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# Toxicological Significance of Renal Bcrp: Another Potential Transporter in the Elimination of Mercuric Ions from Proximal Tubular Cells

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## Abstract

Secretion of inorganic mercury ($Hg^{2+}$) from proximal tubular cells into the tubular lumen has been shown to involve the multidrug resistance-associated protein 2 (Mrp2). Considering similarities in localization and substrate specificity between Mrp2 and the breast cancer resistance protein (Bcrp), we hypothesize that Bcrp may also play a role in the proximal tubular secretion of mercuric species. In order to test this hypothesis, the uptake of $Hg^{2+}$ was examined initially using inside-out membrane vesicles containing Bcrp. The results of these studies suggest that Bcrp may be capable of transporting certain conjugates of $Hg^{2+}$. To further characterize the role of Bcrp in the handling of mercuric ions and in the induction of $Hg^{2+}$-induced nephropathy, Sprague-Dawley and Bcrp knockout (*bcrp*$^{-/-}$) rats were exposed intravenously to a non-nephrotoxic (0.5 μmol • $kg^{-1}$), a moderately nephrotoxic (1.5 μmol • $kg^{-1}$) or a significantly nephrotoxic (2.0 μmol • $kg^{-1}$) dose of $HgCl_2$. In general, the accumulation of $Hg^{2+}$ was greater in organs of *bcrp*$^{-/-}$ rats than in Sprague-Dawley rats, suggesting that Bcrp may play a role in the export of $Hg^{2+}$ from target cells. Within the kidney, cellular injury and necrosis was more severe in *bcrp*$^{-/-}$ rats than in controls. The pattern of necrosis, which was localized in the inner cortex and the outer stripe of the outer medulla was significantly different from that observed in Mrp2-deficient animals. These findings suggest that Bcrp may be involved in the cellular export of select mercuric species and that its role in this export may differ from that of Mrp2.

### Keywords

Breast cancer resistance protein; Multidrug resistance-associated protein 2; mercury; proximal tubule; kidney

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## 1.0 INTRODUCTION

Inorganic mercury ($Hg^{2+}$) accumulates predominately in the kidneys, specifically in the epithelial cells lining the S1, S2 and S3 segments of proximal tubules (Rodier and Kates, 1988; Rodier *et al.*, 1988; Zalups, 1991; Zalups, 2000). Within biological systems, mercuric ions are thought to bind preferentially to thiol-containing molecules to form thiol *S*-conjugates of $Hg^{2+}$ (Zalups, 2000; Bridges and Zalups, 2010). Thiol-*S*-conjugates of $Hg^{2+}$ have been shown to be taken up by transport mechanisms present in luminal and basolateral membranes of proximal tubular epithelial cells (Zalups, 2000; Bridges and Zalups, 2010).

Once mercuric ions gain access to the intracellular compartment of target cells, they tend to be retained within the intracellular compartment due to complex binding reactions of these ions with protein- and non-protein thiols (Zalups, 2000; Clarkson *et al.*, 2007). Mercuric ions are powerful electrophiles and thus, retention of these ions within cells may lead to serious deleterious effects in target cells. Indeed, exposure to moderate (1.5 $\mu mol \cdot kg^{-1}$ in rats) levels of $Hg^{2+}$ can lead to acute renal tubular changes, which can be characterized by loss of membrane integrity, atrophy and subsequent death of the epithelial cells lining the proximal tubule. In cases of mild to moderate intoxication, cellular injury and death occur primarily in S2 segments located at the cortico-medullary junction and in S3 segments in the outer stripe of the outer medulla (OSOM) (Zalups and Diamond, 1987; Zalups *et al.*, 1991; Zalups, 1997; Zalups, 2000; Bridges and Zalups, 2010). In cases of severe nephropathy, cellular necrosis may be evident in other segments of the nephron, including S1 segments in the cortex.

A number of recent studies have identified specific mechanisms that are involved in the entry of mercuric species into proximal tubular epithelial cells (Zalups, 2000; Bridges and Zalups, 2010). However, little is known about the precise mechanisms involved in the export of mercuric ions from target cells. *In vivo* and *in vitro* studies have recently implicated the multidrug resistance-associated protein 2 (Mrp2), localized in the luminal membrane of proximal tubular cells (Schaub *et al.*, 1999), in the export of certain mercuric species from within proximal tubular cells into the tubular lumen (Bridges *et al.*, 2008a; Bridges *et al.*, 2008b; Zalups and Bridges, 2009; Bridges and Zalups, 2010; Bridges *et al.*, 2011). The results of these studies suggest that additional transport proteins may also be involved in the proximal tubular secretion of mercuric species into the tubular lumen. One potential candidate for this secretion is the breast cancer resistance protein (Bcrp; Abcg2). Like, Mrp2, Bcrp is localized in the apical membrane of proximal tubular epithelial cells (Huls *et al.*, 2008) and it has been shown to be involved in the transport of a wide variety of drugs and xenobiotics (Leslie *et al.*, 2005; Vlaming *et al.*, 2009; Konig *et al.*, 2013). Considering the similarities in localization and substrate specificity between Bcrp and Mrp2, we hypothesize that Bcrp may also play a role in the export of mercuric species from within proximal tubular epithelial cells. To test this hypothesis we 1) assessed the transport of mercuric species in inside-out membrane vesicles containing Bcrp; and 2) examined the disposition and nephrotoxicity of various doses of mercuric chloride ($HgCl_2$) in control and Bcrp knockout rats.

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# 2.0 METHODS

## 2.1 Animals

Male Bcrp (Abcg2) knockout rats (SD-*Abcg2*$^{tm1sage}$; *bcrp*$^{-/-}$) were obtained from Sage Labs (Huang *et al.*, 2012; Zamek-Gliszczynski *et al.*, 2012). Male Sprague-Dawley (SD) rats were used as controls and were obtained from Charles River Laboratories. Rats were housed in the Mercer University School of Medicine animal facility. Animals were provided a commercial laboratory diet (Teklad Global Soy Protein Free Extruded Rodent Diet, Harlan Laboratories) and water *ad libitum* throughout all aspects of the present study. All procedures involving animals were reviewed and approved by the Mercer University Institutional Animal Care and Use Committee. Animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health.

## 2.2 Intravenous Injections

SD and *bcrp*$^{-/-}$ rats, weighing 225–250 g, were injected intravenously (i.v.) with either a non-nephrotoxic (0.5 μmol • kg$^{-1}$ • 2 mL$^{-1}$ normal saline), a moderately nephrotoxic dose (1.5 μmol • kg$^{-1}$ • 2 mL$^{-1}$ normal saline) or a significantly nephrotoxic (2.0 μmol • kg$^{-1}$ • 2 mL$^{-1}$ normal saline) dose of $HgCl_2$ according to our previously published protocol (Bridges *et al.*, 2008a; Bridges *et al.*, 2008b). $HgCl_2$ is an inorganic salt to which humans and animals may be exposed. The injection solution contained radioactive mercury ([$^{203}Hg^{2+}$]) and was designed to deliver 1 μCi [$^{203}Hg^{2+}$] to each animal. [$^{203}Hg^{2+}$] was generated by neutron activation of mercuric oxide for four weeks at the University of Missouri Research Reactor (MURR) (Belanger *et al.*, 2001; Bridges *et al.*, 2008a).

At the time of injection, each animal was anesthetized with isoflurane and a small incision was made in the skin in the mid-ventral region of the thigh to expose the femoral vein and artery. A 0.5-μmol, 1.5-μmol or 2.0-μmol • kg$^{-1}$ dose of $HgCl_2$ was administered into the vein. The wound was closed using two 9-mm stainless steel wound clips. Animals were then housed individually in metabolic cages. Forty-eight hours after injection with $HgCl_2$, animals were sacrificed and organs and tissues were harvested.

## 2.3 Collection of Organs

At the time of euthanasia, animals were anesthetized with an intraperitoneal (i.p.) injection of ketamine (70 mg • kg$^{-1}$) and xylazine (30 mg • kg$^{-1}$). A 1-mL sample of blood was obtained from the inferior vena cava and set aside for determination of [$^{203}Hg^{2+}$] content. A separate sample of blood was placed in a Microtainer plasma separation tube in order to estimate content of [$^{203}Hg^{2+}$] in plasma and cellular fractions. The total volume of blood was estimated to be 6% of body weight (Lee and Blaufox, 1985).

The liver and kidneys were also removed from each rat. Each kidney was trimmed of fat and fascia, weighed, and cut in half along the mid-traverse plane. One-half of the right kidney was placed in fixative (40% formaldehyde, 50% glutaraldehyde in 96.7 mM $NaH_2PO_4$ and 67.5 mM NaOH) as preparation for histological analyses. The remaining half was frozen in liquid nitrogen for future RNA analyses. One-half of the left kidney was utilized for

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estimation of [$^{203}Hg^{2+}$] content. A 3-mm traverse slice was obtained from the remaining half and was used for dissection of renal zones (cortex, outer stripe of the outer medulla (OSOM), inner stripe of the outer medulla, and inner medulla). Each sample was weighed and placed in a separate tube for estimation of [$^{203}Hg^{2+}$]. The liver was weighed and a 1-g sample was removed for determination of [$^{203}Hg^{2+}$] content.

Urine and feces were collected in 24-h periods throughout the duration of the experiment. At the end of each 24-h collection period, a 1-mL sample of urine was weighed and placed in a tube for estimation of [$^{203}Hg^{2+}$] content. All of the feces excreted during each 24-h collection period were counted for estimation of [$^{203}Hg^{2+}$] content. The content of [$^{203}Hg^{2+}$] in each sample was determined by counting in a Wallac Wizard 3 automatic gamma counter (Perkin Elmer, Boston, MA) and the content of $Hg^{2+}$ in each sample was estimated using standard computational methods.

### 2.4 Histological Analyses

Kidneys were fixed in 40% formaldehyde, 50% glutaraldehyde in 96.7 mM $NaH_2PO_4$ and 67.5 mM NaOH for 48 hours at 4°C. Following fixation, kidneys were washed twice with normal saline and placed in 70% ethanol. Tissues were processed in a Tissue-Tek VIP processor using the following sequence: 95% ethanol for 30 min (twice); 100% ethanol for 30 min (twice); 100% xylene (twice). Tissue was subsequently embedded in POLY/Fin paraffin (Fisher) and 5 μm sections were cut using a Leitz 1512 microtome and were mounted on glass slides. Sections were stained with hematoxylin and eosin (H & E) and were viewed using an Olympus IX70 microscope. Images were captured with a Jenoptix Progress C12 digital camera.

### 2.5 Measurement of Creatinine and Blood Urea Nitrogen

Plasma creatinine and blood urea nitrogen (BUN) levels were assessed in order to estimate alterations in renal function. Following separation of plasma from cellular components of blood, an aliquot of plasma was stored at −20°C. For determination of plasma creatinine, 30 μL of plasma was utilized and the concentration of creatinine was assessed using the QuantiChrome creatinine assay (BioAssay). Similarly, using a 5 μL sample of plasma, the concentration of BUN was determined using the QuantiChrome urea assay (BioAssay).

### 2.6 Vesicular Transport Assays

Inside-out membrane vesicles made from Sf9 cells containing mouse Bcrp were purchased from Solvo Biotechnology. Control membrane vesicles made from normal Sf9 cells were also purchased from Solvo Biotechnology. Bcrp transport activity was validated by measuring the uptake of 100 nM [$^{3}$-H]-estrone sulfate. Cysteine (Cys)-*S*-conjugates of $Hg^{2+}$ were utilized for these experiments because there is substantial *in vivo* and *in vitro* evidence implicating this mercuric species in the luminal and basolateral uptake of $Hg^{2+}$ by proximal tubular cells (Zalups, 2000; Bridges *et al.*, 2004; Bridges and Zalups, 2010). Mercuric conjugates of 2,3-dimercapto-1-propane succinate (DMPS) were also examined since this species of Hg has been shown to be transported by the multidrug resistance-associated protein 2 (MRP2) as a means of eliminating mercuric ions from proximal tubular cells (Bridges *et al.*, 2008a; Bridges *et al.*, 2008b; Bridges *et al.*, 2011). Vesicular transport assays

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were carried out using a rapid filtration method according to a published protocol (Van Aubel *et al.*, 1999; El-Sheikh *et al.*, 2007; Bridges *et al.*, 2013). Briefly, DMPS- and Cys-*S*-conjugates of $Hg^{2+}$ were formed by mixing 5 μM (5 nmol • $mL^{-1}$) [$^{203}$Hg] with 12.5 μM (12.5 nmol • $mL^{-1}$) DMPS or Cys, respectively, in incubation buffer (250 mM sucrose, 10 mM Tris/HCl, pH 7.4) supplemented with 10 mM $MgCl_2$, 10 mM creatine phosphate and 100 μg/ml creatine phosphokinase in the presence of 4 mM ATP or AMP. Incubation buffer containing mercuric conjugate or estrone sulfate was added to vesicle mixture (7.5 μg protein) and incubated for 30 seconds at 37°C. Following incubation, ice-cold buffer containing 1 mM DMPS (to remove bound Hg) was added and each sample was filtered through a Multiscreen plate (0.45 μm; Millipore, Billarica, MA). Filters were removed and radioactivity contained on filter was determined using liquid scintillation spectroscopy.

In order to assess ATP-dependent transport, the amount of estrone sulfate or [$^{203}$Hg] associated with vesicles in the presence of AMP was subtracted from that in the presence of ATP.

### 2.7 Western Blot Analyses

Western blot analyses utilized kidney sections from three different male *bcrp*$^{-/-}$ and SD rats that were not exposed to $HgCl_2$. Animals were anesthetized with ketamine (70 mg • $kg^{-1}$) and xylazine (30 mg • $kg^{-1}$) and both kidneys were removed. A three-mm slice was obtained from each kidney and the cortex, OSOM, inner stripe of the outer medulla and inner medulla were isolated. Each section was placed in an individual tube and frozen immediately in liquid nitrogen. At the time of protein extraction, tissue sections were pulverized in liquid nitrogen using a mortar and pestle. Following pulverization, RIPA buffer (Sigma, St. Louis, MO), protease inhibitor cocktail and phosphatase inhibitor cocktail were added to the powdered tissue. Samples were mixed and incubated on ice for 45 min. Samples were homogenized, centrifuged and the supernatant was collected for analysis. Protein concentrations were determined using Bradford's method and the concentration of each sample was adjusted so that 20 μg of protein in Laemmli buffer with β-mercaptoethanol was loaded into each well of a 7.5% Tris-HCl gel (BioRad). The proteins were transferred to a PDVF membrane (BioRad, Hercules, CA) using a Criterion blotter. The membrane was incubated in blocking buffer (BioRad) for one h, followed by incubation with mouse, anti-rat Mrp2 antibody (1:500; Abcam); mouse, anti-rat Bcrp antibody (1:500; Abcam); or mouse, anti-rat β-actin antibody (1:1000; Abcam). The membrane was washed and subsequently incubated with goat, anti-mouse IgG (1:3000; BioRad) and StrepTactin-AP (1:5000, BioRad). After washing, the membrane was incubated in alkaline phosphatase substrate solution (BioRad) and exposed to X-ray film for one min. Band intensity was analyzed three times using Image J software.

### 2.8 Statistical Analyses

Means for the control and Bcrp-containing vesicles were assessed by the unpaired, Student's t-test. For each group of vesicles, a mean was generated from three replicate means for each triplicate set of data. A p-value of $\leq 0.05$ was considered statistically significant.

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Data for each rat experiment were analyzed first with the Kolmogorov-Smirnov test for normality and then with Leven's test for homogeneity of variances. Data were then analyzed using a 3 x 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 $\leq 0.05$ was considered statistically significant. Each group of animals contained four rats and each experiment was conducted two times.

## 3.0 RESULTS

### 3.1 Uptake of Mercuric Conjugates into Inside-out Membrane Vesicles

In order to confirm normal functionality of inside-out membrane vesicles expressing Bcrp, the uptake of [$^3$H]-estrone sulfate was measured in both control and Bcrp-containing vesicles. The ATP-dependent uptake of estrone sulfate was significantly greater in Bcrp-containing vesicles than in control vesicles (Figure 1A).

When the uptake of Cys-*S*-conjugates of $Hg^{2+}$ was measured in control and Bcrp-containing vesicles, uptake was significantly greater in Bcrp-containing vesicles than in controls (Figure 1B). Similarly, when the uptake of DMPS-*S*-conjugates of $Hg^{2+}$ was measured in control and Bcrp-containing vesicles, the uptake of this conjugate was greater in Bcrp-containing vesicles than in controls (Figure 1C).

### 3.2 Disposition of Mercuric Ions in Renal Tissue and Urine

When rats were exposed to 0.5, 1.5 or 2.0 μmol • $kg^{-1}$ $HgCl_2$, the burden of $Hg^{2+}$ in the total renal mass (nmol • $g^{-1}$) was significantly greater in *bcrp*$^{-/-}$ rats at each dose than in corresponding SD controls (Figure 2). Similarly, the amount of $Hg^{2+}$ in the renal cortex after each dose was significantly greater in *bcrp*$^{-/-}$ rats than in corresponding SD rats (Figure 3A). Interestingly, in rats exposed to 0.5 or 1.5 μmol • $kg^{-1}$ $HgCl_2$, the amount of $Hg^{2+}$ in the OSOM was not significantly different between SD and *bcrp*$^{-/-}$ rats (Figure 3B). However, when rats were exposed to 2.0 μmol • $kg^{-1}$ $HgCl_2$, the amount of $Hg^{2+}$ in the OSOM was significantly greater in *bcrp*$^{-/-}$ rats than in SD controls (Figure 3B).

Urine volumes are shown in Table 1. In rats exposed to 0.5 or 1.5 μmol • $kg^{-1}$ $HgCl_2$, the urinary excretion of mercuric ions was significantly lower in *bcrp*$^{-/-}$ rats than that of corresponding SD rats (Figure 4). In contrast, when rats were exposed to 2.0 μmol • $kg^{-1}$ $HgCl_2$, the urinary excretion of $Hg^{2+}$ was significantly greater in *bcrp*$^{-/-}$ rats than in controls.

### 3.3 Disposition of $Hg^{2+}$ in Blood, Liver and Feces

The hematologic burden of $Hg^{2+}$ in blood was significantly greater in *bcrp*$^{-/-}$ rats exposed to 1.5 or 2.0 μmol • $kg^{-1}$ $HgCl_2$ than in corresponding SD rats (Figure 5). The amount of $Hg^{2+}$ in blood following exposure to 0.5 μmol • $kg^{-1}$ $HgCl_2$ was not significantly different between SD and *bcrp*$^{-/-}$ rats.

The hepatic burden of $Hg^{2+}$ was significantly greater in *bcrp*$^{-/-}$ rats following exposure to 1.5 and 2.0 μmol • $kg^{-1}$ $HgCl_2$ than in corresponding SD rats (Figure 6A). There was no

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significant difference in the hepatic burden of $Hg^{2+}$ between SD and *bcrp*$^{-/-}$ rats exposed to 0.5 μmol • kg$^{-1}$ $HgCl_2$.

The amount of $Hg^{2+}$ excreted in the feces was significantly greater in *bcrp*$^{-/-}$ rats exposed to the 1.5 or 2.0-μmol • kg$^{-1}$ dose of $HgCl_2$ than in corresponding groups of SD rats (Figure 6B). There was no significant difference in fecal elimination of $Hg^{2+}$ between SD and *bcrp*$^{-/-}$ rats exposed to the 0.5-μmol • kg$^{-1}$ dose of $HgCl_2$.

### 3.4 Histological Analyses of Renal Pathology

No pathological changes were observed in the cortex (data not shown) or the OSOM of SD (Figure 7A) or *bcrp*$^{-/-}$ (Figure 7B) rats following exposure to 0.5 μmol • kg$^{-1}$ $HgCl_2$. In contrast, when SD rats were exposed to 1.5 μmol • kg$^{-1}$ $HgCl_2$, small focal areas of cellular injury, characterized by pyknotic nuclei and eosinophilic cytoplasm, were observed throughout the cortex (data not shown) and OSOM (Figure 7C). In corresponding *bcrp*$^{-/-}$ rats, cellular injury and death in the OSOM was more widespread and more severe than that of SD rats (Figure 7D). Necrosis was not observed in the cortex of these animals (data not shown). When SD rats were exposed to 2.0 μmol • kg$^{-1}$ $HgCl_2$, cellular injury and death were more severe than that of corresponding rats exposed to lower doses of $HgCl_2$ (Figure 7E). Areas of cellular necrosis were severe and were widespread through the cortex (data not shown) and OSOM (Figure 7E). In *bcrp*$^{-/-}$ rats exposed to the 2.0-μmol • kg$^{-1}$ dose of Hg (Figure 7F), cellular necrosis was more severe than that of corresponding SD rats. In addition, the injury and necrosis observed in the OSOM (Figure 7F) was much more severe than that of the cortex (data not shown).

### 3.5 Analyses of Serum Creatinine and Blood Urea Nitrogen

Levels of serum creatinine increased significantly in SD and *bcrp*$^{-/-}$ rats following exposure to the 1.5- or the 2.0-μmol • kg$^{-1}$ dose of $HgCl_2$, compared with the 0.5-μmol dose (Table 2). In rats exposed to 2.0 μmol • kg$^{-1}$ $HgCl_2$, plasma creatinine was significantly greater in *bcrp*$^{-/-}$ rats than in SD rats. Similarly, BUN levels increased significantly in SD rats exposed to 2.0 μmol • kg$^{-1}$ $HgCl_2$ and in *Bcrp*$^{-/-}$ rats exposed to 1.5 or 2.0 μmol • kg$^{-1}$ $HgCl_2$ (Table 1). BUN levels in *bcrp*$^{-/-}$ rats exposed to the 1.5- or 2.0-μmol • kg$^{-1}$ dose of $HgCl_2$ were significantly greater than that of corresponding SD rats.

### 3.6 Western Blot Analyses of Bcrp and Mrp2 in Renal Zones

Representative Western blots are shown in Figure 8. Western blot analyses demonstrated that the expression of Bcrp in SD rats (not exposed to $HgCl_2$) is localized primarily in the cortex and OSOM (Figure 8A). Bcrp protein was not detected in the inner stripe of the outer medulla or in the inner medulla. Expression of Bcrp was normalized against β-actin levels.

The expression of Mrp2 was compared in SD and *bcrp*$^{-/-}$ rats. This protein was detected in the cortex and OSOM of both stains of rats. The expression of Mrp2 was not significantly different between SD and *bcrp*$^{-/-}$ rats (Figure 8B).

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## 4.0 DISCUSSION

Recent studies provide evidence that Mrp2 plays an important role in the proximal tubular secretion of mercuric ions (Bridges *et al.*, 2008a; Bridges *et al.*, 2008b; Zalups *et al.*, 2014). Given the similarities in function and substrate specificity between Mrp2 and Bcrp (Leslie *et al.*, 2005; Noguchi *et al.*, 2014), we hypothesized that Bcrp may also play a role in the export of mercuric ions from within proximal tubular cells.

Initially, we chose to utilize inside-out membrane vesicles containing Bcrp as a means to test directly whether conjugates of $Hg^{2+}$ are transportable substrates of this carrier. We examined the transport of Cys- and DMPS-*S*-conjugates of $Hg^{2+}$ since these conjugates have been shown previously to be transported by Mrp2 (Bridges *et al.*, 2008b; Bridges *et al.*, 2008a). Furthermore, the Cys-S-conjugate of $Hg^{2+}$ appears to be the form of $Hg^{2+}$ that is taken up most readily at the apical membrane of proximal tubular cells (Bridges and Zalups, 2010). Both of the conjugates tested in the current study were transported into inside-out membrane vesicles containing Bcrp suggesting that these two species of $Hg^{2+}$ may be transportable substrates of Bcrp *in vivo*.

We then examined the disposition and toxicity of $Hg^{2+}$ in *bcrp*$^{-/-}$ and SD rats by exposing rats to 0.5 μmol, 1.5 μmol or 2.0 μmol • kg$^{-1}$ $HgCl_2$. Following administration of each dose of $HgCl_2$, the renal burden of mercury was significantly greater in *bcrp*$^{-/-}$ rats than in corresponding SD rats, suggesting that the absence of Bcrp leads to a retention of $Hg^{2+}$ in the kidneys. The increased renal accumulation of $Hg^{2+}$ in *bcrp*$^{-/-}$ rats correlates well with the diminished urinary excretion of $Hg^{2+}$ in these rats. Interestingly, in rats exposed to 2.0 μmol • kg$^{-1}$ $HgCl_2$, more $Hg^{2+}$ was excreted in urine by *bcrp*$^{-/-}$ rats than that in urine of corresponding SD rats. This finding is most likely related to the observation that the severity of cellular necrosis in kidneys of *bcrp*$^{-/-}$ rats was greater than that in kidneys of SD rats. Extensive destruction of tubular cells, like that in *bcrp*$^{-/-}$ rats, can lead to the release of cellular contents, including $Hg^{2+}$, into the tubular lumen and urine. This pattern of renal $Hg^{2+}$ accumulation and excretion is similar to that observed previously in Mrp2-deficient animals (Bridges *et al.*, 2008a; Bridges *et al.*, 2008b; Zalups *et al.*, 2014).

When the distribution of $Hg^{2+}$ within each of the four zones of the kidney was examined, we found that $Hg^{2+}$ was localized primarily in the cortex and OSOM. The amount of $Hg^{2+}$ detected in the cortex of *bcrp*$^{-/-}$ rats was greater than that of SD rats, suggesting that the absence of Bcrp leads to a decrease in the export of $Hg^{2+}$ and increased retention in S1 and S2 proximal tubular segments. In contrast, the accumulation of $Hg^{2+}$ in the OSOM was similar in SD and *bcrp*$^{-/-}$ rats exposed to 0.5 or 1.5 μmol • kg$^{-1}$ $HgCl_2$. Since some proteins have been shown to be upregulated in *bcrp*$^{-/-}$ rats (Zamek-Gliszczynski *et al.*, 2013), we initially postulated that the expression of Mrp2 may also be increased in order to compensate for the loss of Bcrp activity. Surprisingly, our Western blot data suggest that Mrp2 protein is not greater in *bcrp*$^{-/-}$ rats. Another possible explanation for the similar accumulation of $Hg^{2+}$ in the OSOM of SD and *bcrp*$^{-/-}$ rats is that the activity, but not the number, of Mrp2 transporters is increased in *bcrp*$^{-/-}$ rats in order to compensate for the absence of Bcrp. Indeed, Mrp2 activity has been shown to be enhanced (without an increase in protein) by the presence of certain substrates (El-Sheikh *et al.*, 2013; Guyot *et al.*, 2014).

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A third possible explanation is that additional, yet unknown, transport mechanisms for $Hg^{2+}$ are present in S3 segments. It is possible that these mechanisms are involved in the export of mercuric species from within proximal tubules located in the OSOM and that this export is able to account for the absence of Bcrp activity.

The current histological analyses show that in kidneys of *bcrp*$^{-/-}$ rats exposed to the 1.5- or 2.0-μmol • kg$^{-1}$ dose of $HgCl_2$, cellular necrosis occurs in S2 and S3 segments of proximal tubules located in the inner cortex and the OSOM, which is the typical pattern of $Hg^+$-induced renal injury in normal rats (Diamond and Zalups, 1998; Zalups, 2000; Zalups *et al.*, 2014). Necrosis was more severe in *bcrp*$^{-/-}$ rats than in SD rats but the localization was similar in both strains of rats. The severity of necrosis correlated well with the current analyses of plasma creatinine and BUN. Interestingly, the localization of tubular necrosis in the current study differs from that of our previous study in which *mrp2*$^{-/-}$ mice and TR$^-$ rats were exposed to nephrotoxic doses of $HgCl_2$ (Zalups *et al.*, 2014). In our previous study, the absence of Mrp2 altered the pattern of nephropathy so that the majority of cellular necrosis was present in the S1 segments of proximal tubules located in the outer cortex (Zalups *et al.*, 2014). Since Mrp2 is localized primarily in S1 segments of proximal tubules, it was hypothesized that the absence of this transporter led to enhanced retention of mercuric ions within cells of S1 and early S2 segments. This retention not only resulted in cellular necrosis in these segments but also protected terminal S2 and S3 segments in that mercuric ions were unable to be secreted from S1 segments and thus, $Hg^{2+}$ was unable to cause injury to downstream segments of the proximal tubule (Zalups *et al.*, 2014).

Exposure of *bcrp*$^{-/-}$ rats to nephrotoxic doses of $HgCl_2$ resulted in necrosis in the inner cortex and OSOM rather than in the outer cortex like in Mrp2-deficient animals. We suggest that these differences in the pattern of nephropathy may relate to differences in 1) differences in the distribution of Bcrp and Mrp2 along the proximal tubule, 2) axial heterogeneity in the synthesis and secretion of GSH along the proximal tubule, and/or 3) the ability of Bcrp and Mrp2 to transport mercuric species. Previous and current Western blot analyses suggest that the amount of Mrp2 protein in the cortex (S1 and S2 segments) is significantly greater than that in the OSOM (Zalups *et al.*, 2014). In contrast, the current Western blot analyses indicate that the level of Bcrp protein is similar in cortex and OSOM. Considering that Mrp2 is localized primarily in the cortex and that it has been shown to transport mercuric species, we suggest that it plays a major role in the secretion of mercuric ions from within cells of S1 and S2 proximal tubules. Mrp2 has been shown to utilize GSH as a co-factor for the transport of certain substrates (Leslie *et al.*, 2001; Leslie *et al.*, 2005); therefore, it is possible that GSH-*S*-conjugates of $Hg^{2+}$ formed within proximal tubular cells can be secreted into the lumen via Mrp2. It is important to note that the synthesis and secretion of GSH have been shown to be greater in S1 segments than in S2 and S3 segments (Parks *et al.*, 1998). Thus, it is possible that $Hg^{2+}$ within cells of S1 segments binds to GSH to form a GSH-*S*-conjugate, which can then be transported into the tubular lumen via Mrp2. Currently, there is no evidence to suggest that Bcrp utilizes GSH as a co-factor for transport; therefore, we suggest that the affinity of Bcrp for GSH-*S*-conjugates of $Hg^{2+}$ may be lower than that of Mrp2. If this is indeed the case, Mrp2 may play a predominant role in the tubular secretion of $Hg^{2+}$ (as a conjugate of GSH) from within S1 segments of proximal

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tubules while Bcrp may play a more minor role. Consequently, the absence of Mrp2 would greatly reduce the cellular secretion of mercuric ions and enhance the nephropathy in S1 segments while the absence of Bcrp may have less of an effect on this process. Indeed, the absence of Mrp2 led to a 2.5-fold increase in the cortical burden of Hg (Zalups *et al.*, 2014) while the absence of Bcrp led to only a 50% increase, suggesting that Mrp2 may play a greater role than Bcrp in the secretion of mercuric ions from cells of S1 proximal tubular segments.

In the OSOM (S3 segments) in *bcrp*$^{-/-}$ rats, the accumulation of $Hg^{2+}$ and the cellular necrosis was somewhat confounding. In *bcrp*$^{-/-}$ rats, necrosis was present throughout the inner cortex and OSOM. This pattern was in contrast to that in *mrp2*$^{-/-}$ mice and TR$^{-}$ rats where no necrosis was observed in the OSOM (Zalups *et al.*, 2014). We suggest that this pattern of nephropathy may be because Mrp2 (in S1 and S2 segments) remains functional in *bcrp*$^{-/-}$ rats and continues to transport mercuric conjugates out of cells lining S1/early S2 segments into the lumen. Once in the lumen, mercuric conjugates are delivered to and taken up by cells of the late S2 and S3 segments, a process that may subsequently lead to cellular necrosis in these segments. Interestingly, necrosis was more severe in *bcrp*$^{-/-}$ rats than in SD rats. This finding was surprising considering that the amount of $Hg^{2+}$ in the OSOM was similar in these two strains of rats. Therefore, we suggest that S3 segments of the proximal tubule, particularly in *bcrp*$^{-/-}$ rats, may be more sensitive to the effects of $Hg^{2+}$ than segments in other zones of the kidney. Indeed, under certain conditions, S3 segments of proximal tubules have been shown to be more sensitive to heavy metal injury than other segments (Hultman and Enestrom, 1986; Zhang *et al.*, 2008).

When the hematologic burden of $Hg^{2+}$ was examined, the amount of $Hg^{2+}$ in the total blood volume of *bcrp*$^{-/-}$ rats after the 1.5 μmol- and 2.0-μmol • kg$^{-1}$ doses was significantly greater than that of corresponding SD rats. This difference may be due, in part, to a "saturation" effect in target organs. In other words, once the burden of Hg in a particular organ reaches a certain level, the organ appears to be incapable of taking up additional Hg. This has been shown previously when rats were exposed to higher doses of Hg (Zalups, 1997; Zalups *et al.*, 2014). This "saturation" may be due to 1) saturation of transport mechanisms involved in the uptake of Hg or 2) intoxication of cells and consequent inactivity of transport mechanisms.

In liver, the burden of $Hg^{2+}$ was significantly greater in *bcrp*$^{-/-}$ rats exposed to 1.5 or 2.0 μmol • kg$^{-1}$ $HgCl_2$ than in corresponding SD rats. This finding is not surprising considering that Bcrp is localized in the canalicular membrane of hepatocytes and is thought to participate in the export of drugs and metabolites from within hepatocytes into bile for elimination (Maliepaard *et al.*, 2001). Thus, the absence of Bcrp in hepatocytes of *bcrp*$^{-/-}$ rats may be related directly to the enhanced accumulation of mercuric ions in these cells. Interestingly, there were no differences in the hepatic burden of $Hg^{2+}$ between SD and *bcrp*$^{-/-}$ rats following the 0.5-μmol • kg$^{-1}$ dose of $HgCl_2$. The reason for this finding is currently unclear, but it may be due to the compensatory activity of another transport protein.

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Surprisingly, fecal elimination of mercuric ions was greater in *bcrp*$^{-/-}$ rats than in corresponding SD rats. This finding may be related to the hematologic burden of $Hg^{2+}$, which was greater in *bcrp*$^{-/-}$ rats than in SD rats. A higher hematologic burden of $Hg^{2+}$ may lead to increased exposure of enterocytes to $Hg^{2+}$. Previous studies have shown that mercuric ions can be secreted into the lumen of the intestine for elimination in the feces (Zalups, 1998); therefore, it is possible that the increased levels of $Hg^{2+}$ in blood may lead to increased uptake by enterocytes and eventual secretion and/or leak into the lumen of the intestines.

Multiple mechanisms have been identified for the uptake of $Hg^{2+}$ into proximal tubular cells. Therefore, it is logical that multiple mechanisms may also be involved in the proximal tubular elimination of $Hg^{2+}$. The current study provides the first evidence that Bcrp may play a role in the elimination of $Hg^{2+}$ from target cells. It appears that additional mechanisms, including Mrp2, may also play important roles in the proximal tubular elimination of $Hg^{2+}$. Defining and characterizing these mechanisms will provide potential therapeutic targets for the treatment of $Hg^{2+}$ intoxication.

## Acknowledgments

This work was supported by the National Institutes of Health (National Institute of Environmental Health Sciences) grant awarded to Dr. Bridges (ES019991).

## Abbreviations

| | |
|---|---|
| **Bcrp** | breast cancer resistance protein |
| **Mrp2** | multidrug-associated resistance protein 2 |
| **DMPS** | 2,3 dimercapto-1-propane succinate |
| **$Hg^{2+}$** | inorganic mercury |
| **OSOM** | outer stripe of the outer medulla |
| **Cys** | cysteine |
| **SD** | Sprague-Dawley |
| ***bcrp*$^{-/-}$** | Bcrp knockout |
| ***mrp2*$^{-/-}$** | Mrp2 knockout |

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## Highlights

- Bcrp may mediate transport of mercury out of proximal tubular cells
- Hg-induced nephropathy was more severe in Bcrp knockout rats
- Bcrp and Mrp2 may differ in their ability to transport Hg

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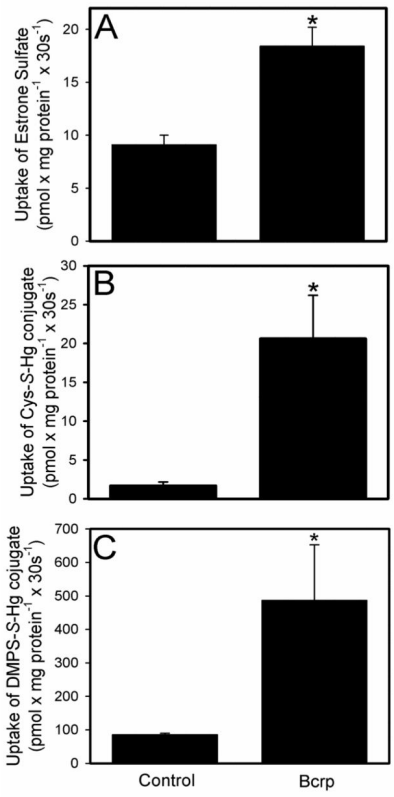


**Figure 1.**

The uptake of 100 nM estrone sulfate (A), 5 μM Cys-*S*-conjugate of $Hg^{2+}$ (B), and 5 μM DMPS-*S*-conjugate of $Hg^{2+}$ (C) was measured in control and Bcrp-expressing inside-out membrane vesicles prepared from Sf9 cells. *, significantly different ($p < 0.05$) from the corresponding mean for control vesicles treated with the same compound.

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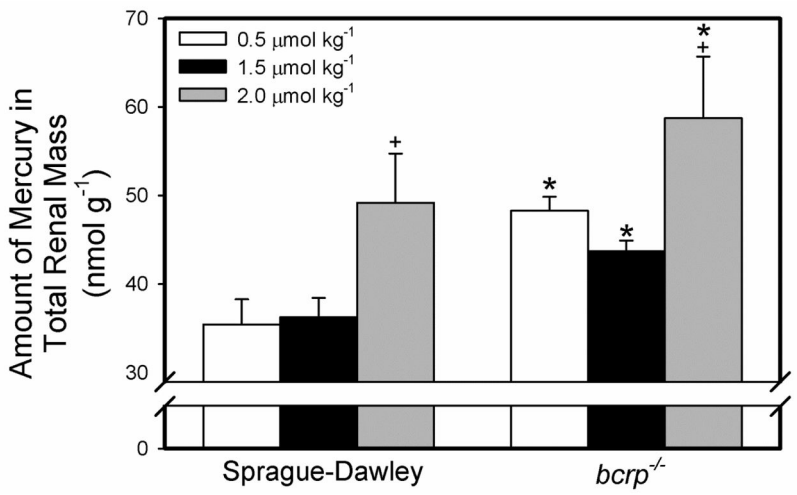


**Figure 2.**

Amount of $Hg^{2+}$ in the total renal mass (nmol • $g^{-1}$) in SD and *bcrp*$^{-/-}$ rats. Rats were injected (i.v.) with 0.5, 1.5, or 2.0 μmol • $kg^{-1}$ $HgCl_2$ and kidneys were harvested for determination of $Hg^{2+}$ content 48 hours later. Data represent mean ± SE of four rats. *, significantly different ($p < 0.05$) from the corresponding mean for the SD rats exposed to the same dose. +, significantly different ($p < 0.05$) from the corresponding mean for rats of the same strain exposed to the 0.5-μmol • $kg^{-1}$ dose of $HgCl_2$.

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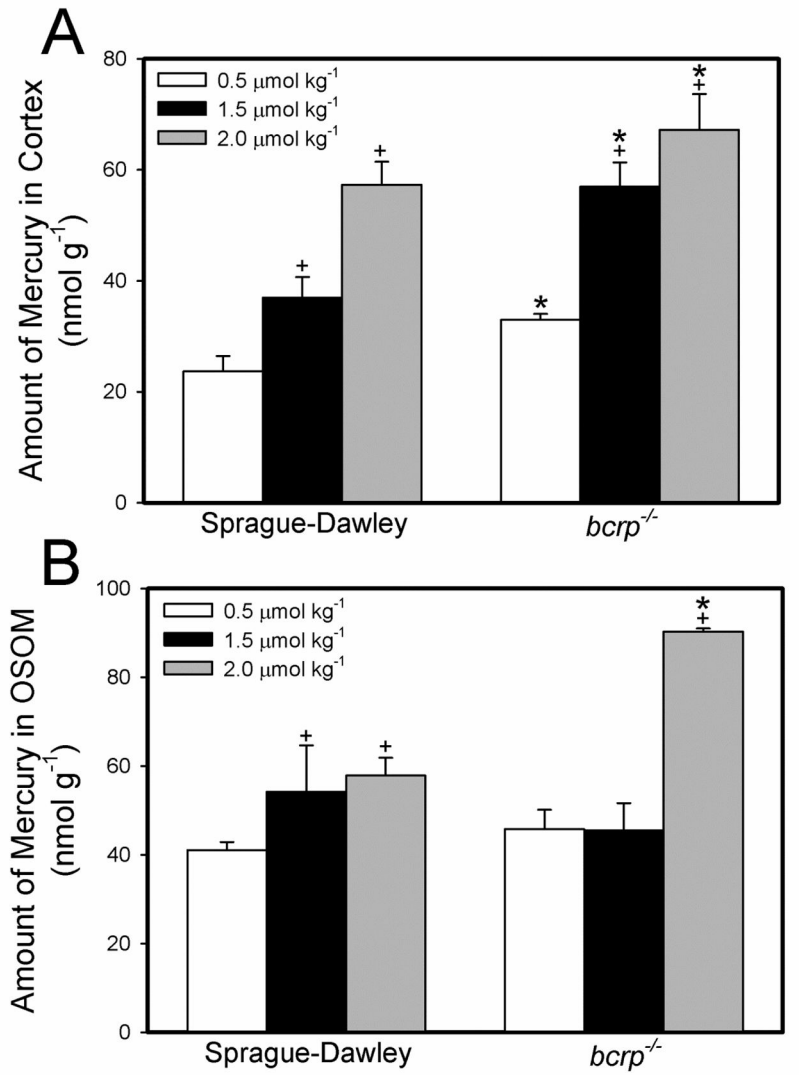


**Figure 3.**

Amount of $Hg^{2+}$ (nmol • $g^{-1}$) in the cortex (A) and the outer stripe of the outer medulla (OSOM) (B) in SD and *bcrp*$^{-/-}$ rats. Rats were injected (i.v.) with 0.5, 1.5, or 2.0 µmol • $kg^{-1}$ $HgCl_2$ and kidneys were harvested for determination of $Hg^{2+}$ content 48 hours later. Data represent mean ± SE of four rats. *, significantly different ($p < 0.05$) from the corresponding mean for the SD rats exposed to the same dose. +, significantly different ($p < 0.05$) from the corresponding mean for rats of the same strain exposed to the 0.5-µmol • $kg^{-1}$ dose of $HgCl_2$.

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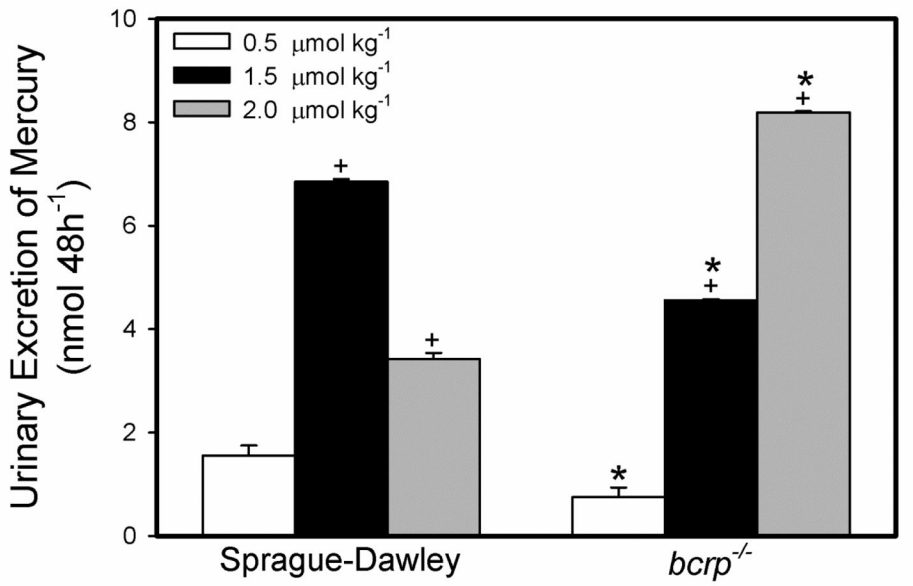


**Figure 4.**

Amount of $Hg^{2+}$ (nmol • $48h^{-1}$) excreted in urine of SD and *bcrp*$^{-/-}$ rats. Rats were injected (i.v.) with 0.5, 1.5, or 2.0 µmol • $kg^{-1}$ $HgCl_2$ and urine was collected in two 24-h time periods following injection of $HgCl_2$. Data represent mean ± SE of four rats. *, significantly different ($p < 0.05$) from the corresponding mean for the SD rats exposed to the same dose. +, significantly different ($p < 0.05$) from the corresponding mean for rats of the same strain exposed to the 0.5-µmol • $kg^{-1}$ dose of $HgCl_2$.

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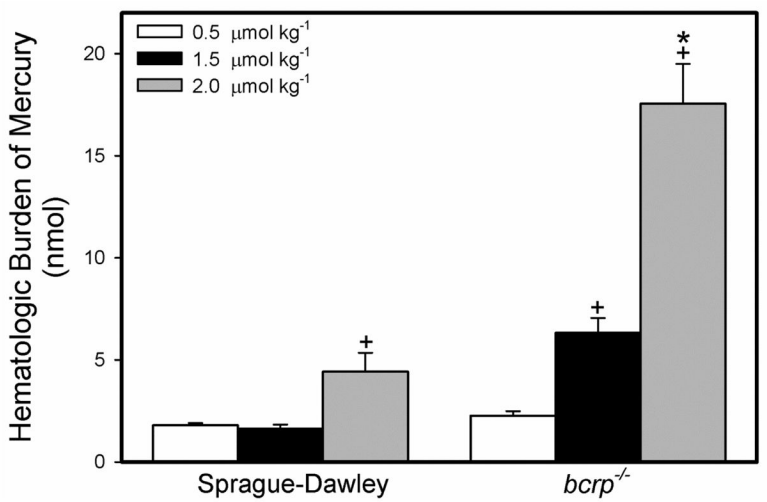


**Figure 5.**

Amount of $Hg^{2+}$ (nmol) in the total blood volume of SD and *bcrp*$^{-/-}$ rats. Rats were injected (i.v.) with 0.5, 1.5, or 2.0 µmol • $kg^{-1}$ $HgCl_2$ and were euthanized 48 h later. Blood was collected for determination of $Hg^{2+}$ content. Data represent mean ± SE of four rats. *, significantly different ($p < 0.05$) from the corresponding mean for the SD rats exposed to the same dose. +, significantly different ($p < 0.05$) from the corresponding mean for rats of the same strain exposed to the 0.5-µmol • $kg^{-1}$ dose of $HgCl_2$.

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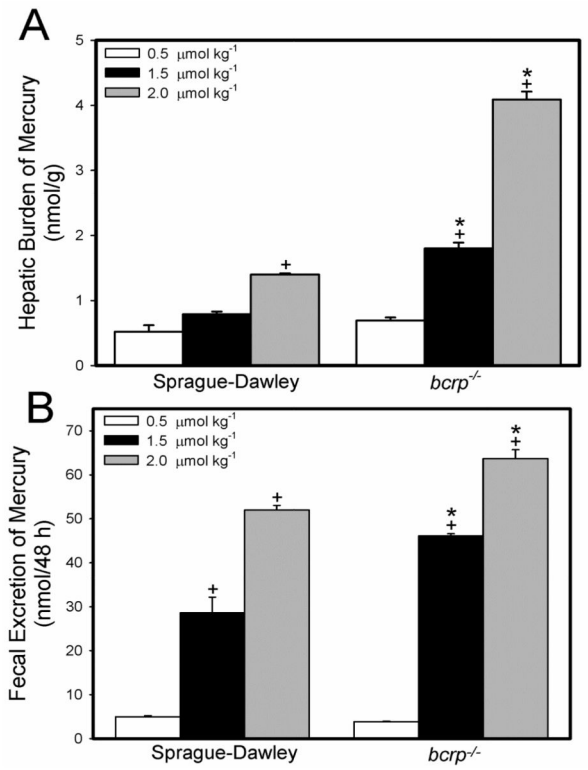


**Figure 6.**

Amount of $Hg^{2+}$ in the liver (A) (nmol • $g^{-1}$) and feces (B) (nmol • $48h^{-1}$) of SD and *bcrp*$^{-/-}$ rats. Rats were injected (i.v.) with 0.5, 1.5, or 2.0 μmol • $kg^{-1}$ $HgCl_2$ and were euthanized 48 h later, at which time the liver was harvested. Feces were collected in two 24-h time periods. Data represent mean ± SE of four rats. *, significantly different ($p < 0.05$) from the corresponding mean for the SD rats exposed to the same dose. +, significantly different ($p < 0.05$) from the corresponding mean for rats of the same strain exposed to the 0.5-μmol • $kg^{-1}$ dose $HgCl_2$.

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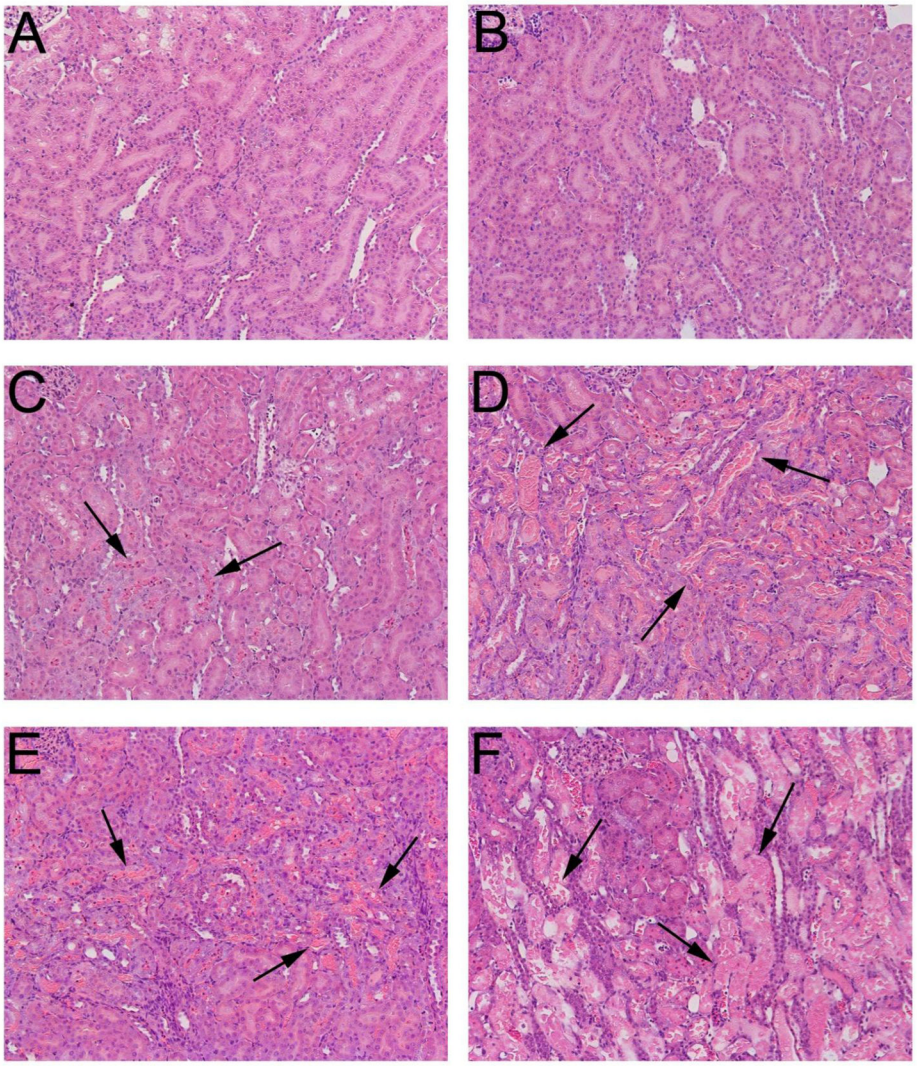


**Figure 7.**

Histological analyses of kidneys from SD and *bcrp*$^{-/-}$ rats injected (i.v.) with 0.5, 1.5, or 2.0 μmol • kg$^{-1}$ $HgCl_2$. No pathological changes were observed in the outer stripe of the outer medulla (OSOM) of SD (A) or *bcrp*$^{-/-}$ (B) rats exposed to 0.5 μmol • kg$^{-1}$ $HgCl_2$. When SD rats were exposed to 1.5 μmol • kg$^{-1}$ $HgCl_2$, focal areas of cellular injury and death (arrows) were observed in the OSOM (C). In *bcrp*$^{-/-}$ rats exposed to 1.5 μmol • kg$^{-1}$ $HgCl_2$, cellular necrosis (arrows) was widespread throughout the OSOM and inner cortex (D). In SD rats exposed to 2.0 μmol • kg$^{-1}$ $HgCl_2$, cellular necrosis (arrows) was localized to the OSOM and inner cortex and was more severe than that in kidneys of corresponding rats exposed to the 1.5-μmol • kg$^{-1}$ dose mercury (E). In *bcrp*$^{-/-}$ rats exposed to 2.0 μmol • kg$^{-1}$ $HgCl_2$, cellular necrosis (arrows) was more severe than that of corresponding rats exposed to the 1.5-μmol • kg$^{-1}$ dose mercury.

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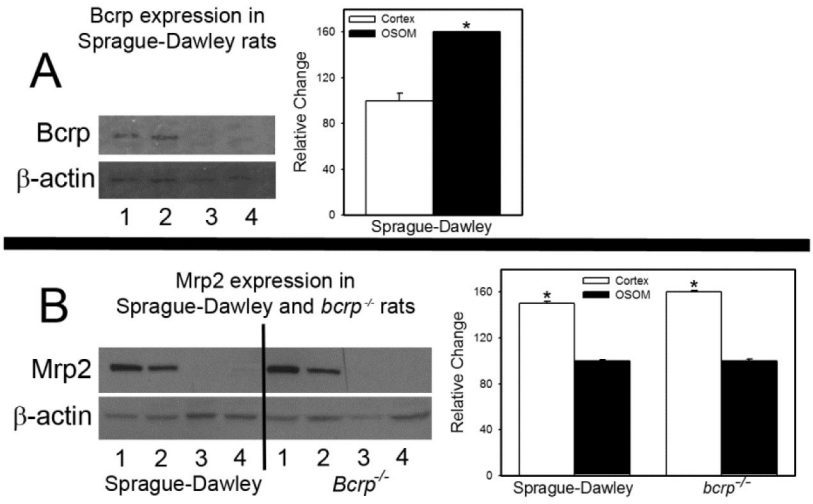


**Figure 8.**

Representative Western blot analyses of Bcrp in SD rats (A) showing that this protein is localized exclusively in the cortex and the outer stripe of the outer medulla (OSOM). The protein levels of Mrp2 (B) were also examined in SD and *bcrp*$^{-/-}$ rats. Blot results are representative of three different rats. Lane 1: cortex; Lane 2: OSOM; Lane 3: inner stripe of the outer medulla; Lane 4: inner medulla

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## Table 1

Plasma creatinine and blood urea nitrogen (BUN) levels in Sprague-Dawley and *bcrp*$^{-/-}$ rats exposed to various doses of $HgCl_2$.

| Strain | Dose | Creatinine (mg/dL) | BUN (mg/dL) |
|---|---|---|---|
| Sprague-Dawley | 0 | 0.34 ± 0.03 | 12.20 ± 0.4 |
| *bcrp*$^{-/-}$ | 0 | 0.32 ± 0.01 | 7.88 ± 0.1 |
| Sprague-Dawley | 0.5 μmol • kg$^{-1}$ | 0.33 ± 0.01 | 12.91 ± 0.5 |
| *bcrp*$^{-/-}$ | 0.5 μmol • kg$^{-1}$ | 0.40 ± 0.03 | 8.05 ± 0.1 |
| Sprague-Dawley | 1.5 μmol • kg$^{-1}$ | 1.19 ± 0.64$^{+}$ | 6.40 ± 1.4 |
| *bcrp*$^{-/-}$ | 1.5 μmol • kg$^{-1}$ | 2.53 ± 0.97$^{+}$ | 41.04 ± 1.5$^{+*}$ |
| Sprague-Dawley | 2.0 μmol • kg$^{-1}$ | 2.05 ± 0.84$^{+}$ | 24.08 ± 7.0$^{+}$ |
| *bcrp*$^{-/-}$ | 2.0 μmol • kg$^{-1}$ | 3.62 ± 0.8$^{+*}$ | 31.0 ± 5.4$^{+*}$ |

$^{*}$significantly different from corresponding Sprague-Dawley rats exposed to same dose;

$^{+}$significantly different from rats of same strain rats not exposed to $HgCl_2$.