MRP2 involvement in renal proximal tubular elimination of methylmercury mediated by DMPS or DMSA

Rudolfs K. Zalups *, Christy C. Bridges

Division of Basic Medical Sciences, Mercer University School of Medicine, 1550 College St., Macon, GA 31207, USA

ABSTRACT

2, 3-Dimercaptopropane-1-sulfonic acid (DMPS) and meso-2, 3-Dimercaptosuccinic acid (DMSA) are dithiols used to treat humans exposed to methylmercury (CH_3Hg^+). After treatment, significant amounts of mercury are eliminated rapidly from the kidneys and are excreted in urine. In the present study, we extended our previous studies by testing the hypothesis that MRP2 mediates the secretion of DMPS or DMSA S-conjugates of CH_3Hg^+. To test this hypothesis, the disposition of mercury was assessed in control and Mrp2-deficient (TR⁻) rats exposed intravenously to a 5.0-mg/kg dose of CH_3HgCl. Twenty-four and 28 h after exposure, groups of four control and four TR⁻ rats were injected with saline, DMPS, or DMSA. Tissues were harvested 48 h later. Renal and hepatic contents of mercury were greater in saline-injected TR⁻ rats than in controls. In contrast, the amounts of mercury excreted in urine and feces by TR⁻ rats were less than those by controls. DMPS and DMSA significantly reduced the renal and hepatic content of mercury in both groups of rats, with the greatest reduction in controls. A significant increase in urinary and fecal excretion of mercury (which was greater in the controls) was also observed. Our findings in inside-out membrane vesicles prepared from hMRP2-transfected Sf9 cells show that uptake of DMPS and DMSA S-conjugates of CH_3Hg^+ was greater in the vesicles containing hMRP2 than in control vesicles. Overall, these dispositional findings indicate that MRP2 does play a role in DMPS- and DMSA-mediated elimination of mercury from the kidney.

© 2008 Elsevier Inc. All rights reserved.

Introduction

Methylmercury (CH_3Hg^+) is the form of mercury to which humans are exposed most often. Following exposure to CH^3Hg^+, significant accumulation of mercuric ions occurs along renal proximal tubules (Zalups, 2000; Rodier et al., 1988). When the burden of mercury exceeds the capacity of proximal tubular epithelial cells to cope, cellular degeneration and necrosis ensues (Zalups, 2000). Thus, pharmacological intervention promoting cellular extraction of mercuric ions in the proximal tubular epithelium is crucial in limiting the amount of pathological effects in the kidneys. A number of chelating agents have been used to minimize the deleterious effects of organic mercuric compounds in humans. Two compounds used widely in both clinical and experimental settings are the water soluble, dithiol chelators, 2, 3-Dimercaptopropane-1-sulfonic acid (DMPS) and meso-2, 3-Dimercaptosuccinic acid (DMSA) (Aposhian, 1983; Aposhian et al., 1992; Planas-Bohne, 1981; Ruprecht, 1997; Zalups, 2000; Zalups et al., 1992). Although these two molecules are distinct structurally and chemically, they both possess vicinal thiol groups on carbons 2 and 3, which facilitate the formation of highly stable DMPS or DMSA S-conjugates with mercuric ions. Despite the extensive international use

of these two compounds, little is known about the molecular mechanisms involved in the proximal tubular extraction and urinary excretion of mercuric ions mediated by either DMPS or DMSA.

There is substantive evidence indicating that reduced and oxidized forms of DMPS (but not DMPS S-conjugates of inorganic mercury (Hg^{2+})) are taken up at the basolateral membrane of proximal tubular cells by OAT1, and possibly OAT3 (Bahn et al., 2002; Islinger et al., 2001; Zalups, 2000; Zalups et al., 1998). In contrast, it appears that DMSA is taken up at the basolateral membrane of these cells by the sodium-dependent dicarboxylate transporter, NaC2 (Burckhardt et al., 2002). Once in the intracellular environment, reduced DMPS and DMSA compete for mercuric ions bonded to intracellular thiols (Diamond et al., 1988; Stewart and Diamond, 1987, 1988). Subsequently, the newly formed DMPS or DMSA S-conjugates of mercury appear to be exported across the luminal plasma membrane into the tubular lumen for elimination in the urine.

We have demonstrated recently that the export protein, MRP2, participates in the process of secreting DMPS and DMSA S-conjugates of Hg^{2+} from the intracellular compartment of proximal tubular cells into the lumen (Bridges et al., 2008). MRP2 has also been implicated in the transport of N-acetylcysteine S-conjugates of CH_3Hg^+ (Madejczyk et al., 1997). However, the role of MRP2 in the transfer of DMPS or DMSA S-conjugates of CH_3Hg^+ from the intracellular compartment to the luminal compartment of proximal tubular segments has not yet been determined.

* Corresponding author. Fax: +1 478 301 5489.
E-mail address: Zalups_rk@mercer.edu (R.K. Zalups).

MRP2 is a member of the ATP-binding cassette transporter superfamily and, in the kidneys, is localized exclusively in the luminal plasma membrane of proximal tubular cells (Schaub et al., 1997). This transporter is believed to play a role in the excretion of various metabolic wastes and xenobiotics that have been extracted from blood (Zalups, 2000; Leslie et al., 2005). Given these previous findings, it is likely that MRP2 plays a role in the normal proximal tubular elimination (without chelation therapy) of mercuric ions following exposure to CH_3Hg^+.

Owing to significant differences in the toxico- and pharmaco-kinetics of Hg^{2+} and CH_3Hg^+, it is important to examine the disposition of CH_3Hg^+ under conditions similar to those in our previous studies. Therefore, the purpose of the current study was to assess the influence of DMPS and DMSA on the renal and corporal disposition of mercuric ions following exposure to a single dose of CH_3Hg^+. In addition, we tested the hypothesis that the export protein, MRP2 mediates the secretion of DMPS *S*- or DMSA *S*-conjugates of CH_3Hg^+ from within proximal tubular cells into the tubular lumen. We tested this hypothesis by studying the disposition of mercuric ions in Wistar (control) and MRP2-deficient (TR⁻) rats exposed to radiolabeled CH_3Hg^+ and treated subsequently with saline, DMPS, or DMSA. Moreover, we studied the transport of DMPS or DMSA *S*-conjugates of CH_3Hg^+ in inside-out membrane vesicles prepared from Sf9 cells containing functional hMRP2 protein in the plasma membrane. The experimental design used in the present study was the same as that used in our recent study on the role of MRP2 in the cellular elimination of Hg^{2+} (Bridges et al., 2008). This design was chosen so that the findings from our previous study could be compared with the findings of the present study. The results of the current study provide the first substantive line of evidence implicating MRP2 in the cellular secretion of DMPS or DMSA *S*-conjugates of CH_3Hg^+ in the kidneys.

Materials and methods

Generation of $^{203}Hg^{2+}$ and $CH_3[^{203}Hg^+]$

$^{203}Hg^{2+}$ was generated by the method described previously (Bridges et al., 2004; Belanger et al., 2001). Briefly, 3 mg of mercuric oxide were doubly sealed in quartz tubing with an acetylene torch. Subsequently, the sealed sample of mercuric oxide was sent to the Missouri University Research Reactor (MURR) facility to be irradiated via neutron activation for four weeks. After we received the irradiated sample from the MURR facility, the solid content of mercury was determined by plasma-coupled elemental mass spectrometry and the radioactivity of $^{203}Hg^{2+}$ in a solution of 1 N HCl was determined by counting in a Wallac Wizard 3 automatic gamma counter (Perkin Elmer, Gaithersburg, MD). The specific activities of the $^{203}Hg^{2+}$ ranged from 6 to 12 mCi/mg.

$CH_3[^{203}Hg^+]$ was generated later by following a protocol adapted from Rouleau and Block (1997). Two mCi of $^{203}Hg^{2+}$ were diluted to 40 μL in de-ionized water. Subsequently, 670 μL of 2 M acetate buffer and 2 mL of methylcobalamin were added to the solution. Methylcobalamin served as the donor of methyl groups. This mixture was incubated for 24 h at room temperature in a fume hood. Following incubation, 16.7 mL of 30% potassium chloride in 4% hydrochloric acid was added. $CH_3[^{203}Hg^+]$ was extracted with five washes of 8.3 mL of dichloromethane (DCM). The collected DCM was evaporated by bubbling nitrogen gas into the solution. Afterwards, the $CH_3[^{203}Hg^+]$ was collected and stored at −20 °C. The specific activity was calculated to be approximately 5 mCi/mg. The purity of the extracted $CH_3[^{203}Hg^+]$ has been confirmed previously by thin layer chromatography (Rouleau and Block, 1997).

Animals

Transport deficient (TR⁻) rats, characterized initially by Jansen et al. (1985), have hereditary conjugated hyperbilirubinemia, a condition

that has since been attributed to a mutation in the *Mrp2* gene (Mayer et al., 1995; Paulusma et al., 1996). Due to this mutation, the MRP2 protein is truncated, and thus, is non-functional in the tissues and organs of these animals (Mayer et al., 1995). TR⁻ rats represent a reliable model that has been used to study hepatic and renal secretion of various MRP2 substrates (de Vries et al., 1989; Masereeuw et al., 2003; Smeets et al., 2004).

Male TR⁻ and normal (control) Wistar rats were purchased from Harlan Laboratories (Indianapolis, IN). All animals were provided a commercial laboratory diet (Tekland 6% rat diet, Harlan Laboratories) and water *ad libitum* throughout all aspects of animal experimentation.

Experimental design

DMPS experiments. In the first set of experiments, control and TR⁻ rats were used to examine the disposition of CH_3Hg^+ in the kidneys, liver, brain and blood 48 h after intravenous injection of a non-toxic, 5 mg/kg, dose of CH_3HgCl. Two groups of control and two groups of TR⁻ rats (four animals per group) were selected randomly from a pool of control rats and TR⁻ rats, respectively. One group of control rats and one group of TR⁻ rats served as controls injected twice with 2 mL/kg normal saline (0.9% NaCl w/v in doubly distilled and de-ionized water). One group of control rats and one group of TR⁻ rats served as experimental animals that received two 100 mg/kg, intraperitoneal doses of DMPS (with each dose administered in 2 mL/kg normal saline). Saline and DMPS (Sigma Chemical Co., St. Louis, MO) were injected at both 24 h and 28 h after exposure to CH_3HgCl.

DMSA experiments. In a separate set of experiments, control and TR⁻ rats were used to examine the effects of DMSA on the disposition of mercury in the kidneys, liver, brain and blood 48 h after the intravenous injection of a non-toxic, 5 mg/kg dose of CH_3HgCl. Two groups of four control and two groups of four TR⁻ rats were selected randomly from the pool of control rats and TR⁻ rats, respectively. One group of control rats and one group of TR⁻ rats served as controls injected with two, 2 mL/kg volumes of normal saline. In an additional set of four control rats and four TR⁻ rats, two 100 mg/kg, intraperitoneal doses of DMSA (Sigma Chemical Co., St. Louis, MO) (with each dose administered in 2 mL/kg normal saline) were administered. Saline and DMSA were injected at both 24 h and 28 h after exposure to CH_3HgCl.

Intravenous injections

Intravenous injections were administered according to the method outlined previously (Zalups, 1993; Zalups et al., 1992). In brief, each animal was first anesthetized lightly with diethyl ether. Then, a small incision was made in the skin in the mid-ventral region of the thigh to expose the femoral vein and artery. The fascia around the femoral vein was trimmed away which permitted easy access to the femoral vein. After the injection of CH_3HgCl, pressure was applied briefly over the injection site. Subsequently, the incised skin was approximated using two 9-mm stainless steel wound clips. Animals were then placed individually in plastic metabolic cages.

Collection of tissues, organs, urine and feces

Forty eight hours after injection of CH_3HgCl, all rats were anesthetized with an intraperitoneal overdose of sodium-pentobarbital (100 mg/kg in 2 mL saline). Once each animal was in a state of deep anesthesia, two 1-mL samples of blood were obtained from the inferior vena cava with a 3-mL syringe and a 20-gauge needle. One of the samples was placed in a polystyrene tube for determination of ^{203}Hg content, while the other sample was placed in a Microtainer tube (Becton Dickenson and Co., Franklin Lakes, NJ), which was

centrifuged at 21,000 ×g for 90 s. Subsequently, the cellular and plasma fractions were removed and placed in separate polystyrene tubes for estimation of Hg content.

The right and left kidneys were also removed from each animal. Each kidney was weighed and cut in half along the mid- transverse plain. From one half of the left kidney, a 3-mm transverse slice was utilized for separation of cortex, outer stripe of outer medulla, inner stripe of outer medulla and inner medulla. Each zone of the kidney was weighed and placed in a polystyrene tube for estimation of ^{203}Hg content. Following removal of the kidneys, the liver and brain were excised carefully and weighed. A 1-g section of liver was removed for determination of ^{203}Hg content.

Urine and feces were collected throughout the duration of each experiment. After 24 h, and again after 48 h, the total volume of the urine excreted by each animal was collected. Subsequently the urine from each animal was mixed by vortexing and a 1-mL sample was weighed and placed in a polystyrene tube for estimation of ^{203}Hg content. All of the feces excreted by each animal during each 24-h period were counted to determine accurately the total fecal content of ^{203}Hg.

Determination of mercury content in samples of tissue, organs, urine, and feces

All samples were placed in 12×75 mm polystyrene tubes, which were sealed immediately to prevent evaporation or desiccation. The content of ^{203}Hg in each sample was determined by counting the samples in a Wallac Wizard 3 automatic gamma counter (Perkin Elmer, Boston, MA). The total content of Hg in the total renal mass, liver, brain, and blood is expressed as percent of administered dose. Concentrations of Hg in the renal zones are expressed as percent of dose per gram of tissue. Total blood volume was estimated to be 6% of body weight. Urinary and fecal excretion of Hg is expressed as percent of administered dose per 24 or 48 h.

Membrane vesicle transport assays

Transport of DMPS or DMSA S-conjugates of CH_3Hg^+ was studied in inside-out membrane vesicles containing the human isoform of MRP2. The inside-out membrane vesicles isolated from Sf9 cells transfected with *hMRP2* (cDNA) were obtained from Xenotech (Lenexa, KS). Transport assays were performed using a rapid filtration method described previously (Bridges et al., 2008; Chancy et al., 2000; Cui et al., 1999). These assays utilized inside-out vesicles prepared from both control and *hMRP2*-transfected Sf9 cells (Xenotech, Lenexa, KS). Prior to use, vesicles were centrifuged at 100,000 ×g for 40 min at 4 °C to remove the storage buffer. Vesicles were re-suspended in ice-cold incubation buffer (250 mM sucrose, 10 mM Tris/HCl, 10 mM $MgCl_2$, pH 7.4) by passing the suspension through a 27-gauge needle 10 times.

Mercuric conjugates were formed by mixing 5 μM CH_3Hg^+ containing $CH_3[^{203}Hg^+]$ with 12.5 μM DMPS or DMSA in the incubation buffer, which was supplemented with 10 mM creatine phosphate (Sigma) and 100 μg/mL creatine phosphokinase (Sigma) in the presence or absence of 5 mM ATP (Sigma). Prior to each experiment, membrane vesicles and mercuric conjugates were incubated separately for 15 min at 37 °C. Transport was initiated by the addition of 40 μL of membrane vesicle solution (20 μg) to 160 μL of buffer containing either species of mercuric conjugate. Transport was allowed to proceed for 15 min, after which, vesicles were collected on pre-wet Tuffryn filter discs (pore size, 0.2 μm; Pall, East Hills, NY). Filters were washed with two changes (8 mL each) of ice-cold stop buffer (250 mM sucrose, 10 mM Tris/HCl, 100 mM NaCl, 1 mM DMPS, pH 7.4). Filters were placed in 20-mL scintillation vials containing 1% SDS in 0.5 N NaOH. The amount of radioactivity present in each sample was measured in a Wallac Wizard 3", 1480 automatic gamma counter.

Data analyses

Data for each experiment were analyzed first with the Kolmogorov–Smirnov test for normality and then with Levene's test for homogeneity of variances. Data were then analyzed using a 2×2 two-way analysis of variance (ANOVA) to assess differences among the means. When statistically significant *F*-values were obtained with ANOVA, the data were analyzed using Tukey's *post hoc* multiple comparison test. A *P*-value of <0.05 was considered statistically significant.

Results

Effect of DMPS on the disposition of mercury

Renal disposition of mercury

In control and TR$^-$ rats exposed to CH_3HgCl and then treated subsequently with saline, the renal burden of mercury was approximately 6.8% and 7.3% of the administered dose, respectively (Fig. 1A). When control rats were treated with DMPS following exposure to CH_3HgCl, the renal burden of mercury (2.9%) was reduced significantly. Similarly, the renal burden of mercury in TR$^-$ rats (approximately 5.1% of the dose) was reduced significantly following treatment with DMPS. It is important to note that the renal burden of mercury was significantly greater in TR$^-$ rats than in control rats.
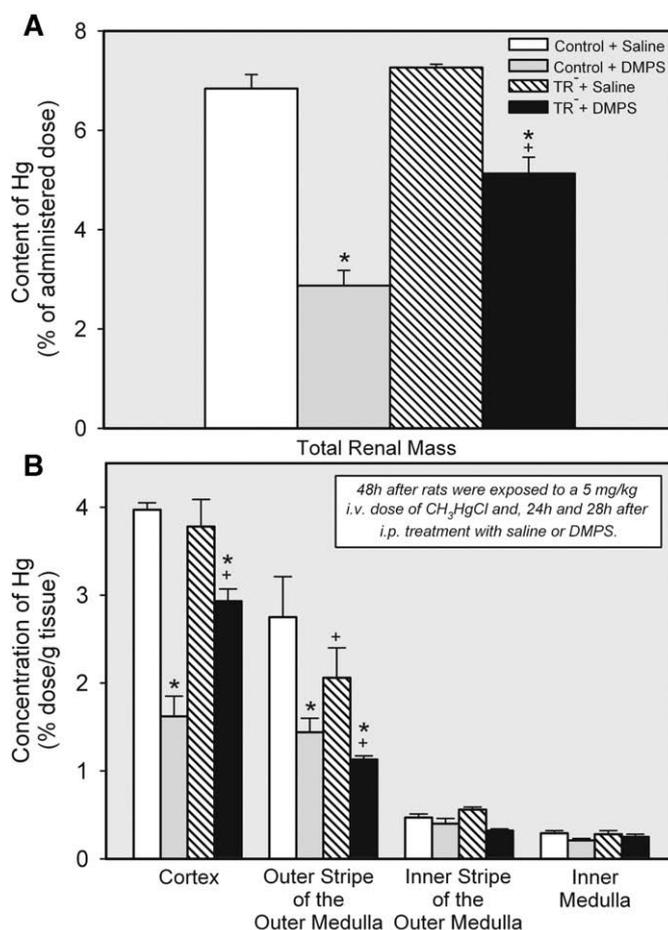

Fig. 1. Content of mercury in the total renal mass (A) and the concentration of mercury in renal zones (B) from control and TR$^-$ rats injected (i.p.) with 2,3-Dimercaptopropane-1-sulfonic acid (DMPS) or saline 24 and 28 h after exposure to a 5 mg/kg of CH_3HgCl (i.v.). Values are represented as mean±SEM. * = significantly different (*P*<0.05) from the mean for the corresponding group of rats treated with saline. + = significantly different (*P*<0.05) from the mean for the corresponding group treated in the same manner.

Concentrations of mercury in the renal cortex were greatest in the saline-treated rats (both control and TR⁻) (Fig. 1B). In control and TR⁻ rats treated with DMPS, the mean concentration of mercury in the renal cortex was significantly less than that in corresponding control and TR⁻ rats treated with saline. The mean value for the renal concentration of mercury in DMPS-treated TR⁻ rats was significantly greater than that in the corresponding group of control rats treated with DMPS.

The pattern for the accumulation of mercury in the outer stripe of the outer medulla among the four groups of rats was different from that detected in the renal cortex (Fig. 1B). The concentration of mercury in the renal outer stripe of the outer medulla in saline-treated TR⁻ rats was significantly less than that in the corresponding group of saline-treated control rats. In addition, the concentration of mercury in the outer stripe of the outer medulla of DMPS-treated TR⁻ rats was significantly less than that of DMPS-treated control rats. However, the concentration of mercury in the outer stripe of the medulla of DMPS-treated control and TR⁻ rats was significantly less than that for the corresponding groups of saline-treated control and TR⁻ rats, respectively.

No significant differences in the concentration of mercury in the inner stripe of the outer medulla or in the inner medulla were detected among the four groups of rats studied (Fig. 1B).

Disposition of mercury in the liver and blood

The hepatic burden of mercury of saline-treated TR⁻ rats (which was about 14.4% of the dose) was more than three times greater than that (about 4.2% of the dose) of saline-treated control rats (Fig. 2). Similarly, the amount of mercury present in the liver of TR⁻ rats treated with DMPS (approximately 9.4% of the dose) was significantly greater than that in control rats treated with DMPS.

The hematological burden of mercury in saline-treated control rats was significantly greater (approximately 33% of the dose) than in corresponding TR⁻ rats (approximately 30% of the dose) (Fig. 2). The amount of mercury present in blood of both, control and TR⁻ rats, was reduced significantly by treatment with DMPS. In all groups of rats, over 99% of the mercury detected in blood was associated with the cellular fraction, while less than 1% was present in plasma (data not shown).

Fig. 3. Amount of mercury excreted in the urine and feces in 48 h (% of administered dose) by control and TR⁻ rats injected (i.p.) with 2,3-Dimercaptopropane-1-sulfonic acid (DMPS) or saline 24 and 28 h after exposure to a 5 mg/kg of CH₃HgCl (i.v.). Values are represented as mean±SEM. * = significantly different ($P<0.05$) from the mean for the corresponding group of rats treated with saline. + = significantly different ($P<0.05$) from the mean for the corresponding group treated in the same manner.

Urinary and fecal excretion of mercury

Control and TR⁻ rats treated with saline excreted less than one percent of the administered dose of mercury in the urine during the 48 h subsequent to CH₃HgCl exposure (Fig. 3). Treatment of both strains of rat with DMPS increased the total amount of mercury excreted in the urine in 48 h to 20% of the administered dose. There was no significant difference in the mean values for the urinary excretion of mercury between control and TR⁻ rats treated with DMPS.

The fecal excretion of mercury was not significantly different between saline-treated control rats and saline-injected TR⁻ rats (Fig. 3). In these two groups, only about 2.5% of the administered dose was excreted in the feces during the 48-h period of study. Treatment with DMPS significantly increased the fecal excretion of mercuric ions in control and TR⁻ rats. Fecal excretion of mercury was significantly greater in the DMPS-treated TR⁻ rats than in the corresponding DMPS-treated controls.

Disposition of mercury in the brain

Very little of the administered dose of mercury was detected in the brain of the rats used in the present study (Fig. 4). The greatest burden of mercury was detected in the control and TR⁻ rats treated with saline. In these rats, the burden of mercury in the brain was approximately 0.2% of the administered dose. Treatment of control and TR⁻ rats with DMPS reduced the content of mercury in the brain by more than 50%.

Effects of DMSA on the disposition of mercury

Renal disposition of mercury

The patterns for the renal disposition of mercury of rats treated with DMSA (Figs. 5A and B) were similar to those of rats treated with DMPS (Figs. 1A and B). Treatment of control and TR⁻ rats with DMSA reduced the renal burden of CH₃Hg⁺ in both strains of rats. Even so, the renal burden of Hg²⁺ remained significantly greater in TR⁻ rats than in control rats.

Disposition of mercury in the liver and blood

The disposition of mercury in the liver and blood of rats treated with DMSA (Fig. 6) was similar to that of rats treated with DMPS (Fig. 2). Treatment with DMSA reduced significantly the hepatic and hematologic burden of CH₃Hg⁺ in both, control and TR⁻ rats.
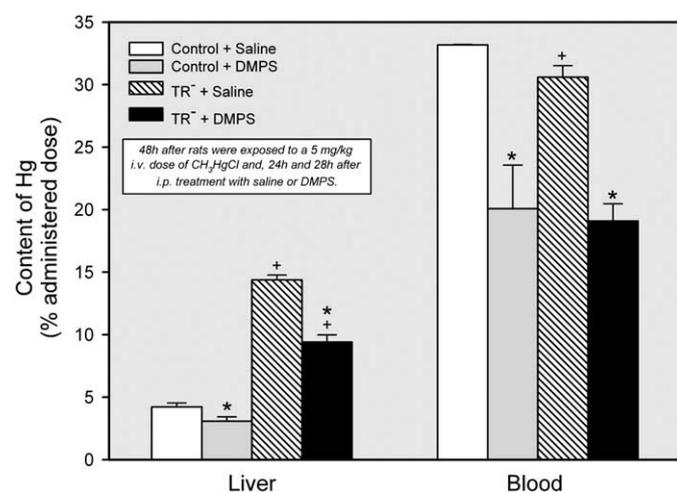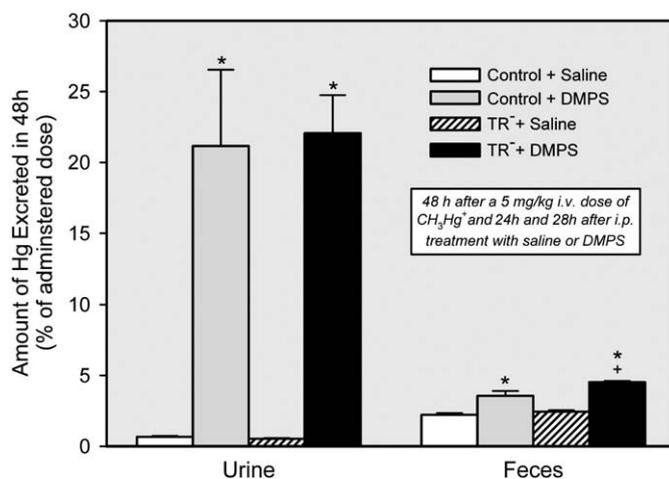

Fig. 2. Content of mercury in the liver and blood (% of administered dose) from control and TR⁻ rats injected (i.p.) with 2,3-Dimercaptopropane-1-sulfonic acid (DMPS) or saline 24 and 28 h after exposure to a 5 mg/kg of CH₃HgCl (i.v.). Values are represented as mean±SEM. * = significantly different ($P<0.05$) from the mean for the corresponding group of rats treated with saline. + = significantly different ($P<0.05$) from the mean for the corresponding group treated in the same manner. Content of blood in each animal was estimated to be 6% of body weight.
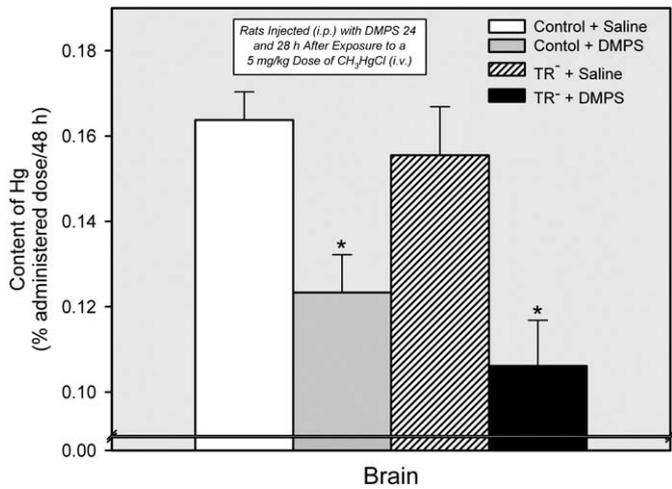
Fig. 4. Content of mercury in the brain (% of administered dose) of control and TR⁻ rats injected (i.p.) with 2,3-Dimercaptopropane-1-sulfonic acid (DMPS) or saline 24 and 28 h after exposure to a 5 mg/kg of CH_3HgCl (i.v.). Values are represented as mean± SEM. * = significantly different ($P<0.05$) from the mean for the corresponding group of rats treated with saline.
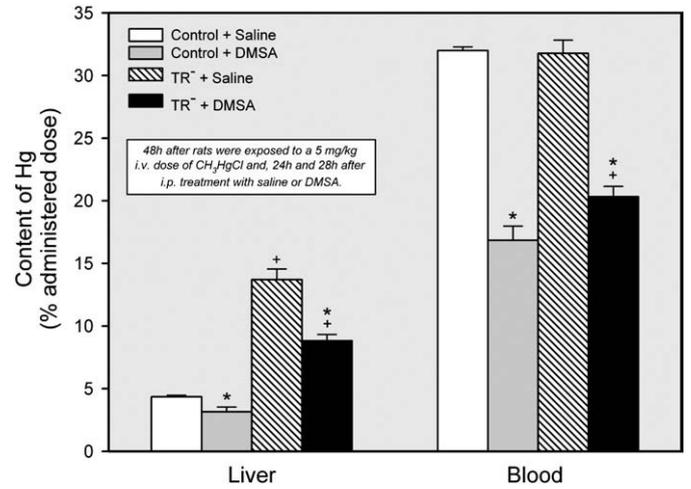

Fig. 6. Content of mercury in the liver and blood (% of administered dose) from control and TR⁻ rats injected (i.p.) with 2,3-Dimercaptosuccinic acid (DMSA) or saline 24 and 28 h after exposure to a 5 mg/kg of CH_3HgCl (i.v.). Values are represented as mean±SEM. * = significantly different ($P<0.05$) from the mean for the corresponding group of rats treated with saline. + = significantly different ($P<0.05$) from the mean for the corresponding group treated in the same manner. Content of blood in each animal was estimated to be 6% of body weight.

Urinary and fecal excretion of mercury

Urinary excretion of mercury was similar in both TR⁻ and control saline-treated rats, which excreted less than one percent of the administered dose of mercury in the urine during the 48 h after exposure to CH_3HgCl (Fig. 7). Unlike TR⁻ rats treated with DMPS (Fig. 3), the amount of mercury excreted in urine of TR⁻ rats after treatment with DMSA (Fig. 7) was significantly less than that in corresponding control rats treated with DMSA.

The fecal excretion of mercury was significantly greater in control rats treated than in TR⁻ rats (Fig. 7). Treatment of rats with DMPS did not alter significantly the fecal excretion of mercury in control or TR⁻ rats.

Disposition of mercury in the brain

The greatest burden of mercury in the brain was detected in control rats treated with saline (Fig. 8). Treatment with DMSA reduced
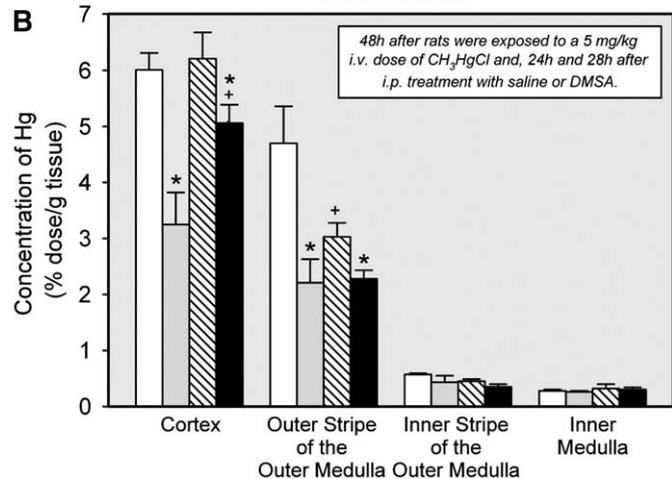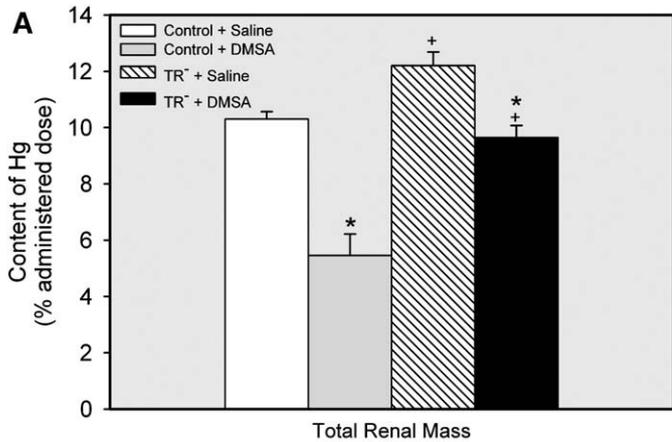

Fig. 5. Content of mercury in the total renal mass (A) and the concentration of mercury in renal zones (B) from control and TR⁻ rats injected (i.p.) with 2,3-Dimercaptosuccinic acid (DMSA) or saline 24 and 28 h after exposure to a 5 mg/kg of CH_3HgCl (i.v.). Values are represented as mean±SEM. * = significantly different ($P<0.05$) from the mean for the corresponding group of rats treated with saline. + = significantly different ($P<0.05$) from the mean for the corresponding group treated in the same manner.
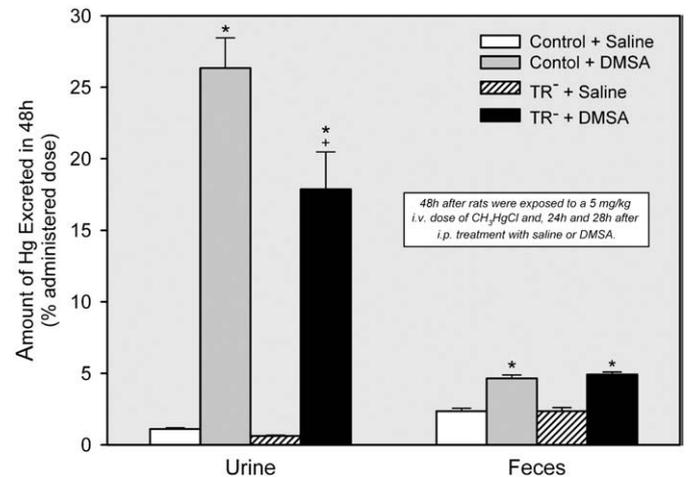

Fig. 7. Amount of mercury excreted in the urine and feces in 48 h (% of administered dose) by control and TR⁻ rats injected (i.p.) with 2,3-Dimercaptosuccinic acid (DMSA) or saline 24 and 28 h after exposure to a 5 mg/kg of CH_3HgCl (i.v.). Values are represented as mean±SEM. * = significantly different ($P<0.05$) from the mean for the corresponding group of rats treated with saline. + = significantly different ($P<0.05$) from the mean for the corresponding group treated in the same manner.

the content of mercury in the brain of both control and TR⁻ rats. The content of mercury in the brain of control treated with DMSA was not significantly different from that of TR⁻ rats treated with DMSA.

Transport of DMPS and DMSA S-conjugates of CH₃Hg⁺ in inside-out membrane vesicles containing the human isoform of MRP2

Uptake of CH_3Hg^+, when co-delivered with DMPS, was significantly greater in membrane-vesicles from Sf9 cells containing hMRP2 than in the membrane vesicles from control Sf9 cells (Fig. 9).

Similarly, when CH_3Hg^+ was co-delivered with DMSA, uptake of CH_3Hg^+ was significantly greater in the membrane vesicles from the Sf9 cells containing hMRP2 than in the membrane vesicles from control Sf9 cells (Fig. 9).

Discussion

In the present study, we used Wistar (control) and TR⁻ rats exposed to a single dose of CH_3Hg^+ to examine the potential role of the transport protein, MRP2, in reducing the renal cellular burden of mercury subsequent to treatment with the dithiol chelators, DMPS or DMSA. The present findings, especially the membrane vesicle and whole animal renal data, provide substantive support for our hypothesis that DMPS and DMSA S-conjugates of CH_3Hg^+ are exported out of the intracellular compartment of proximal tubular epithelial cells into the tubular lumen via MRP2.

Our whole animal data demonstrate that treatment with DMPS or DMSA significantly decreases the renal burden of mercury in both control and TR⁻ rats. Interestingly, treatment with DMPS or DMSA reduced the renal content of mercury to a much greater extent in control rats than in corresponding TR⁻ rats. The differences in the renal disposition of mercury between corresponding control and TR⁻ rats suggest strongly that MRP2 and at least one additional transport system participate in reducing the renal cellular burden of mercury following treatment with DMPS or DMSA in rats exposed previously to CH_3Hg^+. Overall, the whole animal renal findings suggest that the activity of MRP2 accounts for approximately 51% and 45% of the decrease in the renal burden of mercury mediated by DMPS and DMSA, respectively, in the control rats. It is not known at present which additional transporter(s) might account for the additional decreases in the renal burden of mercury mediated by either DMPS or DMSA. However, MRP4, another multidrug resistance protein present in the luminal plasma membrane of proximal tubular epithelial cells
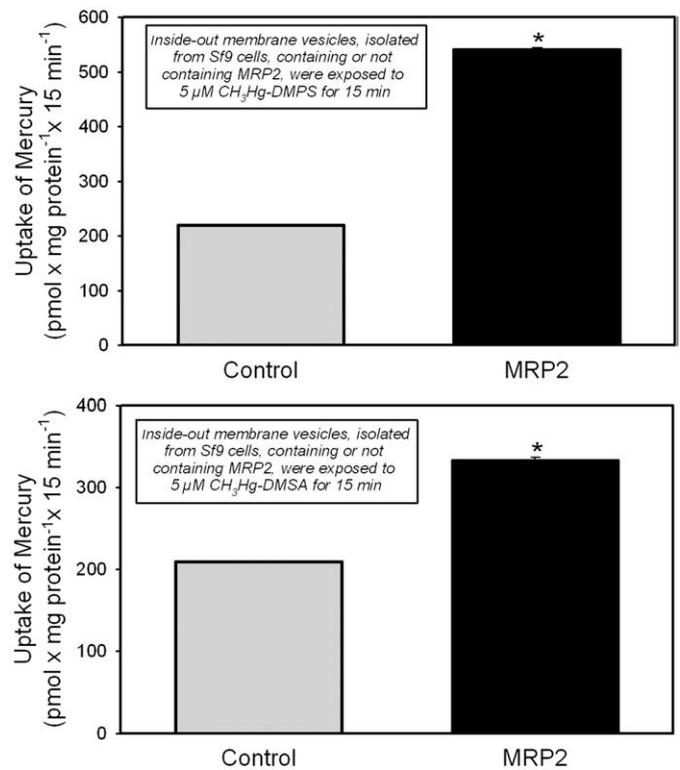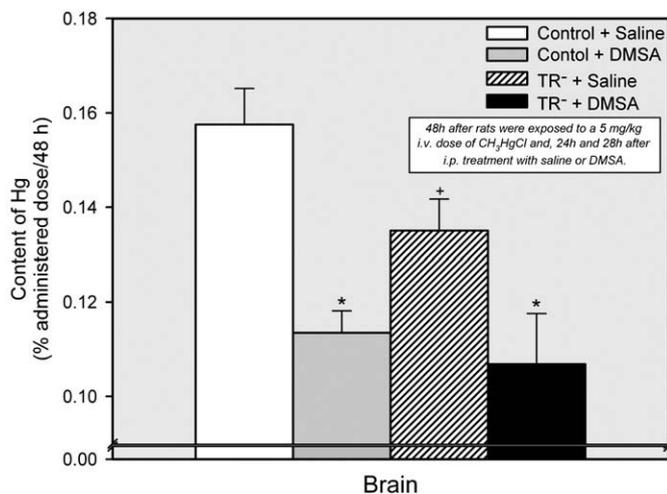

Fig. 8. Content of mercury in the brain (% of administered dose) of control and TR⁻ rats injected (i.p.) with 2,3-Dimercaptosuccinic acid (DMSA) or saline 24 and 28 h after exposure to a 5 mg/kg of CH_3HgCl (i.v.). Values are represented as mean±SEM. * = significantly different ($P<0.05$) from the mean for the corresponding group of rats treated with saline. + = significantly different ($P<0.05$) from the means for all other groups.

Fig. 9. Rate of uptake of CH_3Hg^+ in inside-out membrane vesicles from control Sf9 cells or Sf9 cells transfected with cDNA for *hMRP2*. Membrane vesicles from both types of Sf9 cells were co-exposed to 5 μM CH_3Hg^+ and 12.5 μM 2,3-Dimercaptopropane-1-sulfonic acid (DMPS) or 2,3-Dimercaptosuccinic acid (DMSA) for a period of 15 min. Values represent mean±SEM. * = significantly different from the mean for the corresponding group of membrane vesicles isolated from control Sf9 cells.

(Van Aubel et al., 2002), is a potential candidate. MRP4 has been identified in proximal tubules of TR⁻ rats (Chen et al., 2005; Johnson et al., 2006). Interestingly, it has been suggested that the protein expression of MRP4 is increased in these rats (Chen et al., 2005); however, this notion has been contradicted by other studies (Johnson et al., 2006).

Additionally, the current data show that treatment with DMPS or DMSA decrease the renal burden of mercury (in both control and TR⁻ rats) by diminishing the content of mercury in tubular segments of the cortex and outer stripe of the outer medulla. Since MRP2 in the kidneys is located exclusively in luminal plasma membrane of proximal tubular cells, and since S1 and S2 segments of the proximal tubule are present in the cortex and S3 segments are present only in the outer stripe of the outer medulla, one can conclude that the reductions in the renal cellular burden of mercury mediated by either DMPS or DMSA occur along the entire length of the proximal tubule. Additional support for this notion comes from the dispositional findings showing that DMPS or DMSA did not affect significantly the content of mercury in the inner stripe of the outer medulla or inner medulla, which contain various segments of the nephron beyond the proximal tubule and normally accumulate very little mercury.

The current renal data are consistent, in part, with the findings obtained recently in DMPS- and DMSA-treated control and TR⁻ rats exposed to a non-toxic dose of Hg^{2+} (Bridges et al., 2008). It should be noted, however, that there were significant differences between our present study and the previous one. In the present study, treatment with DMPS and DMSA reduced the total pool of renal mercury in both control and TR⁻ rats, indicating that MRP2 plus one or more transport mechanisms participate in extracting DMPS or DMSA S-conjugates mercury from proximal tubular cells following exposure to CH_3Hg^+. Interestingly, in our previous study in which animals were exposed to

Hg^{2+}, DMSA did not alter significantly the renal burden of mercury in TR^- rats, suggesting that, under these conditions, MRP2 may be the sole or primary mechanism responsible for transporting DMSA S-conjugates of Hg^{2+} from the intracellular compartment into the luminal compartment of proximal tubules.

As mentioned above, the *in vitro* findings from inside-out membrane vesicles containing hMRP2 indicate that MRP2 is capable of transporting DMPS or DMSA S-conjugates of CH_3Hg^+. More specifically, uptake of DMPS or DMSA S-conjugates of CH_3Hg^+ was significantly greater in inside-out vesicles containing hMRP2 than in corresponding control vesicles. Of all the findings in the present study, these data provide the most definitive evidence supporting a role for MRP2 in the transport of DMPS- and DMSA S-conjugates of CH_3Hg^+.

Along with the diminution in the renal burden of mercury during the 24 h following treatment with DMPS or DMSA (in both control and TR^- rats), there was a tremendous increase in the urinary excretion of mercury. However, the amount of mercury extracted from the renal parenchyma accounts for only a small percentage of the enhanced urinary excretion of mercury mediated by either chelator. The precise mechanisms involved in the profound increase in the urinary excretion of mercury subsequent to treatment with either DMPS or DMSA are not known at present, but may be related to chelation of CH_3Hg^+ in blood, which would provide a form of CH_3Hg^+ that is filtered freely at the glomerulus and excreted readily in the urine.

The hepatic burden of mercury in TR^- rats was significantly greater than that in control rats. This finding is most likely related to the absence of MRP2 on the canalicular membrane of hepatocytes, which would reduce or prevent hepatobiliary secretion of mercuric species. Sugawara et al. (1998) have also reported decreased hepatobiliary elimination of Hg^{2+} in TR^- rats. When rats were treated with DMPS or DMSA, each chelator reduced the hepatic content of mercury, with the greatest absolute, but not fractional, decrease detected in the TR^- rats. In addition, fecal excretion of mercury increased in the control and TR^- rats treated with either chelator. The ability of DMPS and DMSA to reduce the hepatic burden of mercury is consistent with that reported previously in Sprague–Dawley rats exposed to Hg^{2+} (Zalups, 1993).

Levels of mercury in the blood of control and TR^- rats were substantial, accounting for as much as 33% of the administered dose. Following treatment with either DMPS or DMSA, the levels of mercury in blood were reduced significantly in both control and TR^- rats. This finding is consistent with the notion that DMPS or DMSA S-conjugates of mercury form in blood, and as they enter into renal circulation, they are filtered freely in renal glomeruli and are excreted subsequently in urine.

Interestingly, 48 hours after exposure to Hg^{2+}, less than 1% of the administered dose of Hg^{2+} is present in the total volume of blood (Bridges et al., 2008). The increased presence of mercuric ions in blood after exposure to CH_3Hg^+ may be related to the presence of multiple transport systems for CH_3Hg^+ in erythrocytes (Wu, 1996).

It is also important to note that treatment with either DMPS or DMSA reduced significantly the content of mercury in the brain, although the amount of mercury in the brain in control saline-treated rats was only about 0.16% of the administered dose. In control rats, treatment with either chelator reduced the levels of mercury in the brain by 25–29%. In contrast, treatment of TR^- rats with DMPS or DMSA resulted in an even greater level of extraction of mercury from the brain. More specifically, there was a 31–34% decrease in the content of mercury in the brain in these animals. At present, however, it is not clear how either chelator diminished the content of mercury in the brain. It is possible that some of the decrease may be related to chelation of mercuric ions within the vascular compartment of the brain.

Numerous studies in humans have demonstrated that DMPS and DMSA reduce effectively the body burden of mercury (Aposhian, 1983). Overall, the findings from the present study (particularly those obtained from the control rats) are consistent with the effects of DMPS and DMSA found in humans. It has also been shown in humans and animals that following exposure to Hg^{2+}, treatment with DMPS or DMSA promotes the urinary excretion of both Hg^{2+} and the respective chelator used (Maiorino et al., 1991; Ruprecht, 1997; Zalups, 1993). Our current data from membrane vesicles support the theory that DMPS or DMSA S-conjugates of mercury are species of mercury that are transported out of proximal tubular cells by MRP2 for elimination in the urine.

The actual structures of the DMPS and DMSA S-conjugates of Hg^{2+} or CH_3Hg^+ formed in proximal tubular cells are unclear presently. However, several studies have shown that stable DMPS S- or DMSA S-conjugates of Hg^{2+} are formed by a single mercuric ion bonded to the vicinal thiol groups on a single molecule of DMPS or DMSA, respectively (Aposhian and Aposhian, 1990; Aposhian et al., 1995; Maiorino et al., 1991; Rivera et al., 1989; Ruprecht, 1997). Based on X-ray spectroscopy and computational analyses, George and colleagues (2004), argue that at least two molecules of DMPS or DMSA and two inorganic mercuric ions are required to form respective DMPS S- or DMSA S-conjugates. Irrespective of the exact species of DMPS or DMSA conjugates formed intracellularly, it is clear that DMPS and DMSA are each capable of effectively reducing the renal and body burden of Hg^{2+} (Aposhian, 1983; Aposhian et al., 1992; Planas-Bohne, 1981; Ruprecht, 1997; Zalups et al., 1992).

In summary, the findings from the present study show that both DMPS and DMSA reduce significantly the renal, hepatic and hematological burden of mercury in control and TR^- rats exposed to a non-toxic dose of CH_3Hg^+. The current findings also show that treatment with DMPS or DMSA promotes the urinary and fecal excretion of mercury. Overall, the data obtained from the animals and membrane vesicles represent the first line of evidence supporting a role for MRP2 in the DMPS- and DMSA-mediated elimination of mercuric ions following exposure to CH_3Hg^+.

References

Aposhian, H.V., 1983. DMSA and DMPS — water soluble antidotes for heavy metal poisoning. Annu. Rev. Pharmacol. Toxicol. 23, 193–215.

Aposhian, H.V., Aposhian, M.M., 1990. Meso-2,3-dimercaptosuccinic acid: chemical, pharmacological, and toxicological properties of an orally effective metal chelating agent. Annu. Rev. Pharmacol. Toxicol. 30, 279–306.

Aposhian, H.V., Maiorino, R.M., Rivera, M., Bruce, D.C., Dart, R.C., Hurlbut, K.M., Levine, D.J., Zheng, W., Fernando, Q., Carter, D., Aposhian, M.M., 1992. Human studies with the chelating agents DMPS and DMSA. Clin. Toxicol. 30, 505–528.

Aposhian, H.V., Maiorino, R.M., Gonzalez-Ramirez, D., Zuniga-Charles, M., Xu, Z., Hurlbut, K.M., Junco-Munoz, P., Dart, R.C., Aposhian, M.M., 1995. Mobilization of heavy metals by newer, therapeutically useful chelating agents. Toxicology 97, 23–38.

Bahn, A., Knabe, M., Hagos, Y., Rodiger, M., Godehardt, S., Graber-Neufeld, D.S., Evans, K.K., Burckhardt, G., Wright, S.H., 2002. Interaction of the metal chelator 2,3-dimercapto-1-propanesulfonate with the rabbit multispecific organic anion transporter 1 (rbOAT1). Mol. Pharmacol. 62, 1128–1136.

Belanger, M., Westin, A., Barfuss, D.W., 2001. Some health physics aspects of working with ^{203}Hg in university research. Health Phys. 80 (Suppl. 1), S28–S30.

Bridges, C.C., Bauch, C., Verrey, F., Zalups, R.K., 2004. Mercuric conjugates of cysteine are transported by the amino acid transporter system $b^{0,+}$: implications of molecular mimicry. J. Am. Soc. Nephrol. 15, 663–673.

Bridges, C.C., Joshee, L., Zalups, R.K., 2008. Multidrug resistance proteins and the renal elimination of inorganic mercury mediated by DMPS or DMSA. J. Pharmacol. Exp. Ther. 324, 383–390.

Burckhardt, B.C., Drinkuth, B., Menzel, C., Konig, A., Steffgen, J., Wright, S.H., Burckhardt, G., 2002. The renal Na⁺-dependent dicarboxylate transporter, NaDC-3 translocates dimethyl- and disulfhydryl-compounds and contributes to renal heavy metal detoxification. J. Am. Soc. Nephrol. 13, 2628–2638.

Chancy, C.D., Kekuda, R., Huang, W., Prasad, P., Kuhnel, J.M., Sirotnak, F.M., Roon, P., Ganapathy, V., Smith, S.B., 2000. Expression and differential polarization of the reduced-folate transporter-1 and the folate receptor α in mammalian retinal pigment epithelium. J. Biol. Chem. 275, 20676–20684.

Chen, C., Slitt, A.L., Dieter, M.Z., Tanaka, Y., Scheffer, G.L., Klaassen, C.D., 2005. Up-regulation of Mrp4 expression in kidney or Mrp2-deficent TR⁻ rats. Biochem. Pharmacol. 70, 1088–1095.

Cui, Y., König, J., Buchholz, U., Spring, H., Leier, I., Keppler, D., 1999. Drug resistance and ATP-dependent transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells. Mol. Pharmacol. 55, 929–937.

de Vries, M.H., Redegeld, F.A.M., Koster, A.S., Noordhoek, J., de Haan, J.G., Oude Elferink, R.P.J., Jansen, P.L.M., 1989. Hepatic, intestinal and renal transport of 1-naphthol-β-D-glucuronide in mutant rats with hereditary-conjugated hyperbilirubinemia. Arch. Pharm. 340, 588–592.

Diamond, G.L., Klotzbach, J.M., Stewart, J.R., 1988. Complexing activity of 2,3-dimercapto-1-propanesulfonate and its disulfide auto-oxidation product in rat kidney. J. Pharmacol. Exp. Ther. 246, 270–274.

George, G.N., Prince, R.C., Gailer, J., Buttigieg, G.A., Denton, M.B., Harris, H.H., Pickering, I.J., 2004. Mercury binding to the chelation therapy agents DMSA and DMPS and the rational design of custom chelators for mercury. Chem. Res. Toxciol. 17, 999–1006.

Islinger, F., Gekle, M., Wright, S.H., 2001. Interaction of 2,3-dimercatpo-1-propane sulfonate with the human organic anion transporter hOAT1. J. Pharmacol. Exp. Ther. 299, 741–747.

Jansen, P.L.M., Peters, W.H., Lamers, W.H., 1985. Hereditary chronic conjugated hyperbilirubinemia in mutant rats caused by defective hepatic anion transport. Hepatology 5, 573–579.

Johnson, B.M., Zhang, P., Schuetz, J.D., Brouwer, K.L.R., 2006. Characterization of transport protein expression in multidrug resistance-associated protein (MRP) 2-deficient rats. Drug Metab. Dispos. 34, 556–562.

Leslie, E.M., Deeley, R.G., Cole, S.P.C., 2005. Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. Toxicol. Appl. Pharmacol. 204, 216–237.

Madejczyk, M.S., Aremu, D.A., Simmons-Willis, T.A., Clarkson, T.W., Ballatori, N., 1997. Accelerated urinary excretion of methylmercury following administration of its antidote N-acetylcysteine requires Mrp2/Abcc2, the apical multidrug resistance-associated protein. J. Pharmacol. Exp. Therap. 322, 378–384.

Maiorino, R.M., Dart, R.C., Carter, D.E., Aposhian, H.V., 1991. Determination and metabolism of dithiol chelating agents. XII. Metabolism and pharmokinetics of sodium 2,3-dimercaptopropane-1-sulfonate in humans. J. Pharmacol. Exp. Therap. 259, 808–814.

Masereeuw, R., Notenboom, S., Smeets, P.H.E., Wouterse, A.C., Russel, F.G.M., 2003. Impaired renal secretion of substrates for the multidrug resistance protein 2 in mutant transport-deficient (TR⁻) rats. J. Am. Soc. Nephrol. 14, 2741–2749.

Mayer, R., Kartenbeck, J., Buchler, M., Jedlitschky, G., Leier, I., Keppler, D., 1995. Expression of the MRP gene-encoded conjugate export pump in liver and its selective absence from the canalicular membrane in transport-deficient mutant hepatocytes. J. Cell Biol. 131, 137–150.

Paulusma, C.C., Bosma, P.J., Zaman, G.T., Bakker, C.T., Otter, M., Scheffer, G.L., Scheper, R.J., Borst, P., Oude Elferink, R.P., 1996. Congenital jaundice in rats with a mutation in a multidrug resistance-associated protein gene. Science 271, 1126–1128.

Planas-Bohne, F., 1981. The effect of 2,3-dimercaptopropane-1-sulfonate and dimer-captosuccinic acid on the distribution and excretion of mercuric chloride in rats. Toxicology 19, 275–278.

Rivera, M., Zheng, W., Aposhian, V., Fernando, Q., 1989. Determination and metabolism of dithiol chelating agents. VIII. Metal complexes of meso-dimercaptosuccinic acid. Toxicol. Appl. Pharmacol. 100, 96–106.

Rodier, P.M., Kates, B., Simons, R., 1988. Mercury localization in mouse kidney over time: autoradiography versus silver staining. Toxicol. Appl. Pharmacol. 257, 235–245.

Rouleau, C., Block, M., 1997. Fast and high yield synthesis of radioactive $CH_3^{203}Hg(II)$. Appl. Organomet. Chem. 2, 751–753.

Ruprecht, J., 1997. Scientific Monograph for Dimaval (DMPS), 6th Ed. Heyltex Corporation, Houston, TX.

Schaub, T.P., Kartenbeck, J., Konig, J., Vogel, O., Witzgall, R., Kriz, W., Keppler, D., 1997. Expression of the conjugate export pump encoded by the mrp2 gene in the apical membrane of kidney proximal tubules. J. Am. Soc. Nephrol. 8, 1213–1221.

Smeets, P.H.E., Van Aubel, R.A.M.H., Wouterse, A.C., Van Den Heuvel, J.J.M.W., Russel, F.G.M., 2004. Contribution of multidrug resistance protein 2 (MRP2/ABCC2) to the renal excretion of p-aminohippurate (PAH) and identification of MRP4 (ABCC4) as a novel PAH transporter. J. Am. Soc. Nephrol. 15, 2828–2835.

Stewart, J.R., Diamond, G.L., 1987. Renal tubular secretion of the alkanesulfonates 2,3-dimercapto-1-propane sulfonate. Am. J. Physiol. 252, F800–F810.

Stewart, J.R., Diamond, G.L., 1988. In vivo renal tubular secretion and metabolism of the disulfide of 2,3-dimercaptopropane-1-sulfonate. Drug Metab. Dispos. 16, 189–195.

Sugawara, N., Lai, Y.R., Sugaware, C., Arizono, K., 1998. Decreased hepatobiliary secretion of inorganic mercury, its deposition and toxicity in the Eisai hyperbilirubinemic rat with no hepatic canalicular organic anion transporter. Toxicology 126, 23–31.

Van Aubel, R.A., Smeets, P.H., Peters, J.G., Bindels, R.J., Russel, F.G., 2002. The MRP4/ABCC4 gene encodes a novel apical organic anion transporter in human kidney proximal tubules: putative efflux pump for urinary cAMP and cGMP. J. Am. Soc. Nephrol. 13, 595–603.

Wu, G., 1996. Methylmercury-cysteine uptake by rat erythrocytes: evidence for several transport systems. J. Appl. Toxicol. 16, 77–83.

Zalups, R.K., 1993. Influence of 2,3-dimercaptopropane-1-sulfonate (DMPS) and meso-2,3-dimercaptosuccinic acid (DMSA) on the renal disposition of mercury in normal and uninephrectomized rats exposed to inorganic mercury. J. Pharmacol. Exp. Ther. 267, 791–800.

Zalups, R.K., 2000. Molecular interaction with mercury in the kidney. Pharmacol. Rev. 52, 113–143.

Zalups, R.K., Barfuss, D.W., Kostyniak, P.J., 1992. Altered intrarenal accumulation of mercury in uninephrectomized rats treated with methylmercury chloride. Toxicol. Appl. Pharmacol. 115, 174–182.

Zalups, R.K., Parks, L.D., Cannon, V.T., Barfuss, D.W., 1998. Mechanisms of action of 2,3-dimercaptopropane-1-sulfonate and the transport, disposition, and toxicity of inorganic mercury in isolated perfused segments of rabbit proximal tubules. Mol. Pharmacol. 54, 353–363.