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## Minimally cytotoxic doses of temozolomide produce radiosensitization in human glioblastoma cells regardless of MGMT expression<sup>1</sup>

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### Abstract

Concurrent treatment with the methylating agent temozolomide (TMZ) during radiotherapy (RT) has yielded the first significant improvement in survival of adult glioblastomas (GBMs) in the last three decades. However, improved survival is observed in a minority of patients, most frequently those whose tumors display CpG methylation of the *MGMT* (*O*<sup>6</sup>-methylguanine-DNA methyltransferase) promoter, and adult GBMs remain invariably fatal. Some, though not all, pre-clinical studies have shown that TMZ can increase radiosensitivity in GBM cells that lack MGMT, the sole activity in human cells that removes *O*<sup>6</sup>-meG from DNA. Here, we systematically examined the TMZ dose dependence of radiation killing in established GBM cell lines that differ in ability to remove *O*<sup>6</sup>-meG or tolerate its lethality. Our results show that minimally cytotoxic doses of TMZ can produce dose-dependent radiosensitization in MGMT-deficient cells, MGMT-proficient cells, and MGMT-deficient cells that lack mismatch repair, a process that renders cells tolerant of the lethality of *O*<sup>6</sup>-meG. In cells that either possess or lack MGMT activity, radiosensitization requires exposure to TMZ before but not after radiation, and is accompanied by formation of double-strand breaks within 45 min of radiation. Moreover, suppressing alkyladenine-DNA glycosylase, the only activity in human cells that excises 3-meA from DNA, reduces the TMZ dose dependence of radiosensitization, indicating that radiosensitization is mediated by 3-meA as well as by *O*<sup>6</sup>-meG. These results provide novel information on which to base further mechanistic study of radiosensitization by TMZ in human GBM cells, and to develop strategies to improve the outcome of concurrent TMZ-RT.

### Keywords

Alkyladenine-DNA glycosylase; brain tumor; 3-methyladenine; *O*<sup>6</sup>-methylguanine

### Introduction

Adult glioblastomas (GBMs; WHO grade IV astrocytomas) are invariably fatal due, in part, to resistance to post-surgical therapy. Historically, median survival following surgery and adjuvant therapy has been 9 to 12 months (1). While the efficacy of adjuvant radiotherapy (RT)

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in prolonging survival has long been recognized, the benefit of alkylating agent-based chemotherapy has been equivocal (reviewed in 2). Recent clinical trials have demonstrated that median survival can be significantly prolonged and the 2-year survival rate significantly increased by administering the methylating agent temozolomide (TMZ) during RT, and continuing TMZ as a single agent after completion of RT (3). As a consequence, concurrent TMZ-RT is now the accepted standard of adjuvant care for newly diagnosed GBMs (4).

TMZ is an orally administered agent that readily crosses the blood-brain barrier and has relatively low toxicity (5). It undergoes spontaneous hydrolysis at physiological pH to form an active metabolite that produces approximately 12 base adducts in DNA (6,7), including the cytotoxic lesions O<sup>6</sup>-methylguanine (O<sup>6</sup>-meG) and 3-methyladenine (3-meA). Improved survival following concurrent TMZ-RT is more frequent in the 40–45% of GBMs that exhibit promoter methylation of the gene for O<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT) (8), indicative of epigenetic silencing of expression (9). MGMT is the sole activity that removes O<sup>6</sup>-meG from DNA (10), suggesting that unrepaired O<sup>6</sup>-meG promotes the efficacy of TMZ-RT. Yet, TMZ-RT is no more effective than RT alone in more than half of tumors that exhibit *MGMT* promoter methylation, while *ca.* 10–20% of GBMs lacking promoter methylation display superior outcome (3,8), suggesting that adducts in addition to O<sup>6</sup>-meG may affect treatment outcome.

While concurrent TMZ-RT has produced the first significant advance in the treatment of newly diagnosed GBMs in the last 30 years, more than half of patients derive no benefit from inclusion of TMZ, and the prognosis remains dismal, with only a minority of patients surviving 2 years (3). Thus, there is urgent need to understand the mechanisms responsible for the efficacy of concurrent TMZ-RT, in order to develop strategies to improve outcome and to extend the benefit of this therapy to a larger fraction of patients. To date, there is a small but growing number of pre-clinical studies (*e.g.*, 11–15) that address the effects of TMZ on radiation sensitivity of human GBM cells. These studies differ widely in experimental procedures, especially in the concentration of TMZ and the duration of TMZ exposure prior to irradiation, and are notable for varying results concerning how TMZ affects radiosensitivity. For example, some studies (*e.g.*, 13–15) report that TMZ has supra-additive effects on cell killing (*i.e.*, that TMZ is a radiosensitizing agent) while others (*e.g.*, 11, 12) report simply additive effects. At present, in accord with the clinical observations noted above, enhanced radiosensitivity has been observed only in cells that express little or no MGMT. To pursue these findings, we have systematically examined the TMZ dose dependence of radiation killing in established GBM cell lines that differ in their ability to remove O<sup>6</sup>-meG or to tolerate its lethality. We also examined the effect of suppressing alkyladenine-DNA glycosylase, the sole DNA repair activity in human cells that excises 3-meA (16), on killing by combined TMZ and radiation. Our findings indicate that minimally cytotoxic doses of TMZ can produce radiosensitization in MGMT-proficient (MGMT<sup>+</sup>) as well as in MGMT-deficient (MGMT<sup>-</sup>) GBM cell lines, and that radiosensitization is mediated by 3-meA as well as by O<sup>6</sup>-meG. Our results, and their possible implications, provide new insights into how TMZ may increase radiation cytotoxicity in human GBM cells.

## Materials and Methods

### Cell culture

The human GBM-derived cell lines A1235 and A1235MR4 (hereafter MR4; provided by Dr. J. Allalunis-Turner, University of Alberta), SNB19 and SF767 (Brain Tumor Research Laboratory, UCSF) and T98G (ATCC) were grown as previously described (17).

## TMZ, O<sup>6</sup>-BG, radiation and clonogenic survival

We have previously described in detail procedures for use of TMZ and O<sup>6</sup>-BG, and determination of proliferative survival by clonogenic assay (17–19). Briefly, unless otherwise stated, 1000 to 2000 cells were incubated for 2 hr with TMZ prior to <sup>137</sup>Cs- $\gamma$ -ray irradiation at 1 Gy/min under ambient conditions. Incubation was continued for 22 hr after irradiation before changing to fresh medium to allow formation of colonies  $\geq$  50 cells. Survival (mean  $\pm$  SD) is the ratio of colony-forming ability of treated cells to that of untreated cells. For experiments defining the TMZ dose dependence of enhanced radiation cytotoxicity, radiosensitization is the ratio of the survival expected if the effects of TMZ and radiation were additive to the survival observed. For example, if treatment with TMZ alone and  $\gamma$ -rays alone produces 95% and 80% survival, respectively, while treatment with both TMZ and  $\gamma$ -rays reduces survival to 45%, radiosensitization would be  $75\%/45\% = 1.7$ -fold. For full  $\gamma$ -ray survival curves, cytotoxicity was quantitated by linear regression analysis of plots of log surviving fraction vs. radiation dose to obtain the three resistance parameters, LD<sub>10</sub>, D<sub>T</sub> and D<sub>37</sub> as we have described previously in detail (18). Survival was determined in 3 separate experiments in which every dose was assayed in triplicate (i.e., 9 determinations per dose) in order to achieve statistical significance.

## Western analysis of $\gamma$ -H2AX in whole cell extracts

$\gamma$ -H2AX content of 50,000 to 200,000 cells solubilized in Laemmli buffer was estimated by Western blotting (20). Detection was by chemiluminescence using standard techniques; a CCD camera imaging system was used to produce digital images of blots for analysis of signal intensity.  $\gamma$ -H2AX signal intensity was normalized to that of  $\beta$ -actin, as a loading control. The ratio was then normalized to that for untreated cells, a control for  $\gamma$ -H2AX expression due to endogenous processes (e.g., mitosis; 21), to permit comparison between separate determinations. Values are the mean  $\pm$  SD of 4 experiments.

## Antisense suppression of AAG

We have previously provided detailed description of antisense and sense oligonucleotides targeting AAG, cationic lipid-mediated transfection and controls for specificity (17). Forty-eight hr after transfection, when suppression of activity is maximal (17), cells were sub-cultured for cytotoxicity analyses.

## Statistical analysis

Data analysis and statistical procedures were performed using Microsoft Excel. Comparison of means was by Student's t-test assuming unequal variances. Relationships between continuous variables were assessed by regression analysis. Statistically significant relationships were determined at the 95% confidence level.

## Results

### TMZ increases $\gamma$ -ray cytotoxicity in MGMT-deficient GBM cell lines

We began this study by examining the TMZ dose dependence of sensitization to  $\gamma$ -rays of two human GBM lines that express no detectable MGMT activity ( $< 0.25$  fmol/10<sup>6</sup> cells; 17,18). These lines, SNB19 and A1235, are highly sensitive to TMZ, with LD<sub>10</sub>'s for a 24 hr exposure of  $37 \pm 4$   $\mu$ M and  $23 \pm 2$   $\mu$ M, respectively (our unpublished data). In these experiments, we sought to mimic clinical practice in which a single daily treatment consists of oral TMZ at 75 mg/m<sup>2</sup> taken shortly before treatment with 2 Gy radiation (4). We therefore incubated cells with TMZ for 2 hr, the interval reported to produce peak cerebral spinal fluid (CSF) concentration in non-human primates (22), prior to radiation with 2 Gy <sup>137</sup>Cs  $\gamma$ -rays. As the half-life of TMZ in aqueous solution is similar to that in serum *in vivo* (1.5 vs. 1.8 hr; 23), we

continued incubation in the presence of TMZ for 22 hr after irradiation to simulate exposure during a single treatment fraction. To further approximate conditions that may prevail *in situ*, we used TMZ doses within the range attainable in CSF, *i.e.*, 10 to 25  $\mu\text{M}$  (22,24).

Fig. 1A shows that incubating SNB19 cells for 2 hr with TMZ doses up to 10  $\mu\text{M}$  had little effect on cell survival (>90%), while 2 Gy  $\gamma$ -rays alone reduced survival to  $81 \pm 4\%$ . Figs. 1A,B show that treatment with minimally cytotoxic doses of TMZ in combination with radiation produced supra-additive killing that was TMZ dose-dependent. At 10  $\mu\text{M}$  TMZ, survival of combined treatment was decreased  $1.8 \pm 0.2$  fold relative to radiation alone ( $81 \pm 4\%$  vs.  $46 \pm 9\%$ ;  $P \leq 0.001$ ; Table 1). However, at 15  $\mu\text{M}$  TMZ, a dose that reduced survival to approximately 40% (Fig. 1A), the enhancement of radiation killing was diminished to 1.2-fold (Fig. 1B). A similar pattern of TMZ-mediated sensitization to killing by 2 Gy  $\gamma$ -rays was observed for A1235 cells (Figs. 1C,D with maximal enhancement observed at 5  $\mu\text{M}$  TMZ (Table 1). These data show that non-lethal or minimally cytotoxic doses of TMZ can sensitize established MGMT<sup>-</sup> cell lines to killing by 2 Gy  $\gamma$ -rays. The observations are important because the TMZ concentrations that produced supra-additive killing are likely attainable in GBM cells *in situ* (24).

### TMZ increases $\gamma$ -ray cytotoxicity in GBM cells deficient in both MGMT and mismatch repair

Mismatch repair (MMR) mediates the cytotoxicity of O<sup>6</sup>-meG, and inactivation of MMR renders cells insensitive to killing by this adduct (10). To investigate the possibility that MMR contributes to TMZ-mediated radiosensitization, we examined MR4 cells. MR4 is a well-characterized human GBM cell line that lacks both MGMT and MMR activities (25). It was derived from MGMT<sup>-</sup> A1235 cells by selection for methylation resistance (26); in accord, the LD<sub>10</sub> for a 24 hr exposure to TMZ,  $1339 \pm 77 \mu\text{M}$ , is 58-fold greater than that for the parental A1235 line (our unpublished data). As shown in Fig. 1E, MR4 cells are insensitive to TMZ at doses as high as 200  $\mu\text{M}$ , while exposure to 2 Gy  $\gamma$ -rays reduced survival to  $77 \pm 8\%$ . Treatment with TMZ at doses that sensitized A1235 and SNB19 cells to radiation (*i.e.*,  $\leq 10 \mu\text{M}$ ) had no discernible effect on  $\gamma$ -ray killing in MR4; however increased sensitivity was detectable at TMZ concentrations  $\geq 25 \mu\text{M}$  (Figs. 1E,F). Treatment with 100  $\mu\text{M}$  TMZ and 2 Gy  $\gamma$ -rays reduced survival  $1.8 \pm 0.3$ -fold compared to radiation alone ( $77 \pm 8\%$  vs.  $43 \pm 7\%$ ;  $P \leq 2 \times 10^{-6}$ ; Table 1). This finding indicates that MMR mediates radiosensitization by TMZ in MGMT<sup>-</sup> GBM cells.

### TMZ increases $\gamma$ -ray cytotoxicity in MGMT-proficient GBM cells

Based on the TMZ dose dependence of radiosensitization of MGMT<sup>-</sup>MMR<sup>-</sup> MR4 cells (Figs. 1E,F), we examined the effect of a range of minimally cytotoxic TMZ doses on radiation killing in the MGMT<sup>+</sup> GBM line SF767. SF767 cells contain  $61 \pm 12 \text{ fmol}/10^6 \text{ cells}$  (*i.e.*,  $\sim 37,000$  molecules/cell) of MGMT (18), and exhibit an LD<sub>10</sub> of  $1119 \pm 62 \mu\text{M}$  for a 24 hr TMZ exposure (our unpublished data). Fig. 1G illustrates the effect of TMZ dose on killing following irradiation with 2 Gy  $\gamma$ -rays. TMZ alone at doses up to 100  $\mu\text{M}$  did not detectably reduce survival, reflecting the TMZ resistance of MGMT<sup>+</sup> cells, while radiation alone reduced survival to  $76\% \pm 6\%$ . Treatment with both TMZ and  $\gamma$ -rays produced supra-additive killing that was TMZ dose-dependent (Fig. 1H). Relative to radiation alone, survival was decreased  $1.7 \pm 0.2$ -fold at 100  $\mu\text{M}$  ( $76 \pm 6\%$  vs.  $45 \pm 5\%$ ;  $P \leq 2 \times 10^{-6}$ ; Table 1). TMZ concentrations that sensitized MGMT<sup>-</sup> cells to radiation (e.g.,  $\leq 10 \mu\text{M}$ ; Figs. 1A–D) had little or no effect on  $\gamma$ -ray killing. We also observed TMZ-mediated radiosensitization in the MGMT<sup>+</sup> GBM line T98G (Figs. 1K,L; Table 1), with maximal enhancement of killing between 50 and 100  $\mu\text{M}$  TMZ. The results for T98G are notable in that this line is insensitive to killing by 2 Gy. These data show that essentially non-lethal doses of TMZ can produce supra-additive radiation cytotoxicity in MGMT<sup>+</sup> GBM cells, albeit at concentrations higher than required in MGMT<sup>-</sup> cells.

To quantify the contribution of MGMT to protection of SF767 cells from TMZ-enhanced radiation killing, we ablated MGMT activity with the substrate analog inhibitor O<sup>6</sup>-benzylguanine (O<sup>6</sup>-BG). Treatment with O<sup>6</sup>-BG reduces MGMT to undetectable levels (<0.25 fmol/10<sup>6</sup> cells) and decreases LD<sub>10</sub> for TMZ *ca.* 12-fold (1119 ± 62 *vs.* 91 ± 6 μM; our unpublished data). As shown in FIG. 1I, ablating MGMT activity greatly reduced the TMZ dose required to produce radiosensitization (compare the X axes in Fig. 1G *vs.* 1I). Exposure to TMZ alone had only a small effect on survival (> 90% at all doses) while exposure to radiation alone reduced survival to 77 ± 7%. Relative to cells receiving radiation only, maximal decrease in survival, 1.7 ± 0.2-fold, was observed at doses as low as 5 μM TMZ (77 ± 7% *vs.* 45 ± 7%; *P* ≤ 0.0004; Fig. 1J and Table 1). These results are similar to those for the MGMT<sup>-</sup> lines SNB19 and A1235 (Figs. 1A–D), and strongly indicate that unrepaired O<sup>6</sup>-meG plays a prominent role in enhancing radiation-induced cytotoxicity.

### Dose dependence of radiation cytotoxicity in the absence and presence of TMZ

In order to further characterize the effect of TMZ on radiation killing, we examined the dose dependence of  $\gamma$ -ray survival of MGMT<sup>-</sup> SNB19, MGMT<sup>+</sup> SF767, and MGMT<sup>-</sup>MMR<sup>-</sup> MR4 cells in the absence and presence of TMZ (Fig. 2). In the absence of TMZ, the lines differ little in radiosensitivity, displaying similar values for LD<sub>10</sub>, the dose that reduces survival to 10%; for D<sub>T</sub>, the threshold dose that defines a shoulder of resistance below which cells are insensitive to killing; and for D<sub>37</sub>, a measure of the rate of cell killing. In these three lines, exposure to minimally cytotoxic concentrations of TMZ [10 μM for SNB19 and 100 μM for SF767 and MR4 (Table 1)] for 2 hr before and 22 hr after irradiation increased radiosensitivity as evidenced by statistically significant 1.5- to 1.9-fold reductions in LD<sub>10</sub> (Fig. 2). Lower LD<sub>10</sub> reflected [1] a large 7.2- to 17-fold reduction in D<sub>T</sub> and [2] a 1.5- to 2.0-fold reduction in D<sub>37</sub>. These data provide further, strong evidence that TMZ can act as a radiosensitizing agent, and together with the data for MGMT<sup>+</sup> (Figs. G,H,K,L) and MGMT<sup>-</sup>MMR<sup>-</sup> cells in Figs. 1E,F, show that removal or tolerance of O<sup>6</sup>-meG does not preclude radiosensitization.

### Radiosensitization requires TMZ treatment before but not after radiation

It has been reported that TMZ-enhanced killing of MGMT<sup>-</sup> GBM cells requires exposure to the drug prior to radiation (*e.g.*, 13–15). To determine whether this is the case for MGMT<sup>+</sup> cells, we analyzed SF767 cells treated with 100 μM TMZ for different intervals before and after irradiation. As shown in Table 2, TMZ alone did not reduce survival, and 2 Gy  $\gamma$ -rays alone reduced survival to 76 ± 0.3%. Incubation with 100 μM TMZ for 2 hr before and for 22 hr after radiation reduced survival to 46 ± 3%, relative to radiation alone, a statistically significant reduction (*P* ≤ 0.005). Limiting TMZ treatment to 2 hr prior to radiation reduced survival to the same extent (48 ± 7%), while exposure to TMZ for 22 hr immediately after radiation did not reduce survival relative to radiation alone (75 ± 3%). As also shown in Table 2, essentially the same results were observed for SF767 cells treated with 5 μM TMZ and O<sup>6</sup>-BG. These data provide evidence that radiosensitization requires the action of TMZ prior to irradiation in MGMT<sup>+</sup> cells as well as cells that lack MGMT, and that TMZ exposure after irradiation does not produce supra-additive killing. They also suggest that methylation damage that would otherwise be repaired or tolerated is converted to lethal lesions by  $\gamma$ -rays.

### TMZ increases double-strand break content within 45 min after radiation

Histone H2AX is rapidly phosphorylated at serine 139 to form  $\gamma$ -H2AX in response to double-strand breaks (DSBs) produced by ionizing radiation (21).  $\gamma$ -H2AX functions in the cellular response to DSBs by promoting chromatin remodeling and assembly of repair proteins, and serves as a sensitive marker of DSBs. Fig. 3A shows a representative determination of  $\gamma$ -H2AX and  $\beta$ -actin content, assessed by Western blotting of extracts of MGMT<sup>+</sup> SF767 following no treatment; incubation for 2 hr with 100 μM TMZ; irradiation with 2 Gy  $\gamma$ -rays alone; or

incubation for 2 hr with 100  $\mu$ M TMZ prior to 2 Gy  $\gamma$ -rays. For all treatments, cells were harvested and extracts prepared *ca.* 30–45 min after irradiation. Digital image analysis of this blot together with that of 3 additional experiments (Fig. 3B) revealed that  $\gamma$ -H2AX content, normalized to actin, was the same in cells treated with TMZ alone as in untreated cells. In cells exposed to 2 Gy  $\gamma$ -rays alone,  $\gamma$ -H2AX content was  $2.0 \pm 0.6$ -fold greater than in untreated cells, while the content of cells treated with TMZ and  $\gamma$ -rays was  $2.8 \pm 0.8$ -fold greater, a difference that is statistically significant ( $P < 0.02$ ). Comparable results were also observed for SF767 treated with O<sup>6</sup>-BG (Fig. 3C), the MGMT<sup>+</sup> line T98G (Fig. 3D) and the MGMT<sup>-</sup>MMR<sup>-</sup> line MR4 (Fig. 3E). In all cases, the difference in  $\gamma$ -H2AX content between cells treated with radiation *vs.* TMZ plus radiation was statistically significant ( $P \leq 0.05$ ). These findings indicate that rapid elevation of DSB content accompanies enhanced sensitivity to radiation killing in TMZ-treated GBM cells.

### Suppressing alkyladenine-DNA glycosylase reduces the TMZ dose dependence of radiosensitization

Our observation that TMZ increases  $\gamma$ -ray sensitivity in MGMT<sup>+</sup> and MGMT<sup>-</sup>MMR<sup>-</sup> GBM cells at doses 10–20-fold greater than those required in MGMT<sup>-</sup> cells suggests that a TMZ-induced adduct in addition to O<sup>6</sup>-meG may contribute to radiosensitization. To assess this possibility, we determined the effect of suppressing the DNA repair enzyme alkyladenine-DNA glycosylase (AAG) on the TMZ dose dependence of radiosensitization. The preferred substrate of AAG is the cytotoxic lesion 3-meA (16) which comprises ~10% of TMZ base adducts (6,7). AAG is the only repair activity in human cells that is known to excise 3-meA from DNA (16). We have previously reported (17) that suppressing AAG activity increases the sensitivity of human GBM cells, including SF767 and MR4, to methyl-lexitropsin, an agent that produces almost exclusively 3-meA in DNA (27). The dose dependence of TMZ-induced sensitization to 2 Gy  $\gamma$ -rays in MGMT<sup>+</sup> SF767 transfected with antisense oligonucleotides (ASO) targeting AAG or sense oligonucleotides (SO) is shown in Fig. 4. Transfection with SO had little or no effect on either killing by TMZ or  $\gamma$ -rays alone, or on TMZ enhanced  $\gamma$ -ray killing compared to untransfected cells (compare Fig. 4A,C *vs.* Fig. 1G,H). Transfection with ASO, which reduces AAG activity 1.8-fold (17), had no effect on radiation killing and only a modest effect on TMZ sensitivity, reducing survival at 100  $\mu$ M from 100% to 90% (compare Figs. 4A *vs.* B). ASO treatment, however, did produce 1.2- to 1.4-fold, statistically significant ( $P \leq 0.05$ ) decreases in survival (Figs. 4A *vs.* B) compared to SO-treated cells at TMZ doses  $\geq 25$   $\mu$ M. The effect of ASO is more clearly illustrated by the statistically significant ( $P \leq 2 \times 10^{-6}$ ; ANOVA) 1.5- to 2-fold reductions in TMZ dose dependence for radiosensitization that accompanied ASO treatment (Fig. 4C): For example, the TMZ dose required to produce a 1.5-fold sensitization was 1.8-fold lower (55  $\mu$ M *vs.* 100  $\mu$ M) in ASO- *vs.* SO-treated cells. Comparable results were observed for MGMT<sup>-</sup>MMR<sup>-</sup> MR4 cells (Figs. 4D–F), in which the TMZ dose required to produce a 1.5-fold radiosensitization was 2.5-fold lower (30  $\mu$ M *vs.* 75  $\mu$ M) in ASO- *vs.* SO-treated cells. These findings indicate that reduced excision of 3-meA can contribute to TMZ-induced radiosensitization in human GBM cells.

### Discussion

The long-term goal of the present work is to better understand the mechanisms underlying the improved efficacy of TMZ-RT, relative to RT alone, in the adjuvant care of GBMs. Toward this end, we compared the TMZ dose dependence of sensitization to killing by  $\gamma$ -rays in GBM cell lines that differ in their ability to remove or tolerate O<sup>6</sup>-meG. Our results show that [1] minimally cytotoxic doses of TMZ can produce supra-additive cytotoxicity, suggestive of an interaction between methylation- and radiation-induced DNA damage; [2] TMZ can enhance radiosensitivity in MGMT<sup>-</sup>, MGMT<sup>-</sup>MMR<sup>-</sup> and MGMT<sup>+</sup> GBM cells, the dose dependence being 10- to 20-fold lower in MGMT<sup>-</sup> cells, indicative of a role for O<sup>6</sup>-meG and MMR in

radiosensitization; [3] enhanced radiation killing requires treatment with TMZ before, but not after irradiation and is accompanied by elevated DSB content within 45 min after irradiation; [4] suppressing AAG increases radiosensitivity, indicating that the DNA adduct 3-meA can promote radiation cytotoxicity. These findings strongly support the conclusion that TMZ is a radiosensitizing agent, and suggest that a common mechanism(s) of radiosensitization may operate in MGMT<sup>+</sup>, MGMT<sup>-</sup>MMR<sup>-</sup> and MGMT<sup>-</sup> GBM cell lines.

### O<sup>6</sup>-meG is a radiosensitizing lesion

In accord with previous studies (*e.g.*, 13–15), we observed radiosensitization by TMZ in two GBM lines that do not express MGMT (Figs. 1 A–D; Table 1). In the absence of MGMT activity, maximal enhancement of killing by 2 Gy  $\gamma$ -rays, the standard fractionated RT dose, was observed at TMZ doses below those attainable in CSF (*i.e.*, 10–25  $\mu$ M; 22,24). In fact, an increase in radiation killing was detectable with as little as 2.5  $\mu$ M TMZ, suggesting that unrepaired O<sup>6</sup>-meG is a potent sensitizing lesion. This conclusion is supported by the finding that ablating MGMT activity in MGMT<sup>+</sup> cells with O<sup>6</sup>-BG reduces the TMZ dose dependence for radiosensitization by 20-fold (Figs. 1 G,H vs. ,I,J). Additional evidence for a role for O<sup>6</sup>-meG is provided by the 20-fold increase in the TMZ dose required for radiosensitization in the MGMT<sup>-</sup>MMR<sup>-</sup> line MR4 relative to its MGMT<sup>-</sup> A1235 parent line (Figs. 1 C,D vs. ,E,F). This latter observation indicates that radiosensitization by O<sup>6</sup>-meG, like the cytotoxicity of this lesion (10), involves the activity of MMR. Lastly, we observed radiosensitization in 2 MGMT<sup>+</sup> lines (Figs. 1 G,H,K,L), at TMZ doses at least 10-times greater than required in MGMT<sup>-</sup> cells (Table 1). The higher TMZ doses may be necessary to produce enough O<sup>6</sup>-meG and/or additional radiosensitizing lesions to escape repair. The foregoing results provide mechanistic support for the clinical observation that GBMs displaying CpG methylation in the MGMT promoter, a presumptive marker of gene silencing (9), are more likely to benefit from concurrent TMZ-RT (3,8).

With one exception (13), our studies differ from previous work in that we examined the interaction of minimally cytotoxic doses of TMZ (*i.e.*, < 10% reduction in survival) with  $\gamma$ -radiation, an experimental approach that greatly facilitated detection of radiosensitization. Our data show a decrease in the enhancement of radiation killing when the contribution of TMZ to cytotoxicity exceeds 20% (Figs. 1 A–D; Figs. 1 K,L). These findings suggest that in our experimental protocol higher doses of TMZ may obscure detection of radiosensitization by producing a high background of cell killing by TMZ alone; some earlier studies that did not observe radiosensitization in some MGMT<sup>-</sup> GBM lines *e.g.*, 11,14) used TMZ concentrations that reduced survival by 40% or greater.

### 3-meA and radiosensitization

Our data indicate that 3-meA is a radiosensitizing adduct and that TMZ-induced potentiation of radiation killing can occur independently of O<sup>6</sup>-meG. These conclusions are based on the statistically significant reduction in the TMZ dose required for radiosensitization that accompanies transfection of MGMT<sup>+</sup> SF767 and MGMT<sup>-</sup>MMR<sup>-</sup> MR4 cells with antisense directed against AAG (Fig. 4). While AAG is the only DNA repair activity in human cells that excises 3-meA, it has a broad substrate specificity, excising a variety of alkyl and oxidative base lesions, although less efficiently than 3-meA in almost all cases (16). Thus, it is possible that increased radiosensitization in antisense-treated cells may reflect, in part, the action of additional lesions.

### A scenario of TMZ radiosensitization

The tumoricidal activity of radiation and clinically utilized alkylating agents is mediated almost exclusively by DSBs (10,28,29). Hence, the goal of therapy with these and other DNA damaging agents is to cause sufficient DSBs to exceed the repair capacity of tumor cells.

Current evidence indicates that the cytotoxicity of O<sup>6</sup>-meG and 3-meA is mediated by the interaction of these adducts with DNA replication (10), suggesting a parsimonious explanation of how exposure to TMZ prior to irradiation may yield supra-additive cytotoxicity in GBM cell lines: O<sup>6</sup>-meG and 3-meA produce single-strand gaps that are converted into DSBs upon subsequent irradiation. In the case of O<sup>6</sup>-meG, recognition of O<sup>6</sup>-meG:T mispairs by MMR is followed by exonucleolytic degradation, extending from the 3' terminus of the newly replicated strand to a position 5' to the mispair (30). It has been proposed that repair synthesis to fill the gap produces another mispair, thus eliciting another round of excision (reviewed in 10). This "futile cycle" would in effect produce a persistent single-strand gap. In the case of 3-meA, diverse evidence strongly indicates that this adduct impedes DNA replication fork progression (31–34), which can result in single-strand gaps, as shown in yeast and frog oocytes (35,36), and in mammalian cells (37). Additional evidence that O<sup>6</sup>-meG and 3-meA are the precursors of single-strand gaps is that both adducts elicit sister chromatid exchange, a hallmark of DNA gap filling (10). The single-strand gaps produced by the action of MMR or blocked replication fork progression are believed to range in length from ~150 nucleotides to more than 300 nucleotides, respectively (30,36). Gaps of this size would greatly increase the vulnerability of DNA to DSB formation, since strand scission by radiation-induced oxidative free radicals at any nucleotide in the single-stranded region converts the gap into a DSB. The resulting susceptibility to DSB formation could account for the supra-additive increases in  $\gamma$ -ray cytotoxicity that accompany treatment with minimally cytotoxic doses of TMZ.

The foregoing scenario provides a simple mechanism to account for the elevation of DSB content observed shortly after irradiation (Fig. 3) and the requirement that TMZ exposure precede irradiation. It also offers a facile explanation for the greater TMZ dose dependence of radiosensitization observed in MGMT<sup>-</sup>MMR<sup>-</sup> MR4 compared to its MGMT<sup>-</sup> parental line A1235, *i.e.*, the inability of O<sup>6</sup>-meG to produce gaps in the absence of MMR necessitates greater concentrations of TMZ to yield compensatory levels of 3-meA and other radiosensitizing lesions. Conversely, the reduction of TMZ dose dependence accompanying ablation of MGMT or transfection with anti-AAG antisense reflects the greater likelihood of a DNA replication fork encountering O<sup>6</sup>-meG or 3-meA in repair-compromised cells. Finally, the scenario accounts for the large, TMZ-induced reduction in the radiation dose tolerated without cytotoxicity, exhibited as the shoulder on the survival curves in Fig. 2 and quantitated as the parameter D<sub>T</sub>, by providing a mechanism by which otherwise innocuous levels of methylation-induced single-strand gaps are converted into levels of DSBs that saturate repair capacity.

Our findings do not rule out the possibility that the radiosensitizing effects of TMZ reflect decreased repair of radiation-induced DSBs or other radiation lesions such as single-strand breaks as has been suggested in earlier studies (13,14). Conceivably, the presence of O<sup>6</sup>-meG or 3-meA near the end of DSBs may prevent damage recognition or processing. Such a mechanism is consistent with the large decrease in D<sub>T</sub> mentioned above. However, it is difficult to imagine that the minimally cytotoxic doses of TMZ would yield enough adducts to interact with the small number of DSBs produced by 2 Gy  $\gamma$ -rays (~75–150 per diploid nucleus; 38). Moreover, decreased repair does not necessitate exposure to TMZ prior to irradiation, and does not explain the large increase in TMZ dose dependence that accompanies loss of MMR in MGMT<sup>-</sup> cells.

### Multifactorial resistance to TMZ-RT and clinical implications

Our studies suggest that resistance to TMZ-RT is multi-factorial, and that MMR and AAG, in addition to MGMT, may influence treatment outcome. Repeated episodes of methylation strongly select for MMR-deficient variants of GBM cell lines that do not express MGMT (*e.g.*, 26), and accumulating evidence suggests that recurrence in GBMs treated with TMZ-RT

can be accompanied by epigenetic and mutational inactivation of MMR (*e.g.*,39,40). Notably, MMR-defective variants arising in MGMT-deficient GBM cells prior to treatment with TMZ-RT would display CpG methylation of the MGMT promoter, and our findings with MR4 suggest that promoter methylation in the absence of MMR might lead to false prediction of favorable clinical response.

The mechanistic scenario described above suggests several additional potential mechanisms of resistance to TMZ-RT. These include activation of the intra-S-phase checkpoint (41) in response to the single strand gaps generated by O<sup>6</sup>-meG and 3-meA. Checkpoint activation reduces the rate of firing of new origins of replication and slows the rate of elongation of ongoing forks. Intra-S-phase arrest would promote resistance to TMZ-RT by providing additional time for adduct removal from template DNA, thereby reducing encounters of DNA replication with methyl adducts. Processes that mediate gap filling and replication restart at stalled forks are other potential resistance mechanisms (35,42). Among the latter, the Werner syndrome helicase promotes recovery of stalled replication forks (42), and increases resistance to O<sup>6</sup>-meG and 3-meA in human GBM cells (19).

Circumventing DNA repair-mediated resistance to TMZ is likely to increase the clinical efficacy of concurrent TMZ-RT, and our findings suggest that targeting repair of O<sup>6</sup>-meG and/or 3-meA during RT may be promising anti-resistance strategies. Inhibiting removal of O<sup>6</sup>-meG is an important priority. Clinical trials have shown that O<sup>6</sup>-BG can deplete MGMT activity in high-grade gliomas, and additional trials are now ongoing to evaluate the effect on outcome of ablating MGMT during alkylating agent treatment (reviewed in 4). Our data suggest that inhibiting repair of 3-meA may complement depletion of MGMT in overcoming resistance to TMZ. Repair of 3-meA is an especially attractive anti-resistance target in MGMT<sup>-</sup> GBM cells that have acquired TMZ resistance via inactivation of MMR. Base excision repair pathways (10) that process 3-meA present multiple targets for anti-resistance intervention. The development of inhibitors of AAG and other members of base excision repair pathways, *e.g.*, Ape1/Ref-1 and poly(ADP-ribose) polymerase (PARP), are ongoing (4,43). Inhibition of PARP has been shown to sensitize human GBM xenografts to TMZ alone (44) and to TMZ used with radiation (45).

## Abbreviations

AAG	alkyladenine DNA-glycosylase
ASO	antisense oligonucleotide
CSF	cerebral spinal fluid
DSB	double-strand break
GBM	glioblastoma
O <sup>6</sup> -BG	O <sup>6</sup> -benzylguanine
3-meA	3-methyladenine
O <sup>6</sup> -meG	O <sup>6</sup> -methylguanine
MGMT	O <sup>6</sup> -methylguanine-DNA methyltransferase
MMR	mismatch repair
RT	radiotherapy
SO	sense oligonucleotide
TMZ	temozolomide

## Acknowledgments

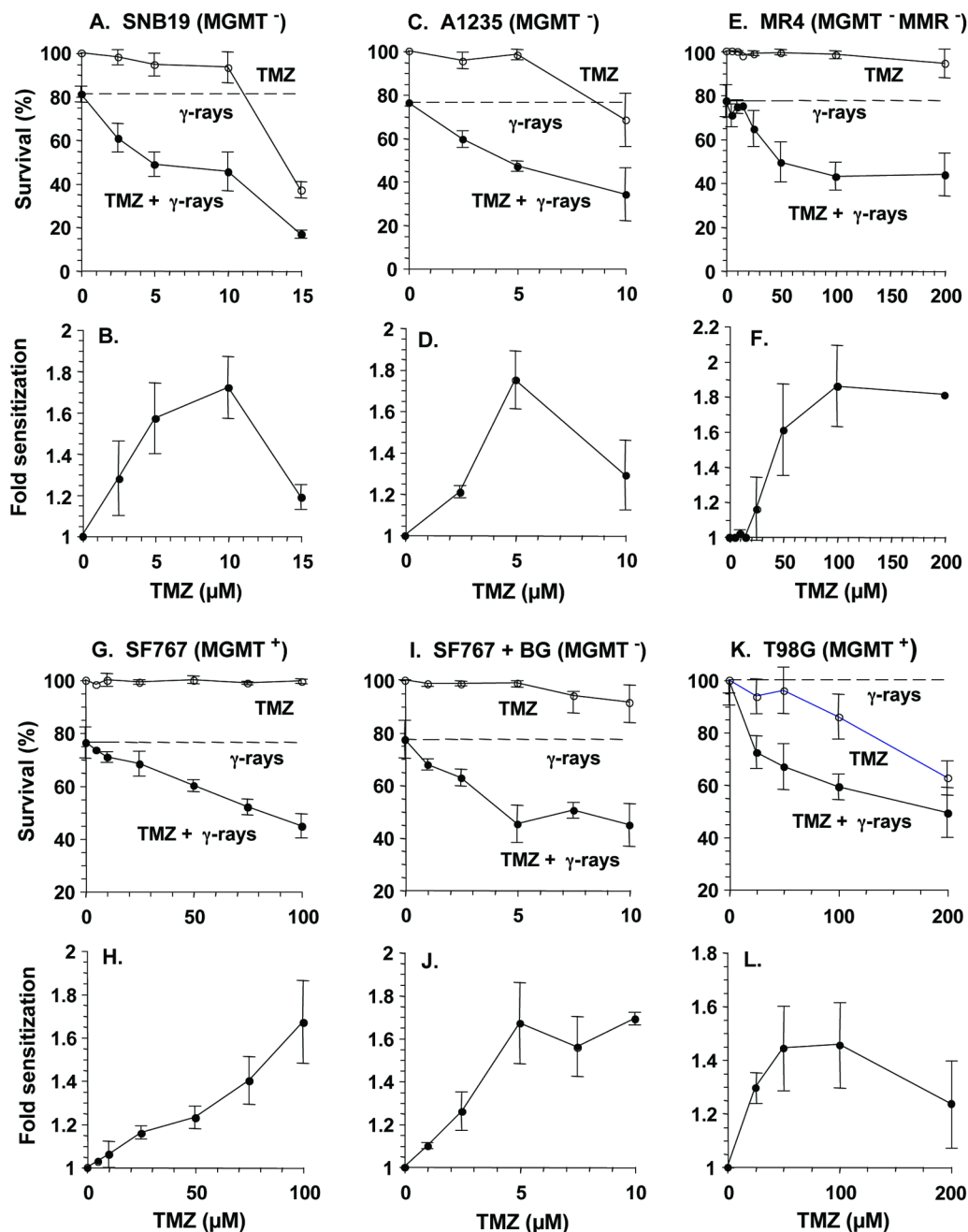
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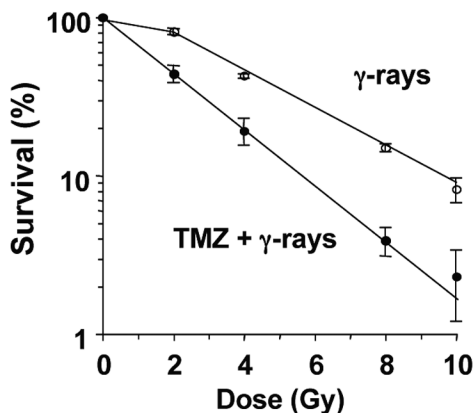
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**Fig. 1. The effect of TMZ on  $\gamma$ -ray killing in MGMT<sup>-</sup> GBM cell lines that possess or lack mismatch repair and in MGMT<sup>+</sup> cell lines in the absence and presence of O<sup>6</sup>-BG**

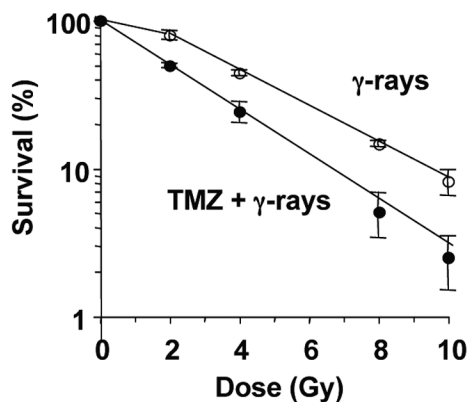
Cells were incubated with TMZ for 2 hr prior to irradiation with 2 Gy <sup>137</sup>Cs- $\gamma$ -rays and for 22 hr after. Survival, determined by clonogenic assay, is the mean  $\pm$  SD of triplicate determinations in each of 3 separate experiments. **A, C, E, G, I and K** show the effect of TMZ dose on survival of unirradiated ( $\circ$ ) and irradiated ( $\bullet$ ) cells. The dashed lines indicate survival after 2 Gy only. In **I and J**, cells were incubated with 20  $\mu$ M O<sup>6</sup>-BG for 2 hr prior to and during the course of TMZ exposure to ablate MGMT activity. **B, D, F, H, J and L** illustrate the fold radiosensitization (*i.e.*, survival expected if killing by TMZ and radiation were additive divided

by the observed survival) as a function of TMZ dose. Where not shown, error bars are too small to be seen.



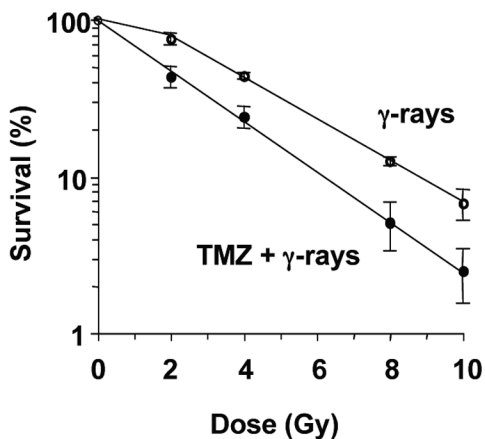
### A. SNB19 (MGMT<sup>-</sup>)

	No TMZ	10 μM TMZ	-TMZ/+TMZ	<i>P</i> ≤
LD <sub>10</sub>	10 ± 0.40	5.3 ± 1.1	1.9	0.006
D <sub>T</sub>	1.2 ± 0.12	0.07 ± 0.01	17	0.001
D <sub>37</sub>	4.5 ± 0.20	2.3 ± 0.50	2.0	0.001



### B. SF767 (MGMT<sup>+</sup>)

	No TMZ	100 μM TMZ	-TMZ/+TMZ	<i>P</i> ≤
LD <sub>10</sub>	10 ± 0.06	6.4 ± 0.40	1.6	0.001
D <sub>T</sub>	1.3 ± 0.36	0.18 ± 0.02	7.2	0.003
D <sub>37</sub>	4.5 ± 0.06	2.8 ± 0.15	1.6	0.003

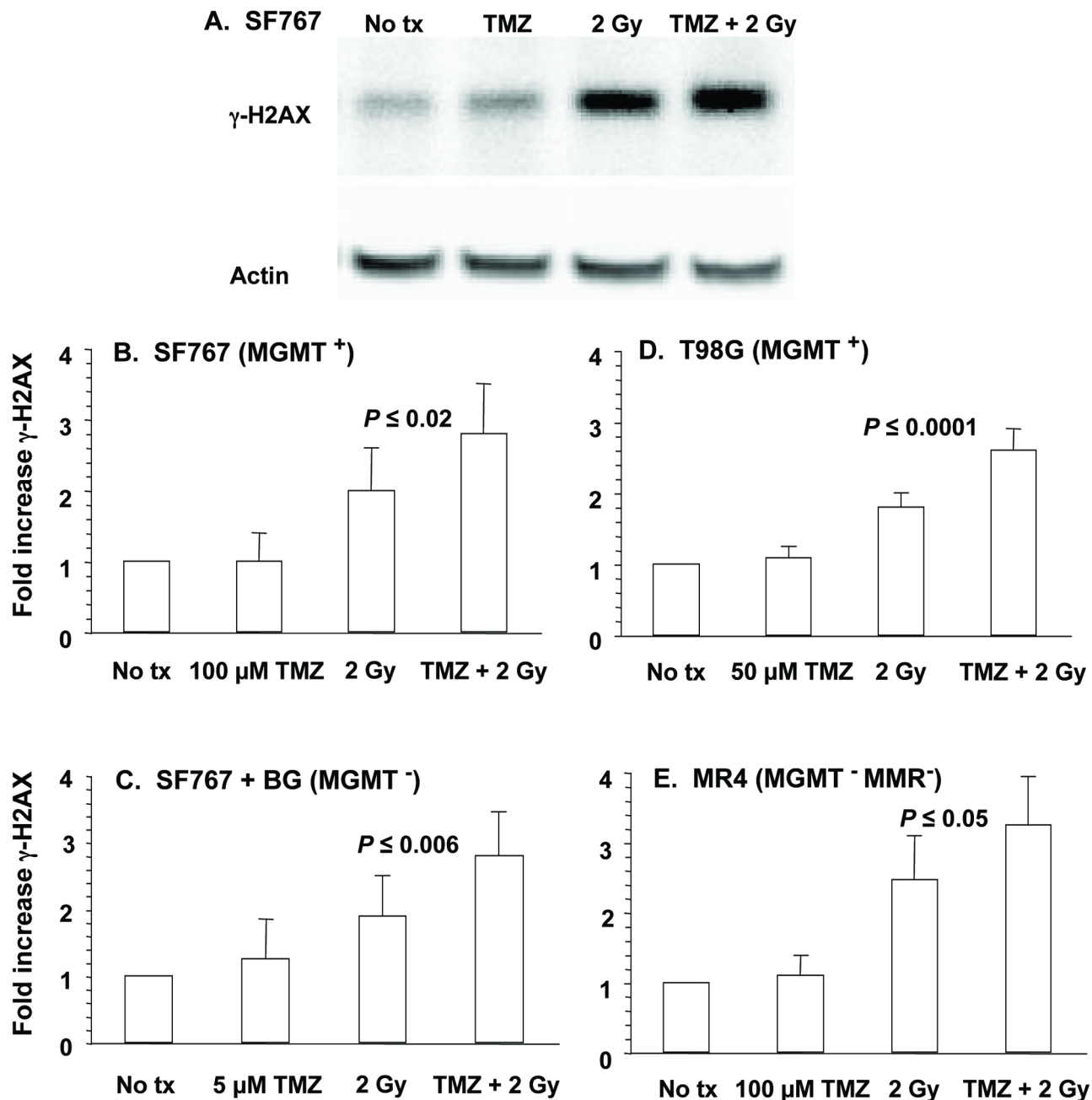


### C. MR4 (MGMT<sup>-</sup> MMR<sup>-</sup>)

	No TMZ	100 μM TMZ	-TMZ/+TMZ	<i>P</i> ≤
LD <sub>10</sub>	9.1 ± 0.93	5.9 ± 0.20	1.5	0.03
D <sub>T</sub>	0.9 ± 0.3	0.09 ± 0.02	10	0.03
D <sub>37</sub>	4.0 ± 0.36	2.6 ± 0.06	1.5	0.03

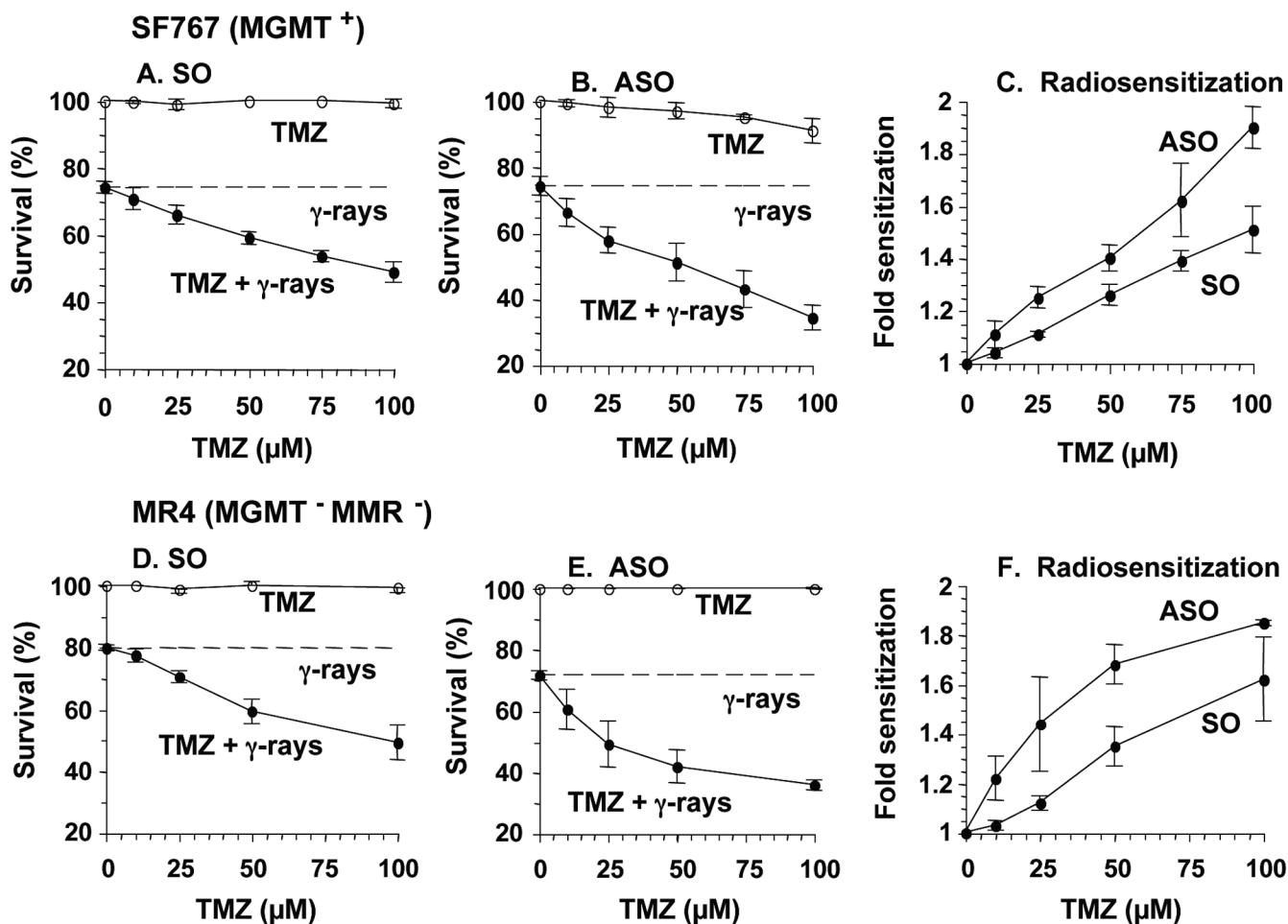
**Fig. 2. Dose dependence of radiation killing in the absence and presence of TMZ**

Cells were incubated in the absence (○) or presence (●) of TMZ for 2 hr prior to irradiation with 2 Gy  $\gamma$ -rays and for 22 hr after. Clonogenic survival is the mean  $\pm$  SD of triplicate determinations in each of 3 separate experiments. The final linear portion of the curves was constructed by linear regression analysis. The survival variables LD<sub>10</sub>, D<sub>T</sub> and D<sub>37</sub> were derived by linear regression analysis of the final linear portion of the curves as we have described (18).



**Fig. 3.  $\gamma$ -H2AX content is rapidly elevated in GBM cells treated with TMZ and  $\gamma$ -rays**

**A**, digital image of  $\gamma$ -H2AX in a representative experiment with actin as a loading control. Sub-confluent cultures of SF767 cells received either no treatment, or were incubated with 100  $\mu$ M TMZ for 2 hr, or exposed to 2 Gy  $\gamma$ -rays, or treated with TMZ for 2 hr prior to exposure to 2 Gy  $\gamma$ -rays. Approximately 30–45 min after treatment, cultures were harvested and extracted for determination of  $\gamma$ -H2AX and actin content by Western blotting. **B–E**, fold increase in  $\gamma$ -H2AX signal intensity, normalized to that of actin, and shown relative to untreated cells, is illustrated for MGMT<sup>+</sup> SF767 (**B**), SF767 + O<sup>6</sup>-BG (**C**), MGMT<sup>+</sup> T98G (**D**) and MGMT<sup>-</sup>MMR<sup>-</sup> MR4 (**E**) cells. *P* values refer to the difference between cells treated with 2 Gy vs. TMZ + 2 Gy. Results are the mean  $\pm$  SD of 4 separate experiments for each line.



**Fig. 4. Antisense against AAG enhances TMZ-radiation killing**  
 MGMT<sup>+</sup> SF767 (A–C) and MGMT<sup>-</sup>MMR<sup>-</sup> MR4 (D–F) cells were incubated with TMZ for 2 hr prior to irradiation with 2 Gy <sup>137</sup>Cs γ-rays. The cells were changed to drug-free medium immediately after irradiation. The effect of TMZ on survival in unirradiated (○) and irradiated (●) cells treated with either SO (A,D) or ASO (B,E) is shown. Dashed lines indicate the survival of cells receiving 2 Gy only. C,F show the fold radiosensitization (*i.e.*, the survival expected if the effects of TMZ and radiation were additive divided by the observed survival) as a function of TMZ dose. Points are the mean ± SD of triplicate determinations at each dose in 3 separate experiments.

Table 1

Radiosensitization by TMZ in MGMT<sup>-</sup>, MGMT<sup>-</sup>MMR<sup>-</sup> and MGMT<sup>+</sup> GBM cells<sup>a</sup>

Cell line	TMZ (μM)	Survival (%)			Fold Sensitization	<i>p</i> <sup>b</sup>
		TMZ only	2 Gy only	TMZ + 2 Gy		
MGMT <sup>-</sup>						
SNB19	10	97 ± 7	81 ± 4	46 ± 9	1.8 ± 0.2	≤ 0.001
A1235	5	98 ± 2	76 ± 1	47 ± 2	1.6 ± 0.1	≤ 0.001
SF767 + O <sup>6</sup> -BG	5	99 ± 2	77 ± 7	45 ± 7	1.7 ± 0.2	0.0004
MGMT <sup>-</sup> MMR <sup>-</sup>						
MR4	100	99 ± 2	77 ± 8	43 ± 7	1.8 ± 0.3	≤ 2 × 10 <sup>-6</sup>
MGMT <sup>+</sup>						
SF767	100	100	76 ± 6	45 ± 5	1.7 ± 0.2	≤ 2 × 10 <sup>-6</sup>
T98G	50	96 ± 9	100	67 ± 9	1.4 ± 0.2	≤ 0.003

<sup>a</sup>Results are the mean ± SD of triplicate determinations in 3 separate experiments, i.e., 9 determinations. Fold sensitization is the ratio of survival expected if the effects of TMZ and radiation were additive to survival actually observed.

<sup>b</sup>Student's *t*-test assuming unequal variances.

**Table 2**

TMZ yields radiosensitization of MGMT<sup>+</sup> cells when given before but not after  $\gamma$ -rays<sup>a</sup>

	Survival (%)			
	100 $\mu$ M TMZ	<i>p</i> <sup>b</sup>	5 $\mu$ M TMZ + BG	<i>p</i> <sup>b</sup>
TMZ alone	98 $\pm$ 3	-	98 $\pm$ 1	-
2 Gy alone	76 $\pm$ 0.3	-	77 $\pm$ 5	-
TMZ before and after 2 Gy	46 $\pm$ 3	$\leq$ 0.005	42 $\pm$ 5	$\leq$ 0.002
TMZ before 2 Gy	48 $\pm$ 7	$\leq$ 0.02	43 $\pm$ 7	$\leq$ 0.003
TMZ after 2 Gy	75 $\pm$ 3	-	76 $\pm$ 7	-

<sup>a</sup>MGMT<sup>+</sup> SF767 cells were exposed to TMZ and/or 2 Gy  $\gamma$ -rays as indicated in Column 1. Cells were either treated with TMZ for two hr; with 2 Gy  $\gamma$ -rays; with TMZ for 2 hr before and 22 hr after radiation; with TMZ for two hr prior to radiation; or with TMZ for 22 hr after radiation. Columns 2 and 3 show results for cells treated with 100  $\mu$ M TMZ; columns 4 and 5 show results for treatment with 5  $\mu$ M TMZ and O<sup>6</sup>-BG. Results are the mean  $\pm$  SD of triplicate determinations in separate experiments, i.e., 9 determinations.

<sup>b</sup>Student's *t*-test assuming unequal variances.