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ORIGINAL ARTICLE

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Role of glutathione and nucleotide excision repair in modulation of cisplatin activity with O^6-benzylguanine

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Abstract *Purpose*: Modulation of platinating agent cytotoxicity has important clinical implications as a result of their widespread use in the treatment of many different cancers. O^6-Benzylguanine (BG) enhances the cytotoxicity of cisplatin against several human tumor lines. The purpose of our work was to elucidate whether BG affects pathways prior to DNA damage (i.e., glutathione, GSH) or following DNA damage (i.e., nucleotide excision repair, NER). *Methods*: In efforts to determine the mechanism of enhancement we: (1) evaluated whether different sequences of BG plus cisplatin treatment differed in their ability to enhance cisplatin-induced cytotoxicity and DNA platination; (2) determined the effect of BG on GSH and glutathione S-transferase (GST) activity and; (3) determined whether BG enhanced cisplatin-induced cytotoxicity in cells lacking specific enzymes in the NER pathway. Colony-forming assay, atomic absorption spectroscopy and HPLC were employed to measure tumor cell growth inhibition, quantitate the amount of platinum on DNA, and determine intracellular GSH concentrations, respectively. *Results*: Increased cytotoxicity and platination of DNA was observed when cells were exposed to BG prior to and/or during cisplatin treatment and not when BG followed cisplatin treatment. BG did not significantly alter GST activity with minimal depletion of GSH. In contrast, buthionine sulfoximine (BSO) caused a much more dramatic decrease in GSH than BG that was not accompanied by a dramatic increase in sensitivity to cisplatin. Furthermore, BG enhanced the cytotoxicity of cisplatin in a series of cell lines deficient in NER. *Conclusions*: Overall, our results suggest that the mechanism of enhancement involves neither the GSH nor the NER pathways, but triggers an event prior to DNA platination damage that ultimately results in increased cytotoxicity, apoptosis and increased platination levels.

Keywords Cisplatin · Modulation · Chemotherapy

Abbreviations AGT: O^6-alkylguanine-DNA alkyltransferase · BG: O^6-Benzylguanine · BSO: Buthionine sulfoximine · CDK: Cyclin-dependent kinase · CSA: Cockayne syndrome A · CSB: Cockayne syndrome B · ERCC-1/XPF: Excision repair cross-complementing 1/xeroderma pigmentosum F · GSH: Glutathione · GST: Glutathione S-transferase · HPLC: High-pressure liquid chromatography · NER: Nucleotide excision repair · TC-NER: Transcription-coupled NER · XP: Xeroderma pigmentosum

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Introduction

Chemotherapeutic DNA-platinating agents, including cisplatin, carboplatin, and oxaliplatin, are effective against a variety of cancers such as lymphomas, testicular, ovarian, lung, and head and neck carcinomas [30]. In particular, cisplatin in combination with etoposide and bleomycin is considered a curative treatment for testicular cancer [14]. Despite their activity against testicular cancer, the activity of platinating agents against other cancers is more limited. For example, cisplatin and carboplatin elicit low response rates against head and neck cancer, yet remain standard components of common multiagent regimens for advanced primary and metastatic disease [15, 32]. Although patients may respond initially to cisplatin or carboplatin treatment, acquired resistance is frequent. 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 [9, 23, 35].

O^6-Benzylguanine (BG) is a potent, specific inactivator of the DNA repair protein, AGT, that in cells expressing AGT enhances the cytotoxicity of agents such as alkylnitrosoureas and alkyltriazenes that produce toxic lesions at the O^6 position of guanine [11]. In AGT-proficient and AGT-deficient head and neck cancer cell lines, we have demonstrated that BG, in combination with cisplatin, results in greater cytotoxicity, a higher percentage of cells undergoing apoptosis, and significantly higher levels of platinum lesions/DNA compared to cisplatin treatment alone [19]. Platinating agents are not known to produce O^6-substituted guanine adducts in DNA which could possibly serve as substrates for AGT repair. Treating the AGT-deficient cell line, SQ20b, with BG does not enhance the cytotoxicity of agents that cause double strand breaks (radiation) or monoadducts (temozolomide), suggesting that the bifunctionality of damage (DNA crosslinking) is important for the enhancement of cisplatin cytotoxicity [19].

To improve our understanding of the modulation of cisplatin activity by BG we investigated: (1) whether the effect of BG on cisplatin-induced cytotoxicity was sequence-dependent; (2) whether BG was affecting the GSH pathway; and (3) whether BG increased the sensitivity of cell lines deficient in nucleotide excision repair (NER). Cisplatin has been shown to be a substrate for the GSH pathway [8, 28]. Using human liver tumor cells, Zhang et al. [50] investigated which step in the GSH pathway was most important for modulating cisplatin activity. They found that inhibition of glutathione S-transferases (GSTs) or GSH conjugate export pump has no effect on cisplatin cytotoxicity, but inhibition of GSH synthesis results in more DNA interstrand crosslinks and an increase in cisplatin-induced cytotoxicity. Evidence exists that modulation of the GSH pathway via inhibition of GSH synthesis results in an increase in the sensitivity of cells to cisplatin [29, 31, 41]. We hypothesized that BG could modulate the GSH pathway by interacting with GSH and thereby increase the intracellular concentration of cisplatin. Our observed increase in total DNA platination immediately following treatment with BG plus cisplatin [19] is consistent with this hypothesis.

Another possibility is that BG inhibits repair of platinum lesions through direct inhibition of NER proteins. The lesions generated by cisplatin on DNA are monoadducts, intrastrand crosslinks including 1,2-d(GpG), 1,2-d(ApG), and 1,3-d(GpNpG) and interstrand crosslinks [35], with intrastrand crosslinks accounting for about 90% of the lesions [13, 18]. Furthermore, a consistent difference between testicular cancer cells which are sensitive to cisplatin and cancer cells that are considered resistant to cisplatin is the lower expression level of two proteins important in the NER pathway, XPA and ERCC1-XPF [39]. To ascertain whether BG directly interacts with NER, resulting in an increase in the sensitivity of tumor cells to cisplatin, we tested the effect of BG on cisplatin cytotoxicity in several NER-deficient cell lines derived from XP patients from complementation group C, A, F, and G, and CSA and CSB patients. The results of these various studies are presented here.

Methods

Cell lines

The head and neck cancer cell line SQ20b was kindly provided by Dr. Michael Beckett (Department of Radiation and Cellular Oncology, University of Chicago). NER-deficient lines were purchased from Coriell Cell Repository (Camden, N.J.): primary human XPC fibroblasts (GM00677), human transformed XPF fibroblasts (GM08437), primary human XPG fibroblasts (GM13370), primary human CSA fibroblasts (GM01856), and primary human CSB fibroblasts (GM01098). The cell lines were maintained in media as indicated by Coriell Cell Repository. Primary human XPA (XP12BE) cells were purchased from the American Type Cell Culture (XP1223), and SL89 (human primary foreskin fibroblasts) were derived from the foreskin of a normal neonate by V.M.M. The head and neck cancer cell line SQ20b was maintained at 37°C in an atmosphere containing 5% CO_2 in Dulbecco's MEM/F12 (50/50 mixture, Mediatech, Herndon, Va.) supplemented with 20% fetal bovine serum (Hyclone, Logan, Utah), 0.4 μg/ml hydrocortisone, and 1% penicillin/streptomycin (Mediatech). Normal fibroblasts, SL89 were maintained in MEM Eagle-Earle BSS and supplemented with 10% fetal bovine serum (Hyclone), nonessential amino acids, and sodium pyruvate (Mediatech).

Materials

Cisplatin, chlorodinitrobenzene (CDNB), GSH, BSO, acivicin, and perchloric acid were purchased from Sigma

Aldrich (St Louis, Mo.). Monobromobimane was purchased from Calbiochem (San Diego, Calif.). BG was synthesized as described previously [10, 43]. BG and cisplatin were dissolved in DMSO, while BSO was dissolved in PBS. Apoptosis assay kits including annexin-V, FITC, and PI (propidium iodide) were purchased from BD Biosciences Clontech (Palo Alto, Calif.).

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 BG (100 μ*M*) for 2 h prior to addition of up to 50 μ*M* cisplatin for 2 h. Following incubation with BG and cisplatin at 37°C, the cells were washed twice with PBS, trypsinized, and replated in the appropriate medium in triplicate at various densities between 150 and 3000 cells per 100-mm dish. After approximately 12 days, the medium was discarded, plates were stained with methylene blue (0.1%), and colonies scored. Percentage survival was calculated based on the plating efficiency of the appropriate set of control cells exposed to vehicle alone. For GSH modulation, cells were treated with BSO (100 μ*M*) for 16 h followed by the treatment protocol described above for BG and cisplatin.

Trypan blue assay

To evaluate cell growth in the CS lines after cisplatin treatment, approximately 2000 cells/well were plated in six-well plates and allowed to attach overnight. Exponentially growing cells were treated with BG (100 μ*M*) for 2 h prior to the addition of increasing concentrations of cisplatin. Following incubation with BG and cisplatin at 37°C, the cells were washed twice with PBS, and serum-containing medium was added to each well. After approximately 96 h, the medium was discarded, cells were trypsinized and live cells were counted with a hemocytometer. Viable cells were quantitated using trypan blue staining. Percentage survival was calculated based on the appropriate set of control cells exposed to vehicle alone.

Measurement of platinum on DNA

Atomic absorption spectroscopy was used to quantitate platinum on DNA. Exponentially growing cells were treated with BG (100 μ*M*) or vehicle for 2 h and then 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, pelleted by centrifugation, washed twice in ice-cold PBS, and incubated in lysis buffer (10 m*M* Tris-HCl, pH 8.0, 0.1 *M* NaCl, 0.1 m*M* EDTA, 0.5% SDS, and 20 μg/ml RNase) for 5 h at 37°C. The cell lysate was then incubated overnight at 50°C after adding 100 μg/ml proteinase K. Total genomic DNA was isolated by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. Platinum concentrations were determined 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 [16].

GST assay

Exponentially growing cells were pretreated with BG (100 μ*M*, 4 h), or vehicle for 4 h. Cells were collected at 0 h and 3 h after completion of BG treatment. Cells were harvested using a cell scraper, pelleted by centrifugation, and washed twice in cold PBS containing 1 g/l D-glucose twice. Activity assay was conducted as previously described [27]. Briefly, pellets were resuspended in 0.1 *M*KPO_4 (pH 6.5) on ice and sonicated. Following sonication, cell lysates were centrifuged (20,000 *g*, 30 min) at 4°C. After determining protein concentration, activity was measured at 340 nm using the following concentrations: 1.0 m*M* CDNB, 1.0 m*M* GSH, and protein concentrations to achieve a change in absorbance that was a linear function of enzyme concentration and of time for at least 3 min when the rate of absorbance change was limited to less than 0.05 min^{-1} [26].

GSH assay

Exponentially growing cells were pretreated with BSO (100 μ*M*, 16 h), BG (100 μ*M*, 4 h), or vehicle for 2 h, and cisplatin (50 μ*M*) was added for an additional 2 h. Cells were collected at 0 h and 3 h after completion of cisplatin treatment. Cell pellets were washed with PBS and resuspended in PBS containing 20 μ*M* acivicin, an inhibitor used to block GSH breakdown catalyzed by gamma-glutamyltranspeptidase. Monobromobimane was added as a trapping reagent for GSH in further HPLC analysis, and the cells were incubated for 5–10 min at 37°C. Perchloric acid (0.5–1 *M*) was added to stop the reaction and to precipitate proteins. Cells were lysed by sonication to ensure all organelles were disrupted and then centrifuged to sediment denatured proteins. The supernatant was mixed with 0.5–2 *M* KOH to bring the pH to between 4 and 7, and the resulting perchlorate salts sedimented by centrifugation. Supernatants were analyzed for GSH content by HPLC. Separation was performed on a Hewlett-Packard RX C18 Zorbax column (Palo Alto, Calif.) with a Waters

Millennium 32 System (Milford, Mass.). Elution was carried out with a gradient formed between mobile phase A (acetonitrile) and mobile phase B (0.1% acetic acid, 10% methanol, 89.9% water). The following elution program was used (linear gradients) at a flow rate of 1.0 ml/min: 0–5 min 7% A; 8 min 100% A; 8–13 min 100% A; 18 min 7% A. GSH-bimane was monitored at excitation and emission wavelengths of 350 nm and 470 nm, respectively. GSH concentrations were determined by comparison with a standard curve performed on the same day as the assay.

Fluorescein-conjugated annexin-V (annexin-V-FITC)/ propidium iodide (PI) staining

To analyze cells for apoptosis, annexin-V-FITC antibody and PI were added to the cells according to the manufacturer's indicated protocol (BD Biosciences Clontech, Palo Alto, Calif.). Samples were analyzed by flow cytometry using FACS DiVa (Becton Dickinson, San Jose, Calif.). As described previously, cells that were annexin-V-FITC-positive, PI-negative were considered positive for apoptosis [19].

Statistical analysis

For the cell survival assays, analysis of variance (ANOVA) models were used to test for an overall effect of the dose of cisplatin, BG treatment, and the interaction between cisplatin dose and BG treatment. Experiment was treated as a blocking factor to control for experiment to experiment variability. The variable outcome employed was the natural logarithm of the proportion of cells surviving. For the measurement of DNA platination, an ANOVA model was fitted to the platinum concentrations to test for an overall effect of time (0, 24, and 48 h after cisplatin treatment), BG treatment, and the interaction between these two factors. Experiment was again used as a blocking factor. BG prior to cisplatin was not included in the ANOVA model due to the small number in that group. If a statistically significant main effect of BG or interaction was found in an ANOVA model (i.e., the P value <0.05), then pairwise comparisons were made between BG treatments to determine which treatments differed. Of primary interest were the comparisons of cisplatin treatment alone to each of the BG treatment groups.

Results

Effect of various BG treatment schedules on cisplatin-induced cytotoxicity

To determine whether the sequence of administration of BG was critical for enhancement of cisplatin cytotoxicity, we compared various treatment schedules. All cells were treated with cisplatin for 2 h. The treatment groups were as follows: none, without BG; Post, BG for 4 h following cisplatin; During, BG for 2 h during cisplatin; Pre, BG 2 h prior to cisplatin; and Pre/Dur, BG 2 h prior to cisplatin and during 2 h cisplatin treatment. ANOVA demonstrated that cell survival was different across the groups ($P=0.0006$). Based on subsequent pairwise comparisons of the various cisplatin plus BG schedules versus cisplatin alone, exposure to BG only before (BG Pre) or following (BG Post) cisplatin treatment did not enhance cisplatin-induced cytotoxicity (Fig. 1a; $P=0.089$ and $P=0.17$, respectively). The most effective enhancement occurred with BG exposure 2 h prior to and during the 2-h cisplatin treatment ($P=0.0002$). However, BG only during (BG During) cisplatin treatment also resulted in marginal, yet

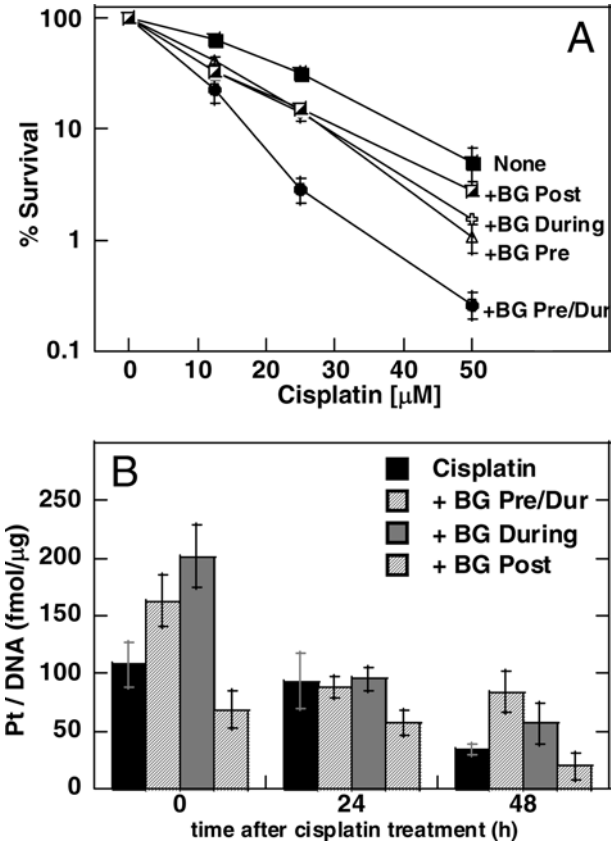


Fig. 1 Effect of various BG treatment schedules on cisplatin cytotoxicity and DNA platination levels. **a** SQ20b cells were treated with 100 μ*M* BG using the indicated treatment schedule or vehicle (0.1% DMSO, *closed squares*) plus increasing concentrations of cisplatin. Cytotoxicity was measured as colony forming ability 10–12 days after treatment. Each data point represents the mean ± SE from at least three separate experiments, and each experiment represents six replicate dishes per treatment group. **b** Platination on DNA of SQ20b cells treated with the indicated treatment schedule plus 50 μ*M* cisplatin or vehicle (0.1% DMSO, *closed squares*) plus 50 μ*M* cisplatin. Cells were collected 0, 24, and 48 h after cisplatin treatment. Total DNA platinum was measured using atomic absorption spectrometry as described in "Materials and methods." Each data point represents the mean ± SE from at least four separate experiments

significant enhancement of cisplatin-induced cytotoxicity ($P = 0.04$). We concluded therefore that enhancement of cisplatin cytotoxicity by BG is dependent on the sequence of drug administration (Fig. 1a).

We also used atomic absorption spectroscopy to compare the total amount of DNA platination in SQ20b cells after exposure to BG and cisplatin under the various conditions described for cytotoxicity. Again, ANOVA detected significant effects of the BG treatment ($P = 0.0052$). Consistent with our cytotoxicity data, BG treatment before and during cisplatin exposure resulted in higher levels of platination compared to cisplatin alone ($P = 0.022$), and when BG was administered immediately following cisplatin treatment, levels of platination decreased ($P = 0.044$; Fig. 1b). Platination levels of cells exposed to BG during the 2-h cisplatin treatment was statistically significantly higher ($P = 0.023$) than following cisplatin treatment alone. Because BG treatment after cisplatin did not enhance cisplatin-induced cytotoxicity, suggesting that BG acts before DNA damage, BG was evaluated for its effect on GST enzymes and GSH concentration.

Role of GST and GSH in BG-enhanced cisplatin cytotoxicity

The GST activity assay was employed to determine if BG was enhancing cisplatin cytotoxicity through inhibition of the GST enzymes. As shown in Fig. 2a, BG treatment for 4 h at 100 μ*M* did not alter GST enzyme activity as measured immediately following or 3 h following treatment in SQ20b cells.

To investigate the role of GSH modulation on the enhancement of cisplatin-induced cytotoxicity by BG, we quantified the concentration of GSH in cells treated with vehicle only (control), BG alone (100 μ*M*, 4 h), cisplatin alone (50 μ*M*, 2 h), and the combination of BG plus cisplatin (BG: 100 μ*M*, 2 h before and 2 h during cisplatin). As shown in Fig. 2b, the GSH concentration after treatment with cisplatin was slightly reduced at both time-points. Cisplatin has been shown to be a

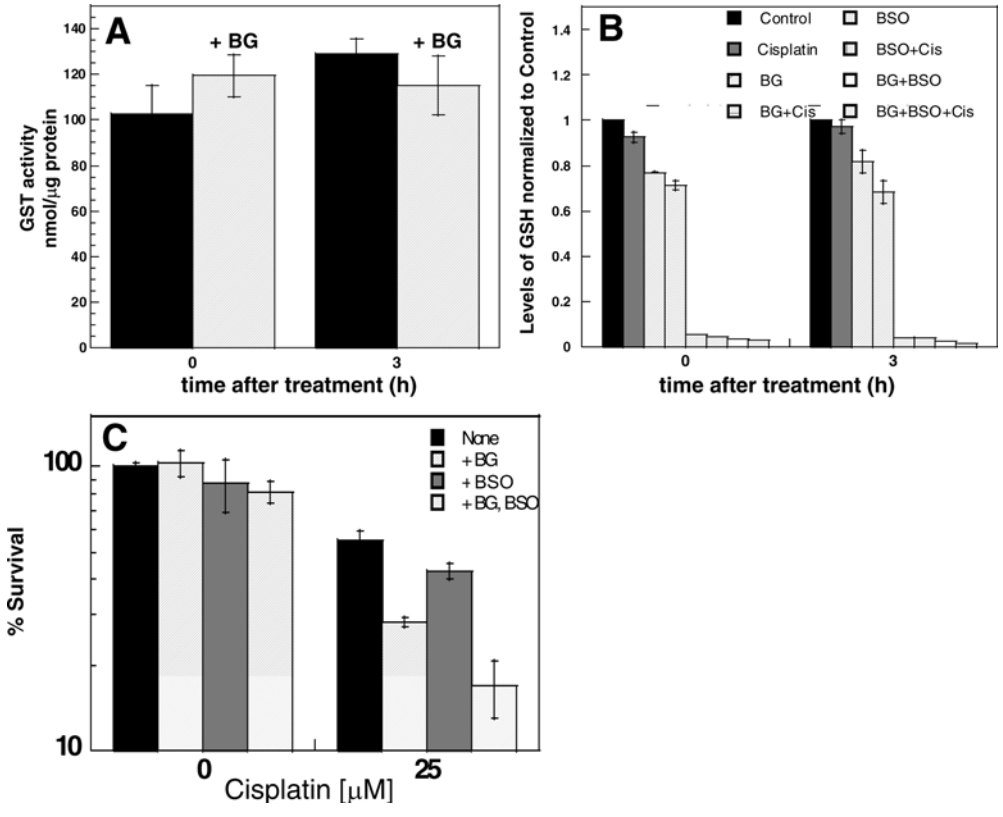


Fig. 2 Effect of BG and cisplatin treatment on the GSH pathway. **a** SQ20b cells were treated with vehicle (0.1% DMSO) or BG (100 μ*M*, 4 h) and collected directly 0 h and 3 h after treatment. Lysates were analyzed for GST activity as described in "Materials and methods." **b** SQ20b cells were treated with vehicle (0.1% DMSO); cisplatin (50 μ*M*, 2 h); BG (100 μ*M*, 4 h); BG + cisplatin (100 μ*M* BG, 2 h prior to and during 50 μ*M* cisplatin, 2 h); BSO (100 μ*M*, 16 h); BSO + cisplatin (100 μ*M* BSO, 16 h prior to 50 μ*M* cisplatin, 2 h); BG + BSO (100 μ*M* BG, 4 h; 100 μ*M* BSO, 16 h prior to BG); and BG + BSO + cisplatin (100 μ*M* BSO, 16 h prior to BG; 100 μ*M* BG, 2 h prior to and during 50 μ*M* cisplatin, 2 h). Lysates were analyzed for GSH content by HPLC as described in "Materials and methods." **c** Effect of BG and BSO on cisplatin-induced cytotoxicity against SQ20b cells. The following treatments are shown (*bars from left to right*): vehicle or cisplatin (0.1% DMSO or 25 μ*M*, 2 h); + BG (100 μ*M*, 2 h before and during cisplatin); + BSO (100 μ*M* BSO, 16 h prior to cisplatin); and + BG and BSO (100 μ*M* BSO, 16 h prior to BG; 100 μ*M* BG, 2 h prior to and during cisplatin). Cytotoxicity was measured as colony forming ability 10–12 days after treatment. Shown is one representative experiment done in triplicate. Each experiment represents six replicate dishes per treatment group

substrate for the GSH pathway [8, 28]. Therefore, we expected the GSH concentrations to be reduced and speculated that GSH concentrations would have actually begun to recover by the end of the 2-h cisplatin exposure. There were decreases in GSH concentrations of approximately 14% and 25% compared to control after treatment with 100 μ*M* BG in the absence and presence of cisplatin, respectively (Fig. 2b).

BSO, an inhibitor of GSH synthesis [25], is known to result in lower GSH levels and increase the sensitivity of cells to cisplatin. We, therefore, compared the degree of GSH depletion and degree of enhancement of cisplatin cytotoxicity induced by treatment with BSO and BG. To evaluate the effect of GSH depletion on cisplatin-induced cytotoxicity and to determine whether a 25% decrease in GSH could account for the observed enhancement of cisplatin cytotoxicity, we treated cells with BSO in the presence and absence of cisplatin plus BG. BSO-induced depletion of GSH in SQ20b cells did not eliminate the effect of BG on cisplatin cytotoxicity, suggesting that the mechanism of enhancement is unlikely to be entirely through depletion of GSH (Fig. 2c). Furthermore, quantification of GSH by HPLC demonstrated a 25% reduction in GSH concentrations after treatment with 100 μ*M* BG, and a 95% reduction in GSH concentration after treatment with BSO in the absence of cisplatin (Fig. 2b). Although BSO caused a much more dramatic decrease in GSH, this was not accompanied by an equally dramatic increase in sensitivity to 25 μ*M* cisplatin (approximately 1.2-fold as compared to a 2-fold increase in cytotoxicity with BG), further arguing against GSH depletion as a mechanism.

ANOVA demonstrated that cell survival was different across the groups ($P = 0.013$). Based on subsequent pairwise comparisons of the various cisplatin plus BG and/or BSO treatments versus cisplatin alone, exposure to BG only or to the combination of BG and BSO resulted in significant enhancement of cytotoxicity (Fig. 2c; $P = 0.014$ and $P = 0.0038$, respectively). Exposure to BSO only did not result in significantly greater enhancement over cisplatin alone ($P = 0.31$).

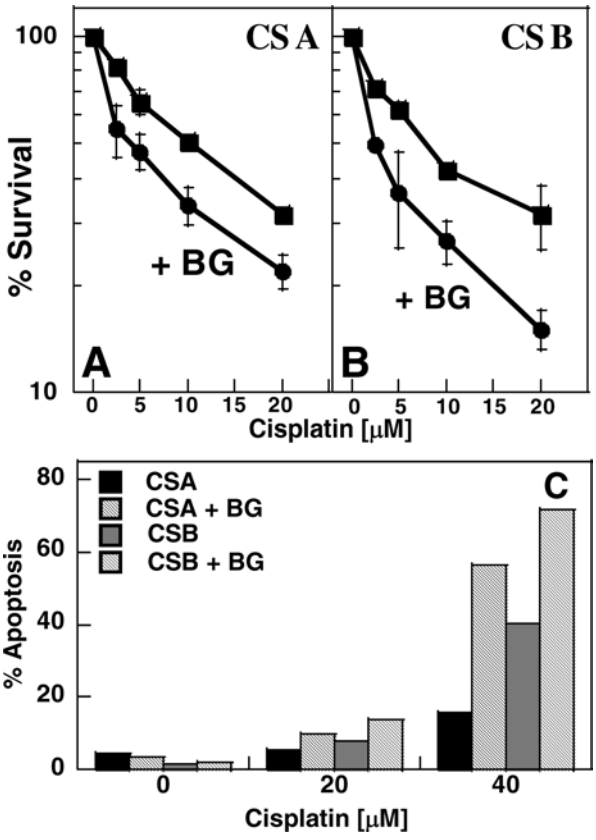
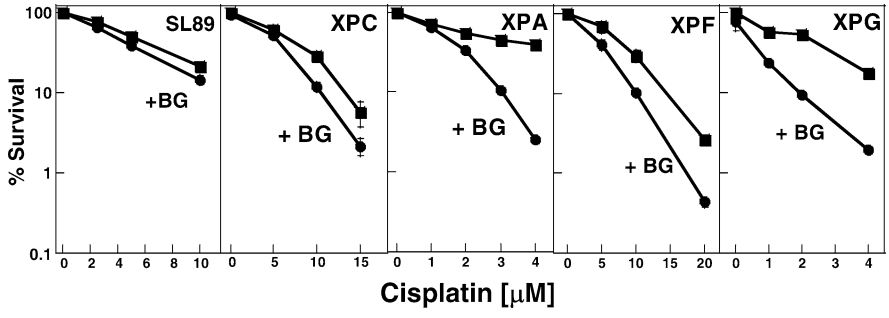


Fig. 4 Effect of BG on cisplatin-induced cytotoxicity and apoptosis in TC-NER-deficient lines. **a**, **b** CS cells were treated with 100 μ*M* BG 2 h prior to and during cisplatin treatment (*closed circles*) or vehicle (0.1% DMSO, *closed squares*) plus cisplatin. Cytotoxicity was measured as percentage of live cells (trypan blue exclusion) 96 h after cisplatin treatment. Each data point represents the mean ± SD from at least two separate experiments. **c** A representative experiment of CS cells stained with annexin-V and PI 96 h following treatment

Fig. 3 Effect of BG on cisplatin-induced cytotoxicity in NER-deficient lines. SL89 and XP cells were treated with 100 μ*M* BG 2 h prior to and during cisplatin treatment (*closed circles*) or vehicle (0.1% DMSO, *closed squares*) plus cisplatin. Cytotoxicity was measured as colony forming ability 10–12 days after treatment. Each data point represents the mean ± SE from at least three separate experiments, and each experiment represents six replicate dishes per treatment group

NER-deficient lines and cisplatin cytotoxicity

To determine the effect of BG on cisplatin-induced cytotoxicity in cell lines deficient in genes important in NER, the combination was evaluated in NER-deficient XP lines. SL89 cells, human primary foreskin



fibroblasts, were included as a control. BG enhanced the sensitivity (ED_{50}) of SL89 cells (1.3-fold, $P = 0.021$), XPC cells (1.25-fold, $P = 0.0081$), XPA cells (1.5-fold, $P = 0.006$), XPF cells (1.6-fold, $P = 0.0001$), and XPG cells (6.7-fold, $P = 0.002$) to the cytotoxic effects of cisplatin treatment (Fig. 3). In all cell survival experiments regardless of the NER status, the slopes of the lines were statistically significantly different comparing cisplatin treatment in the presence and absence of BG. It is interesting to note that the most dramatic enhancement was in the NER cell line deficient in XPG. The combination of BG plus cisplatin was also evaluated in cell lines deficient in transcription-coupled NER (TC-NER). BG enhanced the sensitivity (ED_{50}) of primary human CSA cells (3-fold) and CSB cells (2.5-fold) to cisplatin treatment (Fig. 4a,b). Increased levels of apoptosis were also correlated with the increased sensitivity of CSA and CSB lines in the presence of BG (Fig. 4c).

Discussion

We have previously demonstrated that BG, in combination with cisplatin, results in greater cytotoxicity, a higher percentage of cells undergoing apoptosis, an increase in the amount of DNA platination, and more DNA damage in several human tumor lines [19]. Further investigation of the mechanism involved in this enhancement is presented here. To summarize, our results argue against the following potential mechanisms of BG enhancement of cisplatin cytotoxicity:

(1) Inactivation of AGT: enhancement was observed in cell lines devoid of AGT and with agents that are known not to produce O^6 adducts [6, 19, 46].
(2) Modulation of the GSH pathway: BG treatment did not result in inhibition of GST enzymes, and BSO depleted cells of GSH to a much greater extent than BG, yet did not enhance the cytotoxicity of cisplatin to the extent observed with BG.
(3) Direct inhibition of NER proteins XPC, XPA, XPF, XPG, CSA, and CSB: BG did indeed enhance the cytotoxicity of cisplatin in these NER-deficient cell lines.

Another important finding was that the observed enhancement of cisplatin cytotoxicity by BG was sequence specific. The dramatic increase in cytotoxicity and DNA platination was only observed in cells incubated with BG prior to and during cisplatin exposure and not in cells exposed to BG only before, only during, or immediately following cisplatin treatment. This indicates that a requirement for the observed enhancement is the presence of BG prior to and during DNA damage by cisplatin. Lastly, we present data demonstrating that cisplatin-induced cytotoxicity was enhanced by BG in NER-deficient lines, implying that in tumors with varying levels of NER proteins, cisplatin activity would be expected to be modulated by BG.

Modulation of platinating agent cytotoxicity has important clinical implications as a result of their widespread use in the treatment of many different cancers. Modulators previously tested in vitro include F11782, cyclopentenylcytosine, arabinosyl-2-fluoroadenine, lactacystin, and UCN-01. These modulators have been shown to enhance cisplatin cytotoxicity in vitro through pathways involved in the repair or removal of cisplatin-induced crosslinks [2, 22, 33, 38, 49]. Several of the modulators of platinum resistance that are being evaluated clinically target drug accumulation and efflux. Drugs such as cyclosporin, dipyridamole, amphotericin B, and trifluoperazine are all under investigation to test their efficacy in increasing cisplatin uptake or inhibiting cisplatin efflux [23].

Another way to modulate platinating agents in cancer treatment is through targeting the cell cycle. The cell cycle inhibitor, flavopiridol, exhibits synergy with cisplatin independent of the sequence of administration in A549 cells, a human non-small-cell lung carcinoma cell line [3]. However, in the non-small-cell lung cancer line NCI-H661, cytotoxic synergy was only observed when cisplatin treatment was followed by flavopiridol [40]. In contrast, the combination did not exhibit synergistic or antagonistic toxicity in bladder cancer cells, indicating that the effect may be cell type-dependent [7]. A problem with each of these modulators is that they are often toxic on their own. An advantage of BG is that as a single agent, it does not induce an apoptotic response nor does it exhibit significant cytotoxicity, even at doses as high as 100 μM, in the cell lines we have tested to date [19]. Furthermore, it has been used clinically and shown to have little or no toxicity in humans when administered alone [12, 20, 21, 45, 47].

Due to the finding that BG following cisplatin treatment did not enhance cisplatin cytotoxicity, we speculated that BG was acting upstream of DNA damage. Modulation of the GSH pathway is an upstream event that could explain BG-mediated enhancement of cisplatin cytotoxicity and increased levels of platinum on the DNA. To investigate this, we measured GST activity and GSH concentrations after treatment with BG alone. BSO, an inhibitor of GSH synthesis, was used to evaluate the effect of GSH depletion on cisplatin-induced cytotoxicity [25]. The enhancement of cisplatin-induced cytotoxicity by BSO was not statistically significant when compared to cisplatin treatment alone, whereas enhancement with BG and BG + BSO was significant. SQ20b cells were dramatically depleted (approximately 95%) of GSH following BSO treatment, and GSH concentrations have been shown to be more important than GST activity or GSH export pump activity in the resistance of human liver tumor cells to cisplatin treatment [50]. In SQ20b cells, BG did not have any effect on GST activity, and dramatic depletion of GSH concentrations did not result in a dramatic enhancement of cisplatin cytotoxicity. Therefore, it does not appear that modulation of the GSH pathway by BG contributed significantly to the enhancement observed.

The importance of NER in response to cisplatin treatment has been clearly demonstrated in resistant cells with increased levels of repair enzymes [17, 35] and in sensitive cells such as testicular cancer cells with decreased levels of critical NER proteins [39]. There are two genetically distinct pathways involved in the repair of cisplatin-induced crosslinks: TC-NER and global genome NER. TC-NER repairs transcription-blocking lesions in transcribed DNA strands of active genes [4, 42]. Repair of lesions in the nontranscribed strand of active genes and nontranscribing genome is carried out by global genome NER. Masters and Koberle [39] recently investigated several determinants of cisplatin resistance in testicular cancer cells including drug transport, drug activation and detoxification, and DNA damage responses (p53, apoptosis, and repair). The lower expression level of two proteins important in the NER pathway, XPA and ERCC1-XPF, in testicular tumor cells was the only consistent difference between testicular cancer cells that are sensitive to cisplatin and other cancer cells that are considered resistant to cisplatin [39].

Cells deficient in either NER or homologous recombination (HR) demonstrate increased sensitivity to cisplatin, while cells deficient in mismatch repair (MMR) are rendered more resistant to cisplatin treatment [1] either by failure to induce the futile cycle that leads to strand breaks and eventually apoptosis [5] or because MMR is involved in the lethality of replication-arresting DNA damage through its ability to edit and abort intermediates generated during recombinational repair of double strand breaks [34].

These findings indicate the importance of repair in sensitivity and resistance to cisplatin. Using XPC, XPA, XPF, XPG, CSA, and CSB repair-deficient cell lines in addition to SL89 normal cell line and SQ20b tumor cell line, we found that BG significantly enhanced the cytotoxicity of cisplatin regardless of NER activity. The extent of BG-mediated cisplatin enhancement of ED_{50} was fairly consistent in SL89, XPC, XPA, and XPF (about 1.5-fold). However, the effect of BG on cisplatin ED_{50} was more dramatic in CSA (3-fold), CSB (2.5-fold), and XPG (6-fold). Interestingly, XPG patients frequently display clinical characteristics of CS as well as XP [44]. Furthermore, XPG has been shown to be involved in repair of oxidative damage [36] and in yeast is important in transcription [37]. Perhaps modulation of cisplatin cytotoxicity by BG is even greater in these repair-deficient lines due to gene expression changes associated with CS. It is important to note that these cells are from an affected individual with deficiencies in repair, and the phenotype may be attributed to other genetic changes apart from the known deficiency in XPG, CSA, or CSB. Although we have ruled out inhibition of these important repair proteins as the mechanism of enhancement of cisplatin cytotoxicity by BG, the clinical implications are that BG would effectively enhance cisplatin activity regardless of the NER status of the tumor.

It is also possible that BG has an indirect effect on repair resulting from its effect on the cell cycle. BG is known to inhibit CDK1/cyclin B and CDK2/cyclin A presumably by competing for the ATP binding domain in the CDK enzyme [24, 48]. Enhancement occurs in a sequence-dependent manner when other cell cycle inhibitors are combined with anticancer drugs such as paclitaxel, cytarabine, topotecan, doxorubicin, and etoposide [3], and gemcitabine [40]. Perhaps, BG, through inhibition of CDK enzymes, perturbs the cell cycle and in a more indirect way affects the expression of cell cycle-regulated genes, thereby enhancing the cytotoxicity of platinating agents. We will investigate the potential mechanism of enhancement of cisplatin-induced cytotoxicity by BG and determine the role of perturbation of cell cycle progression by BG in increasing the cytotoxicity of platinating agents, either through inhibition of CDKs or through dysregulation of cell cycle-regulated genes such as histones.

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