Enhancement of cisplatin cytotoxicity by $O^6$-benzylguanine involves endoplasmic reticulum stress

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The abbreviations used are: BG, O$^6$-benzylguanine; ER, endoplasmic reticulum; cisplatin, cis-diammine dichloroplatinum (II); 9-methyl-BG, 9-methyl-O$^6$-benzylguanine; HPV, human papilloma virus; GADD, growth arrest and DNA damage inducible; Grp, glucose-regulated protein; qRT-PCR, quantitative real-time PCR; γH2AX, phosphorylated histone H2AX; PBS, phosphate buffered saline; PP1, protein phosphatase 1.

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ABSTRACT

$O^6$-Benzylguanine (BG) enhances cisplatin-induced cytotoxicity and apoptosis in head and neck cancer cell lines by an unknown mechanism. We investigated the effect of cisplatin with and without BG on two targets of damage: DNA and the endoplasmic reticulum (ER). We chose three cancer cell lines to ascertain the mechanism of BG-enhanced cytotoxicity: SQ20b head and neck and SKOV-3x ovarian cancer cell lines where BG enhanced cisplatin cytotoxicity, and A549 non-small cell lung cancer line where BG did not enhance cisplatin cytotoxicity. All three lines had an increase in DNA damage when BG was added to cisplatin treatment as evidenced by increased platination and $\gamma$H2AX formation. The increase in cisplatin-induced DNA damage following treatment with BG plus cisplatin is not sufficient to increase cytotoxicity or apoptosis. We evaluated the effect of cisplatin on the ER and observed increased caspase 12 cleavage in SQ20b and SKOV-3x cells, but not in A549 cells, following treatment with BG plus cisplatin versus cisplatin alone. GADD153, an ER stress response gene, is upregulated following treatment with BG plus cisplatin compared to cisplatin alone in SQ20b and SKOV-3x cells, but not in A549 cells. ER stress-induced apoptosis is an integral part of the mechanism by which BG enhances cisplatin. Inhibition of ER stress in the SQ20b cell line by salubrinal, an inhibitor of eIF2$\alpha$ dephosphorylation, or GADD153 siRNA, abrogated BG-enhancement of cisplatin cytotoxicity and apoptosis through caspase 3 and 12 cleavage. These data indicate GADD153 upregulation plays an important role in BG-enhanced cisplatin cytotoxicity and apoptosis.
INTRODUCTION

Platinating agents have been used extensively over the past thirty years for treatment of carcinomas, including head and neck, lung, testicular, and gynecologic cancers, and relapsed lymphomas (Hartmann and Lipp, 2003). The main cytotoxic effect of cisplatin (cis-diamminedichloroplatinum (II)) is attributed to formation of crosslinks on DNA, with the prevalent 1,2-GpG intrastrand crosslink thought to be the major cytotoxic lesion (Zorbas and Keppler, 2005). Resistance, both acquired and intrinsic, is a major problem of cisplatin treatment. The initial response rate to cisplatin is only 25-30% in patients with head and neck cancers (Jacobs et al., 1992), and 48% of responding patients with Stage III-IV disease relapse within five years (Arnold, 2006). Approximately 95% of patients with small cell lung carcinoma relapse following treatment with platinating agents (Siddik, 2003). Even in ovarian carcinomas, in which 70% of patients initially respond to cisplatin, the five-year survival rate for responding patients is less than 25%. In platinum-resistant recurrent ovarian cancer, the original regimen of paclitaxel plus a platinating agent is ineffective (Moss and Kaye, 2002). These observations underscore the need to develop modulators of platinum agents to effectively overcome resistance.

The guanine analogue O6-benzylguanine (BG) enhances cisplatin-induced cytotoxicity in head and neck cancer cell lines (Fishel et al., 2003). BG was originally developed as a potent inactivator of O6-alkylguanine DNA alkyltransferase (AGT) (Dolan and Pegg, 1997); however, its enhancement of cisplatin cytotoxicity is independent of its ability to inactivate AGT (Fishel et al., 2003). Structural modifications to BG have resulted in more potent (O6-cyclohexylmethyl guanine) as well as essentially inactive (9-methyl-O6-benzylguanine) compounds, indicating the importance of various structural features on the ability to enhance cisplatin-induced cytotoxicity.
(Fishel et al., 2005b). The mechanism by which BG enhances cisplatin-induced cytotoxicity is as yet unknown, but among the mechanisms ruled out are detoxification by glutathione (GSH) in the cytosol and increased DNA repair of platinum adducts through enzymes within nucleotide excision repair (NER) (Fishel et al., 2005a).

An additional mechanism by which cisplatin can cause apoptosis is through induction of the endoplasmic reticulum (ER) stress pathway (Mandic et al., 2003; Nawrocki et al., 2005; Fribley et al., 2006). One possible mechanism of inducing the ER stress pathway is through oxidative stress in the ER itself (Liu and Baliga, 2003; Liu and Baliga, 2005). Several laboratories have begun to investigate the effect of cisplatin on the ER stress response and modulation of cisplatin activity via the ER response (Linder and Shoshan, 2005; Nawrocki et al., 2005; Fribley et al., 2006). The proteasome inhibitor bortezomib has been shown to enhance cisplatin-induced ER stress in both murine and human cancer cell lines (Nawrocki et al., 2005; Fribley et al., 2006). Bortezomib upregulates Grp78 (BiP) and GADD153 (growth arrest and DNA damage 153, also known as CHOP) expression, thereby signaling downstream in a pro-apoptotic pathway. Grp78 (glucose-regulated protein 78) binds to misfolded proteins in the ER during the unfolded protein response (UPR), whereas GADD153 acts as a transcription factor that is thought to downregulate expression of the anti-apoptotic protein Bcl-2 (McCullough et al., 2001). GADD34 is a cytoplasmic protein that is involved in potentiation of the ER stress pathway (Kojima et al., 2003). Previously, we observed that BG plus cisplatin treatment resulted in the upregulation of GADD34 gene expression in head and neck cancer cell lines (Fishel et al., 2006), further indicating that the mechanism of enhancement of cisplatin-induced cytotoxicity by BG involves potentiation of the ER stress response.
While DNA lesions have been presumed to be the predominant mechanism for cisplatin-induced cytotoxicity, apoptosis can also be initiated through the ER stress pathway (Mandic et al., 2003). Here, we investigate the differences in damage to nuclear DNA and the ER following treatment with BG plus cisplatin compared to cisplatin alone. We examined the tissue and cell line specificity of these types of damage. Our results provide a better understanding of the mechanism of increased cisplatin-induced cytotoxicity caused by BG as well as a better understanding of the cellular targets of cisplatin and the importance of the ER in response to cisplatin.

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, Chicago, IL). The SKOV-3x ovarian cancer cell line was kindly provided by Dr. Robert Bigsby (Department of Obstetrics and Gynecology, Indiana University School of Medicine, Indianapolis, IN). The following cell lines were purchased from ATCC (Manassas, VA): A549, H460, H520, SKOV-3, C33-A, HEC-1-A, and PaCa-2. Media and serum were purchased from Mediatech, Inc. (Herndon, VA) and Hyclone (Logan, UT), respectively. SQ20b cells were maintained in Dulbecco’s MEM/Ham’s F12 (50/50 mixture), supplemented with 20% fetal bovine serum (FBS) and 0.4 μg/ml hydrocortisone (BD Biosciences, Bedford, MA). The A549 lung carcinoma cell line was maintained in Ham’s F12 medium supplemented with 10% FBS. H460, a large cell lung cancer line, and H520, a squamous cell lung carcinoma cell line, were maintained in RPMI 1640 supplemented with 10% FBS, 1.5 g/L sodium bicarbonate (Sigma-Aldrich, St. Louis, MO), 4.5 g/L glucose (Sigma-Aldrich, St. Louis, MO), 10 mM HEPES (Mediatech), and 1.0 mM
sodium pyruvate (Mediatech). The C33-A cervical carcinoma line was maintained in Eagle’s minimum essential medium, supplemented with 10% FBS, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate. The HEC-1-A endometrial carcinoma cell line and SKOV-3X and SKOV-3 ovarian carcinoma cell lines were maintained in McCoy’s 5A medium supplemented with 10% FBS, 1.5 g/L sodium bicarbonate, and 1.0 mM sodium pyruvate. The pancreatic cancer cell line PaCa-2 was maintained in Dulbecco’s modified Eagle medium supplemented with 10% FBS. All cell lines were grown as monolayers at 37°C and 5% CO₂.

**Drugs.** Cisplatin was purchased from Sigma-Aldrich and freshly prepared for each experiment by dissolving it in 100% dimethyl sulfoxide (DMSO), so that the final DMSO concentration was less than 0.1% for the cell experiments. The structure of cisplatin can be found in Rabik, et al. (Rabik, et al., 2006). BG and 9-methyl-O⁶-BG (9-methyl-BG) was kindly provided by the late Dr. Robert C. Moschel (National Cancer Institute at Frederick, Frederick, MD). Structures of BG and 9-methyl-BG are found in Fishel, et al. (Fishel, et al., 2006). Salubrinal was purchased from Calbiochem (San Diego, CA) and dissolved in 100% DMSO as recommended by the manufacturer, with the stock solution being 10 mM. Salubrinal was used in cells at 25 μM, with the final DMSO concentration being less than 0.1%.

**Colony Formation Assay.** Cell survival after drug treatment was determined using the colony formation assay as previously described (Fishel et al., 2003). Briefly, exponentially growing cells were exposed to BG (2 h, 100 μM) prior to the addition of increasing concentrations of cisplatin. Following a 2-h incubation with BG and cisplatin at 37°C, cells were replated in triplicate at
densities varying between 150 and 3000 cells per 100-mm dish. After approximately 10-14 days, colonies were stained with methylene blue (0.1% w/v) and scored. Percentage survival was calculated based on the plating efficiency of cells exposed to vehicle alone.

**DNA Platination Analysis.** Atomic absorption spectroscopy was used to quantify total platinum on DNA, as described previously (Fishel et al., 2003). Cells were treated with 9-methyl-BG (50 μM) or BG (100 μM) with or without cisplatin (25 or 50 μM) as described above, and pellets were collected at 0, 24, or 48 h post-treatment. DNA was isolated either using the Invitrogen ChargeSwitch® gDNA Mini Tissue Kit (A549) (Invitrogen, Carlsbad, CA) or as previously described using phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation (SQ20b, SKOV-3x) (Fishel et al., 2003). Platinum concentration for all samples was assessed with a Perkin-Elmer model 1100 flameless atomic absorption spectrometer (Perkin-Elmer, Norwalk, CT), monitoring 265.9 nm. Platinum concentrations were determined by comparison with a standard curve of known platinum concentrations performed on the same day as the assay (Erkmen et al., 1995). Due to inter-experimental variation, statistical analysis was performed on normalized samples. A two-tailed Student’s t-test assuming unequal variance was performed for statistical analysis. All results were obtained from at least three biological replicates.

**Western Blots.** Cancer cell lines (SQ20b, A549, PaCa-2, SKOV-3x) were treated with vehicle, BG alone, cisplatin alone, and BG plus cisplatin as described above. After drug treatment, exponentially growing cells were harvested and lysed in radioimmunoprecipitation assay (RIPA) buffer containing phosphatase and protease inhibitors. Phosphorylation of histone H2AX was used as a marker to quantify formation of DNA double strand breaks (DSB). This assay was chosen preferentially over the comet assay (single cell gel electrophoresis) because of its
specificity for DSB. While the comet assay also measures DSB formation, it also identifies DNA single strand breaks (Johansson et al., 2008) and apurinic sites (Fatur et al., 2003). Because H2AX phosphorylation is specific for DSB formation, and does not measure other types of DNA damage (Johansson et al., 2008), this assay was preferential for these studies. Phosphorylation of histone H2AX at Ser139 (γH2AX) was measured with a phosphorylation-specific H2AX antibody from Upstate Cell Signaling Solutions (Waltham, MA) as previously described (Rogakou et al., 1998; Fishel et al., 2007). Mouse monoclonal anti-phospho-histone H2AX (1:1000) or goat anti-actin antibody (1:1000, as a loading control) was used to probe for protein levels. For GADD153 westerns, rabbit monoclonal anti-GADD153 (1:500) (Abcam, Cambridge MA) or mouse anti-actin antibody (1:1000, as a loading control) (Abcam) was used to probe for protein levels. Bands were detected using a chemiluminescence kit from Roche Applied Biosciences (Indianapolis, IN), visualized either from autoradiographic films or directly from the blot using the Bio-Rad ChemiDoc (Hercules, CA), and quantified using either QuantityOne® (Bio-Rad) or Sigma Scan Pro 5.0 (Leesburg, VA) (Vasko et al., 2004).

Caspase 3 and 12 Activity. Apoptosis was determined by measuring cleavage of caspase 3 and caspase 12. Exponentially growing cells were treated with vehicle, cisplatin (25 μM), BG (100 μM), BG plus cisplatin, 9-methyl-BG (50 μM), or 9-methyl-BG plus cisplatin as described above. Following treatment, normal growth medium was added back to cells, and cells were incubated for another 24, 48, or 72 h. Following the desired incubation, cells were collected and stained by incubating with 1 of 2 fluorescein isothiocyanate (FITC)-conjugated small molecules, which bind irreversibly to activated caspases: ZEVD-FITC (caspase 3) or ATAD-FITC (caspase
12). (BioVision, Mountain View, CA). After incubation, cells were washed and fluorescence was measured using the FL-1 channel of a FACScan (Becton Dickinson, Franklin Lakes, NJ).

Quantitative Real-Time PCR (qRT-PCR). SQ20b, SKOV-3x, and A549 cells were treated for 2 h with BG (100 μM) or vehicle in serum-free media. After 2 h, 25 μM cisplatin was added for 2 h, after which cells were washed in phosphate-buffered saline (PBS), trypsinized, pelleted, and flash frozen in liquid nitrogen. Pellets were stored at -80°C until RNA isolation. Total RNA was isolated from cells using a combination of the Qiagen QiaShredder kit and Qiagen RNeasy Mini kit (Valencia, CA), following the manufacturer’s protocol. To analyze samples for RNA transcript levels, the LightCycler® RNA Amplification SYBR Green I kit for qRT-PCR was purchased from Roche Applied Science (Indianapolis, IN), and samples were run on the SmartCycler® (Cepheid, Sunnyvale, CA). The protocol used was in accordance with the manufacturer’s indicated specifications. Primers were designed for GADD153 with the forward primer 5’-AACAGAGTGTCATTCCC-3’ and the reverse primer 5’-TTCCCTGCTTGAGCCGTTC-3’. β-Actin was used as the endogenous control with forward primer 5’-ATTGCCGACAGGATGCAGA-3’ and reverse primer 5’-GCTCAGGAGGAGCAATGAGCTT-3’. Standard curves for β-actin and GADD153 were prepared from RNA isolated from exponentially growing cells which ranged in RNA concentration from 0.064 to 1000 ng/μL and had an r² value ≥ 0.985. Thermocycler parameters were as follows: β-actin: 55°C x 1800 sec, 95°C x 600 sec, cycle of 95°C x 1sec - 58°C x 10 sec – 72°C x 6 sec (repeated 45 times), followed by a melting curve from 60°C to 95°C, moving at 0.1°C per sec; GADD153: 55°C x 1800 sec, 95°C x 600 sec, cycle of 95°C x 1 sec - 58°C x 10 sec - 72°C x 6 sec - 82°C x 6 sec (repeated 45 times), followed by a melting curve from 58°C.
to 95°C moving at 0.1°C per sec. Optics were on during the last stage of the cycle and the melting curve. Expression was detected using SYBR green master mix from the kit. RNA concentration in control and drug-treated samples was calculated using the comparative cycle threshold (C_T) values. GADD153 expression was normalized using β-actin, and each experiment was conducted in biological triplicate with freshly treated cells. A one-tailed Student’s t-test was used to compare the control group with treatment groups, and a two-tailed Student’s t-test was used for any comparison between treatment groups. All results were obtained from at least three separate experiments.

**siRNA Transfection.** SQ20b cells were transfected with GADD153 siRNA using the Amaxa 96-well shuttle nucleofection system (Amaxa Biosystems, Gaithersburg, MD) using 250,000 cells/well. The siGENOME ON-Target plus SMARTpool GADD153 siRNA (Dharmacon RNA Technologies, Lafayette, CO) or ON-Target plus Non-Targeting pool was used at a concentration of 1.25 μM, with the total volume of each well being 20 μL. Nucleofection was performed using the Amaxa 96-well Nucleofector Kit SE and nucleofector program DS-113. Following nucleofection, cells were allowed to recover for 1 h before addition of BG. Treatment was then performed as described above, with the following exceptions. For the colony forming assays, higher concentrations of cells were plated to account for the increase in cell death following nucleofection. Cells were plated at concentrations 1.5 times higher than those used for cells that were not transfected. For caspase 3 and caspase 12 assays, apoptosis was evaluated at 48 h post-treatment based on optimization conditions.

**Statistical Analysis.** All statistical analyses were performed using Student’s t-test assuming unequal variance, with the exception of those described below. For colony-forming assays in
which the main effect (difference in slopes) was evaluated (salubrinal and siRNA assays), analysis of variance (ANOVA) models were used with cisplatin dose (0, 6, 12.5, and 25 μM) and modulator condition as factors. The cisplatin dose by modulator condition interaction was also tested. A significant interaction would indicate that the cell survival rates differ significantly by modulator treatment. Experiment-to-experiment variability was controlled for by including experiment as a factor in each ANOVA model. For analytic purposes, the outcome of interest was the proportion of cells surviving (i.e., colony count / number plated). Due to the skewness of the data, the arcsine transformation was employed as appropriate. A p-value <0.05 was considered to be statistically significant. Analyses were performed using Stata, Version 10 (StataCorp LP, College Station, TX).

RESULTS

Cell-line specificity of BG plus cisplatin enhancement. To examine the potential tissue-specificity of cisplatin plus BG treatment, we tested a number of cancer cell lines representing tumor types treated with platinating agents in a clinical setting. Using a clonogenic assay, we observed significant enhancement of cisplatin-induced cytotoxicity following BG treatment in the endometrial carcinoma cell line HEC-1-A, the pancreatic tumor cell line PaCa-2, and the ovarian cancer cell line SKOV-3x (p<0.05, Table 1). In the cervical cancer cell line C33-A and the ovarian cancer cell line SKOV-3, BG enhancement of cisplatin-induced cytotoxicity trended toward significance (Table 1). In two non-small cell lung carcinoma cell lines (H460 and H520), there was no enhancement of cisplatin cytotoxicity by BG (Table 1). We previously observed that treatment of five head and neck cancer cell lines with BG prior to and during cisplatin
treatment results in increased cytotoxicity (Fishel et al., 2003), and this enhancement was not observed in the A549 non-small cell lung cancer cell line (Fishel et al., 2006).

We chose three of these cell lines to act as positive and negative controls for further study to better understand the mechanism of cellular damage caused by cisplatin in the presence of BG. Cell lines chosen as positive for enhancement by BG were the SQ20b head and neck cancer cell line and the SKOV-3x ovarian cancer cell lines, while the A549 non-small cell lung cancer cell line was chosen as a cell line negative for enhancement by BG.

Effect of BG on cisplatin-induced DNA platination. Previously, we observed an increase in total DNA platination in the SQ20b head and neck cancer cell line following treatment with BG plus cisplatin as compared with cisplatin alone (Fishel et al., 2003). Using atomic absorption spectroscopy, we evaluated DNA platination as a marker for DNA damage in head and neck (SQ20b, positive), ovarian (SKOV-3x, positive), and lung (A549, negative) cancer cell lines (Figure 1). We observed increased levels of total platination in BG plus cisplatin-treated samples from all three cell lines analyzed, even though cisplatin-induced cytotoxicity is not enhanced in the A549 lung cancer cell line by BG. Directly following 2 h of cisplatin treatment, there were 1.4, 1.5, and 1.4 fold increases in total DNA platination in SQ20b, SKOV-3x, and A549 cell lines, respectively, when cells were treated with BG (Figure 1). This increase in total platination was significant in the SQ20b cell line at all three timepoints and in the A549 cell line at the latter two timepoints. We further evaluated platinum levels with a structurally similar agent, 9-methyl-\(O^6\)-benzylguanine (9-methyl-BG), that does not enhance cisplatin cytotoxicity (Fishel et al., 2005b). We observed an enhancement of total DNA platination (1.6-fold at 0 h post-treatment) in the SQ20b head and neck cell line when using 9-methyl-BG, similar to that observed following
treatment with BG plus cisplatin (Figure 1A); however, this enhancement following treatment with 9-methyl-BG plus cisplatin was not significant at the time points evaluated (0, 24, and 48 h post-treatment). We concluded that an increase in total DNA platination in these cell lines did not correlate with an increase in cytotoxicity.

Effect of BG on cisplatin-induced DSB. To determine whether double-strand DNA breaks (DSB) were increased upon the addition of BG to cisplatin, we used an antibody specific for phosphorylated histone H2AX (γH2AX) which cisplatin alone has been shown to increase (Bosco et al., 2004). In three cell lines we tested which were positive for BG-enhancement of cisplatin cytotoxicity (SQ20b, SKOV-3x, and PaCa-2), the addition of BG to cisplatin treatment resulted in increased levels of γH2AX formation compared to treatment with cisplatin alone (Figure 2 and data not shown). In SQ20b and SKOV-3x cells, 4-fold and 3.3-fold more γH2AX formed, respectively, in cells treated with BG plus cisplatin (50 μM) compared to cells treated with cisplatin (50 μM) alone (Figure 2). To determine whether this was specific to cell lines in which BG enhances cisplatin cytotoxicity, we also examined γH2AX formation in the A549 lung cancer cell line and found 2.5-fold enhanced γH2AX formation with BG plus cisplatin as compared to treatment with cisplatin alone (Figure 2). We also observed that treatment of the SQ20b cell line with cisplatin plus 9-methyl-BG resulted in 2.76-fold enhancement in γH2AX formation as compared with cisplatin alone (data not shown).

Effect of BG ± cisplatin on endoplasmic reticulum stress. Cisplatin has been shown to result in endoplasmic reticulum (ER) stress leading to apoptosis (Mandic et al., 2003), and we have observed upregulation of the ER stress gene GADD34 upon treatment with BG plus cisplatin.
(Fishel et al., 2006). To investigate further the role of ER stress in the response of cancer cell lines to cisplatin in the presence and absence of BG, we utilized two markers of ER stress: caspase 12 activity and induction of GADD153.

**Caspase 3 and 12 activity assays.** To determine whether the mechanism of enhancement of cisplatin-induced cytotoxicity by BG involved preferentially increasing apoptosis due to ER stress, we investigated cleavage of two different caspases: caspase 12 (ER stress specific) and caspase 3 (general downstream) in all three cell lines. FITC-labeled small molecule inhibitors of the active caspases were used to measure caspase cleavage following treatment with BG ± cisplatin. These inhibitors bind irreversibly to activated caspases, providing a measurement of active caspase within the cell. Using flow cytometry, we observed that in the SQ20b and SKOV-3x cell lines cisplatin alone caused ER-stress induced apoptosis (caspase 12 cleavage) that contributed to cellular apoptosis (caspase 3 cleavage). In contrast, apoptosis in the A549 cell line was independent of ER stress, as no caspase 12 cleavage is observed (Figure 3). Previous reports have shown that A549 cells are capable of activating caspase 12, indicating that this pathway is not defective in these cells (Bitko and Barik, 2001). Cisplatin did induce apoptosis in the A549 cell line, presumably through DNA damage, as indicated by measurable caspase 3 cleavage. However, consistent with cell survival of A549 cells, there was no enhancement in caspase 3 cleavage when comparing samples treated with BG plus cisplatin to those treated with cisplatin alone (Figure 3F). BG significantly increased the level of cisplatin-induced caspase 12 cleavage only in those cell lines in which BG enhanced cisplatin-induced cytotoxicity (SQ20b and SKOV-3x) (Figure 3A, C). Treatment with 9-methyl-BG (negative control) plus cisplatin did not enhance caspase 12 or 3 cleavage over the levels observed with cisplatin alone in any of the three
cell lines (Figure 3). Neither 9-methyl-BG nor BG treatment without cisplatin resulted in increased caspase cleavage over vehicle (data not shown).

**Induction of GADD153 mRNA.** To confirm further the role of ER stress, we evaluated the upregulation of an important gene in the ER stress pathway, GADD153, immediately following treatment of SQ20b, SKOV-3x, and A549 cell lines with BG plus cisplatin. Upon treatment of SQ20b cells with BG alone, there was an 11-fold increase over vehicle-treated cells in GADD153 expression (p<0.05). GADD153 RNA levels significantly increased over vehicle control from 2-fold for treatment with cisplatin alone to 23-fold for BG plus cisplatin treatment (p<0.05) (Figure 4). Consistent with caspase results, 9-methyl-BG plus cisplatin did not upregulate GADD153 expression in the SQ20b head and neck cancer cell line as compared with vehicle control (data not shown). In the SQ20b cell line, upregulation of GADD153 was initiated during treatment with BG alone, and was further upregulated following addition of cisplatin to BG treatment; however, this difference was not significant (Figure 4).

Results similar to those in SQ20b cells were observed in the SKOV-3x ovarian cancer cell line. The following increases in GADD153 expression in SKOV-3x cells were observed: 1.4-fold (cisplatin alone), 28-fold BG alone, and 39-fold BG plus cisplatin upon comparison to vehicle control (p<0.05). Furthermore, there was a significant increase due to BG plus cisplatin versus cisplatin alone in both SQ20b and SKOV-3x cells. In the A549 lung cancer cell line, BG in the presence or absence of cisplatin did not upregulate GADD153 expression (Figure 4).

**Induction of GADD153 protein.** As upregulation of mRNA transcripts does not always correlate to increases in protein, we confirmed the above results by performing Western blots on SQ20b, SKOV-3x, and A549 cell lines treated with BG plus cisplatin. While we did not observe the same fold increase observed using qRT-PCR, the overall trends remained the same. Immediately
following treatment, SQ20b cells treated with BG plus cisplatin had 2.2-fold more GADD153 protein than did those treated with cisplatin alone (Figure 5A); in SKOV-3x cells, BG plus cisplatin-treated cells had 1.8 times more GADD153 than did the corresponding cells treated with cisplatin alone (p<0.05) (Figure 5B). In SQ20b cells this increased expression was also observed when comparing BG plus cisplatin-treated cells to vehicle-treated controls 6 h following treatment (data not shown). Notably, we did not observe a continued upregulation of GADD153 protein in SKOV-3x cells at 6 h (data not shown), potentially indicating a different timeframe of ER stress induction than in the SQ20b cell line. No increase in GADD153 expression was observed in A549 lung cancer cells treated with BG plus cisplatin as compared to control at both the 0 and 6 h timepoints, further indicating that BG plus cisplatin did not cause ER stress in this cell line (Figure 5C, data not shown).

Inhibition of ER stress by salubrinal and GADD153 siRNA. To determine whether the increased enhancement of cisplatin-induced cytotoxicity was due to activation of the ER stress pathway, we evaluated how inhibiting the ER stress pathway affected the treatment of SQ20b cells with BG plus cisplatin. We utilized two different approaches. The first involved pretreatment with salubrinal, a small molecule inhibitor of eIF2α dephosphorylation, and the second used siRNA targeted against GADD153.

Salubrinal. Salubrinal acts by inhibiting the dephosphorylation of eIF2α (Boyce et al., 2005). Therefore, treatment with salubrinal should provide a translational repression of GADD153 induction. We confirmed significant levels of translational repression of the ER stress-specific protein, GADD153, following treatment in samples treated with BG, cisplatin, and salubrinal, as well as in samples treated with salubrinal plus cisplatin (Supplementary Figure 1). Cells treated
with salubrinal alone did not experience a significant decrease in GADD153 protein levels, which was expected because salubrinal would prevent induction of GADD153 protein but would not affect the baseline level already present in the cell (Supplementary Figure 1). Cells treated with either cisplatin alone or BG plus cisplatin exhibited significantly lower levels as compared to vehicle control. This is likely due to the complete inhibition of protein translation during eIF2α phosphorylation, which would prevent both baseline and induced expression of GADD153 (Supplementary Figure 1). Cells were evaluated for the effect of salubrinal on cytotoxicity of BG ± cisplatin (Figure 6A). Salubrinal treatment did slightly decrease cisplatin cytotoxicity in cells without BG; however, this decrease was not statistically significant. Salubrinal significantly decreased, albeit did not eliminate, the enhancement in cytotoxicity observed following treatment with BG plus cisplatin (Figure 6A).

We then determined apoptosis by measuring both caspase 3 and caspase 12 cleavage in SQ20b cell lines treated with or without salubrinal, BG, and cisplatin (Figure 6B, C). In samples treated with BG, cisplatin, and salubrinal, no enhancement of cisplatin-induced apoptosis was observed in either caspase 12 or caspase 3 cleavage. This indicates that the enhanced apoptosis observed in Figure 3 was due primarily to ER stress-induced apoptosis, as salubrinal is specific for inhibition of ER stress-induced cell death. Corresponding to the results observed in cytotoxicity assays, there was a slight, but not significant, reduction in caspase 3 and 12 cleavage in samples treated with cisplatin plus salubrinal as compared to those treated with cisplatin alone at 72 h (Figure 6B, C). The combination of cisplatin plus salubrinal did not eliminate the induction of apoptosis via caspase 12 or caspase 3. Therefore, cisplatin-induced apoptosis is more dependent upon DNA damage and is less affected by inhibition of the ER stress pathway.
This indicates a potential difference in the mechanism by which cells undergo apoptosis and cytotoxicity between cells treated with cisplatin alone and those treated with BG plus cisplatin. **GADD153 siRNA.** Previous research has indicated the importance of GADD153 expression to potentiation of the ER stress pathway (McCullough et al., 2001). To verify the importance of GADD153 in the mechanism of BG-enhanced cisplatin cytotoxicity, and because of the potential off-target effects of salubrinal in the cell, we evaluated how downregulating GADD153 induction via siRNA would affect the ability of BG to enhance cisplatin-induced cytotoxicity and apoptosis. Because we had demonstrated a correlation between increased GADD153 mRNA expression and protein expression (Figures 4 and 5), we used qRT-PCR to evaluate the effect of siRNA on GADD153. As a control, a scrambled, non-targeting (NT) siRNA was used as a determinant of any off-target effects caused by the siRNA molecule. qRT-PCR indicated that SQ20b cells transfected with NT siRNA prior to BG with or without cisplatin treatment had upregulated GADD153, similar to that observed in untransfected cells (Supplementary Figure 2). As in mock and untransfected cells, treatment with BG in NT-transfected cells resulted in upregulation of GADD153, but there was not a significant difference between cells treated with BG alone and those treated with BG plus cisplatin (Supplementary Figure 2). However, cells transfected with siRNA against GADD153 showed significant downregulation of GADD153 expression, beginning immediately following treatment and continuing through at least 24 h post-treatment (Supplementary Figure 2).

The ability of GADD153 siRNA to abrogate the enhancement of cisplatin cytotoxicity by BG was evaluated using long-term clonogenic assays. While NT-transfected cells showed significant enhancement of cisplatin-induced cytotoxicity when treated with BG plus cisplatin versus cisplatin alone (p<0.05), cells transfected with GADD153 siRNA and treated with BG...
plus cisplatin did not exhibit any enhanced cytotoxicity as compared to treatment with cisplatin alone (Figure 7A, B). Notably, there was no significant difference in cytotoxicity between NT- and GADD153-transfected cells treated with cisplatin alone, nor was there a significant difference between NT-transfected cells treated with cisplatin alone and GADD153 siRNA-transfected cells treated with BG plus cisplatin. There were significant differences between NT- and GADD153-transfected cells treated with BG plus cisplatin at 6, 12.5, and 25 μM cisplatin (p<0.05).

ER stress-induced apoptosis was evaluated following transfection with GADD153 siRNA. Untransfected cells (Figure 4) and cells transfected with either NT or GADD153 siRNA were analyzed for caspase cleavage following treatment with BG plus cisplatin (Figure 7C, D). Similar to the salubrinal experiments, there was not a significant difference in the percentage of cells undergoing apoptosis between cells transfected with GADD153 siRNA, NT siRNA, or untransfected cells (data not shown) following cisplatin treatment (Figures 4 and 7C, D). We observed no difference in caspase 3 or 12 activation between NT-transfected cells and untransfected cells with any of the treatments (data not shown). However, for both caspase 12 and caspase 3 cleavage, cells with reduced levels of GADD153 that were treated with BG plus cisplatin showed significantly lower caspase cleavage than did untransfected or NT-transfected cells, indicating a reduction in the activation of apoptotic pathways (Figure 7C, D).

DISCUSSION

Cisplatin remains a vital component of chemotherapy, and overcoming its intrinsic and acquired resistance is important for improving patient outcome. Cisplatin cytotoxicity can be modulated in head and neck, gynecologic, and pancreatic carcinoma cell lines by administration
of BG. This effect is not observed in non-small cell lung cancer cell lines. Irrespective of BG-enhanced cytotoxicity, we observed an increase in total DNA platination and in formation of DSB as measured by $\gamma$H2AX following treatment with BG plus cisplatin as compared to treatment with cisplatin alone in SQ20b, SKOV-3x, and A549 cell lines. In contrast, we observed a significant increase in ER stress-induced apoptosis as measured by induction of GADD153 and caspase 12 specific cleavage specific to cell lines that demonstrated enhancement of cisplatin-induced cytotoxicity by BG. Inhibition of this pathway by either salubrinal or downregulation of GADD153 expression significantly diminished this effect, indicating the importance of ER stress-induced damage in the mechanism of enhanced cisplatin cytotoxicity by BG.

The primary mechanism of cisplatin-induced cytotoxicity is presumed to be through the formation of platinum adducts on DNA. Our data indicate that increased platination of DNA, as well as an increase in DSB, are effects that may not result in greater cytotoxicity, as lung cancer cells exposed to BG plus cisplatin exhibit increases in DNA platination and DSB similar to those observed in SQ20b and SKOV-3x cells, without a corresponding increase in cytotoxicity. Therefore, the enhancement of cisplatin-induced cytotoxicity by BG cannot be attributed simply to greater platinum-associated DNA damage and subsequent double strand break as a result of the damage. Additionally, the rates of decrease in DNA platination levels following cisplatin exposure were not significantly different between cells treated with BG plus cisplatin and those treated with cisplatin alone, implying that inhibition of repair is unlikely involved in the mechanism.

Our data demonstrating that an increased amount of platinum adducts on DNA is not sufficient for the increased cytotoxicity observed with BG treatment led us to investigate the role of ER stress. Several laboratories have demonstrated that the ER plays a role in the cellular
response to apoptosis (Mandic et al., 2003; Liu and Baliga, 2005). Cisplatin has been shown to induce apoptosis through ER stress by Mandic, et al., who observed inhibition of cisplatin-induced caspase 12 cleavage and apoptosis in colorectal cancer cell lines after treatment with the small molecule calpeptin (Mandic et al., 2003). Cisplatin may cause ER stress through oxidative damage (Liu and Baliga, 2003; Liu and Baliga, 2005), and high levels of the cytochrome P450 isoform CYP2E1 correlate with increased levels of cisplatin-induced oxidative stress in the ER, as this isoform of P450 is present in the ER and generates reactive oxygen species (Liu and Baliga, 2005). Previous data implicated ER stress in the mechanism of BG, because GADD34, the regulatory subunit of protein phosphatase 1 (Kojima et al., 2003), was upregulated in SQ20b cells following treatment with BG plus cisplatin as compared to cisplatin (Fishel et al., 2006). This was not observed in SQ20b cells treated with 9-methyl-BG plus cisplatin, nor in A549 cells treated with BG plus cisplatin (Fishel et al., 2006). We now demonstrate that BG plus cisplatin treatment results in ER stress-induced apoptosis and induction of GADD153 expression in head and neck and ovarian cancer cell lines, but not in the A549 non-small cell lung cancer cell line. The A549 cell line is capable of undergoing ER stress-induced apoptosis through caspase 12 cleavage when treated with other agents, such as the respiratory syncytial virus (Bitko and Barik, 2001), indicating that the lack of ER stress observed following treatment with cisplatin is not due to an intrinsic defect in this pathway in this cell line.

Interestingly, BG alone also increased transcript levels of GADD153 in cell lines where BG is effective in modulating cisplatin activity. BG is not cytotoxic as a single agent (Fishel et al., 2003), and therefore, increased GADD153 mRNA levels alone are not sufficient for cytotoxicity. However, increased levels of GADD153 exacerbate the oxidative stress that cisplatin causes in the ER of these cell lines, resulting in cumulative apoptosis through the ER
stress, whereas in cells treated with BG alone, the lack of additional stress from cisplatin does not elevate levels of apoptosis nor does it result in cytotoxicity.

We utilized two approaches to mechanistically determine the role of ER stress in the response of cells to BG plus cisplatin. We inhibited activation of the ER stress pathway through either pretreatment with salubrinal or downregulation of GADD153. Salubrinal was discovered as an inhibitor of ER stress in a chemical screen, and was observed to specifically inhibit apoptosis caused by known ER stressors, including tunicamycin (Boyce et al., 2005). Salubrinal acts by inducing phosphorylation of eIF2α, then inhibiting its dephosphorylation, most likely through a direct interaction with the GADD34/PP1 complex (Boyce et al., 2005), and is specific for eIF2α (Boyce et al., 2005). In rat hippocampal neurons, salubrinal decreased the level of ER stress-induced apoptosis by reducing caspase 12 cleavage and increasing cell survival (Sokka et al., 2007). In human leukemia cells treated with curcumin, a phytochemical which induces apoptosis through the ER stress pathway in many cancer cell lines, pretreatment with salubrinal resulted in a significant decrease in curcumin-induced apoptosis and downregulation of GADD153 expression (Pae et al., 2007). In our studies, salubrinal treatment completely ablated enhancement of cisplatin by BG as measured by caspase cleavage, with partial mitigation of enhancement in long-term cytotoxicity experiments.

Salubrinal may also have other effects in the cell, as recent work has suggested that salubrinal can also act as a cellular protector of Bcl-2, preventing loss of this anti-apoptotic protein (Kessel, 2006). This effect would also lead to a decrease in caspase activity and an increase in cell survival, as we observed following treatment with salubrinal, BG, and cisplatin. Because of this, GADD153 siRNA was also evaluated. GADD153 has been used extensively both as a marker of ER stress-induced apoptosis and as a target for siRNA to inhibit ER stress-
induced apoptosis (Pae et al., 2007). In addition to salubrinal, Pae, et al., utilized siRNA targeted against GADD153 to inhibit curcumin-induced ER stress (Pae et al., 2007); GADD153 siRNA was as effective as salubrinal at inhibiting apoptosis following treatment with curcumin (Pae et al., 2007). GADD153 knockdown by siRNA was utilized in cervical cancer cells to inhibit GADD153-dependent apoptosis following treatment with celecoxib (Kim et al., 2006). Similarly, in a colorectal cancer cell line, GADD153 siRNA attenuated resveratrol-induced apoptosis (Woo et al., 2007).

Inhibition of the ER stress pathway by both approaches at least partially inhibited the enhancement of cisplatin-induced cytotoxicity observed following treatment with BG plus cisplatin as compared to treatment with cisplatin alone. Neither downregulation of GADD153 nor addition of salubrinal significantly affected the percentage of cells undergoing apoptosis in cells treated with cisplatin alone. However, salubrinal resulted in slightly more cellular resistance to cisplatin, as measured by clonogenic assays. This raises the question of the importance of ER stress-induced apoptosis in SQ20b cells treated with cisplatin alone. It may be that the relatively modest amount of caspase 12 cleavage observed following cisplatin treatment is not enough to be affected by salubrinal, which may have other effects in the cell. Therefore, we believe that BG is acting at least partially through augmentation of cisplatin-induced apoptosis via the ER stress pathway.

While cisplatin remains a vital mainstay of chemotherapy, the incidences of recurrence and platinum resistance lead to poor survival rates for head and neck, ovarian, and non-small cell lung cancer patients (Jacobs et al., 1992; Moss and Kaye, 2002; Hartmann and Lipp, 2003; Siddik, 2003; Arnold, 2006), underscoring the need to develop effective modulators of platinating agents. We have shown that BG enhances the efficacy of cisplatin by activating the
ER stress pathway to increase apoptosis. Further insight into the role of cisplatin in the ER stress response will yield information about the targets of cisplatin and BG in this pathway. The ability to increase apoptosis though the ER stress pathway may circumvent known resistance mechanisms commonly observed with regimens that include platinum-based agents. BG may affect the type and/or quantity of damage that cisplatin causes in the cell; we have shown that BG quantitatively increases the amount of platinum on DNA, and it appears that BG induces greater cisplatin damage in the ER. BG enhances the ability of cisplatin to cause ER stress, leading to increased apoptosis. This should be examined further along with the identification of the cellular target of BG that leads to the increase in ER stress.
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REFERENCES


FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Effect of BG on cisplatin-induced DNA platination in SQ20b head and neck, SKOV-3x ovarian, and A549 non-small cell lung cancer cell lines. Cells were treated with 100 μM BG or vehicle followed by 50 μM cisplatin. Cells were collected at various time points following treatment. DNA was analyzed for platinum adducts using atomic absorption spectroscopy. Closed circles represent cisplatin treatment alone, closed squares represent cisplatin plus BG, and closed triangles represent cisplatin plus 9-Me-BG. A. SQ20b cell line (cisplatin alone, BG plus cisplatin results previously published in (Fishel et al., 2003)), B. SKOV-3x cell line, C. A549 cell line. Each point represents the mean ± SEM from at least 3 experiments. Statistical analysis was performed on normalized samples due to inter-experiment variation; *: p<0.05.

Figure 2. γH2AX Western blot following treatment with BG ± cisplatin. A. Cells (SQ20b, SKOV-3x, A549) were treated with 100 μM BG or vehicle followed by cisplatin (25, 50 or 100 μM). Cells were collected 24 h after treatment. Protein was isolated using RIPA extraction, and Western blots were probed using an antibody specific for γH2AX. β-Actin was used as a loading control. B. Quantitation of γH2AX formation. Dosimetry was performed using the QuantityOne® software. Each bar represents the mean ± SEM for at least 3 separate experiments. For each cell line, samples were normalized to cisplatin, 50 μM, which was set at 1.0. *: p<0.05.

Figure 3. Effect of BG, 9-methyl-BG, and cisplatin alone and in combination on cleavage of caspase 12 and caspase 3. Cells (A, B SQ20b, C, D SKOV-3x, E, F A549) were treated with 100 μM BG, 50 μM 9-methyl-BG, or vehicle, followed by cisplatin (25 μM). Cells were
collected 24, 48, or 72 h after treatment and caspase 12 (A, C, E) and caspase 3 (B, D, F) cleavage was analyzed by flow cytometry. Symbols: open circle, vehicle only; closed circle, cisplatin; closed square, BG plus cisplatin; closed triangle, 9-methyl-BG plus cisplatin. Each point represents the mean ± SEM from at least three experiments. In the SQ20b cell line (caspase 3, 12) and SKOV-3x cell line (caspase 3, 12), all samples treated with cisplatin, regardless of modulator, were significantly higher than in control cells treated with vehicle only (p<0.05). Similarly, in the A549 cell line, treatment with cisplatin alone resulted in significantly higher levels of caspase 3 cleavage at 48 and 72 h than observed in control cells treated with vehicle only (p<0.05).

Figure 4. Effect of BG and cisplatin, alone and in combination, on GADD153 mRNA expression in SQ20b (A), SKOV-3x (B), and A549 (C) cancer cell lines. Cells were treated with 100 μM BG or vehicle, followed by cisplatin (25 μM). Cells were collected immediately after treatment and GADD153 mRNA expression was assessed by RT-PCR. Each bar represents the mean ± SEM for at least 3 replicates. The * signifies that the treatment is significantly different from vehicle (p<0.05) and † signifies that the treatment is significantly different from cisplatin alone (p<0.05).

Figure 5. Effect of BG and cisplatin, alone and in combination, on GADD153 protein expression in SQ20b (A), SKOV-3x (B), and A549 (C) cancer cell lines. Cells were treated with 100 μM BG or vehicle, followed by cisplatin (50 μM). Cells were collected immediately after treatment and GADD153 protein expression was assessed by Western blot. Each bar represents the mean ± SEM for at least 3 replicates. Cell lines were normalized to β-actin expression and then to
vehicle. The * signifies that the treatment is significantly different from vehicle (p<0.05) and † signifies that the treatment is significantly different from cisplatin alone (p<0.05).

Figure 6. Effect of salubrinal on BG-enhanced cisplatin cytotoxicity and apoptosis. **A.** SQ20b cells were treated with either cisplatin alone (closed square), BG (100 μM) plus cisplatin (open square), salubrinal (25 μM) plus cisplatin (closed circle), or salubrinal plus BG plus cisplatin (open circle). Total treatment was 5 h, with salubrinal alone for 1 h, salubrinal plus BG for 2 h, and salubrinal, BG, and cisplatin for 2 h. Each point represents the mean ± SEM from at least 3 experiments, with each experiment representing 6 dishes per treatment group. p<0.05 for the following comparisons of cell survival rates: vehicle vs. BG; vehicle vs. Sal + BG; Sal vs. Sal + BG; BG vs. Sal + BG. **B and C.** SQ20b cells were treated with either vehicle (closed squares) or salubrinal (25 μM) (open squares), cisplatin (25 μM) (closed circles), salubrinal plus cisplatin (open circles), BG (100 μM) plus cisplatin (closed triangles), or salubrinal plus BG plus cisplatin (open triangles). Each point represents the mean ± SEM from at least 3 separate experiments. Cells were analyzed for caspase 12 (A) and 3 (B) cleavage by flow cytometry. *:p<0.05 BG plus cisplatin vs salubrinal plus BG plus cisplatin samples. †:p<0.01 BG plus cis vs salubrinal plus BG samples.

Figure 7. Effect of GADD153 knockdown on enhancement of cisplatin cytotoxicity and apoptosis by BG. **A and B.** SQ20b cells transfected with either non-targeting (NT) (A) or GADD153 (B) siRNA were treated with either vehicle or BG (100 μM) plus cisplatin. Each point represents the mean ± SEM from at least 3 experiments, with each experiment representing 6 dishes per treatment group. p<0.05 for the following comparisons: NT vs. NT-BG. **C and D.**
SQ20b cells were transfected with either non-targeting (black bars) or GADD153 (white bars) siRNA. One hour post-transfection, SQ20b cells were treated with either vehicle, cisplatin (25 μM), BG (100 μM), or BG plus cisplatin. Forty-eight hours after treatment, cells were analyzed for caspase 12 (C) and 3 (D) cleavage by flow cytometry. Each bar represents the mean ± SEM from at least 3 separate experiments. *p<0.05.
Table 1. IC$_{50}$ values for cell lines treated with cisplatin ± BG.

<table>
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<th>Cell Line</th>
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<th>p-value</th>
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<td>+ BG</td>
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</table>
Figure 2

A

SQ20b
Cisplatin μM 0 25 50 100 + BG 0 25 50 100
Actin γH2AX

SKOV-3x
Cisplatin μM 0 50 100 + BG 0 50 100
Actin γH2AX

A549
Cisplatin μM 0 50 100 + BG 0 50 100
Actin γH2AX

B

Fold Enhancement of γH2AX formation

SQ20b
SKOV-3x
A549
Cis +BG Cis +BG
50 μM 100 μM

*
Figure 3
Figure 4

A

Fold Increase in GADD153 Expression by RT-PCR

SQ20b

*†

B

SKOV-3x

*†

C

A549

Vehicle  Cis  BG  BG + Cis

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Figure 5

(A) SQ20b

(B) SKOV-3x

(C) A549

Fold Increase in GADD153 Expression by Western Blot
Figure 6

**Cisplatin (μM) vs. % Survival**

- SQ20b (A) and SQ20b + Sal (A)
- Vehicle (A)
- SQ20b + BG (A)
- SQ20b + Sal (B)
- BG/Cis (B)
- Sal + Cis (B)
- Cis (B)
- Sal (B)

**Time post-treatment (h) vs. % Caspase Cleavage**

- Caspase 12 (B)
- Caspase 3 (B)
- BG/Cis (C)
- Sal + Cis (C)
- Cis (C)
- Sal (C)
- Vehicle (C)
Figure 7

A. 

B.

SQ20b
NTsiRNA

GADD153 siRNA

% Survival

C.

D.

Control
Cisplatin
BG
BG + Cis

% Caspase Cleavage

Caspase 12

Caspase 3

Treatment (48 h post-treatment)