Altered Disposition of Acetaminophen in Mice With a Disruption of the *Mrp3* Gene

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MRP3 is an ABC transporter localized in the basolateral membrane of epithelial cells such as hepatocytes and enterocytes. In this study, the role of Mrp3 in drug disposition was investigated. Because Mrp3 preferentially transports glucuronide conjugates, we investigated the *in vivo* disposition of acetaminophen (APAP) and its metabolites. *Mrp3*$^{+/+}$ and *Mrp3*$^{-/-}$ knockout mice received APAP (150 mg/kg), and bile was collected. Basolateral and canalicular excretion of APAP was also assessed in the isolated perfused liver. In separate studies, mice received 400 mg APAP/kg for assessment of hepatotoxicity. No differences were found in the biliary excretion of APAP, APAP-sulfate, and APAP-glutathione between *Mrp3*$^{+/+}$ and *Mrp3*$^{-/-}$ mice. However, 20-fold higher accumulation of APAP-glucuronide (APAP-GLUC) was found in the liver of *Mrp3*$^{-/-}$ mice. Concomitantly, plasma APAP-GLUC content in *Mrp3*$^{-/-}$ mice was less than 10% of that in *Mrp3*$^{+/+}$ *mice*. In addition, APAP-GLUC excretion in bile of *Mrp3*$^{-/-}$ mice was tenfold higher than in *Mrp3*$^{+/+}$ mice. In the isolated perfused liver, we also found a strong decrease of APAP-GLUC secretion into the perfusate of *Mrp3*$^{-/-}$ livers. Plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), and histopathology showed that *Mrp3*$^{-/-}$ mice are more resistant to APAP hepatotoxicity than *Mrp3*$^{+/+}$ mice, which is most likely a result of the faster repletion of hepatic GSH. In conclusion, basolateral excretion of APAP-GLUC in mice is nearly completely dependent on the function of Mrp3. In its absence, sufficient hepatic accumulation occurs to redirect some of the APAP-GLUC to bile. This altered disposition in *Mrp3*$^{-/-}$ mice is associated with reduced hepatotoxicity. (HEPATOLOGY 2005;42: 1091-1098.)

Basolateral excretion is an essential function that prevents the intracellular accumulation of potentially toxic endobiotic and xenobiotic substances in the liver. Export of drugs and their metabolites out of cells commonly involves transport proteins located in the plasma membrane. Several members of the multidrug resistance protein (MRP) subfamily of ABC transporters are localized in the basolateral membrane of epithelial cells in many tissues.[1] MRP2 (apical transporter) and MRP1 and 3 (basolateral transporters) are the most fully characterized in terms of substrate specificity, role in multidrug resistance, and changes in expression by xenobiotics and pathological conditions.

Substrates exported from hepatocytes by MRP1-3 include conjugates of glutathione, glucuronide, and sulfate as well as organic amphiphilic anions. MRP3, which is expressed in liver and gastrointestinal tract,[2,3] prefers glucuronide over glutathione conjugates.[4,5] Mice lacking *Mrp3* were recently developed; these mice are viable, fertile, and have no apparent phenotype.[2] Because of the potential role of Mrp3 in basolateral secretion of glucuronidated drugs, the disposition of acetaminophen (APAP) in *Mrp3*$^{-/-}$ mice was investigated.

Acetaminophen is a popular analgesic and antipyretic that can produce acute liver failure with excessive dosing.[6,7] This drug is metabolized in the liver by conjugation with glucuronic acid and sulfate, while a reactive metabolite generated by CYP450 is detoxified by conjugation

Abbreviations: MRP, multidrug resistance protein; APAP, acetaminophen; GSH, glutathione; APAP-CYS, acetaminophen cysteine; APAP-GLUC, acetaminophen glucuronide; APAP-GSH, acetaminophen glutathione; APAP-SULF, acetaminophen sulfate; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PCA, perchloric acid; TR^{-}*, transport-deficient hyperbilirubinemic rat; NAPQI,* N*-acetyl-p-benzoquinoneimine; TUDCA, tauroursodeoxycholic acid.*

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with glutathione (GSH). Glucuronidation is quantitatively the most important detoxification pathway for this drug, accounting for over 60% of the metabolism of a toxic dose administered. This percentage varies depending on the dose and the species examined.[8-10]

The hepatobiliary disposition of APAP was recently investigated in transport-deficient (TR^-) hyperbilirubinemic rats lacking Mrp2, the canalicular transporter for organic anions. The biliary excretion of several APAP conjugates was significantly decreased in TR^- rats. Notably, APAP-GSH was virtually absent in the bile of TR^- rats. Associated with this, hepatic accumulation of this conjugate was detected in these mutants.[11] The biliary elimination of APAP-glucuronide (APAP-GLUC) was also reduced in TR^- rats, but this was not accompanied by retention of this conjugate in hepatocytes as seen for APAP-GSH. Furthermore, the APAP-GLUC content of the urine of TR^- rats was higher than that of normal rats.[11] Xiong et al.[12] observed that basolateral excretion of APAP-GLUC is 7.4-fold elevated in the isolated perfused livers from TR^- rats as compared with wild-type rats, whereas hepatic APAP-GLUC concentrations were not increased. This result strongly suggested enhanced active basolateral extrusion of this glucuronide. On the basis of these data, APAP-GLUC may be transported from the liver to the blood via Mrp3, because the hepatic level of this protein is elevated in TR^- rats.[13-15] Using membrane vesicles from *Mrp3*-expressing *Sf*9 insect cells Xiong et al.[14] showed that rat Mrp3 has a very low affinity for APAP-GLUC (K_m = 0.9 mmol/L). However, recent findings show that the transport activity of some MRP proteins can be greatly increased by simultaneous interaction with other compounds.[16,17] Hence, how efficient Mrp3-mediated transport of APAP-GLUC is *in vivo* remains to be determined. We have therefore investigated the *in vivo* transport capacity of Mrp3 for glucuronide conjugates by analyzing the hepatobiliary disposition of APAP in $Mrp3^{+/+}$ and $Mrp3^{-/-}$ mice. We extended our studies by determining whether changes in APAP disposition in the absence of Mrp3 result in altered susceptibility to APAP hepatotoxicity.

Materials and Methods

Acetaminophen and propylene glycol were obtained from Sigma Chemical Co. (St. Louis, MO). Perchloric acid, ammonium formate, high-pressure liquid chromatography (HPLC) grade methanol, glutathione, TUDCA, EDTA, NADPH, DTNB, Hypnorm, and Dormicum for mouse anesthesia were obtained from Janssen (Beerse, Belgium) and Roche (Woerden, Netherlands), respectively. All other chemicals were reagent grade or better.

Animals

Biliary Cannulation Studies. $Mrp3^{+/+}$ and $Mrp3^{-/-}$ mice (both strains against a mixed FVB/N and 129/OLA background) were bred at the animal institute of the Academic Medical Center. The production and characterization of the $Mrp3^{-/-}$ mouse is described in Zelcer et al.[2] Mice were anesthetized with a combination of Hypnorm (VetaPharma, Leeds, UK; 17.5 mg/kg fluanisone and 0.55 mg/kg fentanyl citrate) and Dormicum (Roche; 8.75 mg/kg midazolam) and the gallbladder was cannulated with PE10 polyethylene tubing. Body temperature was maintained between 35°C and 37°C by keeping the mice on thermostatted heating pads. Ten minutes after cannulation, animals received a nontoxic dose of APAP (150 mg/kg, 30 mg/mL in 20% propylene glycol/saline) injected via the tail vein. Control animals were injected with vehicle. Bile was collected at 20-minute intervals, and volume was determined gravimetrically. At the end of the experiment, blood was obtained by cardiac puncture for plasma isolation, and the liver was removed. All animal procedures have been approved by the institutional ethical committee.

In Situ *Closed-System Mouse Liver Perfusion Experiments.* Surgical and liver perfusion techniques for mice were performed as previously described.[18] Briefly, mice underwent anesthesia as described, and the gallbladder, vena cava superior, and portal vein were cannulated. Orthograde perfusion was performed with Krebs-bicarbonate buffer in a perfusion cabinet at 37°C. The perfusion medium was gassed with carbogen (5% CO_2, 95% O_2, Hoek Loos, Schiedam, the Netherlands) using an oxygenator. Immediately after perfusions began, a 4-mmol/L TUDCA solution in physiological saline was infused through a three-way connector attached to the portal vein cannula at a rate of 0.05 mL/min. TUDCA was infused throughout the entire perfusion period to ensure constant and proper bile flow. After stabilization on single-pass perfusion mode, the system was switched to recirculating perfusion mode with a total volume of perfusion medium of 100 mL. During the first 20 minutes of recirculating perfusion, baseline bile and perfusate samples were collected. Immediately thereafter, a 2.5-mmol/L APAP solution (in physiological saline) was infused through the portal vein cannula at a flow rate of 0.5 mL/min for 1 minute. This infusion delivered 187 μg (1.25 μmol) of APAP directly into the liver. Bile and perfusate samples were collected every 10 minutes for 90 minutes.

Analysis of APAP and Its Metabolites in Liver, Bile, Plasma, and Perfusate Samples. Liver samples were homogenized (1:10 wt/vol) in ice-cold HPLC grade

methanol. Homogenates were centrifuged at 1,200*g* for 30 minutes, and the supernatants were further diluted (1:10) with 20 mmol/L ammonium formate pH 3.5 (HPLC mobile phase A, see below). Bile samples were diluted with two volumes of ice-cold methanol and then centrifuged at 1,200*g* for 30 minutes. Supernatants were diluted with 9 volumes of mobile phase A. Plasma samples were diluted in 2 volumes of 10% perchloric acid (PCA). After centrifugation, the supernatants were dissolved in 3 to 5 volumes redistilled water and further diluted in 9 volumes mobile phase solvent A before analysis. Perfusate samples were analyzed undiluted. The HPLC method of Oliveira et al.[19] was used for the analysis of APAP and its metabolites, with an HPLC equipped with an Omnis-Spher C18 column (3 μm, 100 mm $\times$ 4.6 mm inner diameter; Chromopack Bergen op Zoom, The Netherlands), Gynkotek pumps (Germering, Germany), an Applied Biosystems absorbance detector (Ramsey, NJ), and a Spark-Holland autosampler (Emmen, The Netherlands). The mobile phase consisted of 20 mmol/L ammonium formate buffer, pH 3.5 (solvent A) and methanol (solvent B). The mobile phase was first maintained at 4% solvent B for 5 minutes, followed by a linear gradient of 24 minutes, ending at 90% solvent B. Flow rate was kept constant at 0.8 mL/min, and peaks were detected at 254 nm. The concentration of APAP and its metabolites was calculated using an APAP standard curve.[20]

Analysis of Liver, Bile, and Perfusate Content of GSH. Liver samples were homogenized in 10% PCA and centrifuged at 1,200*g* for 30 minutes. Aliquots of the supernatant were neutralized (pH 7.4) with 3 mol/L K_3PO_4 and centrifuged again. Bile samples were diluted (1:200) with 10% PCA, and the pH was similarly neutralized after centrifugation. Perfusate samples were analyzed undiluted. GSH content was determined immediately after sample processing according to the recycling method of Tietze.[21]

Toxicity Studies. *Mrp3*$^{-/-}$ and *Mrp3*$^{+/+}$ mice were fasted overnight and received 400 mg APAP per kg body weight, intraperitoneally, in a 50% propylene glycol/saline solution. Twelve hours after APAP treatment, blood was collected ($<$0.2 mL) into heparinized tubes by sinus retro-orbital puncture under light carbon dioxide anesthesia for plasma isolation and determination of liver aminotransferases. The same volume of blood collected was replaced by intraperitoneal injection of sterile physiological saline. At 24 hours after dosing, animals were anesthetized as described, and blood was again collected by cardiac puncture for analysis of liver aminotransferases. Liver samples were also collected for histopathology.

Plasma Markers of Hepatotoxicity and Histopathology. Plasma samples were analyzed for the following aminotransferases: alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase. Samples were analyzed by standard clinical chemical procedures at the Academic Medical Center. Portions of liver were stored in 10% phosphate-buffered formalin, embedded, sectioned, and stained with hematoxylin-eosin for examination under a light microscope. Tissue sections were scored using a scale from 0 to 5 according to the severity of degeneration and necrosis as previously described.[22,23]

Statistical Analysis. Data are expressed as mean $\pm$ standard error of the mean ($n > 3$). Statistically significant differences between *Mrp3*$^{+/+}$ and *Mrp3*$^{-/-}$ mice were determined using two-tailed Student *t* test. Differences in histology scores were analyzed by the Mann-Whitney test. The significance level was set at $P \leq .05$.

Results

Biliary Disposition of Acetaminophen and Its Metabolites. On intravenous administration of 150 mg/kg APAP, no differences in the biliary excretion of unchanged APAP were found between *Mrp3*$^{+/+}$ and *Mrp3*$^{-/-}$ mice. The parent compound accounted for less than 1% of the total dose of APAP found in bile after 120 minutes (Fig. 1A). APAP-GSH was the most prominent metabolite found in bile, accounting for over 90% of all APAP and metabolites appearing in bile or 16% to 18% of the administered dose (Fig. 1B). Biliary excretion of APAP-GSH was slightly higher in *Mrp3*$^{-/-}$ compared with *Mrp3*$^{+/+}$ mice but this was only significant at 20 minutes after administration. At the end of the experiment, the total accumulative amount of biliary APAP-GSH was not significantly different. No differences in the biliary concentrations of the cysteine-conjugate (APAP-CYS) were detected between strains (data not shown). Biliary excretion of APAP-GSH has been proposed as a reliable indicator of *in vivo* formation of N-acetyl-*p*-benzoquinoneimine (NAPQI).[24] Hence, these results suggest that *Mrp3*$^{-/-}$ and *Mrp3*$^{+/+}$ mice do not generate significantly different amounts of the APAP reactive intermediate NAPQI.

The biliary concentrations of APAP-sulfate were also the same between *Mrp3*$^{+/+}$ and *Mrp3*$^{-/-}$ mice at all time points (data not shown), with a total cumulative excretion of approximately 0.2% of the administered APAP dose. In contrast, very large and significant differences in the biliary excretion of APAP-GLUC were detected between genotypes at all time points (Fig. 1C). APAP-GLUC elimination in bile of *Mrp3*$^{-/-}$ gradually increased to approximately 6% of the total administered dose after 120 minutes, whereas *Mrp3*$^{+/+}$ mice only excreted 0.5% of

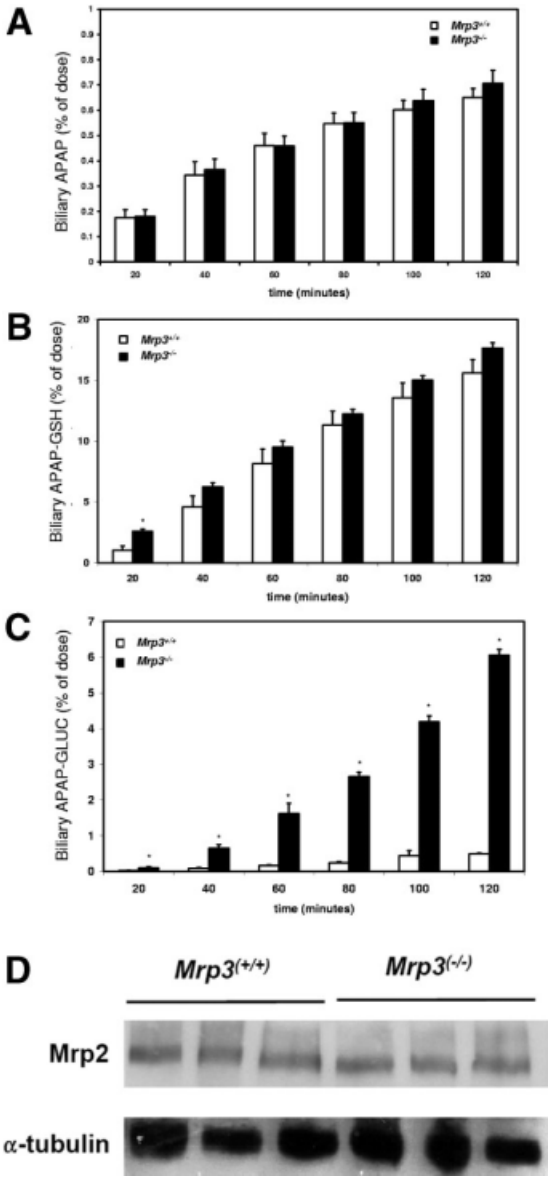

Fig. 1. Biliary excretion of APAP and its metabolites in Mrp3$^{+/+}$ and Mrp3$^{-/-}$ mice. Animals received 150 mg APAP/kg after bile duct cannulation. Bile was collected at 20-minute intervals for 120 minutes. Bile samples were processed and analyzed as described in Materials and Methods. Data are expressed as cumulative percentage of administered dose recovered in bile as unmetabolized APAP. Data represent means ± SE (*$P \leq .05$, n = 3-4). (A) APAP; (B) APAP-GSH; (C) APAP-GLUC. (D) Western blot of liver homogenates from *Mrp3*$^{+/+}$ and *Mrp3*$^{-/-}$ that was probed with a monoclonal antibody against Mrp2 (M_2III-5). APAP, acetaminophen; APA-GSH, acetaminophen glutathione; APAP-GLUC, acetaminophen glucuronide.

the dose as this conjugate into bile (Fig. 1C). This 10-fold increase in biliary excretion indicates that in the absence of Mrp3, the disposition of a portion of the glucuronide conjugate is shifted into the biliary system. Western blotting revealed similar levels of Mrp2 protein in *Mrp3*$^{-/-}$ and *Mrp3*$^{+/+}$ livers (Fig. 1D), demonstrating that the increased biliary APAP-GLUC excretion was not caused by compensatory upregulation of Mrp2.

Hepatic Concentration of APAP and Its Metabolites. Concentrations of unmetabolized APAP and its conjugates were also measured in liver at the end of the bile collection period (Fig. 2A; please note that the Y-axis of this figure is logarithmic). Nearly equal concentrations of APAP-GLUC and APAP-GSH were found in livers from *Mrp3*$^{+/+}$ mice (237 ± 25 and 284 ± 50 nmol/g tissue, respectively). These were the 2 most abundant metabolites found in *Mrp3*$^{+/+}$ livers. No differences in APAP-GSH, APAP-SULF, or APAP-CYS concentrations were seen between wild types and knockouts, whereas unmetabolized APAP was slightly, but significantly higher in *Mrp3*$^{-/-}$ livers. In contrast, *Mrp3*$^{-/-}$ mice showed a dramatic accumulation of APAP-GLUC. Its hepatic concentration was 4.9 ± 1.1 μmol/g liver. This 20-fold difference in hepatic APAP-GLUC content represents retention of approximately 20% of the administered APAP dose in *Mrp3*$^{-/-}$, in contrast to 1% found in *Mrp3*$^{+/+}$ livers. The strong accumulation of APAP-GLUC in the liver of *Mrp3*$^{-/-}$ mice shows that Mrp3 is the only effective basolateral transporter for this conjugate. Overall, *Mrp3*$^{-/-}$ mice retained approximately 25% of the APAP dose in their livers, mostly in the form of APAP-GLUC, whereas *Mrp3*$^{+/+}$ livers retained only 3% to 4% of the dose.

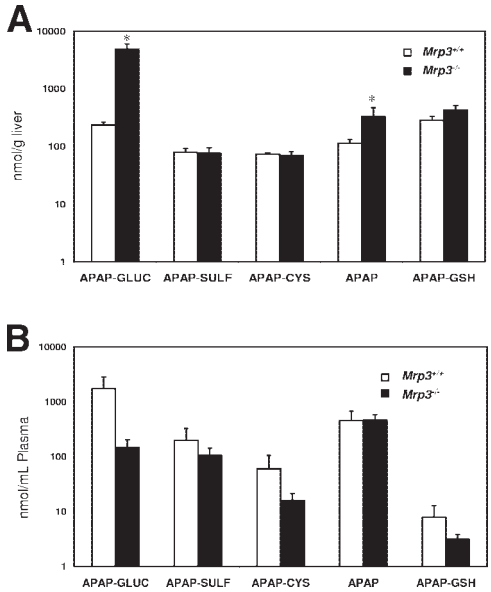

Fig. 2. Hepatic and plasma concentrations of APAP and its metabolites in bile duct–cannulated Mrp3$^{+/+}$ and Mrp3$^{-/-}$ mice. At the end of the biliary excretion experiments, livers were collected and analyzed for APAP and its metabolites as described in Materials and Methods. For details on experimental procedures, see legend to Fig. 1. (A) Hepatic concentrations expressed as nmol/g liver; (B) Plasma concentrations (nmol/mL). Data are expressed as means ± SE. (*$P \leq .05$, n = 3-4). Please note that the Y-axis of both graphs has a logarithmic scale. APAP, acetaminophen.

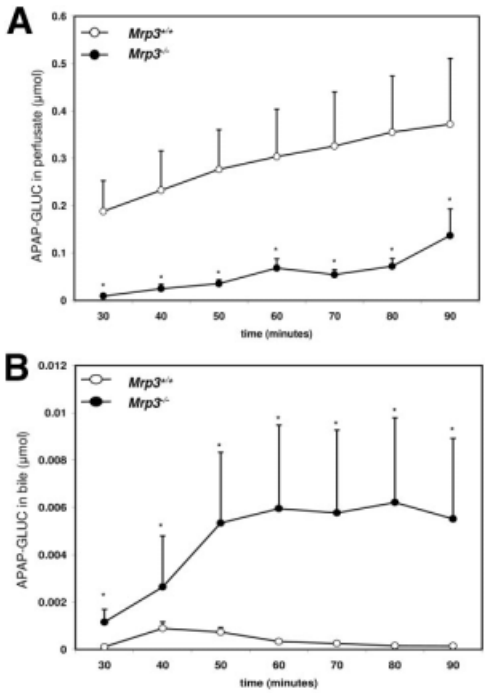

Fig. 3. Basolateral and canalicular excretion of APAP-GLUC in isolated perfused livers from Mrp3$^{+/+}$ and Mrp3$^{-/-}$ mice. Isolated livers from Mrp3$^{+/+}$ and Mrp3$^{-/-}$ mice were perfused with Krebs-bicarbonate buffer as described in Materials and Methods. APAP (2.5 μmol/L) was infused into the portal cannula at a flow rate of 0.5 mL/min for 1 minute. APAP-GLUC was measured in bile and perfusate samples collected at 10-minute intervals for 90 minutes. Data are expressed as micromoles APAP-GLUC in perfusate (A) and bile (B) $\pm$ S.E. (*$P \leq .05$, n = 4). APAP, acetaminophen; APAP-GLUC, acetaminophen glucuronide.

Plasma Concentrations of APAP and Its Conjugates. Plasma concentrations of APAP and its conjugates are shown in Fig. 2B (please note that the Y-axis of this figure is logarithmic). Plasma APAP-GLUC concentration was more than 10-fold lower in *Mrp3*$^{-/-}$ mice than in *Mrp3*$^{+/+}$, which is consistent with the strong accumulation of the glucuronide in the liver. Unmetabolized APAP was the second most abundant form of the compound found in plasma, but this was present at similar concentrations in both strains. As expected, only small amounts of APAP-GSH were found in plasma of *Mrp3*$^{+/+}$ and *Mrp3*$^{-/-}$ mice (7.9 $\pm$ 5.1 vs. 3.13 $\pm$ 0.07 nmol/mL, respectively), because this conjugate is preferentially excreted into bile via Mrp2. No differences in plasma levels for all remaining metabolites were detected.

In Situ *Liver Perfusion Studies.* Sinusoidal secretion of APAP and its metabolites was assessed in the recirculating perfused liver system. On infusion of APAP, the appearance of APAP-GLUC in the perfusate of *Mrp3*$^{-/-}$ livers was greatly reduced compared to *Mrp3*$^{+/+}$ livers (Fig. 3A). This was accompanied by a substantial increase in biliary APAP-GLUC excretion in *Mrp3*$^{-/-}$ livers (Fig. 3B). During the entire perfusion, there were no differences in basolateral or biliary excretion of unmetabolized APAP or any of the other metabolites, including APAP-GSH (not shown). These results are in full agreement with those of the biliary cannulation studies *in vivo*. In the isolated perfused liver, we found no differences in sinusoidal or canalicular GSH excretion during APAP treatment between *Mrp3*$^{+/+}$ and *Mrp3*$^{-/-}$ mice (not shown).

Toxicity Studies. We also examined the susceptibility of *Mrp3*$^{-/-}$ mice to APAP toxicity. In a pilot study using 250 mg APAP/kg, adminstered intraperitoneally, the 12- and 24-hour plasma ALT and AST values were lower in *Mrp3*$^{-/-}$ mice when compared with *Mrp3*$^{+/+}$, but the differences were not statistically significant (data not shown). A higher dose of APAP (400 mg/kg) produced 24-hour plasma AST and ALT values of 2,850 $\pm$ 650 and 4,600 $\pm$ 1,350 U/L, respectively, in *Mrp3*$^{+/+}$ mice (Fig. 4). In contrast, *Mrp3*$^{-/-}$ mice had significantly lower values for both enzymes (by approximately 70%). Plasma alkaline phosphatase activity was generally similar in both strains at either time.

Histological analysis of liver sections obtained at 24 hours after APAP were in line with the results of the 24-hour plasma ALT and AST values (Table 1). Liver sections were scored using a scale from 0 to 5, depending on the severity of centrilobular hepatocellular degeneration and necrosis. Livers with scores higher than 2 are considered to exhibit significant damage.[22,23] In *Mrp3*$^{+/+}$ mice, 60% of the animals had scores higher than 2, whereas none of the knockouts showed significant damage by this criteria. Collectively, the significantly lower increases in plasma ALT and AST along with lower histology scores in *Mrp3*$^{-/-}$ mice indicate that these knockouts are more resistant to APAP hepatotoxicity.

NAD(P)H:quinone oxidoreductase 1 is able to reduce NAPQI to the parent compound APAP, thereby reducing

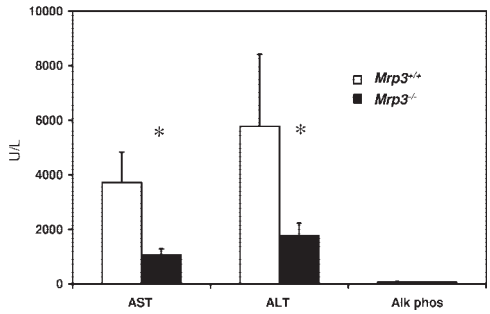

Fig. 4. Susceptibility of Mrp3$^{+/+}$ and Mrp3$^{-/-}$ mice to APAP hepatotoxicity. Mrp3$^{+/+}$ and Mrp3$^{-/-}$ mice received 400 mg APAP/kg after overnight fasting. Plasma was isolated 24 hours later for analysis of aminotransferases (ALT and AST) and alkaline phosphatase as indicators of liver injury. Results are expressed as U/L $\pm$ SE for 5 to 6 animals per group. Results are expressed as means $\pm$ SE (*$P \leq .05$; n = 5-6). APAP, acetaminophen; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Table 1. Liver Pathology Scores in APAP-Treated *Mrp3*$^{-/-}$ and *Mrp3*$^{+/+}$ Mice

	Histology scores					
Strain	**0**	**1**	**2**	**3**	**4**	**5**
Mrp3$^{+/+}$	0	0	2	3	0	0
Mrp3$^{-/-}$ *	0	2	4	0	0	0

The table indicates the number of mice that received the indicated histology score. *Mrp3*$^{+/+}$ and *Mrp3*$^{-/-}$ mice were fasted overnight and received 400 mg/kg APAP in 50% propylene glycol. 24 hours later, mice were euthanized and a portion of the liver was stored in 10% phosphate-buffered formalin, embedded, sectioned and stained with hematoxylin and eosin for examination under a light microscope. Tissue sections were scored (from 0 to 5) according to the severity of necrosis as previously described.[22,23] Scores greater than 2 are indicative of significant necrosis (*$P \leq .05$; n = 5–6).

toxicity of the drug.[25] Activity of this enzyme was similar in livers of *Mrp3*$^{+/+}$ and *Mrp3*$^{-/-}$ mice (34.8 ± 8.1 and 37.6 ± 0.5 nmol/minutes/mg protein, respectively). Hence, increased resistance in *Mrp3*$^{-/-}$ mice was not caused by increased activity of this enzyme.

Hepatic GSH Content in Mrp3$^{-/-}$ Mice After APAP

Biliary Disposition Studies

Because GSH plays an important role in the detoxification of APAP and in modulating transport function of several MRP isoforms, hepatic GSH concentration was also determined in both strains at the end of the biliary disposition experiments (2 hours after APAP administration). Untreated *Mrp3*$^{+/+}$ and *Mrp3*$^{-/-}$ mice had similar hepatic GSH contents (Table 2). APAP treatment led to a more than 90% depletion of the GSH content in *Mrp3*$^{+/+}$ mice. However, this depletion was less severe in livers from *Mrp3*$^{-/-}$ mice, which had almost 3 times higher residual GSH content than *Mrp3*$^{+/+}$ (Table 2). This finding suggests that either less hepatic GSH utilization, less excretion, or more synthesis occurs in response to APAP in knockout mice. Table 2 shows that the basal GSH levels in bile are similar in both strains. Furthermore, GSH secretion into bile after APAP administration (50 minutes after injection) is not significantly different between *Mrp3*$^{+/+}$ and *Mrp3*$^{-/-}$ mice. These observations indicate that the higher GSH content in *Mrp3*$^{-/-}$ mouse liver after APAP exposure is not caused by basal differences in hepatobiliary GSH excretion.

Table 2. Hepatic and Biliary GSH Concentration in *Mrp3*$^{+/+}$ and *Mrp3*$^{-/-}$ Mice

	Mrp3$^{+/+}$	*Mrp3*$^{-/-}$
Hepatic GSH content untreated mice	6430 ± 810	7600 ± 370
Hepatic GSH content APAP treated mice	520 ± 120	1420 ± 470*
Biliary [GSH] untreated mice	1.6 ± 0.2	2.2 ± 0.5
Biliary [GSH] APAP treated mice (at t = 50 min)	0.44 ± 0.21	0.72 ± 0.13

Mrp3$^{+/+}$ and *Mrp3*$^{-/-}$ mice received 150 mg APAP/kg after bile duct cannulation. Bile was collected at 20 min intervals for 120 min. At the end of the experiment, livers were collected for GSH analysis as described in Materials and Methods. Liver and bile GSH concentrations were also analyzed in non-APAP treated bile duct-cannulated mice. Hepatic GSH concentration is expressed as moles/g liver ± SE and biliary GSH concentration is expressed as nmol/mL of bile ± SE (*$P \leq .05$; n = 3–4).

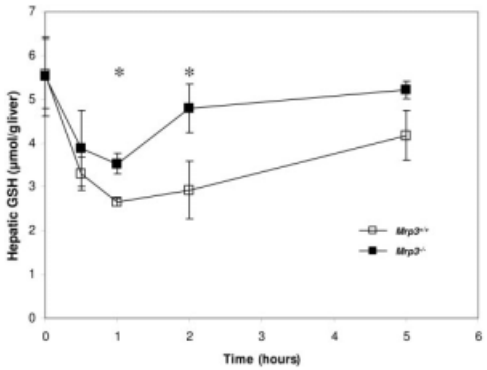

Fig. 5. GSH depletion on APAP administration. Mrp3$^{+/+}$ and Mrp3$^{-/-}$ mice received 150 mg APAP/kg intravenously. At the indicated intervals, animals were sacrificed and the liver was harvested. GSH was determined and expressed per gram liver weight. Each point represents the mean ± SD (*$P \leq .05$; n = 3). GSH, glutathione; APAP, acetaminophen.

Subsequently, we investigated whether the difference in residual hepatic GSH was caused by increased repletion. To this end, we measured hepatic GSH concentrations at various times after administration of APAP (150 mg/kg). Figure 5 shows that hepatic GSH levels were identical before APAP administration and similarly depleted at 0.5 hours after administration. However, recovery to normal GSH levels was significantly more rapid in *Mrp3*$^{-/-}$ mice compared with *Mrp3*$^{+/+}$ mice. These data indicate that glutathione synthesis is activated more rapidly in *Mrp3*$^{-/-}$ mice than in controls.

Discussion

The physiological role of MRP3 has been studied by indirect approaches, that is, by determination of tissue localization, upregulation in pathological conditions, and substrate specificity in *in vitro* assays. The *Mrp3* knockout mouse model provides a direct way to investigate the role of Mrp3 in the disposition of endogenous compounds and xenobiotics. We have looked at the disposition of APAP in *Mrp3*$^{-/-}$ mice, because glucuronidation is quantitatively the most important detoxification pathway for APAP, and Mrp3 has been characterized as a transporter with preference for glucuronides. Indeed, we found that Mrp3 is indispensable for the basolateral excretion of APAP-GLUC from hepatocytes. In the absence of Mrp3, sinusoidal APAP-GLUC excretion was strongly reduced.

Concomitantly, APAP-GLUC accumulated within the liver, with a small fraction of this conjugate being redirected into the bile. Mrp3 thus appears to be the only basolateral transporter that can efficiently secrete APAP-GLUC into blood.

Of all the metabolites generated in animals receiving APAP (APAP-GLUC, APAP-GSH, APAP-CYS, and APAP-SULF), only the biliary disposition of APAP-GLUC was significantly altered in knockout mice. This shows that Mrp3 is not involved in excretion of non-glucuronidated APAP metabolites.

Our results combined with those of other reports demonstrate that rats and mice handle APAP-GLUC differently. Experiments with the TR$^-$ rat, which lacks Mrp2, have shown that this transporter is responsible for the excretion of APAP-GLUC into bile.[11,14] We assume that Mrp2 is also responsible for biliary APAP-GLUC excretion in the mouse. We show here that Mrp3 exclusively mediates APAP-GLUC secretion into blood in the mouse, and it probably does so in the rat as well.[13-15] In rat liver, approximately 50% of APAP-GLUC is eliminated into bile, whereas the other 50% ends up in sinusoidal blood.[26] In contrast, we show that most APAP-GLUC formed in normal mouse liver is excreted into blood. The difference must be caused by a different balance of Mrp2 and Mrp3 activity in the hepatocyte. In the rat, *Mrp3* expression is extremely low under basal conditions, but it is highly induced under cholestatic conditions as well as in the Mrp2-deficient TR$^-$ rat.[13,14,27,28] In contrast, *Mrp3* expression in FVB mice is already high under basal conditions and not induced further on bile duct ligation.[2] Thus, in the mouse, Mrp3 competes with Mrp2 for transport of APAP-GLUC, whereas in the rat the low level of Mrp3 limits transport into the blood, as already suggested by Xiong et al.[14]

Strikingly, the affinities of Mrp2 and Mrp3 for APAP-GLUC in *in vitro* transport assays appear to be very low. The K_m of APAP-GLUC for rat Mrp3 was found to be 0.9 mmol/L and in the same type of vesicular assays APAP-GLUC transport via rat Mrp2 could even not be detected, but APAP-GLUC weakly inhibited transport of another substrate with a K_i of 4 to 5 mmol/L.[14] K_m values from *in vitro* transport assays with Mrps should be interpreted with caution, however, as these transporters can be allosterically stimulated by a variety of compounds, some of which may be present in the liver.[16,17] Our data strongly suggest that the "*in vivo* affinity" of mouse Mrp2 for APAP-GLUC must be lower than that of rat Mrp2. In mice, biliary secretion occurs only on accumulation of APAP-GLUC to 5 μmol/g liver (corresponding to more than 5 mmol/L in the hepatocyte cytosol). In rats, however, no such accumulation occurs while half of APAP-GLUC is excreted into bile. Conversely, the affinities of rat and mouse Mrp3 may be similar, as both species efficiently secrete APAP-GLUC into blood under conditions of high *Mrp3* expression (*i.e.,* in the TR$^-$ rat and in *Mrp3*$^{+/+}$ mice under basal conditions, respectively).

Unexpectedly, the hepatotoxicity of APAP was reduced in the *Mrp3*$^{-/-}$ mice. The levels of liver aminotransferases in plasma as well as the hepatic histopathology clearly show that the lack of Mrp3 results in a significant reduction in the severity of hepatotoxicity by APAP. This might be attributed to a higher glucuronidation activity in *Mrp3*$^{-/-}$ mice, an explanation that would be in line with the higher hepatic APAP-GLUC concentrations. We are confident, however, that this is not the case because the biliary excretion of APAP-GSH and APAP-CYS was practically the same for both genotypes. Biliary excretion of APAP-GSH has been proposed as a reliable indicator of *in vivo* formation of NAPQI.[24] This indicates that the CYP450-mediated oxidation of APAP to the reactive intermediate is not altered in *Mrp3*$^{-/-}$ mice. A pronounced enhancement in APAP glucuronidation in the knockouts should have resulted in a substantial reduction in CYP450 metabolism of APAP. Our APAP-GSH biliary excretion data do not support such a shift in APAP metabolism in mice lacking *Mrp3*.

Conversely, we attribute the lower APAP toxicity in *Mrp3*$^{-/-}$ mice to a more rapid repletion of hepatic GSH levels (Fig. 5), which is important in neutralizing the reactive intermediate of APAP, NAPQI, and in mitigating oxidative stress. The more rapid repletion in the *Mrp3*$^{-/-}$ mice must be the consequence of faster GSH synthesis, as we showed that sinusoidal as well as biliary glutathione excretion are equal in *Mrp3*$^{+/+}$ and *Mrp3*$^{-/-}$ mice. Also, the total formation of APAP-GSH is not significantly different in these animals. We hypothesize that increased GSH synthesis is due to either rapid induction of activity or expression of γ-glutamylcysteine synthase, the rate-controlling enzyme in glutathione synthesis, but this remains to be determined. Compounds causing oxidative stress have been shown to induce expression of γ-glutamylcysteine synthase heavy chain as fast as within 30 minutes.[29]

In conclusion, our studies show that the sinusoidal elimination of APAP-GLUC is almost completely dependent on Mrp3 function. In the absence of Mrp3, APAP-GLUC accumulates in the liver sufficiently to redirect a fraction of its excretion through low-affinity canalicular transport involving Mrp2. The absence of Mrp3 also results in lower susceptibility to APAP hepatotoxicity through unknown mechanisms.

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References

1. Borst P, Oude Elferink R. Mammalian ABC transporters in health and disease. Annu Rev Biochem 2002;71:537-592.
2. Zelcer N, van de Wetering K, Smith A, Scheffer A, Marschall H-U, Wielinga P, et al. Mice lacking Mrp3 (Abcc3) have normal bile acid transport, but display altered hepatic transport of endogenous glucuronides. J Hepatol 2005 (in press).
3. Scheffer GL, Kool M, de Haas M, de Vree JM, Pijnenborg AC, Bosman DK, et al. Tissue distribution and induction of human multidrug resistant protein 3. Lab Invest 2002;82:193-201.
4. Hirohashi T, Suzuki H, Sugiyama Y. Characterization of the transport properties of cloned rat multidrug resistance-associated protein 3 (MRP3). J Biol Chem 1999;274:15181-15185.
5. Zelcer N, Saeki T, Bot I, Kuil A, Borst P. Transport of bile acids in multidrug-resistance-protein 3-overexpressing cells co-transfected with the ileal Na^+-dependent bile-acid transporter. Biochem J 2003;369:23-30.
6. Davidson DGD, Eastham WN. Acute liver necrosis following overdose with paracetamol. Br Med J 1966;2:497-499.
7. Clark R, Thompson RPH, Borirakchanyavat V, Widdop B, Davidson AR, Williams R Hepatic damage and death from overdose of paracetamol. Lancet 1973;i:66-70.
8. Hjelle J, Klaassen CD. Glucuronidation and biliary excretion of acetaminophen in rats. J Pharm Pharmacol 1984;228:407-413.
9. Gregus Z, Madhu C, Klaassen CD. Species variation in toxication and detoxification of acetaminophen in vivo: a comparative study of biliary and urinary excretion of acetaminophen metabolites. J Pharmacol Exp Ther 1988;244:91-99.
10. Manautou JE, Tveit A, Hoivik DJ, Khairallah EA, Cohen SD. Protection by clofibrate against acetaminophen hepatotoxicity in male CD-1 mice is associated with an early increase in biliary concentration of APAP-GSH adducts. Toxicol Appl Pharmacol 1996;140:30-38.
11. Chen C, Hennig GE, Manautou JE. Hepatobiliary excretion of acetaminophen glutathione conjugate and its derivatives in transport-deficient (TR-) hyperbilirubinemic rats. Drug Metab Dispos 2003;31:798-804.
12. Xiong H, Turner C, Ward ES, Jansen PLM, Brouwer KLR. Altered hepatobiliary disposition of acetaminophen glucuronide in isolated perfused livers from multidrug resistance-associated protein 2-deficient TR^- rats. J Pharmacol Exp Ther 2000;295:512-518.
13. Ogawa K, Suzuki H, Hirohashi T, Ishikawa T, Meier PJ, Hirose K, et al. Characterization of inducible nature of MRP3 in rat liver. Am J Physiol 2000;278:G438-G446.
14. Xiong H, Suzuki H, Sugiyama Y, Meier PJ, Pollack GM, Brouwer KLR. Mechanism of impaired biliary excretion of acetaminophen glucuronide after acute phenobarbital treatment or phenobarbital pretreatment. Drug Metab Dispos 2002;30:962-969.
15. Slitt AL, Cherrington NJ, Maher JM, Klaassen CD. Induction of multidrug resistance protein 3 in rat liver is associated with altered vectorial excretion of acetaminophen metabolites. Drug Metab Dispos 2003;31:1176-1186.
16. Zelcer N, Huisman MT, Reid G, Wielinga P, Breedveld P, Kuil A, et al. Evidence for two interacting ligand binding sites in human multidrug resistance protein 2 (ATP binding cassette C2). J Biol Chem 2003;278:23538-23544.
17. Chu XY, Huskey SE, Braun MP, Sarkadi B, Evans DC, Evers R. Transport of ethinylestradiol glucuronide and ethinylestradiol sulfate by the multidrug resistance proteins MRP1, MRP2, and MRP3. J Pharmacol Exp Ther 2004;309:156-164.
18. Groen AK, Van Wijland MJ, Frederiks WM, Smit JJ, Schinkel AH, Oude Elferink RP. Regulation of protein secretion into bile: studies in mice with a disrupted mdr2 p-glycoprotein gene. Gastroenterology 1995;109:1997-2006.
19. Oliveira EJ, Watson GD, Morton NS. A simple microanalytical technique for the determination of paracetamol and its main metabolites in blood spots. J Pharm Biomed Anal 2002;29:803-809.
20. Howie D, Adriaenssens PI, Prescott LF. Paracetamol metabolism following overdosage: application of high performance liquid chromatography. J Pharm Pharmacol 1977;29:235-237.
21. Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal Biochem 1969;27:502-522.
22. Manautou JE, Hoivik DJ, Tveit A, Emeigh Hart SG, Khairallah EA, Cohen SD. Clofibrate pretreatment diminishes acetaminophen's selective covalent binding and hepatotoxicity. Toxicol Appl Pharmacol 1994;129:252-263.
23. Manautou JE, Silva VM, Hennig GE, Whiteley HE. Repeated dosing with the peroxisome proliferator clofibrate decreases the toxicity of model hepatotoxic agents in male mice. Toxicology 1998;172:1-10.
24. Madhu C, Gregus Z, Klaassen CD. Biliary excretion of acetaminophen-glutathione as an index of toxic activation of acetaminophen: effect of chemicals that alter acetaminophen hepatotoxicity. J Pharmacol Exp Ther 1989;248:1069-1077.
25. Gonzalez FJ. The study of xenobiotic-metabolizing enzymes and their role in toxicity in vivo using targeted gene disruption. Toxicol Lett 1998;102-103:161-166.
26. Brouwer KL, Jones JA. Altered hepatobiliary disposition of acetaminophen metabolites after phenobarbital pretreatment and renal ligation: evidence for impaired biliary excretion and a diffusional barrier. J Pharmacol Exp Ther 1990;252:657-664.
27. Soroka CJ, Lee JM, Azzaroli F, Boyer JL. Cellular localization and up-regulation of multidrug resistance-associated protein 3 in hepatocytes and cholangiocytes during obstructive cholestasis in rat liver. HEPATOLOGY 2001;33:783-791.
28. Donner MG, Keppler D. Up-regulation of basolateral multidrug resistance protein 3 (Mrp3) in cholestatic rat liver. HEPATOLOGY 2001;34:351-359.
29. Day RM, Suzuki Y, Lum JM, White AC, Fanburg BL. Bleomycin upregulates expression of γ-glutamylcysteine synthetase in pulmonary artery endothelial cells. Am J Physiol 2002;282:L1349-L1357.