JPET Fast Forward. Published on May 15, 2003 as DOI:10.1124/jpet.103.052225

JPET #52225

Role of Cysteine S-Conjugate β-Lyase in the Metabolism of Cisplatin*

Lei Zhang and Marie H. Hanigan

Department of Cell Biology, University of Oklahoma Health Sciences Center,

Oklahoma City, Oklahoma 73104

Running Title: Cysteine S-Conjugate β-lyase and cisplatin

Corresponding author: Marie H. Hanigan, Ph.D., Biomedical Research Center Room 264, 975 N.E. 10th Street, Oklahoma City, Oklahoma 73104, USA.

Telephone: (405) 271-3832, FAX:(405) 271-3813, Email: marie-hanigan@ouhsc.edu

Number of text pages: 32

Number of tables: 1

Number of figures: 5

Number of references: 36

Number of words in the Abstract: 250

Number of words in the Introduction: 742

Number of words in the Discussion: 888

Abbreviations: GGT, gamma-glutamyl transpeptidase; PLP, pyridoxal 5'-phosphate; GTK, glutamine transaminase K; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; DCVC, S-(1,2-dichlorovinyl)-L-cysteine; DNP, 2,4-dinitrophenylhydrazine.

Recommended section: Toxicology

JPET Fast Forward. Published on May 15, 2003 as DOI: 10.1124/jpet.103.052225
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Cisplatin is nephrotoxic, but the mechanism by which cisplatin kills renal proximal tubule cells is not well defined. Inhibition of gamma-glutamyl transpeptidase or pyridoxal 5'-phosphate (PLP)-dependent enzymes blocks the nephrotoxicity. Our hypothesis is that cisplatin is metabolized to a renal toxin through a platinum-glutathione conjugate to a reactive sulfur-containing compound. The final step in this bioactivation is the conversion of a platinum-cysteine S-conjugate to a reactive thiol by a PLP-dependent cysteine S-conjugate β-lyase. LLC-PK_1 cells, a proximal tubule cell line with low cysteine S-conjugate β-lyase activity, are used to study cisplatin nephrotoxicity. We proposed that the β-elimination reaction catalyzed by cysteine S-conjugate β-lyase is the rate-limiting step in the metabolism of cisplatin to a toxin in these cells. In this study, LLC-PK_1 cells were transfected with human glutamine transaminase K, which catalyzes the β-elimination reaction. Cisplatin was significantly more toxic in confluent monolayers of cells with increased cysteine S-conjugate β-lyase activity. In contrast, carboplatin, a non-nephrotoxic derivative of cisplatin, was 20-fold less toxic than cisplatin in confluent cells and its toxicity was not altered by overexpression of cysteine S-conjugate β-lyase. We propose that carboplatin is not nephrotoxic because it is not metabolized through this pathway. Dividing cells were more sensitive to both cisplatin and carboplatin toxicity. Overexpression of cysteine S-conjugate β-lyase activity had no effect on the toxicity of either drug. These data demonstrate that cisplatin kills quiescent renal cells by a mechanism that is distinct from the mechanism by which it kills dividing cells and that the renal toxicity of cisplatin is dependent on cysteine S-conjugate β-lyase activity.

Cisplatin is one of the most effective anti-cancer agents in the treatment of many solid tumors, including testicular and ovarian cancer (O'Dwyer et al., 1999). The therapeutic effects of the drug are significantly improved by dose escalation. However, high-dose therapy with cisplatin is limited by its cumulative nephrotoxicity and neurotoxicity (O'Dwyer et al., 1999). Carboplatin, a second generation platinum drug, has also proven effective in the treatment of ovarian tumors (Fig. 1). The dose-limiting toxicity of carboplatin is bone-marrow suppression with cumulative anemia (McKeage, 2000). Carboplatin can be administered at doses 5-fold higher than cisplatin without evidence of nephro- or neurotoxicity. The molecular mechanism underlying the difference in the dose-limiting toxicities of these two platinum-based compounds has heretofore been unknown.

Both cisplatin and carboplatin bind DNA, which is toxic to dividing tumor cells (Fink and Howell, 2000; Perez, 1998). Chemotherapeutic drugs that disrupt pathways essential to dividing cells are generally dose-limited by their toxicity toward the rapidly dividing cells in the bone-marrow, as is observed with carboplatin. The toxicity of cisplatin towards the non-dividing proximal tubule cells in the kidney suggests that cisplatin kills the renal cells by a mechanism other than DNA crosslinking.

Data from our previous studies have shown that cisplatin is metabolized to a nephrotoxin. Inhibition of the enzyme gamma-glutamyl transpeptidase (GGT) or pyridoxal 5'-phosphate (PLP)-dependent enzymes blocks the nephrotoxicity of cisplatin (Hanigan et al., 1994; Hanigan et al., 2001; Townsend and Hanigan, 2002). Inhibition of these enzymes also blocks the nephrotoxicity of hexachlorobutadiene and other nephrotoxic halogenated alkenes (deCeaurriz

and Ban, 1990; Lash et al., 1994; Elfarra et al., 1986). The halogenated alkenes are metabolized to a nephrotoxin through a glutathione-conjugate(Anders and Dekant, 1998). One of the halogen moieties is displaced by the sulfur of glutathione. The resulting glutathione-conjugate is cleaved to a cysteinyl-glycine conjugate by GGT, the cysteinyl-glycine conjugate is then cleaved to a cysteine-conjugate by aminopeptidase N (EC 3.4.11.2, also known as aminopeptidase M). The cysteine-conjugate is metabolized to a reactive thiol by a PLP-dependent cysteine S-conjugate β-lyase. The β-elimination reaction that defines a cysteine S-conjugate β-lyase enzyme can be catalyzed by several PLP-dependent enzymes (Cooper et al., 2002). The enzymes are aminotransferases active in amino acid metabolism and vary in their substrate specificity for the β-lyase reaction. The enzyme that is responsible for the metabolism of each of the cysteine S-conjugates of the halogenated alkenes *in vivo* has not yet been identified. However, the PLP-dependent enzyme glutamine transaminase K (GTK) has high specific activity as a cysteine S-conjugate β-lyase with the cysteine-conjugates of trichloroethylene and tetrafluoroethylene (Cooper et al., 2002). Overexpression of GTK in the renal proximal tubule cell line LLC-PK_1 increased the toxicity of S-(1,2-dichlorovinyl)-L-cysteine (DCVC), the cysteine conjugate of trichloroethylene (Goldfarb et al., 1996).

We have shown that the initial steps in the bioactivation of cisplatin to a nephrotoxin are the same as the reactions that activate the halogenated alkenes. Cisplatin-glutathione conjugates, cisplatin-cysteinyl-glycine conjugates and cisplatin-N-acetyl-cysteine conjugates are more toxic to LLC-PK_1 cells than cisplatin (Townsend et al., 2003a). In animals and in cell culture, inhibition of GGT or inhibition of PLP-dependent enzymes blocks the nephrotoxicity of cisplatin (Hanigan et al., 1994; Hanigan et al., 2001; Townsend and Hanigan, 2002). In this study, we

investigated the role of a PLP-dependent cysteine S-conjugate β-lyase in the activation of cisplatin to a nephrotoxin. In LLC-PK_1 cells, GGT and aminopeptidase N activity is similar to activity in the kidney; but the cysteine S-conjugate β-lyase activity is significantly lower than *in vivo* levels(Townsend et al., 2003a). Our hypothesis is that the β-elimination reaction catalyzed by cysteine-S-conjugate β-lyase is the final step in the activation of cisplatin to a nephrotoxin, and that the relatively low levels of cysteine S-conjugate β-lyase activity in LLC-PK_1 cells makes this reaction rate-limiting for the metabolism of cisplatin in these cells (Fig. 2). To test this hypothesis, human GTK cDNA was isolated and transfected into LLC-PK_1 cells. Cells were exposed to cisplatin or carboplatin according to a protocol that mimics the *in vivo* exposure. *In vivo* the proximal tubules are exposed to the highest levels of cisplatin during the first 3 hr after administration (Cornelison and Reed, 1993). Renal toxicity becomes apparent at 3 to 4 days. Confluent cells were used as a model for the non-dividing monolayer of epithelial cells lining the renal proximal tubules. The cells were exposed to cisplatin for 3 hr and toxicity was evaluated at 3 days. In this study, the contribution of GTK to cisplatin and carboplatin toxicity was tested in both dividing and quiescent cells.

Materials and Methods

Cloning of Cytosolic Glutamine Transaminase K. A full length cDNA of cytosolic GTK was isolated from a human kidney QUICK-Clone cDNA library (Clontech Laboratories, Inc., Palo Alto, CA) with two rounds of PCR amplification. The oligonucleotide primers were designed based on the published sequence of the human cytoplasmic GTK (Perry et al., 1995, National Center for Biotechnology Information, GenBank accession number: NM_004059). The first round of PCR was performed with a pair of external primers, 5' GTGAAGCGGCCCAGGTGAGGCTCG 3' and 5' ATGTCAGGGCCAAGGCGTGACTTC 3'. The PCR product from the first round was used as a template for the second round of PCR with a pair of internal primers, 5' ATGGCCAAACAGCTGCAGGCCCGA 3' and 5' CTAGAGTTCCACCTTCCACTTCCG 3'. The PCR product from the second round was ligated to pT-Adv vector (Clontech Laboratories, Inc.). TOP 10F' *E. coli* (Clontech Laboratories, Inc.) were transfected with the ligation mixture and amplified. The GTK cDNA was isolated and cleaved from the pT-Adv vector with *Hind III* and *Xho I*, then subcloned into the same restriction sites in the mammalian expression vector pcDNA3.1 (+) (Invitrogen, Carlsbad, CA). The plasmid was transfected into TOP 10F' *E.coli* and amplified. The orientation of the insert was confirmed by *Pst I* digestion of the plasmid. The GTK cDNA was sequenced with a T7 primer and three internal primers (Oklahoma Medical Research Foundation DNA Sequencing Facility, Oklahoma City, OK). The GTK cDNA had 6 codons that encoded amino acids that differed from the published sequence (Perry et al., 1995). A Quikchange multisite-directed mutagenesis system (Stratagene, La Jolla, CA) was used to correct the sequence in the cDNA so that it encoded a protein identical to the published amino acid sequence.

Transfection of LLC-PK$_1$ cells with GTK cDNA. LLC-PK$_1$ (ATCC CRL 1392), a pig kidney proximal tubule cell line, was obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen), with 5% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT), 50 units/ml Penicillin and 50 μg/ml streptomycin (Invitrogen) at 37 °C in a 5% CO_2 atmosphere. For transfection, 5 x 10^5 LLC-PK$_1$ cells were plated on a P100 dish in 1:1 mixture of F12:DMEM, with 10% FBS . Twenty-four hr after plating, 10 μg GTK/pcDNA3.1 plasmid were precipitated by calcium phosphate and transfected into LLC-PK$_1$ cells (Calcium Phosphate Eukaryotic Transfection Kit, Stratagene). Control cells were transfected with pcDNA3.1 vector alone. Stable transformants were selected with 2 mg/ml G418 (Invitrogen). Individual colonies were picked and grown into cell lines in DMEM, 5% FBS, and 2 mg/ml G418. The transfected cell line with the highest level of cysteine-S-conjugate β-lyase activity was selected and named LLC-PK$_1$/GTK. The control cell line was named LLC-PK$_1$/C1. The two cell lines were maintained in the DMEM medium containing 5% FBS and 400 μg/ml G418.

Preparation of Cell Lysate. LLC-PK$_1$/GTK and LLC-PK$_1$/C1 cells were seeded in P100 tissue culture plates at a density of 2 x 10^5 cells/plate. On day 7, the cells reached confluence and the medium was changed to fresh medium. On day 10, the cells were trypsinized from the plates, rinsed twice with phosphate-buffered saline, and resuspended in 100 μl 10 mM Tris-HCl, 0.25 M sucrose (pH 7.5). The cells were freeze-thawed twice, sonicated twice for 10 sec with a 30 sec cooling interval on ice, and centrifuged at 3000 x *g* for 5 min (Perry et al.,

1993). The supernatant was used the same day to measure enzyme activities and aliquots were stored at -80 °C for Western Blot analysis and protein concentration determination.

Cysteine S-Conjugate β-Lyase Activity. The substrate DCVC was synthesized according to the method of McKinney et al. (McKinney et al., 1959). Trichloroethylene was reacted with cysteine in the presence of metallic sodium in a liquid ammonia solution. The ammonia was evaporated and the crude product was purified by crystallization. Identity of the product was confirmed by ultraviolet spectrum, proton nuclear magnetic resonance spectroscopy and fast atom bombardment mass spectrometry (OU Chem/Biochem Mass Spectrometry Laboratory, Norman OK). The cysteine S-conjugate β-lyase assay was a modification of a previously published assay (Cooper et al., 2001). Briefly, 20 µl reaction mixture containing 100 mM potassium phosphate buffer (pH 7.2), 5 mM DCVC, 10 µM PLP, and the cell lysate was incubated at 37 °C. The reaction was stopped and the pyruvate released was converted to pyruvate 2,4-dinitrophenylhydrazone by addition of 20 µl of 5 mM 2,4-dinitrophenylhydrazine (DNP) in 2 M HCl. The solution was further incubated at 37 °C for 5 min, 160 µl of 1 M KOH was then added. The mixture was immediately transferred to a 96-well plate and the absorbance of pyruvate 2,4-dinitrophenylhydrazone was measured at 450 nm. The background absorbance was determined by adding the cell lysates immediately after the addition of the DNP. The molar extinction coefficient of pyruvate 2,4-dinitrophenylhydrazone under these conditions was 15,000. One unit of activity was defined as the amount of enzyme that released one µmole of pyruvate per min at 37 °C. Protein concentrations were determined by BCA protein assay (Pierce, Rockford, IL).

GTK Activity. GTK activity was measured with a multi-well plate assay (Cooper et al., 2001). A 50 µl reaction mixture contained 100 mM ammediol-HCl (pH 9.0), 5 mM α-keto-γ-methiolbutyrate, 20 mM L-phenylalanine, and cell lysate. The reaction was incubated at 37 °C and terminated by the addition of 150 µl 1M KOH. The absorbance was read immediately at 322 nm. The background absorbance was determined by adding the cell lysate after the KOH. The molar extinction coefficient of phenylpyruvate (enol) under these conditions is 16,000. One unit of activity was defined as the amount of enzyme that released one µmole of phenylpyruvate per min at 37 °C. Protein concentrations were determined by the BCA protein assay.

Western Blot Analysis. The western blot was performed based on a procedure from Cell Signaling (Beverly, MA). The cell lysates (25 µg per lane) were diluted with 1% SDS and 60 µM dithiothreitol and boiled for 5 min. The proteins were separated on 12% SDS-polyacrylamide gel electrophoresis. They were transferred onto nitrocellulose membranes (Osmonics, Inc., Minnetonka MN). The membranes were blocked for 1 hr at 25 °C with 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, and 0.05% Tween-20 (TTBS) containing 5% non-fat dry milk (blocking buffer). Membranes were washed three times for 10 min each in TTBS at 25 °C, then incubated overnight at 4 °C with rabbit anti-rat kidney GTK antibody (a generous gift from Dr. Arthur Cooper, Weill Medical College of Cornell University, NY; (Abraham and Cooper, 1996). The antibody was diluted 1:500 with the blocking buffer. Membranes were washed three times for 10 min each with TTBS and incubated for 2 hr at 25 °C with peroxidase-conjugated goat anti-rabbit antibody (Sigma, St. Louis, MO) diluted 1:10,000 in blocking buffer. After three washes with TTBS, the immunolabeled bands were visualized with an enhanced

chemiluminescence kit (Amersham, Piscataway, NJ) according to the manufacturer's instructions.

Cisplatin and Carboplatin Toxicity in Confluent Cells. The nephrotoxicity of cisplatin and carboplatin was assessed in an *in vitro* assay system that is a model for *in vivo* exposure (Townsend et al., 2003a). Briefly, LLC-PK_1/GTK and LLC-PK_1/C1 cells were seeded in 96-well plates at 10^4 cells/well in DMEM medium containing 5% FBS and 400 μg/ml G418. The cells became confluent on day 3 and the medium was replaced with fresh medium. On day 7, the medium was removed and replaced with cisplatin or carboplatin diluted in DMEM. Cisplatin (Sigma) was prepared as a 1mg/ml stock solution in 0.9% NaCl. A fresh stock solution of carboplatin (Sigma), 1mg/ml DMEM, was prepared each day. The stock solutions were diluted in the DMEM less than 30 min before they were added to the cells. The cells were incubated in the cisplatin or carboplatin containing medium for 3 hr at 37°C in 5% CO_2. Cells incubated in DMEM alone served as controls. After 3 hr, the media containing the drugs were removed and replaced with DMEM medium containing 5% FBS and 400 μg/ml G418. The cells were incubated at 37 °C in 5% CO_2 for an additional 69 hr. The number of viable cells was determined by the MTT assay (Mosmann, 1983).

Cisplatin and Carboplatin Toxicity in Dividing Cells. The toxic effects of cisplatin and carboplatin in dividing transfected cells were assessed with the *in vitro* assay system described above. LLC-PK_1/GTK and LLC-PK_1/C1 cells were seeded in 96-well plates at 5×10^3 cells/well in DMEM medium containing 5% FBS and 400 μg/ml G418. The next day, the medium was removed and the cells were treated for 3 hr with cisplatin or carboplatin diluted in

DMEM. At the end of incubation, the drugs were removed and replaced with DMEM medium containing 5% FBS and 400 μg/ml G418. The cells were incubated at 37 °C in 5% CO_2 for additional 69 hr. The number of viable cells was determined by the MTT assay.

Data Analysis. Statistically significant differences between the cell lines in the levels of enzyme activity were detected with *t*-tests. Differences were considered significant if $p < 0.05$. Each cell culture toxicity experiment was repeated at least three times. In each experiment all points were done in triplicate. The standard deviation (SD) from the mean was computed for each point. LD_{50} and its 95% confidence intervals were calculated with Prism sigmoidal dose-response (variable slope) curve fit (GraphPad Software, Inc., San Diego, CA). Statistically significant differences in toxicity between cell lines were detected with *t*-tests. Differences were considered significant if $p < 0.05$.

JPET Fast Forward. Published on May 15, 2003 as DOI: 10.1124/jpet.103.052225
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Expression of Cysteine S-Conjugate β-Lyase in LLC-PK$_1$ Cells. The full length cDNA of cytGTK was isolated from a human kidney cDNA library and modified by site-directed mutagenesis to encode a protein identical to the published sequence for cytGTK. The pcDNA3.1 containing the GTK cDNA was transfected into LLC-PK$_1$ cells. More than three hundred colonies grew out in the presence of 2 mg/ml G418; six were picked and grown into individual cell lines. The cell lines were assayed for cysteine S-conjugate β-lyase activity eight to ten weeks after the transfection. The highest β-lyase activity of the GTK transfected LLC-PK$_1$ cells was two-fold higher than that of the control lines. The colony that exhibited the highest cysteine S-conjugate β-lyase activity was chosen for further studies and named LLC-PK$_1$/GTK. The β-lyase activity of LLC-PK$_1$/GTK increased as the cells were passaged. Fifteen weeks after transfection the enzyme activity was 4.32 $\pm$ 0.37 mU/mg protein which is 2.8-fold higher than the level of activity in mouse kidney (Townsend et al., 2003a). A second set of cells transfected with the pcDNA3.1 vector served as a control and was named LLC-PK$_1$/C1. Both transfected cell lines maintained the same growth rate as the parental cell line.

Glutamine Transaminase K Activity and Cysteine S-Conjugate β-Lyase Activity. Four months after transfection, the GTK activity of LLC-PK$_1$/GTK was 56-fold higher than that of LLC-PK$_1$/C1 (Table 1). The level of activity remained constant as the cells were passaged over three months for use in these studies. GTK also catalyzes the β-elimination reaction that defines a cysteine S-conjugate β-lyase. The cysteine S-conjugate β-lyase activity was measured in the whole cell lysates of LLC-PK$_1$/GTK and LLC-PK$_1$/C1. The cysteine S-conjugate β-lyase

activity, with DCVC as a substrate, was 4.32 $\pm$ 0.37 mU/mg protein (Table 1). The level of activity in LLC-PK_1/C1 was at or below the detection limit of the assay and could not be measured reliably. These data demonstrate that the GTK transfected into LLC-PK_1/GTK cells was active as a glutamine transaminase. The enzyme was able to catalyze the cysteine S-conjugate β-lyase reaction and was the primary enzyme responsible for this activity in LLC-PK_1/GTK cells.

Western Blot Analysis of GTK Expression. Western blot analysis with a polyclonal rabbit anti-rat kidney GTK antibody was used to detect human kidney GTK in the transfected LLC-PK_1/GTK cells. The amino acid sequence similarity between the rat and human kidney GTK is 83%. A protein band at 48 kD was detected by the antibody in LLC-PK_1/GTK cells, but not in the control cells (Fig. 3). The molecular weight of this band is consistent with human GTK. One subunit of human GTK (homodimer) deduced from amino acid sequence is 47.9 kD (Perry et al., 1995). For both cell lines, there was a minor band at 34 kD. LLC-PK_1 is a porcine cell line with low endogenous GTK activity. The amino acid sequence of porcine GTK has not yet been reported. The minor band may be the endogenous porcine GTK, or another porcine protein with an antigenic site similar to rat GTK. The faint bands at approximately 70 to 80 kD were nonspecific bands, the intensity of which did not change when the amount of lysate loaded on the gel was reduced by 5-fold.

Toxicity of Cisplatin in Confluent GTK Transfected Cells. Confluent monolayers of LLC-PK_1/GTK and LLC-PK_1/C1 cells were treated with cisplatin according to our standard protocol: 3 hr in cisplatin with cell viability assayed 3 days after treatment. The data showed that

cisplatin was more toxic to the cells transfected with GTK than to vector-transfected controls (Fig. 4A). LD_{50} of cisplatin in LLC-PK_1/GTK cells was 111 μM with 95% confidence intervals ranging from 108 to 114 μM. LD_{50} of cisplatin in LLC-PK_1/C1 cells was 164 μM with 95% confidence intervals ranging from 159 to 169 μM. The two LD_{50} were significantly different ($p < 0.0001$), and there was also a significant difference between the slopes of the two dose curves ($p = 0.0006$). These studies demonstrate that in confluent monolayers of LLC-PK_1 cells the β-elimination reaction catalyzed by cysteine S-conjugate β-lyase is an important step in the bioactivation of cisplatin to a nephrotoxin.

Toxicity of Carboplatin in Confluent GTK Transfected Cells. Confluent monolayers of LLC-PK_1/GTK and LLC-PK_1/C1 cells were treated with carboplatin. Carboplatin was less toxic towards these proximal tubule cells than cisplatin (Fig. 4B). The LD_{50} for carboplatin in LLC-PK_1/C1 cells was 3.38 mM with 95% confidence intervals ranging from 3.13 to 3.66 mM, more than 20-fold higher than the LD_{50} for cisplatin in these cells. LD_{50} of carboplatin in LLC-PK_1/GTK cells was 3.25 mM with 95% confidence intervals ranging from 2.95 to 3.58 mM. The overexpression of human GTK had no effect on the LD_{50} of carboplatin ($p = 0.34$). The slopes of the two dose curves were not significantly different ($p = 0.18$). Thus, carboplatin is not bioactivated by a cysteine S-conjugate β-lyase reaction in LLC-PK_1 cells.

Toxicity of Cisplatin in Dividing GTK Transfected Cells. Dividing LLC-PK_1/C1 cells and LLC-PK_1/GTK cells were treated with cisplatin (Fig. 5A). Dividing LLC-PK_1/C1 cells were 4.3-fold more sensitive to cisplatin than quiescent monolayers (Fig. 4A and Fig. 5A). LD_{50} of cisplatin in dividing LLC-PK_1/C1 cells was 38.0 μM with 95% confidence intervals ranging

JPET Fast Forward. Published on May 15, 2003 as DOI: 10.1124/jpet.103.052225
This article has not been copyedited and formatted. The final version may differ from this version.

from 30.5 to 47.2 μM. LD_{50} of cisplatin in dividing LLC-PK_1/GTK cells was 41.7 μM with 95% confidence intervals ranging from 37.9 to 45.9 μM. There was no significant difference between the LD_{50} of the dividing LLC-PK_1/C1 and LLC-PK_1/GTK cells toward cisplatin ($p = 0.36$). The slopes of the two dose curves were not significantly different ($p = 0.84$). These data demonstrate overexpression of cysteine S-conjugate β-lyase does not alter the toxicity of cisplatin toward dividing LLC-PK_1 cells. This result indicates that metabolism of cisplatin through a cysteine S-conjugate β-lyase dependent pathway does not make a significant contribution to the toxicity of cisplatin toward dividing cells.

Toxicity of Carboplatin in Dividing GTK Transfected Cells. The toxicity of carboplatin toward dividing LLC-PK_1/C1 and LLC-PK_1/GTK cells is shown in Fig. 5B. Carboplatin was 2.2-fold more toxic towards dividing LLC-PK_1/C1 cells than confluent cells (Fig. 4B and Fig. 5B). The LD_{50} of carboplatin in dividing LLC-PK_1/C1 cells was 1.56 mM with 95% confidence intervals ranging from 1.36 to 1.79 mM. The LD_{50} of carboplatin in dividing LLC-PK_1/GTK cells was 1.99 mM with 95% confidence intervals ranging from 1.54 to 2.57 mM. There was no significant difference between the LD_{50} of the dividing LLC-PK_1/C1 and LLC-PK_1/GTK cells toward carboplatin ($p = 0.07$). The slopes of the two dose curves were not significantly different ($p = 0.55$). These data are consistent with the hypothesis that a cysteine S-conjugate β-lyase dependent pathway does not play a role in the toxicity of carboplatin toward either confluent or dividing cells.

JPET Fast Forward. Published on May 15, 2003 as DOI: 10.1124/jpet.103.052225
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

The data from these studies confirm the role of a cysteine S-conjugate β-lyase in the metabolism of cisplatin to a nephrotoxin. Our hypothesis is that the β-elimination reaction catalyzed by cysteine S-conjugate β-lyase is the final step in the activation of cisplatin to a nephrotoxin and that the low level of cysteine S-conjugate β-lyase activity in LLC-PK_1 cells make this reaction rate-limiting for the metabolism of cisplatin to a nephrotoxin. Our data show that overexpression of GTK significantly increased the cysteine S-conjugate β-lyase activity in LLC-PK_1 cells. In support of our hypothesis, cisplatin was significantly more toxic toward confluent monolayers of LLC-PK_1 cells that overexpressed GTK than toward control cells. The data show that carboplatin was 20-fold less toxic to confluent monolayers of LLC-PK1 cells than cisplatin, which correlates with its lack of nephrotoxicity in the clinic. Overexpression of GTK had no effect on the toxicity of carboplatin in confluent monolayers of LLC-PK_1 cells. We propose that carboplatin is not bioactivated through the glutathione-conjugate pathway and this differential metabolism explains the difference in renal toxicity between cisplatin and carboplatin.

A comparison of the structures of cisplatin and carboplatin shows features that would contribute to the differential metabolism of the two compounds. The chlorines in cisplatin have been substituted with a 1,1-cyclobutanedicarboxylate ligand in carboplatin (Fig. 1). The stability of the carboplatin ligand renders carboplatin less reactive than cisplatin toward sulfur-based nucleophiles (Dedon and Borch, 1987). The second-order rate constant for the substitution reaction of carboplatin with glutathione is more than 14-fold less than the rate of the

reaction of cisplatin with glutathione. The reduced reactivity of carboplatin with glutathione would limit its metabolism through the proposed bioactivation pathway. In addition, we have shown that a monoplatinum-monoglutathione conjugate of cisplatin is the conjugate that is metabolized to a nephrotoxin (Townsend et al., 2003b). A monoplatinum-monoglutathione conjugate of carboplatin would have a bulky cyclobutanedicarboxylate side chain and may not be a substrate for the enzymes in the pathway.

The data also show that dividing cells are more sensitive than quiescent monolayers of cells to the toxicity of both cisplatin and carboplatin. The level of cysteine S-conjugate β-lyase activity had no effect on the toxicity of either drug toward dividing cells. These data are in agreement with the hypothesis that the mechanism by which cisplatin kills dividing cells is different from the mechanism by which it kills quiescent renal cells. Data from other investigators indicate that death is triggered in dividing cells by cisplatin or carboplatin-induced DNA damage (Fink and Howell, 2000; Perez, 1998). Cysteine S-conjugate β-lyase is not part of pathways through which DNA damage induces apoptosis.

Cysteine S-conjugate β-lyases are PLP-dependent enzymes that catalyze the β-elimination of the cysteine conjugates (Cooper, 1998). The products of this reaction include pyruvate, ammonia, and a sulfur-containing metabolite. The sulfur containing metabolites are highly reactive and thioacylate proteins (Hayden et al., 1991). The lysine residues of proteins are particularly susceptible to the thioacylating products and inactivation of essential proteins would result in cell death (Birner et al., 1994; Cooper et al., 2002). Both mitochondrial and cytosolic proteins are modified after treatment with the nephrotoxic halogenated alkene, trichloroethene or

its cysteine conjugate (Birner et al., 1994). Aminooxyacetic acid, an inhibitor of PLP-dependent enzymes, inhibits the formation of thioacylated proteins.

Cysteine S-conjugate β-lyase activity has been found in cytosolic, mitochondrial, and microsomal fractions of the human kidney tissue, but the cytosolic fraction has the highest activity (Lash et al., 1990). It is still not known which enzyme catalyzes the β-elimination reaction of cysteine-conjugates of halogenated alkenes *in vivo*. Nine mammalian enzymes have been identified that catalyze this reaction (Cooper et al., 2002). Five are cytosolic. They are rat kidney cytGTK, rat liver kynureninase, pig heart aspartate aminotransferase, pig heart alanine aminotransferase and human cytosolic branched-chain amino acid aminotransferase (Stevens et al., 1986; Stevens, 1985; Gaskin et al., 1995; Adcock et al., 1996; Cooper et al., 2002). The mitochondrial enzymes that possess cysteine S-conjugate β-lyase activities are human mitochondrial branched-chain amino acid aminotransferase, rat liver mitochondrial aspartate aminotransferase, rat kidney mitochondrial alanine-glyoxylate aminotransferase II and a high-M_r mitochondrial protein (Cooper et al., 2002; Abraham et al., 1995; Abraham and Cooper, 1991). CytGTK has the highest specific activity of the enzymes that catalyze the cysteine S-conjugate β-elimination reaction with the cysteine conjugates of the halogenated alkenes (Cooper et al., 2002).

CytGTK has been detected immunohistochemically in the S1, S2, and S3 regions of the kidney proximal tubules, but not the other regions of the kidney (Jones et al., 1988). Trevisan and coworkers treated rats with S1-S2 and S3 specific nephrotoxicants and measured GTK excretion in urine (Trevisan et al., 1998). The results showed that the enzyme was distributed

along the whole proximal tubule. The proximal tubules are the major targets of the nephrotoxicity caused by both the halogenated alkenes and cisplatin. Overexpression of GTK in LLC-PK_1 cells increased the sensitivity to the cysteine conjugate DCVC (Goldfarb et al., 1996).

Results in this study confirm our hypothesis that cisplatin is converted to a nephrotoxin via a cysteine S-conjugate β-lyase-dependent pathway. Future studies will focus on identifying the cysteine S-conjugate β-lyase that bioactivates the cysteine conjugate of cisplatin and the critical targets of the reactive thiol produced by this reaction. Understanding the molecular pathways that produce the dose-limiting nephrotoxicity associated with some platinum-based drugs will aid in the rational design of new platinum-based chemotherapy drugs.

Acknowledgments

We gratefully acknowledge Dr. Arthur Cooper (Weill Medical College of Cornell University, NY) for providing us with GTK antibody and for advice regarding studies of cysteine S-conjugate β-lyase. We acknowledge Dr. Paul F. Cook, Sebastian Daum (Department of Chemistry and Biochemistry, University of Oklahoma) and the staff of OU Chem/Biochem Mass Spectrometry Laboratory for their assistance with the structural analysis of the DCVC. We also acknowledge the Oklahoma Medical Research Foundation DNA Sequencing Facility for sequencing the GTK cDNA.

References

Abraham DG and Cooper AJ (1991) Glutamine transaminase K and cysteine S-conjugate beta-lyase activity stains. *Anal.Biochem.* **197**:421-427.

Abraham DG and Cooper AJ (1996) Cloning and expression of a rat kidney cytosolic glutamine transaminase K that has strong sequence homology to kynurenine pyruvate aminotransferase. *Arch.Biochem.Biophys.* **335**:311-320.

Abraham DG, Patel PP, and Cooper AJ (1995) Isolation from rat kidney of a cytosolic high molecular weight cysteine- S-conjugate beta-lyase with activity toward leukotriene E4. *J.Biol.Chem.* **270**:180-188.

Adcock HJ, Gaskin PJ, Shaw PN, Teesdale-Spittle PH, and Buckberry LD (1996) Novel sources of mammalian C-S lyase activity. *J.Pharm.Pharmacol.* **48**:150-153.

Anders MW and Dekant W (1998) Glutathione-dependent bioactivation of haloalkenes. *Annu.Rev.Pharmacol.Toxicol.* **38**:501-537.

Birner G, Richling C, Henschler D, Anders MW, and Dekant W (1994) Metabolism of tetrachloroethene in rats: identification of N epsilon- (dichloroacetyl)-L-lysine and N epsilon-(trichloroacetyl)-L-lysine as protein adducts. *Chem.Res.Toxicol.* **7**:724-732.

Cooper AJ (1998) Mechanisms of cysteine S-conjugate beta-lyases. *Adv.Enzymol.Relat Areas Mol.Biol.* **72**:199-238.

Cooper AJ, Bruschi SA, and Anders MW (2002) Toxic, halogenated cysteine S-conjugates and targeting of mitochondrial enzymes of energy metabolism. *Biochem.Pharmacol.* **64**:553-564.

Cooper AJ, Wang J, Gartner CA, and Bruschi SA (2001) Co-purification of mitochondrial HSP70 and mature protein disulfide isomerase with a functional rat kidney high-M(r) cysteine S-conjugate beta-lyase. *Biochem.Pharmacol.* **62**:1345-1353.

Cornelison TL and Reed E (1993) Nephrotoxicity and hydration management for cisplatin, carboplatin, and ormaplatin. *Gynecol.Oncol.* **50**:147-158.

deCeaurriz J and Ban M (1990) Role of gamma-glutamyl transpeptidase and beta-lyase in the nephrotoxicity of hexachloro-1,3-butadiene and methyl mercury in mice. *Tox.Lett.* **50**:249-256.

Dedon PC and Borch RF (1987) Characterization of the reactions of platinum antitumor agents with biologic and nonbiologic sulfur-containing nucleophiles. *Biochem.Pharmacol.* **36**:1955-1964.

Elfarra AA, Jakobson I, and Anders MW (1986) Mechanism of S-(1,2-dichlorovinyl)glutathione-induced nephrotoxicity. *Biochem.Pharmacol.* **35**:283-288.

Fink D and Howell SB (2000) How does cisplatin kill cells?, in *Platinum-based drugs in cancer therapy* (Kelland LR and Favilli F eds) pp 149-167, Humana Press, Totowa, New Jersey.

Gaskin PJ, Adcock HJ, Buckberry LD, Teesdale-Spittle PH, and Shaw PN (1995) The C-S lysis of L-cysteine conjugates by aspartate and alanine aminotransferase enzymes. *Hum.Exp.Toxicol.* **14**:422-427.

Goldfarb P, Perry S, Harries H, Scholfield C, Lock T, King L, and Gibson G (1996) Molecular cloning and expression of cDNAs for rat and human kidney cysteine conjugate beta-lyase. *Biochem.Soc.Trans.* **24**:330S.

Hanigan MH, Gallagher BC, Taylor PTJr, and Large MK (1994) Inhibition of gamma-glutamyl transpeptidase activity by acivicin in vivo protects the kidney from cisplatin-induced toxicity. *Cancer Res.* **54**:5925-5929.

Hanigan MH, Lykissa ED, Townsend DM, Ou C, Barrios R, and Lieberman MW (2001) Gamma-glutamyl transpeptidase-deficient mice are resistant to the nephrotoxicity of cisplatin. *Am.J.Pathol.* **159**:1889-1894.

Hayden PJ, Ichimura T, McCann DJ, Pohl LR, and Stevens JL (1991) Detection of cysteine conjugate metabolite adduct formation with specific mitochondrial proteins using antibodies raised against halothane metabolite adducts. *J.Biol.Chem.* **266**:18415-18418.

Jones TW, Chen Q, Schaeffer VH, and Stevens JL (1988) Immunohistochemical localization of glutamine transaminase k, a rat kidney cysteine conjugate b-lyase, and the relationship to the segment specificity of cysteine conjugate nephrotoxicity. *Mol.Pharmacol.* **34**:621-627.

Lash LH, Elfarra AA, Rakiewicz-Nemeth D, and Anders MW (1990) Bioactivation mechanism of cytotoxic homocysteine S-conjugates. *Arch.Biochem.Biophys.* **276**:322-330.

Lash LH, Sausen PJ, Duescher RJ, Cooley AJ, and Elfarra AA (1994) Roles of cysteine conjugate b-lyase and s-oxidase in nephrotoxicity: studies with s-(1,2-dichlorovinyl)-l-cysteine and s-(1,2-dichlorovinyl)-l-cysteine sulfoxide. *The Journal of Pharmacology and Experimental Therapeutics* **269**:374-383.

McKeage MJ (2000) Clinical toxicology of platinum-based cancer chemotherapeutic agents, in *Platinum-based drugs in cancer therapy* (Kelland LR and Farrell NP eds) pp 251-275, Humana Press, Totowa, New Jersey.

McKinney LL, Picken Jr. JC, Weakley FB, Eldridge AC, Campbell RE, Cowan JC, and Biester HE (1959) Possible toxic factor of trichloroethylene-extracted soybean oil meal. *J.Am.Chem.Soc.* **81**:909-915.

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival:Application to proliferation and cytotoxicity assays. *J.Immunol.Methods* **65**:55-63.

O'Dwyer PJ, Stevenson JP, and Johnson SW (1999) Clinical Status of Cisplatin, Carboplatin and Other Platinum-Based Antitumor Drugs, in *Cisplatin:Chemistry and Biochemistry of a Leading Anticancer Drug* (Lippert B ed) pp 29-70, Wiley-VCH, Zurich.

Perez RP (1998) Cellular and molecular determinants of cisplatin resistance. *Eur.J.Cancer* **34**:1535-1542.

Perry S, Harries H, Scholfield C, Lock T, King L, Gibson G, and Goldfarb P (1995) Molecular cloning and expression of a cDNA for human kidney cysteine conjugate beta-lyase. *FEBS Lett.* **360**:277-280.

Perry SJ, Schofield MA, MacFarlane M, Lock EA, King LJ, Gibson GG, and Goldfarb PS (1993) Isolation and expression of a cDNA coding for rat kidney cytosolic cysteine conjugate beta-lyase. *Mol.Pharmacol.* **43**:660-665.

Sadowitz PD, Hubbard BA, Dabrowiak JC, Goodisman J, Tacka KA, Aktas MK, Cunningham MJ, Dubowy RL, and Souid AK (2002) Kinetics of cisplatin binding to cellular DNA and modulations by thiol- blocking agents and thiol drugs. *Drug Metab Dispos.* **30**:183-190.

Stevens JL (1985) Isolation and characterization of a rat liver enzyme with both cysteine conjugate beta-lyase and kynureninase activity. *J.Biol.Chem.* **260**:7945-7950.

Stevens JL, Robbins JD, and Byrd RA (1986) A purified cysteine conjugate beta-lyase from rat kidney cytosol. Requirement for an alpha-keto acid or an amino acid oxidase for activity and identity with soluble glutamine transaminase K. *J.Biol.Chem.* **261**:15529-15537.

Townsend DM, Deng M, Zhang L, Lapus MG, and Hanigan MH (2003a) Metabolism of cisplatin to a nephrotoxin in proximal tubule cells. *Journal of the American Society of Nephrology* **14** :1-10.

Townsend DM and Hanigan MH (2002) Inhibition of gamma-glutamyl transpeptidase or cysteine S-conjugate beta-lyase activity blocks the nephrotoxicity of cisplatin in mice. *J.Pharmacol.Exp.Ther.* **300**:142-148.

Townsend DM, Marto JA, Deng M, Macdonald TJ, and Hanigan MH (2003b) High pressure liquid chromatography and mass spectrometry characterization of the nephrotoxic biotransformation products of cisplatin. *Drug Metab.Dispos.* **in press**.

Trevisan A, Cristofori P, Fanelli G, Bicciato F, and Stocco E (1998) Glutamine transaminase K intranephron localization in rats determined by urinary excretion after treatment with segment-specific nephrotoxicants. *Arch.Toxicol.* **72**:531-535.

Footnotes

* This work was supported by Grant R01CA57530 from the National Cancer Institute.

Reprint requests to Marie H. Hanigan, Ph.D., Biomedical Research Center, Room 264, 975 N.E. 10th Street, Oklahoma City, Oklahoma 73104, USA.

Legends for figures

Figure 1. The structures of cisplatin (left) and carboplatin (right).

Figure 2. Metabolism of cisplatin to a nephrotoxin. The proposed pathway of cisplatin activation is based on the metabolism of the glutathione-conjugates of the halogenated-alkenes to nephrotoxins (Anders and Dekant, 1998). The location of intracellular and extracellular reactions that bioactivate cisplatin to a nephrotoxin are shown (left). Cisplatin enters cells and binds to DNA, which is toxic to dividing cells but not to quiescent cells such as the proximal tubules in the kidney. Formation of cisplatin-glutathione conjugates reduces the amount of platinum bound to DNA and protects dividing cells from cisplatin toxicity (Sadowitz et al., 2002). The formation of platinum-glutathione conjugates and their transport out of the cell are the first steps in the metabolism to a nephrotoxin. The platinum-glutathione conjugate is cleaved to a platinum-cysteinyl-glycine conjugate by GGT on the cell surface (Hanigan et al., 2001; Townsend et al., 2003a). The platinum-conjugate is further cleaved to a platinum-cysteine conjugate by aminodipeptidase N on the cell surface. The platinum-cysteine conjugate is taken up into the cell where it is converted to a highly reactive thiol by cysteine S-conjugate β-lyase. Binding of the reactive thiol to essential proteins within the cell is toxic. The reaction catalyzed by cysteine S-conjugate β-lyase is shown (right).

Figure 3. Western blot analysis of LLC-PK_1/GTK and LLC-PK_1/C1 cell lysates. Lane 1: LLC-PK_1/GTK (25 μg). Lane 2: LLC-PK_1/C1 (25 μg). A protein band with MW 48 kD (arrow) was

detected in LLC-PK_1/GTK cell line, but not in LLC-PK_1/C1 cell line. The molecular weight of this band is consistent with that of GTK monomer.

Figure 4. Toxicity of cisplatin and carboplatin in confluent LLC-PK_1/GTK and LLC-PK_1/C1 cells. Confluent monolayers of LLC-PK_1/GTK (empty diamond) or LLC-PK_1/C1 (solid diamond) were treated with cisplatin (A) or carboplatin (B) in DMEM for 3 hr. The cisplatin and carboplatin solutions were removed at the end of the 3 hr exposure and replaced with fresh DMEM, containing 5% FBS and 400 μg/ml G418. The viability of the cells was measured at 72 hr. Each point represents mean of three points ± S.D.

Figure 5. Toxicity of cisplatin and carboplatin in dividing LLC-PK_1/GTK and LLC-PK_1/C1 cells. Dividing cells of LLC-PK_1/GTK (empty diamond) or LLC-PK_1/C1 (solid diamond) were treated with cisplatin (A) or carboplatin (B) in DMEM for 3 hr. The cisplatin and carboplatin solutions were removed at the end of the 3 hr exposure and replaced with fresh DMEM, containing 5% FBS and 400 μg/ml G418. The viability of the cells was measured at 72 hr. Each point represents mean of three points ± S.D.

JPET Fast Forward. Published on May 15, 2003 as DOI: 10.1124/jpet.103.052225
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1. Specific activities of enzymes in LLC-PK_1/GTK and LLC-PK_1/C1 cell lines.

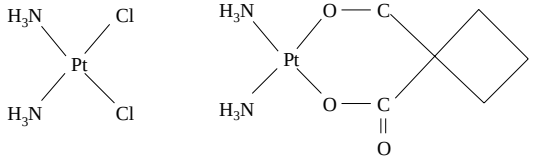
	Enzyme Activities	
Cell Lines	GTK	Cysteine S-Conjugate β-Lyase
LLC-PK_1/C1	1.5 ± 0.27 [a]	<0.2 [b]
LLC-PK_1/GTK	84 ± 8.5	4.3 ± 0.37

[a] All enzyme units are expressed as mU/mg protein ± S.D.

[b] The detection limit of the assay is 0.2 mU/mg protein

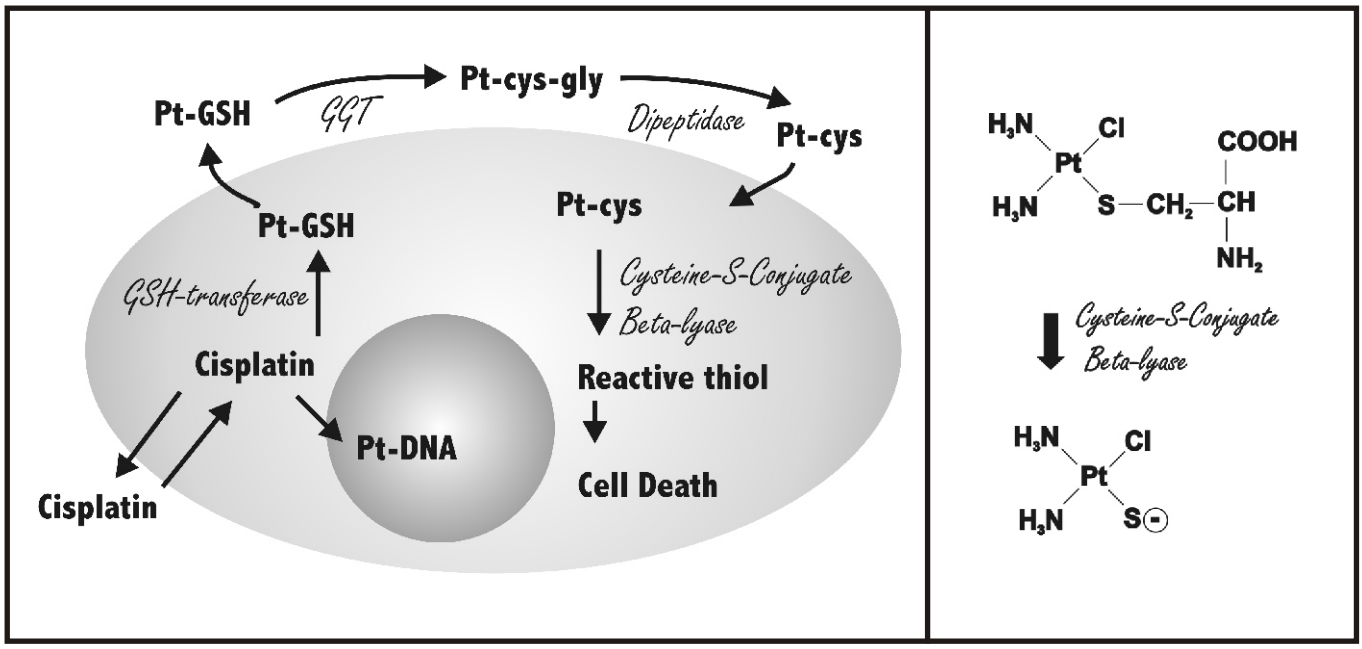
JPET #52225

Figure 1



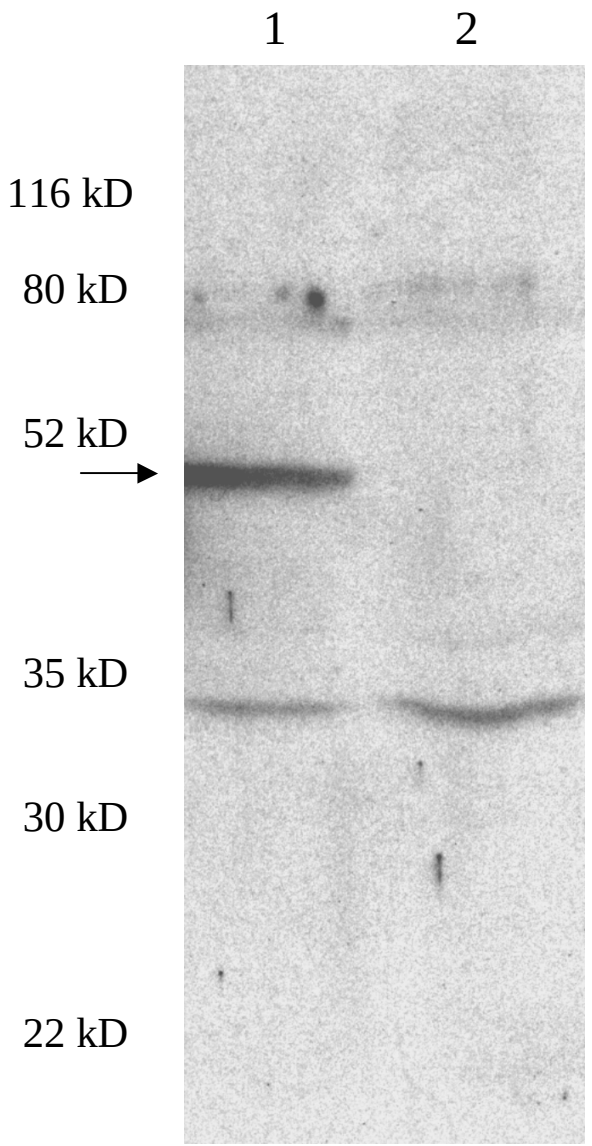
Cisplatin **Carboplatin**

JPET Fast Forward. Published on May 15, 2003 as DOI: 10.1124/jpet.103.052225
This article has not been copyedited and formatted. The final version may differ from this version.



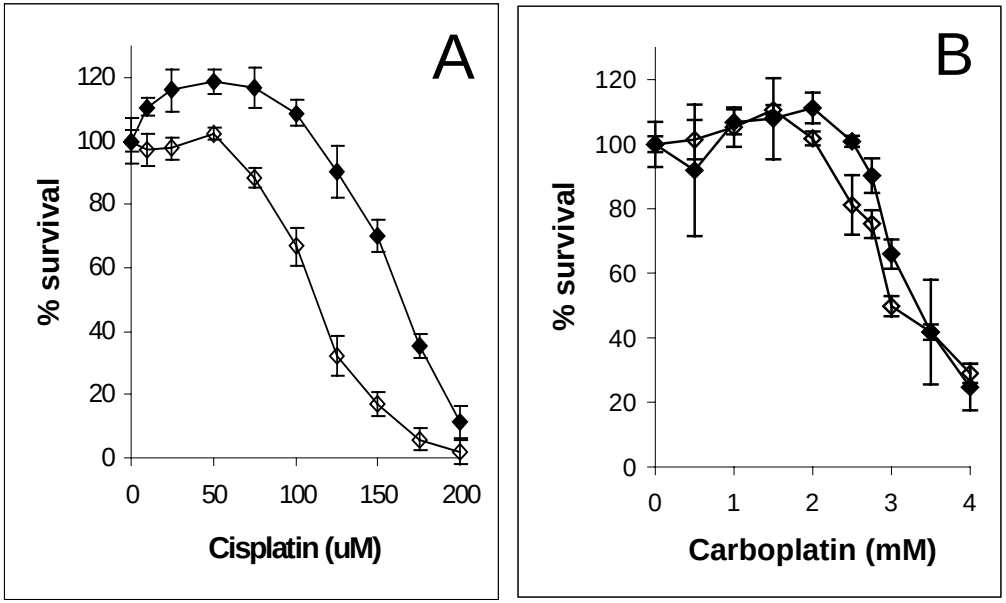
JPET #52225

Figure 3



JPET #52225

Figure 4



JPET #52225

Figure 5