substituted heteroalkyl, unsubstituted heteroalkyl, diphosphates, triphosphates, acyl, $C(O)R^{12}R^{13}$, $C(O)OR^{12}$, $C(O)NR^{12}R^{13}$, $P(O)(OR^{12})_2$, , $C(O)CHR^{12}R^{13}$, SR^{12} and $SiR^{12}R^{13}R^{14}$, in which R^{12}, R^{13}, and R^{14} are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl, or R^{12} and R^{13} together with the nitrogen or carbon atom to which they are attached are joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members;

R^6 is a single bond which is either present or absent and when present R^6 and R^7 are joined to form a cyclopropyl ring; and

R^7 is CH_2-X^1 or –CH_2- joined in said cyclopropyl ring with R^6, wherein X^1 is a leaving group,

R^4 , $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, CR^{15}=NR^{16}, and $O(CH_2)_nNR^{24}R^{25}$ wherein n is an integer from 1 to 20;

R^{15} and R^{16} are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, and substituted or unsubstituted peptidyl, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

and R^{24} and R^{25} are independently selected from unsubstituted alkyl, and

wherein at least one of R^4 , $R^{4'}$, R^5 and $R^{5'}$ is $O(CH_2)_nNR^{24}R^{25}$.

126. The compound of claim 125, wherein R^4 is $O(CH_2)_nNR^{24}R^{25}$.

127. The compound of claim 126, wherein R^4 is $O(CH_2)_nN(CH_3)_2$.

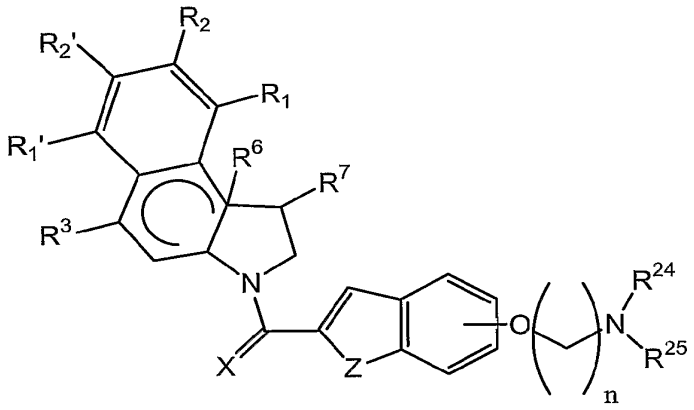
128. The compound of claim127, wherein $R^{4'}$, R^5 and $R^{5'}$ are H.

129. The compound according to any one of claims 125 to 128, wherein R^6 is absent and R^7 is CH_2-X^1, wherein X^1 is F, Cl, or Br.

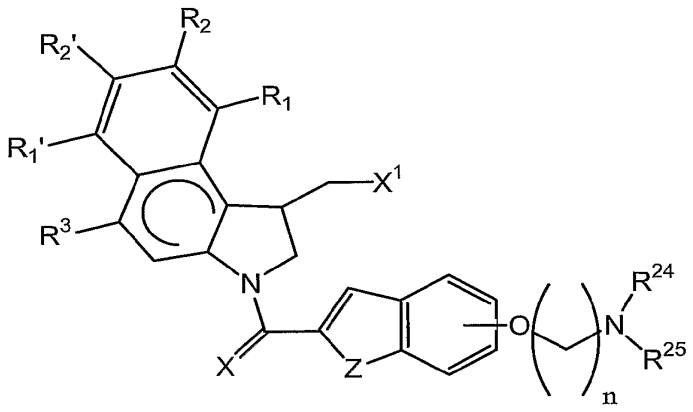
130. The compound according to any one of claims 125 to 129, wherein R^1, $R^{1'}$, R^2, and $R^{2'}$ are H.

131. The compound according to any one of claims 125 to 130, wherein X is O and Z is O.

132. The compound according to any one of claims 125 to 131, wherein the compound has the following formula:

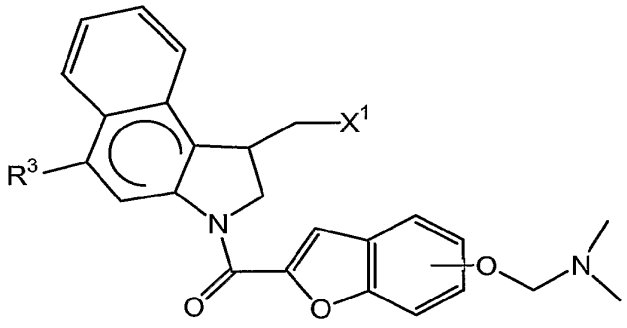


133. The compound according to any one of claims 125 to 132, wherein the compound has the following formula:

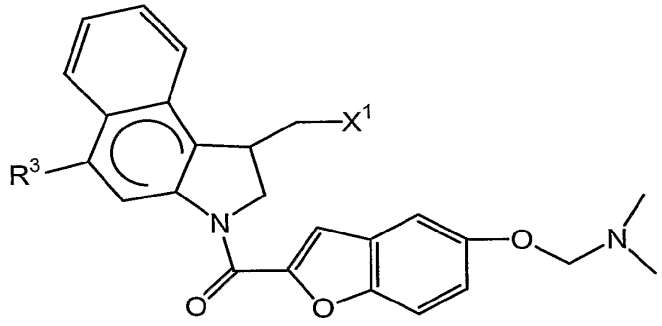


wherein X^1 is F, Cl, or Br.

134. The compound according to any one of claims 125 to 133, wherein the compound has the following formula:



135. The compound according to any one of claims 125 to 134, wherein the compound has the following formula:



136. A pharmaceutical formulation comprising a compound according to any one of claims 1 to 135 and a pharmaceutically acceptable carrier.

137. A method of killing a cell, said method comprising administering to said cell an amount of a compound according to to any one of claims 1 to 135 sufficient to kill said cell.

138. The method of claim 137, wherein the cell is a tumor cell.

139. A method of retarding or stopping the growth of a tumor in a mammalian subject, comprising administering to said subject an amount of a compound according to to any one of claims 1 to 135, sufficient to retard or stop the growth.

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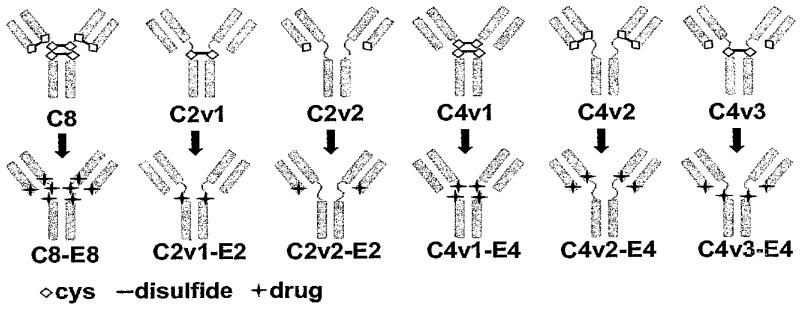
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(54) **Title:** ENGINEERED ANTIBODIES AND IMMUNOCONJUGATES



(57) **Abstract:** Antibody drug conjugates with predetermined sites and stoichiometries of drug attachment are provided. Also provided are methods of using antibody drug conjugates.

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ENGINEERED ANTIBODIES AND IMMUNOCONJUGATES

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application No. 60/631,757, filed November 29, 2004, and of U.S. Provisional Patent Application No. 60/673,146, filed April 19, 2005, each of which is hereby incorporated by reference herein in its entirety.

Background

The present invention is directed to engineered antibodies with predetermined points of attachment for an active moiety. In particular, the invention is directed to antibodies with predetermined points of attachment for active moieties by selective substitution of an amino acid residue(s) of the antibody.

The use of targeting monoclonal antibodies conjugated to radionuclides or other cytotoxic agents offers the possibility of delivering such agents directly to the tumor site, thereby limiting the exposure of normal tissues to the agents (see, e.g., Goldenberg, Semin. Nucl. Med. 19: 332 (1989)). In recent years, the potential of antibody-based therapy and its accuracy in the localization of tumor-associated antigens have been demonstrated both in the laboratory and clinical studies (see, e.g., Thorpe, TIBTECH 11:42 (1993); Goldenberg, Scientific American, Science & Medicine 1:64 (1994); Baldwin et al., U.S. Pat. Nos. 4,925,922 and 4,916,213; Young, U.S. Pat. Nos. 4,918,163 and 5,204,095; Irie et al., U.S. Pat. No. 5,196,337; Hellstrom et al., U.S. Pat. Nos. 5,134,075 and 5,171,665). In general, the use of radiolabeled antibodies or antibody fragments against tumor-associated markers has been more successful for localization of tumors than for therapy, in part because antibody uptake by the tumor is generally low, ranging from only 0.01% to 0.001% of the total dose injected (Vaughan et al., Brit. J. Radiol. 60:567 (1987)). Increasing the concentration of the radiolabel to increase the dosage to the tumor is generally counterproductive because this also increases exposure of healthy tissue to radioactivity.

Monoclonal antibodies can be conjugated to a variety of agents, other than radionuclides, to form immunoconjugates for use in diagnosis and therapy. These agents include chelates, which allow the immunoconjugate to form a stable bond with

radioisotopes, and cytotoxic agents such as toxins and chemotherapy drugs. For example, cytotoxic agents that normally would be too toxic to patients if administered in a systemic fashion can be conjugated to anti-cancer antibodies in such a manner that their toxic effects become directed only to the tumor cells bearing the target antigens. The diagnostic or therapeutic efficacy of immunoconjugates depends upon several factors. Among these factors are the molar ratio of the agent to the antibody and the binding activity of the immunoconjugate.

Researchers have found that the maximum number of agents that can be directly linked to an antibody is limited by the number of modifiable sites on the antibody molecule and the potential loss of immunoreactivity of the antibody. For example, Kulkarni et al. (Cancer Research 41:2700-2706 (1981)) have reported that there is a limit to the number of drug molecules that can be incorporated into an antibody without significantly decreasing antigen-binding activity. Kulkarni et al. found that the highest incorporation obtained for methotrexate was about ten methotrexate molecules per-molecule of antibody, and that attempts to increase the drug-antibody molar ratio over about ten decreased the yield of immunoconjugate and damaged antibody activity. Kanellos et al. (JNCI 75:319-329 (1985)) have reported similar results.

For monoclonal antibodies to function as the delivery vehicles for drugs and radionuclides, it is important to develop methods for their site-specific conjugations, with minimal perturbation of the resultant immunoreactivities. Most commonly, the conjugation of drugs and radionuclides is accomplished through covalent attachments to side chains of amino acid residues. Due to the non-site-restricted nature of these residues, it is difficult to avoid undesirable couplings at residues that lie within or are in close vicinity to the antigen binding site (ABS), leading to reduced affinity and heterogeneous antigen-binding properties. Alternatively, conjugation can be directed at sulfhydryl groups. However, direct labeling relies on the reduction of disulfide (S-S) bonds, with the possible risk of protein fragmentation. Incomplete reduction of such bonds can lead to heterogeneous patterns of attachment.

For example, early preclinical versions of the cAC10 antibody drug conjugate (directed to CD30) involved linkage of eight MMAE (monomethyl auristatin E) drug molecules to the antibody via the cysteine residues. The cysteine residues were obtained by reduction of the four interchain disulfide bonds (Doronina et al., Nat. Biotechnol.

21(7):778-84 (2003)). A recent report has described the effects of drug multiplicity on the in vivo parameters of cAC10 ADCs (Hamblett et al., Clin. Cancer Res.15: 7063-7070 (2004)). cAC10 MMAE drug conjugates with 4 drug molecules attached per antibody (designated C8-E4, where C# indicates the number of interchain cysteine residues available for conjugation and E# indicates the average number of drug molecules attached per antibody molecule) have been shown to have a greater therapeutic window than cAC10 drug conjugates with 8 drugs attached per antibody (designated C8-E8) in animal models. C8-E4 displays similar pharmacokinetic properties to cAC10 alone, while C8-E8 is cleared from circulation more rapidly (Hamblett et al., supra). These characteristics suggest that C8-E4 may be a candidate for clinical development.

The preparation of C8-E4 from cAC10 may result in low yields and heterogeneity of drug attachment, depending on the method of conjugation. One method used to obtain MMAE conjugates with less than eight drugs loaded per antibody utilizes partial reduction of cysteine residues (Hamblett et al., supra). This conjugation process results in a mixture of species with zero, two, four, six or eight drug molecules per antibody molecule (designated C8-E0, C8-E2, C8-E4, C8-E6 and C8-E8, respectively), of which approximately 30% is C8-E4. This conjugate mixture can be separated by hydrophobic interaction chromatography to obtain pure C8-E4, but this process results in a further reduction in overall yield and remaining heterogeneity because the drugs are distributed over eight possible conjugation sites. Further, reduction of the heavy to light chain disulfide bond occurs at approximately double the frequency of the heavy to heavy disulfide bonds, resulting in a 2:1 ratio of the respective C8-E4 isomers. (See, e.g., Sun, et al., Bioconjug Chem 16:1282-1290 (2005).)

Thus, there is a need for antibodies having one or more predetermined sites for stoichiometric drug attachment. These and other limitations and problems of the past are solved by the present invention.

BRIEF SUMMARY OF THE INVENTION

The invention relates to engineered antibodies and immunoconjugates. The invention provides engineered antibodies and immunoconjugates and methods of preparing such engineered antibodies and immunoconjugates. The invention also provides

pharmaceutical compositions of immunoconjugates and methods of using immunoconjugates to treat or diagnose a variety of conditions and diseases.

In one aspect, the invention provides immunoconjugates including engineered antibodies having a functionally active antigen-binding site for a target antigen, at least one interchain cysteine residue, at least one amino acid substitution of an interchain cysteine residue, and a diagnostic, preventative or therapeutic agent conjugated to at least one interchain cysteine residue. In one embodiment, the invention provides immunoconjugates having four interchain cysteine residues and four amino acid substitutions of interchain cysteine residues. In a related embodiment, the invention provides immunoconjugates having two interchain cysteine residues and six amino acid substitutions of interchain cysteine residues. In another embodiment, the invention provides immunoconjugates that are of the IgG1 or IgG4 isotype. The amino acid substitutions can be, for example, cysteine to serine amino acid substitutions of the interchain cysteine residues.

In another aspect, the invention provides immunoconjugates as described above in which a therapeutic agent is conjugated to at least one interchain cysteine residue. In one embodiment, the therapeutic agent is an auristatin or auristatin derivative. In some embodiments, the auristatin derivative is dovaline-valine-dolaisoleunine-dolaproine-phenylalanine (MMAF) or monomethyauristatin E (MMAE).

In another aspect, the invention provides immuoconjugates as described above in which a diagnostic agent is conjugated to at least one interchain cysteine residue. The diagnostic agent can be, for example, a radioactive agent, an enzyme, a fluorescent compounds or an electron transfer agent.

In another aspect, the invention provides immunoconjugates as described above in which the antibody has a functionally active antigen-binding site for a target antigen. The antibody can bind to, for example, CD20, CD30, CD33, CD40, CD70 or Lewis Y. The antibody also can bind to an immunoglobulin gene superfamily member, a TNF receptor superfamily member, an integrin, a cytokine receptor, a chemokine receptor, a major histocompatibility protein, a lectin, or a complement control protein. In other examples, the antibody binds to a microbial antigen, or viral antigen. The antibody also can be an anti-nuclear antibody, anti-ds DNA antibody, anti-ss DNA antibody, anti-cardiolipin

antibody IgM or IgG, anti-phospholipid antibody IgM or IgG, anti-SM antibody, anti-mitochondrial antibody, anti-thyroid antibody, anti-microsomal antibody, anti-thyroglobulin antibody, anti-SCL 70 antibody, anti-Jo antibody, anti-U1RNP antibody, anti-La/SSB antibody, anti-SSA antibody, anti-SSB antibody, anti-perital cells antibody, anti-histone antibody, anti-RNP antibody, anti-C ANCA antibody, anti-P ANCA antibody, anti-centromere antibody, anti-fibrillarin antibody, or anti-GBM antibody.

In another aspect, the invention provides immunoconjugates as described above in which the antibody is an antibody fragment. In one embodiment, the antibody fragment is Fab, Fab' or scFvFc.

In another aspect, the invention provides immuoconjugates of the following formula:

$$Ab_z\left(A_a-W_w-Y_y-D\right)_p$$

or a pharmaceutically acceptable salt or solvate thereof,
wherein:
Ab is an antibody,
A is a stretcher unit,
a is 0 or 1,
each W is independently a linker unit,
w is an integer ranging from 0 to 12,
Y is a spacer unit, and
y is 0, 1 or 2,
p ranges from 1 to about 20, and
D is a diagnostic, preventative and therapeutic agent, and
z is the number of predetermined conjugation sites on the protein.

In some embodiments, the immunoconjugates are of the formula: Ab-MC-vc-PAB-MMAF, Ab-MC-vc-PAB-MMAE, Ab-MC-MMAE or Ab-MC-MMAF.

In another aspect, the invention provides pharmaceutical compositions containing the immunoconjugates described above and a pharmaceutical acceptable carrier. In an

embodiment, the immunoconjugate is formulated with a pharmaceutically acceptable parenteral vehicle. In another embodiment, the immunoconjugate is formulated in a unit dosage injectable form. In a related aspect, the invention provides an article of manufacture having an immunoconjugate conjugated to a therapeutic agent, a container, and a package insert or label indicating that the compound can be used to treat cancer characterized by the overexpression of at least one of CD20, CD30, CD33, CD40, CD70 and Lewis Y.

In another aspect, the invention provides methods of treating a variety of conditions or diseases using immunoconjugates described above that are conjugated to a therapeutic agent. In one embodiment, the methods involve killing or inhibiting the proliferation of tumor cells or cancer cells by treating tumor cells or cancer cells with an amount the immunoconjugate, or a pharmaceutically acceptable salt or solvate, effective to kill or inhibit the proliferation of the tumor cells or cancer cells. In another embodiment, the methods involve treating cancer by administering to a patient an amount of immunoconjugate, or a pharmaceutically acceptable salt or solvate, effective to treat cancer. In another embodiment, the methods involve treating an autoimmune disease by administering to a patient an amount of immunoconjugate, or a pharmaceutically acceptable salt or solvate, effective to treat the autoimmune disease. In yet another embodiment, the methods involve treating an infectious disease by administering to a patient an amount of an immunoconjugate, or a pharmaceutically acceptable salt or solvate, effective to treat the infectious disease.

In another aspect, the invention provides methods of diagnosing a variety of conditions or diseases using immunoconjugates described above that are conjugated to a diagnostic agent. In one embodiment, the methods involve diagnosing cancer by administering to a patient an effective amount of immunoconjugate that binds to an antigen overexpressed by the cancer, and detecting the immunoconjugate in the patient. In another embodiment, the methods involve diagnosing an infectious disease by administering to a patient an effective amount of the immunoconjugate that binds to a microbial or viral antigen, and detecting the immunoconjugate in the patient. In yet another embodiment, the methods involve diagnosing an autoimmune disease in a patient by administering an effective amount of immunoconjugate that binds to an antigen

associated with the autoimmune disease, and detecting the immunoconjugate in the patient.

In another aspect, the invention provides methods of preparing an immunoconjugate involving culturing a host cell expressing an engineered antibody having a functionally active antigen-binding region for a target antigen, at least one interchain cysteine residue, and at least one amino acid substitution of an interchain cysteine residue. The host cell can be transformed or transfected with an isolated nucleic acid encoding the engineered antibody. The antibody can be recovered from the cultured host cells or the culture medium, and conjugated to a diagnostic, preventative or therapeutic agent via at least one interchain cysteine residue. In an embodiment, the antibody is an intact antibody or an antigen-binding fragment. In a preferred embodiment, the antigen binding fragment is an Fab, Fab' or scFvFc.

The invention will best be understood by reference to the following detailed description of the preferred embodiment, taken in conjunction with the accompanying drawings. The discussion below is descriptive, illustrative and exemplary and is not to be taken as limiting the scope defined by any appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the design and analysis of antibody Cys→Ser variants and corresponding antibody drug conjugates (ADCs). (A) Schematic representation of antibody variants and drug conjugates highlighting the location of accessible cysteines (diamonds), inter-chain disulfide bonds (-) and subsequently conjugated drugs (+). Antibodies and ADCs are identified by their variant name (see Table 1), and loading stoichiometry with the drug, MMAE. For example, C8-E8 denotes the ADC in which all eight solvent accessible interchain cysteine residues in the cAC10 parent antibody (C8) are conjugated to MMAE (E8). (B) SDS-PAGE analysis of antibody variants under non-reducing conditions. HHLL, HH, HL, H and L indicate migration patterns for antibody heavy-light chain tetramer, heavy chain dimer, heavy-light chain dimer, heavy chain and light chain, respectively. (C) SDS-PAGE analysis of antibody variant conjugates with MMAE under reducing conditions.

Figure 2 shows titration profiles of a growth proliferation assay using antibody cysteine variants and parent cAC10 antibody conjugated to MC-vcMMAE. (A) Serial dilutions of cAC10 ADCs C2v1-E2, C4v1-E4, C4v2-E4, C6v1-E6 and C8-E4 were incubated with Karpas-299 cells for 96 hours. [H^3]-TdR was then added and its incorporation measured. (B) Karpas-299 cells were incubated with cAC10 ADCs C2v1-E2, C2v2-E2 and C8-E2 for 96 hours. Resazurin was then added and dye reduction measured.

Figure 3 shows single dose efficacy studies on SCID mice bearing Karpas-299 subcutaneous xenografts that were treated with antibody cysteine variants and parent cAC10 antibody conjugated to MC-vcMMAE. Mice were treated with a single dose of C2v1-E2and C8-E2 at 2 mg/kg (A) and C4v1-E4, C4v2-E4, and C8-E4 at 1 mg/kg (B).

Figure 4 shows plasmid map pBSSK AC10H.

Figure 5 shows plasmid map pBSSK AC10 L.

Figure 6 shows reverse phase HPLC analysis of ADCs under reducing conditions. (A) C8-E4M. (B) C8-E4. (C) C4v1-E4. (D) C4v2-E4. (See Table 1). Peaks were identified by the ratio of their absorbances at wavelengths of 248 nm and 280 nm. L-E0 and L-E1 are used to denote light chains loaded with 0 or 1 equivalents of MMAE, respectively, whereas H-E0, H-E1, H-E2 and H-E3 indicate heavy chains loaded with 0, 1, 2, or 3 equivalents of MMAE, respectively.

Figure 7 shows single dose efficacy studies on SCID mice bearing L540cy subcutaneous xenografts. Mice were treated 12 days post tumor implant with a single dose of C2v1-E2, C2v2-E2 and C8-E2 at 6 mg/kg (A) or 12 mg/kg (B). Mice were dosed with C4v1-E4, C4v2-E4, C8-E4 and C8-E4M at 3 mg/kg (C) and 6 mg/kg (D).

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art pertinent to the methods and compositions described. As used herein, the following terms and phrases have the meanings ascribed to them unless specified otherwise.

Antibody. As used herein, "antibody" refers to monoclonal antibodies, such as murine, chimeric, human, or humanized antibodies, mixtures of antibodies, as well as antigen-binding fragments thereof. Such fragments include Fab, Fab', $F(ab)_2$, and $F(ab')_2$. Antibody fragments also include isolated fragments consisting of the light chain variable region, "Fv" fragments consisting of the variable regions of the heavy and light chains, and recombinant single chain polypeptide molecules in which light and heavy variable regions are connected by a peptide linker (e.g., scFv and scFvFc). In some embodiments, the antibody comprises at least one interchain cysteine residue.

Intact Antibody. An "intact" antibody is one which comprises a V_L and V_H antigen-binding variable regions as well as light chain constant domain (C_L) and heavy chain constant domains, C_H1, C_H2, C_H3, and C_H4. The constant domains may be native sequence constant domains (e.g., human native sequence constant domains) or amino acid sequence variants thereof.

Interchain Cysteine Residue: As used herein, "interchain cysteine residue" or "interchain cysteine" refer to a cysteine residue of an antibody chain that can be involved in the formation of an interchain disulfide bond with a cysteine residue of another chain of the unengineered antibody. The interchain cysteine residues are located in the C_L domain of the light chain, the C_H1 domain of the heavy chain, and in the hinge region. The number of interchain cysteine residues in an antibody can vary. For example, human IgG1, IgG2, IgG3 and IgG4 isotypes have 4, 6, 13 and 4 interchain cysteine bonds, respectively. In a specific example, by reference to antibody cAC10, the interchain cysteine thiols are located at amino acid position 214 of the light chain and at amino acid positions 220, 226 and 229 of the heavy chain, according to the numbering scheme of Kabat (Kabat et al., Sequences of Proteins of Immunological Interest, 5th ed. NIH, Bethesda, MD (1991)).

Interchain Disulfide Bond. The term "interchain disulfide bond," in the context of an antibody, refers to a disulfide bond between two heavy chains, or a heavy and a light chain.

Engineered Antibody. As used herein, an "engineered antibody" refers to a nonnaturally occurring intact antibody or antigen-binding fragment having at least one amino acid substitution of an interchain cysteine residue for another amino acid residue

(e.g., a cysteine to serine substitution), and retaining at least one unsubstituted interchain cysteine residue.

Isomer. The term "isomer" in the context of an antibody refers to an antibody having a particular pattern or order of amino acid substitutions of interchain cysteine residues. In the context of an immunoconjugate, the term "isomer" refers to an antibody having a particular pattern or order of amino acid substitutions of interchain cysteine residues and/or a particular pattern of sites of conjugation of an active moiety or moieties. An isomer of an antibody can be referred to by the nomenclature C#v#, where C# indicates the number of interchain cysteine residues available for conjugation and v# refers to a particular pattern or order of interchain cysteine residues. An isomer of an immunoconjugate can be referred to by the nomenclature C#v#-Y, where C# and v# have the same meaning as stated above and Y refers to the average number of diagnostic, preventative or therapeutic agents attached per antibody molecule.

Fully-Loaded. The term "fully-loaded" refers to an antibody in which the predetermined points of conjugation of a particular type and/or of similar reactivity are conjugated to an active moiety, resulting in a homogeneous population of the immunoconjugate (C# = Y).

Partially-Loaded. The term "partially-loaded" refers to an antibody in which only some of the predetermined points of conjugation of a particular type and/or of a similar reactivity are conjugated to an active moiety, resulting in formation of a certain isomer or isomers of the immunoconjugate (C# > Y).

Diagnostic, Preventative or Therapeutic Agent. As used herein, a "diagnostic, preventative or therapeutic agent" is an active moiety such as a macromolecule, molecule or atom which is conjugated to an antibody to produce an immunoconjugate which is useful for diagnosis, prevention and/or for therapy. Examples of diagnostic, preventative or therapeutic agents include drugs, toxins, and detectable labels.

Immunoconjugate. As used herein, an "immunoconjugate" is a molecule comprising an antibody conjugated directly or indirectly to at least one diagnostic, preventative and/or therapeutic agent, or a chelating agent that binds the diagnostic, preventative and/or therapeutic agent. An immunoconjugate retains the immunoreactivity of the antibody, e.g., the antibody has approximately the same, or only slightly reduced,

ability to bind the antigen after conjugation as before conjugation. As used herein, an immunoconjugate is also referred to as an antibody drug conjugate (ADC).

Functionally Active. The term "functionally active,"in the context of an antibody means the antibody immunospecifically binds to a target antigen.

Isolated. The term "isolated," in the context of a molecule or macromolecule (e.g., an antibody or nucleic acid) is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with the desired use (e.g., diagnostic or therapeutic) of the molecule, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In some embodiments, an isolated molecule or macromolecule will be purified (1) to greater than 95%, or greater than 99%, by weight of the molecule or macromolecule as determined by, for example, the Lowry or Bradford methods, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions determined by, for example, Coomassie blue or, preferably, silver staining methods. Isolated molecules and macromolecules include the molecule and macromolecule in situ within recombinant cells since at least one component of the molecules' and macromolecules' natural environment will not be present. Ordinarily, however, isolated molecules and macromolecules will be prepared by at least one purification step.

Structural gene. As used herein, a "structural gene" is a DNA molecule having a sequence that is transcribed into messenger RNA (mRNA) which is then translated into a sequence of amino acids characteristic of a specific polypeptide.

Promoter. As used herein, a "promoter" is a sequence of a nucleic acid that directs the transcription of a structural gene to produce mRNA. Typically, a promoter is located in the 5' region of a gene, proximal to the start codon of a structural gene. If a promoter is an inducible promoter, then the rate of transcription increases in response to an inducing agent. In contrast, the rate of transcription is not regulated by an inducing agent if the promoter is a constitutive promoter.

Enhancer. As used herein, an "enhancer" is a promoter element that can increase the efficiency with which a particular gene is transcribed into mRNA, irrespective of the distance or orientation of the enhancer relative to the start site of transcription.

Complementary DNA (cDNA). As used herein, "complementary DNA" is a single-stranded DNA molecule that is formed from an mRNA template by the enzyme reverse transcriptase. Typically, a primer complementary to a portion(s) of mRNA is employed for the initiation of reverse transcription. Those skilled in the art also use the term "cDNA" to refer to a double-stranded DNA molecule consisting of such a single-stranded DNA molecule and its complement.

Expression. As used herein, "expression" is the process by which a polypeptide is produced from a structural gene or cDNA molecule. The process involves transcription of the coding region into mRNA and the translation of the mRNA into a polypeptide(s).

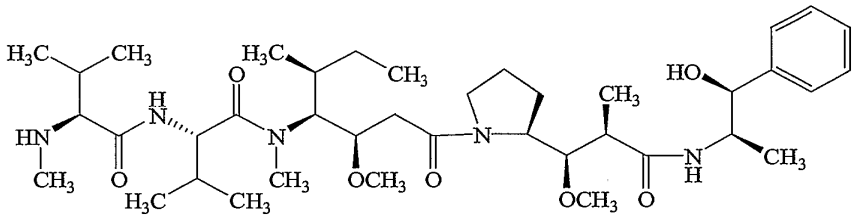
Cloning vector. As used herein, a "cloning vector" is a DNA molecule, such as a plasmid, cosmid, or bacteriophage, which has the capability of replicating autonomously in a host cell and which is used to transform cells for gene manipulation. Cloning vectors typically contain one or a small number of restriction endonuclease recognition sites at which foreign DNA sequences may be inserted in a determinable fashion without loss of an essential biological function of the vector, as well as a marker gene which is suitable for use in the identification and selection of cells transformed with the cloning vector. Marker genes typically include genes that provide tetracycline resistance or ampicillin resistance.

Expression vector. As used herein, an "expression vector" is a DNA molecule comprising a heterologous structural gene or cDNA encoding a foreign protein which provides for the expression of the foreign protein in a recombinant host. Typically, the expression of the heterologous gene is placed under the control of (i.e., operably linked to) certain regulatory sequences such as promoter and/or enhancer sequences. Promoter sequences may be either constitutive or inducible.

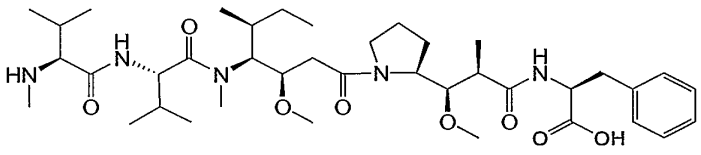
Recombinant Host. A "recombinant host" may be any prokaryotic or eukaryotic cell for expression of a heterologous (foreign) protein. In some embodiments, the recombinant host contains a cloning vector or an expression vector. This term is also meant to include those prokaryotic or eukaryotic cells that have been genetically

engineered to contain a nucleic acid encoding the heterologous protein in the chromosome or genome of the host cell. For examples of suitable hosts, see, e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989); Sambrook et al., Molecular Cloning, A Laboratory Manual, Third Edition, Cold Spring Harbor Publish., Cold Spring Harbor, New York (2001); and Ausubel et al., Current Protocols in Molecular Biology, 4th ed., John Wiley and Sons, New York (1999); all of which are incorporated by reference herein.

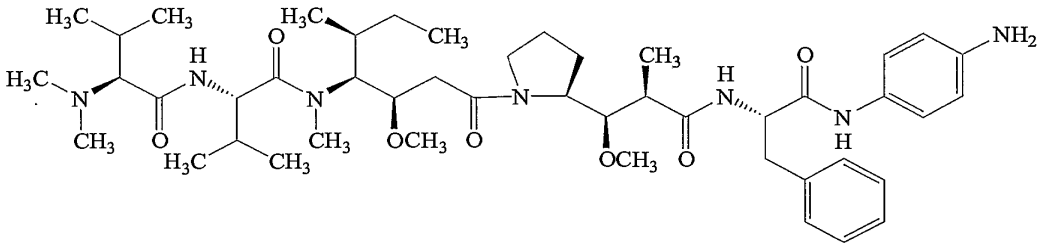
MMAE. The abbreviation "MMAE" refers to monomethyl auristatin E:



MMAF. The abbreviation "MMAF" refers to dovaline-valine-dolaisoleucine-dolaproline-phenylalanine:



AFP. The abbreviation "AFP" refers to dimethylvaline-valine-dolaisoleucine-dolaproline-phenylalanine-p-phenylenediamine:



AEB. The abbreviation "AEB" refers to an ester produced by reacting auristatin E with paraacetyl benzoic acid.

AEVB. The abbreviation "AEVB" refers to an ester produced by reacting auristatin E with benzoylvaleric acid.

Patient. A "patient" includes, but is not limited to, a human, rat, mouse, guinea pig, monkey, pig, goat, cow, horse, dog, cat, bird and fowl.

Effective Amount. The term "effective amount" refers to an amount of a diagnostic, preventative or therapeutic agent sufficient for diagnosis, prevention or treatment of a disease or disorder in a mammal.

Therapeutically Effective Amount. The term "therapeutically effective amount" refers to an amount of a drug , toxin or other molecule effective to prevent or treat a disease or disorder in a mammal. In the case of cancer, the therapeutically effective amount may reduce the number of cancer cells; reduce the tumor size; inhibit (i.e., slow to some extent and preferably stop) cancer cell infiltration into peripheral organs; inhibit (i.e., slow to some extent and preferably 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. To the extent the drug, toxin or other molecule may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic. For cancer therapy, efficacy can, for example, be measured by assessing the time to disease progression (TTP) and/or determining the response rate (RR).

The phrase "pharmaceutically acceptable salt," as used herein, refers to pharmaceutically acceptable organic or inorganic salts of a molecule or macromolecule. Acid addition salts can be formed with amino groups. Exemplary salts include, but are not limited, to sulfate, citrate, acetate, oxalate, chloride, bromide, iodide, nitrate, bisulfate, phosphate, acid phosphate, isonicotinate, lactate, salicylate, acid citrate, tartrate, oleate, tannate, pantothenate, bitartrate, ascorbate, succinate, maleate, gentisinate, fumarate, gluconate, glucuronate, saccharate, formate, benzoate, glutamate, methanesulfonate, ethanesulfonate, benzenesulfonate, p-toluenesulfonate, and pamoate (i.e., 1,1' methylene bis -(2-hydroxy 3-naphthoate)) salts. A pharmaceutically acceptable salt may involve the inclusion of another molecule such as an acetate ion, a succinate ion or other counterion. The counterion may be any organic or inorganic moiety that stabilizes the charge on the parent compound. Furthermore, a pharmaceutically acceptable salt may have more than one charged atom in its structure. Where multiple charged atoms are part of the pharmaceutically acceptable salt, the salt can have multiple counter ions. Hence, a pharmaceutically acceptable salt can have one or more charged atoms and/or one or more counterion.

"Pharmaceutically acceptable solvate" or "solvate" refer to an association of one or more solvent molecules and a molecule or macromolecule. Examples of solvents that form pharmaceutically acceptable solvates include, but are not limited to, water, isopropanol, ethanol, methanol, DMSO, ethyl acetate, acetic acid, and ethanolamine.

DETAILED DESCRIPTION

The present invention provides engineered antibodies and immunoconjugates, and methods of preparing such antibodies and immunoconjugates. The engineered antibodies have at least one predetermined site for conjugation to an active moiety, such as a diagnostic, preventative or therapeutic agent. In some aspects, the engineered antibodies can be stoichiometrically conjugated to a diagnostic, preventative or therapeutic agent to form immunoconjugates with predetermined average loading of the agent. The immunoconjugates can be used therapeutically, diagnostically (e.g., in vitro or in vivo), for in vivo imaging, and for other uses. For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the subsections which follow.

Engineered Antibodies

In one aspect, engineered antibodies are provided. An engineered antibody has an amino acid substitution of at least one interchain cysteine residue, while retaining at least one interchain cysteine residue for conjugation to a diagnostic, preventative or therapeutic agent.

In some embodiments, the antibody is an intact antibody. The antibody can be, for example, of the IgG, IgA, IgM, IgD or IgE class, and within these classes, various subclasses, such as an IgG1, IgG2, IgG3, IgG4, IgA1 or IgA2 isotypes. For example, in some embodiments, the antibody can be an IgG, such as an IgG1, IgG2, IgG3 or IgG4.

In some embodiments, the engineered antibody comprises at least one amino acid substitution replacing an interchain cysteine residue with another amino acid. The interchain cysteine residue can be involved in the formation of an interchain disulfide bond between light and heavy chains and/or between heavy chains. Thus, the amino acid substitution can be in the interchain cysteine residues in the C_L domain of the light chain, the C_H1 domain of the heavy chain, and/or in the hinge region. For example, with reference to antibody cAC10, the interchain cysteine residues are at amino acid positions

214 of the light chain and at amino acid positions 220 (C_H1) and 226 and 229 (hinge region) in the heavy chain in the numbering scheme of Kabat (Kabat et al., Sequences of Proteins of Immunological Interest, 5th ed. NIH, Bethesda, MD (1991)). One or more of these interchain cysteine residues in cAC10 can be substituted.

In some embodiments, the amino acid substitution is a serine for a cysteine residue. In some embodiments, the amino acid substitution introduces is a serine or threonine residue. In some embodiments, the amino acid substitution introduces is a serine, threonine or glycine residue. In some embodiments, the amino acid substitution introduces a neutral (e.g., serine, threonine or glycine) or hydrophilic (e.g., methionine, alanine, valine, leucine or isoleucine) residue. In some embodiments, the amino acid substitution introduces a natural amino acid, other than a cysteine residue.

The engineered antibody retains at least one unsubstituted interchain cysteine residue for conjugation to an active moiety. The number of retained intercysteine residues in an engineered antibody is greater than zero but less than the total number of interchain cysteine residues in the parent (non-engineered) antibody. Thus, in some embodiments, the engineered antibody has at least one, at least two, at least three, at least four, at least five, at least six or at least seven interchain cysteine residues. In typical embodiments, the engineered antibody has an even integral number of interchain cysteine residues (e.g., at least two, four, six or eight reactive sites). In some embodiments, the engineered antibody has less than eight interchain cysteine residues.

In a typical embodiment, the interchain cysteine residues are substituted in a pairwise manner, in which both cysteine residues involved in the formation of an interchain disulfide bond are substituted. (Such interchain cysteine residues can be referred to as "complementary" interchain cysteine residues.) For example, if the C_L interchain cysteine residue(s) are substituted, the complementary C_H1 interchain cysteine residue(s) might also be substituted. In another example, each pair of the interchain cysteine residues in the hinge region can be substituted or remain unsubstituted in a pairwise manner. In other embodiments, an interchain cysteine residue can be substituted while the complementary residue can remain unsubstituted.

In some embodiments, the engineered antibody comprises light chains each having an amino acid substitution of the C_L interchain cysteine residue and heavy chains each

having an amino acid substitution of the C_H1 interchain cysteine residue and retaining the interchain cysteine residues in the hinge region. In a related embodiment, an immunoconjugate of the engineered antibody has active moieties conjugated to the interchain cysteine residues of the hinge region.

In some embodiments, the engineered antibody comprises light chains each having an amino acid substitution of the C_L interchain cysteine residue and heavy chains each having an amino acid substitution of the C_H1 interchain cysteine residue and an amino acid substitution of at least one of the interchain cysteine residues in the hinge region. In a related embodiment, an immunoconjugate of the engineered antibody has active moieties conjugated to the remaining interchain cysteine residues of the hinge region.

In some embodiments, the engineered antibody comprises light chains each having the C_L interchain cysteine residue and heavy chains each retaining the C_H1 interchain cysteine residue and having amino acid substitutions of the hinge region interchain cysteine residues. In a related embodiment, an immunoconjugate of such an engineered antibody has active moieties conjugated to the C_L interchain cysteine residues and heavy chains C_H1 interchain cysteine residues.

In some embodiments, the engineered antibody comprises light chains each having the C_L interchain cysteine residue and heavy chains each retaining the C_H1 interchain cysteine residue and having amino acid substitutions of at least one but less than all of the hinge region interchain cysteine residues. In a related embodiment, an immunoconjugate of such an engineered antibody has active moieties conjugated to the C_L interchain cysteine residues, to heavy chains C_H1 interchain cysteine residues and to the remaining interchain cysteine residues.

In some embodiments, the engineered antibody comprises light chains each having the C_L interchain cysteine residue and heavy chains each having an amino acid substitution of the C_H1 interchain cysteine residue and an amino acid substitution of at least one of the hinge region interchain cysteine residues. In a related embodiment, an immunoconjugate of the engineered antibody has active moieties conjugated to the C_L interchain cysteines and to the remaining interchain cysteine residues of the hinge region.

In some embodiments, the engineered antibody comprises light chains each having the C_L interchain cysteine residue and heavy chains each having an amino acid

substitution of the C_H1 interchain cysteine residue and an amino acid substitution of the hinge region interchain cysteine residues. In a related embodiment, an immunoconjugate of the engineered antibody has active moieties conjugated to the C_L interchain cysteine residues.

In some embodiments, the engineered antibody comprises light chains each having an amino acid substitution of the C_L interchain cysteine residue and heavy chains each having the C_H1 interchain cysteine residue and the hinge region interchain cysteine residues. In a related embodiment, an immunoconjugate of the engineered antibody has active moieties conjugated to the C_H1 interchain cysteine residues and to the interchain cysteine residues of the hinge region.

In some embodiments, the engineered antibody comprises light chains each having an amino acid substitution of the C_L interchain cysteine residue and heavy chains each having the C_H1 interchain cysteine residue and having an amino acid substitution of at least one of the hinge region interchain cysteine residues. In a related embodiment, an immunoconjugate of the engineered antibody has active moieties conjugated to the C_H1 interchain cysteine residues and to the remaining interchain cysteine residues of the hinge region.

In some embodiments, the engineered antibody comprises light chains each having an amino acid substitution of the C_L interchain cysteine residue and heavy chains each having the C_H1 interchain cysteine residue and having an amino acid substitution of the hinge region interchain cysteine residues. In a related embodiment, an immunoconjugate of the engineered antibody has active moieties conjugated to the C_H1 interchain cysteine residues.

In an exemplary embodiment where the parent antibody has eight interchain cysteine residues, the engineered antibody comprises light chains each having an amino acid substitution of the C_L interchain cysteine residue and heavy chains each having an amino acid substitution of the C_H1 interchain cysteine residue and retaining the interchain cysteine residues in the hinge region. In a related embodiment, an immunoconjugate of the engineered antibody has four active moieties conjugated to the interchain cysteine residues of the hinge region.

In an exemplary embodiment where the parent antibody has eight interchain cysteine residues, the engineered antibody comprises light chains each having the C_L interchain cysteine residue and heavy chains each retaining the C_H1 interchain cysteine residue and having amino acid substitutions of both hinge region interchain cysteine residues. In a related embodiment, an immunoconjugate of such an engineered antibody has four active moieties conjugated to the C_L interchain cysteine residues and heavy chains C_H1 interchain cysteine residues.

In an exemplary embodiment where the parent antibody has eight interchain cysteine residues, the engineered antibody comprises light chains each having the C_L interchain cysteine residue and heavy chains each having an amino acid substitution of the C_H1 interchain cysteine residue and an amino acid substitution of one of the hinge region interchain cysteine residues. In a related embodiment, an immunoconjugate of the engineered antibody has four active moieties conjugated to the C_L interchain cysteines and to the remaining interchain cysteine residues of the hinge region.

In an exemplary embodiment where the parent antibody has eight interchain cysteine residues, the engineered antibody comprises light chains each having an amino acid substitution of the C_L interchain cysteine residue and heavy chains each having an amino acid substitution of the C_H1 interchain cysteine residue and a substitution of one of the hinge region interchain cysteine residues. In a related embodiment, an immunoconjugate of the engineered antibody has two active moieties conjugated to the remaining interchain cysteine residues of the hinge region.

In an exemplary embodiment where the parent antibody has eight interchain cysteine residues, the engineered antibody comprises light chains each having the C_L interchain cysteine residue and heavy chains each having an amino acid substitution of the C_H1 interchain cysteine residue and an amino acid substitution of both hinge region interchain cysteine residues. In a related embodiment, an immunoconjugate of the engineered antibody has two active moieties conjugated to the remaining interchain cysteine residues of the hinge region.

In an exemplary embodiment where the parent antibody has eight interchain cysteine residues, the engineered antibody comprises light chains each having the C_L interchain cysteine residue and heavy chains each having the C_H1 interchain cysteine

residue and an amino acid substitution of one of the hinge region interchain cysteine residues. In a related embodiment, an immunoconjugate of the engineered antibody has six active moieties conjugated to the C_L interchain cysteine residues and to the remaining interchain cysteine residues of the hinge region.

In an exemplary embodiment where the parent antibody has eight interchain cysteine residues, the engineered antibody comprises light chains each having the C_L interchain cysteine residue and heavy chains each having an amino acid substitution of the C_H1 interchain cysteine residue and retaining both of the hinge region interchain cysteine residues. In a related embodiment, an immunoconjugate of the engineered antibody has six active moieties conjugated to the C_L interchain cysteine residues and to the interchain cysteine residues of the hinge region.

In an exemplary embodiment where the parent antibody has eight interchain cysteine residues, the engineered antibody comprises light chains each having an amino acid substitution of the C_L interchain cysteine residue and heavy chains each retaining the C_H1 interchain cysteine residue and both of the hinge region interchain cysteine residues. In a related embodiment, an immunoconjugate of the engineered antibody has six active moieties conjugated to the C_H1 interchain cysteine residues and to the interchain cysteine residues of the hinge region.

The antibody also can be an antigen-binding antibody fragment such as, for example, a Fab, a F(ab'), a $F(ab')_2$, a Fd chain, a single-chain Fv (e.g., scFv and scFvFc), a single-chain antibody, a disulfide-linked Fv (sdFv), a fragment comprising either a V_L or V_H domain, a minibody, a maxibody, an $F(ab')_3$, or fragments produced by a Fab expression library. Antigen-binding antibody fragments, including single-chain antibodies, can comprise the variable region(s) alone or in combination with the entirety or a portion of the following: hinge region, C_H1, C_H2, C_H3, C_H4 and/or C_L domains. Also, antigen-binding fragments can comprise any combination of variable region(s) with a hinge region, C_H1, C_H2, C_H3, C_H4 and/or C_L domains. See also Holliger and Hudson, Nat. Biotechnol. 23:1126-1136 (2005), the disclosure of which is incorporated by reference herein.

In some embodiments, an antibody fragment comprises at least one domain, or part of a domain, that includes at least one interchain cysteine residue. For example, the

antibody fragment can include a hinge region, a C_L and C_H1 domains, C_L and C_H1 domains and a hinge region, or the like.

The antibody fragment can be of any suitable antibody class (e.g., IgG, IgA, IgM, IgD and IgE) and subclass (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2).

Typically, the antibodies are human, rodent (e.g., mouse, rat or hamster), donkey, sheep, rabbit, goat, guinea pig, camelid, horse, or chicken. As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin libraries, from human B cells, or from animals transgenic for one or more human immunoglobulins, as described infra and, for example in Reichert et al. (Nat. Biotechnol. 23:1073-8 (2005)) and in U.S. Patent Nos. 5,939,598 and 6,111,166. The antibodies may be monospecific, bispecific, trispecific, or of greater multispecificity.

The antibody is typically a monoclonal antibody but also can be a mixture of monoclonal antibodies. When the subject is a human subject, the antibody may be obtained by immunizing any animal capable of mounting a usable immune response to the antigen. The animal may be a mouse, rat, goat, sheep, rabbit or other suitable experimental animal. The antigen may be presented in the form of a naturally occurring immunogen, or a synthetic immunogenic conjugate of a hapten and an immunogenic carrier. The antibody producing cells of the immunized animal may be fused with "immortal" or "immortalized" human or animal cells to obtain a hybridoma which produces the antibody. If desired, the genes encoding one or more of the immunoglobulin chains may be cloned so that the antibody may be produced in different host cells, and if desired, the genes may be mutated so as to alter the sequence and hence the immunological characteristics of the antibody produced. (See also Teng et al. Proc. Natl. Acad. Sci. USA. 80:7308-7312 (1983); Kozbor et al., Immunology Today 4:72-79 (1983); and Olsson et al., Meth. Enzymol. 92:3-16 (1982)). Human monoclonal antibodies may be made by any of numerous techniques known in the art, such as phage display (see, e.g., Hoogenboom, Nat. Biotechnol. 23:1105-16 (2005); transgenic mice expressing human immunoglobulin genes (see, e.g., Lonberg, Nat. Biotechnol. 23:1117-25 (2005)); ribosome-, mRNA- and yeast-display libraries (see, e.g., Hoogenboom, supra), and human hybridomas from patients (Brändlein et al., Histol. Histopathol. 19:897-905 (2004); and Illert et al., Oncol. Rep. 13:765-70 (2005)), and/or single-antigen selected lymphocytes

(see, e.g., Lagerkvist et al., Biotechniques 18:862-9 (1995); and Babcook et al., Proc. Natl. Acad. Sci. USA 93:7843-8 (1996)).

The antibody can be, for example, a murine, a chimeric, humanized, or fully human antibody produced by techniques well-known to one of skill in the art. Recombinant antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, are useful antibodies. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine monoclonal and human immunoglobulin constant regions. (See, e.g., Cabilly et al., U.S. Patent No. 4,816,567; and Boss et al., U.S. Patent No. 4,816,397, which are incorporated herein by reference in their entirety.) In some embodiments, the antibody light chain constant region domain is not chimeric. In some embodiments, the antibody heavy chain constant region is not chimeric. In this context, "chimeric" refers to a constant region or constant region domain composed of portions from two different species.

The antibody can also be a bispecific antibody. Methods for making bispecific antibodies are known in the art. Traditional production of full-length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Milstein et al., Nature 305:537-539 (1983)). For further details for generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology 121:210 (1986); Rodrigues et al., J. Immunology 151:6954-6961 (1993); Carter et al., Bio/Technology 10:163-167 (1992); Carter et al., J. Hematotherapy 4:463-470 (1995); Merchant et al., Nature Biotechnology 16:677-681 (1998)). Using such techniques, bispecific antibodies can be prepared for use in the treatment or prevention of disease. Bifunctional antibodies are also described in European Patent Publication No. EPA 0 105 360. Hybrid or bifunctional antibodies can be derived either biologically, i.e., by cell fusion techniques, or chemically, especially with cross-linking agents or disulfide-bridge forming reagents, and may comprise whole antibodies or fragments thereof. Methods for obtaining such hybrid antibodies are disclosed for example, in International Publication WO 83/03679, and European Patent Publication No. EPA 0 217 577, both of which are incorporated herein by reference.

In some embodiments, the antibody constant domains have effector function. The term "antibody effector function(s)," or AEC, as used herein refers to a function contributed by an Fc domain(s) of an Ig. Such function can be effected by, for example, binding of an Fc effector domain(s) to an Fc receptor on an immune cell with phagocytic or lytic activity or by binding of an Fc effector domain(s) to components of the complement system. The effector function can be, for example, "antibody-dependent cellular cytotoxicity" or ADCC, "antibody-dependent cellular phagocytosis" or ADCP, "complement-dependent cytotoxicity" or CDC. In other embodiments, the constant domain(s) lacks one or more effector functions.

The antibodies may be directed against an antigen of interest, such as diagnostic preventative and/or therapeutic interest. For example, the antigen can be one associated with infectious pathogens (such as but not limited to viruses, bacteria, fungi, and protozoa), parasites, tumor cells, or particular medical conditions. In the case of a tumor-associated antigen (TAA), the cancer may be of the immune system, lung, colon, rectum, breast, ovary, prostate gland, head, neck, bone, or any other anatomical location. In some embodiments, the antigen is CD2, CD20, CD22, CD30, CD33, CD38, CD40, CD52, CD70, HER2, EGFR, VEGF, CEA, HLA-DR, HLA-Dr10, CA125, CA15-3, CA19-9, L6, Lewis X, Lewis Y, alpha fetoprotein, CA 242, placental alkaline phosphatase, prostate specific membrane antigen, prostate specific antigen, prostatic acid phosphatase, epidermal growth factor, MAGE-1, MAGE-2, MAGE-3, MAGE-4, anti-transferrin receptor, p97, MUC1, gp100, MART1, IL-2 receptor, human chorionic gonadotropin, mucin, P21, MPG, and Neu oncogene product.

Some specific useful antibodies include, but are not limited to, BR96 mAb (Trail et al., Science 261:212-215 (1993)), BR64 (Trail et al., Cancer Research 57:100-105 (1997)), mAbs against the CD 40 antigen, such as S2C6 mAb (Francisco et al., Cancer Res. 60:3225-3231 (2000)), and mAbs against the CD30 antigen, such as AC10 (Bowen et al., J. Immunol. 151:5896-5906 (1993)). Many other internalizing antibodies that bind to tumor specific antigens can be used, and have been reviewed (see, e.g., Franke et al., Cancer Biother. Radiopharm. 15:459-76 (2000); Murray, Semin Oncol. 27:64-70 (2000); Breitling et al., Recombinant Antibodies, John Wiley, and Sons, New York, 1998). The disclosures of these references are incorporated by reference herein.

In some embodiments, the antigen is a "tumor-specific antigen." A "tumor-specific antigen" as used herein refers to an antigen characteristic of a particular tumor, or strongly correlated with such a tumor. However, tumor-specific antigens are not necessarily unique to tumor tissue, i.e., antibodies to tumor-specific antigens may cross-react with antigens of normal tissue. Where a tumor-specific antigen is not unique to tumor cells, it frequently occurs that, as a practical matter, antibodies binding to tumor-specific antigens are sufficiently specific to tumor cells to carry out the desired procedures without unwarranted risk or interference due to cross-reactions. Many factors contribute to this practical specificity. For example, the amount of antigen on the tumor cell may greatly exceed the amount of the cross-reactive antigen found on normal cells, or the antigen on the tumor cells may be more effectively presented. Therefore the term "tumor-specific antigen" relates herein to a specificity of practical utility, and is not intended to denote absolute specificity or to imply an antigen is unique to the tumor.

The nucleotide sequence encoding antibodies that are immunospecific for tumor associated or tumor specific antigens can be obtained, e.g., from the GenBank database or a database like it, commercial sources, literature publications, or by routine cloning and sequencing.

In some embodiments, the antibodies are directed against an antigen for the diagnosis, treatment or prevention of an autoimmune disease. Antibodies immunospecific for an antigen of a cell that is responsible for producing autoimmune antibodies can be obtained from the GenBank database or a database like it, a commercial or other source or produced by any method known to one of skill in the art such as, e.g., chemical synthesis or recombinant expression techniques.

In some embodiments, the antibody is an anti-nuclear antibody; anti-ds DNA; anti-ss DNA, anti-cardiolipin antibody IgM, IgG; anti-phospholipid antibody IgM, IgG; anti-SM antibody; anti-mitochondrial antibody; thyroid antibody; microsomal antibody; thyroglobulin antibody; anti-SCL 70; anti-Jo; anti-U1RNP; anti-La/SSB; anti-SSA; anti-SSB; anti-perital cells antibody; anti-histones; anti-RNP; anti-C ANCA; anti-P ANCA; anti-centromere; anti-fibrillarin, or an anti-GBM antibody.

In some embodiments, the antibody can bind to a receptor or a receptor complex expressed on a target cell (e.g., an activated lymphocyte). The receptor or receptor

complex can comprise an immunoglobulin gene superfamily member, a TNF receptor superfamily member, an integrin, a cytokine receptor, a chemokine receptor, a major histocompatibility protein, a lectin, or a complement control protein. Non-limiting examples of suitable immunoglobulin superfamily members are CD2, CD3, CD4, CD8, CD19, CD22, CD28, CD79, CD90, CD152/CTLA 4, PD 1, and ICOS. Non-limiting examples of suitable TNF receptor superfamily members are CD27, CD40, CD95/Fas, CD134/OX40, CD137/4 1BB, TNF R1, TNF R2, RANK, TACI, BCMA, osteoprotegerin, Apo2/TRAIL R1, TRAIL R2, TRAIL R3, TRAIL R4, and APO 3. Non-limiting examples of suitable integrins are CD11a, CD11b, CD11c, CD18, CD29, CD41, CD49a, CD49b, CD49c, CD49d, CD49e, CD49f, CD103, and CD104. Non-limiting examples of suitable lectins are C type, S type, and I type lectin. In other embodiments, the receptor is CD70.

In some embodiments, the antibody is immunospecific for a viral or a microbial antigen. As used herein, the term "viral antigen" includes, but is not limited to, any viral peptide, polypeptide protein (e.g., HIV gp120, HIV nef, RSV F glycoprotein, influenza virus neuraminidase, influenza virus hemagglutinin, HTLV tax, herpes simplex virus glycoprotein (e.g., gB, gC, gD, and gE) and hepatitis B surface antigen) that is capable of eliciting an immune response. As used herein, the term "microbial antigen" includes, but is not limited to, any microbial peptide, polypeptide, protein, saccharide, polysaccharide, or lipid molecule (e.g., a bacterial, fungi, pathogenic protozoa, or yeast polypeptide including, e.g., LPS and capsular polysaccharide 5/8) that is capable of eliciting an immune response.

Antibodies immunospecific for a viral or microbial antigen can be obtained commercially, for example, from BD Biosciences (San Francisco, CA), Chemicon International, Inc. (Temecula, CA), or Vector Laboratories, Inc. (Burlingame, CA) or produced by any method known to one of skill in the art such as, e.g., chemical synthesis or recombinant expression techniques. The nucleotide sequence encoding antibodies that are immunospecific for a viral or microbial antigen can be obtained, e.g., from the GenBank database or a database like it, literature publications, or by routine cloning and sequencing.

Examples of antibodies available useful for the diagnosis or treatment of viral infection or microbial infection include, but are not limited to, SYNAGIS (MedImmune, Inc., MD) which is a humanized anti-respiratory syncytial virus (RSV) monoclonal

antibody useful for the treatment of patients with RSV infection; PRO542 (Progenics Pharmaceuticals, Inc., NY) which is a CD4 fusion antibody useful for the treatment of HIV infection; OSTAVIR (Protein Design Labs, Inc., CA) which is a human antibody useful for the treatment of hepatitis B virus; PROTOVIR (Protein Design Labs, Inc., CA) which is a humanized IgG1 antibody useful for the treatment of cytomegalovirus (CMV); and anti-LPS antibodies.

Other antibodies include, but are not limited to, antibodies against the antigens from pathogenic strains of bacteria (e.g., Streptococcus pyogenes, Streptococcus pneumoniae, Neisseria gonorrheae, Neisseria meningitidis, Corynebacterium diphtheriae, Clostridium botulinum, Clostridium perfringens, Clostridium tetani, Hemophilus influenzae, Klebsiella pneumoniae, Klebsiella ozaenas, Klebsiella rhinoscleromotis, Staphylococc aureus, Vibrio colerae, Escherichia coli, Pseudomonas aeruginosa, Campylobacter (Vibrio) fetus, Aeromonas hydrophila, Bacillus cereus, Edwardsiella tarda, Yersinia enterocolitica, Yersinia pestis, Yersinia pseudotuberculosis, Shigella dysenteriae, Shigella flexneri, Shigella sonnei, Salmonella typhimurium, Treponema pallidum, Treponema pertenue, Treponema carateneum, Borrelia vincentii, Borrelia burgdorferi, Leptospira icterohemorrhagiae, Mycobacterium tuberculosis, Pneumocystis carinii, Francisella tularensis, Brucella abortus, Brucella suis, Brucella melitensis, Mycoplasma spp., Rickettsia prowazeki, Rickettsia tsutsugumushi, Chlamydia spp.); pathogenic fungi (e.g., Coccidioides immitis, Aspergillus fumigatus, Candida albicans, Blastomyces dermatitidis, Cryptococcus neoformans, Histoplasma capsulatum); protozoa (Entomoeba histolytica, Toxoplasma gondii, Trichomonas tenas, Trichomonas hominis, Trichomonas vaginalis, Tryoanosoma gambiense, Trypanosoma rhodesiense, Trypanosoma cruzi, Leishmania donovani, Leishmania tropica, Leishmania braziliensis, Pneumocystis pneumonia, Plasmodium vivax, Plasmodium falciparum, Plasmodium malaria); or Helminiths (Enterobius vermicularis, Trichuris trichiura, Ascaris lumbricoides, Trichinella spiralis, Strongyloides stercoralis, Schistosoma japonicum, Schistosoma mansoni, Schistosoma haematobium, and hookworms).

Other antibodies include, but are not limited to, antibodies against antigens of pathogenic viruses, including as examples and not by limitation: Poxviridae, Herpesviridae, Herpes Simplex virus 1, Herpes Simplex virus 2, Adenoviridae, Papovaviridae, Enteroviridae, Picornaviridae, Parvoviridae, Reoviridae, Retroviridae,

influenza viruses, parainfluenza viruses, mumps, measles, respiratory syncytial virus, rubella, Arboviridae, Rhabdoviridae, Arenaviridae, Hepatitis A virus, Hepatitis B virus, Hepatitis C virus, Hepatitis E virus, Non A/Non B Hepatitis virus, Rhinoviridae, Coronaviridae, Rotoviridae, and Human Immunodeficiency Virus.

Methods for Introducing an Amino Acid Substitution into an Antibody by Altering the Nucleic Acid Sequence Encoding the Protein

An amino acid substitution can be introduced into a nucleic acid sequence encoding an antibody by any suitable method. Such methods include polymerase chain reaction-based mutagenesis, site-directed mutagenesis, gene synthesis using the polymerase chain reaction with synthetic DNA oligomers, and nucleic acid synthesis followed by ligation of the synthetic DNA into an expression vector, comprising other portions of the heavy and/or light chain, as applicable. (See also Sambrook et al. and Ausubel et al., supra)

A nucleotide sequence encoding an antibody can be obtained, for example, from the GenBank database or a similar database, literature publications, or by routine cloning and sequencing. Examples of some methods that can be used for directed mutagenesis are as follows: oligonucleotide directed mutagenesis with M13 DNA, oligonucleotide directed mutagenesis with plasmid DNA, and PCR-amplified oligonucleotide directed mutagenesis. (See, e.g., Glick et al., Molecular Biotechnology: Principles and Applications of Recombinant DNA, Second Edition, ASM Press, pp. 171-182 (1998). An example of mutagenesis and cloning is described in Example 1.

Detailed protocols for oligonucleotide-directed mutagenesis and related techniques for mutagenesis of cloned DNA are well-known (see, e.g., Zoller and Smith, Nucleic Acids Res. 10:6487-6500 (1982); see also Sambrook et al., supra; and Ausubel et al., supra).

In some embodiments, the amino acid substitution is a serine for a cysteine residue. In some embodiments, the amino acid substitution introduces is a serine or threonine residue. In some embodiments, the amino acid substitution introduces a neutral (e.g., serine, threonine or glycine) or hydrophilic (e.g., methionine, alanine, valine, leucine or isoleucine) residue. In some embodiments, the amino acid substitution introduces a natural amino acid, other than a cysteine residue.

Although the present invention provides methods for introducing an amino acid substitution of an interchain cysteine residue (e.g., a cysteine to serine substitution) into an

antibody or antibody fragment, it will be understood that the present invention is not so limited. It will occur to those of ordinary skill in the art that it is possible to introduce/remove other amino acids for conjugation, such as lysine residues, at other positions of the antibody or antibody fragment. Also, a sulfhydryl group(s) can also be recombinantly introduced into an antibody at an amino acid other than an interchain cysteine residue. Suitable alternative mutagenesis sites for conjugation can be identified using molecular modeling techniques that are well-known to those of skill in the art. See, for example, Lesk et al., "Antibody Structure and Structural Predictions Useful in Guiding Antibody Engineering," in Antibody Engineering: A Practical Guide, C. Borrebaeck (ed.), W. H. Freeman and Company, pp. 1-38 (1992); Cheetham, "Engineering Antibody Affinity," Antibody Engineering: A Practical Guide (supra) at pp. 39-67. See generally Sambrook et al., Molecular Cloning, A Laboratory Manual, 3rd ed., Cold Spring Harbor Publish., Cold Spring Harbor, New York (2001); Ausubel et al., Current Protocols in Molecular Biology, 4th ed., John Wiley and Sons, New York (1999) (all of which are incorporated by reference herein), for methods for site-directed mutagenesis.

Methods for Expressing and Isolating the Protein Product of an Engineered Antibody DNA Sequence

A. Methods for Expressing an Engineered Antibody

After altering the nucleotide sequence, the nucleic acid is inserted into a cloning vector for further analysis, such as confirmation of the nucleic acid sequence. To express the polypeptide encoded by the nucleic acid, the nucleic acid can be operably linked to regulatory sequences controlling transcriptional expression in an expression vector, then introduced into a prokaryotic or eukaryotic host cell. In addition to transcriptional regulatory sequences, such as promoters and enhancers, expression vectors may include translational regulatory sequences and/or a marker gene which is suitable for selection of cells that contain the expression vector.

Promoters for expression in a prokaryotic host can be repressible, constitutive, or inducible. Suitable promoters are well-known to those of skill in the art and include, for example, promoters for T4, T3, Sp6 and T7 polymerases, the P_R and P_L promoters of bacteriophage lambda, the trp, recA, heat shock, and lacZ promoters of E. coli, the alpha-amylase and the sigma$_{28}$ -specific promoters of B. subtilis, the promoters of the bacteriophages of Bacillus, Streptomyces promoters, the int promoter of bacteriophage

lambda, the bla promoter of the beta-lactamase gene of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene. Prokaryotic promoters are reviewed by Glick, J. Ind. Microbiol. 1:277-282 (1987); Watson et al., Molecular Biology Of The Gene, Fourth Edition, Benjamin Cummins (1987); Ausubel et al., supra; and Sambrook et al., supra.

In some embodiments, the prokaryotic host is E. coli. Suitable strains of E. coli include, for example, Y1088, Y1089, CSH18, ER1451 and ER1647 (see, e.g., Brown (Ed.), Molecular Biology Labfax, Academic Press (1991)). An alternative host is Bacillus subtilus, including such strains as BR151, YB886, MI119, MI120 and B170 (see, e.g., Hardy, "Bacillus Cloning Methods," in DNA Cloning: A Practical Approach, Glover (Ed.), IRL Press (1985)).

Methods for producing antibody fragments in E. coli are well-known to those in the art. See, for example, Huse, "Combinatorial Antibody Expression Libraries in Filamentous Phage," in Antibody Engineering: A Practical Guide, C. Borrebaeck (Ed.), W. H. Freeman and Company, pp. 103-120 (1992); Ward, "Expression and Purification of Antibody Fragments Using Escherichia coli as a Host," id. at pp. 121-138 (1992). Fv fragments can also be produced by methods known in the art. See, e.g., id. See also Whitlow et al., "Single-Chain Fv Proteins and their Fusion Proteins," in New Techniques In Antibody Generation, Methods 2(2) (1991). Moreover, certain expression systems for cloning antibodies in prokaryotic cells are commercially available.

In some embodiments, the nucleic acid sequence is expressed in eukaryotic cells, and especially mammalian, insect, and yeast cells. In one embodiment, the eukaryotic host is a mammalian cell. Mammalian cells provide post-translational modifications to the cloned polypeptide including proper folding and glycosylation. For example, such mammalian host cells include COS-7 cells (e.g., ATCC CRL 1651), non-secreting myeloma cells (e.g., SP2/0-AG14; ATCC CRL 1581), Chinese hamster ovary cells (e.g., CHO-K1, ATCC CCL 61; CHO-DG44, Urlaub et al., Somat Cell Mol Genet. 12(6):555-66 (1986)), rat pituitary cells (e.g., GH_1; ATCC CCL 82), HeLa S3 cells (e.g., ATCC CCL 2.2), and rat hepatoma cells (e.g., H-4-II-E; ATCC CRL 1548).

For a mammalian host, the transcriptional and translational regulatory signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, and simian virus. In addition, promoters from mammalian cells, such as actin, collagen, or myosin, can be employed. Alternatively, a prokaryotic promoter (such as the bacteriophage T3 RNA polymerase promoter) can be employed, wherein the prokaryotic promoter is

regulated by a eukaryotic promoter (for example, see Zhou et al., Mol. Cell. Biol. 10:4529-4537 (1990); Kaufman et al., Nucl. Acids Res. 19:4485-4490 (1991)). Transcriptional initiation regulatory signals may be selected which allow for repression or activation, so that expression of the genes can be modulated.

In general, eukaryotic regulatory regions will include a promoter region sufficient to direct the initiation of RNA synthesis. Such a eukaryotic promoter can be, for example, the promoter of the mouse metallothionein I gene (Hamer et al., J. Mol. Appl. Gen. 1:273-288 (1982)); the TK promoter of Herpes virus (McKnight, Cell 31:355-365 (1982)); the SV40 early promoter (Benoist et al., Nature (London) 290:304-310 (1981)); the Rous sarcoma virus promoter ((Gorman, "High Efficiency Gene Transfer into Mammalian cells," in DNA Cloning: A Practical Approach, Volume II, Glover (Ed.), IRL Press, pp. 143-190 (1985)); the cytomegalovirus promoter (Foecking et al., Gene 45:101 (1980)); the yeast gal4 gene promoter (Johnston et al., Proc. Natl. Acad. Sci. USA 79:6971-6975 (1982); Silver et al., Proc. Natl. Acad. Sci. USA 81:5951-5955 (1984)); and the IgG promoter (Orlandi et al., Proc. Natl. Acad. Sci. USA 86:3833-3837 (1989)).

Strong regulatory sequences can be used. Examples of such regulatory sequences include the SV40 promoter-enhancer (Gorman, "High Efficiency Gene Transfer into Mammalian cells," in DNA Cloning: A Practical Approach, Volume II, Glover (Ed.), IRL Press, pp. 143-190 (1985)), the hCMV-MIE promoter-enhancer (Bebbington et al., Bio/Technology 10:169-175 (1992)), Chinese Hamster EF-1α promoter (see, e.g., U.S. Patent No. 5,888,809) and antibody heavy chain promoter (Orlandi et al., Proc. Natl. Acad. Sci. USA 86:3833-3837 (1989)). Also included are the kappa chain enhancer for the expression of the light chain and the IgH enhancer (Gillies, "Design of Expression Vectors and Mammalian Cell Systems Suitable for Engineered Antibodies," in Antibody Engineering: A Practical Guide, C. Borrebaeck (Ed.), W. H. Freeman and Company, pp. 139-157 (1992); Orlandi et al., supra).

The engineered antibody-encoding nucleic acid and an operably linked promoter may be introduced into eukaryotic cells as a non-replicating DNA molecule, which may either be a linear molecule or a circular molecule. Since such molecules are incapable of autonomous replication, the expression of the protein may occur through the transient expression of the introduced sequence. In one aspect, permanent expression occurs through the integration of the introduced sequence into the host chromosome.

In some embodiments, the introduced nucleic acid will be incorporated into a plasmid or viral vector that is capable of autonomous replication in the recipient host. Numerous possible vector systems are available for this purpose. One class of vectors utilize DNA elements which provide autonomously replicating extra-chromosomal plasmids, derived from animal viruses such as bovine papilloma virus, polyoma virus, adenovirus, or SV40 virus. A second class of vectors relies upon the integration of the desired genomic or cDNA sequences into the host chromosome. Additional elements may also be needed for optimal synthesis of mRNA. These elements may include splice signals, as well as transcription promoters, enhancers, and termination signals. The cDNA expression vectors incorporating such elements include those described by Okayama, Mol. Cell. Biol. 3:280 (1983), Sambrook et al., supra, Ausubel et al., supra, Bebbington et al., supra, Orlandi et al., supra, Fouser et al., Bio/Technology 10:1121-1127 (1992); and Gillies, supra. Genomic DNA expression vectors which include intron sequences are described by Orlandi et al., supra. Also, see generally, Lerner et al. (Eds.), New Techniques In Antibody Generation, Methods 2(2) (1991).

To obtain mammalian cells that express intact antibody, the expression vector comprising a nucleic acid encoding an antibody light chain can be co-transfected or transfected into mammalian cells with an antibody heavy chain expression vector. Alternatively, mammalian cells containing a heavy chain expression vector can be transfected with an antibody light chain expression vector, or mammalian cells containing an antibody light chain expression vector can be transfected with an antibody heavy chain expression vector. Moreover, mammalian cells can be transfected with a single expression vector comprising nucleic acid (e.g., DNA) fragments that encode an antibody light chain, as well as nucleic acid (e.g., DNA) fragments that encode antibody heavy chain. See, for example, Gillies, supra; Bebbington et al., supra. Any of these approaches will produce transfected cells that express whole engineered antibody molecules. Standard transfection and transformation techniques are well known in the art. See, for example, Sambrook et al., supra; Ausubel et al., supra.

An example of cell line development and protein expression is described in Example 1.

B. Methods for Isolating an Engineered Antibody from Transfected Cells

Transformed or transfected cells that carry the expression vector are selected using the appropriate drug. For example, G418 can be used to select transfected cells carrying

an expression vector having the aminoglycoside phosphotransferse gene. (See, e.g., Southern et al., J. Mol. Appl. Gen. 1:327-341 (1982).) Alternatively, hygromycin-B can be used to select transfected cells carrying an expression vector having the hygromycin-B-phosphotransferase gene. (See, e.g., Palmer et al., Proc. Natl. Acad. Sci. USA 84:1055-1059 (1987).) Aminopterin and mycophenolic acid can be used to select transfected cells carrying an expression vector having the xanthine-guanine phosphoribosyltransferase gene. (See, e.g., Mulligan et al., Proc. Natl. Acad. Sci. USA 78:2072-2076 (1981).) Methotrexate can be used to select transformed cells carrying an expression vector having the dihydrofolate reductase gene. (See, e.g., Wigler et al., Proc Natl. Acad. Sci. USA 77(6):3567-70 (1980).)

Transformed or transfected cells that produce the engineered antibody can be identified using a variety of methods. For example, any immunodetection assay can be used to identify such "transfectomas."

After transformants or transfectants have been identified, the cells are cultured and antibodies are isolated from the cells and/or the culture supernatants. Isolation techniques include affinity chromatography with Protein-A Sepharose, size-exclusion chromatography, and ion-exchange chromatography. For example, see Coligan et al. (eds.), Current Protocols In Immunology, John Wiley and Sons (1991), for detailed protocols.

Methods for Preparing Immunoconjugates

A. Preparation of Antibody Fragments

The present invention also provides immunoconjugates of engineered antibodies or from antigen-binding antibody fragments. Antibody fragments can be obtained from, for example, recombinant host cells (e.g., transformants or transfectants) and/or by proteolytic cleavage of intact engineered antibodies. Antibody fragments can be obtained directly from transformants or transfectants by transfecting cells with a heavy chain structural gene that has been mutated. For example, transfectomas can produce Fab fragments if a stop codon is inserted following the sequence of the C_H1 domain. Alternatively, transfectomas can produce Fab' or $F(ab')_2$ fragments if a stop codon is inserted after the sequence encoding the hinge region of the heavy chain.

Alternatively, antibody fragments can be prepared from intact antibodies using well-known proteolytic techniques. For example, see, Coligan et al., supra. Moreover,

$F(ab')_2$ fragments can be obtained using pepsin digestion of intact antibodies. Divalent fragments can be cleaved to monovalent fragments using conventional disulfide bond reducing agents, e.g., dithiothreitol (DTT) and the like.

B. Methods of Conjugation

A wide variety of diagnostic, preventative and therapeutic agents can be advantageously conjugated to the antibodies of the invention. In some embodiments, can antibody can be stoichiometrically or fully-loaded (i.e., C# = Y, where Y refers to the average number of active moieties attached to each antibody molecule). In other embodiments, an antibody can be partially-loaded (i.e., C# > Y).

Immunoconjugates can be prepared by conjugating a diagnostic, preventative or therapeutic agent to an intact antibody, or antigen-binding fragment thereof. Such techniques are described in Shih et al., Int. J. Cancer 41:832-839 (1988); Shih et al., Int. J. Cancer 46:1101-1106 (1990); Shih et al., U.S. Pat. No. 5,057,313; Shih Cancer Res. 51:4192, International Publication WO 02/088172; U.S. Pat. No. 6,884,869; International Patent Publication WO 2005/081711; and U.S. Published Application 2003-0130189 A1, all of which are incorporated by reference herein.

In addition, those of skill in the art will recognize numerous possible variations of conjugation methods. For example, it is possible to construct a "divalent immunoconjugate" by attaching a diagnostic or therapeutic agent to a carbohydrate moiety and to a free sulfhydryl group.

In some embodiments, the interchain cysteine residues are present as a disulfide bond as a result of the oxidation of the thiol (--SH) side groups of the cysteine residues. Treatment of the disulfide bond with a reducing agent can causes reductive cleavage of the disulfide bonds to leave free thiol groups.

In some embodiments, the agent has, or is modified to include, a group reactive with an interchain cysteine residue. For example, an agent can be attached by conjugation to thiols. For examples of chemistries that can be used for conjugation, see, e.g., Current Protocols in Protein Science (John Wiley & Sons, Inc.), Chapter 15 (Chemical Modifications of Proteins) (the disclosure of which is incorporated by reference herein in its entirety).

For example, when chemical activation of the antibody results in formation of free thiol groups, the protein may be conjugated with a sulfhydryl reactive agent. In some embodiments, the agent is one which is substantially specific for free thiol groups. Such

agents include, for example, malemide, haloacetamides (e.g., iodo, bromo or chloro), haloesters (e.g., iodo, bromo or chloro), halomethyl ketones (e.g., iodo, bromo or chloro), benzylic halides (e.g., iodide, bromide or chloride), vinyl sulfone and pyridyithio.

In specific embodiments, the sulfyhydryl reactive agent can be an alpha-haloacetyl compounds such as iodoacetamide, maleimides such as N-ethylmaleimide, mercury derivatives such as 3,6-bis-(mercurimethyl)dioxane with counter ions of acetate, chloride or nitrate, and disulfide derivatives such as disulfide dioxide derivatives, polymethylene bismethane thiosulfonate reagents and crabescein (a fluorescent derivative of fluorescein containing two free sulfhydryl groups which have been shown to add across disulfide bonds of reduced antibody).

Alpha-haloacetyl compounds such as iodoacetate readily react with sulfhydryl groups to form amides. These compounds have been used to carboxymethylate free thiols. They are not strictly SH specific and will react with amines. The reaction involves nucleophilic attack of the thiolate ion resulting in a displacement of the halide. The reactive haloacetyl moiety, X--CH_2CO--, has been incorporated into compounds for various purposes. For example, bromotrifluoroacetone has been used for F-19 incorporation, and N-chloroacetyliodotyramine has been employed for the introduction of radioactive iodine into proteins.

Maleimides such as N-ethylmaleimide are considered to be fairly specific to sulfhydryl groups, especially at pH values below 7, where other groups are protonated. Thiols undergo Michael reactions with maleimides to yield exclusively the adduct to the double bond. The resulting thioether bond is very stable. They also react at a much slower rate with amino and imidazoyl groups. At pH 7, for example, the reaction with simple thiols is about 1,000 fold faster than with the corresponding amines. The characteristic absorbance change in the 300 nm region associated with the reaction provides a convenient method for monitoring the reaction. These compounds are stable at low pH but are susceptible to hydrolysis at high pH. See generally Wong, Chemistry of Protein Conjugation and Cross-linking; CRC Press, Inc., Boca Raton, 1991: Chapters 2 and 4.

An agent (such as a drug) which is not inherently reactive with sulfhydryls may still be conjugated to the chemically activated antibody by means of a bifunctional crosslinking agent which bears both a group reactive with the agent and a sulfhydryl reactive group. The cross-linking agent may be reacted simultaneously with both the molecule of interest (e.g., through an amino, carboxy or hydroxy group) and the

chemically activated protein, or it may be used to derivatize the molecule of interest to form a partner molecule which is then sulfhydryl reactive by virtue of a moiety derived from the agent, or it may be used to derivatize the chemically activated protein to make it reactive with the molecule of interest.

The agent also can be linked to an antibody by a linker. Suitable linkers include, for example, cleavable and non-cleavable linkers. A cleavable linker is typically susceptible to cleavage under intracellular conditions. Suitable cleavable linkers include, for example, a peptide linker cleavable by an intracellular protease, such as lysosomal protease or an endosomal protease. In exemplary embodiments, the linker can be a dipeptide linker, such as a valine-citrulline (val-cit) or a phenylalanine-lysine (phe-lys) linker. Other suitable linkers include linkers hydrolyzable at a pH of less than 5.5, such as a hydrazone linker. Additional suitable cleavable linkers include disulfide linkers.

A linker can include a group for linkage to the antibody. For example, a linker can include a sulfhydryl reactive group(s) (e.g., malemide, haloacetamides (e.g., iodo, bromo or chloro), haloesters (e.g., iodo, bromo or chloro), halomethyl ketones (e.g., iodo, bromo or chloro), benzylic halides (e.g., iodide, bromide or chloride), vinyl sulfone and pyridyithio). See generally Wong, Chemistry of Protein Conjugation and Cross-linking; CRC Press, Inc., Boca Raton, 1991.

In certain embodiments, the immunoconjugate has the following formula:

$$Ab_z\left(A_a-W_w-Y_y-D\right)_p$$

or pharmaceutically acceptable salts or solvates thereof,

wherein:

Ab is an antibody,

A is a stretcher unit,

a is 0 or 1,

each W is independently a linker unit,

w is an integer ranging from 0 to 12,

Y is a spacer unit, and

y is 0, 1 or 2,

p ranges from 1 to about 20, and

D is a diagnostic, preventative and therapeutic agent,

z is the number of predetermined conjugation sites on the protein.

When the antibody is fully loaded, p=z. When the antibody is partially loaded, p<z. In some embodiments, p is an even integer. In specific embodiments, p = 2, 4, 6 or 8. In a specific embodiment, p = z = 4. In other embodiments, 0 < p <8.

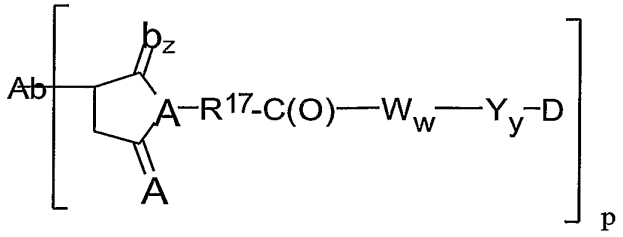
A stretcher unit can is capable of linking a linker unit to an antibody. The stretcher unit has a functional group that can form a bond with an interchain cysteine residue of the antibody. Useful functional groups include, but are not limited to, sulfhydryl reactive groups, as described above..

The linker unit is typically an amino acid unit, such as for example a dipeptide, tripeptide, tetrapeptide, pentapeptide, hexapeptide, heptapeptide, octapeptide, nonapeptide, decapeptide, undecapeptide or dodecapeptide unit. The linker unit can be cleavage or non-cleavable inside the cell.

In one embodiment, the amino acid unit is valine-citrulline. In another embodiment, the amino acid unit is phenylalanine-lysine. In yet another embodiment, the amino acid unit is N-methylvaline-citrulline. In yet another embodiment, the amino acid unit is 5-aminovaleric acid, homo phenylalanine lysine, tetraisoquinolinecarboxylate lysine, cyclohexylalanine lysine, isonepecotic acid lysine, beta-alanine lysine, glycine serine valine glutamine and isonepecotic acid. In certain embodiments, the Amino Acid unit can comprise natural amino acids. In other embodiments, the Amino Acid unit can comprise non-natural amino acids.

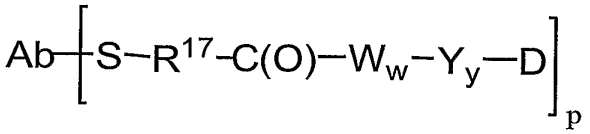
A spacer unit, if present, links a linker unit to D. Alternately, a spacer unit can link a stretcher unit to a drug moiety when the linker unit is absent. The spacer unit can also link a diagnostic, preventative and therapeutic agents to an antibody when both the linker unit and stretcher unit are absent. In one embodiment, the spacer unit is a p-aminobenzyl alcohol (PAB) unit, a p-aminobenzyl ether unit, or p-aminobenzyl carbamoyl unit. (See, e.g., U.S. Patent Publication Nos. 2003-0130189).

In some embodiments, the immunoconjugate has the formula:



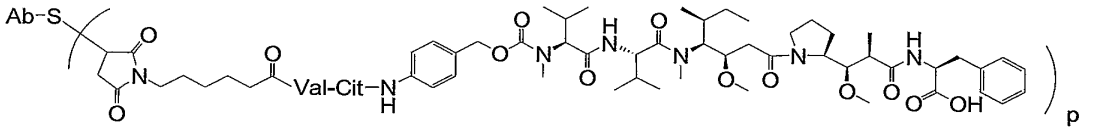
wherein R^{17} is selected from -C_1-C_{10} alkylene-, -C_3-C_8 carbocyclo-, -O-(C_1-C_8 alkyl)-, -arylene-, -C_1-C_{10} alkylene-arylene-, -arylene-C_1-C_{10} alkylene-, -C_1-C_{10} alkylene-(C_3-C_8 carbocyclo)-, -(C_3-C_8 carbocyclo)-C_1-C_{10} alkylene-, -C_3-C_8 heterocyclo-, -C_1-C_{10} alkylene-(C_3-C_8 heterocyclo)-, -(C_3-C_8 heterocyclo)-C_1-C_{10} alkylene-, -$(CH_2CH_2O)_r$-, and -$(CH_2CH_2O)_r$-CH_2-. In some embodiments, R^{17} is -$(CH_2)_5$- or $(CH_2CH_2O)_r$-CH_2- and r is 2.

In another embodiment, the immunoconjugate has the formula:

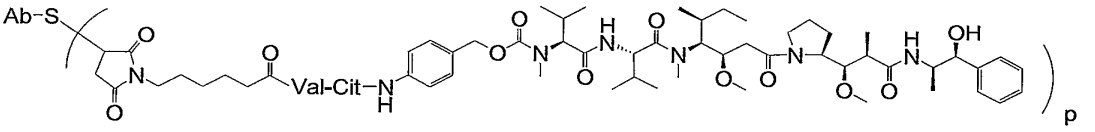


wherein R^{17} is as defined above.

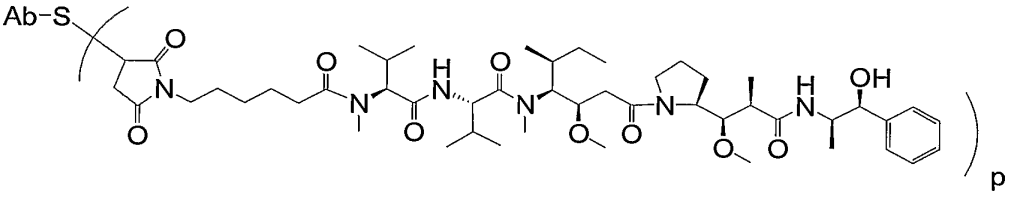
In additional embodiments, the immunoconjugate has one of the following formulae:



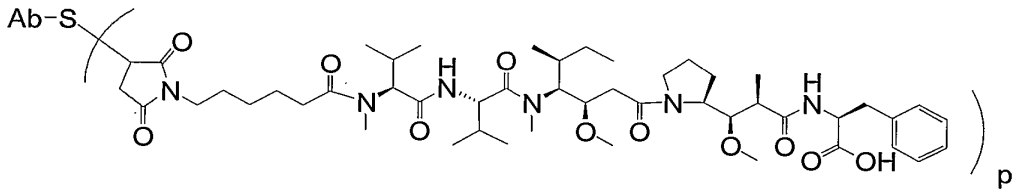
Ab-MC-vc-PAB-MMAF



Ab-MC-vc-PAB-MMAE



Ab-MC-MMAE



Ab-MC-MMAF

The final immunoconjugate may be purified using conventional techniques, such as sizing chromatography on Sephacryl S-300, affinity chromatography such as protein A or protein G sepharose, or the like.

Examples of protein purification and conjugation is described in Examples 1 and 2.

Use of Immunoconjugates for Diagnosis and Therapy

A. Use of Immunoconjugates for Diagnosis

The immunoconjugates can be used for diagnostic imaging. For example, the immunoconjugate can be a radiolabeled monoclonal antibody. See, for example, Srivastava (ed.), Radiolabeled Monoclonal Antibodies For Imaging And Therapy, Plenum Press (1988); Chase, "Medical Applications of Radioisotopes," in Remington's Pharmaceutical Sciences, 18th Edition, Gennaro et al. (eds.), Mack Publishing Co., pp. 624-652 (1990); and Brown, "Clinical Use of Monoclonal Antibodies," in Biotechnology and Pharmacy, Pezzuto et al. (eds.), Chapman and Hall, pp. 227-249 (1993). This technique, also known as immunoscintigraphy, uses a gamma camera to detect the location of gamma-emitting radioisotopes conjugated to monoclonal antibodies. Diagnostic imaging can be used to diagnose cancer, autoimmune disease, infectious disease and/or cardiovascular disease. (See, e.g., Brown, supra.)

In one example, the immunoconjugates can be used to diagnose cardiovascular disease. For example, immunoconjugates comprising anti-myosin antibody fragments can be used for imaging myocardial necrosis associated with acute myocardial infarction. Immunoconjugates comprising antibody fragments that bind to platelets or fibrin can be used for imaging deep-vein thrombosis. Moreover, immunoconjugates comprising antibody fragments that bind to activated platelets can be used for imaging atherosclerotic plaque.

Immunoconjugates can also be used in the diagnosis of infectious diseases. For example, immunoconjugates comprising antibody fragments that bind specific bacterial antigens can be used to localize abscesses. In addition, immunoconjugates comprising

antibody fragments that bind granulocytes and inflammatory leukocytes can be used to localize sites of bacterial infection.

Numerous studies have evaluated the use of monoclonal antibodies for scintigraphic detection of cancer. See, for example, Brown, supra. Investigations have covered the major types of solid tumors such as melanoma, colorectal carcinoma, ovarian carcinoma, breast carcinoma, sarcoma, and lung carcinoma. Thus, the present invention also contemplates the detection of cancer using immunoconjugates comprising antibody fragments that bind tumor markers to detect cancer. Examples of such tumor markers include carcinoembryonic antigen, alpha-fetoprotein, oncogene products, tumor-associated cell surface antigens, and necrosis-associated intracellular antigens, as well as the tumor-associated antigens and tumor-specific antigens discussed infra.

In addition to diagnosis, monoclonal antibody imaging can be used to monitor therapeutic responses, detect recurrences of a disease, and guide subsequent clinical decisions.

For diagnostic and monitoring purposes, radioisotopes may be bound to antibody fragments either directly or indirectly by using an intermediary functional group. Such intermediary functional groups include, for example, DTPA (diethylenetriaminepentaacetic acid) and EDTA (ethylene diamine tetraacetic acid). The radiation dose delivered to the patient is typically maintained at as low a level as possible. This may be accomplished through the choice of isotope for the best combination of minimum half-life, minimum retention in the body, and minimum quantity of isotope which will permit detection and accurate measurement. Examples of radioisotopes which can be bound to antibodies and are appropriate for diagnostic imaging include ^{99}mTc and ^{111}In.

Studies indicate that antibody fragments, particularly Fab and Fab', provide suitable tumor/background ratios. (See, e.g., Brown, supra.)

The immunoconjugates also can be labeled with paramagnetic ions for purposes of in vivo diagnosis. Elements which are particularly useful for Magnetic Resonance Imaging include Gd, Mn, Dy, and Fe ions.

The immunoconjugates can also detect the presence of particular antigens in vitro. In such immunoassays, the immunoconjugates may be utilized in liquid phase or bound to a solid-phase carrier. For example, an intact antibody, or antigen-binding fragment thereof, can be attached to a polymer, such as aminodextran, in order to link the antibody component to an insoluble support such as a polymer-coated bead, plate, or tube.

Alternatively, the immunoconjugates can be used to detect the presence of particular antigens in tissue sections prepared from a histological specimen. Such in situ detection can be accomplished, for example, by applying a detectably-labeled immunoconjugate to the tissue sections. In situ detection can be used to determine the presence of a particular antigen and to determine the distribution of the antigen in the examined tissue. General techniques of in situ detection are well known to those of ordinary skill. (See, e.g., Ponder, "Cell Marking Techniques and Their Application," in Mammalian Development: A Practical Approach, Monk (ed.), IRL Press, pp. 115-138 (1987); Coligan et al., supra.)

Detectable labels such as enzymes, fluorescent compounds, electron transfer agents, and the like can be linked to a carrier by conventional methods well known to the art. These labeled carriers and the immunoconjugates prepared from them can be used for in vitro immunoassays and for in situ detection, much as an antibody conjugate can be prepared by direct attachment of the labels to antibody. The loading of the immunoconjugate with a plurality of labels can increase the sensitivity of immunoassays or histological procedures, where only a low extent of binding of the antibody, or antibody fragment, to target antigen is achieved.

B. Use of Immunoconjugates for Therapy

Immunoconjugates can be used to treat viral and bacterial infectious diseases, cardiovascular disease, autoimmune disease, and cancer. The objective of such therapy is to deliver cytotoxic or cytostatic doses of an active agent (e.g., radioactivity, a toxin, or a drug) to target cells, while minimizing exposure to non-target tissues.

A radioisotope can be attached to an intact antibody, or antigen-binding fragment thereof, directly or indirectly, via a chelating agent. For example, ^{67}Cu can be conjugated to an antibody component using the chelating agent, p-bromo-acetamidobenzyl-tetraethylaminetetraacetic acid (TETA). (See, e.g., Chase, supra.)

Moreover, immunoconjugates can be prepared in which the therapeutic agent is a toxin or drug. Useful toxins for the preparation of such immunoconjugates include ricin, abrin, pokeweed antiviral protein, gelonin, diphtherin toxin, and Pseudomonas endotoxin. Useful chemotherapeutic drugs for the preparation of immunoconjugates include auristatin, dolastatin, MMAE, MMAF, AFP, AEB, doxorubicin, daunorubicin, methotrexate, melphalin, chlorambucil, vinca alkaloids, 5-fluorouridine, mitomycin-C, taxol, L-asparaginase, mercaptopurine, thioguanine, hydroxyurea, cytarabine,

cyclophosphamide, ifosfamide, nitrosoureas, cisplatin, carboplatin, mitomycin, dacarbazine, procarbizine, topotecan, nitrogen mustards, cytoxan, etoposide, BCNU, irinotecan, camptothecins, bleomycin, idarubicin, dactinomycin, plicamycin, mitoxantrone, asparaginase, vinblastine, vincristine, vinorelbine, paclitaxel, and docetaxel and salts, solvents and derivatives thereof. Other suitable agents include chelators, such as DTPA, to which detectable labels such as fluorescent molecules or cytotoxic agents such as heavy metals or radionuclides can be complexed; and toxins such as Pseudomonas exotoxin, and the like.

In some embodiments, the diagnostic, preventative or therapeutic agent is auristatin E (also known in the art as dolastatin-10) or a derivative thereof as well as pharmaceutically salts or solvates thereof. Typically, the auristatin E derivative is, e.g., an ester formed between auristatin E and a keto acid. For example, auristatin E can be reacted with paraacetyl benzoic acid or benzoylvaleric acid to produce AEB and AEVB, respectively. Other typical auristatin derivatives include AFP, MMAF, and MMAE. The synthesis and structure of auristatin E and its derivatives, as well as linkers, are described in U.S. Patent Application No. 09/845,786 (U.S. Patent Application Publication No. 20030083263), U.S. Patent Application Publication No. 2005-0238629; International Patent Application No. PCT/US03/24209; International Patent Application No. PCT/US02/13435; International Patent Application No. PCT/US02/13435; International Patent Publication No. WO 04/073656; and U.S. Patent Nos. 6,884,869; 6,323,315; 6,239,104; 6,214,345; 6,034,065; 5,780,588; 5,665,860; 5,663,149; 5,635,483; 5,599,902; 5,554,725; 5,530,097; 5,521,284; 5,504,191; 5,410,024; 5,138,036; 5,076,973; 4,986,988; 4,978,744; 4,879,278; 4,816,444; and 4,486,414 (all of which are incorporated by reference herein in their entirety).

In some embodiments, the anti-cancer agent includes, but is not limited to, a drug listed in Drug Table below.

Drug Table

Alkylating agents	
Nitrogen mustards:	cyclophosphamide
	Ifosfamide
	trofosfamide
	Chlorambucil
Nitrosoureas:	carmustine (BCNU)
	Lomustine (CCNU)
Alkylsulphonates	busulfan

	Treosulfan
Triazenes:	Dacarbazine
Platinum containing compounds:	Cisplatin
	carboplatin
Plant Alkaloids	
Vinca alkaloids:	vincristine
	Vinblastine
	Vindesine
	Vinorelbine
Taxoids:	paclitaxel
	Docetaxol
DNA Topoisomerase Inhibitors	
Epipodophyllins:	etoposide
	Teniposide
	Topotecan
	9-aminocamptothecin
	camptothecin
	crisnatol
mitomycins:	Mitomycin C
Anti-metabolites	
Anti-folates:	
DHFR inhibitors:	methotrexate
	Trimetrexate
IMP dehydrogenase Inhibitors:	mycophenolic acid
	Tiazofurin
	Ribavirin
	EICAR
Ribonuclotide reductase Inhibitors:	hydroxyurea
	deferoxamine
Pyrimidine analogs:	
Uracil analogs	5-Fluorouracil
	Floxuridine
	Doxifluridine
	Ratitrexed
Cytosine analogs	cytarabine (ara C)
	Cytosine arabinoside
	fludarabine
Purine analogs:	mercaptopurine
	Thioguanine
Hormonal therapies:	
Receptor antagonists:	
Anti-estrogen	Tamoxifen
	Raloxifene
	megestrol
LHRH agonists:	goscrclin
	Leuprolide acetate
Anti-androgens:	flutamide

	bicalutamide
Retinoids/Deltoids	
Vitamin D3 analogs:	EB 1089
	CB 1093
	KH 1060
Photodynamic therapies:	vertoporfin (BPD-MA)
	Phthalocyanine
	photosensitizer Pc4
	Demethoxy-hypocrellin A
	(2BA-2-DMHA)
Cytokines:	Interferon- α
	Interferon- γ
	Tumor necrosis factor
Others:	
Isoprenylation inhibitors:	Lovastatin
Dopaminergic neurotoxins:	1-methyl-4-phenylpyridinium ion
Cell cycle inhibitors:	staurosporine
Actinomycins:	Actinomycin D
	Dactinomycin
Bleomycins:	bleomycin A2
	Bleomycin B2
	Peplomycin
Anthracyclines:	daunorubicin
	Doxorubicin (adriamycin)
	Idarubicin
	Epirubicin
	Pirarubicin
	Zorubicin
	Mitoxantrone
MDR inhibitors:	verapamil
Ca^{2+}ATPase inhibitors:	thapsigargin

In some embodiments, the diagnostic, preventative or therapeutic agent is not a radioisotope.

In some embodiments, an immunoconjugate can be used to treat one of the following particular types of cancer:

Solid tumors, including but not limited to:

sarcoma

fibrosarcoma

myxosarcoma

liposarcoma

chondrosarcoma

osteogenic sarcoma

chordoma
angiosarcoma
endotheliosarcoma
lymphangiosarcoma
lymphangioendotheliosarcoma
synovioma
mesothelioma
Ewing's tumor
leiomyosarcoma
rhabdomyosarcoma
colon cancer
colorectal cancer
kidney cancer
pancreatic cancer
bone cancer
breast cancer
ovarian cancer
prostate cancer
esophogeal cancer
stomach cancer (e.g., gastrointestinal cancer)
oral cancer
nasal cancer
throat cancer
squamous cell carcinoma (e.g., of the lung)
basal cell carcinoma
adenocarcinoma (e.g., of the lung)
sweat gland carcinoma
sebaceous gland carcinoma
papillary carcinoma
papillary adenocarcinomas
cystadenocarcinoma
medullary carcinoma
bronchogenic carcinoma
renal cell carcinoma

hepatoma
bile duct carcinoma
choriocarcinoma
seminoma
embryonal carcinoma
Wilms' tumor
cervical cancer
uterine cancer
testicular cancer
small cell lung carcinoma
bladder carcinoma
lung cancer
non-small cell lung cancer
epithelial carcinoma
glioma
glioblastoma multiforme
astrocytoma
medulloblastoma
craniopharyngioma
ependymoma
pinealoma
hemangioblastoma
acoustic neuroma
oligodendroglioma
meningioma
skin cancer
melanoma
neuroblastoma
retinoblastoma
blood-borne cancers, including but not limited to:
acute lymphoblastic leukemia "ALL"
acute lymphoblastic B-cell leukemia
acute lymphoblastic T-cell leukemia
acute myeloblastic leukemia "AML"

acute promyelocytic leukemia "APL"
acute monoblastic leukemia
acute erythroleukemic leukemia
acute megakaryoblastic leukemia
acute myelomonocytic leukemia
acute nonlymphocyctic leukemia
acute undifferentiated leukemia
chronic myelocytic leukemia "CML"
chronic lymphocytic leukemia "CLL"
hairy cell leukemia
multiple myeloma
acute and chronic leukemias:
lymphoblastic
myelogenous
lymphocytic
myelocytic leukemias
Lymphomas:
Hodgkin's disease
non-Hodgkin's Lymphoma
Multiple myeloma
Waldenström's macroglobulinemia
Heavy chain disease
Polycythemia vera
Other cancers:
Peritoneal cancer
Hepatocellular cancer
Hepatoma
Salivary cancer
Vulval cancer
Thyroid
Penile cancer
Anal cancer
Head and neck cancer
Renal cell carcinoma

Acute anaplastic large cell carcinoma

Cutaneous anaplastic large cell carcinoma

In some embodiments, an immunoconjugate can be used to treat one of the following particular types of autoimmune disease:

Active Chronic Hepatitis

Addison's Disease

Allergic Alveolitis

Allergic Reaction

Allergic Rhinitis

Alport's Syndrome

Anaphlaxis

Ankylosing Spondylitis

Anti-phosholipid Syndrome

Arthritis

Ascariasis

Aspergillosis

Atopic Allergy

Atropic Dermatitis

Atropic Rhinitis

Behcet's Disease

Bird-Fancier's Lung

Bronchial Asthma

Caplan's Syndrome

Cardiomyopathy

Celiac Disease

Chagas' Disease

Chronic Glomerulonephritis

Cogan's Syndrome

Cold Agglutinin Disease

Congenital Rubella Infection

CREST Syndrome

Crohn's Disease

Cryoglobulinemia

Cushing's Syndrome
Dermatomyositis
Discoid Lupus
Dressler's Syndrome
Eaton-Lambert Syndrome
Echovirus Infection
Encephalomyelitis
Endocrine opthalmopathy
Epstein-Barr Virus Infection
Equine Heaves
Erythematosis
Evan's Syndrome
Felty's Syndrome
Fibromyalgia
Fuch's Cyclitis
Gastric Atrophy
Gastrointestinal Allergy
Giant Cell Arteritis
Glomerulonephritis
Goodpasture's Syndrome
Graft v. Host Disease
Graves' Disease
Guillain-Barre Disease
Hashimoto's Thyroiditis
Hemolytic Anemia
Henoch-Schonlein Purpura
Idiopathic Adrenal Atrophy
Idiopathic Pulmonary Fibritis
IgA Nephropathy
Inflammatory Bowel Diseases
Insulin-dependent Diabetes Mellitus
Juvenile Arthritis
Juvenile Diabetes Mellitus (Type I)
Lambert-Eaton Syndrome

Laminitis
Lichen Planus
Lupoid Hepatitis
Lupus
Lymphopenia
Meniere's Disease
Mixed Connective Tissue Disease
Multiple Sclerosis
Myasthenia Gravis
Pernicious Anemia
Polyglandular Syndromes
Presenile Dementia
Primary Agammaglobulinemia
Primary Biliary Cirrhosis
Psoriasis
Psoriatic Arthritis
Raynauds Phenomenon
Recurrent Abortion
Reiter's Syndrome
Rheumatic Fever
Rheumatoid Arthritis
Sampter's Syndrome
Schistosomiasis
Schmidt's Syndrome
Scleroderma
Shulman's Syndrome
Sjorgen's Syndrome
Stiff-Man Syndrome
Sympathetic Ophthalmia
Systemic Lupus Erythematosis
Takayasu's Arteritis
Temporal Arteritis
Thyroiditis
Thrombocytopenia

Thyrotoxicosis
Toxic Epidermal Necrolysis
Type B Insulin Resistance
Type I Diabetes Mellitus
Ulcerative Colitis
Uveitis
Vitiligo
Waldenstrom's Macroglobulemia
Wegener's Granulomatosis

The use of the immunoconjugates for the treatment of other cancers or autoimmune disorders is also contemplated and within the scope of the present invention.

C. Administration of Immunoconjugates

Generally, the dosage of administered immunoconjugate will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition, and previous medical history. Typically, it is desirable to provide the recipient with a dosage of immunoconjugate which is in the range of from about 1 pg/kg to 20 mg/kg (amount of agent/body weight of patient), although a lower or higher dosage may also be administered. For example, many studies have demonstrated successful diagnostic imaging with doses of 0.1 to 1.0 milligram, while other studies have shown improved localization with doses in excess of 10 milligrams. (See, e.g., Brown, supra.)

For therapeutic applications, generally about 10-200 milligrams of immunoconjugate will be administered, depending on protocol. In some embodiments, a dose is from about 0.5 mg/kg to about 20 mg/kg, or about 1 mg/kg to about 10 mg/kg or about 15 mg/kg. Some protocols provide for the administration daily for a period of several days, several weeks or several months. In some embodiments, an immunoconjugate is administered daily, 1-3 times per week, weekly, biweekly or monthly. To reduce patient sensitivity, it may be necessary to reduce the dosage and/or use antibodies from other species and/or use hypoallergenic antibodies, e.g., hybrid human or primate antibodies.

Administration of immunoconjugates to a patient can be intravenous, intraarterial, intraperitoneal, intramuscular, subcutaneous, intrapleural, intrathecal, by perfusion through a regional catheter, or by direct intralesional injection. When administering

immunoconjugates by injection, the administration may be by continuous infusion, or by single or multiple boluses.

The immunoconjugates can be formulated according to known methods to prepare pharmaceutically useful compositions, such as a medicament, whereby immunoconjugates are combined in a mixture with a pharmaceutically acceptable carrier. A composition is said to be a "pharmaceutically acceptable carrier" if its administration can be tolerated by a recipient patient. Sterile phosphate-buffered saline is one example of a pharmaceutically acceptable carrier. Other suitable carriers are well-known to those in the art. (See, e.g., Remington's Pharmaceutical Sciences, 18th Ed. (1990).)

For purposes of immunotherapy, an immunoconjugate and a pharmaceutically acceptable carrier are administered to a patient in a therapeutically effective amount. A "therapeutically effective amount" is the amount administered that is physiologically significant. An agent is physiologically significant if its presence results in a detectable change in the physiology of a recipient patient.

Additional pharmaceutical methods may be employed to control the duration of action of an immunoconjugate in a therapeutic application. Control release preparations can be prepared through the use of polymers to complex or adsorb an immunoconjugate. For example, biocompatible polymers include matrices of poly(ethylene-co-vinyl acetate) and matrices of a polyanhydride copolymer of a stearic acid dimer and sebacic acid. (See, e.g., Sherwood et al., Bio/Technology 10:1446-1449 (1992).) The rate of release of an immunoconjugate from such a matrix depends upon the molecular weight of the immunoconjugate, the amount of immunoconjugate within the matrix, and the size of dispersed particles. (See, e.g., Saltzman et al., Biophysical. J. 55:163-171 (1989); and Sherwood et al., supra.) Other solid dosage forms are described in Remington's Pharmaceutical Sciences, 18th Ed. (1990).

The present invention is not to be limited in scope by the specific embodiments described herein. Various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

The invention is further described in the following examples, which are not intended to limit the scope of the invention. Cell lines described in the following examples were maintained in culture according to the conditions specified by the American Type Culture Collection (ATCC) or Deutsche Sammlung von Mikroorganismen

und Zellkulturen GmbH, Braunschweig, Germany (DMSZ). Cell culture reagents were obtained from Invitrogen Corp., Carlsbad, CA.

EXAMPLES

Example 1.

Construction and Expression of cAC10 Cysteine Variants

Procedures

Construction of chimeric AC10 (cAC10) from the AC10 hybridoma and expression of cAC10 in a CHO cell line has been described (Wahl et al., Cancer Res. 62(13): 3736-42 (2002)).

(i) Mutagenesis and Cloning

Mutants of cAC10 were generated in pBluescript vectors containing cDNAs for either cAC10 heavy (SEQ ID NO:6) (in pBSSK-AC10H) or cAC10 light (SEQ ID NO:8) (in pBSSK-AC10L) chain and encoding the cAC10 heavy (SEQ ID NO:7) or cAC10 light (SEQ ID NO:9) chain, respectively . Mutagenesis was performed using the Quikchange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. A pBluescript vector containing the cAC10 heavy chain cDNA, pBSSK AC10H shown in Figure 4, was used as a template to generate heavy chain C226S, C229S double mutant (having cysteine to serine substitutions are positions 226 and 229). (Residue numbering is of the mature cAC10 heavy and light chains, excluding the signal sequences.) Primer C226S:C229S: 5'GACAAAACTCACACA**TCC**CCACCG**TCC**CCAGCACCTGAACTC (SEQ ID NO:1) and its reverse complement partner were used to introduce the amino acid substitutions (the mutated codons are underlined). The resulting plasmid was called pBSSK AC10H226,229, containing the cDNA for cAC10 H226/229 (SEQ ID NO: 14) and encoding the cAC10 heavy chain C226S, C229S double mutant (SEQ ID NO:15).

A cAC10 heavy chain C220S mutant was generated using pBSSK AC10H as a template and primer C220S: 5'GTTGAGCCCAAATCT**TCT**GACAAAACTCA-CACATGCCC (SEQ ID NO:2) and its reverse complement partner (the mutated codon is underlined) to produce construct pBSSK AC10H220 containing the cDNA for cAC10 H220 (SEQ ID NO:10) and encoding the cAC10 C220S mutant (SEQ ID NO:11). pBSSK AC10H220 was used as a template to generate heavy chain C220S, C226S double mutant

using primer C226S: 5'GACAAAACTCACACA**TCC**CCACCG-TGCCCAGC (SEQ ID NO:3) and its reverse complement partner (the second mutated codon is underlined). The resulting plasmid was called pBSSK AC10H220,226, containing the cDNA for cAC10 H220/226 (SEQ ID NO:12) and encoding the cAC10 heavy chain C220S, C226S double mutant (SEQ ID NO:13). pBSSK AC10 H226,229 was used as a template to generate heavy chain C220S, C226S, C229S mutant using primer C220S: 5'GTTGAGCCCAAATCT**TCT**GACAAAACTCACACATCCCC (SEQ ID NO:4) and its reverse complement partner (the mutated codon is underlined). The resulting plasmid was called pBSSK AC10 H220,226,229, containing the cDNA for cAC10 H220/226/229 (SEQ ID NO:16) and encoding the cAC10 heavy chain C220S, C226S, C229S mutant (SEQ ID NO:17).

A pBluescript vector containing the cAC10 light chain cDNA, pBSSK AC10L as shown in Figure 5, was used as a template to generate light chain C218S mutant pBSSK AC10L218 using primer C218S: 5'CTTCAACAGGGGAGAG**TCT**TAGACGCG-TATTGG (SEQ ID NO:5) and its reverse complement partner (the mutated codon is underlined). The resulting plasmid was called pBSSK AC10L218, containing the cDNA for cAC10 L218 (SEQ ID NO:18) and encoding the cAC10 light chain C218S mutant (SEQ ID NO:19).

cAC10 heavy chain parent and cysteine variant cDNAs were released from pBluescript by cleavage with restriction enzymes *XhoI* and *XbaI* and ligated into the pDEF38 expression vector (Running Deer and Allison, Biotechnol Prog. 20(3):880-9 (2004)) downstream of the CHEF EF-1α promoter. cAC10 light chain parent and cysteine variant cDNAs were released from pBluescript with *MluI* and cloned into the *MluI* site of pDEF38 downstream of the CHEF EF-1α promoter.

(ii) Stable cell line development and protein expression

The cAC10 variants were stably expressed in a CHO-DG44 cell line as previously described for the cAC10 parent antibody (Wahl et al., Cancer Res. 62:3736-3742 (2002)). pDEF38 expression constructs were linearized with restriction enzyme PvuI prior to transfection. Fifty micrograms of linearized pDEF38 cAC10 H chain parent or the cysteine variant construct was cotransfected with 50 μg of linearized pDEF38 cAC10 L chain parent or the cysteine variant construct into CHO-DG44 cells (Urlaub et al., Somat

Cell Mol Genet. 12(6):555-66 (1986)) by electroporation. Following electroporation, the cells were allowed to recover for 2 days in EX-CELL 325 PF CHO medium containing hypoxanthine and thymidine (JRH Bioscience, Lenexa, KS) and 4 mM L-glutamine (Invitrogen, Carlsbad, CA). After 2 days, stable cell lines expressing the cAC10 variants were selected by replacing the medium with selective medium without hypoxanthine and thymidine. Only cells that incorporated the plasmid DNA, which includes the selectable marker, were able to grow in the absence of hypoxanthine and thymidine. After cells were recovered, stable pools were scaled up to 30 ml shake flask cultures. Cell cloning was performed using a limited dilution method in a background of non-transfected CHO-DG44 feeder cells. Briefly, 0.5 transfected cells and 1000 non-transfected cells were plated per well of a microtiter plate in EX-CELL 325 PF CHO medium in the absence of hypoxanthine and thymidine. Following 7-10 days incubation individual colonies were picked and expanded. High titer clones were selected and cultured in spinners at a final volume of 2.5 L or WAVE bioreactors (WAVE Biotech LLC, Bridgewater, NJ) at a final volume of 5-10 L.

Results

cAC10, is a chimeric IgG_1 that binds to human CD30 (Wahl et al., supra). Antibody cAC10, has 4 solvent accessible inter-chain disulfide bonds that are readily reducible and conjugated to vcMMAE, a thiol-reactive auristatin drug in near quantitative yield (Doronina et al., Nat. Biotechnol. 21:778-784 (2003)). This ADC comprising the cAC10 parent antibody with all 8 accessible cysteines and loaded with vcMMAE is designated here as C8-E8 (Figure 1A). The accessible cysteines in cAC10 were systematically mutated to a homologous residue, serine, to generate antibody variants with either 4 (C4v1, C4v2 and C4v3) or 2 (C2v1 and C2v2) remaining accessible cysteines (Table 1 and Figure 1A). In addition, antibody variant C6v1 with heavy chain cysteine residue 226 changed to serine (not shown) had six accessible cysteines. These engineered antibody variants provided a starting point to create conjugates with precisely defined stoichiometry and site of drug attachment.

All antibody variants were expressed in stable CHO-DG44 cell lines at titers of 25-125 mg/L. The antibody variants were purified from 2.5 to 10 L cultures by protein A affinity and ion exchange chromatography (Table 1) and then analyzed by size exclusion chromatography and SDS-PAGE. All antibody variants, except C4v3, were estimated to be > 98 % monomeric by size exclusion chromatography (Table 2). All variants

electrophoresed under reducing conditions gave rise to two major bands consistent with the presence of heavy and light chains (data not shown). As for SDS-PAGE under non-reducing conditions, all antibody variants (except C4v3) gave electrophoretic patterns (Figure 1B) consistent with the anticipated inter-chain disulfide bonding pattern (Figure 1A). Antibody variant C4v3 was excluded from the remainder of these studies on the basis of its unanticipated electrophoretic behavior and a size exclusion chromatography profile that suggested significant aggregation.

Table 1
Generation and Characterization of Antibody Variants

cAC10 variant*	Location of Cys→Ser mutations†	Competition binding to Karpas-299 (IC_{50}, nM) #
C8	none (parent)	2.8 ± 0.1
C2v1	L214, H220, H226	2.2 ± 0.4
C2v2	H220, H226, H229	2.6 ± 0.4
C4v1	L214, H220	3.2 ± 0.4
C4v2	H226, H229	2.4 ± 0.1
C4v3	H220, H226	nd

*cAC10 variants are identified by the number of solvent accessible cysteine residues and, where necessary, a variant number. E.g., C2v1 denotes a cAC10 variant containing 2 solvent accessible cysteine residues (Figure 1A).
†L, light chain; H, heavy chain; numbering scheme of Kabat et al. (Sequences of Proteins of Immunological Interest, 5th ed. NIH, Bethesda, MD (1991)).
Mean (± SEM) for ≥ 3 independent experiments.
‖nd, not determined due to presence of shoulder and broadening of peak

Table 2
Protein recovery for each cAC10 cysteine variant following Protein A purification and results from size exclusion chromatography analysis.

cAC10 cys variant	**Protein recovery in mg/L culture (Protein A purified)**	**% monomer**
C2v1	120	98.8%
C2v2	92	99.1%
C4v1	33	99.5%
C4v2	36	94.6%
C4v3	37	*
C6v1	28	97.3%

* not determined due to presence of shoulder and broadening of peak.

Purified proteins were analyzed by SDS-PAGE under reducing and non-reducing conditions. All variants except cAC10 C4v3 displayed the expected banding pattern under non-reducing conditions as shown in Table 3 and Figure 1B.

Table 3
Expected band patterns and molecular weights for variants analyzed under non-reducing conditions by SDS-PAGE

cAC10 Variant	Cysteine mutations	Non-reduced band pattern	MWs (kDa)
C2v1	L218, H220/226	HH + L	98 + 24
C2v2	H220/226/229	H + L	49 + 24
C4v1	L218, H220	HH + L	98 + 24
C4v2	H226/229	HL	73
C4v3	H220/226	HH + L	98 + 24
C6v1	H226	HHLL	146

Aggregation was assessed by size exclusion high-performance liquid chromatography and all variants except cAC10 C4v3 were determined to be > 94 % monomeric. cAC10 C4v3 was found to be heterogeneous by both non-reducing SDS-PAGE and size exclusion analysis. The banding pattern of cAC10 C4v3 under non-reducing conditions included the expected heavy-heavy chain dimer and light chain bands as shown in Table 3 but also a heavy-light chain dimer and heavy chain alone suggesting that the free light chain cysteine was capable of forming a disulfide bond with the heavy chain cysteine at position H229.

Preparation and analysis of antibody drug conjugates

Procedures

(i) Preparation of antibody drug conjugates

cAC10 parent and cysteine variant antibodies were purified using Protein A chromatography and analyzed by SDS-PAGE and size exclusion chromatography. All cAC10 cysteine variants except cAC10 C6v1 were reduced using 10 mM dithiothreitol (DTT; Sigma, St Louis, MO), which was an excess over antibody of approximately 100X, in 0.025 M sodium borate pH 8, 0.025 M NaCl, and 1 mM diethylenetriaminepentaacetic acid (DTPA; Aldrich, Milwaukee, WI) for 1 h at 37 °C. The reduced antibody was diluted to 150 mL with water and applied to a 70 mL hydroxyapatite column (Macroprep ceramic

type I 40 μm, BioRad) at a flow rate of 10 mL/min. The column was previously equilibrated with 5 column volumes of 0.5 M sodium phosphate pH 7, 10 mM NaCl and 5 column volumes of 10 mM sodium phosphate pH 7, 10 mM NaCl. Following application, the column was washed with 5 column volumes of 10 mM sodium phosphate pH 7, 10 mM NaCl and then eluted with 100 mM sodium phosphate pH 7, 10 mM NaCl. Reduced antibody was titrated with 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB; Pierce) to determine the concentration of antibody-cysteine thiols. Reduced antibody was cooled to 0 °C and treated with 2.75 equivalents of maleimidocaproyl-valine-citrulline-p-aminobenyzoyl-MMAE (vcMMAE) in DMSO. The final DMSO concentration was 10% to ensure that the drug was fully soluble. After 40 min at 0 °C, excess cysteine was added to quench any unreacted vcMMAE and the mixture was diluted to 250 mL with water. The conjugate was purified on a hydroxyapatite column as described above. Antibody-drug conjugates were concentrated and the buffer changed to PBS using 15 mL Amicon Ultrafree 30K cutoff spin concentration devices. cAC10 C6v1 was reduced with a limited number of equivalents of TCEP (tris(2-carboxyethyl)phosphine, Acros) and conjugated to vcMMAE without removal of excess TCEP as follows: 35 mL of C6v1 (2.1 mg/mL or 14.3 μM; 74 mg) were treated with 4.0 equivalents of TCEP (57.1 μM, from 100 mM stock) in PBS with 1 mM DTPA for 2.5 h at 37 °C.

The extent of reduction was checked by purifying a small amount of the reduction reaction through a PD-10 column (Amersham Biosciences) and titrating the number of antibody-cysteine thiols with DTNB, yielding 5.7 per C6v1 The reduced antibody was then cooled to 0 °C and treated with 8.0 equivalents of vcMMAE (the concentration of antibody thiols was 73.5 μM and the vcMMAE concentration was 103.2 μM) in 3.9 mL of DMSO. After 135 min at 0 °C, 0.4 mL of 100 mM cysteine was added to quench any unreacted vcMMAE and the mixture was diluted to 250 mL with water. The conjugate was purified on a hydroxyapatite column as described above. cAC10-C6v1-vcMMAE (20 mL of 2.4 mg/mL; 48 mg) (C6v1-E6) was concentrated and the buffer changed to PBS using 15 mL Amicon Ultrafree 30K cutoff spin concentration devices.

The generation of parent cAC10 antibody drug conjugates (ADCs) with two and four MMAE molecules per antibody, C8-E2 and C8-E4 respectively, has been described (Hamblett et al., Clin. Cancer Res. 15 7063-7070 (2004)). Briefly, the method involved a partial reduction of the mAb to expose ~4 reduced Cys per Ab followed by reaction with

vc-MMAE. Partially loaded cAC10-Val-Cit-MMAE referred to as C8-E4-Mixture (or C8-E4M) was obtained.

C8-E2 and C8-E4 were prepared from C8-E4M by preparative HIC (hydrophobic interaction chromatography) fractionation on a Toyopearl Phenyl-650M HIC resin (Tosoh Bioscience, Montgomeryville, PA) equilibrated with >5 column volumes of Buffer A (50 mM sodium phosphate, 2 M NaCl, pH 7.0). To prepare the sample for loading onto the column, 39 ml of the C8-E4-Mixture (12.9 mg/ml) was mixed with an equivalent volume of Buffer A' (50 mM sodium phosphate, 4 M NaCl, pH 7.0). Following sample loading, the column was washed with Buffer A until an A_{280} baseline was achieved. C8-E2 was eluted and collected with a step gradient consisting of 65% Buffer A / 35% Buffer B (80% v/v 50 mM sodium phosphate, pH 7.0, 20% v/v acetonitrile). After baseline was again achieved, C8-E4 was eluted and collected with a step gradient consisting of 30% Buffer A / 70% Buffer B. Both C8-E2 and C8-E4 peaks were collected to ~20% of their respective peak heights. The fractions of interest were buffer exchanged into PBS using Ultrafree-15 centrifugal filter devices with a molecular weight cutoff of 30 kDa (Millipore, Billerica, MA).

(ii) Analysis of Drug Loading

Drug loading was determined by measuring the ratio of the absorbance at 250 and 280 nm (A250/280). The number of vcMMAE per cAC10 was empirically determined to be (A250/A280 – 0.36)/0.0686. ADCs were analyzed by hydrophobic interaction chromatography (HIC) using a Tosoh Bioscience Ether-5PW column (part 08641) at a flow rate of 1 mL/min and a column temperature of 30 °C. Solvent A was 50 mM sodium phosphate pH 7 and 2.5 M NaCl. Solvent B was 80% 50 mM sodium phosphate pH 7, 10% 2-propanol, and 10% acetonitrile. Isocratic 0% B for 15 min, a 50-min linear gradient from 0 to 100% B, a 0.1-min linear gradient from 100 to 0% B, and isocratic 0% B for 14.9 min. Injections (typically 90-100 µL) were 1 volume of ADC (concentration of at least 3 mg/mL) and 1 volume of 5 M NaCl.

ADCs from HIC chromatography were analyzed using an Agilent Bioanalyzer. A protein 200 chip was used under denaturing but nonreducing conditions as described by the manufacturer. Briefly, 4 µL of 1 mg/mL ADC were mixed with 2 µL of nonreducing

loading buffer and heated to 100 °C for 5 min. Water (84 µL) was added and 6 µL of this mixture was loaded into each well of the chip.

ADCs were analyzed on a PLRP-S column (Polymer Laboratories part 1912-1802: 1000 A, 8 u, 2.1x50 mm). The flow rate was 1 mL/min and the column temperature was 80 °C. Solvent A was 0.05% trifluoroacetic acid in water and solvent B was 0.04% trifluoroacetic acid in acetonitrile. Isocratic 25% B for 3 min, a 15-min linear gradient to 50% B, a 2-min linear gradient to 95% B, a 1-min linear gradient to 25% B, and isocratic 25% B for 2 min. Injections were 10-20 µL of 1 mg/mL ADC previously reduced with 20 mM DTT at 37 °C for 15 min to cleave the remaining interchain disulfides.

Results

MMAE conjugates of cAC10 cysteine variants were generated by reduction of the antibody followed by alkylation with vcMMAE. Analysis of each conjugated cAC10 cysteine variant by both UV-VIS analysis at an absorbance of 280 nm and PLRP chromatography demonstrated that close to the expected drug loading was achieved as shown in Table 4.

Table 4
Summary of the analysis of cAC10 cysteine variant vcMMAE conjugates

cAC10 Variant	Conc. (mg/ml)	Volume (ml)	Total (mg)	DTNB RSH/Ab	PLRP Drug/ab	% monomer	Free drug
C2v1-E2	41	2.3	94.3	2.3	2.1	98.0%	<0.05%
C2v2-E2	5.6	0.6	3.4	2.3	2.0	99.4%	<0.05%
C4v1-E4	12.0	3.8	45.6	4.2	3.7	99.1%	<0.05%
C4v2-E4	12.4	4.0	49.6	4.0	3.5	98.7%	<0.05%
C4v3-E4	0.8	0.4	0.3	7.2	3.7	99%	<0.05%
C6v1-E6	12.5	3.3	40.6	5.7	5.7	98.7%	<0.05%

Analysis by size exclusion chromatography demonstrated that all conjugates consisted of 98% monomer or greater as shown in Table 4. The control molecules described in this study were parent cAC10 conjugated with either two molecules of MMAE (C8-E2), or four molecules of MMAE (C8-E4). These two and four drug-loaded MMAE conjugates were generated by partial reduction of the parent cAC10 antibody and analyzed as previously described in Hamblett et al., Clin. Cancer Res. 15:7063-7070 (2004).

In vitro cytotoxicity of cAC10 cysteine variant conjugates

Procedures

Growth inhibition of CD 30^+ Karpas 299 cells treated with cAC10 cysteine variant conjugates was determined by measuring DNA synthesis. Conjugates were incubated with cells for 92 hours followed by labeling with [^{3}H]-thymidine, 0.5 μCi/well for 4 hours at 37 °C. Cells were harvested onto filters and mixed with scintillation fluid and radioactivity was measured using a Topcount Scintillation counter (Packard Instruments, Meriden, CT). The percent radioactivity incorporated relative to the untreated controls was plotted versus conjugate concentration and the data were fit to a sigmoidal dose-response curve using Prism 4 software (GraphPad Software Inc, San Diego, CA). Alternatively, 50 μM resazurin was added to Karpas 299 cells following the 92 hour incubation period with conjugate. After a 4 hour incubation period dye reduction was measured using a Fusion HT fluorescent plate reader (Packard Instruments, Meriden, CT).

Results

The cytotoxicities of the AC10 cysteine variant C2v1, C4v1, C4v2, and C6v1 MMAE conjugates (C2v1-E2, C4v1-E4, C4v2-E4, and C6v1-E6, respectively) were tested using a [^{3}H]-thymidine incorporation assay on $CD30^+$ Karpas 299 cells. The control conjugate used was the four drug-loaded parent cAC10 (C8-E4) which has been shown to have potency that lies between the fully loaded parent cAC10 MMAE conjugate (C8-E8) (which is the most potent), and the two-drug loaded conjugate (C8-E2). C6v1-E6 had the lowest IC_{50} value of 0.012 nM, while the four drug-loaded cysteine variants C4v1-E4 and C4v2-E4 and the four drug-loaded parent cAC10 conjugate C8-E4 had very similar IC_{50}s of 0.020 nM, 0.027 nM and 0.018 nM, respectively, as shown in Figure 2A. As shown in Figure 2B, the C2v1-E2 MMAE conjugate had an IC_{50} of 0.029 nM. Subsequently, the in vitro cytotoxic activities of both C2v1-E2 and C2v2-E2 MMAE drug conjugates on Karpas 299 cells were evaluated. Cytotoxicity was assessed by reduction of resazurin dye which was introduced to the culture following 92 hours continuous exposure to conjugate. cAC10 conjugated with two MMAE drug molecules per antibody (C8-E2) was used as the control. All three conjugates had similar IC_{50} values of 52.4 ng/ml, 39.8 ng/ml and 39.8 ng/ml for C2v1-E2, C2v2-E2 and C8-E2, respectively. These data demonstrate that the cysteine variant conjugates compare closely in activity to partially loaded MMAE conjugates generated from the parent cAC10 antibody by partial reduction.

Antitumor activity of cAC10 cysteine variant conjugates in vivo using a Xenograft model of human ALCL

Procedures

To establish a subcutaneous disease model of ALCL $5x10^6$ Karpas-299 cells were implanted into the right flank of C.B-17 SCID mice (Harlan, Indianapolis, IN). Therapy with ADCs was initiated when the tumor size in each group of 6-10 animals averaged 100 mm^3. Treatment consisted of a single injection. Tumor volume was calculated using the formula (length x $width^2$)/2. A tumor that decreased in size such that it was impalpable was defined as a complete regression (CR). A complete regression that lasted beyond 100 days post tumor implant was defined as a cure. Animals were euthanized when tumor volumes reached approximately 1000 mm^3.

Results

The efficacies of the cAC10 cysteine variant drug conjugates were assessed in a subcutaneous xenograft model of ALCL in SCID mice. Karpas 299 cells were implanted into the flanks of SCID mice and tumors were grown to an average volume of 100 mm^3. Tumor bearing mice were randomly divided into groups of eight to ten animals and either left untreated or were treated with cAC10 cysteine variant MMAE conjugates C2v1-E2, C4v1-E4 or C4v2-E4 or partially MMAE loaded parent cAC10 conjugates C8-E2 and C8-E4 in a single dose study. ADC doses were normalized so an equal concentration of MMAE was injected per group with 1 mg/kg, 1.14 mg/kg and 1.05 mg/kg injected for C8-E4, C4v2-E4 and C4v1-E4, respectively, and 2 mg/kg and 1.9 mg/kg for C8-E2 and C2v1-E2, respectively. As shown in Figure 3A, C2v1-E2 showed similar antitumor activity to C8-E2 with complete tumor regressions occurring in all animals treated with C8-E2 and six of eight animals treated with C2v1-E2. As shown in Figure 3B, C4v1-E4 and C4v2-E4 displayed similar antitumor activities to C8-E4. Complete regressions occurred in eight of ten animals for C8-E4 and C4v2-E4 and six of ten animals for C4v1-E4.

In summary, the two and four drug loaded ADCs generated from the cysteine variants have similar in vivo activity to the C8-E4 and C8-E2 conjugates produced by the partial reduction method.

Example 2

Preparation and Analysis of Antibody Conjugates

Procedures

The cAC10 parent and variant antibodies prepared as described in Example 1 were purified by protein A followed by anion exchange chromatography using an ÄKTAexplorer (GE Healthcare, Piscataway, NJ). Briefly, the antibody-containing conditioned media were concentrated ~10-fold and buffer-exchanged into PBS, pH 7.4 by tangential flow filtration (Millipore). The concentrated samples were treated with 0.5% (v/v) Triton X-100 (Sigma, St. Louis, MO) with gentle stirring overnight at 4°C for endotoxin removal, before loading onto protein A (GE Healthcare) pre-equilibrated with PBS, pH 7.4. The column was washed with PBS, pH 7.4, 2-3 column volumes (CV) 0.5% v/v Triton X-100, 1 M NaCl in PBS, pH 7.4 then with PBS, pH 7.4 until a stable baseline was reached. Bound antibody was eluted from protein A with 30 mM sodium acetate, pH 3.6 and then dialyzed against 20 mM Tris-HCl, 10 mM NaCl, 1 mM EDTA, pH 8.0 (buffer A). The pooled antibody was then loaded on to Q sepharose (GE Healthcare) pre-equilibrated with buffer A and washed with 2-3 CV buffer A, 5-10 CV buffer A containing 0.5% (v/v) Triton X-100 with incubation and 5 CV buffer A. Antibodies were eluted from Q sepharose by either step or linear NaCl gradient (from 10-500 mM NaCl in buffer A) and dialyzed against PBS, pH 7.4. Purified antibodies were analyzed by SDS-PAGE and by TSK-Gel G3000SW HPLC size exclusion chromatography (Tosoh Bioscience, Montgomeryville, PA).

Conjugation of cAC10 Cys→Ser antibody variants with either 2 (C2v1-E2, C2v2-E2) or four (C4v1-E4, C4v2-E4 and C4v3-E4) equivalents of MMAE molecules involved reduction with a few (2.5 to 4) equivalents of tris(2-carboxyethyl)phosphine (Acros Organics, Geel, Belgium) and conjugation to maleimidocaproyl-valine-citrulline-p-aminobenyzoyl-MMAE (vcMMAE) (Doronina et al., supra) without removal of excess tris(2-carboxyethyl)phosphine. Prior to drug addition the extent of reduction was assessed by purifying a small amount of the reduction reaction through a PD-10 column (GE Healthcare) and titrating the number of antibody-cysteine thiols with 5,5′-dithio-bis(2-nitrobenzoic acid) (Ellman, Arch. Biochem. Biophys. 74:443-450 (1958)). The reduced antibodies were reacted with vcMMAE for 60 min at 0°C and excess N-acetylcysteine (Acros Organics) was then added to quench any unreacted maleimidocaproyl-Val-Cit-MMAE. The reaction mixture was then diluted 5-fold with water and then loaded on to hydroxyapatite column equilibrated with 10 mM sodium phosphate pH 7.0, 10 mM NaCl. The column was washed with 5 CV of the same buffer and the conjugate eluted with 100

mM sodium phosphate pH 7.0, 10 mM NaCl. The conjugates were concentrated and buffer-exchanged into PBS using Amicon Ultrafree centrifugal filter units (Millipore).

The generation of parent cAC10 conjugates with a mean stoichiometry of 4 drugs per antibody (range of 0 to 8 drugs), C8-E4 mixture (C8-E4M), and 2 drugs per antibody, C8-E2M, have been described (Hamblett et al., supra) (Sun et al., Bioconjug. Chem. 16:1282-1290 (2005)). C8-E2M was subjected to hydrophobic interaction chromatography to isolate conjugates loaded with 4 (C8-E4) and 2 (C8-E2) MMAE molecules per antibody, as previously described (Hamblett et al., supra).

ADCs were analyzed to determine the stoichiometry of drug loading using the molar extinction coefficients at wavelengths of 248 nm and 280 nm for the antibody (9.41 x 10^4 and 2.34 x 10^5 M^{-1} cm^{-1}, respectively) and drug (1.50 x 10^3 and 1.59 x 10^4 M^{-1} cm^{-1}, respectively), as previously described (Hamblett et al., supra). The location of drug attachment to the antibody heavy and light chains was investigated by reverse phase HPLC using a PLRP-S column (Polymer Laboratories, Amherst, MA; #1912-1802: 1000 Å, 8 μm, 2.1 x 150 mm) and solvents A (0.05% (v/v) trifluoroacetic acid in water) and solvent B (0.04% (v/v) trifluroacetic acid in acetonitrile). The running conditions (1 ml/min, 80°C) were: isocratic 25% solvent B (3 min), linear gradient to 50% solvent B (25 min), linear gradient to 95% solvent B (2 min), linear gradient to 25% solvent B (1 min), and isocratic 25% solvent B for 2 min. Prior to chromatography ADC samples (10-20 μl, 1 mg/ml) were reduced with 20 mM DTT at 37 °C for 15 min to cleave the remaining inter-chain disulfide bonds.

Results

The cAC10 parent antibody (C8) was partially reduced to yield a mean of 2 or 4 sulfhydryl groups per antibody and then reacted with vcMMAE. The corresponding conjugation products, C8-E2M and C8-E4M, have a mean loading of 2 and 4 equivalents of MMAE respectively. C8-E2M and C8-E4M are mixtures of species loaded with 0, 2, 4, 6 or 8 equivalents of MMAE per antibody (Hamblett et al., supra). Conjugates with uniform stoichiometry of either 2 (C8-E2), or 4 (C8-E4) equivalents of MMAE were purified from the C8-E2M mixture by hydrophobic interaction chromatography as previously described (Hamblett et al., supra). MMAE conjugates of the engineered antibody variants were generated by reduction of the antibody followed by reaction with vcMMAE.

For each ADC, the observed drug loading stoichiometry by spectrophotometric (Hamblett et al., supra) and reverse phase HPLC analyses (Sun et al., supra) closely

matched those expected. Peak area analysis following size exclusion chromatography suggested that all ADCs were ≥ 98% monomeric (Doronina et al., supra). The yield of the Cys→Ser variant conjugates (89-96%) was greatly improved compared to the conjugates C8-E4 (11%) and C8-E2 (27%) purified from C8-E2M. SDS-PAGE analysis of the ADCs under reducing conditions showed the expected reduced motility of the MMAE conjugated light chains in C2v2-E2, C8-E2, C4v2-E4, C8-E4 and C8-E4M compared to the unconjugated light chains in the other ADCs. The decreased motility of the conjugated heavy chains was less pronounced but the heterogeneity of conjugated heavy chains in C8-E2 and C8-E4M was apparent (Figure 1C).

Reverse phase HPLC under reducing conditions was used to evaluate ADC heterogeneity. This method resolves light chains loaded with 0 or 1 equivalents of MMAE (L-E0 and L-E1, respectively) as well as heavy chains loaded with 0, 1, 2, or 3 equivalents of MMAE (H-E0, H-E1, H-E2 and H-E3, respectively). C8-E4M (Figure 6A) is the most heterogeneous conjugate containing all 6 possible species. Purification of C8-E4M to generate C8-E4 reduces the heterogeneity down to 4 species: L-E0, L-E1, H-E1 and H-E2 (Figure 6B). The homogeneity of cAC10 Cys→Ser variants is demonstrated by the presence of the anticipated single light and heavy chain peaks, L-E0 plus H-E2, and L-E1 plus H-E1, for C4v1-E4 (Figure 6C) and C4v2-E4 (Figure 6D), respectively.

In vitro characterization of cAC10 variants and drug conjugates

Procedures

CD30-positive ALCL line Karpas-299 and CD30-negative WSU-NHL were obtained from the Deutsche Sammlung von Mikroorganism und Zellkulturen GmbH (Braunschweig, Germany). L540cy, a derivative of the HD line L540 adapted to xenograft growth, was developed by Dr. Harald Stein (Institüt für Pathologie, University Veinikum Benjamin Franklin, Berlin, Germany). Cell lines were grown in RPMI-1640 media (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum.

Competition binding of the cAC10 variants and their corresponding ADCs was undertaken to assess the impact of mutations and drug conjugation upon antigen binding. Briefly, CD30-positive Karpas-299 cells were combined with serial dilutions of the cAC10 parent antibody, variants or corresponding ADC (prepared as described in Example 1), in the presence of 1 μg/ml cAC10 labeled with europium (Perkin Elmer, Boston, MA) in staining medium (50 mM Tris-HCl pH 8.0, 0.9% NaCl (w/v), 0.5% bovine serum albumin (w/v), 10 μM EDTA) for 30 min on ice then washed twice with ice-

cold staining medium. Labeled cells were detected using a Fusion HT microplate reader (Perkin-Elmer). Sample data were baseline-corrected and reported as the percent of maximum fluorescence as calculated by the sample fluorescence divided by the fluorescence of cells stained with 1 μg/ml cAC10-europium alone.

Growth inhibition of CD30-positive Karpas 299 or L540cy cells or CD30-negative WSU-NHL cells treated with cAC10 Cys→Ser variant conjugates was determined by incubating conjugates with cells for 92 h followed by incubation with 50 μM resazurin for 4 h at 37 °C. Dye reduction was measured using a Fusion HT microplate reader. Data were analyzed by a non-least squares 4-parameter fit using Prism v4.01 (GraphPad Software Inc, San Diego, CA).

Results

Competition binding experiments revealed that neither Cys→Ser mutations (Table 1) nor MMAE conjugation (Table 5) impaired antigen binding. Next the cytotoxicities of the cAC10 Cys→Ser variant conjugates were assessed on CD30 positive (Karpas-299 and L540cy) and negative (WSU-NHL) cell lines. The C2v1-E2 and C2v2-E2 conjugates had very similar potency to C2-E2 on both CD30 positive cell lines (Table 5). Similarly, C4v1-E4, C4v2-E4, C8-E4M and C8-E4 displayed similar activity on both CD30 positive cell lines tested (Table 5). Thus, precisely defining the site of stoichiometry of drug attachment did not significantly impact the cytotoxic activity of the conjugate. Increasing the amount drug loading from 2 to 4 MMAE/Ab increased the potency (reduced IC_{50} values) consistent with previous observations (Hamblett et al., supra). CD30 negative WSU-NHL cells were insensitive to all cAC10 ADCs.

Table 5
Generation and Characterization of Antibody Drug Conjugates

cAC10 drug conjugate*	Percentage yield of conjugate†	Drugs per IgG: method 1, method 2‡	Percentage monomer§	Competition binding to Karpas-299 (IC_{50}, nM) #	Karpas-299 cytotoxicity (IC_{50}, nM)	L540cy cytotoxicity (IC_{50}, nM)	Single dose MTD (mg/kg)
C2v1-E2	92.7	2.0, 1.9	98.4	2.9 ± 0.3	0.26 ± 0.12	0.28 ± 0.03	40
C2v2-E2	88.9	2.1, 1.8	98.5	2.5 ± 0.1	0.46 ± 0.30	0.27 ± 0.02	60
C8-E2	27.4‖	2.0, 2.0	99.7	2.8 ± 0.2	0.32 ± 0.21	0.28 ± 0.02	40
C8-E2M	97.3	2.0, 2.0	n/d	n/d	n/d	n/d	n/d
C4v1-E4	90.6	4.0, 3.8	99.2	3.2 ± 0.1	0.07 ± 0.02	0.13 ± 0.01	20
C4v2-E4	96.0	4.1, 3.8	99.0	2.8 ± 0.2	0.07 ± 0.02	0.12 ± 0.01	< 20
C8-E4	10.8‖	4.0, 4.0	99.5	2.4 ± 0.3	0.07 ± 0.01	0.18 ± 0.04	20
C8-E4M	95	4.4, 4.4	98.8	3.0 ± 0.4	0.03 ± 0.01	0.07 ± 0.02	< 20

Free drug in all ADC preparations was below the detection limit (< 0.05 %)

*ADCs are identified by their cAC10 variant name (see Table 1) loading level with the drug, MMAE, and whether the drug stoichiometry is variable (M) or fixed. For example, C8-E4M and C8-E2M denotes the parent antibody, cAC10, loaded with a mean of 4 (range 0 to 8) and 2 (range of 0 to 8) equivalents of MMAE per IgG, respectively. The fixed stoichiometry ADCs, C8-E4 and C8-E2, were obtained by purification of the variable stoichiometry ADC, C8-E2M, by hydrophobic interaction chromatography.

†Yield of conjugate obtained as a percentage of purified antibody.

‡ Methods 1 and 2 refer to the ratio of absorbance at wavelengths of 248 nm and 280 nm (Hamblett et al., 2004) and reverse phase HPLC analysis under reducing conditions (Figure 6).

§Estimated from the peak areas in size exclusion chromatography.

‖Percentage yield after hydrophobic interaction chromatography based on starting cAC10 protein.

Mean (± SEM) for ≥ 3 independent experiments.

Antitumor activity of antibody Cys→Ser variant conjugates in vivo

Xenograft models

5 x 10^6 Karpas-299 or L540cy cells were implanted into the right flank of C.B-17 SCID mice (Harlan, Indianapolis, IN) to establish a subcutaneous disease model of anaplastic large cell lymphoma or Hodgkin's disease, respectively. Tumor volume was calculated using the formula (A x B^2)/2, where A and B are the largest and second largest perpendicular tumor dimensions, respectively. Tumor bearing mice were randomly divided into groups of 8-10 animals when the mean tumor volume was 100 mm^3. Mice groups were either left untreated or treated with a single intravenous dose of an ADC. For the L540cy xenograft studies the doses used were 6.0 and 12.0 mg/kg for the 2 drug/Ab conjugates and 3.0 and 6.0 mg/kg for the 4/drug Ab conjugates. For the Karpas 299 xenograft model doses used were 0.5, 1.0 and 2.0 mg/kg for the 2 drug/Ab conjugates and 0.5 and 1.0 mg/kg for the 4 drug/Ab conjugates. A tumor that decreased in size such that it was impalpable was defined as a complete regression. A complete regression that lasted beyond 100 d post tumor implant was defined as a "cure". Animals were euthanized when tumor volumes reached ~1000 mm^3.

Results

The efficacies of the cAC10 Cys→Ser variant drug conjugates, C2v1-E2, C2v2-E2, C4v1-E4 and C4v2-E4, were compared to conjugates of the parent antibody, C8-E2, C8-E4 and C8-E4M, in subcutaneous xenograft models of anaplastic large cell lymphoma (Karpas-299) and Hodgkin's disease (L540cy) in SCID mice. Briefly, mice bearing 100 mm^3 L540cy tumors (mean size) were dosed once with a 2 drug/Ab conjugate (6.0 or 12.0 mg/kg) or a 4 drug/Ab conjugate (3.0 or 6.0 mg/kg) or left untreated. Responses to treatment with C2v1-E2 and C2v2-E2 were comparable and complete regressions were induced at both 6.0 and 12.0 mg/kg doses (Figure 7A, B). C8-E2 was slightly more potent than C2v1-E2 and C2v2-E2 with cures achieved at both dose levels (Figure 3A, B). Karpas 299 xenograft models treated with single doses of the 2-drug loaded conjugates showed similar response trends with 3 of the 10 animals achieving complete regressions for C2v1-E2 and C2v2-E2 and 8 of 10 complete regressions for C8-E2 at a 1 mg/kg dose (data not shown). Treatment of L540cy xenograft models with C4v1-E4, C4v2-E4, C8-E4 and C8-E4M resulted in comparable responses with cures achieved at both 3 and 6 mg/kg for each ADC (Figure 7C, D). Treatment of Karpas 299 models with the 4-drug loaded

variants at 0.5 and 1 mg/kg also showed no discrimination between molecules (data not shown).

Determination and Analysis of Maximum tolerated dose

The single dose tolerability of each ADC was determined in Sprague-Dawley rats (Harlan, IN). Groups of three rats were injected with 40-80 mg/kg of C2v1-E2, C2v2-E2 and C8-E2 and 20-40 mg/kg of C4v1-E4, C4v2-E4, C8-E4 and C8-E4M via the tail vein to determine the single dose maximum tolerated dose (MTD). Rats were monitored daily for 14 d, and both weight and clinical observations were recorded. Rats that developed significant signs of distress were euthanized. The maximum tolerated dose was defined as the highest dose that did not induce > 20% weight loss or severe signs of distress in any of the animals.

For the 2-drug loaded conjugates rats were dosed at 40, 60 and 80 mg/kg. The 40 mg/kg dose was well tolerated while the 60 mg/kg dose was only well tolerated by rats treated with C2v2-E2. One animal injected with 60 mg/kg of C2v1-E2 was sacrificed on day 7 while the remaining 2 animals had a maximum weight loss 6% on day 8 after which weight loss was recovered. One animal dosed with 60mg/kg of C8-E2 displayed 11% weight loss and was found dead on day 11. The 80 mg/kg dose of each 2-loaded ADC was not well tolerated. Based on these data the MTDs of C2v1-E2, C2v2-E2 and C8-E2 were determined to be 40, 60 and 40 mg/kg, respectively. The 4-drug loaded ADCs were each dosed at 20, 30 and 40 mg/kg. Animals injected with the 20 mg/kg dose of C4v1-E4 and C8-E4 experienced no adverse effects while several animals in the groups treated with the 20 mg/kg doses of C4v2-E4 and C8-E4M showed signs of distress and one from each group was sacrificed on day 9. The higher doses of 30 and 40 mg/kg of each 4-drug loaded ADC were not tolerated. The MTDs for C4v1-E4 and C8-E4 were determined to be 20 mg/kg while the MTDs for C4v2-E4 and C8-E4M were determined to be < 20 mg/kg.

No license is expressly or implicitly granted to any patent or patent applications referred to or incorporated herein. The discussion above is descriptive, illustrative and exemplary and is not to be taken as limiting the scope defined by any appended claims.

Various references, including patent applications, patents, and scientific publications, are cited herein, the disclosures of each of which is incorporated herein by reference in its entirety.

Claims:

1. An immunoconjugate comprising:

an engineered antibody having (a) a functionally active antigen-binding region for a target antigen, (b) at least one interchain cysteine residue, (c) at least one amino acid substitution of an interchain cysteine residue, and (d) a diagnostic, preventative or therapeutic agent conjugated to at least one interchain cysteine residue.

2. The immunoconjugate of claim 1, having four interchain cysteine residues and four amino acid substitutions of interchain cysteine residues.

3. The immunoconjugate of claim 1, comprising two interchain cysteine residues and six amino acid substitutions of interchain cysteine residues.

4. The immunoconjugate of claim 1, which is an IgG1 or an IgG4.

5. The immunoconjugate of claim 1, wherein each the amino acid substitutions is a cysteine to serine substitution.

6. The immunoconjugate of claim 1, wherein the diagnostic, preventative or therapeutic agent is a therapeutic agent.

7. The immunoconjugate of claim 6, wherein the therapeutic agent is an auristatin or an auristatin derivative.

8. The immunoconjugate of claim 7, wherein the auristatin derivative is dovaline-valine-dolaisoleunine-dolaproine-phenylalanine (MMAF) or monomethyauristatin E (MMAE).

9. The immunoconjugate of claim 1, wherein the diagnostic, preventative or therapeutic agent is a diagnostic agent.

10. The immunoconjugate of claim 9, wherein the diagnostic agent is a radioactive agent, an enzyme, a fluorescent compound or an electron transfer agent.

11. The immunoconjugate of claim 1, wherein the antibody binds to CD20, CD30, CD33, CD40, CD70 or Lewis Y.

12. The immunoconjugate of claim 1, wherein the antibody binds to an immunoglobulin gene superfamily member, a TNF receptor superfamily member, an integrin, a cytokine receptor, a chemokine receptor, a major histocompatibility protein, a lectin, or a complement control protein.

13. The immunoconjugate of claim 1, wherein the antibody binds to a microbial antigen.

14. The immunoconjugate of claim 1, wherein the antibody binds to a viral antigen.

15. The immunoconjugate of claim 1, wherein the antibody is an anti-nuclear antibody, anti-ds DNA antibody, anti-ss DNA antibody, anti-cardiolipin antibody IgM or IgG, anti-phospholipid antibody IgM or IgG, anti-SM antibody, anti-mitochondrial antibody, anti-thyroid antibody, anti-microsomal antibody, anti-thyroglobulin antibody, anti-SCL 70 antibody, anti-Jo antibody, anti-U1RNP antibody, anti-La/SSB antibody, anti-SSA antibody, anti-SSB antibody, anti-perital cells antibody, anti-histone antibody, anti-RNP antibody, anti-C ANCA antibody, anti-P ANCA antibody, anti-centromere antibody, anti-fibrillarin antibody, or anti-GBM antibody.

16. The immunoconjugate of claim 1, wherein the antibody is an antibody fragment.

17. The immunoconjugate of claim 16, wherein the antibody fragment is selected from Fab, Fab' and scFvFc.

18. The immunoconjugate of claim 17, wherein the fragment is an Fab' or an scFvFc.

19. The immunoconjugate of claim 1, having the following formula:

$$Ab_z-(A_a-W_w-Y_y-D)_p$$

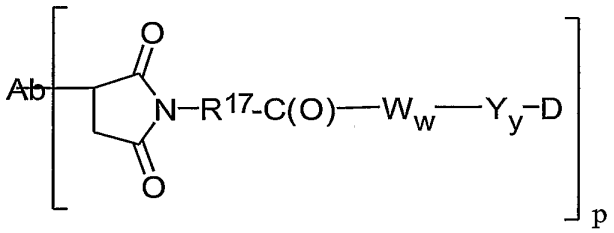
or a pharmaceutically acceptable salt or solvate thereof,

wherein:

Ab is an antibody,

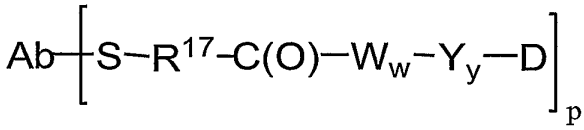
A is a stretcher unit,
a is 0 or 1,
each W is independently a linker unit,
w is an integer ranging from 0 to 12,
Y is a spacer unit, and
y is 0, 1 or 2,
p ranges from 1 to about 20, and
D is a diagnostic, preventative and therapeutic agent, and
z is the number of predetermined conjugation sites on the protein.

20. The immunoconjugate of claim 19, having the formula:



wherein R^{17} is selected from -C_1-C_{10} alkylene-, -C_3-C_8 carbocyclo-, -O-(C_1-C_8 alkyl)-, -arylene-, -C_1-C_{10} alkylene-arylene-, -arylene-C_1-C_{10} alkylene-, -C_1-C_{10} alkylene-(C_3-C_8 carbocyclo)-, -(C_3-C_8 carbocyclo)-C_1-C_{10} alkylene-, -C_3-C_8 heterocyclo-, -C_1-C_{10} alkylene-(C_3-C_8 heterocyclo)-, -(C_3-C_8 heterocyclo)-C_1-C_{10} alkylene-, -$(CH_2CH_2O)_r$-, and -$(CH_2CH_2O)_r$-CH_2-.

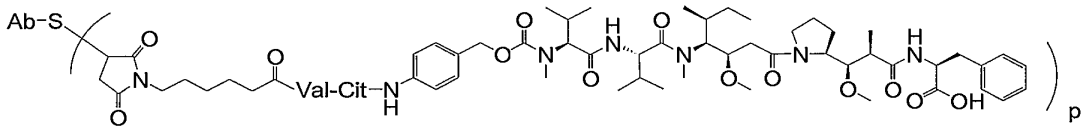
21. The immunoconjugate according to claim 19, having the following formula:



wherein R^{17} is selected from -C_1-C_{10} alkylene-, -C_3-C_8 carbocyclo-, -O-(C_1-C_8 alkyl)-, -arylene-, -C_1-C_{10} alkylene-arylene-, -arylene-C_1-C_{10} alkylene-, -C_1-C_{10} alkylene-(C_3-C_8 carbocyclo)-, -(C_3-C_8 carbocyclo)-C_1-C_{10} alkylene-, -C_3-C_8 heterocyclo-, -C_1-C_{10}

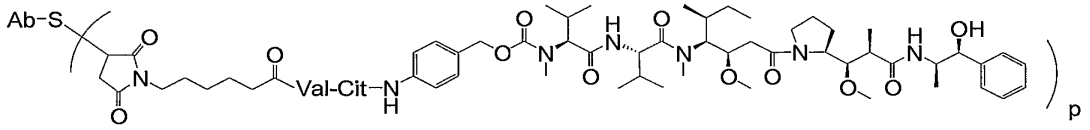
alkylene-(C_3-C_8 heterocyclo)-, -(C_3-C_8 heterocyclo)-C_1-C_{10} alkylene-, -$(CH_2CH_2O)_r$-, and -$(CH_2CH_2O)_r$-CH_2-.

22. The immunoconjugate of claim 19, having the formula:



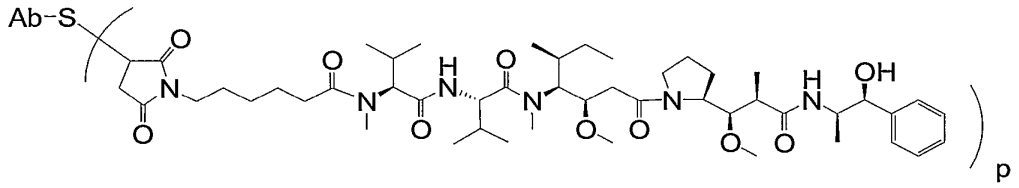
Ab-MC-vc-PAB-MMAF

23. The immunoconjugate of claim 19, having the formula:



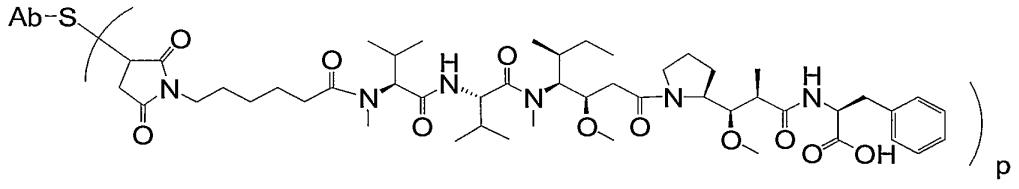
Ab-MC-vc-PAB-MMAE

24. The immunoconjugate of claim 19, having the formula:



Ab-MC-MMAE

25. The immunoconjugate of claim 19, having the formula:



Ab-MC-MMAF

26. A pharmaceutical composition comprising the immunoconjugate of claim 1 and a pharmaceutically acceptable carrier.

27. The pharmaceutical composition of claim 26, wherein the immunoconjugate is formulated with a pharmaceutically acceptable parenteral vehicle.

28. The pharmaceutical composition of claim 26, wherein the immunoconjugate is formulated in a unit dosage injectable form.

29. A method for killing or inhibiting the proliferation of tumor cells or cancer cells comprising treating tumor cells or cancer cells with an amount of the immunoconjugate of claim 6, or a pharmaceutically acceptable salt or solvate thereof, being effective to kill or inhibit the proliferation of the tumor cells or cancer cells.

30. A method for treating cancer comprising administering to a patient an amount of the immunoconjugate of claim 6 or a pharmaceutically acceptable salt or solvate thereof, said amount being effective to treat cancer.

31. A method for treating an autoimmune disease, comprising administering to a patient an amount of the immunoconjugate of claim 6 or a pharmaceutically acceptable salt or solvate thereof, the amount being effective to treat the autoimmune disease.

32. A method for treating an infectious disease, comprising administering to a patient an amount of the immunoconjugate of claim 6 or a pharmaceutically acceptable salt or solvate thereof, the amount being effective to treat the infectious disease.

33. An article of manufacture comprising

an antibody drug conjugate compound of claim 6;

a container; and

a package insert or label indicating that the compound can be used to treat cancer characterized by the overexpression of at least one of CD20, CD30, CD33, CD40, CD70 and Lewis Y.

34. A method for the diagnosis of cancer, comprising administering an effective amount of the immunoconjugate of claim 9 to a patient, wherein the

immunoconjugate binds to an antigen overexpressed by the cancer; and detecting the immunoconjugate in the patient.

35. A method for the diagnosis of an infectious disease, comprising administering an effective amount of the immunoconjugate of claim 9 to a patient, wherein the immunoconjugate binds to a microbial or viral antigen; and detecting the immunoconjugate in the patient.

36. A method for the diagnosis of an autoimmune disease, comprising administering an effective amount of the immunoconjugate of claim 9 to a patient, wherein the immunoconjugate binds to an antigen associated with the autoimmune disease; and detecting the immunoconjugate in the patient.

37. A method for preparing an immunoconjugate, comprising:

(a) culturing a host cell expressing an engineered antibody, the engineered antibody comprising (i) a functionally active antigen-binding region for a target antigen, (ii) at least one interchain cysteine residue, and (iii) at least one amino acid substitution of an interchain cysteine residue, the host cells being transformed or transfected with an isolated nucleic acid encoding the engineered antibody;

(b) recovering the antibody from the cultured host cells or the culture medium; and

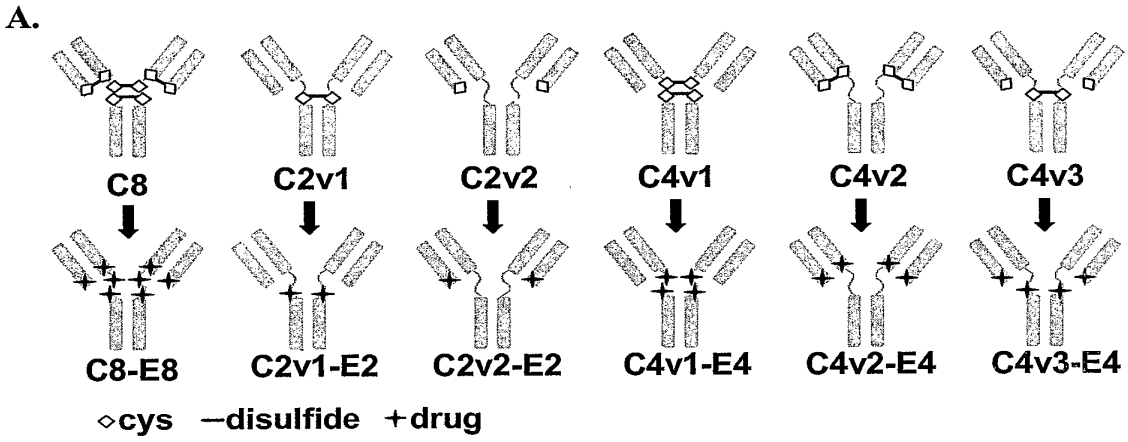
(c) conjugating a diagnostic, preventative or therapeutic agent to the at least one interchain cysteine residue.

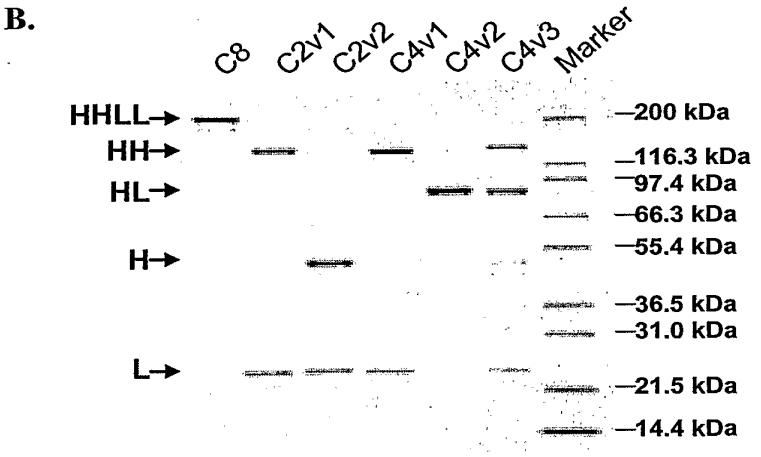
38. The method of claim 37, wherein the amino acid substitution is a cysteine to serine substitution.

39. The method of claim 37, wherein the antibody is an intact antibody or an antigen-binding fragment.

40. The method of claim 39, wherein the antigen binding fragment is an Fab, Fab' or scFvFc.

Figure 1


A.
C8
C2v1
C2v2
C4v1
C4v2
C4v3
C8-E8
C2v1-E2
C2v2-E2
C4v1-E4
C4v2-E4
C4v3-E4
◇cys —disulfide +drug


B.
C8
C2v1
C2v2
C4v1
C4v2
C4v3
Marker
HHLL
HH
HL
H
L
200 kDa
116.3 kDa
97.4 kDa
66.3 kDa
55.4 kDa
36.5 kDa
31.0 kDa
21.5 kDa
14.4 kDa

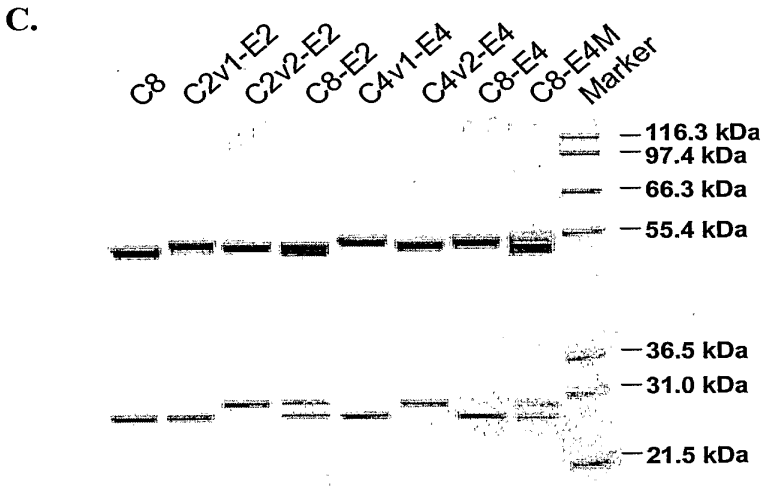
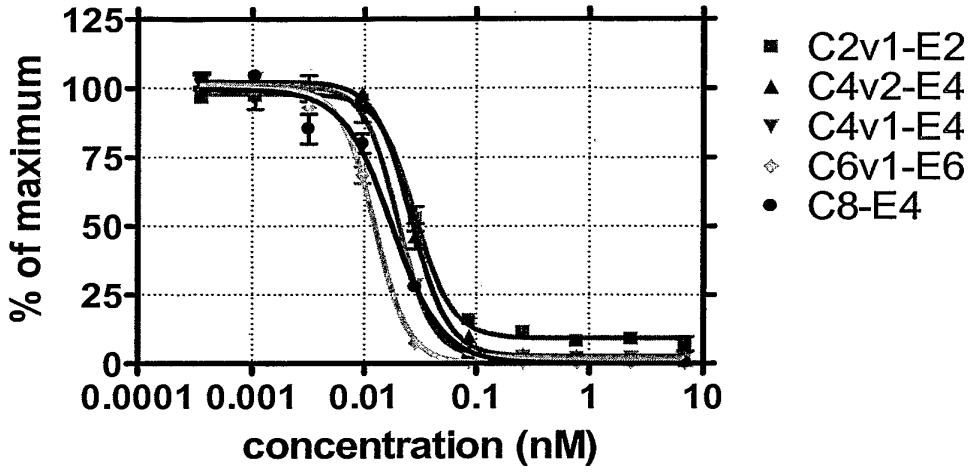

C.
C8
C2v1-E2
C2v2-E2
C8-E2
C4v1-E4
C4v2-E4
C8-E4
C8-E4M
Marker
116.3 kDa
97.4 kDa
66.3 kDa
55.4 kDa
36.5 kDa
31.0 kDa
21.5 kDa

Figure 2

A.


125
100
75
50
25
0
% of maximum
0.0001 0.001 0.01 0.1 1 10
concentration (nM)
C2v1-E2
C4v2-E4
C4v1-E4
C6v1-E6
C8-E4

B.

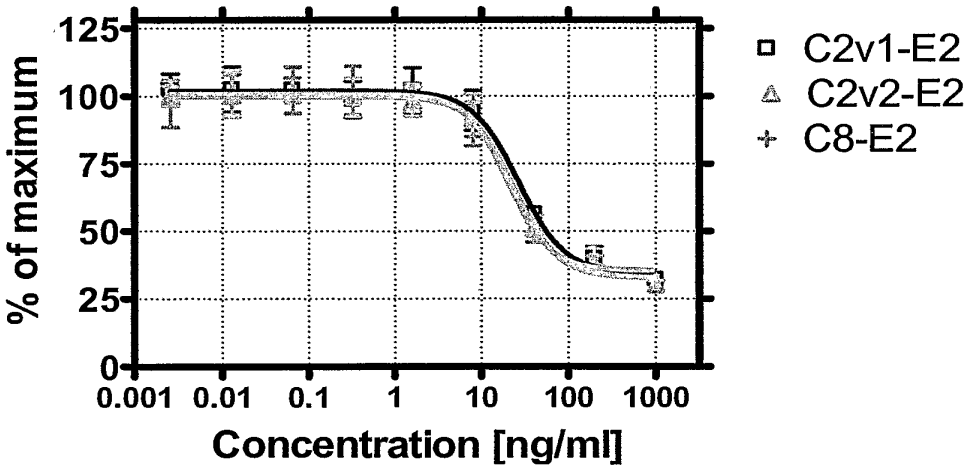
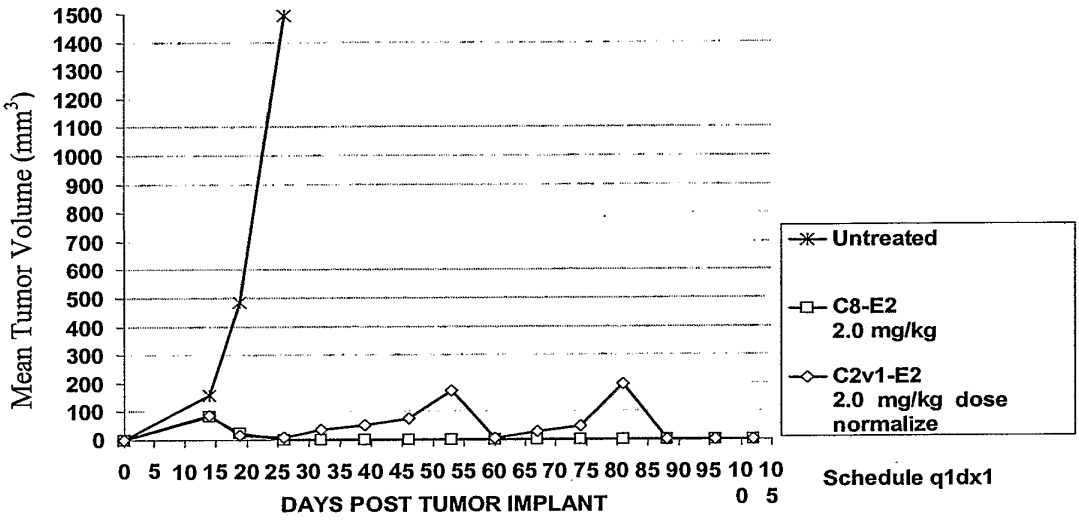

125
100
75
50
25
0
% of maximum
0.001 0.01 0.1 1 10 100 1000
Concentration [ng/ml]
C2v1-E2
C2v2-E2
C8-E2

Figure 3

A.


Mean Tumor Volume (mm3)
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 10 10
0 5
DAYS POST TUMOR IMPLANT
Untreated
C8-E2
2.0 mg/kg
C2v1-E2
2.0 mg/kg dose
normalize
Schedule q1dx1

B.

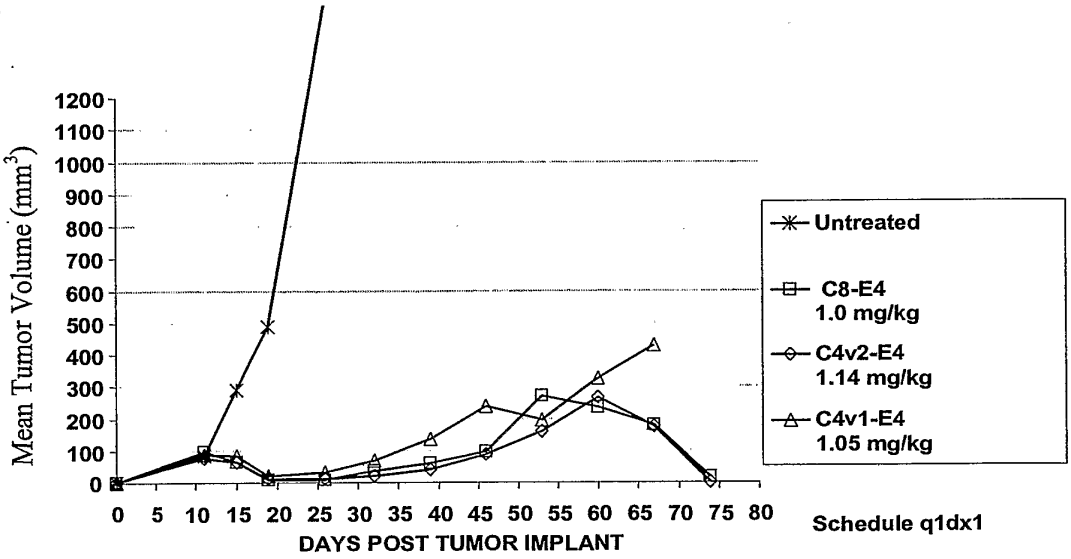

Mean Tumor Volume (mm3)
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80
DAYS POST TUMOR IMPLANT
Untreated
C8-E4
1.0 mg/kg
C4v2-E4
1.14 mg/kg
C4v1-E4
1.05 mg/kg
Schedule q1dx1

Figure 4

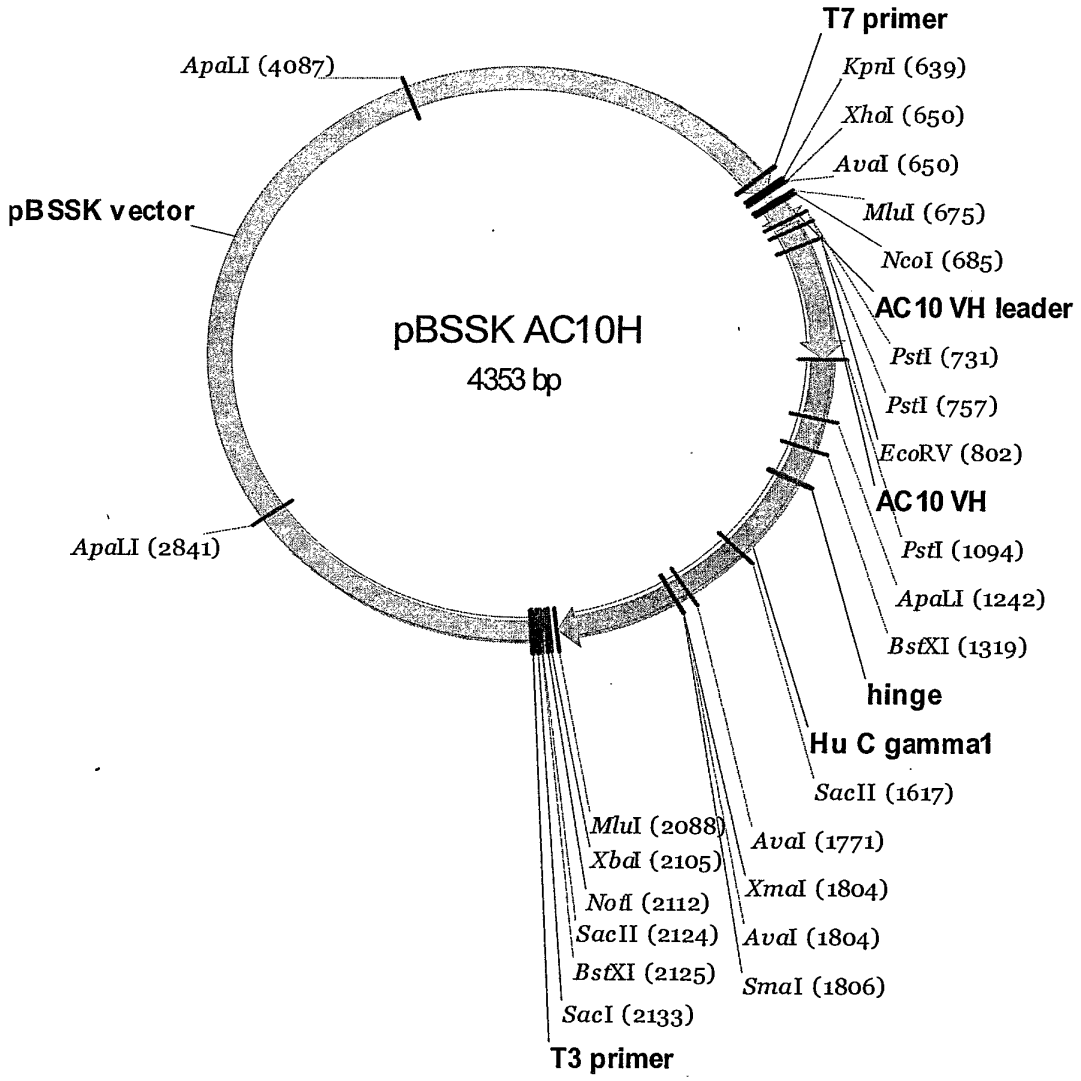

T7 primer
ApaLI (4087)
KpnI (639)
XhoI (650)
AvaI (650)
MluI (675)
NcoI (685)
pBSSK vector
AC10 VH leader
pBSSK AC10H
4353 bp
PstI (731)
PstI (757)
EcoRV (802)
AC10 VH
ApaLI (2841)
PstI (1094)
ApaLI (1242)
BstXI (1319)
hinge
Hu C gamma1
SacII (1617)
MluI (2088)
AvaI (1771)
XbaI (2105)
XmaI (1804)
NotI (2112)
SacII (2124)
AvaI (1804)
BstXI (2125)
SmaI (1806)
SacI (2133)
T3 primer

Figure 5

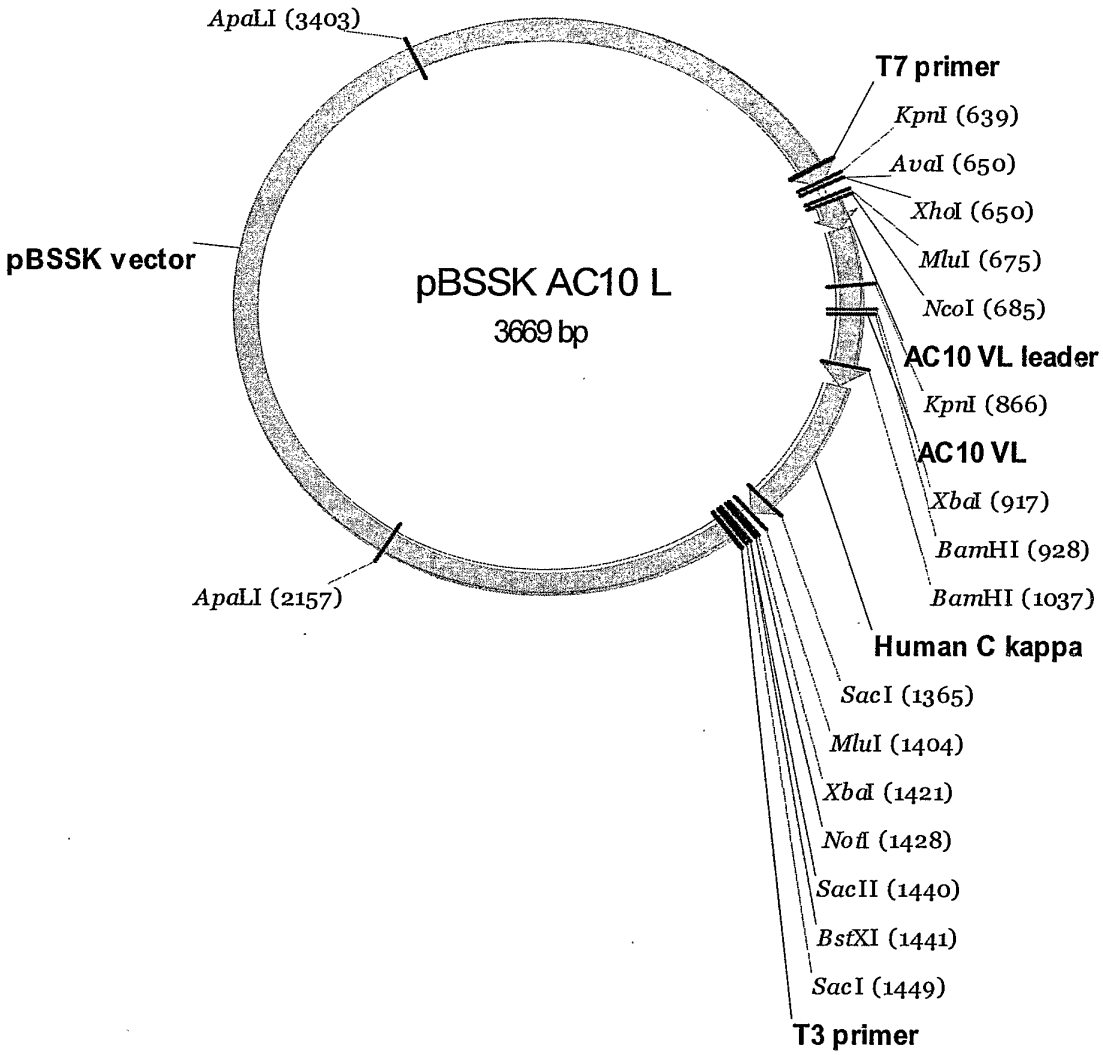

ApaLI (3403)
T7 primer
KpnI (639)
AvaI (650)
XhoI (650)
MluI (675)
NcoI (685)
AC10 VL leader
KpnI (866)
AC10 VL
XbaI (917)
BamHI (928)
BamHI (1037)
Human C kappa
SacI (1365)
MluI (1404)
XbaI (1421)
NotI (1428)
SacII (1440)
BstXI (1441)
SacI (1449)
T3 primer
pBSSK vector
pBSSK AC10 L
3669 bp
ApaLI (2157)

Figure 6

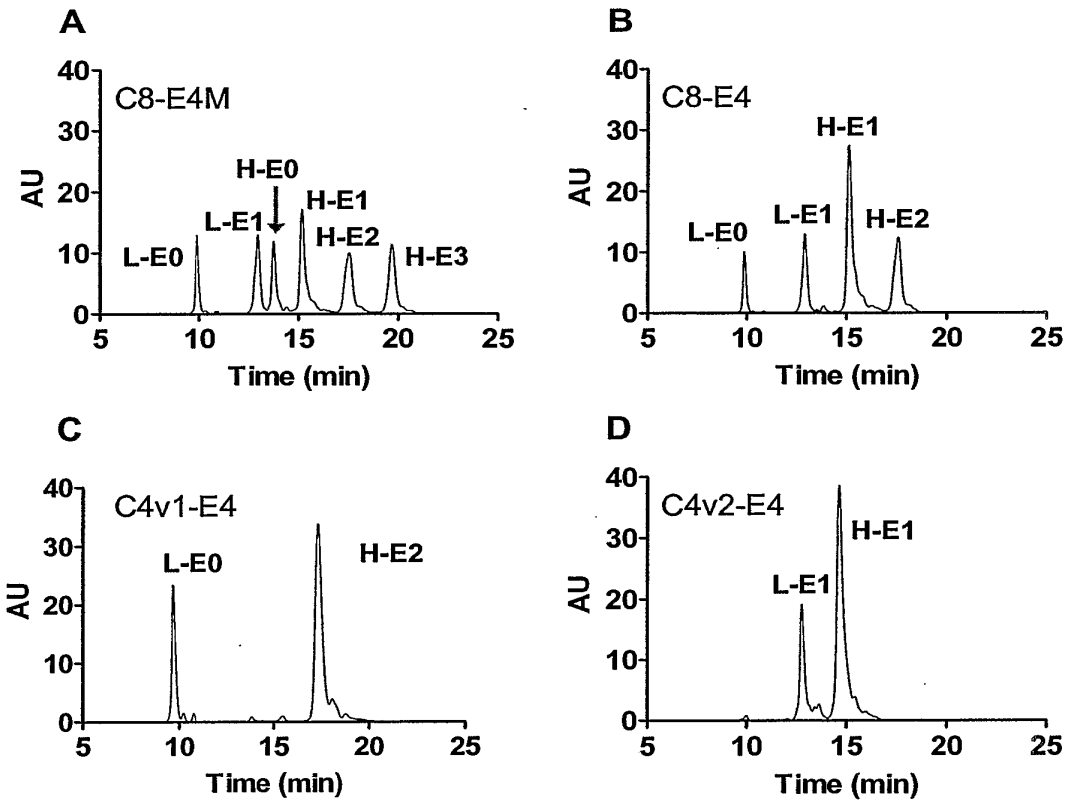
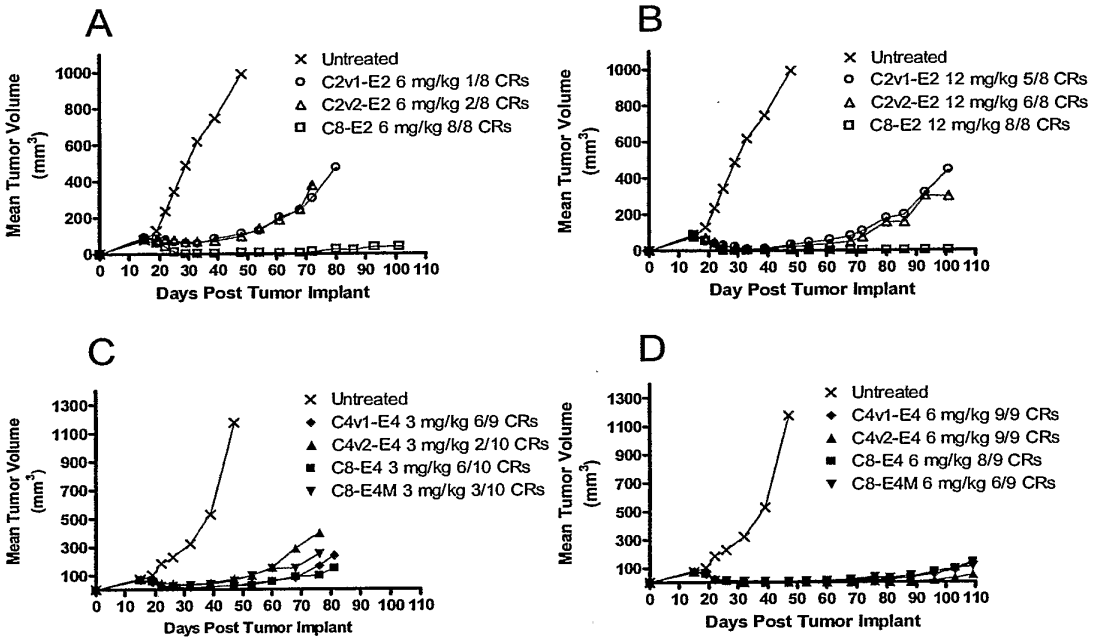

A
C8-E4M
AU
L-E0
L-E1
H-E0
H-E1
H-E2
H-E3
Time (min)
B
C8-E4
AU
L-E0
L-E1
H-E1
H-E2
Time (min)
C
C4v1-E4
AU
L-E0
H-E2
Time (min)
D
C4v2-E4
AU
L-E1
H-E1
Time (min)

Figure 7


A
Mean Tumor Volume (mm3)
Days Post Tumor Implant
Untreated
C2v1-E2 6 mg/kg 1/8 CRs
C2v2-E2 6 mg/kg 2/8 CRs
C8-E2 6 mg/kg 8/8 CRs
B
Mean Tumor Volume (mm3)
Day Post Tumor Implant
Untreated
C2v1-E2 12 mg/kg 5/8 CRs
C2v2-E2 12 mg/kg 6/8 CRs
C8-E2 12 mg/kg 8/8 CRs
C
Mean Tumor Volume (mm3)
Days Post Tumor Implant
Untreated
C4v1-E4 3 mg/kg 6/9 CRs
C4v2-E4 3 mg/kg 2/10 CRs
C8-E4 3 mg/kg 6/10 CRs
C8-E4M 3 mg/kg 3/10 CRs
D
Mean Tumor Volume (mm3)
Days Post Tumor Implant
Untreated
C4v1-E4 6 mg/kg 9/9 CRs
C4v2-E4 6 mg/kg 9/9 CRs
C8-E4 6 mg/kg 8/9 CRs
C8-E4M 6 mg/kg 6/9 CRs

SEQUENCE LISTING

<110> Seattle Genetics, Inc.
McDonagh, Charlotte
Carter, Paul

<120> Engineered Antibody Drug Conjugates

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<212> DNA
<213> Artificial

<220>
<223> cAC10 H mouse/human chimera

<400> 6
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atccagctgc agcagtctgg acctgaggtg gtgaagcctg gggcttcagt gaagatatcc 120

tgcaaggctt ctggctacac cttcactgac tactatataa cctgggtgaa gcagaagcct 180

ggacagggac ttgagtggat tggatggatt tatcctggaa gcggtaatac taagtacaat 240

gagaagttca agggcaaggc cacattgact gtagacacat cctccagcac agccttcatg 300

cagctcagca gcctgacatc tgaggacact gctgtctatt tctgtgcgaa ctatggtaac 360

tactggtttg cttactgggg ccaagggact caggtcactg tctctgcagc tagcaccaag 420

ggcccatcgg tcttcccccc ggcaccctcc tccaagagca cctctggggg cacagcggcc 480

ctgggctgcc tggtcaagga ctacttcccc gaaccggtga cggtgtcgtg gaactcaggc 540

gccctgacca gcggcgtgca caccttcccg gctgtcctac agtcctcagg actctactcc 600

ctcagcagcg tggtgaccgt gccctccagc agcttgggca cccagaccta catctgcaac 660

gtgaatcaca agcccagcaa caccaaggtg gacaagaaag ttgagcccaa atcttgtgac 720

aaaactcaca catgcccacc gtgcccagca cctgaactcc tgggggggacc gtcagtcttc 780

ctcttccccc caaaacccaa ggacaccctc atgatctccc ggacccctga ggtcacatgc 840

gtggtggtgg acgtgagcca cgaagaccct gaggtcaagt tcaactggta cgtggacggc 900

gtggaggtgc ataatgccaa gacaaagccg cgggaggagc agtacaacag cacgtaccgt 960

gtggtcagcg tcctcaccgt cctgcaccag gactggctga atggcaagga gtacaagtgc 1020

aaggtctcca acaaagccct cccagccccc atcgagaaaa ccatctccaa agccaaaggg 1080

cagccccgag aaccacaggt gtacaccctg cccccatccc gggatgagct gaccaagaac 1140

caggtcagcc tgacctgcct ggtcaaaggc ttctatccca gcgacatcgc cgtggagtgg 1200

gagagcaatg ggcagccgga gaacaactac aagaccacgc ctcccgtgct ggactccgac 1260

ggctccttct tcctctacag caagctcacc gtggacaaga gcaggtggca gcaggggaac 1320

gtcttctcat gctccgtgat gcatgaggct ctgcacaacc actacacaca gaagagcctc 1380

tccctgtctc cgggtaaatg a 1401

<210> 7
<211> 466
<212> PRT
<213> Artificial

<220>
<223> cAC10 H mouse/human chimera

<400> 7

Met Gly Trp Ser Trp Ile Phe Leu Phe Leu Leu Ser Gly Thr Ala Gly
1 5 10 15

Val His Cys Gln Ile Gln Leu Gln Gln Ser Gly Pro Glu Val Val Lys
20 25 30

Pro Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe
35 40 45

Thr Asp Tyr Tyr Ile Thr Trp Val Lys Gln Lys Pro Gly Gln Gly Leu
50 55 60

Glu Trp Ile Gly Trp Ile Tyr Pro Gly Ser Gly Asn Thr Lys Tyr Asn
65 70 75 80

Glu Lys Phe Lys Gly Lys Ala Thr Leu Thr Val Asp Thr Ser Ser Ser
85 90 95

Thr Ala Phe Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Thr Ala Val
100 105 110

Tyr Phe Cys Ala Asn Tyr Gly Asn Tyr Trp Phe Ala Tyr Trp Gly Gln
115 120 125

```
Gly Thr Gln Val Thr Val Ser Ala Ala Ser Thr Lys Gly Pro Ser Val
    130                 135                 140

Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala
145                 150                 155                 160

Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser
                165                 170                 175

Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
            180                 185                 190

Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro
        195                 200                 205

Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys
    210                 215                 220

Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp
225                 230                 235                 240

Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly
                245                 250                 255

Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile
            260                 265                 270

Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu
        275                 280                 285

Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His
    290                 295                 300

Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg
305                 310                 315                 320

Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys
                325                 330                 335

Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu
            340                 345                 350

Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr
        355                 360                 365

Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu
```

370 375 380

Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp
385 390 395 400

Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val
405 410 415

Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp
420 425 430

Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His
435 440 445

Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro
450 455 460

Gly Lys
465

<210> 8
<211> 717
<212> DNA
<213> Artificial

<220>
<223> cAC10 L mouse/human chimera

<400> 8
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gacattgtgc tgacccaatc tccagcttct ttggctgtgt ctctagggca gagggccacc 120

atctcctgca aggccagcca aagtgttgat tttgatggtg atagttatat gaactggtac 180

caacagaaac caggacagcc acccaaagtc ctcatctatg ctgcatccaa tctagaatct 240

gggatcccag ccaggtttag tggcagtggg tctgggacag acttcaccct caacatccat 300

cctgtggagg aggaggatgc tgcaacctat tactgtcagc aaagtaatga ggatccgtgg 360

acgttcggtg gaggcaccaa gctggaaatc aaacgaactg tggctgcacc atctgtcttc 420

atcttcccgc catctgatga gcagttgaaa tctggaactg cctctgttgt gtgcctgctg 480

aataacttct atcccagaga ggccaaagta cagtggaagg tggataacgc cctccaatcg 540

ggtaactccc aggagagtgt cacagagcag gacagcaagg acagcaccta cagcctcagc 600

agcaccctga cgctgagcaa agcagactac gagaaacaca aagtctacgc ctgcgaagtc 660

acccatcagg gcctgagctc gcccgtcaca aagagcttca acaggggaga gtgttag 717

<210> 9
<211> 238
<212> PRT
<213> Artificial

<220>
<223> cAC10 L mouse/human chimera

<400> 9

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Met Glu Thr Asp Thr Ile Leu Leu Trp Val Leu Leu Leu Trp Val Pro
1               5                  10                  15

Gly Ser Thr Gly Asp Ile Val Leu Thr Gln Ser Pro Ala Ser Leu Ala
            20                  25                  30

Val Ser Leu Gly Gln Arg Ala Thr Ile Ser Cys Lys Ala Ser Gln Ser
        35                  40                  45

Val Asp Phe Asp Gly Asp Ser Tyr Met Asn Trp Tyr Gln Gln Lys Pro
    50                  55                  60

Gly Gln Pro Pro Lys Val Leu Ile Tyr Ala Ala Ser Asn Leu Glu Ser
65                  70                  75                  80

Gly Ile Pro Ala Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr
                85                  90                  95

Leu Asn Ile His Pro Val Glu Glu Glu Asp Ala Ala Thr Tyr Tyr Cys
            100                 105                 110

Gln Gln Ser Asn Glu Asp Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu
        115                 120                 125

Glu Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro
    130                 135                 140

Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu
145                 150                 155                 160

Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn
                165                 170                 175

Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser
            180                 185                 190

Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala
        195                 200                 205
```

Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly
210 215 220

Leu Ser Ser Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
225 230 235

<210> 10
<211> 1401
<212> DNA
<213> Artificial

<220>
<223> cAC10 H220 cDNA mouse/human chimera

<400> 10
atgggatgga gctggatctt tcttttcctc ctgtcaggaa ctgcaggtgt ccattgtcag 60

atccagctgc agcagtctgg acctgaggtg gtgaagcctg gggcttcagt gaagatatcc 120

tgcaaggctt ctggctacac cttcactgac tactatataa cctgggtgaa gcagaagcct 180

ggacagggac ttgagtggat tggatggatt tatcctggaa gcggtaatac taagtacaat 240

gagaagttca agggcaaggc cacattgact gtagacacat cctccagcac agccttcatg 300

cagctcagca gcctgacatc tgaggacact gctgtctatt tctgtgcgaa ctatggtaac 360

tactggtttg cttactgggg ccaagggact caggtcactg tctctgcagc tagcaccaag 420

ggcccatcgg tcttcccccc ggcaccctcc tccaagagca cctctggggg cacagcggcc 480

ctgggctgcc tggtcaagga ctacttcccc gaaccggtga cggtgtcgtg gaactcaggc 540

gccctgacca gcggcgtgca caccttcccg gctgtcctac agtcctcagg actctactcc 600

ctcagcagcg tggtgaccgt gccctccagc agcttgggca cccagaccta catctgcaac 660

gtgaatcaca agcccagcaa caccaaggtg gacaagaaag ttgagcccaa atcttctgac 720

aaaactcaca catgcccacc gtgcccagca cctgaactcc tgggggggacc gtcagtcttc 780

ctcttccccc caaaacccaa ggacaccctc atgatctccc ggacccctga ggtcacatgc 840

gtggtggtgg acgtgagcca cgaagaccct gaggtcaagt tcaactggta cgtggacggc 900

gtggaggtgc ataatgccaa gacaaagccg cgggaggagc agtacaacag cacgtaccgt 960

gtggtcagcg tcctcaccgt cctgcaccag gactggctga atggcaagga gtacaagtgc 1020

aaggtctcca acaaagccct cccagccccc atcgagaaaa ccatctccaa agccaaaggg 1080

cagccccgag aaccacaggt gtacaccctg cccccatccc gggatgagct gaccaagaac 1140

caggtcagcc tgacctgcct ggtcaaaggc ttctatccca gcgacatcgc cgtggagtgg 1200

gagagcaatg ggcagccgga gaacaactac aagaccacgc ctcccgtgct ggactccgac 1260

ggctccttct tcctctacag caagctcacc gtggacaaga gcaggtggca gcaggggaac 1320

gtcttctcat gctccgtgat gcatgaggct ctgcacaacc actacacaca gaagagcctc 1380

tccctgtctc cgggtaaatg a 1401

<210> 11
<211> 466
<212> PRT
<213> Artificial

<220>
<223> cAC10 H220 mouse/human chimera protein

<400> 11

Met Gly Trp Ser Trp Ile Phe Leu Phe Leu Leu Ser Gly Thr Ala Gly
1 5 10 15

Val His Cys Gln Ile Gln Leu Gln Gln Ser Gly Pro Glu Val Val Lys
20 25 30

Pro Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe
35 40 45

Thr Asp Tyr Tyr Ile Thr Trp Val Lys Gln Lys Pro Gly Gln Gly Leu
50 55 60

Glu Trp Ile Gly Trp Ile Tyr Pro Gly Ser Gly Asn Thr Lys Tyr Asn
65 70 75 80

Glu Lys Phe Lys Gly Lys Ala Thr Leu Thr Val Asp Thr Ser Ser Ser
85 90 95

Thr Ala Phe Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Thr Ala Val
100 105 110

Tyr Phe Cys Ala Asn Tyr Gly Asn Tyr Trp Phe Ala Tyr Trp Gly Gln
115 120 125

Gly Thr Gln Val Thr Val Ser Ala Ala Ser Thr Lys Gly Pro Ser Val
130 135 140

Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala
145 150 155 160

Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser
165 170 175

```
Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
            180                 185                 190

Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro
        195                 200                 205

Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys
    210                 215                 220

Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Ser Asp
225                 230                 235                 240

Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly
                245                 250                 255

Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile
            260                 265                 270

Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu
        275                 280                 285

Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His
    290                 295                 300

Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg
305                 310                 315                 320

Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys
                325                 330                 335

Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu
            340                 345                 350

Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr
        355                 360                 365

Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu
    370                 375                 380

Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp
385                 390                 395                 400

Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val
                405                 410                 415

Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp
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420 425 430

Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His
435 440 445

Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro
450 455 460

Gly Lys
465

<210> 12
<211> 1401
<212> DNA
<213> Artificial

<220>
<223> cAC10 H220/226 mouse/human chimera

<400> 12
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atccagctgc agcagtctgg acctgaggtg gtgaagcctg gggcttcagt gaagatatcc 120

tgcaaggctt ctggctacac cttcactgac tactatataa cctgggtgaa gcagaagcct 180

ggacagggac ttgagtggat tggatggatt tatcctggaa gcggtaatac taagtacaat 240

gagaagttca agggcaaggc cacattgact gtagacacat cctccagcac agccttcatg 300

cagctcagca gcctgacatc tgaggacact gctgtctatt tctgtgcgaa ctatggtaac 360

tactggtttg cttactgggg ccaagggact caggtcactg tctctgcagc tagcaccaag 420

ggcccatcgg tcttcccccct ggcaccctcc tccaagagca cctctggggg cacagcggcc 480

ctgggctgcc tggtcaagga ctacttcccc gaaccggtga cggtgtcgtg gaactcaggc 540

gccctgacca gcggcgtgca caccttcccg gctgtcctac agtcctcagg actctactcc 600

ctcagcagcg tggtgaccgt gccctccagc agcttgggca cccagaccta catctgcaac 660

gtgaatcaca agcccagcaa caccaaggtg gacaagaaag ttgagcccaa atcttctgac 720

aaaactcaca catccccacc gtgcccagca cctgaactcc tgggggggacc gtcagtcttc 780

ctcttccccc caaaacccaa ggacaccctc atgatctccc ggacccctga ggtcacatgc 840

gtggtggtgg acgtgagcca cgaagaccct gaggtcaagt tcaactggta cgtggacggc 900

gtggaggtgc ataatgccaa gacaaagccg cgggaggagc agtacaacag cacgtaccgt 960

gtggtcagcg tcctcaccgt cctgcaccag gactggctga atggcaagga gtacaagtgc 1020

aaggtctcca acaaagccct cccagccccc atcgagaaaa ccatctccaa agccaaaggg 1080

cagccccgag aaccacaggt gtacaccctg cccccatccc gggatgagct gaccaagaac 1140

caggtcagcc tgacctgcct ggtcaaaggc ttctatccca gcgacatcgc cgtggagtgg 1200

gagagcaatg ggcagccgga gaacaactac aagaccacgc ctcccgtgct ggactccgac 1260

ggctccttct tcctctacag caagctcacc gtggacaaga gcaggtggca gcaggggaac 1320

gtcttctcat gctccgtgat gcatgaggct ctgcacaacc actacacaca gaagagcctc 1380

tccctgtctc cgggtaaatg a 1401

<210> 13
<211> 466
<212> PRT
<213> Artificial

<220>
<223> cAC10 H220/226 mouse/human chimera

<400> 13

Met Gly Trp Ser Trp Ile Phe Leu Phe Leu Leu Ser Gly Thr Ala Gly
1 5 10 15

Val His Cys Gln Ile Gln Leu Gln Gln Ser Gly Pro Glu Val Val Lys
20 25 30

Pro Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe
35 40 45

Thr Asp Tyr Tyr Ile Thr Trp Val Lys Gln Lys Pro Gly Gln Gly Leu
50 55 60

Glu Trp Ile Gly Trp Ile Tyr Pro Gly Ser Gly Asn Thr Lys Tyr Asn
65 70 75 80

Glu Lys Phe Lys Gly Lys Ala Thr Leu Thr Val Asp Thr Ser Ser Ser
85 90 95

Thr Ala Phe Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Thr Ala Val
100 105 110

Tyr Phe Cys Ala Asn Tyr Gly Asn Tyr Trp Phe Ala Tyr Trp Gly Gln
115 120 125

Gly Thr Gln Val Thr Val Ser Ala Ala Ser Thr Lys Gly Pro Ser Val
130 135 140

Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala
145 150 155 160

```
Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser
                165                 170                 175

Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
            180                 185                 190

Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro
        195                 200                 205

Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys
    210                 215                 220

Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Ser Asp
225                 230                 235                 240

Lys Thr His Thr Ser Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly
                245                 250                 255

Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile
            260                 265                 270

Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu
        275                 280                 285

Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His
    290                 295                 300

Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg
305                 310                 315                 320

Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys
                325                 330                 335

Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu
            340                 345                 350

Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr
        355                 360                 365

Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu
    370                 375                 380

Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp
385                 390                 395                 400
```

Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val
405 410 415

Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp
420 425 430

Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His
435 440 445

Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro
450 455 460

Gly Lys
465

<210> 14
<211> 1401
<212> DNA
<213> Artificial

<220>
<223> cAC10 H226/229 mouse/human chimera

<400> 14
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atccagctgc agcagtctgg acctgaggtg gtgaagcctg gggcttcagt gaagatatcc 120

tgcaaggctt ctggctacac cttcactgac tactatataa cctgggtgaa gcagaagcct 180

ggacagggac ttgagtggat tggatggatt tatcctggaa gcggtaatac taagtacaat 240

gagaagttca agggcaaggc cacattgact gtagacacat cctccagcac agccttcatg 300

cagctcagca gcctgacatc tgaggacact gctgtctatt tctgtgcgaa ctatggtaac 360

tactggtttg cttactgggg ccaagggact caggtcactg tctctgcagc tagcaccaag 420

ggcccatcgg tcttcccccct ggcaccctcc tccaagagca cctctggggg cacagcggcc 480

ctgggctgcc tggtcaagga ctacttcccc gaaccggtga cggtgtcgtg gaactcaggc 540

gccctgacca gcggcgtgca caccttcccg gctgtcctac agtcctcagg actctactcc 600

ctcagcagcg tggtgaccgt gccctccagc agcttgggca cccagaccta catctgcaac 660

gtgaatcaca agcccagcaa caccaaggtg gacaagaaag ttgagcccaa atcttgtgac 720

aaaactcaca catccccacc gtccccagca cctgaactcc tgggggggacc gtcagtcttc 780

ctcttccccc caaaacccaa ggacaccctc atgatctccc ggacccctga ggtcacatgc 840

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gtggaggtgc ataatgccaa gacaaagccg cgggaggagc agtacaacag cacgtaccgt 960

gtggtcagcg tcctcaccgt cctgcaccag gactggctga atggcaagga gtacaagtgc 1020

aaggtctcca acaaagccct cccagccccc atcgagaaaa ccatctccaa agccaaaggg 1080

cagccccgag aaccacaggt gtacaccctg cccccatccc gggatgagct gaccaagaac 1140

caggtcagcc tgacctgcct ggtcaaaggc ttctatccca gcgacatcgc cgtggagtgg 1200

gagagcaatg ggcagccgga gaacaactac aagaccacgc ctcccgtgct ggactccgac 1260

ggctccttct tcctctacag caagctcacc gtggacaaga gcaggtggca gcaggggaac 1320

gtcttctcat gctccgtgat gcatgaggct ctgcacaacc actacacaca gaagagcctc 1380

tccctgtctc cgggtaaatg a 1401

<210> 15
<211> 466
<212> PRT
<213> Artificial

<220>
<223> cAC10 H226/229 mouse/human chimera

<400> 15

Met Gly Trp Ser Trp Ile Phe Leu Phe Leu Leu Ser Gly Thr Ala Gly
1 5 10 15

Val His Cys Gln Ile Gln Leu Gln Gln Ser Gly Pro Glu Val Val Lys
20 25 30

Pro Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe
35 40 45

Thr Asp Tyr Tyr Ile Thr Trp Val Lys Gln Lys Pro Gly Gln Gly Leu
50 55 60

Glu Trp Ile Gly Trp Ile Tyr Pro Gly Ser Gly Asn Thr Lys Tyr Asn
65 70 75 80

Glu Lys Phe Lys Gly Lys Ala Thr Leu Thr Val Asp Thr Ser Ser Ser
85 90 95

Thr Ala Phe Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Thr Ala Val
100 105 110

Tyr Phe Cys Ala Asn Tyr Gly Asn Tyr Trp Phe Ala Tyr Trp Gly Gln
115 120 125

Gly Thr Gln Val Thr Val Ser Ala Ala Ser Thr Lys Gly Pro Ser Val

```
    130                 135                 140

Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala
145                 150                 155                 160

Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser
                165                 170                 175

Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
            180                 185                 190

Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro
        195                 200                 205

Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys
    210                 215                 220

Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp
225                 230                 235                 240

Lys Thr His Thr Ser Pro Pro Ser Pro Ala Pro Glu Leu Leu Gly Gly
                245                 250                 255

Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile
            260                 265                 270

Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu
        275                 280                 285

Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His
    290                 295                 300

Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg
305                 310                 315                 320

Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys
                325                 330                 335

Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu
            340                 345                 350

Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr
        355                 360                 365

Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu
    370                 375                 380
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Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp
385 390 395 400

Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val
405 410 415

Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp
420 425 430

Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His
435 440 445

Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro
450 455 460

Gly Lys
465

<210> 16
<211> 1401
<212> DNA
<213> Artificial

<220>
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Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro
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(12) NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT) VERÖFFENTLICHTE INTERNATIONALE ANMELDUNG

(19) Weltorganisation für geistiges Eigentum
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— *ohne internationalen Recherchenbericht und erneut zu veröffentlichen nach Erhalt des Berichts*

Zur Erklärung der Zweibuchstaben-Codes und der anderen Abkürzungen wird auf die Erklärungen ("Guidance Notes on Codes and Abbreviations") am Anfang jeder regulären Ausgabe der PCT-Gazette verwiesen.

WO 2006/092230 A2

(54) **Title:** PROTEIN-BINDING CAMPTOTHECIN PEPTIDE DERIVATIVES AND MEDICAMENTS COMPRISING THE SAME

(54) **Bezeichnung:** PROTEINBINDENDE CAMPTOTHECIN-PEPTID-DERIVATE UND DIESE ENTHALTENDE ARZNEIMITTEL

(57) **Abstract:** The invention relates to camptothecin peptide derivatives, comprising a protein-binding group, which may be enzymatically cleaved in vivo with release of the active ingredient or a low-molecular weight active ingredient derivative.

(57) **Zusammenfassung:** Die Erfindung betrifft Camptothecin-Peptid-Derivate, die eine proteinbindende Gruppe enthalten und enzymatisch im Körper unter Freisetzung des Wirkstoffs oder eines niedermolekularen Wirkstoff-Derivates spaltbar sind.

Proteinbindende Camptothecin-Peptid-Derivate und diese enthaltende Arzneimittel

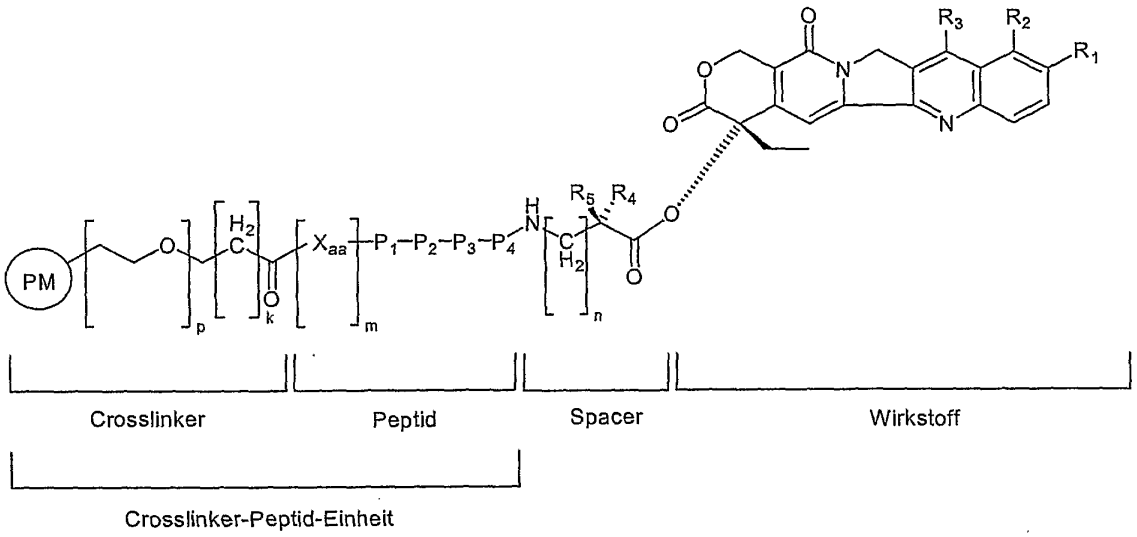
Beschreibung

Die Erfindung betrifft Camptothecin-Peptid-Derivate, die eine proteinbindende Gruppe enthalten und unter Beteiligung der Cathepsine B und/oder D enzymatisch im Körper unter Freisetzung des Wirkstoffs oder eines niedermolekularen Wirkstoff-Derivates spaltbar sind.

Camptothecin (CPT) ist ein Alkaloid mit tumorhemmenden Eigenschaften (Wall M. E., et al. *J. Am. Chem. Soc.* **1966**, *88*, 3888-3890), dessen Derivate wie Topotecan oder Irinotecan zur Behandlung verschiedener Krebserkrankungen eingesetzt werden (Kollmannsberger C., et al. *Oncology* **1999**, *56*, 1-12, Rothenberg M. L. *Oncologist* **2001**, *6*, 66-80). Jedoch sind Therapien mit diesen Arzneistoffen von Nebenwirkungen begleitet. Um das Nebenwirkungsprofil und die Wirksamkeit von CPT bzw. CPT-Derivaten zu verbessern, wurden makromolekulare Transportformen von CPT durch Kopplung des Wirkstoffs an synthetische Polymere wie Poly(ethylenglykol) (Greenwald R. B., et al. *Bioorg. Med. Chem.* **1998**, *6*, 551-562, Conover C. D., et al. *Anti-Cancer Drug Design* **1999**, *14*, 499-506), HPMA-Copolymere (Fraier D., et al. *J. Pharm. Biomed. Anal.* **2000**, *22*, 505-514) oder HPMA-Copolymere in Verbindung mit Peptid-Spacern entwickelt (Caiolfa V. R., et al. *J. Controlled Release* **2000**, *65*, 105-119, Zamai M., et al. *Mol. Cancer Ther.* **2003**, 2, 29-40).

Der Erfindung liegt die Aufgabe zugrunde, Prodrugs von CPT zu schaffen, die CPT oder CPT-Derivate im Tumor freisetzen.

Diese Aufgabe wird erfindungsgemäß gelöst durch Camptothecin-Peptid-Derivate der allgemeinen Formel

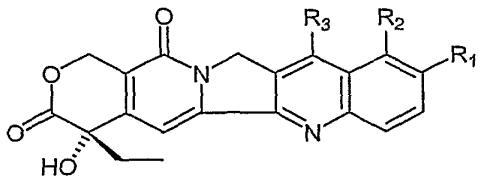


worin R_1 = H oder OH, R_2 = H, NH_2, NO_2 oder *N,N*-Dimethylaminomethyl, R_3 = H oder Ethyl, R_4 = H, Alkyl oder Aryl, R_5 = H, Alkyl oder Aryl, von denen die beiden letztgenannten gegebenenfalls substituiert sein können, P_1–P_4 = L- oder D-Aminosäuren, X_{aa} eine löslichkeitsvermittelnde Aminosäure, n = 0 bis 3, m = 1 bis 3, k = 0 bis 5, p = 0 bis 6 bedeuten und PM eine proteinbindende Gruppe ist.

Eine integrierte hydrolytisch oder enzymatisch spaltbare Sollbruchstelle erlaubt hierbei eine kontrollierte Freisetzung des Wirkstoffs oder eines Aminoacyl-Wirkstoff-Derivates *in vivo*, sodass Camptothecin-Derivate der vorliegenden Erfindung Prodrugs darstellen.

Die erfindungsgemäßen CPT-Derivate sind aus einer antitumoral wirksamen Camptothecin-Komponente, einem Spacermolekül, einer Peptidkette und einem heterobifunktionellen Crosslinker aufgebaut. Im Folgenden wird dieser Aufbau näher erläutert:

Die antitumoral wirksame CPT-Komponente ist ein Wirkstoff der allgemeinen Formel



worin

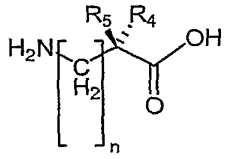
R_1 = H, OH

R_2 = H, NH_2, NO_2 oder *N,N*-Dimethylaminomethyl

R_3 = H, Ethyl

bedeuten, welcher eine freie 20-Hydroxygruppe aufweist. Bevorzugte Wirkstoffe sind Camptothecin, Topotecan, 10-Hydroxycamptothecin, 9-Aminocamptothecin und 9-Nitrocamptothecin.

Das Spacermolekül ist eine Aminocarbonsäure der allgemeinen Formel



in der

R_4 = H, Alkyl oder Aryl, bevorzugt H

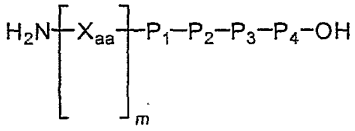
R_5 = H, Alkyl oder Aryl, gegebenenfalls substituiert, bevorzugt H oder Methyl

n = 0 bis 3

bedeuten.

Besonders bevorzugte Spacer sind 4-Aminobutansäure, 5-Aminopentansäure, 3-Aminopropansäure sowie L- und D-Alanin.

Die Peptidkette setzt sich aus einer durch die Cathepsine B und/oder D spaltbaren Sequenz sowie einer *N*-terminalen löslichkeitsvermittelnden Komponente zusammen und hat die allgemeine Formel



in der

P_1–P_4 = L- oder D-Aminosäuren

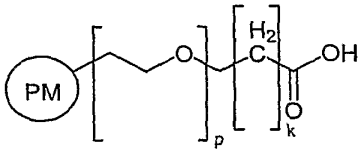
X_{aa} = eine Aminosäure mit basischer Seitenkette

bedeuten.

Die Aminosäuren P_1 und P_3 sind bevorzugt ausgewählt unter den Aminosäuren Alanin, Glycin, Leucin, Isoleucin, Norleucin, Valin und/oder Phenylalanin. Die Aminosäuren P_2 und P_4 sind bevorzugt ausgewählt unter den Aminosäuren Leucin, Isoleucin, Norleucin und/oder Phenylalanin.

Die löslichkeitsvermittelnde Gruppe Xaa ist bevorzugt ausgewählt unter den Aminosäuren Arginin, Lysin und Histidin. Eine besonders bevorzugte Gruppe ist Arginin.

Der heterobifunktionelle Crosslinker ist eine Carbonsäure mit einer proteinbindenden Gruppe der allgemeinen Formel



in der

k = 0 bis 5

p = 0 bis 6

PM = proteinbindende Gruppe

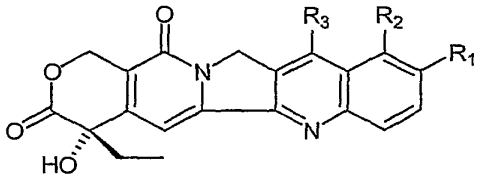
bedeuten.

Die proteinbindende Gruppe (PM) ist bevorzugt ausgewählt unter einer 2-Dithiopyridylgruppe, einer Halogenacetamidgruppe, einer Halogenacetatgruppe, einer Disulfidgruppe, einer Acrylsäureestergruppe, einer Monoalkylmaleinsäureestergruppe, einer Monoalkylmaleaminsäureamidgruppe, einer *N*-Hydroxysuccinimidylestergruppe, einer Isothiocyanatgruppe, einer Aziridingruppe oder einer Maleinimidgruppe. Eine besonders bevorzugte proteinbindende Gruppe ist die Maleinimidgruppe.

Wirkstoff und Spacermolekül sind durch eine Esterbindung zwischen der 20-Hydroxygruppe des Wirkstoffs und einer Carboxylgruppe des Spacermoleküls verbunden. Die Bindung zwischen Spacermolekül und der Crosslinker-Peptid-Einheit besteht aus einer Amidbindung zwischen der Aminogruppe des Spacermoleküls und der C-terminalen Carboxylgruppe der Crosslinker-Peptid-Einheit. Die Bindung zwischen Crosslinker und der Peptidkette besteht aus einer Amidbindung zwischen dem N-Terminus der Peptidkette und der Carboxylgruppe des Crosslinkers.

Eine wesentliche Eigenschaft der erfindungsgemäßen CPT-Derivate besteht darin, dass die Bindungen zwischen Wirkstoff und Spacermolekül sowie zwischen Spacermolekül und dem Crosslinker hydrolytisch und/oder enzymatisch unter Beteiligung der Cathepsine B und/oder D spaltbar sind, wodurch eine kontrollierte Freisetzung des Wirkstoffs oder eines Aminoacyl-Wirkstoff-Derivates in Tumorgewebe ermöglicht wird. Die Cathepsine B und D werden in vielen humanen Tumoren überexprimiert, wodurch sie einen idealen Angriffspunkt für eine Target-orientierte, enzymatische Aktivierung von Prodrugs darstellen (Yan, S., et al. *Biol. Chem.* **1998**, 2, 113-123, Leto, G., et al. *Clin. Exp. Metastasis* **2004**, 91-106). Des Weiteren zeigen die erfindungsgemäßen CPT-Derivate eine rasche Spaltung in experimentellen Tumorhomogenaten (siehe Beispiel 5).

Die Herstellung der erfindungsgemäßen CPT-Derivate erfolgt bevorzugt durch Kondensation von Camptothecin-Derivaten der allgemeinen Formel



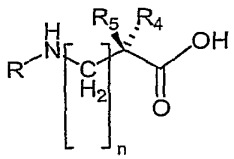
in der

R_1 = H oder OH

R_2 = H, NH_2, NO_2 oder *N,N*-Dimethylaminomethyl

R_3 = H oder Ethyl

bedeuten, mit Spacern der allgemeinen Formel



worin

R = eine dem Fachmann geläufige Schutzgruppe für Amine, bevorzugt Boc oder Fmoc

R_4 = H, Alkyl oder Aryl, bevorzugt H

R_5 = H, Alkyl oder Aryl, gegebenenfalls substituiert, bevorzugt H oder Methyl

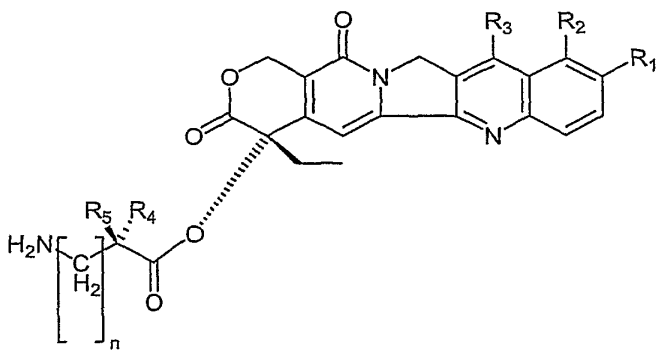
n = 0 bis 3

bedeuten.

Dabei werden als Reagenzien zur Aktivierung der Carboxylgruppe des Spacers vorzugsweise *N,N'*-Dicyclohexylcarbodiimid (DCC), *N,N'*-Diisopropylcarbodiimid (DIPC), (Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium-Hexafluorophosphat (BOP), *O*-(Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium-Hexafluorophosphat (HATU) oder 2-Chlor-1-methylpyridiniumiodid unter Zusatz gängiger Katalysatoren bzw. Hilfsbasen wie z. B. Trialkylamine, Pyridin, 4-Dimethylaminopyridin (DMAP) oder Hydroxybenzotriazol (HOBt) und/oder Scandium(III)trifluormethansulfonat eingesetzt. Die Umsetzung erfolgt zweckmäßig in einem polar-aprotischen organischen Lösungsmittel, vorzugsweise in Dichlormethan, *N,N*-Dimethylformamid und Tetrahydrofuran. Die Umsetzungen werden zweckmäßig bei Temperaturen zwischen –10 °C und Raumtemperatur durchgeführt, wobei die Reaktionszeit normalerweise zwischen 30 Minuten und 48 Stunden liegt. Die Aufarbeitung kann z. B. durch Umkristallisation aus Methanol oder Ethanol, oder durch Säulenchromatographie z. B. an Kieselgel erfolgen. Hierbei verwendete Laufmittel sind bevorzugt Chloroform-Methanol-Gemische, die gegebenenfalls Zusätze von Aminen oder Carbonsäuren enthalten können.

Anschließend wird die Schutzgruppe der Amino-Funktion entfernt. Diese Abspaltung kann z. B. durch Behandlung mit Trifluoressigsäure/Dichlormethan-Gemischen oder Piperidin erreicht werden. In einer für das Verfahren bevorzugten Ausführungsweise wird die Boc-geschütze Prodrugvorstufe 30 min mit Trifluoressigsäure/Dichlormethan 1:1 behandelt und das Produkt durch Fällung mit Diethylether isoliert.

Danach wird die erhaltene Prodrugvorstufe, der allgemeinen Formel



worin

R_1 = H oder OH

R_2 = H, NH_2, NO_2 oder *N,N*-Dimethylaminomethyl

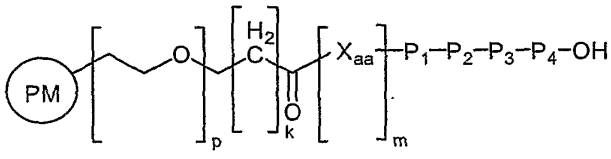
R_3 = H oder Ethyl

R_4 = H, Alkyl oder Aryl, bevorzugt H

R_5 = H, Alkyl oder Aryl, gegebenenfalls substituiert, bevorzugt H, Methyl

n = 0 bis 3

bedeuten, durch Kondensation umgesetzt mit einer Crosslinker-Peptid-Einheit der allgemeinen Formel



worin

m = 1 bis 3

k = 0 bis 5

p = 0 bis 6

PM = proteinbindende Gruppe

bedeuten.

Dabei werden als Reagenzien zur Aktivierung der Carboxylgruppe der Crosslinker-Peptid-Einheit vorzugsweise *O*-(Azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium-Hexafluorophosphat (HATU), (Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium-Hexafluorophosphat (BOP), *N,N'*-Diisopropylcarbodiimid (DIPC), *N,N'*-Dicyclohexylcarbodiimid (DCC) oder 2-Chlor-1-methylpyridiniumiodid unter Zusatz gängiger Katalysatoren bzw. Hilfsbasen wie z. B. *N*-Ethyldiisopropylamin (DIEA),

Trialkylamine, Pyridin, 4-Dimethylaminopyridin (DMAP) oder Hydroxybenzotriazol (HOBt) eingesetzt. Die Umsetzung erfolgt zweckmäßig in einem polaren organischen Lösungsmittel, vorzugsweise in *N,N*-Dimethylformamid. Die Umsetzungen werden zweckmäßig bei Temperaturen zwischen –10 °C und Raumtemperatur durchgeführt, wobei die Reaktionszeit normalerweise zwischen 30 Minuten und 48 Stunden liegt. Die Aufarbeitung erfolgt z. B. durch Säulenchromatographie bevorzugt an C_{18}-RP-Kieselgel. Bevorzugt verwendete Laufmittel sind hierbei Acetonitril-Wasser-Gemische, die gegebenenfalls Zusätze von Aminen oder Carbonsäuren, bevorzugt Trifluoressigsäure, enthalten können.

Nach einer bevorzugten Ausführungsweise wird Camptothecin zuerst mit Boc-geschützter 4-Aminobutansäure (Boc-GABA) und nachfolgender Abspaltung der Schutzgruppe zum Camptothecin-20-O-(4-aminobutanoat)-Trifluoracetat umgesetzt, welches anschließend mit EMC-Arg-Arg-Ala-Leu-Ala-Leu-OH (EMC = 6-Maleinimido-capronsäure) unter Benutzung von HATU als Kopplungsreagenz kondensiert wird (siehe Beispiel 1).

Durch die Verwendung löslichkeitsvermittelnder Argininggruppen lässt sich die Wasserlöslichkeit gegenüber CPT signifikant steigern. Solche Verbindungen weisen typischerweise eine gegenüber CPT um mehr als 400-fach gesteigerte Löslichkeit in isotonischer Kochsalzlösung auf (siehe Beispiel 2).

Die erfindungsgemäßen proteinbindenden Camptothecin-Derivate werden parenteral, bevorzugt intravenös appliziert. Dazu werden die erfindungsgemäßen CPT-Derivate als Lösungen, Feststoffe oder Lyophilisate, gegebenenfalls unter Verwendung üblicher Hilfsstoffe bereitgestellt. Solche Hilfsstoffe sind beispielsweise Polysorbate, Glucose, Lactose, Mannitol, Dextrane, Zitronensäure, Tromethamol, Triethanolamin, Aminoessigsäure und/oder synthetische Polymere. Bevorzugt werden die erfindungsgemäßen CPT-Derivate in einem isotonischen Puffer gelöst appliziert. Die Löslichkeit des CPT-Derivates kann gegebenenfalls durch pharmazeutische Lösungsmittel wie etwa 1,2-Propandiol, Ethanol, Isopropanol, Glycerol und/oder Poly(ethylenglykol) mit einem Molekulargewicht von 200 bis 600 g/mol, bevorzugt Poly(ethylenglykol) mit einem Molekulargewicht von 600 g/mol,

und/oder Löslichkeitsvermittler wie z. B. Tween 80, Cremophor oder Polyvinylpyrrolidon verbessert werden.

Ein wesentliches Merkmal der erfindungsgemäßen CPT-Derivate liegt in einer raschen kovalenten Bindung an Serumproteine über die proteinbindende Gruppe, wodurch eine makromolekulare Transportform des Wirkstoffs generiert wird. Von Serumproteinen wie Transferrin, Albumin und LDL ist eine erhöhte Aufnahme in Tumorgewebe bekannt (Kratz F.; Beyer U. *Drug Delivery* **1998**, *5*, 281-299), sodass diese im Rahmen der Erfindung als endogene Träger für Zytostatika herangezogen werden können. Ein besonders bevorzugtes Serumprotein ist zirkulierendes Humanserumalbumin (HSA), das mit einer durchschnittlichen Konzentration von 30 bis 50 g/L die Hauptprotein-Komponente des menschlichen Blutes bildet (Peters T. *Adv. Protein Chem.* **1985**, *37*, 161-245) und eine freie Cysteingruppe (Cystein-34-Gruppe) an der Oberfläche des Proteins aufweist, welche zur Anbindung von thiolbindenden Gruppen wie Maleinimiden oder Disulfiden geeignet ist (WO 00/76551). Dass das erfindungsgemäße Maleinimid-funktionalisierte CPT-Derivate schnell und selektiv an HSA binden, zeigt Beispiel 3. Die Reaktion der neuen CPT-Derivate mit Serumproteinen kann auch extrakorporal durchgeführt werden, z. B. mit einer zur Infusion vorgesehenen Albumin-, Blut- oder Serummenge.

Gegenüber Camptothecin-Konjugaten mit synthetischen Polymeren als Trägersystemen besitzen die der Erfindung zugrunde liegenden CPT-Peptid-Derivate den Vorteil, chemisch eindeutig definiert zu sein.

Die folgenden Beispiele erläutern die Erfindung in Verbindung mit der beigefügten Zeichnung näher.

In der Zeichnung stellen dar:

Abbildung 1 ein Chromatogramm von humanem Blutplasma; bei λ = 370 nm ist lediglich eine geringfügige Absorption zu beobachten.

Abbildung 2 ein Chromatogramm von EMC-Arg-Arg-Ala-Leu-Ala-Leu-GABA-CPT, das zuvor 30 min mit humanem Plasma inkubiert wurde (Detektion bei λ = 280 und 370 nm).

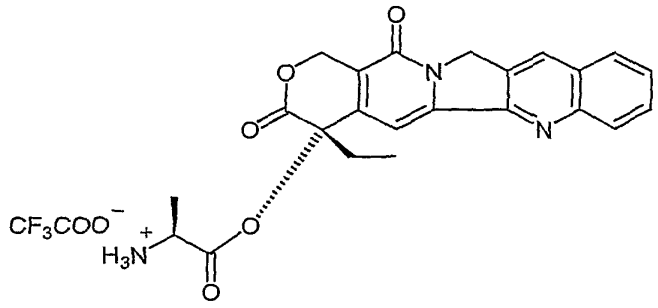
Abbildung 3 ein Chromatogramm des HSA-Konjugats von EMC-Arg-Arg-Ala-Leu-Ala-Leu-GABA-CPT nach Spaltung durch Cathepsin B.

Abbildung 4 ein Chromatogramm des HSA-Konjugats von EMC-Arg-Arg-Ala-Leu-Ala-Leu-GABA-CPT nach Spaltung in HT29-Kolorektaltumor-Homogenat bei pH 5,0.

Abbildung 5 ein Chromatogramm des HSA-Konjugats von EMC-Arg-Arg-Ala-Leu-Ala-Leu-GABA-CPT nach Spaltung in HT29-Kolorektaltumor-Homogenat bei pH 7,4.

Beispiel 1

Herstellung von Camptothecin-20-*O*-(L-Alaninat)-Trifluoracetat

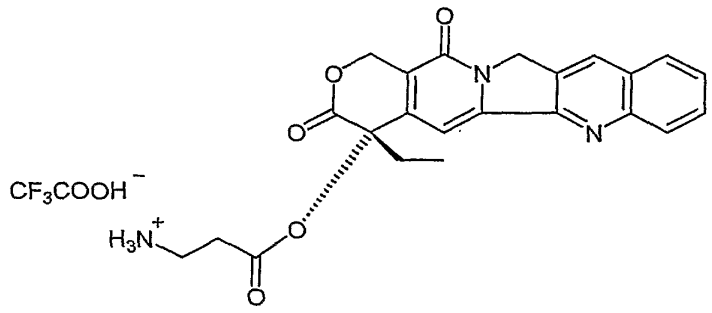


Zu einer Suspension von Boc-L-Alanin (489 mg, 2.58 mmol), Camptothecin (300 mg, 0.861 mmol), 4-Dimethylaminopyridin (330 mg, 2.58 mmol) sowie Scandium(III)-trifluormethansulfonat (256 mg, 1.46 mmol) in 30 mL Dichlormethan wird unter Stickstoffatmosphäre bei -8°C *N,N'*-Diisopropylcarbodiimid (426 µL, 2.76 mmol)

gegeben und 30 Minuten gerührt. Anschließend wird die Reaktionsmischung 2 Stunden bei Raumtemperatur gerührt, die Feststoffe abfiltriert und das Filtrat jeweils einmal mit 60 mL 0.1N HCl, 0.1M $NaHCO_3$ und destilliertem Wasser gewaschen. Die organische Phase wird über Magnesiumsulfat getrocknet und das Lösungsmittel im Vakuum entfernt. Nach Umkristallisation des Rückstands aus Methanol und Abspaltung der Boc-Schutzgruppe durch 30 minütige Behandlung mit 1 mL Trifluoressigsäure/Dichlormethan (1:1) erhält man 292 mg Camptothecin-20-*O*-(L-alaninat)-Trifluoracetat als blassgelben Feststoff.

Beispiel 2

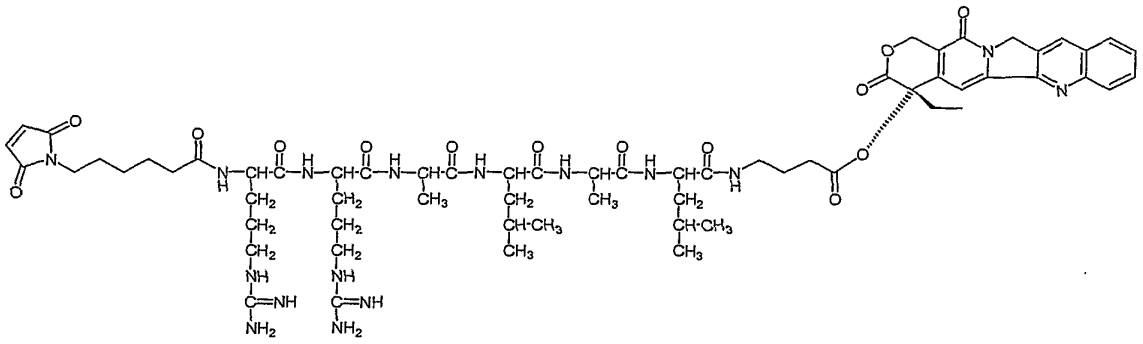
Herstellung von Camptothecin-20-*O*-(3-Aminopropanoat)-Trifluoracetat



Zu einer Lösung von Boc-3-Aminopropansäure (489 mg, 2.87 mmol) in 100 mL Dichlormethan werden bei 0°C Camptothecin (300 mg, 0.861 mmol), 4-Dimethylaminopyridin (210 mg, 1.72 mmol) sowie *N,N'*-Diisopropylcarbodiimid (420 µL, 2.87 mmol) unter Stickstoffatmosphäre gegeben und die entstandene Suspension sechzehn Stunden bei Raumtemperatur gerührt, wobei eine klare Lösung entsteht. Anschließend wird zweimal mit 100 mL 0.1N HCl gewaschen, die organische Phase über Magnesiumsulfat getrocknet und das Lösungsmittel im Vakuum entfernt. Nach Umkristallisation des Rückstands aus Methanol und Abspaltung der Boc-Schutzgruppe durch 30 minütige Behandlung mit 1 mL Trifluoressigsäure/Dichlormethan (1:1) erhält man 310 mg Camptothecin-20-O-(4-aminobutanoat)-Trifluoracetat als blassgelben Feststoff.

Beispiel 3

Herstellung von Camptothecin-20-O-[(((((((6-maleinimidohexanoyl)arginyl)-arginyl)alanyl)leucyl)alanyl)leucyl)-4-aminobutanoat] (abgekürzt EMC-Arg-Arg-Ala-Leu-Ala-Leu-GABA-CPT)



Zu einer Lösung von Boc-4-aminobutansäure (583 mg, 2.87 mmol) in 100 mL Dichlormethan werden bei 0°C Camptothecin (300 mg, 0.861 mmol), 4-Dimethylaminopyridin (210 mg, 1.72 mmol) sowie *N,N'*-Diisopropylcarbodiimid (420 µL, 0.861 mmol) unter Stickstoffatmosphäre gegeben und die entstandene Suspension sechzehn Stunden bei Raumtemperatur gerührt, wobei eine klare Lösung entsteht. Anschließend wird zweimal mit 100 mL 0.1N HCl gewaschen, die organische Phase über Magnesiumsulfat getrocknet und das Lösungsmittel im Vakuum entfernt. Nach Umkristallisation des Rückstands aus Methanol und Abspaltung der Boc-Schutzgruppe durch 30 minütige Behandlung mit 1 mL Trifluoressigsäure/Dichlormethan (1:1) erhält man 310 mg Camptothecin-20-*O*-(4-aminobutanoat)-Trifluoracetat als blassgelben Feststoff.

Anschließend wird zu einer Lösung von Camptothecin-20-*O*-(4-aminobutanoat)-Trifluoracetat (43 mg, 0.079 mmol), [(((((6-(Maleinimidohexanoyl)arginyl)arginyl)-alanyl)leucyl)alanyl]leucin-Trifluoracetat (100 mg, 0.087 mmol) und *N*-Ethyldiisopropylamin (54 µL, 0.316 mmol) in 1.5 mL *N,N*-Dimethylformamid, *O*-(Azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium-Hexafluorophosphat (33 mg, 0.087 mmol) unter Stickstoffatmosphäre gegeben und 35 Minuten bei Raumtemperatur gerührt. Das Produkt wird mit Diethylether gefällt und der Niederschlag im Vakuum getrocknet. Die Reinigung des Rohprodukts erfolgt durch präparative C_{18}-RP-HPLC (Acetonitril/Wasser 40:60, 0.1 % Trifluoressigsäure). Nach Lyophilisation erhält man 96 mg der Zielverbindung als leuchtend gelben Feststoff.

ESI-MS (4.0 kV, MeOH): *m/z* (%) 1307.6 ($[M + H]^+$, 100), 1329.5 ($[M + Na]^+$, 96)
HPLC-Reinheit (λ = 370 nm, C_{18}-RP-Säule, Acetonitril/Wasser 40:60, 0.1 % TFA): >95%

Beispiel 4

Löslichkeit von EMC-Arg-Arg-Ala-Leu-Ala-Leu-GABA-CPT in isotonischer Kochsalzlösung.

Verbindung	molare Masse [g/mol]	Löslichkeit [µmol/L]	[µg/mL]
CPT	348.36	11 (±1)	3.8 (±0.3)
EMC-Arg-Arg-Ala-Leu-Ala-Leu-GABA-CPT	1556	>4500	>7000

Ergebnis:

Die erfindugsgemäßen CPT-Derivate zeigen somit gegenüber CPT eine um mehr als 400-fach gesteigerte Löslichkeit in isotonischer Kochsalzlösung.

Beispiel 5

Bindung von EMC-Arg-Arg-Ala-Leu-Ala-Leu-GABA-CPT an HSA im Humanplasma

Humanplasma wurde durch Chromatographie an einer C_{18}-RP-HPLC-Säule (Symmetry® 300-5 4.6 x 250 mm von Waters) durch Gradientenelution (Fluss: 1.2–1.8 mL/min; Eluent A: 30 % 200 mM K_2HPO_4 pH 7, 70 % Acetonitril; Eluent B: 72.5 % 200 mM K_2HPO_4 pH 7, 28.5 % Acetonitril; Gradient: 26 min Eluent B isokrat., 15 min 0–100 % Eluent A linear) in seine Proteinbestandteile aufgetrennt und bei 254 nm detektiert (siehe Abbildung 1).

Nun werden 1.56 mg EMC-Arg-Arg-Ala-Leu-Ala-Leu-GABA-CPT in 200 µL Tris-Puffer (pH 7.4) bei Raumtemperatur gelöst (5 mM Lösung) und 10 µL dieser Lösung mit 490 µL Humanplasma 30 min lang bei 37 °C inkubiert und die Probe

anschließend auf die C_{18}-RP-HPLC-Säule aufgetragen (Methode s. oben). Bei Messung der Absorption bei 370 nm zeigt sich, dass der überwiegende Teil von EMC-Arg-Arg-Ala-Leu-Ala-Leu-GABA-CPT an Albumin gebunden vorliegt (siehe Abbildung 2).

Beispiel 6

Stabilität von HSA-Konjugaten von EMC-Arg-Arg-Ala-Leu-Ala-Leu-GABA-CPT- in Humanplasma

Die Plasmastabilität der durch Inkubation von EMC-Arg-Arg-Ala-Leu-Ala-Leu-GABA-CPT mit humanem Blutplasma generierten HSA-Konjugate wird mit der in Beispiel 3 beschriebenen HPLC-Methode durch Messung der Abnahme der Peakflächen (λ = 370 nm) bestimmt:

Konjugat mit EMC-Arg-Arg-Ala-Leu-Ala-Leu-GABA-CPT: nach 48 h sind noch >95% des HSA-Konjugats vorhanden.

Somit zeigt das Konjugat eine gute Stabilität in Humanplasma.

Beispiel 7

Enzymatische Spaltung von EMC-Arg-Arg-Ala-Leu-Ala-Leu-GABA-CPT durch Cathepsin B

1.56 mg EMC-Arg-Arg-Ala-Leu-Ala-Leu-GABA-CPT werden in 200 µL Tris-Puffer pH 7.4 (für Cathepsin B) gelöst. 10 µL dieser Lösung werden über 30 Minuten mit 40 µL einer 20 %igen HSA-Lösung bei 37°C inkubiert. Nachfolgend werden 10 µL Cathepsin-B-Lösung (71.7 µg/ml) zugegeben und jeweils mit Puffer auf ein Volumen von 500 µL verdünnt.

Die Bestimmung der Spaltprodukte erfolgt mit der in Beispiel 3 beschriebenen HPLC-Methode (Abbildung 3).

Ergebnis:

Nach nur zweistündiger Spaltung mit Cathepsin B ist nach einer Retentionszeit von 13 Minuten ein niedermolekulares CPT-Derivat, sowie nach 5 Minuten CPT-GABA erkennbar.

Beispiel 8

Enzymatische Spaltung von EMC-Arg-Arg-Ala-Leu-Ala-Leu-GABA-CPT in HT29-Kolorektaltumor-Homogenat

1.56 mg EMC-Arg-Arg-Ala-Leu-Ala-Leu-GABA-CPT werden in 200 µL Tris-Puffer pH 7.4 bzw. Natriumacetat-Puffer pH 5.0 gelöst. 10 µL dieser Lösung werden über 30 Minuten mit 40 µL einer 20 %igen HSA-Lösung bei 37°C inkubiert. Nachfolgend werden 10 µL HT29-Kolorektaltumor-Homogenat zugegeben und mit Puffer auf ein Volumen von 500 µL verdünnt.

Herstellung des Tumor-Homogenats: Das Tumormaterial wird mittels eines Skalpells stark zerkleinert und 200 mg der Masse werden mit 800 µL Puffer (Tris-Puffer pH 7.4 bzw. Natriumacetat-Puffer pH 5.0) unter Zusatz von 3–4 Glasperlen im Schüttler homogenisiert. Anschließend wird bei 4 °C zentrifugiert und der Überstand auf 200 µL aliquotiert.

Die Bestimmung der Spaltprodukte erfolgt mit der in Beispiel 3 beschriebenen HPLC-Methode (Abbildung 4 und 5).

Ergebnis:

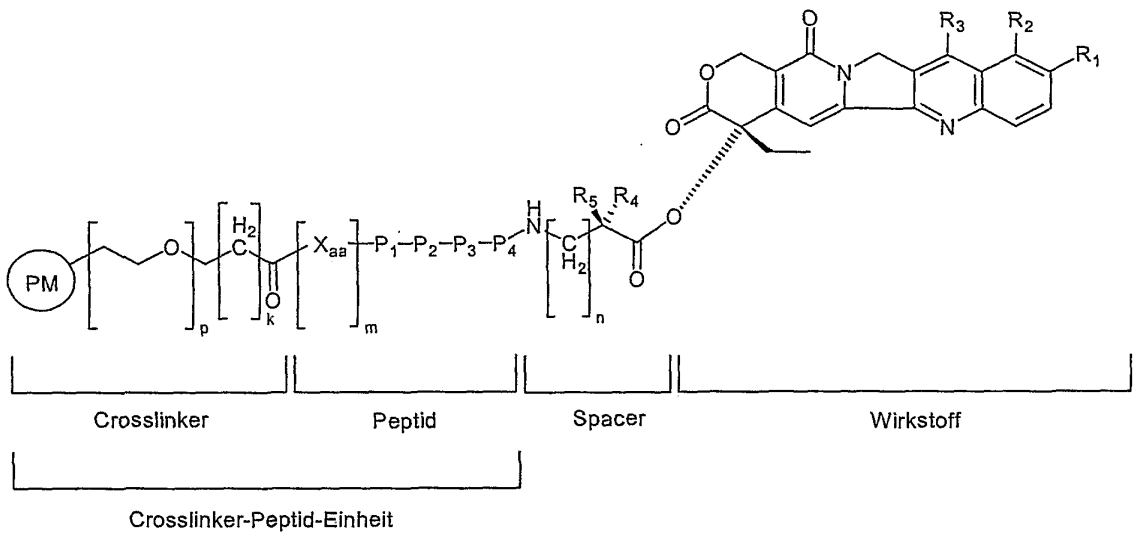
Bei pH 5.0 ist nach 2 Stunden CPT-GABA erkennbar.

Bei pH 7.4 ist nach 2 Stunden ebenfalls CPT-GABA sowie nach 24 Stunden CPT als Spaltprodukt erkennbar.

Somit ist eine Aktivierung des erfindungsgemäßen CPT-Derivates im experimentellen Tumor-Homogenat in seine Wirkformen nachgewiesen.

Ansprüche

1. Ein Camptothecin-Derivat der Formel I:



worin

R_1 = H oder OH

R_2 = H, NH_2, NO_2 oder *N,N*-Dimethylaminomethyl

R_3 = H oder Ethyl

R_4 = H, Alkyl oder Aryl

R_5 = H, gegebenenfalls substituiertes Alkyl oder Aryl

P_1, P_3 = Alanin, Glycin, Leucin, Isoleucin, Norleucin, Valin und/oder Phenylalanin

P_2, P_4 = Leucin, Isoleucin, Norleucin und/oder Phenylalanin

X_{aa} = Aminosäure mit basischer Seitenkette

n = 0 bis 3

m = 1 bis 3

k = 0 bis 5

p = 0 bis 6

PM eine proteinbindende Gruppe bedeutet.

2. Camptothecin-Derivat gemäß Anspruch 1, wobei PM eine Maleinimidgruppe, eine 2-Dithiopyridylgruppe, eine Halogenacetamidgruppe, eine Halogen-

acetatgruppe, eine Disulfidgruppe, eine Acrylsäureestergruppe, eine Monoalkylmaleinsäureestergruppe, eine Monoalkylmaleaminsäureamidgruppe, eine *N*-Hydroxysuccinimidylestergruppe, eine Isothiocyanatgruppe oder eine Aziridingruppe ist, die gegebenenfalls substituiert sein kann.

3. Camptothecin-Derivat nach Anspruch 2, wobei PM eine Maleinimidgruppe ist, die gegebenenfalls substituiert sein kann.

4. Camptothecin-Derivat nach Anspruch 1, worin R_1 = OH, R_2 = *N*,*N*-Dimethylaminomethyl und R_3 = H ist.

5. Camptothecin-Derivat nach Anspruch 3, worin R_1 = H, R_2 = H und R_3 = H ist.

6. Camptothecin-Derivat nach einem der vorhergehenden Ansprüche, worin R_4 = H, R_5 = H, Methyl, Hydroxymethyl, Phenylmethyl, 4-Hydroxyphenylmethyl, Imidazolylmethyl, Indolylmethyl, $CH(CH_3)_2$, $CH_2CH(CH_3)_2$, $CH(CH_3)CH_2CH_3$, $CH(OH)CH_3$, CH_2COOH, CH_2CH_2COOH, CH_2CONH_2, $CH_2CH_2CONH_2$, $CH_2(CH_2)_3NH_2$, $CH_2CH_2SCH_3$ oder $CH_2(CH_2)_2NHC(NH)NH_2$ ist.

7. Camptothecin-Derivat nach einem der vorhergehenden Ansprüche, worin P_1 und P_3 gleich oder unterschiedlich sind und Alanin, Glycin, Leucin, Isoleucin, Norleucin, Valin oder Phenylalanin bedeuten.

8. Camptothecin-Derivat nach einem der vorhergehenden Ansprüche, worin P_2 und P_4 gleich oder unterschiedlich sind und Leucin, Isoleucin, Norleucin oder Phenylalanin bedeuten.

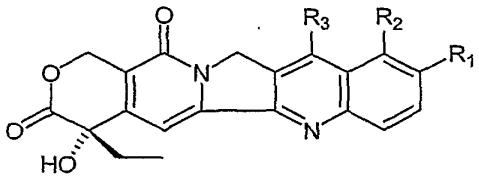
9. Camptothecin-Derivat nach einem der vorhergehenden Ansprüche, worin X_{aa} = Arginin, Lysin oder Histidin bedeuten.

10. Camptothecin-Derivat gemäß einem der vorhergehenden Ansprüche, worin n = 0 bis 3, m = 1 bis 3, k = 0 bis 5 und p = 0 bis 6 ist.

11. Camptothecin-Derivat nach einem der vorhergehenden Ansprüche, worin X_{aa} = Arginin und m = 2 bedeuten.

12. Camptothecin-Derivat nach einem der vorhergehenden Ansprüche, worin P_1, P_3 = Alanin und P_2, P_3 = Leucin bedeuten.

13. Verfahren zur Herstellung von Camptothecin-Derivaten nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, dass ein Camptothecin-Derivat der allgemeinen Formel II



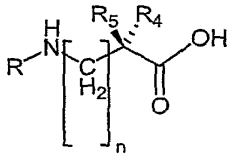
in der

R_1 = H oder OH

R_2 = H, NH_2, NO_2 oder *N,N*-Dimethylaminomethyl

R_3 = H oder Ethyl

bedeuten, wobei etwaige nukleophile Gruppen an R_1, R_2 und R_5 gegebenenfalls durch geeignete Schutzgruppen geschützt vorliegen, mit einem Spacer der allgemeinen Formel III



in der

R = Boc-Schutzgruppe oder andere gängige Aminoschutzgruppe

R_4 = H, Alkyl oder Aryl

R_5 = H, Alkyl oder Aryl

n = 0 bis 3

PM = eine proteinbindende Gruppe

bedeuten, in Gegenwart von Carbonsäure-Aktivierungsreagenzien und unter Zusatz gängiger Katalysatoren bzw. Hilfsbasen umgesetzt werden.

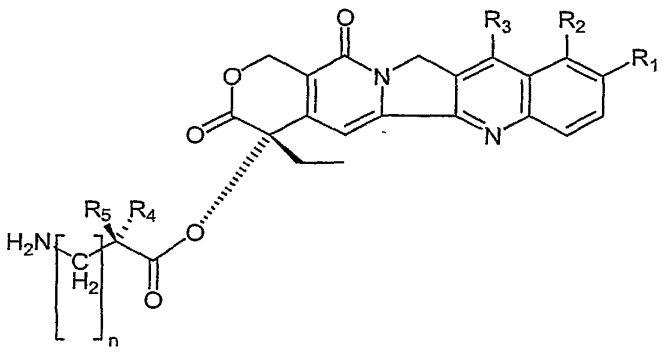
14. Verfahren nach Anspruch 13, dadurch gekennzeichnet, dass als Carbonsäure-Aktivierungsreagenzien *N,N'*-Diisopropylcarbodiimid, *N,N'*-Dicyclohexylcarbodiimid, (Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium-Hexafluorophosphat oder 2-Chlor-1-methylpyridiniumiodid eingesetz werden.

15. Verfahren nach Anspruch 14, dadurch gekennzeichnet, dass als Katalysatoren bzw. Hilfsbasen Trialkylamine, Pyridin, 4-Dimethylaminopyridin (DMAP) oder Hydroxybenzotriazol (HOBt) und/oder Scandium(III)trifluormethansulfonat eingesetzt werden.

16. Verfahren gemäß Anspruch 13, dadurch gekennzeichnet, dass Camptothecin mit einer Boc-Aminocarbonsäure unter Einsatz von *N,N'*-Diisopropylcarbodiimid und Dimethylaminopyridin umgesetzt wird.

17. Verfahren gemäß Anspruch 13, dadurch gekennzeichnet, dass Camptothecin mit Boc-4-Aminobutansäure unter Einsatz von *N,N'*-Diisopropylcarbodiimid und Dimethylaminopyridin umgesetzt wird.

18. Verfahren zur Herstellung von Camptothecin-Derivaten nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, dass ein Camptothecin-*O*-acyl-Derivat der allgemeinen Formel IV



in der

R_1 = H oder OH

R_2 = H, NH_2, NO_2 oder *N,N*-Dimethylaminomethyl

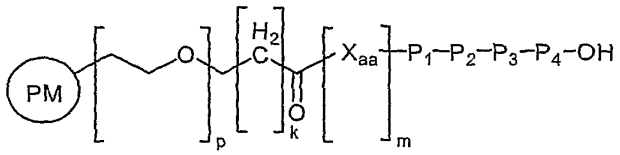
R_3 = H oder Ethyl

R_4 = H, Alkyl oder Aryl

R_5 = H, gegebenenfalls substituiertes Alkyl oder Aryl

n = 0 bis 3

bedeuten, wobei etwaige nukleophile Gruppen an R_1, R_2 und R_5 gegebenenfalls durch geeignete Schutzgruppen geschützt vorliegen, mit einer Crosslinker-Peptid-Einheit der allgemeinen Formel V



in der

P_1 und P_3 gleich oder unterschiedlich sind und Alanin, Glycin, Leucin, Isoleucin, Norleucin, Valin oder Phenylalanin bedeuten

P_2 und P_4 gleich oder unterschiedlich sind und Leucin, Isoleucin, Norleucin oder Phenylalanin bedeuten

X_{aa} = Arginin, Lysin oder Histidin

m = 1 bis 3

k = 0 bis 5

p = 0 bis 6

PM eine proteinbindende Gruppe

bedeutet, in Gegenwart von Carbonsäure-Aktivierungsreagenzien und unter Zusatz gängiger Katalysatoren bzw. Hilfsbasen umgesetzt werden.

19. Verfahren nach Anspruch 18, dadurch gekennzeichnet, dass als Carbonsäure-Aktivierungsreagenzien *N,N'*-Diisopropylcarbodiimid, *N,N'*-Dicyclohexylcarbodiimid, (Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium-Hexafluorophosphat, 2-Chlor-1-methylpyridiniumiodid oder *O*-(Azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium-Hexafluorophosphat verwendet werden.

20. Verfahren nach Anspruch 18, dadurch gekennzeichnet, dass als Katalysatoren bzw. Hilfsbasen *N*-Ethyldiisopropylamin (DIEA), Trialkylamine, Pyridin, 4-Dimethylaminopyridin (DMAP) oder Hydroxybenzotriazol (HOBt) eingesetzt werden.

21. Verfahren nach Anspruch 18, dadurch gekennzeichnet, dass als Carbonsäure-Aktivierungsreagenz *O*-(Azabenzotriazol-1-yl)-*N*,*N*,*N*´,*N*´-tetramethyluronium-Hexafluorophosphat in Verbindung mit *N*-Ethyldiisopropylamin (DIEA) verwendet wird.

22. Verfahren gemäß Anspruch 18, dadurch gekennzeichnet, dass ein Camptothecin-*O*-acyl-Trifluoracetat mit [((((6-(Maleinimido-hexanoyl)arginyl)arginyl)alanyl)leucyl)alanyl]leucin-Trifluoracetat unter Einsatz von *O*-(Azabenzotriazol-1-yl)-N,N,N´,N´-tetramethyluronium-Hexafluorophosphat in Verbindung mit *N*-Ethyldiisopropylamin (DIEA) umgesetzt wird.

23. Verfahren gemäß Anspruch 18, dadurch gekennzeichnet, dass Camptothecin-*O*-4-aminobutanoat-Trifluoracetat mit [((((6-(Maleinimido-hexanoyl)arginyl)arginyl)alanyl)leucyl)alanyl]leucin-Trifluoracetat unter Einsatz von *O*-(Azabenzotriazol-1-yl)-N,N,N´,N´-tetramethyluronium-Hexafluorophosphat in Verbindung mit *N*-Ethyldiisopropylamin (DIEA) umgesetzt wird.

24. Arzneimittel enthaltend ein Camptothecin-Derivat gemäß einem der Ansprüche 1 bis 12, gegebenenfalls zusammen mit üblichen Hilfsstoffen und/oder pharmazeutischen Lösungsmitteln.

25. Verwendung eines Camptothecin-Derivates nach einem der Ansprüche 1 bis 12 zur Behandlung von Krebskrankheiten.

26. Verfahren zur Herstellung eines Arzneimittels für die Behandlung von Krebskrankheiten, dadurch gekennzeichnet, dass man eine Verbindung gemäß einem der Ansprüche 1 bis 12 in eine therapeutisch annehmbare Lösung überführt.

Abbildung 1

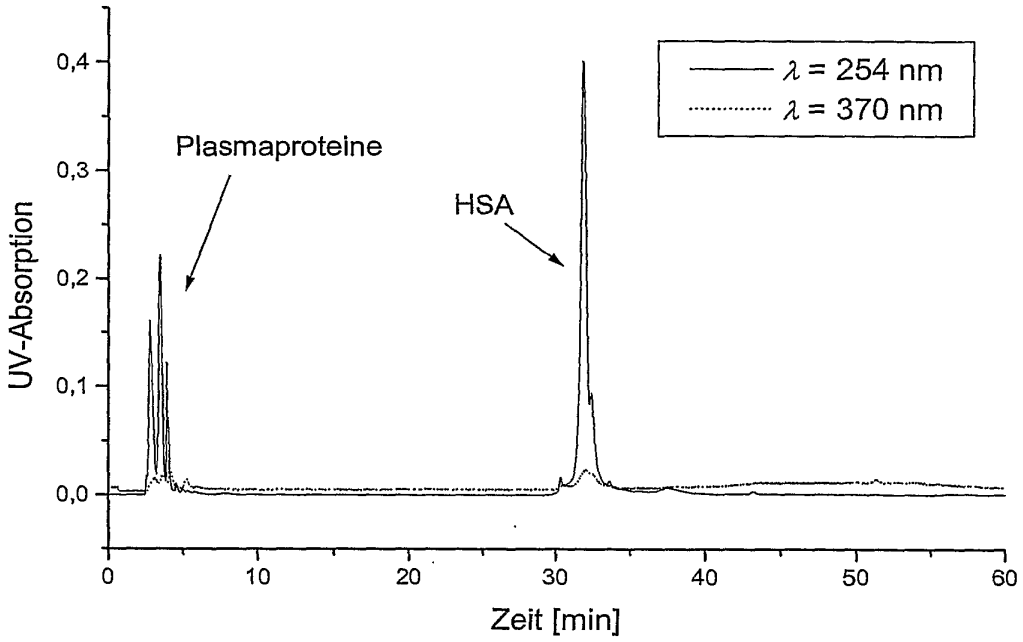

λ = 254 nm
λ = 370 nm
Plasmaproteine
HSA
UV-Absorption
0,0
0,1
0,2
0,3
0,4
0
10
20
30
40
50
60
Zeit [min]

Abbildung 2

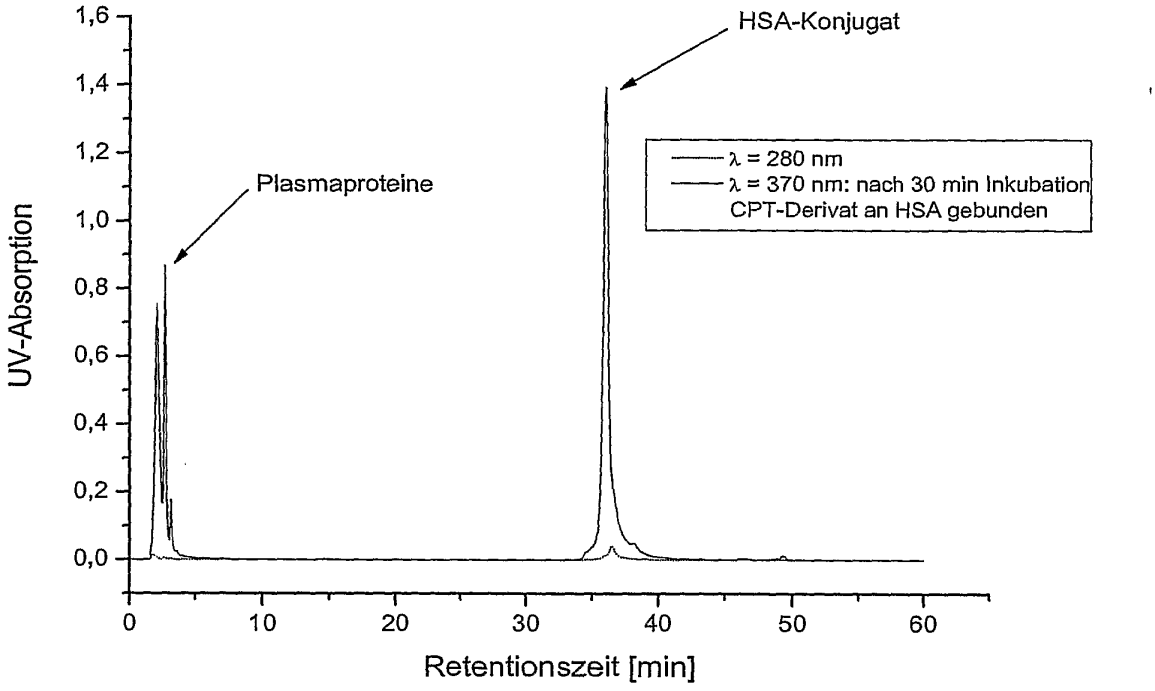

HSA-Konjugat
Plasmaproteine
λ = 280 nm
λ = 370 nm: nach 30 min Inkubation
CPT-Derivat an HSA gebunden
UV-Absorption
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
0
10
20
30
40
50
60
Retentionszeit [min]

Abbildung 3

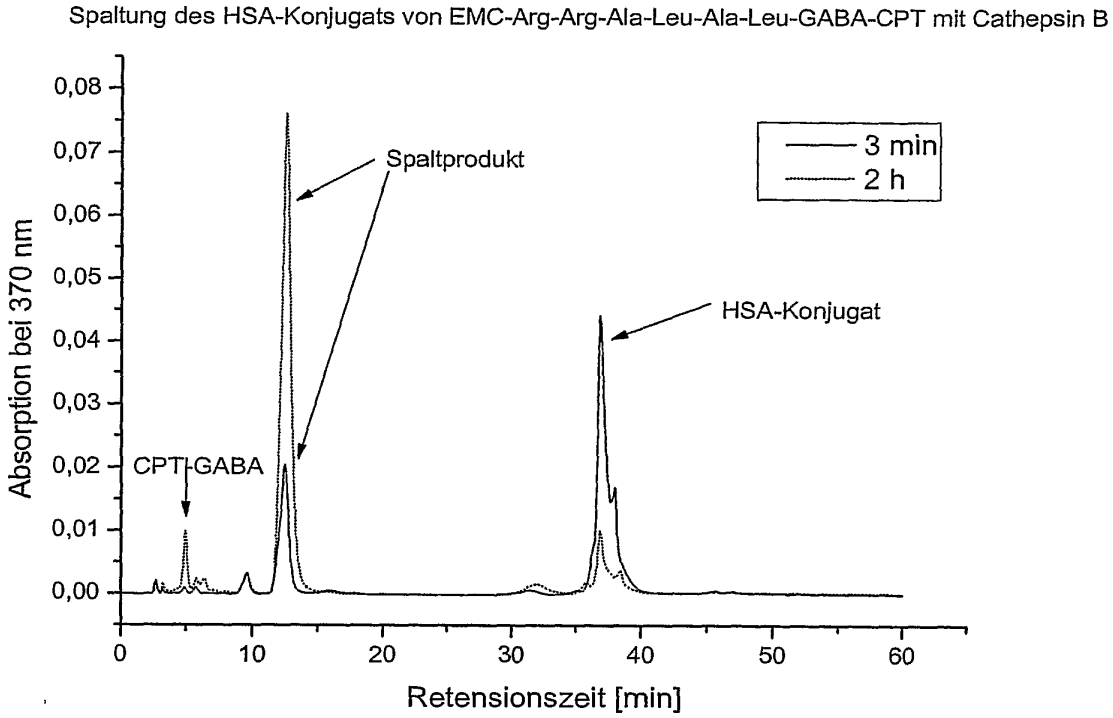

Spaltung des HSA-Konjugats von EMC-Arg-Arg-Ala-Leu-Ala-Leu-GABA-CPT mit Cathepsin B
Absorption bei 370 nm
0,08
0,07
0,06
0,05
0,04
0,03
0,02
0,01
0,00
0
10
20
30
40
50
60
Retensionszeit [min]
3 min
2 h
Spaltprodukt
HSA-Konjugat
CPT-GABA

Abbildung 4

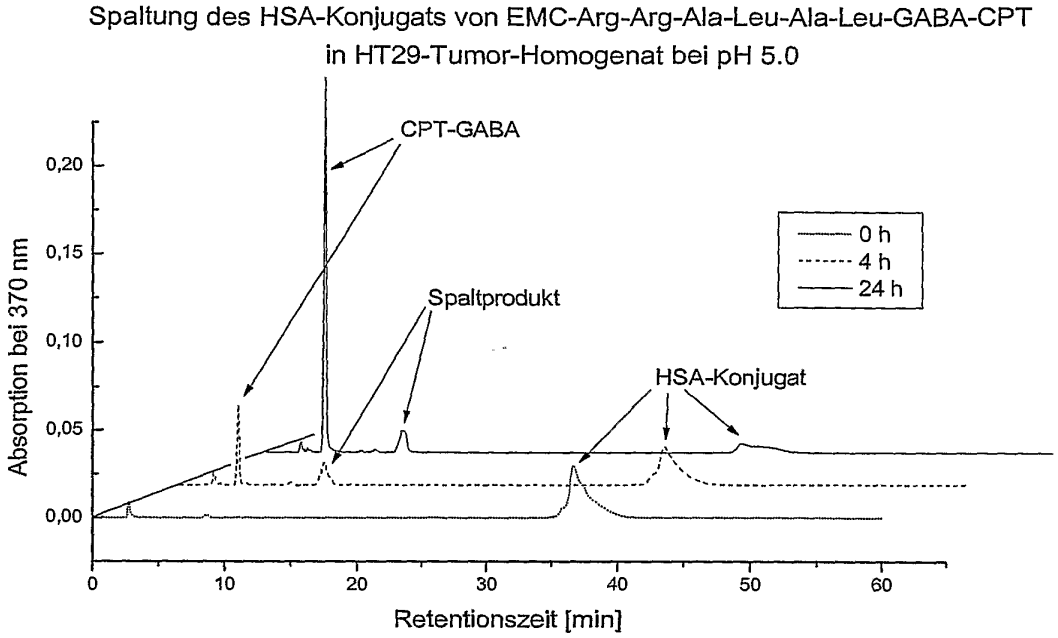
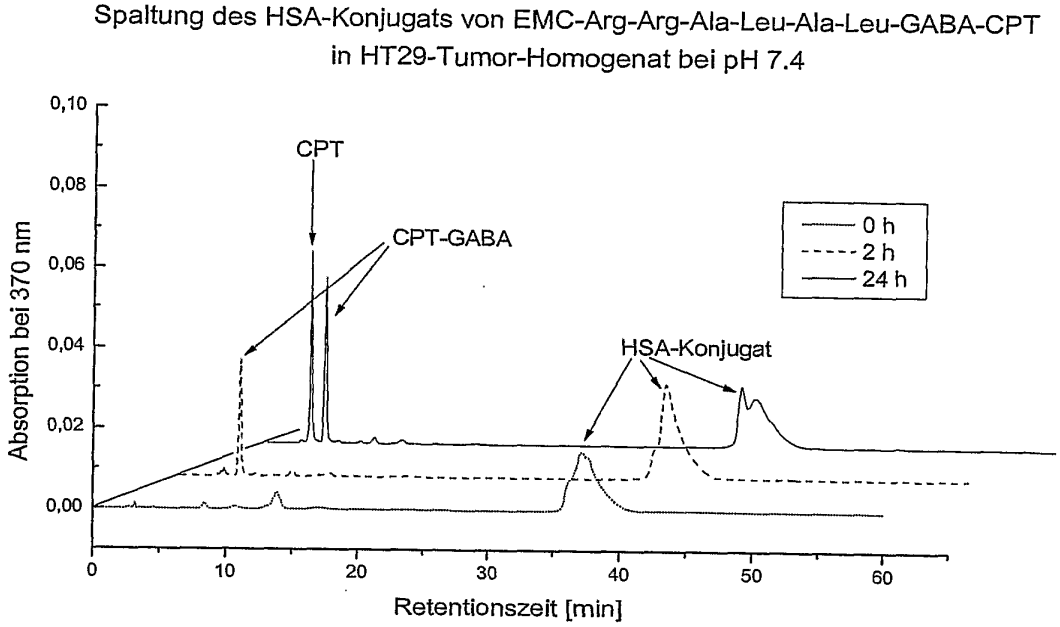

Spaltung des HSA-Konjugats von EMC-Arg-Arg-Ala-Leu-Ala-Leu-GABA-CPT
in HT29-Tumor-Homogenat bei pH 5.0
CPT-GABA
Spaltprodukt
HSA-Konjugat
0 h
4 h
24 h
Absorption bei 370 nm
0,00
0,05
0,10
0,15
0,20
0
10
20
30
40
50
60
Retentionszeit [min]

Abbildung 5


Spaltung des HSA-Konjugats von EMC-Arg-Arg-Ala-Leu-Ala-Leu-GABA-CPT
in HT29-Tumor-Homogenat bei pH 7.4
CPT
CPT-GABA
HSA-Konjugat
0 h
2 h
24 h
Absorption bei 370 nm
0,10
0,08
0,06
0,04
0,02
0,00
0
10
20
30
40
50
60
Retentionszeit [min]

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(54) Title: ANTIBODIES DIRECTED TO HER-3 AND USES THEREOF

(57) Abstract: The present invention relates to binding proteins that bind to HER-3 and polynucleotides encoding the same. Expression vectors and host cells comprising the same for the production of the binding protein of the invention are also provided. In addition, the invention provides compositions and methods for diagnosing and treating diseases associated with HER-3 mediated signal transduction and/or its ligand heregulin.

Antibodies directed to HER-3 and uses thereof

DESCRIPTION

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to binding proteins including antibodies and binding fragments thereof that bind to HER-3 and polynucleotides encoding the same. Expression vectors and host cells comprising the same for the production of the binding protein of the invention are also provided. In addition, the invention provides compositions and methods for diagnosing and treating diseases associated with HER-3 mediated signal transduction and/or its ligand heregulin.

2. Background of the Technology

The human epidermal growth factor receptor 3 (HER-3, also known as ErbB3) is a receptor protein tyrosine kinase and belongs to the epidermal growth factor receptor (EGFR) subfamily of receptor protein tyrosine kinases, which also includes HER-1 (also known as EGFR), HER-2, and HER-4 (Plowman *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 87 (1990), 4905-4909; Kraus *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 86 (1989), 9193-9197; and Kraus *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 90 (1993), 2900-2904). Like the prototypical epidermal growth factor receptor, the transmembrane receptor HER-3 consists of an extracellular ligand-binding domain (ECD), a dimerization domain within the ECD, a transmembrane domain, an intracellular protein tyrosine kinase domain (TKD) and a C-terminal phosphorylation domain.

The ligand Heregulin (HRG) binds to the extracellular domain of HER-3 and activates the receptor-mediated signaling pathway by promoting dimerization with other human epidermal growth factor receptor (HER)

family members and transphosphorylation of its intracellular domain. Dimer formation between HER family members expands the signaling potential of HER-3 and is a means not only for signal diversification but also signal amplification. For example the HER-2/HER-3 heterodimer induces one of the most important mitogenic signals among HER family members.

HER-3 has been found to be overexpressed in several types of cancer such as breast, gastrointestinal and pancreatic cancers. Interestingly a correlation between the expression of HER-2/HER-3 and the progression from a non-invasive to an invasive stage has been shown (Alimandi *et al.*, *Oncogene* 10,1813-1821; deFazio *et al.*, *Cancer* 87, 487-498; Naidu *et al.*, *Br. J. Cancer* 78, 1385-1390). Accordingly, agents that interfere with HER-3 mediated signaling are desirable. Murine or chimeric HER-3 antibodies have been reported, such as in US5968511, US5480968 and WO03013602.

A humanized monoclonal antibody against HER-2, Herceptin®, has recently been shown to interfere with HER-2 mediated signaling and is therapeutically effective in humans (Fendly *et al.*, *Hybridoma* 6, 359-370; Hudziak *et al.*, *Mol. Cell. Biol.* 9, 1165-1172; Stebbing *et al.*, *Cancer Treat. Rev.* 26, 287-290). Herceptin® has been shown to act through two different mechanisms, i.e. the engagement of the effector cells of the immune system as well as a direct cytotoxic, apoptosis inducing effect.

However, only patients with highly amplified HER-2 respond significantly to Herceptin® therapy, thus limiting the number of patients suitable for therapy. In addition the development of resistance to drugs or a change in the expression or epitope sequence of HER-2 on tumor cells may render even those approachable patients unreactive with the antibody and therefore abrogating its therapeutic benefits. Therefore more drugs for target based therapies approaching further members of the HER family, such as HER-3, are needed.

BRIEF DESCRIPTION OF THE DRAWING FIGURES

Fig. 1 shows the extent of HER-3 expression in a panel of human cancer cell lines and demonstrates that HER-3 is expressed in a variety of human cancers.

Fig. 2 shows the results of the FACS analysis of HER-3 antibody binding to either Ratl cells stably expressing the different members of the HER family or only empty vector.

Fig. 3 shows antibody binding competition bins mapped to HER3 domains.

Fig. 4. show the results of the indirect FACS Scatchard antibody affinity analysis performed with anti-HER-3 antibodies of the invention. The analysis indicates that the anti-HER-3 antibodies of the invention possess high affinities and strong binding constants for HER-3 expressed on the cell surface

Fig. 5 shows the accelerated endocytosis of HER-3 induced by anti-HER-3 antibodies of the invention.

Figs. 6 a-e show the results of a ligand competition assay performed with anti-HER-3 antibodies of the invention. The results demonstrate that the antibodies of the invention specifically reduce binding of [^{125}I]-α-HRG/[^{125}I]-β-HRG to cells expressing endogenous HER-3.

Fig. 7a shows the results of a HER-3 phosphotyrosine ELISA performed with anti-HER-3 antibodies of the invention. Antibodies according to the present invention were able to inhibit β-HRG-mediated HER-3 activation as indicated by increased receptor tyrosine phosphorylation. Furthermore fig. 7b shows representative results of this experiment with titrated antibody.

Fig. 8 shows the result of a p42/p44 MAP-Kinase ELISA performed with

anti-HER-3 antibodies of the invention. Antibodies according to the present invention were able to reduce β-HRG-mediated p42/p44 MAP-Kinase activation as indicated by increased MAP-Kinase phosphorylation.

Fig. 9 shows the result of a phospho-AKT ELISA performed with anti-HER-3 antibodies of the invention. Antibodies according to the present invention were able to reduce β-HRG-mediated AKT activation as indicated by AKT phosphorylation.

Fig. 10 shows the inhibition of MCF7 cell proliferation by human anti-HER-3 antibodies of the invention. Antibodies according to the present invention inhibit HRG-induced cell growth in human cancer cells.

Fig. 11 shows the transmigration of MCF7 cells inhibited by human anti-HER-3 antibodies of the invention.

Figs. 12a-i shows the inhibition of the anchorage independent cell growth by human HER-3 antibodies of the invention.

Fig. 13 shows the inhibition of xenograft growth of T47D human breast cancer cells by a human anti-HER-3 antibody of the invention.

Fig. 14 shows the reduction of BxPC3 human pancreas cancer cells in mice after administration of anti Her3 (U1-59 and U1-53) or anti EGFR (Erbitux) antibodies.

Fig. 15 shows the reduction of xenograft growth of BxPC3 human pancreas cancer cells by a human anti-HER-3 antibody of the invention and in combination with anti EGFR (Erbitux) antibodies.

Fig. 16 demonstrates that antibodies of the invention delay human melanoma (HT144) cell growth in nu/nu mice.

Fig. 17 shows the reduction of xenograft growth of HT-29 human colon carcinoma cells by human HER-3 antibodies of the invention (U1-53, U1-59 and U1-7).

Fig. 18 shows the reduction of xenograft growth of Calu-3 human lung cancer cells by human anti-HER-3 antibodies of the invention (U1-59, U1-53 and U1-7).

Fig. 19 shows the reduction of xenograft growth of BxPC-3 human pancreas cancer cells by human anti-HER-3 antibodies of the invention (U1-7, U1-59 and U1-53).

Fig. 20 demonstrates that an antibody of the invention (U1-59) causes suppression of HER-3 in BxPC3 human pancreas cancer xenografts.

SUMMARY OF THE INVENTION

A first aspect of the present invention relates to an isolated binding protein that binds to HER-3.

In one embodiment of the present invention, an isolated binding protein of the invention comprises a heavy chain amino acid sequence comprising at least one of the CDR´s selected from the group consisting of: (a) CDRH1´s as shown in SEQ ID NOs: 2, 6, 10, 14, 18, 22, 26, 30, 34, 36, 40, 42, 46, 50, 54, 60, 62, 66, 70, 74, 78, 80, 84, 88, 92, 96, 100, 104, 108, 112, 116, 120, 122, 126, 130, 134, 138, 142, 146, 150, 154, 158, 162, 166, 170, 174, 178, 182, 186, 190, 194, 198, 202, 206, 210, 214, 218, 222, 226 and 230, (b) CDRH2´s as shown in SEQ ID NOs: 2, 6, 10, 14, 18, 22, 26, 30, 34, 36, 40, 42, 46, 50, 54, 60, 62, 66, 70, 74, 78, 80, 84, 88, 92, 96, 100, 104, 108, 112, 116, 120, 122, 126, 130, 134, 138, 142, 146, 150, 154, 158, 162, 166, 170, 174, 178, 182, 186, 190, 194, 198, 202, 206, 210, 214, 218, 222, 226 and 230, and (c) CDRH3´s as shown in SEQ ID NOs: 2, 6, 10, 14, 18, 22, 26, 30, 34, 36, 40, 42, 46, 50, 54, 60, 62, 66, 70, 74, 78, 80, 84, 88, 92, 96, 100,

104, 108, 112, 116, 120, 122, 126, 130, 134, 138, 142, 146, 150, 154, 158, 162, 166, 170, 174, 178, 182, 186, 190, 194, 198, 202, 206, 210, 214, 218, 222, 226 and 230, and/or a light chain amino acid sequence comprising at least one of the CDR´s selected from the group consisting of: (d) CDRL1´s as shown in SEQ ID NOs: 4, 8, 12, 16, 20, 24, 28, 32, 38, 44, 48, 52, 56, 58, 64, 68, 72, 76, 82, 86, 90, 94, 98, 102, 106, 110, 114, 118, 124, 128, 132, 136, 140, 144, 148, 152, 156, 160, 164, 168, 172, 176, 180, 184, 188, 192, 196, 200, 204, 208, 212, 216, 220, 224, 228 and 232, (e) CDRL2´s as shown in SEQ ID NOs: 4, 8, 12, 16, 20, 24, 28, 32, 38, 44, 48, 52, 56, 58, 64, 68, 72, 76, 82, 86, 90, 94, 98, 102, 106, 110, 114, 118, 124, 128, 132, 136, 140, 144, 148, 152, 156, 160, 164, 168, 172, 176, 180, 184, 188, 192, 196, 200, 204, 208, 212, 216, 220, 224, 228 and 232, and (f) CDRL3´s as shown in SEQ ID NOs: 4, 8, 12, 16, 20, 24, 28, 32, 38, 44, 48, 52, 56, 58, 64, 68, 72, 76, 82, 86, 90, 94, 98, 102, 106, 110, 114, 118, 124, 128, 132, 136, 140, 144, 148, 152, 156, 160, 164, 168, 172, 176, 180, 184, 188, 192, 196, 200, 204, 208, 212, 216, 220, 224, 228 and 232.

In another embodiment of the present invention, an isolated binding protein of the invention comprises a heavy chain amino acid sequence selected from the group consisting of SEQ ID Nos: 2, 6, 10, 14, 18, 22, 26, 30, 34, 36, 40, 42, 46, 50, 54, 60, 62, 66, 70, 74, 78, 80, 84, 88, 92, 96, 100, 104, 108, 112, 116, 120, 122, 126, 130, 134, 138, 142, 146, 150, 154, 158, 162, 166, 170, 174, 178, 182, 186, 190, 194, 198, 202, 206, 210, 214, 218, 222, 226 and 230, and/or a light chain amino acid sequence selected from the group consisting of SEQ ID NOs: 4, 8, 12, 16, 20, 24, 28, 32, 38, 44, 48, 52, 56, 58, 64, 68, 72, 76, 82, 86, 90, 94, 98, 102, 106, 110, 114, 118, 124, 128, 132, 136, 140, 144, 148, 152, 156, 160, 164, 168, 172, 176, 180, 184, 188, 192, 196, 200, 204, 208, 212, 216, 220, 224, 228 and 232.

In yet another embodiment of the present invention, an isolated binding protein of the invention comprises a heavy chain amino acid sequence and a light chain amino acid sequence as shown in SEQ ID NOs: 2 and 4, 6 and 8, 10 and 12, 14 and 16, 18 and 20, 22 and 24, 26 and 28, 30 and 32, 36 and

38, 42 and 44, 46 and 48, 50 and 52, 54 and 56, 60 and 58, 62 and 64, 66 and 68, 70 and 72, 74 and 76, 78 and 82, 80 and 82, 84 and 86, 88 and 90, 92 and 94, 96 and 98, 100 and 102, 104 and 106, 108 and 110, 112 and 114, 116 and 118, 122 and 124, 126 and 128, 130 and 132, 134 and 136, 138 and 140, 142 and 144, 146 and 148, 150 and 152, 154 and 156, 158 and 160, 162 and 164, 166 and 168, 170 and 172, 174 and 176, 178 and 180, 182 and 184, 186 and 188, 190 and 192, 194 and 196, 198 and 200, 202 and 204, 206 and 208, 210 and 212, 214 and 216, 218 and 220, 222 and 224, 226 and 228, 230 and 232, or a heavy chain amino acid sequence as shown in SEQ ID NOs: 34, 40, 60, 62 or 120, or a light chain amino acid sequence as shown in SEQ ID NOs: 58 or 64.

According to the present invention, an isolated binding protein that is capable of binding to HER-3 interacts with at least one epitope in the extracellular part of HER-3. The epitopes are preferably located in domain L1 (aa 19-184) which is the amino terminal domain, in domain S1 (aa 185-327) and S2 (aa 500-632) which are the two Cysteine-rich domains, or in domain L2 (328-499) which is flanked by the two Cysteine-rich domains. The epitopes may also be located in combinations of domains such as but not limited to an epitope comprised by parts of L1 and S1. Further preferred is an isolated binding protein that binds to a three-dimensional structure formed by amino acid residues 1-160, 161-358, 359-575, 1-358 and/or 359-604 of mature HER-3, particularly of mature human HER-3.

Preferably, an isolated binding protein of the invention is a scaffold protein having an antibody like binding activity or an antibody, e.g. an anti-HER-3 antibody. In particular, the anti-HER-3 antibody is selected from the group consisting of U1-1 antibody, U1-2 antibody, U1-3 antibody, U1-4 antibody, U1-5 antibody, U1-6 antibody, U1-7 antibody, U1-8 antibody, U1-9 antibody, U1-10 antibody, U1-11 antibody, U1-12 antibody, U1-13 antibody, U1-14 antibody, U1-15 antibody, U1-16 antibody, U1-17 antibody, U1-18 antibody, U1-19 antibody, U1-20 antibody, U1-21 antibody, U1-22 antibody, U1-23 antibody, U1-24 antibody, U1-25 antibody, U1-26 antibody, U1-27 antibody,

U1-28 antibody, U1-29 antibody, U1-30 antibody, U1-31 antibody, U1-32 antibody, U1-33 antibody, U1-34 antibody, U1-35 antibody, U1-36 antibody, U1-37 antibody, U1-38 antibody, U1-39 antibody, U1-40 antibody, U1-41 antibody, U1-42 antibody, U1-43 antibody, U1-44 antibody, U1-45 antibody, U1-46 antibody, U1-47 antibody, U1-48 antibody, U1-49 antibody, U1-50 antibody, U1-51 antibody, U1-52 antibody, U1-53 antibody, U1-55.1 antibody, U1-55 antibody, U1-57.1 antibody, U1-57 antibody, U1-58 antibody, U1-59 antibody, U1-61.1 antibody, U1-61 antibody, U1-62 antibody or an antibody having at least one heavy or light chain of one of said antibodies. Especially preferred are the antibodies U1-49 (SEQ ID NO: 42/44), U1-53 (SEQ ID NO: 54/56) and U1-59 (SEQ ID NO: 70/72) or an antibody having at least one heavy or light chain of one of said antibodies.

In addition, further embodiments of the present invention provide an isolated binding protein coupled to a labelling group or effector group. Preferably, such an binding protein is useful for the treatment of hyperproliferative diseases, particularly oncological diseases such as breast cancer, gastrointestinal cancer, pancreatic cancer, prostate cancer, ovarian cancer, stomach cancer, endometrial cancer, salivary gland cancer, lung cancer, kidney cancer, colon cancer, colorectal cancer, thyroid cancer, bladder cancer, glioma, melanoma, testis cancer, soft tissue sarcoma, head and neck cancer, or other HER-3 expressing or overexpressing cancers, and the formation of tumor metastases.

Other aspects of the present invention relate to an isolated nucleic acid molecule encoding a binding protein of the invention, a vector having a nucleic acid molecule encoding the binding protein of the invention, and a host cell, e.g. a CHO cell, an NS/0 myeloma cell, transformed with such nucleic acid molecule or vector.

A further aspect of the present invention relates to a method for producing a binding protein of the invention by preparing said binding protein from a host cell that secretes the binding protein. Preferably, the binding protein of the invention is prepared from a hybridoma cell line that secretes a binding

protein or a CHO or other cell type transformed with a nucleic acid molecule encoding a binding protein of the invention.

Another aspect of the present invention relates to a method for producing a binding protein of the invention by preparing said binding protein from a tissue, product or secretion of an animal, plant or fungus transgenic for a nucleic acid molecule or nucleic acid molecules encoding the binding protein of the invention. Preferably, a binding protein of the invention is prepared from the tissue, product or secretion of a transgenic animal such as cow, sheep, rabbit, chicken or other mammalian or avian species, a transgenic plant such as corn, tobacco or other plant, or a transgenic fungus such as *Aspergillus*, *Pichia* or other fungal species.

Another aspect of the present invention pertains to a pharmaceutical composition comprising as an active agent at least one binding protein of the invention in admixture with pharmaceutically acceptable carriers, diluents and/or adjuvants. In another preferred embodiment of the present invention, the pharmaceutical composition of the invention additionally contains at least one other active agent, e.g. at least one antineoplastic agent. Yet another aspect of the present invention pertains to the use of at least one binding protein of the invention, and optionally at least one other active agent, e.g. at least one antineoplastic agent, in admixture with pharmaceutically acceptable carriers, diluents and/or adjuvants for the preparation of a pharmaceutical composition. The pharmaceutical composition is suitable for diagnosing, preventing or treating a hyperproliferative disease, particularly an oncological disease such as breast cancer, gastrointestinal cancer, pancreas cancer, prostate cancer, ovarian cancer, stomach cancer, endometrial cancer, salivary gland cancer, lung cancer, kidney cancer, colon cancer, colorectal cancer, thyroid cancer, bladder cancer, glioma, melanoma or other HER-3 expressing or overexpressing cancers, and the formation of tumor metastases.

Moreover, the present invention relates in a further aspect to a method for

diagnosing diseases or conditions associated with the expression of HER-3, comprising contacting a sample with at least one binding protein of the invention, and detecting the presence of HER-3. Preferred diseases or conditions include the hyperproliferative diseases mentioned above.

Still another aspect of the present invention is a method for preventing or treating diseases or conditions associated with the expression of HER-3 in a patient in need thereof, comprising administering to the patient an effective amount of at least one binding protein of the invention and optionally at least one other active agent, e.g. at least one neoplastic agent. Preferably, the patient is a mammalian patient, more preferably a human patient. Preferred diseases or conditions associated with the expression of HER-3 are the hyperproliferative diseases mentioned above.

A further aspect of the present invention relates to a kit for the diagnosis, prevention or treatment diseases or conditions associated with the expression of HER-3, comprising at least one binding protein, and/or nucleic acid molecule and/or vector of the invention. Optionally, the kit of the invention can further comprise at least one other active agent, e.g. at least one anti neoplastic agent. Preferably, the diseases or conditions associated with the expression of HER-3 are the hyperproliferative diseases mentioned above.

DETAILED DESCRIPTION

A first aspect of the present invention relates to an isolated binding protein that binds to HER-3.

In one embodiment of the present invention, the isolated binding protein of the invention comprises a heavy chain amino acid sequence comprising at least one of the CDR´s selected from the group consisting of: (a) CDRH1's as shown in SEQ ID NOs: 2, 6, 10, 14, 18, 22, 26, 30, 34, 36, 40, 42, 46, 50, 54, 60, 62, 66, 70, 74, 78, 80, 84, 88, 92, 96, 100, 104, 108, 112, 116, 120,

122, 126, 130, 134, 138, 142, 146, 150, 154, 158, 162, 166, 170, 174, 178, 182, 186, 190, 194, 198, 202, 206, 210, 214, 218, 222, 226 and 230, (b) CDRH2´s as shown in SEQ ID NOs: 2, 6, 10, 14, 18, 22, 26, 30, 34, 36, 40, 42, 46, 50, 54, 60, 62, 66, 70, 74, 78, 80, 84, 88, 92, 96, 100, 104, 108, 112, 116, 120, 122, 126, 130, 134, 138, 142, 146, 150, 154, 158, 162, 166, 170, 174, 178, 182, 186, 190, 194, 198, 202, 206, 210, 214, 218, 222, 226 and 230, and (c) CDRH3´s as shown in SEQ ID NOs: 2, 6, 10, 14, 18, 22, 26, 30, 34, 36, 40, 42, 46, 50, 54, 60, 62, 66, 70, 74, 78, 80, 84, 88, 92, 96, 100, 104, 108, 112, 116, 120, 122, 126, 130, 134, 138, 142, 146, 150, 154, 158, 162, 166, 170, 174, 178, 182, 186, 190, 194, 198, 202, 206, 210, 214, 218, 222, 226 and 230, and/or a light chain amino acid sequence comprising at least one of the CDR´s selected from the group consisting of: (d) CDRL1´s as shown in SEQ ID NOs: 4, 8, 12, 16, 20, 24, 28, 32, 38, 44, 48, 52, 56, 58, 64, 68, 72, 76, 82, 86, 90, 94, 98, 102, 106, 110, 114, 118, 124, 128, 132, 136, 140, 144, 148, 152, 156, 160, 164, 168, 172, 176, 180, 184, 188, 192, 196, 200, 204, 208, 212, 216, 220, 224, 228 and 232, (e) CDRL2´s as shown in SEQ ID NOs: 4, 8, 12, 16, 20, 24, 28, 32, 38, 44, 48, 52, 56, 58, 64, 68, 72, 76, 82, 86, 90, 94, 98, 102, 106, 110, 114, 118, 124, 128, 132, 136, 140, 144, 148, 152, 156, 160, 164, 168, 172, 176, 180, 184, 188, 192, 196, 200, 204, 208, 212, 216, 220, 224, 228 and 232, and (f) CDRL3´s as shown in SEQ ID NOs: 4, 8, 12, 16, 20, 24, 28, 32, 38, 44, 48, 52, 56, 58, 64, 68, 72, 76, 82, 86, 90, 94, 98, 102, 106, 110, 114, 118, 124, 128, 132, 136, 140, 144, 148, 152, 156, 160, 164, 168, 172, 176, 180, 184, 188, 192, 196, 200, 204, 208, 212, 216, 220, 224, 228 and 232.

In another embodiment of the present invention, the isolated binding protein of the invention comprises a heavy chain amino acid sequence selected from the group consisting of SEQ ID NOs: 2, 6, 10, 14, 18, 22, 26, 30, 34, 36, 40, 42, 46, 50, 54, 60, 62, 66, 70, 74, 78, 80, 84, 88, 92, 96, 100, 104, 108, 112, 116, 120, 122, 126, 130, 134, 138, 142, 146, 150, 154, 158, 162, 166, 170, 174, 178, 182, 186, 190, 194, 198, 202, 206, 210, 214, 218, 222, 226 and 230, and/or a light chain amino acid sequence selected from the group consisting of SEQ ID NOs: 4, 8, 12, 16, 20, 24, 28, 32, 38, 44, 48, 52,

56, 58, 64, 68, 72, 76, 82, 86, 90, 94, 98, 102, 106, 110, 114, 118, 124, 128, 132, 136, 140, 144, 148, 152, 156, 160, 164, 168, 172, 176, 180, 184, 188, 192, 196, 200, 204, 208, 212, 216, 220, 224, 228 and 232.

In yet another embodiment of the present invention, the isolated binding protein of the invention comprises a heavy chain amino acid sequence and a light chain amino acid sequence as shown in SEQ ID NOs: 2 and 4, 6 and 8, 10 and 12, 14 and 16, 18 and 20, 22 and 24, 26 and 28, 30 and 32, 36 and 38, 42 and 44, 46 and 48, 50 and 52, 54 and 56, 60 and 58, 62 and 64, 66 and 68, 70 and 72, 74 and 76, 78 and 82, 80 and 82, 84 and 86, 88 and 90, 92 and 94, 96 and 98, 100 and 102, 104 and 106, 108 and 110, 112 and 114, 116 and 118, 122 and 124, 126 and 128, 130 and 132, 134 and 136, 138 and 140, 142 and 144, 146 and 148, 150 and 152, 154 and 156, 158 and 160, 162 and 164, 166 and 168, 170 and 172, 174 and 176, 178 and 180, 182 and 184, 186 and 188, 190 and 192, 194 and 196, 198 and 200, 202 and 204, 206 and 208, 210 and 212, 214 and 216, 218 and 220, 222 and 224, 226 and 228, 230 and 232, or a heavy chain amino acid sequence as shown in SEQ ID NOs: 34, 40, 60, 62 or 120, or a light chain amino acid sequence as shown in SEQ ID NOs: 58 or 64.

In accordance with the present invention, it is to be understood, that the amino acid sequence of the binding protein of the invention is not limited to the twenty conventional amino acids (See *Immunology - A Synthesis* (2nd Edition, E.S. Golub and D.R. Gren, Eds., Sinauer Associates, Sunderland, Mass. (1991)), which is incorporated herein by reference). For example, the amino acids may include stereoisomers (e.g. D-amino acids) of the twenty conventional amino acids, unnatural amino acids such as α-,α-disubstituted amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids. Examples of unconventional amino acids, which may also be suitable components for the binding protein of the invention, include: 4-hydroxyproline, γ-carboxyglutamate, ε-N,N,N-trimethyllysine, ε-N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, σ-N-methylarginine, and other similar

amino acids and imino acids, e.g. 4-hydroxyproline.

Furthermore, in accordance with the present invention, minor variations in the amino acid sequences shown in SEQ ID NOs: 1-232 are contemplated as being encompassed by the present invention, providing that the variations in the amino acid sequence maintain at least 75 %, more preferably at least 80 %, 90 %, 95 %, and most preferably 99 % of the sequences shown in SEQ ID NOs: 1-232. The variations may occur within the framework regions (i.e. outside the CDRs), within the CDRs, or within the framework regions and the CDRs. Preferred variations in the amino acid sequences shown in SEQ ID NOs: 1-232, i.e. deletions, insertions and/or replacements of at least one amino acid, occur near boundaries of functional domains. Structural and functional domains can be identified by comparison of the nucleotide and/or amino acid sequence data to public or proprietary sequence databases. Computerized comparison methods can be used to identify sequence motifs or predicted protein conformation domains that occur in other binding proteins of known structure and/or function. Methods to identify protein sequences that fold into a known three-dimensional structure are known. See e.g. Bowie *et al.*, *Science* 253, 164 (1991); *Proteins, Structures and Molecular Principles* (Creighton, Ed., W. H. Freeman and Company, New York (1984)); *Introduction to Protein Structure* (C. Branden and J. Tooze, eds., Garland Publishing, New York, N.Y. (1991)); and Thornton et at., *Nature* 354, 105 (1991), which are all incorporated herein by reference. Thus, those of skill in the art can recognize sequence motifs and structural conformations that may be used to define structural and functional domains in accordance with the invention.

Especially preferred variations in the amino acid sequences shown in SEQ ID NOs: 1-174 and 1-232 are those that lead to a reduced susceptibility to proteolysis or oxidation, alter glycosylation patterns or alter binding affinities or confer or modify other physicochemical or functional properties of the binding protein. In particular, conservative amino acid replacements are contemplated. Conservative replacements are those that take place within a

family of amino acids that are related in their side chains. Preferred amino acid families are the following: acidic family = aspartate, glutamate; basic family = lysine, arginine, histidine; non-polar family = alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and uncharged polar family = glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. More preferred families are: aliphatic-hydroxy family = serine and threonine; amide-containing family = asparagine and glutamine; aliphatic family = alanine, valine, leucine and isoleucine; and aromatic family = phenylalanine, tryptophan, and tyrosine. For example, it is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid will not have a major effect on the binding or properties of the resulting binding protein, especially if the replacement does not involve an amino acid within a framework site. However, all other possible amino acid replacements are also encompassed by the present invention. Whether an amino acid change results in a functional binding protein, i.e. in a binding protein that binds to HER-3 and reduces signal transduction of HER family members, can readily be determined by assaying the specific activity of the resulting binding protein in ELISA or FACS for binding to HER-3 or in vitro or in vivo functional assay.

According to the present invention, the binding protein of the invention interacts with at least one epitope in the extracellular part of HER-3. The epitopes are preferably located in domain L1 (aa 19-184), which is the amino terminal domain, in domain S1 (aa 185-327) and S2 (aa 500-632), which are the two Cysteine-rich domains, in domain L2 (328-499), which is flanked by the two Cysteine-rich domains or in a combination of HER-3 domains. The epitopes may also be located in combinations of domains such as but not limited to an epitope comprised by parts of L1 and S1. Moreover, the binding protein of the invention is further characterized in that its binding to HER-3 reduces HER-3-mediated signal transduction. In accordance with the present invention, a reduction of HER-3-mediated signal

transduction may, e.g. be caused by a downregulation of HER-3 resulting in an at least partial disappearance of HER-3 molecules from the cell surface or by a stabilization of HER-3 on the cell surface in a substantially inactive form, i.e. a form which exhibits a lower signal transduction compared to the non-stabilized form. Alternatively, a reduction of HER-3-mediated signal transduction may also be caused by influencing, e.g. decreasing or inhibiting, the binding of a ligand or another member of the HER family to HER-3, of GRB2 to HER-2 or of GRB2 to SHC, by inhibiting receptor tyrosine phosphorylation, AKT phosphorylation, PYK2 tyrosine phosphorylation or ERK2 phosphorylation, or by decreasing tumor invasiveness. Alternatively, a reduction of HER-3 mediated signal transduction may also be caused by influencing, e.g., decreasing or inhibiting, the formation of HER-3 containing dimers with other HER family members. One example among others may be the decreasing or inhibiting of the HER3-EGFR protein complex formation.

Preferably, the binding protein of the invention is a scaffold protein having an antibody like binding activity or an antibody, i.e. an anti-HER-3 antibody.

Within the context of the present invention, the term "scaffold protein", as used herein, means a polypeptide or protein with exposed surface areas in which amino acid insertions, substitutions or deletions are highly tolerable. Examples of scaffold proteins that can be used in accordance with the present invention are protein A from *Staphylococcus aureus*, the bilin binding protein from *Pieris brassicae* or other lipocalins, ankyrin repeat proteins, and human fibronectin (reviewed in Binz and Plückthun, *Curr Opin Biotechnol*,16, 459-69). Engineering of a scaffold protein can be regarded as grafting or integrating an affinity function onto or into the structural framework of a stably folded protein. Affinity function means a protein binding affinity according to the present invention. A scaffold can be structurally separable from the amino acid sequences conferring binding specificity. In general, proteins appearing suitable for the development of such artificial affinity reagents may be obtained by rational, or most commonly, combinatorial protein engineering techniques such as panning

against HER-3, either purified protein or protein displayed on the cell surface, for binding agents in an artificial scaffold library displayed in vitro, skills which are known in the art (Skerra, J. Mol. Recog., 2000; Binz and Plückthun, 2005). In addition, a scaffold protein having an antibody like binding activity can be derived from an acceptor polypeptide containing the scaffold domain, which can be grafted with binding domains of a donor polypeptide to confer the binding specificity of the donor polypeptide onto the scaffold domain containing the acceptor polypeptide. Said inserted binding domains may be, for example, the complementarity determining region (CDR) of an antibody, in particular an anti-HER-3 antibody. Insertion can be accomplished by various methods known to those skilled in the art including, for example, polypeptide synthesis, nucleic acid synthesis of an encoding amino acid as well by various forms of recombinant methods well known to those skilled in the art.

Moreover, the term "antibody" or "anti-HER-3 antibody", as used herein, means a monoclonal antibody, a polyclonal antibody, a recombinant antibody, a humanized antibody (Jones *et al.*, *Nature* 321 (1986), 522-525; Riechmann *et al.*, *Nature* 332 (1988), 323-329; and Presta, *Curr. Op. Struct. Biol.* 2 (1992), 593-596), a chimeric antibody (Morrison *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 81 (1984), 6851-6855), a multispecific antibody (e.g. a bispecific antibody) formed from at least two antibodies, or an antibody fragment thereof. The term "antibody fragment" comprises any portion of the afore-mentioned antibodies, preferably their antigen binding or variable regions. Examples of antibody fragments include Fab fragments, Fab' fragments, $F(ab')_2$ fragments, Fv fragments, diabodies (Hollinger *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 90 (1993), 6444-6448), single chain antibody molecules (Plückthun in: The Pharmacology of Monoclonal Antibodies 113, Rosenburg and Moore, EDS, Springer Verlag, N.Y. (1994), 269-315) and other fragments as long as they exhibit the desired capability of binding to HER-3.

In addition, the term "antibody" or "anti-HER-3 antibody", as used herein, may include antibody-like molecules that contain engineered sub-domains of

antibodies or naturally occurring antibody variants. These antibody-like molecules may be single-domain antibodies such as VH-only or VL-only domains derived either from natural sources such as camelids (Muyldermans et al., *Reviews in Molecular Biotechnology* 74, 277-302) or through in vitro display of libraries from humans, camelids or other species (Holt et al., *Trends Biotechnol.*, 21, 484-90).

In accordance with the present invention, the "Fv fragment" is the minimum antibody fragment that contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. It is in this configuration that the three CDR´s of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six CDR´s confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDR´s specific for an antigen) has the ability to recognize and bind the antigen, although usually at a lower affinity than the entire binding site. The "Fab fragment" also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. The "Fab fragment" differs from the "Fab' fragment" by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. The "$F(ab')_2$ fragment" originally is produced as a pair of "Fab' fragments" which have hinge cysteines between them. Methods of preparing such antibody fragments, such as papain or pepsin digestion, are known to those skilled in the art.

In a preferred embodiment of the present invention, the anti-HER-3 antibody of the invention is of the IgA-, IgD-, IgE, IgG- or IgM-type, preferably of the IgG- or IgM-type including, but not limited to, the IgG1-, IgG2-, IgG3-, IgG4-, IgM1- and IgM2-type. In most preferred embodiments, the antibody is of the IgG1-, IgG2- or IgG4- type.

In another preferred embodiment of the present invention, the anti-HER-3

antibody of the invention is an anti-HER-3 antibody directed against the extracellular domain (ECD) of HER-3.

In certain respects, e.g. in connection with the generation of antibodies as therapeutic candidates against HER-3, it may be desirable that the anti-HER-3 antibody of the invention is capable of fixing complement and participating in complement-dependent cytotoxicity (CDC). There are a number of isotypes of antibodies that are capable of the same including without limitations the following: murine IgM, murine IgG2a, murine IgG2b, murine IgG3, human IgM, human IgG1, human IgG3, and human IgA. It will be appreciated that antibodies that are generated need not initially possess such an isotype but, rather the antibody as generated can possess any isotype and the antibody can be isotype switched by appending the molecularly cloned V region genes or cDNA to molecularly cloned constant region genes or cDNAs in appropriate expression vectors using conventional molecular biological techniques that are well known in the art and then expressing the antibodies in host cells using techniques known in the art. The isotype-switched antibody may also possess an Fc region that has been molecularly engineered to possess superior CDC over naturally occurring variants (Idusogie et al., *J Immunol.*, 166, 2571-2575) and expressed recombinantly in host cells using techniques known in the art. Such techniques include the use of direct recombinant techniques (see e.g. U.S. Patent No. 4,816,397), cell-cell fusion techniques (see e.g. U.S. Patent Nos. 5,916,771 and 6,207,418), among others. In the cell-cell fusion technique, a myeloma or other cell line such as CHO is prepared that possesses a heavy chain with any desired isotype and another myeloma or other cell line such as CHO is prepared that possesses the light chain. Such cells can, thereafter, be fused and a cell line expressing an intact antibody can be isolated. By way of example, a human anti-HER-3 IgG4 antibody, that possesses the desired binding to the HER-3 antigen, could be readily isotype switched to generate a human IgM, human IgG1 or human IgG3 isotype, while still possessing the same variable region (which defines the antibody's specificity and some of its affinity). Such molecule might then be

capable of fixing complement and participating in CDC.

Moreover, it may also be desirable for the anti-HER-3 antibody of the invention to be capable of binding to Fc receptors on effector cells, such as monocytes and natural killer (NK) cells, and participate in antibody-dependent cellular cytotoxicity (ADCC). There are a number of isotypes of antibodies that are capable of the same, including without limitations the following: murine IgG2a, murine IgG2b, murine IgG3, human IgG1 and human IgG3. It will be appreciated that antibodies that are generated need not initially possess such an isotype but, rather the antibody as generated can possess any isotype and the antibody can be isotype switched by appending the molecularly cloned V region genes or cDNA to molecularly cloned constant region genes or cDNAs in appropriate expression vectors using conventional molecular biological techniques that are well known in the art and then expressing the antibodies in host cells using techniques known in the art. The isotype-switched antibody may also possess an Fc region that has been molecularly engineered to possess superior ADCC over naturally occurring variants (Shields et al. *J Biol Chem.*, 276, 6591-6604) and expressed recombinantly in host cells using techniques known in the art. Such techniques include the use of direct recombinant techniques (see e.g. U.S. Patent No. 4,816,397), cell-cell fusion techniques (see e.g. U.S. Patent Nos. 5,916,771 and 6,207,418), among others. In the cell-cell fusion technique, a myeloma or other cell line such as CHO is prepared that possesses a heavy chain with any desired isotype and another myeloma or other cell line such as CHO is prepared that possesses the light chain. Such cells can, thereafter, be fused and a cell line expressing an intact antibody can be isolated. By way of example, a human anti-HER-3 IgG4 antibody, that possesses the desired binding to the HER-3 antigen, could be readily isotype switched to generate a human IgG1 or human IgG3 isotype, while still possessing the same variable region (which defines the antibody's specificity and some of its affinity). Such molecule might then be capable of binding to FcγR on effectors cells and participating in ADCC.

Furthermore, according to the present invention, it is appreciated that the anti-HER-3 antibody of the invention is a fully human or humanized antibody. Human antibodies avoid certain of the problems associated with xenogeneic antibodies, for example antibodies that possess murine or rat variable and/or constant regions. The presence of xenogeneic-dervied proteins such murine or rat derived proteins can lead to the generation of an immune response against the antibody by a patient, subsequently leading to the rapid clearance of the antibodies, loss of therapeutic utility through neutralization of the antibody and/or severe, even life-threatening, allergic reactions.

Preferably, the anti-HER-3 antibody of the invention is selected from the group consisting of U1-1 antibody, U1-2 antibody, U1-3 antibody, U1-4 antibody, U1-5 antibody, U1-6 antibody, U1-7 antibody, U1-8 antibody, U1-9 antibody, U1-10 antibody, U1-11 antibody, U1-12 antibody, U1-13 antibody, U1-14 antibody, U1-15 antibody, U1-16 antibody, U1-17 antibody, U1-18 antibody, U1-19 antibody, U1-20 antibody, U1-21 antibody, U1-22 antibody, U1-23 antibody, U1-24 antibody, U1-25 antibody, U1-26 antibody, U1-27 antibody, U1-28 antibody, U1-29 antibody, U1-30 antibody, U1-31 antibody, U1-32 antibody, U1-33 antibody, U1-34 antibody, U1-35 antibody, U1-36 antibody, U1-37 antibody, U1-38 antibody, U1-39 antibody, U1-40 antibody, U1-41 antibody, U1-42 antibody, U1-43 antibody, U1-44 antibody, U1-45 antibody, U1-46 antibody, U1-47 antibody, U1-48 antibody, U1-49 antibody, U1-50 antibody, U1-51 antibody, U1-52 antibody, U1-53 antibody, U1-55.1 antibody, U1-55 antibody, U1-57.1 antibody, U1-57 antibody, U1-58 antibody, U1-59 antibody, U1-61.1 antibody, U1-61 antibody, U1-62 antibody.

In a preferred embodiment of the present invention, a binding protein of the invention is coupled to a labelling group. Such a binding protein is particularly suitable for diagnostic applications. As used herein, the term "labelling group" refers to a detectable marker, e.g. a radiolabelled amino acid or biotinyl moiety that can be detected by marked avidin (e.g. streptavidin bound to a fluorescent marker or enzymatic activity that can be detected by optical or

colorimetric methods). Various methods for labelling polypeptides and glycoproteins, such as antibodies, are known in the art and may be used in performing the present invention. Examples of suitable labelling groups include, but are not limited to, the following: radioisotopes or radionuclides (e.g. ^{3}H, ^{14}C, ^{15}N, ^{35}S, ^{90}Y, ^{99}Tc, ^{111}In, ^{125}I, ^{131}I), fluorescent groups (e.g. FITC, rhodamine, lanthanide phosphors), enzymatic groups (e.g. horseradish peroxidase, β-galactosidase, luciferase, alkaline phosphatase), chemiluminescent groups, biotinyl groups, or predetermined polypeptide epitopes recognized by a secondary reporter (e.g. leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags). In certain respects, it may be desirable that the labelling groups are attached by spacer arms of various lengths to reduce potential steric hindrance.

Alternatively, a binding protein of the invention may be coupled to an effector group in another preferred embodiment of the invention. Such a binding protein is especially suitable for therapeutic applications. As used herein, the term "effector group" refers to a cytotoxic group such as a radioisotope or radionuclide, a toxin, a therapeutic group or other effector group known in the art. Examples for suitable effector groups are radioisotopes or radionuclides (e.g. ^{3}H, ^{14}C, ^{15}N, ^{35}S, ^{90}Y, ^{99}Tc, ^{111}In, ^{125}I, ^{131}I), calicheamicin, dolastatin analogs such as auristatins, and chemotherapeutic agents such as geldanamycin and maytansine derivates, including DM1. In certain respects, it may be desirable that the effector groups are attached by spacer arms of various lengths to reduce potential steric hindrance.

A second aspect of the present invention relates to a process for preparing an isolated binding protein of the invention, comprising the step of preparing the binding protein from a host cell that secretes the binding protein. Host cells, that may be used according to the present invention, are hybridomas; eukaryotic cells such as mammalian cells, e.g. hamster, rabbit, rat, pig, mouse or other animal cells, plant cells, fungal cells cells, e.g. *Saccharomyces cerevisiae*, *Pichia pastoris;* prokaryotic cells such as *E. coli*;

and other cells used in the art for the production of binding proteins. Various methods for preparing and isolating binding proteins, such as scaffold proteins or antibodies, from host cells are known in the art and may be used in performing the present invention. Moreover, methods for preparing binding protein fragments, e.g. scaffold protein fragments or antibody fragments, such as papain or pepsin digestion, modern cloning techniques, techniques for preparing single chain antibody molecules (Plückthun in: The Pharmacology of Monoclonal Antibodies 113, Rosenburg and Moore, EDS, Springer Verlag, N.Y. (1994), 269-315) and diabodies (Hollinger *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 90 (1993), 6444-6448), are also known to those skilled in the art and may be used in performing the present invention.

In a preferred embodiment of the present invention, a binding protein of the invention is prepared from a hybridoma that secretes the binding protein. See e.g. Köhler *et al.*, *Nature* 256 (1975), 495.

In a further preferred embodiment of the present invention, a binding protein of the invention is prepared recombinantly by optimizing and/or amplifying expression of the binding protein in a host cell and isolating the binding protein from said host cell. To this end, the host cells are transformed or transfected with DNA encoding a binding protein or a vector containing DNA encoding the binding protein and cultured under appropriate conditions to produce the binding protein of the invention. See e.g. U.S. Patent No. 4,816,567. Preferred host cells may be CHO cells, NS/0 myeloma cells, human embryonic kidney 293 cells, *E. coli* and *Saccharomyces cerevisiae*.

With regard to binding proteins that are antibodies, these antibodies may be prepared from animals genetically engineered to make fully human antibodies or from an antibody display library made in bacteriophage, yeast, ribosome or *E. coli*. See e.g. Clackson *et al.*, *Nature* 352 (1991), 624-628, Marks *et al.*, *J. Mol. Biol.* 222 (1991), 581-597, Feldhaus and Siegel *J Immunol Methods*. 290, 69-80, Groves and Osbourn, *Expert Opin Biol Ther.*, 5, 125-135 and Jostock and Dubel, *Comb Chem High Throughput Screen*. 8,

127-133.

Human antibodies avoid some of the problems associated with antibodies that possess murine or rat variable and/or constant regions. The presence of such murine or rat derived proteins can lead to the rapid clearance of the antibodies or can lead to the generation of an immune response against the antibody by a patient. In order to avoid the utilization of murine or rat derived antibodies, fully human antibodies can be generated through the introduction of functional human antibody loci into a rodent, other mammal or animal so that the rodent, other mammal or animal produces fully human antibodies.

One method for generating fully human antibodies is through the use of XENOMOUSE® strains of mice that have been engineered to contain 245 kb and 190 kb-sized germline configuration fragments of the human heavy chain locus and kappa light chain locus. Other XenoMouse strains of mice contain 980 kb and 800 kb-sized germline configuration fragments of the human heavy chain locus and kappa light chain locus. Still other XenoMouse strains of mice contain 980 kb and 800 kb-sized germline configuration fragments of the human heavy chain locus and kappa light chain locus plus a 740 kb-sized germline configured complete human lambda light chain locus. *See* Mendez *et al. Nature Genetics* 15:146-156 (1997) and Green and Jakobovits *J. Exp. Med.* 188:483-495 (1998). The XENOMOUSE® strains are available from Abgenix, Inc. (Fremont, CA).

The production of the XENOMOUSE® mice is further discussed and delineated in U.S. Patent Application Serial Nos. 07/466,008, filed January 12, 1990, 07/610,515, filed November 8, 1990, 07/919,297, filed July 24, 1992, 07/922,649, filed July 30, 1992, filed 08/031,801, filed March 15,1993, 08/112,848, filed August 27, 1993, 08/234,145, filed April 28, 1994, 08/376,279, filed January 20, 1995, 08/430, 938, April 27, 1995, 08/464,584, filed June 5, 1995, 08/464,582, filed June 5, 1995, 08/463,191, filed June 5, 1995, 08/462,837, filed June 5, 1995, 08/486,853, filed June 5, 1995,

08/486,857, filed June 5, 1995, 08/486,859, filed June 5, 1995, 08/462,513, filed June 5, 1995, 08/724,752, filed October 2, 1996, and 08/759,620, filed December 3, 1996, U.S. Patent Publication 2003/0217373, filed November 20, 2002, and U.S. Patent Nos. 6,833,268, 6,162,963, 6,150,584, 6,114,598, 6,075,181, and 5,939,598 and Japanese Patent Nos. 3 068 180 B2, 3 068 506 B2, and 3 068 507 B2. *See also* European Patent No., EP 0 463 151 B1, grant published June 12, 1996, International Patent Application No., WO 94/02602, published February 3, 1994, International Patent Application No., WO 96/34096, published October 31, 1996, WO 98/24893, published June 11, 1998, WO 00/76310, published December 21, 2000. The disclosures of each of the above-cited patents, applications, and references are hereby incorporated by reference in their entirety.

In an alternative approach, others, including GenPharm International, Inc., have utilized 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 V_H genes, one or more D_H genes, one or more J_H genes, a mu constant region, and a second constant region (preferably a gamma constant region) are formed into a construct for insertion into an animal. This approach is described in U.S. Patent No. 5,545,807 to Surani *et al.* and U.S. Patent Nos. 5,545,806, 5,625,825, 5,625,126, 5,633,425, 5,661,016, 5,770,429, 5,789,650, 5,814,318, 5,877,397, 5,874,299, and 6,255,458 each to Lonberg and Kay, U.S. Patent No. 5,591,669 and 6,023.010 to Krimpenfort and Berns, U.S. Patent Nos. 5,612,205, 5,721,367, and 5,789,215 to Berns *et al.*, and U.S. Patent No. 5,643,763 to Choi and Dunn, and GenPharm International U.S. Patent Application Serial Nos. 07/574,748, filed August 29, 1990, 07/575,962, filed August 31, 1990, 07/810,279, filed December 17, 1991, 07/853,408, filed March 18, 1992, 07/904,068, filed June 23, 1992, 07/990,860, filed December 16, 1992, 08/053,131, filed April 26, 1993, 08/096,762, filed July 22, 1993, 08/155,301, filed November 18, 1993, 08/161,739, filed December 3, 1993, 08/165,699, filed December 10, 1993, 08/209,741, filed March 9, 1994, the disclosures of which are hereby incorporated by reference. *See*

also European Patent No. 0 546 073 B1, International Patent Application Nos. WO 92/03918, WO 92/22645, WO 92/22647, WO 92/22670, WO 93/12227, WO 94/00569, WO 94/25585, WO 96/14436, WO 97/13852, and WO 98/24884 and U.S. Patent No. 5,981,175, the disclosures of which are hereby incorporated by reference in their entirety. *See further* Taylor *et al.*, 1992, Chen *et al.*, 1993, Tuaillon *et al.*, 1993, Choi *et al.*, 1993, Lonberg *et al.*, (1994), Taylor *et al.*, (1994), and Tuaillon *et al.*, (1995), Fishwild *et al.*, (1996), the disclosures of which are hereby incorporated by reference in their entirety.

Kirin has also demonstrated the generation of human antibodies from mice in which, through microcell fusion, large pieces of chromosomes, or entire chromosomes, have been introduced. *See* European Patent Application Nos. 773 288 and 843 961, the disclosures of which are hereby incorporated by reference. Additionally, KM™– mice, which are the result of cross-breeding of Kirin's Tc mice with Medarex's minilocus (Humab) mice have been generated. These mice possess the HC transchromosome of the Kirin mice and the kappa chain transgene of the Medarex mice (Ishida *et al.*, Cloning Stem Cells, (2002) 4:91-102).

Human antibodies can also be derived by *in vitro* methods. Suitable examples include, but are not limited to, phage display (as commercialized by Cambridge Antibody Technology, Morphosys, Dyax, Biosite/Medarex, Xoma, Symphogen, Alexion (formerly Proliferon), Affimed) ribosome display (as commercialized by Cambridge Antibody Technology), yeast display, and the like.

Antibodies, as described herein, were prepared through the utilization of the XENOMOUSE® technology, as described below. Such mice, then, are capable of producing human immunoglobulin molecules and antibodies and are deficient in the production of murine immunoglobulin molecules and antibodies. Technologies utilized for achieving the same are disclosed in the patents, applications, and references disclosed in the background

section herein. In particular, however, a preferred embodiment of transgenic production of mice and antibodies therefrom is disclosed in U.S. Patent Application Serial No. 08/759,620, filed December 3, 1996 and International Patent Application Nos. WO 98/24893, published June 11, 1998 and WO 00/76310, published December 21, 2000, the disclosures of which are hereby incorporated by reference. *See also* Mendez *et al. Nature Genetics* 15:146-156 (1997), the disclosure of which is hereby incorporated by reference.

Through the use of such technology, fully human monoclonal antibodies to a variety of antigens have been produced. Essentially, XENOMOUSE® lines of mice are immunized with an antigen of interest (*e.g.* HER-3), lymphatic cells (such as B-cells) are recovered from the mice that expressed antibodies, and the recovered cell lines are fused with a myeloid-type cell line to prepare immortal hybridoma cell lines. These hybridoma cell lines are screened and selected to identify hybridoma cell lines that produced antibodies specific to the antigen of interest. Provided herein are methods for the production of multiple hybridoma cell lines that produce antibodies specific to HER-3. Further, provided herein are characterization of the antibodies produced by such cell lines, including nucleotide and amino acid sequence analyses of the heavy and light chains of such antibodies.

In general, antibodies produced by the fused hybridomas were human IgG1 heavy chains with fully human kappa light chains. Antibodies described herein possess human IgG4 heavy chains as well as IgG1 heavy chains. Antibodies can also be of other human isotypes, including IgG2 or IgG3. The antibodies possessed high affinities, typically possessing a K_D of from about 10^{-6} through about 10^{-13} M or below, when measured by solid phase and cell-based techniques.

Another aspect of the present invention relates to an isolated nucleic acid molecule encoding a binding protein of the invention. Within the context of the present invention, the term "isolated nucleic acid molecule", as used

herein, means a polynucleotide of genomic, cDNA, or synthetic origin or some combination thereof, which by virtue of its origin, the "isolated nucleic acid molecule" (1) is not associated with all or a portion of a polynucleotide in which the "isolated polynucleotide" is found in nature, (2) is operably linked to a polynucleotide which it is not linked to in nature, or (3) does not occur in nature as part of a larger sequence. Further, the term "nucleic acid molecule", as referred to herein, means a polymeric form of nucleotides of at least 10 bases in length, either ribonucleotides or deoxynucleotides or a modified form of either type of nucleotide, such as nucleotides with modified or substituted sugar groups and the like. The term also includes single and double stranded forms of DNA.

In a one embodiment of the present invention, a nucleic acid molecule of the invention is operably linked to a control sequence. The term "control sequence", as used herein, refers to polynucleotide sequences that are necessary to effect the expression and processing of coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism. In prokaryotes, such control sequences generally include promoters, ribosomal binding sites, and transcription termination sequences. In eukaryotes, generally, such control sequences include promoters and transcription termination sequences. In accordance with the present invention, the term "control sequence" is intended to include, at a minimum, all components whose presence is essential for expression and processing, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences. Furthermore, the term "operably linked", as used herein, refers to positions of components so described which are in a relationship permitting them to function in their intended manner. Moreover, according to the present invention, an expression control sequence operably linked to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the expression control sequence.

A further aspect of the present invention is a vector comprising a nucleic acid molecule that encodes a binding protein of the invention. The nucleic acid molecule can be operably linked to a control sequence. Furthermore, the vector may additionally contain a replication origin or a selection marker gene. Examples of vectors that may be used in accordance with the present invention are e.g. plasmids, cosmids, phages, viruses, etc.

Another aspect of the present invention relates to a host cell transformed with a nucleic acid molecule or vector of the invention. Transformation could be done by any known method for introducing polynucleotides into a host cell, including for example packaging the polynucleotide in a virus (or into a viral vector) and transducing a host cell with the virus (or vector) or by transfection procedures known in the art, as exemplified by U.S. Patent Nos. 4,399,216, 4,912,040, 4,740,461, and 4,959,455, which patents are hereby incorporated herein by reference. Particularly, methods for introducing heterologous polynucleotides into mammalian cells are well known in the art and include dextran-mediated transfection, calcium phosphate precipitation, polybrene mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei. Examples of host cells that may be used according to the present invention are hybridomas eukaryotic cells such as mammalian cells, e.g. hamster, rabbit, rat, pig, mouse or other animal cells; plant cells and fungal cells, e.g. corn, tobacco, *Saccharomyces cerevisiae, Pichia pastoris*; prokaryotic cells such as *E. coli*; and other cells used in the art for the production of antibodies. Especially mammalian cell lines available as hosts for expression are well known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC), including but not limited to Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (e.g. Hep G2), and a number of other cell lines.

Yet another aspect of the present invention is a pharmaceutical composition

comprising as an active agent at least one binding protein of the invention and pharmaceutically acceptable carriers, diluents and/or adjuvants. The term "pharmaceutical composition", as used herein, refers to a chemical compound or composition capable of inducing a desired therapeutic effect when properly administered to a patient (*The McGraw-Hill Dictionary of Chemical Terms*, Parker, S., Ed., McGraw-Hill, San Francisco (1985), incorporated herein by reference). In accordance with the present invention, the potency of the pharmaceutical composition of the invention is based on the binding of the at least one binding protein to HER-3. Preferably, this binding leads to a reduction of the HER-3-mediated signal transduction.

Furthermore, the term "carriers", when used herein, includes carriers, excipients, or stabilizers that are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed. Often the physiologically acceptable carrier is an aqueous pH buffered solution or a liposome (a small vesicle composed of various types of lipids, phospholipids and/or surfactants which is useful for delivery of a drug to a mammal). Examples of physiologically acceptable carriers include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; 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, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TWEEN™, polyethylene glycol (PEG), and PLURONICS™.

In a one embodiment of the present invention, the at least one binding protein of the invention contained in the pharmaceutical composition is coupled to an effector, such as calicheamicin, Auristatin-PE, a radioisotope or a toxic chemotherapeutic agent such as geldanamycin and maytansine. In particular, these binding protein conjugates are useful in targeting cells, e.g.

cancer cells, expressing HER-3 for elimination.

Moreover, linking binding proteins of the invention to radioisotopes e.g. provides advantages to tumor treatments. Unlike chemotherapy and other forms of cancer treatment, radioimmunotherapy or the administration of a radioisotope-binding protein combination directly targets the cancer cells with minimal damage to surrounding normal, healthy tissue. With this "magic bullet", the patient can be treated with much smaller quantities of radioisotopes than other forms of treatment available today. Preferred radioisotopes include yttrium^{90} (^{90}Y), indium^{111} (^{111}In), ^{131}I, ^{99}mTc, radiosilver-111, radiosilver-199, and Bismuth^{213}. The linkage of radioisotopes to binding proteins of the invention may e.g. be performed with conventional bifunctional chelates. Since silver is monovalent, for radiosilver-111 and radiosilver-199 linkage, sulphur-based linkers may be used (Hazra *et al.*, *Cell Biophys.* 24-25, 1-7 (1994)). Linkage of silver radioisotopes may involve reducing the immunoglobulin with ascorbic acid. Furthermore, tiuxetan is an MX-DTPA linker chelator attached to ibritumomab to form ibritumomab tiuxetan (Zevalin) (Witzig, T.E, *Cancer Chemother. Pharmacol.* 48 Suppl 1, 91-5 (2001). Ibritumomab tiuxetan can react with radioisotypes such as indium^{111} (^{111}In) or ^{90}Y to form ^{111}In-ibritumomab tiuxetan and ^{90}Y-ibritumomab tiuxetan, respectively.

Furthermore, a binding protein of the invention, particularly when used to treat cancer, may be conjugated with toxic chemotherapeutic drugs such as calicheamicin (Hamann *et al.*, *Bioconjug. Chem.* 13(1), 40-6 (2002), geldanamycin (Mandler *et al.*, *J. Natl. Cancer Inst.,* 92(19), 1549-51 (2000)) and maytansine, for example, the maytansinoid drug, DM1 (Liu *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 93:8618-8623 (1996)). Different linkers that release the drugs under acidic or reducing conditions or upon exposure to specific proteases may be employed with this technology. According to the present invention, a binding protein of the invention may be conjugated as described in the art.

Auristatin-PE, e.g. is an antimicrotubule agent that is a structural modification of the marine, shell-less mollusk peptide constituent dolastatin 10. Auristatin-PE has both anti-tumor activity and anti-tumor vascular activity (Otani *et al.*, *Jpn. J. Cancer Res.* 91(8), 837-44 (2000)). For example, auristatin-PE inhibits cell growth and induces cell cycle arrest and apoptosis in pancreatic cancer cell lines (Li *et al.*, *Int. J. Mol. Med.* 3(6), 647-53 (1999)). Accordingly, to specifically target the anti-tumor activity and anti-tumor vascular activities of auristatin-PE to particular tumors, auristatin-PE may be conjugated to the binding protein of the invention.

In a one embodiment of the present invention, the pharmaceutical composition comprises at least one further active agent. Examples for further active agents, which may be used in accordance with the present invention, are antibodies or low molecular weight inhibitors of other receptor protein kinases, such as EGFR, HER-2, HER-4, IGFR-1, or c-met, receptor ligands such as vascular endothelial factor (VEGF), cytotoxic agents, such as doxorubicin, cis-platin or carboplatin, cytokines or antineoplatic agents. Many antineoplastic agents are presently known in the art. In one embodiment, the antineoplastic agent is selected from the group of therapeutic proteins including, but not limited to, antibodies or immunomodulatory proteins. In another embodiment the anti-neoplastic agent is selected from the group of small molecule inhibitors or chemotherapeutic agents consisting of mitotic inhibitors, kinase inhibitors, alkylating agents, anti-metabolites, intercalating antibiotics, growth factor inhibitors, cell cycle inhibitors, enzymes, topoisomerase inhibitors, histone deacetylase inhibitors, anti-survival agents, biological response modifiers, anti-hormones, e.g. anti-androgens, and anti-angiogenesis agents. When the anti-neoplastic agent is radiation, treatment can be achieved either with an internal (brachytherapy BT) or external (external beam radiation therapy: EBRT) source.

The pharmaceutical composition of the present invention is especially suitable for the diagnosis, prevention or treatment of a hyperproliferative disease. The hyperproliferative disease may be, e.g., associated with

increased HER family signal transduction. Particularly, the disease can be associated with increased HER-3 phosphorylation and/or increased complex formation between HER-3 and other members of the HER family and/or increased PI_3 kinase activity and/or increased c-jun terminal kinase activity and/or AKT activity and/or increased ERK2 activity and/or PYK2 activity. Preferably, the hyperproliferative disease is selected from the group consisting of breast cancer, gastrointestinal cancer, pancreatic cancer, prostate cancer, ovarian cancer, stomach cancer, endometrial cancer, salivary gland cancer, lung cancer, kidney cancer, colon cancer, colorectal cancer, thyroid cancer, bladder cancer, glioma, melanoma or other HER-3 expressing or overexpressing cancers, and the formation of tumor metastases.

In accordance with the present invention, the term "prevention or treatment", when used herein, refers to both therapeutic treatment and prophylactic or preventative measures, wherein the patient in need is to prevent or slow down (lessen) the targeted pathologic condition or disorder. Those in need of prevention or treatment include those already with the disorder as well as those prone to have the disorder or those in whom the disorder is to be prevented. The patient in need of prevention or treatment is a mammalian patient, i.e. any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, cats, cattle, horses, sheep, pigs, goats, rabbits, etc. Preferably, the patient in need of treatment is a human patient.

According to the present invention, the pharmaceutical composition of the invention may be formulated by mixing the active agent(s) with physiologically acceptable carriers, diluents and /or adjuvants, and optionally other agents that are usually incorporated into formulations to provide improved transfer, delivery, tolerance, and the like. The pharmaceutical composition of the invention may be formulated e.g. in the form of lyophilized formulations, aqueous solutions, dispersions or solid preparations, such as tablets, dragees or capsules. A multitude of appropriate formulations can be found in the formulary known to all pharmaceutical chemists: Remington's

Pharmaceutical Sciences (18th ed, Mack Publishing Company, Easton, PA (1990)), particularly Chapter 87 by Block, Lawrence, therein. These formulations include, for example, powders, pastes, ointments, jellies, waxes, oils, lipids, lipid (cationic or anionic) containing vesicles (such as Lipofectin™), DNA conjugates, anhydrous absorption pastes, oil-in-water and water-in-oil emulsions, emulsions carbowax (polyethylene glycols of various molecular weights), semi-solid gels, and semi-solid mixtures containing carbowax. Any of the foregoing mixtures may be appropriate in treatments and therapies in accordance with the present invention, provided that the active agent in the formulation is not inactivated by the formulation and the formulation is physiologically compatible and tolerable with the route of administration. See also Baldrick P., "Pharmaceutical excipient development: the need for preclinical guidance.", *Regul. Toxicol. Pharmacol.* 32(2), 210-218 (2000); Wang W., "Lyophilization and development of solid protein pharmaceuticals.", *Int. J. Pharm.* 203(1-2), 1-60 (2000); Charman W.N., "Lipids, lipophilic drugs, and oral drug delivery-some emerging concepts.", *J. Pharm. Sci.* 89(8), 967-978 (2000); Powell *et al.*, "Compendium of excipients for parenteral formulations", *PDA J. Pharm. Sci. Technol.* 52, 238-311 (1998); and the citations therein for additional information related to formulations, excipients and carriers well known to pharmaceutical chemists.

Another aspect of the present invention pertains to the use of at least one isolated binding protein of the invention, and optionally at least one other active agent, e.g. at least one anti-neoplastic agent as described above, in admixture with pharmaceutically acceptable carriers, diluents and/or adjuvants, for the manufacture of a pharmaceutical composition for the diagnosis, prevention or treatment of a hyperproliferative disease. Preferably, the pharmaceutical composition is a pharmaceutical composition as described above and the hyperproliferative disease is a hyperproliferative disease as mentioned above.

Yet another aspect of the present invention is concerned with a method for

diagnosing diseases or conditions associated with the expression of HER-3, comprising contacting a sample with a binding protein of the invention, and detecting the presence of HER-3 in the sample. The sample may be a cell that shows expression of HER-3, such as a tumor cell, a blood sample or another suitable sample. In a preferred embodiment of the present invention, the diseases or conditions associated with the expression of HER-3 are the hyperproliferative diseases defined above.

According to the present invention, the method may, e.g., be used for the detection of HER-3 antigen in a cell, for the determination of HER-3 antigen concentration in patients suffering from a hyperproliferative disease as mentioned above or for the staging of said hyperproliferative disease in a patient. In order to stage the progression of a hyperproliferative disease in a subject under study, or to characterize the response of the subject to a course of therapy, a sample of blood can, e.g., be taken from the subject and the concentration of the HER-3 antigen present in the sample is determined. The concentration so obtained is used to identify in which range of concentrations the value falls. The range so identified correlates with a stage of progression or a stage of therapy identified in the various populations of diagnosed subjects, thereby providing a stage in the subject under study. A biopsy of the disease, e.g. cancer, tissue obtained from the patient may also be used assess the amount of HER-3 antigen present. The amount of HER-3 antigen present in the disease tissue may be assessed by immunohistochemistry, ELISA or antibody arrays using HER3 antibodies of the invention. Other parameters of diagnostic interest are the dimerization state as well as the dimerization partners of the HER3 protein and the activation state of it and its partners. Protein analytical methods to determine those parameters are well known in the art and are among others western blot and immunoprecipitation techniques, FACS analysis, chemical crosslinking, bioluminescence resonance energy transfer (BRET), fluorescence resonance energy transfer (FRET) and the like (e.g. Price et al, Methods in Molecular Biology, 218: 255-268 (2002) or the eTag technology (WO0503707, WO04091384, WO04011900).

Furthermore, the present invention relates in another aspect to a method for preventing or treating diseases or conditions associated with the expression of HER-3 in a patient, comprising administering to a patient in need thereof an effective amount of at least one binding protein of the invention. Preferably, the diseases or conditions associated with the expression of HER-3 are the hyperproliferative diseases defined above. The patient in need of prevention or treatment is a mammalian patient, i.e. any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, cats, cattle, horses, sheep, pigs, goats, rabbits, etc. Preferably, the patient in need is a human patient.

In a preferred embodiment of the present invention, the method for preventing or treating a hyperproliferative disease in a patient in need thereof comprises administering to the patent an effective amount of at least one binding protein of the invention and additionally at least one other active agent, e.g., at least one antineoplastic agent as mentioned above. Preferably, the method is for inhibiting abnormal cell growth, migration or invasion.

In addition to classical modes of administration of potential binding protein therapeutics, e.g. via the above mentioned formulations, newly developed modalities of administration may also be useful according to the present invention. For example, local administration of ^{131}I-labeled monoclonal antibody for treatment of primary brain tumors after surgical resection has been reported. Additionally, direct stereotactic intracerebral injection of monoclonal antibodies and their fragments is also being studied clinically and pre-clinically. Intracarotid hyperosmolar perfusion is an experimental strategy to target primary brain malignancy with drug conjugated human monoclonal antibodies.

Depending on the type and severity of the condition to be treated, about 1 µg/kg to 15 mg/kg of the at least one binding protein of the invention may be

administered to a patient in need thereof, e.g. by one or more separate administrations or by continuous infusion. A typical daily dosage might range from about 1 µg/kg to about 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition to be treated, the treatment is sustained until a desired suppression of disease symptoms occurs.

The dose of the at least one antineoplastic agent administered depends on a variety of factors. These are, for example, the nature of the agent, the tumor type or the route of administration. It should be emphasized that the present invention is not limited to any dose.

Finally, the present invention relates in a further aspect to a kit for the diagnosis, prevention or treatment of hyperproliferative diseases associated with HER-3 mediated signal transduction, comprising the at least one binding protein and/or nucleic acid molecule and/or vector of the invention. In addition, the kit of the invention can further comprise at least one other active agent, e.g. at least one other antineoplastic agent as mentioned above.

Further, the present invention shall be explained by the following Examples and the accompanying drawing figures.

EXAMPLES

The following examples, including the experiments conducted and results achieved, are provided for illustrative purposes only and are not to be construed as limiting upon the present invention.

EXAMPLE 1: HER-3 antigen and cell line preparation

In the present study, recombinant HER-3 proteins were prepared. The extracellular domain of HER-3 (ECD) cDNA was cloned by polymerase chain

reaction (PCR) from pcDNA3-HER-3 (expression vector with full length human HER-3, C.Wallasch et al., *EMBO J.* 14, 4267-4275) with primers based on the sequence of HER-3 (Genebank AccNr. NM_001982).

The primers used for the amplification of HER-3 were as follows:

Forward primer: 5'-CGGGATCCATGTCCTAGCCTAGGGGC-3' (SEQ ID NO: 233)

Reverse primer:
5'-GCTCTAGATTAATGATGATGATGATGATGTTGTCCTAAA CAGTCTTG-3' (SEQ ID NO: 234)

The PCR product was digested with BamH1 and Xbal and ligated into pcDNA3 (Invitrogen) digested with BamH1 and Xbal. Plasmids were transfected into HEK293 cells using a $CaPO_4$ method. The HER-3-HIS fusion protein was purified from harvested conditioned media via Ni-NTA affinity chromatography.

Ratl HER-3 cells were generated by retroviral gene transfer. Briefly, GP+E 86 cells ($3x10^5$) were seeded on a 60 mm culture disc and transfected with 2 µg/ml plXSN vector or plXSN-HER-3 cDNA (C. Wallasch, PhD Thesis, Max-Planck Insitute of Biochemistry, Martinsried, Germany) using the calcium phosphate method. After 24h medium was replaced by fresh medium and the GP+E 86 cells were incubated for 4-8 hrs. Subconfluent Rat1 cells ($2x10^5$ cells per 6 cm dish) were then incubated with supernatants of GP+E 86 cells releasing high titer pLXSN or pLXSN-HER-3, p virus (>1 X 106 G418 c.f.u./ml; m.o.i. of 10) for 4-12 h in the presence of Polybrene (4 mg/ml; Aldrich). After changing the medium, selection of Ratl cells with G418 was started. Usually, stable clones were picked after selection for 21 days.

EXAMPLE 2: HER-3 expression in human cancer cell lines

Receptor tyrosine kinases, as for example HER-3, play a crucial role in the initiation and progression of hyperproliferative diseases such as the transition from benign hyperplastic cell growth towards a malignant carcinoma. Since HER-3 expression varies between tumor cells and normal tissue an analysis of HER-3 expression is a critical factor for identification of patient subgroups that would benefit from treatment with binding proteins of the invention. Thus, HER-3 expression was quantified in a panel of human cancer cell lines to elucidate the role of HER-3 in human cancer formation. Cancer cell lines were grown as recommended by the ATCC. In detail, 10^5 cells were harvested with 10 mM EDTA in PBS, washed once with FACS buffer (PBS, 3 % FCS, 0.4 % azide) and seeded on a 96-well round bottom plate. The cells were spun for 3 min at 1000 rpm to remove supernatant and then resuspended with α-HER-3 antibody 2D1D12 (WO03013602) (3 µg/ml). Cell suspensions were incubated on ice for 1 hr, washed twice with FACS buffer and resuspended with secondary antibody (100 µl/well) donkey-anti-human-PE (Jackson) diluted 1:50 in FACS buffer. The cell suspensions were incubated on ice and in the dark for 30 min, washed twice with FACS buffer and analyzed (FACS, Beckman Coulter). Fig. 1 shows representative results of the analysis and demonstrates that HER-3 is expressed in a variety of human cancers.

EXAMPLE 3: Immunization and titering

The HER-3 ECD protein, that was prepared as described in Example 1 and C32 cells (Human melanoma; ATCC #CRL-1585) were used as an antigen. Monoclonal antibodies against HER-3 were developed by sequentially immunizing XenoMouse® mice (XenoMouse® strains: XMG1 and XMG4, Abgenix, Inc. Fremont, CA). XenoMouse® animals were immunized via footpad route for all injections. The total volume of each injection was 50 µl per mouse, 25 µl per footpad.

For cohort #1 (10 XMG1 mice), the initial immunization was with 10 µg of

HER-3 ECD protein admixed 1:1 (v/v) with TITERMAX GOLD® (Sigma, Oakville, ON) per mouse. The subsequent five boosts were made with 10 µg of HER-3 ECD protein admixed 1:1 (v/v) with 100 µg alum gel (Sigma, Oakville, ON) in pyrogen-free D-PBS. The sixth boost consisted of 10 µg of HER-3 ECD protein admixed 1:1 (v/v) with TITERMAX GOLD®. The seventh injection consisted of 10 µg of HER-3 ECD protein admixed 1:1 v/v with 100 µg alum gel. A final boost was made with 10 µg HER-3 ECD protein in pyrogen-free DPBS, without adjuvant. The XenoMouse® mice were immunized on days 0, 4, 7, 11, 15, 20, 24, and 29 for this protocol and fusions were performed on day 33. The two bleeds were made through Retro-Orbital Bleed procedure on day 13 after the fourth boost, on day 19 after the sixth boost. There was no cohort #2.

For Cohort #3 (10 XMG1 mice) and Cohort #4 (10 XMG4 mice), the first injection was with 10^7 C32 cells in pyrogen-free Dulbecco's PBS (DPBS) admixed 1:1 (v/v) with TITERMAX GOLD® per mouse. The next four boosts were with 10^7 C32 cells in pyrogen-free DPBS, admixed with 25 µg of Adju-Phos and 10 µg CpG per mouse. The sixth boost was with 10^7 C32 cells in pyrogen-free DPBS, admixed 1:1 (v/v) with TITERMAX GOLD® per mouse. The seventh, eighth, ninth boosts were with 10^7 C32 cells in pyrogen-free DPBS, admixed with 25 µg of Adju-Phos and 10 µg CpG per mouse. From tenth to fourteen boosts were 5 µg of HER-3 ECD protein in pyrogen-free DPBS, admixed with 25 µg of Adju-Phos and 10 µg CpG per mouse. A final boost consisted of 5 µg of HER-3 ECD protein in pyrogen-free DPBS, without adjuvant. Both Cohort #3 and #4, the XenoMouse® mice were immunized on days 0, 3, 7, 11, 14, 17, 21, 24, 28, 33, 35, 38, 42 and 45 for this protocol and fusions were performed on day 49. The three bleeds were made through Retro-Orbital Bleed procedure on day 12 after the fourth boost, on day 19 after the sixth boost and on day 40 after twelfth boost.

Selection of animals for harvest by titer

For cohort #1, anti-HER-3 antibody titers in the serum from immunized

XenoMouse® mice were determined by ELISA against HER-3 ECD protein. The specific titer of each XenoMouse® animal was determined from the optical density at 650 nm and is shown in Table 1 below. The titer value is the reciprocal of the greatest dilution of sera with an OD reading two-fold that of background. Therefore, the higher the number, the greater was the humoral immune response to HER-3 ECD.

TABLE 1

Cohort #1, XMG1

Mouse ID	After 4 inj.	After 6 inj.
P3421	8,000	11,000
P3422	850	2,600
P3423	2,700	5,200
P3424	3,200	9,100
P3425	5,400	2,500
P3426	700	1,500
P3427	5,800	7,000
P3428	3,900	4,300
P3429	2,200	2,500
P34210	600	850
NC	250	175
PC	377,000	311,000
NC	mAb IL-8, D39.2.1	
PC	xHER-3-2D1D12	

For cohort #3 and #4, anti-HER-3 antibody titers in the serum from immunized XenoMouse® mice were determined by FACS using Rat1/HER-3 cells (antigen positive cell line) cells and Rat1/pLSXN cells (antigen negative cell line). Data are presented as geometric mean (GeoMean) fluorescent intensity of cell anti-HER-3 cell staining by serial dilutions of serum samples.

TABLE 2

Cohort #3, XMG1

Mouse ID	Sample dilution	After 6 inj.		After 12 inj.	
		pos cells GeoMean	neg cells GeoMean	pos cells GeoMean	neg cells GeoMean
Q832-1	1:50	9	10	11	10
	1:250	6	9	6	6
	1:1250	6	7	4	4
Q832-2	1:50	8	10	29	42
	1:250	7	8	11	11
	1:1250	5	6	6	5
Q832-3	1:50	7	12	11	9
	1:250	5	7	5	5
	1:1250	5	5	4	4
Q832-4	1:50	6	10	9	9
	1:250	6	6	5	5
	1:1250	5	5	4	4
Q832-5	1:50	11	11	17	13
	1:250	10	9	7	6
	1:1250	6	8	5	4
Q832-6	1:50	7	11	15	14
	1:250	7	7	7	6
	1:1250	5	6	6	4
Q832-7	1:50	8	11	7	15
	1:250	6	7	5	5
	1:1250	5	5	4	4
Q832-8	1:50	7	8	11	20
	1:250	6	6	7	8
	1:1250	5	5	5	4
Q832-9	1:50	7	12	15	16
	1:250	6	8	6	5
	1:1250	6	6	4	4
Q832-10	1:50	8	13	34	38
	1:250	6	8	9	8
	1:1250	6	6	5	4

TABLE 3

Cohort #4, XMG4

Mouse ID	Sample dilution	After 6 inj.		After 12 inj.	
		pos cells GeoMean	neg cells GeoMean	pos cells GeoMean	neg cells GeoMean
Q856-1	1:50	4	6	91	44
	1:250	4	5	32	18
	1:1250	4	4	19	10
Q856-2	1:50	4	8	148	54
	1:250	4	5	89	23
	1:1250	4	4	42	9
Q856-3	1:50	4	5	72	14
	1:250	4	4	28	6
	1:1250	4	4	18	4
Q856-4	1:50	4	5	11	49
	1:250	4	5	10	17
	1:1250	4	4	8	7
Q856-5	1:50	4	4	74	20
	1:250	4	4	30	14
	1:1250	4	4	16	6
Q856-6	1:50	4	5	86	21
	1:250	4	4	32	10
	1:1250	4	4	16	5
Q856-7	1:50	5	6	74	32
	1:250	4	5	32	14
	1:1250	4	4	16	6
Q856-8	1:50	4	5	106	14
	1:250	4	4	45	6
	1:1250	4	4	22	4
Q856-9	1:50	5	6	53	22
	1:250	4	4	17	11
	1:1250	4	4	11	5
Q856-10	1:50	4	5	72	53
	1:250	4	4	26	17
	1:1250	4	4	15	7

EXAMPLE 4: Recovery of lymphocytes, B-Cell isolations, fusions and generation of hybridomas

Immunized mice were sacrificed and the lymph nodes were harvested and pooled from each cohort. The lymphoid cells were dissociated by grinding in DMEM to release the cells from the tissues, and the cells were suspended in DMEM. The cells were counted, and 0.9 ml DMEM per 100 million lymphocytes was added to the cell pellet to resuspend the cells gently but completely. Using 100 µl of CD90+ magnetic beads per 100 million cells, the cells were labeled by incubating the cells with the magnetic beads at 4 °C for 15 min. The magnetically-labeled cell suspension containing up to 10^8 positive cells (or up to $2x10^9$ total cells) was loaded onto a LS+ column and the column washed with DMEM. The total effluent was collected as the CD90-negative fraction (most of these cells were expected to be B cells).

The fusion was performed by mixing washed enriched B cells from above and nonsecretory myeloma P3X63Ag8.653 cells purchased from ATCC (Cat. No. CRL 1580) (Kearney et al, J. Immunol. 123, 1979, 1548-1550) at a ratio of 1:1. The cell mixture was gently pelleted by centrifugation at 800 g. After complete removal of the supernatant, the cells were treated with 2 to 4 ml of pronase solution (CalBiochem, Cat. No. 53702; 0.5 mg/ml in PBS) for no more than 2 min. Then 3 to 5 ml of FBS was added to stop the enzyme activity and the suspension was adjusted to 40 ml total volume using electro cell fusion solution, ECFS (0.3 M sucrose, Sigma, Cat. No. S7903, 0.1 mM magnesium acetate, Sigma, Cat. No. M2545, 0.1 mM calcium acetate, Sigma, Cat. No. C4705). The supernatant was removed after centrifugation and the cells were resuspended in 40 ml ECFS. This wash step was repeated and the cells again were resuspended in ECFS to a concentration of $2x10^6$ cells/ml.

Electro-cell fusion was performed using a fusion generator, model ECM2001, Genetronic, Inc., San Diego, CA. The fusion chamber size used was 2.0 ml, using the following instrument settings: Alignment condition:

voltage: 50 V, time: 50 sec; membrane breaking at: voltage: 3000 V, time: 30 µsec; post-fusion holding time: 3 sec.

After ECF, the cell suspensions were carefully removed from the fusion chamber under sterile conditions and transferred into a sterile tube containing the same volume of Hybridoma Culture Medium (DMEM (JRH Biosciences), 15 % FBS (Hyclone), supplemented with L-glutamine, pen/strep, OPI (oxaloacetate, pyruvate, bovine insulin) (all from Sigma) and IL-6 (Boehringer Mannheim). The cells were incubated for 15 to 30 min at 37 °C, and then centrifuged at 400 g for five min. The cells were gently resuspended in a small volume of Hybridoma Selection Medium (Hybridoma Culture Medium supplemented with 0.5x HA (Sigma, Cat. No. A9666)), and the volume was adjusted appropriately with more Hybridoma Selection Medium, based on a final plating of $5x10^6$ B cells total per 96-well plate and 200 µl per well. The cells were mixed gently and pipetted into 96-well plates and allowed to grow. On day 7 or 10, one-half the medium was removed, and the cells were re-fed with Hybridoma Selection Medium.

EXAMPLE 5: Selection of candidate antibodies by ELISA

After 14 days of culture, primary screening of hybridoma supernatants from the cohort #1 (mice in cohort one were split arbitrarily into fusion #1 and #2) for HER-3-specific antibodies was performed by ELISA using purified his-tagged HER-3 ECD and counter-screening against an irrelevant his-tagged protein by ELISA using goat anti-huIgGFc-HRP (Caltag Inc., Cat. No. H10507, using concentration was 1:2000 dilution) to detect human IgG binding to HER-3 ECD immobilized on ELISA plates. The old culture supernatants from the positive hybridoma cells growth wells based on primary screen were removed and the HER-3 positive hybridoma cells were suspended with fresh hybridoma culture medium and were transferred to 24-well plates. After 2 days in culture, these supernatants were ready for a secondary confirmation screen. In the secondary confirmation screen for HER-3 specific fully human IgGk antibodies, the positives in the first

screening were screened by ELISA with two sets of detective antibodies: goat anti-huIgGFc-HRP (Caltag Inc., Cat. No. H10507, using concentration was 1:2000 dilution) for human gamma chain detection and goat anti-hIg kappa-HRP (Southern Biotechnology, Cat. No. 2060-05) for human kappa light chain detection. There were 91 fully human IgG/kappa HER-3 specific monoclonal antibodies that were generated from cohort #1.

EXAMPLE 6: Selection of candidate antibodies by FMAT/FACS

After 14 days of culture, hybridoma supernatants from the cohort #3 and #4 (fusion #3 and #4) were screened for HER-3-specific monoclonal antibodies by FMAT. In the primary screen, hybridoma supernatants at 1:10 final dilution were incubated with Rat1-Her3 cells expressing human HER-3 and 400 ng/ml Cy5-conjugated Goat F(ab')2 anti-human IgG, Fc-specific antibody (Jackson ImmunoResearch, Cat. No. 109-176-098) at room temperature for 6 hr. The binding of antibodies and detection antibodies complex to cells were measured by FMAT (Applied Biosystems). Non-specific binding of antibodies to the cells was determined by their binding to parental Rat1 cells. A total of 420 hybridomas producing HER-3-specific antibodies were selected from primary screen of fusion #3. The supernatants from these expanded cultures were tested again using the same FMAT protocol and 262 of them were confirmed to bind to HER-3 expressing cells specifically. A total of 193 hybridomas producing HER-3 specific antibodies were selected from primary screen of fusion #4. The supernatants from these expanded cultures were tested by FACS and 138 of them were confirmed to bind to HER-3 expressing cells specifically. In the FACS confirmation assay, Rat1-Xher3 cells and parental Rat1 cells (as negative control) were incubated with hybridoma supernatants at 1:2 dilution for 1 hr at 40C in PBS containing 2 % FBS. Following washing with PBS, the binding of antibodies to the cells were detected by 2.5 µg/ml Cy5-conjugated Goat F(ab')2 anti-human IgG, Fc-specific antibody (JIR#109-176-098) and 5 µg/ml PE-conjugated Goat F(ab')2 anti-human kappa-specific antibody (SB# 2063-09). After removing the unbound antibodies by washing with PBS, the

cells were fixed by cytofix (BD# 51-2090KZ) at 1:4 dilution and analyzed by FACSCalibur.

EXAMPLE 7: Selection of hybridomas for cloning

Antibodies from cohorts 1 and 2 were selected for hybridoma cloning based on specificity for HER-3 over HER1 (EGFR), HER-2 and HER-4 in ELISA using purified recombinant extra-cellular domains available from, for example R&D Biosystems, and FACS-based analysis of human tumor cell lines expressing different HER family members, and a > 5-time increase in mean fluorescent intensity in FACS staining for HER-3 positive cells over background. Based on these criteria, a total of 23 hybridoma lines were selected for cloning by limiting dilution cell plating.

Antibodies from cohorts 3 and 4 were selected for hybridoma cloning based on specificity for HER-3 over HER-1 (EGFR), HER-2 and HER-4 plus three other criteria. The first criterion was an ELISA screen for antibodies with epitopes contained within the L2 domain of HER-3 (see Example "Structural Analysis of anti-HER-3 Antibodies in the Invention).

The second criterion was neutralization of binding of biotinylated heregulin-alpha to HER-3 expressing cells in a FACS based assay. SKBR-3 cells were harvested, washed in culture medium, pelleted via centrifugation and resuspended in culture medium. Resuspended cells were aliquoted into 96-well plates. The plates were centrifuged to pellet the cells. Test antibodies in exhaust hybridoma supernatants were added at 25µl/well and incubated for 1 hr on ice to allow antibody binding. Fifty µl of a 10 nM heregulin-alpha (R&D Biosystems, Minneapolis, MN) solution was added to each well for a final concentration of 5 nM and incubated on ice for 1.5 hr. Cells were washed in 150 µl PBS, pelleted by centrifugation and the supernatant removed. Cells were resuspended in 50 µl of goat anti-HRG-alpha polyclonal antibody at 10 µg/ml and incubated for 45 min of ice. Cells were washed in 200 µl PBS, pelleted by centrifugation and the supernatant

removed. Fifty µl of a solution of rabbit Cy5-labeled anti-goat polyclonal antibody at 5 µg/ml plus 7AAD at 10 µg/ml was added and incubated on ice for 15 min. Cells were washed in 200 µl PBS, pelleted by centrifugation and the supernatant removed. The cells were resuspended in 100 µl of FACS buffer and read in the FACS. Test HER-3 antibodies that reduced binding of heregulin-alpha were those that had lowest fluorescence intensity. As positive controls, 1:5 serial dilutions from 10,000 ng/ml to 16 ng/ml of a mouse HER-3 mAb (105.5) or the human IgG1 HER-3 mAb, U1-49 was used. Negative controls were heregulin-alpha alone, cells alone, goat anti-heregulin-alpha polyclonal antibody alone and rabbit Cy5-labeled anti-goat polyclonal antibody alone.

The third criterion was relative ranking for affinity and/or higher relative mean fluorescence intensity in FACS using HER-3 expressing cell lines. Relative ranking for affinity was performed by normalizing HER-3-specific antibody concentrations and plotting versus data from limiting antigen ELISA as follows.

Normalization of antigen specific antibody concentrations using high antigen ELISA

Using an ELISA method, supernatants for concentration of antigen specific antibody were normalized. Using two anti-HER-3 human IgG1 antibodies from cohort 1 of known concentration titrated in parallel, a standard curve was generated and the amount of antigen specific antibody in the test hybridoma supernatants from cohorts 3 and 4 were compared to the standard. In this way, the concentration of human HER3 IgG antibody in each hybridoma culture was estimated.

Neutravidin plates were made by coating neutravidin @ 8 µg/ml in 1XPBS/0.05% sodium azide on Costar 3368 medium binding plates at 50 ul/well with overnight incubation at 4° C. The next day the plates were blocked with 1XPBS/1% skim milk. Photobiotinylated his-tagged-HER-3 ECD @ 500 ng/ml in 1XPBS/1% skim milk was bound to the neutravidin

plates by incubating for 1 hour at room temperature. Hybridoma supernatant, serially diluted 1:2.5 from a starting dilution of 1:31 to a final dilution of 1:7568 in1XPBS/1% skim milk/0.05% azide, was added at 50 µl/well, and then incubated for 20 hours at room temperature. Serially dilutions were used to ensure obtaining OD readings for each unknown in the linear range of the assay. Next, a secondary detection antibody, goat anti human IgG Fc HRP at 400 ng/ml in 1XPBX/1%skim milk was added at 50 ul/well. After 1 hour at room temperature, the plates were again washed 5 times with water and 50µL of one-component TMB substrate were added to each well. The reaction was stopped after 30 minutes by the addition of 50 µl of 1M hydrochloric acid to each well and the plates were read at wavelength 450nm. A standard curve was generated from the two IgG1 HER-3 mAbs from cohort 1, serially diluted at 1:2 from 1000 ng/ml to 0.06 ng/ml and assessed in ELISA using the above protocol. For each unknown, OD readings in the linear range of the assay were used to estimate the concentration of human HER-3 IgG in each sample.

The limited antigen analysis is a method that affinity ranks the antigen-specific antibodies prepared in B-cell culture supernatants relative to all other antigen-specific antibodies. In the presence of a very low coating of antigen, only the highest affinity antibodies should be able to bind to any detectable level at equilibrium. (*See, e.g.,* PCT Publication WO/03048730A2 entitled "IDENTIFICATION OF HIGH AFFINITY MOLECULES BY LIMITED DILUTION SCREENING" published on June 12, 2003). In this instance, two mAbs from cohort 1, both of known concentration and known KD, were used as benchmarks in the assay.

Neutravidin plates were made by coating neutravidin at 8 µg/ml in 1XPBS/0.05% sodium azide on Costar 3368 medium binding plates at 50 ul/well with overnight incubation at 4° C. The next day the plates were blocked with 1XPBS/1% skim milk. Biotinylated his-tagged-HER-3 ECD (50 µl/well) was bound to 96-well neutravidin plates at five concentrations: 125, 62.5, 31.2, 15.6, and 7.8 ng/ml in 1XPBS/1% skim milk for 1 hour at room

temperature. Each plate was washed 5 times with water. Hybridoma supernatants diluted 1:31 in 1XPBS/1%skim milk/0.05% azide were added at 50 ul/well. After 20 hours incubation at room temperature on a shaker, the plates were again washed 5 times with dH_2O. Next, a secondary detection antibody, goat anti human IgG Fc HRP (Horsh Radish Peroxidase) at 400 ng/ml in 1XPBS/1%skim milk was added at 50 µl/well. After 1 hour at room temperature, the plates were again washed 5 times with dH_2O and 50µL of one-component TMB substrate were added to each well. The reaction was stopped after 30 minutes by the addition of 50µL of 1M hydrochloric acid to each well and the plates were read at wavelength 450nm. OD readings from an antigen concentration that yielded OD values in the linear range were used in for data analysis.

Plotting the high antigen data, which comparatively estimates specific antibody concentration (see above for details), versus the limited antigen OD illustrated the relatively higher affinity antibodies, e.g., those that bound had higher OD in the limited antigen assay while having lower amounts of IgG HER-3 antibody in the supernatant.

Hybridomas from cohorts 3 and 4 for the 33 best performing antibodies in these sets of assays were advanced to cloning by limiting dilution hybridoma plating.

Alternatively, FACS analysis of HER-3 expression of Ratl/pLXSN and Ratl/HER-3 cells showed similar results (no crossreactivity with endogenous rat epitopes) (Fig. 2) .

In detail $1x10^5$ cells were harvested with 10 mM EDTA in PBS, washed once with FACS buffer (PBS, 3 % FCS, 0.4 % azide) and seeded on a 96-well round bottom plate. The cells were spun for 3 min at 1000 rpm to remove supernatant and then resuspended with the specific HER-family antibodies (3 µg/ml). Cell suspensions were incubated on ice for 45 min, washed twice with FACS buffer and resuspended with secondary antibody (100 µl/well)

donkey-anti-human-PE (Jackson Immunoresearch, PA) diluted 1:50 in FACS buffer. The cell suspensions were incubated on ice and in the dark for 30 min, washed twice with FACS buffer and analyzed (FACS, Beckman Coulter).

EXAMPLE 8: Structural analysis of anti-HER-3 antibodies of the invention

In the following discussion, structural information related to antibodies prepared in accordance with the invention is provided. In order to analyze structures of antibodies produced in accordance with the present invention, genes encoding the heavy and light chain fragments were amplified out of the particular hybridoma. Sequencing was accomplished as follows:

The VH and VL transcripts were amplified from individual hybridoma clones in 96 well plate using reverse transcriptase polymerase chain reaction (RT-PCR). Poly(A)+-mRNA was isolated from approximately $2x10^5$ hybridoma cells using a Fast-Track kit (Invitrogen). Four PCR reactions were run for each Hybridoma: two for light chain (kappa (κ), and two for gamma heavy chain (γ). The QIAGEN OneStep room temperature-PCR kit was used for amplification (Qiagen, Catalog No.210212). In the coupled room temperature-PCR reactions, cDNAs were synthesized with blend of room temperature enzymes (Omniscript and Sensiscript) using antisense sequence specific primer corresponded to C-κ, or to a consensus of the CH1 regions of Cγ genes. Reverse transcription was performed at 50 °C for 1 hr followed by PCR amplification of the cDNA by HotStarTaq DNA Polymerase for high specificity and sensitivity. Each PCR reaction used a mixture of 5'-sense primers; primer sequences were based on the leader sequences of VH and VK available at the Vbase website (http://vbase.mrc-cpe.cam.ac.uk/).

PCR reactions were run at 94 °C for 15 min, initial hot start followed by 40 cycles of 94 °C for 30 sec (denaturation), 60 °C for 30 sec (annealing) and 72 °C for 1 min (elongation).

PCR products were purified and directly sequenced using forward and reverse PCR primers using the ABI PRISM BigDye terminator cycle sequencing ready reaction Kit (Perkin Elmer). Both strands were sequenced using Prism dye-terminator sequencing kits and an ABI 377 sequencing machine.

Sequence analysis

Analyses of human V heavy and V kappa cDNA sequences of the HER3 antibodies were accomplished by aligning the HER-3 sequences with human germline V heavy and V kappa sequences using Abgenix in-house software (5AS). The software identified the usage of the V gene, the D gene and the J gene as well as nucleotide insertions at the recombination junctions and somatic mutations. Amino acid sequences were also generated in silico to identify somatic mutations. Similar results could be obtained with commercially available sequence analysis software and publicly available information on the sequence of human V, D, and J genes, e.g., Vbase (http://vbase.mrc-cpe.cam.ac.uk/).

Molecular cloning of mAb U1-59

Total RNA was extracted from the tissue culture well containing multiple hybridomas lineages, including the hybridoma lineage secreting antibody U1-59. A heavy chain variable region was amplified using 5'-leader VH family specific primers, with 3'-C-gamma primer. A major band was amplified using a VH4 primer, no other bands were visible. The VH4-34 gamma fragment was cloned into pCDNA expression vector in frame with a human gamma 1 constant region gene.

An IgM heavy chain variable region was amplified using 5' VH family specific primers with 3' mu constant region primer. A major band was amplified using VH2 primer, no other bands were visible. The VH2-5 mu fragment was cloned into pCDNA expression vector in frame with a human mu constant

region gene. V kappa chains were amplified and sequenced. Four kappa chain RT-PCR products were identified. The products were sequenced and after sequence analysis via in silico translation, only three of them had open-reading frames. These three functional kappa chains were cloned out of the oligoclonal U1-59 hybridoma well identified based on V kappa gene usage as (1) VK1 A3-JK2, (2) VK1 A20-JK3 and (3) B3-JK1. All V-kappa were cloned into pCDNA expression vector in frame with a human kappa light chain constant region gene.

Transfections:

Each heavy chain was transfected with each of the kappa chains in transient transfections for a total of 6 heavy chain/kappa light chain pairs. The transfection of the gamma chain with the A20 kappa chain gave poor antibody expression, while no antibody was secreted or detected when the A20 kappa chain was co-transfected with the mu chain. A total of three IgG sups and two IgM sups were available for HER-3 binding assay.

Chain	VH	D	J	Constant	ORF
Heavy	VH4-34	D1-20	JH2	Gamma	yes
Heavy	VH2-5	D6-6	JH4b	Mu	yes
Light	A3		JK2	Kappa	yes
Light	A20		JK3	Kappa	yes
Light	B3		JK1	Kappa	yes
Light	A27		JK3	Kappa	NO

Binding activity to HER-3+ cell lines was detected in FACS with the IgG1 mAb consisting of the VH4-34 and the B3 kappa chain. No other VH/Vk combinations gave fluorescence signal above background in FACS using HER-3+ cell lines.

Binding competition of the anti-HER-3 antibodies

Multiplexed competitive antibody binning was performed as published in Jia et al. *J Immunol Methods.* 288, 91-98 (2004) to assess clusters of HER-3 antibodies that competed for binding to HER-3. Tested HER-3 antibodies

from cohort 1 clustered into 5 bins based on competition for binding.

Bin#1	Bin#2	Bin#3	Bin#4	Bin#5
U1-42	U1-48	U1-52	U1-38	U1-45
U1-44	U1-50		U1-39	U1-40
U1-62	U1-51			U1-41
U1-46				U1-43
U1-47	U1-49			U1-61
U1-58				U1-53
				U1-55

Epitope characterization of anti-HER-3 antibodies

The epitopes of human anti-HER-3 antibodies of the invention were characterized. First a dot blot analysis of the reduced, denatured HER-3-His tagged purified ECD protein showed absence of binding by the anti-HER-3 antibodies tested (U1-59, U1-61, U1-41, U1-46, U1-53, U1-43, U1-44, U1-47, U1-52, U1-40, U1-49)) demonstrating that all had epitopes sensitive to reduction of disulfide bonds, suggesting that all had discontinuous epitopes. Next, the antibodies were mapped to defined domains in the HER-3 molecule by engineering various human-rat HER-3 chimeric molecules, based on the division of the HER-3 extra-cellular domain into four domains:

1) L1 (D1): the minor ligand-binding domain,
2) S1 (D2): the first cysteine-rich domain,
3) L2 (D3): the major ligand-binding domain, and
4) S2 (D4): the sec cysteine-rich domain.

The extra-cellular domain (ECD) of Human HER-3 cDNA was amplified from RAT1-HER-3 cells. The rat HER-3 cDNAs was amplified by RT-PCR from rat liver RNA and confirmed by sequencing. The cDNAs expressing the ECD of human and rat Her3 were cloned into mammalian expression vectors as V5-His fusion proteins. Domains from the human HER-3 ECD were swapped into the scaffold provided by the rat HER-3 ECD by using the Mfe1, BstX1 and DraIII internal restriction sites. By this means, various chimeric

rat/human HER-3 ECD HIS fusion proteins (amino acids 1-160, 161-358, 359-575, 1-358, 359-604) were constructed and expressed via transient transfection of HEK 293T cells. Expression of the constructs was confirmed using a rat polyclonal antibody against human HER-3. The human monoclonal antibodies were tested in ELISA for binding to the secreted chimeric ECDs.

Two of the human antibodies, including antibody U1-59, cross-reacted with rat HER-3. To assign binding domains, these mAbs were tested against a truncated form of HER-3 consisting of L1-S1-V5his tagged protein purified from the supernatant of HEK 293T cells transfected with a plasmid DNA encoding the expression of the L1-S1 extra-cellular domains of HER3. mAb U1-59 bound to the L1-S1 protein in ELISA, implying that its epitope is in L1-S1. mAb 2.5.1 did not bind to the L1-S1 protein, implying that its epitope is in L2-S2. Further mapping of antibody U1-59 was accomplished using SELDI time of flight mass spectroscopy with on-chip proteolytic digests of mAb-HER-3 ECD complexes.

Mapping U1-59 epitopes using SELDI

Further mapping of antibody U1-59 was accomplished using a SELDI time of flight mass spectroscopy with on-chip proteolytic digests of mAb-HER-3 ECD complexes. Protein A was covalently bound to a PS20 protein chip array and used to capture mAb U1-59. Then the complex of the PS20 protein chip and the monoclonal antibody was incubated with HER-3-His purified antigen. Next the antibody-antigen complex was digested with high concentration of Asp-N. The chip was washed, resulting in retention of only the HER-3 peptide bound to the antibody on the chip. The epitope was determined by SELDI and identified by mass of the fragment. The identified 6814 D fragment corresponds to two possible expected peptides generated from a partial digest of the HER-3-his ECD. Both overlapping peptides map to the domain S1. By coupling SELDI results with binding to a HER-3 deletion construct, the epitope was mapped to residues 251 to 325.

The location of the binding domains in the extracellular part of HER-3 that are recognized by the human anti-HER-3 mAbs of the invention are summarized in Table 4. The epitope domain mapping results were consistent with results from antibody competition binding competition bins, with antibodies that cross-competed each other for binding to HER-3 also mapping to the same domains on HER-3 (Fig. 3).

TABLE 4

A summary of mAb's binding domain based on ELISA assay results

mAb	Rat XR	Binding domain	mAb	Rat XR	Binding domain
U1-59	Yes	S1	U1-2	No	L2
U1-61	No	L2	U1-7	No	L2
U1-41	No	L2	U1-9	No	L2
U1-46	No	S1	U1-10	No	L2
U1-53	No	L2	U1-12	No	L2
U1-43	No	L2	U1-13	No	L2
U1-44	No	S1	U1-14	No	L2
U1-47	No	S1	U1-15	No	L2
U1-52	Yes	L2S2	U1-19	No	L2
U1-40	No	L2	U1-20	No	L2
U1-49	No	L1	U1-21	No	L2
U1-21	No	L2	U1-28	No	L2
U1-22	No	L2	(U1-31)	No	L2
U1-23	No	L2	U1-32	No	L2
U1-24	No	L2	(U1-35)	No	L2
U1-25	No	L2	U1-36	No	L2
U1-26	No	L2	(U1-37)	No	L2
U1-27	No	L2			

XR = cross-reactive

EXAMPLE 9: Determination of canonical classes of antibodies

Chothia, *et al.* have described antibody structure in terms of "canonical

classes" for the hypervariable regions of each immunoglobulin chain (*J. Mol. Biol.*, 1987 Aug 20, 196(4):901-17). The atomic structures of the Fab and VL fragments of a variety of immunoglobulins were analyzed to determine the relationship between their amino acid sequences and the three-dimensional structures of their antigen binding sites. Chothia, *et al.* found that there were relatively few residues that, through their packing, hydrogen bonding or the ability to assume unusual phi, psi or omega conformations, were primarily responsible for the main-chain conformations of the hypervariable regions. These residues were found to occur at sites within the hypervariable regions and in the conserved β-sheet framework. By examining sequences of immunoglobulins having unknown structure, Chothia, *et al.* show that many immunoglobulins have hypervariable regions that are similar in size to one of the known structures and additionally contained identical residues at the sites responsible for the observed conformation.

Their discovery implied that these hypervariable regions have conformations close to those in the known structures. For five of the hypervariable regions, the repertoire of conformations appeared to be limited to a relatively small number of discrete structural classes. These commonly occurring main-chain conformations of the hypervariable regions were termed "canonical structures." Further work by Chothia, *et al.* (*Nature,* 1989 Dec 21-28, 342 (6252):877-83) and others (Martin, *et al. J. Mol. Biol.,* 1996 Nov 15, 263(5): 800-15) confirmed that there is a small repertoire of main-chain conformations for at least five of the six hypervariable regions of antibodies.

The CDRs of each antibody described above were analyzed to determine their canonical class. As is known, canonical classes have only been assigned for CDR1 and CDR2 of the antibody heavy chain, along with CDR1, CDR2 and CDR3 of the antibody light chain. The tables below summarizes the results of the analysis. The canonical class data is in the form of HCDR1-HCDR2-LCDR1-LCDR2-LCDR3, wherein "HCDR" refers to the heavy chain CDR and "LCDR" refers to the light chain CDR. Thus, for example, a canonical class of 1-3-2-1-5 refers to an antibody that has a

HCDR1 that falls into canonical class 1, a HCDR2 that falls into canonical class 3, a LCDR1 that falls into canonical class 2, a LCDR2 that falls into canonical class 1, and a LCDR3 that falls into canonical class 5.

Assignments were made to a particular canonical class where there was 70 % or greater identity of the amino acids in the antibody with the amino acids defined for each canonical class. The amino acids defined for each antibody can be found, for example, in the articles by Chothia, *et al.* referred to above. Table 5 and Table 6 report the canonical class data for each of the HER-3 antibodies. Where there was less than 70 % identity, the canonical class assignment is marked with an asterisk ("*") to indicate that the best estimate of the proper canonical class was made, based on the length of each CDR and the totality of the data. Where there was no matching canonical class with the same CDR length, the canonical class assignment is marked with a letter s and a number, such as "s18", meaning the CDR is of size 18. Where there was no sequence data available for one of the heavy or light chains, the canonical class is marked with "Z".

TABLE 5

Antibody (sorted)	H1-H2-L1-L2-L3	H3length	Antibody (sorted)	H1-H2-L1-L2-L3	H3length
U1-38	3-1-4-1-1	9	U1-7	3-1-2-1-1	12
U1-39	1-1-4-1*-1	6	U1-9	3-1-2-1-1	12
U1-40	3-1-4-1-1	15	U1-10	3-1-2-1-1	12
U1-41	3-1-2-1-1	15	U1-12	3-1-2-1-1	12
U1-42	1-2-2-1-1	9	U1-13	3-1-4-1-1	7
U1-43	3-1-2-1-1	17	U1-14	3-1-2-1-1	12
U1-44	1-2-2-1-1	9	U1-15	3-1-8-1-1	14
U1-45	1-2*-2-1-1	16	U1-19	3-1-Z-Z-Z	12
U1-46	3-s18-Z-Z-Z	17	U1-20	3-1-2-1-1	19
U1-47	3-s18-2-1-1	16	U1-21	3-1-2-1-1	12
U1-48	1-1-Z-Z-Z	16	U1-22	3-1-2-1-1	12
U1-49	1-3-4-1-1	17	U1-23	3-1-2-1-1	12
U1-50	3-1-2-1-1	17	U1-24	3-1-2-1-1	12
U1-51	1-1-3-1-1	19	U1-25	3-1-2-1-1	12
U1-52	3-1-8-1-1	15	U1-26	3-1-2-1-1	12
U1-53	1-3-2-1-1	10	U1-27	3-1-2-1-1	12
U1-55	3-1-4-1-1	15	U1-28	3-1-2-1-1	12
U1-57	3-1-4-1-1	15	U1-31	1-2-2-1-1	13
U1-58	1-3-2-1-1	12	U1-32	3-1-2-1-1	12
U1-59	1-1-3-1-1	9	U1-35	1-3-2-1-1	14
U1-61.1	3-1*-2-1-1	16	U1-36	3-1-2-1-1	12
U1-62	1-2-8-1-1	12	U1-37	1-2-Z-Z-Z	13
U1-2	3-1-2-1-1	12			

Table 7 is an analysis of the number of antibodies per class. The number of antibodies having the particular canonical class designated in the left column is shown in the right column. The four mAbs lacking one chain sequence data and thus having "Z" in the canonical assignment are not included in this counting.

The most commonly seen structure is 3-1-2-1-1: Twenty-one out of forty-one mAbs having both heavy and light chain sequences had this combination.

TABLE 6

H1-H2-L1-L2-L3	Count
1-1-3-1-1	2
1-1-4-1*-1	1
1-2-2-1-1	4
1-2-8-1-1	1
1-3-2-1-1	3
1-3-4-1-1	1
3-1-2-1-1	21
3-1-4-1-1	5
3-1-8-1-1	2
3-s18-2-1-1	1

EXAMPLE 10: Determination of antibody affinity

Affinity measurements of anti-HER-3 antibodies of the invention were performed by indirect FACS Scatchard analysis. Therefore, 10^5 cells of interest or SK-Br 3 cells were harvested with 10 mM EDTA in PBS, washed once with FACS buffer (PBS, 3 % FCS, 0.4 % azide) and seeded on a 96-well round bottom plate. The cells were spun for 3 min at 1000 rpm to remove supernatant and then resuspended with α-HER-3 antibody (3 µg/ml) or with antibody dilutions (100 µl/well) starting with 20 µg/ml human monoclonal antibody in FACS buffer, diluted in 1:2 dilution steps. Cell suspensions were incubated on ice for 1 hr, washed twice with FACS buffer and resuspended with secondary antibody (100 µl/well) donkey-anti-human-PE (Jackson) diluted 1:50 in FACS buffer. The cell suspensions were incubated on ice and in the dark for 30 min, washed twice with FACS buffer and analyzed (FACS, Beckman Coulter). According to the FACS Scatchard analysis, the fluorescence mean was calculated for each measurement. Background staining (= without 1st antibody) was subtracted from each fluorescence mean. Scatchard plot with x-value = fluorescence mean and y-value = fluorescence mean/concentration of mAb (nM) was generated. The KD was taken as the absolute value of 1/m of linear equation. Fig. 4 shows a kinetic analysis using the U1-59 antibody of the invention. In the following table 8 affinity measurements for certain antibodies of the invention selected in this manner are provided.

TABLE 7

clone	KD (nM)
U1-38	n.d.
U1-39	102
U1-40	6,7
U1-41	0,18
U1-42	n.d.
U1-43	0,57
U1-44	4
U1-52	16,8
U1-61	0,13
U1-62	20,4
U1-46	13,8
U1-47	9,38
U1-49	1
U1-50	39,3
U1-51	131,6
U1-53	0,082
U1-55.1	3,7
U1-58	6,4
U1-59	3,69
U1-24	0,06
U1-7	0,02

EXAMPLE 11: Anti-HER-3 antibodies of the invention induce HER-3 receptor endocytosis

HER-3 has been identified as a factor that can influence initiation and progression of hyperproliferative diseases through serving as an important gatekeeper of HER family mediated cell signaling. Thus, if HER-3 is effectively cleared from the cell surface/membrane by receptor internalization, cell signaling and therefore transformation and/or maintenance of cells in malignancy can be ultimately diminished or suppressed.

In order to investigate whether anti-HER-3 antibodies of the invention are

capable of inducing accelerated endocytosis of HER-3, the relative amount of HER-3 molecules on the cell surface after 0.5 and 4 hr incubation of the cells with anti-HER-3 antibodies of the invention were compared. $3x10^5$ cells were seeded in normal growth medium in 24-well dish and left to grow overnight. Cells were preincubated with 10 µg/ml anti-HER-3 mAbs in normal growth medium for the indicated times at 37 °C. Cells were detached with 10 mM EDTA and incubated with 10 µg/ml anti-HER-3 mAbs in wash buffer (PBS, 3 % FCS, 0.04 % azide) for 45 min at 4 °C. Cells were washed twice with wash buffer, incubated with donkey-anti-human-PE secondary antibody (Jackson) diluted 1:100 for 45 min at 4 °C, washed twice with wash buffer and analyzed by FACS (BeckmanCoulter, EXPO).

Data shown in Fig. 5 demonstrate that treatment of cells with anti-HER-3 antibodies leads to internalization of the receptor. Data are shown as % internalization and refer to the reduction of the mean fluorescence intensity of anti-HER3 treated samples relative to control-treated samples.

EXAMPLE 12: Inhibition of ligand binding to human cancer cells SKBr3 by human anti-HER-3 antibodies of the invention

Radioligand competition experiments were performed in order to quantitate the ability of the anti-HER-3 antibodies of the invention to inhibit ligand binding to HER-3 in a cell based assay. Therefore, the HER-3 receptor binding assay was performed with $4x10^5$ SK-BR-3 cells which were incubated with varying concentrations of antibodies for 30 min on ice. 1.25 nM [I^{125}]-α-HRG/[^{125}I]-β-HRG were added to each well and the incubation was continued for 2 hr on ice. The plates were washed five times, air-dried and counted in a scintillation counter. Figs. 6a-e show the results of these experiments performed with representative anti-HER-3 antibodies of the invention and demonstrate that the antibodies of the invention are capable of specifically reducing the binding of [^{125}I]-α-HRG/[^{125}I]-β-HRG to cells expressing endogenous HER-3.

EXAMPLE 13: Inhibition of ligand-induced HER-3 phosphorylation by human anti-HER-3 antibodies of the invention

ELISA experiments were performed in order to investigate whether the antibodies of the invention are able to block ligand β-HRG-mediated activation of HER-3. Ligand-mediated HER-3 activation was detected by increased receptor tyrosine phosphorylation.

Day 1: 1 x 96 well dish was coated with 20 µg/ml Collagen I in 0,1 M acetic acid for 4 hr at 37 °C. $2.5x10^5$ cells were seeded in normal growth medium

Day 2: Cells were starved in 100 µl serum free medium for 24 hr.

Day 3: Cells were preincubated with 10 µg/ml anti-HER-3 mAbs for 1 hr at 37 °C and then treated with 30 ng/ml β-HRG-EGF domain (R&D Systems) for 10 min. Medium was flicked out and cells were fixed with 4 % formaldehyde solution in PBS for 1 hr at room temperature. 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 and incubated for 20 min at room temperature, then blocked with NET-Gelantine for 5 hr at 4 °C. Primary antibody phospho-HER-3 (Tyr1289) (polyclonal rabbit; Cell signaling #4791; 1:300) was added overnight at 4 °C.

Day 4: The plate was washed 3x with wash buffer, then incubated with anti-rabbit-POD diluted 1:3000 in PBS - 0.5 % BSA was added to each well and incubated for 1.5 hr at room temperature. The plate was washed 3x with wash buffer and once with PBS. Tetramethylbenzidine (TMB, Calbiochem) was added and monitored at 650 nm. The reaction was stopped by addition of 100 µl 250 nM HCl and the absorbance was read at 450 nm with a reference wavelength of 650 nm using a V_{max} plate reader (Thermo Lab Systems).

Fig. 7a shows representative results of this experiment, demonstrating that

anti-HER-3 antibodies of the invention were able to reduce ligand-mediated HER-3 activation as indicated by decreased receptor tyrosine phosphorylation. Data are shown as percent reduction by therapeutic antibodies relative to a control antibody.

To test potency of mAb U1-53 to inhibit ligand induced HER-3 activation, MCF-7 cells were starved for 24 hr, incubated with mAb U1-53 for 1 hr at 37 °C and stimulated with 10 nM HRG-β for 10 min. Lysates were transferred to 1B4 (mouse anti-HER-3 mAb) ELISA plates and phosphorylation of HER-3 was analysed with antibody 4G10. As shown in Fig. 7b phosphorylation of HER-3 was almost completely inhibited in a dose dependent manner with an IC50 of 0.14 nM.

EXAMPLE 14: Inhibition of ligand-induced p42/p44 MAP-Kinase phosphorylation by human anti-HER-3 antibodies of the invention

Next ELISA experiments were performed in order to investigate whether the antibodies of the invention are able to block ligand β-HRG-mediated activation of p42/p44 MAP-Kinase. Ligand-mediated HER-3 activation was detected by increased protein (Thr202/Tyr204) phosphorylation.

Day 1: 1 x 96 well dish was coated with 20 µg/ml Collagen I in 0,1 M acetic acid for 4 hr at 37 °C. $3x10^5$ cells were seeded in normal growth medium

Day 2: Cells were starved in 100 µl serum free medium for 24 hr.

Day 3: Cells were preincubated with 5 µg/ml anti-HER-3 mAbs for 1 hr at 37 °C and then treated with 20 ng/ml β-HRG-EGF domain (R&D Systems) for 10 min. Medium was flicked out and cells were fixed with 4 % formaldehyde solution in PBS for 1 hr at room temperature. 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 and incubated for 20 min at room temperature, then blocked with PBS/0.5 %

BSA for 5 hr at 4 °C. Primary antibody phospho-p44/p42 MAP Kinase (Thr202/Tyr204) (polyclonal rabbit; Cell signaling #9101; 1:3000) was added overnight at 4 °C.

Day 4: The plate was washed 3x with wash buffer, then incubated with anti-rabbit-HRP diluted 1:5000 in PBS - 0.5 % BSA was added to each well and incubated for 1.5 hr at room temperature. The plate was washed 3x with wash buffer and once with PBS. Tetramethylbenzidine (TMB, Calbiochem) was added and monitored at 650 nm. The reaction was stopped by addition of 100 µl 250 nM HCl and The absorbance was read at 450 nm with a reference wavelength of 650 nm using a V_{max} plate reader (Thermo Lab Systems).

Fig. 8 shows representative results of this experiment. The antibodies of the invention were able to reduce ligand-mediated p42/p44 MAP-Kinase activation as indicated by decreased phosphorylation. Data are shown as percent reduction by therapeutic antibodies relative to a control antibody.

EXAMPLE 15: Inhibition of β-HRG-induced phospho-AKT phosphorylation by human anti-HER-3 antibodies of the invention

In the following ELISA experiment we investigated whether the anti-HER-3 antibodies of the invention are able to block ligand β-HRG-mediated activation of AKT-Kinase. Ligand-mediated AKT activation was detected by increased protein (Ser473) phosphorylation.

Day 1: 1 x 96 well dish was coated with 20 µg/ml Collagen I in 0,1 M acetic acid for 4 hr at 37 °C. $3x10^5$ cells were seeded in normal growth medium

Day 2: Cells were starved in 100 µl serum free medium for 24 hr.

Day 3: Cells were preincubated with 5 µg/ml anti-HER-3 mAbs for 1 hr at 37 °C and then treated with 20 ng/ml β-HRG-EGF domain (R&D Systems)

for 10 min. Medium was flicked out and cells were fixed with 4 % formaldehyde solution in PBS for 1 hr at room temperature. 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 and incubated for 20 min at room temperature, then blocked with PBS/0.5 % BSA for 5 hr at 4 °C. Primary antibody phospho-Akt (Ser473) (polyclonal rabbit; Cell signaling #9217; 1:1000) was added overnight at 4 °C.

Day 4: The plate was washed 3x with wash buffer, then incubated with anti-rabbit-HRP diluted 1:5000 in PBS-0.5 % BSA was added to each well and incubated for 1.5 hr at room temperature. The plate was washed 3x with wash buffer and once with PBS. Tetramethylbenzidine (TMB, Calbiochem) was added and monitored at 650 nm. The reaction was stopped by addition of 100 µl 250 nM HCl and The absorbance was read at 450 nm with a reference wavelength of 650 nm using a Vmax plate reader (Thermo Lab Systems).

Fig. 9 shows representative results of this experiment. The anti-HER-3 antibodies of the invention were able to reduce β-HRG-mediated AKT as indicated by decreased phosphorylation. Data are shown as percent reduction by therapeutic antibodies relative to a control antibody.

EXAMPLE 16: Inhibition of α-HRG/β-HRG-mediated MCF7 cell proliferation by human anti-HER-3 antibodies of the invention

In vitro experiments were conducted in order to determine the ability of the antibodies of the invention to inhibit HRG-stimulated cell proliferation. 2000 MCF7 cells were seeded in FCS-containing medium on 96-well plates overnight. Cells were preincubated in quadruplicates with antibody diluted in medium with 0.5 % FCS for 1 hr at 37 °C. Cells were stimulated with 30 ng/ml α- or 20 ng/ml β-HRG (R&D Systems) by adding ligand directly to antibody solution and were then left to grow for 72 hr. AlamarBlue™ (BIOSOURCE) was added and incubated at 37 °C in the dark. Absorbance

was measured at 590 nm every 30 min. The data were taken 90 min after addition of alamar blue. The results as indicated in Fig. 10 show that representative antibodies of the invention inhibit HRG-induced cell growth in human cancer cells. Data are shown as percent reduction by therapeutic antibodies relative to a control antibody.

EXAMPLE 17: Inhibition of β-HRG-induced MCF7 cell migration by human anti-HER-3 antibodies of the invention

Transmigration experiments were performed in order to investigate whether the antibodies of the invention block cell migration. Serum-starved MCF7 cells were preincubated by adding the indicated amount of antibody to the cell suspension and incubating both for 45 min at 37 °C. 500 µl cell suspension (50,000 cells) was then placed in the top chamber of collagen I-coated transwells (BD Falcon, 8 µm pores). 750 µl medium (MEM, amino acids, Na-pyruvate, Pen.-Strept., 0,1 % BSA, without fetal calf serum) alone or containing the ligands β-HRG-EGF domain (R&D Systems) were used in the bottom chamber. Cells were left to migrate for 8 hr at 37 °C and were stained with DAPI.

Stained nuclei were counted manually; percent inhibiton was expressed as inhibition relative to a control antibody.

Fig. 11 shows the result of the experiment demonstrating that representative anti-HER-3 antibodies of the invention reduce HRG-induced cell migration.

EXAMPLE 18: Colony formation assay (soft agar assay)

Soft agar assays were conducted in order to investigate the ability of the anti-HER-3 antibodies of the invention to inhibit anchorage independent cell growth. The soft agar colony formation assay is a standard *in vitro* assay to test for transformed cells, as only such transformed cells can grow in soft agar.

750 to 2000 cells (depending on the cell line) were preincubated with indicated antibodies at 10 µg/ml in IMDM medium (Gibco) for 30 min and resuspended in 0.4 % Difco noble agar. The cell suspension was plated on 0.75 % agarose underlayer containing 20 % FCS in quadruplicate in a 96-well plate. Colonies were allowed to form for 14 days and were then stained with 50 µl MTT (0.5 mg/ml in PBS) overnight. Figs. 12 a-i show the results of these experiments performed with three representative antibodies of the invention. These results demonstrate that anti-HER-3 antibodies of the invention reduce anchorage independent cell growth of MDA-MB361 and NCI-ADR breast cancer cells (Fig. 12a,b), MKN-28 gastric cancer (Fig. 12c), HT144 melanoma cells (Fig. 12d), Skov3 ovary carcinoma cells (Fig. 12e), PPC-1 prostate cancer cells (Fig. 12f),BX-PC3 pancreas cancer cells (Fig. 12g), A431 epidermoid carcinoma cells (Fig. 12h) and lung carcinoma cells (Fig. 12i). Colonies were counted with a Scanalyzer HTS camera system (Lemnatec, Wuerselen).

EXAMPLE 19: Human anti-HER-3 antibodies inhibit human breast carcinoma growth in nude mice

The anti-tumor efficacy of therapeutic antibodies is often evaluated in human xenograft tumor studies. In these studies, human tumors grow as xenografts in immunocompromised mice and therapeutic efficacy is measured by the degree of tumor growth inhibition. In order to determine, if the anti-HER-3 antibodies of the invention interfere with tumor growth of human breast cancer cells in nude mice, $5x10^6$ T47D cells were implanted in female NMRI nude/nude mice. Tumors were subcutaneous, grown on the back of the animal. Treatments began when tumors reached a mean volume of 20 mm^3; eight days post implantation. Prior to first treatment, mice were randomized and statistical tests performed to assure uniformity in starting tumor volumes (mean, median and standard deviation) across treatment groups. Treatment started with a loading dose of 50 mg/kg followed by 25 mg/kg injections once a week by intraperitoneal injection. A control arm received doxorubicin (pharmaceutical grade). All animals were supplemented with 0.5

mg/kg/week oestrogen injected i.p.

Details of the treatment groups are given below.

Gr.	N	1st Compound	Loading (mg/kg)	Weekly dose (mg/kg)	Route	Schedule
1.	10	PBS		--	i.p.	once/week
2.	10	doxorubicin		8mg/kg	i.v.	once/week*
3.	10	U1-53	50mg/kg 20ml/kg	25mg/kg 10ml/kg	i.p.	once/week

* doxorubin treatment as described by Boven *et al.*, Cancer Research, 1992.

Data for median tumor volume (Fig. 13) demonstrated that administration of an anti-HER-3 antibody of the invention resulted in reduction of tumor growth.

EXAMPLE 20: Human anti-HER-3 antibodies inhibit human pancreatic tumor growth in SCID mice

To test the therapeutic potential of anti-HER3 antibodies in other solid tumor types the anti-HER-3 antibodies, U1-53 and U1-59, were tested in mice with established tumors derived from the human pancreatic tumor cell line BxPC3. As controls sets of mice treated with either the vehicle control, PBS, or the established therapeutic antibody, Erbitux, were included. $5x10^6$ BxPC3 cells were inoculated subcutaneously without Matrigel into CB17 SCiD mice. Mice bearing established tumors with a mean volume of $140mm^2$ received 50mg/kg of U1-53, U1-59, Erbitux or the equivalent volume of PBS via intraperitoneal injection. Thereafter the mice received once weekly 25mg/kg injections for the duration of the study.

The results for this experiment are shown in Fig. 14. U1-53 and U1-59 reduced the growth of the human pancreatic tumors in a cytostatic fashion. Notably, in this experiment, U1-53 and U1-59 were more effective than the EGFR-targeting antibody Erbitux at delaying tumor growth. These data demonstrated the therapeutic efficacy of anti-HER-3 antibodies of the

invention in comparison to a benchmark therapeutic agent.

EXAMPLE 21: Combining the human anti-HER-3 antibodies with anti-EGFR antibodies increases anti-tumor activity

The monotherapy of hyperproliferative diseases with targeted antibodies is often hampered by problems such as, on the one hand, the development of resistance to drugs, and on the other hand, a change in the antigenicity. For example, loss of antigenicity after prolonged treatment may render tumor cells insensitive to therapeutic antibodies, since those tumor cells that do not express or have lost the targeted antigen have a selective growth advantage. These problems might be evaded by using the antibodies of the invention in combination with a therapeutic antibody that targets a different receptor on the tumor cells, or another antineoplastic agent. Intervening in multiple signaling pathways or even related pathways but at multiple intervention steps might also provide therapeutic benefit. These combined treatment modalities are likely to be more efficacious, because they combine two anti-cancer agents, each operating via a different mechanism of action.

In order to demonstrate the feasibility of the anti-HER-3 antibodies of the invention, U1-53 and U1-59, as suitable combination agents, we compared monotherapeutic administrations of U1-53 or U1-59 with those in which either U1-53 or U1-59 was combined with the anti-EGR specific antibody, Erbitux. $5x10^6$ BxPC3 cells were inoculated subcutaneously with Matrigel into CB17 SCID mice. After tumor volumes had reached 200 mm^3, mice were randomized into individual treatment groups. Weekly intraperitoneal administrations of U1-53, U1-59 and Erbitux as single agents or combinations of either anti-HER3 antibodies with Erbitux or as a cocktail of two anti HER-3 antibodies were performed. All antibodies were dosed at a single loading doses of 50 mg/kg/week, followed by weekly injections of 25 mg/kg for six weeks. Control arms received bi-weekly administrations of Gemcitabine (120 mg/kg), weekly pooled human IgG or weekly vehicle (PBS) injections. The regimens are detailed below.

Gr.	N	Compound	Loading dose (mg/kg)	Weekly dose (mg/kg)	Route	Schedule
4.	12	PBS	20ml/kg	10ml/kg	q7d	i.p.
5.	12	Pooled human IgG	50mg/kg	25mg/kg	q7d	i.p.
6.	12	U1-53	50mg/kg	25mg/kg	q7d	i.p.
7.	12	U1-59	50mg/kg	25mg/kg	q7d	i.p.
8.	12	Erbitux	50mg/kg	25mg/kg	q7d	i.p.
9.	12	U1-53 + Erbitux	25mg/kg each	12.5mg/kg each	q7d	i.p.
10.	12	U1-59 + Erbitux	25mg/kg each	12.5mg/kg each	q7d	i.p.
11.	12	U1-53 + U1-59	25mg/kg each	12.5mg/kg each	q7d	i.p.
12.	12	Gemcitabine	none	120 mg/kg	2x weekly	ip

The results for this experiment are shown in Fig. 15. Antibodies U1-53 and U1-59, when administered as single agents, delayed the growth of the human pancreatic tumors to the same degree as Gemcitabine, which is often used as a standard anti-pancreatic cancer chemotherapy. Co-administration of Erbitux with U1-53 or U1-59 resulted in a significantly greater reduction of tumor growth than observed with either single agent administration of U1-53, U1-59 or Erbitux. Thus, a beneficial therapeutic response can be achieved by combining the anti-HER-3 antibodies of the invention with suitable antibodies that target separate tumor antigens.

In summary, the anti-HER-3 antibodies of the invention have potent therapeutic efficacy against human tumors in vivo. They can be effectively combined with other anti-neoplastic therapeutics for increased anti-tumor activity.

EXAMPLE 22: Human anti-HER-3 antibodies inhibit human melanoma tumor growth in nu/nu mice

Members of the erbB family of receptors, including Her3, are abnormally expressed in a large variety of epithelial cancers and they are known to play important roles in the growth and survival of many these solid tumors. These tumors include melanomas, head and neck squamous cell cancers, non-small cell lung cancers and prostate, glioma, gastric, breast, colorectal, pancreatic, ovarian cancers. In order to verify, that the anti-Her3 antibodies of the invention are not restricted in their anti-cancer activity to individual tumor types, e.g. pancreatic cancers (see Example 21), but can be used as therapeutics against many HER-3-dependent tumors, we tested U1-53 and U1-59 in additional xenograft studies. One example is shown in Fig. 16. 5 x 10^5 human melanoma cells, HT144, were injected subcutaneously into CB17 SCID mice, followed by immediate subsequent intraperitoneal injection of 50mg/kg of U1-53 and U1-59, the equivalent volume of PBS or Dacarbacin (DITC) at 200mg/kg. Thereafter, mice received 25mg/kg of U1-53 or U1-59 once weekly, whereas DITC was given once every two weeks at 200mg/kg.

The median tumor volumes from each treatment group are shown in Figure 16. Administration of the antibodies of the invention resulted in growth reduction of the human melanomas when compared to tumors that had been treated with the vehicle control. These results demonstrate that the antibodies of the invention are not restricted in their therapeutic potential and target a wide variety of HER-3 expressing cancers.

EXAMPLE 23: Human anti-HER-3 antibodies inhibit growth of colon carcinoma xenografts in mice

HT-29 human colon carcinoma cells were suspended in medium with 2:1 ratio of Matrigel to a final concentration of 10 x 10^6 cells/ml. 0.2 ml of cell suspension were injected s.c. into the right flank of 4-5-week-old CD1 nu/nu mice. A total of 95 mice were used.

The mice were randomly assigned to control and treatment groups. The treatment started on the same day. Duration of treatment was 29 days. Upon completion of the study, three tumours per group were collected 3 hours after administration of treatment. The tumours were fast-frozen and kept at -80 °C.

The following treatment protocol was carried out:

Control group: non-specific human IgG 25 mg/kg, twice weekly, intraperitoneal

Treatment group: antibody U1-53, 25 mg/kg, twice weekly, intraperitoneal

Treatment group: antibody U1-7, 25 mg/kg, twice weekly, intraperitoneal

Treatment group: antibody U1-59, 25 mg/kg, twice weekly, intraperitoneal

Treatment group 5-FU: 5-fluorouracil, 50 mg/kg, 9d x 5, intraperitoneal

The median tumor volumes from each group are shown in Fig. 17. Administration of the antibodies of the invention resulted in growth reduction of the HT-29 colon carcinoma tumors when compared to tumors that had been treated with non-specific human IgG1.

EXAMPLE 24: Human anti-HER-3 antibodies inhibit lung cancer growth in mice

Calu-3 human non-small cell lung cancer cells were suspended in medium with 1:1 ratio of Matrigel to a final concentration of 5×10^6 cells/ml. 0.05 ml of cell suspension were injected s.c. into the right flank of 9-week-old female CB17 scid mice. A total of 60 mice were used.

The mice were randomly selected to control and treatment groups. Treatment started on the same day. The duration of treatment was 32 days.

The following treatment protocol was carried out:

PBS vehicle group

hG control group: non-specific human IgG: 25 mg/kg, twice weekly, intraperitoneal

Treatment group antibody U1-53, 25 mg/kg, twice weekly, intraperitoneal

Treatment group antibody U1-7, 25 mg/kg, twice weekly, intraperitoneal

Treatment group antibody U1-59, 25 mg/kg, twice weekly, intraperitoneal

The median tumor volumes from each control and treatment group are shown in Fig. 18. Administration of the antibodies of the invention resulted in growth reduction of the human non-small lung cancer xenografts when compared to tumors that had been treated with the PBS vehicle control or non-specific human IgG.

EXAMPLE 25: Human anti-HER-3 antibodies inhibit human pancreatic tumor growth in Balb/C-mice

Human pancreatic BxPC3 tumor cells were suspended in medium with a 2:1 ratio of Matrigel to a final concentration of 5 x 10^6 cells per ml. 0.2 ml of cell suspension were injected s.c. into the right flank of 5-7- week-old female BalbC nu/nu mice. A total of 100 mice were used.

The mice were randomly distributed into control and treatment groups. The treatment started on the same day. The treatment duration was 27 days.

The following treatment protocol was carried out:

hIgG control group: non-specific human IgG2, 25 mg/kg, twice weekly, intraperitoneal

Treatment group antibody U1-53, 25 mg/kg, twice weekly, intraperitoneal

Treatment group antibody U1-7, 25 mg/kg, twice weekly, intraperitoneal

Treatment group antibody U1-59, 25 mg/kg, weekly, intraperitoneal

Gemzar treatment group, gemcitabine, 80 mg/kg, weekly, intraperitoneal

The median tumor volumes from each control and treatment group are shown in Fig. 19. Administration of the antibodies of the invention resulted in growth reduction of the human pancreatic tumors when compared to tumors that had been treated with non-specific human IgG or with Gemzar.

The inhibition of HER-3 in the human pancreatic tumors could also be shown in a pharmacodynamic experiment. The BxPC3 tumor xenografts were grown as described above. 3 mice were treated with 500 µg of an IgG1 control antibody and 3 mice were treated with 500 µg of the anti-HER-3 antibody U1-59. The mice were treated on day 1 and day 4 and then sacrificed on day 5 to measure the antibody-dependent inhibition of HER-3 phosphorylation (pHER-3).

The tumors were homogenized in a standard RIPA buffer with protease inhibitors. 50 µg clear lysate was separated on a 4-20 % Tris-glycine gel, transferred onto a nitrocellulose membrane and blocked in 3 % bovine serum albumin (BSA). Immunoblotting was performed using an anti-pHER-3 antibody (antibody 21D3, Cell Signaling technology). An anti-actin antibody (AB a-2066, Sigma) was used as a control.

The expression was detected by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). The images were captured with the Versadoc 5000 Imaging System (BioRad, Hercules, CA).

The results are shown in Fig. 20. After administration of the human anti-HER-3-antibody U1-59 phosphorylation of HER-3 was no longer detectable. Thus, the antibodies of the invention are capable of significantly reducing HER-3 activation in human pancreatic tumor cells.

EXAMPLE 26: Use of anti-HER-3 antibodies of the invention as a diagnostic agent

Anti-HER-3 mAb can be used in the diagnostic of malignant diseases. HER-3 is expressed on tumor cells in a very distinct way compared to normal tissue and, therefore, an expression analysis of HER-3 would assist in the primary diagnosis of solid tumors, staging and grading of solid tumors, assessment of prognostic criteria for proliferative diseases and neoplasias and risk management in patients with HER-3 positive tumors.

A. Detection of HER-3 antigen in a sample

An Enzyme-Linked Immunosorbent Assay (ELISA) for the detection of HER-3 antigen in a sample is developed. In the assay, wells of a microtiter plate, such as a 96-well microtiter plate or a 384-well microtiter plate, are adsorbed for several hr with a first fully human monoclonal antibody directed against the HER-3 antigen. The immobilized antibody serves as a capture antibody for any of the HER-3 antigen that may be present in a test sample. The wells are rinsed and treated with a blocking agent such as milk protein or albumin to prevent nonspecific adsorption of the analyte.

Subsequently the wells are treated with a test sample suspected of containing the HER-3 antigen, or with a solution containing a standard amount of the HER-3 antigen. Such a sample is, for example, a serum

sample from a subject suspected of having levels of circulating HER-3 antigen considered to be diagnostic of a pathology. After rinsing away the test sample or standard, the wells are treated with a second fully human monoclonal anti-HER-3 antibody of the invention that is labelled by conjugation with biotin. The labeled anti-HER-3 antibody serves as a detecting antibody. After rinsing away excess secondary antibody, the wells are treated with avidin-conjugated horseradish peroxidase (HRP) and a suitable chromogenic substrate. The concentration of the HER-3 antigen in the test samples is determined by comparison with a standard curve developed from the standard samples.

B. Detection of HER3-antigen in Immunohistochemistry (IHC)

In order to determine HER3-antigen in tissue sections by IHC, Paraffin-embedded tissues are first deparaffinized in xylene for 2 x 5 min and then hydrated with 100% Ethanol 2 x 3 min, 95% Ethanol 1 min and rinsed in distilled water. Antigenic epitopes masked by formalin-fixation and paraffin-embedding are exposed by epitope unmasking, enzymatic digestion or saponin. For epitope unmasking paraffin sections are heated in a steamer, water bath or microwave oven for 20-40 min in a epitope retrieval solution as for example 2N HCl solution (pH 1.0). In the case of an enzyme digestion, tissue sections are incubated at 37°C for 10-30 minutes in different enzyme solutions such as protienase K, trypsin, pronase, pepsin etc.

After rinsing away the epitope retrieval solution or excess enzyme, tissue sections are treated with a blocking buffer to prevent unspecific interactions. The primary antibody is incubated at appropriate dilutions in dilution buffer for 1 hour at room temperature or overnight. Excess primary antibody is rinsed away and sections are incubated in peroxidase blocking solution for 10 min at room temperature. After another washing step, tissue sections are incubated with a secondary antibody labelled with a group that might serve as an anchor for an enzyme. Examples therefore are biotin labelled secondary antibodies that are recognized by streptavidin coupled

horseradish peroxidase. Detection of said antibody/enzyme complex is achieved by incubating with a suitable chromogenic substrate.

C. Determination of HER-3 antigen concentration in serum of patients

A sandwich ELISA is developed to quantify HER-3 levels in human serum. The two fully human monoclonal anti-HER-3 antibodies used in the sandwich ELISA, recognized different domains on the HER-3 molecule and do not compete for binding, for example, see Example 8. The ELISA is performed as follows: 50 µl of capture anti-HER-3 antibody in coating buffer (0.1 M $NaHCO_3$, pH 9.6) at a concentration of 2 µg/ml were coated on ELISA plates (Fisher). After incubation at 4 °C overnight, the plates are treated with 200 µl of blocking buffer (0.5 % BSA, 0.1 % Tween 20, 0.01 % Thimerosal in PBS) for 1 hr at 25 °C. The plates were washed (3x) using 0.05 % Tween 20 in PBS (washing buffer, WB). Normal or patient sera (Clinomics, Bioreclaimation) are diluted in blocking buffer containing 50 % human serum. The plates are incubated with serum samples overnight at 4 °C, washed with WB, and then incubated with 100 µl/well of biotinylated detection anti-HER-3 antibody for 1 hr at 25 °C. After washing, the plates are incubated with HRP-Streptavidin for 15 min, washed as before, and then treated with 100 µl/well of o-phenylenediamine in H_2O_2 (Sigma developing solution) for color generation. The reaction is stopped with 50 µl/well of H_2SO_4 (2 M) and analyzed using an ELISA plate reader at 492 nm. The concentration of HER-3 antigen in serum samples is calculated by comparison to dilutions of purified HER-3 antigen using a four parameter curve fitting program.

Staging of cancer in a patient

Based on the results set forth and discussed under items A, B and C., through use of the present invention, it is possible to stage a cancer in a subject based on expression levels of the HER-3 antigen. For a given type of cancer, samples of blood are taken from subjects diagnosed as being at

various stages in the progression of the disease, and/or at various points in the therapeutic treatment of the cancer. The concentration of the HER-3 antigen present in the blood samples is determined using a method that specifically determines the amount of the antigen that is present. Such a method includes an ELISA method, such as the method described under items A. and B. Using a population of samples that provides statistically significant results for each stage of progression or therapy, a range of concentrations of the HER-3 antigen that may be considered characteristic of each stage is designated.

In order to stage the progression of the cancer in a subject under study, or to characterize the response of the subject to a course of therapy, a sample of blood is taken from the subject and the concentration of the HER-3 antigen present in the sample is determined. The concentration so obtained is used to identify in which range of concentrations the value falls. The range so identified correlates with a stage of progression or a stage of therapy identified in the various populations of diagnosed subjects, thereby providing a stage in the subject under study.

EXAMPLE 27: Uses of anti-HER-3 antibodies and antibody conjugates of the invention for treatment or prevention of hyperproliferative diseases

Many solid tumors are driven by HER family mediated signalling and it has been demonstrated that HER-3 is a crucial partner through complex formation between HER-1, HER-2 and HER-4. Therefore, a reduction or elimination of HER-3 mediated signaling would impact all other HER family members and impair cell signaling leading to a wide window of therapeutic interventions and potential in combination therapy with other targeted agents, biologics and cytotoxic agents. Thus, anti-HER-3 antibodies of the invention can be used for treatment of certain hyperproliferative or HER-3 associated disorders, that are based on a number of factors as for example HER-3 expression. Tumor types as breast cancer, gastrointestinal cancer, pancreas cancer, prostate cancer, ovarian cancer, stomach cancer,

endometrial cancer, salivary gland cancer, lung cancer, kidney cancer, colon cancer, colorectal cancer, thyroid cancer, bladder cancer, glioma, melanoma, other HER-3 expressing or overexpressing cancers, appear to present preferred indications, but indications are not limited to those on the preceding list. In addition the following groups of patients will benefit from anti-HER-3 directed mAb treatment:

- Patients with resistance to anti-HER-2 mAb treatment
- Patients not eligible for the treatment with anti-HER-2 mAb
- Patients with resistance to anti-HER-1 mAb or small molecule anti-EGFR inhibitor
- Patients with non-small cell lung cancer resistant to erlotinib or gefitinib.

Anti-HER-3 antibodies of the invention would be used either as a monotherapy or in combination with one or more agent in a so called "combination therapy". Said combination therapy may include, but is not limited to, agents that were specified previously in the invention. Combination therapy with anti-HER3 antibodies and other agents may extend patient survival, time to tumor progression or quality of patient life. Protocol and administration design will address therapeutic efficacy as well as the ability to reduce the usual doses of standard therapies, as for example chemo- or radiation therapy.

Treatment of humans with anti-HER-3 antibodies of the invention

To determine the *in vivo* effects of anti-HER-3 antibody treatment in human patients with tumors, such human patients are injected over a certain amount of time with an effective amount of anti-HER-3 antibody of the invention. At periodic times during the treatment, the human patients are monitored to determine whether their tumors progress, in particular, whether the tumors grow and metastasize.

A tumor patient treated with the anti-HER-3 antibodies of the invention has a lower level of tumor growth and/or metastasis compared to the level of tumor growth and metastasis in tumor patients treated with the current standard of

care therapeutics.

Treatment with anti-HER-3 antibody conjugates of the invention

To determine the *in vivo* effects of anti-HER-3 antibody conjugates of the invention, human patients or animals exhibiting tumors are injected over a certain amount of time with an effective amount of anti-HER-3 antibody conjugate of the invention. For example, the anti-HER-3 antibody conjugate administered is DM1-anti-HER-3 antibody conjugate, an auristatin-anti-HER-3 antibody conjugate or radioisotope-anti-HER-3 antibody conjugate. At periodic times during the treatment, the human patients or animals are monitored to determine whether their tumors progress, in particular, whether the tumors grow and metastasize.

A human patient or animal exhibiting tumors and undergoing treatment with, for example, DM1-anti-HER-3 antibody or radioisotope-anti-HER-3 antibody conjugates has a lower level of tumor growth and metastasis when compared to a control patient or animal exhibiting tumors and undergoing treatment with an alternate therapy. Control DM1-antibodies that may be used in animals include conjugates comprising DM1 linked to antibodies of the same isotype of the anti-HER-3 antibodies of the invention, but more specifically, not having the ability to bind to HER-3 tumor antigen. Control radioisotope-antibodies that may be used in animal tests include conjugates comprising radioisotope linked to antibodies of the same isotype of the anti-HER-3 antibodies of the invention, but more specifically, not having the ability to bind to HER-3 tumor antigen. Note: the control conjugates would not be administered to humans.

GENERAL REMARKS

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the construct deposited, since the deposited

embodiment is intended as a single illustration of certain objects of the invention and any constructs that are functionally equivalent are within the scope of this invention. The deposit of material herein does not constitute an admission that the written description herein contained is inadequate to enable the practice of any object of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustrations that it represents.

The foregoing description and Examples detail certain preferred embodiments of the invention and describes the best mode contemplated by the inventors. It will be appreciated, however, that no matter how detailed the foregoing may appear in text, the invention may be practiced in many ways and the invention should be construed in accordance with the appended claims and any equivalents thereof.

Furthermore, unless otherwise defined, scientific and technical terms used in connection with the present invention shall have the meanings that are commonly understood by those of ordinary skill in the art. Moreover, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. Generally, nomenclatures utilized in connection with, and techniques of, cell and tissue culture, molecular biology, and protein and oligo- or polynucleotide chemistry and hybridization described herein are those well known and commonly used in the art. Standard techniques are used for recombinant DNA, oligonucleotide synthesis, and tissue culture and transformation (e.g. electroporation, lipofection). Enzymatic reactions and purification techniques are performed according to manufacturer's specifications or as commonly accomplished in the art or as described herein. The foregoing techniques and procedures are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification. See e.g. Sambrook *et al. Molecular Cloning: A Laboratory Manual* (3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (2001)), which is

incorporated herein by reference. The nomenclatures utilized in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well known and commonly used in the art. Standard techniques are used for chemical syntheses, chemical analyses, pharmaceutical preparation, formulation, and delivery, and treatment of patients.

INCORPORATION BY REFERENCE

All references cited herein, including patents, patent applications, papers, text books, and the like, including the references cited therein, are hereby incorporated herein by reference in their entirety.

SEQUENCE LISTING

Antibody U1-39

1 Heavy Chain DNA:
GAGGTGCAGCTGGTGGAGTCTGGAGGAGGCTTGATCCAGCCTGGGGGGTCCCTGAGACTC
TCCTGTGCAGCCTCTGGGTTCACCGTCAGTAGCAACTACATGAGCTGGGTCCGCCAGGCT
CCAGGGAAGGGGCTGGATTGGGTCTCAGTTATTTATAGCGGTGGTAGCACATACTACGCA
GACTCCGTGAAGGGCCGATTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTT
CAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTGTATTACTGTGCGAGAGGGCAGTGG
CTGGACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCA

2 Heavy Chain Protein:
EVQLVESGGGLIQPGGSLRLSCAASGFTVSSNYMSWVRQAPGKGLDWVSVIYSGGSTYYA
DSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARGQWLDVWGQGTTVTVSS

3 Light Chain DNA:
GATATTGTGATGACTCAGTCTCCACTCTCCCTGCCCGTCACCCCTGGAGAGCCGGCCTCC
ATCTCCTGCAGGTCAAGTCAGAGCCTCCTGCATAGTAATGGATACAACTATTTGGATTGG
TACCTGCAGAGGCCAGGGCAGTCTCCACAACTCCTGTTCTATTTGGGTTTTCATCGGGCC
TCCGGGGTCCCTGACAGGTTCAGTGGCAGTGGATCAGGCACAGATTTTACACTGAAAATC
AGCAGAGTGGAGGCTGAGGATGTTGGGGTTTATTACTGCAGGCAAGCTCTACAAACTCCG
CTCACTTTCGGCGGAGGGACCAAGGTGGAGATCAAA

4 Light Chain Protein:
DIVMTQSPLSLPVTPGEPASISCRSSQSLLHSNGYNYLDWYLQRPGQSPQLLFYLGFHRA
SGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCRQALQTPLTFGGGTKVEIK

Antibody U1-40

5 Heavy Chain DNA:
CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCACAGACCCTGTCCCTC
ACCTGTACTGTCTCTGGTGGCTCCATCAGCAGTGGTGGTTACTACTGGAGCTGGATCCGC
CAGCACCCAGGGAAGGGCCTGGAGTGGATTGGGTACATCTATTCCAGTGGGAGCACCTAC
TACAACCCGTCCCTCAAGAGTCGAGTTACCATATCAGTAGACACGTCTAAGAACCAGTTC
TCCCTGAAGCTGAGCTCTGTGACTGCCGCGGACACGGCCGTGTATTACTGTGCGAGAGAT
AGGGAACTGGAACTTTACTACTACTACTACGGTATGGACGTCTGGGGCCAAGGGACCACG
GTCACCGTCTCCTC

6 Heavy Chain Protein:
QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGGYYWSWIRQHPGKGLEWIGYIYSSGSTY
YNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARDRELELYYYYYGMDVWGQGTT
VTVS

7 Light Chain DNA:
GATATTGTGATGACTCAGTCTCCACTCTCCCTGCCCGTCACCCCTGGAGAGCCGGCCTCC
ATCTCCTGCAGGTCTAGTCAGAGCCTCCTGTATAGTAATGGATACAACTATTTGGATTGG
TACCTGCAGAAGCCAGGGCAGTCTCCACAGCTCCTGATCTATTTGGGTTCTAATCGGGCC
TCCGGGGTCCCTGACAGGTTCAGTGGCAGTGGATCAGGCACAGATTTTACACTGAAAATC
AGCAGAGTGGAGGCTGAGGATGTTGGGATTTATTACTGCATGCAAGCTCTACAAACTCCG
CTCACTTTCGGCGGAGGGACCAAGGTGGAGATCAAA

8 Light Chain Protein:
DIVMTQSPLSLPVTPGEPASISCRSSQSLLYSNGYNYLDWYLQKPGQSPQLLIYLGSNRA
SGVPDRFSGSGSGTDFTLKISRVEAEDVGIYYCMQALQTPLTFGGGTKVEIK

Antibody U1-38

9 Heavy Chain DNA:
CAGATCACCTTGAAGGAGTCTGGTCCTACGCTGGTGAAACCCACACAGACCCTCACGCTG
ACCTGCACCTTCTCTCTGGGTTCTCACTCAGCACTAGTGGAGTGGGTGTGGGCTGGATCCGT
CAGCCCCCAGGAAAGGCCCTGGACTGGCTTGCACTCATTTATTGGAATGATGATAAGCGC

TACAGCCCATCTCTGAAGAGCAGGCTCACCATCACCAAGGACACCTCCAAAAACCAGGTG
GTCCTTACAATGACCAACATGGATCTTGTGGACACAGCCACATATTACTGTGTACACAGA
GACGAAGTTCGAGGGTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA

10 Heavy Chain Protein:
QITLKESGPTLVKPTQTLTLTCTFSGFSLSTSGVGVGWIRQPPGKALDWLALIYWNDDKR
YSPSLKSRLTITKDTSKNQVVLTMTNMDLVDTATYYCVHRDEVRGFDYWGQGTLVTVSS

11 Light Chain DNA:
GATGTTGTGATGACTCAGTCTCCACTCTCCCTGCCCGTCACCCTTGGACAGCCGGCCTCC
ATCTCCTGCAGGTCTAGTCAAAGCCTCGTATACAGTGATGGATACACCTACTTGCATTGG
TTTCAGCAGAGGCCAGGCCAATCTCCAAGGCGCCTTATTTATAAGGTTTCTAACTGGGAC
TCTGGGGTCCCAGACAGATTCAGCGGCAGTGGGTCAGGCACTGATTTCACACTGAAAATC
AGCAGGGTGGAGGCTGAGGATGTTGGGGTTTATTACTGCATGCAAGGTGCACACTGGCCG
ATCACCTTCGGCCAAGGGACACGACTGGAGATTAAA

12 Light Chain Protein:
DVVMTQSPLSLPVTLGQPASISCRSSQSLVYSDGYTYLHWFQQRPGQSPRRLIYKVSNWD
SGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCMQGAHWPITFGQGTRLEIK

Antibody U1-41

13 Heavy Chain DNA:
CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCACAGACCCTGTCCCTC
ACCTGCACTGTCTCTGGTGGCTCCATCAGCAGTGGTGGGTACTACTGGAGCTGGATCCGC
CAGCACCCAGGGAAGGGCCTGGAGTGGATTGGGTACATCTATTACAGTGGGAGCACCTAC
TACAACCCGTCCCTCAAGAGTCGAGTTACCATATCAGTAGACACGTCTAAGAACCAGTTC
TCCCTGAAGCTGAGCTCTGTGACTGCCGCGGACACGGCCGTGTATTTCTGTGCGAGAGAT
CGGGAACTTGAGGGTTACTCCAACTACTACGGTGTGGACGTCTGGGGCCAAGGGACCACG
GTCACCGTCTCCTC

14 Heavy Chain Protein:
QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGGYYWSWIRQHPGKGLEWIGYIYYSGSTY
YNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYFCARDRELEGYSNYYGVDVWGQGTT
VTVS

15 Light Chain DNA:
GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACC
ATCACTTGCCGGGCAAGTCAGGCCATTAGCAACTATTTAAATTGGTATCAGCAGAAACCA
GGGAAAGCCCCTAAGCTCCTGATCTATGCTGCATCCAGTTTGCAAAGTGGGGTCCCATCA
AGGTTCAGTGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGCAGCCTGCAGCCT
GAAGATTTTGCAACTTATTACTGTCAACAGAATAATAGTCTCCCGATCACCTTCGGCCAA
GGGACACGACTGGAGATTAAA

16 Light Chain Protein:
DIQMTQSPSSLSASVGDRVTITCRASQAISNYLNWYQQKPGKAPKLLIYAASSLQSGVPS
RFSGSGSGTDFTLTISSLQPEDFATYYCQQNNSLPITFGQGTRLEIK

Antibody U1-42

17 Heavy Chain DNA:
GAGGTGCAGCTGGTGCAGTCTGGAGCAGAGGTGAAAAAGCCCGGGGAGTCTCTGAAGATC
TCCTGTAAGGGTTCTGGATACAGCTTTACCAGCTACTGGATCGGCTGGGTGCGCCAGATG
CCCGGGAAAGGCCTGGAGTGGATGGGGATCATCTATCCTGGTGACTCTGATACCAGATAC
AGCCCGTCCTTCCAAGGCCAGGTCACCATCTCAGCCGACAAGTCCATCAGCACCGCCTAC
CTGCAGTGGAGCAGCCTGAAGGCCTCGGACACCGCCATGTATTACTGTGCGAGACATGAA
AACTACGGTGACTACAACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA

18 Heavy Chain Protein:
EVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWIGWVRQMPGKGLEWMGIIYPGDSDTRY
SPSFQGQVTISADKSISTAYLQWSSLKASDTAMYYCARHENYGDYNYWGQGTLVTVSS

19 Light Chain DNA:

GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTCGGAGACAGAGTCACC
ATCACTTGCCGGGCAAGTCAGAGCATTCGCAGCTATTTAAATTGGTATCAGCAGAAACCA
GGGAAAGCCCCTAAGCTCCTGATCTATGCTGCTTCCAGTTTGCAAAGTGGGGTCCCATCA
AGGTTCAGTGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGCAGTCTGCAACCT
GAAGATTTTGCACTTTACTGCTGTCAACAGAGTAACGGTTCCCCGCTCACTTTCGGCGGA
GGGACCAAGGTGGAGATCAAA

20 Light Chain Protein:
DIQMTQSPSSLSASVGDRVTITCRASQSIRSYLNWYQQKPGKAPKLLIYAASSLQSGVPS
RFSGSGSGTDFTLTISSLQPEDFALYCCQQSNGSPLTFGGGTKVEIK

Antibody U1-43

21 Heavy Chain DNA:
CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCACAGACCCTGTCCCTC
ACCTGCACTGTCTCTGGTGGCTCCATCAGCAGTGGTGGTTACTACTGGAGCTGGATCCGC
CAGCACCCAGGGAAGGGCCTGGAGTGGATTGGGTACATCTATTACAGTGGGAGCACCTAC
TACAACCCGTCCCTCAGGAGTCGAGTTACCATATCAGTAGACACGTCTAAGAACCAGTTC
TCCCTGAAGCTGAGCTCTGTGACTGCCGCGGACACGGCCGTGTATTACTGTGCGAGAGAT
AGAGAGAGAGAGTGGGATGATTACGGTGACCCCCAAGGTATGGACGTCTGGGGCCAAGGG
ACCACGGTCACCGTCTCCTC

22 Heavy Chain Protein:
QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGGYYWSWIRQHPGKGLEWIGYIYYSGSTY
YNPSLRSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARDRREREWDDYGDPQGMDVWGQG
TTVTVS

23 Light Chain DNA:
GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACC
ATCACTTGCCGGGCAAGTCAGAGCATTAGCAGCTATTTACATTGGTATCAGCAGAAACCA
GGGAAAGCCCCTAAGCTCCTGATCCATGCTGCATCCAGTTTACAAAGTGGGGTCCCATCA
AGGTTCAGTGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGTAGTCTGCAACCT
GAAGATTTTGCAACTTACTACTGTCAACAGAGTTACAGTAACCCGCTCACTTTCGGCGGA
GGGACCAAGGTGGAGATCCAA

24 Light Chain Protein:
DIQMTQSPSSLSASVGDRVTITCRASQSISSYLHWYQQKPGKAPKLLIHAASSLQSGVPS
RFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSNPLTFGGGTKVEIQ

Antibody U1-44

25 Heavy Chain DNA:
GAGGTGCAGCTGGTGCAGTCTGGAGCAGAGGTGAAAAAGCCCGGGGAGTCTCTGAAGATC
TCCTGTAAGGGTTCTGGATACAGCTTTACCAGCTACTGGATCGGCTGGGTGCGCCAGATG
CCCGGGAAAGGCCTGGAGTGGATGGGGATCATCTGGCCTGGTGACTCTGATACCATATAC
AGCCCGTCCTTCCAAGGCCAGGTCACCATCTCAGCCGACAAGTCCATCAGCACCGCCTAC
CTGCAGTGGAGCAGCCTGAAGGCCTCGGACACCGCCATGTATTACTGTGCGAGACATGAA
AACTACGGTGACTACAACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA

26 Heavy Chain Protein:
EVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWIGWVRQMPGKGLEWMGIIWPGDSDTIY
SPSFQGQVTISADKSISTAYLQWSSLKASDTAMYYCARHENYGDYNYWGQGTLVTVSS

27 Light Chain DNA:
GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTGGGAGACAGAGTCACC
ATCACTTGCCGGGCAAGTCAGAGCATTCGAAGTTATTTAAATTGGTATCAGCAGAAACCG
GGGAATGCCCCTAAACTCCTGATCTATGCTGCATCCAGTTTGCAAAGTGGGGTCCCATCA
AGGTTCAGTGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGCAGTCTGCAACCT
GAAGATTTTGCACTTTACTACTGTCAACAGAGTATCAGTTCCCCGCTCACTTTCGGCGGA
GGGACCAAGGTGGAGATCAAA

28 Light Chain Protein:
DIQMTQSPSSLSASVGDRVTITCRASQSIRSYLNWYQQKPGNAPKLLIYAASSLQSGVPS
RFSGSGSGTDFTLTISSLQPEDFALYYCQQSISSPLTFGGGTKVEIK

Antibody (U1-45)

29 Heavy Chain DNA:
CAGGTGCAGCTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCTGGGGCCTCAGTGAAGGTC
TCCTGCAAGGCTTCTGGATACACCTTCACCAGTTATGATATCAACTGGGTGCGACAGGCC
ACTGGACAAGGGCTTGAGTGGATGGGATGGATGAACCCTAACAGTGGTGACACTGGCTAT
GCACAGGTGTTCCAGGGCAGAGTCACCATGACCTGGAACACCTCCATAAGCACAGCCTAC
ATGGAACTGAGCAGCCTGAGATCTGAGGACACGGCCGTGTATTACTGTGCGAGATTTGGG
GATCTCCCGTATGACTACAGTTACTACGAATGGTTCGACCCCTGGGGCCAGGGAACCCTG
GTCACCGTCTCCTC

30 Heavy Chain Protein:
QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYDINWVRQATGQGLEWMGWMNPNSGDTGY
AQVFQGRVTMTWNTSISTAYMELSSLRSEDTAVYYCARFGDLPYDYSYYEWFDPWGQGTL
VTVS

31 Light Chain DNA:
GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACC
ATCACTTGCCGGGCAAGCCAGAGCATTAGCAGCTATTTAAATTGGTATCAGCAGAGACCA
GGGAAAGCCCCTAAGCTCCTGATCTATGCAGCATCCAGTTTGCAAAGTGGGGTCCCATCA
AGGTTCAGTGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGCAGTCTGCAACCT
GAAGATTTTGCAACTTACTACTGTCAACAGAGTTACAGTACCCCGCTCACTTTCGGCGGA
GGGACCAAGGTGGAGATCAAA

32 Light Chain Protein:
DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQRPGKAPKLLIYAASSLQSGVPS
RFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPLTFGGGTKVEIK

Antibody (U1-46

33 Heavy Chain DNA:
CAGGTACAGCTGCAGCAGTCAGGTCCAGGACTGGTGAAGCCCTCGCAGACCCTCTCACTC
ACCTGTGCCATCTCCGGGGACAGTGTCTCTAGCAACAGTGCTGCTTGGAACTGGATCAGG
CAGTCCCCATCGAGAGGCCTTGAGTGGCTGGGAAGGACATACTACAGGTCCAAGTGGTAT
AATGATTATGCAGTATCTGTGAAAAGTCGAATAACCATCAACCCAGACACATCCAAGAAC
CAGTTCTCCCTGCAGCTGAACTCTGTGACTCCCGAGGACACGGCTGTGTATTACTGTGCA
AGAGATCTCTACGATTTTTGGAGTGGTTATCCCTACTACTACGGTATGGACGTCTGGGGC
CAAGGGACCACGGTCACCGTCTCCTC

34 Heavy Chain Protein:
QVQLQQSGPGLVKPSQTLSLTCAISGDSVSSNSAAWNWIRQSPSRGLEWLGRTYYRSKWY
NDYAVSVKSRITINPDTSKNQFSLQLNSVTPEDTAVYYCARDLYDFWSGYPYYYGMDVWG
QGTTVTVS

Antibody U1-47

35 Heavy Chain DNA:
CAGGTACAGCTGCAGCAGTCAGGTCCAGGACTGGTGAAGCCCTCGCAGACCCTCTCACTC
ACCTGTGCCATCTCCGGGGACAGTGTCTCTAGCAACAGTGCTGCTTGGAACTGGATCAGG
CAGTCCCCATCGAGAGGCCTTGAGTGGCTGGGAAGGACATACTACAGGTCCAAGTGGTAT
AATGATTATGCAGTATCTGTGAAAAGTCGAATAACCATCAACCCAGACACATCCAAGAAC
CAGTTCTCCCTGCAGCTGAACTCTGTGACTCCCGAGGACACGGCTGTGTATTACTGTGCA
AGAGATTACTATGGTTCGGGGAGTTTCTACTACTACTACGGTATGGACGTCTGGGGCCAA
GGGACCACGGTCACCGTCTCCTC

36 Heavy Chain Protein:
QVQLQQSGPGLVKPSQTLSLTCAISGDSVSSNSAAWNWIRQSPSRGLEWLGRTYYRSKWY

NDYAVSVKSRITINPDTSKNQFSLQLNSVTPEDTAVYYCARDYYGSGSFYYYYGMDVWGQ
GTTVTVS

37 Light Chain DNA:
GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACC
ATCACTTGCCGGGCAAGTCAGAGCATTAGCAGCTATTTAAATTGGTATCAGCAGAAACCA
GGGAAAGCCCCTAAGGTCCTGATCTATGCTGCATCCAATTTGCAAAGTGGGGTCCCATCA
AGGTTCAGTGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGCAGTCTGCAACCT
GAAGATTTTGCAACTTACTACTGTCAACAGAGTTACAGTACCCCTCGGACGTTCGGCCAA
GGGACCAAGGTGGAAATCAAA

38 Light Chain Protein:
DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKVLIYAASNLQSGVPS
RFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPRTFGQGTKVEIK

Antibody U1-48

39 Heavy Chain DNA:
CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCGGAGACCCTGTCCCTC
ACCTGCACTGTCTCTGGTGGCTCCATCAGTAGTTACTACTGGAGCTGGATCCGGCAGCCC
GCCGGGAAGGGACTGGAGTGGATTGGGCATATCTATACCAGTGGGAGCACCAACTACAAC
CCCTCCCTCAAGAGTCGAGTCACCATGTCAGTAGACACGTCCAAGAACCAGTTCTCCCTG
AAGCTGAGCTCTGTGACCGCCGCGGACACGGCCGTGTATTACTGTGCGAGAGAAGCGATT
TTTGGAGTGGGCCCCTACTACTACTACGGTATGGACGTCTGGGGCCAAGGGACCACGGTC
ACCGTCTCCTC

40 Heavy Chain Protein:
QVQLQESGPGLVKPSETLSLTCTVSGGSISSYYWSWIRQPAGKGLEWIGHIYTSGSTNYN
PSLKSRVTMSVDTSKNQFSLKLSSVTAADTAVYYCAREAIFGVGPYYYYGMDVWGQGTTV
TVS

Antibody U1-49

41 Heavy Chain DNA:
CAGGTGCAGCTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCTGGGGCCTCAGTGAAGGTC
TCCTGCAAGGCTTCTGGATACACCTTCACCGGCTACTATATGCACTGGGTGCGACAGGCC
CCTGGACAAGGGCTTGAGTGGATGGGATGGATCAACCCTAATATTGGTGGCACAAACTGT
GCACAGAAGTTTCAGGGCAGGGTCACCATGACCAGGGACACGTCCATCAGCACAGCCTAC
ATGGAGCTGAGCAGGCTGAGATCTGACGACACGGCCGTGTATTACTGTGCGAGAGGGGGA
CGGTATAGCAGCAGCTGGTCCTACTACTACTACGGTATGGACGTCTGGGGCCAAGGGACC
ACGGTCACCGTCTCCTC

42 Heavy Chain Protein:
QVQLVQSGAEVKKPGASVKVSCKASGYTFTGYYMHWVRQAPGQGLEWMGWINPNIGGTNC
AQKFQGRVTMTRDTSISTAYMELSRLRSDDTAVYYCARGGRYSSSWSYYYYGMDVWGQGT
TVTVS

43 Light Chain DNA:
GATATTCTGATGACCCAGACTCCACTCTCTCTGTCCGTCACCCCTGGACAGCCGGCCTCC
ATCTCCTGCAAGTCTAGTCAGAGCCTCCTGCTTAGTGATGGAGGGACCTATTTGTATTGG
TACCTGCAGAAGCCAGGCCAGCCTCCACAGCTCCTGATCTATGAAGTTTCCAACCGGTTC
TCTGGAGTGCCAGATAGGTTCAGTGGCAGCGGGTCAGGGACAGATTTCACACTGAAAATC
AGCCGGGTGGAGGCTGAGGATGTTGGGGTTTATTACTGCATGCAAAGTATGCAGCTTCCG
ATCACCTTCGGCCAAGGGACACGACTGGAAATTAAA

44 Light Chain Protein:
DILMTQTPLSLSVTPGQPASISCKSSQSLLLSDGGTYLYWYLQKPGQPPQLLIYEVSNRF
SGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCMQSMQLPITFGQGTRLEIK

Antibody U1-50

45 Heavy Chain DNA:

CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCGGAGACCCTGTCCCTC
ACCTGCACTGTCTCTGGTGGCTCCGTCAGCAGTGGTGGTTACTACTGGAGCTGGATCCGG
CAGCCCCCAGGGAAGGGACTGGAGTGGATTGGGTATATCTATTACAGTGGGAGCACCAAC
TACAACCCCTCCCTCAAGAGTCGAGTCACCATATCAGTAGACACGTCCAAGAACCAGTTC
TCCCTGAAGCTGAGCTCTGTGACCGCTGCGGACACGGCCGTGTATTACTGTGCGAGAGGG
GGGGACAGTAACTACGAGGATTACTACTACTACTACGGTATGGACGTCTGGGGCCAAGGG
ACCACGGTCACCGTCTCCTC

46 Heavy Chain Protein:
QVQLQESGPGLVKPSETLSLTCTVSGGSVSSGGYYWSWIRQPPGKGLEWIGYIYYSGSTN
YNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARGGDSNYEDYYYYGMDVWGQG
TTVTVS

47 Light Chain DNA:
GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACC
ATCACTTGCCGGGCAAGTCAGAGCATTAGCATCTATTTACATTGGTATCAGCAGAAACCA
GGGAAAGCCCCTAAGCTCTTGATCTCTGCTGCATCCAGTTTGCAAAGTGGGGTCCCGTCA
AGGTTCAGTGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGAAGTCTGCAACCT
GAAGATTTTGCAACTTACTACTGTCAACAGAGTTACACTTCCCCGATCACCTTCGGCCAA
GGGACACGACTGGAGATTAAA

48 Light Chain Protein:
DIQMTQSPSSLSASVGDRVTITCRASQSISIYLHWYQQKPGKAPKLLISAASSLQSGVPS
RFSGSGSGTDFTLTIRSLQPEDFATYYCQQSYTSPITFGQGTRLEIK

49 Antibody U1-51
Heavy Chain DNA:
CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCGGAGACCCTGTCCCTC
ACCTGCACTGTCTCTGGTGGCTCCATCAGTAGTTACTACTGGAGCTGGATCCGGCAGCCC
CCAGGGAAGGGACTGGAGTGGATTGGGTATATCTATTACAGTGGGAGCACCAACTACAAC
CCCTCCCTCAAGAGTCGAGTCACCATATCAGTAGACACGTCCAAGCACCAGTTCTCCCTG
AAGCTGAGCTCTGTGACCGCTGCGGACACGGCCGTGTATTACTGTGCGAGAGATTCGAGT
TACTATGATAGTAGTGGTTATTACTTATACTACTACGCTATGGACGTCTGGGGCCAAGGG
ACCACGGTCACCGTCTCCTC

50 Heavy Chain Protein:
QVQLQESGPGLVKPSETLSLTCTVSGGSISSYYWSWIRQPPGKGLEWIGYIYYSGSTNYN
PSLKSRVTISVDTSKHQFSLKLSSVTAADTAVYYCARDSSYYDSSGYYLYYYAMDVWGQG
TTVTVS

51 Light Chain DNA:
GACATCGTGATGACCCAGTCTCCAGACTCCCTGGCTGTGTCTCTGGGCGAGAGGGCCACC
ATCAACTGCAAGTCCAGCCAGAGTGTTTTATACAGCTCCAACAATAAGAACTACTTAGCT
TGGTACCAGCAGAAACCAGGACAGCCTCCTAAGCTGCTCATTTCCTGGGCATCTACCCGG
GAATCCGGGGTCCCTGACCGATTCAGTGGCAGCGGGTCTGGGACAGATTTCACTCTCACC
ATCAGCAGCCTGCAGGCTGAAGATGTGGCAGTTTATTACTGTCAGCAATATTATACTACT
CCTCTCACTTTCGGCCCTGGGACCAAAGTGGATATCAAA

52 Light Chain Protein:
DIVMTQSPDSLAVSLGERATINCKSSQSVLYSSNNKNYLAWYQQKPGQPPKLLISWASTR
ESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQQYYTTPLTFGPGTKVDIK

Antibody U1-53
53 Heavy Chain DNA:
GAGGTGCAACTGGTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTC
TCCTGTGCAGCCTCTGGATTCACCTTCAGTATCTATAGCATGAACTGGGTCCGCCAGGCT
CCAGGGAAGGGGCTGGAGTGGGTTTCATACATTAGTAGTAGTAGTAGTACCATATACTAC
GCAGACTCTGTGAAGGGCCGATTCACCATCTCCAGAGACAATGCCAAGAACTCACTGTAT
CTGCAAATGAACAGCCTGAGAGACGAGGACACGGCTGTGTATTACTGTGCGAGAGATAGG

GGTGACTTCGATGCTTTTGATATCTGGGGCCAAGGGACAATGGTCACCGTCTCTTCA

54 Heavy Chain Protein:
EVQLVESGGGLVQPGGSLRLSCAASGFTFSIYSMNWVRQAPGKGLEWVSYISSSSSTIYY
ADSVKGRFTISRDNAKNSLYLQMNSLRDEDTAVYYCARDRGDFDAFDIWGQGTMVTVSS

55 Light Chain DNA:
GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACC
ATCACTTGCCAGGCGAGTCAGGACATTACCAACTATTTGAATTGGTATCAGCAGAAACCA
GGGAAAGCCCCTAAGCTCCTGATCTACGATGCATCCAATTTGGAAACAGGGGTCCCATCA
AGGTTCAGTGGAAGTGGATCTGGGACAGATTTTACTTTCACCATCAGCAGCCTGCAGCCT
GAAGATATTGCAACATATAACTGTCAACAGTGTGAAAATTTCCCGATCACCTTCGGCCAA
GGGACACGACTGGAGATTAAA

56 Light Chain Protein:
DIQMTQSPSSLSASVGDRVTITCQASQDITNYLNWYQQKPGKAPKLLIYDASNLETGVPS
RFSGSGSGTDFTFTISSLQPEDIATYNCQQCENFPITFGQGTRLEIK

Antibody U1-55

57 Light Chain DNA:
GATATTGTGATGACTCAGTCTCCACTCTCCCTGCCCGTCACCCCTGGAGAGCCGGCCTCC
ATCTCCTGCAGGTCTAGTCAGAGCCTCCTGTATAGTAATGGATACAAGTATTTGGATTGG
TACCTGCAGAAGCCAGGGCAGTCTCCACAGCTCCTGATCTATTTGGGTTCTAATCGGGCC
TCCGGGGTCCCTGACAGGTTCAGTGGCAGTGGATCAGGCACAGATTTTACACTGAAAATC
AGCAGAGTGGAGGCTGAGGATGTTGGGGTTTATTATTGCATGCAGGCTCTACAAACTCCG
ATCACCTTCGGCCAAGGGACACGACTGGAGATTAAA

58 Light Chain Protein:
DIVMTQSPLSLPVTPGEPASISCRSSQSLLYSNGYKYLDWYLQKPGQSPQLLIYLGSNRA
SGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCMQALQTPITFGQGTRLEIK

Antibody (U1-55.1)

59 Heavy Chain DNA:
CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCGGAGACCCTGTCCCTC
ACCTGCACTGTCTCTGGTGGCTCCGTCAGCAGTGGTGGTTACTACTGGAACTGGATCCGG
CAGCCCCCAGGGAAGGGACTGGAGTGGATTGGGTATATCAATTACAGTGGGAGCACCAAC
TACAACCCCTCCCTCAAGAGTCGAGTCACCATATCAGTAGACACGTCCAAGAACCAGTTC
TCCCTGAAGCTGAGCTCTGTGACCGCTGCGGACACGGCCGTGTATTACTGTGCGAGAGAT
CGAGAACTGGAACTTTACTACTACTACTACGGTATGGACGTCTGGGGCCAAGGGACCACG
GTCACCGTCTCCTC

60 Heavy Chain Protein:
QVQLQESGPGLVKPSETLSLTCTVSGGSVSSGGYYWNWIRQPPGKGLEWIGYINYSGSTN
YNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARDRELELYYYYYGMDVWGQGTT
VTVS

Antibody (U1-57)

61 Heavy Chain DNA:
CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCTGAGACCCTGTCCCTC
ACCTGCACTGTCTCTGGTGGCTCCGTCAGCAGTGGTGGTTACTACTGGAACTGGATCCGG
CAGCCCCCAGGGAAGGGACTGGAGTGGATTGGGTATATCAATTACAGTGGGAGCACCAAC
TACAACCCCTCCCTCAAGAGTCGAGTCACCATATCAGTAGACACGTCCAAGAACCAGTTC
TCCCTGAAGCTGAGCTCTGTGACCGCTGCGGACACGGCCGTGTATTACTGTGCGAGAGAT
CGAGAACTGGAACTTTACTACTACTACTACGGTATGGACGTCTGGGGCCAAGGGACCACG
GTCACCGTCTCCTC

62 Heavy Chain Protein:
QVQLQESGPGLVKPSETLSLTCTVSGGSVSSGGYYWNWIRQPPGKGLEWIGYINYSGSTN
YNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARDRELELYYYYYGMDVWGQGTT

VTVS

Antibody U1-57.1

63 Light Chain DNA:
GATATTGTGATGACTCAGTCTCCACTCTCCCTGCCCGTCACCCCTGGAGAGCCGGCCTCC
ATCTCCTGCAGGTCTAGTCAGAGCCTCCTGTATAGTAATGGATACAAGTATTTGGATTGG
TACCTGCAGAAGCCAGGGCAGTCTCCACAGCTCATGATCTATTTGGGTTCTAATCGGGCC
TCCGGGGTCCCTGACAGGTTCAGTGGCAGTGGATCAGGCACAGATTTTACACTGAAAATC
AGCAGAGTGGAGGCTGAGGATGTTGGGGTTTATTATTGCATGCAGGCTCTACAAACTCCG
ATCACCTTCGGCCAAGGGACACGACTGGAGATTAAA

64 Light Chain Protein:
DIVMTQSPLSLPVTPGEPASISCRSSQSLLYSNGYKYLDWYLQKPGQSPQLMIYLGSNRA
SGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCMQALQTPITFGQGTRLEIK

Antibody U1-58

65 Heavy Chain DNA:
CAGGTGCAGCTGGTGGAGTCTGGGGGAGGCGTGGTCCAGCCTGGGAGGTCCCTGAGACTC
TCCTGTGCAGCGTCTGGATTCACCTTCAGTAGCTATGGCATGCACTGGGTCCGCCAGGCT
CCAGGCAAGGGGCTGGAGTGGGTGGCAGTTATATGGTATGATGGAAGTAATAAATACTAT
GCAGACTCCGTGAAGGGCCGATTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTAT
CTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCTGTGTATTACTGTGCGAGAGCAGCT
CGCCTTGACTACTACTACGGTATGGACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCC
TCA

66 Heavy Chain Protein:
QVQLVESGGGVVQPGRSLRLSCAASGFTFSSYGMHWVRQAPGKGLEWVAVIWYDGSNKYY
ADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARAARLDYYYGMDVWGQGTTVTVS
S

67 Light Chain DNA:
GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCTCC
ATCACTTGCCGGGCAAGTCAGAGCATTAACAGCTATTTAAATTGGTTTCAGCAGAAGCCA
GGGAAAGCCCCTCAGCTCCTGATCTTTGGTGCATCCGGTTTGCAAAGTGGGGTCCCATCA
AGGTTCAGTGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAACAGTCTGCAACCT
GAAGATTTTGCAACTTACTACTGTCAACAGAGTTACAGTTCCCCGCTCACCTTCGGCCAA
GGGACACGACTGGAGATTAAA

68 Light Chain Protein:
DIQMTQSPSSLSASVGDRVSITCRASQSINSYLNWFQQKPGKAPQLLIFGASGLQSGVPS
RFSGSGSGTDFTLTINSLQPEDFATYYCQQSYSSPLTFGQGTRLEIK

Antibody U1-59

69 Heavy Chain DNA:
CAGGTGCAGCTACAGCAGTGGGGCGCAGGACTGTTGAAGCCTTCGGAGACCCTGTCCCTC
ACCTGCGCTGTCTATGGTGGGTCCTTCAGTGGTTACTACTGGAGCTGGATCCGCCAGCCC
CCAGGGAAGGGGCTGGAGTGGATTGGGGAAATCAATCATAGTGGAAGCACCAACTACAAC
CCGTCCCTCAAGAGTCGAGTCACCATATCAGTAGAAACGTCCAAGAACCAGTTCTCCCTG
AAGCTGAGCTCTGTGACCGCCGCGGACACGGCTGTGTATTACTGTGCGAGAGATAAGTGG
ACCTGGTACTTCGATCTCTGGGGCCGTGGCACCCTGGTCACTGTCTCCTCA

70 Heavy Chain Protein:
QVQLQQWGAGLLKPSETLSLTCAVYGGSFSGYYWSWIRQPPGKGLEWIGEINHSGSTNYN
PSLKSRVTISVETSKNQFSLKLSSVTAADTAVYYCARDKWTWYFDLWGRGTLVTVSS

71 Light Chain DNA:
GACATCGAGATGACCCAGTCTCCAGACTCCCTGGCTGTGTCTCTGGGCGAGAGGGCCACC
ATCAACTGCAGGTCCAGCCAGAGTGTTTTATACAGCTCCAGCAATAGGAACTACTTAGCT
TGGTACCAGCAGAACCCAGGACAGCCTCCTAAGCTGCTCATTTACTGGGCTTCTACCCGG

GAATCCGGGGTCCCTGACCGATTCAGTGGCAGCGGGTCTGGGACAGATTTCACTCTCACC
ATCAGCAGCCTGCAGGCTGAAGATGTGGCAGTTTATTACTGTCAGCAATATTATAGTACT
CCTCGGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAA

72 Light Chain Protein:
DIEMTQSPDSLAVSLGERATINCRSSQSVLYSSSNRNYLAWYQQNPGQPPKLLIYWASTR
ESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQQYYSTPRTFGQGTKVEIK

Antibody U1-52

73 Heavy Chain DNA:
CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCACAGACCCTGTCCCTC
ACCTGCACTGTCTCTGGTGGCTCCATCAGCAGTGGTGGTTACTACTGGAGCTGGATCCGC
CAGCACCCAGGGAAGGGCCTGGAGTGGATGGGGAACATCTATTACAGTGGGAGCACCTAC
TACAACCCGTCCCTCAAGAGTCGAGTTACCATATCAGTAGACACGTCTGAGAACCAGTTC
TCCCTGAAGCTGAACTCTGTGACTGCCGCGGACACGGCCGTATATTACTGTGCGAGAGGG
GGAACTGGAACCAATTACTACTACTACTACGGTATGGACGTCTGGGGCCAAGGGACCACG
GTCACCGTCTCCTC

74 Heavy Chain Protein:
QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGGYYWSWIRQHPGKGLEWMGNIYYSGSTY
YNPSLKSRVTISVDTSENQFSLKLNSVTAADTAVYYCARGGTGTNYYYYYGMDVWGQGTT
VTVS

75 Light Chain DNA:
GAAATTGTGTTGACGCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACC
CTCTCCTGCAGGGCCAGTCAGAGTGTTAGCAGCAGCTACTTAGCCTGGTACCAGCAGAAA
CCTGGCCAGGCTCCCAGGCTCCTCATCTATGGTGCATCCAGCTGGGCCACTGGCATCCCA
AACAGGTTCAGTGGCAGTGGGTCTGGGACAGACTTCACTCTCACCATCAGCAGACTGGAG
CCTGAAGATTTTGCAGTGTATTACTGTCAGCAGTATGGTAGCTCACCGCTCACTTTCGGC
GGAGGGACCAAGGTGGAGATCAAA

76 Light Chain Protein:
EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSWATGIP
NRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSPLTFGGGTKVEIK

Antibody U1-61

77 Heavy Chain DNA:
CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCACAGACCCTGTCCCTC
ACCTGCACTGTCTCTGGTGTCTCCATCAGCAGTGGTGGTTACTACTGGAGCTGGATCCGC
CAGCACCCAGGGATGGGCCTGGAGTGGATTGGGTACATCTATTACAGTGGGAGCACCTAC
TACAACCCGTCCCTCAAGAGTCGAGTCACCATATCAGAAGACACGTCTAAGAACCAGTTC
TCCCTGAAGCTGAGCTCTGTGACTGCCGCGGACACGGCCGTGTATTACTGTGCGAGAGAT
TCCGAGTCCGAGTATAGCAGCTCGTCGAACTACGGTATGGACGTCTGGGGCCAAGGGACC
ACGGTCACCGTCTCCTC

78 Heavy Chain Protein:
QVQLQESGPGLVKPSQTLSLTCTVSGVSISSGGYYWSWIRQHPGMGLEWIGYIYYSGSTY
YNPSLKSRVTISEDTSKNQFSLKLSSVTAADTAVYYCARDSESEYSSSSNYGMDVWGQGT
TVTVS

Antibody U1-61.1

79 Heavy Chain DNA:
CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCACAGACCCTGTCCCTC
ACCTGCACTGTCTCTGGTGTCTCCATCAGCAGTGGTGGTTACTACTGGAGCTGGATCCGC
CAGCACCCAGGGATGGGCCTGGAGTGGATTGGGTACATCTATTACAGTGGGAGCACCTAC
TACAACCCGTCCCTCAAGAGTCGAGTCACCATATCAGAAGACACGTCTAAGAACCAGTTC
TCCCTGAAGCTGAGCTCTGTGACTGCCGCGGACACGGCCGTGTATTACTGTGCGAGAGAT
TCCGAGTCCGAGTATAGCAGCTCGTCGAACTACGGTATGGACGTCTGGGGCCAAGGGACC

ACGGTCACCGTCTCCTC

80 Heavy Chain Protein:
QVQLQESGPGLVKPSQTLSLTCTVSGVSISSGGYYWSWIRQHPGMGLEWIGYIYYSGSTY
YNPSLKSRVTISEDTSKNQFSLKLSSVTAADTAVYYCARDSESEYSSSSNYGMDVWGQGT
TVTVS

81 Light Chain DNA:
GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAATCACC
ATCACTTGCCGGGCAAGTCAGACCATTAGCAGCTATTTAAATTGGTATCAGCAGAAACCA
GGGAAAGCCCCTAAGCTCCTGATCTATGCTGCATCCAGTTTGCAAGGTGGGGTCCCATCA
AGGTTCAGTGGCAGTGTATCTGGGACAGATTTCACCCTCACCGTCAGCAGTCTGCAACCT
GAAGATTTTGCAACTTACTACTGTCAACAGAGTTACAGTAACCCGCTCACTTTCGGCGGA
GGGACCAAGGTGGAGATCAAA

82 Light Chain Protein:
DIQMTQSPSSLSASVGDRITITCRASQTISSYLNWYQQKPGKAPKLLIYAASSLQGGVPS
RFSGSVSGTDFTLTVSSLQPEDFATYYCQQSYSNPLTFGGGTKVEIK

Antibody U1-62 (2.9.1)

83 Heavy Chain DNA:
GAGGTGCAGCTGGTGCAGTCTGGAGCAGAGGTGAAAAAGCCCGGGGAGTCTCTGAAGATC
TCCTGTAAGGGTTCTGGATACAGTTTTACCAGCTACTGGATCGGCTGGGTGCGCCAGATG
CCCGGGAAAGGCCTGGAGTGGATGGGGATCATCTATCCTGGTGACTCTGATACCAGATAC
AGCCCGTCCTTCCAAGGCCAGGTCACCATGTCAGCCGACAAGTCCATCAGTACCGCCTAC
CTGCAGCTGAGCAGCCATGAAGGCCTCGGACACCGCCATGTATTACTGTGCGAGACAGAT
GGCTGGAAACTACGTACATCACGGGTGATCGAGACGTCCTGGGGCCAAGGGACCACGGTC
ACCGTCTCCTC

84 Heavy Chain Protein:
EVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWIGWVRQMPGKGLEWMGIIYPGDSDTRY
SPSFQGQVTMSADKSISTAYLQLSSHEGLGHRHVLLCETDGWKLRTSRVIETSWGQGTTV
TVS

85 Light Chain DNA:
GAAATTGTGTTGACGCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACC
CTCTCCTGCAGGGCCAGTCAGAGTGTTATCAGCATCTACTTAGCCTGGTACCAGCAGAAA
CCTGGCCAGGCTCCCAGGCTCCTCATCTATGGTGCATCCAGCAGGGCCACTGGCATCCCA
GACAGGTTCAGTGGCAGTGGGTCTGGGACAGACTTCACTCTCACCATCAGCAGACTGGAG
CCTGAAGATTTTGCAGTGTATTACTGTCAGCAGTATGGTAGCTCACCGTGCAGTTTTGGC
CAGGGGACCAAACTGGAGATCAAA

86 Light Chain Protein:
EIVLTQSPGTLSLSPGERATLSCRASQSVISIYLAWYQQKPGQAPRLLIYGASSRATGIP
DRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSPCSFGQGTKLEIK

Antibody U1-2

87 Heavy Chain DNA:
CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCACAGACCCTGTCCCTC
ACCTGCACTGTCTCTGGTGGCTCCATCAGCAGTGGTGATTACTACTGGAGCTGGATCCGC
CAGCACCCAGGGAAGGGCCTGGAGTGGATTGGGTACATCTATTACAGTGGGAGCACCTAC
TACAACCCGTCCCTCAGGAGTCGAGTTACCATATCAGTAGACACGTCTAAGAACCAGTTC
TCCCTGAAGCTGAGCTCTGTGACTGCCGCGGACACGGCCGTGTATTACTGTGCGAGAGCG
GATTACGATTTTTGGAGTGGTTATTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTC
TCCTCA

88 Heavy Chain Protein:
QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGDYYWSWIRQHPGKGLEWIGYIYYSGSTY
YNPSLRSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARADYDFWSGYFDYWGQGTLVTV
SS

89 Light Chain DNA:
GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACC
ATCACTTGCCGGGCAAGTCAGGGCATTAGAAATGATTTAGGCTGGTATCAGCAGATACCT
GGGAAAGCCCCTAAGCGCCTGATCTATGCTGCATCCAGTTTGCAAAGTGGGGTCCCATCA
AGGTTCAGCGGCAGTGGATCTGGGACAGAATTCACTCTCACAATCAACAGCCTGCAGCCT
GAAGATTTTGCAACTTATTACTGTCTACAGCATAATGGTTACCCGTGGACGTTCGGCCAA
GGGACCAAGGTGGAAATCAAAC

90 Light Chain Protein:
DIQMTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQIPGKAPKRLIYAASSLQSGVPS
RFSGSGSGTEFTLTINSLQPEDFATYYCLQHNGYPWTFGQGTKVEIK

Antibody U1-7

91 Heavy Chain DNA:
CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCACAGACCCTGTCCCTC
ACCTGCACTGTCTCTGGTGGCTCCATCAGCAGTGGTGATTACTACTGGAGCTGGATCCGC
CAGCACCCAGGGAAGGGCCTGGAGTGGATTGGATACATCTATTACAGTGGGAGCACCTAC
TACAACCCGTCCCTCAAGAGTCGAGTTACCATATCAGTAGACACGTCTAAGAACCAGTTC
TCCCTGAAGCTGAGCTCTGTGACTGCCGCGGACACGGCCGTGTATTACTGTGCGAGAGCG
GATTACGATTTTTGGAGTGGTTATTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTC
TCCTCA

92 Heavy Chain Protein:
QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGDYYWSWIRQHPGKGLEWIGYIYYSGSTY
YNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARADYDFWSGYFDYWGQGTLVTV
SS

93 Light Chain DNA:
GACTTCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACC
ATCACTTGCCGGGCAAGTCAGGACATTCGAAATGATTTAGGCTGGTATCGGCAGAAACCT
GGGAAAGCCCCTAAGCGCCTGATCTATGCTGCATCCAGTTTGCAAAGTGGGGTCCCATCA
AGGTTCAGCGGCAGTGGATCTGGGACAGAATTCACTCTCACAATCAGCAGCCTGCAGCCT
GAAGATTTTGCAACTTATTACTGTCTACAGCATAATAGTTACCCGTGGACGTTCGGCCAA
GGGACCAAGGTGGAAATCAAAC

94 Light Chain Protein:
DFQMTQSPSSLSASVGDRVTITCRASQDIRNDLGWYRQKPGKAPKRLIYAASSLQSGVPS
RFSGSGSGTEFTLTISSLQPEDFATYYCLQHNSYPWTFGQGTKVEIK

Antibody U1-9

95 Heavy Chain DNA:
CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCACAGACCCTGTCCCTC
ACCTGCACTGTCTCTGGTGGCTCCATCAGCAGTGGTGATTACTACTGGAGCTGGATCCGC
CAGCACCCAGGGAAGGGCCTGGAGTGGATTGGATACATCTATTACAGTGGGAGCACCTAC
TACAACCCGTCCCTCAAGAGTCGAGTTACCATATCAATAGACACGTCTAAGAACCAGTTC
TCCCTGAAGCTGAGCTCTGTGACTGCCGCGGACACGGCCGTGTATTACTGTGCGAGAGCG
GATTACGATTTTTGGAATGGTTATTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTC
TCCTCA

96 Heavy Chain Protein:
QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGDYYWSWIRQHPGKGLEWIGYIYYSGSTY
YNPSLKSRVTISIDTSKNQFSLKLSSVTAADTAVYYCARADYDFWNGYFDYWGQGTLVTV
SS

97 Light Chain DNA:
GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACC
ATCACTTGCCGGGCAAGTCAGGACATTAGAAATGATTTAGGCTGGTATCGGCAGAAACCT
GGGAAAGCCCCTAAGCGCCTGATCTATGCTGCATCCAGTTTGCAAAGTGGGGTCCCATCA
AGGTTCAGCGGCAGTGGATCTGGGACAGAATTCACTCTCACAATCAGCAGCCTGCAGCCT

GAAGATTTTGCAACTTATTACTGTCTACAGCATAATAGTTACCCGTGGACGTTCGGCCAA
GGGACCAAGGTGGAAATCAAA

98 Light Chain Protein:
DIQMTQSPSSLSASVGDRVTITCRASQDIRNDLGWYRQKPGKAPKRLIYAASSLQSGVPS
RFSGSGSGTEFTLTISSLQPEDFATYYCLQHNSYPWTFGQGTKVEIK

Antibody U1-10

99 Heavy Chain DNA:
CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTACACAGACCCTGTCCCTC
ACCTGCACTGTCTCTGGTGGCTCCATCAGCAGTGGTGATTACTACTGGAGCTGGATCCGC
CAGCACCCAGGGAAGGGCCTGGAGTGGATTGGGTACATCTATTACAGTGGGAGCACCTAC
TACAACCCGTCCCTCAAGAGTCGACTTACCATATCAGTAGACACGTCTAAGAACCAGTTC
TCCCTGAAGCTGAGCTCTGTGACTGCCGCGGACACGGCCGTGTATTACTGTGCGAGAGCA
GATTACGATTTTTGGAGTGGTTACTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTC
TCCTCA

100 Heavy Chain Protein:
QVQLQESGPGLVKPTQTLSLTCTVSGGSISSGDYYWSWIRQHPGKGLEWIGYIYYSGSTY
YNPSLKSRLTISVDTSKNQFSLKLSSVTAADTAVYYCARADYDFWSGYFDYWGQGTLVTV
SS

101 Light Chain DNA:
GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACC
ATCACTTGCCGGGCAAGTCAGGGCATTAGAAATGATTTAGGCTGGTATCAGCAGAAACCA
GGGAAAGCCCCTAAGCGCCTGATCTATGCTGCATCCAGTTTGCAAAGTGGGGTCCCATCA
AGGTTCAGCGGCAGTGGATCTGGGACAGAATTCACTCTCACAATCAGCAGCCTGCAGCCT
GAAGATTTTGCAACTTATTACTGTCTACAGCATAATAATTACCCGTGGACGTTCGGCCAA
GGGACCAAGGTGGAAATCAAA

102 Light Chain Protein:
DIQMTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKRLIYAASSLQSGVPS
RFSGSGSGTEFTLTISSLQPEDFATYYCLQHNNYPWTFGQGTKVEIK

Antibody U1-12

103 Heavy Chain DNA
CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCACAGACCCTGTCCCTC
ACCTGCACTGTCTCTGGTGGCTCCATCAGTAGTGGTGATTACTACTGGAGCTGGATCCGC
CAGCACCCAGGGAAGGGCCTGGAGTGGATTGGGTACATCTATTACAGTGGGAGCACCTAC
TACAACCCGTCCCTCAAGAGTCGAGTTACCATATCAGTAGACACGTCTAAGAACCAGTTC
TCCCTGAAGTTGAGCTCTGTGACTGCCGCGGACACGGCCGTGTATTACTGTGCGAGAGCC
GATTACGATTTTTGGAGTGGTTATTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTC
TCCTCA

104 Heavy Chain Protein:
QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGDYYWSWIRQHPGKGLEWIGYIYYSGSTY
YNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARADYDFWSGYFDYWGQGTLVTV
SS

105 Light Chain DNA:
GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACC
ATCACTTGCCGGGCAAGTCAGGGCATTAGAAATGATTTAGGCTGGTATCAGCAGAAACCA
GGGAAAGCCCCTAAGCGCCTGATCTATGCTGCATCCAGTTTGCAAAGTGGGGTCCCATCA
AGGTTCAGCGGCAGTGGATCTGGGACAGAATTCACTCTCACAATCAGCAGCCTGCAGCCT
GAAGATTTTGCAACTTATTACTGTCTACAGCATAATAATTACCCGTGGACGTTCGGCCAA
GGGACCAAGGTGGAAATCAAA

106 Light Chain Protein:
DIQMTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKRLIYAASSLQSGVPS
RFSGSGSGTEFTLTISSLQPEDFATYYCLQHNNYPWTFGQGTKVEIK

Antibody U1- 13

107 Heavy Chain DNA:
CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCACAGACCCTGTCCCTC
ACCTGCACTGTCTCTGGTGGCTCCATCAGCAGTGGTGGTTACTACTGGAGCTGGATCCGC
CAGCACCCAGGGAAGGGCCTGGAGTGGATTGGGTACATCTATTACAGTGGGAGCACCTAC
TACAACCCGTCCCTCAAGAGTCGAGTTACCATATCAGTAGACACGTCTAAGAACCAGTTC
TCCCTGAAGCTGAGCTCTGTGACTGCCGCGGACACGGCCGTGTATTACTGTGCGAGAGAG
GACGACGGTATGGACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCA

108 Heavy Chain Protein:
QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGGYYWSWIRQHPGKGLEWIGYIYYSGSTY
YNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCAREDDGMDVWGQGTTVTVSS

109 Light Chain DNA:
GATATTGTGATGACTCAGTCTCCACTCTCCCTGCCCGTCACCCCTGGAGAGCCGGCCTCC
ATTTCCTGCAGGTCTAGTCAGAGCCTCCTGCATAGTAATGGATACAACTATTTGGAATGG
TACCTGCAGAAGCCAGGGCAGTCCCCACAGTTCATGATTTATTTGGGGTCTAATCGGGCC
TCCGGGGTCCCTGACAGGTTCAGTGGCAGTGGATCAGGCACAGATTTTACACTGAAAATC
AGCAGAGTGGAGGCTGAGGATGTTGGGGTTTATTACTGCATGCAAGCTCTACAAACTCCG
ATCACCTTCGGCCAAGGGACACGACTGGAGATTAAA

110 Light Chain Protein:
DIVMTQSPLSLPVTPGEPASISCRSSQSLLHSNGYNYLEWYLQKPGQSPQFMIYLGSNRA
SGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCMQALQTPITFGQGTRLEIK

Antibody U1-14

111 Heavy Chain DNA:
CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCACAGACCCTGTCCCTC
ACCTGCACTGTCTCTGGTGGCTCCATCAGCAGTGGTGATTACTACTGGAGCTGGATCCGC
CAGTACCCAGGGAAGGGCCTGGAGTGGATTGGGTACATCTATTACAGTGGGAGCACCTAC
TACAACCCGTCCCTCAAGAGTCGAGTTACCATATCAGTAGACACGTCTAAGAACCAGTTC
TCCCTGAAGCTGAGGTCTGTGACTGCCGCGGACACGGCCGTGTATTACTGTGCGAGAGCG
GATTACGATTTTTGGAGTGGTTATTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTC
TCCTCA

112 Heavy Chain Protein:
QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGDYYWSWIRQYPGKGLEWIGYIYYSGSTY
YNPSLKSRVTISVDTSKNQFSLKLRSVTAADTAVYYCARADYDFWSGYFDYWGQGTLVTV
SS

113 Light Chain DNA:
GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACC
ATCACTTGCCGGGCAAGTCAGGGCATTAGAAATGATTTAGGCTGGTATCAGCAGAAACCA
GGGAAAGCCCCTAAGCGCCTGATCTATGCTGCATCCAGTTTGCAAAGTGGGGTCCCATCA
AGGTTCAGCGGCAGTGGATCTGGGACAGAATTCACTCTCACAATCAGCAGCCTGCAGCCT
GAAGATTTTGCAACTTATTACTGTCTACAGCATAATACTTACCCGTGGACGTTCGGCCAA
GGGACCAAGGTGGAAATCAAAC

114 Light Chain Protein:
DIQMTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKRLIYAASSLQSGVPS
RFSGSGSGTEFTLTISSLQPEDFATYYCLQHNTYPWTFGQGTKVEIK

Antibody U1-15

115 Heavy Chain DNA:
CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCGGAGACCCTGTCCCTC
ACCTGCACTGTCTCTGGTGGCTCCGTCAGCAGTGGTGGTTACTACTGGAGCTGGATCCGG
CAGCCCCCAGGGAAGGGACTGGAGTGGATTGGGTATATCTATTACAGTGGGAGCACCAAC
TACAACCCCTCCCTCAAGAGTCGAGTCACCATATCAGTAGACACGTCCAAGAACCAGTTC

TCCCTGAAGCTGAGCTCTGTGACCGCTGCGGACACGGCCGTGTATTACTGTGCGAGAGAT
GGGGACGTGGATACAGCTATGGTCGATGCTTTTGATATCTGGGGCCAAGGGACAATGGTC
ACCGTCTCCTCA

116 Heavy Chain Protein:
QVQLQESGPGLVKPSETLSLTCTVSGGSVSSGGYYWSWIRQPPGKGLEWIGYIYYSGSTN
YNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARDGDVDTAMVDAFDIWGQGTMV
TVSS

117 Light Chain DNA:
GAAATTGTATTGACGCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACC
CTCTCCTGCAGGGCCAGTCAGAGTTTAAGCGGCAACTACTTAGCCTGGTACCAGCAGAAG
CCTGGCCAGGCTCCCAGGCTCATCATCTGTGGTGCATCCAGCAGGGCCACTGGCATCCCA
GACAGGTTCAGTGGCAGTGGGTCTGGGACAGACTTCACTCTCACCATCACAAGACTGGAG
CCTGAAGATTTTGCAGTGTATTACTGTCAGCAGTATGATAGGTCACCGCTCACTTTCGGC
GGAGGGACCAAGGTGGAGATCAAA

118 Light Chain Protein:
EIVLTQSPGTLSLSPGERATLSCRASQSLSGNYLAWYQQKPGQAPRLIICGASSRATGIP
DRFSGSGSGTDFTLTITRLEPEDFAVYYCQQYDRSPLTFGGGTKVEIK

Antibody U1-19

119 Heavy Chain DNA:
CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCACAGACCCTGTCCCTC
ACCTGCACTGTCTCTGGTGGCTCCATCAGCAGTGGTGATTACTACTGGAGCTGGATCCGC
CAGCACCCAGGGAAGGGCCTGGAGTGGATTGGGTACATCTATTACAGTGGGAGCACCTAC
TACAACCCGTCCCTCAAGAGTCGAGTTACCATATCAGTAGACACGTCTAAGAACCAGTTC
TCCCTGAAGCTGAGCTCTGTGACTGCCGCGGACACGGCCGTGTATTACTGTGCGAGAGGA
GATTACGATTTTTGGAGTGGAGAGTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTC
TCCTCA

120 Heavy Chain Protein:
QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGDYYWSWIRQHPGKGLEWIGYIYYSGSTY
YNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARGDYDFWSGEFDYWGQGTLVTV
SS

Antibody U1-20

121 Heavy Chain DNA:
CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCACAGACCCTGTCCCTC
ACCTGCACTGTCTCTGGTGGCTCCATCAGCAGTGGTGGTTACTACTGGAGCTGGATCCGC
CAGCACCCAGGGAAGGGCCTGGAGTGGATTGGGTACATCTATGACAGTGGGAGCACCTAC
TACAACCCGTCCCTCAAGAGTCGAGTTACCATATCAGTAGACACGTCTAAGAACCAGTTC
TCCCTGAAGCTGAGGTCTGTGACTGCCGCGGACACGGCCGTGTATTACTGTGCGAGAGAT
CAGGGGCAGGACGGATACAGCTATGGTTACGGCTACTACTACGGTATGGACGTCTGGGGC
CAAGGGACCACGGTCACCGTCTCCTC

122 Heavy Chain Protein:
QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGGYYWSWIRQHPGKGLEWIGYIYDSGSTY
YNPSLKSRVTISVDTSKNQFSLKLRSVTAADTAVYYCARDQGQDGYSYGYGYYYGMDVWG
QGTTVTVS

123 Light Chain DNA:
GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACC
ATCACTTGCCAGGCGAGTCAGGACATTAGCAATTATTTAAATTGGTATCAGCAGAAACCA
GGGAAAGCCCCTAAACTCCTGATCTACGTTGCATCCAATTTGGAAACAGGGGTCCCATCA
AGGTTCAGTGGAAGTGGATCTGGGACAGATTTTACTTTCACCATCAGCAGCCTGCAGCCT
GAAGATATTGCAACATATTACTGTCAACAGTGTGATAATCTCCCTCTCACTTTCGGCGGA
GGGACCAAGGTGGAGATCAAA

124 Light Chain Protein:

124 Light Chain Protein:
DIQMTQSPSSLSASVGDRVTITCQASQDISNYLNWYQQKPGKAPKLLIYVASNLETGVPS
RFSGSGSGTDFTFTISSLQPEDIATYYCQQCDNLPLTFGGGTKVEIK

Antibody U1-21

125 Heavy Chain DNA:
CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCACAGACCCTGTCCCTC
ACCTGCACTGTCTCTGGTGGCTCCATCAGCAGTGGTGATTACTACTGGAGCTGGATCCGC
CAGCACCCAGGGAAGGGCCTGGAGTGGATTGGATACATCTATTACAGTGGGAGCACCTAC
TACAACCCGTCCCTCAAGAGTCGAGTTACCATATCAGTAGACACGTCTAAGAACCAGTTC
TCCCTGAAGCTGAGCTCTGTGACTGCCGCGGACACGGCCGTGTATTACTGTGCGAGAGCG
GATTACGATTTTTGGAGTGGTTATTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTC
TCCTC

126 Heavy Chain Protein:
QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGDYYWSWIRQHPGKGLEWIGYIYYSGSTY
YNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARADYDFWSGYFDYWGQGTLVTV
S

127 Light Chain DNA:
GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACC
ATCACTTGCCGGGCAAGTCAGGACATTAGAAATGATTTAGGCTGGTATCGGCAGAAACCT
GGGAAAGCCCCTAAGCGCCTGATCTATGCTGCATCCCGTTTGCAAAGTGGGGTCCCATCA
AGGTTCAGCGGCAGTGGATCTGGGACAGAATTCACTCTCACAATCAGCAGCCTGCAGCCT
GAAGATTTTGCAACTTATTACTGTCTACAGCATAATAGTTACCCGTGGACGTTCGGCCAA
GGGACCAAGGTGGAAATCAAAC

128 Light Chain Protein:
DIQMTQSPSSLSASVGDRVTITCRASQDIRNDLGWYRQKPGKAPKRLIYAASRLQSGVPS
RFSGSGSGTEFTLTISSLQPEDFATYYCLQHNSYPWTFGQGTKVEIK

Antibody U1-22

129 Heavy Chain DNA:
CAGGTGCAGCTGCAGCAGTCGGGCCCAGGACTGGTGAAGCCTTCACAGACCCTGTCCCTC
ACCTGCACTGTCTCTGGTGGCTCCATCAGCAGTGGTGATTACTACTGGAGCTGGATCCGC
CAGCACCCAGGGAAGGGCCTGGAGTGGATTGGGTACATCTATTACAGTGGGAGCACCTAC
TACAACCCGTCCCTCAAGAGTCGAGTTACCATATCAGTAGACACGTCTAAGAACCAGTTC
TCCCTGAAGCTGAGCTCTGTGACTGCCGCGGACACGGCCGTGTATTACTGTGCGAGAGCC
GATTACGATTTTTGGAGTGGTTATTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTC
TCCTCA

130 Heavy Chain Protein:
QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGDYYWSWIRQHPGKGLEWIGYIYYSGSTY
YNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARADYDFWSGYFDYWGQGTLVTV
SS

131 Light Chain DNA:
GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACC
ATCACTTGCCGGGCAAGTCAGGGCATTAGAAATGATTTAGGCTGGTATCAGCAGAAACCA
GGGAAAGCCCCTAAGCGCCTGATCTATGCTGCATCCAGTTTGCAAAATGGGGTCCCATCA
AGGTTCAGCGGCAGTGGATCTGGGACAGAATTCACTCTCACAATCAGCAGCCTGCAGCCT
GAAGATTTTGCAACTTATTACTGTCTACAGCATAATAGTTACCCGTGGACGTTCGGCCAA
GGGACCAAGGTGGAAATCAAAC

132 Light Chain Protein:
DIQMTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKRLIYAASSLQNGVPS
RFSGSGSGTEFTLTISSLQPEDFATYYCLQHNSYPWTFGQGTKVEIK

Antibody U1-23

Antibody U1-23

133 Heavy Chain DNA:
CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCACAGACCCTGTCCCTC
ACCTGCACTGTCTCTGGTGGCTCCATCAGCAGTGGTGATTACTACTGGAGCTGGATCCGC
CAGCACCCAGGGAAGGGCCTGGAGTGGATTGGGTACATCTATTACAGTGGGAGCACCTAC
TACAACCCGTCCCTCAAGAGTCGAGTTACCATATCAGTAGACACGTCTAAGAACCAGTTC
TCCCTGAAGCTGAGCTCTGTGACTGCCGCGGACACGGCCGTGTATTACTGTGCGAGAGCG
GATTACGATTTTTGGAGTGGTTATTTTGACTACTGGGGCCAGGGAATCCTGGTCACCGTC
TCCTC

134 Heavy Chain Protein:
QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGDYYWSWIRQHPGKGLEWIGYIYYSGSTY
YNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARADYDFWSGYFDYWGQGILVTV
S

135 Light Chain DNA:
GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACC
ATCACTTGCCGGGCAAGTCAGGGCATTAGAAATGATTTAGGCTGGTATCAGCAGAAACCA
GGGAAAGCCCCTAAGCGCCTGATTTATGCTGCATCCAGTTTGCAAAGTGGGGTCCCATCA
AGGTTCAGCGGCAGTGGATCTGGGACAGAATTCACTCTCACAATCAGCAGCCTGCAGCCT
GAAGATTTTGCAACTTATTACTGTCTACAGCATAATAGTTACCCGTGGACGTTCGGCCAA
GGGACCAAGGTGGAAATCAAAC

136 Light Chain Protein:
DIQMTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKRLIYAASSLQSGVPS
RFSGSGSGTEFTLTISSLQPEDFATYYCLQHNSYPWTFGQGTKVEIK

Antibody U1-24

137 Heavy Chain DNA:
CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCACAGACCCTGTCCCTC
ACCTGCACTGTCTCTGGTGGCTCCATCAGTAGTGGTGATTACTACTGGAGCTGGATCCGC
CAGCACCCAGGGAAGGGCCTGGAGTGGATTGGGTACATCTATTACAGTGGGAGCACCTAC
TACAACCCGTCCCTCAAGAGTCGAGTTACCATATCAGTAGACACGTCTAAGAACCAGTTC
TCCCTGAAGTTGAGCTCTGTGACTGCCGCGGACACGGCCGTGTATTACTGTGCGAGAGCC
GATTACGATTTTTGGAATGGTTATTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTC
TCCTCA

138 Heavy Chain Protein:
QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGDYYWSWIRQHPGKGLEWIGYIYYSGSTY
YNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARADYDFWNGYFDYWGQGTLVTV
SS

139 Light Chain DNA:
GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACC
ATCACTTGCCGGGCAAGTCAGGGCATTAGAAATGATTTAGGCTGGTATCAGCAGAAACCA
GGGAAAGCCCCTAAGCGCCTGATCTATGCTGCATCCAGTTTGCAAAGTGGGGTCCCATCA
AGGTTCAGCGGCAGTGGATCTGGGACAGAATTCACTCTCACAATCAGCAGCCTGCAGCCT
GAAGATTTTGCAACTTATTACTGTCTACAGCATAATAATTACCCGTGGACGTTCGGCCAA
GGGACCAAGGTGGAAATCAAA

140 Light Chain Protein:
DIQMTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKRLIYAASSLQSGVPS
RFSGSGSGTEFTLTISSLQPEDFATYYCLQHNNYPWTFGQGTKVEIK

Antibody U1-25

141 Heavy Chain DNA:
CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCACAGACCCTGTCCCTC
ACCTGCACTGTCTCTGGTGGCTCCATCAGCAGTGGTGATTACTACTGGAGCTGGATCCGC
CAGCACCCAGGGAAGGGCCTGGAGTGGATTGGGTACATCTATTACAGTGGGAGCACCTAC

TACAACCCGTCCCTCAAGAGTCGAGTTACCATATCAGTAGACACGTCTAAGAACCAGTTC
TCCCTGAAGCTGAGCTCTGTGACTGCCGCGGACACGGCCGTGTATTACTGTGCGAGAGCC
GATTACGATTTTTGGAGTGGTTATTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTC
TCCTCA

142 Heavy Chain Protein:
QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGDYYWSWIRQHPGKGLEWIGYIYYSGSTY
YNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARADYDFWSGYFDYWGQGTLVTV
SS

143 Light Chain DNA:
GACATCCAGCTGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACC
ATCACTTGCCGGGCAAGTCAGGGCATTAGAAATGATTTAGGCTGGTATCAGCAGAAACCA
GGGAAAGCCCCTAAGCGCCTGATCTATGCTGCATCCAGTTTGCAAAATGGGGTCCCATCA
AGGTTCAGCGGCAGTGGATCTGGGACAGAATTCACTCTCACAATCAGCAGCCTGCAGCCT
GAAGATTTTGCAACTTATTACTGTCTACAGCATAATAGTTACCCGTGGACGTTCGGCCAA
GGGACCAAGGTGGAAATCAAAC

144 Light Chain Protein:
DIQLTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKRLIYAASSLQNGVPS
RFSGSGSGTEFTLTISSLQPEDFATYYCLQHNSYPWTFGQGTKVEIK

Antibody U1-26

145 Heavy Chain DNA:
CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCACAGACCCTGTCCCTC
ACCTGCACTGTCTCTGGTGGCTCCATCAGCAGTGGTGATTACTACTGGAGCTGGATCCGC
CAGTACCCAGGGAAGGGCCTGGAGTGGATTGGGTACATCTATTACAGTGGGAGCACCTAC
TACAACCCGTCCCTCAAGAGTCGAGTTACCATATCAGTAGACACGTCTAAGAACCAGTTC
TCCCTGAAGCTGGGCTCTGTGACTGCCGCGGACACGGCCGTGTATTTCTGTGCGAGAGCC
GATTACGATTTTTGGAGTGGTTATTTTGACTTCTGGGGCCAGGGAACCCTGGTCACCGTC
TCCTC

146 Heavy Chain Protein:
QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGDYYWSWIRQYPGKGLEWIGYIYYSGSTY
YNPSLKSRVTISVDTSKNQFSLKLGSVTAADTAVYFCARADYDFWSGYFDFWGQGTLVTV
S

147 Light Chain DNA:
GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACC
ATCACTTGCCGGGCAAGTCAGGGCATTAGAAATGATTTAGGCTGGTATCAGCAGAAACCA
GGGAAAGCCCCTAAGCGCCTGATCTATGCTGCATCCAGTTTGCAAAGTGGGGTCCCATCA
AGGTTCAGCGGCAGTGGATCTGGGACAGAATTCACTCTCACAATCAGCAGCCTGCAGCCT
GAAGATTTTGCAACTTATTACTGTCTACAGCATAATGGTTACCCGTGGACGTTCGGCCAA
GGGACCAAGGTGGAAATCAAAC

148 Light Chain Protein:
DIQMTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKRLIYAASSLQSGVPS
RFSGSGSGTEFTLTISSLQPEDFATYYCLQHNGYPWTFGQGTKVEIK

Antibody U1-27

149 Heavy Chain DNA:
CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCACAGACCCTGTCCCTC
ACCTGCACTGTCTCTGGTGGCTCCATCAGCAGTGGTGATTACTACTGGAGCTGGATCCGC
CAGTACCCAGGGAAGGGCCTGGAGTGGATTGGGTACATCTATTACAGTGGGAGCACCTAC
TACAACCCGTCCCTCAAGAGTCGAGTTACCATATCAGTAGACACGTCTAAGAACCAGTTC
TCCCTGAAGCTGGGCTCTGTGACTGCCGCGGACACGGCCGTGTATTTCTGTGCGAGAGCC
GATTACGATTTTTGGAGTGGTTATTTTGACTTCTGGGGCCAGGGAACCCTGGTCACCGTC
TCCTC

150 Heavy Chain Protein:

QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGDYYWSWIRQYPGKGLEWIGYIYYSGSTY
YNPSLKSRVTISVDTSKNQFSLKLGSVTAADTAVYFCARADYDFWSGYFDFWGQGTLVTV
S

151 Light Chain DNA:
GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACC
ATCACTTGCCGGGCAAGTCAGGGCATTAGAAATGATTTAGGCTGGTATCAGCAGAAACCA
GGGAAAGCCCCTAAGCGCCTGATCTATGCTGCATCCAGTTTGCAAAGTGGGGTCCCATCA
AGGTTCAGCGGCAGTGGATCTGGGACAGAATTCACTCTCACAATCAGCAGCCTGCAGCCT
GAAGATTTTGCAACTTATTACTGTCTACAGCATAATGGTTACCCGTGGACGTTCGGCCAA
GGGACCAAGGTGGAAATCAAAC

152 Light Chain Protein:
DIQMTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKRLIYAASSLQSGVPS
RFSGSGSGTEFTLTISSLQPEDFATYYCLQHNGYPWTFGQGTKVEIK

Antibody U1-28

153 Heavy Chain DNA:
CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCACAGACCCTGTCCCTC
ACCTGCACTGTCTCTGGTGGCTCCATCAGTAGTGGTGATTACTACTGGAGCTGGATCCGC
CAGCACCCAGGGAAGGGCCTGGAGTGGATTGGGTACATCTATTACAGTGGGAGCACCTAC
TACAACCCGTCCCTCAAGAGTCGAGTTACCATATCAGTAGACACGTCTAAGAACCAGTTC
TCCCTGAAGCTGAGCTCTGTGACTGCCGCGGACACGGCCGTGTATTACTGTGCGAGAGCG
GATTACGATTTTTGGAGTGGTTATTTTGACTCCTGGGGCCAGGGAACCCTGGTCACCGTC
TCCTCA

154 Heavy Chain Protein:
QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGDYYWSWIRQHPGKGLEWIGYIYYSGSTY
YNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARADYDFWSGYFDSWGQGTLVTV
SS

155 Light Chain DNA:
GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACC
ATCACTTGCCGGGCAAGTCAGGGCATTAGAAATGATTTAGGCTGGTATCAGCAGATACCT
GGGAAAGCCCCTAAGCGCCTGATCTATGCTGCATCCAGTTTGCAAAGTGGGGTCCCATCA
AGGTTCAGCGGCAGTGGATCTGGGACAGAATTCACTCTCACAATCAGCAGCCTGCAGCCT
GAAGATTTTGCAACTTATTACTGTCTACAGCATAATGGTTACCCGTGGACGTTCGGCCAA
GGGACCAAGGTGGAAATCAAA

156 Light Chain Protein:
DIQMTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQIPGKAPKRLIYAASSLQSGVPS
RFSGSGSGTEFTLTISSLQPEDFATYYCLQHNGYPWTFGQGTKVEIK

Antibody U1-31

157 Heavy Chain DNA:
CAGGTTCAGCTGGTGCAGTCTGGAGCTGAGGTGAAGAAGCCTGGGGCCTCAGTGAAGGTC
TCCTGCAAGGCTTCTGGTTACACCTTTACCAACTATGGTATCAGCTGGGTGCGGCAGGCC
CCTGGACAAGGGCTTGAGTGGATGGGATGGATCAGCGCTTACGATGGTTACAGAAACTAT
GCACAGAAGCTCCAGGGCAGAGTCACCATGACCACAGACACATCCACGACCACTGCCTAC
ATGGAGCTGAGGAGCCTGAGATCTGACGACACGGCCGTGTATTACTGTGCGAGAGATGTT
CAAGACTACGGTGACTACGACTACTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTC
TCCTCA

158 Heavy Chain Protein:
QVQLVQSGAEVKKPGASVKVSCKASGYTFTNYGISWVRQAPGQGLEWMGWISAYDGYRNY
AQKLQGRVTMTTDTSTTTAYMELRSLRSDDTAVYYCARDVQDYGDYDYFDYWGQGTLVTV
SS

159 Light Chain DNA:
GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACC

ATCACTTGCCGGGCAAGTCAGAGCATTAGCAGTTATTTAAATTGGTATCAGCAGAAACCA
GGGAAAGCCCCTAACCTCCTGATCTATGCTGCATCCAGTTTGCAAAGTGGGGTCCCATCA
AGATTCAGGGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGCAGTCTGCAACCT
GAAGATTTTGCAACTTACTACTGTCAACAGAGTTACAGTACCCCCATCACCTTCGGCCAA
GGGACACGACTGGAGATTAAA

160 Light Chain Protein:
DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPNLLIYAASSLQSGVPS
RFRGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPITFGQGTRLEIK

Antibody U1-32

161 Heavy Chain DNA:
CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTTACAGACCCTGTCCCTC
ACCTGCACTGTCTCTGGTGGCTCCATCAGCAGTGGTGATTACTACTGGAGCTGGATCCGC
CAGCACCCAGGGAAGGGCCTGGAGTGGATTGGGTACATCTATTACAGTGGGACCACCTAC
TACAACCCGTCCCTCAAGAGTCGAGTTACCATATCAGTAGACACGTCTAAGAACCAGTTC
GCCCTGAAGCTGAACTCTGTGACTGCCGCGGACACGGCCGTGTATTACTGTGCGAGAGCC
GATTACGATTTTTGGAGTGGTTATTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTC
TCCTCA

162 Heavy Chain Protein:
QVQLQESGPGLVKPLQTLSLTCTVSGGSISSGDYYWSWIRQHPGKGLEWIGYIYYSGTTY
YNPSLKSRVTISVDTSKNQFALKLNSVTAADTAVYYCARADYDFWSGYFDYWGQGTLVTV
SS

163 Light Chain DNA:
GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACC
ATCACTTGCCGGGCAGGTCAGGGCATTAGAAATGATTTAGGCTGGTATCAGCAGAAACCA
GGGAAAGCCCCTCAGCGCCTGATCTATGCTGCATCCAGTTTGCAAAGTGGGGTCCCATCA
AGGTTCAGCGGCAGTGGATCTGGGACAGAATTCTCTCTCACAATCTCCAGCCTGCAGCCT
GAAGATTTTGCAACTTATTACTGTCTACAGCATAATAGTTACCCGTGGACGTTCGGCCAA
GGGACCAAGGTGGAAATCAAAC

164 Light Chain Protein:
DIQMTQSPSSLSASVGDRVTITCRAGQGIRNDLGWYQQKPGKAPQRLIYAASSLQSGVPS
RFSGSGSGTEFSLTISSLQPEDFATYYCLQHNSYPWTFGQGTKVEIK

Antibody U1-35

165 Heavy Chain DNA:
CAGGTGCAGCTGGTGGAGTCTGGGGGAGGCTTGGTCAAGCCTGGAGGGTCCCTGAGACTC
TCCTGTGCAGCCTCTGGATTCACCTTCAGTGACTACTACATGAGCTGGATCCGCCAGGCT
CCAGGGAAGGGGCTGGAGTGGGTTTCATATATTAGTAGTAGTGGTAATAACATATACCAC
GCAGACTCTGTGAAGGGCCGATTCACCATCTCCAGGGACAACGCCAAGAACTCACTGTAT
CTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTGTATTACTGTGCGAGAGAGAGA
TATAGTGGCTACGACGACCCTGATGGTTTTGATATCTGGGGCCAAGGGACAATGGTCACC
GTCTCTTCA

166 Heavy Chain Protein:
QVQLVESGGGLVKPGGSLRLSCAASGFTFSDYYMSWIRQAPGKGLEWVSYISSSGNNIYH
ADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARERYSGYDDPDGFDIWGQGTMVT
VSS

167 Light Chain DNA:
GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACC
ATCACTTGCCAGGCGAGTCAGGACATTAGCAACTATTTAAGTTGGTTTCAGCAGAAACCA
GGGAAAGCCCCTAAGCTCCTGATCCACGATGCATCCAATTTGGAAACAGGGGTCCCTTCA
AGGTTCAGTGGAAGTGGATCTGGGACAGATTTTACTTTCACCATCAGCAGCCTGCAGCCT
GAAGATATTGCAACATATTACTGTCAACAGTATGATAATCCCCCGTGCAGTTTTGGCCAG
GGGACCAAGCTGGAGATCAAA

168 Light Chain Protein:
DIQMTQSPSSLSASVGDRVTITCQASQDISNYLSWFQQKPGKAPKLLIHDASNLETGVPS
RFSGSGSGTDFTFTISSLQPEDIATYYCQQYDNPPCSFGQGTKLEIK

Antibody U1-36
169 Heavy Chain DNA:
CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCACAGACCCTGTCCCTC
ACCTGCACTGTCTCTGGTGGCTCCATCAGCAGTGGTTATTACTACTGGAGCTGGATCCGC
CAGCACCCAGGGAAGGGCCTGGAGTGGATTGGGTACATCTATTACAGTGGGACCACCTAC
TACAATCCGTCCTTCAAGAGTCGAGTTACCATATCAGTAGACACGTCTAAGAACCAGTTC
TCCCTGAAACTGAGCTCTGTGACTGCCGCGGACACGGCCGTGTATTACTGTGCGAGAGCC
GATTACGATTTTTGGAGTGGTCACTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTC
TCCTCA
170 Heavy Chain Protein:
QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGYYYWSWIRQHPGKGLEWIGYIYYSGTTY
YNPSFKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARADYDFWSGHFDYWGQGTLVTV
SS
171 Light Chain DNA:
GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACC
ATCACTTGCCGGGCAAGTCAGGGCATTAGAAATGATTTAGGCTGGTATCAGCAGAAACCA
GGGAAAGCCCCTAAGCGCCTGATCTATGCTGCATCCAGTTTGCAAAGTGGGGTCCCATCA
AGGTTCAGCGGCAGTGGATCTGGGACAGAATTCACTCTCACAATCAGCAGCCTGCAGCCT
GAAGATTTTGCAACTTATTACTGTCTACAGCATAATAGTTACCCGTGGACGTTCGGCCAA
GGGACCAAGGTGGAAATCAAA
172 Light Chain Protein:
DIQMTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKRLIYAASSLQSGVPS
RFSGSGSGTEFTLTISSLQPEDFATYYCLQHNSYPWTFGQGTKVEIK

Antibody U1-37
173 Heavy Chain DNA:
CAGGTTCAGCTGGTGCAGTCTGGAGCTGAGGTGAAGAAGCCTGGGGCCTCAGTGAAGGTC
TCCTGCAAGGCTTCTGGTTACACCTTTACCAGCTATGGTATCAGCTGGGTGCGACAGGCC
CCTGGACAAGGACTTGAGTGGATGGGATGGATCAGCGCTTACGATGGTCACACAAACTAT
GCACAGAAGCTCCAGGGCAGAGTCACCATGACCACAGACACATCCACGAACACAGCCTAC
ATGGAGCTGAGGAGCCTGAGATCTGACGACACGGCCGTTTATTACTGTGCGAGAGACCCC
CATGACTACAGTAACTACGAGGCTTTTGACTTCTGGGGCCAGGGAACCCTGGTCACCGTC
TCCTC
174 Heavy Chain Protein:
QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYGISWVRQAPGQGLEWMGWISAYDGHTNY
AQKLQGRVTMTTDTSTNTAYMELRSLRSDDTAVYYCARDPHDYSNYEAFDFWGQGTLVTV
S

175 Light Chain DNA
atgaggtcccctgctcagctcctggggctcctgctactctggctccgaggtgccagatgtg
acatccagatgacccagtctccatcctccctgtctgcatctgtaggagacagagtcaccat
cacttgccgggcaagtcagagcattagcagttatttaaattggtatcagcagaaaccaggg
aaagcccctaacctcctgatctatgctgcatccagtttgcaaagtggggtcccatcaagat
tcagtggcagtggatctgggacagatttcactctcaccatcagcagtctgcaacctgaaga
ttttgcaacttactactgtcaacagagttacagtaccccatcaccttcggccaagggaca
cgactggagattaaacgaactgtggctgcaccatctgtcttcatcttcccgccatctgatg
agcagttgaaatctggaactgcctctgttgtgtgcctgctgaataacttctatcccagaga
ggccaaagtacagtggaaggtggataacgcc

176 Light Chain Protein:
DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPNLLIYAASSLQSGVPSR
FSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPITFGQGTRLEIK

Antibody U1-34

177 Heavy Chain DNA:
accatggactggacctggagggtccttttcttggtggcagcagcaacaggtgcccactccca
ggttcagctggtgcagtctggagctgaggtgaagaagcctggggcctcagtgaaggtctcct
gcaaggcttctggttacacctttaccaactatggtatcagctgggtgcggcaggcccctgga
caagggcttgagtggatgggatggatcagcgcttacgatggttacagaaactatgcacagaa
gctccagggcagagtcaccatgaccacagacacatccacgaccactgcctacatggagctga
ggagcctgagatctgacgacacggccgtgtattactgtgcgagagatgttcaagactacggt
gactacgactactttgactactggggccagggaaccctggtcaccgtctcctcagcttccac
caagggcccatccgtcttccccctggtgccctgctccaggagcacctccgagagcacagccg
ccctgggctgcctggtcaaggactacttccccgaaccg

178 Heavy Chain Protein
QVQLVQSGAEVKKPGASVKVSCKASGYTFTNYGISWVRQAPGQGLEWMGWISAYDGYRNYA
QKLQGRVTMTTDTSTTTAYMELRSLRSDDTAVYYCARDVQDYGDYDYFDYWGQGTLVTVSS

179 Light Chain DNA:
cagctcctggggctcctgctactctggctccgaggtgccagatgtgacatccagatgaccc
agtctccatcctccctgtctgcatctgtaggagacagagtcaccatcacttgccgggcaag
tcagagcattagcagttatttaaattggtatcagcagaaaccagggaaagcccctaacctc
ctgatctatgctgcatccagtttgcaaagtggggtcccatcaagattcagtggcagtggat
ctgggacagatttcactctcaccatcagcagtctgcaacctgaagattttgcaacttacta
ctgtcaacagagttacagtacccccatcaccttcggccaagggacacgactggagattaaa
cgaactgtggctgcaccatctgtcttcatcttcccgccatctgatgagcagttgaaatctg
gaactgcctctgttgtgtgcctgctgaataacttctatcccagagaggccaaagtacagtg
gaaggtggataacgcc

180 Light Chain Protein:
DIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPNLLIYAASSLQSGVPSR
FSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPITFGQGTRLEIK

Antibody U1-1

181 Heavy Chain DNA:
catctgtggttcttcctcctgctggtggcagctcccagatgggtcctgtcccaggtgcagc
tgcaggagtcgggcccaggactggtgaagccttcacagaccctgtccctcacctgcactgt
ctctggtggctccatcaacagtggtgattactactggagctggatccgccagcacccaggg
aagggcctggagtggattgggtacatctattacagtgggagcacctactacaacccgtccc
tcaagagtcgagttaccatatcagtagacacgtctaagaaccagttctccctgaagctgag
ctctgtgactgccgcggacacggccgtgtattactgtgcgagagcagattacgatttttgg
agtggttactttgactactggggccagggaaccctggtcaccgtctcctcagcctccacca
agggcccatcggtcttccccctggcaccctcctccaagagcacctctgggggcacaacggc
cctgg

182 Heavy Chain Protein
QVQLQESGPGLVKPSQTLSLTCTVSGGSINSGDYYWSWIRQHPGKGLEWIGYIYYSGSTYY
NPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARADYDFWSGYFDYWGQGTLVTVSS

183 Light Chain DNA:
atgagggtccctgctcagctcctggggctcctgctgctctggttcccaggtgccaggtgtga
catccagatgacccagtctccatcctccctgtctgcatctgtaggagacagagtcaccatca
cttgccgggcaagtcagggcattagaaatgatttaggctggtatcagcagaaaccagggaaa
gcccctaagcgcctgatctatgctgcatccagtttgcaaagtggggtcccatcaaggttcag
cggcagtggatctgggacagaattcactctcacaatcagcagcctgcagcctgaagattttg
caacttattactgtctacagcataatagttacccgtggacgttcggccaagggaccaaggtg
gaaatcaaacgaactgtggctgcaccatctgtcttcatcttcccgccatctgatgagcagtt
gaaatctggaactgcctctgttgtgtgcctgctgaataacttctatcccagagaggccaaag
tacagtggaaggtggataacgc

184 Light Chain Protein
DIQMTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKRLIYAASSLQSGVPSR
FSGSGSGTEFTLTISSLQPEDFATYYCLQHNSYPWTFGQGTKVEIK

Antibody U1-3
185 Heavy Chain DNA:
tggttcttccttctgctggtggcagctcccagatgggtcctgtcccaggtgcagctgcagga
gtcgggcccaggactggtgaagccttcacagaccctgtccctcacctgcactgtctctggtg
gctccatcagcagtggtggttactactggagctggatccgccagcacccagggaagggcctg
gagtggattgggtacatctattacagtgggagcacctactacaacccgtccctcaagagtcg
agttaccatatcagtagacacgtctaagaaccagttctccctgaagctgagctctgtgactg
ccgcggacacggccgtgtattactgtgcgagagatggctatgatagtagtggttattaccac
ggctactttgactactggggccagggaaccctggtcaccgtctcctcagcctccaccaaggg
cc

186 Heavy Chain Protein
QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGGYYWSWIRQHPGKGLEWIGYIYYSGSTYY
NPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARDGYDSSGYYHGYFDYWGQGTLVT
VSS

187 Light Chain DNA:
H3_130_1N1K
caggtcttcatttctctgttgctctggatctctggtgcctacggggacatcgtgatgaccc
agtctccagactccctggctgtgtctctgggcgagagggccaccatcaactgcaagtccag
ccagagtgttttatacagctccaacaataagaactacttagcttggtaccagcagaaacca
ggacagcctcctaagctgctcatttactgggcatctacccgggaatccggggtccctgacc
gattcagtggcagcgggtctgggacagatttcactctcaccatcagcagcctgcaggctga
agatgtggcagtttattactgtcagcaatattatagtactccgctcactttcggcggaggg
accaaggtggagatcaaacgaactgtggctgcaccatctgtcttcatcttcccgccatctg
atgagcagttgaaatctggaactgcctctgttgtgtgcctgctgaataacttctatcccag
agaggccaaagtacagtggaaggtggataacgc

188 Light Chain Protein:
DIVMTQSPDSLAVSLGERATINCKSSQSVLYSSNNKNYLAWYQQKPGQPPKLLIYWASTRE
SGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQQYYSTPLTFGGGTKVEIK

Antibody U1-4
H3_133_1N1G1

189 Heavy Chain DNA
ctgtggttcttcctcctgctggtggcagctcccagatgggtcctgtcccaggtgcagctgca
ggagtcgggcccaggactggtgaagccttcacagaccctgtccctcacctgcactgtctctg
gtggctccatcagtagtggtgattactactggagctggatccgccagcacccagggaagggc
ctggagtggattgggtacatctattacagtgggagcacctactacaacccgtccctcaagag
tcgagttaccatatcagtagacacgtctaagaaccagttctccctgaagttgagctctgtga
ctgccgcggacacggccgtgtattactgtgcgagagccgattacgatttttggagtggttat
tttgactactggggccagggaaccctggtcaccgtctcctcagcctccaccaagggcccatc
ggtcttcccccctggcaccctc

190 Heavy Chain Protein
QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGDYYWSWIRQHPGKGLEWIGYIYYSGSTYY
NPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARADYDFWSGYFDYWGQGTLVTVSS

191 Light Chain DNA
H3_133_1N1K
gtgcccgctcagcgcctggggctcctgctgctctggttcccaggtgccaggtgtgacatcc
agatgacccagtctccatcctccctgtctgcatctgtaggagacagagtcaccatcacttg
ccgggcaagtcagggcattagaaatgatttaggctggtatcagcagaaaccagggaaagcc
cctaagcgcctgatctatgctgcatccagtttgcaaagtggggtcccatcaaggttcagcg
gcagtggatctgggacagaattcactctcacaatcagcagcctgcagcctgaagattttgc
aacttattactgtctacagcataataattacccgtggacgttcggccaagggaccaaggtg
gaaatcaaacgaactgtggctgcaccatctgtcttcatcttcccgccatctgatgagcagt
tgaaatctggaactg

192 Light Chain Protein
DIQMTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKRLIYAASSLQSGVPSRF
SGSGSGTEFTLTISSLQPEDFATYYCLQHNNYPWTFGQGTKVEIK

Antibody U1-5

193 Heavy Chain DNA:
H3_138_1N1G1
tggttcttccttctgctggtggcagctcccagatgggtcctgtcccaggtgcagctgcagga
gtcgggcccaggactggtgaagccttcacagaccctgtccctcacctgcactgtctctggtg
gctccatcagcagtggtgattactactggagctggatccgccagcacccagggaagggcctg
gagtggattgggtacatctattacagtgggagcacctactacaacccgtccctcaagagtcg
agttaccatatcagtagacacgtctaagaaccagttctccctgaagctgagctctgtgactg
ccgcggacacggccgtgtatttctgtgcgagagccgattacgatttttggagtggttatttt
gactactggggccagggaaccctggtcaccgtctcctcagcctccaccaagggcc

194 Heavy Chain Protein
QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGDYYWSWIRQHPGKGLEWIGYIYYSGSTYYN
PSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYFCARADYDFWSGYFDYWGQGTLVTVSS

195 Light Chain DNA:
H3_138_1N1K
atgagggtccccgctcagctcctggggctcctgctgctctggttcccaggtgccaggtgtga
catccagatgacccagtctccatcctccctgtctgcatctgtaggagacagagtcaccatca
cttgccgggcaagtcagggcattagaaatgatttaggctggtatcagcagaaaccagggaaa

gcccctaagcgcctgatctatgctgcatccagtttgcaaagtggggtcccatcaaggttcag
cggcagtggatctgggacagaattcactctcacaatcagcagcctgcagcctgaagattttg
caacttattactgtctacagcataatacttacccgtggacgttcggccaagggaccaaggtg
gaaatcaaacgaactgtggctgcaccatctgtcttcatcttcccgccatctgatgagcagtt
gaaatctggaactgcctctgttgtgtgcctgctgaataacttctatcccagagaggccaaag
tacagtggaaggtggataacgc

196 Light Chain Protein
DIQMTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKRLIYAASSLQSGVPS
RFSGSGSGTEFTLTISSLQPEDFATYYCLQHNTYPWTFGQGTKVEIK

Antibody U1-6

197 Heavy Chain DNA:
H3_162_1N1G1
tggttcttccttctgctggtggcagctcccagatgggtcctgtcccaggtgcagctgcagga
gtcgggcccaggactggtgaagccttcacagaccctgtccctcacctgcactgtctctggtg
gctccatcagcagtggtgattactactggagctggatccgccagcacccagggaagggcctg
gagtggattgggtacatctattacagtgggagcacctactacaacccgtccctcaagagtcg
agttaccatatcagtagacacgtctaagaaccagttctccctgaagctgagctctgtgactg
ccgcggacacggccgtgtatttctgtgcgagagccgattacgatttttggaatggttatttt
gactactggggccagggaaccctggtcaccgtctcctcagcctccaccaagggccc

198 Heavy Chain Protein
QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGDYYWSWIRQHPGKGLEWIGYIYYSGSTY
YNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYFCARADYDFWNGYFDYWGQGTLVTV
SS

199 Light Chain DNA:
H3_162_1N1K
atgagggtccccgctcagctcctggggctcctgctgctctggttcccaggtgccaggtgtga
catccagatgacccagtctccatcctccctgtctgcatctgtaggagacagagtcaccatca
cttgccgggcaagtcagggcattagaaatgatttaggctggtatcagcagaaaccagggaaa
gcccctaagcgcctgatctatgctgcttccagtttgcaaagtggggtcccatcaaggttcag
cggcagtggatctgggacagaattcactctcacaatcagcagcctgcagcctgaagattttg
caacttattactgtctacagcataatacttacccgtggacgttcggccaagggaccaaggtg
gaaatcaaacgaactgtggctgcaccatctgtcttcatcttcccgccatctgatgagcagtt
gaaatctggaactgcctctgttgtgtgcctgctgaataacttctatcccagagaggccaaag
tacagtggaaggtggataacgcc

200 Light Chain Protein
DIQMTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKRLIYAASSLQSGVPS
RFSGSGSGTEFTLTISSLQPEDFATYYCLQHNTYPWTFGQGTKVEIK

Antibody U1-8

201 Heavy Chain DNA:
H3_174_1N1G1
ttggtggcagcagctacaggcacccacgcccaggtccagctggtacagtctggggctgaggt
gaagaagcctggggcctcagtgaaggtctcctgcaaggtttccggatacaccctcactgaat
tatccatgtactgggtgcgacaggctcctggaaaagggcttgagtggatgggaggttttgat

cctgaagatggtgaaacaatctacgcacagaagttccagggcagagtcaccatgaccgagga
cacatctacagacacagcctacatggagctgagcagcctgagatctgaggacacggccgtgt
attactgtgcaactgggtggaactacgtctttgactactggggccagggaaccctggtcacc
gtctcctcagcctccaccaagggccc

202 Heavy Chain Protein
QVQLVQSGAEVKKPGASVKVSCKVSGYTLTELSMYWVRQAPGKGLEWMGGFDPEDGETIYA
QKFQGRVTMTEDTSTDTAYMELSSLRSEDTAVYYCATGWNYVFDYWGQGTLVTVSS

203 Light Chain DNA:
H3_174_1N1K
ggatccagtggggatattgtgatgactcagtctccactctccctgcccgtcacccctggaga
gccggcctccatctcctgcaggtccagtcagagcctcctgcatagtaatggatacaactatt
tggattggtacctgcagaagccagggcagtctccacagctcctgatctatttggattctcat
cgggcctccggggtccctgacaggttcagtggcagtggatcaggcacagattttacactgaa
aatcagcagagtggaggctgaggatgttggggtttattactgcatgcaagctctacaaactc
cgctcactttcggcggagggaccaaggtggagatcaaacgaactgtggctgcaccatctgtc
ttcatcttcccgccat

204 Light Chain Protein
DIVMTQSPLSLPVTPGEPASISCRSSQSLLHSNGYNYLDWYLQKPGQSPQLLIYLDSHRA
SGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCMQALQTPLTFGGGTKVEIK

Antibody U1-11
205 Heavy Chain DNA:
H3_178_1N1G1
tggttcttccttctgctggtggcagctcccagatgggtcctgtcccaggtgcagctgcagga
gtcgggcccaggactggtgaagccttcacagaccctgtccctcacctgcactgtctctggtg
gctccatcagcagtggtgattactactggagctggatccgccagcacccagggaagggcctg
gagtggattgggtacatctattacagtgggagcacctactacaacccgtccctcaagagtcg
agttaccatatcagtagacacgtctaagaaccagttctccctgaagctgagctctgtgactg
ccgcggacacggccgtgtatttctgtgcgagagccgattacgatttttggagtggttatttt
gactactggggccagggaaccctggtcaccgtctcctcagcctccaccaagggcccatcgag
tcttcccccctgg

206 Heavy Chain Protein
QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGDYYWSWIRQHPGKGLEWIGYIYYSGSTY
YNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYFCARADYDFWSGYFDYWGQGTLVTV
SS

207 Light Chain DNA:
H3_178_1N1K
atgagggtccccgctcagctcctggggctcctgctgctctggttcccaggtgccaggtgtg
acatccagatgacccagtctccatcctccctgtctgcatctgtaggagacagagtcaccat
cacttgccgggcaagtcagggcattagaaatgatttaggctggtatcagcagaaaccaggg
aaagcccctaagcgcctgatctatgctgcatccagtttgcaaagtggggtcccatcaaggt
tcagcggcagtggatctgggacaaaattcactctcactatcagcagcctgcagcctgaaga
ttttgcaacttattactgtctacagcataatacttacccgtggacgttcggccaagggacc
aaggtggaaatcagacgaactgtggctgcaccatctgtcttcatcttcccgccatctgatg
agcagttgaaatctggaactgcctctgttgtgtgcctgctgaataacttctatcccagaga
ggccaaagtacagtggaaggtggataacgcc

208 Light Chain Protein
DIQMTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKRLIYAASSLQSGVP
SRFSGSGSGTKFTLTISSLQPEDFATYYCLQHNTYPWTFGQGTKVEIR

Antibody U1-16
209 Heavy Chain DNA:
H3_221_1N1G1
accatgaaacatctgtggttcttcctcctgctggtggcagctcccagatgggtcctgtccc
aggtgcagctgcaggagtcgggcccaggactggtgaagccttcacagaccctgtccctcac
ctgcactgtctctggtggctccatcagcagtggtgattactactggagctggatccgccag
cacccagggaagggcctggagtggattgggtacatctattacagtgggagcacctactaca
acccgtccctcaagagtcgagttaccatatcagtagacacgtctaagaaccagttctccct
gaagctgagctctgtgactgccgcggacacggccgtgtattactgtgcgagagcggattac
gatttttggagtggttattttgactactggggccagggaatcctggtcaccgtctcctcag
cctccaccaagggcccatcggtcttccccctggcaccctcctccaagaacacctctggggg
cacagcggccctgggctgcctggtcaaggactacttccccgaaccggtgacggtgtcctgg
aactcaggcgccctg

210 Heavy Chain Protein
QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGDYYWSWIRQHPGKGLEWIGYIYYSGSTYY
NPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARADYDFWSGYFDYWGQGILVTVSS

211 Light Chain DNA:
H3_221_1N1K
atgagggtccccgctcagctcctggggctcctgctgctctggttcccaggtgccaggtgt
gacatccagatgacccagtctccatcctccctgtctgcatctgtaggagacagagtcacc
atcacttgccgggcaagtcagggcattagaaatgatttaggctggtatcagcagaaacca
gggaaagcccctaagcgcctgatctatgctgcatccagtttgcaaagtggggtcccatca
aggttcagcggcagtggatctgggacagaattcactctcacaatcagcagcctgcagcct
gaagattttgcaacttattactgtctacagcataatagttacccgtggacgttcggccaa
gggaccaaggtggaaatcaaacgaactgtggctgcaccatctgtcttcatcttcccgcca
tctgatgagcagttgaaatctggaactgcctctgttgtgtgcctgctgaataacttctat
cccagagaggccaaagtacagtggaaggtggataacgcc

212 Light Chain Protein
DIQMTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKRLIYAASSLQSGVPSR
FSGSGSGTEFTLTISSLQPEDFATYYCLQHNSYPWTFGQGTKVEIK

Antibody U1-17
213 Heavy Chain DNA:
H3_224_1N1G1
tggttcttccttctgctggtggcagctcccagatgggtcctgtcccaggtgcagctgcagg
agtcgggcccaggactggtgaagccttcacagaccctgtccctcacctgcactgtctctgg
tggctccatcagcagtggtgattactactggagctggatccgccagcacccagggaagggc
ctggagtggattggatacatctattacagtgggagcacctactacaattcgtccctcaaga
gtcgagttaccatatcagtagacacgtctaagaaccagttctccctgaagctgagctctgt
gactgccgcggacacggccgtgtattactgtgcgagagcggattacgatttttggagtggt
tattttgactactggggccagggaaccctggtcaccgtctcctcagcctccaccaagggcc
catcg

214 Heavy Chain Protein
QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGDYYWSWIRQHPGKGLEWIGYIYYSGSTYY
NSSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARADYDFWSGYFDYWGQGTLVTVSS

215 Light Chain DNA:
H3_224_1N1K
ggtgccaggtgtgacatccagatgacccagtctccatcctccctgtctgcatctgtaggag
acagagtcaccatcacttgccgggcaagtcagggcattagaaatgatttaggctggtatca
gcagaaacctgggaaagcccctaagcgcctgatctatgctgcatccagtttgcaaagtggg
gtcccatcaaggttcagcggcagtggatctgggacagaattcactctcacaatcagcagcc
tgcagcctgaagattttgcaacttattactgtctacagcacaatagttacccgtggacgtt
cggccaagggaccaaggtggaaatcaaacgaactgtggctgcaccatctgtcttcatcttc
ccgcca

216 Light Chain Protein
DIQMTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKRLIYAASSLQSGVPS
RFSGSGSGTEFTLTISSLQPEDFATYYCLQHNSYPWTFGQGTKVEIK

Antibody U1-18
217 Heavy Chain DNA:
H3_227_1N1G1
aggttcttccttctgctggtggcagctcccagatgggtcctgtcccaggtgcagctgcagg
agtcgggcccaggactggtgaagccttcacagaccctgtccctcacctgcactgtctctgg
tggctccatcagcagtggtgattactactggagctggatccgccagcacccagggaagggc
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gtcgagttaccatatcagtagacacgtctaagaaccagttctccctgaagctgagctctgt
gactgccgcggacacggccgtgtattactgtgcgagagccgattacgatttttggagtggt
tattttgactactggggccagggaaccctggtcaccgtctcctcagcctccaccaagggcc
catcggtcttcccctggcaccctcctccaagagcacctctgggggcacagcggccctggg
ctgcctggtcaaggactacttccccgaaccggtgacggtgtcgtggaactcaggcgccct

218 Heavy Chain Protein
QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGDYYWSWIRQHPGKGLEWIGYIYYSGSTYY
NPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARADYDFWSGYFDYWGQGTLVTVSS

219 Light Chain DNA:
H3_227_1N1K
atgagggtccccgctcagctcctggggctcctgctgctctggttcccaggtgccaggtgtga
catccagatgacccagtctccatcctccctgtctgcatctgtaggagacagagtcaccatca
cttgccgggcaagtcagggcattagaaatgatttaggctggtatcagcagaaaccagggaaa
gcccctaagcgcctgatctatgctgcatccagtttgcaaagtggggtcccatcaaggttcag
cggcagtggatctgggacagaattcactctcacaatcagcagcctgcagcctgaagattttg
caacttattactgtctacagcataatagttacccgtggacgttcggccaagggaccaaggtg
gaaatcaaacgaactgtggctgcaccatctgtcttcatcttcccgccatctgatgagcagtt
gaaatctggaactgcctctgttgtgtgcctgctgaataacttctatcccagagaggccaaag
tacagtggaaggtggataacg

220 Light Chain Protein
DIQMTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKRLIYAASSLQSGVPS
RFSGSGSGTEFTLTISSLQPEDFATYYCLQHNSYPWTFGQGTKVEIK

Antibody U1-33

221 Heavy Chain DNA:
H4_14_1N1G4
ctgtggttcttccttctgctggtggcagctcccagatgggtcctgtcccaggtgcagctgc
aggagtcgggcccaggactggtgaagccttcacagaccctgtccctcacctgcactgtctc
tggtggctccatcagcagtggtgattactactggagctggatccgccagcacccagggaag
ggcctggagtggattgggtacatctattacagtgggagcacctactacaacccgtccctca
agagtcgagttaccatgtcagtagacacgtctaagaaccagttctccctgaagctgagctc
tgtgactgccgcggacacggccgtgtattactgtgcgagagccgattacgatttttggagt
ggtcactttgactgctggggccagggaaccctggtcaccgtctcctcagcttccaccaagg
gccccatccgtcttccccc

222 Heavy Chain Protein
QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGDYYWSWIRQHPGKGLEWIGYIYYSGSTYY
NPSLKSRVTMSVDTSKNQFSLKLSSVTAADTAVYYCARADYDFWSGHFDCWGQGTLVTVSS

223 Light Chain DNA:
H4_14_1N1K
atgagggtccccgctcagctcctggggctcctgctgctctggttcccaggtgccaggtgtga
catccagatgacccagtctccatcctccctgtctgcatctgtaggagacagagtcaccatca
cttgccgggcaagtcagggcattagagatgatttaggctggtatcagcagaaaccagggaaa
gcccctaagcgcctgatctatgctgaatccagtttgcaaagtggggtcccatcaaggttcag
cggcagtggatctgggacagaattcactctcacaatcagcagcctgcagcctgaagattttg
caacttattactgtctacagcatcatagttacccgtggacgttcggccaagggaccaaggtg
gaaatcaaacgaactgtggctgcaccatctgtcttcatcttcccgcc

224 Light Chain Protein
DIQMTQSPSSLSASVGDRVTITCRASQGIRDDLGWYQQKPGKAPKRLIYAESSLQSGVPSR
FSGSGSGTEFTLTISSLQPEDFATYYCLQHHSYPWTFGQGTKVEIK

Antibody U1-29

225 Heavy Chain DNA:
H4_107_1N1G4
tggctgagctgggttttcctcgttgctcttttaagaggtgtccagtgtcaggtgcagctgg
tggagtctgggggaggcgtggtccagcctgggaggtccctgagactctcctgtgcagcgtc
tggattcaccttcaatagctatgacatgcactgggtccgccaggctccaggcaaggggctg
gagtgggtggcagttatatggtatgatggaagtaataaatactatgcagactccgtgaagg
gccgattcaccatctctagagacaattccaagaacacgctgtatctgcaaatgaacagcct
gagagccgaggacacggctgtgtattactgtgcgagagaccgcttgtgtactaatggtgta
tgctatgaagactacggtatggacgtctggggccaagggaccacggtcaccgtctcctcag
cttccaccaagggcccatccgtcttcccccctggcgccctgctccaggagcacctccgagag
cacagccgccctgggc

226 Heavy Chain Protein
QVQLVESGGGVVQPGRSLRLSCAASGFTFNSYDMHWVRQAPGKGLEWVAVIWYDGSNKYYA
DSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARDRLCTNGVCYEDYGMDVWGQGTTV
TVSS

227 Light Chain DNA:
H4_107_1N1K
atgagggtccctgctcagctcctggggctcctgctgctctggctctcaggtgccagatgtga
catccagatgacccagtctccatcctccctgtctgcatctgtaggagacagagtcaccatca

cttgccaggcgagtcaggacattagcaactatttaaattggtatcagcagaaaccagggaaa
gcccctaaggtcctgatctacgatgcatccaatttggaaacaggggtcccatcaaggttcag
tggaagtggatctgggacagattttactttcaccatcagcagcctgcagcctgaagatgttg
caacatattactgtcaacactatgatactctcccgctcactttcggcggagggaccaaggtg
gagatcaaacgaactgtggctgcaccatctgtcttcatcttcccgccatctgatgagcagtt
gaaatctggaactgcctctgttgtgtgcctgctgaataacttctatcccagagaggccaaag
tacagtgg

228 Light Chain Protein
DIQMTQSPSSLSASVGDRVTITCQASQDISNYLNWYQQKPGKAPKVLIYDASNLETGVPSR
FSGSGSGTDFTFTISSLQPEDVATYYCQHYDTLPLTFGGGTKVEIK

Antibody U1-30
229 Heavy Chain DNA:
H4_116_1_1N1G4
ggactgtgcaagaacatgaaacacctgtggttcttcctcctgctggtggcagctcccagatg
ggtcctgtcccaggtgcagctgcaggagtcgggcccaggactggtgaagcctttacagaccc
tgtccctcacctgcactgtctctggtggctccatcagcagtggtgattactactggagctgg
atccgccagcacccagggaagggcctggagtggattgggtacatctattacagtgggaccac
ctactacaacccgtccctcaagagtcgagttaccatatcagtagacacgtctaagaaccagt
tcgccctgaagctgaactctgtgactgccgcggacacggccgtgtattactgtgcgagagcc
gattacgatttttggagtggttattttgactactggggccagggaaccctggtcaccgtctc
ctcagcttccaccaagggcccatccgtcttcccccctgg

230 Heavy Chain Protein
QVQLQESGPGLVKPLQTLSLTCTVSGGSISSGDYYWSWIRQHPGKGLEWIGYIYYSGTTYY
NPSLKSRVTISVDTSKNQFALKLNSVTAADTAVYYCARADYDFWSGYFDYWGQGTLVTVSS

231 Light Chain DNA:
H4_116_1_1N1K
atgagggtccctgctcagctcctggggctcctgctgctctggttcccaggtgccaggtgtg
acatccagatgacccagtctccatcctccctgtctgcatctgtaggagacagagtcaccat
cacttgccgggcaggtcagggcattagaaatgatttaggctggtatcagcagaaaccaggg
aaagcccctcagcgcctgatctatgctgcatccagtttgcaaagtggggtcccatcaaggt
tcagcggcagtggatctgggacagaattctctctcacaatctccagcctgcagcctgaaga
ttttgcaacttattactgtctacagcataatagttacccgtggacgttcggccaagggacc
aaggtggaaatcaaacgaactgtggctgcaccatctgtcttcatcttcccgccatctgatg
agcagttgaaatctggaactgcctctgttgtgtgcctgctgaataacttctatcccagaga
ggccaaagtacagtggaaggtggataacgcccttccaatcggg

232 Light Chain Protein
DIQMTQSPSSLSASVGDRVTITCRAGQGIRNDLGWYQQKPGKAPQRLIYAASSLQSGVPSR
FSGSGSGTEFSLTISSLQPEDFATYYCLQHNSYPWTFGQGTKVEIK

SEQUENCE LISTING

Primer (SEQ ID NO: 233):
CGGGATCCATGTCCTAGCCTAGGGGC

Primer (SEQ ID NO: 234):
GCTCTAGATTAATGATGATGATGATGATGTTGTCCTAAA

Table of CDR Sequences

Ab chain	Pat. ID:	CDR1	CDR2	CDR3
heavy	U1-1	GGSINSGDYYWS	YIYYSGSTYYNPSLKS	ADYDFWSGYFDY
light		RASQGIRNDLG	AASSLQS	LQHNSYPWT
heavy	U1-2	GGSISSGDYYWS	YIYYSGSTYYNPSLRS	ADYDFWSGYFDY
light		RASQGIRNDLG	AASSLQS	LQHNGYPWT
heavy	U1-3	GGSISSGGYYWS	YIYYSGSTYYNPSLKS	DGYDSSGYYHGYFDY
light		KSSQSVLYSSNNKNYLA	WASTRES	QQYYSTPLT
heavy	U1-4	GGSISSGDYYWS	YIYYSGSTYYNPSLKS	ADYDFWSGYFDY
light		RASQGIRNDLG	AASSLQS	LQHNNYPWT
heavy	U1-5	GGSISSGDYYWS	YIYYSGSTYYNPSLKS	ADYDFWSGYFDY
light		RASQGIRNDLG	AASSLQS	LQHNTYPWT
heavy	U1-6	GGSISSGDYYWS	YIYYSGSTYYNPSLKS	ADYDFWNGYFDY
light		RASQGIRNDLG	AASSLQS	LQHNTYPWT
heavy	U1-7	GGSISSGDYYWS	YIYYSGSTYYNPSLKS	ADYDFWSGYFDY
light		RASQDIRNDLG	AASSLQS	LQHNSYPWT
heavy	U1-8	GYTLTELSMY	GFDPEDGETIYAQKFQG	GWNYVFDY
light		RSSQSLLHSNGYNYLD	LDSHRAS	MQALQTPLT
heavy	U1-9	GGSISSGDYYWS	YIYYSGSTYYNPSLKS	ADYDFWNGYFDY
light		RASQDIRNDLG	AASSLQS	LQHNSYPWT
heavy	U1-10	GGSISSGDYYWS	YIYYSGSTYYNPSLKS	ADYDFWSGYFDY
light		RASQGIRNDLG	AASSLQS	LQHNNYPWT
heavy	U1-11	GGSISSGDYYWS	YIYYSGSTYYNPSLKS	ADYDFWSGYFDY
light		RASQGIRNDLG	AASSLQS	LQHNTYPWT
heavy	U1-12	GGSISSGDYYWS	YIYYSGSTYYNPSLKS	ADYDFWSGYFDY
light		RASQGIRNDLG	AASSLQS	LQHNNYPWT
heavy	U1-13	GGSISSGGYYWS	YIYYSGSTYYNPSLKS	EDDGMDV
light		RSSQSLLHSNGYNYLE	LGSNRAS	MQALQTPIT
heavy	U1-14	GGSISSGDYYWS	YIYYSGSTYYNPSLKS	ADYDFWSGYFDY
light		RASQGIRNDLG	AASSLQS	LQHNTYPWT

heavy	U1-15	GGSVSSGGYYWS	YIYYSGSTNYNPSLKS	DGDVDTAMVDAFDI
light		RASQSLSGNYLA	GASSRAT	QQYDRSPLT
heavy	U1-16	GGSISSGDYYWS	YIYYSGSTYYNPSLKS	ADYDFWSGYFDY
light		RASQGIRNDLG	AASSLQS	LQHNSYPWT
heavy	U1-17	GGSISSGDYYWS	YIYYSGSTYYNSSLKS	ADYDFWSGYFDY
light		RASQGIRNDLG	AASSLQS	LQHNSYPWT
heavy	U1-18	GGSISSGDYYWS	YIYYSGSTYYNPSLKS	ADYDFWSGYFDY
light		RASQGIRNDLG	AASSLQS	LQHNSYPWT
heavy	U1-19	GGSISSGDYYWS	YIYYSGSTYYNPSLKS	GDYDFWSGEFDY
light		sequence not available		
heavy	U1-20	GGSISSGGYYWS	YIYDSGSTYYNPSLKS	DQGQDGYSYGYGYYYGMDV
light		QASQDISNYLN	VASNLET	QQCDNLPLT
heavy	U1-21	GGSISSGDYYWS	YIYYSGSTYYNPSLKS	ADYDFWSGYFDY
light		RASQDIRNDLG	AASRLQS	LQHNSYPWT
heavy	U1-22	GGSISSGDYYWS	YIYYSGSTYYNPSLKS	ADYDFWSGYFDY
light		RASQGIRNDLG	AASSLQN	LQHNSYPWT
heavy	U1-23	GGSISSGDYYWS	YIYYSGSTYYNPSLKS	ADYDFWSGYFDY
light		RASQGIRNDLG	AASSLQS	LQHNSYPWT
heavy	U1-24	GGSISSGDYYWS	YIYYSGSTYYNPSLKS	ADYDFWNGYFDY
light		RASQGIRNDLG	AASSLQS	LQHNNYPWT
heavy	U1-25	GGSISSGDYYWS	YIYYSGSTYYNPSLKS	ADYDFWSGYFDY
light		RASQGIRNDLG	AASSLQN	LQHNSYPWT
heavy	U1-26	GGSISSGDYYWS	YIYYSGSTYYNPSLKS	ADYDFWSGYFDF
light		RASQGIRNDLG	AASSLQS	LQHNGYPWT
heavy	U1-27	GGSISSGDYYWS	YIYYSGSTYYNPSLKS	ADYDFWSGYFDF
light		RASQGIRNDLG	AASSLQS	LQHNGYPWT
heavy	U1-28	GGSISSGDYYWS	YIYYSGSTYYNPSLKS	ADYDFWSGYFDS
light		RASQGIRNDLG	AASSLQS	LQHNGYPWT
heavy	U1-29	GFTFNSYDMH	VIWYDGSNKYYADSVKG	DRLCTNGVCYEDYGMDV
light		QASQDISNYLN	DASNLET	QHYDTLPLT
heavy	U1-30	GGSISSGDYYWS	YIYYSGTTYYNPSLKS	ADYDFWSGYFDY
light		RAGQGIRNDLG	AASSLQS	LQHNSYPWT

heavy	U1-31	GYTFTNYGIS	WISAYDGYRNYAQKLQG	DVQDYGDYDYFDY
light		RASQSISSYLN	AASSLQS	QQSYSTPIT
heavy	U1-32	GGSISSGDYYWS	YIYYSGTTYYNPSLKS	ADYDFWSGYFDY
light		RAGQGIRNDLG	AASSLQS	LQHNSYPWT
heavy	U1-33	GGSISSGDYYWS	YIYYSGSTYYNPSLKS	ADYDFWSGHFDC
light		RASQGIRDDLG	AESSLQS	LQHHSYPWT
heavy	U1-34	GYTFTNYGIS	WISAYDGYRNYAQKLQG	DVQDYGDYDYFDY
light		RASQSISSYLN	AASSLQS	QQSYSTPIT
heavy	U1-35	GFTFSDYYMS	YISSSGNNIYHADSVKG	ERYSGYDDPDGFDI
light		QASQDISNYLS	DASNLET	QQYDNPPCS
heavy	U1-36	GGSISSGYYYWS	YIYYSGTTYYNPSFKS	ADYDFWSGHFDY
light		RASQGIRNDLG	AASSLQS	LQHNSYPWT
heavy	U1-37	GYTFTSYGIS	WISAYDGHTNYAQKLQG	DPHDYSNYEAFDF
light		RASQSISSYLN	AASSLQS	QQSYSTPIT
heavy	U1-38	GFSLSTSGVGVG	LIYWNDDKRYSPSLKS	RDEVRGFDY
light		RSSQSLVYSDGYTYLH	KVSNWDS	MQGAHWPIT
heavy	U1-39	GFTVSSNYMS	VIYSGGSTYYADSVKG	GQWLDV
light		RSSQSLLHSNGYNYLD	LGFHRAS	RQALQTPLT
heavy	U1-40	GGSISSGGYYWS	YIYSSGSTYYNPSLKS	DRELELYYYYYGMDV
light		RSSQSLLYSNGYNYLD	LGSNRAS	MQALQTPLT
heavy	U1-41	GGSISSGGYYWS	YIYYSGSTYYNPSLKS	DRELEGYSNYYGVDV
light		RASQAISNYLN	AASSLQS	QQNNSLPIT
heavy	U1-42	GYSFTSYWIG	IIYPGDSDTRYSPSFQG	HENYGDYNY
light		RASQSIRSYLN	AASSLQS	QQSNGSPLT
heavy	U1-43	GGSISSGGYYWS	YIYYSGSTYYNPSLRS	DRREREWDDYGDPQGMDV

light		RASQSISSYLH	AASSLQS	QQSYSNPLT
heavy	U1-44	GYSFTSYWIG	IIWPGDSDTIYSPSFQG	HENYGDYNY
light		RASQSIRSYLN	AASSLQS	QQSISSPLT
heavy	U1-45	GYTFTSYDIN	WMNPNSGDTGYAQVFQG	FGDLPYDYSYYEWFDP
light		RASQSISSYLN	AASSLQS	QQSYSTPLT
heavy	U1-46	GDSVSSNSAAWN	RTYYRSKWYNDYAVSVKS	DLYDFWSGYPYYYGMDV
light		sequence not available		
heavy	U1-47	GDSVSSNSAAWN	RTYYRSKWYNDYAVSVKS	DYYGSGSFYYYYGMDV
light		RASQSISSYLN	AASNLQS	QQSYSTPRT
heavy	U1-48	GGSISSYYWS	HIYTSGSTNYNPSLKS	EAIFGVGPYYYYGMDV
light		sequence not available		
heavy	U1-49	GYTFTGYYMH	WINPNIGGTNCAQKFQG	GGRYSSSWSYYYYGMDV
light		KSSQSLLLSDGGTYLY	EVSNRFS	MQSMQLPIT
heavy	U1-50	GGSVSSGGYYWS	YIYYSGSTNYNPSLKS	GGDSNYEDYYYYYGMDV
light		RASQSISIYLH	AASSLQS	QQSYTSPIT
heavy	U1-51	GGSISSYYWS	YIYYSGSTNYNPSLKS	DSSYYDSSGYYLYYYAMDV
light		KSSQSVLYSSNNKNYLA	WASTRES	QQYYTTPLT
heavy	U1-52	GGSISSGGYYWS	NIYYSGSTYYNPSLKS	GGTGTNYYYYYGMDV
light		RASQSVSSSYLA	GASSWAT	QQYGSSPLT
heavy	U1-53	GFTFSIYSMN	YISSSSSTIYYADSVKG	DRGDFDAFDI
light		QASQDITNYLN	DASNLET	QQCENFPIT
heavy	U1-55.1	GGSVSSGGYYWN	YINYSGSTNYNPSLKS	DRELELYYYYYGMDV
light		will be same as U1-55		
heavy	U1-55	will be same as U1-55.1		
light		RSSQSLLYSNGYKYLD	LGSNRAS	MQALQTPIT
heavy	U1-57.1	will be same as U1-57		

light		RSSQSLLYSNGYKYLD	LGSNRAS	MQALQTPIT
heavy	U1-57	GGSVSSGGYYWN	YINYSGSTNYNPSLKS	DRELELYYYYYGMDV
light		will be same as U1-57.1		
heavy	U1-58	GFTFSSYGMH	VIWYDGSNKYYADSVKG	AARLDYYYGMDV
light		RASQSINSYLN	GASGLQS	QQSYSSPLT
heavy	U1-59	GGSFSGYYWS	EINHSGSTNYNPSLKS	DKWTWYFDL
light		RSSQSVLYSSSNRNYLA	WASTRES	QQYYSTPRT
heavy	U1-61.1	GVSISSGGYYWS	YIYYSGSTYYNPSLKS	DSESEYSSSSNYGMDV
light		RASQTISSYLN	AASSLQG	QQSYSNPLT
heavy	U1-61	GVSISSGGYYWS	YIYYSGSTYYNPSLKS	DSESEYSSSSNYGMDV
light		will be the same as U1-61.1		
heavy	U1-62	GYSFTSYWIG	IIYPGDSDTRYSPSFQG	QMAGNYYYGMDV
light		RASQSVISIYLA	GASSRAT	QQYGSSPCS

Claims

1. An isolated binding protein that binds to HER-3, comprising:

a heavy chain amino acid sequence that comprises at least one of the CDR's selected from the group consisting of (a) CDRH1's as shown in SEQ ID NOs: 2, 6, 10, 14, 18, 22, 26, 30, 34, 36, 40, 42, 46, 50, 54, 60, 62, 66, 70, 74, 78, 80, 84, 88, 92, 96, 100, 104, 108, 112, 116, 120, 122, 126, 130, 134, 138, 142, 146, 150, 154, 158, 162, 166, 170, 174, 178, 182, 186, 190, 194, 198, 202, 206, 210, 214, 218, 222, 226 and 230; (b) CDRH2's as shown in SEQ ID NOs: 2, 6, 10, 14, 18, 22, 26, 30, 34, 36, 40, 42, 46, 50, 54, 60, 62, 66, 70, 74, 78, 80, 84, 88, 92, 96, 100, 104, 108, 112, 116, 120, 122, 126, 130, 134, 138, 142, 146, 150, 154, 158, 162, 166, 170, 174, 178, 182, 186, 190, 194, 198, 202, 206, 210, 214, 218, 222, 226 and 230; and (c) CDRH3's as shown in SEQ ID NOs: 2, 6, 10, 14, 18, 22, 26, 30, 34, 36, 40, 42, 46, 50, 54, 60, 62, 66, 70, 74, 78, 80, 84, 88, 92, 96, 100, 104, 108, 112, 116, 120, 122, 126, 130, 134, 138, 142, 146, 150, 154, 158, 162, 166, 170, 174, 178, 182, 186, 190, 194, 198, 202, 206, 210, 214, 218, 222, 226 and 230.

2. An isolated binding protein that binds to HER-3, comprising:

a light chain amino acid sequence that comprises at least one of the CDR´s selected from the group consisting of: (d) CDRL1´s as shown in SEQ ID NOs: 4, 8, 12, 16, 20, 24, 28, 32, 38, 44, 48, 52, 56, 58, 64, 68, 72, 76, 82, 86, 90, 94, 98, 102, 106, 110, 114, 118, 124, 128, 132, 136, 140, 144, 148, 152, 156, 160, 164, 168, 172, 176, 180, 184, 188, 192, 196, 200, 204, 208, 212, 216, 220, 224, 228 and 232; (e) CDRL2´s as shown in SEQ ID NOs: 4, 8, 12, 16, 20, 24, 28, 32, 38, 44, 48, 52, 56, 58, 64, 68,

72, 76, 82, 86, 90, 94, 98, 102, 106, 110, 114, 118, 124, 128, 132, 136, 140, 144, 148, 152, 156, 160, 164, 168, 172, 176, 180, 184, 188, 192, 196, 200, 204, 208, 212, 216, 220, 224, 228 and 232; and (f) CDRL3´s as shown in SEQ ID NOs: 4, 8, 12, 16, 20, 24, 28, 32, 38, 44, 48, 52, 56, 58, 64, 68, 72, 76, 82, 86, 90, 94, 98, 102, 106, 110, 114, 118, 124, 128, 132, 136, 140, 144, 148, 152, 156, 160, 164, 168, 172, 176, 180, 184, 188, 192, 196, 200, 204, 208, 212, 216, 220, 224, 228 and 232.

3. The isolated binding protein of Claim 1 or 2, wherein said binding protein comprises:

a heavy chain amino acid sequence that comprises at least one of the CDR's selected from the group consisting of (a) CDRH1´s as shown in SEQ ID NOs: 2, 6, 10, 14, 18, 22, 26, 30, 34, 36, 40, 42, 46, 50, 54, 60, 62, 66, 70, 74, 78, 80, 84, 88, 92, 96, 100, 104, 108, 112, 116, 120, 122, 126, 130, 134, 138, 142, 146, 150, 154, 158, 162, 166, 170, 174, 178, 182, 186, 190, 194, 198, 202, 206, 210, 214, 218, 222, 226 and 230; (b) CDRH2´s as shown in SEQ ID NOs: 2, 6, 10, 14, 18, 22, 26, 30, 34, 36, 40, 42, 46, 50, 54, 60, 62, 66, 70, 74, 78, 80, 84, 88, 92, 96, 100, 104, 108, 112, 116, 120, 122, 126, 130, 134, 138, 142, 146, 150, 154, 158, 162, 166, 170, 174, 178, 182, 186, 190, 194, 198, 202, 206, 210, 214, 218, 222, 226 and 230; and (c) CDRH3´s as shown in SEQ ID NOs: 2, 6, 10, 14, 18, 22, 26, 30, 34, 36, 40, 42, 46, 50, 54, 60, 62, 66, 70, 74, 78, 80, 84, 88, 92, 96, 100, 104, 108, 112, 116, 120, 122, 126, 130, 134, 138, 142, 146, 150, 154, 158, 162, 166, 170, 174, 178, 182, 186, 190, 194, 198, 202, 206, 210, 214, 218, 222, 226 and 230, and

a light chain amino acid sequence that comprises at least one of the CDR´s selected from the group consisting of: (d) CDRL1´s as shown in SEQ ID NOs: 4, 8, 12, 16, 20, 24, 28, 32, 38, 44, 48, 52, 56, 58, 64, 68, 72, 76, 82, 86, 90, 94, 98, 102, 106, 110, 114, 118, 124, 128, 132, 136, 140, 144, 148, 152, 156, 160, 164, 168, 172, 176, 180, 184, 188, 192, 196, 200, 204, 208, 212, 216, 220, 224, 228 and 232; (e) CDRL2´s as shown in SEQ

ID NOs: 4, 8, 12, 16, 20, 24, 28, 32, 38, 44, 48, 52, 56, 58, 64, 68, 72, 76, 82, 86, 90, 94, 98, 102, 106, 110, 114, 118, 124, 128, 132, 136, 140, 144, 148, 152, 156, 160, 164, 168, 172, 176, 180, 184, 188, 192, 196, 200, 204, 208, 212, 216, 220, 224, 228 and 232; and (f) CDRL3's as shown in SEQ ID NOs: 4, 8, 12, 16, 20, 24, 28, 32, 38, 44, 48, 52, 56, 58, 64, 68, 72, 76, 82, 86, 90, 94, 98, 102, 106, 110, 114, 118, 124, 128, 132, 136, 140, 144, 148, 152, 156, 160, 164, 168, 172, 176, 180, 184, 188, 192, 196, 200, 204, 208, 212, 216, 220, 224, 228 and 232.

4. The isolated binding protein of Claim 1 or 2, wherein said binding protein comprises a heavy chain amino acid sequence that comprises a CDRH1, a CDRH2, and a CDRH3 of any of SEQ ID NOs:2, 6, 10, 14, 18, 22, 26, 30, 34, 36, 40, 42, 46, 50, 54, 60, 62, 66, 70, 74, 78, 80, 84, 88, 92, 96, 100, 104, 108, 112, 116, 120, 122, 126, 130, 134, 138, 142, 146, 150, 154, 158, 162, 166, 170, 174, 178, 182, 186, 190, 194, 198, 202, 206, 210, 214, 218, 222, 226 and 230, or a light chain amino acid sequence that comprises a CDRL1, a CDRL2, and a CDRL3 of any of SEQ ID NOs:4, 8, 12, 16, 20, 24, 28, 32, 38, 44, 48, 52, 56, 58, 64, 68, 72, 76, 82, 86, 90, 94, 98, 102, 106, 110, 114, 118, 124, 128, 132, 136, 140, 144, 148, 152, 156, 160, 164, 168, 172, 176, 180, 184, 188, 192, 196, 200, 204, 208, 212, 216, 220, 224, 228 and 232.

5. The isolated binding protein of Claim 3, wherein said binding protein comprises a heavy chain amino acid sequence that comprises a CDRH1, a CDRH2, and a CDRH3 of any of SEQ ID NOs: 2, 6, 10, 14, 18, 22, 26, 30, 34, 36, 40, 42, 46, 50, 54, 60, 62, 66, 70, 74, 78, 80, 84, 88, 92, 96, 100, 104, 108, 112, 116, 120, 122, 126, 130, 134, 138, 142, 146, 150, 154, 158, 162, 166, 170, 174, 178, 182, 186, 190, 194, 198, 202, 206, 210, 214, 218, 222, 226 and 230, and a light chain amino acid sequence that comprises a CDRL1, a CDRL2, and a CDRL3 of any of SEQ ID NOs:4, 8, 12, 16, 20, 24, 28, 32, 38, 44, 48, 52, 56, 58, 64, 68, 72, 76, 82, 86, 90, 94, 98, 102, 106, 110, 114, 118, 124, 128, 132, 136, 140, 144, 148, 152, 156, 160, 164, 168, 172, 176, 180, 184, 188, 192, 196, 200, 204, 208, 212, 216,

220, 224, 228 and 232.

6. The isolated binding protein of Claim 1, comprising:

a heavy chain amino acid sequence selected from the group consisting of SEQ ID NOs: 2, 6, 10, 14, 18, 22, 26, 30, 34, 36, 40, 42, 46, 50, 54, 60, 62, 66, 70, 74, 78, 80, 84, 88, 92, 96, 100, 104, 108, 112, 116, 120, 122, 126, 130, 134, 138, 142, 146, 150, 154, 158, 162, 166, 170, 174, 178, 182, 186, 190, 194, 198, 202, 206, 210, 214, 218, 222, 226 and 230.

7. The isolated binding protein of Claim 2 comprising:

a light chain amino acid sequence selected from the group consisting of SEQ ID NOs: 4, 8, 12, 16, 20, 24, 28, 32, 38, 44, 48, 52, 56, 58, 64, 68, 72, 76, 82, 86, 90, 94, 98, 102, 106, 110, 114, 118, 124, 128, 132, 136, 140, 144, 148, 152, 156, 160, 164, 168, 172, 176, 180, 184, 188, 192, 196, 200, 204, 208, 212, 216, 220, 224, 228 and 232.

8. The isolated binding protein of Claim 1, comprising:

a heavy chain amino acid sequence selected from the group consisting of SEQ ID NOs: 2, 6, 10, 14, 18, 22, 26, 30, 34, 36, 40, 42, 46, 50, 54, 60, 62, 66, 70, 74, 78, 80, 84, 88, 92, 96, 100, 104, 108, 112, 116, 120, 122, 126, 130, 134, 138, 142, 146, 150, 154, 158, 162, 166, 170, 174, 178, 182, 186, 190, 194, 198, 202, 206, 210, 214, 218, 222, 226 and 230; and

a light chain amino acid sequence selected from the group consisting of SEQ ID NOs: 4, 8, 12, 16, 20, 24, 28, 32, 38, 44, 48, 52, 56, 58, 64, 68, 72, 76, 82, 86, 90, 94, 98, 102, 106, 110, 114, 118, 124, 128, 132, 136, 140, 144, 148, 152, 156, 160, 164, 168, 172, 176, 180, 184, 188, 192, 196, 200, 204, 208, 212, 216, 220, 224, 228 and 232.

9. The isolated binding protein of Claim 1, comprising a CDRH3 of any of SEQ ID NOs: 2, 6, 10, 14, 18, 22, 26, 30, 34, 36, 40, 42, 46, 50, 54, 60, 62, 66, 70, 74, 78, 80, 84, 88, 92, 96, 100, 104, 108, 112, 116, 120, 122, 126, 130, 134, 138, 142, 146, 150, 154, 158, 162, 166, 170, 174, 178, 182, 186, 190, 194, 198, 202, 206, 210, 214, 218, 222, 226 and

230.

10. The isolated binding protein according to Claim 9, comprising a light chain amino acid sequence of any of SEQ ID NOs: 4, 8, 12, 16, 20, 24, 28, 32, 38, 44, 48, 52, 56, 58, 64, 68, 72, 76, 82, 86, 90, 94, 98, 102, 106, 110, 114, 118, 124, 128, 132, 136, 140, 144, 148, 152, 156, 160, 164, 168, 172, 176, 180, 184, 188, 192, 196, 200, 204, 208, 212, 216, 220, 224, 228 and 232.

11. The isolated binding protein according of any one of Claims 1-9 comprising the heavy chain amino acid sequence of SEQ ID NO:42 and the light chain amino acid sequence of SEQ ID NO:44.

12. The isolated binding protein according of any one of Claims 1-9 comprising the heavy chain amino acid sequence of SEQ ID NO:54 and the light chain amino acid sequence of SEQ ID NO:56.

13. The isolated binding protein according of any one of Claims 1-9 comprising the heavy chain amino acid sequence of SEQ ID NO:70 and the light chain amino acid sequence of SEQ ID NO:72.

14. An isolated binding protein that binds to a three-dimensional structure formed by amino acid residues 1-160; 161-358; 359-575; 1-358; 359-604 of mature HER-3, particularly of mature human HER-3.

15. The isolated binding protein of any of Claims 1-14, wherein the binding protein is directed against the extracellular domain of HER-3.

16. The isolated binding protein of any one of Claims 1-15, wherein the binding of the binding protein to HER-3 reduces HER-3-mediated signal transduction.

17. The isolated binding protein of any one of Claims 1-15, wherein the binding of the binding protein to HER-3 reduces HER-3 phosphorylation.

18. The isolated binding protein of any one of Claims 1-15, wherein the binding of the binding protein to HER-3 reduces cell proliferation.

19. The isolated binding protein of any one of Claims 1-15, wherein the binding of the binding protein to HER-3 reduces cell migration.

20. The isolated binding protein of any one of Claims 1-15, wherein the binding of the binding protein to HER-3 increases the downregulation of HER-3.

21. The isolated binding protein of any one of Claims 1-20 which is an antibody.

22. The isolated binding protein of Claims 21, wherein the antibody is a monoclonal antibody, a polyclonal antibody, a recombinant antibody, a humanized antibody, a human antibody, a chimeric antibody, a multispecific antibody, or an antibody fragment thereof.

23. The isolated binding protein of Claim 22, wherein the antibody fragment is a Fab fragment, a Fab' fragment, a $F(ab')_2$ fragment, a Fv fragment, a diabody, or a single chain antibody molecule.

24. The isolated binding protein of any one of Claims 1-23, wherein said isolated binding protein is of the IgG1-, IgG2-, IgG3- or IgG4-type.

25. The isolated binding protein of any one of Claims 1-

24, wherein the binding protein is coupled to a labelling group.

26. The isolated binding protein of Claim 25, wherein the labelling group is a radioisotope or radionuclide, a fluorescent group, an enzymatic group, a chemiluminescent group, a biotinyl group, or a predetermined polypeptide epitope.

27. The isolated binding protein of any one of Claims 1-24, wherein the binding protein is coupled to an effector group.

28. The isolated binding protein of Claim 27, wherein the effector group is a radioisotope or radionuclide, a toxin, or a therapeutic or chemotherapeutic group.

29. The isolated binding protein of Claim 28, wherein the therapeutic or chemotherapeutic group is selected from the group consisting of calicheamicin, auristatin-PE, geldanamycin, maytansine and derivatives thereof.

30. An isolated nucleic acid molecule encoding an binding protein of any one of Claims 1-29.

31. The isolated nucleic acid molecule of Claim 30, wherein the nucleic acid molecule is operably linked to a control sequence.

32. A vector comprising the nucleic acid molecule of Claim 30.

33. A vector comprising the nucleic acid molecule of Claim 31.

34. A host cell transformed with the vector of Claim 32.

35. A host cell transformed with the vector of Claim 33.

36. A process for preparing the isolated binding protein of any one of Claims 1-29, comprising the step of isolating said binding protein from a host cell

37. The process of Claim 36, wherein the host cell is a mammalian cell, a plant cell, a fungal cell, or a prokaryotic cell.

38. A pharmaceutical composition comprising as an active agent at least one isolated binding protein of any one of Claims 1-29, and a pharmaceutically acceptable carrier, diluent or adjuvant.

39. The composition of Claim 37 or 38 for therapeutic use.

40. The composition of Claim 37 or 38 for diagnostic use.

41. A method for treating or preventing a disease associated with HER-3 in a patient, comprising administering a pharmaceutically effective amount of a pharmaceutical composition of Claim 38 or 39 to a subject in need thereof.

42. The method of Claim 41, wherein the disease is a hyperproliferative disease.

43. The method of Claim 42, wherein said hyperproliferative disease is selected from the group consisting of breast cancer, gastrointestinal cancer, pancreas cancer, prostate cancer, ovarian cancer, stomach cancer, endometrial cancer, salivary gland cancer, lung cancer, kidney cancer, colon cancer, colorectal cancer, thyroid cancer, bladder cancer, glioma, melanoma, testis cancer, soft tissue sarcoma, head and neck cancer, other HER-3 expressing or overexpressing cancers, and formation of tumor metastases.

44. The method of Claim 42 or 43, wherein said hyperproliferative disease is associated with increased HER-3 phosphorylation, increased HER-2/HER-3 heterodimerization or an increased activity of PI_3-kinase, c-jun-terminal kinase, AKT, ERK2 and/or PYK2.

45. A method for diagnosing a disease associated with HER-3, comprising:

(a) contacting a sample with the binding protein of any one of Claims 1-26, under conditions suitable to allow binding of said binding protein to HER-3; and

(c) identifying binding of said binding protein to HER-3.

46. The method of Claim 45, wherein the disease is a hyperproliferative disease.

47. The method of Claim 46, wherein said hyperproliferative disease is selected from the group consisting of breast cancer, gastrointestinal cancer, pancreas cancer, prostate cancer, ovarian cancer, stomach cancer, endometrial cancer, salivary gland cancer, lung cancer, kidney cancer, colon cancer, colorectal cancer, thyroid cancer, bladder cancer, glioma, melanoma, other HER-3 expressing or overexpressing cancers, testis cancer, soft tissue sarcoma, head and neck cancer, and formation of tumor metastases.

48. The method of Claim 46 or 47, wherein said hyperproliferative disease is associated with increased HER-3 phosphorylation, increased HER-2/HER-3 heterodimerization or an increased activity of PI_3-kinase, c-jun-terminal kinase, AKT, ERK2 and/or PYK2.

49. A kit comprising the isolated binding protein of any one of Claims 1-28.

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Ex. 1002

50. The kit of Claim 49, comprising a further therapeutic agent.

51. The kit of Claim 50 wherein the further therapeutic agent is an antineoplastic agent.

52. The kit of Claim 51, wherein the anti-neoplastic agent is an anti-tumor antibody or a chemotherapeutic agent.

Fig. 1

cell line	tissue	expression level
Hs578T	breast	++
T47D	breast	++
SKBr3	breast	+++
ZR 75-1	breast	++
MDA-MB-435s	breast	+++
MCF-7	breast	++
BT474	breast	+++
MDA-MB 453	breast	+++
MDA-MB 361	breast	++
HCT116	colon	+
HT29	colon	++
LoVo		+
CaCo-2	colon	+
SW480	colon	++
A431	epidermoid	+
HT1080	fibrosarcoma	O/+
CaKi-1	kidney	O
CaKi-2	kidney	O
Calu6	lung	O
A549	lung	O
M19	melanoma	++
M14	melanoma	+++

cell line	tissue	expression level
RPMI-7951	melanoma	O/+
MelJuso	melanoma	++
MelGerlach	melanoma	++
C32	melanoma	+++
HT144	melanoma	+++
SK-Mel2	melanoma	+++
MALME3	melanoma	+++
Hs294T	melanoma	+++
A375p	melanoma	+++
SK-Mel3	melanoma	+++
Sk-Mel5	melanoma	++
KB	nasopharynx	+
SCC9	Squamous cell carcinoma	
SKOV3	ovary	+
Capan-1	pancreas	+
Capan-2	pancreas	+
Bx-PC-3	pancreas	+
LNCap	prostate	++
DU145	prostate	+
PC-3	prostate	+
BM1604	prostate	++
TSU	prostate	+

0=no expression; +=weak; ++=medium; +++=strong

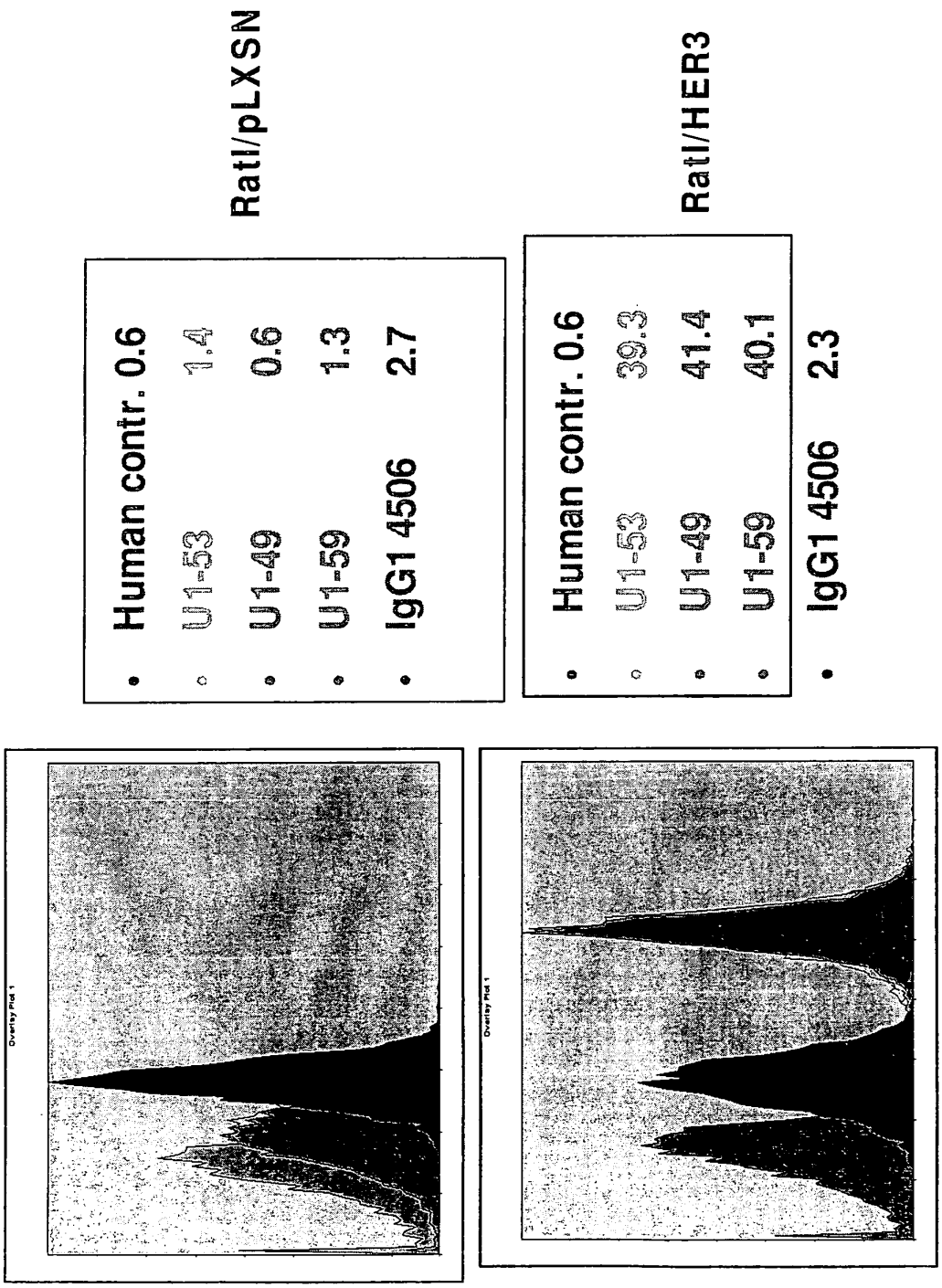

RatI/pLXSN
Human contr. 0.6
U1-53 1.4
U1-49 0.6
U1-59 1.3
IgG1 4506 2.7
RatI/HER3
Human contr. 0.6
U1-53 39.3
U1-49 41.4
U1-59 40.1
IgG1 4506 2.3

Fig. 2

Fig. 3 antibody bins

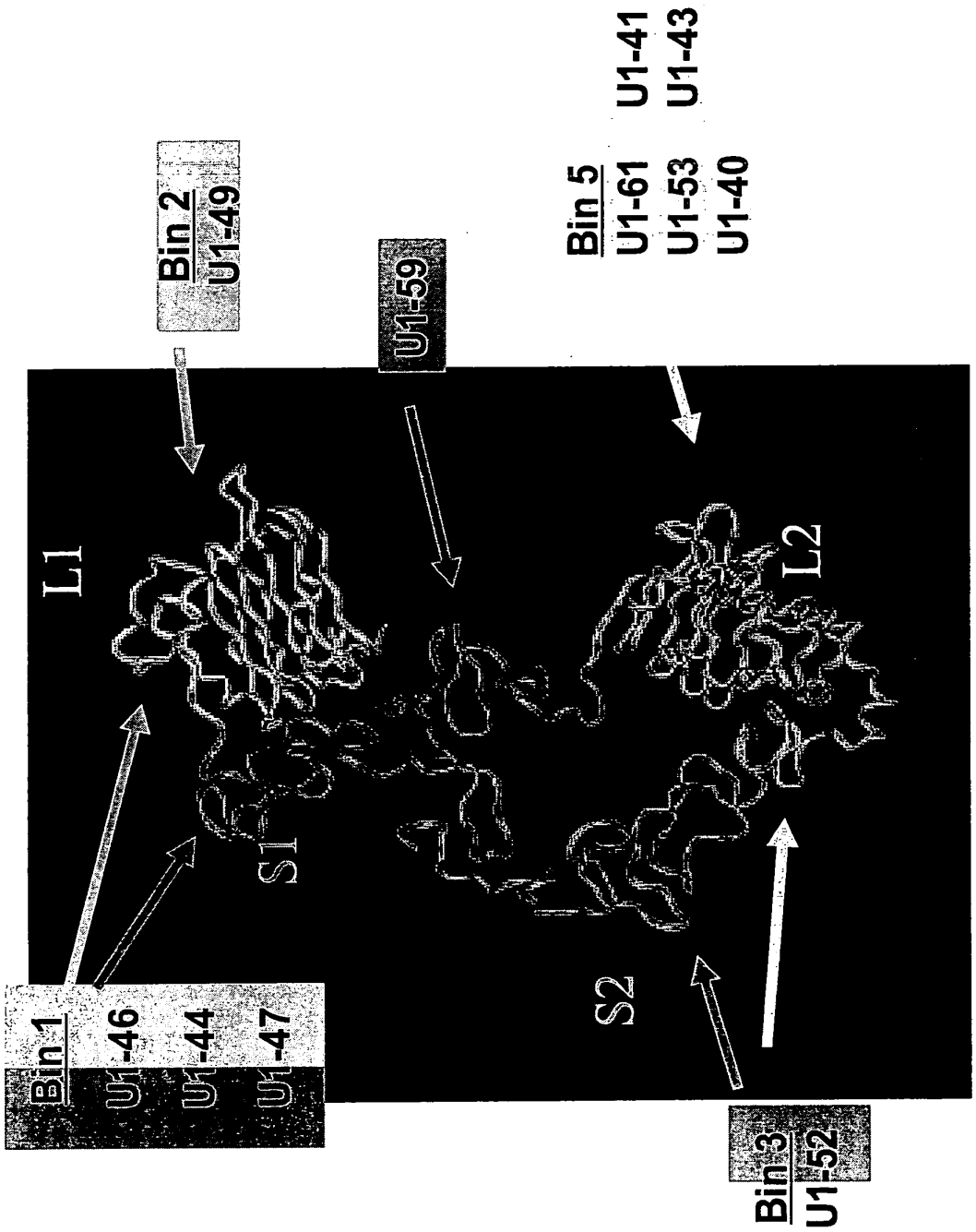
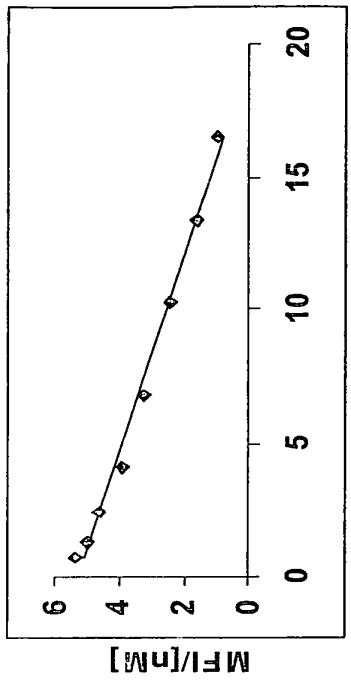


Fig. 4 FACS scatchard



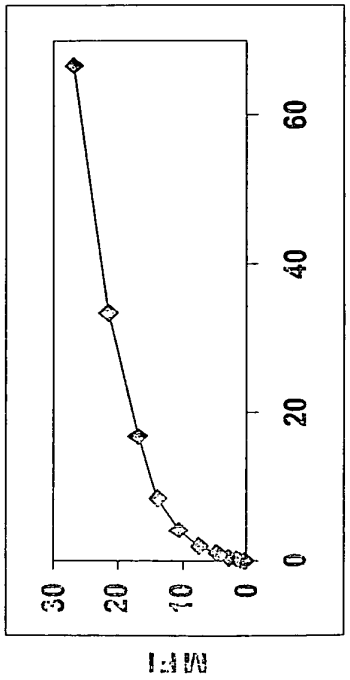


Fig. 5 internalization

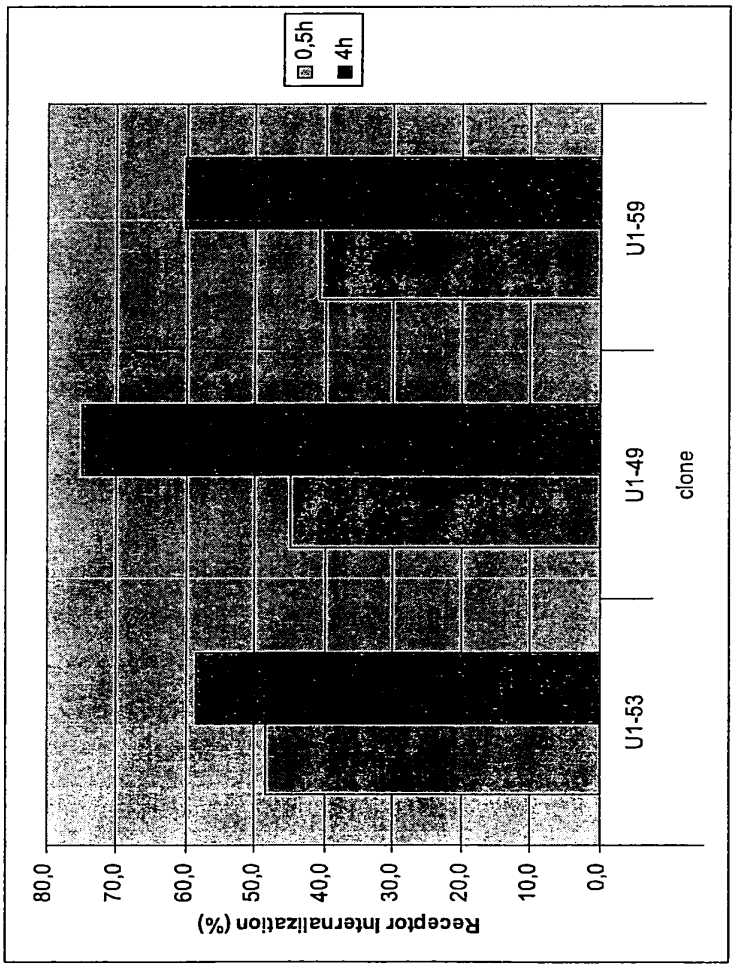
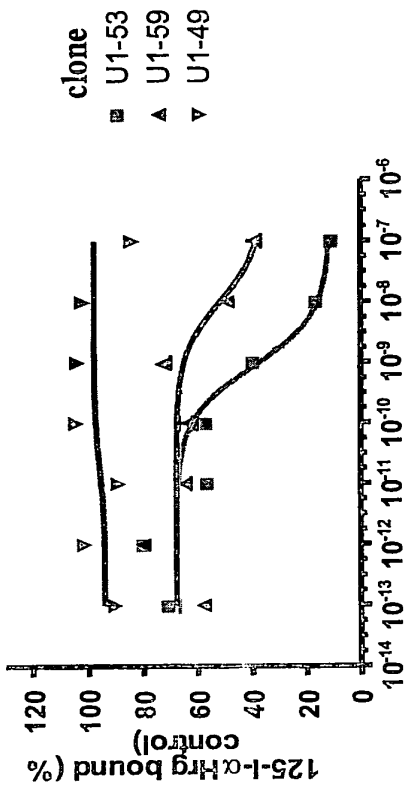
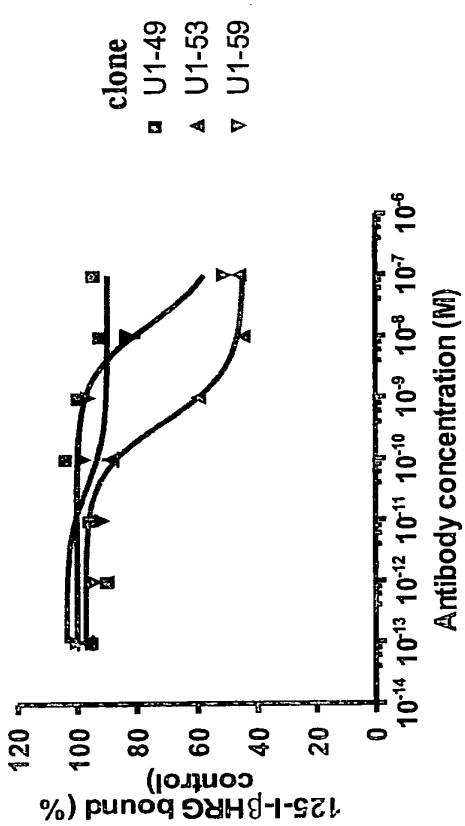


Fig. 6a HRG blocking



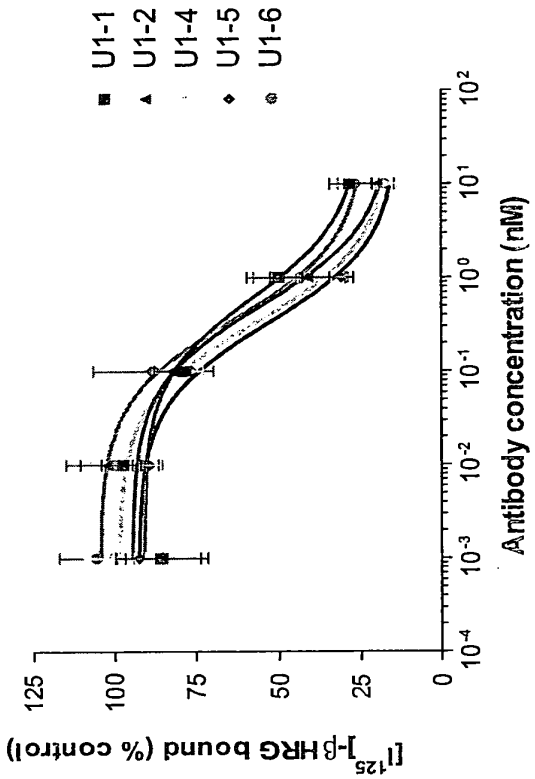
clone	EC50 (nM)
U1-59	9,65
U1-49	-
U1-53	0,71

Fig. 6b HRG blocking



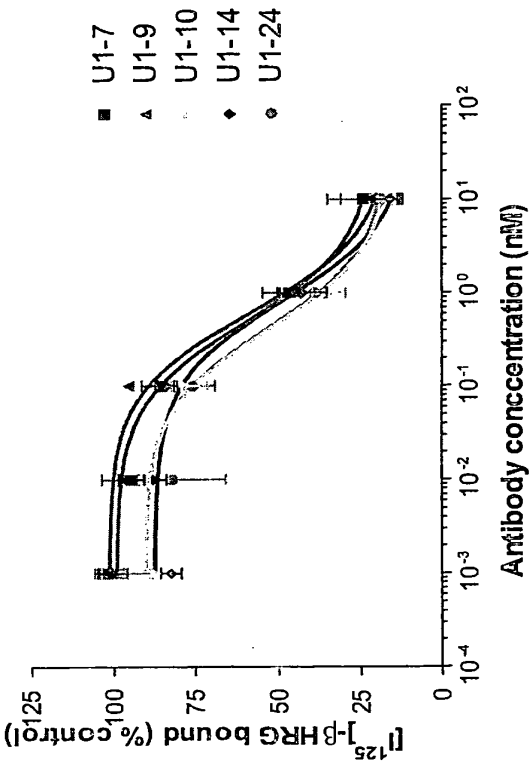
clone	EC50 (nM)
U1-59	32,1
U1-49	-
U1-53	0,49

Fig. 6C



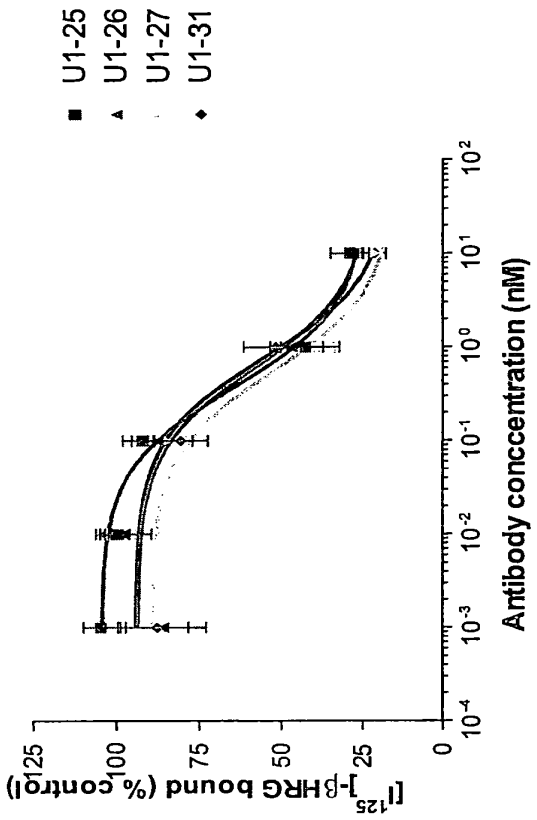
clone	EC50 (nM)
U1-1	0.61
U1-2	0.51
U1-4	0.32
U1-5	0.33
U1-6	0.35

Fig. 6d



clone	EC50 (nM)
U1-7	0.49
U1-9	0.60
U1-10	0.40
U1-14	0.83
U1-24	0.41

Fig. 6e



clone	EC50 (nM)
U1-25	0.38
U1-26	0.60
U1-27	0.56
U1-31	0.77

Fig. 7a

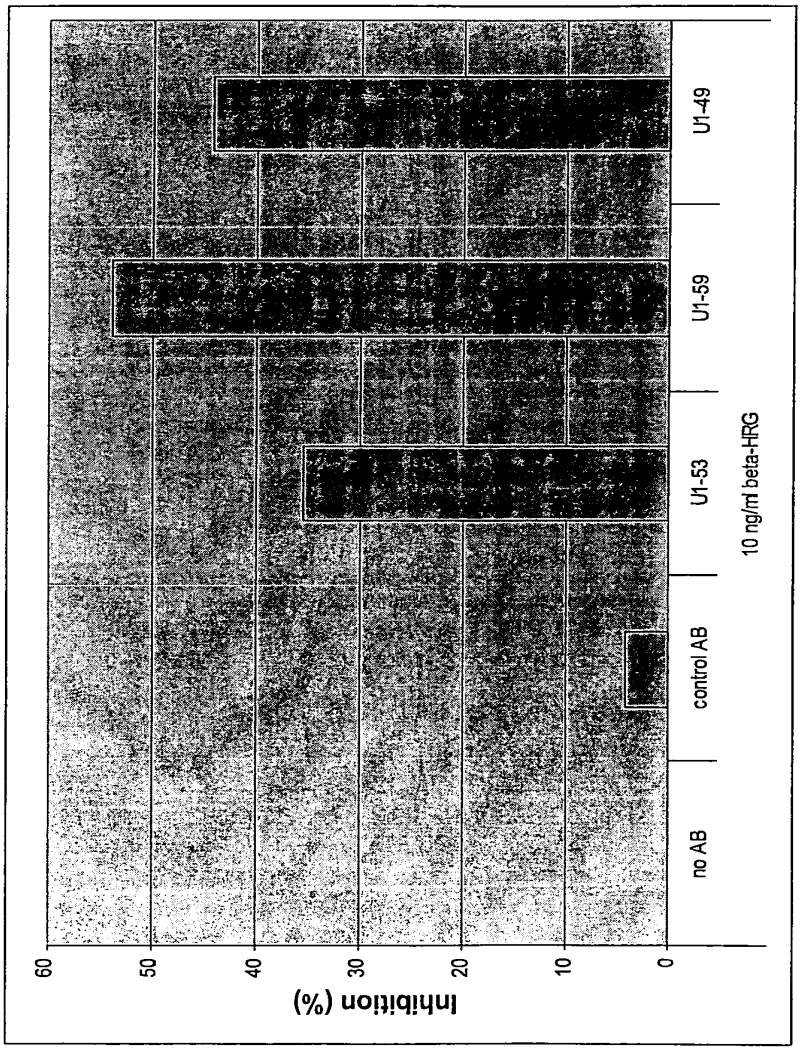

HER3 tyrosine phosphorylierung
Inhibition (%)
0
10
20
30
40
50
60
no AB
control AB
U1-53
U1-59
U1-49
10 ng/ml beta-HRG

Fig. 7b

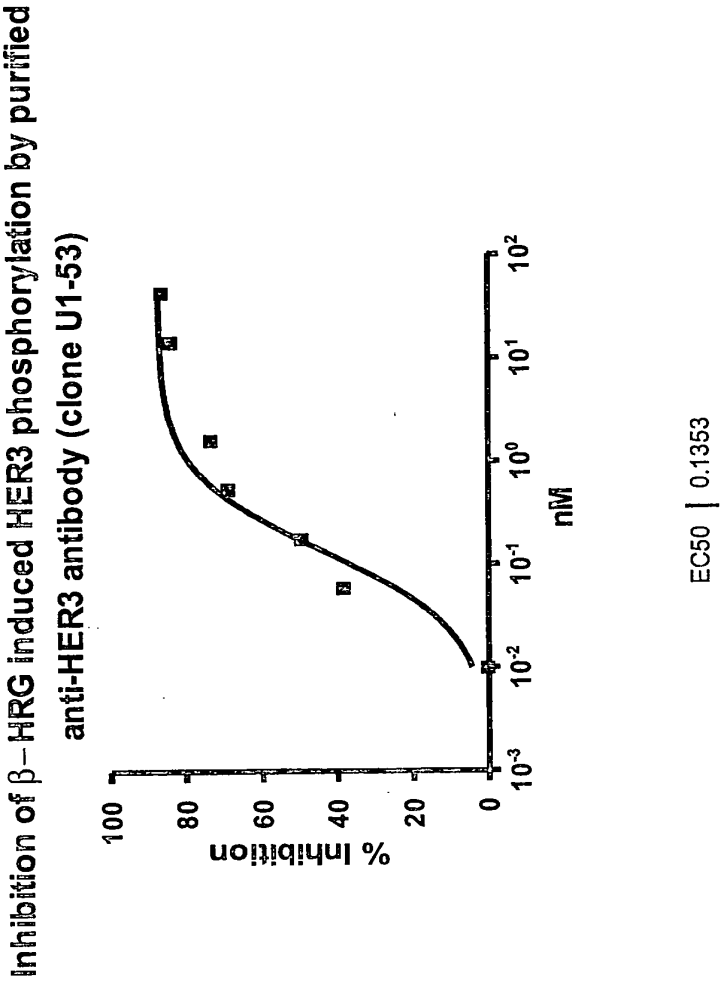

HER3 tyrosine phosphorylation
Inhibition of β–HRG induced HER3 phosphorylation by purified anti-HER3 antibody (clone U1-53)
% Inhibition
100
80
60
40
20
0
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
nM
EC50 | 0.1353

Fig. 8 MAP kinase

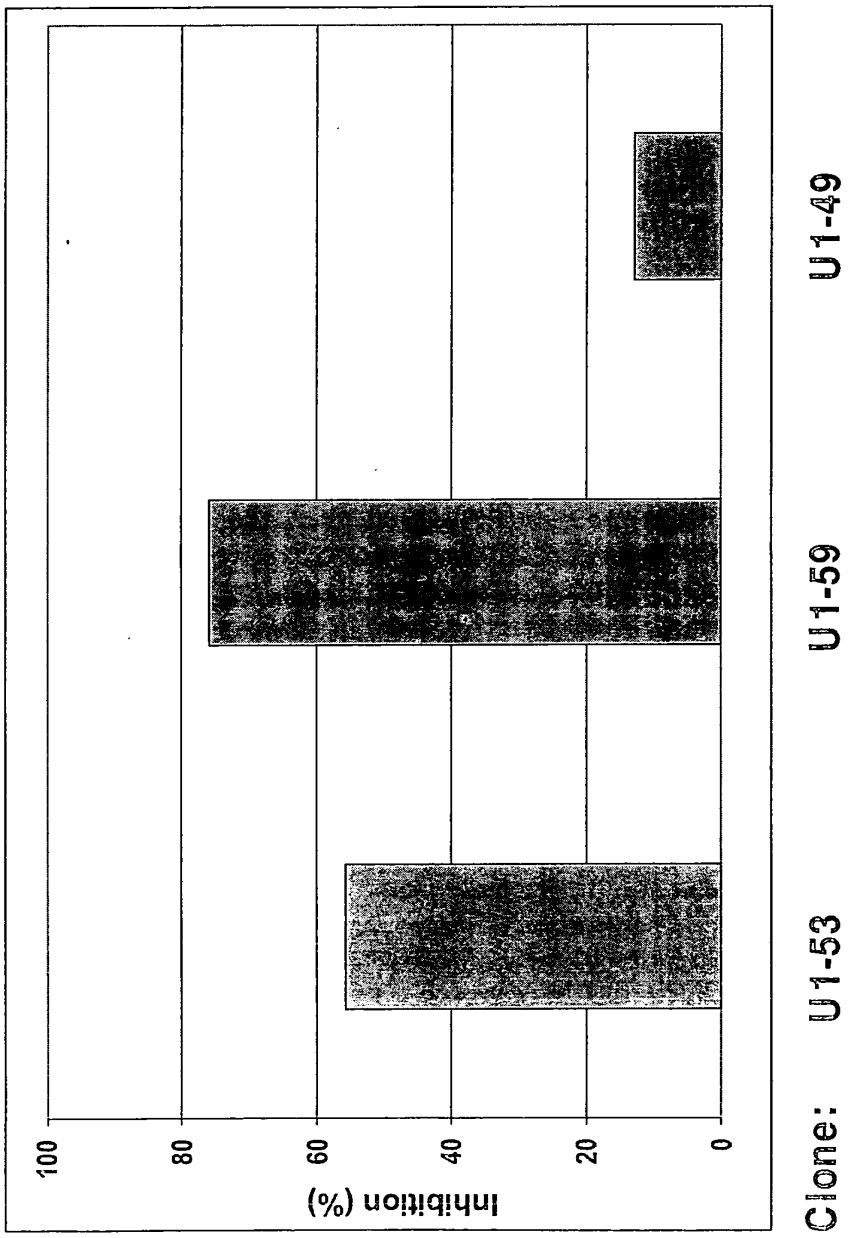


Fig. 9 AKT

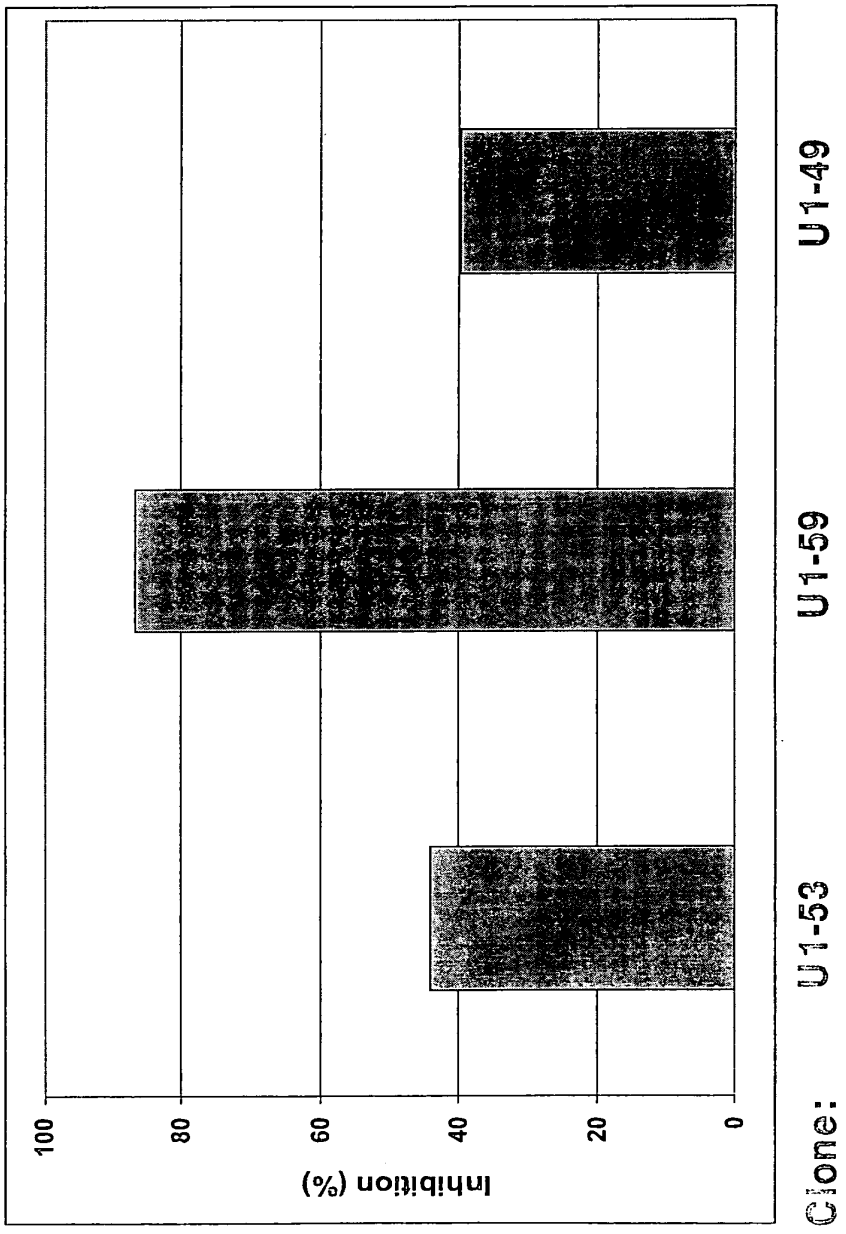


Fig. 10 proliferation

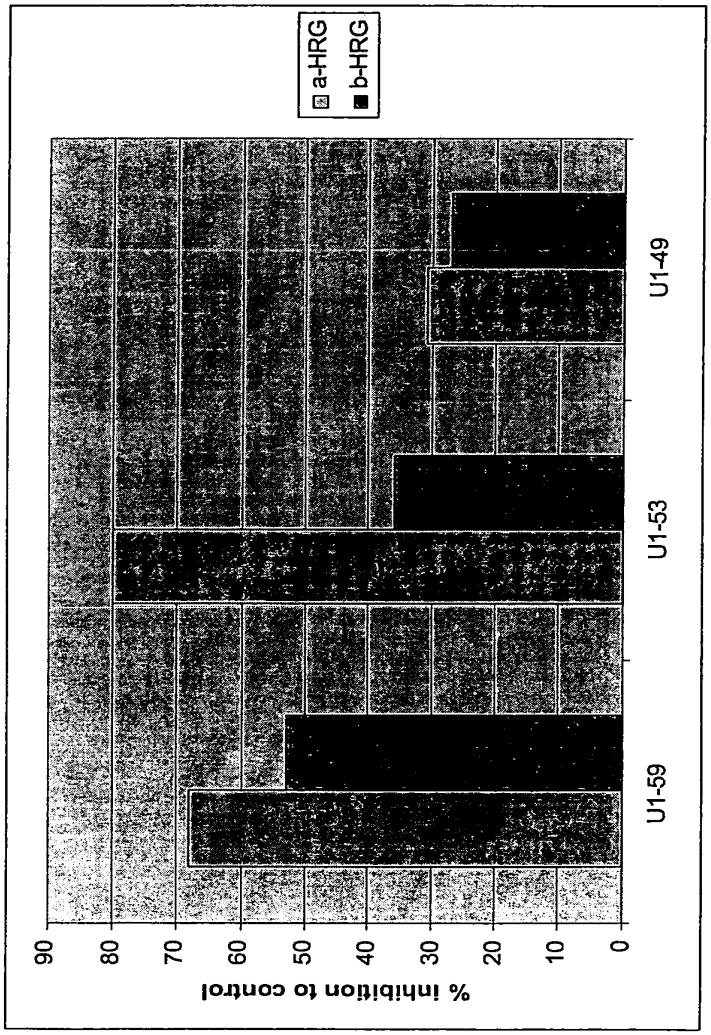


Fig. 11

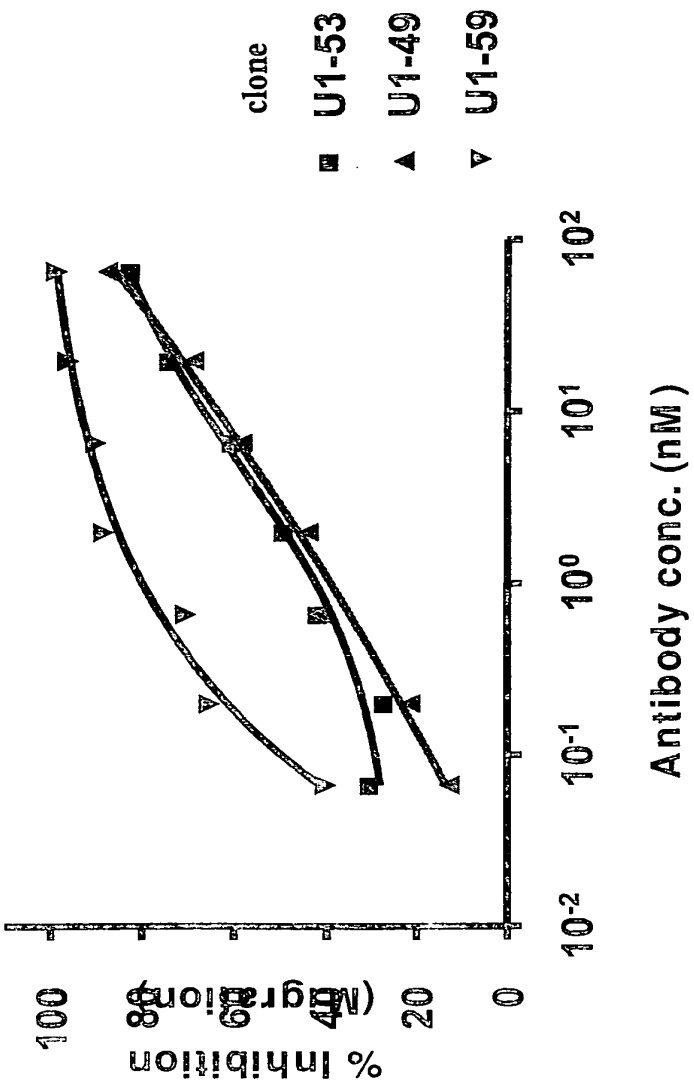

clone
U1-53
U1-49
U1-59
% inhibition (migration)
100
80
40
20
0
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
Antibody conc. (nM)

Fig. 12a Soft Agar MB361

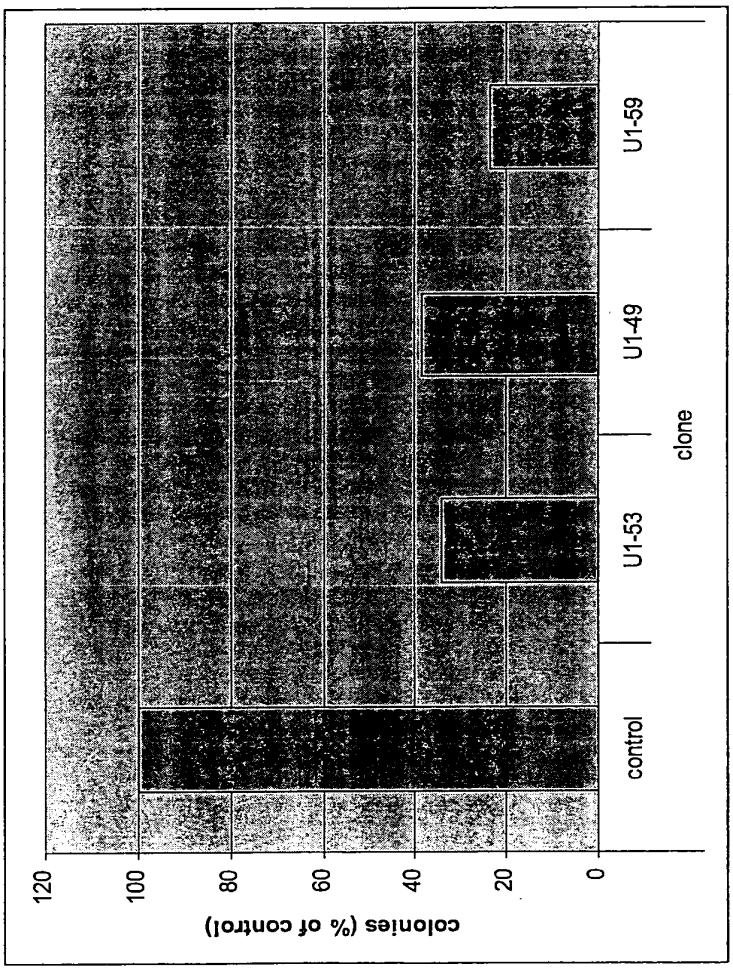


Fig. 12b Soft Agar NCI

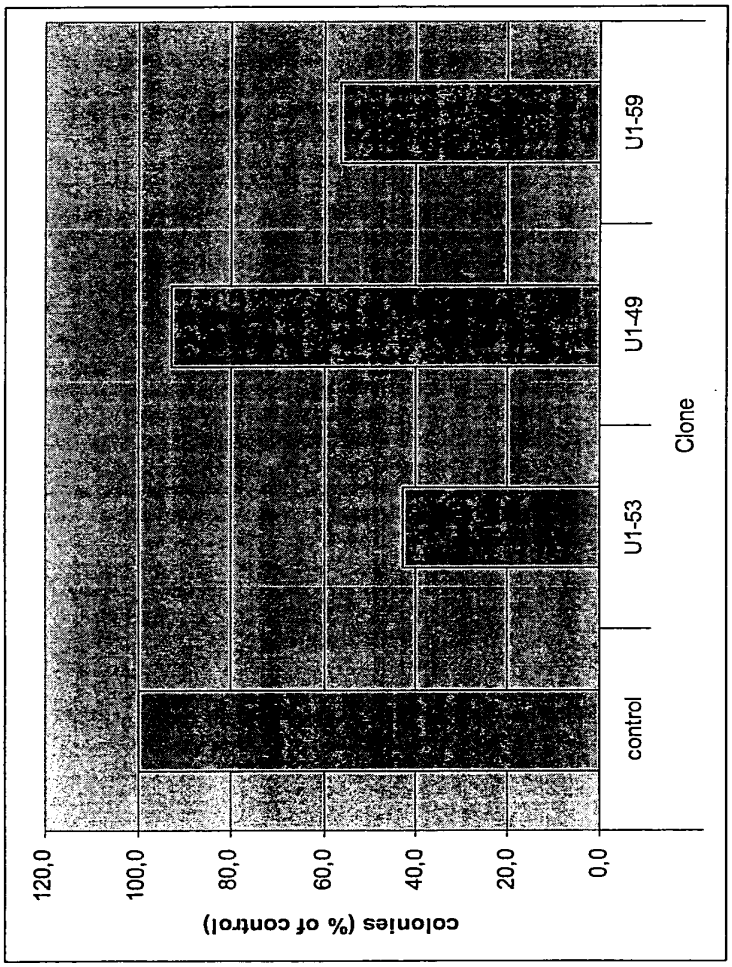


Fig. 12c Soft Agar MKN-28

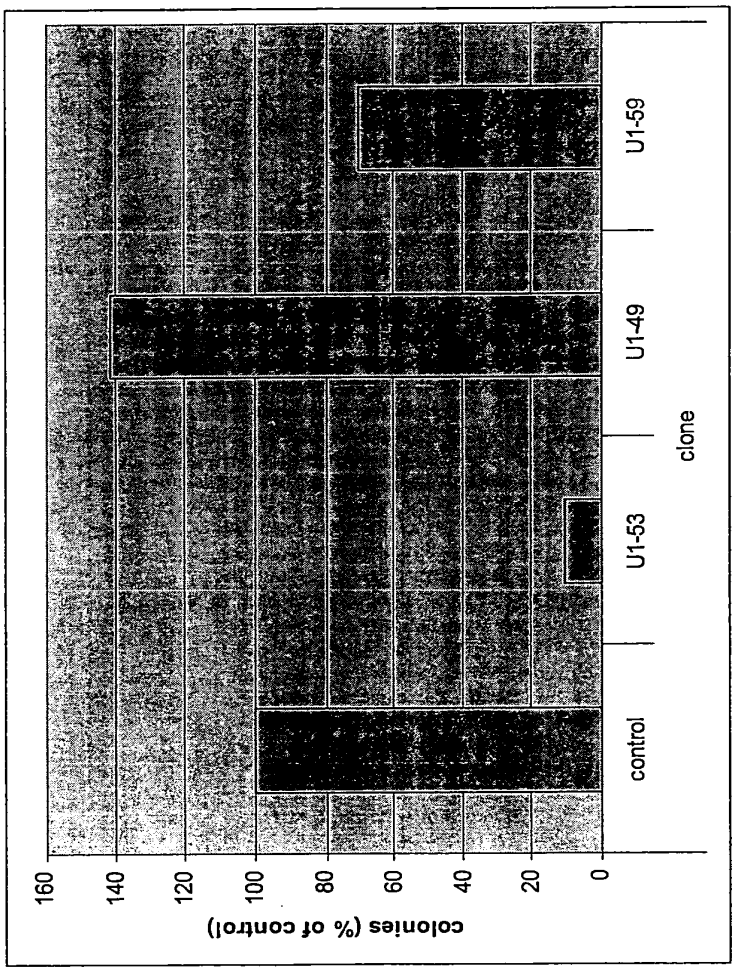


Fig. 12d Soft Agar HT-144

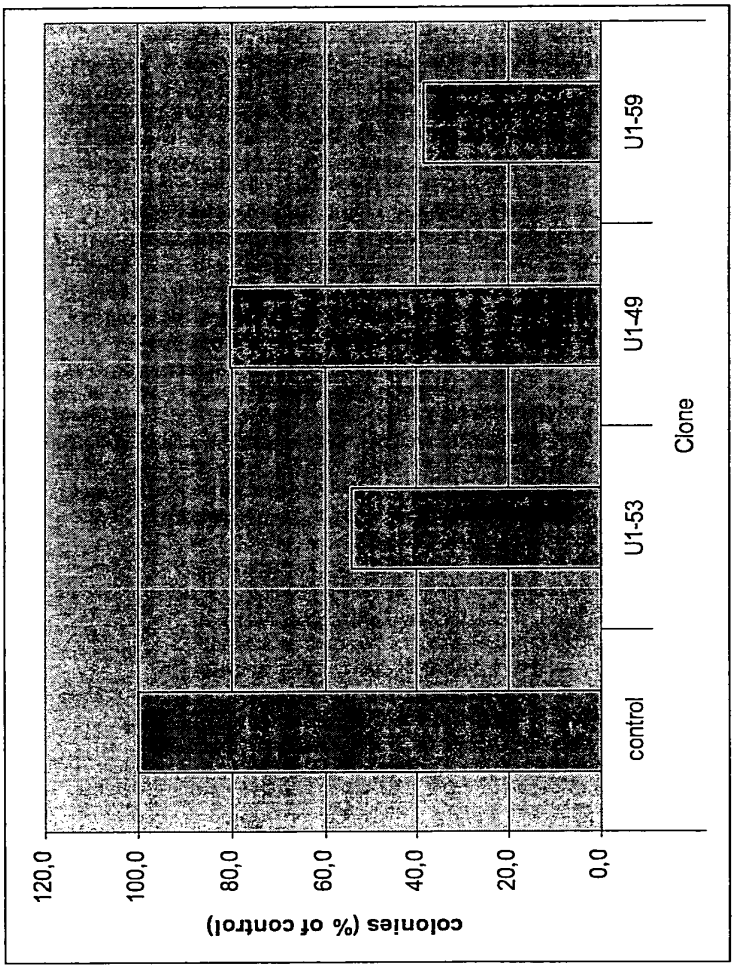


Fig. 12e Soft Agar Skov-3

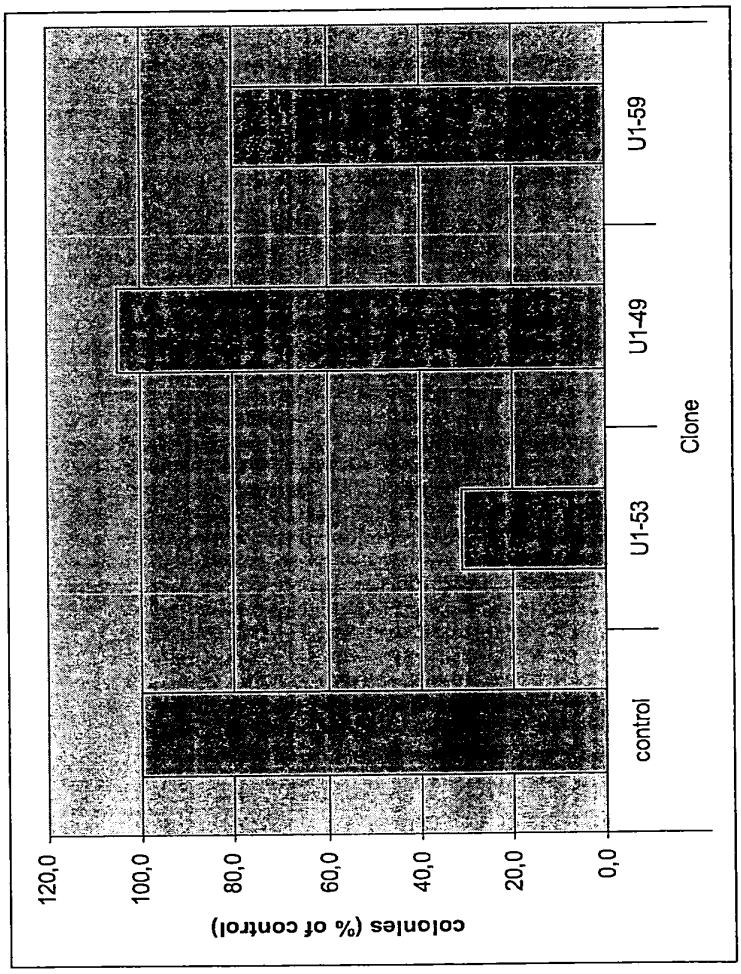


Fig. 12f Soft Agar PPC1

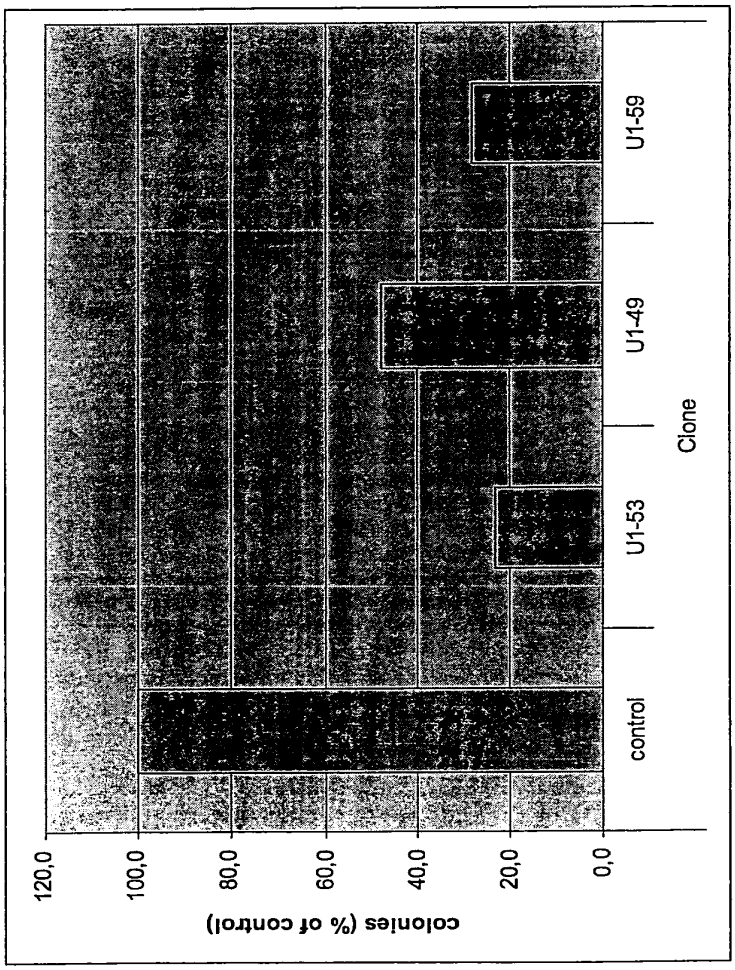


Fig. 12g Soft Agar BX-PC3

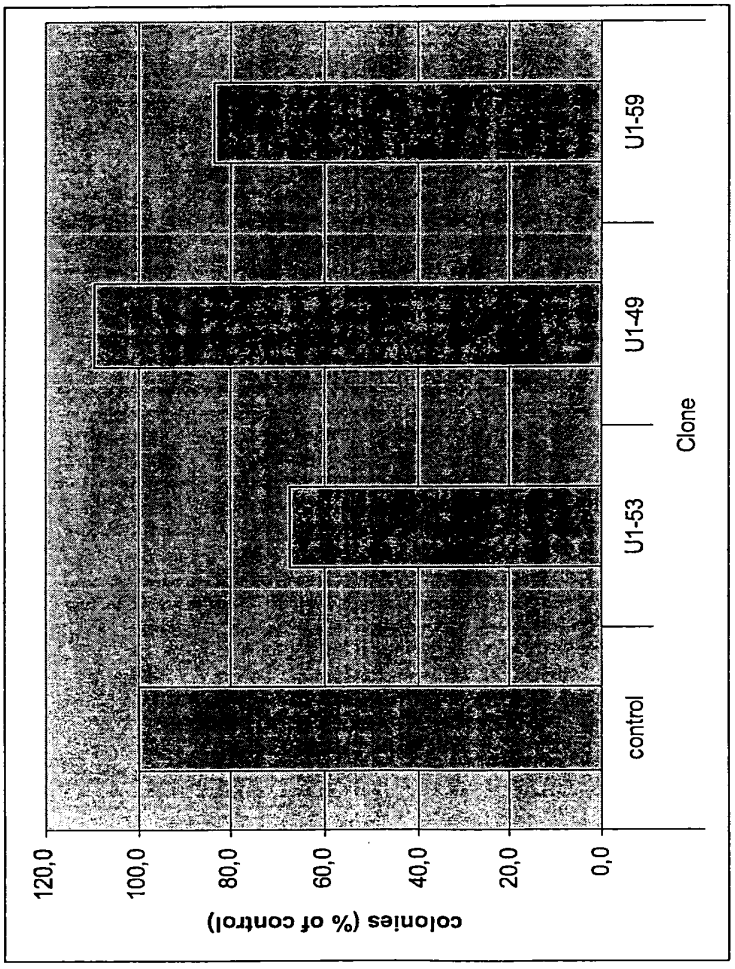


Fig. 12h Soft Agar A549

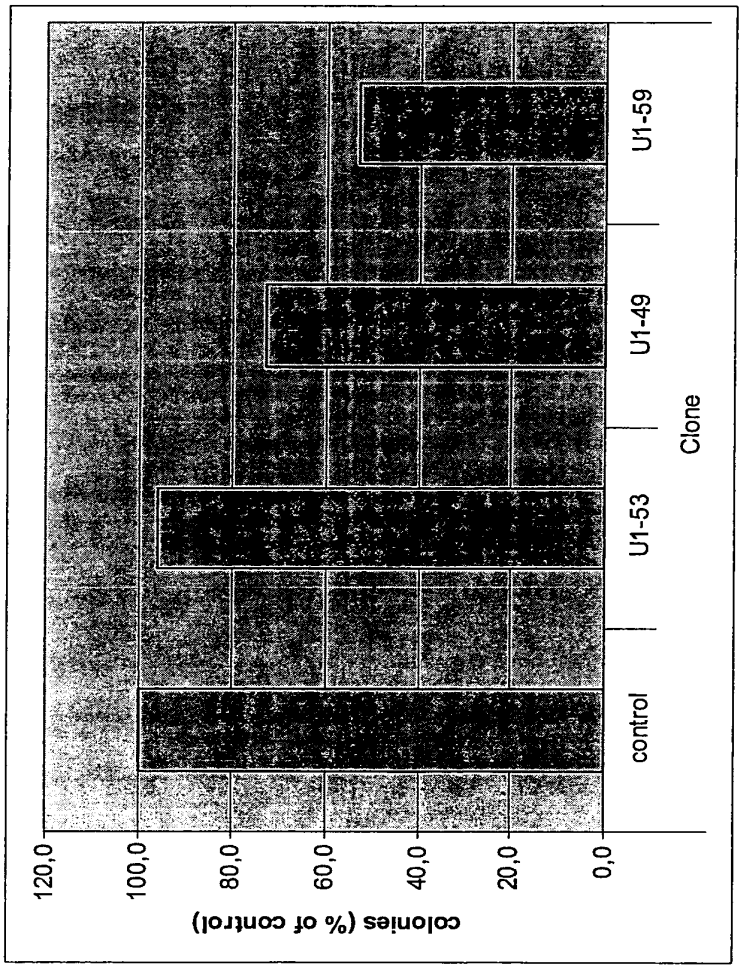


Fig. 12i Soft Agar epidermoid

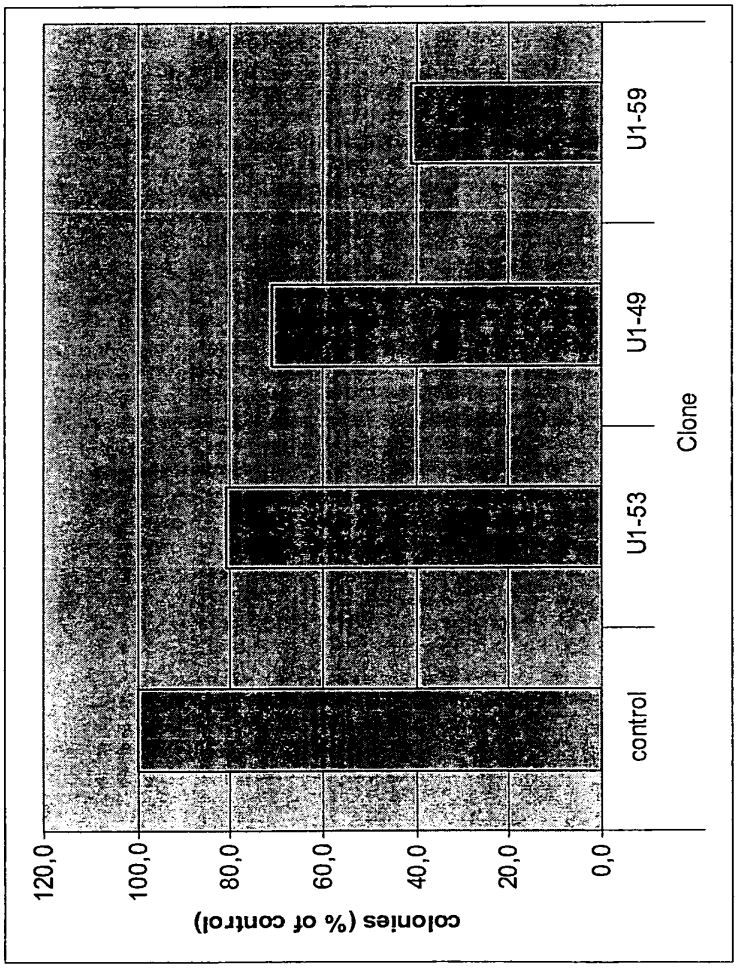


Fig. 13 in vivo T47D

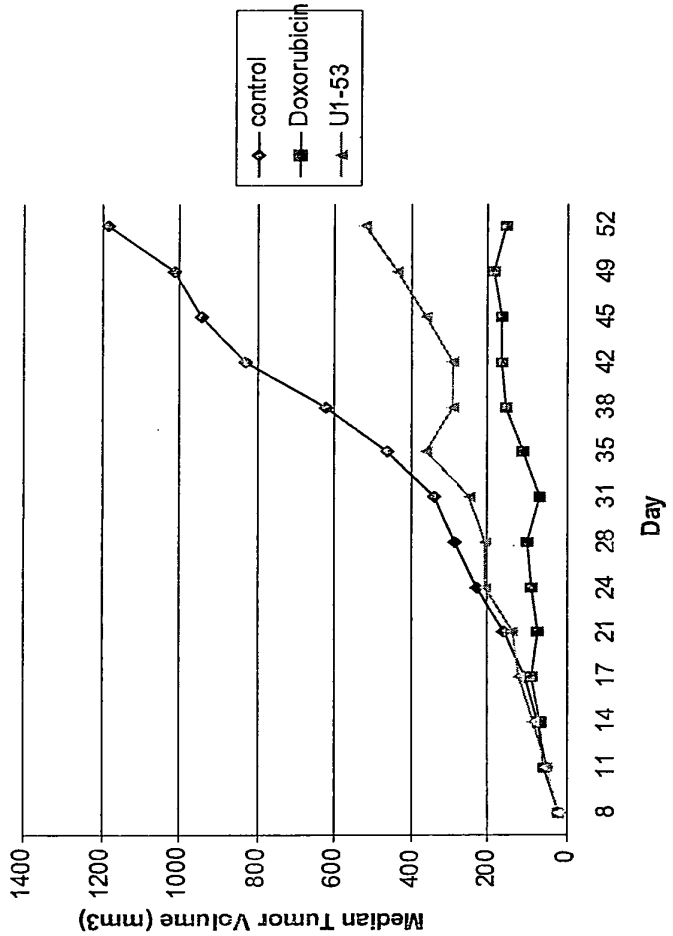


Fig. 14

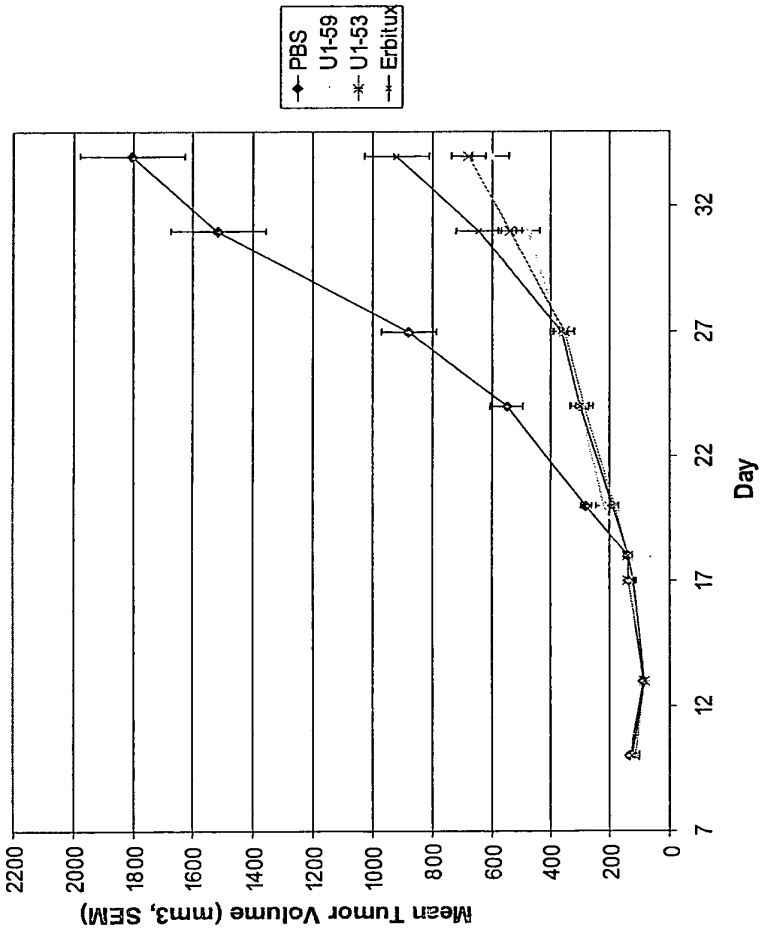

BxPC3
PBS
U1-59
U1-53
Erbitux
Mean Tumor Volume (mm3, SEM)
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
7
12
17
22
27
32
Day

Fig. 15 BxPC3 combination

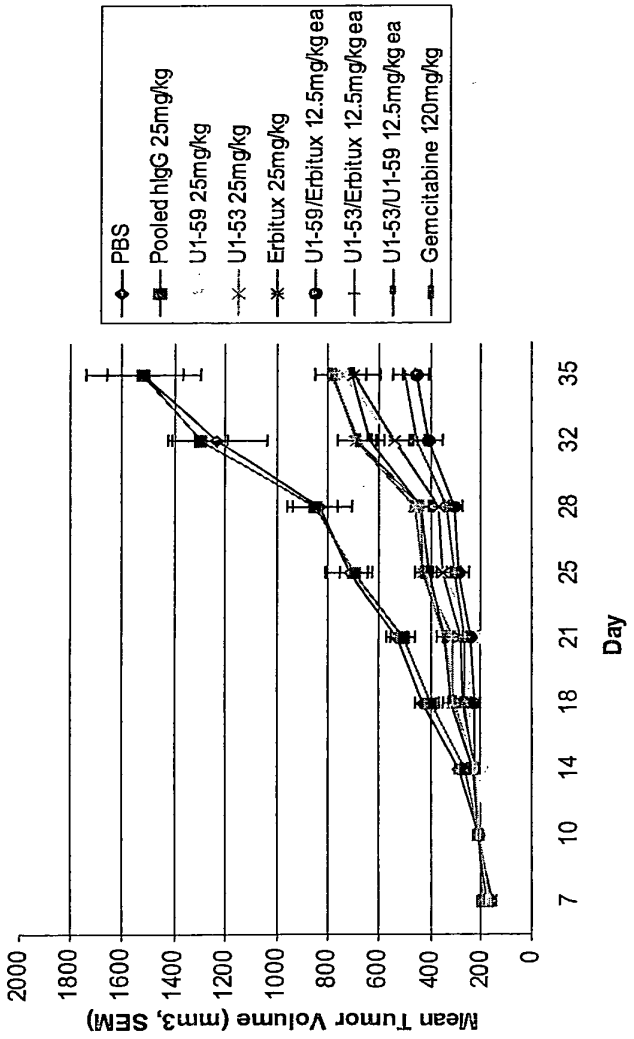


Fig. 16 HT144

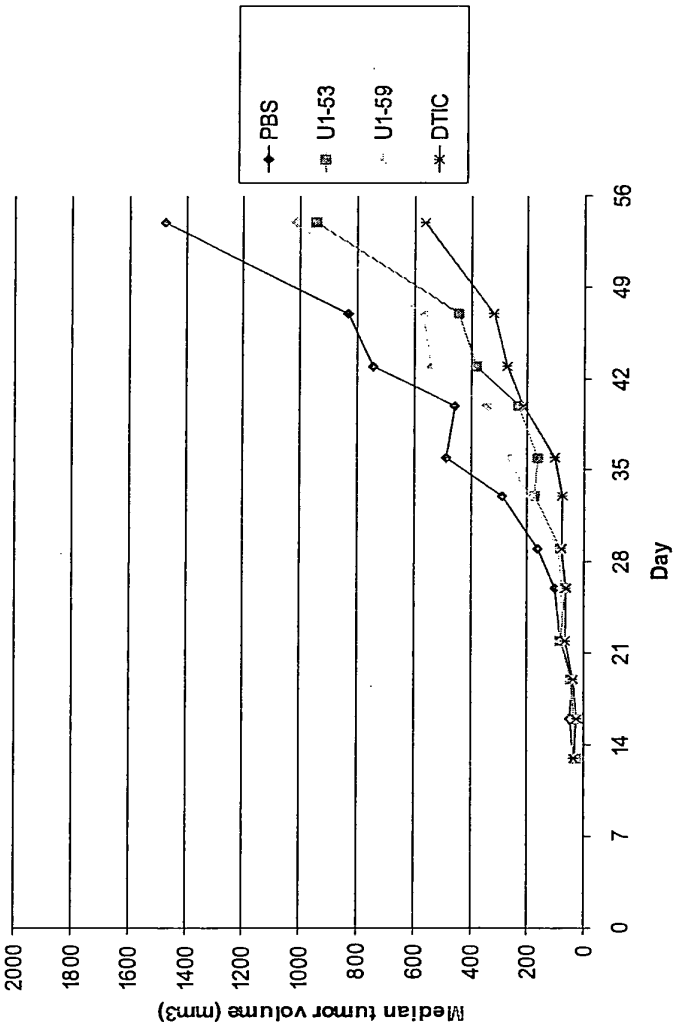
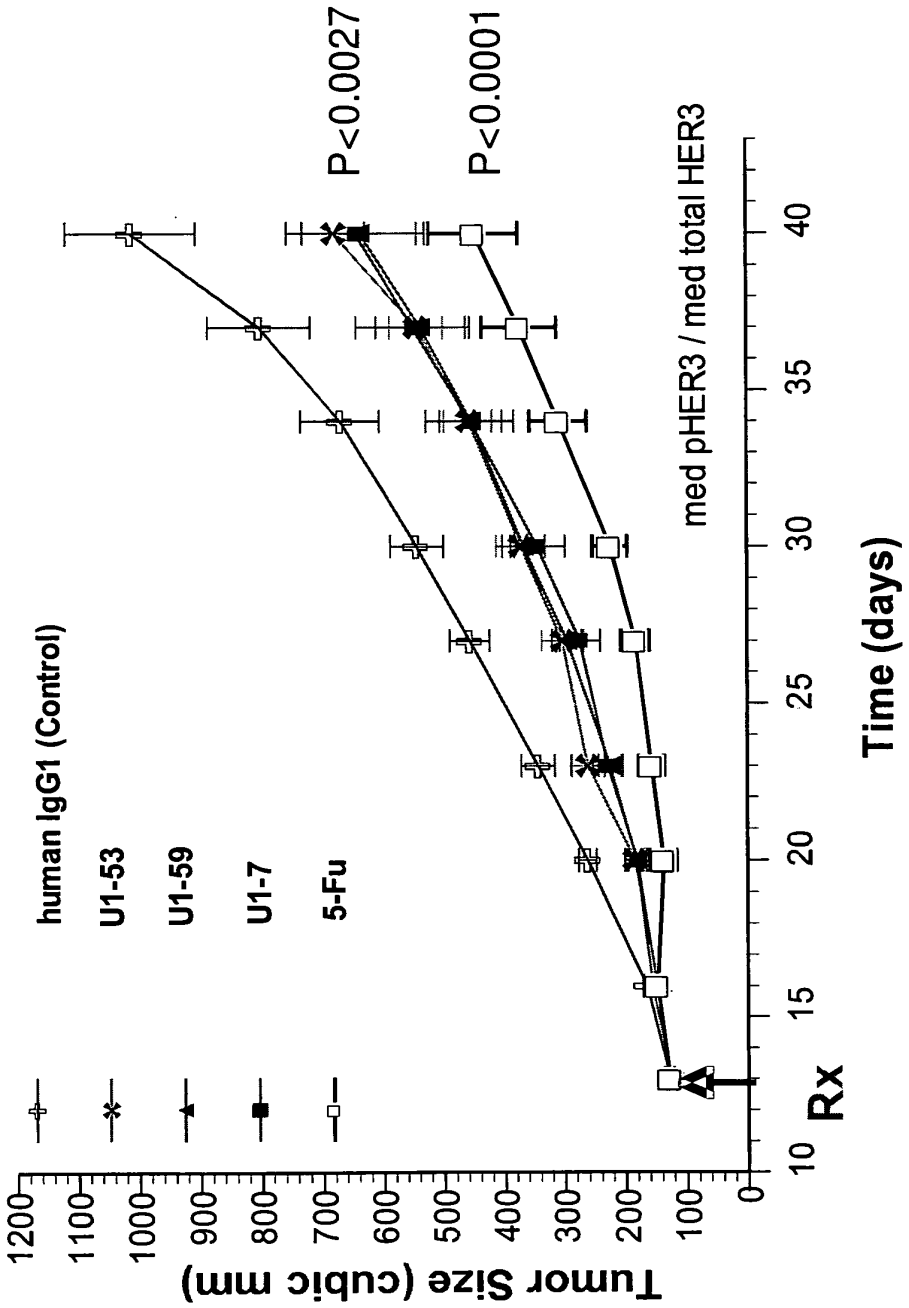

PBS
U1-53
U1-59
DTIC
Median tumor volume (mm3)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
7
14
21
28
35
42
49
56
Day

Fig. 17


Tumor Size (cubic mm)
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
10
15
20
25
30
35
40
Time (days)
Rx
human IgG1 (Control)
U1-53
U1-59
U1-7
5-Fu
P<0.0027
P<0.0001
med pHER3 / med total HER3

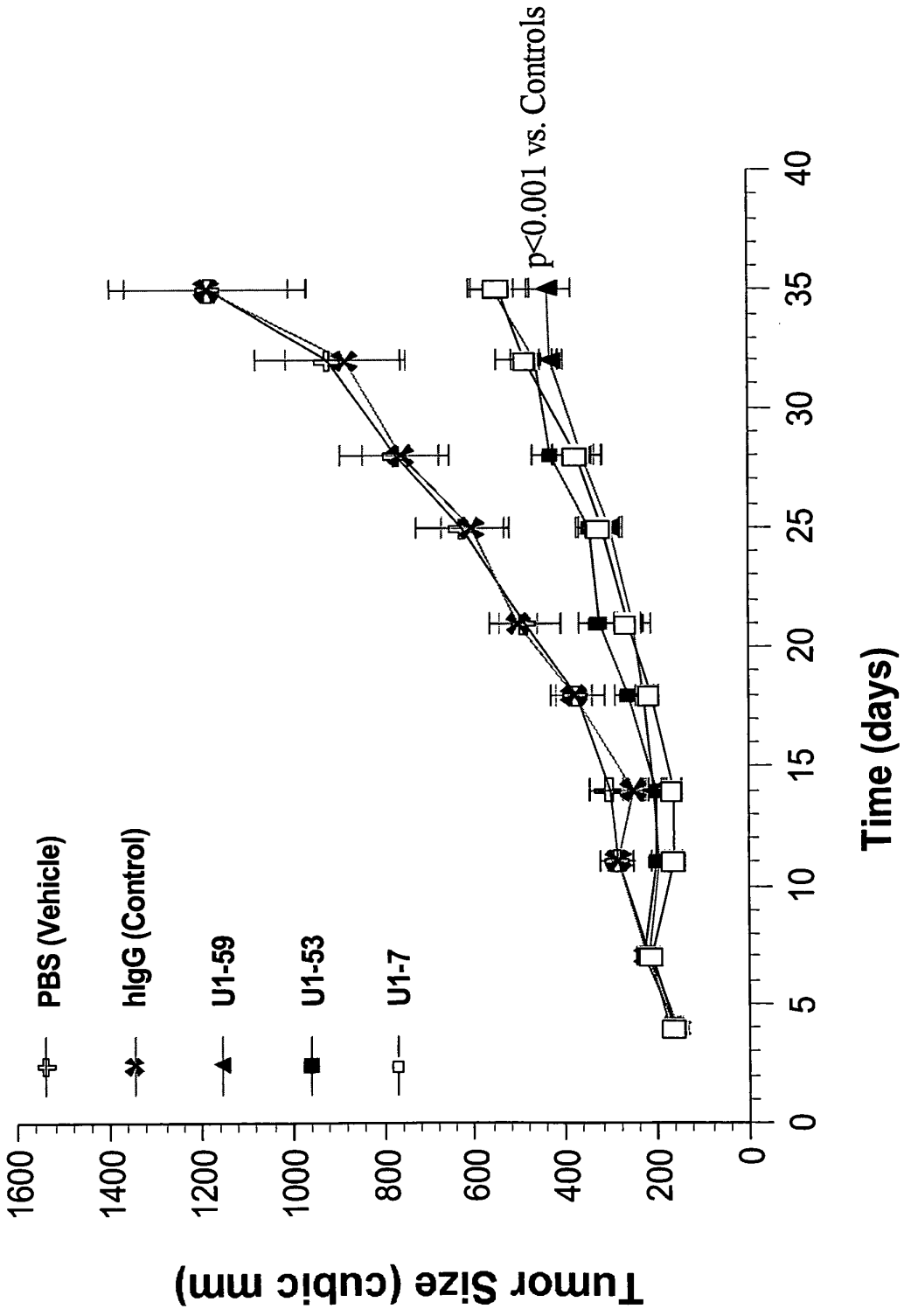
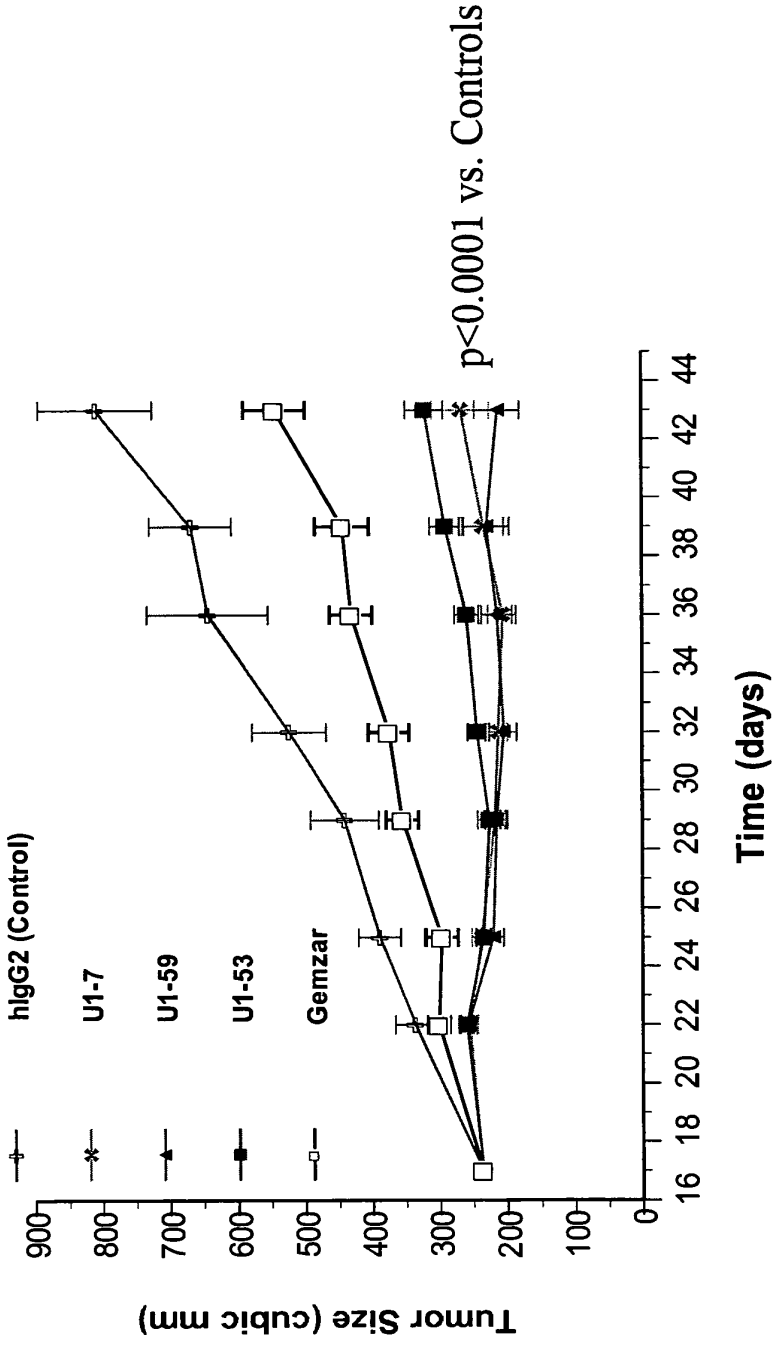

Tumor Size (cubic mm)
1600
1400
1200
1000
800
600
400
200
0
0
5
10
15
20
25
30
35
40
Time (days)
PBS (Vehicle)
hIgG (Control)
U1-59
U1-53
U1-7
p<0.001 vs. Controls

Fig. 18

Fig. 19


p<0.0001 vs. Controls
hIgG2 (Control)
U1-7
U1-59
U1-53
Gemzar
Tumor Size (cubic mm)
Time (days)
0
100
200
300
400
500
600
700
800
900
16
18
20
22
24
26
28
30
32
34
36
38
40
42
44

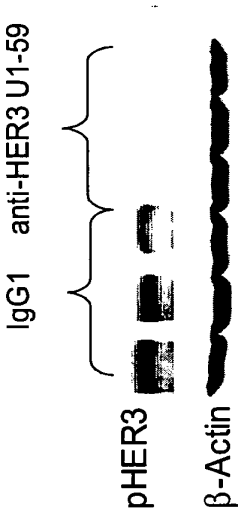


Fig. 20

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(54) Title: ANTIBODIES AGAINST ERBB3 AND USES THEREOF

(57) Abstract: The present invention provides a novel class of monoclonal antibodies which bind ErbB3 receptor and inhibits various ErbB3 functions. For example, the antibodies described herein are capable of binding to ErbB3 and inhibiting EGF-like ligand mediated phosphorylation of the receptor.

ANTIBODIES AGAINST ERBB3 AND USES THEREOF

Background of the Invention

The ErbB/HER subfamily of polypeptide growth factor receptors include the epidermal growth factor (EGF) receptor (EGFR, ErbB1/HER1), the *neu* oncogene product (ErbB2/HER2), and the more recently identified ErbB3/HER3 and ErbB4/HER4 receptor proteins (see, *e.g.*, Hynes *et. al.* (1994) *Biochim. Biophys. Acta Rev. Cancer* 1198, 165–184). Each of these receptors is predicted to consist of an extracellular ligand-binding domain, a membrane-spanning domain, a cytosolic protein tyrosine kinase (PTK) domain and a C-terminal phosphorylation domain (see, *e.g.*, Kim *et al.*, (1998) *Biochem. J.* 334, 189-195).

Experiments *in vitro* have indicated that the protein tyrosine kinase activity of the ErbB3 protein is attenuated significantly relative to that of other ErbB/HER family members and this attenuation has been attributed, in part, to the occurrence of non-conservative amino acid substitutions in the predicted catalytic domain of ErbB3 (see, *e.g.*, Guy *et al.* (1994) *Proc. Natl. Acad. Sci. USA.* 91, 8132–8136; Sierke *et al.* (1997) Biochem. J. 322, 757–763). However, the ErbB3 protein has been shown to be phosphorylated in a variety of cellular contexts. For example, ErbB3 is constitutively phosphorylated on tyrosine residues in a subset of human breast cancer cell lines overexpressing this protein (see, *e.g.*, Kraus *et al.* (1993) *Proc. Natl. Acad. Sci. USA.* 90, 2900–2904; and Kim *et al.* Supra; see, also, Schaefer et al. (2006) Neoplasia 8(7):613-22 and Schaefer et al. Cancer Res (2004) 64(10):3395-405).

Although, the role of ErbB3 in cancer has been explored (see, *e.g.*, Horst *et al.* (2005) 115, 519-527; Xue *et al.* (2006) *Cancer Res.* 66, 1418-1426), ErbB3 remains largely unappreciated as a target for clinical intervention. Current immunotherapies primarily focus on inhibiting the action of ErbB2 and, in particular, heterodimerization of ErbB2/ErbB3 complexes (see, *e.g.*, Sliwkowski *et al.* (1994) J. Biol. Chem. 269(20):14661-14665 (1994)). Accordingly, it is an object of the present invention to provide improved immunotherapies that effectively inhibit ErbB3 signaling, and can be used to treat and diagnose a variety of cancers.

Summary of the Invention

The present invention provides a novel class of monoclonal antibodies which binds to the ErbB3 receptor and inhibits various ErbB3 functions. For example, the antibodies described herein are capable of binding to ErbB3 and inhibiting EGF-like ligand mediated phosphorylation of the receptor. As described herein, EGF-like ligands include EGF, TGF-α, betacellulin, heparin-binding epidermal growth factor, biregulin

and amphiregulin, which bind to EGFR and induce dimerization of EGFR with ErbB3. This dimerization, in turn, causes phosphorylation of ErbB3, and activates signaling through the receptor. Monoclonal antibodies of the present invention, thus, are useful for treating and diagnosing a variety of cancers associated with ErbB3-mediated cellular signaling. Accordingly, in one embodiment, the present invention provides monoclonal antibodies (and antigen binding portions thereof) which bind to ErbB3 and inhibit EGF-like ligand mediated phosphorylation of ErbB3.

In another embodiment, the antibodies are further characterized by one or more of the following properties: (i) inhibition of ErbB3 ligand-mediated signaling, including signaling mediated by binding of ErbB3 ligands, such as heregulin, epiregulin, epigen and BIR, to ErbB3; (ii) inhibition of proliferation of cells expressing ErbB3; (iii) the ability to decrease levels of ErbB3 on cell surfaces (*e.g.*, by inducing internalization of ErbB3); (iv) inhibition of VEGF secretion of cells expressing ErbB3; (v) inhibition of the migration of cells expressing ErbB3; (vi) inhibition of spheroid growth of cells expressing ErbB3; and/or (vii) binding to an epitope located on domain I (residues 20-209) of ErbB3, for example, an epitope involving or spanning residues 20-202 of the amino acid sequence of ErbB3.

Particular monoclonal antibodies and antigen binding portions thereof of the present invention exhibit a K_D of 50 nM or less, as measured by a surface plasmon resonance assay or a cell binding assay

In further embodiments, particular monoclonal antibodies and antigen binding portions thereof of the present invention include a heavy chain variable region comprising an amino acid sequence which is at least 80% (*e.g.*, 85%, 90%, 95%, 96%, 97%, 98% or 99%) identical to the heavy chain variable region amino acid sequence set forth in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:35, or SEQ ID NO: 37. Other particular monoclonal antibodies and antigen binding portions thereof of the present invention include a light chain variable region comprising an amino acid sequence which is at least 80% (*e.g.*, 85%, 90%, 95%, 96%, 97%, 98% or 99%) identical to the light chain variable region amino acid sequence set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:36, or SEQ ID NO:38. The antibodies may also include both of the aforementioned heavy chain and light chain variable regions.

The variable heavy and light chain regions of the antibodies or antigen binding portions thereof typically include one or more complementarity determining regions (CDRs). These include one or more CDR1, CDR2, and CDR3 regions. Accordingly, other particular antibodies and antigen binding portions thereof of the present invention include one or more CDR sequences selected from a heavy chain variable region CDR1 comprising SEQ ID NO:7; a heavy chain variable region CDR2 comprising SEQ ID NO:8; a heavy chain variable region CDR3 comprising SEQ ID NO:9; a light chain

variable region CDR1 comprising SEQ ID NO:10; a light chain variable region CDR2 comprising SEQ ID NO:11; a light chain variable region CDR3 comprising SEQ ID NO:12; and combinations thereof.

Still other particular antibodies and antigen binding portions thereof of the present invention include one or more CDR sequences selected from a heavy chain variable region CDR1 comprising SEQ ID NO:13; a heavy chain variable region CDR2 comprising SEQ ID NO:14; a heavy chain variable region CDR3 comprising SEQ ID NO:15; a light chain variable region CDR1 comprising SEQ ID NO:16; a light chain variable region CDR2 comprising SEQ ID NO:17; a light chain variable region CDR3 comprising SEQ ID NO:18; and combinations thereof.

Still other particular antibodies and antigen binding portions thereof of the present invention include; or one or more CDR sequences selected from a heavy chain variable region CDR1 comprising SEQ ID NO:19; a heavy chain variable region CDR2 comprising SEQ ID NO:20; a heavy chain variable region CDR3 comprising SEQ ID NO:21; a light chain variable region CDR1 comprising SEQ ID NO:22; a light chain variable region CDR2 comprising SEQ ID NO:23; a light chain variable region CDR3 comprising SEQ ID NO:24; and combinations thereof.

Still other particular antibodies and antigen binding portions thereof of the present invention include; or one or more CDR sequences selected from a heavy chain variable region CDR1 comprising SEQ ID NO:39; a heavy chain variable region CDR2 comprising SEQ ID NO:40; a heavy chain variable region CDR3 comprising SEQ ID NO:41; a light chain variable region CDR1 comprising SEQ ID NO:42; a light chain variable region CDR2 comprising SEQ ID NO:43; a light chain variable region CDR3 comprising SEQ ID NO:44; and combinations thereof.

Still other particular antibodies and antigen binding portions thereof of the present invention include; or one or more CDR sequences selected from a heavy chain variable region CDR1 comprising SEQ ID NO:45; a heavy chain variable region CDR2 comprising SEQ ID NO:46; a heavy chain variable region CDR3 comprising SEQ ID NO:47; a light chain variable region CDR1 comprising SEQ ID NO:48; a light chain variable region CDR2 comprising SEQ ID NO:49; a light chain variable region CDR3 comprising SEQ ID NO:50; and combinations thereof.

The antibodies and antigen binding portions thereof may also comprise one or more CDRs which are at least 80% (*e.g.*, 85%, 90%, 95%, 96%, 97%, 98% or 99%) identical to any of the aforementioned CDRs, or combinations of CDRs.

In one embodiment, the antibodies and antibody portions thereof are fully human (*i.e.*, contains human CDR and framework sequences). Particular human antibodies of the present invention include those having a heavy chain variable region that is from a

human VH3 germ line gene, and/or a light chain variable region from human VL2 germ line gene.

Also encompassed by the present invention are monoclonal antibodies and portions thereof that bind to the same or overlapping epitopes bound by any of the antibodies or portions thereof described herein (e.g., an epitope located on domain I of ErbB3, such as an epitope involving or spanning, residues 20-202 of the amino acid sequence of ErbB3). Antibodies which have the same activity as the antibodies described herein, e.g., antibodies having the same sequence as Ab #6, are also encompassed by the present invention.

Antibodies of the present invention include all known forms of antibodies and other protein scaffolds with antibody-like properties. For example, the antibody can be a human antibody, a humanized antibody, a bispecific antibody, a chimeric antibody or a protein scaffold with antibody-like properties, such as fibronectin or Ankyrin repeats. The antibody also can be a Fab, Fab'2, ScFv, SMIP, affibody, nanobody, or a domain antibody. The antibody also can have any of the following isotypes: IgG1, IgG2, IgG3, IgG4, IgM, IgA1, IgA2, IgAsec, IgD, and IgE.

In yet another embodiment, the present invention further provides compositions comprising combinations of antibodies or antigen binding portions described herein, formulated with an acceptable carrier and/or adjuvant. In a particular embodiment, the composition comprises two or more antibodies that bind different epitopes on ErbB3 or antibodies described herein combined with anti-cancer antibodies which do not bind ErbB3.

In still another embodiment, the present invention provides isolated nucleic acids encoding the antibodies and antigen binding portions thereof described herein. In particular embodiments, the nucleic acid encodes a heavy chain variable region comprising a nucleotide sequence which is at least 80% (*e.g.*, 85%, 90%, 95%, 96%, 97%, 98% or 99%) identical to, or which hybridizes under high stringency conditions to, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:29, SEQ ID NO:35, or SEQ ID NO:37; or a light chain variable region comprising a nucleotide sequence which is at least 80% (*e.g.*, 85%, 90%, 95%, 96%, 97%, 98% or 99%) identical to, or which hybridizes under high stringency conditions to, SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:30, SEQ ID NO:36, or SEQ ID NO:38; or combinations of such heavy and light variable regions.

The present invention further provides transgenic non-human mammals, hybridomas, and transgenic plants that express and/or produce the antibodies and antigen binding portions described herein.

Also provided by the invention are kits comprising one or more isolated monoclonal antibodies or antigen binding portions thereof described herein and,

optionally, instructions for use in treating or diagnosing a disease associated with ErbB3 dependent signaling, such as cancers.

Antibodies and antigen binding portions thereof of the present invention can be used in a broad variety of therapeutic and diagnostic applications, particularly oncological applications. Accordingly, in another aspect, the invention provides method for inhibiting EGF-like ligand mediated phosphorylation of ErbB3 in a subject by administering one or more antibodies or antigen binding portions thereof described herein in an amount sufficient to inhibit EGF-like mediated phosphorylation of ErbB3. The invention further provides methods for treating a variety of cancers in a subject, including, but not limited to, melanoma, breast cancer, ovarian cancer, renal carcinoma, gastrointestinal/colon cancer, lung cancer, clear cell sarcoma, and prostate cancer, by administering one or more antibodies or antigen binding portions thereof described herein in an amount sufficient to treat the cancer. The antibodies or antigen binding portions thereof can be administered alone or in combination with other therapeutic agents, such as anti-cancer agents, *e.g.*, other antibodies, chemotherapeutic agents and/or radiation.

In yet other embodiments, the invention provides methods for diagnosing and prognosing diseases (*e.g.*, cancers) associated with ErbB3. In one embodiment, this is achieved by contacting antibodies or antigen binding portions of the invention (*e.g.*, *ex vivo* or *in vivo*) with cells from the subject, and measuring the level of binding to ErbB3 on the cells, wherein abnormally high levels of binding to ErbB3 indicate that the subject has a cancer associated with ErbB3.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description of the Drawings

Figures 1A and 1B are bar graphs depicting the binding of various anti-ErbB3 antibody candidates (Fabs, referred to as Abs herein) to ErbB3 expressed on MALME-3M melanoma cells using a goat anti-human Alexa 647 secondary antibody.

Figures 2A-2D are graphs depicting the K_D values of various anti-ErbB3 antibody candidates. Figures 2A and 2B are graphs depicting the K_D value of Antibody # 6 (referred to as Ab #6) and Antibody # 3 (referred to as Ab #3), respectively, as measured using surface plasmon resonance (SPR) technology. Figures 2C and 2D are graphs depicting the K_D values of Ab #6 and Ab #3, respectively, as measured using a cell binding assay using MALME-3M melanoma cells.

Figure 3 is a graph depicting the binding specificity of an anti-ErbB3 antibody (Ab #6) to ErbB3 using ELISA. EGFR, BSA and TGF-α were used as controls.

Figure 4 is a graph depicting the ability of an anti-ErbB3 antibody (Ab #6) to decrease total ErbB3 levels in MALME-3M melanoma cells *in vitro*, as measured using ELISA.

Figures 5A and 5B are graphs depicting the ability of an anti-ErbB3 antibody (Ab #6) to downregulate ErbB3 receptors on MALME-3M cells, measured using FACS analysis. Figure 5A shows the results using an IgG1 isotype of the antibody. Figure 5B shows the results using an IgG2 isotype of the antibody.

Figures 6A-6D are graphs depicting the timecourse of antibody-mediated ErbB3 downregulation (Ab #6), as measured using FACS analysis.

Figure 7 is a bar graph depicting the ability of various anti-ErbB3 antibodies to downregulate ErbB3 in melanoma cells *in vivo*.

Figure 8 is a bar graph depicting the ability of an anti-ErbB3 antibody (Ab #6) to downregulate ErbB3 in ADRr xenografts *in vivo*.

Figure 9 is a graph depicting the ability of an anti-ErbB3 antibody (Ab #6) to inhibit proliferation of MALME-3M cells in a Cell Titer Glow Assay.

Figure 10 is a graph depicting the ability of an anti-ErbB3 antibody (Ab #6) to inhibit cell proliferation in an ovarian cell line, ADRr.

Figure 11 is a graph depicting the ability of an anti-ErbB3 antibody (Ab #6) to inhibit proliferation of ACHN cells.

Figure 12 is a bar graph depicting the ability of an anti-ErbB3 antibody (Ab #6) to inhibit ErbB3 phosphorylation in ADRr xenografts *in vivo*.

Figures 13A-13C are graphs depicting the ability of an anti-ErbB3 antibody (Ab #6) to inhibit betacellulin and heregulin-mediated phosphorylation of ErbB3 in ADRr cells.

Figures 14A-14B are graphs depicting the ability of an anti-ErbB3 antibody (Ab #6 IgG2 isotype) to inhibit ErbB3 phosphorylation in ovarian tumor cell lines OVCAR 5 and OVCAR 8.

Figures 15A-15C are graphs depicting the ability of betacellulin (BTC) to bind ErbB1 as shown by a lack of binding to ErbB1 negative MALME-3M cells (Figure 17A); binding to ErbB1 positive ADRr cells at concentrations of 10 nM (Figure 17B) and 200 nM (Figure 17B), respectively, and the inhibition of such binding by Erbitux.

Figures 16A-16B are graphs depicting the ability of an anti-ErbB3 antibody (Ab #6 IgG2 isotype) to inhibit heregulin-mediated signaling in MALME-3M cells. Figure 16A depicts the ability of the Ab #6 to inhibit heregulin-mediated phosphorylation of ErbB3 in MALME-3M cells and 16B depicts the ability of Ab #6 to inhibit phosphorylation of AKT in MALME-3M cells.

Figures 17A-D are graphs depicting the ability of an anti-ErbB3 antibody (Ab #6) to inhibit (A) ovarian (ADRr cells), (B) prostate Du145 cells), (C) ovarian (OvCAR8 cells), and (D) pancreatic (Colo357 cells) tumor growth via xenograft studies.

Figures 18A and 18B are graphs depicting the ability of Ab #6 (Figure 18A) and Fab for Ab #3 (Figure 18B) to inhibit heregulin binding to ErbB3 on MALME-3M cells, as measured using FACS analysis.

Figures 19A and 19B are graphs depicting the ability of Ab #6 to inhibit the binding of epiregulin to ErbB3 on ADRr cells. Figure 19A depicts the binding of epiregulin to ADRr cells, and Figure 19B depicts the ability of both Erbitux and Ab #6 to inhibit epiregulin binding to ADRr cells.

Figures 20A and 20B are graphs depicting the ability of heparin binding epidermal growth factor (HB-EGF) to bind ErbB on ADRr cells (Figure 20A) and the inability of an anti-ErbB3 antibody (Ab #6) to inhibit such binding (Figure 20B).

Figures 21A-21C show the amino acid sequences of the variable heavy and light chain regions of antibodies: Ab #6, Ab #3, Ab #14, Ab #17, and Ab #19.

Figures 22A-22B show the nucleotide sequences of the variable heavy and light chain regions of antibodies: Ab #6, Ab #3, and Ab #14.

Figure 23 shows the amino acid sequences of the variable light chain regions of antibodies: Ab #6, Ab #17, and Ab #19, which have been reverted to the corresponding germline amino acid sequence. Amino acid residue changes are underlined.

Figures 24A-24C are graphs showing the ability of Ab #6 to inhibit VEGF secretion of tumor cells.

Figure 25 is a graph showing the effect of Ab #6 on cell migration.

Figures 26A-C are graphs showing (A) inhibition of spheroid growth in AdrR cells, (B) inhibition of HRG induced spheroid growth in AdrR, and (C) inhibition of HRG induced spheroid growth in Du145 cells.

Figures 27 A and B are graphs showing the effect of Ab #6 on (A) HRG and (B) BTC binding to AdrR cells.

Figure 28 is a graph showing the effect of Ab #6 on HGF induced ErbB3 phosphorylation.

Figures 29 A and B show the effect of Ab #6 on phosphorylation of (A) pErbB1 and pErbB3 and (B) HRG induced ErbB2/3 complex formation.

Figure 30 is a graph showing Ab #6 binds amino acid residues 20-202 of ErbB3.

Detailed Description of the Invention

In order that the present invention may be more readily understood, certain terms are first defined. Additional definitions are set forth throughout the detailed description.

I. Definitions

The terms "ErbB3," "HER3," "ErbB3 receptor," and "HER3 receptor," as used interchangeably herein, refer human ErbB3 protein, as described in U.S. Pat. No. 5,480,968 and Plowman *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:4905-4909 (1990); see, also, Kani et al., Biochemistry 44:15842-857 (2005), Cho and Leahy, Science 297:1330-1333 (2002)).

The term "EGF-like ligand," as used herein, refers to ligands of epidermal growth factor receptor (EGFR), including epidermal growth factor (EGF) and closely related proteins, such as transforming growth factor-α (TGF-α), betacellulin (BTC), heparin-binding epidermal growth factor (HB-EGF), biregulin (BIR) and amphiregulin (AR), which bind to EGFR on the surface of cells and stimulate the receptor's intrinsic protein-tyrosine kinase activity. Specifically, EGF-like ligands induce formation of EGFR (also referred to as ErbB1) and ErbB3 protein complex (see *e.g.*, Kim *et al.*, (1998) *Biochem J.*, 334:189-195), which results in phosphorylation of tyrosine residues in the complex.

The antibodies and antigen binding portions thereof of the present invention inhibit EGF-like ligand mediated phosphorylation of ErbB3 and, in certain embodiments, exhibit one or more of the following additional properties: (i) inhibition of one or more of heregulin, epiregulin, epigen and biregulin (BIR)-mediated signaling through ErbB3; (ii) inhibition of proliferation of cells expressing ErbB3; (iii) the ability to decrease levels of ErbB3 on cell surfaces; (iv) inhibition of VEGF secretion of cells expressing ErbB3; (v) inhibition of the migration of cells expressing ErbB3; (vi) inhibition of spheroid growth of cells expressing ErbB3; and/or (vii) binding to an epitope located on domain I of ErbB3, e.g., an epitope which involves or spans residues 20-202 of the amino acid sequence of ErbB3.

The term "inhibition" as used herein, refers to any statistically significant decrease in biological activity, including full blocking of the activity. For example, "inhibition" can refer to a decrease of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% in biological activity.

Accordingly, the phrase "inhibition of EGF-like ligand mediated phosphorylation of ErbB3," as used herein, refers to the ability of an antibody or antigen binding portion to statistically significantly decrease the phosphorylation of ErbB3 induced by an EGF-like ligand, relative to the phosphorylation in an untreated (control) cell. The cell which expresses ErbB3 can be a naturally occurring cell or cell line or can be recombinantly produced by introducing nucleic acid encoding ErbB3 into a host cell. In one embodiment, the antibody or antigen binding portion thereof inhibits EGF-like ligand mediated phosphorylation of ErbB3 by at least 10%, or at least 20%, or at least 30%, or at least 40%, or at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least

90%, or 100%, as determined, for example, by Western blotting followed by probing with an anti-phosphotyrosine antibody as described in Kim *et al.*, (1998) *Biochem J.*, 334:189-195 and the Examples *infra*.

The phrase "inhibition of heregulin, epiregulin, epigen or biregulin-mediated signaling through ErbB3," as used herein, refers to the ability of an antibody or an antigen-binding portion thereof to statistically significantly decrease signaling mediated by an ErbB3 ligand (*e.g.*, heregulin, epiregullin, epigen and biregulin) through ErbB3, relative to the signaling in the absence of the antibody (control). ErbB3-ligands are also referred to herein as "heregulin-like ligands." This means that, in the presence of the antibody or antigen binding portion thereof, a signal mediated in a cell expressing ErbB3 by one or more of heregulin, epiregulin, epigen and biregulin, relative to a control (no antibody), is statistically significantly decreased. An ErbB3-ligand mediated signal can be measured by assaying for the level or activity of an ErbB3 substrate, and/or a protein which is present in a cellular cascade involving ErbB3. In one embodiment, the antibody or antigen binding portion thereof decreases the level or activity of an ErbB3 substrate and/or that of a protein in a cellular cascade involving ErbB3, by at least 10%, or at least 20%, or at least 30%, or at least 40%, or at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or 100% relative to the level or activity in the absence of such antibody or antigen binding portion thereof (control). Such ErbB3-ligand mediated signaling can be measured using art recognized techniques which measure the level or activity of a substrate of ErbB3 (*e.g.*, SHC or PI3K) or a protein in a cellular cascade involving ErbB3 (*e.g.*, AKT) using kinase assays for such proteins (see, *e.g.*, Horst *et al. supra*, Sudo *et al.* (2000) *Methods Enzymol*, 322:388-92; and Morgan *et al.* (1990) *Eur. J. Biochem.*, 191:761-767).

In a particular embodiment, the antibody or antigen binding portion thereof inhibits ErbB3-ligand (*e.g.*, heregulin, epiregulin, epigen or biregulin) mediated signaling through ErbB3 by inhibiting the binding of the ErbB3-ligand (*e.g.*, one or more of heregulin, epiregulin, epigen or biregulin) to ErbB3. Some ligands (*e.g.*, biregulin or BIR) function both as EGF-like ligands (*i.e.*, bind to EGFR/ErbB1) as well as ErbB3-like ligands (*i.e.*, bind to ErbB3).

The phrase "inhibition of heregulin, epiregulin, epigen or biregulin binding to ErbB3," as used herein, refers to the ability of an antibody or an antigen-binding portion thereof to statistically significantly decrease the binding of an ErbB3 ligand (*e.g.*, one or more of heregulin, epiregulin, epigen or biregulin) to ErbB3, relative to the binding in the absence of the antibody (control). This means that, in the presence of the antibody or antigen binding portion thereof, the amount of the ErbB3-ligand (*e.g.*, heregulin, epiregulin, epigen or biregulin) which binds to ErbB3 relative to a control (no antibody), is statistically significantly decreased. The amount of an ErbB3 ligand which binds

ErbB3 may be decreased in the presence of an antibody or antigen binding portion thereof of the invention by at least 10%, or at least 20%, or at least 30%, or at least 40%, or at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or 100% relative to the amount in the absence of the antibody or antigen binding portion thereof (control). A decrease in ErbB3-ligand binding can be measured using art recognized techniques which measure the level of binding of labeled ErbB3-ligand (*e.g.*, radiolabelled heregulin, epiregulin, epigen or biregulin) to cells expressing ErbB3 in the presence or absence (control) of the antibody or antigen binding portion thereof.

The phrase "inhibition of proliferation of a cell expressing ErbB3," as used herein, refers to the ability of an antibody or an antigen-binding portion thereof to statistically significantly decrease proliferation of a cell expressing ErbB3 relative to the proliferation in the absence of the antibody. In one embodiment, the proliferation of a cell expressing ErbB3 (*e.g.*, a cancer cell) may be decreased by at least 10%, or at least 20%, or at least 30%, or at least 40%, or at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or 100% when the cells are contacted with the antibody or antigen binding portion thereof of the present invention, relative to the proliferation measured in the absence of the antibody or antigen binding portion thereof (control). Cellular proliferation can be assayed using art recognized techniques which measure rate of cell division, the fraction of cells within a cell population undergoing cell division, and/or rate of cell loss from a cell population due to terminal differentiation or cell death (*e.g.*, using a cell titer glow assay or thymidine incorporation).

The phrase "the ability to decrease levels of ErbB3 on cell surfaces," as used herein, refers to the ability of an antibody or antigen binding portion thereof to statistically significantly reduce the amount of ErbB3 found on the surface of a cell which has been exposed to the antibody relative to an untreated (control) cell. For example, a decrease in levels of ErbB3 on cell surfaces may result from increased internalization of ErbB3 (or increased ErbB3 endocytosis). In one embodiment, the antibody or antigen binding portion thereof decreases cell surface expression of ErbB3 by at least 10%, or at least 20%, or at least 30%, or at least 40%, or at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or 100% and/or increases internalization of the ErbB3 receptor by at least 10%, or at least 20%, or at least 30%, or at least 40%, or at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or 100% relative to the cell surface expression or internalization in the absence of the antibody or antigen binding portion thereof (control). The levels of ErbB3 on surfaces of cells and/or internalization of the ErbB3 receptor in the absence and the presence of an antibody or antigen-binding portion thereof can be readily measured using art recognized techniques, such as those described in Horst *et al.*, *supra* and in the examples herein.

The phrase "inhibition of VEGF secretion of cells expressing ErbB3," as used herein, refers to the ability of an antibody or an antigen-binding portion thereof to statistically significantly decrease VEGF secretion of a cell expressing ErbB3 relative to the VEGF secretion in the absence of the antibody. In one embodiment, the VEGF secretion of a cell expressing ErbB3 (*e.g.*, a cancer cell) may be decreased by at least 10%, or at least 20%, or at least 30%, or at least 40%, or at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or 100% when the cells are contacted with the antibody or antigen binding portion thereof of the present invention, relative to the VEGF secretion measured in the absence of the antibody or antigen binding portion thereof (control). VEGF secretion can be assayed using art recognized techniques, such as those described herein.

The phrase "inhibition of the migration of cells expressing ErbB3," as used herein, refers to the ability of an antibody or an antigen-binding portion thereof to statistically significantly decrease the migration of a cell expressing ErbB3 relative to the migration of the cell in the absence of the antibody. In one embodiment, the migration of a cell expressing ErbB3 (*e.g.*, a cancer cell) may be decreased by at least 10%, or at least 20%, or at least 30%, or at least 40%, or at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or 100% when the cells are contacted with the antibody or antigen binding portion thereof of the present invention, relative to cell migration measured in the absence of the antibody or antigen binding portion thereof (control). Cell migration can be assayed using art recognized techniques, such as those described herein.

The phrase "inhibition of spheroid growth of cells expressing ErbB3," as used herein, refers to the ability of an antibody or an antigen-binding portion thereof to statistically significantly decrease the migration of a cell expressing ErbB3 relative to the migration of the cell in the absence of the antibody. In one embodiment, the migration of a cell expressing ErbB3 (*e.g.*, a cancer cell) may be decreased by at least 10%, or at least 20%, or at least 30%, or at least 40%, or at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or 100% when the cells are contacted with the antibody or antigen binding portion thereof of the present invention, relative to cell migration measured in the absence of the antibody or antigen binding portion thereof (control). Cell migration can be assayed using art recognized techniques, such as those described herein. The term "antibody" or "immunoglobulin," as used interchangeably herein, includes whole antibodies and any antigen binding fragment (*i.e.*, "antigen-binding portion") or single chains thereof. An "antibody" comprises at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as V_H) and a heavy chain constant region. The heavy chain constant region is comprised of three

domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as V_L) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The V_H and V_L regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and V_L is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (*e.g.*, effector cells) and the first component (Clq) of the classical complement system. Exemplary antibodies of the invention include antibodies#1, 3 and 14, and antigen-binding portions thereof.

The term "antigen-binding portion" of an antibody (or simply "antibody portion"), as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen (*e.g.*, ErbB3). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the V_L, V_H, CL and CH1 domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the V_H and CH1 domains; (iv) a Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody, (v) a dAb including VH and VL domains; (vi) a dAb fragment (Ward *et al.* (1989) *Nature* 341, 544-546), which consists of a V_H domain; (vii) a dAb which consists of a VH or a VL domain; and (viii) an isolated complementarity determining region (CDR) or (ix) a combination of two or more isolated CDRs which may optionally be joined by a synthetic linker. Furthermore, although the two domains of the Fv fragment, V_L and V_H, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules (known as single chain Fv (scFv); see *e.g.*, Bird *et al.* (1988) *Science* 242, 423-426; and Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85, 5879-5883). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. These antibody fragments are obtained using conventional techniques known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies. Antigen-binding portions can be produced by recombinant DNA techniques, or by enzymatic or chemical cleavage of intact immunoglobulins.

The term "monoclonal antibody" as used herein 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 conventional (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. Monoclonal antibodies can be prepared using any art recognized technique and those described herein such as, for example, a hybridoma method, as described by Kohler *et al.* (1975) *Nature*, 256:495, a transgenic animal, as described by, for example, (see *e.g.*, Lonberg, *et al.* (1994) Nature 368(6474): 856-859), recombinant DNA methods (see, *e.g.*, U.S. Pat. No. 4,816,567), or using phage antibody libraries using the techniques described in, for example, Clackson *et al.*, *Nature*, 352:624-628 (1991) and Marks *et al.*, *J. Mol. Biol.*, 222:581-597 (1991). Monoclonal antibodies include chimeric antibodies, human antibodies and humanized antibodies and may occur naturally or be recombinantly produced.

The term "recombinant antibody," refers to antibodies that are prepared, expressed, created or isolated by recombinant means, such as (a) antibodies isolated from an animal (*e.g.*, a mouse) that is transgenic or transchromosomal for immunoglobulin genes (*e.g.*, human immunoglobulin genes) or a hybridoma prepared therefrom, (b) antibodies isolated from a host cell transformed to express the antibody, *e.g.*, from a transfectoma, (c) antibodies isolated from a recombinant, combinatorial antibody library (*e.g.*, containing human antibody sequences) using phage display, and (d) antibodies prepared, expressed, created or isolated by any other means that involve splicing of immunoglobulin gene sequences (*e.g.*, human immunoglobulin genes) to other DNA sequences. Such recombinant antibodies may have variable and constant regions derived from human germline immunoglobulin sequences. In certain embodiments, however, such recombinant human antibodies can be subjected to *in vitro* mutagenesis and thus the amino acid sequences of the V_H and V_L regions of the recombinant antibodies are sequences that, while derived from and related to human germline V_H and V_L sequences, may not naturally exist within the human antibody germline repertoire *in vivo*.

The term "chimeric immunoglobulin" or antibody refers to an immunoglobulin or antibody whose variable regions derive from a first species and whose constant regions derive from a second species. Chimeric immunoglobulins or antibodies can be constructed, for example by genetic engineering, from immunoglobulin gene segments belonging to different species.

The term "human antibody," as used herein, is intended to include antibodies having variable regions in which both the framework and CDR regions are derived from human germline immunoglobulin sequences as described, for example, by Kabat et al. (See Kabat, *et al.* (1991) *Sequences of proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242). Furthermore, if the antibody contains a constant region, the constant region also is derived from human germline immunoglobulin sequences. The human antibodies may include amino acid residues not encoded by human germline immunoglobulin sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*). However, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

The human antibody can have at least one ore more amino acids replaced with an amino acid residue, *e.g.*, an activity enhancing amino acid residue which is not encoded by the human germline immunoglobulin sequence. Typically, the human antibody can have up to twenty positions replaced with amino acid residues which are not part of the human germline immunoglobulin sequence. In a particular embodiment, these replacements are within the CDR regions as described in detail below.

The term "humanized immunoglobulin" or "humanized antibody" refers to an immunoglobulin or antibody that includes at least one humanized immunoglobulin or antibody chain (*i.e.*, at least one humanized light or heavy chain). The term "humanized immunoglobulin chain" or "humanized antibody chain" (*i.e.*, a "humanized immunoglobulin light chain" or "humanized immunoglobulin heavy chain") refers to an immunoglobulin or antibody chain (*i.e.*, a light or heavy chain, respectively) having a variable region that includes a variable framework region substantially from a human immunoglobulin or antibody and complementarity determining regions (CDRs) (*e.g.*, at least one CDR, preferably two CDRs, more preferably three CDRs) substantially from a non-human immunoglobulin or antibody, and further includes constant regions (*e.g.*, at least one constant region or portion thereof, in the case of a light chain, and preferably three constant regions in the case of a heavy chain). The term "humanized variable region" (*e.g.*, "humanized light chain variable region" or "humanized heavy chain variable region") refers to a variable region that includes a variable framework region substantially from a human immunoglobulin or antibody and complementarity determining regions (CDRs) substantially from a non-human immunoglobulin or antibody.

A "bispecific" or "bifunctional antibody" is an artificial hybrid antibody having two different heavy/light chain pairs and two different binding sites. Bispecific

antibodies can be produced by a variety of methods including fusion of hybridomas or linking of Fab' fragments. See, *e.g.*, Songsivilai & Lachmann, (1990) *Clin. Exp. Immunol.* 79, 315-321; Kostelny *et al.* (1992) *J. Immunol.* 148, 1547-1553. In a particular embodiment, a bispecific antibody according to the present invention includes binding sites for both ErbB3 and IGF1-R (*i.e.*, insulin-like growth factor 1-receptor). In another embodiment, a bispecific antibody according to the present invention includes binding sites for both ErbB3 and C-MET. In other embodiments, a bispecific antibody includes a binding site for ErbB3 and a binding site for ErbB2, ERbB3, ErbB4, EGFR, Lewis Y, MUC-1, EpCAM, CA125, prostate specific membrane antigen, PDGFR-α, PDGFR-β, C-KIT, or any of the FGF receptors.

As used herein, a "heterologous antibody" is defined in relation to the transgenic non-human organism or plant producing such an antibody.

An "isolated antibody," as used herein, is intended to refer to an antibody which is substantially free of other antibodies having different antigenic specificities (*e.g.*, an isolated antibody that specifically binds to ErbB3 is substantially free of antibodies that specifically bind antigens other than ErbB3). In addition, an isolated antibody is typically substantially free of other cellular material and/or chemicals. In one embodiment of the invention, a combination of "isolated" monoclonal antibodies having different ErbB3 binding specificities are combined in a well defined composition.

As used herein, "isotype" refers to the antibody class (*e.g.*, IgM or IgGl) that is encoded by heavy chain constant region genes. In one embodiment, an antibody or antigen binding portion thereof is of an isotype selected from an IgG1, an IgG2, an IgG3, an IgG4, an IgM, an IgA1, an IgA2, an IgAsec, an IgD, or an IgE antibody isotype. In some embodiments, a monoclonal antibody of the invention is of the IgG1 isotype. In other embodiments, a monoclonal antibody of the invention is of the IgG2 isotype.

As used herein, "isotype switching" refers to the phenomenon by which the class, or isotype, of an antibody changes from one Ig class to one of the other Ig classes.

As used herein, "nonswitched isotype" refers to the isotypic class of heavy chain that is produced when no isotype switching has taken place; the CH gene encoding the nonswitched isotype is typically the first CH gene immediately downstream from the functionally rearranged VDJ gene. Isotype switching has been classified as classical or non-classical isotype switching. Classical isotype switching occurs by recombination events which involve at least one switch sequence regions in a gene encoding an antibody. Non-classical isotype switching may occur by, for example, homologous recombination between human σ_μ and human Σ_μ (δ-associated deletion). Alternative non-classical switching mechanisms, such as intertransgene and/or interchromosomal recombination, among others, may occur and effectuate isotype switching.

As used herein, the term "switch sequence" refers to those DNA sequences responsible for switch recombination. A "switch donor" sequence, typically a μ switch region, will be 5' (*i.e.*, upstream) of the construct region to be deleted during the switch recombination. The "switch acceptor" region will be between the construct region to be deleted and the replacement constant region (*e.g.*, γ, ε, etc.). As there is no specific site where recombination always occurs, the final gene sequence will typically not be predictable from the construct.

An "antigen" is an entity (*e.g.*, a proteinaceous entity or peptide) to which an antibody or antigen-binding portion thereof binds. In various embodiments of the present invention, an antigen is ErbB3 or a ErbB3-like molecule. In a particular embodiment according to the invention, an antigen is human ErbB3.

The term "epitope" or "antigenic determinant" refers to a site on an antigen to which an immunoglobulin or antibody specifically binds. Epitopes can be formed both from contiguous amino acids or noncontiguous amino acids juxtaposed by tertiary folding of a protein. Epitopes formed from contiguous amino acids are typically retained on exposure to denaturing solvents, whereas epitopes formed by tertiary folding are typically lost on treatment with denaturing solvents. An epitope typically includes at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acids in a unique spatial conformation. Methods of determining spatial conformation of epitopes include techniques in the art and those described herein, for example, x-ray crystallography and 2-dimensional nuclear magnetic resonance. See, *e.g.*, *Epitope Mapping Protocols in Methods in Molecular Biology*, Vol. 66, G. E. Morris, Ed. (1996).

Also encompassed by the present invention are antibodies that bind the same or an overlapping epitope as the antibodies of the present invention, *i.e.*, antibodies that compete for binding to ErbB3, or bind epitopes which overlap with epitopes bound by the antibodies described herein, i.e., an epitope located on domain I of ErbB3. Antibodies that recognize the same epitope can be identified using routine techniques such as an immunoassay, for example, by showing the ability of one antibody to block the binding of another antibody to a target antigen, *i.e.*, a competitive binding assay. Competitive binding is determined in an assay in which the immunoglobulin under test inhibits specific binding of a reference antibody to a common antigen, such as ErbB3. Numerous types of competitive binding assays are known, for example: solid phase direct or indirect radioimmunoassay (RIA), solid phase direct or indirect enzyme immunoassay (EIA), sandwich competition assay (see Stahli *et al.*, (1983) *Methods in Enzymology* 9:242); solid phase direct biotin-avidin EIA (see Kirkland *et al.*, (1986) *J. Immunol.* 137:3614); solid phase direct labeled assay, solid phase direct labeled sandwich assay (see Harlow and Lane, (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Press); solid phase direct label RIA using I-125 label (see Morel *et al.*,

(1988) *Mol. Immunol.* 25(1):7); solid phase direct biotin-avidin EIA (Cheung *et al.*, (1990) *Virology* 176:546); and direct labeled RIA. (Moldenhauer *et al.*, (1990) *Scand. J. Immunol.* 32:77). Typically, such an assay involves the use of purified antigen (*e.g.*, ErbB3) bound to a solid surface or cells bearing either of these, an unlabeled test immunoglobulin and a labeled reference immunoglobulin. Competitive inhibition is measured by determining the amount of label bound to the solid surface or cells in the presence of the test immunoglobulin. Usually the test immunoglobulin is present in excess. Usually, when a competing antibody is present in excess, it will inhibit specific binding of a reference antibody to a common antigen by at least 50-55%, 55-60%, 60-65%, 65-70% 70-75% or more.

As used herein, the terms "specific binding," "specifically binds," "selective binding," and "selectively binds," mean that an antibody or antigen-binding portion thereof, exhibits appreciable affinity for a particular antigen or epitope and, generally, does not exhibit significant cross-reactivity with other antigens and epitopes. "Appreciable" or preferred binding includes binding with an affinity of at least 10^6, 10^7, 10^8, 10^9 M^{-1}, or 10^{10} M^{-1}. Affinities greater than 10^7 M^{-1}, preferably greater than 10^8 M^{-1} are more preferred. Values intermediate of those set forth herein are also intended to be within the scope of the present invention and a preferred binding affinity can be indicated as a range of affinities, for example, 10^6 to 10^{10} M^{-1}, preferably 10^7 to 10^{10} M^{-1}, more preferably 10^8 to 10^{10} M^{-1}. An antibody that "does not exhibit significant cross-reactivity" is one that will not appreciably bind to an undesirable entity (*e.g.*, an undesirable proteinaceous entity). For example, in one embodiment, an antibody or antigen-binding portion thereof that specifically binds to ErbB3 will appreciably bind that ErbB3 molecule but will not significantly react with other ErbB molecules and non-ErbB proteins or peptides. Specific or selective binding can be determined according to any art-recognized means for determining such binding, including, for example, according to Scatchard analysis and/or competitive binding assays.

The term "K_D," as used herein, is intended to refer to the dissociation equilibrium constant of a particular antibody-antigen interaction or the affinity of an antibody for an antigen. In one embodiment, the antibody or antigen binding portion thereof according to the present invention binds an antigen (*e.g.*, ErbB3) with an affinity (K_D) of 50 nM or better (i.e., or less) (*e.g.*, 40 nM or 30 nM or 20 nM or 10 nM or less), as measured using a surface plasmon resonance assay or a cell binding assay. In a particular embodiment, an antibody or antigen binding portion thereof according to the present invention binds ErbB3 with an affinity (K_D) of 8 nM or better (*e.g.*, 7 nM, 6 nM, 5 nM, 4 nM, 2 nM, 1.5 nM, 1.4 nM, 1.3 nM, 1nM or less), as measured by a surface plasmon resonance assay or a cell binding assay. In other embodiments, an antibody or antigen binding portion thereof binds an antigen (*e.g.*, ErbB3) with an affinity (K_D) of approximately less than

10^{-7} M, such as approximately less than 10^{-8} M, 10^{-9} M or 10^{-10} M or even lower when determined by surface plasmon resonance (SPR) technology in a BIACORE 3000 instrument using recombinant ErbB3 as the analyte and the antibody as the ligand, and binds to the predetermined antigen with an affinity that is at least two-fold greater than its affinity for binding to a non-specific antigen (*e.g.*, BSA, casein) other than the predetermined antigen or a closely-related antigen.

The term "K_{off}," as used herein, is intended to refer to the off rate constant for the dissociation of an antibody from the antibody/antigen complex.

The term "EC50," as used herein, refers to the concentration of an antibody or an antigen-binding portion thereof, which induces a response, either in an *in vitro* or an *in vivo* assay, which is 50% of the maximal response, *i.e.*, halfway between the maximal response and the baseline.

As used herein, "glycosylation pattern" is defined as the pattern of carbohydrate units that are covalently attached to a protein, more specifically to an immunoglobulin protein.

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

The term "rearranged" as used herein refers to a configuration of a heavy chain or light chain immunoglobulin locus wherein a V segment is positioned immediately adjacent to a D-J or J segment in a conformation encoding essentially a complete V_H or V_L domain, respectively. A rearranged immunoglobulin gene locus can be identified by comparison to germline DNA; a rearranged locus will have at least one recombined heptamer/nonamer homology element.

The term "unrearranged" or "germline configuration" as used herein in reference to a V segment refers to the configuration wherein the V segment is not recombined so as to be immediately adjacent to a D or J segment.

The term "nucleic acid molecule," as used herein, is intended to include DNA molecules and RNA molecules. A nucleic acid molecule may be single-stranded or double-stranded, but preferably is double-stranded DNA.

The term "isolated nucleic acid molecule," as used herein in reference to nucleic acids encoding antibodies or antibody portions (*e.g.*, V_H, V_L, CDR3) that bind to ErbB3, is intended to refer to a nucleic acid molecule in which the nucleotide sequences encoding the antibody or antibody portion are free of other nucleotide sequences encoding antibodies that bind antigens other than ErbB3, which other sequences may naturally flank the nucleic acid in human genomic DNA.

The term "modifying," or "modification," as used herein, is intended to refer to changing one or more amino acids in the antibodies or antigen-binding portions thereof. The change can be produced by adding, substituting or deleting an amino acid at one or more positions. The change can be produced using known techniques, such as PCR mutagenesis. For example, in some embodiments, an antibody or an antigen-binding portion thereof identified using the methods of the invention can be modified, to thereby modify the binding affinity of the antibody or antigen-binding portion thereof to ErbB3.

The present invention also encompasses "conservative amino acid substitutions" in the sequences of the antibodies of the invention, *i.e.*, nucleotide and amino acid sequence modifications which do not abrogate the binding of the antibody encoded by the nucleotide sequence or containing the amino acid sequence, to the antigen, *i.e.*, ErbB3. Conservative amino acid substitutions include the substitution of an amino acid in one class by an amino acid of the same class, where a class is defined by common physicochemical amino acid side chain properties and high substitution frequencies in homologous proteins found in nature, as determined, for example, by a standard Dayhoff frequency exchange matrix or BLOSUM matrix. Six general classes of amino acid side chains have been categorized and include: Class I (Cys); Class II (Ser, Thr, Pro, Ala, Gly); Class III (Asn, Asp, Gln, Glu); Class IV (His, Arg, Lys); Class V (Ile, Leu, Val, Met); and Class VI (Phe, Tyr, Trp). For example, substitution of an Asp for another class III residue such as Asn, Gln, or Glu, is a conservative substitution. Thus, a predicted nonessential amino acid residue in an anti-ErbB3 antibody is preferably replaced with another amino acid residue from the same class. 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.* 32:1180-1187 (1993); Kobayashi *et al. Protein Eng.* 12(10):879-884 (1999); and Burks *et al. Proc. Natl. Acad. Sci. USA* 94:.412-417 (1997)).

The term "non-conservative amino acid substitution" refers to the substitution of an amino acid in one class with an amino acid from another class; for example, substitution of an Ala, a class II residue, with a class III residue such as Asp, Asn, Glu, or Gln.

Alternatively, in another embodiment, mutations (conservative or non-conservative) can be introduced randomly along all or part of an anti-ErbB3 antibody coding sequence, such as by saturation mutagenesis, and the resulting modified anti-ErbB3 antibodies can be screened for binding activity.

A "consensus sequence" is a sequence formed from the most frequently occurring amino acids (or nucleotides) in a family of related sequences (See *e.g.*, Winnaker, From Genes to Clones (Verlagsgesellschaft, Weinheim, Germany 1987). In a family of proteins, each position in the consensus sequence is occupied by the amino

acid occurring most frequently at that position in the family. If two amino acids occur equally frequently, either can be included in the consensus sequence. A "consensus framework" of an immunoglobulin refers to a framework region in the consensus immunoglobulin sequence.

Similarly, the consensus sequence for the CDRs of can be derived by optimal alignment of the CDR amino acid sequences of ErbB3 antibodies of the present invention.

For nucleic acids, the term "substantial homology" indicates that two nucleic acids, or designated sequences thereof, when optimally aligned and compared, are identical, with appropriate nucleotide insertions or deletions, in at least about 80% of the nucleotides, usually at least about 90% to 95%, and more preferably at least about 98% to 99.5% of the nucleotides. Alternatively, substantial homology exists when the segments will hybridize under selective hybridization conditions, to the complement of the strand.

The percent identity between two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, % homology = # of identical positions/total # of positions x 100), taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm, as described in the non-limiting examples below.

The percent identity between two nucleotide sequences can be determined using the GAP program in the GCG software, using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. The percent identity between two nucleotide or amino acid sequences can also be determined using the algorithm of E. Meyers and W. Miller (CABIOS, 4:11-17 (1989)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. In addition, the percent identity between two amino acid sequences can be determined using the Needleman and Wunsch (*J. Mol. Biol.* (48):444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package, 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, or 6.

The nucleic acid and protein sequences of the present invention can further be used as a "query sequence" to perform a search against public databases to, for example, identify related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, *et al.* (1990) *J. Mol. Biol.* 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100,

wordlength = 12 to obtain nucleotide sequences homologous to the nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to the protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.*, (1997) *Nucleic Acids Res.* 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used.

The nucleic acids may be present in whole cells, in a cell lysate, or in a partially purified or substantially pure form. A nucleic acid is "isolated" or "rendered substantially pure" when purified away from other cellular components or other contaminants, *e.g.*, other cellular nucleic acids or proteins, by standard techniques, including alkaline/SDS treatment, CsCl banding, column chromatography, agarose gel electrophoresis and others well known in the art. *See*, F. Ausubel, *et al.*, ed. Current Protocols in Molecular Biology, Greene Publishing and Wiley Interscience, New York (1987).

The nucleic acid compositions of the present invention, while often in a native sequence (except for modified restriction sites and the like), from either cDNA, genomic or mixtures thereof may be mutated, in accordance with standard techniques to provide gene sequences. For coding sequences, these mutations, may affect amino acid sequence as desired. In particular, DNA sequences substantially homologous to or derived from native V, D, J, constant, switches and other such sequences described herein are contemplated (where "derived" indicates that a sequence is identical or modified from another sequence).

The term "operably linked" refers to a nucleic acid sequence placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the

sequence. With respect to transcription regulatory sequences, operably linked means that the DNA sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame. For switch sequences, operably linked indicates that the sequences are capable of effecting switch recombination.

The term "vector," as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid," which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors"(or simply, "expression vectors"). In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. The terms, "plasmid" and "vector" may be used interchangeably. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The term "recombinant host cell" (or simply "host cell"), as used herein, is intended to refer to a cell into which a recombinant expression vector has been introduced. It should be understood that such terms are intended to refer not only to the particular subject cell but to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term "host cell" as used herein.

The terms "treat," "treating," and "treatment," as used herein, refer to therapeutic or preventative measures described herein. The methods of "treatment" employ administration to a subject, an antibody or antigen binding portion of the present invention, for example, a subject having a disease or disorder associated with ErbB3 dependent signaling or predisposed to having such a disease or disorder, in order to prevent, cure, delay, reduce the severity of, or ameliorate one or more symptoms of the disease or disorder or recurring disease or disorder, or in order to prolong the survival of a subject beyond that expected in the absence of such treatment.

The term "disease associated with ErbB3 dependent signaling," or "disorder associated with ErbB3 dependent signaling," as used herein, includes disease states and/or symptoms associated with a disease state, where increased levels of ErbB3 and/or activation of cellular cascades involving ErbB3 are found. It is understood that ErbB3 heterodimerizes with other ErbB proteins such as, EGFR and ErbB2, when increased levels of ErbB3 are found. Accordingly, the term "disease associated with ErbB3 dependent signaling," also includes disease states and/or symptoms associated with disease states where increased levels of EGFR/ErbB3 and/or ErbB2/ErbB3 heterodimers are found. In general, the term "disease associated with ErbB3 dependent signaling," refers to any disorder, the onset, progression or the persistence of the symptoms of which requires the participation of ErbB3. Exemplary ErbB3-mediated disorders include, but are not limited to, for example, cancer.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastric cancer, pancreatic cancer, glial cell tumors such as glioblastoma and neurofibromatosis, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, melanoma, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, renal cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer. In a particular embodiment, a cancer treated or diagnosed using the methods of the present invention is selected from melanoma, breast cancer, ovarian cancer, renal carcinoma, gastrointestinal/colon cancer, lung cancer, and prostate cancer.

The term "effective amount," as used herein, refers to that amount of an antibody or an antigen binding portion thereof that binds ErbB3, which is sufficient to effect treatment, prognosis or diagnosis of a disease associated with ErbB3 dependent signaling, as described herein, when administered to a subject. A therapeutically effective amount will vary depending upon the subject and disease condition being treated, the weight and age of the subject, the severity of the disease condition, the manner of administration and the like, which can readily be determined by one of ordinary skill in the art. The dosages for administration can range from, for example, about 1 ng to about 10,000 mg, about 5 ng to about 9,500 mg, about 10 ng to about 9,000 mg, about 20 ng to about 8,500 mg, about 30 ng to about 7,500 mg, about 40 ng to about 7,000 mg, about 50 ng to about 6,500 mg, about 100 ng to about 6,000 mg, about 200 ng to about 5,500 mg, about 300 ng to about 5,000 mg, about 400 ng to about 4,500 mg, about 500 ng to about 4,000 mg, about 1 μg to about 3,500 mg, about 5 μg to about

3,000 mg, about 10 μg to about 2,600 mg, about 20 μg to about 2,575 mg, about 30 μg to about 2,550 mg, about 40 μg to about 2,500 mg, about 50 μg to about 2,475 mg, about 100 μg to about 2,450 mg, about 200 μg to about 2,425 mg, about 300 μg to about 2,000, about 400 μg to about 1,175 mg, about 500 μg to about 1,150 mg, about 0.5 mg to about 1,125 mg, about 1 mg to about 1,100 mg, about 1.25 mg to about 1,075 mg, about 1.5 mg to about 1,050 mg, about 2.0 mg to about 1,025 mg, about 2.5 mg to about 1,000 mg, about 3.0 mg to about 975 mg, about 3.5 mg to about 950 mg, about 4.0 mg to about 925 mg, about 4.5 mg to about 900 mg, about 5 mg to about 875 mg, about 10 mg to about 850 mg, about 20 mg to about 825 mg, about 30 mg to about 800 mg, about 40 mg to about 775 mg, about 50 mg to about 750 mg, about 100 mg to about 725 mg, about 200 mg to about 700 mg, about 300 mg to about 675 mg, about 400 mg to about 650 mg, about 500 mg, or about 525 mg to about 625 mg, of an antibody or antigen binding portion thereof, according to the invention. Dosage regiments may be adjusted to provide the optimum therapeutic response. An effective amount is also one in which any toxic or detrimental effects (*i.e.*, side effects) of an antibody or antigen binding portion thereof are minimized and/or outweighed by the beneficial effects.

The term "patient" includes human and other mammalian subjects that receive either prophylactic or therapeutic treatment.

As used herein, the term "subject" includes any human or non-human animal. For example, the methods and compositions of the present invention can be used to treat a subject having cancer. In a particular embodiment, the subject is a human. The term "non-human animal" includes all vertebrates, *e.g.*, mammals and non-mammals, such as non-human primates, sheep, dog, cow, chickens, amphibians, reptiles, *etc.*

The term "sample" refers to tissue, body fluid, or a cell from a patient or a subject. Normally, the tissue or cell will be removed from the patient, but *in vivo* diagnosis is also contemplated. In the case of a solid tumor, a tissue sample can be taken from a surgically removed tumor and prepared for testing by conventional techniques. In the case of lymphomas and leukemias, lymphocytes, leukemic cells, or lymph tissues can be obtained and appropriately prepared. Other patient samples, including urine, tear drops, serum, cerebrospinal fluid, feces, sputum, cell extracts *etc.* can also be useful for particular tumors.

The terms "anti-cancer agent" and "antineoplastic agent" refer to drugs used to treat malignancies, such as cancerous growths. Drug therapy may be used alone, or in combination with other treatments such as surgery or radiation therapy. Several classes of drugs may be used in cancer treatment, depending on the nature of the organ involved. For example, breast cancers are commonly stimulated by estrogens, and may be treated with drugs which inactive the sex hormones. Similarly, prostate cancer may

be treated with drugs that inactivate androgens, the male sex hormone. Anti-cancer agents of the present invention include, among others, the following agents:

Anti-Cancer Agent	Comments	Examples
Antibodies (a) antibodies other than anti-ErbB3 antibodies; and (b) anti-ErbB3 antibodies which bind different epitopes	Antibodies which bind IGF-1R (insulin-like growth factor type 1 receptor), which is expressed on the cell surface of must human cancers	A12 (fully humanized mAb) 19D12 (fully humanized mAb) CP751-871 (fully humanized mAb) H7C10 (humanized mAb) alphaIR3 (mouse) scFV/FC (mouse/human chimera) EM/164 (mouse)
	Antibodies which bind EGFR (epidermal growth factor receptor); Mutations affecting EGFR expression or activity could result in cancer	Matuzumab (EMD72000) Erbitux® / Cetuximab (Imclone) Vectibix® / Panitumumab (Amgen) mAb 806 Nimotuzumab (TheraCIM)
	Antibodies which bind cMET (Mesenchymal epithelial transition factor); a member of the MET family of receptor tyrosine kinases)	AVEO (AV299) (AVEO) AMG102 (Amgen) 5D5 (OA-5D5) (Genentech)
	Anti-ErbB3 antibodies which bind different epitopes	Ab #14 (MM 121-14) described herein Herceptin® (Trastuzumab; Genentech) 1B4C3; 2D1D12 (U3 Pharma AG)
Small Molecules Targeting IGF1R	IGF-1R (insulin-like growth factor type 1 receptor), which is expressed on the cell surface of must human cancers	NVP-AEW541-A BMS-536,924 (1H-benzoimidazol-2-yl)-1H-pyridin-2-one) BMS-554,417 Cycloligan TAE226 PQ401
Small Molecules Targeting EGFR	EGFR (epidermal growth factor receptor); Mutations affecting EGFR expression or activity could result in cancer	Iressa® / Gefitinib (AstraZeneca) CI-1033 (PD 183805) (Pfizer) Lapatinib (GW-572016) (GlaxoSmithKline) Tykerb® / Lapatinib Ditosylate (SmithKline Beecham) Tarceva®/ Erlotinib HCL (OSI-774) (OSI Pharma) PKI-166 (Novartis)

		PD-158780 EKB-569 Tyrphostin AG 1478(4-(3-Chloroanillino)-6,7-dimethoxyquinazoline)
Small Molecules Targeting cMET	cMET (Mesenchymal epithelial transition factor); a member of the MET family of receptor tyrosine kinases)	PHA665752 ARQ 197
Antimetabolites	An antimetabolite is a chemical with a similar structure to a substance (a metabolite) required for normal biochemical reactions, yet different enough to interfere with the normal functions of cells, including cell division.	Flourouracil (5-FU) Capecitabine / XELODA® (HLR Roche) 5-Trifluoromethyl-2'-deoxyuridine Methotrexate sodium (Trexall) (Barr) Raltitrexed / Tomudex® (AstraZaneca) Pemetrexed / Alimta® (Lilly) Tegafur Cytosine Arabinoside (Cytarabine, Ara-C) / Thioguanine® (GlaxoSmithKline) 5-azacytidine 6-mercaptopurine (Mercaptopurine, 6-MP) Azathioprine / Azasan® (AAIPHARMA LLC) 6-thioguanine (6-TG) / Purinethol® (TEVA) Pentostatin / Nipent® (Hospira Inc.) Fludarabine phosphate / Fludara® (Bayer Health Care) Cladribine (2-CdA, 2-chlorodeoxyadenosine) / Leustatin® (Ortho Biotech)
Alkylating agents	An alkylating antineoplastic agent is an alkylating agent that attaches an alkyl group to DNA. Since cancer cells generally proliferate unrestrictively more than do healthy cells they are more sensitive to DNA damage, and alkylating agents are used clinically to treat a variety of tumours.	Ribonucleotide Reductase Inhibitor (RNR) Cyclophosphamide / Cytoxan (BMS) Neosar (TEVA) Ifosfamide /Mitoxana® (ASTA Medica) Thiotepa (Bedford, Abraxis, Teva) BCNU→ 1,3-bis(2-chloroethyl)-1-nitrosourea CCNU→ 1,-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (methyl CCNU) Hexamethylmelamine (Altretamine, HMM) / Hexalen® (MGI Pharma Inc.) Busulfan / Myleran (GlaxoSmithKline) Procarbazine HCL / Matulane (Sigma Tau Pharmaceuticals, Inc.) Dacarbazine (DTIC) Chlorambucil / Leukaran® (SmithKline Beecham) Melphalan / Alkeran® (GlaxoSmithKline)

		Cisplatin (Cisplatinum, CDDP) / Platinol (Bristol Myers) Carboplatin / Paraplatin (BMS) Oxaliplatin / Eloxitan® (Sanofi-Aventis US)
Topoisomerase inhibitors	Topoisomerase inhibitors are chemotherapy agents designed to interfere with the action of topoisomerase enzymes (topoisomerase I and II), which are enzymes that control the changes in DNA structure by catalyzing the breaking and rejoining of the phosphodiester backbone of DNA strands during the normal cell cycle.	Doxorubicin HCL / Doxil® (Alza) Daunorubicin citrate / Daunoxome® (Gilead) Mitoxantrone HCL/Novantrone (EMD Serono) Actinomycin D Etoposide / Vepesid® (BMS)/ Etopophos® (Hospira, Bedford, Teva Parenteral, Etc.) Topotecan HCL / Hycamtin® (GlaxoSmithKline) Teniposide (VM-26) / Vumon® (BMS) Irinotecan HCL(CPT-11) / Camptosar® (Pharmacia & Upjohn)
Microtubule targeting agents	Microtubules are one of the components of the cytoskeleton. They have diameter of ~ 24 nm and length varying from several micrometers to possibly millimeters in axons of nerve cells. Microtubules serve as structural components within cells and are involved in many cellular processes including mitosis, cytokinesis, and vesicular transport.	Vincristine / Oncovin® (Lilly) Vinblastine sulfate/Velban®(discontinued) (Lilly) Vinorelbine tartrate / Navelbine® (PierreFabre) Vindesine sulphate / Eldisine® (Lilly) Paclitaxel / Taxol® (BMS) Docetaxel / Taxotere® (Sanofi Aventis US) Nanoparticle paclitaxel (ABI-007) / Abraxane® (Abraxis BioScience, Inc.) Ixabepilone / IXEMPRA™ (BMS)
Kinase inhibitors	Tyrosine kinases are enzymes within the cell that function to attach phosphate groups to the amino acid tyrosine. By blocking the ability of protein tyrosine kinases to function, these compounds provide a tool for controlling cancerous	Imatinib mesylate / Gleevec (Novartis) Sunitinib malate / Sutent® (Pfizer) Sorafenib tosylate / Nexavar® (Bayer) Nilotinib hydrochloride monohydrate / Tasigna® (Novartis)

	cell growth.	
Protein synthesis inhibitors	Induces cell apoptosis	L-asparaginase / Elspar® (Merck & Co.)
Immunotherapeutic agents	Induces cancer patients to exhibit immune responsiveness	Alpha interferon Angiogenesis Inhibitor / Avastin® (Genentech) IL-2→ Interleukin 2 (Aldesleukin) / Proleukin ® (Chiron) IL-12→ Interleukin 12
Hormones	Hormone therapies associated with menopause and aging seek to increase the amount of certain hormones in your body to compensate for age- or disease-related hormonal declines. Hormone therapy as a cancer treatment either reduces the level of specific hormones or alters the cancer's ability to use these hormones to grow and spread.	Toremifene citrate / Fareston® (GTX, Inc.) Fulvestrant / Faslodex® (AstraZeneca) Raloxifene HCL / Evista® (Lilly) Anastrazole / Arimidex® (AstraZeneca) Letrozole / Femara® (Novartis) Fadrozole (CGS 16949A) Exemestane / Aromasin® (Pharmacia & Upjohn) Leuprolide acetate / Eligard® (QTL USA) Lupron® (TAP Pharm.) Goserelin acetate / Zoladex® (AstraZeneca) Triptorelin pamoate / Trelstar® (Watson Labs) Buserelin / Suprefact® (Sanofi Aventis) Nafarelin Cetrorelix / Cetrotide® (EMD Serono) Bicalutamide / Casodex® (AstraZeneca) Nilutamide / Nilandron® (Aventis Pharm.) Megestrol acetate / Megace® (BMS) Somatostatin Analogs (Octreotide acetate / Sandostatin® (Novartis))
Glucocorticoids	Anti-inflammatory drugs used to reduce swelling that causes cancer pain.	Predinsolone Dexamethasone / Decadron® (Wyeth)
Aromatose inhibitors	Includes imidazoles	Ketoconazole
mTOR inhibitors	The mTOR signaling pathway was originally discovered during studies of the immunosuppressive agent rapamycin. This highly conserved pathway regulates cell proliferation and	Sirolimus (Rapamycin) / Rapamune® (Wyeth) Temsirolimus (CCI-779) / Torisel® (Wyeth) Deforolimus (AP23573) (Ariad Pharm.) Everolimus (RAD001) /Certican® (Novartis)

	metabolism in response to environmental factors, linking cell growth factor receptor signaling via phosphoinositide-3-kinase (PI-3K) to cell growth, proliferation, and angiogenesis.
Chemotherapeutic agents	Adriamycin, 5-Fluorouracil, Cytoxin, Bleomycin, Mitomycin C, Daunomycin, Carminomycin, Aminopterin, Dactinomycin, Mitomycins, Esperamicins

One or more anti-cancer agents may be administered either simultaneously or before or after administration of an antibody or antigen binding portion thereof of the present invention.

Various aspects of the invention are described in further detail in the following subsections.

II. Methods for Producing Antibodies of the Invention

(i) Monoclonal Antibodies

Monoclonal antibodies of the invention can be produced using a variety of known techniques, such as the standard somatic cell hybridization technique described by Kohler and Milstein (1975) *Nature* 256: 495, viral or oncogenic transformation of B lymphocytes or phage display technique using libraries of human antibody genes. In particular embodiments, the antibodies are fully human monoclonal antibodies.

Accordingly, in one embodiment, a hybridoma method is used for producing an antibody that binds ErbB3. In this method, a mouse or other appropriate host animal can be immunized with a suitable antigen in order to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the antigen used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes can then be fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, Monoclonal Antibodies: Principles and Practice, pp.59-103 (Academic Press, 1986)). Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, Monoclonal Antibodies:Principles and Practice, pp. 59-103 (Academic Press, 1986)). 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. The monoclonal antibodies secreted by the subclones can be separated from the culture medium, ascites

fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

In another embodiment, antibodies and antibody portions that bind ErbB3 can be isolated from antibody phage libraries generated using the techniques described in, for example, McCafferty *et al.*, *Nature*, 348:552-554 (1990). Clackson *et al.*, Nature, 352:624-628 (1991), Marks *et al.*, *J. Mol. Biol.*, 222:581-597 (1991) and Hoet *et al* (2005) *Nature Biotechnology* 23, 344-348 ; U.S. Patent Nos. 5,223,409; 5,403,484; and 5,571,698 to Ladner *et al.*; U.S. Patent Nos. 5,427,908 and 5,580,717 to Dower *et al.*; U.S. Patent Nos. 5,969,108 and 6,172,197 to McCafferty *et al.*; and U.S. Patent Nos. 5,885,793; 6,521,404; 6,544,731; 6,555,313; 6,582,915 and 6,593,081 to Griffiths *et al.*. Additionally, production of high affinity (nM range) human antibodies by chain shuffling (Marks *et al.*, *Bio/Technology*, 10:779-783 (1992)), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large phage libraries (Waterhouse *et al.*, *Nuc. Acids. Res.*, 21:2265-2266 (1993)) may also be used.

In a particular embodiment, the monoclonal antibody or antigen binding portion thereof that binds ErbB3 is produced using the phage display technique described by Hoet *et al.*, *supra*. This technique involves the generation of a human Fab library having a unique combination of immunoglobulin sequences isolated from human donors and having synthetic diversity in the heavy-chain CDRs is generated. The library is then screened for Fabs that bind to ErbB3.

In yet another embodiment, human monoclonal antibodies directed against ErbB3 can be generated using transgenic or transchromosomic mice carrying parts of the human immune system rather than the mouse system (see *e.g.*, Lonberg, *et al.* (1994) Nature 368(6474): 856-859; Lonberg, N. *et al.* (1994), *supra*; reviewed in Lonberg, N. (1994) *Handbook of Experimental Pharmacology* 113:49-101; Lonberg, N. and Huszar, D. (1995) *Intern. Rev. Immunol.* 13: 65-93, and Harding, F. and Lonberg, N. (1995) *Ann. N.Y. Acad. Sci.* 764:536-546. See further, U.S. Patent Nos. 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,789,650; 5,877,397; 5,661,016; 5,814,318; 5,874,299; and 5,770,429; all to Lonberg and Kay; U.S. Patent No. 5,545,807 to Surani *et al.*; PCT Publication Nos. WO 92/03918, WO 93/12227, WO 94/25585, WO 97/13852, WO 98/24884 and WO 99/45962, all to Lonberg and Kay; and PCT Publication No. WO 01/14424 to Korman *et al.*).

In another embodiment, human antibodies of the invention can be raised using a mouse that carries human immunoglobulin sequences on transgenes and transchomosomes, such as a mouse that carries a human heavy chain transgene and a human light chain transchromosome (see *e.g.*, PCT Publication WO 02/43478 to Ishida *et al.*).

Still further, alternative transgenic animal systems expressing human immunoglobulin genes are available in the art and can be used to raise anti-ErbB3 antibodies of the invention. For example, an alternative transgenic system referred to as the Xenomouse (Abgenix, Inc.) can be used; such mice are described in, for example, U.S. Patent Nos. 5,939,598; 6,075,181; 6,114,598; 6, 150,584 and 6,162,963 to Kucherlapati *et al.*

Moreover, alternative transchromosomic animal systems expressing human immunoglobulin genes are available in the art and can be used to raise anti-ErbB3 antibodies of the invention. For example, mice carrying both a human heavy chain transchromosome and a human light chain tranchromosome can be used; as described in Tomizuka *et al.* (2000) *Proc. Natl. Acad. Sci. USA* 97:722-727. Furthermore, cows carrying human heavy and light chain transchromosomes have been described in the art (Kuroiwa *et al.* (2002) *Nature Biotechnology* 20:889-894) and can be used to raise anti-ErbB3 antibodies of the invention.

In yet another embodiment, antibodies of the present invention can be prepared using a transgenic plant and/or cultured plant cells (such as, for example, tobacco, maize and duckweed) that produce such antibodies. For example, transgenic tobacco leaves expressing antibodies or antigen binding portions thereof can be used to produce such antibodies by, for example, using an inducible promoter (see, *e.g.*, Cramer *et al.*, *Curr. Top. Microbol. Immunol.* 240:95 118 (1999)). Also, transgenic maize can be used to express such antibodies and antigen binding portions thereof (see, *e.g.*, Hood *et al.*, *Adv. Exp. Med. Biol.* 464:127 147 (1999)). Antibodies can also be produced in large amounts from transgenic plant seeds including antibody portions, such as single chain antibodies (scFv's), for example, using tobacco seeds and potato tubers (see, *e.g.*, Conrad *et al.*, *Plant Mol. Biol.* 38:101 109 (1998)). Methods of producing antibodies or antigen binding portions in plants can also be found in, *e.g.*, Fischer *et al.*, *Biotechnol. Appl. Biochem.* 30:99 108 (1999), Ma *et al.*, *Trends Biotechnol.* 13:522 7 (1995); Ma *et al.*, *Plant Physiol.* 109:341 6 (1995); Whitelam *et al.*, *Biochem. Soc. Trans.* 22:940 944 (1994) and U.S. Patent Nos. 6,040,498 and 6,815,184.

The binding specificity of monoclonal antibodies or portions thereof that bind ErbB3 prepared using any technique including those disclosed here, can be determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). The binding affinity of a monoclonal antibody or portion thereof also can be determined by the Scatchard analysis of Munson *et al.*, *Anal. Biochem.*, 107:220 (1980).

In certain embodiments, an ErbB3 antibody or portion thereof produced using any of the methods discussed above may be further altered or optimized to achieve a

desired binding specificity and/or affinity using art recognized techniques, such as those described herein.

In one embodiment, partial antibody sequences derived from an ErbB3 antibody may be used to produce structurally and functionally related antibodies. For example, antibodies interact with target antigens predominantly through amino acid residues that are located in the six heavy and light chain complementarity determining regions (CDRs). For this reason, the amino acid sequences within CDRs are more diverse between individual antibodies than sequences outside of CDRs. Because CDR sequences are responsible for most antibody-antigen interactions, it is possible to express recombinant antibodies that mimic the properties of specific naturally occurring antibodies by constructing expression vectors that include CDR sequences from the specific naturally occurring antibody grafted onto framework sequences from a different antibody with different properties (see, *e.g.*, Riechmann, L. *et al.*, 1998, *Nature* 332:323-327; Jones, P. *et al.*, 1986, *Nature* 321:522-525; and Queen, C. *et al.*, 1989, *Proc. Natl. Acad. See.* U.S.A. 86:10029-10033). Such framework sequences can be obtained from public DNA databases that include germline antibody gene sequences.

Thus, one or more structural features of an anti-ErbB3 antibody of the invention, such as the CDRs, can be used to create structurally related anti-ErbB3 antibodies that retain at least one functional property of the antibodies of the invention, e.g., inhibiting EGF-like ligand mediated phosphorylation of ErbB3; inhibiting one or more of heregulin, epiregulin, epigen or biregulin-mediated signaling through ErbB3; inhibiting proliferation or cells expressing ErbB3; and/or decreasing levels of ErbB3 on cell surfaces.

In a particular embodiment, one or more CDR regions selected from SEQ ID NOs:7-12, SEQ ID NOs:13-18, SEQ ID NOs:19-24, SEQ ID NOs:39-44, and SEQ ID NOs:45-50 is combined recombinantly with known human framework regions and CDRs to create additional, recombinantly-engineered, anti-ErbB3 antibodies of the invention. The heavy and light chain variable framework regions can be derived from the same or different antibody sequences.

It is well known in the art that antibody heavy and light chain CDR3 domains play a particularly important role in the binding specificity/affinity of an antibody for an antigen (*see*, Hall *et al.*, *J. Imunol.*, 149:1605-1612 (1992); Polymenis *et al.*, *J. Immunol.*, 152:5318-5329 (1994); Jahn *et al.*, *Immunobiol.*, 193:400-419 (1995); Klimka *et al.*, *Brit. J. Cancer*, 83:252-260 (2000); Beiboer *et al.*, *J. Mol. Biol*, 296:833-849 (2000); Rader *et al.*, *Proc. Natl. Acad. Sci. USA*, 95:8910-8915 (1998); Barbas *et al.*, *J. Am. Chem. Soc.*, 116:2161-2162 (1994); Ditzel *et al.*, *J. Immunol.*, 157:739-749 (1996)). Accordingly, in certain embodiments, antibodies are generated that include the heavy and/or light chain CDR3s of the particular antibodies described herein (*e.g.*, SEQ ID

NOs:9, 15, 21, 41, 47 and/or SEQ ID NOs:12, 18, 24, 44, 50). The antibodies can further include the heavy and/or light chain CDR1 and/or CDR2s of the antibodies of the present invention (*e.g.*, SEQ ID NOs:7-8 and/or SEQ ID NOs:10-11; SEQ ID NOs:13-14 and/or SEQ ID NOs:16-17; SEQ ID NOs:20-21 and/or SEQ ID NOs:22-23; SEQ ID NOs:39-40 and/or SEQ ID NOs:42-43; or SEQ ID NOs:45-46 and/or SEQ ID NOs:48-49).

The CDR1, 2, and/or 3 regions of the engineered antibodies described above can comprise the exact amino acid sequence(s) as those disclosed herein (*e.g.*, CDRs of Ab #6, Ab #3, Ab #14, Ab #17, or Ab #19, set forth in SEQ ID NOs:7-12, 13-18, 19-24, 39-44, and 45-50, respectively). However, the ordinarily skilled artisan will appreciate that some deviation from the exact CDR sequences may be possible while still retaining the ability of the antibody to bind ErbB3 effectively (*e.g.*, conservative amino acid substitutions). Accordingly, in another embodiment, the engineered antibody may be composed of one or more CDRs that are, for example, 90%, 95%, 98%, 99% or 99.5% identical to one or more CDRs of Ab #6, Ab #3 or Ab #14.

In another embodiment, one or more residues of a CDR may be altered to modify binding to achieve a more favored on-rate of binding. Using this strategy, an antibody having ultra high binding affinity of, for example, 10^{10} M^{-1} or more, can be achieved. Affinity maturation techniques, well known in the art and those described herein, can be used to alter the CDR region(s) followed by screening of the resultant binding molecules for the desired change in binding. Accordingly, as CDR(s) are altered, changes in binding affinity as well as immunogenicity can be monitored and scored such that an antibody optimized for the best combined binding and low immunogenicity are achieved.

In addition to, or instead of, modifications within the CDRs, modifications can also be made within one or more of the framework regions, FR1, FR2, FR3 and FR4, of the heavy and/or the light chain variable regions of an antibody, so long as these modifications do not eliminate the binding affinity of the antibody.

In another embodiment, the antibody is further modified with respect to effector function, so as to enhance the effectiveness of the antibody in treating cancer, for example. For example cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron *et al.*, *J. Exp Med.* 176:1191-1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff *et al.* *Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered

which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson *et al. Anti-Cancer Drug Design* 3:219-230 (1989).

Also encompassed by the present invention are bispecific antibodies and immunoconjugates, as discussed below.

(ii) Bispecific Antibodies

Bispecific antibodies of the present invention include at least one binding specificity for ErbB3 and at least one binding specificity for another antigen, such as the product of an oncogene. 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 well known in the art (see, e.g., WO 05117973 and WO 06091209). For example, production of full length bispecific antibodies can be based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (see, *e.g.*, Millstein *et al.*, *Nature*, 305:537-539 (1983)). Further details of generating bispecific antibodies can be found, for example, in Suresh *et al.*, *Methods in Enzymology*, 121:210 (1986) and in Brennan *et al.*, *Science*, 229: 81 (1985), which describes a chemical linkage process for making bispecific antibodies. Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers (see, e.g., Kostelny *et al.*, *J. Immunol.*, 148(5):1547-1553 (1992)). Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported (see, *e.g.*, Gruber *et al.*, *J. Immunol.*, 152:5368 (1994)).

In a particular embodiment, the bispecific antibody comprises a first antibody or binding portion thereof which binds to ErbB3 and a second antibody or binding portion thereof which binds to ErbB2, ERbB3, ErbB4, EGFR, IGF1-R, C-MET, Lewis Y, MUC-1, EpCAM, CA125, prostate specific membrane antigen, PDGFR-α, PDGFR-β, C-KIT, or any of the FGF receptors.

(iii) Immunoconjugates

Immunoconjugates of the present invention can be formed by conjugating the antibodies or antigen binding portions thereof described herein to another therapeutic agent. Suitable agents include, for example, a cytotoxic agent (e.g., a chemotherapeutic agent), a toxin (*e.g.* an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof), and/or a radioactive isotope *(i.e.*, a radioconjugate). Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A

chain (from Pseudomonas aeruginosa), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated anti-ErbB3 antibodies. Examples include ^{212}Bi, ^{131}I, ^{131}In, ^{90}Y and ^{186}Re.

Immunoconjugates of the invention can be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta *et al.*, *Science* 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody (see, *e.g.*, WO94/11026).

III. Methods for Screening Antibodies of the Invention

Subsequent to producing antibodies or antigen binding portions that bind ErbB3, such antibodies, or portions thereof, can be screened for various properties, such as those described herein, using a variety of assays that are well known in the art.

In one embodiment, the antibodies or antigen binding portions thereof are screened for the ability to inhibit EGF-like ligand mediated phosphorylation of ErbB3. This can be done by treating cells expressing ErbB3 with an EGF-like ligand in the presence and absence of the antibody or antigen binding portion thereof. The cells can then be lysed and the crude lysates can be centrifuged to remove insoluble material. ErbB3 phosphorylation can be measured, for example, by Western blotting followed by probing with an anti-phosphotyrosine antibody as described in Kim *et al.*, *supra* and the Examples below.

In other embodiments, the antibodies and antigen binding portions are further screened for one or more of the following properties: (1) inhibition of ErbB3-ligand (*e.g.*, heregulin, epiregulin, epigen or biregulin) mediated signaling through ErbB3; (2) inhibition of proliferation of cells expressing ErbB3; (3) the ability to decrease levels of ErbB3 on cell surface (*e.g.*, by inducing internalization of ErbB3), (4) inhibition of VEGF secretion of cells expressing ErbB3; (5) inhibition of the migration of cells

expressing ErbB3; (6) inhibition of spheroid growth of cells expressing ErbB3; and/or (7) binding to an epitope located on domain I of ErbB3, each of which can be readily measured using art recognized techniques and those discussed herein.

Inhibition of one or more of heregulin, epiregulin, epigen or biregulin-mediated signaling through ErbB3 can be readily measured using routine assays, such as, described in Horst *et al. supra*. For example, the ability of an antibody or antigen binding portion thereof to inhibit heregulin, epiregulin, epigen or biregulin-mediated signaling through ErbB3 can be measured by kinase assays for known substrates of ErbB3 such as, for example, SHC and PI3K, as described in, for example, Horst *et al. supra*, Sudo *et al.*, (2000) *Methods Enzymol*, 322:388-92; and Morgan *et al.* (1990) *Eur. J. Biochem.*, 191:761-767, following stimulation by one or more of heregulin, epiregulin, epigen or biregulin. Accordingly, cells expressing ErbB3 can be stimulated with one or more of heregulin, epiregulin, epigen or biregulin, and incubated with a candidate antibody or antigen-binding portion thereof. Cell lysates subsequently prepared from such cells can be immunoprecipitated with an antibody for a substrate of ErbB3 (or a protein in a cellular pathway involving ErbB3) such as, for example, an anti-JNK-1 antibody, and assayed for kinase activity (*e.g.*, JNK kinase activity or PI3-kinase activity) using art recognized techniques. A decrease in or complete disappearance in level or activity (*e.g.*, kinase activity) of a ErbB3 substrate or protein in a pathway involving ErbB3 in the presence of the antibody or antigen binding portion thereof, relative to the level or activity in the absence of the antibody or antigen binding portion thereof, is indicative of an antibody or antigen binding portion which inhibits one or more of heregulin, epiregulin, epigen or biregulin-mediated signaling.

In certain embodiments, the antibody or antigen binding portion thereof inhibits ErbB3-ligand (*e.g.*, heregulin, epiregulin, epigen or biregulin) mediated signaling by decreasing the binding of one or more of heregulin, epiregulin, epigen or biregulin to ERbB3.

In order to select for those antibodies or antigen binding portions thereof which inhibit the binding of one or more of heregulin, epiregulin, epigen or biregulin to ErbB3, cells which express ErbB3 (*e.g.* MALME-3M cells, as described in the Examples *infra)*, can be contacted with a labeled ErbB3-ligand (*e.g.*, radiolabeled heregulin, epiregulin, epigen or biregulin) in the absence (control) or presence of the anti-ErbB3 antibody or antigen binding portion thereof. If the antibody or antigen binding portion thereof inhibits heregulin, epiregulin, epigen or biregulin binding to ErbB3, then a statistically significantly decrease in the amount of label recovered (*e.g.*, radiolabeled heregulin, epiregulin, epigen or biregulin), relative to the amount in the absence of the antibody or antigen binding portion thereof, will be observed.

The antibody or antigen binding portion thereof may inhibit the binding of the ErbB3-ligand (*e.g.*, heregulin, epiregulin, epigen or biregulin) by any mechanism. For example, the antibody or antigen binding portion thereof may inhibit binding of the ErbB3 ligand (*e.g.*, one or more of heregulin, epiregulin, epigen or biregulin) to ErbB3 by binding to the same site or an overlapping site on ErbB3 as the ErbB3 ligand. Alternatively, the antibody or antigen binding portion thereof may inhibit binding of an ErbB3 ligand by altering or distorting the conformation of ErbB3, such that it is unable to bind to the ErbB3 ligand.

Antibodies and antigen binding portions thereof that decrease levels of ErbB3 on cell surfaces can be identified by their ability to downregulate ErbB3 on tumor cells. In certain embodiments, the antibodies or antigen binding portions thereof decrease ErbB3 cell surface expression by inducing internalization (or increasing endocytosis) of Erbb3. To test this, ErbB3 can be biotinylated and the number of ErbB3 molecules on the cell surface can be readily determined, for example, by measuring the amount of biotin on a monolayer of cells in culture in the presence or absence of an antibody or antigen binding portion thereof, for example, as described in, *e.g.*, Waterman *et al.*, J. Biol. Chem. (1998), 273:13819-27, followed by immunoprecipitation of ErbB3 and probing with streptavidin. A decrease in detection of biotinylated ErbB3 over time in the presence of an antibody or antigen binding portion is indicative of an antibody which decreases ErbB3 levels on cell surfaces.

Antibodies or antigen binding portions thereof of the present invention can also be tested for their ability to inhibit proliferation of cells expressing ErbB3, for example, tumor cells, using art recognized techniques, such as the Cell Titer Glow Assay described in the Examples below (also see, *e.g.*, Macallan *et al.*, *Proc. Natl. Acad. Sci.* (1998) 20;95(2):708-13; Perez *et al.* (1995) *Cancer Research* 55, 392-398).

In another embodiment, the antibodies or antigen binding portions thereof are screened for the ability to inhibit VEGF secretion of cells expressing ErbB3. This can be done by using well-known assays, such as the VEGF ELISA kit available from R&D Systems (Minneapolis, MN, Cat.#DY293B). Similarly, the antibodies or portions can be screened for the ability to inhibit the migration of cells expressing ErbB3 (*e.g.*, MCF-7 cells) using a trans-well assay (Millipore Corp., Billerica, MA, Cat # ECM552) as described herein.

In another embodiment, the antibodies or antigen binding portions thereof are screened for the ability to inhibit spheroid growth of cells expressing ErbB3. This can be done by using an assay which approximates conditions of a developing tumor growth (see, e.g., Herman et al. (2007) Journal of Biomolecular Screening Electronic publication) as described herein.

Antibodies or antigen binding portions thereof that bind to the same or overlapping epitopes as one or more antibodies of the present invention can also be identified using standard techniques known in the art and described herein. For example, in order to screen for antibodies which bind to the same or an overlapping epitope on ErbB3 bound by an antibody of interest, a cross-blocking assay, such as that described in *Antibodies*, A Laboratory Manual, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed.

IV. Pharmaceutical Compositions

In another aspect, the present invention provides a composition, *e.g.*, a pharmaceutical composition, containing one or a combination of monoclonal antibodies, or antigen-binding portion(s) thereof, of the present invention, formulated together with a pharmaceutically acceptable carrier. In one embodiment, the compositions include a combination of multiple (*e.g.*, two or more) isolated antibodies of the invention, which bind different epitopes on ErbB3.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Preferably, the carrier is suitable for intravenous, intramuscular, subcutaneous, parenteral, spinal or epidermal administration (*e.g.*, by injection or infusion). Depending on the route of administration, the active compound, *i.e.*, antibody, bispecific and multispecific molecule, may be coated in a material to protect the compound from the action of acids and other natural conditions that may inactivate the compound.

A "pharmaceutically acceptable salt" refers to a salt that retains the desired biological activity of the parent compound and does not impart any undesired toxicological effects (see *e.g.*, Berge, S.M., *et al.* (1977) *J. Pharm. Sci.* 66:1-19). 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.

Pharmaceutical compositions of the invention can be administered alone or in combination therapy, *i.e.*, combined with other agents. For example, the combination

therapy can include a composition of the present invention with at least one or more additional therapeutic agents, such as the anti-cancer agents described infra. The pharmaceutical compositions of the invention can also be administered in conjunction with radiation therapy and/or surgery.

A composition of the present invention can be administered by a variety of methods known in the art. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results. The active compounds can be prepared with carriers that will protect the compound against rapid release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Many methods for the preparation of such formulations are patented or generally known to those skilled in the art. *See, e.g., Sustained and Controlled Release Drug Delivery Systems*, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978.

To administer a compound of the invention by certain routes of administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation. For example, the compound may be administered to a subject in an appropriate carrier, for example, liposomes, or a diluent. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Liposomes include water-in-oil-in-water CGF emulsions as well as conventional liposomes (Strejan *et al.* (1984) *J. Neuroimmunol.* 7:27).

Pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the pharmaceutical compositions of the invention is contemplated. Supplementary active compounds can also be incorporated into the compositions.

Therapeutic compositions typically must 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 will be preferable 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, the preferred methods of preparation are 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.

Dosage regimens are adjusted to provide the optimum desired response (*e.g.*, a therapeutic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. For example, the human antibodies of the invention may be administered once or twice weekly by subcutaneous injection or once or twice monthly by subcutaneous injection.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated; each unit contains a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

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) oil-soluble 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.

For the therapeutic compositions, formulations of the present invention include those suitable for oral, nasal, topical (including buccal and sublingual), rectal, vaginal and/or parenteral administration. The formulations may conveniently be presented in

unit dosage form and may be prepared by any methods known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the subject being treated, and the particular mode of administration. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the composition which produces a therapeutic effect. Generally, out of one hundred per cent, this amount will range from about 0.001 per cent to about ninety percent of active ingredient, preferably from about 0.005 per cent to about 70 per cent, most preferably from about 0.01 per cent to about 30 per cent.

Formulations of the present invention which are suitable for vaginal administration also include pessaries, tampons, creams, gels, pastes, foams or spray formulations containing such carriers as are known in the art to be appropriate. Dosage forms for the topical or transdermal administration of compositions of this invention include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The active compound may be mixed under sterile conditions with a pharmaceutically acceptable carrier, and with any preservatives, buffers, or propellants which may be required.

The phrases "parenteral administration" and "administered parenterally" as used herein means 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.

Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the invention 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. Particular examples of adjuvants which are well-known in the art include, for example, inorganic adjuvants (such as aluminum salts, e.g., aluminum phosphate and aluminumhydroxide), organic adjuvants (e.g., squalene), oil-based adjuvants, virosomes (e.g., virosomes which contain a membrane-bound heagglutinin and neuraminidase derived from the influenza virus).

Prevention of presence of microorganisms may be ensured both by sterilization procedures, supra, 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.

When the compounds of the present invention are administered as pharmaceuticals, to humans and animals, they can be given alone or as a pharmaceutical composition containing, for example, 0.001 to 90% (more preferably, 0.005 to 70%, such as 0.01 to 30%) of active ingredient in combination with a pharmaceutically acceptable carrier.

Regardless of the route of administration selected, the compounds of the present invention, which may be used in a suitable hydrated form, and/or the pharmaceutical compositions of the present invention, are formulated into pharmaceutically acceptable dosage forms by conventional methods known to those of skill in the art.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of the present invention 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 will depend upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention 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 well known in the medical arts. A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the compounds of the invention employed in the pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved. In general, a suitable daily dose of a composition of the invention will be that amount of the compound which is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above. It is preferred that administration be intravenous, intramuscular, intraperitoneal, or subcutaneous, preferably administered proximal to the site of the target. If desired, the effective daily dose of a therapeutic

composition may be administered as two, three, four, five, six or more sub-doses administered separately at appropriate intervals throughout the day, optionally, in unit dosage forms. While it is possible for a compound of the present invention to be administered alone, it is preferable to administer the compound as a pharmaceutical formulation (composition).

Therapeutic compositions can be administered with medical devices known in the art. For example, in a preferred embodiment, a therapeutic composition of the invention can be administered with a needleless hypodermic injection device, such as the devices disclosed in U.S. Patent Nos. 5,399,163, 5,383,851, 5,312,335, 5,064,413, 4,941,880, 4,790,824, or 4,596,556. Examples of well-known implants and modules useful in the present invention include: U.S. Patent No. 4,487,603, which discloses an implantable micro-infusion pump for dispensing medication at a controlled rate; U.S. Patent No. 4.,486,194, which discloses a therapeutic device for administering medications through the skin; U.S. Patent No. 4,447,233, which discloses a medication infusion pump for delivering medication at a precise infusion rate; U.S. Patent No. 4,447,224, which discloses a variable flow implantable infusion apparatus for continuous drug delivery; U.S. Patent No. 4,439,196, which discloses an osmotic drug delivery system having multi-chamber compartments; and U.S. Patent No. 4,475,196, which discloses an osmotic drug delivery system. Many other such implants, delivery systems, and modules are known to those skilled in the art.

In certain embodiments, the monoclonal antibodies of the invention 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 of the invention cross the BBB (if desired), they can be formulated, for example, in liposomes. For methods of manufacturing liposomes, see, *e.g.*, U.S. Patents 4,522,811; 5,374,548; and 5,399,331. The liposomes may comprise one or more moieties which are selectively transported into specific cells or organs, thus enhance targeted drug delivery (*see, e.g.*, V.V. Ranade (1989) *J. Clin. Pharmacol.* 29:685). Exemplary targeting moieties include folate or biotin (see, *e.g.*, U.S. Patent 5,416,016 to Low *et al.*); mannosides (Umezawa *et al.*, (1988) *Biochem. Biophys. Res. Commun.* 153:1038); antibodies (P.G. Bloeman *et al.* (1995) *FEBS Lett.* 357:140; M. Owais *et al.* (1995) *Antimicrob. Agents Chemother.* 39:180); surfactant protein A receptor (Briscoe *et al.* (1995) *Am. J. Physiol.* 1233:134), different species of which may comprise the formulations of the inventions, as well as components of the invented molecules; p120 (Schreier *et al.* (1994) *J. Biol. Chem.* 269:9090); see also K. Keinanen; M.L. Laukkanen (1994) *FEBS Lett.* 346:123; J.J. Killion; I.J. Fidler (1994) *Immunomethods* 4:273.

V. Methods of Using Antibodies of the Invention

The present invention also provides methods of using antibodies and antigen-binding portions thereof that bind ErbB3 in a variety of *ex vivo* and *in vivo* diagnostic and therapeutic applications. For example, antibodies of the invention can be used for treating a disease associated with ErbB3 dependent signaling, including a variety of cancers.

In one embodiment, the present invention provides a method for treating a disease associated with ErbB3 dependent signaling by administering to a subject an antibody or antigen binding portion thereof of the invention in an amount effective to treat the disease. Suitable diseases include, for example, a variety of cancers including, but not limited to, melanoma, breast cancer, ovarian cancer, renal carcinoma, gastrointestinal cancer, colon cancer, lung cancer, and prostate cancer.

The antibody can be administered alone or with another therapeutic agent which acts in conjunction with or synergistically with the antibody to treat the disease associated with ErbB3 mediated signaling. Such therapeutic agents include, for example, the anticancer agents described infra (e.g., cytotoxins, chemotherapeutic agents, small molecules and radiation).

In another embodiment, the present invention provides a method for diagnosing a disease (*e.g.*, a cancer) associated with ErbB3 upregulation in a subject, by contacting antibodies or antigen binding portions of the invention (e.g., *ex vivo* or *in vivo*) with cells from the subject, and measuring the level of binding to ErbB3 on the cells. Abnormally high levels of binding to ErbB3 indicate that the subject has a disease associated with ErbB3 upregulation.

Also within the scope of the present invention are kits comprising antibodies and antigen binding portions thereof of the invention which optionally include instructions for use in treating or diagnosing a disease associated with ErbB3 upregulation and/or ErbB3 dependent signaling. The kits may include a label indicating the intended use of the contents of the kit. The term label includes any writing, marketing materials or recorded material supplied on or with the kit, or which otherwise accompanies the kit.

Other embodiments of the present invention are described in the following Examples.

The present invention is further illustrated by the following examples which should not be construed as further limiting. The contents of Sequence Listing, figures and all references, patents and published patent applications cited throughout this application are expressly incorporated herein by reference.

Examples

Materials and Methods

Throughout the examples, the following materials and methods were used unless otherwise stated.

In general, the practice of the present invention employs, unless otherwise indicated, conventional techniques of chemistry, molecular biology, recombinant DNA technology, immunology (especially, *e.g.*, antibody technology), and standard techniques in polypeptide preparation. See, *e.g.*, Sambrook, Fritsch and Maniatis, *Molecular Cloning: Cold Spring Harbor Laboratory Press* (1989); *Antibody Engineering Protocols (Methods in Molecular Biology)*, 510, Paul, S., Humana Pr (1996); *Antibody Engineering: A Practical Approach* (Practical Approach Series, 169), McCafferty, Ed., Irl Pr (1996); *Antibodies: A Laboratory Manual*, Harlow *et al.*, C.S.H.L. Press, Pub. (1999); and *Current Protocols in Molecular Biology*, eds. Ausubel *et al.*, John Wiley & Sons (1992). *In vitro* and *in vivo* model systems for assaying HCV biology are described, for example, in *Cell culture models and animal models of viral hepatitis. Part II: hepatitis C, Lab. Anim.* (NY).;34(2):39-47 (2005) and in *The chimpanzee model of hepatitis C virus infections, ILAR J.*;42(2):117-26 (2001).

Cell Lines

All the cell lines used in the experiments described below were obtained from the National Cancer Institute or provided by investigators, as indicated.

Cell Lines:

MCF7- ATCC cat. No. HTB-22

T47D- ATCC cat. No. HTB-133

Colo357- These cells were obtained from an academic investigator and are described by Kolb et al. (2006) Int. J. Cancer, 120:514-523.

Du145- ATCC cat. No. HTB-81

OVCAR8- source already described in provisional application.

H1975 ATCC cat. No. CRL-5908

Pulverization of Tumor Cells

A cryopulverizer (Covaris Inc) was used for the pulverization of tumors. Tumors were stored in special bags (pre-weighed before the addition of the tumor) and placed in liquid nitrogen while handling them. For small tumors, 200 uL of Lysis buffer was first added to the bag containing the tumor, frozen in liquid nitrogen and then pulverized to improve the recovery of the tumor from the bag. Pulverized tumors were transferred to 2 mL Eppendorf tubes and placed in liquid nitrogen until ready for further processing

Lysis of Tumor Cells

Tumors were lysed in Lysis buffer supplemented with protease and phosphatase inhibitors. Lysis Buffer was added to the tumor aliquots in a final concentration of about 62.5 mg/mL. Tumor samples were homogenized by vortexing for 30 sec and incubating on ice for about 30 min. The lysates were spun for about 10 min in Qiagen Qiashredder columns for further homogenization of the samples. Cleared lysates were aliquoted into fresh tubes for further processing.

BCA Assay

BCA assay (Pierce) was performed following the manufacturer's protocol on all tumor samples. The total protein concentration (in mg/mL) of each tumor sample was later used in the normalization of the ELISA results

ELISA Assay

All ELISA reagents for the total and phospho-ErbB3 ELISAs were purchased from R&D Systems as Duoset kits. 96-well Nunc Maxisorb plates were coated with 50 uL of an antibody and incubated overnight at room temperature. Next morning, plates were washed 3 times with 1000 µl/well in the BioTek plate washer with PBST (0.05% Tween-20). Plates were subsequently blocked for about an 1 hr at room temperature with 2 % BSA in PBS. The plates were washed 3 times with 1000 µl/well in the BioTek plate washer with PBST (0.05%Tween-20). 50 µL of cell lysates and standards diluted in 50% Lysis buffer and 1%BSA were used in duplicates for further processing. Samples were incubated for 2hrs at 4°C on a plate shaker and washed 3 times with 1000 µl/well in the BioTek plate washer with PBST (0.05%Tween-20). About 50 µl of a detection antibody diluted in 2% BSA, PBST was added and incubated for about 1 hr at room temperature. For phosphor-ErbB3, the detection antibody was directly conjugated to horse radish peroxidase (HRP) and incubated for 2 hrs at room temperature. The plate was washed 3 times with 1000 µl/well in the BioTek plate washer with PBST (0.05%Tween-20). About 50 µl of Streptavidin-HRP was added and incubate for 30 min at room temperature (except for pErbB3). The plates were washed 3 times with 1000 µl/well in the BioTek plate washer with PBST (0.05%Tween-20). About 50 µL of Supersignal Pico ELISA substrate was added and the plate was read using a Fusion plate reader. The data was analysed using EXCEL. Duplicate samples were averaged and the error bars were used to represent the standard deviation between the two replicates.

Example 1: Production of Antibodies Using Phage Display

In order to obtain human anti-ErbB3 antibodies referred to herein as Ab #6, Ab #3, Ab #14, Ab #17, and Ab #19, a human Fab-phage library including a unique combination of immunoglobulin sequences obtained from human donors (Hoet *et al. supra*, incorporated by reference in its entirety herein) was initially screened for ErbB3 binders.

Using purified ErbB3 and a Chinese hamster ovary cell line expressing cell surface ErbB3, 73 unique Fab sequences from the library were identified. These 73 clones were then reformatted as Fab only without the phage. Using high throughput methods, these Fabs were expressed on a small scale and tested for binding using ELISA and the Flexchip method which is a high-throughput surface plasmon resonance (SPR) technology. The 73 Fabs without the phage were spotted on a chip surface and the binding kinetics and epitope blocking to a ErbB3-his fusion target protein or a ErbB3-Fcprotein (R & D Systems) were measured. The equilibrium binding constant and on/off rates for the Fabs were calculated from the data obtained.

Binding of the various Fabs to MALME-3M cells was next examined using about 500 nM of the Fabs and a 1:750 dilution of a goat anti-human Alexa 647 secondary antibody. As shown in Figures 1A and 1B, several candidate Fabs exhibited appreciable staining of MALME-3M cells.

Example 2: Optimization of anti-ErbB3 Fabs

Subsequent to the identification of Fabs which blocked the binding of ErbB3 ligand, heregulin, to ErbB3, the VH and VL sequences of the Fabs were codon-optimized as follows.

Specifically, the VH and VL regions were reformatted using expression constructs for expression as an IgG1 or IgG2 isotype. The constructs included a Selexis backbone which has a cassette designed for substitution of the appropriate heavy and light chain sequences. The Selexis vectors included a CMV promoter and a matching poly-A signal.

The nucleic acid sequences for the codon-optimized VH and VL of Ab #6 are set forth in SEQ ID NOs:25 and 26, respectively, and those for Ab #3 are set forth in SEQ ID NOs:27 and 28, respectively, as shown in Figure 22.

Example 3: Binding Affinity for ErbB3

The dissociation constants of the anti-ErbB3 antibodies were measured using two independent techniques, *i.e.*, a Surface Plasmon Resonance Assay and a cell binding assay using MALME-3M cells.

Surface Plasmon Resonance Assay

The Surface Plasmon Resonance Assay (also referred to as the Flexchip assay) was performed as described in Wassaf *et al.* (2006) *Analytical Biochem.*, 351:241-253. The K_D value was calculated based on the formula $K_D = K_d/K_a$.

The K_D values of Ab #6 and Ab #3, respectively, as measured using the Surface Plasmon Resonance Assay, are depicted in Figures 2A and 2B. Ab #6 had a K_D value of

about 4 nM and Ab #3 had a K_D value of about 8 nM, as depicted in Figures 2A and 2B, respectively.

Cell Binding Assay

The cell binding assay for determining the K_D values of Ab #6 and Ab #3 was performed as follows.

MALME-3M cells were detached with 2 mls trypsin-EDTA + 2 mls RMPI + 5mM EDTA at room temperature for 5 minutes. Complete RPMI (10 mls) was added immediately to the trypsinized cells, resuspended gently and spun down in a Beckman tabletop centrifuge at 1100 rpm for 5 minutes. Cells were resuspended in BD stain buffer (PBS + 2% fetal bovine serum + 0.1% sodium azide, Becton Dickinson) at a concentration of 2×10^6 cells per ml and 50 µl (1×10^5 cells) aliquots were plated in a 96-well titer plate.

A 150 µl solution of 200 nM anti-ErbB3 antibody (Ab #6 or Ab #3) in BD stain buffer was prepared in an eppendorf tube and serially diluted 2-fold into 75 µl BD stain buffer. The concentrations of the diluted antibody ranged from 200 nM to 0.4 nM. 50 µl aliquots of the different protein dilutions were then added directly to the 50 ul cell suspension giving the final concentrations of 100 nM, 50 nM, 25 nM, 12 nM, 6 nM, 3 nM, 1.5 nM, 0.8 nM, 0.4 nM and 0.2 nM of the antibody.

Aliquoted cells in the 96-well plate were incubated with the protein dilutions for 30 minutes at room temperature on a platform shaker and washed 3 times with 300 µl BD stain buffer. Cells were then incubated with 100 µl of a 1:750 dilution of Alexa 647-labeled goat anti-human IgG in BD stain buffer for 45 minutes on a platform shaker in the cold room. Finally, cells were washed twice, pelleted and resuspended in 250 µl BD stain buffer + 0.5 µg/ml propidium iodide. Analysis of 10,000 cells was done in a FACScalibur flow cytometer using the FL4 channel. MFI values and the corresponding concentrations of the anti-ErbB3-antibodies were plotted on the y-axis and x-axis, respectively. The K_D of the molecule was determined by GraphPad Prism using the one-site binding model for a non-linear regression curve.

The K_D value was calculated based on the formula $Y=Bmax^* X/ K_D + X$ (Bmax = fluorescence at saturation. X= antibody concentration. Y = degree of binding). As shown in Figures 2C and 2D, Ab # 6 and Ab #3 had K_D values of about 4 nM and 1.3 nM, respectively, in a cell binding assay using MALME-3M cells.

Example 4: Binding Specificity / Epitope Binding for ErbB3

The binding specificity of an IgG2 isotype of Ab #6 to ErbB3 was assayed using ELISA as follows. Identification of the epitope bound by Ab #6 was also analyzed.

Specifically, 96-well Nunc Maxisorb plates were coated with 50 µl of 5 µg/ml protein (recombinant human ErbB3, recombinant human EGFR or unrelated protein (BSA)) and incubated overnight at room temperature. The next morning, plates were washed 3 times with 1000 µl/well of PBST (0.05% Tween-20) in the BioTek plate washer. The wells were blocked for 1 hr at room temperature with 2 % BSA in PBS. The plates were washed 3 times with 1000 µl/well of PBST (0.05%Tween-20) in the BioTek plate washer. About 50 µL of the Ab #6 was added at several dilutions (1 µM and serial 2 fold dilutions) in 2% BSA, PBST. All samples were run in duplicate and incubated for 2hrs at 4°C on a plate shaker. The plates were washed plates 3 times with 1000 µl/well of PBST (0.05%Tween-20) in the BioTek plate washer. 50 µl of human IgG detection antibody (HRP conjugated (Bethyl Inc; 1:75000 dilution in 2% BSA, PBST)) was added and the plates were incubated for 1 hr at room temperature. The plates were washed plate 3 times with 1000 µl/well of PBST (0.05%Tween-20) in the BioTek plate washer. 50 µL of Supersignal Pico ELISA substrate was added and the plate was read on the Fusion plate reader. The data was analyzed using the EXCEL program. Duplicate samples were averaged and the error bars represent the standard deviation between the two replicates.

As shown in Figure 3, Ab #6 bound recombinant ErbB3 in an ELISA, but did not show any appreciable binding to EGFR, BSA or TGF-α.

A fragment (truncation mutant) corresponding to amino acid residues 20-202 of ErbB3 was cloned into the yeast display vector pYD2 (a modified version of pYD1 (Invitrogen) with a stop codon engineered in front of the His tag) between the Nhe and BsiWI restriction sites. The plasmid was transformed into the yeast strain EBY100 (Invitrogen) and clones containing the plasmid selected on Trp- selective medium. The clone was grown in glucose containing medium overnight at 30°C and expression of the ErbB3 truncation mutant was induced by transfer to a galactose-containing medium for 2 days at 18°C. Yeast displaying the ErbB3 truncation mutant were stained with 50 nM of Ab #6, followed by a goat anti-human antibody labeled with Alexa dye-647. A separate sample was stained with the goat anti-human antibody only to show that there is no non-specific binding to yeast of the secondary antibody. Analysis was performed by flow cytometry on the FACS Calibur cell sorter (BD Biosciences).

As shown in Figure 30, Ab #6 bound to the truncation mutant, i.e., amino acid residues 20-202 of ErbB3.

Example 5: Downregulation of Total ErbB3 on Tumor Cells

The ability of Ab #6 to downregulate ErbB3 expression both *in vitro* and *in vivo* in tumor cells was tested as follows.

MALME-3M cells were seeded in 96 well tissue culture plates and grown in RPMI-1640 media supplemented with antibiotics, 2mM L-glutamine and 10% fetal bovine serum (FBS) for 24 hours at 37°C and 5% carbon dioxide. Media was then switched to RPMI-1640 MEDIA with antibiotics, 2mM L-glutamine with and without the antibody at concentrations of 1uM, 250nM, 63nM, 16nM, 4.0nM, 1.0nM, 240pM, 61pM and 15pM. Cells were grown for 24 hours at 37°C and 5% carbon dioxide, washed with cold PBS, then harvested with mammalian protein extract (MPER) lysis (Pierce, 78505) buffer containing 150mM NaCl, 5mM sodium pyrophosphate, 10uM bpV (phen), 50uM phenalarsine, 1mM sodium orthovanadate, and protease inhibitor cocktail (Sigma, P714). Cell lysates were diluted two-fold with 4% bovine serum albumin in phosphate buffered saline with 0.1% tween-20, then analyzed by ELISA with mouse anti-human ErbB3 capture antibody and biotinylated mouse anti-human ErbB3 secondary detection antibody. Signal was generated with streptavidin conjugated to horseradish-peroxidase reacted with chemiluminescent substrate (Pierce, 37070). ELISAS were visualized using a luminometer.

As shown in Figure 4, Ab #6 decreased total ErbB3 levels by about 46.9% in MALME-3M cells *in vitro*, as measured by ELISA. Media containing no serum and antibody was used as control.

In a further experiment, the downregulation of ErbB3 receptors on MALME-3M cells using IgG1 and IgG2 isotypes of Ab #6 was examined using FACS analysis. MALME-3M cells were trypsinized from a 15 cm dish and washed once with RPMI + 10% fetal bovine serum. Cell pellets were resuspended at a density of 1×10^6 cells per ml. Two aliquots of 2×10^5 cells were added to a 12-well tissue culture plate and resuspended in a final volume of 800 ul RPMI + 10% fetal bovine serum. To one well, Ab #6 IgG1 or Ab #6IgG2 isotype was added to a final concentration of 100 nM (treated sample) and to the other well, an equivalent volume of PBS was (untreated sample) was added.

The following day, treated and untreated cells were trypsinized, washed and incubated with 100 nM of Ab #6 in BD stain buffer for 30 minutes on ice. Cells were washed twice with 1 ml BD stain buffer and incubated with 100 ul of a 1:500 dilution of Alexa 647-labeled goat anti-human Alexa 647 for 45 minutes on ice. Cells were then washed and resuspended in 300 ul BD stain buffer + 0.5 ug/ml propidium iodide. Analysis of 10,000 cells was done in a FACScalibur flow cytometer using the FL4 channel.

As shown in Figures 5A and 5B, both IgG1 and IgG2 isotypes of Ab #6 downregulated ErbB3 on MALME-3M cells by about 62% and about 66%, respectively.

In order to determine whether this decrease was due to internalization of the ErbB3 receptor on the surface of MALME-3M cells, the expression of ErbB3 in the

presence of the antibody over time was measured. Specifically, MALME-3M cells were trypsinized from a 15 cm dish and washed once with RPMI + 10% fetal bovine serum. Cell pellets were resuspended at a density of 1 x 10^6 cells per ml. Two aliquots of 2 x 10^5 cells were added to a 12-well tissue culture plate and resuspended in a final volume of 800 µl RPMI + 10% fetal bovine serum. To one well, the anti-ErbB3 antibody was added to a final concentration of 100 nM (treated sample) and to the other well, an equivalent volume of PBS was (untreated sample) was added. The following day, treated and untreated cells were trypsinized, washed and incubated with 100 nM anti-ErbB3 antibody in BD stain buffer for 30 minutes on ice. Cells were washed twice with 1 ml BD stain buffer and incubated with 100 µl of a 1:500 dilution of Alexa 647-labeled goat anti-human Alexa 647 for 45 minutes on ice. Cells were then washed and resuspended in 300 µl BD stain buffer + 0.5 µg/ml propidium iodide. Analysis of 10,000 cells was done in a FACScalibur flow cytometer using the FL4 channel.

As shown in Figure 6, downregulation of ErbB3 in the presence of Ab #6 was measured at 0 hour (Figure 6A), 0.5 hour (Figure 6B), 2 hour (Figure 6C) and 24 hours (Figure 6D). As shown in Figure 6A-6D, about 50% of the cell surface ErbB3 receptors were downregulated after about 30minutes and at about 24 hours, about 93% of the cell surface receptors were downregulation.

The ability of Ab #6 to cause ErbB3 downregulation *in vivo* in melanoma cells was also examined as follows.

Briefly, T-cell deficient nu/nu mice (3-4 week old female mice originated at NIH; outbred; albino background) were purchased from Charles River Labs (Wilmington, MA). MALME-3M cells for implantation were grown in culture (RPMI media, 10% FBS, L-glutamine and antibiotics, 37°C, 5% CO2) to about 80% confluency before harvesting. Cells were kept on ice until implantation. Mice were implanted via subcutaneous injection with 100ul MALME-3M cells on the right flank and allowed to recover while being monitored for initial tumor growth.

The tumors were measured (length by width) by digital caliper and the mice were dosed with IgG2a (Sigma, M7769-5MG) by intravenous injection. Mice were dosed intra-peritoneally every other day with either 15 µg or 100 µg of antibody number 6 and tumors were measured three times per week and recorded in a Microsoft EXCEL spreadsheet.

Final tumor measurements (L x W) were taken, the mice were euthanized by CO_2 asphyxiation and tumors were excised, snap frozen in liquid nitrogen, and were stored at -80^{0}C (for biochemical analysis). Final tumor measurements were analyzed and graphed by tumor area and tumor volume as described, for example, in Burtrum *et al.*, (2003) *Cancer Res.*, 63:8912-8921. The data was also analyzed by "normalized' and "non-normalized" means for both tumor volume and tumor area. For the

"normalization" of the data, at each time point of measurement, each tumor in each group was divided by the initial tumor size determined by caliper measurement.

As shown in Figure 7, among the various antibodies tested in this assay, Ab #6 caused downregulation of total ErbB3 as soon as 24 hours post-injection in tumors treated with either IgG1 or IgG2 isotype of Ab #6). PBS was used as a control

In a further experiment, the ability of Ab #6 to downregulate ErbB3 in ADRr xenografts *in vivo* was examined.

Briefly, the samples were pulverized in a cryopulverizer (Covaris Inc). Tumors were stored in special bags (pre-weighed before the addition of the tumor) and placed in liquid nitrogen while handling them. For small tumors, 200 µL of Lysis buffer was first added to the bag with the tumor, frozen in liquid nitrogen and then pulverized to improve the recovery of the tumor from the bag. Pulverized tumors were transferred to 2 ml Eppendorf tubes and placed in liquid nitrogen until lysed. Tumors were lysed in Lysis buffer supplemented with protease and phosphotase inhibitors. Lysis Buffer was added to the tumor aliquots in a final concentration of 62.5 mg/ml. Tumor samples were homogenized by vortexing for 30 seconds and letting them sit on ice for 30 min. The lysates were spun for 10 minutes in Qiagen Qiashredder columns for further homogenization of the samples. Cleared lysates were aliquoted into fresh tubes.

The BCA assay was performed as set forth in the materials and methods section supra.

The total levels of ErbB3 were determined by ELISA. The ELISA reagents were purchased from R&D Systems as Duoset kits. 96-well Nunc Maxisorb plates were coated with 50 µl of respective capture antibody and incubated overnight at room temperature. The next morning, the plates were washed 3 times with 1000 µl/well in a BioTek plate washer with PBST (0.05% Tween-20) and then blocked for 1 hour at room temperature with 2 % BSA in PBS. The plates were then washed three times with 1000 µl/well in the BioTek plate washer with PBST (0.05%Tween-20). Lysates (50 µl) and standards were diluted in 50% Lysis buffer and 1%BSA; all samples were run in duplicate. Plates were incubated for 2 hours at 4° C on a plate shaker and then washed three times with 1000 µl/well in a BioTek plate washer with PBST (0.05%Tween-20). Fifty microliters of detection antibody diluted in 2% BSA, PBST was added and the plates were incubated for 1 hour at room temperature. Plates were washed three times with 1000 µl/well in the BioTek plate washer with PBST (0.05%Tween-20). Fifty microliters of Streptavidin-HRP was added and the plates were incubated for 30 miutes at room temperature. Plates were washed again three times with 1000 µl/well in a BioTek plate washer with PBST (0.05%Tween-20). Fifty microliters of Supersignal Pico ELISA substrate was added and readout was performed on a Fusion plate reader.

Data was analyzed using EXCEL. Duplicate samples were averaged and the error bars represent the standard deviation between the two replicates.

The results of this experiment are shown in Figure 8. As shown in Figure 8, Ab #6 downregulated ErbB3 in ADRr xenografts *in vivo*.

Example 6: Inhibition of Tumor Cell Proliferation

The ability of Ab #6 to inhibit cellular proliferation of cells expressing ErbB3 (*e.g.*, cancer cells) was examined as follows.

MALME3M, ACHN and NCI/ADRr cells were seeded in 96 well tissue culture plates and grown in RPMI-1640 media supplemented with antibiotics, 2mM L-glutamine and 10% fetal bovine serum (FBS) for 24 hours at 37 degrees Celsius and 5% carbon dioxide. Media was then switched to RPMI-1640 media with antibiotics, 2mM L-glutamine and with and without the antibody at 1uM, 250nM, 63nM, 16nM, 4.0nM, 1.0nM, 240pM, 61pM and 15pM concentrations. Cells were grown for 96 hours at 37°C and 5% carbon dioxide, then harvested with CellTiter-Glo® Luminescent Cell Viability Assay (Promega, G7573) and analyzed on a luminometer. Media containing no serum and antibody was used as control.

As shown in Figures 9, 10 and 11, Ab #6 inhibited proliferation of MALME-3M cells (Figure 9), ADRr ovarian cancer cells (Figure 10) and ACHN cells (Figure 11) which express ErbB3. Specifically, Ab # 6 inhibited proliferation of MALME-3M cells by about 19.6%, as measured using the Cell Titer Glow assay, and inhibited proliferation of ADRr ovarian cancer cells by about 30.5%. Also, as shown in Figure 11, Ab # 6 inhibited proliferation of ACHN cells by about 25.4%.

Example 7: Inhibition of ErbB3 Phosphorylation in Tumor Cells

The ability of Ab #6 to inhibit ErbB3 phosphorylation *in vivo* was examined as follows.

The samples were pulverized using the technique described in Example 5 *supra*, with respect to Figure 8. The BCA assay was performed as set forth in the Materials and Methods section *supra*, and the ELISA assay was performed as described in Example 5 *supra* with respect to Figure 8.

The results of this experiment are shown in Figure 12. As shown in Figure 12, Ab #6 significantly inhibited ErbB3 phosphorylation in ADRr ovarian xenografts *in vivo*, as measured by the amount of phosphorylated ErbB3 (pErbB3) in ng/mg of total protein.

The ability of Ab #6 to inhibit betacellulin (BTC) or heregulin (HRG) induced ErbB3 phosphorylation was also examined, as follows.

Ovarian ADRr cells were preincubated with Ab #6 for 30 minutes prior to stimulation with 50 mM BTC, 10 mM HRG or 333 nM TGF-α. Following pre incubation, the media was removed and the cells were stimulated for 5 minutes at 37^{0}C, 5% CO2 with 50nM BTC or 333nM TGF-α (for PE498). HRG controls (5 minutes, 5nM), 10% serum and 0% serum controls were also used. Cells were washed with 1X cold PBS and lysed in 30µl cold lysis buffer (M-PER buffer plus sodium vanadate (NaVO4, Sigma), 2-glycerophosphate, phenylarsine oxide, BpV and protease inhibitors) by incubating on ice for 30 minutes. Lysates were stored overnight at -80^{0}C.

As shown in Figures13A-13C, Ab #6 significantly inhibited both betacellulin and heregulin-mediated phosphorylation of ErbB3.

In a further experiment, the ability of Ab #6 to inhibit ErbB3 phosphorylation in ovarian tumor cell lines OVCAR 5 and OVCAR 8 was examined as follows.

The OVCAR 5 and OVCAR 8 cell lines were obtained from the National Cancer Institute, Division of Cancer Treatment and Diagnostics ("DCTD"). The ELISA was performed as described in the Materials and Methods section *supra*.

The results of this experiment are depicted in Figures 14A and 14B. As depicted in Figures 14A and 14B, Ab #6 inhibited ErbB3 phosphorylation in both OVCAR 5 and OVCAR 8 ovarian cancer cell lines.

As discussed above, Ab #6 inhibits betacellulin-mediated phosphorylation of ErbB3. In order to investigate whether betacellulin-mediated phosphorylation of ErbB3 occurs through ErbB1 or ErbB3, the following experiment was performed.

ADRr cells or MALME-3M cells (1 x 10^5) were pre-incubated with 25µM of anti-ErbB3 Ab # 6 or 25 µM of Erbitux (as control) in 50µl BD stain buffer for 30 minutes on ice. After 30 minutes, 50 µl of 400 nM biotinylated BTC was added to the cells and incubated for another 30 minutes on ice. This gave a final concentration of 12.5 µM antibodies and 200 nM BTC. Cells were then washed twice with 500 µl BD stain buffer and incubated with 100 µl of a 1:200 dilution of streptavidin-PE (PE=phycoerythrin) (Invitrogen) in BD stain buffer for 45 minutes. Finally, cells were washed twice, resuspended in 300 µl of BD stain buffer and analyzed in a FACScalibur flow cytometer. As a positive control, 1 x 10^5 ADRr or MALME-3M cells were incubated with 200 nM BTC for 30 minutes on ice, washed twice and incubated with a 1:200 dilution of streptavidin-PE for 45 minutes. To assess background staining from the streptavidin-PE conjugate, cells were incubated with 100 µl of a 1:200 dilution of streptavidin-PE only for 45 minutes.

The results of this experiment are depicted in Figures 15A-15C. As shown in Figure 15A, betacellulin (BTC) does not show any appreciable binding to ErbB1 negative MALME-3M cells. However, as depicted in Figures 15B and 15C, BTC does show binding to ErbB1 positive ADRr cells.

Also, as shown in Figures 15B and 15C, this binding was blocked by Erbitux, which is an anti-EGFR antibody which specifically binds EGFR and was included as a control to demonstrate that EGF-like ligands bind to EGFR, and which is described in *e.g.*, Adams *et al.* (2005), *Nature Biotechnology* 23, 1147 – 1157.

Example 8: Inhibition of Heregulin-Mediated Signaling in Tumor Cells

The ability of Ab # 6 to inhibit heregulin-mediated tumor cell signaling was investigated as follows.

MALME-3M cells were seeded in 96 well tissue culture plates and grown in RPMI-1640 media supplemented with antibiotics, 2mM L-glutamine and 10% fetal bovine serum (FBS) for 24 hours at 37°C and 5% carbon dioxide. Cells were serum starved in RPMI-1640 media with antibiotics and 2mM L-glutamine for 24 hours at 37°C and 5% carbon dioxide. Cells were pre-treated with and without the anti-ErbB3 antibody (IgG2 isotype of Ab #6) at 1μM, 250nM, 63nM, 16nM, 4.0nM, 1.0nM, 240pM and 61pM concentrations for 30 minutes then stimulated with HRG1-beta1-ECD for 10 minutes at 37°C and 5% carbon dioxide. Cells were washed with cold PBS then harvested with mammalian protein extract (MPER) lysis (Pierce, 78505) buffer containing 150mM NaCl 5mM sodium pyrophosphate, 10uM bpV (phen), 50μM phenalarsine, 1mM sodium orthovanadate, and protease inhibitor cocktail (Sigma, P714). Cell lysates were diluted two-fold with 4% bovine serum albumin in phosphate buffered saline with 0.1% tween-20, then analyzed by ELISA for AKT (a downstream effector of ErbB3) and ErbB3 phosphorylation.

In order to test for AKT phosphorylation, lysates were run on an ELISA plate with a capture antibody specific for AKT and biotinylated detection antibody specific to the phosphorylation site on serine 473 of AKT. Signal was generated with streptavidin conjugated to horseradish-peroxidase reacted with chemiluminescent substrate (Pierce, 37070). In order to assay for ErbB3 phosphorylation, lysates were run on an ELISA plate with a capture antibody specific for ErbB3 and an anti-phosphotyrosine detection antibody conjugated to horseradish-peroxidase. This was then reacted with chemiluminescent substrate (Pierce, 37070). ELISAs were visualized using a luminometer.

As shown in Figures 16A and 16B, Ab #6 was a potent inhibitor of heregulin-mediated signaling in MALME-3M cells, as measured by decreased phosphorylation of ErbB3 (Figure 16A) and AKT (Figure 16B). Notably, Ab #6 inhibited the phosphorylation of AKT by almost 100%.

Example 9: Inhibition of Ovarian, Prostate, and Pancreatic Tumor Growth

To assess the efficacy of Ab #6 *in vivo*, several xenograft models of human cancer were established in nude mice and the inhibition of tumor growth was assessed at multiple doses. For example, T-cell deficient nu/nu mice (3-4 week old female mice originated at NIH; outbred; albino background) were purchased from Charles River Labs (Wilmington, MA) for xenograft studies. ADRr cells for implantation were grown in culture (RPMI media, 10% FBS, L-glutamine and antibiotics, 37°C, 5% CO2) to about 85% confluency before harvesting. Cells were kept on ice until implantation. Mice were implanted via subcutaneous injection with 100µl ADRr cells on the right flank and allowed to recover while being monitored for initial tumor growth.

Tumors were measured (length by width) by digital caliper and the mice were dosed with IgG2a (Sigma, M7769-5MG) by intravenous injection. Mice were dosed intra-peritoneally every third day with either 30 µg or 300 µg of Ab #6 and tumors were measured three times per week and recorded in a Microsoft Excel spreadsheet.

Final tumor measurements (L x W) were taken, the mice were euthanized by C02 asphyxiation and tumors were excised, snap frozen in liquid nitrogen, and were stored at -80^{0}C (for biochemical analysis). Final tumor measurements were analyzed and graphed by tumor area and tumor volume, as described in Burtrum *et al.*, supra. . The data was also analyzed by "normalized' and "non-normalized" means for both tumor volume and tumor area. For the "normalization" of the data, at each time point of measurement, each tumor in each group was divided by the initial tumor size determined by caliper measurement.

The data from three different models derived from human tumor cell lines, ADRr (ovarian), Du145 (prostate) and OvCAR8 (ovarian) are shown in Figures 17A-C and Colo357 xenograft study is shown in Figure 17D. The data from these studies demonstrated that a 300ug dose of Ab #6 every three days (Q3d) results in significant inhibition of tumor growth ($p<0.05$ for multiple time points during the studies). Moreover, this inhibitory effect of Ab #6 was further elevated when the dose was increased to 600ug, Q3d, in the Du145 prostate cancer model as well as a renal and a pancreatic carcinoma xenograft model (ACHN and COLO357). However, further elevating the dose to 1500ug Q3d did not result in increased efficacy (OvCAR8-Figure 17; COLO357) suggesting that the 600ug is saturating in regards to tumor growth inhibition. Pharmacokinetic (PK) analyses of the serum from the animals from these studies demonstrate a dose-dependent increase in the serum retention of Ab #6. Similarly, biochemical analysis of the intra-tumoral levels of Ab #6 from these different studies showed a dose-dependent range of 0 to ~6 pg MM121/ug of total tumor lysate (data not shown).

Example 10: Inhibition of binding of ErbB3 Ligands to ErbB3 on Tumor Cells

In a further experiment, the specificity of the antibodies of the invention to inhibit the binding of ErbB3 ligands to ErbB3, and not EGF-like ligands to EGFR, was investigated as follows.

In one experiment, the specificity of Ab #6 and a Fab version of Ab #3 (Ab/Fab #3) to inhibit the binding of ErbB3 ligands (*e.g.*, heregulin and epiregulin) to ErbB3 was investigated.

In order to investigate the ability of Ab #6 and Ab/Fab #3 to inhibit the binding of heregulin to ErbB3, the following experiment was performed.

ADRr cells (1×10^5) were incubated with 10 μM of an anti-ErbB3 antibody (*e.g.*, Ab #6 or Ab/Fab # 3) in 50 μl BD stain buffer for 30 minutes on ice. After 30 minutes, 50 μl of 40 nM biotinylated heregulin EGF was added to the cells and incubated for another 10 minutes on ice. This gave a final concentration of 5 μM antibody and 20 nM heregulin EGF. Cells were then washed twice with 500 μl BD stain buffer and incubated with 100 μl of a 1:200 dilution of streptavidin-PE (PE=phycoerythrin) (Invitrogen) in BD stain buffer for 45 minutes. Finally, cells were washed twice, resuspended in 300 μl of BD stain buffer and analyzed in a FACScalibur flow cytometer. As a positive control, 1×10^5 ADRr cells were incubated with 20 nM heregulin EGF for 10 minutes on ice, washed twice and incubated with a 1:200 dilution of streptavidin-PE for 45 minutes. In order to assess background staining from the streptavidin-PE conjugate, 1×10^5 ADRr cells were incubated with 100 μl of a 1:200 dilution of streptavidin-PE only for 45 minutes.

The results of this experiment are shown in Figures 18A and 18B. As depicted in Figures 18A and 18B, both Ab #6 and Ab/Fab #3 were able to inhibit heregulin binding to ErbB3.

Similarly, the ability of Ab #6 to inhibit the binding of another ErbB3-ligand, epiregulin, to ErbB3, was examined as follows.

ADRr cells (1×10^5) were pre-incubated with 25μM of Ab #6 or 25 μM of Erbitux (as control) in 50 μl BD stain buffer for 30 minutes on ice. After 30 minutes, 50 μl of 2 μM biotinylated Epi was added to the cells and incubated for another 30 minutes on ice. This gave a final concentration of 12.5 μM antibodies and 1 μM Epi. Cells were then washed twice with 500 μl BD stain buffer and incubated with 100 μl of a 1:200 dilution of streptavidin-PE (PE=phycoerythrin) (Invitrogen) in BD stain buffer for 45 minutes. Finally, cells were washed twice, resuspended in 300 μl of BD stain buffer and analyzed in a FACScalibur flow cytometer. As a positive control, 1×10^5 ADRr cells were incubated with 1 μM Epi for 30 minutes on ice, washed twice and incubated with a 1:200 dilution of streptavidin-PE for 45 minutes. To assess background staining from

the streptavidin-PE conjugate, cells were incubated with 100 µl of a 1:200 dilution of streptavidin-PE only for 45 minutes.

The results of this experiment are depicted in Figures 19A and 19B. As shown in Figure 19A, epiregulin binds to ErbB3 positive ADRr cells. Further, as shown in Figure 19B, this binding is inhibited by both Erbitux and Ab #6, suggesting that epiregulin may bind to both EGFR and ErbB3.

A further experiment was performed to investigate whether Ab #6 is able to inhibit the binding of an EGF-like ligand (*e.g.*, HB-EGF) to tumor cells.

ADRr cells (1 x 10^5) were pre-incubated with 25µM of Ab #6 or 25 µM of Erbitux (as control) in 50 µl BD stain buffer for 30 minutes on ice. After 30 minutes, 50 µl of 400 nM biotinylated HB-EGF was added to the cells and incubated for another 30 minutes on ice. This gave a final concentration of 12.5 µM antibodies and 200 nM HB-EGF. Cells were then washed twice with 500 µl BD stain buffer and incubated with 100 µl of a 1:200 dilution of streptavidin-PE (PE=phycoerythrin) (Invitrogen) in BD stain buffer for 45 minutes. Finally, cells were washed twice, resuspended in 300 µl of BD stain buffer and analyzed in a FACScalibur flow cytometer. As a positive control, 1 x 10^5 ADRr cells were incubated with 200 nM HB-EGF for 30 minutes on ice, washed twice and incubated with a 1:200 dilution of streptavidin-PE for 45 minutes. To assess background staining from the streptavidin-PE conjugate, cells were incubated with 100 µl of a 1:200 dilution of streptavidin-PE only for 45 minutes.

As shown in Figures 20A and 20B, HB-EGF binds to ErbB on ADRr cells and Ab #6 does not inhibit this binding, evidencing that Ab #6 is specific for inhibiting the binding of ErbB3 ligands (*e.g.*, heregulin and epiregulin) to ErbB3.

Example 11: Inhibition of VEGF Secretion in Tumor Cells

The ability of Ab #6 to inhibit VEGF secretion of cells expressing ErbB3 (*e.g.*, cancer cells) was examined using VEGF secretion assay (VEGF ELISA kit available from R&D Systems, Minneapolis, MN, Cat.#DY293B). First, the ability of Ab #6 to inhibit VEGF secretion in the untreated and HRG –beta1 treated MCF-7, T47D, and COLO-357 cells was analyzed. These studies revealed that COLO-357 secreted the highest amount of VEGF into the media. As these cells also had very high HRG levels (data not shown), addition of HRG to the media was not able to further induce VEGF secretion (Fig. 24A). In contrast, HRG was able to induce VEGF secretion in MCF-7 and T47D cells.

Ab #6 shows a potent inhibitory effect at high levels in all three cell lines with the highest being in COLO-357 (Fig. 24A). Ab #6 also shows a similar effect in vivo by inhibiting VEGF secretion in three different xenografts, the highest being in COLO-357 xenograft (Fig. 24B). Inhibition of VEGF correlates with inhibition of ErbB3

phosphorylation (Fig. 24 C). Inhibition of VEGF secretion also correlates with inhibition of angiogenesis of the tumor cells. In particular, it has been identified that myeloma cell-secreted factors, such as VEGF and bFGF, trigger angiogenesis (see, e.g., Leung et al. (1989) Science 246(4935):1306-9; Yen et al. (2000) Oncogene 19(31):3460-9).

Example 12: Inhibition of Cell Migration

The ability of Ab #6 to inhibit the migration of cells expressing ErbB3 (*e.g.*, MCF-7 cells) was examined using a trans-well assay (Millipore Corp., Billerica, MA, Cat # ECM552). First, MCF-7 cells were serum-starved overnight and then incubated in the presence or absence of Ab #6 (8uM final concentration) for 15 minutes at room temperature. The cells were then transferred to an upper chamber that is separated from a lower chamber by a collagen type I-coated membrane through which the cells can migrate. 10%FBS was added to media in the lower chamber to act as a chemoattractant in the presence of absence of Ab #6. The chambers were incubated at 37°C for 16 hours and then the cells that migrated through the membrane were removed using a detachment buffer and incubated with a cell-binding fluorescent dye. Fluorescence was quantitated using a fluorescent plate reader. The average fluorescence ± SEM (n=2) is shown in Figure 25.

As shown in Figure 25, 10% FBS stimulates cell migration (lane 3) as compared to untreated control (lane 1) and 8uM Ab #6 inhibits the FBS induced cell migration (lane 4).

Example 13: Inhibition of Spheroid Growth

The ability of Ab #6 to inhibit the spheroid growth of cells expressing ErbB3 was examined using an assay which approximates conditions of a developing tumor growth (Herman et al. (2007) Journal of Biomolecular Screening Electronic publication). AdrR and DU145 spheroids were initiated at a frequency of 1 spheroid per well of a 96 well plate using the hanging drop method (Herrman et al., 2008). Individual spheroids were then treated with either Ab #6 (8 uM final concentration), Heregulin-β1 EGF domain (R&D Systems, Minneapolis, MN, Cat # 396-HB, 3.4 nM final concentration), or a combination of both, as indicated. The diameters of the spheroids were measured using light microscopy (10X objective) at day 1 and day 13.

Ab #6 inhibits spheroid growth in AdrR cells (Fig. 26A). In addition, 3.4nM HRG stimulates spheroid growth and Ab #6 inhibits the HRG effect (Fig. 26B). Spheroids derived from DU145 did not increase in size during 13 days of the

experiment; however, growth was significantly stimulated by HRG1-beta 1. In these cells, 8uM Ab #6 inhibits HRG induced spheroid growth (Fig. 26C).

Example 14: Inhibition of Signaling

The ability of Ab #6 to inhibit the signaling induced by different ligands was examined. For example, the effect of Ab #6 on HRG and BTC binding to AdrR cells expressing ErbB3 receptor was tested. As shown in Figures 27A and B, using FACS analysis, Ab #6 competes with HRG and not BTC for binding to AdrR cells. Accordingly, blocking by Ab #6 of HRG binding to ErbB3 would prevent signaling induced by HRG.

Additionally, various ligands were tested for inducement of ErBb3 phosphorylation. Three ligands, HRG, BTC, and HGF, were able to stimulate ErbB3 induced phosphorylation in AdrR cells, while EGF could not. As shown in Figure 28, Ab #6 inhibits HGF induced pErbB3 phosphorylation in AdrR cells (Fig. 28). Further, as known in the art (see, e.g., Wallenius et al. (2000) Am J Pathol. 156 (3):821-9 10702398), enhanced HGF signaling has been found in various epithelial and non-epithelial tumors.

ErbB3/cMET interaction and the role of Ab #6 in modulating this interaction

It has been shown that non-small-cell lung cancers carrying activating mutations in the epidermal growth factor receptor (EGFR) develop resistance to tyrosine kinase inhibitors by recruiting MET and HER3 and thus activating the PI3K-AKT cell survival pathway (Engelmann et al. (2007) Science 316: 1039-1043; Gou (2007) PNAS: 105(2): 692-697). The association between EGFR and c-MET in cell lines that carry activating EGFR mutations has been well established by co-immunoprecipitation (Engelmann et al. 2007; Gou 2007). Guo et al. recently demonstrated that c-MET and ErbB3 also exist in a complex in a gastric cell line MKN45 known to be dependent on amplified c-MET, using co-immunoprecipitation.

This c-MET-erbB3 interaction occurs also in AdrR cells carrying the wild type EGFR and is not dependent on amplified c-MET. HGF (Hepatocyte Growth Factor) induces ErbB3 phosphorylation in AdrR cells in a dose dependent manner as shown in Figure 28. In addition, Ab #6 inhibits HGF induced erbB3 phosphorylation.

The effect of HRG and BTC on both ErbB1 and ErbB3 phosphorylation has also been investigated, and HRG and BTC were found to induce phosphorylation of both ErbB1 and ErbB3. HRG was found to be a more potent inducer of ErbB3 phosphorylation while BTC was a potent inducer of ErbB1 phosphorylation (Fig. 29).

This phosphorylation is likely to be driven by the complex between ErbB1 and ErbB3. Briefly, HRG binding to ErbB3 induces complex formation between ErbB1 and ErbB3, leading to the activation of both receptors. The same phenomenon appears likely for BTC, where BTC binding to ErbB1 stimulates complex formation between ErbB1 and ErbB3, leading to the phosphorylation of both ErbB1 and ErbB3.

Antibody Inhibition of ligand (HRG, BTC, EGF, and HGF) stimulated ErbB3 phosphorylation.

The ability of Ab #6 to inhibit ligand (HRG, BTC, EGF, and HGF) induced ErbB3 phosphorylation was examined based on the following method:

1. AdrR cells were plated into 96 well plate at a density of 30,000 cells/well/100 uL in RPMI medium containing 10% FBS and allowed to grow overnight;
2. The next day, cells were serum-starved by changing medium to FBS-free medium and allowed to grow overnight;
3. Cells were pre-treated with different concentrations of Ab #6 (from 0.01 nM to 100nM), or buffer (control), for 2hours;
4. The cells were then stimulated with 10 nM HRG and HGF for 10 minutes, or 10 nM BTC and EGF for 5 minutes;
5. The reaction was stopped by removing the culture medium and washing the cells once with ice cold PBS;
6. The cells were then lysed in 25 mM Tris, pH+7.5, 150 mM NaCl, 1mM EDTA, 1.0% Triton X-100, 1.0% CHAPS, 10% v/v glycerol, containing 1X protease inhibitor and 1X phosphatase inhibitor; and
7. ErbB3 phosphorylation was measured in cell lysates using Human Phospho-ErbB3 ELISA kit (R&D Systems, Minneapolis, MN, Cat. No. DYC1769) according to manufacturer's instructions.

Antibody Inhibition of ErbB2-ErbB3 protein complex formation.

AdrR cells were pre-incubated with buffer (control), or 250 nM Ab #6 for 60 minutes at room temperature, then treated with 10 nM HRG or 10 nM BTC or control buffer for 10 minutes. The cells were lysed in 25 mM Tris, pH+7.5, 150 mM NaCl, 1mM EDTA, 1.0% Triton X-100, 1.0% CHAPS, 10% v/v glycerol, containing 0.2mM PMSF, 50mTU/mL aprotinin, and 100 uM leupeptin, and the crude lysate were centrifuged briefly to remove insoluble material. Supernatant was transferred to a new eppendorf tube, and anti-ErbB3 antibody (Santa Cruz sc-285) was added at 1:500 dilution. Supernatants were incubated overnight with gentle shaking at 4C.

60 ul of Immobilized Protein A/G agarose beads (Pierce, Rockford, IL, Cat# 20421) was first washed with 1X PBS. The cell lysate-antibody mixture was added to the PBS washed beads, and incubated for 2 hours with gentle shaking at 4°C. The immunoprecipitates were then washed with ice-cold lysis buffer 3 times, resuspended in 30 ul of 2X SDS sample buffer, heat denatured at 95°C for 7 minutes and run on 4-12% Bis-Tris Gels. SDS-PAGE and electro-transferred to PVDF membrane in Tri-Glycine buffer with 10% MeOH. The membrane was blocked for 1hour in 10ml of blocking buffer (Li-Cor Biosciences, Lincoln, NE, Cat# 927-40000) and then incubated with the anti-ErbB2 antibody at 1:1000 (Cell Signaling Technology, Danvers, MA, Cat # 29D8) in 10 ml of blocking buffer (Li-Cor Biosciences, Cat# 927-40000). The signal was detected using goat anti-rabbit IRDye800 at 1:5000 (2ul) in 10ml of blocking buffer (Li-Cor Biosciences, Cat# 927-40000).

Ab #6 was also shown to completely inhibit HRG stimulated ErbB2/3 complex formation (Fig.29B).

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims. Any combination of the embodiments disclosed in the dependent claims are contemplated to be within the scope of the invention.

Incorporation by Reference

All publications, patents, and pending patent applications referred to herein are hereby incorporated by reference in their entirety.

We claim:

1. An isolated monoclonal antibody or antigen binding portion thereof that binds to ErbB3 and inhibits EGF-like ligand mediated phosphorylation of ErbB3.

2. An isolated monoclonal antibody or antigen binding portion thereof that binds to ErbB3, wherein the antibody or antigen binding portion thereof exhibits one or more of the following properties:

(i) inhibition of heregulin, epiregulin, betacellulin, epigen or biregulin-mediated signaling through ErbB3;

(ii) inhibition of proliferation of cells expressing ErbB3;

(iii) the ability to decrease levels of ErbB3 on cell surfaces;

(iv) inhibition of VEGF secretion of cells expressing ErbB3;

(v) inhibition of the migration of cells expressing ErbB3;

(vi) inhibition of spheroid growth of cells expressing ErbB3; and/or

(vii) binding to an epitope located on domain I of ErbB3 .

3. The antibody or antigen binding portion thereof of any one of the preceding claims, wherein the antibody or antigen binding portion thereof binds to ErbB3 with a K_D of at least about 8 nM or better, as measured by a surface plasmon resonance assay or a cell binding assay.

4. The antibody or antigen binding portion of any one of the preceding claims, wherein the EGF-like ligand is selected from the group consisting of EGF, TGF-α, betacellulin, heparin-binding epidermal growth factor, amphiregulin and biregulin.

5. An isolated monoclonal antibody or antigen binding portion thereof that binds to ErbB3, wherein the antibody or antigen binding portion thereof comprises a heavy chain variable region comprising an amino acid sequence at least 80% identical to the heavy chain variable region amino acid sequence set forth in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:35, or SEQ ID NO:37.

6. An isolated monoclonal antibody or antigen binding portion thereof that binds to ErbB3, wherein the antibody or antigen binding portion thereof comprises a light chain variable region comprising an amino acid sequence at least 80% identical to the light chain variable region amino acid sequence set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:36, or SEQ ID NO:38.

7. An isolated monoclonal antibody or antigen binding portion thereof that binds to ErbB3, wherein the antibody or antigen binding portion thereof comprises a heavy chain variable region comprising an amino acid sequence at least 95% identical to the heavy chain variable region amino acid sequence set forth in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:35, or SEQ ID NO:37.

8. An isolated monoclonal antibody or antigen binding portion thereof that binds to ErbB3, wherein the antibody or antigen binding portion thereof comprises a light chain variable region comprising an amino acid sequence at least 95% identical to the light chain variable region amino acid sequence set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:36, or SEQ ID NO:38.

9. The antibody or antigen binding portion thereof of claim 7, wherein the antibody or antigen binding portion thereof further comprises a light chain variable region comprising an amino acid sequence at least 95% identical to the light chain variable region amino acid sequence set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:36, or SEQ IDNO:38.

10. An isolated antibody or antigen binding portion thereof that binds to the epitope which is same or overlapping with the epitope bound by the antibody of any one of the preceding claims.

11. The antibody or antigen binding portion thereof of any of the preceding claims, which binds to residues 20-202 of the amino acid sequence of ErbB3.

12. An isolated monoclonal antibody or antigen binding portion thereof that binds to ErbB3, wherein the antibody or antigen binding portion thereof comprises:

a heavy chain variable region comprising CDR1, CDR2, and CDR3 sequences; and

a light chain variable region comprising CDR1, CDR2, and CDR3 sequences, wherein

the heavy chain variable region CDR3 sequence comprises an amino acid sequence selected from the group consisting of SEQ ID NOs:9, 15, 21, 41, 47 and conservative amino acid substitutions thereof.

13. The antibody or antigen binding portion thereof of claim 12, wherein the light chain variable region CDR3 sequence comprises an amino acid sequence selected from the group consisting of SEQ ID NOs:12, 18, 24, 44, 50 and conservative sequence modifications thereof.

14. The antibody or antigen binding portion thereof of claim 12 or 13, wherein the heavy chain variable region CDR2 sequence comprises an amino acid sequence selected from the group consisting of SEQ ID NOs:8, 14, 20, 40, 46 and conservative sequence modifications thereof.

15. The antibody or antigen binding portion thereof of claims 12-14, wherein the light chain variable region CDR2 sequence comprises an amino acid sequence selected from the group consisting of SEQ ID NOs:11, 17, 23, 43, 49 and conservative sequence modifications thereof.

16. The antibody or antigen binding portion thereof of claims 12-15, wherein the heavy chain variable region CDR1 sequence comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 7, 13, 19, 39, 45 and conservative sequence modifications thereof.

17. The antibody or antigen binding portion thereof of claims 12-16, wherein the light chain variable region CDR1 sequence comprises an amino acid sequence selected from the group consisting of SEQ ID NOs:10, 16, 22, 42, 48 and conservative sequence modifications thereof.

18. An isolated monoclonal antibody or antigen binding portion thereof that binds to ErbB3 and comprises heavy and light chain variable region CDR1, CDR2 and CDR3 sequences selected from the group consisting of:

(a) a heavy chain variable region CDR1 comprising SEQ ID NO:7;
a heavy chain variable region CDR2 comprising SEQ ID NO:8;
a heavy chain variable region CDR3 comprising SEQ ID NO:9;
a light chain variable region CDR1 comprising SEQ ID NO:10;
a light chain variable region CDR2 comprising SEQ ID NO:11;
a light chain variable region CDR3 comprising SEQ ID NO:12;
and combinations thereof;

(b) a heavy chain variable region CDR1 comprising SEQ ID NO:13;
a heavy chain variable region CDR2 comprising SEQ ID NO:14;
a heavy chain variable region CDR3 comprising SEQ ID NO:15;
a light chain variable region CDR1 comprising SEQ ID NO:16;
a light chain variable region CDR2 comprising SEQ ID NO:17;
a light chain variable region CDR3 comprising SEQ ID NO:18;
and

combinations thereof;

(c) a heavy chain variable region CDR1 comprising SEQ ID NO:19;
a heavy chain variable region CDR2 comprising SEQ ID NO:20;
a heavy chain variable region CDR3 comprising SEQ ID NO:21;
a light chain variable region CDR1 comprising SEQ ID NO:22;
a light chain variable region CDR2 comprising SEQ ID NO:23;
a light chain variable region CDR3 comprising SEQ ID NO:24;

and

(d) a heavy chain variable region CDR1 comprising SEQ ID NO:39;
a heavy chain variable region CDR2 comprising SEQ ID NO:40;
a heavy chain variable region CDR3 comprising SEQ ID NO:41;
a light chain variable region CDR1 comprising SEQ ID NO:42;
a light chain variable region CDR2 comprising SEQ ID NO:43;
a light chain variable region CDR3 comprising SEQ ID NO:44;

and

(e) a heavy chain variable region CDR1 comprising SEQ ID NO:45;
a heavy chain variable region CDR2 comprising SEQ ID NO:46;
a heavy chain variable region CDR3 comprising SEQ ID NO:47;
a light chain variable region CDR1 comprising SEQ ID NO:48;
a light chain variable region CDR2 comprising SEQ ID NO:49;
a light chain variable region CDR3 comprising SEQ ID NO:50;

19. An isolated monoclonal antibody or antigen binding portion thereof that binds to ErbB3 and comprises:

a heavy chain variable region CDR1 comprising SEQ ID NO:7;
a heavy chain variable region CDR2 comprising SEQ ID NO:8;
a heavy chain variable region CDR3 comprising SEQ ID NO:9;
a light chain variable region CDR1 comprising SEQ ID NO:10;
a light chain variable region CDR2 comprising SEQ ID NO:11;

and

a light chain variable region CDR3 comprising SEQ ID NO:12.

20. An isolated monoclonal antibody or antigen binding portion thereof that binds to ErbB3 and comprises:

a heavy chain variable region comprising CDR1, CDR2, and CDR3 sequences; and

a light chain variable region comprising CDR1, CDR2, and CDR3 sequences, wherein:

the heavy chain variable region CDR3 sequence comprises an amino acid sequence which is at least 90% identical to the amino acid sequence selected from the group consisting of SEQ ID NOs:9, 15 and 21.

21. The antibody of claim 20, wherein the light chain variable region CDR3 sequence comprises an amino acid sequence which is at least 90% identical to the amino acid sequence selected from the group consisting of SEQ ID NOs:12, 18 and 24.

22. The antibody of claim 20 or 21, wherein the heavy chain variable region CDR2 sequence comprises an amino acid sequence which is at least 90% identical to the amino acid sequence selected from the group consisting of SEQ ID NOs:8, 14 and 20.

23. The antibody of claims 20-22, wherein the light chain variable region CDR2 sequence comprises an amino acid sequence which is at least 90% identical to the amino acid sequence selected from the group consisting of SEQ ID NOs:11, 17 and 23.

24. The antibody of claims 20-23, wherein the heavy chain variable region CDR1 sequence comprises an amino acid sequence which is at least 90% identical to the amino acid sequence selected from the group consisting of SEQ ID NOs:7, 13 and 19.

25. The antibody of claims 20-24, wherein the light chain variable region CDR1 sequence comprises an amino acid sequence which is at least 90% identical to the amino acid sequence selected from the group consisting of SEQ ID NOs:10, 16 and 22.

26. An isolated monoclonal antibody or antigen binding portion thereof that binds to ErbB3, wherein the antibody or antigen binding portion thereof:

comprises a heavy chain variable region that is from a human VH3 germline gene.

27. The antibody or antigen binding portion thereof of claim 26, wherein the antibody or antigen binding portion thereof further comprises a light chain variable region from human VL2 germline gene.

28. The antibody or antigen binding portion thereof of any one of the preceding claims, wherein the antibody is selected from the group consisting of a human antibody, a humanized antibody, a bispecific antibody and a chimeric antibody.

29. The antibody or antigen binding portion thereof of any one of the preceding claims, wherein the antibody or antigen binding portion thereof is selected from the group consisting of a Fab, Fab'2, ScFv, SMIP, affibody, avimer, nanobody, and a domain antibody.

30. The antibody or antigen binding portion thereof of any one of the preceding claims, wherein the antibody isotype is selected from the group consisting of an IgG1, an IgG2, an IgG3, an IgG4, an IgM, an IgA1, an IgA2, an IgAsec, an IgD, and an IgE antibody.

31. A composition comprising the antibody or antigen binding portion of any one of the preceding claims in a pharmaceutically acceptable carrier.

32. A composition comprising two or more antibodies of any one of the preceding claims, wherein the antibodies bind to different epitopes on ErbB3.

33. An isolated nucleic acid encoding a variable region of a human antibody that binds ErbB3 comprising a sequence at least 90% identical to the heavy chain variable region of SEQ ID NO:25, the light chain variable region of SEQ ID NO:26, the heavy chain variable region of SEQ ID NO:27, the light chain variable region of SEQ ID NO:28, the heavy chain variable region of SEQ ID NO:29, the light chain variable region of SEQ ID NO:30, or combinations thereof.

34. An isolated nucleic acid encoding a variable region of a human antibody that binds ErbB3 comprising a sequence that hybridizes under high stringency to the nucleotide sequence of heavy chain variable region of SEQ ID NO:25, the light chain variable region of SEQ ID NO:26, the heavy chain variable region of SEQ ID NO:27, the light chain variable region of SEQ ID NO:28, the heavy chain variable region of SEQ ID NO:29, or the light chain variable region of SEQ ID NO:30.

35. An expression vector comprising the nucleic acid of claim 33 or 34.

36. A host cell comprising the nucleic acid of claim 35.

37. A transgenic non-human mammal which expresses a monoclonal antibody or antigen binding portion thereof that binds the same epitope as the antibody or antigen binding portion of any one of the preceding claims.

38. A transgenic plant which expresses a monoclonal antibody or antigen binding portion thereof that binds the same epitope as the antibody or antigen binding portion of any one of the preceding claims.

39. A hybridoma which produces an antibody or antigen binding portion thereof of any one of the preceding claims.

40. A hybridoma which produces a human antibody that binds to ErbB3, wherein the antibody is encoded by:

(a) a heavy chain variable region nucleotide sequence set forth in SEQ ID NO:25 and a light chain variable region nucleotide sequence set forth in SEQ ID NO:26, and conservative sequence modifications thereof;

(b) a heavy chain variable region nucleotide sequence set forth in SEQ ID NO:27 and a light chain variable region nucleotide sequence set forth in SEQ ID NO:28, and conservative sequence modifications thereof; or

(c) a heavy chain variable region nucleotide sequence set forth in SEQ ID NO:29 and a light chain variable region nucleotide sequence set forth in SEQ ID NO:30, and conservative sequence modifications thereof.

41. A kit comprising one or more isolated monoclonal antibodies or antigen binding portions thereof of any one of the preceding claims, and optionally comprising instructions for use in treating or diagnosing a disease associated with ErbB3 dependent signaling.

42. The kit of claim 41, wherein the disease is a cancer.

43. A method of inhibiting EGF-like ligand mediated phosphorylation of ErbB3 in a subject, comprising administering to the subject an isolated monoclonal antibody or antigen binding portion thereof of any one of the preceding claims, in an amount sufficient to inhibit EGF-like mediated phosphorylation of ErbB3.

44. A method of treating cancer in a subject, comprising administering to the subject a therapeutically effective amount of an isolated monoclonal antibody or antigen binding portion thereof of any one of the preceding claims.

45. The method of claim 44, wherein the cancer is selected from the group consisting of melanoma, breast cancer, ovarian cancer, renal carcinoma, gastrointestinal/colon cancer, lung cancer, clear cell sarcoma ,and prostate cancer.

46. The method of any one of claims 43-45, wherein the subject is human.

47. The method of any one of claims 43-45, wherein the antibody or antigen binding portion thereof is administered intravenously, intramuscularly, or subcutaneously to the subject.

48. The method of any one of claims 44-47, wherein the antibody or antigen binding portion thereof is administered in combination with a second therapeutic agent.

49. The method of claim 48, wherein the second agent is a second antibody or antigen binding portion thereof.

50. The method of claim 48, wherein the second agent is an anti-cancer agent.

51. The method of claim 50, wherein the anti-cancer agent is selected from the group consisting of an antibody, a small molecule, an antimetabolite, an alkylating agent, a topoisomerase inhibitor, a microtubule-targeting agent, a kinase inhibitor, a protein synthesis inhibitor, an immunotherapeutic, a hormone or analog thereof, a somatostatin analog, a glucocortocoid, an aromatose inhibitor, and an mTOR inhibitor.

52. The method of claim 51, wherein the antibody is an anti-IGF1R antibody, an anti-EGFR antibody, or an anti-cmet antibody.

53. The method of claim 51, wherein the small molecule binds IGF1R, EGFR, or cmet,

54. A method of diagnosing a cancer associated with ErbB3 in a subject, comprising (a) contacting *ex vivo* or *in vivo* cells from the subject with an isolated monoclonal antibody or antigen binding portion thereof of any of claims 1-30, and (b) measuring the level of binding to ErbB3 on the cells, wherein abnormally high levels of binding to ErbB3 indicate that the subject has a cancer associated with ErbB3.

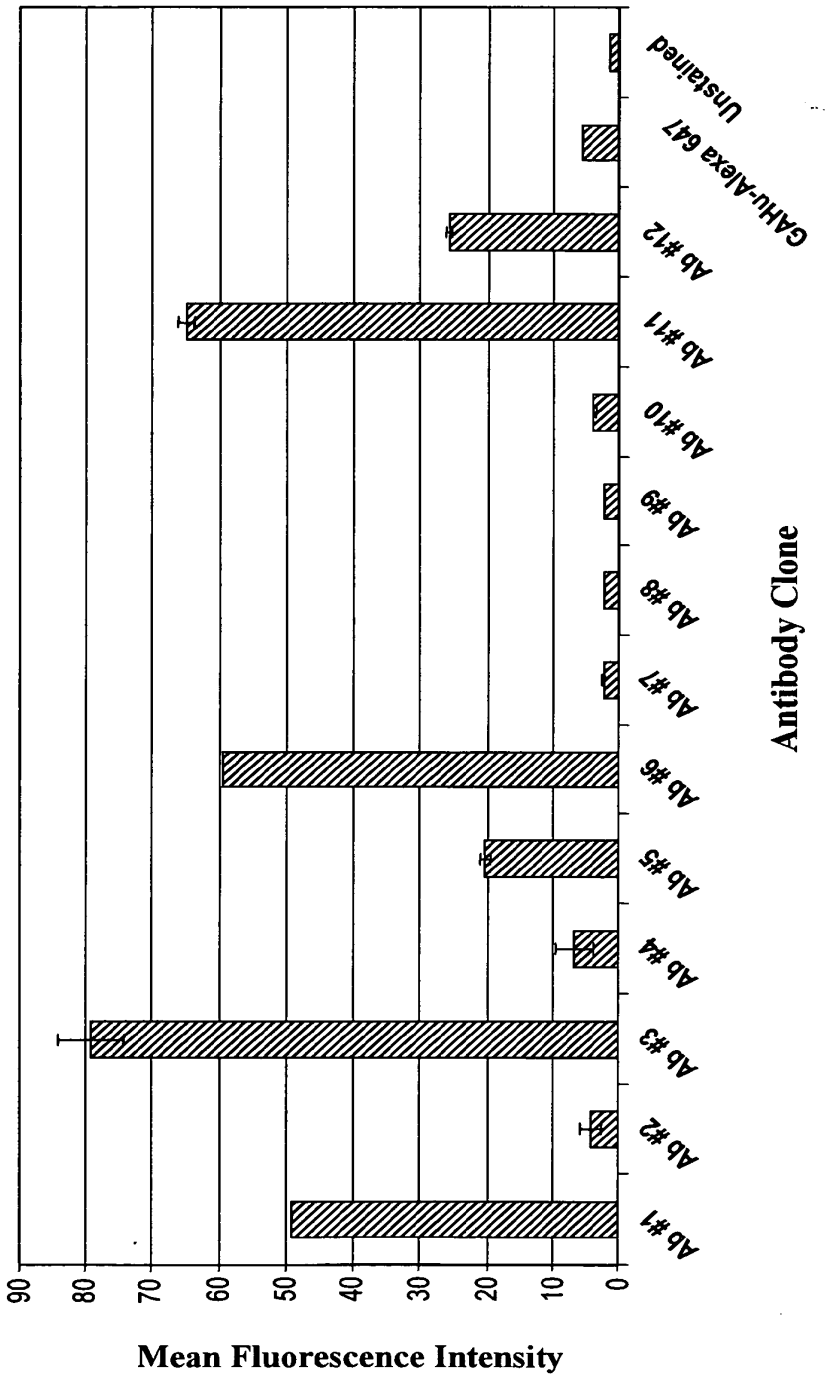

Unstained
GAHu-Alexa 647
Ab #12
Ab #11
Ab #10
Ab #9
Ab #8
Ab #7
Ab #6
Ab #5
Ab #4
Ab #3
Ab #2
Ab #1
0
10
20
30
40
50
60
70
80
90
Mean Fluorescence Intensity
Antibody Clone

Fig. 1A

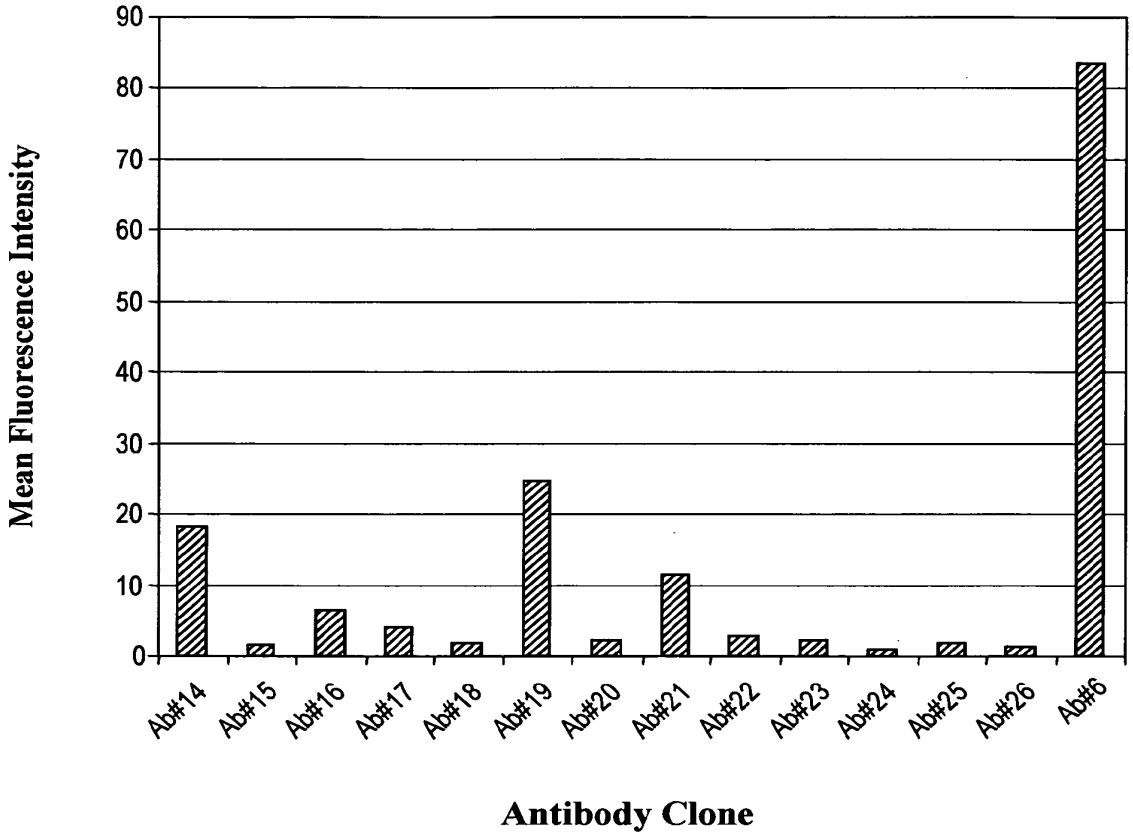

Mean Fluorescence Intensity
90
80
70
60
50
40
30
20
10
0
Ab#14
Ab#15
Ab#16
Ab#17
Ab#18
Ab#19
Ab#20
Ab#21
Ab#22
Ab#23
Ab#24
Ab#25
Ab#26
Ab#6
Antibody Clone

Fig. 1B

Binding of Antibodies to ErbB3 as Measured by SPR

$$K_D = k_d / k_a$$

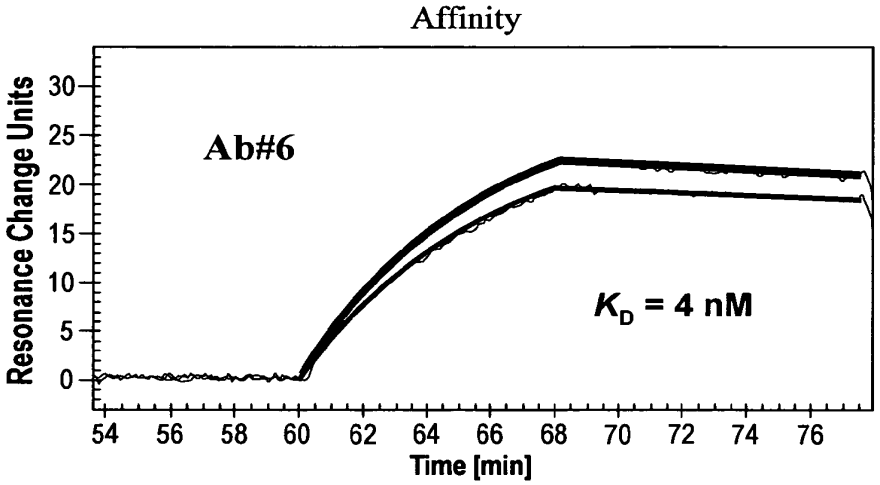


Fig. 2A

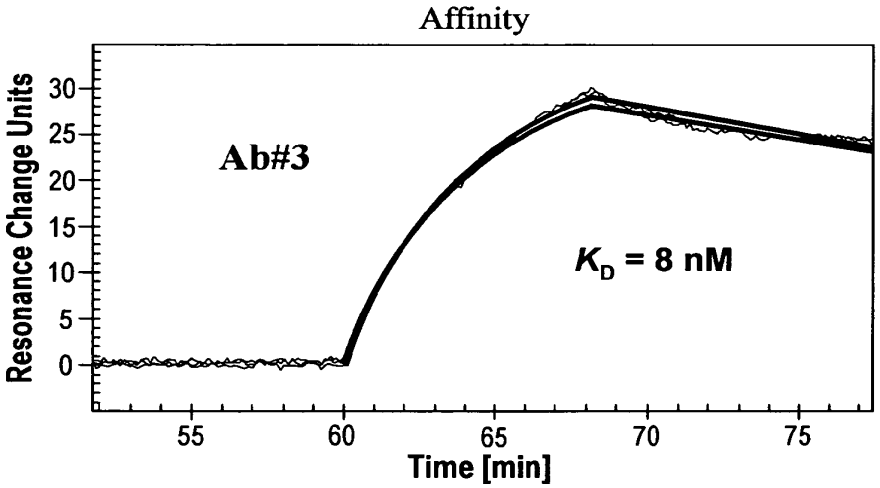


Fig. 2B

Cell Binding Assay for Antibodies to MALME-3M cells

$$Y=Bmax*X/K_D+X$$

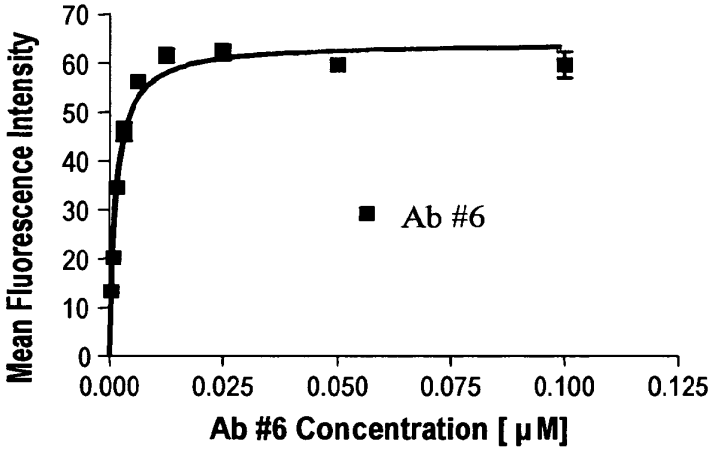


Fig. 2C

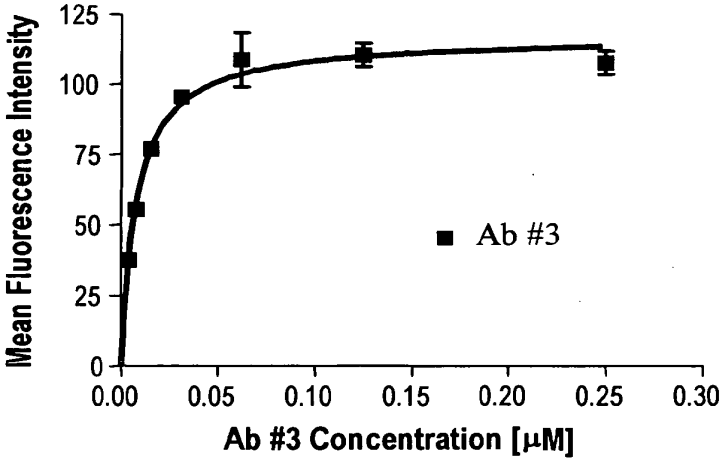


Fig. 2D

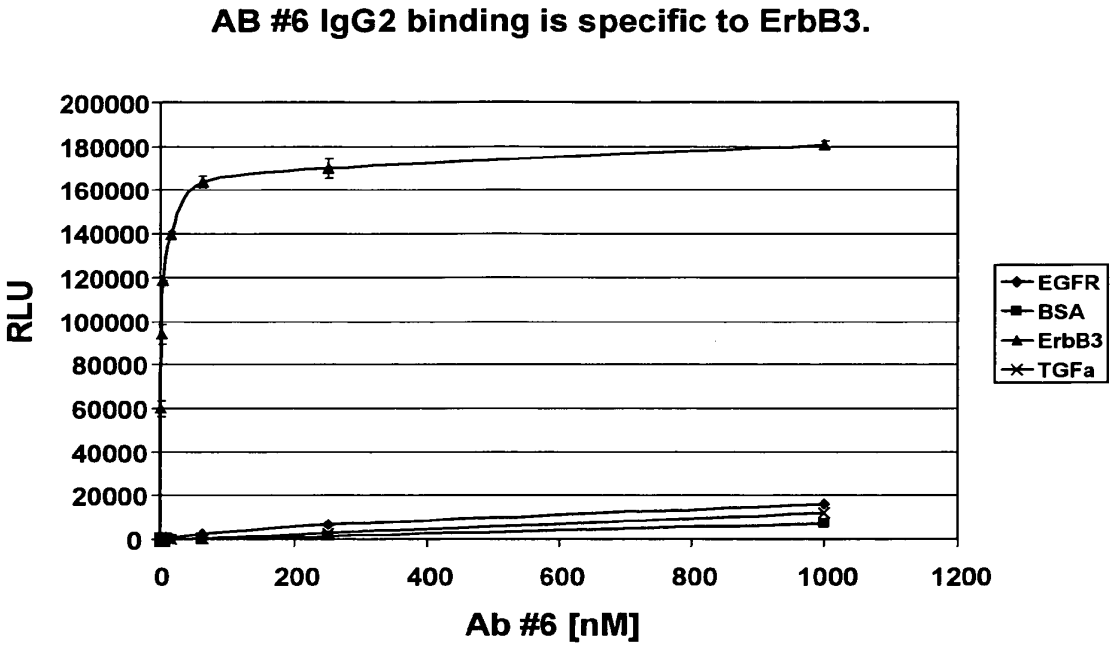

AB #6 IgG2 binding is specific to ErbB3.
RLU
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
0
200
400
600
800
1000
1200
Ab #6 [nM]
EGFR
BSA
ErbB3
TGFa

Fig. 3

Antibody#6 causes a decrease in total ErbB3 levels in MALME 3M melanoma cells *in vitro* measured by ELISA

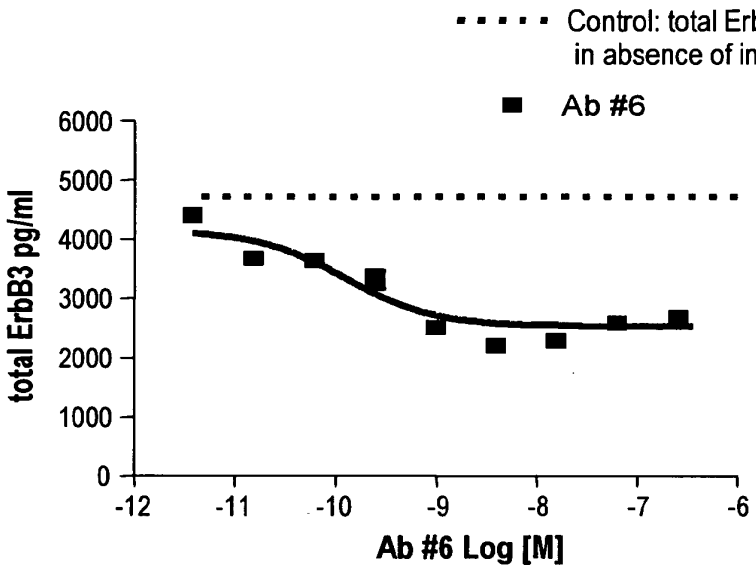


Fig. 4

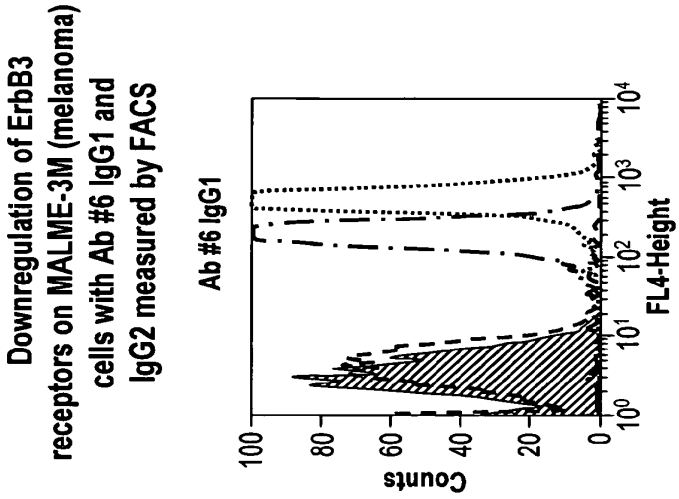

Downregulation of ErbB3 receptors on MALME-3M (melanoma) cells with Ab #6 IgG1 and IgG2 measured by FACS
Ab #6 IgG1
Counts
FL4-Height
Control
20nM_Ab #6_IgG2
Secondary Ab
20nM_Ab #6_IgG2

Fig. 5A

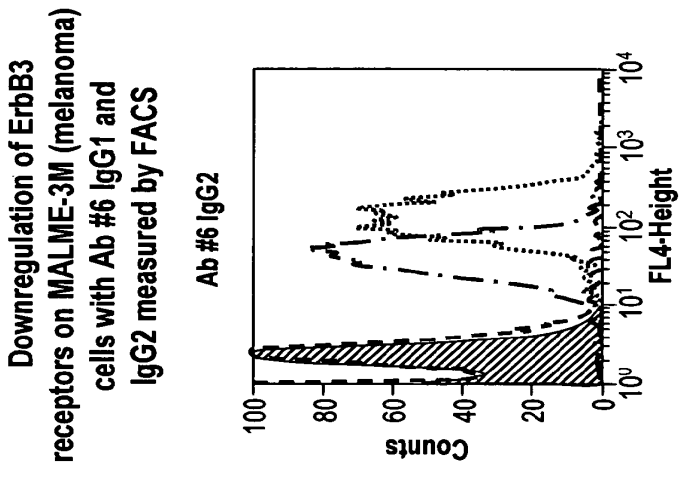

Downregulation of ErbB3 receptors on MALME-3M (melanoma) cells with Ab #6 IgG1 and IgG2 measured by FACS
Ab #6 IgG2
Counts
FL4-Height
Control
20nM_Ab #6_IgG2
Secondary Ab
20nM_Ab #6_IgG2

Fig. 5B

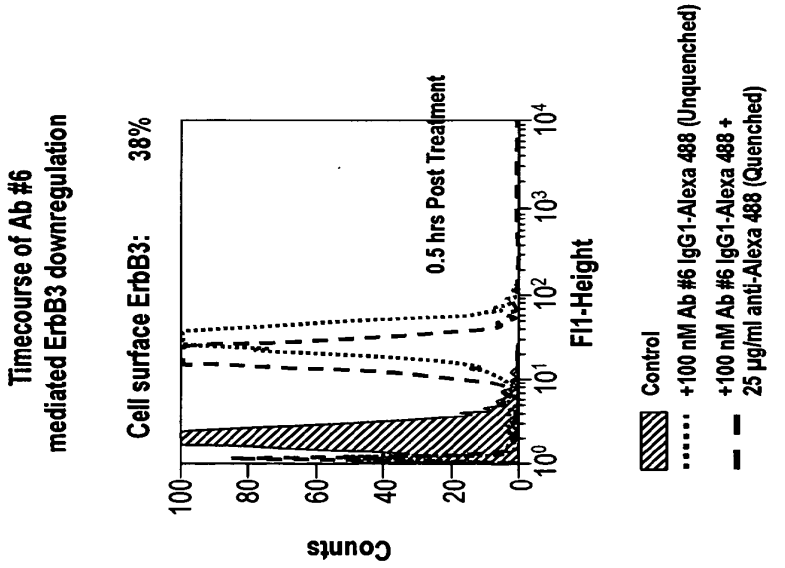

Timecourse of Ab #6
mediated ErbB3 downregulation
Cell surface ErbB3: 38%
0.5 hrs Post Treatment
Counts
Fl1-Height
Control
+100 nM Ab #6 IgG1-Alexa 488 (Unquenched)
+100 nM Ab #6 IgG1-Alexa 488 +
25 µg/ml anti-Alexa 488 (Quenched)

Fig. 6B

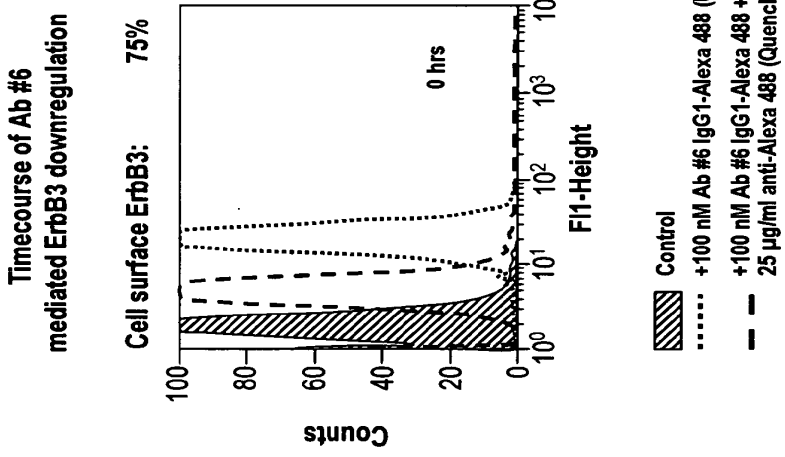

Timecourse of Ab #6
mediated ErbB3 downregulation
Cell surface ErbB3: 75%
0 hrs
Counts
Fl1-Height
Control
+100 nM Ab #6 IgG1-Alexa 488 (Unquenched)
+100 nM Ab #6 IgG1-Alexa 488 +
25 µg/ml anti-Alexa 488 (Quenched)

Fig. 6A

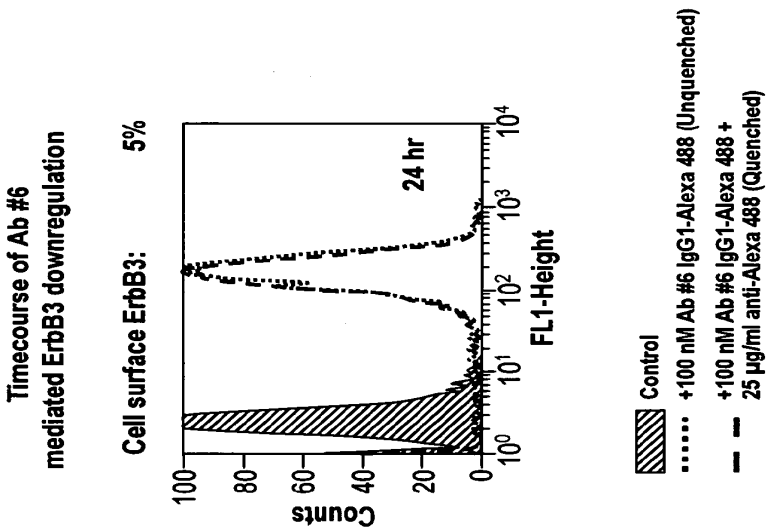

Timecourse of Ab #6
mediated ErbB3 downregulation
Cell surface ErbB3: 5%
24 hr
Counts
FL1-Height
Control
+100 nM Ab #6 IgG1-Alexa 488 (Unquenched)
+100 nM Ab #6 IgG1-Alexa 488 +
25 µg/ml anti-Alexa 488 (Quenched)

Fig. 6D

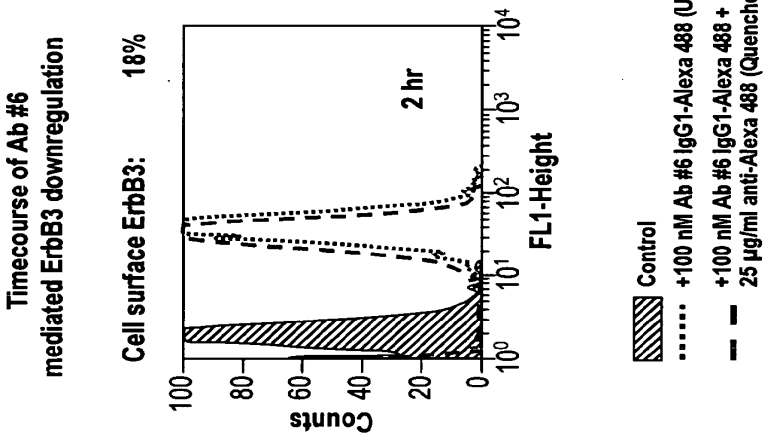

Timecourse of Ab #6
mediated ErbB3 downregulation
Cell surface ErbB3: 18%
2 hr
Counts
FL1-Height
Control
+100 nM Ab #6 IgG1-Alexa 488 (Unquenched)
+100 nM Ab #6 IgG1-Alexa 488 +
25 µg/ml anti-Alexa 488 (Quenched)

Fig. 6C

Ab #6 causes ErbB3 downregulation in melanoma cells in vivo

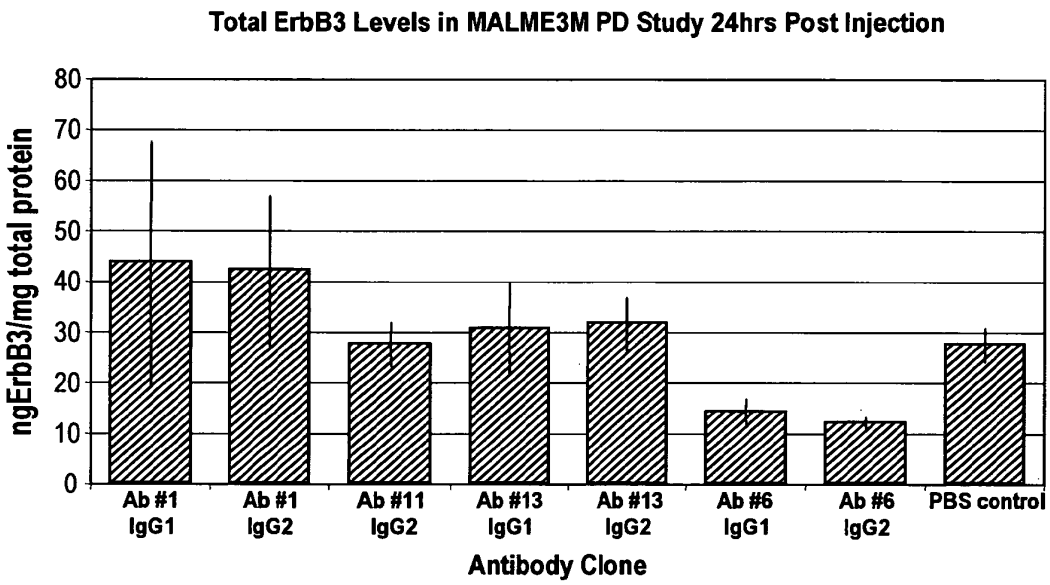


Fig. 7

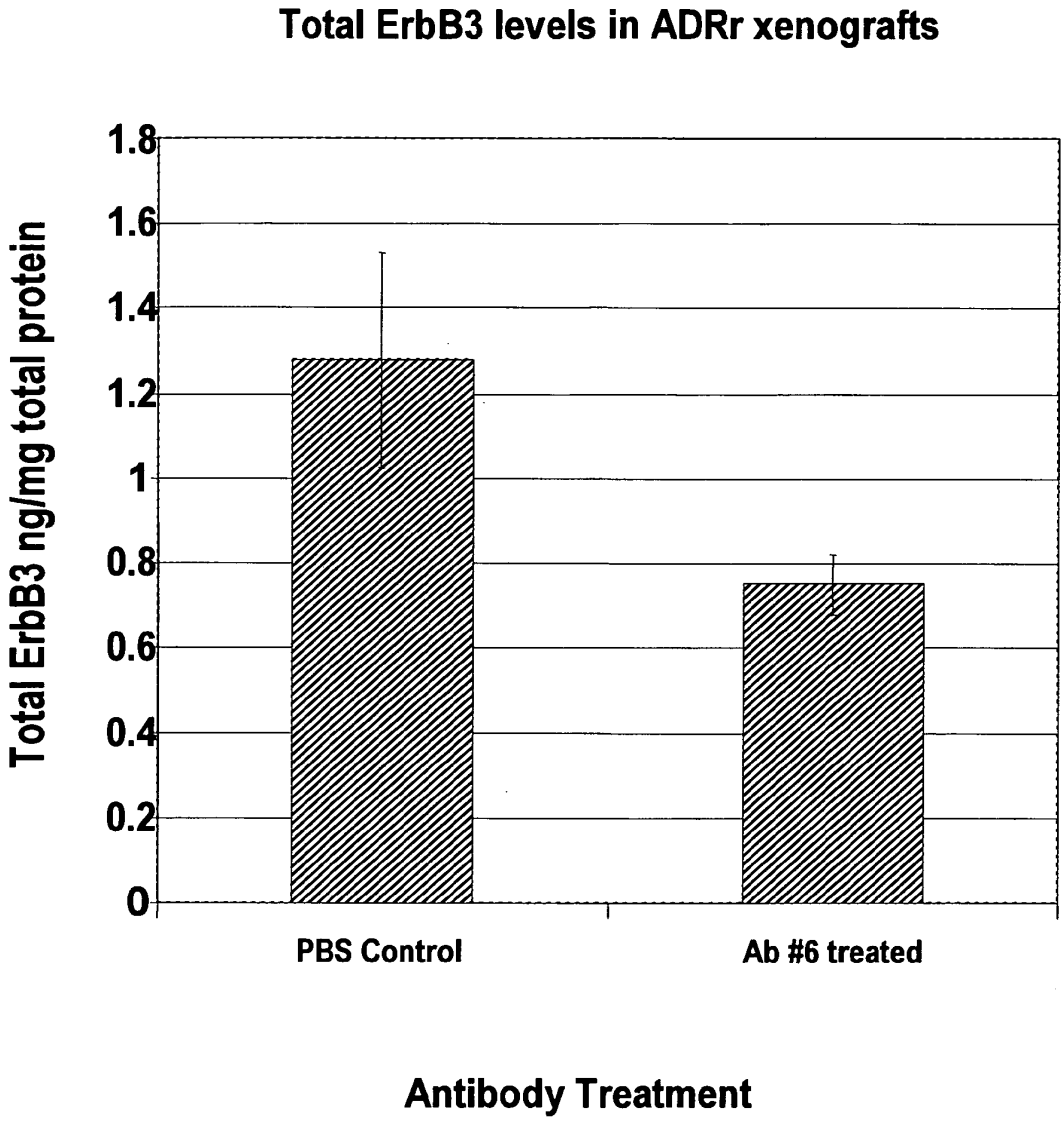

Total ErbB3 levels in ADRr xenografts
Total ErbB3 ng/mg total protein
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
PBS Control
Ab #6 treated
Antibody Treatment

Fig. 8

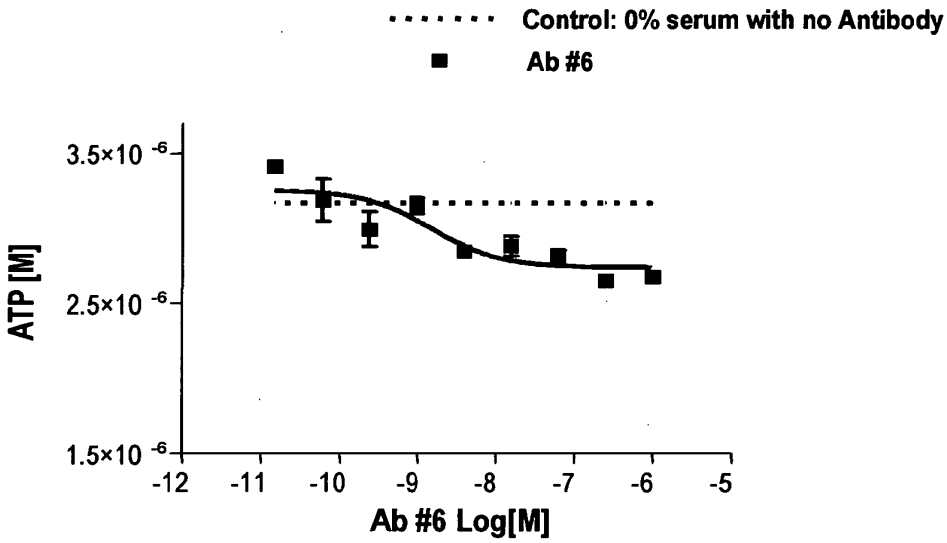

Ab #6 inhibits proliferation in MALME 3M melanoma cells measured by Cell Titer Glow assay
Control: 0% serum with no Antibody
Ab #6
ATP [M]
3.5×10 -6
2.5×10 -6
1.5×10 -6
-12
-11
-10
-9
-8
-7
-6
-5
Ab #6 Log[M]

Fig. 9

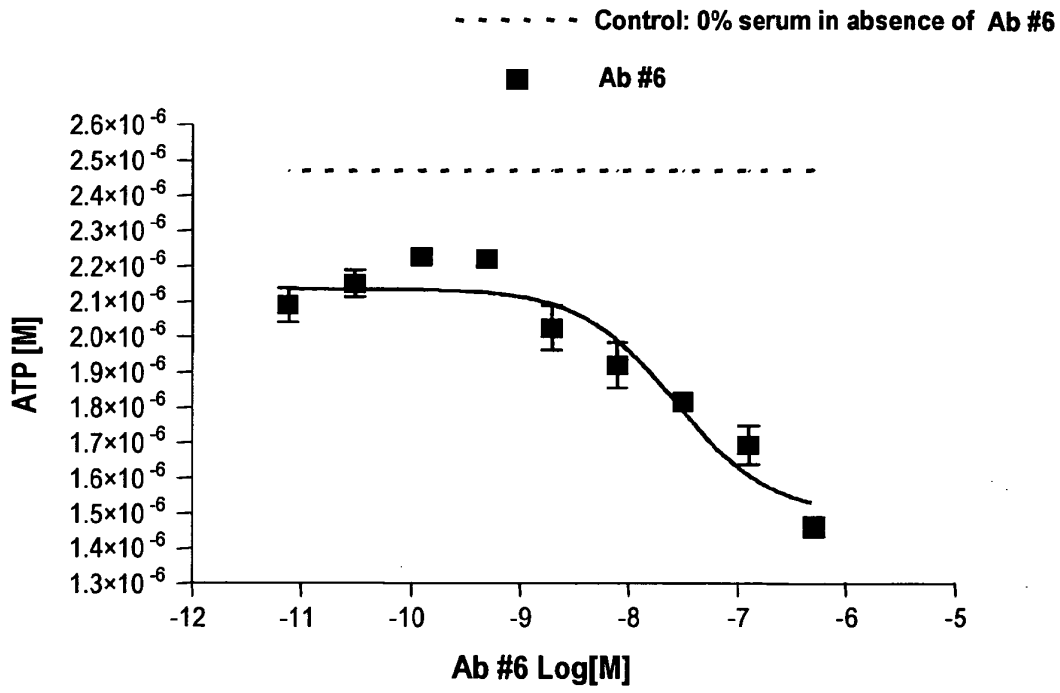

Ab #6 inhibits cell proliferation in ADRr (ovarian) cell line
Control: 0% serum in absence of Ab #6
Ab #6
ATP [M]
2.6×10-6
2.5×10-6
2.4×10-6
2.3×10-6
2.2×10-6
2.1×10-6
2.0×10-6
1.9×10-6
1.8×10-6
1.7×10-6
1.6×10-6
1.5×10-6
1.4×10-6
1.3×10-6
-12
-11
-10
-9
-8
-7
-6
-5
Ab #6 Log[M]

Fig. 10

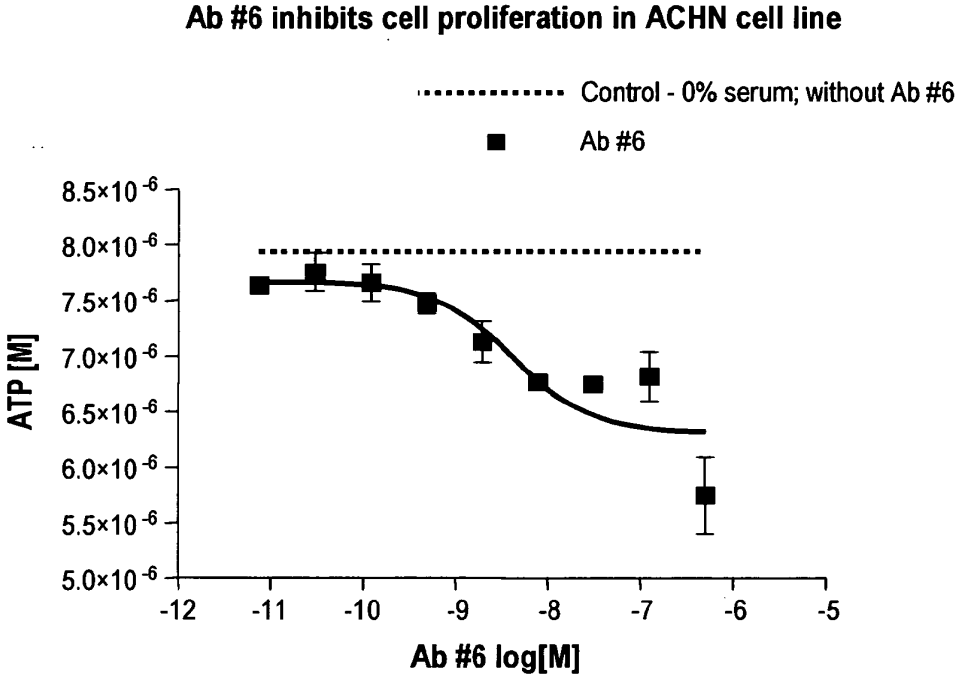

Ab #6 inhibits cell proliferation in ACHN cell line
Control - 0% serum; without Ab #6
Ab #6
ATP [M]
8.5×10-6
8.0×10-6
7.5×10-6
7.0×10-6
6.5×10-6
6.0×10-6
5.5×10-6
5.0×10-6
-12
-11
-10
-9
-8
-7
-6
-5
Ab #6 log[M]

Fig. 11

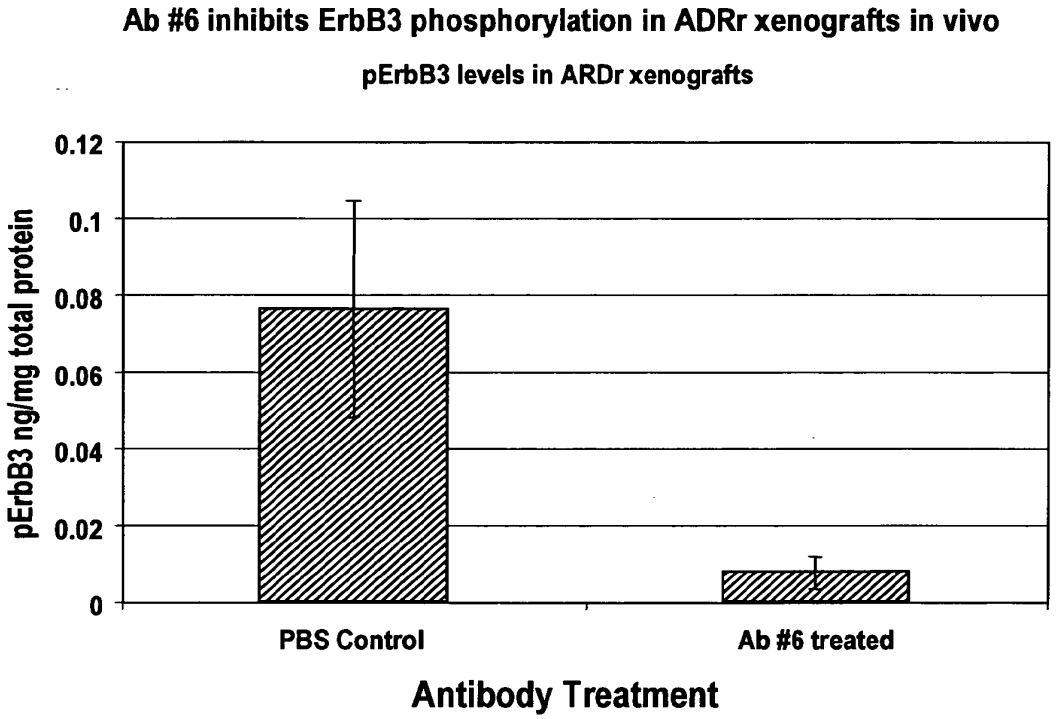

Ab #6 inhibits ErbB3 phosphorylation in ADRr xenografts in vivo
pErbB3 levels in ARDr xenografts
pErbB3 ng/mg total protein
0.12
0.1
0.08
0.06
0.04
0.02
0
PBS Control
Ab #6 treated
Antibody Treatment

Fig. 12

Ab #6 IgG2/BTC

IC50	8.37e-10 M
95% CI	5.32e-10 to 1.32e-9

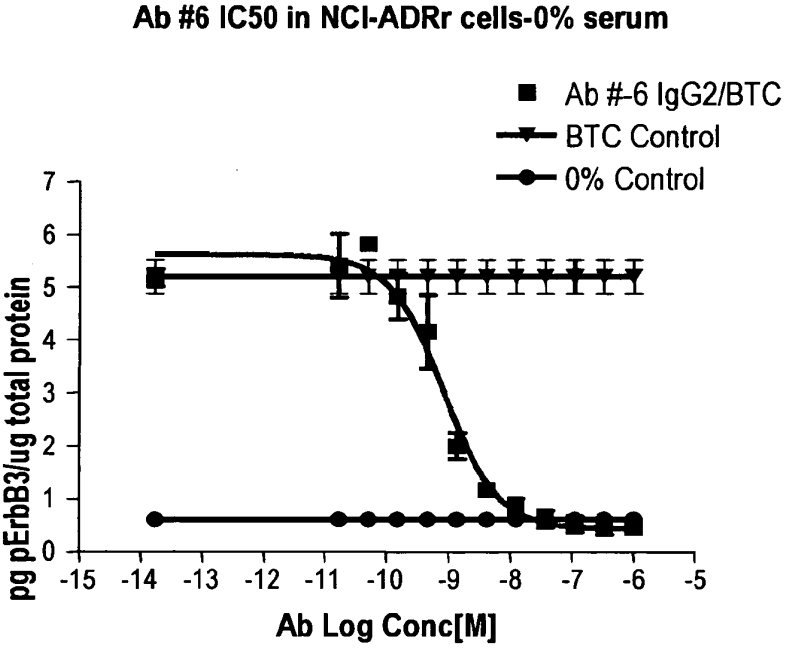


Fig. 13A

Ab #6 IgG2/BTC	
IC50	2.488e-009
95% CI	1.051e-009 to 5.889e-009

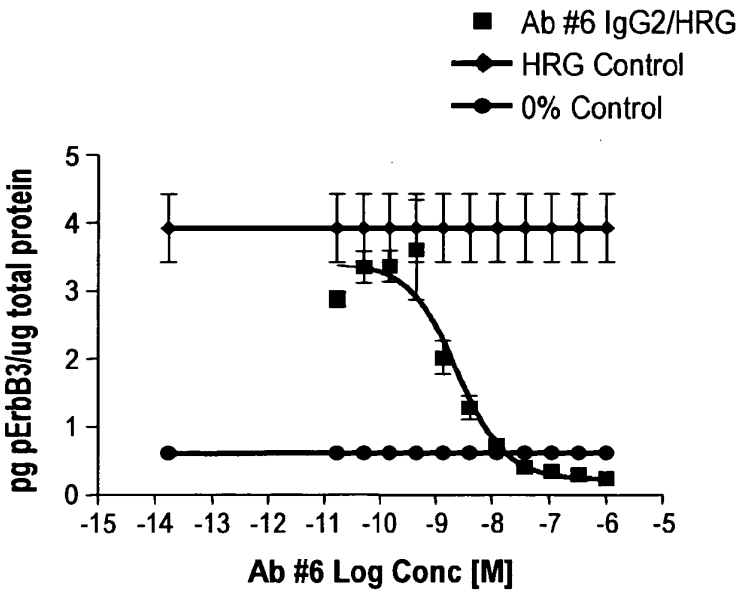


Fig. 13B

Ab #6 IgG2/TGFa	
IC50	4.901e-009
95% CI	1.965e-009 to 1.222e-008

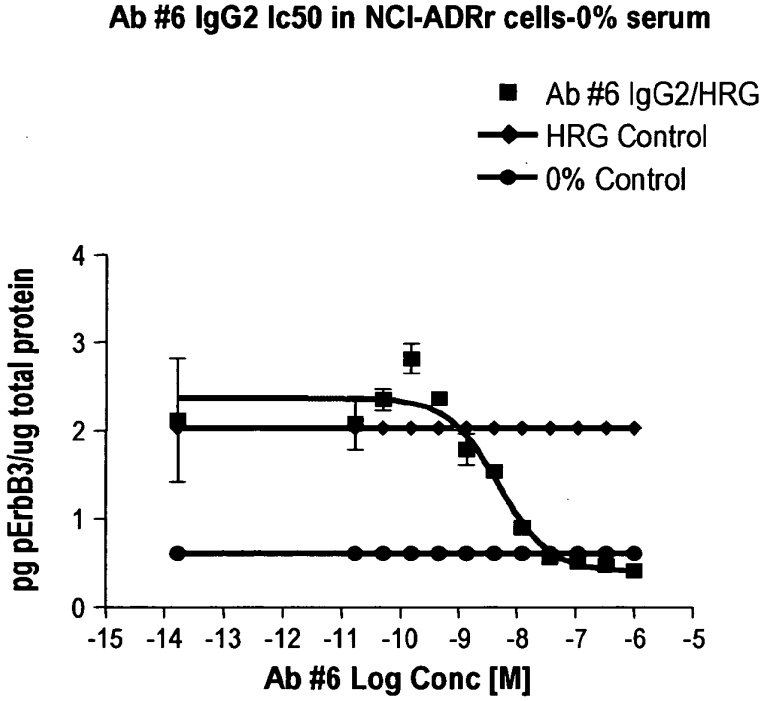


Fig. 13C

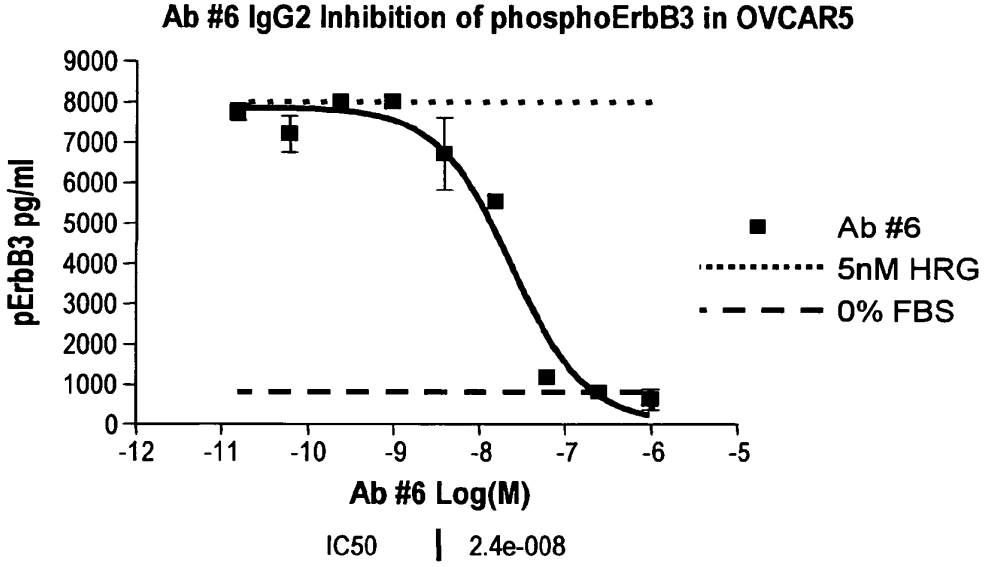


Fig. 14A

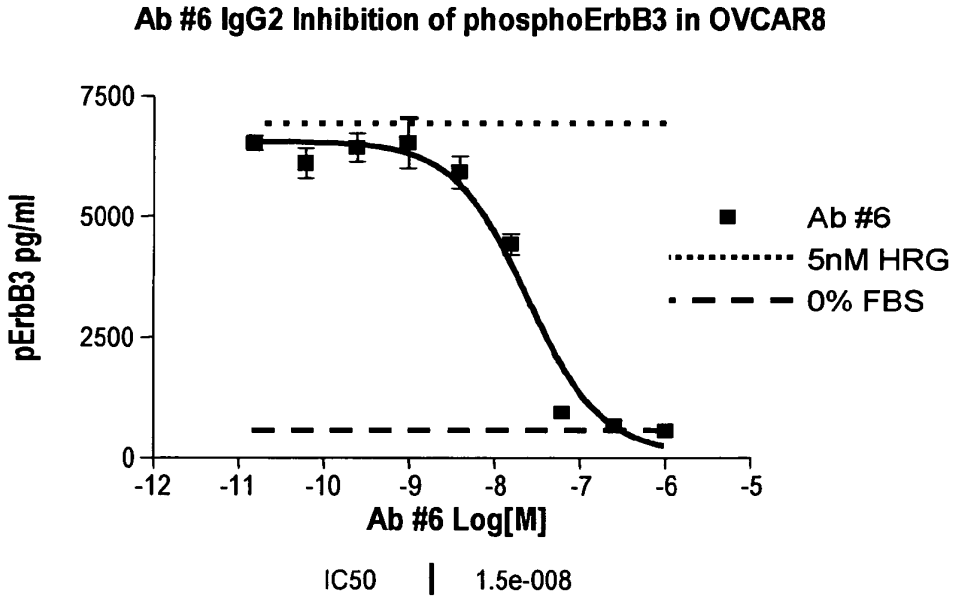


Fig. 14B

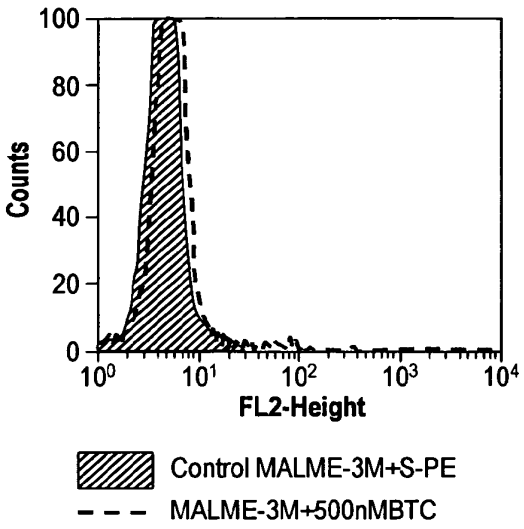


Fig. 15A

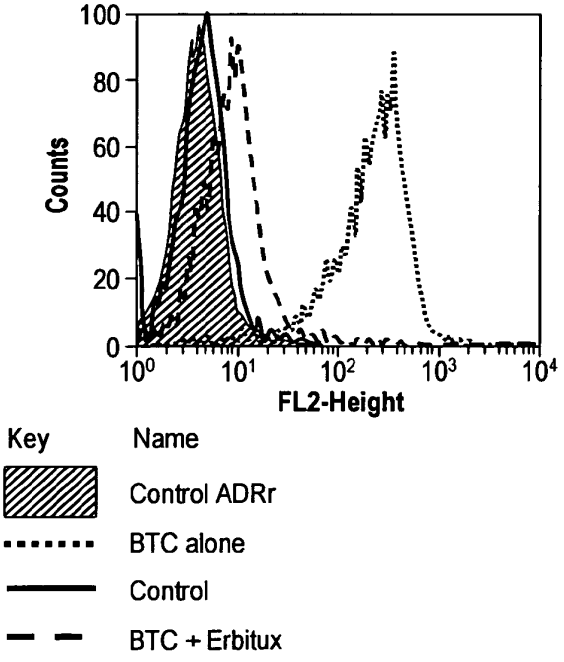


Fig. 15B

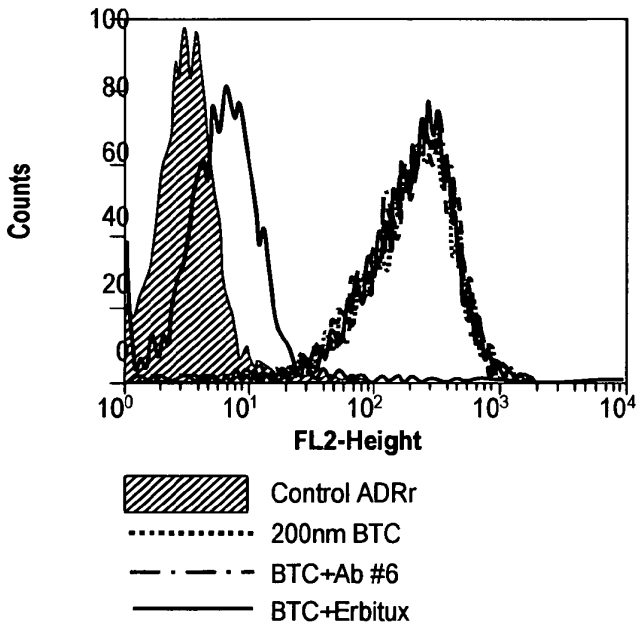

Blocking BTC Binding by Erbitux but not Ab #6
Counts
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
FL2-Height
Control ADRr
200nm BTC
BTC+Ab #6
BTC+Erbitux

Fig. 15C

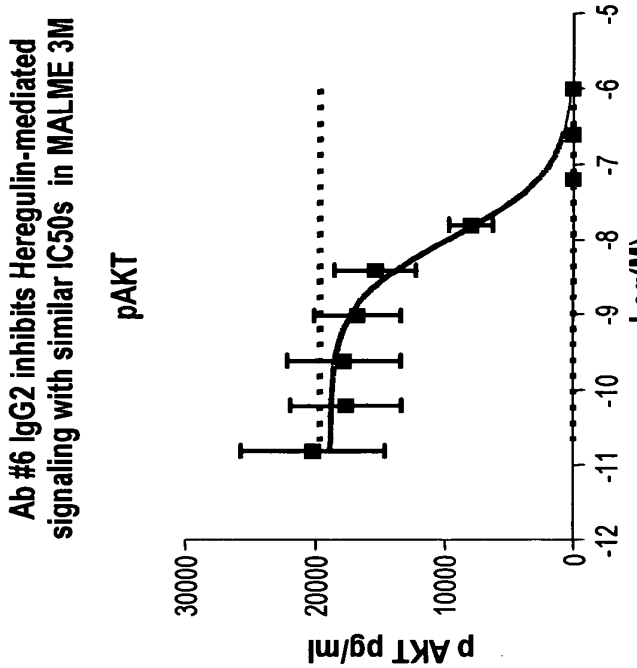


Fig. 16B

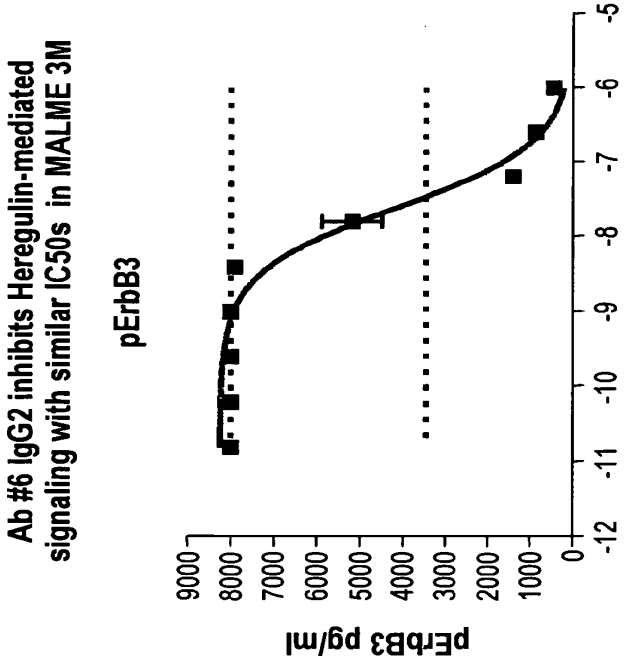


Fig. 16A

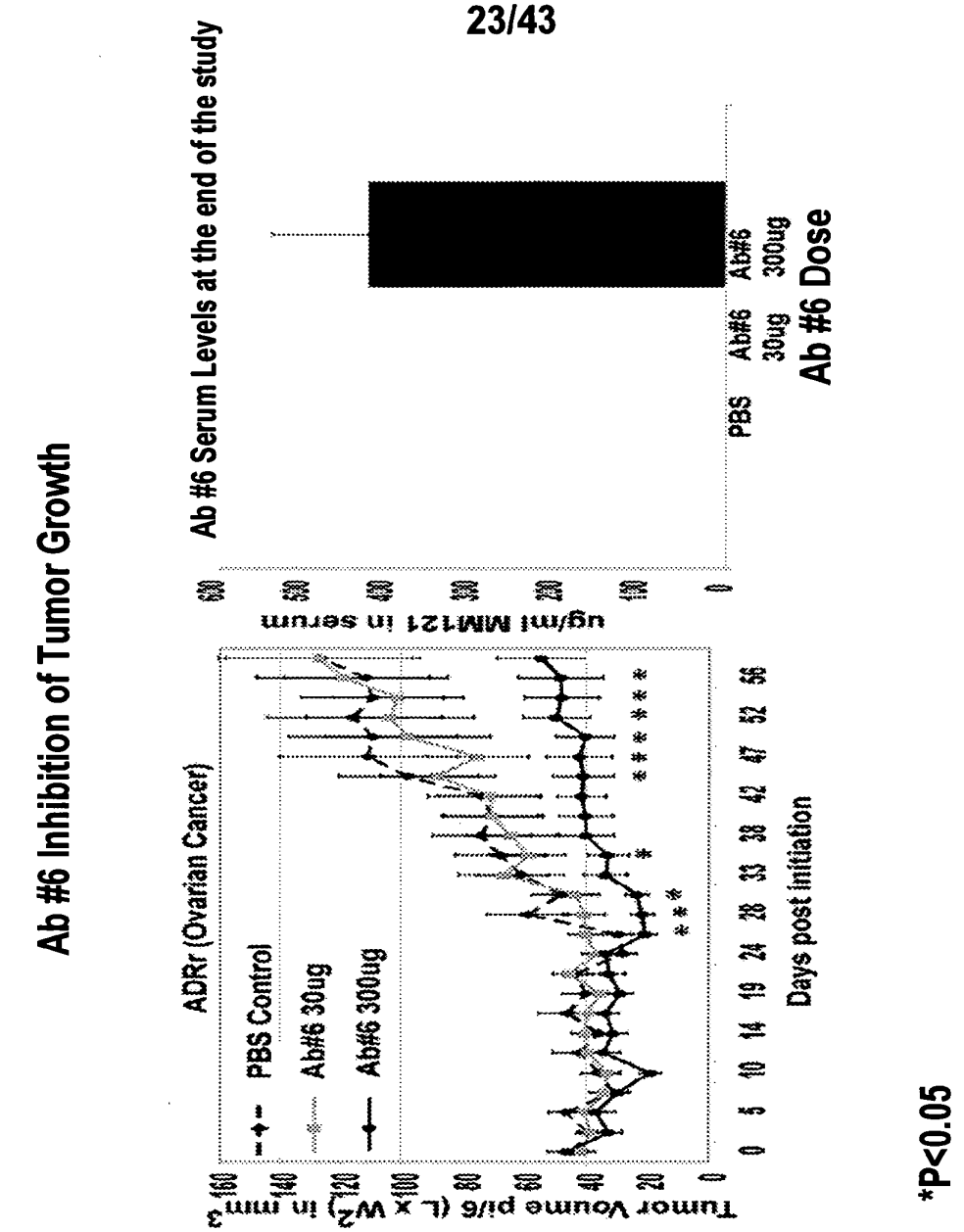

Ab #6 Inhibition of Tumor Growth
Ab #6 Serum Levels at the end of the study
ug/ml MM121 in serum
PBS
Ab#6 30ug
Ab#6 300ug
Ab #6 Dose
ADRr (Ovarian Cancer)
PBS Control
Ab#6 30ug
Ab#6 300ug
Tumor Volume p/6 (L x W2) in mm3
Days post initiation
*P<0.05

Fig. 17A

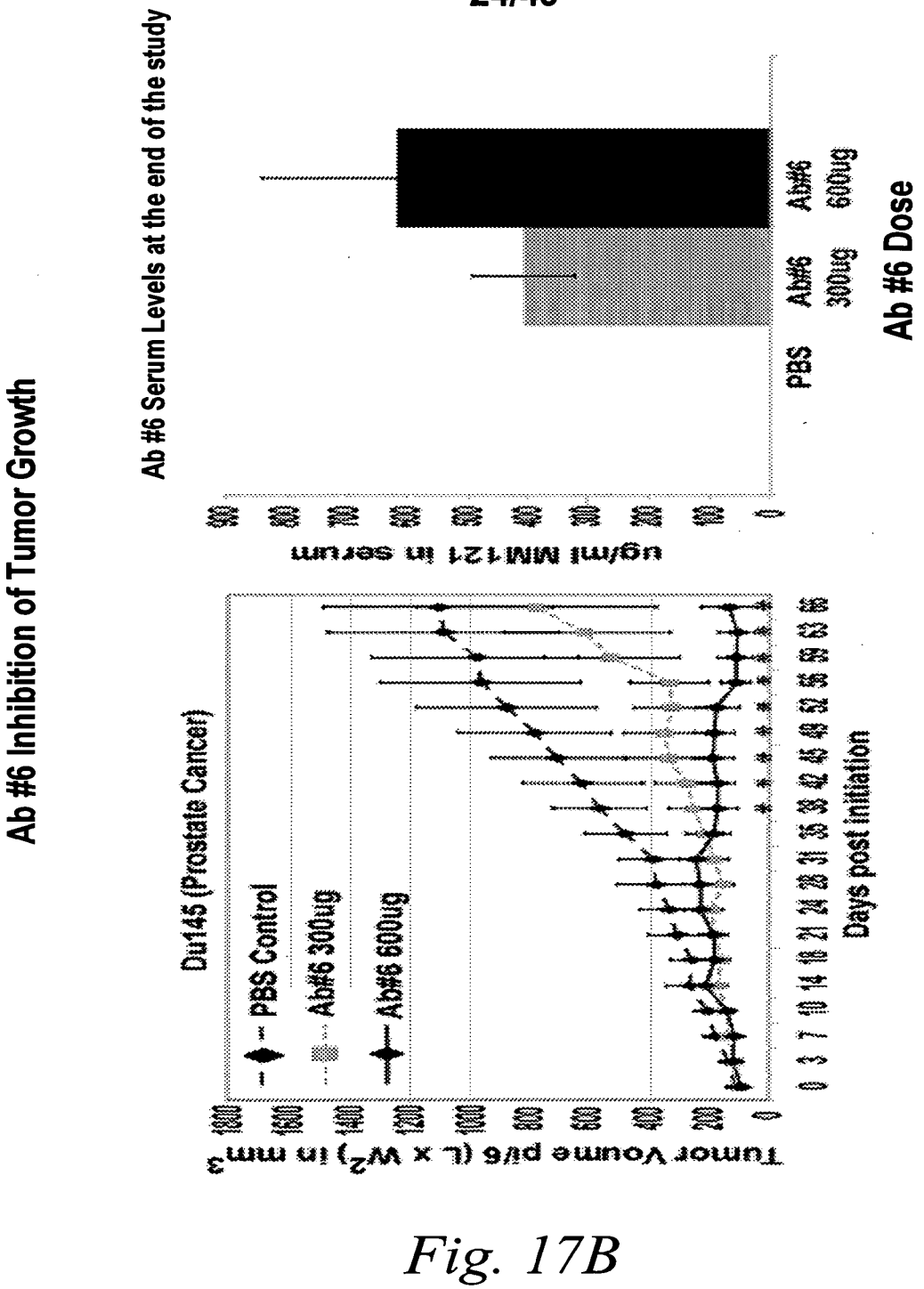

Ab #6 Inhibition of Tumor Growth
Du145 (Prostate Cancer)
PBS Control
Ab#6 300ug
Ab#6 600ug
Tumor Volume p/6 (L x W2) in mm3
Days post initiation
0 3 7 10 14 18 21 24 28 31 35 38 42 45 49 52 56 59 63 66
1800 1600 1400 1200 1000 800 600 400 200 0
Ab #6 Serum Levels at the end of the study
ug/ml MM121 in serum
1000 900 800 700 600 500 400 300 200 100 0
PBS
Ab#6 300ug
Ab#6 600ug
Ab #6 Dose
*P<0.05

Fig. 17B

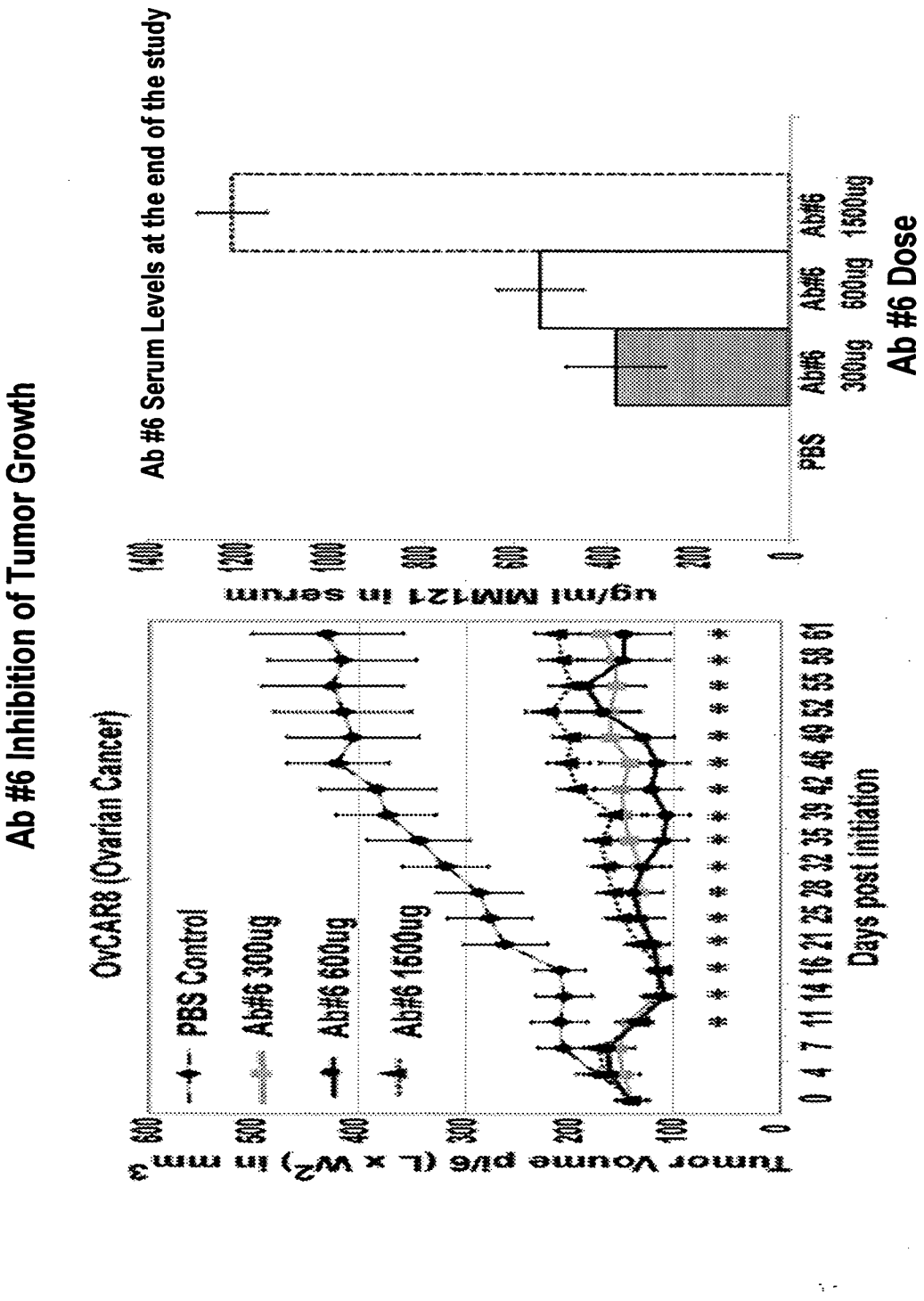

Ab #6 Inhibition of Tumor Growth
OvCAR8 (Ovarian Cancer)
PBS Control
Ab#6 300ug
Ab#6 600ug
Ab#6 1600ug
Tumor Voume p/6 (L x W2) in mm3
Days post initiation
0 4 7 11 14 16 21 25 28 32 35 39 42 46 49 52 55 58 61
0 100 200 300 400 500 600
* * * * * * * * * * * * * * * * * * *
ug/ml MM121 in serum
0 200 400 600 800 1000 1200 1400
Ab #6 Serum Levels at the end of the study
PBS
Ab#6 300ug
Ab#6 600ug
Ab#6 1600ug
Ab #6 Dose
*P<0.05

Fig. 17C

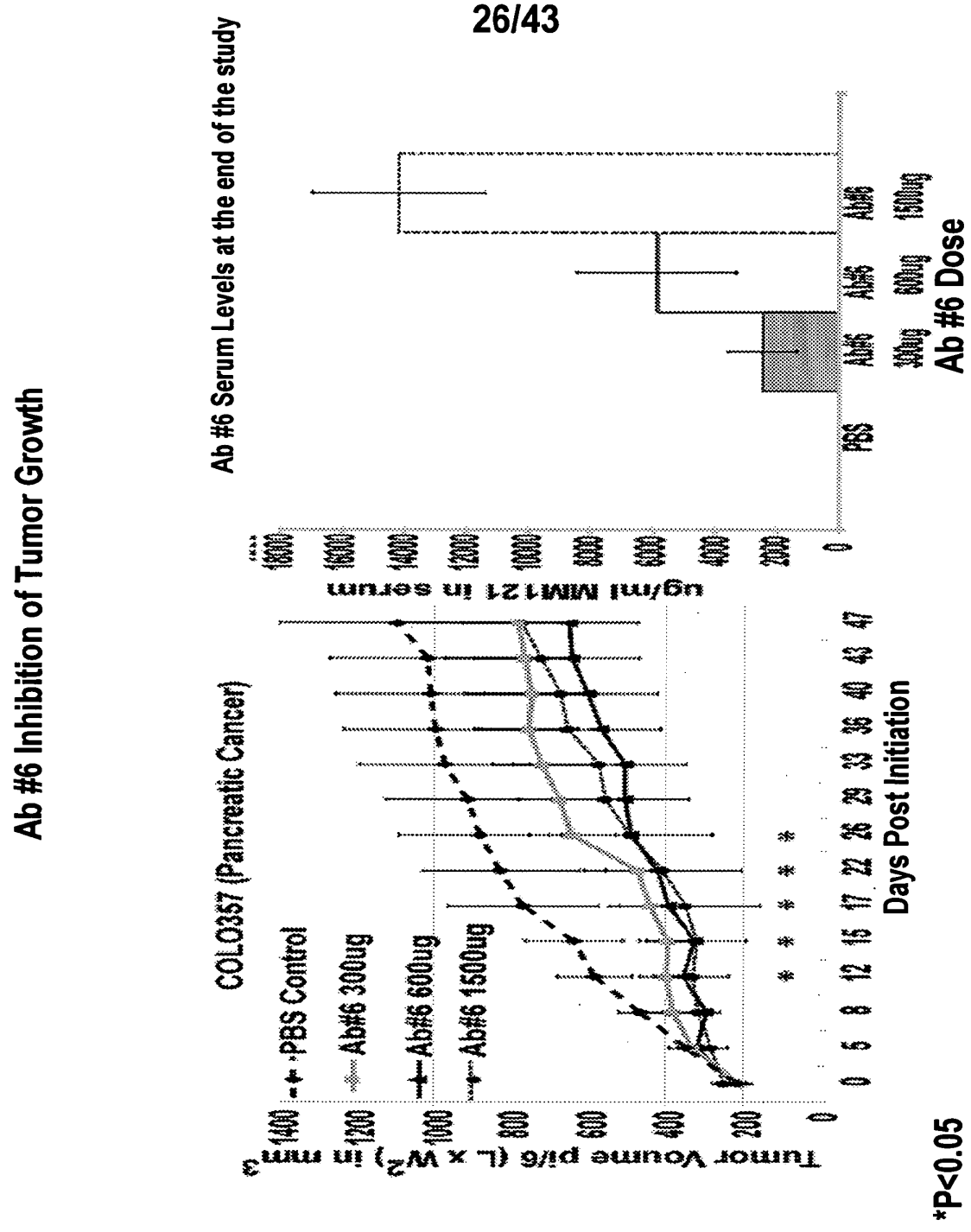

Ab #6 Inhibition of Tumor Growth
COLO357 (Pancreatic Cancer)
PBS Control
Ab#6 300ug
Ab#6 600ug
Ab#6 1500ug
Tumor Voume p/6 (L x W²) in mm³
Days Post Initiation
*P<0.05
Ab #6 Serum Levels at the end of the study
ug/ml MM121 in serum
PBS
Ab#6 300ug
Ab#6 600ug
Ab#6 1500ug
Ab #6 Dose

Fig. 17D

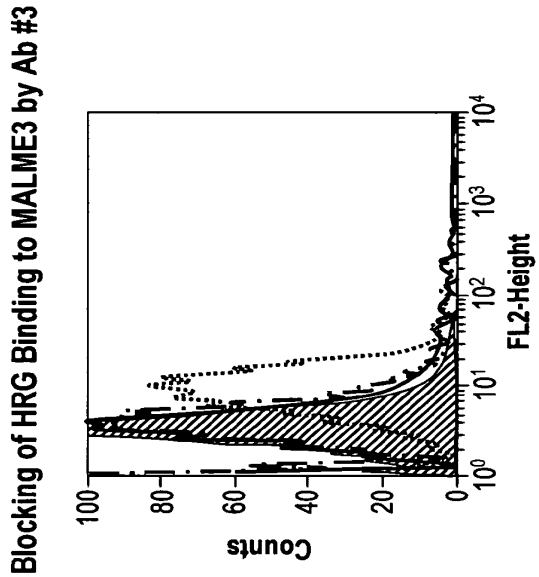


Fig. 18B

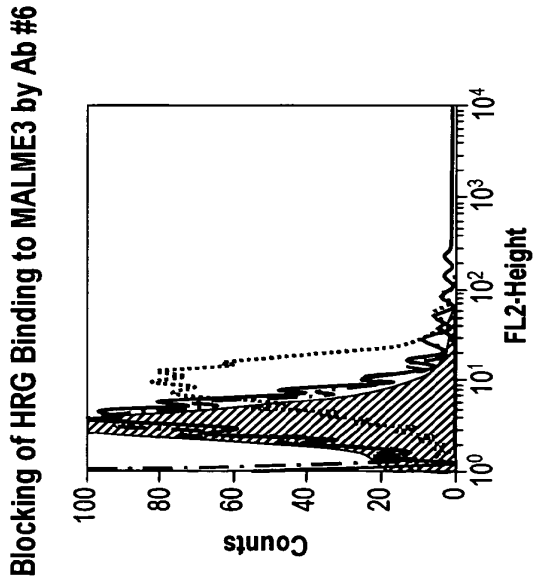


Fig. 18A

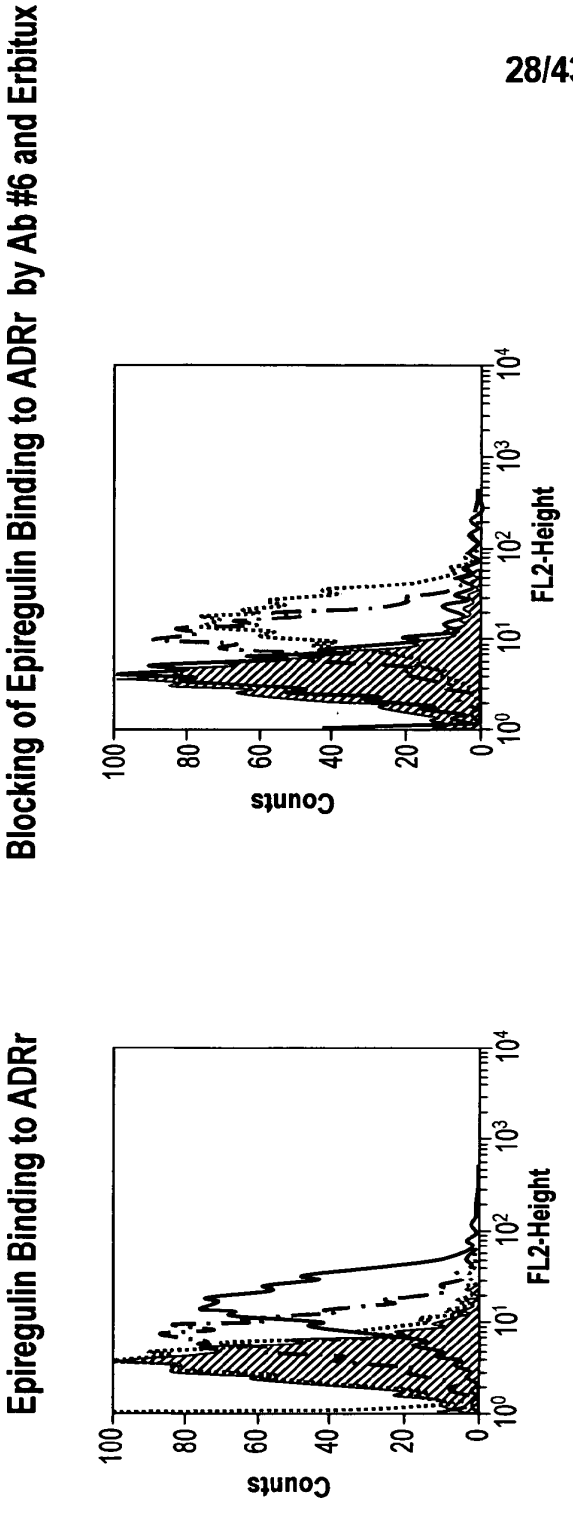


Fig. 19A

Fig. 19B

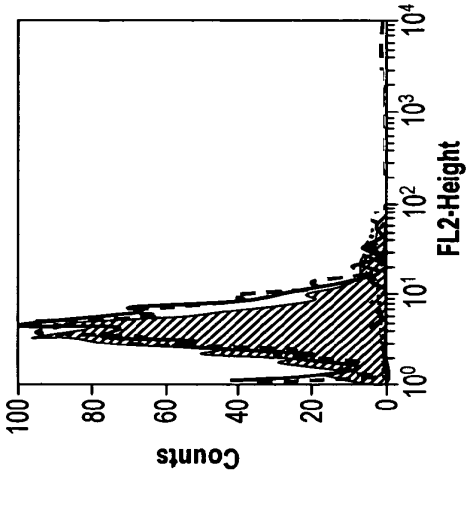


Fig. 20B

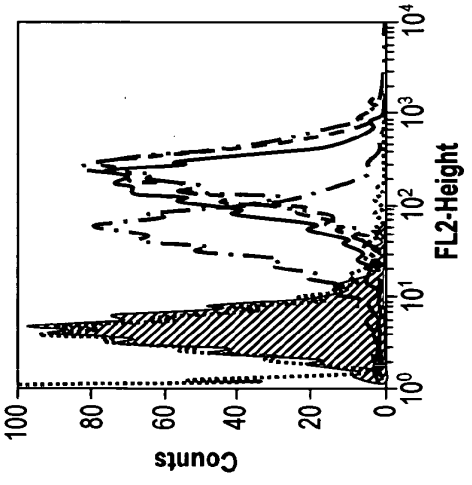


Fig. 20A

Ab # 6 VH amino acid sequence (SEQ ID NO:1)
EVQLLESGGGLVQPGGSLRLSCAASGFTFSHYVMAWVRQAPGKGLEWVSSISSS
GGWTLYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCTRGLKMATIFD
YWGQGTLVTVSS

Ab # 6 VL amino acid sequence (SEQ ID NO:2)
QSALTQPASVSGSPGQSITISCTGTSSDVGSYNVVSWYQQHPGKAPKLIIYEVSQR
PSGVSNRFSGSKSGNTASLTISGLQTEDEADYYCCSYAGSSIFVIFGGGTKVTVL

Ab # 3 VH amino acid sequence (SEQ ID NO:3)
EVQLLESGGGLVQPGGSLRLSCAASGFTFSAYNMRWVRQAPGKGLEWVSVIYPS
GGATRYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARGYYYYGMDV
WGQGTLVTVSS

Ab # 3 VL amino acid sequence (SEQ ID NO:4)
QSVLTQPPSASGTPGQRVTISCSGSDSNIGRNYIYWYQQFPGTAPKLLIYRNNQRP
SGVPDRISGSKSGTSASLAISGLRSEDEAEYHCGTWDDSLSGPVFGGGTKLTVL

Ab # 14 VH amino acid sequence (SEQ ID NO:5)
EVQLLESGGGLVQPGGSLRLSCAASGFTFSAYGMGWVRQAPGKGLEWVSYISPS
GGHTKYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKVLETGLLVD
AFDIWGQGTMVTVSS

Ab # 14 VL amino acid sequence (SEQ ID NO:6)
QYELTQPPSVSVYPGQTASITCSGDQLGSKFVSWYQQRPGQSPVLVMYKDKRRP
SEIPERFSGSNSGNTATLTISGTQAIDEADYYCQAWDSSTYVFGTGTKVTVL

Ab # 17 VH amino acid sequence (SEQ ID NO:35)
EVQLLESGGGLVQPGGSLRLSCAASGFTFSWYGMGWVRQAPGKGLEWVSYISPS
GGITVYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARLNYYYGLDV
WGQGTTVTVSS

Ab # 17 VL amino acid sequence (SEQ ID NO:36)
QDIQMTQSPSSLSASVGDRITITCQASQDIGDSLNWYQQKPGKAPRLLIYDASNLE
TGVPPRFSGSGSGTDFTFTFRSLQPEDIATYFCQQSANAPFTFGPGTKVDIK

Ab # 19 VH amino acid sequence (SEQ ID NO:37)
EVQLLESGGGLVQPGGSLRLSCAASGFTFSRYGMWWVRQAPGKGLEWVSYIGSS
GGPTYYVDSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAGGRGTPYYFDS
WGQGTLVTVSS

Ab # 19 VL amino acid sequence (SEQ ID NO:38)
QYELTQPASVSGSPGQSITISCTGTSSDIGRWNIVSWYQQHPGKAPKLMIYDVSNRPSGVSNRF
SGSKSGNTASLTISGLQAEDEADYYCSSYTSSSTWVFGGGTKLTVL

Fig. 21A

Ab # 6 VH CDR1 (SEQ ID NO:7)
HYVMA

Ab # 6 VH CDR2 (SEQ ID NO:8)
SISSSGGWTLYADSVKG

Ab # 6 VH CDR3 (SEQ ID NO:9)
GLKMATIFDY

Ab # 6 VL CDR1 (SEQ ID NO:10)
TGTSSDVGSYNVVS

Ab # 6 VL CDR2 (SEQ ID NO:11)
EVSQRPS

Ab # 6 VL CDR3 (SEQ ID NO:12)
CSYAGSSIFVI

Ab # 3 VH CDR1 (SEQ ID NO:13)
AYNMR

Ab # 3 VH CDR2 (SEQ ID NO:14)
VIYPSGGATRYADSVKG

Ab # 3 VH CDR3 (SEQ ID NO:15)
GYYYYGMDV

Ab # 3 VL CDR1 (SEQ ID NO:16)
SGSDSNIGRNYIY

Ab # 3 VL CDR2 (SEQ ID NO:17)
RNNQRPS

Ab # 3 VL CDR3 (SEQ ID NO:18)
GTWDDSLSGPV

Fig. 21B

Ab # 14 VH CDR1 (SEQ ID NO:19)
AYGMG

Ab # 14 VH CDR2 (SEQ ID NO:20)
YISPSGGHTKYADSVKG

Ab # 14 VH CDR3 (SEQ ID NO:21)
VLETGLLVDAFDI

Ab # 14 VL CDR1 (SEQ ID NO:22)
SGDQLGSKFVS

Ab # 14 VL CDR2 (SEQ ID NO:23)
YKDKRRPS

Ab # 14 VL CDR3 (SEQ ID NO:24)
QAWDSSTYV

Ab # 17 VH CDR1 (SEQ ID NO:39)
WYGMG

Ab # 17 VH CDR2 (SEQ ID NO:40)
YISPSGGITVYADSVKG

Ab # 17 VH CDR3 (SEQ ID NO:41)
LNYYYGLDV

Ab # 17 VL CDR1 (SEQ ID NO:42)
QASQDIGDSLN

Ab # 17 VL CDR2 (SEQ ID NO:43)
DASNLET

Ab # 17 VL CDR3 (SEQ ID NO:44)
QQSANAPFT

Ab # 19 VH CDR1 (SEQ ID NO:45)
RYGMW

Ab # 19 VH CDR2 (SEQ ID NO:46)
YIGSSGGPTYYVDSVKG

Ab # 19 VH CDR3 (SEQ ID NO:47)
GRGTPYYFDS

Ab # 19 VL CDR1 (SEQ ID NO:48
TGTSSDIGRWNIVS

Ab # 19 VL CDR2 (SEQ ID NO:49)
DVSNRPS

Ab # 19 VL CDR3 (SEQ ID NO:50)
SSYTSSSTWV

Fig. 21C

Ab # 6 VH Codon Optimized Nucleic Acid Sequence (SEQ ID NO:25)

gaggtgcagctgctggagagcggcggagggctggtccagccaggcggcagcctgaggctgtcctgcgccgccagcggcttcac
cttcagccactacgtgatggcctgggtgcggcaggccccaggcaagggcctggaatgggtgtccagcatcagcagcagcggcgg
ctggaccctgtacgccgacagcgtgaagggcaggttcaccatcagcagggacaacagcaagaacaccctgtacctgcagatgaac
agcctgagggccgaggacaccgccgtgtactactgcaccaggggcctgaagatggccaccatcttcgactactggggccagggc
accctggtgaccgtgagcagc

Ab # 6 VL Codon Optimized Nucleic Acid Sequence (SEQ ID NO:26)

cagtccgccctgacccagcccgccagcgtgagcggcagcccaggccagagcatcaccatcagctgcaccggcaccagcagcga
cgtgggcagctacaacgtggtgtcctggtatcagcagcaccccggcaaggcccccaagctgatcatctacgaggtgtcccagagg
cccagcggcgtgagcaacaggttcagcggcagcaagagcggcaacaccgccagcctgaccatcagcggcctgcagaccgagg
acgaggccgactactactgctgcagctacgccggcagcagcatcttcgtgatcttcggcggagggaccaaggtgaccgtccta

Ab # 3 VH Codon Optimized Nucleic Acid Sequence (SEQ ID NO:27)

gaggtgcagctgctggaaagcggcggagggctggtgcagccaggcggcagcctgaggctgtcctgcgccgccagcggcttcac
cttcagcgcctacaacatgagatgggtgcggcaggccccaggcaagggcctggaatgggtgtccgtgatctaccccagcggcgg
agccaccagatacgccgacagcgtgaagggcaggttcaccatcagcagggacaacagcaagaacaccctgtacctgcagatgaa
cagcctgagggccgaggacaccgccgtgtactactgcgccaggggctactactactacggcatggacgtgtggggccagggcac
cctggtgaccgtgagcagc

Ab # 3 VL Codon Optimized Nucleic Acid Sequence (SEQ ID NO:28)

cagagcgtgctgacccagcccccaagcgccagcggcaccccaggccagagggtgaccatcagctgcagcggcagcgacagca
acatcggcaggaactacatctactggtatcagcagttccccggcaccgcccccaagctgctgatctacaggaacaaccagaggccc
agcggcgtgcccgacaggatcagcggcagcaagagcggcaccagcgccagcctggccatcagcggcctgagaagcgaggac
gaggccgagtaccactgcggcacctgggacgacagcctgagcggcccagtgttcggcggagggaccaagctgaccgtccta

Fig. 22A

Ab # 14 VH Nucleic Acid Sequence (SEQ ID NO:29)

gaagttcaattgttagagtctggtggcggtcttgttcagcctggtggttctttacgtctttcttgcgctgcttccggattcactttctct
gcttacggtatgggttgggttcgccaagctcctggtaaaggtttggagtgggtttcttatatctctccttctggtggccatactaag
tatgctgactccgttaaaggtcgcttcactatctctagagacaactctaagaatactctctacttgcagatgaacagcttaagggc
tgaggacacggccgtatattactgtgcgaaagtactggaaactggcttattggttgatgcttttgatatctggggccaagggaca
atggtcaccgtctcaagc

Ab # 14 VL Nucleic Acid Sequence (SEQ ID NO:30)

cagtacgaattgactcagccaccctcagtgtccgtgtacccaggacagacagccagcatcacctgctctggagatcaattggg
gagtaaatttgtttcctggtatcagcagaggccaggccagtcccctgtgttggtcatgtataaagataaaaggcggccgtcaga
gatccctgagcgattctctggctccaactctgggaacacagccactctgaccatcagcgggacccaggctatagatgaggct
gactattattgtcaggcgtgggacagcagcacttatgtcttcggcactgggaccaaggtcaccgtccta

Ab # 6 VH Pre-Optimization Nucleic Acid Sequence (SEQ ID NO:31)

gaagttcaattgttagagtctggtggcggtcttgttcagcctggtggttctttacgtctttcttgcgctgcttccggattcactttctct
cattacgttatggcttgggttcgccaagctcctggtaaaggtttggagtgggtttcttctatctcttcttctggtggctggactctttat
gctgactccgttaaaggtcgcttcactatctctagagacaactctaagaatactctctacttgcagatgaacagcttaagggctg
aggacacagccgtgtattactgtactagaggtctcaagatggctacaatttttgactactggggccagggcaccctggtcaccg
tctcaagc

Ab # 6 VL Pre-Optimization Nucleic Acid Sequence (SEQ ID NO:32)

cagagcgctttgactcagcctgcctccgtgtctgggtctcctggacagtcgatcaccatctcctgcactggaaccagcagtgat
gttgggagttataatgttgtctcctggtaccaacaacacccaggcaaagcccccaaactcatcatttatgaggtcagtcagcgg
ccctcaggggtttctaatcgcttctctggctccaagtctggcaacacggcctccctgacaatctctgggctccagactgaggac
gaggctgattattactgctgctcatatgcaggtagtagtattttcgtgatattcggcggagggaccaaggtgaccgtccta

Ab # 3 VH Pre-Optimization Nucleic Acid Sequence (SEQ ID NO:33)

gaagttcaattgttagagtctggtggcggtcttgttcagcctggtggttctttacgtctttcttgcgctgcttccggattcactttctct
gcttacaatatgcgttgggttcgccaagctcctggtaaaggtttggagtgggtttctgttatctatccttctggtggcgctactcgtt
atgctgactccgttaaaggtcgcttcactatctctagagacaactctaagaatactctctacttgcagatgaacagcttaagggct
gaggacacggccgtgtattactgtgcgagagggtactactactacggtatggacgtctggggccaaggcaccctggtcaccg
tctcaagc

Ab # 3 VL Pre-Optimization Nucleic Acid Sequence (SEQ ID NO:34)

cagagcgtcttgactcagccaccctcagcgtctgggacccccgggcagagggtcaccatctcgtgttctggaagcgactcca
acatcggaagaaattatatatattggtaccagcaattcccaggaacggcccccaagctcctcatctataggaataatcagcggc
cctcaggggtccctgaccgaatctctggctccaagtctggcacctcagcctccctggccatcagtgggctccggtccgaggat
gaggctgagtatcactgtggaacatgggatgacagcctgagtggtccggtattcggcggagggactaagctgaccgtccta

Fig. 22B

- **Ab #6 VLb**:
- QSALTQPASVSGSPGQSITISCTGTSSDVGSYNVVSWYQQHPGKAPK LMIYEVSKRPSGVSNRFSGSKSGNTASLTISGLQAEDEADYYCCSYA GSSIFVIFGGGTKVTVL (SEQ ID NO:51)

- **Ab #17 VK1b:**
- QDIQMTQSPSSLSASVGDRITITCQASQDIGDSLNWYQQKPGKAPRLL IYDASNLETGVPPRFSGSGSGTDFTFTFRSLQPEDIATYFCQQSANAP FTFGPGTKVDIR (SEQ ID NO:52)

- **Ab #19 VL2b:**
- QYELTQPASVSGSPGQSITISCTGTSSDIGRWNIVSWYQQHPGKAPK LMIYDVSNRPSGVSNRFSGSKSGNTASLTISGLQAEDEADYYCSSYTS SSTVVFGGGTKLTVL (SEQ ID NO:53)

Fig. 23

OnCusp
Ex. 1002

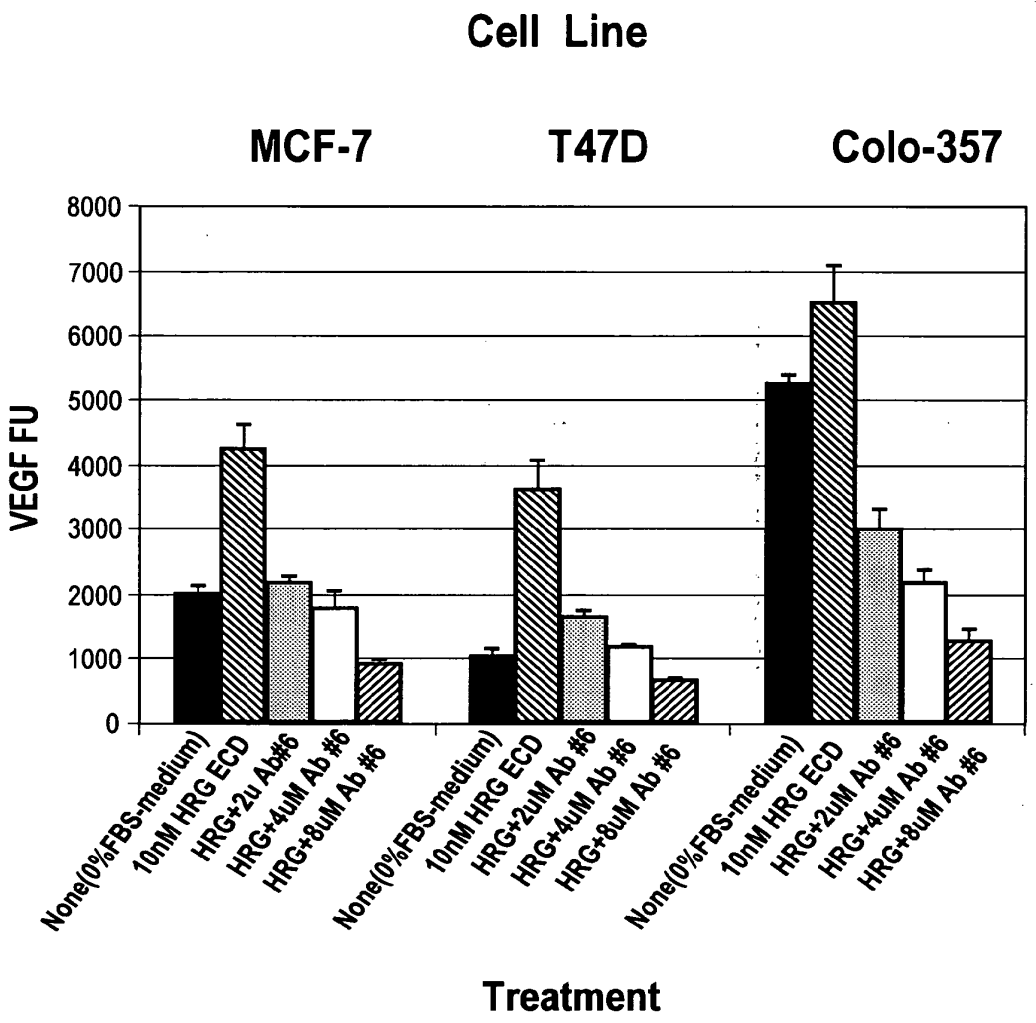


Fig. 24A

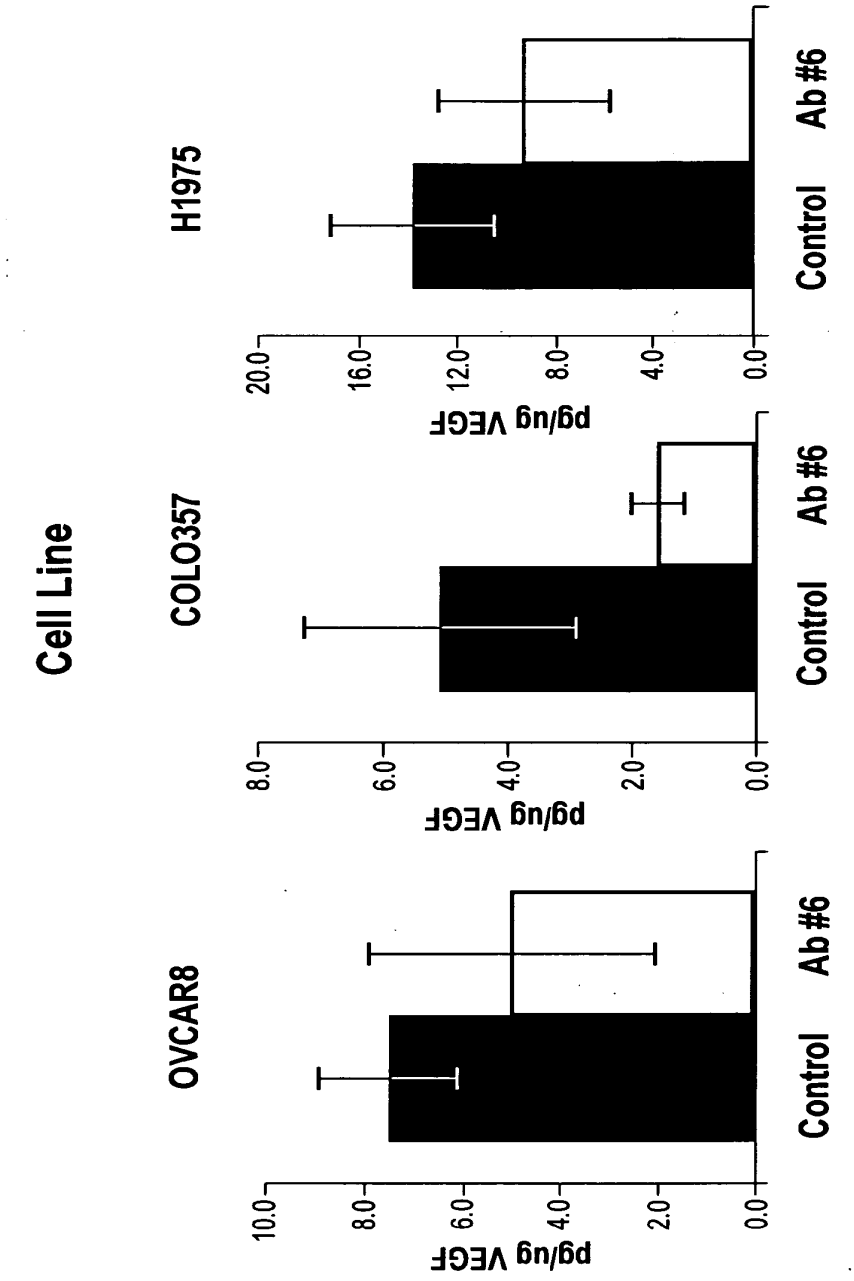

Cell Line
OVCAR8
COLO357
H1975
pg/ug VEGF
10.0
8.0
6.0
4.0
2.0
0.0
20.0
16.0
12.0
8.0
4.0
Control
Ab #6
Treatment

Fig. 24B

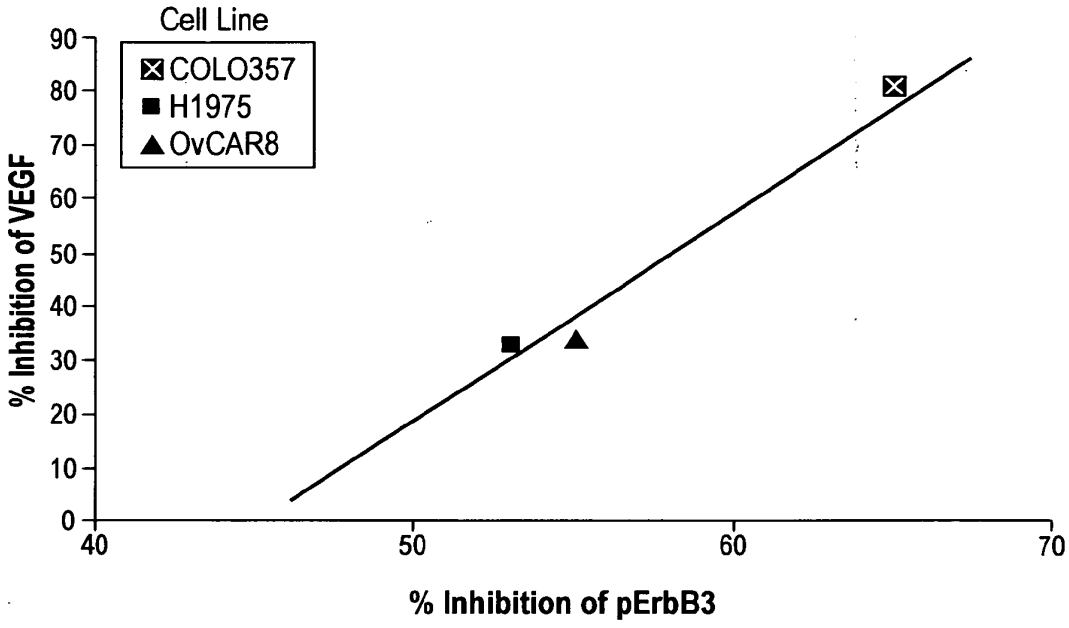

Correlation of phosphoErbB3 inhibition and inhibition of VEGF Secretion
Cell Line
COLO357
H1975
OvCAR8
% Inhibition of VEGF
% Inhibition of pErbB3
0
10
20
30
40
50
60
70
80
90
40
50
60
70

Fig. 24C

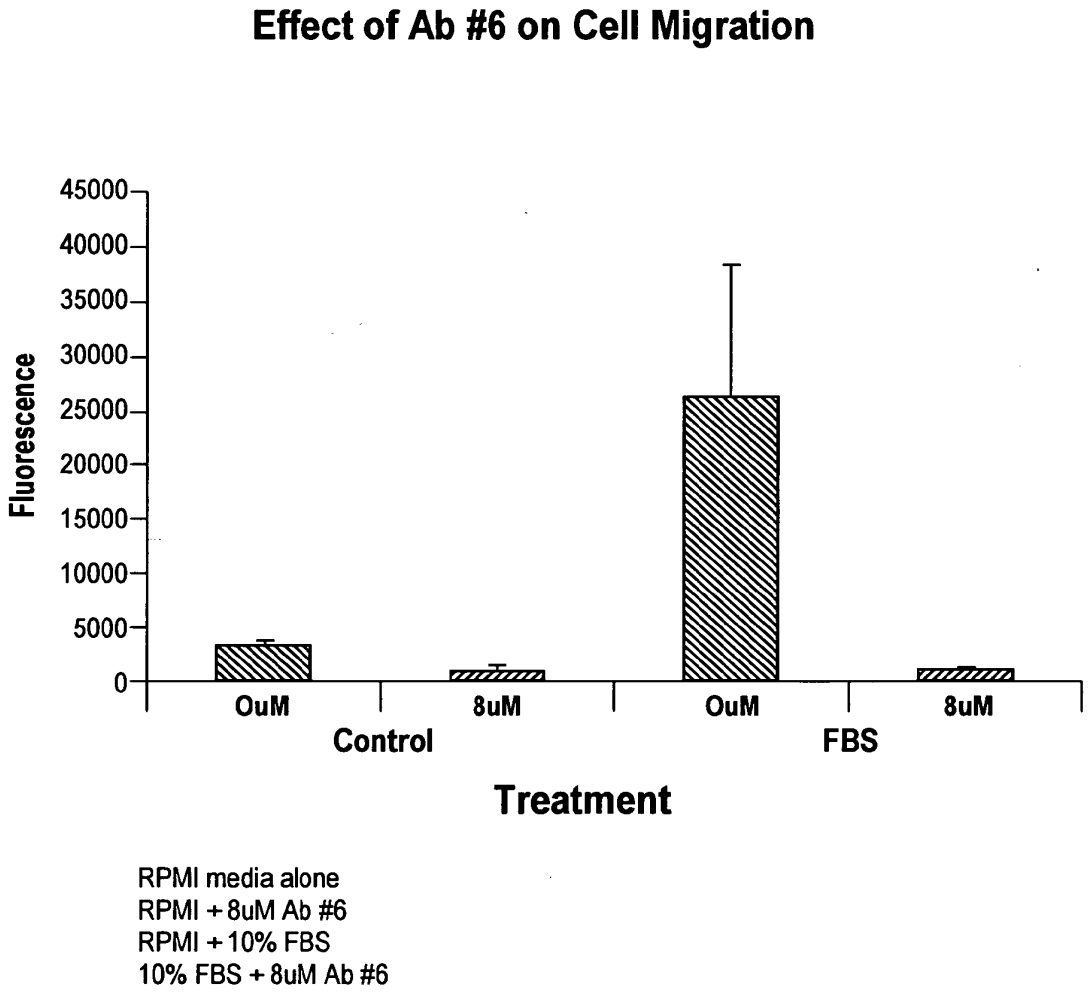

Effect of Ab #6 on Cell Migration
Fluorescence
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
OuM
8uM
OuM
8uM
Control
FBS
Treatment
RPMI media alone
RPMI + 8uM Ab #6
RPMI + 10% FBS
10% FBS + 8uM Ab #6

Fig. 25

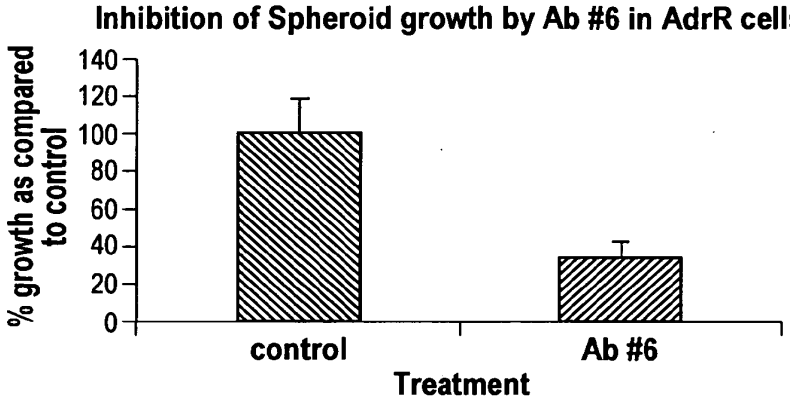


Fig. 26A

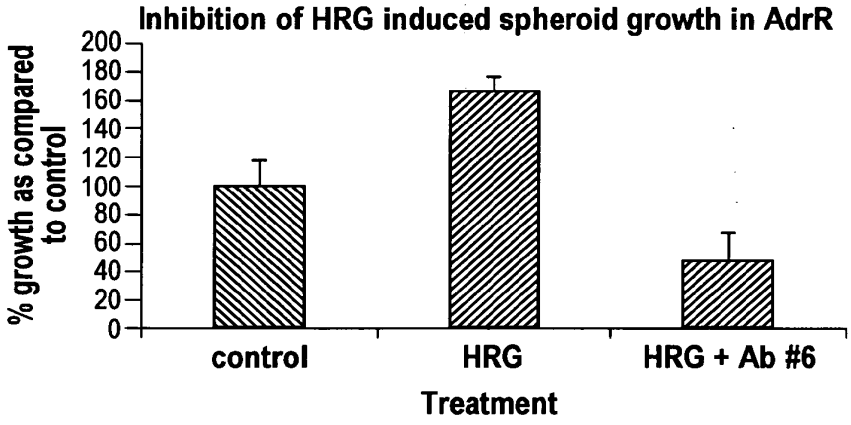


Fig. 26B

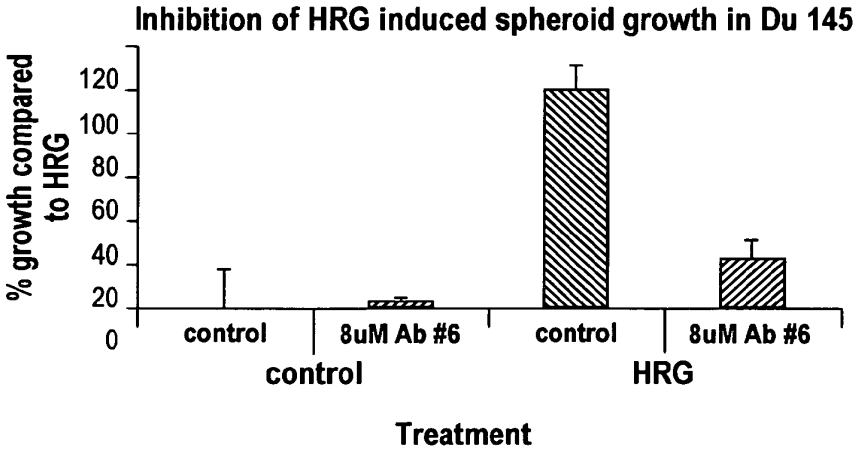


Fig. 26C

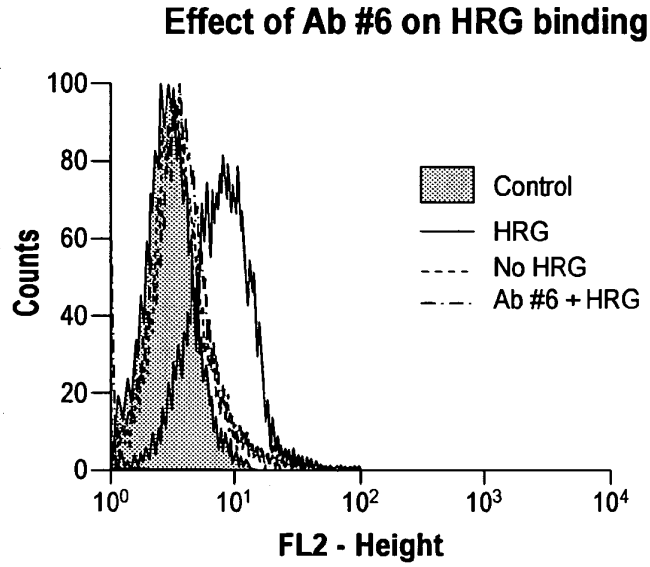


Fig. 27A

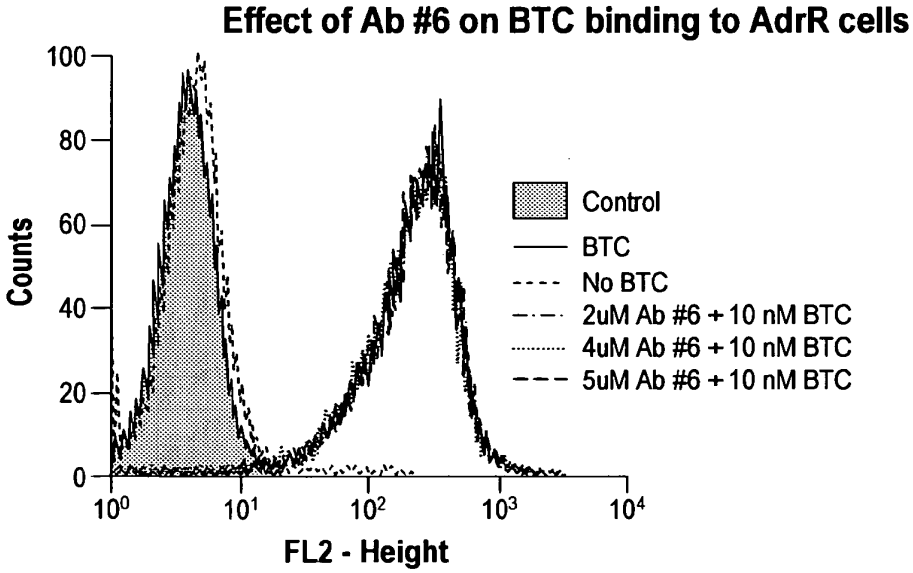


Fig. 27B

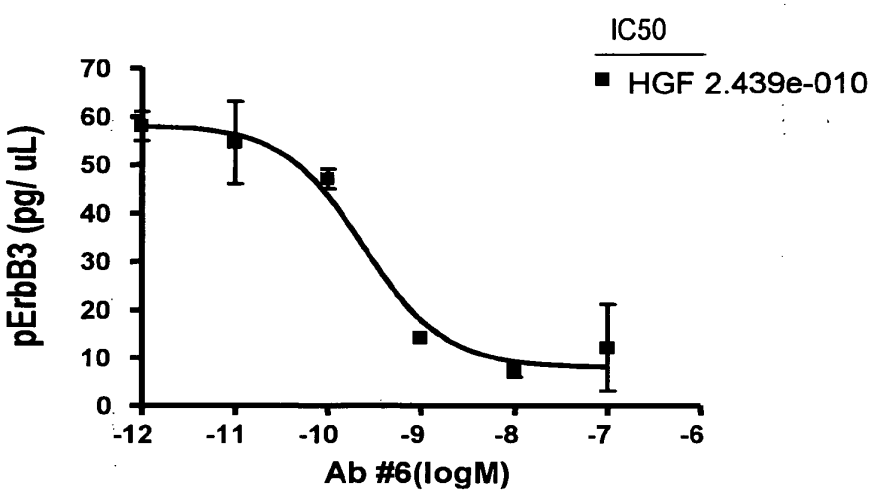


Fig. 28

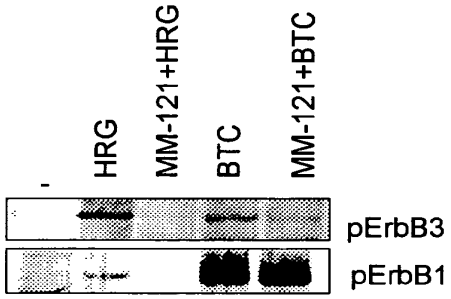


Fig. 29A

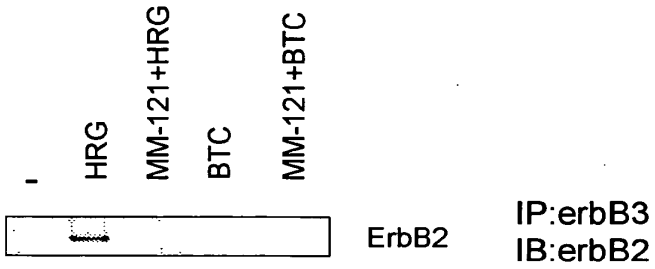
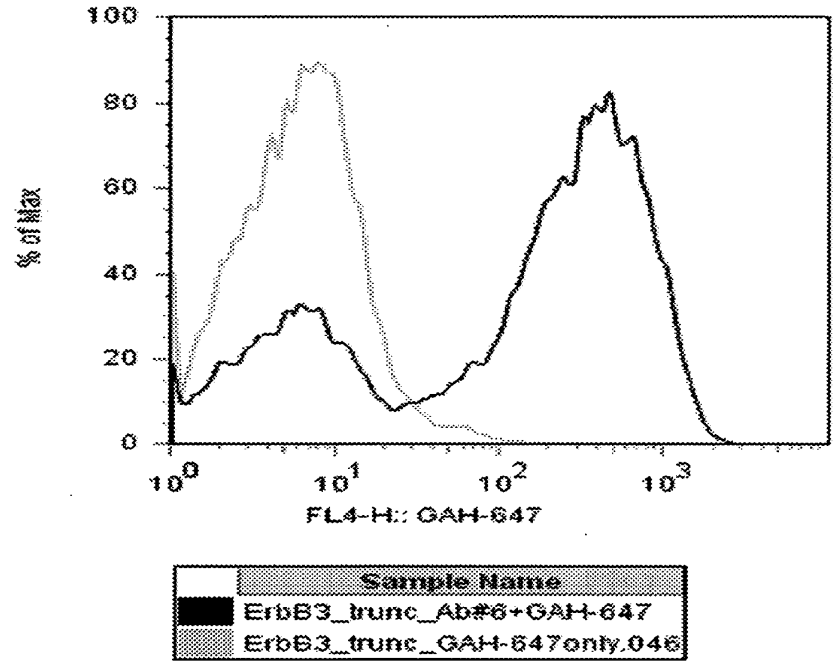


Fig. 29B

Fig. 30


100
80
60
40
20
0
% of Max
10^0
10^1
10^2
10^3
FL4-H:: GAH-647
Sample Name
ErbB3_trunc_Ab#6+GAH-647
ErbB3_trunc_GAH-647only.046

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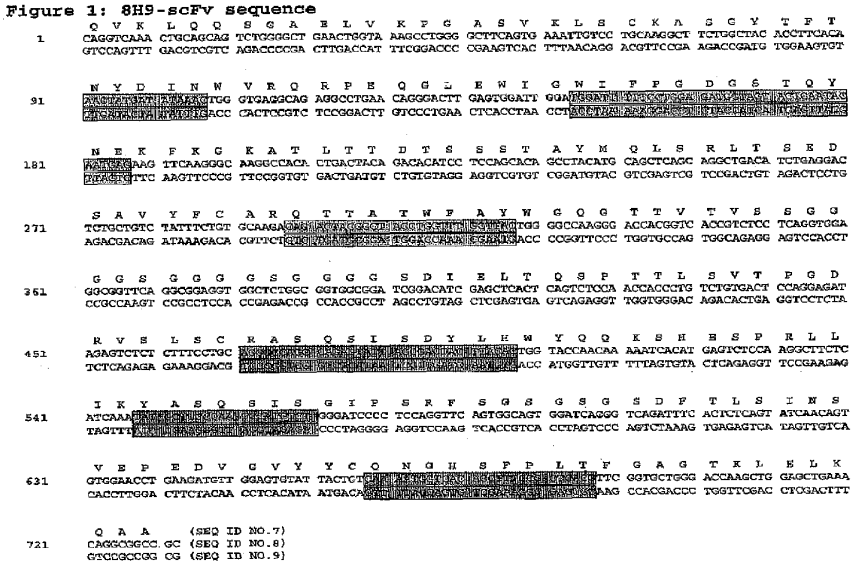
Declarations under Rule 4.17:

— *as to the identity of the inventor (Rule 4.17(i))*
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*
— *of inventorship (Rule 4.17(iv))*

[Continued on next page]

(54) **Title:** USES OF MONOCLONAL ANTIBODY 8H9

Figure 1: 8H9-scFv sequence



(57) **Abstract:** The present invention discloses monoclonal antibody 8H9 which binds to the 4Ig domain isoform of the human B7-homolog 3, 4Ig-B7H3. The present invention provides a method of improving the prognosis or prolonging the survival of a subject bearing tumor cells, the method comprises administering to the subject a composition comprising an effective amount of an agent capable of binding to an antigen recognized by monoclonal antibody 8H9.

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USES OF MONOCLONAL ANTIBODY 8H9

[0001] This application claims the benefit of priority of U.S. Serial No. 60/896,416, filed March 22, 2007, and U.S. Serial No. 60/915,672, filed May 2, 2007. The entire contents and disclosures of the preceding applications are incorporated by reference into this application.

FIELD OF THE INVENTION

[0002] This invention relates to uses of monoclonal antibody 8H9 or derivates thereof in treating cancer patients.

BACKGROUND OF THE INVENTION

[0003] Tumor-restricted surface antigens may be targets for diagnosis and immune-based therapies. An ideal tumor antigen for targeted immunotherapy should be absent on normal tissues and abundantly expressed on tumor cell surface. Moreover, a "generic" tumor-specific antigen expressed on tumor cells of varying lineage recognized by monoclonal antibodies may have broader utility in antibody-based strategies. A novel 58 kD surface tumor-associated antigen recognized by a murine monoclonal antibody 8H9 has been reported previously (*see e.g.* U.S. patent application publication US 2005/0169932). The antigen recognized by 8H9 was expressed on cell membranes of a broad spectrum of tumors of neuroectodermal, mesenchymal and epithelial origin, with restricted distribution on normal tissues. This novel antibody-antigen system is very promising for tumor targeting and immunotherapy.

[0004] Monoclonal antibody 8H9 can be used for tumor targeting and imaging, and purging of tumor cells. The 8H9 antigen is also a potential target for antibody-based immunotherapy against a broad spectrum of human cancers,

including neuroblastoma, brain tumors, desmoplastic small round cell tumor, rhabdomyosarcoma, osteosarcoma, Ewings sarcoma, PNET, melanoma, sarcoma, wilm's tumor, hepatoblastoma, and carcinomas of various tissue origins. Construction of 8H9 single chain antibody and antibody-fusion constructs have also been described (*see e.g.* U.S. patent application publication US 2005/0169932).

[0005] The present disclosure provides further data on using monoclonal antibody 8H9 to improve the prognosis and/or prolong the survival of a subject bearing tumor cells.

[0006] Throughout this application, various references are cited. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains.

SUMMARY OF THE INVENTION

[0007] The present invention provides a method of improving the prognosis or prolonging the survival of a subject bearing a tumor, comprising administering to the subject a composition comprising an effective amount of an agent capable of binding to an antigen recognized by mononclonal antibody 8H9.

[0008] The present invention also provides a method of improving the prognosis or prolonging the survival of a subject bearing a tumor expressing an antigen recognized by monoclonal antibody 8H9. This method comprises administering to the subject a composition comprising an effective amount of an agent capable of binding to an antigen recognized by mononclonal antibody 8H9.

[0009] The present invention also provides a method of screening for antibodies that have the same or similar binding specificity as monoclonal antibody 8H9, comprising the step of contacting candidate antibodies with a polypeptide comprising the sequence of SEQ ID NO.15, or a fragment thereof, wherein antibodies that bind to the polypeptide are antibodies that have the same or similar binding specificity as monoclonal antibody 8H9. The present invention also provides an antibody identified by the above screening method.

[0010] The present invention also provides an antigen which is recognized by monoclonal antibody 8H9, wherein the antigen has at least about 10%, preferably between 10% and 99% homology to SEQ ID NO.15.

DETAILED DESCRIPTION OF THE FIGURES

[0011] **FIGURE 1** shows 8H9 scFv amino acid sequence (SEQ ID NO.7) and gene sequences (sense and complementary, SEQ ID NOs. 8-9). Complementary determining regions (CDR) are marked in boxes in the following order: CDR-1 (HC, heavy chain), CDR-2 (HC), CDR-3 (HC), CDR-1 (LC, light chain), CDR-2 (LC), CDR-3 (LC).

[0012] **FIGURE 2** shows nucleotide and amino acid sequences of 8H9scFv (SEQ ID NOs.10-12). Mutated 8H9 scFv carries the following site-directed mutagenesis (VH: K13E and VL: R18Q, R45Q, K103E, K107E) to decrease PI from 6.4 to 4.8, and net charge from -1 to -9, a strategy to decrease nonspecific normal tissue adherence.

[0013] **FIGURE 3** shows non-reduced SDS-PAGE of 8H9 Western Blot.

[0014] **FIGURE 4** shows 8H9 affinity purification (non-reduced SDS-PAGE, Western Blot).

[0015] **FIGURE 5** shows 8H9 affinity purification (non-reduced SDS-PAGE, silver stain).

[0016] **FIGURE 6** shows HLA-I (MHC class I) and B7H3 protein expression on K562 cell surface analyzed by FACS.

[0017] **FIGURE 7** shows the cytolytic activity of NK92 cells against K562 and HTB82 cells (results of Chromium release assay for cell-mediated cytolysis).

[0018] **FIGURE 8** shows the cytolytic activity of NK cells against HTB82 cells (results of Chromium release assay for cell-mediated cytolysis). NK92MI: parental NK cells; NK92MI/NTGLS-8H: NK92MI transduced with 8H9scFv.

[0019] **FIGURE 9** shows the cytolytic activity of NK cells against K562 cells (results of Chromium release assay for cell-mediated cytolysis). NK92MI: parental NK cells; NK92MI/NTGLS-8H: NK92MI transduced with 8H9scFv.

DETAILED DESCRIPTION OF THE INVENTION

[0020] The present invention provides a method of improving the prognosis or prolonging the survival of a subject bearing a tumor, comprising administering to the subject a composition comprising an effective amount of an agent capable of binding to an antigen recognized by mononclonal antibody 8H9. As used herein, "improving the prognosis" refers to early detection of cancer and early initiation of treatment that would lead to a future course of disease with possible recovery or cure of the disease, whereas "prolonging the survival" refers to

increasing the life expectancy after the diagnosis of cancer. In one embodiment, the tumor expresses an antigen recognized by mononclonal antibody 8H9.

[0021] In one embodiment, the antigen recognized by mononclonal antibody 8H9 is a polypeptide comprising the sequence of SEQ ID NO.15. In another embodiment, the antigen is a polypeptide homolog of SEQ ID NO.15. In general, there is at least about 10% homology, or at least about 15% homology, or at least about 25% homology, or at least about 35% homology, or at least about 45% homology, or at least about 55% homology, or up to 100% homology to SEQ ID NO.15. One of ordinary skill in the art would readily a homolog or ortholog of SEQ ID NO.15 (see e.g. Table 1).

TABLE 1
Orthologs of CD276

Organism	Gene	Description	Human Similarity	NCBI accessions
dog (Canis familiaris)	CD276[1]	CD276 molecule	90.65*(n)* 93.97*(a)*	487638 XM_849111.1 XP_854204.1
chimpanzee (Pan troglodytes)	LOC467818[1]	CD276 molecule	99*(n)* 98.88*(a)*	467818 XM_523213.2 XP_523213.2
rat (Rattus norvegicus)	Cd276[1]	CD276 antigen	89.35*(n)* 93.47*(a)*	315716 NM_182824.2 NP_877976.1
mouse (Mus musculus)	Cd2764 Cd276[1]	CD276 antigen[1,4]	88.89*(n)*[1] 92.78*(a)*[1]	102657[1] NM_133983.3[1] NP_598744.1[1] AI415625[4] AI593640[4] (see all 16)
chicken (Gallus gallus)	CD276[1]	CD276 molecule	73.36*(n)* 68.51*(a)*	415315 XM_413702.2 XP_413702.2
zebrafish (Danio rerio)	LOC572193[1]	similar to CD276 antigen	62.37*(n)* 55.68*(a)*	572193 XM_695881.2 XP_700973.2
African clawed frog (Xenopus laevis)	XI.15387[1~]	Xenopus laevis transcribed sequence with weak similarity more	68.63*(n)*	CB207657.1

Human similarity showing % homology at the nucleotide level (n) or amino acid level (a).

[0022] In one embodiment, the agent in the above composition is a polypeptide comprising Complementary Determining Regions (CDRs) derived from monoclonal antibody 8H9. Examples of such polypeptide include, but are not limited to, single chain antibody or antibody-fusion construct. As used herein, "single chain antibody" refers to reduction of an immunoglobulin molecule (4 peptide chains) into a single peptide that retains immunoreactivity and specificity for the antigen or for the tumor, usually in the form of a single peptide incorporating the heavy chain and the light chain of the immunoglobulin, whereas "antibody-fusion construct" refers to chemically or genetically linking such single chain antibody to another protein or peptide to form a novel antibody-fusion construct.

[0023] In one embodiment, such polypeptide comprises CDRs of SEQ ID NOs.1-3, 4-6, or 1-6. Preferably, sequences other than the CDRs on the above polypeptides are of human origin. In another embodiment, the polypeptide has an amino acid sequence of SEQ ID NO. 7 or 12. Moreover, the agent in the above composition can be directly or indirectly coupled to a labeling agent or a cytotoxic agent. Representative examples of such labeling agent or cytotoxic agent include, but are not limited to, radioisotopes and toxins such as pseudomonas exotoxin.

[0024] In general, the above composition can be administered intraperitoneally, intravenously, intrathecally, by Ommaya reservoir or by spinal tap, intraparenchymally into the tumors (either primary or metastatic), or into tissues surrounding the tumor.

[0025] The agent of the above compositions, when labeled with a radioisotope, may be used for both therapeutic purposes

and for imaging purposes. In one embodiment, such agent in the above composition is administered at 0.01 mg to 20 mg per injection, carrying 1 mCi to 100 mCi of 131-Iodine, and in a preferred embodiment is used therapeutically.

[0026] In another embodiment, the agent in the above composition is administered at 0.01 mg to 20 mg per injection, carrying 1 mCi to 100 mCi of 124-Iodine, and in a preferred embodiment is used for imaging and dosimetry purposes.

[0027] In another embodiment, the agent in the above composition is administered at 0.01 mg to 20 mg per injection, carrying biologically equivalent radioactive doses of beta-emitters or alpha emitters to 1 mCi to 100 mCi of 131-Iodine, wherein such beta-emitters or alpha emitters may be 213-Bismuth, 212-Bismuth, 111-Indium, 118-Rhenium, 90-Yttrium, 225-Actinium, and 177-Lutetium, or 85-Astatine.

[0028] In another embodiment, the agent in the above composition is administered at 0.01 mg to 20 mg per injection, carrying biologically equivalent radioactive doses of positron-emitters to 1 mCi to 100 mCi of 124-Iodine, wherein such positron-emitters may be 94m-Technetium, 64-Copper, 89-Zirconium, 68-Gallium, 66-Gallium, 76-Bromium, 86-Yttrium, 82-Rubidium, 110m-Indium, 13-Nitrogen, 11-Carbon or 18-Fluorine.

[0029] In a preferred embodiment, the above composition is administered after the subject has been treated with one or more other cancer treatments. In a further embodiment, the above composition is administered simultaneously or sequentially while the subject is being treated with one or more other cancer treatments. Examples of such other cancer treatments include, but are not limited to, surgery, chemotherapy, and radiation.

[0030] The present invention also provides use of an agent, which has the characteristics described above (e.g. capable of binding to an antigen recognized by monoclonal antibody 8H9) as a medicament for improving the prognosis or prolonging the survival of a subject bearing a tumor. In one embodiment, the tumor expresses an antigen recognized by mononclonal antibody 8H9. The routes and doses of administrating a composition comprising the agent can be readily determined by one of ordinary skill in the art. For example, the composition can be administered according to the doses and routes of administration described above.

[0031] The present invention also provides a method of screening for antibodies that have the same or similar binding specificity as monoclonal antibody 8H9, comprising the step of contacting candidate antibodies with a polypeptide comprising the sequence of SEQ ID NO.15, or a fragment thereof, wherein antibodies that bind to the polypeptide are antibodies that have the same or similar binding specificity as monoclonal antibody 8H9. The present invention also comprises an antibody identified by the method described herein.

[0032] The present invention also provides an antigen which is recognized by monoclonal antibody 8H9, wherein the antigen has at least about 10%, preferably between 10% and 99% homology to SEQ ID NO.15.

[0033] The present invention also provides a method of upregulating anti-metastatic immune response in NK/T cells comprising the steps of blocking B7H3 receptors present on NK/T cells with an appropriate agent.

[0034] This invention also provides methods for screening agents which competitively inhibit the binding of monoclonal antibody 8H9 to its target, comprising steps of contacting candidate with the target in conditions permitting the binding of the candidate and the target. In a preferred embodiment, the above method further comprises detection of formation of a complex and the candidate and the target. In this embodiment, the target is B7H3, also known as CD276, and the agent may be an antibody, a peptide, a cell surface protein, or a ligand.

[0035] The invention will be better understood by reference to the Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative, and are not meant to limit the invention as described herein, which is defined by the claims which follow thereafter.

EXAMPLE 1

Improved Outcome With Combined Modality Including 131-Iodine-8H9 Radioimmunotherapy Delivered Through The Cerebrospinal Fluid

[0036] **Background:** Primary brain tumors and cancers metastatic to the CNS (brain parenchyma or leptomeninges [LM]) are difficult to control. Antibody-based targeted therapies administered through the cerebrospinal fluid (CSF) compartment have therapeutic potentials. Monoclonal antibody 8H9 is a murine IgG1 antibody reactive with a wide spectrum of human solid tumors. 131-Iodine-8H9 administered through the Ommaya has favorable pharmacokinetics in non-human primates with minimal toxicities.

[0037] **Methods:** In a phase I study, 15 patients (pts, ages 2-34 years) (1 melanoma, 3 recurrent ependymoma, 8 relapsed

CNS neuroblastoma [NB], 3 recurrent medulloblastoma) received 2 mCi intra-Ommaya 131I-8H9 for dosimetry followed 1 week later by an intra-Ommaya treatment dose of 10 (n=3 pts), 20 (n=3), 30 (n=6), or 40 (n=3) mCi. Serial cerebrospinal fluid (CSF) and blood were sampled for dosimetry calculations. Nuclear scans were performed at 24 hours post to study 131I-8H9 localization. The 131-Iodine-8H9 dosimetry and treatment doses were repeated after 1 month if the pt had no PD.

[0038] **Results:** Side effects included grade 1 or 2 fever, headache or vomiting; one had a transient grade 3 ALT elevation on first injection (30 mCi). Calculated mean radiation dose to the CSF was 35.7 (range 15-79) cGy/mCi; mean blood dose was 2.4 cGy/mCi. Of the 15 pt, 8 (group #1) had primary diagnosis of neuroblastoma. When they developed CNS metastasis (at median age of 3.8 years) they were treated with a salvage regimen which included 131-Iodine-8H9. All 8 pts remain alive progression-free (3+, 10+, 16+, 16+, 18+, 18+, 20+, 30+ months since 131-Iodine-8H9, and 5-43+ months since CNS/LM relapse); in one pt, 131-Iodine-8H9 achieved CR of LM disease. In contrast, median time to death from the onset of CNS/LM NB was 5.4 months for 27 historical controls. Acute side effects were self-limited; at 40 mCi dose no DLT was seen.

[0039] Conclusion: Similar to CNS metastases in most other solid tumors, conventional therapies have been ineffective for NB-CNS. Intra-Ommaya 131-Iodine-8H9 (1) is safe, (2) has favorable dosimetry to CSF and marrow, and (3) may have clinical utility when added to salvage therapy using conventional modalities in the treatment of 8H9-positive LM/CNS cancers.

EXAMPLE 2

Improved Resolution and Contrast Imaging of CNS Tumors Using 124-Iodine-8H9 in PET/CT Scans

[0040] **Background:** As stated in Example 1, antibody-based targeted therapies administered through the cerebrospinal fluid (CSF) compartment have therapeutic potential, and radiolabeled 131-Iodine-8H9 can be used to treat metastatic disease. A patient's prognosis will be improved not only with improved treatment but also with improved detection of the metastatic disease and improved dosimetry. The following example describes an improved means of detection of neuroblastoma.

[0041] **Methods:** Five patients were injected intrathecally with 124-Iodine-8H9 and serial PET/CT imaging and CSF sample was performed. Patients had CNS tumors (choroid plexus carcinoma, metastatic rhabdomyosarcoma, and metastatic neuroblastoma. 1.7-2 mCi 124-Iodine-8H9 was administered through an Ommaya reservoir. PET/CT scans were obtained at approximately 4, 24, and 48 hours post-injection. Serial cerebrospinal fluid (CSF) samples through 48 hours were obtained. Images were analyzed by placing regions of interest on the spinal column at all three time points. PET images provided direct activity measurements within the CSF compartment.

[0042] **Results:** 124-Iodine-8H9 PET scans provided high resolution images of the antibody distribution with targeting of disease in the 2 patients with structural lesions on MRI. At 24 hours, most of the antibody had cleared from the ventricles and had distribution through the thecal sac and around the cerebral convexities. The distribution corresponded well with pre-treatment ^{111}In-DTPA cisternography.

Systemic activity in liver, spleen and bladder was seen at 24 and 48 hours. The biological T1/2 clearance ranged from 8.9 hours to 64.6 hours with corresponding doses of 14.1 ti 92.9 cGy/mCi to CSF.

[0043] Conclusion: 124-Iodine-8H9 PCT/CT provides higher resolution and contrast images than SPECT with 131-Iodine-8H9 for distribution, targeting and dosimetry.

EXAMPLE 3

8H9 Antibody Recognizes The 4Ig Domain Isoform of The Human B7-Homolog 3, 4Ig-B7H3

[0044] The following example describes biochemical characterization of the antigen recognized by the 8H9 antibody. The identity of the antigen is the 4Ig domain isoform of the human B7-homolog 3, 4Ig-B7H3.

[0045] Cell Culture. The human neuroblastoma cell line LAN-1 was provided by Dr. Robert Seeger (Children's Hospital of Los Angeles, Los Angeles, CA). Human rhabdomyosarcoma cell line HTB82, osteosarcoma cell line U2OS, and Burkitt's lymphoma cell line Daudi were purchased from American Type Culture Collection (Bethesda, MD). All cell lines were grown in RPMI 1640 medium supplemented with 10% bovine calf serum, 2mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a 5% CO2 incubator.

[0046] Monoclonal Antibodies. Both 8H9 and control MoAb 5F9 are murine IgG1 and were produced against human neuroblastoma. They were purified by protein A (GE Healthcare, Piscataway, NJ) affinity chromatography before use.

[0047] **Whole Cell Lysates and Western Blot.** 8H9-positive cell lines (LAN-1, HTB82 and U2OS) and 8H9-negative cell line (Daudi) were grown to ~ 80% confluence. Cells were harvested using 2 mM EDTA and washed with ice-cold PBS.

[0048] Native PAGE was performed using NativePAGE Novex Bis-Tris Gel System (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, cells were lysed on ice (20 min) in NativePAGE 1X Sample Buffer plus 1% detergent (either Triton-X100 or n-dodecyl-β-D-maltoside (DDM)) and protease inhibitor cocktail tablets (Roche Applied Science, Germany). The lysates were clarified by centrifugation at 14,000 rpm for 20 min at 4°C. 50 µg whole cell lysates were analyzed by NativePAGE Novex 4-16% Bis-Tris Gels.

[0049] SDS-PAGE under nonreducing or reducing conditions was performed using Tris-Glycine Ready Gel System (Bio-Rad, Hercules, CA). Briefly, cells were lysed on ice (20 min) in Triton Lysis Buffer (50 mM Tris-HCl, pH 7.2, 50 mM NaCl, 10% glycerol, 1% Triton X-100, and protease inhibitor cocktail tablets). The lysates were clarified as above. 25 ~ 50 µg whole cell lysates were analyzed by 4-15% Tris-HCl Gels.

[0050] After electrophoresis in either PAGE, samples were transferred onto Immun-Blot PVDF Membrane (Bio-Rad), blocked for one hour at room temperature (RT) with 10% dry milk in TBST, and incubated with primary antibodies (8H9 at 10-20 µg/ml, 5F9 at 20 µg/ml) for 3 hrs at RT. The membrane was then washed with TBST, and incubated with secondary Peroxidase-conjugated AffiniPure Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch, West Grove, PA). Bands were detected with SuperSignal West Pico Chemiluminescent Substrate (PIERCE, Rockford, IL).

[0051] Subcellular Fractionation. For crude membrane preparation, LAN-1 cells were pipetted off the tissue culture dish, washed with ice-cold PBS, and lysed on ice in sucrose buffer (0.25 M sucrose, 5 mM Tris-HCl, pH 7.2, and protease inhibitor cocktail tablets) with a Dounce homogenizer (Kontes, Vineland, NJ). Centrifugation for 10 min at 1000 g pelleted all nuclei, as judged microscopically. The 1000 g supernatant was ultracentrifuged at 100,000 g for 30 min in a Beckman L-70K (25,000 rpm, SW41Ti rotor) to give membrane particulate (P100) and cytosolic (S100) fractions. Cytosolic fraction was adjusted to 1% Triton, while crude nuclear and membrane fractions were resuspended in Triton Lysis Buffer and clarified before use.

[0052] 8H9 Antigen Affinity Purification. 8H9 antigen was purified from LAN-1 cell extracts by immuno-affinity chromatography using MoAb 8H9. The 8H9 affinity column was prepared using Pierce's Protein G IgG Plus Orientation Kit (PIERCE, Rockford, IL) according to the manufacturer's instructions.

[0053] Four mg of LAN-1 whole cell lysates or equivalent membrane fraction prepared as above were incubated overnight at 4°C with 20 µl 8H9-protein G Sepharose (covalently crosslinked with disuccinimidyl suberate (DSS), 3 mg bound 8H9/ml beads). After extensive washing with Triton Lysis Buffer, the column was eluted sequentially with 50 mM Tris-HCl, pH 7.2 containing 1M NaCl, 0.1 M Glycine-HCl, pH 2.8 and pH 2.0, SDS Sample Buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.005% Bromophenol blue), and SDS Sample Buffer plus boiling in water for 5 min. A small aliquot of eluates was monitored for the presence of 8H9 antigen by Western Blot analysis under nonreducing conditions using 8H9 antibody. One-fourth of the eluates was also analyzed by silver staining

(SilverQuest Silver Staining Kit, Invitrogen). Finally, one-half of the 8H9 antigen-positive eluate (0.1 M Glycine-HCl, pH 2.0 eluted fraction) was analyzed by colloidal Coomassie blue staining (GelCode Blue Stain Reagent, PIERCE), and the 8H9 antigen-positive band was sent for mass spectrometric identification by MSKCC Microchemistry and Proteomics Core Facility.

Results

[0054] Western Blot Detection of 8H9 Antigen. 8H9 antigen was first detected by 8H9 MoAb under native conditions using NativePAGE Novex Bis-Tris Gel system. A single band was detected in all 8H9-positve cell lines (LAN-1, HTB82 and U2OS) but not 8H9-negative cell line (Daudi), as defined by flow cytometry analysis, using 1% nonionic detergent (either Triton-X100 or DDM) (data not shown). The detection was specific since 5F9, a control MoAb against Ku70 protein, detected a band with a different size (data not shown).

[0055] Later, 8H9 antigen was also detected by 8H9 MoAb under nonreducing conditions using Tris-Glycine Ready Gel SDS-PAGE system. Just like under native conditions, a single band (~ 85 KD using Invitrogen SeeBlue Plus2 Pre-Stained Standard as protein molecular weight marker) was detected in all 8H9-positve cell lines (LAN-1, HTB82 and U2OS) but not 8H9-negative cell line (Daudi), using 1% Triton Lysis Buffer (Fig. 3, and data not shown). The detection was specific, since 5F9 (an IgG1 specific for Ku70) did not detect a band at the same size (data not shown). The size of 8H9 antigen detected is consistent with previous data using 8H9 radio-immunoprecipitation. We were unable to detect 8H9 antigen by Western Blot analysis under reducing conditions (data not shown), suggesting 8H9 recognize a conformational sensitive epitope.

[0056] After subcellular fractionation, 8H9 antigen was detected predominantly in membrane fraction (Fig. 3), which is consistent with previous data that 8H9 antigen is a cell surface antigen. Enrichment of 8H9 antigen in the membrane fraction was then undertaken using affinity purification.

[0057] **Affinity Purification of the 8H9 Antigen.** LAN-1 cell line was selected for antigen purification because its relatively high level expression of 8H9 antigen and ability of growing rapidly in tissue culture. 8H9 affinity column was prepared by covalently conjugating Fc portion of 8H9 to protein G of the gel matrix in a defined orientation, allowing exposure of a higher number of free antibody binding sites for antigen binding. Utilizing the NHS-ester DSS in place of the traditional imidoester DMP for crosslinking also significantly prevents the leaching of antibody from the support.

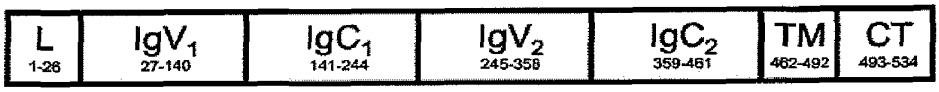
[0058] After incubating either LAN-1 (and Daudi as negative control) whole cell lysates or LAN-1 membrane fraction with 8H9-protein G Sepharose overnight, a significant portion (> 50%) of 8H9 antigen was bound to the Sepharose (Fig. 4, and data not shown). 8H9 antigen was eluted specifically and predominantly in 0.1 M Glycine-HCl, pH 2.0 as monitored by Western Blot analysis (Fig. 4, and data not shown), suggesting a very strong interaction between 8H9 antibody and its antigen. After silver staining the same eluate, a clear band was detected accordingly only in LAN-1 cell extracts but not in Daudi cell extracts (Fig. 5). The eluate was also clean enough in the 85 KD vicinity for mass spec analysis. Finally, enough quantity of 8H9 antigen in the band (~ 10 ng, visible with colloidal Coomassie staining, data not shown) was collected and sent for mass spectrometric identification.

[0059] Mass spectrometric identification Tryptic digests were subjected to a micro-clean-up procedure using 2 µL bed-volume of Poros 50 R2 (PerSeptive) reversed-phase beads, packed in an Eppendorf gel-loading tip. Mass spectrometry (MALDI-ReTOF) was performed on peptide pools (16 & 30% MeCN) recovered from the RP-microtip column using a Bruker Ultraflex TOF/TOF instrument with delayed extraction. For mass fingerprinting, experimental masses (m/z) combined from both MALDI-ReTOF experiments were used to search a non-redundant human protein database (NR; ~192,489 entries; NCBI; Bethesda, MD), using the PeptideSearch (Matthias Mann, Max-Planck Institute for Biochemistry, Martinsried, Germany) algorithm. A molecular weight range twice the predicted weight was covered, with a mass accuracy restriction better than 50 ppm, and maximum one missed cleavage site allowed per peptide. Mass spectrometric sequencing (MALDI-TOF-MS/MS) of selected peptides from partially fractionated pools was done on a Bruker Ultraflex TOF/TOF instrument in 'LIFT' mode, and the fragment ion spectra taken to search human databases using the MASCOT MS/MS Ion Search program (Matrix Science). Two peptide sequences from the peptide digest were identified: NPVLQQDAHSSVTITPQR (SEQ ID NO.13), and SPTGAVEVQVPEDPVVALVGTDATLR (SEQ ID NO.14).

[0060] These yielded an unequivocal identification of the antigen as 4Ig-B7H3, the 4Ig domain isoform of the human B7-homolog 3, also named CD276, accession number of NM_001024736.1, which codes for a peptide of 534 amino acids, of 57235 kD molecular weight. The gene is located on chromosome 15q24.1. The amino acid sequence of the mature human protein is as follows (with the potential N-glycosylation sites underlined):

[0061] MLRRRGSPGMGVHVGAALGALWFCLTGALEVQVPEDPVVALVGTDATLCCSF SPEPGFSLAQLNLIWQLTDTKQLVHSFAEGQDQGSAYANRTALFPDLLAQGNASLRLQRVRV ADEGSFTCFVSIRDFGSAAVSLQVAAPYSKPSMTLEPNKDLRPGDTVTITCSSYQGYPEAEV FWQDGQGVPLTGNVTTSQMANEQGLFDVHSILRVVLGANGTYSCLVRNPVLQQDAHSSVTIT PQRSPTGAVEVQVPEDPVVALVGTDATLRCSFSPEPGFSLAQLNLIWQLTDTKQLVHSFTEG RDQGSAYANRTALFPDLLAQGNASLRLQRVRVADEGSFTCFVSIRDFGSAAVSLQVAAPYSK PSMTLEPNKDLRPGDTVTITCSSYRGYPEAEVFWQDGQGVPLTGNVTTSQMANEQGLFDVHS VLRVVLGANGTYSCLVRNPVLQQDAHGSVTITGQPMTFPPEALWVTVGLSVCLIALLVALAF VCWRKIKQSCEEENAGAEDQDGEGEGSKTALQPLKHSDSKEDDGQEIA (SEQ ID NO.15)

TABLE 2



B7 Member	Action On Immunity	Receptor
B7H1 (CD274)	inhibitory	PD-1 (CD279)
B7DC (CD273)	stimulatory/inhibitory	PD-1 (CD279)
B7H2 (LICOS)	TH2 skewing	ICOS
B7H3 (CD276)	stimulatory/inhibitory	??
B7H4 (B7X)	inhibitory	??

[0062] To date, the new B7s include B7H1, B7DC, B7H2, B7H3, and B7H4 (see Table 2).[4] Athough their mRNAs are fairly ubiquitously, these protein molecules may be differentially regulated at the post-transcriptional level. B7H3 was first cloned by Chapoval et al[5] as a member of the B7 costimulatory family of proteins. Later it was determined to exist as a type I membrane protein with four instead of two Ig-like domains, and hence given the new name of 4Ig-B7H3 (see Table 2).[6] In vitro 4Ig-B7H3 was more inhibitory than costimulatory for T-cell activation.[6] B7H3 protein expression has been detected in gastric, NSCLC, neuroblasotma and many human tumor cell lines.[5,7,8] Human neuroblastoma tumors and cell lines

expressing 4Ig-B7H3 may inhibit an NK-mediated immune response.[7] B7H3 was found to be expressed on 59% of gastric carcinoma and 100% of gastric adenoma samples,[9] and appears to correlate with better survival. In murine models[10,11] and human melanoma,[12] B7H3 appears to evince anti-tumor response. Murine B7H3 promotes acute and chronic allograft rejection.[13] B7H3 probably plays a role in potentiating tumor immunosurveillance while the 4Ig-B7H3 exerts an inhibitory effect.[4] It is of interest that 4Ig-B7H3 is the major isoform in most issues except brain and placenta.[14] In the placenta B7H3 is a 110kd double band and a 60kd single band by western blot.[15] It was most prominent on the extravillous trophoblast throughout gestation. B7H3 is also thought to play a role in bone formation.[16]

[0063] The identification of 4Ig-B7H3 as the antigen for 8H9 suggests that this glycoprotein is highly expressed among human solid tumors. The epitope that 8H9 recognizes appears to be restricted to tumors versus normal tissues. Based on the mRNA work published to date, one would have inevitably concluded that this antigen is ubiquitous and unsuitable to be a tumor target. We, however, found otherwise. We postulate that antibody directed at 4Ig-B7H3 can be safely administered without major side effects as was seen recently with anti-CD28 antibodies or with anti-CTLA4 where T-cell are targeted. We believe that 4Ig-B7H3 is an immune coinhibitory molecule, and antibodies like 8H9 can modulate its function and potentiate host anti-tumor immune response across a spectrum of human cancers.

EXAMPLE 4

Isolation and Identification of B7H3 Receptors on Activated NK/T Cells Using 8H9 Monoclonal Antibodies

[0064] Monoclonal antibody 8H9 recognizes the 4Ig domain isoform of the human B7-homolog 3, 4Ig-B7H3. Human B7-homolog 3 (B7H3), which is also known as CD276, is a molecule which is believed to provide negative signals to the immune system, in particular providing negative signals to NK/T cells, allowing tumor cells to escape immune response. The identified antigen 4Ig-B7H3 targeted by monoclonal antibody 8H9 is the dominant variant form of B7H3 (CD276). 4Ig-B7H3 is a dominantly expressed form of human B7H3 containing a splice variation that duplicates the V-like and C-like Ig domain.[14,6]

[0065] As an immune modulator, both positive and negative immunologic functions of B7H3 have been reported. Reports describing the 2Ig-B7H3 variant demonstrated that the role of B7H3 was to promote T cell activation and IFN-γ production by binding to a putative receptor on activated T cells.[5] Antitumor response was enhanced by B7H3 expression in murine tumor models.[11] In patients, B7H3 positivity in gastric carcinoma was correlated with increased survival.[9] Conversely, the coinhibitory role of B7H3 was supported by reports that both 2Ig-B7H3 and 4Ig-B7H3 inhibited T cell proliferation and cytokine production[6], that B7H3 preferentially downregulated TH1-mediated immune response in B7H3-deficient mice[17], and that 4Ig-B7H3 inhibited NK-mediated lysis of neuroblastoma cells by interacting with a putative inhibitory receptor in the surface of NK cells[7]. The contradictory findings were possibly explained by the antagonistic B7H3 receptors.

[0066] The following example describes how B7H3 receptors on activated NK/T cells may be identified and isolated. This experiment has not yet been performed.

[0067] B7H3 receptors on NK/T cells are purified by affinity chromatography using both 2Ig-B7H3-Fc and 4Ig-B7H3-Fc

as the baits. A B7H3-Fc fusion protein is created in the following manner: 2Ig-B7H3-Fc is purchased from R & D Systems while the cDNA sequence encoding the extracellular domain of human 4Ig-B7H3 is fused to the Fc region of mouse IgG2a using the pFUSE-mIg-G2a-Fc2 expression vector. The fusion protein is expressed in the CG44-CHO cell line and purified by affinity chromatography using protein A Sepharose. Purity and functionality of the fusion protein are evaluated by coomassie blue staining and anti-B7H3 Western blot.

[0068] NK/T cells positive for the B7H3 receptor are selected for. The established NK/T cell lines NK92, NKL, NK3.3, YT, TALL-104, as well as activated NK/T cells enriched from fresh peripheral blood mononuclear cells (PBMC) are incubated with B7H3-Fc with subsequent staining with fluorescence-conjugated secondary antibody, and analyzed by fluorescence activated cell sorting (FACS). The positive cells are further confirmed by B7H3-Fc Western blot.

[0069] The NK/T cells selected as being positive for B7H3 receptors are used for affinity purification. A B7H3-Fc affinity column is prepared by covalently conjugating the Fc portion of B7H3-Fc to protein G on the gel matrix using Protein G IgG Plus Orientation Kit (Pierce Biotechnology). Cell extracts from B7H3 receptor-positive cells are incubated with the Sepharose beads on the column. The column is washed extensively and eluted. The presence and purity of B7H3 receptor is monitored by B7H3-Fc Western blot and silver staining. B7H3 receptor-positive bands of over 20 ng are sent for mass spectrometric identification.

EXAMPLE 5

Use of the 8H9 Monoclonal Antibody For Blockage of Inhibitory B7H3 (CD276) and Subsequent Enhanced NK/T Cell-Mediated Cytolysis of Tumor Cells

[0070] Immune responses to tumors have been found to be enhanced by blocking inhibitory receptors on T cells with monoclonal antibodies specific to said inhibitory receptors. A known example of this phenomenon is the enhancement of immune response through the blockade of CTLA-4 inhibitory receptor on T cells using anti-CTLA-4 monoclonal antibodies. The following example describes how a blockade of B7H3 receptor on NK/T cells with 8H9 antibody sensitizes tumor cells to NK/T cell-mediated toxicity. This experiment has not yet been performed.

[0071] Cell-Mediated Cytolysis (Chromium release) Assay: For the NK cell-mediated cytolysis assay, human CML cell line K562 is chosen for the target cells. As demonstrated by FACS analysis, K562 has low expression of HLA-1 and B7H3 proteins. Rhabdomyosarcoma HTB82 cells are used as a control. In a standard 4 hour ^{51}Cr-release assay, while only less than 10% of rhabdomyosarcoma HTB82 cells were lysed by NK92 cells, up to 60% of K562 cells were effectively killed by NK92 effector cells. One group of the target cell population of K562 is transfected with nucleic acids encoding for the splice forms 4Ig-B7H3 in order that B7H3 be overexpressed in this cell population. The K562 target cells are radiolabeled with 100 µCi $^{51}Cr/10^6$ cells for 1 hour at 37°C. The monoclonal antibody 8H9 is incubated with the transfected target cells while controls are incubated with HLA-1 mAb HB95, and cytolysis assays are performed using effector cells positive for the coinhibitory B7H3 receptor. NK92 effector cells are incubated in 96-well plates with target cells in 250 µl for 4 hours at

37°C. Cytolytic activity of NK92 effector cells against B7H3 transfected K562 will be decreased vis-à-vis non-transfected K562. Restored cytolytic activities will be observed after blocking of the coinhibitory B7H3.

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What is claimed is:

1. A method of improving the prognosis or prolonging the survival of a subject bearing a tumor, comprising administering to the subject a composition comprising an effective amount of an agent capable of binding to an antigen recognized by monoclonal antibody 8H9.

2. The method of claim 1, wherein the agent is a polypeptide comprising Complementary Determining Regions (CDRs) that have the sequences of SEQ ID NOs.1-6.

3. The method of claim 1, wherein the agent is a polypeptide comprising Complementary Determining Regions (CDRs) that have the sequences of SEQ ID NOs.1-3 or SEQ ID NOs.4-6.

4. The method of claim 1, wherein the agent is an antibody construct comprising Complementary Determining Regions (CDRs) of monoclonal antibody 8H9.

5. The method of claim 4, wherein the antibody construct is a single chain antibody or an antibody-fusion construct.

6. The method of claim 4, wherein sequences other than the CDRs are of human origin.

7. The method of claim 4, wherein the antibody has an amino acid sequence of SEQ ID NO. 7 or 12.

8. The method of claim 1, wherein the antigen recognized by monoclonal antibody 8H9 is CD276, or specifically the 4Ig domain isoform of the human B7-homolog 3, 4Ig-B7H3.