Temozolomide-Mediated Radiation Enhancement in Glioblastoma: A Report on Underlying Mechanisms

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Abstract

Purpose: In this study, we investigated the mechanisms by which temozolomide enhances radiation response in glioblastoma cells.

Experimental Design: Using a panel of four primary human glioblastoma cell lines with heterogeneous O^6-methylguanine-DNA methyltransferase (MGMT) protein expression, normal human astrocytes, and U87 xenografts, we investigated (*a*) the relationship of MGMT status with efficacy of temozolomide-based chemoradiation using a panel of *in vitro* and *in vivo* assays; (*b*) underlying mechanisms by which temozolomide enhances radiation effect in glioblastoma cells; and (*c*) strategies to overcome resistance to radiation + temozolomide.

Results: Temozolomide enhances radiation response most effectively in glioblastomas without detectable MGMT expression. On concurrent radiation + temozolomide administration in MGMT-negative glioblastomas, there seems to be decreased double-strand DNA (dsDNA) repair capacity and enhanced dsDNA damage compared either with radiation alone or with sequentially administered temozolomide. Our data suggest that O^6-benzylguanine can enhance the antitumor effects of concurrent radiation + temozolomide in MGMT-positive cells by enhancing apoptosis and the degree of dsDNA damage. O^6-Benzylguanine was most effective when administered concurrently with radiation + temozolomide and had less of an effect when administered with temozolomide in the absence of radiation or when administered sequentially with radiation. Our *in vivo* data using U87 xenografts confirmed our *in vitro* findings.

Conclusions: The present study shows that temozolomide enhances radiation response most effectively in MGMT-negative glioblastomas by increasing the degree of radiation-induced double-strand DNA damage. In MGMT-positive glioblastomas, depletion of MGMT by the addition of O^6-benzylguanine significantly enhances the antitumor effect of concurrent radiation + temozolomide. These are among the first data showing mechanisms of synergy between radiation and temozolomide and the effect of MGMT.

Glioblastomas are the most common malignant primary brain tumor in adults and are among the most aggressive of all human tumors. The standard of care, until recently, has involved maximum possible resection, followed by adjuvant irradiation (1, 2). More recent data suggest that combining temozolomide with radiotherapy followed by maintenance temozolomide improves outcome compared with radiation alone (3–5). A randomized phase III trial by the European Organization for Research and Treatment of Cancer/National Cancer Institute of Canada (EORTC 26981-22981/NCIC CE.3) confirmed these findings, and the combination of temozolomide + radiation is now considered the standard of care in the treatment of glioblastomas (3, 4).

Temozolomide, an alkylating agent, is a prodrug, which at a physiologic pH spontaneously converts to the active metabolite methyltriazeno-imidazole-arboxamide (MTIC), inducing methylation in multiple locations on DNA. The methylation of the O^6 position of GMP in DNA, although relatively infrequent (6-7%), is usually regarded as the lethality-causing lesion (6–10). Temozolomide is taken orally and is absorbed rapidly and completely after oral administration. Clinical and laboratory studies show that temozolomide crosses the blood-brain barrier. A significant proportion of the drug ($\geq30\%$) enters the cerebrospinal fluid rapidly, and repetitive daily dosing is feasible as the drug does not have a long half-life and therefore shows no significant accumulation with repeat dosing. Approximately 20% of the serum area under the curve can be detected in the cerebrospinal fluid (11). Hegi et al. (12) did a correlative analysis on specimens from EORTC 26981/NCIC CE.3 to

examine the association of O^6-methylguanine-DNA methyltransferase (*MGMT*) gene promoter methylation status with survival. Methyl adducts at the O^6 position on guanine in DNA are repaired by the cytoprotective DNA repair protein, MGMT, which transfers the methyl group to an internal cysteine acceptor residue. This reaction results in irreversible inactivation of MGMT, requiring increased *de novo* protein synthesis to restore repair activity. Restoration of MGMT activity is a relatively rapid event, usually occurring within several hours, both in peripheral blood cells (13) and in human malignant brain tumors (14). The effectiveness of temozolomide, therefore, would be assumed to have significant schedule dependency, with daily repetitive dosing schedules predicted to be most successful at depleting MGMT and thereby enhancing its cytotoxicity.

Hegi et al. reported that the effect on survival of combining radiation + temozolomide was most marked in glioblastoma patients whose tumors had *MGMT* promoter methylation. Glioblastoma patients with unmethylated *MGMT* promoters did not seem to derive a survival benefit from the addition of temozolomide to radiation. The 2-year survival rates for the four patient groups, unmethylated and methylated *MGMT* promoters treated with radiotherapy alone or radiotherapy plus temozolomide are 2%, 14%, 23%, and 46%, respectively. These data raise a number of critical questions. First, patients with methylated MGMT had superior survival compared with patients whose tumors were unmethylated. Does this imply that *MGMT* methylation is a prognostic marker, and that these patients will do better irrespective of therapy? Or, does this finding imply that *MGMT* methylation is a predictive marker for both therapies and therefore these tumors will respond better not only to temozolomide but also to radiotherapy? Second, does temozolomide actually enhances radiation response per se or is the observed benefit in the EORTC 26981/NCIC CE.3 study secondary to the independent effect of temozolomide? Third, if there is an additive or synergistic effect between radiation + temozolomide in *MGMT*-methylated glioblastomas, what are the underlying mechanisms behind this benefit? Fourth, does combined radiation + temozolomide result in increased toxicity in normal human astrocytes compared with radiation alone? Finally, are there effective strategies to overcome resistance mechanisms to radiation + temozolomide in *MGMT*-unmethylated cells? These questions are the subject of the present study.

Materials and Methods

Cell lines

The four cell lines used in this study (UN10-UN13) were established in culture from human glioblastoma tumors using previously described techniques (15) under an Institutional Review Board–approved protocol. Briefly, the glioblastoma tissues were obtained during open resection, mechanically dissociated, and the dispersed cells and fragments were cultured and passaged. All four glioblastoma tumor cell lines had undetectable levels of p16 and phosphatase and tensin homologue, with presence of wild-type p53, by Western blot analysis. Although all had low levels of detectable epidermal growth factor receptor (EGFR) expression, none expressed EGFR variant III, implying that the EGFR pathway was not up-regulated. Therefore, the tumor cell lines all could be deduced to have no significant up-regulation of signaling through EGFR, constitutive up-regulation of signaling through

phosphatidylinositol 3-kinase (because of the absence of phosphatase and tensin homologue), and the presence of wild-type p53. The normal human astrocytic cell line used in this study had detectable levels of p16, p53, and phosphatase and tensin homologue and undetectable levels of EGFR expression.

Clonogenic survival assays

Clonogenic survival assays were done as previously described (16 – 18). Treatment of cells included either temozolomide in doses of 100 μmol/L administered 2 hours before and during radiation (concurrent arm) or temozolomide in doses of 100 μmol/L administered 6 hours after radiation exposure (adjuvant arm) and continued for a period of 24 hours. In brief, plating efficiencies of all four primary cell lines were initially determined. Survival fraction at each radiation dose level was calculated, with plating efficiency taken into consideration. Colonies were defined as >50 cells. Each set of experiments was done in duplicate.

MGMT Western blot analysis

Lysates were generated by placing cells in radioimmunoprecipitation assay lysis buffer. Bradford assays were done to determine total protein concentrations, which were normalized to 1 μg/μL for all samples. Samples were then prepared in sample buffer and heated to 95°C for 5 minutes. These samples were then run on 16% polyacrylamide gels. Protein lysates (15 μL) in sample buffer from each tissue were loaded within each well. Gels were run at constant current (40 mA) for 3 to 4 hours for maximum separation. Wet transfer was done for 4 hours at constant voltage (40 V) using polyvinylidene difluoride membrane presoaked in methanol. The membrane was blocked in 5% milk in 0.2% TBST. The membrane was then washed in 0.2% TBST × 3 for 15 minutes each. The membranes were then incubated overnight with primary antibodies directed at MGMT (Abcam, Cambridge, MA). Subsequently, the membranes were washed in 0.2% TBST × 3 for 15 minutes each. The membrane was then incubated with secondary antibody for 45 minutes. Chemiluminescent (Bio-Rad, Hercules, CA) detection was then used to detect expression of MGMT. Actin levels served as internal loading controls. Western blot analysis for phospho-H2ax (also known as γ-H2ax) was done in a similar manner using a primary antibody directed against the phospho-S139 site (Abcam), diluted 1:1,000 and incubated overnight. The remainder of the procedure is as described above.

Annexin V apoptosis assay

Cells were treated with either radiation alone (6 Gy) or radiation (6 Gy) in combination with temozolomide (100 μmol/L). After various time intervals after radiation, detection of membrane externalization of phosphatidylserine was done with Annexin V-FITC conjugate (Caltag, Burlingame, CA). Both adherent and floating cells were harvested at various intervals after treatment and resuspended in PBS solution. Propidium iodide was added to a final concentration of 1 μg/mL. This was analyzed using blue light excitation; green fluorescence of FITC was measured at 530 ± 20 nm and red fluorescence was measured at >600 nm.

Comet assay

The primary glioblastoma cell lines were treated with radiation (6 Gy) with or without temozolomide (100 μmol/L) and subsequently evaluated for extent of double-strand DNA (dsDNA) damage using the Comet (Single Cell Gel Electrophoresis) assay at various time points after radiation exposure (5 minutes-96 hours). DsDNA breaks, which are known to be lethal events leading to radiation cell kill (19), were evaluated using neutral lysis and electrophoresis. Single cells embedded in agarose were lysed to remove proteins and then underwent electrophoresis. Staining was done with ethidium bromide with quantitative image analysis of DNA image length and tail length, which has been found to be associated with the degree of dsDNA damage (19).

U87 xenograft experiments. U87 tumor cells (5×10^6) were injected into the flanks of nude mice and resulting tumors were allowed to reach 0.5 cm in size. It should be mentioned that these U87 cells were isogenic cells, expressing EGFR variant III. U87 xenografts were injected with temozolomide (3 mg/kg) alone or temozolomide (3 mg/kg) in combination with O^6-benzylguanine (3 mg/kg). The tumors were then irradiated to a dose of 25 Gy in a single fraction. Untreated tumor xenografts served as controls in these experiments. Tumor viability and minimum tumor volume measurements were indexed to these non-treated controls. Representative xenografts from each treatment arm were examined histopathologically to evaluate for microscopic evidence of cell death including necrosis and fibrosis. Representative xenografts were also flash-frozen and plated to evaluate percent viability using

trypan blue exclusion. The remaining xenografts from each treatment arm were measured on a daily basis to evaluate maximum reduction in tumor volume.

Statistical analysis. All statistical analyses were done using the GraphPad Prism software (San Diego, CA). Comparisons were done using the ANOVA model. When possible, experiments were done in duplicates or triplicates, with calculation of 95% confidence interval and *P* values in relevant comparisons.

Results

Temozolomide enhances radiation response in MGMT-methylated glioblastoma cells. Our panel of four primary glioblastoma cell
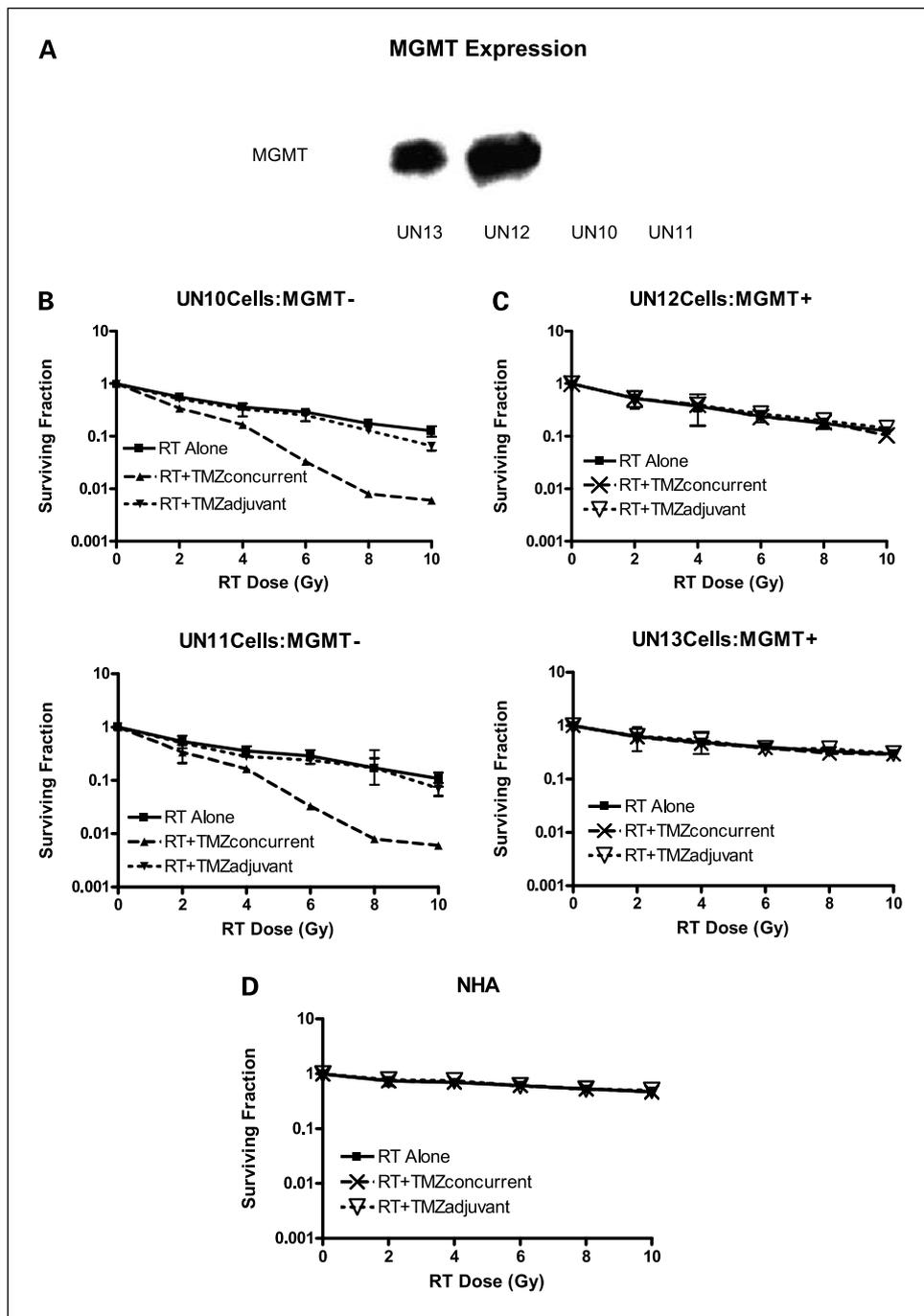

Fig. 1. Association of MGMT protein expression with response to radiation and temozolomide. *A,* Western blot analysis showing expression of MGMT in a panel of four primary glioblastoma cell lines. UN12 and UN13 lines have detectable levels of MGMT. MGMT expression levels in UN10 and UN11 cell lines are below detectable levels. *B,* MGMT-negative UN10 and UN11 cells show significantly decreased clonogenic survivals when treated with temozolomide administered concurrently with radiation (*TMZ + RTconcurrent*) compared with adjuvantly administered temozolomide + radiation (*TMZ + RTadjuvant*) and radiation alone (*RT Alone*). *C* and *D,* MGMT-positive UN12 and UN13 cell lines, as well as normal human astrocytes (*NHA*), respectively, show no differences in clonogenic survival with temozolomide either when administered concurrently with radiation or adjuvantly with radiation, versus radiation alone.

Fig. 2. Association of MGMT protein expression with treatment-induced apoptosis. *A*, MGMT-negative UN10 cells have a bimodal apoptotic peak, occurring 4 and 48 hours after radiation when temozolomide is concurrently administered. This effect is not seen with radiation alone and is not enhanced by coadministration of O^6-benzylguanine. *B*, MGMT-positive UN12 cells have a single apoptotic peak after radiation or after radiation + temozolomide administration. Coadministration of O^6-benzylguanine with radiation + temozolomide results in a bimodal apoptotic peak, as seen in the MGMT-negative cells. *C*, concurrent administration of temozolomide most significantly enhances radiation-mediated apoptosis compared with adjuvantly administered temozolomide for MGMT-negative UN10 cells. Administration of O^6-benzylguanine does not seem to have any additional effects. *D*, same findings as described in (*C*) for MGMT-negative UN11 cells. *E*, no enhancement of radiation-mediated apoptosis in MGMT-positive UN12 cells with either concurrently or adjuvantly administered temozolomide. Coadministration of O^6-benzylguanine enhances peak apoptosis values of temozolomide especially when concurrently administered, versus adjuvantly administered, with radiation in UN12 cells. *F* and *G*, same findings as in (*E*) for MGMT-positive UN13 cells as well as normal human astrocytes.
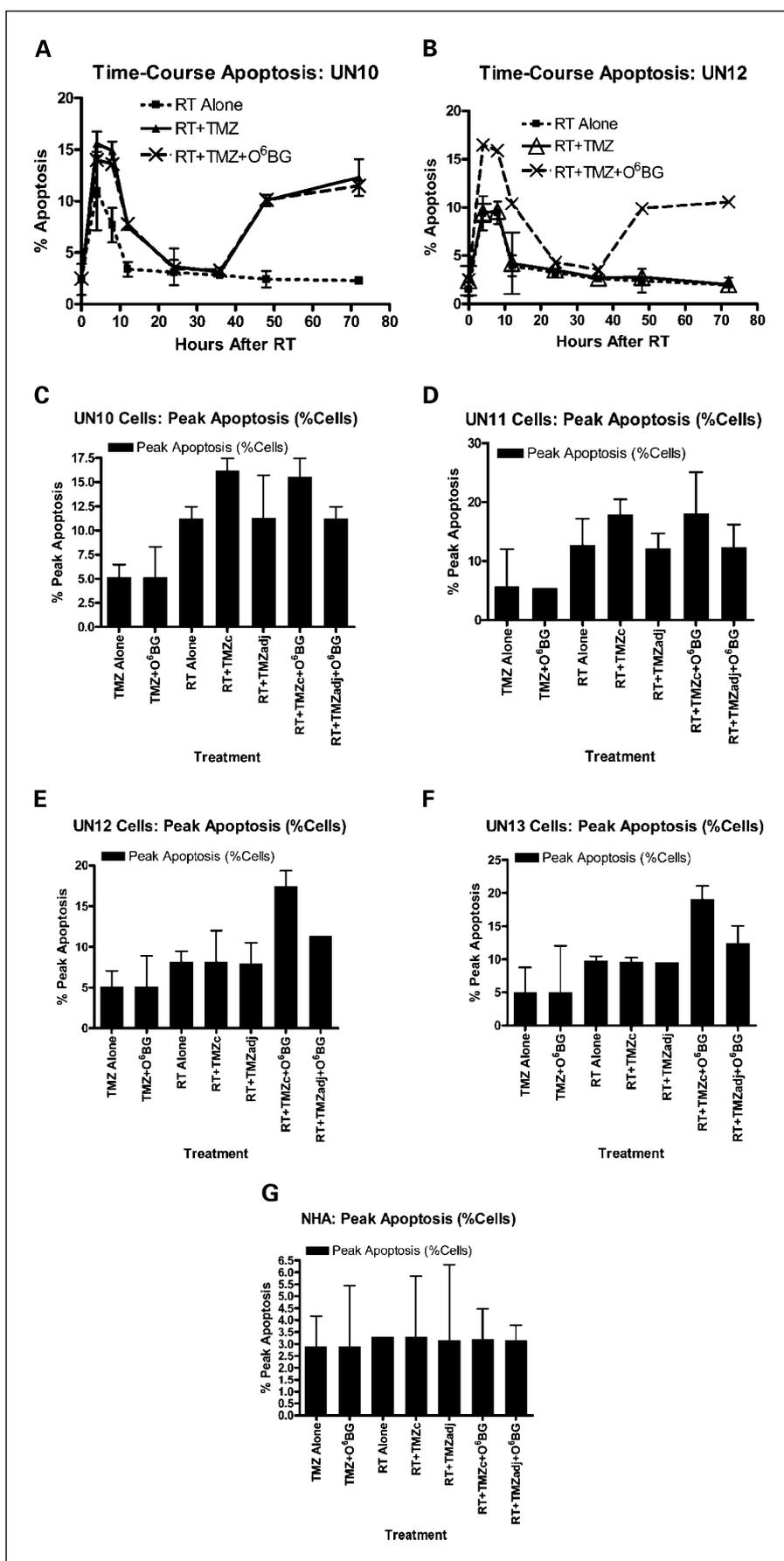
lines (UN10-UN13) differed in MGMT expression. UN10 and UN11 had undetectable MGMT protein expression levels. UN12 and UN13 both had high expression levels of MGMT protein (Fig. 1A). The clonogenic survival data suggest that for UN10 and UN11 cells, the addition of concurrent temozolomide to radiation significantly reduced clonogenic survival compared with radiation alone ($P < 0.001$; Fig. 1B). Sequentially administered temozolomide with radiation did not seem to have as pronounced effect on clonogenic survival for UN10 and UN11 cells (Fig. 1B). For UN12 and UN13 (MGMT-expressing) cell lines, there did not seem to be significant differences in clonogenic survival with the addition of temozolomide to radiation, either concurrently or sequentially ($P > 0.05$; Fig. 1C). Therefore, temozolomide seems to enhance radiation effect more prominently in MGMT-deficient cell lines versus MGMT-expressing cell lines, and further, this enhancement is most pronounced with temozolomide given concurrently with radiation; in fact, adjuvant temozolomide seems to have minimal effect on the surviving fraction, beyond the effect of radiotherapy alone. For normal human astrocytes, addition of temozolomide to radiation did not appreciably affect clonogenic survival, either when administered adjuvantly or concurrently ($P > 0.05$; Fig. 1D).

Temozolomide enhances radiation-induced apoptosis in MGMT-negative glioblastoma cells. We next examined the mechanisms by which temozolomide enhanced radiation effect in UN10 and UN11 cell lines. Time-course measurements of apoptosis were undertaken using the Annexin V assay. It was apparent that there was a bimodal apoptosis peak in both UN10 and UN11 cell lines after concurrent radiation + temozolomide administration (Fig. 2A). The first peak was within 4 hours of radiation administration and was seen in both the radiation + temozolomide and radiation alone arms, although somewhat more prominent in the former. There seemed to be a second wave of apoptosis that occurred beyond 36 hours of treatment, which was more pronounced in the UN10 and UN11 cells treated with radiation + temozolomide versus those treated with radiation alone ($P < 0.001$). In contrast, in MGMT-expressing UN12 and UN13 cells, there seemed to be only one early apoptotic peak in the radiation + temozolomide and radiation alone arms, respectively (Fig. 2B). There was an absence of a delayed apoptotic peak that was seen with the MGMT-negative cells. Furthermore, the differences in peak apoptosis levels in the radiation + temozolomide versus the radiation alone arm did not seem to be significant (Fig. 2E and F). Likewise, in normal human astrocytes, there did not seem to be a significant difference in peak apoptosis levels in radiation + temozolomide versus radiation alone treatment arms, again arguing against additive toxicity with combined radiation + temozolomide compared with radiation alone in normal brain cells (Fig. 2G).

Temozolomide enhances dsDNA damage in MGMT-negative cells. As the extent of dsDNA damage is thought to determine the degree of cell death after radiotherapy, we investigated whether adding temozolomide to radiation enhanced dsDNA breaks using the Comet assay done under neutral elution conditions. The length of the Comet tail of the migrating DNA is indicative of the degree of double-strand breaks after treatment. We found that in UN10 and UN11 cells there was a significantly enhanced degree of dsDNA damage when temozolomide was added to radiation compared with radiation alone ($P < 0.001$; Fig. 3A and B). In contrast, in UN12 and UN13 cells,

there was no apparent increase in dsDNA damage with coadministration of radiation + temozolomide compared with temozolomide alone (Fig. 3A and C). Likewise, in normal human astrocytes, there was no increase in the degree of dsDNA damage observed after combined radiation + temozolomide compared with radiation alone (Fig. 3D). Again, there is lack of evidence in normal human astrocytes that combined radiation + temozolomide results in enhanced cytotoxicity compared with radiation alone.

To examine the effects of combined radiation + temozolomide on the repair process itself, we investigated expression levels of phospho-H2ax, which is a histone that rapidly associates with the DNA repair machinery on exposure to radiation when phosphorylated, as determined by quantitative Western blot analysis. It became apparent that combined radiation + temozolomide treatments led to significantly decreased levels of p-H2ax, indicating a suppression of dsDNA break repair compared with the radiation alone treatment arm ($P < 0.001$; Fig. 3E). Therefore, the enhanced dsDNA breaks observed with the Comet assay in the radiation + temozolomide treatment arm may be secondary to either enhanced dsDNA breaks induced by the combined therapy or a reduction in dsDNA break repair capacity of glioblastoma cells (as evidenced by decreased p-H2ax levels), or a combination of both effects.

Administration of O^6-benzylguanine enhances response to radiation + temozolomide. O^6-Benzylguanine is a substrate analogue of MGMT, which has been reported to deplete MGMT (6–10). We investigated whether O^6-benzylguanine could enhance the effect of temozolomide, either when administered alone or in combination with radiation. For MGMT-negative cells, the addition of O^6-benzylguanine failed to further enhance sensitivity to radiation + temozolomide (beyond the enhancement already observed by adding temozolomide to radiation), as measured through the Annexin V apoptosis assay (Fig. 2A, C, and D). However, in MGMT-expressing glioblastoma cells, there seemed to be significant enhancement of treatment-induced apoptosis on coadministration of O^6-benzylguanine ($P < 0.001$; Fig. 2B, E, and F). Further, in MGMT-expressing UN12 and UN13 cells, increased dsDNA damage was observed after incorporation of O^6-benzylguanine with radiation + temozolomide compared with radiation + temozolomide (Fig. 3C). Again, this was not apparent in our MGMT-negative UN10 and UN11 cells (Fig. 3B).

In vivo confirmation of in vitro findings. We proceeded to investigate whether some of the *in vitro* observations above could be validated *in vivo* using our U87 xenograft model. Our U87 cells were found to express MGMT protein. We initially investigated whether the addition of temozolomide to radiation could enhance radiation effect. We found that addition of temozolomide to radiation did not appreciably enhance radiation effect *in vivo*, which confirmed the findings from our MGMT-expressing cell lines. Tumor viability was not significantly reduced in the radiation + temozolomide arm compared with the radiation alone arm *in vivo*, determined both histopathologically and by quantitative analysis (Fig. 4B). Likewise, tumor volume was not significantly reduced in the radiation + temozolomide arm compared with the radiation alone arm (Fig. 4C). DsDNA breaks were also found to be equivalent between the two treatment arms as determined by Comet assay (Fig. 4D).

We next investigated whether the addition of O^6-benzylguanine could enhance the effect of temozolomide, either given

Fig. 3. Lack of MGMT expression, as well as suppression of MGMT activity pharmacologically in MGMT-positive glioblastomas, is associated with increased dsDNA damage following treatment of primary glioblastoma cells with temozolomide concurrently administered with radiation. *A,* representative sample photographs showing the increase in comet tail length of MGMT-negative UN10 cells treated with temozolomide concurrently administered with radiation compared with radiation alone, as measured using the Comet assay. MGMT-positive UN12 cells do not show changes in comet tail length in temozolomide + radiation – treated cells compared with radiation alone. *B,* as measured using the Comet assay, MGMT-negative UN10 and UN11 cells show increased mean comet tail length when treated with temozolomide administered concurrently with radiation. Administration of O^6-benzylguanine had no effect on mean comet tail length. *C,* as measured using the Comet assay, MGMT-positive UN12 and UN13 cells show no differences in mean comet tail length when comparing temozolomide-, radiation-, or temozolomide + radiation – treated cells. Administration of O^6-benzylguanine in MGMT-positive UN12 and UN13 cell lines is associated with increased mean comet tail length when treated with temozolomide administered concurrently with radiation. Levels of mean comet tail length obtained after O^6-benzylguanine administration are similar to those seen in MGMT-negative UN10 and UN11 cells treated with temozolomide administered concurrently with radiation. *D,* neither temozolomide treatment, radiation, nor O^6-benzylguanine administration influenced mean comet tail length in normal human astrocytes. *E* and *F,* suppressed levels of the DNA damage repair marker pH2ax are observed in MGMT-negative UN10 and UN11 glioblastoma cells treated with temozolomide concurrently administered with radiation compared with treatment with radiation alone, indicative of decreased DNA repair capacity. MGMT-positive UN12 and UN13 cells show no effect of radiation + temozolomide on relative pH2ax levels and, hence, no effect on DNA repair capacity. *E,* quantitative data in this regard from Western blot analysis normalized to an internal actin loading control; *F,* representative Western blot from this analysis.
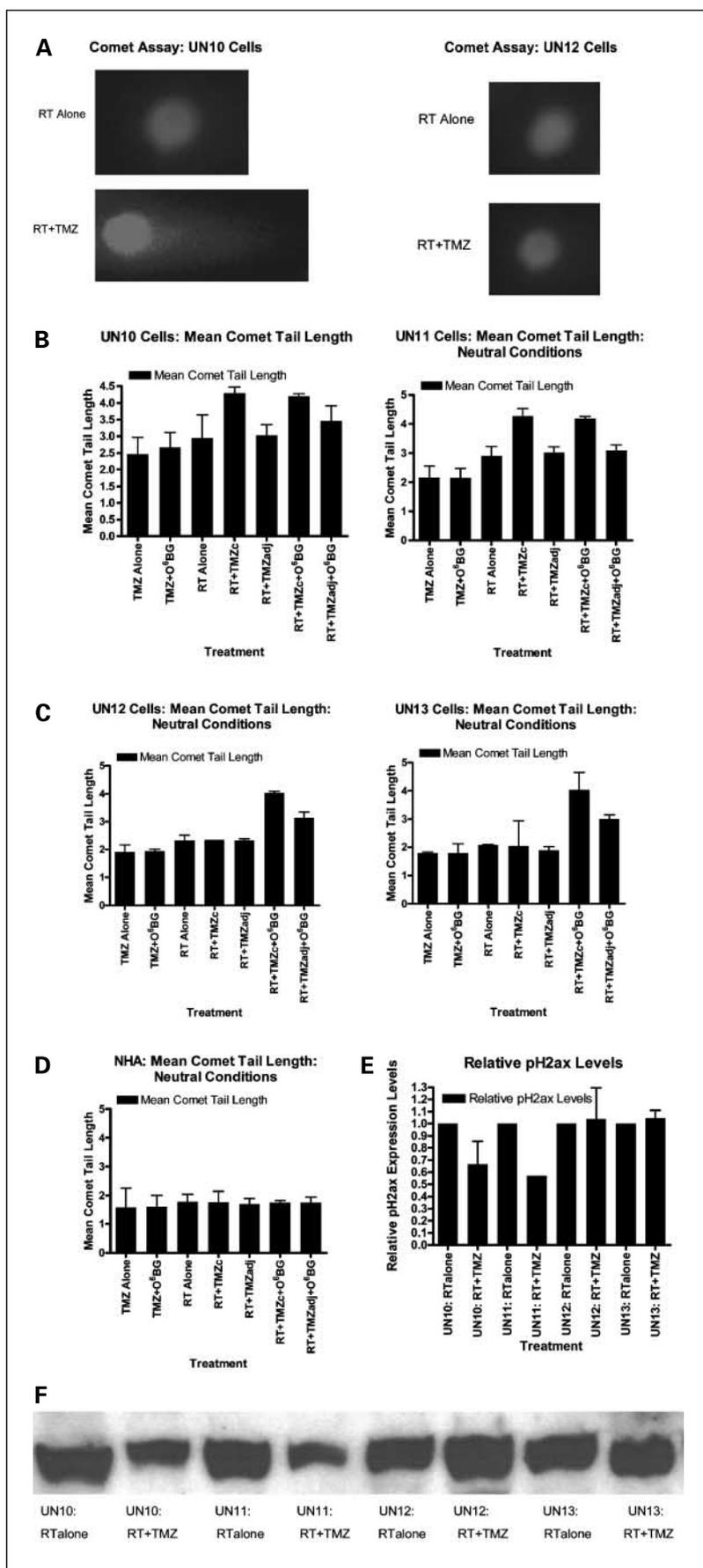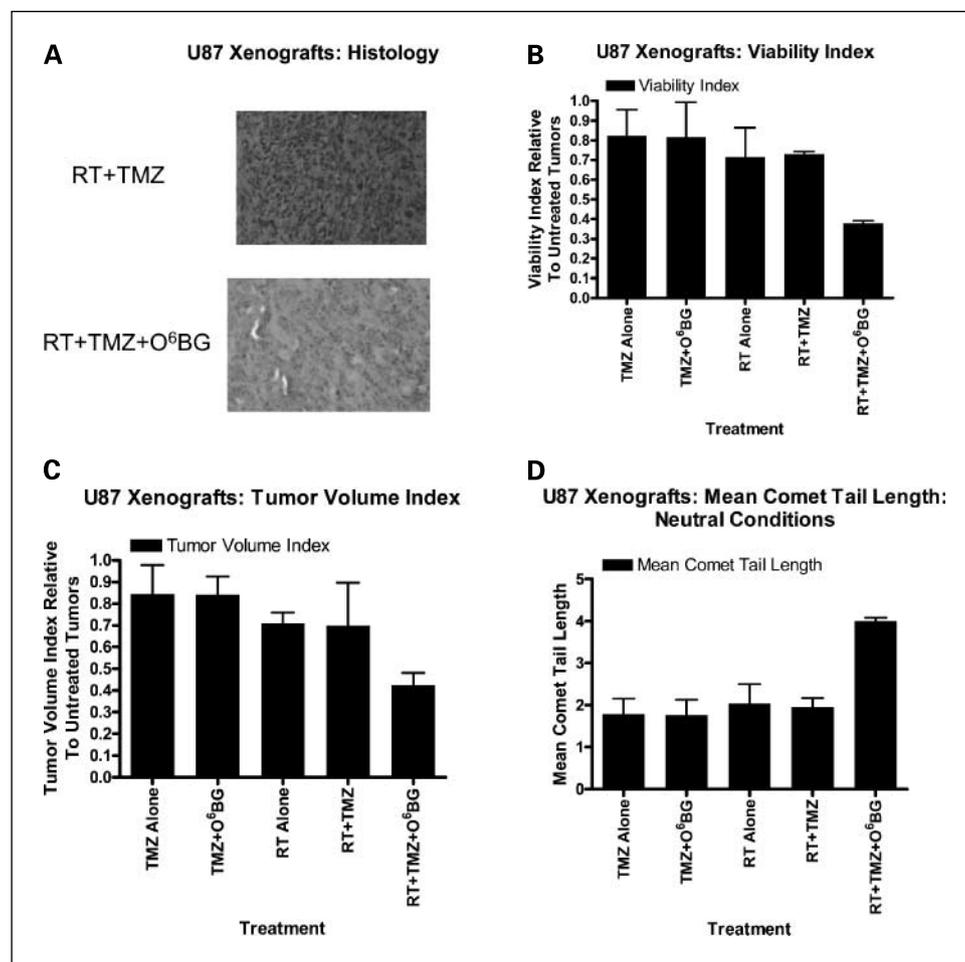
Fig. 4. When treated with MGMT inhibitor O^6-benzylguanine, MGMT-positive U87 tumor cell xenografts show decreased viability, tumor volume, and dsDNA damage *in vivo* when treated with temozolomide + radiation compared with temozolomide alone as well as the other treatment arms. *A*, representative U87 xenograft tumor sample sections treated with temozolomide + radiation + O^6-benzylguanine show fewer viable cells and increased fibrosis and necrosis compared with tumors treated with radiation + temozolomide (determined 2 weeks following treatment). *B*, U87 xenografts treated with temozolomide + radiation that also received O^6-benzylguanine show a significantly reduced viability index values compared with other treatment arms (determined 2 weeks following treatment). *C*, U87 xenografts treated with temozolomide + radiation that also received O^6-benzylguanine show significantly reduced tumor volume than do all other treatments (determined 2 weeks following treatment). *D*, cells taken from U87 xenografts treated with temozolomide + radiation that also received O^6-benzylguanine show a significantly increased mean comet tail length compared with all other treatments (determined 6 hours after treatment).

alone or in combination with radiation. It was determined that O^6-benzylguanine did not significantly enhance the effect of temozolomide administered alone (no radiation) with regards to either reducing tumor cell viability or overall tumor volume (Fig. 4B and C). However, when temozolomide was administered concurrently with radiation, O^6-benzylguanine significantly reduced both tumor cell viability and tumor volume compared with radiation alone ($P < 0.001$; Fig. 4B and C). We found that for temozolomide administered sequentially after radiation, there was no added effect of O^6-benzylguanine administration (data not shown). As the histologic sections illustrate, tumors treated with concurrent radiation + temozolomide + O^6-benzylguanine show larger areas of fibrosis and necrosis compared with concurrent radiation + temozolomide (Fig. 4A). Further, we found that there was a significantly greater degree of dsDNA damage in tumors treated with radiation + temozolomide + O^6-benzylguanine compared with radiation + temozolomide, also suggesting enhancement of radiation effect, in addition to the possible enhancement of temozolomide cytotoxicity($P < 0.001$; Fig. 4D).

Discussion

The results of EORTC 26981/NCIC CE.3 show that the addition of temozolomide to radiation followed by maintenance temozolomide significantly improves outcome for certain glioblastoma patients (4). *MGMT* methylation status seems to

identify a subset of glioblastoma patients who benefit from the addition of temozolomide to radiation (12). Hegi et al. reported that glioblastoma patients with tumors showing *MGMT* methylation derived the greatest survival advantage from the concurrent and sequential administration of temozolomide with radiation versus radiation alone. However, this relationship is not universally observed, in that only less than half the patients with *MGMT* methylation treated with temozolomide + radiation became long-term survivors, implying that about half the patients did not benefit. Conversely, a smaller proportion of patients (about a quarter) without *MGMT* methylation survived for ≥2 years with the same regimen (although theory would predict that these patients should not benefit from the treatment). Clearly, *MGMT* methylation status may be an important but not an exclusive factor.

The present study suggests that concurrently administered temozolomide seems to have significantly greater antitumor effect compared with sequential administration. The benefit is most pronounced in cells deficient in functional MGMT protein, and this benefit seems to occur from enhancement of the cytotoxic effects both of temozolomide and radiation. The mechanisms of action seem to involve an increased degree of radiation-induced apoptosis and enhanced dsDNA damage, which have been reported to be critical factors underlying radiation-induced cell death. Our data suggest that the observed increase in dsDNA damage may be secondary to either decreased repair capacity or increased degree of breaks on combined

temozolomide + radiation administration. The observed biphasic apoptotic peaks in the radiation + temozolomide–treated MGMT-negative cells suggest that there seems to be an early and a delayed pattern of cell death. The pattern of cell death in tumor cells treated with radiation alone is characterized primarily by this early phase of cell death. It is possible that with the additional degree of dsDNA damage observed in radiation + temozolomide–treated MGMT-negative glioblastoma cells, temozolomide acts as a catalyst in promoting this latter phase of cell death. Calvert et al. have shown in a colon cancer cell line that absence or inactivation of MGMT protein alone is insufficient for cytotoxicity; in fact, their research suggests that an intact mismatch repair pathway is necessary for this as do other reports (20, 21). In cells with an intact mismatch repair, the sustained alkylation on the O^6 position of guanine leads to futile DNA cycling, which ultimately causes cytotoxicity; this could be speculated to be the second delayed wave of apoptosis with combination temozolomide and radiation. More recent studies suggest that high doses of S_N1 type methylating agents such as temozolomide can activate DNA damage signaling cascades, which may be independent of mismatch repair (22). It is also revealing that when the opposite question is asked (i.e., whether radiation can enhance the sensitivity of temozolomide), a previous report suggests that sequential administration of these agents does not result in enhanced synergism (23), much like the current study. Therefore, the concurrent administration of these agents seems to result in optimum benefit versus sequential administration.

The question of whether drugs like temozolomide actually enhance the effects of radiation or cause additive cytotoxicity has been controversial. Temozolomide is known to act through methylation of N^7-guanine (70% of adducts), N^3-adenine (9%), and O^6-guanine (5% of adducts), respectively (6–10). Although accounting for a small percentage of methylation adducts, the formation of O^6-methylguanine has been reported to be especially cytotoxic. The mechanism of cytotoxic action of temozolomide is thought to be related to the failure of the DNA mismatch repair system to find a complementary base for the methylated guanine. This results in long-lived nicks in the DNA, which can accumulate, preventing progression through the cell cycle and ultimately leading to apoptotic cell death.

The results of the present study suggest that temozolomide enhances the therapeutic efficacy of radiation in glioblastoma cells when administered concurrently. Sequential administration of temozolomide after radiation seemed to have a more marginal benefit compared with radiation alone. Possible mechanisms by which temozolomide enhances radiation effect include enhancement of radiation-mediated dsDNA breaks and suppression of dsDNA repair pathways after radiation exposure. Time-course measurements of dsDNA breaks reveal that combined radiation + temozolomide results in enhanced double-strand breaks at each time point compared with radiation alone. Although temozolomide is not known to

cause the same extent of dsDNA breaks as radiation, several possible mechanisms may explain our findings. The first involves temozolomide producing single-strand breaks in close proximity to radiation-induced single-strand breaks on adjacent strands of DNA. Although radiation is known to produce lethality through induction of dsDNA breaks, the ratio of single to double-strand breaks after radiation approximates 2:1 (24). The primary mechanism by which radiation induces cell death is secondary to DNA damage produced by production of hydroxyl radicals (^POH), which damage DNA. The abstraction of a hydrogen atom from the deoxyribose moiety by an ^POH radical contributes to radiation-induced strand breaks. Should these radiation-induced breaks physically approximate those produced by temozolomide, this could be a possible mechanism of enhanced dsDNA breaks observed with combined modality therapy.

Alternatively, concurrent administration of temozolomide with radiation may inhibit repair of dsDNA damage. The observation that combined administration of temozolomide and radiation results in reduced levels of pH2ax despite a more extensive degree of dsDNA damage (as determined by Comet assay) suggests that there may be suppression of the dsDNA damage repair resulting from concurrent administration of radiation + temozolomide. Recent reports have suggested that histone variants have important functions beyond their well-characterized roles in cellular metabolism and maintenance of genomic stability. It has been found that the histone H2ax becomes rapidly phosphorylated on a highly conserved serine residue four amino acids from the COOH terminus on dsDNA damage (25). It is also known that pH2ax is present in focal aggregates at sites of dsDNA damage and complexes with other important repair molecules. pH2ax is required for foci formation for numerous factors including p53, NBS1, BRCA1, and MDC1. pH2ax has been reported to physically interact with p53, NBS1, and MDC1 (25). Therefore, the observation that combined temozolomide + radiation significantly reduces levels of pH2ax in MGMT-negative glioblastomas suggests that concurrently administered temozolomide may not only interfere with the generation of pH2ax but perhaps also with its ability to mediate dsDNA repair. It is curious that in MGMT-expressing tumors, concurrent temozolomide + radiation fails to significantly reduce pH2ax levels compared with radiation alone. However, on treatment with O^6-benzylguanine, concurrent temozolomide significantly reduces levels of pH2ax in MGMT-expressing glioblastomas. The ability of O^6-benzylguanine to penetrate the blood-brain barrier has not been well characterized, which may limit its therapeutic efficacy if there are severe limitations in this regard. The precise relationship between MGMT and H2ax phosphorylation status remains largely unknown. However, given the present observations, the answer to this question may be critical in identifying the underlying mechanisms by which temozolomide and other cytotoxic drugs enhance the efficacy of radiation in glioblastoma cells.

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