EMOZOLIDE is a relatively new DNA methylating agent in Phase II and III clinical trials for the treatment of various lesions, including malignant brain tumors. Because TMZ is generally well tolerated, can be administered orally, readily crosses the blood–brain barrier, and has shown efficacy against human gliomas in clinical trials, there is considerable interest in using this compound to treat brain tumors.

The cytotoxic action of TMZ, like that of other DNA methylating agents, is dependent on the creation of DNA adducts and on the cellular response to the adducts. It has been reported that TMZ, like other chemotherapeutic methylating agents, creates a variety of DNA adducts, including N7-methylguanine, MG, and N3-methyladenine. Although all of these adducts may contribute to the cytotoxic potential of TMZ, the best evidence to date indicates that the mutagenic and toxic effects of TMZ are directly related to the formation of O6 position of G, and to the cellular response to the lesion. The TMZ-induced MG mispairs with T instead of C during DNA replication, resulting in the formation of MG:T mismatches. The MG:T mismatches are substrates for the DNA MMR system, which excises the mismatched T, only to reinsert T opposite of MG during repair resynthesis. Therefore, although the MMR system accurately repairs G:T mismatches, its activation in response to MG:T mismatches results in futile cycles of MMR that persist as long as MG exists in the DNA. In MMR-proficient cells (which include all normal cells and most glioblastoma lines), the initiation of multiple rounds of futile repair and resynthesis is temporally associated with a variety of cellular events, including the induction of p53/p21, activation of the G2 checkpoint, and ultimately the loss of proliferative capacity. Conversely, cells deficient in MMR accumulate MG lesions and MG:T mismatches after exposure to...
methylyating agents, but because of the lack of MMR they avoid cytotoxic consequences.13,24

Although the loss of MMR can protect tumor cells from methylyating agent-induced cytotoxicity, repair of MG lesions before their mispairing and triggering of futile MMR can serve the same purpose. Repair of MG is accomplished by MGMT, a protein that irreversibly transfers the methyl group from MG in DNA to a cysteine in its active site, after which the protein undergoes ubiquitination and degradation.11,12,25 Whereas cells with even modest levels of MGMT avoid the cytotoxic effects of methylyating agents, cells lacking MGMT or depleted of its activity by MG lesions themselves, or by a selective inhibitor of MGMT such as BG, are sensitized to the cytotoxic effects of methylyating agents.3,4

Although both MMR and MGMT can influence the cytotoxic actions of methylyating agents, most glioma cells have a functional MMR system that cannot be readily manipulated. The relative ease with which MGMT can be depleted in cells therefore makes it the more therapeutically relevant controller of the chain of events that leads from TMZ-induced MG lesions to cytotoxicity.

Although it is clear that MGMT depletion sensitizes cells to TMZ-induced cytotoxicity, the potential use of the TMZ+BG combination in the treatment of gliomas may be complicated by the unique response of these tumors. In lymphoid cells, which to date remain the most studied system, the induction of TMZ-induced futile MMR is followed closely by induction of p53 and apoptotic cell death, with minimal G2–M arrest noted.2,9,14,18 Therefore, in lymphoid cells, TMZ activates a nearly seamless process leading from DNA damage to apoptosis. In glioma cells incubated with TMZ, however, we noted that they did not die immediately but rather underwent a prolonged (5–10-day) G2–M arrest in association with activation of the Chk1-mediated G2 checkpoint.16,17 Although the glioma cells ultimately lost clonogenicity, the point along this path at which the commitment to loss of viability occurred, and the events associated with it, were unclear. Furthermore, because activation of the G2–M checkpoint is believed to allow cells time to repair otherwise fatal DNA damage,28,29 it is possible that the extended G2–M arrest in response to TMZ may provide gliomas with a prolonged period of time in which to replenish MGMT levels and avoid TMZ-induced cytotoxicity. This prolonged period of repair might, in turn, necessitate prolonged MGMT depletion to achieve maximal TMZ sensitization in gliomas. To address these issues, the actions of TMZ were monitored in MMR-proficient SF767 glioma cells depleted of MGMT by BG, and in cells in which BG was removed at various times after TMZ exposure.

Materials and Methods

Cell Culture and Treatment

The human glioblastoma cell line SF767 was cultured in Dulbecco modified Eagle H-21 medium supplemented with 10% fetal calf serum (Gibco/BRL, Gaithersburg, MD) at 37°C in a 5% CO2 atmosphere. Cells were plated at least 2 days before drug treatment.

The TMZ and BG were supplied by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, MD. The TMZ was dissolved in dimethyl sulfoxide (Sigma Chemical Co., St. Louis, MO), and the BG was dissolved in ethanol. Unsynchronized, subconfluent cells were pretreated with 20 μM of BG for 2 hours, followed by incubation with 100 μM of TMZ for 3 hours in the presence of BG (20 μM). The cells were then washed and incubated in media containing 5 μM of BG for 0 to 10 days, with BG being replenished every day. For all studies, the final concentration of dimethyl sulfoxide or ethanol in the media did not exceed 0.1% (vol/vol).

Cell Cycle Studies

At each time point, the cells were trypsinized and collected; fixation and staining were performed as described elsewhere.25 Stained nuclei were then analyzed using a Becton-Dickinson FACScan apparatus (San Jose, CA), with 20,000 events per determination.

Immunoblot Analyses

The protein samples were prepared and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by electroblotting onto an Immobilon-P membrane (Millipore, Bedford, MA), as described elsewhere.27 The membrane was blocked in 5% nonfat milk (BioRad Laboratories, Richmond, CA) in Tris-buffered saline/ Tween (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), human p21 (187; Santa Cruz Biotechnology) or human actin (Santa Cruz Biotechnology) for 1 hour at room temperature. For immunoblotting of tyrosine-15 phosphorylated cdc2, the membrane was blocked with 5% bovine serum albumin (Sigma Chemical Co.). In Tris-buffered saline/Tween at 4°C overnight and probed with anti-human phospho-cdc2 (New England Biolabs, Beverly, MA). Bound antibody was detected with horseradish peroxidase–conjugated secondary immunoglobulin G (Santa Cruz Biotechnology) by using enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech, Inc., Piscataway, NJ).

Measurement of MGMT Activity

Control (untreated), BG-treated, or BG+TMZ-treated cells (1–5 × 105) were harvested, washed twice with cold PBS, and resuspended in 0.5 ml of assay buffer (50 mM Tris-HCl [pH 7.5], 0.5 mM EDTA, 1 mM dithiothreitol, and 5% glycerol). Cell suspensions were then sonicated on ice (5 × 5 seconds, setting 4) in a sonicator (VirSonic 300; Virstis, Inc., Gardiner, NY) and centrifuged at 14,000 G for 30 minutes at 4°C. The supernatant was recovered and analyzed for protein concentration as previously described. For analysis of MGMT activity, 0, 5, 10, or 25 μg of protein extracted from each sample of interest was added to 0.2 pmol of a double-stranded DNA probe containing an MG lesion in an internal PvuII restriction enzyme recognition sequence, and end-labeled with a fluorescent HEX dye. After incubation at 37°C for 2 hours, the samples were sequentially extracted with phenol and phenol/chloroform, after which the DNA was precipitated and recovered by centrifugation. The fluorescent/labeled DNA was then resuspended in PvuII digestion buffer (50 mM Tris HCl [pH 7.4], 50 mM KCl, and 0.1 mM EDTA) along with 5 U PvuII, and incubated at 37°C for 2 hours. Five microliters of the digestion reaction mixture was then added to 2 μl of formamide, heated at 100°C for 2 minutes, and cooled on ice. The DNA was then analyzed using an automated DNA sequencer (ABI 3700; Applied Biosystems, Foster City, CA). The extent of cleavage of the labeled probe (and hence the extent of MG repair) was determined by comparing the amount of PvuII-cleaved product to the amount of cleaved plus uncleaved product. The limit of detection of the system was 1% cleavage, and the maximal cleavage in control SF767 cells (50 μg) was 52%. The extent of cleavage was linear over the range of 5 to 35 μg of SF767 protein extract.

Measurement of Viable Cells and Colony Formation Efficiency

The SF767 cells (105) were placed into 35-mm culture dishes 2 days before TMZ treatment. The cells were prepared as described earlier and incubated in media with or without BG for the period indicated. Viable cells were counted with a hemocytometer by using the trypan blue dye exclusion method.

Assessment of colony formation efficiency was performed as described by Sarkar, et al.19 The cells pretreated with BG and treated

Y. Hirose, et al.
Arrest of G₂–M provides limited protection from TMZ cytotoxicity

with TMZ were trypsinized, counted, and plated at a concentration of 100 cells/well onto six-well culture plates. These cultures were then incubated with or without BG as indicated, stained with methylene blue (Sigma Chemical Co.) 17 days after BG removal, and colonies larger than 50 cells were counted. Colonies of the cells treated with TMZ only were counted 14 days after the drug treatment.

**Single-Cell Gel Electrophoresis (Comet Assay)**

The DNA damage in the cells was measured using a comet assay kit (Trevigen, Gaithersburg, MD) according to the manufacturer’s protocol. The BG-pre-treated cells were treated with TMZ and incubated in media containing BG for the period indicated. After trypsinization, cells were washed with ice-cold PBS and suspended in a 0.5% (wt/vol) solution of low-temperature-melting agarose in PBS (pH 7.4) at 37°C and immediately pipetted onto Comet Slides (Trevigen). The agarose was allowed to set for 30 minutes at 4°C and the slides were incubated in a lysis solution (2.5 M NaCl, 100 mM EDTA [pH 10], 10 mM Tris base, 1% sodium lauryl sarcosinate, and 0.01% Triton X-100; provided by Trevigen) at 4°C for 30 minutes, and in alkali buffer (0.3 M NaOH and 0.001 M EDTA) for 30 minutes at room temperature in the dark. For the alkali comet assay, the slides were then subjected to electrophoresis in alkali buffer at 1 V/cm for 30 minutes. For neutral comet assay, the alkali-treated slides were rinsed twice in Tris/borate EDTA for 5 minutes before electrophoresis in Tris/borate EDTA at 1 V/cm for 15 minutes. After electrophoresis, the slides were dried, stained using a Comet Slides silver-staining kit (Trevigen), and viewed using a bright-field microscope. The presence of a tail (comet) reflects DNA damage in the cell (comet cell). For each condition (alkaline and neutral), comet cells were counted in a total of more than 200 cells.

**Results**

*The TMZ-Induced G₂–M Cell Cycle Arrest in SF767 Glioma Cells Depleted of MGMT by BG*

Cell cycle analysis was first performed using MGMT-proficient SF767 cells treated only with TMZ. Unsynchronized cells were treated with TMZ (100 μM for 3 hours) and incubated for 1 to 4 days without it. The drug concentration used was based on our previous study of MGMT-deficient U87MG cells and the published data on the concentration used was based on our previous study of MGMT-arrest.

Because MGMT-depleted SF767 cells showed prolonged G₂–M arrest after TMZ exposure, we examined

**F I G . 1.** Histograms showing results of cell cycle analysis of SF767 cultures treated with TMZ and/or BG. A: Histograms showing results of FACS analysis of SF767 cells treated with either TMZ or BG alone. Unsynchronized cells were treated with TMZ (100 μM for 3 hours) or BG (20 μM for 2 hours, followed by 5 μM replenished every 24 hours) and harvested at the indicated times. B: Histograms showing results of FACS analysis of MGMT-depleted SF767 cells treated with TMZ. Unsynchronized cells were pretreated with BG (20 μM for 2 hours), treated with TMZ (100 μM for 3 hours), and continuously posttreated with BG (5 μM, replenished every 24 hours), and harvested at the indicated time points. Data shown are representative of three experiments.

The G₂–M arrest induced by TMZ in MGMT-depleted SF767 cells was also associated with a variety of other TMZ-induced effects. Exposure of MGMT-depleted cells to TMZ led to phosphorylation of cdc2 (Fig. 2), the G₂ checkpoint protein that in its unphosphorylated form complexes with cyclin B1 and stimulates progression into mitosis. Phosphorylation of cdc2 was noted within 1 day of TMZ treatment and persisted for at least 9 days after the agent’s removal. The TMZ exposure also resulted in induction of p53 and p21 at 2 and 3 days posttreatment, respectively (Fig. 2), with induction sustained for up to 10 days after TMZ exposure.

**Effects of MGMT Repletion on TMZ-Induced G₂–M Arrest and Cell Cycle Regulatory Proteins**

Because MGMT-depleted SF767 cells showed prolonged G₂–M arrest after TMZ exposure, we examined
whether repletion of MGMT during the period of G2–M arrest could reverse the cellular response to TMZ. For these studies, MGMT-depleted cells were treated with TMZ and returned to BG-containing media. Two to 5 days later the cells were washed with culture media to remove BG and allow MGMT repletion. Analysis of MGMT activity by using a sensitive restriction enzyme assay showed that BG pretreatment alone (20 μM for 2 hours) completely depleted MGMT activity in SF767 cells and that continuous BG exposure (5 μM, replenished every 24 hours) prevented recovery of the enzyme activity for up to 10 days (Table 1). In cells exposed only to BG, the MGMT activity rapidly recovered to near control levels within 1 day of the agent’s removal, even after 5 days of BG exposure, indicating that washing cells was sufficient to remove the agent and allow MGMT repletion. Removal of BG from the media of BG/TMZ–treated cells similarly allowed for detectable repletion of MGMT levels within 1 day of the removal (Table 1), regardless of how long the cells had been kept in BG-containing media, although the extent of repletion in TMZ-treated cells never exceeded 20%. This level of MGMT repletion, however, was associated in all cases with resolution of G2–M arrest, because in all cell groups that were washed free of BG and that contained detectable levels of MGMT, G2–M arrest resolved within 2 days of BG removal and MGMT repletion (Fig. 3). Whereas repletion of MGMT allowed for resolution of G2–M arrest, regardless of how long cells had been in the arrested state, the effects of MGMT repletion on cell cycle regulatory proteins and TMZ-induced effects on DNA integrity were more variable. As with G2–M arrest, removal of BG (and repletion of MGMT) 2 or 5 days after TMZ treatment allowed for a reduction of phospho-cdc2 levels to nearly those noted in control cells (Fig. 4). The TMZ-induced increases in p21 levels were reversible if BG was removed 2 days after TMZ exposure, but were irreversible if the agent was removed 5 days after exposure. In contrast, accumulation of p53 did not appear to be reversible even if BG was removed as soon as 2 days after TMZ exposure.

Cell Viability and Colony Formation Efficiency

To address the fate of TMZ-treated cells in which G2–M arrest had been reversed by MGMT repletion, we assessed the number of viable cells after TMZ drug treatment by using the trypan blue dye exclusion method. In the presence of BG, TMZ-treated cells increased in number for 2 days, and then started to decrease after 3 days.
and then the number of viable cells gradually decreased (Fig. 5A). If BG was removed from the culture media 2 days after TMZ treatment, the cells remained viable and proliferative. If BG was removed 5 days after TMZ treatment, however, cells remained viable but relatively constant in number. Assessment of clonogenicity with a colony formation efficiency assay showed that the colony-forming ability of cells pretreated with BG, exposed to TMZ, re-exposed to BG for 2 days, and then allowed to replenish MGMT in BG-free media was not significantly different from that of untreated or TMZ-only treated cells (Fig. 5B). Keeping cells depleted of MGMT for even 1 day longer before removing BG and allowing repletion of MGMT, however, resulted in a dramatic loss of colony-forming ability. These data indicate that although SF767 glioma cells undergo a prolonged and reversible G2–M arrest after TMZ exposure, a series of events leads to irreversible cell damage 2 to 3 days after drug exposure.

Events Associated With Loss of Clonogenicity After TMZ Exposure

To address the events associated with irreversible loss of clonogenicity after TMZ exposure, we monitored DNA damage in TMZ+BG–treated cells with a comet assay. The presence of tails in nuclei electrophoresed under alkaline conditions reflects DNA with abasic sites, single-strand breaks, and double-strand breaks, whereas the presence of tails in nuclei electrophoresed under neutral conditions mostly reflects double-strand breaks.23 The TMZ treatment resulted in the creation of DNA damage that was detectable under alkaline conditions by comet assay (Fig. 6A). Damage occurred in nearly every cell within 1 day of TMZ exposure, but was not apparent in TMZ-treated MGMT-proficient cells (data not shown). The percentage of nuclei containing damaged DNA, however, decreased in response to removal of BG from media 2 or 3 days after TMZ exposure (Fig. 6A). Exposure of MGMT-depleted cells to TMZ also resulted in the appearance of DNA damage that was detectable under neutral conditions by comet assay. This damage, which is typically associated with DNA double-strand breaks, however, began 2 to 3 days after TMZ+BG exposure, was sustained throughout the 5 days of analysis, which was consistent with the onset of TMZ-induced cytotoxicity, and could not be reversed by BG removal (Fig. 6B). The creation of DNA double-strand breaks but not single-strand breaks therefore appeared to be associated with the onset of irreversible cytotoxicity in TMZ+BG–treated cells.

Discussion

Although considerable effort has gone into the creation of novel therapies for brain tumors, alkylating agents remain the mainstay of glioma therapy. Of the alkylating agents, TMZ, an orally available DNA-methylating agent, shows promise and is likely to have widespread clinical use. Knowledge of the mechanism of action of TMZ has led to the recognition that the DNA repair protein MGMT inhibits the effectiveness of the drug by limiting the formation of MG, a key cytotoxic adduct. Fortunately, effective inhibitors of MGMT, such as the nontoxic analog BG, are avail-

Fig. 5. Graphs showing cell viability and colony formation efficiency of TMZ-treated SF767 cells with MGMT depletion for various periods. A: Graph showing changes in number of viable cells after TMZ treatment measured by the trypan blue dye exclusion method. Cells were pretreated with BG (20 μM for 2 hours), treated with TMZ (100 μM for 3 hours), and posttreated with BG (5 μM, replenished every 24 hours) for 2 (BG-2d) or 5 (BG-5d) days or continuously until harvest. B: Bar graph showing results of colony formation efficiency assay. Cells were pretreated with BG and then treated with TMZ as described, and posttreated with BG for the indicated period. These cultures were stained with methylene blue 17 days after BG removal, and colonies of more than 50 cells were counted. Colonies of the cells treated only with TMZ were counted 14 days after the drug treatment. UT = untreated.

Fig. 4. Immunoblot analysis of the protein levels of p53, p21, and phospho-cdc2 in BG-pretreated SF767 cells that were then treated with TMZ, followed by BG posttreatment for various periods. Cells were treated as described in the legend to Fig. 3. The actin level was used as a standard for each sample. Data are representative of three experiments.
cates that G2–M arrest is likely to be related to the creation of MG lesions and the onset of futile MMR in the unsynchronized cell population used in our study. Similarly, because the generation of DNA damage detectable by using the alkali comet assay was only noted in TMZ-treated cells depleted of MGMT, this action of TMZ also appears to be a response to MG adducts and/or their processing. Although cdc2 phosphorylation, alkali-labile DNA damage, and G2–M arrest appear to be consistent responses of glioma cells to TMZ, these events do not in and of themselves seem to be cytotoxic, because they all are readily reversible on repletion of MGMT. The events that ultimately trigger TMZ-induced cytotoxicity therefore appear to lie downstream of TMZ-induced G2–M arrest.

After induction of cdc2 phosphorylation, concomitantly with G2–M arrest, TMZ exposure induced expression of p53. Induction of p53 in response to TMZ again appeared to be dependent on depletion or absence of MGMT, because no induction was noted in TMZ-treated SF767 cells not exposed to BG. As with G2–M arrest, however, induction of p53 did not appear in and of itself to drive the cytotoxic actions of TMZ, because p53 levels remained elevated even in cells in which TMZ-induced cytotoxicity was averted by repletion of MGMT. Therefore, although it has been shown that p53 can influence the duration of TMZ-induced G2–M arrest and ultimately the fate of TMZ-treated cells,17 induction of p53 in response to TMZ does not appear to ensure cell death.

Exposure to TMZ also induced expression of p21, although this was a later event, occurring at approximately 3 days after TMZ exposure. The effects of TMZ on p21 are interesting; TMZ-induced increases in p21 levels and its cytotoxicity could be reversed if MGMT was replenished within 2 days of exposure. The additional observation that neither p21 induction nor cytotoxicity could be averted after 5 days of TMZ exposure indicates that the actions of this agent may be tied to its ability to induce p21. Previous studies in p53-deficient cells, however, showed that even in the absence of p21 induction, TMZ-treated glioma cells underwent transient G2–M arrest and mitotic catastrophe, suggesting that the role of p21 in TMZ-induced cytotoxicity is dispensable.

A final event associated with the onset of TMZ-induced cytotoxicity is the creation of DNA double-strand breaks. The DNA double-strand breaks were not apparent in the first 2 days after TMZ exposure, that is, in the time period in which MGMT repletion could avert TMZ-induced cytotoxicity. The creation of DNA double-strand breaks at 2 to 3 days after TMZ exposure, however, mirrored the loss of clonogenicity in the cells. Furthermore, the irreversibility of the DNA double-strand breaks on MGMT repletion mirrored the irreversible loss of clonogenicity. Although a temporal link between TMZ-induced DNA single-strand breaks and toxicity exists, several questions remain. For instance, although DNA strand breakage appears to precede loss of viability, it might be possible that strand breakage is a consequence, not a cause, of cell death. Although DNA double-strand breaks could be caused by cells undergoing apoptosis, the subG1 fraction of the TMZ+BG–treated SF767 cells being used rarely exceeded 5%, indicating that apoptosis was not a common route of death in the cells examined. Glioma cells, however, have also been shown to respond to TMZ by undergoing mitotic catastrophe,18 an

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**Fig. 6.** Graphs showing DNA damage in SF767 cells treated with TMZ and BG. Cells were pretreated with BG (20 µM for 2 hours), treated with TMZ (100 µM for 3 hours), and posttreated with BG (5 µM, replenished every 24 hours) for 2 or 3 days or continuously until harvest. Collected cells were subjected to single-cell gel electrophoresis (comet assay) under alkaline (A) and neutral (B) conditions. For each condition, cells with a tail, namely comet cells, were counted in a total of more than 200 cells. Under alkaline conditions comet cells reflect DNA with abasic sites, single-strand breaks, and double-strand breaks; under neutral conditions comet cells reflect double-strand breaks.

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**TABLE 5.** Graph showing DNA damage in SF767 cells treated with TMZ and BG. Cells were pretreated with BG (20 µM for 2 hours), treated with TMZ (100 µM for 3 hours), and posttreated with BG (5 µM, replenished every 24 hours) for 2 or 3 days or continuously until harvest. Collected cells were subjected to single-cell gel electrophoresis (comet assay) under alkaline (A) and neutral (B) conditions. For each condition, cells with a tail, namely comet cells, were counted in a total of more than 200 cells. Under alkaline conditions comet cells reflect DNA with abasic sites, single-strand breaks, and double-strand breaks; under neutral conditions comet cells reflect double-strand breaks.

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**TABLE 6.** Graph showing DNA damage in SF767 cells treated with TMZ and BG. Cells were pretreated with BG (20 µM for 2 hours), treated with TMZ (100 µM for 3 hours), and posttreated with BG (5 µM, replenished every 24 hours) for 2 or 3 days or continuously until harvest. Collected cells were subjected to single-cell gel electrophoresis (comet assay) under alkaline (A) and neutral (B) conditions. For each condition, cells with a tail, namely comet cells, were counted in a total of more than 200 cells. Under alkaline conditions comet cells reflect DNA with abasic sites, single-strand breaks, and double-strand breaks; under neutral conditions comet cells reflect double-strand breaks.
event that could also lead to DNA double-strand breaks. In other studies, MGMT-negative U87MG cells treated with TMZ and the G2 checkpoint abrogator UCN-01 were shown to die of mitotic catastrophe, and 4,6-diamino-2-phenylindole-dihydrochloride staining of the cultures used in our study showed an accumulation of cells with abnormal nuclei characteristic of mitotic catastrophe (data not shown). Statistically significant numbers of cells with abnormal nuclei, however, were not apparent until 4 days after TMZ exposure (data not shown). Additionally, in cultures of TMZ + BG–treated cells, the presence of cells floating in the media was not apparent until 5 days after TMZ exposure (data not shown). The DNA damage noted on the comet assay, therefore, appeared to precede cell death and did not seem to be a consequence of activation of cell death pathways.

Another question concerns the fact that, because cells exposed to TMZ are arrested at the G2–M boundary for long periods of time beginning at 2 days after TMZ exposure, it is unclear how DNA double-strand breaks are generated in arrested cells. Nonetheless, our data indicate that a temporal pathway exists that leads from DNA single-strand breaks to G2–M arrest, induction of p53/p21, and ultimately DNA double-strand breaks and cell death. The pathway, however, is not continuous and is irreversible at or before the onset of G2–M arrest, but only for a brief period thereafter. Together, these data indicate that TMZ-induced early events (cdc2 phosphorylation, DNA single-strand breaks, and G2–M arrest) are reversible at any time by MGMT repletion, and that late events are irreversible once established (DNA double-strand breaks associated with marked induction of p21, and loss of clonogenicity related to mitotic catastrophe).

The clearer definition of the pathway leading from TMZ treatment to cell death provided in this study may have a direct impact on the potential use of TMZ clinically. As noted, glioma cells depleted of MGMT undergo a prolonged G2–M arrest in response to TMZ. Because the G2 checkpoint is thought to allow cells time to repair damage before mitosis, the possibility exists that prolonged G2–M arrest in response to TMZ allows cells time to avert TMZ-induced cytotoxicity. The data presented in our study support this notion, at least in principle. In cells with modest levels of MGMT, exposure to high concentrations of endogenous or exogenous methylating agents could deplete levels of MGMT to the point at which MG adducts could be created and could persist. Because MGMT is regenerated at a relatively slow rate (repletion after complete depletion takes ~ 24 hours), a period of G2–M arrest of approximately 1 day might allow cells to replenish MGMT levels to the extent that any adducts formed could be repaired before replication and mitosis. If MGMT levels were depleted beyond this time frame, however, other pathways might be activated that would lead to cell elimination. Such a system would therefore allow repair and recovery from damage induced by modest exposure to methylating agents, but would dictate cell elimination after more severe or prolonged exposure. This model, which is supported by our data, indicates that in addition to allowing the formation of more MG lesions, prolonged depletion of MGMT by BG allows the conversion of potentially repairable TMZ-induced lesions into irreversibly cytotoxic ones. Because this conversion takes very little time (< 1 day), MGMT depletion for the extended period of TMZ-induced G2–M arrest appears to be unnecessary for maximal sensitization of cells to TMZ.

Therapeutically, this finding has multiple implications. First, because the cytotoxic actions of TMZ are confined within a limited period of exposure to this agent, MGMT repletion may be less of a hindrance to alkylating agent therapy than supposed. Second, because the period of protection from TMZ-induced cytotoxicity afforded by G2–M arrest may vary between normal and tumor cells, it may be possible to find TMZ + BG dosing strategies that allow preferential protection of normal cells. Finally, because G2–M arrest has been shown in this study to provide a small window of time for averting TMZ toxicity, our results indicate the possibility of alternate mechanisms of resistance to the cytotoxic actions of methylating agents. To date, resistance to these agents has been ascribed either to high levels of MGMT or lack of MMR. Although clinical trials of TMZ for gliomas in adults showed that many tumors were or became resistant to this agent, previous studies have revealed that very few gliomas in adults have either excessively high levels of MGMT or are MMR deficient. The mechanism of resistance that we posit based on results in the present study, namely loss of factors that link MMR to formation of double-strand breaks, may therefore be important in gliomas and may warrant further examination.

Conclusions

Our results showed that the prolonged period of G2–M arrest in SF767 glioma cells after TMZ exposure provides only a brief period in which the cytotoxic actions of TMZ can be averted. Because the conversion of a DNA lesion from repairable to lethal damage takes very little time, MGMT depletion for the extended period of TMZ-induced G2–M arrest appears unnecessary for maximal sensitization of cells to TMZ, and this finding has multiple implications in BG + TMZ–based chemotherapeutic regimens for gliomas. Further studies will be needed to determine if this is unique to SF767 cells, or if it also applies to other glioma cells.

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