ABSTRACT

Temozolomide (TMZ) is a DNA-methylating agent that has recently been introduced into Phase II and III trials for the treatment of gliomas. TMZ produces O\textsuperscript{6}-methylguanine in DNA, which mispairs with thymine during the next cycle of DNA replication. Subsequent futile cycles of DNA mismatch repair can lead to a p53-associated apoptotic cell death, although this mechanism has been described mostly in hematopoietic neoplasms. We studied the action of TMZ in gliomas and the role p53 might play by using U87 glioma cells that were either p53-wild-type or p53-deficient (by virtue of expression of the viral oncoprotein E6). LN-Z308 cells, in which p53 gene is deleted, were also used. p53-proficient U87 MG cells underwent a prolonged, p53- and p21Waf1/Cip1-associated G\textsubscript{2}-M arrest beginning 2 days after TMZ treatment. Although very few of these cells underwent apoptosis, most underwent senescence over a 10-day period. p53-deficient (E6-transfected U87 and LN-Z308) cells similarly underwent G\textsubscript{2}-M arrest in response to TMZ, but this arrest was accompanied by only minor changes in p53 or p21Waf1/Cip1, and was reversed within 7 days of TMZ treatment in association with the appearance of cells with either 8n or subG\textsubscript{1} DNA content. These results suggest that glioma cells respond to TMZ by undergoing G\textsubscript{2}-M arrest. p53 is not necessary for this G\textsubscript{2}-M arrest to occur but is important in the duration of G\textsubscript{2}-M arrest and in the ultimate fate of TMZ-treated cells. Therefore, the integrity of the G\textsubscript{2}-M cell cycle checkpoint may be important in the cytotoxicity of TMZ in glioma cells.

INTRODUCTION

Astrocytic tumors are the most common primary brain tumors. Of the astrocytic tumors, GB\textsuperscript{3} is the most malignant form and has the worst prognosis. Complete surgical resection of GB is difficult, and the tumor generally recurs within a year after radiation and chemotherapy regardless of the initial response to these treatment modalities (1). Of the chemotherapeutic agents used to treat GB, alkylating agents are the most widely used. Even with these agents, however, the patient response is poor. TMZ is a second-generation imidazotetrazolo[1,5-a]pyrimidine derivative, which was found to be generally well tolerated and easily administrated p.o. (2, 3). Recent Phase II and III trials for recurrent high grade gliomas revealed that 50–60% of GB showed objective response or stable disease to this compound (4–6). The ability of TMZ to delay tumor progression, along with its ease of administration, has increased interest in using TMZ in the treatment of GB (7).

The action of TMZ has been extensively studied, primarily in leukemia and lymphoma cells. TMZ spontaneously decomposes in an aqueous solution to form the cytotoxic methylating agent, 5-(3-methyltriazolen-1-y1) imidazole-4-carboxamide (8). The cytotoxicity of TMZ appears to be mediated mainly through addition of a methyl group to O\textsuperscript{6} position of guanine in genomic DNA (9–11). The methyl group can be removed from O6-meG by MGMT (12, 13). If MGMT is deficient in the cell, however, O6-meG is not repaired. Incorporation of a T rather than a cytosine opposite the O6-meG during the next cycle of DNA replication leads to the formation of GT mismatches in DNA (14). This triggers the DNA MMR system that removes the T, only to have the T reinserted during repair synthesis. Futile cycles of MMR triggered by GT mismatches can lead to a variety of outcomes in TMZ-treated cells (15). In MGMT-deficient, MMR-proficient lymphocytes, TMZ-induced O6-meG lesions, GT mispairs, and futile cycles of MMR result in growth arrest, induction of p53/p21Waf1/Cip1, and apoptosis (16–18). In MGMT-deficient, MMR-deficient lymphocytes, O6-meG lesions and GT mispairs are tolerated, do not lead to futile cycles of MMR or accumulation of p53, and, although mutagenic, are not toxic. Therefore, the action of TMZ appears to rely on a functional MMR system. The observation that lymphoid cells with wt p53 were more likely to undergo TMZ-induced apoptosis than p53-deficient cells, however, also suggests that p53 plays a contributing role in the response to TMZ-induced futile MMR. Although published studies have helped define the action of TMZ in hematopoietic neoplasms, the action of the compound in glioma cells remains largely undefined. Few gliomas display alterations in MMR (19, 20), and, as such, the early actions of TMZ in gliomas may resemble those in lymphocytes. However, gliomas have been shown to be relatively resistant to apoptosis induced by DNA damage (21), and, as such, it is not clear if TMZ could induce apoptosis in gliomas. Finally, because p53 mutations are a common event even in the low-grade gliomas (22–24) and because p53 has been suggested to play a role in the activity of TMZ in hematopoietic cells (25, 26), p53 status might influence the action of TMZ in gliomas.

To address the action of TMZ in gliomas and to determine the role of p53 in this action, we incubated glioma cells that were MGMT-negative, MMR-proficient, and either p53-wt or p53-deficient with TMZ and monitored the effects of TMZ on cell cycle progression and survival.

MATERIALS AND METHODS

Cell Culture and Treatment. The human GB cell lines U87 MG and LN-Z308 were cultured in DMEM-H:21 supplemented with 10% FCS (Life Technologies, Inc.) at 37°C in a 5% CO\textsubscript{2} (g) atmosphere. U87 MG cells expressing human papilloma virus (type 16) E6 protein (U87 MG-E6) were a generous gift from Dr. Daphne Haas-Kogan (Brain Tumor Research Center, University of California, San Francisco). Cells were plated at least 2 days before the drug treatment.

TMZ was supplied by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, Maryland and was dissolved in DMSO (Sigma Chemical Co., St. Louis, MO). Unsynchronized cells were treated with 100 μM TMZ for 3 h, with the final DMSO concentration not exceeding 0.1% (v/v). After treatment, cells were gently washed, incubated in fresh media at 37°C, and harvested at subconfluent conditions at various time points.

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2 To whom requests for reprints should be addressed, at Department of Neurological Surgery, University of California San Francisco, 2340 Sutter Street, Rm N261, Box 0875, San Francisco, CA 94143-0875. Phone: (415) 502-7132; Fax: (415) 502-6779; E-mail: rpieper@cc.ucsf.edu.

3 The abbreviations used are: GB, glioblastoma; TMZ, temozolomide; O6-meG, O\textsuperscript{6}-methylguanine; MGMT, O\textsuperscript{6}-methylguanine-DNA methyl transferase; MMR, mismatch repair; T, thymine; GT, guanine: thymine; wt, wild-type; SA-gal, senescence-associated β-galactosidase; FACS, fluorescence-activated cell sorter.

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Cell Cycle Studies. At each time point, the cells attached to the culture dish were trypsinized and collected together with the cells floating in the media. These cells were washed in PBS, fixed in 70% (v/v) ethanol, and stored for up to 2 weeks at −20°C. The cells were washed once with PBS, followed by incubation in PBS containing 40 µg/ml propidium iodide (Sigma Chemical Co.) and 200 µg/ml RNase A (Sigma Chemical Co.) for 1 h at room temperature in the dark. Stained nuclei were then analyzed using a Becton Dickinson FACScan (San Jose, CA) with 20,000 events/determination. ModFit LT software (Verity Software House, Inc., Topsham, ME) was used to assess cell cycle distribution.

Immunoblot Analyses. Cells were washed with ice-cold PBS, scraped from the culture dish, and incubated in tissue lysis buffer containing 10 mM KCl, 1 mM sucrose, 2 mM MgCl2, 0.5% Igepal CA-630, 1 mM EDTA, 1 mM DTT, 10 mM β-glycerophosphate, 1 mM Na3VO4, 10 mM NaF, 100 µg/ml phenylmethylsulfonyl fluoride, and 10 µg/ml aprotonin (all of the reagents was purchased from Sigma Chemical Co.) for 30 min on ice. The cell lysate was centrifuged, and the supernatant was stored at −80°C until use. The protein concentration of extracts was measured using Protein Assay reagent (Bio-Rad Laboratories, Hercules, CA). Protein (40 µg) was subjected to SDS-PAGE (27) and electroblotted onto Immobilon-P membrane (Millipore, Bedford, MA). The membrane was blocked in 5% nonfat skim milk (Bio-Rad Laboratories)/TBST [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Tween 20] at 4°C overnight and probed with mouse monoclonal antibodies against human p53 (DO-1; Santa Cruz Biotechnology, Santa Cruz, CA) or human p21Waf1/Cip1 (187; Santa Cruz Biotechnology) for 1 h at room temperature. Bound antibody was detected with goat antimouse IgG (Santa Cruz Biotechnology) enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Densitometric measurement of immunoreactive bands was performed using Alpha Imager 2200 (Alpha Innotech Corporation, San Leandro, CA).

Measurement of Viable Cells and Colony Formation Efficiency. U87 MG and U87 MG-E6 cells (1 × 104) were plated onto a 35-mm dish 2 days before TMZ treatment. After a 3-h incubation in TMZ-containing (100 µM) media, the cells were incubated in TMZ-free media for the period indicated. Viable, trypan blue-excluding cells were counted using a hemocytometer.

Assessment of colony formation efficiency was performed as described by Sarkar et al. (28). Briefly, on the day before TMZ treatment, SF126 cells were irradiated (40 Gy) and plated in 6-well plates at a density of 5 × 103 cells/well to function as a feeder layer. On the day after the cells, the clonogenicity of which was to be evaluated, were treated with TMZ at various concentration for 3 h, trypsinized, counted, and plated at a concentration of 100 cells/well into wells containing an irradiated feeder layer. These cultures were then incubated for 20 days, and colonies of over 50 cells were counted.

SA-β-gal Staining. The method of SA-β-gal staining was performed as described by Dimri et al. (29). Briefly, after incubation for the period indicated, the cells were washed in PBS, fixed with 2% formaldehyde/0.2% glutaraldehyde in PBS for 5 min at room temperature, and incubated overnight at 37°C in fresh 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside solution (1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM MgCl2 in 40 mM citric acid/sodium phosphate buffer (pH 6.0)). The next day, cells were rinsed with PBS, and the percentages of SA-β-gal positive (blue) cells were determined after scoring 500 cells for each sample using a bright-field microscope.

RESULTS

TMZ-induced G2-M Cell Cycle Arrest in U87 MG Glioblastoma Cells in Association with p53- and p21-Accumulation. To clarify the effect of TMZ in glioma cells, we first performed cell cycle analysis of U87 MG cells treated with TMZ. Unsynchronized cells were treated with TMZ (100 µM for 3 h) and incubated for 1–10 days in the absence of TMZ. The drug concentration used was based on the published data of the plasma concentration of TMZ in patients involved in its clinical trials (2, 3). Harvested cells were subjected to analysis by FACS, which used DNA content as a measure of progression in the cell cycle and as means of detecting cells with a sub-G1 DNA content (apoptotic cells). There was no significant difference in the percentage of cells in each phase of the cell cycle between untreated U87 MG cells and the cells harvested at 1 day after TMZ treatment (Fig. 1A). However, cells began to accumulate at the G2-M boundary (4n DNA content) 2 days after TMZ treatment. This G2-M arrest (here defined as a greater percentage of cells in G2-M than G1) was sustained for at least 10 days after TMZ treatment and was associated with the gradual appearance of hyperploid (>4n DNA content) cells and the gradual loss of cells with 2n DNA content (G1 cells). Although TMZ-treated cells underwent cell cycle arrest, the sub-G1 population, which represents apoptotic cells, was small and did not significantly increase throughout the 10 days after TMZ treatment. We hypothesized that TMZ-induced G2-M arrest in glioblastoma cells was associated with alteration of the proteins that play a key role in cell cycle regulation. Therefore, the protein level of p53 and p21Waf1/Cip1 were investigated by immunoblotting. Consistent with the G2-M arrest data, p53 and p21Waf1/Cip1 levels were increased approximately 2–4-fold at 2 days after TMZ treatment with the accumulation persisting at least 10 days after the treatment (Fig. 1B).

p53-deficient Glioblastoma Cells Showed Transient G2-M Arrest in Response to TMZ. Because the data from U87 MG cells suggested that p53 and p21Waf1/Cip1 might play an important role in TMZ-induced G2-M cell cycle, we next performed similar experiments using E6-expressing U87 (U87 MG-E6) cells. These cells have low levels of p53 because of E6-mediated ubiquitination and destruction of p53 (30). As in U87 MG cells, G2-M arrest in TMZ-treated U87 MG-E6 cells began 2 days after the drug treatment (note the higher percentage of cells in G2-M compared with G1; Fig. 2A). Unlike the persistent G2-M arrest in U87 MG cells, however, the G2-M arrest in TMZ-treated U87 MG-E6 cells began decreasing by 3 days after TMZ treatment and was considerably lessened by 10 days after treatment. The reduction in G2-M arrest in these cells was also associated with a gradual increase in cells with a sub-G1 (less than 2n) and a slight increase in cells with >4n DNA content, although the increase in polyploid cells was not as remarkable as that observed in the experiments using U87 MG cells. As expected, untreated U87 MG-E6 cells had very low level of p53 and p21Waf1/Cip1 (Fig. 2B). Although the levels rose slightly after TMZ treatment, they never exceeded those in untreated U87 MG cells and were considerably less than those in TMZ-treated U87 MG cells (Fig. 2B). These results suggest that although p53 is not necessary for the initiation of TMZ-induced G2-M arrest, it appears to be involved in sustaining G2-M arrest induced by TMZ.

Because U87 MG and U87 MG-E6 cells share a common genetic background except for expression of E6, comparison of the responses of these cells provides more useful information than the comparison of responses of cells with completely different genetic backgrounds. However, it was possible that the E6 protein had effects on cells other than those mediated by p53 inactivation (31). Therefore, we also investigated the action of TMZ on LN-Z308 cells that lack the p53 gene but have an intact p21Waf1/Cip1 (32–34). Like TMZ-treated U87 MG and U87 MG-E6 cells, TMZ-treated LN-Z308 cells showed G2-M arrest within 2 days of TMZ treatment (Fig. 3A). As in the TMZ-treated U87 MG-E6 cells, G2-M arrest was not sustained, and by 7 days after TMZ treatment, G2-M arrest was lessened in connection with an increase in the number of cells with a sub-G1 DNA content. Immunoblot analyses revealed that neither p53 nor p21Waf1/Cip1 was accumulated to any significant degree in LN-Z308 cells. Although TMZ-treated cells underwent cell cycle arrest, the sub-G1 population, which represents apoptotic cells, was small and did not significantly increase throughout the 10 days after TMZ treatment. We hypothesized that TMZ-induced G2-M arrest in glioblastoma cells was associated with alteration of the proteins that play a key role in cell cycle regulation. Therefore, the protein level of p53 and p21Waf1/Cip1 were investigated by immunoblotting. Consistent with the G2-M arrest data, p53 and p21Waf1/Cip1 levels were increased approximately 2–4-fold at 2 days after TMZ treatment with the accumulation persisting at least 10 days after the treatment (Fig. 1B).

Cell Viability and SA-β-gal Positivity. To address the fate of TMZ-treated glioma cells, we assessed the number of viable U87 MG and U87 MG-E6 cells 0–12 days after the drug treatment using the
trypan blue dye-exclusion method. U87 MG cells increased in number for 2 days after TMZ treatment, after which cell number remained constant over the next 12 days (Fig. 4A). In contrast, U87 MG-E6 cells showed transient growth arrest 2–4 days after TMZ treatment, after which the cells proliferated for a few days and eventually lost viability. We also performed a colony formation efficiency assay. U87 MG cells showed a dose-dependent decrease in clonogenicity (Fig. 4B) with a colony formation efficiency of 30% (relative to untreated cells) after exposure to 100 μM TMZ (the concentration used in most of the studies in this manuscript). On the other hand, U87 MG-E6 cells formed no colonies when they were treated with $100 \mu M$ TMZ (Fig. 4B).

Because U87 MG cells that had a reduced proliferative capacity after TMZ treatment also showed accumulation of p21Waf1/Cip1, we checked whether senescence-like events (35, 36) occurred in TMZ-treated cells. SA-β-gal activity was induced in TMZ-treated U87 MG cells, and after 10 days culture, more than 50% of the cells were positive for SA-β-gal activity (Fig. 5A). Most of SA-β-gal positive cells (blue cells in Fig. 5B) also exhibited senescence-like morphology with increased size and flattened shape. In contrast, few of the TMZ-treated U87 MG-E6 cells displayed either SA-β-gal activity or senescence-like morphology. These results indicated that TMZ-induced prolonged growth arrest promoted a senescence-like program in U87 MG cells.

**DISCUSSION**

In hematopoietic cells, the action of TMZ has clearly been shown to be dependent on the creation of O6-meG lesions (9–11), the mispairing of O6-meG with T, the triggering of the MMR system (14), the initiation of futile cycles of MMR (15), and, ultimately, p53-associated apoptosis (17, 18). The importance of TMZ in the treatment of gliomas, along with the relative lack of information concerning its action in glial cells, led us to study the action of TMZ in glioma cells and the role of p53 might play. The results of these studies suggest that the most prominent effect of TMZ in gliomas is G2-M arrest, which is either sustained and associated with senescence in p53-wt cells or fatally bypassed in p53-deficient cells.

The action of TMZ in glioma cells stands in contrast to the previously described action of TMZ in hematopoietic cells. The action of TMZ has been studied in lymphoid cells because of the use of TMZ in the treatment of lymphoid malignancies (37) and because of the availability of lymphoid cell lines differing only in MMR proficiency (16, 38). The actions of TMZ in the glioma cells used in the present study were similar to those in lymphoid cells in that no effects were noted until the second day (approximately the second cell cycle) after drug treatment (17, 18), consistent with the timeframe of creation of O6-meG:T mispairs and the triggering of MMR. Lymphoid cells, however, are considered to be extremely sensitive to a variety of

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**Fig. 1.** Cellular responses of U87 MG cells to TMZ treatment. A, cell cycle analysis of TMZ-treated U87 MG cells by FACS. Histograms and cell cycle distributions shown are representative of three experiments. B, analysis of protein levels of p53 and p21Waf1/Cip1 by immunoblotting. Fold-induction of protein level was based on densitometric measurements and is shown below each immunoreactive band (protein levels in untreated U87 MG cells were defined as 1.0).
apoptotic stimuli, whereas glioma cells have been shown to be relatively resistant to apoptosis induced by radiation (21). The low level of apoptosis induced by TMZ in glioma cells relative to lymphoid cells, therefore, likely results from both the relative ease with which lymphoid cells undergo apoptosis and the relative lack of ability of glioma cells to do so. On the other hand, it should be noted that the U87 MG cells used in this study can be induced to undergo apoptosis by treatment with various DNA-damaging agents (39–41) and that a small percentage of apoptotic cells were noted after TMZ treatment of p53-deficient cells. Therefore, although some U87 cells underwent apoptosis after TMZ treatment, the predominant effect of TMZ in the cells examined was not apoptosis but rather was G2-M arrest.

The role of p53 in the action of TMZ in gliomas also stands in contrast to that described in lymphoid cells. In HL60 cells, the presence of wt-p53 increased the ability of TMZ to trigger apoptosis, although p53-independent apoptosis was also noted (42). In glioma cells used in the present studies, p53 did not enhance apoptosis induced by TMZ and similarly had little effect on initiation of G2-M arrest. G2-M arrest in response to TMZ has been noted in both lymphoid (17, 18, 42) and glioma (43) cells, although the duration and resolution of the arrest have not been carefully followed. In U87 MG cells, the duration of the G2-M arrest was associated with p53 status, i.e., p53-wt cells underwent prolonged arrest associated with elevation of p53 levels, whereas p53-deficient cells underwent a more transient arrest. The ability of TMZ-treated U87 MG-E6 and LN-Z308 cells to undergo G2-M arrest in the absence of p53/p21Waf1/Cip1 induction is consistent with studies in fibroblasts showing that inhibition of the kinase activity of cyclin B1 (an early G2 checkpoint event) is p21Waf1/Cip1 independent (44, 45). The transient nature of the G2-M arrest in U87 MG-E6 and LN-Z308 cells is also consistent with studies in which both p53 and p21Waf1/Cip1 were shown to be required to sustain G2-M arrest after γ irradiation in colon cancer cells (46). Therefore, it appears that although the effects of p53 in the regulation of apoptosis can be noted in cells sensitive to apoptotic stimuli, e.g., lymphoid cells, the effects of p53, p21Waf1/Cip1, and/or other p53-regulated proteins on TMZ-induced G2-M arrest become more apparent and important in cells less sensitive to apoptotic stimuli, e.g., glioma cells.

As well as being associated with the duration of TMZ-induced arrest in glioma cells, the p53 status of cells was also associated with the ultimate fate of the cells. p53-wt cells underwent a prolonged G2-M arrest that left the majority of cells viable yet nonproliferative. These cells had high levels of p21Waf1/Cip1 expression, exhibited SA-β-gal activity, and underwent morphological changes consistent with senescence. The proportion of cells with a >4n DNA content was also increased, consistent with the observation that in the absence
of a functional p16/pRb pathway (as is the case in U87 MG cells; Ref. 47) p21 can induce endoreduplication (36, 48, 49). In contrast, p53-deficient cells underwent a more transient arrest and, within 12 days, lost viability in a manner consistent with mitotic catastrophe. Mitotic catastrophe, in turn, may have eliminated those cells that underwent endoreduplication, which might explain the relative lack of cells U87 MG-E6 cells with >4n DNA content. In other p53-deficient human glioblastoma cell lines (SF126 and U251; Ref. 32), TMZ also induced transient G2-M arrest and a combination of mitotic catastrophe and apoptosis (data not shown). Therefore, although both p53-wt and
p53-deficient cells became nonproliferative in response to TMZ, the means by which this was accomplished differed in a manner consistent with p53 status.

The results of the studies presented may have implications for the use of TMZ in the treatment of gliomas. As noted, gliomas are variable in p53 status with approximately 40% of tumors lacking wt-p53 (22–24). p53-deficient gliomas are more likely than p53-wt gliomas to die as a result of bypass of TMZ-induced G2-M arrest. Of note, however, is that the reentry of p53-deficient TMZ-treated cells into the cell cycle may result in the creation of cells that, by virtue of the low level of p53 and their ability to bypass G2-M arrest, may divide and undergo further alterations that give rise to TMZ-resistant cells. In contrast, p53-wt tumor cells are likely to undergo permanent G2-M arrest, although it is unclear if this arrest is dependent on the continued presence of the O6-meG:T mismatch or simply the initiation of futile MMR. Whereas approximately half of the G2-M arrested cells in our study underwent a senescence-like event, the fate of the remaining half remains unclear. Although the number of p53-wt TMZ-treated cells appeared not to increase after drug treatment, a significant percentage of the cells retained proliferative capacity as assessed by colony formation assay. Therefore, it may be possible that given enough time, the TMZ-induced lesion that triggered G2-M arrest can be reversed or bypassed. In this sense, the bypass of G2-M arrest noted in p53-deficient cells appears to be therapeutically desirable in that it may force cells to perform fatal replicative events. The events associated with the TMZ-induced G2-M arrest have not been studied, although it is reasonable to suspect that a G2 checkpoint mechanism is involved. Inhibitors of this pathway have been suggested to act synergistically with DNA-damaging agents (50–52). Given the action of TMZ noted in glioma cells in this study, it may be reasonable to combine TMZ with a G2 checkpoint abrogator, such as UCN-01 (53–56).

In summary, the studies in this manuscript suggest that the G2-M checkpoint plays an important role in the action of TMZ in gliomas. The resolution of the G2-M arrest is, in turn, dependent on the p53 status of the cells. This knowledge may be useful in designing better TMZ-based therapeutic regimens for the treatment of gliomas.

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Fig. 5. SA-β-gal activity at 10 days after drug treatment. The photographs were taken at ×100 magnification (50 μm scale bar). β-Galactosidase positive cells are stained blue.


p53 Effects Both the Duration of G2/M Arrest and the Fate of Temozolomide-treated Human Glioblastoma Cells

Yuichi Hirose, Mitchel S. Berger and Russell O. Pieper


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