US008771687B2

(12) **United States Patent**
Ferrone et al.

(10) **Patent No.: US 8,771,687 B2**
(45) **Date of Patent: Jul. 8, 2014**

(54) **METHODS FOR TREATING A TUMOR USING AN ANTIBODY THAT SPECIFICALLY BINDS GRP94**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 245 days.

(21) Appl. No.: **13/309,490**

(22) Filed: **Dec. 1, 2011**

(65) **Prior Publication Data**

US 2012/0148598 A1 Jun. 14, 2012

Related U.S. Application Data

(60) Provisional application No. 61/419,208, filed on Dec. 2, 2010.

(51) **Int. Cl.**
A61K 39/395 (2006.01)

(52) **U.S. Cl.**
USPC **424/133.1**; 424/138.1

(58) **Field of Classification Search**
None
See application file for complete search history.

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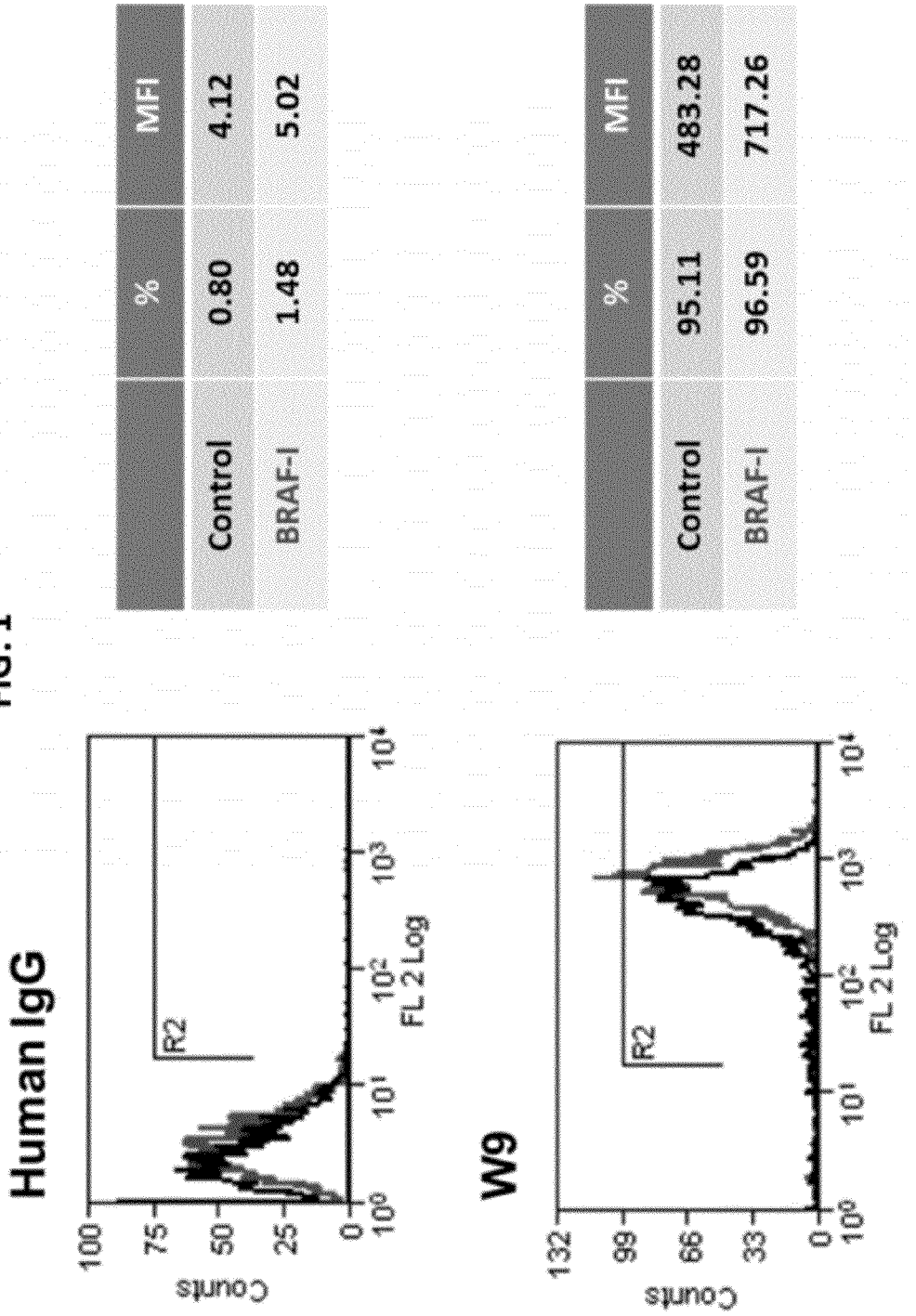
(57) **ABSTRACT**

Combinations of agents that have a synergistic effect for the treatment of a tumor are disclosed herein. These combinations of agents can be used to treat tumors, wherein the cells of the cancer express a mutated BRAF. Methods are disclosed for treating a subject diagnosed with a tumor that expresses a mutated BRAF. The methods include administering to the subject (1) a therapeutically effective amount of an antibody or antigen binding fragment thereof that specifically binds glucose regulated protein (GRP) 94; and (2) a therapeutically effective amount of a BRAF inhibitor. In some embodiments, the tumor is melanoma. In some embodiments the method includes selecting a subject with primary or secondary resistance to a BRAF inhibitor. In further embodiments, treating the tumor comprises decreasing the metastasis of the tumor. In additional embodiments, the BRAF inhibitor comprises PLX4032 or PLX4720.

22 Claims, 9 Drawing Sheets

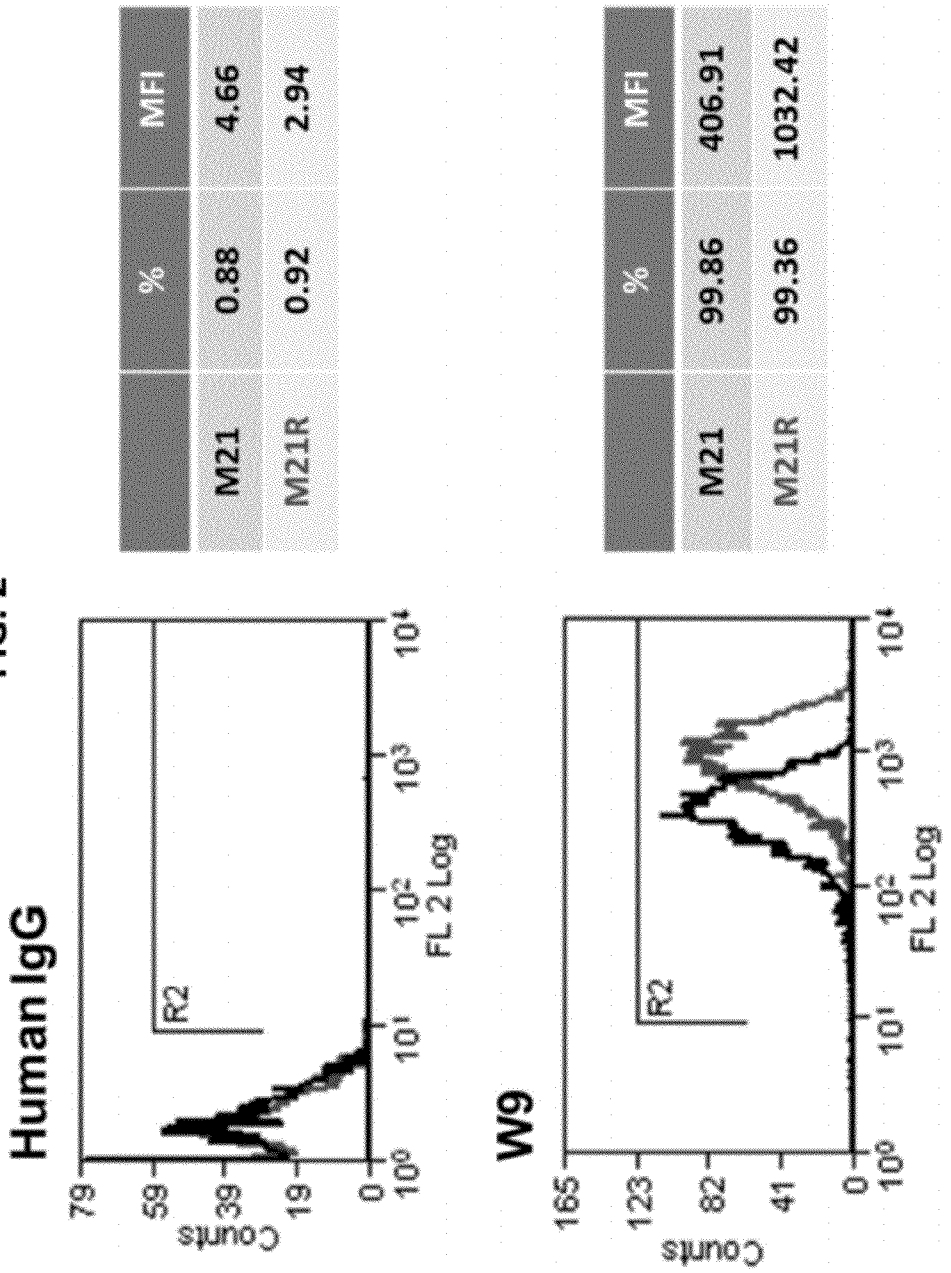
FIG. 1



	%	MFI
Control	0.80	4.12
BRAF-I	1.48	5.02

	%	MFI
Control	95.11	483.28
BRAF-I	96.59	717.26

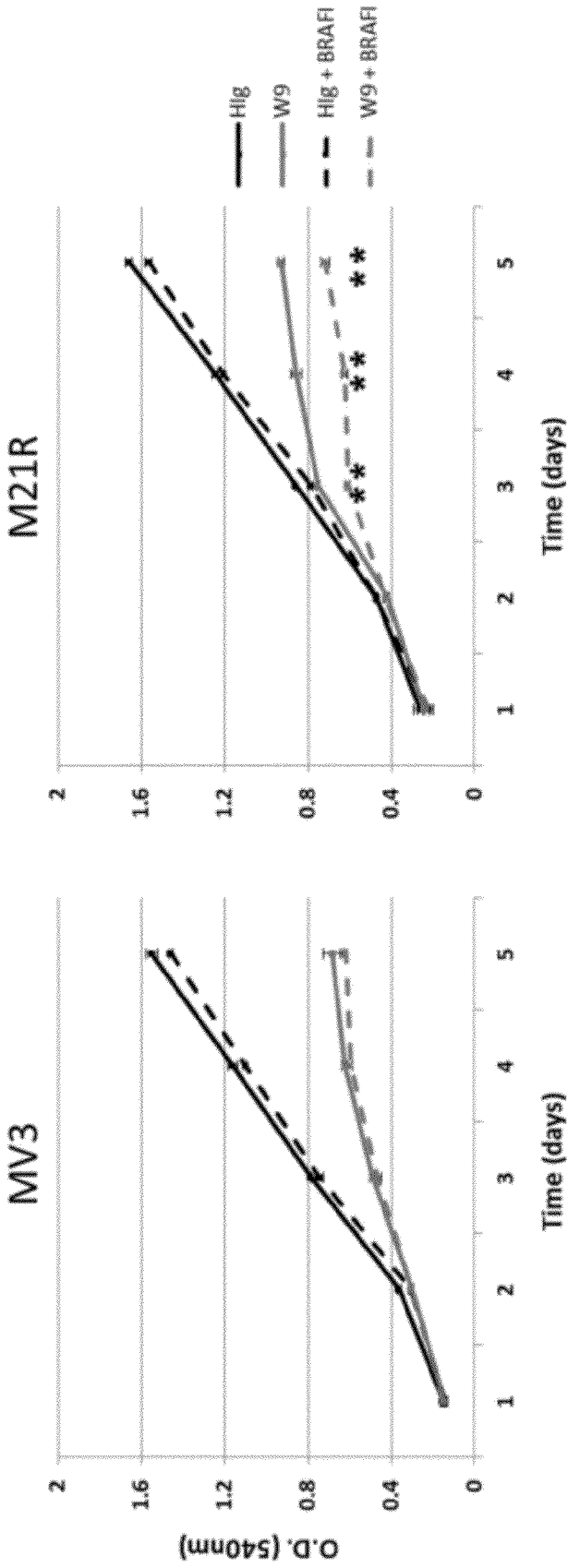
FIG. 2



	%	MFI
M21	0.88	4.66
M21R	0.92	2.94

	%	MFI
M21	99.86	406.91
M21R	99.36	1032.42

FIG. 3


M21R
MV3
Hlg
W9
Hlg + BRAFI
W9 + BRAFI
O.D. (540nm)
Time (days)
Time (days)
0
0.4
0.8
1.2
1.6
2
1
2
3
4
5
**
**
**

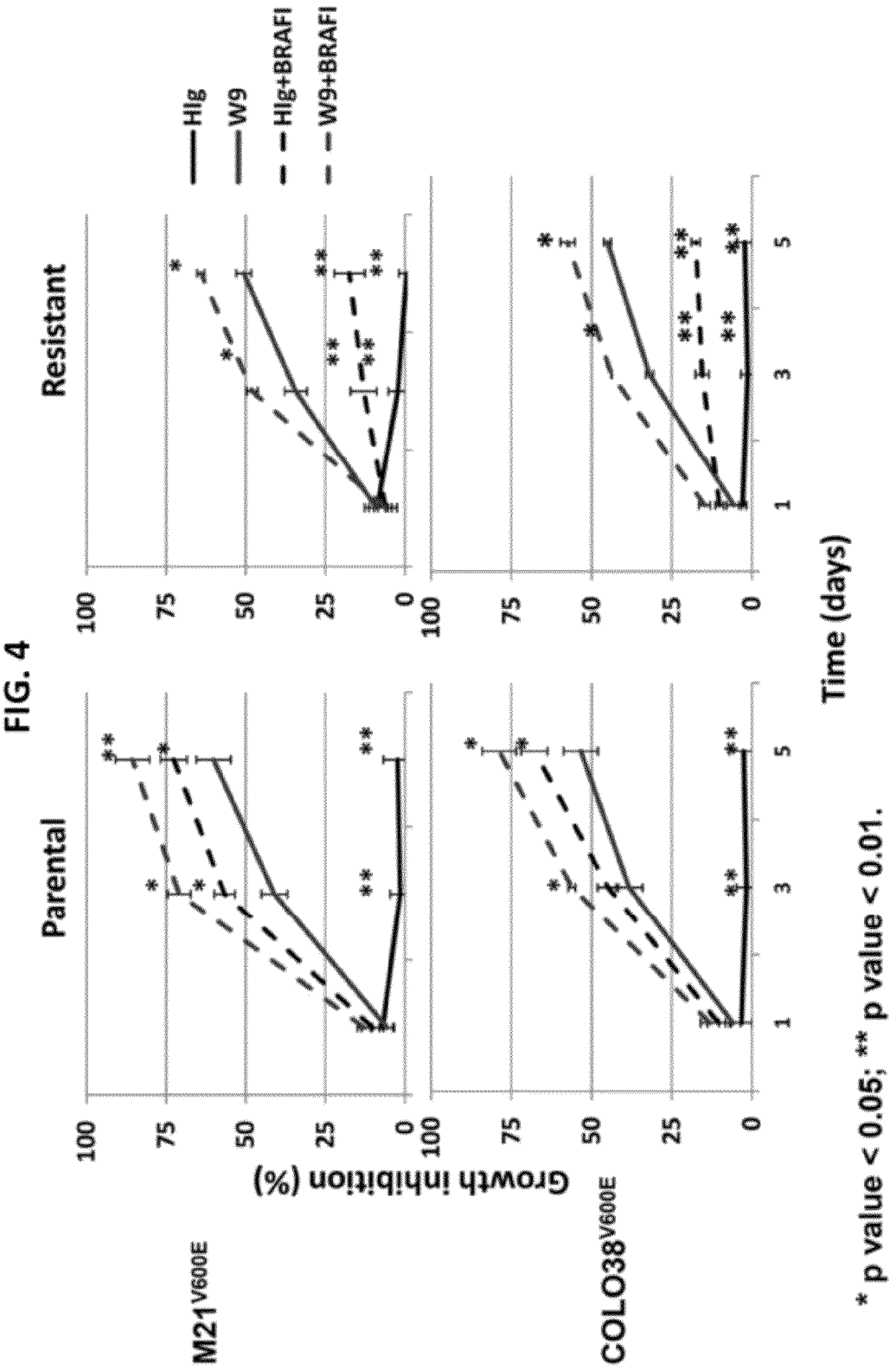

Hlg
W9
Hlg+BRAFI
W9+BRAFI
Parental
Resistant
M21V600E
COLO38V600E
Growth inhibition (%)
Time (days)
100
75
50
25
0
1
3
5
* p value < 0.05; ** p value < 0.01.

FIG. 4

FIG. 5

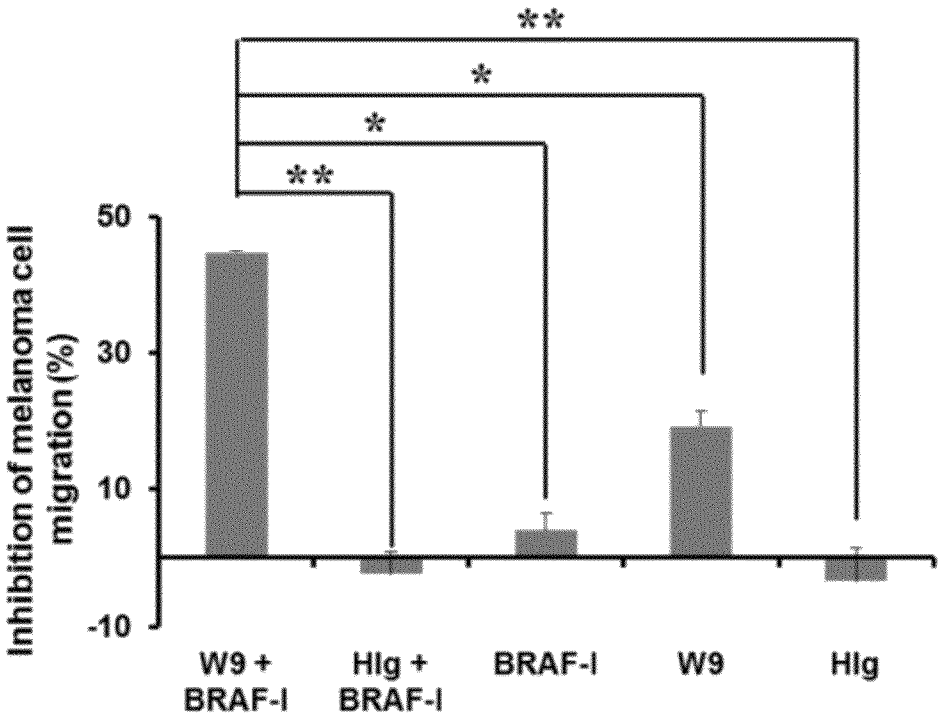

**
*
*
**
50
30
10
-10
Inhibition of melanoma cell migration (%)
W9 + BRAF-I
Hlg + BRAF-I
BRAF-I
W9
Hlg

FIG. 6

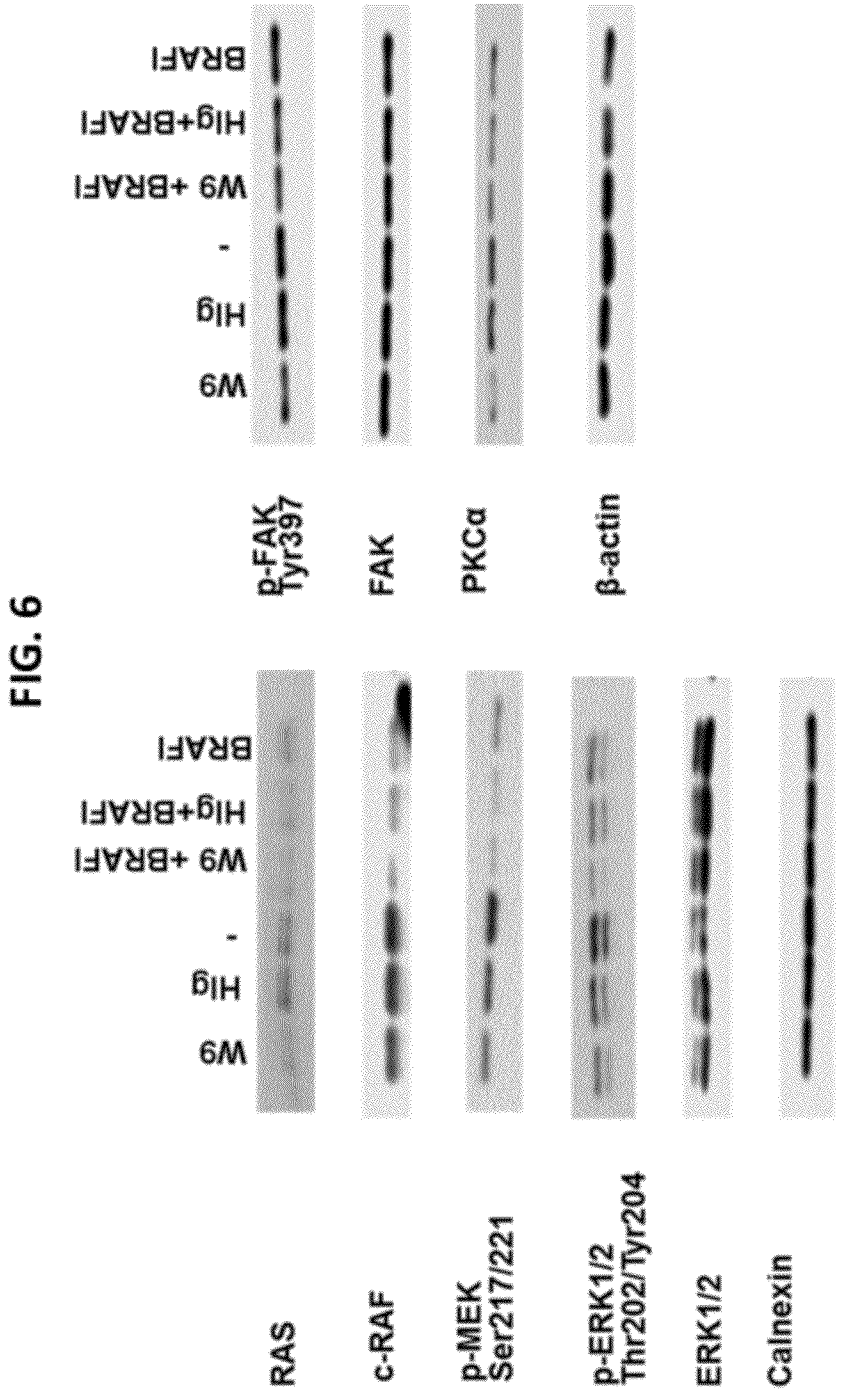

W9
Hlg
-
W9 +BRAFi
Hlg+BRAFi
BRAFi
RAS
c-RAF
p-MEK Ser217/221
p-ERK1/2 Thr202/Tyr204
ERK1/2
Calnexin
p-FAK Tyr397
FAK
PKCα
β-actin

FIG. 7

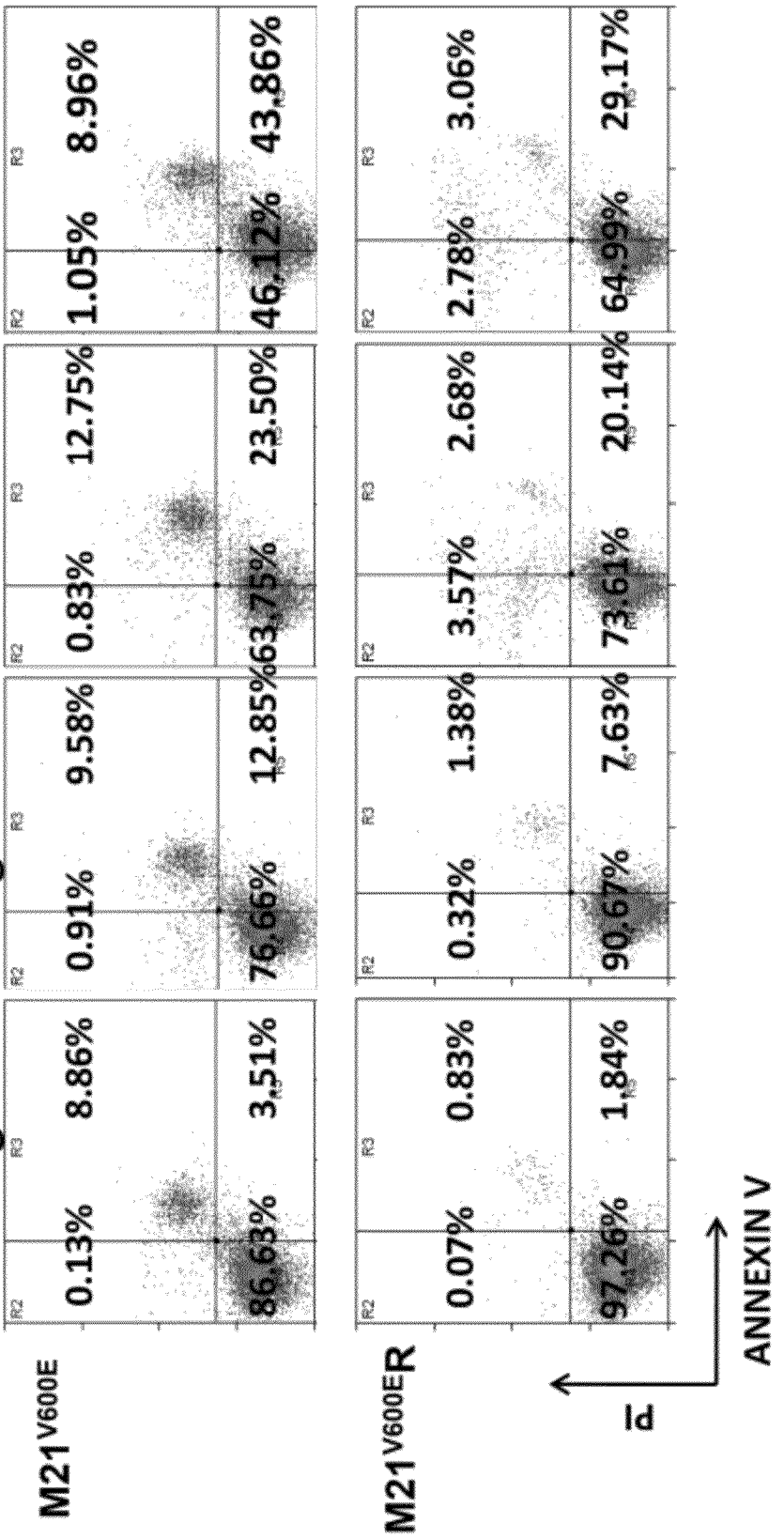

HIg
HIg + BRAF-I
W9
W9 + BRAF-I
M21V600E
M21V600ER
R2
R3
0.13%
8.86%
86.63%
3.51%
0.91%
9.58%
76.66%
12.85%
0.83%
12.75%
63.75%
23.50%
1.05%
8.96%
46.12%
43.86%
0.07%
0.83%
97.26%
1.84%
0.32%
1.38%
90.67%
7.63%
3.57%
2.68%
73.61%
20.14%
2.78%
3.06%
64.99%
29.17%
PI
ANNEXIN V

FIG. 8

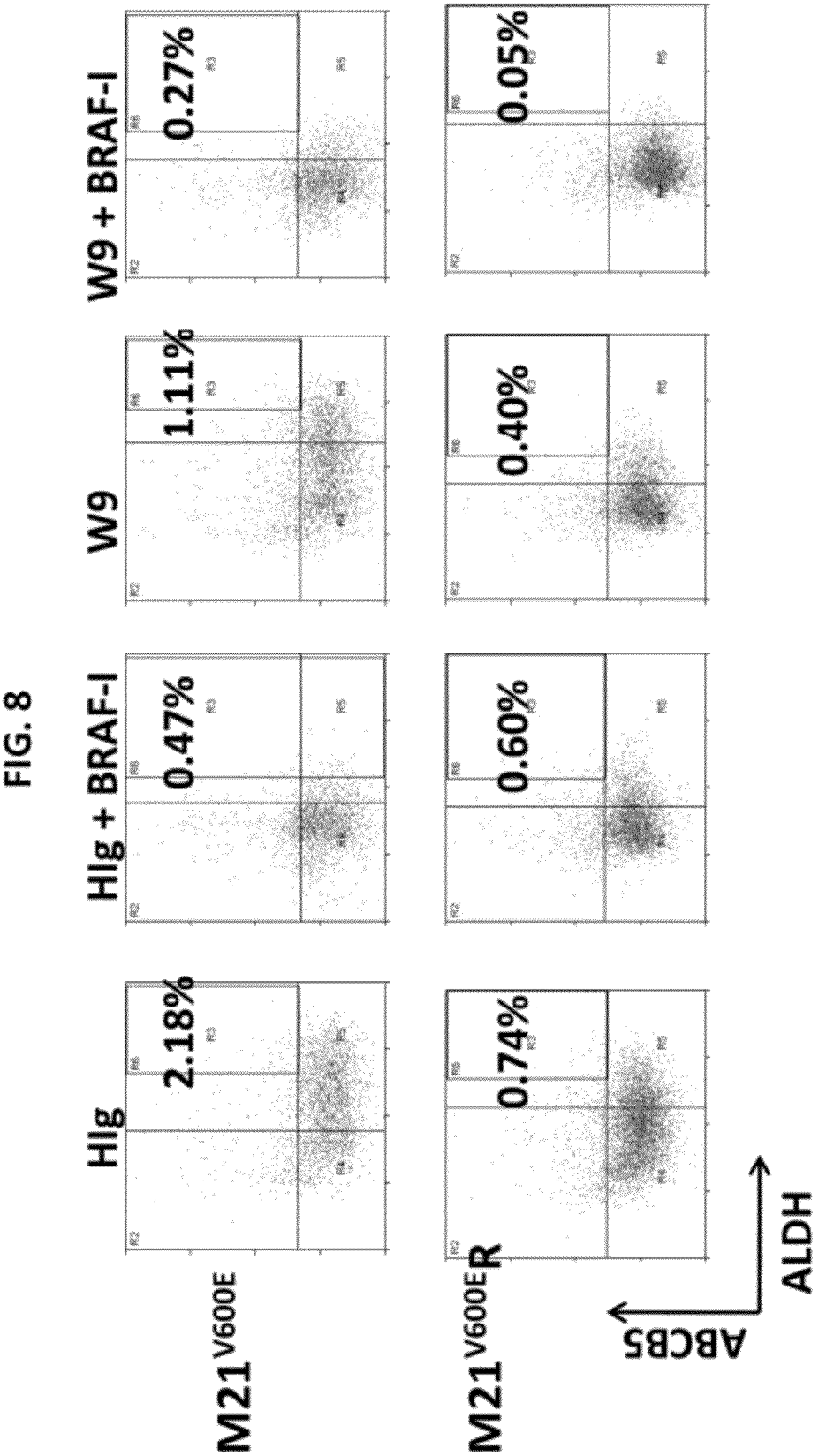
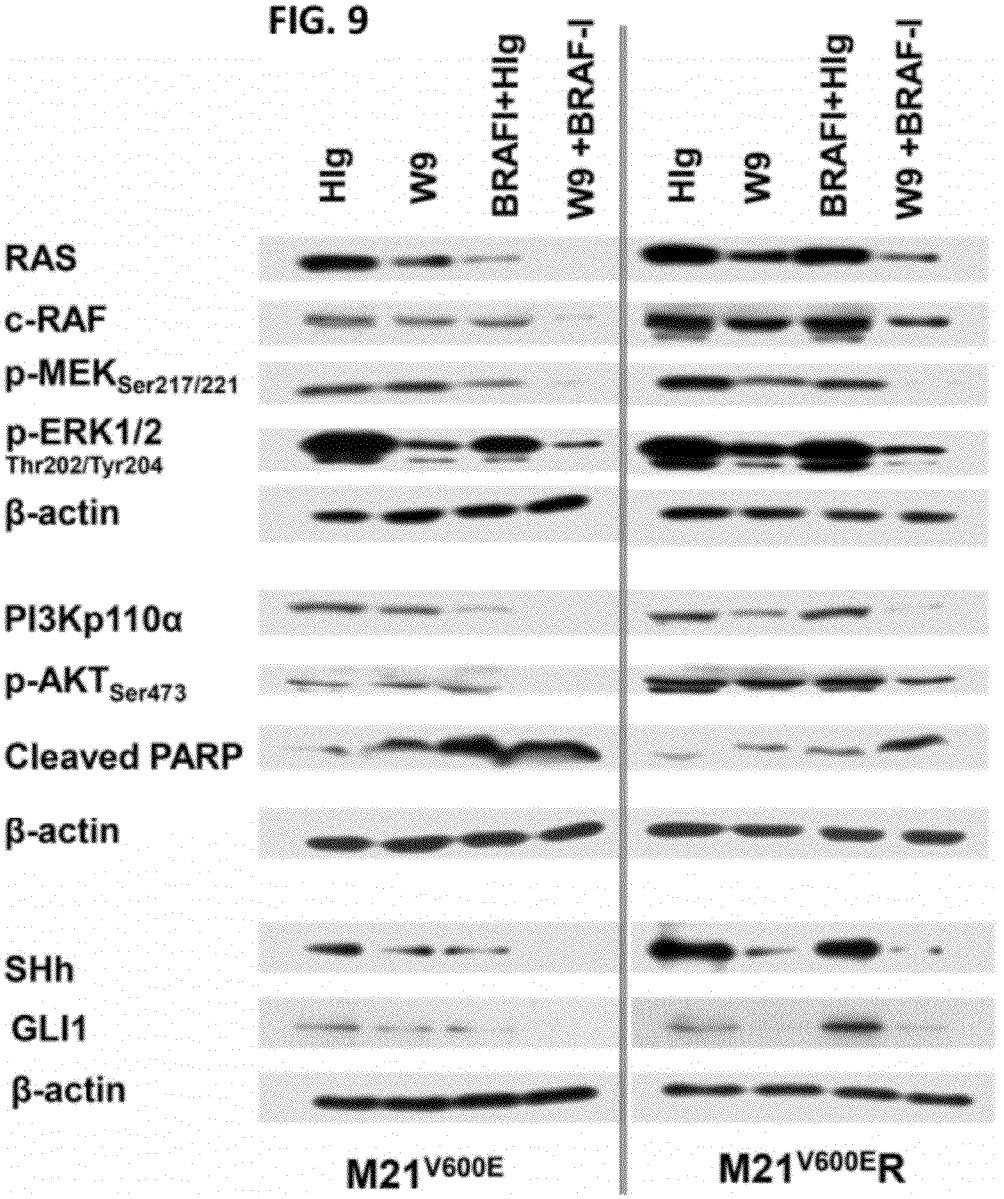

Hlg
Hlg + BRAF-I
W9
W9 + BRAF-I
2.18%
0.47%
1.11%
0.27%
0.74%
0.60%
0.40%
0.05%
M21V600E
M21V600ER
ABCB5
ALDH

FIG. 9


Hlg
W9
BRAFI+Hlg
W9 +BRAF-I
Hlg
W9
BRAFI+Hlg
W9 +BRAF-I
RAS
c-RAF
p-MEKSer217/221
p-ERK1/2
Thr202/Tyr204
β-actin
PI3Kp110α
p-AKTSer473
Cleaved PARP
β-actin
SHh
GLI1
β-actin
M21V600E
M21V600ER

METHODS FOR TREATING A TUMOR USING AN ANTIBODY THAT SPECIFICALLY BINDS GRP94

PRIORITY CLAIM

This claims the benefit of U.S. Provisional Application No. 61/419,208, filed Dec. 2, 2010, which is incorporated by reference herein.

FIELD

This application relates to the treatment of cancer, specifically to the use of a combination of an antibody that specifically binds glucose regulated protein (GRP) 94 and a BRAF inhibitor.

BACKGROUND

Melanoma is a malignant tumor of melanocytes that are predominately found in skin, but can also be found in the bowel and eye. Although melanoma is not the most common form of skin cancer, it causes the majority of skin cancer related deaths. Melanoma incidence and mortality rates in fair-skinned populations are increasing worldwide. Approximately 160,000 cases of malignant melanoma are diagnosed in the world each year. Current treatments include surgical removal of the tumor, adjuvant treatment, chemotherapy, immunotherapy and radiation therapy.

RAF protein kinases are key components of signal transduction pathways by which specific extracellular stimuli elicit precise cellular responses in mammalian cells. Activated cell surface receptors activate ras/rap proteins at the inner aspect of the plasma-membrane which in turn recruit and activate Raf proteins. Activated RAF proteins phosphorylate and activate the intracellular protein kinases MEK1 and MEK2. In turn, activated MEKs catalyze phosphorylation and activation of p42/p44 mitogen-activated protein kinase (MAPK). Several cytoplasmic and nuclear substrates of activated MAPK are known that directly or indirectly contribute to the cellular response to environmental change. Three distinct genes have been identified in mammals that encode Raf proteins: ARAF, BRAF and CRAF (also known as RAF-1).

Inhibitors of RAF kinases have been suggested for use in disruption of tumor cell growth and in the treatment of cancers, such as histiocytic lymphoma, adenocarcinoma, small cell lung cancer, melanoma and pancreatic and breast carcinoma. Specific inhibitors of BRAF mutants, such as V600E ($BRAF^{V600E}$ mutant) are known and have been used for the treatment of cancer. However, some subjects are refractory to treatment with BRAF inhibitors. Furthermore, some subjects develop secondary resistance to BRAF inhibitors, such that regression induced by a BRAF inhibitor is only temporary. Thus, a need remains for agents that augment the effect of BRAF inhibitors, such as a combination of agents of use for treating cancer and for inhibiting secondary resistance to a BRAF inhibitor.

SUMMARY

Combinations of agents that have a synergistic effect for the treatment of cancer are disclosed herein. These combinations of agents can be used to treat cancers, wherein the cells of the cancer express a mutated BRAF. The compositions include a therapeutically effective amount of a BRAF inhibitor.

Methods are disclosed for treating a subject diagnosed with a tumor that expresses a mutated BRAF. The methods include administering to the subject (1) a therapeutically effective amount of an antibody or antigen binding fragment thereof that specifically binds glucose regulated protein (GRP) 94; and (2) a therapeutically effective amount of a BRAF inhibitor.

In some embodiments, the tumor is melanoma. In further embodiments, treating the tumor comprises decreasing the metastasis of the tumor. In additional embodiments, the BRAF inhibitor is vemurafenib (PLX4032). The disclosed methods are of use to treat a subject that has primary or secondary resistance to the BRAF inhibitor. The cells in the tumor can have a BRAF V600E mutation.

In some embodiments, the methods include administering to the subject a therapeutically effective amount of an antibody or antigen binding fragment thereof that specifically binds GRP94. For example, the antibody can be a monoclonal antibody, wherein the heavy chain of the antibody comprises the amino acid sequence set forth as amino acids 26-33 of SEQ ID NO: 3 (CDR1), amino acids 51-58 of SEQ ID NO: 3 (CDR2), and amino acids 97-103 of SEQ ID NO: 3 (CDR3) and/or wherein the light chain of the antibody comprises the amino acid sequence set forth as amino acids 27-32 of SEQ ID NO: 4 (CDR1), amino acids 50-52 of SEQ ID NO: 4 (CDR2), and amino acids 89-97 of SEQ ID NO: 4 (CDR3).

The foregoing and other objects, features, and advantages of the invention will become more apparent from the following detailed description, which proceeds with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1**. Effect of BRAF-I on GRP94 expression by M21 melanoma cell line. Cells (2×10^5/ml) were incubated in RPMI 1640 medium containing 10% FCS with 1 μM of BRAF-I for 14 days. Cells were harvested, stained with GRP94-specific fully human antibody (mAb) W9, human IgG (HIg) as negative control, and analyzed by flow cytometry. Untreated cells were used as control. Percentage of stained cells and mean fluorescence intensity (MFI) are indicated.

FIG. **2**. GRP94 expression in BRAF-I resistant M21 melanoma cell line. The melanoma cell line M21 acquired resistance to BRAF-I PLX4720 following repeated exposures to this inhibitor namely M21R. BRAF-I PLX4720 resistant M21R cells (red) and parental melanoma cells M21 (black) were cell surface stained with GRP94-specific mAb W9. Human immunoglobulin (HIg) was used as control. Stained cells were analyzed with a flow cytometer. Percentage of stained cells and mean fluorescence intensity (MFI) are indicated.

FIG. **3**. Synergic anti-cell growth effect of GRP94-specific mAb W9 combined with BRAF-I PLX 4720 on melanoma cells. Human melanoma cells (MV3, M21R) were seeded (2.5×10^3 cells per well) in a 96-well plate (RPMI 1640 media plus 1% FCS) and treated with GRP94-specific mAb W9, HIg (negative control) in presence of BRAF inhibitor PLX4720 (5 μM) for 1, 2, 3, 4, 5 days at 37° C. in a 5% CO2 atmosphere. Cells were then tested by MTT assay. The O.D. values at 540 nm indicate the living cells. *p value<0.05; **p value<0.01 (W9 vs W9+BRAF-1).

FIG. **4**. Synergic anti-cell growth effect of GRP94-specific mAb W9 combined with BRAF-I PLX 4032 on melanoma cells. Human melanoma cells (M21, M21R, Colo38, Colo38R) were seeded (2.5×10^3 cells per well) in a 96-well plate (RPMI 1640 media plus 1% FCS) and treated with

GRP94-specific mAb W9, HIg (negative control) in presence of BRAF inhibitor PLX4032 (500 nM) for 1, 3, 5 days at 37° C. in a 5% CO2 atmosphere. Cells were then tested by MTT assay. The O.D. values at 540 nm indicate the living cells. *p value<0.05; **p value<0.01 (W9 vs W9+BRAF-1).

FIG. **5**. Inhibition by GRP94-specific antibody W9 combined with BRAF inhibitor PLX4720 of M21R cells migration. M21R cells were incubated in RPMI 1640 medium containing 1% FCS with W9 Ab, HIg, BRAF Inhibitor PLX4720 (BRAF-I) combined with W9 Ab, BRAF-I combined with HIg, or BRAF-I in a 24-trans-well plate (2.5×10^4 per well) for 3 days in a migration assay. Cells incubated in RPMI 1640 medium containing 1% FCS was used as a reference for 100% cell migration. The results are expressed as % inhibition of migration, utilizing the values obtained in RPMI 1640 medium containing 1% FCS without Ab as a reference. *p value<0.05; **p value<0.01.

FIG. **6**. Inhibition of melanoma cell M21R signaling pathways RAS-MEK-ERK and FAK by BRAF-I in combination with GRP94-specific Ab W9. The human melanoma cell M21R was serum starved for 3 days then seeded at the concentration of 1.0×10^5 per well in a 6-well plate in RPMI 1640 medium without serum and incubated with W9 Ab, the human IgG (HIg), or untreated in presence of BRAF inhibitor PLX4720 (5 μM) for 72 hours at 37° C. Cell lysate were tested in Western blot with anti-RAS, c-RAF, phosphorylated (p)-MEK, p-ERK1/2, ERK1/2, (p)-FAK (Tyr397), FAK, and PKCα mAbs. Calnexin and β-actin was used as the loading control. The density of resultant bands was determined with IMAGEJ™ software, normalized to that of Calnexin and β-actin, shown below the respective bands. Data are expressed as the percentage of the expression in untreated control cells.

FIG. **7**. Synergic pro-apoptosis of GRP94-specific mAb W9 combined with BRAF-I PLX 4032 on melanoma cells. Human melanoma cells (M21 and M21R) were starved for 12 hours and seeded at a density of 2×105/ml in a 6-well plate and treated for 6 hours with BRAF-I PLX4032 (500 nM) and Grp94-specific mAb W9 (20 μg/ml) in RPMI 1640 medium plus 1.5% FCS. Cells were stained with Annexin V-FITC and PI, and evaluated for apoptosis by flow cytometry according to the manufacturer's protocol (BD PharMingen, San Diego, Calif., USA). The early apoptotic cells (annexin V-positive, PI-negative) were determined using a flow cytometer.

FIG. **8**. Synergic inhibition by Grp94-specific mAb W9 combined with BRAF-I of BRAFV600E mutant and BRAF-I resistant melanoma CICs proliferation in vitro. Cells growing in the exponential phase were seeded at a density of 2×105/ml. The cells were treated for 3 days with BRAF-I PLX4032 (500 nM) and Grp94-specific mAb W9 (20 μg/ml) in RPMI 1640 medium plus with 1.5% FCS. Cells were stained with ALDEFLUOR® according to the manufacturer's protocol (Stem Cell Technologies). Incubation of cells with ALDEFLUOR® in the presence the ALDH1-specific inhibitor diethylaminobenzaldehyde (DEAB), was used as a negative staining control for the assay. Then cells were stained with ABCB5-specific mAb RK1 (1 μg/ml) for 30 min at 4° C., and incubated with APC-conjugated secondary mAb (1:200) (Jackson Immunoresearch).

FIG. **9**. Synergic targeting multiple signaling pathways by GRP94-specific mAb W9 combined with BRAF-I PLX 4032 on melanoma cells. Cells growing in the exponential phase were seeded at a density of 2×10^5/ml. The cells were treated for 3 days with BRAF-I PLX4032 (500 nM) and Grp94-specific mAb W9 (5 μg/ml) in RPMI 1640 medium plus with 2% FCS. Then the 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, Calif.)]. Equal amount of proteins (80 μg per well) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane of 0.45 μm pore size (Millipore, Bedford, Mass.). After blocking the membranes with 5% nonfat dry milk plus 2% BSA at room temperature for 2 hrs, membranes were incubated overnight at 4° C. with anti-RAS, c-RAF, phosphorylated (p)-MEK (Ser217/221), p-ERK (Thr202/Tyr204), p-AKT, PI3 Kp110α, cleaved PARP, SHg, GLI and β-actin mAb. The appropriate peroxidase-conjugated secondary mAb (Cell signaling technology) was added and incubation was continued at room temperature for an additional 1 hr. After washing the membrane, the bound antibodies were detected using ECL PLUS™ Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK), and bands were visualized using the FOTO/ANALYST® Investigator Eclipse System (Fotodyne Incorporate, Hartland, Wis.). The β-actin was used as the protein loading control.

SEQUENCE LISTING

The Sequence Listing is submitted as an ASCII text file [8123-86316-03_Sequence_Listing.txt, Dec. 1, 2011, 21.8 KB], which is incorporated by reference herein.

The nucleic and amino acid sequences listed are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids, as defined in 37 C.F.R. 1.822. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand.

SEQ ID NO: 1 is the amino acid sequence of GRP94.

SEQ ID NO: 2 is an exemplary nucleic acid sequence encoding GRP94.

SEQ ID NO: 3 is the amino acid sequence of a heavy chain of an antibody that specifically binds GRP94.

SEQ ID NO: 4 is the amino acid sequence of a light chain of an antibody that specifically binds GRP94.

SEQ ID NO: 5 is the amino acid sequence of a *Pseudomonas* exotoxin.

SEQ ID NOs: 6-7 are the amino acid sequence of *Pseudomonas* exotoxin motifs.

DETAILED DESCRIPTION

Disclosed herein are methods for treating a tumor in a subject. The methods include selecting a subject with a BRAF mutation, and administering to the subject a therapeutically effective amount of 1 an antibody that specifically binds glucose regulated protein (GRP) 94; and 2) a BRAF inhibitor. The use of a combination of an antibody that specifically binds GRP94 and a BRAF inhibitor for the treatment of cancer provides an unexpectedly superior result for the treatment of a tumor, wherein cells in the tumor comprise a BRAF mutation. In some embodiments, the tumor is a melanoma. In other embodiments, the BRAF mutation is a V600E mutation. In additional embodiments, the subject has resistance to the BRAF inhibitor. In some specific non-limiting examples, the BRAF inhibitor is PLX4032 or PLX4720.

Disclosed herein are methods to treat a subject diagnosed with a tumor, such as a tumor that expresses GRP94. Methods are also provided for treating a melanoma. Melanoma includes spreading melanoma, nodular melanoma, acral lentiginous melanoma, and lentigo maligna (melanoma). However, the methods disclosed herein can also be used to treat other cancers, such breast cancer, prostate cancer, ovarian

cancer, thyroid cancer, colon cancer, stomach cancer, pancreatic cancer, glioma, chordoma, chondrosarcoma, glioma or a squamous cell carcinoma. Squamous cell carcinomas include, but are not limited to head and neck squamous cell carcinoma, and squamous cell cancers of the skin, lung, prostate, esophagus, vagina and cervix.

In some embodiments, the disclosed methods can also be used to prevent metastasis or decrease the number of micrometastases, such as micrometastases to regional lymph nodes.

Pharmaceutical compositions are also provided that include a therapeutically effective amount of an antibody that specifically binds GRP94 and a therapeutically effective amount of a BRAF inhibitor. In specific, non-liming examples, the BRAF inhibitor is PLX4032 or PLX4720.

TERMS

Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found in Benjamin Lewin, *Genes V*, published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew et al. (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

In order to facilitate review of the various embodiments of this disclosure, the following explanations of specific terms are provided:

Animal:

Living multi-cellular vertebrate organisms, a category that includes, for example, mammals and birds. The term mammal includes both human and non-human mammals. Similarly, the term "subject" includes both human and veterinary subjects.

Antibody:

A polypeptide ligand comprising at least a light chain or heavy chain immunoglobulin variable region which specifically recognizes and specifically binds an epitope of an antigen, such as GRP94, or a fragment thereof. Antibodies are composed of a heavy and a light chain, each of which has a variable region, termed the variable heavy (V_H) region and the variable light (V_L) region. Together, the V_H region and the V_L region are responsible for binding the antigen recognized by the antibody.

Antibodies include intact immunoglobulins and the variants and portions of antibodies well known in the art, such as Fab fragments, Fab' fragments, $F(ab)'_2$ fragments, single chain Fv proteins ("scFv"), and disulfide stabilized Fv proteins ("dsFv"). A scFv protein is a fusion protein in which a light chain variable region of an immunoglobulin and a heavy chain variable region of an immunoglobulin are bound by a linker, while in dsFvs, the chains have been mutated to introduce a disulfide bond to stabilize the association of the chains. The term also includes genetically engineered forms such as chimeric antibodies (for example, humanized murine antibodies), heteroconjugate antibodies (such as, bispecific antibodies). See also, *Pierce Catalog and Handbook,* 1994-1995 (Pierce Chemical Co., Rockford, Ill.); Kuby, J., *Immunology,* 3rd Ed., W.H. Freeman & Co., New York, 1997.

Typically, a naturally occurring immunoglobulin has heavy (H) chains and light (L) chains interconnected by disulfide bonds. There are two types of light chain, lambda (λ) and kappa (k). There are five main heavy chain classes (or isotypes) which determine the functional activity of an antibody molecule: IgM, IgD, IgG, IgA and IgE.

Each heavy and light chain contains a constant region and a variable region, (the regions are also known as "domains"). In combination, the heavy and the light chain variable regions specifically bind the antigen. Light and heavy chain variable regions contain a "framework" region interrupted by three hypervariable regions, also called "complementarity-determining regions" or "CDRs." The extent of the framework region and CDRs has been defined (see, Kabat et al., *Sequences of Proteins of Immunological Interest*, U.S. Department of Health and Human Services, 1991, which is hereby incorporated by reference). The Kabat database is now maintained online. The sequences of the framework regions of different light or heavy chains are relatively conserved within a species, such as humans. The framework region of an antibody, that is the combined framework regions of the constituent light and heavy chains, serves to position and align the CDRs in three-dimensional space.

The CDRs are primarily responsible for binding to an epitope of an antigen. The CDRs of each chain are typically referred to as CDR1, CDR2, and CDR3, numbered sequentially starting from the N-terminus, and are also typically identified by the chain in which the particular CDR is located. Thus, a V_H CDR3 is located in the variable domain of the heavy chain of the antibody in which it is found, whereas a V_L CDR1 is the CDR1 from the variable domain of the light chain of the antibody in which it is found. An antibody that binds GRP94 will have a specific V_H region and the V_L region sequence, and thus specific CDR sequences. Antibodies with different specificities (i.e. different combining sites for different antigens) have different CDRs. Although it is the CDRs that vary from antibody to antibody, only a limited number of amino acid positions within the CDRs are directly involved in antigen binding. These positions within the CDRs are called specificity determining residues (SDRs).

References to "V_H" or "VH" refer to the variable region of an immunoglobulin heavy chain, including that of an Fv, scFv, dsFv or Fab. References to "V_L" or "VL" refer to the variable region of an immunoglobulin light chain, including that of an Fv, scFv, dsFv or Fab.

A "monoclonal antibody" is an antibody produced by a single clone of B-lymphocytes or by a cell into which the light and heavy chain genes of a single antibody have been transfected. Monoclonal antibodies are produced by methods known to those of skill in the art, for instance by making hybrid antibody-forming cells from a fusion of myeloma cells with immune spleen cells. Monoclonal antibodies include humanized monoclonal antibodies.

A "chimeric antibody" has framework residues from one species, such as human, and CDRs (which generally confer antigen binding) from another species, such as a murine antibody that specifically binds GRP94.

A "human" antibody (also called a "fully human" antibody) is an antibody that includes human framework regions and all of the CDRs from a human immunoglobulin. In one example, the framework and the CDRs are from the same originating human heavy and/or light chain amino acid sequence. However, frameworks from one human antibody can be engineered to include CDRs from a different human antibody. A "humanized" immunoglobulin is an immunoglobulin including a human framework region and one or more CDRs from a non-human (for example a mouse, rat, or synthetic) immunoglobulin. The non-human immunoglobulin providing the CDRs is termed a "donor," and the human immunoglobulin providing the framework is termed an "acceptor." In one embodiment, all the CDRs are from the

donor immunoglobulin in a humanized immunoglobulin. Constant regions need not be present, but if they are, they must be substantially identical to human immunoglobulin constant regions, i.e., at least about 85-90%, such as about 95% or more identical. Hence, all parts of a humanized immunoglobulin, except possibly the CDRs, are substantially identical to corresponding parts of natural human immunoglobulin sequences. A "humanized antibody" is an antibody comprising a humanized light chain and a humanized heavy chain immunoglobulin. A humanized antibody binds to the same antigen as the donor antibody that provides the CDRs. The acceptor framework of a humanized immunoglobulin or antibody may have a limited number of substitutions by amino acids taken from the donor framework. Humanized or other monoclonal antibodies can have additional conservative amino acid substitutions which have substantially no effect on antigen binding or other immunoglobulin functions. Humanized immunoglobulins can be constructed by means of genetic engineering (see for example, U.S. Pat. No. 5,585,089).

Binding affinity: Affinity of an antibody for an antigen. In one embodiment, affinity is calculated by a modification of the Scatchard method described by Frankel et al., *Mol. Immunol.,* 16:101-106, 1979. In another embodiment, binding affinity is measured by an antigen/antibody dissociation rate. In another embodiment, a high binding affinity is measured by a competition radioimmunoassay. In another embodiment, binding affinity is measured by ELISA. An antibody that "specifically binds" an antigen, such as GRP94 with a high affinity and does not significantly bind other unrelated antigens.

BRAF:

A member of the Raf kinase family of serine/threonine-specific protein kinases. This protein plays a role in regulating the MAP kinase/ERKs signaling pathway, which affects cell division, differentiation, and secretion. BRAF transduces cellular regulatory signals from Ras to MEK in vivo. BRAF is also referred to as v-raf murine sarcoma viral oncogene homolog B1.

BRAF Mutant:

A mutated form of BRAF that has increased basal kinase activity relative to the basal kinase activity of wild type BRAF is also an activated form of BRAF. More than 30 mutations of the BRAF gene that are associated with human cancers have been identified. The frequency of BRAF mutations in melanomas and nevi are 80%. In 90% of the cases, a Glu for Val substitution at position 600 (referred to as V600E) in the activation segment has been found in human cancers. This mutation is observed in papillary thyroid cancer, colorectal cancer and melanoma. Other mutations which have been found are R462I, I463S, G464E, G464V, G466A, G466E, G466V, G469A, G469E, N581S, E585K, D594V, F595L, G596R, L597V, T599I, V600D, V600K, V600R, K601E or A728V. Most of these mutations are clustered to two regions: the glycine-rich P loop of the N lobe and the activation segment and flanking regions. A mutated form of BRAF that induces focus formation more efficiently than wild type BRAF is also an activated form of BRAF.

Breast Cancer:

A neoplastic condition of breast tissue that can be benign or malignant. The most common type of breast cancer is ductal carcinoma. Ductal carcinoma in situ is a non-invasive neoplastic condition of the ducts. Lobular carcinoma is not an invasive disease but is an indicator that a carcinoma may develop. Infiltrating (malignant) carcinoma of the breast can be divided into stages (I, IIA, IIB, IIIA, IIIB, and IV).

Chemotherapeutic Agents:

Any chemical agent with therapeutic usefulness in the treatment of diseases characterized by abnormal cell growth. Such diseases include tumors, neoplasms, and cancer as well as diseases characterized by hyperplastic growth such as psoriasis. In one embodiment, a chemotherapeutic agent is an agent of use in treating a melanoma or another tumor. In one embodiment, a chemotherapeutic agent is a radioactive compound. One of skill in the art can readily identify a chemotherapeutic agent (see for example, Slapak and Kufe, *Principles of Cancer Therapy*, Chapter 86 in Harrison's Principles of Internal Medicine, 14th edition; Perry et al., *Chemotherapy*, Ch. 17 in Abeloff, Clinical Oncology 2nd ed., © 2000 Churchill Livingstone, Inc; Baltzer, L., Berkery, R. (eds.): *Oncology Pocket Guide to Chemotherapy,* 2nd ed. St. Louis, Mosby-Year Book, 1995; Fischer, D. S., Knobf, M. F., Durivage, H. J. (eds): *The Cancer Chemotherapy Handbook,* 4th ed. St. Louis, Mosby-Year Book, 1993). Combination chemotherapy is the administration of more than one agent to treat cancer. One example is the administration of an antibody that binds GRP94, used in combination with a BRAF inhibitor, such as a chemical compound.

Decrease in Survival:

As used herein, "decrease in survival" refers to a decrease in the length of time before death of a patient, or an increase in the risk of death for the patient. A decrease in survival also can refer to a decrease in the average time to death in a group, such as a group of patients diagnosed with a cancer, such as melanoma.

Diagnosing:

Refers to the process of identifying the nature or cause of a disease or disorder.

Effector Molecule:

The portion of a chimeric molecule that is intended to have a desired effect on a cell to which the chimeric molecule is targeted. Effector molecule is also known as an effector moiety (EM), therapeutic agent, or diagnostic agent, or similar terms.

Therapeutic agents include such compounds as nucleic acids, proteins, peptides, amino acids or derivatives, glycoproteins, radioisotopes, lipids, carbohydrates, or recombinant viruses. Nucleic acid therapeutic and diagnostic moieties include antisense nucleic acids, derivatized oligonucleotides for covalent cross-linking with single or duplex DNA, and triplex forming oligonucleotides. Alternatively, the molecule linked to a targeting moiety, such as an anti-GRP94 antibody, may be an encapsulation system, such as a liposome or micelle that contains a therapeutic composition such as a drug, a nucleic acid (such as an antisense nucleic acid), or another therapeutic moiety that can be shielded from direct exposure to the circulatory system. Means of preparing liposomes attached to antibodies are well known to those of skill in the art (see, for example, U.S. Pat. No. 4,957,735; and Connor et al., *Pharm. Ther.* 28:341-365, 1985). Diagnostic agents or moieties include radioisotopes and other detectable labels. Detectable labels useful for such purposes are also well known in the art, and include radioactive isotopes such as ^{35}S, ^{11}C, ^{13}N, ^{15}O, ^{18}F, ^{19}F, ^{99m}Tc, ^{131}I, ^{3}H, ^{14}C, ^{15}N, ^{90}Y, ^{99}Tc, ^{111}In and ^{125}I, fluorophores, chemiluminescent agents, and enzymes.

Epitope:

An antigenic determinant. These are particular chemical groups or peptide sequences on a molecule that are antigenic, i.e. that elicit a specific immune response. An antibody specifically binds a particular antigenic epitope on a polypeptide, such as GRP94.

Glucose Regulated Protein (GRP)94 (Also Known as Endoplasmin):

A protein which is the endoplasmic reticulum (ER)-resident member of the heat-shock-protein 90 (Hsp90) family. In vivo, hsp90 and GRP94 interact with client proteins and function to protect them from ubiquitin-dependent proteasomal degradation. Although the GRP94 protein is expressed constitutively in all cell types, its expression is up-regulated under various stress conditions including low glucose levels, low extracellular pH, expression of mutated proteins, and viral infections. Heat-shock proteins have a cytoprotective function and modulate apoptosis directly or indirectly.

It has been shown that expression of GRP94 is increased in tumor cells, including hepatocellular carcinoma, colorectal carcinoma and lung cancer cells, and that GRP94 has an anti-apoptotic effect on some tumor cells. Moreover, increased levels of GRP94 were observed when a chronic hepatitis B virus (HBV) infection progressed to cirrhosis and hepatocellular carcinoma (HCC). Inhibitors of Hsp90 and GRP94 (such as geldanamycin (GA) and its less toxic derivative 17-AAG) have been investigated for efficacy in cancer treatment.

GRP94 (endoplasmin) may be encoded by the following genes, but not limited thereto: GENBANK® Accession Nos. NM_003299, BC066656 (*Homo sapiens*); NM_011631 (*Mus musculus*); NM_001045763: (*Xenopus* (*Silurana*) *tropicalis*); NM_214103 (*Sus scrofa*) NM_98210 (*Danio rerio*); NM_001012197 (*Rattus norvegicus*); NM_001134101: Pongo abelii; NM_001003327 (*Canis lupus familiaris*) heat shock protein 90 kDa beta (GRP94); NM_204289 (*Gallus gallus*).

Expression Control Sequences:

Nucleic acid sequences that regulate the expression of a heterologous nucleic acid sequence to which it is operatively linked. Expression control sequences are operatively linked to a nucleic acid sequence when the expression control sequences control and regulate the transcription and, as appropriate, translation of the nucleic acid sequence. Thus expression control sequences can include appropriate promoters, enhancers, transcription terminators, a start codon (i.e., ATG) in front of a protein-encoding gene, splicing signal for introns, maintenance of the correct reading frame of that gene to permit proper translation of mRNA, and stop codons. The term "control sequences" is intended to include, at a minimum, components whose presence can influence expression, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences. Expression control sequences can include a promoter.

A promoter is a minimal sequence sufficient to direct transcription. Also included are those promoter elements which are sufficient to render promoter-dependent gene expression controllable for cell-type specific, tissue-specific, or inducible by external signals or agents; such elements may be located in the 5' or 3' regions of the gene. Both constitutive and inducible promoters are included (see e.g., Bitter et al., *Methods in Enzymology* 153:516-544, 1987). For example, when cloning in bacterial systems, inducible promoters such as pL of bacteriophage lambda, plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like may be used. In one embodiment, when cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the retrovirus long terminal repeat; the adenovirus late promoter; the vaccinia virus 7.5K promoter) can be used. Promoters produced by recombinant DNA or synthetic techniques may also be used to provide for transcription of the nucleic acid sequences.

Inhibitor:

As used herein, an "inhibitor" refers to any compound that is capable of reducing or altering the expression or activity of a target molecule. In some embodiments, the inhibitor is an inhibitor of BRAF.

Isolated:

An "isolated" biological component, such as a nucleic acid, protein (including antibodies) or organelle, has been substantially separated or purified away from other biological components in the environment (such as a cell) in which the component naturally occurs, i.e., other chromosomal and extra-chromosomal DNA and RNA, proteins and organelles. Nucleic acids and proteins that have been "isolated" include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acids.

Melanoma:

A form of cancer that originates in melanocytes (cells that make the pigment melanin). Melanocytes are found primarily in the skin, but are also present in the bowel and eye. As used herein, "melanoma" refers to any stage of melanoma, or any subtype of melanoma, such as superficial spreading melanoma, nodular melanoma, acral lentiginous melanoma, lentigo maligna, melanoma-in-situ, mucosal melanoma and uveal melanoma. Melanoma in the skin includes superficial spreading melanoma, nodular melanoma, acral lentiginous melanoma, and lentigo maligna (melanoma). Any of the above types may produce melanin or can be amelanotic. Similarly, any subtype may show desmoplasia (dense fibrous reaction with neurotropism) which is a marker of aggressive behavior and a tendency to local recurrence. Other melanomas include clear cell sarcoma, mucosal melanoma and uveal melanoma.

Features that affect prognosis are tumor thickness in millimeters (Breslow's depth), depth related to skin structures (Clark level), type of melanoma, presence of ulceration, presence of lymphatic/perineural invasion, presence of tumor infiltrating lymphocytes (if present, prognosis is better), location of lesion, presence of satellite lesions, and presence of regional or distant metastasis. When melanomas have spread to the lymph nodes, one of the most important factors is the number of nodes with malignancy. The extent of malignancy within a node is also important; micrometastases in which malignancy is only microscopic have a more favorable prognosis than macrometastases. When there is distant metastasis, the five year survival rate is less than 10 percent; the median survival is 6 to 12 months. Metastases to skin and lungs have a better prognosis. Metastases to brain, bone and liver are associated with a worse prognosis.

Melanoma can be staged as follows:

Stage 0: Melanoma in Situ (Clark Level I), 100% Survival

Stage I/II: Invasive Melanoma, 85-95% Survival

- T1a: Less than 1.00 mm primary, w/o Ulceration, Clark Level II-III
- T1b: Less than 1.00 mm primary, w/Ulceration or Clark Level IV-V
- T2a: 1.00-2.00 mm primary, w/o Ulceration

Stage II: High Risk Melanoma, 40-85% Survival

- T2b: 1.00-2.00 mm primary, w/Ulceration
- T3a: 2.00-4.00 mm primary, w/o Ulceration
- T3b: 2.00-4.00 mm primary, w/Ulceration
- T4a: 4.00 mm or greater primary w/o Ulceration
- T4b: 4.00 mm or greater primary w/Ulceration

Stage III: Regional Metastasis, 25-60% Survival
N1: Single Positive Lymph Node
N2: 2-3 Positive Lymph Nodes OR Regional Skin/In-Transit Metastasis
N3: 4 Positive Lymph Nodes OR Lymph Node and Regional Skin/In Transit Metastases
Stage IV: Distant Metastasis, 9-15% Survival
M1a: Distant Skin Metastasis, Normal lactate dehydrogenase (LDH)
M1b: Lung Metastasis, Normal LDH
M1c: Other Distant Metastasis OR Any Distant Metastasis with Elevated LDH

Metastasis:

Refers to the spread of cancer cells from the original tumor to other sites in the body.

Monoclonal Antibody:

An antibody produced by a single clone of B-lymphocytes or by a cell into which the light and heavy chain genes of a single antibody have been transfected. Monoclonal antibodies are produced by methods known to those of skill in the art, for instance by making hybrid antibody-forming cells from a fusion of myeloma cells with immune spleen cells. Monoclonal antibodies include humanized and fully human monoclonal antibodies. As used herein a monoclonal antibody includes antibody fragments, such as, but not limited to scFv, Fv, dsRv, or Fab.

Mutation:

Any change of the DNA sequence within a gene or chromosome. In some instances, a mutation will alter a characteristic or trait (phenotype), but this is not always the case. Types of mutations include base substitution point mutations (e.g., transitions or transversions), deletions and insertions. Missense mutations are those that introduce a different amino acid into the sequence of the encoded protein; nonsense mutations are those that introduce a new stop codon. In the case of insertions or deletions, mutations can be in-frame (not changing the frame of the overall sequence) or frame shift mutations, which may result in the misreading of a large number of codons (and often leads to abnormal termination of the encoded product due to the presence of a stop codon in the alternative frame).

This term specifically encompasses variations that arise through somatic mutation, for instance those that are found only in disease cells (such as cancer cells), but not constitutionally, in a given individual. Examples of such somatically-acquired variations include the point mutations that frequently result in altered function of various genes that are involved in development of cancers. This term also encompasses DNA alterations that are present constitutionally, that alter the function of the encoded protein in a readily demonstrable manner, and that can be inherited by the children of an affected individual. In this respect, the term overlaps with "polymorphism," as discussed below, but generally refers to the subset of constitutional alterations that have arisen within the past few generations in a kindred and that are not widely disseminated in a population group.

In some embodiments, a mutation in BRAF refers to a nucleotide substitution in the BRAF gene or cDNA, or an amino acid substitution in the BRAF protein.

Neoplasia, Malignancy, Cancer or Tumor:

The result of abnormal and uncontrolled growth of cells. A neoplasm is an abnormal growth of tissue or cells that results from excessive cell division. Neoplastic growth can produce a tumor, malignancy, cancer and tumor are often used interchangeably. The amount of a tumor in an individual is the "tumor burden" which can be measured as the number, volume, or weight of the tumor. A tumor that does not metastasize is referred to as "benign." A tumor that invades the surrounding tissue and/or can metastasize is referred to as "malignant." A "non-cancerous tissue" is a tissue from the same organ wherein the malignant neoplasm formed, but does not have the characteristic pathology of the neoplasm. Generally, noncancerous tissue appears histologically normal. A "normal tissue" is tissue from an organ, wherein the organ is not affected by cancer or another disease or disorder of that organ. A "cancer-free" subject has not been diagnosed with a cancer of that organ and does not have detectable cancer.

Examples of hematological tumors include leukemias, including acute leukemias (such as 11q23-positive acute leukemia, acute lymphocytic leukemia, acute myelocytic leukemia, acute myelogenous leukemia and myeloblastic, promyelocytic, myelomonocytic, monocytic and erythroleukemia), chronic leukemias (such as chronic myelocytic (granulocytic) leukemia, chronic myelogenous leukemia, and chronic lymphocytic leukemia), polycythemia vera, lymphoma, Hodgkin's disease, non-Hodgkin's lymphoma (indolent and high grade forms), multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, myelodysplastic syndrome, hairy cell leukemia and myelodysplasia.

Examples of solid tumors, such as sarcomas and carcinomas, include fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, and other sarcomas, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, lymphoid malignancy, pancreatic cancer, breast cancer (including basal breast carcinoma, ductal carcinoma and lobular breast carcinoma), lung cancers, ovarian cancer, prostate cancer, hepatocellular carcinoma, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, medullary thyroid carcinoma, papillary thyroid carcinoma, pheochromocytomas sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, Wilms' tumor, cervical cancer, testicular tumor, seminoma, bladder carcinoma, and CNS tumors (such as a glioma, astrocytoma, medulloblastoma, craniopharyogioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, menangioma, melanoma, neuroblastoma and retinoblastoma).

In several examples, a tumor is melanoma, breast cancer, prostate cancer, glioma or a squamous cell carcinoma, such as head and neck cancer.

Nevi:

Melanocytic lesions that can be considered regional melanocytic hyperplasias. The term "nevi" includes all types of nevi, such as congenital nevi, acquired nevi, intradermal nevi, compound nevi, dysplastic nevi, atypical nevi, and junctional nevi.

Nevus:

The term "nevus" encompasses one or more nevi, including one or more in vivo nevi cells and one or more in vitro nevi cells. A "nevus" also encompasses one or more melanocytic lesions that can be considered regional melanocytic hyperplasias. The term "nevus" as used herein includes all types of nevi, such as congenital nevi, acquired nevi, intradermal nevi, compound nevi, dysplastic nevi, atypical nevi, and junctional nevi.

Nucleic Acid:

A polymer composed of nucleotide units (ribonucleotides, deoxyribonucleotides, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof) linked via phosphodiester bonds, related naturally occurring structural variants, and synthetic non-naturally

occurring analogs thereof. Thus, the term includes nucleotide polymers in which the nucleotides and the linkages between them include non-naturally occurring synthetic analogs, such as, for example and without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs), and the like. Such polynucleotides can be synthesized, for example, using an automated DNA synthesizer. The term "oligonucleotide" typically refers to short polynucleotides, generally no greater than about 50 nucleotides. It will be understood that when a nucleotide sequence is represented by a DNA sequence (i.e., A, T, G, C), this also includes an RNA sequence (i.e., A, U, G, C) in which "U" replaces "T."

Conventional notation is used herein to describe nucleotide sequences: the left-hand end of a single-stranded nucleotide sequence is the 5'-end; the left-hand direction of a double-stranded nucleotide sequence is referred to as the 5'-direction. The direction of 5' to 3' addition of nucleotides to nascent RNA transcripts is referred to as the transcription direction. The DNA strand having the same sequence as an mRNA is referred to as the "coding strand;" sequences on the DNA strand having the same sequence as an mRNA transcribed from that DNA and which are located 5' to the 5'-end of the RNA transcript are referred to as "upstream sequences;" sequences on the DNA strand having the same sequence as the RNA and which are 3' to the 3' end of the coding RNA transcript are referred to as "downstream sequences."

"cDNA" refers to a DNA that is complementary or identical to an mRNA, in either single stranded or double stranded form.

"Encoding" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (i.e., rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA produced by that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and non-coding strand, used as the template for transcription, of a gene or cDNA can be referred to as encoding the protein or other product of that gene or cDNA. Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. Nucleotide sequences that encode proteins and RNA may include introns.

"Recombinant nucleic acid" refers to a nucleic acid having nucleotide sequences that are not naturally joined together. This includes nucleic acid vectors comprising an amplified or assembled nucleic acid which can be used to transform a suitable host cell. A host cell that comprises the recombinant nucleic acid is referred to as a "recombinant host cell." The gene is then expressed in the recombinant host cell to produce, such as a "recombinant polypeptide." A recombinant nucleic acid may serve a non-coding function (such as a promoter, origin of replication, ribosome-binding site, etc.) as well.

A first sequence is an "antisense" with respect to a second sequence if a polynucleotide whose sequence is the first sequence specifically hybridizes with a polynucleotide whose sequence is the second sequence.

Terms used to describe sequence relationships between two or more nucleotide sequences or amino acid sequences include "reference sequence," "selected from," "comparison window," "identical," "percentage of sequence identity," "substantially identical," "complementary," and "substantially complementary."

For sequence comparison of nucleic acid sequences, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters are used. Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison can be conducted, for example, by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482, 1981, by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443, 1970, by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444, 1988, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by manual alignment and visual inspection (see for example, *Current Protocols in Molecular Biology* (Ausubel et al., eds 1995 supplement)).

One example of a useful algorithm is PILEUP. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle, *J. Mol. Evol.* 35:351-360, 1987. The method used is similar to the method described by Higgins & Sharp, *CABIOS* 5:151-153, 1989. Using PILEUP, a reference sequence is compared to other test sequences to determine the percent sequence identity relationship using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps. PILEUP can be obtained from the GCG sequence analysis software package, such as version 7.0 (Devereaux et al., *Nuc. Acids Res.* 12:387-395, 1984.

Another example of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and the BLAST 2.0 algorithm, which are described in Altschul et al., *J. Mol. Biol.* 215:403-410, 1990 and Altschul et al., *Nucleic Acids Res.* 25:3389-3402, 1977. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The BLASTN program (for nucleotide sequences) uses as defaults a word length (W) of 11, alignments (B) of 50, expectation (E) of 10, M=5, N=−4, and a comparison of both strands. The BLASTP program (for amino acid sequences) uses as defaults a word length (W) of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915, 1989).

Operably Linked:

A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter, such as the CMV promoter, is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein-coding regions, in the same reading frame.

Patient:

As used herein, the term "patient" includes human and non-human animals. The preferred patient for treatment is a human.

Pharmaceutically Acceptable Vehicles:

The pharmaceutically acceptable carriers (vehicles) useful in this disclosure are conventional. *Remington's Pharmaceutical Sciences*, by E. W. Martin, Mack Publishing Co., Easton, Pa., 15th Edition (1975), describes compositions and formulations suitable for pharmaceutical delivery of one or more therapeutic compounds, molecules or agents.

In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (for example, powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to biologically-neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

Pharmaceutical Agent:

A chemical compound or composition capable of inducing a desired therapeutic or prophylactic effect when properly administered to a subject or a cell.

Polymorphism:

Variant in a sequence of a gene, or any genomic sequence, usually carried from one generation to another in a population. Polymorphisms can be those variations (nucleotide sequence differences) that, while having a different nucleotide sequence, produce functionally equivalent gene products, such as those variations generally found between individuals, different ethnic groups, and geographic locations. The term polymorphism also encompasses variations that produce gene products with altered function, i.e., variants in the gene sequence that lead to gene products that are not functionally equivalent. This term also encompasses variations that produce no gene product, an inactive gene product, a truncated gene product, or increased or increased activity gene product.

Polymorphisms can be referred to, for instance, by the nucleotide position at which the variation exists, by the change in amino acid sequence caused by the nucleotide variation, or by a change in some other characteristic of the nucleic acid molecule or protein that is linked to the variation (e.g., an alteration of a secondary structure such as a stem-loop, or an alteration of the binding affinity of the nucleic acid for associated molecules, such as polymerases, RNAses, a change in the availability of a site for cleavage by a restriction endonuclease, either the formation of a new site, or lose of a site, and so forth).

Polypeptide:

A polymer in which the monomers are amino acid residues which are joined together through amide bonds. When the amino acids are alpha-amino acids, either the L-optical isomer or the D-optical isomer can be used. The terms "polypeptide" or "protein" as used herein are intended to encompass any amino acid sequence and include modified sequences such as glycoproteins. The term "polypeptide" is specifically intended to cover naturally occurring proteins, as well as those which are recombinantly or synthetically produced.

The term "residue" or "amino acid residue" includes reference to an amino acid that is incorporated into a protein, polypeptide, or peptide.

Conservative amino acid substitutions are those substitutions that, when made, least interfere with the properties of the original protein, that is, the structure and especially the function of the protein is conserved and not significantly changed by such substitutions. Examples of conservative substitutions are shown in the following table:

Original Residue	Conservative Substitutions
Ala	Ser
Arg	Lys
Asn	Gln, His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
His	Asn; Gln
Ile	Leu, Val
Leu	Ile; Val
Lys	Arg; Gln; Glu
Met	Leu; Ile
Phe	Met; Leu; Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp; Phe
Val	Ile; Leu

Conservative substitutions generally maintain (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain.

The substitutions which in general are expected to produce the greatest changes in protein properties will be non-conservative, for instance changes in which (a) a hydrophilic residue, for example, seryl or threonyl, is substituted for (or by) a hydrophobic residue, for example, leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, for example, lysyl, arginyl, or histadyl, is substituted for (or by) an electronegative residue, for example, glutamyl or aspartyl; or (d) a residue having a bulky side chain, for example, phenylalanine, is substituted for (or by) one not having a side chain, for example, glycine.

Preventing, Treating or Ameliorating a Disease:

"Preventing" a disease (such as metastatic melanoma) refers to inhibiting the full development of a disease. "Treating" refers to a therapeutic intervention that ameliorates a sign or symptom of a disease or pathological condition after it has begun to develop. "Ameliorating" refers to the reduction in the number or severity of signs or symptoms of a disease.

Prognosis:

The likelihood of the clinical outcome for a subject afflicted with a specific disease or disorder. With regard to cancer, the prognosis is a representation of the likelihood (probability) that the subject will survive (such as for one, two, three, four or five years) and/or the likelihood (probability) that the tumor will metastasize. A "poor prognosis" indicates a greater than 50% chance that the subject will not survive to a specified time point (such as one, two, three, four or five years), and/or a greater than 50% chance that the tumor will metastasize. In several examples, a poor prognosis indicates that there is a greater than 60%, 70%, 80%, or 90% chance that the subject will not survive and/or a greater than 60%, 70%, 80% or 90% chance that the tumor will metastasize. Conversely, a "good prognosis" indicates a greater than 50% chance that the subject will survive to a specified time point (such as one, two, three, four or five years), and/or a

greater than 50% chance that the tumor will not metastasize. In several examples, a good prognosis indicates that there is a greater than 60%, 70%, 80%, or 90% chance that the subject will survive and/or a greater than 60%, 70%, 80% or 90% chance that the tumor will not metastasize.

Proliferation:

One or more cellular events that result in cell growth. Proliferation includes any of a number of growth activities including increase in the number of cells, increase in the rate of cell division, increase in the number of cell divisions, increase in the size of a cell, change in cellular differentiation, transformation to a malignant state, metastatic transformation, change in cell cycle phase to a more mitotically active cell cycle phase (e.g., S phase), or a combination of two or more of those activities. Cell growth (either in vitro or in vivo) can be a hyper-proliferative condition, such as is characteristic of certain disorders or diseases, for instance neoplasia or tumor formation.

Inhibiting proliferation includes any of a number of anti-growth activities that reduce or even eliminate the ability of a cell to proliferate. Inhibiting proliferation includes, for instance, decreasing cell number, decreasing colony forming ability, decreasing the rate of cell division, decreasing the number of cell divisions, stopping cell division, inducing apoptosis, inducing senescence, inducing quiescence, changing cell cycle phase to a less mitotically active cell cycle phase, decreasing cellular de-differentiation, preventing transformation to a malignancy, decreasing malignant potential, decreasing metastatic ability or potential or a combination of two or more of those activities.

Resistance:

The lack of response of a disease, such as a cancer, to a therapeutic agent. In some embodiment, the agent is a BRAF inhibitor. Primary resistance is the lack of a response to a therapeutic agent upon initial treatment with the agent. Secondary resistance is the lack of a response to a therapeutic agent, wherein the cancer in the subject is initially susceptible to treatment with the agent. Resistance of a cancer to an agent can be measured by an increase in tumor burden, an increase in the number of metastases, or an increase in the amount of a tumor marker present in the subject.

Sample:

A biological specimen containing genomic DNA, RNA, protein, or combinations thereof, obtained from a subject. Examples include, but are not limited to, peripheral blood, urine, saliva, tissue biopsy (such as skin tissue), surgical specimen, and autopsy material. In one example, a sample includes a biopsy of a melanoma tumor or a sample of normal tissue, such as skin tissue (from a subject not afflicted with a known disease or disorder, such as a cancer-free subject).

Somatic Mutation:

An acquired mutation that occurs in a somatic cell (as opposed to a germ cell).

Subject:

Living multi-cellular vertebrate organisms, a category that includes both human and non-human mammals. In some embodiments, the subject is a human subject.

Therapeutic Agent:

A chemical compound, small molecule, or other composition, such as an antisense compound, antibody, peptide or nucleic acid molecule capable of inducing a desired therapeutic or prophylactic effect when properly administered to a subject. For example, therapeutic agents for melanoma include agents that prevent or inhibit development or metastasis of melanoma. In some embodiments, the therapeutic agent is an inhibitor of BRAF.

Therapy:

The mode of treatment or care of a patient. In some cases, therapy refers to administration of a therapeutic agent. In some embodiments herein, therapy includes administration of a BRAF inhibitor. In other examples, therapy includes surgery, such as surgical resection of a melanoma tumor, chemotherapy, radiation therapy, or any combination thereof.

Therapeutically Effective Amount:

A quantity of an agent sufficient to achieve a desired effect in a subject or a cell being treated. For instance, this can be the amount necessary to inhibit or to measurably reduce B-Raf activity in a nevi or to inhibit melanoma proliferation. A therapeutically effective amount of an agent may be administered in a single dose, or in several doses, for example daily or more often, during a course of treatment. However, the effective amount will be dependent on the particular agent applied, the subject being treated, the severity and type of the affliction, and the manner of administration.

Treating:

Includes inhibiting or preventing the partial or full development or progression of a disease or medical condition or abnormal biological state in a subject, for example in a person who is known to have a predisposition to or to be at risk for the disease or medical condition or abnormal biological state, or a cell or a lesion, for instance a nevus or a melanoma. Furthermore, "treating" refers to a therapeutic intervention that ameliorates at least one sign or symptom of a disease or pathological condition, or interferes with a pathophysiological process, after the disease or pathological condition has begun to develop. The therapeutic intervention can be prophylactic inhibition of a disease or medical condition or biological state, and therapeutic interventions to alter the natural course of an untreated disease process or medical condition or a biological state, such as a tumor growth. The therapeutic intervention can be surgical, including but not limited to cryosurgery or ablation, such as laser ablation, and administration of agents, systemically, regionally, or topically or locally.

Unless otherwise explained, 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 disclosure belongs. The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of this disclosure, suitable methods and materials are described below. The term "comprises" means "includes." All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including explanations of terms, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Antibodies that Specifically Bind GRP94, Antigen Binding Fragments and Immunotoxins

Antibodies have been produced that specifically bind GRP94 (also known as endoplasmin) including monoclonal

antibodies, such as fully human monoclonal antibodies. These antibodies and/or antigen binding fragments thereof can be used in the methods disclosed herein. These antibodies can be conjugated to labels or effector molecules. The antibodies used in the disclosed methods can be fully human monoclonal antibodies and functional fragments thereof that specifically bind GRP 94 (endoplasmin).

Exemplary GRP94 antibodies of use in the methods and compositions disclosed herein include those listed in the following table:

Name	Source	Clonality/Source
HSP90B1 antibody (Cat. # 60012-1-Ig)	Proteintech Group	monoclonal, mouse
HSP90B1 antibody (Cat. # 14700-1-AP)	Proteintech Group	polyclonal, rabbit
HSP90B1 antibody (Cat. # 10979-1-AP)	Proteintech Group	polyclonal, rabbit
Human TRA-1-60 MAb (clone TRA-1-60)	R & D Systems	monoclonal, mouse
Human TRA-1-85 MAb (clone TRA-1-85)	R & D Systems	monoclonal, mouse
Human TRA-1-85 phycoerythrin MAb (clone TRA-1-85)	R & D Systems	monoclonal, mouse
GRP94 polyclonal antibody	Enzo Life Sciences	polyclonal, rabbit
GRP94 monoclonal antibody (9G10), PE	Enzo Life Sciences	monoclonal, mouse
GRP94 monoclonal antibody (9G10), DyLight 488	Enzo Life Sciences	monoclonal, rat
Anti-human TRA1 antibody (clone 2H3)	ATGen	monoclonal, mouse
HSP90B1 antibody	Epitomics	polyclonal, rabbit
HSP90B antibody	Epitomics	polyclonal, rabbit
GRP94 antibody	Epitomics	monoclonal, rabbit
HSP90B1 antibody	OriGene	polyclonal, goat
Anti-GRP94/TRA1 antibody	Everest Biotech	polyclonal, goat
Anti-HSP90B1 antibody	AbDSerotec	polyclonal, rabbit
Anti-human GRP94 antibody	AbDSerotec	polyclonal, rabbit
Anti-human TRA1 antibody	MyBioSource	polyclonal, goat
Anti-human TRA1 antibody (clone 2H3)	MyBioSource	monoclonal, mouse
Anti-GRP94 antibody	MyBioSource	monoclonal, rat
HSP90B1 antibody	GeneTex	polyclonal, rabbit
GRP94 antibody	Abcam	monoclonal, mouse
Alexa Fluor ™ 488 anti-human TRA-1-60-R	BioLegend	monoclonal, mouse
PE anti-human TRA-1-60-R	BioLegend	monoclonal, mouse
Heat Shock Protein 94 antibody	Thermo Fisher Scientific	polyclonal, rabbit
Glucose-Regulated Protein 94	Thermo Fisher Scientific	polyclonal, rabbit
Anti-human Heat Shock Protein 90 kDa Beta (GRP94) Member 1 antibody	ProSpec	monoclonal, mouse
Anti-GRP94	StressMarq Biosciences	monoclonal, rat
GRP94 antibody	Abbiotec	polyclonal, rabbit
HSP90B1 antibody (N-term)	Abgent	polyclonal, rabbit
GRP94 antibody	US Biological	polyclonal, rabbit
TRA-1-60 antibody	US Biological	monoclonal, mouse
GRP94 antibody	US Biological	polyclonal, goat
GRP94 antibody	Novus Biologicals	polyclonal, rabbit
TRA-1-60 antibody (MG38)	Novus Biologicals	monoclonal, mouse
TRA-1-60(S) antibody	Cell Signaling Technology	monoclonal, mouse
TRA-1-85 mAb	Cell Signaling Technology	monoclonal, mouse
HSP90B1 anti-mouse polyclonal antibody	LifeSpan Biosciences	polyclonal, rabbit
HSP90B1 anti-human polyclonal antibody	LifeSpan Biosciences	polyclonal, goat
HSP90B1 anti-chicken monoclonal antibody (9G10)	LifeSpan Biosciences	monoclonal, rat
GRP 94 (H-212)	Santa Cruz Biotechnology	polyclonal, rabbit
GRP 94 (C-19)	Santa Cruz Biotechnology	polyclonal, goat
GRP 94 (4E89)	Santa Cruz Biotechnology	monoclonal, rabbit
Glucose Regulated Protein 94 antibody	Biotrend	polyclonal, rat
Anti-TRA-1-60, clone TRA-1-60	Millipore	monoclonal, mouse

Humanized forms and antigen binding fragments of these antibodies are also of use in the presently disclosed methods.

In one example, human GRP94 (also known as endoplasmin) has an amino acid sequence set forth as:

MRALWVLGLCCVLLTFGSVRADDEVDVDGTVEEDLGKSREGSRT

DDEVVQREEEAIQLDGLNASQIRELREKSEKFAFQAEVNRMMKLIINSL

YKNKEIFLRELISNASDALDKIRLISLTDENALSGNEELTVKIKCDKEK

NLLHVTDTGVGMTREELVKNLGTIAKSGTSEFLNKMTEAQEDGQSTSEL

IGQFGVGFYSAFLVADKVIVTSKHNNDTQHIWESDSNEFSVIADPRGNT

LGRGTTITLVLKEEASDYLELDTIKNLVKKYSQFINFPIYVWSSKTETV

EEPMEEEEAAKEEKEESDDEAAVEEEEEEKKPKTKKVEKTVWDWELMND

IKPIWQRPSKEVEEDEYKAFYKSFSKESDDPMAYIHFTAEGEVTFKSIL

-continued

FVPTSAPRGLFDEYGSKKSDYIKLYVRRVFITDDFHDMMPKYLNFVKGV

VDSDDLPLNVSRETLQQHKLLKVIRKKLVRKTLDMIKKIADDKYNDTFW

KEFGTNIKLGVIEDHSNRTRLAKLLRFQSSHHPTDITSLDQYVERMKEK

QDKIYFMAGSSRKEAESSPFVERLLKKGYEVIYLTEPVDEYCIQALPEF

DGKRFQNVAKEGVKFDESEKTKESREAVEKEFEPLLNWMKDKALKDKIE

KAVVSQRLTESPCALVASQYGWSGNMERIMKAQAYQTGKDISTNYYASQ

KKTFEINPRHPLIRDMLRRIKEDEDDKTVLDLAVVLFETATLRSGYLLP

DTKAYGDRIERMLRLSLNIDPDAKVEEEPEEEPEETAEDTTEDTEQDED

EEMDVGTDEEEETAKESTAEKDEL

SEQ ID NO: 1, See also GENBANK® Accession No. NM_003299 incorporated herein by reference.

In another example, the GRP94 is encoded by the nucleic acid sequence set forth as:

gtgggcggac cgcgcggctg gaggtgtgag gatccgaacc caggggtggg gggtggaggc

ggctcctgcg atcgaagggg acttgagact caccggccgc acgccatgag ggccctgtgg

gtgctgggcc tctgctgcgt cctgctgacc ttcgggtcgg tcagagctga cgatgaagtt

gatgtggatg gtacagtaga agaggatctg ggtaaaagta gagaaggatc aaggacggat

gatgaagtag tacagagaga ggaagaagct attcagttgg atggattaaa tgcatcacaa

ataagagaac ttagagagaa gtcggaaaag tttgccttcc aagccgaagt taacagaatg

atgaaactta tcatcaattc attgtataaa aataaagaga ttttcctgag agaactgatt

tcaaatgctt ctgatgcttt agataagata aggctaatat cactgactga tgaaaatgct

ctttctggaa atgaggaact aacagtcaaa attaagtgtg ataaggagaa gaacctgctg

catgtcacag acaccggtgt aggaatgacc agagaagagt tggttaaaaa ccttggtacc

atagccaaat ctgggacaag cgagttttta aacaaaatga ctgaagcaca ggaagatggc

cagtcaactt ctgaattgat tggccagttt ggtgtcggtt tctattccgc cttccttgta

gcagataagg ttattgtcac ttcaaaacac aacaacgata cccagcacat ctgggagtct

gactccaatg aattttctgt aattgctgac ccaagaggaa acactctagg acggggaacg

acaattaccc ttgtcttaaa agaagaagca tctgattacc ttgaattgga tacaattaaa

aatctcgtca aaaaatattc acagttcata aactttccta tttatgtatg gagcagcaag

actgaaactg ttgaggagcc catggaggaa gaagaagcag ccaaagaaga gaaagaagaa

tctgatgatg aagctgcagt agaggaagaa gaagaagaaa agaaaccaaa gactaaaaaa

gttgaaaaaa ctgtctggga ctgggaactt atgaatgata tcaaaccaat atggcagaga

ccatcaaaag aagtagaaga agatgaatac aaagctttct acaaatcatt ttcaaaggaa

agtgatgacc ccatggctta tattcacttt actgctgaag gggaagttac cttcaaatca

attttatttg tacccacatc tgctccacgt ggtctgtttg acgaatatgg atctaaaaag

agcgattaca ttaagctcta tgtgcgccgt gtattcatca cagacgactt ccatgatatg

atgcctaaat acctcaattt tgtcaagggt gtggtggact cagatgatct ccccttgaat

gtttcccgcg agactcttca gcaacataaa ctgcttaagg tgattaggaa gaagcttgtt

cgtaaaacgc tggacatgat caagaagatt gctgatgata aatacaatga tacttttttgg

aaagaatttg gtaccaacat caagcttggt gtgattgaag accactcgaa tcgaacacgt

cttgctaaac ttcttaggtt ccagtcttct catcatccaa ctgacattac tagcctagac

-continued

cagtatgtgg aaagaatgaa ggaaaaacaa gacaaaatct acttcatggc tgggtccagc

agaaaagagg ctgaatcttc tccatttgtt gagcgacttc tgaaaaaggg ctatgaagtt

atttacctca cagaacctgt ggatgaatac tgtattcagg cccttcccga atttgatggg

aagaggttcc agaatgttgc caaggaagga gtgaagttcg atgaaagtga gaaaactaag

gagagtcgtg aagcagttga gaaagaattt gagcctctgc tgaattggat gaaagataaa

gcccttaagg acaagattga aaaggctgtg gtgtctcagc gcctgacaga atctccgtgt

gctttggtgg ccagccagta cggatggtct ggcaacatgg agagaatcat gaaagcacaa

gcgtaccaaa cgggcaagga catctctaca aattactatg cgagtcagaa gaaaacattt

gaaattaatc ccagacaccc gctgatcaga gacatgcttc gacgaattaa ggaagatgaa

gatgataaaa cagttttgga tcttgctgtg gttttgtttg aaacagcaac gcttcggtca

gggtatcttt taccagacac taaagcatat ggagatagaa tagaaagaat gcttcgcctc

agtttgaaca ttgaccctga tgcaaaggtg gaagaagagc ccgaagaaga acctgaagag

acagcagaag acacaacaga agacacagag caagacgaag atgaagaaat ggatgtggga

acagatgaag aagaagaaac agcaaaggaa tctacagctg aaaaagatga attgtaaatt

atactctcac catttggatc ctgtgtggag agggaatgtg aaatttacat catttctttt

tgggagagac ttgttttgga tgccccctaa tccccttctc ccctgcactg taaaatgtgg

gattatgggt cacaggaaaa agtgggtttt ttagttgaat ttttttttaac attcctcatg

aatgtaaatt tgtactattt aactgactat tcttgatgta aaatcttgtc atgtgtataa

aaataaaaaa gatcccaaat

SEQ ID NO: 2, see also GENBANK® Accession No. NM_003299, incorporated herein by reference.

Once of skill in the art can readily use a nucleic acid sequence to produce a polypeptide, such as GRP94 using standard method in molecular biology (see, for example, *Molecular Cloning: A Laboratory Manual,* 2nd ed., vol. 1-3, ed. Sambrook et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989).

Described herein are methods for using isolated human monoclonal antibodies and fragments thereof that specifically bind human GRP94 (endoplasmin) for the treatment of cancer, such as, but not limited to, melanoma. In some embodiments, the human monoclonal antibody functional fragment is a scFv. Also described are compositions including a monoclonal antibody or antigen fragment thereof, a BRAF inhibitor, and a pharmaceutically acceptable carrier. Nucleic acids encoding these antibodies, expression vectors comprising these nucleic acids, and isolated host cells that express the nucleic acids are also provided. Thus, in other embodiments, compositions are provided that include a nucleic acid encoding a monoclonal antibody or antigen fragment thereof, a BRAF inhibitor, and a pharmaceutically acceptable carrier

In some embodiments, the human monoclonal antibody or functional fragment thereof comprises at least a portion of the variable chain of the heavy chain amino acid sequence set forth as SEQ ID NO: 3 and specifically binds GRP94. For example, the human monoclonal antibody can include the SDRs (specificity determining residues), the CDRs, or the variable region of the amino acid sequence set forth as SEQ ID NO: 3. In the amino acid sequence shown below, the constant region is in bold, and the CDRs are underlined:

(SEQ ID NO: 3)

Q V Q L V Q S G A E V K K P G A S V K V S C K A S G Y T F T S Y A M H W V R Q

A P G Q R L E W M G W I N A G N G N T K Y S Q K F Q G R V T I T R D T S A S T A

Y M E L S S L R S E D T A V Y Y C A R A H F D Y W G Q G T L V T V S **A S T K G P**

S V F P L A P S S K S T S G G T A A L G C L V K D Y F P E P V T V S W N S G A L

T S G V H T F P A V L Q S S G L Y S L S S V V T V P S S S L G T Q T Y I C N V N

H K P S N T K V D K K V E P K S C D K T H T C P P C P A P E L L G G P S V F L

F P P K P K D T L M I S R T P E V T C V V V D V S H E D P E V K F N W Y V D G

V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H Q D W L N G K E Y

K C K V S N K A L P A P I E K T I S K A K G Q P R E P Q V Y T L P P S R E E M T

-continued

K N Q V S L T C L V K G F Y P S D I A V E W E S N G Q P E N N Y K T T P P V L

D S D G S F F L Y S K L T V D K S R W Q Q G N V F S C S V M H E A L H N H Y T

Q K S L S L S P G K

In some embodiments, the monoclonal antibody or functional fragment thereof comprises at least a portion of the heavy chain amino acid sequence set forth as SEQ ID NO: 3 and specifically binds GRP94. The monoclonal antibody can be a human monoclonal antibody. In some examples, at least one of the CDRs of the light chain of the antibody comprises one or more of the amino acid sequences set forth as amino acids 26-33 of SEQ ID NO: 3 (CDR1), amino acids 51-58 of SEQ ID NO: 3 (CDR2), and amino acids 97-103 of SEQ ID NO: 3 (CDR3). In additional examples, the heavy chain of the antibody comprises the amino acid sequence set forth as amino acids 26-33 of SEQ ID NO: 3 (CDR1), amino acids 51-58 of SEQ ID NO: 3 (CDR2), and amino acids 97-103 of SEQ ID NO: 3 (CDR3). In some examples, the variable region of the heavy chain of the antibody can include, or consist of, amino acids 1-113 of SEQ ID NO: 3. The heavy chain of the antibody can include, or consist of, SEQ ID NO: 3.

In some embodiments, the monoclonal antibody or functional fragment thereof comprises at least a portion of the variable region of the light chain amino acid sequence set forth as SEQ ID NO: 4 and specifically binds GRP94. The monoclonal antibody can be a human monoclonal antibody. In the amino acid sequence shown below, the constant region is in bold, and the CDRs are underlined:

(SEQ ED NO: 4)

E I E L T Q S P S S L S A S V G D R V T I T C R A S Q S I S S Y L N W Y Q Q K P G K

A P K L L I Y A A S S L Q S G V P S R F S G S G S G T D F T L T I S S L Q P E D F A

T Y Y C Q Q S Y S T P P T F G Q G T K V E I K **T V A A P S V F I F P P S D E Q L K**

S G T A S V V C L L N N F Y P R E A K V Q W K V D N A L Q S G N S Q E S V T E

Q D S K D S T Y S L S S T L T L S K A D Y E K H K V Y A C E V T H Q G L S S P

V T K S F N R G E C

In some examples, at least one of the CDRs of the light chain of the antibody comprises one or more of the amino acid sequences set forth as amino acids 27-32 of SEQ ID NO: 8 (CDR1), amino acids 50-52 of SEQ ID NO: 4 (CDR2), and amino acids 89-97 of SEQ ID NO: 4 (CDR3). In additional examples, the light chain of the antibody comprises amino acids amino acids 27-32 of SEQ ID NO: 4 (CDR1), amino acids 50-52 of SEQ ID NO: 4 (CDR2), and amino acids 89-97 of SEQ ID NO: 4 (CDR3). The variable region of the light chain of the antibody can include, or consist of, amino acids 1-107 of SEQ ID NO: 4. The light chain of the antibody can include, or consist of, SEQ ID NO: 4.

Fully human monoclonal antibodies include human framework regions. The human framework regions can include the framework regions disclosed in one or both of SEQ ID NO: 3 or SEQ ID NO: 4 (these sequences include CDR sequences as well as framework sequences). However, the framework regions can be from another source. Additional examples of framework sequences that can be used include the amino acid framework sequences of the heavy and light chains disclosed in PCT Publication No. WO 2006/074071 (see, for example, SEQ ID NOs: 1-16), which is herein incorporated by reference.

Chimeric antibodies include CDRs from one species, and framework regions (and/or a constant domain), from another species. In some embodiments, the monoclonal antibody, or antigen binding fragment, is chimeric. In one specific non-limiting example, the monoclonal antibody includes the CDRs from a murine antibody, and a human framework region. In another specific non-liming example, the monoclonal antibody includes the CDRs from a rabbit antibody, and a human framework region.

The monoclonal antibody of use in the disclosed methods can be of any isotype. The monoclonal antibody can be, for example, an IgA, IgM or an IgG antibody, such as IgG_1 or an IgG_2. The class of an antibody that specifically binds GRP94 can be switched with another. In one aspect, a nucleic acid molecule encoding V_L or V_H is isolated using methods well-known in the art, such that it does not include any nucleic acid sequences encoding the constant region of the light or heavy chain, respectively. The nucleic acid molecule encoding V_L or V_H is then operatively linked to a nucleic acid sequence encoding a C_L or C_H from a different class of immunoglobulin molecule. This can be achieved using a vector or nucleic acid molecule that comprises a C_L or C_H chain, as known in the art. For example, an antibody that specifically binds GRP94 that was originally IgM may be class switched to an IgG. Class switching can be used to convert one IgG subclass to another, such as from IgG_1 to IgG_2.

The method disclosed herein can utilize immunoconjugates comprising the monoclonal antibodies or functional fragment thereof that specifically binds human GRP94, such as human monoclonal antibodies. The immunoconjugates can comprise any therapeutic agent, toxin or other moiety. In one example, the toxin is PE or a variant or fragment thereof.

Antibody fragments that specifically bind GRP94 are encompassed by the present disclosure, such as Fab, $F(ab')_2$, and Fv which include a heavy chain and light chain variable region and are capable of binding the epitopic determinant on GRP94. These antibody fragments retain the ability to specifically bind with the antigen. These fragments include:

(1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule, can be produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain;

(2) Fab', the fragment of an antibody molecule can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule;

(3) $(Fab')_2$, the fragment of the antibody that can be obtained by treating whole antibody with the enzyme pepsin

without subsequent reduction; $F(ab')_2$ is a dimer of two Fab' fragments held together by two disulfide bonds;

(4) Fv, a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and

(5) Single chain antibody (such as scFv), defined as a genetically engineered molecule containing the variable region of the light chain, the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule.

(6) A dimer of a single chain antibody ($scFV_2$), defined as a dimer of a scFV. This has also been termed a "miniantibody."

Methods of making these fragments are known in the art (see for example, Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, 1988). In several examples, the variable region included in the antibody is the variable region of M912.

In a further group of embodiments, the antibodies are Fv antibodies, which are typically about 25 kDa and contain a complete antigen-binding site with three CDRs per each heavy chain and each light chain. To produce these antibodies, the V_H and the V_L can be expressed from two individual nucleic acid constructs in a host cell. If the V_H and the V_L are expressed non-contiguously, the chains of the Fv antibody are typically held together by noncovalent interactions. However, these chains tend to dissociate upon dilution, so methods have been developed to crosslink the chains through glutaraldehyde, intermolecular disulfides, or a peptide linker. Thus, in one example, the Fv can be a disulfide stabilized Fv (dsFv), wherein the heavy chain variable region and the light chain variable region are chemically linked by disulfide bonds.

In an additional example, the Fv fragments comprise V_H and V_L chains connected by a peptide linker. These single-chain antigen binding proteins (scFv) are prepared by constructing a structural gene comprising DNA sequences encoding the V_H and V_L domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing scFvs are known in the art (see Whitlow et al., *Methods: a Companion to Methods in Enzymology*, Vol. 2, page 97, 1991; Bird et al., *Science* 242: 423, 1988; U.S. Pat. No. 4,946,778; Pack et al., *Bio/Technology* 11:1271, 1993; and Sandhu, supra). Dimers of a single chain antibody ($scFV_2$), are also contemplated.

The antibody can also be included in a bi-specific antibody. In further embodiments, an engineered antibody domain, including the heavy chain CDRs or the light chain CDRs can be utilized, provided the engineered antibody domain specifically binds GRP94.

Antibody fragments can be prepared by proteolytic hydrolysis of the antibody or by expression in *E. coli* of DNA encoding the fragment. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted $F(ab')_2$. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly (see U.S. Pat. No. 4,036,945 and U.S. Pat. No. 4,331,647, and references contained therein; Nisonhoff et al., *Arch. Biochem. Biophys.* 89:230, 1960; Porter, *Biochem. J.* 73:119, 1959; Edelman et al., *Methods in Enzymology*, Vol. 1, page 422, Academic Press, 1967; and Coligan et al. at sections 2.8.1-2.8.10 and 2.10.1-2.10.4).

Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

One of skill will realize that conservative variants of the antibodies can be produced. Such conservative variants employed in antibody fragments, such as dsFv fragments or in scFv fragments, will retain critical amino acid residues necessary for correct folding and stabilizing between the V_H and the V_L regions, and will retain the charge characteristics of the residues in order to preserve the low pI and low toxicity of the molecules. Amino acid substitutions (such as at most one, at most two, at most three, at most four, or at most five amino acid substitutions) can be made in the V_H and the V_L regions to increase yield. Conservative amino acid substitution tables providing functionally similar amino acids are well known to one of ordinary skill in the art.

In some embodiments, the disclosed methods utilize immunoconjugates. Immunoconjugates include, but are not limited to, molecules in which there is a covalent linkage of a diagnostic or therapeutic agent with an antibody. A therapeutic agent is an agent with a particular biological activity directed against a particular target molecule or a cell bearing a target molecule. Therapeutic agents include various drugs such as vinblastine, daunomycin and the like, and effector molecules such as cytotoxins such as native or modified *Pseudomonas* exotoxin or Diphtheria toxin, encapsulating agents (e.g., liposomes), which themselves contain pharmacological compositions, target moieties and ligands.

The choice of a particular therapeutic agent depends on the particular target molecule or cell and the biological effect desired. Thus, for example, the therapeutic agent may be an effector molecule that is cytotoxin which is used to bring about the death of a particular target cell. Conversely, where it is merely desired to invoke a non-lethal biological response, a therapeutic agent can be conjugated to a non-lethal pharmacological agent or a liposome containing a non-lethal pharmacological agent.

Toxins can be employed with antibodies that bind GRP94 polypeptide and antigen biding antibody fragments, such as a svFv or a dsFv, to yield chimeric molecules, which are of use as immunotoxins. Exemplary toxins include *Pseudomonas* exotoxin (PE), ricin, abrin, diphtheria toxin and subunits thereof, ribotoxin, ribonuclease, saporin, and calicheamicin, as well as botulinum toxins A through F. These toxins are well known in the art and many are readily available from commercial sources (for example, Sigma Chemical Company, St. Louis, Mo.).

Diphtheria toxin is isolated from *Corynebacterium diphtheriae*. Typically, diphtheria toxin for use in immunotoxins is mutated to reduce or to eliminate non-specific toxicity. A mutant known as CRM107, which has full enzymatic activity but markedly reduced non-specific toxicity, has been known since the 1970's (Laird and Groman, *J. Virol.* 19:220, 1976), and has been used in human clinical trials. See, U.S. Pat. No. 5,792,458 and U.S. Pat. No. 5,208,021. As used herein, the term "diphtheria toxin" refers as appropriate to native diphtheria toxin or to diphtheria toxin that retains enzymatic activity but which has been modified to reduce non-specific toxicity.

Ricin is the lectin RCA60 from *Ricinus communis* (Castor bean). The term "ricin" also references toxic variants thereof.

For example, see U.S. Pat. No. 5,079,163 and U.S. Pat. No. 4,689,401. *Ricinus communis* agglutinin (RCA) occurs in two forms designated RCA_{60} and RCA_{120} according to their molecular weights of approximately 65 and 120 kD, respectively (Nicholson & Blaustein, *J. Biochim. Biophys. Acta* 266:543, 1972). The A chain is responsible for inactivating protein synthesis and killing cells. The B chain binds ricin to cell-surface galactose residues and facilitates transport of the A chain into the cytosol (Olsnes et al., *Nature* 249:627-631, 1974 and U.S. Pat. No. 3,060,165).

Ribonucleases have also been conjugated to targeting molecules for use as immunotoxins (see Suzuki et al., *Nat Biotech* 17:265-270, 1999). Exemplary ribotoxins such as α-sarcin and restrictocin are discussed in, e.g., Rathore et al., *Gene* 190:31-35, 1997; and Goyal and Batra, *Biochem* 345 Pt 2:247-254, 2000. Calicheamicins were first isolated from *Micromonospora echinospora* and are members of the enediyne antitumor antibiotic family that cause double strand breaks in DNA that lead to apoptosis (see, e.g., Lee et al., *J. Antibiot* 42:1070-1087. 1989). The drug is the toxic moiety of an immunotoxin in clinical trials (see, e.g., Gillespie et al., *Ann Oncol* 11:735-741, 2000).

Abrin includes toxic lectins from *Abrus precatorius*. The toxic principles, abrin a, b, c, and d, have a molecular weight of from about 63 and 67 kD and are composed of two disulfide-linked polypeptide chains A and B. The A chain inhibits protein synthesis; the B-chain (abrin-b) binds to D-galactose residues (see, Funatsu et al., *Agr. Biol. Chem.* 52:1095, 1988; and Olsnes, *Methods Enzymol.* 50:330-335, 1978).

In one embodiment, the toxin is *Pseudomonas* exotoxin (PE). Native *Pseudomonas* exotoxin A ("PE") is an extremely active monomeric protein (molecular weight 66 kD), secreted by *Pseudomonas aeruginosa*, which inhibits protein synthesis in eukaryotic cells. The native PE sequence and the sequence of modified PE are provided in U.S. Pat. No. 5,602,095, incorporated herein by reference. In one embodiment, native PE has a sequence set forth as:

(SEQ ID NO: 5)

```
AEEAFDLWNE CAKACVLDLK DGVRSSRMSV DPAIADTNGQ

GVLHYSMVLE GGNDALKLAI DNALSITSDG LTIRLEGGVE

PNKPVRYSYT RQARGSWSLN WLVPIGHEKP SNIKVFIHEL

NAGNQLSHMS PIYTIEMGDE LLAKLARDAT FFVRAHESNE

MQPTLAISHA GVSVVMAQTQ PRREKRWSEW ASGKVLCLLD

PLDGVYNYLA QQRCNLDDTW EGKIYRVLAG NPAKHDLDIK

PTVISHRLHF PEGGSLAALT AHQACHLPLE TFTRHRQPRG

WEQLEQCGYP VQRLVALYLAARLSWNQVDQ VIRNALASPG

SGGDLGEAIR EQPEQARLAL TLAAAESERF VRQGTGNDEA

GAANADVVSL TCPVAAGECA GPADSGDALL ERNYPTGAEF

LGDGGDVSFS TRGTQNWTVE RLLQAHRQLE ERGYVFVGYH

GTFLEAAQSI VFGGVRARSQ DLDAIWRGFY IAGDPALAYG

YAQDQEPDAR GRIRNGALLR VYVPRSSLPG FYRTSLTLAA

PEAAGEVERL IGHPLPLRLD AITGPEEEGG RLETILGWPL

AERTVVIPSA IPTDPRNVGG DLDPSSIPDK EQAISALPDY

ASQPGKPPRE DLK
```

The method of action of PE is inactivation of the ADP-ribosylation of elongation factor 2 (EF-2). The exotoxin contains three structural domains that act in concert to cause cytotoxicity. Domain Ia (amino acids 1-252) mediates cell binding. Domain II (amino acids 253-364) is responsible for translocation into the cytosol and domain III (amino acids 400-613) mediates ADP ribosylation of elongation factor 2. The function of domain Ib (amino acids 365-399) remains undefined, although a large part of it, amino acids 365-380, can be deleted without loss of cytotoxicity. See Siegall et al., *J. Biol. Chem.* 264:14256-14261, 1989.

The term "*Pseudomonas* exotoxin" ("PE") as used herein refers as appropriate to a full-length native (naturally occurring) PE or to a PE that has been modified. Such modifications may include, but are not limited to, elimination of domain Ia, various amino acid deletions in domains Ib, II and III, single amino acid substitutions and the addition of one or more sequences at the carboxyl terminus, such as KDEL (SEQ ID NO: 6) and REDL (SEQ ID NO: 7) (see Siegall et al., supra). In several examples, the cytotoxic fragment of PE retains at least 50%, such as about 75%, about 90%, or about 95% of the cytotoxicity of native PE. In one embodiment, the cytotoxic fragment is more toxic than native PE.

Thus, the PE used in the immunotoxins disclosed herein includes the native sequence, cytotoxic fragments of the native sequence, and conservatively modified variants of native PE and its cytotoxic fragments. Cytotoxic fragments of PE include those which are cytotoxic with or without subsequent proteolytic or other processing in the target cell (e.g., as a protein or pre-protein). Cytotoxic fragments of PE known in the art include PE40, PE38, and PE35.

In several embodiments, the PE has been modified to reduce or eliminate non-specific cell binding, typically by deleting domain Ia, as taught in U.S. Pat. No. 4,892,827, although this can also be achieved, for example, by mutating certain residues of domain Ia. U.S. Pat. No. 5,512,658, for instance, discloses that a mutated PE in which Domain Ia is present but in which the basic residues of domain Ia at positions 57, 246, 247, and 249 are replaced with acidic residues (glutamic acid, or "E") exhibits greatly diminished non-specific cytotoxicity. This mutant form of PE is sometimes referred to as PE4E.

PE40 is a truncated derivative of PE (see, Pai et al., *Proc. Nat'l Acad. Sci. U.S.A.* 88:3358-3362, 1991; and Kondo et al., *J. Biol. Chem.* 263:9470-9475, 1988). PE35 is a 35 kD carboxyl-terminal fragment of PE in which amino acid residues 1-279 have deleted and the molecule commences with a met at position 280 followed by amino acids 281-364 and 381-613 of native PE. PE35 and PE40 are disclosed, for example, in U.S. Pat. No. 5,602,095 and U.S. Pat. No. 4,892,827.

In some embodiments, the cytotoxic fragment PE38 is employed. PE38 is a truncated PE pro-protein composed of amino acids 253-364 and 381-613 of SEQ ID NO: 3 which is activated to its cytotoxic form upon processing within a cell (see e.g., U.S. Pat. No. 5,608,039, and Pastan et al., *Biochim. Biophys. Acta* 1333:C1-C6, 1997).

While in some embodiments, the PE is PE4E, PE40, or PE38, any form of PE in which non-specific cytotoxicity has been eliminated or reduced to levels in which significant toxicity to non-targeted cells does not occur can be used in the immunotoxins disclosed herein so long as it remains capable of translocation and EF-2 ribosylation in a targeted cell.

Conservatively modified variants of PE or cytotoxic fragments thereof have at least about 80% sequence identity, such as at least about 85% sequence similarity, at least about 90% sequence identity, or at least about 95% sequence similarity at the amino acid level, with the PE of interest, such as PE38.

Nucleic Acids

Nucleic acids encoding antibodies that specifically bind GRP94, and conjugates and fusion thereof, are provided herein. These nucleic acids can be used in conjunction with a BRAF inhibitor. With the antibodies and immunotoxins herein provided, one of skill can readily construct a variety of clones containing functionally equivalent antibodies, and nucleic acids encoding these antibodies, such as nucleic acids which differ in sequence but which encode the same effector molecule ("EM") or antibody sequence. Thus, nucleic acids encoding antibodies and conjugates and fusion proteins are provided herein.

Nucleic acid sequences encoding the antibodies and/or immunotoxins can be prepared by any suitable method including, for example, cloning of appropriate sequences or by direct chemical synthesis by methods such as the phosphotriester method of Narang, et al., *Meth. Enzymol.* 68:90-99, 1979; the phosphodiester method of Brown et al., *Meth. Enzymol.* 68:109-151, 1979; the diethylphosphoramidite method of Beaucage et al., *Tetra. Lett.* 22:1859-1862, 1981; the solid phase phosphoramidite triester method described by Beaucage & Caruthers, *Tetra. Lens.* 22(20):1859-1862, 1981, e.g., using an automated synthesizer as described in, for example, Needham-VanDevanter et al. *Nucl. Acids Res.* 12:6159-6168, 1984; and, the solid support method of U.S. Pat. No. 4,458,066. Chemical synthesis produces a single stranded oligonucleotide. This may be converted into double stranded DNA by hybridization with a complementary sequence, or by polymerization with a DNA polymerase using the single strand as a template. One of skill would recognize that while chemical synthesis of DNA is limited to sequences of about 100 bases, longer sequences may be obtained by the ligation of shorter sequences.

In one embodiment, the nucleic acid sequences encoding the antibody or immunotoxin are prepared by cloning techniques. Examples of appropriate cloning and sequencing techniques, and instructions sufficient to direct persons of skill through many cloning exercises are found in Sambrook et al., supra, Berger and Kimmel (eds.), supra, and Ausubel, supra. Product information from manufacturers of biological reagents and experimental equipment also provide useful information. Such manufacturers include the SIGMA Chemical Company (Saint Louis, Mo.), R&D Systems (Minneapolis, Minn.), Pharmacia Amersham (Piscataway, N.J.), CLONTECH Laboratories, Inc. (Palo Alto, Calif.), Chem Genes Corp., Aldrich Chemical Company (Milwaukee, Wis.), Glen Research, Inc., GIBCO BRL Life Technologies, Inc. (Gaithersburg, Md.), Fluka Chemica-Biochemika Analytika (Fluka Chemie AG, Buchs, Switzerland), Invitrogen (San Diego, Calif.), and Applied Biosystems (Foster City, Calif.), as well as many other commercial sources known to one of skill.

Nucleic acids can also be prepared by amplification methods. Amplification methods include polymerase chain reaction (PCR), the ligase chain reaction (LCR), the transcription-based amplification system (TAS), the self-sustained sequence replication system (3SR). A wide variety of cloning methods, host cells, and in vitro amplification methodologies are well known to persons of skill.

In one example, an immunotoxin of use is prepared by inserting the cDNA which encodes a variable region into a vector which comprises the cDNA encoding the EM. The insertion is made so that the variable region and the EM are read in frame so that one continuous polypeptide is produced. The polypeptide contains a functional Fv region and a functional EM region. In one embodiment, cDNA encoding a cytotoxin is ligated to a scFv so that the cytotoxin is located at the carboxyl terminus of the scFv. In one example, cDNA encoding a *Pseudomonas* exotoxin ("PE"), mutated to eliminate or to reduce non-specific binding, is ligated to a scFv so that the toxin is located at the amino terminus of the scFv. In another example, PE38 is located at the amino terminus of the scFv. In a further example, cDNA encoding a cytotoxin is ligated to a heavy chain variable region of an antibody that binds the antigen of interest so that the cytoxin is located at the carboxyl terminus of the heavy chain variable region. The heavy chain-variable region can subsequently be ligated to a light chain variable region of the antibody using disulfide bonds. In yet another example, cDNA encoding a cytotoxin is ligated to a light chain variable region of an antibody that binds the antigen (for example, GRP94), so that the cytotoxin is located at the carboxyl terminus of the light chain variable region. The light chain-variable region can subsequently be ligated to a heavy chain variable region of the antibody using disulfide bonds.

Once the nucleic acids encoding the antibody immunotoxin is isolated and cloned, the protein can be expressed in a recombinantly engineered cell such as bacteria, plant, yeast, insect and mammalian cells. One or more DNA sequences encoding an antibody immunotoxin can be expressed in vitro by DNA transfer into a suitable host cell. The cell may be prokaryotic or eukaryotic. The term also includes any progeny of the subject host cell. It is understood that all progeny may not be identical to the parental cell since there may be mutations that occur during replication. Methods of stable transfer, meaning that the foreign DNA is continuously maintained in the host, are known in the art.

Polynucleotide sequences encoding the antibody or immunotoxin can be operatively linked to expression control sequences. An expression control sequence operatively linked to a coding sequence is ligated such that expression of the coding sequence is achieved under conditions compatible with the expression control sequences. The expression control sequences include, but are not limited to appropriate promoters, enhancers, transcription terminators, a start codon (i.e., ATG) in front of a protein-encoding gene, splicing signal for introns, maintenance of the correct reading frame of that gene to permit proper translation of mRNA, and stop codons.

The polynucleotide sequences encoding the antibody or immunotoxin can be inserted into an expression vector including, but not limited to a plasmid, virus or other vehicle that can be manipulated to allow insertion or incorporation of sequences and can be expressed in either prokaryotes or eukaryotes. Hosts can include microbial, yeast, insect and mammalian organisms. Methods of expressing DNA sequences having eukaryotic or viral sequences in prokaryotes are well known in the art. Biologically functional viral and plasmid DNA vectors capable of expression and replication in a host are known in the art.

Transformation of a host cell with recombinant DNA may be carried out by conventional techniques as are well known to those skilled in the art. Where the host is prokaryotic, such as *E. coli*, competent cells which are capable of DNA uptake can be prepared from cells harvested after exponential growth phase and subsequently treated by the $CaCl_2$ method using procedures well known in the art. Alternatively, $MgCl_2$ or RbCl can be used. Transformation can also be performed after forming a protoplast of the host cell if desired, or by electroporation.

When the host is a eukaryote, such methods of transfection of DNA as calcium phosphate coprecipitates, conventional mechanical procedures such as microinjection, electroporation, insertion of a plasmid encased in liposomes, or virus vectors may be used. Eukaryotic cells can also be cotrans-

formed with polynucleotide sequences encoding the immunotoxin, and a second foreign DNA molecule encoding a selectable phenotype, such as the herpes simplex thymidine kinase gene. Another method is to use a eukaryotic viral vector, such as simian virus 40 (SV40) or bovine papilloma virus, to transiently infect or transform eukaryotic cells and express the protein (see for example, *Eukaryotic Viral Vectors*, Cold Spring Harbor Laboratory, Gluzman ed., 1982). One of skill in the art can readily use an expression systems such as plasmids and vectors of use in producing proteins in cells including higher eukaryotic cells such as the COS, CHO, HeLa and myeloma cell lines.

Isolation and purification of recombinantly expressed polypeptide may be carried out by conventional means including preparative chromatography and immunological separations. Once expressed, the recombinant immunotoxins can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, and the like (see, generally, R. Scopes, *Protein Purification*, Springer-Verlag, N.Y., 1982). Substantially pure compositions of at least about 90 to 95% homogeneity are disclosed herein, and 98 to 99% or more homogeneity can be used for pharmaceutical purposes. Once purified, partially or to homogeneity as desired, if to be used therapeutically, the polypeptides should be substantially free of endotoxin.

Methods for expression of single chain antibodies and/or refolding to an appropriate active form, including single chain antibodies, from bacteria such as *E. coli* have been described and are well-known and are applicable to the antibodies disclosed herein. See, Buchner et al., *Anal. Biochem.* 205:263-270, 1992; Pluckthun, *Biotechnology* 9:545, 1991; Huse et al., *Science* 246:1275, 1989 and Ward et al., *Nature* 341:544, 1989, all incorporated by reference herein.

Often, functional heterologous proteins from *E. coli* or other bacteria are isolated from inclusion bodies and require solubilization using strong denaturants, and subsequent refolding. During the solubilization step, as is well known in the art, a reducing agent must be present to separate disulfide bonds. An exemplary buffer with a reducing agent is: 0.1 M Tris pH 8, 6 M guanidine, 2 mM EDTA, 0.3 M DTE (dithioerythritol). Reoxidation of the disulfide bonds can occur in the presence of low molecular weight thiol reagents in reduced and oxidized form, as described in Saxena et al., *Biochemistry* 9: 5015-5021, 1970, incorporated by reference herein, and especially as described by Buchner et al., supra.

Renaturation is typically accomplished by dilution (e.g., 100-fold) of the denatured and reduced protein into refolding buffer. An exemplary buffer is 0.1 M Tris, pH 8.0, 0.5 M 1-arginine, 8 mM oxidized glutathione (GSSG), and 2 mM EDTA.

As a modification to the two chain antibody purification protocol, the heavy and light chain regions are separately solubilized and reduced and then combined in the refolding solution. An exemplary yield is obtained when these two proteins are mixed in a molar ratio such that a 5-fold molar excess of one protein over the other is not exceeded. It is desirable to add excess oxidized glutathione or other oxidizing low molecular weight compounds to the refolding solution after the redox-shuffling is completed.

In addition to recombinant methods, the immunoconjugates, EM, and antibodies disclosed herein can also be constructed in whole or in part using standard peptide synthesis. Solid phase synthesis of the polypeptides of less than about 50 amino acids in length can be accomplished by attaching the C-terminal amino acid of the sequence to an insoluble support followed by sequential addition of the remaining amino acids in the sequence. Techniques for solid phase synthesis are described by Barany & Merrifield, *The Peptides: Analysis, Synthesis, Biology. Vol. 2: Special Methods in Peptide Synthesis, Part A*. pp. 3-284; Merrifield et al., *J. Am. Chem. Soc.* 85:2149-2156, 1963, and Stewart et al., *Solid Phase Peptide Synthesis,* 2nd ed., Pierce Chem. Co., Rockford, Ill., 1984. Proteins of greater length may be synthesized by condensation of the amino and carboxyl termini of shorter fragments. Methods of forming peptide bonds by activation of a carboxyl terminal end (e.g., by the use of the coupling reagent N,N'-dicycylohexylcarbodiimide) are well known in the art.

BRAF Inhibitors

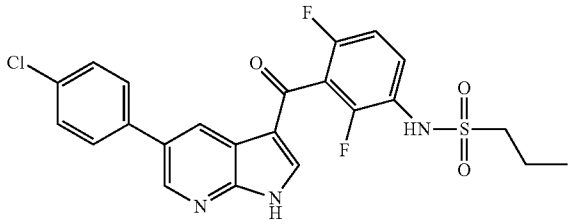
The methods disclosed herein utilize BRAF inhibitors. One exemplary amino acid sequence for human BRAF is provided below.

```
MAALSGGGGG GAEPGQALFN GDMEPEAGAG AGAAASSAAD

PAIPEEVWNI KQMIKLTQEHIEALLDKFGG EHNPPSIYLE

AYEEYTSKLD ALQQREQQLL ESLGNGTDFS VSSSASMDTV

TSSSSSSLSV LPSSLSVFQN PTDVARSNPK SPQKPIVRVF

LPNKQRTVVP ARCGVTVRDSLKKALMMRGL IPECCAVYRI

QDGEKKPIGW DTDISWLTGE ELHVEVLENV PLTTHNFVRK

TFFTLAFCDF CRKLLFQGFR CQTCGYKFHQ RCSTEVPLMC

VNYDQLDLLF VSKFFEHHPI PQEEASLAET ALTSGSSPSA

PASDSIGPQI LTSPSPSKSI PIPQPFRPAD EDHRNQFGQR

DRSSSAPNVH INTIEPVNID DLIRDQGFRG DGGSTTGLSA

TPPASLPGSL TNVKALQKSPGPQRERKSSS SSEDRNRMKT

LGRRDSSDDW EIPDGQITVG QRIGSGSFGT VYKGKWHGDV

AVKMLNVTAP TPQQLQAFKN EVGVLRKTRH VNILLFMGYS

TKPQLAIVTQ WCEGSSLYHHLHIIETKFEM IKLIDIARQT

AQGMDYLHAK SIIHRDLKSN NIFLHEDLTV KIGDFGLATV

KSRWSGSHQF EQLSGSILWM APEVIRMQDK NPYSFQSDVY

AFGIVLYELM TGQLPYSNINNRDQIIFMVG RGYLSPDLSK

VRSNCPKAMK RLMAECLKKK RDERPLFPQI LASIELLARS

LPKIHRSASE PSLNRAGFQT EDFSLYACAS PKTPIQAGGY

GAFPVH
(SEQ ID NO:10, see GENBANK ® Accession
No. ACD11489.1, incorporated herein by
reference)
```

A number of BRAF inhibitors have been previously described (see, for example, PCT Publication Nos. WO 2007/002325, WO 2007/002433, WO 2009/047505, WO 03/086467; WO 2009/143024, WO 2010/104945, WO 2010/104973, WO 2010/111527 and WO 2009/152087; U.S. Pat. Nos. 6,187,799 and 7,329,670; and U.S. Patent Application Publication Nos. 2005/0176740 and 2009/0286783, each of which is herein incorporated by reference).

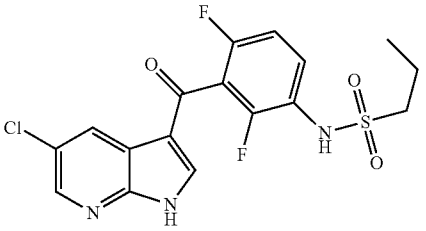
PLX 4032 (also known as RG7204, RO5185426, and Vemurafenib, $C_{23}H_{18}ClF_2N_3O_3S$) is a BRAF small molecule inhibitor being developed by Plexxikon and Roche (Genentech) for the treatment of melanoma. Phase I clinical trials in patients with advanced melanoma demonstrated that PLX

4032 was effective in promoting tumor regression and increasing overall survival in patients with the V600E BRAF mutation. In particular embodiments of the present disclosure, the BRAF inhibitor is PLX 4032:



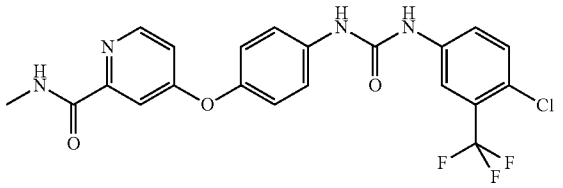
or a salt, solvate or functional derivative thereof. Thus, a therapeutically effective amount of PLX4032 can be used in combination with a therapeutically effective amount of an antibody that specifically binds GRP94 (or a nucleic acid encoding this antibody) for the treatment of tumors.

In other embodiments, the BRAF inhibitor is PLX 4720 ($C_{17}H_{14}ClF_2N_3O_3S$):



or a salt, solvate or functional derivative thereof. Thus, a therapeutically effective amount of PLX4720 can be used in combination with a therapeutically effective amount of an antibody that specifically binds GRP94 (or a nucleic acid encoding this antibody) for the treatment of tumors.

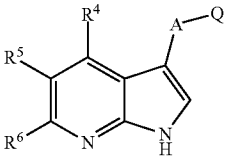
In further embodiments, the BRAF inhibitor is sofafenib ($C_{21}H_{16}ClF_3N_4O_3$):



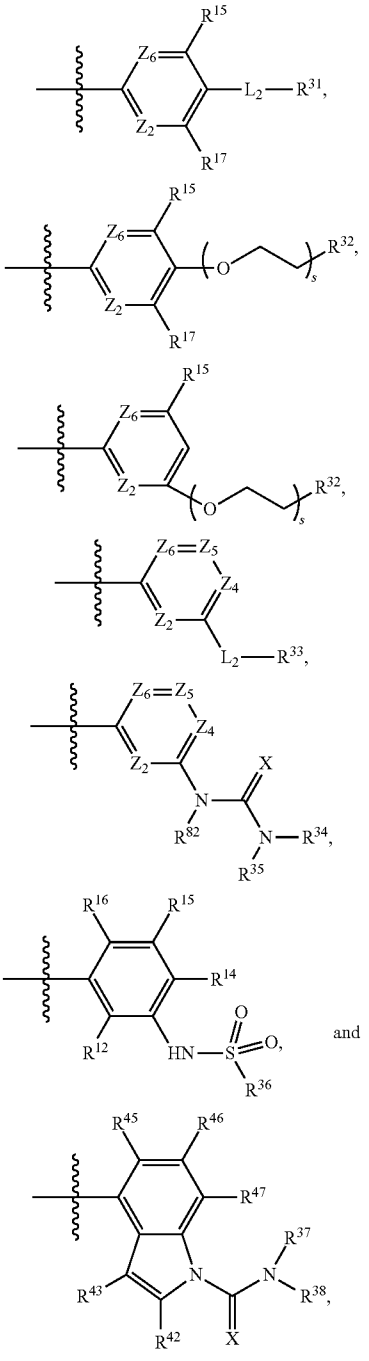
or a salt, solvate or functional derivative thereof. Sofafenib (Nexavar) is used for the treatment of renal cancer, liver cancer, thyroid cancer, lung cancer, glioblastoma and kidney cancer. Thus a therapeutically effective amount of sofafenib can be used in combination with a therapeutically effective amount of an antibody that specifically binds GRP94 (or a nucleic acid encoding this antibody) for the treatment of these tumors.

In some embodiments, the BRAF inhibitors have the structure of Formula III, shown below, or any salts, prodrugs, tautomers or isomers thereof, as described in PCT Publication Nos. WO 2007/002325 and WO 2007/002433 (which are incorporated herein by reference):

Formula III



wherein: Q has a structure selected from the group consisting of



and

in which

indicates the attachment point of Q to A of Formula III;

Z_2 is N or CR^{12}; Z_4 is N or CR^{14}; Z_5 is N or CR^{15}; Z_6 is N or CR^{16};

L2 is selected from the group consisting of $—(CR^{10}R^{11})_p—NR^{25}—(CR^{10}R^{11})_q—$, $—(CR^{10}R^{11})_p—O—(CR^{10}R^{11})_q—$, $—(CR^{10}R^{11})_p—S—(CR^{10}R^{11})_q—$, $—(CR^{10}R^{11})_pC(O)—(CR^{10}R^{11})_q—$, $—(CR^{10}R^{11})_p—C(S)—(CR^{10}R^{11})_q—$, $—(CR^{10}R^{11})_p—S(O)—(CR^{10}R^{11})_q—$, $—(CR^{10}R^{11})_p—S(O)_2—(CR^{10}R^{11})_q—$, $—(CR^{10}R^{11})_p—C(O)NR^{25}—(CR^{10}R^{11})_q—$, $—(CR^{10}R^{11})_p—C(S)NR^{25}—(CR^{10}R^{11})_q—$, $—(CR^{10}R^{11})_p—S(O)_2NR^{25}—(CR^{10}R^{11})_q—$, $—(CR^{10}R^{11})_p—NR^{25}C(O)—(CR^{10}R^{11})_q—$, $—(CR^{10}R^{11})_p—NR^{25}C(S)—(CR^{10}R^{11})_q—$, and $—(CR^{10}R^{11})_p—NR^{25}S(O)_2—(CR^{10}R^{11})_q—$;

p and q are independently 0, 1, or 2 provided, however, that at least one of p and q is 0;

s is 1 or 2;

X is O or S;

A is selected from the group consisting of —O—, —S—, $—CR^aR^b—$, $—NR^1—$, —C(O)—, —C(S)—, —S(O)—, and $—S(O)_2—$;

R^a and R^b at each occurrence are independently selected from the group consisting of hydrogen, fluoro, —OH, $—NH_2$, lower alkyl, lower alkoxy, lower alkylthio, mono-alkylamino, di-alkylamino, and $—NR^8R^9$, wherein the alkyl chain(s) of lower alkyl, lower alkoxy, lower alkylthio, mono-alkylamino, or di-alkylamino are optionally substituted with one or more substituents selected from the group consisting of fluoro, —OH, $—NH_2$, lower alkoxy, fluoro substituted lower alkoxy, lower alkylthio, fluoro substituted lower alkylthio, mono-alkylamino, di-alkylamino, and cycloalkylamino, provided, however, that any substitution of the alkyl chain carbon bound to O of alkoxy, S of thioalkyl or N of mono- or di-alkylamino is fluoro; or

R^a and R^b combine to form a 3-7 membered monocyclic cycloalkyl or 5-7 membered monocyclic heterocycloalkyl, wherein the monocyclic cycloalkyl or monocyclic heterocycloalkyl are optionally substituted with one or more substituents selected from the group consisting of halogen, —OH, $—NH_2$, lower alkyl, fluoro substituted lower alkyl, lower alkoxy, fluoro substituted lower alkoxy, lower alkylthio, fluoro substituted lower alkylthio, mono-alkylamino, di-alkylamino, and cycloalkylamino;

R^1 is selected from the group consisting of hydrogen, lower alkyl, cycloalkyl, heterocycloalkyl, aryl, heteroaryl, $—C(O)R^7$, $—C(S)R^7$, $—S(O)_2R^7$, $—C(O)NHR^7$, $—C(S)NHR^7$, and $—S(O)_2NHR^7$, wherein lower alkyl is optionally substituted with one or more substituents selected from the group consisting of fluoro, —OH, $—NH_2$, lower alkoxy, lower alkylthio, mono-alkylamino, di-alkylamino, and $—NR^8R^9$, wherein the alkyl chain(s) of lower alkoxy, lower alkylthio, mono-alkylamino, or di-alkylamino are optionally substituted with one or more substituents selected from the group consisting of fluoro, —OH, $—NH_2$, lower alkoxy, fluoro substituted lower alkoxy, lower alkylthio, fluoro substituted lower alkylthio, mono-alkylamino, di-alkylamino, and cycloalkylamino, provided, however, that any substitution of the alkyl chain carbon bound to O of alkoxy, S of thioalkyl or N of mono- or di-alkylamino is fluoro, further provided that when R^1 is lower alkyl, any substitution on the lower alkyl carbon bound to the N of $—NR^1—$ is fluoro, and wherein cycloalkyl, heterocycloalkyl, aryl or heteroaryl are optionally substituted with one or more substituents selected from the group consisting of halogen, —OH, $—NH_2$, lower alkyl, fluoro substituted lower alkyl, lower alkoxy, fluoro substituted lower alkoxy, lower alkylthio, fluoro substituted lower alkylthio, mono-alkylamino, di-alkylamino, and cycloalkylamino;

R^7 is selected from the group consisting of lower alkyl, cycloalkyl, heterocycloalkyl, aryl, and heteroaryl, wherein lower alkyl is optionally substituted with one or more substituents selected from the group consisting of fluoro, —OH, $—NH_2$, lower alkoxy, lower alkylthio, mono-alkylamino, di-alkylamino, and $—NR^8R^9$, provided, however, that any substitution of the alkyl carbon bound to the N of $—C(O)NHR^7$, $—C(S)NHR^7$ or $—S(O)_2NHR^7$ is fluoro, wherein the alkyl chain(s) of lower alkoxy, lower alkylthio, mono-alkylamino, or di-alkylamino are optionally substituted with one or more substituents selected from the group consisting of fluoro, —OH, $—NH_2$, lower alkoxy, fluoro substituted lower alkoxy, lower alkylthio, fluoro substituted lower alkylthio, mono-alkylamino, di-alkylamino, and cycloalkylamino, provided, however, that any substitution of the alkyl chain carbon bound to O of alkoxy, S of thioalkyl or N of mono- or di-alkylamino is fluoro, and wherein cycloalkyl, heterocycloalkyl, aryl and heteroaryl are optionally substituted with one or more substituents selected from the group consisting of halogen, —OH, $—NH_2$, lower alkyl, fluoro substituted lower alkyl, lower alkoxy, fluoro substituted lower alkoxy, lower alkylthio, fluoro substituted lower alkylthio, mono-alkylamino, di-alkylamino, and cycloalkylamino;

R^4, R^5, R^6, R^{12}, R^{14}, R^{15}, R^{16}, R^{42}, R^{43}, R^{45}, R^{46} and R^{47} are independently selected from the group consisting of hydrogen, halogen, optionally substituted lower alkyl, optionally substituted lower alkenyl, optionally substituted lower alkynyl, optionally substituted cycloalkyl, optionally substituted heterocycloalkyl, optionally substituted aryl, optionally substituted heteroaryl, —CN, $—NO_2$, $—CR^aR^bR^{26}$, and $-LR^{26}$;

L at each occurrence is independently selected from the group consisting of $-(alk)_a-S-(alk)_b-$, $-(alk)_a-O-(alk)_b-$, $-(alk)_a-NR^{25}—(alk)_b-$, $-(alk)_a-C(O)-(alk)_b-$, $-(alk)_a-C(S)-(alk)_b-$, $-(alk)_a-S(O)-(alk)_b-$, $-(alk)_a-S(O)_2-(alk)_b-$, $-(alk)_a-OC(O)-(alk)_b-$, $-(alk)_a-C(O)O-(alk)_b-$, $-(alk)_a-OC(S)-(alk)_b-$, $-(alk)_a-C(S)O-(alk)_b-$, $-(alk)_a-C(O)NR^{25}-(alk)_b-$, $-(alk)_a-C(S)NR^{25}-(alk)_b-$, $-(alk)_a-S(O)_2NR^{25}-(alk)_b-$, $-(alk)_a-NR^{25}C(O)-(alk)_b-$, $-(alk)_a-NR^{25}C(S)-(alk)_b-$, $-(alk)_a-NR^{25}S(O)_2-(alk)_b-$, $-(alk)_a-NR^{25}C(O)O-(alk)_b-$, $-(alk)_a-NR^{25}C(S)O-(alk)_b-$, $-(alk)_a-OC(O)NR^{25}-(alk)_b-$, $-(alk)_a-OC(S)NR^{25}-(alk)_b-$, $-(alk)_a-NR^{25}C(O)NR^{25}-(alk)_b-$, $-(alk)_a-NR^{25}C(S)NR^{25}-(alk)_b-$, and $-(alk)_a-NR^{25}S(O)_2NR^{25}-(alk)_b-$;

a and b are independently 0 or 1;

alk is C_{1-3} alkylene or C_{1-3} alkylene substituted with one or more substituents selected from the group consisting of fluoro, —OH, $—NH_2$, lower alkyl, lower alkoxy, lower alkylthio, mono-alkylamino, di-alkylamino, and $—NR^8R^9$, wherein lower alkyl or the alkyl chain(s) of lower alkoxy, lower alkylthio, mono-alkylamino or di-alkylamino are optionally substituted with one or more substituents selected from the group consisting of fluoro, —OH, $—NH_2$, lower alkoxy, fluoro substituted lower alkoxy, lower alkylthio, fluoro substituted lower alkylthio, mono-alkylamino, di-alkylamino and cycloalkylamino,

provided, however, that any substitution of the alkyl chain carbon bound to O of alkoxy, S of thioalkyl or N of mono- or di-alkylamino is fluoro;

R^{25} at each occurrence is independently selected from the group consisting of hydrogen, optionally substituted lower alkyl, optionally substituted cycloalkyl, optionally substituted heterocycloalkyl, optionally substituted aryl, and optionally substituted heteroaryl;

R^{26} at each occurrence is independently selected from the group consisting of hydrogen, provided, however, that hydrogen is not bound to any of S(O), $S(O)_2$, C(O) or C(S) of L, optionally substituted lower alkyl, optionally substituted lower alkenyl, provided, however, that when R^{26} is optionally substituted lower alkenyl, no alkene carbon thereof is bound to N, S, O, S(O), $S(O)_2$, C(O) or C(S) of L, optionally substituted lower alkynyl, provided, however, that when R^{26} is optionally substituted lower alkynyl, no alkyne carbon thereof is bound to N, S, O, S(O), $S(O)_2$, C(O) or C(S) of L, optionally substituted cycloalkyl, optionally substituted heterocycloalkyl, optionally substituted aryl, and optionally substituted heteroaryl;

R^{10} and R^{11} at each occurrence are independently selected from the group consisting of hydrogen, fluoro, lower alkyl, and lower alkyl optionally substituted with one or more substituents selected from the group consisting of fluoro, —OH, $—NH_2$, lower alkoxy, fluoro substituted lower alkoxy, lower alkylthio, fluoro substituted lower alkylthio, mono-alkylamino, di-alkylamino, and cycloalkylamino; or any two of R^{10} and R^{11} on the same or adjacent carbon atoms combine to form a 3-7 membered monocycle cycloalkyl or 5-7 membered monocyclic heterocycloalkyl, and any others of R^{10} and R^{11} are independently selected from the group consisting of hydrogen, fluoro, lower alkyl, and lower alkyl optionally substituted with one or more substituents selected from the group consisting of fluoro, —OH, $—NH_2$, lower alkoxy, fluoro substituted lower alkoxy, lower alkylthio, fluoro substituted lower alkylthio, mono-alkylamino, di-alkylamino, and cycloallylamino, and wherein the monocyclic cycloalkyl or monocyclic heterocycloalkyl are optionally substituted with one or more substituents selected from the group consisting of halogen, —OH, $—NH_2$, lower alkyl, fluoro substituted lower alkyl, lower alkoxy, fluoro substituted lower alkoxy, lower alkylthio, fluoro substituted lower alkylthio, mono-alkylamino, di-alkylamino, and cycloalkylamino;

R^8 and R^9 combine with the nitrogen to which they are attached to form a 5-7 membered heterocycloalkyl optionally substituted with one or more substituents selected from the group consisting of fluoro, —OH, $—NH_2$, lower alkyl, fluoro substituted lower alkyl, lower alkoxy, fluoro substituted lower alkoxy, lower alkylthio, and fluoro substituted lower alkylthio;

R^{17} is selected from the group consisting of hydrogen, halogen, optionally substituted lower alkyl and —OR^{18};

R^{31} and R^{33} are independently selected from the group consisting of optionally substituted aryl, optionally substituted heteroaryl, optionally substituted cycloalkyl, and optionally substituted heterocycloalkyl;

R^{36} is selected from the group consisting of substituted methyl, optionally substituted C_{2-6} alkyl, optionally substituted lower alkenyl, provided, however, that when R^{36} is optionally substituted lower alkenyl, no alkene carbon thereof is bound to the $S(O)_2$ of $S(O)_2R^{36}$, optionally substituted lower alkynyl, provided, however, that when R^{36} is optionally substituted lower alkynyl, no alkyne carbon thereof is bound to the $S(O)_2$ of $S(O)_2R^{36}$, optionally substituted cycloalkyl, optionally substituted heterocycloalkyl, optionally substituted aryl, optionally substituted heteroaryl, and —$NR^{19}R^{20}$;

R^{19}, R^{20}, R^{34}, R^{35}, R^{37}, and R^{38} are independently selected from the group consisting of hydrogen, optionally substituted lower alkyl, optionally substituted lower alkenyl, provided, however, that when R^{19}, R^{20}, R^{34}, R^{35}, R^{37}, or R^{38} is optionally substituted lower alkenyl, no alkene carbon thereof is bound to the N of $NR^{19}R^{20}$, $NR^{34}R^{35}$ or $NR^{37}R^{38}$, optionally substituted lower alkynyl, provided, however, that when R^{19}, R^{20}, R^{34}, R^{35}, R^{37}, or R^{38} is optionally substituted subs lower alkynyl, no alkyne carbon thereof is bound to the N of $NR^{19}R^{20}$, $NR^{34}R^{35}$ or $NR^{37}R^{38}$, optionally substituted cycloalkyl, optionally substituted heterocycloalkyl, optionally substituted aryl and optionally substituted heteroaryl; or

R^{34} and R^{35} together with the nitrogen to which they are attached form optionally substituted 5-7 membered heterocycloalkyl or optionally substituted 5 or 7 membered nitrogen containing heteroaryl; or

R^{37} and R^{38} together with the nitrogen to which they are attached form optionally substituted 5-7 membered heterocycloalkyl or optionally substituted 5 or 7 membered nitrogen containing heteroaryl;

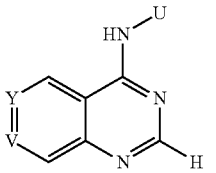
R^{32} is selected from the group consisting of hydrogen, optionally substituted lower alkyl, optionally substituted cycloalkyl, optionally substituted heterocycloalkyl, optionally substituted aryl, optionally substituted heteroaryl, and —OR^{18};

R^{82} is selected from hydrogen or lower alkyl; and

R^{18} is hydrogen or optionally substituted lower alkyl;

In other embodiments of the present disclosure, the BRAF inhibitor comprises a formula as described in PCT Publication No. WO 03/086467 (incorporated herein by reference) as Formula I, Formula II or Formula III:

Formula I—



(I)

or a salt, solvate, physiologically functional derivative thereof;

wherein

Y is CR^1 and V is N;

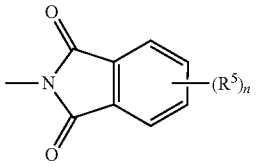
or Y is CR^1 and V is CR^2;

R^1 represents a group $CH_3SO_2CH_2CH_2NHCH_2$—Ar—, wherein Ar is selected from phenyl, furan, thiophene, pyrrole and thiazole, each of which may optionally be substituted by one or two halo, C_{1-4} alkyl or C_{1-4} alkoxy groups;

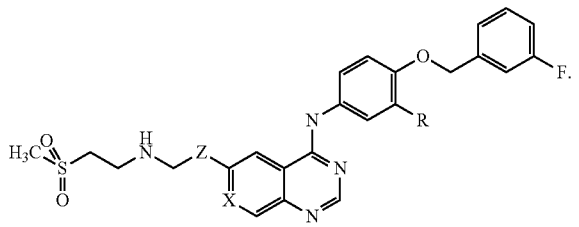
R^2 is selected from the group comprising hydrogen, halo, hydroxy, C_{1-4} alkyl, C_{1-4} alkoxy, C_{1-4} alkylamino and di[C_{1-4} alkyl]amino;

U represents a phenyl, pyridyl, 3H-imidazolyl, indolyl, isoindolyl, indolinyl, isoindolinyl, 1H-indazolyl, 2,3-dihydro-1H-indazolyl, 1H-benzimidazolyl, 2,3-dihydro-1H-benzimidazolyl or 1H-benzotriazolyl group, substituted by an R^3 group and optionally substituted by at least one independently selected R^4 group;

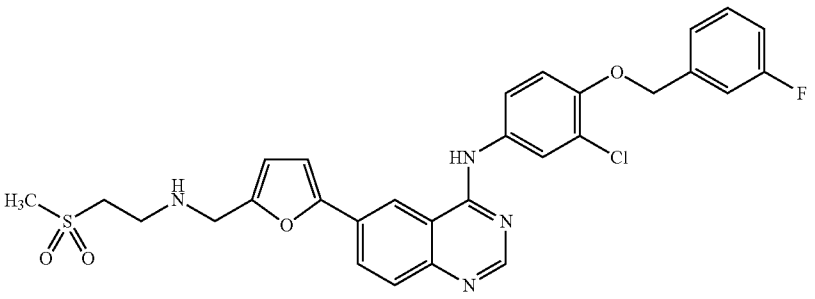
R^3 is selected from a group comprising benzyl, halo-, dihalo- and trihalobenzyl, benzoyl, pyridylmethyl, pyridylmethoxy, phenoxy, benzyloxy, halo-, dihalo- and trihalobenzyloxy and benzenesulphonyl;
or R^3 represents trihalomethylbenzyl or trihalomethylbenzyloxy;
or R^3 represents a group of formula



wherein each R^5 is independently selected from halogen, C_{1-4} alkyl and C_{1-4} alkoxy; and n is 0 to 3;
each R^4 is independently hydroxy, halogen, C_{1-4} alkyl, C_{2-4} alkenyl, C_{2-4} alkynyl, C_{1-4} alkoxy, amino, C_{1-4} alkylamino, di[C_{1-4} alkyl]amino, C_{1-4} alkylthio, C_{1-4} alkylsulphinyl, C_{1-4} alkylsulphonyl, C_{1-4} alkylcarbonyl, carboxy, carbamoyl, C_{1-4} alkoxycarbonyl, C_{1-4} alkanoylamino, N—(C_{1-4} alkyl)carbamoyl, N,N-di(C_{1-4} alkyl) carbamoyl, cyano, nitro and trifluoromethyl.
Additional BRAF inhibitors are shown below:



wherein R is —Cl or —Br, X is CH, N, or CF, and Z is thiazole or furan.



In some embodiments, the BRAF inhibitor is selected from a compound as described in PCT Publication No. WO 2010/104973 which is incorporated by reference herein. These include the following:

In a first aspect, a compound selected from the group consisting of N-[3-(4-cyano-1H-pyrrolo[2,3-b]pyridine-3-carbonyl)-2,4-difluoro-phenyl]-4-trifluorometh)/1-benzenesulfonamide (P-0001), N-[3-(4-ethynyl-1H-pyrrolo[2,3-b]pyridine-3-carbonyl)-2,4-difluoro-phenyl]-4-trifluoromethyl-benzenesulfonamide (P-0002), any salt thereof, any formulation thereof, any conjugate thereof, any derivative thereof, and any form thereof is provided. In certain embodiments P-0001, P-0002, or a salt thereof, formulation thereof, conjugate thereof, derivative thereof, or form thereof is an inhibitor of one or more Raf protein kinases, including A-Raf, B-Raf, and c-Raf-1 (including any mutations of these kinases),

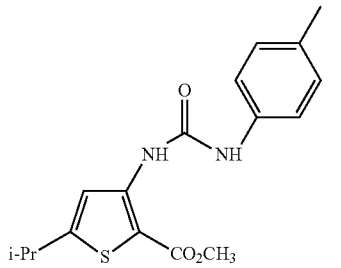
In a second aspect the compound N-[3-(4-cyano-1H-pyrrolo[2,3-b]pyridine-3-carbonyl)-2,4-difluoro-phenyl]-4-trifluoromethyl-benzenesulfonamide (P-0001), or a salt thereof, formulation thereof, conjugate thereof, derivative thereof, or form thereof is provided. In certain embodiments P-0001, or a salt thereof, formulation thereof, conjugate thereof, derivative thereof, or form thereof is an inhibitor of one or more Raf protein kinases, including
A-Raf, B-Raf, and c-Raf-1 (including any mutations of these kinases).

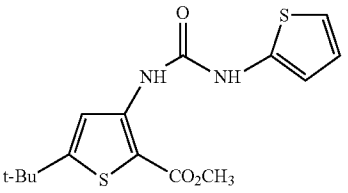
In a third aspect the compound N-[3-(4-ethynyl-1H-pyrrolo[2,3-b]pyridine-3-carbonyl)-2,4-difluoro-phenyl]-4-trifluoromethyl-benzenesulfonamide (P-0002), or a salt thereof, formulation thereof, conjugate thereof, derivative thereof, or form thereof is provided. In certain embodiments P-0002, or a salt thereof, formulation thereof, conjugate thereof, derivative thereof, or form thereof is an inhibitor of one or more Raf protein kinases, including A-Raf, B-Raf, and c-Raf-1 (including any mutations of these kinases).

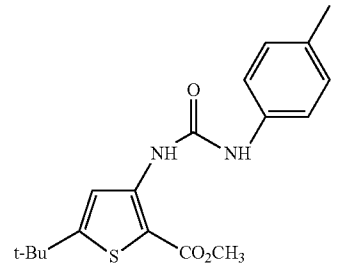
In some embodiments, the BRAF inhibitor is selected from a compound as described in PCT Publication No. WO 2010/104945, which is incorporated by reference herein. These include the following:

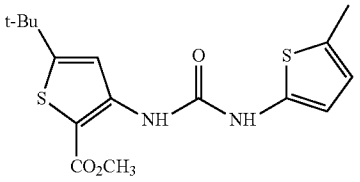
In a first aspect, a compound selected from the group consisting of propane-1-sulfonic acid {2,4-difluoro-3-[5-(2-methoxy-pyrimidin-5-yl)-1H-pyr[tau]olo[2,3-b]pyridine-3-carbonyl]-phenyl}-amide (P-0001), propane-1-sulfonic acid [3-(5-cyano-1H-pyrrolo[2,3-b]pyridine-3-carbonyl)-2,4-difluoro-phenyl]-amide (P-0002), propane-1-sulfonic acid [3-(5-cyano-1H-pyrrolo[2,3-b]pyridine-3-carbonyl)-2-fluoro-phenyl]-amide (P-0003), N-[3-(5-cyano-1H-pyrrolo[2,3-b]pyridine-3-carbonyl)-2,4-difluoro-phenyl]-2,5-diflu[upsilon]r[upsilon]-ben/enesulfonamide (P-0004), N-[3-(5-cyano-1H-pyrrolo[2,3-b]pyridine-3-carbonyl)-2,4-difluoro-phenyl]-3-fluoro-benzenesulfonamide (P-0005), pyrrolidine-1-sulfonic acid [3-(5-cyano-1H-pyi[tau]olo[2,3-b]pyridine-3-carbonyl)-2,4-difluoro-phenyl]-amide (P-0006), N,N-dimethylamino-sulfonic acid [3-(5-cyano-1H-pyrrolo[2,3-b]pyridine-3-carbonyl)-2,4-difluoro-phenyl]-amide (P-0007). any salt thereof, any formulation thereof, any conjugate thereof, any derivative thereof, and any form thereof is provided. In certain embodiments P-0001, P-0002, P-0003, P-0004, P-0005, P-0006, P-0007. or a salt thereof, formulation thereof, conjugate thereof, derivative thereof, or form thereof is an inhibitor of one or more Raf prolei[pi] kinases, including [Lambda]-Raf, R-Raf. and c-Raf-1 (including any mutations of these kinases).

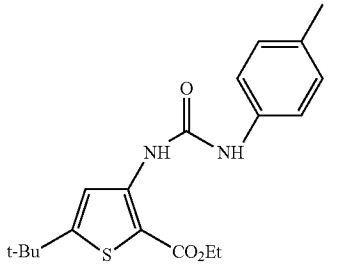
In some embodiments, the BRAF inhibitor is a compound having a formula from U.S. Pat. No. 6,187,799, which is incorporated by reference herein:

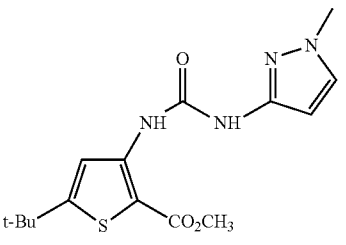












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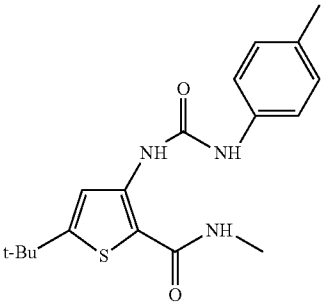

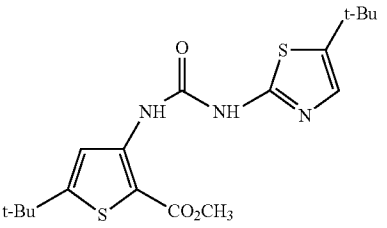

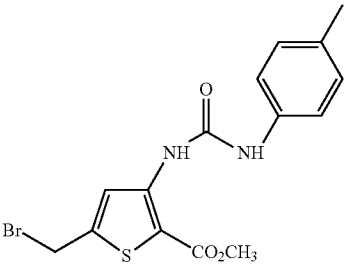

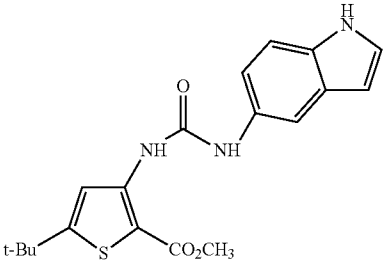

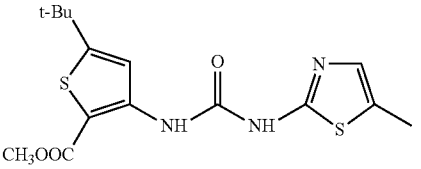

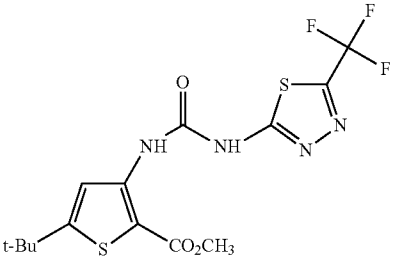

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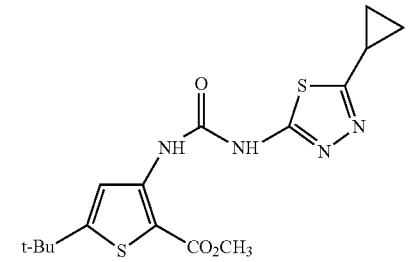

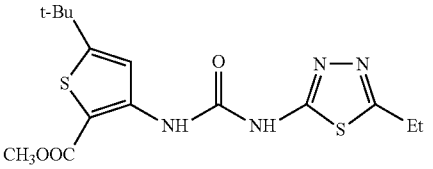

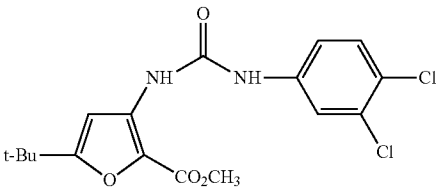

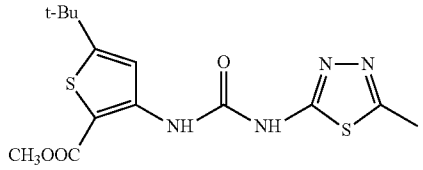

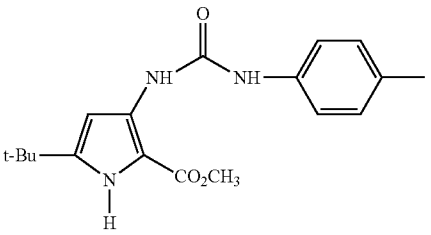

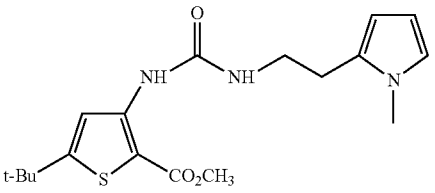

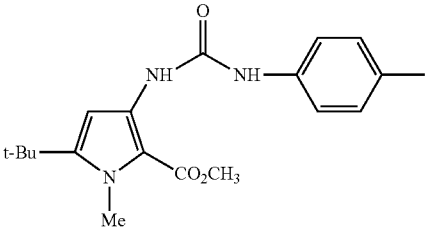

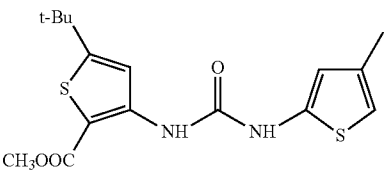

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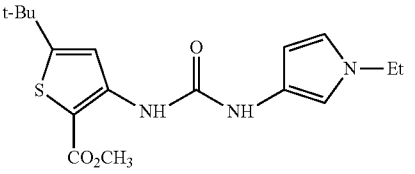

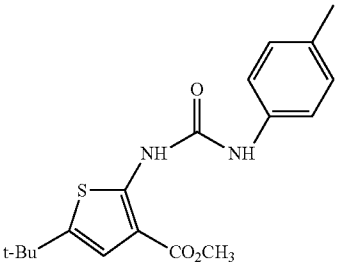

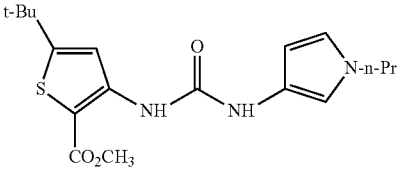

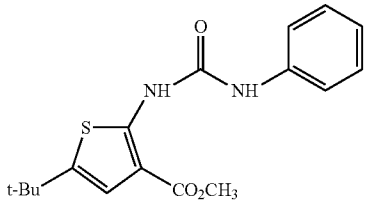

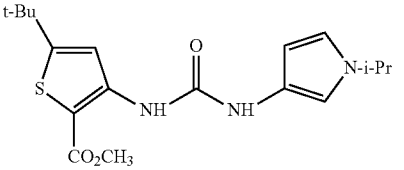

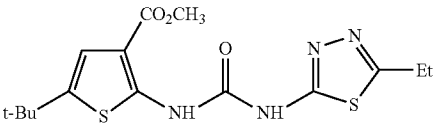

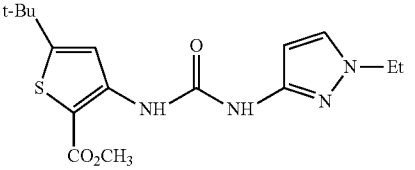

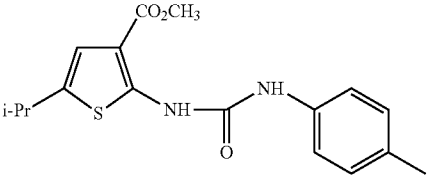

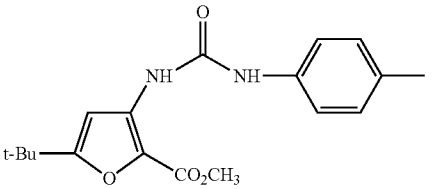

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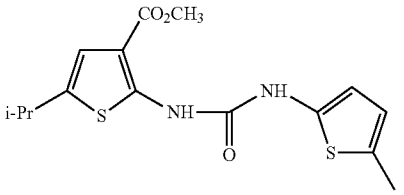
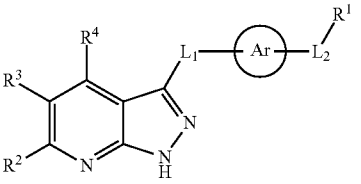


In some embodiments, the BRAF inhibitor is a compound as disclosed in PCT Publication No. WO 2010/111527, which is incorporated by reference herein. For example, the BRAF inhibitor can have a structure according to Formula I of PCT Publication No. WO 2010/111527.

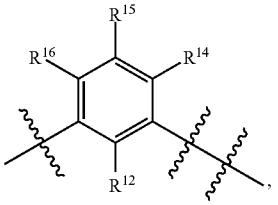
Formula I

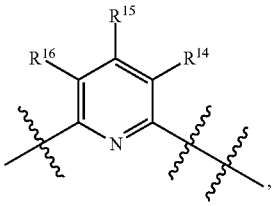


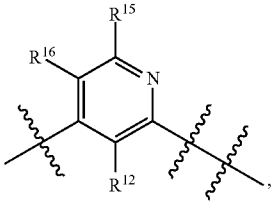
or a salt, a prodrug, a tautomer or an isomer thereof,

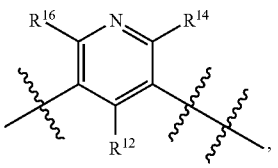
wherein:

Ar is selected from the group consisting of:









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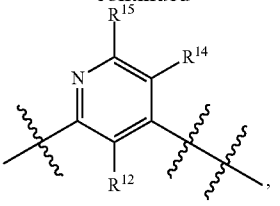
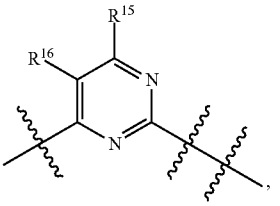
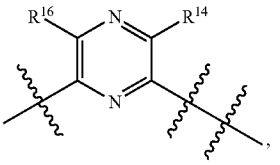
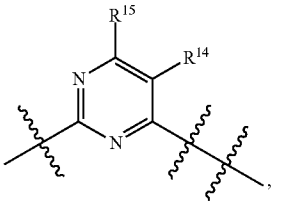
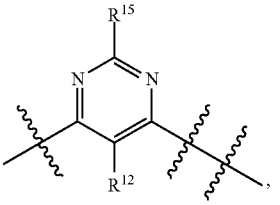
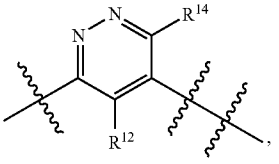


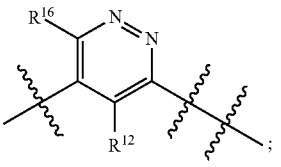








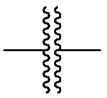




and

wherein

indicates the point of attachment of Ar to L^ of Formula I and



indicates the point of attachment of Ar to L^ of Formula I;

L, is selected from the group consisting Of —C(R^5R^6)—, —C(O)—, —C(S)—, —N(R^7)—, —O—, —S—, —S(O)—, and —S(O)2-;

L2 is selected from the group consisting of —N(R^8)—C(O)—, —N(R^8)—C(S)—, —N(R^8)—S(O)—N(R^8)—S(O)2-, —N(R^8)—C(O)—N(R^8)—, —N(R^8)—C(S)—N(R^8)—, and —N(R^8)—S(O)2-N(R^8)—;

R^1 is selected from the group consisting of optionally substituted lower alkyl, optionally substituted lower alkenyl, optionally substituted lower alkynyl, optionally substituted cycloalkyl, optionally substituted heterocycloalkyl, optionally substituted aryl, and optionally substituted heteroaryl;

R^2 is selected from the group consisting of hydrogen, halogen, optionally substituted lower alkyl, optionally substituted lower alkenyl, optionally substituted lower alkynyl, optionally substituted cycloalkyl, optionally substituted heterocycloalkyl, optionally substituted aryl, optionally substituted heteroaryl, —CN, —NO_2, —O—R^9, —S—R^{11}, —N(R^9)—R^{10}, —C(O)—R^{11}, —C(S)—R^{11}, —C(O)—N(R^9R^{10}, —C(S)—N(R^9)—R^{10}, —C(O)—N(R^{13})—OR^9, —C(S)—N(R^{13})—OR^9, —C(O)—N(R^{13})—S(O_2)—R^{11}, —C(S)—N(R^{13})—S(O_2)—R^{11}, —C(O)—O—R^9, —S(O)—R^{11}, —S(O_2)—R^{11}, —S(O)—N(R^9)—R^{10}, —S(O_2)—N(R^9)—R^{10}, —S(O_2)—N(R^{13})—C(O)R^{11}, —S(O_2)—N(R^{13})—C(S)R^{11}, —N(R^{13})—C(O)—R^{11}, —N(R^{13})—C(S)—R^{11}, —N(R^{13})—S(O)—R^{11}, —N(R^{13})—S(O_2)—R^{11}, —N(R^{13})—C(O)—N(R^9)—R^{10}, N(R^{13})—C(S)—N(R^9)—R^{10}, and —N(R^{13})—S(O_2)—N(R^9)—R^{10};

R^3 is selected from the group consisting of hydrogen, halogen, optionally substituted lower alkyl, optionally substituted lower alkenyl, optionally substituted lower alkynyl, optionally substituted cycloalkyl, optionally substituted heterocycloalkyl, optionally substituted aryl, optionally substituted heteroaryl, —CN, —NO_2, —O—R^{17}, —S—R^{19}, —N(R^{17})—R^{18}, —C(O)—R^{19}, —C(S)—R^{19}, —C(O)—N($R^{17}R^{18}$, —C(S)—N(R^{17})—R^{18}, —C(O)—N(R^{20})—OR^{17}, —C(S)—N(R^{20})—OR^{17}, —C(O)—N(R^{20})—S(O_2)—R^{19}, —C(S)—N(R^{20})—S(O_2)—R^{19}, —C(O)—O—R^{17}, —S(O)—R^{19}, —S(O_2)—R^{19}, —S(O)—N(R^{17})—R^{18}, —S(O_2)—N(R^{17})—R^{18}, —S(O_2)—N(R^{20})—C(O)R^{19}, —S(O_2)—N(R^{20})—C(S)R^{19}, —N(R^{20})—C(O)—R^{19}, —N(R^{20})—C(S)—R^{19}, —N(R^{20})S(O)—R^{19}, —N(R^{20})S(O_2)—R^{19}, —N(R^{20})C(O)—N(R^{17})—R^{18}, —N(R^{20})—C(S)—N(R^{17})—R^{18}, and —N(R^{20}—S(O_2)—N(R^{17})—R^{18};

R^4 is selected from the group consisting of hydrogen, halogen, optionally substituted lower alkyl, optionally substituted lower alkenyl, optionally substituted lower alkynyl, optionally substituted cycloalkyl, optionally substituted heterocycloalkyl, optionally substituted aryl, optionally substituted heteroaryl, —CN, NO_2, —O—R^{21}, —S—R^{23}, —N(R^{21})R^{22}, —C(O)R^{23}, —C(S)R^{23}, —C(O)—N(R^{21})—R^{22}, —C(S)—N(R^{21})—R^{22}, —C(O)—N(R^{24})—OR^{21}, —C(S)—N(R^{24})—OR^{21}, —C(O)—N(R^{24})—S(O_2)—R^{23}, —C(S)—N(R^{24})—S(O_2)—R^{23}, —C(O)—O—R^{21}, —S(O_2)—R^{23}, —S(O_2)—R^{23}, —S(O)—N(R^{21})—R^{22}, —S(O)2-N(R^{21})—R^{22}, —S(O_2)—N(R^{24})—C(O)R^{23}, —S(O_2—N(R^{24})—C(S)R^{23}, —N(R^{24})—C(O)—R^{23}, —N(R^{24})—C(S)—R^{23}, —N(R^{24})—S(O)—R^{23}, —N(R^{24})—S(O_2)—R^{23}, —N(R^{24})—C(O)—N(R^{21})—R^{22}, —N(R^{24})—C(S)—N(R^{21})—R^{22}, and —N(R^{24})—S(O_2)—N(R^{21})—R^{22};

R^5 and R^6 are independently selected from the group consisting of hydrogen, fluoro, —OH, —NH_2, lower alkyl, lower alkoxy, lower alklylthio, mono-alkylamino, di-alkylammo, and —N(R^{25})—R^{26}, wherein the alkyl chain(s) of lower alkyl, lower alkoxy, lower alkylthio, mono-alkylamino, or di-alkylamino are optionally substituted with one or more substituents selected from the group consisting of fluoro, —OH, —NH_2, lower alkoxy, fluoro substituted lower alkoxy, lower alkylthio, fluoro substituted lower alkylthio, mono-alkylamino, di-alkylamino, and cycloalkylammo; or

R^5 and R^6 combine to form a 3-7 membered monocyclic cycloalkyl or 5-7 membered monocyclic heterocycloalkyl, wherein the 3-7 membered monocyclic cycloalkyl or 5-7 membered monocyclic heterocycloalkyl are optionally substituted with one or more substituents selected from the group consisting of halogen, —OH, —NH_2, lower alkyl, fluoro substituted lower alkyl, lower alkoxy, fluoro substituted lower alkoxy, lower alkylthio, fluoro substituted lower alkylthio, mono-alkylammo, di-alkylamino, and cycloalkylammo,

R^7, R^{13}, R^{20}, and R^{24} are independently selected from the group consisting of hydrogen, optionally substituted lower alkyl, optionally substituted cycloalkyl, optionally substituted heterocycloalkyl, optionally substituted aryl, optionally substituted heteroaryl, —C(O)—R^{27}, —C(S)—R^{27}, —S(O)—R^{27}, —S(O_2)—R^{27}, —C(O)—N(H)—R^{27}, —C(S)—N(H)—R^{27}, and —S(O_2)—N(H)—R^{27},

R^8 at each occurrence is independently hydrogen, lower alkyl, or lower alkyl substituted with one or more substituents selected from the group consisting of fluoro, —OH, —NH_2, lower alkoxy, fluoro substituted lower alkoxy, lower alkylthio, fluoro substituted lower alkylthio, mono-alkylamino, fluoro substituted mono-alkylammo, di-alkylamino, fluoro substituted di-alkylamino, and —N(R^{25})—R^{26},

R^{12}, R^{14}, R^{15}, and R^{16} are independently selected from the group consisting of hydrogen, halogen, optionally substituted lower alkyl, —N(R^{28})—R^{29}, —O—R^{28}, and —S—R^{30},

R^{11}, R^{19} and R^{23} are independently selected from the group consisting of optionally substituted lower alkyl, optionally substituted lower alkenyl, optionally substituted lower alkynyl, optionally substituted cycloalkyl, optionally substituted heterocycloalkyl, optionally substituted aryl, and optionally substituted heteroaryl,

R^9, R^{10}, R^{17}, R^{18}, R^{21} and R^{22} are independently selected from the group consisting of hydrogen, optionally substituted lower alkyl, optionally substituted lower alkenyl, optionally substituted lower alkynyl, optionally substituted cycloalkyl, optionally substituted heterocycloalkyl, optionally substituted aryl, and optionally substituted heteroaryl;

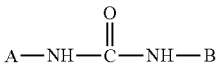
R^{25} and R^{26} at each occurrence combine with the nitrogen to which they are attached to form a 5-7 membered heterocycloalkyl optionally substituted with one or more substituents selected from the group consisting of fluoro, —OH, —NH_2, lower alkyl, fluoro substituted lower alkyl, lower alkoxy, fluoro substituted lower alkoxy, lower alkylthio, and fluoro substituted lower alkylthio;

R^{27} at each occurrence is independently selected from the group consisting of optionally substituted lower alkyl, optionally substituted cycloalkyl, optionally substituted heterocycloalkyl, optionally substituted aryl, and optionally substituted heteroaryl;

R^{28} and R^{29} at each occurrence are independently hydrogen or optionally substituted lower alkyl; and

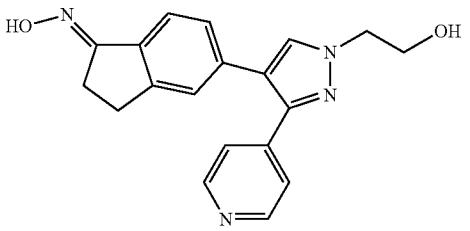
R^{30} at each occurrence is optionally substituted lower alkyl.

In some embodiments, the BRAF inhibitor is a compound having the formula disclosed in U.S. Pat. No. 7,329,670, which is incorporated by reference herein. For example, the BRAF inhibitor can have the following structure



wherein B is generally an unsubstituted or substituted, up to tricyclic, aryl or heteroaryl moiety with up 30 carbon atoms with at least one 5 or 6 member aromatic structure containing 0-4 members of the group consisting of nitrogen, oxygen and sulfur. A is a heteroaryl moiety.

In some examples of the presence disclosure, the BRAF inhibitor is GDC 0789 (Genentech):



In other examples, the inhibitor is PD-325901, XL518, PD-184352, PD-318088, AZD6244 or CI-1040 (see PCT Publication No. WO 2009/047505, incorporated herein by reference).

In further examples, the BRAF inhibitor can be an antibody that specifically binds BRAF, or an antigen binding fragment thereof. This includes monoclonal antibodies and antigen binding fragments that specifically bind BRAF. The BRAF inhibitor can also be an inhibitory RNA, such as, but not limited to, anti-sense RNA, small inhibitor RNA, and shRNA.

Methods of Treatment

Methods are provided herein for treating a subject diagnosed with a tumor that expresses a mutated BRAF. The methods include administering to the subject (1) a therapeutically effective amount of an antibody or antigen binding fragment thereof that specifically binds glucose regulated protein (GRP) 94, or a nucleic acid encoding the antibody or antigen binding fragment; and (2) a therapeutically effective amount of a BRAF inhibitor, thereby treating the cancer in the subject.

These use of a BRAF inhibitor in combination with an antibody or antigen binding fragment thereof that specifically binds GRP94 provide an unexpectedly superior result for the treatment of any tumor, wherein cells in the tumor comprise a BRAF mutation. In other embodiments, the BRAF mutation is a V600E mutation. In other embodiments, the BRAF mutation is R462I, I463S, G464E, G464V, G466A, G466E, G466V, G469A, G469E, N581S, E585K, D594V, F595L, G596R, L597V, T599I, V600D, V600E, V600K, V600R, K601E, and A728V. In further embodiments the BRAF mutation is in one of two regions: the glycine-rich P loop of the N lobe and the activation segment and flanking regions. The cells in the tumor can have more than one BRAF mutation.

In additional embodiments, the subject has a primary or secondary resistance to the BRAF inhibitor. A subject that does not respond initially to a BRAF inhibitor has primary resistance. A subject that initially responds to the BRAF inhibitor, but then ceases to respond, has secondary resistance. In some examples, the subject responds for one, two, three, four, five, six, seven, eight, nine, ten, eleven or twelve months, but then does not respond to the BRAF inhibitor. Thus, the methods can include selecting a subject with a resistance to a BRAF inhibitor, such as selecting a subject with primary resistance to the BRAF inhibitor or secondary resistance to the BRAF inhibitor.

The subject can have a tumor. Thus, in some embodiments, the method includes selecting a subject that has secondary resistance to a BRAF inhibitor, such as a subject with secondary resistance to a BRAF inhibitor that has a tumor, such as melanoma. In other embodiments, the method includes selecting a subject with primary resistance to a BRAF inhibitor, such as a subject with primary resistance to a BRAF inhibitor that has a tumor, such as melanoma.

The disclosed methods can also be used to prevent metastasis or decrease the number of micrometastases, such as micrometastases to regional lymph nodes. Disclosed herein are methods to treat a subject diagnosed with a tumor, such as a melanoma. Melanoma includes spreading melanoma, nodular melanoma, acral lentiginous melanoma, and lentigo maligna (melanoma). However, the methods disclosed herein can also be used to treat other cancers, such breast cancer, prostate cancer, ovarian cancer, colon cancer, stomach cancer, pancreatic cancer, glioma, chordoma, chondrosarcoma, thyroid cancer, colon cancer, glioma or a squamous cell carcinoma. Squamous cells carcinomas include, but are not limited to head and neck squamous cell carcinoma, and squamous cell cancers of the skin, lung, prostate, esophagus, vagina and cervix. The methods disclosed herein can also be used to breast cancer, head and neck squamous cell carcinoma, renal cancer, lung cancer, glioma, bladder cancer, ovarian cancer, colon cancer or pancreatic cancer, wherein cells of the cancer express GRP94. The methods also can be used to treat non-Hodgkin lymphoma, colorectal cancer, papillary thyroid carcinoma, non-small cell lung carcinoma, and adenocarcinoma of lung. Thus, the methods can include selecting a subject with a tumor.

A variety of different types of melanoma may be treated in accordance with the present methods including, such as superficial spreading melanoma, nodular malignant melanoma, acral lentiginous melanoma, lentiginous malignant melanoma, and mucosal lentiginous melanoma. The primary melanoma may also be cutaneous or extracutaneous. Extracutaneous primary malignant melanomas include ocular melanoma and clear-cell sarcoma of the soft tissues. Additional indications include rare melanomas or precancerous lesions where relevance of RTK targets may be implicated. The present methods are also useful in the treatment of melanoma that has metastasized.

A therapeutically effective amount of the agents will depend upon the severity of the disease and the general state of the patient's health. A therapeutically effective amount of the antibodies or antigen binding fragments thereof (or nucleic acids encoding the antibody or antigen binding fragment) and BRAF inhibitor is that which provides either a reduction in tumor burden, decreased metastatic lesions and/or subjective relief of a symptom(s) or an objectively identifiable improvement as noted by the clinician or other qualified observer.

The antibody or antigen binding fragment can be administered in the same formulation or separately. They can be given at the same time or a different time (simultaneously or sequentially), but sufficiently concurrently to have the beneficial effect disclosed herein. Compositions are provided

herein that include a carrier and one or more of the antibodies that specifically bind GRP94 and/or antigen binding fragments thereof, in combination with a BRAF inhibitor. Compositions comprising immunoconjugates or immunotoxins of these antibodies are also provided. The compositions can be prepared in unit dosage forms for administration to a subject. The amount and timing of administration are at the discretion of the treating physician to achieve the desired purposes.

The antibody (or a nucleic acid encoding the antibody) and/or BRAF inhibitor can be formulated for systemic or local (such as intra-tumor) administration. In one example, the antibodies and/or BRAF inhibitor is formulated for parenteral administration, such as intravenous administration. In another example, the antibodies, antigen binding fragments, nucleic acid and/or one or more BRAF inhibitors are formulated for topical administration, subcutaneous or intradermal administration. A BRAF inhibitor and/or and antibody or antigen binding fragment described above (or a nucleic acid encoding the antibody or antigen binding fragment) can be delivered transdermally via, for example, a transdermal delivery device or a suitable vehicle or, such in an ointment base, which may be incorporated into a patch for controlled delivery. Such devices are advantageous, as they may allow a prolonged period of treatment relative to, for example, an oral or intravenous medicament. Examples of transdermal delivery devices may include, for example, a patch, dressing, bandage or plaster adapted to release a compound or substance through the skin of a patient.

The compositions for administration can include a solution of the antibodies that specifically bind GRP94 (or nucleic acids encoding these antibodies) and/or BRAF inhibitor(s) dissolved or suspended in a pharmaceutically acceptable carrier, such as an aqueous carrier. A variety of aqueous carriers can be used, for example, buffered saline and the like. These solutions are sterile and generally free of undesirable matter. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example, sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate and the like. The concentration of antibody in these formulations can vary widely, and will be selected primarily based on fluid volumes, viscosities, body weight and the like in accordance with the particular mode of administration selected and the subject's needs.

A typical pharmaceutical composition for intravenous administration includes about 0.1 to 10 mg of antibody per subject per day. Dosages from 0.1 up to about 100 mg per subject per day may be used, particularly if the agent is administered to a secluded site and not into the circulatory or lymph system, such as into a body cavity or into a lumen of an organ. Actual methods for preparing administrable compositions will be known or apparent to those skilled in the art and are described in more detail in such publications as *Remington's Pharmaceutical Science,* 19th ed., Mack Publishing Company, Easton, Pa. (1995).

Antibodies can be provided in lyophilized form and rehydrated with sterile water before administration, although they are also provided in sterile solutions of known concentration. The antibody solution is then added to an infusion bag containing 0.9% sodium chloride, USP, and typically administered at a dosage of from 0.5 to 15 mg/kg of body weight. Considerable experience is available in the art in the administration of antibody drugs, which have been marketed in the U.S. since the approval of RITUXAN® in 1997. Antibodies can be administered by slow infusion, rather than in an intravenous push or bolus. In one example, a higher loading dose is administered, with subsequent, maintenance doses being administered at a lower level. For example, an initial loading dose of 4 mg/kg may be infused over a period of some 90 minutes, followed by weekly maintenance doses for 4-8 weeks of 2 mg/kg infused over a 30 minute period if the previous dose was well tolerated.

Compositions for administering BRAF inhibitors are known in the art. Suitable BRAF inhibitors and pharmaceutical formations including these inhibitors are disclosed for example, in PCT Publication No. WO2009047505A2, PCT Publication No. WO03086467A1, U.S. Published Patent Application US20050176740A1, U.S. Pat. No. 6,187,799, U.S. Pat. No. 7,329,670, PCT Publication No. WO2009143024A2, PCT Publication No. WO2010104945A1, PCT Publication No. WO2010104973A1, PCT Publication No. WO2010111527A1, U.S. Published Patent Application No. 20090286783A1 and PCT Publication No. WO2009152087A1, all of which are incorporated by reference herein.

The antibody or antigen binding fragment thereof that specifically binds GRP94 (or a nucleic acid encoding the antibody or antigen binding fragment) and the BRAF inhibitors can be administered to slow or inhibit the growth of cells, such as cancer cells. In these applications, a therapeutically effective amount of an antibody is administered to a subject in an amount sufficient to inhibit growth, replication or metastasis of cancer cells, or to inhibit a sign or a symptom of the cancer. In some embodiments, the compositions are administered to a subject to inhibit or prevent the development of metastasis, or to decrease the size or number of metasases, such as micrometastases, for example micrometastases to the regional lymph nodes (Goto et al., *Clin. Cancer Res.* 14(11): 3401-3407, 2008).

These compositions disclosed herein can be administered in conjunction with another chemotherapeutic agent, either simultaneously or sequentially. Many chemotherapeutic agents are presently known in the art. In one embodiment, the chemotherapeutic agents is selected from the group consisting of mitotic inhibitors, alkylating agents, anti-metabolites, intercalating antibiotics, growth factor inhibitors, cell cycle inhibitors, enzymes, topoisomerase inhibitors, anti-survival agents, biological response modifiers, anti-hormones, e.g. anti-androgens, and anti-angiogenesis agents.

Methods of treating melanoma can further include administering one or more additional anti-cancer drugs for the treatment of melanoma with a compound as defined herein. For example, anti-cancer drugs for the treatment of melanoma, especially metastatic melanoma, may be selected from alkylating anti-cancer drugs such as dacarbazine, temozolomide, mechlorethamine, and nitrosoureas such as carmustine, lomustine, and fotemustine; taxanes, such as paclitaxel and docetaxel; vinca alkaloids, such as vinblastine; topoisomerase inhibitors such as irinotecan; thalidomide; anticancer antibiotics such as streptozocin and dactinomycin; or platinum anti-cancer drugs, such as cisplatin and carboplatin. Compounds of the invention may be added to polychemotherapeutic regimes such as the Dartmouth regime, CVD (cisplatin, vinblastine, and dacarbazine) and BOLD (bleomycin, vincristine, lomustine, and dacarbazine). In some embodiments, the anti-cancer drugs are selected from interferons such as, but not limited to, interferon alpha-2a, interferon alpha-2b, pegylated interferons such as pegylated interferon alpha-2b. Interleukins such as interleukin-2 may also be used in combination with compounds disclosed herein.

In the methods of treating melanoma described herein, the therapeutically effective amount of the compound can range from about 0.25 mg/kg to about 30 mg/kg body weight of the subject. In some embodiments, the therapeutically effective amount of the compound can range from about 0.5 mg/kg to about 30 mg/kg, from about 1 mg/kg to about 30 mg/kg, from about 1 mg/kg to about 25 mg/kg, from about 1 mg/kg to about 15 mg/kg, or from about 1 or 2 mg/kg to about 10 mg/kg. In other embodiments, the amount of the compound administered to the subject ranges from about 25 to about 1500 mg/day and, preferably, from about 100 or 200 mg/day to about 500 or 600 mg/day.

Treatment may also include administering the pharmaceutical formulations of the present methods in combination with other therapies. For example, a pharmaceutical formulations including the antibody and BRAF inhibitor may be administered before, during, or after a surgical procedure and/or radiation therapy.

Anti-angiogenesis agents, such as MMP-2 (matrix-metalloproteinase 2) inhibitors, MMP-9 (matrix-metalloproteinase 9) inhibitors, and COX-II (cyclooxygenase II) inhibitors, can be used in conjunction with the antibody and the BRAF inhibitor. Examples of useful COX-II inhibitors include CELEBREX™ (alecoxib), valdecoxib, and rofecoxib. Examples of useful matrix metalloproteinase inhibitors are described in PCT Publication No. WO 96/33172 (published Oct. 24, 1996), PCT Publication No. WO 96/27583 (published Mar. 7, 1996), European Patent Application No. 97304971.1 (filed Jul. 8, 1997), European Patent Application No. 99308617.2 (filed Oct. 29, 1999), PCT Publication No. WO 98/07697 (published Feb. 26, 1998), PCT Publication No WO 98/03516 (published Jan. 29, 1998), PCT Publication No WO 98/34918 (published Aug. 13, 1998), PCT Publication No WO 98/34915 (published Aug. 13, 1998), PCT Publication No WO 98/33768 (published Aug. 6, 1998), PCT Publication No WO 98/30566 (published Jul. 16, 1998), European Patent Publication 606,046 (published Jul. 13, 1994), European Patent Publication 931,788 (published Jul. 28, 1999), PCT Publication No WO 90/05719 (published May 31, 1990), PCT Publication No WO 99/52910 (published Oct. 21, 1999), PCT Publication No WO 99/52889 (published Oct. 21, 1999), PCT Publication No WO 99/29667 (published Jun. 17, 1999), PCT International Application No. PCT/IB98/01113 (filed Jul. 21, 1998), European Patent Application No. 99302232.1 (filed Mar. 25, 1999), U.S. Pat. No. 5,863,949 (issued Jan. 26, 1999), U.S. Pat. No. 5,861,510 (issued Jan. 19, 1999), and European Patent Publication 780, 386 (published Jun. 25, 1997). In one example, the MMP inhibitors do not induce arthralgia upon administration. In another example, the MMP inhibitor selectively inhibits MMP-2 and/or MMP-9 relative to the other matrix-metalloproteinases (such as MMP-1, MMP-3, MMP-4, MMP-5, MMP-6, MMP-7, MMP-8, MMP-10, MMP-11, MMP-12, and MMP-13). Some specific examples of MMP inhibitors of use are AG-3340, RO 32-3555, RS 13-0830, 3-[[4-(4-fluoro-phenoxy)-benzenesulfonyl]-(1-hydroxycarbamoyl-cyclopentyl)-amino]-propionic acid; 3-exo-3-[4-(4-fluoro-phenoxy)-benzenesulfonylamino]-8-oxa-bicyclo[3.2.1]octane-3-carboxylic acid hydroxyamide; (2R,3R) 1-[4-(2-chloro-4-fluoro-benzyloxy)-benzenesulfonyl]-3-hydroxy-3-methyl-piperidine-2-carboxylic acid hydroxyamide; 4-[4-(4-fluoro-phenoxy)-benzenesulfonylamino]-tetrahydro-pyran-4-carboxylic acid hydroxyamide; 3-[[4-(4-fluoro-phenoxy)-benzenesulfonyl]-(1-hydroxycarbamoyl-cyclobutyl)-amino]-propionic acid; 4-[4-(4-chloro-phenoxy)-benzenesulfonylamino]-tetrahydro-pyran-4-carboxylic acid hydroxyamide; (R)3-[4-(4-chloro-phenoxy)-benzenesulfonylamino]-tetrahydro-pyran-3-carboxylic acid hydroxyamide; (2R,3R) 1-[4-(4-fluoro-2-methyl-benzyloxy)-benzenesulfonyl]-3-hydroxy-3-methyl-piperidine-2-carboxylic acid hydroxyamide; 3-[[4-(4-fluoro-phenoxy)-benzenesulfonyl]-(1-hydroxycarbamoyl-1-methyl-ethyl)-amino]-propionic acid; 3-[[4-(4-fluoro-phenoxy)-benzenesulfonyl]-(4-hydroxycarbamoyl-tetrahydro-pyran-4-yl)-amino]-propionic acid; 3-exo-3-[4-(4-chloro-phenoxy)-benzenesulfonylamino]-8-oxaicyclo[3.2.1]octane-3-carboxylic acid hydroxyamide; 3-endo-3-[4-(4-fluoro-phenoxy)-benzenesulfonylamino]-8-oxa-icyclo[3.2.1] octane-3-carboxylic acid hydroxyamide; and (R)3-[4-(4-fluoro-phenoxy)-benzenesulfonylamino]-tetrahydro-furan-3-carboxylic acid hydroxyamide; and pharmaceutically acceptable salts and solvates of said compounds.

The antibody or antigen binding fragment that specifically bind GRP94 (or nucleic acid encoding the antibody or antigen binding fragment) and the BRAF inhibitor can also be used with signal transduction inhibitors, such as agents that can inhibit EGF-R (epidermal growth factor receptor) responses, such as EGF-R antibodies, EGF antibodies, and molecules that are EGF-R inhibitors; VEGF (vascular endothelial growth factor) inhibitors, such as VEGF receptors and molecules that can inhibit VEGF; and erbB2 receptor inhibitors, such as organic molecules or antibodies that bind to the erbB2 receptor, for example, HERCEPTIN™ (Genentech, Inc.). EGF-R inhibitors are described in, for example in PCT Publication Nos. WO 95/19970 (published Jul. 27, 1995), WO 98/14451 (published Apr. 9, 1998), WO 98/02434 (published Jan. 22, 1998), and U.S. Pat. No. 5,747,498 (issued May 5, 1998). EGFR-inhibiting agents also include, but are not limited to, the monoclonal antibodies C225 and anti-EGFR 22Mab (ImClone Systems Incorporated), ABX-EGF (Abgenix/Cell Genesys), EMD-7200 (Merck KgaA), EMD-5590 (Merck KgaA), MDX-447/H-477 (Medarex Inc. and Merck KgaA), and the compounds ZD-1834, ZD-1838 and ZD-1839 (AstraZeneca), PKI-166 (Novartis), PKI-166/CGP-75166 (Novartis), PTK 787 (Novartis), CP 701 (Cephalon), leflunomide (Pharmacia/Sugen), CI-1033 (Warner Lambert Parke Davis), CI-1033/PD 183,805 (Warner Lambert Parke Davis), CL-387,785 (Wyeth-Ayerst), BBR-1611 (Boehringer Mannheim GmbH/Roche), Naamidine A (Bristol Myers Squibb), RC-3940-II (Pharmacia), BIBX-1382 (Boehringer Ingelheim), OLX-103 (Merck & Co.), VRCTC-310 (Ventech Research), EGF fusion toxin (Seragen Inc.), DAB-389 (Seragen/Lilgand), ZM-252808 (Imperial Cancer Research Fund), RG-50864 (INSERM), LFM-A12 (Parker Hughes Cancer Center), WHI-P97 (Parker Hughes Cancer Center), GW-282974 (Glaxo), KT-8391 (Kyowa Hakko) and EGF-R Vaccine (York Medical/Centro de Immunologia Molecular (CIM)).

VEGF inhibitors, for example SU-5416 and SU-6668 (Sugen Inc.), SH-268 (Schering), and NX-1838 (NeXstar) can also be used in conjunction with an antibody that specifically binds endoplasmin. VEGF inhibitors are described in, for example in PCT Publication No. WO 99/24440 (published May 20, 1999), PCT International Application PCT/IB99/00797 (filed May 3, 1999), PCT Publication No. WO 95/21613 (published Aug. 17, 1995), PCT Publication No. WO 99/61422 (published Dec. 2, 1999), U.S. Pat. No. 5,834,504 (issued Nov. 10, 1998), PCT Publication No. WO 98/50356 (published Nov. 12, 1998), U.S. Pat. No. 5,883,113 (issued Mar. 16, 1999), U.S. Pat. No. 5,886,020 (issued Mar. 23, 1999), U.S. Pat. No. 5,792,783 (issued Aug. 11, 1998), PCT Publication No. WO 99/10349 (published Mar. 4, 1999), PCT Publication No. WO 97/32856 (published Sep. 12, 1997), PCT Publication No. WO 97/22596 (published Jun.

26, 1997), PCT Publication No. WO 98/54093 (published Dec. 3, 1998), PCT Publication No. WO 98/02438 (published Jan. 22, 1998), WO 99/16755 (published Apr. 8, 1999), and PCT Publication No. WO 98/02437 (published Jan. 22, 1998). Other examples of some specific VEGF inhibitors are IM862 (Cytran Inc.); anti-VEGF monoclonal antibody of Genentech, Inc.; and angiozyme, a synthetic ribozyme from Ribozyme and Chiron. These and other VEGF inhibitors can be used in conjunction with the antibodies, antigen binding fragments, and the BRAF inhibitor.

ErbB2 receptor inhibitors, such as GW-282974 (Glaxo Wellcome pic), and the monoclonal antibodies AR-209 (Aronex Pharmaceuticals Inc.) and 2B-1 (Chiron), can furthermore be combined with the antibodies, antigen binding fragments and BRAF inhibitor, for example those indicated in PCT Publication No. WO 98/02434 (published Jan. 22, 1998), PCT Publication No. WO 99/35146 (published Jul. 15, 1999), PCT Publication No. WO 99/35132 (published Jul. 15, 1999), PCT Publication No. WO 98/02437 (published Jan. 22, 1998), PCT Publication No. WO 97/13760 (published Apr. 17, 1997), PCT Publication No. WO 95/19970 (published Jul. 27, 1995), U.S. Pat. No. 5,587,458 (issued Dec. 24, 1996), and U.S. Pat. No. 5,877,305 (issued Mar. 2, 1999). ErbB2 receptor inhibitors of use are also described in U.S. Provisional Application No. 60/117,341, filed Jan. 27, 1999, and in U.S. Provisional Application No. 60/117,346, filed Jan. 27, 1999.

For the treatment of cancer, such as melanoma, the antibody or antigen binding fragment that specifically bind GRP94 (or nucleic acid encoding the antibody or antigen binding fragment) and a BRAF inhibitor can be used with surgical treatment, or with another therapeutic including dacarbazine (also termed DTIC), or interleukin-2 (IL-2) or interferon, such as interferon (IFN). For the treatment of a superficial melanoma, the antibody or antigen binding fragment that specifically bind GRP94 (or nucleic acid encoding the antibody or antigen binding fragment) and a BRAF inhibitor can be used in conjunction with Imiquimod. However, for the treatment of another cancer, such as head and neck squamous cell carcinoma, the antibody or antigen binding fragment that specifically bind GRP94 (or nucleic acid encoding the antibody or antigen binding fragment) and a BRAF inhibitor can be used in conjunction with surgery, radiation therapy, chemotherapy, other antibodies (such as cetuximab and bevacizumab) or small-molecule therapeutics (such as erlotinib). One of skill in the art can readily determine surgical procedures and additional chemotherapeutic agents of use.

Single or multiple administrations of the compositions are administered depending on the dosage and frequency as required and tolerated by the patient. In any event, the composition should provide a sufficient quantity of at least one of the antibodies (or antigen binding fragments thereof) and BRAF inhibitor to effectively treat the patient. The dosage can be administered once but may be applied periodically until either a therapeutic result is achieved or until side effects warrant discontinuation of therapy. The subject can be treated at regular intervals, such as daily, weekly, biweekly, or monthly, until a desired therapeutic result is achieved. Generally, the dose is sufficient to treat or ameliorate symptoms or signs of disease without producing unacceptable toxicity to the patient.

Controlled release parenteral formulations can be made as implants, oily injections, or as particulate systems. For a broad overview of protein delivery systems see, Banga, A. J., *Therapeutic Peptides and Proteins: Formulation, Processing, and Delivery Systems*, Technomic Publishing Company, Inc., Lancaster, Pa., (1995) incorporated herein by reference. Particulate systems include microspheres, microparticles, microcapsules, nanocapsules, nanospheres, and nanoparticles. Microcapsules contain the therapeutic protein, such as a cytotoxin or a drug, as a central core. In microspheres the therapeutic is dispersed throughout the particle. Particles, microspheres, and microcapsules smaller than about 1 μm are generally referred to as nanoparticles, nanospheres, and nanocapsules, respectively. Capillaries have a diameter of approximately 5 μm so that only nanoparticles are administered intravenously. Microparticles are typically around 100 μm in diameter and are administered subcutaneously or intramuscularly. See, for example, Kreuter, J., *Colloidal Drug Delivery Systems*, J. Kreuter, ed., Marcel Dekker, Inc., New York, N.Y., pp. 219-342 (1994); and Tice & Tabibi, *Treatise on Controlled Drug Delivery, A. Kydonieus, ed., Marcel Dekker, Inc. New York, N.Y., pp.* 315-339, (1992) both of which are incorporated herein by reference.

Polymers can be used for ion-controlled release of the antibody compositions disclosed herein. Various degradable and non-degradable polymeric matrices for use in controlled drug delivery are known in the art (Langer, *Accounts Chem. Res.* 26:537-542, 1993). For example, the block copolymer, polaxamer 407, exists as a viscous yet mobile liquid at low temperatures but forms a semisolid gel at body temperature. It has been shown to be an effective vehicle for formulation and sustained delivery of recombinant interleukin-2 and urease (Johnston et al., *Pharm. Res.* 9:425-434, 1992; and Pec et al., *J. Parent. Sci. Tech.* 44(2):58-65, 1990). Alternatively, hydroxyapatite has been used as a microcarrier for controlled release of proteins (Ijntema et al., *Int. J. Pharm.* 112:215-224, 1994). In yet another aspect, liposomes are used for controlled release as well as drug targeting of the lipid-capsulated drug (Betageri et al., *Liposome Drug Delivery Systems*, Technomic Publishing Co., Inc., Lancaster, Pa. (1993)). Numerous additional systems for controlled delivery of therapeutic molecules are known (see U.S. Pat. No. 5,055,303; U.S. Pat. No. 5,188,837; U.S. Pat. No. 4,235,871; U.S. Pat. No. 4,501,728; U.S. Pat. No. 4,837,028; U.S. Pat. No. 4,957,735; U.S. Pat. No. 5,019,369; U.S. Pat. No. 5,055,303; U.S. Pat. No. 5,514,670; U.S. Pat. No. 5,413,797; U.S. Pat. No. 5,268,164; U.S. Pat. No. 5,004,697; U.S. Pat. No. 4,902,505; U.S. Pat. No. 5,506,206; U.S. Pat. No. 5,271,961; U.S. Pat. No. 5,254,342 and U.S. Pat. No. 5,534,496).

The disclosure is illustrated by the following non-limiting Examples.

EXAMPLES

The treatment of tumors, such as metastatic melanoma is changing rapidly due to the great success in translational research from bench to bedside. PLX4032 (RG7204, Plexxikon,) is a selective V600E mutated BRAF inhibitor. A response rate of 70% in 32 BRAF mutated advanced melanoma patients treated by PLX4032 was reported in the phase II study (Flaherty et al, *N Engl J Med* 363:809-819, 2010). Although the significant clinical activity of BRAF selective inhibitors is a major breakthrough in the treatment of this disease, there are still hurdles to overcome to optimize this targeted therapy approach (Flaherty and McArthur, Cancer 116:4902-4913, 2010). Some patients did not respond (primary resistance). Most patients had partial responses. Moreover, the median duration of response is approximately only 8 months (secondary resistance). The next urgent clinical goal is to identify rational therapy to obtain complete response and to improve the longevity of initial response to BRAF-inhibitor (BRAF-I). It is disclosed herein that antibodies that spe-

cifically bind GRP94 have a synergistic effect with BRAF inhibitors to lower viability of tumor cells and decrease metastasis.

Example 1

Material and Methods

Generation of BRAF Inhibitor (BRAF-I) PLX4720 M21 Resistant (M21R) Melanoma Cell Line.

The cell line M21 (harboring the V600E BRAF missense mutation) (4×10^5/well) was cultured in a 6-well plate containing RPMI 1640 medium supplemented with 10% FBS and 2.5 μM of PLX4720. Medium was changed every 4 days. On the 8^{th} day the concentration of PLX4720 was increased to 5 μM. Cells were cultured under these conditions for 12 days changing the medium every 4 days. Then the concentration of PLX4720 in the medium was increased to 10 μM, and the medium has been changed every 3 days. Cells were cultured until when resistant colonies appeared (around 3 weeks). Cells were tested for resistance to BRAF-I PLX4720 by analyzing their growth in the presence of 10 μM of PLX4720, using the MTT assay.

Effect of BRAF-I PLX4720 on GRP94 Expression by M21 Cell Line:

M21 cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 1 μM of PLX4720. Cells were harvested after 14 days of culture, and washed twice with PBS-BSA. Cells (1×10^5/sample), and were stained with 1 μg of anti-GRP94 mAb W9. Human IgG (HIg) and untreated cells were used as controls. Following an incubation of 30 min at 4° C., cells were washed three times with PBS containing 2% of BSA (PBS-BSA). Cells were then incubated with an appropriate dilution of PE-conjugated anti-human IgG for 30 min at 4° C. Cells were washed and fixed with 2% PFA. Immunofluorescence was measured using a Cyan cytofluorimeter.

GRP94 Expression by M21R Melanoma Cell Line:

M21 acquired resistance to BRAF-I PLX4720 following repeated exposures to this inhibitor. BRAF-I PLX4720 resistant melanoma cells M21R and parental melanoma cells M21 were cell surface stained with the GRP94-specific mAb W9. HIg was used as negative control. The staining was performed as previously described. Immunofluorescence was measured using a Cyan cytofluorimeter.

Anti-Cell Growth Effect of GRP94-Specific mAb W9 Combined with BRAF-I:

Cells (1×10^4 cells/ml) were seeded in quadruplicate into 96-well plates and treated with W9 mAb combined with 5 μM of BRAF-I. HIg was used as a control. Cells growth was analyzed at different time points (1, 2, 3, 4, and 5 days) using the MTT assay.

Migration:

BRAF-I PLX4720 resistant melanoma cells M21R (2.5×10^4/well) were seeded in a 24-transwell plate (24-well insert, pore size 8 μm; BD Biosciences) in RPMI 1640 medium containing 1% FCS with W9 antibody (Ab), HIg, BRAF-I combined with W9 Ab, BRAF-I combined with HIg, or BRAF-I only. Cells migrated toward RPMI1640 medium containing 1% FCS and 10 μg/ml fibronectin. After 72 hours, migrated cells were stained with HEMA 3 stain set, taken picture and counted under a Zeiss Inverted Fluorescence Microscope (AxioVision Software). Mean of six independent high power field (100×) were shown as columns. The experiments were performed in triplicates.

Immunoblot:

Human melanoma cell line M21R was serum starved for 3 days then seeded at the concentration of 1.0×10^5 per well in a 6-well plate in RPMI 1640 medium without serum and incubated with the GRP94 mAb, HIg, untreated, BRAF-I PLX4720 (5 μM) combined with W9 mAb, BRAF-I combined with HIg, or BRAF-I only, at 37° C. for an additional 3 days. Cells were 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). Protein concentrations in the lysates were measured utilizing the Bradford reagent (Bio-Rad, Laboratories, Hercules, Calif.). Equal amount of proteins (60 μg per well) from the clarified lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane of 0.45 μm pore size (Millipore). After blocking the membranes with 5% nonfat dry milk plus 2% BSA at room temperature for 2 hours, membranes were incubated overnight at 4° C. with rabbit anti-RAS, C-RAF, phosphorylated (p)-MEK (Ser217/221), ERK, p-ERK1/2 (Thr202/Tyr204), PKCα and β-actin mAb (Cell Signaling Technology), mouse anti-FAK, p-FAK (Tyr397) mAb (BD Transduction Laboratories), mouse anti-calnexin mAb TO-5. Then peroxidase-conjugated secondary antibodies (anti-mouse IgG antibody, or anti-rabbit IgG antibody) were added and incubation was continued at room temperature for an additional 45 minutes. Between the incubations, membranes were washed five times, 5 minutes each, with PBS (pH 7.4) containing 0.1% Tween. Then bound antibodies were detected using ECL Plus Western Blotting Detection System (GE Healthcare), and bands were visualized using the FOTO/Analyst Investigator Eclipse System (Fotodyne Incorporate). The calnexin and β-actin were used as the protein loading controls. The densities of resultant bands were determined with ImageJ software (NIH), normalized to that of Calnexin and β-actin, and are shown below the respective bands. Data are expressed as the percentage of the expression in untreated control cells.

Statistical analysis: Statistical analysis was performed using the t-test. Statistical significance was indicated by $p<0.05$.

Example 2

Effect of BRAF-I PLX4720 on the Expression of GRP94 by M21 Cell Line

M21 cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 1 μM of PLX4720. Cells were harvested after 14 days of culture, and washed twice with PBS-BSA. Cells (1×10^5/sample), and were stained with 1 μg of anti-GRP94 mAb W9. Human IgG (HIg) and untreated cells were used as controls. Following an incubation of 30 min at 4° C., cells were washed three times with PBS containing 2% of BSA (PBS-BSA). Cells were then incubated with an appropriate dilution of PE-conjugated anti-human IgG for 30 min at 4° C. Cells were washed and fixed with 2% PFA. Immunofluorescence was measured using a Cyan cytofluorimeter and the results show treatment of BRAF-I sensitive melanoma cell line M21 with the BRAF-I PLX4720 results in increased expression of GRP94. No change in human IgG binding was detected (FIG. **1**). These data suggest that BRAF-I treatment enhances the anti-tumor effect of GRP94-specific W9 mAb.

Example 3

GRP94 Expression by M21R Melanoma Cell Line M21

BRAF-I PLX4720 resistant melanoma cells M21R and parental melanoma cells M21 were cell surface stained with the GRP94-specific mAb W9. HIg was used as negative control. The staining was performed as previously described. Immunofluorescence was measured using a CYAN™ cytofluorimeter. The results show that BRAF-I resistant melanoma cells display an increased expression of GRP94 when compared to the parental M21 cells (FIG. **2**). These results suggest that BRAF-I resistant melanoma cells are more susceptible to the anti-tumor effect of GRP94-specific mAb.

Example 4

Anti-Cell Growth Effect of GRP94-Specific mAb W9 Combined with BRAF-I

Cells (1×10^4 cells/ml) were seeded in quadruplicate into 96-well plates and treated with W9 mAb combined with 5 μM of BRAF-I PLX4720. HIg was used as a control. Cells growth was analyzed at different time points (1, 2, 3, 4, and 5 days) using the MTT assay. The results show that treatment of BRAF-I resistant M21R cell line with GRP94-specific mAb in combination with BRAF-I results in lower viability than cells treated with mAb W9 alone. No changes are detectable in the viability of BRAF wild type MV3 cells treated with W9 mAb alone or combined with BRAF-I (FIG. **3**). This data shows the synergic anti-proliferative effect of W9 mAb and BRAF-I on BRAF-I resistant cell line.

Example 5

Anti-Cell Growth Effect of GRP94-Specific mAb W9 Combined with BRAF-I

Cells (1×10^4 cells/ml) were seeded in quadruplicate into 96-well plates and treated with W9 mAb combined with 500 nM of BRAF-I PLX4032. HIg was used as a control. Cells growth was analyzed at different time points (1, 3 and 5 days) using the MTT assay. The results show that treatment of M21, M21R, Colo38 and Colo38R cell line with GRP94-specific mAb in combination with BRAF-I results in lower viability than cells treated with mAb W9 alone (FIG. **4**). This data shows the synergic anti-proliferative effect of W9 mAb and BRAF-I on BRAF-I sensitive and resistant cell line.

Example 6

Migration

BRAF-I PLX4720 resistant melanoma cells M21R (2.5×10^4/well) were seeded in a 24-transwell plate (24-well insert, pore size 8 μm; BD Biosciences) in RPMI 1640 medium containing 1% FCS with W9 antibody (Ab), HIg, BRAF-I combined with W9 Ab, BRAF-I combined with HIg, or BRAF-I only. Cells migrated toward RPMI1640 medium containing 1% FCS and 10 μg/ml fibronectin. After 72 hrs incubation, as show in FIG. **5**, GRP94-specific Ab W9 inhibited around 20% of the motility of M21R cells towards fibronectin in a Boyden chamber assay. When cells incubated with GRP94-specific Ab W9 combined with BRAF-I, the inhibition of migration was increased to 45%. This data shows the synergic anti-migration effect of W9 mAb and BRAF-I on BRAF-I resistant cell line.

Example 7

Immunoblot

In vitro incubation with the GRP94-specific W9 mAb showed a decrease in the level of phosphorylated (p)-MEK (Ser217/221), and p-ERK1/2 (Thr202/Tyr204) and p-FAK (Tyr397), in the M21R cell line (FIG. **6**). In addition, the total protein levels of RAS, c-RAF, and PKCα were also decreased. Treatment of BRAF-I resistant M21R cell line with W9 in combination with BRAF-I results in lower level of RAS, c-RAF, p-MEK (Ser217/221) and p-ERK1/2 (Thr202/Tyr204) than those in cells treated with W9 or BRAF-I alone.

Example 8

Combination Therapy with BRAF-I PLX4720 (or PLX4320) and Anti-GRP94 Antibody is More Effective than Either Agent Alone in Inducing Melanoma Cell Apoptosis

To measure cell apoptosis in vitro, cells are seeded (5.0×10^4 cells per well) in a 96-well plate and treated with 5 different regimens as described above for 2, 6 and 24 hours. Cells are then subjected to staining simultaneously with FITC-Annexin V (green fluorescence) (BD Biosciences) and the non-vital dye propidium iodide (red fluorescence) (BD Biosciences), which allows bivariate analysis to discriminate intact cells ($FITC^-PI^-$), early apoptotic ($FITC^+PI^-$) and late apoptotic or necrotic cells ($FITC^+PI^+$). All experiments are performed three independent times.

To investigate the mechanisms of action underlying the effects on cell growth, migration and apoptosis, cells treated as described above are lysed for examining the changes in the level of total and activated signaling protein molecules, such as FAK, RAS, BRAF, ERK1/2, PI3, AKT, PKCα by Western blot. The significance of the difference in cell growth, migration, apoptosis and the level of signaling molecules are analyzed using the Student T test.

The primary resistance of melanoma cells to BRAF-I can be reversed and the secondary resistance of melanoma cells to BRAF-I can be delayed/prevented by targeting multiple signaling pathways with BRAF-I and anti-GRP94 antibody. The $M233^{V600E}$ cell line is naturally, i.e., primarily resistant to BRAF-I (Sondergaard et al., *J Transl Med* 8:39, 2010). In addition to M233 cell line, in order to obtain at least one more BRAF-I primary resistant cell line, additional V600E mutated human melanoma cell lines are screened using a cell growth MTT assay. It is determined that the combination therapy can reverse their resistance to BRAF-I in these two primary resistant cell lines in cell growth assays. Furthermore, the cell lines $M21^{V600E}$ and $SK\text{-}MEL\text{-}5^{V600E}$ are used, both of which are BRAF-I sensitive to confirm that secondary resistance can be delayed or prevented in the presence of anti-GPR94 in cell growth assays.

Cells were starved for 12 hours and seeded at a density of 2×10^5/ml in a E-well plate and treated for 6 hours with BRAF-I PLX4032 (500 nM) and Grp94-specific mAb W9 (20 μg/ml) in RPMI 1640 medium plus 1.5% FCS. Cells were stained with Annexin V-FITC and PI, and evaluated for apoptosis by flow cytometry according to the manufacturer's protocol (BD PharMingen, San Diego, Calif., USA). The early apoptotic cells (annexin V-positive, PI-negative) were determined using a flow cytometer. The results show that

treatment of M21 and M21R cell lines with GRP94-specific mAb in combination with BRAF-I PLX4032 induced more cell apoptosis than cells treated with mAb W9 and BRAF-I PLX4032 alone (FIG. **7**). This data shows the synergic pro-apoptpsis effect of W9 mAb and BRAF-I on BRAF-I sensitive and resistant cell line.

Example 9

Combination Therapy with BRAF-I PLX4032 and Anti-GRP94 Antibody is More Effective than Either Agent Alone in Inhibition of Cancer Stem Cell Proliferation in Vitro

Cells growing in the exponential phase were seeded at a density of 2×10^5/ml. The cells were treated for 3 days with BRAF-I PLX4032 (500 nM) and Grp94-specific mAb W9 (20 μg/ml) in RPMI 1640 medium plus with 1.5% FCS. Cells were stained with ALDEFLUOR® according to the manufacturer's protocol (Stem Cell Technologies). Incubation of cells with ALDEFLUOR® in the presence the ALDH1-specific inhibitor diethylaminobenzaldehyde (DEAB) was used as a negative staining control for the assay. Then cells were stained with ABCB5-specific mAb RK1 (1 μg/ml) for 30 min at 4° C., and incubated with APC-conjugated secondary mAb (1:200) (Jackson Immunoresearch). The results show that treatment of M21 and M21R cell lines with GRP94-specific mAb in combination with BRAF-I PLX 4032 results in lower viability than cells treated with mAb W9 and PLX4032 alone (FIG. **8**). This data shows the synergic inhibition of growth of cancer stem cell effect of W9 mAb and BRAF-I on BRAF-I sensitive and resistant cell line.

Example 10

Inhibition by GRP94-Specific mAb W9 and BRAF-I PLX 4032 of Signaling Pathways in BRAFV600E Mutant and BRAF-I Resistant Melanoma Cells

Cells growing in the exponential phase were seeded at a density of 2×10^5/ml. The cells were treated for 3 days with BRAF-I PLX4032 (500 nM) and Grp94-specific mAb W9 (5 μg/ml) in RPMI 1640 medium plus with 2% FCS. Then the cells were collected and lysed in lysis buffer. The expression and activation of multiple signaling molecules were analyzed by immunoblot. The results show that the combination of GRP94-specific mAb W9 and BRAF-I PLX4032 inhibited the expression and activation of signaling molecules important for cell proliferation (RAS, MER, ERK1/2), for hedgehog signaling pathway (Gill and SHh) as compared to the protein level in cells treated by GRP94-specific mAb W9 and BRAF-I PLX4032 alone (FIG. **9**).

Example 11

V600E Mutated Human Melanoma Cell Lines and Resistance to PLX4720 and/or PLX4320

Several V600E mutated human melanoma cell lines are tested to identify the cell lines which maintain the same growth rate at days 1, 3 and 7 in the presence and absence of BRAF-I PLX4720 (or PLX4320) at 5 μM in MTT assays. The identified cell lines, which are primary resistant to BRAF-I are confirmed with a higher dose (10 μM) of PLX4720 (or PLX4320) in cell growth assays.

The cells are treated with treatment with BRAF-I PLX4720 and an antibody that specifically binds GRP94 (anti-GRP94) to determine that primary resistance of human melanoma cell lines to BRAF-I PLX4720 (or PLX4320) is affected by the combination therapy. In addition to cell line $M233^{V600E}$, the additional primary resistant cell lines are treated in several different regimens and then cell growth, migration and apoptosis is measured. Combination therapy affects the growth, migration and apoptosis of the cell lines.

It is also determined that combination treatment with BRAF-I PLX4720 (or PLX4320) and anti-GRP94 delays and/or prevents secondary resistance of human melanoma cell lines to BRAF-I PLX4720 (or PLX4320). It has been determined that V600E mutated melanoma cell lines, which are sensitive to BRAF-I in vitro treatment, became resistant, i.e., secondary resistant, to PLX4720 (or PLX4320) after 3-4 week of in vitro treatment with increasing doses (2.5-10 μM) of PLX4720 (or PLX4320).

The cells (4×10^5/well) are cultured in a 6-well plate containing 2 ml of RPMI 1640 medium supplemented with 10% FBS and 2.5 μM of PLX-4720 (or PLX4320). Medium is changed every 4 days. On day 8, the dose of PLX4720 (or PLX4320) is increased to 5 μM. Cells are cultured under these conditions for 12 days changing the medium every 4 days. Then the dose of PLX4720 (or PLX4320) is increased to 10 μM, changing the medium every 3 days. Cells are cultured until resistant colonies appear (around 3-4 weeks). Cells are tested for resistance to BRAF-I by testing their growth in the presence of 10 μM of PLX4720 (or PLX4320), using the MTT assay. Simultaneously, cells in one well will be set up in the exact same way except adding anti-GRP94 every 3-4 days at its optimal dose identified as described above. In the control well, everything is not changed but only anti-GRP94 is replaced with the isotype control mAb. The cells, in the presence of both PLX4720 (or PLX4320) and anti-GRP94, are kept in culture as long as needed for monitoring whether and/or when secondary resistance occurs.

To analyze the mechanisms of action of combination treatment in primary and secondary resistances, cells are lysed for examining the changes in the level of total and activated signaling protein molecules by western blot as above. The significance of the difference in cell growth and the level of signaling molecules is analyzed using the Student T test.

It will be apparent that the precise details of the methods or compositions described may be varied or modified without departing from the spirit of the described invention. We claim all such modifications and variations that fall within the scope and spirit of the claims below.

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Lys Asp Glu Leu
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<210> SEQ ID NO 7
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<212> TYPE: PRT
<213> ORGANISM: Pseudomonas aeruginosa

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Arg Glu Asp Leu
1
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The invention claimed is:

1. A method of treating a subject diagnosed with a tumor that expresses a BRAF mutation, comprising

administering to the subject (1) a therapeutically effective amount of a monoclonal antibody or antigen binding fragment thereof that specifically binds glucose regulated protein (GRP) 94; and (2) a therapeutically effective amount of a BRAF inhibitor,

thereby treating the tumor in the subject, wherein the tumor is melanoma.

2. The method of claim **1**, wherein the subject has primary or secondary resistance to the BRAF inhibitor.

3. The method of claim 1, wherein the BRAF inhibitor is PLX4032 or PLX4720.

4. The method of claim 1, comprising administering to the subject a therapeutically effective amount of the antigen binding fragment of the monoclonal antibody that specifically binds GRP94.

5. The method of claim 1, wherein the monoclonal antibody or antigen binding fragment comprises a heavy chain variable domain, and wherein the heavy chain variable domain of the monoclonal antibody comprises the amino acid sequence set forth as amino acids 26-33 of SEQ ID NO: 3, amino acids 51-58 of SEQ ID NO: 3, and amino acids 97-103 of SEQ ID NO: 3.

6. The method of claim 1, wherein the monoclonal antibody or antigen binding fragment comprises a light chain variable domain, wherein the light chain variable domain of the antibody comprises the amino acid sequence set forth as amino acids 27-32 of SEQ ID NO: 4, amino acids 50-52 of SEQ ID NO: 4, and amino acids 89-97 of SEQ ID NO: 4.

7. The method of claim 1, wherein treating the tumor comprises decreasing the metastasis of the tumor.

8. The method of claim 1, wherein the subject is human.

9. The method of claim 1, wherein the melanoma is superficial spreading melanoma, nodular melanoma, acral lentiginous melanoma, lentiginous malignant melanoma, or mucosal lentiginous melanoma.

10. The method of claim, wherein the melanoma is cutaneous or extracutaneous melanoma.

11. The method of claim 1, wherein the melanoma is intraocular or a clear-cell sarcoma of the soft tissues.

12. The method of claim 1, further comprising administering one or more additional chemotherapeutic agents.

13. The method of claim 1, wherein the therapeutically effective amount of the BRAF inhibitor and the therapeutically effective amount of the antibody or antigen binding fragment thereof that specifically binds glucose regulated protein (GRP) 94 are administered simultaneously.

14. The method of claim 1, wherein cells in the tumor comprise a BRAF mutation.

15. The method of claim 14, wherein the BRAF mutation is a V600E mutation.

16. The method of claim 14, wherein the BRAF mutation is R462I, I463S, G464E, G464V, G466A, G466E, G466V, G469A, G469E, N581S, E585K, D594V, F595L, G596R, L597V, T599I, V600D, V600E, V600K, V600R, K601E or A728V.

17. The method of claim 14, further comprising detecting a V600E BRAF mutation in a sample from the subject, wherein the sample comprises cells from the tumor.

18. The method of claim 1, wherein:

the BRAF inhibitor is PLX4032 or PLX4720; and

the monoclonal antibody, or antigen binding fragment thereof comprises a heavy chain variable domain and a light chain variable domain, wherein the heavy chain variable domain of the monoclonal antibody comprises the amino acid sequence set forth as amino acids 26-33 of SEQ ID NO: 3, amino acids 51-58 of SEQ ID NO: 3, and amino acids 97-103 of SEQ ID NO: 3 and the light chain variable domain of the antibody comprises the amino acid sequence set forth as amino acids 27-32 of SEQ ID NO: 4, amino acids 50-52 of SEQ ID NO: 4, and amino acids 89-97 of SEQ ID NO: 4.

19. A pharmaceutical composition comprising (1) a therapeutically effective amount of a monoclonal antibody or antigen binding fragment thereof that specifically binds glucose regulated protein (GRP) 94; and (2) a therapeutically effective amount of a BRAF inhibitor.

20. The pharmaceutical composition of claim 19, wherein the BRAF inhibitor is PLX4032 or PLX4720.

21. The pharmaceutical composition of claim 20, wherein the BRAF inhibitor is PLX4032; and

the monoclonal antibody, or antigen binding fragment thereof comprises a heavy chain variable domain and a light chain variable domain, wherein the heavy chain variable domain of the monoclonal antibody comprises the amino acid sequence set forth as amino acids 26-33 of SEQ ID NO: 3, amino acids 51-58 of SEQ ID NO: 3, and amino acids 97-103 of SEQ ID NO: 3 and the light chain variable domain of the antibody comprises the amino acid sequence set forth as amino acids 27-32 of SEQ ID NO: 4, amino acids 50-52 of SEQ ID NO: 4, and amino acids 89-97 of SEQ ID NO: 4.

22. The pharmaceutical composition of claim 19, formulated for systemic administration.

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