

# Mismatch Repair Mutations Override Alkyltransferase in Conferring Resistance to Temozolomide but not to 1,3-Bis(2-chloroethyl)nitrosourea<sup>1</sup>

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## Abstract

Cells with the mutator phenotype are tolerant to methylating damage from *N*-methylnitrosourea and *N*-methyl-*N'*-nitro-*N*-nitrosoguanine, exhibit replication repair errors, and have recently been found to be mutant in mismatch repair (MMR). However, resistance of cell lines with these defects to clinically used chemotherapeutic agents and the relationship of this resistance to expression of *O*<sup>6</sup>-alkylguanine-DNA alkyltransferase (AGT), which repairs DNA damage caused by methylating agents, has not been demonstrated. We compared resistance to the methylating agent temozolomide (TMZ) and to the chloroethylating agent 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), with and without AGT inhibition by *O*<sup>6</sup>-bG in several colorectal carcinoma cell lines. Two cell lines had known microsatellite instability (replication repair error-positive) and high levels of AGT, as well as a mutation in one of two MMR genes, *hMLH1* (HCT116) or *GTBP* (HCT15). Cell line SW480 had wild-type MMR genes and high AGT, and HCT116+Ch3 has previously been transduced with chromosome 3 (carrying wild-type *hMLH1*) and thus has a "corrected" MMR phenotype. SW480 exhibited the expected sensitivity to TMZ and BCNU and marked potentiation of cytotoxicity by *O*<sup>6</sup>-bG. In contrast, HCT15 and HCT116 were markedly resistant to TMZ and were not sensitized by *O*<sup>6</sup>-bG-mediated inhibition of AGT, whereas the sensitivity pattern in HCT116+Ch3 cells was similar to that in SW480. All cell lines were sensitized to BCNU by *O*<sup>6</sup>-bG. Thus, tumor cells with defects in MMR appear particularly resistant to methylating agents in a manner that overrides dependence on AGT and its inhibition by *O*<sup>6</sup>-bG. However, these cells use AGT for resistance to chloroethylating agents, providing an alternative strategy for alkylating agent therapy.

## Introduction

Colon cancer remains difficult to treat using conventional chemotherapeutic approaches. This is due in part to the complex matrix of resistance mechanisms present in colon cancer cells. In this study, we determined the role of two resistance mechanisms, AGT<sup>3</sup> and MMR, in the cytotoxicity of TMZ and BCNU. These agents form cytotoxic lesions at the *O*<sup>6</sup> position of guanine in DNA. In the past, resistance has been attributed to repair of *O*<sup>6</sup>-alkylguanine lesions by the DNA repair protein, AGT. AGT transfers the alkyl group from the *O*<sup>6</sup> position of guanine in DNA to the active site-cysteine residue, inactivating the enzyme and either restoring DNA to normal, in the case of monoadducts, or preventing DNA cross-links, in the case of chloroethyl adducts (1-3). A number of studies have demonstrated a striking correlation between resistance to BCNU and AGT levels in

cell lines and xenografts expressing high levels of AGT (4, 5). Furthermore, *O*<sup>6</sup>-bG, a potent AGT inhibitor, increases the sensitivity of tumor cells to BCNU (6-9), substantiating the importance of AGT in resistance. Recently, the deficient MMR system has been identified as a mechanism of resistance to methylating agents due to the failure of the defective MMR complex to mediate the cytotoxic response to DNA-methylating damage (10, 11).

The MMR system was first identified in *Escherichia coli*, in which it comprises the products of four genes, *MutH*, *MutL*, *MutS*, and *MutU*. *MutS* is responsible for recognition and binding at the site of the mismatched base pair. *MutH* is a GATC endonuclease that is activated by addition of *MutL*. Excision depends on the cooperative action of the MMR complex with *MutU* (12). The human homologue for *MutS* is *hMSH2*, whereas *hMLH1*, *hPMS1*, and *hPMS2* specify *MutL* homologous (13-15). Another recently identified component, *GTBP*, encodes a protein that binds to G:T mismatches and forms a heteroduplex stabilizing *hMSH2* (16, 17).

MMR defects in humans were initially shown to be responsible for the syndrome of hereditary nonpolyposis colon cancer (18, 19), which accounts for 5-13% of all colon cancer cases. These kindreds inherit one mutant MMR allele and acquire a second mutation in the development of colon cancer. Abnormalities in MMR are recognized by the acquisition of instability at microsatellite repeats, termed RER, which is detected as expansion or contraction of single, di-, or trinucleotide repeats within these regions compared to the germline. Recently, the RER+ phenotype has been described in B-cell lines of a subset of hereditary nonpolyposis colon cancer patients (20), suggesting that dominant negative mutations may exist, which disrupt the function of heterodimeric proteins.

The RER phenotype is present in a number of human colon cancer cell lines. HCT116, which carries an *hMLH1* mutation, is RER+, has a high rate of hypoxanthine-guanine phosphoribosyl-transferase mutations, and is resistant to 6-TG and MNNG (21, 22). Our group [da Costa *et al.* (23)] found that the HCT15 cell line carries mutations in *GTBP* and DNA polymerase L and shows microsatellite instability at single but not di- or trinucleotide repeats. Similarly, Aquilina *et al.* (24) noted that LoVo cells were defective in a G:T mismatch binding protein, were RER+, and exhibited MMR defects. Apparent proof that MMR defects are responsible for the RER+ phenotype was established by Koi *et al.* (22), who transferred human chromosome 3 to HCT116 cells by microcell fusion, restoring a normal *hMLH1* allele. These cells did not show microsatellite instability, were capable of MMR, and were sensitive to MNNG.

Although MMR mutant cells are tolerant of MNNG- and *N*-methylnitrosourea-induced *O*<sup>6</sup>-mG lesions, which would otherwise be lethal (10, 11), studies with methylating carcinogens do not necessarily predict the results with chemotherapeutic methylating agents, such as TMZ, dacarbazine, streptozotocin, or procarbazine. These agents may have more complex mechanisms of cytotoxicity than the carcinogens, perhaps involving lesions other than *O*<sup>6</sup>-mG, such as *N*<sup>7</sup>-methylguanine and *N*<sup>3</sup>-methyladenine, which are repaired by the base excision

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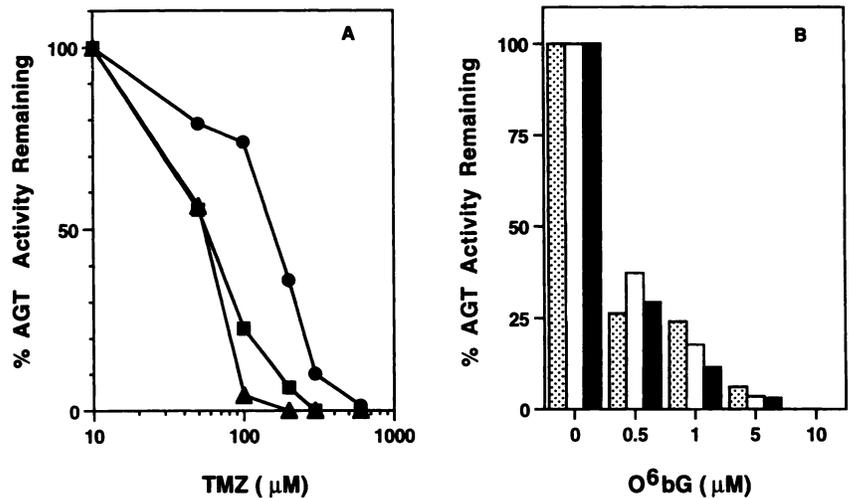
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<sup>3</sup> The abbreviations used are: AGT, *O*<sup>6</sup>-alkylguanine-DNA alkyltransferase; MMR, mismatch repair; TMZ, temozolomide; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; RER, replication error; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanine; *O*<sup>6</sup>-bG, *O*<sup>6</sup>-benzylguanine; *O*<sup>6</sup>-mG, *O*<sup>6</sup>-methylguanine; wt, wild type.

Fig. 1. A, AGT activity in human colon cancer cell lines following treatment with 50–1200  $\mu\text{M}$  TMZ for 2 h.  $\blacktriangle$ , HCT116;  $\blacksquare$ , SW480;  $\bullet$ , HCT15. B, inactivation of AGT in human colon cancer cell lines treated with 0.5–10  $\mu\text{M}$   $O^6$ -bG for 2 h. Columns, level of AGT in each cell line ( $\square$ , SW480;  $\square$ , HCT116;  $\blacksquare$ , HCT15).



repair pathway (25). In the present study, we chose a series of cell lines that express AGT but are either wt or mutant in MMR. We investigated which DNA repair mechanism (AGT or MMR mutations) provided the greatest degree of resistance to methylating and chloroethylating lesions at the  $O^6$  of guanine.

## Materials and Methods

**Chemicals and Reagents.**  $O^6$ -bG was generously provided by Dr. Robert Moschel (Frederick Cancer Research and Development Center, National Cancer Institute). A stock solution was made in DMSO. TMZ and BCNU were obtained from the Drug Synthesis and Chemistry Branch, Drug Therapeutic Program, National Cancer Institute. BCNU was prepared fresh in 0.5 ml of 100% ethanol, diluted in PBS, and used within 10 min.

**Cell Line and Cell Treatment.** All cell lines were cultured in appropriate growth medium. HCT15 and SW480 were obtained from American Type Culture Collection. HCT116 and HCT116+Ch3 were obtained from R. Boland (University of Michigan Medical Center). To measure depletion of AGT after treatment  $O^6$ -bG,  $3 \times 10^5$  cells were treated with  $O^6$ -bG (0.5–10.0  $\mu\text{M}$ ) for 2 h in serum-free medium. Cells were then rinsed with PBS, detached after 5 min of incubation in trypsin, washed twice in PBS/1 mM EDTA, and resuspended in cell extract buffer and frozen for AGT assay.

**Colony Survival Assay.** Two thousand cells/dish were plated, adhered for 18 h, and treated with or without 10  $\mu\text{M}$   $O^6$ -bG in medium for 2 h. TMZ was added at concentrations of 0–2000  $\mu\text{M}$  for 4 h, whereas 0–50  $\mu\text{M}$  BCNU was added for 2 h. After this, the medium was replaced with fresh medium. Cells

exposed previously to  $O^6$ -bG were cultured in fresh medium containing 5  $\mu\text{M}$   $O^6$ -bG. The maintenance of  $O^6$ -bG was required to maintain depletion of AGT and prevent newly synthesized protein from repairing preformed  $O^6$ -alkylguanine DNA adducts as described previously (26). The cells were grown for a further 7 days prior to staining with methylene blue for determination of colonies containing more than 50 cells.

**Alkyltransferase Assay.** The assay of AGT activity in the cell extracts was performed as previously described (27). AGT activity was measured in sonicated cell extracts by the removal of the [<sup>3</sup>H-methyl] group from  $O^6$ -[<sup>3</sup>H-methyl]-guanine present in substrate DNA, which was prepared by incubating calf-thymus DNA with [<sup>3</sup>H]*N*-methylnitrosourea for 1 h at 37°C in a HEPES assay buffer. The alkylated [<sup>3</sup>H-methyl] $O^6$ -mG and *N*<sup>7</sup>-methylguanine bases were separated by HPLC and quantified by liquid scintillation. AGT activity was expressed as fmol of  $O^6$ -mG removed/ $\mu\text{g}$  of DNA.

## Results

AGT inactivation by TMZ and  $O^6$ -bG. The MMR wt, non-RER cell line SW480 and two MMR mutant, RER+ cell lines, HCT116 and HCT15, expressed high levels of AGT ( $6.7 \pm 1.3$ ,  $22.5 \pm 4.5$ , and  $7.0 \pm 0.9$  fmol/ $\mu\text{g}$  of DNA, respectively). We next compared the cell lines for the kinetics of depletion of AGT by TMZ and  $O^6$ -bG. As shown in Fig. 1A, depletion of AGT by TMZ, due to repair of  $O^6$ -mG DNA adducts resulted in an  $\text{EC}_{50}$  of 58  $\mu\text{M}$  in both SW480 and HCT116 but was 180  $\mu\text{M}$  in HCT15, the AGT level of which was

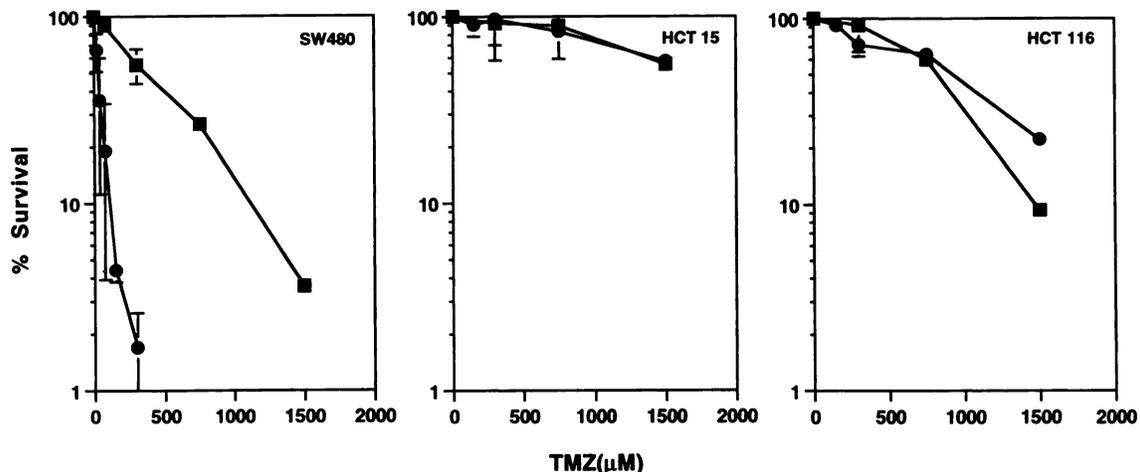


Fig. 2. Cytotoxicity of TMZ plus  $O^6$ -bG in human colon cancer cell lines. Cells were treated with 150–1500  $\mu\text{M}$  TMZ alone or with 10  $\mu\text{M}$   $O^6$ -bG for 2 h prior to 4 h exposure to TMZ.  $\blacksquare$ , TMZ alone;  $\bullet$ ,  $O^6$ -bG plus TMZ; bars, SD.

Table 1 Comparison of cytotoxicity ( $IC_{50}$ ) of TMZ and BCNU with and without  $O^6$ -bG in human colon cancer cell lines

Cell line	MMR phenotype	TMZ ( $\mu$ M)	TMZ + $O^6$ -bG ( $\mu$ M)	BCNU ( $\mu$ M)	BCNU + $O^6$ -bG ( $\mu$ M)
HCT 15	-	1600	1600	53	20
HCT 116	-	875	875	30	7
SW 480	+	350	25	12	3
HCT116+Ch3	+	167	25	29	6

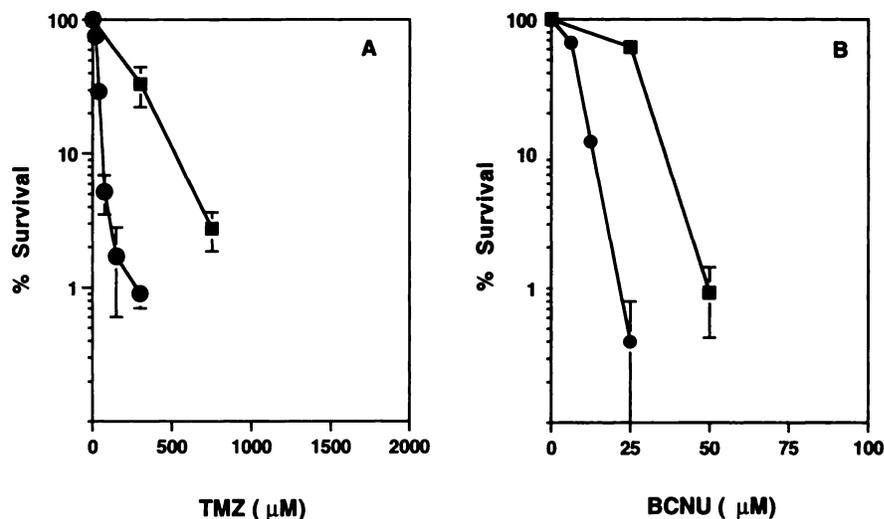


Fig. 3. Comparison of the cytotoxicity induced by  $O^6$ -bG plus TMZ (A) and by BCNU (B) in the human colon cancer cell line HCT116+Ch3. Cells were treated with TMZ or BCNU alone or with TMZ or BCNU plus  $10 \mu$ M  $O^6$ -bG for 2 h prior to 4 h exposure to TMZ or 2 h exposure to BCNU. ■, TMZ or BCNU alone; ●, TMZ or BCNU plus  $O^6$ -bG; bars, SD.

3-fold higher than SW480 and HCT116. Thus, the TMZ  $EC_{50}$  for AGT correlated with AGT activity, which is equivalent to the capacity to remove  $O^6$ -mG DNA adducts formed by TMZ. On the other hand, AGT inactivation by  $O^6$ -bG was similar in these cell lines (Fig. 1B), most likely because this reaction occurs in the presence of excess  $O^6$ -bG. Ten  $\mu$ M  $O^6$ -bG (the concentration used in subsequent experiments) was able to deplete AGT by over 95% in each cell line, indicating that inhibition of AGT by  $O^6$ -bG was independent of the MMR or RER phenotype.

**Cytotoxicity of TMZ.** Clonogenic survival was compared after exposure to TMZ at concentrations of 0–2000  $\mu$ M in the absence or presence of  $10 \mu$ M  $O^6$ -bG (Fig. 2). Because the maximum TMZ serum concentrations in patients is  $75 \mu$ M,<sup>4</sup> concentrations higher than this would correlate with clinical resistance to TMZ. Because recovery of AGT after withdrawal of  $O^6$ -bG allows removal of  $O^6$ -mG lesions induced by TMZ,  $O^6$ -bG was left in the culture medium during the entire period of colony formation to prevent recovery of AGT and repair of DNA adducts. MMR wt SW480 cells were moderately resistant to TMZ, with an  $IC_{50}$  of 350  $\mu$ M, which was reduced 14-fold to 25  $\mu$ M by  $O^6$ -bG pretreatment. Greater resistance to TMZ was observed in the two MMR mutant cell lines even after inhibition of AGT by  $O^6$ -bG. For HCT116 cells, the TMZ  $IC_{50}$  was 875  $\mu$ M, and for HCT15 cells, it was 1600  $\mu$ M (Table 1); neither cell line was sensitized to TMZ by  $O^6$ -bG. The resistance to TMZ observed in HCT116 cells was abrogated in cells carrying the chromosome 3 transfection, HCT116+Ch3 (Fig. 3A). In these cells, the  $IC_{50}$  for TMZ was 160  $\mu$ M, compared to 875  $\mu$ M in HCT116 cells, and after exposure to  $O^6$ -bG, the TMZ  $IC_{50}$  in HCT116+Ch3 cells was 25  $\mu$ M, similar to that in SW480 cells. Thus, in the presence of  $O^6$ -bG, the RER+, MMR mutant cell lines are 35–64-fold more resistant to TMZ than MMR wt and non-RER cell lines.

**Cytotoxicity of BCNU with or without  $O^6$ -bG in Colon Cancer Cell Lines.** Because MMR status was clearly important in resistance to the methylating agent TMZ, we measured the cytotoxicity of

BCNU, which has the potential to induce both DNA monoadduct and cross-links in these cell lines. We were interested in whether recognition of these lesions, particularly the monoadduct, by the MMR complex would influence its cytotoxicity or whether, in contrast to MMR, AGT was a primary mechanism of resistance. Cells were exposed to 0–50  $\mu$ M BCNU with or without  $O^6$ -bG. Relative resistance to BCNU was observed in all cell lines expressing AGT, but all were sensitized to BCNU by  $O^6$ -bG (Fig. 4 and Table 1). Dose modification factors at the  $IC_{50}$  were in the range of 3–4. A similar response to BCNU alone or with  $O^6$ -bG was observed in HCT116+Ch3 (Fig. 3B) and HCT116 (Fig. 4), indicating that restoration of MMR did not have an impact on resistance to BCNU. Thus, there was no evidence that MMR status influenced resistance to BCNU.

## Discussion

The mechanism by which  $O^6$ -mG leads to cell death appears to involve MMR recognition of unrepaired  $O^6$ -mG adducts paired to either cytosine or thymine (28). The initiation of MMR results in DNA strand breaks, removal of one of the strands, and resynthesis followed by ligation. If DNA replication has produced  $O^6$ -mG:thymine base pairs, then the newly synthesized strand containing the thymine is preferentially removed (12) and the “repair” process fails to remove the offending adduct, leading to repetitive efforts at repair, strand breaks, and ultimately cell death. Despite this understanding of the mechanism of MMR, there have not been reports of the role of this pathway in resistance to clinically used methylating agents. This is an important issue because there are a number of DNA adducts formed by these clinical agents that may contribute to cytotoxicity, lessening the impact of  $O^6$ -mG lesions. Thus, heretofore, it has been unclear whether MMR is an important mechanism of cell death in tumor cells exposed to methylating chemotherapeutic agents. Our results show that AGT is the first line of defense against agents that alkylate the  $O^6$  of guanine in DNA and that removal of  $O^6$ -mG prevents cytotoxicity of methylating agents. For cells with normal MMR, high levels of

<sup>4</sup> Unpublished results.

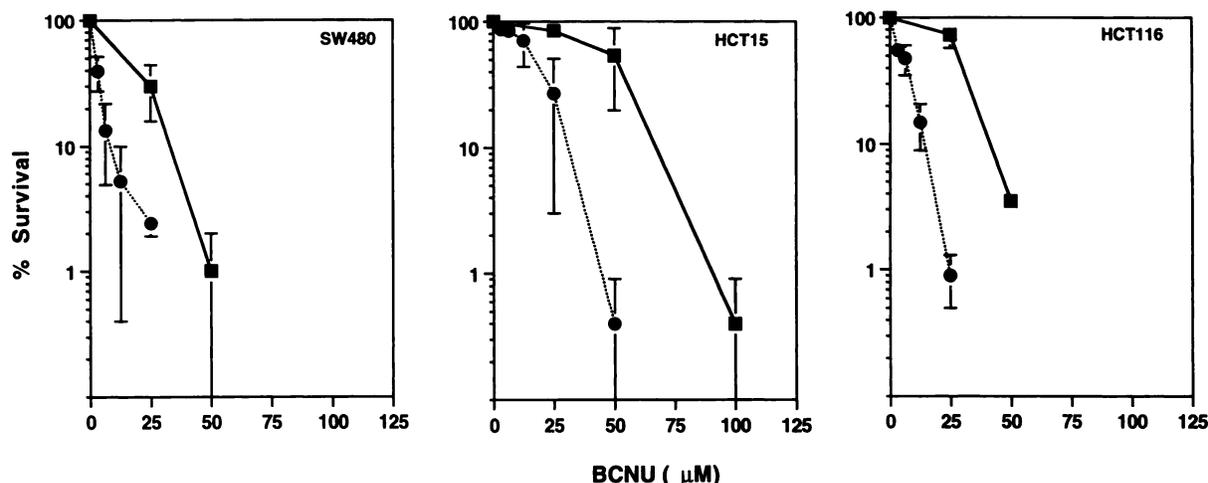


Fig. 4. Survival fraction of human colon cancer cell lines after exposure to BCNU plus  $O^6$ -bG. Cells were treated with 25–100  $\mu$ M BCNU or with 25–100  $\mu$ M BCNU plus 10  $\mu$ M  $O^6$ -bG for 2 h prior to 2 h exposure to BCNU. ■, BCNU alone; ●,  $O^6$ -bG plus BCNU; bars, SD.

AGT prevent cytotoxic sequela by removal of the  $O^6$ -alkylguanine DNA adducts. Inactivation of AGT by a potent inhibitor,  $O^6$ -bG, sensitizes cells to killing by both methylating and chloroethylating agents.

Our observations in MMR-defective cell lines HCT116 and HCT15 indicate that in the absence of MMR, cells are extremely resistant to TMZ and that this resistance is not abated by inhibition of AGT with resultant persistence of  $O^6$ -mG DNA adducts. Of interest, comparing these cell lines, HCT15, which has a defect in *GTBP* and low levels of *hMSH2*, is more resistant to TMZ than HCT116, which has a defect in *hMLH1*. It may be that loss of recognition of the adduct by loss of *hMSH2* and *GTBP* makes cells more resistant than loss of *hMLH1*, which initiates the repair process. It is remarkable that inactivation of AGT by  $O^6$ -bG failed to sensitize cells to TMZ, suggesting that even large numbers of DNA adducts in and of themselves are not cytotoxic, although they may be very mutagenic. Thus, MMR mutations appear to override the AGT mechanism of resistance to methylating agents.

This conclusion is corroborated by results with HCT116+Ch3, which contains a normal copy of *hMLH1* on chromosome 3 and which has been shown to have correction of microsatellite instability (22), indicating that genes present on chromosome 3 (*hMLH1* and perhaps others as well) are responsible for sensitizing the cells to TMZ. HCT116+Ch3 has AGT activity similar to that of HCT116, suggesting that the level of AGT does not account for the observed difference in sensitivity to TMZ, particularly in the presence of  $O^6$ -bG. We predict that in patients with colon and other malignancies, mutations in MMR, which leads to the phenotype of RER+, will lead to clinical resistance to methylating chemotherapeutic agents and, when present, will block the potentiation effect of the AGT inhibitor  $O^6$ -bG. We further predict that screening tumors for the MMR mutator phenotype would identify nonresponders to methylating agent therapy.

BCNU, on the other hand, induces  $O^6$ -chloroethylguanine DNA adducts, which form interstrand DNA cross-links following the formation of the intermediate  $O^6$ -*N*<sup>1</sup>-ethanoguanine (29). In cells with high AGT, few if any cross-links form, and cytotoxicity appears to be due to other processes (30). These cross-links disrupt DNA synthesis and, like methylating agents, give rise to chromosomal aberrations, rearrangements, sister chromatid exchanges, and strand breaks (31, 32), leading to cell death. Of interest, only a few cross-links are required for cytotoxicity, whereas over 6000  $O^6$ -mG lesions are required for cell death following methylating agent exposure (33). We

observed no effect of MMR status on resistance to BCNU or to sensitization by  $O^6$ -bG. In addition, there was no impact of correction of the *hMLH1* defect in HCT116 cells. From this, we conclude that neither the pre-cross-link monoadduct, the cyclic intermediate, nor the cross-link is recognized by the MMR complex, suggesting that the cytotoxicity of BCNU is due entirely to non-MMR-mediated processes. Furthermore, we predict that tumors resistant to methylating agents should be sensitive to BCNU plus  $O^6$ -bG.

In summary, TMZ-induced  $O^6$ -mG lesions appear to be the predominant site responsible for killing colon cancer cells that are wt for MMR. MMR mutant cell lines are remarkably resistant to TMZ, even in the presence of  $O^6$ -bG inhibition of AGT, but in one instance, they are sensitized by introduction of wt *hMLH1*. In MMR wt cells, depletion of AGT potentiated TMZ cytotoxicity by at least 10-fold, which is greater than the potentiation observed with BCNU plus  $O^6$ -bG compared to BCNU alone. Thus,  $O^6$ -bG plus TMZ may be an effective therapy in patients with tumors containing wt MMR. In both MMR wt and MMR mutant cells, BCNU had similar efficacy, and its cytotoxicity was enhanced by AGT depletion. Because MMR activity was not involved in BCNU-mediated cytotoxicity, BCNU and  $O^6$ -bG may be an effective combination in patients with colon cancer regardless of MMR status.

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