Tumor Regression in Pan0146 Pancreatic PDX Model

In vivo efficacy studies of Trop-2 ADC using humanized anti-Trop-2 antibody (h7E6SVG) conjugated with TG6-vc0101 were performed with target-expressing pancreatic cancer patient derived xenograft model (PDX Pan0146) from Jackson Laboratories (Bar Harbor, Maine). For efficacy studies, 1-2 mm^3 of tumor fragments were implanted subcutaneously into the lateral flanks of the animals. Animals were randomized by tumor sizes once they reached ~300 mm^3, and h7E6SVG-TG6-AcLys-vc-PABC-0101 and the control conjugate were administered through bolus tail vein injection. Figure 12A: Single dose of h7E6SVG-TG6-AcLys-vc-PABC-0101 (shown as h7E6SVG-TG6-vc0101 in the Figure) and control conjugate were given at doses indicated. Gemcitabine was given at 75 mg/kg twice weekly for a total of 6 doses. Figure 12B: h7E6SVG-TG6-AcLys-vc-PABC-0101 was given at 0.75 mg/kg weekly for 4 doses, 1.5 mg/kg bi-weekly for two doses or 3.0 mg/kg single dose. Gemcitabine was given once weekly at 75 mg/kg for 4 doses. Tumor volume was measured once a week by a Caliper device and calculated with the following formula: Tumor volume = (length x width^2) / 2. Studies were terminated before their tumor volumes reached 2000 mm^3. The data show that the humanized anti-Trop2 antibody conjugated with TG6-AcLys-vc-PABC-0101 has superior efficacy to gemcitabine to induce tumor regression in Pan0146 pancreatic PDX model and that continuous dosing of h7E6SVG-TG6-AcLys-vc-PABC-0101 resulted in sustained tumor regression in pancreatic PDX Pan0146 xenograft model.

Example 16: Anti-Trop-2 7E6 Auristatin Conjugate Induces Tumor Regression in Pancreatic Pan144607 PDX Model

In vivo efficacy study of Trop-2 ADC using humanized anti-Trop-2 antibody (h7E6SVG) conjugated with vc0101 or PEG6MMAD was performed with target-