Effect of O6-Benzylguanine on Alkylating Agent-induced Toxicity and Mutagenicity in Chinese Hamster Ovary Cells Expressing Wild-Type and Mutant O6-Alkylguanine-DNA Alkyltransferases

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ABSTRACT

The DNA repair protein O6-alkylguanine-DNA alkyltransferase (AGT) has been shown to protect cells from the toxic and mutagenic effect of alkylating agents by removing lesions from the O6 position of guanine. O6-Benzylguanine (BG) is a potent inactivator of AGT, resulting in an increase in the sensitivity of cells to the toxic effects of chemotherapeutic alkylating agents. Chinese hamster ovary (CHO) cells and CHO cells transfected with wild-type AGT (CHOWTAGT) and a mutant AGT [P138M/V139I/P140K (CHO MIK)] known to be resistant to BG were treated with BG and various alkylating agents. BG treatment alone dramatically decreased AGT activity in CHO WTAGT cells but resulted in no depletion in AGT activity in CHO MIK cells. In the absence of AGT, these cells are highly sensitive to the toxic and mutagenic effects of temozolomide and BCNU, and treatment with BG resulted in a significantly higher cell killing and mutation frequency. CHO MIK cells were completely resistant to temozolomide or BCNU in the presence and absence of BG. Both cell killing and mutation frequency of 4-hydroperoxycyclophosphamide (4-HC) in CHO, CHO WTAGT, and CHO MIK cells were increased in the presence of BG. 4-HC generates two active metabolites, phosphoramidate mustard (PM) and acrolein. BG had no effect on 4-hydroperoxycyclophosphamide (which generates acrolein and a nonalkylating form of PM) in CHO cells and CHO MIK cells, but enhancement of toxicity was observed with PM in both these cell lines. Therefore, we attribute the enhancement to the PM metabolite of 4-HC. Our results demonstrate that wild-type AGT plays an important role in protecting against the toxic and mutagenic effect of O6 alkylating agents and that a mutant AGT resistant to inactivation by BG effectively prevents BG-enhanced toxicity and mutagenicity induced by these agents. Expression of the AGT protein contributes to resistance of 4-HC. BG also enhances the toxicity of 4-HC and PM by a mechanism that may not involve the AGT repair protein.

INTRODUCTION

Alkylating agents comprise a broad class of agents that produce a reactive alkylating species capable of reacting with nucleophilic sites on DNA (1–3). Methylation and chloroethylating agents (i.e., temozolomide and BCNU) are known to produce a toxic/mutagenic lesion at the O6 position of guanine. Adducts produced at the O6 position are strongly miscoding and toxic lesions. The DNA repair protein AGT (1) is a unique protein that is able to remove O6-guanine-DNA adducts and restore the original DNA in a single step (4–6). There are no other proteins or cofactors involved in this reaction, and the protein is inactivated in the repair process. By removing O6 adducts, the AGT protein limits the production of mutations and/or toxic lesions in response to carcinogenic and chemotherapeutic alkylating agents.

BG is a potent, specific inactivator of the AGT protein that results in depletion of AGT activity and subsequently increases the sensitivity of tumor cells and tumor xenografts to the antitumor effects of agents that alkylate O6-guanine in DNA (7). BG in combination with BCNU is presently in Phase II clinical trials. The dose-limiting toxicity of the combination of BC and BCNU is bone marrow suppression (8–10). One way to overcome enhanced hematopoietic toxicity and the risk of mutational events that may eventually lead to drug-related leukemias in these patients would be to use gene therapy to express an alkyltransferase gene in the relevant bone marrow stem cells. Expression of mutant AGT protein in hematopoietic progenitor cells by gene therapy techniques has been used to increase their AGT activity and provide a form resistant to BG (11–14). Preclinical evidence suggests that expression of BG-resistant AGTs in normal bone marrow results in protection against alkylating agent-induced toxicity and mutagenicity and may result in an increase in the therapeutic index for treatment of tumors that express wild-type AGT that is sensitive to inactivation by BG.

In efforts to determine the role of wild-type and mutant AGTs in protection against enhanced toxicity and mutagenicity induced by the combination of BG plus alkylating agents, we embarked on cell toxicity and mutagenicity studies of the combination in CHO cells and in CHO cells transfected with wild-type AGT (CHO WTAGT cells) and MIK (CHO MIK cells), a mutant AGT resistant to BG. We focused on three alkylating agents, a chloroethylating agent (BCNU), a methylating agent (temozolomide), and an oxazaphosphorine (4-HC; the activated form of cyclophosphamide). The rationale for evaluating BCNU and temozolomide is that these compounds are being evaluated in combination with BG in clinical trials. Although BG and cyclophosphamide are not in clinical trials, several recent studies suggest a role for AGT in protecting against the toxic and mutagenic effects of this agent (15, 16).

MATERIALS AND METHODS

Materials. Mutant AGT (P138M/V139I/P140K) resistant to inactivation by BG was generated from a library containing a random sequence at positions 138–140 (17). BG was generously provided by Dr. Robert C. Moschel (National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD). The synthesis of 4-HC and 4-HDC have been described previously (18, 19). PM (as the cyclohexylammonium salt), temozolomide, and BCNU were obtained from the National Cancer Institute Drug Synthesis and Chemistry Branch (Bethesda, MD). All other biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Construction of pcDNA-AGT and pcDNA-MIK. Wild-type agt gene was subcloned and constructed as described previously (15). The small fragment defined by SfI and Age I in the wild-type and MIK mutant AGT cDNAs that have additional silent restriction sites (17) was used to replace the

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3 The abbreviations used are: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; AGT, O6-alkylguanine-DNA alkyltransferase; BG, O6-benzylguanine; CHO, Chinese hamster ovary; 6-TG, 6-thioguanine; 4-HC, 4-hydroperoxycyclophosphamide; 4-HDC, 4-hydroperoxydidechlorocyclophosphamide; PM, phosphoramidate mustard.

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corresponding region in pcDNA3-AGT. The mutations have been confirmed by sequencing on an ABI377 DNA sequencer. The final constructs were propagated in Escherichia coli and purified by CsCl-EtBr ultracentrifugation for transfection (Table 1).

**Cell Transfection.** CHO cells were transfected with pcDNA-AGT (CHO\(^{WTAGT}\)) or pcDNA-MIK (CHO\(^{MIK}\)) by electroporation using Electro Cell Manipulator (ECM 600; BTX Electronic Genetics, San Diego, CA) as described previously (20) and cultured to produce a cloned stable cell line.

**Depletion of AGT Activity by BG.** CHO\(^{WTAGT}\) and CHO\(^{MIK}\) cells were plated at a density of 1.4 × 10\(^5\) cells/T75 flask and treated 24 h later with DMSO (control), 25 μM, or 100 μM BG in DMEM supplemented with 10% fetal bovine serum. Extracts were prepared from CHO clones with stable expression of wild-type AGT cDNA or MIK cDNA by homogenization in 50 mM Tris (pH 7.5), 0.1 mM EDTA, 5 mM DTT, and 50 μg of DNA. AGT activity was determined as described previously (21, 22). Briefly, cell extracts were incubated with \(^3\)H-methylated DNA substrate (5.77 Ci/mmol) for 30 min. The DNA was precipitated by adding ice-cold perchloric acid at a final concentration of 0.25% and hydrolyzed in 0.1 N HCl at 70°C for 30 min. The modified bases were eluted on a C\(_{18}\) reverse-phase column. Protein concentration was determined by the method of Bradford (23). The results were expressed as femtomoles of 0\(^\circ\)-methylguanine released from DNA per milligram of protein.

**Assay for Cell Survival.** The cytotoxicity induced by temozolomide, BCNU, 4-HC, 4-HDC, and PM was determined by loss of colony-forming ability as described previously (24). Briefly, CHO, CHO\(^{WTAGT}\), and CHO\(^{MIK}\) cells were plated at a density of 1.4 × 10\(^5\) cells/T75 flask and treated 24 h later with DMSO (control), 25 μM, or 100 μM BG in DMEM supplemented with 10% fetal bovine serum. Extracts were prepared from CHO clones with stable expression of wild-type AGT cDNA or MIK cDNA by homogenization in 50 mM Tris (pH 7.5), 0.1 mM EDTA, 5 mM DTT, and 50 μg of DNA. AGT activity was determined as described previously (21, 22). Briefly, cell extracts were incubated with \(^3\)H-methylated DNA substrate (5.77 Ci/mmol) for 30 min. The DNA was precipitated by adding ice-cold perchloric acid at a final concentration of 0.25% and hydrolyzed in 0.1 N HCl at 70°C for 30 min. The modified bases were eluted on a C\(_{18}\) reverse-phase column. Protein concentration was determined by the method of Bradford (23). The results were expressed as femtomoles of 0\(^\circ\)-methylguanine released from DNA per milligram of protein.

**Assay for Mutation Frequency in Transduced CHO Cells.** Cells were plated and treated as described above, with a slight modification. Posttreatment of cells with BG lasted for 24 h instead of 16 h. The cells were maintained in exponential growth for an additional 7-day expression period before 1 × 10\(^5\) cells were plated into a 100-mm dish with 5 μg/ml 6-TG. Cells were incubated for approximately 10 days to allow the formation of colonies. Mutation frequency was determined by counting 6-TG-resistant colonies and expressed as the number of 6-TG resistant colonies per 10\(^6\) surviving cells.

### RESULTS

**Effect of BG on the Level of AGT Activity in CHO\(^{WTAGT}\) and CHO\(^{MIK}\) Cells.** AGT activity in CHO\(^{WTAGT}\) or CHO\(^{MIK}\) cells is 1913 (15) and 1432 fmol/mg protein, respectively (Fig. 1A, inset). CHO cells have undetectable AGT activity (15). On incubation of CHO\(^{WTAGT}\) cells with 25 or 100 μM BG for 2, 6, or 24 h, there was a dramatic decrease of AGT activity (Fig. 1A). In contrast, no decrease in AGT activity was observed when CHO\(^{MIK}\) cells were treated with 25 or 100 μM BG for 24 h (Fig. 1B).

**Effect of BG on Temozolomide-induced Cytotoxicity and Mutagenicity in CHO, CHO\(^{WTAGT}\), and CHO\(^{MIK}\) Cells.** The effect of BG (100 μM) on temozolomide-induced cytotoxicity and mutagenicity was determined by assaying colony-forming ability and 6-TG-resistant mutants in CHO, CHO\(^{WTAGT}\), and CHO\(^{MIK}\) cells (Fig. 2). As a result of undetectable AGT activity, CHO cells are exquisitely sensitive to the toxic and mutagenic effects of temozolomide, and there is no enhancement of the sensitivity of CHO cells to the effects of temozolomide in the presence of BG (Fig. 2, A and D). In contrast, CHO\(^{WTAGT}\) and CHO\(^{MIK}\) cells are resistant to the toxic and mutagenic effects of temozolomide (Fig. 2, B, C, E, and F). Both the cell killing and mutation frequency induced by temozolomide in CHO\(^{WTAGT}\) cells are substantially increased after treatment with BG (Fig. 2, B and E). The percentage of CHO\(^{WTAGT}\) cell survival after treatment with the combination of 300 μM temozolomide plus BG decreased 7 times compared with cells treated with temozolomide alone (Fig. 2B). The mutation frequency induced by 300 μM temozolomide increased from 7 TG-resistant colonies/10\(^6\) surviving cells to 200 TG-resistant colonies/10\(^6\) surviving cells in the presence of BG (Fig. 2E). Expression of MIK resulted in full protection against the toxic and mutagenic effects of both temozolomide alone and BG combined with temozolomide (Fig. 2, C and F). At 300 μM temozolomide, 100% of cells survived in the presence and absence of BG (Fig. 2C).

<table>
<thead>
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<th>AGTMIK</th>
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+ Bold refers to 3 amino acid change

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**Fig. 1.** Effect of BG on AGT activity in CHO\(^{WTAGT}\) cells and CHO\(^{MIK}\) cells. CHO cells expressing wild-type AGT or MIK were treated with 0 μM (●), 25 μM (▲), and 100 μM BG (▪) for 2, 6, and 24 h. The results are plotted as a percentage of AGT activity in untreated cells. Control AGT activity is expressed in femtomoles/milligram of protein (inset). Each data point represents an average of duplicate samples from two separate experiments.
Effect of BG on BCNU-induced Cytotoxicity and Mutagenicity in CHO, CHO<sup>WTAGT</sup>, and CHO<sup>Mik</sup> Cells. CHO cells lacking AGT activity are equally sensitive to the toxic and mutagenic effects of BCNU in the presence and absence of BG (Fig. 3, A and D). CHO<sup>WTAGT</sup> and CHO<sup>Mik</sup> cells were resistant to the toxic and mutagenic effects of BCNU (Fig. 3, B, C, E, and F). BG dramatically potentiated the cytotoxic and mutagenic effects of BCNU in CHO<sup>WTAGT</sup> cells. Cell survival after treatment with 80 μM BCNU was 87% in the absence of BG and 6% in the presence of BG (Fig. 3B). CHO cells expressing Mik were protected against the toxic and mutagenic effects of BCNU even after treatment with BG (Fig. 3, C and F).

Effect of BG on 4-HC-induced Cytotoxicity and Mutagenicity in CHO, CHO<sup>WTAGT</sup>, and CHO<sup>Mik</sup> Cells. BG enhanced the sensitivity of CHO cells to the toxic and mutagenic effects of 4-HC (Fig. 4, A and D). Wild type and Mik AGTs offered protection against 4-HC-induced cytotoxicity; however, BG increased the sensitivity of cells expressing wild-type and mutant AGT to 4-HC-induced toxicity by 5- and 3-fold, respectively (Fig. 4, B and C). The number of mutants per 10<sup>6</sup> cells increased 3.5 and 3.9 times in wild-type and Mik cells after treatment with BG plus 4-HC (20 μM) compared to treatment with 4-HC alone, respectively (Fig. 4, D and E). One possible explanation for enhancement of 4-HC toxicity by BG in Mik cells is if BG alone or BG plus 4-HC resulted in a depletion of AGT.

Fig. 2. Effect of BG on cytotoxicity and mutagenicity induced by temozolomide. CHO, CHO<sup>WTAGT</sup>, and CHO<sup>Mik</sup> cells were treated with temozolomide in the presence (●) or absence of BG (○) as described in “Materials and Methods.” Each data point represents the mean ± SD from two to three separate experiments. Each experiment represents 5 and 20 replicate dishes/treatment group for survival and mutation, respectively.

Fig. 3. Effect of BG on cytotoxicity and mutagenicity induced by BCNU. CHO, CHO<sup>WTAGT</sup>, and CHO<sup>Mik</sup> cells were treated with BCNU in the presence (●) or absence of BG (○) as described in “Materials and Methods.” Each data point represents the mean ± SD from two to three separate experiments. Each experiment represents 5 and 20 replicate dishes/treatment group for survival and mutation, respectively.
activity. However, there was no difference in AGT activity in CHO\textsuperscript{MK} cells in the presence or absence of either BG alone (Fig. 1B) or the combination (data not shown). AGT does contribute to resistance because there is greater cell killing in CHO cells than in CHO\textsuperscript{WTAGT} and CHO\textsuperscript{MK} cells after treatment with 4-HC. However, a second mechanism unrelated to AGT is contributing to BG-enhanced toxicity of 4-HC as discussed in the next section.

**Effect of BG on PM- and 4-HDC-induced Cytotoxicity in CHO Cells.** 4-HC generates two active metabolites, PM and acrolein (25). To determine which metabolite of 4-HC might be responsible for the observed effect, the toxic effects of each separate metabolite in CHO cells in the presence and absence of BG were examined (Fig. 5). This was accomplished by using authentic PM in one experiment and 4-HDC in another. 4-HDC is an analogue of 4-HC that generates acrolein and a nonalkylating cognate of PM (19). Much higher concentrations of PM and 4-HDC are required for cell toxicity compared to 4-HC (Figs. 5 and 6). At pH 7.4, PM is anionic and has poor membrane permeability, as demonstrated previously by cell perfusion and \textsuperscript{31}P nuclear magnetic resonance techniques (26, 27). There are much data to support the conclusion that PM generated extracellularly will not lead to the same level of therapeutic benefits as that generated intracellularly (25). Higher concentrations of 4-HDC are required for toxicity because acrolein is less toxic than intracellularly generated PM after degradation of 4-HC.

As shown in Fig. 5, the percentage of cell survival in CHO and CHO\textsuperscript{WTAGT} cells after treatment with the combination of BG and 4-HDC was similar to the effect of 4-HDC alone, ruling out acrolein as a substantial contributor to the observed enhancement. In fact, only at the highest dose of 4-HDC is there an apparent increase in cell kill after BG treatment, consistent with a previous observation that AGT contributes to resistance to 4-HDC (15). However, treatment of CHO, CHO\textsuperscript{WTAGT}, and CHO\textsuperscript{MK} cells with the combination of BG and PM resulted in a decrease of cell survival compared to treatment with PM alone, suggesting a mechanism for enhancement of PM toxicity irrespective of AGT activity (Fig. 6).

**DISCUSSION**

Our results demonstrate that both wild-type and mutant forms of AGT play a critical role in protecting against the toxic and mutagenic effects of alkylating agents such as temozolomide and BCNU. BG enhances the sensitivity of cells expressing wild-type AGT protein to

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**Fig. 4.** Effect of BG on cytotoxicity and mutagenicity induced by 4-HC. CHO, CHO\textsuperscript{WTAGT}, and CHO\textsuperscript{MK} cells were treated with 4-HC in the presence (●) or absence of BG (○) as described in “Materials and Methods.” Each data point represents the mean ± SD from two to three separate experiments. Each experiment represents 5 and 20 replicate dishes/treatment group for survival and mutation, respectively.

**Fig. 5.** Effect of BG on 4-HDC-induced cytotoxicity in CHO, CHO\textsuperscript{WTAGT}, and CHO\textsuperscript{MK} cells. CHO (A), CHO\textsuperscript{WTAGT} (B), and CHO\textsuperscript{MK} (C) cells were treated with the compounds in the presence (●) or absence of BG (○) as described in “Materials and Methods.” The data are an average from two to three separate experiments. Each experiment represents five replicate dishes/treatment group.
the biological effects of these alkylating agents, whereas expression of mutant AGT forms resistant to BG offers complete protection against the toxic and mutagenic effects in the presence and absence of BG.

Several studies have demonstrated a role for the AGT protein in protecting against the toxic effects of methylation agents (i.e., temozolomide) and chloroethylylating agents [i.e., BCNU (28–31)]. We now demonstrate that MIK protects against the mutagenic effects of methylation and chloroethylylating agents, suggesting that the introduction of mutant AGT genes, including MIK, into the relevant hematopoietic stem cells may be a useful strategy to protect against mutagenic damage and therapy-related leukemia caused by these agents as well. Although AGT activity was lower in MIK-expressing cells than in wild-type cells, the protection offered by MIK against temozolomide-induced toxicity was greater than that observed in cells expressing wild-type AGT, regardless of whether BG was present or not. One possible explanation is that the activity in the cell is not fully represented by measuring activity in extract. The MIK protein may be less stable in an extract preparation than in the cell. Another possible explanation is that temozolomide may directly inactivate wild-type AGT protein as suggested by Lee et al. (32), whereas mutant MIK may be resistant to direct inactivation by temozolomide. BG enhanced the toxicity of 4-HDC at the highest dose tested in CHO\textsuperscript{WTAGT} cells, but no enhancement was observed in CHO\textsuperscript{MK} and CHO cells. Recently, we demonstrated that the AGT protein plays a role in resistance to the toxic and mutagenic effects of 4-HC and 4-HDC (15). Our present data demonstrating enhancement in wild-type AGT-expressing cells after treatment with BG plus 4-HDC are consistent with this finding. It is possible that AGT repairs lesions introduced by acrolein or that AGT, by directly acting with acrolein, reduces the amount of acrolein available for reaction with DNA (15). However, depletion of AGT by BG does not explain enhanced toxicity and mutagenicity of 4-HC in CHO or CHO\textsuperscript{MK} cells.

BG increases sensitivity to 4-HC and PM but not 4-HDC in CHO cells lacking expression of the AGT protein and in cells expressing wild-type or mutant AGT. Clearly, our data suggest that mechanisms other than inactivation of AGT by BG may be involved in increasing the toxic effects of 4-HC and PM. There are several possible mechanisms for greater toxicity of 4-HC and PM after treatment with BG. One is that BG may enter into the nucleotide pool and be misincorporated in DNA in the process of lesion repair introduced by PM. The DNA repair process would not involve AGT. Another possibility is a direct reaction between the monoadduct of PM and BG, resulting in a cross-link between DNA, PM, and BG. This cross-link may be toxic. A third possibility is that BG directly inhibits a repair mechanism responsible for removing toxic lesions introduced by PM. Lastly, it is possible that BG inhibits cyclin-dependent kinase, resulting in cell cycle arrest and, in concert with a DNA-damaging agent such as PM, produces greater cell killing. Structurally similar compounds including 2,6,9-irinsubstituted purines have recently been shown to inhibit cyclin-dependent kinases (33).

BG is known to enhance the biological effects of alkylating agents by depleting cells of the AGT repair protein and thus increasing the number of toxic/mutagenic lesions at the O\textsuperscript{6} position of guanine. We have now demonstrated that in the absence of AGT, BG enhances the toxic effect of a nitrogen mustard. We are currently investigating whether structurally different nitrogen mustards are also enhanced with BG.

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