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Antibodies directed to the deletion mutants of epidermal growth factor receptor and uses thereof

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(71) Applicant(s)
Amgen Fremont Inc.

(72) Inventor(s)
Keyt, Barry;King, Chadwick T.;Foltz, Ian;Kang, Jaspal;Gudas, Jean;Corvalan Jose;Green, Larry;Foord, Orit;Rathanaswami, Palani;Su, Qiaojuan Jane;Weber, Richard;Raya, Robert;Klakamp, Scott L.;Feng, Xiao;Yang, Xiao Dong;Jia, Xiao-Chi;Liu, Ying

(74) Agent / Attorney
Davies Collison Cave, PO Box 2219 BUSINESS CENTRE, MILTON, QLD, 4064

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ABSTRACT

This invention relates to novel antibodies, particularly antibodies directed against deletion mutants of epidermal growth factor receptor and particularly to the type III deletion mutant, EGFRvIII. The embodiments also relate to human monoclonal antibodies directed against deletion mutants of epidermal growth factor and particularly to EGFRvIII. The embodiments also relate to variants of such antibodies. Diagnostic and therapeutic formulations of such antibodies, and immunoconjugates thereof, are also provided.

Australian Patents Act 1990 – Regulation 3.2

ORIGINAL COMPLETE SPECIFICATION

STANDARD PATENT

Invention Title:

"Antibodies directed to the deletion mutants of epidermal growth factor receptor and uses thereof"

The following statement is a full description of this invention, including the best method of performing it known to us:-

P/00/011

ANTIBODIES DIRECTED TO THE DELETION MUTANTS OF EPIDERMAL GROWTH FACTOR RECEPTOR AND USES THEREOF

This application is a divisional of Australian Patent Application No. 2004259398, the entire content of which is incorporated herein by reference.

Related Applications

This Application claims priority to United States Provisional Applications Serial Number 60/562,453 filed April 15, 2004, Serial Number 60/525570, filed November 26, 2003, and Serial Number 60/483,145, filed June 27, 2003.

FIELD OF THE INVENTION

The present embodiments relate to novel antibodies, particularly antibodies directed against deletion mutants of epidermal growth factor receptor and particularly to the type III deletion mutant, EGFRvIII. The embodiments also relate to human monoclonal antibodies directed against deletion mutants of epidermal growth factor receptor and particularly to EGFRvIII. The embodiments also relate to variants of such antibodies. Diagnostic and therapeutic formulations of such antibodies, and immunoconjugates thereof, are also provided.

BACKGROUND OF THE INVENTION

Tumor specific molecules to aid in better diagnosis and treatment of human and animal cancer have been sought since the last century. Hard evidence of tumor-specific substances, based on molecular structural data, has been difficult to provide in most types of human cancer except those based on virally-induced cancer and involving molecular structures specified by the virus genome. There have been extremely few examples of tumor-specific molecules based on novel molecular structures. In the case of malignant human gliomas and other tumors potentially associated with amplification or changes in the epidermal growth factor receptor molecule, such as carcinoma of the breast and other human carcinomas, there have been no unequivocal demonstrations of structurally altered molecules with unique sequences.

The epidermal growth factor receptor (EGFR) is the 170 kilodalton membrane glycoprotein product of the proto-oncogene c-erb B. The sequence of the EGFR gene is known (Ullrich et al. (1984). Human Epidermal Growth Factor Receptor cDNA Sequence and Aberrant Expression of the Amplified Gene in A431 Epidermoid Carcinoma Cells. Nature 309:418-425). The EGFR gene is the cellular homolog of the erb B oncogene originally identified in avian erythroblastosis viruses (Downward et al. (1984). Close Similarity of Epidermal Growth Factor Receptor and v-erb B Oncogene Protein Sequence. Nature 307:521-527, Ullrich, et al. (1984)). Activation of this oncogene by gene amplification has been observed in a variety of human tumors (Haley et al. (1987A). The Epidermal Growth Factor Receptor Gene in: Oncogenes, Genes, and Growth Factors Edited by: Guroff, G. 12th Edition. Chapter 2. pp. 40-76. Wiley, N.Y.), and in particular, those of glial origin (Libermann et al. (1985). Amplification, Enhanced Expression and Possible Rearrangement of EGF Receptor Gene in Primary Human Brain Tumours of Glial Origin. Nature

313:144-147; Wong et al. (1987). Increased Expression of the Epidermal Growth Factor Receptor Gene in Malignant Gliomas is Invariably Associated with Gene Amplification. Proc. Natl. Acad. Sci. USA 84:6899-6903; Yamazaki et al. (1988). Amplification of the Structurally and Functionally Altered Epidermal Growth Factor Receptor Gene (c-erbB) in Human Brain Tumors. Molecular and Cellular Biology 8:1816-1820; Malden et al., (1988). Selective Amplification of the Cytoplasmic Domain of the Epidermal Growth Factor Receptor Gene in Glioblastoma Multiforme. Cancer Research 4:2711-2714).

EGF-r has been demonstrated to be overexpressed on many types of human solid tumors. Mendelsohn Cancer Cells 7:359 (1989), Mendelsohn Cancer Biology 1:339-344 (1990), Modjtahedi and Dean Int'l J. Oncology 4:277-296 (1994). For example, EGFR overexpression has been observed in certain lung, breast, colon, gastric, brain, bladder, head and neck, ovarian, kidney and prostate carcinomas. Modjtahedi and Dean Int'l J. Oncology 4:277-296 (1994). Both epidermal growth factor (EGF) and transforming growth factor-alpha (TGF-alpha.) have been demonstrated to bind to EGF-r and to lead to cellular proliferation and tumor growth.

One major difference between v-erb B oncogenes and the normal EGFR gene is that the viral oncogenes are amino-truncated versions of the normal receptor; they lack most of the extracytoplasmic domain but retain the transmembrane and tyrosine kinase domains (Fung et al., (1984) Activation of the Cellular Oncogene c-erb B by LTR Insertion: Molecular Basis for Induction of Erythroblastosis by Avian Leukosis Virus. Cell 33:357-368; Yamamoto et al., (1983). A New Avain Erythroblastosis Virus, AEV-H Carries erbB Gene Responsible for the Induction of Both Erythroblastosis and Sarcoma. Cell 34:225-232, Nilsen et al., (1985). c-erbB Activation in ALV-Induced Erythroblastosis: Novel RNA Processing and Promoter Insertion Results in Expression of an Amino-Truncated EGF Receptor. Cell 41:719-726; Gammett et al., (1986). Differences in Sequences Encoding the Carboxy-Terminal Domain of the Epidermal Growth Factor Receptor Correlate with Differences in the Disease Potential of Viral erbB Genes. Proc. Natl. Acad. Sci. USA 83:6053-6057). This results in a protein that is unable to bind epidermal growth factor (EGF) but can still phosphorylate other substrates (Gilmore et al., (1985). Protein Phosphorlytion at Tyrosine is Induced by the v-erb B Gene Product in Vivo and In Vitro. Cell 40:609-618; Kris et al., (1985). Antibodies Against a Synthetic Peptide as a Probe for the Kinase Activity of the Avian EGF Receptor and v-erB Protein. Cell 40:619-625), and has led to speculation that the v-erb B proteins are oncogenic because the kinase domain is unregulated and constitutively active (Downward et al., 1984).

A variety of genetic alterations can occur in viral erb B oncogenes, e.g. amino acid substitutions and deletions in the carboxy terminus of the gene. Available evidence, however, argues that the amino truncation is critical to carcinogenesis. Amino truncations are a feature of all v-erb B oncogenes, including those that arise by promoter insertion or retroviral transduction (Nilsen et al., (1985). c-erbB Activation in ALV-Induced Erythroblastosis: Novel RNA Processing

and Promoter Insertion Results in Expression of an Amino-Truncated EGF Receptor. Cell 41:719-726; Gammett et al., (1986). Differences in Sequences Encoding the Carboxy-Terminal Domain of the Epidermal Growth Factor Receptor Correlate with Differences in the Disease Potential of Viral erbB Genes. Proc. Natl. Acad. Sci. USA 83:6053-6057).

In contrast, carboxy-terminal deletions appear to be associated only with tumors that arise through retroviral transduction and seem to determine host range and tumor type specificity (Gammett et al., 1986; Raines et al., (1985). c-erbB Activation in Avian Leukosis Virus-Induced Erythroblastosis: Clustered Integration Sites and the Arrangement of Provirus in the c-erbB Alleles. Proc. Natl. Acad. Sci. USA 82:2287-2291). Transfection experiments with amino-truncated avian c-erb B genes or chimeric viral oncogene-human EGF receptors demonstrates that this deletion is sufficient alone to create a transforming protein (Pelley et al., (1988). Proviral-Activated c-erbB is Leukemogenic but not Sarcomagenic: Characterization of a Replication–Competent Retrovirus Containing the Activated c-erbB. Journal of Virology 62: 1840-1844; Wells et al., (1988). Genetic Determinants of Neoplastic Transformation by the Retroviral Oncogene v-erbB. Proc. Natl. Acad. Sci. USA 85:7597-7601).

Amplification of the EGFR gene occurs in 40% of malignant human gliomas (Libermann et al., (1985) Amplification, Enhanced Expression and Possible Rearrangement of EGF Receptor Gene in Primary Human Brain Tumours of Glial Origin. Nature 313:144-147; Wong et al., (1987). Increased Expression of the Epidermal Growth Factor Receptor Gene in Malignant Gliomas is Invariably Associated with Gene Amplification. Proc. Natl. Acad. Sci. USA 84:6899-6903), Rearrangement of the receptor gene is evident in many of the tumors with gene amplification. The structural alterations seem to preferentially affect the amino terminal half of the gene (Yamazaki et al., (1985). Amplification, Enhanced Expression and Possible Rearrangement of EGF Receptor Gene in Primary Human Brain Tumours of Glial Origin. Nature 313:144-147; Malden et al., (1988). Selective Amplification of the Cytoplasmic Domain of the Epidermal Growth Factor Receptor Gene in Glioblastoma Multiforme. Cancer Research 4:2711-2714), but the nature of the rearrangements had not at that time been precisely characterized in any tumor.

Size variant EGFR genes and amplification have been reported in several human cancers. (Humphrey et al., (1988). Amplification and Expression of the Epidermal Growth Factor Receptor Gene in Human Glioma Xenografts. Cancer Research 48:2231-2238; Bigner et al., (1988) J. Neuropathol. Exp. Neurol., 47:191-205; Wong et al., (1987). Increased Expression of the Epidermal Growth Factor Receptor Gene in Malignant Gliomas is Invariably Associated with Gene Amplification. Proc. Natl. Acad. Sci. USA 84:6899-6903; and Humphrey et al. Amplification and expression of the epidermal growth factor receptor gene in human glioma xenografts. Cancer Res. 48(8):2231-8 (1988)) There had been no determination, however, of the molecular basis for the altered EGFR molecules in cells.

In 1989, work of Drs. Bigner and Vogelstein elucidated the sequence of a EGF receptor mutant that has become known as the type III mutant (also referred to as delta-EGFr or EGFrvIII). This work is described in U.S. Patent Nos. 6,455,498, 6,127,126, 5,981,725, 5,814,317, 5,710,010, 5,401,828, and 5,212,290.

EGFR variants are caused by gene rearrangement accompanied by EGFR gene amplification. There are eight major variants of EGFr that are known: (i) EGFRvI lacks a majority of the extracellular domain of EGFR, (ii) EGFRvII consists of an 83 aa in-frame deletion in the extracellular domain of EGFR, (iii) EGFRvIII consists of a 267 aa in-frame deletion in the extracellular domain of EGFR, (iv) EGFRvIV contains deletions in the cytoplasmic domain of EGFR, (v) EGFRvV contains deletions in cytoplasmic domain of EGFR, (vi) EGFR.TDM/2-7 contains a duplication of exons 2-7 in the extracellular domain of EGFR, (vii) EGFR.TDM/18-25 contains a duplication of exons 18-26 in the tyrosine kinase domain of EGFR, and (viii) EGFR.TDM/18-26 contains a duplication of exons 18-26 in the tyrosine kinase domain of EGFR (Kuan et al. EGF mutant receptor vIII as a molecular target in cancer therapy. Endocr Relat Cancer. 8(2):83-96 (2001)). In addition, there is a second, more rare, EGFRvIII mutant (EGFRvIII/Δ12-13) that possesses a second deletion that introduces a novel histidine residue at the junction of exons 11 and 14 (Kuan et al. EGF mutant receptor vIII as a molecular target in cancer therapy. Endocr Relat Cancer. 8(2):83-96 (2001)).

EGFRvIII is the most commonly occurring variant of the epidermal growth factor (EGF) receptor in human cancers (Kuan et al. EGF mutant receptor vIII as a molecular target in cancer therapy. Endocr Relat Cancer. 8(2):83-96 (2001)). During the process of gene amplification, a 267 amino acid deletion occurs in the extracellular domain creating a novel junction to which tumor specific monoclonal antibodies can be directed. This variant of the EGF receptor contributes to tumor progression through constitutive signaling in a ligand independent manner. EGFrVIII is not know to be expressed on any normal tissues (Wikstrand, CJ. et al. Monoclonal antibodies against EGFR,III are tumor specific and react with breast and lung carcinomas malignant gliomas. Cancer Research 55(14): 3140-3148 (1995); Olapade-Olaopa, EO. et al. Evidence for the differential expression of a variant EGF receptor protein in human prostate cancer. Br J Cancer. 82(1):186-94 (2000)). Yet, EGFRvIII shows significant expression in tumor cells, *e.g.*, 27~76% breast cancer biopsies express EGFRvIII (Wikstrand, CJ. et al. Monoclonal antibodies against EGFR,III are tumor specific and react with breast and lung carcinomas malignant gliomas. Cancer Research 55(14): 3140-3148 (1995); Ge H. et al. Evidence of high incidence of EGFRvIII expression and coexpression with EGFR in human invasive breast cancer by laser capture microdissection and immunohistochemical analysis. Int J Cancer. 98(3):357-61 (2002)), 50~70% gliomas express EGFRvIII (Wikstrand, CJ. et al. Monoclonal antibodies against EGFR,III are tumor specific and react with breast and lung carcinomas malignant gliomas. Cancer Research 55(14): 3140-3148 (1995); Moscatello, G. et al. Frequent expression of a mutant epidermal growth factor receptor in

multiple human tumors. Cancer Res. 55(23):5536-9 (1995)), 16% NSCL cancers express EGFRvIII (Garcia de Palazzo, IE. et al. Expression of mutated epidermal growth factor receptor by non-small cell lung carcinomas. Cancer Res. 53(14):3217-20 (1993)), 75% ovarian cancers express EGFRvIII (Moscatello, G. et al. Frequent expression of a mutant epidermal growth factor receptor in multiple human tumors. Cancer Res. 55(23):5536-9 (1995)), and 68% prostate cancers express EGFRvIII (Olapade-Olaopa, EO. et al. Evidence for the differential expression of a variant EGF receptor protein in human prostate cancer. Br J Cancer. 82(1):186-94 (2000)).

The deletion of 267 amino acids with a Glycine substitution creates a unique junction that may be capable of antibody targeting. Further, in view of EGFRvIII's expression in certain tumors and its lack of expression in normal tissues, EGFRvIII may be an ideal target for drug targeting in tumor therapy. In particular, EGFRvIII would appear to be an ideal candidate for immunoconjugate therapy of tumors (e.g., an antibody conjugated to an antineoplastic agent or toxin). Another method of treatment of cancers which over-express EGFRvIII involved the use of a tumor-specific ribozyme targeted specifically to the variant receptor which did not cleave normal EGFR. The ribozyme was found to significantly inhibit breast cancer growth in athymic nude mice (Luo et al. Int. J. Cancer. 104(6):716-21 (2003)).

General antibodies for the entire EGFRvIII protein have been described. See International Patent Application No. WO 01/62931 and Kuan et al. EGF mutant receptor vIII as a molecular target in cancer therapy. Endocr Relat Cancer. 8(2):83-96 (2001), Kuan et al. EGFRvIII as a promising target for antibody-based brain tumor therapy. Brain Tumor Pathol. 17(2):71-78 (2000), Kuan et al. Increased binding affinity enhances targeting of glioma xenografts by EGFRvIII-specific scFv. International Journal of Cancer. 88(6):962-969 (2000), Landry et al. Antibody recognition of a conformational epitope in a peptide antigen: Fv-peptide complex of an antibody fragment specific for the mutant EGF receptor, EGFRvIII. Journal of Molecular Biology. 308(5):883-893 (2001), Reist et al. Astatine-211 labeling of internalizing anti-EGFRvIII monoclonal antibody using N-succinimidyl 5-[211At]astato-3-pyridinecarboxylate. Nuclear Medicine and Biology. 26(4):405-411 (1999), Reist et al. In vitro and in vivo behavior of radiolabeled chimeric anti-EGFRvIII monoclonal antibody: comparison with its murine parent. Nuclear Medicine and Biology. 24(7):639-647 (1997), Wikstrand et al. Generation of anti-idiotypic reagents in the EGFRvIII tumor-associated antigen system. Cancer Immunology, Immunotherapy. 50(12):639-652 (2002), Wikstrand et al. Monoclonal antibodies against EGFRvIII are tumor specific and react with breast and lung carcinomas malignant gliomas. Cancer Research. 55(14):3140-3148 (1995), Wikstrand et al. The class III variant of the epidermal growth factor receptor ($EGFR_{vIII}$): characterization and utilization as an immunotherapeutic target. J.Neurovirol. 4(2):148-158 (1998), Wikstrand et al. The class III variant of the epidermal growth factor receptor (EGFRvIII): characterization and utilization as an immunotherapeutic target. J.Neurovirol. 4(2):148-158 (1998), Jungbluth et al. A monoclonal antibody recognizing human cancers with

amplification/overexpression of the human epidermal growth factor receptor. Proc Natl Acad Sci U S A. 100(2):639-44 (2003), Mamot et al. Epidermal Growth Factor Receptor (EGFR)-targeted Immunoliposomes Mediate Specific and Efficient Drug Delivery to EGFR- and EGFRvIII-overexpressing Tumor Cells. Cancer Research 63:3154-3161 (2003)). Each of these above-mentioned antibodies, however, possess or contain murine sequences in either the variable and/or constant regions. The presence of such murine derived proteins can lead to the rapid clearance of the antibodies or can lead to the generation of an immune response against the antibody in a patient. In addition, such antibodies are relatively low affinity, on the order of 2.2×10^{-8} through 1.5×10^{-9}, even after affinity maturation. (Kuan et al. EGF mutant receptor vIII as a molecular target in cancer therapy. Endocr Relat Cancer. 8(2):83-96 (2001)).

In order to avoid the utilization of murine or rat derived antibodies, researchers have introduced human antibody function into rodents so that the rodents can produce fully human antibodies. *See e.g.*, Mendez et al. Functional transplant of megabase human immunoglobulin loci recapitulates human antibody response in mice. Nat Genet.15(2):146-56 (1997). This approach has been used in connection with the generation of successful antibodies directed against wild type EGFR. *See e.g.*, Yang X et al. Development of ABX-EGF, a fully human anti-EGF receptor monoclonal antibody, for cancer therapy. Crit Rev Oncol Hemato 38(1):17-23 (2001); Yang X-D et al. Eradication of Established Tumors by a Fully Human Monoclonal Antibody to the Epidermal Growth Factor Receptor without Concomitant Chemotherapy. Cancer Research 59(6):1236-1243 (1999); and U.S. Patent No. 6,235,883.

SUMMARY OF THE INVENTION

In one embodiment, the invention comprises an isolated human monoclonal antibody that specifically binds to EGFRvIII and a peptide that comprises the sequence L E E K K G N Y V V T D H C (SEQ ID NO: 56). In another embodiment, the invention comprises an isolated human monoclonal antibody that specifically binds to an epitope contained within a sequence comprising L E E K K G N Y V V T D H C (SEQ ID NO: 56), wherein the residues required for binding, as determined by Alanine scanning in a SPOTs array, are selected from the group consisting of EEK, KKNYV, LEK, EKNY and EEKGN.

Further embodiments include an isolated human monoclonal antibody that comprises a heavy chain variable region amino sequence that is encoded by a VH3-33 gene. The heavy chain variable region amino sequence can include an amino acid sequence that is encoded by a JH4b gene, or an amino acid sequence that is encoded by a D gene that is selected from the group consisting of D6-13 and D3-9.

Other embodiments include an isolated human monoclonal antibody that comprises a light chain variable region amino sequence that is encoded by a A23(VK2) gene. The light chain variable region amino sequence can include an amino acid sequence that is encoded by a JK1 gene.

Other embodiments include an isolated antibody, or fragment thereof, that binds to EGFRvIII and that comprises a heavy chain amino acid sequence selected from the group consisting of the heavy chain amino acid sequence of antibody 13.1.2, 131, 170, 150, 095, 250, 139, 211, 124, 318, 342 and 333 as identified in (SEQ ID NO: 138, 2, 4, 5, 7, 9, 10, 12, 13, 15, 16, and 17). The antibody can be a monoclonal antibody, a chimeric antibody, a humanized antibody or a human antibody. The antibody or fragment can be associated with a pharmaceutically acceptable carrier or diluent, and can be conjugated to a therapeutic agent. The therapeutic agent can be a toxin. The therapeutic agent can be a toxin such as DM-1, AEFP, AURISTATIN E, or ZAP. The agent can be associated with the antibody via a linker. The toxin can be associated with the antibody via a secondary antibody. Further embodiments include a hybridoma cell line producing the antibody, and a transformed cell comprising a gene encoding the antibody. The cell can be, for example, a Chinese hamster ovary cell.

Further embodiments include a method of inhibiting cell proliferation associated with the expression of EGFRvIII, comprising treating cells expressing EGFRvIII with an effective amount of the antibody or fragment. In one emobidment, the antibody comprises a heavy chain amino acid sequence selected from the group consisting of the heavy chain amino acid sequence of antibody 13.1.2 (SEQ ID NO: 138), 131 (SEQ ID NO: 2), 170 (SEQ ID NO: 4), 150 (SEQ ID NO: 5), 095 (SEQ ID NO: 7), 250 (SEQ ID NO: 9), 139 (SEQ ID NO: 10), 211 (SEQ ID NO: 12), 124 (SEQ ID NO: 13), 318 (SEQ ID NO: 15), 342 (SEQ ID NO: 16), and 333 (SEQ ID NO: 17). The method can be performed *in vivo*, and performed on a mammal, such as a human, who suffers from a cancer involving epithelial cell proliferation, such as a lung, colon, gastric, renal, prostate, breast, glioblastoma or ovarian carcinoma.

Further embodiments include a method of killing a targeted cell. This is achievd by contacting the targeted cell with an antibody associated with a toxin. The antibody binds to a peptide LEEKKGNY (SEQ ID NO: 133). In one embodiment, the antibody has a binding affinity greater than $1.3*10^{-9}$M to the peptide. In one embodiment the toxin is selected from AEFP, DM-1, and ZAP. In one embodiment, the antibody toxin compound is 10 fold more toxic to targeted cells than to cells without the peptide. In one embodiment, the antibody comprises a heavy chain amino acid sequence selected from the group consisting of the heavy chain amino acid sequence of antibody 13.1.2 (SEQ ID NO: 138), 131 (SEQ ID NO: 2), 170 (SEQ ID NO: 4), 150 (SEQ ID NO: 5), 095 (SEQ ID NO: 7), 250 (SEQ ID NO: 9), 139 (SEQ ID NO: 10), 211 (SEQ ID NO: 12), 124 (SEQ ID NO: 13), 318 (SEQ ID NO: 15), 342 (SEQ ID NO: 16), and 333 (SEQ ID NO: 17). In another embodiment, the antibody is associated with a toxin via a peptide linker or a second antibody.

Further embodiments of the invention include an isolated antibody that binds to EGFRvIII and that comprises a heavy chain amino acid sequence comprising the following complementarity determining regions (CDRs):

(a) CDR1 consisting of a sequence selected from the group consisting of the amino acid sequences for the CDR1 region of antibodies 13.1.2, 131, 170, 150, 095, 250, 139, 211, 124, 318, 342 and 333 as identified in SEQ ID NO: 138, 2, 4, 5, 7, 9, 10, 12, 13, 15, 16, and 17;

(b) CDR2 consisting of a sequence selected from the group consisting of the amino acid sequences for the CDR2 region of antibodies 13.1.2, 131, 170, 150, 095, 250, 139, 211, 124, 318, 342 and 333 as identified in SEQ ID NO: 138, 2, 4, 5, 7, 9, 10, 12, 13, 15, 16, and 17; and

(c) CDR3 consisting of a sequence selected from the group consisting of the amino acid sequences for the CDR3 region of antibodies 13.1.2, 131, 170, 150, 095, 250, 139, 211, 124, 318, 342 and 333 as identified in SEQ ID NO: 138, 2, 4, 5, 7, 9, 10, 12, 13, 15, 16, and 17. In one embodiment, the antibody is a monoclonal antibody, a chimeric antibody, human, or a humanized antibody. In one embodiment, the antibody is associated with a pharmaceutically acceptable carrier, diluent, and/or therapeutic agent. In one embodiment, the therapeuctic agent is a toxin. In one embodiment, the the toxin is DM-1 or Auristatin E.

Also included is an isolated antibody, or fragment thereof, that binds to EGFRvIII and that comprises a light chain amino acid sequence selected from the group consisting of the light chain amino acid sequence of antibody 13.1.2, 131, 170, 150, 123, 095, 139, 250, 211, 318, 342, and 333 as identified in SEQ ID NO: 140, 19, 20, 21, 29, 23, 25, 26, 28, 33, 31 and 32. The antibody can be a monoclonal antibody, a chimeric antibody, a humanized antibody, or a human antibody. It can be associated with a pharmaceutically acceptable carrier or diluent, or conjugated to a therapeutic agent, such as a toxin, for example DM1 or AURISTATIN E. In one embodiment a hybridoma cell line or a transformed cell producing an antibody comprising a light chain amino acid sequence selected from the group consisting of the light chain amino acid sequence of antibody 13.1.2, 131, 170, 150, 123, 095, 139, 250, 211, 318, 342, and 333 as identified in SEQ ID NO: 140, 19, 20, 21, 29, 23, 25, 26, 28, 33, 31 and 32 is contemplated.

Further embodiments include a hybridoma cell line producing such an antibody, and a transformed cell, such as a Chinese hamster ovary cell, comprising a gene encoding the antibody.

Yet another embodiment includes a method of inhibiting cell proliferation associated with the expression of EGFRvIII, comprising treating cells expressing EGFRvIII with an effective amount of the antibodies or fragments described above. The method can be performed *in vivo* and on a mammal, such as a human, who suffers from a cancer involving epithelial cell proliferation such as lung, colon, gastric, renal, prostate, breast, glioblastoma or ovarian carcinoma.

Yet another embodiment includes an isolated antibody that binds to EGFRvIII and that comprises a light chain amino acid sequence comprising the following complementarity determining regions (CDRs):

(a) CDR1 consisting of a sequence selected from the group consisting of the amino acid sequences for the CDR1 region of antibodies 13.1.2, 131, 170, 150, 123, 095, 139, 250, 211, 318, 342, and 333 as identified in SEQ ID NO: 140, 19, 20, 21, 29, 23, 25, 26, 28, 33, 31 and 32;

(b) CDR2 consisting of a sequence selected from the group consisting of amino acid sequences for the CDR1 region of antibodies 13.1.2, 131, 170, 150, 123, 095, 139, 250, 211, 318, 342, and 333 as identified in SEQ ID NO: 140, 19, 20, 21, 29, 23, 25, 26, 28, 33, 31 and 32; and

(c) CDR3 consisting of a sequence selected from the group consisting of amino acid sequences for the CDR1 region of antibodies 13.1.2, 131, 170, 150, 123, 095, 139, 250, 211, 318, 342, and 333 as identified in SEQ ID NO: 140, 19, 20, 21, 29, 23, 25, 26, 28, 33, 31 and 32.

The antibody identified in the previous paragraph can further include a heavy chain amino acid sequence comprising the following complementarity determining regions (CDRs):

(a) CDR1 consisting of a sequence selected from the group consisting of the amino acid sequences for the CDR1 region of antibodies 13.1.2, 131, 170, 150, 095, 250, 139, 211, 124, 318, 342 and 333 as identified in SEQ ID NO: 138, 2, 4, 5, 7, 9, 10, 12, 13, 15, 16, and 17;

(b) CDR2 consisting of a sequence selected from the group consisting of the amino acid sequences for the CDR2 region of antibodies 13.1.2, 131, 170, 150, 095, 250, 139, 211, 124, 318, 342 and 333 as identified in SEQ ID NO: 138, 2, 4, 5, 7, 9, 10, 12, 13, 15, 16, and 17; and

(c) CDR3 consisting of a sequence selected from the group consisting of the amino acid sequences for the CDR3 region of antibodies 13.1.2, 131, 170, 150, 095, 250, 139, 211, 124, 318, 342 and 333 as identified in SEQ ID NO: 138, 2, 4, 5, 7, 9, 10, 12, 13, 15, 16, and 17.

Further embodiments include a method of inhibiting cell proliferation associated with the expression of EGFRvIII, comprising treating cells expressing EGFRvIII with an effective amount of the antibody or fragment described above. The method can be performed *in vivo*, on a mammal, such as a human, suffering from a cancer involving epithelial cell proliferation, such as lung carcinoma, breast carcinoma, head & neck cancer, prostate carcinoma or glioblastoma.

Other embodiments include an isolated polynucleotide molecule comprising a nucleotide sequence encoding a heavy chain amino acid sequence, or a fragment thereof, selected from the group consisting of the heavy chain amino acid sequence of antibodies 13.1.2, 131, 170, 150, 095, 250, 139, 211, 124, 318, 342, and 333 as identified in SEQ ID NO: 138, 2, 4, 5, 7, 9, 10, 12, 13, 15, 16, and 17, or an isolated polynucleotide molecule comprising a nucleotide sequence encoding a light chain amino acid sequence, or a fragment thereof, selected from the group consisting of the light chain amino acid sequence of antibodies 13.1.2, 131, 170, 150, 123, 095, 139, 250, 211, 318, 342, and 333, as identified in SEQ ID NO: 140, 19, 20, 21, 29, 23, 25, 26, 28, 33, 31 and 32.

Further embodiments include an article of manufacture comprising a container, a composition contained therein, and a package insert or label indicating that the composition can be used to treat cancer characterized by the expression of EGFRvIII, wherein the composition comprises an antibody as described above. Such cancers include a lung carcinoma, breast carcinoma, head & neck cancer, prostate carcinoma or glioblastoma. Also included is an assay kit for the detection of EGFRvIII in mammalian tissues or cells in order to screen for lung, colon, gastric, renal, prostate or ovarian carcinomas, the EGFRvIII being an antigen expressed by

epithelial cancers, the kit comprising an antibody that binds the antigen protein and means for indicating the reaction of the antibody with the antigen, if present. The antibody can be a labeled monoclonal antibody, or the antibody can be an unlabeled first antibody and the means for indicating the reaction comprises a labeled second antibody that is anti-immunoglobulin. The antibody that binds the antigen can be labeled with a marker selected from the group consisting of a fluorochrome, an enzyme, a Radionuclide and a radiopaque material. The antibody that binds the antigen can also bind to over-expressed wtEGFR. The kit can be used clinically for patient selection.

A further embodiment includes an antibody which specifically recognizes the epitope of EGFRvIII containing the novel Gly residue.

A further embodiment includes a protein variant of EGFRvIII. The variant may have a pFLAG insert, may consist of the amino acids in SEQ ID NO: 56, and can exist in silico.

Another embodiment includes an antibody, or variant thereof, which binds to the recognition sequence EEKKGNYVVT (SEQ ID NO: 57).

Another embodiment includes an antibody variant that specifically binds to EGFRvIII. The antibody variant can further bind to a peptide that comprises SEQ ID NO: 57. The antibody variant can have residues that interact with residues EKNY or EEKGN in the peptide. In one embodiment, the antibody variant binds to the peptide sequence ten fold more tightly than it does to a wild-type EGFR protein. In one embodiment, the antibody binds specifically binds to EGFRvIII and the peptide of SEQ ID NO: 56. In one embodiment, the isolated antibody or variant has a complementarity determining region comprising a deep cavity, wherein the cavity is created by CDR2 and CDR3 of the heavy chain, CDR3 of the light chain, and a small portion from CDR1 of the , light chain. In one embodiment, the isolated antibody or variant has residues 31, 37, 95-101, 143- 147, 159, 162-166, 169-171, 211-219, 221, and 223 within 5 angstroms of a binding cavity. In one embodiment, the isolated antibody or variant has a complementarity determining region comprising a narrow groove, wherein the groove is created by heavy chain CDR2 and CDR3, and light chain CDR1, CDR2, and CDR3. In one embodiment, the isolated antibody or variant has residues 31, 33, 35-39, 51, 54-56, 58-61, 94-101, 144-148, 160, 163-166, 172, and 211-221 within 5 angstroms of a binding groove. In one embodiment, the isolated antibody or variant has residues 31- 33, 35, 37, 55, 96-101, 148, 163, 165, 170, 172, 178, 217, and 218 within 5 angstroms of a binding groove. In one embodiment, the isolated antibody or variant has a paratope configured so that when the epitope of peptide EEKKGN (SEQ ID NO 127) binds to the paratope of the antibody, at least one bond is formed between two residues selected from the group consisting of E2 and Y172, K3 and H31, K4 and H31, N6 and D33, N6 and Y37, and N6 and K55. In one embodiment, the isolated antibody or variant has a paratope configured so that when the epitope of peptide EEKKGNY (SEQ ID 131) binds to the paratope of the antibody, at least one bond is formed between two residues selected from the group consisting of K4 and Q95, K4 and

Q95, N6 and Q98, G5 and H31, Y7 and H31, Y7 and W165. In one embodiment, the antibody has a structure or interaction with a structure that is determined *in silico*.

Another embodiment provides a method for selecting variants that bind to EGFRvIII with particular binding characteristics, the method comprising the use of a molecular structure to create a paratope, the use of a molecular structure to create an epitope, calculating the interaction energy between the two and comparing that energy level to the energy level of the epitope and a second paratope of a mAb variant, and selecting a variant based on the differences in the energy levels. The method can further include using an interaction energy between a second variant of the paratope and the epitope to determine a third interaction energy and comparing the third interaction energy and the second interaction energy to determine which variant to select. In one embodiment, the variant is created and tested for binding.

Another embodiment provides a method for selecting variants that bind to EGFRvIII with particular binding characteristics, the method comprising examining residues of an epitope which interact with a paratope, selecting important residues to create a recognition sequence, using this sequence to create a EGFRvIII variant, and using the EGFRvIII variant to select the mAb variant.

Another embodiment provides a method for making antibody variants to EGFRvIII, said method comprising analyzing the residues of an epitope which interact with a paratope, selecting the more important residues of an epitope to create a recognition sequence, using the recognition sequence to create an EGFRvIII variant, and using the EGFRvIII variant to select antibody variants. In one embodiment, the selection of the antibodies is achieved *in silico*. In one embodiment, the selection of the antibodies through the use of the EGFRvIII variant is achieved by raising antibodies against EGFRvIII variant.

In the embodiment where the isolated antibody variant binds to EGFRvIII and the peptide of SEQ ID NO: 57, the antibody can further comprise a point mutation of the following: Tyr172Arg, Leu99Glu, Arg101Glu, Leu217Glu, Leu99Asn, Leu99His, L99T, Arg101Asp, or some combination thereof. In one embodiment, the antibody is a monoclonal antibody, a chimeric antibody, a humanized antibody, or a human antibody.

In one embodiment, the antibody or variant thereof binds to the sequence EEKKGNYVVT (SEQ ID NO: 57), and the antibody or variant has subnanomolar binding ability.

In a further embodiment, the antibody binds to EGFRvIII and the antibody has a paratope that binds to an epitope, and the epitope has a set of residues that interact with the paratope that include E, K, N, and Y. In one embodiment, the antibody is antibody 131.

In a further embodiment, the antibody binds to EGFRvIII and the antibody has a paratope that binds to an epitope that has a set of residues that interact with the paratope comprising: E, E, K, G, and N. In one embodiment, the primary structure of the epitope is EEKKGNY (SEQ ID NO: 131). In one embodiment, the antibody is 13.1.2.

In a further embodiment, the antibody that binds to EGFRvIII and has a K_D of less than $1.3*10^{-9}$ M, less than $1.0*10^{-9}$ M, or less than 500 pM. In one embodiment, the antibody is specific for SEQ ID NO: 56 compared to a wild type EGFR peptide. In one embodiment, the nonspecific binding of the antibody to the wild type EGFR peptide (SEQ ID NO: 134) is less than 10% of that of the specific binding of the antibody to EGFRVIII (SEQ ID NO: 135). In one embodiment, the antibody is selected from the group consisting of 131, 139, and 13.1.2. In one embodiment, the antibody is internalized. In one embodiment, the internalization occurs for at least about 70% or at least about 80% of the antibody.

In one embodiment, the variant human monoclonal antibody preferentially binds to an epitope that is substantially unique to an EGFRvIII protein compared to a wild-type EGFR protein or variant thereof (SEQ ID NO: 134). In one embodiment, the variant comprises a heavy chain complementarity determining region (CDR1) corresponding to canonical class 1. In one embodiment, the variant comprises a heavy chain complementarity determining region (CDR2) corresponding to canonical class 3. In one embodiment, the variant comprises a light chain complementarity determining region (CDR1) corresponding to canonical class 4. In one embodiment, the variant comprises a light chain complementarity determining region (CDR2) corresponding to canonical class 1. In one embodiment, the variant comprises a light chain complementarity determining region (CDR3) corresponding to canonical class 1. In one embodiment, the variant comprises a first heavy chain complementarity determining region (CDR1) corresponding to canonical class 1, a second heavy chain complementarity determining region (CDR2) corresponding to canonical class 3, a first light chain complementarity determining region (CDR1) corresponding to canonical class 4, a second light chain complementarity determining region (CDR2) corresponding to canonical class 1; and a third light chain complementarity determining region (CDR3) corresponding to canonical class 1, wherein the complementary determining regions are configured to allow the variant to bind to an epitope that is substantially unique to an EGFRvIII protein as compared to a EGFR protein.

In a further embodiment, a method of inhibiting cell proliferation associated with the expression of EGFRvIII is provided. The method involves treating cells expressing EGFRvIII with an effective amount of an antibody or fragment thereof, wherein said antibody or fragment thereof binds to EGFRvIII, wherein said antibody is conjugated to a toxin and wherein the antibody comprises a heavy chain amino acid sequence selected from the group consisting of the heavy chain amino acid sequence of antibody 13.1.2 (SEQ ID NO: 138), 131 (SEQ ID NO: 2), 170 (SEQ ID NO: 4), 150 (SEQ ID NO: 5), 095 (SEQ ID NO: 7), 250 (SEQ ID NO: 9), 139 (SEQ ID NO: 10), 211 (SEQ ID NO: 12), 124 (SEQ ID NO: 13), 318 (SEQ ID NO: 15), 342 (SEQ ID NO: 16), and 333 (SEQ ID NO: 17). The method can be performed *in vivo*, on a mammal, the mammal can be human, and can be suffering from a cancer involving epithelial cell proliferation, and the cancer

may involve lung, colon, gastric, renal, prostate, breast, glioblastoma or ovarian carcinoma. The toxin may by DM-1, AEFP, MMAE, AURISTATIN E, or ZAP.

In a further embodiment, a method of inhibiting cell proliferation of cells expressing EGFRvIII is provided. The method involves treating cells expressing EGFRvIII with an effective amount of an antibody or fragment thereof, wherein said antibody is conjugated to a toxin, and wherein said antibody has a light chain amino acid sequence selected from the group consisting of the light chain amino acid sequence of antibodies 13.1.2, 131, 170, 150, 123, 095, 139, 250, 211, 342, 333, and 318 as identified in SEQ ID NOs: 19, 20, 21, 29, 23, 25, 26, 28, 33, 31 and 32, wherein said isolated polynucleotide molecule will bind a peptide with the sequence identified in SEQ ID NO: 56. The method can be performed *in vivo*, on a mammal, the mammal can be human, and can be suffering from a cancer involving epithelial cell proliferation, and the cancer may involve lung, colon, gastric, renal, prostate, breast, glioblastoma or ovarian carcinoma. The toxin may by DM-1, AEFP, MMAE, AURISTATIN E, or ZAP.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is an alignment between wild type EGFR and EGFRvIII showing the 267 amino acid deletion and G substition.

FIG. 2 is a diagram of the design of the EGFRvIII PEP3 14-mer peptide. In FIG. 2A, the N-terminal sequence of EGFRvIII with amino acids LEEKK (SEQ ID NO: 58) (1-5) that are identical to the N-terminal seqence of EGFR, followed by the unique Glysine residue, followed by amino acids that are identical to residues 273 through 280 in EGFR. FIG. 2B represents the amino acids of EGFR that are deleted in EGFRvIII (6-272).

FIGs. 3A-L provide sequences of antibodies of the invention. For each antibody provided, a nucleotide and amino acid sequence is provided for both a heavy chain and a light chain variable region. Accordingly, four sequences are provided for every antibody listed.

FIG. 4 is a table comparing the 13.1.2 antibody heavy chain regions to a particular germ line heavy chain region. "-"s indicate that the amino acid residue of the hybridoma heavy chain region is the same as the germ line for that particular position. Deviation from the germline is indicated by the appropriate amino acid residue.

FIG. 5 is a table comparing the 13.1.2 antibody light chain regions to a particular germ line light chain region. "-"s indicate that the amino acid residue of the hybridoma light chain region is the same as the germ line for that particular position. Deviation from the germline is indicated by the appropriate amino acid residue.

FIG. 6 is a table comparing various hybridoma derived antibody heavy chain regions to a particular germ line heavy chain region. "-"s indicate that the amino acid residue of the hybridoma heavy chain region is the same as the germ line for that particular position. Deviation from the germline is indicated by the appropriate amino acid residue.

FIG. 7 is a table comparing various hybridoma derived antibody light chain regions to a particular germ line light chain region. "-"s indicate that the amino acid residue of the hybridoma light chain region is the same as the germ line for that particular position. Deviation from the germline is indicated by the appropriate amino acid residue.

FIG. 8 is a representative figure showing binding of recombinant EGFRvIII mAbs to cells expressing EGFRvIII (NR6 cells). Diamonds represent 95, triangles represent 133, squares represent 139, "x" represent 150, asterixes represent 170, circles represent 221, lines 230, and rectangles represent 250.

FIG. 9A shows FACS staining analysis for a human anti-EGFR antibody (ABX-EGF) to H80.

FIG. 9B shows FACS staining analysis for antibody 131 to H80.

FIG. 9C shows FACS staining analysis for antibody 139 to H80.

FIG. 9D shows FACS staining analysis for antibody 13.1.2 to H80.

FIG. 9E shows FACS staining analysis for ABX-EGF to H1477.

FIG. 9F shows FACS staining analysis for antibody 131 to H1477.

FIG. 9G shows FACS staining analysis for antibody 139 to H1477.

FIG. 9H shows FACS staining analysis for antibody 13.1.2 to H1477.

FIG. 9I shows FACS staining analysis for ABX-EGF to A549.

FIG. 9J shows FACS staining analysis for antibody 131 to A549.

FIG. 9K shows FACS staining analysis for antibody 139 to A549.

FIG. 9L shows FACS staining analysis for antibody 13.1.2 to A549.

FIG. 9M is a graph displaying binding of EGFRvIII mAbs to glioblastoma cells. Filled triangles represent antibody 131 binding to H1477. Filled squares represent antibody 13.1.2 binding to H1477. Empty triangles represent antibody 131 binding to H80. Empty squares represent antibody 13.1.2 binding to H80.

FIG. 9N is a graph displaying the binding of EGFRvIII mAbs to human epidermoid carcinoma cell line A431. The filled squares represent antibody 13.1.2. The filled triangles represent antibody 131.

FIG. 9O is a graph displaying the binding of antibody 13.1.2 to NR6 murine fibroblast cell lines. The squares represent NR6. The triangles represent NR6 with with wild type EDFR. The circles represent NR6 with EGFRvIII.

FIG. 9P is a graph displaying the binding of antibody 131 to murine fibroblast cell lines. The squares represent NR6. The triangles represent NR6 with wild type EGFR. The circles represent NR6 with EGFRvIII.

FIG. 10A shows FACS staining analysis for a human anti-EGFR antibody (ABX-EGF) binding to cells expressing EGFR (A431).

FIG. 10B shows FACS staining analysis for antibody 131 to cells expressing EGFR (A431).

FIG. 10C shows FACS staining analysis for antibody 139 to cells expressing EGFR (A431).

FIG. 10D shows FACS staining analysis for antibody 13.1.2 to cells expressing EGFR (A431).

FIG. 11A shows in vitro toxicities for EGFRvIII antibody 13.1.2 indirectly conjugated to AEFP in EGFRvIII expressing cells (H1477, circles) versus cells that do not express EGFRvIII (H80, squares).

FIG. 11B shows in vitro toxicities for EGFRvIII antibody 13.1.2 indirectly conjugated to DM1 in EGFRvIII expressing cells (H1477, circles) versus cells that do not express EGFRvIII (H80, squares).

FIG. 11C shows in vitro toxicities for EGFRvIII antibody 13.1.2 indirectly conjugated to ZAP in EGFRvIII expressing cells (H1477, circles) versus cells that do not express EGFRvIII (H80, squares).

FIG. 11D shows in vitro toxicities for EGFRvIII antibody 95 indirectly conjugated to AEFP in EGFRvIII expressing cells (H1477, circles) versus cells that do not express EGFRvIII (H80, squares).

FIG. 11E shows in vitro toxicities for EGFRvIII antibody 95 indirectly conjugated to DM1 in EGFRvIII expressing cells (H1477, circles) versus cells that do not express EGFRvIII (H80, squares).

FIG. 11F shows in vitro toxicities for EGFRvIII antibody 95 indirectly conjugated to ZAP in EGFRvIII expressing cells (H1477, circles) versus cells that do not express EGFRvIII (H80, squares).

FIG. 11G shows in vitro toxicities for EGFRvIII antibody 131 indirectly conjugated to AEFP in EGFRvIII expressing cells (H1477, circles) versus cells that do not express EGFRvIII (H80, squares).

FIG. 11H shows in vitro toxicities for EGFRvIII antibody 131 indirectly conjugated to DM1 in EGFRvIII expressing cells (H1477, circles) versus cells that do not express EGFRvIII (H80, squares).

FIG. 11I shows in vitro toxicities for EGFRvIII antibody 131 indirectly conjugated to ZAP in EGFRvIII expressing cells (H1477, circles) versus cells that do not express EGFRvIII (H80, squares).

FIG. 12A shows in vitro toxicities for EGFRvIII antibody 139 indirectly conjugated AEFP in EGFRvIII expressing cells (H1477, circles) versus cells that do not express EGFRvIII (H80, squares).

FIG. 12B shows in vitro toxicities for EGFRvIII antibody 139 indirectly conjugated DM1 in EGFRvIII expressing cells (H1477, circles) versus cells that do not express EGFRvIII (H80, squares).

FIG. 12C shows in vitro toxicities for EGFRvIII antibody 139 indirectly conjugated ZAP in EGFRvIII expressing cells (H1477, circles) versus cells that do not express EGFRvIII (H80, squares).

FIG. 12D shows in vitro toxicities for EGFRvIII antibody 150 indirectly conjugated AEFP in EGFRvIII expressing cells (H1477, circles) versus cells that do not express EGFRvIII (H80, squares).

FIG. 12E shows in vitro toxicities for EGFRvIII antibody 150 indirectly conjugated DM1 in EGFRvIII expressing cells (H1477, circles) versus cells that do not express EGFRvIII (H80, squares).

FIG. 12F shows in vitro toxicities for EGFRvIII antibody 150 indirectly conjugated ZAP in EGFRvIII expressing cells (H1477, circles) versus cells that do not express EGFRvIII (H80, squares).

FIG. 12G shows in vitro toxicities for EGFRvIII antibody 170 indirectly conjugated AEFP in EGFRvIII expressing cells (H1477, circles) versus cells that do not express EGFRvIII (H80, squares).

FIG. 12H shows in vitro toxicities for EGFRvIII antibody 150 indirectly conjugated DM1 in EGFRvIII expressing cells (H1477, circles) versus cells that do not express EGFRvIII (H80, squares).

FIG. 12I shows in vitro toxicities for EGFRvIII antibody 150 indirectly conjugated ZAP in EGFRvIII expressing cells (H1477, circles) versus cells that do not express EGFRvIII (H80, squares).

FIG. 13A shows in vitro toxicities for antibody 211 indirectly conjugated to AEFP in EGFRvIII expressing cells (H1477, circles) versus cells that do not express EGFRvIII (H80, squares).

FIG. 13B shows in vitro toxicities for antibody 211 indirectly conjugated to DM1 in EGFRvIII expressing cells (H1477, circles) versus cells that do not express EGFRvIII (H80, squares).

FIG. 13C shows in vitro toxicities for antibody 211 indirectly conjugated to ZAP in EGFRvIII expressing cells (H1477, circles) versus cells that do not express EGFRvIII (H80, squares).

FIG. 13D shows in vitro toxicities for antibody 250 indirectly conjugated to AEFP in EGFRvIII expressing cells (H1477, circles) versus cells that do not express EGFRvIII (H80, squares).

FIG. 13E shows in vitro toxicities for antibody 250 indirectly conjugated to DM1 in EGFRvIII expressing cells (H1477, circles) versus cells that do not express EGFRvIII (H80, squares).

FIG. 13F shows in vitro toxicities for antibody 250 indirectly conjugated to ZAP in EGFRvIII expressing cells (H1477, circles) versus cells that do not express EGFRvIII (H80, squares).

FIG. 13G shows in vitro toxicities for antibody IgG1, a negative control, indirectly conjugated to AEFP in EGFRvIII expressing cells (H1477, circles) versus cells that do not express EGFRvIII (H80, squares).

FIG. 13H shows in vitro toxicities for antibody IgG1, a negative control, indirectly conjugated to DM1 in EGFRvIII expressing cells (H1477, circles) versus cells that do not express EGFRvIII (H80, squares).

FIG. 13I shows in vitro toxicities for antibody IgG1, a negative control, indirectly conjugated to ZAP in EGFRvIII expressing cells (H1477, circles) versus cells that do not express EGFRvIII (H80, squares).

FIG. 14A is bar graph that demonstrates that EGFRvIII antibodies (13.1.2, 131, and 139) inhibit colony formation in H1477 cells in a clongenic assay when conjugated to AEFP.

FIG. 14B is a bar graph that demonstrates that EGFRvIII antibodies (13.1.2, 131, and 139) inhibit colony formation in H1477 cells in a clongenic assay when conjugated to DM1.

FIG. 15A is a graph showing in vitro toxicities of direct conjugates of anti-EGFRvIII antibodies (13.1.2) with toxin, MMAE, in EGFRvIII expressing cells (H1477, circles) versus cells that do not express EGFRvIII (H80, squares).

FIG. 15B is a graph showing in vitro toxicities of direct conjugates of anti-EGFRvIII antibodies (13.1.2) with toxin, AEFP, in EGFRvIII expressing cells (H1477, circles) versus cells that do not express EGFRvIII (H80, squares).

FIG. 15C is a graph showing in vitro toxicities of direct conjugates of anti-EGFRvIII antibodies (13.1.2) with toxin, DM1, in EGFRvIII expressing cells (H1477) versus cells that do not express EGFRvIII (H80).

FIG. 16 shows the results of an *in vivo* animal model in which mice having an established tumor xenograft were treated with an anti-EGFRvIII (or dEGFR) antibody (13.1.2), that was directly conjugated to a toxin (DM1, MMAE, or AEFP). Filled squares represent 250 micrograms of dEGFR-DM1. Filled upward pointing triangles represent 75 micrograms of the same. Filled downward pointing triangles represents 75 micrograms of dEGFR-MMAE. Diamonds represent 250 micrograms of the same. The lighter square represents 75 micrograms of dEGFR-AEFP. The empty square represents 250 micrograms of the same. The empty downward pointing triangle represents dEGFR and free DM1. The empty upward pointing antibody represents PBS. All antibodies used were 13.1.2. Arrows indicate pro-drug treatments.

FIG. 17 shows the molecular surface of antibody 131 structure model. The six CDRs are shaded different shades to mark their boundries. The binding cavity is located close to the center.

FIG. 18 shows a structural model of the molecular surface of antibody 13.1.2. The six CDR regions are shaded and identified by number. The long groove is located approximately along the vertical centerline.

FIG. 19A is a possible docking model of the 13.1.2 antibody and peptide EEKKGN (SEQ ID NO: 127) complex. The CDR regions are shaded to denote boundries.

FIG. 19B shows the hydrogen bonds in the docking model of the 13.1.2 antibody and peptide EEKKGN (SEQ ID NO: 127) complex. Shading of the CDR loops and residues is the same as in FIG. 18. The peptide residue is numbered from the N-terminus, at the top of the figure, to the C-terminus as 1 through 6. Six hydrogen bonds are indicated by dashed lines. The six pairs of amino acids forming hydrogen bonds are: E2...Y172, K3...H31, K4...H31, N6...D33, N6...Y37, and N6...K55.

FIG. 20 is a graph demonstrating a correlation between the epitope-antibody binding energy and the logrithm of Kd for one of the docking models selected.

FIG. 21 is a depiction of a refined docking model for the peptide-13.1.2 antibody complex. The peptide is rendered in a space-filling manner..

FIG. 22 is a depiction representing the hydrogen bonds in the refined docking model.

FIG. 23 is a graph that depicts the linear fitting of antibody-antigen binding energy versus the logrithm of relative affinities.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

As discussed above, EGFRvIII is a deletion mutant of EGFR in which 267 amino acids in the extracellular domain of EGFr are deleted with a single amino acid substitution of Glycine at the junction. These features are shown in a sequence alignment between wild type EGFR and EGFRvIII in FIG. 1. In view of the amino acid substitution of Glycine at the junction of the deletion, it becomes theoretically possible to generate antibodies to the novel epitope present in EGFRvIII that is not present in wild type EGFR. Thus, a peptide for immunization and screening was designed, termed PEP3, as shown in FIG. 2 (Kuan et al. EGF mutant receptor vIII as a molecular target in cancer therapy. Endocr Relat Cancer. 8(2):83-96 (2001)). Such 14-mer peptide possesses the 5 n-terminal amino acids common to EGFRvIII and wild type EGFR, the unique Glycine junction site, and 8 amino acid residues contained in the conserved sequences between wild type EGFR (corresponding to residues 273-280) and EGFRvIII (corresponding to residues 7-14). In addition, glioblastoma cell and cells (B300.19 cells) transfected with the gene encoding EGFRvIII were also utilized for immunization and screening (sometimes referred to herein as B300.19/EGFRvIII transfectants).

In order to generate human antibodies against EGFRvIII, transgenic XenoMouse® mice were immunized with combinations of glioblastoma cells/EGFRvIII, B300.19/EGFRvIII cells, and

peptides (PEP3) directed to the junction region in the novel extracellular domain represented in EGFRvIII as compared to wild type EGFR. B cells from immunized mice were isolated and either used to produce hybridomas followed by screening for binding to EGFRvIII or used directly in screening for binding to EGFRvIII using XenoMax™/SLAM™ technologies (Babcook et al. A novel strategy for generating monoclonal antibodies from single, isolated lymphocytes producing antibodies of defined specificities. Proc Natl Acad Sci U S A.93(15):7843-8 (1996), and U.S. Patent No. 5,627,052). Antibodies identified that bound to EGFRvIII were screened in a series of assays to ascertain specific recognition of EGFRvIII. Through this process, panels of human monoclonal antibodies that bound to and were specific for EGFRvIII were generated, isolated, and characterized. Subsequent epitope mapping demonstrated unique but overlapping specificities . All antibodies were further evaluated *in vitro* for their ability to be internalized by cells for the purpose of delivering cytotoxic drugs to cells. Antibodies demonstrating efficient drug delivery were directly conjugated with a cytotoxic drug and examined for their ability to kill tumor cells expressing EGFRvIII *in vitro* and *in vivo*. These studies provide the basis for the next generation of antibody drug conjugates for treating cancer in patients whose tumor harbor specific genetic lesions.

Through the processes described above, panels of fully human anti-EGFRvIII antibodies were generated. Using the hybridoma approach, several antibodies, including antibody 13.1, 13.2, 13.3, and 13.4 that were positive on ELISA for binding with the PEP3, were generated with limited cross-reactivity with wild type EGFR. Out of these, antibody 13.1 (and, particularly, its subclone 13.1.2) was selected for further research and development. Using the XenoMax approach a panel of antibodies, including antibody 131, 139, 250, and 095, were generated that were highly specific for binding with the pep3 oligonucleotide and had limited cross-reactivity with wild type EGFR. Of these, the 131 antibody has very interesting properties. The sequences for each of the antibodies are displayed in FIGs. 4-7 (SEQ ID NO: 1-33 and 141-144). A comparison of the sequences and binding abilities of the various antibodies was made and the results are displayed in FIGs. 4-10. As can be seen in FIGs. 9A-9L, and FIGs. 10A-10D antibodies 131, 139, and 13.1.2 all demonstrated superior selectivity for EGFRvIII expressing cells (H1477) as compared to ABX-EGF. Some of the results are shown in graph form in FIGs. 9M-9P, which demonstrates that at least two of the antibodies, 13.1.2 and 131 demonstrated superior specificity for EGFRvIII expressing cells compared to simply EGFRvIII cells. Additionally, several possibile utilities for the antibodies of the current embodiment were examined; the results of which are shown in FIGs. 11-16. Finally, based on predicted structural models, variants of the antibodies were made in order to obtain antibodies with altered binding characteristics.

Further, antibodies of the invention are highly useful for the screening of other antibodies that bind to the same or similar epitopes. Antibodies of the invention can be utilized in cross

competition studies for the elucidation of other antibodies that are expected to have the same or improved effects with respect to characteristics of the antigen-antibody complex that is formed.

Each of the 131 antibody and the 13.1.2 possessed very high affinities for EGFRvIII, were internalized well by cells, and appeared highly effective in cell killing when conjugated to toxins. Intriguingly, both of the antibodies, despite having been generated in different immunizations of XenoMouse mice, and utilizing different technologies, were derived from very similar germline genes. Based upon epitope mapping work, however, each of the antibodies appears to bind to slightly different epitopes on the EGFRvIII molecule and have slightly different residues on EGFRvIII that are essential for binding. These results indicate that the germline gene utilization is of importance to the generation of antibody therapeutics targeting EGFRvIII and that small changes can modify the binding and effects of the antibody in ways that allow for the further design of antibodies and other therapeutics based upon these structural findings.

Antibodies that bind to the same epitope as, or compete for binding with, the 13.1.2 and 131 antibodies are highly desirable. As discussed in more detail below, through Alanine scanning on SPOTs arrays important residues for binding of certain antibodies have been elucidated. Accordingly, antibdodies that share critical binding residues are also highly desirable.

Definitions

Unless otherwise defined, scientific and technical terms used herein shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, 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* (2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989). 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.

The term "isolated polynucleotide" as used herein shall mean a polynucleotide of genomic, cDNA, or synthetic origin or some combination thereof, which by virtue of its origin the "isolated

polynucleotide" (1) is not associated with all or a portion of a polynucleotide in which the "isolated polynucleotide" is found in nature, (2) is 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.

The term "isolated protein" referred to herein means a protein of cDNA, recombinant RNA, or synthetic origin or some combination thereof, which by virtue of its origin, or source of derivation, the "isolated protein" (1) is not associated with proteins found in nature, (2) is free of other proteins from the same source, e.g. free of murine proteins, (3) is expressed by a cell from a different species, or (4) does not occur in nature.

The term "polypeptide" is used herein as a generic term to refer to native protein, fragments, or analogs of a polypeptide sequence. Hence, native protein, fragments, and analogs are species of the polypeptide genus. Preferred polypeptides in accordance with the invention comprise the human heavy chain immunoglobulin molecules and the human kappa light chain immunoglobulin molecules, as well as antibody molecules formed by combinations comprising the heavy chain immunoglobulin molecules with light chain immunoglobulin molecules, such as the kappa light chain immunoglobulin molecules or lambda light chain immunoglobulin molecules, and vice versa, as well as fragments and analogs thereof.

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 or otherwise is naturally-occurring.

The term "operably linked" as used herein refers to positions of components so described are in a relationship permitting them to function in their intended manner. A 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 control sequences.

The term "control sequence" as used herein refers to polynucleotide sequences which 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 promoter, ribosomal binding site, and transcription termination sequence; in eukaryotes, generally, such control sequences include promoters and transcription termination sequence. The term "control sequences" 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.

The term "polynucleotide" 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. The term includes single and double stranded forms of DNA.

The term "oligonucleotide" referred to herein includes naturally occurring, and modified nucleotides linked together by naturally occurring, and non-naturally occurring oligonucleotide linkages. Oligonucleotides are a polynucleotide subset generally comprising a length of 200 bases or fewer. Preferably oligonucleotides are 10 to 60 bases in length and most preferably 12, 13, 14, 15, 16, 17, 18, 19, or 20 to 40 bases in length. Oligonucleotides are usually single stranded, e.g. for probes; although oligonucleotides may be double stranded, e.g. for use in the construction of a gene mutant. Oligonucleotides of the invention can be either sense or antisense oligonucleotides.

The term "naturally occurring nucleotides" referred to herein includes deoxyribonucleotides and ribonucleotides. The term "modified nucleotides" referred to herein includes nucleotides with modified or substituted sugar groups and the like. The term "oligonucleotide linkages" referred to herein includes oligonucleotides linkages such as phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoraniladate, phosphoroamidate, and the like. *See e.g.*, LaPlanche et al. *Nucl. Acids Res.* 14:9081 (1986); Stec et al. *J. Am. Chem. Soc.* 106:6077 (1984); Stein et al. *Nucl. Acids Res.* 16:3209 (1988); Zon et al. *Anti-Cancer Drug Design* 6:539 (1991); Zon et al. *Oligonucleotides and Analogues: A Practical Approach*, pp. 87-108 (F. Eckstein, Ed., Oxford University Press, Oxford England (1991)); Stec et al. U.S. Patent No. 5,151,510; Uhlmann and Peyman *Chemical Reviews* 90:543 (1990). An oligonucleotide can include a label for detection, if desired.

The term "variant" as used herein, is a polypeptide, polynucleotide, or molecule that differs from the recited polypeptide or polynucleotide, but only such that the activity of the protein is not detrimentally altered. There may be variants of epitopes. There may be variants of antibodies. In a preferred embodiment, the ability of a protein variant to bind to the epitope is not detrimentally altered. In one embodiment, the protein variant can bind with 10-500% of the ability of the wild type mAb. For example, the protein variant can bind with 10%, 50%, 110%, 500%, or greater than 500% of the ability of the wild type mAb. In one embodiment, the range of binding abilities between 10-500% is inlcuded. Binding ability may be reflected in many ways, including, but not limited to the k_a, k_d, or K_D of the variant to an epitope. In one preferred embodiment, the epitope is one described in the present specification.

In one embodiment, variant antibodies can differ from the wild-type sequence by substitution, deletion or addition of five amino acids or fewer. Such variants may generally be identified by modifying one of the disclosed polypeptide sequences, and evaluating the binding properties of the modified polypeptide using, for example, the representative procedures described herein. In another embodiment, polypeptide variants preferably exhibit at least about 70%, more preferably at least about 90% and most preferably at least about 95% identity to the identified polypeptides. Preferrably, the variant differs only in conservative substitutions and/or modifications. Variant proteins include those that are structurally similar and those that are functionally equivalent to the protein structures described in the present specification. In another

embodiment, the protein is a variant if it is functionally equivalent to the proteins described in this specification, so long as the paratope of variant is similar to the paratopes described in the specification. In one embodiment, any substance with a shape that is similar to the paratope described in FIG. 17 is a variant. In one embodiment, any substance with a shape that is similar to the paratope described in FIG. 18 is a variant. In one embodiment, any substance that has a shape that is similar to the interaction surface described in FIG. 19A and 19B is a variant.

In one embodiment, the antibody is a variant if the nucleic acid sequence can selectively hybridize to wild-type sequence under stringent conditions. In one embodiment, suitable moderately stringent conditions include prewashing in a solution of 5xSSC; 0.5% SDS, 1.0 mM EDTA (pH 8:0); hybridizing at 50°C-65°C, 5xSSC, overnight or, in the event of cross-species homology, at 45°C with 0.5xSSC; followed by washing twice at 65°C for 20 minutes with each of 2x, 0.5x and 0.2xSSC containing 0.1% SDS. Such hybridizing DNA sequences are also within the scope of this invention, as are nucleotide sequences that, due to code degeneracy, encode an antibody polypeptide that is encoded by a hybridizing DNA sequence.The term "selectively hybridize" referred to herein means to detectably and specifically bind. Polynucleotides, oligonucleotides and fragments thereof in accordance with the invention selectively hybridize to nucleic acid strands under hybridization and wash conditions that minimize appreciable amounts of detectable binding to nonspecific nucleic acids. High stringency conditions can be used to achieve selective hybridization conditions as known in the art and discussed herein. Generally, the nucleic acid sequence homology between the polynucleotides, oligonucleotides, and fragments of the invention and a nucleic acid sequence of interest will be at least 80%, and more typically with preferably increasing homologies of at least 85%, 90%, 95%, 99%, and 100%. Two amino acid sequences are homologous if there is a partial or complete identity between their sequences. For example, 85% homology means that 85% of the amino acids are identical when the two sequences are aligned for maximum matching. Gaps (in either of the two sequences being matched) are allowed in maximizing matching; gap lengths of 5 or less are preferred with 2 or less being more preferred. Alternatively and preferably, two protein sequences (or polypeptide sequences derived from them of at least 30 amino acids in length) are homologous, as this term is used herein, if they have an alignment score of at more than 5 (in standard deviation units) using the program ALIGN with the mutation data matrix and a gap penalty of 6 or greater. *See* Dayhoff, M.O., in *Atlas of Protein Sequence and Structure*, pp. 101-110 (Volume 5, National Biomedical Research Foundation (1972)) and Supplement 2 to this volume, pp. 1-10. The two sequences or parts thereof are more preferably homologous if their amino acids are greater than or equal to 50% identical when optimally aligned using the ALIGN program. The term "corresponds to" is used herein to mean that a polynucleotide sequence is homologous (i.e., is identical, not strictly evolutionarily related) to all or a portion of a reference polynucleotide sequence, or that a polypeptide sequence is identical to a reference polypeptide sequence. In contradistinction, the term "complementary to" is

used herein to mean that the complementary sequence is homologous to all or a portion of a reference polynucleotide sequence. For illustration, the nucleotide sequence "TATAC" corresponds to a reference sequence "TATAC" and is complementary to a reference sequence "GTATA".

The following terms are used to describe the sequence relationships between two or more polynucleotide or amino acid sequences: "reference sequence", "comparison window", "sequence identity", "percentage of sequence identity", and "substantial identity". A "reference sequence" is a defined sequence used as a basis for a sequence comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length cDNA or gene sequence given in a sequence listing or may comprise a complete cDNA or gene sequence. Generally, a reference sequence is at least 18 nucleotides or 6 amino acids in length, frequently at least 24 nucleotides or 8 amino acids in length, and often at least 48 nucleotides or 16 amino acids in length. Since two polynucleotides or amino acid sequences may each (1) comprise a sequence (i.e., a portion of the complete polynucleotide or amino acid sequence) that is similar between the two molecules, and (2) may further comprise a sequence that is divergent between the two polynucleotides or amino acid sequences, sequence comparisons between two (or more) molecules are typically performed by comparing sequences of the two molecules over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window", as used herein, refers to a conceptual segment of at least 18 contiguous nucleotide positions or 6 amino acids wherein a polynucleotide sequence or amino acid sequence may be compared to a reference sequence of at least 18 contiguous nucleotides or 6 amino acid sequences and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions, deletions, substitutions, and the like (i.e., gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith and Waterman *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman and Wunsch *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson and Lipman *Proc. Natl. Acad. Sci. (U.S.A.)* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, (Genetics Computer Group, 575 Science Dr., Madison, Wis.), Geneworks, or MacVector software packages), or by inspection, and the best alignment (i.e., resulting in the highest percentage of homology over the comparison window) generated by the various methods is selected.

The term "sequence identity" means that two polynucleotide or amino acid sequences are identical (i.e., on a nucleotide-by-nucleotide or residue-by-residue basis) over the comparison window. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) or residue occurs in both sequences to yield

the number of matched positions, dividing the number of matched positions by the total number of positions in the comparison window (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. The terms "substantial identity" as used herein denotes a characteristic of a polynucleotide or amino acid sequence, wherein the polynucleotide or amino acid comprises a sequence that has at least 85 percent sequence identity, preferably at least 90 to 95 percent sequence identity, more usually at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 18 nucleotide (6 amino acid) positions, frequently over a window of at least 24-48 nucleotide (8-16 amino acid) positions, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the comparison window. The reference sequence may be a subset of a larger sequence. Amino acids or nucleic acids with substantial identity to the wild-type protein or nucleic acid are examples of variants of the wild-type protein or nucleic acid.

As used herein, the twenty conventional amino acids and their abbreviations follow conventional usage. *See Immunology - A Synthesis* (2nd Edition, E.S. Golub and D.R. Gren, Eds., Sinauer Associates, Sunderland, Mass. (1991)). Stereoisomers (e.g., D-amino acids) of the twenty conventional amino acids, unnatural amino acids such as α-, α-disubstituted amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids may also be suitable components for polypeptides of the present invention. Examples of unconventional amino acids include: 4-hydroxyproline, γ-carboxyglutamate, ε-N,N,N-trimethyllysine, ε-N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, σ-N-methylarginine, and other similar amino acids and imino acids (e.g., 4-hydroxyproline). In the polypeptide notation used herein, the left-hand direction is the amino terminal direction and the right-hand direction is the carboxy-terminal direction, in accordance with standard usage and convention.

Similarly, unless specified otherwise, the left-hand end of single-stranded polynucleotide sequences is the 5' end; the left-hand direction of double-stranded polynucleotide sequences is referred to as the 5' direction. The direction of 5' to 3' addition of nascent RNA transcripts is referred to as the transcription direction; sequence regions on the DNA strand having the same sequence as the RNA and which are 5' to the 5' end of the RNA transcript are referred to as "upstream sequences"; sequence regions on the DNA strand having the same sequence as the RNA and which are 3' to the 3' end of the RNA transcript are referred to as "downstream sequences".

As applied to polypeptides, the term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity, and most preferably at least 99 percent sequence identity. Preferably, residue positions which are not identical differ by conservative amino acid substitutions. Conservative amino acid substitutions refer to the interchangeability of

residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, glutamic-aspartic, and asparagine-glutamine. Polypeptides with substantial identity can be variants.

Variant proteins also include proteins with minor variations. As discussed herein, minor variations in the amino acid sequences of antibodies or immunoglobulin molecules 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%. 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. Genetically encoded amino acids are generally divided into families: (1) acidic=aspartate, glutamate; (2) basic=lysine, arginine, histidine; (3) non-polar=alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar=glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. More preferred families are: serine and threonine are aliphatic-hydroxy family; asparagine and glutamine are an amide-containing family; alanine, valine, leucine and isoleucine are an aliphatic family; and phenylalanine, tryptophan, and tyrosine are an aromatic family. 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 molecule, especially if the replacement does not involve an amino acid within a framework site. Whether an amino acid change results in a functional peptide can readily be determined by assaying the specific activity of the polypeptide derivative. Assays are described in detail herein. Fragments or analogs of antibodies or immunoglobulin molecules can be readily prepared by those of ordinary skill in the art. Preferred amino- and carboxy-termini of fragments or analogs 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. Preferably, computerized comparison methods are used to identify sequence motifs or predicted protein conformation domains that occur in other proteins of known structure and/or function. Methods to identify protein sequences that fold into a known three-dimensional structure are known. Bowie et al. *Science* 253:164 (1991). Thus, the foregoing examples demonstrate that

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 antibodies described herein.

Preferred amino acid substitutions are those which: (1) reduce susceptibility to proteolysis, (2) reduce susceptibility to oxidation, (3) alter binding affinity for forming protein complexes, (4) alter binding affinities, and (4) confer or modify other physicochemical or functional properties of such analogs. Analogs can include various muteins of a sequence other than the naturally-occurring peptide sequence. For example, single or multiple amino acid substitutions (preferably conservative amino acid substitutions) may be made in the naturally-occurring sequence (preferably in the portion of the polypeptide outside the domain(s) forming intermolecular contacts. A conservative amino acid substitution should not substantially change the structural characteristics of the parent sequence (e.g., a replacement amino acid should not tend to break a helix that occurs in the parent sequence, or disrupt other types of secondary structure that characterizes the parent sequence). Examples of art-recognized polypeptide secondary and tertiary structures are described in *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).

The term "polypeptide fragment" as used herein refers to a polypeptide that has an amino-terminal and/or carboxy-terminal deletion, but where the remaining amino acid sequence is identical to the corresponding positions in the naturally-occurring sequence deduced, for example, from a full-length cDNA sequence. Fragments typically are at least 5, 6, 8 or 10 amino acids long, preferably at least 14 amino acids long, more preferably at least 20 amino acids long, usually at least 50 amino acids long, and even more preferably at least 70 amino acids long. The term "analog" as used herein refers to polypeptides which are comprised of a segment of at least 25 amino acids that has substantial identity to a portion of a deduced amino acid sequence. Analogs typically are at least 20 amino acids long, preferably at least 50 amino acids long or longer, and can often be as long as a full-length naturally-occurring polypeptide. Both fragments and analogs are forms of variants

Peptide analogs are commonly used in the pharmaceutical industry as non-peptide drugs with properties analogous to those of the template peptide. These types of non-peptide compound are termed "peptide mimetics" or "peptidomimetics". Fauchere, *J. Adv. Drug Res.* 15:29 (1986); Veber and Freidinger *TINS* p.392 (1985); and Evans et al. *J. Med. Chem.* 30:1229 (1987). Such compounds are often developed with the aid of computerized molecular modeling. Peptide mimetics that are structurally similar to therapeutically useful peptides may be used to produce an equivalent therapeutic or prophylactic effect. Generally, peptidomimetics are structurally similar to a paradigm polypeptide (i.e., a polypeptide that has a biochemical property or pharmacological activity), such as human antibody, but have one or more peptide linkages optionally replaced by a linkage selected from the group consisting of: --CH_2NH--, --CH_2S--, --CH_2-CH_2--, --CH=CH--(cis

and trans), --$COCH_2$--, --$CH(OH)CH_2$--, and --CH_2SO--, by methods well known in the art. Systematic substitution of one or more amino acids of a consensus sequence with a D-amino acid of the same type (e.g., D-lysine in place of L-lysine) may be used to generate more stable peptides. In addition, constrained peptides comprising a consensus sequence or a substantially identical consensus sequence variation may be generated by methods known in the art (Rizo and Gierasch *Ann. Rev. Biochem.* 61:387 (1992)); for example, by adding internal cysteine residues capable of forming intramolecular disulfide bridges which cyclize the peptide. Peptide mimetics and peptidomimetics are both forms of variants.

"Antibody" or "antibody peptide(s)" refer to an intact antibody, or a binding fragment thereof that competes with the intact antibody for specific binding. Binding fragments are produced by recombinant DNA techniques, or by enzymatic or chemical cleavage of intact antibodies. Binding fragments include Fab, Fab', $F(ab')_2$, Fv, and single-chain antibodies. An antibody other than a "bispecific" or "bifunctional" antibody is understood to have each of its binding sites identical. An antibody substantially inhibits adhesion of a receptor to a counterreceptor when an excess of antibody reduces the quantity of receptor bound to counterreceptor by at least about 20%, 40%, 60% or 80%, and more usually greater than about 85% (as measured in an *in vitro* competitive binding assay).

The term "epitope" includes any protein determinant capable of specific binding to an immunoglobulin or T-cell receptor or otherwise interacting with a molecule. Epitopic determinants generally consist of chemically active surface groupings of molecules such as amino acids or carbohydrate or sugar side chains and generally have specific three-dimensional structural characteristics, as well as specific charge characteristics. An epitope may be "linear" or "conformational." In a linear epitope, all of the points of interaction between the protein and the interacting molecule (such as an antibody) occur linearally along the primary amino acid sequence of the protein. In a conformational epitope, the points of interaction occur across amino acid residues on the protein that are separated from one another. An antibody is said to specifically bind an antigen when the dissociation constant is ≤ 1 μM, preferably ≤ 100 nM and more preferably ≤ 10 nM, and even more preferably $\leq$ 1nM. Once a desired epitope on an antigen is determined, it is possible to generate antibodies to that epitope, e.g., using the techniques described in the present invention. Alternatively, during the discovery process, the generation and characterization of antibodies may elucidate information about desirable epitopes. From this information, it is then possible to competitively screen antibodies for binding to the same epitope. An approach to achieve this is to conduct cross-competition studies to find antibodies that competively bind with one another, e.g., the antibodies compete for binding to the antigen. A high throughput process for "binning" antibodies based upon their cross-competition is described in International Patent Application No. WO 03/48731. As will be appreciated by one of skill in the art, practically anything to which an antibody can specificially bind could be an epitope. An epitope can

comprises those residues to which the antibody binds. In one embodiment, the epitope is the EGFRvIII epitope. In a more preferred embodiment, the epitope is that described in Example 4 of this specification. In one embodiment, the epitope is the epitope described in Example 14. In one embodiment, the epitope comprises the sequence LEEKKGNYVVTD (SEQ ID NO: 59). In one embodiment, the epitope comprises the sequence EEKKGNYVVT (SEQ ID NO: 57). In one embodiment, the epitope comprises the sequence EKNY (SEQ ID NO: 60). In one embodiment, the epitope comprises the sequence EEKGN (SEQ ID NO: 61). One of skill in the art will appreciate that these need not be actually assembled in this order on a single peptide, rather, these are the residues that form the eptiope which interacts with the paratope. As will be appreciated by one of skill in the art, the space that is occupied by a residue or side chain that creates the shape of a molecule helps to determine what an epitope is. Likewise, any functional groups associated with the epitope, van der Waals interactions, degree of mobility of side chains, etc. can all determine what an epitope actually is. Thus an epitope may also include energetic interactions.

The term "paratope" is meant to describe the general structure of a binding region that determines binding to an epitope. This structure influences whether or not and in what manner the binding region might bind to an epitope. Paratope can refer to an antigenic site of an antibody that is responsible for an antibody or fragment thereof, to bind to an antigenic determinant. Paratope also refers to the idiotope of the antibody, and the complementary determining region (CDR) region that binds to the epitope. In one embodiment, the paratope is the region of the antibody that is L1 10, L2 30, L3 50, H1 20, H2 40, and H3 60 in FIG. 17. In one embodiment, the paratope is the region of the antibody that comprises the CDR sequences in Example 16 for L1, L2, L3, H1, H2, and H3. In one embodiment, the paratope is the region of the antibody that is L1 110, L2 130, L3 150, H1 120, H2 140, and H3 160 in FIG. 18. In one embodiment, the paratope is the region of the antibody that comprises the CDR sequences in Example 18 for L1, L2, L3, H1, H2, and H3. In one embodiment, the paratope comprises the sequences listed in Example 18. In one embodiment, the paratope comprises the residues that interact with the epitope, as shown in FIG. 19A and FIG. 19B. The solid black structure is the peptide structure. In one embodiment, the paratope comprises residue Tyr172Arg of the 13.1.2 mAb. In one embodiment, the paratope of the 13.1.2 mAb comprises at least one residue selected from the group consisting of: Tyr 172Arg, Arg101Glu, Leu99Asn, Leu99His, Arg101Asp, Leu217Gln, Leu99Thr, Leu217Asn, Arg101Gln, and Asn35Gly. As will be appreciated by one of skill in the art, the paratope of any antibody, or variant thereof, can be determined in the manner set forth by the present application. Residues are considered "important" if they are predicted to contribute 10% of the binding energy. In one embodiment, residues are considered "important" if they are predicted to contribute 2% of the binding energy. In one embodiment, residues are considered "important" if they are predicted to contribute 50% of the binding energy. In one embodiment, residues are considered "important" if they are predicted to

interact with the surface of the epitope, or the surface of the paratope. In one embodiment, residues are considered "important" if changing the residue results in a loss in binding.

The terms "specifically" or "preferrentially" binds to, or similar phrases are not meant to denote that the antibody exclusively binds to that epitope. Rather, what is meant is that the antibody, or variant thereof, can bind to that epitope, to a higher degree than the antibody binds to at least one other substance to which the antibody is exposed to. In one embodiment, the specifically binding antibody will bind to the EGFRvIII protein with an affinity greater than (more tightly, or lower K_D) it will to the EGFR protein. For example, the specifically binding antibody will bind more tightly by at least a minimal increase to 1, 1-2, 2-5, 5-10, 10-20, 20-30, 30-50, 50-70, 70-90, 90-120, 120-150, 150-200, 200-300, 300-500, 500-1000 percent or more.

The shorthand of amino acid, number, amino acid, e.g., Leu217Gln, denotes a mutation at the numbered amino acid, from the first amino acid, to the second amino acid. Thus, Tyr172Arg would mean that while the wild type protein has a tyrosine at position 172, the mutant has an arginine at position 172.

The term "agent" is used herein to denote a chemical compound, a mixture of chemical compounds, a biological macromolecule, or an extract made from biological materials.

"Mammal" when used herein refers to any animal that is considered a mammal. Preferably, the mammal is human.

Digestion of antibodies with the enzyme, papain, results in two identical antigen-binding fragments, known also as "Fab" fragments, and a "Fc" fragment, having no antigen-binding activity but having the ability to crystallize. Digestion of antibodies with the enzyme, pepsin, results in the a $F(ab')_2$ fragment in which the two arms of the antibody molecule remain linked and comprise two-antigen binding sites. The $F(ab')_2$ fragment has the ability to crosslink antigen.

"Fv" when used herein refers to the minimum fragment of an antibody that retains both antigen-recognition and antigen-binding sites. These fragments can also be considered variants of the antibody.

"Fab" when used herein refers to a fragment of an antibody which comprises the constant domain of the light chain and the CH1 domain of the heavy chain.

The term "mAb" refers to monoclonal antibody.

The description of XenoMax method generated antibody sequences is coded as follows: "AB"-referring to antibody, "EGFRvIII"-referring to antibody's binding specificity, "X" referring to XenoMouse mouse derived, "G1"-referring to IgG1 isotype or "G2" referring to IgG2 isotype, the last three digits refer to the single cell number from which the antibody was derived, for example: AB- EGFRvIII -XG1-095 would be an antibody with binding specificity to EGFRvIII from XenoMouse mouse of a IgG1 isotype and cell number 95.

The term "SC" refers to single cell and a particular XenoMax method derived antibody may be referred to as SC followed by three digits, or just three digits, referring to the single cell number from which the antibody was derived herein.

The description of hybridoma derived antibody sequences is coded as follows: "AB"-referring to antibody, "EGFRvIII"-refers to the antibody's binding specificity, "X" refers to XenoMouse mouse derived, "G1"-refers to IgG1 isotype or "G2" refers to IgG2 isotype, "K" refers to kappa, "L' refers to lambda. The last three digits referring to the clone from which the antibody was derived, for example: AB-EGFRvIII-XG1K-13.1.2

"Label" or "labeled" as used herein refers to the addition of a detectable moiety to a polypeptide, for example, a radiolabel, fluorescent label, enzymatic label chemiluminescent labeled or a biotinyl group. Radioisotopes or radionuclides may include ^{3}H, ^{14}C, ^{15}N, ^{35}S, ^{90}Y, ^{99}Tc, ^{111}In, ^{125}I, ^{131}I, fluorescent labels may include rhodamine, lanthanide phosphors or FITC and enzymatic labels may include horseradish peroxidase, β-galactosidase, luciferase, alkaline phosphatase.

The term "pharmaceutical agent or drug" as used herein refers to a chemical compound or composition capable of inducing a desired therapeutic effect when properly administered to a patient. Other chemistry terms herein are used according to conventional usage in the art, as exemplified by *The McGraw-Hill Dictionary of Chemical Terms* (Parker, S., Ed., McGraw-Hill, San Francisco (1985)).

As used herein, "substantially pure" means an object species is the predominant species present (i.e., on a molar basis it is more abundant than any other individual species in the composition), and preferably a substantially purified fraction is a composition wherein the object species comprises at least about 50 percent (on a molar basis) of all macromolecular species present. Generally, a substantially pure composition will comprise more than about 80 percent of all macromolecular species present in the composition, more preferably more than about 85%, 90%, 95%, 99%, and 99.9%. Most preferably, the object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species.

The term "patient" includes human and veterinary subjects.

The term "SLAM® Technology" refers to the "Selected Lymphocyte Antibody Method" (Babcook et al., *Proc. Natl. Acad. Sci. USA*, i93:7843-7848 (1996), and Schrader, US Patent No. 5,627,052).

The term "XenoMax™" refers to the use of SLAM Technology with XenoMouse® mice (as described below).

Antibody Structure

The basic antibody structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kDa) and one "heavy" chain (about 50-70 kDa). The amino-terminal portion of each chain includes a

variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The carboxy-terminal portion of each chain defines a constant region primarily responsible for effector function. Human light chains are classified as kappa and lambda light chains. Heavy chains are classified as mu, delta, gamma, alpha, or epsilon, and define the antibody's isotype as IgM, IgD, IgG, IgA, and IgE, respectively. Within light and heavy chains, the variable and constant regions are joined by a "J" region of about 12 or more amino acids, with the heavy chain also including a "D" region of about 10 more amino acids. *See generally,* Fundamental *Immunology* Ch. 7 (Paul, W., ed., 2nd ed. Raven Press, N.Y. (1989)). The variable regions of each light/heavy chain pair form the antibody binding site.

Thus, an intact antibody has two binding sites. Except in bifunctional or bispecific antibodies, the two binding sites are the same.

The chains all exhibit the same general structure of relatively conserved framework regions (FR) joined by three hyper variable regions, also called complementarity determining regions or CDRs. The CDRs from the two chains of each pair are aligned by the framework regions, enabling binding to a specific epitope. From N-terminal to C-terminal, both light and heavy chains comprise the domains FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4. The assignment of amino acids to each domain is in accordance with the definitions of Kabat *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md. (1987 and 1991)), or Chothia & Lesk *J. Mol. Biol.* 196:901-917 (1987); Chothia et al. *Nature* 342:878-883 (1989).

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 *Clin. Exp. Immunol.* 79: 315-321 (1990), Kostelny et al. *J. Immunol.* 148:1547-1553 (1992). Production of bispecific antibodies can be a relatively labor intensive process compared with production of conventional antibodies and yields and degree of purity are generally lower for bispecific antibodies. Bispecific antibodies do not exist in the form of fragments having a single binding site (e.g., Fab, Fab', and Fv).

In addition to the general structural aspects of antibodies, the more specific interaction between the paratope and the epitope may be examined through structural approaches. In one embodiment, the structure of the CDRs form a paratope, through which an antibody is able to bind to an epitope. The structure of such a paratope may be determined in a number of ways. Traditional structural examination approaches may be used, such as NMR or x-ray crystalography. These approaches may examine the structure of the paratope alone, or while it is bound to the epitope. Alternatively, molecular models may be generated *in silico*. A structure can be generated through homology modeling, aided with a commercial package, such as InsightII modeling package from Accelrys (San Diego, CA). Briefly, one can use the sequence of the antibody to be examined to search against a database of proteins of known structures, such as the Protein Data Bank. After

one identifies homologous proteins with known structures, these homologous proteins are used as modeling templates. Each of the possible templates can be aligned, thus producing structure based sequence alignments amoung the templates. The sequence of the antibody with the unknown structure can then be aligned with these templates to generate a molecular model for the antibody with the unknown structure. As will be appreciated by one of skill in the art, there are many alternative methods for generating such structures *in silico*, any of which may be used. For instance, a process similar to the one described in Hardman et al., issued U.S. Pat. No. 5,958,708 employing QUANTA (Polygen Corp., Waltham, Mass.) and CHARM (Brooks, B. R., Bruccoleri, R. E., Olafson, B. D., States, D. J., Swaminathan, S. and Karplus, M., 1983, J. Comp. Chem,. 4:187) may be used.

Not only is the shape of the paratope important in determining whether and how well a possible paratope will bind to an epitope, but the interaction itself, between the epitope and the paratope is a source of great information in the design of variant antibodies. As appreciated by one of skill in the art, there are a variety of ways in which this interaction can be studied. One way is to use the structural model generated, perhaps as described above, and then to use a program such as InsightII (Accelrys, San Diego, CA), which has a docking module, which, among other things, is capable of performing a Monte Carlo search on the conformational and orientational spaces between the paratope and its epitope. The result is that one is able to estimate where and how the epitope interacts with the paratope. In one embodiment, only a fragment, or variant, of the epitope is used to assist in determining the relevant interactions. In one embodiment, the entire epitope is used in the modeling of the interaction between the paratope and the epitope. As will be appreciated by one of skill in the art, these two different approaches have different advantages and disadvantages. For instance, using only a fragment of the epitope allows for a more detailed examination of the possible variations of each side chain, without taking huge amounts of time. On the other hand, by using only a fragment of the epitope, or simply the epitope instead of the entire protein, it is possible that the characteristics of the epitope fragment may not be the same as the characteristics for the whole epitope, thus possibly increasing the risk of being mislead during the computational modeling. In one embodiment, both approaches are used to a limited extent, in order to cross check the results. In a preferred embodiment, if a variant of an epitope is used, it will be optimized so that the variant of the epitope comprises the most important residues of the epitope. The identity of the most important residues can be determined in any number of ways, for instance as described in Examples 4 and 14 of the present specification.

Through the use of these generated structures, one is able to determine which residues are the most important in the interaction between the epitope and the paratope. Thus, in one embodiment, one is able to readily select which residues to change in order to alter the binding characteristics of the antibody. For instance, it may be apparent from the docking models that the side chains of certain residues in the paratope may sterically hinder the binding of the epitope, thus

altering these residues to residues with smaller side chains may be beneficial. One can determine this in many ways. For example, one may simply look at the two models and esitmate interactions based on functional groups and proximety. Alternatively, one may perform repeated pairings of epitope and paratope, as described above, in order to obtain more favorable energy interactions. One can also determine these interactions for a variety of variants of the antibody to determine alternative ways in which the antibody may bind to the epitope. One can also combine the various models to determine how one should alter the structure of the antibodies in order to obtain an antibody with the particular characteristics that are desired.

The models determined above can be tested through various techniques. For example, the interaction energy can determined with the programs discussed above in order to determine which of the variants to further examine. Also, coulumbic and van der Waals interactions are used to determine the interaction energies of the epitope and the variant paratopes. Also site directed mutagenesis is used to see if predicted changes in antibody structure actually result in the desired changes in binding characteristics. Alternatively, changes may be made to the epitope to verify that the models are correct or to determine general binding themes that may be occurring between the paratope and the epitope.

The above methods for modeling structures can be used to determine what changes in protein structure will result in particular desired characteristics of an antibody. These methods can be used to determine what changes in protein structure will not result in the desired characteristics.

As will be appreciated by one of skill in the art, while these models will provide the guidance necessary to make the antibodies and variants thereof of the present embodiments, it may still be desirable to perform routine testing of the *in silico* models, perhaps through *in vitro* studies. In addition, as will be apparent to one of skill in the art, any modification may also have additional side effects on the activity of the antibody. For instance, while any alteration predicted to result in greater binding, may induce greater binding, it may also cause other structural changes which might reduce or alter the activity of the antibody. The determination of whether or not this is the case is routine in the art and can be achieved in many ways. For Example, the activity can be tested through an ELISA test, as in Example 21. Alternatively, the samples can be tested through the use of a surface plasmon resonance device.

Antibodies Binding, and Variant Antibodies for Superior Binding

In one embodiment, the models described above are used to increase the binding ability of the antibody to its epitope. The antibody can bind to the epitope more readily, and thus have a higher association constant (k_a). Alternatively, the antibody may dissociate from the epitope slower, and thus have a lower dissociation constant (k_d), or the K_D of the epitope-paratope interaction can be smaller in value, thus making the extent of the binding between the epitope and paratope higher.

In some embodiments, the variant antibodies are designed to bind with the opposite characteristics. That is, the antibodies do not bind as tightly or perhaps as quickly.

In other embodiments, the variant antibodies are not different in their K_D from the wild-type antibodies, but the variant antibodies are more specific for a particular epitope. This may mean that the paratopes of the designed antibodies have a lower risk of binding to other epitopes. The antibodies can have other characteristics that have been altered. For example, a variant may be more immune to nonspecific antibody binding or may stay solvated in solution even when the antibody is present in high concentrations. Such a variant may be present in the discussed antibodies. For instance, while the higher concentrations of some variant antibodies examined below resulted in slower binding components in Biacore experiments, for instance 13.1.2 mAb, certain variants did not exhibit this slower component, even at the same concentrations, L217N-2.1, for example.

The variants predicted by the models determined above can be created and then tested to determine if they actually bind with the desired characteristics. Mutants with a greater total interaction energy with the epitope can be selected for further testing. The interaction energy can be determined in a number of ways, one of which is described above.

These variants can be tested in a number of ways. Exemplary options include and are not limited to KinExA (e.g., Lackie, Issued Pat. No. 5,372,783, Dec 13, 1994) (Sapidyne Instruments Inc., ID, Boise), surface plasmon resonance (SPR)(*e.g.*, BIACORE™ Biacore, Inc., Pistcataway, N.J.), stopped-flow fluorescence, resonant mirror, and fluorescence polarization. Many of these options are able to not only record the data, but also provide ready means for fitting the data to various theoretical curves and thus determine the k_a, k_d, and K_D, as well as other properties. It is important to note that the fitting of these curves to the resulting data is not without the possibility for some variation. Because of this, the relevant association, dissociation, and equilibrium constants can be looked at, not only through these curve fitting mechanisms, but also in direct comparison with each other, and in light of the knowledge of one of skill in the art.

Human Antibodies and Humanization of Antibodies

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 human antibody function into a rodent so that the rodent produces fully human antibodies.

The ability to clone and reconstruct megabase-sized human loci in YACs and to introduce them into the mouse germline provides a powerful approach to elucidating the functional components of very large or crudely mapped loci as well as generating useful models of human disease. Furthermore, the utilization of such technology for substitution of mouse loci with their

human equivalents could provide unique insights into the expression and regulation of human gene products during development, their communication with other systems, and their involvement in disease induction and progression.

An important practical application of such a strategy is the "humanization" of the mouse humoral immune system. Introduction of human immunoglobulin (Ig) loci into mice in which the endogenous Ig genes have been inactivated offers the opportunity to study the mechanisms underlying programmed expression and assembly of antibodies as well as their role in B-cell development. Furthermore, such a strategy could provide an ideal source for production of fully human monoclonal antibodies (mAbs)--an important milestone towards fulfilling the promise of antibody therapy in human disease. Fully human antibodies are expected to minimize the immunogenic and allergic responses intrinsic to mouse or mouse-derivatized mAbs and thus to increase the efficacy and safety of the administered antibodies. The use of fully human antibodies can be expected to provide a substantial advantage in the treatment of chronic and recurring human diseases, such as inflammation, autoimmunity, and cancer, which require repeated antibody administrations.

One approach towards this goal was to engineer mouse strains deficient in mouse antibody production with large fragments of the human Ig loci in anticipation that such mice would produce a large repertoire of human antibodies in the absence of mouse antibodies. Large human Ig fragments would preserve the large variable gene diversity as well as the proper regulation of antibody production and expression. By exploiting the mouse machinery for antibody diversification and selection and the lack of immunological tolerance to human proteins, the reproduced human antibody repertoire in these mouse strains should yield high affinity antibodies against any antigen of interest, including human antigens. Using the hybridoma technology, antigen-specific human mAbs with the desired specificity could be readily produced and selected. This general strategy was demonstrated in connection with our generation of the first XenoMouse mouse strains, as published in 1994. (See Green et al. Nature Genetics 7:13-21 (1994)) The XenoMouse strains were engineered with yeast artificial chromosomes (YACs) containing 245 kb and 190 kb-sized germline configuration fragments of the human heavy chain locus and kappa light chain locus, respectively, which contained core variable and constant region sequences. *Id.* The human Ig containing YACs proved to be compatible with the mouse system for both rearrangement and expression of antibodies and were capable of substituting for the inactivated mouse Ig genes. This was demonstrated by their ability to induce B-cell development, to produce an adult-like human repertoire of fully human antibodies, and to generate antigen-specific human mAbs. These results also suggested that introduction of larger portions of the human Ig loci containing greater numbers of V genes, additional regulatory elements, and human Ig constant regions might recapitulate substantially the full repertoire that is characteristic of the human humoral response to infection and immunization. The work of Green et al. was recently extended to the introduction of

greater than approximately 80% of the human antibody repertoire through introduction of megabase sized, germline configuration YAC fragments of the human heavy chain loci and kappa light chain loci, respectively. See Mendez et al. Nature Genetics 15:146-156 (1997) and U.S. patent application Ser. No. 08/759,620, filed Dec. 3, 1996.

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 and U.S. Patent Nos. 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* Mendez et al. *Nature Genetics* 15:146-156 (1997) and Green and Jakobovits *J. Exp. Med.* 188:483-495 (1998). *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, WO 03/47336.

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. *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. *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).

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. Xenerex Biosciences is developing a technology for the potential generation of human antibodies. In this technology, SCID mice are reconstituted with human lymphatic cells, e.g., B and/or T cells. Mice are then immunized with an antigen and can generate an immune response against the antigen. See U.S. Patent Nos. 5,476,996, 5,698,767, and 5,958,765.

Human anti-mouse antibody (HAMA) responses have led the industry to prepare chimeric or otherwise humanized antibodies. While chimeric antibodies have a human constant region and a murine variable region, it is expected that certain human anti-chimeric antibody (HACA) responses will be observed, particularly in chronic or multi-dose utilizations of the antibody. Thus, it would be desirable to provide fully human antibodies against EGFRvIII in order to vitiate concerns and/or effects of HAMA or HACA response.

Antibody Therapeutics

As discussed herein, the function of the EGFRvIII antibody appears important to at least a portion of its mode of operation. By function, it is meant, by way of example, the activity of the EGFRvIII antibody in operation with EGFRvIII. Accordingly, in certain respects, it may be desirable in connection with the generation of antibodies as therapeutic candidates against EGFRvIII that the antibodies be capable of fixing complement and recruiting cytotoxic lymphocytes thus participating in CDC and ADCC. There are a number of isotypes of antibodies that are capable of the same, including, without limitation, the following: murine IgM, murine IgG2a, murine IgG2b, murine IgG3, human IgM, human IgG1, human IgG3, and human IgA. Also, it may be desirable in connection with the generation of antibodies as therapeutic candidates against EGFRvIII that the antibodies be capable of activating antibody-dependent celluclar cytotoxicity (ADCC), through engagement of Fc receptors on effectors cells such as natural killer (NK) cells. There are a number of isotypes of antibodies that are capable of ADCC, including, without limitation, 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 thereafter using conventional techniques that are well known in the art. Such techniques include the use of direct recombinant techniques (*see e.g.*, U.S. Patent No. 4,816,397) and 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 is prepared that possesses a heavy chain with any desired isotype and another myeloma or other cell line 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, certain anti-EGFRvIII antibodies discussed herein are human anti-EGFRvIII IgG1 antibodies. If such antibody possessed desired binding to the EGFRvIII molecule, it could be readily isotype switched to generate a human IgM, human IgG3, or human IgGA while still possessing the same variable region (which defines the antibody's specificity and some of its affinity). Such molecules, including IgG1, would then be capable of fixing complement and participating in CDC, and, if comprising and IgG1 or IgG3 constant region, such molecules would also be capable of participating in antibody-dependent cellular cytotoxicity (ADCC) through recruiting cytotoxic lymphocytes.

Accordingly, as antibody candidates are generated that meet desired "structural" attributes as discussed above, they can generally be provided with at least certain of the desired "functional" attributes through isotype switching.

Design and Generation of Other Therapeutics

Based on the activity of the antibodies that are produced and characterized herein with respect to EGFRvIII, the design of other therapeutic modalities beyond antibody moieties is facilitated. Such modalities include, without limitation, advanced antibody therapeutics, such as bispecific antibodies, immunotoxins, and radiolabeled therapeutics, generation of peptide therapeutics, gene therapies, particularly intrabodies, antisense therapeutics, and small molecules.

In connection with the generation of advanced antibody therapeutics, where complement fixation and recruitment of cytoxic lymphocytes is a desirable attribute, it is possible to enhance cell killing through the use of bispecifics, immunotoxins, or radiolabels, for example.

For example, in connection with bispecific antibodies, bispecific antibodies can be generated that comprise (i) two antibodies one with a specificity to EGFRvIII and another to a second molecule that are conjugated together, (ii) a single antibody that has one chain specific to EGFRvIII and a second chain specific to a second molecule, or (iii) a single chain antibody that has specificity to EGFRvIII and the other molecule. Such bispecific antibodies can be generated using techniques that are well known for example, in connection with (i) and (ii) *see e.g.,* Fanger et al. *Immunol Methods* 4:72-81 (1994) and Wright and Harris, *supra.* and in connection with (iii) *see e.g.,* Traunecker et al. *Int. J. Cancer (Suppl.)* 7:51-52 (1992). In each case, the second specificity can be made to the Fc chain activation receptors, including, without limitation, CD16 or CD64 (*see e.g.,* Deo et al. 18:127 (1997)) CD3 (Micromet's BiTE technology) or CD89 (*see e.g.,* Valerius et al. *Blood* 90:4485-4492 (1997)). Bispecific antibodies prepared in accordance with the foregoing would be likely to kill cells expressing EGFRvIII, and particularly those cells in which the EGFRvIII antibodies of the invention are effective.

In connection with immunotoxins, antibodies can be modified to act as immunotoxins utilizing techniques that are well known in the art. *See e.g.,* Vitetta *Immunol Today* 14:252 (1993).

See also U.S. Patent No. 5,194,594. In connection with the preparation of radiolabeled antibodies, such modified antibodies can also be readily prepared utilizing techniques that are well known in the art. *See e.g.*, Junghans et al. in *Cancer Chemotherapy and Biotherapy* 655-686 (2d edition, Chafner and Longo, eds., Lippincott Raven (1996)). *See also* U.S. Patent Nos. 4,681,581, 4,735,210, 5,101,827, 5,102,990 (RE 35,500), 5,648,471, and 5,697,902. Each of immunotoxins and radiolabeled molecules would be likely to kill cells expressing EGFRvIII, and particularly those cells in which the antibodies described herein are effective.

The antibodies can be designed to bind more quickly, or to dissociate more slowly from the epitope, and thus the antibodies themselves can be designed therapeutics. The altered characterisitics of the antibodies can be used, for example, in the administration of a therapeutic to a patient.

Therapeutic Immunoconjugates

As will be appreciated, antibodies conjugated to drugs, toxins, or other molecules (immunoconjugates or immunotoxins) are highly useful in the targeted killing of cells that express a molecule that can be specifically bound by a specific binding molecule, such as an antibody. As discussed above, EGFRvIII is not known to be expressed on any normal tissues. Further, EGFRvIII shows significant expression in numerous human tumors. Accordingly, EGFRvIII is a highly attractive molecule for targeting with an immunotoxin.

Many reports have appeared on the attempted specific targeting of tumor cells with monoclonal antibody-drug conjugates (Sela et al. in Immunoconjugates 189-216 (C. Vogel, ed. 1987); Ghose et al, in Targeted Drugs 1-22 (E. Goldberg, ed. 1983); Diener et al, in Antibody Mediated Delivery Systems 1-23 (J. Rodwell, ed. 1988); Pietersz et al, in Antibody Mediated Delivery Systems 25-53 (J. Rodwell, ed. 1988); Bumol et al, in Antibody Mediated Delivery Systems 55-79 (J. Rodwell, ed. 1988). Cytotoxic drugs such as methotrexate, daunorubicin, doxorubicin, vincristine, vinblastine, melphalan, mitomycin C, and chlorambucil have been conjugated to a variety of murine monoclonal antibodies. In some cases, the drug molecules were linked to the antibody molecules through an intermediary carrier molecule such as serum albumin (Garnett et al. Cancer Res. 46:2407-2412 (1986); Ohkawa et al. Cancer Immumol. Immunother. 23:81-86 (1986); Endo et al. Cancer Res. 47:1076-1080 (1980)), dextran (Hurwitz et al. Appl. Biochem. 2:25-35 (1980); Manabi et al. Biochem. Pharmacol. 34:289-291 (1985); Dillman et al. Cancer Res. 46:4886-4891 (1986); Shoval et al. Proc. Natl. Acad. Sci. 85: 8276-8280 (1988)), or polyglutamic acid (Tsukada et al. J. Natl. Canc. Inst. 73:721-729 (1984); Kato et al. J. Med. Chem. 27:1602-1607 (1984); Tsukada et al. Br. J. Cancer 52:111-116 (1985)).

A wide array of linker technologies has been employed for the preparation of such immunoconjugates and both cleavable and non-cleavable linkers have been investigated. In most cases, the full cytotoxic potential of the drugs could only be observed, however, if the drug molecules could be released from the conjugates in unmodified form at the target site.

One of the cleavable linkers that has been employed for the preparation of antibody-drug conjugates is an acid-labile linker based on cis-aconitic acid that takes advantage of the acidic environment of different intracellular compartments such as the endosomes encountered during receptor mediated endocytosis and the lysosomes. Shen and Ryser introduced this method for the preparation of conjugates of daunorubicin with macromolecular carriers (Biochem. Biophys. Res. Commun. 102:1048-1054 (1981)). Yang and Reisfeld used the same technique to conjugate daunorubicin to an anti-melanoma antibody (J. Natl. Canc. Inst. 80:1154-1159 (1988)). Recently, Dillman et al. also used an acid-labile linker in a similar fashion to prepare conjugates of daunorubicin with an anti-T cell antibody (Cancer Res. 48:6097-6102 (1988)).

An alternative approach, explored by Trouet et al. involved linking daunorubicin to an antibody via a peptide spacer arm (Proc. Natl. Acad. Sci. 79:626-629 (1982)). This was done under the premise that free drug could be released from such a conjugate by the action of lysosomal peptidases.

In vitro cytotoxicity tests, however, have revealed that antibody-drug conjugates rarely achieved the same cytotoxic potency as the free unconjugated drugs. This suggested that mechanisms by which drug molecules are released from the antibodies are very inefficient. In the area of immunotoxins, conjugates formed via disulfide bridges between monoclonal antibodies and catalytically active protein toxins were shown to be more cytotoxic than conjugates containing other linkers. See, Lambert et al. J. Biol. Chem. 260:12035-12041 (1985); Lambert et al. in Immunotoxins 175-209 (A. Frankel, ed. 1988); Ghetie et al. Cancer Res. 48:2610-2617 (1988). This was attributed to the high intracellular concentration of glutathione contributing to the efficient cleavage of the disulfide bond between an antibody molecule and a toxin. Despite this, there are only a few reported examples of the use of disulfide bridges for the preparation of conjugates between drugs and macromolecules. Shen et al. described the conversion of methotrexate into a mercaptoethylamide derivative followed by conjugation with poly-D-lysine via a disulfide bond (J. Biol. Chem. 260:10905-10908 (1985)). In addition, a report described the preparation of a conjugate of the trisulfide-containing toxic drug calicheamycin with an antibody (Menendez et al. Fourth International Conference on Monoclonal Antibody Immunoconjugates for Cancer, San Diego, Abstract 81 (1989)). Another report described the preparation of a conjugate of the trisulfide-containing toxic drug calicheamycin with an antibody (Hinman et al, 53 Cancer Res. 3336-3342 (1993)).

One reason for the lack of disulfide linked antibody-drug conjugates is the unavailability of cytotoxic drugs that bear a sulfur atom containing moiety that can be readily used to link the drug to an antibody via a disulfide bridge. Furthermore, chemical modification of existing drugs is difficult without diminishing their cytotoxic potential.

Another major drawback with existing antibody-drug conjugates is their inability to deliver a sufficient concentration of drug to the target site because of the limited number of targeted

antigens and the relatively moderate cytotoxicity of cancerostatic drugs like methotrexate, daunorubicin and vincristine. In order to achieve significant cytotoxicity, linkage of a large number of drug molecules either directly to the antibody or through a polymeric carrier molecule becomes necessary. However such heavily modified antibodies often display impaired binding to the target antigen and fast in vivo clearance from the blood stream.

Maytansinoids are highly cytotoxic drugs. Maytansine was first isolated by Kupchan et al. from the east African shrub Maytenus serrata and shown to be 100 to 1000 fold more cytotoxic than conventional cancer chemotherapeutic agents like methotrexate, daunorubicin, and vincristine (U.S. Pat. No. 3,896,111). Subsequently, it was discovered that some microbes also produce maytansinoids, such as maytansinol and C-3 esters of maytansinol (U.S. Pat. No. 4,151,042). Synthetic C-3 esters of maytansinol and analogues of maytansinol have also been reported (Kupchan et al. J. Med. Chem. 21:31-37 (1978); Higashide et al. Nature 270:721-722 (1977); Kawai et al. Chem. Pharm. Bull. 32:3441-3451 (1984)). Examples of analogues of maytansinol from which C-3 esters have been prepared include maytansinol with modifications on the aromatic ring (e.g. dechloro) or at the C-9, C-14 (e.g. hydroxylated methyl group), C-15, C-18, C-20 and C-4,5.

The naturally occurring and synthetic C-3 esters can be classified into two groups:

(a) C-3 esters with simple carboxylic acids (U.S. Pat. Nos. 4,248,870; 4,265,814; 4,308,268; 4,308,269; 4,309,428; 4,317,821; 4,322,348; and 4,331,598), and

(b) C-3 esters with derivatives of N-methyl-L-alanine (U.S. Pat. Nos. 4,137,230; 4,260,608; 5,208,020; and Chem. Pharm. Bull. 12:3441 (1984)).

Esters of group (b) were found to be much more cytotoxic than esters of group (a).

Maytansine is a mitotic inhibitor. Treatment of L1210 cells in vivo with maytansine has been reported to result in 67% of the cells accumulating in mitosis. Untreated control cells were reported to demonstrate a mitotic index ranging from between 3.2 to 5.8% (Sieber et al. 43 Comparative Leukemia Research 1975, Bibl. Haemat. 495-500 (1976)). Experiments with sea urchin eggs and clam eggs have suggested that maytansine inhibits mitosis by interfering with the formation of microtubules through the inhibition of the polymerization of the microtubule protein, tubulin (Remillard et al. Science 189:1002-1005 (1975)).

In vitro, P388, L1210, and LY5178 murine leukemic cell suspensions have been found to be inhibited by maytansine at doses of 10^{-3} to 10^{-1} .mu.g/.mu.l with the P388 line being the most sensitive. Maytansine has also been shown to be an active inhibitor of in vitro growth of human nasopharyngeal carcinoma cells, and the human acute lymphoblastic leukemia line CEM was reported inhibited by concentrations as low as 10^{-7} mg/ml (Wolpert-DeFillippes et al. Biochem. Pharmacol. 24:1735-1738 (1975)).

In vivo, maytansine has also been shown to be active. Tumor growth in the P388 lymphocytic leukemia system was shown to be inhibited over a 50- to 100-fold dosage range which

suggested a high therapeutic index; also significant inhibitory activity could be demonstrated with the L1210 mouse leukemia system, the human Lewis lung carcinoma system and the human B-16 melanocarcinoma system (Kupchan, Ped. Proc. 33:2288-2295 (1974)).

Current methods of conjugation of maytansinoids with cell binding agents (such as antibodies) involve two reaction steps. A cell binding agent, for example an antibody, is first modified with a cross-linking reagent such as N-succinimidyl pyridyldithiopropionate (SPDP) to introduce dithiopyridyl groups into the antibody (Carlsson et al. Biochem. J. 173:723-737 (1978); U.S. Pat. No. 5,208,020). In a second step, a reactive maytansinoid having a thiol group, such as DM1 (formally termed $N^{2'}$ -deacetyl-$N^{2'}$ -(3-mercapto-1-oxopropyl)-maytansine, as the starting reagent., is added to the modified antibody, resulting in the displacement of the thiopyridyl groups in the modified antibodies, and the production of disulfide-linked cytotoxic maytansinoid/antibody conjugates (U.S. Pat. No. 5,208,020). A one-step process for conjugation of maytansinoids is described in U.S. Patent No. 6,441,163. Maytansinoid-based immunotoxin technology is available from Immunogen Corporation (Cambridge, MA).

Another important toxin technology is based upon auristatin toxins. Auristatins are derived from Dolastatin 10 that was obtained from the Indian Ocean sea hare Dolabella, as a potent cell growth inhibitory substance. See U.S. Patent Nos. 4,816,444 and 4,978,744. With respect to other Dolastatins, see also U.S. Patent Nos. 4,414,205 (Dolastatin-1, 2, and 3), 5,076,973 (Dolastatin-3), 4,486,414 (Dolastatin-A and B),; 4,986,988 (Dolastatin-13), 5,138,036 (Dolastatin-14), and 4,879,278 (dolastatin-15). Isolated and synthesized by Dr. Pettit and colleagues at the University of Arizona, a variety of auristatine derivatives have been tested and shown to be highly toxic to cells. See Pettit et al. Antineoplastic agents 337. Synthesis of dolastatin 10 structural modifications. Anticancer Drug Des. 10(7):529-44 (1995), Woyke et al. In vitro activities and postantifungal effects of the potent dolastatin 10 structural modification auristatin PHE. Antimicrobial Agents and Chemotherapy. 45:3580-3584 (2001), Pettit et al. Specific activities of dolastatin 10 and peptide derivatives against Cryptococcus neoformans. Antimicrobial Agents and Chemotherapy. 42:2961-2965 (1998), WoykeThree-dimensional visualization of microtubules during the Cryptococcus neoformans cell cycle and the effects of auristatin PHE on microtubule integrity and nuclear localization. Submitted, Antimicrobial Agents and Chemotherapy.

Recently, additional auristatin derivatives have been developed that appear quite effective when delivered as payloads on antibodies. For example monomethyl auristatin E (MMAE) has been shown as a potent toxin for tumor cells when conjugated to tumor specific antibodies. Doronina et al. Development of potent monoclonal antibody auristatin conjugates for cancer therapy. Nature Biotechnology. (2003) (available online), Francisco et al. cAC10-vcMMAE, an anti-CD30-monomethyl auristatin E conjugate with potent and selective antitumor activity. Blood. (2003) May 8 [Epub ahead of print]. Epub 2003 Apr 24 (available online). In addition to the toxicity of the auristatin molecule, research has shown that peptide-linked conjugates are more

stable , and, thus, more specific and less toxic to normal tissues than other linker technologies in buffers and plasma. Doronina et al. Development of potent monoclonal antibody auristatin conjugates for cancer therapy. Nature Biotechnology. (2003) (available online), Francisco et al. cAC10-vcMMAE, an anti-CD30-monomethyl auristatin E conjugate with potent and selective antitumor activity. Blood. (2003) May 8 [Epub ahead of print]. Epub 2003 Apr 24 (available online). Such linkers are based on a branched peptide design and include, for example, mAb-valine-citrulline-MMAE and mAb-phenylalanine-lysine-MMAE conjugates. Doronina et al. Development of potent monoclonal antibody auristatin conjugates for cancer therapy. Nature Biotechnology. (2003) (available online), Francisco et al. cAC10-vcMMAE, an anti-CD30-monomethyl auristatin E conjugate with potent and selective antitumor activity. Blood. (2003) May 8 [Epub ahead of print]. Epub 2003 Apr 24 (available online). Such designs and conjugation techniques are described, for example, by King et al. Monoclonal antibody conjugates of doxorubicin prepared with branched peptide linkers: inhibition of aggregation by methoxytriethyleneglycol chains. J Med Chem. 45(19):4336-43 (2002) and Dubowchik et al. Cathepsin B-sensitive dipeptide prodrugs. 2. Models of anticancer drugs paclitaxel (Taxol), mitomycin C and doxorubicin. Bioorg Med Chem Lett. 8(23):3347-52 (1998). Auristatin E-based immunotoxin technology based upon the foregoing is available from Seattle Genetics Corporation (Seattle, WA).

There are a large number of novel microtubule effecting compounds obtained from natural sources-extracts, and semisynthetic and synthetic analogs that appear to possess potential as toxins for the generation of immunoconjugates. (see the website at newmedinc "dot" com). Such molecules and examples of drug products utilizing them, include the following: Colchicine-site Binders (Curacin), Combretastatins (AVE806, Combretastatin A-4 prodrug (CA4P), Oxi-4503), Cryptophycins (LY355703), Discodermolide, Dolastatin and Analogs (Auristatin PHE, Dolastatin 10, ILX-651, Symplostatin 1, TZT-1027), Epothilones (BMS-247550, BMS-310705, EPO906, KOS-862, ZK-EPO), Eleutherobin, FR182877, Halichondrin B (E7389), Halimide (NPI-2352 and NPI-2358), Hemiasterlins (HTI-286), Laulimalide, Maytansinoids ("DM1")(Bivatuzumab mertansine, Cantuzumab mertansine, huN901-DM1/BB-10901TAP, MLN591DM1, My9-6-DM1, Trastuzumab-DM1), PC-SPES, Peloruside A, Resveratrol, S-allylmercaptocysteine (SAMC), Spongistatins, Vitilevuamide, Molecular Motor-Kinesins (SB-715992), Designed Colchicine-Site Binders (A-289099, A-293620/A-318315, ABT-751/E7010, D-24851/D-64131, ZD6126), Other Novel Spindle Poisons (2-Methoxyestradiol (2-ME2), Bezimidazole Carbamates (ANG 600 series, Mebendazole), CP248/CP461, HMN-214, R440, SDX-103, T67/T607). Further, additional marine derived toxins are reviewed in Mayer, A.M.S. Marine Pharmacology in 1998: Antitumor and Cytotoxic Compounds. The Pharmacologist. 41(4):159-164 (1999).

Therapeutic Administration and Formulations

A prolonged duration of action will allow for less frequent and more convenient dosing schedules by alternate parenteral routes such as intravenous, subcutaneous or intramuscular injection.

When used for *in vivo* administration, antibody formulations described herein should be sterile. This is readily accomplished, for example, by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution. Antibodies ordinarily will be stored in lyophilized form or in solution. Therapeutic antibody compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having an adapter that allows retrieval of the formulation, such as a stopper pierceable by a hypodermic injection needle.

The route of antibody administration is in accord with known methods, e.g., injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial, intrathecal, inhalation or intralesional routes, or by sustained release systems as noted below. Antibodies are preferably administered continuously by infusion or by bolus injection.

An effective amount of antibody to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it is preferred for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. Typically, the clinician will administer antibody until a dosage is reached that achieves the desired effect. The progress of this therapy is easily monitored by conventional assays or by the assays described herein.

Antibodies as described herein can be prepared in a mixture with a pharmaceutically acceptable carrier. Therapeutic compositions can be administered intravenously or through the nose or lung, preferably as a liquid or powder aerosol (lyophilized). Composition can also be administered parenterally or subcutaneously as desired. When administered systemically, therapeutic compositions should be sterile, pyrogen-free and in a parenterally acceptable solution having due regard for pH, isotonicity, and stability. These conditions are known to those skilled in the art. Briefly, dosage formulations of the compounds are prepared for storage or administration by mixing the compound having the desired degree of purity with physiologically acceptable carriers, excipients, or stabilizers. Such materials are non-toxic to the recipients at the dosages and concentrations employed, and include buffers such as TRIS HCl, phosphate, citrate, acetate and other organic acid salts; antioxidants such as ascorbic acid; low molecular weight (less than about ten residues) peptides such as polyarginine, proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidinone; amino acids such as glycine, glutamic acid, aspartic acid, or arginine; monosaccharides, disaccharides, and other carbohydrates including cellulose or its derivatives, glucose, mannose, or dextrins; chelating agents

such as EDTA; sugar alcohols such as mannitol or sorbitol; counterions such as sodium and/or nonionic surfactants such as TWEEN, PLURONICS or polyethyleneglycol.

Sterile compositions for injection can be formulated according to conventional pharmaceutical practice as described in *Remington's Pharmaceutical Sciences* (18th ed, Mack Publishing Company, Easton, PA, 1990). For example, dissolution or suspension of the active compound in a vehicle such as water or naturally occurring vegetable oil like sesame, peanut, or cottonseed oil or a synthetic fatty vehicle like ethyl oleate or the like may be desired. Buffers, preservatives, antioxidants and the like can be incorporated according to accepted pharmaceutical practice.

Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the polypeptide, which matrices are in the form of shaped articles, films or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (e.g., poly(2-hydroxyethyl-methacrylate) as described by Langer *et al.*, *J. Biomed Mater. Res.*, (1981) 15:167-277 and Langer, *Chem. Tech.*, (1982) 12:98-105, or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman *et al.*, *Biopolymers*, (1983) 22:547-556), non-degradable ethylene-vinyl acetate (Langer *et al.*, *supra*), degradable lactic acid-glycolic acid copolymers such as the LUPRON Depot™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid (EP 133,988).

While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated proteins remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for protein stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Sustained-released compositions also include preparations of crystals of the antibody suspended in suitable formulations capable of maintaining crystals in suspension. These preparations when injected subcutaneously or intraperitoneally can produce a sustain release effect. Other compositions also include liposomally entrapped antibodies. Liposomes containing such antibodies are prepared by methods known per se: U.S. Pat. No. DE 3,218,121; Epstein et al., Proc. Natl. Acad. Sci. USA, (1985) 82:3688-3692; Hwang *et al.*, *Proc. Natl. Acad. Sci. USA*, (1980) 77:4030-4034; EP 52,322; EP 36,676; EP 88,046; EP 143,949; 142,641; Japanese patent application 83-118008; U.S. Pat. Nos. 4,485,045 and 4,544,545; and EP 102,324.

The dosage of the antibody formulation for a given patient will be determined by the attending physician taking into consideration various factors known to modify the action of drugs including severity and type of disease, body weight, sex, diet, time and route of administration, other medications and other relevant clinical factors. Therapeutically effective dosages may be determined by either *in vitro* or *in vivo* methods.

An effective amount of the antibody to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it is preferred that the therapist titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. A typical daily dosage might range from about 0.001 mg/kg to up to 100 mg/kg or more, depending on the factors mentioned above. Typically, the clinician will administer the therapeutic antibody until a dosage is reached that achieves the desired effect. The progress of this therapy is easily monitored by conventional assays or as described herein.

It will be appreciated that administration of therapeutic entities in accordance with the compositions and methods herein will be administered with suitable carriers, excipients, and other agents that are incorporated into formulations to provide improved transfer, delivery, tolerance, and the like. 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 ingredient 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):210-8 (2000), Wang W. "Lyophilization and development of solid protein pharmaceuticals." *Int. J. Pharm.* 203(1-2):1-60 (2000), Charman WN "Lipids, lipophilic drugs, and oral drug delivery-some emerging concepts." *J Pharm Sci* .89(8):967-78 (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.

Preparation of Antibodies

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 herein. In particular, however, a one 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. *See also* Mendez et al. *Nature Genetics* 15:146-156 (1997).

Through use of such technology, fully human monoclonal antibodies to a variety of antigens can be produced. In one embodiement, XenoMouse® lines of mice are immunized with an antigen of interest (e.g. EGFRvIII), lymphatic cells are recovered (such as B-cells) from the mice that expressed antibodies, and such cells are fused with a myeloid-type cell line to prepare immortal hybridoma cell lines, and such hybridoma cell lines are screened and selected to identify hybridoma cell lines that produce antibodies specific to the antigen of interest. Provided herein are methods for the production of multiple hybridoma cell lines that produce antibodies specific to EGFRvIII. Further, provided herein are characterization of the antibodies produced by such cell lines, including nucleotide and amino acid sequences of the heavy and light chains of such antibodies.

Alternatively, instead of being fused to myeloma cells to generate hybridomas, the antibody produced by recovered cells, isolated from immunized XenoMouse® lines of mice, are screened further for reactivity against the initial antigen, preferably EGFRvIII protein. Such screening includes ELISA with EGFRvIII protein, *in vitro* binding to NR6 M cells stably expressing full length EGFRvIII and internalization of EGFRvIII receptor by the antibodies in NR6 M cells. Single B cells secreting antibodies of interest are then isolated using a EGFRvIII-specific hemolytic plaque assay (Babcook et al., *Proc. Natl. Acad. Sci. USA*, i93:7843-7848 (1996)). Cells targeted for lysis are preferably sheep red blood cells (SRBCs) coated with the EGFRvIII antigen. In the presence of a B cell culture secreting the immunoglobulin of interest and complement, the formation of a plaque indicates specific EGFRvIII-mediated lysis of the target cells. The single antigen-specific plasma cell in the center of the plaque can be isolated and the genetic information that encodes the specificity of the antibody is isolated from the single plasma cell. Using reverse-transcriptase PCR, the DNA encoding the variable region of the antibody secreted can be cloned. Such cloned DNA can then be further inserted into a suitable expression vector, preferably a vector cassette such as a pcDNA, more preferably such a pcDNA vector containing the constant domains of immunglobulin heavy and light chain. The generated vector can then be transfected into host cells, preferably CHO cells, and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. Herein, we describe the isolation of multiple single plasma cells that produce antibodies specific to EGFRvIII. Further, the genetic material that encodes the specificity of the anti-EGFRvIII antibody is isolated, introduced into a suitable expression vector that is then transfected into host cells.

B cells from XenoMouse mice may be also be used as a source of genetic material from which antibody display libraries may be generated. Such libraries may be made in bacteriophage, yeast or in vitro via ribosome display using ordinary skills in the art. Hyperimmunized XenoMouse mice may be a rich source from which high-affinity, antigen-reactive antibodies may be isolated. Accordingly, XenoMouse mice hyperimmunized against EGFRvIII may be used to generate antibody display libraries from which high-affinity antibodies against EGFRvIII may be isolated. Such libraries could be screened against the pep3 oligopeptide and the resultingly derived antibodies screening against cells expressing EGFRvIII to confirm specificity for the natively display antigen. Full IgG antibody may then be expressed using recombinant DNA technology. See *e.g.*, WO 99/53049.

In general, antibodies produced by the above-mentioned cell lines possessed fully human IgG1 or IgG2 heavy chains with human kappa light chains. In one embodiment, the antibodies possessed high affinities, typically possessing Kd's of from about 10^{-9} through about 10^{-13} M, when measured by either solid phase and solution phase. In other embodiments the antibodies possessed lower affinities, from about 10^{-6} through about 10^{-8} M.

As appreciated by one of skill in the art, antibodies in accordance with the present embodiments can be expressed in cell lines other than hybridoma cell lines. Sequences encoding particular antibodies can be used for transformation of a suitable mammalian host cell. Transformation can be 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. The transformation procedure used depends upon the host to be transformed. Methods for introduction of 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.

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. Cell lines of particular preference are selected through determining which cell lines have high expression levels and produce antibodies with constitutive EGFRvIII binding properties.

EXAMPLES

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

The strategy for generating EGFRvIII-specific antibodies initially involved immunization of XenoMouse mice with combinations of antigens (peptide, various soluble proteins, antigen-expressing cells) followed by isolation of antibody producing cells, either as through fusions to produce hybridomas or isolation of B cell cells through the XenoMax™/SLAM™ technology. Antibody producing cells were subjected to a primary screen for specificity by ELISA and a secondary screen for cell surface binding by FMAT and/or FACS. Internalization assays were then conducted to identify antibodies that would be useful for drug delivery. Affinities of the antibodies were measured. Certain antibodies were selected for epitope mapping. In addition, certain antibodies were selected for *in vitro* and *in vivo* tests to analyze the efficacy of such antibodies for treatment of cancers.

EXAMPLE 1

Antigen Preparation

A. EGFRvIII PEP3-KLH Antigen Preparation

In connection with Example 2, the 14-mer human EGFRvIII PEP3 (L E E K K G N Y V V T D H C (SEQ ID NO: 56)) peptide was custom synthesized by R&D Systems. The PEP3 peptide was then conjugated to keyhole limpet hemocyanin (KLH), as follows: EGFRvIII PEP3 (200 mcg) (R&D) was mixed with 50 mcg of keyhole limpet hemocyanin (KLH; Pierce, Rockford, IL) to a final volume of 165 mcl using distilled water. 250 mcl of conjugation buffer (0.1M MES, 0.9M NaCl, pH 4.7) was added and EGFRvIII PEP3 and KLH were crosslinked by the addition of 25 mcl of 10 mg/ml stock solution of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC, Pierce, Rockford, IL). Conjugate was incubated for 2 hours at room temperature and the unreacted EDC was removed by centrifugation through a 1 kDa filter (Centrifugal filter; Millipore, Bedford, MA) using PBS pH 7.4.

In connection with Example 3, the 14-mer human EGFRvIII PEP3 (L E E K K G N Y V V T D H C (SEQ ID NO: 56)) peptide was custom synthesized. The PEP3 peptide was then conjugated to KLH, as follows: human EGFRvIII PEP3 (200 mcg) was mixed with 50 mcg of keyhole limpet hemocyanin (KLH; Pierce, Rockford, IL) to a final volume of 165 mcl using distilled water. 250 mcl of conjugation buffer (0.1M MES, 0.9M NaCl, pH 4.7) was added and EGFRvIII PEP3 and KLH were crosslinked by the addition of 25 mcl of 10 mg/ml stock solution of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC, Pierce, Rockford, IL). Conjugate was incubated for 2 hours at room temperature and the unreacted EDC was removed by centrifugation through a 1 kDa filter (Centrifugal filter; Millipore, Bedford, MA) using PBS pH 7.4.

B. B300.19/EGFRvIII Transfectants

In order to prepare the B300.19/EGFRvIII transfectants, wild type EGFR was initially cloned from A431 cells and EGFR gene was modified to code for EGFRvIII to delete the codons encoding residues 6-273, with a codon encoding a Glycine residue created at the junction of the deletion. The deletion occurs within the codons surrounding the deletion GTT (Valine) and CGT (Arginine), such that the resulting codon after the deletion is GGT (Glycine). (Wikstrand et al. J Neurovirol. 4(2):148-58 (1998))

1. Cloning of wild type EGFR Construct:

PolyA+mRNA was extracted from A431 (ATCC) cells usingMicro-fast RNA kit (Invitrogen, Burlington, ON). Total cDNA was synthesized from polyA+ mRNA with random pdN6 primers and M-MuLV reverse transcriptase (NEB, New England Biolabs, Beverly, Mass.). A 2.3kb PCR product was amplified from A431 cDNA with the following primers:

sense 5'-GGATCTCGAGCCAGACCGGAACGACAGGCCACCTC-3'; (SEQ ID NO: 62)

anti-sense 5'-CGGATCTCGAGCCGGAGCCCAGCACTTTGATCTT-3' (SEQ ID NO: 63)

using Pfu DNA polymerase.

The PCR product was digested with XhoI, gel purified and ligated into plasmid pWBFNP (see International Patent Application No. WO 99/45031) linearized with XhoI to yield plasmid Wt-EGFR/pWBFNP.

2. Generation of EGFRvIII Construct:

PCR products amplified from plasmid Wt-EGFR/pWBFNP template with primer pairs C13659/C29538 and C29539/C14288 (BioSource International), in which the C29538 and C29539 were phosphorylated with T4 Polynucleotide kinase (NEB, New England Biolabs, Beverly, Mass.):

C13659: 5'-CGGATGAATTCCCAGACCGGACGACAGGCCACCTC-3'(Sense) (SEQ ID NO: 64);

C29538: 5'-CTTTCTTTTCCTCCAGAGCC-3'(Anti-Sense) (SEQ ID NO: 65);

C29539: 5'-GTAATTATGTGGTGACAGATC-3'(Sense) (SEQ ID NO: 66);

C14288:5'-CGGATCTCGAGCTCAAGAGAGCTTGGTTGGGAGCT-3'(Anti- Sense) (SEQ ID NO: 67).

were ligated to introduce a deletion in the sequence encoding amino acids 6 through 273 of the EGFR extracellular domain and subcloned into expression vector pWBDHFR2 (see International Patent Application No. WO 99/45031).

A 232 bp fragment representing the 5'end of the deletion was generated with primer pair C13659/C29538 from Wt-EGFR/pWBFNP template amplified with Pfu polymerase (NEB, New England Biolabs, Beverly, Mass.). The PCR product was digested with EcoR1 (NEB, New England Biolabs, Beverly, Mass.) and gel purified. A 1273 bp fragment representing the 3'end of the deletion was generated with primer pair C29539/C14288 from Wt-EGFR/pWBFNP and the

template amplified with Pfu polymerase. The PCR product was digested with Xho1 (NEB, New England Biolabs, Beverly, Mass.) and gel purified. Fragments were ligated into EcoR1/Xho1 digested pWBDHFR2 with T4 DNA ligase (NEB, New England Biolabs, Beverly, Mass.) to yield construct EGFRvIII/pWBDHFR

The intracellular domain of EGFR was introduced into the resulting construct as follows: A 1566bp DraIII/XhoI fragment was isolated from plasmid Wt-EGFR/pWBFNP and ligated into DraIII/XhoI digested EGFRvIII/pWBDHFR to yield EGFRvIII-FL/pWBDHFR.

3. Transfection of B300.19 cells with EGFRvIII-FL/pWBDHFR:

B300.19 cells ($8x10^6$) were used per transfection in 700 µl DMEM/HI medium. 20 µg EGFRvIII-FL/pWBDHFR and 2 µg CMV-Puro plasmid DNA were added. Cells were electroporated at 300 volts/960uF with Bio-Rad Gene Pulser. Following electroporation, cells were cooled on ice for 10 minutes and, thereafter, 10 ml non-selection medium (DMEM/HI Glucose, 10% FBS, 50 µM BME, 2mM L-Glutamine, 100 units Penicillin-G/ml, 100 units MCG Streptomycin/ml) was added. Cells were incubated for 48hrs at 37^0C 7.5% CO_2.

Following incubation, cells were split into selection medium (DMEM/HI Glucose, 10% FBS, 2 mM L-Glutamine, 50 µM BME, 100 units Penicillin-G/ml, 100 units MCG Streptomycin/ml, 2ug/ml puromycin) at $2x10^4$, $0.4x10^4$ and $0.08x10^4$ cells/ well in 96 well plate and were selected in selection medium for 14 days to generate stable clones. Puro resistant clones were stained with E752 mAb (an anti-EGFR antibody, described in Yang et al., Crit Rev Oncol Hematol., 38(1):17-23 (2001)) and goat anti-human IgG PE then analyzed on FACS Vantage (Becton Dickinson).

C. Construction of EGFRvIII-RbFc Expression Constructs.

In order to generate the EGFRvIII rabbit Fc fusion, protein, we first constructed a vector containing DNA encoding rabbit Fc. This was ligated with DNA encoding EGFRvIII. This approach is described in more detail below:

1. Construction of RbFc/pcDNA3.1 Hygro:

Primers 1322/867 (below) were used to amplify a 721bp fragment encoding the Hinge-CH2-CH3 domain of rabbit IgG.

#1322 (sense): 5'-GGTGGCGGTACCTGGACAAGACCGTTGCG-3' (SEQ ID NO: 68)

#867 (antisense): 5'-ATAAGAATGCGGCCGCTCATTTACCCGGAGAGCGGGA-3' (SEQ ID NO: 69)

The resulting PCR product was digested with KpnI and NotI, gel purified and ligated into KpnI/NotI digested pcDNA3.1(+)/Hygro (Invitrogen, Burlington, ON) to yield plasmid RbFc/pcDNA3.1 Hygro.

2. Construction of EGFRvIII-RbFc/pCEP4:

Primers 1290/1293 (below) were used to amplify an 1165bp product from EGFRvIII-FL/pWBDHFR plasmid template with Pfu polymerase

#1290 (sense): 5'-CTACTAGCTAGCCACCATGCGACCCTCCGGGA-3' (SEQ ID NO: 70)

#1293 (anti-sense): 5'-CGGGGTACCCGGCGATGGACGGGATC-3' (SEQ ID NO: 71)

The PCR product was digested with NheI and KpnI, gel purified and ligated into NheI/KpnI digested RbFc/pcDNA3.1 Hygro to yield plasmid EGFRvIII-RbFc/pcDNA3.1Hygro.

A 2170 bp SnaBI/XhoI fragment was isolated from EGFRvIII-RbFc/pcDNA3.1Hygro and subcloned into SnaBI/XhoI digested pCEP4 (Invitrogen, Burlington, ON) to yield plasmid EGFRvIII-RbFc/pCEP4.

3. Generation of 293F EGFRvIII-RbFc stable Cell Lines:

Plasmid EGFRvIII-RbFc/pCEP4 was introduced into 293F cells (Gibco, Grand Island, NY) by Calcium Phosphate transfection, as follows: one day prior to transfection, $1x10^6$ 293F cells were plated on a gelatin coated 100mm tissue culture petridish and incubated at 5% CO2, 37°C. Cells were fed with 10ml of fresh non-selective media (DMEM/F12, 10% FBS, 2mM L-Glutamine, 100U/ml Penicillin G, 100U/ml MCG Streptomycin) 2-3 hours before transfection. Transfection reagents were prepared in a microfuge tube, as follow: 10µg of DNA (EGFRvIII-RbFc/pCEP4) was mixed with 62µl of 2M Calcium Phosphate and deionized water to make the final volume 500µl. In another tube pipette 500µl of 2XHBS is drawn and used to transfer the transfection reagents.

The solution in the tube pipette was added to the cells drop by drop, while maintaining proper pH by leaving cells in a 5% CO2 incubator until transfection was performed. 15-20 hours after transfection, cells were washed with PBS and feed with 10ml of fresh 293F non-selective media. Expressing cells were harvested with trypsin 48-72 post-transfection and cells were plated at $0.08x10^4$ cells/well in a 96 well plate in 293F selective media (DMEM/F12, 10% FBS, 2mM L-Glutamine, 100U/ml Penicillin G, 100U/ml MCG Streptomycin, 250ug/ml Hygromycin) for 14 days.

Hygromycin resistent clones were screened by ELISA using anti-EGFR antibody E763 (US Patent No. 6,235,883) as the capture antibody at 1ug/ml and detecting with a goat anti-rabbit IgG HRPO (CalTag) at 1:100 dilution.

D. Conjugation of EGFRvIII PEP3 to OVA via Maleimide Conjugation

The EGFRvIIIpeptide-OVA used for titration of antibodies (Example 3) was produced as follows:

207 µg of EGFRvIII PEP3 was reduced using pre-weighed DTT from Pierce (#20291). One vial of 7.7mg of pre-weighed DTT was dissolved using 100 µL of de-ionized water. The DTT

stock was added to the EGFRvIII PEP3. The volume of the reaction was brought to 600 µL using PBS pH 7.4. The reaction was rotated for 30 minutes at room temperature.

A G10 column was prepared by weighing out 5 grams of G10 sephadex beads and adding 40 mL of PBS, mixing and leaving at room temperature for 10 minutes, and then centrifuging the beads at 1000 rpm for 10 minutes. The supernatant was removed and an additional 20 mL of PBS was added. The beads were centrifuged at 1000 rpm for 10 minutes. The supernatant was removed and enough PBS added to make a 50% slurry of G10 sephadex beads. 5 mL of the 50% slurry mixture was added to a 5mL spin column and the column was placed in a 14mL polypropylene tube. The column was centrifuged at 1000 rpm for 3 minutes. Another 3 mL of PBS was added and the column was cenrifuged again at 1000 rpm for 3 minutes. The polypropylene tube was replaced with a new tube and the columns were now ready to use.

DTT was removed from the reduced peptide. After the 30 minute reaction time for reducing the peptide, 300 µL of the reduced peptide was added per column. The column was centrifuged at 1000rpm for 3 minutes. An additional 250 µL of PBS was added to each column and centrifuged again at 1000 rpm for 3 minutes. The reduced peptide was collected in the 14 mL polypropylene tube.

The reduced peptide was conjugated to maleimide activated OVA and collected in an eppendorf tube. 2 mg of the maleimide activated OVA was dissolved (Pierce: 77126, Rockford IL) with maleimide conjugation buffer to make a 10 mg/mL stock. 414 µg of the maleimide activated OVA was added to the reduced peptide in the eppendorf tube. 500 µL of the maleimide conjugation buffer was added to the reaction. The reaction was allowed to incubate for 2 hours at room temperature and then 2mg of cysteine was added to quench any active maleimide groups that might have been present. The cysteine was allowed to react for 30 additional minutes at room temperature. The conjugate was then washed with a 10K centrifugal column 3 times using 1X PBS pH 7.4. This removed any free peptide that did not conjugate to the OVA and free cysteine. The conjugate was removed from the centrifugal column using gel loading tips and transferred to an eppendorf tube. Finally, the conjugate was brought to the desired concentration using 1X PBS pH 7.4. The conjugate produced had a molar ratio of 14.5:1 (peptide:OVA)

EXAMPLE 2

Production of anti-EGFRvIII Antibodies Through Hybridoma Generation

Eight XenoMouse mice that produce antibodies with a gamma-1 constant region (XenoMouse G1 mice) were immunized on day 0 and boosted on days 11, 21, 32, 44 and 54 for this protocol and fusions were performed on day 58. All immunizations were conducted via subcutaneous administration at the base of tail plus intraperitoneal administartion for all injections. The day 0 immunization was done with 1.5 x 10^7 B300.19/EGFRvIII transfected cells (Example 1A) suspended in pyrogen free DPBS admixed 1:1 v/v with complete Freunds adjuvant (CFA) (Sigma, St. Louis, MO). Boosts on days 11, 21, and 32 were done with 1.5 x 10^7

B300.19/EGFRvIII transfected cells in DPBS admixed 1:1 v/v with incomplete Freunds adjuvant (IFA) (Sigma, St. Louis, MO). The boosts on day 44 was done with 5 μg of the PEP3 (EGFRvIII peptide) – KLH conjugate (Example 1) in DPBS admixed 1:1 v/v with IFA and final boost, on day 54, was done with 5ug PEP3 (EGFRvIII peptide) – KLH conjugate in DPBS without adjuvant.

On day 58, mice were euthanized, and then inguinal and Lumbar lymph nodes were recovered. Lymphocytes were released by mechanical disruption of the lymph nodes using a tissue grinder then depleted of T cells by CD90 negative selection. The fusion was performed by mixing washed enriched B cells and non-secretory myeloma P3X63Ag8.653 cells purchased from ATCC, cat. # CRL 1580 (Kearney et al, J. Immunol. 123:1548-1550 (1979)) 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 2-4 mL of Pronase solution (CalBiochem, cat. # 53702; 0.5 mg/ml in PBS) for no more than 2 minutes. Then, 3–5 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 (0.3M Sucrose, Sigma, Cat# S7903, 0.1mM Magnesium Acetate, Sigma, Cat# M2545, 0.1 mM Calcium Acetate, Sigma, Cat# C4705 (St. Louis, MO)).

The supernatant was removed after centrifugation and the cells washed by resuspension 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, and using the following instrument settings: Alignment condition: voltage: 50 v, time: 50 s, Membrane breaking at: voltage: 3000 v, time: 30 μs, Post-fusion holding time: 3 s. After fusion, the cells were re-suspended in DMEM (JRH Biosciences),15% FCS (Hyclone), containing HAT, and supplemented with L-glutamine, pen/strep, OPI (oxaloacetate, pyruvate, bovine insulin) (all from Sigma, St. Louis, MO) and IL-6 (Boehringer Mannheim) for culture at 37 °C and 10% CO^2 in air.

Cells were plated in flat bottomed 96-well tissue culture plates at $4x10^4$ cells per well. Cultures were maintained in HAT (hypoxanthine, aminopterin and thymidine) supplemented media for 2 weeks before transfer to HT (hypoxanthine and thymidine) supplemented media. Hybridomas were selected for by survival in HAT medium and supernatants were screened for antigen reactivity by ELISA. The ELISA format entailed incubating supernatants on antigen coated plates (EGFRvIII peptide-OVA coated plates and wild type EGFr peptide-OVA coated plates as a counter screen) and detecting EGFRvIII-specific binding using horseradish peroxidase (HRP) labeled mouse anti-human IgG (see Table 2.1).

TABLE _2.1

Plate.Well	Hybridoma	1st OD	2nd OD	
		fusion plate	muEGFr	EGFr
13.2 D10	13.1	4.034	2.653	0.051
13.3 C12	13.2	3.829	2.443	0.049
13.3 F11	13.3	3.874	1.081	0.049
13.6 B11	13.4	3.322	1.311	0.052

Clones	Plate	OD #1	OD #2	
		cloning plate	muEGFr	EGFr
13.1.1	0.5c/w D2	2.614	2.586	0.042
13.1.2	0.5c/w F5	2.248	1.272	0.041

As will be observed, at least four antigen specific hybridomas were detected: 13.1, 13.2, 13.3, and 13.4. These hybridomas that were positive in the ELISA assay EGFRvIII specificity were confirmed by FACS on stably transfected 300.19 cells expressing EGFRvIII versus 300.19 untransfected parental cells.

Cloning was performed on selected antigen-positive wells using limited dilution plating. Plates were visually inspected for the presence of single colony growth and supernatants from single colony wells then screened by antigen-specific ELISAs and FACS confirmation as described above. Highly reactive clones were assayed to verify purity of human gamma and kappa chain by multiplex ELISA using a Luminex instrument. Based on EGFRvIII specificity in the ELISA and FACS assay, Clone 13.1.2 was selected as the most promising candidate for further screening and analysis. The nucleotide and amino acid sequences of the heavy and light chains of 13.1.2 antibody are shown in FIG. 3L and SEQ ID NO: 137 and 139 for heavy and light chain nucleic acids and 138 and 140 for heavy and light chain amino acid sequences.. In addition, a comparison of the 13.1.2 heavy chain and light chain sequences with the germline sequence from which they were derived as shown in FIGs 4 and 5.

EXAMPLE 3

Antibody Generation Through Use of XenoMax Technology

Immunization of XenoMouse animals

Human monoclonal antibodies against human EGFRvIII were developed by sequentially immunizing XenoMouse mice that produce antibodies with a gamma-1 constant region (XenoMouse G1 mice), XenoMouse mice that produce antibodies with gamma-2 constant regions

(XenoMouse XMG2 mice), and XenoMouse mice that produce antibodies with a gamma-4 constant region (XenoMouse G4 mice).

To generate mAbs by through XenoMax technology, cohorts of XenoMouse G1 and XMG2 mice were immunized with EGFRvIII PEP3 (Example 1A) and EGFRvIII-expressing 300.19 cells (Example 1B) or with bacterially expressed extracellular domain of EGFRvIII protein (EGFRvIII-ECD) (Dr. Bigner, Duke University) and EGFRvIII-expressing 300.19 cells or with EGFRvIII-Rabbit Fc fusion protein (EGFRvIII-RbFc) (Example 1C) and EGFRvIII-expressing 300.19 cells or with EGFRvIII-RbFc only via foot pad (FP), or via base of the tail by subcutaneous injection and intraperitoneum (BIP).

For footpad immunizations, the initial immunization was with or without 10 X 10^6 EGFRvIII-expressing 300.19 cells and with or without 10 μg of EGFRvIII PEP3 or EGFRvIII-ECD or EGFRvIII-RbFc mixed 1:1 v/v with Titermax gold (Sigma, Oakville, ON) per mouse. The subsequent boosts were performed with half of the amount of immunogen used in the initial immunization. The first four boosts were done by taking the immunogen mixed with alum (Sigma, Oakville, ON), adsorbed overnight, per mouse as shown in the Table 3.1 below. This was followed by one injection with the respective immunogen in Titermax gold, one injection with alum and then a final boost of the immunogen in PBS as shown in Table 3.1. In particular, animals were immunized on days 0, 3, 7, 10, 14, 17, 21 and 24. The animals were bled on day 19 to obtain sera and determine the titer for harvest selection. The animals were harvested on Day 28.

Table 3.1

Footpad immunization schedule

Group #		1	2	3	4	5	6	7	8
# of animals		5	5	5	5	5	5	5	5
Mouse strain		XMG2	XM3C-3	XMG2	XM3C-3	XMG2	XM3C-3	XMG2	XM3C-3
Boost #	Adjuvant	Immunogen		Immunogen		Immunogen		Immunogen	
1st	Titermax gold	EGFRvIII-300.19 cells + PEP3-KLH		EGFRvIII-300.19 cells + EGFRvIII-ECD		EGFRvIII-300.19 cells + EGFRvIII-RbFc		EGFRvIII-RbFc	
2nd	Alum	EGFRvIII-300.19 cells		EGFRvIII-300.19 cells		EGFRvIII-300.19 cells		EGFRvIII-RbFc	
3rd	Alum	PEP3-KLH		EGFRvIII-ECD		EGFRvIII-ECD		EGFRvIII-RbFc	
4th	Alum	EGFRvIII-300.19 cells		EGFRvIII-300.19 cells		EGFRvIII-300.19 cells		EGFRvIII-RbFc	
5th	Alum	PEP3-KLH		EGFRvIII-ECD		EGFRvIII-ECD		EGFRvIII-RbFc	
6th	Titermax gold	EGFRvIII-300.19 cells		EGFRvIII-300.19 cells		EGFRvIII-300.19 cells		EGFRvIII-RbFc	
7th	Alum	PEP3-KLH		EGFRvIII-ECD		EGFRvIII-ECD		EGFRvIII-RbFc	
8th	PBS	EGFRvIII-300.19 cells + PEP3-KLH		EGFRvIII-300.19 cells + EGFRvIII-ECD		EGFRvIII-300.19 cells + EGFRvIII-RbFc		EGFRvIII-RbFc	
Harvest									

The initial BIP immunization with the respective immunogen, as described for the footpad immunization, was mixed 1:1 v/v with Complete Freund's Adjuvant (CFA, Sigma, Oakville, ON) per mouse. Subsequent boosts were made first with the immunogen respectively, mixed 1:1 v/v with Incomplete Freund's Adjuvant (IFA, Sigma, Oakville, ON) per mouse, followed by a final boost in PBS per mouse. The animals were immunized on days 0, 14, 28, 42, 56, and day 75 (final boost) as shown in Table 3.2 below. The animals were bled on day 63 to obtain sera and determine the titer for harvest selection. The animals were harvested on Day 78.

Table 3.2

Bip Immunization schedule

Group		9	10	11	12	13	14	15	16
# of animals		5	5	5	5	5	5	5	5
Mouse strain		XMG2	XM3C-3	XMG2	XM3C-3	XMG2	XM3C-3	XMG2	XM3C-3
Boost #	Adjuvant	Immunogen		Immunogen		Immunogen		Immunogen	
1st	CFA	EGFRvIII-300.19 cells + PEP3-KLH		EGFRvIII-300.19 cells + EGFRvIII-ECD		EGFRvIII-300.19 cells + EGFRvIII-RbFc		EGFRvIII-RbFc	
2nd	IFA	EGFRvIII-300.19 cells		EGFRvIII-300.19 cells		EGFRvIII-300.19 cells		EGFRvIII-RbFc	
3rd	IFA	PEP3-KLH		EGFRvIII-ECD		EGFRvIII-ECD		EGFRvIII-RbFc	
4th	IFA	EGFRvIII-300.19 cells		EGFRvIII-300.19 cells		EGFRvIII-300.19 cells		EGFRvIII-RbFc	
5th	IFA	PEP3-KLH		EGFRvIII-ECD		EGFRvIII-ECD		EGFRvIII-RbFc	
6th	PBS	EGFRvIII-300.19 cells + PEP3-KLH		EGFRvIII-300.19 cells + EGFRvIII-ECD		EGFRvIII-300.19 cells + EGFRvIII-RbFc		EGFRvIII-RbFc	
Harvest									

Selection of animals for harvest By titer determination

Anti-hEGFRvIII antibody titers were determined by ELISA. EGFRvIII-RbFc (2.5 μg/ml) or a control RbFc (2 μg/ml) or EGFRvIIIpeptide-OVA (2 μg/ml) (Example 1) or control OVA (4 μg/ml) were coated onto Costar Labcoat Universal Binding Polystyrene 96-well plates (Corning, Acton, MA) overnight at four degrees. The solution containing unbound antigen was removed and the plates were treated with UV light (365nm) for 4 minutes (4000 microjoules). The plates were washed five times with dH_2O. Sera from the EGFRvIII immunized XenoMouse® animals, or naïve XenoMouse® animals, were titrated in 2% milk/PBS at 1:2 dilutions in duplicate from a 1:100 initial dilution. The last well was left blank. The plates were washed five times with dH_2O. A goat anti-human IgG Fc-specific horseradish peroxidase (HRP, Pierce, Rockford, IL) conjugated antibody was added at a final concentration of 1 μg/mL for 1 hour at room temperature. The plates were washed five times with dH_2O. The plates were developed with the addition of TMB chromogenic substrate (Gaithersburg, MD) for 30 minutes and the ELISA was stopped by the addition of 1 M phosphoric acid. The specific titer of individual XenoMouse® animals was

determined from the optical density at 450 nm and is shown in Tables 3.3 and 3.4. The titer represents the reciprocal dilution of the serum and therefore the higher the number the greater the humoral immune response to hEGFRvIII.

For the mice immunized via base of the tail by subcutaneous injection and intraperitoneum, the titre was determined exactly as above except the plates were coated with EGFRvIII-RbFc (2.0 µg/ml) or a control RbFc (2.5 µg/ml).

Table 3.3

Group #	Immunization (site and Immunogen)	Mouse Strain and sex	Mouse I.Ds	EGFRvIII -RbFc @ 2.5ug/ml.	Control RbFc @ 2.0ug/ml.	EGFRvIII peptide-OVA coated at 2.0 µg/ml	OVA coated at 4.0 µg/ml
1	FP EGFRvIII-300.19 cells + EGFRvIII PEP3-KLH (see Imm. Sched.)	XMG2	0748-1	330		13549	<100
			0748-2	237		7635	<100
			0748-3	109		9824	<100
			0748-4	714		8014	<100
			0748-5	165		9421	<100
			Naïve	<100		n/a	n/a
2	FP EGFRvIII-300.19 cells + EGFRvIII PEP3-KLH (see Imm. Sched.)	XM3C-3	0741-1	388		347	<100
			0741-2	327		240	<100
			0741-3	385		330	<100
			0741-4	589		227	<100
			0741-5	273		626	<100
			Naïve	<100		n/a	n/a
3	FP EGFRvIII-300.19 cells + EGFRvIII-ECD (see Imm. Sched.)	XMG2	0749-1	552		<100	<100
			0749-2	477		<100	<100
			0749-3	100		<100	<100
			0749-4	100		<100	<100
			0749-5	1631		<100	<100
			Naïve	100		n/a	n/a
4	FP EGFRvIII-300.19 cells + EGFRvIII-ECD (see Imm. Sched.)	XM3C-3	0742-1	372		<100	<100
			0742-2	745		<100	<100
			0742-3	484		<100	<100
			0742-4	530		<100	<100
			0742-5	270		<100	<100
			Naïve	100		n/a	n/a
5	FP EGFRvIII-300.19 cells + EGFRvIII-RbFc (see Imm. Sched.)	XMG2	0750-1	5399	175	<100	<100
			0750-2	3072	151	<100	<100
			0750-3	>6400	358	<100	<100
			0750-4	5845	196	<100	<100
			0750-5	5770	196	<100	<100
			Naïve	100	100	n/a	n/a
6	FP EGFRvIII-300.19 cells + EGFRvIII-RbFc (see Imm. Sched.)	XM3C-3	0743-1	1220	<100	<100	<100
			0743-2	1183	<100	<100	<100
			0743-3	645	<100	<100	<100
			0743-4	759	<100	<100	<100
			0743-5	1260	<100	<100	<100
			Naïve	100	<100	n/a	n/a

Group #	Immunization (site and Immunogen)	Mouse Strain and sex	Mouse I.Ds	EGFRvIII -RbFc @ 2.5ug/ml.	Control RbFc @ 2.0ug/ml.	EGFRvIII peptide-OVA coated at 2.0 µg/ml	OVA coated at 4.0 µg/ml
7	FP EGFRvIII-RbFc (see Imm. Sched.)	XMG2	0745-1	1897	<100	<100	<100
			0745-2	>6400	323	<100	<100
			0745-3	1225	<100	<100	<100
			0745-4	4047	<100	<100	<100
			0745-5	852	<100	<100	<100
			Naïve	100	<100	n/a	n/a
8	FP EGFRvIII-RbFc (see Imm. Sched.)	XM3C-3	0744-1	362	<100	<100	<100
			0744-2	807	<100	<100	<100
			0744-3	479	<100	<100	<100
			0744-4	631	<100	<100	<100
			0744-5	1112	<100	<100	<100
			Naïve	100	<100	n/a	n/a

All the XenoMouse animals from group 5 and XenoMouse animals 0743-5 from group 6 from Table 3.3 were selected for XenoMax harvests based on the serology.

Table 3.4

Group #	Immunization (site and Immunogen)	Mouse Strain and sex	Mouse I.Ds	EGFRvIII -RbFc @ 2.0ug/ml.	Control RbFc @ 2.5ug/ml.	EGFRvIII peptide-OVA coated at 2.0 µg/ml	OVA coated at 4.0 µg/ml
9	BIP EGFRvIII-300.19 cells + EGFRvIII PEP3-KLH (see Imm. Sched.)	XMG2	O695-1	2921		>128000	472
			O695-2	2219		30504	379
			O695-3	4609		>128000	608
			O695-4	>6400		>128000	368
			O695-5	1580		19757	269
			Naïve	<100		n/a	242
10	BIP EGFRvIII-300.19 cells + EGFRvIII PEP3-KLH (see Imm. Sched.)	XM3C-3	O700-1	<100			
			O700-2	<100			
			O700-3	>6400			
			O700-4	5342			
			O700-5	>6400			
			Naïve	<100			
11	BIP EGFRvIII-300.19 cells + EGFRvIII-ECD (see Imm. Sched.)	XMG2	O696-1	<100		561	240
			O696-2	<100		788	326
			O696-3	<100		604	266
			O696-4	143		444	263
			O696-5	<100		303	254
			Naïve	<100			242
12	BIP EGFRvIII-300.19 cells + EGFRvIII-ECD (see Imm.	XM3C-3	O702-1	358			
			O702-2	469			
			O702-3	401			
			O702-4	>6400			
			O702-5	>6400			

Group #	Immunization (site and Immunogen)	Mouse Strain and sex	Mouse I.Ds	EGFRvIII -RbFc @ 2.0ug/ml.	Control RbFc @ 2.5ug/ml.	EGFRvIII peptide-OVA coated at 2.0 µg/ml	OVA coated at 4.0 µg/ml
	Sched.)		Naïve	<100			
13	BIP EGFRvIII-300.19 cells + EGFRvIII-RbFc (see Imm. Sched.)	XMG2	O694-1	>6400	>6400	250	243
			O694-2	>6400	>6400	296	309
			O694-3	>6400	>6400	736	605
			O694-4	>6400	>6400	739	1111
			O694-5	3710	>6400	517	465
			Naïve	<100	>6400		242
14	BIP EGFRvIII-300.19 cells + EGFRvIII-RbFc (see Imm. Sched.)	XM3C-3	O703-1	2740	>6400		
			O703-2	408	>6400		
			O703-3	1406	>6400		
			O703-4	1017	>6400		
			O703-5	403	>6400		
			Naïve	<100	>6400		
15	BIP EGFRvIII-RbFc (see Imm. Sched.)	XMG2	O697-1	>6400	>6400	340	348
			O697-2	>6400	>6400	642	1793
			O697-3	6242	>6400	319	246
			O697-4	1766	>6400	133	<100
			O697-5	>6400	>6400	685	448
			Naïve	<100	>6400	243	242
16	BIP EGFRvIII-RbFc (see Imm. Sched.)	XM3C-3	O701-1	592	>6400		
			O701-2	1118	>6400		
			O701-3	>6400	>6400		
			O701-4	<100	<100		
			O701-5	n/a	n/a		
			Naïve	<100	>6400		

XenoMouse animals (0695-1, 0695-3 and 0695-4) were selected for harvests based on the serology data in Table 3.4.

Selection of B cells.

B-cells from the above-discussed animals were harvested and cultured. Those secreting EGFRvIII-peptide specific antibodies were isolated as described in Babcook et al., *Proc. Natl. Acad. Sci. USA,* 93:7843-7848 (1996). ELISA was used to identify primary EGFRvIII-peptide-OVA -specific wells. About 5 million B-cells were cultured from XenoMouse animals in 245 96 well plates at 500 or 150 or 50 cells/well, and were screened on EGFRvIII-peptide-OVA to identify the antigen-specific wells. About 515 wells showed ODs significantly over background, a representative sample of which are shown in Table 3.5.

Table 3.5

	Total # of plates	Positives above cutoff OD of:															
		0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	1.5	2.0	2.5	3.0	3.5
Cansera 500 cells/ well	12	11 52	63 4	81	56	49	45	38	32	29	26	25	18	11	4	1	0
Sigma 500 cells/ well	13	12 48	77 3	19 5	13 9	11 7	99	80	73	58	53	49	21	9	5	1	0
Sigma 150 cells/ well	20	19 20	13 04	47 8	17 8	91	67	55	47	45	36	33	19	9	5	2	0
Total	45	43 20	27 11	75 4	37 3	25 7	21 1	17 3	15 2	13 2	11 5	10 7	58	29	14	4	0

244 of EGFRvIII-peptide-OVA-Elisa positive wells of OD > 0.5 were screened again on EGFRvIII-peptide-OVA and on OVA to confirm that they were EGFRvIII-peptide specific. A representative example of these results is shown in Table 3.6.

Table 3.6

Plate	Well		1' EGFRvIII peptide-OVA OD	2' EGFRvIII peptide-OVA OD	OVA OD
121	G	1	0.7534	1.4065	0.1355
121	A	7	1.3472	2.1491	0.1268
121	D	8	0.6743	0.4179	0.1531
121	E	8	2.0415	2.6965	0.1498
121	H	10	0.8611	0.4288	0.1595
121	C	12	2.1455	2.6443	0.1404
122	H	1	1.8890	2.5987	0.1164
122	H	5	0.5943	0.8321	0.1572
122	F	8	0.6834	0.7715	0.1450

Limited antigen assay and analysis

The limited antigen analysis is a method that affinity-ranks the antigen-specific antibodies present 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.*, International Patent Application No. WO 03/48730)

EGFRvIII peptide-OVA was coated to plates at three concentrations; 7.5 ng/ml, 1.5 ng/ml and 0.03 ng/ml for overnight at 4^0 C on 96-well Elisa plates. Each plate was washed 5 times with dH_2O, before 50ul of 1% milk in PBS with 0.05% sodium azide were added to the plate, followed by 4 µl of B cell supernatant added to each well. After 18 hours at room temperature on a shaker, the plates were again washed 5 times with dH_2O. To each well was added 50ul of Gt anti-Human (Fc)-HRP at 1 µg/ml. After 1 hour at room temperature, the plates were again washed 5 times with dH_2O and 50 µl of TMB substrate were added to each well. The reaction was stopped by the addition of 50uL of 1M phosphoric acid to each well and the plates were read at wavelength 450nm and the results shown in Table 3.7.

Table 3.7

Culture Plate	Well		Limited Ag						High Antigen (1.0µg/ml)
			0.03ng/ml		1.5ng/ml		7.5ng/ml		
			OD	Rank	OD	Rank	OD	Rank	
133	B	2	0.7670	1	1.189	54	1.871	95	2.050
124	G	12	0.7400	2	1.895	1	3.101	1	3.463
145	C	1	0.715	3	1.552	7	2.671	10	3.194
129	G	10	0.6720	4	1.367	22	2.692	8	2.977
186	B	6	0.657	5	1.842	2	2.859	3	3.411
143	F	12	0.653	6	1.677	3	2.741	6	3.156
136	E	3	0.6340	7	1.468	15	2.683	9	3.280
137	C	11	0.595	8	1.582	5	2.94	2	3.444
139	A	11	0.582	9	1.374	19	2.282	47	2.255
174	F	1	0.573	10	1.577	6	2.775	4	2.364

The results generated from limited antigen analysis were compared to the total OD obtained in high antigen assay. A relative ranking of affinity was done by taking the ratio of the OD obtained in limited antigen assay Vs that obtained in high antigen assay. Antibodies with higher ratio will have the highest affinity. Table 3.7 shows the sample of B-cell culture supernatants that were ranked based on limited antigen assay OD (for the lowest antigen plating concentration of 0.03 ng/ml) Vs the high antigen assay OD.

Native cell binding assay by FMAT

EGFRvIII peptide-OVA-Elisa positive well supernatants were analyzed for their ability to bind to the native form of EGFRvIII stably expressed on NR6 cells (NR6 M cells) (*See,* Batra et al. Epidermal growth factor ligand-independent, unregulated, cell-transforming potential of a naturally

occurring human mutant EGFRvIII gene. Cell Growth Differ. 6(10):1251-9 (1995)). NR 6 M cells were seeded at 8000 cells per well and incubated over night in 96 well FMAT plates. Media was then removed leaving 15 µl in the well. 15 µl B-cell culture supernatants were added and 15 µl anti-human IgG Fc Cy5 at 1 µg/ml final concentration added to wells. It is then left incubated at 4° C for 2 hours. The cells were washed with 150 µl PBS, and fixed before reading on FMAT. The results were expressed as total fluorescent intensity (Table 3.8). Human anti-EGFRvIII mAb 13.1.2 was used as a positive control starting at 1 µg/ml final concentration and negative control was PK 16.3.1 at the same concentration. 134 of the 244 samples tested bound to NR6M cells of which 62 had a total fluorescence of greater than 8000. 6 of these 134 binders were false positives.

The same type of native binding assay was done on NR6 Wt cells (NR6 cells expressing EGF receptor) (See Batra et al. Epidermal growth factor ligand-independent, unregulated, cell-transforming potential of a naturally occurring human mutant EGFRvIII gene. Cell Growth Differ. 6(10):1251-9 (1995)) to eliminate the binding is due to binding to Wt receptor (Table 3.8). ABX-EGF was used as a positive control and PK 16.3.1 at the same concentration was used as a negative control antibody. 3 out the 134 NR6 M binders were binding strongly to NR6 Wt cells. 190 of the 244 wells bound EGFRvIII peptide in Elisa were also bound to the native form on cells. Examples are given in Table 3.8.

Table 3.8

Plate			1' VIII-pep-OVA OD	2' VIII-pep-OVA OD	OVA OD	FMAT native binding to NR6 M cells	FMAT native binding to NR6 Wt cells
174	F	1	2.4945	3.0308	0.1900	138373	1668
187	A	4	1.5337	1.2085	0.1920	128626	202459.8
132	D	8	0.8555	1.2070	0.1649	109379	0
142	C	11	2.2889	2.8194	0.2239	94944	0
129	A	7	2.1501	2.8208	0.1515	84024	0
127	E	1	2.6923	3.1986	0.1219	82031	0
124	G	12	3.2929	3.5634	0.1455	73080	0
141	C	6	0.7512	1.2567	0.1547	60816	814.5
173	C	1	2.5728	2.5714	0.2134	58702	2523.4
128	G	9	0.6293	0.7483	0.1520	49631	0
129	H	6	2.9370	3.0952	0.2582	0	0
183	E	11	2.3450	2.7717	0.1050	0	0

In Table 3.8, supernatant from well 187A4 is identified as a Wt binder and 141C6 was a false positive for NR6 M cells binding. Wells 129H6 and 183E11 are strong peptide binders with no native binding.

Internalization assay

The top 60 native binding B cell culture supernatants were further assayed for their ability to internalize the receptor. NR6 M cells were seeded at 8000 cells/well into 96 well FMAT plates and incubated overnight. Media was removed and 10-15 µl B-Cell culture supernatant in a total volume of 30 µl media, in duplicate was added. Next, 15 µl of secondary antibody (SS Alexa 647 anti-Human IgG Fab at 1.5 µg/ml final concentration) was added and the mixture was incubated on ice for 1 hr. An irrelevant B-Cell Culture supernatant was used to see the effect of the culture media. Human anti-EGFRvIII mAb 13.2.1 was used as a positive control starting at 1 µg/ml (final concentration) and negative control was PK 16.3.1 (human anti-KLH IgG2 antibody) at the same concentration. After incubation, the cells were washed with cold PBS, 50 µl media was added to all of the wells, one of the duplicates were incubated at 37 °C for 30 mins while the other duplicate remained on ice. After the incubations media was removed, 100ul of cold 50 mM glutathione was added to the set incubated at 37 °C and 100 µl of cold media added to the other set, both sets were then left on ice for 1 hr. The cells were then washed with 100 µl cold PBS and then fixed with 1% paraformaldehyde and read in FMAT. The results were expressed as % internalized, calculated as total fluorescence in the presence of glutathione/ total fluorescence in the absence of glutathione X 100. Representative information is given in Table 3.9.

Table 3.9

Well no.	No glutathione FL1xcount	With glutathione FL1xcount	% internalized, (glut+/glut-) X 100
124 C9	1877	1394	74.3%
124 G12	26465	9959	37.6%
125 H1	14608	3686	25.2%
125 D10	2342	1236	52.8%
127 E1	15059	1318	8.7%
127 B9	12444	7109	57.1%
127 E11	6623	0	0.0%
128 G9	10071	1851	18.4%
129 A7	27648	8708	31.5%
130 B4	4558	4354	95.5%
131 H5	9258	2656	28.7%
132 D8	35820	13293	37.1%
133 F9	9773	3621	37.0%
136 F10	2392	0	0.0%
137 G6	5104	1021	20.0%
137 G10	3451	0	0.0%

EGFRvIII-specific Hemolytic Plaque Assay.

A number of specialized reagents were needed to conduct this assay. These reagents were prepared as follows.

1. *Biotinylation of Sheep red blood cells (SRBC).* SRBCs were stored in RPMI media as a 25% stock. A 250 µl SRBC packed-cell pellet was obtained by aliquoting 1.0 ml of SRBC to a fresh eppendorf tube. The SRBC were pelleted with a pulse spin at 8000 rpm (6800 rcf) in microfuge, the supernatant drawn off, the pellet re-suspended in 1.0 ml PBS at pH 8.6, and the centrifugation repeated. The wash cycle was repeated 2 times, then the SRBC pellet was transferred to a 15-ml falcon tube and made to 5 ml with PBS pH 8.6. In a separate 50 ml falcon tube, 2.5 mg of Sulfo-NHS biotin was added to 45 ml of PBS pH 8.6. Once the biotin had completely dissolved, the 5 ml of SRBCs were added and the tube rotated at RT for 1 hour. The SRBCs were centrifuged at 3000rpm for 5 min and the supernatant drawn off. The biotinylated SRBCs were transferred to an eppendorf tube and washed 3 times as above but with PBS pH 7.4 and then made up to 5 ml with immune cell media (RPMI 1640) in a 15 ml falcon tube (5% B-SRBC stock). Stock was stored at 4° C until needed.

2. *Streptavidin (SA) coating of B-SRBC.* 1 ml of the 5% B-SRBC stock was transferred into a fresh eppendorf tube. The B-SRBC cells were washed 3 times as above and resuspended in 1.0 ml of PBS at pH 7.4 to give a final concentration of 5% (v/v). 10 µl of a 10 mg/ml streptavidin (CalBiochem, San Diego, CA) stock solution was added and the tube mixed and rotated at RT for 20 min. The washing steps were repeated and the SA-SRBC were re-suspended in 1ml PBS pH 7.4 (5% (v/v)).

3. *EGFRvIII coating of SA-SRBC.* The SA-SRBCs were coated with biotinylated-EGFRvIIIpetide-OVA at 10 µg/ml, mixed and rotated at RT for 20 min. The SRBC were washed twice with 1.0 ml of PBS at pH 7.4 as above. The EGFRvIII-coated SRBC were re-suspended in RPMI (+10%FCS) to a final concentration of 5% (v/v).

4. *Determination of the quality of EGFRvIIIpeptide-SRBC by immunofluorescence (IF).* 10 µl of 5% SA-SRBC and 10 µl of 5% EGFRvIII peptide-coated SRBC were each added to a separate fresh 1.5 ml eppendorf tube containing 40ul of PBS. A control human anti-EGFRvIII antibody was added to each sample of SRBCs at 45 µg/ml. The tubes were rotated at RT for 25 min, and the cells were then washed three times with 100 µl of PBS. The cells were re-suspended in 50 µl of PBS and incubated with 40 mcg/mL Gt-anti Human IgG Fc antibody conjugated to Alexa488 (Molecular Probes, Eugene, OR). The tubes were rotated at RT for 25 min, and then washed with 100 µl PBS and the cells re-suspended in 10 µl PBS. 10 µl of the stained cells were spotted onto a clean glass microscope slide, covered with a glass coverslip, observed under fluorescent light, and scored on an arbitrary scale of 0-4.

5. *Preparation of plasma cells.* The contents of a single microculture well previously identified by various assays as containing a B cell clone secreting the immunoglobulin of interest

were harvested. Using a 100-1000 µl pipetman, the contents of the well were recovered by adding 37C RPMI (10% FCS). The cells were re-suspended by pipetting and then transferred to a fresh 1.5 ml eppendorf tube (final vol. approx 500-700 µl). The cells were centrifuged in a microfuge at 2500 rpm (660 rcf) for 1 minute at room temperature, and then the tube was rotated 180 degrees and spun again for 1 minute at 2500 rpm. The freeze media was drawn off and the immune cells resuspended in 100 µl RPMI (10% FCS), then centrifuged. This washing with RPMI (10% FCS) was repeated and the cells re-suspended in 60 µl RPMI (10% FCS) and stored on ice until ready to use.

6. *Micromanipulation of plasma cells.* Glass slides (2 x 3 inch) were prepared in advance with silicone edges and allowed to cure overnight at RT. Before use, the slides were treated with approx. 5ul of SigmaCoat (Sigma, Oakville, ON) wiped evenly over glass surface, allowed to dry and then wiped vigorously. To a 60 µl sample of cells was added 60 µl each of EGFRvIIIpeptide-coated SRBC (5% v/v stock), 4x guinea pig complement (Sigma, Oakville, ON) stock prepared in RPMI (10% FCS), and 4x enhancing sera stock (1:150 in RPMI with 10% FCS). The mixture was spotted (10-15 µl) onto the prepared slides and the spots covered with undiluted paraffin oil. The slides were incubated at 37° C for a minimum of 45 minutes. The EGFRvIII-specific plasma cells were identified from plaques and rescued by micromanipulation (see Table 3.10).

Table 3.10

Well ID			Single Cell Number	Total number of Single cells picked
124	G	12	EGFRvIII-SCX-105-116 (LL)	12
129	A	7	EGFRvIII -SCX-117-128 (DM)	12
174	F	1	EGFRvIII -SCX-129-137 (DM)	9
182	A	5	EGFRvIII -SCX-138-149 (LL); 162-169 (OP)	20
125	D	10	EGFRvIII -SCX-170-181 (DM); 194-201 (LL)	20
127	B	9	EGFRvIII -SCX-182-193 (LL); 202-209 (OP)	20
190	D	7	EGFRvIII -SCX-210-229 (LL)	20
130	B	4	EGFRvIII -SCX-230-249 (LL)	20
138	D	2	EGFRvIII -SCX-250-269 (LL)	20
145	C	1	EGFRvIII -SCX-80-92 (DM)	13
172	B	12	EGFRvIII -SCX-93-104 (LL)	12
187	A	4	EGFRvIII -SCX-270-281 (LL)	12
173	C	1	EGFRvIII -SCX-282-293 (BC)	12
127	E	1	EGFRvIII -SCX-294-305 (LL)	12
142	C	11	EGFRvIII -SCX-306-317 (LL)	12

Well ID			Single Cell Number	Total number of Single cells picked
141	A	10	EGFRvIII -SCX-318-329 (BC)	12
132	D	8	EGFRvIII -SCX-330-341 (LL)	12
124	D	4	EGFRvIII -SCX-342-349 (BC)	8

Single cell PCR, Cloning, Expression, Purification and Characterization of Recombinant anti-EGFRvIII Antibodies.

The genes encoding the variable regions were rescued by RT-PCR on the single micromanipulated plasma cells. mRNA was extracted and reverse transcriptase PCR was conducted to generate cDNA. The cDNA encoding the variable heavy and light chains was specifically amplified using polymerase chain reaction. The human variable heavy chain region was cloned into an IgG1 expression vector. This vector was generated by cloning the constant domain of human IgG1 into the multiple cloning site of pcDNA3.1+/Hygro (Invitrogen, Burlington, ON). The human variable light chain region was cloned into an IgK expression vector. These vectors were generated by cloning the constant domain of human IgK into the multiple cloning site of pcDNA3.1+/Neo (Invitrogen, Burlington, ON). The heavy chain and the light chain expression vectors were then co-lipofected into a 60 mm dish of 70% confluent human embryonal kidney 293 cells and the transfected cells were allowed to secrete a recombinant antibody with the identical specificity as the original plasma cell for 24-72 hours. The supernatant (3 mL) was harvested from the HEK 293 cells and the secretion of an intact antibody was demonstrated with a sandwich ELISA to specifically detect human IgG (Table 3.11). Specificity was assessed through binding of the recombinant antibody to EGFRvIII using ELISA (Table 3.11).

Table 3.11

mAb ID	Cell #	Titer	
		Total antibody	Antigen binding
129A7	SC- EGFRvIII –XG1-123/124	>1:64	>1:64
138D2	SC- EGFRvIII -XG1-250	>1:64	>1:64
174F1	SC- EGFRvIII -XG1-131	>1:64	>1:64
182A5	SC- EGFRvIII -XG1-139	>1:64	>1:64
190D7	SC- EGFRvIII -XG1-211	>1:64	>1:64
125D10	SC- EGFRvIII -XG2-170	>1:64	>1:64
182D5	SC- EGFRvIII -XG2-150	>1:64	>1:64
141A10	SC- EGFRvIII -XG1-318	1:64	1:64
132D8	SC- EGFRvIII -XG1-333	>1:64	>1:64
124D4	SC- EGFRvIII -XG1-342	>1:64	>1:64

The secretion ELISA tests were performed as follows. For Ab secretion, 2 μg/mL of Goat anti-human IgG H+L and for antigen binding, 1.5 μg/ml of EGFRvIII-Rab Ig Fc fusion protein was coated onto Costar Labcoat Universal Binding Polystyrene 96 well plates and held overnight at four degrees. The plates were washed five times with dH_2O. Recombinant antibodies were titrated 1:2 for 7 wells from the undiluted minilipofection supernatant. The plates were washed five times with dH_2O. A goat anti-human IgG Fc-specific HRP-conjugated antibody was added at a final concentration of 1 μg/mL for 1 hour at RT for the secretion plates and binding plates detected with 1μg/ml Rb anti Hu Fc for 1 hour at room temperature. The plates were washed five times with dH_2O. The plates were developed with the addition of TMB for 30 minutes and the ELISA was stopped by the addition of 1 M phosphoric acid. Each ELISA plate was analyzed to determine the optical density of each well at 450 nm.

Sequencing and sequence analysis

The cloned heavy and light chain cDNAs were sequenced in both directions and analyzed to determine the germline sequence derivation of the antibodies and identify changes from germline sequence. Such sequences are provided in FIGs. 3A-3K and (SEQ ID NO: 34-55). A comparison of each of the heavy and light chain sequences and the germline sequences from which they are derived is provided in FIGs 4-7. In addition, the sequence of the hybridoma derived 13.1.2 antibody is compared to its germline sequence in FIGs. 4 and 5.

As will be appreciated from the discussion herein, each of the 131 antibody and the 13.1.2 antibody possess very high affinities for EGFRvIII, are internalized well by cells, and appear highly effective in cell killing when conjugated to toxins. Intriguingly, each of the antibodies, despite having been generated in different immunizations of XenoMouse mice, and utilizing different technologies, each are derived from very similar germline genes. Based upon epitope mapping work (described herein), each of the antibodies, however, appear to bind to slightly different epitopes on the EGFRvIII molecule and have slightly different residues on EGFRvIII that are essential for binding. These results indicate that the germline gene utilization is of importance to generation of antibody therapeutics targeting EGFRvIII and that small changes can modify the binding and effects of the antibody in ways that allow further design of antibody and other therapeutics based upon these structural findings.

Binding of Anti-EGFRvIII mAbs to native EGFRvIII expressed on cells

In this example, binding of anti-EGFRvIII antibodies to NR6 M cells was measured. Specifically, unquantitated supernatants of XenoMax derived IgG1 recombinant antibodies were assayed for their ability to bind to NR6 M and NR6 WT cells. Cells were seeded at 10000 / well and incubated overnight at 37 C in FMAT 96 well plates. Media was removed and 40 μl mini lipo supernatant (titrated down) was added, the cells were incubated on ice for 1 hr. The human 13.1.2 EGFRvIII antibodies and ABX EGF (E7.6.3, U.S. Patent No. 6,235,883) antibodies were added as positive controls. The PK 16.3.1 antibody was used as a negative control. The cells were washed

with Cold PBS, secondary antibody was added (SS Alexa antihuman IgG Fc) at 1 μg/ml, 40 μl/well and incubated on ice for 1 hr. The cells were then washed with Cold PBS and fixed and read by FMAT. All antibodies were tested for specificity for binding by counter screening against NR6 WT cells.

Purification of Recombinant Anti-EGFRvIII Antibodies.

For larger scale production, heavy and light chain expression vectors (2.5 μg of each chain/dish) were lipofected into ten 100 mm dishes that were 70% confluent with HEK 293 cells. The transfected cells were incubated at 37°C for 4 days, the supernatant (6 mL) was harvested and replaced with 6 mL of fresh media. At day 7, the supernatant was removed and pooled with the initial harvest (120 mL total from 10 plates). Each antibody was purified from the supernatant using a Protein-A Sepharose (Amersham Biosciences, Piscataway, NJ) affinity chromatography (1 mL). The antibody was eluted from the Protein-A column with 500 mcL of 0.1 M Glycine pH 2.5. The eluate was dialyzed in PBS, pH 7.4 and filter-sterilized. The antibody was analyzed by non-reducing SDS-PAGE to assess purity and yield. Concentration was also measured by UV analysis at OD 250.

Internalization of EGFRvIII receptor by recombinant anti-EGFRvIII mAbs

XenoMax derived IgG1 recombinant antibodies were expressed, purified and quantitated as described previously. Antibodies were further assayed for their ability to internalize the EGFRvIII receptor in NR6 M cells. 250,000 NR6 M cells were incubated with primary antibody (SC95, SC131, SC133, SC139, SC150, SC170, SC211, SC230, SC250 and human 13.1.2 as a control) at 0.25 μg/ml, 7 mins on ice in 96 well v-bottomed plate in triplicate. The cells were washed with cold 10% FCS in PBS and secondary antibody (SS Alexa antihuman IgG Fab) at 3 μg/ml Fab was added and incubated for 7 mins on ice. The cells were washed with cold 10% FCS in PBS once and then resuspended in cold media. Next, two sets of the triplicate were incubated at 37 °C and the remaining set was incubated at 4 °C for 1 hr. After that the cells incubated at 4 °C and one set of the cells incubated at 37 °C were treated with glutathione (as previously mentioned) for 1 hr on ice. Then the cells were washed and resuspended in 100 μl of cold 1% FCS in PBS and analyzed by FACS. The % internalization was calculated from the geometric mean obtained from the FACS analysis [(mean at 37 °C with glutathione – mean at 4 °C with glutathione) / (mean at 37 °C without glutathione – mean at 4 C with glutathione)]. NA means that a FACS analysis was performed but the data was not provided in Table 3.12.

Table 3.12

mAb	FACS Geometric mean			% Internalization
	Without glutathione 37 °C	With glutathione 37 °C	With glutathione 4 °C	
13.1.2	22.12	19.19	5.38	82.5%
sc95	22.56	17.75	5.13	72.4%
sc131	NA	NA	NA	72%
sc133	23.39	18.63	6.24	72.2%
sc139	22.64	19.23	4.88	80.8%
sc150	20.29	7.78	4.66	20.0%
sc170	19.97	7.75	4.67	20.1%
sc211	20.76	8.23	4.78	21.6%
sc230	20.68	7.97	5.02	18.8%
sc250	24.13	8.07	4.84	16.7%

13.1.2 is an antibody that was generated through hybridoma generation (Example 2) that was directed against the EGFRvIII epitope previously and was used as a positive control in this experiment. These results in Table 3.12 demonstrate the presence of two subsets of antibodies, those that are efficiently internalized (70-80%) and those that are not (22% or less).

Example 4

Epitope Mapping Of Human Anti EGFRvIII Antibodies

In order to determine the epitopes to which certain of the antibodies of the present invention bound, the epitopes of 6 human and 3 murine monoclonal antibodies (mabs) against EGFRvIII were mapped using synthetic peptides derived from the specific EGFRvIII peptide sequence. The antibodies mapped were the human hybridoma derived anti-EGFRvIII 13.1.2 antibody, the human XenoMax derived anti-EGFRvIII 131, 139, 250, 095, and 211 antibodies and the murine anti-EGFRvIII H10, Y10, and B9 antibodies (from Dr. D. Bigner, Duke University).

The approach that was used was a custom SPOTs peptide array (Sigma Genosys) to study the molecular interaction of the human anti-EGFrVIII antibodies with their peptide epitope. SPOTs technology is based on the solid-phase synthesis of peptides in a format suitable for the systematic analysis of antibody epitopes. Synthesis of custom arrayed oligopeptides is commerically available from Sigma-Genosys. A peptide array of overlapping oligopeptides derived from the amino-acid sequence of the EGFr VIII variant was ordered from Sigma-Genosys.

A series of nine 12-mer peptides were synthesized as spots on polypropylene membrane sheets. The peptide array spanned residues 1- 20 of the EGFrVIII sequence, representing the deletion of amino acids 6-273 in the extracellular domain of wtEGFr, and the generation of a glycine (G) residue at the junction point. Each consecutive peptide was offset by 1 residue from the

previous one, yielding a nested, overlapping library of arrayed oligopeptides. The membrane carrying the 9 peptides was reacted with 9 different anti EGFrVIII antibodies (1 μg/ml). The binding of the mAbs to the membrane-bound peptides was assessed by an enzyme-linked immunosorbent assay using HRP-conjugated secondary antibody followed by enhanced chemiluminescence (ECL). The array utilized is shown in Table 4.1.

Table 4.1

Spot Array Sequence:

1. ALEEKKGNYVVT (SEQ ID NO:72)
2. LEEKKGNYVVTD (SEQ ID NO: 59)
3. EEKKGNYVVTDH (SEQ ID NO: 73)
4. EKKGNYVVTDHG (SEQ ID NO: 74)
5. KKGNYVVTDHGS (SEQ ID NO: 75)
6. KGNYVVTDHGSC (SEQ ID NO: 76)
7. GNYVVTDHGSCV (SEQ ID NO: 77)
8. NYVVTDHGSCVR (SEQ ID NO: 78)
9. YVVTDHGSCVRA (SEQ ID NO: 79)

In addition, functional epitopes were mapped by combinatorial Alanine scanning. In this process, a combinatorial Alanine-scanning strategy was used to identify amino acids in the EGFrVIII peptide necessary for interaction with anti-EGFRvIII mAbs. To accomplish this, a second set of SPOTs arrays was ordered for Alanine scanning. A panel of variants peptides with alanine substitutions in each of the 12 residues was scanned as above. Spot #1, the unmutated sequence, is a positive control for antibody binding. The array utilized is shown in Table 4.2.

Table 4.2

Alanine Scanning Array:

1. LEEKKGNYVVTD (SEQ ID NO: —59)
2. AEEKKGNYVVTD (SEQ ID NO: 80)
3. LAEKKGNYVVTD (SEQ ID NO: 81)
4. LEAKKGNYVVTD (SEQ ID NO: 82)
5. LEEAKGNYVVTD (SEQ ID NO: 83)
6. LEEKAGNYVVTD (SEQ ID NO: 84)
7. LEEKKANYVVTD (SEQ ID NO: 85)
8. LEEKKGAYVVTD (SEQ ID NO: 86)
9. LEEKKGNAVVTD (SEQ ID NO: 87)
10. LEEKKGNYAVTD (SEQ ID NO: 88)
11. LEEKKGNYVATD (SEQ ID NO: 89)
12. LEEKKGNYVVAD (SEQ ID NO: 90)
13. LEEKKGNYVVTA (SEQ ID NO: 91)

Epitopes of all 9 mAbs to the human EGFrVIII were mapped and identified by SPOTs procedure. All 9 antibodies were reactive with the peptides. The results obtained with 3 murine antibodies and 6 XenoMouse mouse derived human antibodies are presented in Table 4.3. Highlighted residues are those which we mutated to alanine and abrogated binding by the test antibody. These are therefore relevant residues for binding to the antibody.

Table 4.3

EGFR	A	T	C	V	K	K	C	P	R	N	Y	V	V	T	D	H	G	S	C	V	R	A	SEQ ID NO: 92
EGFRvIII				L	E	E	K	K	G	N	Y	V	V	T	D	H	G	S	C	V	R	A	(SEQ ID NO: 93)
13.1.2					E	E	K	K	G	N	Y	V	V	T									(SEQ ID NO: 94)
131					E	E	K	K	G	N	Y	V	V	T									(SEQ ID NO: 94)
139				L	E	E	K	K	G	N	Y	V	V	T	D								(SEQ ID NO: 95)
250				L	E	E	K	K	G	N	Y	V	V	T	D								(SEQ ID NO: 95)
095											Y	V	V	T	D	H							(SEQ ID NO: 96)
211											Y	V	V	T	D								(SEQ ID NO: 97)
H10											Y	V	V	T	D								(SEQ ID NO: 97)
Y10					E	E	K	K	G	N	Y	V	V	T									(SEQ ID NO: 98)
B9									G	N	Y	V	V	T									(SEQ ID NO: 99)

The shaded amino acids shown in Table 4.3 are the most relevant residues in the eiptope for antibody recognition. The minimal lengths of epitopes of all ten of the mAbs were precisely mapped using peptides of overlapping sequences, and the tolerance for mAb binding to mutated epitopes was determined by systematically replacing each residue in the epitope with Alanine.

In Table 4.4, additional characteristics of the antibodies are summarized. Specifcially, a subset of the antibodies were tested for their binding of to lysates of tumor cell lines in Western plates of polyacrylamide gel electrophoresis under either non-reducing or reducing conditions. Purifed recombinant protein is also included. Antibodies binding in both reducing and non-reducing conditions suggest that the epitope is linear. Sample identifications:

EGFRvIII – the rabbit Fc fusion protein

H1477 – H80 human tumor cell line transfected with EGFRvIII expression contruct. These cells express both EGFR and EGFRvIII.

EGFR – purified wild-type EGFR protein

A431 – human tumor cell line expressing only wild-type EGFR

A549 – human tumor cell line expressing only wild-type EGFR

H80 – human tumor cell line expressing only wild-type EGFR

EGFR Biacore – mAbs were tested in Biacore for binding to purifed EGFR as a highly sensitive test for specificity

Table 4.4

mAb	EGFRvIII Western (native)	rEGFRvIII Western (reduced)	EGFRvIII FACS	H1477 Western (native)	H1477 Western (reduced)	pep3 KinExA	EGFR Western (native)	EGFR Western (reduced)
13.1.2	+	+	+	+	+	25 pM	-	-
131	+	+	+	+	+	0.05 pM	-	-
139	?	+	+	ND	ND	ND	ND	ND
095	+	+	+	ND	ND	ND	ND	ND
211	+	+	+	ND	ND	ND	ND	ND
250	+	+	+	ND	ND	ND	ND	ND

MAb	EGFR Blacore	A431 FACS	A431 Western (native)	A431 Western (reduced)	A549 FACS	A549 Western (native)	A549 Western (reduced)	H80 FACS	H80 Western (native)	H80 Western (reduced)
13.1.2	-	-	-	-	-	-	-	-	-	-
131	-	++	-	-	+	-	-	-	-	-
139	N.D.	ND	ND	ND	ND	ND	ND	ND	ND	ND
095	-	ND	ND	ND	ND	ND	ND	ND	ND	ND
211	-	ND	ND	ND	ND	ND	ND	ND	ND	ND
250	-	ND	ND	ND	ND	ND	ND	ND	ND	ND

The results showed that most of these mAbs have essentially the same binding specificity, seven of the mAbs were shown to bind specifically to the EGFrVIII variant, while 2 mAbs cross reacted with wildtype EGFr (murine H10 and human 211) in Western blots of purified protein and in lysate of A431 cells. Note, however, that while antibody 211 binds to both native and reduced purified EGFRvIII in Western blots, it binds slightly more strongly to the non-reduced protein. In tests against a lysate of A431 cells, antibody 211 binds strongly to a band of the size of wild-type EGFR in the non-reduced sample but there is no signal in the reduced sample. This suggests that the binding of antibody 211 is due to a conformational epitope present in wild-type EGFR and represented differently in the EGFRvIII variant. The epitopes of 5 of the mAbs are within residues 2-12 spanning the EGFRvIII variant specific Glycine residue, whereas the epitope of 4 of the mAbs (including H10 and 211) spans residues 7-16 which are common to the EGFRvIII and wildtype EGFr. Antibody 131 binds to A431 and A549 cells in FACS. These cells are apparently negative for expression of EGFRvIII while positive for EGFR expression. Antibody 131 does not bind to non-reduced or non-reduced purified EGFR or to reduced or non-reduced lysates of A43 and A549 cells in Westerns suggesting that antibody 131 may be binding to a variant of EGFR expressed on the cell surface of some human tumor cell lines. This variant would be sensitive to denaturation.

EXAMPLE 5

Characterization of Specificity of Anti-EGFRvIII Antibodies *in Vitro*

The specificity of the purified antibodies was ascertained by performing FACS analysis on NR6 cells that were transfected with either wild type or mutant EGFR. Cells were incubated on ice with 5 μg/ml of the respective antibody for 1 hr, washed in FACs buffer and subsequently incubated with PE-conjugated goat anti-human IgG.

EXAMPLE 6

Cross-reactivity with amplified EGFR

Antibodies directed to variant EGF receptors have been shown to cross-react with subsets of wild type EGF receptors on cells in which gene amplification has occurred (Johns et al., Int. J. Cancer. 98: 398, 2002). To determine whether the human EGFRvIII antibodies identified had similar properties, they were tested for their ability to recognize wild type EGF receptors on a variety of cells in culture. Antibodies were incubated with the indicated cell lines at 4°C. After washing in FACS buffer, a secondary antibody conjugated with phyoerythrin was added and the incubation was continued. All cell lines analyzed expressed wild type EGFR. A subset of wild type EGFRs was recognized by the antibody XG1-131 on both A431 and SF-539 cells but not on A498 or SKRC-52 cells. Another antibody to EGFRvIII, 13.1.2, did not recognize this subset of wild type EGFRs. When considered together these data indicate that only a subset of antibodies directed to the mutant EGFRvIII are able to recognize wild type EGFRs on the surface of cells. The ability of certain antibodies directed to mutant EGFRvIII to recognize a subpopulation of wild type EGF receptors is not dependent on total EGFR density but likely represents a novel conformational epitope that is unique to tumor cells. The ability of antibodies directed to EGFRvIII to cross-react with subpopulations of wild type receptors may be determined by both the specific epitope within the junction of the mutant receptor and the affinity of the antibody for this unique epitope (See the results of the epitope mapping and affinity determination section herein).

EXAMPLE 7

Characterization of Specificity of Anti-EGFRvIII Antibodies *in vitro*: Binding of the Antibodies to Cell Lines

The specificity of the purified antibodies was ascertained by performing FACS analysis on a panel of cell lines. H80, a human glioblastoma line, and H1477 (H80-EGFRvIII) that expresses high levels of EGFRvIII, A431, a human epidermoid carcinoma line, and A549, a human lung carcinoma cell line were used as the cell lines. All cell lines were from Dr. Bigner except A431 and A549, which were from ATCC (Rockville, MD, U.S.A.). Cells were incubated on ice with 10 μg/ml of the respective antibody for 30 min., washed in FACS buffer and subsequently incubated with PE-conjugated goat anti-human IgG from Jackson ImmunoResearch (West Grove, PA, U.S.A.). In FIGs. 9A-9L and 10A-10D, the darkened histogram indicates cells stained with an irrelevant IgG, the outlined, or white histogram, represents the staining of the relevant antibodies.

The anti-EGFRvIII antibodies 13.1.2, 131 and 139 bind to the EGFRvIII protein on the transfected cell lines. A graph summarizing some of the results is displayed in FIGs. 9M-9P.

Antibodies directed to variant EGF receptors have been shown to cross-react with subsets of wild type EGF receptors on cells in which gene amplification has occurred (Johns et al., Int. J. Cancer. 98: 398, 2002). In this example, A431 and A549 stained by XG1-131 and XG1-139. FIG. 10B and FIG. 10 C show that 131 and 139 have certain cross reactivity with the wild type EGFR instead of just recognizing a subset of population in H80, A431 and A549 line. However, this cross reactivity is only at 10% of the level of ABX-EGF (E7.6.3) staining on these cell lines. The results are provided in FIGs 9A-9P and 10A-10D.

Antibodies directed to cell surface antigens can be used as delivery vehicles that specifically transport drugs or toxins into cells. If the antibody stimulates internalization of antigen, the drug or toxin can result in death of the cell, perhaps after the drug or toxin is cleaved from the antibody. Such a mechanism can be utilized to specifically kill tumor cells in animals and in patients. One way to select antibodies that can deliver drugs to cells is through secondary cytotoxicity assays. In these assays the primary antibody binds to the cell surface and a secondary antibody that is conjugated with a drug or toxin is added. If the primary antibody stimulates antigen internalization, the secondary antibody will be co-internalized and upon cleavage of the drug or toxin result in cell killing.

EXAMPLE 8

Secondary Cytotoxicity Assays

In the following studies, EGFRvIII-specific antibodies were used to direct toxins conjugated secondary antibodies into glioblastoma cell line (H80) and EGFRvIII transfected glioblastoma cell line (H1477). Mouse anti-human IgG (cat # 555784) from Pharmingen (BD Biosciences Pharmingen) were conjugated with toxins AEFP (Seattle Genetics, Inc.) and maytansine (DM1, Immunogen Inc.) to generate mah-AEFP (murine anti-human IgG-AEFP and mah-DM1 (murine anti-human IgG-DM1). A saporin conjugated goat anti-human IgG, Hum-ZAP (TM, cat # IT-22-250, affinity-purified goat anti-human IgG-saporin) is from Advanced Targeting Systems (San Diego, CA, U.S.A.). H80 and H1477 cells were plated out in 96-well plates with 1000 cells in 100 µl growth medium per well. After 24 hours, primary antibodies were mixed with conjugated secondary antibodies at 1:3, serially diluted at 1:5 over 6 wells. 100 µl of diluted primary and toxin secondary antibody mixtures were added into wells of cells at final starting concentrations of 0.1 µg/ml of primary antibodies and 0.3 µg/ml of secondary antibodies. The plate was allowed to continue to culture for three days. On the fourth day, CellTiter-Glo reagents (cat #G7571) from Promega (Madison, WI, U.S.A.) were added and luminescence was read. FIGs. 11A-11I, 12A-12I, and 13A-13I demonstrate the results from this experiment. Hum-ZAP mediated antigen specific killings in H1477 (filled circle) compared to H80 (filled square) in most EGFRvIII specific mAbs tested. MAbs XG1-131 and XG1-139 generated antigen specific secondary killings

with mah-AEFP, at less extent with mah-DM1. Among the antibodies tested, XG1-131 performed at least one log better than 13.1.2, XG1-095, XG1-139, XG1-150, XG1-170, XG1-250 and XG1-211. IgG1 was used as a negative control and antigen positive cells (H1477) were compared to antigen negative cells (H80).

The amount of specific killing required can vary depending upon the particular use. In one embodiment, any reduction in possibly cancerous cells is sufficient. For example, a reduction of 0-1, 1-5, 5-10, 10-20, 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90, 90-95, 95-99, or 100% of the target cells will be sufficient. In another embodiment, the desired reduction in target cell number is also a function of the nonspecific lethality of the antibody combination. For example, antibody/toxin combinations that only have a 10 % decrease in target cell number may be sufficient, if there is very little nonspecific targeting and lethality by the antibody. For example, the antibody toxin combination kills less than 10% of a non-target population. Again, the particular amount will depend on the particular need and situation. Particularly useful are antibodies that are highly selective for a target cell (e.g., H1477) and bind well to the target cell, or proteins associated with the cell. In one embodiment, the target is the EGFRvIII protein, or a fragment thereof. In one embodiment, antibodies that are human, or humanized, efficient at being internalized, specific to the EGFRvIII protein or fragments thereof, associate tightly with the EGFRvIII protein or fragment, and are associated with an effective toxin, are taught from these examples.

EXAMPLE 9

Secondary Cytotoxicity Clonogenic Assays

In addition to the secondary Cytotoxicity assays, EGFRvIII-specific antibodies were tested in the clonogenic assays. Specific EGFRvIII antibodies direct toxin conjugated secondary antibodies into EGFRvIII transfected glioblastoma cell line (H1477), toxins are released inside the cells and eventually reduced the cell's ability to proliferate to form colonies. Thus, the application of these EGFRvIII antibody-toxins generated reduced number of clones when cells were re-plated after primary and secondary toxin antibody treatments. In this example, H80 and H1477 cells were plated out in 6 well plates at 30,000 cells per well and incubated overnight. The primary antibody and secondary toxin antibody were mixed at a ratio of 1:3. This this antibody mixture was added into the proper wells at a final concentration of primary antibody at 0.5 μg/ml and secondary toxin antibody at 1.5 μg/ml. This was the incubated at 37 °C overnight. After incubation, the toxin mixture was disposed of properly and thecells were detached from the wells with 1X trypsin solution. The cells were counted and plated at 200 cells per well into new 6-well plates. Triplicates wells were plated for each treatment group. These plates were incubated in a 37 °C incubator for 2-3 weeks until the colonies formed and could be identified by eye or under a microscope. The medium was aspirated and 5M Methylene Blue in methanol was added for 1 hour. The plate was rinsed in water and the colonies were counted. FIG. 14A and FIG. 14B show the results from this

experiment. As can be seen, mab-AEFP secondary toxin antibody inhibited colony formation with the three EGFRvIII antibodies tested.

EXAMPLE 10

Anti-EGFRvIII antibody (13.1.2) Direct Conjugates in the Cytotoxicity Assays

Further evidence that an antibody is capable of delivering a drug to a tumor cells is provided by direct conjugation of the antibody with a cytotoxic drug. In the following example EGRvIII antibody 13.1.2 was directly conjugated with auristatin E MMAE and AEFP conjugates via peptide linkers (both MMAE and AEFP are available from Seattle Genetics and described above) and and maytansine (DM1) was conjugated with a thiol linker (DM1 is available from Immunogen and described above). Upon addition of the conjugate to cells that expressed the EGFRvIII antigen, H1477, specific cytoxicity was observed. Cells that did not express the antigen, H80, were only killed when exposed to very high concentrations of the antibody. Results from this experiment are shown in FIGs. 15A-15C.

Direct conjugation of the EGFRvIII antibodies with the drugs or toxins is a particularly advantageous method for therapeutic use. Thus, this initial experiment showed that such conjugates do result in specific killing of EGFRvIII- expressing cells.

EXAMPLE 11

In vivo Anti-EGFRvIII Antibodies Characterization.

An optional method to determine if an antibody is capable of delivering a cytotoxic drug to a cell is to evaluate the effect of the conjugated antibody on the growth of human tumors *in vivo*. This example presents one such method. H1477 glioblastoma cells were cultured *in vitro*, harvested by trypsinization and subsequently embedded in Matrigel as explained below. Five million cells were injected subcutaneously into female nude mice and tumors allowed to develop until they reached a size of approximately 0.5 cm^3. At this time the animals were randomized into groups and treatment with the indicated concentration of conjugated antibody intravenously every 4 days was initiated. The results in FIG. 16 demonstrate that antibody 13.1.2 can cause regression of glioblastoma tumors when conjugated with maytansine (dEGFR- DM1) or auristatin E (dEGFR-MMAE). If the antibody is administered with an equivalent amount of unconjugated drug, (Group2), there is no effect on tumor growth proving that targeting the tumor cells in vivo requires the antibody conjugation with the toxin.

The animal model used above was developed by injecting H1477 cell xenografts into nude mice. Various amounts of the cells were injected with or without MATRIGEL into 8 week old nu/nu female mice and the tumor implantation over days analyzed. From this analysis, the number of cells that would allow for an appropriately sized tumor was identified as 5 million cells in MATRIGEL for approximately 22 days. Group G8 was included as a control to show that the killing was antibody specific. Group G7 was included as a negative control.

Thus a protocol was developed for the toxin study as follows:

Day 1: tumor implantation of 5 million cells in MATRIGEL into 8 week-old nu/nu female mice.

Day 22: antibody pro-drug treatments every 4 days, via I.V., as shown in Table 11.1

Table 11.1

Group	Number of Mice	Treatment
G1	8	13.1.2-DM1 250 μg every 4 days, via I.V.
G2	8	13.1.2-DM1 75 μg every 4 days, via I.V.
G3	9	13.1.2-MMAE 75 μg every 4 days, via I.V.
G4	8	13.1.2-MMAE 250 μg every 4 days, via I.V.
G5	9	13.1.2-AEFP 75 μg every 4 days, via I.V.
G6	8	13.1.2-AEFP 250 μg every 4 days, via I.V.
G7	8	PBS every 4 days, via I.V.
G8	9	13.1.2 (unconjugated) 250 μg + Maytansine 4μg

The results are shown in FIG. 16 with the arrows indicating the addition of drug. Groups G1, G6, and G4 showed effective killing. Group G3 showed a lesser amount of killing. Groups G8 and G7 showed no killing. Certain toxicity may have been observed in the high dose vc-AEFP group, Group G8. These animals received 2 treatments at 250 μg and 1 treatment at 125 μg.

EXAMPLE 12

Expression of EGFRvIII in Cancer Patients/Human Tumors

The expression of EGFRvIII on human tumors was determined by staining frozen tissue sections from a variety of cancer patients with a combination of 2 murine monoclonal antibodies (B9, IgG1 and Y10, IgG2 (Dr. Bigner, Duke University)) known to bind specifically to EGFRvIII. The same sections were stained with isotype matched control antibodies. A summary of the staining results obtained from all patient samples is presented in Table 12.1.

Table 12.1

Tumor type	Sample Size (N)	EGFRvIII>+	EGFRvIII>++
Glioblastoma	8	100%	100%
Breast Cancer	100	31%	24%
NSCL cancer	51	47%	39%
Head & neck Cancer	21	42%	38%
Prostate Cancer	22	4.5%	4.5%

EGFRvIII>+: include all tumors that express EGFRvIII

EGFRvIII>++: include only those tumors that express at least 10% or more EGFRvIII

The expression was found primarily on the cell membrane and/or cytoplasm. Significant numbers of breast (31%), NSCL (47%), and head & neck (42%) cancer specimens stained positively for EGFRvIII. In certain instances, in order to obtain high quality IHC staining, the use of two antibodies can be better than the use of one antibody. Frozen tissue specimens were superior over fixed tissues.

As appreciated by one of skill in the art, it may be advantageous to test patients before using therapeutic antibodies to ensure that the tumor which is being treated expresses EGFRvIII.

EXAMPLE 13

In vivo Anti-EGFRvIII Antibodies Characterization.

The method of Example 11 will be used to treat lung cancer and gliomas. This will be broadly examined by producing animal models. Animal models for glioblastoma and lung cancer are developed as follows: lung cancer cells that express wt-EGFR are transfected with EGFRvIII. The cells are injected into the lungs of nu/nu mice and tumors allowed to progress to a comparable stage to that above. Anti-EGFRvIII conjugates will then be injected intravenously as above every 1 to 10 days as needed. The size and prevention or suppression of continued growth of these cancer cells will then be monitored, to determine the effectiveness of these Anti-EGFRvIII antibodies and antibody-toxins combinations. As appreciated by one of skill in the art, this can be done for any of the antibodies disclosed herein.

EXAMPLE 14

Functional Characterization of Epitopes by Substitional Analyses

In order to further resolve the identity of those amino acid residues that are indispensable for binding within the EGFRvIII epitope, substitutional analyses of the amino acids in the epitope peptides were performed. The starting point was the sequence that was derived from Example 4, **LEEKKGNYVVTD** (SEQ ID NO 59). In this example each amino acid of the mapped epitope was substituted one-at-a-time by all 20 L-amino acids, thus, all possible single site substitution analogs were synthesized and screened to provide detailed information on the mode of peptide binding. Discrete substitution patterns were identified for mAbs 131 and 13.1.2. The results from the substitutions are summarized in Table 14.1.

Table 14.1

mAbs	Recognition sequence
131	E E K K G N Y V V T (SEQ ID NO: 57)
13.1.2	**E E** K **K G N** Y V V T (SEQ ID NO: 57)

It appears that for mAb 13.1.2, 5 residues are important for binding (bold), while only 4 residues are essential for the binding of mAb 131. The rest of the residues were replaced by various amino acids without significant loss of binding. Although the 131 and 13.1.2 epitopes are identical

by sequence and length, the binding pattern for each appears different. Binding of mAb 131 is strongly dependent on the residues EKNY (SEQ ID NO: 60). On the other hand, the data revealed that residues EEKGN (SEQ ID NO: 61) are involed in binding of mAb 13.1.2.

Example 15

mAbs Chain Shuffling

Heavy and light chains of mAbs 131 and 13.1.2 were shuffled and transiently transfected into 293T cells. Seventy-two hours later supernatants were collected and assayed for secretion and binding to EGFrVIII antigen by ELISA.

The results demonstrated that antibodies derived from expression of 131 heavy chain with 13.1.2 kappa chain, and vice versa were expressed well but binding activity was reduced by 75% probably due to the different binding pattern of these two mAbs to EGFrVIII antigen. (data not shown). This demonstrates the difference between the two paratopes of the 131 and 13.1.2 mAbs, again suggesting that the structural characteristics of the epitope selected for between the two mAbs are different.

Example 16

Molecular Modeling of 131 and its Paratope

This example demonstrates how three-dimensional structures can be generated for the proteins of the embodiments. The three-dimensional structure model of the variable region of antibody 131 was generated through a homology modeling approach using the InsightII modeling package from Accelrys (San Diego, CA). The model was built from the variable region sequences described below, Table 16.1. The residue numbering starts with the light chain amino acids, and continues to heavy chain amino acids.

Table 16.1

Light chain variable region	
DTVMTQTPLSSHVTLGQPASISC	(SEQ ID NO: 100)
RSSQSLVHSDGNTYLS (CDR1)	(SEQ ID NO: 101)
WLQQRPGPPRLLIY	(SEQ ID NO: 102)
RISRRFS (CDR2)	(SEQ ID NO: 103)
GVPDRFSGSGAGTDFTLEISRVEAEDVGVYYC	(SEQ ID NO: 104)
MQSTHVPRT (CDR3)	(SEQ ID NO: 105)
FGQTKVEIK	(SEQ ID NO: 106)
Heavy chain variable region	
QVQLVESGGGVVQSGRSLRLSCAASGFTFR	(SEQ ID NO: 107)
NYGMH (CDR1)	(SEQ ID NO: 108)
WVRQAPGKGLEWVA	(SEQ ID NO: 109)

VIWYDGSDKYYADSVRG (CDR2)	(SEQ ID NO: 110)
RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR	(SEQ ID NO: 111)
DGYDILTGNPRDFDY (CDR 3)	(SEQ ID NO: 112)
WGQGTLVTVSS	(SEQ ID NO: 113)

Antibody 131 sequences were used to search against the Protein Data Bank to identify homologous antibodies and their structures. Based on the homologous antibodies' sequence similarity to the 131 antibody, several structures were selected. The structures selected for modeling samples from the Protein Data Bank had the Protein Data Bank identifications of 1HEZ, 2H1P, 1AQK, 1DQL, 1MF2 and 1FLR. These template structures were then aligned by superposition and used to generate structure-based sequence alignments among the templates. The sequences of antibody 131's variable region were then aligned to the template sequences. The structure and sequence alignments were used to generate the molecular model for the variable region of the 131 antibody. The sequence for CDR1, light chain was: RSSQSLVHSDGNTYLS (SEQ ID NO 101). The sequence for CDR2, light chain was: RISRRFS (SEQ ID NO 103). The sequence for CDR3, light chain was: MQSTHVPRT (SEQ ID NO 105). The sequence for CDR1, heavy chain was: NYGMH (SEQ ID NO 108). The sequence for CDR2, heavy chain was: VIWYDGSDKYYADSVRG (SEQ ID NO 110). The sequence for CDR3, heavy chain was: DGYDILTGNPRDFDY (SEQ ID NO 112).

The interaction surface for antibody 131 was calculated from the structure model and shown in FIG. 17. The various CDRs are identified as follows: L1 (light CDR1) **10**, H1 (heavy CDR1) **20**, L2 **30**, H2 **40**, L3 **50** and H3 **60**. A prominent feature on the predicted antibody 131 interaction surface is a deep cavity. The cavity is mainly surrounded by heavy chain CDR2, CDR3 and light chain CDR3, with a small portion contributed by light chain CDR1. The cavity is probably the binding pocket. Within 5 Angstroms of the binding cavity are residues 31, 37, 95-101, 143-147, 159, 162-166, 169-171, 211-219, 221 and 223. These residues are likely to comprise the paratope and make key contacts in the binding of EGFRvIII epitope. It is also likely that the residues provide important structural features to the binding site in general.

Example 17

Site-directed mutagenesis confirming the model for antibody 131

This example demonstrates one method by which models that suggest residues that are important in binding may be tested. The Example also results in several antibody variants. Antibody variants of the 131 clone were generated by single residue mutations introduced to the heavy and the light chain of mAb 131. These variants were then analyzed to determine how the altered side chains from the point mutation contributed to antigen binding.

Changes were made in the heavy and light chains of mAb 131. On the heavy chain L216 was changed by site directed mutagenesis to R. On the light chain, V99 was changed to F. Both

mutations affected the expression and secretion of the variant antibodies compared to the wildtype sequence. Both mutations resulted in a loss of binding of the mAb variant to the EGFRvIII antigen. This demonstrates L216 and V99 probably have significant contacts with the EGFRvIII antigen since substitutions of these residues to R and F respectively resulted in reduced activity. Of course, it is always an option that these substitutions are disruptive to the antibody's general structure.

Example 18

Molecular Modeling of 13.1.2 and its Paratope

The three-dimensional structure model of the variable region of the 13.1.2 antibody was generated through homology modeling approach with the InsightII modeling package from Accelrys (San Diego, CA). The model was built from the variable region sequences, shown below in Table 18.1, using the published x-ray crystal structures as templates.

Table 18.1

Light chain variable region (1-113)	
DIVMTQTPLSSPVTLGQPASISC	(SEQ ID NO: 114)
RSSQSLVHSDGNTYLS (CDR1)	(SEQ ID NO:101)
WLHQRPGQPPRLLIY	(SEQ ID NO: 115)
KISNRFS (CDR2)	(SEQ ID NO: 116).
GVPDRFSGSGAGTAFTLKISRVEAEDVGVYYC	(SEQ ID NO: 117)
MQATQLPRT (CDR3)	(SEQ ID NO: 118)
FGQGTKVEIKR	(SEQ ID NO: 119)
Heavy chain variable region (114-234)	
QVQLVESGGGVVQPGRSLRLSCAASGFTFS	(SEQ ID NO: 120)
SYGMH (CDR1)	(SEQ ID NO: 121)
WVRQAPGKGLEWVA	(SEQ ID NO: 122)
VIWYDGSNKYYVDSVKG (CDR2)	(SEQ ID NO: 123)
RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR	(SEQ ID NO: 124)
DGWQQLAPFDY (CDR3)	(SEQ ID NO: 125)
WGQGTLVTVSSA	(SEQ ID NO: 126)

The sequence for CDR1, light chain was: RSSQSLVHSDGNTYLS (SEQ ID NO: 101). The sequence for CDR2, light chain was: KISNRFS (SEQ ID NO: 116). The sequence for CDR3, light chain was: MQATQLPRT (SEQ ID NO: 118). The sequence for CDR1, heavy chain was: SYGMH (SEQ ID NO: 121). The sequence for CDR2, heavy chain was:

VIWYDGSNKYYVDSVKG (SEQ ID NO: 123). The sequence for CDR3, heavy chain was: DGWQQLAPFDY (SEQ ID NO: 125).

Antibody 13.1.2 sequences were used to search the Protein Data Bank to identify homologous antibodies. The structures with the Protein Data Bank identifications of 1HEZ, 2H1P, 8FAB and 1AQK were selected as modeling templates, based on their sequence similarity to antibody 13.1.2. The template structures were aligned by superposition and used to generate structure-based sequence alignments among the templates. The sequences of the variable regions of the 13.1.2 antibody were then aligned to the template sequences. The structure and sequence alignments were used to generate the molecular model for antibody 13.1.2 variable region.

The interaction surface was calculated for the model and is shown in FIG. 18. A major feature of the 13.1.2 model is a long and narrow groove on the surface of the CDR region. The groove is outlined by heavy chain CDR2 **140**, CDR3 **160**, and light chain CDR1 **110**, CDR2 **130** and CDR3 **150**. One end of the groove touches the rest of light chain CDR3 **150**, and the other end opens to the wider area of heavy chain CDR3 **160** near the heavy chain-light chain interface. The groove is probably the binding pocket for the antigen. Within 5 Angstroms of the binding groove are residues 31, 33, 35-39, 51, 54-56, 58-61, 94-101, 144-148, 160, 163-166, 172, and 211-221. These residues are likely to comprise the paratope for the binding of EGFRvIII epitope. It is also likely that the residues provide important structural features to the binding site in general.

Example 19

Docking Models of a Peptide to an Antibody

The epitope mapping studies in Example 14 revealed that the relevant amino acids required for binding of the epitope to the paratope of 13.2.1 mAb reside in the six-residue peptide EEKKGN (SEQ ID NO: 127). Therefore, docking models of this six-residue peptide complexed to the CDR region of 13.1.2 structure model were generated. First, a model of the peptide EEKKGN (SEQ ID NO: 127) was produced. This was done, similarly as described before, except this time using the x-ray crystal structure of 1I8I, as identified in the Protein Data Bank, as the template. Next, this peptide structure was manually placed into the long groove to form an initial assembly complex. A Monte Carlo search in the conformational and orientational spaces were then automatically performed with the Docking module in InsightII. The peptide conformation was allowed to be flexible by giving each of the Phi, Psi and Chi angles full rotational freedom. During the docking process, the residues within 5 Angstroms of the binding groove were allowed to move while the other residues of the antibody were fixed. The plausible configurations found by Monte Carlo search were subjected to simulated annealing and energy minimization to reach the final complex structure models. For each docking model obtained, the interaction energy between the antibody and the peptide was calculated with the Discover_3 module of InsightII package. The interaction energies for all docking models were assessed and the model with the strongest overall antibody-peptide interaction was examined and is shown in FIG. 19A and 19B.

In this docking model, there are six hydrogen bonds between peptide EEKKGN (SEQ ID NO: 127) and antibody 13.1.2, as shown in FIG. 19B. The peptide residue number is labeled from N-terminus to the C-terminus as 1 through 6. Six hydrogen bonds are indicated by green dashed lines. The six pairs of amino acids forming hydrogen bonds are: E2...Y172, K3...H31, K4...H31, N6...D33, N6...Y37, and N6...K55. In this docking model, the peptide is bound to the groove in an extended β-strand conformation. Residues in the peptide alternately face the solvent and the antibody surface. The residues facing the binding groove with the most significant contacts to the antibody are E2, K4 and N6. This indicates that these three residues may be important to peptide binding, consistent with the epitope mapping results. The interaction energies for each of the six peptide residues with the 13.1.2 paratope was calculated with the Discover_3 module and the results are shown in Table 19.1. Table 19.1 shows the interaction energies for each of the six peptide residues with the 13.1.2 paratope. All energies are in the unit of kcal/mol.

The residues with the strongest interaction energies are in the order of N6, K4 and E2, confirming that these residues are key contributors on the antigen side in the antibody-antigen interaction, again consistent with experimental data. These data provided strong evidence to support the docking model. In this embodiment, the paratope is defined as the residues within 5 Angstroms of the docked peptide. The 20 residues comprising the paratope as so defined are residues 31-33, 35, 37, 55, 96-101, 148, 163, 165, 170, 172, 178 and 217-218. To evaluate, on an individual residue basis, the contribution of each of these residues of the antibody in the antibody-antigen interaction, the interaction energy between the paratope residues and the peptide EEKKGN (SEQ ID NO: 127) was calculated for each of the above 20 residues. The results are listed in Table 19.2. Table 19.2 shows the interaction energies for each of the 20 paratope residues with the peptide EEKKGN (SEQ ID NO:127). All energies are in the unit of kcal/mol. The residues with the strongest interaction energies with the peptide are Lys55 and His31, followed by Tyr172, Ala96, Asp33, Tyr37, Leu99, Thr97, Gln98, Lys178 and Asn170.

Table 19.1

Peptide Residue	Coulumbic	VdW	Total
E1	-2.013	-3.738	-5.751
E2	-10.661	-0.617	-11.278
K3	-9.816	-0.493	-10.309
K4	-11.123	-0.968	-12.091
G5	-1.241	-1.468	-2.709
N6	-16.504	-0.181	-16.685

Table 19.2

13.1.2 Residue	Coulumbic	VdW	Total
His31	-12.835	3.033	-9.801
Ser32	2.857	-1.062	1.794
Asp33	-4.181	-0.698	-4.879
Asn35	0.253	-1.009	-0.756
Tyr37	-2.058	-2.463	-4.521
Lys55	-14.363	1.568	-12.794
Ala96	-6.077	0.896	-5.182
Thr97	-2.739	-1.431	-4.171
Gln98	-2.542	-1.548	-4.09
Leu99	-1.507	-2.779	-4.286
Pro100	0.439	-0.379	0.061
Arg101	3.992	-0.549	3.443
His148	0.101	-0.083	0.018
Val163	-0.104	-0.237	-0.342
Trp165	1.358	-1.122	0.236
Asn170	-2.102	-0.487	-2.589
Tyr172	-8.7	0.896	-7.804
Lys178	-3.614	-0.03	-3.644
Leu217	0.761	-1.426	-0.664
Ala218	-0.071	-0.281	-0.352

Example 20

Rational Design for Affinity-Improved Antibodies

This Example demonstrates how the docking model can be used as the basis of rational design for affinity-improved antibodies by site-directed mutagenesis. Each of the 13.1.2 paratope residues was mutated to all 19 other amino acids *in silico*, resulting in a total of 19x20 or 380 virtual mutants. The mutation was done by residue replacement followed by 50 steps of energy minimization to account for any local conformational changes that could be induced by the side chain change. The interaction energy between the whole peptide and the whole paratope was calculated for each mutant. Mutants with a total interaction energy stronger than the wild type 13.1.2 could potentially have a higher affinity for the peptide EEKKGN (SEQ ID NO: 127), and perhaps even the whole EGFRvIII protein. These mutants mostly have stronger coulumbic interactions than the wild type 13.1.2, but some of them have weaker van der Waals (VdW) interactions than the wild type antibody. Considering that in the wild type 13.1.2 antibody, the VdW interacting energy is –9.689 kcal/mol, mutants with VdW interaction energy weaker than –8.5 kcal/mol were filtered out. The rest of the mutants that have stronger, total interaction energy, than the wild type 13.1.2 are listed in Table 20.1. The wild type data are listed at the bottom for comparison. All energies are in the units of kcal/mol.

Table 20.1

Mutant	Coulumbic	VdW	Total
Tyr172Arg	-93.004	-8.702	-101.706
Leu99Glu	-79.897	-8.506	-88.403
Arg101Glu	-77.984	-8.833	-86.817
Leu217Glu	-75.124	-8.998	-84.123
Leu99Asn	-73.337	-9.894	-83.231
Leu99His	-73.631	-9.008	-82.639
Arg101Asp	-71.983	-9.877	-81.861
Leu217Gln	-70.263	-9.795	-80.058
Leu99Thr	-69.882	-10.153	-80.035
Gln98Glu	-70.651	-9.257	-79.908
Leu217Asn	-70.989	-8.769	-79.758
Arg101Gln	-69.432	-10.164	-79.596
Leu217Asp	-69.934	-9.643	-79.578
Asn35Gly	-69.016	-10.191	-79.207
Tyr172His	-69.312	-9.509	-78.820
Val163Asn	-68.841	-9.944	-78.784
Tyr172Asn	-68.896	-9.871	-78.767
Ala218Lys	-70.024	-8.570	-78.594
Asn35Arg	-68.989	-9.604	-78.593
Trp165Lys	-69.578	-8.766	-78.344
Trp165Arg	-68.814	-9.216	-78.030
Leu99Tyr	-67.052	-10.464	-77.517
Tyr172Thr	-68.146	-9.225	-77.371
Ala96Thr	-67.534	-9.623	-77.158
Ala96Ser	-67.222	-9.822	-77.045
Pro100Trp	-67.399	-9.496	-76.894
Leu217Ser	-66.676	-10.133	-76.810
Ser32Ile	-66.700	-10.077	-76.777
Tyr172Ser	-67.588	-9.146	-76.734
His31Glu	-67.070	-9.461	-76.531
Leu217Tyr	-65.605	-10.726	-76.331
Val163His	-67.236	-9.064	-76.300
His148Ser	-66.780	-9.495	-76.274
His148Val	-66.634	-9.629	-76.263
His148Ala	-66.770	-9.473	-76.243
His148Gly	-66.762	-9.456	-76.217
His148Thr	-66.700	-9.508	-76.209
Leu99Ser	-66.126	-10.006	-76.132
Pro100Asp	-66.153	-9.787	-75.940
Trp165Glu	-66.665	-9.267	-75.932
His148Asn	-66.010	-9.889	-75.899
Pro100Gln	-65.873	-9.871	-75.745
Leu217Thr	-66.045	-9.672	-75.717
Ser32Val	-65.845	-9.854	-75.699
Ser32Pro	-65.807	-9.813	-75.620
Pro100Gly	-65.841	-9.774	-75.615
Pro100Ala	-65.889	-9.712	-75.601
Ser32Ala	-65.497	-10.089	-75.586
Ser32Thr	-65.723	-9.861	-75.584

Mutant	Coulumbic	VdW	Total
Ala218Thr	-66.054	-9.505	-75.560
Pro100Ser	-65.831	-9.699	-75.530
Val163Gly	-65.993	-9.536	-75.529
Gln98Thr	-66.162	-9.277	-75.438
Pro100Met	-65.811	-9.602	-75.412
Ser32Met	-66.252	-9.153	-75.406
Ser32Gly	-65.509	-9.891	-75.399
Pro100Asn	-65.729	-9.655	-75.384
Tyr37Phe	-66.253	-9.020	-75.272
Val163Ala	-65.713	-9.543	-75.255
Leu217Ile	-65.479	-9.759	-75.238
Wild type 13.1.2	-65.517	-9.689	-75.205

The mutants listed in Table 20.1 could be candidates for engineering of affinity-improved antibodies. For the top 14 candidates in the list, per residue contributions on the antigen side and on the antibody side were further analyzed to examine the impact of the proposed modifications. The 10 mutants selected for *in vitro* site-directed mutagenesis were Tyr172Arg, Arg101Glu, Leu99Asn, Leu99His, Arg101Asp, Leu217Gln, Leu99Thr, Leu217Asn, Arg101Gln and Asn35Gly. The results can be seen in Example 21.

Example 21

Site-directed mutagenesis confirming the model for 13.1.2

This example demonstrates one method by which the above models, which suggest residues that are important in binding, can be tested. The Example also results in several antibody variants. Antibody variants of 13.1.2 were generated by single residue mutations introduced into the heavy and the light chains of the 13.1.2 mAb. The variants were analyzed to determine the contribution that the various side chains had in antigen binding. A list of the mutations introduced by site directed mutagenesis are summarized in Table 21.1.

Table 21.1

	Chain	Mutation
1	Light chain (CDR3)	Arg101Asp
2	Light chain (CDR3)	Arg101Gln
3	Light chain (CDR3)	Arg101Glu
4	Light chain (CDR1)	Asn35Gly
5	Heavy chain (CDR3)	Leu217Asn
6	Heavy chain (CDR3)	Leu217Gln
7	Light chain (CDR3)	Leu99Asn
8	Light chain (CDR3)	Leu99His
9	Light chain (CDR3)	leu99Thr
10	heavy chain (CDR2)	Tyr172Arg

Each of the 10 mutations in Table 21.1 was introduced into the heavy or light chain of the 13.1.2 mAb. Each mutated chain was then transfected with the complementary wild-type chain in

293 cells. Supernatants were then tested for expression and secretion of human IgG antibodies, and for binding to EGFrVIII antigen. The results, as determined by an ELISA, are summarized in Table 21.2.

Table 21.2

	Mutation	Binding Energy	Expression	Binding
1	Arg101Asp	-81.861	Yes	No
2	Arg101Gln	-79.596	Yes	No
3	Arg101Glu	-86.817	Yes	No
4	Asn35Gly	-79.207	Yes	Yes
5	Leu217Asn	-79.758	Yes	Yes
6	Leu217Gln	-80.058	Yes	Yes
7	Leu99Asn	-83.231	Yes	Yes
8	Leu99His	-82.639	Yes	Yes
9	Leu99Thr	-80.035	Yes	Yes
10	Tyr172Arg	-101.706	Yes	Yes
11	WT	-75.205	Yes	Yes

Example 22

Preparation of EGFRvIII/pFLAG variant construct

This example demonstrates how a variant to EGFRvIII can be made. A 1092bp fragment encoding the extracellular domain of EGFRvIII was generated with primer pairs 9712 and 9713 (Qiagen, Valencia, CA):

Primer # 9712: 5'-ataaaagcttctggaggaaaagaaaggtaatta-3' (sense) (SEQ ID NO 128)

Primer # 9713: 5'-TTATTGGTACCTCAGGCGATGGACGGGATCTTA- 3' (antisense) (SEQ ID NO 129)

from plasmid template EGFRvIII-rbIgG/pCEP4 (as described above) amplified using Pfu DNA polymerase enzyme (Stratagene, La Jolle, CA). Primer # 9712 introduced a HindIII site and primer # 9713 introduced a KpnI site. The PCR product was column purified (Qiagen column purification kit, Valencia, CA) digested with HindIII and KpnI (NEB, New England Biolabs, Beverly, Mass.) and gel purified (Qiagen gel purification kit, Valencia, CA). Fragments were ligated with T4 DNA Ligase (NEB, New England Biolabs, Beverly, Mass.) into pFLAG-CMV-1 (Sigma, St. Louis, MO) linearized with HindIII and KpnI (NEB, New England Biolabs, Beverly, Mass.). The resulting vector was designated EGFRvIII/pFLAG-CMV-1 # 1.

Example 23

Preparation of EGFRvIII/pFLAG Recombinant Protein

This example demonstrates how a variant EGFRvIII protein can be made. First, 500 µg of EGFRvIII/pFLAG-CMV-1# 1 plasmid DNA was resuspended in 25 ml of Opti-MEMI (Invitrogen, Burlington, ON) and combined with 500 µl of 293fectin (Invitrogen, Burlington, ON) resuspended in 25 ml of Opti-MEMI. The mixture was incubated for 20 min at room temperature then mixed with 293T cells ($1x10^9$) prepared in 1L 293 FreeStyle media (Invitrogen, Burlington, ON),

supplemented with 2% FBS and 50 μg/ml G418 (Invitrogen, Burlington, ON). Cells are grown for 7 days at 37°C in 8% CO_2 with shaking at 125 rpm.

EGFRvIII-FLAG fusion protein purification was carried out with Anti-FLAG M2 Affinity Chromatography kit (Sigma, St. Louis, MO) according to the manufacture's protocol.

Monomeric fusion protein was produced as follows. First, purified protein (1508 μg), was reduced with DTT in a final concentration of 10 mM for 30 minutes at 55 °C. Then IAA (iodoacetic acid) (Sigma, St. Louis, MO) was added to 22 mM and incubated 15 minutes at room temperature in the dark then dialyzed against PBS at 4 °C in 7k MWCO dialysis cassettes (Pierce, Rockford, Ill.).

Examples 24-30

Binding Studies of Antibody Variants

The following examples involve Biacore experiments (surface plasmon resonance) and KinExA experiments. These examples demonstrate how one can test the various antibodies and variants thereof produced by the above examples to determine if they have the desired binding characteristics. All of the variants examined were variants in the 13.1.2 background.

Instrumentation.

All surface plasmon resonance experiments were performed using Biacore 2000 optical biosensors (Biacore, Inc., Piscataway, NJ). All Kinetic Exclusion Assays were performed using a KinExA 3000 instrument (Sapidyne Instruments, Inc., Boise, ID).

Reagents.

Pep-3, NH_2-LEEKKGNYVVTDHG-OH (MW = 1590 Da) (SEQ ID NO: 130), was custom synthesized and purchased from Anatech, Inc. (San Jose, CA). All mAbs were prepared in-house. The antigen EGFRvIIIpflag (iodoacetic acid reacted in order to block aggregation through free sulfhdryl groups), MW 39,907, was prepared in-house. Bovine serum albumin (BSA) fraction V (#BP1605-100) was purchased from Fisher Scientific (Pittsburgh, PA). All other general reagents were purchased from Sigma-Aldrich, Inc (St. Louis, MO).

All antigen and mAb samples for Biacore and KinExA analysis were prepared in vacuum-degassed HBS-P buffer (0.01 M HEPES, 0.15 M NaCl, 0.005% surfactant P-20, Biacore Inc., Uppsala, Sweden) containing 100 μg/mL BSA. Biacore amine-coupling reagents, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), and ethanolamine were purchased from Biacore, Inc. Biacore surface regeneration was with a 12 second pulse of 26 mM NaOH for the pep-3/mAb 131 experiment. All other mAbs dissociated to baseline within 20 minutes. Research grade CM5 biosensor chips were purchased from Biacore, Inc.

The KinExA detection antibody was Cy5-labeled goat anti-human IgG, Fcγ specific (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, #109-175-008) and was diluted 1000-fold in HEPES buffer (0.01 M HEPES, 0.15 M NaCl, pH 7.2) from a 0.5 mg/mL stock (1 X PBS, pH 7.4). The solid phase particles used for the KinExA experiments were NHS-activated

Sepharose 4 Fast Flow beads (Pharmacia Biotech AB, Uppsala, Sweden, #17-0906-01). Prior to reacting the sepharose beads with antigen, a bead stock aliquot of 1.5 mL in a microcentrifuge tube was spun down and washed at least six times with cold deionized H_2O. After rinsing the beads once with sodium carbonate buffer (0.05 M, pH 9.3), antigen (~40 μg) in sodium carbonate buffer was added to the sepharose beads. The sepharose/antigen tube was rocked overnight at 4°C. After rocking, the sepharose was spun and rinsed twice with 1 M Tris buffer, pH 8.3. The antigen-coated beads were then rocked for 1 hour at room temperature in 1 M Tris buffer with 2% BSA.

Biacore Measurements.

Standard EDC/NHS and carbohydrate coupling was used to covalently immobilize mAbs to a CM5 sensor chip. To minimize mass transport and crowding mAbs were immobilized at levels that gave a maximum antigen binding response (R_{max}) of no more than 50 – 100 RU. A reference flow cell on each chip was activated and blocked with no mAb immobilization to serve as a control.

All Biacore kinetic experiments were conducted at 23°C. For each experiment, a series of six to eight antigen concentrations (starting with 1.01 μM pep-3) was prepared using 2-fold dilutions. Antigen samples were randomly injected over the biosensor surface in triplicate at 100 μL/min. Several buffer blanks were injected intermittently over the course of an experiment for double referencing. Each pep-3 concentration and blank were injected for 90 seconds. Dissociation was followed for 13 to 180 minutes. Dissociation data for pep-3 binding to mAb 131 were acquired by alternating three additional injections of 251 nM pep-3 with three additional blank injections and following the dissociation phase for 3-4 hours.

All Biacore sensorgrams were processed using Scrubber software (Version 1.1f, BioLogic Software, Australia). Sensorgrams were first zeroed on the y-axis and then x-aligned at the beginning of the injection. Bulk refractive index changes were removed by subtracting the reference flow cell responses. The average response of all blank injections was subtracted from all analyte and blank sensorgrams to remove systematic artifacts between the experimental and reference flow cells. CLAMP biosensor data analysis software (Version 3.40, BioLogic Software, Australia) was used to determine k_a and k_d from the processed data sets. Data from all flow cells were globally fit to a 1:1 bimolecular binding model that included a mass transport term. For several of the mAbs the injections corresponding to the first or second concentration of the pep-3 series were excluded in the nonlinear kinetic fit where it was obvious that the sensorgrams were not described well by a 1:1 interaction model. The K_D was calculated from the quotient k_d/k_a.

KinExA Equilibrium Measurements.

All KinExA experiments were conducted at room temperature (~23°C). For all equilibrium experiments, antigen was serially diluted into solutions having a constant mAb binding site concentration. For the first 10 titration points the dilutions were 2-fold and the 11^{th} and 12^{th} serial dilutions were 10-fold. The sample flow rate for all experiments was 0.25 mL/min and the labeling antibody flow rate was 0.5 mL/min. Antigen/antibody samples were then allowed to reach

equilibrium, which took ~48-72 hr to reach. For the pep-3/mAb 131 KinExA experiment the starting concentration of pep-3 in the K_D-controlled titration was 352 nM and the constant [mAb binding site] = 219 pM; for the mAb-controlled titration the starting [pep-3] = 251 nM and the [mAb binding site] = 11 nM. During the K_D-controlled experiment with pep-3/mAb 131, 1.25 mL of each sample was drawn through the flow cell. A sample volume of 250 µL was analyzed for the antibody-controlled experiment. Two or three replicates of each sample were measured for all equilibrium experiments. The equilibrium titration data were fit in a dual curve analysis to a 1:1 binding model using KinExA software (Version 2.4, Sapidyne Instruments).

The EGFRvIIIpflag/mAb 131 complex was studied with KinExA under K_D-controlled conditions only. The starting [EGFRvIIIpflag] was 198 nM and the [mAb binding site] was 150 pM. A sample volume of 1 mL was drawn through the flow cell. Duplicate measurements were collected for all samples. The equilibrium titration data were fit in a dual curve analysis to a 1:1 binding model using KinExA software (Version 2.4, Sapidyne Instruments). See Example 28 below for results and predicted equilibrium constant.

For the KinExA titrations of the EGFRvIIIpflag/mAb 13.1.2 complex the starting concentration of EGFRvIII was 5.26 µM (mAb-controlled), 230.1 nM (K_D-controlled) and [mAb binding site] = 9.59 nM (mAb-controlled), 498 pM (K_D-controlled). During the K_D-controlled experiment, 1.30 mL of each sample was drawn through the flow cell. A sample volume of 250 µL was analyzed for the antibody-controlled experiment. Two or three replicates of each sample were measured for all equilibrium experiments. The equilibrium titration data were fit in a dual curve analysis to a 1:1 binding model using KinExA software (Version 2.4, Sapidyne Instruments).

Example 24

In vitro determination of binding constants for antibodies

The binding kinetics of the wild type mAb 131 was observed by using a Surface Plasmon Resonance (SPR) instrument from Biacore. The K_D was very low, 380 pM, owing to the very slow k_d and a rapid k_a. Estimates for the other kinetic parameters, derived from curve fitting, were $k_a=2.246*10^6$ and $k_d=8.502*10^{-4}$.

In one embodiment, improved or variant antibodies with improved kinetics are taught. By improved kinetics, it is meant that one of the kinetic elements of antibody binding to an epitope is superior to the same element in previously known antibodies for the same epitope. For example, an antibody that binds to pep-3 with a K_D of greater (in binding ability) than $1.3*10^{-9}$ M would be an improved antibody. As such, antibodies with a K_D of less than 500 nM, 500-300 nM, 300-100nM, 100-1nM, 1.3 nM, 1.3 nM to 1000 pM, 1000 pM to 900 pM, 900-500pM, 500-400 pM, 400-300 pM, 300-100 pM, 100-50 pM, 50-1 pM, or smaller K_D are contemplated.

Example 25

In vitro determination of binding constants for antibodies

Similar to Example 24, the binding kinetics of mAb13.1.2 to Pep-3 (EGFRvIII epitope) were examined. The estimated K_D was 67nM, but varied slightly between experiments. Estimates, for the other kinetic parameters, derived from curve fitting, were $k_a=2.835*10^5$ and $k_d=0.01922$.

Example 26

In vitro determination of binding constants for antibodies

Similar to Example 24, the binding kinetics of mAb 095 to Pep-3 (EGFRvIII epitope) were examined. The estimated K_D was 66nM. Estimates, for the kinetic parameters, derived from curve fitting, were $k_a=1.491*10^5$ and $k_d=9.927*10^{-3}$.

Example 27

In vitro determination of binding constants for antibodies

Similar to Example 24, the binding kinetics of mAb 139 to Pep-3 (EGFRvIII epitope) were examined. The estimated K_D was 290nM. Estimates, for the kinetic parameters, derived from curve fitting, were $k_a=10328$ and $k_d=2.981*10^{-3}$.

Example 28

In vitro determination of binding constants for antibodies

In order to more fully analyze the binding characterisitics of the antibodies, KinExA experiments were performed to determine the binding characterisitics of the mAb 131. The K_D determined from a dual curve analysis was $1.74*10^{-10}$. In a KinExA experiment, the K_D for EGFRvIIIpflag to mAb 131 was $6.266*10^{-11}$.

Example 29

In vitro determination of binding constants for variant antibodies

In order to more fully analyze the binding characterisitics of the 13.1.2 antibodies, a KinExA experiment was performed to deteremine the binding characterisitics of the mAb 13.1.2. The K_D determined from a dual curve analysis was $7.538*10^{-10}$. Additionally, the antigen in this example was the EGFRvIIIpflag variant and was reacted with iodoacetic acid (IAA).

Example 30

Comparison of Biacore results and KinExA results

The results of the previous Examples and the KinExA tests are presented in Table 30.1 below. Numbers in parentheses in Table 30.1 are 95% confidence intervals. "ND," means not determined and "*" denotes binding to EGFRvIIIpflag (iodoacetic acid reacted), instead of pep-3.

As is evidenced by the rate constants, mAb 131 appears to have the greatest association constant and the lowest dissociation constant, thus giving mAb 131 the lowest K_D.

TABLE 30.1

MAb	K_a ($M^{-1}s^{-1}$)	K_d (s^{-1})	K_D (nM)	KinExA K_D (nM)
131	2.25×10^8	8.50×10^{-4}	0.380	0.174 (0.0627 on EGFRvIIIpflag)
13.1.2	$2.10 (0.58) \times 10^5$	0.016 (0.003)	75 (14)	0.75 (on EGFRvIIIpflag (IAA reacted))
095	1.49×10^5	9.90×10^{-3}	66	ND
139	1.03×10^4	2.98×10^{-3}	290	ND

Example 31

In vitro determination of binding constants for L99T-5.3 variant antibodies

The binding kinetics of mAb L99T-5.3 to Pep-3 (EGFRvIII epitope) were examined. The first step was to immobilize 5,600 resonance units (RU) to 8,000 RU of mAb L99T-5.3 to two flow cells (Fc) of a CM5 sensor chip and 5,600 resonance units (RU) to 8,000 RU of mAb 13.1.2 to one Fc using standard EDC/NHS coupling chemistry. This surface density yielded a binding signal with pep-3 of less than 100 RU. Two CM5 sensor chips were used in total to immobilize both mAbs. With the previously collected data, this produced a total of 5 independent experiments for both antibodies that allows the 95% confidence intervals to be calculated. Biacore 2000 optical biosensors were used for all studies.

Next, pep-3 was flowed across the mAb immobilized biosensor surfaces. The starting concentration of pep-3 was 1.25 μM, which was followed with eight two-fold serial dilutions in randomized triplicate injections. Blank injections were run every sixth sample throughout the injection series for double referencing purposes.

Finally, the biosensor data was processed with Scrubber and the data was fit to curves utilizing Clamp with a 1:1 interaction model with a term included for mass transport. The high concentration injections, 1.25 μM, were excluded from the kinetic fits because it was apparent that the data was not consistent with a 1:1 interaction model. Most likely, this deviation is caused by non-specific interactions occurring at high concentrations of pep-3. All the kinetic data fit a 1:1 interaction model satisfactorily.

The estimated K_D varied from 54-70 nM. Estimates, for the other kinetic parameters, which also varied slightly between runs, were $k_a=2.238*10^5$ and $k_d=0.01217$.

Examples 32-38

Examples 32-38 further examined the binding kinetics of the variant mAbs through the use of a Biacore device. The first step in these examples involved the immobilization of 5,600 resonance units (RU) to 8,000 RU of each mAb tested to one flow cell (Fc) of a CM5 sensor chip using standard EDC/NHS coupling chemistry. This surface density yielded a binding signal with pep-3 of less than 100 RU. Three CM5 sensor chips were used in total to immobilize all mutant mAbs with a unique mAb immobilized to each flow cell. MAb 13.1.2 was included on one flow

cell for two out of the three CM5 sensor chips. Biacore 2000 optical biosensors were used for all studies.

Next, pep-3 was run across the mAb immobilized biosensor surfaces. The starting concentration of pep-3 was 4.98 μM, followed by eight to eleven two-fold serial dilutions in randomized duplicate or triplicate injections. Blank injections were run every sixth sample throughout the injection series for double referencing purposes.

Finally, the biosensor data was processed with Scrubber and fitted utilizing Clamp with a 1:1 interaction model with a term included for mass transport. Some high concentration injections (4.98 – 1.25 μM), depending upon the mAb and its affinity, were excluded from the kinetic fits when it was apparent that the data was not consistent with a 1:1 interaction model. Most likely, this deviation is caused by non-specific interactions occurring at high concentrations of pep-3. All the kinetic data fit a 1:1 interaction model.

Example 32

In vitro determination of binding constants for L217Q-10.1 variant antibodies

The binding kinetics of mAb L217Q-10.1 to Pep-3 (EGFRvIII epitope) were examined. The estimated K_D was 92 nM. Estimates, for the other kinetic parameters, derived from curve fitting, were $k_a=2.04*10^5$ and $k_d=0.01885$.

Example 33

In vitro determination of binding constants for L217N-2.1 variant antibodies

Similar to Example 32, the binding kinetics of mAb L217N-2.1 to Pep-3 (EGFRvIII epitope) were examined. The estimated K_D was 185 nM. Estimates, for the other kinetic parameters, derived from curve fitting, were $k_a=2.198*10^5$ and $k_d=0.04069$.

Example 34

In vitro determination of binding constants for N35G-3.1 variant antibodies

Similar to Example 32, the binding kinetics of mAb N35G-3.1 to Pep-3 (EGFRvIII epitope) were examined. The estimated K_D was 204 nM. Estimates, for the other kinetic parameters, derived from curve fitting, were $k_a=1.497*10^5$ and $k_d=0.03057$.

Example 35

In vitro determination of binding constants for variant antibodies

Similar to Example 32, the binding kinetics of mAb L99H-9.2 to Pep-3 (EGFRvIII epitope) were examined. The estimated K_D was 395 nM. Estimates, for the other kinetic parameters, derived from curve fitting, were $k_a=83390$ and $k_d=0.03293$.

Example 36

In vitro determination of binding constants for variant antibodies

Similar to Example 32, the binding kinetics of mAb Y172R-1.2 to Pep-3 (EGFRvIII epitope) were examined. The estimated K_D was 927 nM. Estimates, for the other kinetic parameters, derived from curve fitting, were $k_a=82237$ and $k_d=0.07622$.

Example 37

In vitro determination of binding constants for variant antibodies

Similar to Example 32, the binding kinetics of mAb L99N-4.1 to Pep-3 (EGFRvIII epitope) were examined. The estimated K_D was 1.4 μM. MAb L99N-4.1 was fit using a steady-state (equilibrium) binding model in order to determine the K_D because the kinetics were too fast to be fitted.

Example 38

Comparison of 13.1.2 with designed variants

As can be seen in Table 38.1 a mAb with improved binding characteristics was developed. The 95% confidence intervals are shown in parentheses. L99T-5.3 exhibited an enhanced k_a, a decreased k_d, and thus a slower K_D overall. While statistically there appears to be little if any significant difference in the equilibrium dissociation constants and kinetic rate constants of Pep-3 binding to mAbs 13.1.2 and L99T-5.3 (at the 95% confidence interval), there still seems to be an intuitive bias for a marginal increase in affinity for Pep-3 binding to L99T-5.3. Moreover, when the same biosensor chip was used, L99T-5.3 seemed to always have a higher affinity than 13.1.2.

Table 38.1

MAb	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (nM)
13.1.2	$2.10\ (0.58) \times 10^5$	0.016 (0.003)	75 (14)
L99T-5.3	$2.16\ (0.12) \times 10^5$	0.013 (0.001)	60 (10)
L217Q-10.1	2.04×10^5	0.019	92
L217N-2.1	2.20×10^5	0.040	185
N35G-3.1	1.50×10^5	0.030	204
L99H-9.2	8.34×10^4	0.033	395
Y172R-1.2	8.22×10^4	0.076	927
L99N-4.1	ND	ND	1,400*

Additional Docking Models and Methods of Selecting Models and Predicting Binding Affinity

In other embodiments, the examples described above can be performed with various length peptides rather than just peptides that are 6 amino acids in length, as long as the key binding residues are included in the peptide. For example, instead of the six amino acid peptide, EEKKGN (SEQ ID NO: 127), a seven amino acid peptide, EEKKGNY (SEQ ID NO: 131) can be used. Any size peptide for the epitope can be used. In other embodiments, the peptide is selected from the following peptides: LEEKKGNYVVTDHC (SEQ ID NO: 56), LEEKKGNYVVTD (SEQ ID NO: 59), LEEKKGNYVVT (SEQ ID NO:132), and EEKKGNYVVT (SEQ ID NO:57). Any sized peptide between the short fragments disclosed herein, to the full length peptide, or variants thereof, can be used.

As appreciated by one of skill in the art, the presence of additional amino acids can alter the manner in which the peptide binds to the antibody. Not only does the presence of the additional

amino acid allow for alternative and additional bonds to be formed between the peptide and the antibody, but the additional amino acid can change the structure of the peptide and the structure of the antibody upon binding of the peptide with the antibody. Thus, in one embodiment, various lengths of the epitope peptide, *e.g.* EEKKGN (SEQ ID NO: 127) and EEKKGNY (SEQ ID NO: 131), can be examined for binding properties and binding optimization. Not only will the longer fragments of the peptide provide an accurate depiction of the peptide-antibody interaction for longer segments of the peptide, but an examination of the changes in binding strength and the residues involved in binding will allow additional information concerning longer peptides to be extrapolated from the data.

In addition, and perhaps complementary to the testing of longer peptide fragments, additional filtering steps can be performed on the various docking models in order to select a refined docking model. An additional filtering step can allow one to filter through numerous docking models to find those that are consistent with available experimental data.

In one embodiment, the filter is based on fine-resolution epitope mapping data, e.g., the experimentally characterized individual residue binding profile, which could be correlated with the computed binding energy profile for each amino acid in the peptide. The binding energy profile of a seven amino acid peptide, for example, can be used to select docking models that contain similar binding energy profiles. A binding energy profile is an assignment of the binding energy of each amino acid in a peptide to the particular amino acid to create a profile of the peptide in terms of each amino acid's binding energy in that model. For example, in one docking model, given a peptide comprising amino acids A and B, where A has a binding energy of -5 and B has a binding energy of -20, one would have a profile of A1 (at -5) and B2 (at -20). This profile could be used as a filter to select other docking models. For example, the use of this binding energy profile as a filter or "template" would result in other docking models being selected if the peptide in the candidate model had a relatively low value attributed to position A, and a relatively high (larger negative, higher absolute value) value attributed to position B. In an alternative embodiment, the template requires additional limitations; for example, that the value at position B is four fold higher than the value at position A.

One can compare the binding energy profile template with the profiles of the peptide in the other docking models in a variety of ways. If the binding energy profile template is similar to the desired binding energy profile, then the filter can be used to pick out favorable docking models for further examination. If the binding energy profile template is dissimilar to the desired binding energy profile, then the filter can be used to eliminate unfavorable docking models. In one embodiment, the filtering process includes a template with both favorable and unfavorable binding energies and the filter is used to both select and exclude docking models. As appreciated by one of skill in the art, there are many possible different binding energy profiles, and thus many different binding energy profile templates that can be used depending upon the situation.

In one embodiment, one can define a binding energy profile template as a template that has a series of relatively high binding energies at particular positions in the peptide. In a preferred embodiment, the binding energy profile template, and the binding energy profile selected by the template, will have relatively high binding energy at position 2, 4, or 6 of the peptide, EEKKGNY (SEQ ID NO: 131). In another embodiment, the binding energy profile template will have a relatively high binding energy at positions 2, 4, and 6 of the peptide EEKKGNY (SEQ ID NO: 131). In another embodiment, the binding energy profile template will have a relatively low binding energy attributed to position 3 of the peptide EEKKGNY (SEQ ID NO: 131). In the above discussion, the positions are assigned as follows: E1, E2, K3, K4 G5, N6, Y7.

In one embodiment, the filtering process first involves a comparison of the binding energies at K3 and K4. Docking models that result in a relatively higher binding energy for K4 compared to K3 are selected, while docking models that result in a lower binding energy for K4 compared to K3 are filtered out. Thus, by "relatively high," it is meant that the binding energy for K4 is greater (more negative value, larger absolute value) than K3. Next, the docking models are again filtered through the binding energy profile template, this time, those binding models with relatively higher energies at positions 2, 4, and 6 are selected for, while the other models can be removed. Thus, by "relatively high," it is meant that the binding energy at positions 2, 4, and 6 are higher (more negative value, larger absolute value) than the lowest binding energy in the peptide. Thus, in this embodiment, the binding energy profile template could be summarized as follows: E1 can be any value, E2 should be greater than the lowest value, K3 should be less than K4, K4 should be greater than the lowest value, G5 can be any value, N6 should be greater than the lowest value, Y7 can be any value. Thus, E1, G5, and Y7 could be any value, as long as at least one (or K3) is lower than at least one of E2, K4, and N6. In another embodiment, "relatively high" can be set to a standard value as determined through modeling or experimentation. In one embodiment, that the docking models pass the first filter is more important than the docking model pass the second filtering step. As appreciated by one of skill in the art, one need not perform these two steps sequentially and they can be performed simultaneously.

Additionally, these profile templates for filtering through results will vary depending upon the peptide, the antibody, and the binding conditions. One of skill in the art, given the present disclosure, especially with reference to Example 14, could determine the appropriate binding energy profile template. For example, as shown in Table 14.1, there are several possible important residues for peptide binding both in the 131 and in the 13.1.2 antibody. In the 131 mAb, positions E2, K4, N6, and Y7 are important for the particular peptide tested. In the 13.1.2 mAb, positions E1, E2, K4, G5, and N6 are important for the particular peptide tested. Those residues that are important can be residues involved in the creation of a binding energy profile template. As clear from the discussion below, the binding energy profile template in Example 39 appears to be different from that suggested by an analysis of Example 14. Example 39 is a less stringent version

of the template that allows more models to pass through the screening step. If one wanted to reduce the number of models that made it through the screening step, one could further add requirements concerning E1 and G5.

The following example demonstrates both the use of a longer peptide, how it can alter the results demonstrated above, what such changes can mean, as well as demonstrating the use of one of the above filters for selecting particular docking models.

Example 39

Epitope-Antibody Docking Model for a Seven Amino Acid Peptide

This example demonstrates the generation of a set of docking models for a seven-residue peptide complexed to the CDR region of 13.1.2 structure model. Additionally, this example demonstrates methods for selecting one docking model over another docking model.

First, a structural model for the seven-residue peptide EEKKGNY (SEQ ID NO: 131) was built in an extended conformation and energy minimized with Discover_3 module in InsightII modeling package. Next, the peptide structure was manually placed into the combining site to form an initial assembly. A Monte Carlo search in the translational and rotational spaces was then automatically performed with relaxed energy constraints in the Docking module in InsightII. During the docking process, the residues within 5 Angstroms of the binding groove were allowed to move while other antibody residues were fixed. The peptide was constrained to within 5 Angstroms of the starting position. The plausible configurations found by the Monte Carlo search were followed by simulated annealing and energy minimization to reach the final complex structure models. A total of 63 docking models were obtained.

For each docking model, the interaction energy between the antibody and the individual residue in the peptide was calculated with Discover_3. The profile of individual residue contribution in epitope-antibody binding was inspected to select the docking models that are consistent with the fine-resolution epitope mapping data, *i.e.*, "the binding energy profile template." 19 out of 63 docking models passed this check. A typical individual residue binding energy profile is shown in Table 39.1. Consistent with the epitope mapping data, in Example 14, the binding energy for K4 is prominent, and those for N6 and E2 are large. This binding energy profile template placed particular emphasis on the fact that K4 is greater than K3. This binding energy profile also placed an emphasis on the requirement that E2, K4, and N6 are relatively large. In other words, the binding energies of E2, K4, and N6 were not the lowest (least negative or smallest absolute value) binding energies in the peptide.

Table 39.1. Binding energy profile for individual residue in the seven-residue peptide to antibody 13.1.2 is consistent with epitope mapping data in Example 14.

E1	E2	K3	K4	G5	N6	Y7	Total
-10.97	-19.34	-13.46	-24.26	-10.1	-18.19	-15.15	-111.45

For the 19 models that passed the filter based on the binding energy profile, epitope-antibody binding energetics simulations were performed on each of the seven mutants with affinity data (Tyr172Arg, Example 36; Leu217Asn, Example 33; Leu217Gln, Example 32; Asn35Gly, Example 34; Leu99Asn, Example 37; Leu99His, Example 35; and Leu99Thr, Example 31). Since the extent of electrostatic interaction in this complex had to be approximated, a number of different dielectric constants were used in a series of calculations. The mutation was done with residue replacement followed by 30-100 steps of energy minimization to account for any local conformational changes that are induced by the side chain change. For each docking model, the interaction energy between the seven-residue peptide and the whole antibody was calculated for each mutant for the selected parameters. For each set of 8 binding energies (7 mutants plus the wild type), a linear fitting procedure was done on each set of the binding data, in comparison with the logarithm of Kd. A correlation coefficient was calculated for each linear fitting. The best correlation was obtained for one model, the model with the data described in Table 39.1, with a dielectric constant 1*r and 50 steps of energy minimization. The epitope-antibody binding energies for this model are shown in Table 39.2. The correlation coefficient was 0.80 with all the data. As the K_D for Leu99Asn was not measured with high degree of accuracy, see Example 37 above, a separate linear fitting was performed excluding the data for Leu99Asn. An excellent correlation coefficient of 0.91 was obtained, as shown in FIG. 20. The refined docking model is thus well represented by the above selected model. The model with space-filling peptide is shown in FIG. 21, and the hydrogen bonds are shown in FIG. 22. L3 **150** is the lower section and H3 **160** is the upper section on FIG. 22. H2 **140** is to the right of the peptide binding area. The peptide itself is placed into the binding site with E1 being positioned at the top of the page in a light shade, down through K3, K4, G5, N6, and Y7, progressively getting darker. The antibody residues involved in hydrogen bonding are shown in FIG. 22. The model produced from this example demonstrates that there are seven hydrogen bonds: K4...Q95, K4...Q95, N6...Q98, G5...H31, Y7...H31, and Y7...W165.

Table 39.2.

Simulation of epitope-antibody binding energetics, in comparison with logarithm of Kd.

mutant	coulumbic	vdw	total	Ln(Kd)
172Arg	-19.103	-27.962	-47.065	-13.891
217Asn	-19.003	-28.715	-47.718	-15.503
217Gln	-18.977	-28.73	-47.707	-16.201
35Gly	-19.095	-28.431	-47.526	-15.405
99Asn	-18.719	-28.778	-47.497	(-13.479)
99His	-18.837	-28.719	-47.556	-14.744
99Thr	-19.155	-28.704	-47.859	-16.475
WT	-18.981	-28.728	-47.708	-16.269

As can be seen from the model selected in Example 39, which is represented in FIG. 21, the docking model revealed some unexpected results. One interesting result is that while residues E2, K4, and N6 are important residues in the binding of the peptide as a whole, not all of these amino acids are modeled as involved in forming H-bonds with the antibody. It appears that K4 is involved in the formation of two H-bonds, both with Q95, which is consistent with K4's importance in the binding energy profile and profile template. It also appears that N6 is modeled to bond to Q98; however, in this particular model, E2 does not appear to form H-bonds in the model. One interesting trend that is consistent is that each of the key residues from the binding energy profile template (*e.g.*, E2, K4, and N6) are mostly buried and thus in close contact with the antibody binding groove. Thus, this docking model selection can account for the fact that these key residues are important because of their close interaction with the antibody. Additionally, it is possible that E1 is involved in a hydrogen bond with W214.

Example 39 also demonstrates that the above described method results in a strong correlation between binding energy and K_D, suggesting that models created by this method will also allow optimization or at least a prediction of the K_D of the antibody-peptide complex.

As can be seen from a comparison of Example 39 and Example 19, there are some residues that are important between the two models, some residues that appear only in the seven amino acid docking model, as well as some residues that do not appear to be as important in the seven amino acid docking model. For example, the seven peptide epitope appears to create H bonds between K4...Q95, K4...Q95, N6...Q98, G5...H31, Y7...H31, and Y7...W165. On the other hand, the six peptide epitope appears to create H bonds between E2...Y172, K3...H31, K4...H31, N6...D33, N6...Y37, and N6...K55. As can be seen from the above data, both the six and the seven amino acid peptide models emphasize the importance of H 31, as both models involve H31 forming two hydrogen bonds with the peptide. While there are other possible trends between the two data sets, it also appears that many of the binding interactions have changed from the six amino acid model to

the seven amino acid model. However, these examples demonstrate that variations due to epitope size can be detected with these models and thus the scaling up from shorter to longer epitope peptides should not be problematic in light of the present disclosure. The presence of amino acids that consistently demonstrate their importance in various binding models allows one to bias the importance of the various interactions accordingly so that shorter peptide models can be more representative of longer peptide interactions.

As appreciated by one of skill in the art, any of the above discussion or examples concerning the six amino acid peptide, EEKKGN (SEQ ID NO: 127), can also be applied towards the seven amino acid peptide, EEKKGNY (SEQ ID NO: 131), or any longer peptide. For instance, Example 20 can be repeated with the information from Example 39 for rational design for affinity-improved antibodies by site-directed mutagenesis. Furthermore, Example 21 can be repeated, using the results of Example 39, following an attempt of rational design for affinity-improved antibodies by site-directed mutagenesis to test any new antibodies derived from Example 20.

In one embodiment, the results from Example 39 are used to redefine the interaction area between the antibody and the peptide. For example, the paratope, for EEKKGNY (SEQ ID NO: 131), can be defined as including the other residues on the antibody that are predicted to interact with the peptide, for example, residue 95. Alternatively, as in Example 19, the paratope can be defined as all residues within 5 Angstroms of the docked peptide.

In silico affinity maturation in different proteins

Antibody affinity maturation has been successfully done *in vitro* in a number of different studies. Typically, randomized mutant libraries need to be constructed by molecular biology methods and selection/screening assays need to be developed to enrich the clones with good binding capability. Selected variants then need to be purified to determine affinities. This process requires a series of lengthy and laborious experiments. The following example demonstrates that it is possible to accurately predict affinity maturation through *in silico* selection utilizing an antibody-antigen complex structure alone.

Example 40

In silico Affinity Maturation through Antibody-Antigen Binding Energetics Simulations

This Example demonstrates that *in silico* antibody-antigen binding energetics simulations can be used for affinity maturation. In particular, this example demonstrates that the binding kinetics of a Fab-12 (IgG form known as rhuMAb VEGF) to VEGF (vascular endothelial growth factor) can be predicted through the above described *in silico* process.

The crystal structure of the VEGF-Fab complex used was located in the PDB database with the accession number 1BJ1, at a resolution of 2.4 angstroms. Published experimental affinity data for a series of mutants of an anti-VEGF Fab were used to test the concept. The 3-D coordinates of the VEGF-Fab structure were used for carrying out *in silico* mutation for the following mutants: H97Y, S100aT, T28D, 28D31H, 28D31H97Y100aT, N31H, Y53W, 71I73K, 71V73V. The

affinity data were obtained from the paper by Chen, Y et al., (J Mol Biol., 293(4):865-81 (1999)). The energetics simulations were carried out between the various VEGF-Fab mutants, as described in Example 39. The results are listed in Table 40.1. The results from this example demonstrate that a significant correlation between the binding energy and affinity ranking was obtained through this process. The linear fitting of the binding energy versus logarithm of the relative affinity is shown in FIG. 23. The correlation coefficient of –0.91 indicates that the *in silico* simulation accurately captures the detailed interaction at the atomic level.

Table 40.1. Antibody-antigen binding energy simulation compared with affinity data.

Kabat Number	Sequence Number	Relative Affinity	Ln(Relative Affinity)	BindingEnergy
H97Y	101Y	14	2.639	-59.065
S100aT	105T	1.9	0.642	-57.465
T28D	28D	1.4	0.336	-57.647
28D31H	28D31H	3.1	1.131	-57.699
28D31H97Y100aT	28D31H101Y105T	20	2.996	-59.518
N31H	31H	3.6	1.281	-57.724
Y53W	54W	1.3	0.262	-57.504
71I73K	72I74K	0.9	-0.105	-57.158
71V73V	72V74V	0.3	-1.204	-57.314
WT	WT	1	0.000	-57.404

As is clear from the Examples above, the simulation can be extrapolated to identify higher affinity mutants without the use of *in vitro* experimentation. Additionally, it is clear that this approach is useful for different antibodies and for different peptides. This methodology can be generally applied to perform affinity maturation *in silico*, using only a high-resolution antibody-antigen complex structure. In one embodiment, this use of *in silico* affinity maturation will save tremendous amounts of time and resource.

Example 41

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 beta-sheet framework. By examining sequences of immunoglobulins having unknown structure, Chothia, et al show that many immunoglobuins 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 that there is a small repertoire of main-chain conformations for at least five of the six hypervariable regions of antibodies.

Some of the antibodies described above were analyzed to determine the canonical class for each of the antibody's complementarity determining regions (CDRs). 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 table below (41.1) 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.

TABLE 41.1

mAb	H1-H2-L1-L2-L3
139	1-3-2-1-1
250	1-3-2-1-1
123	1-3-4-1-1
131	1-3-4-1-1
13_1_2	1-3-4-1-1
211	1-3-4-1-1
318	1-3-4-1-1
333	1-3-4-1-1
342	1-3-4-1-1
95	3-1-4-1-1
150	3-Y-4-1-1
170	3-Y-4-1-1

Each CDR (except for H3) was assigned to a canonical structure if it satisfies the length requirement and matches the key residues defined in the canonical class. The amino acids defined for each antibody can be found, for example, in the articles by Chothia, et al. referred to above.

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Equivalents

[0412] 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.

[0413] Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

[0414] The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. An isolated human monoclonal antibody that comprises a heavy chain polypeptide and a light chain polypeptide, wherein the antibody is conjugated to a toxin selected from the group consisting of: Maytansinoid (DM-1), saporin, Auristatin E toxin (AEFP), monomethyl auristatin E (MMAE), and Auristatin E, wherein at least one of the heavy or light chain polypeptides comprises an amino acid sequence that is at least 90% identical to the amino acid sequence selected from the group consisting of: SEQ ID NO: 2, SEQ ID NO: 19, SEQ ID NO: 142, SEQ ID NO: 144, and any combination thereof.

2. The antibody of Claim 1, wherein the toxin is selected from the group consisting of: Maytansinoid (DM-1) and saporin.

3. The antibody of Claim 1, wherein the toxin comprises Maytansinoid (DM-1).

4. The antibody of Claim 1, wherein the toxin is selected from the group consisting of: Auristatin E toxin (AEFP), monomethyl auristatin E (MMAE), and Auristatin E.

5. The isolated human monoclonal antibody of Claim 1, wherein the heavy chain polypeptide comprises the amino acid sequence of SEQ ID NO: 2.

6. The isolated human monoclonal antibody of Claim 1, wherein the light chain polypeptide comprises the amino acid sequence of SEQ ID NO: 142.

7. The isolated human monoclonal antibody of Claim 1, wherein the antibody binds to a peptide that consists of the sequence LEEKKGNYVVTDHC (SEQ ID NO: 56).

8. The isolated human monoclonal antibody of Claim 1, wherein the heavy chain polypeptide comprises an amino acid sequence that is at least about 95% identical to the amino acid sequence of SEQ ID NO: 2 and the light chain polypeptide comprises an amino acid sequence that is at least about 95% identical to the amino acid sequence of SEQ ID NO: 19.

9. The isolated human monoclonal antibody of Claim 1, wherein the heavy chain polypeptide comprises at least one of the following amino acid sequences: SEQ ID NO: 108, SEQ ID NO: 110, and SEQ ID NO: 112.

10. The isolated human monoclonal antibody of Claim 1, wherein the heavy chain polypeptide comprises all three of the following amino acid sequences: SEQ ID NO: 108, SEQ ID NO: 110, and SEQ ID NO: 112.

11. The isolated human monoclonal antibody of Claim 1, wherein the light chain polypeptide comprises at least one of the following amino acid sequences: SEQ ID NO: 101, SEQ ID NO: 103, and SEQ ID NO: 105.

12. The isolated human monoclonal antibody of Claim 1, wherein the light chain polypeptide comprises all three of the following amino acid sequences: SEQ ID NO: 101, SEQ ID NO: 103, and SEQ ID NO: 105.

13. The isolated human monoclonal antibody of Claim 1, wherein the heavy chain polypeptide comprises an amino acid sequence that is at least about 95% identical to the amino acid sequence of SEQ ID NO: 142 and the light chain polypeptide comprises an amino

acid sequence that is at least about 95% identical to the amino acid sequence of SEQ ID NO: 144.

14. The isolated human monoclonal antibody of Claim 1, wherein the heavy chain polypeptide comprises at least one of the following amino acid sequences: SEQ ID NO: 121, SEQ ID NO: 123, and SEQ ID NO: 125.

15. The isolated human monoclonal antibody of Claim 1, wherein the heavy chain polypeptide comprises all three of the following amino acid sequences: SEQ ID NO: 121, SEQ ID NO: 123, and SEQ ID NO: 125.

16. The isolated human monoclonal antibody of Claim 1, wherein the light chain polypeptide comprises at least one of the following amino acid sequences: SEQ ID NO: 101, SEQ ID NO: 116, and SEQ ID NO: 118.

17. The isolated human monoclonal antibody of Claim 1, wherein the light chain polypeptide comprises all three of the following amino acid sequences: SEQ ID NO: 101, SEQ ID NO: 116, and SEQ ID NO: 118.

18. An isolated human monoclonal antibody that binds to EGFRvIII comprising:

a heavy chain polypeptide comprising the following complementarity determining regions (CDRs): a heavy chain CDR1 that is a CDR1 in SEQ ID NO: 142; a heavy chain CDR2 that is a CDR2 in SEQ ID NO: 142; a heavy chain CDR3 that is a CDR3 in SEQ ID NO: 142;

a light chain polypeptide comprising the following CDRs: a light chain CDR1 that is a CDR1 in SEQ ID NO: 144; a light chain CDR2 that is a CDR2 in SEQ ID NO: 144; and a light chain CDR3 that is a CDR3 in SEQ ID NO: 144; and

a toxin conjugated to the antibody, wherein the toxin is selected from the group consisting of: Maytansinoid (DM-1), saporin, Auristatin E toxin (AEFP), monomethyl auristatin E (MMAE), and Auristatin E.

19. The antibody of Claim 18, wherein the toxin is selected from the group consisting of: Maytansinoid (DM-1) and saporin.

20. The antibody of Claim 18, wherein the toxin comprises Maytansinoid (DM-1).

21. The antibody of Claim 18, wherein the toxin is selected from the group consisting of: Auristatin E toxin (AEFP), monomethyl auristatin E (MMAE), and Auristatin E.

22. The isolated human monoclonal antibody of Claim 18, wherein the heavy chain polypeptide comprises all three of the following amino acid sequences: SEQ ID NO: 121, SEQ ID NO: 123, and SEQ ID NO: 125.

23. The isolated human monoclonal antibody of Claim 18, wherein the light chain polypeptide comprises all three of the following amino acid sequences: SEQ ID NO: 101, SEQ ID NO: 116, and SEQ ID NO: 118.

24. An isolated human monoclonal antibody that binds to EGFRvIII

comprising:

a heavy chain polypeptide comprising the following complementarity determining regions (CDRs): a heavy chain CDR1 that is a CDR1 in SEQ ID NO: 2; a heavy chain CDR2 that is a CDR2 in SEQ ID NO: 2; a heavy chain CDR3 that is a CDR3 in SEQ ID NO: 2;

a light chain polypeptide comprising the following CDRs: a light chain CDR1 that is a CDR1 in SEQ ID NO: 19; a light chain CDR2 that is a CDR2 in SEQ ID NO: 19; and a light chain CDR3 that is a CDR3 in SEQ ID NO: 19; and

a toxin conjugated to the antibody, wherein the toxin is selected from the group consisting of: Maytansinoid (DM-1), saporin, Auristatin E toxin (AEFP), monomethyl auristatin E (MMAE), and Auristatin E.

25. The antibody of Claim 24, wherein the toxin is selected from the group consisting of: Maytansinoid (DM-1) and saporin.

26. The antibody of Claim 24, wherein the toxin comprises Maytansinoid (DM-1).

27. The antibody of Claim 24, wherein the toxin is selected from the group consisting of: Auristatin E toxin (AEFP), monomethyl auristatin E (MMAE), and Auristatin E.

28. The isolated human monoclonal antibody of Claim 24, wherein the heavy chain polypeptide comprises all three of the following amino acid sequences: SEQ ID NO: 108, SEQ ID NO: 110, and SEQ ID NO: 112.

29. The isolated human monoclonal antibody of Claim 24, wherein the light chain polypeptide comprises all three of the following sequences: SEQ ID NO: 101, SEQ ID NO: 103, and SEQ ID NO: 105.

30. An isolated human monoclonal antibody that binds to an epitope on an EGFRvIII protein, wherein said antibody binds to the amino acid sequence LEEKKGNYVVTDHC (SEQ ID NO: 56), wherein the second lysine in SEQ ID NO: 56 is part of said epitope, and wherein the antibody is conjugated to a toxin selected from the group consisting of: Maytansinoid (DM-1), saporin, Auristatin E toxin (AEFP), monomethyl auristatin E (MMAE), and Auristatin E.

31. The antibody of Claim 30, wherein the toxin is selected from the group consisting of: Maytansinoid (DM-1) and saporin.

32. The antibody of Claim 30, wherein the toxin comprises Maytansinoid (DM-1).

33. The antibody of Claim 30, wherein the toxin is selected from the group consisting of: Auristatin E toxin (AEFP), monomethyl auristatin E (MMAE), and Auristatin E.

34. An isolated human monoclonal antibody that binds to an EGFRvIII protein, wherein said antibody competes for binding to EGFRvIII with the antibody of Claim 1, and wherein the antibody is conjugated to a toxin selected from the group consisting of: Maytansinoid (DM-1), saporin, Auristatin E toxin (AEFP), monomethyl auristatin E (MMAE), and Auristatin E.

35. The antibody of Claim 34, wherein the toxin is selected from the group

consisting of: Maytansinoid (DM-1) and saporin.

36. The antibody of Claim 34, wherein the toxin comprises Maytansinoid (DM-1).

37. The antibody of Claim 34, wherein the toxin is selected from the group consisting of: Auristatin E toxin (AEFP), monomethyl auristatin E (MMAE), and Auristatin E.

38. A method of killing a targeted cell comprising contacting the targeted cell with at least one of the antibodies from Claim 1 to Claim 37.

39. The method of Claim 38, wherein the method is performed *in vivo*.

40. The method of Claim 39, wherein the method is performed on mammal.

41. The method of Claim 40, wherein the mammal is a human.

42. The method of Claim 40, wherein the mammal suffers from a cancer involving epithelial cell proliferation.

43. The method of Claim 42, wherein the cancer comprises a lung carcinoma, breast carcinoma, head & neck cancer, prostate carcinoma or glioblastoma.

44. The method of Claim 38, wherein the method is performed *in vitro*.

45. The method of Claim 38, wherein said antibody associated with said toxin is at least ten fold more toxic to said targeted cell than to cells that do not express said peptide.

46. The method of Claim 38, wherein the antibody is associated with the toxin via a peptide linker.

47. The method of Claim 38, wherein the antibody is associated with the toxin via a second antibody.

48. Use of at least one of the antibodies of Claims 1 to 37, in the preparation of a medicament for the treatment of cancer cells expressing Epidermal Growth Factor Receptor viii (EGFRvIII).

49. The use of Claim 42, wherein said cancer cells are epithelial cells.

50. The use of Claim 42, wherein said cancer cells are selected from the group consisting of lung, colon, gastric, renal, prostate, breast, glioblastoma or ovarian cancer cells.

51. An isolated human monoclonal antibody according to any one of claims 1 to 37, or a method of killing a targeted cell according to any one of claims 38 to 47, or the use according to any one of claims 48 to 50, substantially as hereinbefore described with reference to the Figures and/or Examples.

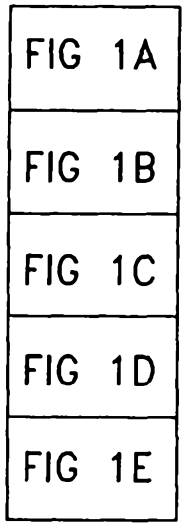

FIG. 1


```
EGFR wt    1    LEEKKVCQGTSNKLTQLGTFEDHFLSLQRMFNNCEVVLGNLEITYVQRNYDLSFLKTIQE 60
EGFRvIII   1    LEEKK---------------------------------------------------- 1

EGFR wt    61   VAGYVLIALNTVERIPLENLQIIRGNMYYENSYALAVLSNYDANKTGLKELPMRNLQEIL 120
EGFRvIII   1    ---------------------------------------------------------- 1

EGFR wt    121  HGAVRFSNNPALCNVESIQWRDIVSSDFLSNMSMDFQNHLGSCQKCDPSCPNGSCWGAGE 180
EGFRvIII   1    ---------------------------------------------------------- 1

EGFR wt    181  ENCQKLTKIICAQQCSGRCRGKSPSDCCHNQCAAGCTGPRESDCLVCRKFRDEATCKDTC 240
EGFRvIII   1    ---------------------------------------------------------- 1
```

FIG. 1A

FIG. 1B

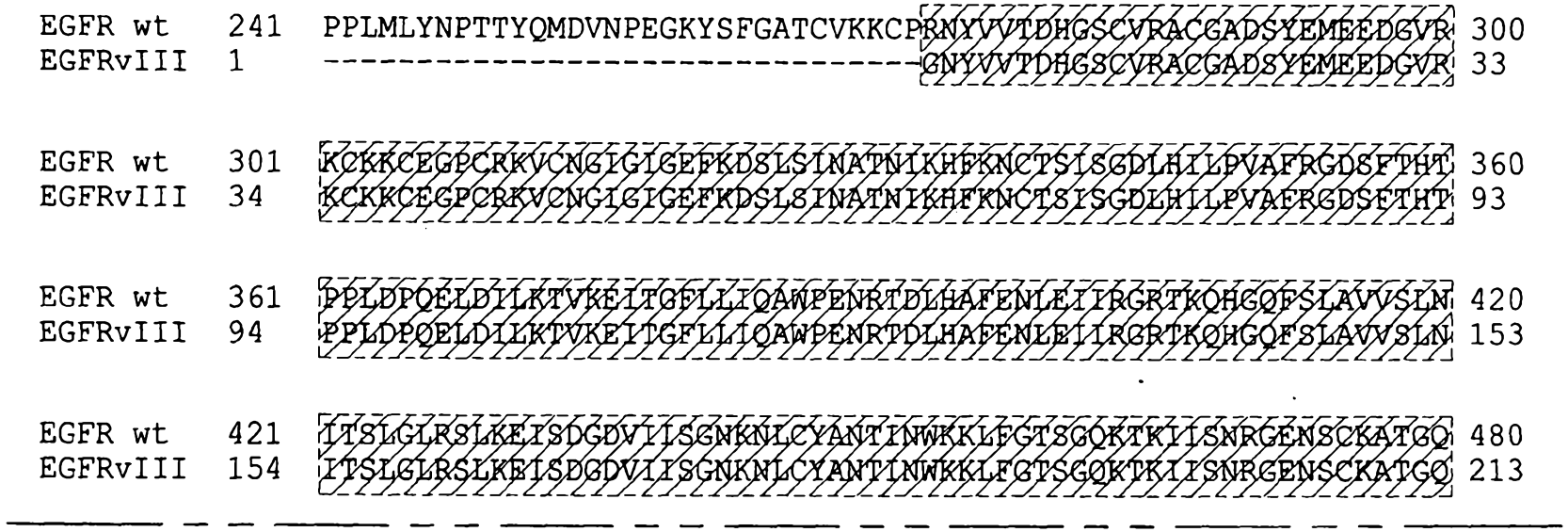
EGFR wt 241 PPLMLYNPTTYQMDVNPEGKYSFGATCVKKCPRNYVVTDHGSCVRACGADSYEMEEDGVR 300
EGFRvIII 1 ----------GNYVVTDHGSCVRACGADSYEMEEDGVR 33
EGFR wt 301 KCKKCEGPCRKVCNGIGIGEFKDSLSINATNIKHFKNCTSISGDLHILPVAFRGDSFTHT 360
EGFRvIII 34 KCKKCEGPCRKVCNGIGIGEFKDSLSINATNIKHFKNCTSISGDLHILPVAERGDSETHT 93
EGFR wt 361 PPLDPQELDILKTVKEITGFLLIQAWPENRTDLHAFENLEIIRGRTKQHGQFSLAVVSLN 420
EGFRvIII 94 PPLDPQELDILKTVKEITGFLLIQAWPENRTDLHAFENLEIIRGRTKQHGQFSLAVVSLN 153
EGFR wt 421 ITSLGLRSLKEISDGDVIISGNKNLCYANTINWKKLFGTSGQKTKIISNRGENSCKATGQ 480
EGFRvIII 154 ITSLGLRSLKEISDGDVIISGNKNLCYANTINWKKLFGTSGQKTKIISNRGENSCKATGQ 213

FIG. 1C

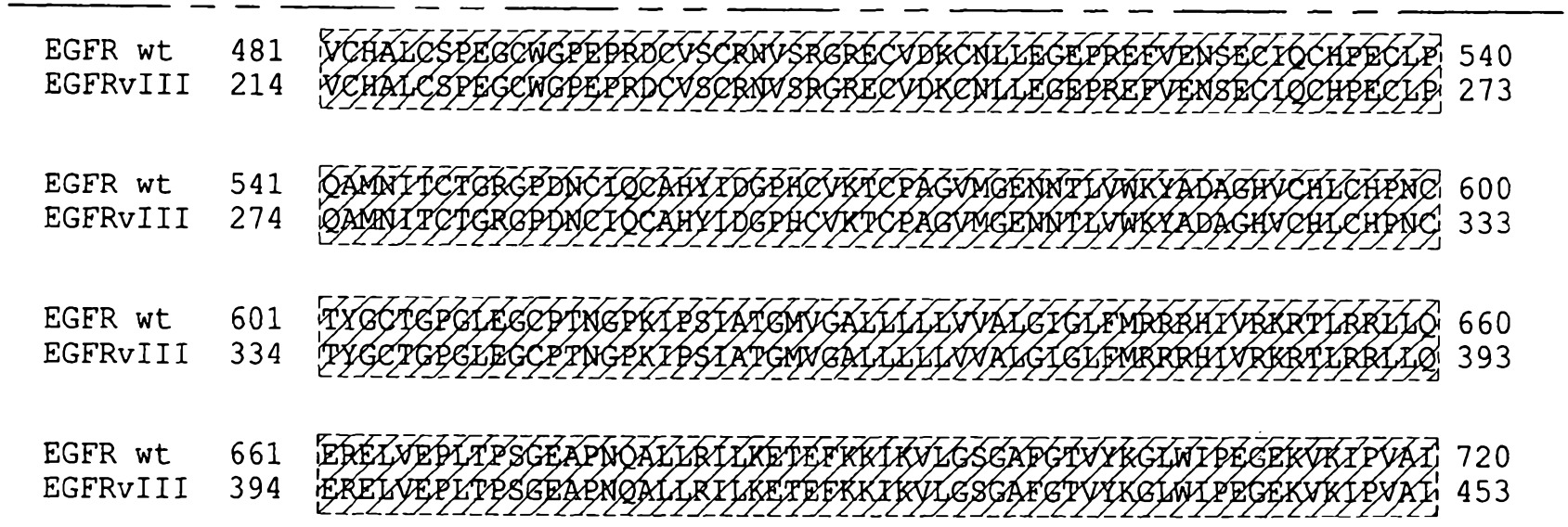
EGFR wt 481 VCHALCSPEGCWGPEPRDCVSCRNVSRGRECVDKCNLLEGEPREFVENSECIQCHPECLP 540
EGFRvIII 214 VCHALCSPEGCWGPEPRDCVSCRNVSRGRECVDKCNLLEGEPREFVENSECIQCHPECLP 273
EGFR wt 541 QAMNITCTGRGPDNCIQCAHYIDGPHCVKTCPAGVMGENNTLVWKYADAGHVCHLCHPNC 600
EGFRvIII 274 QAMNITCTGRGPDNCIQCAHYIDGPHCVKTCPAGVMGENNTLVWKYADAGHVCHLCHPNC 333
EGFR wt 601 TYGCTGPGLEGCPTNGPKIPSIATGMVGALLLLLVVALGIGLFMRRRHIVRKRTLRRLLQ 660
EGFRvIII 334 TYGCTGPGLEGCPTNGPKIPSIATGMVGALLLLLVVALGIGLFMRRRHIVRKRTLRRLLQ 393
EGFR wt 661 ERELVEPLTPSGEAPNQALLRILKETEFKKIKVLGSGAFGTVYKGLWIPEGEKVKIPVAI 720
EGFRvIII 394 ERELVEPLTPSGEAPNQALLRILKETEFKKIKVLGSGAFGTVYKGLWIPEGEKVKIPVAI 453

FIG. 1D

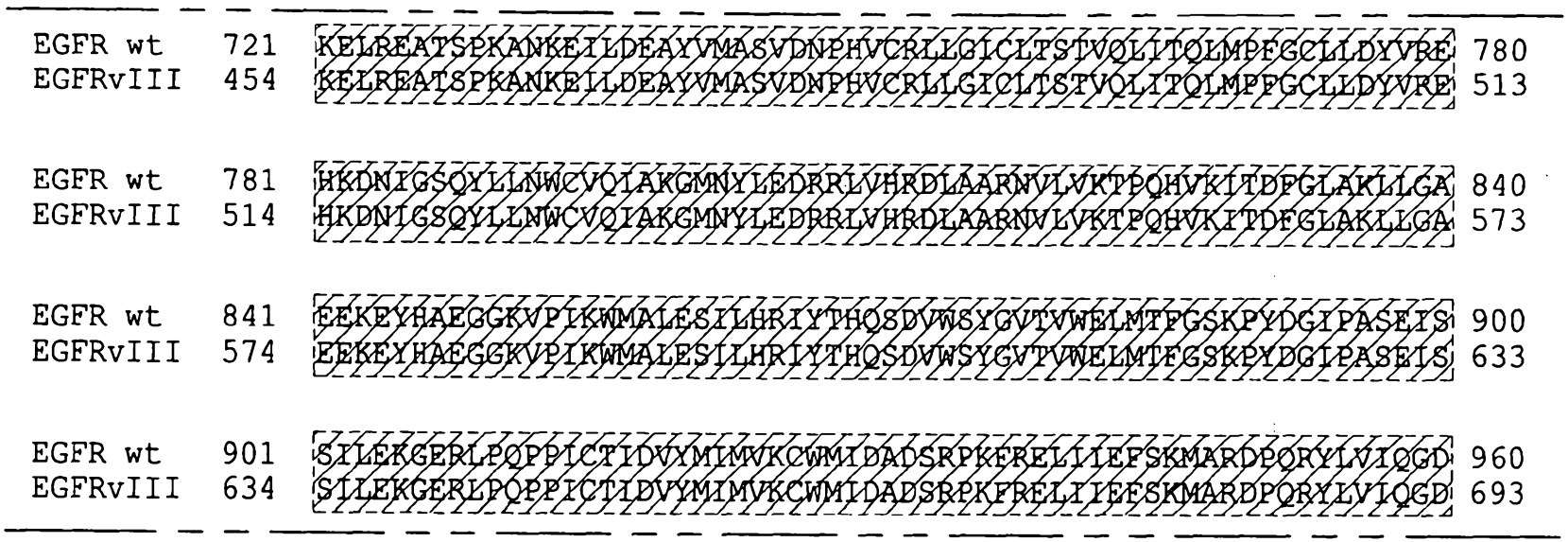
EGFR wt 721 KELREATSPKANKEILDEAYVMASVDNPHVCRLLGICLTSTVQLITQLMPFGCLLDYVRE 780
EGFRvIII 454 KELREATSPKANKEILDEAYVMASVDNPHVCRLLGICLTSTVQLITQLMPFGCLLDYVRE 513
EGFR wt 781 HKDNIGSQYLLNWCVQIAKGMNYLEDRRLVHRDLAARNVLVKTPQHVKITDFGLAKLLGA 840
EGFRvIII 514 HKDNIGSQYLLNWCVQIAKGMNYLEDRRLVHRDLAARNVLVKTPQHVKITDFGLAKLLGA 573
EGFR wt 841 EEKEYHAEGGKVPIKWMALESILHRIYTHQSDVWSYGVTVWELMTFGSKPYDGIPASEIS 900
EGFRvIII 574 EEKEYHAEGGKVPIKWMALESILHRIYTHQSDVWSYGVTVWELMTFGSKPYDGIPASEIS 633
EGFR wt 901 SILEKGERLPQPPICTIDVYMIMVKCWMIDADSRPKFRELIIEFSKMARDPQRYLVIQGD 960
EGFRvIII 634 SILEKGERLPQPPICTIDVYMIMVKCWMIDADSRPKFRELIIEFSKMARDPQRYLVIQGD 693

FIG. 1E

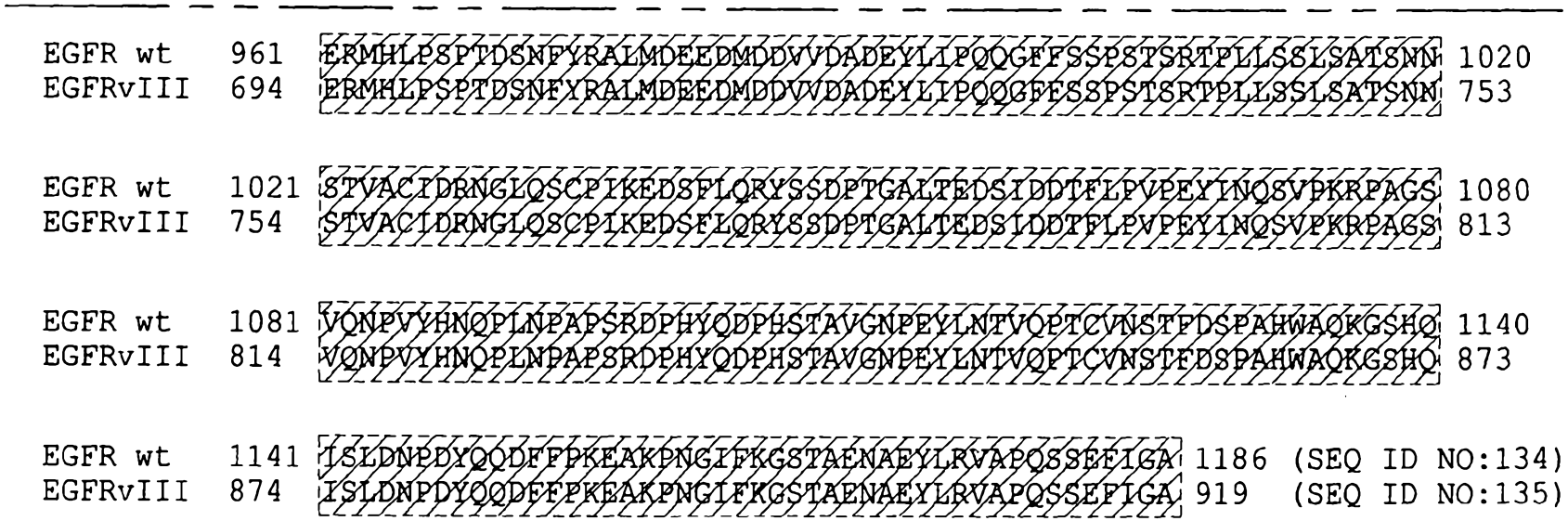
EGFR wt 961 ERMHLPSPTDSNFYRALMDEEDMDDVVDADEYLIPQQGFFSSPSTSRTPLLSSLSATSNN 1020
EGFRvIII 694 ERMHLPSPTDSNFYRALMDEEDMDDVVDADEYLIPQQGFFSSPSTSRTPLLSSLSATSNN 753
EGFR wt 1021 STVACIDRNGLQSCPIKEDSFLQRYSSDPTGALTEDSIDDTFLPVPEYINQSVPKRPAGS 1080
EGFRvIII 754 STVACIDRNGLQSCPIKEDSFLQRYSSDPTGALTEDSIDDTFLPVPEYINQSVPKRPAGS 813
EGFR wt 1081 VQNPVYHNQPLNPAPSRDPHYQDPHSTAVGNPEYLNTVQPTCVNSTFDSPAHWAQKGSHQ 1140
EGFRvIII 814 VQNPVYHNQPLNPAPSRDPHYQDPHSTAVGNPEYLNTVQPTCVNSTFDSPAHWAQKGSHQ 873
EGFR wt 1141 ISLDNPDYQQDFFPKEAKPNGIFKGSTAENAEYLRVAPQSSEFIGA 1186 (SEQ ID NO:134)
EGFRvIII 874 ISLDNPDYQQDFFPKEAKPNGIFKGSTAENAEYLRVAPQSSEFIGA 919 (SEQ ID NO:135)

FIG. 2A

1 2 3 4 5 273 275 277 279
L-E-E-K-K-G-N-Y-V-V-T-D-H-C (SEQ ID NO.56)
278 280

6
VCQGTSNKLTQLGTFEDHFLSLQRMFNNCEVVLGNLEITYVQRNYDLSFLKTIQEV
AGYVLIALNTVERIPLENLQIIRGNMYYENSYALAVLSNYDANKTGLKELPMRNLQ
EILHGAVRFSNNPALCNVESIQWRDIVSSDFLSNMSMDFQNHLGSCQKCDPSCPNG
SCWGAGEENCQKLTKIICAQQCSGRCRGKSPSDCCHNQCAAGCTGPRESDCLVCRK
FRDEATCKDTCPPLMLYNPTTYQMDVNPEGKYSFGATCVKKCPR
272

(SEQ ID NO.136)

FIG. 2B

XenoMax Derived Human anti- EGFRvIII Antibody Nucleotide and Amino Acid Sequences

095

Nucleotide sequence of heavy chain variable region:

5'CAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCACAGACCCTGT
CCCTCACCTGCACTGTCTCTGGTGGCTCCATCAGCAGTGGTGGTTACTACTGGAGCT
GGATCCGCCAGCACCCAGGGAAGGGCCTGGAGTGGATTGGGTTCATCTATTACAGA
GGGAACACCTACTACAACCCGTCCCTCAAGAGTCGAGTTACCATATCAGTTGACACG
TCTAAGAACCAGTTCTCCCTGAAGCTGAGCTCTGTGACTGCCGCGGACACGGCCGTG
TATTACTGTGCGCGAGACGGATATTGTAGTAGAACCGGCTGCTATGGCGGCTGGTTC
GACCCCTGGGGCCAGGGAACCCTGGTCACGTCTCCT**3' (SEQ ID NO:35)**

Amino Acid sequence of heavy chain variable region:

QVQLQESGPFLVKPSQTLSLTCTVSGGSISSGGYYWSWIRQHPGKGLEWIGFIYYRGNTY
YNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARDGYCSRTGCYGGWFDPWGQ
GTLVTVSP **(SEQ ID NO:7)**

Nucleotide sequence of light chain variable region:

5'GATATTGTGATGACCCAGACTCCACTCTCCTCACCTGTCACCCTTGGACAGCCGGC
CTCCATCTCCTGCAGGTCTAGTCAAAGCCTCATACACACTGATGGAAACATCTATTT
GAGTTGGCTTCAGCAGAGGCCAGGCCAGCCTCCAAGACTCCTAATTTATAAGATTTC
TAATCGGTTCTCTGGGGTCCCAGACAGATTCAGTGGCAGTGGGGCAGGGACAGATTT
CACACTGAAGATCAGCAGGGTGGAAGCTGAGGATGTGGGGGTTTATTACTGCATGC
AAGGTACACAATTTCCTATCACCTTCGGCCAAGGGACACGACTGGAGATTAAA**3'(SEQ ID NO:34)**

Amino Acid sequence of light chain variable region:

DIVMTQTPLSSPVTLGQPASISCRSSQSLIHTDGNIYLSWLQQRPGQPPRLLIYKISNRFSG
VPDRFSGSGAGTDFTLKISRVEAEDVGVYYCMQGTQFPITFGQGTRLEIK **(SEQ ID NO:23)**

FIG. 3A

123k/124g

Nucleotide sequence of heavy chain variable region:

5'CAGGTGCAGCTGGTGGAGTCTGGGGGAGGCGTGGTCCAGCCTGGGAGGTCCCTGA
GACTCTCCTGTGCAGCCTCCGGATTCACCCTCAGTAGCTATGGCATGCACTGGGTCC
GCCAGGCTCCAGGCAAGGGGCTGGAGTGGGTGGCAGTTATGTCATATGATGGAAGT
AAAGAAGACTATGCAGACTCCGTGAAGGGCCGATTCACCATCTCTAGAGACAATTC
CGAGAACATGCTGTATCTGCAAATGAACAGCCTGAGAGCTGAGGACACGGCTGTAT
ATTACTGTGTGAGCGAAGGATATTGTAGTAGTCGTAGCTGCTATAAGTACTACTACT
ACGGCATGGACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCA**3'** **(SEQ ID NO:37)**

Amino Acid sequence of heavy chain variable region:

QVQLVESGGGVVQPGRSLRLSCAASGFTLSSYGMHWVRQAPGKGLEWVAVMSYDGS
KEDYADSVKGRFTISRDNSENTLYLQMNSLRAEDTAVYYCVSEGYCSSRSCYKYYYYG
MDVWGQGTTVTVSS **(SEQ ID NO:13)**

Nucleotide sequence of light chain variable region:

5'ATATTGTGATGACTCAGTCTCCACTCTCCCTGCCCGTCACCCCTGGAGAGCCGGCC
TCCATCTCCTGCAGGTCTAGTCAGAGCCTCCTGTATAGAAATGGAAACAACTATTTG
GATTGGTATCTGCAGAAGCCAGGGCAGTCTCCACAGCTCCTGATCTATTTGGGTTCT
AATCGGGCCTCCGGGGTCCCTGACAGGTTCAGTGGCAGTGGATCGGGCACAGATTTT
ACACTGAACATCAGCAGAGTGGAGGCTGAGGATGTTGGGCATTATTACTGCATGCA
GGCTCTACAAACTCCTCGGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAA**3'**
(SEQ ID NO:36)

Amino Acid sequence of light chain variable region:

DIVMTQSPLSLPVTPGEPASISCRSSQSLLYRNGNNYLDWYLQKPGQSPQLLIYLGSNRA
SGVPDRFSGSGSGTDFTLNISRVEAEDVGHYYCMQALQTPRTFGQGTKVEIK **(SEQ ID NO:29)**

FIG. 3B

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Nucleotide sequence of heavy chain variable region:

5'CAGGTGCAGCTGGTGGAGTCTGGGGGAGGCGTGGTCCAGTCTGGGAGGTCCCTGA
GACTCTCCTGTGCAGCGTCTGGATTCACCTTCAGAAACTATGGCATGCACTGGGTCC
GCCAGGCTCCAGGCAAGGGGCTGGAGTGGGTGGCAGTTATATGGTATGATGGAAGT
GATAAATACTATGCAGACTCCGTGAGGGGCCGATTCACCATCTCCAGAGACAATTCC
AAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCTGTGTA
TTACTGTGCGAGAGATGGCTACGATATTTTGACTGGTAATCCTAGGGACTTTGACTA
CTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA3' **(SEQ ID NO:39)**

Amino Acid sequence of heavy chain variable region:

QVQLVESGGGVVQSGRSLRLSCAASGFTFRNYGMHWVRQAPGKGLEWVAVIWYDGS
DKYYADSVRGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARDGYDILTGNPRDFDYW
GQGTLVTVSS **(SEQ ID NO:2)**

Nucleotide sequence of light chain variable region:

5'GATACTGTGATGACCCAGACTCCACTCTCCTCACATGTAACCCTTGGACAGCCGGC
CTCCATCTCCTGCAGGTCTAGTCAAAGCCTCGTACACAGTGATGGAAACACCTACTT
GAGTTGGCTTCAGCAGAGGCCAGGCCAACCTCCAAGACTCCTAATTTATAGGATTTC
TAGGCGGTTCTCTGGGGTCCCAGACAGATTCAGTGGCAGTGGGGCAGGGACAGATT
TCACACTGGAAATCAGCAGGGTGGAGGCTGAGGATGTCGGGGTTTATTACTGCATG
CAATCTACACACGTTCCTCGGACGTTCGGCCAAGGGACCAAGGTGGAGATCAAA3'
(SEQ ID NO:38)

Amino Acid sequence of light chain variable region:

DTVMTQTPLSSHVTLGQPASISCRSSQSLVHSDGNTYLSWLQQRPGQPPRLLIYRISRRFS
GVPDRFSGSGAGTDFTLEISRVEAEDVGVYYCMQSTHVPRTFGQGTKVEIK **(SEQ ID NO:19)**

FIG. 3C

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Nucleotide sequence of heavy chain variable region:

5'GAGGTGCAGGTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGA
GACTCTCCTGTGCAGCCTCTGGATTCACCTTTAGCAGCTATGCCATGAGCTGGGTCC
GCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCGGCTATTAGTGGTAGTGGTGGT
AGTACAAACTACGCAGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTC
CAAGAACACACTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTCT
ATTACTGTGCTGGGAGCAGTGGCTGGTCCGAGTACTGGGGCCAGGGAACCCTGGTC
ACCGTCTCCTCG**3' (SEQ ID NO:41)**

Amino Acid sequence of heavy chain variable region:

EVQVLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAISGSGGST
NYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAGSSGWSEYWGQGTLVTVS
S **(SEQ ID NO:10)**

Nucleotide sequence of light chain variable region:

5'GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGT
CACCATCACTTGCCGGGCTAGTCAGGGCATTAGAAATAATTTAGCCTGGTATCAGCA
GAAACCAGGGAAAGCCCCTAAGCGCCTGATCTATGCTGCCTCCAATTTGCAAAGTG
GGGTCCCATCAAGGTTCACCGGCAGTGGATCTGGGACAGAATTCACTCTCATAGTCA
GCAGCCTGCAGCCTGAAGATTTTGCGACTTATTACTGTCTACAGCATCACAGTTACC
CGCTCACTTCCGGCGGAGGGACCAAGGTGGAGATCAAA**3' (SEQ ID NO:40)**

Amino Acid sequence of light chain variable region:

DIQMTQSPSSLSASVGDRVTITCRASQGIRNNLAWYQQKPGKAPKRLIYAASNLQSGVPS
RFTGSGSGTEFTLIVSSLQPEDFATYYCLQHHSYPLTSGGGTKVEIK **(SEQ ID NO:25)**

FIG. 3D

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Nucleotide sequence of heavy chain variable region:

5'CAGGTACAGCTGCAGCAGTCAGGTCCAGGACTGGTGAAGCCCTCGCAGACCCTCT
CACTCACCTGTGCCATCTCCGGGGACAGTGTCTCTAGCTACAGTTCTGCTTGGAACT
GGATCAGGCAGTCCCCATCGAGAGGCCTTGAGTGGCTGGGAAGGGCATATCACAGG
TCCAGGTGGTATTACGAGTATGCAGTATCGGTGAAAAGTCGAATAAACATCACCCC
AGACACATCCAAGAACCAGTTCTCCCTGCAGCTGAACTCTGTGACTCCCGAGGACAC
GGCTGTGTATTACTGTGCAAGAGGCAGTCGCTTTGACTACTGGGGCCAGGGAACCCT
GGTCACCGTCTCCTCA**3' (SEQ ID NO:43)**

Amino Acid sequence of heavy chain variable region:

QVQLQQSGPGLVKPSQTLSLTCAISGDSVSSYSSAWNWIRQSPSRGLEWLGRAYHRSRW
YYEYAVSVKSRINITPDTSKNQFSLQLNSVTPEDTAVYYCARGSRFDYWGQGTLVTVSS
(SEQ ID NO:5)

Nucleotide sequence of light chain variable region:

5'GATATTGTGATGACCCAGACTCCACTCTCCTCACCTGTCACCCTTGGACAGCCGGC
CTCCATCTCCTGCAGGTCTAGTCAAAGCCTCGTACACAGGGATGGAAATACCTACTT
GAGTTGGCTTCAGCAGAGGCCAGGCCAGCCTCCAAGACTCCTAATTTATAAGATTTC
TAACCGGTTCTCTGGGGTCCCAGACAGATTCAGTGGCAGTGGGGCAGGGACAGATT
TCACACTGAAAATTAGCAGGGTGGAAGCTGAGGATGTCGGGATTTATTTCTGCATGC
ATACTACACAATTTCCTTGGACGTTCGGCCAAGGGACCAGGGTGGAAATCAAA**3'**
(SEQ ID NO:42)

Amino Acid sequence of light chain variable region:

DIVMTQTPLSSPVTLGQPASISCRSSQSLVHRDGNTYLSWLQQRPGQPPRLLIYKISNRFS
GVPDRFSGSGAGTDFTLKISRVEAEDVGIYFCMHTTQFPWTFGQGTRVEIK **(SEQ ID NO:21)**

FIG. 3E

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Nucleotide sequence of heavy chain variable region:

5'CAGGTACAGCTGCAGCAGTCAGGTCCAGGACTGGTGAAGCCCTCGCAGACCCTCT
CACTCACCTGTGCCATCTCCGGGGACAGTGTCTCTAGCAACAATGCTGCTTGGAACT
GGATCAGGCAGTCCCCAGCGAGAGGCCTTGAGTGGCTGGGAAGGACATACTACAGG
TCCAAGTGGTATAATGATTATGTAGTATCTGTGAAAAGTCGAATAACCATCAACCCA
GACACATCCAAGAACCAGTTCTCCCTGCAGCTGAACTCTGTGACTCCCGAGGACACG
GCTGTGTATTACTGTGTAAGAGGCAGTCGCTTTGACTACTGGGGCCAGGGAACCCTG
GTCACCGTCTCCTCA**3' (SEQ ID NO:45)**

Amino Acid sequence of heavy chain variable region:

QVQLQQSGPGLVKPSQTLSLTCAISGDSVSSNNAAWNWIRQSPARGLEWLGRTYYRSK
WYNDYVVSVKSRITINPDTSKNQFSLQLNSVTPEDTAVYYCVRATAFDYWGQGTLVTV
SS **(SEQ ID NO:4)**

Nucleotide sequence of light chain variable region:

5'GCTATTGTGTTGACCCAGACTCCACTCTCCTCACCTGTCACCCTTGGACAGCCGGC
CTCCATCTCCTGCAGGTCTAGTCAAAGCCTCGTTCACAGGGATGGAAACACCTACTT
GAGTTGGCTTCAGCAGAGGCCAGGCCAGCCTCCAAGACTCCTAATTTATAAGATTTC
TAACCGGTTCTCTGGGGTCCCAGACAGATTCAGTGGCAGTGGGGCAGGGACAGATT
TCACACTGAAAATCAGCAGGGTGGAACCTGACGATGTCGGGGTTTATTACTGCATGC
ATACTACACAACTTCCTTGGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAA**3'**
(SEQ ID NO:44)

Amino Acid sequence of light chain variable region:

AIVLTQTPLSSPVTLGQPASISCRSSQSLVHRDGNTYLSWLQQRPGQPPRLLIYKISNRFSG
VPDRFSGSGAGTDFTLKISRVEPDDVGVYYCMHTTQLPWTFGQGTKVEIK **(SEQ ID NO:20)**

FIG. 3F

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Nucleotide sequence of heavy chain variable region:

5'CAGGTGCAGCTGGTGGAGTCTGGGGGAGGCGTGGTCCAGCCTGGGAGGTCCCTGA
GACTCTCCTGTGTAGCCTCTGGATTCACCCTCAGTAGCTATGGCATGCACTGGGTCC
GCCAGGCTCCAGGCAAGGGGCTGGAGTGGGTGGCAGTGACATCATATGATGGAAGT
AAAAAAGACTATGCAGACTCCGCGAAGGGCCGATTCACCATCTCCAGAGACAATTC
CAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCTGAGGACACGGCTGTGT
ATTACTGTGTGAGCGAAGGATATTGTAGTAGTAGTAGCTGCTATAAGTACTACTATT
ACGGTATGGACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCTTCA**3'** **(SEQ ID NO:47)**

Amino Acid sequence of heavy chain variable region:

QVQLVESGGGVVQPGRSLRLSCVASGFTLSSYGMHWVRQAPGKGLEWVAVTSYDGSK
KDYADSAKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCVSEGYCSSSSCYKYYYYGM
DVWGQGTTVTVSS **(SEQ ID NO:12)**

Nucleotide sequence of light chain variable region:

5'GATATTGTGATGACTCAGTCTCCACTCTCCCTGCCCGTCACCCCTGGAGAGCCGGC
CTCCATCTCCTGCAGGTCTAGTCAGAGCCTCCTATATAGAAATGGAAACAACTATTT
GGATTGGTATCTGCAGAGGCCAGGGCAGTCTCCACAACTCCTGATCTATTTGGGTTC
TAATCGGGCCTCCGGGGTCCCTGACAGGTTCAGTGGCAGTGGATCAGGCACAGATTT
TACATTGAAAATCGGCAGAGTGGAGGCTGAGGATGTTGGGGTTTATTACTGCATGCA
GGCTCTACAAACTCCTCGGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAA**3'**
(SEQ ID NO:46)

Amino Acid sequence of light chain variable region:

DIVMTQSPLSLPVTPGEPASISCRSSQSLLYRNGNNYLDWYLQRPGQSPQLLIYLGSNRA
SGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCMQALQTPRTFGQGTKVEIK **(SEQ ID NO:28)**

FIG. 3G

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Nucleotide sequence of heavy chain variable region:

5'GAGGGGCAGCTGTTGGAGTCTGGGGGAGGCTGGGTACAGCCTGGGGAGTCCCTGA
GACTCTCCTGTGCAGCCTCTGGATTCACCTTTAGCAGCTATGCCATGAGCTGGGTCC
GCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCGGCTATTAGTGGTAGTGGTGGT
AGCACAAATTACGCAGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTC
CAAGAACACGCTGTATCTGCAAGTGAACAGCCTGAGAGTCGAGGACACGGCCGTAT
ATTACTGTGCTGGGAGCAGTGGCTGGTCCGAGTACTGGGGCCAGGGAACCCTGGTC
ACCGTCTCCTCA3' **(SEQ ID NO:49)**

Amino Acid sequence of heavy chain variable region:

EGQLLESGGGWVQPGESLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAISGSGGST
NYADSVKGRFTISRDNSKNTLYLQVNSLRVEDTAVYYCAGSSGWSEYWGQGTLVTVSS
(SEQ ID NO:9)

Nucleotide sequence of light chain variable region:

5'GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGCGT
CACCATCACTTGCCGGACAAGTCAGGGCATTAGAAAAAATTTAGGCTGGTATCAGC
AGAAACCAGGGAAAGCCCCTAAGCGCCTGATCTATGCTGCATCCAGTTTACAAAGT
GGGGTCCCATCAAGGTTCAGCGGCAGTGGATCTGGGACAGAATTCACTCTCACAATC
CGCAGCCTGCAGCCTGAAGATTTTGCAACTTATTACTGTCTCCAGCATCATAGTTAC
CCGCTCACTTTCGGCGGAGGGACCAGGGTGGAGATCAGA3' **(SEQ ID NO:48)**

Amino Acid sequence of light chain variable region:

DIQMTQSPSSLSASVGDSVTITCRTSQGIRKNLGWYQQKPGKAPKRLIYAASSLQSGVPS
RFSGSGSGTEFTLTISRLQPEDFATYYCLQHHSYPLTFGGGTRVEIR **(SEQ ID NO:26)**

FIG. 3H

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Nucleotide sequence of heavy chain variable region:

5'CAGGTGCAGCTGGTGGAGTCTGGGGGAGGCGTGGTCCAGCCTGGGAGGTCCCTGA
GACTCTCCTGTGTAGCCTCTGGATTCACCCTCAGTAGCTATGGCATGCACTGGGTCC
GCCAGGCTCCAGGCAAGGGGCTGGAGTGGGTGGCAGTGACATCATATGATGGAAGT
AAAAAAGACTATGCAGACTCCGTGAAGGGCCGATTCACCATCTCCAGAGACAATTC
CAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCTGAGGACACGGCTGTGT
ATTACTGTGTGAGCGAAGGATATTGTGATAGTAGTAGCTGCTATAAGTACTACTACT
ACGGTATGGACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCTTCA3' **(SEQ ID NO:51)**

Amino Acid sequence of heavy chain variable region:

QVQLVESGGGVVQPGRSLRLSCVASGFTLSSYGMHWVRQAPGKGLEWVAVTSYDGSK
KDYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCVSEGYCDSSSCYKYYYYG
MDVWGQGTTVTVSS **(SEQ ID NO:15)**

Nucleotide sequence of light chain variable region:

5'GATATTGTGATGACTCAGTCTCCACTCTCCCTGCCCGTCACCCCTGGAGAGCCGGC
CTCCATCTCCTGCAGGTCTAGTCAGAGCCTCCTATATAGAAATGGAAACAACTATTT
GGATTGGTATCTGCAGAGGCCAGGGCAGTCTCCACAACTCCTGATCTATTTGGGTTC
TAATCGGGCCTCCGGGGTCCCTGACAGGTTCAGTGGCAGTGGATCAGGCACAGATTT
TACACTGAAAATCAGCAGAGTGGAGGCTGAGGATGTTGGGGTTTATTACTGCATGC
AGGCTCTACAAACTCCTCGGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAA3'
(SEQ ID NO:50)

Amino Acid sequence of light chain variable region:

DIVMTQSPLSLPVTPGEPASISCRSSQSLLYRNGNNYLDWYLQRPGQSPQLLIYLGSNRA
SGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCMQALQTPRTFGQGTKVEIK **(SEQ ID NO:33)**

FIG. 3I

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333

Nucleotide sequence of heavy chain variable region:

5'CAGGTGCAGCTGGTGGAGTCTGGGGGAGGCGTGGTCCAGCCTGGGAGGTCCCTGA
GACTCTCCTGTGTAGCCTCTGGATTCACCCTCAGTAGCTATGGCATGCACTGGGTCC
GCCAGGCTCTAGGCAAGGGGCTGGAGTGGGTGGCAGTGACATCATATGATGGAAGT
AAAAAAGACTATGCAGACTCCGTGAAGGGCCGATTCACCATCTCCAGAGACAATTC
CAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCTGAGGACACGGCTGTGT
ATTACTGTGTGAGCGAAGGATATTGTGATAGTACTAGTTGCTATAAGTACTACTACT
ACGGTATGGACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCTTCA3' **(SEQ ID NO:53)**

Amino Acid sequence of heavy chain variable region:

QVQLVESGGGVVQPGRSLRLSCVASGFTLSSYGMHWVRQALGKGLEWVAVTSYDGSK
KDYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCVSEGYCDSTSCYKYYYYG
MDVWGQGTTVTVSS **(SEQ ID NO:17)**

Nucleotide sequence of light chain variable region:

5'GATATTGTGATGACTCAGTCTCCACTCTCCCTGCCCGTCACCCCTGGAGAGCCGGC
CTCCATCTCCTGCAGGTCTAGTCAGAGCCTCCTATATAGAAATGGAAACAACTATTT
GGATTGGTATCTGCAGAGGCCAGGGCAGTCTCCACAACTCCTGATCTATTTGGGTTC
TAATCGGGCCTCCGGGGTCCCTGACAGGTTCAGTGGCAGTGGATCAGGCACAGATTT
TACACTGAAAATCAGCAGAGTGGAGGCTGAGGATGTTGGGGTTTATTACTGCATGC
AGGCTCTACAAACTCCTCGGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAA3'
(SEQ ID NO:52)

Amino Acid sequence of light chain variable region:

DIVMTQSPLSLPVTPGEPASISCRSSQSLLYRNGNNYLDWYLQRPGQSPQLLIYLGSNRA
SGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCMQALQTPRTFGQGTKVEIK **(SEQ ID NO:32)**

FIG. 3J

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342

Nucleotide sequence of heavy chain variable region:

5'CAGGTGCAGCTGGTGGAGTCTGGGGGAGGCGTGGTCCAGCCTGGGAGGTCCCTGA
GACTCTCCTGTGTAGCCTCTGGATTCACCCTCAGTAGCTATGGCATGCACTGGGTCC
GCCAGGCTCCAGGCAAGGGGCTGGAGTGGGTGGCAGTGACATCATATGATGGAAGT
AAAAAAGACTATGCAGACTCCGTGAAGGGCCGATTCACCATCTCCAGAGACAATTC
CAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCTGAGGACACGGCTGTGT
ATTACTGTGTGAGCGAAGGATATTGTGATAGTACTAGCTGCTATAAGTACTACTACT
ACGGTATGGACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCTTCA3' **(SEQ ID NO:55)**

Amino Acid sequence of heavy chain variable region:

QVQLVESGGGVVQPGRSLRLSCVASGFTLSSYGMHWVRQAPGKGLEWVAVTSYDGSK
KDYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCVSEGYCDSTSCYKYYYYG
MDVWGQGTTVTVSS **(SEQ ID NO:16)**

Nucleotide sequence of light chain variable region:

5'GATATTGTGATGACTCAGTCTCCACTCTCCCTGCCCGTCACCCCTGGAGAGCCGGC
CTCCATCTCCTGCAGGTCTAGTCAGAGCCTCCTATATAGAAATGGAAACAACTATTT
GGATTGGTATCTGCAGAGGCCAGGGCAGTCTCCACAACTCCTGATCTATTTGGGTTC
TAATCGGGCCTCCGGGGTCCCTGACAGGTTCAGTGGCAGTGGATCAGGCACAGATTT
TACACTGAAAATCAGCAGAGTGGAGGCTGAGGATGTTGGGGTTTATTACTGCATGC
AGGCTCTACAAACTCCTCGGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAA3'
(SEQ ID NO:54)

Amino Acid sequence of light chain variable region:

DIVMTQSPLSLPVTPGEPASISCRSSQSLLYRNGNNYLDWYLQRPGQSPQLLIYLGSNRA
SGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCMQALQTPRTFGQGTKVEIK **(SEQ ID NO:31)**

FIG. 3K

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13.1.2

Nucleotide sequence of heavy chain variable region:
CAGGTGCAGCTGGTGGAGTCTGGGGGAGGCGTGGTCCAGC
CTGGGAGGTCCCTGAGACTCTCCTGTGCAGCGTCTGGATT
CACCTTCAGTAGCTATGGCATGCACTGGGTCCGCCAGGCT
CCAGGCAAGGGGCTGGAGTGGGTGGCAGTTATATGGTATG
ATGGAAGTAATAAATACTATGTAGACTCCGTGAAGGGCCG
ATTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTAT
CTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCTGTGT
TGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA
GCCTCCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCT
CTAGCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTG
CCTGGTCAAGGACTACTTCCCCGAACCGGTGACGGTGTCG
TGGAACTCAGGCGCCCTGACCAGCGGCGTGCA **(SEQ ID NO: 137)**

Amino Acid sequence of heavy chain variable region:
QVQLVESGGGVVQPGRSLRLSCAASGFTFS
SYGMHWVRQAPGKGLEWVAVIWYDGSNKYY
VDSVKGRFTISRDNSKNTLYLQMNSLRAED
TAVYYCARDGWQQLAPFDYWGQGTLVTVSS
ASTKGPSVFPLAPSSKSTSGGTAALGCLVK
DYFPEPVTVSWNSGALTSGV **(SEQ ID NO: 138)**

Nucleotide sequence of light chain variable region:
GATATTGTGATGACCCAGACTCCACTCTCCTCACCTGTCA
CCCTTGGACAGCCGGCCTCCATCTCCTGCAGGTCTAGTCA
AAGCCTCGTGCATAGTGATGGAAACACCTACTTGAGTTGG
CTTCACCAGAGGCCAGGCCAGCCTCCAAGACTCCTAATTT
ATAAGATTTCTAACCGGTTCTCTGGGGTCCCAGACAGATT
CAGTGGCAGTGGGGCAGGGACAGCTTTCACACTGAAAATC
AGCAGGGTGGAAGCTGAGGATGTCGGGGTTTATTACTGCA
TGCAAGCTACACAACTTCCTCGGACGTTCGGCCAAGGGAC
CAAGGTGGAAATCAAACGAACTGTGGCTGCACCATCTGTC
TTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAA
CTGCTAGCGTTGTGTGCCTGCTGAATAACTTCTATCCCAG
AGAGGCCAAAGTACAGTGGAAGGTGGATAACGCCCTCCAA
TCGGGTAACTCCCAGG **(SEQ ID NO: 139)**

Amino Acid sequence of light chain variable region:
DIVMTQTPLSSPVTLGQPASISCRSSQSLV
HSDGNTYLSWLHQRPGQPPRLLIYKISNRF
SGVPDRFSGSGAGTAFTLKISRVEAEDVGV
YYCMQATQLPRTFGQGTKVEIKRTVAAPSV
FIFPPSDEQLKSGTASVVCLLNNFYPREAK
VQWKVDNALQSGNSQ **(SEQ ID NO: 140)**

FIG. 3L

FIG. 4

HeavyChain					SEQ ID NO
Chain Name	V	D	J	FR1	
		Germline		QVQLVESGGGVVQPGRSLRLSCAAS	**SEQ ID NO 141**
13_1_2	VH3-33	D6-13	JH4B	----------	**SEQ ID NO 142**

				SEQ ID NO
	CDR1	FR2	CDR2	
	GFTFSSYGMH	WVRQAPGKGLEWVA	VIWYDGSNKYYADSVKG	**SEQ ID NO 141**
13_1_2	---------	----------	----------V-----	**SEQ ID NO 142**

				SEQ ID NO
	FR3	CDR3	J	
	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR		WGQGTLVTVSSA	**SEQ ID NO 141**
13_1_2	----------	DGWQQLAPFDY	----------	**SEQ ID NO 142**

FIG. 5

LightChain				SEQ ID NO
Chain Name	V	J	FR1	
		Germline	DIVMTQTPLSSPVTLGQPASISC	**SEQ ID NO 143**
13_1_2	A23	JK1	----------	**SEQ ID NO 144**

				SEQ ID NO
	CDR1	FR2	CDR2	
	RSSQSLVHSDGNTYLS	WLQQRPGQPPRLLIY	KISNRFS	**SEQ ID NO 143**
13_1_2	----------	--H----------	-------	**SEQ ID NO 144**

					SEQ ID NO
	FR3	CDR3	J		
	GVPDRFSGSGAGTDFTLKISRVEAEDVGVYYC	MQATQFPRT	FGQGTKVEIKR		**SEQ ID NO 143**
13_1_2	----------A----------	-----L---	----------		**SEQ ID NO 144**

FIG 6A1	FIG 6A2
FIG 6A3	FIG 6A4

FIG. 6

Well	Single Cell	V Heavy/D/J	FR1
-	-	Germline	QVQLVESGGGVVQPGRSLRLSCAASGFTFS
174F1	131	VH-33/D3-9/JH4b	----------S----------R
-	-	Germline	QVQLQQSGPGLVKPSQTLSLTCAISGDSVS
125D10	170	VH6-01/D2-21/JH4b	----------
182D5	150		----------
-	-	Germline	QVQLQESGPFLVKPSQTLSLTCTVSGGSIS
172B12	095	VH4-31/D4/JH5b	----------
-	-	Germline	EVQLLESGGGLVQPGGSLRLSCAASGFTFS
138D2	250	VH3-23/D6-19/JH5b	-G--------W----E----------
182A5	139		---V----------
-	-	Germline	QVQLVESGGGVVQPGRSLRLSCAASGFTFS
190D7	211	VH3-30/D2-15/JH6b	----------V-----L-
129A7	124		----------L-
-	-	Germline	QVQLVESGGGVVQPGRSLRLSCAASGFTFS
141A10	318	VH3-30/D2-2/JH6b	----------V-----L-
124D4	342		----------V-----L-
132D8	333		----------V-----L-

FIG. 6A1

FIG. 6A[2]

CDR1	FR2	SEQ ID NO.
SYGMH	WVRQAPGKGLEWVA	1
N----	--------------	2
SNSAAWN	WIRQSPSRGLEWLG	3
--N----	------A-------	4
-Y-S---	--------------	5
SGGYYWS	WIRQHPGKGLEWIG	6
-------	--------------	7
SYAMS	WVRQAPGKGLEWVS	8
-----	--------------	9
-----	--------------	10
SYGMH	WVRQAPGKGLEWVA	11
-----	--------------	12
-----	--------------	13
SYGMH	WVRQAPGKGLEWVA	14
-----	--------------	15
-----	--------------	16
-----	-----L--------	17

Well	Single Cell	CDR2	FR3
-	-	VIWYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR
174F1	131	-------D-------R-	----------
-	-	RTYYRSKWYNDYAVSVKS	RITINPDTSKNQFSLQLNSVTPEDTAVYYCAR
125D10	170	----------V-----	----------V-
182D5	150	-A-H--R--YE-------	--N-T----------
-	-	YIYYSGSTYYNPSLKS	RVTISVDTSKNQFSLKLSSVTAADTAVYYCAR
172B12	095	F---R-N---------	----------
-	-	AISGSGGSTYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK
138D2	250	---------N-------	----------V----V---------G
182A5	139	---------N-------	----------G
-	-	VISYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK
190D7	211	-T-----K-D-------	----------VS
129A7	124	-M-----KED-------	---------E----------VS
-	-	VISYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK
141A10	318	-T-----K-D-------	----------VS
124D4	342	-T-----K-D-------	----------VS
132D8	333	-T-----K-D-------	----------VS

FIG. 6A3

FIG. 6A4

CDR3	FR4	SEQ ID NO.
	WGQGTLVTVSS	1
DGYDILTGNPRDFDY	----------	2
	WGQGTLVTVSS	3
ATAFDY	----------	4
GSR---	----------	5
	WGQGTLVTVSS	6
DGYCSRTGCYGGWFDP	----------	7
	WGQGTLVTVSS	8
SSGWSEY	----------	9
-------	----------	10
	WGQGTLVTVSS	11
EGYCSSSSCYKYYYYGMDV	----------	12
------R----------	----------	13
	WGQGTLVTVSS	14
EGYCDSSSCYKYYYYGMDV	----------	15
------T----------	----------	16
------T----------	----------	17

FIG 7A1	FIG 7A2
FIG 7A3	FIG 7A4

FIG. 7

Well	Single Cell	V Kappa/J	FR1
-	-	Germline	DIVMTQTPLSSPVTLGQPASISC
174F1	131	A23(VK2)/JK1	-T---------H----------
125D10	170		A--L-------------------
182D5	150		----------------------
-	-	Germline	DIVMTQTPLSSPVTLGQPASISC
172B12	095	A23(Vk2)/JK5	----------------------
-	-	Germline	DIQMTQSPSSLSASVGDRVTITC
182A5	139	A30(VK1)/JK4	----------------------
138D2	250		----------------S-----
-	-	Germline	DIVMTQSPLSLPVTPGEPASISC
190D7	211	A3(Vk2)/JK1	----------------------
129A7	123		----------------------
-	-	Germline	DIVMTQSPLSLPVTPGEPASISC
124D4	342	A3(Vk2)/JK1	----------------------
132D8	333		----------------------
141A10	318		----------------------

FIG. 7A1

FIG. 7A2

CDR1	FR2	SEQ ID NO.
RSSQSLVHSDGNTYLS	WLQQRPGQPPRLLIY	18
----------	----------	19
--------R-------	----------	20
--------R-------	----------	21
RSSQSLVHSDGNTYLS	WLQQRPGQPPRLLIY	22
------I-T---I---	----------	23
RASQGIRNDLG	WYQQKPGKAPKRLIY	24
--------N-A	----------	25
-T-----KN--	----------	26
RSSQSLLHSNGYNYLD	WYLQKPGQSPQLLIY	27
-------YR--N----	----R-----------	28
-------YR--N----	----------	29
RSSQSLLHSNGYNYLD	WYLQKPGQSPQLLIY	30
-------YR--N----	----R-----------	31
-------YR--N----	----R-----------	32
-------YR--N----	----R-----------	33

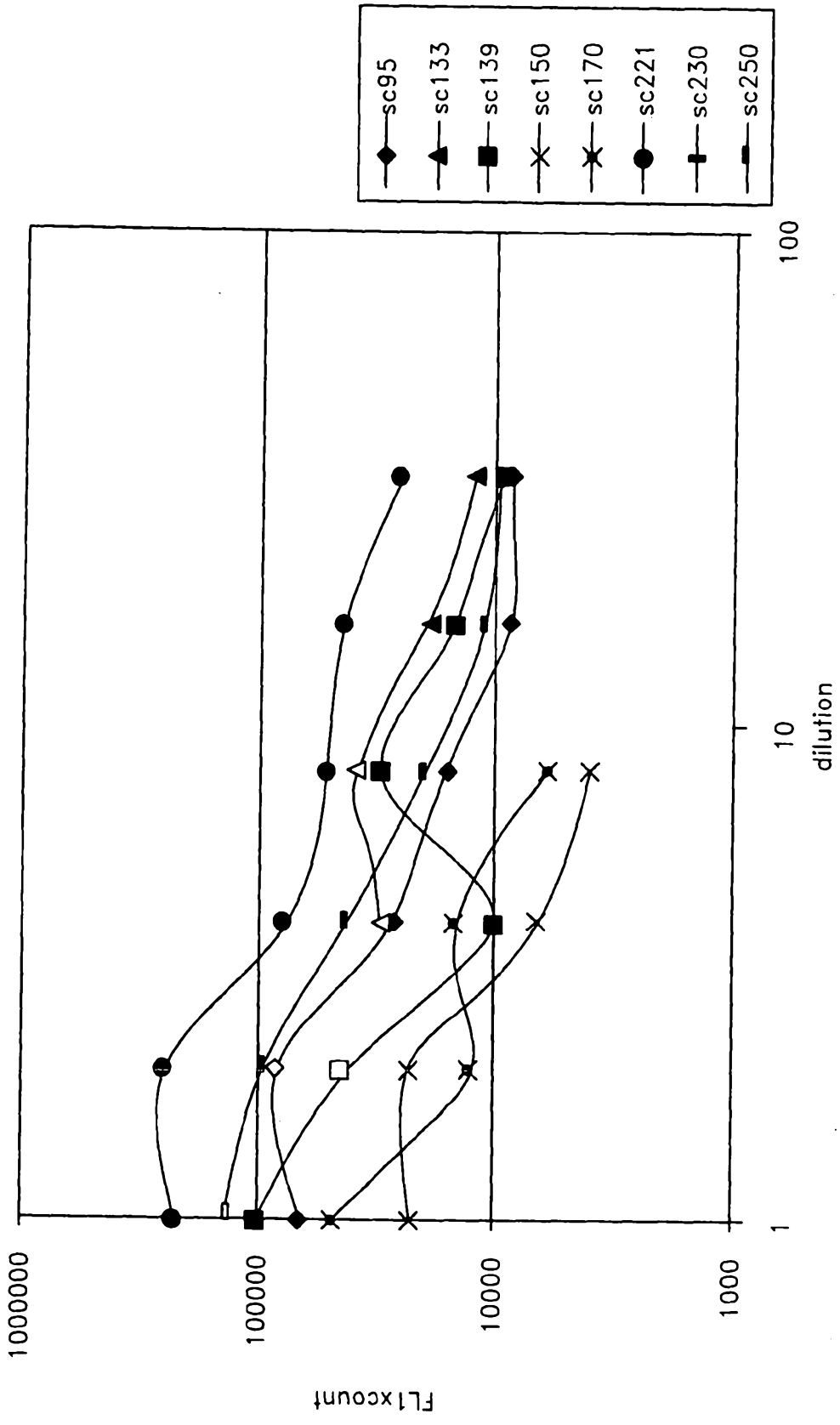
Well	Single Cell	CDR2	FR3
-	-	KISNRFS	GVPDRFSGSGAGTDFTLKISRVEAEDVGVYYC
174F1	131	R--R---	----------E----------
125D10	170	-------	----------PD-------
182D5	150	-------	----------I-F-
-	-	KISNRFS	GVPDRFSGSGAGTDFTLKISRVEAEDVGVYYC
172B12	095	-------	----------
-	-	AASSLQS	GVPSRFSGSGSGTEFTLTISSLQPEDFATYYC
182A5	139	---N---	------T----------IV----------
138D2	250	-------	----------R----------
-	-	LGSNRAS	GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC
190D7	211	-------	----------
129A4	123	-------	----------N----------H---
-	-	LGSNRAS	GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC
124D4	342	-------	----------
132D8	333	-------	----------
141A10	318	-------	----------

FIG. 7A3

FIG. 7A4

CDR3	FR4	SEQ ID NO.
MQATQFPWT	FGQGTKVEIK	18
--S-HV-R-	----------	19
-HT--L---	----------	20
-HT------	-----R----	21
MQATQFPIT	FGQGTRLEIK	22
--G------	----------	23
LQHNSYPLT	FGGGTKVEIK	24
---H-----	S---------	25
---H-----	-----R---R	26
MQALQTPWT	FGQGTKVEIK	27
-------R-	---------	28
-------R-	---------	29
MQALQTPWT	FGQGTKVEIK	30
-------R-	----------	31
-------R-	----------	32
-------R-	----------	33

FIG. 8


Native binding of recombinant EGFRvIII mAbs to NR6M cells
FL1xcount
1000000
100000
10000
1000
1
10
100
dilution
sc95
sc133
sc139
sc150
sc170
sc221
sc230
sc250

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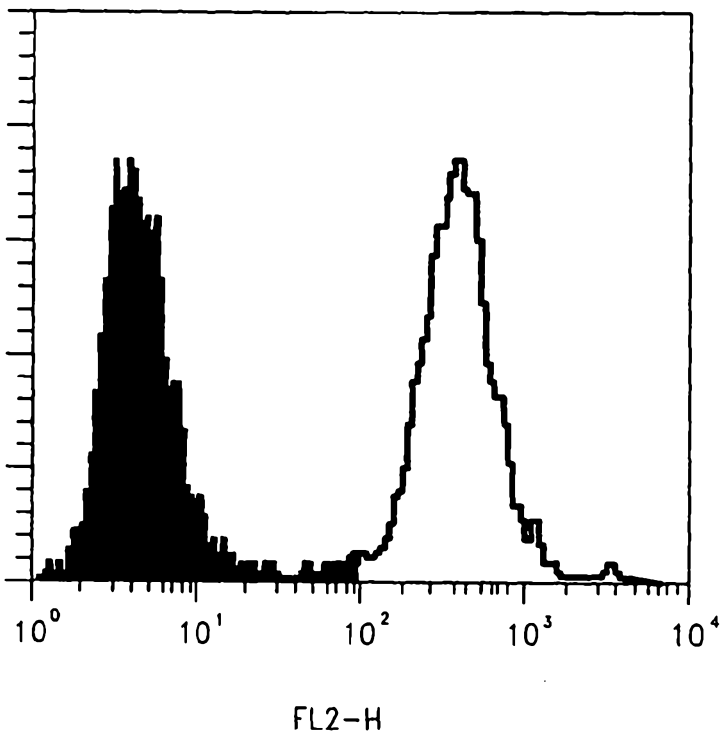

FIG. 9A

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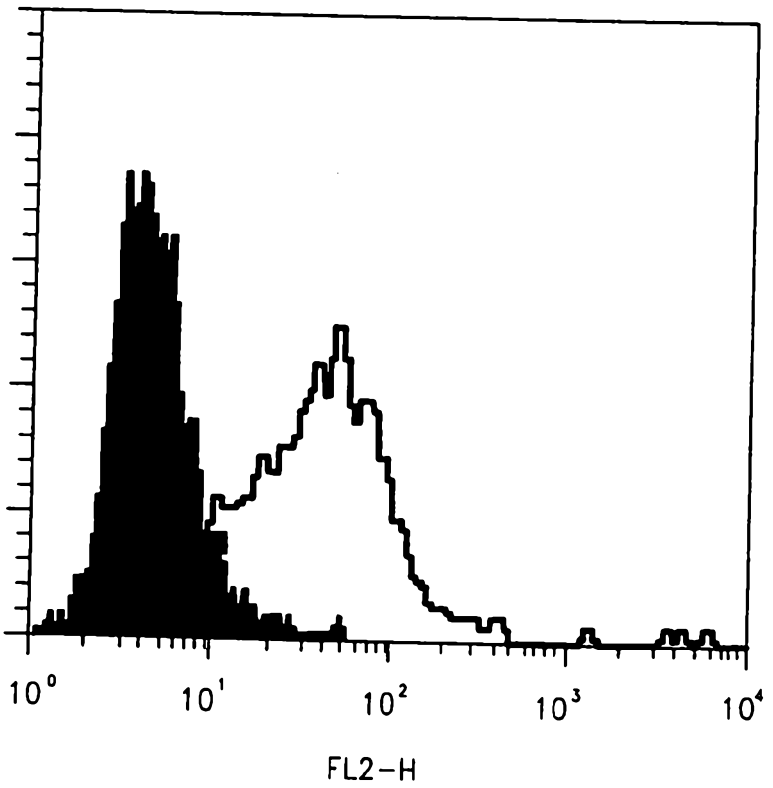

FIG. 9B

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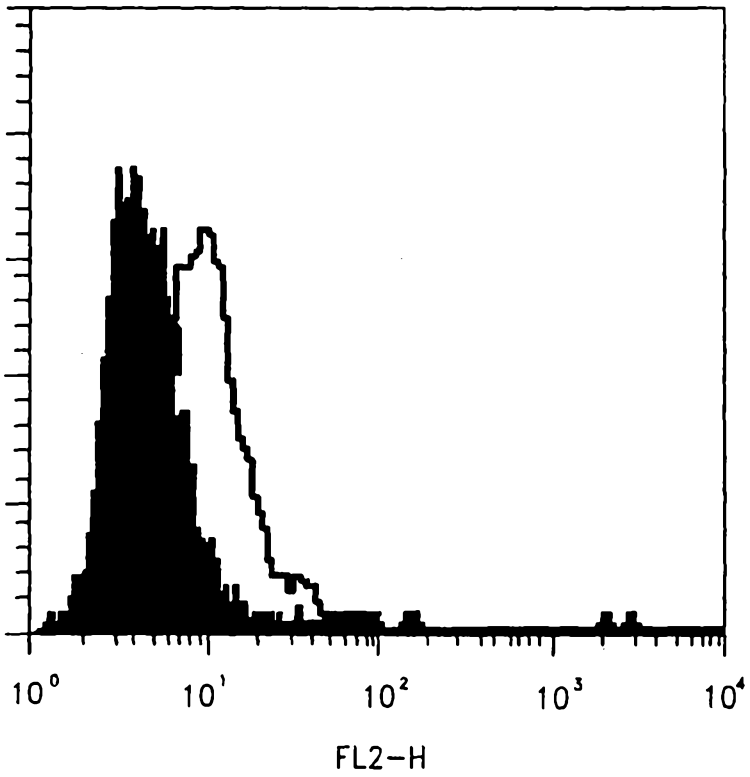

FIG. 9C

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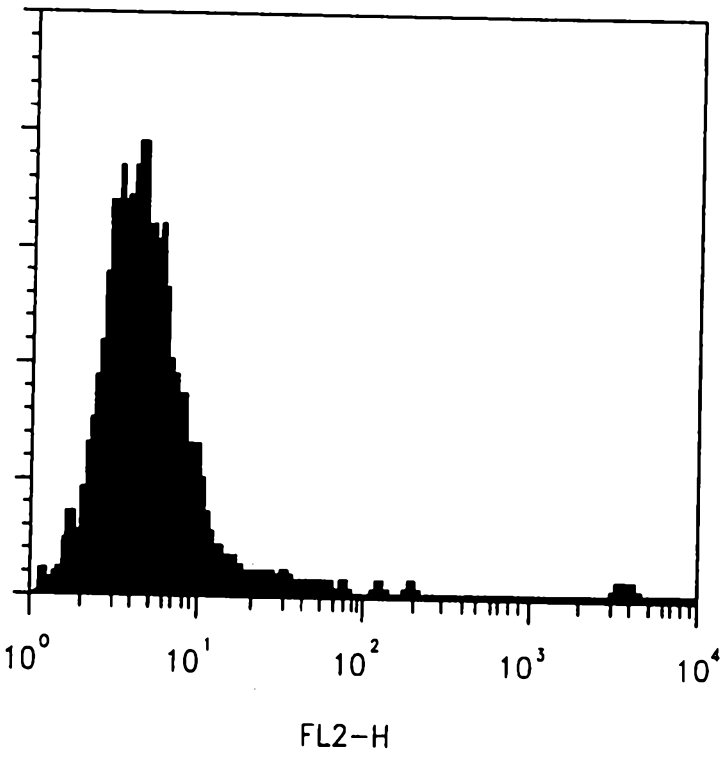

FIG. 9D

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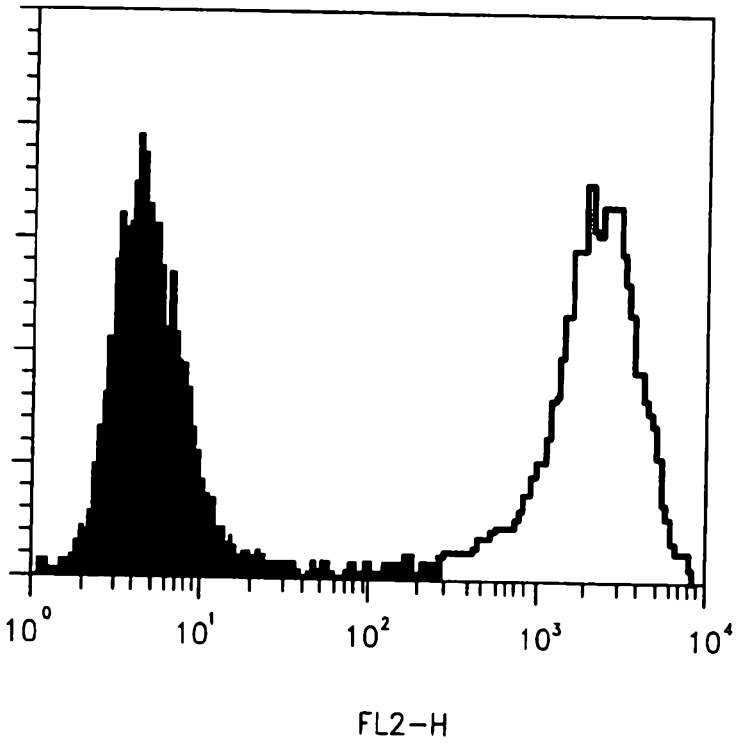

FIG. 9E

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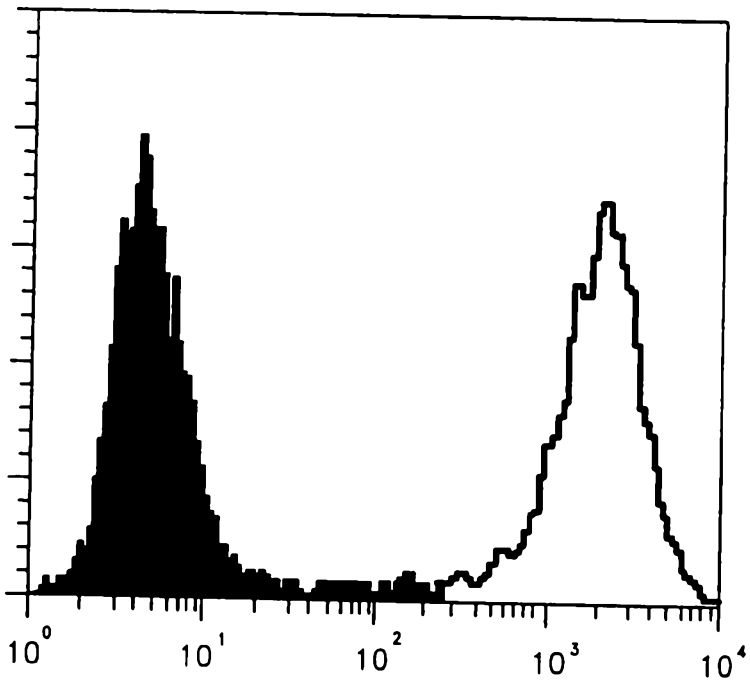

FIG. 9F

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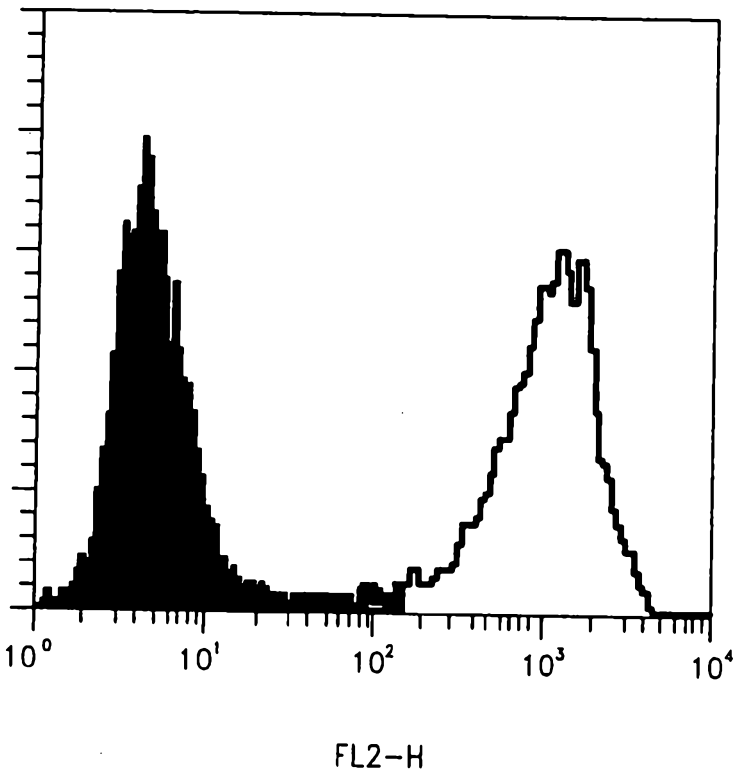

FIG. 9G

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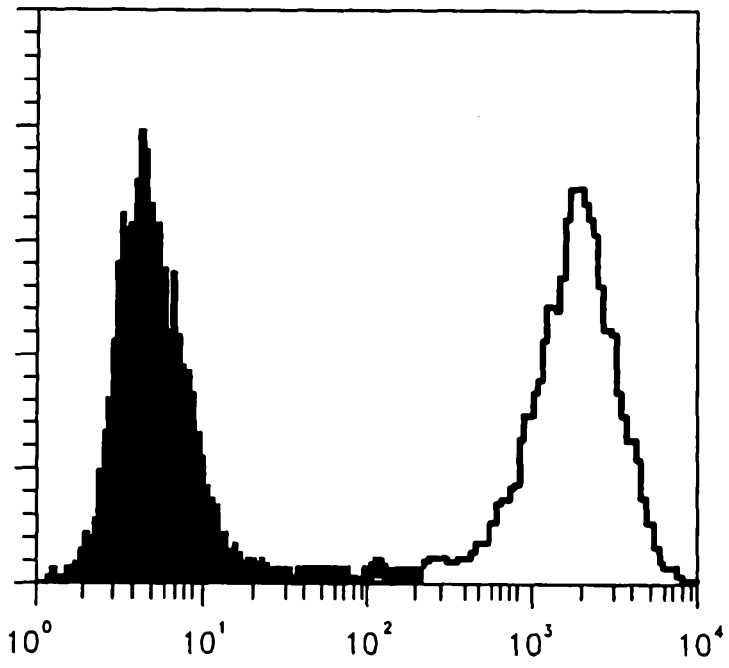

FIG. 9H

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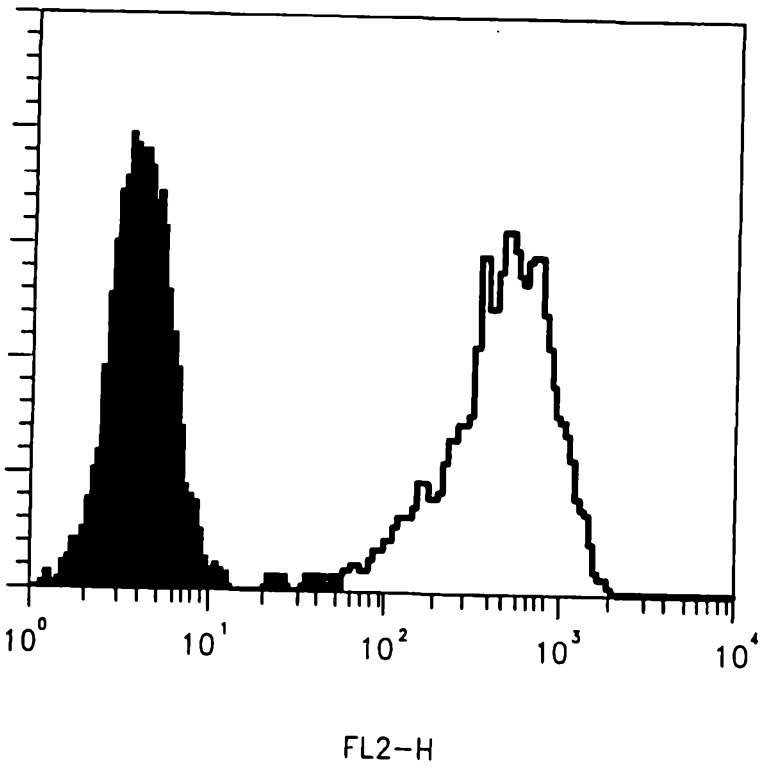

FIG. 9I

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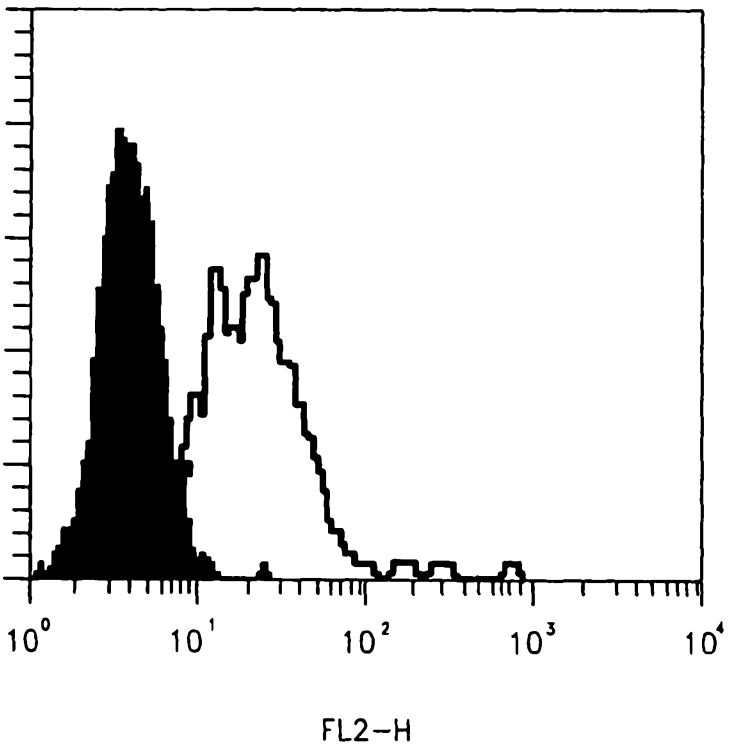

FIG. 9J

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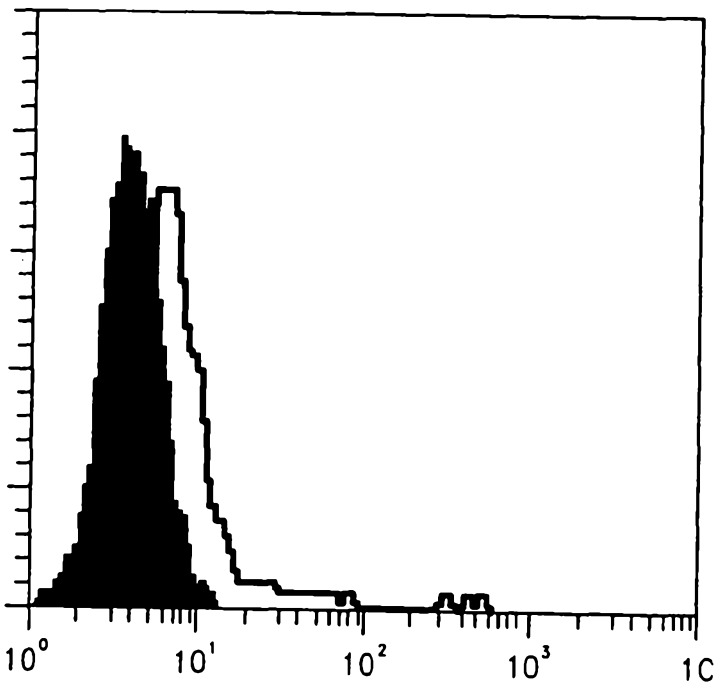

FIG. 9K

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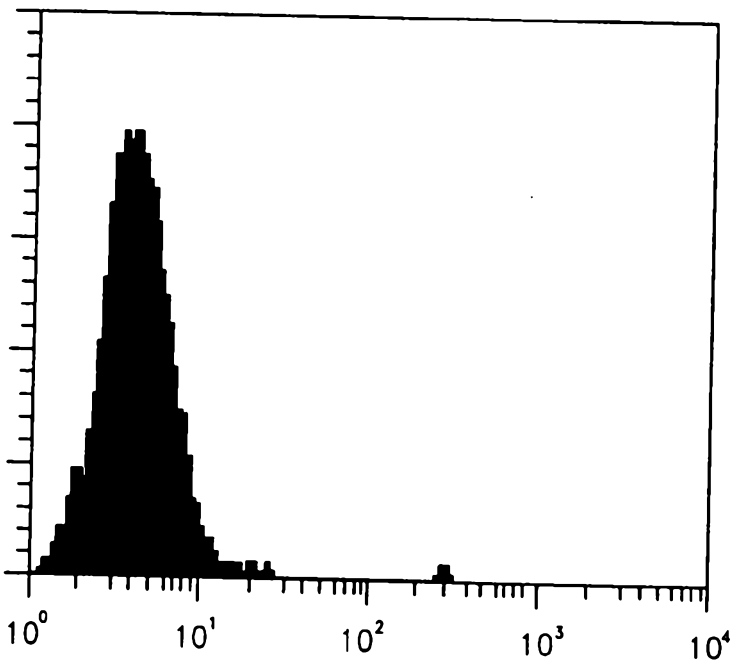

FIG. 9L

FIG. 9M

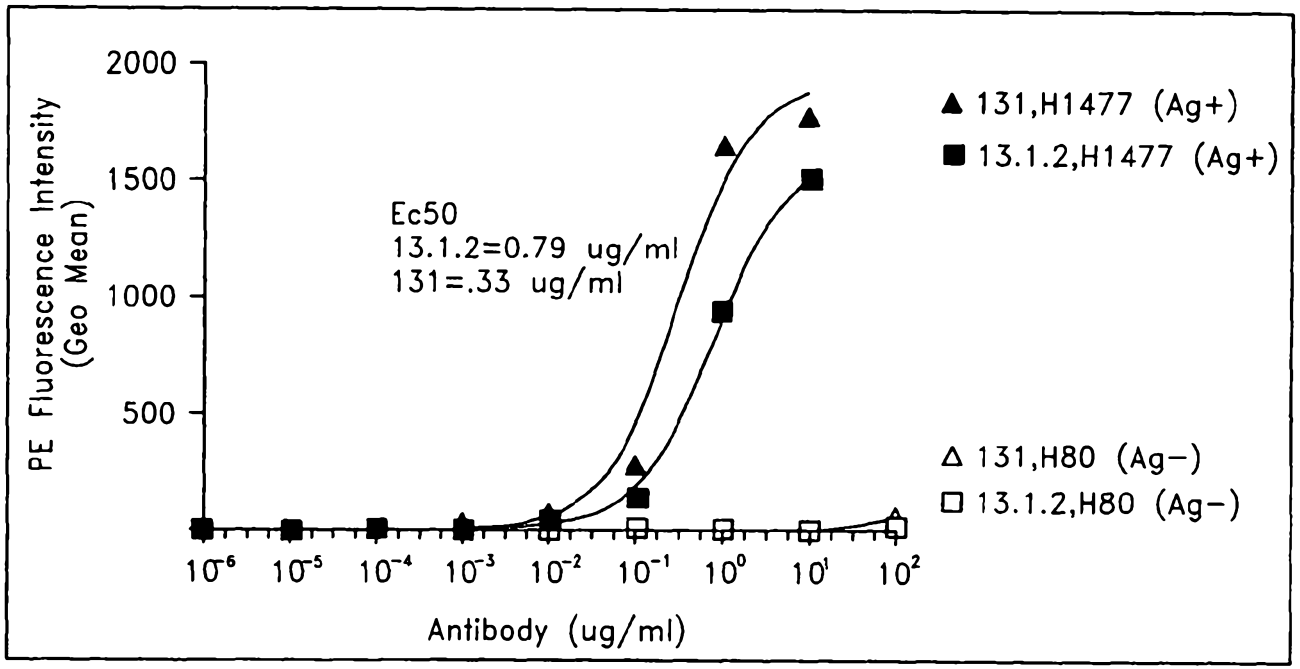
2000
1500
1000
500
PE Fluorescence Intensity (Geo Mean)
Ec50
13.1.2=0.79 ug/ml
131=.33 ug/ml
▲ 131,H1477 (Ag+)
■ 13.1.2,H1477 (Ag+)
△ 131,H80 (Ag−)
□ 13.1.2,H80 (Ag−)
10⁻⁶ 10⁻⁵ 10⁻⁴ 10⁻³ 10⁻² 10⁻¹ 10⁰ 10¹ 10²
Antibody (ug/ml)

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FIG. 9N

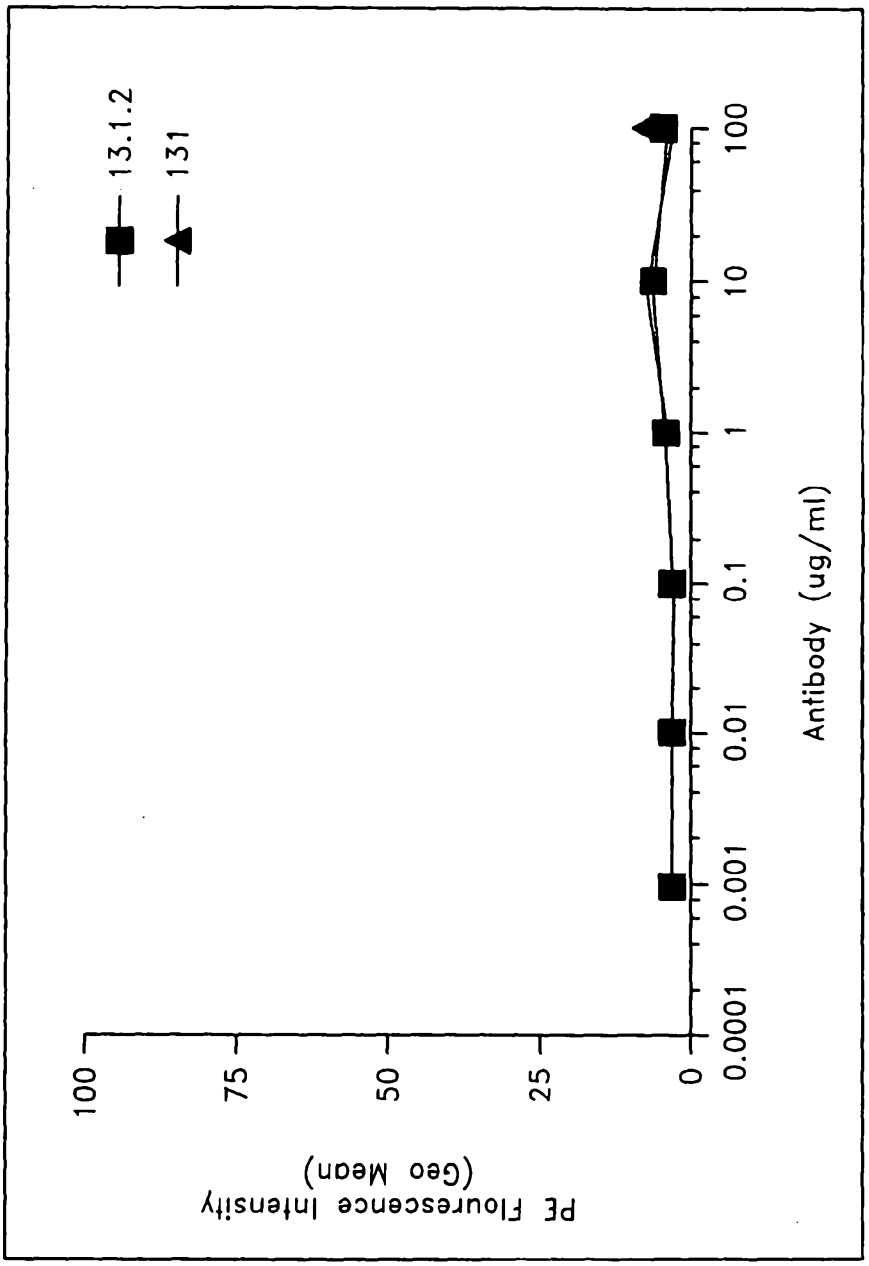

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FIG. 9O

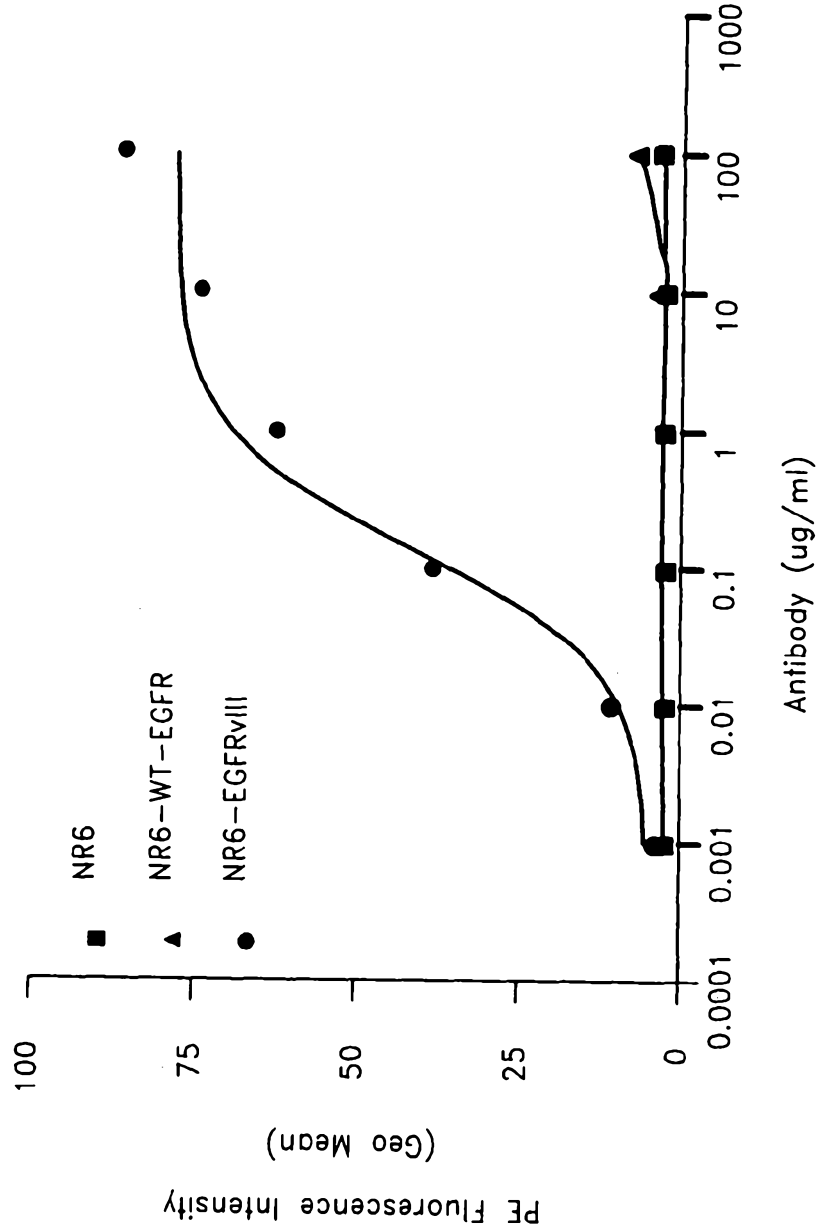

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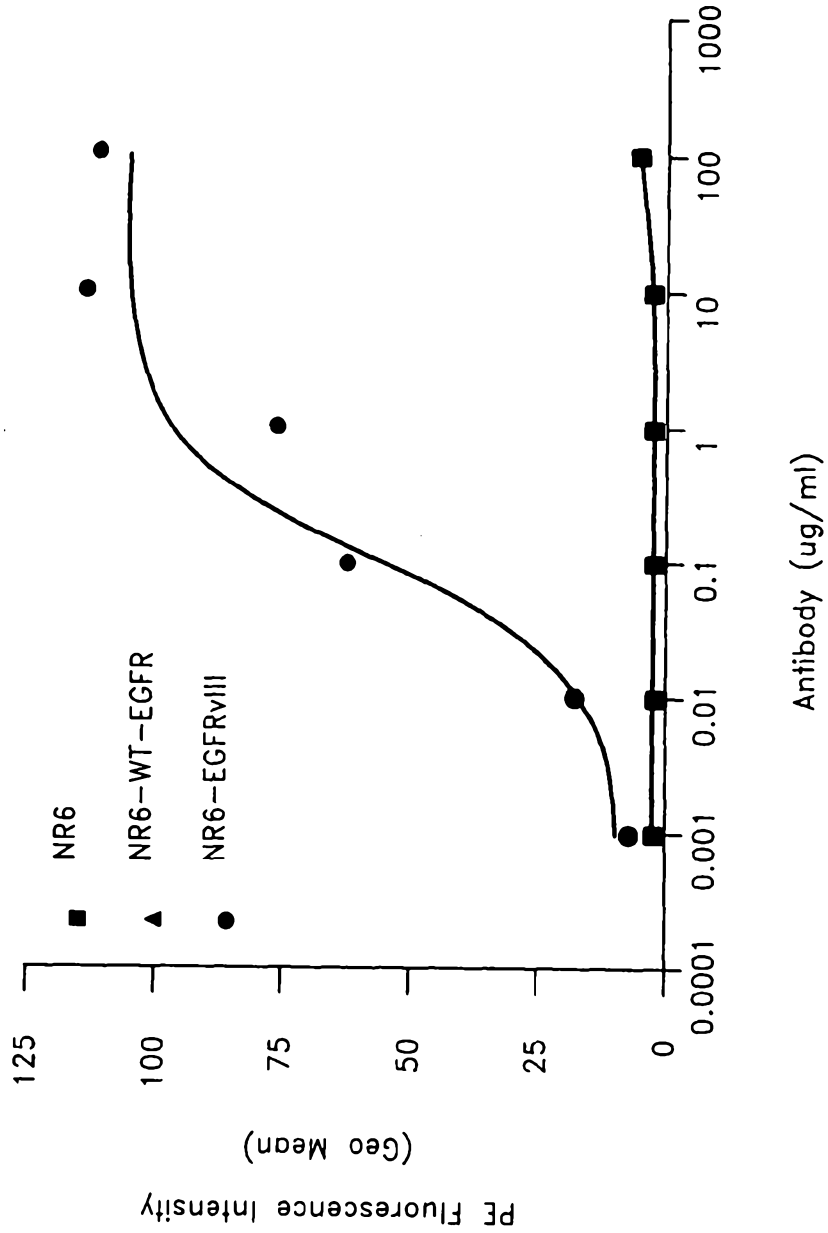
FIG. 9P



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ABX-EGF

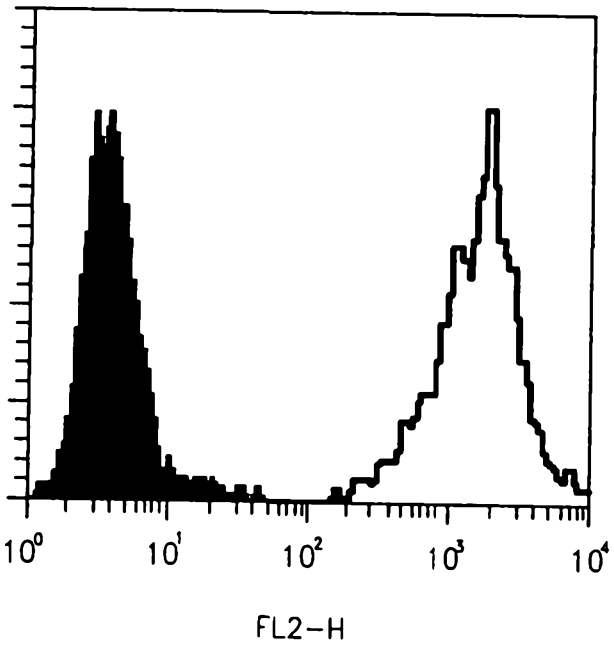

FIG. 10A

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XG1-131

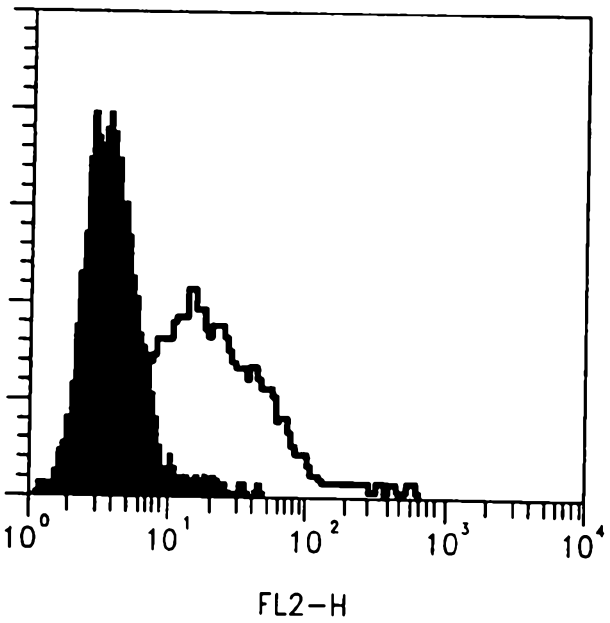

FIG. 10B

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XG1-139

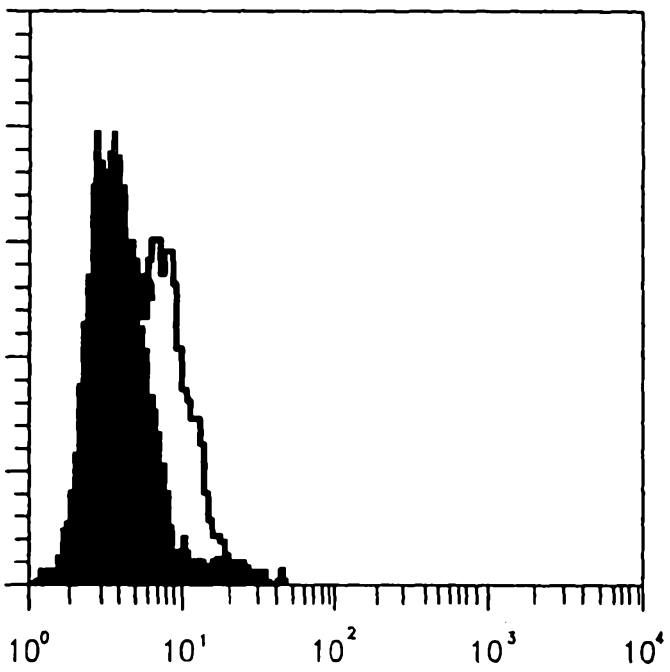

FIG. 10C

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dEGFR13.1.2

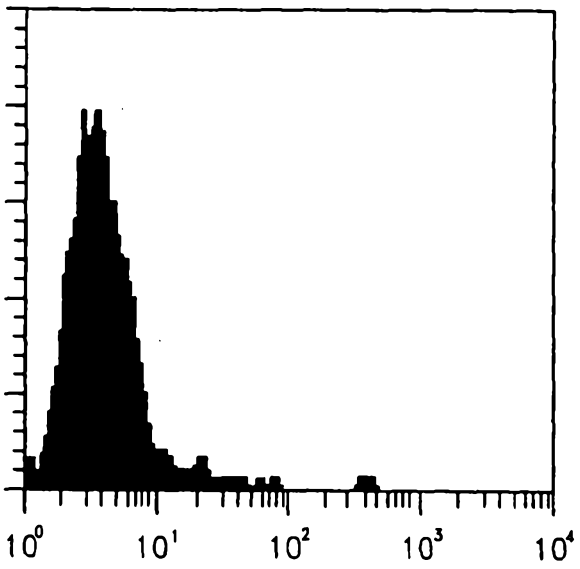

FIG. 10D

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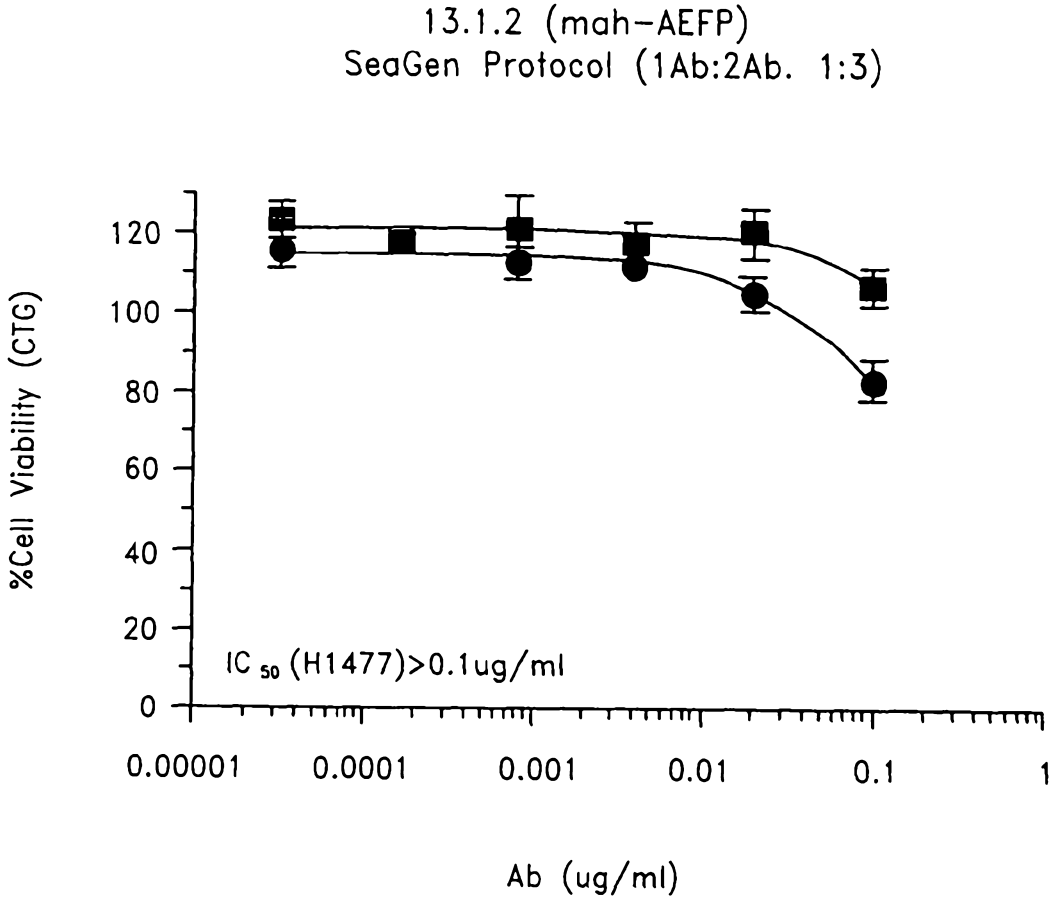

FIG. 11A

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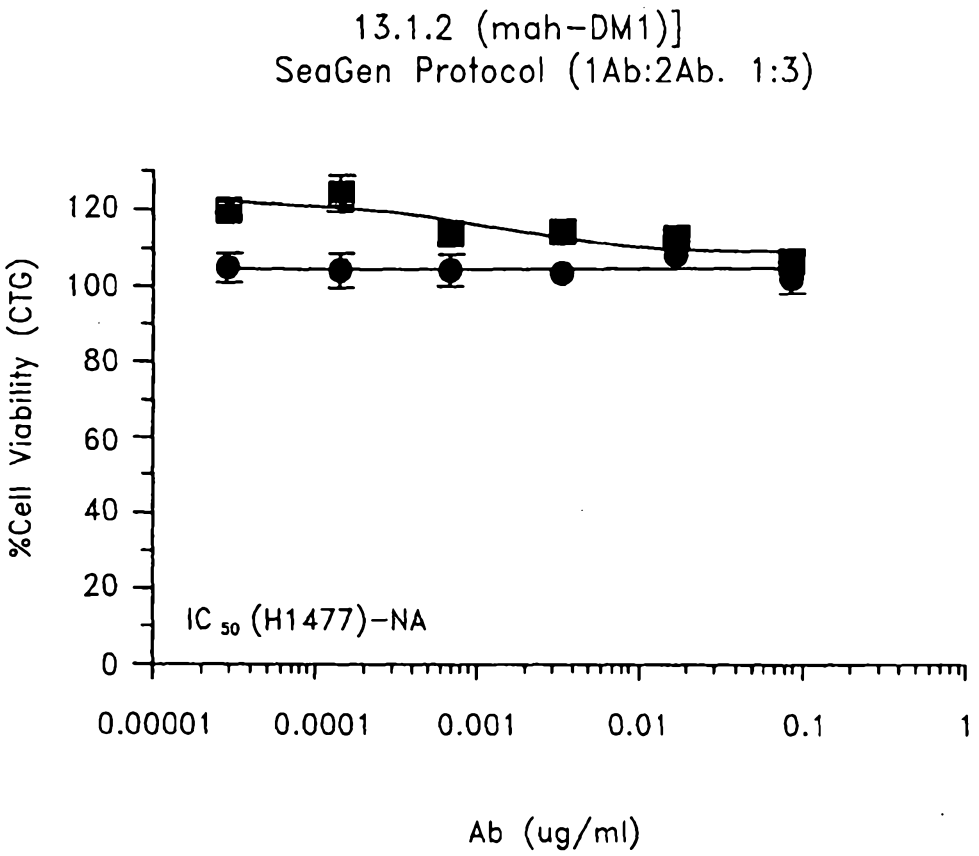

FIG. 11B

2011265359 20 Dec 2011

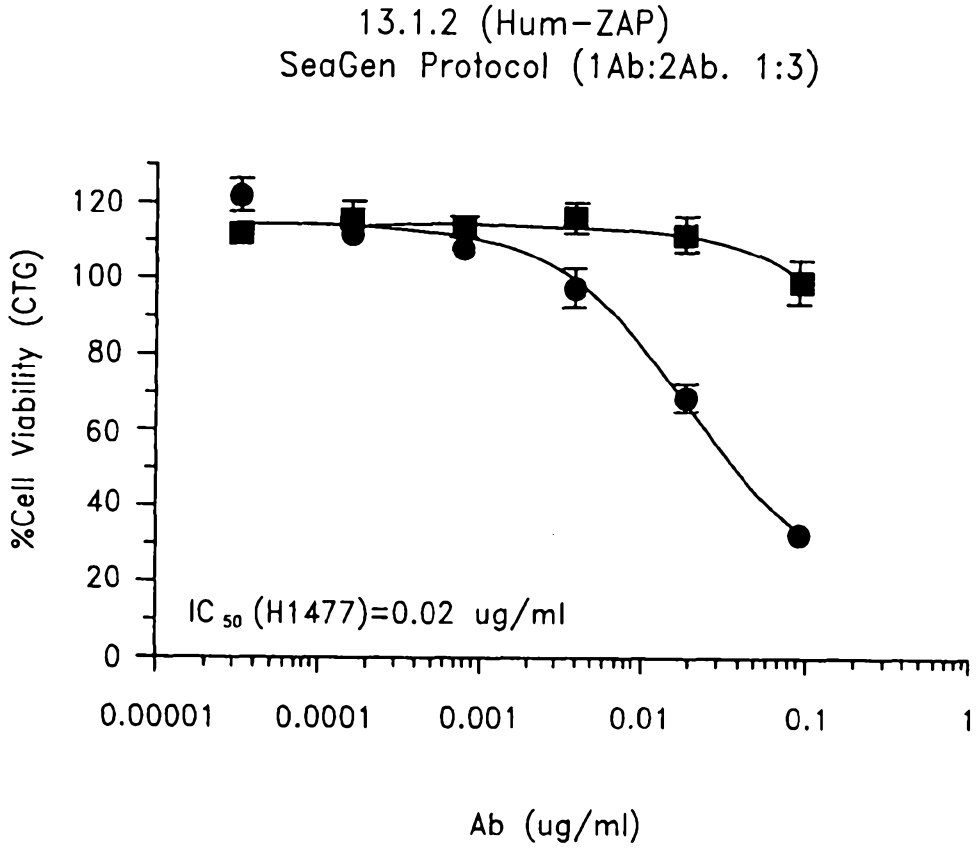

FIG. 11C

2011265359 20 Dec 2011

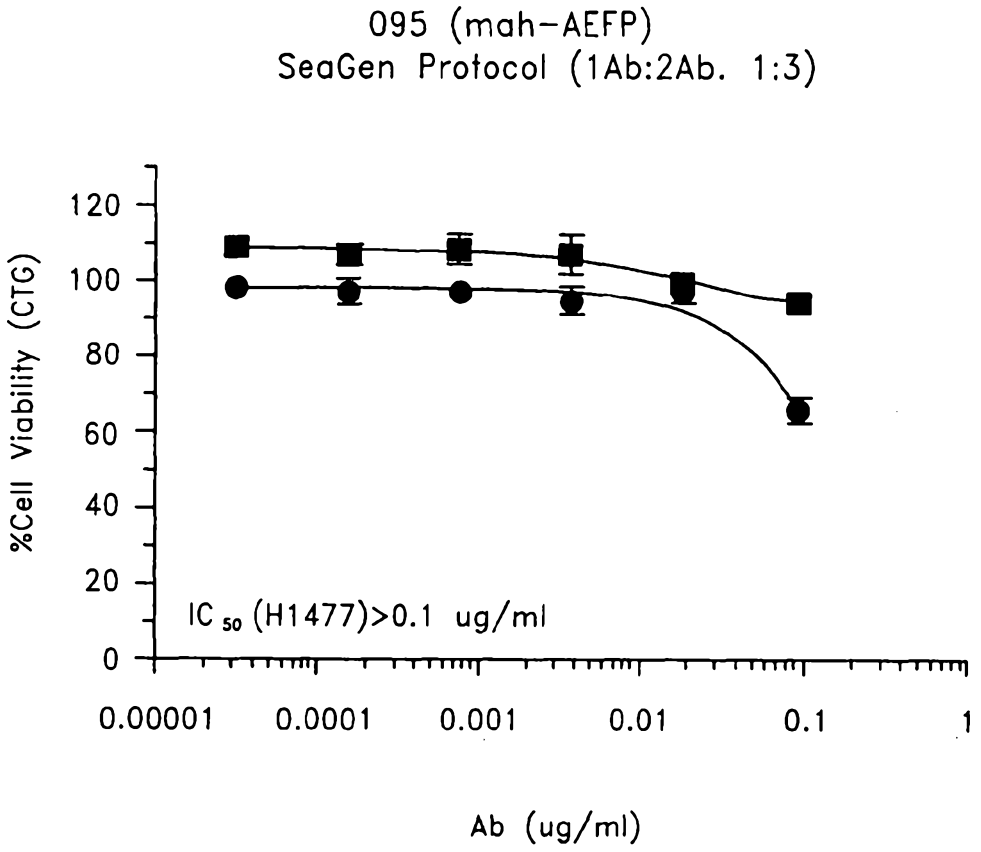

FIG. 11D

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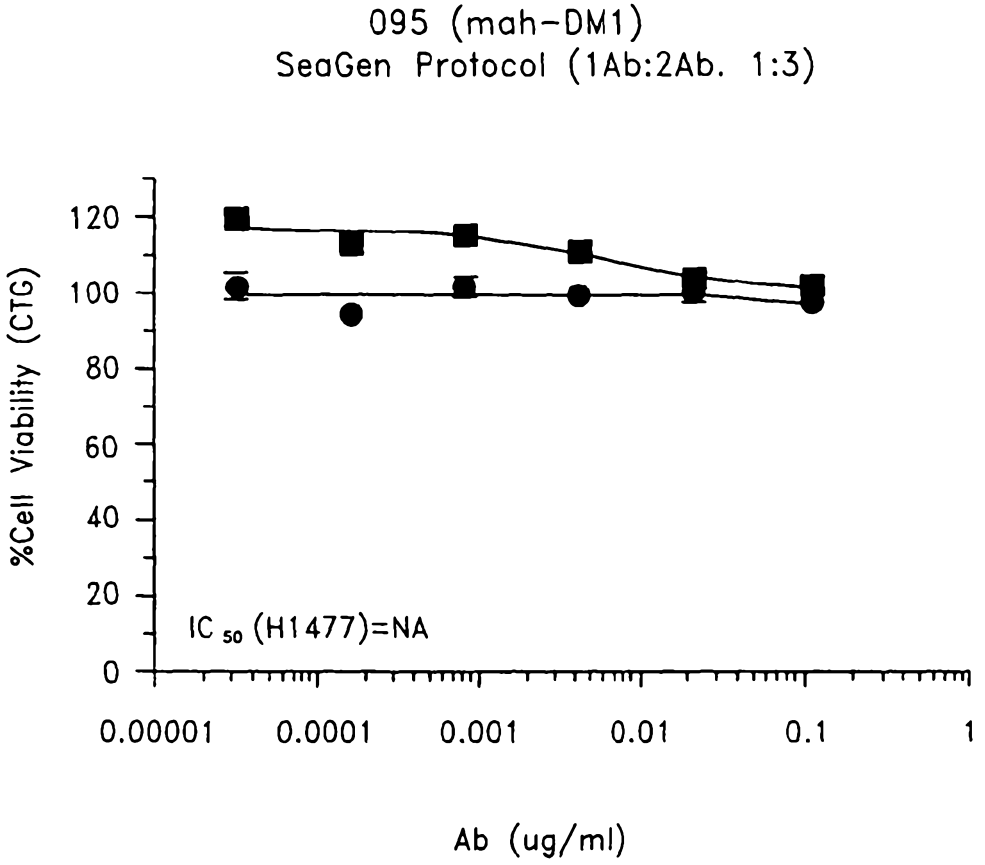

FIG. 11E

2011265359 20 Dec 2011

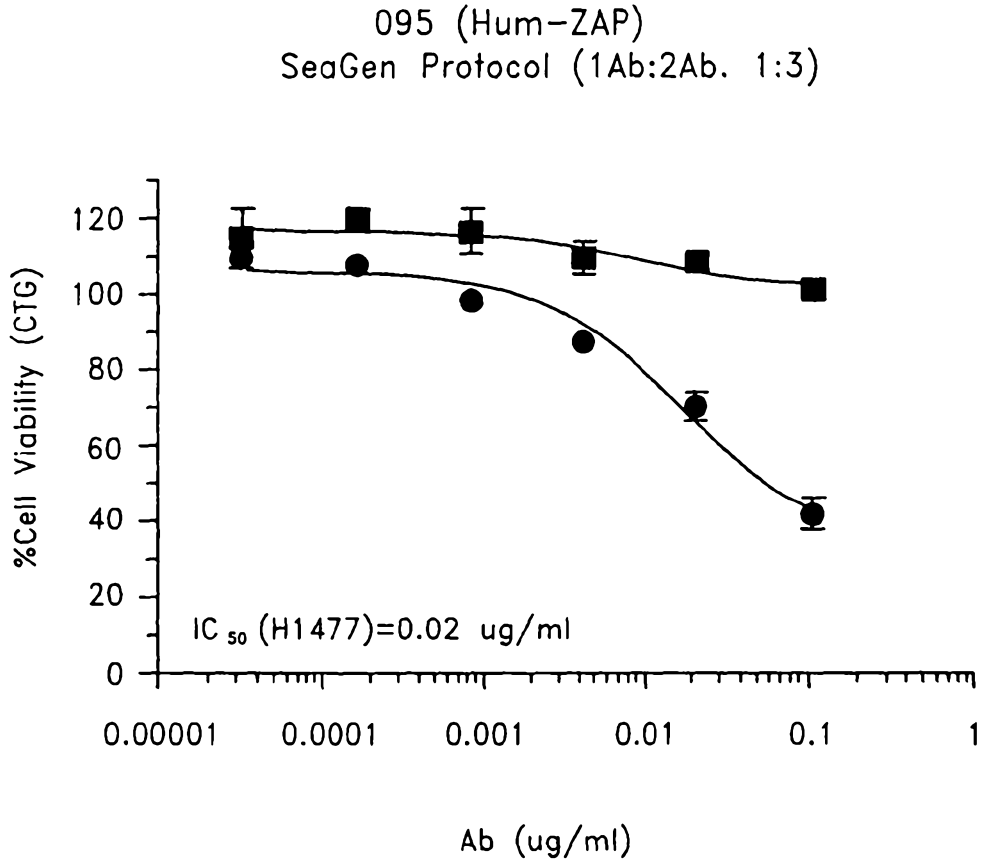

FIG. 11F

2011265359 20 Dec 2011

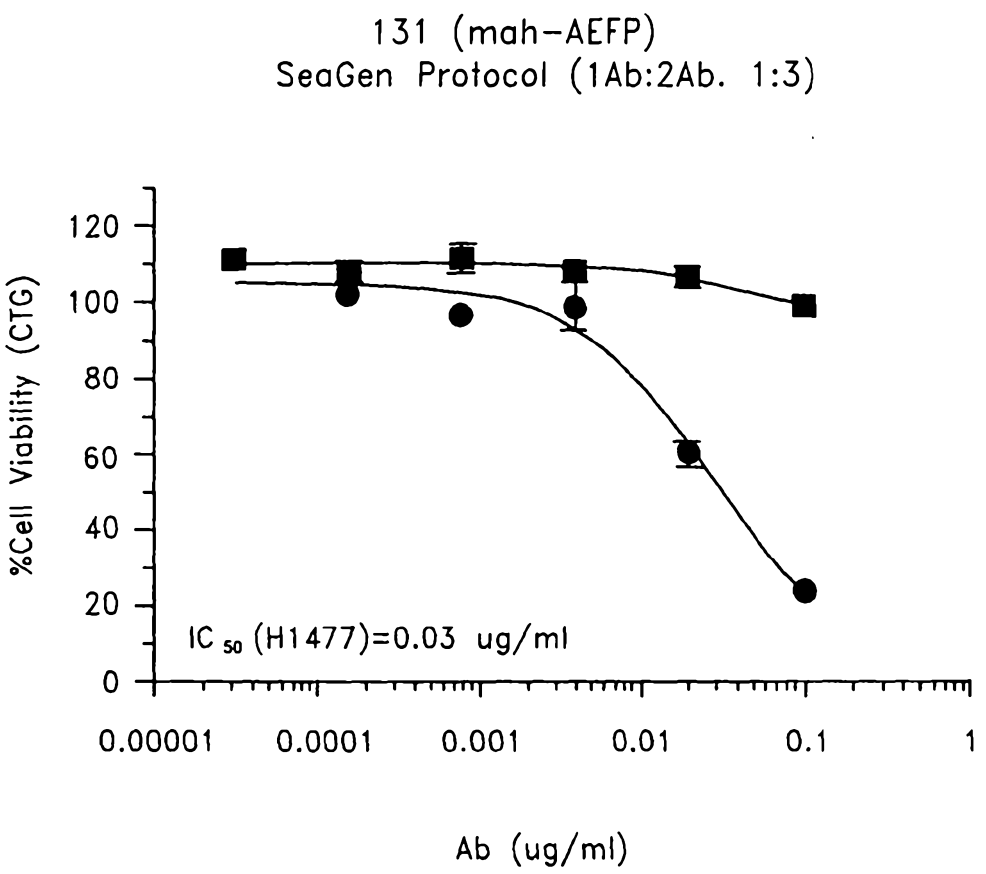

FIG. 11G

2011265359 20 Dec 2011

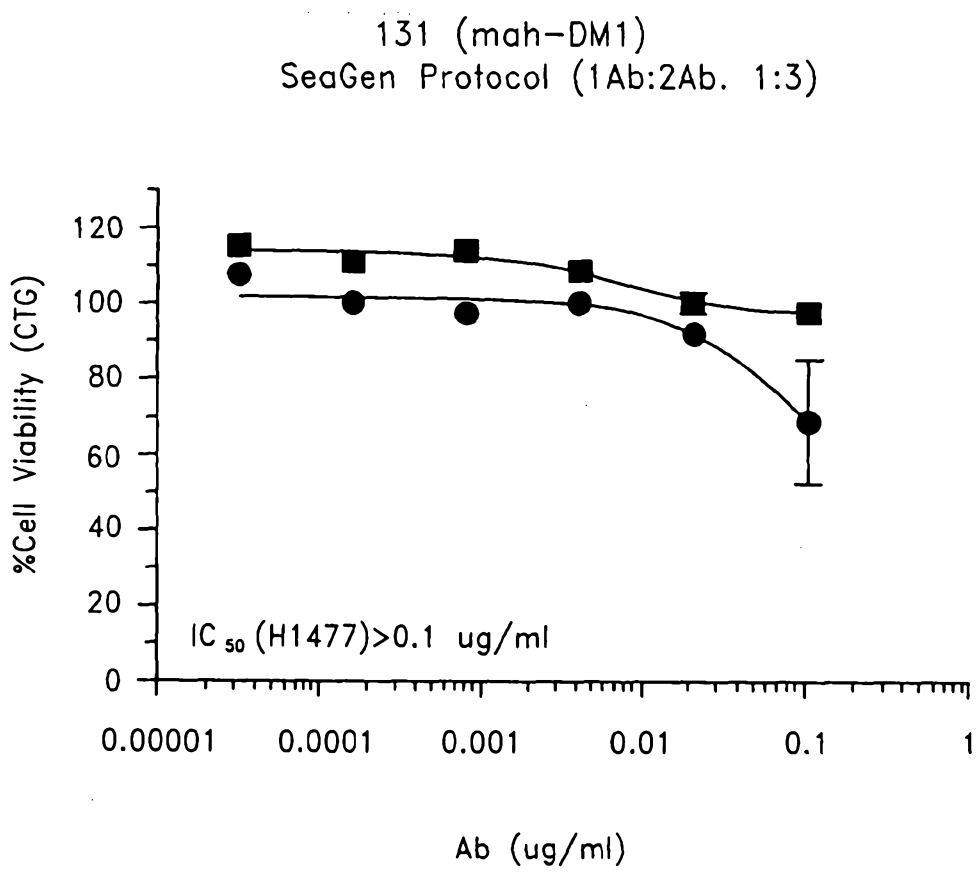

FIG. 11H

2011265359 20 Dec 2011

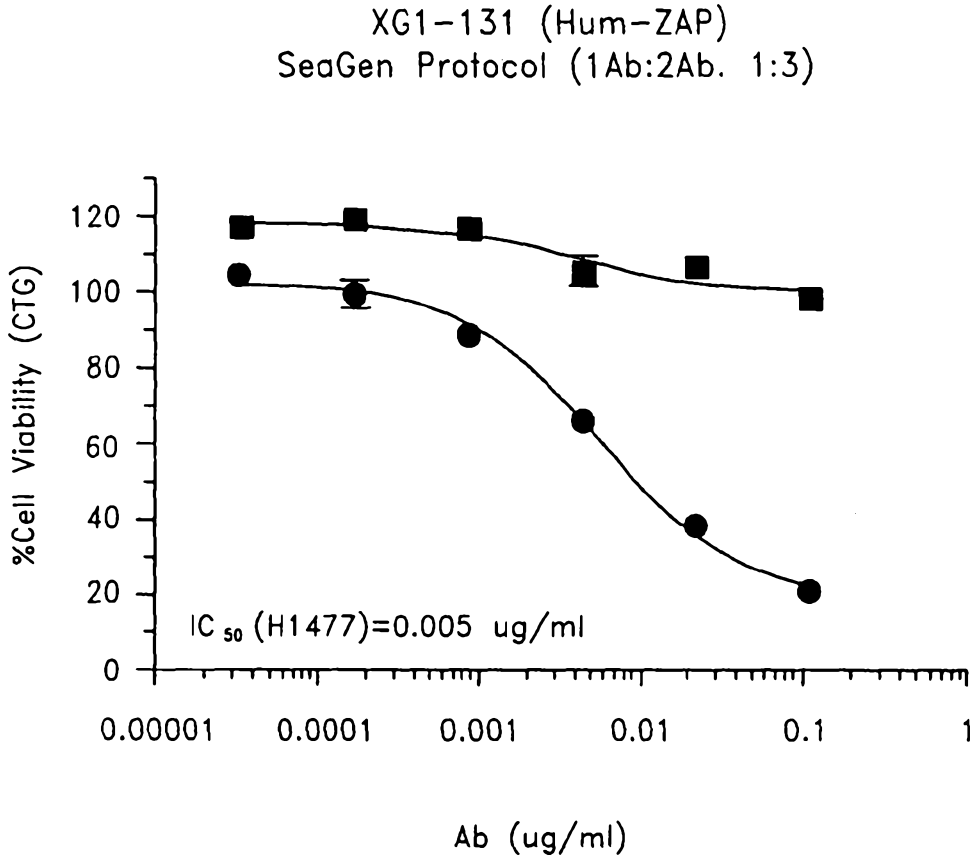

FIG. 11I

2011265359 20 Dec 2011

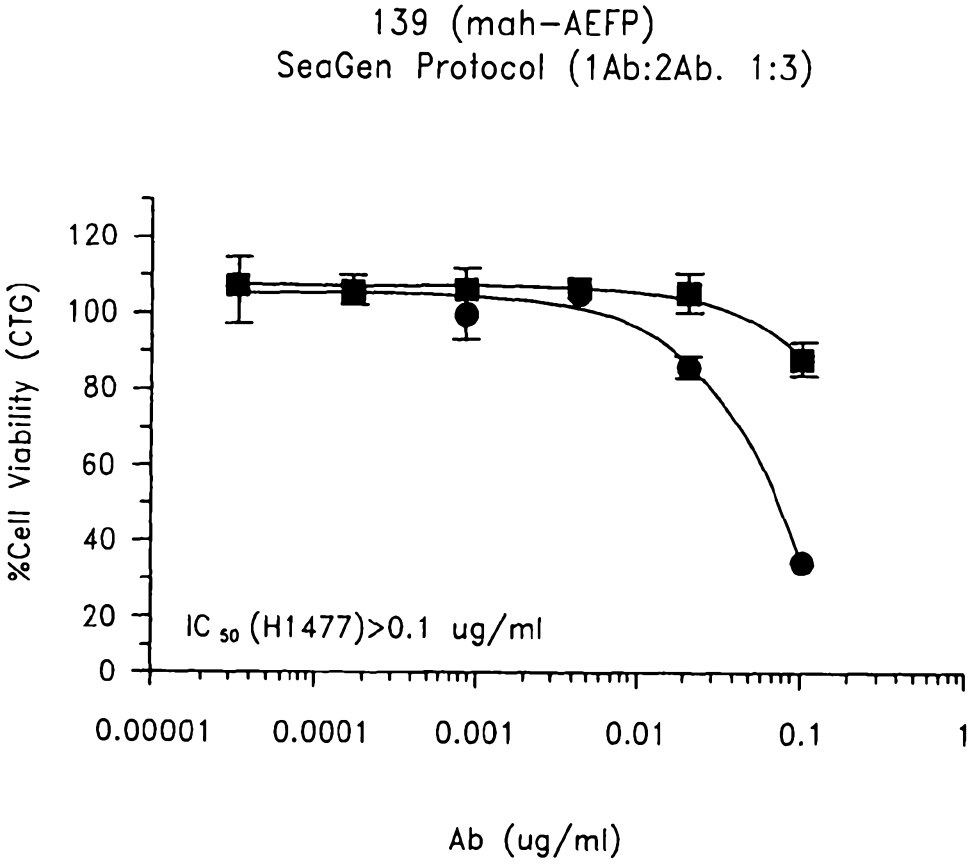

FIG. 12A

2011265359 20 Dec 2011

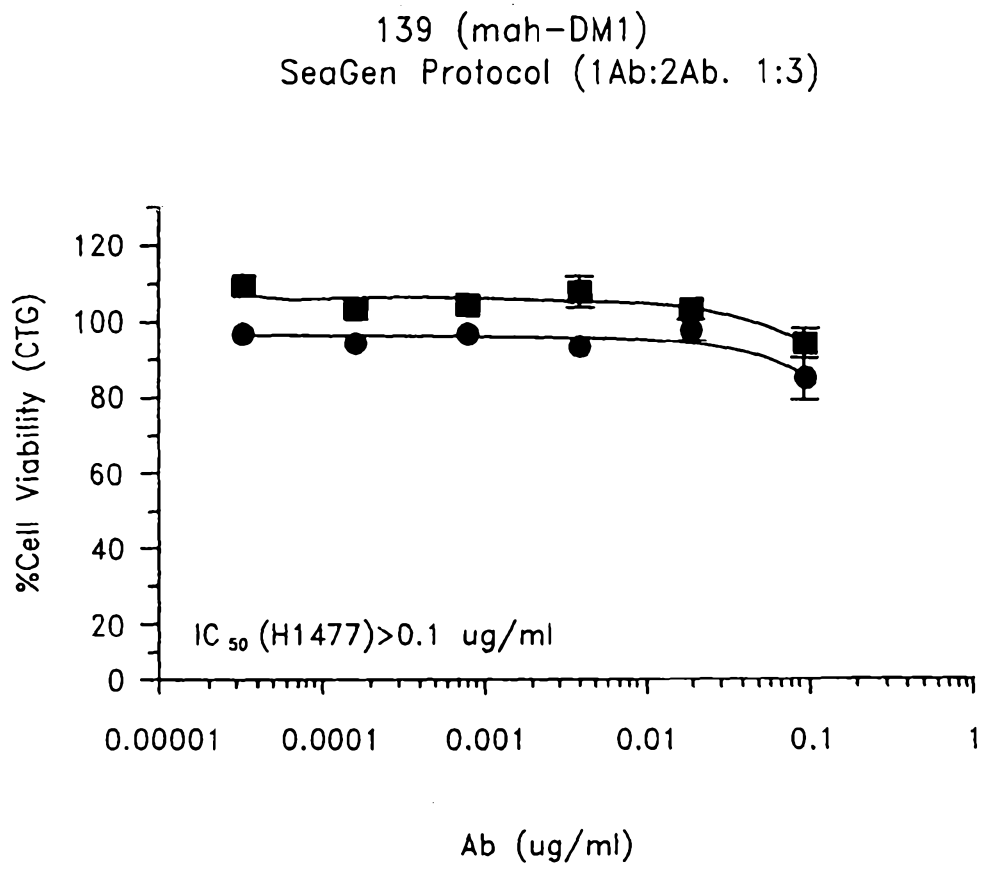

FIG. 12B

2011265359 20 Dec 2011

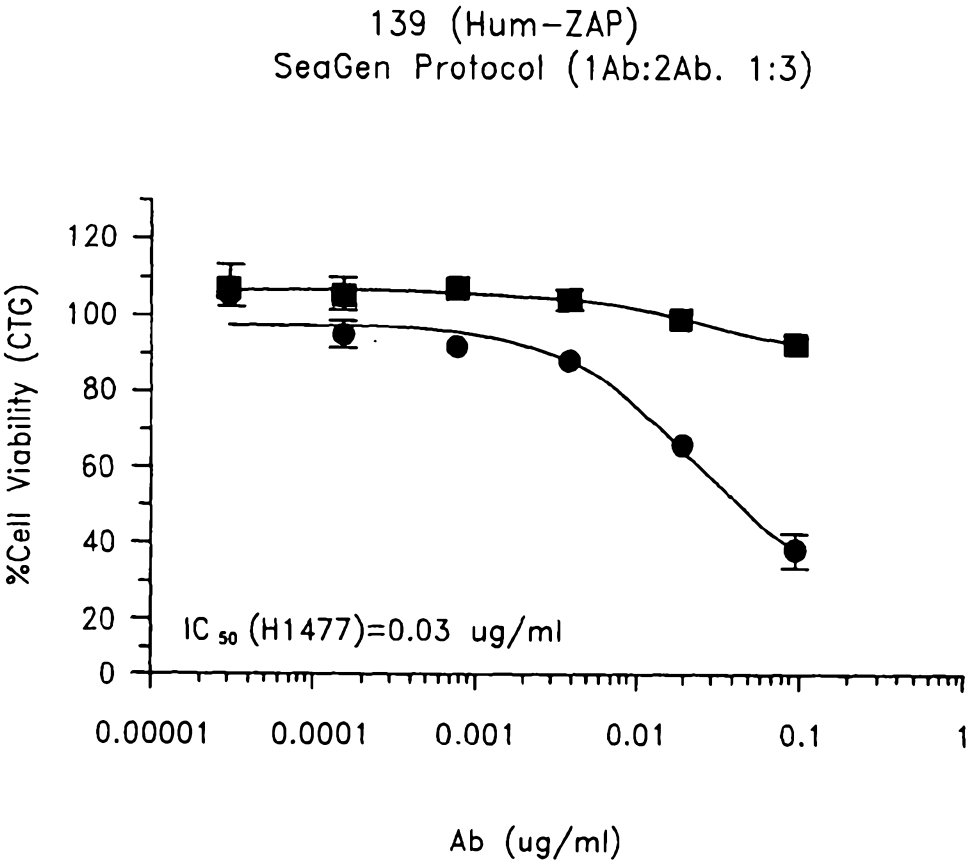

FIG. 12C

2011265359 20 Dec 2011

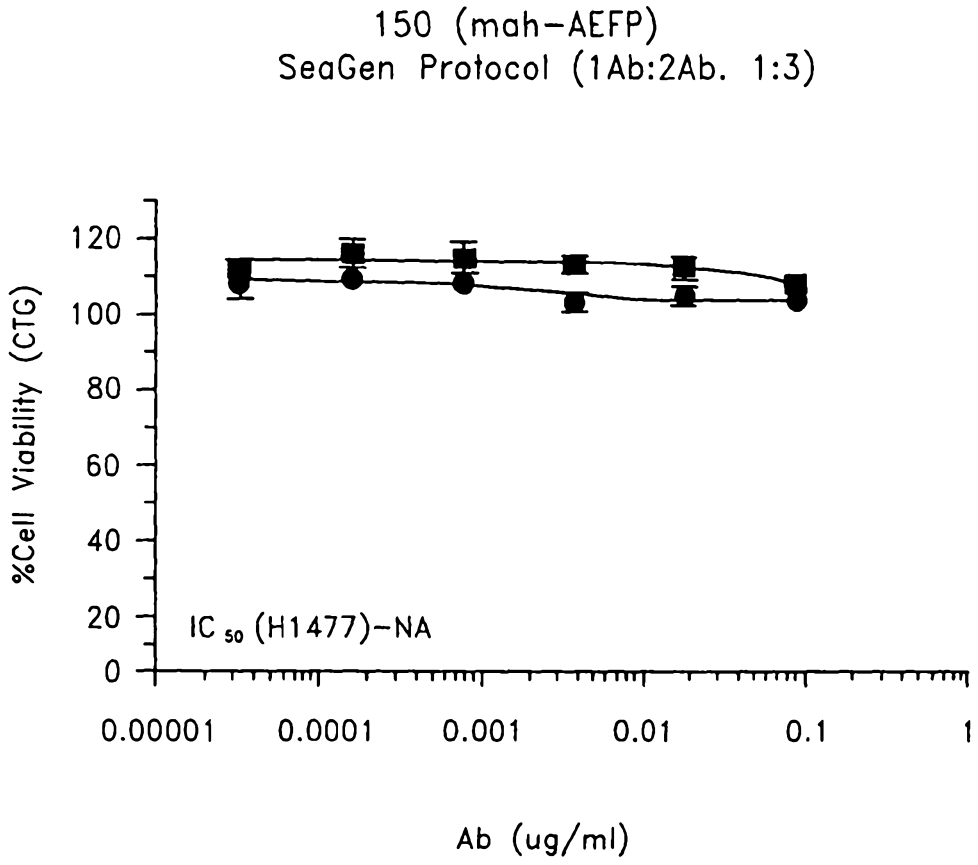

FIG. 12D

2011265359 20 Dec 2011

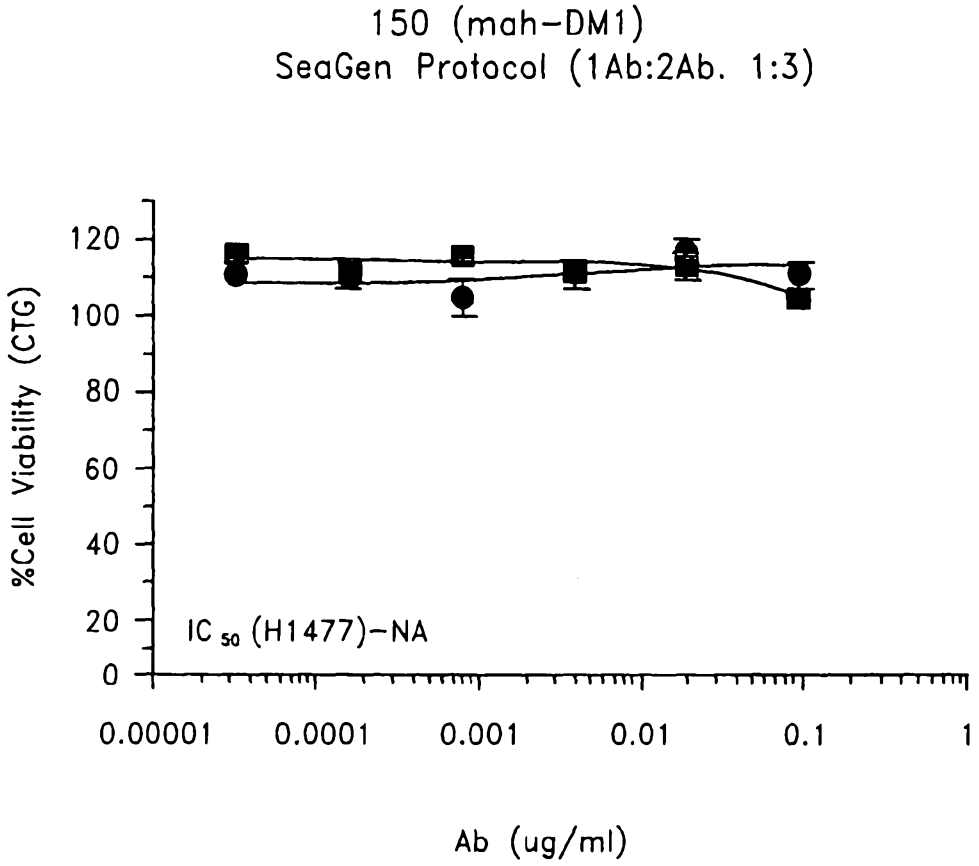

FIG. 12E

2011265359 20 Dec 2011

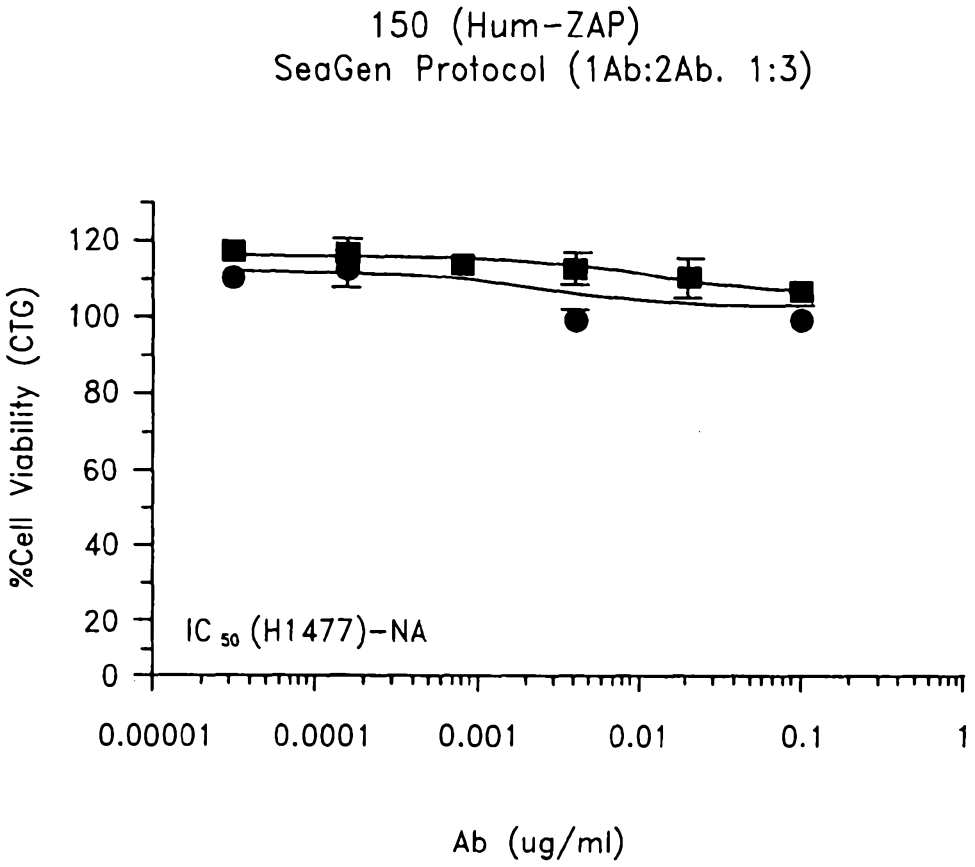

FIG. 12F

2011265359 20 Dec 2011

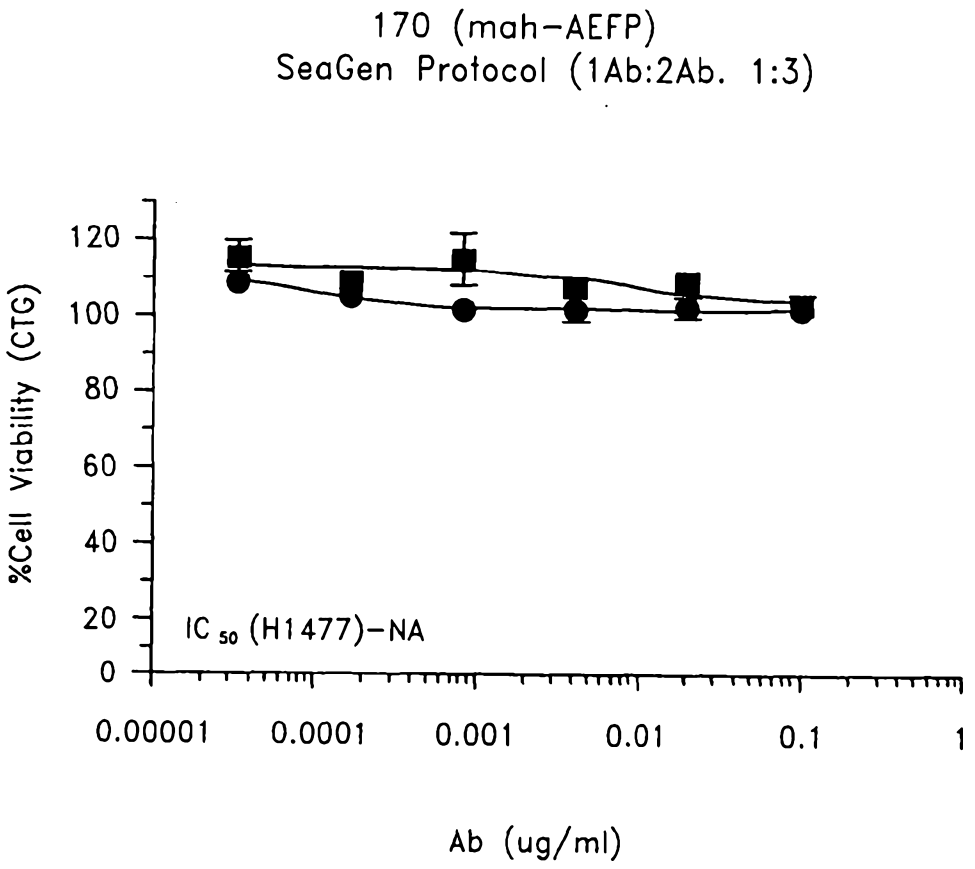

FIG. 12G

2011265359 20 Dec 2011

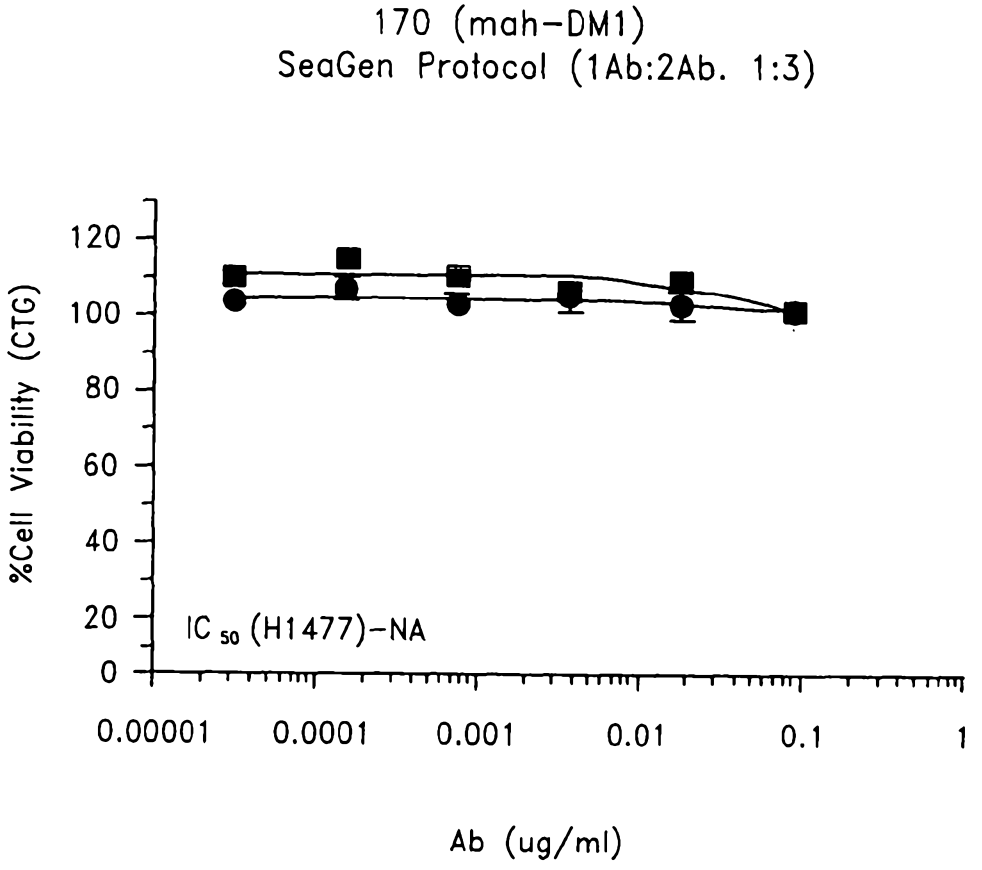

FIG. 12H

2011265359 20 Dec 2011

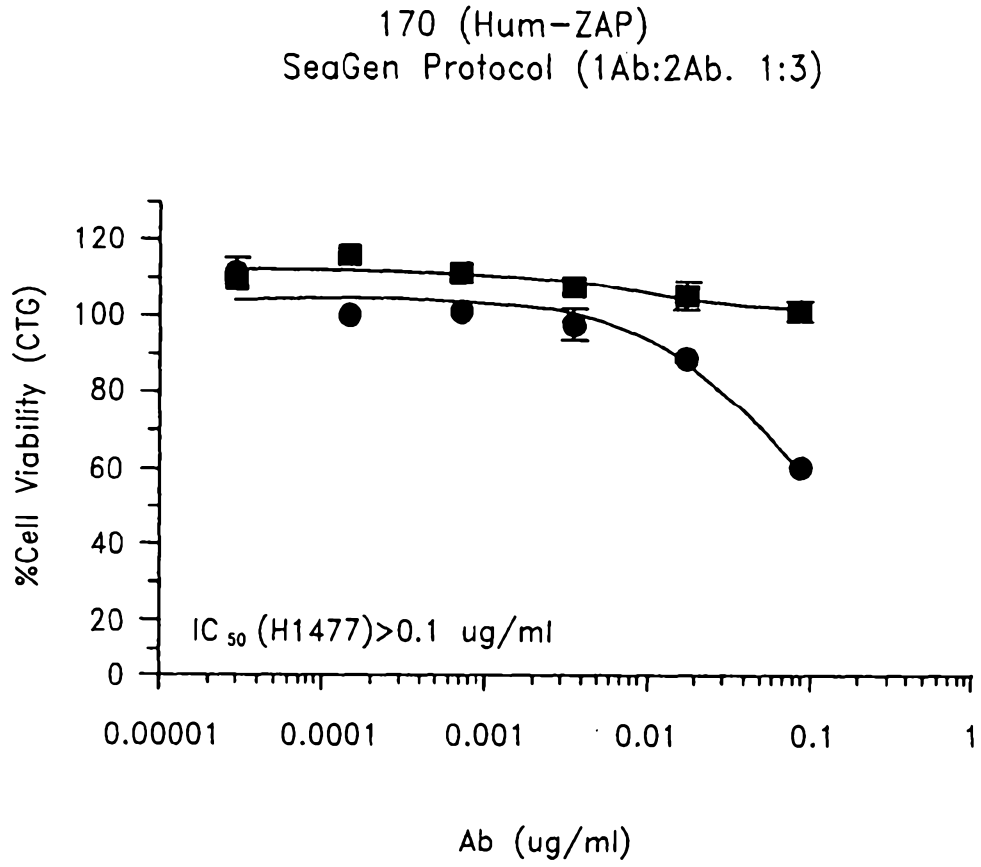

FIG. 12I

2011265359 20 Dec 2011

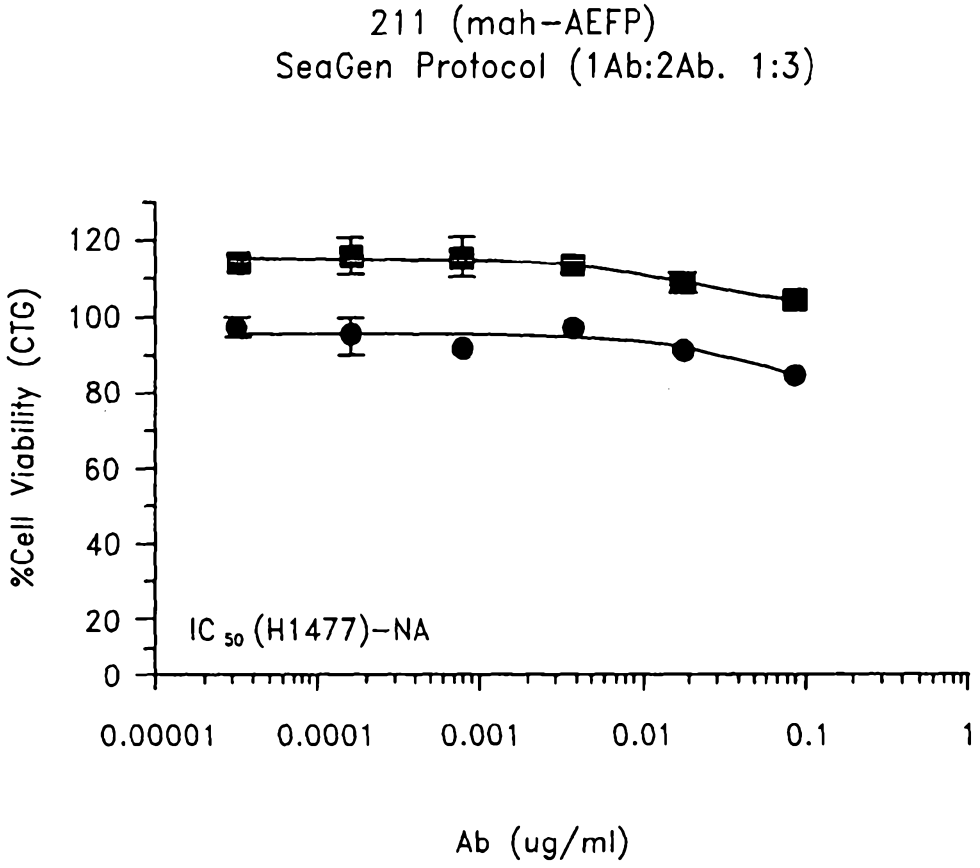

FIG. 13A

2011265359 20 Dec 2011

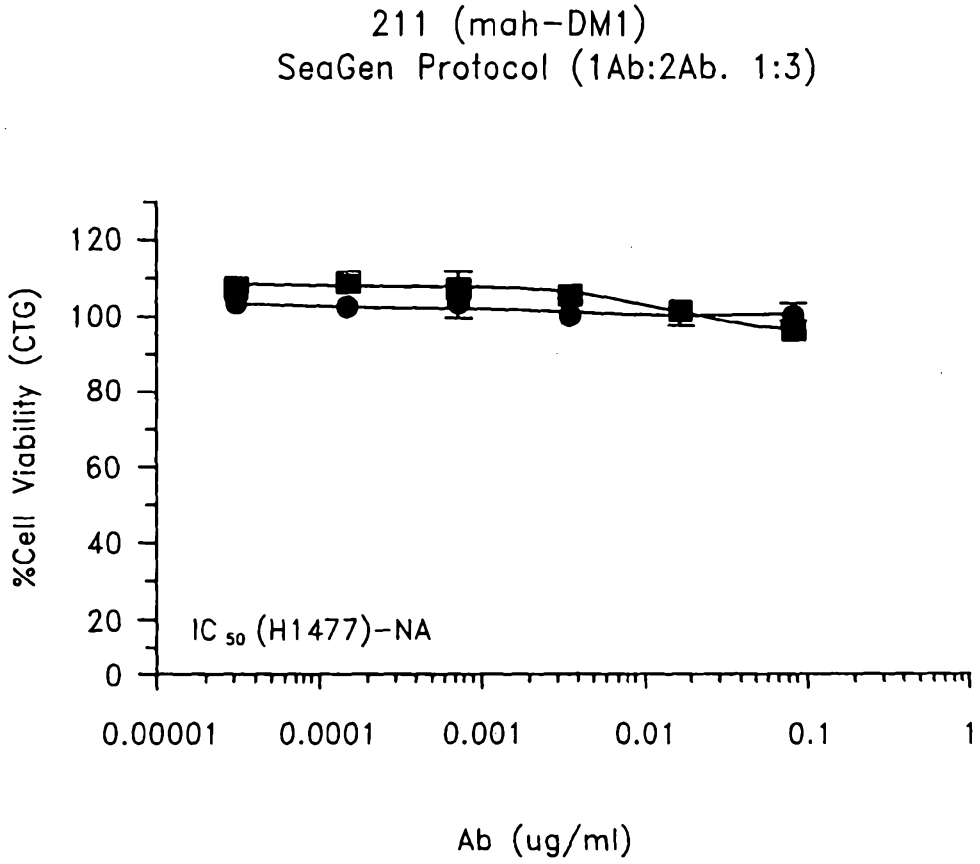

FIG. 13B

2011265359 20 Dec 2011

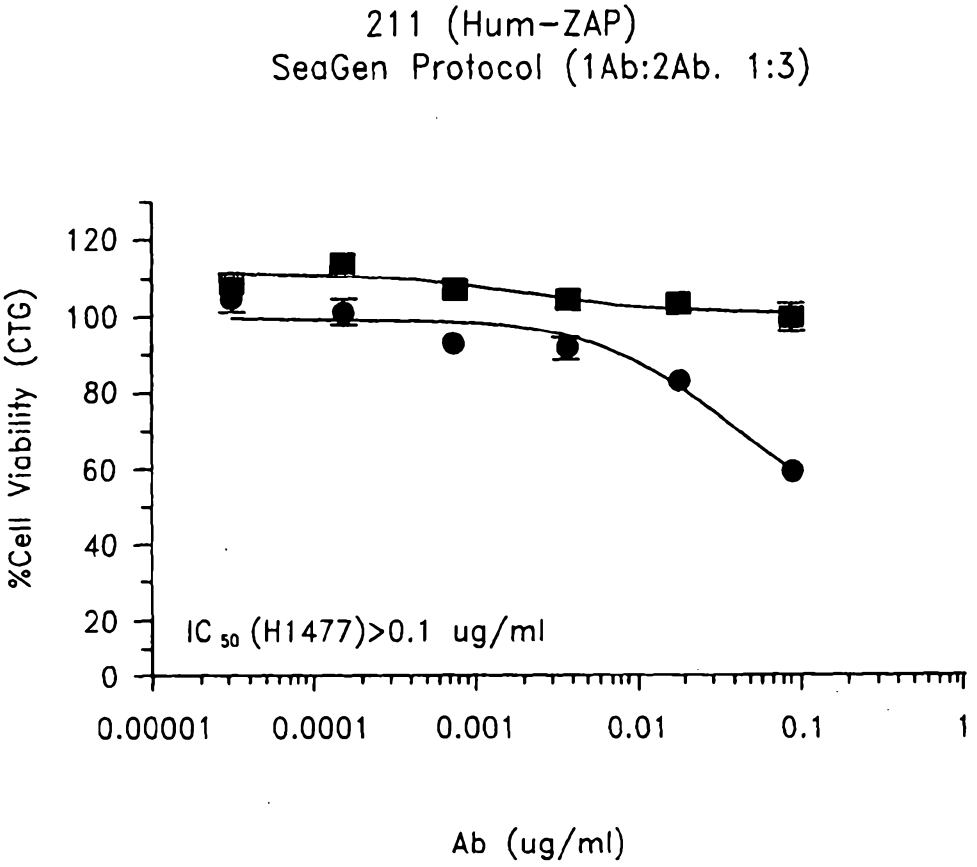

FIG. 13C

2011265359 20 Dec 2011

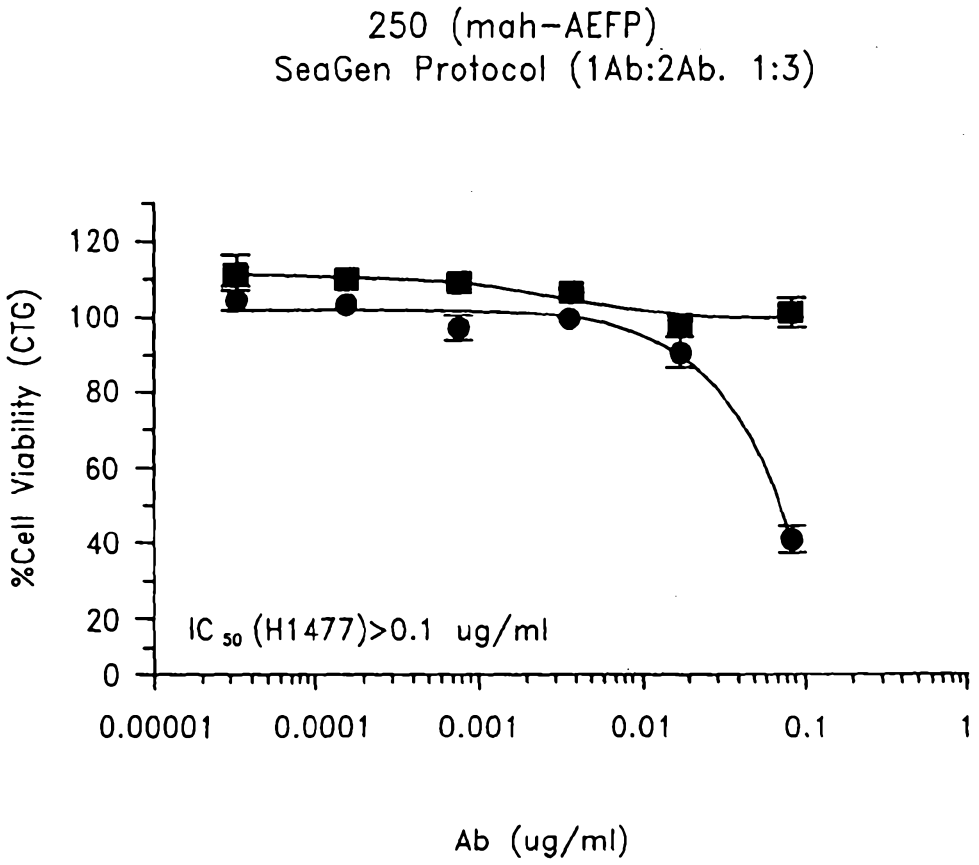

FIG. 13D

2011265359 20 Dec 2011

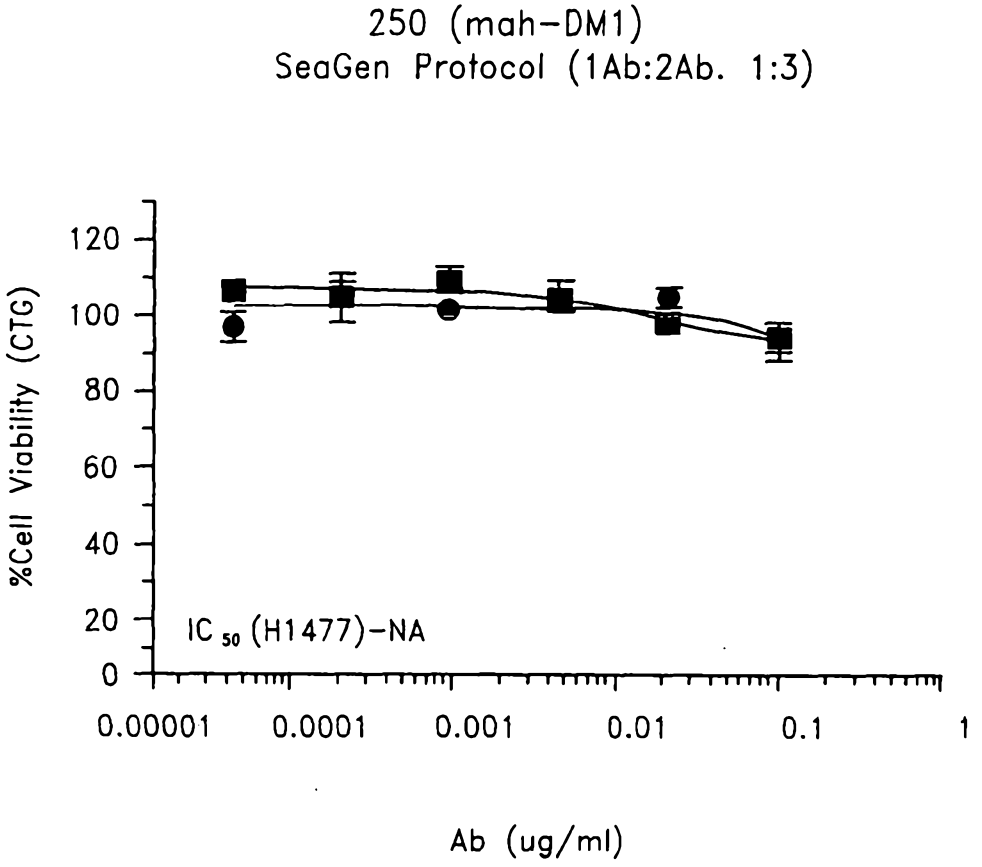

FIG. 13E

2011265359 20 Dec 2011

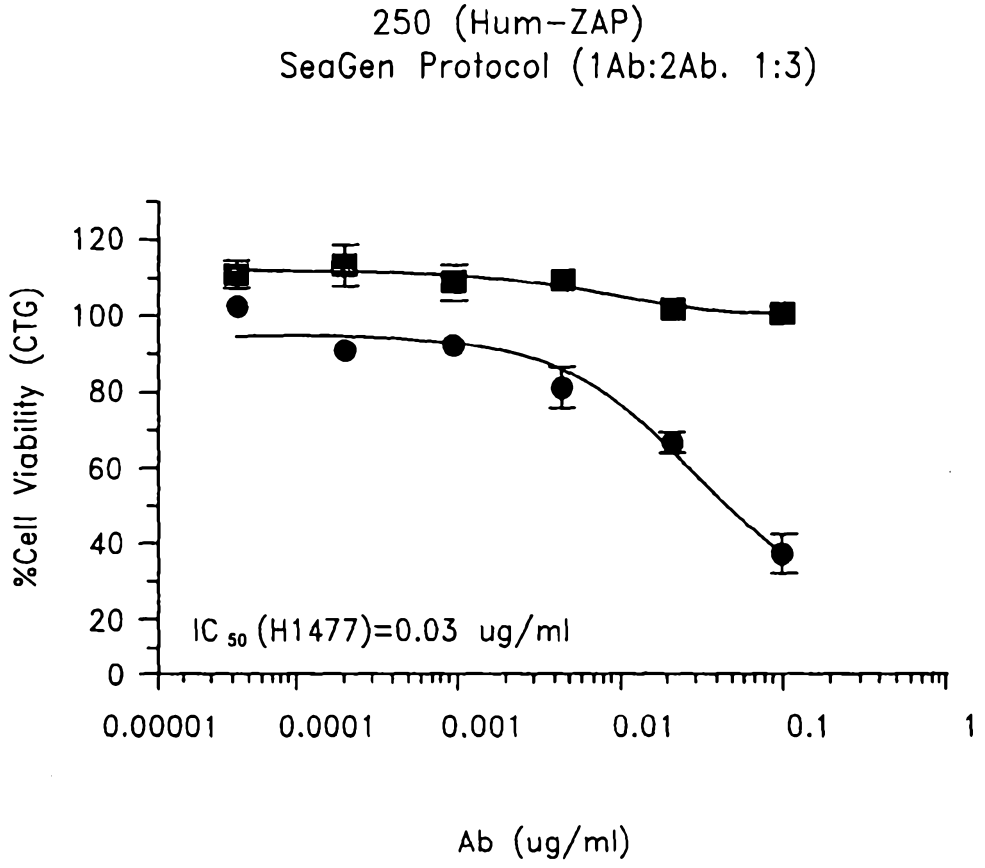

FIG. 13F

2011265359 20 Dec 2011

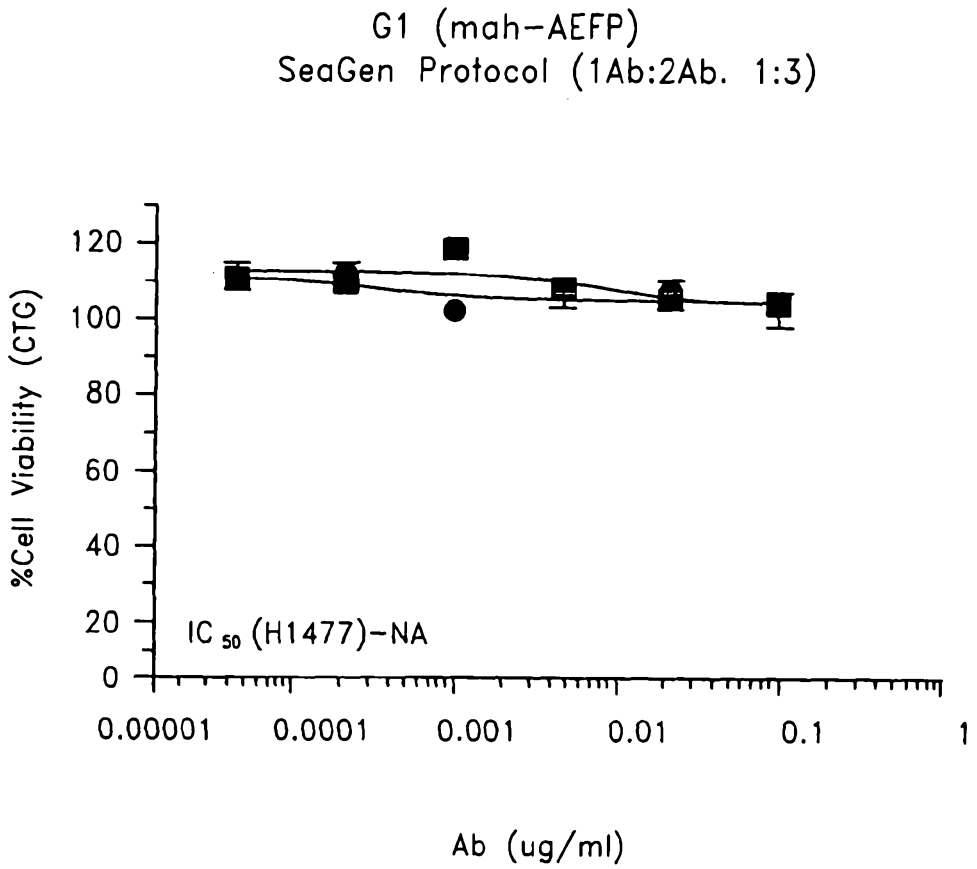

FIG. 13G

2011265359 20 Dec 2011

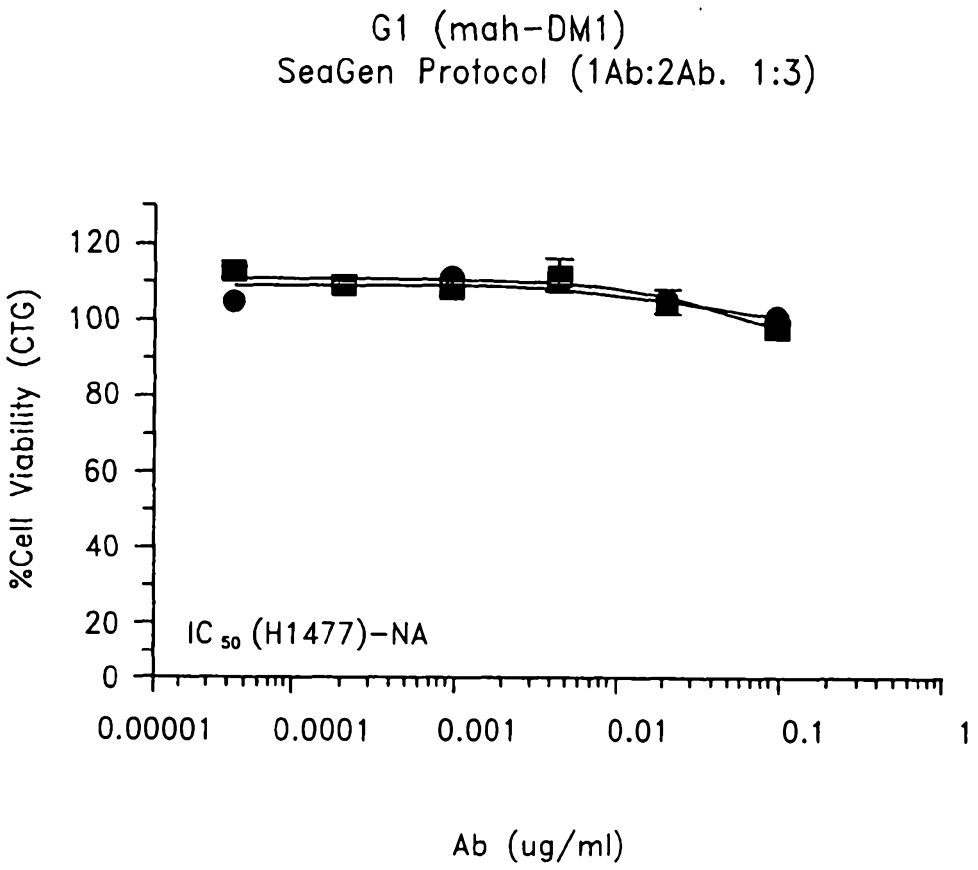

FIG. 13H

2011265359 20 Dec 2011

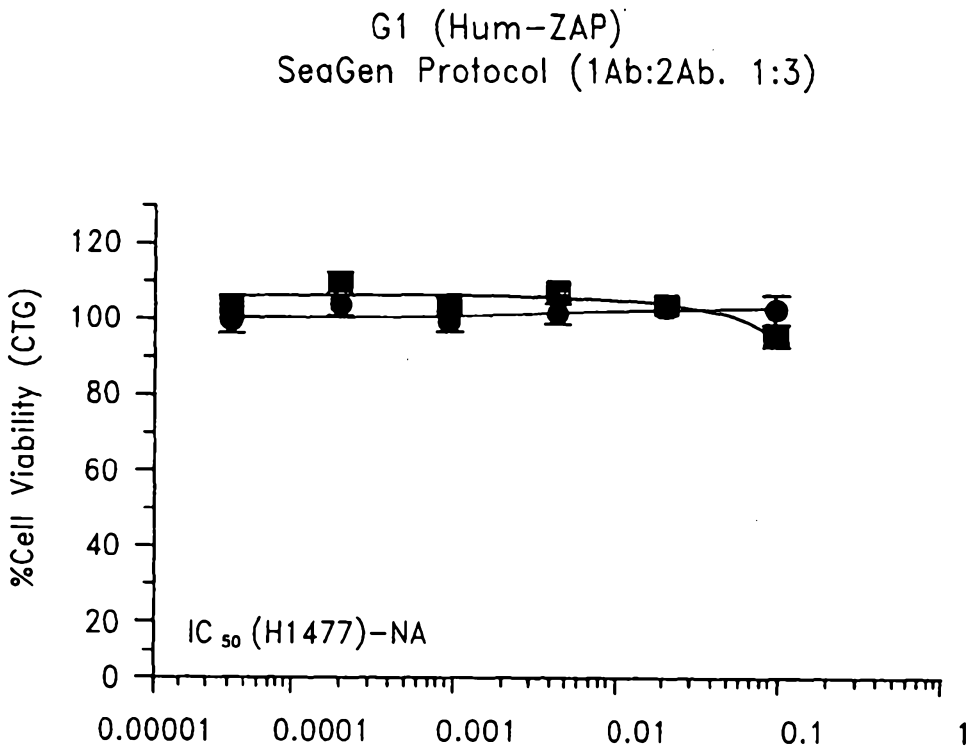

FIG. 13I

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FIG. 14A

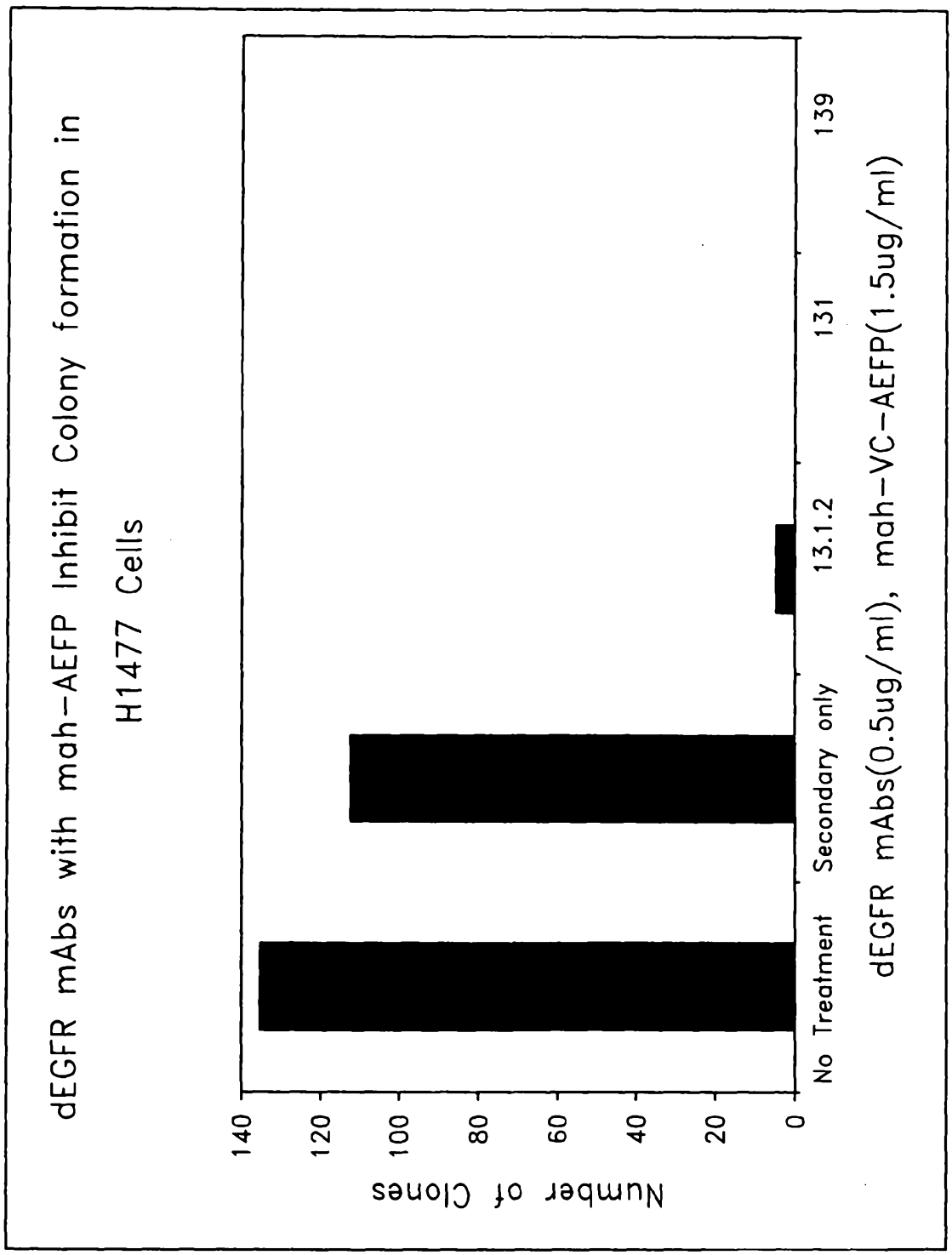

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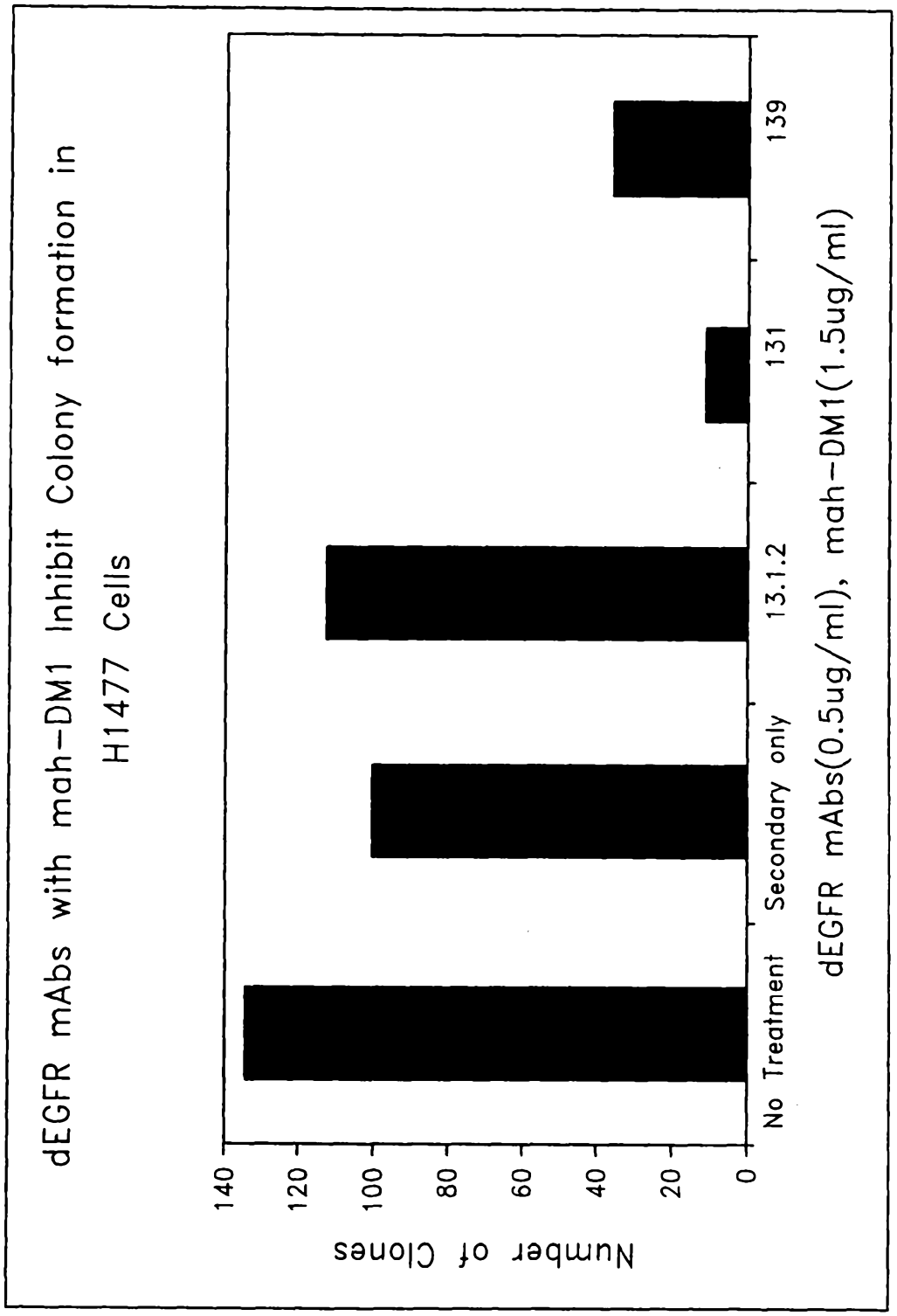
FIG. 14B



2011265359 20 Dec 2011

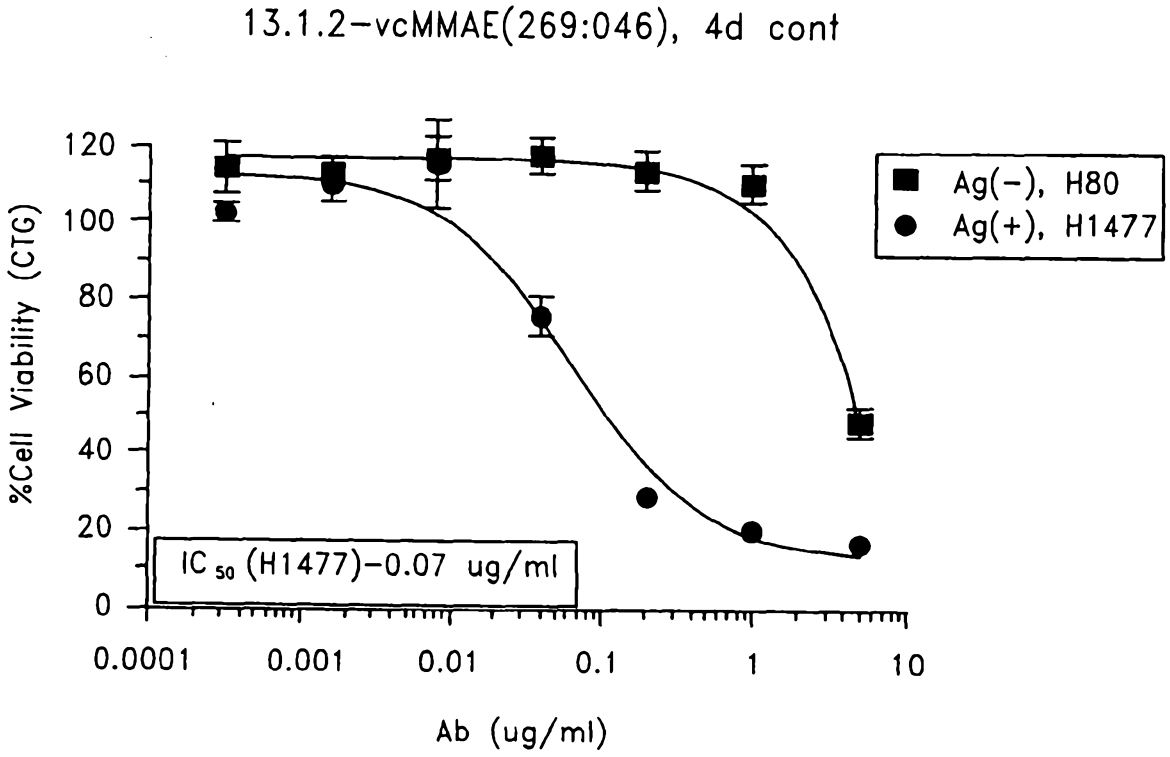


FIG. 15A

2011265359 20 Dec 2011

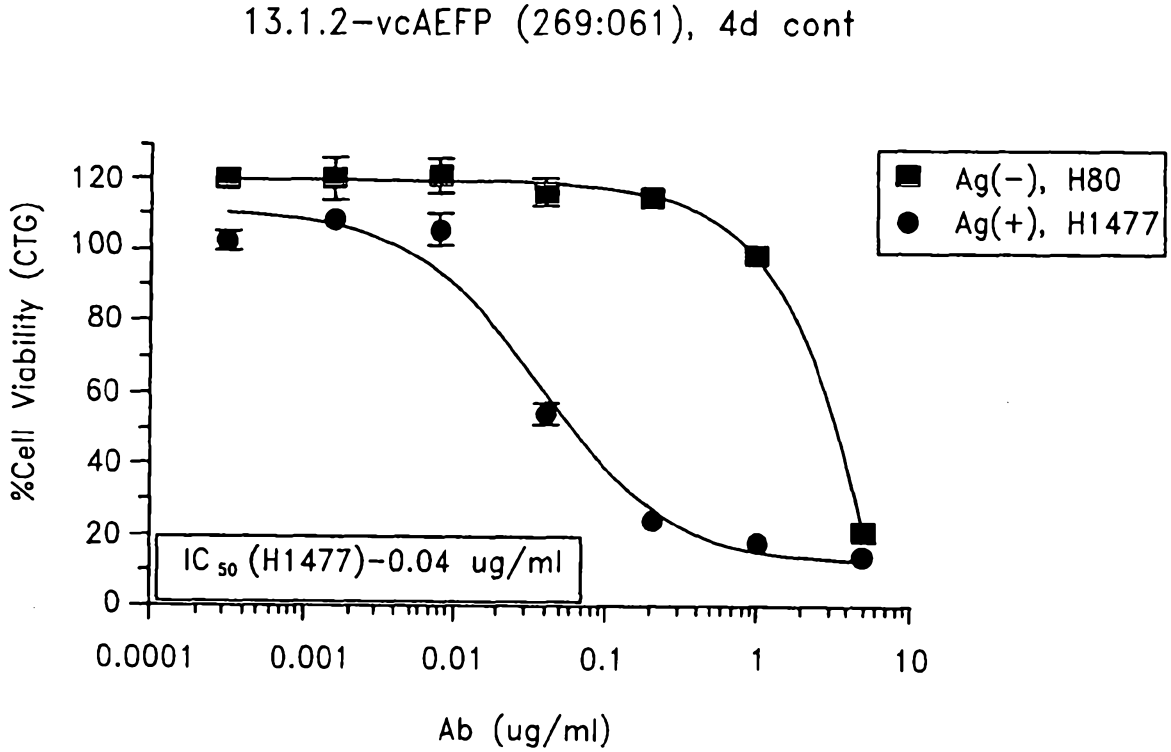


FIG. 15B

2011265359 20 Dec 2011

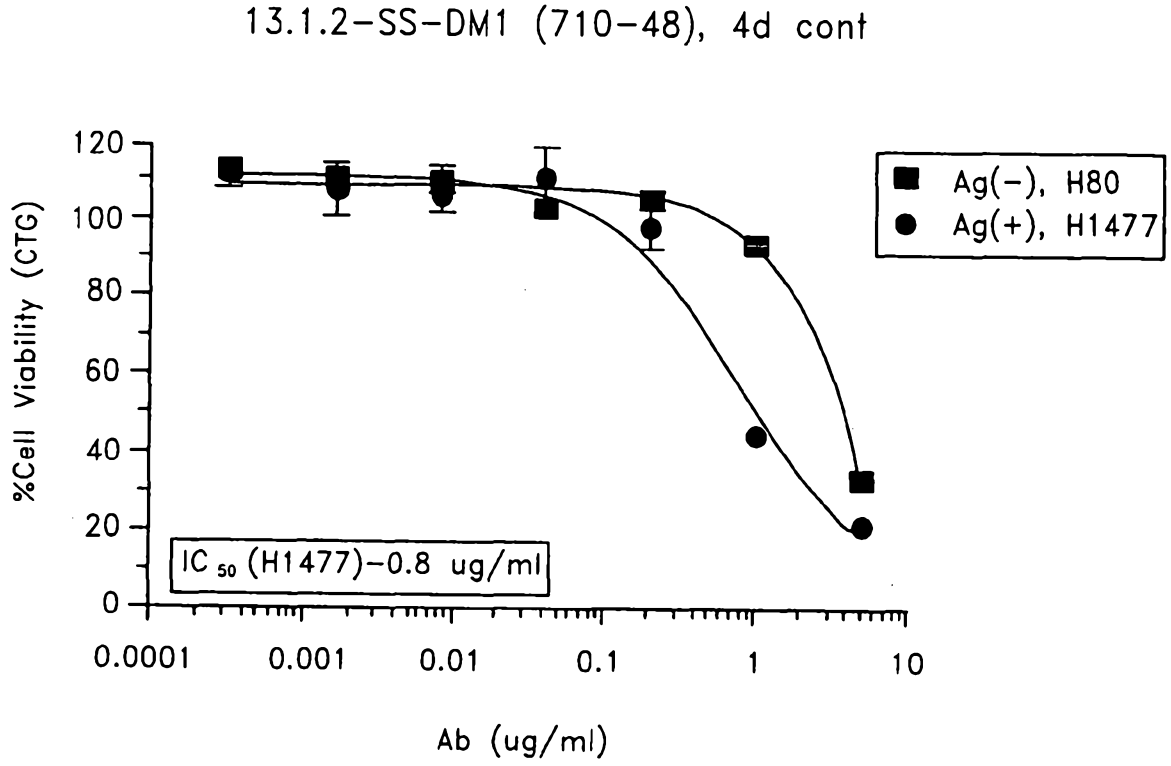


FIG. 15C

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13.1.2-pro-drug mediate tumor eradication

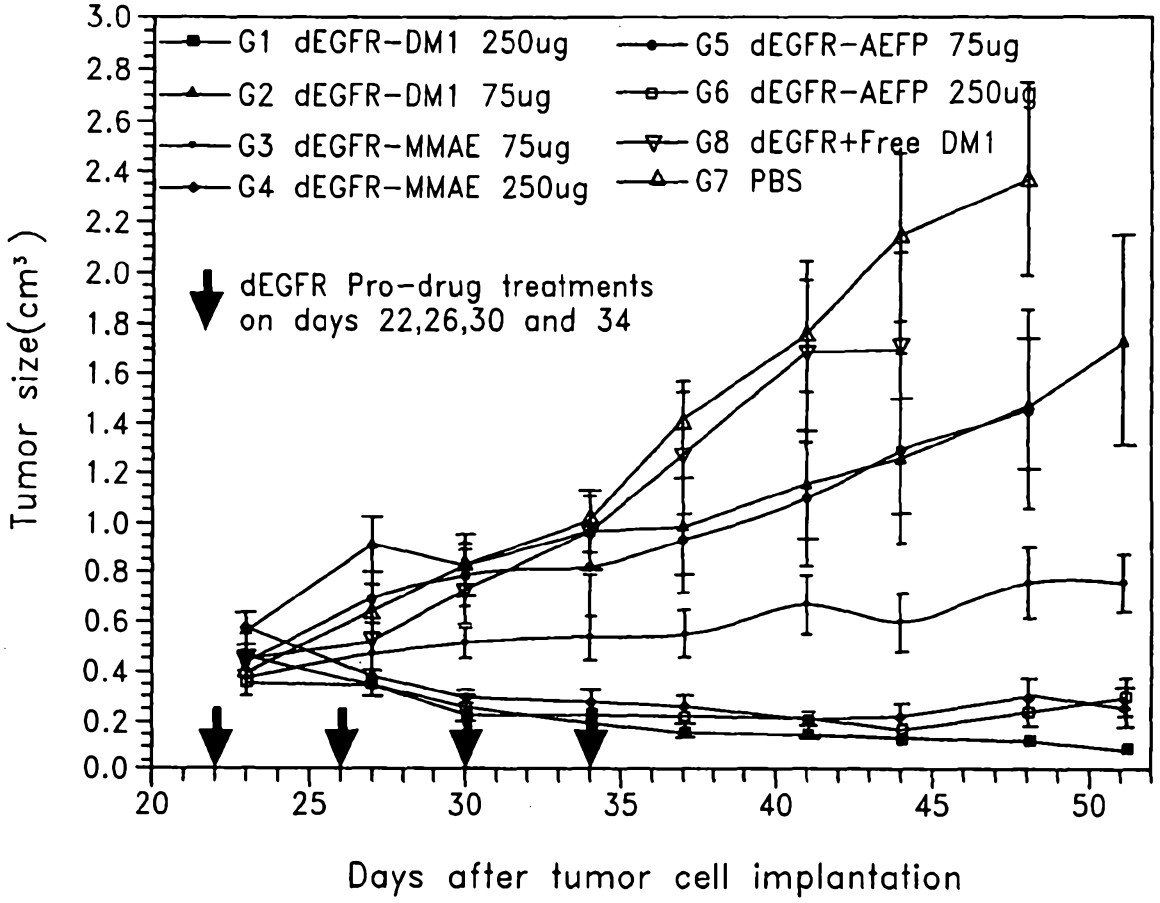

FIG. 16

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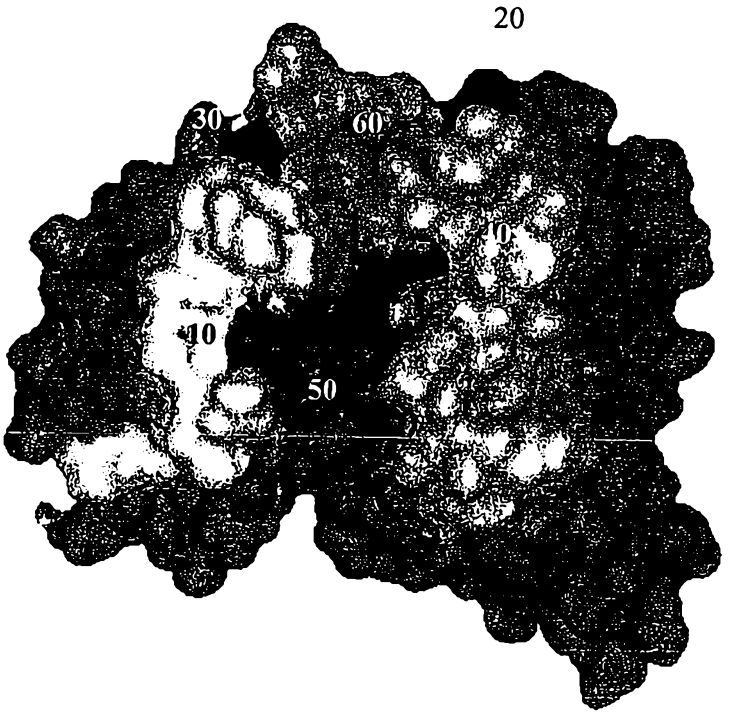

FIG. 17

2011265359 20 Dec 2011

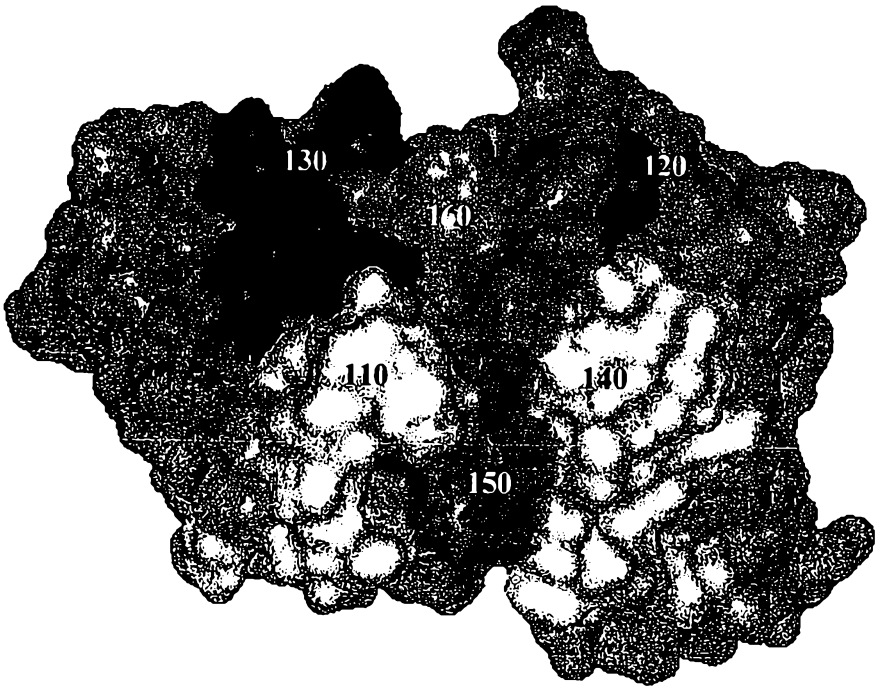

FIG. 18

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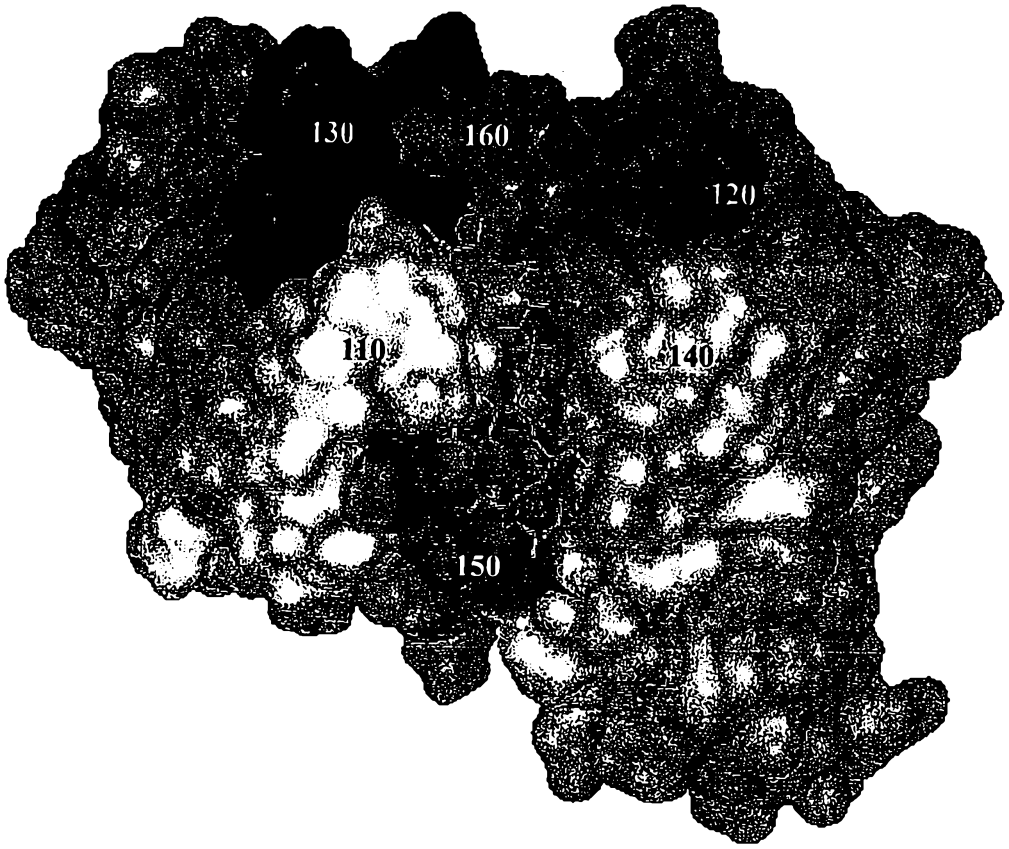

FIG. 19A

2011265359 20 Dec 2011

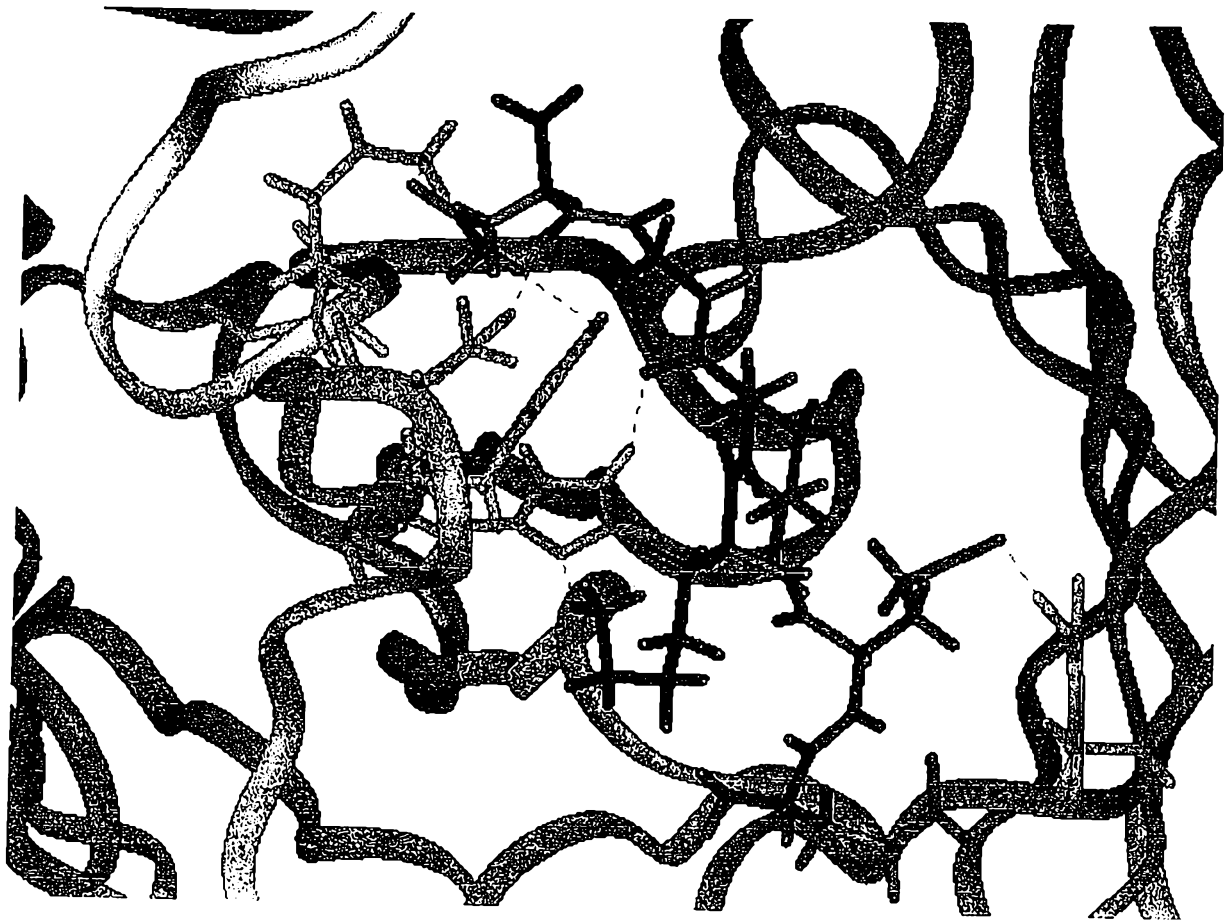

FIG. 19B

2011265359 20 Dec 2011

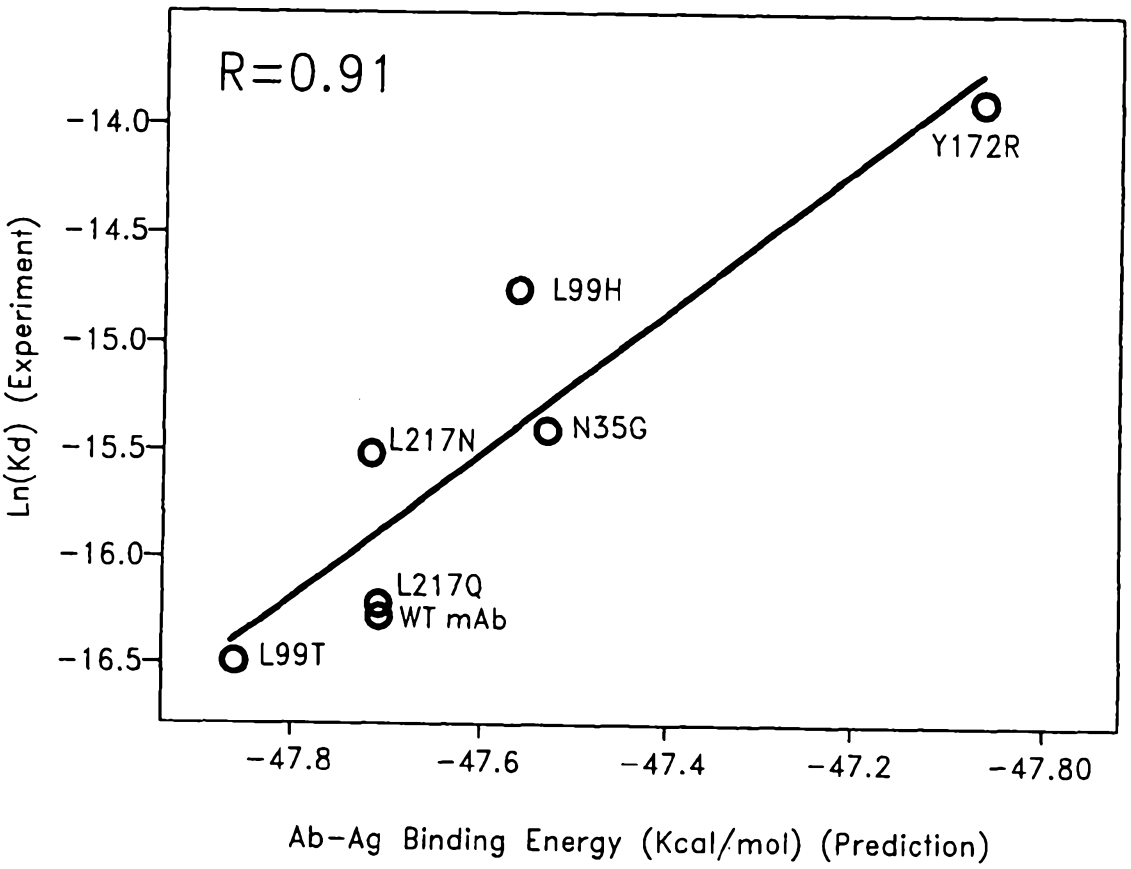

FIG. 20

2011265359 20 Dec 2011

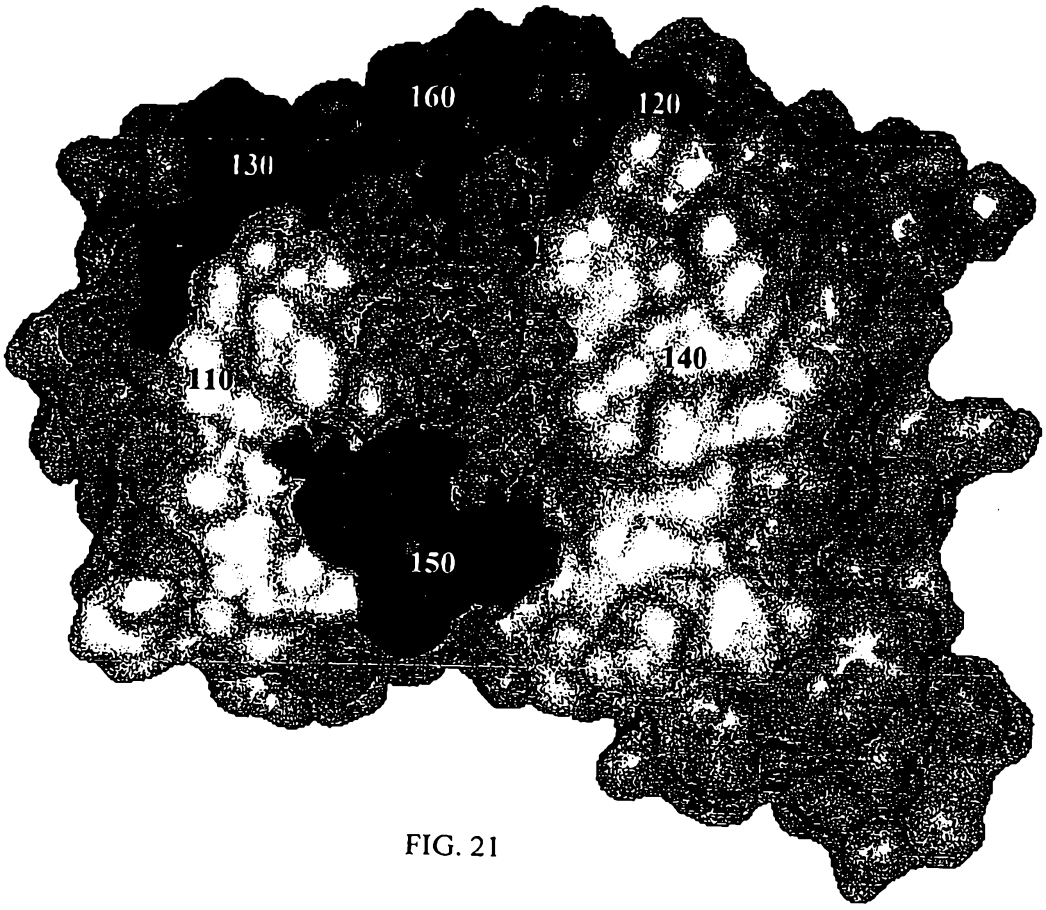

FIG. 21

2011265359 20 Dec 2011

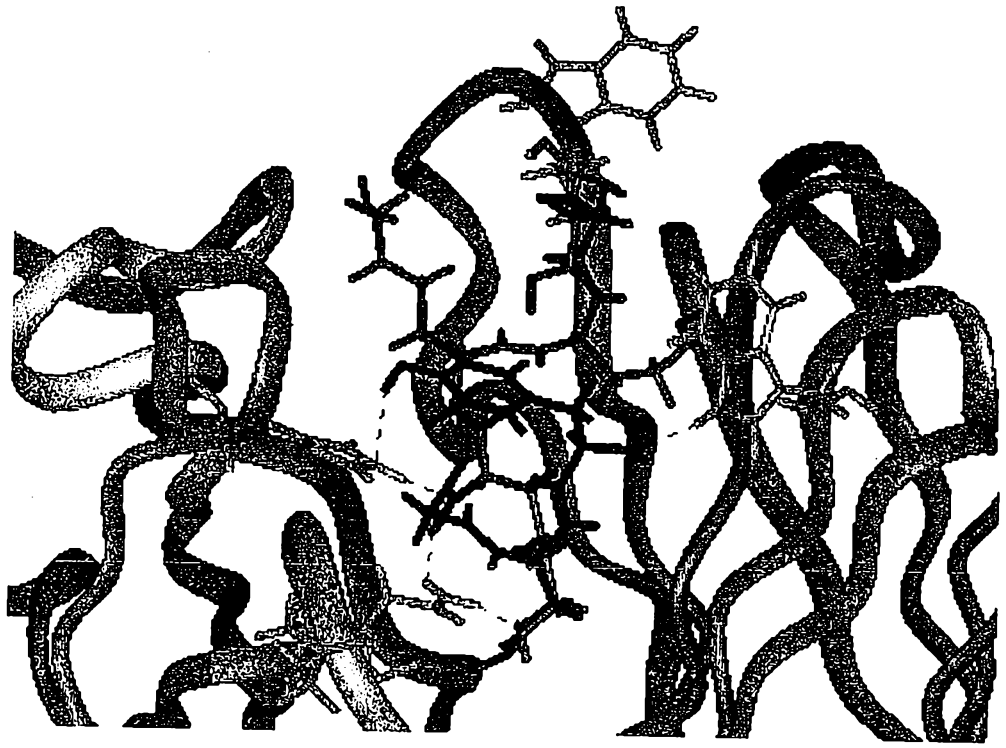

FIG. 22

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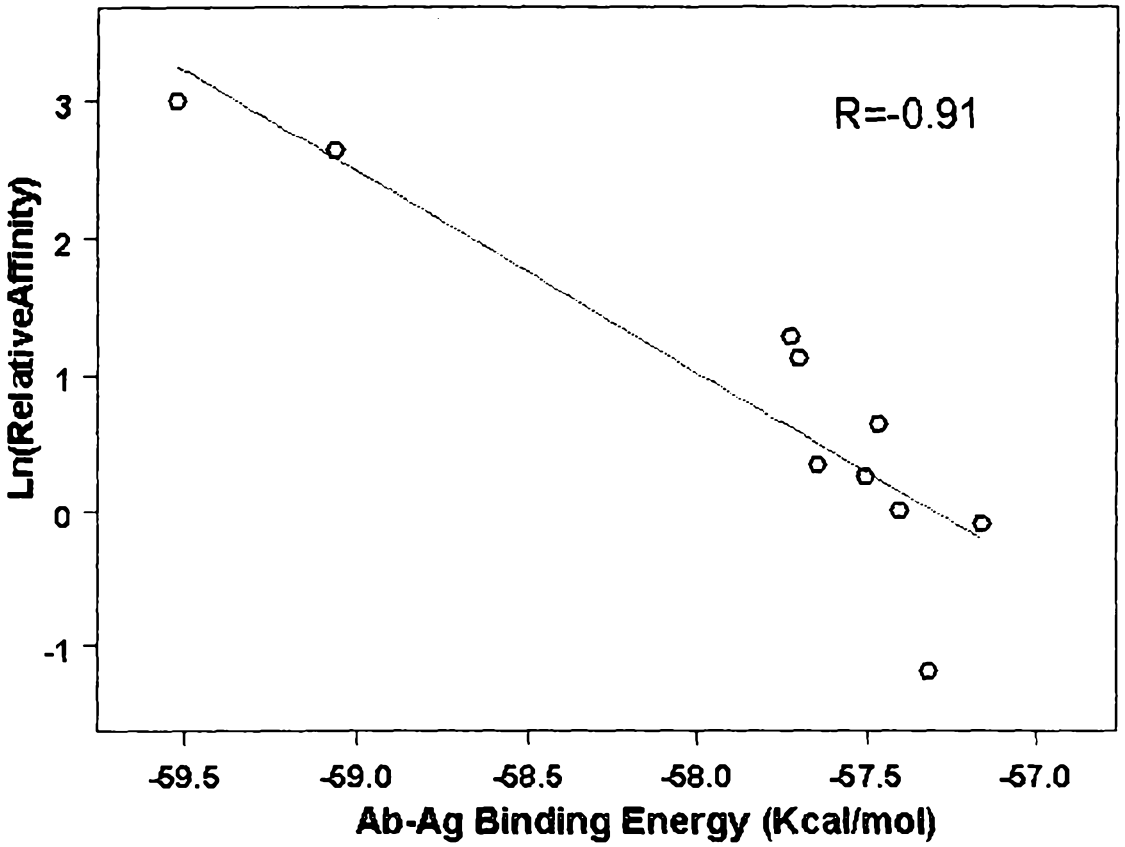

FIG. 23

SEQUENCE LISTING

<110> Weber, Richard
Feng, Xiao
Foord, Orit
Green, Larry
Gudas, Jean
Keyt, Bruce
Liu, Ying
Rathanaswami, Palaniswami
Raya, Robert
Yang, Xiao Dong
Corvalan, Jose
Foltz, Ian
Jia, Xiao-Chi
Kang, Jaspal
King, Chadwick T.
Klakamp, Scott L.
Su, Qiaojuan Jane

<120> ANTIBODIES DIRECTED TO THE DELETION MUTANTS OF EPIDERMAL GROWTH FACTOR RECEPTOR AND USES THEREOF

<130> ABGENIX.087A

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<151> 2003-06-27

<150> 60/525,570
<151> 2003-11-26

<150> 60/562,453
<151> 2004-04-15

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<213> Homo sapiens

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Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
            20                  25                  30
Gly Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45
Ala Val Ile Trp Tyr Asp Gly Ser Asn Lys Tyr Tyr Ala Asp Ser Val
    50                  55                  60
```

```
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80
Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95
Ala Arg Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
            100                 105
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<210> 2
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Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Arg Asn Tyr
            20                  25                  30
Gly Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45
Ala Val Ile Trp Tyr Asp Gly Ser Asp Lys Tyr Tyr Ala Asp Ser Val
    50                  55                  60
Arg Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80
Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95
Ala Arg Asp Gly Tyr Asp Ile Leu Thr Gly Asn Pro Arg Asp Phe Asp
            100                 105                 110
Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
        115                 120
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<210> 3
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Thr Leu Ser Leu Thr Cys Ala Ile Ser Gly Asp Ser Val Ser Ser Asn
            20                  25                  30
Ser Ala Ala Trp Asn Trp Ile Arg Gln Ser Pro Ser Arg Gly Leu Glu
        35                  40                  45
Trp Leu Gly Arg Thr Tyr Tyr Arg Ser Lys Trp Tyr Asn Asp Tyr Ala
    50                  55                  60
Val Ser Val Lys Ser Arg Ile Thr Ile Asn Pro Asp Thr Ser Lys Asn
65                  70                  75                  80
Gln Phe Ser Leu Gln Leu Asn Ser Val Thr Pro Glu Asp Thr Ala Val
                85                  90                  95
Tyr Tyr Cys Ala Arg Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
            100                 105                 110
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<210> 4
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Thr Leu Ser Leu Thr Cys Ala Ile Ser Gly Asp Ser Val Ser Ser Asn
            20                  25                  30
Asn Ala Ala Trp Asn Trp Ile Arg Gln Ser Pro Ala Arg Gly Leu Glu
        35                  40                  45
Trp Leu Gly Arg Thr Tyr Tyr Arg Ser Lys Trp Tyr Asn Asp Tyr Val
    50                  55                  60
Val Ser Val Lys Ser Arg Ile Thr Ile Asn Pro Asp Thr Ser Lys Asn
65                  70                  75                  80
Gln Phe Ser Leu Gln Leu Asn Ser Val Thr Pro Glu Asp Thr Ala Val
                85                  90                  95
Tyr Tyr Cys Val Arg Ala Thr Ala Phe Asp Tyr Trp Gly Gln Gly Thr
            100                 105                 110
Leu Val Thr Val Ser Ser
        115
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<210> 5
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Thr Leu Ser Leu Thr Cys Ala Ile Ser Gly Asp Ser Val Ser Ser Tyr
            20                  25                  30
Ser Ser Ala Trp Asn Trp Ile Arg Gln Ser Pro Ser Arg Gly Leu Glu
        35                  40                  45
Trp Leu Gly Arg Ala Tyr His Arg Ser Arg Trp Tyr Tyr Glu Tyr Ala
    50                  55                  60
Val Ser Val Lys Ser Arg Ile Asn Ile Thr Pro Asp Thr Ser Lys Asn
65                  70                  75                  80
Gln Phe Ser Leu Gln Leu Asn Ser Val Thr Pro Glu Asp Thr Ala Val
                85                  90                  95
Tyr Tyr Cys Ala Arg Gly Ser Arg Phe Asp Tyr Trp Gly Gln Gly Thr
            100                 105                 110
Leu Val Thr Val Ser Ser
        115
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<210> 6
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 1               5                  10                  15
Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Gly Ser Ile Ser Ser Gly
            20                  25                  30
Gly Tyr Tyr Trp Ser Trp Ile Arg Gln His Pro Gly Lys Gly Leu Glu
        35                  40                  45
```

```
Trp Ile Gly Tyr Ile Tyr Tyr Ser Gly Ser Thr Tyr Tyr Asn Pro Ser
    50                  55                  60
Leu Lys Ser Arg Val Thr Ile Ser Val Asp Thr Ser Lys Asn Gln Phe
65                  70                  75                  80
Ser Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr
                85                  90                  95
Cys Ala Arg Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
            100                 105                 110


<210> 7
<211> 126
<212> PRT
<213> Homo sapiens

<400> 7
Gln Val Gln Leu Gln Glu Ser Gly Pro Phe Leu Val Lys Pro Ser Gln
 1               5                  10                  15
Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Gly Ser Ile Ser Ser Gly
            20                  25                  30
Gly Tyr Tyr Trp Ser Trp Ile Arg Gln His Pro Gly Lys Gly Leu Glu
        35                  40                  45
Trp Ile Gly Phe Ile Tyr Tyr Arg Gly Asn Thr Tyr Tyr Asn Pro Ser
    50                  55                  60
Leu Lys Ser Arg Val Thr Ile Ser Val Asp Thr Ser Lys Asn Gln Phe
65                  70                  75                  80
Ser Leu Lys Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr
                85                  90                  95
Cys Ala Arg Asp Gly Tyr Cys Ser Arg Thr Gly Cys Tyr Gly Gly Trp
            100                 105                 110
Phe Asp Pro Trp Gly Gln Gly Thr Leu Val Thr Val Ser Pro
        115                 120                 125


<210> 8
<211> 109
<212> PRT
<213> Homo sapiens

<400> 8
Glu Val Gln Leu Leu Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
 1               5                  10                  15
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
            20                  25                  30
Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45
Ser Ala Ile Ser Gly Ser Gly Gly Ser Thr Tyr Tyr Ala Asp Ser Val
    50                  55                  60
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80
Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95
Ala Lys Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
            100                 105


<210> 9
```

<211> 116
<212> PRT
<213> Homo sapiens

<400> 9
Glu Gly Gln Leu Leu Glu Ser Gly Gly Gly Trp Val Gln Pro Gly Glu
 1   5   10   15
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
            20   25   30
Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35   40   45
Ser Ala Ile Ser Gly Ser Gly Gly Ser Thr Asn Tyr Ala Asp Ser Val
    50   55   60
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65   70   75   80
Leu Gln Val Asn Ser Leu Arg Val Glu Asp Thr Ala Val Tyr Tyr Cys
                85   90   95
Ala Gly Ser Ser Gly Trp Ser Glu Tyr Trp Gly Gln Gly Thr Leu Val
            100   105   110
Thr Val Ser Ser
        115

<210> 10
<211> 116
<212> PRT
<213> Homo sapiens

<400> 10
Glu Val Gln Val Leu Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
 1   5   10   15
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
            20   25   30
Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35   40   45
Ser Ala Ile Ser Gly Ser Gly Gly Ser Thr Asn Tyr Ala Asp Ser Val
    50   55   60
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65   70   75   80
Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85   90   95
Ala Gly Ser Ser Gly Trp Ser Glu Tyr Trp Gly Gln Gly Thr Leu Val
            100   105   110
Thr Val Ser Ser
        115

<210> 11
<211> 109
<212> PRT
<213> Homo sapiens

<400> 11
Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
 1   5   10   15
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
            20   25   30

```
Gly Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45
Ala Val Ile Ser Tyr Asp Gly Ser Asn Lys Tyr Tyr Ala Asp Ser Val
    50                  55                  60
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80
Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95
Ala Lys Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
            100                 105
```

<210> 12
<211> 128
<212> PRT
<213> Homo sapiens

<400> 12

```
Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
 1               5                  10                  15
Ser Leu Arg Leu Ser Cys Val Ala Ser Gly Phe Thr Leu Ser Ser Tyr
            20                  25                  30
Gly Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45
Ala Val Thr Ser Tyr Asp Gly Ser Lys Lys Asp Tyr Ala Asp Ser Ala
    50                  55                  60
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80
Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95
Val Ser Glu Gly Tyr Cys Ser Ser Ser Ser Cys Tyr Lys Tyr Tyr Tyr
            100                 105                 110
Tyr Gly Met Asp Val Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
        115                 120                 125
```

<210> 13
<211> 128
<212> PRT
<213> Homo sapiens

<400> 13

```
Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
 1               5                  10                  15
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Leu Ser Ser Tyr
            20                  25                  30
Gly Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45
Ala Val Met Ser Tyr Asp Gly Ser Lys Glu Asp Tyr Ala Asp Ser Val
    50                  55                  60
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Glu Asn Thr Leu Tyr
65                  70                  75                  80
Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95
Val Ser Glu Gly Tyr Cys Ser Ser Arg Ser Cys Tyr Lys Tyr Tyr Tyr
            100                 105                 110
Tyr Gly Met Asp Val Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
```

115 120 125

<210> 14
<211> 109
<212> PRT
<213> Homo sapiens

<400> 14
Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
1 5 10 15
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
20 25 30
Gly Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45
Ala Val Ile Ser Tyr Asp Gly Ser Asn Lys Tyr Tyr Ala Asp Ser Val
50 55 60
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65 70 75 80
Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95
Ala Lys Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
100 105

<210> 15
<211> 128
<212> PRT
<213> Homo sapiens

<400> 15
Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
1 5 10 15
Ser Leu Arg Leu Ser Cys Val Ala Ser Gly Phe Thr Leu Ser Ser Tyr
20 25 30
Gly Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45
Ala Val Thr Ser Tyr Asp Gly Ser Lys Lys Asp Tyr Ala Asp Ser Val
50 55 60
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65 70 75 80
Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95
Val Ser Glu Gly Tyr Cys Asp Ser Ser Ser Cys Tyr Lys Tyr Tyr Tyr
100 105 110
Tyr Gly Met Asp Val Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
115 120 125

<210> 16
<211> 128
<212> PRT
<213> Homo sapiens

<400> 16
Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
1 5 10 15

```
Ser Leu Arg Leu Ser Cys Val Ala Ser Gly Phe Thr Leu Ser Ser Tyr
            20                  25                  30
Gly Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45
Ala Val Thr Ser Tyr Asp Gly Ser Lys Lys Asp Tyr Ala Asp Ser Val
    50                  55                  60
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80
Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95
Val Ser Glu Gly Tyr Cys Asp Ser Thr Ser Cys Tyr Lys Tyr Tyr Tyr
            100                 105                 110
Tyr Gly Met Asp Val Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
        115                 120                 125


<210> 17
<211> 128
<212> PRT
<213> Homo sapiens

<400> 17
Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
 1               5                  10                  15
Ser Leu Arg Leu Ser Cys Val Ala Ser Gly Phe Thr Leu Ser Ser Tyr
            20                  25                  30
Gly Met His Trp Val Arg Gln Ala Leu Gly Lys Gly Leu Glu Trp Val
        35                  40                  45
Ala Val Thr Ser Tyr Asp Gly Ser Lys Lys Asp Tyr Ala Asp Ser Val
    50                  55                  60
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80
Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95
Val Ser Glu Gly Tyr Cys Asp Ser Thr Ser Cys Tyr Lys Tyr Tyr Tyr
            100                 105                 110
Tyr Gly Met Asp Val Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
        115                 120                 125


<210> 18
<211> 112
<212> PRT
<213> Homo sapiens

<400> 18
Asp Ile Val Met Thr Gln Thr Pro Leu Ser Ser Pro Val Thr Leu Gly
 1               5                  10                  15
Gln Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Val His Ser
            20                  25                  30
Asp Gly Asn Thr Tyr Leu Ser Trp Leu Gln Gln Arg Pro Gly Gln Pro
        35                  40                  45
Pro Arg Leu Leu Ile Tyr Lys Ile Ser Asn Arg Phe Ser Gly Val Pro
    50                  55                  60
Asp Arg Phe Ser Gly Ser Gly Ala Gly Thr Asp Phe Thr Leu Lys Ile
65                  70                  75                  80
Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln Ala
```

85 90 95
Thr Gln Phe Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
100 105 110

<210> 19
<211> 112
<212> PRT
<213> Homo sapiens

<400> 19
Asp Thr Val Met Thr Gln Thr Pro Leu Ser Ser His Val Thr Leu Gly
1 5 10 15
Gln Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Val His Ser
20 25 30
Asp Gly Asn Thr Tyr Leu Ser Trp Leu Gln Gln Arg Pro Gly Gln Pro
35 40 45
Pro Arg Leu Leu Ile Tyr Arg Ile Ser Arg Arg Phe Ser Gly Val Pro
50 55 60
Asp Arg Phe Ser Gly Ser Gly Ala Gly Thr Asp Phe Thr Leu Glu Ile
65 70 75 80
Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln Ser
85 90 95
Thr His Val Pro Arg Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
100 105 110

<210> 20
<211> 112
<212> PRT
<213> Homo sapiens

<400> 20
Ala Ile Val Leu Thr Gln Thr Pro Leu Ser Ser Pro Val Thr Leu Gly
1 5 10 15
Gln Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Val His Arg
20 25 30
Asp Gly Asn Thr Tyr Leu Ser Trp Leu Gln Gln Arg Pro Gly Gln Pro
35 40 45
Pro Arg Leu Leu Ile Tyr Lys Ile Ser Asn Arg Phe Ser Gly Val Pro
50 55 60
Asp Arg Phe Ser Gly Ser Gly Ala Gly Thr Asp Phe Thr Leu Lys Ile
65 70 75 80
Ser Arg Val Glu Pro Asp Asp Val Gly Val Tyr Tyr Cys Met His Thr
85 90 95
Thr Gln Leu Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
100 105 110

<210> 21
<211> 112
<212> PRT
<213> Homo sapiens

<400> 21
Asp Ile Val Met Thr Gln Thr Pro Leu Ser Ser Pro Val Thr Leu Gly
1 5 10 15

```
Gln Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Val His Arg
            20                  25                  30
Asp Gly Asn Thr Tyr Leu Ser Trp Leu Gln Gln Arg Pro Gly Gln Pro
        35                  40                  45
Pro Arg Leu Leu Ile Tyr Lys Ile Ser Asn Arg Phe Ser Gly Val Pro
    50                  55                  60
Asp Arg Phe Ser Gly Ser Gly Ala Gly Thr Asp Phe Thr Leu Lys Ile
65                  70                  75                  80
Ser Arg Val Glu Ala Glu Asp Val Gly Ile Tyr Phe Cys Met His Thr
                85                  90                  95
Thr Gln Phe Pro Trp Thr Phe Gly Gln Gly Thr Arg Val Glu Ile Lys
            100                 105                 110
```

<210> 22
<211> 112
<212> PRT
<213> Homo sapiens

<400> 22

```
Asp Ile Val Met Thr Gln Thr Pro Leu Ser Ser Pro Val Thr Leu Gly
 1               5                  10                  15
Gln Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Val His Ser
            20                  25                  30
Asp Gly Asn Thr Tyr Leu Ser Trp Leu Gln Gln Arg Pro Gly Gln Pro
        35                  40                  45
Pro Arg Leu Leu Ile Tyr Lys Ile Ser Asn Arg Phe Ser Gly Val Pro
    50                  55                  60
Asp Arg Phe Ser Gly Ser Gly Ala Gly Thr Asp Phe Thr Leu Lys Ile
65                  70                  75                  80
Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln Ala
                85                  90                  95
Thr Gln Phe Pro Ile Thr Phe Gly Gln Gly Thr Arg Leu Glu Ile Lys
            100                 105                 110
```

<210> 23
<211> 112
<212> PRT
<213> Homo sapiens

<400> 23

```
Asp Ile Val Met Thr Gln Thr Pro Leu Ser Ser Pro Val Thr Leu Gly
 1               5                  10                  15
Gln Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Ile His Thr
            20                  25                  30
Asp Gly Asn Ile Tyr Leu Ser Trp Leu Gln Gln Arg Pro Gly Gln Pro
        35                  40                  45
Pro Arg Leu Leu Ile Tyr Lys Ile Ser Asn Arg Phe Ser Gly Val Pro
    50                  55                  60
Asp Arg Phe Ser Gly Ser Gly Ala Gly Thr Asp Phe Thr Leu Lys Ile
65                  70                  75                  80
Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln Gly
                85                  90                  95
Thr Gln Phe Pro Ile Thr Phe Gly Gln Gly Thr Arg Leu Glu Ile Lys
            100                 105                 110
```

<210> 24
<211> 107
<212> PRT
<213> Homo sapiens

<400> 24
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1 5 10 15
Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Gly Ile Arg Asn Asp
20 25 30
Leu Gly Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Arg Leu Ile
35 40 45
Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly
50 55 60
Ser Gly Ser Gly Thr Glu Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65 70 75 80
Glu Asp Phe Ala Thr Tyr Tyr Cys Leu Gln His Asn Ser Tyr Pro Leu
85 90 95
Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
100 105

<210> 25
<211> 107
<212> PRT
<213> Homo sapiens

<400> 25
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1 5 10 15
Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Gly Ile Arg Asn Asn
20 25 30
Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Arg Leu Ile
35 40 45
Tyr Ala Ala Ser Asn Leu Gln Ser Gly Val Pro Ser Arg Phe Thr Gly
50 55 60
Ser Gly Ser Gly Thr Glu Phe Thr Leu Ile Val Ser Ser Leu Gln Pro
65 70 75 80
Glu Asp Phe Ala Thr Tyr Tyr Cys Leu Gln His His Ser Tyr Pro Leu
85 90 95
Thr Ser Gly Gly Gly Thr Lys Val Glu Ile Lys
100 105

<210> 26
<211> 107
<212> PRT
<213> Homo sapiens

<400> 26
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1 5 10 15
Asp Ser Val Thr Ile Thr Cys Arg Thr Ser Gln Gly Ile Arg Lys Asn
20 25 30
Leu Gly Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Arg Leu Ile
35 40 45

```
Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60
Ser Gly Ser Gly Thr Glu Phe Thr Leu Thr Ile Ser Arg Leu Gln Pro
65                  70                  75                  80
Glu Asp Phe Ala Thr Tyr Tyr Cys Leu Gln His His Ser Tyr Pro Leu
                85                  90                  95
Thr Phe Gly Gly Gly Thr Arg Val Glu Ile Arg
            100                 105
```

<210> 27
<211> 111
<212> PRT
<213> Homo sapiens

<400> 27

```
Asp Ile Val Met Thr Gln Ser Pro Leu Leu Pro Val Thr Pro Gly Glu
 1               5                  10                  15
Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Leu His Ser Asn
            20                  25                  30
Gly Tyr Asn Tyr Leu Asp Trp Tyr Leu Gln Lys Pro Gly Gln Ser Pro
        35                  40                  45
Gln Leu Leu Ile Tyr Leu Gly Ser Asn Arg Ala Ser Gly Val Pro Asp
    50                  55                  60
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile Ser
65                  70                  75                  80
Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln Ala Leu
                85                  90                  95
Gln Thr Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
            100                 105                 110
```

<210> 28
<211> 112
<212> PRT
<213> Homo sapiens

<400> 28

```
Asp Ile Val Met Thr Gln Ser Pro Leu Ser Leu Pro Val Thr Pro Gly
 1               5                  10                  15
Glu Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Leu Tyr Arg
            20                  25                  30
Asn Gly Asn Asn Tyr Leu Asp Trp Tyr Leu Gln Arg Pro Gly Gln Ser
        35                  40                  45
Pro Gln Leu Leu Ile Tyr Leu Gly Ser Asn Arg Ala Ser Gly Val Pro
    50                  55                  60
Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65                  70                  75                  80
Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln Ala
                85                  90                  95
Leu Gln Thr Pro Arg Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
            100                 105                 110
```

<210> 29
<211> 112
<212> PRT

<213> Homo sapiens

<400> 29

```
Asp Ile Val Met Thr Gln Ser Pro Leu Ser Leu Pro Val Thr Pro Gly
 1               5                  10                  15
Glu Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Leu Tyr Arg
            20                  25                  30
Asn Gly Asn Asn Tyr Leu Asp Trp Tyr Leu Gln Lys Pro Gly Gln Ser
        35                  40                  45
Pro Gln Leu Leu Ile Tyr Leu Gly Ser Asn Arg Ala Ser Gly Val Pro
    50                  55                  60
Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Asn Ile
65                  70                  75                  80
Ser Arg Val Glu Ala Glu Asp Val Gly His Tyr Tyr Cys Met Gln Ala
                85                  90                  95
Leu Gln Thr Pro Arg Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
            100                 105                 110
```

<210> 30
<211> 112
<212> PRT
<213> Homo sapiens

<400> 30

```
Asp Ile Val Met Thr Gln Ser Pro Leu Ser Leu Pro Val Thr Pro Gly
 1               5                  10                  15
Glu Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Leu His Ser
            20                  25                  30
Asn Gly Tyr Asn Tyr Leu Asp Trp Tyr Leu Gln Lys Pro Gly Gln Ser
        35                  40                  45
Pro Gln Leu Leu Ile Tyr Leu Gly Ser Asn Arg Ala Ser Gly Val Pro
    50                  55                  60
Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65                  70                  75                  80
Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln Ala
                85                  90                  95
Leu Gln Thr Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
            100                 105                 110
```

<210> 31
<211> 112
<212> PRT
<213> Homo sapiens

<400> 31

```
Asp Ile Val Met Thr Gln Ser Pro Leu Ser Leu Pro Val Thr Pro Gly
 1               5                  10                  15
Glu Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Leu Tyr Arg
            20                  25                  30
Asn Gly Asn Asn Tyr Leu Asp Trp Tyr Leu Gln Arg Pro Gly Gln Ser
        35                  40                  45
Pro Gln Leu Leu Ile Tyr Leu Gly Ser Asn Arg Ala Ser Gly Val Pro
    50                  55                  60
Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65                  70                  75                  80
```

```
Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln Ala
                85                  90                  95
Leu Gln Thr Pro Arg Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
            100                 105                 110


<210> 32
<211> 112
<212> PRT
<213> Homo sapiens

<400> 32
Asp Ile Val Met Thr Gln Ser Pro Leu Ser Leu Pro Val Thr Pro Gly
 1               5                  10                  15
Glu Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Leu Tyr Arg
            20                  25                  30
Asn Gly Asn Asn Tyr Leu Asp Trp Tyr Leu Gln Arg Pro Gly Gln Ser
        35                  40                  45
Pro Gln Leu Leu Ile Tyr Leu Gly Ser Asn Arg Ala Ser Gly Val Pro
    50                  55                  60
Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65                  70                  75                  80
Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln Ala
                85                  90                  95
Leu Gln Thr Pro Arg Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
            100                 105                 110


<210> 33
<211> 112
<212> PRT
<213> Homo sapiens

<400> 33
Asp Ile Val Met Thr Gln Ser Pro Leu Ser Leu Pro Val Thr Pro Gly
 1               5                  10                  15
Glu Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Leu Tyr Arg
            20                  25                  30
Asn Gly Asn Asn Tyr Leu Asp Trp Tyr Leu Gln Arg Pro Gly Gln Ser
        35                  40                  45
Pro Gln Leu Leu Ile Tyr Leu Gly Ser Asn Arg Ala Ser Gly Val Pro
    50                  55                  60
Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65                  70                  75                  80
Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln Ala
                85                  90                  95
Leu Gln Thr Pro Arg Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
            100                 105                 110


<210> 34
<211> 336
<212> DNA
<213> Homo sapiens

<400> 34
gatattgtga tgacccagac tccactctcc tcacctgtca cccttggaca gccggcctcc 60
```

atctcctgca ggtctagtca aagcctcata cacactgatg gaaacatcta tttgagttgg 120
cttcagcaga ggccaggcca gcctccaaga ctcctaattt ataagatttc taatcggttc 180
tctggggtcc cagacagatt cagtggcagt ggggcaggga cagatttcac actgaagatc 240
agcagggtgg aagctgagga tgtgggggtt tattactgca tgcaaggtac acaatttcct 300
atcaccttcg gccaagggac acgactggag attaaa 336

<210> 35
<211> 375
<212> DNA
<213> Homo sapiens

<400> 35
caggtgcagc tgcaggagtc gggcccagga ctggtgaagc cttcacagac cctgtccctc 60
acctgcactg tctctggtgg ctccatcagc agtggtggtt actactggag ctggatccgc 120
cagcacccag ggaagggcct ggagtggatt gggttcatct attacagagg gaacacctac 180
tacaacccgt ccctcaagag tcgagttacc atatcagttg acacgtctaa gaaccagttc 240
tccctgaagc tgagctctgt gactgccgcg gacacggccg tgtattactg tgcgcgagac 300
ggatattgta gtagaaccgg ctgctatggc ggctggttcg acccctgggg ccagggaacc 360
ctggtcacgt ctcct 375

<210> 36
<211> 335
<212> DNA
<213> Homo sapiens

<400> 36
atattgtgat gactcagtct ccactctccc tgcccgtcac ccctggagag ccggcctcca 60
tctcctgcag gtctagtcag agcctcctgt atagaaatgg aaacaactat ttggattggt 120
atctgcagaa gccagggcag tctccacagc tcctgatcta tttgggttct aatcgggcct 180
ccggggtccc tgacaggttc agtggcagtg gatcgggcac agattttaca ctgaacatca 240
gcagagtgga ggctgaggat gttgggcatt attactgcat gcaggctcta caaactcctc 300
ggacgttcgg ccaagggacc aaggtggaaa tcaaa 335

<210> 37
<211> 384
<212> DNA
<213> Homo sapiens

<400> 37
caggtgcagc tggtggagtc tgggggaggc gtggtccagc ctgggaggtc cctgagactc 60
tcctgtgcag cctccggatt caccctcagt agctatggca tgcactgggt ccgccaggct 120
ccaggcaagg ggctggagtg ggtggcagtt atgtcatatg atggaagtaa agaagactat 180
gcagactccg tgaagggccg attcaccatc tctagagaca attccgagaa catgctgtat 240
ctgcaaatga acagcctgag agctgaggac acggctgtat attactgtgt gagcgaagga 300
tattgtagta gtcgtagctg ctataagtac tactactacg gcatggacgt ctggggccaa 360
gggaccacgg tcaccgtctc ctca 384

<210> 38
<211> 336
<212> DNA
<213> Homo sapiens

<400> 38
gatactgtga tgacccagac tccactctcc tcacatgtaa cccttggaca gccggcctcc 60
atctcctgca ggtctagtca aagcctcgta cacagtgatg gaaacaccta cttgagttgg 120
cttcagcaga ggccaggcca acctccaaga ctcctaattt ataggatttc taggcggttc 180
tctggggtcc cagacagatt cagtggcagt ggggcaggga cagatttcac actggaaatc 240

agcagggtgg aggctgagga tgtcggggtt tattactgca tgcaatctac acacgttcct 300
cggacgttcg gccaagggac caaggtggag atcaaa 336

<210> 39
<211> 372
<212> DNA
<213> Homo sapiens

<400> 39
caggtgcagc tggtggagtc tgggggaggc gtggtccagt ctgggaggtc cctgagactc 60
tcctgtgcag cgtctggatt caccttcaga aactatggca tgcactgggt ccgccaggct 120
ccaggcaagg ggctggagtg ggtggcagtt atatggtatg atggaagtga taaatactat 180
gcagactccg tgaggggccg attcaccatc tccagagaca attccaagaa cacgctgtat 240
ctgcaaatga acagcctgag agccgaggac acggctgtgt attactgtgc gagagatggc 300
tacgatattt tgactggtaa tcctagggac tttgactact ggggccaggg aaccctggtc 360
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<210> 40
<211> 348
<212> DNA
<213> Homo sapiens

<400> 40
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tcctgtgcag cctctggatt cacctttagc agctatgcca tgagctgggt ccgccaggct 120
ccagggaagg ggctggagtg ggtctcggct attagtggta gtggtggtag tacaaactac 180
gcagactccg tgaagggccg gttcaccatc tccagagaca attccaagaa cacactgtat 240
ctgcaaatga acagcctgag agccgaggac acggccgtct attactgtgc tgggagcagt 300
ggctggtccg agtactgggg ccagggaacc ctggtcaccg tctcctcg 348

<210> 41
<211> 321
<212> DNA
<213> Homo sapiens

<400> 41
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atcacttgcc gggctagtca gggcattaga aataatttag cctggtatca gcagaaacca 120
gggaaagccc ctaagcgcct gatctatgct gcctccaatt tgcaaagtgg ggtcccatca 180
aggttcaccg gcagtggatc tgggacagaa ttcactctca tagtcagcag cctgcagcct 240
gaagattttg cgacttatta ctgtctacag catcacagtt acccgctcac ttccggcgga 300
gggaccaagg tggagatcaa a 321

<210> 42
<211> 336
<212> DNA
<213> Homo sapiens

<400> 42
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cttcagcaga ggccaggcca gcctccaaga ctcctaattt ataagatttc taaccggttc 180
tctggggtcc cagacagatt cagtggcagt ggggcaggga cagatttcac actgaaaatt 240
agcagggtgg aagctgagga tgtcgggatt tatttctgca tgcatactac acaatttcct 300
tggacgttcg gccaagggac cagggtggaa atcaaa 336

<210> 43

<211> 354
<212> DNA
<213> Homo sapiens

<400> 43
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cagtccccat cgagaggcct tgagtggctg ggaagggcat atcacaggtc caggtggtat 180
tacgagtatg cagtatcggt gaaaagtcga ataaacatca ccccagacac atccaagaac 240
cagttctccc tgcagctgaa ctctgtgact cccgaggaca cggctgtgta ttactgtgca 300
agaggcagtc gctttgacta ctggggccag ggaaccctgg tcaccgtctc ctca 354

<210> 44
<211> 354
<212> DNA
<213> Homo sapiens

<400> 44
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cagtccccag cgagaggcct tgagtggctg ggaaggacat actacaggtc caagtggtat 180
aatgattatg tagtatctgt gaaaagtcga ataaccatca acccagacac atccaagaac 240
cagttctccc tgcagctgaa ctctgtgact cccgaggaca cggctgtgta ttactgtgta 300
agaggcagtc gctttgacta ctggggccag ggaaccctgg tcaccgtctc ctca 354

<210> 45
<211> 336
<212> DNA
<213> Homo sapiens

<400> 45
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cttcagcaga ggccaggcca gcctccaaga ctcctaattt ataagatttc taaccggttc 180
tctggggtcc cagacagatt cagtggcagt ggggcaggga cagatttcac actgaaaatc 240
agcagggtgg aacctgacga tgtcggggtt tattactgca tgcatactac acaacttcct 300
tggacgttcg gccaagggac caaggtggaa atcaaa 336

<210> 46
<211> 336
<212> DNA
<213> Homo sapiens

<400> 46
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tatctgcaga ggccagggca gtctccacaa ctcctgatct atttgggttc taatcgggcc 180
tccggggtcc ctgacaggtt cagtggcagt ggatcaggca cagattttac attgaaaatc 240
ggcagagtgg aggctgagga tgttggggtt tattactgca tgcaggctct acaaactcct 300
cggacgttcg gccaagggac caaggtggaa atcaaa 336

<210> 47
<211> 384
<212> DNA
<213> Homo sapiens

<400> 47

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tcctgtgtag cctctggatt caccctcagt agctatggca tgcactgggt ccgccaggct 120
ccaggcaagg ggctggagtg ggtggcagtg acatcatatg atggaagtaa aaaagactat 180
gcagactccg cgaagggccg attcaccatc tccagagaca attccaagaa cacgctgtat 240
ctgcaaatga acagcctgag agctgaggac acggctgtgt attactgtgt gagcgaagga 300
tattgtagta gtagtagctg ctataagtac tactattacg gtatggacgt ctggggccaa 360
gggaccacgg tcaccgtctc ttca 384

<210> 48
<211> 348
<212> DNA
<213> Homo sapiens

<400> 48
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tcctgtgcag cctctggatt cacctttagc agctatgcca tgagctgggt ccgccaggct 120
ccagggaagg ggctggagtg ggtctcggct attagtggta gtggtggtag cacaaattac 180
gcagactccg tgaagggccg gttcaccatc tccagagaca attccaagaa cacgctgtat 240
ctgcaagtga acagcctgag agtcgaggac acggccgtat attactgtgc tgggagcagt 300
ggctggtccg agtactgggg ccagggaacc ctggtcaccg tctcctca 348

<210> 49
<211> 321
<212> DNA
<213> Homo sapiens

<400> 49
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atcacttgcc ggacaagtca gggcattaga aaaaatttag gctggtatca gcagaaacca 120
gggaaagccc ctaagcgcct gatctatgct gcatccagtt tacaaagtgg ggtcccatca 180
aggttcagcg gcagtggatc tgggacagaa ttcactctca caatccgcag cctgcagcct 240
gaagattttg caacttatta ctgtctccag catcatagtt acccgctcac tttcggcgga 300
gggaccaggg tggagatcag a 321

<210> 50
<211> 336
<212> DNA
<213> Homo sapiens

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tatctgcaga ggccagggca gtctccacaa ctcctgatct atttgggttc taatcgggcc 180
tccggggtcc ctgacaggtt cagtggcagt ggatcaggca cagattttac actgaaaatc 240
agcagagtgg aggctgagga tgttggggtt tattactgca tgcaggctct acaaactcct 300
cggacgttcg gccaagggac caaggtggaa atcaaa 336

<210> 51
<211> 384
<212> DNA
<213> Homo sapiens

<400> 51
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tcctgtgtag cctctggatt caccctcagt agctatggca tgcactgggt ccgccaggct 120
ccaggcaagg ggctggagtg ggtggcagtg acatcatatg atggaagtaa aaaagactat 180
gcagactccg tgaagggccg attcaccatc tccagagaca attccaagaa cacgctgtat 240

ctgcaaatga acagcctgag agctgaggac acggctgtgt attactgtgt gagcgaagga 300
tattgtgata gtagtagctg ctataagtac tactactacg gtatggacgt ctggggccaa 360
gggaccacgg tcaccgtctc ttca 384

<210> 52
<211> 336
<212> DNA
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tatctgcaga ggccagggca gtctccacaa ctcctgatct atttgggttc taatcgggcc 180
tccggggtcc ctgacaggtt cagtggcagt ggatcaggca cagattttac actgaaaatc 240
agcagagtgg aggctgagga tgttggggtt tattactgca tgcaggctct acaaactcct 300
cggacgttcg gccaagggac caaggtggaa atcaaa 336

<210> 53
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<212> DNA
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tcctgtgtag cctctggatt caccctcagt agctatggca tgcactgggt ccgccaggct 120
ctaggcaagg ggctggagtg ggtggcagtg acatcatatg atggaagtaa aaaagactat 180
gcagactccg tgaagggccg attcaccatc tccagagaca attccaagaa cacgctgtat 240
ctgcaaatga acagcctgag agctgaggac acggctgtgt attactgtgt gagcgaagga 300
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gggaccacgg tcaccgtctc ttca 384

<210> 54
<211> 336
<212> DNA
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<400> 54
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tatctgcaga ggccagggca gtctccacaa ctcctgatct atttgggttc taatcgggcc 180
tccggggtcc ctgacaggtt cagtggcagt ggatcaggca cagattttac actgaaaatc 240
agcagagtgg aggctgagga tgttggggtt tattactgca tgcaggctct acaaactcct 300
cggacgttcg gccaagggac caaggtggaa atcaaa 336

<210> 55
<211> 384
<212> DNA
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<400> 55
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ccaggcaagg ggctggagtg ggtggcagtg acatcatatg atggaagtaa aaaagactat 180
gcagactccg tgaagggccg attcaccatc tccagagaca attccaagaa cacgctgtat 240
ctgcaaatga acagcctgag agctgaggac acggctgtgt attactgtgt gagcgaagga 300
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gggaccacgg tcaccgtctc ttca 384

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<220>
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1 5 10

<210> 57
<211> 10
<212> PRT
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<220>
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<400> 57
Glu Glu Lys Lys Gly Asn Tyr Val Val Thr
1 5 10

<210> 58
<211> 5
<212> PRT
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<220>
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<400> 58
Leu Glu Glu Lys Lys
1 5

<210> 59
<211> 12
<212> PRT
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<220>
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<400> 59
Leu Glu Glu Lys Lys Gly Asn Tyr Val Val Thr Asp
1 5 10

<210> 60
<211> 4
<212> PRT
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<220>
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<400> 60
Glu Lys Asn Tyr
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<210> 61
<211> 5
<212> PRT
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<220>
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<400> 61
Glu Glu Lys Gly Asn
1 5

<210> 62
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
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<400> 62
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<210> 63
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic oligonucleotide primer sequence

<400> 63
cggatctcga gccggagccc agcactttga tctt 34

<210> 64
<211> 35
<212> DNA
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<220>
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<210> 65
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<212> DNA
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<220>
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<400> 65
ctttcttttc ctccagagcc 20

<210> 66
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<220>
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<400> 66
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<210> 67
<211> 35
<212> DNA
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<220>
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<400> 67
cggatctcga gctcaagaga gcttggttgg gagct 35

<210> 68
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<212> DNA
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<210> 69
<211> 37
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ataagaatgc ggccgctcat ttacccggag agcggga 37

<210> 70
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ctactagcta gccaccatgc gaccctccgg ga 32

<210> 71
<211> 26
<212> DNA
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<220>
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<210> 72
<211> 12
<212> PRT
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Ala Leu Glu Glu Lys Lys Gly Asn Tyr Val Val Thr
1 5 10

<210> 73
<211> 12
<212> PRT
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<220>
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<400> 73
Glu Glu Lys Lys Gly Asn Tyr Val Val Thr Asp His
1 5 10

<210> 74
<211> 12
<212> PRT
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<400> 74
Glu Lys Lys Gly Asn Tyr Val Val Thr Asp His Gly
1 5 10

<210> 75
<211> 12
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<220>
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<400> 75
Lys Lys Gly Asn Tyr Val Val Thr Asp His Gly Ser
1 5 10

<210> 76
<211> 12
<212> PRT
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<400> 76
Lys Gly Asn Tyr Val Val Thr Asp His Gly Ser Cys
1 5 10

<210> 77
<211> 12
<212> PRT
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<220>
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<400> 77
Gly Asn Tyr Val Val Thr Asp His Gly Ser Cys Val
1 5 10

<210> 78
<211> 12
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<400> 78
Asn Tyr Val Val Thr Asp His Gly Ser Cys Val Arg
1 5 10

<210> 79
<211> 12
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Tyr Val Val Thr Asp His Gly Ser Cys Val Arg Ala
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<210> 80
<211> 12
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<400> 80
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1 5 10

<210> 81
<211> 12
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<400> 81
Leu Ala Glu Lys Lys Gly Asn Tyr Val Val Thr Asp
1 5 10

<210> 82
<211> 12
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<400> 82
Leu Glu Ala Lys Lys Gly Asn Tyr Val Val Thr Asp
1 5 10

<210> 83
<211> 12
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<400> 83
Leu Glu Glu Ala Lys Gly Asn Tyr Val Val Thr Asp

1 5 10

<210> 84
<211> 12
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<220>
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<400> 84
Leu Glu Glu Lys Ala Gly Asn Tyr Val Val Thr Asp
1 5 10

<210> 85
<211> 12
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<400> 85
Leu Glu Glu Lys Lys Ala Asn Tyr Val Val Thr Asp
1 5 10

<210> 86
<211> 12
<212> PRT
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<400> 86
Leu Glu Glu Lys Lys Gly Ala Tyr Val Val Thr Asp
1 5 10

<210> 87
<211> 12
<212> PRT
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<220>
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<400> 87
Leu Glu Glu Lys Lys Gly Asn Ala Val Val Thr Asp
1 5 10

<210> 88
<211> 12

<212> PRT
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<220>
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<400> 88
Leu Glu Glu Lys Lys Gly Asn Tyr Ala Val Thr Asp
1 5 10

<210> 89
<211> 12
<212> PRT
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<220>
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<400> 89
Leu Glu Glu Lys Lys Gly Asn Tyr Val Ala Thr Asp
1 5 10

<210> 90
<211> 12
<212> PRT
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<220>
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<400> 90
Leu Glu Glu Lys Lys Gly Asn Tyr Val Val Ala Asp
1 5 10

<210> 91
<211> 12
<212> PRT
<213> Artificial Sequence

<220>
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<400> 91
Leu Glu Glu Lys Lys Gly Asn Tyr Val Val Thr Ala
1 5 10

<210> 92
<211> 22
<212> PRT
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<220>
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1 5 10 15
Gly Ser Cys Val Arg Ala
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<210> 93
<211> 19
<212> PRT
<213> Artificial Sequence

<220>
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<400> 93
Leu Glu Glu Lys Lys Gly Asn Tyr Val Val Thr Asp His Gly Ser Cys
1 5 10 15
Val Arg Ala

<210> 94
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
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<400> 94
Glu Glu Lys Lys Gly Asn Tyr Val Val Thr
1 5 10

<210> 95
<211> 12
<212> PRT
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<220>
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<400> 95
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1 5 10

<210> 96
<211> 6
<212> PRT
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<400> 96
Tyr Val Val Thr Asp His
1 5

<210> 97
<211> 5
<212> PRT
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<220>
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<400> 97
Tyr Val Val Thr Asp
1 5

<210> 98
<211> 10
<212> PRT
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<220>
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<400> 98
Glu Glu Lys Lys Gly Asn Tyr Val Val Thr
1 5 10

<210> 99
<211> 6
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<220>
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<400> 99
Gly Asn Tyr Val Val Thr
1 5

<210> 100
<211> 23
<212> PRT
<213> Artificial Sequence

<220>
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1 5 10 15
Gln Pro Ala Ser Ile Ser Cys
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<210> 101
<211> 16
<212> PRT
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<220>
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<400> 101
Arg Ser Ser Gln Ser Leu Val His Ser Asp Gly Asn Thr Tyr Leu Ser
1 5 10 15

<210> 102
<211> 14
<212> PRT
<213> Artificial Sequence

<220>
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<400> 102
Trp Leu Gln Gln Arg Pro Gly Pro Pro Arg Leu Leu Ile Tyr
1 5 10

<210> 103
<211> 7
<212> PRT
<213> Artificial Sequence

<220>
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<400> 103
Arg Ile Ser Arg Arg Phe Ser
1 5

<210> 104
<211> 32
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic peptide sequence

<400> 104
Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ala Gly Thr Asp Phe Thr
1 5 10 15
Leu Glu Ile Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys
20 25 30

<210> 105

<211> 9
<212> PRT
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<220>
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<400> 105
Met Gln Ser Thr His Val Pro Arg Thr
 1   5

<210> 106
<211> 9
<212> PRT
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<220>
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<400> 106
Phe Gly Gln Thr Lys Val Glu Ile Lys
 1   5

<210> 107
<211> 30
<212> PRT
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<220>
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<400> 107
Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Ser Gly Arg
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Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Arg
            20   25   30

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<211> 5
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<400> 108
Asn Tyr Gly Met His
 1   5

<210> 109
<211> 14
<212> PRT
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<220>
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<400> 109
Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Ala
1 5 10

<210> 110
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<212> PRT
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<400> 110
Val Ile Trp Tyr Asp Gly Ser Asp Lys Tyr Tyr Ala Asp Ser Val Arg
1 5 10 15
Gly

<210> 111
<211> 32
<212> PRT
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<220>
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<400> 111
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1 5 10 15
Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg
20 25 30

<210> 112
<211> 15
<212> PRT
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<400> 113
Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
1 5 10

<210> 114
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<212> PRT
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Gln Pro Ala Ser Ile Ser Cys
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<210> 115
<211> 15
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<223> Synthetic peptide sequence

<400> 115
Trp Leu His Gln Arg Pro Gly Gln Pro Pro Arg Leu Leu Ile Tyr
1 5 10 15

<210> 116
<211> 7
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic peptide sequence

<400> 116
Lys Ile Ser Asn Arg Phe Ser
1 5

<210> 117
<211> 32
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic peptide sequence

<400> 117
Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ala Gly Thr Ala Phe Thr
1 5 10 15
Leu Lys Ile Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys
20 25 30

<210> 118
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic peptide sequence

<400> 118
Met Gln Ala Thr Gln Leu Pro Arg Thr
1 5

<210> 119
<211> 11
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic peptide sequence

<400> 119
Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
1 5 10

<210> 120
<211> 30
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic peptide sequence

<400> 120
Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
1 5 10 15
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
20 25 30

<210> 121
<211> 5
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic peptide sequence

<400> 121

Ser Tyr Gly Met His
1 5

<210> 122
<211> 14
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic peptide sequence

<400> 122
Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Ala
1 5 10

<210> 123
<211> 17
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic peptide sequence

<400> 123
Val Ile Trp Tyr Asp Gly Ser Asn Lys Tyr Tyr Val Asp Ser Val Lys
1 5 10 15
Gly

<210> 124
<211> 32
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic peptide sequence

<400> 124
Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr Leu Gln
1 5 10 15
Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg
20 25 30

<210> 125
<211> 11
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic peptide sequence

<400> 125
Asp Gly Trp Gln Gln Leu Ala Pro Phe Asp Tyr

1 5 10

<210> 126
<211> 12
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic peptide sequence

<400> 126
Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala
1 5 10

<210> 127
<211> 6
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic peptide sequence

<400> 127
Glu Glu Lys Lys Gly Asn
1 5

<210> 128
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic peptide sequence

<400> 128
ataaaagctt ctggaggaaa agaaaggtaa tta 33

<210> 129
<211> 33
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic peptide sequence

<400> 129
Thr Thr Ala Thr Thr Gly Gly Thr Ala Cys Cys Thr Cys Ala Gly Gly
1 5 10 15
Cys Gly Ala Thr Gly Gly Ala Cys Gly Gly Gly Ala Thr Cys Thr Thr
20 25 30
Ala

<210> 130
<211> 14
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic peptide sequence

<400> 130
Leu Glu Glu Lys Lys Gly Asn Tyr Val Val Thr Asp His Gly
1 5 10

<210> 131
<211> 7
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic peptide sequence

<400> 131
Glu Glu Lys Lys Gly Asn Tyr
1 5

<210> 132
<211> 11
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic peptide sequence

<400> 132
Leu Glu Glu Lys Lys Gly Asn Tyr Val Val Thr
1 5 10

<210> 133
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic peptide sequence

<400> 133
Leu Glu Glu Lys Lys Gly Asn Tyr
1 5

<210> 134
<211> 1186
<212> PRT
<213> Homo sapiens

<400> 134

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Leu Glu Glu Lys Lys Val Cys Gln Gly Thr Ser Asn Lys Leu Thr Gln
 1               5                  10                  15
Leu Gly Thr Phe Glu Asp His Phe Leu Ser Leu Gln Arg Met Phe Asn
            20                  25                  30
Asn Cys Glu Val Val Leu Gly Asn Leu Glu Ile Thr Tyr Val Gln Arg
        35                  40                  45
Asn Tyr Asp Leu Ser Phe Leu Lys Thr Ile Gln Glu Val Ala Gly Tyr
    50                  55                  60
Val Leu Ile Ala Leu Asn Thr Val Glu Arg Ile Pro Leu Glu Asn Leu
65                  70                  75                  80
Gln Ile Ile Arg Gly Asn Met Tyr Tyr Glu Asn Ser Tyr Ala Leu Ala
                85                  90                  95
Val Leu Ser Asn Tyr Asp Ala Asn Lys Thr Gly Leu Lys Glu Leu Pro
            100                 105                 110
Met Arg Asn Leu Gln Glu Ile Leu His Gly Ala Val Arg Phe Ser Asn
        115                 120                 125
Asn Pro Ala Leu Cys Asn Val Glu Ser Ile Gln Trp Arg Asp Ile Val
    130                 135                 140
Ser Ser Asp Phe Leu Ser Asn Met Ser Met Asp Phe Gln Asn His Leu
145                 150                 155                 160
Gly Ser Cys Gln Lys Cys Asp Pro Ser Cys Pro Asn Gly Ser Cys Trp
                165                 170                 175
Gly Ala Gly Glu Glu Asn Cys Gln Lys Leu Thr Lys Ile Ile Cys Ala
            180                 185                 190
Gln Gln Cys Ser Gly Arg Cys Arg Gly Lys Ser Pro Ser Asp Cys Cys
        195                 200                 205
His Asn Gln Cys Ala Ala Gly Cys Thr Gly Pro Arg Glu Ser Asp Cys
    210                 215                 220
Leu Val Cys Arg Lys Phe Arg Asp Glu Ala Thr Cys Lys Asp Thr Cys
225                 230                 235                 240
Pro Pro Leu Met Leu Tyr Asn Pro Thr Thr Tyr Gln Met Asp Val Asn
                245                 250                 255
Pro Glu Gly Lys Tyr Ser Phe Gly Ala Thr Cys Val Lys Lys Cys Pro
            260                 265                 270
Arg Asn Tyr Val Val Thr Asp His Gly Ser Cys Val Arg Ala Cys Gly
        275                 280                 285
Ala Asp Ser Tyr Glu Met Glu Glu Asp Gly Val Arg Lys Cys Lys Lys
    290                 295                 300
Cys Glu Gly Pro Cys Arg Lys Val Cys Asn Gly Ile Gly Ile Gly Glu
305                 310                 315                 320
Phe Lys Asp Ser Leu Ser Ile Asn Ala Thr Asn Ile Lys His Phe Lys
                325                 330                 335
Asn Cys Thr Ser Ile Ser Gly Asp Leu His Ile Leu Pro Val Ala Phe
            340                 345                 350
Arg Gly Asp Ser Phe Thr His Thr Pro Pro Leu Asp Pro Gln Glu Leu
        355                 360                 365
Asp Ile Leu Lys Thr Val Lys Glu Ile Thr Gly Phe Leu Leu Ile Gln
    370                 375                 380
Ala Trp Pro Glu Asn Arg Thr Asp Leu His Ala Phe Glu Asn Leu Glu
385                 390                 395                 400
Ile Ile Arg Gly Arg Thr Lys Gln His Gly Gln Phe Ser Leu Ala Val
                405                 410                 415
Val Ser Leu Asn Ile Thr Ser Leu Gly Leu Arg Ser Leu Lys Glu Ile
            420                 425                 430
Ser Asp Gly Asp Val Ile Ile Ser Gly Asn Lys Asn Leu Cys Tyr Ala
        435                 440                 445
```

```
Asn Thr Ile Asn Trp Lys Lys Leu Phe Gly Thr Ser Gly Gln Lys Thr
    450                 455                 460
Lys Ile Ile Ser Asn Arg Gly Glu Asn Ser Cys Lys Ala Thr Gly Gln
465                 470                 475                 480
Val Cys His Ala Leu Cys Ser Pro Glu Gly Cys Trp Gly Pro Glu Pro
                485                 490                 495
Arg Asp Cys Val Ser Cys Arg Asn Val Ser Arg Gly Arg Glu Cys Val
            500                 505                 510
Asp Lys Cys Asn Leu Leu Glu Gly Glu Pro Arg Glu Phe Val Glu Asn
        515                 520                 525
Ser Glu Cys Ile Gln Cys His Pro Glu Cys Leu Pro Gln Ala Met Asn
    530                 535                 540
Ile Thr Cys Thr Gly Arg Gly Pro Asp Asn Cys Ile Gln Cys Ala His
545                 550                 555                 560
Tyr Ile Asp Gly Pro His Cys Val Lys Thr Cys Pro Ala Gly Val Met
                565                 570                 575
Gly Glu Asn Asn Thr Leu Val Trp Lys Tyr Ala Asp Ala Gly His Val
            580                 585                 590
Cys His Leu Cys His Pro Asn Cys Thr Tyr Gly Cys Thr Gly Pro Gly
        595                 600                 605
Leu Glu Gly Cys Pro Thr Asn Gly Pro Lys Ile Pro Ser Ile Ala Thr
    610                 615                 620
Gly Met Val Gly Ala Leu Leu Leu Leu Leu Val Val Ala Leu Gly Ile
625                 630                 635                 640
Gly Leu Phe Met Arg Arg Arg His Ile Val Arg Lys Arg Thr Leu Arg
                645                 650                 655
Arg Leu Leu Gln Glu Arg Glu Leu Val Glu Pro Leu Thr Pro Ser Gly
            660                 665                 670
Glu Ala Pro Asn Gln Ala Leu Leu Arg Ile Leu Lys Glu Thr Glu Phe
        675                 680                 685
Lys Lys Ile Lys Val Leu Gly Ser Gly Ala Phe Gly Thr Val Tyr Lys
    690                 695                 700
Gly Leu Trp Ile Pro Glu Gly Glu Lys Val Lys Ile Pro Val Ala Ile
705                 710                 715                 720
Lys Glu Leu Arg Glu Ala Thr Ser Pro Lys Ala Asn Lys Glu Ile Leu
                725                 730                 735
Asp Glu Ala Tyr Val Met Ala Ser Val Asp Asn Pro His Val Cys Arg
            740                 745                 750
Leu Leu Gly Ile Cys Leu Thr Ser Thr Val Gln Leu Ile Thr Gln Leu
        755                 760                 765
Met Pro Phe Gly Cys Leu Leu Asp Tyr Val Arg Glu His Lys Asp Asn
    770                 775                 780
Ile Gly Ser Gln Tyr Leu Leu Asn Trp Cys Val Gln Ile Ala Lys Gly
785                 790                 795                 800
Met Asn Tyr Leu Glu Asp Arg Arg Leu Val His Arg Asp Leu Ala Ala
                805                 810                 815
Arg Asn Val Leu Val Lys Thr Pro Gln His Val Lys Ile Thr Asp Phe
            820                 825                 830
Gly Leu Ala Lys Leu Leu Gly Ala Glu Glu Lys Glu Tyr His Ala Glu
        835                 840                 845
Gly Gly Lys Val Pro Ile Lys Trp Met Ala Leu Glu Ser Ile Leu His
    850                 855                 860
Arg Ile Tyr Thr His Gln Ser Asp Val Trp Ser Tyr Gly Val Thr Val
865                 870                 875                 880
Trp Glu Leu Met Thr Phe Gly Ser Lys Pro Tyr Asp Gly Ile Pro Ala
                885                 890                 895
Ser Glu Ile Ser Ser Ile Leu Glu Lys Gly Glu Arg Leu Pro Gln Pro
```

```
            900                 905                 910
Pro Ile Cys Thr Ile Asp Val Tyr Met Ile Met Val Lys Cys Trp Met
        915                 920                 925
Ile Asp Ala Asp Ser Arg Pro Lys Phe Arg Glu Leu Ile Ile Glu Phe
    930                 935                 940
Ser Lys Met Ala Arg Asp Pro Gln Arg Tyr Leu Val Ile Gln Gly Asp
945                 950                 955                 960
Glu Arg Met His Leu Pro Ser Pro Thr Asp Ser Asn Phe Tyr Arg Ala
                965                 970                 975
Leu Met Asp Glu Glu Asp Met Asp Asp Val Val Asp Ala Asp Glu Tyr
            980                 985                 990
Leu Ile Pro Gln Gln Gly Phe Phe Ser Ser Pro Ser Thr Ser Arg Thr
        995                 1000                1005
Pro Leu Leu Ser Ser Leu Ser Ala Thr Ser Asn Asn Ser Thr Val Ala
    1010                1015                1020
Cys Ile Asp Arg Asn Gly Leu Gln Ser Cys Pro Ile Lys Glu Asp Ser
1025                1030                1035                1040
Phe Leu Gln Arg Tyr Ser Ser Asp Pro Thr Gly Ala Leu Thr Glu Asp
                1045                1050                1055
Ser Ile Asp Asp Thr Phe Leu Pro Val Pro Glu Tyr Ile Asn Gln Ser
            1060                1065                1070
Val Pro Lys Arg Pro Ala Gly Ser Val Gln Asn Pro Val Tyr His Asn
        1075                1080                1085
Gln Pro Leu Asn Pro Ala Pro Ser Arg Asp Pro His Tyr Gln Asp Pro
    1090                1095                1100
His Ser Thr Ala Val Gly Asn Pro Glu Tyr Leu Asn Thr Val Gln Pro
1105                1110                1115                1120
Thr Cys Val Asn Ser Thr Phe Asp Ser Pro Ala His Trp Ala Gln Lys
                1125                1130                1135
Gly Ser His Gln Ile Ser Leu Asp Asn Pro Asp Tyr Gln Gln Asp Phe
            1140                1145                1150
Phe Pro Lys Glu Ala Lys Pro Asn Gly Ile Phe Lys Gly Ser Thr Ala
        1155                1160                1165
Glu Asn Ala Glu Tyr Leu Arg Val Ala Pro Gln Ser Ser Glu Phe Ile
    1170                1175                1180
Gly Ala
1185


<210> 135
<211> 919
<212> PRT
<213> Homo sapiens

<400> 135
Leu Glu Glu Lys Lys Gly Asn Tyr Val Val Thr Asp His Gly Ser Cys
 1               5                  10                  15
Val Arg Ala Cys Gly Ala Asp Ser Tyr Glu Met Glu Glu Asp Gly Val
            20                  25                  30
Arg Lys Cys Lys Lys Cys Glu Gly Pro Cys Arg Lys Val Cys Asn Gly
        35                  40                  45
Ile Gly Ile Gly Glu Phe Lys Asp Ser Leu Ser Ile Asn Ala Thr Asn
    50                  55                  60
Ile Lys His Phe Lys Asn Cys Thr Ser Ile Ser Gly Asp Leu His Ile
65                  70                  75                  80
Leu Pro Val Ala Phe Arg Gly Asp Ser Phe Thr His Thr Pro Pro Leu
                85                  90                  95
```

```
Asp Pro Gln Glu Leu Asp Ile Leu Lys Thr Val Lys Glu Ile Thr Gly
            100                 105                 110
Phe Leu Leu Ile Gln Ala Trp Pro Glu Asn Arg Thr Asp Leu His Ala
        115                 120                 125
Phe Glu Asn Leu Glu Ile Ile Arg Gly Arg Thr Lys Gln His Gly Gln
    130                 135                 140
Phe Ser Leu Ala Val Val Ser Leu Asn Ile Thr Ser Leu Gly Leu Arg
145                 150                 155                 160
Ser Leu Lys Glu Ile Ser Asp Gly Asp Val Ile Ile Ser Gly Asn Lys
                165                 170                 175
Asn Leu Cys Tyr Ala Asn Thr Ile Asn Trp Lys Lys Leu Phe Gly Thr
            180                 185                 190
Ser Gly Gln Lys Thr Lys Ile Ile Ser Asn Arg Gly Glu Asn Ser Cys
        195                 200                 205
Lys Ala Thr Gly Gln Val Cys His Ala Leu Cys Ser Pro Glu Gly Cys
    210                 215                 220
Trp Gly Pro Glu Pro Arg Asp Cys Val Ser Cys Arg Asn Val Ser Arg
225                 230                 235                 240
Gly Arg Glu Cys Val Asp Lys Cys Asn Leu Leu Glu Gly Glu Pro Arg
                245                 250                 255
Glu Phe Val Glu Asn Ser Glu Cys Ile Gln Cys His Pro Glu Cys Leu
            260                 265                 270
Pro Gln Ala Met Asn Ile Thr Cys Thr Gly Arg Gly Pro Asp Asn Cys
        275                 280                 285
Ile Gln Cys Ala His Tyr Ile Asp Gly Pro His Cys Val Lys Thr Cys
    290                 295                 300
Pro Ala Gly Val Met Gly Glu Asn Asn Thr Leu Val Trp Lys Tyr Ala
305                 310                 315                 320
Asp Ala Gly His Val Cys His Leu Cys His Pro Asn Cys Thr Tyr Gly
                325                 330                 335
Cys Thr Gly Pro Gly Leu Glu Gly Cys Pro Thr Asn Gly Pro Lys Ile
            340                 345                 350
Pro Ser Ile Ala Thr Gly Met Val Gly Ala Leu Leu Leu Leu Leu Val
        355                 360                 365
Val Ala Leu Gly Ile Gly Leu Phe Met Arg Arg Arg His Ile Val Arg
    370                 375                 380
Lys Arg Thr Leu Arg Arg Leu Leu Gln Glu Arg Glu Leu Val Glu Pro
385                 390                 395                 400
Leu Thr Pro Ser Gly Glu Ala Pro Asn Gln Ala Leu Leu Arg Ile Leu
                405                 410                 415
Lys Glu Thr Glu Phe Lys Lys Ile Lys Val Leu Gly Ser Gly Ala Phe
            420                 425                 430
Gly Thr Val Tyr Lys Gly Leu Trp Ile Pro Glu Gly Glu Lys Val Lys
        435                 440                 445
Ile Pro Val Ala Ile Lys Glu Leu Arg Glu Ala Thr Ser Pro Lys Ala
    450                 455                 460
Asn Lys Glu Ile Leu Asp Glu Ala Tyr Val Met Ala Ser Val Asp Asn
465                 470                 475                 480
Pro His Val Cys Arg Leu Leu Gly Ile Cys Leu Thr Ser Thr Val Gln
                485                 490                 495
Leu Ile Thr Gln Leu Met Pro Phe Gly Cys Leu Leu Asp Tyr Val Arg
            500                 505                 510
Glu His Lys Asp Asn Ile Gly Ser Gln Tyr Leu Leu Asn Trp Cys Val
        515                 520                 525
Gln Ile Ala Lys Gly Met Asn Tyr Leu Glu Asp Arg Arg Leu Val His
    530                 535                 540
Arg Asp Leu Ala Ala Arg Asn Val Leu Val Lys Thr Pro Gln His Val
```

```
545                 550                 555                 560
Lys Ile Thr Asp Phe Gly Leu Ala Lys Leu Leu Gly Ala Glu Glu Lys
                565                 570                 575
Glu Tyr His Ala Glu Gly Gly Lys Val Pro Ile Lys Trp Met Ala Leu
            580                 585                 590
Glu Ser Ile Leu His Arg Ile Tyr Thr His Gln Ser Asp Val Trp Ser
        595                 600                 605
Tyr Gly Val Thr Val Trp Glu Leu Met Thr Phe Gly Ser Lys Pro Tyr
    610                 615                 620
Asp Gly Ile Pro Ala Ser Glu Ile Ser Ser Ile Leu Glu Lys Gly Glu
625                 630                 635                 640
Arg Leu Pro Gln Pro Pro Ile Cys Thr Ile Asp Val Tyr Met Ile Met
                645                 650                 655
Val Lys Cys Trp Met Ile Asp Ala Asp Ser Arg Pro Lys Phe Arg Glu
            660                 665                 670
Leu Ile Ile Glu Phe Ser Lys Met Ala Arg Asp Pro Gln Arg Tyr Leu
        675                 680                 685
Val Ile Gln Gly Asp Glu Arg Met His Leu Pro Ser Pro Thr Asp Ser
    690                 695                 700
Asn Phe Tyr Arg Ala Leu Met Asp Glu Glu Asp Met Asp Asp Val Val
705                 710                 715                 720
Asp Ala Asp Glu Tyr Leu Ile Pro Gln Gln Gly Phe Phe Ser Ser Pro
                725                 730                 735
Ser Thr Ser Arg Thr Pro Leu Leu Ser Ser Leu Ser Ala Thr Ser Asn
            740                 745                 750
Asn Ser Thr Val Ala Cys Ile Asp Arg Asn Gly Leu Gln Ser Cys Pro
        755                 760                 765
Ile Lys Glu Asp Ser Phe Leu Gln Arg Tyr Ser Ser Asp Pro Thr Gly
    770                 775                 780
Ala Leu Thr Glu Asp Ser Ile Asp Asp Thr Phe Leu Pro Val Pro Glu
785                 790                 795                 800
Tyr Ile Asn Gln Ser Val Pro Lys Arg Pro Ala Gly Ser Val Gln Asn
                805                 810                 815
Pro Val Tyr His Asn Gln Pro Leu Asn Pro Ala Pro Ser Arg Asp Pro
            820                 825                 830
His Tyr Gln Asp Pro His Ser Thr Ala Val Gly Asn Pro Glu Tyr Leu
        835                 840                 845
Asn Thr Val Gln Pro Thr Cys Val Asn Ser Thr Phe Asp Ser Pro Ala
    850                 855                 860
His Trp Ala Gln Lys Gly Ser His Gln Ile Ser Leu Asp Asn Pro Asp
865                 870                 875                 880
Tyr Gln Gln Asp Phe Phe Pro Lys Glu Ala Lys Pro Asn Gly Ile Phe
                885                 890                 895
Lys Gly Ser Thr Ala Glu Asn Ala Glu Tyr Leu Arg Val Ala Pro Gln
            900                 905                 910
Ser Ser Glu Phe Ile Gly Ala
        915
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<210> 136
<211> 268
<212> PRT
<213> Homo sapiens

<400> 136

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Val Cys Gln Gly Thr Ser Asn Lys Leu Thr Gln Leu Gly Thr Phe Glu
 1               5                  10                  15
```

```
Asp His Phe Leu Ser Leu Gln Arg Met Phe Asn Asn Cys Glu Val Val
            20                  25                  30
Leu Gly Asn Leu Glu Ile Thr Tyr Val Gln Arg Asn Tyr Asp Leu Ser
        35                  40                  45
Phe Leu Lys Thr Ile Gln Glu Val Ala Gly Tyr Val Leu Ile Ala Leu
    50                  55                  60
Asn Thr Val Glu Arg Ile Pro Leu Glu Asn Leu Gln Ile Ile Arg Gly
65                  70                  75                  80
Asn Met Tyr Tyr Glu Asn Ser Tyr Ala Leu Ala Val Leu Ser Asn Tyr
                85                  90                  95
Asp Ala Asn Lys Thr Gly Leu Lys Glu Leu Pro Met Arg Asn Leu Gln
            100                 105                 110
Glu Ile Leu His Gly Ala Val Arg Phe Ser Asn Asn Pro Ala Leu Cys
        115                 120                 125
Asn Val Glu Ser Ile Gln Trp Arg Asp Ile Val Ser Ser Asp Phe Leu
    130                 135                 140
Ser Asn Met Ser Met Asp Phe Gln Asn His Leu Gly Ser Cys Gln Lys
145                 150                 155                 160
Cys Asp Pro Ser Cys Pro Asn Gly Ser Cys Trp Gly Ala Gly Glu Glu
                165                 170                 175
Asn Cys Gln Lys Leu Thr Lys Ile Ile Cys Ala Gln Gln Cys Ser Gly
            180                 185                 190
Arg Cys Arg Gly Lys Ser Pro Ser Asp Cys Cys His Asn Gln Cys Ala
        195                 200                 205
Ala Gly Cys Thr Gly Pro Arg Glu Ser Asp Cys Leu Val Cys Arg Lys
    210                 215                 220
Phe Arg Asp Glu Ala Thr Cys Lys Asp Thr Cys Pro Pro Leu Met Leu
225                 230                 235                 240
Tyr Asn Pro Thr Thr Tyr Gln Met Asp Val Asn Pro Glu Gly Lys Tyr
                245                 250                 255
Ser Phe Gly Ala Thr Cys Val Lys Lys Cys Pro Arg
            260                 265
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<210> 137
<211> 512
<212> DNA
<213> Homo sapiens

<400> 137
caggtgcagc tggtggagtc tgggggaggc gtggtccagc ctgggaggtc cctgagactc 60
tcctgtgcag cgtctggatt caccttcagt agctatggca tgcactgggt ccgccaggct 120
ccaggcaagg ggctggagtg ggtggcagtt atatggtatg atggaagtaa taaatactat 180
gtagactccg tgaagggccg attcaccatc tccagagaca attccaagaa cacgctgtat 240
ctgcaaatga acagcctgag agccgaggac acggctgtgt attactgtgc gagagatgga 300
tggcagcagc tggccccctt tgactactgg ggccagggaa ccctggtcac cgtctcctca 360
gcctccacca agggcccatc ggtcttcccc ctggcaccct ctagcaagag cacctctggg 420
ggcacagcgg ccctgggctg cctggtcaag gactacttcc ccgaaccggt gacggtgtcg 480
tggaactcag gcgccctgac cagcggcgtg ca 512

<210> 138
<211> 170
<212> PRT
<213> Homo sapiens

<400> 138
Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg

```
 1               5                  10                  15
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
            20                  25                  30
Gly Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45
Ala Val Ile Trp Tyr Asp Gly Ser Asn Lys Tyr Tyr Val Asp Ser Val
    50                  55                  60
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80
Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95
Ala Arg Asp Gly Trp Gln Gln Leu Ala Pro Phe Asp Tyr Trp Gly Gln
            100                 105                 110
Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val
        115                 120                 125
Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala
    130                 135                 140
Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser
145                 150                 155                 160
Trp Asn Ser Gly Ala Leu Thr Ser Gly Val
                165                 170
```

<210> 139
<211> 496
<212> DNA
<213> Homo sapiens

<400> 139
gatattgtga tgacccagac tccactctcc tcacctgtca cccttggaca gccggcctcc 60
atctcctgca ggtctagtca aagcctcgtg catagtgatg gaaacaccta cttgagttgg 120
cttcaccaga ggccaggcca gcctccaaga ctcctaattt ataagatttc taaccggttc 180
tctggggtcc cagacagatt cagtggcagt ggggcaggga cagctttcac actgaaaatc 240
agcagggtgg aagctgagga tgtcggggtt tattactgca tgcaagctac acaacttcct 300
cggacgttcg gccaagggac caaggtggaa atcaaacgaa ctgtggctgc accatctgtc 360
ttcatcttcc cgccatctga tgagcagttg aaatctggaa ctgctagcgt tgtgtgcctg 420
ctgaataact tctatcccag agaggccaaa gtacagtgga aggtggataa cgccctccaa 480
tcgggtaact cccagg 496

<210> 140
<211> 165
<212> PRT
<213> Homo sapiens

<400> 140
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Asp Ile Val Met Thr Gln Thr Pro Leu Ser Ser Pro Val Thr Leu Gly
 1               5                  10                  15
Gln Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Val His Ser
            20                  25                  30
Asp Gly Asn Thr Tyr Leu Ser Trp Leu His Gln Arg Pro Gly Gln Pro
        35                  40                  45
Pro Arg Leu Leu Ile Tyr Lys Ile Ser Asn Arg Phe Ser Gly Val Pro
    50                  55                  60
Asp Arg Phe Ser Gly Ser Gly Ala Gly Thr Ala Phe Thr Leu Lys Ile
65                  70                  75                  80
Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln Ala
                85                  90                  95
```

```
Thr Gln Leu Pro Arg Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
            100                 105                 110
Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu
        115                 120                 125
Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe
    130                 135                 140
Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln
145                 150                 155                 160
Ser Gly Asn Ser Gln
                165
```

<210> 141
<211> 110
<212> PRT
<213> Homo sapiens

<400> 141

```
Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
 1               5                  10                  15
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
            20                  25                  30
Gly Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45
Ala Val Ile Trp Tyr Asp Gly Ser Asn Lys Tyr Tyr Ala Asp Ser Val
    50                  55                  60
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80
Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95
Ala Arg Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala
            100                 105                 110
```

<210> 142
<211> 121
<212> PRT
<213> Homo sapiens

<400> 142

```
Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
 1               5                  10                  15
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
            20                  25                  30
Gly Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45
Ala Val Ile Trp Tyr Asp Gly Ser Asn Lys Tyr Tyr Val Asp Ser Val
    50                  55                  60
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80
Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95
Ala Arg Asp Gly Trp Gln Gln Leu Ala Pro Phe Asp Tyr Trp Gly Gln
            100                 105                 110
Gly Thr Leu Val Thr Val Ser Ser Ala
        115                 120
```

<210> 143
<211> 113
<212> PRT
<213> Homo sapiens

<400> 143
Asp Ile Val Met Thr Gln Thr Pro Leu Ser Ser Pro Val Thr Leu Gly
1 5 10 15
Gln Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Val His Ser
20 25 30
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Pro Arg Leu Leu Ile Tyr Lys Ile Ser Asn Arg Phe Ser Gly Val Pro
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Asp Arg Phe Ser Gly Ser Gly Ala Gly Thr Asp Phe Thr Leu Lys Ile
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Arg

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35 40 45
Pro Arg Leu Leu Ile Tyr Lys Ile Ser Asn Arg Phe Ser Gly Val Pro
50 55 60
Asp Arg Phe Ser Gly Ser Gly Ala Gly Thr Ala Phe Thr Leu Lys Ile
65 70 75 80
Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln Ala
85 90 95
Thr Gln Leu Pro Arg Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
100 105 110
Arg