

(12) STANDARD PATENT
(19) AUSTRALIAN PATENT OFFICE

(11) Application No. AU 2012332587 B2

(54) Title
Antibodies and methods of treating cancer

(51) International Patent Classification(s)
C07K 16/28 (2006.01) **A61K 39/395** (2006.01)

(21) Application No: **2012332587** (22) Date of Filing: **2012.10.31**

(87) WIPO No: **WO13/067054**

(30) Priority Data

(31) Number (32) Date (33) Country
61/554,436 **2011.11.01** **US**
61/554,440 **2011.11.01** **US**

(43) Publication Date: **2013.05.10**
(44) Accepted Journal Date: **2017.02.23**

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(56) Related Art
US 2011/0176995 A1 (FUNAHASHI) 21 July 2011
SASAKI, Y et al Biochemical and Biophysical Research Communications, Vol. 394, No. 3, page 498-502. 9 April 2010

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization

International Bureau



(10) International Publication Number

WO 2013/067054 A1

(43) International Publication Date

10 May 2013 (10.05.2013)

WIPO | PCT

(51) International Patent Classification:

C07K 16/28 (2006.01) A61K 39/395 (2006.01)

(21) International Application Number:

PCT/US2012/062861

(22) International Filing Date:

31 October 2012 (31.10.2012)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

61/554,436 1 November 2011 (01.11.2011) US
61/554,440 1 November 2011 (01.11.2011) US

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

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

Published:

- with international search report (Art. 21(3))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: ANTIBODIES AND METHODS OF TREATING CANCER

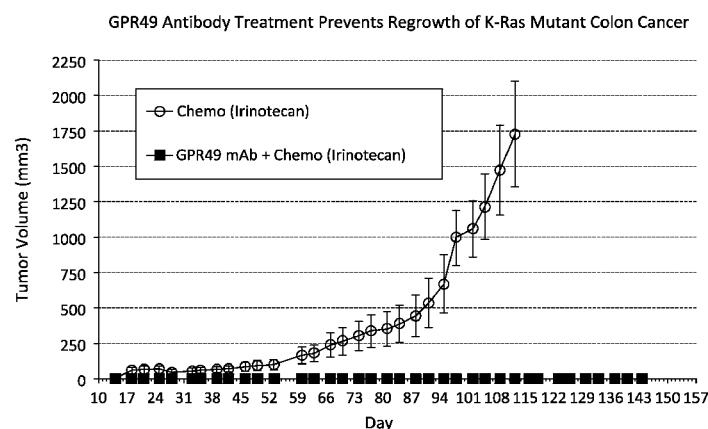


FIG. 23

(57) Abstract: Described herein are antibodies against GPR49 and uses of such antibodies. Various aspects relate to monoclonal, humanized, or fully human antibodies against GPR49, hybridomas or other cell lines expressing such antibodies, nucleic acids and vectors comprising nucleic acids encoding for such antibodies, and methods of treating cancer with such antibodies.

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ANTIBODIES AND METHODS OF TREATING CANCER

REFERENCE TO SEQUENCE LISTING

[0001] The present application is filed with a Sequence Listing in Electronic format. The Sequence Listing is provided as a file entitled BIONO.002WO_Sequence.txt, created October 26, 2012, which is approximately 31 kb in size. The information in the electronic format of the sequence listing is incorporated herein by reference in its entirety.

CROSS-REFERENCE TO RELATED APPLICATIONS

[0002] This application claims priority to U.S. Provisional Patent Application 61/554,436, filed on November 1, 2011 and entitled METHODS OF TREATING CANCER, and this application also claims priority to U.S. Provisional Patent Application 61/554,440, filed on November 1, 2011 and entitled ANTI-GPR49 ANTIBODIES, the entirety of which are hereby incorporated by reference herein.

FIELD

[0003] This application relates generally to the field of cancer biology. More particularly, embodiments are drawn to antibodies against GPR49 and uses of such antibodies. Several embodiments relate to monoclonal, humanized, or fully human antibodies against GPR49, hybridomas or other cell lines expressing such antibodies, nucleic acids and vectors comprising nucleic acids encoding for such antibodies, and methods of treating cancer with such antibodies.

BACKGROUND

[0004] G-Protein-coupled Receptor 49 (GPR49), also known as LGR5/HG38/FEX, belongs to the leucine-rich repeat containing G-protein-coupled receptors (LGRs) structurally similar to glycoprotein hormone receptors. LGRs are divided into three subgroups: (1) glycoprotein hormone receptors including thyroid-stimulating hormone (TSH) receptor, follicle-stimulating hormone (FSH) receptor, and luteinizing hormone (LH) receptor; (2) relaxin receptors LGR7 and LGR8; and (3) GPR48, GPR49, and LGR6. GPR49 is expressed in several tissues including the intestine, skeletal muscle, placenta, brain, and spinal cord. However, little is known about the function of GPR49.

SUMMARY

[0005] Several embodiments relate to a method of treating cancer in a mammal, comprising administering to the mammal a therapeutic amount of a monoclonal antibody that binds to G-Protein Coupled Receptor 49 (GPR49) polypeptide with a K_d of less than 1×10^{-9} M, wherein the GPR49 polypeptide has an amino acid sequence of SEQ ID NO: 1 and the therapeutic amount is sufficient to treat the cancer. In one aspect, the therapeutic amount of the antibody is sufficient to reduce tumor growth in the mammal. In another aspect, the reduced tumor growth is measured as reduced tumor volume. In a further aspect, the therapeutic amount of the antibody is sufficient to increase survival of the mammal compared to control. In an additional aspect, the antibody binds to GPR49 polypeptide with a K_d of less than 1×10^{-12} M.

[0006] In other aspects, the antibody is an IgG class antibody, an IgG1 class antibody, a human antibody, or a mouse antibody. In additional aspects, the GPR49 polypeptide consists of the amino acid sequence of SEQ ID NO: 1.

[0007] In further aspects, the antibody is monoclonal antibody 78F05, 5D6.3, 1B3.5, 14A8.1, 76C12, 18G7.1, 5B10.1, 14F7.1, 5F2.5, or 7C3.4.

[0008] In various aspects, the antibody competitively inhibits a monoclonal antibody selected from the group consisting of 71C10, 86C11, 66D05, 76C12, 78F05, and 76B04, or a monoclonal antibody produced by a hybridoma cell selected from the group consisting of monoclonal antibody 2B5.5, 7F8.2, 1B3.5, 9C6.4, 6H5.4, 10A6.7, 10A9.2, 2G8.1, 6C10.5, 6G10.3, 8H8.1, 6B10.2, 3B8.11, 2F12.5, 5G2.11, 1F10.5, 10E1.1, 7C3.4, 2H9.2, 5B12.4, 3G8.1, 5F2.5, 6G10.1, 14H9.1, 12G5.1, 6E10.1, 14F7.1, 4A10.2, 3F11.1, 11F6.1, 5B10.1, 14A8.1, 8E9.1, 9C7.1, 4F6.2, 1B8.1, 18G7.1, 12E3.1, 6H5.1, 2P69.2, 17C9.1, 2H5.1, and 10A9.2.

[0009] Certain embodiments are drawn to a method of producing an antibody or fragment thereof which specifically binds GPR49, comprising culturing a host cell comprising a vector which comprises a polynucleotide sequence encoding the antibody of claim 1; and recovering said antibody, or fragment thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] Figure 1 is a bar graph showing binding of anti-GPR49 Fabs to human GPR49-Fc by ELISA.

[0011] Figure 2 is a bar graph showing binding of anti-GPR49 Fabs to human GPR49-HA transfected HEK293E cells by FACS.

[0012] Figure 3 is a graph showing the EC50 values of 3 Dyax Fab candidates on CHO-GPR49 cells (Fig. 3A) or parental CHO cells (Fig. 3B) by FACS binding.

[0013] Figure 4 is a set of bar graphs showing binding of GPR49 Fabs to SW620 colon tumor cells as measured by geometric mean (Fig. 4A) or percent positive cells (Fig. 4B).

[0014] Figure 5A is a bar graph showing binding of GPR49 Fabs to SW480 colon tumor cells as measured by FACS geometric mean.

[0015] Figure 5B is a bar graph showing binding of GPR49 Fabs to SW480 colon tumor cells as measured by FACS percent positive cells.

[0016] Figure 5C is a panel of FACS histograms of various concentrations of the 76C12 Fab used to bind SW480 colon tumor cells.

[0017] Figure 6A is a bar graph showing binding of GPR49 Fabs to HCT116 colon tumor cells as measured by FACS geometric mean.

[0018] Figure 6B is a bar graph showing binding of GPR49 Fabs to HCT116 colon tumor cells as measured by FACS percent positive cells.

[0019] Figure 6C is a panel of FACS histograms of various concentrations of the 76C12 Fab used to bind HCT116 colon tumor cells.

[0020] Figures 7A-C are graphs showing binding of murine GPR49 antibodies to CHO GPR49-Flag-His expressing cells by FACs.

[0021] Figures 8A and 8C are graphs showing binding of murine GPR49 antibodies to CHO GPR49-Flag-His expressing cells by FACs. Figures 8B and 8D are tables indicating the EC50 of the antibodies of Figures 8A and 8C, respectively.

[0022] Figures 9A, 9C, 9E, and 9G are graphs showing binding of second generation mouse antibodies to GPR49 by direct ELISA. Figures 9B, 9D, 9F, and 9H are tables indicating the EC50 of the antibodies of Figures 9A, 9C, 9E, and 9G, respectively.

[0023] Figure 10A is a bar graph showing the average number of tumorspheres from sorted GPR49+, sorted GPR49-, or unsorted colon tumor cells. Figure 10B is a microscopic image of GPR49+ and GPR49- cells in the tumorsphere assay.

[0024] Figure 11 is a graph showing tumor volume (Fig. 11A) and body weight (Fig. 11B) over time of GPR49+ MoFlo sorted colon tumor cells inoculated into mice.

[0025] Figure 12A is a graph showing the serum concentration of GPR49 antibody 76C12 over time in mice. Figure 12B is a graph showing the simulated serum concentration of a once weekly administered dose of anti-GPR49 antibody 76C12 over time in mice. Figure 12C is a table showing the pharmacokinetic parameters of anti-GPR49 antibody C12 IgG1 in mice after a single intraperitoneal administration.

[0026] Figure 13A is a graph showing primary colon tumor volume over time in mice treated with IgG1 control mAb, anti-GPR49 mAb 76C12 (C12), or Irinotecan. Figure 13B is a graph showing the T/C values at day 62.

[0027] Figure 14 is a set of graphs showing tumor volume over time in mice treated with IDEC152 control antibody, 76C12 (ET CSC mAb), control mAb with chemo vehicle control, or ET CSC mAb with chemo vehicle control.

[0028] Figure 15 is a Kaplan-Meier curve showing the percent survival over time of untreated mice or mice treated with Irinotecan and IgG1 control, 76C12 (ET mAb), or Irinotecan and 76C12 (ET mAb).

[0029] Figure 16A is a graph showing DLD-1 colon xenograft tumor growth over time in mice treated with IDEC152 control antibody, Irinotecan and IDEC152 control antibody, or 76C12 (C12) antibody.

[0030] Figure 16B is a graph showing DLD-1 colon xenograft tumor growth over time in mice treated with dextrose control, Irinotecan and control IgG1, or Irinotecan and 76C12 (ET12) antibody.

[0031] Figure 17 is a graph showing Tumor Growth Inhibition in a primary colon cancer model having K-Ras, PI3K, PTEN, and p53 mutations

[0032] Figure 18 is a chart showing Percentage Tumor Growth Inhibition.

[0033] Figure 19 is a chart showing Percentage Tumor Inhibition by GPR49 mAb + Irinotecan combination treated CRC tumors with K-Ras, PI3K, PTEN, and p53 mutations.

[0034] Figure 20 is a graph showing Tumor Growth Inhibition in primary colon cancer model with K-Ras, PI3K, PTEN, H-Ras, APC, TP53, FGFR2, VANGL2, STK11, JAK2, and RB1 mutations.

[0035] Figure 21A is a chart showing Anti-GPR49 antibodies reduce cancer stem cell frequency in colon cancer tumors with K-Ras, PI3K, PTEN, and p53 mutations.

[0036] Figure 21B is a graph showing that Anti-GPR49 antibody treatment inhibits the ability of CRC tumors with K-Ras, PI3K, PTEN, and p53 mutations from re-forming new tumors when transplanted into secondary recipient mice.

[0037] Figure 22 is a chart showing that GPR49 mAb 18G.7.1 treatment reduces the number of cancer stem cells from CRC tumors with K-Ras, PI3K, PTEN, H-Ras, APC, TP53, FGFR2, VANGL2, STK11, JAK2, and RB1 mutations. CSC frequency was measured in a serial re-implantation, limiting dilution assay with primary CRC tumor cells previously treated as indicated.

[0038] Figure 23 is a graph showing that Anti-GPR49 antibodies + Irinotecan treatment inhibits the ability of CRC tumors having K-Ras, PI3K, PTEN, H-Ras, APC, TP53, FGFR2, VANGL2, STK11, JAK2, and RB1 mutations to grow upon re-implantation in secondary recipient mice.

DETAILED DESCRIPTION

[0039] Several embodiments of the present application are drawn to antibodies against GPR49 and treating cancer with such antibodies. Various embodiments relate to humanized or fully human antibodies against GPR49, hybridomas or other cell lines expressing such antibodies, nucleic acids and vectors comprising nucleic acids encoding for such antibodies, and methods of treating cancer with such antibodies.

Anti-GPR49 Antibodies

[0040] Several embodiments are drawn to anti-GPR49 antibodies. As used herein, GPR49 includes, but is not limited to, human GPR49 including the polypeptide of NCBI Accession No. NP_003658.1 (SEQ ID NO: 1), which is encoded by the coding nucleotide sequence within NM_003667.2 (SEQ ID NO: 2), or fragments thereof. The amino acid sequence of NCBI Accession No. NP_003658.1 and nucleotide sequence of NM_003667.2 are fully incorporated by reference in their entireties. Examples of GPR49 fragments contemplated herein include the GPR49 ectodomain, transmembrane domain, or intracellular domain and portions thereof.

[0041] As used herein, the term “antibody” includes, but is not limited to, synthetic antibodies, monoclonal antibodies, recombinantly produced antibodies, intrabodies, multispecific antibodies (including bi-specific antibodies), human antibodies, humanized antibodies, chimeric antibodies, synthetic antibodies, single-chain Fvs (scFv), Fab fragments, F(ab') fragments, disulfide-linked Fvs (sdFv) (including bi-specific sdFvs), and anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. The antibodies of several embodiments provided herein may be monospecific, bispecific, trispecific or of greater multispecificity. Multispecific antibodies may be specific for different epitopes of a polypeptide or may be specific for both a polypeptide as well as for a heterologous epitope, such as a heterologous polypeptide or solid support material. See, e.g., PCT publications WO 93/17715; WO 92/08802; WO91/00360; WO 92/05793; Tutt, et al., *J. Immunol.* 147:60-69 (1991); U.S. Pat. Nos. 4,474,893; 4,714,681; 4,925,648; 5,573,920; 5,601,819; Kostelny et al., *J. Immunol.* 148:1547-1553 (1992); each of which is incorporated herein by reference in its entirety.

[0042] Other anti-GPR49 antibodies contemplated include “oligoclonal” antibodies. As used herein, the term “oligoclonal” antibodies” refers to a predetermined mixture of distinct monoclonal antibodies. Methods for generating oligoclonal antibodies are known in the art. See, e.g., “Examples Section”, example 1, PCT publication WO 95/20401; U.S. Pat. Nos. 5,789,208 and 6,335,163; each of which is incorporated herein by reference in its entirety. In certain

embodiments, oligoclonal antibodies consist of a predetermined mixture of antibodies against one or more epitopes generated in a single cell. In other embodiments, oligoclonal antibodies comprise a plurality of heavy chains capable of pairing with a common light chain to generate antibodies with multiple specificities (e.g., PCT publication WO 04/009618, which is incorporated herein by reference in its entirety). Oligoclonal antibodies are particularly useful when it is desired to target multiple epitopes on a single target molecule (e.g., GPR49). Those skilled in the art will know or can determine what type of antibody or mixture of antibodies is applicable for an intended purpose and desired need. In particular, antibodies of several embodiments include immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site that specifically binds to a GPR49 antigen (e.g., one or more complementarity determining regions (CDRs) of an anti-GPR49 antibody). It is also specifically contemplated that the antibodies of several embodiments include immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site that specifically binds to a GPR49 antigen (e.g., one or more complementarity determining regions (CDRs) of an anti-GPR49 antibody).

[0043] Several embodiments are drawn to the anti-GPR49 human Fabs produced as described in the Examples below, including antibodies 71C10, 86C11, 66D05, 76C12, 78F05, and 76B04, which bind to a human GPR49-Fc ectodomain (GPR49-RFc) (SEQ ID NO: 3). Several embodiments are drawn to the full-length human IgGs of these Fabs as described in the Examples.

[0044] Several embodiments relate to the mouse monoclonal antibodies raised against the human GPR49 ectodomain (GPR49-His) (SEQ ID NO: 4) as described in the Examples below, including antibodies 2B5.5, 7F8.2, 1B3.5, 9C6.4, 6H5.4, 10A6.7, 10A9.2, 2G8.1, 6C10.5, 6G10.3, 8H8.1, 6B10.2, 3B8.11, 2F12.5, 5G2.11, 1F10.5, 10E1.1, 7C3.4, 2H9.2, 5B12.4, 3G8.1, 5F2.5, and 6G10.1.

[0045] Several embodiments are drawn to the mouse monoclonal antibodies raised against full-length human GPR49 as described in the Examples below, including antibodies 14H9.1, 12G5.1, 6E10.1, 14F7.1, 4A10.2, 3F11.1, 11F6.1, 5B10.1, 14A8.1, 8E9.1, 9C7.1, 4F6.2, 1B8.1, 18G7.1, 12E3.1, 6H5.1, 2P69.2, 17C9.1, 2H5.1, and 10A9.2.

[0046] The anti-GPR49 antibodies of several embodiments can be of any type (e.g., IgG, IgE, IgM, IgD, IgA and IgY), class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule. Immunoglobulins may have both a heavy and light chain. An array of IgG, IgE, IgM, IgD, IgA, and IgY heavy chains may be paired with a light chain of the kappa or lambda forms.

[0047] Several embodiments relate to a hybridoma that produces the light chain and/or the heavy chain of an anti-GPR49 antibody, including any one of the anti-GPR49 antibodies designated as 2B5.5, 7F8.2, 1B3.5, 9C6.4, 6H5.4, 10A6.7, 10A9.2, 2G8.1, 6C10.5, 6G10.3, 8H8.1, 6B10.2, 3B8.11, 2F12.5, 5G2.11, 1F10.5, 10E1.1, 7C3.4, 2H9.2, 5B12.4, 3G8.1, 5F2.5, 6G10.1, 14H9.1, 12G5.1, 6E10.1, 14F7.1, 4A10.2, 3F11.1, 11F6.1, 5B10.1, 14A8.1, 8E9.1, 9C7.1, 4F6.2, 1B8.1, 18G7.1, 12E3.1, 6H5.1, 2P69.2, 17C9.1, 2H5.1, and 10A9.2 produced and described in the Examples below. In one aspect, the hybridoma produces the light chain and/or the heavy chain of a humanized or fully human monoclonal antibody.

[0048] Some embodiments are drawn to a nucleic acid molecule encoding the light chain or the heavy chain of an anti-GPR49 antibody, including any one of the anti-GPR49 antibodies designated as 2B5.5, 7F8.2, 1B3.5, 9C6.4, 6H5.4, 10A6.7, 10A9.2, 2G8.1, 6C10.5, 6G10.3, 8H8.1, 6B10.2, 3B8.11, 2F12.5, 5G2.11, 1F10.5, 10E1.1, 7C3.4, 2H9.2, 5B12.4, 3G8.1, 5F2.5, 6G10.1, 14H9.1, 12G5.1, 6E10.1, 14F7.1, 4A10.2, 3F11.1, 11F6.1, 5B10.1, 14A8.1, 8E9.1, 9C7.1, 4F6.2, 1B8.1, 18G7.1, 12E3.1, 6H5.1, 2P69.2, 17C9.1, 2H5.1, and 10A9.2 produced and described in the Examples below. In one aspect, a nucleic acid molecule encodes the light chain or the heavy chain of a humanized or fully human monoclonal antibody.

[0049] Various embodiments are directed to a vector comprising a nucleic acid molecule or molecules encoding a light chain and/or a heavy chain of an anti-GPR49 antibody, including any one of the anti-GPR49 antibodies designated as 2B5.5, 7F8.2, 1B3.5, 9C6.4, 6H5.4, 10A6.7, 10A9.2, 2G8.1, 6C10.5, 6G10.3, 8H8.1, 6B10.2, 3B8.11, 2F12.5, 5G2.11, 1F10.5, 10E1.1, 7C3.4, 2H9.2, 5B12.4, 3G8.1, 5F2.5, 6G10.1, 14H9.1, 12G5.1, 6E10.1, 14F7.1, 4A10.2, 3F11.1, 11F6.1, 5B10.1, 14A8.1, 8E9.1, 9C7.1, 4F6.2, 1B8.1, 18G7.1, 12E3.1, 6H5.1, 2P69.2, 17C9.1, 2H5.1, and 10A9.2 produced and described in the Examples below.

[0050] Other embodiments relate to a method of making an anti-GPR49 antibody comprising transfecting at least one host cell with at least one nucleic acid molecule encoding an anti-GPR49 antibody, expressing the nucleic acid molecule in said host cell and isolating said antibody. In several aspects, such anti-GPR49 antibody includes any one of the anti-GPR49 antibodies designated as 2B5.5, 7F8.2, 1B3.5, 9C6.4, 6H5.4, 10A6.7, 10A9.2, 2G8.1, 6C10.5, 6G10.3, 8H8.1, 6B10.2, 3B8.11, 2F12.5, 5G2.11, 1F10.5, 10E1.1, 7C3.4, 2H9.2, 5B12.4, 3G8.1, 5F2.5, 6G10.1, 14H9.1, 12G5.1, 6E10.1, 14F7.1, 4A10.2, 3F11.1, 11F6.1, 5B10.1, 14A8.1, 8E9.1, 9C7.1, 4F6.2, 1B8.1, 18G7.1, 12E3.1, 6H5.1, 2P69.2, 17C9.1, 2H5.1, and 10A9.2 produced and described in the Examples below.

[0051] In several embodiments, antibodies can specifically bind GPR49 and antigenic fragments thereof with a dissociation constant or K_d (k_{off}/k_{on}) of less than 10^{-5} M, or

of less than 10^{-6} M, or of less than 10^{-7} M, or of less than 10^{-8} M, or of less than 10^{-9} M, or of less than 10^{-10} M, or of less than 10^{-11} M, or of less than 10^{-12} M, or of less than 10^{-13} M.

[0052] In another embodiment, the antibody can bind to GPR49 and/or antigenic fragments thereof with a K_{off} of less than 1×10^{-3} s $^{-1}$. In other embodiments, the antibody binds to GPR49 and antigenic fragments thereof with a K_{off} of less than 10^{-3} s $^{-1}$, less than 5×10^{-3} s $^{-1}$, less than 10^{-4} s $^{-1}$, less than 5×10^{-4} s $^{-1}$, less than 10^{-5} s $^{-1}$, less than 5×10^{-5} s $^{-1}$, less than 10^{-6} s $^{-1}$, less than 5×10^{-6} s $^{-1}$, less than 10^{-7} s $^{-1}$, less than 5×10^{-7} s $^{-1}$, less than 10^{-8} s $^{-1}$, less than 5×10^{-8} s $^{-1}$, less than 10^{-9} s $^{-1}$, less than 5×10^{-9} s $^{-1}$, or less than 10^{-10} s $^{-1}$.

[0053] In another embodiment, the antibody binds to GPR49 and/or antigenic fragments thereof with an association rate constant or k_{on} rate of at least 10^{-5} M $^{-1}$ s $^{-1}$, at least 5×10^{-5} M $^{-1}$ s $^{-1}$, at least 10^{-6} M $^{-1}$ s $^{-1}$, at least 5×10^{-6} M $^{-1}$ s $^{-1}$, at least 10^{-7} M $^{-1}$ s $^{-1}$, at least 5×10^{-7} M $^{-1}$ s $^{-1}$, or at least 10^{-8} M $^{-1}$ s $^{-1}$, or at least 10^{-9} M $^{-1}$ s $^{-1}$.

[0054] An additional embodiment includes antibodies that have certain preferred biochemical characteristics such as a particular isoelectric point (pI) or melting temperature (Tm).

[0055] In one embodiment, the high affinity antibodies have a pI ranging from 5.5 to 9.5. In one embodiment, the high affinity antibodies of several embodiments have a Tm ranging from about 65° C to about 120° C.

[0056] Antibodies of several embodiments also encompass those that have half-lives (e.g., serum half-lives) in a mammal, (e.g., a human), of greater than 1 day, greater than 2 days, greater than 3 days, greater than 4 days, greater than 5 days, greater than 6 days, greater than 7 days, greater than 8 days, greater than 9 days, greater than 10 days, greater than 15 days, greater than 20 days, greater than 25 days, greater than 30 days, greater than 35 days, greater than 40 days, greater than 45 days, greater than 2 months, greater than 3 months, greater than 4 months, or greater than 5 months. The increased half-lives of the antibodies in a mammal, (e.g., a human), results in a higher serum titer of said antibodies or antibody fragments in the mammal, and thus, reduces the frequency of the administration of said antibodies or antibody fragments and/or reduces the concentration of said antibodies or antibody fragments to be administered. Antibodies having increased *in vivo* half-lives can be generated by techniques known to those of skill in the art. For example, antibodies with increased *in vivo* half-lives can be generated by modifying (e.g., substituting, deleting or adding) amino acid residues identified as involved in the interaction between the Fc domain and the FcRn receptor (see, e.g., International Publication Nos. WO 97/34631; WO 04/029207; U.S. Pat. No. 6,737,056 and U.S. Patent Publication No. 2003/0190311 and discussed in more detail below); each of which is incorporated herein by reference in its entirety.

[0057] In several embodiments, the antibodies may comprise modifications/substitutions and/or novel amino acids within their Fc domains such as, for example, those disclosed in Ghetie et al., 1997, *Nat. Biotech.* 15:637-40; Duncan et al, 1988, *Nature* 332:563-564; Lund et al., 1991, *J. Immunol.* 147:2657-2662; Lund et al, 1992, *Mol Immunol* 29:53-59; Alegre et al, 1994, *Transplantation* 57:1537-1543; Hutchins et al., 1995, *Proc Natl. Acad Sci USA* 92:11980-11984; Jefferis et al, 1995, *Immunol Lett.* 44:111-117; Lund et al., 1995, *Faseb J* 9:115-119; Jefferis et al, 1996, *Immunol Lett* 54:101-104; Lund et al, 1996, *J Immunol* 157:4963-4969; Armour et al., 1999, *Eur J Immunol* 29:2613-2624; Idusogie et al, 2000, *J Immunol* 164:4178-4184; Reddy et al, 2000, *J Immunol* 164:1925-1933; Xu et al., 2000, *Cell Immunol* 200:16-26; Idusogie et al, 2001, *J Immunol* 166:2571-2575; Shields et al., 2001, *J Biol Chem* 276:6591-6604; Jefferis et al, 2002, *Immunol Lett* 82:57-65; Presta et al., 2002, *Biochem Soc Trans* 30:487-490); U.S. Pat. Nos. 5,624,821; 5,885,573; 5,677,425; 6,165,745; 6,277,375; 5,869,046; 6,121,022; 5,624,821; 5,648,260; 6,194,551; 6,737,056; 6,821,505; 6,277,375; U.S. patent application Ser. No. 10/370,749 and PCT Publications WO 94/2935; WO 99/58572; WO 00/42072; WO 02/060919, WO 04/029207; each of which is incorporated herein by reference in its entirety. Other modifications/substitutions of the Fc domain will be readily apparent to one skilled in the art.

[0058] Antibodies can comprise modifications/substitutions and/or novel amino acid residues in their Fc regions that can be generated by numerous methods well known to one skilled in the art. Non-limiting examples include, isolating antibody coding regions (e.g., from hybridoma) and making one or more desired substitutions in the Fc region of the isolated antibody coding region. Alternatively, the variable regions of an antibody may be subcloned into a vector encoding an Fc region comprising one or modifications/substitutions and/or novel amino acid residues.

[0059] Antibodies of several embodiments may also be modified to alter glycosylation, again to alter one or more functional properties of the antibody.

[0060] In various embodiments, the glycosylation of the antibodies can be modified. For example, an aglycosylated antibody can be made (i.e., the antibody lacks glycosylation). Glycosylation can be altered to, for example, increase the affinity of the antibody for a target antigen. Such carbohydrate modifications can be accomplished by, for example, altering one or more sites of glycosylation within the antibody sequence. For example, one or more amino acid substitutions can be made that result in elimination of one or more variable region framework glycosylation sites to thereby eliminate glycosylation at that site. Such aglycosylation may increase the affinity of the antibody for antigen. Such an approach is described in further detail

in U.S. Pat. Nos. 5,714,350 and 6,350,861; each of which is incorporated herein by reference in its entirety.

[0061] Additionally or alternatively, an antibody of several embodiments can be made that has an altered type of glycosylation, such as a hypofucosylated antibody having reduced amounts of fucosyl residues or an antibody having increased bisecting GlcNAc structures. Such altered glycosylation patterns have been demonstrated to increase the ADCC ability of antibodies. Such carbohydrate modifications can be accomplished by, for example, expressing the antibody in a host cell with altered glycosylation machinery. Cells with altered glycosylation machinery have been described in the art and can be used as host cells in which to express recombinant antibodies to thereby produce an antibody with altered glycosylation. See, for example, Shields, R. L. et al. (2002) *J. Biol. Chem.* 277:26733-26740; Umana et al. (1999) *Nat. Biotech.* 17:176-1, as well as, European Patent No: EP 1,176,195; PCT Publications WO 03/035835; WO 99/54342; each of which is incorporated herein by reference in its entirety.

[0062] The antibodies of several embodiments can be used either alone or in combination with other compositions. The antibodies may further be recombinantly fused to a heterologous polypeptide at the N- or C-terminus or chemically conjugated (including covalent and non-covalent conjugations) to polypeptides or other compositions. For example, antibodies can be recombinantly fused or conjugated to molecules useful as labels in detection assays and effector molecules such as heterologous polypeptides, drugs, radionuclides, or toxins. See, e.g., PCT publications WO 92/08495; WO 91/14438; WO 89/12624; U.S. Pat. No. 5,314,995; and EP 396,387; each of which is incorporated herein by reference in its entirety.

[0063] Antibodies provided herein can include derivatives that are modified, i.e., by the covalent attachment of any type of molecule to the antibody such that covalent attachment does not prevent the antibody from binding an GPR49 polypeptide or fragment thereof and/or generating a desired response. For example, but not by way of limitation, the antibody derivatives include antibodies that have been modified, e.g., by glycosylation, acetylation, pegylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, etc. Any of numerous chemical modifications may be carried out by known techniques, including, but not limited to specific chemical cleavage, acetylation, formylation, metabolic synthesis of tunicamycin, etc. Additionally, the derivative may contain one or more non-classical amino acids.

[0064] In several embodiments, the antibodies specifically bind a polypeptide comprising or consisting of a GPR49 polypeptide having at least 60% identity, or at least 70% identity, or at least 80% identity, at least 85% identity, at least 90% identity, at least 95% identity, or at least at least 97% identity, or at least 99% identity, or 100% identity to the human

GPR49 polypeptide of NCBI Accession Nos. NP_003658.1 (SEQ ID NO: 1) or fragments thereof. Such fragments can, for example, be at least about 5, 10, 15, 20, 25, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, or 900 contiguous or non-contiguous amino acids of SEQ ID NO: 1, or any number of contiguous or non-contiguous amino acids in between any of the aforementioned lengths.

[0065] The percent identity of two amino acid sequences (or two nucleic acid sequences) can be determined, for example, by aligning the sequences for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first sequence). The amino acids or nucleotides at corresponding positions are then compared, and the percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity = # of identical positions/total # of positions ×100). The actual comparison of the two sequences can be accomplished by well-known methods, for example, using a mathematical algorithm. A specific, non-limiting example of such a mathematical algorithm is described in Karlin et al., Proc. Natl. Acad. Sci. USA, 90:5873-5877 (1993), which is incorporated herein by reference in its entirety. Such an algorithm is incorporated into the BLASTN and BLASTX programs (version 2.2) as described in Schaffer et al., Nucleic Acids Res., 29:2994-3005 (2001), which is incorporated herein by reference in its entirety. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., BLASTN) can be used. See <http://www.ncbi.nlm.nih.gov>, as available on Apr. 10, 2002. In one embodiment, the database searched is a non-redundant (NR) database, and parameters for sequence comparison can be set at: no filters; Expect value of 10; Word Size of 3; the Matrix is BLOSUM62; and Gap Costs have an Existence of 11 and an Extension of 1.

[0066] Several embodiments also encompass variants of the above described antibodies, including any one of the anti-GPR49 antibodies designated as 2B5.5, 7F8.2, 1B3.5, 9C6.4, 6H5.4, 10A6.7, 10A9.2, 2G8.1, 6C10.5, 6G10.3, 8H8.1, 6B10.2, 3B8.11, 2F12.5, 5G2.11, 1F10.5, 10E1.1, 7C3.4, 2H9.2, 5B12.4, 3G8.1, 5F2.5, 6G10.1, 14H9.1, 12G5.1, 6E10.1, 14F7.1, 4A10.2, 3F11.1, 11F6.1, 5B10.1, 14A8.1, 8E9.1, 9C7.1, 4F6.2, 1B8.1, 18G7.1, 12E3.1, 6H5.1, 2P69.2, 17C9.1, 2H5.1, and 10A9.2 produced and described in the Examples below, comprising one or more amino acid residue substitutions in the variable light (V_L) domain and/or variable heavy (V_H) domain. Several also encompass variants of the above described antibodies with one or more additional amino acid residue substitutions in one or more V_L CDRs and/or one or more V_H CDRs. The antibody generated by introducing substitutions in the V_H domain, V_H CDRs, V_L domain and/or V_L CDRs of the above described antibodies can be tested *in vitro* and *in vivo*, for example, for its ability to bind to GPR49 (by, e.g., immunoassays including, but not limited to ELISAs and BIACore).

[0067] In other embodiments, antibodies can have at least one, at least two, at least three, at least four, at least five, or at least six of the CDRs of the antibodies described above, including any one of the anti-GPR49 antibodies designated as 2B5.5, 7F8.2, 1B3.5, 9C6.4, 6H5.4, 10A6.7, 10A9.2, 2G8.1, 6C10.5, 6G10.3, 8H8.1, 6B10.2, 3B8.11, 2F12.5, 5G2.11, 1F10.5, 10E1.1, 7C3.4, 2H9.2, 5B12.4, 3G8.1, 5F2.5, 6G10.1, 14H9.1, 12G5.1, 6E10.1, 14F7.1, 4A10.2, 3F11.1, 11F6.1, 5B10.1, 14A8.1, 8E9.1, 9C7.1, 4F6.2, 1B8.1, 18G7.1, 12E3.1, 6H5.1, 2P69.2, 17C9.1, 2H5.1, and 10A9.2 produced and described in the Examples below.

[0068] Various embodiments include antibodies that specifically bind to GPR49 comprising derivatives of the V_H domains, V_H CDRs, V_L domains, or V_L CDRs of anti-GPR49 antibodies, such as any one of the anti-GPR49 antibodies designated as 2B5.5, 7F8.2, 1B3.5, 9C6.4, 6H5.4, 10A6.7, 10A9.2, 2G8.1, 6C10.5, 6G10.3, 8H8.1, 6B10.2, 3B8.11, 2F12.5, 5G2.11, 1F10.5, 10E1.1, 7C3.4, 2H9.2, 5B12.4, 3G8.1, 5F2.5, 6G10.1, 14H9.1, 12G5.1, 6E10.1, 14F7.1, 4A10.2, 3F11.1, 11F6.1, 5B10.1, 14A8.1, 8E9.1, 9C7.1, 4F6.2, 1B8.1, 18G7.1, 12E3.1, 6H5.1, 2P69.2, 17C9.1, 2H5.1, and 10A9.2 produced and described in the Examples below, that specifically bind to GPR49. Standard techniques known to those of skill in the art can be used to introduce mutations (e.g., additions, deletions, and/or substitutions) in the nucleotide sequence encoding an antibody, including, for example, site-directed mutagenesis and PCR-mediated mutagenesis are routinely used to generate amino acid substitutions. In one embodiment, the V_H and/or V_L CDRs derivatives include less than 25 amino acid substitutions, less than 20 amino acid substitutions, less than 15 amino acid substitutions, less than 10 amino acid substitutions, less than 5 amino acid substitutions, less than 4 amino acid substitutions, less than 3 amino acid substitutions, or less than 2 amino acid substitutions relative to the original V_H and/or V_L CDRs. In another embodiment, the V_H and/or V_L CDRs derivatives have conservative amino acid substitutions (e.g. *supra*) made at one or more predicted non-essential amino acid residues (i.e., amino acid residues which are not critical for the antibody to specifically bind to GPR49). Alternatively, mutations can be introduced randomly along all or part of the V_H and/or V_L CDR coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for biological activity to identify mutants that retain activity. Following mutagenesis, the encoded antibody can be expressed and the activity of the antibody can be determined.

[0069] Several embodiments also encompass antibodies that specifically bind to GPR49 or a fragment thereof, said antibodies comprising an amino acid sequence of a variable heavy chain and/or variable light chain that is at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% identical to the amino acid sequence of the variable heavy chain and/or light chain of any of the antibodies described herein including any one of the anti-GPR49

antibodies designated as 2B5.5, 7F8.2, 1B3.5, 9C6.4, 6H5.4, 10A6.7, 10A9.2, 2G8.1, 6C10.5, 6G10.3, 8H8.1, 6B10.2, 3B8.11, 2F12.5, 5G2.11, 1F10.5, 10E1.1, 7C3.4, 2H9.2, 5B12.4, 3G8.1, 5F2.5, 6G10.1, 14H9.1, 12G5.1, 6E10.1, 14F7.1, 4A10.2, 3F11.1, 11F6.1, 5B10.1, 14A8.1, 8E9.1, 9C7.1, 4F6.2, 1B8.1, 18G7.1, 12E3.1, 6H5.1, 2P69.2, 17C9.1, 2H5.1, and 10A9.2 produced and described in the Examples below.

[0070] Various embodiments further encompass antibodies that specifically bind to GPR49 or a fragment thereof, said antibodies or antibody fragments comprising an amino acid sequence of one or more CDRs that is at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% identical to the amino acid sequence of one or more CDRs of the antibodies described herein including any one of the anti-GPR49 antibodies designated as 2B5.5, 7F8.2, 1B3.5, 9C6.4, 6H5.4, 10A6.7, 10A9.2, 2G8.1, 6C10.5, 6G10.3, 8H8.1, 6B10.2, 3B8.11, 2F12.5, 5G2.11, 1F10.5, 10E1.1, 7C3.4, 2H9.2, 5B12.4, 3G8.1, 5F2.5, 6G10.1, 14H9.1, 12G5.1, 6E10.1, 14F7.1, 4A10.2, 3F11.1, 11F6.1, 5B10.1, 14A8.1, 8E9.1, 9C7.1, 4F6.2, 1B8.1, 18G7.1, 12E3.1, 6H5.1, 2P69.2, 17C9.1, 2H5.1, and 10A9.2 produced and described in the Examples below. The determination of percent identity of two amino acid sequences can be determined by any method known to one skilled in the art, including BLAST protein searches.

[0071] Another embodiment includes the introduction of conservative amino acid substitutions in any portion of an anti-GPR49 antibody, such as any one of the anti-GPR49 antibodies designated as 2B5.5, 7F8.2, 1B3.5, 9C6.4, 6H5.4, 10A6.7, 10A9.2, 2G8.1, 6C10.5, 6G10.3, 8H8.1, 6B10.2, 3B8.11, 2F12.5, 5G2.11, 1F10.5, 10E1.1, 7C3.4, 2H9.2, 5B12.4, 3G8.1, 5F2.5, 6G10.1, 14H9.1, 12G5.1, 6E10.1, 14F7.1, 4A10.2, 3F11.1, 11F6.1, 5B10.1, 14A8.1, 8E9.1, 9C7.1, 4F6.2, 1B8.1, 18G7.1, 12E3.1, 6H5.1, 2P69.2, 17C9.1, 2H5.1, and 10A9.2 produced and described in the Examples below. It is well known in the art that “conservative amino acid substitution” refers to amino acid substitutions that substitute functionally-equivalent amino acids. Conservative amino acid changes result in silent changes in the amino acid sequence of the resulting peptide. For example, one or more amino acids of a similar polarity act as functional equivalents and result in a silent alteration within the amino acid sequence of the peptide. Substitutions that are charge neutral and which replace a residue with a smaller residue may also be considered “conservative substitutions” even if the residues are in different groups (e.g., replacement of phenylalanine with the smaller isoleucine). Families of amino acid residues having similar side chains have been defined in the art. Several families of conservative amino acid substitutions are shown in Table 1.

TABLE 1

Families of Conservative Amino Acid Substitutions

Family	Amino Acids
non-polar	Trp, Phe, Met, Leu, Ile, Val, Ala, Pro
uncharged polar	Gly, Ser, Thr, Asn, Gln, Tyr, Cys
acidic/negatively charged	Asp, Glu
basic/positively charged	Arg, Lys, His
Beta-branched	Thr, Val, Ile
residues that influence chain orientation	Gly, Pro
aromatic	Trp, Tyr, Phe, His

[0072] The term “conservative amino acid substitution” also refers to the use of amino acid analogs or variants.

Methods of generating antibodies

[0073] Antibodies that specifically bind to a GPR49 polypeptide can be identified, for example, by immunoassays, BIACore, or other techniques known to those of skill in the art.

[0074] The antibodies of several embodiments may be generated by any suitable method known in the art. Polyclonal antibodies to an antigen-of-interest can be produced by various procedures well known in the art. For example, a GPR49 polypeptide can be administered to various host animals including, but not limited to, rabbits, mice, rats, etc. to induce the production of sera containing polyclonal antibodies specific for the antigen. Various adjuvants may be used to increase the immunological response, depending on the host species, and include but are not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*. Such adjuvants are also well known in the art.

[0075] Monoclonal antibodies can be prepared using a wide variety of techniques known in the art including the use of hybridoma, recombinant, and phage display technologies, or a combination thereof. For example, monoclonal antibodies can be produced using hybridoma techniques including those known in the art and taught, for example, in Harlow et al., *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling, et al., in: *Monoclonal Antibodies and T-Cell Hybridomas* 563-681 (Elsevier, N.Y., 1981); each of which is incorporated herein by reference in its entirety. The term “monoclonal antibody” (abbreviated as “mAb”) as used herein is not limited to antibodies produced through

hybridoma technology. The term “monoclonal antibody” refers to an antibody that is derived from a single clone, including any eukaryotic, prokaryotic, or phage clone, and not the method by which it is produced. A “monoclonal antibody” may comprise, or alternatively consist of, two proteins, i.e., a heavy and a light chain.

[0076] Methods for producing and screening for specific antibodies using hybridoma technology are routine and well known in the art. In a non-limiting example, mice can be immunized with a GPR49 polypeptide or a cell expressing such peptide. Once an immune response is detected, e.g., antibodies specific for the antigen are detected in the mouse serum, the mouse spleen is harvested and splenocytes isolated. The splenocytes are then fused by well-known techniques to any suitable myeloma cells, for example cells from cell line SP20 available from the ATCC. Hybridomas are selected and cloned by limited dilution. The hybridoma clones are then assayed by methods known in the art for cells that secrete antibodies capable of binding a GPR49 polypeptide. Ascites fluid, which generally contains high levels of antibodies, can be generated by immunizing mice with positive hybridoma clones.

[0077] Accordingly, several embodiments provide methods of generating monoclonal antibodies as well as antibodies produced by the method comprising culturing a hybridoma cell secreting an antibody wherein the hybridoma is generated by fusing splenocytes isolated from a mouse immunized with a GPR49 antigen with myeloma cells and then screening the hybridomas resulting from the fusion for hybridoma clones that secrete an antibody able to bind a GPR49 polypeptide.

[0078] Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, Fab and F(ab')2 fragments of several embodiments may be produced by proteolytic cleavage of immunoglobulin molecules, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')2 fragments). F(ab')2 fragments contain the variable region, the light chain constant region and the CH1 domain of the heavy chain.

[0079] The antibodies of various embodiments can also be generated using various phage display methods known in the art. In phage display methods, functional antibody domains are displayed on the surface of phage particles which carry the polynucleotide sequences encoding them. In a particular embodiment, such phage can be utilized to display antigen-binding domains expressed from a repertoire or combinatorial antibody library (e.g., human or murine). Phage expressing an antigen binding domain that binds the antigen of interest can be selected or identified with antigen, e.g., using labeled antigen or antigen bound or captured to a solid surface or bead. Phage used in these methods are typically filamentous phage including fd and M13 binding domains expressed from phage with Fab, Fv or disulfide stabilized Fv antibody domains recombinantly fused to either the phage gene III or gene VIII protein.

Examples of phage display methods that can be used to make the antibodies of several embodiments include those disclosed in PCT application No. PCT/GB91/01134; PCT publications WO 90/02809; WO 91/10737; WO 92/01047; WO 92/18619; WO 93/11236; WO 95/15982; WO 95/20401; and U.S. Patent Nos. 5,698,426; 5,223,409; 5,403,484; 5,580,717; 5,427,908; 5,750,753; 5,821,047; 5,571,698; 5,427,908; 5,516,637; 5,780,225; 5,658,727; 5,733,743 and 5,969,108; each of which is incorporated herein by reference in its entirety.

[0080] As described in the above references, after phage selection, the antibody coding regions from the phage can be isolated and used to generate whole antibodies, including human antibodies, or any other desired antigen binding fragment, and expressed in any desired host, including mammalian cells, insect cells, plant cells, yeast, and bacteria, e.g., as described in detail below. For example, techniques to recombinantly produce Fab, Fab' and F(ab')2 fragments can also be employed using methods known in the art such as those disclosed in PCT publication WO 92/22324; Mullinax et al., *BioTechniques* 12(6):864-869 (1992); and Sawai et al., *AJRI* 34:26-34 (1995); and Better et al., *Science* 240:1041-1043 (1988), each of which is incorporated herein by reference in its entirety.

[0081] Examples of techniques which can be used to produce single-chain Fvs and antibodies include those described in U.S. Pat. Nos. 4,946,778 and 5,258,498; Huston et al., *Methods in Enzymology* 203:46-88 (1991); Shu et al., *PNAS* 90:7995-7999 (1993); and Skerra et al., *Science* 240:1038-1040 (1988), each of which is incorporated herein by reference in its entirety.

Human Antibodies and Humanization of Antibodies

[0082] For some uses, including *in vivo* use of antibodies in humans and *in vitro* detection assays, it may be desirable to use chimeric, humanized, or human antibodies. A chimeric antibody is a molecule in which different portions of the antibody are derived from different animal species, such as antibodies having a variable region derived from a murine monoclonal antibody and a human immunoglobulin constant region. Methods for producing chimeric antibodies are known in the art. See, e.g., Morrison, *Science* 229:1202 (1985); Oi et al., *BioTechniques* 4:214 (1986); Gillies et al., (1989) *J. Immunol. Methods* 125:191-202; U.S. Pat. Nos. 5,807,715; 4,816,567; and 4,816,397; each of which is incorporated herein by reference in its entirety. Humanized antibodies are antibody molecules from non-human species antibody that bind the desired antigen having one or more complementarity determining regions (CDRs) from the non-human species and a framework region from a human immunoglobulin molecule. Often, framework residues in the human framework regions will be substituted with the corresponding residue from the CDR donor antibody to alter, preferably improve, antigen

binding. These framework substitutions are identified by methods well known in the art, e.g., by modeling of the interactions of the CDR and framework residues to identify framework residues important for antigen binding and sequence comparison to identify unusual framework residues at particular positions. (See, for example, Queen et al., U.S. Pat. No. 5,585,089; Riechmann et al., *Nature* 332:323 (1988); each of which is incorporated herein by reference in its entirety).

[0083] 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, antibodies can be humanized using a variety of techniques known in the art including, for example, CDR-grafting (EP 239,400; PCT publication WO 91/09967; U.S. Pat. Nos. 5,225,539; 5,530,101; and 5,585,089; each of which is incorporated herein by reference in its entirety), veneering or resurfacing (EP 592,106; EP 519,596; Padlan, *Molecular Immunology* 28(4/5):489-498 (1991); Studnicka et al., *Protein Engineering* 7(6):805-814 (1994); Roguska. et al., *PNAS* 91:969-973 (1994)), de-immunization (U.S. Patent Publication No. 20030153043) and chain shuffling (U.S. Pat. No. 5,565,332), each of which is incorporated herein by reference in its entirety.

[0084] Completely human antibodies can be used for therapeutic treatment of human patients. Human antibodies can be made by a variety of methods known in the art including phage display methods described above using antibody libraries derived from human immunoglobulin sequences. See also, U.S. Pat. Nos. 4,444,887 and 4,716,111; and PCT publications WO 98/46645, WO 98/50433, WO 98/24893, WO 98/16654, WO 96/34096, WO 96/33735, and WO 91/10741, each of which is incorporated herein by reference in its entirety.

[0085] Human antibodies can also be produced using transgenic mice which are incapable of expressing functional endogenous immunoglobulins, but which can express human immunoglobulin genes. For example, the human heavy and light chain immunoglobulin gene complexes may be introduced randomly or by homologous recombination into mouse embryonic stem cells. Alternatively, the human variable region, constant region, and diversity region may be introduced into mouse embryonic stem cells in addition to the human heavy and light chain genes. The mouse heavy and light chain immunoglobulin genes may be rendered non-functional separately or simultaneously with the introduction of human immunoglobulin loci by homologous recombination. In particular, homozygous deletion of the JH region prevents endogenous antibody production. The modified embryonic stem cells are expanded and microinjected into blastocysts to produce chimeric mice. The chimeric mice are then bred to produce homozygous offspring which express human antibodies. The transgenic mice are

immunized in the normal fashion with a selected antigen, e.g., all or a portion of a GPR49 polypeptide. Monoclonal antibodies directed against the antigen can be obtained from the immunized, transgenic mice using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies. For an overview of this technology for producing human antibodies, see Lonberg and Huszar, *Int. Rev. Immunol.* 13:65-93 (1995), which is incorporated herein by reference in its entirety. For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, see, e.g., PCT publications WO 98/24893; WO 92/01047; WO 96/34096; WO 96/33735; European Patent No. 0 598 877; U.S. Pat. Nos. 5,413,923; 5,625,126; 5,633,425; 5,569,825; 5,661,016; 5,545,806; 5,814,318; 5,885,793; 5,916,771; and 5,939,598; each of which is incorporated herein by reference in its entirety. In addition, companies such as Abgenix, Inc. (Fremont, CA) and Genpharm (San Jose, CA) can be engaged to provide human antibodies directed against a selected antigen using technology similar to that described above. Abgenix, Inc. (Fremont, CA) provides XenoMouse® strains of mice that have been engineered to contain up to but less than 1000 kb-sized germline configured fragments of the human heavy chain locus and kappa light chain locus. See Mendez *et al.* *Nature Genetics* 15:146-156 (1997) and Green and Jakobovits *J. Exp. Med.* 188:483-495 (1998).

[0086] 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 usually a second constant region (preferably a gamma constant region) are formed into a construct for insertion into an animal. This approach is described in U.S. Patent No. 5,545,807 to Surani *et al.* and U.S. Patent Nos. 5,545,806, 5,625,825, 5,625,126, 5,633,425, 5,661,016, 5,770,429, 5,789,650, 5,814,318, 5,877,397, 5,874,299, and 6,255,458 each to Lonberg and Kay, U.S. Patent No. 5,591,669 and 6,023,010 to Krimpenfort and Berns, U.S. Patent Nos. 5,612,205, 5,721,367, and 5,789,215 to Berns *et al.*, and U.S. Patent No. 5,643,763 to Choi and Dunn, and GenPharm International U.S. Patent Application Serial Nos. 07/574,748, filed August 29, 1990, 07/575,962, filed August 31, 1990, 07/810,279, filed December 17, 1991, 07/853,408, filed March 18, 1992, 07/904,068, filed June 23, 1992, 07/990,860, filed December 16, 1992, 08/053,131, filed April 26, 1993, 08/096,762, filed July 22, 1993, 08/155,301, filed November 18, 1993, 08/161,739, filed December 3, 1993, 08/165,699, filed December 10, 1993,

08/209,741, filed March 9, 1994, the disclosures of which are hereby incorporated by reference. *See also* European Patent No. 0 546 073 B1, International Patent Application Nos. WO 92/03918, WO 92/22645, WO 92/22647, WO 92/22670, WO 93/12227, WO 94/00569, WO 94/25585, WO 96/14436, WO 97/13852, and WO 98/24884 and U.S. Patent No. 5,981,175, the disclosures of which are hereby incorporated by reference in their entirety. *See further* Taylor *et al.*, 1992, Chen *et al.*, 1993, Tuailon *et al.*, 1993, Choi *et al.*, 1993, Lonberg *et al.*, (1994), Taylor *et al.*, (1994), and Tuailon *et al.*, (1995), Fishwild *et al.*, (1996), the disclosures of which are hereby incorporated by reference in their entirety.

[0087] Human antibodies can be produced from transgenic mice that have had large pieces of chromosomes or entire chromosomes introduced through microcell fusion. *See* European Patent Application Nos. 773 288 and 843 961, the disclosures of which are hereby incorporated by reference. Additionally, KMTM– mice, which are the result of cross-breeding of Tc mice with Medarex's minilocus (Humab) mice have been generated. These mice possess the human IgH transchromosome of the Kirin mice and the kappa chain transgene of the Genpharm mice (Ishida *et al.*, Cloning Stem Cells, (2002) 4:91-102).

[0088] Completely human antibodies which recognize a selected epitope can be generated using a technique referred to as “guided selection.” In this approach a selected non-human monoclonal antibody, e.g., a mouse antibody, is used to guide the selection of a completely human antibody recognizing the same epitope. (Jespers *et al.*, *Bio/technology* 12:899-903 (1988), which is incorporated herein by reference in its entirety).

[0089] Further, antibodies to GPR49 polypeptides can, in turn, be utilized to generate anti-idiotype antibodies that “mimic” GPR49 polypeptides using techniques well known to those skilled in the art (See, e.g., Greenspan & Bona, *FASEB J.* 7(5):437-444; (1989) and Nissinoff, *J. Immunol.* 147(8):2429-2438 (1991); each of which is incorporated herein by reference in its entirety).

[0090] In several embodiments, antibodies provided herein can be used therapeutically *in vivo*. Accordingly, the antibody can be modified to make it less immunogenic in the individual. For example, if the individual is human the antibody can be “humanized” where the complementarity determining region(s) of the antibody is transplanted into a human antibody (for example, as described in Jones *et al.*, *Nature* 321:522-525, 1986; and Tempest *et al.*, *Biotechnology* 9:266-273, 1991), which is incorporated herein by reference in its entirety.

[0091] Phage display technology can also be utilized to select antibody genes with binding activities towards the polypeptide either from repertoires of PCR amplified v-genes of lymphocytes from humans screened for possessing anti-GPR49 antibodies or from naive libraries (McCafferty *et al.*, *Nature* 348:552-554, 1990; and Marks, *et al.*, *Biotechnology* 10:779-

783, 1992, which is incorporated herein by reference in its entirety). The affinity of these antibodies can also be improved by chain shuffling (Clackson et al., *Nature* 352: 624-628, 1991, which is incorporated herein by reference in its entirety).

Methods of Producing Antibodies

[0092] The antibodies of several embodiments can be produced by any method known in the art for the synthesis of antibodies, in particular, by chemical synthesis or preferably, by recombinant expression techniques.

[0093] Recombinant expression of an antibody or fragment, derivative or analog thereof, (e.g., a heavy or light chain of an antibody or a single chain antibody), requires construction of an expression vector containing a polynucleotide that encodes the antibody. Once a polynucleotide encoding an antibody molecule or a heavy or light chain of an antibody, or portion thereof (preferably containing the heavy or light chain variable domain) has been obtained, the vector for the production of the antibody molecule may be produced by recombinant DNA technology using techniques well known in the art. Thus, methods for preparing a protein by expressing a polynucleotide containing an antibody encoding nucleotide sequence are described herein. Methods which are well known to those skilled in the art can be used to construct expression vectors containing antibody coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. Various embodiments thus provide replicable vectors comprising a nucleotide sequence encoding an antibody molecule, or a heavy or light chain thereof, or a heavy or light chain variable domain, operably linked to a promoter. Such vectors may include the nucleotide sequence encoding the constant region of the antibody molecule (see, e.g., PCT Publication WO 86/05807; PCT Publication WO 89/01036; and U.S. Pat. No. 5,122,464; each of which is incorporated herein by reference in its entirety) and the variable domain of the antibody may be cloned into such a vector for expression of the entire heavy or light chain.

[0094] The expression vector is transferred to a host cell by conventional techniques and the transfected cells are then cultured by conventional techniques to produce an antibody. Thus, several embodiments include host cells containing a polynucleotide encoding an antibody, or a heavy or light chain thereof, or a single chain antibody, operably linked to a heterologous promoter. Vectors encoding both the heavy and light chains may be co-expressed in the host cell for expression of the entire immunoglobulin molecule, as detailed below.

[0095] A variety of host-expression vector systems may be utilized to express the antibody molecules described herein. Such host-expression systems represent vehicles by which

the coding sequences of interest may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, express an antibody molecule *in situ*. These include but are not limited to microorganisms such as bacteria (e.g., *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing antibody coding sequences; yeast (e.g., *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing antibody coding sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing antibody coding sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing antibody coding sequences; or mammalian cell systems (e.g., COS, CHO, BHK, 293, NS0, 3T3, PerC6 cells) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter). Bacterial cells such as *Escherichia coli*, and eukaryotic cells can be used for the expression of a recombinant antibody molecule. For example, mammalian cells such as Chinese hamster ovary cells (CHO), in conjunction with a vector such as the major intermediate early gene promoter element from human cytomegalovirus are an effective expression system for antibodies (Foecking et al., *Gene* 45:101 (1986); Cockett et al., *Bio/Technology* 8:2 (1990)). Also see, e.g., U.S. Pat. Nos. 5,827,739, 5,879,936, 5,981,216, and 5,658,759, each of which is incorporated herein by reference in its entirety.

[0096] In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the antibody molecule being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions of an antibody molecule, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the *E. coli* expression vector pUR278 (Ruther et al., *EMBO J.* 2:1791 (1983)), which is incorporated herein by reference in its entirety, in which the antibody coding sequence may be ligated individually into the vector in frame with the lacZ coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, *Nucleic Acids Res.* 13:3101-3109 (1985); Van Heeke & Schuster, *J. Biol. Chem.* 24:5503-5509 (1989)), each of which is incorporated herein by reference in its entirety; and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption and binding to matrix glutathione-agarose beads followed by elution in the presence of free

glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

[0097] In an insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The antibody coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter).

[0098] In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the antibody coding sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the antibody molecule in infected hosts (e.g., see Logan & Shenk, *Proc. Natl. Acad. Sci. USA* 81:355-359 (1984), which is incorporated herein by reference in its entirety). Specific initiation signals may also be required for efficient translation of inserted antibody coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner et al., *Methods in Enzymol.* 153:51-544 (1987), which is incorporated herein by reference in its entirety).

[0099] In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERY, BHK, Hela, COS, MDCK, 293, 3T3, W138, NS0, Per.C6 and in particular, breast cancer cell lines such as, for example, BT483, Hs578T, HTB2,

BT20 and T47D, and normal mammary gland cell lines such as, for example, CRL7030 and Hs578Bst.

[0100] For long-term, high-yield production of recombinant proteins, stable expression can be used. For example, cell lines which stably express the antibody molecule may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the antibody molecule. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that interact directly or indirectly with the antibody molecule.

[0101] A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler et al., *Cell* 11:223 (1977)), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, *Proc. Natl. Acad. Sci. USA* 89:202 (1992)), and adenine phosphoribosyltransferase (Lowy et al., *Cell* 22:817 (1980)) genes can be employed in tk-, hgprt- or aprt-cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler et al., *Proc Natl. Acad. Sci. USA* 77:357 (1980); O'Hare et al., *Proc. Natl. Acad. Sci. USA* 78:1527 (1981)); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, *Proc. Natl. Acad. Sci. USA* 78:2072 (1981)); neo, which confers resistance to the aminoglycoside G-418 *Clinical Pharmacy* 12:488-505; Wu and Wu, *Biotherapy* 3:87-95 (1991); Tolstoshev, *Ann. Rev. Pharmacol. Toxicol.* 32:573-596 (1993); Mulligan, *Science* 260:926-932 (1993); and Morgan and Anderson, *Ann. Rev. Biochem.* 62:191-217 (1993); May, 1993, *TIB TECH* 11(5):155-215); and hygro, which confers resistance to hygromycin (Santerre et al., *Gene* 30:147 (1984), each of which is incorporated herein by reference in its entirety). Methods commonly known in the art of recombinant DNA technology may be routinely applied to select the desired recombinant clone, and such methods are described, for example, in Ausubel et al. (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, NY (1993); Kriegler, *Gene Transfer and Expression, A Laboratory Manual*, Stockton Press, NY (1990); and in Chapters 12 and 13, Dracopoli et al. (eds), *Current Protocols in Human Genetics*, John Wiley & Sons, NY

(1994); Colberre-Garapin et al., *J. Mol. Biol.* 150:1 (1981), each of which is incorporated herein by reference in its entirety.

[0102] The expression levels of an antibody molecule can be increased by vector amplification (for a review, see Bebbington and Hentschel, *The use of vectors based on gene amplification for the expression of cloned genes in mammalian cells in DNA cloning*, Vol. 3. (Academic Press, New York, 1987), which is incorporated herein by reference in its entirety). When a marker in the vector system expressing antibody is amplifiable, increase in the level of inhibitor present in culture of host cell will increase the number of copies of the marker gene. Since the amplified region is associated with the antibody gene, production of the antibody will also increase (Crouse et al., *Mol. Cell. Biol.* 3:257 (1983), which is incorporated herein by reference in its entirety).

[0103] The host cell may be co-transfected with two expression vectors, the first vector encoding a heavy chain derived polypeptide and the second vector encoding a light chain derived polypeptide. The two vectors may contain identical selectable markers which enable equal expression of heavy and light chain polypeptides. Alternatively, a single vector may be used which encodes, and is capable of expressing, both heavy and light chain polypeptides. In such situations, the light chain should be placed before the heavy chain to avoid an excess of toxic free heavy chain (Proudfoot, *Nature* 322:562 (1986); Kohler, *Proc. Natl. Acad. Sci. USA* 77:2197 (1980), which is incorporated herein by reference in its entirety). The coding sequences for the heavy and light chains may comprise cDNA or genomic DNA.

[0104] Once an antibody molecule has been produced by an animal, chemically synthesized, or recombinantly expressed, it may be purified by any method known in the art for purification of an immunoglobulin molecule, for example, by chromatography (e.g., ion exchange, affinity, particularly by affinity for the specific antigen after Protein A, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. In addition, the antibodies or fragments thereof can be fused to heterologous polypeptide sequences described herein or otherwise known in the art, to facilitate purification.

[0105] Moreover, the antibodies or fragments thereof can be fused to marker sequences, such as a peptide to facilitate purification. In certain embodiments, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, Calif., 91311), among others, many of which are commercially available. As described in Gentz et al., *Proc. Natl. Acad. Sci. USA* 86:821-824 (1989), which is incorporated herein by reference in its entirety, for instance, hexa-histidine provides for convenient purification of the fusion protein. Other peptide tags useful for

purification include, but are not limited to, the “HA” tag, which corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson et al., *Cell* 37:767 (1984), which is incorporated herein by reference in its entirety) and the “flag” tag.

[0106] The antibodies described herein include derivatives that are modified (e.g., by the covalent attachment of any type of molecule to the antibody). For example, but not by way of limitation, the antibody derivatives include antibodies that have been modified, e.g., by glycosylation, acetylation, pegylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, etc. Any of numerous chemical modifications may be carried out by known techniques, including, but not limited to, specific chemical cleavage, acetylation, formylation, metabolic synthesis of tunicamycin, etc. Additionally, the derivative may contain one or more non-classical amino acids.

[0107] Antibodies or fragments thereof with increased *in vivo* half-lives can be generated by attaching to said antibodies or antibody fragments polymer molecules such as high molecular weight polyethyleneglycol (PEG). PEG can be attached to said antibodies or antibody fragments with or without a multifunctional linker either through site-specific conjugation of the PEG to the N- or C-terminus of said antibodies or antibody fragments or via epsilon-amino groups present on lysine residues. Linear or branched polymer derivatization that results in minimal loss of biological activity will be used. The degree of conjugation will be closely monitored by SDS-PAGE and mass spectrometry to ensure proper conjugation of PEG molecules to the antibodies. Unreacted PEG can be separated from antibody-PEG conjugates by, e.g., size exclusion or ion-exchange chromatography.

[0108] Further, antibodies can be conjugated to albumin in order to make the antibody or antibody fragment more stable *in vivo* or have a longer half life *in vivo*. The techniques are well known in the art, see e.g., International Publication Nos. WO 93/15199, WO 93/15200, and WO 01/77137; and European Patent No. EP 413, 622, each of which is incorporated herein by reference in its entirety. Embodiments provided herein encompass the use of antibodies or fragments thereof conjugated or fused to one or more moieties, including but not limited to, peptides, polypeptides, proteins, fusion proteins, nucleic acid molecules, small molecules, mimetic agents, synthetic drugs, inorganic molecules, and organic molecules.

[0109] Various embodiments encompass the use of antibodies or fragments thereof recombinantly fused or chemically conjugated (including both covalent and non-covalent conjugations) to a heterologous protein or polypeptide (or fragment thereof, specifically to a polypeptide of at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90 or at least 100 amino acids) to generate fusion proteins. In some

embodiments, the antibodies or fragments thereof can be recombinantly fused or chemically conjugated (including both covalent and non-covalent conjugations) to a heterologous protein or polypeptide (or fragment thereof, specifically to a polypeptide of at least about 10, at least about 20, at least about 30, at least about 40, at least about 50, at least about 60, at least about 70, at least about 80, at least about 90 or at least about 100 amino acids) to generate fusion proteins. The fusion does not necessarily need to be direct, but may occur through linker sequences. For example, antibodies may be used to target heterologous polypeptides to particular cell types, either *in vitro* or *in vivo*, by fusing or conjugating the antibodies to antibodies specific for particular cell surface receptors. Antibodies fused or conjugated to heterologous polypeptides may also be used in *in vitro* immunoassays and purification methods using methods known in the art. See e.g., International publication No. WO 93/21232; European Patent No. EP 439,095; Naramura et al., 1994, *Immunol. Lett.* 39:91-99; U.S. Pat. No. 5,474,981; Gillies et al., 1992, *PNAS* 89:1428-1432; and Fell et al., 1991, *J. Immunol.* 146:2446-2452, each of which is incorporated herein by reference in its entirety.

[0110] Several embodiments include formulations comprising heterologous proteins, peptides or polypeptides fused or conjugated to antibody fragments. For example, the heterologous polypeptides may be fused or conjugated to a Fab fragment, Fd fragment, Fv fragment, F(ab)2 fragment, a VH domain, a VL domain, a VH CDR, a VL CDR, or fragment thereof. Methods for fusing or conjugating polypeptides to antibody portions are well known in the art. See, e.g., U.S. Pat. Nos. 5,336,603, 5,622,929, 5,359,046, 5,349,053, 5,447,851, and 5,112,946; European Patent Nos. EP 307,434 and EP 367,166; International publication Nos. WO 96/04388 and WO 91/06570; Ashkenazi et al., 1991, *Proc. Natl. Acad. Sci. USA* 88: 10535-10539; Zheng et al., 1995, *J. Immunol.* 154:5590-5600; and Vil et al., 1992, *Proc. Natl. Acad. Sci. USA* 89:11337-11341, each of which is incorporated herein by reference in its entirety.

[0111] Additional fusion proteins of antibodies that specifically bind GPR49 or fragments thereof (e.g., *supra*), may be generated through the techniques of gene-shuffling, motif-shuffling, exon-shuffling, and/or codon-shuffling (collectively referred to as “DNA shuffling”). DNA shuffling may be employed to alter the activities of antibodies or fragments thereof (e.g., antibodies or fragments thereof with higher affinities and lower dissociation rates). See, generally, U.S. Pat. Nos. 5,605,793; 5,811,238; 5,830,721; 5,834,252; and 5,837,458, and Patten et al., 1997, *Curr. Opinion Biotechnol.* 8:724-33; Harayama, 1998, *Trends Biotechnol.* 16(2): 76-82; Hansson, et al., 1999, *J. Mol. Biol.* 287:265-76; and Lorenzo and Blasco, 1998, *Biotechniques* 24(2): 308-313, each of which is incorporated herein by reference in its entirety. Antibodies or fragments thereof, or the encoded antibodies or fragments thereof, may be altered

by being subjected to random mutagenesis by error-prone PCR, random nucleotide insertion or other methods prior to recombination. One or more portions of a polynucleotide encoding an antibody or antibody fragment, which portions specifically bind to a C/CLP may be recombined with one or more components, motifs, sections, parts, domains, fragments, etc. of one or more heterologous molecules.

[0112] Moreover, the antibodies or fragments thereof can be fused to marker sequences, such as a peptide to facilitate purification. In certain embodiments, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, Calif., 91311), among others, many of which are commercially available. As described in Gentz et al., 1989, Proc. Natl. Acad. Sci. USA 86:821-824, which is incorporated herein by reference in its entirety, for instance, hexa-histidine provides for convenient purification of the fusion protein. Other peptide tags useful for purification include, but are not limited to, the hemagglutinin “HA” tag, which corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson et al., 1984, Cell 37:767, which is incorporated herein by reference in its entirety) and the “flag” tag.

[0113] Various embodiments further encompass antibodies or fragments thereof conjugated to a diagnostic or therapeutic agent. The antibodies can be used diagnostically to, for example, monitor the development or progression of a tumor as part of a clinical testing procedure to, e.g., determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, radioactive materials, positron emitting metals using various positron emission tomographies, and nonradioactive paramagnetic metal ions. The detectable substance may be coupled or conjugated either directly to the antibody (or fragment thereof) or indirectly, through an intermediate (such as, for example, a linker known in the art) using techniques known in the art. See, for example, U.S. Pat. No. 4,741,900, which is incorporated herein by reference in its entirety, for metal ions which can be conjugated to antibodies for use as diagnostics. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferon, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin; and examples of suitable radioactive material include but are not limited to, ¹²⁵I, ¹³¹I, ¹¹¹In or ⁹⁹Tc, in addition positron emitting metals using various positron emission

tomographies, nonradioactive paramagnetic metal ions, and molecules that are radiolabelled or conjugated to specific radioisotopes can be conjugated to the antibodies described herein.

[0114] Further, an antibody or fragment thereof may be conjugated to a therapeutic moiety such as a cytotoxin, e.g., a cytostatic or cytoidal agent, a therapeutic agent or a radioactive metal ion, e.g., alpha-emitters such as, for example, ^{213}Bi . A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Examples include paclitaxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Therapeutic agents include, but are not limited to, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioepa, chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclothosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum(II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g., vincristine and vinblastine). A more extensive list of therapeutic moieties can be found in PCT publication WO 03/075957, which is incorporated herein by reference in its entirety.

[0115] The conjugates can be used for modifying a given biological response, the therapeutic agent or drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, an apoptotic agent or an anti-angiogenic agent.

[0116] Antibodies may also be attached to solid supports, which are particularly useful for immunoassays or purification of the target antigen. Such solid supports include, but are not limited to, glass, cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene.

[0117] Techniques for conjugating such therapeutic moiety to antibodies are well known, see, e.g., Amon et al., "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in *Monoclonal Antibodies And Cancer Therapy*, Reisfeld et al. (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom et al., "Antibodies For Drug Delivery", in *Controlled Drug Delivery* (2nd Ed.), Robinson et al. (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in *Monoclonal Antibodies '84: Biological And Clinical Applications*, Pinchera et al. (eds.), pp.

475-506 (1985); “Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody in Cancer Therapy”, in Monoclonal Antibodies For Cancer Detection And Therapy, Baldwin et al. (eds.), pp. 303-16 (Academic Press 1985), and Thorpe et al., “The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates”, *Immunol. Rev.* 62:119-58 (1982), each of which is incorporated herein by reference in its entirety.

[0118] The antibodies can be conjugated to other polypeptides. Methods for fusing or conjugating antibodies to polypeptide moieties are known in the art. See, e.g., U.S. Pat. Nos. 5,336,603; 5,622,929; 5,359,046; 5,349,053; 5,447,851, and 5,112,946; EP 307,434; EP 367,166; PCT Publications WO 96/04388 and WO 91/06570; Ashkenazi et al., 1991, PNAS USA 88:10535; Zheng et al., 1995, *J Immunol* 154:5590; and Vil et al., 1992, PNAS USA 89:11337, each of which is incorporated herein by reference in its entirety. The fusion of an antibody to a moiety does not necessarily need to be direct, but may occur through linker sequences. Such linker molecules are commonly known in the art and described in Denardo et al., 1998, *Clin Cancer Res* 4:2483; Peterson et al., 1999, *Bioconjug Chem* 10:553; Zimmerman et al., 1999, *Nucl Med Biol* 26:943; Garnett, 2002, *Adv Drug Deliv Rev* 53:171. Alternatively, an antibody can be conjugated to a second antibody to form an antibody heteroconjugate as described by Segal in U.S. Pat. No. 4,676,980, each of which is incorporated herein by reference in its entirety.

Methods of Treating Cancer with Anti-GPR49 Antibodies

[0119] Several embodiments are drawn to treating cancer in a subject with anti-GPR49 antibodies. Various embodiments relate to treating colon cancer in a subject with anti-GPR49 antibodies. In some embodiments, a method of treating cancer, such as colon cancer, comprises administering an effective amount of an anti-GPR49 antibody to a subject sufficient to treat the cancer.

[0120] As used herein, “subject” includes organisms which are capable of suffering from a cancer treatable with an anti-GPR49 antibody, such as human and non-human animals. Preferred animals include human subjects. The term “non-human animals” of the invention includes all vertebrates, mammals, rodents (e.g. mice and rats), and non-human primates (e.g. monkeys and macaques). The term “administration” or “administering” includes routes of introducing an anti-GPR49 antibody to a subject to perform its intended function.

[0121] As used with respect to treating cancer, the term “effective amount” refers to an amount of anti-GPR49 antibody sufficient to treat cancer, which can be measured by a number of different parameters including, but not limited to, reduction in the size of a tumor in a subject having cancer, reduction in the growth rate or proliferation rate of a tumor in a subject

having cancer, preventing metastasis or reducing the extent of metastasis, or extending the survival of a subject having cancer compared to control. In several embodiments, an effective amount of an anti-GPR49 antibody will be sufficient to treat cancer in a subject as measured by reduction in the size of a tumor by about 5%, 10%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%, or any number in between the aforementioned percentages. In some aspects, the anti-GPR49 antibody is any one or combination of the anti-GPR49 antibodies designated as 2B5.5, 7F8.2, 1B3.5, 9C6.4, 6H5.4, 10A6.7, 10A9.2, 2G8.1, 6C10.5, 6G10.3, 8H8.1, 6B10.2, 3B8.11, 2F12.5, 5G2.11, 1F10.5, 10E1.1, 7C3.4, 2H9.2, 5B12.4, 3G8.1, 5F2.5, 6G10.1, 14H9.1, 12G5.1, 6E10.1, 14F7.1, 4A10.2, 3F11.1, 11F6.1, 5B10.1, 14A8.1, 8E9.1, 9C7.1, 4F6.2, 1B8.1, 18G7.1, 12E3.1, 6H5.1, 2P69.2, 17C9.1, 2H5.1, and 10A9.2 produced and described in the Examples below.

[0122] In several embodiments, the effective amount of the anti-GPR49 antibody sufficient to treat cancer in a subject can be administered in a dosage of about 1 μ g/kg, 50 μ g/kg, 100 μ g/kg, 150 μ g/kg, 200 μ g/kg, 250 μ g/kg, 300 μ g/kg, 350 μ g/kg, 400 μ g/kg, 500 μ g/kg, 550 μ g/kg, 600 μ g/kg, 700 μ g/kg, 800 μ g/kg, 900 μ g/kg, 1 mg/kg, 5 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg, 25 mg/kg, 30 mg/kg, 35 mg/kg, 40 mg/kg, 45 mg/kg, 50 mg/kg, 75 mg/kg, 100 mg/kg, 125 mg/kg, 150 mg/kg, 175 mg/kg, 200 mg/kg, 225 mg/kg, 250 mg/kg, 275 mg/kg, 300 mg/kg, 325 mg/kg, 350 mg/kg, 375 mg/kg, 400 mg/kg, 425 mg/kg, 450 mg/kg, 425 mg/kg, 450 mg/kg, 475 mg/kg, 500 mg/kg, 750 mg/kg, 1000 mg/kg, or any number in between any two of the aforementioned dosages (mass of antibody/mass of subject). In some aspects, the anti-GPR49 antibody is any one or combination of the anti-GPR49 antibodies designated as 2B5.5, 7F8.2, 1B3.5, 9C6.4, 6H5.4, 10A6.7, 10A9.2, 2G8.1, 6C10.5, 6G10.3, 8H8.1, 6B10.2, 3B8.11, 2F12.5, 5G2.11, 1F10.5, 10E1.1, 7C3.4, 2H9.2, 5B12.4, 3G8.1, 5F2.5, 6G10.1, 14H9.1, 12G5.1, 6E10.1, 14F7.1, 4A10.2, 3F11.1, 11F6.1, 5B10.1, 14A8.1, 8E9.1, 9C7.1, 4F6.2, 1B8.1, 18G7.1, 12E3.1, 6H5.1, 2P69.2, 17C9.1, 2H5.1, and 10A9.2 produced and described in the Examples below.

[0123] In some embodiments, the effective amount of an anti-GPR49 antibody sufficient to treat cancer is a blood or serum concentration in a subject of about 1 nM, 50 nM, 75 nM, 100 nM, 150 nM, 200 nM, 250 nM, 300 nM, 350 nM, 400 nM, 500 nM, 550 nM, 600 nM, 700 nM, 800 nM, 900 nM, 1 μ M, 5 μ M, 10 μ M, 15 μ M, 20 μ M, 25 μ M, 30 μ M, 35 μ M, 40 μ M, 45 μ M, 50 μ M, 55 μ M, 60 μ M, 65 μ M, 70 μ M, 75 μ M, 80 μ M, 85 μ M, 90 μ M, 95 μ M, 100 μ M, 150 μ M, 200 μ M, 250 μ M, 300 μ M, 350 μ M, 400 μ M, 500 μ M, 550 μ M, 600 μ M, 700 μ M, 800 μ M, 900 μ M, 1 mM, or any number in between any two of the aforementioned concentrations. In some aspects, the anti-GPR49 antibody is any one or combination of the anti-GPR49 antibodies designated as 2B5.5, 7F8.2, 1B3.5, 9C6.4, 6H5.4, 10A6.7, 10A9.2, 2G8.1, 6C10.5, 6G10.3, 8H8.1, 6B10.2, 3B8.11, 2F12.5, 5G2.11, 1F10.5, 10E1.1, 7C3.4, 2H9.2, 5B12.4, 3G8.1,

5F2.5, 6G10.1, 14H9.1, 12G5.1, 6E10.1, 14F7.1, 4A10.2, 3F11.1, 11F6.1, 5B10.1, 14A8.1, 8E9.1, 9C7.1, 4F6.2, 1B8.1, 18G7.1, 12E3.1, 6H5.1, 2P69.2, 17C9.1, 2H5.1, and 10A9.2 produced and described in the Examples below.

[0124] In another embodiment, the effective amount of an anti-GPR49 antibody can be administered as a fixed dosage irrespective of the subject's mass. For example, the effective amount of the anti-GPR49 antibody sufficient to treat cancer in a subject can be a fixed dose of about 1 µg, 50 µg, 75 µg, 100 µg, 150 µg, 200 µg, 250 µg, 300 µg, 350 µg, 400 µg, 500 µg, 550 µg, 600 µg, 700 µg, 800 µg, 900 µg, 1 mg, 5 mg, 10 mg, 15 mg, 20 mg, 25 mg, 30 mg, 35 mg, 40 mg, 45 mg, 50 mg, 75 mg, 100 mg, 200 mg, 300 mg, 400 mg, 500 mg, 600 mg, 700 mg, 800 mg, 900 mg, 1000 mg, 1250 mg, 1500 mg, 1750 mg, 2000 mg, 2250 mg, 2500 mg, 2750 mg, 3000 mg, 3500 mg, 4000 mg, 4500 mg, 1000 mg, 1500 mg, 2000 mg, 2500 mg, 3000 mg, 3500 mg, 4000 mg, 4500 mg, 5000 mg, 5500 mg, 6000 mg, 6500 mg, 7000 mg, 7500 mg, 8000 mg, 8500 mg, 9000 mg, 9500 mg, 10,000 mg, or any number in between any two of the aforementioned fixed doses. In some aspects, the anti-GPR49 antibody is any one or combination of the anti-GPR49 antibodies designated as 2B5.5, 7F8.2, 1B3.5, 9C6.4, 6H5.4, 10A6.7, 10A9.2, 2G8.1, 6C10.5, 6G10.3, 8H8.1, 6B10.2, 3B8.11, 2F12.5, 5G2.11, 1F10.5, 10E1.1, 7C3.4, 2H9.2, 5B12.4, 3G8.1, 5F2.5, 6G10.1, 14H9.1, 12G5.1, 6E10.1, 14F7.1, 4A10.2, 3F11.1, 11F6.1, 5B10.1, 14A8.1, 8E9.1, 9C7.1, 4F6.2, 1B8.1, 18G7.1, 12E3.1, 6H5.1, 2P69.2, 17C9.1, 2H5.1, and 10A9.2 produced and described in the Examples below.

Administration and Pharmaceutical Forms

[0125] The anti-GPR49 antibodies can be administered in a variety of ways and pharmaceutical forms in the embodiments provided herein for treating cancer. As such, several embodiments are drawn to pharmaceutical compositions comprising any one or combination of the anti-GPR49 antibodies described above and in the Examples below, and a pharmaceutically acceptable carrier or diluent depending on the route and form of administration.

[0126] Examples of routes of administration that may be used include injection (subcutaneous, intravenous, parenterally, intraperitoneally, intrathecal), or oral routes. The pharmaceutical preparations may be given by forms suitable for each administration route. For example, these preparations can be administered in tablets or capsule form, by injection or orally. The injection can be bolus or can be continuous infusion. The anti-GPR49 antibodies can be administered alone, or in conjunction with either another agent or agents known in the art for treating cancer or with a pharmaceutically-acceptable carrier, or both.

[0127] "Carriers" as used herein include pharmaceutically acceptable carriers, excipients, or stabilizers which are nontoxic to subject being exposed thereto at the dosages and

concentrations employed. Often the physiologically acceptable carrier is an aqueous pH buffered solution. Examples of physiologically acceptable carriers include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TWEEN, polyethylene glycol (PEG).

[0128] Solid dosage forms for oral administration include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the antibodies can be admixed with at least one inert pharmaceutically acceptable carrier such as sucrose, lactose, or starch. Such dosage forms can also comprise, as is normal practice, additional substances other than inert diluents, for example, lubricating agents such as magnesium stearate. In the case of capsules, tablets, and pills, the dosage forms may also comprise buffering agents. Tablets and pills can additionally be prepared with enteric coatings known in the art.

[0129] Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, solutions, suspensions, syrups, with the elixirs containing inert diluents commonly used in the art, such as water. Besides such inert diluents, compositions can also include adjuvants, such as wetting agents, emulsifying and suspending agents, and sweetening, flavoring, and perfuming agents.

[0130] The anti-GPR49 antibodies can also be administered parenterally. The phrases “parenteral administration” and “administered parenterally” as used herein includes, for example, modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticulare, subcapsular, subarachnoid, intraspinal and intrasternal injection and infusion. Parenteral administration can include sterile aqueous or non-aqueous solutions, suspensions, or emulsions. Examples of non-aqueous solvents or vehicles are propylene glycol, polyethylene glycol, vegetable oils, such as olive oil and corn oil, gelatin, and injectable organic esters such as ethyl oleate. Such dosage forms may also contain adjuvants such as preserving, wetting, emulsifying, and dispersing agents. They may be sterilized by, for example, filtration through a bacteria retaining filter, by incorporating sterilizing agents into the compositions, by irradiating the compositions, or by heating the compositions. They can also be manufactured using sterile water, or some other sterile injectable medium, immediately before use.

[0131] For parenteral administration, the antibodies can be, for example, formulated as a solution, suspension, emulsion or lyophilized powder in association with a pharmaceutically acceptable parenteral vehicle. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Liposomes and nonaqueous vehicles such as fixed oils may also be used. The vehicle or lyophilized powder may contain additives that maintain isotonicity (for example, sodium chloride, mannitol) and chemical stability (for example, buffers and preservatives). The formulation is sterilized by commonly used techniques. For example, a parenteral composition suitable for administration by injection is prepared by dissolving 1.5% by weight of active ingredient in 0.9% sodium chloride solution.

[0132] The pharmaceutical compositions described herein can be administered as a single dose or in multiple doses; administered either as individual therapeutic agents or in combination with other therapeutic agents; and combined with conventional therapies, which may be administered sequentially or simultaneously.

Combination Therapy

[0133] Any of the anti-GPR49 antibodies described above can be administered in combination with each other and/or with any therapeutic agent known to be useful for treating cancer. Non-limiting classes of therapeutic agents that can be combined with an anti-GPR49 antibody described above include, but are not limited to alkylating agents, antimetabolites, antibiotics, plant-derived antitumor agents, camptothecin derivatives, topoisomerase inhibitors, tyrosine kinase inhibitors, hormonal therapy agents, antibodies, and interferons.

[0134] Alkylating agents include, but are not limited to, nitrogen mustard N-oxide, cyclophosphamide, ifosfamide, melphalan, busulfan, mitobronitol, carboquone, thiotepa, ranimustine, nimustine, temozolomide, AMD-473, altretamine, AP-5280, apaziquone, brostallicin, bendamustine, carmustine, estramustine, fotemustin, glufosfamide, ifosfamide, KW-2170, mafosfamide, and mitolactol; platinum-coordinated alkylating compounds include but are not limited to, cisplatin, carboplatin, eptaplatin, lobaplatin, nedaplatin, oxaliplatin or satrplatin.

[0135] Antimetabolites include but are not limited to, methotrexate, 6-mercaptopurine riboside, mercaptopurine, 5-fluorouracil (5-FU) alone or in combination with leucovorin, tegafur, UFT, doxifluridine, carmofur, cytarabine, cytarabine ocfosfate, enocitabine, S-1, gemcitabine, fludarabin, 5-azacitidine, capecitabine, cladribine, clofarabine, decitabine, eflornithine, ethynylcytidine, cytosine arabinoside, hydroxyurea, TS-1, melphalan, nelarabine, nolatrexed, ocfosfate, disodium premetrexed, pentostatin, pelitrexol, raltitrexed, triapine, trimetrexate, vidarabine, vincristine, or vinorelbine.

[0136] Antibiotics include but are not limited to: aclarubicin, actinomycin D, amrubicin, annamycin, bleomycin, daunorubicin, doxorubicin, elsamitruclin, epirubicin, galarubicin, idarubicin, mitomycin C, memorubicin, neocarzinostatin, peplomycin, pirarubicin, rebeccamycin, stimalamer, srteptozocin, valrubicin or zinostatin.

[0137] Hormonal therapy agent include exemestane, anastrozole, doxercalciferol, fadrozole, formestane, anti-estrogens such as tamoxifen citrate and fulvestrant, toremifene, raloxifene, lasofoxifene, letrozole, or anti-androgens such as bicalutamide, flutamide, mifepristone, nilutamide, and 4'-cyano-3-(4-fluorophenylsulphonyl)-2-hydroxy-2-methyl-3'-(trifluoromethyl)propionanilide).

[0138] Plant derived anti-tumor substances include for example those selected from nitotic inhibitors, for example vinblastine, docetaxel, and paclitaxel.

[0139] Cytotoxic topoisomerase inhibiting agents include one or more agents selected from the group consisting of aclarubicin, amonafide, belotecan, camptothecin, 10-hydroxycamptothecin, 9-aminocamptothecin, diflomotecan, irinotecan, edotecarin, epirubicin, etoposide, exatecan, gimatecan, lurtotecan, mitoxantrone, pirarubicin, pixantrone, rubitecan, sobuzoxane, SN-38, tafluposide, and topotecan, and combinations thereof.

[0140] Immunologicals include interfeons and numerous other immune enhancing agents. Interferons include interferon alpha, interferon alpha-2a, interferon, alpha-2b, interferon beta, interferon gamma-1a or interferon gamma-n1.

[0141] Other anticancer agents include PF3512676, filgrastim, lentinan, sizofilan, ubenimex, WF-10, aldesleukin, alemtuzumab, BAM-002, dacarbazine, daclizumab, denileukin, gemtuzumab ozogamicin, ibritumomab, imiquimod, lenograstim, lentinan, melgramostin, sargramostim, tasonermin, tecleukin, thymalasin, tositumomab, Virulizin, Z-100, epratuzumab, mitumomab, oregovomab, pemtumomab, alitretinoin, ampligen, atrasentan, bexarotene, bortezomib, calcitrol, exisulind, finasteride, fotemustine, ibandronic acid, miltefosine, mitoxantrone, l-asparaginase, procarbazine, dacarbazine, hydroxycarbamide, pegaspargase, pentostatin, tazarotene, TLK-286, or tretinoin.

[0142] Anti-angiogenic compounds include acitretin, fenretinide, thalidomide, zoledronic acid, angiostatin, aplidine, cilengtide, combretastatin A-4, endostatin, halofuginone, rebimastat, removab, squalamine, and ukrain.

[0143] A more extensive list of therapeutic agents that can be used in combination with an anti-GPR49 antibody can be found in PCT publication WO 03/075957, which is incorporated herein by reference in its entirety.

[0144] While the present embodiments have been described in some detail for purposes of clarity and understanding, one skilled in the art will appreciate that various changes in form and detail can be made without departing from the true scope of the invention.

EXAMPLES

[0145] Having generally described embodiments drawn to antibodies against GPR49, hybridomas or other cell lines expressing such antibodies, nucleic acids and vectors comprising nucleic acids encoding for such antibodies, and methods of treating cancer with such antibodies, a further understanding can be obtained by reference to certain specific examples which are provided for purposes of illustration only and are not intended to be limiting.

Example 1

Selection of human Fabs specific to human GPR49 from Phage display libraries

[0146] Human antibodies that specifically recognize the extracellular domain of a human GPR49 receptor were isolated using phage display technology.

Part I: Phage-display Panning

[0147] Methods: Recombinant human GPR49-Fc ectodomain (GPR49-Fc) (SEQ ID NO: 3) was used to screen a human naive phagemid Fab library containing 3.5×10^{10} unique clones (Hoet, R. M., et al. NatBiotechnol. 23(3): 344-8 (2005)). Prior to incubation with the phage library, a biotinylated anti-Fc antibody was captured on magnetic beads, followed by capture of the GPR49-Fc fusion protein. Selections were performed as described in Hoet et al. After 3 rounds of panning, the 479 bp gene III stump was removed by MluI digestion, and the vector was relegated for soluble Fab expression in T25 cells.

[0148] Results: 61 unique clones were isolated in this panning. Unique clones were subsequently purified and binding was reconfirmed.

Part II: ELISA

[0149] Binding of Fabs to recombinant human GPR49-Fc ectodomain was demonstrated by ELISA. Methods: In brief, soluble GPR49-Fc fusion protein at 2.5 µg/ml in 0.025 M carbonate buffer, pH 9.6 was coated at 50 µl/well in a 96-well (IMMULON2 HB, Dynex Technologies, Inc., Cat. #3455) plate and incubated overnight at 4° C. The plate was washed with phosphate-buffered saline (PBS, Irvine Scientific, Cat#9240), pH 7.4 plus 0.025% Tween 20 in the Skan Washer 300 (Skatron Instruments), blocked with buffer containing 1% nonfat milk, 0.05% Tween 20 in PBS, pH 7.4, and then incubated at room temperature for 1 hour. After

incubation the plate was washed with PBS plus 0.025% Tween 20 in the Skan Washer 300. For the assay, the GPR49-coated plate was next incubated with the control and test antibodies of varied concentrations, diluted in 1% nonfat milk, 0.05% Tween 20 in PBS at 50 ul/well. Following one hour incubation at room temperature, plate washed with PBS plus 0.025% Tween 20 in the Skan Washer 300. A 2000-fold dilution in 1% nonfat milk, 0.05% Tween 20 in PBS of goat anti-human Kappa—HRP (Southern Biotech Cat#2060-05) was added 50 ul/well to detect bound Fab. Plate incubated for 1 hour at room temperature washed with PBS plus 0.025% Tween 20 in the Skan Washer 300. TMB solution (KIRKEGAARD & PERRY LABS, INC. cat: 50-76-00) was added 100 ul/well, and the reaction was stopped with 50 ul/well of 4N H2S04 (LabChem, Cat#LC25830-I) after two minutes. The absorbance was measured at 450 nm, background 540 nm for TMB using the Molecular Devices plate reader. Data was analyzed using the SOFTMAX PRO software package version 4.3 LS (Molecular Devices Corp.) (Figure 1).

[0150] Results: This resulted in six Fabs with titratable binding: 71C10, 86C11, 66D05, 76C12, 78F05, and 76B04.

Part III: FACS Analysis

[0151] Methods: The six GPR49 Fabs were diluted 1:20, 1:40, and 1:80 and tested for binding to HEK293E transfected with HA-GPR49 by FACS. Twenty-four to forty-eight hours post-transfection, cells are collected in suspension and incubated on ice with anti-GPR49 antibodies or control IgG. The cells were washed and primary antibodies detected with anti-mouse secondary antibodies conjugated to a fluorescent chromophore. Labeled cells were then sorted by FACS to identify anti-GPR49 antibodies that specifically recognize expression of native cell-surface GPR49 protein.

[0152] Results: All six prospective FACS positive GPR49 Fabs showed a decrease in binding to HA-GPR49-HEK293E with increasing Fab dilution and none showed binding to HEK293E (Figure 2). Low geometric means may be due to Fab epitopes not being very accessible, low expression of GPR49 on cell surface, or Fabs may be low affinity. Additional testing by FACS of GPR49 Fabs (diluted 2-fold from 400nM down to zero) on CHO-GPR49 (50nM MTX) compared to parental CHO revealed three Fabs with approximate EC50 < 10nM: 76C12, 76B04, and 78F05 (Figures 3A and 3B). Testing of the Fabs against tumor cell lines (SW480, SW620 and HCT116) revealed only Fab 76C12 is capable of binding by FACS (Figures 4A, 4B, 5A-C, and 6A-C).

Part IV: Biacore Analysis

[0153] Methods: Three particular Fabs (76C12, 78F05 and 76B04) were identified that specifically bound the human GPR49 receptor with less than 10nM affinity by FACS. To analyze the binding kinetics, biotinylated anti-human IgG Fc antibody was immobilized on a Biacore SA chip to a level of 2950 RUs. GPR49-Fc was then captured to a density of ~400 RUs in flow cell 2 with flow cell 1 used as a reference. Purified Fabs (100, 50, 25 nM in HSP-EP) were injected at 30 μ l/min for 7 minutes with 20 minutes allowed for dissociation. The data were analyzed with BIAevaluation software (v4.1) assuming a 1:1 model. To test the cross reactivity of the Fabs with the Fc domain, IgG1 Fc was then captured to a density of ~250 RUs in flow cell 2 with flow cell 1 used as a reference. All Fabs were then tested under the same conditions as above. All biacore experiments were performed at 25°C on a BIACore 3000 instrument.

[0154] Results: Fabs 76C12 and 78F05 bound with K_D 's of 3.4 nM and 1.7 nM respectively. Fab 76B04 exhibited weak binding.

Example 2

Construction of Full-Length Anti-GPR49 IgGs

[0155] Methods: Three Fabs were converted to human IgG1 and expressed in CHO cells. DNA sequences encoding three distinct anti-GPR49 Fabs 76C12, 78F05 and 76B04 were selected from a human antibody phage library (Dyax Corp) by biopanning against a recombinant human GPR49 ectodomain-Fc fusion protein. The Fab gene sequences were used to construct expression plasmids encoding full-length antibodies using the pV90AS expression vector system for antibody production in mammalian cells. pV90AS is a modified pV90 expression vector designed to generate two transcripts from a single promoter through alternate splicing of a primary transcript (Reference: USPTO Application WO2005/089285). The natural CMV splice donor is spliced either to a partially impaired splice acceptor to generate an antibody light chain-encoding transcript, or to a natural CMV splice acceptor to generate the antibody heavy chain-encoding transcript. The partially impaired splice acceptor has been engineered to result in similar amounts of both heavy and light chain transcripts. Light chain Variable (VL) and Constant (CL) regions were amplified by PCR. The 5' light chain PCR primer included a Sfi I restriction endonuclease site followed by sequence encoding an immunoglobulin light chain signal peptide MDMRVPAQLLGLLLWLPGARC (SEQ ID NO: 5) in frame to sequences corresponding to the amino-terminus of the VL region according to the methods described in Nakamura T, et al., Int J Immunopharmacol. 22:131-41 (2000), which is incorporated herein by reference in its entirety. The PCR product was purified by agarose gel electrophoresis and extraction using the

QIAquick GelExtraction kit protocol (QIAGEN CA), digested with restriction endonucleases Sfi I and Asc I and ligated with the Sfi I/Asc I digested pHLP025 vector (Holly Prentice). The pHLP025 vector contains Sfi I/Asc I restriction endonuclease sites for receiving antibody light chain (signal peptide-VL-CL) as a Sfi I/Asc I digested PCR fragment in addition to the natural CMV splice donor site sequence, a partially impaired splice acceptor site sequence, and a poly A signal sequence (Reference: USPTO Application WO2005/089285).

[0156] The heavy chain Variable (VH) region of each anti-GPR49 Fab (76C12, 78F05, 76B04) was amplified by PCR. The 5' heavy chain VH PCR included a Nco I restriction endonuclease site followed by sequence encoding synthetic heavy chain signal peptide MGWSLILLFLVAVATRVL (SEQ ID NO: 6) in frame to sequences corresponding to the aminotermminus of the VH region as described above. The 3' heavy chain VH PCR primer included sequence corresponding to the carboxyl-terminus of the VH region and an Sfi I site. The PCR product was purified by agarose gel electrophoresis and extraction using the QIAquick GelExtraction kit protocol (QIAGEN, CA), digested with restriction endonucleases Nco I and Sfi I and ligated with the Nco I/Sfi I digested pHLP029 vector (Holly Prentice). The pHLP029 vector contains Nco I/Sfi I sites for receiving the antibody signal peptide-VH sequence as a Nco I/Sfi I digested PCR fragment in addition to an upstream poly A signal sequence, a natural CMV splice acceptor site sequence, and a downstream poly A signal sequence (Reference: USPTO Application WO2005/089285).

[0157] The gene sequences coding for (Sfi I site-light chain signal peptide-anti-GPR49 VL and CL) in pHLP025 and (heavy chain signal peptide-anti-GPR49 VH-Sfi I site) in pHLP029 were assembled into a single DNA fragment by PCR amplification through common overlapping sequences present in both vectors using the 5' light chain and 3' heavy chain VH PCR primers described above. The resulting PCR product was purified by agarose gel electrophoresis and extraction using the QIAquick GelExtraction kit protocol (QIAGEN, CA), digested with restriction endonuclease Sfi I and ligated with the Dra III digested IgG1 parental vector.

[0158] Results: The resulting plasmid produces a bi-cistronic precursor transcript that upon alternative splicing results in translationally active antibody heavy and light chain mRNAs in approximately stoichiometric quantities. Correct sequences were confirmed by DNA sequence analysis. Expression of full-length in mammalian cells resulted in production of stable, human IgG1 antibodies.

Example 3Construction of Full-Length Anti-GPR49 IgGs for Improved Expression in Mammalian Cells

[0159] To improve antibody expression yields and product quality the original VH gene sequences from anti-GPR49 Fabs 76C12, 78F05, 76B04 were modified.

[0160] Methods: First, anti-GPR49 VH sequences were analyzed for sequences containing putative splice sites with public sequence recognition programs (www.tigr.org/tdb/GeneSplicer/gene_spl.html (The Institute for Genomic Research, 9712 Medical Center Drive, Rockville, Md. 20850), www.fruitfly.org/seq_tools/splice.html). (Martin G. Reese and Frank H. Eeckman, Lawrence Berkeley National Laboratory, Genome Informatics Group, 1 Cyclotron Road, Berkeley, Calif, 94720; see also, Reese M G, Eeckman, F H, Kulp, D, Haussler, D, 1997. "Improved Splice Site Detection in Genie". *J Comp Biol* 4(3), 311-23.). Second, codons in the heavy chain variable region of the anti-GPR49 Fabs were replaced with codons corresponding to the identical Kabat positions from antibodies that have been successfully expressed in CHO cells without encountering any changes in the original anti-GPR49 VH polypeptide sequence. This second step mostly removes putative splice sites but an additional splice site analysis followed by synonymous codon exchange was performed to reduce the predicted likelihood of a putative splice site being present.

[0161] DNA fragments encoding synthetic heavy chain leader in frame with sequence-optimized VH sequences of anti- GPR49Fabs were obtained as chemically synthesized doublestranded DNA sequences from a commercial provider (Blue Heron Biotechnology, Inc. Bothell Wash.). The Nco I and Sfi I restriction endonuclease sites at 5' and 3' were included in the synthesized fragments. The leader and anti-GPR49 sequence-optimized VH region fragments were cloned into the Nco I/Sfi I digested the pHLP029 vector as described in Example 2 above. Recombination with the appropriate corresponding light chains in pHLP025 and subsequent cloning of the single fragment is as described in Example 2 above. Correct sequences were confirmed by DNA sequence analysis.

[0162] Results: Expression of full-length antibodies from this plasmid series in mammalian cells results in increased production of stable, human IgG1 antibodies.

Example 4Transient Expression and Characterization of GPR49 Antibodies

[0163] Methods: Plasmid DNAs were used to transform CHO DG44 cells for transient production of antibody protein. 20 u.g of plasmid DNA was combined with 4x10⁶ cells in a volume of 0.4 mL of 1xPBS. The mixture was added to a 0.4 cm cuvette (BioRad) and placed on ice for 15 min. The cells were electroporated at 600 uF and 350 volts with a Gene

Pulser electroporator (BioRad). The cells were placed into a T-25 flask containing CHO-SSFM II media plus 100 uM Hypoxanthine and 16 uM Thymidine and incubated at 37° for 4 days. In addition, plasmid DNA was also used to transfect 293E cells for transient expression of antibody protein. 1.2 u.g of each (heavy and light) plasmid DNA was transfected into 2xl06 cells with Qiagen's Effectene Transfection Protocol (Qiagen, CA). Cells were incubated at 37° C. for 3 days.

[0164] Results: Supernatant was harvested and full-length antibody confirmed by both Western Blot and ELISA methods. The ability of full IgG1 to bind to GPR49 was confirmed by ELISA.

Example 5

Development of Anti-GPR49 Antibody Producing CHO Cell Line

[0165] This example gives a detailed description of expression of the anti-GPR49 antibody comprising the binding domain of the Fab 76C12 as full-length IgG1. The other Fabs described herein, i.e., those listed in Example 1, were expressed in a similar manner.

[0166] Methods: The variable and constant regions of 76C12 are of human sequence origin. The entire light chain and heavy chain variable regions are derived from a Fab generated against human GPR49 by the DYAX phage display technology. The variable, as well as the light chain constant regions were subcloned into an alternate splice expression vector. The alternate splice configuration links the light and heavy chain through the usage of a single splice donor with two splice acceptors where each splice acceptor generates a transcript encoding one of the two chains. The expression vector DNA encoding the immunoglobulin genes was electroporated into insulin independent Chinese hamster ovary cells (CHO DG44i). A CHO transfectoma was selected for production purposes.

[0167] Complementary DNA from the corresponding variable (VL) and constant (CL) domains of the light chain gene of 76C12 and the variable (VH) domain of the heavy chain gene of 76C12 was cloned into an expression vector. The vector contains cloning sites for inserting the entire light chain and variable heavy cDNAs directly upstream of the human heavy chain constant region. In addition to the Ig genes, this expression vector contains a dihydrofolate reductase (DHFR) gene that can be used for selection in mammalian cells. The resulting expression vector was then transfected into CHO cells to initiate the generation of the anti-GPR49 secreting CHO cell lines.

[0168] The expression vector was electroporated into CHO cells. Immunoglobulin light chain specific PCR primers were used to PCR amplify the Fab light chain cDNA. The 5' specific oligo sequence included the native signal peptide from the light chain of the Biogen

Idec anti-CD23 molecule. The 5' and 3' oligos contain Sfi I and Asc I restriction endonuclease recognition sequences, respectively, for subcloning into an intermediate vector. The VH cDNA was PCR amplified using a 5' oligo that included a synthetic heavy chain signal peptide. The 5' and 3' oligos contain Nco I and Sfi I restriction endonuclease recognition sequences, respectively, for subcloning into an intermediate vector.

[0169] Overlapping PCR using the light chain 5' and VH 3' oligos as templates was employed to combine the light chain and the VH region as one cDNA segment. The resultant product was subcloned into the Dra III site thus creating the final alternate splice expression vector. The alternate splice configuration generates two transcripts from a single promoter through alternate splicing of the primary transcript. The natural CMV splice donor is spliced either to a suboptimal splice acceptor to generate a light chain-encoding transcript, or to a natural CMV splice acceptor to generate the heavy chain-coding transcript. The sub-optimal splice acceptor has been designed to generate similar amounts of both transcripts.

[0170] The final DNA vector was prepared in HEBS buffer at a concentration of 700 ng/uL prior to electroporation in to CHO cells. Five electroporations were performed using various concentrations of DNA (15, 20, 30, 40, and 45 ug). Each electroporation was done in a disposable 0.4 cm cuvette (Invitrogen) containing 4x106 log phase CHO cells in 0.7 ml sterile HEBS buffer and DNA in 0.1 mL HEBS (0.8 mL total volume). Cells were shocked using a Bio-Rad Gene Pulser XCELL, set at 290 volts, 950 micro Faradays. Shocked cells were then allowed to stand at room temperature for 10 minutes then mixed with 10 mL room temp insulin free CHOM16 medium, centrifuged (3'@ 1000 rpm), and aspirated. Cells were then resuspended in 12 mL insulin free CHOM16 medium (RT) and transferred to a T-75 tissue culture flask.

[0171] Cells and Media: prior to electroporation the CHO cells were grown in serum free media (CHOM24) with the addition of 1x nucleosides. CHOM24 is a chemically defined in-house media formulation that does not contain any animal components. Methotrexate selection was performed in nucleoside free CHOM16 and CHOM24 chemically defined media.

[0172] Following electroporation, 4x106 CHO cells were pooled into a T-75 flask. Selection for DHFR expression began immediately as the cells were inoculated in nucleoside free medium. Cells were eventually expanded to 125 mL shake flasks in CHOM24 (~3 weeks). To isolate clonal cell lines, the transfected stable pools were diluted and plated at 1 cell/well in 200 uL CHOM16 on four 96-well plates. Plates were maintained at 37° C until they were screened for antibody titer.

[0173] CHO colonies were screened for immunoglobulin production by assaying cell supernatants using an ELISA specific for the human kappa chain (day 21 to day 28 after plating). The capture antibody used in the ELISA was a polyclonal goat anti-human IgG

(SouthernBiotech) and the detection antibody was a polyclonal goat anti-human kappa conjugated to horseradish peroxidase (SouthernBiotech). Colonies secreting the highest amount of immunoglobulin were expanded.

[0174] Results: High-expressing CHO cell lines were developed that resulted in anti-GPR49 mAbs with expected biochemical and biophysical properties suitable for scale-up and manufacturing.

Example 6

Purification and Characterization of Fully Human Anti-GPR49 IgG1 Antibodies

[0175] The antibody produced in CHO cells were purified and characterized by methods described below.

[0176] Methods: Protein A Capture: A Protein A column was pre-equilibrated with 1xPBS (equilibration buffer) at 100-150 cm/hr with 3 column volumes. Supernatant was loaded at 150 cm/hr with a maximum of 10 mg of GPR49 mAb per milliliter of resin. After loading, the column was washed with 5 column volumes of equilibration buffer. Then, the column was step eluted in an upflow direction with 100 mM Glycine, pH 3.0. Desired fractions were collected and titrated to neutral pH with 2M Tris base. Collected fractions were dialyzed against 1xPBS and concentrate material to prepare for the size exclusion step. A Size Exclusion aggregate removal step involved equilibration of SUPERDEX 200 with 1xPBS with 1.5 column volumes at a flow rate of 36 cm/hr followed by loading of protein and collecting desired fractions.

[0177] Identity testing was performed as follows:

[0178] 1) Intact mass analysis by mass spectrometry where molecular mass measurements were performed on an electrospray mass spectrometer (ESI-MSD). Prior to analysis, the sample was reduced to remove disulfide bonds. The deconvoluted mass spectrum represents the masses of the heavy and light chains.

[0179] 2) N-terminal sequence analysis was performed by Edman degradation using an ABI protein sequencer equipped with an on-line PTH analyzer. The sequences for the initial amino acids of the light chain and heavy chain were identified.

[0180] 3) Peptide mapping with mass spectrometric analysis: tryptic or/and EndoLysC peptide maps were performed to obtain complete sequence coverage by analysis of the LC/MS data generated from each peptide. In addition, determination of sites and amounts of oxidation and deamidation were detected.

[0181] Purity testing was performed by; 1) SDS-Page or CE-SDS: Reduced and non-reduced samples, this technique is used to measure antibody fragmentation, aggregation and impurities, 2) SEC-HPLC with LS and RI technique was used to measure aggregation and

fragmentation and light scattering determines the molar mass of sample components. 3) SDS gel or capillary IEF method was used to determine the isoelectric focusing pattern and pi distribution of charge isoforms that can result from C- and N-terminal heterogeneity and/or deamidation. Finally, endotoxin concentrations were measured by the Limulus amoebocyte lysate (LAL) kinetic turbidometric method.

[0182] Results: The purification of anti-GPR49 mAbs resulted in greater than 99% monomer, endotoxin free mAbs in gram quantities with properties suitable for scale-up and manufacturing.

Example 7

Production of mouse antibodies to human GPR49 ectodomain

Part I: Hybridoma selection

[0183] Methods: To produce antibodies against the ectodomain of GPR49, mice were immunized three times with purified and endotoxin free GPR49-His (SEQID 2) using standard techniques. Blood from individual mice was screened for antigen recognition using ELISA and FACS analysis. The animals with the highest antibody titers were then selected for final antigen boost after which spleen cells were isolated for hybridoma production. Approximately 1,000 clones were transferred from 4x24-well fusion plates to 10x96-well culture plates. 199 positive clones were selected by GPR49-Fc capture ELISA, and transferred to 48-well plates. From these, 100 positive clones were not selected because they either showed GPR49-CHO negative or positive binding to parental CHO by FACS. 50 of the 99 positive clones were then selected depending upon isotype.

[0184] Results: 36 clones were sub-cloned (single or mixed IgGs bands, such as, IgG1/k, IgG2a/G and IgG1/2b/k). 8 clones lost expression from the parental to subclones. Monoclonal antibodies (mAbs) from 24 selected subclones were purified from the hybridoma supernatant using protein A or protein G agarose chromatography and antibodies were tested by FACS as described below.

Part II: FACS Analysis

[0185] Methods: The 24 murine GPR49 mAbs were serially diluted and tested for binding to CHO transfected with GPR49-Flag-His and parental CHO by FACS (standard methods).

[0186] Results: The results are summarized in Table 2 (data shown in Figures 7A-C)

Table 2

GPR49 ms mAb	FACs EC50 (nM)
2B5.5	~0.2
7F8.2	~0.2
1B3.5	0.8129
9C6.4	0.8297
6H5.4	~1
10A6.7	1.428
10A9.2	4.522
2G8.1	9.766
6C10.5	9.978
6G10.3	~10
8H8.1	11.19
6B10.2	11.2
3B8.11	12.14
2F12.5	14.64
5G2.11	20.94
1F10.5	24.54
10E1.1	29.49
7C3.4	34.51
2H9.2	43.27
5B12.4	43.56
3G8.1	63.83
5F2.5	530.5
6G10.1	2658

Part III: Biacore Analysis

[0187] The binding kinetics of the mouse mAbs were analyzed by Biacore.

[0188] Methods: Biotinylated anti-human IgG Fc antibody was immobilized on a Biacore SA chip to a level of 2950 RUs. GPR49-Fc was then captured to a density of ~400 RUs in flow cell 2 with flow cell 1 used as a reference. Purified murine mAbs (100, 50, 25 nM in HSP-EP) were injected at 30 μ l/min for 7 minutes with 20 minutes allowed for dissociation. The

data were analyzed with BIAevaluation software (v4.1) assuming a 1:1 model. All biacore experiments were performed at 25°C on a BIACore 3000 instrument.

[0189] Results: Murine mAbs bound to GPR49-Fc with 64nM to less than 1 nM affinities (K_D). Data are shown in Table 3.

Table 3

	BIACore
mAb	K_D nM
1B3-5	1.11
5B12.4	7.52
6C10.5	0.68
9C6.4	1.44
5G2.11	1.59
6G10_3	2.72
10E1_1	3.98
7F8.2	0.91
10A9.2	<100pM
6B10_2	3.05
2H9-2	0.71
2G8.1	N/A
6H5.4	0.17
10A6_7	<100pM
3B8-11	3.92 uM
3G8.1	0.99
7C3_4	6.32
2B5-5	1.67
8H8_1	1.54
2F12.5	1.29
1F10.5	64.0
5F2_5	5.52
6G10.1	0.14

Example 8**Production of mouse antibodies to full-length human GPR49****Part I: Hybridoma selection**

[0190] Methods: To produce antibodies against the full length receptor, mice were immunized three times with 10ug/mouse of DNA vector encoding the full-length cDNA clone of the GPR49 gene mixed with gold particles. Blood from individual mice was screened approximately 75 days after initial immunization for antigen recognition using ELISA and FACS analysis. The animals with the highest antibody titers were then selected for final antigen boost (25ug GPR49-Fc, 10ug GPR49-DNA/gold particle, and 5×10^6 GPR49-CHO cells) after which spleen cells were isolated for hybridoma production. Approximately 10,000 clones were generated with approximately 200 positive clones were selected by GPR49-CHO capture ELISA.

[0191] Results: 67 clones were confirmed by ELISA and GPR49-CHO FACS. 22 of the positive clones were then sub-cloned (single or mixed IgGs bands, such as, IgG1/k, IgG2a/G and IgG1/2b/k). Monoclonal antibodies (mAbs) from 19 selected subclones were purified from the hybridoma supernatant using protein A or protein G agarose chromatography and antibodies were tested by FACS as described below.

Part II: FACS Analysis

[0192] Methods: The 19 murine GPR49 mAbs were serially diluted and tested for binding to CHO transfected with GPR49-Flag-His and parental CHO by FACS (standard methods).

[0193] Results: Murine mAbs bound to GPR49 with 17nM to less than 1 nM (EC50's). The results are summarized in Table 4 (data shown in Figures 8A-D). Antibody 10A9.1 was used as a control.

Table 4

mAb	EC50 (nM, CHO-GPR49)
14H9.1	0.9
12G5.1	1.2
6E10.1	1.3
14F7.1	1.5
4A10.2	1.7

3F11.1	1.8
11F6.1	1.9
5B10.1	1.9
14A8.1	2.1
8E9.1	2.2
9C7.1	2.2
4F6.2	2.2
1B8.1	2.2
18G7.1	2.5
12E3.1	2.6
6H5.1	3.0
2P69.2	5.1
17C9.1	12.5
2H5.1	17.5
10A9.2	(1st gen mAb) 6.2

Part III: GPR49 direct binding ELISA

[0194] To characterize the binding of the mouse antibodies to GPR49, a direct binding ELISA assay was performed.

[0195] Methods: Soluble GPR49-Fc fusion protein at 2.5 ug/ml in 0.025 M carbonate buffer, pH 9.6 was coated at 50 ul/well in a 96-well (IMMULON2 HB, Dynex Technologies, Inc., Cat. #3455) plate and incubated overnight at 4° C. The plate washed with phosphate-buffered saline (PBS, Irvine Scientific, Cat#9240), pH 7.4 plus 0.025% Tween 20 in the Skan Washer 300 (Skatron Instruments), blocked with buffer containing 1% nonfat milk, 0.05% Tween 20 in PBS, pH 7.4, and then incubated at room temperature for 1 hour. After incubation plate was washed with PBS plus 0.025% Tween 20 in the Skan Washer 300. For the assay, the GPR49-coated plate was next incubated with the control and test antibodies of varied concentrations, diluted in 1% nonfat milk, 0.05% Tween 20 in PBS at 50 ul/well. Following one hour incubation at room temperature, plate washed with PBS plus 0.025% Tween 20 in the Skan Washer 300. A 2000-fold dilution in 1% nonfat milk, 0.05% Tween 20 in PBS of goat anti-mouse-Fc—HRP (Southern Biotech Cat#2060-05) was added 50 ul/well to detect bound Fab. Plate incubated for 1 hour at room temperature washed with PBS plus 0.025% Tween 20 in the Skan Washer 300. TMB solution (KIRKEGAARD & PERRY LABS, INC. cat: 50-76-00) was added 100 ul/well, and the reaction was stopped with 50 ul/well of 4N H2S04 (LabChem,

Cat#LC25830-l) after two minutes. The absorbance was measured at 450 nm, background 540 nm for TMB using the Molecular Devices plate reader. Data was analyzed using the SOFTMAX PRO software package version 4.3 LS (Molecular Devices Corp.) EC50 values were obtained as a result.

[0196] Results: Murine mAbs bound to GPR49-Fc with EC50's in the range of 224nM to less than 1 nM (Table 5; Figures 9A-D).

Table 5

EC50 [nM]	(ELISA) Hybridoma
0.2	8E9.1
0.24	11F6.1
0.25	12G5.1
0.27	5B10.1
0.28	6H5.1
0.38	14A8.1
0.5	9C7.1
0.53	4A10.2
0.55	14H9.1
0.55	14E3.1
0.57	14F7.1
0.65	4F6.2
0.657	6E10.1
0.67	2P69.2
0.88	3F11.1
0.95	17C9.1
1.34	1B8.1

1.35	18G7.1
2.86	2H5.1
225.4	18D9.1

Example 9

Cancer tumors can be labeled with anti-GPR49 antibodies

[0197] Methods: The expression of GPR49 in colon and other tumor cells was measured by flow cytometry (FACs) using the anti-GPR49 mAbs 76C12 and 78F05.

[0198] Results: GPR49 was found to be expressed in multiple colon tumors (CT1, CT3, LS174T, Sw480, HCT116, SW620, DLD-1, Lovo), gastric tumor N87, lung tumor A549, and the positive control GPR49-CHO stable transfectants. In addition, the specificity of anti-GPR49 mAb binding to GPR49 was confirmed by RNAi knockdown of GPR49 in LS174T cells, which significantly inhibited the binding of GPR49 mAbs 76C12, 76B04, 78F05 to bind LS174T cells.

Example 10

Validation of GPR49 as a marker of colon cancer stem cells (CSC)

Part I:

[0199] Methods: CT1 primary colon tumor cells maintained in cancer stem cell tumorsphere conditions were sorted by FACs using GPR49 mAbs 76C12. GPR49 positive (GPR49+), GPR49 negative (GPR49-) and unsorted live PI (propidium iodide) negative cells were seeded at 1 cell per well in 96 well plates, and analyzed 3 weeks later for sphere forming ability (i.e. number of cancer stem cells).

[0200] Results: The sorted GPR49 positive (GPR49+) colon tumor cells were highly enriched for cancer stem cell activity compared to GPR49 negative and unsorted cells (Figures 10A and 10B). GPR49 sorted LS174T colon tumor cells were similarly highly enriched for cancer stem cells in 2 separate, independent experiments.

Part II:

[0201] Methods: To further corroborate these results, we performed an additional experiment where colon cancer stem cell tumorspheres were treated with GPR49 or control RNAi-1 and then seeded in 96-well plates at 250 cells per well.

[0202] Results: Treatment with GPR49 RNAi-1 but not control RNAi significantly reduced the number of colon cancer stem cells measured 14 days after treatment.

Example 11

GPR49 positive colon tumor cells have cancer stem cell properties in vivo

[0203] Methods: To show that isolated GPR49+ cells have cancer stem cell properties, in vivo cancer stem cell assays comparing the in vivo growth of GPR49+ versus GPR49- cells were performed. GPR49+ cells from the primary colon tumor line CT1 were sorted with 76C12 using a MoFlo, and implanted at 1000 cells per mouse. Mice were analyzed for tumor formation and weight loss over the next 50 days.

[0204] Results: GPR49+ primary colon tumor cells cause aggressive tumor growth (Figure 11A) and rapid weight loss (Figure 11B) in a primary tumor xenograft study. In contrast, GPR49- cells grew very slowly and did not cause rapid weight loss. These findings demonstrate that GPR49 high expressing colon tumor cells are highly tumorigenic, an important feature of cancer stem cells.

Example 12

R-spondins bind to GPR49 with high affinity but do not activate GPR49-mediated signalling

[0205] **Part I:** GPR49 is an orphan G protein-coupled receptor (GPCR) related to the glycoprotein hormone receptor family (eg. receptors for FSH, TSH, LH) and is an intestinal and stomach stem cell marker that was identified by screening Wnt target genes in the gut (Barker et al., *Nature*, 2007). To identify the natural ligand of GPR49, binding studies were focused on known Wnt-pathway modulators Noggin, Gremlin1, DAN, Chordin-like 1, Cerberus1, PRDC, stanniocalcin-1, COCO, Chordin, R-spondin1-3, BMP2 and BMP4.

[0206] Methods: Ligand binding activity to GPR49-Fc was determined in a biolayer interometry-based assay. In brief, GPR49-Fc and test ligands (all purchased from R& D systems) were all diluted in OB buffer (PBS, pH 7.4, 0.01% (w/v) NaN3, 1 mg/ml BSA, 0.02 % (v/v) Tween 20). GPR49-Fc was captured on anti-human IgG Octet tips (ForteBio, Inc., Menlo Park, CA; Part# 18-5001), using an Octet Red System (ForteBio, Inc., Menlo Park, CA). Tips were washed in OB buffer and moved to wells containing test ligand in OB buffer. Binding of test ligand to GPR49-Fc was recorded as biointerferometry signals during an association phase (120 seconds) and dissociation phase (120 seconds). In addition, the ligands were tested against murine GPR49-Fc.

[0207] Results: R-spondin (RSPO) family members interact to human GPR49-Fc, but show a non-specific component that makes interpretation difficult. Specifically, the two-

phase association and dissociation observed suggests multiple binding events. RSPO family members do not interact with mouse GPR49-Fc [88% identity (91% similar)].

[0208] Part II: Although the GPR49-Fc used is a dimer of GPR49 ecto-domain in solution (due to interacting Fc halves), the bi-phasic binding cannot be explained by this due to the immobilization of the GPR49-Fc to the tip and the monomeric state of the test RSPO molecules. Therefore, the bi-phasic nature of the RPOS binding to GPR49-Fc was further investigated by a solution affinity surface plasmon resonance assay.

[0209] Methods: Binding of RSPO to GPR49 was analyzed using solution affinity surface plasmon resonance (Day ES, et al. Biochemistry. 2005 Feb 15;44(6):1919-31.). The method utilizes conditions of so-called "mass-transport-limited" binding, in which the initial rate of ligand binding (protein binding to the sensor chip) is proportional to the concentration of ligand in solution (BIAApplications Handbook (1994) Chapter 6: Concentration measurement, pp 6-1-6-10, Pharmacia Biosensor AB). Under these conditions, binding of the soluble analyte (protein flowing over chip surface) to the immobilized protein on the chip is fast compared to the diffusion of the analyte into the dextran matrix on the chip surface. Therefore, the diffusion properties of the analyte and the concentration of analyte in solution flowing over the chip surface determine the rate at which analyte binds to the chip. In this experiment, the concentration of free RSPO-1 in solution is determined by the initial rate of binding to a CM5 Biacore chip containing immobilized GPR49-Fc. Into these RSPO-1 solutions were titrated the competing GPR49-Fc.

[0210] Results: Initial binding rates were obtained from raw sensorgram data. R-spondin-1 showed a bi-phasic binding profile suggesting multiple cooperative binding sites for RSPO on GPR49.

[0211] Part III. To test for the effects of RSPO on GPR49 signaling, multiple assays were utilized.

[0212] Methods: Cyclic AMP, Calcium flux and β -arrestin assays were conducted by standard methods. In addition, a β -catenin/TCF reporter assay which is a transcription-based reporter assay that characterizes the Wnt/beta-catenin signaling pathway. The β -catenin/TCF reporter assay utilized transfected cells with SABioscience TCF/LEF reporter or negative construct. After 24 hour transfection, cells were counted and aliquoted (2×10^4 cells/well) into a 96 well plate. Cells were then starved with Opti-MEM+0.5%FBS+0.1mM NEAA+1mM sodium pyruvate+1x antibiotic media for 6 hours. Cells were then treated with titrated amount of RSPO and mWnt3a (200ng/ml) with or without antibody for 18 hours. Dual luciferase activities are developed with Promega dual luciferase kit. In addition, GPR49 RNAi was used

to examine the specific contribution of GPR49 to RSPO dependent β -catenin/TCF reporter activity.

[0213] Results: No measurable activity was observed in the cAMP, calcium flux or β -arrestin activity assays for RSPO. However, RSPO drove β -catenin/TCF reporter activity in a dose-dependent manner that was knocked down by GPR49 RNAi.

Example 13

Stimulation of GPR49-3T3 cells with R-spondin-1

[0214] Methods: To test the effects of R-spondin on the proliferation of GPR49 overexpressed cells, 2500 NIH 3T3 fibroblast cells stably transfected with GPR49 (GPR49-3T3 clone 50) were incubated with R-spondin 1 for 2 days and the Cell Titer Glo ATP assay used to assess cell proliferation. GPR49-3T3 clone 50 was previously shown to express high levels of cell surface GPR49 by FACs with anti-GPR49 mAb 76C12.

[0215] Results: R-spondin 1 stimulation caused a 25-40% increase in cell proliferation of GPR49-3T3 cells compared to control stimulation with Stanniocalcin in serum free conditions. This result was confirmed in a second independent experiment using a different clone, GPR49-3T3 clone 28, which showed a 4-fold increase in proliferation in response to R-spondin 1 compared to Stanniocalcin control stimulated cells.

Example 14

Inhibition of R-spondin binding by GPR49 mAbs

[0216] The ability of the anti-GPR49 mAbs detailed in this invention to block RSPO binding to soluble GPR49-Fc was determined.

[0217] Methods: The ligand blocking ability of the anti-GPR49 antibodies was determined by a solution phase competition surface plasmon resonance assay. Briefly, the antibodies (1uM) co-incubated with 200nM RSPO for 45 minutes on ice. RSPO alone (200nM) or in combination with incubated antibodies were then flowed over a CM5 Biacore chip immobilized with GPR49-Fc (as detailed in Example 1). The Rmax, which is the binding signal at the end of the association phase of 60 seconds (Rmax) was used as a measure of the fraction bound at steady state/equilibrium.

[0218] Results: Anti-GPR49 antibodies 3B8.11, 10A6.7, 2B5.5, 6C10.5 all decreased Rmax greater than 20%. 3G8.1, 6H5.4 and 7F8.2 all decreased the Rmax by less than 20%.

Example 15GPR49 mAbs cross-reactivity and specificity to related family members GPR48 (LGR4) and LGR6

[0219] Methods: In order to determine the specificity of the anti-GPR49 antibodies detailed in this invention for related family members, GPR48 (LGR4) and LGR6, recombinant GPR48, GPR49 and LGR6 were independently expressed in mammalian cells (HEK293T). Binding of antibodies to cells overexpressing the receptors was assessed by FACS (standard methods) and compared to cells transfected with control vector (pV100).

[0220] Results: 76C12 antibody binds to GPR48 but not to LGR6 expressed in mammalian cells. None of the other antibodies test bind to either GPR48 or LGR6 (Table 6)

Table 6

	Human GPR48 (LGR4)	Human LGR6
76C12	Y	N
78F05	N	N
1B8.1	N	N
14E3.1	N	N
14A8.1	N	N
14F7.1	N	N
18G7.1	N	N
6H5.4	N	N
7C3.4	N	N
7F8.2	N	N
14H9.1	N	N
9C7.3	N	N
1B3.5	N	N

Example 16Internalization of GPR49 by Fully Human Anti-GPR49 Antibodies

[0221] Methods: Lovo colon tumor cells were seeded at 50,000 cells per well into 8 well chamber slides (Becton Dickinson Collagen Type 1 coated culture slides, BD BioCoat™#354630) 48 hours prior to staining procedures. Cells were routinely maintained below 20 passages. On day of staining procedures, culture media was discarded from each well and replaced with 500 ul cold incubation buffer (MEM Eagle ATCC #30-2003+1% BSA). Cells were washed 2x with this buffer for 3 min each wash. 250 ul of each mAb (76C12, 78F05 and murine 10A9.2) to be tested was then added to the appropriate well at a concentration of 10 ug/ml, diluted in incubation media, and incubated on ice for 1 hour. A human anti-human-IGF-IR antibody was used as a positive control to compare degree of internalization. Antibody 5A7 (anti-Id mAb), IDEC152 (anti-CD23 IgG1 mAb) and no antibody were used as negative controls. After the 45 minutes incubation on ice, the time zero (t=0') slide washed 3x with 500 ul of cold wash buffer (PBS+1% BSA+2% Goat serum) for 3 min each wash (slides always kept on ice). The t=0 slide was then fixed with 500 ul 14% paraformaldehyde (diluted with PBS from 16% stock; EMS #15710) for 15 minutes at room temperature. The t=0 slide was then washed again 3x with cold wash buffer for 3 minutes each wash, then left on ice. Meanwhile, the remaining slides were put into a 37° C. incubator for their designated time points (15 and 60 minutes). At the end of their incubation time each slide followed the same procedures as above—washes and fixation, and put on ice. All slides were then permeabilized with 200 ul cold permeabilization buffer (Wash buffer+0.5% Triton-X) for 10 minutes on ice. All slides were then washed 3x with 500 ml cold wash buffer for 3 minutes each wash. The secondary antibody was prepared at a 1:1000 dilution (AlexaFluor 488 Goat-anti-mouse IgG (H+ L), Molecular Probes #A11029 for the mAbs and AlexaFluor 488 Goat-anti-human IgG (H+L), Molecular Probes #A11013 for G4 antibodies) in wash buffer, after an initial spin of the stock vial at 10,000 rpm for 10 min at 4° C. 250 ul of the diluted secondary antibody was added to each well and incubated for 40 min at room temperature in the dark (covered). Slides were again washed 3x with 500 ul cold wash buffer. On the final wash, the buffer was discarded and all wells were left empty. The chambers were then disassembled from the slide using the provided disassembly tool, and cover slips were mounted with Vectashield mounting medium containing DAPI (Vector #H-1500, Hard Set™). Slides were stored at 4° C. in the dark overnight to allow the mounting medium to dry. Pictures of the slides were taken with a confocal microscope using the LaserSharp 2000 program (BioRad v5.2) and represented as a merge of blue and green components from Kalman 10 average.

[0222] Results: 76C12, 78F05 and 10A9.2 all showed rapid internalization of GPR49 in 60 min. As expected the positive control, IGF1R C06, showed internalized of the IGF1R receptor whereas isotype matched negative controls (mouse 5A7, IDEC152 (primatized anti-CD23 antibody)) did not bind or internalize.

Example 17

GPR49 antibodies bind to murine GPR49

[0223] Methods: In order to determine the specificity of the anti-GPR49 antibodies described herein to murine GPR49, recombinant murine GPR49 was expressed in mammalian cells (HEK293T). Binding of antibodies to cells overexpressing the receptors was assessed by FACS (standard methods) and compared to cells transfected with control vector (pV100).

[0224] Results: 76C12 binds to murine GPR49 expressed in mammalian cells with high affinity. Multiple additional GPR49 mAbs bind to murine GPR49 (Table 7).

Table 7

	murine GPR49 (Lgr5)
76C12	HI
78F05	LO
1B8.1	MED
14E3.1	MED
14A8.1	MED
14F7.1	MED
18G7.1	LO
6H5.4	LO
7C3.4	LO
7F8.2	LO
14H9.1	LO
9C7.3	+/-

1B3.5	+/-
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Example 18Epitope grouping of anti-GPR49 mAbs

[0225] An equilibrium binding assay was developed and used to determine common epitope binding groups for the panel of GPR49 antibodies detailed in this invention. Cross-blocking of binding to GPR49 was used to define groups of antibodies that bind to distinct binding epitopes of GPR49.

[0226] Methods: GPR49-Fc binding activity to the test anti-GPR49 mAb was compared to that of GPR49-Fc pre-incubated with secondary test mAb (either self or different mAb) in a biolayer interometry-based assay. Primary anti-GPR49 test mAb (primary mAb) was biotinylated with a Thermo Scientific EZ-Link Sulfo-NHS-Biotin (Thermo Scientific, # 21425) according to manufacturer's protocol. Biotinylated primary mAb, GPR49-Fc, and GPR49-Fc plus secondary test anti-GPR49 mAb (secondary mAb) were all diluted in OB buffer (PBS, pH 7.4, 0.01% (w/v) NaN₃, 1 mg/ml BSA, 0.02 % (v/v) Tween 20). Biotinylated primary mAbs were captured on streptavidin Octet tips (ForteBio, Inc., Menlo Park, CA; Part# 18-5001), using an Octet Red System (ForteBio, Inc., Menlo Park, CA). Tips were washed in OB buffer and moved to wells containing GPR49-Fc in OB buffer. Binding of GPR49-Fc to the primary mAb on the tips was recorded as biointerferometry signals to saturation, during an association phase (120 seconds) and the binding signal at the end of the association phase (R_{max}) was used as a measure of the fraction bound at steady state/equilibrium. Binding during the dissociation phase (120 seconds) was also recorded. To measure the cross-blocking ability of the various mAbs to each other, a second binding experiment was performed in which the GPR49-Fc outlined above, was preincubated with a 5 fold molar excess of secondary test mAb (unbiotinylated). This was then bound to the tips pre-load with biotinylated primary mAb to determine the R_{max'}. The R_{max'} prime was then compared to the R_{max} and the percentage of R_{max'} /R_{max} was calculated to determine the amount of blocking the secondary mAb had on the ability of GPR49-Fc to bind to the primary mAb pre-bound to the tip. Cross-blocking (R_{max'}/R_{max} x 100) of secondary to primary mAbs was recorded as follows: 0-25% full cross-blocking, 25-50% partial cross-blocking, 50-75% low cross blocking, 75-100% no cross-blocking. All mAbs were tested against self (primary and secondary mAb were the same test mAb) to ensure proper analysis.

[0227] Results: Six distinct epitope binding groups were observed for the anti-GPR49 mAbs tested. The groups are: group 1 (76C12, 1B3.5, 6B10.2, 4F6.2), group 2 (18G7.1, 14A8.1, 5B10.1, 14F7.1, 11F6.1, 14E3.1, 1B8.1), group 3 (5F2.5, 6B10.2), group 4 (3F11.1), group 5 (10A.2) and group 6 (6E10.1). Cross-blocking by self-mAbs is shown in Table 8 and 9.

Table 8

	18G7.1	1B3.5	76C12	14A8.1	5B10.1	14F7.1	5D6.3	5F2.5
18G7.1	11%	100%	100%	20%	28%	14%	62%	100%
1B3.5	100%	4%	7%	100%	100%	100%	47%	100%
76C12	100%	4%	5%	100%	100%	100%	25%	100%
14A8.1	5%	100%	100%	6%	9%	4%	99%	100%
5B10.1	13%	100%	100%	16%	11%	14%	76%	100%
14F7.1	7%	100%	100%	7%	11%	5%	100%	100%
5D6.3	100%	96%	81%	100%	100%	100%	66%	97%
5F2.5	100%	100%	100%	100%	100%	100%	79%	13%
11F6.1	9%	100%	100%					93%
3F11.1	78%	68%	100%					100%
14E3.1	5%	88%	95%					83%
1B8.1	6%	85%	96%					85%

Table 9

	11F6.1	3F11.1	14E3.1	1B8.1	10A9.2	6B10.2	4F6.2	6E10.1
18G7.1								
1B3.5								
76C12					100%	4%	6%	100%
14A8.1								
5B10.1								
14F7.1								
5D6.3								
5F2.5					100%	8%	100%	100%
11F6.1	4%	99%	0%	1%	100%	100%	100%	100%
3F11.1	100%	5%	86%	75%	100%	100%	100%	100%
14E3.1	4%	87%	2%	0%				
1B8.1	5%	86%	2%	3%				

Example 19Pharmacokinetic properties of GPR49 mAbs 76C12

[0228] Methods: To determine the pharmacokinetic properties of GPR49 antibody 76C12 in mice and the appropriate dosing schedule for upcoming study with 76C12 in the CT1 model, a single dose of 76C12, I.P, with 15 or 30mg/kg was given to CRL SCID Beige mice. Bleeds were taken at 15min, 30min and 60min, 2,6,24, and 48 hours and after 4, 7, 9, 11, and 14 days. Serum concentrations determined by ELISA.

[0229] Results: 76C12 exhibits a half-life of approximately 4 days (Figure 12A-C).

Example 20In vivo inhibition of primary tumor growth in a primary colon cancer model

[0230] Methods: Single agent in vivo efficacy of 76C12 IgG1 antibody was evaluated in a primary colon tumor xenograft model system using CT1 (primary colon cancer) cells. CT1 is a colon tumor cell line that originated from a colon tumor patient sample, and was used to establish a colon cancer stem cell (tumorsphere) line. It is considered a “primary” line because it is low-passage (<10 passages) derived recently from the patient primary sample. It is considered a cancer stem cell line because it is grown under established cancer stem cell conditions (serum-free medium) and low-attachment plates (cells grow in suspension). CRL SCID-beige female mice were inoculated with 1000 cells and monitored for tumor growth. Mean tumor volume at the start of the therapy was approximately 130 mm³. The 76C12mAb was administered intraperitoneally (i.p.) at 30 and 15 mg/kg administered one time per week for 4 weeks. The 76C12 mAb was also tested at 15 and 7.5 mg/kg twice-weekly for 4 weeks. An isotype matched antibody, CE9.1 (IgG1), was administered as a negative control at 15 mg/kg one time per week for 4 weeks while Irinotecan was dosed at 15mpk once/week for 4 weeks. Tumors were extracted at the indicated intervals post-inoculation and total tumor volume was measured.

[0231] Results: The fully human 76C12 antibody inhibited tumor growth in a dose dependent manner (Figures 13A & 13B). The antibody demonstrated statistically significant single agent efficacy at 30 mg/kg administered weekly for 4 weeks.

Example 21In vivo inhibition of primary tumor growth using combination therapy

[0232] Methods: Single agent in vivo efficacy of 76C12 IgG1 antibody was evaluated in a primary colon tumor xenograft model system using CT1 (primary colon cancer) cells. CRL SCID-beige female mice were inoculated with 1000 cells and monitored for tumor

growth. Mean tumor volume at the start of the therapy was approximately 130 mm³. The 76C12 mAb was administered intraperitoneally (i.p.) at 30 and 15 mg/kg administered bi-weekly time per week for 4 weeks as a single agent and in combination with Irinotecan administered according to the current standard of care (i.e., 125 mg/m² every 7 days for 4 weeks). Untreated, an isotype matched antibody IDEC152 (IgG1), a chemo vehicle control and IDEC152 plus Irinotecan (control for combination) were administered as negative controls at 15 mg/kg one time per week for 4 weeks while Irinotecan was dosed at 15mpk once/week for 4 weeks. Tumors were extracted at the indicated intervals post-inoculation and total tumor volume was measured.

[0233] Results: Anti-GPRR49 mAb76C12 antibody and Irinotecan as a single agent (i.e., administered alone) showed similar efficacy. In combination with Irinotecan, the 76C12 antibody at 30 mg/kg (Figure 14) and at 15mg/kg on twice a week schedule showed additive efficacy compared to the single agent treatments. In addition, combination with 15 mg/kg also showed additive efficacy.

Example 22

In vivo inhibition of primary tumor growth using combination therapy

[0234] Methods: From Example 21.

[0235] Results: Addition benefit of treatment of GPR49 antibody was observed on overall survival of mice (from experiment described in Example 21). GPR49 antibody 76C12 treatment significantly decreased weight loss and increased animal survival by 50% in the cachexic CT1 model (Figure 15). In addition, no toxicity was observed with anti-GPR49 mAb treatment (from Examples 16 and 18, 76C12 cross-reacts with and binds endogenous mouse GPR49); this finding and the increased survival of GPR49 mAb treated mice support both the high potential and safety of GPR49 antibody blockade in cancer.

Example 23

In vivo inhibition of tumor line DLD-1 growth using combination therapy

[0236] Methods: Single agent *in vivo* efficacy of 76C12 IgG1 antibody was evaluated in colon tumor xenograft model DLD-1. CRL SCID-beige female mice were inoculated with 1000 cells and monitored for tumor growth. Mean tumor volume at the start of the therapy was approximately 130 mm³. The 76C12 mAb was administered intraperitoneally (i.p.) at 15 mg/kg administered bi-weekly time per week for 4 weeks as a single agent and in combination with Irinotecan administered according to the current standard of care. A chemo vehicle control and IDEC152 plus Irinotecan (control for combination) were administered as

negative controls at 15 mg/kg one time per week for 4 weeks. Tumors were extracted at the indicated intervals post-inoculation and total tumor volume was measured.

[0237] Results: 76C12 antibody showed inhibition of tumor growth. Enhancing efficacy was observed with the combination with Irinotecan. (Figures 16A & 16B)

Example 24

Treatment of Human Cancer Using Anti-GPR49 Antibodies

[0238] This example describes methods for treating cancer using antibodies against GPR49 to target malignant cells, for example, cancer stem cells or tumor initiating cells in which GPR49 expression has been detected.

[0239] In certain embodiments, anti-GPR49 antibodies of the present invention is purified and formulated with a suitable pharmaceutical vehicle for injection. A human patient with a hyperproliferative disorder is given multiple doses of a fully human or humanized anti-GPR49 antibody of the present embodiments, by intravenous infusion at about 1 mg/kg body weight to about 100 mg/kg body weight, e.g., once per every two weeks or once a month, for at least six months. Intervals can also be irregular as indicated by measuring prognostic indicators in the patient.

[0240] Antibodies are administered prior to, concurrently with, or after standard radiotherapy regimens. The patient is monitored to determine whether treatment has resulted in an anti-tumor response, for example, based on tumor regression, reduction in the incidences of new tumors, lower tumor antigen expression, improved prognosis free survival or extension of overall survival or other means of evaluating disease prognosis.

Example 25

In vivo inhibition of primary colon cancer tumors having K-Ras, PI3K, PTEN, and p53 mutations using GPR49 antibodies

[0241] We performed an experiment to determine if GPR49 antibodies were effective *in vivo* to inhibit colon cancers that had particular mutations that can lead to resistance to known cancer therapies. For example, patients with the KRAS mutation may have a poor response to panitumumab (Vectibix®) and cetuximab (Erbitux®) therapy in colorectal cancer. Thus, we wanted to determine if the GPR49 antibodies could provide a useful treatment for colon cancers with these known therapeutic resistance markers.

[0242] **Methods:** Single agent *in vivo* efficacy of murine antibodies 14F7.1, 18G7.1, 5B10.1, 14A8.1, 1B3.5 was evaluated in a primary colon tumor xenograft model system using

CT1 primary xenograft cells. CT1 is a primary colorectal cancer (CRC) *in vivo* xenograft tumor established from a fresh CRC patient tumor sample. The mutational status of the tumor cells was determined by Ion Torrent® (Life Technologies, Carlsbad, CA) deep sequencing to identify oncogene mutations. Approximately 5-15% of CT1 tumors express GPR49. SCID beige female mice were inoculated with CT1 tumor cells and monitored for tumor growth. Mice were randomized into groups of 10 when mean tumor volume reached 175 mm³. The antibodies were administered intraperitoneally (i.p.) at 15 mg/kg, twice weekly for 4 weeks. An isotype matched antibody IgG1 was administered as a negative control at 15 mg/kg twice per week for 4 weeks. Tumor volumes and body weights were measured 2x per week.

[0243] Results: The antibodies inhibited tumor growth up to 40% compared to control (Figure 17 and 18). Antibodies 14F7 and 18G7 exhibited the highest tumor growth inhibition (40% and 34% tumor inhibition, respectively). The 34% and 40% tumor growth inhibition by the antibodies is an “outsized” effect since GPR49 is only expressed on approximately 5-15% of the tumor cells. This outsized effect suggests that antibody inhibition of GPR49 positive cancer stem cells is targeting the source of the proliferating tumor cells *in vivo*.

Example 26

In vivo inhibition of primary colon tumors having K-Ras, PI3K, PTEN, and p53 mutations by GPR49 antibodies in combination with the chemotherapeutic Irinotecan

[0244] We also performed an experiment to determine if GPR49 antibodies in combination with well-known cancer therapeutic agents would be effective *in vivo* to inhibit colon cancers that had particular mutations that can lead to resistance to known cancer therapies.

[0245] Methods: Single agent *in vivo* efficacy of murine antibodies 14F7.1, 18G7.1, 5B10.1, 14A8.1, 1B3.5 was evaluated in a primary colon tumor xenograft model system using CT1 primary xenograft cells. CT1 is a primary colorectal (CRC) *in vivo* xenograft tumor established from a fresh CRC patient tumor sample. The mutational status was determined by Ion Torrent deep sequencing to identify oncogene mutations. Approximately 5-15% of CT1 tumors express GPR49. SCID beige female mice were inoculated with CT1 tumor cells and monitored for tumor growth. Mice were randomized at day 0 into groups of 10 when mean tumor volume reached 175 mm³. Chemotherapeutic Irinotecan was administered IP at 10 mg/kg once per day for the first 5 days. The antibodies were administered intraperitoneally (i.p.) at 15 mg/kg administered twice weekly for 4 weeks. Tumors were measured at the indicated intervals post-inoculation for total tumor volume.

[0246] **Results:** Anti-GPR49 antibodies in combination with irinotecan inhibited tumor growth by 57% to 65%, compared to 45% tumor inhibition by Irinotecan alone (Figure 17, Figure 19). GPR49 antibodies therefore enhanced the anti-tumor activity of Irinotecan by 27% to 44% in colon cancers known to be resistant to certain available therapeutics.

Example 27

In vivo inhibition of primary colon cancer tumors having K-Ras , PI3K, PTEN, H-Ras, APC, TP53, FGFR2, VANGL2, STK11, JAK2, and RB1 mutations

[0247] We performed an experiment to determine whether GPR49 antibodies would be effective as single agent inhibitors of colon cancer tumors that had particular mutations that can lead to resistance to known cancer therapies. We had determined, above, that GPR49 antibodies in combination with well-known cancer therapeutic agents were effective. In this experiment we determined the relative inhibition of tumor growth upon treatment with GPR49 antibodies compared to no treatment control.

[0248] **Methods:** Single agent *in vivo* efficacy of murine antibodies 18G7.1 and 7C3.4 was evaluated in a primary colon tumor xenograft model system using CT3 (primary colon cancer) cells. CT3 is a primary colon tumor xenograft derived from a fresh CRC patient tumor sample, and maintained *in vivo* at low passage number ($p < 4$). The mutational status was determined by Ion Torrent deep sequencing to identify oncogene mutations. Approximately 15-20% of CT3 tumors express GPR49. CB17-Scid female mice were inoculated with CT3 cells and monitored for tumor growth. Mice were randomized into groups of 10 when mean tumor volume reached 130 mm^3 (Day 0). Antibodies were then dosed intraperitoneally (i.p.) at 15 mg/kg twice weekly for 4 weeks. An isotype matched antibody IgG1 was administered as a negative control at 15 mg/kg twice per week for 4 weeks. Tumor volume and body weight was measured twice per week until study completion.

[0249] **Results:** The antibodies inhibited tumor growth up to 43%, compared to control treatment (Figure 20). This >40% tumor growth inhibition is an “outsized” effect given the GPR49 antibodies only bind to a 15-20% subpopulation of the CT3 tumor cells. This outsized effect suggests that inhibition of GPR49 positive cancer stem cells is targeting the source of the proliferating tumor cells *in vivo*.

Example 28

GPR49 mAb Treatment Reduces Cancer Stem Cell Frequency *In Vivo* in primary colon tumors having K-Ras , PI3K, PTEN, and p53 mutations

[0250] We performed an experiment to assay for the impact of GPR49 antibodies on cancer stem cell frequency in cells derived from colon cancer tumors that had particular mutations that can lead to resistance to known cancer therapies. We had observed that GPR49 antibodies demonstrated a relative impact on tumor growth that was 'outsized' in comparison to the proportion of tumor cells bound. This experiment was to determine the effect of these antibodies on cancer stem cell frequency specifically.

[0251] **Methods:** Isolated CT1 tumor cells from control, GPR49 mAb, Irinotecan and GPR49 mAb in combination with Irinotecan treatment and control groups from the *in vivo* studies outlined in Example 1 and 2 were harvested, pooled, dissociated and re-implanted in a limiting dilution secondary transplant assay to measure cancer stem cell frequency. For each treatment group, 8 mice were implanted with 30, 100 and 300 cells. Tumor formation (i.e. tumor take) and growth rate were monitored on a bi-weekly basis for 8 weeks. To calculate the frequency of cancer stem cells in each treatment group, linear regression analysis was performed with Prism GraphPadTM to calculate the frequency of cancer stem cells in each treatment group. This assay is considered the gold-standard for measuring CSCs, because it is a functional CSC assay that measures the frequency of CSC clones in any given tumor that can give rise to a new tumor in a secondary host. In this assay, tumors containing the CSCs to be measured were serially transplanted into secondary recipients in limiting dilution assay. The assay is thus a functional *in vivo* measurement of self-renewal capacity, a key component of any stem cell. It does not rely on the as yet incomplete understanding and characterization of cell surface markers or enzyme assays that are often used to measure CSCs *in vitro*.

[0252] **Results:** Tumor re-growth from GPR49 mAb and GPR49 mAb in combination with Irinotecan pre-treated tumors was significantly inhibited (Figure 21). Greater than 62% (5/8) of mice implanted with GPR49 mAb or GPR49 mAb +Irinotecan pre-treated tumors failed to show tumor formation (i.e. were tumor-free) 8 weeks post-transplant. In contrast, only 1/8 mice (13%) implanted with control treated tumors remained tumor-free 8 weeks post-transplant. Linear regression analysis showed a 3 fold reduction in the number of CSCs after GPR49 antibody treatment compared to controls (Figure 21).

Example 29

GPR49 mAb Treatment Reduces Cancer Stem Cell Frequency In Vivo in primary colon tumors having K-Ras , PI3K, PTEN, H-Ras, APC, TP53, FGFR2, VANGL2, STK11, JAK2, and RB1 mutations.

[0253] We performed an experiment to determine if GPR49 antibodies had a similar impact on cancer cells harboring a larger array of mutations that lead to resistances to known cancer therapies. Expanding on the results above, we determined the effect of these antibodies on cancer stem cell frequency in cancer cell lines harboring a larger number of mutations.

[0254] **Methods:** Isolated CT3 primary tumor cells from control, GPR49 mAb, Irinotecan and GPR49 mAb + Irinotecan treated tumors from the treatment and control groups in the *in vivo* study outlined in Example 3 were harvested, pooled, dissociated and re-implanted in a limiting dilution secondary transplant assay to measure cancer stem cell frequency. For each treatment group, 8 mice were implanted with 10, 30, 100 cells. Tumor formation and growth rate were monitored on a bi-weekly basis for 12 weeks. To calculate the frequency of cancer stem cells in each treatment group, linear regression analysis was performed with Prism Graph Pad to calculate the frequency of cancer stem cells in each treatment group. This assay is considered the gold-standard for measuring CSCs, because it is a functional CSC assay that measures the frequency of CSC clones in any given tumor that can give rise to a new tumor in a secondary host. In this assay, tumors containing the CSCs to be measured were serially transplanted into secondary recipients in limiting dilution assay. The assay is thus a functional *in vivo* measurement of self-renewal capacity, a key component of any stem cell. It does not rely on the as yet incomplete understanding and characterization of cell surface markers or enzyme assays that are often used to measure CSCs *in vitro*.

[0255] **Results:** GPR49 mAb treatment reduced CSC frequency by 5-fold compared to control (Figure 22).

Example 30

GPR49 mAb treatment in combination with the chemotherapeutic irinotecan prevents colon tumor cells from forming new tumors in secondary recipients.

[0256] We performed an experiment to determine whether *in vivo* treatment of colon tumor xenografts with GPR49 mAb in combination with irinotecan would prevent the colon tumor cells from forming new tumors in secondary recipients. New secondary tumor formation in subjects implanted with previously-treated tumor cells is a proxy measurement of the frequency of tumor stem cells in the cancer cell population. We wanted to determine the effect of GPR49 antibodies and irinotecan in combination on secondary tumor formation in subjects receiving previously treated colon tumor cells.

[0257] **Methods:** Isolated tumor cells from control or GPR49 mAb + irinotecan treated tumors from the *in vivo* study outlined in Example 3 were harvested, pooled, dissociated

and re-implanted in a limiting dilution secondary transplant assay to measure cancer stem cell frequency. For each treatment group, 8 mice were implanted with 10, 30, 100 cells. Tumor formation was monitored on a bi-weekly basis for 12 weeks.

[0258] Results: After a 140 day follow-up, no tumor growth was observed from a GPR49 mAb + irinotecan group (0/8 mice with tumors). In contrast, 5/8 animals implanted with control irinotecan treated mice formed tumors with an average size of 1729 mm³ (Figure 23). This showed that treatment with GPR49 antibodies destemmed the tumors and greatly reduced the capability of the cancer stem cells to continue proliferating, even after treatment with the GPR49 antibodies was discontinued.

WHAT IS CLAIMED:

1. A method of inhibiting growth of a solid tumor in a mammal, comprising administering to the mammal a therapeutic amount of a monoclonal antibody that binds to G-Protein Coupled Receptor 49 (GPR49) polypeptide, wherein the GPR49 polypeptide has an amino acid sequence of SEQ ID NO: 1 and the therapeutic amount is sufficient to inhibit growth of the tumor, and wherein the antibody has antitumor activity against human tumor cells in vivo, and the antibody has activity capable of causing internalization of GPR49 in cells contacted with the antibody.
2. The method of claim 1, wherein the therapeutic amount of the antibody is sufficient to reduce the rate of tumor growth in the mammal.
3. The method of claim 2, wherein the reduced rate of tumor growth comprises a reduced rate in the increase of tumor volume.
4. The method of claim 2, wherein the therapeutic amount of the antibody is sufficient to increase survival of the mammal compared to control.
5. The method of claim 1, wherein the antibody is an IgG-class antibody.
6. The method of claim 2, wherein the antibody is an IgG1-class antibody.
7. The method of claim 2, wherein the antibody is a mouse antibody.
8. The method of claim 1, wherein the monoclonal antibody binds to GPR49 polypeptide with a K_d in the range of 10×10^{-9} M and 1×10^{-12} M.
9. The method of claim 1, wherein the antibody lacks specific binding to human GPR48 and to human leucine-rich repeat containing G-protein-coupled receptor (LGR6).
10. The method of claim 1, wherein the monoclonal antibody lacks specific binding to leucine-rich repeat containing G-protein-coupled receptor 4 (LGR4).
11. The method of claim 1, wherein the monoclonal antibody lacks specific binding to leucine-rich repeat containing G-protein-coupled receptor 6 (LGR6).
12. The method of claim 1, wherein the monoclonal antibody specifically binds to murine GPR49.
13. The method of claim 1, wherein the tumor comprises colon cancer cells.
14. The method of claim 1, wherein the tumor comprises colon adenocarcinoma cells.
15. The method of claim 1, wherein the tumor comprises colon cancer stem cells.

16. 1, The method of claims 1, wherein the tumor comprises a mutation in a human gene represented by a gene symbol selected from the group consisting of K-Ras, PI3K, PTEN, p53, H-Ras, APC, TP53, FGFR2, VANGL2, STK11, JAK2, and RB1.



FIG. 1

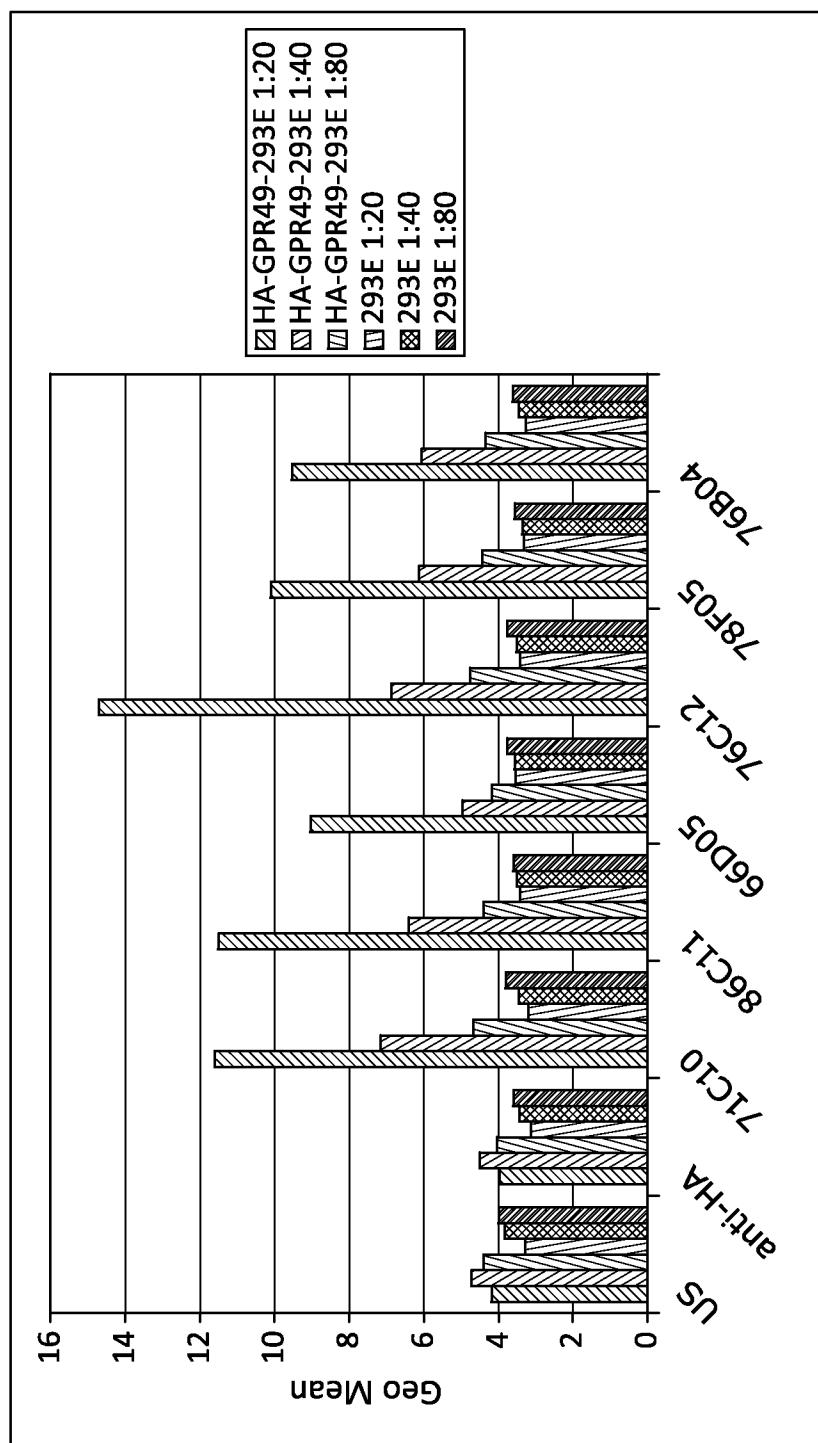


FIG. 2

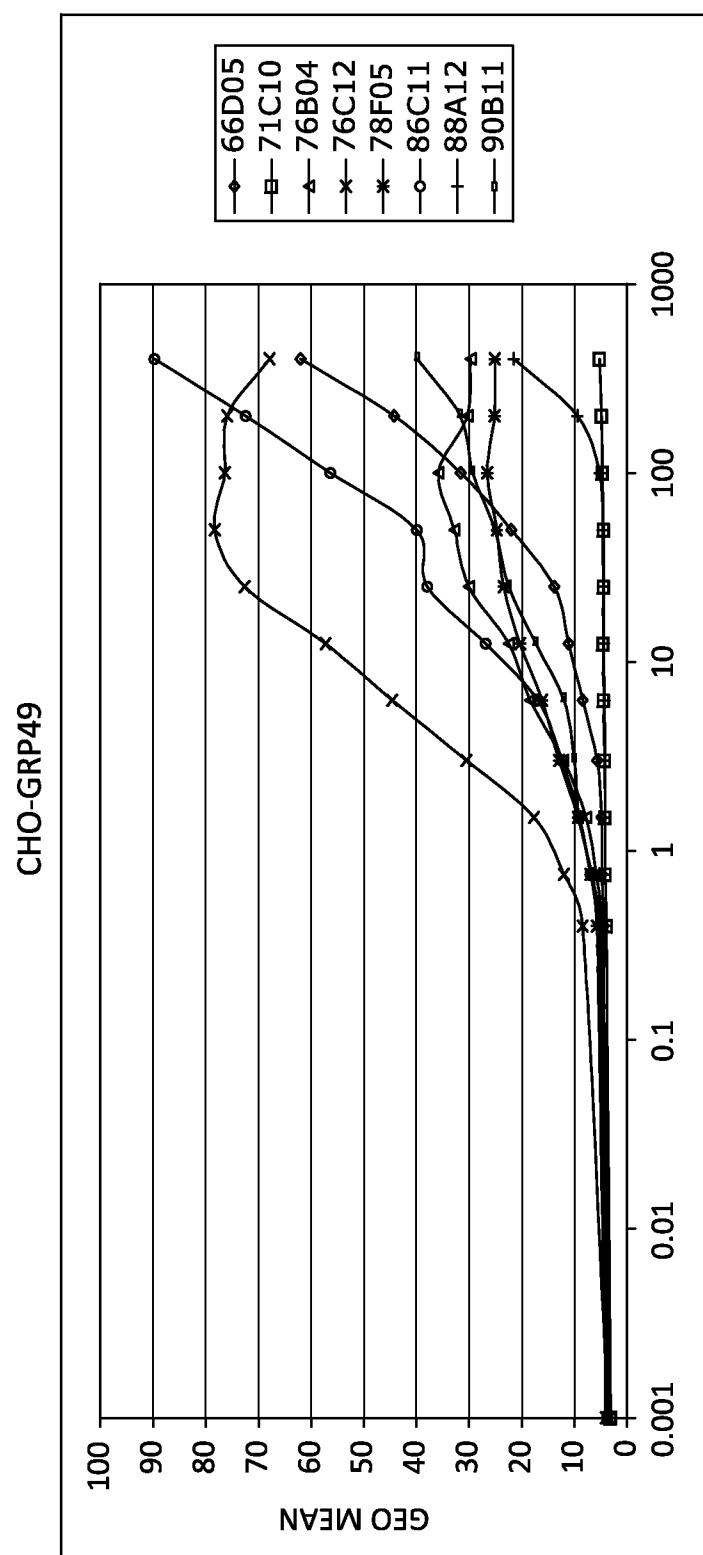


FIG. 3A

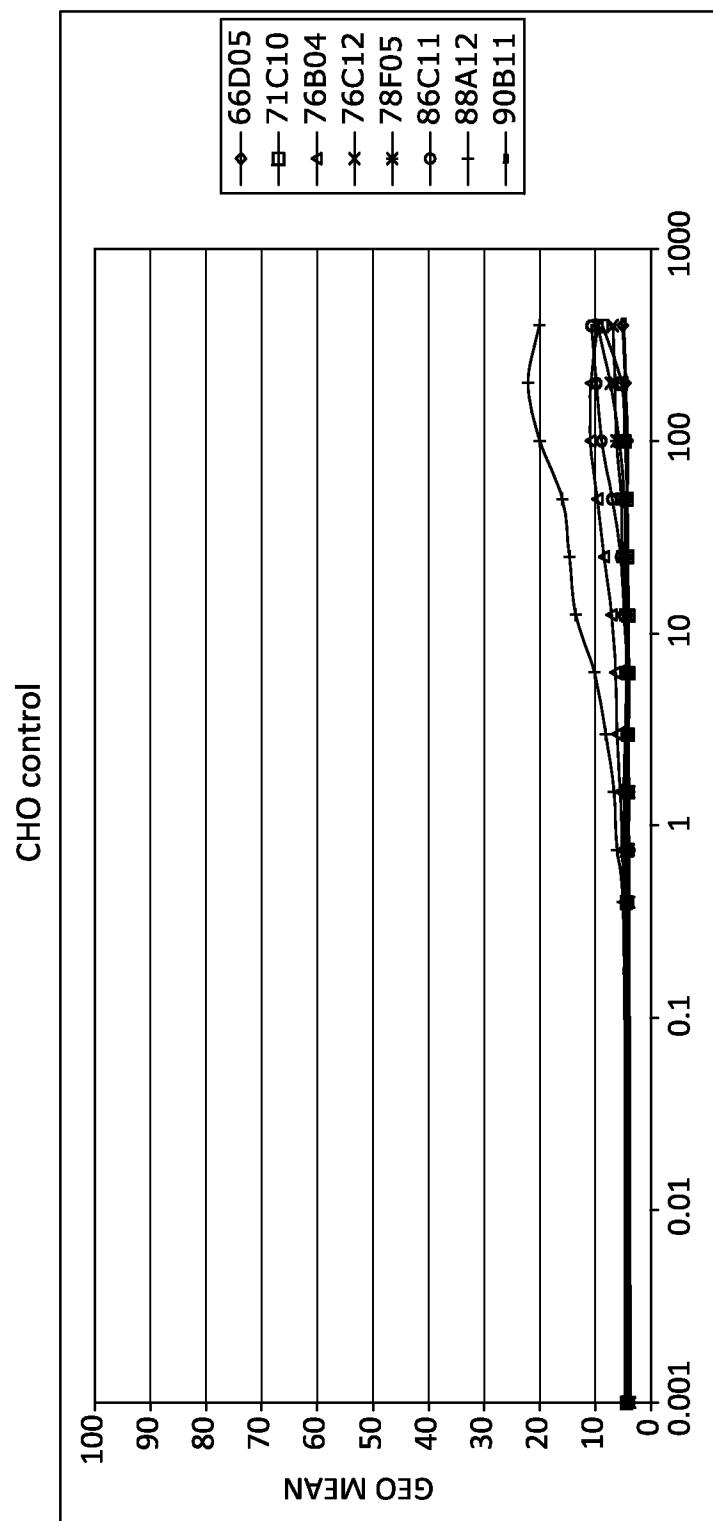
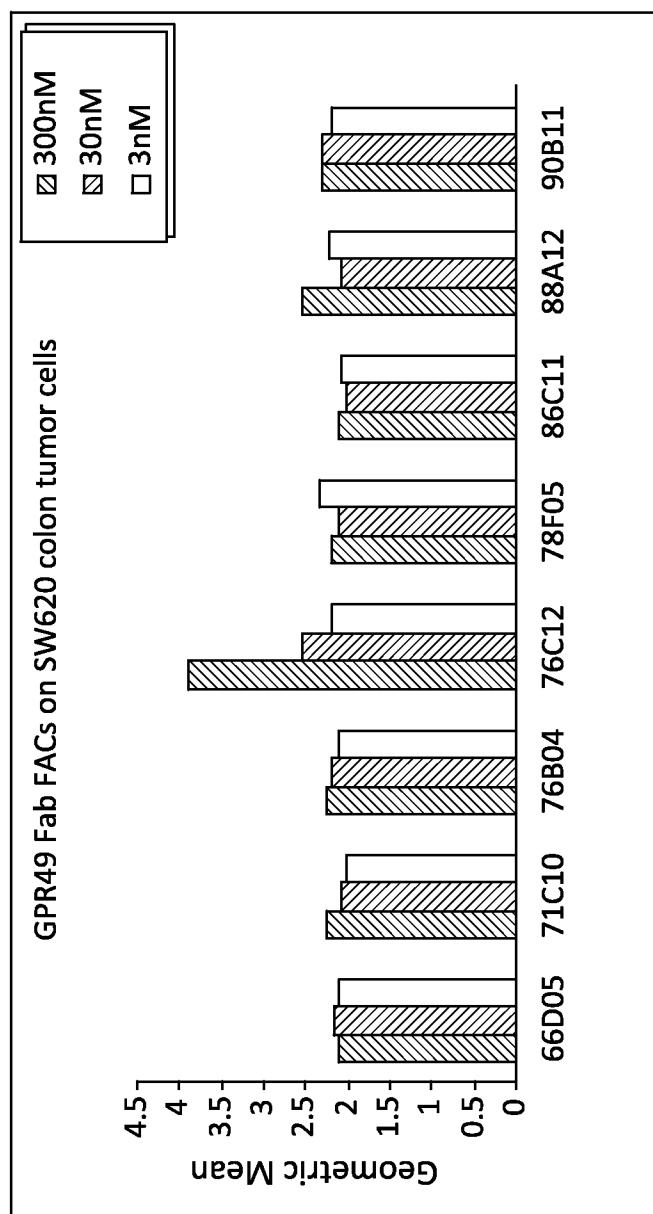
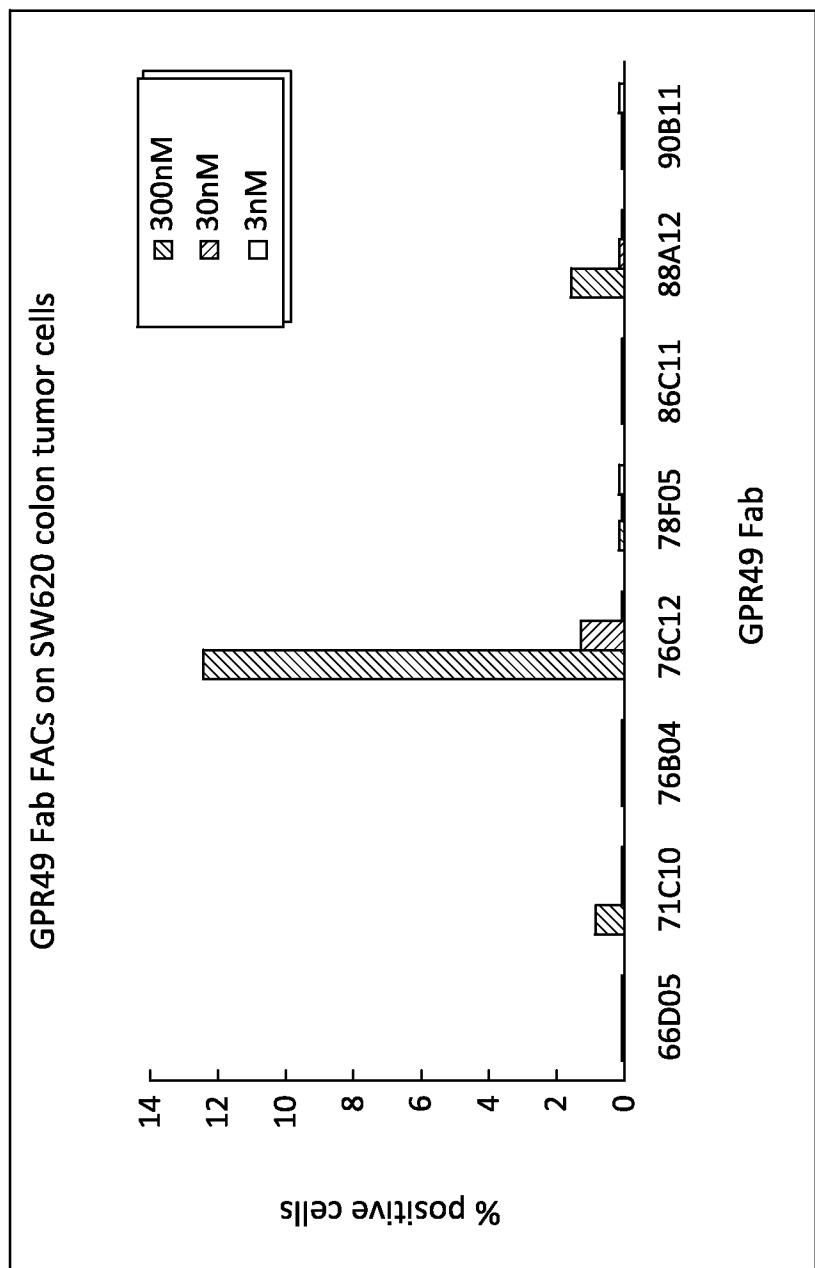
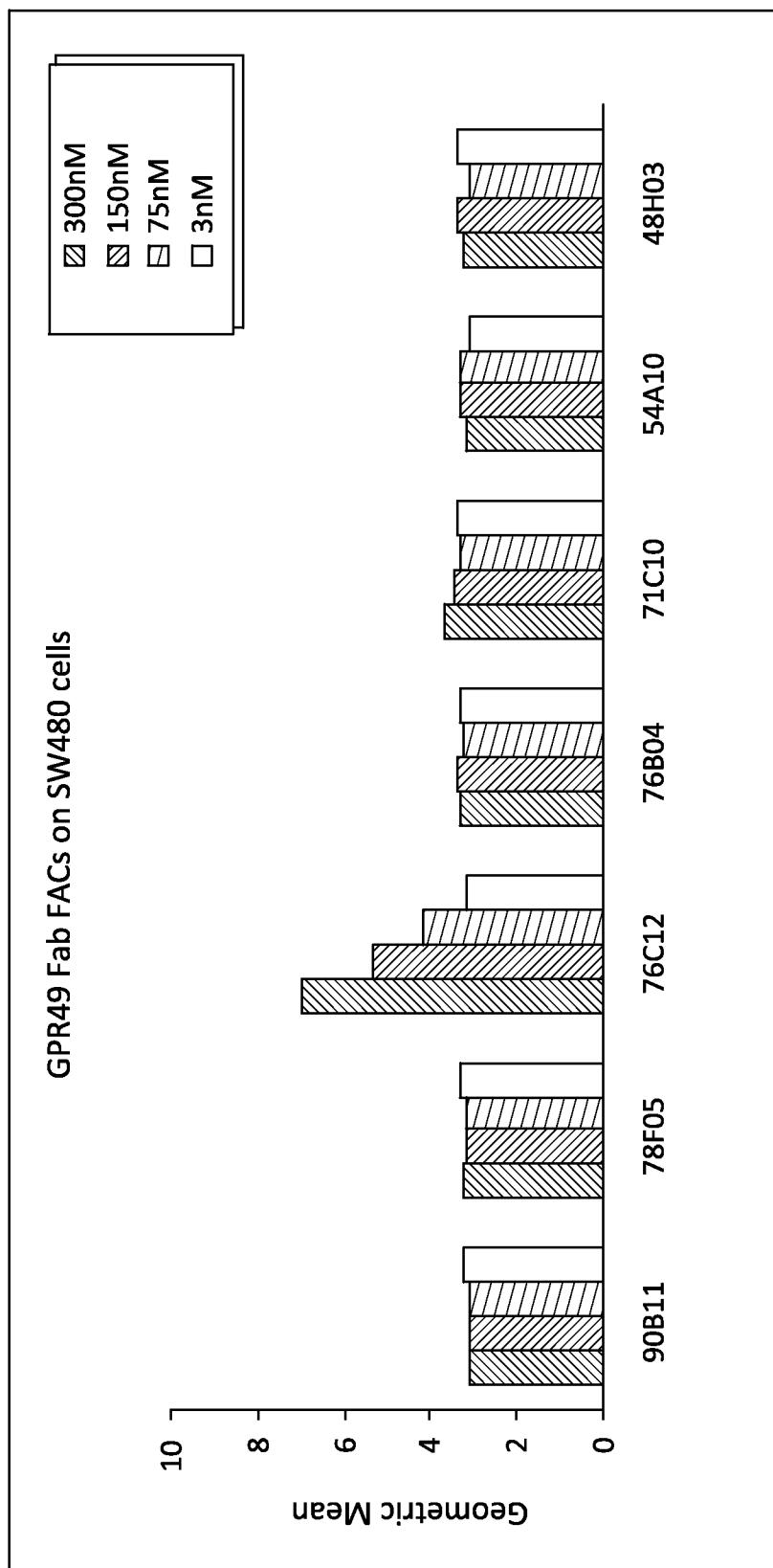
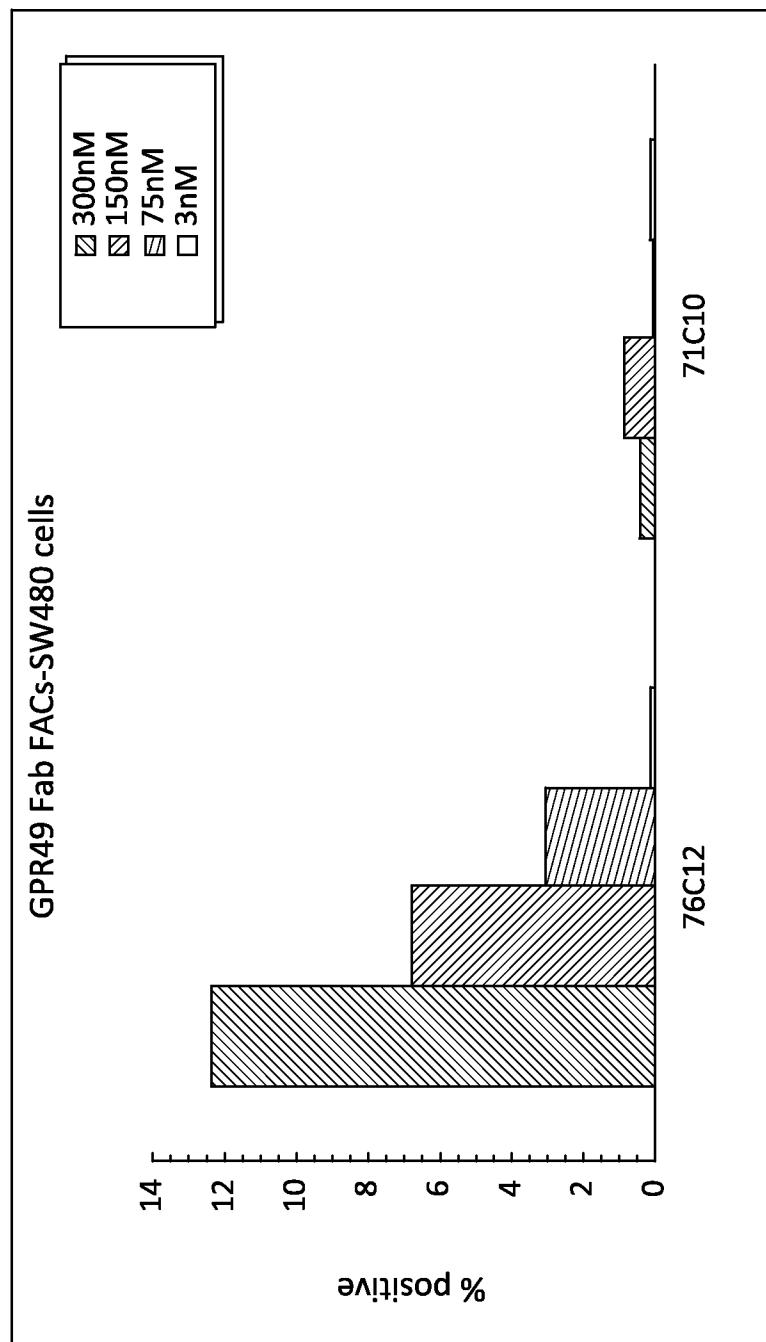


FIG. 3B









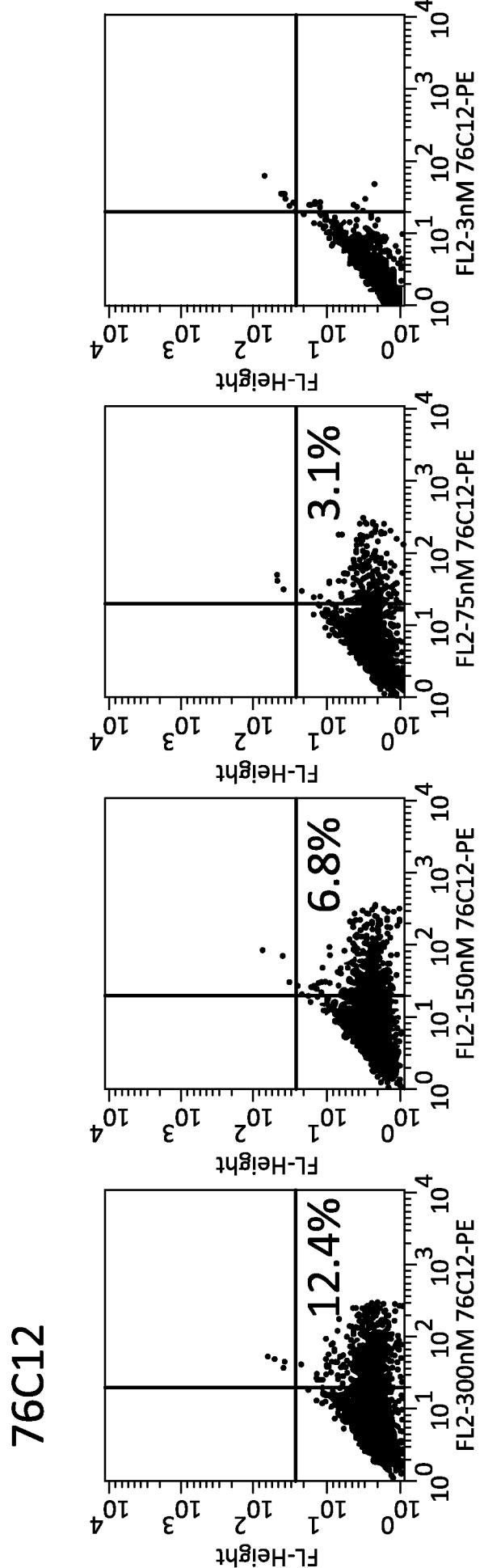
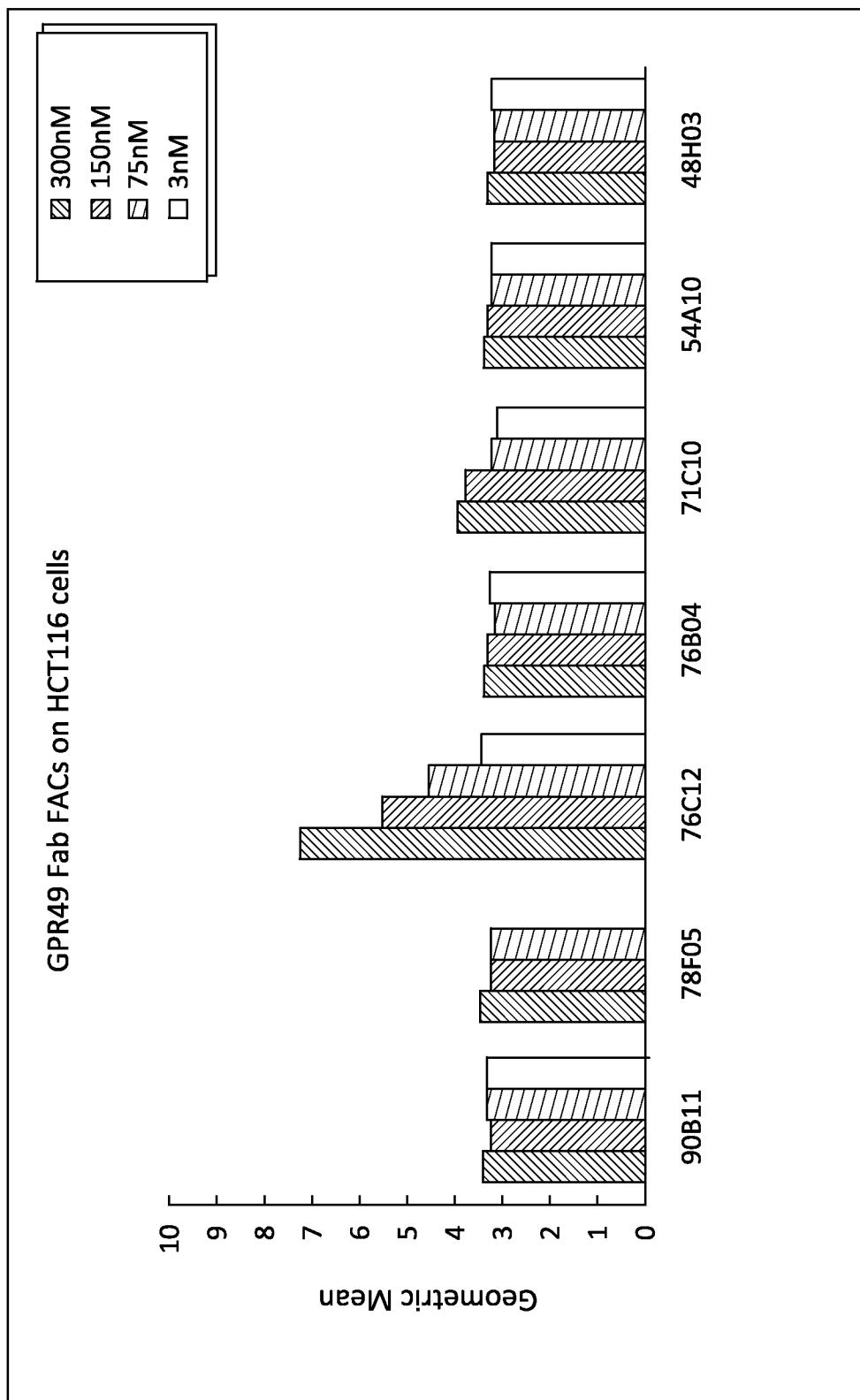


FIG. 5C



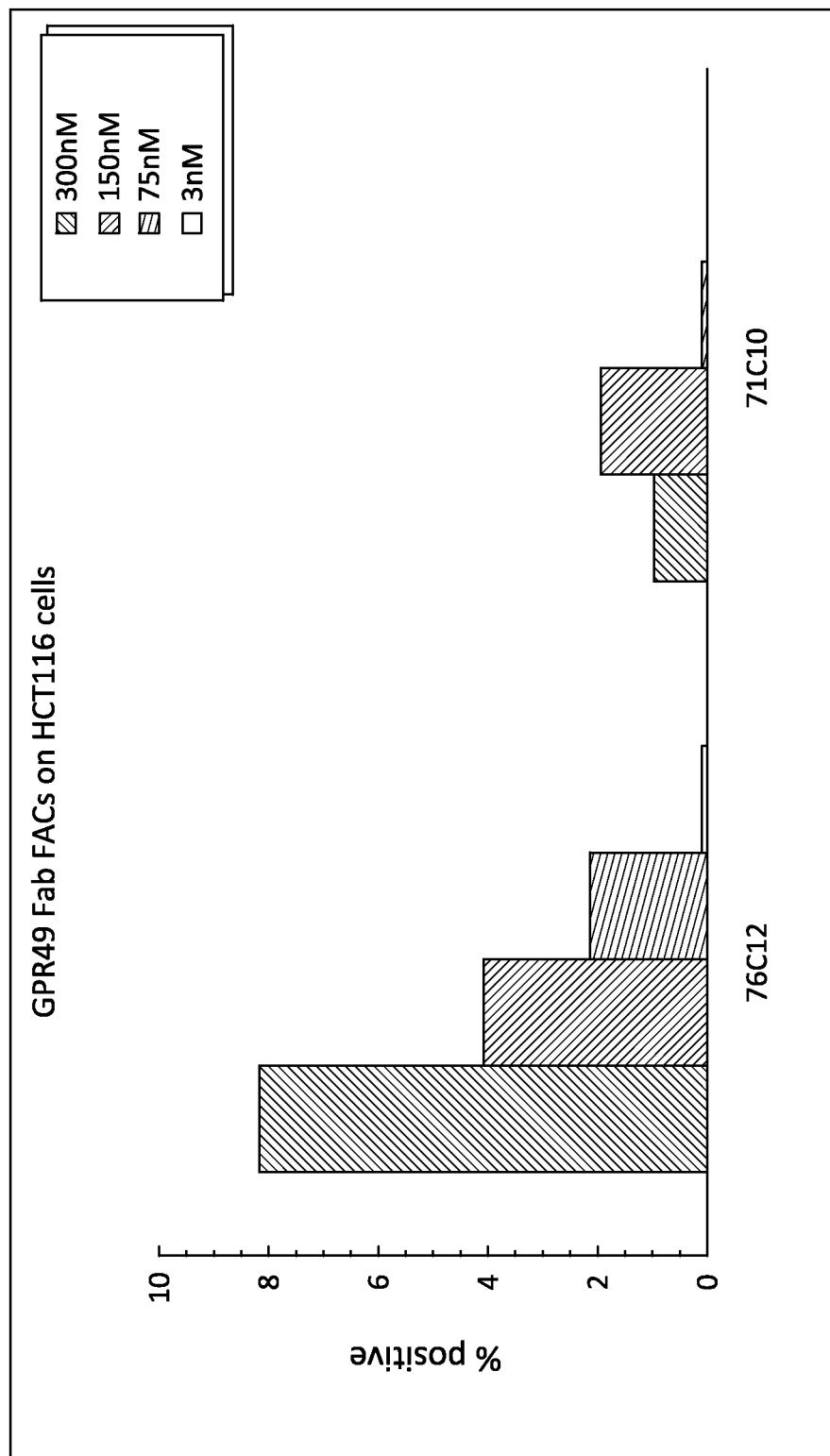


FIG. 6B

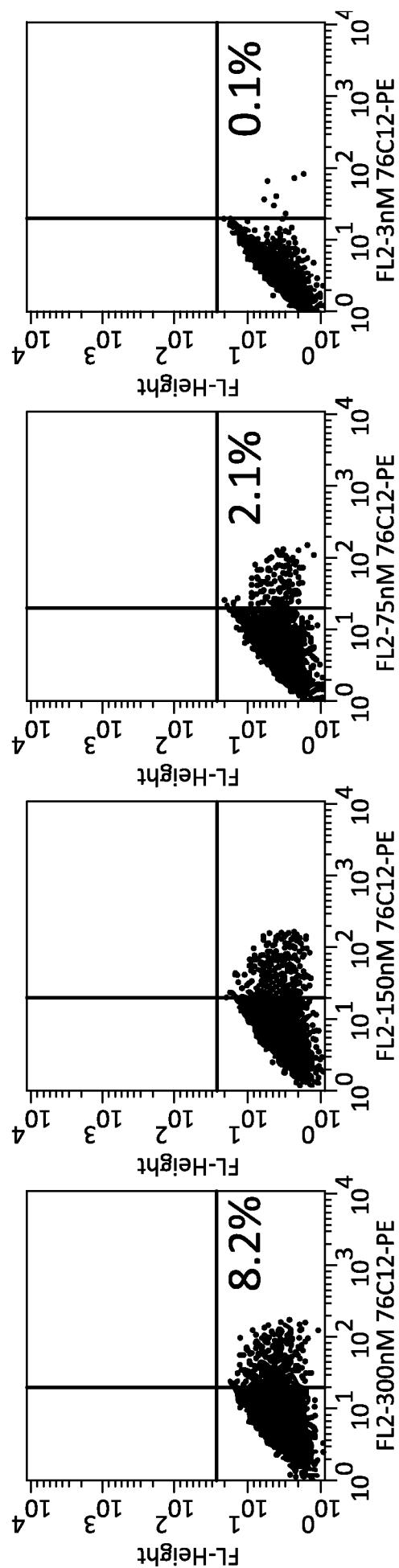


FIG. 6C

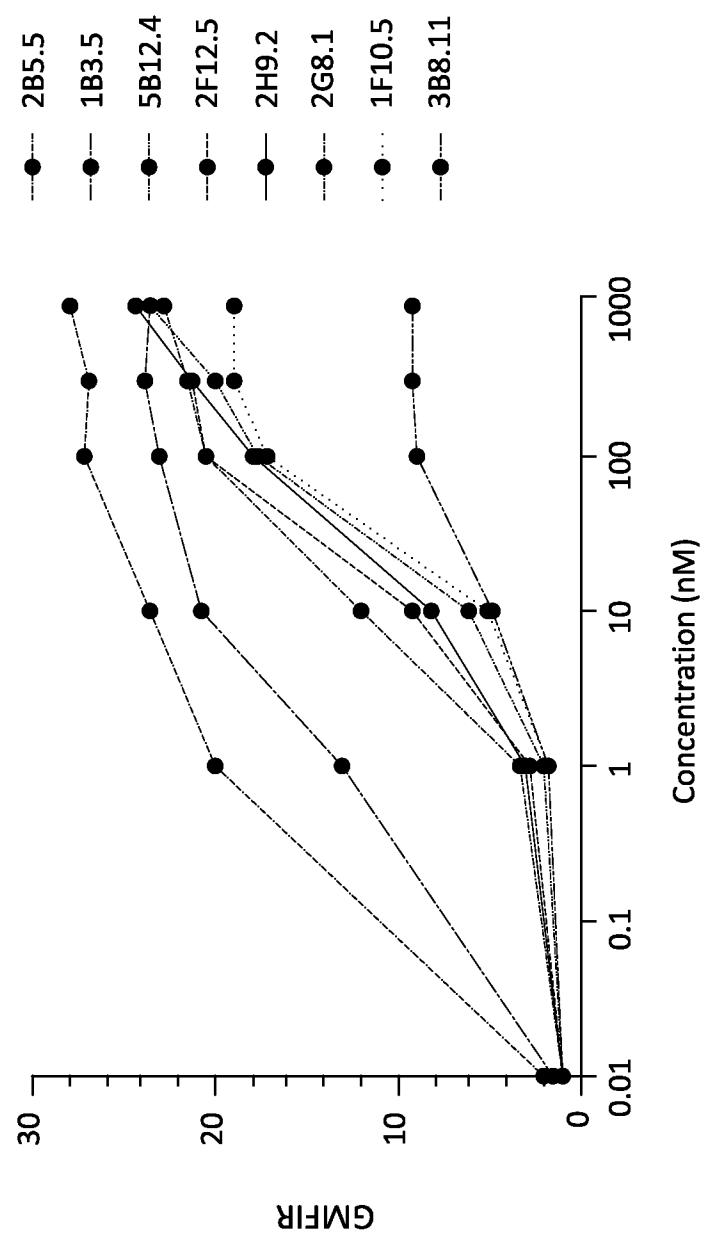


FIG. 7A

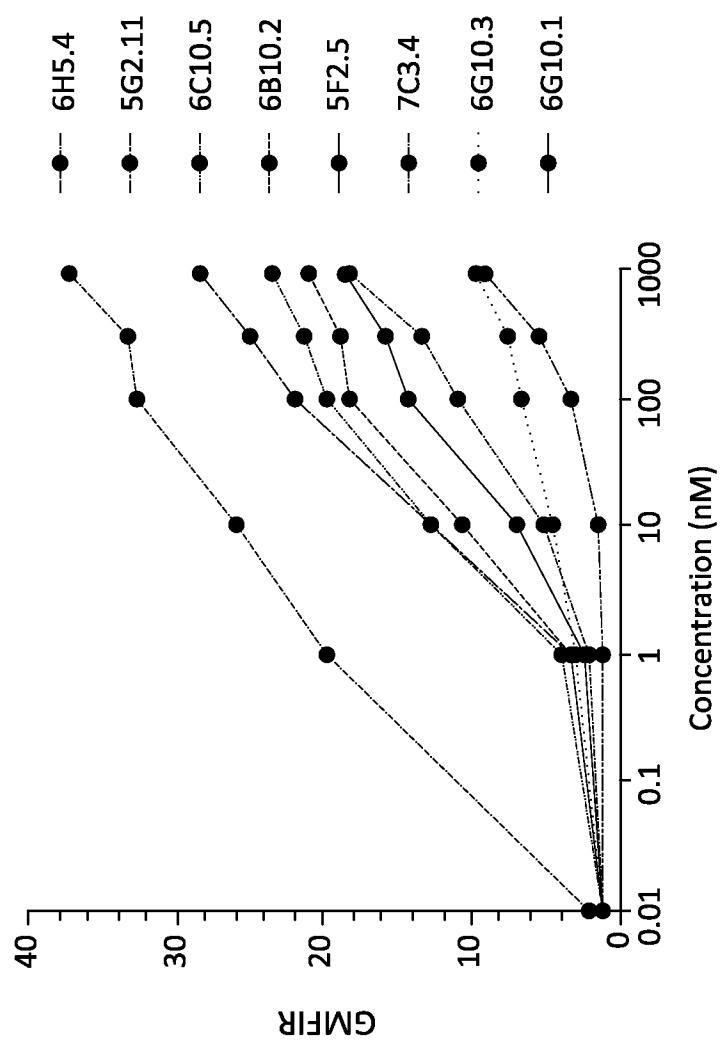


FIG. 7B

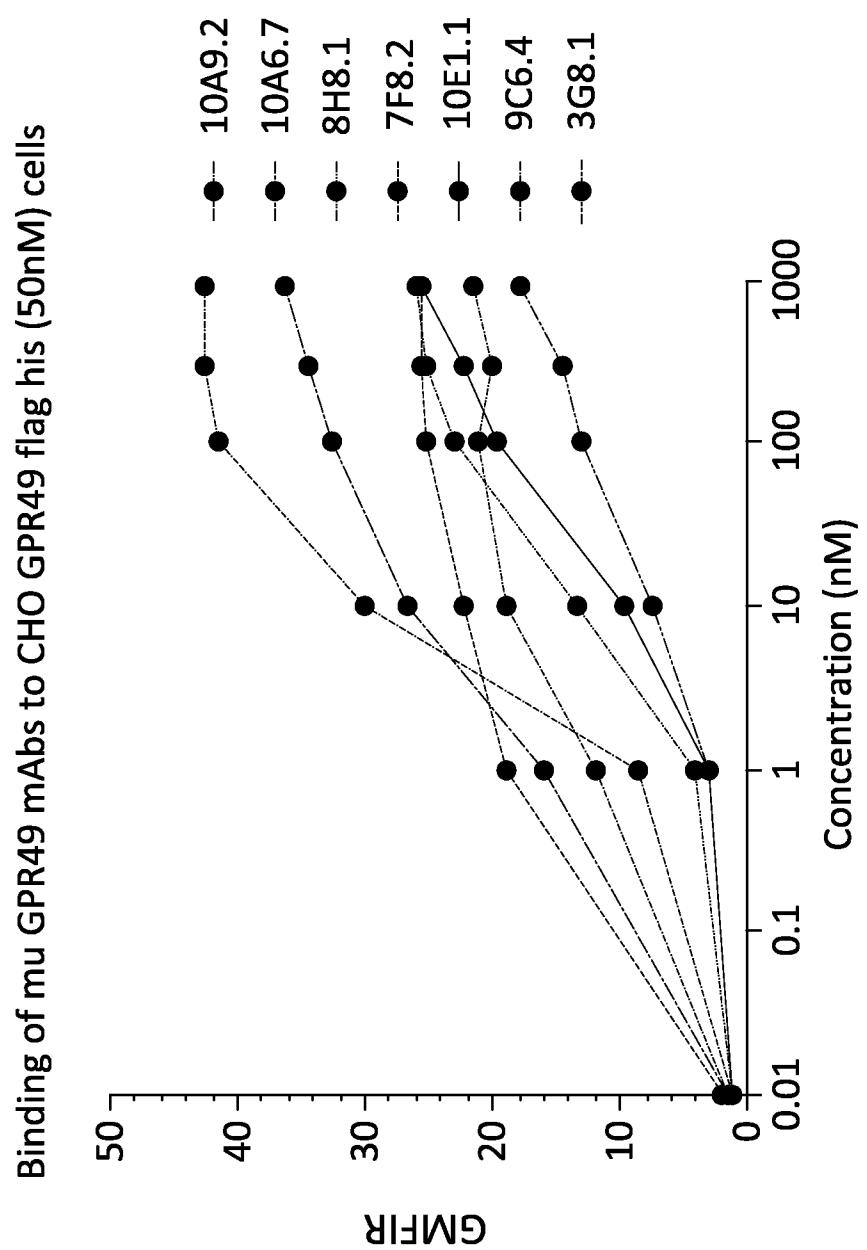


FIG. 7C

DISCLOSURE INFORMATION UNCLASSIFIED BY AD'S CORP/44

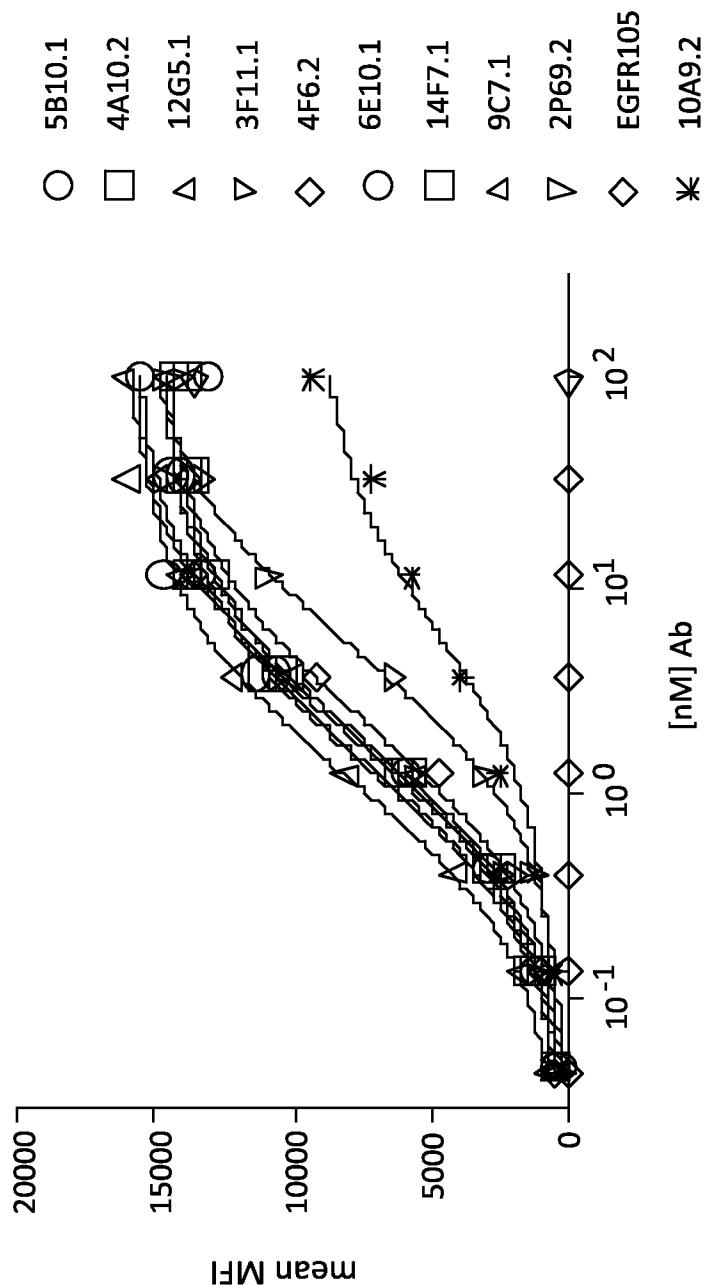


FIG. 8A

	5B10.1	4A10.2	12G5.1	3F11.1	4F6.2	6E10.1	14F7.1	9C7.1	2P69.2	EGFR105	10A9.2
EC50	1.941	1.716	1.210	1.750	2.206	1.322	1.476	2.205	5.116	0.2681	6.218

FIG. 8B

Direct FACS for mouse ABs to GPR49

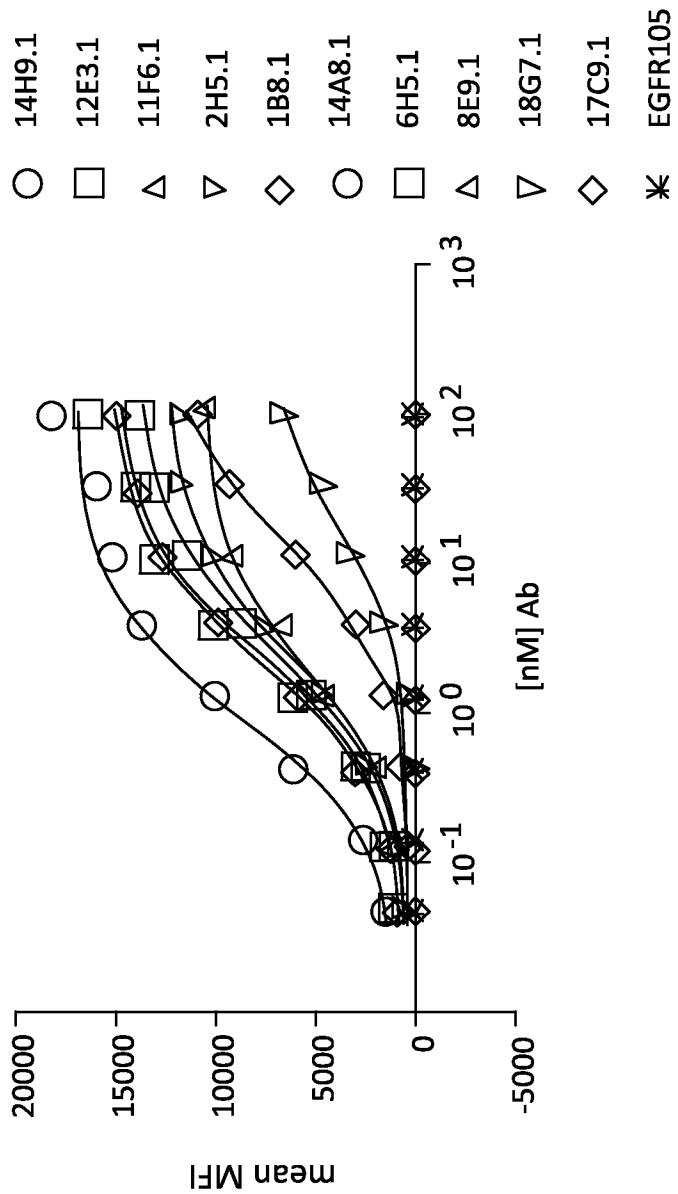


FIG. 8C

	14H9.1	12E3.1	11F6.1	2H5.1	1B8.1	14A8.1	6H5.1	8E9.1	18G7.1	17C9.1
EC50	0.9161	2.632	1.853	17.48	2.239	2.143	2.991	2.170	2.460	12.46

FIG. 8D

Direct ELISA for 2nd gen. mouse ABs to GPR49

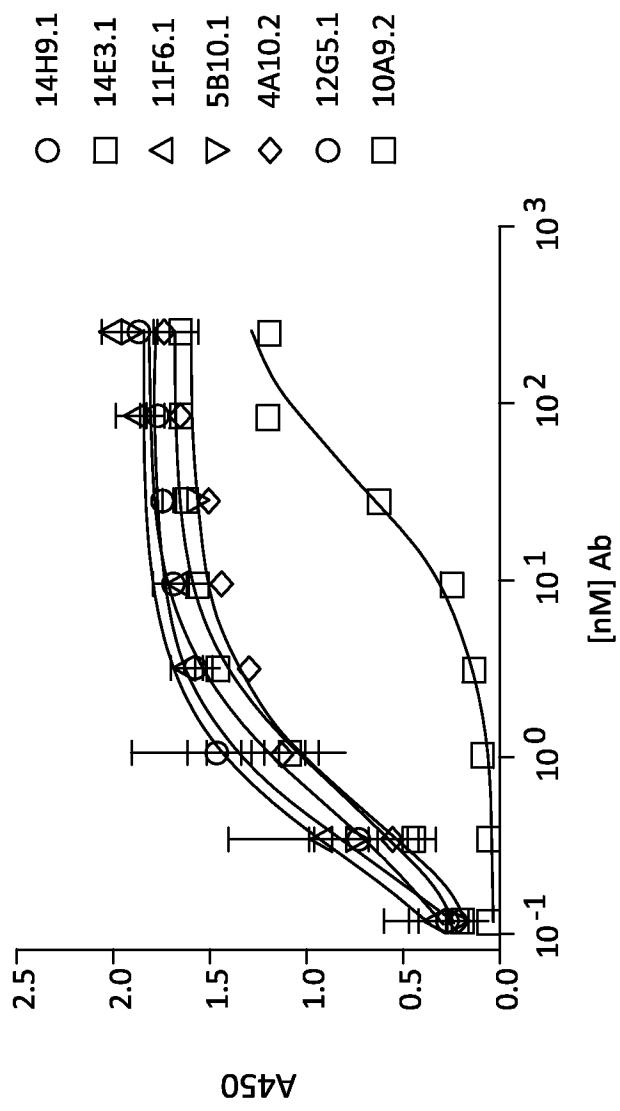
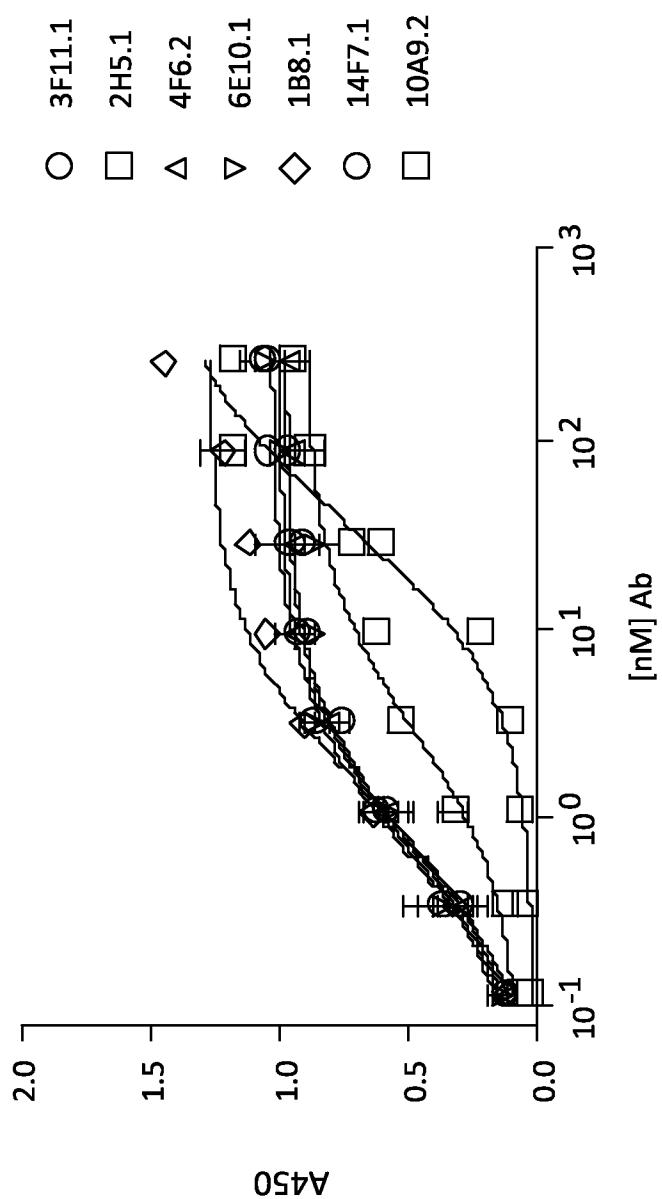


FIG. 9A

	14H9.1	14E3.1	11F6.1	5B10.1	4A10.2	12G5.1	10A9.2
EC50	0.5523	0.5587	0.2429	0.2737	0.5311	0.2565	35.22

FIG. 9B

Direct ELISA for 2nd gen. mouse ABs to GPR49



	3F11.1	2H5.1	4F6.2	6E10.1	1B8.1	14F7.1	10A9.2
EC50	0.8837	2.866	0.6506	0.6577	1.335	0.5695	35.22

FIG. 9C

FIG. 9D

Direct ELISA for 2nd gen. mouse ABs to GPR49

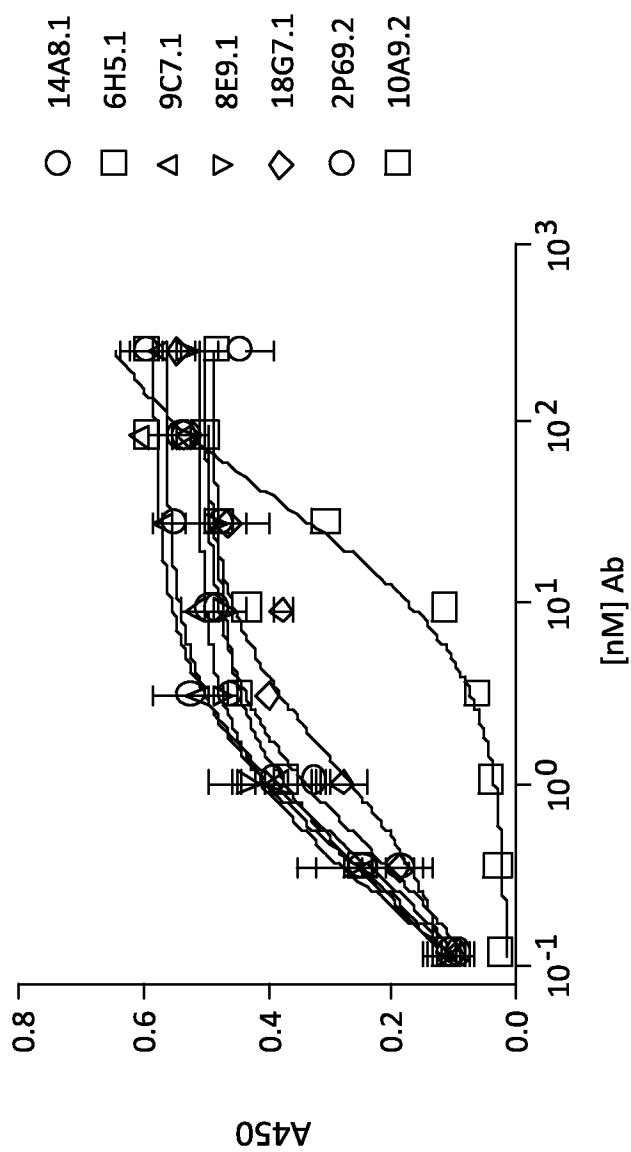


FIG. 9E

	14A8.1	6H5.1	9C7.1	8E9.1	18G7.1	2P69.2	10A9.2
EC50	0.3792	0.2844	0.4986	0.2036	1.352	0.4662	35.22

FIG. 9F

Direct ELISA for 2nd gen. mouse ABs to GPR49

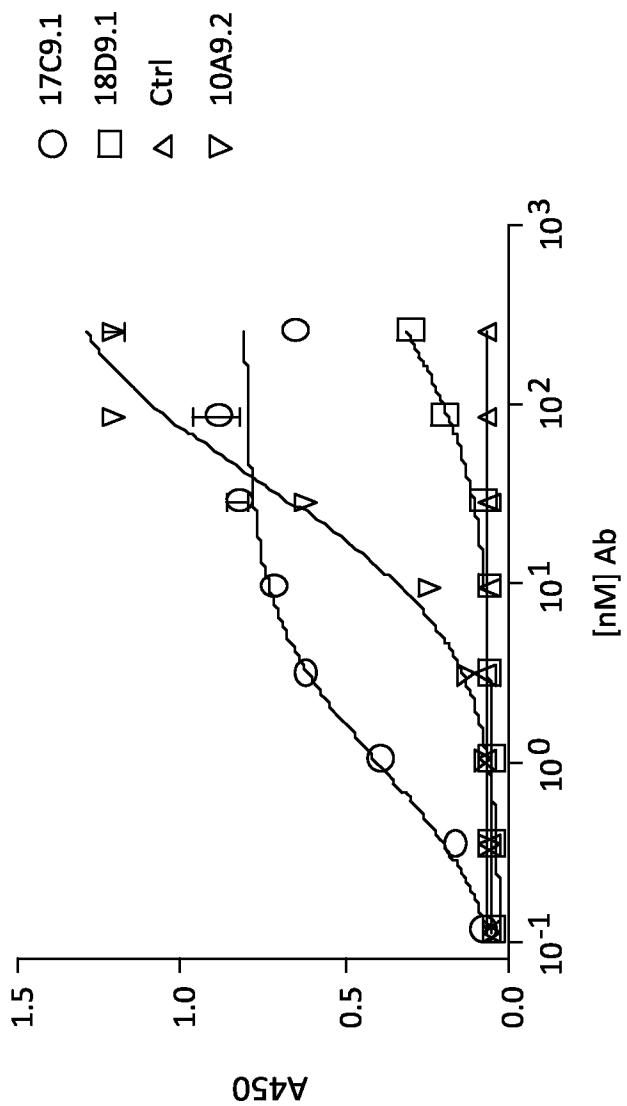


FIG. 9G

	17C9.1	18D9.1	Ctrl	10A9.2
EC50	0.9520	225.4	1579	35.22

FIG. 9H

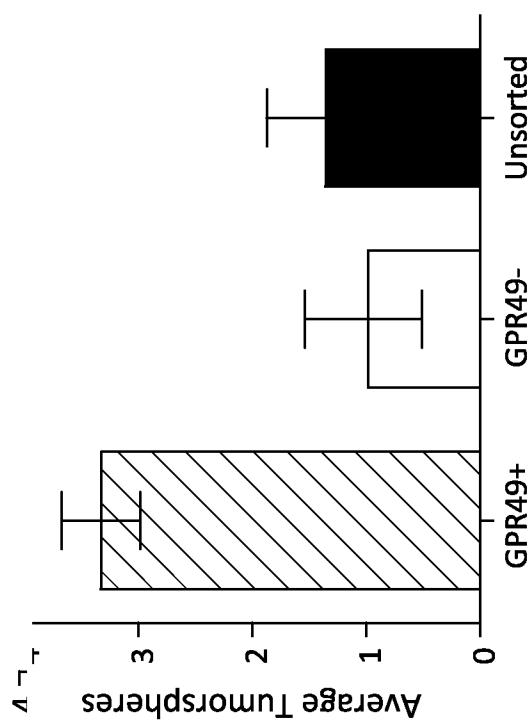


FIG. 10A

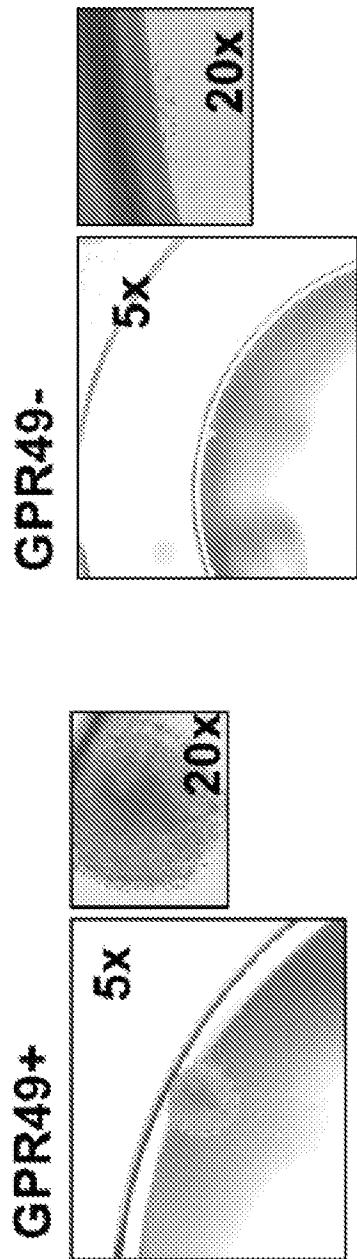


FIG. 10B

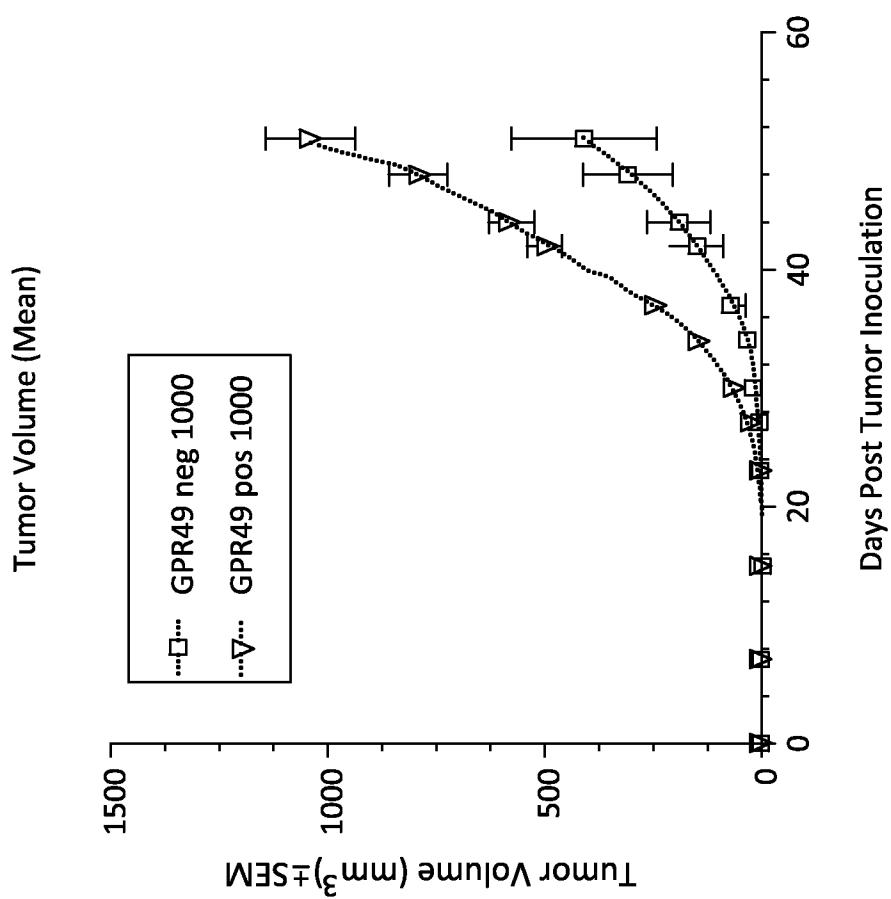


FIG. 11A

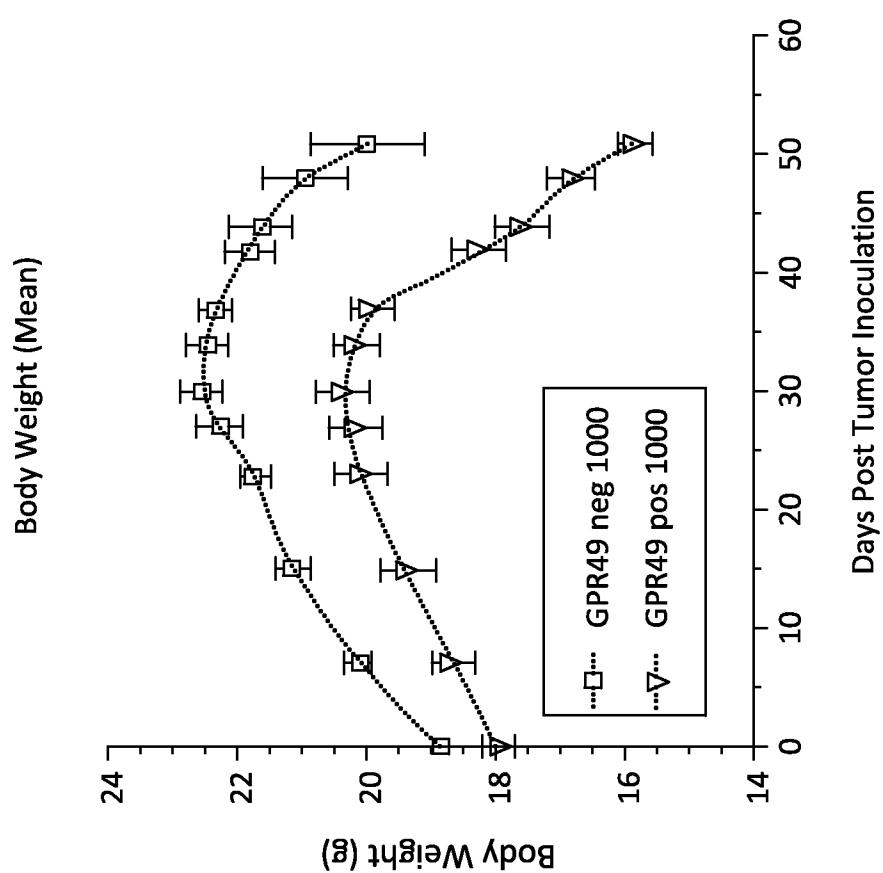


FIG. 11B

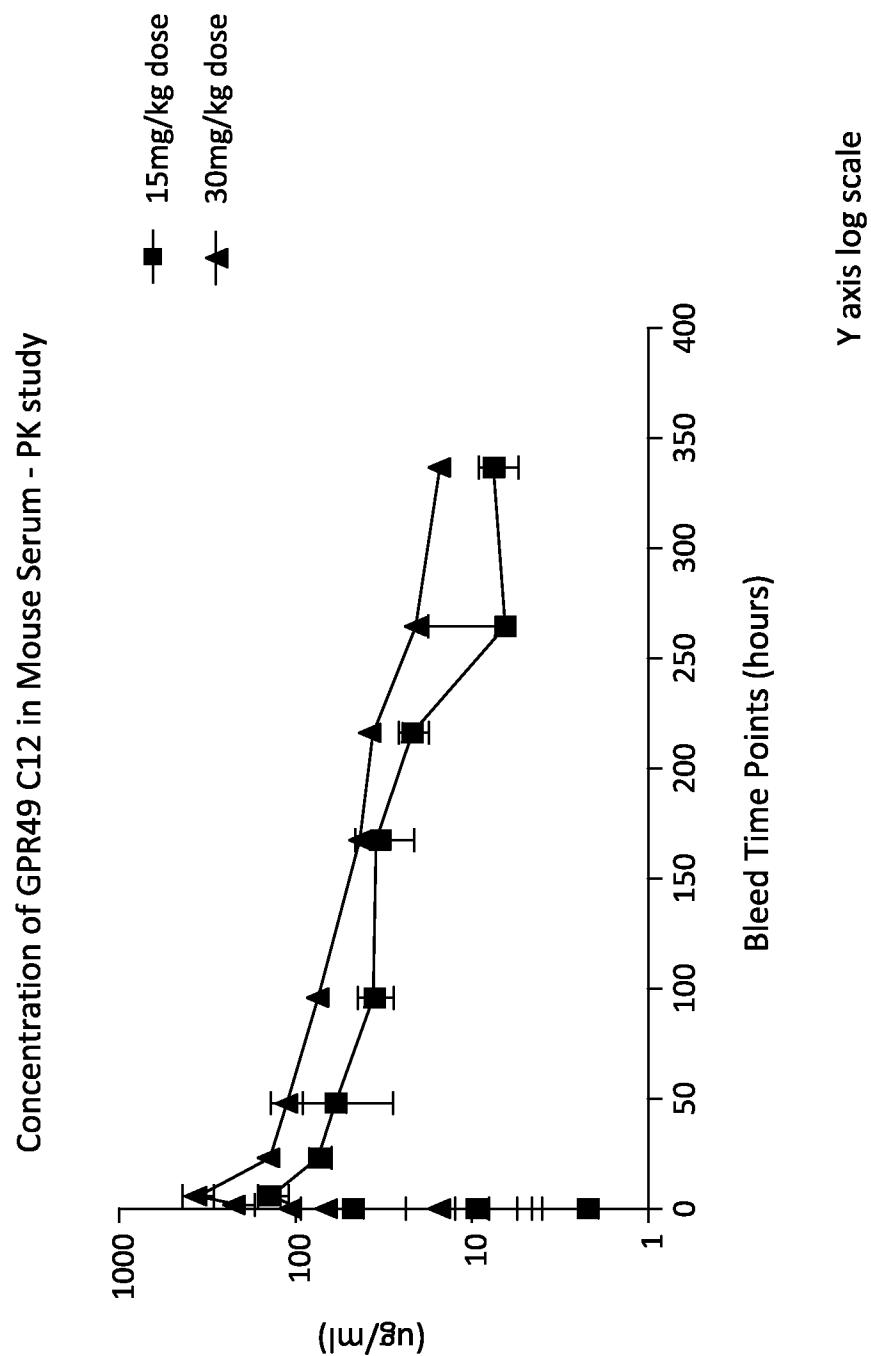


FIG. 12A

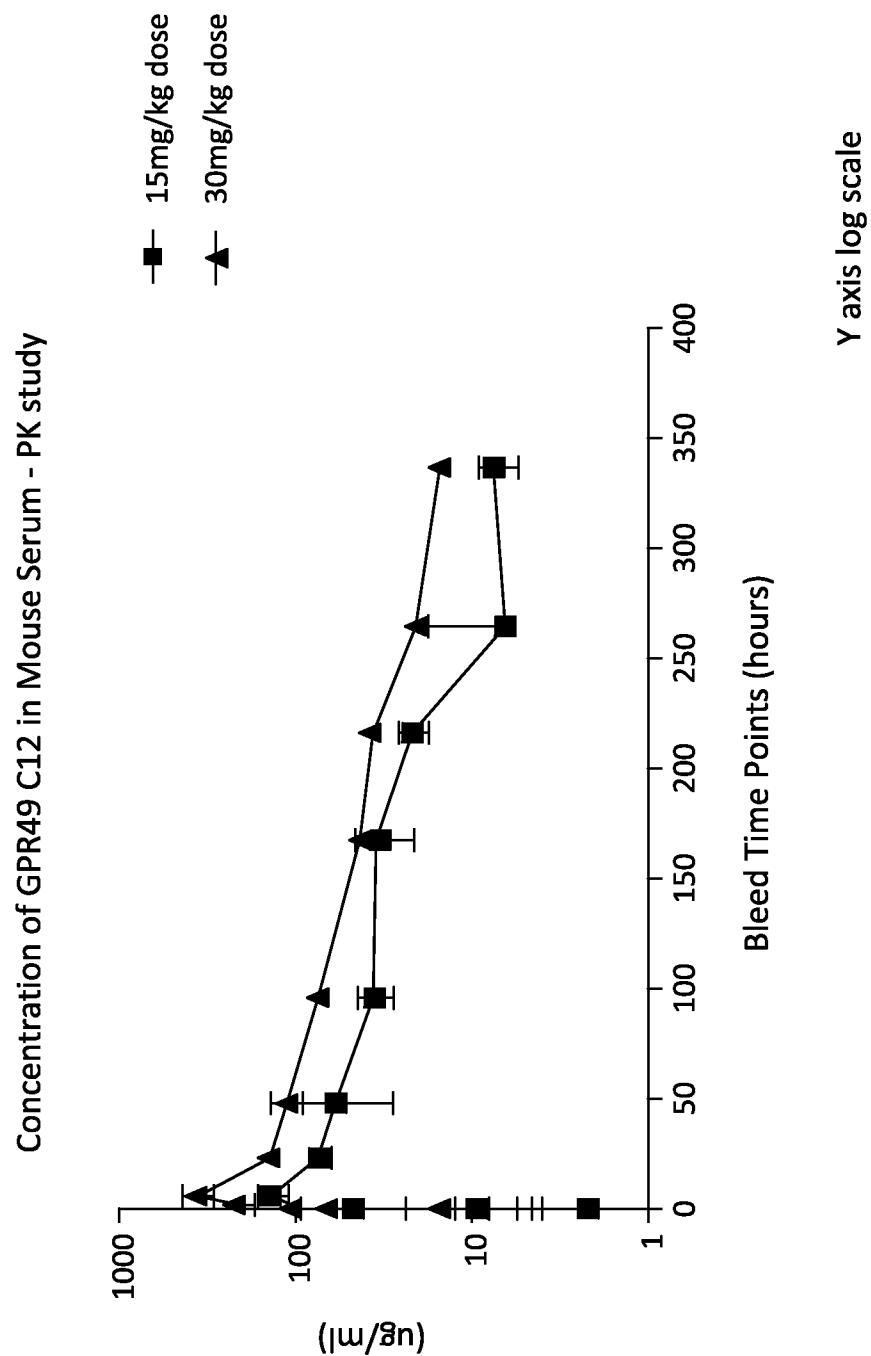


FIG. 12B

Pharmacokinetic Parameters of GRP49 (C12, IgG1) in SCID Beige Mice after a Single IP Administration of 15 or 30 mg/kg

Dose	T _{max} Hr	C _{max} -g/mL	t _{1/2} Hr	AUC _{0-336hr} Hr*mg/L	AUC _{0-INF} Hr*mg/L	AUC %Extrap	CL / F mL/hr/kg	Vz / F mL/kg
15	6	135	90	11,310	12,276	8	1.24	162
30	6	359	104	21,853	24,162	10	1.25	187

FIG. 12C

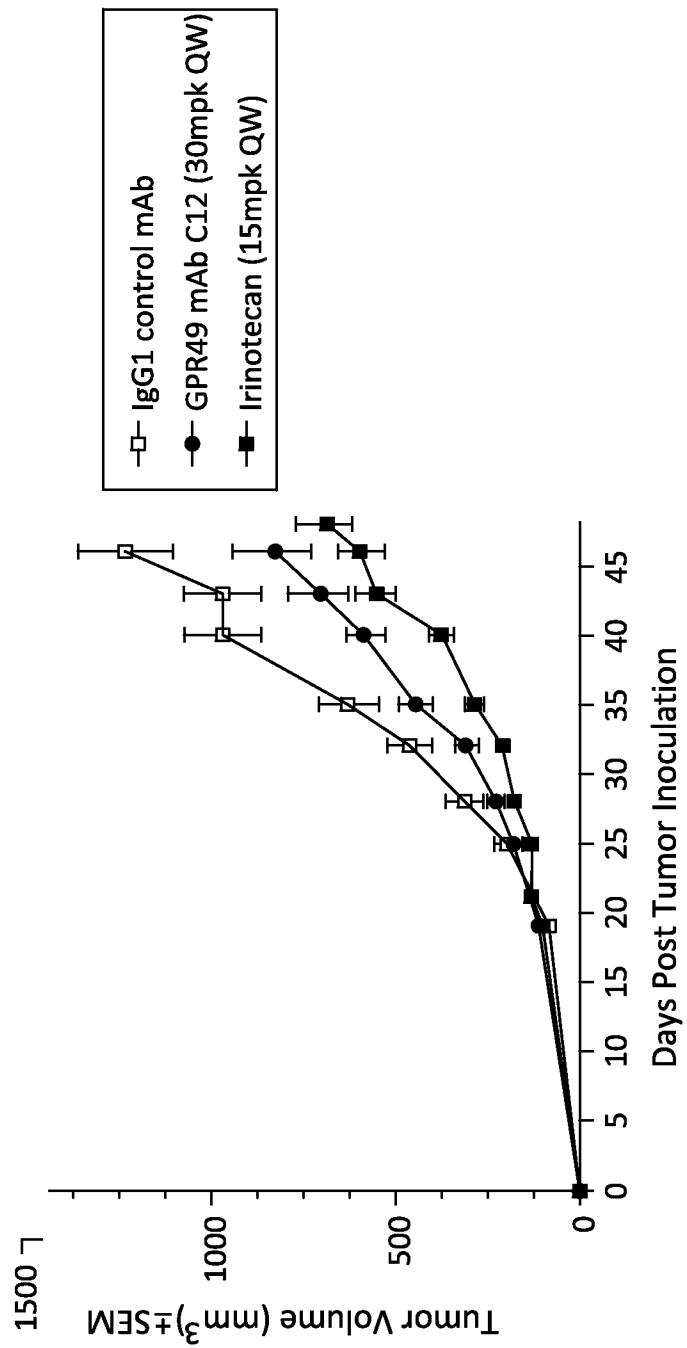


FIG. 13A

T/C Values @ Day 62

Treatment	p-value	%T/C Day 62
ET12 7.5mpk BIW	0.025	67
ET12 15mpk QW	0.188	81
ET12 15mpk BIW	0.008	61
ET12 30mpk QW	0.005	59

FIG. 13B

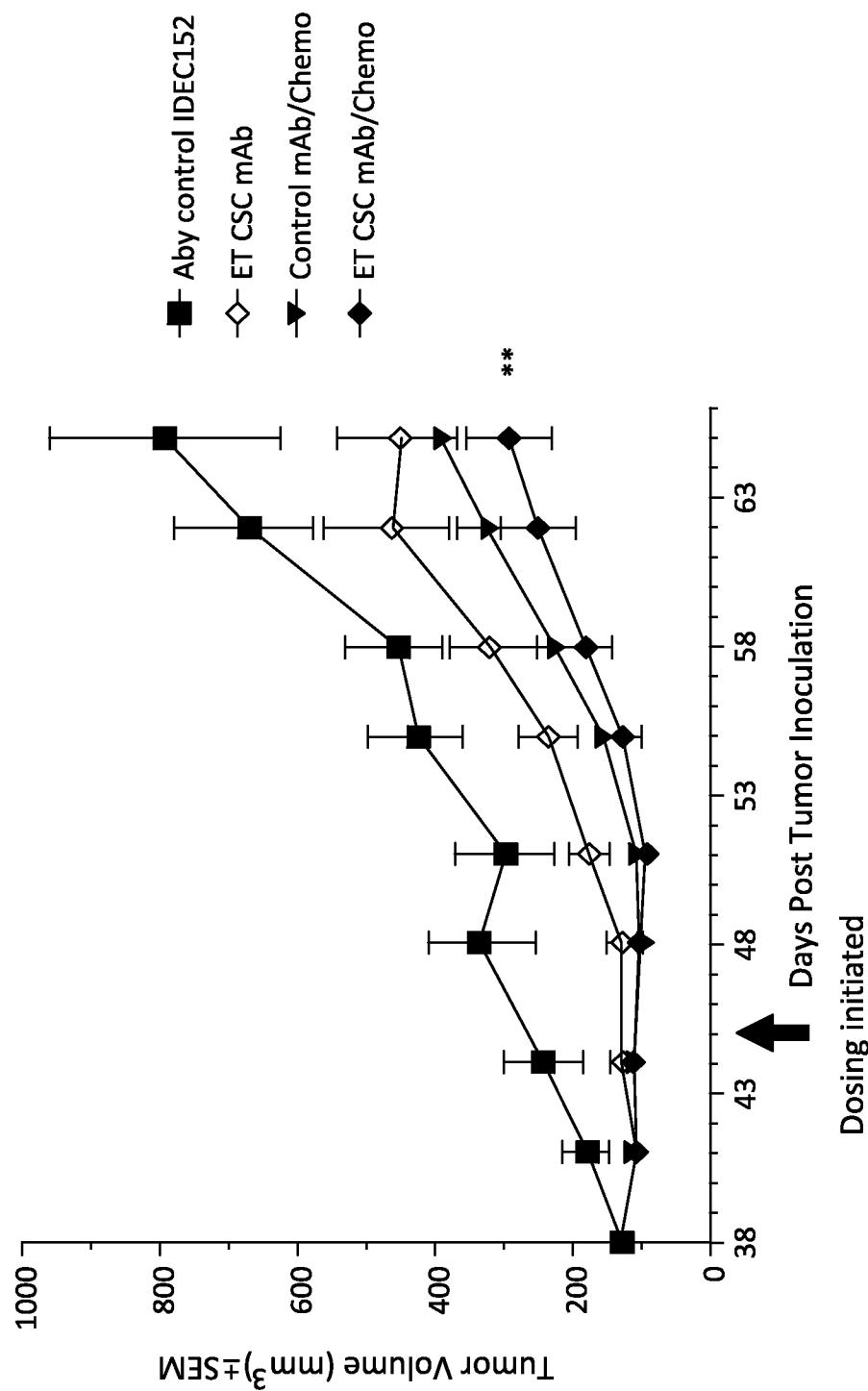


FIG. 14

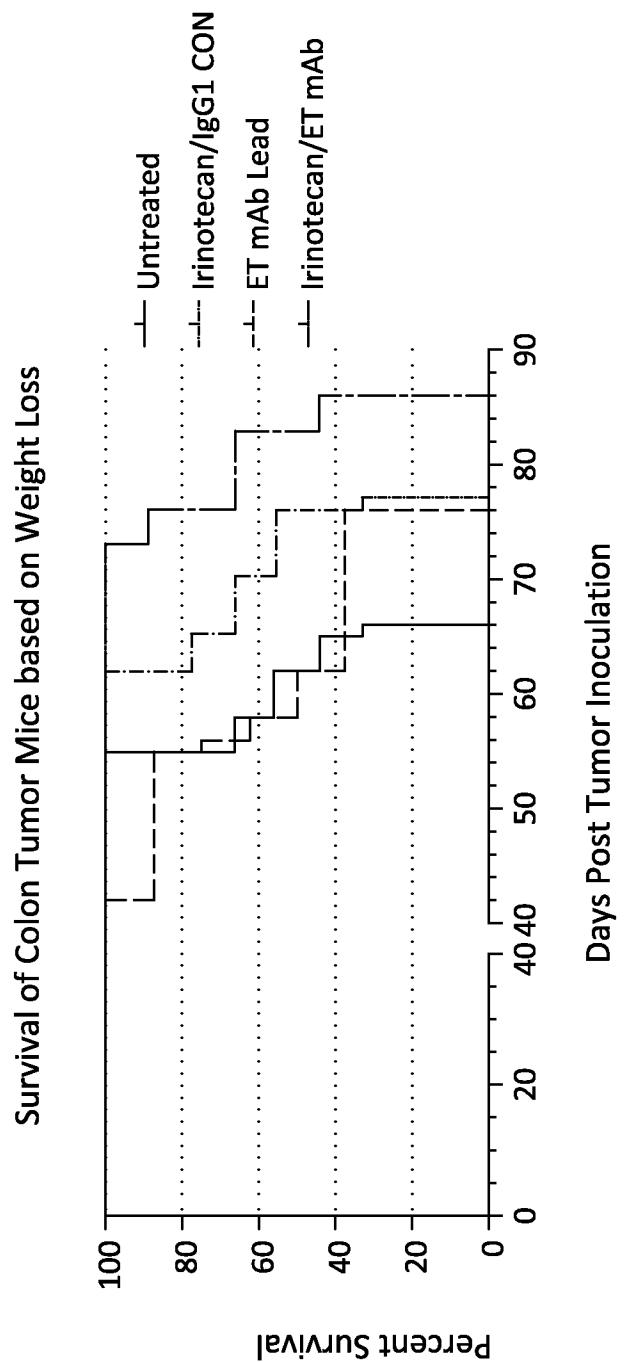


FIG. 15

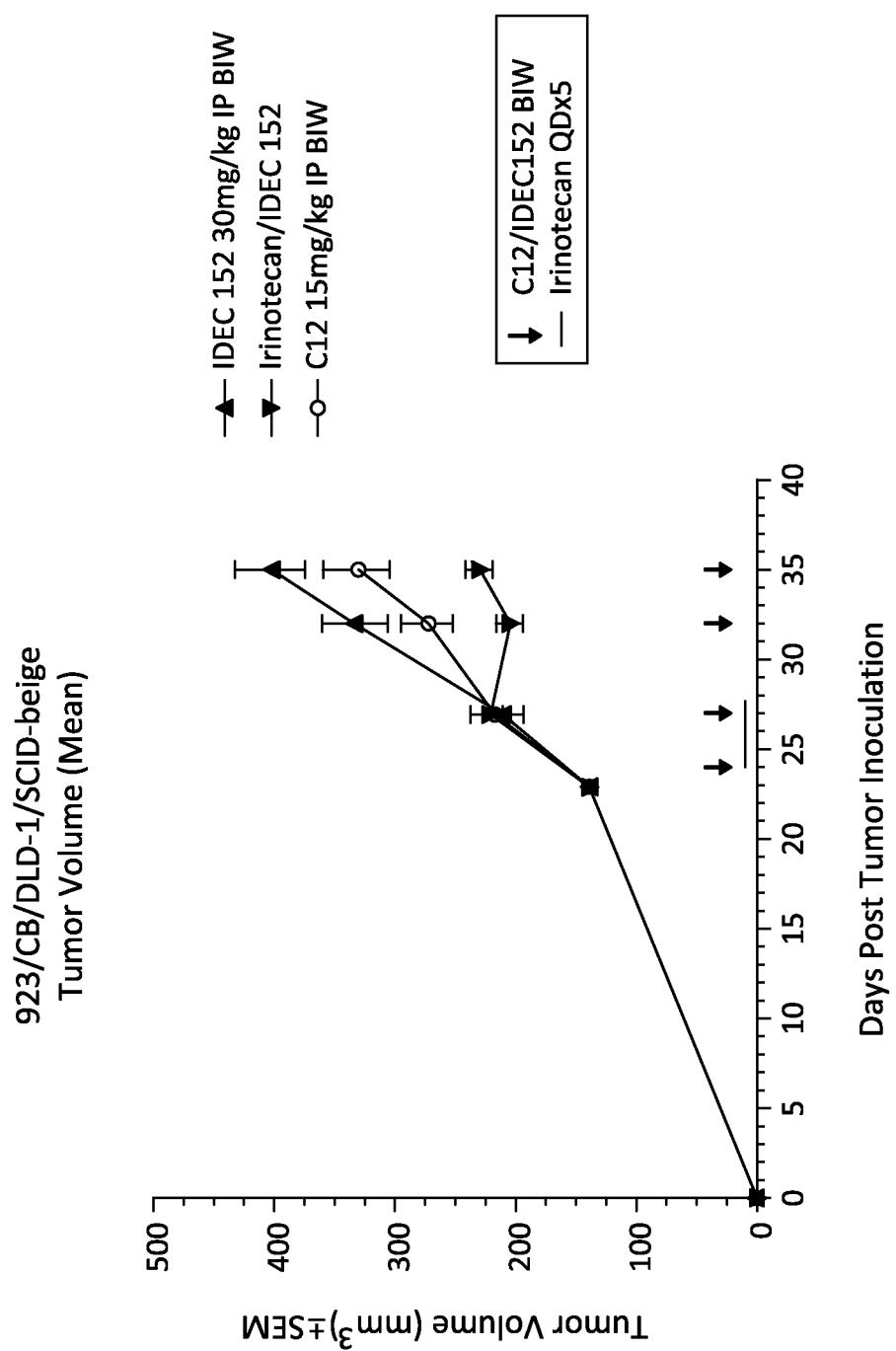


FIG. 16A

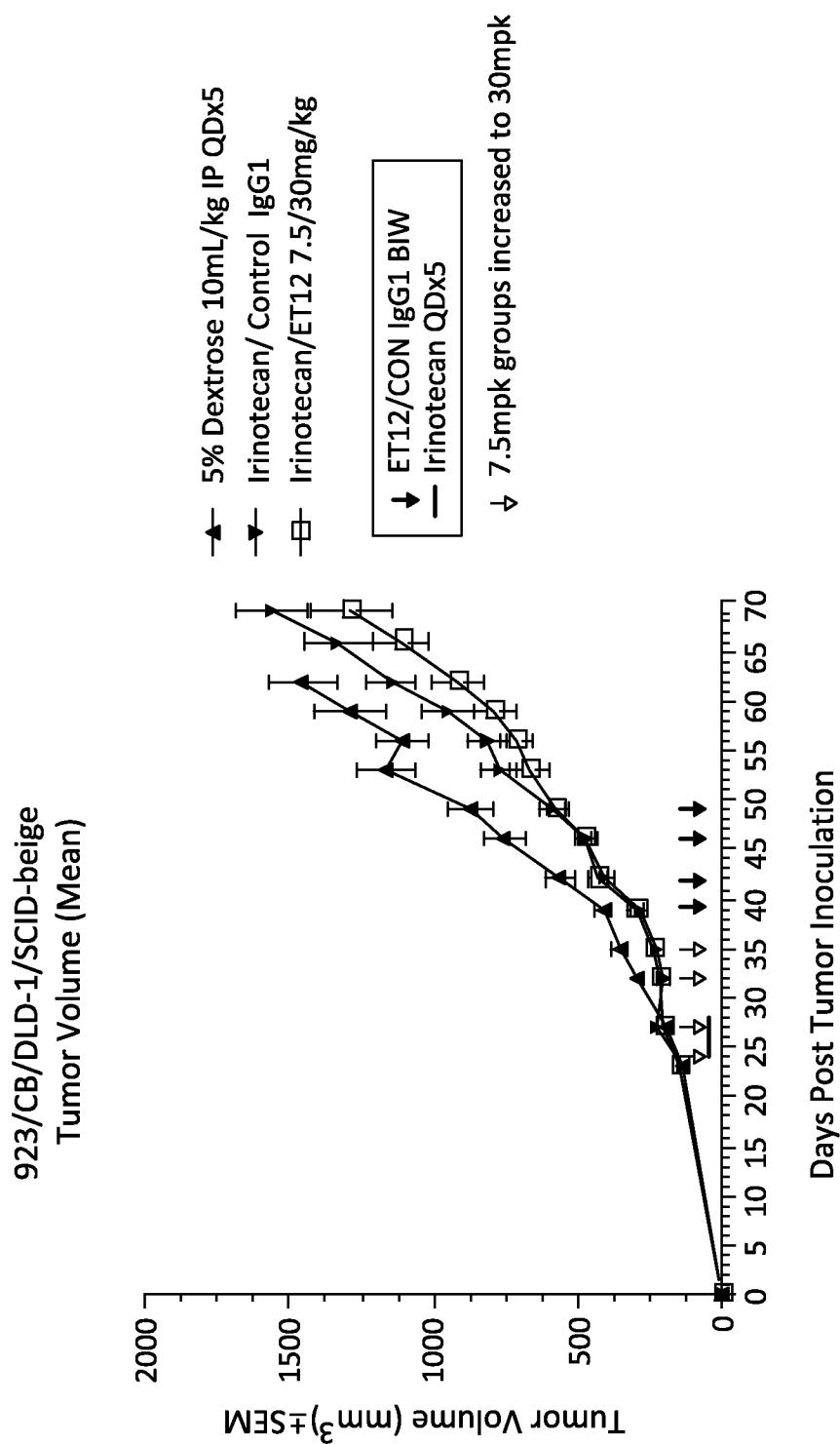


FIG. 16B

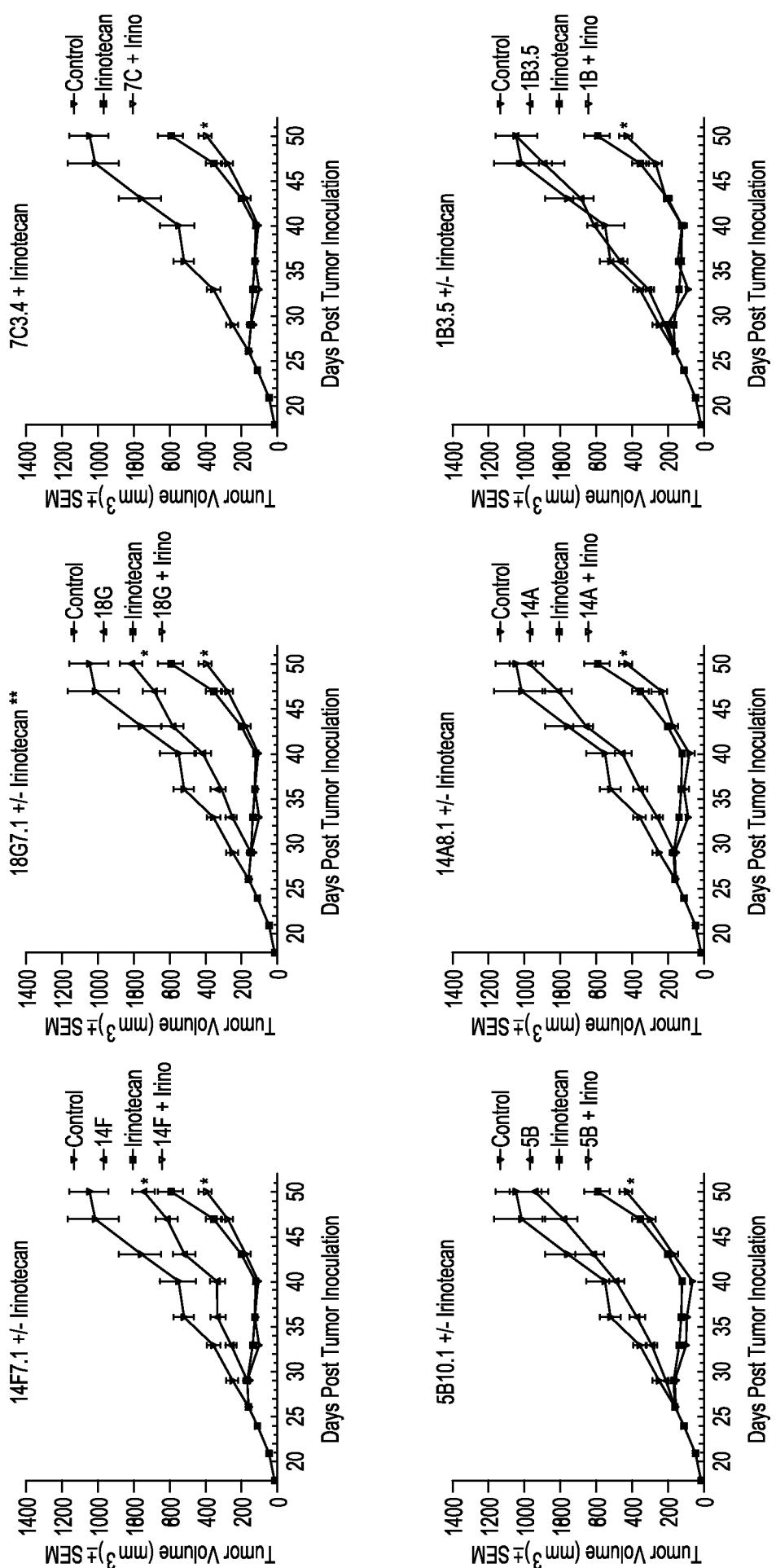


FIG. 17

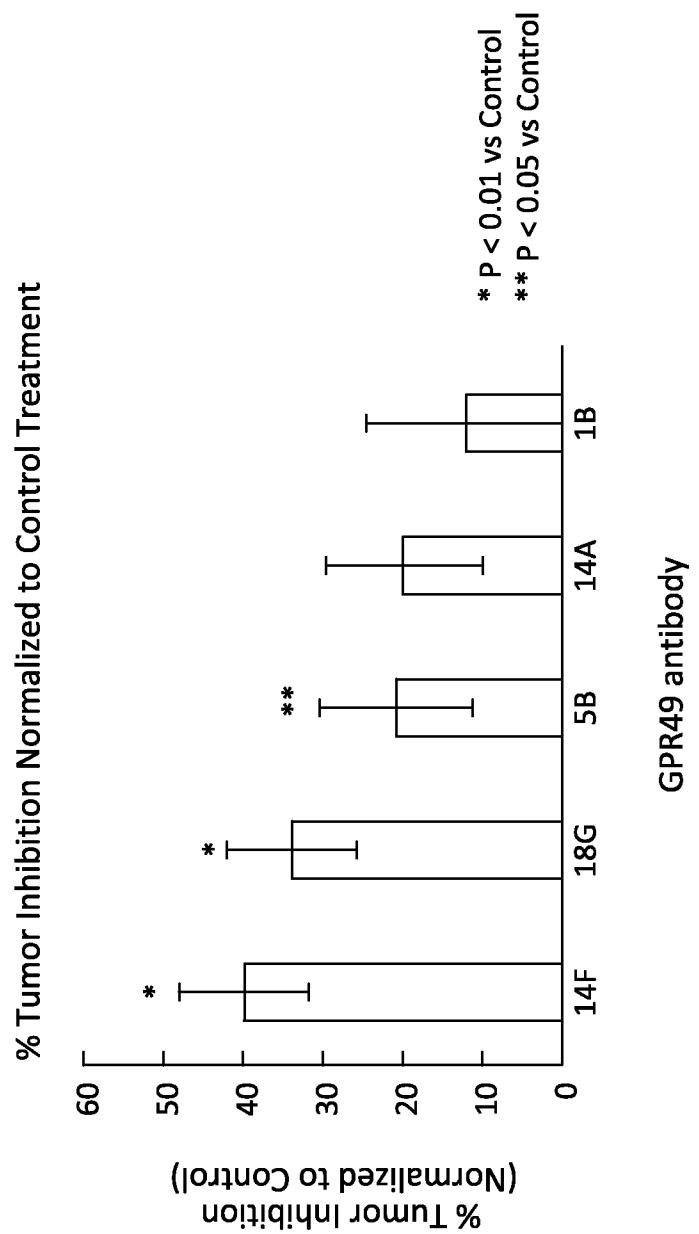


FIG. 18

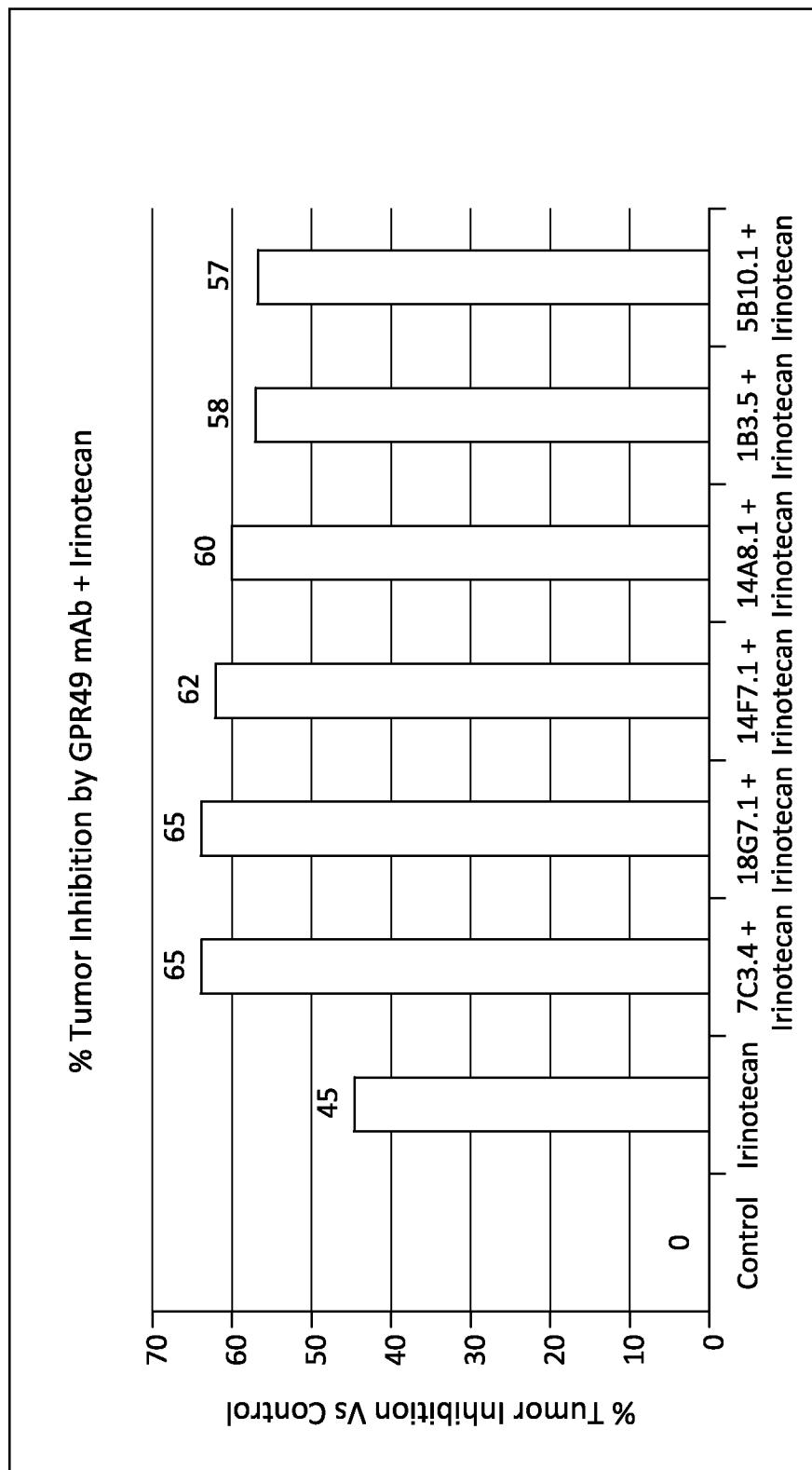


FIG. 19

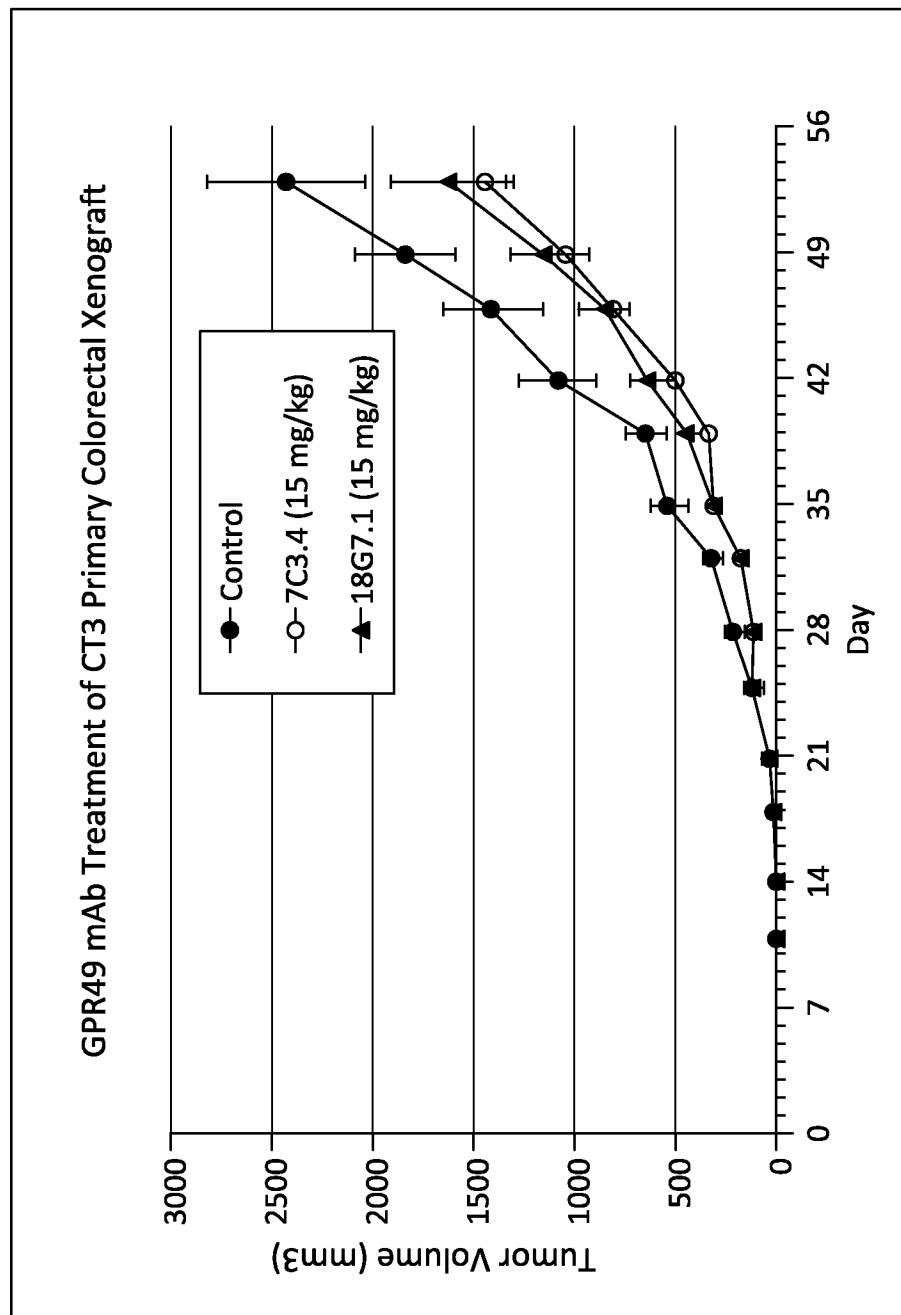


FIG. 20

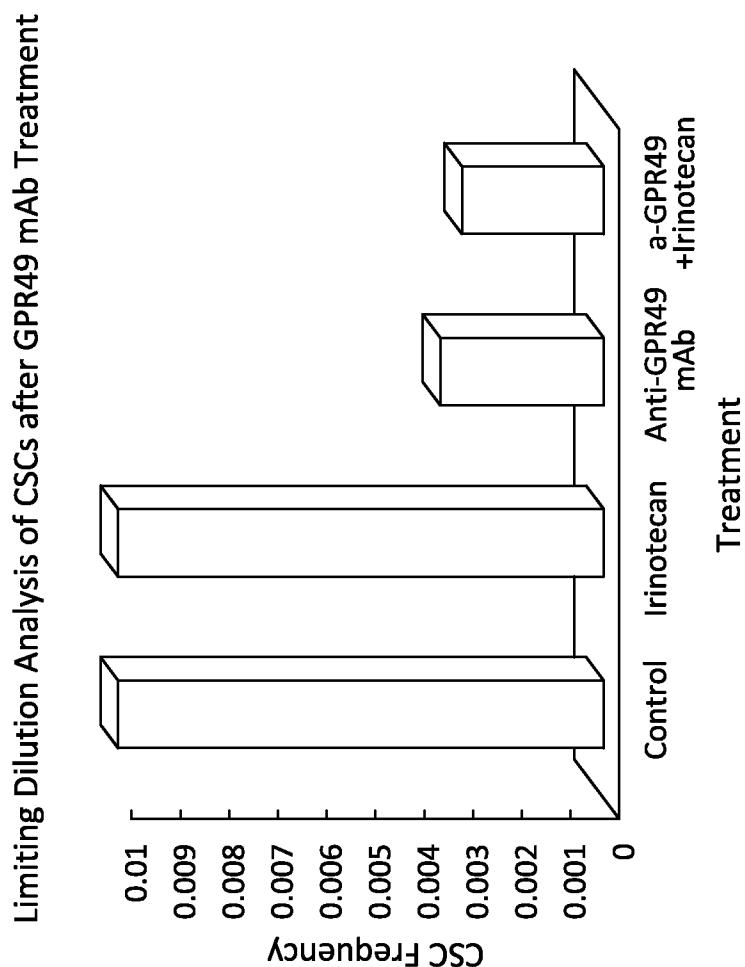
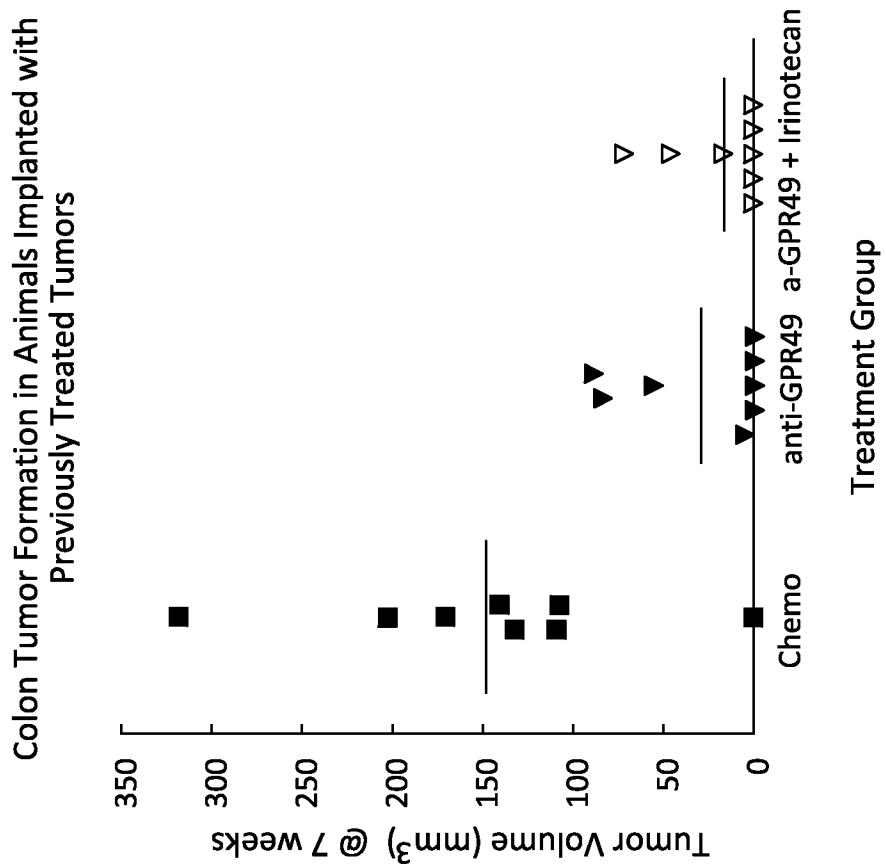


FIG. 21B

FIG. 21A

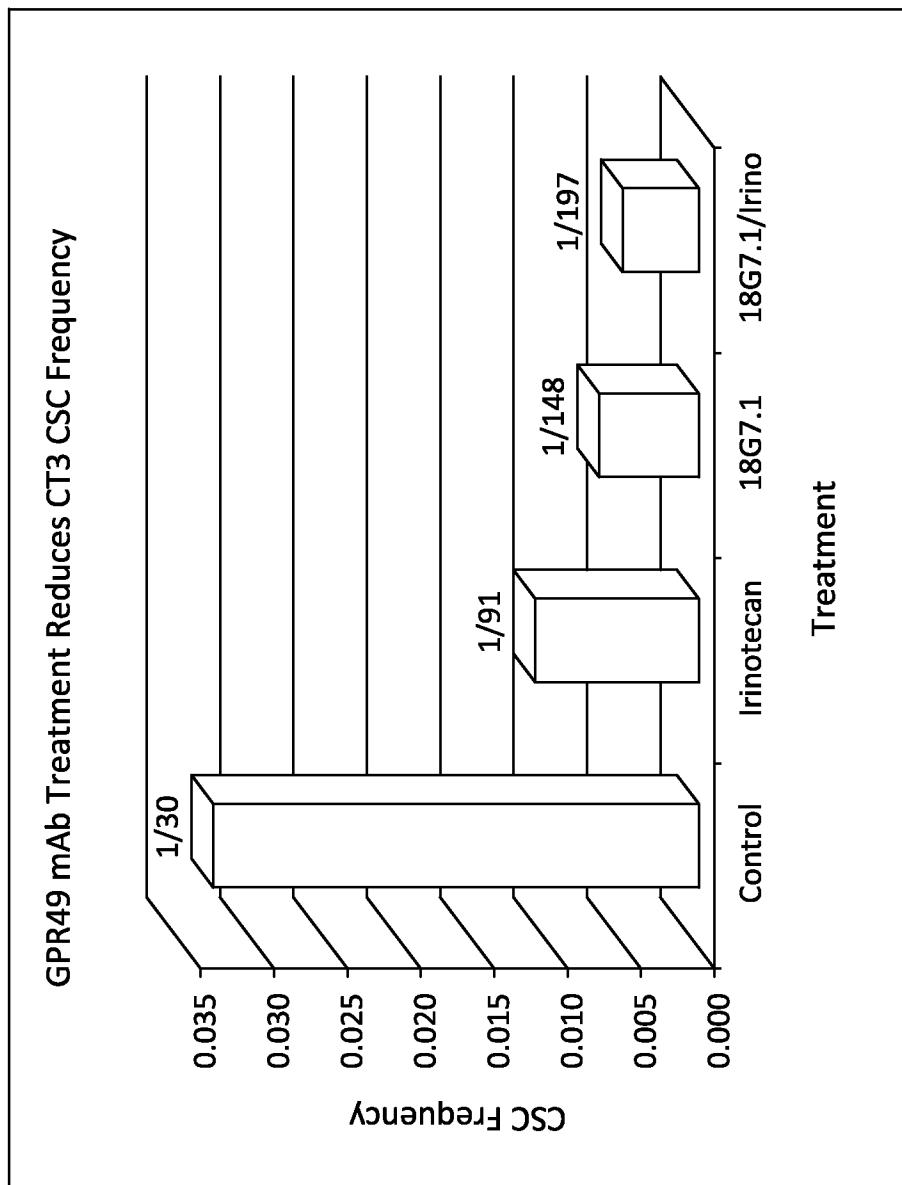


FIG. 22

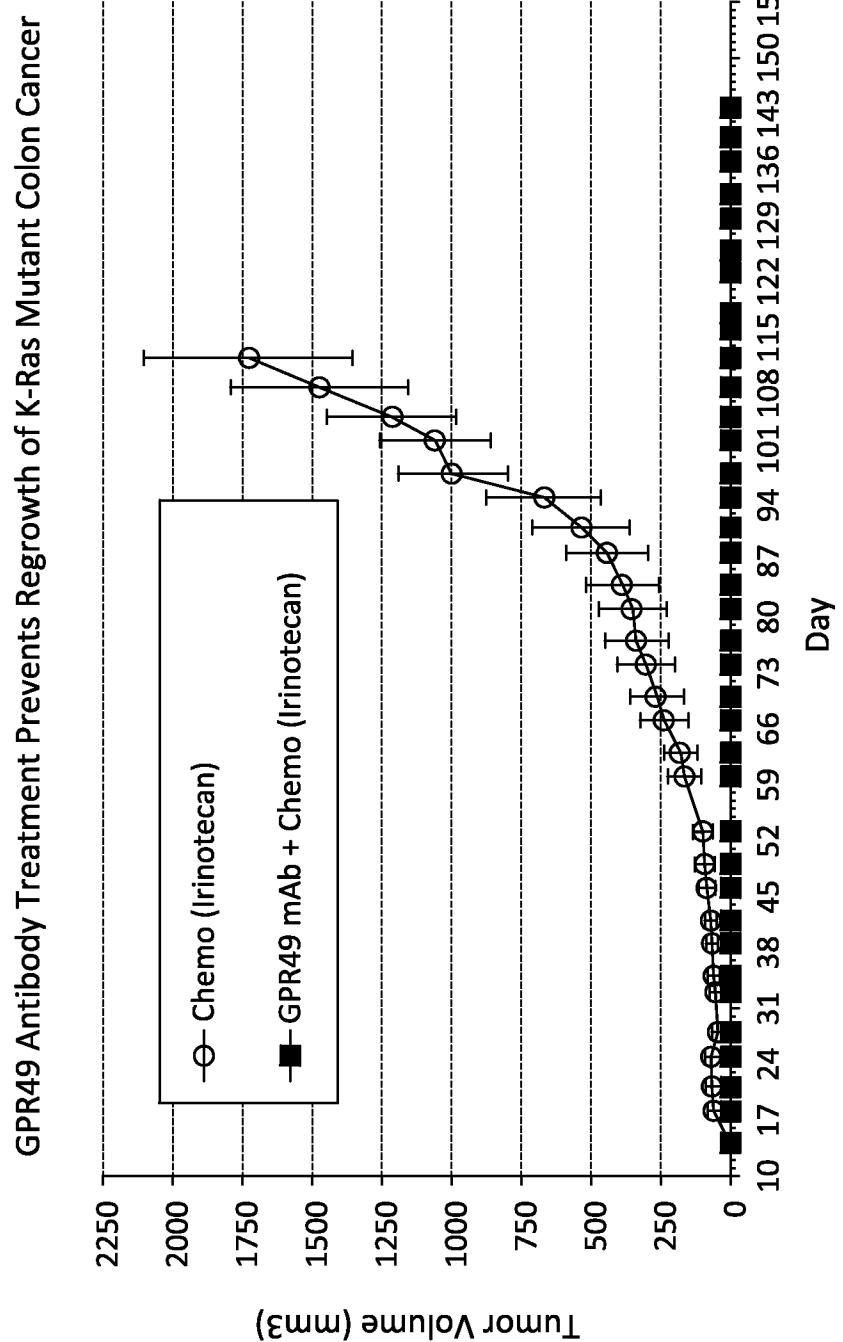


FIG. 23

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Val	Cys	Gl n	Lys	Leu	Gl n	Lys	Ile	Asp	Leu	Arg	His	Asn	Gl u	Ile	Tyr	
		375					380						385			
gaa	att	aaa	gtt	gac	act	ttc	cag	cag	ttg	ctt	agc	ctc	cga	tcg	ctg	1257
Gl u	Ile	Lys	Val	Asp	Thr	Phe	Gl n	Gl n	Leu	Leu	Ser	Leu	Arg	Ser	Leu	
		390			395						400					
aat	ttg	gct	tgg	aac	aaa	att	gct	att	att	cac	ccc	aat	gca	ttt	tcc	1305
Asn	Leu	Al a	Trp	Asn	Lys	Ile	Al a	Ile	Ile	His	Pro	Asn	Al a	Phe	Ser	
		405			410					415						
act	ttg	cca	tcc	cta	ata	aag	ctg	gac	cta	tcg	tcc	aac	ctc	ctg	tcg	1353
Thr	Leu	Pro	Ser	Leu	Ile	Lys	Leu	Asp	Leu	Ser	Ser	Asn	Leu	Leu	Ser	
		420			425				430					435		
tct	ttt	cct	ata	act	ggg	tta	cat	ggt	tta	act	cac	tta	aaa	tta	aca	1401
Ser	Phe	Pro	Ile	Thr	Gly	Leu	His	Gly	Leu	Thr	His	Leu	Lys	Leu	Thr	
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gga aat cat gcc tta cag agc ttg ata tca tct gaa aac ttt cca gaa	1449
Gly Asn His Ala Leu Glu Ser Leu Ile Ser Ser Glu Asn Phe Pro Glu	
455 460 465	
ctc aag gtt ata gaa atg cct tat gct tac cag tgc tgt gca ttt gga	1497
Leu Lys Val Ile Glu Met Pro Tyr Ala Tyr Glu Cys Cys Ala Phe Glu	
470 475 480	
gtg tgt gag aat gcc tat aag att tct aat caa tgg aat aaa ggt gac	1545
Val Cys Glu Asn Ala Tyr Lys Ile Ser Asn Glu Trp Asn Lys Glu Asp	
485 490 495	
aac agc agt atg gac gac ctt cat aag aaa gat gct gga atg ttt cag	1593
Asn Ser Ser Met Asp Asp Leu His Lys Lys Asp Ala Glu Met Phe Glu	
500 505 510 515	
gct caa gat gaa cgt gac ctt gaa gat ttc ctg ctt gac ttt gag gaa	1641
Ala Glu Asp Glu Arg Asp Leu Glu Asp Phe Leu Leu Asp Phe Glu Glu	
520 525 530	
gac ctg aaa gcc ctt cat tca gtg cag tgt tca cct tcc cca ggc ccc	1689
Asp Leu Lys Ala Leu His Ser Val Glu Cys Ser Pro Ser Pro Glu Pro	
535 540 545	
ttc aaa ccc tgt gaa cac ctg ctt gat ggc tgg ctg atc aga att gga	1737
Phe Lys Pro Cys Glu His Leu Leu Asp Glu Trp Leu Ile Arg Ile Glu	
550 555 560	
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Val Trp Thr Ile Ala Val Leu Ala Leu Thr Cys Asn Ala Leu Val Thr	
565 570 575	
tca aca gtt ttc aga tcc cct ctg tac att tcc ccc att aaa ctg tta	1833
Ser Thr Val Phe Arg Ser Pro Leu Tyr Ile Ser Pro Ile Lys Leu Leu	
580 585 590 595	
att ggg gtc atc gca gca gtg aac atg ctc acg gga gtc tcc agt gcc	1881
Ile Glu Val Ile Ala Ala Val Asn Met Leu Thr Glu Val Ser Ser Ala	
600 605 610	
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Val Leu Ala Glu Val Asp Ala Phe Thr Phe Glu Ser Phe Ala Arg His	
615 620 625	
ggt gcc tgg tgg gag aat ggg gtt ggt tgc cat gtc att ggt ttt ttg	1977
Gly Ala Trp Trp Glu Asn Glu Val Glu Cys His Val Ile Glu Phe Leu	
630 635 640	
tcc att ttt gct tca gaa tca tct gtt ttc ctg ctt act ctg gca gcc	2025
Ser Ile Phe Ala Ser Glu Ser Val Phe Leu Leu Thr Leu Ala Ala	
645 650 655	
ctg gag cgt ggg ttc tct gtg aaa tat tct gca aaa ttt gaa acg aaa	2073
Leu Glu Arg Glu Phe Ser Val Lys Tyr Ser Ala Lys Phe Glu Thr Lys	
660 665 670 675	
gct cca ttt tct agc ctg aaa gta atc att ttg ctc tgt gcc ctg ctg	2121
Ala Pro Phe Ser Ser Leu Lys Val Ile Ile Leu Leu Cys Ala Leu Leu	
680 685 690	
gcc ttg acc atg gcc gca gtt ccc ctg ctg ggt ggc agc aag tat ggc	2169
Ala Leu Thr Met Ala Ala Val Pro Leu Leu Glu Glu Ser Lys Tyr Glu	
695 700 705	
gcc tcc cct ctc tgc ctg cct ttg cct ttt ggg gag ccc agc acc atg	2217
Ala Ser Pro Leu Cys Leu Pro Leu Pro Phe Glu Glu Pro Ser Thr Met	
710 715 720	

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ggc tac atg gtc gct ctc atc ttg ctc aat tcc ctt tgc ttc ctc atg	2265
Gly Tyr Met Val Ala Leu Ile Leu Leu Asn Ser Leu Cys Phe Leu Met	
725 730 735	
atg acc att gcc tac acc aag ctc tac tgc aat ttg gac aag gga gac	2313
Met Thr Ile Ala Tyr Thr Lys Leu Tyr Cys Asn Leu Asp Lys Gly Asp	
740 745 750 755	
ctg gag aat att tgg gac tgc tct atg gta aaa cac att gcc ctg ttg	2361
Leu Glu Asn Ile Trp Asp Cys Ser Met Val Lys His Ile Ala Leu Leu	
760 765 770	
ctc ttc acc aac tgc atc cta aac tgc cct gtg gct ttc ttg tcc ttc	2409
Leu Phe Thr Asn Cys Ile Leu Asn Cys Pro Val Ala Phe Leu Ser Phe	
775 780 785	
tcc tct tta ata aac ctt aca ttt atc agt cct gaa gta att aag ttt	2457
Ser Ser Leu Ile Asn Leu Thr Phe Ile Ser Pro Glu Val Ile Lys Phe	
790 795 800	
atc ctt ctg gtg gta gtc cca ctt cct gca tgt ctc aat ccc ctt ctc	2505
Ile Leu Leu Val Val Pro Leu Pro Ala Cys Leu Asn Pro Leu Leu	
805 810 815	
tac atc ttg ttc aat cct cac ttt aag gag gat ctg gtg agc ctg aga	2553
Tyr Ile Leu Phe Asn Pro His Phe Lys Glu Asp Leu Val Ser Leu Arg	
820 825 830 835	
aag caa acc tac gtc tgg aca aga tca aaa cac cca agc ttg atg tca	2601
Lys Glu Thr Tyr Val Trp Thr Arg Ser Lys His Pro Ser Leu Met Ser	
840 845 850	
att aac tct gat gat gtc gaa aaa cag tcc tgt gac tca act caa gcc	2649
Ile Asn Ser Asp Asp Val Glu Lys Glu Ser Cys Asp Ser Thr Glu Ala	
855 860 865	
ttg gta acc ttt acc agc tcc agc atc act tat gac ctg cct ccc agt	2697
Leu Val Thr Phe Thr Ser Ser Ile Thr Tyr Asp Leu Pro Pro Ser	
870 875 880	
tcc gtg cca tca cca gct tat cca gtg act gag agc tgc cat ctt tcc	2745
Ser Val Pro Ser Pro Ala Tyr Pro Val Thr Glu Ser Cys His Leu Ser	
885 890 895	
tct gtg gca ttt gtc cca tgt ctc taa ttaatatgtg aaggaaaatg	2792
Ser Val Ala Phe Val Pro Cys Leu *	
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 Gly Cys Pro Thr His Cys His Cys Glu Pro Asp Gly Arg Met Leu Leu
 35 40 45
 Arg Val Asp Cys Ser Asp Leu Gly Leu Ser Glu Leu Pro Ser Asn Leu

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Leu	Leu	Pro	Asn	Pro	Leu	Pro	Ser	Leu	Arg	Phe	Leu	Glu	Glu	Leu	Arg
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Leu	Ala	Gly	Asn	Ala	Leu	Thr	Tyr	Ile	Pro	Lys	Gly	Ala	Phe	Thr	Gly
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Leu	Tyr	Ser	Leu	Lys	Val	Leu	Met	Leu	Gln	Asn	Asn	Gln	Leu	Arg	His
							115	120				125			
Val	Pro	Thr	Glu	Ala	Leu	Gln	Asn	Leu	Arg	Ser	Leu	Gln	Ser	Leu	Arg
						130	135			140					
Leu	Asp	Ala	Asn	His	Ile	Ser	Tyr	Val	Pro	Pro	Ser	Cys	Phe	Ser	Gly
					145	150			155				160		
Leu	His	Ser	Leu	Arg	His	Leu	Trp	Leu	Asp	Asp	Asn	Ala	Leu	Thr	Glu
						165			170				175		
Ile	Pro	Val	Gln	Ala	Phe	Arg	Ser	Leu	Ser	Ala	Leu	Gln	Ala	Met	Thr
					180			185					190		
Leu	Ala	Leu	Asn	Lys	Ile	His	His	Ile	Pro	Asp	Tyr	Ala	Phe	Gly	Asn
					195			200				205			
Leu	Ser	Ser	Leu	Val	Val	Leu	His	Leu	His	Asn	Asn	Arg	Ile	His	Ser
					210			215			220				
Leu	Gly	Lys	Lys	Cys	Phe	Asp	Gly	Leu	His	Ser	Leu	Glu	Thr	Leu	Asp
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Leu	Asn	Tyr	Asn	Asn	Leu	Asp	Glu	Phe	Pro	Thr	Ala	Ile	Arg	Thr	Leu
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Ser	Asn	Leu	Lys	Glu	Leu	Gly	Phe	His	Ser	Asn	Asn	Ile	Arg	Ser	Ile
					260			265				270			
Pro	Glu	Lys	Ala	Phe	Val	Gly	Asn	Pro	Ser	Leu	Ile	Thr	Ile	His	Phe
					275			280			285				
Tyr	Asp	Asn	Pro	Ile	Gln	Phe	Val	Gly	Arg	Ser	Ala	Phe	Gln	His	Leu
					290			295			300				
Pro	Glu	Leu	Arg	Thr	Leu	Thr	Leu	Asn	Gly	Ala	Ser	Gln	Ile	Thr	Glu
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Phe	Pro	Asp	Leu	Thr	Gly	Thr	Ala	Asn	Leu	Glu	Ser	Leu	Thr	Leu	Thr
					325			330				335			
Gly	Ala	Gln	Ile	Ser	Ser	Leu	Pro	Gln	Thr	Val	Cys	Asn	Gln	Leu	Pro
					340			345				350			
Asn	Leu	Gln	Val	Leu	Asp	Leu	Ser	Tyr	Asn	Leu	Leu	Glu	Asp	Leu	Pro
					355			360			365				
Ser	Phe	Ser	Val	Cys	Gln	Lys	Leu	Gln	Lys	Ile	Asp	Leu	Arg	His	Asn
					370			375			380				
Glu	Ile	Tyr	Glu	Ile	Lys	Val	Asp	Thr	Phe	Gln	Gln	Leu	Leu	Ser	Leu
					385			390			395			400	
Arg	Ser	Leu	Asn	Leu	Ala	Trp	Asn	Lys	Ile	Ala	Ile	Ile	His	Pro	Asn
						405			410				415		
Ala	Phe	Ser	Thr	Leu	Pro	Ser	Leu	Ile	Lys	Leu	Asp	Leu	Ser	Ser	Asn
					420			425				430			
Leu	Leu	Ser	Ser	Phe	Pro	Ile	Thr	Gly	Leu	His	Gly	Leu	Thr	His	Leu
						435			440			445			
Lys	Leu	Thr	Gly	Asn	His	Ala	Leu	Gln	Ser	Leu	Ile	Ser	Ser	Glu	Asn
						450			455			460			
Phe	Pro	Glu	Leu	Lys	Val	Ile	Glu	Met	Pro	Tyr	Ala	Tyr	Gln	Cys	Cys
						465			470			475			480
Ala	Phe	Gly	Val	Cys	Glu	Asn	Ala	Tyr	Lys	Ile	Ser	Asn	Gln	Trp	Asn
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Lys	Gly	Asp	Asn	Ser	Ser	Met	Asp	Asp	Leu	His	Lys	Lys	Asp	Ala	Gly
						500			505				510		
Met	Phe	Gln	Ala	Gln	Asp	Glu	Arg	Asp	Leu	Glu	Asp	Phe	Leu	Leu	Asp
						515			520			525			
Phe	Glu	Glu	Asp	Leu	Lys	Ala	Leu	His	Ser	Val	Gln	Cys	Ser	Pro	Ser
						530			535			540			
Pro	Gly	Pro	Phe	Lys	Pro	Cys	Glu	His	Leu	Leu	Asp	Gly	Trp	Leu	Ile
						545			550			555			560
Arg	Ile	Gly	Val	Asp	Lys	Thr	His	Thr	Cys	Pro	Pro	Cys	Pro	Ala	Pro
						565			570				575		
Glu	Leu	Leu	Gly	Gly	Pro	Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys
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Asp	Thr	Leu	Met	Ile	Ser	Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val

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		595		600		605									
Asp	Val	Ser	His	Glu	Asp	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp
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Gly	Val	Glu	Val	His	Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gl n	Tyr
625				630					635						640
Asn	Ser	Thr	Tyr	Arg	Val	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gl n	Asp
				645					650						655
Trp	Leu	Asn	Gly	Lys	Glu	Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu
				660					665						670
Pro	Ala	Pro	Ile	Glu	Lys	Thr	Ile	Ser	Lys	Ala	Lys	Gly	Gl n	Pro	Arg
				675					680						685
Glu	Pro	Gl n	Val	Tyr	Thr	Leu	Pro	Pro	Ser	Arg	Asp	Glu	Leu	Thr	Lys
				690					695						700
Asn	Gl n	Val	Ser	Leu	Thr	Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp
				705					710						720
Ile	Ala	Val	Glu	Trp	Glu	Ser	Asn	Gly	Gl n	Pro	Glu	Asn	Asn	Tyr	Lys
				725					730						735
Thr	Thr	Pro	Pro	Val	Leu	Asp	Ser	Asp	Gly	Ser	Phe	Phe	Leu	Tyr	Ser
				740					745						750
Lys	Leu	Thr	Val	Asp	Lys	Ser	Arg	Trp	Gl n	Gl n	Gly	Asn	Val	Phe	Ser
				755					760						765
Cys	Ser	Val	Met	His	Glu	Ala	Leu	His	Asn	His	Tyr	Thr	Gl n	Lys	Ser
				770					775						780
Leu	Ser	Leu	Ser	Pro	Gly	Lys	Gly	Gly	Ser	Asp	Tyr	Lys	Asp	Asp	Asp
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				20				25						30	
Gly	Cys	Pro	Thr	His	Cys	His	Cys	Glu	Pro	Asp	Gly	Arg	Met	Leu	Leu
				35				40						45	
Arg	Val	Asp	Cys	Ser	Asp	Leu	Gly	Leu	Ser	Glu	Leu	Pro	Ser	Asn	Leu
				50				55						60	
Ser	Val	Phe	Thr	Ser	Tyr	Leu	Asp	Leu	Ser	Met	Asn	Asn	Ile	Ser	Gl n
				65				70						75	
Leu	Leu	Pro	Asn	Pro	Leu	Pro	Ser	Leu	Arg	Phe	Leu	Glu	Gl u	Leu	Arg
				85				90						95	
Leu	Ala	Gly	Asn	Ala	Leu	Thr	Tyr	Ile	Pro	Lys	Gly	Ala	Phe	Thr	Gly
				100				105						110	
Leu	Tyr	Ser	Leu	Lys	Val	Leu	Met	Leu	Gl n	Asn	Asn	Gl n	Leu	Arg	His
				115				120						125	
Val	Pro	Thr	Glu	Ala	Leu	Gl n	Asn	Leu	Arg	Ser	Leu	Gl n	Ser	Leu	Arg
				130				135						140	
Leu	Asp	Ala	Asn	His	Ile	Ser	Tyr	Val	Pro	Pro	Ser	Cys	Phe	Ser	Gly
				145				150						160	
Leu	His	Ser	Leu	Arg	His	Leu	Trp	Leu	Asp	Asp	Asn	Ala	Leu	Thr	Gl u
				165				170						175	
Ile	Pro	Val	Gl n	Ala	Phe	Arg	Ser	Leu	Ser	Ala	Leu	Gl n	Ala	Met	Thr
				180				185						190	
Leu	Ala	Leu	Asn	Lys	Ile	His	Ile	Pro	Asp	Tyr	Ala	Phe	Gly	Asn	
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Leu	Ser	Ser	Leu	Val	Val	Leu	His	Leu	His	Asn	Asn	Arg	Ile	His	Ser
				210				215						220	
Leu	Gly	Lys	Lys	Cys	Phe	Asp	Gly	Leu	His	Ser	Leu	Glu	Thr	Leu	Asp
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Ser Asn Leu Lys Glu Leu Gly Phe His Ser Asn Asn Ile Arg Ser Ile
260 265 270
Pro Glu Lys Ala Phe Val Gly Asn Pro Ser Leu Ile Thr Ile His Phe
275 280 285
Tyr Asp Asn Pro Ile Gln Phe Val Gly Arg Ser Ala Phe Gln His Leu
290 295 300
Pro Glu Leu Arg Thr Leu Thr Leu Asn Gly Ala Ser Gln Ile Thr Glu
305 310 315 320
Phe Pro Asp Leu Thr Gly Thr Ala Asn Leu Glu Ser Leu Thr Leu Thr
325 330 335
Gly Ala Gln Ile Ser Ser Leu Pro Gln Thr Val Cys Asn Gln Leu Pro
340 345 350
Asn Leu Gln Val Leu Asp Leu Ser Tyr Asn Leu Leu Glu Asp Leu Pro
355 360 365
Ser Phe Ser Val Cys Gln Lys Leu Gln Lys Ile Asp Leu Arg His Asn
370 375 380
Glu Ile Tyr Glu Ile Lys Val Asp Thr Phe Gln Gln Leu Leu Ser Leu
385 390 395 400
Arg Ser Leu Asn Leu Ala Trp Asn Lys Ile Ala Ile Ile His Pro Asn
405 410 415
Ala Phe Ser Thr Leu Pro Ser Leu Ile Lys Leu Asp Leu Ser Ser Asn
420 425 430
Leu Leu Ser Ser Phe Pro Ile Thr Gly Leu His Gln Leu Thr His Leu
435 440 445
Lys Leu Thr Gly Asn His Ala Leu Gln Ser Leu Ile Ser Ser Glu Asn
450 455 460
Phe Pro Glu Leu Lys Val Ile Glu Met Pro Tyr Ala Tyr Gln Cys Cys
465 470 475 480
Ala Phe Gly Val Cys Glu Asn Ala Tyr Lys Ile Ser Asn Gln Trp Asn
485 490 495
Lys Gly Asp Asn Ser Ser Met Asp Asp Leu His Lys Lys Asp Ala Gly
500 505 510
Met Phe Gln Ala Gln Asp Glu Arg Asp Leu Glu Asp Phe Leu Leu Asp
515 520 525
Phe Glu Glu Asp Leu Lys Ala Leu His Ser Val Gln Cys Ser Pro Ser
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Arg Ile Gly Gly His His His His His
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Leu Pro Gly Ala Arg Cys
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Val Leu Ser