Abrogation of the Chk1-mediated G2 Checkpoint Pathway Potentiates Temozolomide-induced Toxicity in a p53-independent Manner in Human Glioblastoma Cells

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ABSTRACT

Temozolomide (TMZ) produces O6-methylguanine in DNA, which in turn mispairs with thymine, triggering futile DNA mismatch repair (MMR) and ultimately cell death. We found previously that in p53-proficient human glioma cells, TMZ-induced futile DNA MMR resulted not in apoptosis but rather in prolonged, p53- and p21-associated G2-M arrest and senescence. Additionally, p53-deficient cells were relatively more TMZ resistant than p53-deficient glioma cells, which underwent only transient G2-M arrest before death by mitotic catastrophe. These results suggested that prolonged G2-M arrest might protect cells from TMZ-induced cytotoxicity. In the present study, we therefore focused on the mechanism by which TMZ induces G2-M arrest and on whether inhibition of such G2-M arrest might sensitize glioma cells to TMZ-induced toxicity. U87MG glioma cells treated with TMZ underwent G2-M arrest associated with Chk1 activation and phosphorylation of both cdc25C and cdc2. These TMZ-induced effects were inhibited by the Chk1 kinase inhibitor UCN-01. Although not in itself toxic, UCN-01 increased the cytotoxicity of TMZ 5-fold, primarily by inhibiting cellular senescence and increasing the percentage of cells bypassing G2-M arrest and undergoing mitotic catastrophe. In addition to enhancing TMZ-induced cytotoxicity in p53-proficient cells, UCN-01 also blocked TMZ-induced Chk1 activation and transient G2-M arrest in p53-deficient U87MG-E6 cells and similarly enhanced TMZ-induced mitotic catastrophe and cell death. Taken together, these results indicate that Chk1 links TMZ-induced MMR to G2-M arrest. Furthermore, inhibition of the cytoprotective G2-M arrest pathway sensitizes cells to TMZ-induced cytotoxicity and may represent a novel, mechanism-based means of increasing TMZ efficacy in both p53 wild-type and p53 mutant glioma cells.

INTRODUCTION

Gliomas are the most common brain tumor, and grade IV glioblastomas are almost universally fatal (1, 2). Treatment of glioblastomas typically involves surgical resection in combination with radiation and alkylating agent-based adjuvant chemotherapy. However, even in instances where complete surgical resection of these tumors is possible, the tumor generally recurs within a year regardless of the initial response to treatment (3). A new alkylating agent, TMZ,3 has recently been introduced into Phase II and III trials for treatment of recurrent high-grade gliomas and has been shown to yield objective response or stable disease in 50–60% of glioblastomas (4–7). Furthermore, TMZ is generally well tolerated and, unlike other alkylating agents, easily penetrates the blood-brain barrier. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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3 The abbreviations used are: TMZ, temozolomide; O6-meG, O6-methylguanine; MMR, mismatch repair; wt, wild-type; MGMT, O6-methylguanine-DNA methyltransferase; FACS, fluorescence-activated cell-sorting; SA-β-gal, senescence-associated β-galactosidase; DAPI, 4′,6-diamidino-2-phenylindole.

The cytotoxicity of TMZ appears to be mediated mainly through the creation of O6-meG in genomic DNA (10, 11). In the absence of MGMT, a repair enzyme that can remove methyl groups from the O6 position of guanine (12, 13), unpaired TMZ-induced O6-meG mispairs with thymine rather than cytosine during replication (14). The resultant guanine/thymine mismatches in DNA trigger the DNA MMR system. The DNA MMR system, however, consistently removes a portion of the DNA strand containing the mismatched thymine, only to reinsert thymine opposite the unpaired O6-meG during repair DNA synthesis. As a result, TMZ-induced unpaired O6-meG lesions are believed to lead to futile cycles of DNA MMR.

Futile cycles of MMR triggered by TMZ-induced guanine/thymine mismatches can in turn lead to a variety of outcomes in TMZ-treated cells (15). Whereas TMZ has been shown to induce apoptosis in hematopoietic cells (16, 17), the results of our previous studies indicate that the most prominent effect of TMZ in gliomas is G2-M arrest (18). TMZ-induced G2-M arrest in glioma cells is initiated in a p53-independent manner and is either sustained and associated with senescence in p53-wt glioma cells or bypassed and associated with mitotic catastrophe in isogenic p53-deficient cells. Whereas both p53-wt and p53-deficient cells initiate G2-M arrest and become nonproliferative in response to TMZ, p53-wt cells that undergo prolonged G2-M arrest are less sensitive than p53-deficient cells to the cytotoxic actions of TMZ (18). One possible explanation for this effect is that the prolonged G2-M arrest noted in p53-wt cells allows more time for reversal of the cytotoxic effects of the drug before entry into mitosis and death by mitotic catastrophe. G2-M arrest in response to TMZ may therefore represent a defense mechanism against the cytotoxic actions of TMZ and other methylating agents.

Whereas TMZ-induced G2-M arrest may protect cells from TMZ-induced cytotoxicity, the linkage between TMZ exposure and the activation of the G2 checkpoint remains unclear. Because MMR-induced G2-M arrest (and cytotoxicity) is only noted in MMR-proficient cells, TMZ-induced G2-M arrest has been suggested to result from DNA damage associated with futile MMR of TMZ-induced lesions (19–21). Whereas the linkage between TMZ-induced DNA damage and G2-M arrest has not been explored, the linkage between irradiation-induced DNA damage and G2-M arrest has been shown to involve a pathway controlling the cyclin-dependent kinase cdc2. Studies have shown that DNA damage induced by irradiation, topoisomerase inhibitors, or hydroxyurea activates Chk1 and Chk2 (22–27). Chk1 kinase phosphorylates cdc25C phosphatase at serine 216, which enhances the binding of 14-3-3 proteins and the export of the cdc25C/14-3-3 complex to the cytoplasm (28). The cytoplasmic sequestration of phosphorylated cdc25C in turn eliminates the potential cdc25C-mediated dephosphorylation of cdc2 (29, 30). cdc2 therefore remains bound to cyclin B in an inactive, phosphorylated state, in which form the cdc2/cyclin B complex is sequestered into the cytoplasm by...
14-3-3σ (31). The end result of DNA damage-induced Chk1 activation therefore is the sequestration of phosphorylated cdc2 and its binding partner cyclin B in the cytoplasm and the arrest of cells with damaged DNA at the G2-M boundary (32). The ability of TMZ to induce guanine/thymine mismatches that can be processed by futile MMR into the equivalent of DNA damage suggests that like other DNA-damaging agents, TMZ might initiate G2-M arrest via a Chk1-dependent pathway.

If Chk1 activation is critical in activation of G2-M arrest in TMZ-treated cells, and if G2-M arrest provides the opportunity for cells to avoid TMZ-induced cytotoxicity, inhibition of cdc2-dependent G2 arrest should sensitize cells to TMZ. A variety of small molecule inhibitors have recently been developed, including the clinically approved staurosorpin drug compound UCN-01 (33–35). Whereas a relatively nonspecific protein kinase inhibitor at high concentrations (36), UCN-01 at lower concentrations has been shown to inhibit Chk1-mediated phosphorylation of cdc25C and cdc2 (37–39). UCN-01 has also been shown to sensitize cells to various DNA-damaging agents, although it is not clear whether this sensitization is related to effects on G2-M arrest (40–43). We therefore used the clinically approved UCN-01 as a pharmacological tool to assess the linkage between TMZ exposure and G2-M arrest, to determine whether G2-M arrest protects cells from TMZ-induced cytotoxicity, and to determine whether Chk1 inhibitors might represent a way to sensitize cells to TMZ.

MATERIALS AND METHODS

Cell Culture and Treatment. A pool population of U87MG cells expressing human papilloma virus E6 (U87MG-E6) was a generous gift from Dr. Daphne Haas-Kogan (Brain Tumor Research Center, University of California, San Francisco, CA). The human glioblastoma cell lines U87MG and U87MG-E6 were cultured in DME H-21 medium supplemented with 10% FCS (Life Technologies, Inc.) at 37°C in a 5% CO2 atmosphere. Cells were plated at least 2 days prior to the drug treatment.

TMZ and UCN-01 were supplied by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (Bethesda, MD), and was dissolved in DMSO (Sigma Chemical Co., St. Louis, MO). Unsynchronized cells were treated with 100 μM TMZ for 3 h, and the final DMSO concentration did not exceed 0.1% (v/v). After TMZ treatment, cells were washed gently and incubated in fresh media containing UCN-01 at 37°C. UCN-01 was left in the culture media for up to 10 days and, in select experiments, was washed out with drug-free media 4 days after TMZ treatment. The cells were harvested at subconfluence at various time points.

Western Blot Analyses. Preparation of protein samples and Western blot were performed as described previously (18). Forty μg of protein were subjected to SDS-PAGE and electroblotted onto an Immobilon-P membrane (Millipore, Bedford, MA). The membrane was blocked in 5% nonfat skim milk (Bio-Rad Laboratories, Hercules, CA) for 1 h in TBS-T (Tris-buffered saline with 0.1% Tween-20). Membranes were incubated with primary antibodies—an anti-human Chk1 (Cell Signaling Technology, Beverly, MA) or anti-pTyr-15-phosphorylated cdc2 (Cell Signaling Technology) for 1 h at 4°C. After washing, the membrane was incubated in secondary antibodies conjugated to horseradish peroxidase (Bio-Rad Laboratories) for 1 h at room temperature. Blots were developed using the chemiluminescence detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ). Densitometric measurement of immunoreactive bands was performed using AlphaEase 2000 (Alpha Innotech Corp., San Leandro, CA), and fold-induction of the protein level was determined using the level of actin as a standard.

Cell Cycle Studies. At each time point, cells attached to culture dishes were trypsinized and collected together with the cells floating in the media. Cells were then 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 per determination.

SA-β-gal Staining. SA-β-gal staining was performed as described by Dimiri et al. (44). 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 washed with PBS, and the percentages of SA-β-gal-positive (blue-stained) cells were determined after scoring 500 cells for each sample using a bright-field microscope.

DAPI Staining. After 4 days of incubation with UCN-01 followed by incubation in media with or without UCN-01 for 2 and 6 days (i.e., 6 and 10 days after TMZ treatment), the cells were fixed with 4% paraformaldehyde/PBS for 1 h at room temperature, rinsed with PBS, incubated with 0.5% Igepal CA-630/PBS for 1 h at room temperature, and stained with DAPI. Nuclei were visualized by fluorescence microscopy, and more than 100 nuclei were examined for each experiment.

Colony Formation Efficiency. Assessment of colony formation efficiency was performed as described previously (18). The cells treated with TMZ were trypsinized, counted, and plated at a concentration of 100 cells/well into wells of 6-well culture plates. These cultures were then incubated with UCN-01 at the concentration indicated for 4 days, washed with drug-free media, and allowed to form colonies. Seventeen days after TMZ exposure, cells were stained with methylene blue (Sigma Chemical Co.), and colonies of >50 cells were counted. From the data on colony formation efficiency, ID50 was determined as the TMZ concentration that reduced the number of colonies to 50% of control cells (cells without TMZ or UCN-01 treatment).

Statistical Analyses. Differences in SA-β-gal and percentage of abnormal nuclei between cells with and without UCN-01 treatment were analyzed statistically using paired t test.

RESULTS

TMZ Induces G2-M Cell Cycle Arrest in Glioblastoma Cells in Association with Activation of the G2 Checkpoint Pathway. To better define how TMZ induces G2-M arrest, we first analyzed alterations in levels of G2 checkpoint-associated proteins in TMZ-treated cells. Unsynchronized glioblastoma cells were treated with TMZ (100 μM for 3 h) and incubated for 1–10 days in the absence of TMZ. Drug concentrations used were based on the published data of the plasma concentration of TMZ in patients involved in clinical trials (8, 9) and on levels previously shown to induce G2-M arrest in U87MG and U87MG-E6 cells (18). Immunoblot analyses revealed that protein levels of Chk1 increased in a transient manner, rising at 1–2 days after TMZ exposure and returning to subcontrol levels by 10 days after TMZ exposure (Fig. 1A). Levels of the Chk1 substrate cdc25C (both phosphorylated and unphosphorylated forms) also increased in U87MG and U87MG-E6 cells in response to TMZ in a transient manner similar to that noted for Chk1, with induction in U87MG-E6 cells occurring in most cases slightly more rapidly than that in U87MG cells (Fig. 1, A and B). Whereas total levels of cdc2 were unchanged or only slightly increased after TMZ treatment (Fig. 1A), levels of phosphorylated cdc2 were transiently increased in both U87MG and U87MG-E6 cells (Fig. 1B) in a timeframe and manner similar to that noted for induction of Chk1 and phosphorylated cdc25C. Although it is unclear why the levels of Chk1, cdc25C, and phosphorylated cdc2 decreased to less than control values, these results do suggest that TMZ exposure is associated in a p53-independent manner with activation/phosphorylation of the known G2 checkpoint-regulatory proteins Chk1 kinase, cdc25C, and cdc2.
UCN-01 Inhibits TMZ-induced Phosphorylation of cdc25C and cdc2. To more directly test the association between TMZ and alterations in G₂ checkpoint proteins, U87MG and U87MG-E6 cells were treated with TMZ and then exposed to UCN-01 for 3 days immediately after TMZ removal. Immunoblot analyses of cells harvested 3 days after TMZ treatment revealed that UCN-01 at 10 nM reduced the level of phosphorylated cdc25C and cdc2 in U87MG cells and that the level of these proteins was reduced to control levels by UCN-01 at ≥25 nM (Fig. 2). In U87MG-E6 cells, 25 nM UCN-01 also inhibited accumulation of the phosphorylated form of these proteins, although this action was only complete when cells were treated with ≥50 nM UCN-01. Because the Chk1-selective effects of UCN-01 can be lost at higher concentrations, we used 25 nM UCN-01 in subsequent experiments.

UCN-01 Abrogates TMZ-induced G₂-M Arrest in Human Glioma Cells. To assess the effects of UCN-01 on TMZ-induced G₂-M arrest, unsynchronized U87MG and U87MG-E6 cells were treated with TMZ (100 μM, 3 h), after which cells were incubated for 4 days with 25 nM UCN-01 (UCN-01 was left in the culture media for 4 days because TMZ could promote phosphorylation of cdc2 for up to 4 days after drug treatment; Fig. 1B). Cells were then cultured in media with or without UCN-01 for an additional 6 days and harvested at various time points for FACS analysis. Numbers below the histograms represent the day after TMZ exposure on which the FACS analysis was performed. Histograms and cell cycle distributions shown are representative of three experiments.

Effects of UCN-01 on TMZ-induced p53 and p21 Levels and on cdc2 Phosphorylation. To examine the basis for TMZ-induced G₂-M arrest and the abrogation of this arrest by UCN-01, levels of G2 checkpoint proteins, U87MG and U87MG-E6 cells incubated with TMZ (100 μM, 3 h). A, immunoblot analysis of protein levels of Chk1, cdc25C, and cdc2. B, immunoblot analysis of Ser-216-phosphorylated cdc25C and Tyr-15-phosphorylated cdc2. Fold induction of protein level was based on densitometric measurements and is shown below each immunoreactive band. Actin level was used as a standard for each sample, and protein levels in untreated cells were defined as 1.0. Data are representative of three analyses.
p53, p21, and phosphorylated cdc2 were measured in TMZ-treated U87MG cells incubated with 25 nM UCN-01 for 4 days (i.e., UCN-01 was removed from culture media on the 5th day of TMZ treatment) and in those treated with 25 nM UCN-01 until cell harvest (i.e., without UCN-01 removal). In U87MG cells exposed to TMZ alone, levels of p53 were increased 3 days after TMZ exposure (far right lane, Fig. 4A) and remained high throughout the next 10 days (data not shown; Ref. 18). UCN-01 had no effect on this TMZ-induced p53 induction. UCN-01 did, however, delay the induction of p21 noted in TMZ-treated U87MG cells from 2–3 days (far right lane, Fig. 4A; Ref. 18) to 6–8 days (Fig. 4A), suggesting that UCN-01 affected some factor that was important in p53-dependent induction of p21. Consistent with the ability of UCN-01 to inhibit the initiation of G2-M arrest, levels of phosphorylated cdc2 were clearly reduced in TMZ-treated cells as long as UCN-01 was present in culture media, but they increased immediately (and transiently) after removal of UCN-01 (Fig. 4A). This transient increase in phosphorylated cdc2 after UCN-01 removal in TMZ-treated cells was consistent with the transient G2-M arrest noted in these cells after UCN-01 removal (Fig. 3) and occurred even in the presence of persistently high levels of p53 and p21 (Fig. 4A). These results suggest that by blocking cdc2 phosphorylation, UCN-01 blocked the ability of U87MG cells to undergo TMZ-induced, p53-associated prolonged G2-M arrest. We also performed similar experiments using U87MG-E6 cells. Because neither p53 nor p21 accumulates in U87MG-E6 cells after TMZ exposure (18), UCN-01 posttreatment had no effect on p53 or p21 levels (data not shown). UCN-01 did, however, reduce the TMZ-induced increase in phosphorylated cdc2 by approximately 40% three days after TMZ treatment (compare the phosphorylated cdc2 level in cells exposed to TMZ + 25 nM UCN-01 with that in cells treated with TMZ only) and also reduced the duration of accumulation of phosphorylated cdc2 from 4 days (Fig. 1B) to 3 days (Fig. 4B). As in U87MG cells, removal of UCN-01 promoted a transient increase in phosphorylated cdc2 in U87MG-E6 cells in connection with G2-M arrest (Fig. 4B).

**UCN-01 Reduces TMZ-induced SA-β-gal Activity and Enhances Mitotic Catastrophe.** TMZ was previously shown to induce either a senescence-like state (in p53-wt U87MG cells) or mitotic catastrophe (in p53-deficient U87MG-E6 cells). To address the effects of reduced G2-M arrest on these TMZ-induced antiproliferative effects, cells were incubated with TMZ (100 μM, 3 h) or TMZ followed by UCN-01 (25 nM UCN-01, 4 days). Ten days after TMZ treatment, approximately 50% of U87MG cells not exposed to UCN-01 were positive for SA-β-gal activity. Post-TMZ exposure to UCN-01 significantly reduced this percentage of SA-β-gal-positive U87MG cells (Fig. 5). UCN-01 posttreatment had little or no effect on the percentage of TMZ-treated U87MG-E6 cells that were SA-β-gal positive (Fig. 5). Next we examined the nuclear morphology of cells treated with TMZ and UCN-01. Staining of TMZ/UCN-01-treated U87MG and U87MG-E6 cells with DAPI revealed the presence of fragmented or lobulated nuclei in association with micronuclei, characteristics consistent with mitotic catastrophe (Refs. 31, 45, and 46; Fig. 6A). Counting of such abnormal nuclei under a fluorescence microscope revealed that both 6 and 10 days after TMZ exposure, UCN-01 significantly increased the proportion of abnormal nuclei in both U87MG and U87MG-E6 cells (Fig. 6B). Inhibition of G2-M arrest therefore decreased TMZ-induced senescence in p53-wt U87MG cells but enhanced TMZ-induced mitotic catastrophe in both p53-proficient and p53-deficient U87MG-E6 cells.

**UCN-01 Increases TMZ-induced Cytotoxicity in Both p53-wt and p53-deficient Glioma Cells.** To assess the effects of UCN-01 on TMZ-induced loss of clonogenicity, we performed colony formation efficiency assays using TMZ- and TMZ/UCN-01-treated cells. Cells exposed to UCN-01 alone (25–100 nM, 4 days) had a colony formation efficiency no different from that of untreated cells (data not shown). In both U87MG and U87MG-E6 cells, however, UCN-01 potentiated TMZ-induced cytotoxicity in a dose-dependent manner (Fig. 7), reducing the TMZ ID50 approximately 2–5-fold in both cell groups across the UCN-01 exposure range of 25–100 nM (Fig. 7; Table 1).

**DISCUSSION**

We reported previously that G2-M arrest was the prominent response of human glioblastoma cells to TMZ-induced DNA mismatch
Whereas it is known that various types of DNA damage can induce G2-M arrest of mammalian cells through activation of Chk1 and the G2 checkpoint pathway (22–27), the means by which TMZ induces G2-M arrest have not been examined. In the present study, we found that TMZ-induced activation of MMR promoted induction of Chk1 and phosphorylation of the Chk1 kinase substrate cdc25C. TMZ-induced phosphorylation and inactivation of the cdc25C phosphatase were also temporally associated with phosphorylation of cdc2 and with the onset of TMZ-induced G2-M arrest. Furthermore, incubation of cells with nontoxic doses of the Chk1 inhibitor UCN-01 reversibly blocked TMZ-induced cdc25C and cdc2 phosphorylation and also significantly reduced TMZ-induced G2-M arrest. TMZ-induced activation of Chk1 in response to TMZ-induced futile MMR appeared to be a p53-independent process, occurring in both p53-wt and p53-deficient cells. Furthermore, inhibition of Chk1 by UCN-01 blocked cdc2 phosphorylation and initiation of G2-M arrest without blocking TMZ-induced p53/p21 induction in p53-wt cells. These results suggest that Chk1 activation and cdc2 phosphorylation link TMZ-induced MMR to the initiation of G2-M arrest.

Having established an association between TMZ exposure, Chk1 activation, and TMZ-induced G2-M arrest, we examined the hypothesis that G2-M arrest is a protective response of cells to TMZ-induced cytotoxicity and that elimination of the G2 checkpoint might sensitize cells (and in particular p53-wt cells) to TMZ. In p53-wt cells, which undergo a p53-associated prolonged G2-M arrest and senescence in response to TMZ, UCN-01 posttreatment greatly reduced the extent of G2-M arrest, reduced the percentage of cells undergoing TMZ-induced senescence, and increased the percentage of cells prematurely entering mitosis and undergoing mitotic catastrophe. Therefore, at concentrations that alone had little effect on cell viability, UCN-01 altered the response of cells and mediated a 5-fold decrease in TMZ ID50. These results clearly suggest that in p53-proficient glioma cells, the G2 checkpoint serves a protective function and that elimination of the checkpoint is associated with an increase in the number of cells that die by premature entry into mitosis. However, UCN-01 not only sensitized p53-wt cells but also sensitized p53-deficient cells, which underwent only a transient G2-M arrest in response to TMZ. This sensitization did not involve changes in TMZ-induced senescence.
CHEMOTHERAPEUTIC METHYLATING AGENTS SUCH AS TMZ WITH G2 CHECKPOINT SENSITIVITY. Whereas issues relating to duration of exposure, drug se-
tion of a Chk1 kinase inhibitor enhances the action of TMZ not only in p53-deficient cells but also in p53-deficient cells. Because approx-
ably two-thirds of gliomas have defects in the p53 pathway (50–
2), the ability of UCN-01 to sensitize cells regardless of p53 status also increases the range of tumors for which this approach might be effective. Whereas issues relating to duration of exposure, drug se-
quencing, and the events that link MMR to Chk1 activation remain to be examined, the present studies suggest that the combinations of chemotherapeutic mutagens as agents such as TMZ with G2 checkpoint inhibitors such as UCN-01 might be useful additions to existing therapies for brain tumors.

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