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ORIGINAL ARTICLE

Role of copper transporters in resistance to platinating agents

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Abstract Copper transporters have been proposed to be involved in cellular import and export of platinating agents. Expression of the human copper transporter 1 (hCtr1) is thought to result in increased sensitivity to cisplatin, whereas expression of ATP7A and ATP7B are thought to be involved in resistance to cisplatin either by sequestering drug away from its targets (ATP7A) or by exporting the drug from the cell (ATP7B). In this study, we evaluated the sensitivity of cells expressing copper transporters to cisplatin, carboplatin and oxaliplatin. We also examined whether O^6-benzylguanine, a modulator of platinating agent cytotoxicity, enhanced sensitivity of cells with or without the transporters to cisplatin. Overexpression of hCtr1 in the HEK293 cell line did not result in increased sensitivity to cisplatin, either alone or following treatment with O^6-benzylguanine. In contrast, overexpression of ATP7A and ATP7B in Me32a fibroblasts resulted in increased resistance to cisplatin, but not to carboplatin or oxaliplatin. ATP7A-expressing cells (MeMNK) showed a significant enhancement of cisplatin cytotoxicity following O^6-benzylguanine treatment, but ATP7B-expressing cells (MeWND) did not. Notably, expression of either ATP7A or ATP7B did not result in a change in total cytoplasmic platinum levels following treatment with BG plus cisplatin. The mechanism of BG enhancement of cisplatin cytotoxicity is not likely through regulation of copper transporters.

Keywords Cisplatin · O^6-Benzylguanine · hCtr1 · ATP7A · ATP7B · Carboplatin

Abbreviations

Cisplatin	*Cis*-platinum (II) diammine dichloride
Carboplatin	Cyclobutane-1,1-dicarboxylic acid platinum (II)
Oxaliplatin	Oxalate (*trans*-L-1,2-diamminocyclohexane) platinum (II)
BG	O^6-Benzylguanine
hCtr1	Human copper transporter 1

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LR	Low resolution
MR	Medium resolution
ICP/MS	Inductively coupled plasma mass spectrometer
ppt	Parts per trillion
MEFs	Mouse embryonic fibroblasts
SN-38	7-Etyl-10-hydroxy-camptothecin
HEK	Human embryonic kidney

Introduction

Platinating agents have been used for more than 30 years to treat a number of carcinomas, and they remain a mainstay of chemotherapeutic treatment [1]. *Cis*-platinum(II) diammine dichloride (cisplatin) is used clinically for head and neck carcinomas, testicular cancer, and germ cell tumors, while cyclobutane-1,1-dicarboxylic acid platinum (II) (carboplatin) has supplanted cisplatin in non-small cell lung carcinoma and most gynecologic cancers. Oxalate (*trans*-L-1,2-diamminocyclohexane) platinum (II) (oxaliplatin) adds to the spectrum of the platinating agents with its activity against colorectal cancer. The cytotoxicity of platinating agents is attributed to the formation of platinum crosslinks on DNA, with the 1,2-GpG intrastrand crosslink thought to be the major cytotoxic lesion [2]. Cisplatin and carboplatin share cross-resistance with each other, but not with oxaliplatin.

O^6-Benzylguanine (BG) is a small molecule initially designed as an inactivator of O^6-alkylguanine-DNA alkyltransferase [3]; however, through a mechanism independent of alkyltransferase inactivation, BG enhances cisplatin- and carboplatin-induced cytotoxicity in head and neck cancer cell lines [4]. This enhancement is independent of traditional resistance mechanisms, including nucleotide excision repair, glutathione, and cell cycle inhibition [5, 6].

Until recently, transport of cisplatin was presumed to be passive; however, recent studies have indicated that cisplatin may be mediated by copper transport proteins (reviewed in [7]). The copper transporters hCtr1, ATP7A, and ATP7B have all been implicated in transport of cisplatin, carboplatin, and, to a lesser extent, oxaliplatin. hCtr1 has been proposed to be important for the import of cisplatin into cells. In a non-small cell lung carcinoma made resistant to cisplatin by intermittent exposure to the drug, overexpression of hCtr1 resulted in increased uptake of cisplatin, though not to the levels of the sensitive counterpart [8], indicating the complex, multigenic nature of cisplatin resistance. Mouse embryonic fibroblasts (MEFs) in which both alleles of CTR1 are deleted are 3.2-fold more resistant to cisplatin than wild-type MEFs expressing endogenous transporter [9].

Two copper exporters, ATP7A and ATP7B, have also been proposed to be involved in cellular resistance to cisplatin. ATP7A is thought to sequester cisplatin, carboplatin, and oxaliplatin in intracellular compartments, preventing their reaction with nuclear DNA [10]. ATP7A is overexpressed in some cisplatin-resistant ovarian carcinoma cell lines [11]. Additionally, ovarian cancer patients with ATP7A expression have a lower survival rate than patients with undetectable levels of expression, as determined by ATP7A histochemical staining [12].

Overexpression of ATP7B in an epidermoid carcinoma cell line resulted in resistance to cisplatin, with only 60% of the cisplatin accumulation present in ATP7B-expressing cells compared to vector control [13, 14]. In a number of primary ovarian carcinomas and ovarian carcinoma cell lines, a correlation between ATP7B expression and resistance to cisplatin has been observed [14, 15].

The fibroblast cell line Me32a, lacking both ATP7A and ATP7B, has been shown to be more sensitive to cisplatin and carboplatin than cell lines stably transfected with either ATP7A or ATP7B using a short-term proliferation assay [16]. Here, we investigate the role of these copper transporters both in resistance to cisplatin, carboplatin, and oxaliplatin, and in their sensitivity to modulation by BG. The objective of this work was to better understand the role of copper transporters in BG-enhanced platinating agent cytotoxicity. Because numerous sources [9, 10, 12, 16–22] suggest that copper transport is vital for sensitivity and resistance to cisplatin, we hypothesized that BG acted to enhance cisplatin-induced cytotoxicity through one or more of these copper transport proteins.

Materials and methods

Maintenance of cell lines

The human embryonic kidney (HEK) cell line was created by transforming cells from a normal embryonic kidney with adenovirus. HEK293-based Flp-In™ T-Rex™ cell line was purchased from Invitrogen (Carlsbad CA) and maintained in Dulbecco's Minimum Essential Medium with 10% FBS and 2 mM L-glutamine. In cells containing the hCtr1 expression plasmid, the media was supplemented with 12 μg/ml blasticidin and 350 μg/ml hygromycin (Invitrogen, Carlsbad, CA). For induction of hCtr1 expression, 1 μg/ml tetracycline (Sigma Aldrich, St Louis, MO) was included in the media 48 h prior to use of the cells. Me32a, MeMNK, and MeWND were maintained in Minimum Essential Medium alpha supplemented with 10% FBS and 2 mM L-glutamine. Transfection of empty pCMB77 vector into Me32a cells and vector containing ATP7A and ATP7B into MeMNK and MeWND cell lines, respectively, has been previously described [16, 23, 24]. MeMNK and MeWND were maintained with 500 μg/mL G418 sulfate

(Mediatech, Inc., Herndon, VA). Cells were evaluated by Western blot for ATP7A and ATP7B expression prior to use (data not shown). Notably, previous work has shown that G418 treatment does not contribute to the development of cisplatin resistance [25], and all experiments were performed in the absence of G418. All cell lines were grown as a monolayer at 37°C and 5% CO_2. Medium and serum were purchased from Mediatech, Inc. (Herndon, VA) and Hyclone (Logan, UT), respectively.

hCtr1 cloning and transfection

The hCtr1 open reading frame [26] was cloned into the pcDNA5/FRT/TO© inducible expression vector (Invitrogen, Carlsbad, CA) as a BamHI-ApaI fragment [27]. Transfection of the hCtr1 expression plasmid into the Flp-In™ T-Rex™ cell line was performed using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Cells containing hCtr1 at the expression site were selected using hygromycin and blasticidin, then tested for regulated hCtr1 expression by growing in media with or without 1 μg/ml tetracycline for 48 h.

Drugs

Cisplatin, carboplatin, and oxaliplatin were purchased from Sigma Aldrich (St. Louis, MO) and prepared fresh for each experiment. Cisplatin was prepared by dissolution in 100% dimethyl sulfoxide (DMSO) with the final concentration being less than 0.1% for the cell experiments. Carboplatin and oxaliplatin were prepared by dissolution in distilled water. BG was a generous gift from Dr. Robert C. Moschel (NCI, Frederick, MD) and was prepared by dissolution in 100% DMSO (stock solution 100 mM), with the final DMSO concentration being less than 0.1% for the cell experiments.

Colony formation assay

We evaluated cell survival after drug treatment using the colony formation assay as previously described [4]. Briefly, exponentially growing cells were exposed to vehicle or BG (2 h, 100 μM) prior to the addition of increasing concentrations of either cisplatin (0–25 μM), carboplatin (0–400 μM), or oxaliplatin (0–20 μM) for 2 h. Following incubation with BG and cisplatin at 37°C, cells were replated in triplicate at varying densities between 100 and 2,400 per 100-mm dish (HEK293) or T25 flask (Me32a, MeMNK, MeWND). At the time of plating, there was no significant cell death noted between vehicle-treated samples and those treated with high doses of platinating agent as determined by cell count. After approximately 14 days, colonies were stained with methylene blue (0.1% w/v) and scored. Number of cells plated following treatment were optimized to achieve at least 30 colonies per plate in cells treated with cisplatin alone. Percentage survival was calculated based on the plating efficiency of the appropriate set of control cells exposed to vehicle alone.

Western blots

Western blots for hCtr1 expression were performed as previously described [27]. Briefly, total membranes were prepared from cells scraped off 10-cm culture dishes. Cells were homogenized with a dounce homogenizer, and the post-nuclear supernatant was pelleted at 100,000 × *g* for 20 min to obtain microsomal membranes. Microsomal protein (25 μg/lane) was loaded in 12% SDS-polyacrylamide gels [28]. Gels were transferred to Immobilon-P membranes (Millipore, USA) in 10 mM Caps buffer, pH 11.0 (Fisher Scientific). Membranes were blocked and incubated sequentially in primary and secondary antibodies in PBS pH 7.4 with 0.1% Tween 20 (Fisher Scientific) containing 5% W/V powdered milk. Membranes were washed after both antibody treatments four times with PBS plus 0.1% Tween 20. Proteins were detected using Supersignal® West Pico detection system and CL-Xposure™ film (Pierce, Rockford, IL). Anti-hCtr1 antibody raised against the C terminus was previously described [26]. Anti-rabbit secondary antibody was from Pierce (Rockford, IL). The relative level of endogenous and overexpressed protein was measured using a Chemi-Doc XRS system (Bio-Rad Laboratories, Hercules, CA). Relative band intensity was determined using Quantity One® Software (Bio-Rad).

^{64}Copper uptake assays for hCtr1

Functionality of the hCtr1 transporter was analyzed using ^{64}Cu uptake assays as described in [27]. Briefly, 48 h prior to the assay, HEK293 cells were induced with tetracycline to induce hCtr1 expression. One day prior to the assay, cells were seeded in 12-well tissue culture plates. On the day of the assay, cells were washed in media (without antibiotics) and then incubated for 5 min at room temperature or 45 min at 37°C in media containing 2.5 μM $CuCl_2$ and trace amounts of ^{64}Cu. For inhibition studies, 12.5 μM or 25 μM cisplatin or 25 μM silver chloride (AgCl) was also added to the incubation media. The reaction was stopped by adding ice-cold buffer, followed by two washes in buffer and eventual dissolution in 1 ml 0.1 N NaOH. Following collection, half the volume was counted in a Beckman LS 6500 scintillation counter, with a portion of the remaining lysate used for protein determination. ^{64}Cu uptakes from 5-min incubations were subtracted from 45-min incubations to correct for nonspecific binding.

Cytoplasmic platinum assays

Total cytoplasmic platinum levels were determined in Me32a, MeMNK, and MeWND cell lines following treatment with either cisplatin, BG plus cisplatin, or vehicle (control). Treatment with cisplatin was performed using the IC_{50} concentration of cisplatin alone. Cells were treated as described above, and pellets were isolated immediately following treatment with cisplatin and flash frozen. Approximately 5 ml of 5% v/v HNO_3 was added to a clean test tube containing the cell pellets. The tubes were placed in a water bath held at just below 100°C for 1 h. After 1 h in the water bath, the tubes were ultrasonicated for 1 h. The contents of the tubes were transferred to 60 ml clean polypropylene bottles. The solutions were diluted with 5% v/v HNO_3 until the total volume was approximately 20 ml. All additions of the 5% v/v HNO_3 were done gravimetrically and weights were recorded.

The solutions were analyzed in low resolution (LR) and medium resolution (MR) on a Thermo Finnigan Element 2 High-Resolution inductively coupled plasma mass spectrometer (ICP-MS). A three point standard addition calibration curve was generated for each sample (average r^2 for LR = 0.9996 and for MR = 0.9996) yielding 1 sigma external precision of 5 and 6% for LR and MR, respectively (for samples with Pt above detection limit). Detection limits for Pt were 0.19 parts per trillion (ppt) for LR and 0.31 ppt for MR. Machine drift and matrix effects were monitored using 203Tl internal standard at 2 ng/g. Signal suppression of approximately 5% due to matrix effects were observed and corrected for using the internal standard.

Statistical analysis

For analyses, the outcome of interest was the proportion of cells surviving (i.e., colony count/number plated). However, for the comparison of the Me32a and MeWND cell lines, due to the highly differential plating efficiency of the two lines (30 vs. 80%, respectively), the proportion surviving in the treated cell groups was expressed relative to that of the untreated cells. Due to the skewness of the data, the natural logarithmic or arcsine transformation was employed as appropriate. Two-way analysis of variance (ANOVA) models were used with drug (cisplatin, carboplatin, or oxaliplatin) dose as one factor and the presence of BG or transporter/cell line type as the other factor. Interaction terms were also tested. A significant interaction term would indicate that the cell survival rates differ significantly by presence of BG, cell line, or transporter type. Experiment to experiment variability was controlled for by including experiment as a factor in each ANOVA model. A P value <0.05 was considered to be statistically significant. All P values reported are two-sided. Analyses were performed using Stata, Version 9 (StataCorp LP, College Station, TX).

Results

Effect of overexpression of hCtr1 in the HEK293 cell line

HEK293 "Flp-in" cell lines containing a tetracycline-inducible hCtr1 gene were grown in the presence or absence of tetracycline. Total membranes from these HEK293 cells were analyzed by Western blot to show the level of wild-type hCtr1 (overexpressed) following induction with 1 μg/mL tetracycline for 48 h (Fig. 1a). In four independent experiments, the level of overexpressed hCtr1 protein averaged 12.7 ± 2.7-fold higher than endogenous hCtr1 protein. Copper uptake experiments showed that the overexpressed hCtr1 protein is functional (Fig. 1b). We also investigated whether cisplatin inhibits copper uptake via the hCtr1 transport protein in the HEK293 cell line. Copper uptake assays were performed in the presence of 12.5 or 25 μM cisplatin or in the presence of 25 μM silver, a known inhibitor of hCtr1 [29]. Cisplatin did not block copper transport by hCtr1 that is effectively blocked by silver ions (Fig. 1b), showing that cisplatin does not enter HEK293 cells via the copper transport pathway that has been previously characterized. Some studies have shown data consistent with an endocytic mechanism for hCtr1-mediated cisplatin entry. However, this was not the case in HEK293 cells overexpressing hCtr1 [30].

The overexpression of copper transporter hCtr1 did not result in an increase of cisplatin-induced cytotoxicity following a 2-h incubation with cisplatin, as determined by colony-forming assays (interaction $P = 0.97$) (Fig. 2a; Table 1). We also investigated whether pre-incubation with BG before cisplatin treatment would result in enhanced cisplatin-induced cytotoxicity in the line expressing hCtr1 as compared with control (Fig. 2b, c). We observed a similar degree of enhancement resulting from BG plus cisplatin treatment in HEK293 cell line regardless of the level of hCtr1 expression (Table 1), suggesting that the enhancement of cisplatin by BG is through a mechanism independent of the hCtr1 transporter. Notably, overexpression of hCtr1 did not affect the plating efficiency of the HEK293 cell line, which was approximately 40% in cells with and without hCtr1 expression.

Role of ATP7A and ATP7B in resistance to cisplatin, carboplatin, and oxaliplatin

We investigated the Me32a fibroblast cell line and two derived lines, one expressing ATP7A (MeMNK) and one expressing ATP7B (MeWND). All three cell lines were

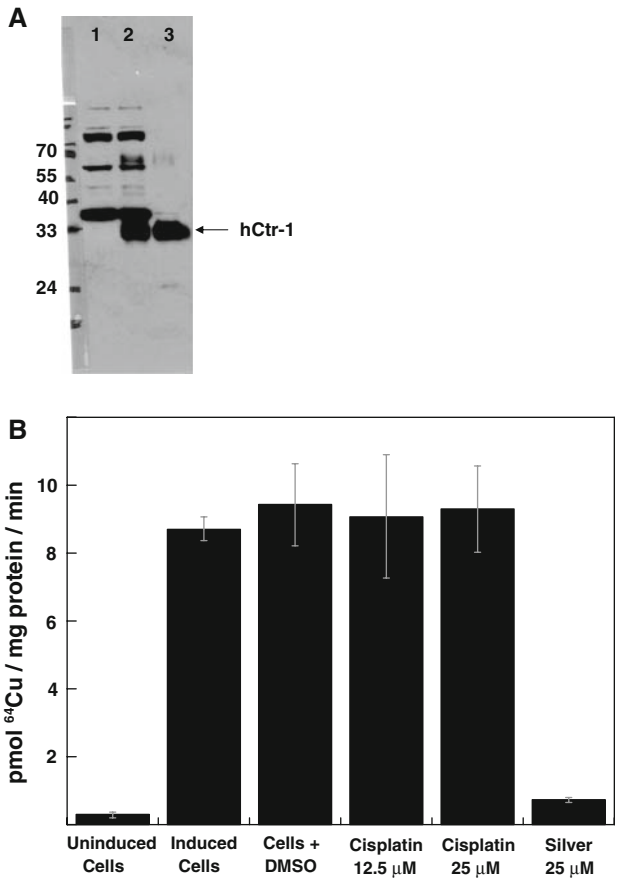

Fig. 1 Functionality of transfected hCtr1 vector in HEK293 cell line. **a** Western blot showing hCtr1 expression. Lane *1* HEK293 uninduced total membranes. Lane *2* HEK293 induced total membranes. Lane *3* purified hCtr1 protein. **b** ^{64}Cu uptake assay in HEK293 cells. Copper uptake as pmol/mg per min in the uninduced cell line or in cells overexpressing hCtr1. Statistical analysis was performed using an unpaired *t* test, and induced cells, induced cells plus DMSO, and induced cells plus cisplatin (12.5 and 25 μM) all exhibited significantly higher ($P < 0.05$) copper uptake than uninduced cells or induced cells treated with silver (25 μM). Each *bar* represents the mean ± SEM from three separate experiments, with the assay performed in triplicate for each experiment

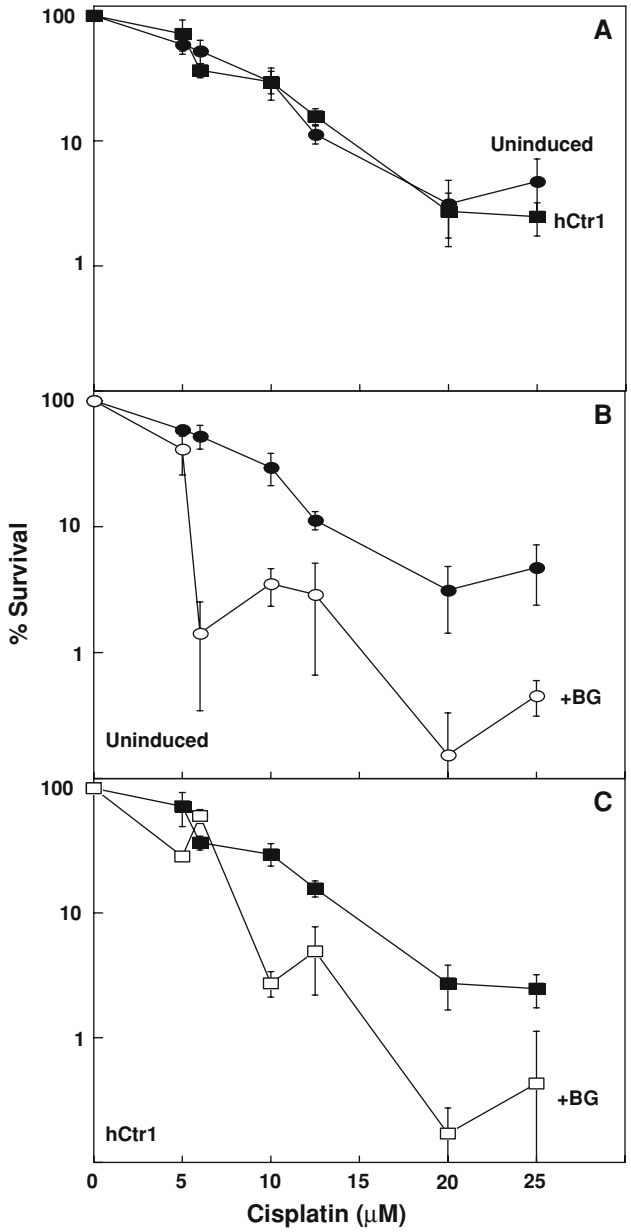

Fig. 2 Sensitivity of uninduced or hCtr1-expressing HECK293 cells to cisplatin, in the presence or absence of BG. **a** Colony formation of HEK293 cells treated with cisplatin for 2 h. Uninduced cells (*closed circle*) and hCtr1-expressing cells (*closed square*). **b** Colony formation of uninduced HEK293 cells treated with vehicle (*closed circle*) or BG (*open circle*) for 2 h prior to cisplatin treatment. **c** Colony formation of hCtr1-expressing HEK293 cells treated with vehicle (*closed square*) or BG (*open square*) for 2 h prior to cisplatin treatment. Each point represents the mean ± SEM from at least three experiments, with each experiment representing six dishes per treatment group

evaluated by Western blot for ATP7A and ATP7B expression, and it was observed that Me32a did not express either transport protein, while MeMNK and MeWND expressed either ATP7A or ATP7B, respectively (data not shown). The MeMNK (IC_{50} = 8.0 μM) and MeWND (IC_{50} = 6.4 μM) cell lines were resistant, while the Me32a parent fibroblast line was extremely sensitive to cisplatin (IC_{50} = 1.75 μM) (Fig. 3a; Table 2). At 10-μM cisplatin, for example, the Me32a cell line had >99% cell kill, compared with approximately 70% for MeMNK and 73% for the MeWND cell lines (for Me32a vs. MeWND; interaction $P < 0.005$). We also examined the sensitivity of these three lines to the other commonly used platinating agents, carboplatin and oxaliplatin (Table 2). The Me32a parent line was not significantly more sensitive to carboplatin than the MeMNK cell line (interaction $P = 0.85$); however, there was a significant difference between the Me32a cell line and the MeWND cell line (interaction $P = 0.001$), with the MeWND cell line showing significantly greater sensitivity to carboplatin than the parent line (Fig. 3b). Although ATP7A (MeMNK) and ATP7B (MeWND) expression

Table 1 IC_{50} values ± SEM for cell lines treated with cisplatin ± BG

Cell line	IC_{50} (cisplatin) (μM)	IC_{50} (BG + cisplatin) (μM)	Fold enhancement
HEK293			
Vector	7.0 ± 0.4	4.5 ± 0.9	1.6 ($P < 0.05$)
hCtr1	6.5 ± 0.8	3.0 ± 0.9	2.2 ($P < 0.05$)
Me32a			
Vector	1.3 ± 0.6	0.8 ± 0.7	1.7 ($P = 0.17$)
MNK (ATP7A)	8.0 ± 0.1	3.5 ± 0.9	2.3 ($P = 0.03$)
WND (ATP7B)	6.0 ± 0.3	4.0 ± 0.1	1.5 ($P = 0.07$)

Table 2 IC_{50} values ± SEM for cell lines treated with platinating agents

Cell line	IC_{50} (μM)		
	Cisplatin[a]	Carboplatin	Oxaliplatin
Me32a			
Vector	1.3 ± 0.6	95 ± 5	1.5 ± 0.2
MNK (ATP7A)	8.0 ± 0.1	110 ± 4	3.8 ± 0.1
WND (ATP7B)	6.0 ± 0.3	70 ± 6	4.3 ± 0.2

[a] Cisplatin values also listed in Table 1

confers some resistance to oxaliplatin when comparing IC_{50} values (Table 2), these differences are not significant (interaction $P = 0.37$ for comparison across all three cell lines) and ATP7A and ATP7B appear to be more sensitive at higher concentrations of drug (Fig. 3c). Results from short-term growth inhibition assays using Me32a-based cell lines treated with cisplatin were consistent with colony forming experiments (data not shown).

The parent Me32a cell line showed no statistically significant difference in survival rates between cells treated with cisplatin alone versus those treated with BG plus cisplatin (interaction $P = 0.17$) (Table 1). ATP7A expression, as in the MeMNK cell line, results in a statistically significant difference in cell survival rates in the presence and absence of BG treatment (interaction $P < 0.05$); however, this enhancement does not sensitize MeMNK cells to the level of the vector control cell line (Me32a) (Fig. 4a). Expression of the ATP7B transporter in the MeWND cell line showed a trend toward a significant difference in cell survival rates between these treatments (interaction $P = 0.07$) (Fig. 4b); again, this enhancement does not sensitize MeWND cells to the level of vector control (Me32a). These results suggest that BG enhancement of cisplatin cytotoxicity occurs in the presence of ATP7A, with only weak evidence to support the involvement of ATP7B in the mechanism for BG-induced cisplatin enhancement. However, at the IC_{50} dosage of cisplatin alone, fold enhancement of cytotoxicity by BG was determined to be 1.7 (Me32a), 2.3 (ATP7A-MeMNK), and 1.5 (ATP7B-MeWND) (Table 1), indicating similar enhancement of cisplatin-induced cytotoxicity by BG in all three cell lines.

Correspondingly, total cellular platinum levels, as measured by ICP/MS, are not significantly different in ATP7A and ATP7B-expressing cells treated with cisplatin versus those treated with BG plus cisplatin (Table 3). Interestingly, total cytoplasmic platinum levels are not significantly different between Me32a cells and MeWND cells, indicating that the presence of the ATP7B protein does not lead to significantly decreased cytoplasmic cisplatin concentrations. Fold difference determined by comparing cells treated with BG plus cisplatin to those treated with cisplatin alone indicates that BG significantly affects the level of cellular cisplatin only in the Me32a cell line ($P < 0.05$), in which no copper transporters are present (Table 3). Our data indicates that BG is not inhibiting either the ATP7A or ATP7B transporter, resulting in an increase in cytoplasmic platinum levels and subsequent cytotoxicity.

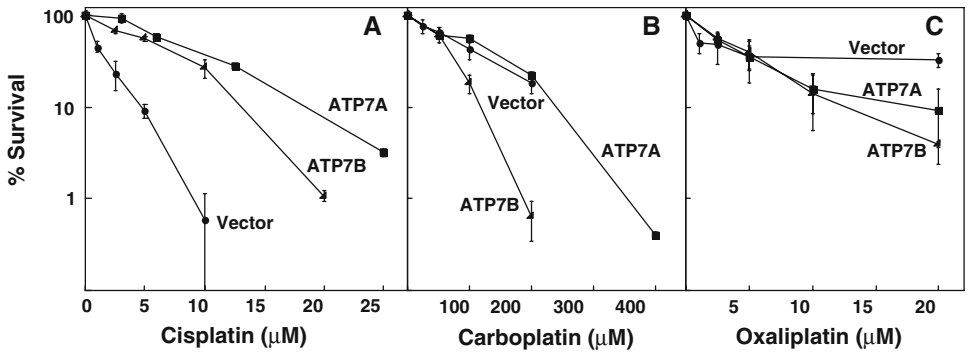

Fig. 3 Sensitivity of Me32a fibroblast cell line transfected with either vector, ATP7A or ATP7B to platinating agents. Colony formation of the vector control Me32a (*closed circle*); MeMNK (ATP7A) (*closed square*); MeWND (ATP7B) (*closed triangle*) treated with 2 h **a** Cisplatin, **b** carboplatin, and **c** oxaliplatin. Each point represents the mean ± SEM from at least three experiments, with each experiment representing six dishes per treatment group

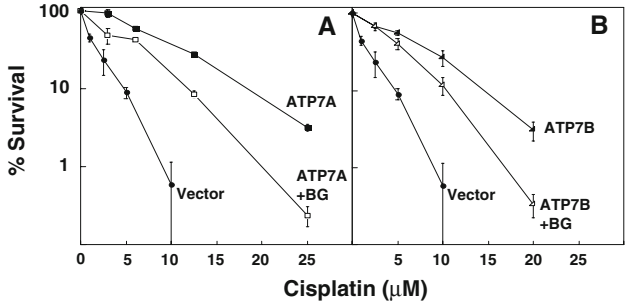

Fig. 4 Effect of BG on sensitivity of ATP7A- and ATP7B-expressing cell lines to platinating agents. Colony formation of Me32a-based cells treated with vehicle (*closed symbol*) or BG (*open symbol*) for 2 h prior to cisplatin treatment (2 h) as compared with the Me32a vector control cell line (*circle*) without BG in **a** MeMNK (ATP7A) (*squares*) and **b** MeWND (ATP7B) (triangles). Each point represents the mean ± SEM from at least three experiments, with each experiment representing six dishes per treatment group

Table 3 Total cytoplasmic platinum ± SEM in cell lines treated with cisplatin ± BG

Cell line	Total cytoplasmic platinum (ng Pt/g protein)	Fold difference (BG + cisplatin vs cisplatin)
Me32a		
Control	0.007 ± 0.001	
Cisplatin	1.86 ± 0.01	
BG + cisplatin	2.41 ± 0.21	1.30 ($P < 0.05$)
MeMNK		
Control	0.004 ± 0.001	
Cisplatin	1.08 ± 0.03	
BG + cisplatin	1.16 ± 0.02	1.07 ($P = 0.75$)
MeWND		
Control	0.007 ± 0.001	
Cisplatin	2.17 ± 0.09	
BG + cisplatin	2.31 ± 0.20	1.06 ($P = 0.8$)

Cells were treated with either vehicle or BG (100 μM) for 2 h, followed by a 2-h incubation with the IC_{50} concentration of cisplatin alone for that cell line as shown in Table 1

Discussion

In this work, we have sought to clarify the contribution of copper transporters to resistance and sensitivity to platinating agents, and to the mechanism of BG-enhanced cisplatin cytotoxicity. Although exogenously expressed hCtr1 was clearly competent to transport copper, it did not appear to facilitate delivery of cisplatin such that increased cell killing resulted, nor did cisplatin affect copper transport. We also found that both ATP7A and ATP7B contribute to cisplatin resistance, but not to resistance to either carboplatin or oxaliplatin. The role of these transport proteins in the mechanism of BG-enhanced cisplatin-induced cytotoxicity was also examined. Only in ATP7A-expressing cell lines did we observe a statistically significant enhancement in cisplatin-induced cytotoxicity following treatment with BG plus cisplatin as compared with cisplatin alone, although the fold enhancement was similar in all three lines, regardless of transport expression, as were the total cytoplasmic platinum levels, leading us to conclude that BG's enhancement of cisplatin is not through an interaction with these transporters.

Cellular and clinical resistance to platinating agent is multifactorial and varies in different tissues. hCtr1 has been proposed to be a substantial contributor to the influx of cisplatin [31]. The import of cisplatin via hCtr1 is mechanistically different from the influx of copper via the same transport protein, as cisplatin can be imported by an hCtr1 mutant that is defective in copper uptake [32]. Cisplatin transport by hCtr1 is dependent on the presence of eight N-terminal methionine-rich motifs [32]. MEFs null for hCtr1 provide increased resistance to cisplatin and carboplatin, but not oxaliplatin, compared to MEFs with hCtr1 expression, using a short-term growth inhibition assay [9]. In a comparison between resistant (SR2) and sensitive (SCLC) small cell lung cancer cell lines, loss of hCtr1 expression was shown to be one mechanism for cellular resistance to platinating agents, using a short-term assay [8]. This effect may be cell line specific or assay specific, as our results in the HEK293 cell line do not demonstrate a role for hCtr1 in sensitivity to cisplatin. One reason for the discrepancy may be that established tumor cell lines behave differently in many cellular processes, such as the glutathione state of the cytosol [33] and DNA replication [34]; thus, it is possible that hCtr1 does affect cisplatin entry or toxicity in some cells and other more prominent mechanisms of resistance mask the effect in other cell lines. Additionally, it may be that while the hCtr1 transporter does enhance the entry of cisplatin, the higher cisplatin levels do not correlate with cytotoxicity, as previously observed in the A2780 cell line [18].

Previous studies have suggested that cisplatin may be processed differently between endogenously and exogenously expressed hCtr1 [18]. It is conceivable that the overexpressed hCtr1 protein in the HEK293 cell line used here does not mediate cisplatin uptake or toxicity in the same manner as does endogenous hCtr1, although the overexpressed protein clearly transports copper, and this transport is blocked by addition of silver ions. It has been previously shown by Maryon, et al. [27] that overexpressed hCtr1 proteins expressed in this cell line traffic normally to the cell surface. The inability of cisplatin to inhibit copper uptake in our studies indicates that cisplatin may have effects on the maintenance of copper levels in the cell independent of

hCtr1 inhibition. Finally, studies in MEFs which led to conclusions of a role for hCtr1 in cisplatin transport were performed with short-term assays following a 36-h exposure to the drug [9], while our readout was a colony forming assay approximately 14 days after a 2-h exposure to cisplatin. Cells may show an initial sensitivity to drug in the presence of the transporter, but this effect may be diluted out after time by other resistance and repair mechanisms present in the cell.

The ATP7A and ATP7B transport proteins have been proposed to contribute to resistance to cisplatin. Overexpression of ATP7A above the endogenous levels in the 2008 ovarian cancer cell line was shown to result in increased resistance to cisplatin, carboplatin, and oxaliplatin through increased cellular sequestration of the drugs [10]. Additionally, in primary ovarian tumors, a correlation was observed between ATP7A histological staining and a lower overall patient survival rate [12]. ATP7B expression has been correlated with resistance to cisplatin in both ovarian carcinoma cell lines and in primary ovarian tumors [14, 15]. ATP7A overexpression in CHO cells confers only a slight resistance to cisplatin as compared to parental, nontransfected cells [35]. The mechanism by which ATP7A confers cellular resistance to all of these drugs appears to be similar; through sequestration of drugs with the Golgi apparatus, followed by an increase in drug efflux. Additionally, ATP7A expression appears to be only one of several factors important for resistance to cisplatin in these colorectal cancer biopsies [35].

Recent studies on the transport of cisplatin by ATP7B indicate that cisplatin is transported similar to copper, in that both require necessary formation of an acyl phosphate intermediate, albeit at much slower rates for cisplatin than for copper [21]. However, these studies were performed at an acidic pH of 4.6, and the relevance of this acidic pH to clinical responses to platinum drugs should be further analyzed. Additionally, it appears that cisplatin may be able to inhibit its own export, because it binds to ATP7B with high affinity [21], implying that expression of ATP7B (and presumably expression of ATP7A) may not be sufficient to cause clinical resistance to platinating agents.

Our data presented here for cisplatin and oxaliplatin are in agreement with those previously reported: expression of both ATP7A and ATP7B provide increased cellular resistance to cisplatin, while cells became hypersensitive to oxaliplatin [16]; however, we observed a discrepancy when comparing the carboplatin results [16]. It had been reported that expression of both ATP7A and ATP7B provided increased cellular resistance to carboplatin in both primary ovarian tumors and ovarian carcinoma cell lines [14, 15], as well as the ATP7A and ATP7B-expressing cell lines used here; however, we observed no difference in resistance upon treatment with carboplatin in cells with ATP7A expression, and hypersensitivity to carboplatin in cells with ATP7B expression. One possibility for this difference is the time frame of the assay; while we used a 14-day readout clonogenic assay, previous results were reported 72-h post-treatment, which may have a different dependence on expression of the export proteins than the later time points. There are additional differences in the spectrum of toxicities associated with different platinating agents, as carboplatin is devoid of nephrotoxicity, less toxic to the gastrointestinal tract, and less neurotoxic than cisplatin. Although the DNA adducts formed from cisplatin and carboplatin are similar, a 20–40-fold higher concentration of carboplatin than cisplatin is required, with the rate of carboplatin adduct formation tenfold slower than that of cisplatin [36]. Possibly, cisplatin is carried through the ATP7B transporter; while carboplatin may actually inhibit or inactivate ATP7B, as described above, allowing more carboplatin to react in the cell with DNA and other targets instead of being exported or sequestered away from the cytosol.

O^6-benzylguanine enhances cytotoxicity of platinating agents in head and neck and ovarian cancer cell lines [4]. It is unlikely that the lack of significant enhancement of cisplatin cytotoxicity observed in Me32a-based cells is due to the utilization of a fibroblast cell line. While we have observed that enhancement of cisplatin cytotoxicity by BG is not universal [37], previous work in fibroblast-based cell lines by our laboratory has indicated that BG does enhance cisplatin-induced cytotoxicity in some fibroblast cell lines [5]. We have ruled out hCtr1 and ATP7B as being critical factors for the mechanism of BG based on our data. Previous studies have shown that the level of platinum damage on DNA is not altered with ATP7A or ATP7B expression [16], and we have shown that BG does not affect the total cellular platinum levels in these cell lines; it is therefore likely that an alternate damage pathway, such as the endoplasmic reticulum stress pathway, may be responsible for some cisplatin-induced apoptosis. In addition, we have shown that BG increases the level of cisplatin-induced DNA platination in head and neck cancer cells [4]; thereby, making it increasingly unlikely that ATP7A acts as a main target for BG in the cell.

The mechanism of cisplatin transport appears to be tissue-specific. While cisplatin can be transported in some tissue types by some of these transporters, a complete story has yet to be determined. The potential tissue specificity of the hCtr1 and ATP7A/B transporters should be analyzed through both overexpression and knockdown experiments in tumor cell lines to determine the role of these transporters in clinical resistance and sensitivity to platinating agents. In some tumor types, these transporters may be vital in providing resistance to platinating agents, and this can be targeted in developing new therapies to modulate cisplatin

treatment. BG may be important in enhancing the activity of cisplatin in cells resistant to cisplatin as a result of the expression of ATP7A and ATP7B. Combining BG with specific inhibitors of these transport proteins could result in an even greater enhancement of cisplatin.

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