



ANIMAL MODELS

Transgenic Expression of the Human MRP2 Transporter Reduces Cisplatin Accumulation and Nephrotoxicity in *Mrp2*-Null Mice

Xia Wen,^{*} Brian Buckley,[†] Elizabeth McCandlish,[†] Michael J. Goedken,^{*} Samira Syed,^{*} Ryan Pelis,[‡] José E. Manautou,[§] and Lauren M. Aleksunes^{*†}

From the Department of Pharmacology and Toxicology* and the Environmental and Occupational Health Sciences Institute,[†] Rutgers, The State University of New Jersey, Piscataway, New Jersey; the Department of Pharmacology,[‡] Dalhousie University, Halifax, Nova Scotia, Canada; and the Department of Pharmaceutical Sciences,[§] University of Connecticut, Storrs, Connecticut

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Address correspondence to
Lauren M. Aleksunes, Pharm.D.,
Ph.D., Department of Pharma-
cology and Toxicology, Rutgers
University, 170 Frelinghuysen
Rd, Piscataway, NJ 08854.
E-mail: aleksunes@cohsi.rutgers.edu.

The chemotherapeutic drug cisplatin is actively transported into proximal tubules, leading to acute renal injury. Previous studies suggest that the multidrug resistance-associated protein 2 (Mrp2) transporter may efflux cisplatin conjugates from cells. We sought to determine whether the absence of Mrp2 alters the accumulation and toxicity of platinum in the kidneys of mice and whether transgenic expression of the human *MRP2* gene could protect against cisplatin injury *in vivo*. Plasma, kidneys, and livers from vehicle- and cisplatin-treated wild-type and *Mrp2*-null mice were collected for quantification of platinum and toxicity. By 24 hours, twofold higher concentrations of platinum were detected in the kidneys and livers of *Mrp2*-null mice compared with wild types. Enhanced platinum concentrations in *Mrp2*-null mice were observed in DNA and cytosolic fractions of the kidneys. Four days after cisplatin treatment, more extensive proximal tubule injury was observed in *Mrp2*-null mice compared with wild-type mice. Kidneys from naive *Mrp2*-null mice had elevated glutathione S-transferase mRNA levels, which could increase the formation of cisplatin-glutathione conjugates that may be metabolized to toxic thiol intermediates. Transgenic expression of the human *MRP2* gene in *Mrp2*-null mice reduced the accumulation and nephrotoxicity of cisplatin to levels observed in wild-type mice. These data suggest that deficiency in *Mrp2* lowers platinum excretion and increases susceptibility to kidney injury, which can be rescued by the human *MRP2* ortholog. (*Am J Pathol* 2014, 184: 1299–1308; <http://dx.doi.org/10.1016/j.ajpath.2014.01.025>)

Cisplatin is commonly used in chemotherapy regimens for the treatment of solid cancers. The success of cisplatin therapy is limited, in part, by kidney injury. Up to 37% of patients develop signs of nephrotoxicity after receiving a single dose of cisplatin despite strategies such as hydration to limit renal exposure.¹ This is problematic for patients because kidney injury can delay further treatment and limit the total number of chemotherapy cycles received, thereby reducing the overall efficacy of cisplatin-containing regimens.

Previous studies have identified the contribution of uptake and efflux transporters to the renal secretion of cisplatin. The organic cation transporter 2 (Oct2; gene *Slc22a2*) is highly expressed in the kidneys and mediates the renal uptake of cisplatin.^{2–5} Transgenic mice lacking Oct1 and Oct2

transporters exhibit decreased platinum (Pt) excretion and reduced nephrotoxicity.⁶ In addition, patients with cancer with a loss-of-function polymorphism in OCT2 (808G>T) are protected against cisplatin renal injury.^{6,7} Additional data suggest that the copper transporter 1 (Ctr1; gene *Slc31a1*) participates in the renal uptake of cisplatin,^{8–12} although

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Table 1 Methods for Inductively Coupled Plasma Mass Spectrometry Analysis

Matrix	Approximate weight (g) or volume (μL)	Nitric acid (mL)	Microwave program, 300 W
Urine*	20–100 μL	0.25–0.5	50% power, 5 minutes, once, then 75% power, 5 minutes, 5 \times
Liver ^{†‡}	0.1–0.25 g	1–1.5	75% power, 5 minutes, once, then 100% power, 5 minutes, 5 \times , then 100% power, 10 minutes, 1–2 \times
Kidney [†]	0.1–0.25 g	1–1.5	Same as liver
Plasma* ^{†§}	50 μL	0.25	Same as urine
Vesicles	100 μL	0.15	Same as urine

*The approximate rule for a largely aqueous matrix is at least two parts acid to one part matrix by volume.

[†]Microwaving was discontinued when the samples were clear and pale yellow.

[‡]Hydrogen peroxide (0.5 mL, 30% H_2O_2) was occasionally added to complete the digestion of liver. If so, an additional microwave treatment (100% power, 10 minutes) was used to decompose the residual hydrogen peroxide.

[§]Samples in the 0.25-mL range can be microwaved in 15-mL centrifuge tubes provided that less time and lower percentage power are used.

recent work has found conflicting results.¹³ After uptake of cisplatin by Oct2 or Ctr1, the subsequent efflux of cisplatin into the renal lumen is mediated by the multidrug and toxin extrusion protein 1 (Mate1; gene *Slc47a1*).¹⁴ As expected, Mate1 knockout mice have increased renal concentrations of cisplatin and enhanced nephrotoxicity.¹⁴

Once inside the renal cell, cisplatin is biotransformed by spontaneous hydrolysis to monoaquated and diaquated species. Formation of cisplatin aquated intermediates leads to electrophilic attack of cellular macromolecules, such as DNA and protein. The tripeptide glutathione (GSH) can also bind cisplatin and has been suggested to form a variety of nontoxic cisplatin conjugates, including monoplatinum-monogluthathione, diplatinum-monogluthathione, and bis-(glutathionato)-platinum.^{15,16} It has been postulated that these conjugates have the potential for metabolism to reactive thiol intermediates. Previous studies suggest that the multidrug resistance-associated transporter 2 (Mrp2; gene *Abcc2*) can efflux GSH conjugates of cisplatin from cancer cells and confer resistance to cytotoxicity.^{15,17,18} Overexpression of human (h)MRP2 increases cisplatin resistance by 10-fold in human embryonic kidney cells¹⁷ and reduces cisplatin accumulation by 30% in porcine kidney LLC-PK1 cells.¹⁸ Moreover, primary hepatocytes isolated from transport-deficient rats (*Mrp2*-deficient strain) exhibit increased binding of Pt to DNA and enhanced susceptibility to cisplatin cytotoxicity.¹⁹ However, the ability of Mrp2 to reduce the accumulation of cisplatin and/or its conjugates and limit renal injury *in vivo* is not well understood. Therefore, the purpose of this study was to determine whether loss of Mrp2 function enhances renal Pt accumulation and nephrotoxicity in mice and to evaluate the ability of the human *MRP2* gene to rescue *Mrp2*-null mice from heightened cisplatin-induced nephrotoxicity *in vivo*.

Materials and Methods

Chemicals

Unless otherwise specified, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Vesicle Transport Assays

Inside-out *Sf9* insect cell plasma membrane vesicles expressing mouse (m)Mrp2 or hMRP2 were purchased from Life Technologies (Carlsbad, CA). To assess the ability of cisplatin to inhibit Mrp2/MRP2-mediated transport of 5,6-carboxy-2',7'-dichlorofluorescein (CDCF), 20- μg vesicles were incubated with 10 $\mu\text{mol/L}$ CDCF, 4 mmol/L adenosine triphosphate, 2 mmol/L GSH, and increasing concentrations of cisplatin (50 to 200 $\mu\text{mol/L}$) in reaction buffer at 37°C for 10 minutes according to the manufacturer's protocol. Vesicles were washed, vacuum filtered, and solubilized with 50% methanol. Fluorescence was read at excitation wavelength 504 nm; emission wavelength 529 nm.

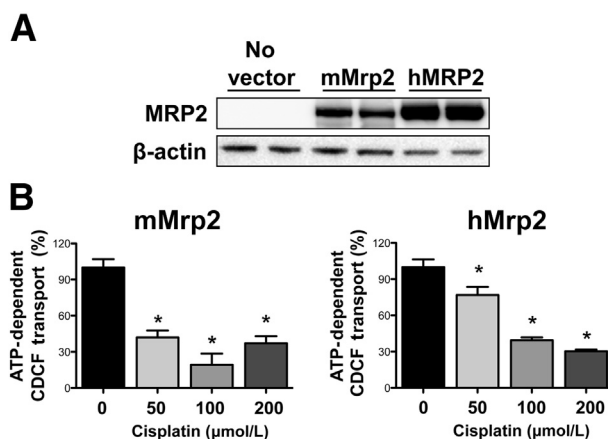


Figure 1 Interaction of mMrp2 and hMRP2 transporters with cisplatin *in vitro*. **A:** Western blot analysis of commercially available mMrp2 and hMRP2 vesicles. β -Actin was used as a loading control. **B:** Twenty micrograms of inverted Mrp2- and MRP2-expressing vesicles were incubated with 10 $\mu\text{mol/L}$ CDCF for 10 minutes in the presence and absence of adenosine triphosphate (ATP), GSH, and increasing concentrations of cisplatin. The total mean transport rates of CDCF in mMrp2- and hMRP2-expressing vesicles were 1051 and 1933 pmol/min/mg of protein, respectively (data not shown). The ATP-independent transport rates of CDCF in mMrp2- and hMRP2-expressing vesicles were 12 and 222 pmol/min/mg protein, respectively (data not shown). Data are presented as means \pm SEM ($n = 4$ to 5) normalized to ATP-dependent MRP2 transport of CDCF (no cisplatin). * $P < 0.05$ compared with 0 $\mu\text{mol/L}$ cisplatin.

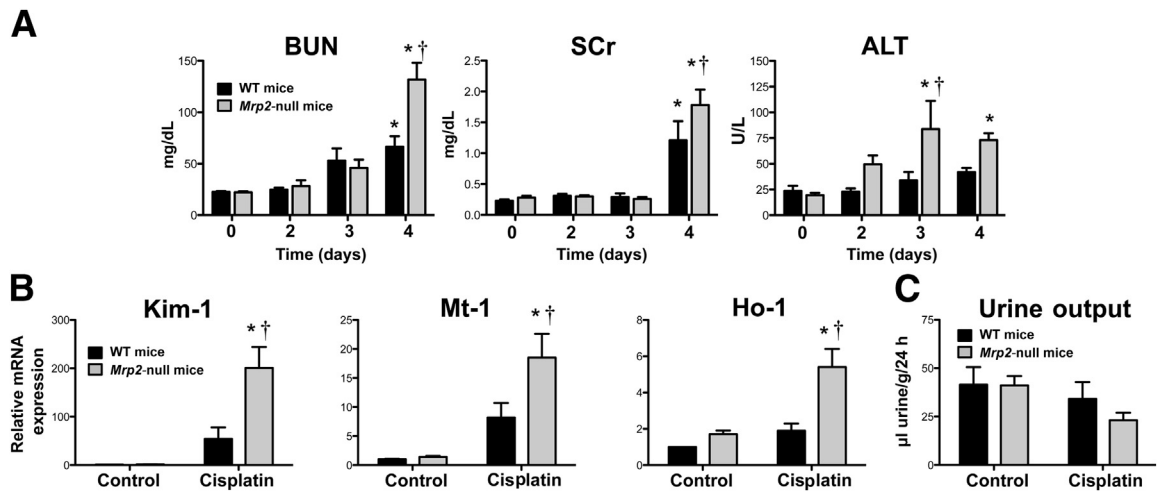


Figure 2 Renal and hepatic injury markers in wild-type (black bars) and *Mrp2*-null (gray bars) mice treated with cisplatin. **A:** BUN, serum creatinine (SCr), and alanine aminotransferase (ALT) levels in plasma from WT and *Mrp2*-null mice 2 through 4 days after 20 mg/kg of i.p. cisplatin treatment ($n = 5$ to 15). **B:** mRNA expression of kidney injury molecule-1 (Kim-1), metallothionein-1 (Mt-1), and heme oxygenase-1 (Ho-1) was quantified using total kidney RNA from control and 20-mg/kg cisplatin-treated WT and *Mrp2*-null mice on day 4 ($n = 4$ to 11). **C:** Urine flow rate of control and 20-mg/kg cisplatin-treated WT and *Mrp2*-null mice from day 3 to 4. Urine volume was quantified from mice in metabolic cages for 24 hours and was normalized to body weight ($n = 3$ to 8). Data are presented as means \pm SEM. mRNA data were normalized to WT control mice. * $P < 0.05$, compared with genotype control mice; † $P < 0.05$, compared with cisplatin-treated WT mice.

Animal Treatment

Wild-type (WT) C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). *Mrp2*-null mice were obtained from Taconic Laboratories (Hudson, NY) and were backcrossed until they were >99% congenic with the C57BL/6 strain. Congenic analysis was performed by the Biomomics Research and Technology Core at Rutgers University (New Brunswick, NJ). hMRP2 mice on a C57BL/6 background were purchased from Taconic. Cisplatin was dissolved in saline after heating to 50°C. Groups of 9- to 14-week-old adult male mice were injected i.p. with 5 mL/kg of saline vehicle or 20 mg/kg of cisplatin after overnight fasting. The doses of cisplatin used in this study were similar to those used clinically.¹ Feed was returned 4 hours after cisplatin injection. Kidney, liver, and plasma samples were collected 1 hour to 4 days after cisplatin treatment. Groups of mice were placed into metabolism cages for quantification of urine Pt concentrations (24-hour periods between 0 and 3 days) and urine output (24-hour period from 3 to 4 days). Tissues were fixed in 10% formalin or were snap frozen.

Analytes and Pathology

Blood urea nitrogen (BUN) and serum creatinine levels were quantified as indicators of renal injury (ThermoTrace, Melbourne, Australia; and Pointe Scientific, Canton, MI). Total and direct bilirubin concentrations as well as alanine aminotransferase activity were also quantified (Pointe Scientific). GSH was quantified using the GSH/GSSG-Glo assay (Promega, Madison, WI). Five-micron paraffin-embedded kidney sections were stained with H&E and were examined

for histopathological changes by a board-certified anatomic veterinary pathologist (M.J.G.) using a published grading scale.²⁰

RNA Isolation and mRNA Quantification

Total RNA was isolated using RNA-Bee reagent (Tel-Test Inc., Friendswood, TX). The mRNA expression of mouse kidney injury molecule-1, heme oxygenase-1,

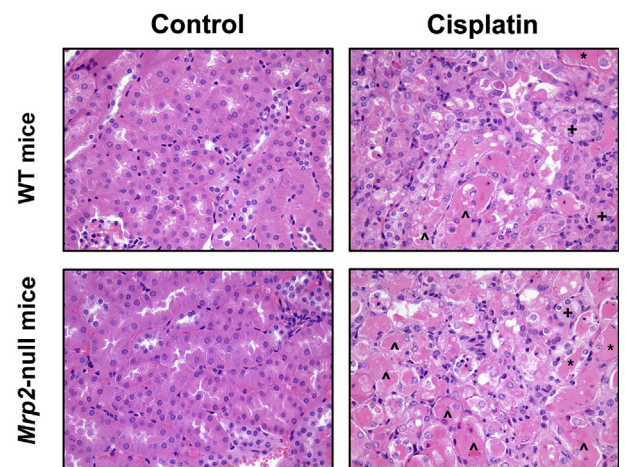


Figure 3 Histopathologic injury in WT and *Mrp2*-null mice treated with cisplatin. WT and *Mrp2*-null mice were treated with vehicle or 20 mg/kg of i.p. cisplatin, and kidneys were collected on day 4. Samples were fixed in zinc formalin before routine processing and paraffin embedding. Five-micron sections of kidneys were stained with H&E and were examined by light microscopy for the presence and severity of renal cast formation (*), proximal tubule degeneration (+), apoptosis (A), and necrosis (N) as well as neutrophil infiltration. Original magnification, $\times 40$.

Table 2 Histopathologic Analysis of Kidneys from WT and *Mrp2*-Null Mice after Cisplatin Injection

Mice and time after injection	Histopathologic grade						Mice with grade ≥ 2 (%)
	0	1	2	3	4	5	
WT							
Control	13	0	0	0	0	0	0
2 Days	1	6	0	0	0	0	0*
3 Days	1	2	2	0	0	0	40*
4 Days	4	3	6	1	0	0	50*
<i>Mrp2</i>-null							
Control	13	0	0	0	0	0	0
2 Days	1	4	2	0	0	0	29*
3 Days	1	1	3	0	0	0	60*
4 Days	0	1	3	6	2	0	91* [†]

Kidneys were removed 2 to 4 days after 20 mg/kg of i.p. cisplatin or vehicle injection and were fixed in zinc formalin before paraffin embedding and staining with H&E. Kidney slices were evaluated for the severity of degeneration and necrosis in proximal tubule segments. Histopathologic scoring of renal proximal tubule degeneration and necrosis by a veterinary pathologist was as follows: grade 0, no injury; grade 1, minimal injury involving <10% of cells with degeneration or necrosis; grade 2, mild injury involving 10% to 25% of cells; grade 3, moderate injury involving >25% to 40% of cells; grade 4, marked injury involving >40% to 50% of cells; grade 5, severe injury involving >50% of cells. The number of mice with a particular histopathologic grade is shown in each column. Mice with grades ≥ 2 are considered to have significant kidney injury. The ratios of mice with grades ≥ 2 compared with the total number of mice are presented as percentages. Histopathologic grades were rank ordered before statistical analysis.

* $P < 0.05$ compared with genotype control mice.

[†] $P < 0.05$ compared with cisplatin-treated WT mice.

metallothionein-1, glutathione *S*-transferase (*Gst*) isoforms, glutamate cysteine ligase catalytic subunit, *Oct2*, *Mrp4*, breast cancer resistance protein (*Bcrp*), and multidrug resistance protein 1a was quantified using the branched DNA 1.0 signal amplification assay (Affymetrix Inc., Santa Clara, CA).²¹ *Oct1*, *Mate1*, and *Ctr1* mRNAs were quantified by quantitative PCR. cDNA was generated using the SuperScript first-strand cDNA synthesis kit (Life Technologies). Specific forward and reverse primers (Integrated DNA Technologies, Coralville, IA) were added to 1 μ g of cDNA from each sample. The following primers (forward and reverse, respectively) were used for mouse *Mate1* (5'-GTTGGCCTTACGGAGAGGAC-3' and 5'-AATCCCACCCACCAAGACTAA-3'), mouse *Ctr1* (5'-ACACACAAAAGTGTGGGCAG-3' and 5'-AGAGAAAGTATCCCGTCCCAG-3'), mouse *Oct1* (5'-TGTCGGCTCTGGCTACAGGAGA-3' and 5'-GGGGG-ATTCTGGGACAAACCAGTAA-3'), and mouse ribosomal protein 1 13a (*Rpl13a*) (5'-CAAGAAAAGCGGATGGTGG-3' and 5'-TCCGTAACCTCAAGATCTGC-3'). SYBR Green (Applied Biosystems, Carlsbad, CA) was used for the detection of amplified products. Quantitative PCR was performed in a 384-well plate format using the ABI 7900HT PCR system (Applied Biosystems). C_T values were converted to $\Delta\Delta C_T$ values by comparing with a reference gene, *Rpl13a*.

Inductively Coupled Plasma Mass Spectrometry

Total Pt Analysis

Concentrated EMD OmniTrace Ultra high-purity nitric acid (VWR, Radnor, PA) was added to all the samples to a final concentration of 5%, followed by digestion using a CEM Mars X microwave system (CEM Corp, Matthews, NC) (Table 1). Large samples were pretreated in an ultrasonic bath, whereas samples not completely digested were reacted, postdigestion, with 30% hydrogen peroxide (Trace-Select, Fluka; Sigma-Aldrich). Digested samples were analyzed by inductively coupled plasma mass spectrometry using a Thermo-Elemental X5 instrument (Thermo Fisher). Multiple Pt ions were monitored for quality control. Reported concentrations were determined using $m/z = 195$. This method has been used previously for metals analysis in blood samples.²²

Fractionation Pt Studies

Kidneys were homogenized in 250 mmol/L sucrose–10 mmol/L Tris buffer and were centrifuged at $100,000 \times g$ for the collection of cytosol and crude membrane fractions. DNA was isolated using the DNeasy blood and tissue kit (Qiagen, Valencia, CA).

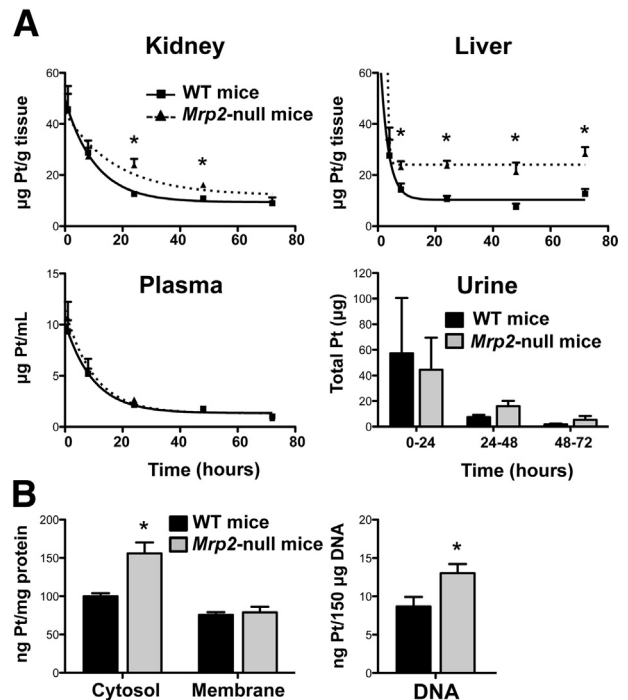
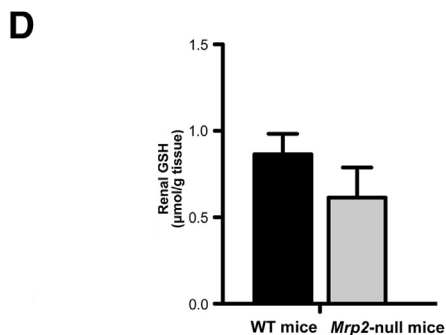
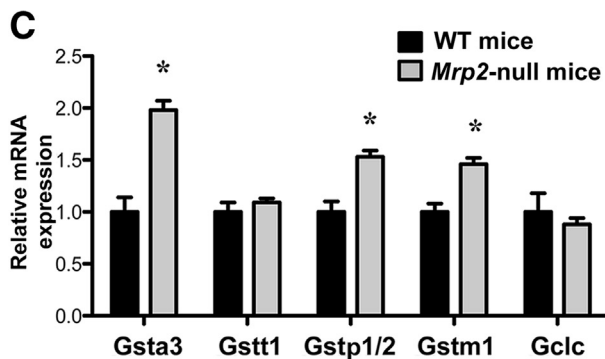
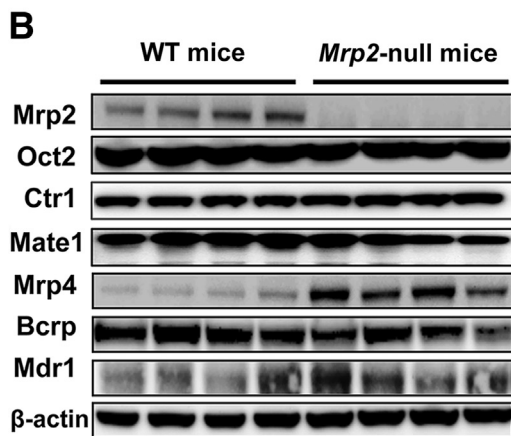
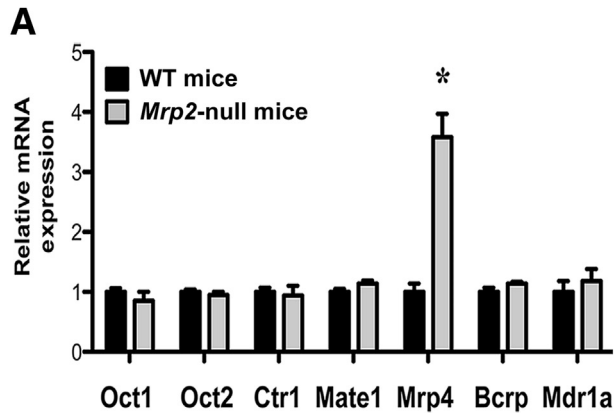


Figure 4 Pt concentrations in WT and *Mrp2*-null mice treated with cisplatin. Quantification of Pt concentrations by inductively coupled plasma mass spectrometry in the kidneys, livers, plasma, and urine (A) and cytosol, crude membrane, and DNA fractions from kidneys (B) of WT and *Mrp2*-null mice treated with 20 mg/kg of i.p. cisplatin. There were no differences in urine output between WT and *Mrp2*-null mice up to 72 hours. Data are presented as means \pm SEM. $n = 4$ to 10 (A); $n = 5$ (B). * $P < 0.05$ compared with cisplatin-treated WT mice.

Western Blot Analysis

Kidneys were homogenized in sucrose-Tris buffer containing protease inhibitors. Western blot staining was performed



as described elsewhere.²³ The following antibodies were used: Mrp2 (M₂III-5), Mrp4 (M₄I-10), and Bcrp (BXP-53) from Enzo Life Sciences (Farmingdale, NY); Oct2 (sc292622) and Mate1 (sc138983) from Santa Cruz Biotechnology (Santa Cruz, CA); and Ctr1 (ab129067), multidrug resistance protein 1a/b (C219), and β-actin (ab8227) from Abcam Inc. (Cambridge, MA).

Immunofluorescence

Immunofluorescence analysis was performed for hMRP2 (M₂III-6), mMrp2 (EAG15), and mouse Bcrp (Bxp-53). Sections were washed and incubated with goat anti-mouse or anti-rabbit IgG Alexa 488 IgG and anti-rat 563 IgG antibodies (Invitrogen, Carlsbad, CA). Images were acquired using a Zeiss Observer D1 microscope with an X-Cite series 120Q fluorescent illuminator (Zeiss Inc., Thornwood, NY) and a ProgRes camera with CapturePro version 2.8 software (Jenoptik, Easthampton, MA). Negative controls without primary antibody were included to ensure minimal nonspecific staining (data not shown).

Statistical Analysis

The software program GraphPad Prism version 5 (GraphPad Software Inc., La Jolla, CA) was used for statistical analysis. Pharmacokinetic curves were generated using area-under-the-curve analysis with one-phase nonlinear decay. Differences among groups were evaluated by Student's unpaired *t*-test (two groups) or one-way analysis of variance followed by the Newman-Keuls multiple comparison tests (three or more groups). Histopathological data were rank ordered before analysis. Statistical significance was set at $P < 0.05$.

Results

Interaction of mMrp2 and hMRP2 Transporters with Cisplatin *in Vitro*

An indirect approach (competition for transport) was used to investigate cisplatin as a potential Mrp2/MRP2 substrate in the presence of GSH using inverted plasma membrane vesicles. Commercial vesicles expressed the Mrp2 and MRP2 proteins, with notably lower expression of mMrp2 compared with hMRP2 protein (Figure 1A). The probe Mrp2/MRP2 substrate, CDCF, was used for inhibition assays as previously described.^{24,25} Cisplatin inhibited the adenosine

Figure 5 Basal expression of transporters and GSH-related enzymes in kidneys of naive WT and MRP2-null mice. **A:** mRNA levels of uptake and efflux transporters in WT and MRP2-null mice. **B:** Western blot analysis of transporter proteins in WT and MRP2-null mice. β-Actin was used as a loading control. mRNA expression of glutathione *S*-transferase (Gst) and glutamate cysteine ligase catalytic subunit (Gclc) (**C**) and GSH concentrations (**D**) in WT and MRP2-null mice. Data are presented as means ± SEM. $n = 4$ to 5 (**A**); $n = 4$ to 6 (**C** and **D**). mRNA data were normalized to WT mice. * $P < 0.05$ compared with naive WT mice.

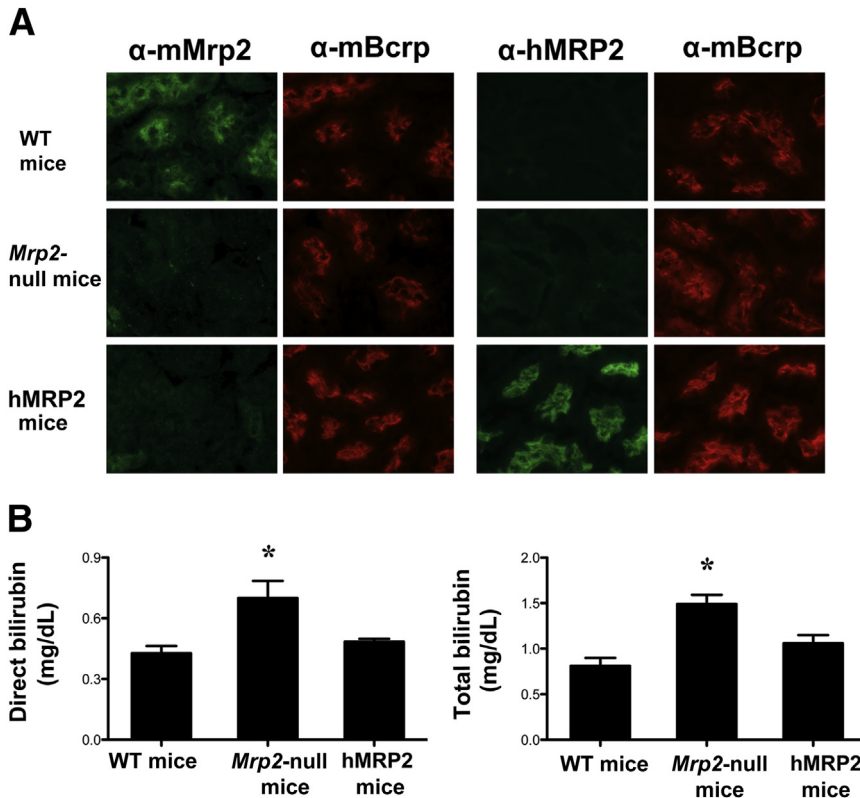


Figure 6 Characterization of hMRP2 mice. Indirect immunofluorescence against mMrp2 (EAG15 antibody) or hMRP2 (M₂III-6 antibody) (green) was conducted on 6- μ m kidney cryosections from control WT, *Mrp2*-null, and hMRP2 mice. Brush border localization of mMrp2 and hMRP2 proteins was confirmed by co-localization with mouse Bcrp protein (mBcrp; Bxp-53 antibody) (red). **A**: Representative cortex regions are shown. **B**: Direct and total bilirubin concentrations were quantified in plasma from control WT, *Mrp2*-null, and hMRP2 mice. Data are presented as means \pm SEM. $n = 3$ to 5. * $P < 0.05$ compared with naive WT mice. Original magnification, $\times 64$ (**A**).

triphosphate-dependent transport of CDCF in plasma membrane vesicles (Figure 1B). Transport of CDCF by both orthologs was inhibited up to 70% to 80% at 100 to 200 μ mol/L cisplatin. mMrp2-mediated transport was also markedly inhibited at 50 μ mol/L cisplatin. Note that serum concentrations as high as 20 μ mol/L have been observed clinically,²⁶ although it is unclear how this value translates to intracellular tubule levels.

Renal and Hepatic Injury

Cisplatin caused time-dependent renal injury in WT and *Mrp2*-null mice (Figure 2A). BUN levels increased similarly in both genotypes on day 3. By 4 days, BUN levels in cisplatin-treated *Mrp2*-null mice were twofold higher than those in WT mice. Likewise, elevations in serum creatinine levels were observed only on day 4, with a greater increase in *Mrp2*-null mice. Mild increases in alanine aminotransferase activity were also seen in cisplatin-treated *Mrp2*-null mice, with no change detected in WT mice.

Cisplatin-treated *Mrp2*-null mice exhibited significant elevations in the renal mRNA expression of kidney injury molecule-1 (fourfold), metallothionein-1 (twofold), and heme oxygenase-1 (2.5-fold) on day 4 compared with WT mice (Figure 2B). In addition, *Mrp2*-null mice had a 44% reduction in urine output between 3 and 4 days, although this decrease was not statistically significant (Figure 2C).

Histopathologic evaluation of kidneys from cisplatin-treated mice revealed S₁/S₂ proximal tubule degeneration and necrosis as well as sloughing of tubule epithelium that

was more severe in *Mrp2*-null mice at 4 days (Figure 3 and Table 2). Necrotic tubules contained renal casts with eosinophilic material and were more numerous in *Mrp2*-null mice. Livers from WT and *Mrp2*-null mice showed no evidence of hepatocellular degeneration and/or necrosis (data not shown).

Pt Concentrations

Kidney concentrations of Pt in *Mrp2*-null mice were elevated above WT levels at 24 and 48 hours (30% increase in the area under the curve) (Figure 4A). Similar to the kidneys, Pt concentrations were higher in the livers of *Mrp2*-null mice through 72 hours. The clearance of Pt from the plasma was similar between WT and *Mrp2*-null mice, suggesting similar uptake between genotypes. Using metabolic cages, total Pt excretion into urine was high and variable in both genotypes at 24 hours and decreased by 48 and 72 hours, with no differences observed (Figure 4A). Fractionation of kidneys from WT and *Mrp2*-null mice 24 hours after cisplatin treatment revealed 50% increases in Pt levels in cytosol and DNA but not in the crude membrane fraction, suggesting specificity in the subcellular localization of cisplatin intermediates and/or adducts (Figure 4B).

Kidney Uptake and Efflux Transporters

Because additional proteins can transport cisplatin, we quantified the basal expression of Oct2, Ctr1, and MATE1 mRNAs and proteins as well as additional apical efflux transporters, Mrp4, Bcrp, and multidrug resistance protein 1

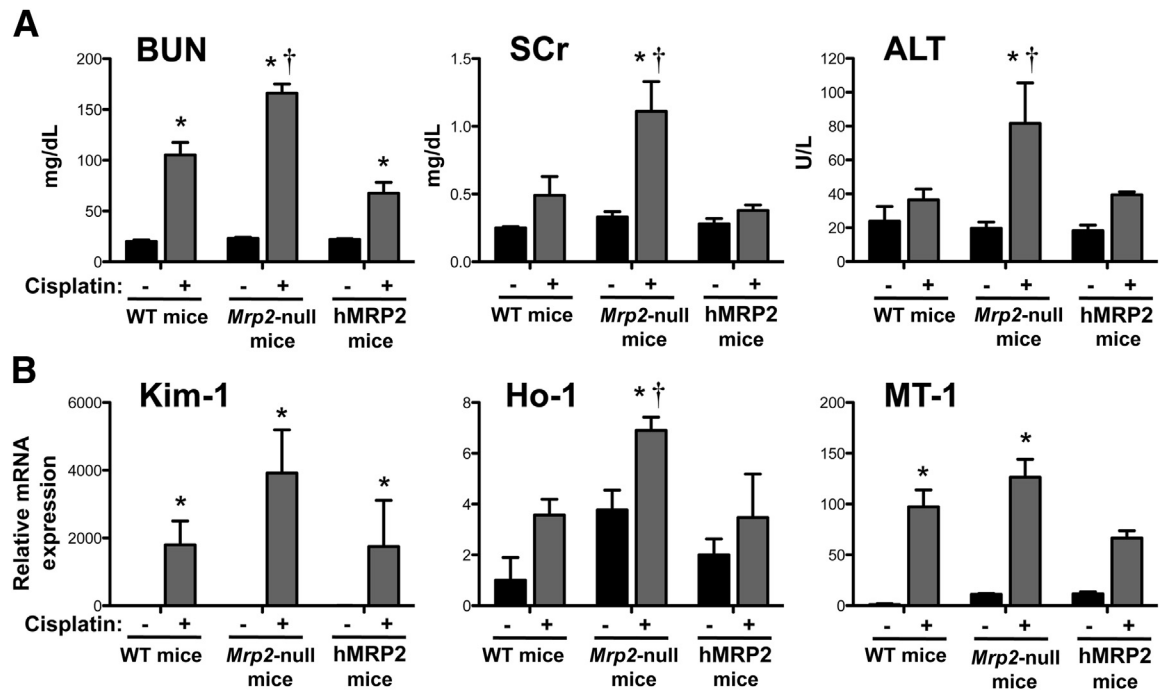


Figure 7 Renal and hepatic injury markers in WT, *Mrp2*-null, and hMRP2 mice treated with cisplatin. **A:** BUN, serum creatinine (Scr), and alanine aminotransferase (ALT) levels in plasma from WT, *Mrp2*-null, and hMRP2 mice 4 days after 20-mg/kg i.p. cisplatin treatment. **B:** mRNA expression of kidney injury molecule-1 (Kim-1), metallothionein-1 (Mt-1), and heme oxygenase-1 (Ho-1) was quantified using total kidney RNA from control and 20 mg/kg cisplatin-treated WT and *Mrp2*-null mice on day 4. Data are presented as means \pm SEM. $n = 3$ to 9 (**A** and **B**) * $P < 0.05$ compared with genotype control mice; † $P < 0.05$ compared with cisplatin-treated WT mice.

in both genotypes. Expression of Oct2, Ctr1, Mdr1, Bcrp, and multidrug resistance protein 1 mRNA and protein was similar in kidneys from naive WT and *Mrp2*-null mice (Figure 5, A and B). Because of the high expression of Oct1 mRNA in mice,²⁷ mRNA levels of this isoform were also assessed and unchanged in *Mrp2*-null mice (Figure 5A). Oct3 mRNA levels were very low in both genotypes (data not shown). *Mrp4* mRNA and protein levels were elevated in *Mrp2*-null mice, similar to previous reports.^{28,29} It is unclear whether *Mrp4* can transport cisplatin; however, this basal increase does not seem to compensate for reduced Pt excretion and enhanced toxicity in *Mrp2*-null mice.

Kidney GSH Pathways

Basal differences in the mRNA expression of enzymes involved in GSH production and conjugation were also quantified. Compared with WT mice, expression of *Gsta3*, *p1/2*, and *m1* was elevated 50% to 100% in kidneys from naive *Mrp2*-null mice (Figure 5C). There were no notable differences in *Gstt1* and glutamate cysteine ligase catalytic subunit mRNA levels between genotypes. Renal GSH concentrations were not significantly different between WT and *Mrp2*-null mice (Figure 5D).

hMRP2 Mice

hMRP2 mice have been recently generated as a transgenic knock-in in *Mrp2*-null mice.³⁰ To confirm proper trafficking

of hMRP2 protein to the brush border membrane of tubules, we used species-specific antibodies against *Mrp2*/MRP2 for co-localization with the mouse *Bcrp* transporter (Figure 6A). As expected, the anti-rodent *Mrp2* antibody detected protein in WT mice only, which co-localized with mouse *Bcrp* on the apical membrane of tubules. The anti-human MRP2 antibody detected MRP2 protein in tubules that co-localized with mouse *Bcrp* in the humanized mice. Neither m*Mrp2* nor hMRP2 protein was detected in the *Mrp2*-null mice. Similar insertion of hMRP2 protein into the canalicular membrane of hepatocytes was seen in humanized mice (Supplemental Figure S1). Bilirubin is a known *Mrp2*/MRP2 substrate. Similar to patients with Dubin-Johnson syndrome, mice lacking *Mrp2* have increased direct and total bilirubin levels.^{28,29} We similarly observed hyperbilirubinemia in *Mrp2*-null mice that was reduced in humanized mice (Figure 6B), confirming that the hMRP2 protein is functional in the transgenic animals.

Similar to Figure 2, administration of cisplatin to *Mrp2*-null mice increased BUN, serum creatinine, and alanine aminotransferase levels as well as kidney injury molecule-1 and heme oxygenase-1 mRNA levels to a greater extent than in WT mice (Figure 7). Expression of MRP2 in humanized mice restored these end points to levels observed in WT mice. Likewise, Pt levels were 41% higher in the kidneys of *Mrp2*-null mice compared with WT and hMRP2 mice (Figure 8A). No differences in plasma Pt levels were detected between genotypes (Figure 8B).

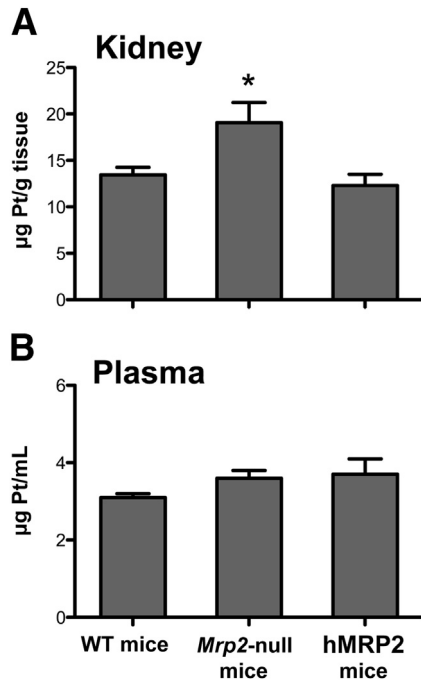


Figure 8 Pt concentrations in WT, *Mrp2*-null, and hMRP2 mice treated with cisplatin. Pt concentrations were quantified by inductively coupled plasma mass spectrometry in the kidneys (A) and plasma (B) of WT, *Mrp2*-null, and hMRP2 mice 24 hours after treatment with 20 mg/kg of i.p. cisplatin. Data are presented as means \pm SEM. $n = 6$ to 9 (A and B). * $P < 0.05$ from cisplatin-treated WT mice.

Discussion

We investigated the role of *Mrp2* in the disposition and toxicity of cisplatin in mice. We have shown enhanced susceptibility of *Mrp2*-null mice to renal injury by multiple end points (histopathology, serum analytes, urine output, and biomarker mRNAs). Differences in toxicity are likely due to higher concentrations of Pt in the kidneys of *Mrp2*-null mice. It can be suggested that elevated tissue Pt concentrations are due to impaired efflux rather than enhanced uptake because levels of Oct2 and Ctr1 mRNA and protein as well as Pt clearance from plasma were similar between genotypes. The present findings are in contrast to a recent study that concluded that *Mrp2* does not participate in the renal excretion and toxicity of cisplatin in mice.³¹ This conclusion was based on histopathological evaluation and urinary Pt concentrations in cisplatin-treated WT and *Mrp2*-null mice at 3 days. Note that these data are consistent with the present findings because we did not observe differences in renal injury between genotypes until 4 days. Likewise, quantification of Pt concentrations in the kidneys and liver may be a more sensitive measure of pharmacokinetics in mice. These findings strongly support the use of time course studies for comparison of toxicity end points. Furthermore, these data are consistent with previous *in vitro* studies demonstrating a role for MRP2 in cytoprotection against cisplatin in isolated hepatocytes,¹⁹ esophageal squamous

carcinoma cells,³² and hepatocellular carcinoma cells³³ but extends this function to normal kidneys.

Cisplatin-GSH is proposed to be metabolized by γ -glutamyltranspeptidase to a cysteinyl-glycine conjugate and aminopeptidase to a cysteine conjugate on the extracellular surface of the brush border membrane (Figure 9).^{34–36} The cysteine conjugates are then transported into proximal tubules and are metabolized by β -lyase to highly reactive thiols. The mechanism(s) for cisplatin conjugate reabsorption is not well-known. Neutral amino acid transporter complexes, such as system L, may mediate influx, similar to observations in carcinoma cells.³⁷ However, additional experiments are needed to identify the exact carriers involved in intact proximal tubules.

Although conjugation with GSH is generally a detoxification reaction, cisplatin conjugated to GSH, cysteinyl-glycine, and N-acetylcysteine is more toxic to cultured LLC-PK1 kidney cells than cisplatin itself.³⁴ This is further supported by studies demonstrating that pharmacological inhibition of γ -glutamyltranspeptidase and β -lyase protects mice from nephrotoxicity^{35,36} and that γ -glutamyltranspeptidase-null mice are resistant to cisplatin injury.³⁵ Because of the important role of GSH conjugation in the formation of reactive Pt metabolites, the higher expression of Gsts, despite similar GSH levels in *Mrp2*-null mice, may contribute to the higher susceptibility of these mice to cisplatin toxicity. Therefore, as more sensitive technologies

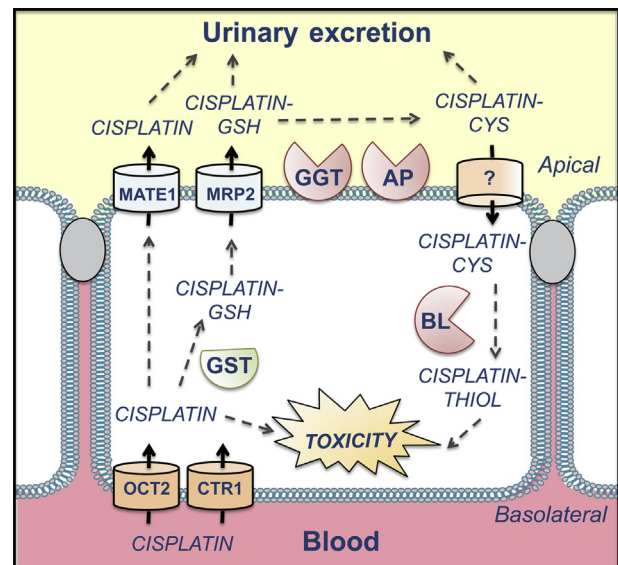


Figure 9 Proposed metabolism and transport of cisplatin renal proximal tubules. Cisplatin is removed from the blood by OCT2 and CTR1. Cisplatin can then be excreted by MATE1. Cisplatin also hydrolyzes spontaneously to aquated species that can bind DNA and cause toxicity or that can be conjugated with GSH by glutathione S-transferases (GSTs). We propose that the MRP2 effluxes cisplatin-GSH to the tubule lumen, where it can be cleaved by γ -glutamyltranspeptidase (GGT) and aminodipeptidase (AP) to cisplatin-cysteine (CYS). The cisplatin-CYS conjugate is then reabsorbed by the proximal tubule through an unknown mechanism. Cisplatin-CYS can then be metabolized by cysteine S-conjugate β -lyase (BL) to form a reactive thiol that contributes to toxicity.

for detecting cisplatin conjugates *in vivo* are developed, it will be important to identify the cisplatin species that are retained in the cytosol of *Mrp2*-null mice.

The hMRP2 protein traffics to the brush border and canalicular membranes and restores normal circulating bilirubin concentrations in *Mrp2*-null mice.³⁰ We have demonstrated that hMRP2 mice have a similar susceptibility to cisplatin nephrotoxicity as WT mice. In fact, BUN and serum creatinine levels were slightly lower in hMRP2 mice. This may be due to the higher MRP2 mRNA level in humanized mice compared with WT mice.³⁰ The ability of hMRP2 to protect against the toxicity of various pharmaceuticals has been demonstrated in multiple tissues based on single nucleotide polymorphism analyses. For example, hMRP2 polymorphisms alter the sensitivities of individuals to tenofovir nephrotoxicity,³⁸ irinotecan-induced diarrhea,³⁹ doxorubicin-induced cardiotoxicity,⁴⁰ and carbamazepine-induced neurotoxicity.⁴¹ A preliminary investigation revealed that MRP2 polymorphisms do not predict cisplatin pharmacokinetics and toxicity; however, this study was underpowered, and few variants were detected in the study population.³¹ Although nephrotoxicity was not evaluated, a polymorphism in the *MRP2* gene has been shown to predict greater cisplatin efficacy in patients with non-small cell lung cancer.⁴² Based on these findings, further work is needed to investigate MRP2 polymorphisms in predicting cisplatin efficacy and toxicity.

We observed elevated Pt concentrations in the kidneys and livers of *Mrp2*-null mice compared with WT mice (Figure 4A). In fact, the higher hepatic Pt concentrations persisted through 72 hours. The ability of the kidneys to ultimately excrete the Pt in *Mrp2*-null mice at a faster rate than the liver may reflect the higher expression of *Mate1* mRNA in the kidneys.⁴³ The greater hepatic burden of Pt in the livers of *Mrp2*-null mice did not result in toxicity. Although mild increases in serum alanine aminotransferase activity were observed between 3 and 4 days in the null mice, no histopathologic changes consistent with hepatocyte degeneration or necrosis were observed. This difference in organ sensitivity to cisplatin may reflect the unique ability of the kidneys to generate reactive thiols via β -lyase-mediated metabolism or possibly enhanced detoxification mechanisms in the liver.

The present data add to the existing literature regarding the transporters that participate in the renal disposition and toxicity of cisplatin (Figure 9). In conclusion, the absence of *Mrp2* exacerbates cisplatin-induced nephrotoxicity in mice due to elevated concentrations of Pt in the DNA and cytosol, and possibly due to enhanced *Gst* mRNA expression. Moreover, the *MRP2* gene protects *Mrp2*-null mice from cisplatin toxicity, demonstrating a renoprotective role for the human ortholog *in vivo*.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2014.01.025>.

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