

Enhancement of Platinum-induced Cytotoxicity by O⁶-Benzylguanine¹

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Abstract

O⁶-Benzylguanine (O⁶-BG), a potent inactivator of the DNA repair protein O⁶-alkylguanine-DNA alkyltransferase (AGT), is presently in clinical trials combined with alkylating agents that modify the O⁶ position of DNA guanine residues, *i.e.*, 1,3-bis(2-chloroethyl)-1-nitrosourea and temozolomide. Previous work demonstrated that O⁶-BG also enhances the cytotoxicity of cyclophosphamide, ifosfamide, and nitrogen mustards in Chinese hamster ovary cells. We have extended this study to include other clinically relevant agents that form interstrand and intrastrand cross-links including cisplatin and carboplatin. Pretreatment of a series of head and neck tumor cell lines (*i.e.*, SQ20b, JSQ3, SCC25, SCC35, and SCC61), Chinese hamster ovary cells, and HT29 human colon tumor cells with O⁶-BG (100 μM for 2 h before treatment and 2 h during treatment) resulted in a 2-fold decrease in the ED₅₀ of cisplatin and a concomitant increase in the percentage of cells undergoing apoptosis. The enhancement was independent of AGT activity. Similar enhancement was observed with carboplatin, but no enhancement was seen in AGT-deficient cell lines with radiation or temozolomide, demonstrating the dependence of the effect on bifunctional, cross-linking agents. Furthermore, levels of platinum on DNA after treatment with cisplatin increased 1.4-fold in SQ20b cells and 4.5-fold in JSQ3 cells immediately after treatment with O⁶-BG plus cisplatin and remained elevated for 48 h. Consistent with greater cytotoxicity and apoptosis is the ~2-fold higher amount of DNA damage when cells are treated with O⁶-BG plus cisplatin compared with cisplatin

alone. Modulation of cisplatin therapy with O⁶-BG might improve the prognosis of patients with head and neck, ovarian, testicular, or lung cancer who are treated with this drug.

Introduction

Chemotherapeutic DNA platinating agents including cisplatin, carboplatin, and oxaliplatin are effective against a variety of cancers such as lymphomas and testicular, ovarian, lung, and head and neck carcinomas. In particular, cisplatin in combination with etoposide and bleomycin is considered curative treatment for testicular cancer (1, 2). Despite their activity against testicular cancer, the activity of platinating agents against other cancers is more limited. For example, cisplatin and carboplatin have low response rates against head and neck cancer yet remain standard components of common multiagent regimens for advanced primary and metastatic disease (3, 4). In comparison with cisplatin, carboplatin is associated with lower rates of nephrotoxicity, ototoxicity, nausea, and vomiting (5, 6). Although patients may initially respond to cisplatin or carboplatin treatment, a common problem is acquired resistance. This acquired resistance, as well as intrinsic resistance in some patients, has multifactorial etiologies including contributions from drug uptake, cellular detoxification systems, and DNA repair mechanisms (7, 8). The desire to improve and prolong clinical response to platinating agent therapy has prompted a search for modulators to overcome resistance to these drugs.

Platinating agents produce a cytotoxic effect by introducing intrastrand and interstrand cross-links in DNA that can block replication or inhibit transcription (9, 10). The lesions generated by cisplatin on DNA from most abundant to least abundant are intrastrand cross-links including 1,2-d(GpG), 1,2-d(ApG), and 1,3-d(GpNpG) and interstrand cross-links (7). There are two pathways believed to be involved in the repair of these lesions: NER³ and HR. NER is the main mechanism for removing cisplatin intrastrand cross-links, whereas HR appears to play a more prominent role in the repair of interstrand cross-links. There are two pathways in NER, TC-NER and GG-NER. TC-NER repairs transcription-blocking lesions in transcribed DNA strands of active genes (11, 12), whereas the repair of lesions in the nontranscribed strand of active genes and nontranscribing genome is carried out by GG-NER. Recently Furuta *et al.* (13) reported the importance of TC-NER and irrelevance of GG-NER in cellular response to cisplatin.

Received 2/3/03; revised 4/11/03; accepted 5/14/03.

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¹ Supported in part by NIH Grant CA81485.

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³ The abbreviations used are: NER, nucleotide excision repair; HR, homologous recombination repair; TC-NER, transcription-coupled NER; GG-NER, global genome-NER; O⁶-BG, O⁶-benzylguanine; AGT, O⁶-alkylguanine-DNA alkyltransferase; CHO, Chinese hamster ovary; TMZ, temozolomide; XP, xeroderma pigmentosum; ERCC1, excision repair cross-complementing 1; CDK, cyclin-dependent kinase; PI, propidium iodide.

O^6 -BG is a potent, specific inactivator of the DNA repair protein AGT and thereby enhances the cytotoxicity of agents that produce toxic lesions at the O^6 position of guanine such as alkylnitrosoureas and alkyltriazenes (14). However, we observed that the cytotoxicity of active forms of cyclophosphamide, ifosfamide, and nitrogen mustards was also enhanced by O^6 -BG (15, 16). Because these agents are not known to produce O^6 adducts in DNA, and because the enhancement of nitrogen mustard toxicity was observed in CHO cells, which are devoid of AGT, the mechanism of O^6 -BG enhancement was unclear. Therefore, we have extended our studies of possible O^6 -BG-enhanced cytotoxicity to other clinically relevant DNA-damaging agents, *i.e.*, cisplatin, carboplatin, radiation, and TMZ, to improve our understanding of the mechanism. We demonstrate that pretreatment of head and neck cancer cell lines with O^6 -BG results in greater cisplatin cytotoxicity coincident with an increase in the percentage of cells undergoing apoptosis, more total platination of DNA, and more DNA damage. O^6 -BG-enhanced cytotoxicity was also observed with carboplatin, but not with ionizing radiation, indicating that O^6 -BG may be an effective adjuvant for chemotherapy with platinating agents or cross-linking agents in general.

Materials and Methods

Materials. Cisplatin and carboplatin were purchased from Sigma Aldrich (St. Louis, MO). O^6 -BG was synthesized as described previously (17, 18). Apoptosis assay kit including Annexin-V-FITC and PI was purchased from BD Biosciences Clontech (Palo Alto, CA). All cell culture supplies were purchased from Mediatech, Inc. (Herndon, VA).

Cell Lines. All head and neck cell lines (SQ20b, JSQ3, SCC25, SCC35, and SCC61) were kindly provided by Dr. Michael Beckett (Radiation Oncology, The University of Chicago) and maintained in DMEM:Ham's F-12 (50:50 mixture; Mediatech, Inc.) supplemented with 20% fetal bovine serum (Hyclone, Logan, UT) and 0.4 μ g/ml hydrocortisone. HT29 cells were purchased from American Type Culture Collection (Manassas, VA) and maintained in McCoy's 5A medium (Mediatech, Inc.) supplemented with 10% fetal bovine serum. CHOA8 cells were purchased from American Type Culture Collection and maintained in DMEM (Mediatech, Inc.) supplemented with 10% fetal bovine serum. All cell lines were grown with the addition of 1% penicillin/streptomycin (Mediatech, Inc.) at 37°C and 5% CO₂.

Western Blot Analysis with AGT Monoclonal Antibody. To analyze cell lines for AGT protein expression, cells were collected from a confluent flask, centrifuged for 5 min at 1200 rpm to pellet the cells, washed once with PBS (Invitrogen, Gaithersburg, MD), and lysed with Laemmli buffer (Bio-Rad Laboratories, Hercules, CA). The samples were boiled to denature proteins, and protein concentration was quantitated using the Bradford protein assay (Bio-Rad Laboratories). Equal amounts of protein (50 μ g) were separated on a 12% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membrane. The membrane was blocked in 5% milk (Pierce, Rockford, IL). Primary AGT monoclonal antibody (Chemicon, Temecula, CA) was added at a dilution of 1:1000. After primary antibody incubation at room tem-

perature for 2 h, the filter was washed, and the mouse secondary antibody was added. The ECL Plus Chemiluminescence kit (Amersham Bioscience Corp., Piscataway, NJ) was used for detection of AGT protein. After these solutions were added to the membrane, the image was captured using the Chemidoc Imaging system (Bio-Rad Laboratories).

AGT Activity Assay. Cellular extracts were prepared in 50 mM Tris (pH 7.5), 0.1 mM EDTA, and 5 mM DTT buffer. Samples were sonicated for 1 min and centrifuged at 14,000 $\times g$ for 30 min. The assay for AGT activity was performed as described previously (18). Briefly, AGT activity was measured as the removal of O^6 -[³H]methylguanine from a ³H-methylated DNA substrate (18 Ci/mmol) after incubation with tissue extract at 37°C for 30 min. The DNA was precipitated by adding ice-cold perchloric acid (0.25N) and hydrolyzed by the addition of 0.1N HCl at 70°C for 30 min. After filtration using a microfilter apparatus, the modified bases were separated by Beckman Ultrasphere C₁₈ (Beckman Coulter, Fullerton, CA) reverse-phase high-performance liquid chromatography with 0.05 M ammonium formate (pH 4.5) containing 10% methanol. Protein was determined by the method of Bradford, and the amount of O^6 -methylguanine released from the DNA substrate per milligram of protein was calculated.

Colony Formation Assay. To evaluate cell survival after drug treatment, approximately 350,000 cells were plated in a T25 flask and allowed to attach overnight. Exponentially growing cells were treated with O^6 -BG (100 μ M) for 2 h before the addition of cytotoxic agent or radiation (0–50 μ M cisplatin, 0–400 μ M carboplatin, 0–20 μ M TMZ, or 0–800 Gy of radiation). After incubation with modulator and DNA-damaging agent at 37°C, the cells were washed twice with PBS, trypsinized, and replated in the appropriate medium in triplicate at varying densities (150–3,000 cells/100-mm dish). After approximately 12 days at 37°C, 5% CO₂, the medium was discarded, plates were stained with methylene blue (0.1%), and the colonies were scored. Percentage survival was calculated based on the plating efficiency of the appropriate set of control cells exposed to vehicle alone.

Annexin-V-FITC/PI Staining. To analyze the cells for apoptosis, approximately 350,000 cells were plated in a T25 flask and allowed to attach overnight. Cells were treated with modulator and platinating agent as described for the cell survival assays. After drug treatment, cells were washed with PBS and cultured in normal growth medium for 72 or 96 h, after which apoptosis was assessed using Annexin-V-FITC and PI staining. Cells were trypsinized and centrifuged at room temperature at 1200 rpm for 5 min. The pellet was washed once in binding buffer provided by the manufacturer (BD Biosciences Clontech), centrifuged as described above, and resuspended in binding buffer. The medium in which the cells were growing was also collected and centrifuged to ensure that any apoptotic cells that had lifted off the flask were included in the analysis. Annexin-V-FITC antibody and PI were added to the cells according to the manufacturer's indicated protocol (BD Biosciences Clontech). Samples were analyzed by flow cytometry using CellQuest software (Becton Dickinson, San Jose, CA). Bivariate flow cytometry was used to simultaneously measure log green fluorescence

(FL1-height) versus log red fluorescence (FL2-height). The following controls were used: unstained cells; cells stained with Annexin-V-FITC only; and cells stained with PI only. Cells that were Annexin-V-FITC+/PI- were considered positive for apoptosis.

Measurement of Total Platinum in DNA. Atomic absorption spectroscopy was used to quantitate total platinum on DNA. Exponentially growing cells were treated with O^6 -BG (100 μ M) or vehicle for 2 h and then treated with 50 μ M cisplatin for an additional 2 h. Cells were collected at 0, 24, or 48 h after completion of cisplatin treatment. Cells were trypsinized, sedimented by centrifugation, washed twice in ice-cold PBS, and incubated in lysis buffer [10 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 0.1 mM EDTA, 0.5% SDS, and 20 μ g/ml RNase] for 5 h at 37°C. The cell lysate was then incubated overnight at 50°C after adding 100 μ g/ml proteinase K. Total genomic DNA was isolated by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. Platinum concentration was assessed with a Perkin-Elmer model 1100 flameless atomic absorption spectrometer (Perkin-Elmer, Norwalk, CT) monitoring 265.9 nm. The temperature program used was as follows: ramp over 30 s to 90°C and hold for 30 s; ramp over 10 s to 110°C and hold for 10 s; ramp over 30 s to 300°C and hold for 30 s; ramp over 45 s to 1500°C and hold for 60 s; and atomize at 2700°C with no ramping. Argon gas flow was 800 ml/min during all heating steps except atomization, when it was interrupted. Platinum concentrations were determined by comparison with a standard curve performed on the same day as the assay (19).

Comet Assay. Single cell gel electrophoresis or the Comet assay was used to determine the degree of DNA damage in the cells. The kit and protocol used in these experiments were purchased from Trevigen, Inc. (Gaithersburg, MD). Briefly, 350,000 cells were plated in T25 flasks and allowed to attach overnight. The next day, cells were treated with cisplatin \pm BG, washed twice with PBS, incubated in serum-containing medium for 4 h, and then trypsinized. Next, cells were pelleted, washed once with ice-cold PBS, resuspended in cold PBS at a concentration of $\sim 2-4 \times 10^5$ cells/ml, and then suspended in low-melt agarose (42°C) at a 1:10 dilution. Immediately, 75 μ l of the cell-agarose suspension were pipetted onto the CometSlide (pretreated slides provided in the Trevigen kit). The agarose was allowed to adhere to the slide at 4°C in the dark for ~ 30 min. Next, the slide was placed into chilled lysis buffer [2.5 M NaCl, 100 mM EDTA (pH 10), 10 mM Tris, 1% sodium lauryl sarcosinate, and 0.01% Triton X-100] for 30 min at 4°C. Slides were placed into alkali buffer (0.3 M NaOH and 1 mM EDTA) for another 45 min at room temperature in the dark. Slides were washed twice in $1 \times$ Tris base, boric acid, and EDTA (TBE) for 5 min and placed in TBE electrophoresis buffer. A voltage of 1 V/cm measured electrode to electrode was applied for 20 min. To fix the cells for storage, the slides were immersed into 100% ethanol for 5 min. After the slides were dry, they were stored at room temperature with dessicant until staining. To view the cells and Comet tails, the slides were stained with the Silver Staining Kit from Bio-Rad

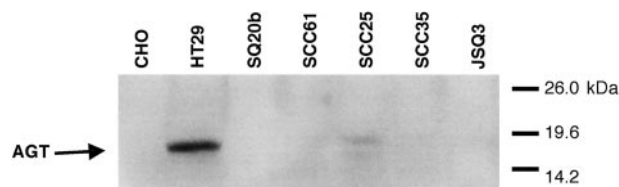


Fig. 1. Expression of AGT in head and neck cancer cell lines determined by Western blot.

Laboratories. NIH Image, a free software for image analysis,⁴ was used in addition to a macro for calculating tail moment (20) provided by Dr. Herbert M. Geller (Department of Pharmacology, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, Piscataway, NJ) to score the extent of DNA damage (*i.e.*, tail moment).

Results

AGT Expression in Head and Neck Cancer Cell Lines.

Because O^6 -BG is used as an AGT inactivator, we probed the five head and neck carcinoma lines with AGT monoclonal antibody to determine whether AGT inactivation by O^6 -BG might contribute to results in these cells. CHO and HT29 cells were used as negative and positive controls, respectively. AGT protein was detected in HT29 cells, as expected, and in the head and neck cell line SCC25. AGT protein could not be detected in 50 μ g of whole cell lysate from the other cell lines (Fig. 1). The results of a direct assay for AGT activity in these cell lines were consistent with the Western results and indicated that SCC25 cells had AGT activity (175 fmol/mg protein), whereas activity was undetectable in SQ20b, JSQ3, and SCC35 cells. A very low but reproducible amount of activity was measured in SCC61 cells (37 fmol/mg protein).

Effect of O^6 -BG on Sensitivity of Head and Neck Cell Lines to Cisplatin.

The effect of O^6 -BG on platinating agents in cell lines without AGT activity (CHO; AGT-) and with high AGT activity (HT29 cells; AGT+) was evaluated (Fig. 2). O^6 -BG dramatically enhanced the cytotoxicity of both cell lines to cisplatin, implying that O^6 -BG was working through a mechanism independent of AGT. These studies were extended to several human head and neck cancer lines with varying AGT activity. As shown in Fig. 3, *top panel*, O^6 -BG enhanced the cytotoxicity of cisplatin in head and neck cancer cell lines including SQ20b (2.6-fold), JSQ3 (1.8-fold), SCC25 (1.7-fold), SCC35 (2.2-fold), and SCC61 (2.5-fold). The fold increase is based on evaluation of the ED₅₀.

Apoptotic Response of Cells Treated with O^6 -BG and Cisplatin.

Using an Annexin-V antibody to stain for apoptotic cells, the effect of O^6 -BG on induction of apoptotic cell death after drug treatment was studied. In all cell lines tested, there was a concurrent increase in the percentage of cells undergoing apoptosis when O^6 -BG was added to cisplatin treatment as compared with cisplatin alone (Fig. 3, *bottom panel*). O^6 -BG treatment alone did not induce an apoptotic

⁴ <http://rsb.info.nih.gov/nih-image>.

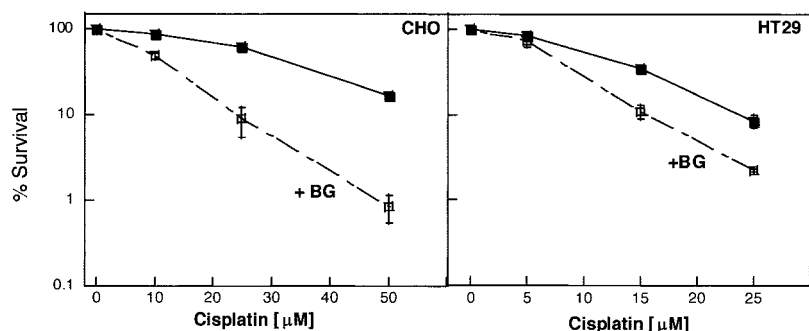
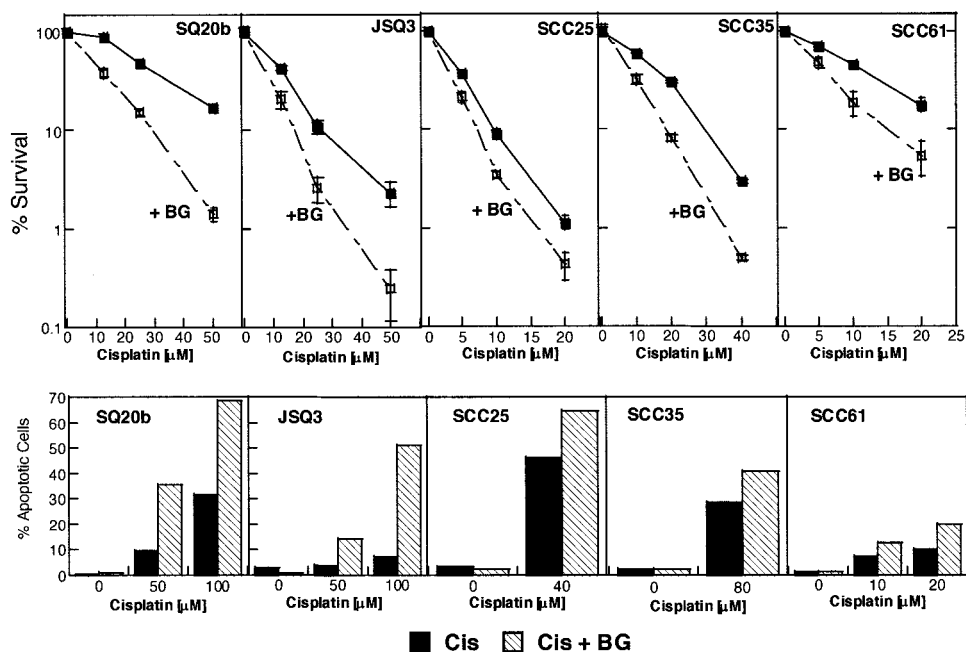


Fig. 2. Effect of O^6 -BG on cisplatin cytotoxicity in CHO (AGT $^-$) and HT29 (AGT $^+$) cells. Cells were treated with 100 μ M O^6 -BG (\square) or vehicle (0.01% DMSO; \blacksquare) for 2 h before and during cisplatin exposure. Each data point represents the mean \pm SD from at least three separate experiments. Each experiment represents 6 replicate dishes/treatment group.

Fig. 3. Effect of O^6 -BG on cisplatin cytotoxicity and apoptosis in head and neck cancer cell lines. Cells were treated with 100 μ M O^6 -BG (\square) or vehicle (0.01% DMSO; \blacksquare) for 2 h before and during cisplatin exposure. *Top panel*, cytotoxicity was measured as colony-forming ability 10–12 days after treatment. Each data point represents the mean \pm SD from at least three separate experiments. Each experiment represents 6 replicate dishes/treatment group. *Bottom panel*, cells were stained with Annexin-V and PI 96 h after treatment. Each experiment was done in triplicate. Shown here is a representative experiment.



response. As measured 96 h after treatment, O^6 -BG increased the percentage of cells undergoing apoptosis in SQ20b (2.2-fold), JSQ3 (6.8-fold), SCC25 (1.4-fold), SCC35 (1.4-fold), and SCC61 cells (1.9-fold).

Effect of O^6 -BG on Platination of DNA after Treatment with Cisplatin. To ascertain whether the increased cytotoxicity and apoptosis correlated with higher levels of DNA damage, we used atomic absorption spectroscopy to evaluate the total amount of platination of DNA in SQ20b and JSQ3 cells. O^6 -BG-treated cells had higher levels of platination at 0, 24, and 48 h (Fig. 4). Immediately after treatment, there was a difference of 1.4- and 4.5-fold in the amount of platinum/ μ g DNA in SQ20b cells and JSQ3, respectively. There is a 3-fold change in scale from the *top panel* to *bottom panel* (Fig. 4); however the increase in platination is observed in both cell lines. This difference in platination remained constant up to 48 h.

Effect of O^6 -BG on DNA Damage after Treatment with Cisplatin. Single cell gel electrophoresis (Comet assay) was used to measure the amount of DNA damage in SQ20b cells treated with cisplatin in the presence and

absence of O^6 -BG. O^6 -BG-treated cells demonstrated a \sim 2-fold increase in tail moment compared with cells treated with cisplatin alone, indicating more DNA damage on a single cell level (Fig. 5). As expected, a dose effect is observed with cisplatin alone at 50 and 100 μ M, and in the presence of O^6 -BG the relative tail moment doubled. Although this difference is reproducible, the tail moment measured in cells treated with cisplatin alone or in the presence of O^6 -BG is small relative to tail moments observed with monoalkylators or agents that cause single-strand breaks. This is expected due to the type of lesions generated by cisplatin. One possible explanation consistent with these data is that the increase in platinum on DNA after O^6 -BG treatment seen in Fig. 4 results in an increase in single strand breaks due to initiation of repair of platinum lesions. Another possibility is that O^6 -BG is interfering with the repair process resulting in incomplete gap filling.

Effect of O^6 -BG on Sensitivity of Head and Neck Cancer Cell Lines to Carboplatin. O^6 -BG also enhanced the activity of carboplatin against several head and neck cancer

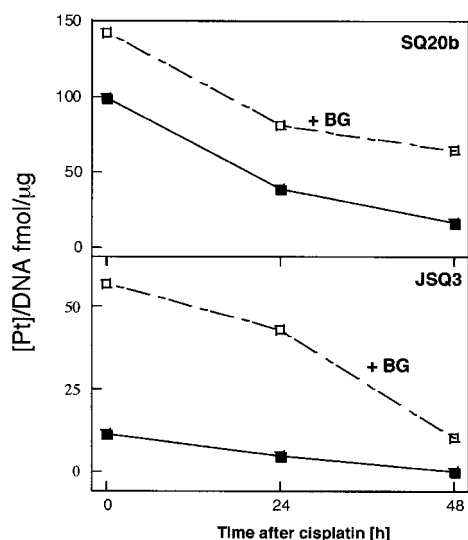


Fig. 4. Effect of O^6 -BG \pm cisplatin on total platinumated DNA. A representative experiment of SQ20b (top panel) and JSQ3 (bottom panel) cells treated with $100 \mu\text{M}$ O^6 -BG (\square) or vehicle (0.01% DMSO; \blacksquare) for 2 h before and during cisplatin exposure is shown. DNA was isolated using phenol-chloroform, and platinum was measured using atomic absorption spectrometry.

cell lines (Fig. 6). O^6 -BG enhanced carboplatin activity (ED_{50}) against SQ20b (2.8-fold), JSQ3 (1.8-fold), SCC35 (1.2-fold), and SCC61 cells (1.9-fold).

TMZ and Radiation Sensitivity of SQ20b Cells after Treatment with O^6 -BG. The cytotoxicity of TMZ and radiation was not enhanced by O^6 -BG treatment of SQ20b cells, supporting the hypothesis that O^6 -BG enhances the activity of agents capable of forming intrastrand or interstrand cross-links (Fig. 7) and not agents that generate monoalkylated bases (in the absence of AGT) or single- and double-strand breaks. This lack of enhancement of TMZ activity by O^6 -BG is common to cells lacking AGT activity such as SQ20b cells.

Discussion

Our results demonstrate that O^6 -BG enhances the cytotoxicity of cisplatin and carboplatin against head and neck cancer lines, concomitant with an increase in the percentage of cells undergoing apoptosis. O^6 -BG combined with cisplatin results in higher levels and persistence of platinum lesions on DNA as well as more DNA damage as measured by the Comet assay 4 h posttreatment. This enhancement is independent of AGT status and specific to bifunctional, DNA-interactive agents. O^6 -BG does not have any effect on the sensitivity of cells to radiation, nor does it affect TMZ activity in cells lacking AGT. These findings have important clinical implications due to the wide use of platinum drugs in head and neck cancer and other tumors such as testicular, ovarian, and lung cancers.

The mechanism by which O^6 -BG enhances cisplatin and carboplatin cytotoxicity is not fully understood. One possible explanation is that O^6 -BG is converted to a benzylated nucleotide that is either misincorporated into DNA while undergoing NER or disrupts the pool of unaltered nucleosides

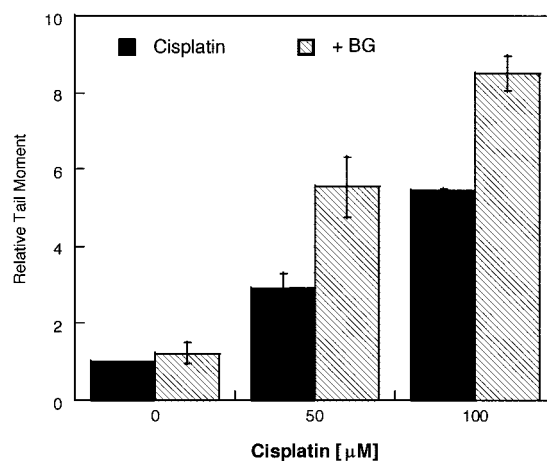


Fig. 5. Effect of O^6 -BG \pm cisplatin on DNA damage. SQ20b cells were treated with $100 \mu\text{M}$ O^6 -BG or vehicle (0.01% DMSO) for 2 h before and during cisplatin (50 and $100 \mu\text{M}$) exposure. Cells were allowed to recover for 4 h and then assayed for DNA damage.

required for repair. Although we demonstrated that inactivation of AGT by O^6 -methylguanidine did not require incorporation of the base into DNA (21), to our knowledge there have been no studies to evaluate whether O^6 -BG at the concentrations used in this study is misincorporated into DNA or disrupts the pool of unaltered nucleosides. Another reasonable explanation is that these compounds inhibit the removal of interstrand and/or intrastrand cross-links formed in DNA. Although there is more platinum on DNA, the rate of repair of platinum lesions does not appear to be changed in the presence or absence of O^6 -BG (Fig. 4). Our data does support the requirement of bifunctional agents that generate intra- and interstrand cross-links, which are repaired by NER and/or HR repair machinery. We previously reported enhancement by O^6 -BG of the activity of a bifunctional phosphoramidate mustard, but lack of enhancement of propylphosphoramidate mustard, a monofunctional analogue of phosphoramidate mustard (16). NER proteins are thought to be primarily responsible for removal of the intrastrand adducts such as 1,3-d(GpTpG), 1,2-d(GpG), and 1,2-d(ApG) (22). Several studies suggest a correlation between NER proficiency and resistance to cisplatin and between NER deficiency and sensitivity to cisplatin. Cells deficient in ERCC1/XP-F, XP-A, XP-D, XP-G, Cockayne syndrome group A, and Cockayne syndrome group B are more sensitive to cisplatin than normal cells (13, 23–29). Testicular tumor cells known to be sensitive to cisplatin have been shown to have lower levels of XP-A and ERCC1/XP-F (30). Conversely, ovarian cancer patients who had high levels of XP-A and ERCC1 mRNA were more resistant to a platinum-based regimen than were patients with lower levels of XP-A and ERCC1 mRNA (31). Little is known about the repair of cisplatin interstrand cross-links. However, De Silva *et al.* (29) recently suggested that unlike other conventional cross-linking agents, repair of cisplatin lesions does not involve induction of DNA double-strand breaks. HR is likely initiated before repair of this type of lesion. To repair an interstrand cross-link, it is possible that a combination of the two pathways, NER and HR, acts sequentially or that proteins from both pathways are involved. Thus, the inhibition of any of

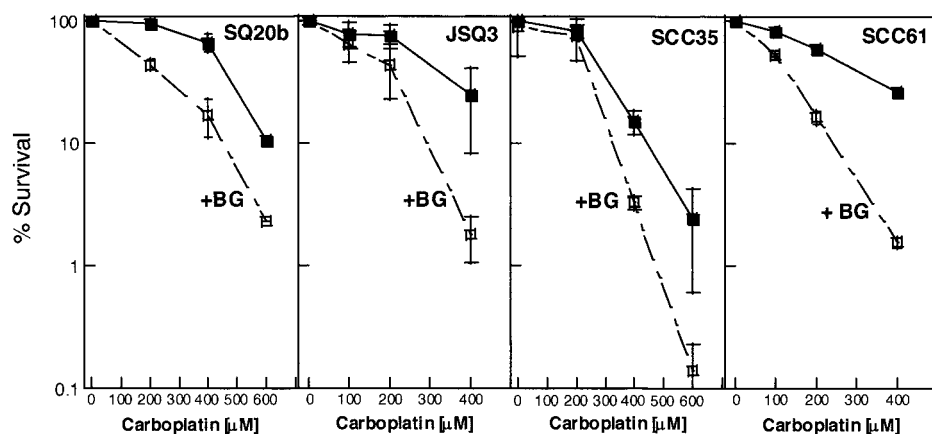
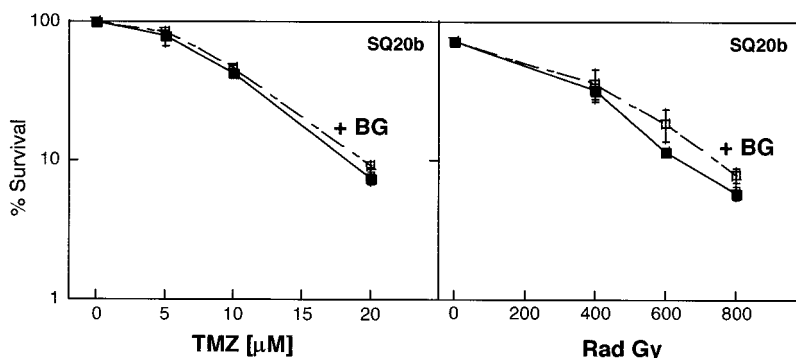


Fig. 6. Effect of O^6 -BG on carboplatin cytotoxicity against head and neck cancer cell lines. Cells were treated with $100 \mu\text{M}$ O^6 -BG (\square) or vehicle (0.01% DMSO; \blacksquare) for 2 h before and during carboplatin exposure. Each data point represents the mean \pm SD from at least three separate experiments. Each experiment represents 6 replicate dishes/treatment group.

Fig. 7. Effect of O^6 -BG on radiation- and TMZ-induced cytotoxicity against the head and neck cancer cell line SQ20b. Cells were treated with $100 \mu\text{M}$ O^6 -BG (\square) or vehicle (0.01% DMSO; \blacksquare) for 2 h before and during radiation or TMZ exposure. Each data point represents the mean \pm SD from at least three separate experiments. Each experiment represents 6 replicate dishes/treatment group.



these enzymes or their interaction with each other by O^6 -BG could explain the enhanced sensitivity to cisplatin.

Another hypothesis to explain enhancement of cisplatin cytotoxicity by O^6 -BG is through cell cycle inhibition. We reported previously that O^6 -BG treatment for approximately 16 h resulted in a dramatic G_0 - G_1 arrest in CHO cells (16) and speculated that perturbation of the cell cycle by O^6 -BG caused an increase in the cytotoxicity of bifunctional alkylating agents. In support of this, a recent report by Gibson *et al.* (32) demonstrated the ability of O^6 -BG to competitively inhibit CDK1 and CDK2. The IC_{50} for O^6 -BG was 24 and 35 μM for CDK1/cyclin B and CDK2/cyclin A, respectively (32). At the 100 μM concentration used in our experiments, it is likely that both CDK enzymes are inhibited by O^6 -BG. However, we did not observe an accumulation of cells in G_0 - G_1 (as was observed with the 16-h O^6 -BG exposure) following the abbreviated treatment schedule of O^6 -BG used in these studies (data not shown). The increase in sensitivity to cisplatin was nevertheless observed with the abbreviated treatment schedule. This suggests that the increase in cytotoxicity is not entirely dependent on a G_0 - G_1 arrest. Inhibition of G_0 - G_1 may be coincident or a result, rather than a cause of persistence of platinum lesions on DNA. We could also speculate that inhibition of CDK1 and/or CDK2 would prevent a protein important for DNA damage recognition and/or repair from being phosphorylated and thereby cause persistence of platinum lesions.

A third possible explanation for the increase in cisplatin cytotoxicity when O^6 -BG is added to the treatment is that

O^6 -BG is blocking the inactivation of cisplatin by glutathione and/or metallothionein by binding to these biomolecules. Both glutathione and metallothionein have been implicated in cisplatin resistance (7). In a cell-free system and *in vitro*, cisplatin was shown to bind to glutathione (33). In cisplatin-resistant human ovarian tumor lines, glutathione levels were increased 13–50-fold (34). Metallothioneins also bind to cisplatin (35), and overexpression of metallothioneins has been associated with cisplatin resistance in tissue culture cells (7, 36). Increased levels of platinumated DNA in SQ20b and JSQ3 cells immediately after incubation with O^6 -BG and cisplatin are consistent with reaction of O^6 -BG with glutathione or metallothionein allowing more cisplatin to bind to DNA.

Modulation of cisplatin cytotoxicity has important clinical implications because of the widespread use of cisplatin in the treatment of many different cancers. Modulators tested previously include F11782 and cyclopentenylcytosine. F11782 is a fluorinated epipodophylloid thought to act through inhibition of ERCC1/XP-F or XP-G endonuclease activity of NER (37). In contrast, cyclopentenylcytosine sensitized HT29 cells to cisplatin both *in vitro* and in a tumor xenograft model through a step subsequent to excision of platinum-cross-linked DNA (38). Another approach to modulating NER activity is to overexpress a truncated form of the XP-A protein, a protein involved in recognition of bulky DNA adducts. The overexpression of truncated XP-A protein sensitized cells to cisplatin, presumably by acting as a decoy and diminishing the interaction of full-length XP-A and

ERCC1/XP-F needed to repair the cisplatin lesions (39). An advantage of O^6 -BG over other modulators is that it has been used clinically and shown to have little to no toxicity when administered alone (40–44). The caveat is that the O^6 -BG concentration required to enhance alkylnitrosourea toxicity through AGT inactivation is lower than that required for cisplatin modulation. Therefore, higher doses of O^6 -BG or a more potent substrate may be necessary *in vivo*.

Apoptosis is a potentially important mechanism for translating cisplatin-related DNA damage into cell death. Considerable evidence indicates that cisplatin can kill cells through the induction of apoptosis (45–47). Cisplatin activates a robust apoptotic response involving the activation of the c-Jun NH_2 -terminal kinase pathway in cisplatin-sensitive ovarian cells, whereas it fails to elicit such a response in the cisplatin-resistant cells (48). Although it is still debatable whether the clinical success of cisplatin relies primarily on its ability to trigger apoptosis, at least two distinct pathways have been proposed to contribute to cisplatin-induced apoptosis *in vitro*. One involves the tumor suppressor protein p53, and the other is mediated by the p53-related protein p73 (49–51). The head and neck cancer cell lines used in these experiments vary in their p53 status: SQ20b, JSQ3, and SCC25 are p53 mutant; SCC35 and SCC61 are p53 wild type (52). It follows that p53 status of the cells is not a determinant of the ability of O^6 -BG to enhance cisplatin cytotoxicity. Cisplatin lesions that are not repaired induce the mitochondrial apoptotic pathway, most likely through a decrease in the levels of the antiapoptotic protein Bcl-2 (53). We also observed an increase in apoptosis and persistence of platinum in the DNA of cells treated with O^6 -BG and cisplatin, which supports the induction of apoptosis when the cisplatin lesions are unrepaired.

The major limitation to successful treatment of malignancies with platinum drugs is the emergence of drug-resistant tumor cells. Cellular resistance to these drugs is multifactorial and consists of mechanisms that limit the formation of lethal platinum-DNA adducts. There is a need for the discovery of modulators that result in increased platinum DNA damage and greater apoptosis. Modulation of cisplatin resistance has been limited by the availability of nontoxic repair inhibitors. Some examples include aphidicolin, cytarabine, hydroxyurea, gemcitabine, and arabinosyl-2-fluoroadenine (54). Although these agents have demonstrated synergy with cisplatin, each contributes its own toxicity. We show here that O^6 -BG, a modulator in Phase II and III clinical trials, results in increased levels of platinum-DNA adducts in a series of head and neck cancer cell lines. To date, there have been no significant toxicities associated with O^6 -BG (40–44). The combination of O^6 -BG and cisplatin may result in increased response rates in patients with tumors that are sensitive to cisplatin and carboplatin such as head and neck, ovarian, lung, and testicular cancers. Perhaps O^6 -BG could be effective in cisplatin-resistant as well as cisplatin-sensitive tumors, and the combination of O^6 -BG and DNA cross-linking agents is being investigated further in human tumor xenograft studies.

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