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Role of GADD34 in modulation of cisplatin cytotoxicity

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Abbreviations:
BG, O^6-benzylguanine
9-CH_3-BG, O^6-benzyl-9-methylguanine
qRT-PCR, quantitative real time polymerase chain reaction
GADD, growth arrest and DNA damage inducible
ATF4, activating transcription factor 4
XBP1, X-box binding protein 1
ER, endoplasmic reticulum
eIF2α, eukaryotic initiation factor 2α

ABSTRACT

Cisplatin and carboplatin are widely used clinical chemotherapeutic agents, especially against testicular, ovarian, and head and neck cancers. O^6-Benzylguanine (BG) has been shown to result in enhanced cytotoxicity, apoptosis, and DNA platination when used in conjunction with cisplatin and carboplatin in head and neck cancer cell lines. Microarray expression data indicated overexpression of 19 genes and underexpression of 22 genes specific to treatment with the combination of BG ± cisplatin compared to cisplatin alone treatment in SQ20b head and neck cancer cells ($p < 0.05$) using the Affymetrix HG-U133A GeneChip®. Among the overexpressed probe sets were genes involved in DNA damage and apoptosis, including GADD34, DDIT4, ATF4, and PTHLH. A similarly structured analog, 9-CH_3-BG, does not enhance cisplatin-induced cytotoxicity or apoptosis nor is there enhanced expression of GADD34 in cisplatin or 9-CH_3-BG ± cisplatin-treated cells compared to control cells. Analysis of cells exposed to 9-CH_3-BG ± cisplatin allowed us to focus our array list on 32 probe sets specific to BG + cisplatin versus cisplatin, ruling out differentially expressed probe sets common to 9-CH_3-BG + cisplatin versus cisplatin. Similarly, 14 probe sets were specific to BG ± cisplatin versus BG, ruling out differentially expressed probe sets common to 9-CH_3-BG ± cisplatin versus 9-CH_3-BG. Quantitative real-time PCR demonstrated a dose dependent increase in GADD34 expression in cells exposed to BG ± cisplatin with levels approximately >2-fold for cells exposed to BG + cisplatin compared to cisplatin alone. Levels of GADD34 transcripts were determined with both cisplatin and BG + cisplatin at several different time points concomitant with and following drug treatment. At all timepoints, GADD34 transcript levels are approximately two-fold elevated in cells treated with BG + cisplatin compared to cisplatin alone. Furthermore, significant changes in GADD34 expression levels in SQ20b, SCC35, and SCC61 cells, with approximately three-fold, two-fold, and 3.5-fold increases in expression, respectively, upon treatment with BG ± cisplatin compared with control. Elucidation of these molecular pathways will aid in our goal of synthesizing more powerful modulators to increase efficacy of platinum agents.

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1. Introduction

Cisplatin and carboplatin are platinating agents that are effective against a variety of malignancies such as testicular, metastatic lung, relapsed lymphomas, head and neck, and gynecologic cancers [1]. However, their efficacy is limited by acquired and intrinsic resistance observed in patients treated with platinum agents underscoring the importance of developing effective modulators for this therapy. Understanding the changes that occur at the molecular and cellular level after cisplatin treatment will have a direct impact on the development of therapeutic options for these patients.

Our laboratory has observed that nontoxic concentrations of O^6-benzylguanine (BG) and other similarly structured guanine derivatives, such as O^6-cyclohexylmethylguanine, potentiate cisplatin and carboplatin cytotoxicity [2]. BG was originally developed as a potent inactivator of the O^6-alkylguanine-DNA alkyltransferase (AGT) repair protein [3] and found later to be an inhibitor of cyclin-dependent kinases (CDK1 and 2) [4]. To better understand the mechanism behind this enhancement of platinum agent cytotoxicity, we initially evaluated candidate gene products such as glutathione and nucleotide excision repair (NER) genes [5]. However, our findings indicate that the mechanism is independent of AGT inactivation, glutathione concentration, and direct inhibition of several NER proteins. Therefore, we employed global gene expression analysis to determine the possible genes or pathway(s) involved in the modulation of cisplatin activity. We selected both an effective and an ineffective modulator of cisplatin-induced cytotoxicity, BG and 9-CH_3-BG, respectively (Fig. 1), to gain insight into the changes specific to modulation of cisplatin. Growth and DNA damage response genes, including GADD34, were found to be specifically overexpressed with BG ± cisplatin treatment in a time- and concentration-dependent manner, but not with 9-CH_3-BG ± cisplatin. Consistent with a role for GADD34 in BG-enhanced cisplatin cytotoxicity, we did not observe significant upregulation of GADD34 expression levels in a lung tumor cell line that showed no enhanced cytotoxicity when BG was combined with cisplatin.

2. Materials and methods

2.1. Cell lines

Head and neck cancer cell lines, SQ20b, SCC61, SCC35 were kindly provided by Dr. Michael Beckett (Department of Radiation and Cellular Oncology, University of Chicago). These cell lines were maintained at 37 °C and 5% CO_2 in Dulbecco's MEM/F12 (50/50 mixture) supplemented with 20% fetal bovine serum, 0.4 μg/ml hydrocortisone (BD Biosciences, Bedford, MA), and 1% penicillin/streptomycin. A549, lung carcinoma cell line, was purchased from ATCC (Manassas, VA) and maintained at 37 °C and 5% CO_2 in Ham's F12 media supplemented with 10% fetal bovine serum. Medium and fetal bovine serum were purchased from Mediatech Inc. (Herndon, VA) and Hyclone (Logan, UT), respectively.

2.2. Drugs

Cisplatin was purchased from Sigma–Aldrich (St. Louis, MO) and prepared fresh for each experiment by dissolving in 100% dimethyl sulfoxide (DMSO) with the final DMSO concentration being less than 0.1% for the cell experiments. BG and 9-CH_3-BG were synthesized as described previously [6,7].

2.3. Colony formation assay

To evaluate cell survival after drug treatment, a colony formation assay was used as previously described [2]. Briefly, exponentially growing cells were exposed to guanine derivative at concentrations indicated for 2 h prior to the addition of increasing concentrations of cisplatin. Following incubation with guanine derivative and cisplatin at 37 °C, the cells were replated in triplicate at varying densities between 150

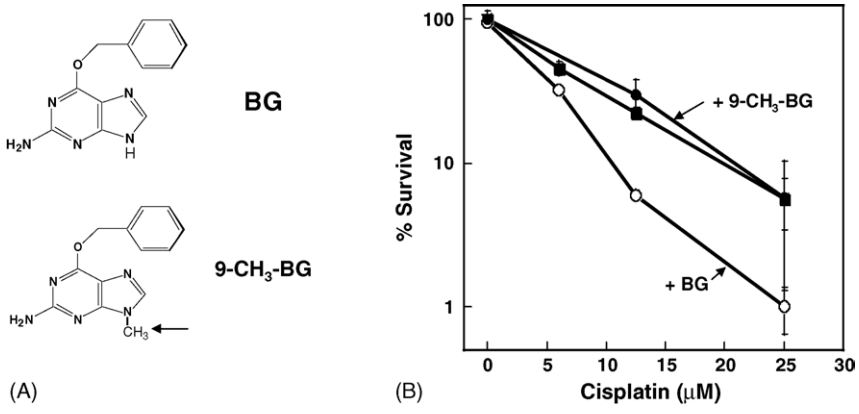

Fig. 1 – Effect of BG and 9-CH_3-BG on cisplatin cytotoxicity in SQ20b cells. (A) Structures of BG and 9-CH_3-BG. (B) Colony formation assay of SQ20b cells treated with vehicle (closed square), BG (open circle), and 9-CH_3-BG (closed circle) at 50 μM for 2 h prior to and during cisplatin exposure (2 h). Each data point represents the mean ± S.E. from at least three separate experiments with each experiment representing six replicate dishes per treatment group. This data is taken from [19].

and 3000 cells per 100-mm dish. After approximately 12 days, colonies were stained with methylene blue (0.1%, w/v) and scored. Percentage survival was calculated based on the plating efficiency of the appropriate set of control cells exposed to vehicle alone.

2.4. Microarray expression analysis

For both microarray experiments, exponentially growing SQ20b cells were treated with BG or 9-CH_3-BG for 2 h prior to addition of cisplatin for 2 h. For experiment 1, cells were collected at 0 and 6 h after completion of BG (100 μM) ± cis-cisplatin (25 μM) treatment, and for experiment 2, cells were collected 0 h after completion of BG (50 μM) or 9-CH_3-BG (50 μM) ± cisplatin (25 μM). Following incubation at 37 °C, the cells were washed twice with phosphate-buffered saline (PBS) and either collected immediately for RNA isolation or had normal media replaced in the flask until the desired time point of collection. Cells were trypsinized, pelleted by centrifugation, washed in ice-cold PBS, flash frozen, and stored at −80 °C until RNA isolation. Total RNA was isolated from cells using the combination of Qiagen QiaShredder kit and Qiagen RNeasy Mini kit (Valencia, CA) following the manufacturer's protocol. The Functional Genomic Facility at the University of Chicago conducted the hybridization using the Human Genome U133A Genechip® containing greater than 22,000 transcripts from Affymetrix Inc. (Santa Clara, CA). Prior to hybridization, the integrity of the total RNA samples was determined using the Agilent 2100 Bioanalyzer microfluidic based assay (Agilent Technologies, Palo Alto, CA). The target preparation protocol followed the Affymetrix GeneChip® Expression Analysis Manual (Santa Clara, CA).

2.5. Microarray data analysis

Two separate software programs were used to analyze the expression data: GeneSpring® 7.1 purchased from Agilent Silicon Genetics (Redwood City, CA) and DNA-Chip Analyzer (dCHIP) available from Harvard University (http://www.dchip.org). Data analyses were performed using DNA-Chip Analyzer 1.3 [8] with the .CEL files obtained from Microarray Suite Analysis (MAS) 5.0 (Affymetrix). In dChip, we used a Perfect Match-only model to estimate gene expression level. The invariant set approach was used for normalization. The chromosomal regions with significantly enriched, differentially expressed genes were identified using "Classify genes" function in dCHIP.

The data presented in Tables 1 and 2 were analyzed using GeneSpring® 7.1, and gene ontologies assigned using Locus link Genebank, GO databases, and the search engine GeneInfoViz [9]. All samples from experiments 1 and 2 were normalized to minimize per chip and per gene variation. Values below 0.01 were set to 0.01. Each measurement was divided by the 50th percentile of all measurements in that sample. Each gene was divided by the median of its measurements in all samples. If the median of the raw values was below 10 then each measurement for that gene was divided by 10. Median normalized data was then filtered for background noise to create a list of probe sets with present or marginal signals and intensity of ≥100 over the baseline signal. Using samples from BG ± cisplatin and cisplatin alone from experiments 1 and 2 at the 2 h time point, a list was created elucidating probe sets with a difference in expression by a factor of 1.5-fold or greater and at a significance level <0.05 between BG ± cisplatin and cisplatin alone groups. The rationale for choosing 1.5-fold as significant cutoff was based on our empirically established, signal-dependent thresholds [10]. This list was then compared to a list created using probe sets found differentially expressed from experiment 2 between the 9-CH_3-BG ± cis-cisplatin versus cisplatin alone list. This list was used to elucidate the changes attributed to BG ± cisplatin treatment combination without including nonspecific changes due to the addition of an altered guanine derivative that did not result in enhanced cytotoxicity.

2.6. Quantitative real time PCR (qRT-PCR)

Cells including SQ20b, SCC61, SCC35, and A549 were treated for 2 h with modulator (BG 100 μM, BG 50 μM, or 9-CH_3-BG 50 μM) in serum-free media. After 2 h, cisplatin was added (25 μM in all lines except SCC61, in which the concentration was 20 μM). At the conclusion of two additional hours, cells were washed in PBS, trypsinized, pelleted, and flash frozen in liquid nitrogen. Pellets were stored at −80 °C until RNA isolation was begun. Total RNA was isolated from cells using the combination of Qiagen QiaShredder kit and Qiagen RNeasy Mini kit (Valencia, CA) following the manufacturer's protocol. To analyze SQ20b cells for RNA transcript levels and verify microarray data, a 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 protocol. Briefly, primers were designed for GADD34 with the forward primer 5′-TCAATTTGCAGATGGCCAGCGTGCTCC-3′ and the reverse primer 5′-CCTCGGCTTTCTCCTCCCCTGGGTTCTTAT-3′. β-actin was used as our endogenous control with forward primer 5′-ATTGCCGACAGGATGCAGA-3′ and reverse primer 5′-GCTCAGGAGGAGCAATGATCTT-3′. Standard curves were prepared from RNA isolated from exponentially growing cells for β-actin and GADD34, which ranged in RNA concentration from 0.064 to 1000 ng/μl and had an r^2 value ≥0.985. Thermocycler parameters were as follows: β-actin – 55° × 1800 s, 95° × 600 s, cycle of 95° × 1 s – 58° × 10 s – 72° × 6 s (repeated 45 times), followed by a melting curve from 60° to 95° moving at 0.1°/s; GADD34 – 55° × 1800 s, 95° × 600 s, cycle of 95° × 1 s – 58° × 10 s – 72° × 6 s – 62° × 6 s (repeated 45 times), followed by a melting curve from 60° to 95° moving at 0.1°/s. Optics were on during the last stage of the cycle and the melting curve. Expression was detected using SYBR green master mix provided in the Roche kit. RNA concentration in control and drug-treated samples was calculated using the comparative cycle threshold (C_T) values. GADD34 expression was normalized using β-actin, and each experiment was conducted in triplicate with freshly treated cells and freshly isolated RNA. A one-tailed Student's *t*-test was used to compare the control group with treatment group, 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.

Table 1 – Genes differentially expressed ($p < 0.05$) upon comparison between BG + cisplatin vs. cisplatin alone showing a fold difference of >1.5

Gene name	Locus link	Average normalized expression		Average fold difference	ANOVA p-value	Gene ontology/biological function
		BG + cisplatin	Cisplatin			
AKAP1	**8165**	**−1.87**	**1.01**	**1.89**	**0.0075**	**RNA binding**
ARL7	**10123**	**−1.75**	**1.03**	**1.81**	**0.0081**	**GTP binding; GTPase activity**
ATF4	**468**	**1.74**	**1.02**	**1.71**	**0.018**	**DNA binding; RNAPII transcription factor**
BTG1	**694**	**1.68**	**−1.15**	**1.93**	**0.0026**	**Kinase binding; transcription cofactor activity**
CDKN1B	1027	1.60	−1.12	1.79	0.014	CDK inhibitor; protein binding; TGFβ receptor
CSF1	**1435**	**−1.36**	**1.24**	**1.67**	**0.046**	**Macrophage colony stimulating factor activity**
CYB561	**1534**	**−1.36**	**1.24**	**1.69**	**0.020**	**Cytochrome-b5 reductase activity**
DDIT4	**54541**	**2.00**	**−1.37**	**2.74**	**0.019**	**DNA damage inducible transcript 4**
DDX11	**1663**	**−1.31**	**1.47**	**1.93**	**0.049**	**ATP-dependent DNA helicase activity**
GADD34	**23645**	**1.76**	**−1.08**	**1.90**	**0.033**	**Apoptosis; DNA damage response; cell cycle**
GORASP1	64689	1.02	−1.92	1.95	0.0047	Protein binding and transport
GPR109B	**8843**	**−1.62**	**1.22**	**1.98**	**0.017**	**G-protein coupled purinergic receptor activity**
HYPE	**11153**	**1.53**	**1.01**	**1.51**	**0.042**	**Protein binding; cell cycle regulation**
INPP5B	**3633**	**−1.28**	**1.18**	**1.52**	**0.031**	**Inositol-polyphosphate 5-phosphatase activity**
IRS2	**8660**	**1.41**	**−1.30**	**1.83**	**0.011**	**Insulin receptor binding; signal transduction**
IRX4	**50805**	**−1.67**	**1.02**	**1.70**	**0.017**	**Transcription factor; heart development**
KIAA1033	**23325**	**1.72**	**−1.02**	**1.76**	**0.050**	**Hypothetical protein LOC23325**
KLF4	**9314**	**1.78**	**−1.08**	**1.92**	**0.023**	**Nucleic acid binding; transcription factor**
LOC90355	**90355**	**1.10**	**−1.38**	**1.52**	**0.0017**	**Hypothetical protein LOC90355**
MEIS2	**4212**	**−1.44**	**1.22**	**1.76**	**0.0090**	**RNAPII transcription factor activity**
MPEG1	**219972**	**−1.63**	**1.12**	**1.83**	**0.048**	**Macrophage expressed gene**
MTHFD2	**10797**	**1.39**	**−1.26**	**1.75**	**0.011**	**Folic acid biosynthesis; oxidoreductase activity**
NAP1L1	4673	1.17	−1.38	1.61	0.047	DNA replication; nucleosome assembly
NFATC2IP	84901	−1.07	1.44	1.54	0.027	Protein modification
NPHP4	**261734**	**1.58**	**−1.02**	**1.61**	**0.048**	**Signal transduction; cell–cell adhesion**
NUP62	**23636**	**−1.55**	**1.09**	**1.69**	**0.046**	**Nuclear pore transport**
OSTM1	28962	1.37	−1.19	1.63	0.011	Integral membrane protein; osteopetrosis
PAK6	**56924**	**−1.64**	**1.13**	**1.85**	**0.039**	**Serine/threonine kinase activity; ATP binding**
PCK2	**5106**	**2.03**	**−1.20**	**2.44**	**0.013**	**Phosphoenolpyruvate carboxylase activity-GTP**
PFAAP5	**10443**	**−1.73**	**−1.01**	**1.71**	**0.029**	**Phosphonoformate immuno-associated protein**
PJA2	9867	1.51	−1.15	1.74	0.049	Protein ubiquitination; ubiquitin ligase activity
PTHLH	**5744**	**−1.52**	**1.09**	**1.66**	**0.035**	**Cell signaling; cell proliferation; cAMP metab.**
PTPN3	**5774**	**−1.80**	**1.19**	**2.14**	**0.046**	**Protein tyrosine phosphatase activity**
RAP2C	57826	1.25	−1.20	1.50	0.032	GTPase mediated signal transduction
RPUSD2	**27079**	**−1.32**	**1.14**	**1.50**	**0.049**	**RNA binding; pseudouridine synthetase activity**
TAOK2	9344	−1.36	1.27	1.73	0.046	Serine/threonine kinase; apoptosis
TCFL4	**6945**	**−1.40**	**1.15**	**1.61**	**0.041**	**DNA-dependent transcription factor**
TSAP6	**55240**	**−1.25**	**1.31**	**1.64**	**0.043**	**Golgi to plasma membrane transport**
UBE2D1*	7321	1.48	−1.27	1.88	0.037	Ubiquitin cycle; ubiquitin conjugating enzyme
USP14	**9097**	**−1.34**	**1.16**	**1.55**	**0.000073**	**Ubiquitin-dependent protein catabolism**
ZNF339	**58495**	**−1.63**	**−1.01**	**1.62**	**0.021**	**DNA binding; zinc ion binding; transcription**

Average normalized expression levels for both treatment groups, as well as the absolute fold difference between BG + cisplatin vs. cisplatin groups, from two independent experiments are shown. Bold rows indicate transcripts that are specific to BG + cisplatin vs. cisplatin treatment and not significantly differentially expressed upon treatment with 9-CH_3-BG (50 μM) + cisplatin vs. cisplatin. An asterick indicates that this gene was identified as significant by the above criteria by more than one probe set on the Affymetrix U133A chip.

3. Results

3.1. *Effect of BG on cisplatin-induced cytotoxicity*

Our laboratory previously demonstrated that BG, at 50 and 100 μM, significantly ($p < 0.001$) increased the sensitivity of SQ20b, head and neck cancer cells to cisplatin, and increased the percentage of cells undergoing apoptosis [2,5]. In contrast, 9-CH_3-BG at 50 μM did not increase the sensitivity of SQ20b cells to cisplatin and had no effect on the percentage of cells undergoing apoptosis (Fig. 1 and data not shown). BG and 9-CH_3-BG at 50 μM were not toxic in the absence of cisplatin treatment, 95.7 ± 5.1% and 105 ± 10.4% survival compared to control cells, respectively.

3.2. *Effect of BG and cisplatin on global gene expression*

We profiled expression of probe sets using the Human Genome U133A GeneChip® from Affymetrix Inc. and compared the expression of SQ20b cells that were treated with vehicle, cisplatin (25 μM), BG (50 and 100 μM), and BG (50 and 100 μM) + cisplatin (25 μM) immediately following treatment (0 h) and 6 h later. Probe sets significantly differentially expressed ($p < 0.05$) upon comparison between both concen-

Table 2 – Genes differentially expressed ($p < 0.05$) upon comparison between BG + cisplatin vs. BG alone showing a fold difference of >1.5

Gene name	Locus link	Average normalized expression		Average fold difference	ANOVA p-value	Gene ontology/biological function
		BG + cisplatin	Cisplatin			
C20orf45	51012	1.37	−1.18	1.16	0.0348	Chromosome 20 open reading frame 45
CEBPD	**1052**	**−1.09**	**−1.67**	**1.53**	**0.0210**	**DNA binding; regulation of transcription**
ETS1	**2113**	**1.07**	**−1.71**	**1.83**	**0.0472**	**Regulation of transcription, DNA-dependent**
FGFR1	**2260**	**1.44**	**−1.22**	**1.76**	**0.0161**	**MAPKKK cascade**
KPNA4	**3840**	**1.07**	**−1.56**	**1.67**	**0.0499**	**Intracellular protein transport**
MYLIP	**29116**	**1.65**	**1.00**	**1.65**	**0.0348**	**Cell motility; protein ubiquitination**
NAB2	**4665**	**1.58**	**−1.09**	**1.72**	**0.0456**	**Regulation of transcription, DNA-dependent**
OSTM1	**28962**	**1.37**	**−1.15**	**1.58**	**0.0037**	**Integral membrane protein; osteopetrosis**
PPP2R3A	5523	1.26	−1.52	1.92	0.0073	Protein amino acid dephosphorylation
PTPNS1	**140885**	**−1.34**	**1.18**	**1.58**	**0.0288**	**Protein tyrosine phosphatase**
RRN3	**54700**	**−1.01**	**−1.52**	**1.50**	**0.0092**	**Transcription from RNA polymerase**
RWDD3	**25950**	**1.00**	**−1.64**	**1.64**	**0.0203**	**RWD domain containing 3**
SUMO2	6613	1.08	−1.52	1.64	0.0179	Protein modification; ubiquitin cycle
TAF12	**6883**	**1.09**	**−1.48**	**1.61**	**0.0233**	**Transcription initiation**
TCEB1	**6921**	**1.33**	**−1.24**	**1.65**	**0.0110**	**Transcription/ubiquitin cycle**
TEAD1	**7003**	**−1.35**	**1.22**	**1.65**	**0.0310**	**Regulation of transcription, DNA-dependent**
TPI1	**7167**	**−1.05**	**−1.72**	**1.64**	**0.0079**	**Gluconeogenesis; isomerase activity**

Average normalized expression levels for both treatment groups, as well as the absolute fold difference between BG + cisplatin vs. BG groups, from two independent experiments are shown. Bold rows indicate transcripts that are specific to BG + cisplatin vs. BG treatment and not significantly differentially expressed upon treatment with 9-CH_3-BG (50 μM) + cisplatin vs. 9-CH_3-BG.

trations of BG + cisplatin and cisplatin alone using a fold difference of >1.5 are listed in Table 1. These 41 probe sets include 22 underexpressed genes, and 19 overexpressed genes. Gene ontology and biological function is also listed; the functions include transcription factors, DNA damage-induced transcripts, and signal transduction. In addition to these genes, a group of genes on chromosome 6p corresponding to several different histone isoforms was found to be differentially expressed following BG treatment using DNA-Chip Analyzer software.

3.3. *Expression changes specific to BG-enhanced cisplatin cytotoxicity*

In the second microarray experiment, 9-CH_3-BG was included as a control to rule out gene expression changes that resulted from exposure to a guanine derivative that did not result in increased cisplatin cytotoxicity. This allowed us to focus on the expression changes associated with enhanced cytotoxicity and apoptosis. The genes specific only to the BG ± cisplatin versus cisplatin alone, not those that came up using the same criteria and experimental design but replacing BG with 9-CH_3-BG are identified in bold (Table 1). From these lists in Table 1, we observed that genes involved in apoptosis and the unfolded protein response (GADD34), in negative regulation of cell proliferation (BTG1, KLF4), in electron transport (MTHFD2), in transcription or DNA binding (ATF4, LOC90355, TCFL4), in signal transduction (ARL7), in gluconeogenesis (PCK2), and in cell cycle regulation (DDX11) appear to be important in modulating cisplatin activity by BG. In a similar manner, we identified probe sets specific to BG ± cisplatin versus BG alone, and not those that came up using the same criteria and experimental design but replacing BG with 9-CH_3-BG (Table 2 identified in bold). Several of these genes were involved in transcription (CEBPD, ETS1, RRN3, NAB2, TAF12, TCEB1, TEAD1).

3.4. *Confirming GADD34 expression changes using quantitative real time PCR in SQ20b cells*

Cisplatin has been shown to result in cell death by means of the endoplasmic reticulum (ER) pathway [11]. We observed changes in GADD34, an important component of ER stress pathway signaling [12] and important in response to DNA damage, in both array experiments comparing BG + cisplatin versus cisplatin alone and chose to confirm these changes using qRT-PCR. Consistent with expression array, GADD34 upregulation increased more dramatically with BG alone or BG ± cisplatin treatment than cisplatin treatment alone. As shown in Fig. 2, there were statistically significant differences between control and BG (100 μM) and control and BG (100 μM) + cisplatin ($p < 0.05$) as well as differences between cisplatin versus BG alone (50 μM, $p < 0.05$; 100 μM, $p < 0.001$) and cisplatin versus BG (50, 100 μM) + cisplatin ($p < 0.05$). Fig. 2 illustrates a dose dependent increase in GADD34 RNA expression following treatment with 50 and 100 μM BG. qRT-PCR confirmed no changes in GADD34 RNA levels following treatment of cells with 9-CH_3-BG ± cisplatin implying that an increase in GADD34 RNA is coincident with modulation of cisplatin cytotoxicity and apoptosis.

3.5. *Determination of GADD34 expression changes upon treatment with BG ± cisplatin*

To ascertain whether upregulation of GADD34 was dependent on the concentration of cisplatin, we evaluated GADD34 expression following exposure to increasing concentrations of BG ± cisplatin (Fig. 3A). We found that GADD34 increased in a dose dependent manner for both treatments with a 1.9–5.4 times higher level if cells were exposed to BG + cisplatin compared to cisplatin alone. GADD34 expression was also evaluated over time following exposure to vehicle, BG alone,

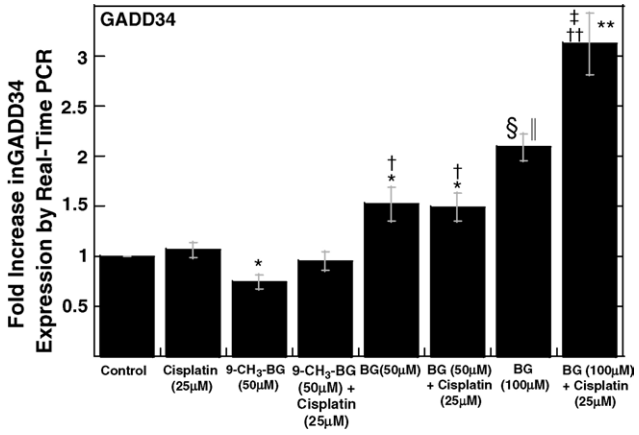

Fig. 2 – Effect of vehicle, cisplatin, BG ± cisplatin, and 9-CH_3-BG ± cisplatin on GADD34 RNA expression in SQ20b head and neck cancer cell line. Each bar represents the mean ± S.E. from at least three separate experiments. (*$p < 0.05$ between control and treatment; ‡$p < 0.05$ between BG (100 μM) alone and BG (100 μM) + cisplatin; †$p < 0.05$ between cisplatin alone and treatment; **$p < 0.01$ between control and treatment; ††$p < 0.01$ between cisplatin and treatment; §$p < 0.001$ between control and treatment; ‖$p < 0.001$ between cisplatin alone and treatment.)

cisplatin alone, or BG + cisplatin (Fig. 3B). Exposure to BG alone or BG + cisplatin resulted in a time-dependent increase in GADD34 that begins 30 min following BG treatment and remains significantly higher than control for 2 h after cisplatin treatment. GADD34 levels drop to basal levels between 2 and 5 h following BG alone treatment. Similarly, normalized GADD34 expression in BG-treated and cisplatin-treated samples had essentially returned to baseline while GADD34 expression in cisplatin + BG-treated samples remained elevated in our first microarray experiment (data not shown). GADD34 levels for cells treated with cisplatin alone remained at basal levels except at 2 h post-treatment (two-fold increase). These data show that increases in GADD34 occur within 30 min of BG + cisplatin treatment and remain elevated up to 5 h post-cisplatin compared to BG alone or cisplatin alone. BG + cisplatin levels of transcript are significantly higher than cisplatin alone at $t = -1.5$ ($p < 0.05$) and $t = 0$ ($p < 0.01$). At 20 h post-treatment, BG + cisplatin levels of transcript are 1.9 times higher than levels with cisplatin alone (data not shown).

3.6. *Quantitative real time PCR with GADD34 in SCC61 and SCC35 cells*

We have previously observed a similar increase in cisplatin-induced cytotoxicity and apoptosis with BG treatment in additional head and neck cancer cell lines (SCC61 and SCC35) [2]. Therefore, we extended our studies evaluating GADD34 expression levels in SCC61 and SCC35 cells. Similar to SQ20b cells, there is a statistically significant induction of GADD34 RNA in these head and neck cancer cell lines upon treatment with BG alone or in combination with cisplatin (Fig. 4).

3.7. *Lack of enhancement of cisplatin-induced cytotoxicity or GADD34 expression by BG in A549 lung carcinoma cells*

To begin to understand the importance of GADD34 upregulation in enhancement of cisplatin cytotoxicity, we evaluated another cancer cell line, A549 lung cancer cells. In contrast to head and neck cancer cell lines, there was no enhancement in cisplatin-induced cytotoxicity in A549 cells (Fig. 5A). As shown in Fig. 5B, concomitant with a lack of enhancement in cytotoxicity, there is no enhancement in GADD34 expression with the combination of BG ± cisplatin treatment even though a significant enhancement (1.8-fold, $p < 0.05$) was observed with BG treatment alone. BG alone can induce GADD34 expression in head and neck cell lines (SQ20b, SCC35,

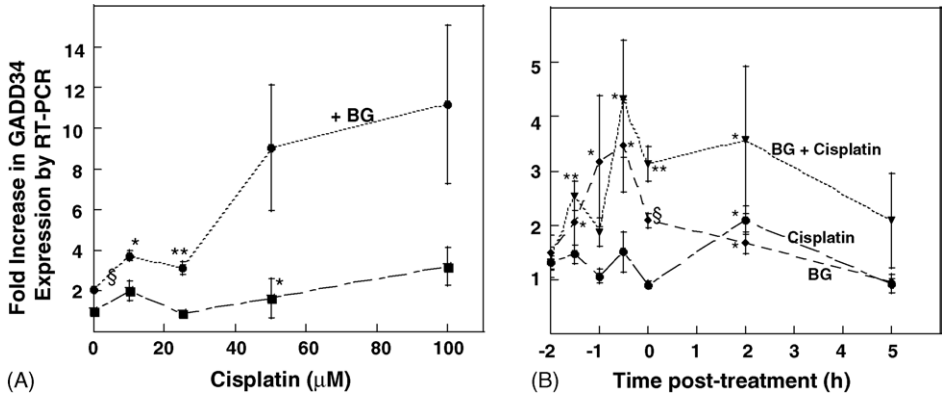

Fig. 3 – Effect of BG ± cisplatin on GADD34 expression over time. (A) Cells were treated for 2 h with vehicle or BG (100 μM) ± cisplatin (10, 25, 50, 100 μM) for an additional 2 h and RNA isolated immediately following treatment. Each data point represents the mean ± S.E. from at least three separate experiments. (B) Cells were treated with vehicle, cisplatin alone (25 μM), BG alone (100 μM) or BG (100 μM) ± cisplatin (25 μM). Cells were exposed to BG for 2 h prior to a 2 h exposure to cisplatin and time = 0 represents the end of the cisplatin exposure. RNA was isolated 0.5, 1 and 1.5 after start of cisplatin treatment and 0, 2 and 5 h following treatment. Baseline expression level was set at 1.0. Each data point represents the mean ± S.E. from at least three separate experiments. (*$p < 0.05$ between baseline (control) and treatment; **$p < 0.01$ between baseline and treatment; §$p < 0.001$ between baseline and treatment.)

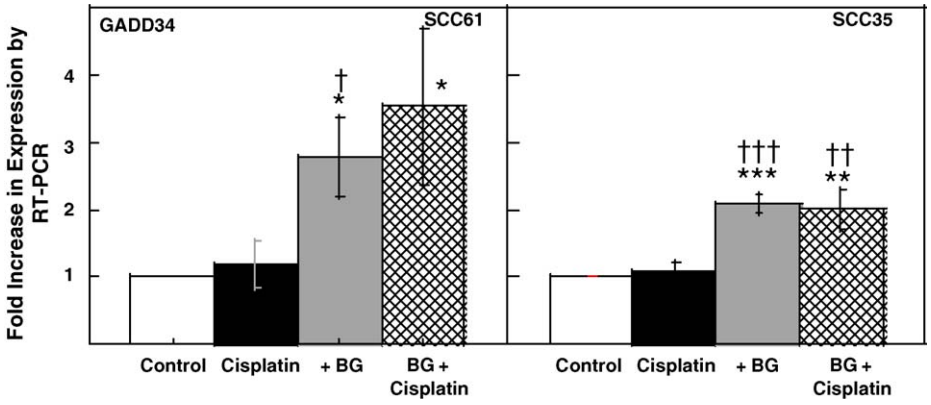

Fig. 4 – Effect of BG on GADD34 RNA expression in SCC61 and SCC35, head and neck cancer lines. Cells were treated with vehicle, cisplatin alone (SCC61: 20 μM cisplatin, SCC35: 25 μM cisplatin, BG (100 μM), and BG + cisplatin, and RNA isolated immediately following cisplatin treatment. Each data point represents the mean ± S.E. from at least three separate experiments. (*$p < 0.05$ between control and treatment; †$p < 0.05$ between cisplatin alone and treatment; **$p < 0.01$ between control and treatment; ††$p < 0.01$ between cisplatin alone and treatment; ***$p < 0.001$ between control and treatment; †††$p < 0.001$ between cisplatin alone and treatment.)

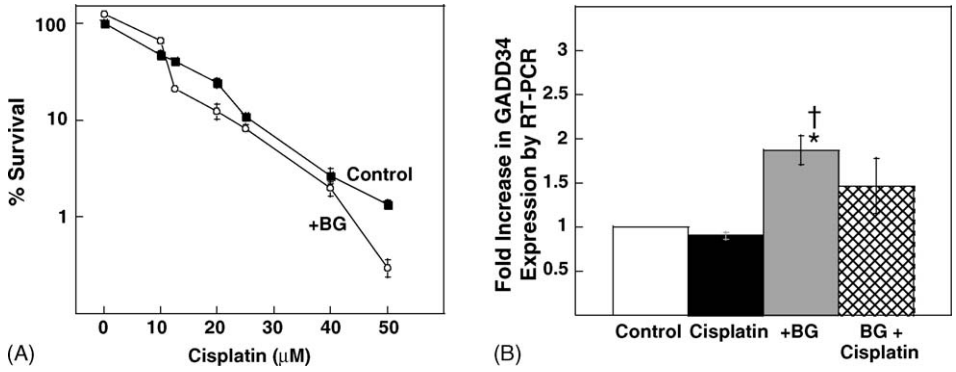

Fig. 5 – Effect of BG on cisplatin cytotoxicity and GADD34 RNA expression in A549 cells. (A) Colony formation assay of A549 cells treated with BG at 100 μM for 2 h prior and during cisplatin exposure (2 h). Vehicle-treated cells (closed squares) and BG-treated cells (open circles). The lines represent the mean ± S.E. from at least three separate experiments with each experiment representing six replicate dishes per treatment group. (B) qRT-PCR was utilized to investigate the changes in RNA levels of GADD34 in A549 cells. A549 cells were treated as described for cytotoxicity and harvested for RNA isolation immediately following cisplatin exposure. Each data point represents the mean ± S.E. from at least three separate experiments. (*$p < 0.05$ between control and BG alone; †$p < 0.05$ between cisplatin alone and BG alone.)

SCC61) and lung (A549), but is not coincident with enhanced cytotoxicity. GADD34 upregulation after treatment with BG ± cisplatin is only observed in the cell lines we tested with enhanced cisplatin cytotoxicity, however, more cell lines will need to be evaluated to determine the significance of this result in A549 cells.

4. Discussion

Our long-term objective is to increase the anti-tumor activity of platinum agents through an understanding of the mechanism of modulation by guanine derivatives. Here, we use Affymetrix microarray to identify global expression changes that are associated with modulation of cisplatin activity. We have found that several classes of genes are consistent with this effect: apoptosis, cell cycle, signal transduction, transcription/DNA binding, and chromosome organization. 9-CH_3-BG was utilized as our negative control because it is structurally similar, yet does not enhance cisplatin-induced cytotoxicity or apoptosis. Ruling out genes that were in common between lists containing BG ± cisplatin versus cisplatin-treated cells and 9-CH_3-BG ± cisplatin versus cisplatin-treated cells allowed us to exclude nonspecific changes due to exposure to a guanine derivative but not specific to enhanced cytotoxicity and apoptosis. GADD34, an apoptosis gene, was validated using qRT-PCR in three head and neck cancer cell lines. GADD34 is known to be overexpressed upon DNA damage, and thus makes it a reasonable candidate for apoptosis caused by cisplatin. Consistent with this, we demonstrated a dose dependent increase in GADD34 up to 100 μM cisplatin with two- to three-fold higher levels in cells

treated with BG + cisplatin. GADD34 transcript levels increase within 30 min of exposure to BG and remains high for 5 h post-treatment for cells that are also treated with cisplatin. Although increases in GADD34 are observed with BG treatment alone, the increases are not as dramatic and return to control levels over time as demonstrated in the timecourse in Fig. 3B. Therefore, we believe that GADD34 upregulation at the time of DNA damage may be important for the synergistic effect observed with cisplatin. In addition, A549 cells, in which cisplatin-induced cytotoxicity is not enhanced by addition of BG, do not demonstrate induction of GADD34 RNA after BG ± cisplatin treatment. In A549 cells, similar to SQ20b cells, BG treatment alone induced GADD34 expression, however, BG is non-toxic to cells, therefore, we believe that GADD34 upregulation is not sufficient for cytotoxicity. GADD34 RNA is no longer induced in A549 cells upon the addition of cisplatin. This lends support to the combination of GADD34 upregulation and DNA damage for cisplatin enhanced cytotoxicity.

GADD34 (PPP1R15A), a cytoplasmic protein that comprises the regulatory subunit of protein phosphatase 1, is involved in potentiation of the ER stress pathway [13]. Protein phosphatase 1 (PP1) is responsible for dephosphorylating many cellular proteins, including p53. Upon sensation of ER cellular stress, eukaryotic initiation factor 2α (eIF2α) is immediately phosphorylated, thus halting protein translation. To enable upregulation of ER stress genes, eIF2α must be dephosphorylated to allow translation to occur. GADD34 brings the catalytic subunit of PP1 into close proximity with eIF2α, enabling its dephosphorylation [12]. In GADD34-deficient cells, levels of eIF2α phosphorylation are high after treatment with ER stressors and remain high even hours later, while in wild-type cells, eIF2α phosphorylation was both immediate and transient [13]. This indicates the importance of GADD34 in the ER stress pathway. GADD34 is also known to act with protein phosphatase 1 in the TGFβ signaling pathway. It is thought that this pathway is also important in the cellular stress response [14].

Other microarray reports in head and neck cancer cell lines treated with cisplatin which focused on expression changes important in acquired resistance to cisplatin did not identify GADD genes to be important in the acquired resistance to cisplatin [15–17]. In YES-2, an esophageal squamous cell carcinoma line, treated with cisplatin, out of 44 differentially expressed genes, 34% encoded ribosomal proteins that were underexpressed in the cisplatin-resistant YES-2 cells [16]. Kihara et al. also demonstrated that gene expression profiles may be used to predict sensitivity of esophageal tumors to cisplatin [18]. Our results here in SQ20b head and neck cancer cell line indicate the importance of several classes of genes including the GADD genes in the modulation of cisplatin cytotoxicity. We will continue to investigate further the roles of the GADDs, histones, and other overexpressed genes in the modulation of cisplatin activity. It is likely that a particular combination of genes rather than one single gene product is essential for the observed enhancement or that a single transcription factor controls expression of several genes important in enhancement of cytotoxicity. The vast amount of data generated in these microarray studies will help to delineate pathways or groups of genes that dictate this response.

The ER stress pathway is one possibility for the mechanism of BG-enhanced cisplatin cytotoxicity based on the upregulation of ER stress response transcripts in microarray experiments, including GADD34 and ATF4. In addition, cisplatin has been shown to induce ER stress [11]. Using a human melanoma cell line, Mandic et al. demonstrated that in enucleated cytoplasts, cisplatin could induce caspase-3 and caspase-12 activity, indicative of activation of the ER stress pathway. The specificity of these results for cisplatin was demonstrated by performing similar experiments using etoposide, a chemotherapeutic agent that eliminated the activation of caspase-3 and upregulation of Grp78. Further work will focus on these transcripts and other mechanisms to determine the exact cellular targets of BG, with a goal of establishing a mechanism for BG-enhanced cisplatin cytotoxicity. A need for more effective and rationally based combinations clearly exists in the clinic, and a better understanding of the regulation of genes and/or pathways important in platinating agent anti-tumor activity will aid in the design of future therapies.

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