Effect of $O^6$-Benzylguanine on Nitrogen Mustard-induced Toxicity, Apoptosis, and Mutagenicity in Chinese Hamster Ovary Cells

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Abstract

$O^6$-Benzylguanine (BG) inactivates $O^6$-alkylguanine-DNA alkyltransferase (AGT), resulting in an increase in the sensitivity of cells to the toxic effects of $O^6$-alkylating agents. BG significantly enhances the cytotoxicity and decreases the mutagenicity of nitrogen mustards [i.e., phosphoramidate mustard (PM), melphalan, and chlorambucil], a group of alkylating agents not known to produce $O^6$-adducts in DNA. The enhancement is observed in cells irrespective of AGT activity. Exposure of Chinese hamster ovary cells to $100 \mu M$ BG results in enhancement in the cytotoxicity of PM (300 $\mu M$), chlorambucil (40 $\mu M$), and melphalan (10 $\mu M$) by 9-, 7-, and 18-fold, respectively. In contrast, mutation frequency after treatment with $300 \mu M$ PM is decreased from 259 mutants/10$^6$ cells to 22 mutants/10$^6$ cells when cells are pretreated with BG. The enhancement of toxicity of these bis-alkylating agents appears to involve cross-link formation, because neither cytotoxicity nor mutagenicity of a mono-alkylating PM analogue is significantly altered when combined with BG. Enhanced cytotoxicity and decreased mutagenicity is concomitant with a dramatic increase in the number of cells undergoing apoptosis when BG is combined with PM, melphalan, or chlorambucil at 72–94 h after treatment. Cell cycle analysis demonstrates that BG alone or combined with nitrogen mustards arrests cells in G$_1$ phase of the cell cycle. At 16 h after treatment, 11 and 57% of cells treated with PM alone or with BG plus PM are in G$_1$ phase, respectively. Our data suggest that treatment with BG causes G$_1$ arrest and drives noncycling cells treated with nitrogen mustards into apoptosis, thus protecting against mutagenic DNA damage introduced by nitrogen mustards.

Introduction

Nitrogen mustards (i.e., cyclophosphamide, chlorambucil, and melphalan) are bifunctional alkylating agents used extensively for >30 years in the treatment of neoplastic and autoimmune diseases (1, 2). All nitrogen mustards induce single-strand guanine N7 adducts, as well as interstrand N7–N7 cross-links involving the two guanines in GNC–GNC (5’–3’/5’–3’) sequences (3, 4). In addition, the aromatic mustards melphalan and chlorambucil also induce substantial alkylation at adenine N3 (5), whereas cyclophosphamide forms phosphotriesters with relatively high frequency (6). Cyclophosphamide is metabolized to PM$^3$ and acrolein. ICLs are a result of the reaction between PM, or other nitrogen mustards, and DNA. Although cross-linked adducts comprise only a small fraction of total adducts, there is strong evidence that ICLs are the critical cytotoxic adducts produced by nitrogen mustards (7).

The precise mechanism by which ICLs are repaired is still not clear. Studies using yeast mutants point to excision repair and recombination as playing important roles in removing ICLs introduced by nitrogen mustards (8, 9). In mammalian cells, nucleotide excision is also important in repair, as evidenced by cells defective in ERCC1 and ERCC4 that are more sensitive to the toxic properties of nitrogen mustards (10, 11). DSBs are produced in cycling cells treated with nitrogen mustards, probably as a result of activities that act to process a stalled replication fork, and these breaks have been proposed to initiate homologous recombination (12). Muller et al. (13) showed that the activity of the DNA-dependent protein kinase complex is a determinant in the cellular response to nitrogen mustards.

Recently, we observed a significant increase in the cytotoxicity of 4-hydroperoxycyclophosphamide (an activated form of cyclophosphamide and a PM generator) and PM alone after treatment with nontoxic concentrations of BG in CHO cells expressing wild-type AGT, mutant AGT, or lacking AGT expression (14). BG is a potent, specific inactivator of AGT, a protein that repairs $O^6$-guanine lesions in DNA. BG increases the sensitivity of tumor cells and tumor xenografts...
to the antitumor effects of agents such as nitrosoureas and alkyltriazenes (15). Combination treatments of BG and alkyl-nitrosoureas [1,3-bis(2-chloroethyl)-1-nitrosourea, Gliadel] or alkyltriazenes (temozolomide) are presently in human clinical trials. However, BG enhancement of nitrogen mustard toxicity must involve mechanisms other than inactivation of AGT because the observation with 4-hydroperoxycyclophosphamide and PM was made in cells irrespective of AGT activity.

We investigated our earlier observation by evaluating the effect of BG on nitrogen mustard-induced mutagenicity and found that BG decreases the mutagenicity of this class of compounds. In an effort to determine the mechanism by which BG enhances the toxicity and decreases the mutation frequency of nitrogen mustards, we studied the effect of BG on the cytotoxicity, mutational spectrum, cell cycle distribution, and apoptosis induced by nitrogen mustards.

Materials and Methods

Materials. BG was generously provided by Dr. Robert C. Moschel (National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD). PM (as the cyclohexylammonium salt) was 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).

Synthesis of propyl-PM

**N-(2-Chloroethyl)-N-propylamine Hydrochloride.** Thionyl chloride (0.4 mol, 29 ml) was added dropwise to a solution of N-propylaminoethanol (20 mmol, 2.3 ml) in CH₂CN (dry, distilled, 74 ml). After being stirred for 26 h under a drying tube, the reaction mixture was added to ether (550 ml), resulting in the precipitation of product. The white solid was collected by vacuum filtration and dried under high vacuum to give the product CH₃CH₂CH₂NHCH₂CH₂Cl.HCl in 70% yield (14 mmol, 2.2 g).

**N-(2-Chloroethyl)-N-(propyl)phosphorodiamidic Acid Phenylmethyl Ester.** A solution of benzyl alcohol (12 mmol, 1.24 ml) and triethylamine (12 mmol, 1.67 ml) in THF (dry, distilled, 12 ml) was added dropwise to a solution of phosphorus oxychloride (12 mmol, 1.13 ml) in THF (18 ml) at −23°C (CCl₄/dry ice bath) and under N₂. Upon complete addition, the reaction mixture was stirred at −23°C for 30 min. Then, the CCl₄/dry ice bath was exchanged for one of ethylene glycol/dry ice (−15°C). N-(2-Chloroethyl)-N-propylamine hydrochloride (12 mmol, 1.09 g) was added as a solid to the reaction mixture followed by THF (20 ml) and, slowly, triethylamine (24 mmol, 3.35 ml). The bath was allowed to come to room temperature gradually, and then the mixture was stirred overnight. The flask was then cooled to 5°C (ice bath), and NH₃ was bubbled through the mixture for 15 min. The flask was stoppered, and the mixture was allowed to sit at room temperature for 4 h. TLC analysis suggested incomplete product formation; therefore, NH₃ was again passed through the reaction mixture for 10 min at 5°C. The well-stoppered flask was then stored at room temperature for 5 days. (Note: this was for convenience; overnight probably would have been sufficient.) Excess NH₃ was removed by pulling a low vacuum on the reaction flask (5 min), and then deionized water (15 ml) was added to the mixture. THF was removed from the resultant solution on a rotary evaporator, and the residual, biphase mixture was extracted with CH₂Cl₂ (3 × 35 ml). The organic layers were combined, dried (MgSO₄), gravity filtered, and concentrated on a rotary evaporator. Crude product was flash chromatographed on silica gel (230–400 mesh, 200 ml, 1.7 inch × 5 inch column) using hexane-ethyl acetate (5:95) as eluent; 20-ml fractions were collected. The fractions containing product [Rₚ 0.2 in hexane-ethyl acetate (5:95) and 0.67 in CH₂Cl₂-CH₃OH (9:1)] were combined and concentrated on a rotary evaporator. The residual oil was dissolved in CH₂Cl₂ (50 ml) and washed with water (2 × 20 ml). The organic layer was dried (MgSO₄), filtered, and concentrated on a rotary evaporator and then a high vacuum pump. The product, C₇H₉Cl₂O₃P(ONH₃)[N(CH₂CH₂Cl)(CH₂CH₂CH₃)], was obtained as an oil in 46% yield (5.5 mmol, 1.59 g). 1H NMR (300 MHz, CDCl₃, δ): 7.41–7.27 (m, 5H, aromatic), 5.08–4.90 (m, 2H, CH₂Cl), 3.62–3.52 (m, 2H, CH₂Cl), 3.43–3.32 (m, 2H, NCH₂CH₂Cl), 3.07–2.95 (m, 2H, NCH₂CH₂CH₃), 2.75–2.56 (br s, 2H, NH₂), 1.53 (apparent sextuplet, J = 8 Hz, 2H, CH₂CH₂), and 0.86 (t, J = 7 Hz, 3H, CH₃). 31P (121 MHz, CDCl₃, δ): 16.4 (relative to capillary insert of 5% H₃PO₄).

**propyl-PM.** A solution of N-(2-chloroethyl)-N-(propyl)phosphorodiamidic acid phenylmethyl ester (5.5 mmol, 1.59 g) in 1,4-cyclohexadiene-absolute ethanol (6:94, 230 ml) was subjected to catalytic transfer hydrogenation using a palladium black column as described previously for similar benzyl esters (16). The solution was passed through the column (1 cm × 8 cm) at a rate of 1 ml/min. Six fractions were collected in 50-ml increments, and each immediately upon collection was treated with cyclohexylamine (0.28 ml). The treated fractions were allowed to sit for 10 min and were then concentrated on a rotary evaporator at ambient temperature. The residual oil was dissolved in minimal absolute ethanol, and to this were added several volumes of ether. After storage overnight at −20°C, the product was collected as a white solid (0.7 mmol, 0.2 g, 13% yield, melting point 37–43°C). Elemental analysis for C₁₀H₁₂Cl₂N₂O₃P, found (theory): C, 44.06 (43.97); H, 9.10 (8.90); N, 14.02 (13.91). 1H NMR (300 MHz, D₂O, δ): 3.65 (t, J = 7 Hz, 2H, CH₂Cl), 3.35–3.22 (m, 2H, NCH₂CH₂Cl), 2.98–2.85 (m, 2H, NCH₂CH₂CH₃), 1.53 (apparent sextuplet, J = 7 Hz, 2H, CH₂CH₂), and 0.85 (t, J = 7 Hz, 3H, CH₃). For cyclohexylamine: δ 3.22–3.06 and 2.16–1.08 (various multiplets). 31P (121 MHz, CDCl₃, δ): 13.4 (relative to external 25% H₃PO₄).  

**Alkylation Kinetics.** The alkylation kinetics of propyl-PM were determined by 31P NMR using procedures similar to those previously described kinetic analyses of phosphorodiamidic mustards (17). In brief, an 11 mM solution of the phosphorodiamidic acid in 0.22 M BisTris (pH 7.4) was monitored by 31P NMR at 37°C. Kinetic experiments were run in the presence (10 equivalents) and absence of glutathione; in each case, the half-lives were identical (within experimental error), as would be expected for a first-order reaction involving intramolecular cyclization of the chloroethylamido functionality. The average half-life was 5.5 ± 0.3 min. Under the same conditions (0.22 M BisTris, pH 7.4, 37°C), the half-life for PM was 18 min (17).
Cells
CHO/AA8, CHO/UV41, and CHO/UV135 cell lines were obtained from American Type Culture Collection (Rockville, MD). Cells were maintained as exponentially growing monolayer cultures in DMEM (CHO/AA8) or α-MEM (CHO/UV41 and CHO/UV135) supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml) in a humidified incubator at 37°C with 5% CO2.

Assay for Cell Survival
Cytotoxicity induced by PM, melphalan, chlorambucil, and propyl-PM was determined by loss of colony-forming ability as described previously (18). All nitrogen mustards used in these studies are reactive; therefore, solutions were made fresh immediately before use. BG (100 mM), 8-oxoBG (50 mM), and N7-BG (50 mM) were made up as stock solutions in DMSO and diluted appropriately with medium. Briefly, CHO cells were plated at a density of 1.4 × 10⁵ cells/T75 flask. On the following day, cells were treated with 100 μM BG or an analogue or vehicle (0.1% DMSO) in serum-free medium for 2 h prior to and 1 h during exposure to a nitrogen mustard. Medium containing 10% serum with or without 100 μM BG was placed on cells for an additional 16 h after alkylating agent treatment. After treatment, cells were plated in DMEM with 10% serum plus 0.01% DMSO (control) or 10 μM BG at a density of 200 or 400 cells/100 mm dish for 10–12 days. Cell colonies (>50 cells) were counted after staining with 0.15% methylene blue. Colony-forming efficiency was expressed as a percentage of the number of cells surviving treatment with drug relative to treatment with vehicle.

Assay for Mutation Frequency in CHO Cells
Cells were plated and treated as described above with slight modification. Posttreatment of cells with 100 μM BG lasted for 24 h instead of 16 h. The cells were maintained in exponential growth in the absence of BG for an additional 7-day expression period before 1 × 10⁶ cells were plated into 100-mm dishes with 5 μg/ml 6-TG. Cells were incubated for ~10 days to allow the formation of colonies. Mutation frequency was determined by counting 6-TG-resistant colonies and expressed as a number of 6-TG resistant colonies per 10⁶ surviving cells.

Mutation Spectrum Analysis
Cells were treated as described above for the mutation frequency. After the 10-day incubation period, a single mutant colony from each dish was replated for further culturing to determine the mutational spectrum. Cells were grown to confluency in 100-mm² tissue culture dishes. RNA was extracted using the method supplied with Qiagen RNeasy Mini kit. (Valencia, CA). The oligonucleotides 5’-CTCACCGCCT-TCTCGTGCGCTGCGC-3’ (CD1), 5’-GTACTAAGCAGATG-GCTGAGAGAC-3’ (CD2), and 5’-AGGACATAATGGCACAC-3’ (CDS3) were synthesized by Genosys Biotechnologies, Inc. (The Woodlands, TX). Synthesis and PCR amplification of the hprt CDNA was completed using the Qiagen One Step RT-PCR kit using CD1 and CD2 primers. Thirty-five step cycles of amplification were performed in a Perkin-Elmer GeneAmp PCR System 9700 thermocycler (reverse transcription: 50°C, 30 min; PCR reaction: 95°C, 15 min; 35 cycles: 94°C, 40 s; 64°C, 40 s; 72°C, 40 s followed by 72°C, 10 min). PCR products were purified from agarose gel (Life Technologies, Inc., Gaithersburg, MD) using the QiAquick Gel Extraction kit from Qiagen. Purified products were sequenced by cycle sequencing using the ABI Prism Big Dye Terminator Cycle Sequencing kit with primers CD1 and CDS3. The sequence data were generated on the ABI 377 sequencer from PE Applied Biosystems (Foster City, CA).

Fig. 1. Structures of nitrogen mustards used in these studies.

GeneAmp PCR System 9700 thermocycler (reverse transcription: 50°C, 30 min; PCR reaction: 95°C, 15 min; 35 cycles: 94°C, 40 s; 64°C, 40 s; 72°C, 40 s followed by 72°C, 10 min). PCR products were purified from agarose gel (Life Technologies, Inc., Gaithersburg, MD) using the QiAquick Gel Extraction kit from Qiagen. Purified products were sequenced by cycle sequencing using the ABI Prism Big Dye Terminator Cycle Sequencing kit with primers CD1 and CDS3. The sequence data were generated on the ABI 377 sequencer from PE Applied Biosystems (Foster City, CA).

Assay for Cell Cycle Distribution of CHO Cells Treated with Nitrogen Mustards
At various time points up to 116 h after treatment (as described above) with 100 μM BG and/or a nitrogen mustard, cells were harvested, and 1 × 10⁶ cells were resuspended in 0.5 ml of cold PBS. Cells were fixed in ice-cold 75% alcohol and kept overnight at 4°C. The following day, cells were centrifuged at 1500 rpm for 10 min, and pellets were suspended in propidium iodide solution containing 0.125 mg/ml RNase. Incubation was carried out on ice for 30 min to 1 h. Cells were then analyzed by flow cytometry using a FACScan (Becton Dickinson, NJ).

Assay for Determining Apoptosis in CHO Cells
At various time points after treatment with 100 μM BG plus 300 μM PM, 40 μM chlorambucil, 9.8 μM melphalan, or 450 μM propyl-PM analogue, cells were harvested and washed with DMEM supplemented with 10% FBS. Apoptotic cells were detected by incubating cells with Annexin V conjugated with FITC (ClonTech, Palo Alto, CA), followed by flow cytometric analysis using a single laser emitting excitation light at 488 nm as described by Martin et al. (20).

Results
Structure of Alkylating Agents. Fig. 1 illustrates the structures of the bifunctional (PM, chlorambucil, and melphalan) and monofunctional (propyl-PM) nitrogen mustards used in this study.
Effect of BG on Cytotoxicity and Mutagenicity Induced by Nitrogen Mustards in CHO Cells. CHO cells were treated with 100 μM BG before, during, and 16 h after treatment with increasing concentrations of PM, chlorambucil, and melphalan. BG alone at this concentration resulted in 89% cell survival. The effective PM concentration required to kill 90% of CHO cells decreased from 357 to 199 μM in the presence of BG (Fig. 2A). Similarly, CHO cells treated with BG in combination with chlorambucil or melphalan had significantly lower cell survival than cells treated with chlorambucil or melphalan alone (Fig. 2, B and C). The cells treated with the combination of BG and 40 μM chlorambucil resulted in seven times greater cell killing compared with cells treated with chlorambucil alone (Fig. 2B). At a concentration of 10 μM melphalan, the percentage of survival of cells in the presence of BG was 17 times less than in the absence of BG (Fig. 2C).

In an effort to ascertain whether BG-enhanced toxicity occurred as a result of cross-links or monoadducts, we determined the effect of BG on propyl-PM, an analogue of PM in which one chloroethyl group was replaced with a propyl group. The resultant propyl-PM analogue had only one alkylating moiety and, therefore, any cell toxicity had to be consistent with monoadducts. CHO cells were much less sensitive to the effects of this propyl-PM analogue, as expected for a monoalkylating agent, and BG did not enhance the cytotoxic effects of this compound (Fig. 2A). Although the half-life of propyl-PM is shorter than that of PM at 37°C and pH 7.4 (i.e., 5.5 and 18 min, respectively), the incubation time of 1 h allowed for several half-lives of both drugs.

Concomitantly with increased cytotoxicity, BG treatment resulted in a significant decrease in mutation frequency induced by nitrogen mustards. At a dose of 300 μM PM, the number of mutations/10⁶ cells decreased by a factor of 12 in the presence of BG (Fig. 2D). Similarly, cells treated with 80 μM chlorambucil resulted in 157 mutants/10⁶ cells, yet when combined with BG, the mutation frequency decreased to 13 mutants/10⁶ cells. Although melphalan is in general less mutagenic than PM and chlorambucil, BG treatment still resulted in a decrease in mutation frequency by 2-fold. The propyl-PM analogue was much less mutagenic than PM; moreover, BG slightly reduced the mutation frequency induced by the monoalkylator.

The spectrum of mutations at the hprt locus of 12 mutant colonies after treatment with PM as well as that of 10 mutant colonies after treatment with the combination of PM and BG were determined (Table 1). All 12 colonies evaluated after PM treatment contained only deletions, whereas only 2 of 10 colonies treated with PM and BG contained deletions. These 2 colonies also contained transversions and transitions. The remaining colonies treated with the combination of PM and BG contained only point mutations. The majority of the deletions (8 of 12) were at the position 413–478, which may represent a hot spot. Because mutant colonies were taken from separate dishes, the mutational spectrum represents different cell colo-
nies. None of the 7 colonies evaluated after treatment with BG plus propyl-PM contained deletions, whereas 6 of 13 treated with propyl-PM alone contained deletions. All other mutant colonies contained point mutations.

Comparison of the Effect of Increasing Concentrations of BG and BG Analogues on PM-induced Cytotoxicity. Fig. 3 illustrates an increase in the sensitivity of cells to the toxic effects of PM in a dose-dependent manner with 50% enhancement at a dose of 10 μM BG. Altering the structure of the modulator by using N7-BG (instead of benzyl at O6, it is at the N7 position) abolished the effect (Fig. 3). Similarly, guanine had no effect on PM-induced toxicity (data not shown); however, other O6-benzylated guanine derivatives (i.e., O6-benzyl-8-oxoguanine) increased the sensitivity of cells to PM (Fig. 3).

Effect of BG on Apoptotic Cell Death in CHO Cells Treated with Nitrogen Mustards. To determine whether BG induces apoptosis of CHO cells treated with nitrogen mustards, an Annexin V-propidium iodide binding assay was performed 24–116 h after treatment with nitrogen mustards. As shown in Fig. 4A, BG markedly increased the percentage of PM-treated cells undergoing apoptosis from 2 to 24% at 94 h. Increases in the percentage of Annexin V-positive cells were observed when BG was combined with melphalan and chlorambucil as well (Fig. 4, B and C). The highest percentage of apoptotic cells were seen at 90–94 h after treatment with all three nitrogen mustard compounds. No significant increase in the percentage of apoptotic cells was observed in cells 24–72 h or at 116 h after treatment with the combination of BG and nitrogen mustard (data not shown). Consistent with our cytotoxic results, BG did not increase the percentage of cells undergoing apoptosis after treatment with the monoalkylating, propyl-PM analogue (data not shown).

Effect of BG on Cytotoxicity Induced by PM in ERCC4- and ERCC5-deficient CHO Cells. One possible explanation for enhanced apoptosis of PM-treated cells after treatment with BG is inhibition of nucleotide excision repair by BG, thus causing a greater number of cross-links with the combination. To determine whether BG inhibited nucleotide excision repair, the effect of BG on PM toxicity and apoptosis was evaluated in two mutant cell lines, UV41-CHO cells (deficient in ERCC4) and UV135-CHO cells (deficient in ERCC5). Fig. 5 illustrates a greater sensitivity of UV41-CHO cells to the cytotoxic effect of PM compared with UV135-CHO cells, as expected. BG significantly enhanced the sensitivity of both cell lines to the toxicity of PM. The percentage of cells undergoing apoptotic cell death also increased significantly after treatment with BG and PM for both UV41 (Fig. 5A, inset) and UV135 cells (Fig. 5B, inset). Therefore, the mechanism of BG-enhanced toxicity is unrelated to inhibition of ERCC4.

Effect of BG on CHO Cell Cycle Progression after Treatment with Nitrogen Mustards. To determine whether the effect of BG-enhanced apoptosis was related to cell cycle progression, CHO cells treated with vehicle, PM alone, BG alone, or the combination of BG and PM were analyzed by flow cytometric analysis. As shown in Fig. 6, 80% of
PM-treated cells accumulated in late S-phase, as measured 16 h after treatment. Previous reports demonstrate an accumulation of cells in S-phase after treatment with nitrogen mustards (21, 22). Cells treated with BG with or without PM accumulated in G1, resulting in 57 and 68% cells in G1, respectively, compared with 11% in G1 after treatment with PM alone at 16 h. Similar results were observed for BG in combination with chlorambucil and melphalan (data not shown). N7-BG did not increase the cytotoxicity of PM and failed to arrest cells in G1 with or without PM (data not shown).

**Discussion**

Our data demonstrate that BG, a nontoxic modulator of O6-guanine alkylating compounds, enhances the toxicity and reduces the mutagenicity of nitrogen mustards including PM, chlorambucil, and melphalan in CHO cells. This is in contrast to the effect of BG on alkyltransferase and alkyltransferase, in which both toxicity and mutagenicity are enhanced as a result of AGT inactivation, ultimately increasing the number of toxic/mutagenic lesions at the O6-position of guanine (14, 15). The mechanism of the interaction of BG and nitrogen mustards is unrelated to depletion of AGT because the observed effect occurs in CHO cells deficient in AGT activity. We report that BG treatment results in a significantly greater percentage of PM-treated cells undergoing apoptosis compared with cells treated with PM alone. This may be related to the effect of BG on cell cycle kinetics. BG causes G1 arrest at 16 h after treatment. The significance of our finding is that greater toxicity and lower mutagenicity may translate into increased efficacy and a lower incidence of therapy-related leukemia with this clinically important class of alkylating agents. The mechanism by which BG arrests cells in G1 has not been elucidated. One possibility for G1 arrest is that BG inhibits CDK. Structurally related alkylated purines including O6-cyclohexylmethylguanine were found to be competitive inhibitors of CDK1 and CDK2 (23). The difference in structure between BG and O6-cyclohexylmethylguanine consists of a benzyl group instead of a cyclohexylmethyl group at the O6-position. The purine analogs were found to form a triplet of hydrogen bonds (i.e., NH-9, N-3, and 2-NH2) with CDK2. The triplet of hydrogen bonds could be reproduced with BG but not with N7-BG. In support of this, our data show that N7-BG did not cause G1 arrest and failed to enhance the toxic effects of nitrogen mustards. CDKs play an important role in cell cycle regulation, and the sequential activation of CDKs ensures the correct timing and ordering of events required for cell cycle progression (24–26). CHOAA8 cells are functionally defective in p53 (27, 28); therefore, BG-induced G1 arrest is independent of typical p53-induced G1 arrest.

Our data, in support of previous reports (21, 22), show an accumulation of cells in late S-phase after treatment with nitrogen mustards alone. BG alone or combined with PM arrests cells in stationary phase (i.e., G0) at 16 h after treatment. One hypothesis to explain our data is that an accumulation of PM-induced damage, particularly DNA ICLs, results in greater toxicity and less mutagenicity by virtue of the
intermediates formed during repair of these lesions in G1 compared with S-phase. Evidence in yeast and mammalian cells suggests differences in repair of ICLs in replicating and nonreplicating cells (9, 12). De Silva et al. (12) showed that in mammalian cells, DSBs are induced in response to ICLs, but this is dependent on factors found mainly in dividing cells (12). In stationary phase, very few DSBs are produced. PM and other nitrogen mustards are known to cause mutating deletions (29). The possibility exists that processing of ICLs in G1 results in fewer deletions because of the lack of DSBs as intermediates. Another possibility is that ICLs are not repaired in G1, and their presence eventually signals an apoptotic pathway. Whatever the mechanism, the work presented here suggests that exposure to BG provides a route for increasing the chemosensitivity and decreasing the mutagenicity of several nitrogen mustards.

Murray and Meyn (30) reported previously that cell populations enriched in G1 were more sensitive to nitrogen mustards than those enriched in late S-phase-G2 as determined by clonogenic assay. There was no significant difference in the levels of either DNA interstrand or DNA-protein cross-links induced in either phase of the cell cycle 6 h after treatment as measured by alkaline elution after nitrogen mustard treatment. The authors (30) concluded that neither differences in DNA damage nor extent of repair could explain the differential cytotoxicity of nitrogen mustard toward cells in the different cell cycle phases. Our data are consistent with greater toxicity observed in G1 than S-phase for nitrogen mustards. However, because cross-links were only evaluated at one time point in this study and it is likely that cross-links form up to 24 h, the possibility of a persistence of ICLs in the G1 phase as a result of lack of repair of these lesions cannot be excluded.

There are previous reports of modulating the toxicity of nitrogen mustards by altering cell cycle kinetics. Lau and Pardee (31) reported that high concentrations of caffeine (2 mM) increased the lethality of nitrogen mustards by 5–10-fold by preventing G2 arrest, thereby allowing cells to divide without completing the repair process. Caffeine combined with nitrogen mustards caused cells to undergo apoptosis. However, the extremely high concentrations of caffeine required limit the clinical utility of this approach. BG is extensively metabolized to 8-oxoBG in humans (32). Both BG and 8-oxoBG at micromolar concentrations enhance nitrogen mustard toxicity, suggesting clinical utility. Human tumor xenograft studies are under way to evaluate the antitumor activity of BG and nitrogen mustards. It is possible that more potent cell cycle inhibitors will produce the same affect at even lower concentrations.

Bonatti et al. (33) showed that O6-ethylguanine and O6-methylguanine at very high concentrations induced apoptosis and inhibited p70^S6K^ activity (a mitogen-activated kinase involved in G2-G1 transition) while activating the MAPK pathway (33). BG is similar in structure to these purine derivatives and might have similar effects on p70^S6K^ activity; however, the concentrations of O6-alkylguanine reported in that study were 10-fold higher than concentrations used in our study. At concentrations used in the present study, we did not observe apoptosis after BG treatment alone.

Previously, we demonstrated a slight increase in the number of mutants per 10^6 cells when CHO cells were treated with BG plus 4-hydroperoxycyclophosphamide (activated form of cyclophosphamide that produces PM and acrolein) compared with treatment with 4-hydroperoxycyclophosphamide alone (14). This would suggest that although BG protects against PM-, chlorambucil-, and melphalan-induced mutagenicity, it does not protect against cyclophosphamide-induced mutations. DNA damage introduced by acrolein or the combination of damage by acrolein and PM may be more mutagenic in the presence of BG. Whether this is specific to BG or to modulators that produce G1 arrest remains to be determined.

Our laboratory has demonstrated that by exposure of CHO cells to BG, nitrogen mustards (specifically PM, melphalan, and chlorambucil) are more toxic and less mutagenic. By elucidating the mechanism, we may find more potent inhibitors or drug combinations that will improve the efficacy and reduce the mutagenic effects of this important class of alkylating agents.

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