

Metabolism of Cisplatin to a Nephrotoxin in Proximal Tubule Cells

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Abstract. Cisplatin, a commonly used chemotherapeutic agent, is nephrotoxic. The mechanism by which cisplatin selectively kills the proximal tubule cells was heretofore unknown. Recent studies in mice and rats have shown that the nephrotoxicity of cisplatin can be blocked by acivicin or (aminoxy)acetic acid, the same enzyme inhibitors that block the metabolic activation of a series of nephrotoxic halogenated alkenes. In this study, it was hypothesized that cisplatin is activated in the kidney to a toxic metabolite through the same pathway that has been shown to activate the halogenated alkenes. This activation begins with the formation of a glutathione-conjugate that is metabolized to a cysteinyl-glycine-conjugate, to a cysteine-conjugate, and finally to a reactive thiol. In this study, a protocol was developed in which confluent monolayers of LLC-PK₁ cells were exposed to clinically relevant concentra-

tions of cisplatin or cisplatin-conjugate for 3 h. Cell viability was assayed at 72 h. The role of gamma-glutamyl transpeptidase (GGT) and cysteine-S-conjugate beta-lyase in the metabolism of each of the cisplatin-conjugates was investigated. Pre-incubation of cisplatin with glutathione, cysteinyl-glycine, or N-acetyl-cysteine to allow for the spontaneous formation of cisplatin-conjugates increased the toxicity of cisplatin toward LLC-PK₁ cells. Inhibition of GGT activity showed that GGT was necessary only for the toxicity of the cisplatin-glutathione-conjugate. Inhibition of cysteine-S-conjugate beta-lyase reduced the toxicity of each of the cisplatin-conjugates. These data demonstrate that metabolism of cisplatin in proximal tubule cells is required for its nephrotoxicity. The elucidation of this pathway provides new targets for the inhibition of cisplatin nephrotoxicity.

Cisplatin is a potent antitumor agent currently used in the treatment of germ cell tumors, head and neck tumors, cervical cancer, and as a salvage treatment for other solid tumors (1). The dose of cisplatin that can be administered is limited by its nephrotoxicity (2). The mechanism by which cisplatin kills the proximal tubule cells in the kidney has been the focus of intense investigation for many years. In tumors and other dividing cells, cisplatin-DNA crosslinks are thought to be the cytotoxic lesion (3). Nonproliferating cells are less sensitive to the toxicity of DNA-damaging agents, yet the quiescent proximal tubule cells are selectively killed by cisplatin. High concentrations of cisplatin induce necrotic cell death in confluent monolayers of proximal tubule cells, whereas lower concentrations of cisplatin induce apoptosis through a caspase-9-dependent pathway (4,5). The molecular mechanism by which cisplatin kills these nonproliferating cells has been unclear.

Our studies in rats and mice have shown that the nephrotoxicity of cisplatin can be blocked by inhibiting either of two

enzymes expressed in the proximal tubules, gamma-glutamyl transpeptidase (GGT) or cysteine-S-conjugate beta-lyase (6–8). Data from these *in vivo* studies have led to the hypothesis that the nephrotoxicity of cisplatin is the result of the metabolic activation of the cisplatin in the kidney to a more potent toxin. We propose that this activation is through a pathway that includes GGT and cysteine-S-conjugate beta-lyase. These two enzymes have been shown to be required for the nephrotoxicity of a series of halogenated alkenes that include hexachloro-1,3-butadiene, trichloroethylene, and tetrafluoroethylene (9–13). The metabolism of these compounds is initiated by their conjugation to glutathione (Figure 1). As the glutathione-conjugates pass through the kidney, they are cleaved to cysteinyl-glycine-conjugates by GGT expressed on the surface of the proximal tubule cells (14). GGT cleaves gamma-glutamyl bonds in extracellular glutathione and glutathione-conjugates (15). The cysteinyl-glycine-conjugates are further metabolized to cysteine-conjugates by aminodi-peptidases, also expressed on the surface of the proximal tubule cells (16). Both the GGT and aminodi-peptidase catalyzed reactions take place extracellularly. The cysteine-conjugates are transported into the proximal tubule cells, where they are further metabolized by cysteine-S-conjugate beta-lyase to the highly reactive thiols (17). The toxicity of N-acetyl-cysteine-conjugates of the halogenated alkenes is similar to the cysteine-conjugates, and both are metabolized by cysteine-S-conjugate beta-lyase to reactive thiols (18). The reactive electrophilic metabolites bind to cellular

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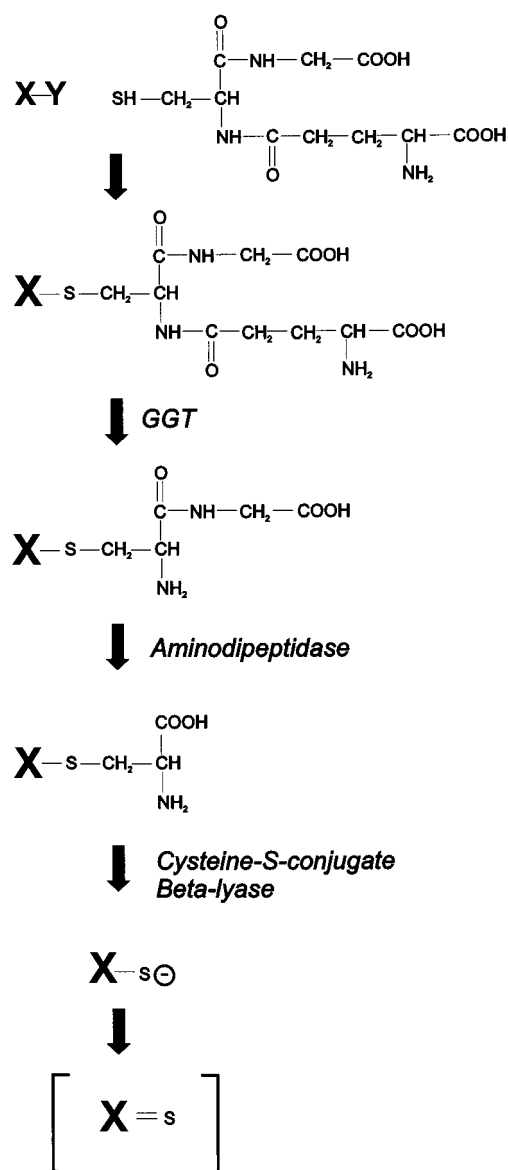


Figure 1. Metabolic activation of glutathione-conjugates to reactive thiols. Halogenated alkenes (X represents the alkene and Y a halogen molecule: fluorine, chlorine, or bromine) have been shown to be metabolized to nephrotoxins via this pathway (9,12,60). The halogenated alkenes form glutathione-S-conjugates, which are cleaved to cysteinyl-glycine-conjugates by GGT. Aminodipeptidase cleaves the cysteinyl-glycine-conjugates to cysteine-conjugates. Cysteine-S-conjugate beta-lyase catalyzes the production of unstable reactive thiols, which are toxic. We propose that cisplatin is metabolized through this same pathway. In the proposed pathway, Y would represent one of the chlorines in cisplatin and X would represent the remainder of the cisplatin molecule. In the proposed pathway, the sulfur of the glutathione molecule binds to the platinum displacing the chlorine.

macromolecules, triggering an increase in cytosolic free calcium and ultimately cell death (19). The toxicity caused by the nephrotoxic halogenated-alkenes is localized to the proximal convoluted tubules in the outer renal cortex, the same cells killed by cisplatin (10,20–22).

Cisplatin is not a substrate for either GGT or cysteine-S-

conjugate beta-lyase. However, cisplatin has been shown to form glutathione-conjugates spontaneously in solution (23,24). Dissociation of one of the chlorines from the cisplatin molecule results in a positive charge on the platinum that will attract the negatively charged sulfur on the cysteine moiety of the glutathione molecule. Cisplatin-glutathione-conjugates have been isolated from cells treated with cisplatin and from the serum of cisplatin-treated rats (24,25). Pretreating rats with an inhibitor of glutathione-S-transferases reduced the nephrotoxicity of cisplatin, suggesting that *in vivo* glutathione-S-transferases may catalyze the conjugation of cisplatin to glutathione (26). Conjugation to glutathione is a means of detoxifying electrophilic compounds. However, as noted above, the glutathione-conjugates of some compounds can be further metabolized to nephrotoxins. We propose cisplatin is bioactivated in the kidney by the same pathway that activates the halogenated alkenes. Our hypothesis is that GGT cleaves the gamma-glutamyl group of the glutathione-conjugate, and aminodipeptidase cleaves the cysteinyl-glycine bond, resulting in a platinum-cysteine-conjugate. Finally the cysteine-conjugate is metabolized by cysteine-S-conjugate beta-lyase to a reactive thiol.

In this study, we have used LLC-PK₁ cells, a porcine proximal tubule cell line, to test our hypothesis. LLC-PK₁ cells were used to investigate the bioactivation of halogenated alkenes to nephrotoxins (12,27–30). Confluent monolayers of LLC-PK₁ cells have the morphologic and metabolic characteristics of proximal tubules (31,32). There have been several studies of cisplatin-induced toxicity that have used dividing LLC-PK₁ cells (33–35). Cisplatin binds DNA, forming interstrand and intrastrand crosslinks that inhibit cell division (36,37). Dividing cells can be killed by low concentrations of cisplatin. These studies have not addressed the mechanism of cisplatin nephrotoxicity because *in vivo* the proximal tubule cells are not dividing. They form a confluent monolayer of epithelial cells lining the tubules. The proximal tubules are exposed to cisplatin during its biphasic excretion into the urine (38). The highest levels of exposure are during the first 3 h after administration. The toxicity is dose- and time-dependent (22). Damage to the proximal tubules is first observed 3 to 4 d after the administration of the drug. Montine and Borch (39) evaluated the toxicity of cisplatin and its non-nephrotoxic derivative, carboplatin, in confluent monolayers of LLC-PK₁ cells. They showed that cisplatin toxicity was dose- and time-dependent, whereas carboplatin was not toxic even at tenfold the LC₅₀ dose of cisplatin.

We began our studies by developing a treatment protocol that closely mimicked the *in vivo* exposure of proximal tubule cells to cisplatin. Confluent monolayers of cells were exposed to cisplatin for 3 h. The cisplatin was removed and replaced with tissue culture media. The cells were maintained in culture for 3 d and then assayed for viability. With this protocol, we observed toxicity at 50 μ M cisplatin. The serum concentrations of cisplatin in patients and experimental animals treated with nephrotoxic levels of cisplatin reaches 30 to 50 μ M (40,41); whereas, in non-nephrotoxic regimens, the serum concentration rarely exceeds 10 μ M platinum (42). We incubated cisplatin with glutathione, cysteinyl-glycine, or NAC to allow for

the formation of cisplatin-conjugates. Previous studies have shown that cisplatin will spontaneously form conjugates with glutathione and cysteine (23,43,44). These conjugates are the intermediates along the proposed pathway of metabolic activation to a nephrotoxin. The enzymes in the proposed pathway, GGT and cysteine-S-conjugate beta-lyase, were inhibited. The effect of this inhibition on the toxicity of each conjugate was evaluated.

Materials and Methods

Cell Line

LLC-PK₁ (ATCC CRL 1392), a proximal tubule cell line isolated from pig kidney, was purchased from American Type Culture Collection (Rockville, MD). Cells were maintained in Dulbecco Modified Eagle's Medium (DMEM; Life Technologies/BRL, Grand Island, NY), 5% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT), 50 units penicillin G, and 50 µg streptomycin/ml (Life Technologies/BRL) at 37°C in a 5% CO₂ atmosphere. Subconfluent cultures were passaged every 3 to 4 d. For experiments, LLC-PK₁ cells were seeded in 96-well plates at 1 × 10⁴ cells/well. On the third day after plating, confluent monolayers formed and the media was replaced with fresh media. Cells were used for experiments on day 7.

Time Course of Cisplatin Toxicity

A fresh stock solution of 3.33 mM cis-platinum(II)-diamine dichloride (cisplatin, Sigma Chemical Co.) was prepared in 0.9% sodium chloride solution the day of the experiment. For the experiments in which cells were exposed continuously to cisplatin for up to 3 d, the cisplatin was diluted in DMEM medium containing 10% FBS, 50 units penicillin G, and 50 µg streptomycin/ml. At the time points indicated, the number of viable cells was determined by the MTT assay (45). Wells with untreated cells served as controls. A standard curve was developed relating cell number to the OD₅₇₀ value obtained in the MTT assay.

For experiments in which cells were exposed to cisplatin for 3 h, the cisplatin was diluted in Hanks Balanced Salt Solution (HBSS, Cat. No. 11201, Life Technologies) with 5 mM N-[2-Hydroxyethyl]piperazine-N-[2-ethane sulfonic acid] (HEPES, Sigma Chemical Co.), pH 7.2. Medium was removed from the cells and replaced with cisplatin diluted in HBSS with HEPES, pH 7.2. The cells were incubated at 37°C. After 3 h, the cisplatin solution was removed from the cells and replaced with DMEM medium containing 5% FBS, penicillin, and streptomycin. The cells were incubated at 37°C in 5% CO₂. In the time course experiments, the MTT assay was started at the time indicated. In all other experiments, cell viability was determined 72 h after the start of the experiment (69 h after the drug was removed).

Treatment with Glutathione, Cysteinyl-Glycine, and N-Acetyl Cysteine-Glutathione Solutions

Solutions containing 50 µM cisplatin and equimolar glutathione, cysteinyl-glycine (Bachem, King of Prussia, PA) or N-acetyl cysteine (NAC) in HBSS with 5 mM HEPES, pH 7.2, were incubated at 37°C for 30 min to allow for the spontaneous formation of cisplatin-conjugates (23,43). The medium was removed from the cells, and 150 µl of the incubation mixture was added to each well. The cells were incubated at 37°C. The mixture was removed after 3 h and replaced with fresh DMEM medium containing 5% FBS and penicillin and streptomycin. The cells were incubated for an additional 69 h at 37°C in 5% CO₂. The number of viable cells was determined 72 h after the start of the experiment by the MTT assay.

Kidney Homogenates

Kidneys from 5-mo-old female Balb/c mice were harvested and stored at –80°C. Kidneys were thawed, homogenized in 0.9% sodium chloride, and assayed for GGT and aminopeptidase N activity. Kidneys to be assayed for cysteine-S-conjugate beta-lyase activity were homogenized in 3 vol of 10 mM Tris-HCl, 0.25 M sucrose (pH 7.5) in a Potter-Elvehjem homogenizer, 4°C. The homogenate was freeze-thawed twice, followed by sonication twice for 10 s with a 30-s cooling interval, then centrifuged at 3000 × g for 5 min. The supernatant was used to assay enzyme activity.

GGT Assay

GGT activity was assayed as described previously (14). LLC-PK₁ cells were trypsinized off the plates and assayed for GGT activity. One unit of GGT activity was defined as the amount of enzyme that released 1 µmol of p-nitroaniline per min at 25°C. The protein concentrations of the kidney homogenates and LLC-PK₁ monolayers were determined with the BCA assay (Pierce, Rockford, IL).

Aminopeptidase Assay

The activity of aminopeptidase N (EC 3.4.11.2), previously referred to as aminopeptidase M, was measured by the method of Hughey and co-workers with S-benzylglycine-p-nitroanilide as the substrate as described previously (16,46). To determine the level of aminopeptidase N activity in confluent monolayers of LLC-PK₁ cells, the media was removed, the intact monolayers were rinsed with phosphate-buffered saline (PBS) and the reaction mixture for the assay was added directly to the cells. The plates were incubated at 37°C. The reaction was stopped by removing the assay solution and boiling it. One unit of aminopeptidase N activity is defined as the amount of enzyme that released 1 µmol of p-nitroaniline per min at 37°C. The protein concentrations of the kidney homogenates and LLC-PK₁ monolayers were determined with the BCA assay.

Cysteine-S-Conjugate Beta-Lyase Assay

Confluent monolayers of LLC-PK₁ cells were trypsinized off the plates. The cells were rinsed with PBS, resuspended in 10 mM Tris-HCl, 0.25 M sucrose (pH 7.5), freeze-thawed twice, sonicated twice for 10 s with a 30 s cooling interval, then centrifuged at 3000 × g for 5 min as described previously (47). The supernatant was used to assay enzyme activity. S-(1,2-Dichlorovinyl)-L-cysteine (DCVC) was synthesized by the method of McKinney *et al.* (48). Cysteine-S-conjugate beta-lyase activity was measured by a method developed by Dr. Authur Cooper (personal communication), which is a modification of a previously published assay (13). Briefly, 20 µl of reaction mixture was prepared containing 100 mM potassium phosphate buffer (pH 7.2), 5 mM DCVC, 10 µM PLP, and the kidney or cell supernatant. The reaction mixture was incubated at 37°C. Addition of 20 µl of 5 mM 2,4-dinitrophenylhydrazine (DNP) in 2 M HCl stopped the reaction and produced a quantifiable reaction with the pyruvate released during the reaction. The solution was incubated at 37°C for an additional 5 min; then 160 µl of 1 M KOH was added. The mixture was rapidly transferred to a 96-well plate, and the absorbance was measured at 450 nm within 2 min. The background absorbance level was determined by adding the supernatant immediately after the DNP. One unit of activity was defined as the amount of enzyme that released 1 µmol of pyruvate per min at 37°C. Protein concentrations were determined by BCA protein assay.

Inhibition of GGT and Cysteine-S-Conjugate Beta-Lyase in LLC-PK₁ Cells

To inhibit GGT activity in confluent monolayers of LLC-PK₁ cells, the medium was removed 2 h before treatment with cisplatin or its conjugates and replaced with HBSS with 5 mM HEPES, pH 7.2, containing 250 μ M acivicin. During the 3 h treatment of the cells with the cisplatin mixtures, the treatment solution also contained 250 μ M acivicin. To inhibit cysteine-S-conjugate beta-lyase activity, media was removed from the cells 30 min before treatment and replaced with HBSS with 5 mM HEPES, pH 7.2, containing 100 μ M (aminooxy-)acetic acid (AOAA) (12,27–29). During the 3 h treatment, the treatment solution also contained 100 μ M AOAA.

Statistical Analyses

All cell culture experiments were repeated at least three times. In each experiment, all points were done in triplicate. The SD from the mean was computed for each treatment. Statistically significant differences among the mean values were detected by a one-way ANOVA. A Tukey test was used to determine which mean values were significantly different from the control value (T_0 or untreated cells) (49). Statistically significant differences in cell survival due to treatment with acivicin or AOAA were detected by a *t* test.

Results

Cisplatin Toxicity: Continuous Exposure of LLC-PK₁ Cells to Cisplatin

In vivo nephrotoxicity of cisplatin is not apparent until 3 to 5 d after administration of cisplatin (50). We analyzed the viability of LLC-PK₁ cells with time during continuous exposure to cisplatin. Confluent monolayers of LLC-PK₁ cells were treated with 50 μ M or 100 μ M cisplatin. Cells that were incubated in 50 μ M cisplatin did not show any significant decrease in viability during the first 24 h of exposure (Figure

2A). However, there was a statistically significant reduction in cell survival by 48 h when compared with untreated controls ($P < 0.05$). Toxicity increased with time. At 72 h, only 5% of the cells were still alive. The toxicity was both dose- and time-dependent. Cells incubated in 100 μ M cisplatin showed significantly reduced survival by 24 h ($P < 0.05$).

Cisplatin Toxicity: 3 h Exposure of LLC-PK₁ Cells to Cisplatin

Confluent monolayers of LLC-PK₁ cells were incubated in cisplatin for 3 h. The cisplatin was removed and fresh medium was added to the cells. Cell survival was assayed with time after the cisplatin treatment (Figure 2B). As was observed with continuous exposure to cisplatin, there was time- and dose-dependent toxicity. Cells treated with 50 μ M or 100 μ M cisplatin for 3 h did not show any significant reduction in viability until 48 h ($P < 0.05$). Treatment with 150 μ M cisplatin resulted in significant toxicity within 10 h ($P < 0.05$). Continuous incubation in the presence of cisplatin was more toxic to LLC-PK₁ cells than the limited 3 h exposure (Figure 2, A and B). Exposure of the cells to 50 μ M cisplatin for 3 h resulted in a low but significant amount of toxicity at 72 h. This limited exposure to cisplatin closely mimics the *in vivo* exposure.

Enzyme Activity in LLC-PK₁ Cells

GGT activity is induced in LLC-PK₁ cells as they become confluent and form monolayers (27). With the plating conditions used for these experiments, the cells became confluent on day three, and the GGT activity then increased approximately fourfold, reaching a maximum level by day 7 (data not shown).

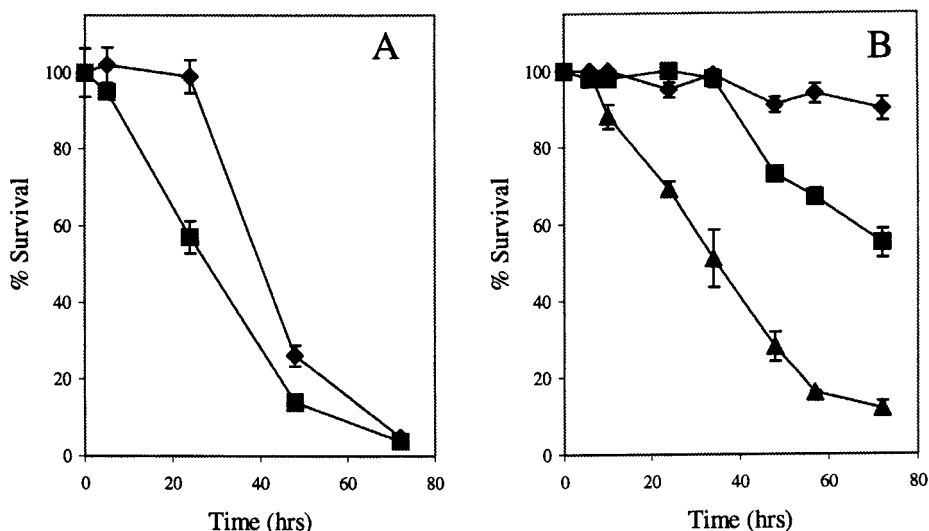


Figure 2. Survival of confluent monolayers of LLC-PK₁ cells after continuous exposure to cisplatin (A) or a 3 h pulse (B). (A) Confluent monolayers of LLC-PK₁ cells were incubated in DMEM containing 50 μ M cisplatin (diamonds) or 100 μ M cisplatin (squares). At each time point, cell survival was determined relative to untreated cells. Each point represents the mean of three points \pm SD. (B) Confluent monolayers of LLC-PK₁ cells were incubated for 3 h in a balanced salt solution containing 50 μ M cisplatin (diamonds), 100 μ M cisplatin (squares), or 150 μ M cisplatin (triangles). The cisplatin was removed at the end of the 3 h exposure and fresh medium added to the cells. At each time point, cell survival was determined relative to the number of viable cells in control wells. Each point represents the average of three means \pm SEM.

The specific activity of GGT in the LLC-PK₁ cells on day 7 is shown in Table 1. A comparison of GGT activity in LLC-PK₁ cells with the activity in mouse kidney showed that the LLC-PK₁ cells have 74% of the level of activity in the kidney. Aminopeptidase N activity was also assayed in both LLC-PK₁ cells and mouse kidney. The specific activity of aminopeptidase N activity was 1.4-fold higher in the LLC-PK₁ cells than the kidney. The largest difference in enzyme activity between LLC-PK₁ cells and kidney was the level of cysteine-S-conjugate beta-lyase activity. The cysteine-S-conjugate beta-lyase activity was measured in low-speed supernatants that included both cytosol and mitochondria. LLC-PK₁ cells had only 16% of the activity measured in mouse kidney. These data demonstrate that all of the enzymes in the proposed pathway are expressed in LLC-PK₁ cells, although the cysteine-S-conjugate beta-lyase activity is lower than in the kidney.

Potential of Cisplatin Toxicity: Formation of Toxic Cisplatin Derivatives

Preincubating cisplatin with equimolar glutathione for 30 min at 37°C significantly increased the toxicity of cisplatin (Figure 3). Confluent monolayers of LLC-PK₁ cells were exposed to 50 μM cisplatin-glutathione or 50 μM cisplatin. When assayed for cell viability at 72 h, the data show that 50 μM cisplatin-glutathione killed 33% ± 2 of the cells versus 19% ± 2 of the cells killed by 50 μM cisplatin ($P < 0.05$). Preincubating cisplatin with equimolar cysteinyl-glycine or *N*-acetyl cysteine at 37°C for 30 min also potentiated the toxicity of cisplatin. Treatment for 3 h with 50 μM cisplatin-cysteinyl-glycine killed 42% ± 2 of the cells versus 19% ± 2 of the cisplatin-treated cells ($P < 0.05$). Treatment with 50 μM cisplatin-*N*-acetyl cysteine killed 41% ± 7 of the cells, significantly more than killed by cisplatin ($P < 0.05$).

Inhibition of GGT

GGT is the first enzyme in the proposed metabolic pathway for activation of cisplatin-glutathione-conjugates. We inhibited GGT activity and determined the effect of this inhibition on the toxicity of cisplatin-glutathione-conjugates and on the toxicity of the two conjugates that are downstream of the GGT reaction, the cisplatin-cysteinyl-glycine-conjugate and the cisplatin-cysteine-conjugate. Preliminary experiments showed that acivicin inhibited GGT in monolayers of LLC-PK₁ cells in a time- and dose-dependent manner (data not shown). For these studies, the cell monolayers were treated with 250 μM acivicin

Table 1. Specific activity of enzymes in confluent monolayers of LLC-PK₁ cells and in mouse kidney

Enzyme	LLC-PK ₁ Cells	Mouse Kidney
GGT	446 ± 37 ^a	603 ± 89
Aminopeptidase N	12.6 ± 0.88	9.07 ± 1.63
Cysteine-S-conjugate beta-lyase	0.244 ± 0.05	1.52 ± 0.30

^a All enzyme units are expressed as mU/mg protein ± SD.

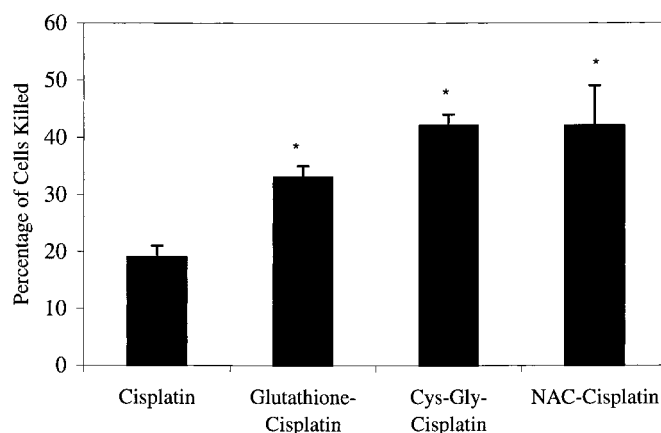


Figure 3. Toxicity of a 3 h treatment with cisplatin and cisplatin-conjugates toward confluent monolayers of LLC-PK₁ cells. LLC-PK₁ cells were exposed for 3 h to 50 μM cisplatin or 50 μM cisplatin preincubated with equimolar glutathione, cysteinyl-glycine, or *N*-acetyl-cysteine. The cisplatin solutions were removed at the end of the 3 h exposure, and fresh medium was added to the cells. At 72 h, the number of cells killed was determined as a percentage of the number of cells in control wells. Each point represents mean of three points ± SD. * differed significantly from the cisplatin-treated cells ($P < 0.05$)

for 2 h before treatment with the cisplatin mixtures. The acivicin inhibited GGT activity by 89%, from 446 ± 37 mU/mg protein to 53 ± 8 mU/mg protein. The toxicity of the cisplatin-glutathione-conjugates was dose-dependent (Table 2). A 3 h treatment of LLC-PK₁ cells with 25 μM cisplatin-glutathione killed 17% ± 5 of the cells, 50 μM killed 33% ± 4 of the cells. Inhibition of GGT reduced the total toxicity to 2% ± 5 and 14% ± 2, respectively. Inhibiting GGT activity eliminated all of the toxicity of the cisplatin-glutathione-conjugate. In the presence of acivicin, the toxicity of 50 μM cisplatin-glutathione-conjugate was less than the toxicity of 50 μM cisplatin (Figure 3). In contrast, inhibition of GGT had no significant effect on the toxicity of the cisplatin-cysteinyl-glycine-conjugate or the cisplatin-NAC-conjugate (Figure 4). These data are consistent with our hypothesis as both the

Table 2. Toxicity of a 3 h treatment with cisplatin-glutathione-conjugates towards LLC-PK₁ cells

Inhibitor	Dose of Cisplatin-Glutathione-Conjugates		
	0	25 μM	50 μM
None	0 ± 3 ^a	17 ± 5	33 ± 4
Acivicin	0 ± 1	2 ± 5 ^b	14 ± 2 ^c
AOAA	0 ± 3	0 ± 6 ^b	14 ± 6 ^c

^a Confluent monolayers of LLC-PK₁ cells were exposed to cisplatin-glutathione-conjugates for 3 h in the presence or absence of acivicin or AOAA. Toxicity was assessed at 72 h. Data are the percentage of cells killed.

^b Values differ significantly from percentage of cells killed in the absence of inhibitor; $P < 0.05$.

^c Values differ significantly from percentage of cells killed in the absence of inhibitor; $P < 0.01$.

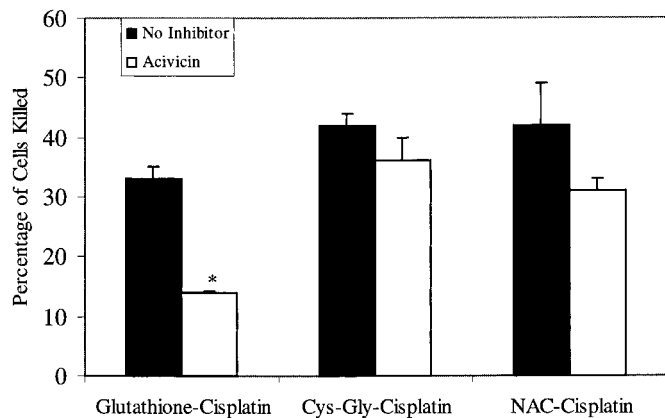


Figure 4. Effect of acivicin on the toxicity of a 3 h treatment of cisplatin-conjugates. LLC-PK₁ cells were exposed for 3 h to 50 μ M cisplatin preincubated with equimolar glutathione, cysteinyl-glycine, or N-acetyl-cysteine. Cells were either treated with no inhibitor (black bars) or were treated with acivicin (white bars) to inhibit GGT activity. The cisplatin solutions and acivicin were removed at the end of the 3 h exposure and fresh medium added to the cells. At 72 h, the number of cells killed by the cisplatin-conjugates was determined as a percentage of the number of cells in control wells. Each point represents mean of three points \pm SD. * treatment with acivicin significantly reduced the percentage of cells killed ($P < 0.001$)

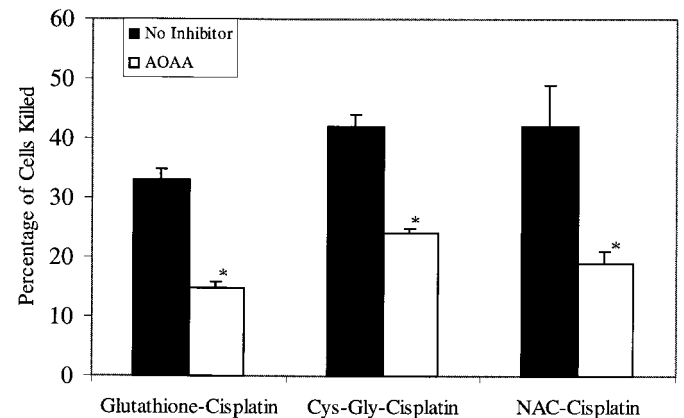


Figure 5. Effect of AOAA on the toxicity of a 3 h treatment of cisplatin-conjugates. LLC-PK₁ cells were exposed for 3 h to 50 μ M cisplatin preincubated with equimolar glutathione, cysteinyl-glycine, or N-acetyl-cysteine. Cells were either treated with no inhibitor (black bars) or were treated with AOAA (white bars) to inhibit cysteine-S-conjugate beta-lyase activity. The cisplatin solutions and AOAA were removed at the end of the 3 h exposure and fresh medium added to the cells. At 72 h, the number of cells killed was determined as a percentage of the number of cells in control wells. Each point represents mean of three points \pm SD. * treatment with AOAA significantly reduced the percentage of cells killed ($P < 0.005$)

cysteinyl-glycine-platinum-conjugate and cysteine-conjugate are downstream of the GGT reaction. Therefore inhibiting GGT should have no effect on their toxicity.

Inhibition of Cysteine-S-Conjugate Beta-Lyase

The beta-lyase reaction is the final reaction in the proposed metabolic activation of a cisplatin-glutathione-conjugate to a nephrotoxin. We inhibited cysteine-S-conjugate beta-lyase activity by pretreating the LLC-PK₁ cells with 100 μ M AOAA for 30 min before exposure to the cisplatin-conjugates (12,28,29,51). Inhibiting cysteine-S-conjugate beta-lyase significantly inhibited the toxicity of each of the cisplatin-conjugates in the proposed pathway (Figure 5). Pretreatment with AOAA reduced the toxicity of the cisplatin-glutathione-conjugate from 33% \pm 2 of LLC-PK₁ cells killed to 15% \pm 1 ($P < 0.001$). The toxicity of both the cisplatin-cysteinyl-glycine and cisplatin-cysteine-conjugate was also significantly reduced by AOAA ($P < 0.005$). These data are consistent with our hypothesis, which predicts that each of the conjugates would be metabolized to a cisplatin-cysteine-conjugate then activated by cysteine-S-conjugate beta-lyase to a nephrotoxin. Controls within these experiments showed that pretreating cells with acivicin and AOAA resulted in a small but significant inhibition of the toxicity of 50 μ M cisplatin from 25% \pm 1 of LLC-PK₁ cells killed to 16% \pm 5 ($P < 0.05$). The toxicity of cisplatin in the presence of the inhibitors is equivalent to the toxicity of the cisplatin-glutathione adducts with the inhibitors (Table 2). These data suggest that in the absence of the inhibitors the LLC-PK₁ cells are conjugating a small portion of the cisplatin to glutathione and metabolizing it through the proposed pathway.

Discussion

The development of a protocol for short-term exposure of a monolayer of kidney cells to clinically relevant concentrations of cisplatin has allowed us to test our hypothesis regarding the metabolic activation of cisplatin to a nephrotoxin. Incubating cisplatin in a balanced salt solution containing equimolar glutathione, cysteinyl-glycine or N-acetyl-cysteine increased the toxicity of the cisplatin. Cisplatin reacts spontaneously in solution with glutathione or cysteine to form cisplatin-glutathione or cisplatin-cysteine-conjugates (23,24,44). Inhibition of GGT blocked the toxicity of the cisplatin-glutathione-conjugate. These data support our hypothesis that cleavage of the cisplatin-glutathione-conjugate to a cisplatin-cysteinyl-glycine-conjugate by GGT is the first step in the metabolism of the cisplatin-glutathione-conjugate to a nephrotoxin. The inhibition of GGT had no effect on the toxicity of the cisplatin-cysteinyl-glycine-conjugate or the cisplatin-cysteine-conjugate, both of which are downstream of the GGT reaction in the proposed pathway. Inhibition of cysteine-S-conjugate beta-lyase reduced the toxicity of the cisplatin-glutathione-conjugate, the cisplatin-cysteinyl-glycine-conjugate, and the cisplatin-cysteine-conjugate. These data are also consistent with our hypothesis that cysteine-S-conjugate beta-lyase catalyzes the final step in the pathway converting the cisplatin-cysteine-conjugate to a reactive thiol.

Data from *in vivo* studies support the hypothesis that the formation of a cisplatin-glutathione-conjugate is an essential component of the nephrotoxicity of cisplatin. Cisplatin-glutathione-conjugates have been identified in rat kidneys (25). Buthionine-sulfoximine, a glutathione-depleting agent, diminished the nephrotoxic effects of cisplatin in rats when injected

2 hrs before treatment (52,53). Several studies have presented data indicating that *in vivo* the formation of cisplatin-glutathione-conjugates is catalyzed by glutathione-S-transferases. Sadzuka *et al.* treated rats with ketoprofen, an inhibitor of glutathione-S-transferase pi, before cisplatin treatment and found a significant decrease in nephrotoxicity (26). Decreased levels of glutathione or glutathione-S-transferase activity would reduce the formation of the cisplatin-glutathione-conjugate, the substrate for GGT in the first step of the proposed activation pathway. We are characterizing the cisplatin-adducts that are formed during the incubation of cisplatin with glutathione, cysteinyl-glycine, or NAC. The incubation mixtures are being analyzed by high-pressure liquid chromatography and mass spectrometry to determine the relative abundance and structure of each adduct. An abstract describing the preliminary results of those studies has been published (54).

Administration of high doses of glutathione within 30 min of cisplatin administration has been shown to protect against cisplatin-induced nephrotoxicity (55,56). The amount of glutathione that is necessary to achieve this protective effect is 83-fold higher than the cisplatin concentration. Whereas these data may appear to contradict our hypothesis, that the formation of a cisplatin-glutathione-conjugate activates cisplatin to a nephrotoxin, we have proposed that the high concentration of glutathione protects against cisplatin nephrotoxicity by serving as a competitive inhibitor of GGT activity (8). Glutathione is the major physiologic substrate for GGT (57). GGT is localized to the cell surface and would be inhibited by high levels of glutathione in the extracellular fluid. By inhibiting GGT activity, glutathione would reduce the metabolism of the cisplatin-glutathione- to a cisplatin-cysteinyl-glycine-conjugate.

Several additional studies indicate that cisplatin is activated to a nephrotoxin via metabolism through a series of intermediate-compounds. Daley-Yates and McBrien reported that within 15 min after injecting rats with a single dose of cisplatin, seven platinum-containing species were present in plasma that could be separated via HPLC (58). The mixture of platinum containing species was more nephrotoxic than cisplatin. Cisplatin-cysteine-conjugates have been identified in the kidneys of cisplatin-treated rats (25). Maines incubated cysteine and cisplatin in a 2:1 molar ratio for 30 min at 37°C (59). He reported that in the kidney the cysteine-platinum incubation mixture was a more potent inhibitor of hemometabolism and glutathione synthesis than cisplatin.

The final step in the proposed activation pathway is the metabolism of the cysteine-S-conjugate to a reactive thiol. Cysteine-S-conjugate beta-lyases catalyze a beta-elimination reaction of the halogenated alkene-cysteine-S-conjugates (60). Several proteins have been identified in kidney that can catalyze this reaction. All are PLP-dependent and are therefore inhibited by AOAA (61). Mitochondria appear to be the primary target of the cysteine-S-conjugate beta-lyase-mediated toxicity of the haloalkenes (62,63). A high-molecular weight protein complex in kidney mitochondria has been shown to have cysteine-S-conjugate beta-lyase activity (13,64). This high-molecular weight complex may contain the dominant enzyme that catalyzes the conversion of the cisplatin-cysteine-

conjugate to a reactive thiol *in vivo*. The enzyme has not yet been identified.

Possible alternative mechanisms of action by the inhibitors have been ruled out by our *in vivo* studies. In our original study of the role of GGT in cisplatin nephrotoxicity, we used acivicin to inhibit GGT *in vivo* (8). Acivicin is a glutamine analogue. It blocks L-glutamine-requiring enzymes and at high concentrations inhibits *de novo* synthesis of purine and pyrimidines (65). To determine whether acivicin affected cisplatin nephrotoxicity by inhibiting GGT or through an alternative mechanism, we assessed the nephrotoxicity of cisplatin in GGT knockout mice (7). The data from both the acivicin studies and the GGT-knockout mice showed that renal cisplatin toxicity is dependent on GGT activity. Inhibition of cysteine-S-conjugate beta-lyase with AOAA in mice demonstrated that inhibition of this enzyme blocked the nephrotoxicity of cisplatin but had no effect on the uptake of platinum into the kidney (6).

While conjugation of cisplatin to glutathione is the first step in the activation of cisplatin to a nephrotoxin, it renders cisplatin inactive as an antitumor drug. Conjugation of cisplatin with glutathione reduces the formation of interstrand and intrastrand platinum DNA adducts, resulting in decreased toxicity of cisplatin in dividing cells (66). This contradictory role of glutathione conjugation in the nephrotoxicity and antitumor activity of the drug is further confirmed by data showing that depletion of glutathione by buthionine-sulfoximine or reduction of glutathione-S-transferase activity potentiated the antitumor activity of cisplatin (66,67). Increased levels of intracellular glutathione or glutathione-S-transferase have been associated with cisplatin resistance in tumor cells (68–72). Expression of GGT also has opposing roles in the nephrotoxicity and antitumor activity of cisplatin. GGT expression is necessary for the metabolism of cisplatin to a nephrotoxin (7,8). However, GGT expression in tumors decreases the antitumor activity of the drug (73). These contradictory effects may be due to differences among tissues in the uptake of the cisplatin-cysteine-conjugates or in expression of the cysteine-conjugate beta-lyase that converts the cisplatin-cysteine-conjugate to a reactive thiol. These issues will be investigated in our future studies.

Thiol compounds are used in clinical practice to mitigate cisplatin-induced nephrotoxicity. Some thiol compounds such as diethyldithiocarbamate bind to cisplatin and inactivate it, reducing both the antitumor and nephrotoxic activity of the drug (74). We propose that these compounds block both the binding of cisplatin to DNA and the binding of cisplatin to glutathione, preventing its further metabolism to a nephrotoxin. A large number of sulfur-containing compounds have been shown to reduce the nephrotoxicity of cisplatin without inhibiting its antitumor effect (75). Some of these agents, such as the prodrug Amifostine, are used in the clinic to protect against cisplatin nephrotoxicity (76). Procainamide, an antiarrhythmic drug, also protects against the nephrotoxicity of cisplatin without altering its antitumor activity (77). The formation of the procainamide-cisplatin complex increases the amount of platinum bound to DNA, which would explain the maintenance of the antitumor activity of cisplatin in the presence of procain-

amide (78). The binding of procainamide to the cisplatin may prevent the formation of a cisplatin-glutathione complex and thereby protect against the metabolism of cisplatin to a nephrotoxin. The thiol agents may be working by the same mechanism as procainamide, forming complexes with cisplatin that do not prevent the binding of the platinum to DNA but do prevent the formation of a glutathione-cisplatin-conjugate.

In this study, we have demonstrated that preincubating cisplatin with glutathione, cysteinyl-glycine, or NAC potentiates its toxicity toward LLC-PK₁ cells. In all of these assays, the cells were incubated in HBSS buffered with HEPES during the 3 h exposure to cisplatin or cisplatin adducts. Preliminary experiments had shown that the toxicity of cisplatin is modulated if the cells are incubated in tissue culture medium during the cisplatin exposure. Cisplatin was less toxic to LLC-PK₁ cells when they were incubated in DMEM, rather than HBSS, during the 3 h cisplatin exposure. However, cisplatin was more toxic when the cells were incubated in RPMI-1640 medium during the exposure period. In a separate study, we have found that the modulation of cisplatin toxicity by the media is due to the interplay of several media components. We are analyzing the effect of each component and determining the mechanism by which it influences cisplatin toxicity. Those data will be presented in a forthcoming paper. To eliminate the confounding variables in the media, all of the studies in this paper have been done with the cells in HBSS, a balanced salt solution, during the 3 h exposure to cisplatin.

It is a common assumption that conjugation to glutathione is a detoxification mechanism; but, as has been shown for the halogenated-alkenes and now cisplatin, conjugation to glutathione is the first step in the pathway that activates some compounds to potent nephrotoxins. The data in this study further define the steps in the metabolism of cisplatin to a nephrotoxin. Delineation of this pathway provides insights into the distinct nephrotoxic and antitumor activity of cisplatin. Strategic inhibition of critical components of this pathway could reduce the nephrotoxicity of cisplatin while potentiating its antitumor effect.

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