

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
14 May 2009 (14.05.2009)

PCT

(10) International Publication Number  
**WO 2009/062128 A2**

(51) International Patent Classification:  
**A61K 48/00** (2006.01)

(21) International Application Number:  
PCT/US2008/082924

(22) International Filing Date:  
8 November 2008 (08.11.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
60/986,525 8 November 2007 (08.11.2007) US

(71) Applicant (for all designated States except US): **INDIANA UNIVERSITY RESEARCH AND TECHNOLOGY CORPORATION** [US/US]; 351 West 10th Street, Indianapolis, IN 46202 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **POLLOK, Karen, E.** [US/US]; Indianapolis, IN (US). **MAYO, Lindsey, D.** [US/US]; Zionsville, IN (US).

(74) Agent: **BARTOL, Michael, C.**; Bose McKinney & Evans LLP, 111 Monument Circle, Suite 2700, Indianapolis, IN 46204 (US).

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

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

Published:

— without international search report and to be republished upon receipt of that report

(54) Title: COMBINATION DRUG THERAPY FOR THE TREATMENT OF CANCER

(57) Abstract: A combination of drugs was used to kill or inhibit the growth of cancer cells. The combination drug therapy includes administering to a human or animal patient diagnosed with cancer at least one drug that inhibits the activity of an intracellular protein that repairs alkylated DNA and a drug that inhibits the interaction between Hdm2 and p53. The combination drug therapy is useful in the treatment of tumors including some tumors that have become drug resistant. In some instances the combination drug therapy allows the drugs to be administered to a patient at therapeutically effective submyeloablative levels. Still another aspect provides a combination drug therapy that includes administering to a cancer patient a therapeutically effective dose of at least one compound that alkylates DNA, at least one compound that inhibits at least one DNA repair protein and at least one compound that prolongs the half-life of p53 by inhibiting the interaction of p53 with HDM2.



WO 2009/062128 A2

**TITLE****COMBINATION DRUG THERAPY FOR THE TREATMENT OF CANCER****PRIORITY CLAIM**

**[0001]** This application claims the priority of U.S. provisional patent application No. 60/986,525, filed on November 8, 2007, which is incorporated herein by reference in its entirety.

**TECHNICAL FIELD**

**[0002]** Various aspects and embodiments relate generally to therapeutic methods for treating cancers, including combination drug therapies intended to treat patients with glioblastoma and drug resistant glioblastoma.

**BACKGROUND**

**[0003]** Glioblastoma multiforme (GBM), also known as grade 4 astrocytoma, is the most common and aggressive type of primary brain tumor, accounting for about 52% of all primary brain tumor cases and about 20% of all intracranial tumors. Current treatments for these and some other diseases involve chemotherapy, radiotherapy and surgery, all of which are acknowledged as being merely palliative. The five year survival rate for patients with GBM has remained unchanged over the past 30 years and stands at less than 3%.

**[0004]** Even with complete surgical resection of the tumor, combined with the best available treatment, the survival rate for patients with GBM remains very low. Accordingly, there is a need for new formulations and therapeutic methods to treat patients with this and similar diseases. Various aspects and embodiments disclosed herein address this need.

## SUMMARY

**[0005]** One aspect is a method of treating cancer in a patient, comprising the steps: identifying a patient in need thereof of a treatment for a cancer; and administering to the patient a therapeutically effective amount a combination drug therapy that includes: a compound that inhibits DNA repair or a prodrug or pharmaceutically acceptable salt thereof; a compound that either methylates or alkylates DNA or a prodrug or pharmaceutically acceptable salt thereof; and a compound that up-regulates p53 mediated apoptosis or a prodrug or a pharmaceutically acceptable salt thereof.

**[0006]** In one embodiment the compound that inhibits DNA repair acts by inhibiting the activity of the enzyme O<sup>6</sup>-methylguanine-DNA-methyltransferase (MGMT). In one embodiment the compound that inhibits DNA repair is O6-Benzylguanine (6BG) or a pharmaceutically acceptable salt thereof. In one embodiment the compound that methylates DNA is selected from the group consisting of: dacarbazine (5-(3,3-Dimethyl-1-triazenyl)imidazole-4-carboxamide), procarbazine (*N*-(1-methylethyl)-4-[(*N'*-methylhydrazino)methyl]benzamide), other triazenes and the like, or pharmaceutically acceptable salts thereof. In still another embodiment the compound that methylates DNA is temozolomide (TMZ), other imidazotrazines and the like or pharmaceutically acceptable salts thereof. In some embodiment the compounds that either methylate or alkylate DNA are delivered at a dose that is submyeloablative. In still another embodiment the alkylating or methylating compounds of the method are delivered at a dose that is myeloablative; in some methods further include a bone marrow transplant.

**[0007]** In another embodiment the method of treating a cancer patient includes administering a compound that alkylates DNA is selected from the group consisting of: carmustine, lomustin (1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-urea), fotemustine diethyl (1-{{(2-chloroethyl) (nitroso) carbamoyl}amino}ethyl) phosphonate, other nitrosourea alkylating agents and the like or pharmaceutically acceptable salts thereof. In one embodiment the method for treating a patient with cancer includes administering a compound that up-regulates p53-mediated apoptosis by changing p53 activity or the expression of p53, in one method this compound is nutlin3 or a pharmaceutically acceptable salt thereof. In one embodiment the compound that up-regulates p53 apoptosis likely interferes with the interaction between HDM2 and p53.

**[0008]** One aspect is a drug combination for treating cancer, comprising: a therapeutically effective amount of a compound that inhibits DNA repair or a prodrug or pharmaceutically acceptable salt thereof; a therapeutically effective amount of a compound that either methylates or alkylates DNA or a prodrug or pharmaceutically acceptable salt thereof; and a therapeutically effective amount of a compound that up-regulates p53-mediated apoptosis or a prodrug or pharmaceutically acceptable salt thereof. In one embodiment wherein the compound that inhibits DNA repair acts by inhibiting the activity of the DNA repair protein MGMT. In one embodiment the compound that inhibits DNA repair is 6BG or a pharmaceutically acceptable salt thereof.

**[0009]** In one embodiment the drug combination for treating a cancer includes at least one compound that methylates or alkylates DNA is selected from the group consisting of: lomustine, foremustine, dacarbazine and procarbazine, carmustine, TMZ or pharmaceutically acceptable salts thereof.

**[0010]** In one embodiment the drug combination for treating a cancer includes at least one compound, that changes p53 activity or expression and up-regulates p53-mediated apoptosis the compound may be, for example nutlin3.

**[0011]** Still another embodiment is a method for treating glioma or other types of malignancies comprising the steps of identifying a human or animal patient with cancer, providing a combination drug regimen and providing a therapeutically effective dose of the drugs to the patient. In one embodiment the method comprises the step of administering an inhibitor of at least one DNA repair protein and a small molecule that inhibits the interaction between HDM2 and p53. (In one embodiment the DNA repair protein targeted is O<sup>6</sup> methylguanine-DNA-methyltransferase MGMT. Inhibitors of the DNA repair proteins that may be used in this treatment regimen include guanine analogues with antineoplastic activity, including, for example, O6-Benzylguanine or similar compounds. One of the small molecule inhibitors of the HDM2/p53 interaction that may be used in this treatment is, for example, nutlin3. In some embodiments, this type of combination drug therapy may eliminate the need for a subsequent bone marrow transplant, thereby making this combination drug therapy an attractive cancer treatment.

**[0012]** Another embodiment is a method for treating glioma or other types of malignancies using a combination drug therapy. In one embodiment the method comprises the

steps of administering the following compounds to a patient in need thereof, an inhibitor of a DNA repair protein such as O-6-Benzylguanine, a DNA alkylating agent pro-drug such as the imidazotetrazine derivative temozolomide, and a molecule such as nutlin3, which inhibits the interaction between HDM2 and p53.

**[0013]** In one embodiment the DNA alkylating agent or its pro-drug is administered as part of a myeloablative regimen of chemotherapeutic agents. Treatments according to this embodiment may include a bone marrow transplant performed after completing at least one round of chemotherapy.

**[0014]** Still another embodiment includes administering to a patient in need thereof a combination of at least one compound that inhibits at least one DNA repair protein, a DNA alkylating agent or pro-drug, and a small molecule that inhibits the interaction between Hdm-2 and p53. In one embodiment the combination chemotherapeutic drug treatment regimen successfully inhibits the replication of cancer cells or destroys cancer cells and has so few side effects that it is not necessary to perform bone marrow transplant after chemotherapy.

#### BRIEF DESCRIPTION OF THE FIGURES

**Table 1.** Overview of some tests carried out in order to measure the efficacy of various combination drug therapies that can be used to treat some types of cancer.

**Table 2.** Summary of the results obtained by measuring the amount of nutlin3 in peripheral blood (PB) and brain tissue.

**Figure 1.** Graph of data illustrating that the enhancement of TMZ-mediated tumor cell killing by inhibition of MGMT by 6BG.

**Figure 2.** Graph of data illustrating the results of tests performed to optimize *in vitro* SF767 tumor-cell killing by using p53 suppressors such as nutlin3 and other compounds such as 6BG and TMZ in combination with one another.

**Figure 3.** Graph of data illustrating that administering the compound nutlin3 decreases cellular metabolism in the presence of low dose TMZ.

**Figure 4.** Graph of data illustrating that nutlin3 enhances TMZ-mediated apoptosis.

**Figure 5.** Graph of data illustrating that nutlin3 significantly decreases cellular metabolism in the presence of the MGMT inhibitor, 6BG, and TMZ or BCNU in glioblastoma cells expressing MGMT.

**Figure 6.** Graph of data illustrating a significant decrease in clonogenic SF767 cells following 6BG/TMZ/nutlin3 treatment.

**Figure 7.** Graph of data illustrating the effect of intermittent nutlin3 administration on ectopic U87 tumor growth.

### DETAILED DESCRIPTION

[0015] For the purposes of promoting an understanding of the principles of the novel technology, reference will now be made to the preferred embodiments thereof, and specific language will be used to describe the same. It will nevertheless be understood that no limitation of the scope of the novel technology is thereby intended, such alterations, modifications, and further applications of the principles of the novel technology being contemplated as would normally occur to one skilled in the art to which the novel technology relates.

[0016] Temozolomide is an imidazotetrazine second generation alkylating agent. It has received accelerated approval from the FDA as a treatment for adult patients with anaplastic astrocytoma who have relapsed after treatment which included a nitrosourea drug carmustine (BCNU) or lomustine (CCNU) and procarbazine. Temozolomide is considered to be the leading compound in a new class of chemotherapeutic agents that enters the cerebrospinal fluid and does not require hepatic metabolism for activation. The pro-drug rapidly undergoes nonenzymatic conversion under physiological condition into the therapeutically reactive compound MTIC (5-(3-methyltriazene-1-yl) imidazole-4-Carboximide).

[0017] Temozolomide (4-methyl-5-oxo-2,3,4,6,8-pentazabicyclo [4.3.0] nona-2,7,9-triene-9-carboxamide) is a DNA alkylating agent. This compound is marketed under the names Temodar® and Temodal® which are registered trademarks of the Schering-Plough Corp. Its cytotoxicity and antiproliferative activity against tumor cells is thought to be due primarily to the alkylation (methylation) of specific guanine rich areas of DNA, alkylating the areas of cellular DNA blocks the initiation of transcription of at least some genes necessary for tumor cell replication and/or survival. Temozolomide has displayed pre-clinical activity against a broad spectrum of murine tumors *in vivo*. Temozolomide, (sometimes abbreviated as TMZ) is currently indicated for the treatment of malignant glioma. It is available as a capsule for oral administration in a number of countries including the US and in Europe. Still other clinical trials of temozolomide suggest that it may be effective in the treatment of patients with metastatic malignant melanoma. However, there are two major obstacles preventing the more widespread

use of this compound and others like it: 1) the drug exhibits a number of serious side effects, including leucopenia, nausea, vomiting with alpecia etc., as well as dose-limiting myelotoxicity following extended exposure to the compound; and 2) many tumors that express DNA repair proteins develop resistance to temozolomide. TMZ resistance renders the drug ineffective over time despite its bioavailability under physiological conditions. Accordingly, there is a need to reduce the amount of temozolomide required to effectively treat cancer patients in order to eliminate or at least reduce its hematological toxicity, and there is a need to reduce resistance to temozolomide in order to treat tumors that have developed resistance to the drug, such as in tumor cells that exhibit high levels of DNA repair activity.

**[0018]** One aspect of the disclosure provides solutions to at least some of the problems associated with using the drug temozolomide to treat glioma or other difficult to treat tumors. Tumors that can be treated using this approach include tumors that exhibit drug resistance and/or tumors that can only be effectively treated by administering myeloablative levels of TMZ or similar acting compounds.

**[0019]** The inability of chemotherapy to effectively kill some tumor cells is largely due to the ability of some cells to acquire drug resistance. Drug resistance may be due to a mutation in at least one pathway required for cell death or proliferation, including pathways such as those involved in apoptosis, angiogenesis, and DNA repair pathways. For example, expression of high levels of the DNA repair protein, O<sup>6</sup>-methylguanine-DNA-methyltransferase (MGMT), is found in many types of tumors including those of the central nervous system. Recent evidence indicates high levels of MGMT expression in the vast majority of pediatric brain tumors analyzed. Disclosed herein is an effective therapeutic method of killing drug resilient brain tumors, this may include the steps of providing at least one compound that inhibits DNA repair and at least one compound that promotes apoptosis in tumor cells.

**[0020]** The methylation of DNA is thought to be one of the mechanisms responsible for temozolomide's cytotoxicity towards malignant cells. Among the lesions produced in DNA by treatment with temozolomide, the most common are probably methylations at the N7 position of guanine, at the O3 position of adenine, and at the O6 position of guanine. The O6 MG adduct, is thought to account for about 5% of the total adducts formed by temozolomide, probably plays a critical role in the antitumor activity of this agent. Evidence for this hypothesis comes from the

correlation between the sensitivity of tumor cell lines to temozolomide and the activity of the DNA repair protein O6 alkylguanine alkyltransferase (AGT) in tumor cells. The protein AGT specifically removes alkyl groups at the O6 position of guanine. Accordingly, one method for treating temozolomide resistance tumor cells is to co-treat the cells with an effective dose of an AGT inhibitor.

**[0021]** One way to reduce AGT activity towards to the cell's alkylated DNA is to administer an effective amount of a non-physiological substrate for AGT, and/or an inhibitor of AGT. The compound O<sup>6</sup>-Benzylguanine (6BG) is a low molecular weight substrate for AGT and a potent inhibitor of AGT. It can be used to mediate the resistance to reagents that act by damaging DNA, reagents such as chloroethylnitrosourea and methylating agents. Although administering 6BG can sensitize cells to chemotherapeutic agents that alkylate DNA, the compound has its own deleterious side effects. One particularly troublesome side effect of 6BG when given in combination with chloroethylnitrosourea and methylating agents is its hematological toxicity. Hematopoietic bone marrow cells have lower levels of AGT activity than many other types of cells. Accordingly treating a patient with a reagent that impairs the cell's ability to repair damaged DNA has a disproportionate impact on hematopoietic bone marrow cells. Administering high levels of AGT inhibitors such as 6BG to a patient may damage, kill or inhibit the growth of hematopoietic bone marrow cells. If enough of the hematopoietic cells are adversely affected, the patient undergoing this type of treatment may require a bone marrow transplant following treatment with compounds such as 6BG.

**[0022]** Currently, the therapeutic goal of killing tumor cells by administering DNA alkylating agents is complicated by the dilemma of either 1) selecting for drug-resistant tumor cells by administering only alkylating agents, or 2) inducing hematological cell death by co-administering a high dose of an AGT inhibitor such as 6BG. One current solution is to administer compounds such as 6BG at a dose high enough to treat the cancer (and inadvertently but almost inevitably damaging the patient's bone marrow) followed by a bone marrow transplant to restore the patient's damaged immune system. Bone marrow transplants after chemotherapy are risky, expensive, painful, and provide no guarantee that additional transplants will not have to be performed in the event that the patient must undergo additional rounds of hematopoietic toxic chemotherapy. Because of the risk to the patient and expenses involved in serial bone marrow transplants patients rarely undergo more than one bone marrow transplant.

**[0023]** Hdm-2 is an oncogene and levels of the protein encoded by this gene are elevated in numerous types of malignant cells. Elevated levels of the protein HDM2 are thought to impede the function of the p53 tumor suppressor gene by binding to p53 and marking p53 for proteosomal degradation. Antagonists to interaction between HDM2 and p53 have been developed, and these antagonists include nutlin3. Nutlin3 has demonstrated promising activity towards disrupting the p53-HDM2 association. Studies indicate that nutlin3 binds to HDM2's p53-binding pocket thereby disrupting the interaction between HDM2 and p53 and preventing p53 from being marked for proteosomal degradation. Accordingly, the presence of a compound such as nutlin3 in a cell may raise the level of p53 in the cell. Functional levels of p53 activity in stressed cells may result in cell cycle arrest, apoptosis, or the inhibition of cellular growth.

**[0024]** As disclosed herein, administering the combination of an ATG inhibitor, a DNA alkylating agent, and a molecule that inhibits the interaction between HDM2 and p53 resulted in a surprisingly high level of tumor cell growth inhibition. Quite unexpectedly, the combination of the ATG inhibitor 6BG and the HDM2-p53 interaction inhibitor nutlin3 significantly inhibited tumor growth even in the absence of a DNA alkylating agent. This later combination of drugs and combinations of compounds that operate to inhibit the same cellular processes may obviate the need for a DNA alkylating agent altogether in the treatment of some cancers. Under some conditions these same drugs and combination of compounds reduce the levels of DNA alkylating agent that must be used to achieve a therapeutic effect to a level that reduces some of the side effects of DNA alkylating agents. When the need for a genotoxic drug regimen such as TMZ is either eliminated altogether or the effective dosage of the drug is reduced to submyeloablative levels the patient may be spared the dangerous and expensive need to undergo a bone marrow transplant after chemotherapy. Either scenario can be of tremendous benefit to patients undergoing chemotherapy.

**[0025]** A number of explanations and experiments are provided by way of explanation and not by limitation. No theory of how the novel technology operates is to be considered limiting, whether proffered by virtue of description, comparison, explanation or example. Accordingly, the following examples and discussion are presented by way of guidance and explanation and not limitation.

## EXAMPLES

**[0026]** The objective of some of these examples is to compare the sensitivity of glioblastoma cell lines to submyeloablative and myeloablative combinations of various compounds. The U87 cell line disclosed and described herein is a good candidate for assessing the effectiveness of submyeloablative therapies because these cells do not express detectable levels of the DNA repair protein, O<sup>6</sup>-methylguanine-DNA-methyltransferase (MGMT). In some instances cells of this line are treated with at least one of the following compounds: TMZ and/or nutlin3.

**[0027]** The SF767 cell line is an excellent platform for testing the efficacy of myeloablative therapies since these cells express high levels of MGMT. SF767 cells are treated with at least one of the following compounds: 6BG, TMZ, or nutlin3. The MGMT inhibitor, 6BG, is used to sensitize the cells to TMZ. See, e.g., Table 1 for a general outline of how these studies are and may be conducted.

### EXAMPLE 1

**[0028]** Effect of myeloablative 6BG/Temozolomide/6BG on growth of the human SF767. In this example, NOD/SCID mice were injected subcutaneously with cells from the human glioblastoma, SF767 cell line, which expressed high levels of MGMT. Tumors were allowed to grow for 12 days. One cycle of treatment consists of 3 consecutive days of dosing with 30 mg/kg 6BG followed one hour later with an 80 mg/kg dose of TMZ, and 6-7 hours later with a 15 mg/kg dose of 6BG. One day later drug-treated mice were transplanted with NOD/SCID bone-marrow cells.

**[0029]** These mice were given two treatment cycles, spaced 2 weeks apart. Referring now to Figure 1, (Figure 1A), the tumor volumes over this time period were measured and were compared to the base line tumor volume determined at the onset of the experiment. The final weight of the tumor was determined (see Figure 1B). Tumor growth decreased significantly over time following a high-dose 6BG/TMZ/6BG compared to tumors treated with only the vehicle used to deliver the active compounds (control)  $p < 0.05$ . Following 2 cycles of therapy, SF767 tumor growth began to increase over time by day 35-42 post-treatment.

## EXAMPLE 2

**[0030]** In this experiment levels of various compounds were tested to determine the effective concentrations *in vitro* that kill SF767 tumor cells. Referring now to Figure 2, drug sensitivity assays were performed on tumor cells using the CellTiter-Glo Luminescent Cell Viability assay (Promega, Madison, WI). Cells exposed to nutlin3 in combination with TMZ and/or 6BG. Cells were treated in triplicate with increasing doses of TMZ in the absence or presence of 6BG and/or nutlin3. At day 7 post-treatment, cell viability was determined using the Celltiter Glo Luminescent Viability assay according to the manufacturer's instructions statistical analysis of the data rendered the following values:  $p < 0.05$ , Control, Nutlin, 6BG, T(200-400 uM), or T(200-400 uM)/N versus B/T(200-400 uM);  $p < 0.05$ , Control, Nutlin, 6BG, T(200-400 uM), B/T(200-400 uM) or T (200-400uM)/N versus B/N/T(200-400 uM);  $p < 0.05$ , Control, Nutlin, 6BG, T (200-400 uM) or T (200-400 uM)/N versus B/N.

**[0031]** These result illustrated a significant decrease in cell viability by day 7 post-treatment, indicating that blocking Hdm2 activity increased the sensitivity of tumor cells to 6BG/TMZ. Surprisingly, the combination of the MGMT inhibitor, 6BG, and nutlin3, alone resulted in a 50% decrease in tumor cell viability, even in the absence of TMZ. These results indicate that combination drug therapies targeting components of the DNA damage/repair networks can be used to treat tumors even without the addition of genotoxic agents such as TMZ.

## EXAMPLE 3

**[0032]** The outlines of one form of the combination therapy disclosed herein are listed in Table 1. This study compares the sensitivity of glioblastoma cell lines to submyeloablative and myeloablative doses of various combination drug therapies disclosed herein. The U87 line is a good candidate for testing submyeloablative chemotherapy regimens since these cells do not express detectable levels of the DNA repair protein, MGMT. In this experiment, TMZ also utilized and nutlin3. The SF767 cell line is a good candidate for testing myeloablative drug regimen combinations since these cells express high levels of MGMT. The experiment utilizes

SF767 and 6BG, TMZ, and nutlin3. The MGMT inhibitor, 6BG, is used to sensitize the cells to TMZ (Table 1).

**[0033]** Methodology. The effect of dosing effect levels of each compound (Nutlin-3, 6BG, and TMZ) is determined by measuring using a CellTiter-Glo Luminescent Cell Viability assay kit (Promega) and colony forming assays using the glioblastoma cell lines-U87 and SF767. To support these tests large quantities of nutlin3 about (1.5 grams) are synthesized. This study uses IC<sub>50</sub> levels of TMZ and 6BG; titration of nutlin3 in combination with TMZ or 6BG alone or in combination to treat cancer cells. Cancer cell viability is measured as a function of drug dosage levels. Apoptotic cell death is followed via caspase-3 cleavage using Western and flow cytometric analyses (caspase 3 Magic Red detection kit; Immunochemistry Technologies). Drug- and vehicle-treated (control) tumors are stained with hematoxylin and eosin (H&E) to look for mitotic catastrophe in drug-treated cells. The contribution of the autophagic process following drug treatment is assessed by monitoring the conversion of the microtubule-associated protein 1 light-chain 3 (MAP1-LC3) to a lipidized form (LC3-II) as described previously. Senescence-associated  $\beta$ -galactosidase staining is performed as described by Dimiri et al. in *Proc Natl Acad Sci USA*, (1995); 92:9363-9367.

#### EXAMPLE 4

**[0034]** Assessment of nutlin3-mediated toxicity *in vivo*. The toxicity of these treatment regimens are studied by using 100 mg/kg nutlin3 delivered twice daily via gavage for 3 consecutive days. This amount of nutlin3 inhibits osteosarcoma growth in a nude mouse model by ~68% and represents a reasonable range to look for evidence of efficacy of combination therapy. If the 100 mg/kg dose levels results in a significant loss of body weight, the dose is decreased to 50 mg/kg and the test is reported. The submyeloablative combination drug therapy regimen consists of 80 mg/kg TMZ for 3 consecutive days, 80 mg/kg TMZ for 3 consecutive days. The results in transient decreases in blood cell counts in NOD/SCID mice. In comparison 75% of the mice did not recover from a dose of 160 mg/kg TMZ. Body weight and survival are monitored. Peripheral blood counts and bone marrow cellularity is determined and organ damage (intestine, lung, kidney, liver, brain, and bone marrow) is assessed by organ weight and the analysis of H&E-stained tissue sections.

### EXAMPLE 5

**[0035]** Sensitivity of glioblastoma xenografts to combination therapy. Ten million glioblastoma cells are implanted into the right flank of NOD/SCID mice by subcutaneous injection. Once tumor volumes reach at least  $\sim 150\text{mm}^3$ , the chemotherapy regimen is administered various regimen are outlined in Table 1. One cycle of treatment is administered to determine efficacy of the regimen. The dosing cycles are optimized to determine the initial signs of glioma cell death. Three mice from each cohort (vehicle- and drug-treated) are sacrificed 7 days post-treatment. Tumors are removed and cell suspensions made by collagenase treatment. Biochemical assays are performed to determine the predominant mechanism(s) of cell death. In the remaining mice, tumor volumes are monitored by caliper measurement [tumor volume = length x (perpendicular width)<sup>2</sup> x 0.5] about 3 times per week, compared to the day 0 measurement. These data are analyzed as % initial tumor volume. In this method each tumor serves as its own reference and the data from multiple experiments is pooled for analysis. At the end of the study (about 5-6 weeks post-treatment), tumor sections are stained to measure the effect of different dosages of these compounds on angiogenesis. Assays for angiogenesis are performed using antibodies specific to endothelial markers such as PECAM1/CD31 (R&D Systems). The experiments outlined in Table 1 are performed twice.

**[0036]** Animal studies use NOD/SCID mice provided by the *In Vivo* Therapeutics Core (IUSCC). Animal studies are approved by an independent regulation of experimental ethics, following all federal guidelines for the humane treatment of animals in laboratory settings.

### EXAMPLE 6

**[0037]** Potentiation of TMZ-sensitivity by nutlin3 in glioblastoma cells. We utilized the p53wt, MGMT-negative U87-MG cell line which is very sensitive to TMZ ( $\text{IC}_{50} = 25\text{ }\mu\text{M}$ ) as an initial screening tool for monitoring drug effects on cellular metabolism. U87-MG cells were set up in triplicate in the absence or presence of increasing concentrations of TMZ (12.5-200  $\mu\text{M}$ ) and nutlin3 (0.5-10.0  $\mu\text{M}$ ). Parallel 96-well plates were set up and incubated under either hypoxic (1%  $\text{O}_2$ ) or normoxic (5%  $\text{O}_2$ ) conditions and cell metabolism evaluated at 4 days post-

treatment by the CellTiter Glo Luminescent Cell Viability assay. At day 4 post-treatment, the relative ATP content was determined by the CellTiter Glo Luminescent Cell Viability Assay.

**[0038]** Referring now to Figure 3, data are the mean  $\pm$  standard deviation.  $*p < 0.05$  for TMZ/nutlin3 treated versus non-treated, nutlin3 (0.5-5  $\mu$ M), or TMZ. A significant increase in TMZ sensitivity was observed when the U87-MG cells were exposed to micromolar concentrations of nutlin3 under hypoxic (1% O<sub>2</sub>) and normoxic conditions (5% O<sub>2</sub>). Analysis of these data using the Dose Effect CalcuSyn software (Biosoft, Ferguson, MO) indicated that the combination index (CI) value at the ED50 was  $< 1$  and indicative of a synergistic effect between nutlin3 and TMZ.

### EXAMPLE 7

**[0039]** To study the mechanism of cell death, we monitored caspase 3/7 cleavage by flow cytometry using caspase-3 Magic Red detection kit (Immunochemistry Technologies). Referring now to Figure 4, U87-MG cells were exposed to TMZ in the absence or presence of nutlin3 and analyzed by flow cytometry for caspase-3 activation at day 3 (A) or for early and late apoptotic cells at day 4 using Annexin V-APC and propidium iodide staining (B).  $*p < 0.05$  for TMZ/nutlin3 treated versus nontreated, nutlin3, or TMZ. A significant increase in apoptotic cells in the presence of TMZ and nutlin3 was observed by day 3 post-treatment (Figure 4A) which correlated with the appearance of early and late apoptotic/necrotic cells by day 4 post-treatment (Figure 4B). These data demonstrate that nutlin3 enhances sensitization of cells to TMZ and leads to an increase in cell death.

### EXAMPLE 8

**[0040]** The SF767 glioblastoma cell line, which is resistant to TMZ and the anticancer drug Carmustine (1,3-bis(2-chloroethyl)-1-nitroso-urea) was tested. Carmustine is commercially available under the trade names BCNU and BiCNU® which is a registered trademark of Bristol-Myers Squibb, Corp. This resistance is thought to be due to high levels of MGMT in these cells. We and others have found that incorporation of 6BG, an inhibitor of MGMT, is necessary to inhibit tumor cell growth to any significant degree either *in vitro* or *in vivo*. First we determined what effect nutlin3 would have on the SF767 cell metabolism. SF767 cells were set up in the CellTiter Glo Luminescent Cell Viability assay in the presence or absence of 6BG, nutlin3, and

BCNU or TMZ. At day 6 post-treatment, the relative ATP content was determined by CellTiter Glo Luminescent Cell Viability assay.

**[0041]** Referring now to Figure 5, the data are expressed as the mean +/- standard deviation of triplicate samples. \* $p < 0.05$  for 6BG/TMZ or 6BG/BCNU vs 6BG/TMZ/nutlin3 or 6BG/BCNU/nutlin3 respectively. Referring now to Figure 5 A, SF767 cells were exposed to 5  $\mu\text{M}$  nutlin3 in the presence of TMZ (0-200  $\mu\text{M}$ ) or 20  $\mu\text{M}$  6BG and TMZ (0-200  $\mu\text{M}$ ). Referring now to Figure 5 B, SF7676 cells were exposed to 5  $\mu\text{M}$  nutlin3 in the presence of BCNU (0-180  $\mu\text{M}$ ) or 20  $\mu\text{M}$  6BG and BCNU (0-180  $\mu\text{M}$ ). In this example the inhibition of MGMT by 6BG was critical for decreasing cellular metabolism. A significant decrease in cellular metabolism in SF767 cells treated with 6BG, nutlin3, and TMZ or BCNU was observed compared to control, single, or double agent treatments.

### EXAMPLE 9

**[0042]** Inhibition of SF767 clonogenic cell growth. To determine the effect of TMZ and nutlin3 on the growth of primitive clonogenic cells, SF767 cells were exposed to clinically obtainable doses of compound (6BG = 20  $\mu\text{M}$ , TMZ = 50  $\mu\text{M}$ , and nutlin3 = 10  $\mu\text{M}$ ). SF767 cells were plated at 200 cells per well and treated with vehicle, 20  $\mu\text{M}$  6BG, 50  $\mu\text{M}$  TMZ, 10  $\mu\text{M}$  nutlin3, or combinations of these drugs. Ten days after treatment, the wells were stained with crystal violet and the number of colonies per well determined. The nutlin3 a/b racemic mixture was used in all experiments.

**[0043]** Referring now to Figure 6, \* $p < 0.001$ , control, vehicle, 6BG, TMZ, nutlin3, or TMZ/nutlin3 vs. 6BG/TMZ or 6BG/TMZ/nutlin3; \*\* $p < 0.001$ , 6BG/TMZ vs. 6BG/TMZ/nutlin3. There was a significant decrease in the number of colonies in both the 6BG/TMZ- and in the 6BG/TMZ/nutlin3-treated cultures compared to vehicle control or single agent addition ( $p < 0.001$ ) (FIG. 6). In addition, there was a 6-fold decrease in the number of colonies in the 6BG/TMZ/nutlin3-treated cultures compared to cultures treated with 6BG/TMZ only. These data indicate the 6BG/TMZ/nutlin3 combination will inhibit the growth of primitive cells residing in the SF767 population.

### EXAMPLE 10

**[0044]** Detection of nutlin3 *in vivo*. The validity and sensitivity of the detection methodology for simultaneously measuring TMZ and nutlin3 in the peripheral blood of NOD/SCID mice was developed. Using this methodology both TMZ and nutlin3 levels can be analyzed from a 25  $\mu$ l aliquot of peripheral blood. The limit of quantification is 1 ng/ml for nutlin3 and 10 ng/ml for TMZ, determined using 10  $\mu$ L of mouse blood. Pilot pharmacokinetic studies in NOD/SCID mice given 100 mg/kg nutlin3 via gavage were recently performed. Analysis of blood samples via HPLC-MS/MS (API 4000; Applied Biosystems) indicated that micromolar levels of nutlin3 ( $13 \mu\text{M} \pm 6 \mu\text{M}$ ) could be detected for at least 7 hours following administration (data not shown). Our approach will be to first develop a strategy in which nutlin3 is given intermittently in combination with TMZ. NOD/SCID mice were injected subcutaneously with U87 cells in matrigel. At 2 weeks post-tumor injection mice were given 100 mg/kg nutlin3 via gavage once per day on days 1 and 5 during weeks 2-3. Tumor volumes were measured over time via caliper. \*p, 0.05 for vehicle versus nutlin3 treated.

**[0045]** Referring now to Figure 7. We initially determined if nutlin3 had any efficacy when administered alone. U87-MG-bearing mice were given 2-weekly doses of nutlin3 for 2 consecutive weeks. Significant delay in tumor progression was evident compared to vehicle-treated tumor-bearing mice.

### EXAMPLE 11

**[0046]** Detection of nutlin3 in brain tissue. We have also developed methodology to detect and quantitate the amount of nutlin3 in brain tissue. Male NOD/SCID/IL-2R $\gamma$ chain null mice were given 100 mg/kg or 200 mg/kg nutlin3 via gavage. Peripheral blood and brain tissue were obtained at 2-6 hours post-delivery and flash-frozen. Tissues were extracted by liquid-liquid extraction followed by HPLC-MS/MS (API4000). Referring now to Table 2, nutlin3 was detected in the peripheral blood at all time points tested. In addition, nutlin3 was detectable in the brain tissue at all time points tested. The amounts of nutlin3 ranged from 8-29 ng nutlin3/g brain tissue and 30-84 ng nutlin3/g brain tissue following delivery of 100 mg/kg nutlin3 and 200 mg/kg nutlin3 respectively. This information, however, will be important as we develop a rational dosing regimen for treatment of orthotopic glioblastoma xenografts using TMZ/nutlin combination therapy.

[0047] While the novel technology has been illustrated and described in detail in the figures and foregoing description, the same is to be considered as illustrative and not restrictive in character, it being understood that only the preferred embodiments have been shown and described and that all changes and modifications that come within the spirit of the novel technology are desired to be protected. As well, while the novel technology was illustrated using specific examples, theoretical arguments, accounts, and illustrations, these illustrations and the accompanying discussion should by no means be interpreted as limiting the novel technology. All patents, patent applications, and references to texts, scientific treatises, publications, and the like referenced in this application are incorporated herein by reference in their entirety.

## CLAIMS

### WHAT IS CLAIMED IS:

1. A method of treating cancer in a patient, comprising the steps:  
identifying a patient in need thereof of a treatment for a cancer; and  
administering to the patient a therapeutically effective amount a combination drug therapy that includes:  
a compound that inhibits DNA repair or a prodrug or pharmaceutically acceptable salt thereof;  
a compound that either methylates or alkylates DNA or a prodrug or pharmaceutically acceptable salt thereof; and  
a compound that up-regulates p53 mediated apoptosis or a prodrug or a pharmaceutically acceptable salt thereof.
2. The method of treating cancer in a patient according to claim 1, wherein the compound that inhibits DNA repair acts by inhibiting the activity of MGMT.
3. The method of treating cancer in a patient according to claim 1, wherein the compound that inhibits DNA repair is 6BG or a pharmaceutically acceptable salt thereof.
4. The method of treating cancer in a patient according to claim 1, wherein the compound that methylates DNA is selected from the group consisting of: decarbazine and procarbazine or pharmaceutically acceptable salts thereof.
5. The method of treating cancer in a patient according to claim 1, wherein the compound that methylates DNA is TMZ or a pharmaceutically acceptable salt thereof.
6. The method of treating cancer in a patient according to claim 1, wherein the compound that alkylates DNA is selected from the group consisting of: lomustin and fotemustine, or pharmaceutically acceptable salts thereof.
7. The method of treating cancer in a patient according to claim 1, wherein the compound that alkylates DNA is carmustine, or a pharmaceutically acceptable salt thereof.
8. The method of treating cancer in a patient according to claim 1, wherein the compound that up-regulates p53 apoptosis activates changes p53 activity or the expression of p53.

9. The method of treating cancer in a patient according to claim 1, wherein the compound that up-regulates p53 apoptosis is nutlin3 or a pharmaceutically acceptable salt thereof.
10. The method of treating cancer in a patient according to claim 1, wherein the compound that up-regulates p53 apoptosis interferes with the interaction between HDM2 and p53.
11. The method of treating cancer in a patient according to claim 10, wherein the compound that interferes with the interaction between HDM2 and p53 is nutlin3 or a pharmaceutically acceptable salt thereof.
12. The method of treating cancer in a patient according to claim 1, wherein the amount of the compound that either methylates or alkylates DNA is submyeloablative.
13. The method of treating cancer in a patient according to claim 1, wherein the therapeutically effective amount of the compound that either methylates or alkylates DNA is myeloablative.
14. The method of treating cancer in a patient according to claim 13, further comprising the step of:
  - performing a bone marrow transplant on the patient.
15. A drug combination for treating cancer, comprising:
  - a therapeutically effective amount of a compounds that inhibits DNA repair or a prodrug or pharmaceutically acceptable salt thereof;
  - a therapeutically effective amount of a compound that either methylates or alkylates DNA or a prodrug or pharmaceutically acceptable salt thereof; and
  - a therapeutically effective amount of a compound that up-regulates p53 mediated apoptosis or a prodrug or pharmaceutically acceptable salt thereof.
16. The drug combination for treating a cancer according to claim 15, wherein the compound that inhibits DNA repair acts by inhibiting the activity of MGMT.
17. The drug combination for treating a cancer according to claim 15, wherein the compound that inhibits DNA repair is 6BG or a pharmaceutically acceptable salt thereof.
18. The drug combination for treating a cancer according to claim 15, wherein the compound that methylates or alkylates DNA is selected from the group consisting of:

lomustine, foremostine, dacarbazine and procarbazine, or pharmaceutically acceptable salts thereof.

19. The drug combination for treating a cancer according to claim 15, wherein the compound that methylates DNA is TMZ, or a pharmaceutically acceptable salt thereof.

20. The drug combination for treating a cancer according to claim 15, wherein the compound that alkylates DNA is carmustine, or a pharmaceutically acceptable salt thereof.

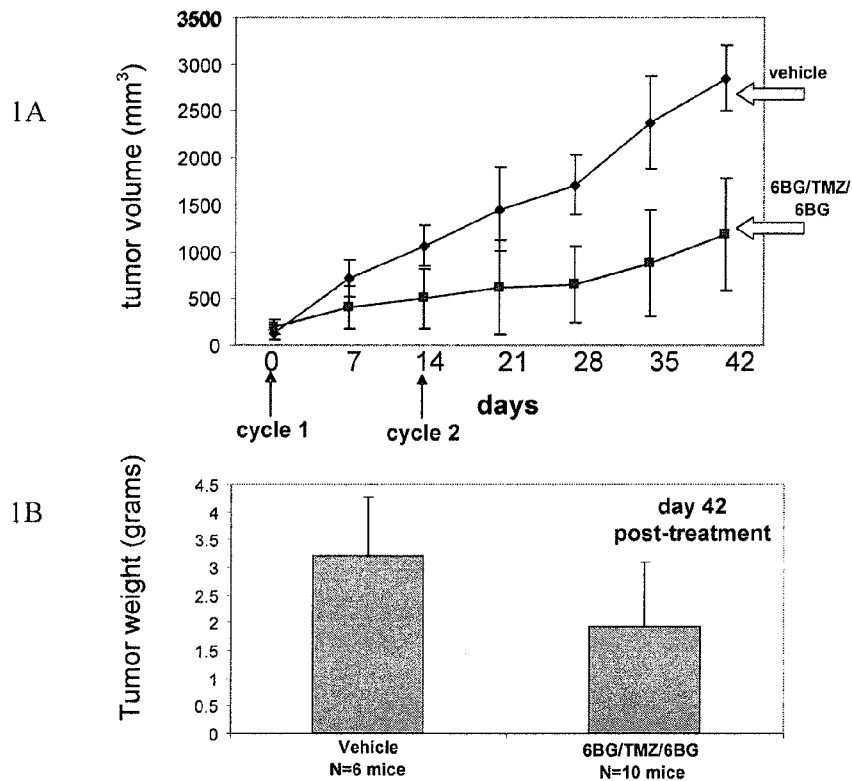
21. The drug combination for treating a cancer according to claim 15, wherein the compound that changes p53 activity or the expression of p53 and up-regulates p53-mediated apoptosis.

22. The drug combination for treating a cancer according to claim 19, wherein the compound that up-regulates p53 induced apoptosis is nutlin3.

1/8

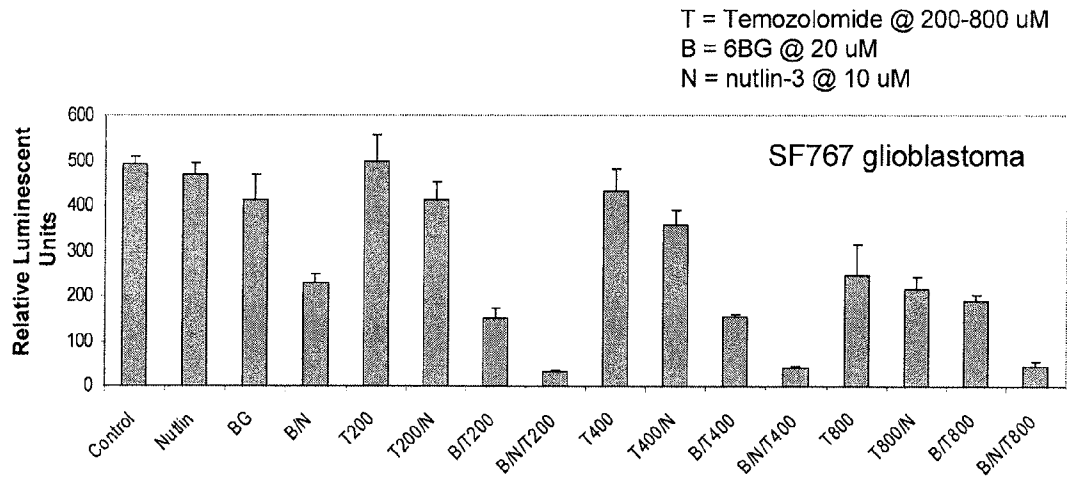
FIG. 1

† Effect of myeloablative 6BG/Temozolomide/6BG on growth of the human glioma SF767



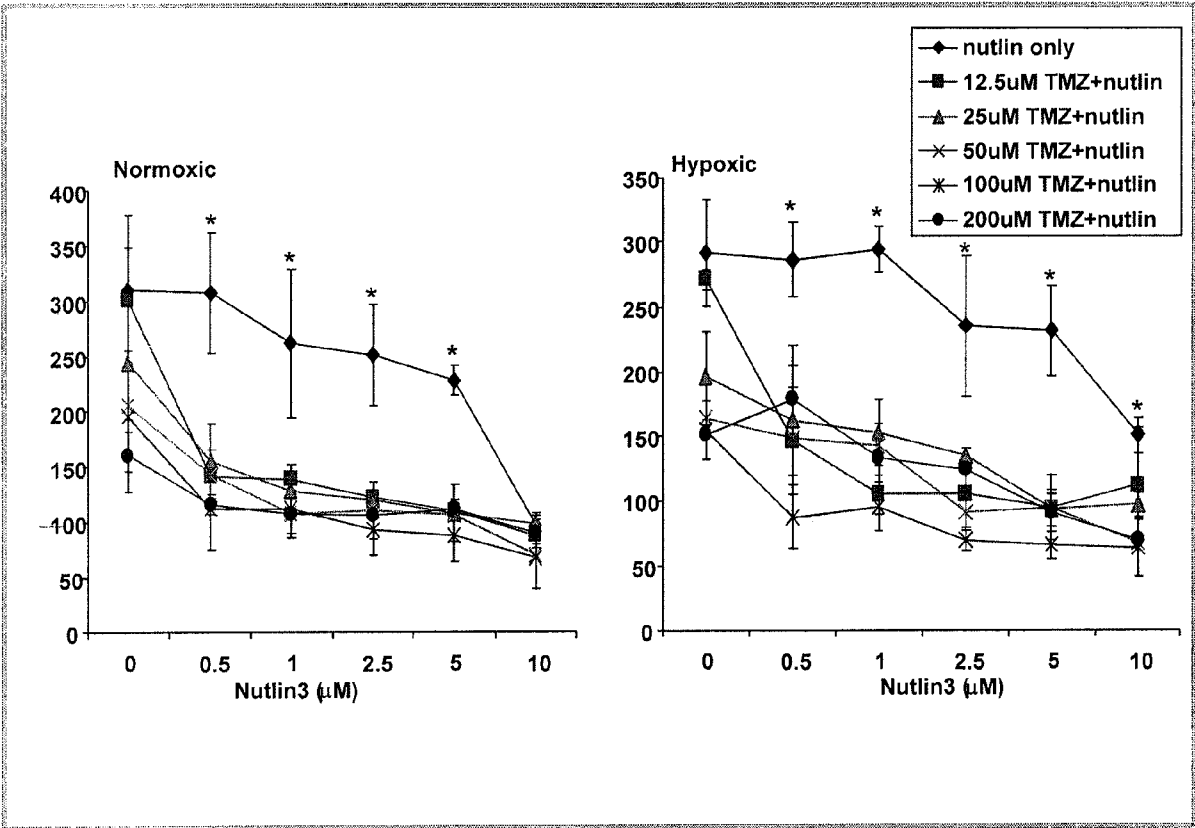
2/8

FIG. 2



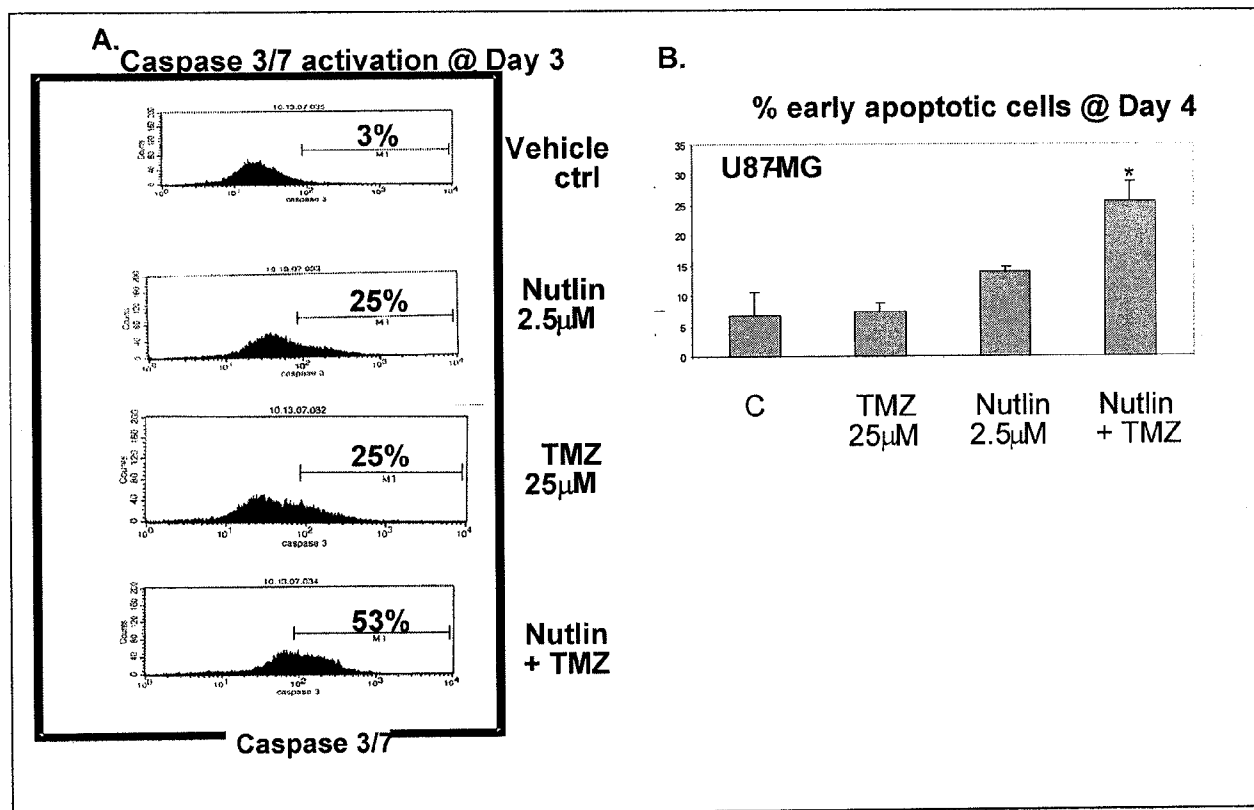
3/8

FIG. 3



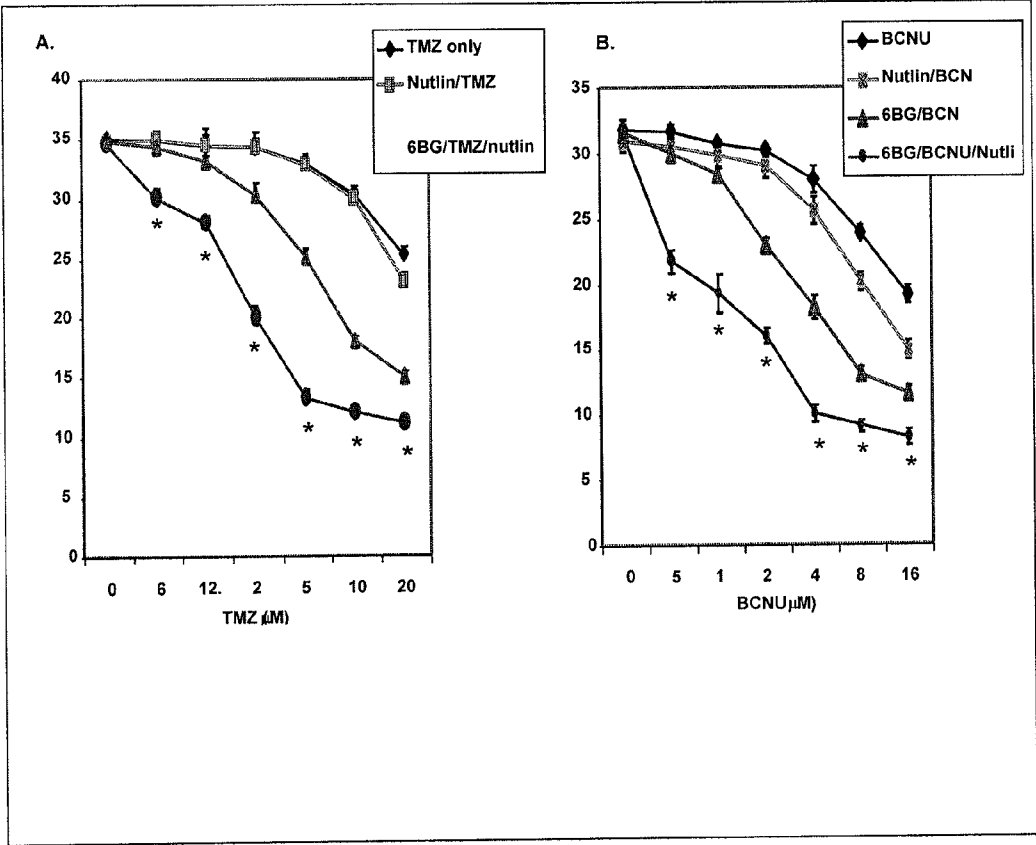
4/8

FIG. 4



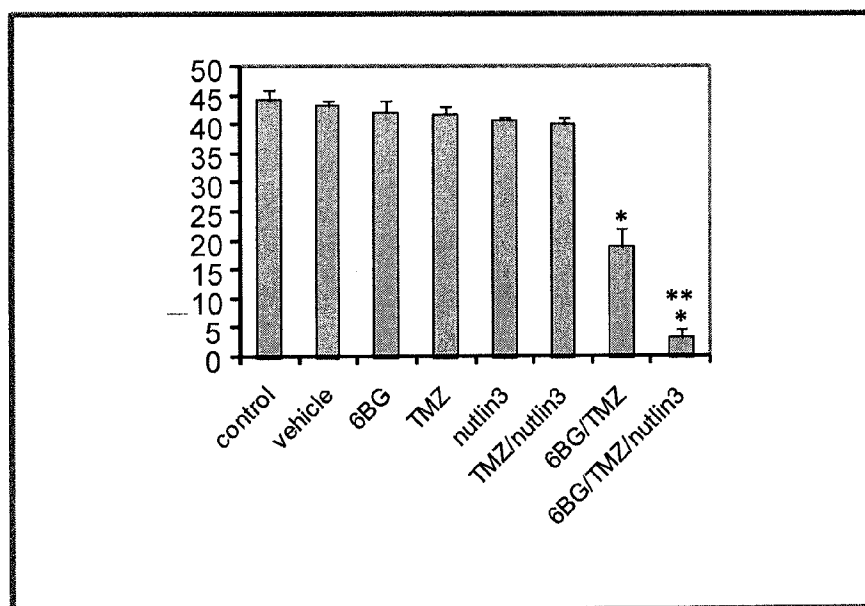
5/8

FIG. 5



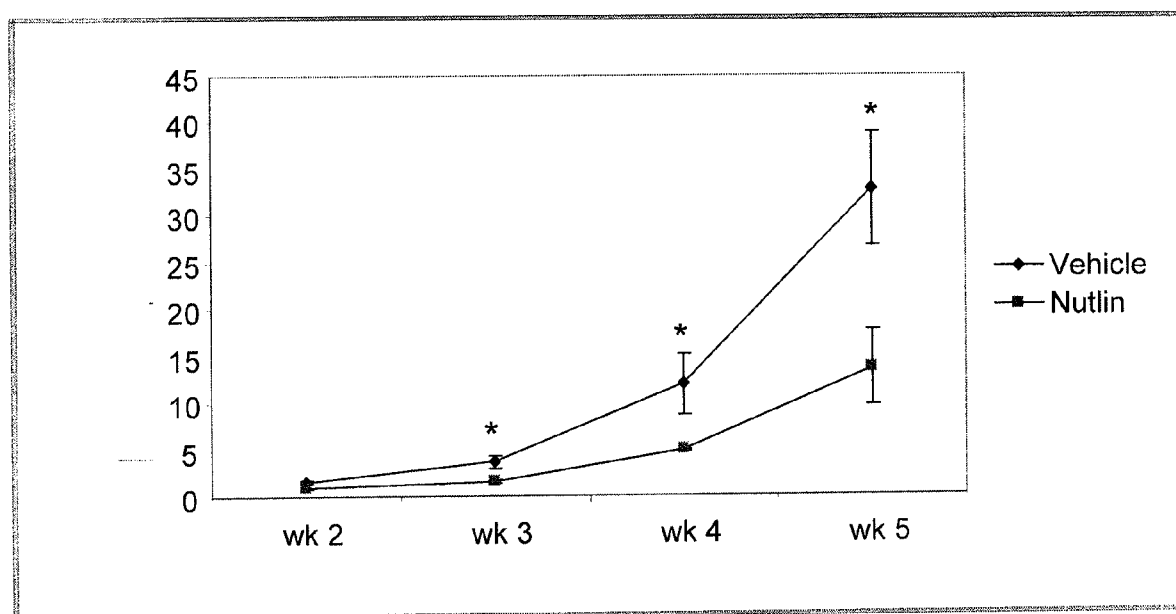
6/8

FIG. 6



7/8

FIG. 7



8/8

Table 1. Outlines of combination therapy

| Cohort              | # of mice | Treatment of U87 xenografts    | Treatment of SF767 xenografts                 |
|---------------------|-----------|--------------------------------|-----------------------------------------------|
| 1                   | 9         | vehicle control for TMZ        | vehicle control for 6BG/TMZ/6BG               |
| 2                   | 9         | vehicle control for Nutlin/TMZ | vehicle control for Nutlin-6BG/TMZ/Nutlin-6BG |
| 3                   | 12        | Nutlin                         | Nutlin                                        |
| 4                   | 12        | TMZ                            | BG/TMZ/BG                                     |
| 5                   | 12        | Nutlin/TMZ                     | Nutlin-BG/TMZ/Nutlin-BG                       |
| 6                   | 5         | -                              | bone marrow donors for transplants            |
| Total with 1 repeat |           | 108 mice                       | 118                                           |

Table 2

## Detection of nutlin3 in peripheral blood (PB) and brain tissue

| Peripheral Blood |                         |      |                             |
|------------------|-------------------------|------|-----------------------------|
| Mouse ID         | Concentration mg/kg/hr. | Time | PB Concentration ng/mL      |
| 1                | control                 | 0    | *below LOQ                  |
| 2                | 100                     | 2    | 5359                        |
| 3                | 100                     | 4    | 7054                        |
| 4                | 100                     | 6    | 4171                        |
| 5                | 200                     | 2    | 11344                       |
| 6                | 200                     | 4    | 7639                        |
| 7                | 200                     | 6    | 10470                       |
| Brain            |                         |      |                             |
| Mouse ID         | Concentration mg/kg/hr. | Time | Tissue concentration (ng/g) |
| 1                | control                 | 0    | *below LOQ                  |
| 2                | 100                     | 2    | 8.16                        |
| 3                | 100                     | 4    | 29.77                       |
| 4                | 100                     | 6    | 28.51                       |
| 5                | 200                     | 2    | 53.34                       |
| 6                | 200                     | 4    | 30.05                       |
| 7                | 200                     | 6    | 83.99                       |

\*LOQ = level of quantification