Cisplatin-Induced Toxicity Is Associated with Platinum Deposition in Mouse Kidney Mitochondria in Vivo and with Selective Inactivation of the α-Ketoglutarate Dehydrogenase Complex in LLC-PK$_1$ Cells†

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ABSTRACT: The anticancer drug cisplatin is nephrotoxic and neurotoxic. Previous data support the hypothesis that cisplatin is bioactivated to a nephrotoxicant. The final step in the proposed bioactivation is the formation of a platinum−cysteine *S*-conjugate followed by a pyridoxal 5′-phosphate (PLP)-dependent cysteine *S*-conjugate *β*-lyase reaction. This reaction would generate pyruvate, ammonium, and a highly reactive platinum (Pt)−thiol compound in vivo that would bind to proteins. In this work, the cellular location and identity of the PLP-dependent cysteine *S*-conjugate *β*-lyase were investigated. Pt was shown to bind to proteins in kidneys of cisplatin-treated mice. The concentration of Pt-bound proteins was higher in the mitochondrial fraction than in the cytosolic fraction. Treatment of the mice with aminooxyacetic acid (AOAA, a PLP enzyme inhibitor), which had previously been shown to block the nephrotoxicity of cisplatin, decreased the binding of Pt to mitochondrial proteins but had no effect on the amount of Pt bound to proteins in the cytosolic fraction. These data indicate that a mitochondrial enzyme catalyzes the PLP-dependent cysteine *S*-conjugate *β*-lyase reaction. PLP-dependent mitochondrial aspartate aminotransferase (mitAspAT) is a mitochondrial enzyme that catalyzes *β*-elimination reactions with cysteine *S*-conjugates of halogenated alkenes. We reasoned that the enzyme might also catalyze a *β*-lyase reaction with the cisplatin−cysteine *S*-conjugate. In this study, mitAspAT was stably overexpressed in LLC-PK$_1$ cells. Cisplatin was significantly more toxic in confluent monolayers of LLC-PK$_1$ cells that overexpressed mitAspAT than in control cells containing vector alone. AOAA completely blocked the cisplatin toxicity in confluent mitAspAT-transfected cells. The Pt−thiol compound could rapidly bind proteins and inactivate enzymes in close proximity of the PLP-dependent cysteine *S*-conjugate *β*-lyase. Treatment with 50 or 100 *μ*M cisplatin for 3 h, followed by removal of cisplatin from the medium for 24 h, resulted in a pronounced loss of α-ketoglutarate dehydrogenase complex (KGDHC) activity in both mitAspAT-transfected cells and control cells. Exposure to 100 *μ*M cisplatin resulted in a significantly greater loss of KGDHC activity in the cells overexpressing mitAspAT than in control cells. Aconitase activity was diminished in both cell types, but only at the higher level of exposure to cisplatin. AspAT activity was also significantly decreased by cisplatin treatment. By contrast, several other enzymes (both cytosolic and mitochondrial) involved in energy/amino acid metabolism were not significantly affected by cisplatin treatment in the LLC-PK$_1$ cells, whether or not mitAspAT was overexpressed. The susceptibility of KGDHC and aconitase to inactivation in kidney cells exposed to cisplatin metabolites may be due to the proximity of mitAspAT to KGDHC and aconitase in mitochondria. These findings support the hypothesis that a mitochondrial cysteine *S*-conjugate *β*-lyase converts the cisplatin−cysteine *S*-conjugate to a toxicant, and the data are consistent with the hypothesis that mitAspAT plays a role in the bioactivation of cisplatin.

Cisplatin[1] has been used successfully to treat a variety of cancers, including testicular cancer, ovarian cancer, and some glioblastomas (*1*). Unfortunately, the drug cannot be administered at high doses because of its toxicity to renal proximal tubules and its neurotoxicity (*2*, *3*). Studies from many laboratories have implicated DNA damage as the primary

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[1] Abbreviations: AOAA, aminooxyacetic acid; AspAT, aspartate aminotransferase; cisplatin, cisdiamminedichloroplatinum(II); cytAspAT, cytosolic aspartate aminotransferase; DCVC, *S*-(1,2-dichlorovinyl)-L-cysteine; DMEM, Dulbecco's modified Eagle's medium; GFAAS, graphite furnace atomic absorption spectrometry; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GDH, glutamate dehydrogenase; GGT, *γ*-glutamyltranspeptidase; GTK, glutamine transaminase K; HBSS, Hanks' balanced salt solution; KGDHC, α-ketoglutarate dehydrogenase complex; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; mitAspAT, mitochondrial aspartate aminotransferase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; pmitAspAT, precursor of mitochondrial aspartate aminotransferase; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; SD, standard deviation; SEM, standard error of the mean; TFEC, *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine.

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mechanism by which cisplatin kills tumor cells and other dividing cells (*4*, *5*). However, the renal proximal tubule cells are well-differentiated, nondividing cells that are not killed by other DNA-damaging agents (*6*). Early work suggested that a metabolite of cisplatin is responsible for the nephrotoxicity (*7*). Several steps in the metabolic pathway through which cisplatin is bioactivated to a nephrotoxicant have recently been identified (*8*−*13*). In this study, we have focused on the identification of the enzyme(s) that catalyzes the final step in the metabolism of cisplatin to a nephrotoxicant.

Inhibition of either γ-glutamyltranspeptidase (GGT) or pyridoxal 5′-phosphate (PLP)-dependent enzymes blocks the nephrotoxicity of cisplatin both in vivo and in vitro (*8*−*13*). Buthionine sulfoximine, which depletes endogenous glutathione by inhibiting glutathione synthesis, also protects against cisplatin-induced nephrotoxicity (*14*). These findings indicate that cisplatin is metabolized to a nephrotoxicant through conversion of the drug to a glutathione *S*-conjugate, followed by steps that involve a GGT- and cysteine *S*-conjugate β-lyase-dependent pathway (*8*−*13*). The enzymatic steps involved in this proposed pathway for the bioactivation of cisplatin are similar to those previously established for the bioactivation of several halogenated alkenes to nephrotoxicants (*15*, *16*). Bioactivation of halogenated alkenes involves the sequential formation of the glutathione, cysteinylglycine, and cysteine *S*-conjugates. The final step in the bioactivation pathway of halogenated alkenes is a β-elimination reaction on the corresponding cysteine *S*-conjugate, catalyzed by cysteine *S*-conjugate β-lyase(s), yielding pyruvate, ammonium, and a highly reactive halogenated thiol-containing fragment. Halogenated thiol-containing fragments are rapidly converted nonenzymatically to products that thioacylate macromolecules in target cells (*17*−*19*).

Cytosolic, mitochondrial, and microsomal fractions of kidney homogenates have been shown to catalyze cysteine *S*-conjugate β-lyase reactions in vitro with halogenated cysteine *S*-conjugates (*20*−*23*). To date, at least 11 enzymes have been reported to catalyze cysteine *S*-conjugate β-lyase reactions with the nephrotoxicants *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC) and *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine (TFEC), which are the cysteine *S*-conjugates of the halogenated alkenes trichloroethylene and tetrafluoroethylene, respectively (*24*, *25*). Cysteine *S*-conjugate β-lyases are PLP-dependent enzymes that are normally involved in amino acid metabolism. However, when the halogenated cysteine *S*-conjugates serve as substrates, the strong electron-withdrawing property of the halogenated moiety bound to the sulfur causes these enzymes to catalyze a nonphysiological β-elimination reaction (*15*, *19*, *24*−*26*). The sulfur-containing fragment produced in the β-lyase reaction with the halogenated cysteine *S*-conjugates, such as DCVC and TFEC, is toxic because it acts as a thioacylating agent particularly of the ϵ-amino group of lysyl residues in proteins (*17*, *18*, *24*, *25*). Several cysteine *S*-conjugate β-lyases are present in the mitochondria, including mitochondrial aspartate aminotransferase (mitAspAT), mitochondrial branched-chain aminotransferase, and alanine-glyoxylate aminotransferase isozyme II (see the Discussion). Others are largely cytosolic, including kynureninase, glutamine transaminase K (GTK), and cytosolic branched-chain aminotransferase (*25*).

In a fashion similar to the bioactivation pathway for halogenated alkenes, the initial step in the proposed bioactivation of cisplatin involves the formation of a cisplatin−glutathione *S*-conjugate (*13*). The sulfur moiety of glutathione replaces one of the chloride ions of cisplatin (*11*, *12*). The glutathione *S*-conjugate is cleaved to a cisplatin−cysteinylglycine *S*-conjugate by GGT, which is highly expressed on the surface of proximal tubule cells (*27*). The cysteinyl−glycine *S*-conjugate is further metabolized to a cisplatin−cysteine *S*-conjugate by aminopeptidases, which are also present on the surface of proximal tubule cells (*28*). Both the GGT- and aminopeptidase-catalyzed reactions take place extracellularly.

We hypothesize that the cisplatin−cysteine *S*-conjugate is transported into the cells, where, because of the strong electron-withdrawing property of the Pt−S moiety, it is converted by cysteine *S*-conjugate β-lyase(s) to pyruvate, ammonium, and a reactive Pt−thiol compound [$(NH_2)_2$Pt(Cl)SH] (eq 1).

$$(H_2N)_2Pt(Cl)SCH_2CH(NH_3^+)CO_2^- + H_2O \rightarrow CH_3C(O)CO_2^- + NH_4^+ + (NH_2)_2Pt(Cl)SH \quad (1)$$

We further hypothesize that this highly reactive Pt−thiol compound rapidly binds to the sulfhydryl moieties of adjacent proteins, thereby suggesting the cellular location of the PLP-dependent cysteine *S*-conjugate β-lyase that metabolizes the cysteine *S*-conjugate of cisplatin.

To test our hypothesis, mice were treated with cisplatin with or without AOAA, a PLP enzyme inhibitor that blocks cysteine *S*-conjugate β-lyase activity and which was previously shown to protect renal proximal tubule cells against cisplatin toxicity both in vivo and in vitro (*10*, *11*). We asked whether AOAA reduced the level of Pt bound to proteins in the mitochondria and/or cytosol of the kidney. After finding that AOAA reduced the level of Pt bound to the mitochondrial protein, we investigated whether mitAspAT [an enzyme previously shown to possess cysteine *S*-conjugate β-lyase activity (*29*)] could catalyze the bioactivation of cisplatin. mitAspAT was transfected into LLC-PK_1 cells, and its effect on cisplatin toxicity was evaluated in both dividing and confluent cells. Finally, we assayed specific cytosolic and mitochondrial enzymes of energy/amino acid metabolism to determine which, if any, are disrupted in LLC-PK_1 cells exposed to cisplatin. The results of these studies are reported here.

EXPERIMENTAL PROCEDURES

Reagents and Enzymes. Pig heart malate dehydrogenase (MDH) (910 units/mg, 5.6 mg/mL), cisplatin, G418, ammediol (2-amino-2-methyl-1,3-propanediol), AOAA, Trizma base (Tris), HEPES, EDTA, EGTA, 2,4-dinitrophenylhydrazine, NADH, NADPH, NAD^+, ADP, L-phenylalanine, trypsin, dithiothreitol, sodium thiomalate, ferrous ammonium sulfate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, thiazolyl blue tetrazolium bromide), bovine serum albumin, cytochrome *c* (horse heart type VI), leupeptin, the monosodium salts of L-aspartate and L-glutamate, and the sodium salts of pyruvate, α-keto-γ-methiolbutyrate, and α-ketoglutarate were obtained from Sigma Chemical Co. (St. Louis, MO). Hank's balanced salt solution (HBSS) was

from GIBCO/BRL. Rabbit anti-rat liver mitAspAT whole serum was a generous gift from A. Iriarte (University of Missouri, Kansas City, MO). TFEC synthesized by the method of Hayden and Stevens (*30*) was a generous gift from S. Bruschi (University of Washington, Seattle, WA). DCVC was synthesized by the method of McKinney et al. (*31*).

Animals. Male C57BL/6 mice (6−8 weeks old) were purchased from Harlan (Indianapolis, IN). Animals were housed in cages in the Animal Resource Facilities at the University of Oklahoma Health Sciences Center (OUHSC). Food and water were provided ad libitum. All treatment protocols were approved by the OUHSC IACUC Committee.

Treatment of Mice with Cisplatin and AOAA. Mice were treated with cisplatin and AOAA according to the same treatment protocol used in previous studies in which AOAA protected against cisplatin-induced renal toxicity (*10*). AOAA has been shown to inhibit kidney cysteine *S*-conjugate β-lyase activity in vivo (*32*). The AOAA treatment protocol used in this study has been used in mice by other investigators to block the nephrotoxicity of hexachloro-1,3-butadiene, a halogenated alkene that is metabolized to a nephrotoxicant by cysteine *S*-conjugate β-lyase (*33*). Cisplatin was prepared as a 1 mg/mL (3.3 mM) stock solution in 0.9% (w/v) NaCl, sterilized by filtration through a 0.22 μm filter. Within 30 min of treatment, AOAA was dissolved in saline at a concentration of 10 mg/mL (110 mM) and sterilized by being filtered through a 0.22 μm filter. The mice were divided into two treatment groups. Animals in group 1, the cisplatin treatment group, were each administered three doses of saline (10 μL/g of body weight) via oral gavage 1 h before, 10 min before, and 5 h after 15 mg of cisplatin/kg of body weight was injected intraperitoneally. Animals in group 2, the AOAA−cisplatin treatment group, were each treated with three doses of AOAA (100 mg of AOAA/kg of body weight) via oral gavage 1 h before, 10 min before, and 5 h after 15 mg of cisplatin/kg of body weight was injected intraperitoneally. Twenty-four hours after cisplatin treatment, the mice were weighed and sacrificed by CO_2 asphyxiation. The kidneys were removed, weighed, and used immediately for isolation of subcellular fractions.

Isolation of Subcellular Fractions from Mouse Kidney. Fractionation of mouse kidneys was carried out according to published methods (*34*, *35*). Briefly, the tissue was homogenized in ice-cold isolation medium [0.3 M sucrose and 25 mM Tris-HCl (pH 7.3)] with a Dounce homogenizer. The homogenate was centrifuged at 500*g* for 15 min. The supernatant was collected and centrifuged at 9000*g* for 15 min. The 9000*g* pellet was rinsed by being resuspended in isolation medium followed by centrifugation at 10000*g* for 15 min. The washed pellet was resuspended in isolation medium and designated M1. The 9000*g* supernatant was centrifuged at 100000*g* for 1 h. The 100000*g* supernatant was the cytosolic fraction. The 100000*g* pellet was resuspended in isolation medium and designated M2. The fractions were stored at −80 °C.

Assays of Marker Enzymes in Subcellular Fractions Prepared from Mouse Kidney. Lactate dehydrogenase (LDH), glutamate dehydrogenase (GDH), and NADPH-linked cytochrome *c* reductase served as markers for cytosol, mitochondria, and microsomes, respectively. LDH and GDH were assayed by continuously monitoring the disappearance of NADH at 340 nm (NADH $\epsilon_{340} = 6230\ M^{-1}\ cm^{-1}$) (*36*). NADPH-linked cytochrome *c* reductase was assayed by a modification of the method of Srinivas et al. (*37*). The reaction mixture (0.2 mL) contained 100 mM Tris-HCl buffer (pH 8.0), 6 μL of cytochrome *c* (20 mg/mL in distilled water), 0.1 mM NADPH, and enzyme. In preliminary experiments in which NADPH was omitted, there was no change in the residual absorbance at 340 nm. Therefore, the blank used routinely to measure the activity of NADPH-linked cytochrome *c* reductase lacked cytochrome *c*. The disappearance of NADPH was continuously monitored at 340 nm (NADPH $\epsilon_{340} = 6230\ M^{-1}\ cm^{-1}$). All three assays were carried out at 37 °C in a SpectraMax 96-well plate spectrophotometer (Molecular Devices, Sunnyvale, CA).

Quantitation of Protein-Bound Pt in Mouse Kidney Fractions. Proteins in the M1, M2, and cytosolic kidney fractions were precipitated with 10% (w/v) trichloroacetic acid for 15 min at 4 °C and pelleted by centrifugation at 13000*g* for 3 min. The pellets were washed once with ice-cold acetone and digested overnight at room temperature in 7.5 M nitric acid. The digested samples were diluted with water to 3.25 M nitric acid, and the amount of Pt bound to proteins was quantified by graphite furnace atomic absorption spectrometry (GFAAS) with a Varian SpectrAA-220Z graphite furnace double-beam atomic absorption spectrophotometer with Zeeman background correction. The lamp current was 10 mA; the wavelength was 265.9 nm, and the slit width was 0.5 nm. Pt standards in 10% HCl (SPEX CertiPrep, Metuchen, NJ) were diluted with 3.25 M nitric acid. Each sample was measured in triplicate and calibrated relative to Pt standards that were measured with each set of samples. Hot injection was performed at 85 °C, and the injection volume was 15 μL. The furnace program consisted of four phases: drying (85 °C for 5 s, 95 °C for 32 s, and 120 °C for 10 s), ashing (1200 °C for 17 s), atomization (2700 °C for 3.5 s), and cleaning (2800 °C for 3 s).

Transfection of LLC-PK$_1$ Cells with Rat Liver mitAspAT cDNA. The cDNA for the rat liver precursor of mitAspAT (pmitAspAT) was a generous gift from A. Iriarte (*38*, *39*). The precursor contains the N-terminal 29-amino acid peptide that targets the protein for translocation into mitochondria (*38*). The targeting sequence is removed during import to form the mature mitAspAT. The cDNA of pmitAspAT was subcloned into the mammalian pcDNA3.1(+) expression vector (Invitrogen, Carlsbad, CA) through *Hin*dIII and *Bam*HI sites. Cell line LLC-PK$_1$ (ATCC CRL 1392), a pig kidney proximal tubule cell line, was obtained from 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. The pmitAspAT cDNA was transfected into LLC-PK$_1$ cells with the calcium phosphate eukaryotic transfection kit (Stratagene, La Jolla, CA) as previously described (*40*). More than 1000 colonies grew in the presence of 2 mg/mL G418. Six colonies were picked and grown into individual cell lines. The AspAT activity of each cell line was measured, and the cell line that exhibited the highest AspAT activity was chosen for the studies presented herein and named LLC-PK$_1$/mitAspAT. The LLC-PK$_1$/mitAspAT cell line maintained the same growth rate as the untransfected LLC-PK$_1$ cells. The pcDNA3.1(+) vector-transfected cell

line (LLC-PK_1/C1) from previous studies was used as the control (*40*). The two cell lines were maintained in DMEM containing 5% FBS and 400 μg/mL G418.

Western Blot Analysis. Cell lysates of LLC-PK_1/mitAspAT and LLC-PK_1/C1 cells were used for SDS−polyacrylamide gel electrophoresis and Western blot analysis as previously described (*40*). Rabbit anti-rat liver mitAspAT whole serum, generously provided by A. Iriarte, was used as the primary antibody at a 1:100000 dilution (*38*, *39*, *41*, *42*).

Cisplatin Toxicity in Confluent LLC-PK_1/mitAspAT Cells and LLC-PK_1/C1 Cells. The toxicity of cisplatin toward LLC-PK_1 cells was determined as described previously (*40*). Briefly, LLC-PK_1/mitAspAT and LLC-PK_1/C1 cells were seeded in 96-well plates at a density of 10^4 cells/well in DMEM 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 diluted in DMEM. As in our previous studies, serum and antibiotics were not included in the medium during the treatment (*40*). The cisplatin stock solution was diluted in DMEM within 30 min of addition to the cells. The cells were incubated in the cisplatin-containing medium for 3 h at 37 °C in 5% CO_2. Cells incubated in DMEM alone served as controls. After 3 h, the medium was removed and replaced with DMEM containing 5% FBS and 400 μg/mL G418. The cells were incubated at 37 °C in 5% CO_2 for an additional 69 h. The number of viable cells was determined by the MTT assay which utilizes dehydrogenase activity in intact cells to assess the number of living cells (*43*). For all experiments, the cells were observed immediately before the MTT assay was started to ensure that MTT data correlated with our observation of cell toxicity.

Treatment of Confluent LLC-PK_1/mitAspAT Cells and LLC-PK_1/C1 Cells with AOAA and Cisplatin. Treatment of the cells with AOAA was carried out by a modification of a previously published method (*11*). After 7 days in culture, a stock of 10 mM AOAA in DMEM was added at various concentrations to the cell medium. The cells were then incubated at 37 °C in 5% CO_2 for 30 min. The medium was removed and replaced with DMEM containing no cisplatin or 120 μM cisplatin and the concentration of AOAA used in the preincubation. The cells were incubated at 37 °C for an additional 3 h. At the end of the 3 h incubation, the medium was replaced with DMEM containing 5% FBS and 400 μg/mL G418. The cells were incubated at 37 °C in 5% CO_2 for an additional 69 h. The number of viable cells was determined by the MTT assay (*43*).

Cisplatin Toxicity toward Dividing Cells. LLC-PK_1/mitAspAT and LLC-PK_1/C1 cells were seeded in 96-well plates at a density of 5×10^3 cells/well in DMEM containing 5% FBS and 400 μg/mL G418. The next day, the medium was removed and replaced with cisplatin diluted in DMEM. The stock solution of cisplatin was diluted in DMEM within 30 min of addition to the cells. The cells were incubated in the cisplatin-containing medium for 3 h at 37 °C in 5% CO_2. Cells incubated in DMEM alone served as controls. At the end of the incubation, the medium was removed and replaced with DMEM containing 5% FBS and 400 μg/mL G418. The cells were then incubated at 37 °C in 5% CO_2 for an additional 69 h. The number of viable cells was determined by the MTT assay (*43*).

Preparation of LLC-PK_1 Cell Lysates for Enzyme Measurements. LLC-PK_1/C1 and LLC-PK_1/mitAspAT cells were seeded in P100 tissue culture plates at a density of 10^6 cells/plate. On day 4, the cells reached confluence and the medium was changed. On day 7, the medium was removed and replaced with 10 mL of incubation buffer [HBSS with 5 mM HEPES (pH 7.2)] containing 50 μM cisplatin, 100 μM cisplatin, or no cisplatin (control), and the mixtures were incubated for 3 h at 37 °C in an air incubator. At the end of the 3 h incubation, all HBSS solutions were replaced with DMEM containing 5% FBS and 400 μg/mL G418. The cells were then incubated at 37 °C in 5% CO_2 for 24 h. After the 24 h incubation, the cells were detached from the plates by trypsinization and resuspended in 600 μL of lysis buffer [50 mM Tris-HCl, 5 mM sodium citrate, 0.6 mM magnesium chloride, 1 mM dithiothreitol, 0.2 mM EGTA, 0.08% (v/v) Triton X-100, and 50 μM leupeptin (pH 7.4) (*44*)]. For measurement of aconitase activity, the enzyme was activated by incubating the lysate for 30 min at 37 °C in a mixture containing 20 mM sodium thiomalate and 4 mM ferrous ammonium sulfate (*44*). Freezing the lysate resulted in variable recoveries of KGDHC, aconitase, glutamine transaminase K (GTK), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activities. Therefore, the activities of these enzymes (and TFEC β-lyase activity) were determined immediately after suspension of the cells in the lysis buffer. AspAT, GDH, LDH, and MDH activities are stable to freezing and thawing and were assayed in lysates that had previously been stored at −80 °C.

Enzyme Measurements in LLC-PK_1 Cell Lysates. The total AspAT activity [cytosolic (cytAspAT) activity plus mitochondrial AspAT (mitAspAT) activity] was assayed as described by Cooper et al. (*29*). The reaction mixture (final volume of 0.2 mL) contained 100 mM HEPES (pH 7.0), 10 mM L-aspartate, 6 mM α-ketoglutarate, 0.1 mM NADH, and 0.6 μg of MDH. The reaction was initiated by addition of a solution containing AspAT (1−10 μL). The disappearance of absorbance of NADH at 340 nm was continuously monitored. Blanks lacked aspartate. To distinguish between cytosolic and mitochondrial isozymes, mitAspAT was inactivated by heating for 10 min at 70 °C in the presence of 4 mM α-ketoglutarate (*45*). Under these conditions, cytAspAT is stable. In cells overexpressing mitAspAT, the cell lysate was diluted 25-fold in distilled water before the assay of AspAT activity.

GTK activity was measured by the procedure of Cooper (*46*) modified for 96-well plate analysis. The assay procedure makes use of GTK as a freely reversible glutamine (methionine) aromatic aminotransferase (*46*, *47*). The reaction mixture (50 μL) contained 5 mM α-keto-γ-methiolbutyrate, 10 mM L-phenylalanine, 100 mM ammediol buffer (pH 9.0), and enzyme. After the mixture had been incubated at 37 °C, the reaction was terminated by the addition of 150 μL of 1 M NaOH. The absorbance was read within 2 min at 320 nm (phenylpyruvate enol $\epsilon_{320} = 16000\ M^{-1}\ cm^{-1}$). The blank contained the complete reaction mixture to which enzyme was added immediately prior to addition of NaOH. The reaction is linear for at least 2 h, provided no more than 25 nmol of α-keto-γ-methiolbutyrate is transaminated.

Cysteine *S*-conjugate β-lyase activity with TFEC as the β-lyase substrate was measured by the procedure of

Table 1: Specific Activities of Marker Enzymes in Subcellular Fractions Isolated from Kidneys of Mice Treated with Cisplatin and Saline or with Cisplatin and Aminooxyacetic Acid (AOAA)[a]

	cytosol	M1	M2
cisplatin and saline			
lactate dehydrogenase	195 ± 25	13.6 ± 1.7	15.1 ± 2.1
glutamate dehydrogenase	0.70 ± 0.03	47.4 ± 1.2	33.2 ± 3.9
cytochrome *c* reductase	1.86 ± 0.10	2.14 ± 0.66	14.8 ± 0.9
cisplatin and AOAA			
lactate dehydrogenase	224 ± 20	16.4 ± 2.1	23.6 ± 0.8
glutamate dehydrogenase	1.44 ± 0.21	33.7 ± 5.5	53.0 ± 6.5
cytochrome *c* reductase	2.86 ± 0.14	4.25 ± 0.32	22.2 ± 2.2

[a] All enzyme specific activities are expressed as mU/mg of protein. $n = 4$ for all determinations. Data are reported as the mean ± SEM.

Cooper and Pinto (*48*). α-Ketoglutarate and α-keto-γ-methiolbutyrate were included in the reaction mixture to prevent accumulation of the pyridoxamine 5′-phosphate (PMP) form of the enzyme which cannot support a β-lyase reaction (*48*). Cysteine *S*-conjugate β-lyase activity with DCVC as the β-lyase substrate was determined in the presence of PLP as previously described (*40*).

Levels of GDH and LDH were measured in the isolated cells as described above for analysis of these enzymes in subcellular fractions. GAPDH was assayed by the method of Cooper et al. (*49*) except that the volume of the assay mixtures was reduced from 1.0 to 0.2 mL. The activity of MDH was assayed according to the method of Park et al. (*36*). The disappearance of NADH (for measurement of MDH activity) or the appearance of NADH (for the measurement of GAPDH activity) was continuously recorded at 340 nm. Aconitase and KGDHC activities were determined by continuous fluorometric procedures coupled to NAD^+/$NADP^+$ reduction (*44*).

All enzyme assays, except those of KGDHC and aconitase, were carried out at 37 °C in a SpectraMax 96-well plate spectrophotometer (Molecular Devices). Aconitase and KGDHC assays were carried out at 30 °C in a SpectraMax 96-well plate fluorometer (Molecular Devices). Each enzyme was assayed in triplicate for each cell harvest (*44*).

Protein and Specific Activity Measurements. Protein concentrations were determined using a micro-Biuret assay kit from Sigma or by the BCA protein assay (Pierce, Rockford, IL). In both cases, bovine serum albumin was used as a standard. For all enzyme activities reported here, 1 unit of enzyme activity is defined as the amount that catalyzes the formation of 1 μmol of product/min. Enzyme specific activity is defined as milliunits (mU) per milligram of protein.

Data Analyses. Significant differences in the levels of protein-bound Pt between AOAA-treated mice and controls were detected with two-tailed *t*-tests (Prism, GraphPad Software, Inc., San Diego, CA). In each cell culture toxicity experiment, all determinations were made in triplicate. Each experiment was repeated at least three times. The mean and standard deviation (SD) were computed for each treatment. LD_{50} and its 95% confidence intervals were calculated with a Prism sigmoidal dose−response (variable slope) curve fit. The two-tailed *t*-test was used to detect significant differences in cisplatin toxicity among the cell lines and to detect changes in enzyme activities in LLC-PK_1 cells overexpressing mit-AspAT. The effects of cisplatin treatment on specific activities of selected enzymes in the cell lysates were analyzed by a one-way ANOVA test using SPSS version 11.5 for Windows. Differences were considered significant if $p \leq 0.05$.

RESULTS

Effect of AOAA on Levels of Protein-Bound Pt in Kidney Subcellular Fractions. Mice were treated with cisplatin, and the distribution of protein-bound Pt in subcellular fractions of the kidney was analyzed. To identify the subcellular location of the cysteine *S*-conjugate β-lyase(s) that activates cisplatin to a reactive thiol, half of the mice were treated with cisplatin and the other half were treated with cisplatin and AOAA. Treatment with AOAA blocks the action of cysteine *S*-conjugate β-lyases and reduces the binding of Pt in the subcellular fractions that contain these enzymes. The kidneys from the cisplatin-treated mice were homogenized and fractionated into three subcellular fractions. The purity of these subcellular fractions was determined by assaying each fraction for LDH-, GDH-, and NADPH-linked cytochrome *c* reductase, which are predominantly localized in the cytosol, mitochondria, and microsomes, respectively. These markers have high specific activities and are not subject to inactivation by AOAA or by freezing and thawing (data not shown). Table 1 shows that the cytosolic fractions were highly enriched with the cytosolic marker LDH and contained low levels of GDH (mitochondrial marker enzyme) or cytochrome *c* reductase (microsomal marker enzyme). The low specific activity of GDH in the cytosolic fraction indicates that minimal rupture of mitochondria occurred during isolation. The M1 fraction was enriched with mitochondria as evidenced by the high specific activity of GDH and low relative specific activities of LDH and cytochrome *c* reductase. The M2 fraction was enriched with microsomes as shown by the high specific activity of NADPH-linked cytochrome *c* reductase in the M2 fraction compared to those in the M1 and cytosolic fractions. The M2 fractions also contained mitochondria, more in the AOAA-treated mice than the saline-treated controls, as evidenced by the relatively high specific activity of GDH in that fraction.

The amount of Pt bound to protein in each of the fractions is shown in Figure 1. In mice treated with cisplatin alone, the concentration of Pt bound to protein was significantly higher in the mitochondrial fraction (M1) than in the cytosolic fraction [67.3 ± 4.0 (SD) ng of Pt/mg of protein vs 32.1 ± 7.6 ng of Pt/mg of protein, $n = 3$, $p = 0.0058$]. The level of Pt bound to protein in the mitochondrial fraction was also higher than that in the M2 fraction, although the difference was not statistically significant, which may be due to the contamination of the microsomal fraction with

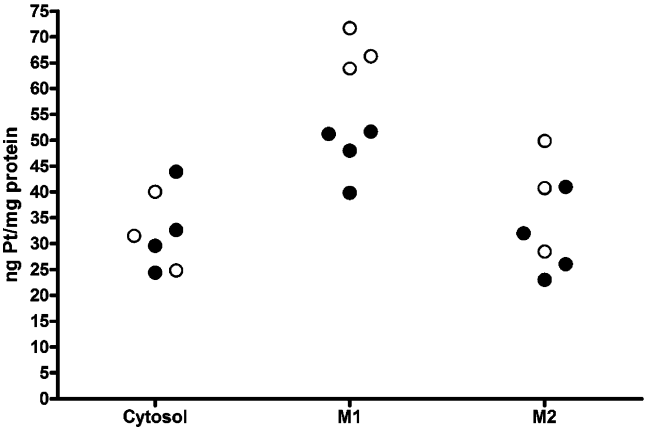

FIGURE 1: Effect of AOAA on binding of Pt to protein in kidneys from mice treated with cisplatin. Mice were pretreated with saline (○) or AOAA (●) followed by an injection of 15 mg of cisplatin/kg. Twenty-four hours after cisplatin treatment, kidney fractions were isolated and the protein in each fraction was precipitated with acid. The amount of Pt bound to protein was measured with GFAAS. There was significantly more Pt bound to protein in the M1 fraction than in the cytosol. AOAA had no effect on the amount of Pt bound to proteins in the cytosol but significantly inhibited the binding to proteins in the M1 fraction. Treatment with AOAA did not significantly reduce the amount of Pt bound to protein in the M2 fraction.

mitochondria (67.3 ± 4.0 and 39.7 ± 10.7, respectively; $p = 0.053$). In mice pretreated with AOAA, there was no significant change in the concentration of Pt bound to protein in the cytosolic fraction (32.1 ± 7.6 and 32.6 ± 8.3, respectively; $n = 4$, $p = 0.93$). However, inhibition of the cysteine *S*-conjugate β-lyase reaction with AOAA significantly decreased the amount of Pt bound to protein in the M1 fraction (67.3 ± 4.0 vs 47.7 ± 5.5; $p = 0.0055$). There was a small, but not significant, reduction in the concentration of Pt bound to protein in the M2 fraction in mice treated with AOAA (39.7 ± 10.7 vs 30.5 ± 7.9; $p = 0.03$). This reduction may be due to the mitochondrial proteins present in the M2 fraction.

In summary, the data show that in the kidney of cisplatin-treated mice, the highest concentration of Pt bound to protein was in the mitochondrial fraction. Inhibition of the nephrotoxicity of cisplatin with AOAA was associated with a significantly decreased concentration of Pt bound to protein in the mitochondria but not in the cytosol or M2 fraction. These data suggest that the cysteine *S*-conjugate β-lyase that metabolizes the cisplatin−cysteine *S*-conjugate to a nephrotoxicant is located in the mitochondria.

Expression of mitAspAT in LLC-PK$_1$/mitAspAT Cells. LLC-PK$_1$ cells were transfected with the cDNA for rat liver mitAspAT, a mitochondrial enzyme with cysteine *S*-conjugate β-lyase activity (*29*). Individual colonies were grown in the presence of G418. A subline that overexpressed mitAspAT was named LLC-PK$_1$/mitAspAT and used for all subsequent studies. A subline of LLC-PK$_1$ cells stably transfected with an empty transfection vector served as a control and was named LLC-PK$_1$/C1. Rat liver mitAspAT is a homodimer with a subunit molecular mass of 45 kDa (*38*, *39*). Expression of rat liver mitAspAT was detected by Western blot analysis in the LLC-PK$_1$/mitAspAT cells (Figure 2, lane 2). The band detected by the anti-rat liver mitAspAT antibody was 45 kDa, which demonstrated that rat liver mitAspAT was correctly processed to mature protein

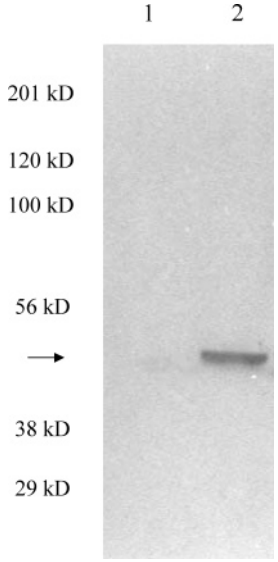

FIGURE 2: Western blot of LLC-PK$_1$/mitAspAT and LLC-PK$_1$/C1 cell lysates. LLC-PK$_1$/C1 (lane 1) or LLC-PK$_1$/mitAspAT (lane 2) cell lysate was loaded in each lane (15 μg of protein per lane). Rabbit anti-rat liver mitAspAT whole serum was used as the primary antibody. A prominent protein band with a molecular mass of ~45 kDa (arrow) was detected in the LLC-PK$_1$/mitAspAT cell line, but not in the LLC-PK$_1$/C1 cell line. The molecular mass of this band is consistent with that of the mitAspAT monomer. The positions of molecular mass markers are indicated.

Table 2: Specific Activities of AspAT and Cysteine *S*-Conjugate β-Lyase(s) (DCVC as Substrate) in LLC-PK$_1$ Cells Transfected with mitAspAT[a]

	LLC-PK$_1$/C1 (control)	LLC-PK$_1$/mitAspAT
total AspAT[b]	341 ± 20 (8)	7240 ± 509 (8)[c]
cytosolic AspAT	170 ± 14 (5)	212 ± 28 (5)
cysteine *S*-conjugate β-lyase	<0.2[d] (6)	25.0 ± 0.7[e] (6)

[a] All enzyme specific activities are expressed as mU/mg of protein. Values in parentheses represent the number of separate cell cultures that were analyzed. Data are reported as means ± SEM. [b] Total AspAT is the sum of mitAspAT and cytAspAT. [c] Significantly different from the control value with a p of <0.04. [d] Below the limit of detection. [e] Significantly different from the control value with a p of <0.001.

in the LLC-PK$_1$ cells. The anti-rat liver mitAspAT antibody did not bind to any proteins in the LLC-PK$_1$/C1 cell line, which indicated that the antibody did not recognize porcine kidney AspAT (Figure 2, lane 1).

Quantitation of total AspAT activity (i.e., mitAspAT plus cytAspAT) shows a 21-fold increase in specific activity in the LLC-PK$_1$/mitAspAT cells compared with the control cell line (Table 2, $p < 0.001$). As shown in Table 2, ~50% of the total AspAT activity in the control cells is due to mitAspAT. Therefore, the increase in mitAspAT specific activity in LLC-PK$_1$/mitAspAT cells relative to control cells is more than 40-fold.

The increase in the specific activity of mitAspAT in the LLC-PK$_1$/mitAspAT cells was accompanied by a large increase in cysteine *S*-conjugate β-lyase specific activity when either DCVC or TFEC was used as a β-lyase substrate (Tables 2 and 3). This result is consistent with our previous findings that rat liver mitAspAT catalyzes a β-lyase reaction with TFEC or DCVC as the substrate (*29*). In the control cells, cysteine *S*-conjugate β-lyase activity toward DCVC was too low to be detected (Table 2). However, a low level

Table 3: Specific Activities (mU/mg) of GTK and Cysteine *S*-Conjugate β-Lyase(s) (TFEC as Substrate) in LLC-PK$_1$ Cells Transfected with mitAspAT and Treated with Cisplatin

		specific activity[a]	
	treatment	LLC-PK$_1$/C1 (control)	LLC-PK$_1$/ mitAspAT
cysteine *S*-conjugate β-lyase	none	1.25 ± 0.21 (4)	16.0 ± 1.6 (4)[b]
	50 μM cisplatin	1.75 ± 0.09 (3)	14.4 ± 0.5 (3)[c]
	100 μM cisplatin	0.96, 2.0 (2)	19.2, 20.6 (2)
GTK	none	0.73 ± 0.11 (4)	1.08 ± 0.16 (5)
	50 μM cisplatin	0.90 ± 0.14 (3)	0.91 ± 0.13 (3)
	100 μM cisplatin	0.69 ± 0.19 (4)	0.82 ± 0.13 (5)

[a] Specific activity in cells 24 h after a 3 h treatment with 100 μM cisplatin. Data are reported as means ± SEM. The numbers of separate cell lysates analyzed for a particular enzyme activity are given in parentheses. [b] Significantly different from that of the LLC-PK$_1$/C1 cells with a p of <0.01. [c] Significantly different from that of the LLC-PK$_1$/C1 cells with a p of <0.05.

of endogenous activity could be detected with TFEC as a substrate (Table 3). Overexpression of mitAspAT resulted in a 12-fold increase in the β-lyase specific activity with TFEC as a substrate.

Purified rat kidney GTK exhibits strong β-lyase activity toward TFEC in vitro (see the Discussion). The specific activity of GTK in the control LLC-PK$_1$ cells, however, is low and was not increased in the cells overexpressing mitAspAT (Table 3). This finding rules out the possibilities that (a) mitAspAT overexpression leads to the concomitant overexpression of GTK and (b) part of the increase in cysteine *S*-conjugate β-lyase activity in the cells overexpressing mitAspAT is due to an increased level of GTK. As noted for TFEC β-lyase activity, cisplatin treatment did not affect GTK activity.

Toxicity of Cisplatin in Confluent mitAspAT-Transfected Cells. Confluent monolayers of LLC-PK$_1$/mitAspAT and LLC-PK$_1$/C1 cells were treated with cisplatin. The cells transfected with mitAspAT were more sensitive to cisplatin-induced toxicity than the vector-transfected controls (Figure 3). The LD$_{50}$ of cisplatin in LLC-PK$_1$/mitAspAT cells was 126 μM with 95% confidence intervals ranging from 116 to 136 μM. The LD$_{50}$ of cisplatin in LLC-PK$_1$/C1 cells was 182 μM with 95% confidence intervals ranging from 177 to 187 μM. There was a significant difference between the LD$_{50}$ of cisplatin toward LLC-PK$_1$/mitAspAT and LLC-PK$_1$/C1 cells ($p < 0.0001$), and the slopes of the two dose curves were also significantly different ($p = 0.0033$). These data demonstrate that overexpression of mitAspAT increases cisplatin-induced toxicity in confluent monolayers of LLC-PK$_1$ cells.

Protection by AOAA against Cisplatin-Induced Toxicity in LLC-PK$_1$/mitAspAT Cells. The effect of AOAA on cisplatin toxicity was assessed in confluent monolayers of LLC-PK$_1$/mitAspAT cells (Figure 4). In the absence of AOAA, 120 μM cisplatin killed 33% of the LLC-PK$_1$/mitAspAT cells ($p < 0.05$). AOAA protected confluent LLC-PK$_1$/mitAspAT cells against cisplatin-induced toxicity ($p < 0.0001$). The protective effect was significant with 1 or 2 mM AOAA ($p < 0.0005$). Addition of 0.1−2 mM AOAA to LLC-PK$_1$/mitAspAT cells had no effect on cell viability in the absence of cisplatin ($p = 0.9$). AOAA completely

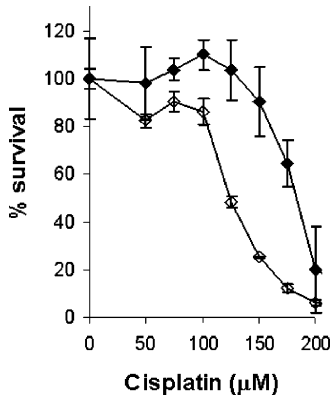

FIGURE 3: Toxicity of cisplatin in confluent LLC-PK$_1$/mitAspAT and LLC-PK$_1$/C1 cells. Confluent monolayers of LLC-PK$_1$/mitAspAT cells (◇) or LLC-PK$_1$/C1 cells (◆) were treated with cisplatin in DMEM for 3 h. The cisplatin was removed at the end of the 3 h exposure and replaced with fresh DMEM, containing 5% FBS and 400 μg/mL G418. The viability of the cells was measured at 72 h. A representative experiment is shown. Each point represents the mean of triplicate samples ± SD.

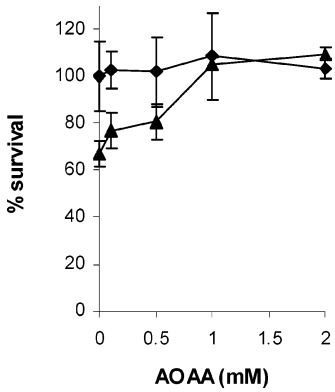

FIGURE 4: Effect of AOAA on cisplatin toxicity in confluent LLC-PK$_1$/mitAspAT cells. Confluent monolayers of LLC-PK$_1$/mitAspAT cells were preincubated with AOAA for 30 min. The cells were then treated for 3 h with DMEM containing no cisplatin (◆) or 120 μM cisplatin (▲) and the concentration of AOAA used in the preincubation. The cisplatin and AOAA were removed at the end of the 3 h exposure and replaced with fresh DMEM, containing 5% FBS and 400 μg/mL G418. The viability of the cells was measured at 72 h. The experiment was carried out three times. A representative experiment is shown. Each point represents the mean of triplicate samples ± SD.

blocked the toxicity of 120 μM cisplatin in mitAspAT-transfected cells (Figure 4).

Toxicity of Cisplatin toward Dividing LLC-PK$_1$ Cells Transfected with mitAspAT. The effect of cisplatin on survival of dividing LLC-PK$_1$/C1 and LLC-PK$_1$/mitAspAT cells is shown in Figure 5. The LD$_{50}$ of cisplatin in dividing LLC-PK$_1$/mitAspAT cells was 25.7 μM with 95% confidence intervals ranging from 21.2 to 31.1 μM. The LD$_{50}$ of cisplatin in dividing LLC-PK$_1$/C1 cells was 28.2 μM with 95% confidence intervals ranging from 22.4 to 35.4 μM. There was no significant difference between the LD$_{50}$ of the dividing LLC-PK$_1$/C1 and LLC-PK$_1$/mitAspAT cells ($p = 0.48$). The slopes of the two dose−response curves were not significantly different ($p = 0.10$). Dividing cells are more sensitive than nondividing cells to the toxicity of DNA-damaging agents such as cisplatin. Both the LLC-PK$_1$/C1 and LLC-PK$_1$/mitAspAT cells were more sensitive to cisplatin toxicity in log growth (Figure 5) than as confluent

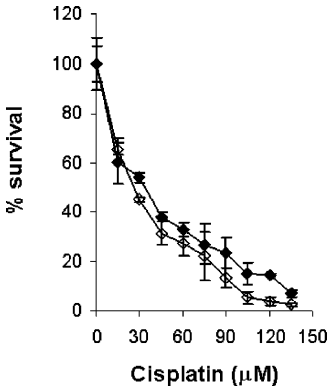

FIGURE 5: Toxicity of cisplatin in dividing LLC-PK_1/mitAspAT and LLC-PK_1/C1 cells. Dividing LLC-PK_1/mitAspAT (◇) or LLC-PK_1/C1 (◆) cells were treated with cisplatin in DMEM for 3 h. The cisplatin was removed at the end of the 3 h exposure and replaced with fresh DMEM, containing 5% FBS and 400 μg/mL G418. The viability of the cells was measured at 72 h. The experiment was carried out three times. A representative experiment is shown. Each point represents the mean of triplicate samples ± SD.

monolayers (Figure 3). The DNA damage-induced apoptosis in theses cell lines is independent of cysteine *S*-conjugate β-lyase activity.

Alteration of the Specific Activities of Some Enzymes of Energy Metabolism when mitAspAT Is Overexpressed in LLC-PK_1 Cells. The specific activities of KGDHC and aconitase in the LLC-PK_1 cells were not significantly affected by overexpression of mitAspAT (Table 4). However, the specific activity of GAPDH in cells overexpressing mitAspAT was significantly increased compared to that of control cells (24.4 ± 6.6 and 12.4 ± 1.8, respectively; $p < 0.05$). The specific activity of total MDH (mitochondrial and cytosolic MDH) was significantly decreased in cells overexpressing mitAspAT compared to controls (314 ± 44 and 486 ± 126, respectively; $p < 0.05$). The specific activity of GDH was increased (62.1 ± 19.5 vs 139 ± 65), but the *p* value did not quite reach significance ($p = 0.07$). Similar trends were observed for enzyme specific activities between LLC-PK_1/mitAspAT and LLC-PK_1/C1 cells treated with cisplatin (Table 4). Thus, overexpression of mitAspAT apparently leads to compensatory changes in the specific activities of some other enzymes of energy metabolism. These changes have to be taken into account when assessing the effects of cisplatin on the specific activities of enzymes of energy metabolism in the cells overexpressing mitAspAT.

Selective Inhibition of KGDHC in LLC-PK_1 Cells Treated with Cisplatin. The specific activities of selected enzymes of energy metabolism were measured in LLC-PK_1 cells exposed to 50 or 100 μM cisplatin for 3 h followed by a 24-h incubation in the absence of cisplatin (Table 4). The specific activities varied considerably among different experiments carried out on different days, accounting for the large SEM. Nevertheless, by comparing relative specific activities of enzymes prepared simultaneously from untreated cells and cisplatin-treated cells lysed on the same day and by using one-way ANOVA, the data in this study showed that KGDHC (a mitochondrial enzyme) is especially sensitive to inactivation by cisplatin (Figure 6). The specific activity of aconitase (another mitochondrial enzyme of energy metabolism) was not significantly affected in the cells treated with 50 μM cisplatin but was strongly inhibited in cells treated with 100 μM cisplatin (Figure 6). There was a significant decline in the specific activity of total AspAT in both the control cells and cells overexpressing mitAspAT treated with either 50 or 100 μM cisplatin. In the presence of 100 μM cisplatin, the relative decline in specific activity, however, was much smaller for total AspAT than for KGDHC and aconitase in both the control and LLC-PK_1/mitAspAT cells (Figure 6). Cisplatin had no significant effect on the specific activities of GDH (a largely mitochondrial enzyme), LDH (a cytosolic enzyme), GAPDH (a cytosolic enzyme), and total MDH in either LLC-PK_1/mitAspAT or control cells (Figure 6). Interestingly, the percent relative KGDHC value in the LLC-PK_1/mitAspAT cells treated with 100 μM cisplatin (15.4 ± 4.1) is significantly lower than that in the LLC-PK_1/C1 cells treated with 100 μM cisplatin (26.6 ± 5.9), as determined by the two-tailed paired *t*-test ($p = 0.008$).

DISCUSSION

Mechanism of Cisplatin-Induced Toxicity toward Mitochondria in Confluent Renal Cells. Our finding that the amount of Pt bound to proteins is greater in renal mitochondria of mice injected with cisplatin than in the cytosolic fraction or the microsomal fraction (Figure 1) is consistent with the previous observation that cisplatin is a mitochondrial toxicant in confluent renal proximal tubule cells (*50*). Our finding leads to the question of how Pt is incorporated into renal mitochondrial proteins. The binding of cisplatin to proteins in the cytosol may be nonenzymatic. When cisplatin enters the cell, the low intracellular chloride concentration can lead to the dissociation of one or more of the chlorides, resulting in the formation of monoaquo and diaquo complexes, *cis*-$[Pt(NH_3)_2(H_2O)Cl]^+$ and *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$, respectively. Platinum forms a coordinate covalent bond with negatively charged molecules such as the sulfur on cysteine. Direct interaction of cisplatin with protein cysteine residues would explain the platination of proteins in the cytosolic fraction in the kidneys of mice treated with cisplatin and the lack of an effect of AOAA on protein platination in this fraction (Figure 1). It is of interest that in vivo cisplatin accumulates in both the liver and the kidney (*51*). However, the accumulation in the liver is transient. In the liver, the cisplatin may be bound to glutathione and excreted from the cell. In the kidney, cisplatin or its *S*-conjugates must traverse the cytosolic compartment before entering the mitochondria. Our data show a decreased level of platination of proteins in the renal mitochondria of AOAA-treated mice but no decrease in the level of protein platination in the renal cytosol of AOAA-treated mice. Our previous studies showed that AOAA protects mice and LLC-PK_1 cells against cisplatin (*10*). AOAA forms an oxime with α-keto acids and with the coenzyme at the active site of many PLP-containing enzymes, inactivating them (*52*). The work presented here provides strong evidence that mitAspAT, acting at least in part as a β-lyase, contributes to the bioactivation of cisplatin to a renal toxicant.

Evidence that mitAspAT Is a Major Cysteine S-Conjugate β-Lyase Involved in the Bioactivation of Halogenated Cysteine S-Conjugates and the Cisplatin−Cysteine S-Conjugate in Kidney Mitochondria. There has been some debate

Table 4: Specific Activities of Several Enzymes of Energy Metabolism in LLC-PK$_1$/C1 and LLC-PK$_1$/mitAspAT Cells Exposed to Cisplatin[a]

enzyme	cells transfected with mitAspAT	no addition	50 μM cisplatin	100 μM cisplatin
total AspAT	no	341 ± 20 (8)[b,c]	247 ± 5 (6)[d]	267 ± 25 (6)[d]
	yes	7240 ± 509 (8)	6470 ± 386 (6)	5420 ± 354 (6)
KGDHC	no	10.2 ± 2.2 (10)	5.18 ± 2.52 (6)	3.41 ± 1.20 (9)[d]
	yes	12.0 ± 3.0 (10)	3.03 ± 1.1 (6)[d]	1.83 ± 0.7 (9)[d]
aconitase	no	5.62 ± 1.32 (7)	5.77 ± 0.14 (5)	2.11 ± 1.14 (4)[d]
	yes	6.99 ± 2.59 (7)	6.30 ± 0.16 (5)	1.82 ± 1.12 (4)[d]
GAPDH	no	12.4 ± 1.8 (7)	12.7 ± 2.0 (3)	9.49 ± 0.50 (5)
	yes	24.4 ± 6.6 (7)[e]	18.5 ± 3.3 (3)	14.9 ± 2.8 (5)
GDH	no	62.1 ± 19.5 (7)	70.9 ± 28.5 (5)	89.2 ± 29.4 (5)
	yes	139 ± 65 (7)	137 ± 61 (5)	164 ± 74 (5)
total MDH	no	486 ± 126 (5)	346 ± 68 (5)	336 ± 31 (3)
	yes	314 ± 44 (5)[e]	191 ± 13 (5)	225 ± 66 (3)
LDH	no	208 ± 28 (4)	197 ± 8 (3)	236 ± 70 (3)
	yes	206 ± 32 (4)	186 ± 19 (3)	185 ± 33 (3)

[a] Cells were treated with 50 or 100 μM cisplatin, and the specific activities of selected enzymes were determined. [b] Specific activity (mU/mg of protein). The data are reported as means ± SEM. [c] The numbers of separate cell lysates analyzed for a particular enzyme activity are given in parentheses. [d] Treatment with cisplatin significantly ($p < 0.05$) lowers the specific activity of the enzyme when compared with that of cells not treated with cisplatin. Relative decreases of the specific activities are shown in Figure 6. [e] The specific activity of the enzyme differs significantly ($p < 0.05$) in cells that overexpress mitAspAT vs that of control cells. The two-tailed *t*-test was used for the statistical analysis.

about which PLP-dependent enzymes are responsible for the bioactivation of halogenated cysteine *S*-conjugates in the kidney in vivo. Purified rat kidney GTK is very active in vitro as a β-lyase with several toxic halogenated cysteine *S*-conjugates (*24*, *26*, *53*), and some authors have given this enzyme the alternative name "cysteine *S*-conjugate β-lyase" as if it were the only such lyase (*54*). However, work from our laboratory and from other groups has shown that mammalian tissues possess at least ten other PLP-containing enzymes capable of catalyzing β-lyase reactions with toxic, halogenated cysteine *S*-conjugates (*24*, *25*).

Both TFEC and DCVC are mitochondrial toxicants, and renal mitochondrial proteins (but not cytosolic proteins) are thioacylated after rats are treated with pharmacological doses of TFEC (*55*−*59*). Moreover, the specific activity of cysteine *S*-conjugate β-lyase(s) capable of converting *S*-(6-guaninyl)-L-cysteine (a cysteine *S*-conjugate) to 6-mercaptoguanine is 45 times higher in a rat kidney mitochondrial fraction than in a rat kidney cytosolic fraction (*60*). These findings suggest the major involvement of mitochondrial cysteine *S*-conjugate β-lyase(s) in the bioactivation of nephrotoxic, halogenated (or prodrug) cysteine *S*-conjugates. In the rat, GTK exists in the cytosolic and mitochondrial compartments. The same gene encodes cytosolic and mitochondrial forms of GTK (*61*). Alternative splicing generates a 34-amino acid mitochondrial targeting sequence in mitGTK, but not in cytGTK (*61*). As a result, ~10% of the total GTK activity in rat kidney homogenates is in the mitochondrial fraction (*62*). Thus, this enzyme may contribute under certain conditions to bioactivation of halogenated cysteine *S*-conjugates and the cisplatin−cysteine *S*-conjugate in rat kidney mitochondria (see below).

In a recent study, we showed that cisplatin was significantly more toxic to LLC-PK$_1$ cells overexpressing human cytosolic GTK than to control LLC-PK$_1$/C1 cells (*40*). In that study, GTK specific activity was increased ~60-fold compared to that in control cells. Rooseboom et al. (*63*) have shown that both TFEC and cisplatin are much more toxic to LLC-PK$_1$ cells overexpressing rat kidney GTK than to control LLC-PK$_1$ cells. Therefore, highly overexpressed GTK can contribute significantly to the bioactivation of cysteine *S*-conjugates in LLC-PK$_1$ cells in culture. However, the results do not reveal the most likely cysteine *S*-conjugate β-lyase contributing to the in vivo mitochondrial toxicity of cisplatin in rat kidneys with normal levels of GTK. We previously showed that GTK does not contribute significantly to total cysteine *S*-conjugate β-lyase activity (with DCVC and TFEC) in isolated rat kidney mitochondria (*64*). Mitochondrial enzymes positively identified as cysteine *S*-conjugate β-lyases to date include a high-M_r β-lyase (*65*), alanine-glyoxylate aminotransferase isozyme II (*66*), mitochondrial branched-chain aminotransferase (*67*), and mitAspAT (*29*). Inasmuch as alanine-glyoxylate aminotransferase II is restricted to kidney and to a lesser extent liver (*66*), this enzyme might contribute to the heightened sensitivity of kidney (and to a lesser extent liver) mitochondria to toxic halogenated cysteine *S*-conjugates. Particularly important, however, are the high-M_r lyase and mitAspAT. The high-M_r lyase was shown to be copurified with HSP70 (*68*) and contain mitAspAT (manuscript in preparation). HSP70 is known to be important in the transport of pmitAspAT into the mitochondria (*69*).

In support of the hypothesis that mitAspAT is an important mitochondrial enzyme involved in the bioactivation of halogenated cysteine *S*-conjugates, at least 15−20% of the cysteine *S*-conjugate β-lyase activity with TFEC as the substrate in crude rat kidney mitochondria is due to mitAspAT (*29*). Moreover, the thioacylated proteins found in kidney mitochondria after rats are treated with TFEC include mitAspAT, HSP70, HSP60, the E2k and E3 subunits of KGDHC, and aconitase. This labeling pattern is entirely consistent with mitAspAT acting as a mitochondrial TFEC β-lyase. Thioacylation of mitAspAT is presumably due to release of a reactive sulfur-containing fragment at the active site of this enzyme. As noted above, the high-M_r lyase contains both HSP70 and mitAspAT, accounting for the thioacylation of HSP70. mitAspAT is thought to be part of a metabolon that also includes KGDHC and aconitase (*24*). KGDHC activity is decreased in the kidneys of TFEC-treated rats (*58*, *59*) and in TFEC-treated PC12 cells (*36*). It was proposed that the susceptibility of KGDHC to inactivation by TFEC and to its thioacylation in rats treated with TFEC

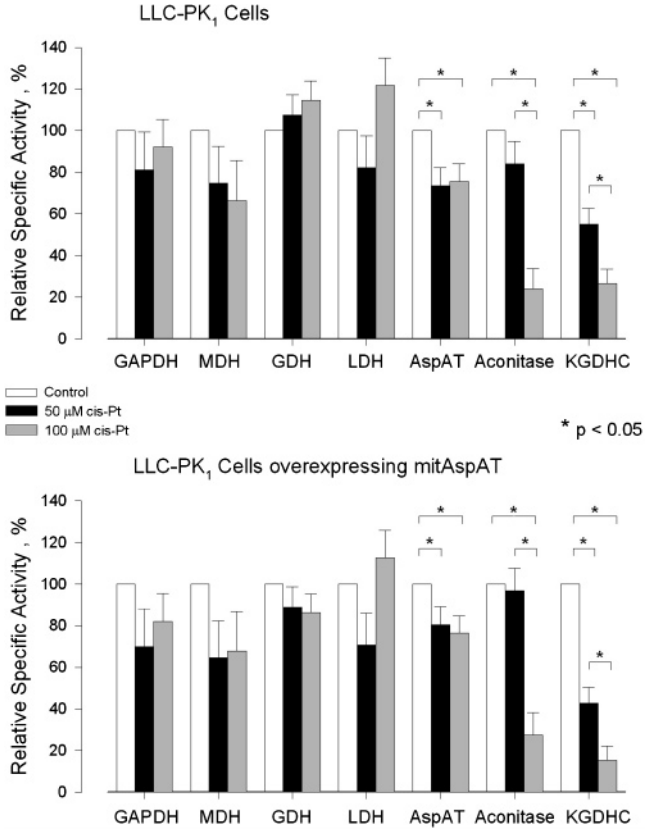

FIGURE 6: Effect of cisplatin on the relative specific activities of several enzymes of energy metabolism in control LLC-PK_1 cells expressing empty vector (LLC-PK_1/C1 cells) and in LLC-PK_1 cells overexpressing mitAspAT (LLC-PK_1/mitAspAT cells). The cells were treated with 50 or 100 μM cisplatin for 3 h. The cisplatin solution was removed, and the cells were incubated in DMEM containing 5% FBS for an additional 24 h. At the end of the incubation, the specific activities of selected enzymes in the cisplatin-treated cells were compared to those of control cells. The *n* and absolute values are as shown in Table 4. AspAT represents mitAspAT plus cytAspAT. The specific activities of the enzymes measured in the cisplatin-treated cells are reported as a percentage of the specific activity of the enzyme in the control cells for each individual cell preparation. Significance values were determined using a one-way ANOVA. The data are shown as the means ± SEM. The asterisk indicates $p < 0.05$. In addition, the two-tailed paired *t*-test indicates that the relative specific activity of KGDHC is significantly lower in the LLC-PK_1/mitAspAT cells treated with 100 μM cisplatin than in the LLC-PK_1/C1 cells treated with 100 μM cisplatin ($p = 0.008$).

is due to the close juxtapositioning of mitAspAT and KGDHC and to toxicant targeting of a reactive sulfur-containing fragment from mitAspAT to KGDHC (*24*, *25*, *59*).

By analogy with the bioactivation of halogenated cysteine *S*-conjugates, we suggest that mitAspAT contributes to the toxicity of cisplatin in kidney mitochondria in vivo. If mitAspAT is able to catalyze a β-elimination reaction with the cisplatin−cysteine *S*-conjugate and β-elimination leads to toxicity, then overexpression of this enzyme in a suitable cell line should lead to increased cisplatin toxicity, which can be attenuated by AOAA. In our experiments, overexpression of mitAspAT in confluent LLC-PK_1 cells leads to increased cisplatin toxicity, which can be overcome by AOAA treatment (Figures 3 and 4). Overexpression of mitAspAT, however, had no effect on cisplatin toxicity in dividing LLC-PK_1 cells (Figure 5). This finding is consistent with the hypothesis that the metabolism of cisplatin to a reactive thiol does not contribute to cisplatin toxicity in dividing cells, but it does so in confluent, nondividing cells.

Comparison of Cisplatin and Halogenated Alkene-Induced Toxicity to Kidney. Halogenated alkenes are metabolized to their corresponding cysteine *S*-conjugates, which are nephrotoxicants. The reactive RSH fragments generated from toxic halogenated cysteine *S*-conjugates induce lipid peroxidation and deplete energy stores (*70*, *71*). The S_3 regions of the kidney proximal tubules are especially susceptible to the effects of toxic cysteine *S*-conjugates (*72*). Mitochondria are the prime targets of toxic halogenated cysteine *S*-conjugates leading to cell death in kidney cells (*24*, *25*).

Toxicity induced by cisplatin is similar to that induced by halogenated alkenes. In the kidney, the proximal tubules are targeted by cisplatin and mitochondria are especially vulnerable (*73*). Cisplatin-induced renal damage results in inhibition of complexes I−IV of the respiratory chain (*73*), dose-dependent inhibition of oxygen consumption and inhibition of Na^+/K^+-ATPase activity (*50*), decreased respiration and oxidative phosphorylation, altered mitochondrial transmembrane potential in renal proximal tubule cells (*74*), and an increased level of ROS production (*74*). These findings are consistent with our hypothesis that the toxicity of cisplatin in confluent cells is the result of the metabolism of a Pt−cysteine *S*-conjugate to a highly reactive thiol by mitochondrial cysteine *S*-conjugate β-lyases. In contrast, Tacka et al. (*75*) showed that exposure of dividing Jurkat cells to low concentrations of cisplatin that are toxic to cells in logarithmic growth had no immediate effect on cellular mitochondrial oxygen consumption. Cellular respiration and viability did not decrease until 24 h as the cells underwent apoptosis (*75*). These data are consistent with the hypothesis that cisplatin-induced DNA damage triggers cell death in dividing cells.

Toxicant Targeting May Contribute to Cisplatin-Induced Damage to Kidney Mitochondria. This work is consistent with the hypothesis that a metabolite of cisplatin generated in the kidney interferes with energy metabolism in the mitochondria. These findings show that KGDHC activity and, to a lesser extent, aconitase activity are selectively decreased in LLC-PK_1 cells exposed to cisplatin. This finding suggests that bioactivation of cisplatin, in a fashion similar to that proposed for TFEC (*24*, *58*), may involve targeting of a reactive fragment from the active site of mitAspAT to a metabolon composed in part of mitAspAT, KGDHC, and aconitase. We suggest that cisplatin is converted to the corresponding cysteine *S*-conjugate, which is a β-lyase substrate of mitAspAT. The Pt−S compound resulting from the lyase reaction then binds to nearby proteins such as KGDHC and aconitase, thereby inactivating them. The Pt−S compound also apparently slowly inactivates mitAspAT. We found a significant decline in total AspAT activity 24 h after LLC-PK_1 cells had been exposed to cisplatin for 3 h. Inhibition of AspAT would lead to disruption of the malate−aspartate shuttle and impede passage of reducing equivalents across the mitochondrial membrane (*52*). In summary, inhibition of mitAspAT, KGDHC, and aconitase may contribute to the cisplatin-induced mitochondrial dysfunction of energy metabolism in kidney cells.

What Accounts for the Selective Toxicity of the Halogenated Alkenes and Cisplatin to the Renal Proximal Tubules? A major question that remains to be answered is why the

renal proximal tubule cells are killed by cisplatin and by nephrotoxic halogenated alkenes while other cells in the kidney and most other nondividing cells throughout the body are not affected to the same extent by these toxicants. mitAspAT is widely distributed among various organs (*45*). Liver, heart, brain, kidney, and skeletal muscles exhibit high levels of mitAspAT activity (*45*). Therefore, the nephrotoxicity of halogenated alkenes and cisplatin cannot be explained on the basis of the tissue expression of this enzyme. Important factors may include uptake of cisplatin into the kidney, activities of glutathione *S*-transferases and export pumps for the cisplatin−glutathione conjugates, activities of the ecto-enzymes γ-glutamyltranspeptidase (*27*) and aminopeptidase N (*76*) on the surface of the renal proximal tubules, and an uptake system for cysteine *S*-conjugates in the kidney (*77*).

Summary. Previous studies have provided evidence that bioactivation of cisplatin involves the action of cisplatin−cysteine *S*-conjugate and PLP-containing cysteine *S*-conjugate β-lyases (*8*−*11*, *13*, *40*). Analogy with the bioactivation of nephrotoxic, halogenated alkenes suggested that the cysteine *S*-conjugate β-lyases generated a reactive sulfur-containing fragment from the cisplatin−cysteine *S*-conjugate and that this fragment bound to proteins (*8*−*11*, *13*, *40*). The work presented here shows that in the kidney, adduction of Pt to proteins in vivo preferentially occurs in the mitochondrial fraction. We also showed that cisplatin-induced toxicity toward kidney mitochondria is associated with targeting of enzymes of energy metabolism, namely KGDHC, and to a lesser extent aconitase. The GGT-dependent, PLP β-lyase-dependent pathway does not play a role in the toxicity of cisplatin to dividing cells (*40*). It might be possible to administer reversible inhibitors to temporarily diminish the binding of the cisplatin−cysteine *S*-conjugate to the active site of mitAspAT. This strategy may allow larger doses of cisplatin to be administered to cancer patients while at the same time minimizing toxicity to the kidneys.

REFERENCES

1. Masters, J. R., and Koberle, B. (2003) Curing metastatic cancer: Lessons from testicular germ-cell tumours, *Nat. Rev. Cancer 3*, 517−525.
2. Arany, I., and Safirstein, R. L. (2003) Cisplatin nephrotoxicity, *Semin. Nephrol. 23*, 460−464.
3. Steeghs, N., de Jongh, F. E., Sillevis Smitt, P. A., and van den Bent, M. J. (2003) Cisplatin-induced encephalopathy and seizures, *Anticancer Drugs 14*, 443−446.
4. Siddik, Z. H. (2003) Cisplatin: Mode of cytotoxic action and molecular basis of resistance, *Oncogene 22*, 7265−7279.
5. Boulikas, T., and Vougiouka, M. (2003) Cisplatin and platinum drugs at the molecular level (review), *Oncol. Rep. 10*, 1663−1682.
6. Chu, C., and Sartorelli, A. C. (2004) Cancer Chemotherapy, in *Basic and Clinical Pharmacology* (Katzung, B. G., Ed.) pp 898−930, Lang Medical Books, McGraw-Hill, New York.
7. Daley-Yates, P. T., and McBrien, D. C. H. (1984) Cisplatin metabolites in plasma: A study of their pharmacokinetics and importance in the nephrotoxic and antitumour activity of cisplatin, *Biochem. Pharmacol. 33*, 3063−3070.
8. Hanigan, M. H., Gallagher, B. C., Taylor, P. T., Jr., and Large, M. K. (1994) Inhibition of γ-glutamyl transpeptidase activity by acivicin in vivo protects the kidney from cisplatin-induced toxicity, *Cancer Res. 54*, 5925−5929.
9. Hanigan, M. H., Lykissa, E. D., Townsend, D. M., Ou, C. N., Barrios, R., and Lieberman, M. W. (2001) γ-Glutamyl transpeptidase-deficient mice are resistant to the nephrotoxic effects of cisplatin, *Am. J. Pathol. 159*, 1889−1894.
10. Townsend, D. M., and Hanigan, M. H. (2002) Inhibition of γ-glutamyl transpeptidase or cysteine S-conjugate β-lyase activity blocks the nephrotoxicity of cisplatin in mice, *J. Pharmacol. Exp. Ther. 300*, 142−148.
11. Townsend, D. M., Deng, M., Zhang, L., Lapus, M. G., and Hanigan, M. H. (2003) Metabolism of cisplatin to a nephrotoxin in proximal tubule cells, *J. Am. Soc. Nephrol. 14*, 1−10.
12. Townsend, D. M., Marto, J. A., Deng, M., Macdonald, T. J., and Hanigan, M. H. (2003) High-pressure liquid chromatography and mass spectrometry characterization of the nephrotoxic biotransformation products of cisplatin, *Drug Metab. Dispos. 31*, 705−713.
13. Hanigan, M. H., and Devarajan, P. (2003) Cisplatin nephrotoxicity: Molecular mechanisms, *Cancer Ther. 1*, 47−61.
14. Mayer, R. D., Lee, K., and Cockett, A. T. K. (1987) Inhibition of cisplatin-induced nephrotoxicity in rats by buthionine sulfoximine, a glutathione synthesis inhibitor, *Cancer Chemother. Pharmacol. 20*, 207−210.
15. Dekant, W., Vamvakas, S., and Anders, M. W. (1995) Formation and fate of nephrotoxic and cytotoxic glutathione S-conjugates: Cysteine conjugate β-lyase pathway, in *Advances in Pharmacology* (Anders, M. W., and Dekant, W., Eds.) Vol. 27, pp 115−162, Academic Press Inc., New York.
16. McGoldrick, T. A., Lock, E. A., Rodilla, V., and Hawksworth, G. M. (2003) Renal cysteine conjugate C−S lyase mediated toxicity of halogenated alkenes in primary cultures of human and rat proximal tubular cells, *Arch. Toxicol. 77*, 365−370.
17. Hayden, P. J., Yang, Y., Ward, A. J., Dulik, D. M., McCann, D. J., and Stevens, J. L. (1991) Formation of difluorothionoacetyl-protein adducts by *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine metabolites: Nucleophilic catalysis of stable lysyl adduct formation by histidine and tyrosine, *Biochemistry 30*, 5935−5943.
18. Anders, M. W., and Dekant, W. (1998) Glutathione-dependent bioactivation of haloalkenes, *Annu. Rev. Pharmacol. Toxicol. 38*, 501−537.
19. Cooper, A. J. L. (1998) Mechanisms of cysteine *S*-conjugate β-lyases, *Adv. Enzymol. 72*, 199−238.
20. Stevens, J. L. (1985) Isolation and characterization of a rat liver enzyme with both cysteine conjugate β-lyase and kynureninase activity, *J. Biol. Chem. 260*, 7945−7950.
21. Lash, L. H., Elfarra, A. A., and Anders, M. W. (1986) Renal cysteine conjugate β-lyase: Bioactivation of nephrotoxic cysteine S-conjugates in mitochondrial outer-membrane, *J. Biol. Chem. 261*, 5930−5935.
22. Jones, T. W., Chen, Q., Schaeffer, V. H., and Stevens, J. L. (1988) Immunohistochemical localization of glutamine transaminase K, a rat kidney cysteine conjugate β-lyase, and the relationship to the segment specificity of cysteine conjugate nephrotoxicity, *Mol. Pharmacol. 34*, 621−627.
23. Stevens, J. L., Ayoubi, N., and Robbins, J. D. (1988) The role of mitochondrial matrix enzymes in the metabolism and toxicity of cysteine conjugates, *J. Biol. Chem. 263*, 3395−3401.
24. Cooper, A. J. L., Bruschi, S. A., and Anders, M. W. (2002) Toxic, halogenated cysteine *S*-conjugates and targeting of mitochondrial enzymes of energy metabolism, *Biochem. Pharmacol. 64*, 553−564.
25. Cooper, A. J. L., and Pinto, J. T. (2006) Cysteine S-conjugate β-lyases, *Amino Acids 30*, 1−15.
26. Stevens, J. L., Robbins, J. D., and Byrd, R. A. (1986) A purified cysteine conjugate β-lyase from rat kidney cytosol. Requirement for an α-keto acid or an amino acid oxidase for activity and identity with soluble glutamine transaminase K, *J. Biol. Chem. 261*, 15529−15537.
27. Hanigan, M. H., and Frierson, H. F., Jr. (1996) Immunohistochemical detection of γ-glutamyl transpeptidase in normal human tissue, *J. Histochem. Cytochem. 44*, 1101−1108.
28. McIntyre, T. M., and Curthoys, N. P. (1982) Renal catabolism of glutathione: Characterization of a particulate rat renal dipeptidase that catalyzes the hydrolysis of cysteinylglycine, *J. Biol. Chem. 257*, 11915−11921.
29. Cooper, A. J. L., Bruschi, S. A., Iriarte, A., and Martinez-Carrion, M. (2002) Mitochondrial aspartate aminotransferase catalyses cysteine S-conjugate β-lyase reactions, *Biochem. J. 368*, 253−261.
30. Hayden, P. J., and Stevens, J. L. (1990) Cysteine conjugate toxicity, metabolism, and binding to macromolecules in isolated rat kidney mitochondria, *Mol. Pharmacol. 37*, 468−476.
31. McKinney, L. L., Picken, J. C. J., Weakley, F. B., Eldridge, A. C., Campbell, R. E., Cowan, J. C., and Biester, H. E. (1959)

Possible toxic factor of trichloroethylene-extracted soybean oil meal, *J. Am. Chem. Soc. 81*, 909−915.
32. Elfarra, A. A., Jakobson, I., and Anders, M. W. (1986) Mechanism of S-(1,2-dichlorovinyl)glutathione-induced nephrotoxicity, *Biochem. Pharmacol. 35*, 283−288.
33. de Ceaurriz, J., and Ban, M. (1990) Role of γ-glutamyltranspeptidase and β-lyase in the nephrotoxicity of hexachloro-1,3-butadiene and methyl mercury in mice, *Tox. Lett. 50*, 249−256.
34. Hensley, K., Kotake, Y., Sang, H., Pye, Q. N., Wallis, G. L., Kolker, L. M., Tabatabaie, T., Stewart, C. A., Konishi, Y., Nakae, D., and Floyd, R. A. (2000) Dietary choline restriction causes complex I dysfunction and increased H_2O_2 generation in liver mitochondria, *Carcinogenesis 21*, 983−989.
35. Wallin, A., Gerdes, R. G., Morgenstern, R., Jones, T. W., and Ormstad, K. (1988) Features of microsomal and cytosolic glutathione conjugation of hexachlorobutadiene in rat liver, *Chem.-Biol. Interact. 68*, 1−11.
36. Park, L. C. H., Gibson, G. E., Bunik, V., and Cooper, A. J. L. (1999) Inhibition of select mitochondrial enzymes in PC12 cells exposed to *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine, *Biochem. Pharmacol. 58*, 1557−1565.
37. Srinivas, K. S., Chandrasekar, G., Srivastava, R., and Puvanakrishnan, R. (2004) A novel protocol for the subcellular fractionation of C3A hepatoma cells using sucrose density gradient centrifugation, *J. Biochem. Biophys. Methods 60*, 23−27.
38. Altieri, F., Mattingly, J. R., Rodriguez-Berrocal, F. J., Youssef, J., Iriarte, A., Wu, T. H., and Martinez-Carrion, M. (1989) Isolation and properties of a liver mitochondrial precursor protein to aspartate-aminotransferase expressed in *Escherichia coli*, *J. Biol. Chem. 264*, 4782−4786.
39. Artigues, A., Crawford, D. L., Iriarte, A., and Martinez-Carrion, M. (1998) Divergent Hsc70 binding properties of mitochondrial and cytosolic aspartate aminotransferase: Implications for their segregation to different cellular compartments, *J. Biol. Chem. 273*, 33130−33134.
40. Zhang, L., and Hanigan, M. H. (2003) Role of cysteine *S*-conjugate β-lyase in the metabolism of cisplatin, *J. Pharmacol. Exp. Ther. 306*, 988−994.
41. Mattingly, J. R., Jr., Youssef, J., Iriarte, A., and Martinez-Carrion, M. (1993) Protein folding in a cell-free translation system. The fate of the precursor to mitochondrial aspartate aminotransferase, *J. Biol. Chem. 268*, 3925−3937.
42. Torella, C., Mattingly, J. R., Jr., Artigues, A., Iriarte, A., and Martinez-Carrion, M. (1998) Insight into the conformation of protein folding intermediate(s) trapped by GroEL, *J. Biol. Chem. 273*, 3915−3925.
43. Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays, *J. Immunol. Methods 65*, 55−63.
44. Bubber, P., Haroutunian, V., Fisch, G., Blass, J. P., and Gibson, G. E. (2005) Mitochondrial abnormalities in Alzheimer brain: Mechanistic implications, *Ann. Neurol. 57*, 695−703.
45. Parli, J. A., Godfrey, D. A., and Ross, C. D. (1987) Separate enzymatic microassays for aspartate-aminotransferase isoenzymes, *Biochim. Biophys. Acta 925*, 175−184.
46. Cooper, A. J. L. (1978) Purification of soluble and mitochondrial glutamine transaminase K from rat kidney. Use of a sensitive assay involving transamination between L-phenylalanine and α-keto-γ-methiolbutyrate, *Anal. Biochem. 89*, 451−460.
47. Cooper, A. J. L., and Meister, A. (1981) Comparative studies of glutamine transaminases from rat tissues, *Comparative 69B*, 137−145.
48. Cooper, A. J. L., and Pinto, J. T. (2005) Aminotransferase, L-amino acid oxidase and β-lyase reactions involving L-cysteine S-conjugates found in allium extracts. Relevance to biological activity? *Biochem. Pharmacol. 69*, 209−220.
49. Cooper, A. J. L., Sheu, K. R., Burke, J. R., Onodera, O., Strittmatter, W. J., Roses, A. D., and Blass, J. P. (1997) Transglutaminase-catalyzed inactivation of glyceraldehyde 3-phosphate dehydrogenase and α-ketoglutarate dehydrogenase complex by polyglutamine domains of pathological length, *Proc. Natl. Acad. Sci. U.S.A. 94*, 12604−12609.
50. Brady, H. R., Kone, B. C., Stromski, M. E., Zeidel, M. L., Giebisch, G., and Gullans, S. R. (1990) Mitochondrial injury: An early event in cisplatin toxicity to renal proximal tubules, *Am. J. Physiol. 258*, F1181−F1187.
51. Lange, R. C., Spencer, R. P., and Harder, H. C. (1973) The antitumor agent cis-$Pt(NH_3)_2Cl_2$: Distribution studies and dose calculations for ^{193m} Pt and ^{195m} Pt, *J. Nucl. Med. 14*, 191−195.
52. Fitzpatrick, S. M., Cooper, A. J. L., and Duffy, T. E. (1983) Use of β-methylene-D,L-aspartate to assess the role of aspartate aminotransferase in cerebral oxidative metabolism, *J. Neurochem. 41*, 1370−1383.
53. Yamauchi, A., Stijntjes, G. J., Commandeur, J. N. M., and Vermeulen, N. P. E. (1993) Purification of glutamine transaminase K/cysteine conjugate β-lyase from rat renal cytosol based on hydrophobic interaction HPLC and gel permeation FPLC, *Protein Expression Purif. 4*, 552−562.
54. Plant, N., Kitchen, I., Goldfarb, P. S., and Gibson, G. G. (1997) Developmental modulation of cysteine conjugate β-lyase/glutamine transaminase K/kynurenine aminotransferase mRNA in rat brain, *Eur. J. Drug Metab. Pharmacokinet. 22*, 335−339.
55. Ho, H. K., Hu, Z. H., Tzung, S. P., Hockenbery, D. M., Fausto, N., Nelson, S. D., and Bruschi, S. A. (2005) BCL-xL overexpression effectively protects against tetrafluoroethylcysteine-induced intramitochondrial damage and cell death, *Biochem. Pharmacol. 69*, 147−157.
56. Chen, Y., Cai, J., Anders, M. W., Stevens, J. L., and Jones, D. P. (2001) Role of mitochondrial dysfunction in *S*-(1,2-dichlorovinyl)-l-cysteine-induced apoptosis, *Toxicol. Appl. Pharmacol. 170*, 172−180.
57. Bruschi, S. A., West, K. A., Crabb, J. W., Gupta, R. S., and Stevens, J. L. (1993) Mitochondrial Hsp60 (P1 protein) and A Hsp70-like protein (mortalin) are major targets for modification during *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine-induced nephrotoxicity, *J. Biol. Chem. 268*, 23157−23161.
58. Bruschi, S. A., Lindsay, J. G., and Crabb, J. W. (1998) Mitochondrial stress protein recognition of inactivated dehydrogenases during mammalian cell death, *Proc. Natl. Acad. Sci. U.S.A. 95*, 13413−13418.
59. James, E. A., Gygi, S. P., Adams, M. L., Pierce, R. H., Fausto, N., Aebersold, R. H., Nelson, S. D., and Bruschi, S. A. (2002) Mitochondrial aconitase modification, functional inhibition, and evidence for a supramolecular complex of the TCA cycle by the renal toxicant *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine, *Biochemistry 41*, 6789−6797.
60. Elfarra, A. A., Duescher, R. J., Hwang, I. Y., Sicuri, A. R., and Nelson, J. A. (1995) Targeting 6-thioguanine to the kidney with *S*-(guanin-6-yl)-L-cysteine, *J. Pharmacol. Exp. Ther. 274*, 1298−1304.
61. Mosca, M., Croci, C., Mostardini, M., Breton, J., Malyszko, J., Avanzi, N., Toma, S., Benatti, L., and Gatti, S. (2003) Tissue expression and translational control of rat kynurenine aminotransferase/glutamine transaminase K mRNAs, *Biochim. Biophys. Acta 1628*, 1−10.
62. Cooper, A. J. L. (1988) Glutamine aminotransferases and amidases, in *Glutamine and Glutamate in Mammals* (Kvamme, E., Ed.) pp 33−52, CRC Press, Boca Raton, FL.
63. Rooseboom, M., Schaaf, G., Commandeur, J. N. M., Vermeulen, N. P. E., and Fink-Gremmels, J. (2002) β-Lyase-dependent attenuation of cisplatin-mediated toxicity by selenocysteine *Se*-conjugates in renal tubular cell lines, *J. Pharmacol. Exp. Ther. 301*, 884−892.
64. Abraham, D. G., Thomas, R. J., and Cooper, A. J. L. (1995) Glutamine transaminase K is not a major cysteine S-conjugate β-lyase of rat kidney mitochondria: Evidence that a high-molecular weight enzyme fulfills this role, *Mol. Pharmacol. 48*, 855−860.
65. Abraham, D. G., Patel, P. P., and Cooper, A. J. L. (1995) Isolation from rat kidney of a cytosolic high-molecular-weight cysteine-*S*-conjugate β-lyase with activity toward leukotriene E(4), *J. Biol. Chem. 270*, 180−188.
66. Cooper, A. J. L., Krasnikov, B. F., Okuno, E., and Jeitner, T. M. (2003) L-Alanine-glyoxylate arninotransferase II of rat kidney and liver mitochondria possesses cysteine *S*-conjugate β-lyase activity: A contributing factor to the nephrotoxicity/hepatotoxicity of halogenated alkenes? *Biochem. J. 376*, 169−178.
67. Cooper, A. J. L., Bruschi, S. A., Conway, M., and Hutson, S. M. (2003) Human mitochondrial and cytosolic branched-chain aminotransferases are cysteine *S*-conjugate β-lyases, but turnover leads to inactivation, *Biochem. Pharmacol. 65*, 181−192.
68. Cooper, A. J. L., Wang, J., Gartner, C. A., and Bruschi, S. A. (2001) Co-purification of mitochondrial HSP70 and mature protein

disulfide isomerase with a functional rat kidney high-M_r cysteine *S*-conjugate β-lyase, *Biochem. Pharmacol. 62*, 1345−1353.

69. Lain, B., Iriarte, A., and Martinez-Carrion, M. (1994) Dependence of the folding and import of the precursor to mitochondrial aspartate-aminotransferase on the nature of the cell-free translation system, *J. Biol. Chem. 269*, 15588−15596.
70. Chen, Q., Jones, T. W., Brown, P. C., and Stevens, J. L. (1990) The mechanism of cysteine conjugate cytotoxicity in renal epithelial cells. Covalent binding leads to thiol depletion and lipid peroxidation, *J. Biol. Chem. 265*, 21603−21611.
71. Anders, M. W. (1995) Mitochondrial bioactivation of cysteine *S*-conjugates and 4-thiaalkanoates: Implications for mitochondrial dysfunction and mitochondrial diseases, *Biochim. Biophys. Acta 1271*, 51−57.
72. Nash, J. A., King, L. J., Lock, E. A., and Green, T. (1984) The metabolism and disposition of hexachloro-1:3-butadiene in the rat and its relevance to nephrotoxicity, *Toxicol. Appl. Pharmacol. 73*, 124−137.
73. Kruidering, M., van de, W. B., de Heer, E., Mulder, G. J., and Nagelkerke, J. F. (1997) Cisplatin-induced nephrotoxicity in porcine proximal tubular cells: Mitochondrial dysfunction by inhibition of complexes I to IV of the respiratory chain, *J. Pharmacol. Exp. Ther. 280*, 638−649.
74. Goodisman, J., Hagrman, D., Tacka, K. A., and Souid, A. K. (2006) Analysis of cytotoxicities of platinum compounds, *Cancer Chemother. Pharmacol. 57*, 257−267.
75. Tacka, K. A., Dabrowiak, J. C., Goodisman, J., Penefsky, H. S., and Souid, A. K. (2004) Effects of cisplatin on mitochondrial function in Jurkat cells, *Chem. Res. Toxicol. 17*, 1102−1111.
76. Kozak, E. M., and Tate, S. S. (1982) Glutathione-degrading enzymes of microvillus membranes, *J. Biol. Chem. 257*, 6322−6327.
77. Wright, S. H., Wunz, T. M., North, J., and Stevens, J. L. (1998) Na-dependent transport of *S*-(1,2-dichlorovinyl)-L-cysteine by renal brush-border membrane vesicles, *J. Pharmacol. Exp. Ther. 285*, 162−169.

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