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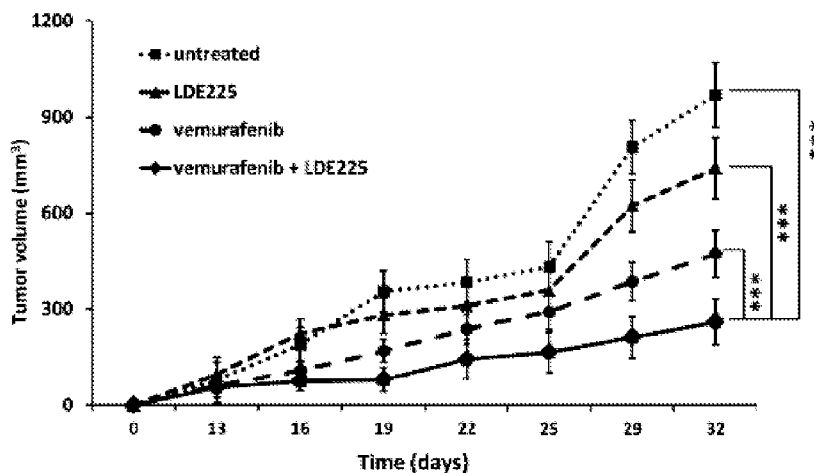
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FIG. 6D

(57) Abstract: Methods for treating cancer using a combination of an inhibitor of the sonic hedgehog signaling pathway (e.g., LDE225) with radiation and a tumor antigen-specific monoclonal antibody (e.g., heat shock protein (HSP) glucose regulated protein of 94000 daltons (Grp94)-specific mAb W9, or chondroitin sulfate proteoglycan 4 (CSPG4)-targeted mAbs), or with a BRAF inhibitor, e.g., in BRAF inhibitor resistant cancers.

COMBINATION TREATMENTS WITH SONIC HEDGEHOG INHIBITORS

CLAIM OF PRIORITY

This application claims the benefit of U.S. Provisional Patent Application Serial No. 61/808,517, filed on April 4, 2013. The entire contents of the foregoing are hereby incorporated by reference.

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with Government support under Grant Nos. RO1CA138188 and RO1CA110249 awarded by the National Cancer Institutes of the National Institutes of Health. The Government has certain rights in the invention.

TECHNICAL FIELD

The present application relates to methods for treating cancer using a combination of an inhibitor of the sonic hedgehog signaling pathway (e.g., LDE225) with a tumor antigen-specific monoclonal antibody (e.g., heat shock protein (HSP) glucose regulated protein of 94000 daltons (Grp94)-specific mAb W9, or chondroitin sulfate proteoglycan 4 (CSPG4)-targeted mAbs), or with a BRAF inhibitor, e.g., in BRAF inhibitor resistant cancers.

BACKGROUND

Combination therapies can enhance the effectiveness of certain treatments, boosting therapeutic efficacy of drugs that, alone, are not clinically useful, or providing new treatment modalities in drug resistant tumors.

SUMMARY

The present invention is based, at least in part, on the discovery that combining Sonic Hedgehog pathway inhibitors, e.g., LDE225, with one or more other treatments such as radiation, tumor-antigen specific monoclonal antibodies, PDGFR α inhibitors, and/or BRAF inhibitors, (BRAF-I) can synergistically increase treatment efficacy.

Thus, in a first aspect, the invention provides methods for treating BRAF inhibitor (BRAF-I)-resistant cancers, e.g., melanoma, sarcoma, or carcinoma, e.g., colon cancer, thyroid cancer, pancreatic adenocarcinoma, triple negative breast cancer,

glioma, sarcoma, or melanoma, comprising administering a therapeutically effective amount of a BRAF-I and a Sonic Hedgehog Inhibitor (Shh-I). In some embodiments, the BRAF-I is selected from the group consisting of Vemurafenib, GDC-0879, PLX-4720, GSK2118436, Sorafenib Tosylate, dabrafenib, and LGX818.

5 In some embodiments of the methods described herein, instead of or in addition to the Shh-I, a PDGFR α inhibitor is used, e.g., Crenolanib (CP-868596); Ki8751; Ponatinib (AP24534); Nintedanib (BIBF 1120); Dovitinib (TKI-258, CHIR-258); Masitinib (AB1010); Imatinib (STI571); Pazopanib; or KRN 633.

10 In some embodiments of the methods described herein, the Shh-I is selected from the group consisting of sulforaphane; vismodegib; TAK-441; itraconazole; and erismodegib.

15 In another aspect, the invention provides methods for treating cancer, e.g., carcinoma, sarcoma, or melanoma, e.g., triple negative breast cancer or pancreatic ductal adenocarcinoma, comprising administering a therapeutically effective amount of an antibody that binds to a tumor antigen, and a Sonic Hedgehog Inhibitor (Shh-I). In some embodiments, the tumor antigen is Grp94 or chondroitin sulfate proteoglycan 4 (CSPG4).

In some embodiments, the cancer is a recurrent cancer, i.e., has recurred after treatment.

20 In some embodiments of the methods described herein, the Shh-I is selected from the group consisting of sulforaphane; vismodegib; TAK-441; itraconazole; and erismodegib.

25 In some embodiments of the methods described herein, instead of or in addition to the Shh-I, a PDGFR α inhibitor is used, e.g., Crenolanib (CP-868596); Ki8751; Ponatinib (AP24534); Nintedanib (BIBF 1120); Dovitinib (TKI-258, CHIR-258); Masitinib (AB1010); Imatinib (STI571); Pazopanib; or KRN 633.

30 Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Methods and materials are described herein for use in the present invention; other, suitable methods and materials known in the art can also be used. The materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, sequences, database entries, and other references mentioned herein are incorporated by reference in their

entirety. In case of conflict, the present specification, including definitions, will control.

Other features and advantages of the invention will be apparent from the following detailed description and figures, and from the claims.

DESCRIPTION OF DRAWINGS

Figure 1. Association of vemurafenib resistance with ERK reactivation, AKT activation and PDGFR α upregulation in BRAF(V600E) melanoma cells.

Colo38, Colo38R, M21, M21R and TPF-10-741 cells were treated with vemurafenib (1 μ M). **A.** Following an incubation at indicated time points at 37°C cells were harvested and lysed. Cell lysates were analyzed by western blot with the indicated mAbs. β -actin- was used as a loading control. **B.** Following a 48 h incubation at 37°C cells were harvested and lysed. Cell lysates were analyzed by western blot with the indicated mAbs. β -actin or Calnexin were used as a loading control. The results presented are representative of the results obtained in three independent experiments.

Figure 2. Restoration by PDGFR α downregulation of vemurafenib sensitivity of BRAF(V600E) melanoma cells with acquired BRAF-I resistance.

A. M21R and TPF-10-741 cells were transduced with PDGFR α -specific shRNA (#4) or with GFP-shRNA, used as a control, lentiviral particles. Transduced cells were treated with vemurafenib (1 μ M). Following a three day incubation at 37°C cells were harvested and lysed. Cell lysates were analyzed by western blot with the indicated mAbs. β -actin was used as a loading control. The results presented are representative of the results obtained in three independent experiments. **B.** PDGFR α -specific shRNA (#4) transduced M21R and TPF-10-741 cells were treated with the indicated vemurafenib concentrations. GFP-shRNA transduced M21R and TPF-10-741 cells were used as controls. Cell proliferation was determined by MTT assay following a three day incubation at 37°C. Percentage of cell proliferation was calculated as the ratio of treated cells to untreated GFP-shRNA transduced cells. Data are expressed as mean $\pm$ SD of the results obtained in three independent experiments. *** indicates $P < 0.01$.

Figure 3. PDGFR α expression in melanoma tumors obtained from patients who acquired BRAF-I resistance.

Tumor biopsies of melanoma removed from patients were performed pre-treatment (day 0), at 10-14 days on treatment, and/or at the time of disease progression following treatment with BRAF-I or with BRAF-I and MEK-I. Frozen sections were fixed with 4% PFA and stained with H&E and PDGFR α -specific rabbit antibody. Scores were recorded semiquantitatively as 1+, 2+, 3+ and 4+, when, 1–25%, 26–50%, 51–75% and >75% of cells were stained, respectively. Patients were divided in two groups based on change of PDGFR α expression at IHC after treatment: those whose PDGFR α staining score had no or 1 point increase after treatment ($\leq 1+$) and those for whose PDGFR α staining score increased 2 or more points after treatment ($>1+$). **A.** Representative IHC staining and immunofluorescent staining of PDGFR α expression in melanoma patients before treatment, on treatment and at the time of disease progression in 5 out of 9 tumor biopsies. Magnification is indicated. **B.** Two groups of patients were graphed based upon RECIST criteria and compared as a percent of the total population of the PDGFR α stain score group. **C.** Two groups of patients were graphed based upon the time to disease progression utilizing Kaplan–Meier methods.

Figure 4. Enhancement by PDGFR α inhibition of the signaling pathway inhibition by vemurafenib in melanoma cells harboring BRAF(V600E).

Enhancement by PDGFR α inhibition of the signaling pathway inhibition by vemurafenib in melanoma cells harboring BRAF(V600E). Colo38, Colo38R, M21, M21R and TPF-10-741 cells were treated with vemurafenib (1 μ M) and/or sunitinib (3 μ M) and/or imatinib (20 μ M) and/or crenolanib (1 μ M). Following an incubation at indicated time points at 37°C cells were harvested and lysed. Cell lysates were analyzed by western blot with the indicated mAbs. β -actin was used as a loading control. The results presented are representative of the results obtained in three independent experiments.

Figure 5. Enhancement by PDGFR α inhibition of the growth inhibition, signaling pathway inhibition and apoptosis induction by vemurafenib of BRAF(V600E) melanoma cells grafted in immunodeficient mice.

M21 and M21R cells were implanted subcutaneously in 20 SCID mice. When tumors became palpable, mice were randomly divided into 4 groups (5 mice/group). One group was treated with vemurafenib (12.5 mg/kg, twice daily), one with sunitinib

(20 mg/kg, once each day) and one with the vemurafenib (12.5 mg/kg, twice daily) in combination with sunitinib (20 mg/kg, once each day). One group of mice was left untreated as a reference for the natural course of the disease. Efficacy data are plotted as mean tumor volume (in mm³) $\pm$ SD. The asterisk (***) indicates $P < 0.001$ (**A, E**).

5 Tumors harvested from untreated and treated mice (three for each group) were lysed and analyzed for expression and activation of the indicated signaling pathways components. β -actin was used as a loading control (**B, F**). Tumor tissue sections were analyzed for the content of mitotic cells by staining with p-Histone H3 (Ser10) protein-specific antibodies. Mitotic tumor cells were quantified by counting 5
10 randomly selected high-power fields per section (magnification $\times 200$) (**C, G**). Tumor tissue sections were analyzed for the content of apoptotic cells by staining with Cleaved Caspase-3 (Asp175)-specific antibodies. Apoptotic tumor cells were quantified by counting 5 randomly selected high-power fields per section (magnification $\times 200$) (**D, H**). Data are presented as means $\pm$ SD. *** indicates $P <$
15 0.001.

Figure 6. Association of PDGFR α upregulation mediating vemurafenib resistance with Gli1 activation in melanoma cells harboring BRAF(V600E).

A. Colo38, Colo38R, M21, M21R and TPF-10-741 cells were treated with vemurafenib (1 μ M). Following an incubation at indicated time points at 37°C cells
20 were harvested and lysed. Cell lysates were analyzed by western blot with the indicated mAbs. β -actin was used as a loading control. **B.** M21 and M21R cells were treated with vemurafenib (1 μ M) and/or LDE225 (10 μ M). Cell growth inhibition was determined by MTT assay following a three day incubation at 37°C. Percentage of cell growth inhibition was calculated as the ratio of treated to untreated cells. Data are
25 expressed as the mean $\pm$ SD of the results obtained in three independent experiments. The asterisk (*) indicates $P < 0.05$. **C.** Colo38, Colo38R, M21 and M21R cells were treated with vemurafenib (1 μ M) and/or LDE225 (10 μ M). Following a 48h incubation at 37°C cells were harvested and lysed. Cell lysates were analyzed by western blot with the indicated mAbs. β -actin was used as a loading control. **D.** M21 cells were
30 implanted subcutaneously in 20 SCID mice. When tumors became palpable, mice were randomly divided into 4 groups (5 mice/group). One group was treated with the BRAF-I vemurafenib (12.5 mg/kg/twice per day), one with LDE225 (40 mg/kg/day) and one with vemurafenib (12.5 mg/kg/twice per day) in combination with LDE225 (40 mg/kg/day). One group of mice was left untreated as a reference for the natural

course of the disease. Efficacy data are plotted as mean tumor volume (in mm³) ± SD. The asterisks (***) indicate $P < 0.001$.

Figure 7. BRAF(V600E) melanoma cell lines, autologous cells made resistant to BRAF-I by continuous exposure to increasing doses of vemurafenib and a melanoma cell line isolated from a patient who acquired BRAF-I resistance.

M21, M21R and TPF-10-741 cells were treated with the indicated concentrations of vemurafenib. Cell growth inhibition was determined by MTT assay following a five day incubation at 37°C. Percentage of cell growth inhibition was calculated as the ratio of treated to untreated cells at each vemurafenib dose. Data are expressed as the mean ± SD of the results obtained in three independent experiments.

Figure 8. Dose dependent effect of sunitinib on the *in vitro* proliferation of BRAF-I sensitive and resistant melanoma cells harboring BRAF(V600E).

Colo38, Colo38R, M21 and M21R cells were treated with the indicated concentrations of sunitinib. Cell growth inhibition was determined by MTT assay following a five day incubation at 37°C. Percentage of cell growth inhibition was calculated as the ratio of treated to untreated cells at each sunitinib dose. Data are expressed as mean ± SD of the results obtained in three independent experiments.

Figure 9. Enhancement by PDGFR α inhibition with sunitinib or imatinib of the *in vitro* anti-proliferative activity of vemurafenib in BRAF-I sensitive and resistant melanoma cells harboring BRAF(V600E).

Colo38, Colo38R, M21, M21R and TPF-10-741 cells were treated with the indicated concentrations of vemurafenib and/or sunitinib or imatinib. Cell growth inhibition was determined by MTT assay following a three (A) and five (B) days of treatment. Percentage of cell growth inhibition was calculated as ratio of treated to untreated cells at each treatment. Data are expressed as mean ± SD of the results obtained in three independent experiments. The asterisk (*) indicates $P < 0.05$.

Figure 10. Enhancement by PDGFR α inhibition of apoptosis induction by vemurafenib in melanoma cells harboring BRAF(V600E).

M21 and M21R cells (A), Colo38 and Colo38R cells (B), and TPF-10-741 cells (C) were treated with vemurafenib (500 nM) and/or sunitinib (3 μ M). Following a 24 h incubation at 37°C cells were harvested and stained with Annexin V and PI. The data presented are representative of the staining obtained in three independent experiments (left panel). The levels of apoptosis are plotted and expressed as mean

fraction of apoptotic cells $\pm$ SD of the results obtained in three independent experiments (**right panel**). The asterisk (*) indicates $P < 0.05$.

Figure 11. Enhancement by PDGFR α inhibition of the growth inhibition by vemurafenib of BRAF(V600E) melanoma cells grafted in immunodeficient mice.

M21 cells were implanted subcutaneously in 20 SCID mice. When tumors became palpable, mice were randomly divided into 4 groups (5 mice/group). One group was treated with vemurafenib (25 mg/kg, twice daily), one with imatinib (100 mg/kg, daily) and one with the vemurafenib (25 mg/kg, twice daily) in combination with imatinib (100 mg/kg, daily). One group of mice was left untreated as a reference for the natural course of the disease. Efficacy data are plotted as mean tumor volume (in mm³) $\pm$ SD. The asterisk (***) indicates $P < 0.001$.

Figure 12. Inhibition by mAb W9, radiation and LDE225 combination of human pancreatic CIC *in vitro* proliferation. Human PDAC2 cells (4×10^5 /ml) were irradiated at the dose of 20Gy and incubated with mAb W9 (20 μ g/ml) and LDE225 (10 μ M) for 72 hrs at 37°C. Cells were then stained with ALDEFLUOR with or without the DEAB inhibitor to identify ALDH^{bright} cells. Non-irradiated cells and HIgG1 (20 μ g/ml) treated cells were used as controls. The percentage of CICs identified as ALDH^{bright} cells are plotted and expressed as mean $\pm$ SD of the results obtained in three independent experiments. Treatment with mAb W9 is enhanced by radiation and LDE225. Furthermore mAb W9, LDE225 and 20Gy combination decrease CIC % to a significantly ($P < 0.01$) greater extent than either of the single or double agents.

Figure 13. Enhancement by CSPG4-specific mAb 225.28 of the CIC growth inhibition induced by LDE225, an inhibitor of the SHH pathway, in the TNBC cell line MDA-MB-231. MDA-MB-231 cells (2.5×10^5 /well) were starved for 6 hrs at 37°C, then incubated with mAb 225.28 (0.25 mg/ml) and LDE225 (10 μ M) for 72 hrs at 37 °C in RPMI 1640 medium + 1.5% FCS. The isotype matched mAb F3-C25 (0.25 mg/ml) was used as a control. Cells were stained with ALDEFLUOR with or without the DEAB inhibitor to identify ALDH^{bright} cells. These results indicate that mAb 225.28 and LDE225 to suppress the growth of TNBC CICs as measured by the percentage of ALDH^{bright} cells.

DETAILED DESCRIPTION

The methods described herein include combination therapies including administration of a sonic hedgehog inhibitor, plus one or more of radiation and a tumor antigen-specific monoclonal antibody (e.g., Grp94-specific mAb W9, or CSPG4-targeted mAbs), and/or a PDGFRa inhibitor.

Methods of Treatment

The methods described herein include methods for the treatment of cancer. In some embodiments, the disorder is melanoma or sarcoma. Generally, the methods include administering a therapeutically effective amount of a sonic hedgehog inhibitor, as described herein, plus one or more of radiation and a tumor antigen-specific monoclonal antibody (e.g., Grp94-specific mAb W9, or CSPG4-targeted

mAbs), and/or a PDGFRa inhibitor, to a subject who is in need of, or who has been determined to be in need of, such treatment.

As used in this context, to “treat” means to ameliorate at least one symptom of the cancer. For example, administration of a therapeutically effective amount of a compound described herein for the treatment of a cancer will result in decreased tumor size, or reduced or delayed tumor growth rate.

As used herein, the terms “cancer”, “hyperproliferative” and “neoplastic” refer to cells having the capacity for autonomous growth, i.e., an abnormal state or condition characterized by rapidly proliferating cell growth. Hyperproliferative and neoplastic disease states may be categorized as pathologic, i.e., characterizing or constituting a disease state, or may be categorized as non-pathologic, i.e., a deviation from normal but not associated with a disease state. The term is meant to include all types of cancerous growths or oncogenic processes, metastatic tissues or malignantly transformed cells, tissues, or organs, irrespective of histopathologic type or stage of invasiveness. “Pathologic hyperproliferative” cells occur in disease states characterized by malignant tumor growth. Examples of non-pathologic hyperproliferative cells include proliferation of cells associated with wound repair.

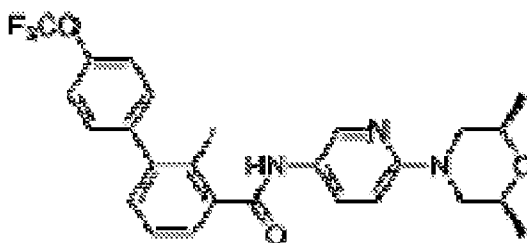
The terms “cancer” or “neoplasms” include malignancies of the various organ systems, such as affecting lung, breast, thyroid, lymphoid, gastrointestinal, and genito-urinary tract, as well as adenocarcinomas which include malignancies such as most colon cancers, renal-cell carcinoma, prostate cancer and/or testicular tumors, non-small cell carcinoma of the lung, cancer of the small intestine and cancer of the esophagus.

The term “carcinoma” is art recognized and refers to malignancies of epithelial or endocrine tissues including respiratory system carcinomas, gastrointestinal system carcinomas, genitourinary system carcinomas, testicular carcinomas, breast carcinomas, prostatic carcinomas, endocrine system carcinomas, and melanomas. In some embodiments, the disease is renal carcinoma or melanoma. Exemplary carcinomas include those forming from tissue of the cervix, lung, prostate, breast, head and neck, colon and ovary. The term also includes carcinosarcomas, e.g., which include malignant tumors composed of carcinomatous and sarcomatous tissues. An “adenocarcinoma” refers to a carcinoma derived from glandular tissue or in which the tumor cells form recognizable glandular structures.

The term “sarcoma” is art recognized and refers to malignant tumors of mesenchymal derivation.

Sonic Hedgehog (Shh) Pathway Inhibitors

A number of Shh inhibitors have been developed, including sulforaphane; vismodegib (GDC-0449; see Van Hoff et al., N Engl J Med 2009;361:1164-72); TAK-441 (Ohashi et al., Bioorg Med Chem. 2012 Sep 15;20(18):5507-17); itraconazole; and erismodegib (also known as LDE225 or NVP-LDE225). The structure of erismodegib is as follows:



In some embodiments, the Shh pathway inhibitor binds to and inhibits the activity of Smoothed homologue (SMO). See, e.g., Romer and Curran, Cancer Res June 15, 2005 65; 4975.

Antibodies against Tumor-Specific Antigens

In some embodiments, the methods described herein include administering a combination of an Shh-I plus one or more antibodies directed against a tumor-specific antigen.

The term “antibody” as used herein refers to an immunoglobulin molecule or an antigen-binding portion thereof. Examples of antigen-binding portions of immunoglobulin molecules include F(ab) and F(ab')₂ fragments, which retain the ability to bind antigen. The antibody can be polyclonal, monoclonal, recombinant, chimeric, de-immunized or humanized, fully human, non-human, (e.g., murine), or single chain antibody. In some embodiments the antibody has effector function and can fix complement. In some embodiments, the antibody has reduced or no ability to bind an Fc receptor. For example, the antibody can be an isotype or subtype, fragment or other mutant, which does not support binding to an Fc receptor, e.g., it has a mutagenized or deleted Fc receptor binding region. Methods for making antibodies and fragments thereof are known in the art, see, e.g., Harlow et. al., editors, Antibodies: A Laboratory Manual (1988); Goding, Monoclonal Antibodies: Principles and Practice, (N.Y. Academic Press 1983); Howard and Kaser, Making and Using

Antibodies: A Practical Handbook (CRC Press; 1st edition, Dec 13, 2006);
Kontermann and Dübel, Antibody Engineering Volume 1 (Springer Protocols)
(Springer; 2nd ed., May 21, 2010); Lo, Antibody Engineering: Methods and Protocols
(Methods in Molecular Biology) (Humana Press; Nov 10, 2010); and Dübel,
5 Handbook of Therapeutic Antibodies: Technologies, Emerging Developments and
Approved Therapeutics, (Wiley-VCH; 1 edition September 7, 2010).

Example of tumor antigens include Grp94 (e.g., targeted using mAb W9) and
CSPG4 (e.g., targeted using CSPG4 specific-mAb 225.28).

Grp94 is a heat shock protein located in the endoplasmic reticulum of all
10 mammalian cells. It is a chaperone essential for the conformational maturation of
proteins, and regulates activation of several signaling pathways (e.g., tumor cell
proliferation and survival). Like other members of the HSP90 family, the molecular
chaperone Grp94 is required for the stability and activity of client proteins involved in
the activation of signaling pathways associated with tumor cell survival and
15 proliferation. Overexpression of Grp94 is associated with cellular transformation and
tumorigenicity, and inhibition of Grp94 leads to depletion of oncogenic client proteins
by the ubiquitin proteasome pathway. Specific inhibitors of Hsp90 have recently
entered clinical trials, but application of Hsp90 inhibitors is experiencing some
difficulties, especially because of their toxicity.

20 MAb W9 has unique specificity for an extracellular epitope of the heat shock
protein (HSP) glucose regulated protein of 94000 daltons (Grp94). mAb W9
recognizes Grp94, since i) mass spectrometry analysis has identified two peptides
unique of Grp94 in the approximately 100 KDa protein immunoprecipitated by mAb
W9 from 5 human cancer cell lines; and ii) human melanoma cells transfected with
25 Grp94 shRNA selectively lost their reactivity with mAb W9. Unlike other Grp94-
specific mAbs described in the literature and/or available commercially, mAb W9 is
able to selectively target malignant cells by recognizing a carbohydrate epitope which
is expressed on malignant cells, but is not detectable on normal cells. See, e.g., US
2012/0148598 and US2012/0009194, both of which are incorporated by reference
30 herein.

CSPG4 is expressed in patient derived Triple-negative breast cancer (TNBC)
lesions. It is expressed at high levels not only on differentiated TNBC cells but also
on TNBC cancer initiating cells (CICs). In TNBC cell lines CICs have been shown to
express high level of Aldehyde Dehydrogenases A1 (ALDH^{bright}; Ginestier et al.,

Cell Stem Cell. 2007;1:555-567; Visus et al., Clinical cancer research : an official journal of the American Association for Cancer Research. 2011;17:6174-6184) in addition to being able to form mammospheres in vitro and to be tumorigenic in SCID mice. In addition, CSPG4 is involved in signaling pathways associated with TNBC cell proliferation, survival and migration.

A number of antibodies against cancer-related antigens are known; exemplary antibodies are described in Ross et al., Am J Clin Pathol 119(4):472-485, 2003), as well as in Tables A-B, below.

Table A. Anticancer Antibodies

Drug Name Generic (Brand)	Source (Partners)	Antibody Type	Target	Approved and Investigational Indications
Alemtuzumab (Campath)	BTG, West Conshohocken, PA (ILEX Oncology, Montville, NJ; Schering AG, Berlin, Germany)	Monoclonal antibody, humanized; anticancer, immunologic; multiple sclerosis treatment; immunosuppressant	CD52	Chronic lymphocytic and chronic myelogenous leukemia; multiple sclerosis, chronic progressive
Daclizumab (Zenapax)	Protein Design Labs, Fremont, CA (Hoffmann-La Roche, Nutley, NJ)	Monoclonal IgG1 chimeric; immunosuppressant; antipsoriatic; antidiabetic; ophthalmologic; multiple sclerosis treatment	IL-2 receptor, CD25	Transplant rejection, general and bone marrow; uveitis; multiple sclerosis, relapsing-remitting and chronic progressive; cancer, leukemia, general; psoriasis; diabetes mellitus, type 1; asthma; ulcerative colitis
Edrecolomab (Panorex)	GlaxoSmithKline, London, England	Monoclonal IgG2A murine; anticancer	Epithelial cell adhesion molecule	Cancer: colorectal
Gemtuzumab (Mylotarg)	Wyeth/AHP, Collegeville, PA	Monoclonal IgG4 humanized	CD33/cal- icheamycin	Acute myelogenous leukemia (patients older than 60 y)

Drug Name Generic (Brand)	Source (Partners)	Antibody Type	Target	Approved and Investigational Indications
Ibritumomab (Zevalin)	IDEC Pharmaceutica ls	Monoclonal IgG1 murine; anticancer	CD20/ yttrium 90	Low-grade lymphoma, follicular lymphoma, transformed non- Hodgkin lymphoma (relapsed or refractory)
Rituximab (Rituxan)	IDEC Pharmaceutica ls, San Diego, CA (Genentech, South San Francisco, CA; Hoffmann-La Roche; Zen- yaku Kogyo, Tokyo, Japan)	Monoclonal IgG1 chimeric; anticancer, immunologic; antiarthritic, immunologic; immunosuppressant	CD20	Non-Hodgkin lymphoma, B-cell lymphoma, chronic lymphocytic leukemia; rheumatoid arthritis; thrombocytopenic purpura
Trastuzumab (Herceptin)	Genentech (Hoffmann-La Roche; ImmunoGen, Cambridge, MA)	Monoclonal IgG1 humanized; anticancer, immunologic	p185neu	Cancer: breast, non-small cell of the lung, pancreas

Table B. Additional Anticancer Antibodies

Drug Name Generic (Brand)	Source	Features	Investigational Indications
2C4 antibody	Genentech	Chimeric monoclonal antibody; anticancer immunologic	Cancer: breast
ABX-EGF	Abgenix, Fremont, CA	Monoclonal antibody, human; anticancer immunologic	Cancer: renal, non- small cell of the lung, colorectal, prostate
ABX-MA1	Abgenix	Humanized monoclonal antibody; anticancer immunologic	Melanoma

Drug Name Generic (Brand)	Source	Features	Investigational Indications
ACA-125	CellControl Biomedical, Martinsried, Germany	Monoclonal antibody; anticancer immunologic	Cancer: ovarian
Anti-HMI.24	Chugai	Chimeric monoclonal antibody; anticancer immunologic	Myeloma
Anti-LCG MAbs	eXegenics, Dallas, TX	Monoclonal antibody; anticancer; imaging agent	Cancer: lung, general; diagnosis of cancer
Anti-PTHrP	Chugai	Chimeric monoclonal antibody; anticancer immunologic; osteoporosis	Hypercalcemia of malignancy; cancer, bone
Apolizumab	Protein Design Labs	Chimeric monoclonal antibody; anticancer immunologic	Non-Hodgkin lymphoma; chronic lymphocytic leukemia
AR54	AltaRex	Murine monoclonal antibody; anticancer immunologic	Cancer: ovarian
Bevacizumab (Avastin)	Genentech, South San Francisco, CA	Anti-VEGF humanized monoclonal antibody; anticancer immunologic; antidiabetic; ophthalmologic	Cancer: colorectal, breast, non-small cell of the lung; diabetic retinopathy
BrevaRex MAb	AltaRex	Murine monoclonal antibody; anticancer immunologic	Cancer: myeloma, breast
CDP-860	Celltech, Slough, England	Humanized monoclonal antibody; anticancer immunologic; cardiovascular	Cancer: general; restenosis
CeaVac	Titan Pharmaceuticals, South San Francisco, CA	Anti-CEA murine monoclonal antibody; anticancer immunologic vaccine	Cancer: colorectal, non-small cell of the lung, breast, liver
Cetuximab (C- 225; Erbitux)	ImClone Systems	Anti-EGFR chimeric monoclonal antibody; anticancer immunologic	Cancer: head and neck, non-small cell of the lung, colorectal, breast, pancreas, prostate

Drug Name Generic (Brand)	Source	Features	Investigational Indications
Edrecolomab	Johnson & Johnson, New Brunswick, NJ	Murine monoclonal antibody; anticancer immunologic	Cancer: colorectal and breast
EMD 72 000	Merck KGaA, Darmstadt, Germany	Chimeric monoclonal antibody; anticancer immunologic	Cancer: stomach, cervical, non-small cell of the lung, head and neck, ovarian
Epratuzumab (LymphoCide)	Immunomedics, Morris Plains, NJ	Chimeric monoclonal antibody; anticancer immunologic; immunosuppressant	Non-Hodgkin lymphoma
G-250, unconjugated	Johnson & Johnson	Chimeric monoclonal antibody; anticancer immunologic	Cancer: renal
GlioMab-H	Viventia Biotech	Humanized monoclonal antibody; imaging agent; anticancer immunologic	Diagnosis of cancer; cancer, brain
H-11 scFv	Viventia Biotech, Toronto, Canada	Humanized monoclonal antibody; anticancer immunologic	Non-Hodgkin lymphoma, melanoma
H22xKi-4	Medarex	Chimeric monoclonal antibody; anticancer immunologic	Hodgkin lymphoma
huJ591 MAb, BZL	Millennium Pharmaceuticals, Cambridge, MA; BZL Biologics, Framingham, MA	Chimeric monoclonal antibody; anticancer immunologic	Cancer: prostate and general
IGN-101	Igeneon, Vienna, Austria	Murine monoclonal antibody; anticancer immunologic	Cancer: non-small cell of the lung, liver, colorectal, esophageal, stomach
IMC-1C11	ImClone Systems	Chimeric monoclonal antibody; anticancer immunologic	Cancer: colorectal
Imuteran	Nonindustrial source	Monoclonal antibody; anticancer immunologic	Cancer: breast, ovarian

Drug Name Generic (Brand)	Source	Features	Investigational Indications
ING-1	Xoma, Berkeley, CA	Chimeric monoclonal antibody; anticancer immunologic	Cancer: breast, lung (general), ovarian, prostate
ior-t1	Center of Molecular Immunology, Havana, Cuba	Murine monoclonal antibody; anticancer immunologic; antipsoriatic; antiarthritic immunologic	T-cell lymphoma; psoriasis; rheumatoid arthritis
KSB-303	KS Biomedix, Guildford, England	Chimeric monoclonal antibody; anticancer immunologic	Diagnosis of cancer; cancer, colorectal
KW-2871	Kyowa Hakko, Tokyo, Japan	Chimeric monoclonal antibody; anticancer immunologic	Melanoma
Labetuzumab	Immunomedics	Chimeric monoclonal antibody; immunoconjugate; anticancer immunologic	Cancer: colorectal, breast, small cell of the lung, ovarian, pancreas, thyroid, liver
Lintuzumab (Zamyl)	Protein Design Labs, Fremont, CA	Chimeric monoclonal antibody; anticancer immunologic	Acute myelogenous leukemia; myelodysplastic syndrome
MAB, AME	Applied Molecular Evolution, San Diego, CA	Chimeric monoclonal antibody; anticancer immunologic; imaging agent; antiarthritic immunologic; ophthalmologic; cardiovascular	Cancer: sarcoma, colorectal; rheumatoid arthritis; psoriatic arthritis
MDX-010	Medarex	Humanized anti- HER-2 monoclonal antibody; anticancer immunologic; immunostimulant	Cancer: prostate, melanoma; infection, general
MDX-210	Medarex, Princeton, NJ; Immuno-Designed Molecules, Havana, Cuba	Bispecific chimeric monoclonal antibody; anti-HER- 2/neu-anti-Fc gamma RI; anticancer immunologic	Cancer: ovarian, prostate, colorectal, renal, breast

Drug Name Generic (Brand)	Source	Features	Investigational Indications
MDX-220	Immuno-Designed Molecules	Chimeric monoclonal antibody; anticancer immunologic	Cancer: prostate, colorectal
Mitumomab	ImClone Systems, New York, NY	Murine monoclonal antibody; anticancer immunologic	Small cell cancer of the lung; melanoma
Monopharm-C	Viventia Biotech	Monoclonal antibody; anticancer immunologic; imaging agent	Cancer: colorectal; diagnosis of cancer
MRA	Chugai Pharmaceutical, Tokyo, Japan	Chimeric monoclonal antibody; antiarthritic immunologic; anticancer immunologic; GI inflammatory and bowel disorders	Rheumatoid arthritis; cancer, myeloma; Crohn disease; Castleman disease
MT-103	Micromet	Murine monoclonal antibody; anticancer immunologic	B-cell lymphoma, non-Hodgkin lymphoma, chronic myelogenous leukemia, acute myelogenous leukemia
MT-201	Micromet, Munich, Germany	Humanized monoclonal antibody; anticancer immunologic	Cancer: prostate, colorectal, stomach, non-small cell of the lung
Onyvax-105	Onyvax, London, England	Monoclonal antibody; anticancer immunologic	Cancer: colorectal; sarcoma, general
Oregovomab	AltaRex, Waltham, MA	Monoclonal antibody, murine; anticancer immunologic; immunoconjugate	Cancer: ovarian
Prostate cancer antibody	Biovation, Aberdeen, Scotland	Monoclonal antibody; anticancer	Cancer: prostate
R3	Center of Molecular Immunology	Chimeric monoclonal antibody; anticancer immunologic; imaging agent; immunoconjugate	Cancer: head and neck; diagnosis of cancer

Drug Name Generic (Brand)	Source	Features	Investigational Indications
SGN-30	Seattle Genetics, Seattle, WA	Monoclonal antibody; anticancer immunologic; multiple sclerosis treatment; immunosuppressant; immunoconjugate	Hodgkin lymphoma
Therex	Antisoma, London, England	Chimeric monoclonal antibody; anticancer immunologic	Cancer: breast
Tositumomab (Bexxar)	Corixa, Seattle, WA	Anti-CD20 murine monoclonal antibody with iodine 131 conjugation	Non-Hodgkin lymphoma
TRAIL-RI MAb, CAT	Cambridge Antibody Technology, Cambridge, England	Humanized monoclonal antibody; anticancer immunologic	Cancer: general
TriAb	Titan Pharmaceuticals	Murine monoclonal antibody; anticancer immunologic	Cancer: breast, non- small cell of the lung, colorectal
TriGem	Titan Pharmaceuticals	Murine monoclonal antibody; anticancer immunologic	Cancer: melanoma, small cell of the lung, brain
Visilizumab	Protein Design Labs	Chimeric monoclonal antibody; immunosuppressant; anticancer immunologic; GI inflammatory and bowel disorders	Transplant rejection, bone marrow; cancer, T-cell lymphoma; ulcerative colitis; myelodysplastic syndrome; systemic lupus erythematosus

BRAF Inhibitors

A number of BRAF inhibitors are known in the art, including Vemurafenib, GDC-0879, PLX-4720, GSK2118436, Sorafenib Tosylate, dabrafenib, and LGX818.

5

PDGFR α Inhibitors

A number of PDGFR α inhibitors are known in the art, including Crenolanib (CP-868596); Ki8751; Ponatinib (AP24534); Nintedanib (BIBF 1120); Dovitinib (TKI-258, CHIR-258); Masitinib (AB1010); Imatinib (STI571); Pazopanib; and KRN

633. PDGFR α inhibitors can be used in any of the methods described herein, e.g., instead of or in addition to a Shh-I.

Treating BRAF-Inhibitor Resistant Cancers

In over 50% of metastatic melanoma patients their melanoma harbors the BRAF(V600E) point mutation (T1799A)^{1,2}. Mutant BRAF(V600E) represents a constitutively active protein serine kinase that leads to the sustained activation of the BRAF→MEK1/2→ERK1/2 MAP kinase pathway^{3,4}. This pathway plays a critical role in the regulation of gene expression, cell proliferation and survival, which contributes to the initiation and progression of melanoma^{5,6}. Clinical trials have demonstrated that the BRAF inhibitor (BRAF-I) PLX4032 (vemurafenib) and other inhibitors in its class (GSK2118436 or dabrafenib) can induce tumor regression in >50% of patients with metastatic melanoma harboring the BRAF(V600E) mutation and improve both progression-free and overall survival^{7,8}. Although the clinical activity of BRAF-I therapy is a major breakthrough in the treatment of metastatic melanoma, the median time to disease progression is less than 7 months, due to acquired resistance⁸. Furthermore complete responses to vemurafenib are only observed in 5% of patients, as a consequence of intrinsic BRAF-I resistance^{7,9}.

The multiple mechanisms underlying melanoma BRAF-I resistance can be classified into two groups. The first includes ERK signaling reactivation, due to a point mutation in MEK1^{10,11}, amplification of mutant BRAF(V600E)¹², elevated CRAF activity¹³, activating NRAS mutation¹⁴, increased levels of COT/Tpl2¹⁵ and/or aberrantly spliced BRAF(V600E)¹⁶. The second category includes activation of alternative pro-tumorigenic pathways due to an increase in receptor tyrosine kinase (RTK) driven signaling, via platelet-derived growth factor receptor β (PDGFR β) overexpression^{14,17}, insulin-like growth factor 1 receptor (IGF1R) activation¹⁸, phosphatase and tensin homolog (PTEN) loss and activation of PI3K/AKT¹⁹. Most of these mechanisms have corroborating clinical evidence^{10,14-16,18} which has led to therapeutic strategies combining BRAF-I with other treatment modalities such as the concurrent administration of BRAF-I and MEK inhibitors (MEK-I)²⁰.

Unfortunately, the mechanisms described to date account for the cause of resistance in less than 50% of patients whose disease relapses while being treated with a BRAF-I. Therefore, identification and characterization of unknown pathways

mediating BRAF-I resistance are essential for the rational design of targeted strategies to prevent and overcome BRAF-I resistance across this entire population of patients.

In this study we demonstrate that both melanoma cell lines with acquired BRAF-I resistance and lesions harvested from patients who acquire vemurafenib resistance develop PDGFR α upregulation implying its contribution in BRAF-I resistance. Furthermore, we demonstrate that the PDGFR α mediated BRAF-I resistance can be overcome by combining vemurafenib with a PDGFR α inhibitor (PDGFR α -I) such as sunitinib²¹, imatinib²², or crenolanib (Dai et al., Clin Cancer Res. 2013 Dec 15;19(24):6935-42) both *in vitro* and *in vivo*. Lastly, we show that activation of the Sonic Hedgehog Homolog pathway (SHH) is a mechanism by which PDGFR α upregulation mediates intrinsic and acquired BRAF-I resistance in melanoma, and that combination therapy with a BRAF-I and a SHH inhibitor (SHH-I) can overcome resistance to BRAF-I monotherapy *in vitro* and *in vivo*.

PDGFR α is also overexpressed in sarcoma and glioma. It is involved in tumor growth, metastasis and neoangiogenesis, as well as in the development of resistance to cytotoxic therapy²⁸. These functional properties of PDGFR α are likely to reflect its ability to engage signaling pathways, such as RAS/RAF/MEK/ERK and PI3K/AKT which play a role in tumor cell proliferation and aggressive tumor phenotypes. The present study demonstrates that PDGFR α is expressed by human melanoma cells both *in vitro* and *in vivo*. Its upregulation in human melanoma cells harboring the BRAF(V600E) mutation is shown for the first time to be associated with the loss of their sensitivity to the anti-proliferative and pro-apoptotic activity of the BRAF-I vemurafenib both *in vitro* and *in vivo*. The association between PDGFR α upregulation and vemurafenib resistance reflects a cause-effect relationship, since sensitivity to vemurafenib is restored in melanoma cells which downregulate PDGFR α expression following transduction with a PDGFR α -specific shRNA. An association between the PDGFR α and BRAF(V600E) mutation is also observed in wild type PDGFR α gastrointestinal stromal tumors which acquire the BRAF(V600E) mutation when they develop resistance to PDGFR α -I²⁹⁻³².

Vemurafenib resistance of melanoma cells harboring a BRAF mutation reflects ERK and AKT activation induced by PDGFR α upregulation, since inhibition of its synthesis by PDGFR α -specific shRNA causes a reduction of ERK and AKT activation and restores sensitivity to BRAF-I. A similar effect has been demonstrated for HGF/c-met mediated resistance to BRAF-I³³. This is corroborated by the *in vitro*

and *in vivo* results obtained by inhibiting the function of PDGFR α with the tyrosine kinase inhibitor sunitinib, imatinib or crenolanib. Vemurafenib in combination with a PDGFR α -I inhibits *in vitro* proliferation and induces apoptosis of melanoma cells with a PDGFR α upregulation mediated BRAF-I resistance. These results are
5 paralleled by our *in vivo* findings. Vemurafenib in combination with the PDGFR α -I inhibited the growth and induced apoptosis in human melanoma cells with PDGFR α upregulation mediated BRAF-I resistance engrafted in immunodeficient mice. These effects are mediated by the inhibition of the RAF/MEK/ERK and PI3K/AKT signaling pathways since the levels of p-ERK and p-AKT were markedly reduced in
10 melanoma cells with PDGFR α upregulation mediated BRAF-I resistance following *in vitro* or *in vivo* treatment with vemurafenib in combination with a PDGFR α -I. It is noteworthy that vemurafenib in combination with a PDGFR α -I has a significantly greater anti-proliferative and pro-apoptotic effect than either agent alone both *in vitro* and *in vivo* also with BRAF-I sensitive human melanoma cells which expresses
15 PDGFR α . Therefore our results suggest that the combinatorial strategy we have developed may overcome not only the acquired, but also the intrinsic BRAF-I resistance if PDGFR α is expressed. Furthermore they confirm that simultaneous inhibition of both the AKT and ERK pathways is more effective in suppressing tumor cell proliferation and in inducing apoptosis in both BRAF-I sensitive and resistant
20 melanoma cells^{18,34-40}.

PDGFR α upregulation has been found to be regulated by the SHH pathway and by Gli1 activation²³⁻²⁶. Our data confirm this association since treatment with BRAF-I as well as for PDGFR α expression induces a Gli1 upregulation. The latter results are associated with PDGFR α upregulation which leads to BRAF-I resistance
25 since treatment with a SHH-I, which inhibits Gli1 activation, downregulates the expression of PDGFR α . Furthermore PDGFR α downregulation by the SHH-I in combination with vemurafenib enhances tumor growth inhibition and decreases ERK and AKT activation in both sensitive and resistant cell lines.

PDGFR α is not the only growth factor receptor which plays a role in BRAF-I
30 resistance. IGFR1¹⁸ and PDGFR β ^{14,17} are involved in the acquired BRAF-I resistance of melanoma. BRAF-I resistance mediated by IGFR and PDGFR β , similar to PDGFR α , is mediated by ERK and AKT activation. However as reported¹⁷ and as found by us the PDGFR α /PDGFR β inhibitors sunitinib and imatinib are not able to overcome BRAF-I resistance mediated by PDGFR β upregulation. The latter finding

reflects the lack of inhibition of ERK activation in spite of the inhibition of AKT activation since the inhibition of these two downstream components of the RAF/MEK/ERK and PI3K/AKT signaling pathways by a PDGFR β -specific shRNA restored sensitivity of melanoma cells to vemurafenib.

5 The clinical relevance of our results is suggested by two lines of evidence. First, PDGFR α expression was upregulated in 5 out of the 9 melanoma lesions with a BRAF(V600E) mutation surgically removed from patients who had developed BRAF-I resistance. Second, the extent of PDGFR α increase in melanoma lesions, as indicated by the IHC staining intensity, was associated with the clinical course of the
10 disease. Specifically a marked increase in PDGFR α was found to be associated with a shorter time to progression and less tumor regression based on RECIST criteria. Notably, baseline expression of PDGFR α did not correlate with response or time to progression. In order to utilize the phenomenon we have observed as a method for patient selection, one would need to monitor PDGFR α upregulation in tumor biopsy
15 specimens or develop a noninvasive or surrogate method to detect upregulation.

 These findings have important translational implications, potentially effective in counteracting the BRAF-I Gli1/PDGFR α -mediated resistance of melanoma. The translation of this approach into the clinic is facilitated by the availability of FDA approved drugs to use in combination. Furthermore these data suggest that PDGFR α
20 may be a biomarker for patients with BRAF-mutant melanoma, predicting who will or will not respond to BRAF-I or combination BRAF-I and MEK-I. The finding of upregulated PDGFR α has therapeutic implications, as these patients may potentially benefit from treatment with BRAF-I in combination with PDGFR α -I or SHH-I.

 Resistance to BRAF inhibitors is a major obstacle to the clinical success of
25 melanoma treatment. Several mechanisms of BRAF inhibitor resistance have been identified. The data presented herein demonstrate for the first time that a broad range of the described mechanisms can be driven by upregulation and/or activation of sonic hedgehog pathway. These resistances can be overcome by combining a sonic hedgehog inhibitor and a BRAF inhibitor.

30 Thus the present methods for treating BRAF inhibitor (BRAF-I)-resistant cancers, e.g., melanoma, carcinoma, e.g., colon cancer, thyroid cancer, pancreatic adenocarcinoma, triple negative breast cancer, glioma, or sarcoma, include the administration of therapeutically effective amounts of at least one BRAF-I plus at least one Shh-I.

Combination Therapy with Sonic Hedgehog Pathways Inhibitors and Tumor Antigen-Specific Monoclonal Antibody-Based Immunotherapy

The recurrence of disease in patients with solid tumors treated with Tumor Antigen-specific monoclonal antibodies in combination with chemo- and/or radio-therapy may reflect the persistence of Cancer initiating cells because according to the cancer stem cell theory these cells are responsible for disease recurrence and metastatic spread. To overcome these limitations we have developed a combinatorial strategy that includes administration of tumor antigen-specific monoclonal antibodies with inhibitor of signaling pathways activated in Cancer initiating cells such as inhibitors of the Sonic Hedgehog pathways in triple negative breast cancer and in pancreatic ductal cancer. We show for the first time that this combinatorial strategy completely eradicates in vitro the so called cancer initiating cells. These effects are mediated by an enhanced apoptosis and inhibition of signaling pathways associated with proliferation and survival of cancer cells including cancer initiating cells.

Dosage

An “effective amount” is an amount sufficient to effect beneficial or desired results. For example, a therapeutic amount is one that achieves the desired therapeutic effect. This amount can be the same or different from a prophylactically effective amount, which is an amount necessary to prevent onset of disease or disease symptoms. An effective amount can be administered in one or more administrations, applications or dosages. A therapeutically effective amount of a therapeutic compound (i.e., an effective dosage) depends on the therapeutic compounds selected. The compositions can be administered one from one or more times per day to one or more times per week; including once every other day. The skilled artisan will appreciate that certain factors may influence the dosage and timing required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of the therapeutic compounds described herein can include a single treatment or a series of treatments.

Dosage, toxicity and therapeutic efficacy of the therapeutic compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD50 (the dose lethal to 50% of the population) and

the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds which exhibit high therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

EXAMPLES

The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

Material and Methods

The following materials and Methods were used in Example 1, below.

Cell cultures

The parental BRAF(V600E) melanoma cell lines Colo38 and M21 were cultured in RPMI 1640 medium (Mediatech, Inc. Herndon, VA) supplemented with 2 mmol/L L-glutamine (Mediatech) and 10% fetal calf serum (FCS; Atlanta Biologicals, Lawrenceville, GA). The BRAF(V600E) melanoma cell line TPF-10-741 was cultured in DMEM (Mediatech) supplemented with 2 mmol/L L-glutamine and 10% FCS. This cell line was started from a cutaneous metastasis of the melanoma patient TPF-10-741 who had developed BRAF-I resistance following treatment with

vemurafenib. Melanoma cell lines with acquired vemurafenib resistance (Colo38R and M21R) were generated by propagating parental Colo38 and M21 cells in increasing concentrations of BRAF-I (up to 2 μ M) to achieve chronic selection. Colo38R and M21R were cultured in RPMI 1640 medium supplemented with 2 mmol/L L-glutamine, 10% FCS and 500 nM vemurafenib. All cells were cultured at 37°C in a 5% CO₂ atmosphere.

Chemical reagents, antibodies and shRNAs

Vemurafenib and LDE225 were purchased from ChemieTek (Indianapolis, IN). Sunitinib, imatinib and crenolanib were purchased from Selleck Chemicals LLC (Houston, TX). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO). Phospho (p)-AKT (Ser473)-, AKT-, p-PI3K p85 (γ 458)-, p-CRAF (S289/296/301)-, p-MEK 1/2 (S217/221)-, p-ERK 1/2 (Thr202/Tyr204)-, ERK1/2-, PDGFR β -, p-PDGFR α -, PDGFR α -, PTEN-, VEGFR2-, Cleaved Caspase-3 (Asp175)-, p-Histone H3 (Ser10)-, Gli1- and β -actin-specific monoclonal antibodies (mAbs) were purchased from Cell Signaling Technology (Danvers, MA). The calnexin-specific mAb TO-5 was developed and characterized as described¹. PDGFR α -specific short hairpin RNA (shRNA) and GFP-shRNA were provided by the- Vector Core Facility of the University of Pittsburgh Cancer Institute (Pittsburgh, PA).

Cell proliferation and MTT assay

Cells were plated in triplicate in 96-well microtiter plates at the density of 2.5 x 10³ per well in 100ul in RPMI 1640 or DMEM medium supplemented with 2% FCS and treated with vemurafenib and/or PDGFR α -I (sunitinib or imatinib or crenolanib) and/or SHH-I LDE225. DMSO (vehicle of the drugs) concentration was maintained at 0.02% in all wells. Cell proliferation was evaluated by MTT assay at indicated time points. MTT assay was carried out as reported elsewhere². Data were expressed as percent of inhibition or percent of proliferation of treated cells compared with the untreated control cells. All experiments were performed three independent times in triplicates.

Western Blot analysis

For samples preparation from cell lines, cells were seeded at the density of 1 x 10⁵ per well in a 6-well plate in medium supplemented with 2% FCS and treated with vemurafenib (1 μ M), sunitinib (3 μ M), imatinib (20 μ M), crenolanib (1 μ M), LDE225 (10 μ M), vemurafenib 10 plus sunitinib or imatinib, and vemurafenib plus LDE225 at

37 °C in a 5% CO₂ atmosphere for the indicated time points. The DMSO (vehicle of the drugs) concentration was maintained at 0.02% in all wells. Untreated cells were used as a control. Cells were collected and lysed in lysis buffer [10 mM Tris-HCl (pH 8.2), 1% NP40, 1 mM EDTA, 0.1% BSA, 150 mM NaCl) containing 1/50 (vol/vol) of protease inhibitor cocktail (Calbiochem, La Jolla, CA). For sample preparation from tumor xenografts, tumors were extracted at the time of killing, harvested and stored at -80°C. Proteins were extracted by homogenization in the presence of 2 to 5 ml lysis buffer. Western blot assay for signaling-related proteins was carried out as described³. The investigator who analyzed the sample from tumor xenografts was blinded to the type of treatment received by the mice used as the source of the tumor.

Transduction of melanoma cells with Lentiviral vectors encoding shRNA

Colo38R, M21R and TPF-10-741 cells were seeded at the density of 6×10^4 per well in a 6-well plate and incubated in culture medium for 24 h at 37°C in a 5% CO₂ atmosphere prior to viral infection. Cells were transduced with PDGFR α -specific shRNAs [Target sequence: CCAGCCTCATATAAGAAGAAA (#1; SEQ ID NO:1), CCAGCTTTCATTACCCTCTAT (#2; SEQ ID NO:2), CGGTGAAAGACAGTGGAGAT (#3; SEQ ID NO:3), CCCAACTTTCTTATCCAACTT (#4; SEQ ID NO:4), CAATGGACTTACCCTGGAGAA (#5; ; SEQ ID NO:5)] or GFP-shRNA, used as a control, lentiviral particles (1×10^6 per well) in presence of polybrene (2 μ g/ml) as described elsewhere⁴. Following an 18 h incubation at 37°C, culture medium was removed and replaced with fresh culture medium. Following an additional incubation for up to 72 h at 37°C, cells were analyzed for GFP expression under the microscope, split, enriched for infected cells by selection with puromycin (2.5 μ g/ml) and collected for further analysis.

Flow Cytometry

These assays were carried out as described elsewhere⁵. In briefly cells were seeded in 6-well plates in triplicate at the density of 1×10^5 per well and treated with vemurafenib and/or sunitinib. Following a 24 h treatment apoptotic cells were identified by staining with Annexin -V and PI (BD Bioscience, San Jose, CA) for 15 min at room temperature. Flow cytometry data were analyzed using Summit v4.3 software (DAKO, Carpinteria, CA).

In vivo studies

C.B-17 severe combined immunodeficient (SCID) female mice (8–10 week old) were purchased from Taconic Farms, Inc. (Hudson, NY). Parental and BRAF-I resistant cell lines M21 and M21R (1×10^6 cells/mouse) were implanted subcutaneously in the right lateral flank of mice. A total of 20 SCID mice was challenged with each cell line. Body weight and tumor volume were measured twice per week. Tumor volume was measured by vernier caliper. Treatment was initiated 10 days after cell inoculation when the tumor developed and had a diameter of around 0.4 cm. Mice were randomly divided into 4 groups of 5 mice each. Mice in Group 1 were treated with vemurafenib (12.5mg/kg/twice per day⁶), those in Group 2 with sunitinib (20mg/kg/day)⁷ and those in Group 3 with vemurafenib (12.5mg/kg/twice per day) plus sunitinib (20mg/kg/day). Mice in Group 4 were left untreated as a reference for the natural course of the disease.

For mice treated with vemurafenib and imatinib combination mice were randomly divided into 4 groups of 5 mice each. Mice in Group 1 were treated with vemurafenib (25mg/kg/twice per day⁶), those in Group 2 with imatinib (100mg/kg/day)⁸ and those in Group 3 with vemurafenib (25mg/kg/twice per day) plus imatinib (100mg/kg/day). Mice in Group 4 were left untreated as a reference for the natural course of the disease.

To test vemurafenib and LD225 combination, mice were randomly divided into 4 groups of 5 mice each. Mice in Group 1 were treated with vemurafenib (12.5 mg/kg/twice per day), those in Group 2 with LDE225 (40 mg/Kg/day) (Fendrich et al., Annals of Surgery. 2011; 254(5):818-823; discussion 823), and those in Group 3 with vemurafenib (12.5 mg/kg/twice per day) plus LDE225 (40 mg/kg/day). Mice in Group 4 were left untreated as a reference for the natural course of the disease.

Drugs were administered by oral gavage. When tumor diameter from untreated mice reached 2.0 cm all mice were sacrificed. Primary tumors and organs were collected for further analysis. Animal studies have been approved by the Institutional Animal Care and Use Committee.

Patient Samples

Patients with metastatic melanoma harboring the BRAF(V600E) mutation (confirmed by genotyping) were enrolled in clinical trials with the BRAF-I (vemurafenib) or with BRAF-I (dabrafenib) and MEK-I (trametinib). Patients were consented for tissue acquisition per IRB-approved protocol. Tumor biopsies were

performed pre-treatment (day 0), at 10-14 days on treatment, and/or at the time of disease progression [as defined by Response Evaluation Criteria In Solid Tumors (RECIST)] if applicable. Formalin-fixed tissue was analyzed to confirm that viable tumor was present via hematoxylin and eosin (H&E) staining.

Immunohistochemistry and Immunofluorescence

Patient biopsies and tumors generated in mice were formalin fixed and paraffin embedded and then used as substrates in immunohistochemical reactions. Five-um thick xenograft tissue sections were fixed on silane-coated glass slides, deparaffinized, and subjected to antigen retrieval (Target retrieval solution, DAKO). Following blocking, slides from mice were incubated with Cleaved Caspase-3 (Asp175) and p-Histone H3 (Ser10) –specific mAbs overnight. Four-um thick sections from patient-derived samples were incubated with PDGFR α -specific mAb (sc-338, Santa Cruz) (1:400) overnight. Sections were then washed with PBS, and the primary antibody was amplified using the VECTASTAIN ABC Kit (Peroxidase rabbit IgG, Vector Laboratories, PK-4001). The detection of this antibody was performed with the DAB Peroxidase Substrate Kit from DAKO and the sections were counterstained with H&E. PDGFR α expression, as measured by its staining intensity, in tumors harvested from BRAF-I treated patients either on treatment or at the time of disease progression was compared to that the staining in pretreatment tumors. Scores were recorded semiquantitatively as 1+, 2+, 3+ and 4+, when , 1–25%, 26–50%, 51–75% and >75% of cells were stained, respectively. Mitotic and apoptotic tumor cells in the sections of primary tumors harvested from mice were detected by staining p-Histone H3 (Ser10) and Cleaved Caspase-3 proteins, respectively, and quantified by counting 5 random fields per section (magnification $\times 200$). Data were expressed as the mean number of mitotic or apoptotic tumor cells in each group. The number of mitotic or apoptotic tumor cells was counted by an investigator who was blinded to the type of treatment received by the mice from which tumors had been harvested.

Statistical analysis

Averages, standard deviations, and unpaired *t*-test were calculated using MS-Excel. Data showed the mean $\pm$ SD of the results obtained in at least three independent experiments. Time of disease progression (time to progression) of BRAF-I treated patients was calculated using the Kaplan-Meier methods. Differences between groups were considered significant when the *P* value was < 0.05 . The asterisk (*) indicates $P < 0.05$.

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Example 1. Sonic Hedgehog Pathway activation and BRAF inhibitor resistance

Control of mutant BRAF(V600E) metastatic melanoma by the selective BRAF inhibitor (BRAF-I) is limited by intrinsic and acquired resistance. We demonstrate for
30 the first time that PDGFR α upregulation causes BRAF-I resistance *in vitro* and *in vivo*. PDGFR α inhibition by PDGFR α -specific shRNA and by PDGFR α inhibitors restores melanoma cells' sensitivity to BRAF-I *in vitro* and *in vivo*. PDGFR α inhibition reverses ERK and AKT mediated proliferation and resistance to apoptosis

which are associated with BRAF-I resistance. PDGFR α upregulation is mediated by activation of the Sonic Hedgehog Homolog (SHH) pathway which is induced by BRAF-I treatment. PDGFR α upregulation is found in melanoma matched biopsies of BRAF-I treated patients and correlates with shorter time to disease progression and less tumor regression. These data suggest that monitoring patients for early PDGFR α upregulation may identify those who may most benefit from the treatment with vemurafenib in combination with PDGFR α inhibitors or SHH inhibitor utilizing currently available agents.

1A. ERK reactivation, AKT activation and PDGFR α upregulation in Colo38R, M21R and TPF-10-741 melanoma cell lines with acquired vemurafenib resistance

To assess the BRAF-I sensitivity of the parental Colo38 and M21 cell lines and those with acquired BRAF-I resistance Colo38R, M21R and TPF-10-741, growth inhibition was analyzed following treatment with vemurafenib. MTT assays demonstrated that Colo38 and M21 cells were exquisitely sensitive to the anti-proliferative activity of vemurafenib, while Colo38R and M21R cells had acquired resistance to the growth inhibitory effects of vemurafenib. TPF-10-741 cells displayed an intermediate sensitivity to vemurafenib (**Fig. 7**). This acquired resistance model was used to investigate the molecular mechanisms underlying disease progression after an initial response to vemurafenib. Since acquired vemurafenib resistance can be mediated by reactivation of the RAF/MEK/ERK pathway or by activation of alternative pathways like PI3K/AKT, signaling through these pathways were evaluated in both parental and resistant cell lines (**Fig.1A**). Western blot analysis demonstrated that following incubation with vemurafenib p-ERK levels were reduced in both Colo38 and M21 cells, but no change in p-ERK levels was detected in Colo38R and M21R cells. A limited effect on TPF-10-741 cells was seen. p-AKT levels were increased in Colo38R and M21R cells compared to Colo38 and M21 cells. P-AKT levels also increased in M21 and TPF-10-741 cells after treatment with BRAF-I. Therefore the decreased sensitivity to the inhibition of the RAF/MEK/ERK pathway by BRAF-I was associated with the reactivation of the RAF/MEK/ERK pathway and with activation of AKT.

Since BRAF-I resistance through activation of the PI3K/AKT pathway can be mediated by RTK upregulation¹⁴, we investigated the role of RTK in acquired

vemurafenib resistance. Western blot analysis (**Fig.1B**) demonstrated that treatment with vemurafenib enhanced PDGFR α activation and expression in Colo38R and M21R cells as compared to the parental cells. PDGFR α was also expressed and activated in TPF-10-741 cells both under basal conditions and following treatment with vemurafenib. PDGFR β was upregulated on TPF-10-741 cells after treatment with vemurafenib, but not in the other cell lines. VEGFR2 expression was not detected in any cell lines before or after treatment with vemurafenib. Further analysis of the RAF/MEK/ERK and PI3K/AKT pathway components demonstrated that PDGFR α upregulation was associated with PI3K, CRAF and MEK activation in Colo38R, M21R, and TPF-10-741 cells. Lastly, analysis of PTEN status demonstrated the lack of its expression in TPF-10-741 cells, but not in the other cells.

1B. PDGFR α mediates acquired vemurafenib resistance in Colo38R, M21R, and TPF-10-741 melanoma cell lines by reactivation of the RAS/RAF/ERK pathway and activation of the PI3K/AKT pathway

To test whether PDGFR α upregulation resulted in vemurafenib resistance in Colo38R, M21R and TPF-10-741, PDGFR α was knocked down in the three cell lines using 5 PDGFR α -specific shRNAs. As shown in **Fig.2A**, lentiviral transduction of M21R cells with a PDGFR α -specific shRNA(#4) construct knocked down PDGFR α protein expression. PDGFR α downregulation was associated with decreased p-ERK and p-AKT. Additionally, the M21R and TPF-10-741 cells transduced with the PDGFR α -specific shRNA(#4) displayed a significantly increased sensitivity to vemurafenib, as compared to the autologous cells transduced with a GFP-shRNA ($P<0.01$) (**Fig.2B**). Lastly, western blot analysis demonstrated that the PDGFR α downregulation caused by the PDGFR α -specific shRNA(#4) in combination with vemurafenib treatment dramatically decreased the levels of p-ERK and p-AKT in M21R cells (**Fig.2A**).

1C. Association of PDGFR α upregulation in melanoma patient derived biopsies with BRAF-I resistance

To validate our *in vitro* findings we compared PDGFR α expression in biopsies obtained from 9 melanoma patients treated with BRAF-I +/- MEK-I (**Table 1**). Tumor biopsies were performed before treatment, on treatment, and at the time of disease progression. Immunohistochemical staining demonstrated PDGFR α upregulation in 5

out of 9 patients following treatment with BRAF-I +/- MEK-I (**Fig.3A**). Interestingly, in 3 of the 5 patients a significant increase in PDGFR α expression ($>1+$) was observed after treatment. Patients with a significant ($>1+$) increase in PDGFR α expression after treatment with BRAF-I +/- MEK-I had less tumor regression based on RECIST criteria (**Fig.3B**) and shorter time to disease progression (**Fig.3C**) when compared to patients who had no change or a small change in expression ($\leq 1+$).

ID#	Sex	Age	Treatment	Objective Response	time to progression (months)	PDGFR α IHC Expression Score (+)		
						Pre-treatment	On-treatment	After Progression of disease
1	M	68	BRAF-I	SD (-25%)	5	1	3	-
7	M	56	BRAF-I + MEK-I	CR (100%)	17	1	-	2
8	M	37	BRAF-I + MEK-I	PR (-30%)	3	4	4	-
10	F	37	BRAF-I + MEK-I	SD (-13%)	3	0	4	-
12	F	31	BRAF-I + MEK-I	PR (-88.9%)	12	3	3	-
13	M	69	BRAF-I + MEK-I	PR (-57.9%)	9	1	2	-
17	M	74	BRAF-I	PR (-71.7%)	15	-	-	1
21	M	61	BRAF-I + MEK-I	PR (-49%)	13	-	-	4
25	M	72	BRAF-I + MEK-I	PR (-64%)	3	1	-	4

1D. Inhibition of PDGFR α increases the anti-tumor activity of vemurafenib in BRAF-I sensitive and resistant melanoma cell lines

To investigate whether the anti-tumor activity of BRAF-I could be enhanced by PDGFR α inhibition, Colo38, Colo38R, M21, M21R and TPF-10-741 cells were treated with vemurafenib and/or PDGFR α -I sunitinib²¹ or imatinib²² or crenolanib (Dai et al., 2013, supra). A dose titration experiment established the dose of PDGFR α -I to be combined with vemurafenib in the 5 cell lines. The IC₅₀ doses of sunitinib, imatinib and crenolanib were found to be 2, 15 and 1 μ M, respectively (**Fig. 8**). The dose of 1.5 μ M and 3 μ M for sunitinib and the dose of 10 μ M and 20 μ M for imatinib were chosen to be tested in combination with vemurafenib for their effect on cell growth (**Fig. 9**) and survival (**Fig. 10**). Cell growth inhibition of Colo38 and M21 by

the MTT assay demonstrated that vemurafenib in combination with PDGFR α -I significantly inhibited proliferation ($p < 0.05$) when compared to each agent alone. Furthermore, PDGFR α -I synergized ($p < 0.05$) with vemurafenib to overcome BRAF-I resistance, increasing the growth inhibition of Colo38R, M21R and TPF-10-741 cells. Analysis of apoptosis by the Annexin-V expression assay demonstrated that treatment with vemurafenib and sunitinib induced apoptosis in a significantly ($p < 0.05$) higher percentage of cells than each agent alone in both BRAF-I sensitive and resistant cell lines. Sunitinib, but not vemurafenib resulted in a significantly higher percentage of apoptotic cells in both BRAF-I sensitive and resistant cell lines ($p < 0.05$).

1E. BRAF and PDGFR α inhibition leads to inhibition of ERK and AKT activation

We next investigated whether vemurafenib in combination with the PDGFR α -I could inhibit ERK and AKT activation in BRAF-I sensitive and resistant cells. Western blot analysis (**Fig.4**) demonstrated that p-ERK levels were decreased when Colo38 and M21 cells were treated with vemurafenib, but to a lesser extent with sunitinib. In addition, p-AKT levels were increased when M21 cells were treated with vemurafenib, but reduced when Colo38 and M21 cells were treated with sunitinib. However, vemurafenib in combination with sunitinib strongly inhibited the levels of both p-ERK and p-AKT in both Colo38 and M21 cells. On the other hand, p-ERK levels were not inhibited by the treatment with vemurafenib in Colo38R, M21R, or TPF-10-741 cells. Treatment with sunitinib minimally inhibited p-ERK levels in Colo38R, M21R and TPF-10-741 cells, but reduced p-AKT levels in Colo38R and TPF-10-741 cells. However, the combination of vemurafenib with sunitinib inhibited both p-ERK and p-AKT levels to a greater extent than each agent alone in all of the BRAF-I resistant cell lines. Similar results were obtained with vemurafenib in combination with imatinib (**Fig.4**).

1F. Melanoma xenograft growth inhibition by the combination of vemurafenib and PDGFR α -I

To assess the *in vivo* relevance of our *in vitro* results, the combination of vemurafenib and the PDGFR α -I sunitinib or imatinib were tested for the ability to inhibit the growth of M21 and M21R cells in SCID mice. The oral administration of the drugs caused no overt side effects. In the mice grafted with the M21 cells (**Fig.5A**)

treatment with vemurafenib (12.5 mg/kg twice daily) or sunitinib (20 mg/kg daily) significantly ($p<0.001$) inhibited tumor growth as compared to untreated mice. However, vemurafenib in combination with sunitinib inhibited tumor growth to a significantly ($p<0.001$) greater extent than each single agent. Similar results were obtained combining vemurafenib (25 mg/kg twice daily) with imatinib (100 mg/kg daily) (**Fig. 11**). Western blot analysis of the tumor lysates removed from treated and untreated mice (**Fig. 5B**) demonstrated that vemurafenib decreased p-ERK levels but increased p-AKT levels, while sunitinib decreased p-ERK and p-AKT levels. This effect was more marked in tumors from mice treated with vemurafenib in combination with sunitinib. Analysis of primary tumors by IHC demonstrated that vemurafenib in combination with sunitinib (**Fig. 5C**) markedly reduced the number of mitotic cells in tumors when compared to tumors from untreated mice ($p<0.001$) or mice treated with the single agents ($p<0.001$). The number of apoptotic cells (**Fig. 5D**) in tumors from mice treated with vemurafenib and sunitinib was significantly higher than in tumors from untreated mice or from mice treated with vemurafenib or sunitinib individually ($p<0.001$). Sunitinib, but not vemurafenib resulted in a significantly higher number of apoptotic cells in the tumors when compared to untreated mice ($p<0.001$).

In the mice grafted with the M21R cells (**Fig. 5E**) vemurafenib did not inhibit tumor growth as compared to untreated mice. In contrast, sunitinib caused a significant inhibition of tumor growth compared to untreated mice ($p<0.001$). Vemurafenib in combination with sunitinib inhibited tumor growth to a significantly ($P<0.001$) greater extent than sunitinib alone. Western blot analysis of tumor lysates (**Fig. 5F**) demonstrated that sunitinib inhibited p-AKT and p-ERK levels. This effect was more marked in tumors from mice treated with vemurafenib in combination with sunitinib. As expected the levels of p-ERK and p-AKT were not affected by treatment with vemurafenib as a single agent. IHC staining (**Fig. 5G**) revealed that treatment with sunitinib markedly decreased the number of mitotic cells in tumors as compared to that in tumors from vemurafenib or untreated mice ($p<0.001$). In addition, treatment with sunitinib caused a strong increase in the number of apoptotic cells in tumors as compared to tumors from vemurafenib treated or untreated mice ($p<0.001$) (**Fig. 5H**). However treatment with vemurafenib in combination with sunitinib decreased the number of mitotic cells and increased the number of apoptotic cells to a significantly ($p<0.001$) greater extent than treatment with sunitinib alone.

1G. SHH pathway activation mediates BRAF-I resistance by PDGFR α upregulation

The previously described role of the SHH pathway and Gli1 activation²³⁻²⁶ in the PDGFR α upregulation associated with MAPK pathway activation prompted us to investigate whether Gli1 activation is involved in PDGFR α upregulation which in turn mediates BRAF-I resistance. Western blot analysis (**Fig.6A**) demonstrated that treatment with vemurafenib enhanced Gli1 expression in Colo38, Colo38R, M21, M21R, and TPF-10-741 cells as compared to untreated cells. The association between Gli1 expression and PDGFR α upregulation mediating BRAF-I resistance was also indicated by the marked inhibition of tumor growth ($p < 0.05$) both in vitro and in vivo and expression level of PDGFR α , p-ERK and p-AKT in the BRAF-I sensitive and resistant cells after treatment with both vemurafenib and LDE225, a novel SHH-I²⁷ (**Figs. 6B-6D**).

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Example 2. Enhancement of the Anti-Tumor Activity of LDE225 by Radiation and Grp94-Specific Monoclonal Antibody Combination Therapy

The mAb W9, which we have recently developed, has a unique specificity because it recognizes an extracellular epitope of the heat shock protein (HSP) glucose regulated protein of 94000 daltons (Grp94). Similar to other members of the HSP90 family, the molecular chaperone Grp94 is required for the stability and activity of client proteins involved in the activation of signaling pathways associated with tumor cell survival and proliferation¹⁻³. These functional properties of Grp94 provide a

molecular mechanism for the anti-proliferative effect and the induction of apoptosis of malignant cells by Grp94 inhibitors⁴. What distinguishes mAb W9 from other Grp94-specific mAbs, described in the literature and/or available commercially, is its ability to selectively target malignant cells by recognizing a carbohydrate epitope that is expressed on malignant cells but is not detectable on normal cells.

Grp94-specific mAb W9 was selected as the target of our combinatorial immunotherapy for the following reasons. First this epitope is expressed in pancreatic ductal adenocarcinoma (PDAC) cell lines and in patient derived PDAC lesions. Second it is expressed at high level not only on differentiated PDAC cells but also on PDAC CICs, defined as cells expressing high level of Aldehyde Dehydrogenases A1 (ALDH^{bright}). As a result we will target not only differentiated PDAC cells, but also pancreatic CICs. According to the literature⁵⁻⁷, and our own unpublished data, ALDH^{bright} cells have the characteristics of CICs, such as self-renewal, chemo- and radio-resistance, aberrant regulation of stem cell signaling pathways (such as SHH and Notch), and high tumorigenicity at low cell numbers in SCID mice⁸⁻¹⁰.

To enhance the therapeutic efficacy of Grp94-targeted mAb-based immunotherapy, we will combine it radiation and LDE225 which is currently undergoing clinical evaluation⁶. This small molecule is a novel oral inhibitor of the Sonic Hedgehog Homolog (SHH) pathway^{11, 12} which is abnormally activated in differentiated PDAC cells and in pancreatic CICs¹³⁻¹⁶. Radiation is a component in the treatment of patients with resectable and locally advanced PDAC. It has been included in our combinatorial strategy, since our preliminary *in vitro* studies demonstrate that radiation markedly enhances Grp94 expression on PDAC cells. Furthermore, inhibition of the functions and/or expression of Grp94 enhances the sensitivity of tumor cells to radiation¹⁷⁻¹⁹. These mechanisms provide an explanation for the enhancement by Grp94 specific-mAb W9 of the ability of LDE225 and radiation to eliminate *in vitro* CICs, detected as ALDH^{bright} cells in PDAC cell lines ($p < 0.05$) (Fig. 12).

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Example 3. Enhancement of the Anti-Tumor Activity of LDE 225 by CSPG4-Specific Monoclonal Antibodies

As noted above, CSPG4 is a good target for combinatorial immunotherapy. As a result, targeting TNBC cells with a CSPG4-specific mAb inhibits their proliferation and metastatic spread in SCID mice by inhibiting ERK and AKT activation, as we have recently described ³.

To enhance the therapeutic efficacy of CSPG4-targeted mAb-based immunotherapy, we will combine it with a novel oral inhibitor (LDE225) ^{4, 5} of the SHH

signaling pathway. This small molecule, which is currently undergoing clinical evaluation⁶, has been selected since the SHH pathways is activated in TNBC cells and especially in TNBC CICs^{7, 8}. Furthermore there is evidence that SHH pathway can cross talk with MAPK and PI3K/AKT pathways which are both inhibited by CSPG4-specific mAbs⁹⁻¹¹. These mechanisms provide an explanation for the enhancement by CSPG4 specific-mAb 225.28 of the ability of LDE225 to eliminate *in vitro* CICs, detected as ALDH^{bright} cells in TNBC cell lines (p<0.05) (**Fig. 1**).

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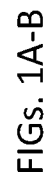
OTHER EMBODIMENTS

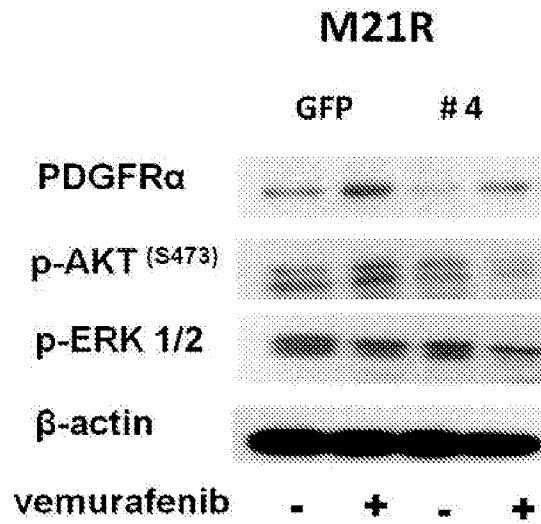
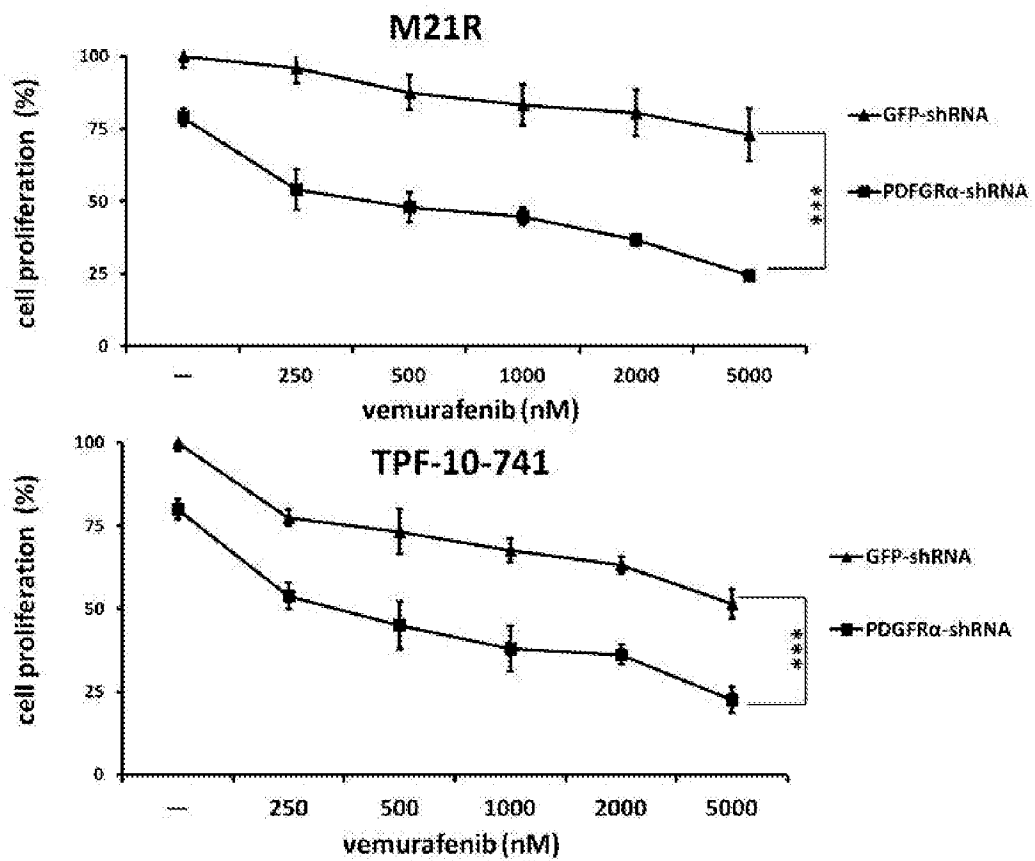
It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of
5 the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

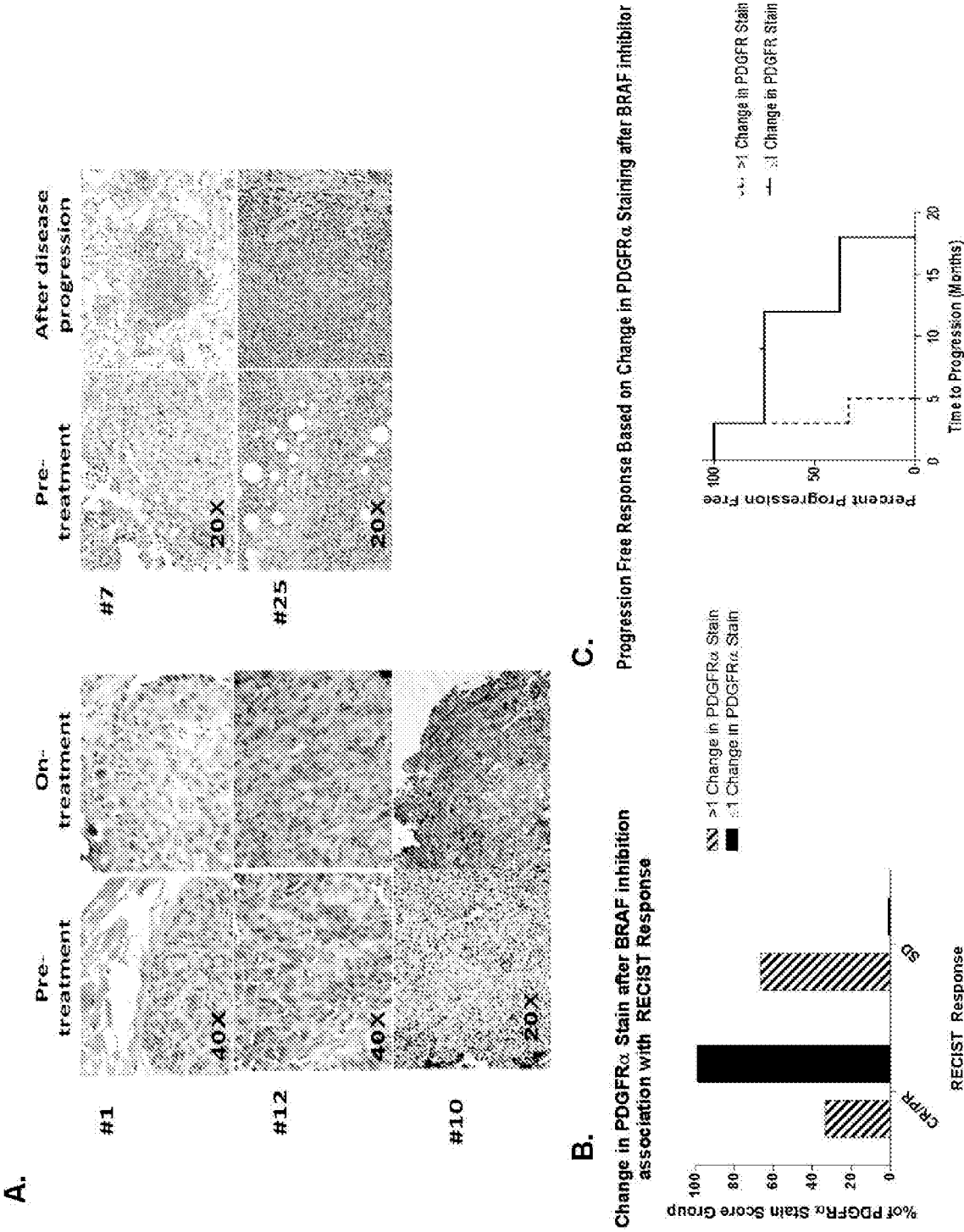
1. A method for treating a BRAF inhibitor (BRAF-I)-resistant cancer, the method comprising administering a therapeutically effective amount of a BRAF-I and a Sonic Hedgehog Inhibitor (Shh-I).
2. Use of a BRAF inhibitor (BRAF-I) and a Sonic Hedgehog Inhibitor (Shh-I) for for treating a BRAF inhibitor (BRAF-I)-resistant cancer.
3. The method of claim 1 or use of claim 2, wherein the BRAF-I-resistant cancer is a melanoma, colon cancer, thyroid cancer, pancreatic adenocarcinoma, triple negative breast cancer, glioma, or sarcoma.
4. The method of claim 1 or use of claim 2, wherein the BRAF-I is selected from the group consisting of Vemurafenib, GDC-0879, PLX-4720, GSK2118436, Sorafenib Tosylate, dabrafenib, and LGX818.
5. A method for treating cancer, the method comprising administering a therapeutically effective amount of an antibody that binds to a tumor-specific antigen and a Sonic Hedgehog Inhibitor (Shh-I).
6. Use of an antibody that binds to a tumor-specific antigen and a Sonic Hedgehog Inhibitor (Shh-I) for for treating cancer.
7. The method of claim 5 or use of claim 6, wherein the cancer is a recurrent cancer.
8. The method of claim 5 or use of claim 6, wherein the cancer is carcinoma or melanoma.
9. The method of claim 5 or use of claim 6, wherein the cancer is triple negative breast cancer or pancreatic ductal adenocarcinoma.
10. The method of claim 5 or use of claim 6, wherein the tumor-specific antigen is Grp94 or chondroitin sulfate proteoglycan 4 (CSPG4).

11. The method or use of any of the preceding claims, wherein the Shh-I is selected from the group consisting of sulforaphane; vismodegib; TAK-441; itraconazole; and erismodegib.



A.**B.**

FIGs. 2A-B



FIGS. 3A-C

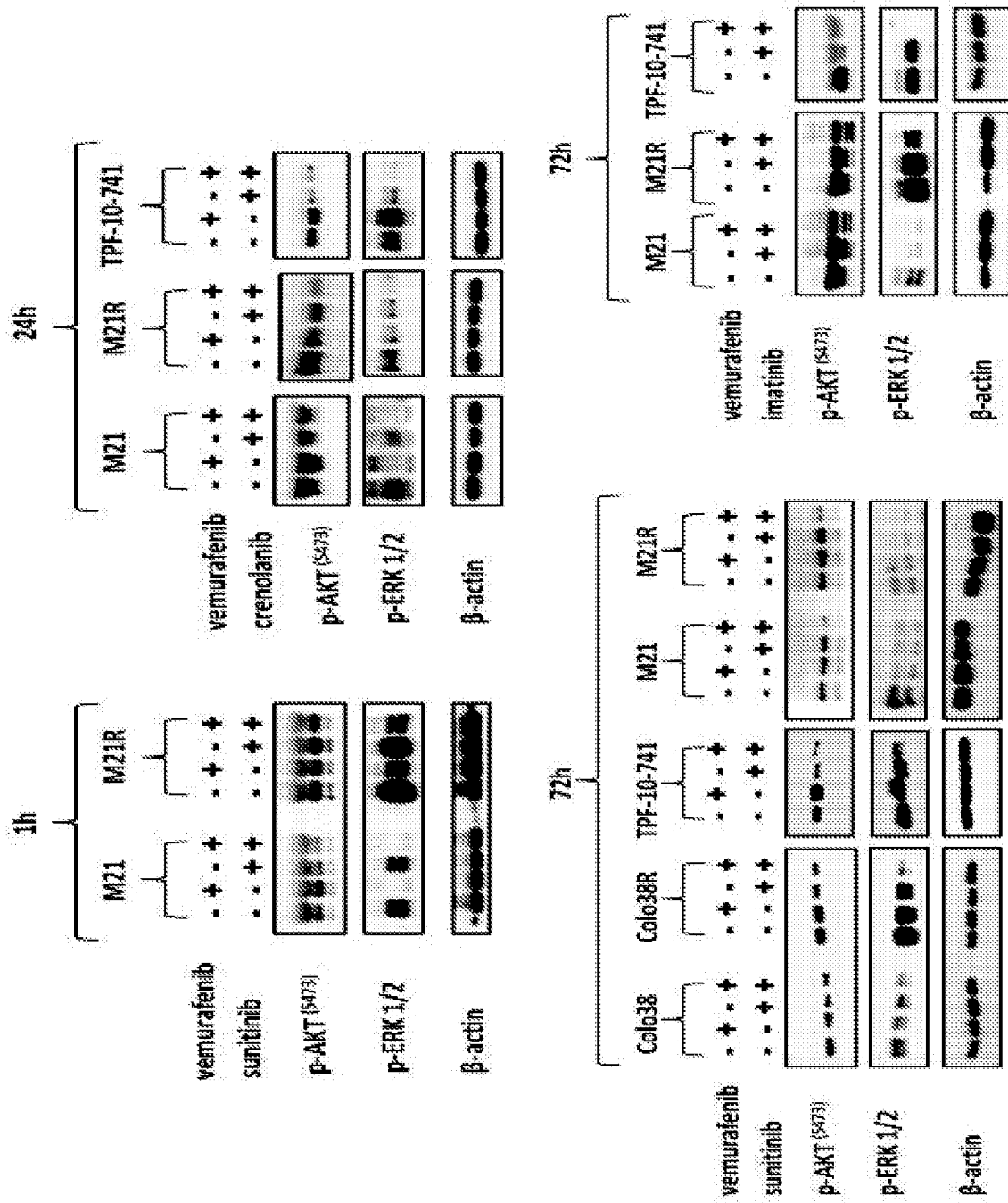
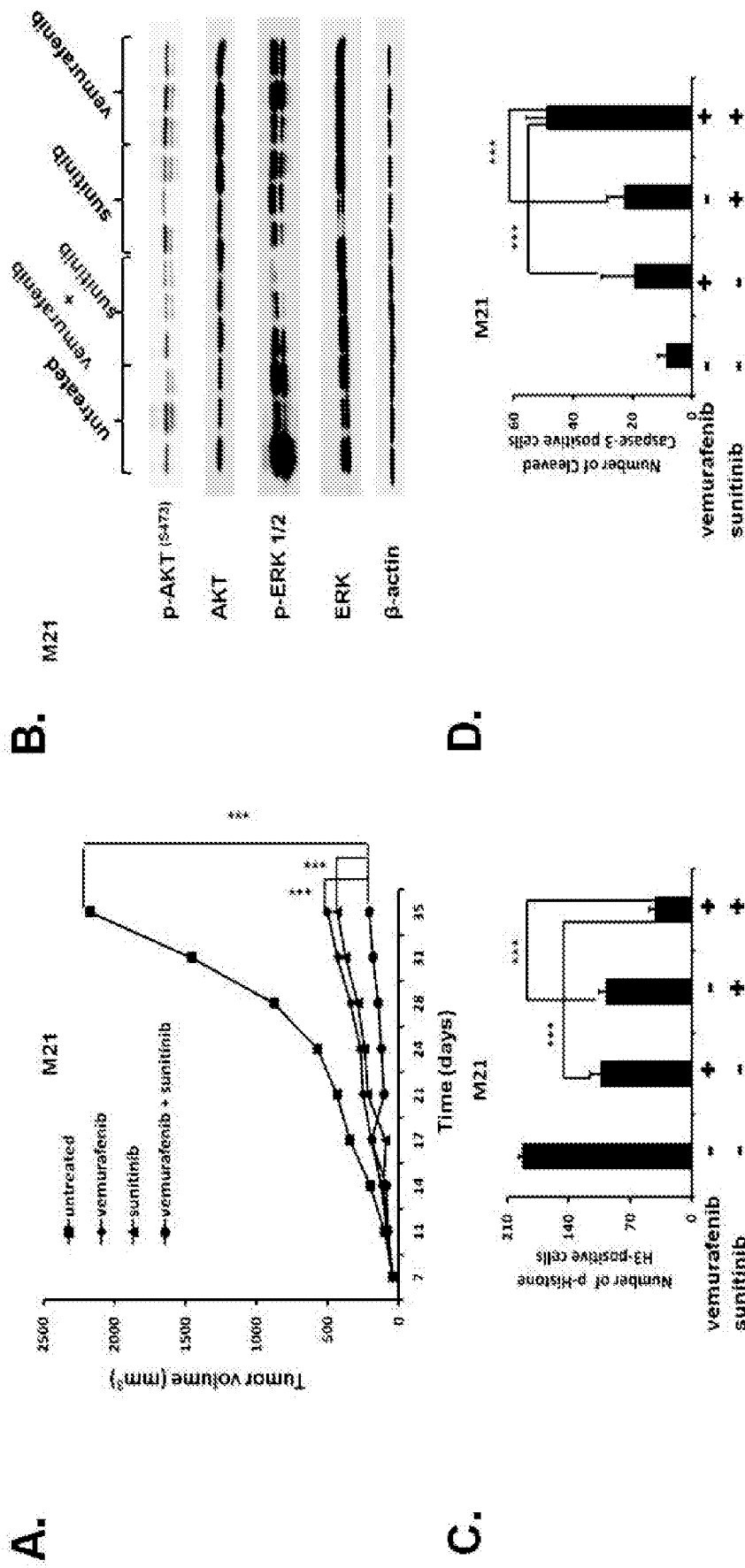
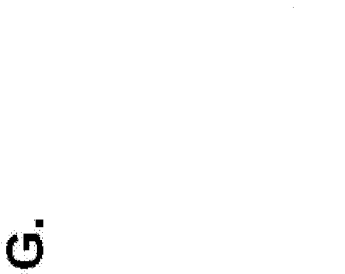
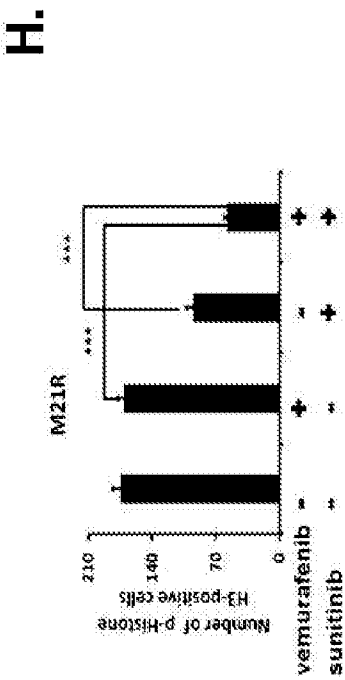
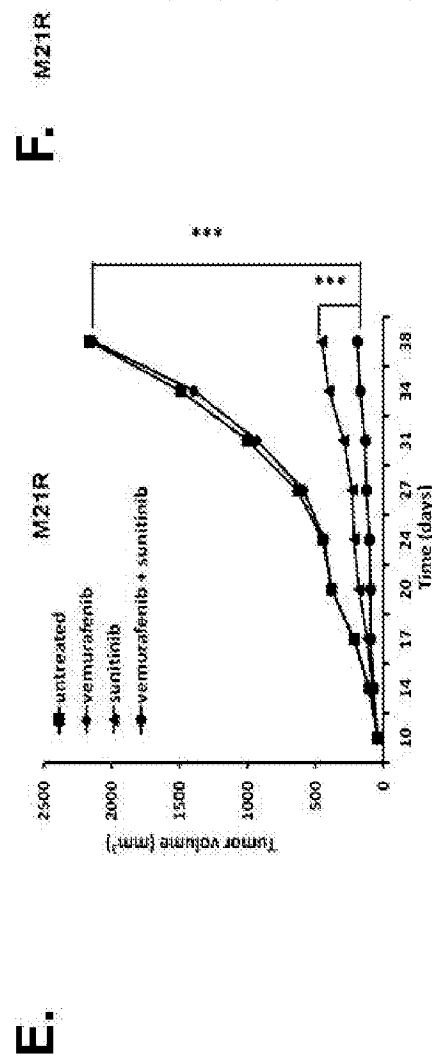
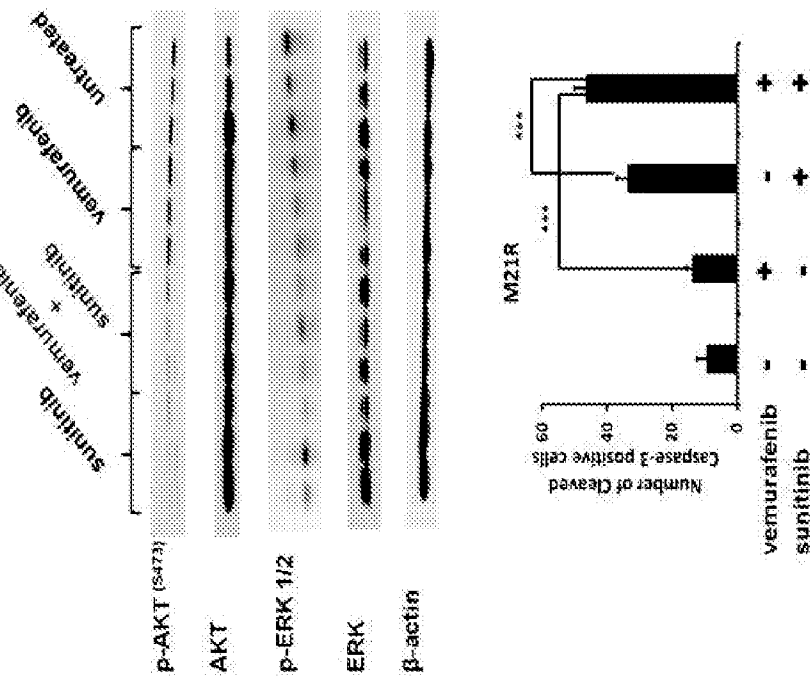


FIG. 4



FIGS. 5A-D



FIGS. 5E-H

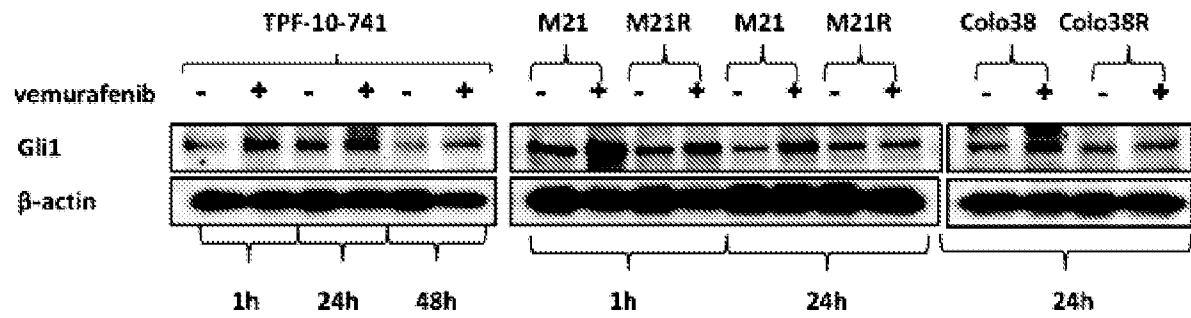


FIG. 6A

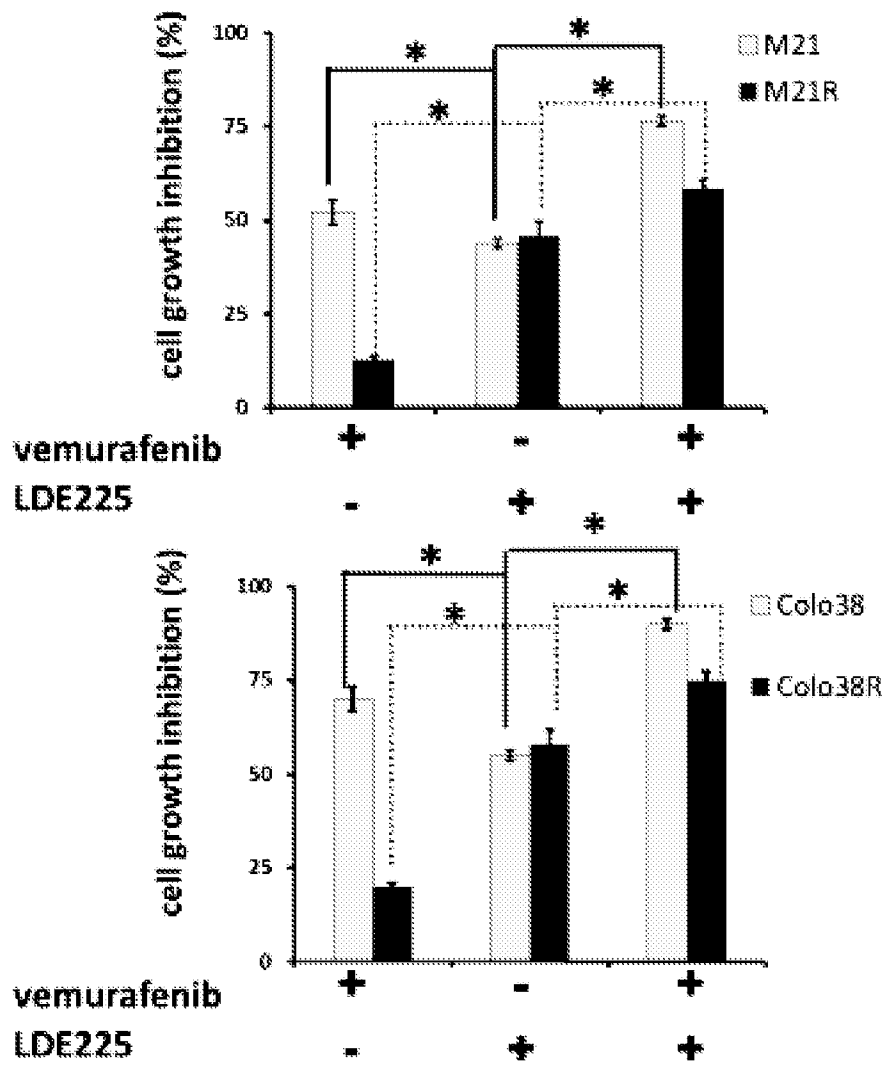


FIG. 6B

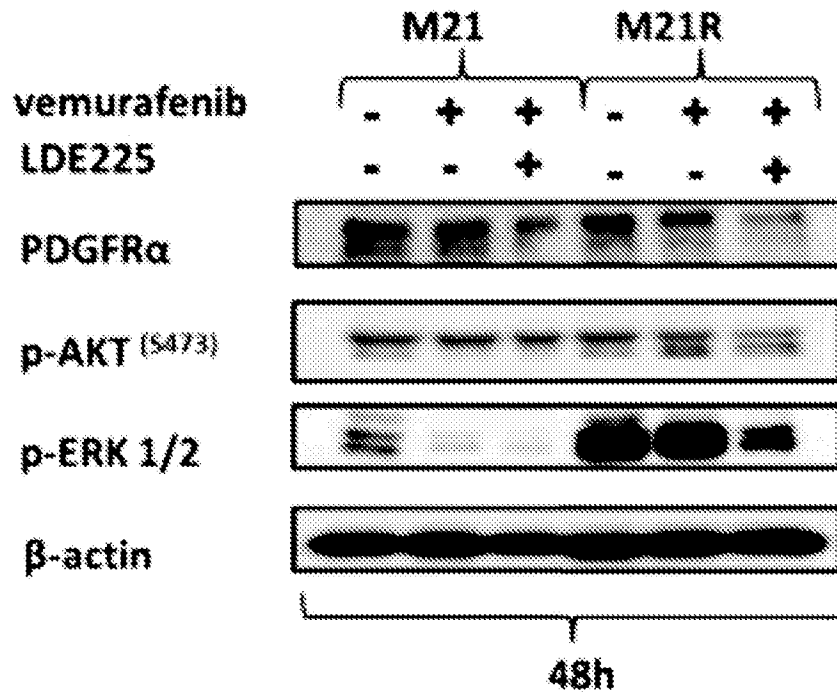


FIG. 6C

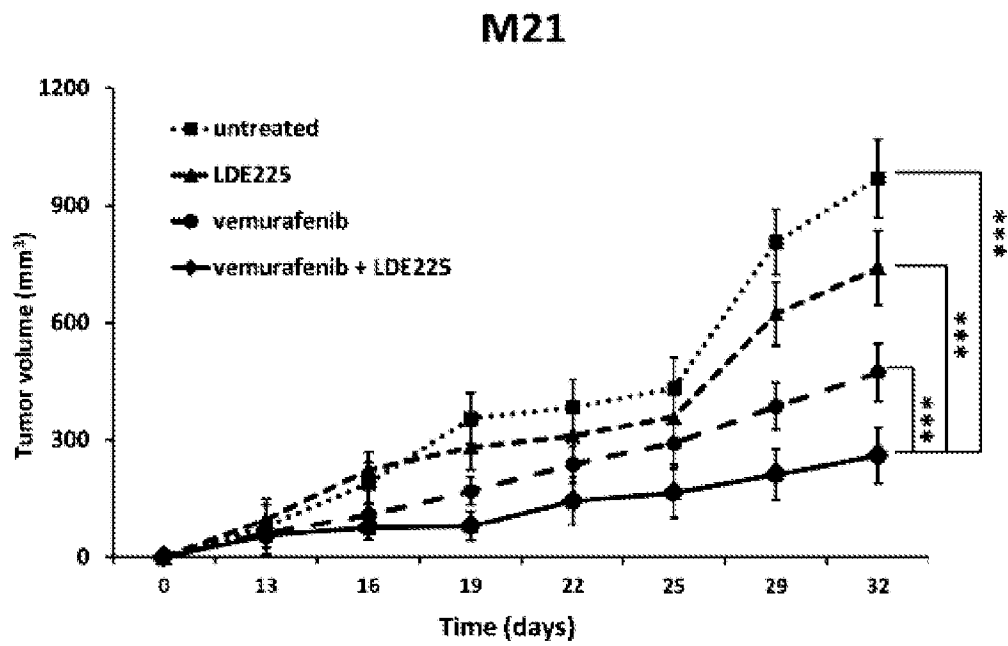


FIG. 6D

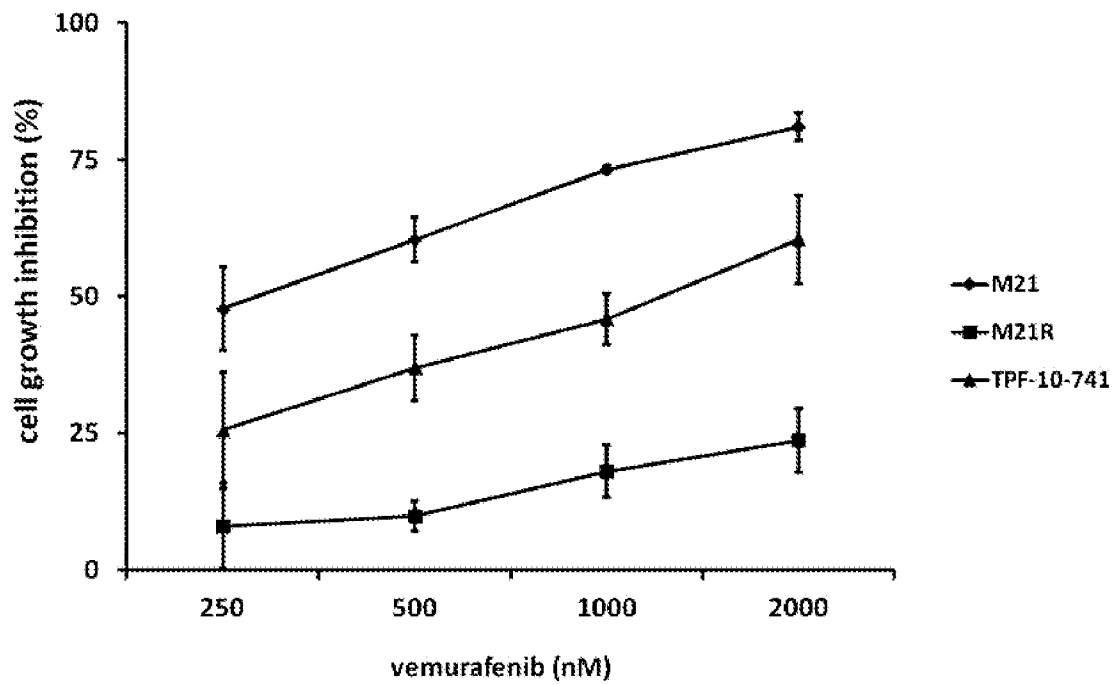


FIG. 7

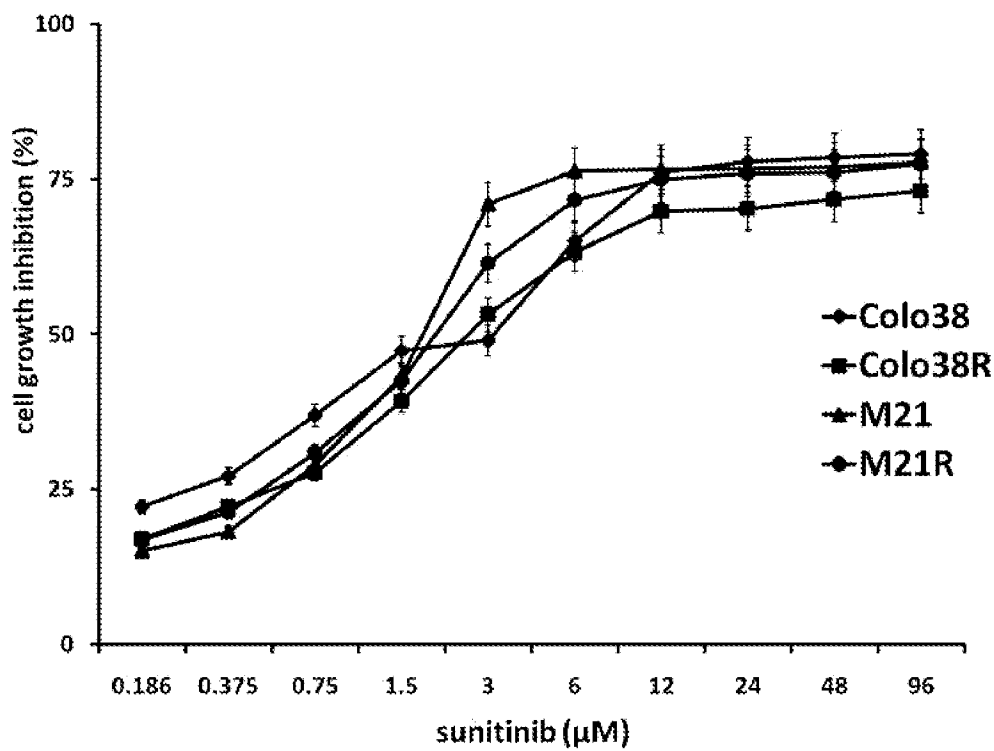


FIG. 8

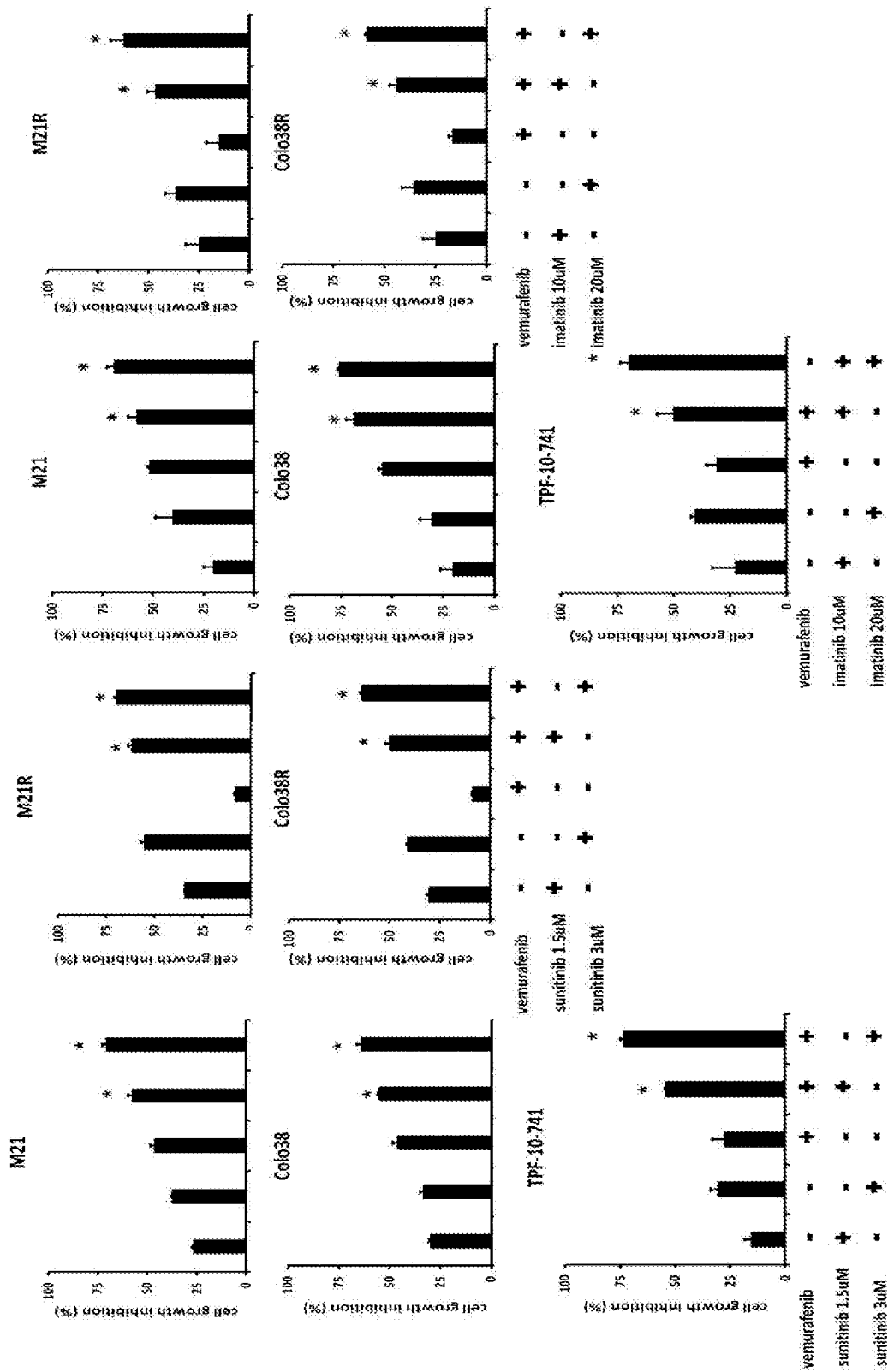


FIG. 9A

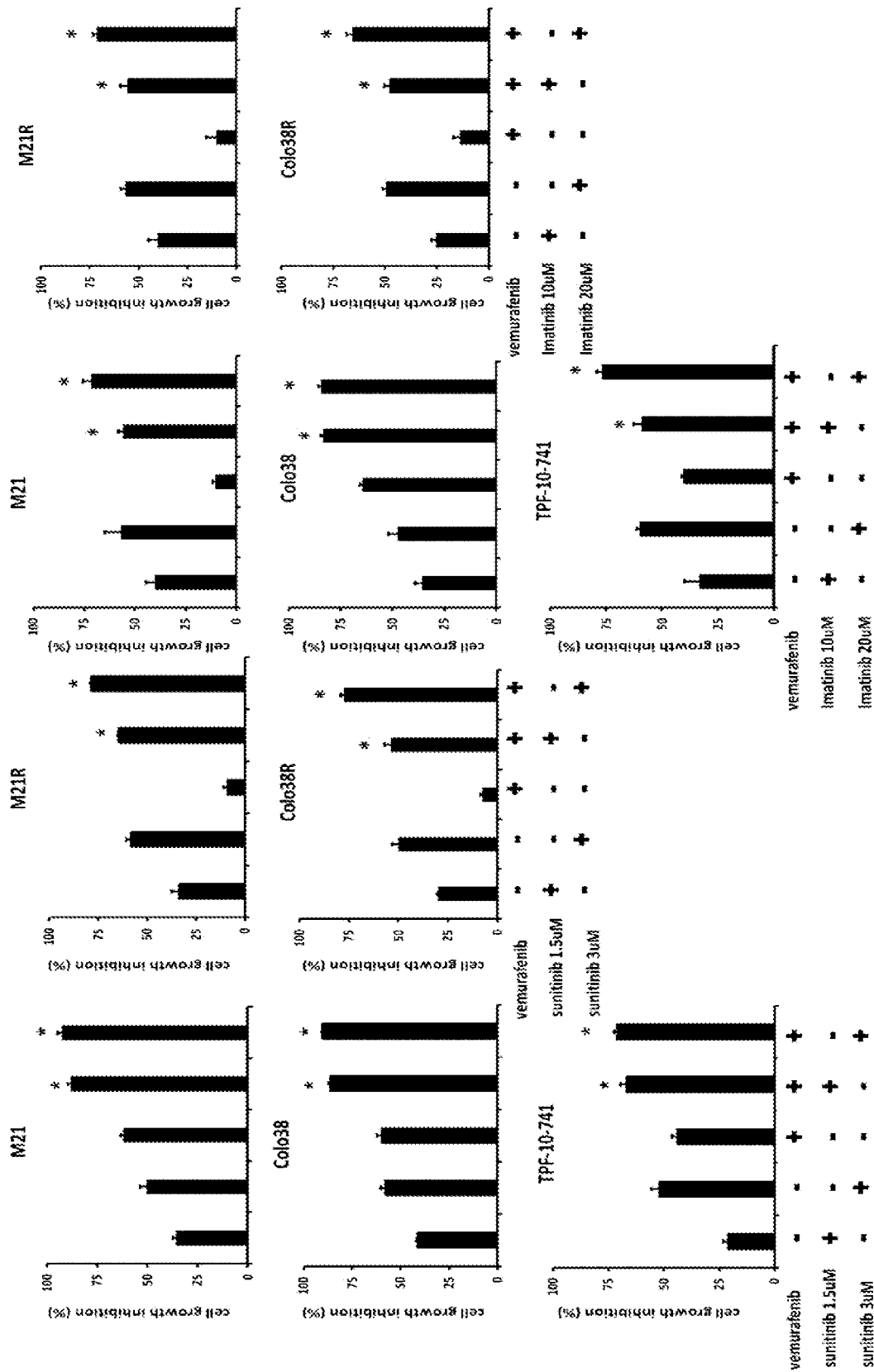


FIG. 9B

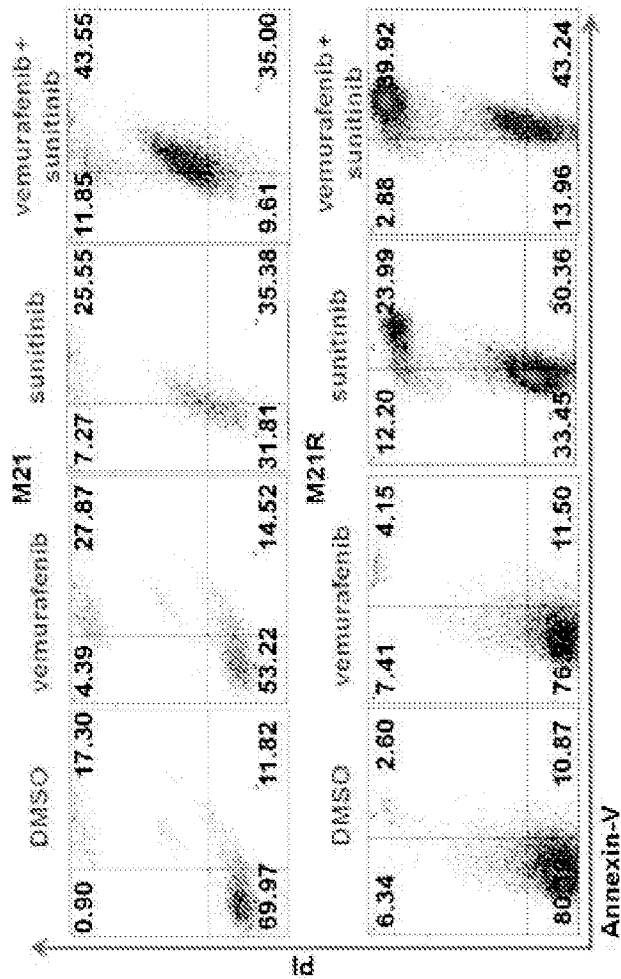
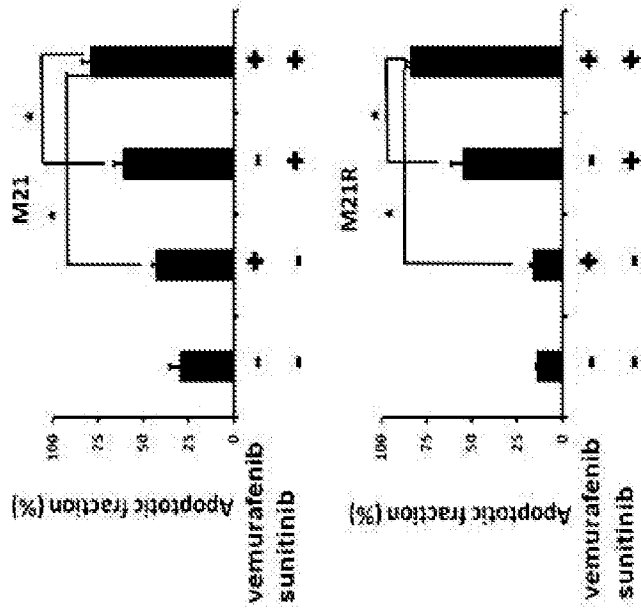


FIG. 10A

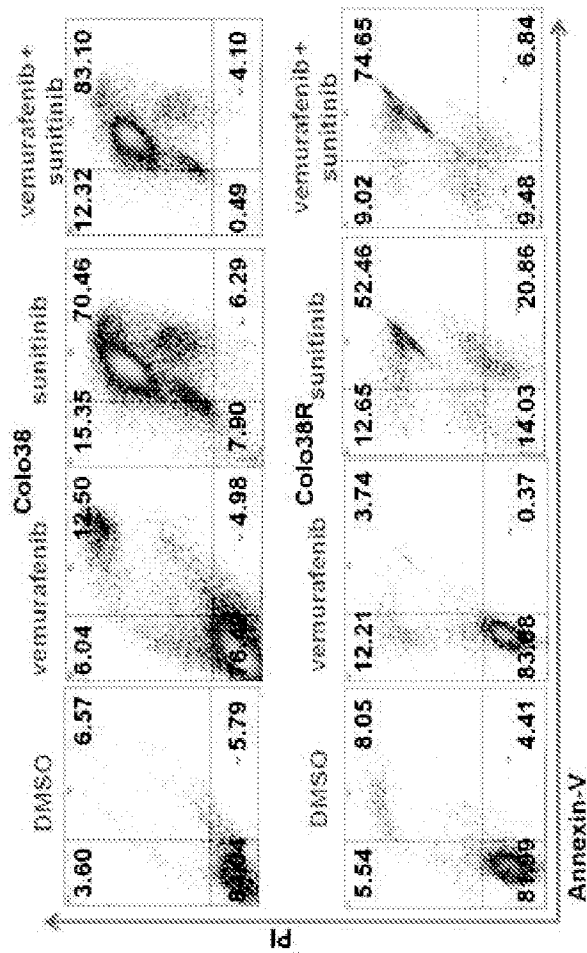
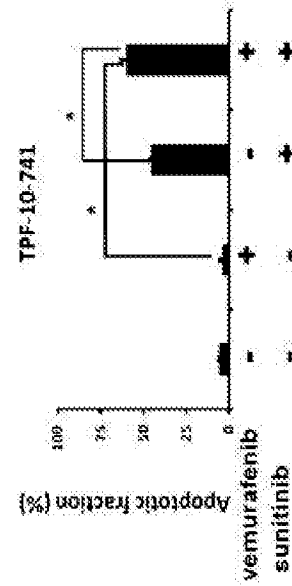
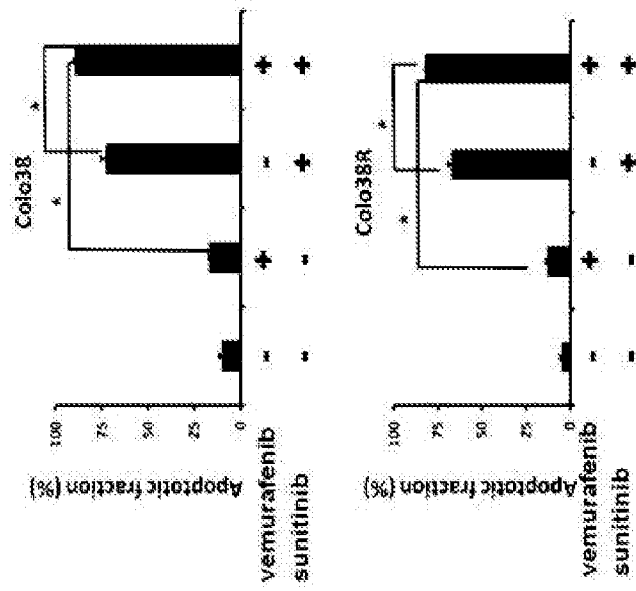


FIG. 10B

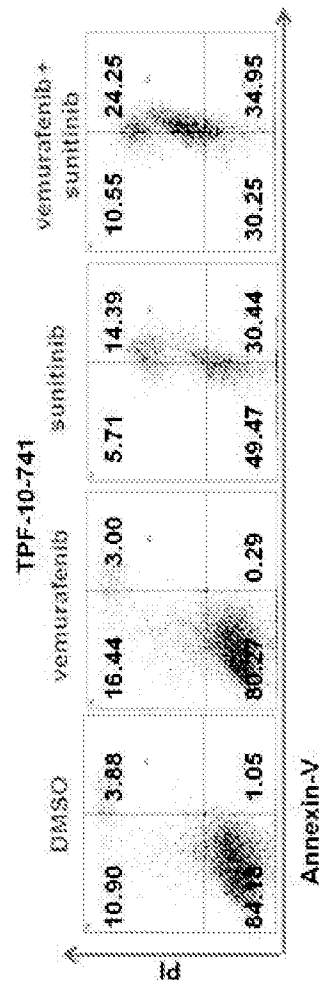


FIG. 10C

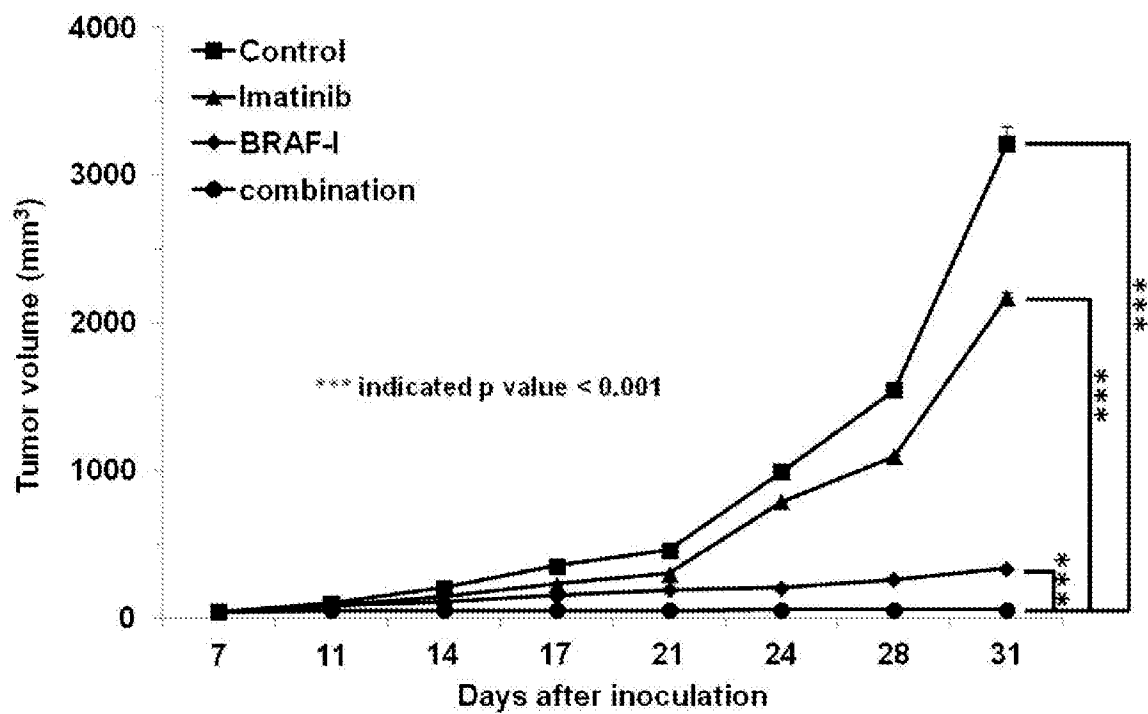


FIG. 11

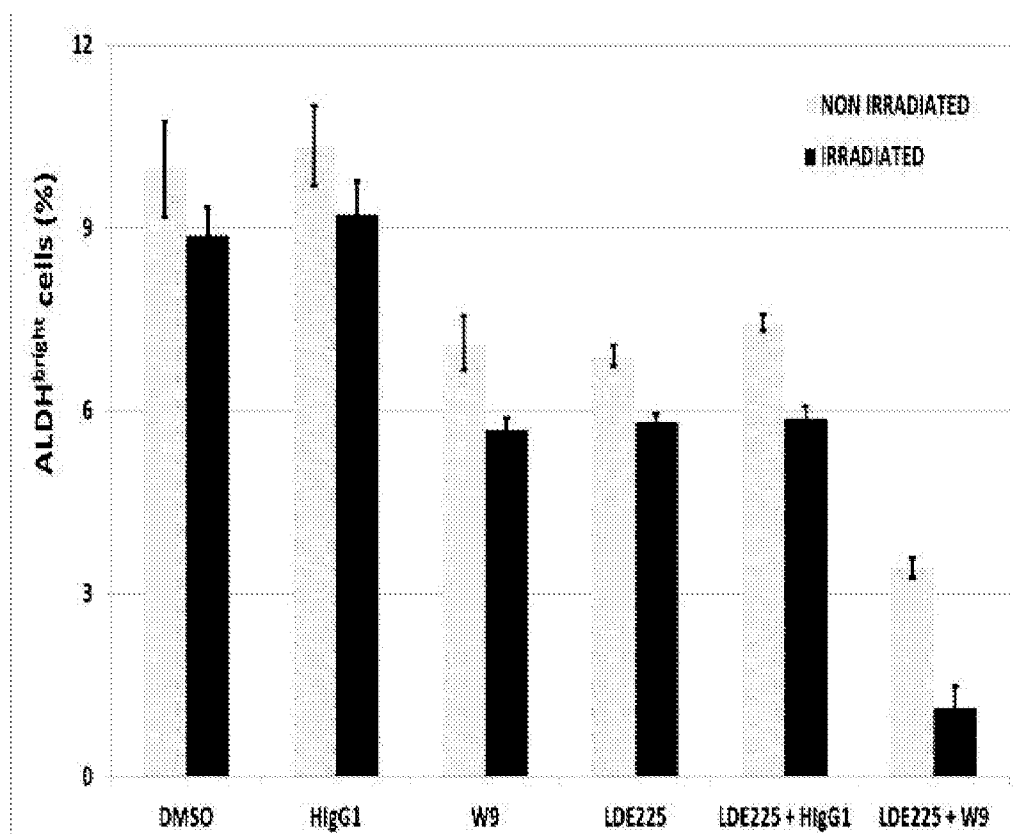


FIG. 12

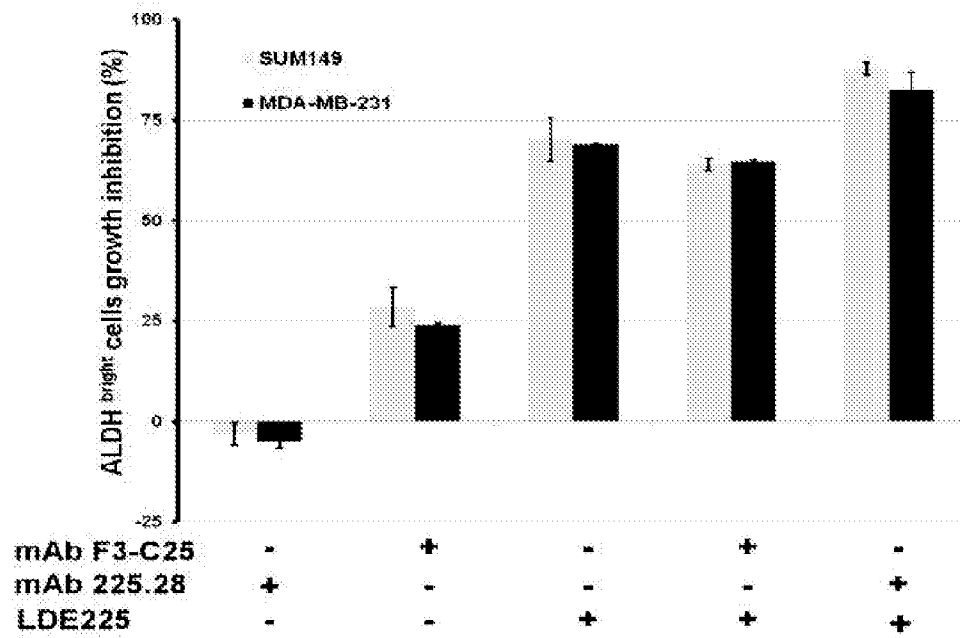


FIG. 13