Differential Involvement of Mrp2 (Abcc2) and Bcrp (Abcg2) in Biliary Excretion of 4-Methylumbelliferyl Glucuronide and Sulfate in the Rat

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ABSTRACT

The hepatic excretion of hydrophilic conjugates, end products of phase II metabolism, is not completely understood. In the present studies, transport mechanism(s) responsible for the biliary excretion of 4-methylumbelliferyl glucuronide (4MUG) and 4-methylumbelliferyl sulfate (4MUS) were studied. Isolated perfused livers (IPLs) from Mrp2-deficient (TR−) Wistar rats were used to examine the role of Mrp2 in the biliary excretion of 4MUG and 4MUS. After a 30-μmol dose of 4-methylumbelliferyl glucuronide, cumulative biliary excretion of 4MUG was extensive in wild-type rat IPLs (25 ± 3 μmol) but was negligible in TR− livers (0.4 ± 0.1 μmol); coadministration of the Bcrp and P-glycoprotein inhibitor GF120918 [N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isouquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide] had no effect on 4MUG biliary excretion in wild-type rat IPLs. In contrast, biliary excretion of 4MUS was partially maintained in Mrp2-deficient rat IPLs. Recovery of 4MUS in bile was ~50 to 60% lower in both control TR− (149 ± 8 nmol) and wild-type IPLs with GF120918 coadministration (176 ± 30 nmol) relative to wild-type control livers (378 ± 37 nmol) and was nearly abolished in TR− IPLs in the presence of GF120918 (13 ± 8 nmol). These changes were the result of decreased rate constants governing 4MUG and 4MUS biliary excretion. In vitro assays and perfused livers from Bcrp and P-glycoprotein gene-knockout mice indicated that 4MUS did not interact with P-glycoprotein but was transported by Bcrp in a GF120918-sensitive manner. In the rat liver, Mrp2 mediates the biliary excretion of 4MUG, whereas both Mrp2 and Bcrp contribute almost equally to the transport of 4MUS into bile.

The liver is the primary site of xenobiotic detoxification in the body. Whereas substrates of hepatic metabolism may gain access to hepatocytes either by passive or carrier-mediated uptake, conjugation with a glucuronide or sulfate group results in a molecule with greatly decreased membrane permeability that requires active transport for hepatic excretion. Several transport proteins have been identified as efflux pumps for hepatic excretion of phase II metabolites (Zamek-Gliszczynski et al., 2006). Multidrug resistance-associated protein 2 (Mrp2) and breast cancer resistance protein (Bcrp) are localized to the hepatic canalicular (apical) membrane, where they pump substrates into bile (Keppler and Konig, 1997; Maliepaard et al., 2001). Basolateral multidrug resistance-associated proteins 3 and 4 (Mrp3 and Mrp4) are responsible for excretion of organic anions into sinusoidal blood for subsequent urinary excretion (Konig et al., 1999; Rius et al., 2003; Zelcer et al., 2003).

Glucuronide and sulfate conjugates are typically water-soluble detoxification and inactivation products that are ex-
created into urine and/or feces. Nonetheless, several examples of pharmacologically and toxico logically active phase II metabolites have been identified (Zamek-Gliszczynski et al., 2006). Given the active nature of these metabolites, a fundamental understanding of transport mechanisms responsible for hepatic excretion of phase II conjugates is important not only from a drug disposition and elimination standpoint but also in some cases for drug pharmacodynamics and toxicity. For example, inhibition of the biliary excretion of SN-38-glucuronide, the conjugate of the active metabolite of irinotecan, ameliorated the gastrointestinal toxicity associated with irinotecan treatment in rats (Horikawa et al., 2002). In Mrp3 gene knockout mice, systemic concentrations of morphine-6-glucuronide were reduced with a concomitant attenuation in pharmacodynamics (Zeleer et al., 2005).

Hepatic drug disposition studies using Mrp2-deficient rats indicated that this canalicular transporter plays an important role in the biliary excretion of anionic phase II conjugates, including glucuronide, sulfate, and glutathione metabolites (Zamek-Gliszczynski et al., 2006). These studies demonstrated that the biliary excretion of the glucuronide conjugates of acetaminophen, phenobarbital, phenolphtalein, mycophenolic acid, bisphenol A, and valproic acid was nearly absent in Mrp2-deficient rats, whereas the sulfate metabolites, where relevant, were excreted at reduced levels (Xiong et al., 2000; Ogasawara and Takikawa, 2001; Chen et al., 2003; Patel et al., 2003; Tanaka et al., 2003; Kobayashi et al., 2004; Wright and Dickinson, 2004; Inoue et al., 2005). However, notable exceptions exist in this generally observed pattern of reduced phase II conjugate biliary excretion in Mrp2-deficient rats. Biliary excretion of troglitazone sulfate and glucuronide was delayed 2 to 4 h but was not affected at later time points in Mrp2-deficient rats (Kostrubsky et al., 2001). Furthermore, the biliary excretion of the glucuronide conjugates of SN-38 and indomethacin was only partially reduced in Mrp2-deficient rats (Chu et al., 1997; Kouzuki et al., 2000).

In addition to Mrp2, Bcrp also is localized to the canalicular plasma membrane domain (Maliepaard et al., 2001), although its physiological importance in biliary excretion remains to be fully elucidated. Using plasma membrane vesicles from cells overexpressing human BCRP, Suzuki et al. (2003) demonstrated that both 4MUS and 4MUG were BCRP substrates, although transport activity of the sulfate was much higher than of the glucuronide conjugate. Furthermore, in situ intestinal perfusion experiments with Mrp2-deficient rats and Bcrp gene-knockout (Abcg2+/−) mice indicated that intestinal secretion of 4MUG and 4MUS was maintained in the absence of Mrp2 but was impaired in the absence of Bcrp (Adachi et al., 2005). In contrast to the gut, renal clearance of 4MUS was not altered in Abcg2+/− mice (Mizuno et al., 2004).

4-Methylumbelliferone is a choleretic agent (Fontaine et al., 1968) found in dietary supplements for stimulation of liver function (Heparvit, Heparmed, and DetoxPro). Although the hydrophilic phase II conjugates of 4-methylumbelliferone seem to be responsible for the observed choleresis (Ratna et al., 1993), transport mechanisms, by which 4MUG and 4MUS are excreted into the bile, have not been elucidated. Studies detailed in this article employed isolated perfused livers (IPLs) from wild-type and Mrp2-deficient (TR−) Wistar rats in the absence or presence of the Bcrp and P-glycoprotein inhibitor GF120918 [N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide] (de Bruin et al., 1999) to test the hypothesis that the biliary excretion of 4MUG and 4MUS in rats is mediated by Mrp2 and/or Bcrp but not by P-glycoprotein. GF120918 was selected because it is a potent inhibitor of Bcrp and P-glycoprotein (de Bruin et al., 1999), but not Mrp2, and it has been previously validated for use in the recirculating rat IPL (Zamek-Gliszczynski et al., 2005). Where GF120918 modulation of hepatic transport was noted, involvement of Bcrp and P-glycoprotein was evaluated in vitro and in Bcrp and P-glycoprotein gene-knockout mice.

Materials and Methods

4-Methylumbelliferone, 4MUG, 4MUS, sodium taurocholate, loperamide, triamterene, propranolol, cimetidine, methadone, sodium orthovanadate, adenosine triphosphatase, prazosin, imipramine, carbamazepine, and Lucifer yellow were purchased from Sigma Chemical Co. (St. Louis, MO). GF120918 was kindly donated by GlaxoSmithKline (Research Triangle Park, NC). Calcein-AM and calcein were obtained from Molecular Probes (Eugene, OR). [3H]Mannitol was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO), and [3H]propranolol and [3H]prazosin were obtained from Amersham Biosciences (Piscataway, NJ). Parallel artificial membrane permeability assay (PAMPA) phospholipid formulation was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). The following primary antibodies were used for immunoblotting: P-glycoprotein (Ab-1) and BCRP (Ab-1 from clone BXP-21) from Oncogene Research (Dallas, TX); Mrp2 (Ab-2 from clone H11022); Abcb1b (Ab-1 from clone H9262), Abcb1a (Ab-1 from clone H11002), and Abcg2 (Ab-1 from clone H11002) from BD Gentest (Woburn, MA), and Abcc7 (clone MDR1) from Chemicon International (Temecula, CA). MDR1-Madin-Darby canine kidney (MDCKII) cells and Bcrp-transfected MDCKII cells were obtained from The Netherlands Cancer Institute (Amsterdam, The Netherlands). All cell culture reagents were purchased from Invitrogen (Carlsbad, CA). Membranes from MDRI cDNA-transfected High Five cells were purchased from Gentest (Bedford, MA).

Male wild-type Wistar rats (275–295 g; Charles River Laboratories, Raleigh, NC) and male TR− Wistar rats (275–295 g; in-house breeding colony originally 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 anesthetized with ketamine/xylazine (60/12 mg/kg intraperitoneally) before surgical manipulation. Male wild-type (age-matched heterozygotes) and Bcrp gene-knockout (Abgc2−/−) C57BL/6 mice (23–29 g), as well as wild-type (age-matched heterozygotes) and P-glycoprotein gene-knockout (Abcb1a+/−/Abcb1b−/−) FVB mice (23–29 g), as well as wild-type (age-matched heterozygotes) and P-glycoprotein gene-knockout (Abgc2+/− Abcb1b−/−) mice were purchased from Taconic farms (Germantown, NY). Mice were anesthetized with ketamine/xylazine (140/8 mg/kg intraperitoneally). The Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill approved all procedures. Recirculating IPLs were conducted using standard techniques (Zamek-Gliszczynski and Brouwer, 2004). In brief, the bile duct and portal vein were cannulated, and the liver was isolated. Recirculating liver perfusion was conducted ex situ in a temperature-controlled chamber with 80 ml of recirculating Krebs’-Henseleit buffer containing 20% (v/v) whole-rat blood. Taurocholate was infused (40 μmol/h) into the perfuse reservoir to maintain bile flow. GF120918 (8 μmol), 0.5 ml of dimethyl sulfoxide, or vehicle was added to the perfuse reservoir 5 min before the commencement of 4-methylum-
belliferone infusion (1 μmol/min, 100 mM in dimethyl sulfoxide, 30 min). The perfusion was continued for an additional 60 min after the end of the 4-methylumbelliferone infusion. Bile was collected continuously and sampled together with perfusate at 10-min intervals; both were analyzed for 4-methylumbelliferone, 4MUG, and 4MUS by liquid chromatography with detection by tandem mass spectrometry.

In brief, 4-methylumbelliferone, 4MUG, and 4MUS, and the internal standard, cimetidine, were eluted from a Primesphere C18 column (2.0 × 30 mm, dp = 5 μm) (Phenomenex, Torrance, CA) using a mobile phase gradient (A, 0.05% formic acid in 1% methanol; B, 0.05% formic acid in 99% methanol; 0–1-min hold at 0% B, 1–2 min linear gradient to 70% B, 2–2.9-min linear gradient to 90% B, 2.9–3-min hold at 90% B, 3–3.1-min linear gradient to 0% B, 3.1–4-min hold at 0% B; flow rate = 0.6 ml/min; 1–4 min directed to mass spectrometer) and were detected in negative ion mode using multiple reaction monitoring: 4-methylumbelliferone, 175 m/z; 4MUG, 237 → 194 m/z; 4MUS, 175 → 133 m/z; and cimetidine, 251 → 157 m/z. Analytes were quantified with standard curves (1–1000 ng/ml) prepared in the appropriate matrix; interday and intraday coefficients of variation were <15%.

A compartmental modeling approach was employed to characterize the disposition of 4-methylumbelliferone and its metabolites in rat IPLs. The compartmental model scheme depicted in Fig. 1 has been optimized previously to describe the disposition of 4-methylumbelliferone, 4MUG, and 4MUS in rat IPLs and was modified by removing the minor 4MUG futile cycling component (Chiba and Pang, 1993; Ratna et al., 1993; Chiba et al., 1998). Differential equations based on perfusate concentration and biliary excretion rate data (weighted 1/y) were resolved simultaneously in all data sets using least-squares regression analysis (WinNonlin 4.1; Pharsight Corporation, Mountain View, CA).

Perfused liver homogenates were prepared from wild-type and TR−/− Wistar rats as described previously (Johnson et al., 2005). Fifty micrograms of total protein per lane was resolved on 4 to 12% Bis-Tris polyacrylamide gels under denaturing conditions (150 V, 2 h) and was transferred to polyvinylidene fluoride membranes (38 V, 3 h). Membranes were blocked with 5% skim milk (0.5 h) and were incubated (2 h) with primary antibodies [1:2000, with the exception of anti-Mrp3 (1:3000), anti-UGT1A1 (1:500), anti-UGT1A1 (1:1000), antisulfotransferase 1A1 (1:1000), and antiactin (1:10,000)] followed by an incubation (2 h) with anti-rabbit or anti-mouse horseradish-peroxidase-conjugated secondary antibody (1:4000–1:10,000). Bands were visualized with SuperSignal West Dura chemiluminescence reagent (Pierce, Rockford, IL). Protein bands of interest were quantified by densitometry using Quantity One version 4.1 (Bio-Rad Laboratories, Hercules, CA). All reported band densities were normalized for the optical density of actin in the same membrane.

PAMPA was conducted in 96-well plate format (Corning Life Sciences, Acton, MA) using 5 μl of Avanti polar lipids in the filter plate, 150 μl of morpholinopropanesulfonate-buffered Hanks’ balanced salt solution, pH 6.5, in the donor chamber, and 250 μl of hydroxyethylpiperazine-ethanesulfonate-buffered Hanks’ balanced salt solution, pH 7.4, in receiver chambers. The initial test compound concentration in the donor chamber was 10 μM. Prazosin, imipramine, and carbamazepine were used as low-, intermediate-, and high-permeability reference compounds, respectively. Assembled PAMPA sandwiches were incubated at 37°C for 6 h when concentrations in donor and receiver chambers were quantified. In brief, analytes and internal standard cimetidine were eluted from a C18 column (1.0 × 15 mm, dp = 20 μm) (Optimize Technologies, Oregon City, OR) using a mobile phase gradient [A, 2 mM ammonium acetate in 2% methanol/acetonitrile (1:1, v/v); B, 2 mM ammonium acetate in 90% methanol/acetonitrile (1:1, v/v); 0–0.4 min linear gradient to 100% B; 0.4–1.3 min hold at 100% B; 1.3–1.4 min linear gradient to 0% B; 1.4–2-min hold at 0% B; flow rate = 1.5 ml/min; 20 s to 2 min directed to mass spectrometer) and were detected in positive (prazosin, imipramine, and carbamazepine) or negative (4-methylumbelliferone and 4MUS) ion mode using multiple reaction monitoring: prazosin, 384 → 247 m/z; imipramine, 281 → 86 m/z; carbamazepine, 237 → 194 m/z; 4-methylumbelliferone, 175 → 133 m/z; 4MUG, 255 → 175 m/z; and cimetidine, 251 → 157 m/z. Analytes were quantified with standard curves (1–1000 ng/ml) prepared in the appropriate matrix; interday and intraday coefficients of variation were <15%.

Passage 7 murine Bcrp-MDCKII cells were seeded on 12-well Transwell polycarbonate (12-well, 1 cm², 0.4-μm pore size; Corning, Inc.) inserts at a density of 300,000 cells/cm² and were cultured for three days when Lucifer yellow apparent permeability (5 ± 2 nm/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 4-methylumbelliferone (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) when medium from both the apical and basolateral chambers was collected. Lucifer yellow was quantified by fluorescence spectrophotometry (λex 430 nm, λem 538 nm). [3H]Prazosin was quantified by liquid scintillation spectrometry. 4MUS and the internal standard cimetidine were eluted from an Aquasil C18 column (2.1 × 50 mm, dp = 5 μm) 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; 4.5–6.4 min linear gradient to 0% B; 6.5–5 min hold at 0% B; flow rate = 0.75 ml/min; and 0.8–5 min directed to mass spectrometer) and were detected in negative ion mode using multiple reaction monitoring: 4MUS, 255 → 175 m/z; and cimetidine, 251 → 157 m/z.

The ability of P-glycoprotein to transport 4MUS was evaluated in the mouse P-gp-MDCKII cell model.

Perfusate Liver 4MUS 4MUS Bile 4MUS 4MUS 4MUS 4MUS 4MUS

Fig. 1. Scheme depicting the compartmental model used for pharmacokinetic analysis of the hepatobiliary disposition of 4-methylumbelliferone (4MU), 4MUG, and 4MUS.
standard procedures (Polli et al., 2001; Zamek-Gliszczynski et al., 2005). In all cases, loperamide and triamterene were used as positive and negative controls, respectively (Polli et al., 2001). In brief, stimulation of P-glycoprotein ATPase activity by 4MUS was measured in membranes from MDRI-transfected High Five cells according to the manufacturer's instructions (BD Gentest, Woburn, MA). The ability of 4MUS to inhibit P-glycoprotein-mediated calcein-AM efflux was assessed in passage 29 MDRI-MDCKII cells as detailed previously (Polli et al., 2001). Transcellular flux of mannitol (paracellular marker; 3.5 μM), propranolol (transcellular marker; 100 μM), loperamide (10 μM), triamterene (10 μM), or 4MUS (10 μM) in the presence of GF120918 (2 μM) or vehicle was determined in passage 34 MDRI-MDCKII cell monolayers over the course of 60 min as described previously (Zamek-Gliszczynski et al., 2005). 4MUS was quantified by reverse-phase high-performance liquid chromatography with detection by tandem mass spectrometry as detailed above in the Bcrp-MDCKII cell monolayer section.

TABLE 1
Percentage recovery (mean ± S.D., n = 4/group) of the 4-methylumbelliferone dose (30 μmol) in bile and perfusate as 4MUG and 4MUS at the end of the IPL experiment

<table>
<thead>
<tr>
<th></th>
<th>Wild-Type Wistar Rat IPLs</th>
<th></th>
<th>GF120918</th>
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<tr>
<td></td>
<td>Bile</td>
<td>Perfusate</td>
<td>Total</td>
</tr>
<tr>
<td>4MUG</td>
<td>83 ± 10</td>
<td>15 ± 1</td>
<td>98 ± 9</td>
</tr>
<tr>
<td>4MUS</td>
<td>1.3 ± 0.1</td>
<td>4 ± 1</td>
<td>5 ± 1</td>
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<tr>
<td>Total recovery</td>
<td>85 ± 10</td>
<td>18 ± 2</td>
<td>103 ± 9</td>
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<th></th>
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<th>Mrp2-Deficient TR− Rat IPLs</th>
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<tr>
<td></td>
<td>Bile</td>
<td>Perfusate</td>
<td>Total</td>
</tr>
<tr>
<td>4MUG</td>
<td>1.2 ± 0.3*</td>
<td>91 ± 9</td>
<td>92 ± 9</td>
</tr>
<tr>
<td>4MUS</td>
<td>0.50 ± 0.03*</td>
<td>6.7 ± 0.2*</td>
<td>7.2 ± 0.2*</td>
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<tr>
<td>Total recovery</td>
<td>1.7 ± 0.4*</td>
<td>97 ± 9</td>
<td>99 ± 9</td>
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* p < 0.05, TR− vs. Wild Type within GF120918 treatment group.
† p < 0.05, GF120918 vs. Control within rat group.

Results

Steady-state 4-methylumbelliferone perfusate concentrations of ~50 to 60 μM were achieved in rat IPLs within ~10 min of the commencement of the 30-min infusion, and complete elimination of 4-methylumbelliferone was observed by the 40-min time point. Steady-state 4-methylumbelliferone concentrations were comparable between wild-type and TR−rat IPLs and were not affected by the presence of GF120918.

The percentage recovery of the 4-methylumbelliferone dose as the glucuronide and sulfate metabolites in IPLs is summarized in Table 1. Biliary excretion of 4MUG was extensive in wild-type Wistar IPLs, insensitive to GF120918 coadministration, and was reduced to negligible levels in TR−rat livers (Fig. 2A). Concentrations of 4MUG in the perfusate of TR−rat IPLs were ~5-fold higher than in wild-type rat IPLs and were not significantly affected by the presence of GF120918 (Fig. 2B).

Relative to control wild-type Wistar rat IPLs, recovery of 4MUS in bile was reduced ~50 to 60% both in control TR−rat livers and in wild-type Wistar rat IPLs in the presence of GF120918. Biliary excretion of 4MUS in TR−rat IPLs in the presence of GF120918 was negligible (Fig. 2C). 4MUS perfusate concentrations were ~2-fold higher in TR−rat IPLs but were not affected by the presence of GF120918 (Fig. 2D).

Pharmacokinetic analysis of IPL data based on the model scheme presented in Fig. 1 was conducted to identify the process(es) responsible for the altered disposition of 4MUG and 4MUS in the presence of GF120918 and in TR−rat livers. A representative fit of the model to IPL perfusate concentrations of 4-methylumbelliferone, 4MUG, as well as 4MUS and 4MUG and 4MUS biliary excretion rates in wild-type and TR−rat IPLs, is presented in Fig. 3. Mean parameter estimates are reported in Table 2. Hepatic uptake clearance of 4-methylumbelliferone (Cl\text{\textsubscript{UP,4MU}}) was slightly lower than hepatic blood flow (20 ml/min) and was comparable between groups. The rate constant governing 4-methylumbelliferone glucuronidation (k\text{\textsubscript{gluc}}) was approximately an order of magnitude greater than the rate constant for sulfation (k\text{\textsubscript{sulf}}). No significant differences in the rate constants governing 4-methylumbelliferone metabolism were noted among different experimental groups, although the rate constant for formation of 4MUS (k\text{\textsubscript{sulf}}) seemed to be higher in TR−rat livers (~2-fold). The rate constant governing hepatic 4MUS desulfation (k\text{\textsubscript{desulf}}) was comparable between groups. The rate constant for hepatic basolateral excretion of 4MUG (k\text{\textsubscript{B,L,4MUG}}) was unaffected by GF120918 coadministration.
but was ~5-fold higher in TR^- rat livers. In contrast, the 4MUS basolateral excretion rate constant \( (k_{B/l,4MUS}) \) was similar in all groups, although a small increase was noted in TR^- rat livers (~2-fold). Hepatic reuptake clearances of 4MUS (Cl_{UP,4MUS}) and 4MUG (Cl_{UP,4MUG}) were low, approximately one and two orders of magnitude lower than hepatic blood flow, respectively, and were similar between groups.

The rate constant for 4MUG biliary excretion \( (k_{\text{bile,4MUG}}) \) was not affected by GF120918 in wild-type Wistar IPLs but was reduced ~100-fold in TR^- rat livers; GF120918 further reduced \( k_{\text{bile,4MUG}} \) ~2.5-fold in TR^- rat IPLs. The rate constant governing 4MUS biliary excretion \( (k_{\text{bile,4MUS}}) \) was significantly reduced by GF120918 in TR^- rat livers.

Immunoblot analysis was conducted to assess the protein
expression of relevant transporters and drug-metabolizing enzymes in wild-type and TR- Wistar rat livers (data not shown). The expression levels of canalicular P-glycoprotein and Bcrp, as well as basolateral Mrp4, were comparable. In contrast, TR- rat livers expressed significantly more basolateral Mrp3 protein (∼13-fold). Protein levels of UGT1A1, the bilirubin UGT, were significantly higher (∼4-fold) in TR- rat livers despite the fact that immunoblot with the nonspecific UGT1A subfamily antibody showed no significant differences. Hepatic expression of phenol sulfotransferase (sulfotransferase 1A1) was slightly higher (2-fold) in TR- rats, but the differences were not significant.

The non-Mrp2-dependent component of 4MUS biliary excretion was investigated further in vitro (Fig. 4). Passive permeability of 4-methylumbelliferone and 4MUS were determined using PAMPA (Fig. 4A). 4-Methylumbelliferone exhibited intermediate permeability, comparable to imipramine. In contrast, 4MUS had essentially no passive membrane permeability. 4MUS permeability (0.01 \times 10^{-6} \text{ cm/s}) was much lower than that of the low permeability marker, prazosin. Transport of 4MUS by Bcrp was evaluated in confluent Bcrp-MDCKII cell monolayers. Monolayer confluence was confirmed using Lucifer yellow flux, which was below 20 nm/s in both the apical-to-basolateral and basolateral-to-apical directions (5 ± 3 and 5 ± 2 nm/s, respectively; not significant). The presence of functional Bcrp transport on the apical membrane of the Bcrp-MDCKII cell monolayer was confirmed with prazosin flux, 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), and sensitive to the presence of GF120918 (the basolateraltol-apical over apical-to-basolateral flux ratio = 19 ± 1 versus 2.2 ± 0.1 (control versus GF120918, respectively, p < 0.05)). Recovery of 4MUS after the incubation of Bcrp-MDCKII confluent cell monolayers with 4-methylumbelliferone (to assure adequate intracellular concentrations of 4MUS due to low permeability of preformed 4MUS) was higher in the apical chamber (Fig. 4B). Preferential apical excretion of 4MUS was not observed in the presence of GF120918. Interactions between 4MUS and P-glycoprotein were evaluated in three validated assays using loperamide and triamterene as positive and negative controls, respectively (Polli et al., 2001). Stimulation of P-glycoprotein ATPase activity was evaluated in membranes from MDR1-transfected High Five cells using orthovanadate, a mechanism-based inhibitor of P-glycoprotein ATPase, which was used to distinguish P-glycoprotein ATPase from nonspecific ATPase activity (data not shown). Loperamide (positive control) stimulated P-glycoprotein ATPase in a concentration-dependent manner (9.2 ± 1.7-fold) in Wild-Type Wistar Rat IPLs 4MUS, whereas neither triamterene (negative control) nor 4MUS (up to 1 mM) stimulated ATPase activity. Inhibition of P-glycoprotein-mediated calcein-AM efflux was assessed in MDR1-MDCKII cells, defining maximal P-glycoprotein inhibition as calcein fluorescence observed in the presence of 2 μM GF120918 (data not shown). Inhibition of P-glycoprotein-mediated calcein-AM efflux from MDR1-MDCKII cells was observed only for loperamide (concentration-dependent, 84 ± 1% at 100 μM); both triamterene and 4MUS

### Table 2

<table>
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<tr>
<th>Parameter Description</th>
<th>Control</th>
<th>GF120918</th>
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<tr>
<td>Cl_{UP,4MU} (ml/min)</td>
<td>17 ± 2</td>
<td>16 ± 1</td>
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<td>k_{d,4MU} (min^{-1})</td>
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<td>3 ± 3</td>
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<td>k_{u,4MU} (min^{-1})</td>
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<tr>
<td>k_{d,4MU,4MUS} (ml/min)</td>
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<td>k_{B/L,4MU,4MUS} (min^{-1})</td>
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<tr>
<td>Cl_{UP,4MUS} (ml/min)</td>
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<td>k_{u,4MUS} (min^{-1})</td>
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<tr>
<td>k_{u,4MUS} (min^{-1})</td>
<td>0.09 ± 0.02</td>
<td>0.08 ± 0.03</td>
</tr>
<tr>
<td>k_{d,4MUS} (min^{-1})</td>
<td>0.10 ± 0.04</td>
<td>0.052 ± 0.002</td>
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* p < 0.05, TR- vs. Wild Type within GF120918 treatment group.
1 p < 0.05, GF120918 vs. Control within rat group.

In vitro characterization of 4-methylumbelliferone sulfate transfer. The effective artificial membrane permeability of 4-methylumbelliferone and its sulfate metabolite were determined using a parallel artificial membrane permeability assay (A). Permeability markers (prazosin (low), imipramine (intermediate), and carbamazepine (high)) are shown for comparison. Mean ± S.D., n = 3/group, ∗, p < 0.05 4-methylumbelliferone (4MU) versus 4MUS. Recovery of 4-methylumbelliferone sulfate in Bcrp-MDCKII confluent cell monolayers (B). Recovery is expressed as the ratio of the amount of sulfate metabolite recovered in apical-to-basolateral (A-to-B) chambers of Bcrp-MDCKII cell monolayers after a 3-h incubation with 4-methylumbelliferone (10 μM) in the absence or presence of 5 μM GF120918. Mean ± S.D., n = 3/group, ∗, p < 0.05 GF120918 versus Control.
Two cumulative biliary excretion: 3.0
C57BL/6 mouse livers was profoundly reduced (60-min cu-
presence of GF120918, biliary excretion of 4MUS in wild-type
C57BL/6 mouse livers lacking Bcrp. Furthermore, in the
In contrast, the biliary excretion of 4MUS was negligible in
wild-type and P-glycoprotein gene-knockout mice (Fig. 5A).
crease of 4MUS was not significantly different between
both Mrp2 and Bcrp contributed approximately equally to
the biliary transport of 4MUS in rats. Biliary excretion of
4MUS was reduced significantly in both wild-type Wistar
rat IPLs by GF120918 coadministration; a similar
decrease was observed in the rate constant governing 4MUS
biliary excretion. 4MUS biliary excretion was reduced to
negligible levels only in TR rat IPLs with GF120918 coad-
ministration. These data indicated that, in the rat liver,
4MUS biliary excretion is mediated by Mrp2, as well as an
Mrp2-independent and GF120918-sensitive mechanism.
GF120918 is a potent inhibitor of both Bcrp and P-glyco-
protein (de Bruin et al., 1999). Thus, the GF120918-sensitive
mechanism necessitated further investigation. Furthermore,
conflicting reports exist regarding whether the plasma mem-
brane represents a permeability barrier for 4MUS (Miyauchi
et al., 1988; Ratna et al., 1993). As would be expected based
on lipophilicity \( \log D_{\text{pH}7.4} \) 4-methylumbelliferone (+2.35)
and 4MUS (−2.59), in the PAMPA assay, 4-methylumbellifer-
one exhibited intermediate membrane permeability, but the
lipid bilayer represented a diffusional barrier for 4MUS.
The hepatic basolateral and canalicular membranes vary
somewhat in their physicochemical properties due to differ-
ences in lipid composition (Wisher and Evans, 1975), and
their composition is also different from the artificial mem-
branes in the PAMPA assay. Nonetheless, these lipid bilay-
ers all represent a lipophilic barrier, such that a hydrophilic
and low-permeability compound like 4MUS would not be able
to passively diffuse across these membranes and would ne-
cessitate carrier-mediated transport. In fact, coadministra-
tion of the P-glycoprotein and Bcrp inhibitor in the absence
of Mrp2 resulted in negligible 4MUS biliary excretion, support-
ing the PAMPA finding that the lipid bilayer represents a
diffusional barrier for 4MUS. Nonetheless, PAMPA is an
artificial lipid bilayer system and provides only an indirect
measure of actual permeability in the intact cell. The results
of in vitro studies suggested that 4MUS did not interact with
P-glycoprotein but was preferentially excreted into the apical
chamber in Bcrp-MDCKII confluent cell monolayers; this
preferential apical excretion was completely reversed by
GF120918. The results demonstrating the presence of a
GF120918-sensitive Bcrp-mediated transport process for
4MUS are in agreement with previous studies in which
4MUS was transported by the recombinant BCRP protein
and 4MUS intestinal secretion was reduced in Bcrp gene
knockout mice (Suzuki et al., 2003; Adachi et al., 2005).
Furthermore, the importance of Mrp2 in biliary excretion of
4MUS may help explain the unexpected finding of unaltered
4MUS renal clearance in Bcrp gene knockout mice (Mizuno
et al., 2004), because 4MUS may be transported into urine by
an Mrp isoform.

Discussion
Hepatic disposition of 4-methylumbelliferone metabolites
demonstrated the differences in transport mechanisms re-
ponsible for the excretion of glucuronide and sulfate metab-
olites from the rat liver. 4MUG biliary excretion was medi-
at ed by Mrp2, whereas 4MUS was excreted into bile by both
Mrp2 and Bcrp. Hepatic basolateral excretion of 4MUG was
increased in TR rat livers, consistent with an increase in
Mrp3 protein expression. In contrast, hepatic basolateral
excretion of 4MUS was influenced only slightly by Mrp3
modulation.

The extensive biliary excretion of 4MUG observed in wild-
type Wistar rat IPLs was reduced to negligible levels in TR rat
livers. The present study clearly demonstrated that the
biliary excretion of 4MUG in the rat is mediated primarily by
Mrp2. Coadministration of GF120918 in wild-type Wistar rat
 IPLs did not result in a noticeable difference in the recovery
of 4MUG in bile or the rate constant governing biliary excre-
In TR rat IPLs, 4MUG biliary excretion accounted for
only ~1% of the dose.

In contrast to Mrp2-mediated 4MUG biliary excretion,
both Mrp2 and Bcrp contributed approximately equally to
the biliary transport of 4MUS in rats. Biliary excretion of
4MUS was reduced significantly in both wild-type Wistar
and TR rat IPLs by GF120918 coadministration; a similar
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4MUS renal clearance in Bcrp gene knockout mice (Mizuno
et al., 2004), because 4MUS may be transported into urine by
an Mrp isoform.

Fig. 5. Cumulative biliary excretion of 4MUS in perfused livers from
wild-type (○) and P-glycoprotein gene-knockout (Abcb1a<sup>−/−</sup>/Abcb1b<sup>−/−</sup>)
(×) FVB mice (A), as well as Bcrp gene-knockout (Abcg2<sup>−/−</sup>) (△) and
wild-type (○) C57BL/6 mice in the absence (open symbols) or presence
(closed symbols) of 10 μM GF120918 (B). Mean ± S.D., n = 3–4/group.
Although the current in vitro data seem to be in good agreement with the IPL data and previous in vivo studies, it is important to note that these in vitro assays provide only indirect evidence and that the ultimate relevance of these findings required demonstration in a higher experimental system, such as transporter gene-knockout mice. Mice livers genetically deficient in P-glycoprotein did not exhibit impaired 4MUS biliary excretion. In contrast, biliary excretion of 4MUS was significantly reduced in mouse livers lacking Bcrp, as well as by GF120918 administration to wild-type mouse livers. Interestingly, unlike in rats where 4MUS biliary excretion was only sensitive in part to GF120918, in mice, the biliary excretion of 4MUS was mediated almost exclusively by GF120918-sensitive Bcrp. This finding may be explained by much higher hepatic expression and function of Mrp2 in rats relative to other preclinical species, including mice (Ishizuka et al., 1999; Ninomiya et al., 2005).

In agreement with previous studies, hepatic Mrp3 protein expression was approximately an order of magnitude higher in TR− rats (Xiong et al., 2002). Up-regulation of basolateral Mrp3 resulted in a concomitant increase in the rat constant governing the basolateral excretion of Mrp3 substrates, such as carboxydichlorofluorescein and acetaminophen glucuronide (Xiong et al., 2000; Zamek-Gliszczynski et al., 2003). In the present study, the rat constant governing 4MUG basolateral excretion was increased ~5-fold in livers from TR− rats. Therefore, the kinetics of 4MUG basolateral excretion are in agreement with Mrp3 modulation, and this basolateral Mrp isoform may be responsible for the hepatic secretion of 4MUG into sinusoidal blood. In contrast, the rat constant governing 4MUS basolateral excretion was increased only slightly (~2-fold, not significant) in TR− rat livers, suggesting that Mrp3 is not the predominant mechanism for the secretion of 4MUS into sinusoidal blood. Basolateral excretion of 4MUS may instead be mediated by a hepatic basolateral transporter with an affinity for sulfate metabolites whose expression is maintained in TR− rat livers, such as Mrp4 (Zelcer et al., 2003). These kinetic findings in the whole liver are in agreement with in vivo studies that demonstrated inhibition of Mrp3-mediated transport of estradiol-17β-(β-D-glucuronic) by 4MUG (IC50 ~39 μM) but not by 4MUS (Hirohashi et al., 1999). In previous hepatic disposition studies using TR− rats, the rat constant governing acetaminophen glucuronide basolateral excretion was increased together with Mrp3 protein levels, but the kinetics of acetaminophen sulfate basolateral excretion were not affected (Xiong et al., 2002; Zamek-Gliszczynski et al., 2005). Likewise, studies using Mrp3 gene knockout mice proved the importance of Mrp3 in the basolateral excretion of the glucuronide metabolites of acetaminophen and morphine; however, the absence of Mrp3 did not significantly decrease the plasma concentrations of acetaminophen sulfate (Manautou et al., 2005; Zelcer et al., 2005). Based on the current and previous findings with glucuronide and sulfate metabolites, Mrp3 probably plays a major role in hepatic basolateral excretion of 4MUG but not 4MUS.

In conclusion, the present studies clearly demonstrated that, in rats, 4MUG biliary excretion is mediated primarily by Mrp2, whereas 4MUS is transported across the hepatic canalicular membrane by two distinct mechanisms. Mrp2 and GF120918-sensitive Bcrp contribute approximately equally to the overall transport of 4MUS into bile. Based on the up-regulation of Mrp3 in TR− rat livers, 4MUG hepatic basolateral excretion may be mediated by hepatic Mrp3. 4MUS seems to be less sensitive to Mrp3 modulation of basolateral excretion. Further studies are underway to elucidate the mechanism(s) responsible for the transport of sulfate metabolites from the liver into sinusoidal blood.

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