MULTIPLE MECHANISMS ARE INVOLVED IN THE BILIARY EXCRETION OF ACETAMINOPHEN SULFATE IN THE RAT: ROLE OF MRP2 AND BCRP1

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ABSTRACT:

Previous reports have demonstrated that sulfate metabolites may be excreted into bile by the multidrug resistance-associated protein 2 (Mrp2, Abcc2). Although recombinant human breast cancer resistance protein (BCRP, ABCG2) has affinity for sulfated xenobiotics and endobiotics, its relative importance in biliary excretion of sulfate metabolites in the intact liver is unknown. In the present studies, the potential contribution of Bcrp1 to the biliary excretion of acetaminophen sulfate (AS) was examined following acetaminophen administration (66 μmol, bolus) to isolated perfused livers (IPLs) from wild-type Wistar and Mrp2-deficient (TR GF120918) rats in the presence or absence of the Bcrp1 and P-glycoprotein inhibitor, GF120918. In vitro and in situ assays indicated that impaired AS biliary excretion in the presence of GF120918 was due to inhibition of Bcrp1, and not P-glycoprotein. In conclusion, Mrp2 and, to a lesser extent, Bcrp1 mediate biliary excretion of AS in the intact liver.

Hepatic sulfation of xenobiotics is a common phase II metabolic reaction that increases hydrophilicity of the molecule prior to biliary excretion or efflux across the hepatic basolateral membrane for subsequent renal clearance. Xenobiotic inactivation and/or detoxification may occur by direct sulfation of the parent compound (e.g., acetaminophen) or may follow phase I oxidation (e.g., phenobarbital). However, hepatic xenobiotic sulfation also can lead to activation of prodrugs [e.g., minoxidil (Buhl et al., 1990)] and hepatotoxins [e.g., covalent microsomal protein binding of phenacetin sulfate (Mulder et al., 1977); formation of DNA adducts with tamoxifen sulfate (Glatt et al., 1998); potent bile salt export pump inhibition by troglitazone glucuronide across the canalicular membrane was negligible in the absence of Mrp2 (Takahata, 2002)].

Thromboxane A2 synthase, E3040, was largely maintained in canaliculi in wild-type TR GF120918 rat livers was 5-fold lower relative to wild-type controls (0.3 ± 0.1 versus 1.5 ± 0.3 μmol). In the presence of GF120918, biliary excretion of AS was decreased ~2-fold in both TR (0.16 ± 0.09 μmol) and wild-type (0.8 ± 0.3 μmol) rat IPLs. These changes were primarily due to alterations in the rate constant governing biliary excretion of AS, which was decreased ~90% in TR relative to wild-type rat IPLs (0.02 ± 0.01 versus 0.2 ± 0.1 h⁻¹) and was further decreased in the presence of GF120918 (0.010 ± 0.003 and 0.12 ± 0.05 h⁻¹; TR and wild-type, respectively). In vitro assays indicated that impaired AS biliary excretion in the presence of GF120918 was due to inhibition of Bcrp1, and not P-glycoprotein. In conclusion, Mrp2 and, to a lesser extent, Bcrp1 mediate biliary excretion of AS in the intact liver.

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ABBREVIATIONS: E3040, 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl)benzothiazole; AS, acetaminophen sulfate; AUC, area under the concentration curve; Bcrp1 (Abcg2), breast cancer resistance protein 1; GF120918, N-(4-[2-[1,2,3,4-tetrahydro-6,7-dimethoxy-2-isooquinolinyl](ethyl)-phenyl]-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide); Recovery of AS in bile of TR⁻⁻⁻⁻⁻ rats was ~5-fold lower relative to wild-type controls (0.3 ± 0.1 versus 1.5 ± 0.3 μmol). In the presence of GF120918, biliary excretion of AS was decreased ~2-fold in both TR⁻⁻⁻⁻⁻ (0.16 ± 0.09 μmol) and wild-type (0.8 ± 0.3 μmol) rat IPLs. These changes were primarily due to alterations in the rate constant governing biliary excretion of AS, which was decreased ~90% in TR⁻⁻⁻⁻⁻ relative to wild-type rat IPLs (0.02 ± 0.01 versus 0.2 ± 0.1 h⁻¹) and was further decreased in the presence of GF120918 (0.010 ± 0.003 and 0.12 ± 0.05 h⁻¹; TR⁻⁻⁻⁻⁻ and wild-type, respectively). In vitro assays indicated that impaired AS biliary excretion in the presence of GF120918 was due to inhibition of Bcrp1, and not P-glycoprotein. In conclusion, Mrp2 and, to a lesser extent, Bcrp1 mediate biliary excretion of AS in the intact liver.

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2003). In contrast, in the absence of Mrp2, acetaminophen sulfate (AS) biliary excretion is maintained partially (~20–30% (Xiong et al., 2000; Chen et al., 2003)). The differential transport properties of glucuronide, glutathione, and sulfate conjugates at the canalicular membrane can be generalized as follows: Mrp2 mediates biliary excretion of glucuronide and glutathione metabolites, whereas sulfate metabolites are transported by Mrp2 and at least one additional pathway.

Recent studies with recombinant breast cancer resistance protein (BCRP, ABCG2) indicated that this transporter, which is localized to the canalicular membrane in hepatocytes, exhibits transport activity for sulfated xenobiotics and endobiotics (Maliepaard et al., 2001; Imai et al., 2003; Suzuki et al., 2003). Suzuki et al. (2003) reported that recombinant BCRP transported dehydroepiandrosterone sulfate, estrone sulfate, 4-methylumbelliferyl sulfate, and E3040 sulfate; BCRP was inhibited by sulfated steroids, bile acids, and xenobiotics, but not by their unconjugated, glucuronidated, or glutathione-conjugated forms. Interestingly, recombinant BCRP also transported glucuronide and glutathione conjugates of xenobiotics that are not excreted into bile of Mrp2-deficient rats. Thus, the physiological relevance of the affinity of sulfate conjugates for BCRP is unclear.

The purpose of this study was to test the hypothesis that both Bcrp1 and Mrp2 contribute to the biliary excretion of AS. At therapeutic concentrations, acetaminophen primarily undergrads direct sulfation and glucuronidation; hepatic clearance via phase I oxidation and excretion of unchanged parent compound into bile are less extensive. However, at toxic concentrations of acetaminophen (>2 mM), sulfation and, eventually, glucuronidation become saturated, resulting in nonlinear systemic disposition of acetaminophen and an increased driving force for oxidation to N-acetyl-p-benzoquinone imine (Galinsky and Levy, 1981; Hjelle and Klaassen, 1984). Unless detoxified by conjugation with glutathione, the toxic electrophilic oxidative metabolite forms covalent adducts with microsomal and cytoplasmic proteins, leading to hepatic necrosis (Dahlin et al., 1984). The glutathione conjugate follows the mercapturic acid degradation pathway by undergoing sequential hydrolysis to form acetaminophen cysteinylglycine and then acetaminophen cysteine; the cysteine metabolite partially undergoes N-acetylation to form acetaminophen mercapturate. Since sulfation and glucuronidation compete with oxidation, and glutathione conjugation inactivates the toxic oxidative metabolite, phase II metabolism of acetaminophen is considered a detoxification mechanism.

Previous transport studies indicated that acetaminophen glucuronide was excreted from the liver into bile by Mrp2 and into sinusoidal blood by multidrug resistance-associated protein 3 (Mrp3, Abcc3) (Xiong et al., 2000, 2002). The glutathione conjugate of the oxidative metabolite was excreted into bile by Mrp2 (Chen et al., 2003). The mechanisms of biliary excretion of AS in the rat liver have not been fully elucidated.

**Materials and Methods**

Acetaminophen, acetaminophen glucuronide, p-aminobenzoic acid, sodium taurocholate, loperamide, triamterene, propranolol, cinetidine, methadone, sodium orthovanadate, adenosine triphosphate, prazosin, and Lucifer yellow were purchased from Sigma-Aldrich (St. Louis, MO). ADP was obtained from BD (Woburn, MA). All other chemicals were of reagent grade and were readily available from commercial sources.

Male wild-type Wistar rats (300–350 g; Charles River Laboratories, Inc., Wilmington, MA) and male Mrp2-deficient (TR) Wistar rats (300–350 g; in-house breeding colony obtained from Dr. Mary Vore, University of Kentucky, Lexington, KY) were used as liver donors. Male wild-type Wistar rats (>400 g) were used as blood donors. Rats were maintained on a 12-h light/dark cycle with access to water and chow ad libitum. Rats were fully anesthetized with ketamine/xylazine (60/12 mg/kg i.p.) prior to surgical manipulation. The Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill approved all procedures.

Recirculating IPL experiments were performed according to standard procedures (Zamek-Gliszczynski and Brouwer, 2004). Briefly, the bice duct was cannulated and the liver perfused through the portal vein with Krebs-Henseleit buffer. The liver was isolated and placed in a humidified, temperature-controlled chamber, and the perfusion was continued ex situ with 80 ml of recirculating Krebs-Henseleit buffer containing 20% (v/v) whole rat blood. Perfusion was continuously oxygenated with the use of a Hamilton pump; perfusate pH was maintained at ~7.4 by addition of sodium bicarbonate as needed. Taurocholate was infused (30 μmol/l) into the perfusate reservoir to maintain bile flow. Livers were allowed to acclimate in the perfusion chamber for 10 min before initiating the experiment. Livers demonstrating acceptable viability (based on portal pressure < 15 cm of water, initial bile flow > 0.8 and > 0.2 μl/min/g liver in wild-type and TR- rat IPIs, respectively, and gross morphology) were used in drug disposition experiments. GF120918 (8 μmol, 0.5 ml of dimethyl sulfoxide) or vehicle was added to the perfusate reservoir 5 min before the acetaminophen dose. Light-sensitive GF120918 was handled in the dark, and all IPI experiments were conducted in the dark after addition of GF120918 or vehicle to the perfusate reservoir. An acetaminophen bolus dose (66 μmol, 1 ml of saline) was administered into the perfusate. Perfusion was sampled every 10 min, and the plasma was used for subsequent analysis. Bile was collected in toto in 10-min intervals. Previous reports documented that similar acetaminophen, GF120918, and dimethyl sulfoxide concentrations did not compromise the viability of isolated perfused rat livers (Booth et al., 1998; Xiong et al., 2000; Zamek-Gliszczynski et al., 2003).

IPL perfusate plasma and bile concentrations of acetaminophen, and its sulfate, glucuronide, glutathione, and cysteinylglycine/cysteine metabolites, as well as the internal standard, p-aminobenzoic acid, were quantified by the reverse-phase high performance liquid chromatography method of Brouwer and colleagues (Brouwer et al., 1990). Briefly, analytes were resolved on a 3 μm Econosil column (4.6 × 250 mm, d5 = 5 μm; Alltech Biotechnology, Nicholasville, KY) and were detected by UV absorbance (λ = 245 nm). Mobile phase (6.5% acetonitrile, 0.75% acetic acid, 25 mM KH2PO4; pH = 3.14) was delivered isocratically at a flow rate of 1.0 ml/min. The overall run time was 30 min. The molar extinction coefficients were assumed to be the same for acetaminophen and the glutathione conjugate of the oxidative metabolite and its hydrolytic products, and the concentrations of these were calculated using the parent compound standard curve (Howie et al., 1977). As reported by others using similar analytical procedures, the method used here was not able to resolve the peaks corresponding to acetaminophen cysteinylglycine and acetaminophen cysteine; thus, these two species were quantified together (Howie et al., 1978; 2003). Acetaminophen mercapturate concentrations were below the limit of detection in all samples and are not reported.

Noncompartmental pharmacokinetic analysis was performed initially to calculate total perfusate clearance of acetaminophen and formation clearances of AS, acetaminophen glucuronide, and N-acetyl-p-benzoquinone imine. The areas under the perfusate acetaminophen concentration–time curves (AUCs) were calculated with WinNonlin 4.1 (Pharsight, Mountain View, CA). Formation clearances of AS, acetaminophen glucuronide, and N-acetyl-p-benzoquinone imine were calculated by dividing the total amount of a given metabolite recovered in perfusate and bile over 90 min by AUCl,90 mmol of...
acacetaminophen in perfusate. Total recovery of N-acetyl-p-benzoquinone imine was calculated using the recovery of the glutathione conjugate and all of its hydrolytic products, which are representative of the extent of acetaminophen oxidation (Madhu et al., 1989). Biliary excretion clearance of the parent compound was calculated by dividing the amount of the parent compound recovered in bile over 90 min by the AUC_{0–90 min} of acetaminophen in perfusate.

A compartmental modeling approach was used to characterize further the disposition of acetaminophen and its sulfate metabolite in wild-type and TRβ-lentiviral IFLPs in the presence or absence of GF120918. Pharmacokinetic models of Studenberg and Brouwer (1992), who described AS formation as a first-order process, and Xiong et al. (2000), who modeled the data using saturable sulfation with the K_{sat} fixed at the value estimated in suspended hepatocytes, were used as starting points in model development for this data set. Differential equations based on perfusate concentrations and biliary excretion rates of acetaminophen and its sulfate conjugate, as well as models modified with various combinations of first-order and saturable processes, were simultaneously resolved using least-squares regression analysis (WinNonlin 4.1; Pharsight). The goodness of fit of each model was evaluated using visual examination of the distribution of residuals, coefficients of variation on estimated parameters, correlation between parameters, rank numbers, and Akaike’s Information Criterion. Based on these criteria, the model presented in Fig. 1A best described the data. The following differential equations were simultaneously resolved to fit the perfusate concentration-time and biliary excretion rate-time profiles of acetaminophen and AS:

\[
\frac{dC_A}{dt} = -k_X C_A - k_{OTHER} C_A + C_A - \frac{X_0}{V}
\]

\[
\frac{dX_{AS}^{intra}}{dt} = k_X C_A V - k_{PAS} X_{AS}^{intra} - k_{BAS} X_{AS}^{intra}, X_{AS}^{intra} = 0
\]

\[
V \times \frac{dC_A}{dt} = k_{PAS} X_{AS}^{intra}, C_{AS}^{intra} = 0
\]

\[
\frac{dX_{AS}^{bili}}{dt} = k_{BAS} X_{AS}^{intra}, X_{AS}^{intra} = 0
\]

The ability of P-glycoprotein (Abcb1, Mdr1) to transport AS was evaluated in three validated in vitro assays: stimulation of P-glycoprotein ATPase activity, inhibition of P-glycoprotein-mediated calcine-AM efflux, and MDR1-MDCKII cell monolayer flux using previously described standard procedures (Polli et al., 2001). P-glycoprotein ATPase activity was measured in membranes from MDR1-transfected High Five cells according to the manufacturer’s instructions (BD Gentest). Briefly, loperamide (positive control; 10, 50, or 100 μM), triamterene (negative control; 10, 50, or 100 μM), and AS (0.1, 0.5, or 1 mM) were incubated with P-glycoprotein membranes (40 μg) and ATP (5 mM), in the absence or presence of the inhibitor of P-glycoprotein ATPase, ouabain (100 μM), in tris-morpholineethanesulfonate buffer (pH = 6.8; 60 μL; total volume) at 37°C for 30 min. The reaction was stopped by addition of sodium dodecyl sulfate (30 μL; 10% v/v) prior to incubation with the detection reagent (180 μL; 4 1/v/10% ascorbic acid; 35 mM ammonium molybdate/15 mM zinc acetate) at 37°C for 20 min and measurement of optical density (λ = 655 nm).

The calcine-AM efflux assay was conducted in passage 29 MDR1-MDCKII cells seeded at a density of 70,000 cells/well in a 96-well plate after 3 days of culture. Inhibition of P-glycoprotein-mediated calcine-AM (5 μM) efflux by loperamide (positive control; 10, 50, or 100 μM), triamterene (negative control; 10, 50, or 100 μM), and AS (0.1, 0.5, or 1 mM) was compared with inhibition observed in the presence of GF120918 (2 μM). After 60-min incubation of cells with calcine-AM and test compounds in phosphate-buffered saline (pH = 7.4; 100 μL, total volume) at room temperature, calcine-associated fluorescence was quantified (λ_ex = 485 nm, λ_em = 530 nm).

Passage 34 MDR1-MDCKII cells were seeded on 12-well Transwell polycarbonate inserts at a density of 200,000 cells/cm² and were cultured for 4 days when mannitol apparent permeability (8 ± 1 mm/s) indicated confluence. Cell monolayers were rinsed with Hanks’ balanced salt solution and were preincubated with GF120918 (2 μM) or vehicle in both apical (0.4 mL) and basolateral (1.5 mL) chambers at 37°C for 30 min. Hanks’ balanced salt solution was replaced in the donor chamber with either mannitol (paracellular marker; 3.5 μM), propranolol (transcellular marker; 100 μM), loperamide (positive control; 10 μM), triamterene (negative control; 10 μM), or AS (10 μM), with GF120918 (2 μM) or vehicle. Monolayers were incubated at 37°C for 60 min with sampling and replacement of the buffer in the acceptor chamber every 20 min. [14C]Mannitol and [3H]propranolol were quantified by liquid scintillation spectroscopy. Loperamide, triamterene, and AS were quantified by reverse-phase high performance liquid chromatography with detection by tandem mass spectrometry (Applied Biosystems API 4000 triple quadrupole with Turbo-IonSpray interface; Applied Biosystems/MDS Sciex, Foster City, CA). Briefly, loperamide, triamterene, and the internal standard, methadone, were eluted from a Phenomenex Synergi Max-RP column (2 × 30 mm, dp = 4 μm; Phenomenex, Torrance, CA) using a mobile phase gradient (A, 10 mM ammonium acetate with 1% 2-propanol; B, methanol; 0–2 min, linear gradient from 5% B to 95% B; 2–2.5 min, hold at 95% B; 2.5–3 min, linear gradient to 5% B; 0- to 2-min flow rate = 0.75 mL/min; 2- to 3-min flow rate = 1.5 mL/min; 0.8–2.5 min, directed to mass spectrometer) and were detected in positive ion mode using multiple reaction monitoring: loperamide, 477.4 → 266.0 m/z; triamterene, 251.1 → 237.2 m/z; methadone, 310.3 → 265.1 m/z; AS and the internal standard, cetidine, were eluted from an Aquasil C18 column (2.1 × 50 mm, dp = 5 μm; Thermo Electron Corporation, Waltham, MA) using a mobile phase gradient (A, 0.05% formic acid; B, acetonitrile; 0–0.75 min, hold at 0% B; 0.75–4 min, linear gradient to 70% B; 4–4.5 min, hold at 70% B; 4.5–4.6 min, linear gradient to 0% B; 4.6–5 min, hold at 0% B; flow rate = 0.75 mL/min; 0.8–5 min, directed to mass spectrometer) and were detected in negative ion mode using multiple reaction monitoring: AS, 229.84 → 149.84 m/z; cetidine, 250.91 → 156.90 m/z.

Passage 7 murine Bcrp1-MDCKII cells were seeded on 12-well Transwell polycarbonate inserts at a density of 300,000 cells/cm² and were cultured for 3 days, when Lucifer yellow apparent permeability (5 ± 2 mm/s) indicated confluence. Cell monolayers were preincubated with Dulbecco’s modified Eagle’s medium containing GF120918 (5 μM) or vehicle in both apical (0.4 mL) and basolateral (1.5 mL) chambers at 37°C for 15 min. The preincubation medium was replaced in the donor chamber with Dulbecco’s modified Eagle’s medium containing test compound [prazosin (positive control, 3 μM) or acetaminophen (10 μM)], GF120918 (5 μM), or vehicle, and Lucifer yellow (paracellular marker; 100 μM). Receiver chamber preincubation medium was replaced with Dulbecco’s modified Eagle’s medium containing GF120918 (5 μM) or vehicle. Monolayers were incubated with shaking at 37°C for 3 h [90 min for prazosin for ease of comparison with historical controls (Polli et al., 2004)], when medium from both the apical and basolateral chambers was collected. Lucifer yellow was quantified by fluorescence spectrophotometry.
and TR GF120918, the biliary excretion of AS in IPLs from both wild-type livers was decreased. In the bile, AS and acetaminophen glucuronide accounted for greater in perfusate than in bile; more extensive recovery in perfusate and bile is summarized in Table 1. In control wild-type rat IPLs, the presence of GF120918 did not affect significantly the fractions of the dose eliminated by sulfation, glucuronidation, and cysteine/cysteinylglycine conjugates in TR rats. In the presence of GF120918, the biliary excretion of AS in IPLs from both wild-type and TR rats was decreased ~50%. GF120918 had no apparent effect on biliary excretion of the other conjugates.

The apparent fractions of the acetaminophen dose that were eliminated by sulfation, glucuronidation, oxidation, and biliary excretion of unchanged acetaminophen are summarized in Table 2. The presence of GF120918 did not affect significantly the fractions of the dose eliminated by sulfation and glucuronidation. The fractions of the dose cleared by sulfation and glucuronidation were significantly increased in TR rat IPLs, whereas the fractions of the dose cleared by oxidation and biliary excretion of the parent compound were decreased.

Clearance values calculated by noncompartamental analysis of the data are reported in Table 3. Total acetaminophen clearance in TR− rat IPLs was significantly higher due to elevated clearance by sulfation and glucuronidation. Acetaminophen oxidative clearance and clearance by biliary excretion of the parent compound were significantly lower in TR− rat IPLs. The presence of GF120918 did not affect significantly the total acetaminophen clearance or clearance by sulfation and glucuronidation.

Representative fits of eqs. 1 to 4 to perfusate concentrations of acetaminophen and AS, as well as biliary excretion rate of AS in wild-type and TR− rat IPLs, are presented in Fig. 2. Mean parameter estimates obtained by fitting eqs. 1 to 4 to all three functions (acetaminophen and AS perfusate concentrations, AS biliary excretion rate) in all data sets simultaneously are presented in Table 4. The rate constant governing the formation of AS was elevated ~5-fold lower in TR− rat IPLs. The presence of GF120918 did not affect significantly the total acetaminophen clearance or clearance by sulfation and glucuronidation.

Cumulative biliary excretion of AS and AS perfusate concentrations in wild-type and TR− rat IPLs in the presence or absence of GF120918 are shown in Fig. 3. Cumulative biliary excretion of AS was ~5-fold lower in Mrp2-deficient rat IPLs. In both wild-type and

### Table 1

<table>
<thead>
<tr>
<th>Wild-Type Wistar Rat IPLs</th>
<th>GF120918</th>
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<tbody>
<tr>
<td><strong>Bile</strong></td>
<td><strong>Perfusate</strong></td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>Sulfate</td>
<td>2.3 ± 0.5</td>
</tr>
<tr>
<td>Glucuronide</td>
<td>16 ± 4</td>
</tr>
<tr>
<td>Glutathione</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.07 ± 0.08</td>
</tr>
<tr>
<td>Mass balance</td>
<td>21 ± 4</td>
</tr>
<tr>
<td>Total recovery</td>
<td>76 ± 5</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Wild-Type Wistar Rat IPLs</th>
<th>Mrp2-Deficient (TR−) Wistar Rat IPLs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bile</strong></td>
<td><strong>Perfusate</strong></td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>0.150 ± 0.009*</td>
</tr>
<tr>
<td>Sulfate</td>
<td>0.5 ± 0.2*</td>
</tr>
<tr>
<td>Glucuronide</td>
<td>0.029 ± 0.005*</td>
</tr>
<tr>
<td>Glutathione</td>
<td>N.D.</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.005 ± 0.006</td>
</tr>
<tr>
<td>Mass balance</td>
<td>0.7 ± 0.2*</td>
</tr>
<tr>
<td>Total recovery</td>
<td>95 ± 11*</td>
</tr>
</tbody>
</table>

N.D., not detectable.

* P < 0.05, comparison of GF120918 to control within rat type.
1 P < 0.05, comparison of TR− to wild-type within GF120918 treatment group.
2 Sum of acetaminophen cysteine and acetaminophen cysteinylglycine.

(λ<sub>ex</sub> = 430 nm, λ<sub>em</sub> = 538 nm). [3H]Prazosin was quantified by liquid scintillation spectroscopy. AS was quantified by reverse-phase high performance liquid chromatography with detection by tandem mass spectrometry as detailed above.

Statistical significance was assessed by analysis of variance with Tukey’s post hoc test, except where the groups being compared had unequal variances or a data set failed the normality test, in which case analysis of variance on ranks with Dunn’s post hoc test was used. In all cases, p < 0.05 was considered to be statistically significant. All data are reported as mean ± S.D., n = 3 to 4 per condition.

### Results

The percentage recovery of acetaminophen and metabolites in perfusate and bile is summarized in Table 1. In control wild-type rat livers, total recovery of acetaminophen and metabolites was ~2-fold greater in perfusate than in bile; more extensive recovery in perfusate relative to bile was noted in the presence of GF120918 and in TR− rat IPLs. In the bile, AS and acetaminophen glucuronide accounted for the majority of the total amount recovered; the glutathione conjugate and its hydrolytic products as well as biliary excretion of the parent compound were relatively minor. Recovery of AS in bile of TR− rat livers was ~5-fold lower relative to wild-type. In contrast, biliary excretion of the glucuronide, glutathione, and cysteine/cysteinylglycine conjugates was negligible in TR− rat livers. In the presence of GF120918, the biliary excretion of AS in IPLs from both wild-type and TR− rats was decreased ~50%. GF120918 had no apparent effect on biliary excretion of the other conjugates.

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Representative fits of eqs. 1 to 4 to perfusate concentrations of acetaminophen and AS, as well as biliary excretion rate of AS in wild-type and TR− rat IPLs, are presented in Fig. 2. Mean parameter estimates obtained by fitting eqs. 1 to 4 to all three functions (acetaminophen and AS perfusate concentrations, AS biliary excretion rate) in all data sets simultaneously are presented in Table 4. The rate constant governing the formation of AS was elevated ~2.5-fold in livers from TR− rats. Rate constants governing AS basolateral excretion were comparable between rat types; however, the rate constant governing biliary excretion was ~10-fold lower in livers from TR− rats. In IPLs from both rat types, GF120918 decreased the AS biliary excretion rate constant ~40%, although this decrease was not statistically significant.

Cumulative biliary excretion of AS and AS perfusate concentrations in wild-type and TR− rat IPLs in the presence or absence of GF120918 are shown in Fig. 3. Cumulative biliary excretion of AS was ~5-fold lower in Mrp2-deficient rat IPLs. In both wild-type and
Clearance values (mean ± S.D., n = 3–4/group) obtained from noncompartmental pharmacokinetic analysis

Values were calculated as described under Materials and Methods.

<table>
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<tr>
<td><strong>Wild-Type Wistar Rat IPLs</strong></td>
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<tr>
<td><strong>CL</strong></td>
</tr>
<tr>
<td><strong>Control</strong></td>
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<td><strong>CL−</strong></td>
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<td><strong>Cl</strong></td>
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<td><strong>Cl</strong></td>
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* P < 0.05, comparison of TR− to wild-type within GF120918 treatment group.
† P < 0.05, comparison of GF120918 to control within rat type.
‡ Cl−biliary excretion refers to biliary excretion of the parent compound.

TR− rat IPLs, GF120918 appeared to reduce the biliary excretion of AS ~2-fold. Per fusate concentrations of AS were higher in TR− rat IPLs. GF120918 appeared to have little or no effect on AS perfusate concentrations.

The potential interaction of AS with P-glycoprotein was evaluated in three validated in vitro assays using loperamide and triamterene as positive and negative controls, respectively (Polli et al., 2001). Unlike loperamide, triamterene and AS did not stimulate vanadate-sensitive ATPase activity in P-glycoprotein membranes from MDRI-transfected High Five cells (Fig. 4A). P-glycoprotein-mediated efflux of calcine-AM was impaired by loperamide, but not by triamterene or AS (Fig. 4B). MDRI-MDCKII cell monolayer efflux of loperamide was ~11-fold higher in the basolateral-to-apical than in the apical-to-basolateral direction, and was GF120918-sensitive. In contrast, triamterene and AS monolayer flux was similar in the basolateral-to-apical and apical-to-basolateral directions, and was GF120918-independent (Fig. 4C). The apparent permeability values of AS (16 ± 7 nm/s) were comparable to those of the paracellular marker, mannitol (8 ± 1 nm/s, not significant) but were significantly lower than permeability values of the transcellular marker, propranolol (626 ± 47 nm/s, p < 0.05).

Monolayer integrity of Bcrp1-MDCKII cells was confirmed with transcellular flux of prazosin, which was significantly higher in the basolateral-to-apical than in the apical-to-basolateral direction (623 ± 61 versus 32 ± 3 nm/s, respectively; not significant). Functional expression of Bcrp1 in the Bcrp1-MDCKII cell line was confirmed with transcellular flux of prazosin, which was significantly higher in the basolateral-to-apical than in the apical-to-basolateral direction (623 ± 61 versus 32 ± 3 nm/s, p < 0.05). In the presence of GF120918, the basolateral-to-apical over apical-to-basolateral transcellular flux ratio of prazosin was significantly decreased (19 ± 1 versus 2.2 ± 0.1, p < 0.05). Recovery of AS in apical and basolateral chambers following incubation of Bcrp1-
TABLE 5

Appearance of acetaminophen sulfate in apical and basolateral chambers of murine Bcrp1-MDCKII confluent cell monolayers

<table>
<thead>
<tr>
<th>Chamber Incubated with Acetaminophen</th>
<th>Presence of GF120918 (5 μM)</th>
<th>Acetaminophen Sulfate Recovery</th>
<th>Recovery Ratio Apical/Basolateral</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Apical Chamber</td>
<td>Basolateral Chamber</td>
</tr>
<tr>
<td>Apical</td>
<td>−</td>
<td>8.2 ± 0.3</td>
<td>&lt;1.5</td>
</tr>
<tr>
<td>Apical</td>
<td>+</td>
<td>4.6 ± 0.7*</td>
<td>3 ± 3</td>
</tr>
<tr>
<td>Basolateral</td>
<td>−</td>
<td>13 ± 1</td>
<td>&lt;1.5</td>
</tr>
<tr>
<td>Basolateral</td>
<td>+</td>
<td>6.4 ± 0.4*</td>
<td>6.8 ± 0.7*</td>
</tr>
</tbody>
</table>

* P < 0.05, GF120918 vs. vehicle.

MDCKII confluent cell monolayers with acetaminophen is summarized in Table 5. In control Bcrp1-MDCKII cells, AS recovery in the apical chambers was significantly higher than in the basolateral chambers. In contrast, in the presence of GF120918, recovery of AS in apical and basolateral chambers was comparable. Furthermore, in the presence of GF120918, AS recovery in apical chambers was significantly lower relative to control Bcrp1-MDCKII cells. GF120918 significantly decreased the apical-to-basolateral recovery ratio of AS.

Discussion

Consistent with previous reports, the biliary excretion of acetaminophen glucuronide and acetaminophen glutathione was negligible in livers from Mrp2-deficient rats (Xiong et al., 2000; Chen et al., 2003). In contrast, the biliary excretion of AS was maintained partially in the absence of Mrp2. Recent reports suggested that BCRP, a transporter localized to the canalicular membrane in hepatocytes, may be capable of transporting sulfate conjugates (Maliepaard et al., 2001; Imai et al., 2003; Suzuki et al., 2003). To evaluate the potential contribution of Bcrp1 to biliary excretion of AS, acetaminophen disposition was studied in IPLs from wild-type Wistar and Mrp2-deficient TR−/−rats in the presence or absence of the Bcrp1 and P-glycoprotein inhibitor, GF120918.

GF120918 is an inhibitor of both P-glycoprotein and Bcrp1, but not Mrp2 (de Bruin et al., 1999; Evers et al., 2000; Shepard et al., 2003). This modulator has been used previously in recirculating IPLs to impair (~10-fold) biliary excretion of doxorubicin, which is excreted into bile by both P-glycoprotein and Bcrp1 (Booth et al., 1998; Shepard et al., 2003). GF120918 is a less potent inhibitor of Bcrp1 than P-glycoprotein, by approximately an order of magnitude (de Bruin et al., 1999). Thus, the dose of GF120918 in these studies was 10-fold higher than in the doxorubicin studies to ensure maximal Bcrp1 inhibition; in vitro, the extent of inhibition of both transporters by GF120918 is similar and almost complete (de Bruin et al., 1999).

GF120918 was selected to evaluate the potential role of Bcrp1 in biliary excretion of AS because a potent and specific inhibitor of Bcrp1 is not readily available. In addition to Bcrp1, GF120918 would have impaired P-glycoprotein function. Based on the well-characterized properties of P-glycoprotein substrates (large molecular weight lipophilic and amphiphilic cations), a small hydrophilic anion, like AS, is a very unlikely P-glycoprotein substrate (Kimura et al., 2004). However, the observed decrease in AS biliary excretion in the presence of GF120918 necessitated investigation of the potential of AS to interact with P-glycoprotein and Bcrp1.

In vitro assays demonstrated that AS did not stimulate P-glycoprotein ATPase activity. However, a lack of stimulation of ATPase activity does not conclusively rule out an interaction with P-glyco-
protein (Polli et al., 2001). Therefore, additional experiments were conducted. AS did not impair P-glycoprotein-mediated cellular efflux of calcine-AM. AS flux in the basolateral-to-apical direction in MDR1-MDCKII monolayers was not enhanced by P-glycoprotein. In confluent Bcrp1-MDCKII monolayers, the presence of functional Bcrp1 was demonstrated by the significantly higher basolateral-to-apical than apical-to-basolateral transcellular flux of prazosin and the attenuation of this difference in the presence of GF120918 (Polli et al., 2004). Incubation of Bcrp1-MDCKII cell monolayers with acetaminophen resulted in preferential excretion of AS into the apical chamber, which was attenuated in the presence of GF120918, indicating transport of AS by a GF120918-sensitive apical transporter, presumably Bcrp1. Therefore, perturbations in AS biliary excretion in the presence of GF120918 should be attributed to inhibition of Bcrp1 and not P-glycoprotein. Negative results for AS in all three P-glycoprotein assays are consistent with previous in vitro studies, which failed to demonstrate transport of sulfate conjugates by P-glycoprotein (Huang et al., 1998). The current findings also are in agreement with previous studies demonstrating that sulfate conjugates are transported by human BCRP (Imai et al., 2003; Suzuki et al., 2003). Nevertheless, these results must be interpreted with caution, given that hepatic disposition was studied in rat livers, and the in vitro transport assays utilized murine Bcrp1 and human P-glycoprotein.

The pharmacokinetic model that best described the IPL data utilized first-order processes for the formation and subsequent excretion of AS from hepatocytes. Relevant rate constant estimates and their variability were in agreement with previous reports (Studenberg and Brouwer, 1992; Xiong et al., 2000). The rate constant governing the formation of AS was significantly higher in TR− rat livers, consistent with the significantly increased AS formation clearance. These changes resulted in an increase in the apparent fraction of the acetaminophen dose converted to AS, suggesting that sulfation is up-regulated in TR− rats. This finding is consistent with that of Xiong et al. (2000), who estimated the VMAX for sulfation of acetaminophen to be 75% higher in TR− rats relative to wild-type rat livers, assuming the same KMAX. Up-regulation of sulfation may serve as a protective mechanism in the Mrp2-deficient rat, since AS is excreted primarily into sinusoidal blood by a mechanism whose function appears to be maintained in the absence of Mrp2.

The rate constant governing biliary excretion of AS was decreased ~10-fold in TR− rat livers. Greatly impaired biliary excretion of AS in livers from Mrp2-deficient rats demonstrates the key role of this transporter in biliary excretion of acetaminophen conjugates. In livers from both wild-type and Mrp2-deficient rats, GF120918 decreased the rate constant governing biliary excretion of AS ~40%, suggesting that Bcrp1 plays a lesser role than Mrp2 in biliary excretion of AS.

The rate constant governing the basolateral excretion of AS was unchanged in TR− rat livers, suggesting that the mechanism responsible for efflux of AS from hepatocytes to blood is maintained in TR− rat livers. Elevated perfusate concentrations of AS in TR− rat IPLs were caused by the significantly higher AS formation clearance and impaired AS biliary excretion that increased the driving force for AS basolateral efflux. In contrast, the elevated perfusate concentrations of acetaminophen glucuronide in TR− rat IPLs are caused primarily by the ~10-fold increase in the rate constant governing its basolateral excretion (Xiong et al., 2000, 2002). Acetaminophen glucuronide is a substrate for Mrp3, the efflux transporter for organic anions localized to the basolateral membrane of hepatocytes, that is primarily responsible for excreting glucuronide conjugates and bile acids into sinusoidal blood (Konig et al., 1999; Xiong et al., 2002). Consistent with the increase in the rate constant governing basolateral excretion of acetaminophen glucuronide, Mrp3 protein levels are up-regulated by at least an order of magnitude in Mrp2-deficient rats (Xiong et al., 2002). The lack of change in the rate constant governing the basolateral excretion of AS in TR− rat IPLs suggests that Mrp3 is not the primary mechanism of AS basolateral excretion.

Two potential mechanisms could explain the lack of agreement between expression levels of Mrp3 and the rate constant governing basolateral excretion of AS: 1) AS passively diffuses across the basolateral membrane into sinusoidal blood, and 2) AS basolateral excretion is mediated primarily by a transporter whose expression is unchanged in TR− rat liver. Although evidence exists for passive diffusion of acetaminophen, a relatively lipophilic compound (log D = 0.34, pH = 7.4), through the plasma membranes of cells, the hydrophilic sulfate metabolite (log D = −3.90, pH = 7.4) is too polar to passively diffuse through the plasma membrane. In contrast, uptake of acetaminophen into hepatocytes is passive and instantaneous, in contrast to hepatic uptake of AS, which has been characterized as carrier-mediated and fairly insignificant in hepatocytes (Iida et al., 1989). This finding was confirmed by perfusion of the liver with preformed AS, where very poor basolateral membrane permeability of AS resulted in the lack of appreciable hepatic uptake (Goresky et al., 1992).

In the present study, MDR1-MDCKII cell monolayer flux of AS was small; apparent permeability values were similar in the basolateral-to-apical and apical-to-basolateral directions, GF120918-independent, and comparable to those of the paracellular marker mannitol, indicating that the cell monolayer represents a diffusional barrier for AS.

In addition to Mrp3, other transport proteins are present on the basolateral membranes of hepatocytes, including several members of the multidrug resistance-associated protein family, bidirectional organic anion-transporting polypeptides, and the organic anion transporters, whose physiological roles in the liver are not yet fully understood (Chandra and Brouwer, 2004). For example, Mrp4 is a transporter localized to the basolateral membrane of hepatocytes that has high affinity for sulfated bile acids (Rius et al., 2003; Zelcer et al., 2003). Unlike Mrp3, Mrp4 efficiently transports sulfate conjugates of steroids (Zelcer et al., 2003). Bidirectional organic anion-transporting polypeptides are expressed on the hepatic basolateral membrane, where they mediate hepatic uptake of structurally diverse substrates, including sulfated steroids (Chandra and Brouwer, 2004). Hepatic basolateral efflux of AS could be mediated by the organic anion-transporting polypeptides, given their bidirectional activity and affinity for sulfated conjugates. Organic anion transporters are expressed in the liver, where they are thought to mediate hepatic uptake of organic anions, including sulfated steroids; however, potential involvement of these transporters in hepatic basolateral excretion remains to be investigated (Chandra and Brouwer, 2004). Given that AS is not adequately lipophilic to passively diffuse through the plasma membrane, and that the transport characteristics of AS are not in agreement with Mrp3 expression, basolateral excretion of AS may be mediated by another transporter, whose expression is maintained in livers of TR− rats.

In conclusion, biliary excretion of AS is mediated primarily by Mrp2 and, to a lesser extent, by Bcrp1. Sulfation of acetaminophen appears to be up-regulated in Mrp2-deficient TR− rat livers. The absence of change in the rate constant governing basolateral excretion of AS in IPLs from Mrp2-deficient rats, which have highly up-regulated Mrp3, suggests that Mrp3 does not mediate basolateral excretion of AS.

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