The Role of Base Excision Repair in the Sensitivity and Resistance to Temozolomide-Mediated Cell Death

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Abstract

DNA-alkylating agents have a central role in the curative therapy of many human tumors; yet, resistance to these agents limits their effectiveness. The efficacy of the alkylating agent temozolomide has been attributed to the induction of O^6-MeG, a DNA lesion repaired by the protein O^6-methylguanine-DNA methyltransferase (MGMT). Resistance to temozolomide has been ascribed to elevated levels of MGMT and/or reduced mismatch repair. However, >80% of the DNA lesions induced by temozolomide are *N*-methylated bases that are recognized by DNA glycosylases and not by MGMT, and so resistance to temozolomide may also be due, in part, to robust base excision repair (BER). We used isogenic cells deficient in the BER enzymes DNA polymerase-β (pol-β) and alkyladenine DNA glycosylase (Aag) to determine the role of BER in the cytotoxic effect of temozolomide. Pol-β–deficient cells were significantly more susceptible to killing by temozolomide than wild-type or Aag-deficient cells, a hypersensitivity likely caused by accumulation of BER intermediates. RNA interference–mediated pol-β suppression was sufficient to increase temozolomide efficacy, whereas a deficiency in pol-ι or pol-λ did not increase temozolomide-mediated cytotoxicity. Overexpression of Aag (the initiating BER enzyme) triggered a further increase in temozolomide-induced cytotoxicity. Enhanced Aag expression, coupled with pol-β knockdown, increased temozolomide efficacy up to 4-fold. Furthermore, loss of pol-β coupled with temozolomide treatment triggered the phosphorylation of H2AX, indicating the activation of the DNA damage response pathway as a result of unrepaired lesions. Thus, the BER pathway is a major contributor to cellular resistance to temozolomide and its efficacy depends on specific *BER* gene expression and activity. (Cancer Res 2005; 65(14): 6394-400)

Introduction

Melanoma and glioblastoma are significant causes of death due to malignancy in the United States. The incidence of malignant melanoma has doubled in the past 10 years and is rising at a rate exceeding all other solid tumors (1). Chemotherapy provides a response rate of 7% to 20% with rare complete remissions. Approximately 17,000 new cases of brain cancer are diagnosed annually, yet therapy remains inadequate and the mortality rate is high (2). Recently, limited success has been shown with temozolomide in the treatment of melanoma, glioblastoma, and some nonglial tumors, such as breast cancer tumors, that have metastasized to the brain (3–10); however, significant resistance to this agent has limited its efficacy. Partial deletion of chromosome 1p seems to predict for temozolomide sensitivity for oligodendroglial tumors, although the mechanism is unclear (11). The DNA repair mechanism that provides resistance to temozolomide as presented in this study may provide additional mechanistic avenues for improvements in efficacy.

Temozolomide is an alkylating agent that causes methylation of guanine on the *N7* and *O*6 atoms and methylation of adenine on the N3 atom (12). The guanine lesion O^6-MeG is repaired by O^6-methylguanine-DNA methyltransferase (MGMT; ref. 13). This has been taken advantage of experimentally and in many clinical trials (14) because MGMT can be inhibited with the O^6-MeG analogue O^6-benzylguanine, among others (15, 16). Elevated levels of MGMT (17) and/or a nonfunctional mismatch repair pathway have been blamed for much of the observed resistance to temozolomide, yet it has also been suggested that repair of O^6-MeG by MGMT is not the sole mechanism responsible for resistance to alkylating agents (18–21). Interestingly, >80% of the temozolomide-induced DNA lesions are substrates for the base excision repair (BER) pathway (12, 13, 22), yet very few studies have addressed the role of BER in temozolomide responsiveness (23–25).

BER is the predominant DNA repair system in mammalian cells for eliminating small DNA base lesions (26). Damaged base residues are removed by a lesion-specific DNA glycosylase, in this case alkyladenine DNA glycosylase (Aag). The resulting abasic site is recognized by an apurinic/apyrimidinic endonuclease, APE1, which incises the damaged strand, leaving 3′-OH and 5′-deoxyribose phosphate (5′dRP) groups at the margins. A DNA polymerase β (pol-β)–mediated DNA synthesis step fills the single-nucleotide gap (22, 27), and the cytotoxic 5′dRP group is removed by the 5′dRP lyase activity of pol-β (22, 28, 29). In the absence of pol-β, cells exhibit a defect in BER and are hypersensitive to the alkylating agent methyl methanesulfonate due to a failure to repair the BER intermediate 5′dRP (28–31). Alternatively, DNA polymerase λ (pol-λ) or DNA polymerase-ι (pol-ι) both encode a 5′dRP lyase activity and may also participate in BER to remove this toxic repair intermediate (32–34). Finally, DNA ligase I or a complex of DNA ligase III and XRCC1 conducts the final, nick-sealing step in the pathway (26).

We report here that temozolomide induces a significant proportion of DNA damage that is repaired by the BER pathway, that at least some of the temozolomide-induced toxicity is caused by unrepaired BER intermediates (due to incomplete repair), and that intrinsic cellular resistance to temozolomide is due, in part, to robust repair of temozolomide-induced DNA lesions by the BER pathway. We present evidence that pol-β–deficient cells are

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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significantly more susceptible to the killing effects of temozolomide than wild-type (Wt) or Aag-deficient cells and that this effect seems to be specific to pol-β–dependent BER as only a deficiency in pol-β and not pol-λ or pol-ι presented an increase in temozolomide-mediated cytotoxicity. We show that RNA interference–mediated pol-β down-regulation was sufficient to increase temozolomide efficacy in an Aag-dependent manner, as overexpressed human Aag mediated a significant increase in the temozolomide-induced cytotoxic effect when pol-β was down-regulated. This is consistent with the findings of Rinne et al. (35), showing that overexpression of Aag alone improves chemotherapeutic sensitivity of alkylating agents. Further, we show that pol-β provides cellular resistance to temozolomide by repairing lesions that trigger activation of the DNA damage response cascade.

Materials and Methods

Chemicals and reagents. Cell culture supplies were from Invitrogen-Gibco (Carlsbad, CA). Temozolomide was from the National Cancer Institute Developmental Therapeutics Program and prepared as a 100 mmol/L stock in DMSO. Methyl methanesulfonate and mitomycin C were purchased from Sigma-Aldrich (St. Louis, MO). We used the following primary antibodies: anti–pol-β (Mab clone 18S), a kind gift from S.H. Wilson (National Institute of Environmental Health Sciences, NIH, RTP, NC); anti-hAag, provided by T.R. O'Connor (City of Hope National Medical Center, Duarte, CA; ref. 36); anti–γ-H2AX (Upstate Biotechnology, Waltham, MA); anti–α-tubulin (Oncogene Research Products, San Diego, CA) and anti–proliferating cell nuclear antigen (PCNA; Santa Cruz Biotechnology, Santa Cruz, CA). All electrophoresis reagents were from Bio-Rad (Hercules, CA). Neomycin and puromycin were purchased from Invitrogen and BD Clontech (Mountain View, CA) respectively.

Plasmid expression vectors and RNA interference development. We used the following mammalian expression vectors: human Aag, pRS1422 (30, 31), and mpolβ-specific small-interfering RNA (siRNA) expression plasmids, pSuper.mpol-$\beta^{463/481}$ and pSuper.mpol-$\beta^{787/805}$. The mpolβ-specific siRNA expression plasmids were developed using the algorithm for siRNA/shRNA design from Oligoengine (Seattle, WA). Oligonucleotides were designed to target murine pol-β mRNA (mpol-$\beta^{463/481}$; sequence 5′-atgc-tgcagatgcaggata-3′ and mpol-$\beta^{787/805}$; sequence 5′-gatcagtactactgtggtg-3′) and cloned into the pSUPER vector (Oligoengine) within the *Bgl*II/*Hind*III restriction sites, yielding pSuper.mpol-$\beta^{463/481}$ and pSuper.mpol-$\beta^{787/805}$.

Cell lines and transfection conditions. Transformed MEF cell lines (92TAg, Wt; 88TAg, pol-β null; 308TAg, Aag null; and 283TAg, pol-β null/Aag null) have been described previously and are available from the American Type Culture Collection (Manassas, VA; ref. 30). Primary cultures of pol-λ null murine embryonic fibroblasts (MEF) were a kind gift from C.A. Reynaud (Faculte de Medecine Necker-Enfants Malades, Paris, France). The pol-ι null MEFs were derived from C129SvJ mice as described previously (31) and identified by PCR (not shown) to confirm the pol-ι null mutation (37). These were immortalized by SV40 large T-antigen (370TAg, pol-λ null; 369TAg, pol-ι null) as described previously (29, 30). Human Aag–over-expressing cell lines were prepared as follows: Briefly, 1.5×10^5 cells were seeded into 60 mm dishes and incubated for 24 to 30 hours at 10% CO_2 at 37°C. The Aag expression plasmid (pRS1422; refs. 30, 31) was transfected using FuGene 6 Transfection Reagent (Roche Diagnostic Corp., Indianapolis, IN) according to the manufacturer's instructions. Stable cell lines were selected in G418 (600 μg/mL) for 2 weeks, individual clones were amplified, and 20 μg of nuclear extract was analyzed by immunoblotting for the expression of human Aag protein and then reprobed for expression of pol-β and PCNA.

Transfection of the pSuper siRNA plasmids were completed as follows: Briefly, 1.5×10^5 cells were seeded into 60 mm dishes and incubated for 24 to 30 hours at 10% CO_2 at 37°C. Plasmids were transfected using FuGene 6 Transfection Reagent (Roche Diagnostic) according to the manufacturer's instructions. Stable cell lines were isolated following transfection as above followed by selection in puromycin (7.5 μg/mL) for 2 weeks. Individual clones were amplified and 20 μg of nuclear extract were analyzed by immunoblotting for the expression of endogenous pol-β protein using the Nucbuster nuclear protein extraction reagent (Novagen, Madison, WI) and then reprobed for expression of PCNA as a loading control.

Culture conditions and cell cytotoxicity assays. Transformed MEFs were cultured at 37°C in a humidified incubator with 10% CO_2 in DMEM supplemented with 10% fetal bovine serum, penicillin (50 units/mL), streptomycin (50 μg/mL), and Glutamax (4 mmol/L). Temozolomide-, methyl methanesulfonate-, and mitomycin C–induced cytotoxicities were determined by growth inhibition assays. Briefly, cells were seeded in 96-well dishes at 1,250 cells/well. After 24 hours, cells (quadruplicate wells) were exposed to serial dilutions of damaging agent in growth media for 1 or 2 hours (or as indicated in the text) at 37°C. Drug-containing medium was replaced with fresh medium and the plates were incubated at 37°C for 48 hours at which point the total cell number was determined by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (MTS; Promega, Madison, WI; ref. 38). Metabolically active cells were quantified by the bioreduction of the MTS tetrazolium compound by recording absorbance at 490 nm using a microplate reader. Results were calculated from the average of four separate experiments and are reported as the percentage of treated cells relative to the cells in control wells (% Control).

Cell extract preparation and immunoblot assays. Nuclear extracts were prepared using the NucBuster nuclear protein extraction reagent (Novagen). Protein concentration was determined by Bio-Rad protein assay reagents, according to the manufacturer's instruction. Nuclear protein (20 μg) was separated by electrophoresis in a 10% SDS-polyacrylamide gel and electrotransferred to a 0.45 μm nitrocellulose membrane (Trans-Blot, Bio-Rad). Antigens were detected using standard protocols. Primary antibodies (anti–pol-β, 1,000×; anti-hAag, 2,000×; anti–α-tubulin, 1,000×; and anti-PCNA, 1,000×) and the horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-mouse HRP or goat anti-rabbit HRP; Bio-Rad, 10,000×) were diluted in TBST/5% milk. Each membrane was stripped and reprobed with anti–α-tubulin or anti-PCNA antibodies to correct for differences in protein loading.

For γ-H2AX immunoblotting, protein was prepared as follows: Briefly, 6×10^5 cells were seeded into 150 mm dishes and incubated for 24 hours at 10% CO_2 at 37°C. Cells were treated with methyl methanesulfonate and temozolomide for 1 or 2 hours, respectively, and whole cells were scraped from the plates in 1× PBS, washed, and immediately placed on dry ice. Thawed pellets were resuspended in a 1:1 ratio of radioimmunoprecipitation assay buffer and Laemmli buffer (39). Samples were boiled for 5 minutes and protein from an equal cell number was added to each well. Protein was separated by electrophoresis in a 12% SDS-polyacrylamide gel and electrotransferred to a 0.45 μm nitrocellulose membrane (Trans-Blot, Bio-Rad). Membranes were blocked for 20 minutes in a 3% dried milk/TBS solution at room temperature and incubated with 0.5 to 1 μg/mL of anti–phospho-H2AX (Serine 139; Upstate Biotechnology) prepared in TBS/3% dried milk and 0.1% Tween 20 over night at 4°C. Membranes were washed twice with water and incubated with HRP-conjugated secondary antibody (goat anti-rabbit HRP; Bio-Rad) at room temperature for 1.5 hour. Following a series of washes with water and TBS, HRP activity was detected using the Super Signal West Femto reagent (Pierce, Rockford, IL). Each membrane was reprobed with anti-PCNA to correct for differences in protein loading, and densitometry was used to quantitate with Quantity One 1-D analysis software (Bio-Rad).

Alkyladenine DNA glycosylase activity assay. Aag activity was measured using a double-stranded oligonucleotide (21 bp) substrate containing a single etheno-adenine (eA) lesion: 5′-cctgccctgagceAgctgtggg-3′ (Trevigen, Gaithersburg, MD), as described previously (30). Twenty micrograms of whole cell protein extract were incubated with ^{32}P-5′-labeled substrate (0.5 pmol) for 0, 15, 30, 45, and 60 minutes at 37°C and product was analyzed by electrophoretic separation on a 16% polyacrylamide gel (7 mol/L urea, Tris-borate EDTA). The reaction product was visualized by autoradiography and quantified by PhosphoImager analysis.

Statistical analysis. For each figure, a two way ANOVA model was built to examine the effect of group (the cell line), dose, and the interaction of each cell line at each dose in a given figure. If the interaction effect was nonsignificant, we conclude that there was no group effect, because at the starting dose (dose = 0) the percentage of control was a constant 100%. If it was significant, then the group effect was assessed at each dose level using multiple comparisons with Bonferroni adjustment (i.e., each pairwise comparison was done at significance level 0.05 / N, where N is the number of comparisons at this dose level). Two-way ANOVA all showed significant interaction (all the F test for the interaction term had $P < 0.0001$), so in all cases, multiple comparisons were done at each dose level to see group difference. The complete results for the statistical analysis are listed in the Supplementary Data.

Results

Enhanced temozolomide-induced cytotoxicity in cells deficient in DNA polymerase-β. To determine whether methylated base lesions induced by temozolomide are repaired by the pol-β–dependent BER pathway, we compared the cytotoxicity of temozolomide in isogenic Wt, pol-β null, Aag null, and pol-β/Aag double-null cell lines (30). Temozolomide exposure conferred no increase in cytotoxicity in Aag null MEF cells (Fig. 1*A*). However, pol-β null cells were significantly more sensitive to temozolomide than either Wt or Aag null cells ($P < 0.008$ for all doses; Fig. 1*A*). Interestingly, pol-β/Aag double-null cells were more resistant to temozolomide than pol-β null cells ($P < 0.008$ for all doses), suggesting that the temozolomide-induced hypersensitivity of pol-β–deficient cells is dependent upon glycosylase-mediated initiation of repair (Fig. 1*A*) and that pol-β protects against accumulation of toxic BER intermediates, such as the toxic 5′dRP BER intermediates (28–30).

Although pol-β is the predominant polymerase participating in BER both *in vitro* and *in vivo* (22, 40), both pol-λ and pol-ι encode a nucleotidyl transferase activity and a 5′dRP lyase activity, similar to that found for pol-β, suggesting that these polymerases may participate in BER (32–34). Therefore, we directly compared cell lines deficient in the expression of pol-β, pol-λ, or pol-ι to define their role in BER *in vivo*. We found that only pol-β null cells are hypersensitive to temozolomide, whereas cells deficient in either pol-λ or pol-ι presented a Wt phenotype (Fig. 1*B*). Similar results were obtained for the alkylating agent methyl methanesulfonate (Supplementary Fig. S1). Pol-β null cells presented an increased sensitivity to temozolomide at all doses, compared with Wt, pol-λ null, or pol-ι null cells ($P < 0.008$). As expected, these cell lines were equally sensitive to the cross-linking agent mitomycin C (Supplementary Fig. S2).

Small interfering RNA mediates long-term pol-β knockdown and induces a temozolomide-hypersensitive phenotype. The above studies suggested that inhibiting pol-β expression (or activity) would be sufficient to induce a temozolomide-hypersensitive cellular phenotype and thereby improve temozolomide efficacy. We investigated whether RNA interference–mediated gene knockdown was sufficient to affect a pol-β null phenotype (e.g., temozolomide hypersensitivity). Pol-β–specific siRNA-expressing plasmids were initially screened in transient transfection assays. One pol-β–specific siRNA construct, pSuper.mpol-$\beta^{787/805}$, caused strong inhibition of expression of pol-β (not shown). We next determined if pSuper.mpol-$\beta^{787/805}$ could lead to significant and long-term knockdown of endogenous pol-β. Wt cells expressing pSuper.mpol-$\beta^{787/805}$ lost from 80% to 99.9% pol-β protein expression (Fig. 2*A*), as measured by Western blot analysis and band intensities determined by densitometric scanning.

It was our expectation that siRNA-mediated knockdown of pol-β would lead to an increase in temozolomide-mediated cell killing. Therefore, we compared the cytotoxic effect of temozolomide in Wt and pol-β null cells with three independently isolated clones (clones 2, 3, and 4) of Wt cells expressing pSuper.mpol-$\beta^{787/805}$ and a Wt cell harboring a control pSuper plasmid. The siRNA-mediated knockdown of pol-β protein expression was sufficient to increase sensitivity to temozolomide ($P < 0.003$), whereas cell lines expressing a control siRNA presented a Wt cell phenotype (Fig. 2*B*). Similar results were obtained for the alkylating agent methyl methanesulfonate (Supplementary Fig. S1). However, increasing temozolomide exposure from 2 to 48 hours increased formation of the active temozolomide-methylating species and resulted in an increase in cellular sensitivity in the pol-β knockdown cells. With this longer treatment time, temozolomide-mediated cytotoxicity for the pol-β knockdown clones was similar to pol-β null cells (Fig. 2*C*). All cell lines were equally sensitive to mitomycin C (Supplementary Fig. S2). We conclude that siRNA is effective as a long-term down-regulator of pol-β and that this down-regulation leads to an increased sensitivity to temozolomide.

Aag overexpression increases the temozolomide-hypersensitive phenotype. A second approach to increase cellular sensitivity to temozolomide is to increase BER initiation via overexpression of human Aag because increasing Aag activity promotes an increase in alkylating agent-induced cytotoxicity in the absence of pol-β (30). Two Wt^{hAag} cell clones (Wt cells overexpressing hAag) and two Wt$^{hAag/pol\beta\text{-}KD.4}$ cell clones (Wt pol-β knockdown cells overexpressing hAag) were isolated for further

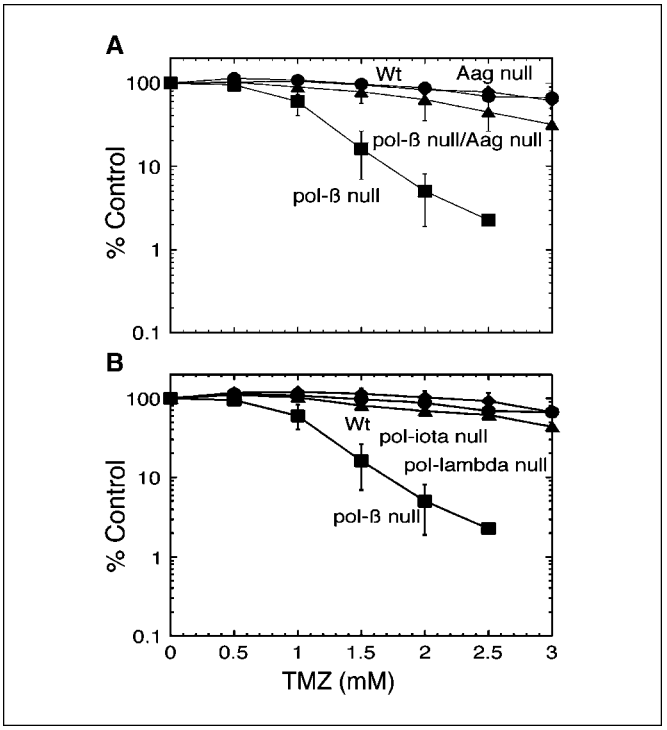

Figure 1. Temozolomide methylation damage is repaired by the pol-β–dependent BER pathway. Cell survival following temozolomide treatment. Cells were cultured for 24 hours, treated with temozolomide for 2 hours, and viable cells were measured after 48 hours by a modified MTT assay (MTS). *A,* Wt cells (●), Aag null cells (♦), pol-β null cells (■), and pol-β null/Aag null cells (▲). *B,* Wt cells (●), pol-β null cells (■), pol-λ null cells (♦), and pol-ι null cells (▲). Means are calculated from quadruplicate values in each experiment. *Points,* mean of four independent experiments; *bars,* SD.

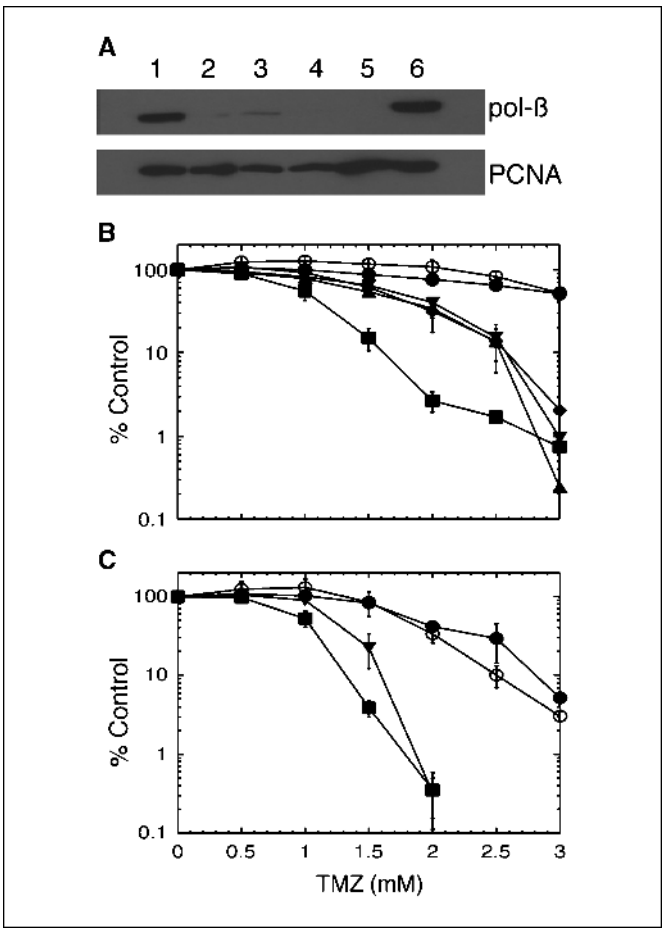

Figure 2. siRNA mediates long-term pol-β knockdown and induces a temozolomide hypersensitive phenotype. *A,* siRNA down-regulation of endogenous pol-β protein expression in Wt cells (92TAg) transfected with an siRNA-expressing plasmid (pSuper.mpol-$\beta^{787/805}$). Stable clones were selected as described in Materials and Methods. Twenty micrograms of nuclear extract were prepared from Wt cells transfected with a control pSuper vector (*lane 1*), Wt cells expressing pSuper.mpol-$\beta^{787/805}$ ($Wt^{polβ\text{-}KD.2}$, $Wt^{polβ\text{-}KD.3}$, and $Wt^{polβ\text{-}KD.4}$; *lanes 2-4*), pol-β null cells (*lane 5*), and parental Wt cells (*lane 6*) and analyzed for pol-β expression by immunoblot (*top*). Blots were reprobed for the expression of PCNA as a loading control. *B* and *C,* cell survival following temozolomide treatment. Cells were cultured for 24 hours and (*B*) cells were treated with temozolomide for 2 hours and viable cells were measured after 48 hours, or (*C*) cells were treated with temozolomide for 48 hours and viable cells were measured immediately. Viability was determined by a modified MTT assay (MTS). Wt cells (●), pol-β null cells (■), Wt cells expressing control siRNA (Wt^{Cont}, ○), Wt cells expressing pol-β–specific siRNA ($Wt^{polβ\text{-}KD.2}$, clone 2, ▲; $Wt^{polβ\text{-}KD.3}$, clone 3, ♦; and $Wt^{polβ\text{-}KD.4}$, clone 4, ▼). Means are calculated from quadruplicate values in each experiment. *Points,* mean of four independent experiments; *bars,* SD.

study; all four clones expressed equivalent levels of human Aag protein as determined by immunoblot analysis (not shown). The parental Wt, pol-β null, Wt^{Cont}, and $Wt^{polβ\text{-}KD.4}$ cells expressed low levels of Aag activity, as measured by a standard *in vitro* glycosylase assay (30). However, the hAag-overexpressing cells harbor a 20- to 35-fold increase in Aag activity (measured at 30-minute incubation time) compared with the parental cells (Supplementary Fig. S3).

Both Aag overexpressing cell lines ($Wt^{hAag.3}$ and $Wt^{hAag.8}$) presented a temozolomide hypersensitivity with 2-hours exposure ($P < 0.008$ at 2.0 and 2.5 mmol/L temozolomide) and an increased hypersensitivity with 48-hour exposure ($P < 0.008$ at temozolomide doses of 1.0 mmol/L and higher; Fig. 3*A* and *B*). Although a direct measurement of accumulated 5′dRP lesions is currently unavailable, this increased cellular sensitivity to temozolomide when hAag is overexpressed suggests that the elevated level of hAag may have generated increased levels of cytotoxic BER intermediates following temozolomide exposure, similar to that reported earlier in MEFs

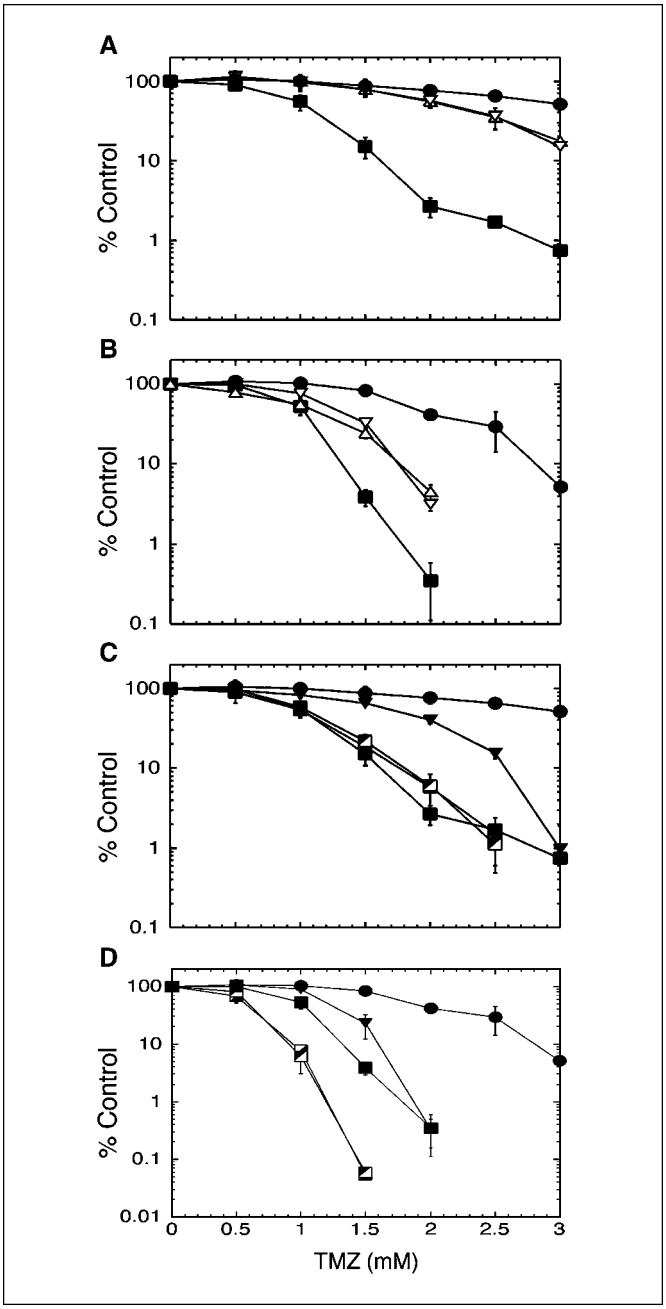

Figure 3. Overexpression of human Aag in pol-β down-regulated cells results in increased sensitivity of temozolomide. Cell survival following temozolomide treatment. Cells were cultured for 24 hours and (*A* and *C*) cells were treated with temozolomide for 2 hours and viable cells were measured after 48 hours, or (*B* and *D*) cells were treated with temozolomide for 48 hours and viable cells were measured immediately. Viability was determined by a modified MTT assay (MTS). *A* and *B,* Wt cells (●), pol-β null cells (■), Wt cells expressing hAag ($Wt^{hAag.3}$, clone 3, △; $Wt^{hAag.8}$, clone 8, ▽); *C* and *D,* Wt cells (●), pol-β null cells (■), Wt cells expressing pol-β–specific siRNA ($Wt^{polβ\text{-}KD.4}$, clone 4, ▼), Wt cells expressing pol-β–specific siRNA and hAag ($Wt^{hAag.2/polβ\text{-}KD.4}$, clone 2, ◪; $Wt^{hAag.3/polβ\text{-}KD.4}$, clone 3, ◩). Means are calculated from quadruplicate values in each experiment. *Points,* mean of four independent experiments; *bars,* SD.

(30) and breast cancer cells (35). The most prominent effect was observed upon the combination of pol-β knockdown and hAag overexpression ($Wt^{hAag.2/pol\beta\text{-}KD.4}$ and $Wt^{hAag.3/pol\beta\text{-}KD.4}$ cells; Fig. 3*C* and *D*). These cells exhibited a pol-β null-like phenotype when exposed to temozolomide for 2 hours, a significant increase in temozolomide efficacy compared with the parental $Wt^{pol\beta\text{-}KD.4}$ cells, presenting an IC_{50} of 1.25 mmol/L, similar to pol-β cells (Fig. 3*C*). Further, when exposed to temozolomide for 48 hours, temozolomide efficacy was improved almost 4-fold (Fig. 3*D*); the IC_{50} for Wt cells is 2.6 mmol/L, whereas the IC_{50} for $Wt^{hAag.3/pol\beta\text{-}KD.4}$ cells is ~0.7 mmol/L. As with the other cell lines, these cells were all equally sensitive to mitomycin C (Supplementary Fig. S2).

Increased phosphorylation of γ-H2AX in DNA polymerase-β–deficient cells exposed to temozolomide. Pol-β is essential for BER *in vivo* (28) and failure to repair alkylation-damage–induced BER intermediates leads to cell death (29). However, the mechanism of BER intermediate–induced cell death has not been determined. Therefore, we investigated if accumulation of BER intermediates (e.g., 5′dRP) leads to activation of the DNA damage response pathway by monitoring the expression of γ-H2AX following both methyl methanesulfonate and temozolomide treatment in Wt and pol-β null cells. We found that pol-β–deficient cells exhibited a clear increase in γ-H2AX expression compared with Wt cells following methyl methanesulfonate (Fig. 4*A*) and temozolomide (Fig. 4*B*) exposure. Quantitative analysis of methyl methanesulfonate- and temozolomide-induced H2AX phosphorylation is shown in Fig. 4. The immunoblots demonstrating methyl methanesulfonate- and temozolomide-induced phosphorylation of γ-H2AX are shown in Supplementary Fig. S4.

Discussion

Temozolomide is a DNA alkylating agent that is moderately effective against glioblastoma and melanoma (3–8); however, significant resistance to this agent has limited its efficacy. In this report, we present evidence that temozolomide-induced DNA damage is efficiently repaired by the pol-β–dependent BER pathway. In particular, the human methylation-specific glycosylase Aag recognizes and removes a majority of the base damage induced by temozolomide, initiating repair. Temozolomide-mediated cellular toxicity requires glycosylase initiation and, therefore, cytotoxicity is not the result of accumulated methylated base lesions. This is consistent with studies using Aag-deficient bone marrow cells that are resistant to alkylation damage (41). Conversely, the mechanism of methylated base–mediated toxicity may vary according to cell type and differentiation state, as other studies have shown that Aag-deficient embryonic stem cells are hypersensitive to alkylating agents (42).

Pol-β is required for efficient repair of methylation damage induced by temozolomide and the absence of pol-β leads to increased sensitivity to temozolomide-induced cellular toxicity. The cytotoxic effect of temozolomide is likely due to the accumulation of the BER intermediate 5′dRP, the lesion reported to mediate hypersensitivity of pol-β null cells to similar alkylating agents (29, 30). The repair of this lesion, however, can be conducted by several DNA polymerases. *In vitro* studies have shown that both pol-λ and pol-ι can participate in BER (32–34). However, we find that pol-β–deficient cells, and not cells deficient in pol-λ or pol-ι, are hypersensitive to temozolomide. These results support earlier observations that pol-β plays the major role in BER *in vivo* (28–30). In support of these findings, Haracska et. al. (43) reported that pol-ι lacks the ability to catalytically remove the cytotoxic 5′dRP lesion.

Cells engineered for RNA interference–mediated knockdown of pol-β lost 80% to 99.9% of endogenous pol-β expression. Most importantly, the siRNA-mediated inhibition of pol-β expression increased the cellular sensitivity of normally resistant Wt cells to temozolomide. It was interesting that siRNA-mediated knockdown of pol-β exhibited an intermediate temozolomide-induced cytotoxic effect, compared with pol-β null cells. One explanation for this phenomenon is that fewer toxic BER intermediates (29) accumulate due to residual pol-β protein expression. However, by increasing temozolomide treatment to allow for the formation of a greater number of temozolomide reactive metabolites, cellular survival decreased to that of pol-β null cells. Furthermore, when we increased BER initiation by increasing Aag expression, cells were further sensitized to temozolomide. Taken together, these data unequivocally shows that the BER pathway functions in the repair of temozolomide-mediated DNA damage and that inhibiting pol-β together with increasing Aag activity significantly increases temozolomide efficacy.

Previous studies have indicated that BER intermediates are DNA synthesis–blocking lesions and are cytotoxic (29, 30). Although many studies have shown that alkylating agents activate the DNA damage response pathway (44–46), the mechanism by which these repair intermediates (e.g., 5′dRP) cause a block to DNA synthesis

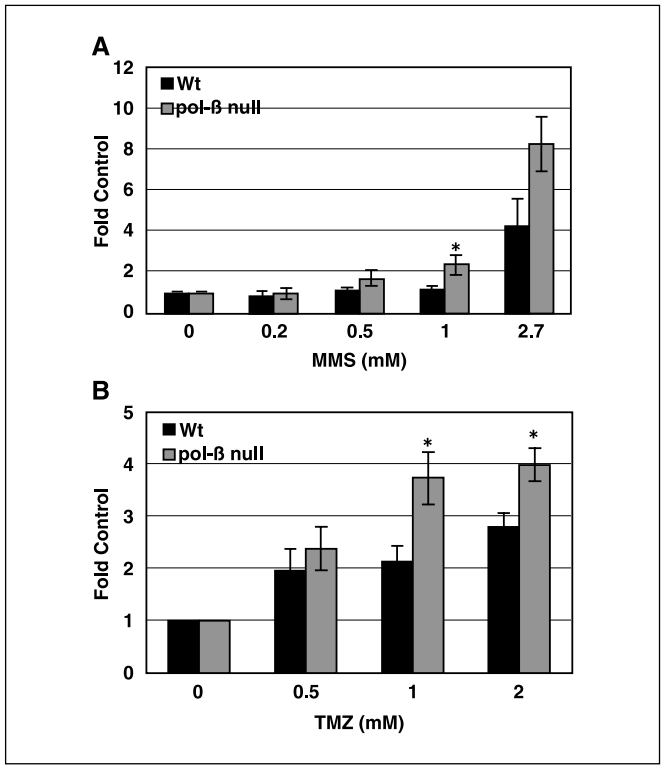

Figure 4. Deletion of pol-β results in increased expression of γ-H2AX. Phosphorylation of H2AX following treatment with methyl methanesulfonate and temozolomide in Wt and pol-β null cells. γ-H2AX expression following increasing concentrations of methyl methanesulfonate (*A*) and temozolomide (*B*) in Wt and pol-β null cells relative to PCNA as a loading control. γ-H2AX expression was quantified using Quantity One analysis software and a Bio-Rad chemi-doc Imager and represented as the fold of control normalized to PCNA expression. *Columns,* means of at least three independent experiments; *bars,* SE. *Statistical significance with $P \leq 0.05$ (two-tailed distribution; two-sample variance) between Wt and pol-β null cells at the indicated dose.

and lead to cellular toxicity is not known. Damaged DNA and blocks to replication fork progression trigger activation of the DNA damage response pathways (44–46). H2AX, a histone protein, is rapidly phosphorylated on Ser^{139} (γ-H2AX) when DNA breaks are introduced in mammalian cells following external damage and replication fork collapse (47, 48). Many of the early components of the DNA damage response colocalize with γ-H2AX at sites of breaks (49–52). Therefore, the detection of γ-H2AX is useful in monitoring the induction of DNA damage response signaling pathways. The activation of the DNA damage response pathway following treatment of pol-β–deficient cells with methylating agents shows that the accumulation of temozolomide-induced BER intermediates triggers the γ-H2AX–mediated DNA damage response. The mechanism by which BER intermediates induce this DNA damage response is unknown at this point. It is possible that the initiation of BER at lesions within clusters of damage or closely spaced lesions on both DNA strands lead to the direct formation of DNA double-strand breaks (53–55). Alternately, unrepaired BER intermediates may lead to replication fork stalling and/or collapse, the formation of a double-strand break at the site of the stalled fork, and the onset of homologous recombination (56, 57). Studies to differentiate these two mechanistic possibilities are currently under way.

These studies address the problem of therapeutic resistance of temozolomide by focusing on biochemical and molecular explanations for the mechanism of cell killing by temozolomide. Overall, we propose that BER contributes significantly to the repair of temozolomide-induced DNA damage and that modulating the BER pathway could enhance the chemotherapeutic index of agents, such as temozolomide, that damage DNA and initiate BER. Base lesions, abasic sites, and strand breaks all exhibit varying degrees of cellular toxicity, suggesting that targeting additional steps in the BER pathway, such as modulating DNA glycosylase expression (35), blocking abasic site repair (23, 25, 35), or inhibition of PARP-1 (24) may offer alternative avenues for increasing temozolomide efficacy via BER deregulation. The experiments presented herein suggest that it will be important to investigate whether an active BER pathway could be partially responsible for the temozolomide-mediated resistance seen in some tumors, and that BER capacity may help predict sensitivity to temozolomide. Finally, these findings may have clinical applicability toward the design of specific pol-β and BER modulators as adjuvant treatments for reversing a temozolomide resistant phenotype.

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