Construction of Triple-Transfected Cells [Organic Anion-Transporting Polypeptide (OATP) 1B1/Multidrug Resistance-Associated Protein (MRP) 2/MRP3 and OATP1B1/MRP2/MRP4] for Analysis of the Sinusoidal Function of MRP3 and MRP4

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ABSTRACT:
Multidrug resistance-associated protein (MRP) 3/ABCC3 and MRP4/ABCC4 are ATP-binding cassette (ABC) transporters expressed in the sinusoidal membrane of hepatocytes. The purpose of the present study was to establish organic anion-transporting polypeptide (OATP) 1B1/MRP2/MRP3 and OATP1B1/MRP2/MRP4 triple transfected as in vitro model of the hepatobiliary transport of anionic drugs. To find in vivo relevant Mrp3 probes, wild-type and Mrp3(-/-) mice were given gemfibrozil, 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl)benzothiazole (E3040), troglitazone, bisphenol A, and 4-methylumbelliferone orally. Plasma concentrations of the glucuronide conjugates were significantly lower in Mrp3(-/-) mice than in wild-type mice. The systemic exposure of gemfibrozil, E3040, and troglitazone were similar in wild-type and Mrp3(-/-) mice. 4-Methylumbelliferone and bisphenol A were undetectable in the plasma. In MRP3-expressing membrane vesicles, ATP-dependent uptakes of the glucuronide conjugates of estradiol, gemfibrozil, E3040, and troglitazone were markedly greater than those in controls, whereas MRP4-expressing membrane vesicles exhibited significant ATP-dependent uptake of gemfibrozil glucuronide and estradiol glucuronide. MRP3 or MRP4 was expressed in the OATP1B1/MRP2 double transfected using adenovirus. The expression levels of OATP1B1 and MRP2 proteins were maintained both in the OATP1B1/MRP2/MRP3 and OATP1B1/MRP2/MRP4 triple transfectants, whereas MRP3 and MRP4 were localized in the basal membrane. Significant reductions in the basal-to-apical flux of the glucuronide conjugates of estradiol, gemfibrozil, E3040, and troglitazone were observed in the OATP1B1/MRP2/MRP3 triple transfected compared with those in the double transfected, whereas significant reduction was observed only for gemfibrozil glucuronide and estradiol glucuronide in the OATP1B1/MRP2/MRP4 triple transfected. These results suggest that MRP3- or MRP4-triple transfected provide a simple and useful in vitro system for evaluating their importance in the hepatobiliary transport of drugs.

One of the major functions of the liver is the removal of various endogenous and exogenous compounds from the blood circulation. This clearance process involves uptake across the basolateral membrane and excretion across the bile canalicular membrane. In addition, sinusoidal efflux is an essential function to prevent the intracellular accumulation of potentially toxic endobiotic and xenobiotic substances and their metabolites in the liver. For instance, glucuronide conjugates are mainly produced in the liver and undergo both sinusoidal efflux followed by urinary excretion and biliary excretion (Klaassen and Watkins, 1984; Suzuki and Sugiyama, 1998). The elimination pathways of the glucuronide conjugates are determined by the transport activities for the canalicular and sinusoidal efflux.

The molecular mechanism of the biliary excretion of many anionic compounds has been well characterized (Gerloff et al., 1998; Suzuki and Sugiyama, 1998; Keppler and König, 2000). It is generally accepted that multidrug resistance-associated protein 2 (MRP2/ABCC2) is responsible for the biliary excretion of various organic anions including glucuronide conjugates (Suzuki and Sugiyama, 1998; König et al., 1999a; Keppler and König, 2000). The importance of MRP2 in the disposition of organic anions has been highlighted by the fact that the biliary excretion of MRP2 substrates is almost completely abolished in MRP2-deficient mutant rats (Büchler et al.,

ABBREVIATIONS: MRP, multidrug resistance-associated protein; MDCK, Madin-Darby canine kidney; OATP, organic anion-transporting polypeptide; ABC, ATP-binding cassette; Gem-G, gemfibrozil 1-O-β-glucuronide; E3040, 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl)benzothiazole; E3040G, E3040 glucuronide; TGGZ-G, troglitazone glucuronide; Bis-AG, bisphenol A glucuronide; E17βG, 17β-estradiol-17β-D-estradiol; LC, liquid chromatography; MS, mass spectrometry; HEK, human embryonic kidney; MOI, multiplicity of infection; 4-MUG, 4-methylumbelliferone glucuronide.


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1996; Paulusma et al., 1996). On the other hand, the molecular mechanism governing sinusoidal efflux is now being gradually clarified. Multidrug resistance-associated protein 3 (MRP3/ABCC3) accepts glucuronide and glutathione conjugates, as well as monovalent (e.g., taurocholate and glycocholate) and sulfated bile salts as substrate (Hirohashi et al., 2000; Akita et al., 2002a). In MRP2-deficient mutant rats, Mrp3 is up-regulated at the sinusoidal membrane in the liver to maintain hepato-cellular homeostasis by pumping into the bloodstream those endo- and xenobiotics that cannot be excreted into bile. Akita et al. (2001, 2002b) have demonstrated that the sinusoidal efflux of taurocholate correlated well with Mrp3 expression in rats. Multidrug resistance-associated protein 4 (MRP4/ABCC4) mediates the basolateral efflux of sulfate conjugates, whereas Mrp4 did not seem to play a major role in the transport of glucuronide conjugates (Zamek-Gliszczynski et al., 2006).

The double-transfected Madin-Darby canine kidney II (MDCK II) cell lines expressing both human organic anion-transporting polypeptide 1B1 (OATP1B1/SLC01B1) or OATP1B3 (SLCO1B3) in the basolateral membrane and canalicul ABC transporters, such as MRP2, P-glycoprotein, and breast cancer resistance protein, in the apical membrane have been established as an in vitro model of hepatic vectorial transport of organic anions including several glucuronide and sulfate conjugates, as well as monoanionic bile salts (Kruh and Belinsky, 2003; Rius et al., 2003; Zelcer et al., 2003; Ci et al., 2007; Hasegawa et al., 2007; Imaoka et al., 2007). Using Abcc4(−/−) mice, Zamek-Gliszczynski et al. (2006) demonstrated that Mrp4 mediates the basolateral efflux of sulfate conjugates, whereas Mrp3 did not seem to play a major role in the transport of glucuronide conjugates (Zamek-Gliszczynski et al., 2006).

Preparation of Gemfibrozil Glucuronide. Gem-G was biosynthesized in vitro with dog liver microsomes. The incubation mixtures (40 × 2 ml) contained dog liver microsomes (2 mg/ml), the zwitterionic detergent CHAPS (10 mM), Tris-HCl (100 mM, pH 7.7 at 37°C), EDTA (2.0 mM), MgCl2 (10 mM), d-saccharic acid 1,4-lactone (100 mM), UDP-glucuronic acid (8.0 mM), and gemfibrozil (4 mM). Incubations were performed for 8 h at 37°C and were stopped by the addition of 60% perchloric acid (w/v) (final concentration of approximately 1.5% v/v). The acidified samples were extracted with ethyl acetate (2 × 4 ml). The organic extracts were pooled, evaporated in a vacuum centrifuge, and reconstituted in 50% acetonitrile-water containing (v/v) 1% formic acid (v/v). After insoluble material was removed by centrifugation, aliquots (400 μl) of the reconstituted samples were subjected to high-performance liquid chromatography on a Supelcosil LC-18, 4.6 × 150 mm column. Gem-G was separated from gemfibrozil with a linear gradient mixture of water and acetonitrile, each of which contained 0.1% formic acid, at a flow rate of 1 ml/min. Gem-G was monitored by fluorescence (λex: 284 nm; λem: 316 nm) and UV detection (235 nm), and its identity was confirmed by comparison with a standard that had been characterized by liquid chromatography (LC)-tandem mass spectrometry and NMR [prepared and characterized as described previously (Ogivie et al., 2006)]. The Gem-G fractions were collected (retention time 6–8 min) and lyophilized. The white solid (approximately 100 mg) was transferred to amber vials and stored desiccated at −20°C.

Materials and Methods
Materials. Gemfibrozil 1-O-β-glucuronide (Gem-G) was prepared by Xenotech, LLC (Lenexa, KS) as described below. E3040 and its glucuronide conjugate (E3040G) were kindly donated by Eisai Co. Ltd. (Tokyo, Japan). Troglitazone and its glucuronide conjugate (TGZ-G) were kindly donated by Sankyo Co. (Tokyo, Japan). Bisphenol A glucuronide (Bis-AG) was kindly provided by Dr. Hiroshi Yokota (Rakuno Gakuen University, Hokkaido, Japan). D-[1H]E2 (51 Ci/mmol) with a purity of 98.5% was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). ATP, creatine phosphate, and creatine phosphokinase were purchased from Sigma-Aldrich (St. Louis, MO). Monoclonal antibodies against MRP2 (M1166) and MRP4 (M41-10) were purchased from Alexis Biochemicals (Grenkau, Germany) and Abcam plc (Cambridge, UK), respectively, and anti-OATP1B1 polyclonal antibody was from Alpha Diagnostic International Inc. (San Antonio, TX). Anti-MRP3 rabbit serum was produced as described previously (Akita et al., 2002a). All other chemicals used were commercially available and of reagent grade.

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Animals. Male Mrp3(−/−) mice and wild-type FVB mice (12–18 weeks) were used in this study. They are fertile and do not exhibit any obvious physiological abnormalities. Mrp3(−/−) mice used in this study were described previously (Zelcer et al., 2006). All animals were treated humanely, and all experiments were reviewed and approved by the Institutional Animal Care Committee of the Graduate School of Pharmaceutical Sciences, The University of Tokyo.

Determination of Plasma Pharmacokinetics of Glucuronide Conjugates in Wild-Type and Mrp3(−/−) Mice. Mice. Mice were given oral doses of genistin (20 mg/kg), E3040 (2 mg/kg), troglitazone (25 mg/kg), bisphenol A (10 mg/kg), and 4-methylumbelliferone (18 mg/kg). Blood samples were collected from the tail artery 10, 20, 30, 60, 120, and 180 min after administration, and an aliquot (8 μl) of each sample was mixed with 16 μl of acetonitrile. After the precipitated protein was removed by centrifugation, an aliquot (20 μl) of the supernatant fraction was diluted with 80 μl of Milli-Q, and 30-μl aliquots were analyzed by LC–mass spectrometry (MS) as described below.

Construction of Recombinant Adenoviruses Containing MRP3 and MRP4. Human MRP3 and MRP4 cDNAs used in this study were cloned previously (Kiuchi et al., 1998; Hasegawa et al., 2007). The MRP3 and MRP4 cDNAs were initially subcloned into the ApaI and KpnI sites of the p shuttle, which has I-CeuI and PstI sites upstream and downstream of the MRP3 and MRP4 expression cassettes, respectively. The I-CeuI/PstI–cleaved-digested fragments of p shuttle-MRP3 and -MRP4 were ligated with I-CeuI/PstI–cleaved-digested AdenoX Viral DNA (BD Biosciences, Palo Alto, CA), resulting in pAd-MRP3 and pAd-MRP4. To generate the recombinant viruses, both pAd-MRP3 and pAd-MRP4 were digested with PacI. Linearized DNAs were transfected into HEK293 cells grown in a 12-well dish with FuGENE 6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer’s instructions. Viruses (Ad-MRP3 and Ad-MRP4) were prepared as described previously (Mizuguchi and Kay, 1998). Recombinant viruses were purified by CsCl gradient centrifugation, dialyzed with a solution containing 10 mM Tris (pH 7.5), 1 mM MgCl₂, and 10% glycerol, and aliquots were stored at −80°C. The resulting virus titer was determined as described previously (Mizuguchi and Kay, 1999).

Preparation of Membrane Vesicles of MRP3 and MRP4. HEK293 cells cultured in a 15-cm dish were infected by recombinant adenoviruses containing MRP3 (10 MOI) and MRP4 (10 MOI). As a negative control, cells were infected with Ad-tTA (10 MOI). Cells were harvested 48 h after infection, and then the membrane vesicles were isolated from 1 to 2 × 10⁶ cells as described in detail previously (Hasegawa et al., 2007). In brief, cells were diluted 40-fold with hypotonic buffer (1 mM Tris-HCl and 0.1 mM EDTA, pH 7.4, at 4°C) and stirred gently for 1 h on ice in the presence of 2 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 1 μM pepstatin, and 5 μg/ml aprotinin. The cell lysate was centrifuged at 100,000 g for 30 min at 4°C, and the resulting pellet was suspended in 10 ml of isotonic TS buffer (10 mM Tris-HCl and 250 mM sucrose, pH 7.4 at 4°C) and homogenized with a Dounce B homogenizer (glass homogenizers). The suspension was centrifuged for 10 min at a speed of 1,500 g at room temperature in a Beckman SW41 rotor centrifuge at 280,000 g for 1 h at 4°C, and the resulting supernatant was filtered through a 0.45-μm HA filter (Millipore Corporation, Billerica, MA) and subjected to centrifugation (Clearsol I; Nacalai Tesque, Tokyo, Japan). Nonradioactive substrates retained on the membrane filter (JH-filter; Millipore Corporation) were dissolved in 1 ml of methanol containing internal standard using sonication for 15 min. After centrifugation, supernatants were concentrated and dissolved in 75 μl of Milli-Q water, and 50-μl aliquots were analyzed by LC–MS as described below. Ligand uptake was normalized to the amount of membrane protein.

Western Blotting. For Western blotting, the isolated crude membranes were dissolved in 3× SDS sample buffer (New England Biolabs, Ipswich, MA) and subjected to electrophoresis on an 8.5% SDS-polyacrylamide gel with a 4.4% stacking gel. The molecular weight was determined with a prestained protein marker (New England BioLabs). Proteins were transferred electrophoretically to a nitrocellulose membrane (Millipore Corporation) with a trans-blotter (Bio-Rad Laboratories, Richmond, CA) at 15 V for 1 h. Nonspecific binding on the membrane was blocked with 2.5% skimmed milk for 1 h at room temperature, after which the membrane was incubated for 1 h at room temperature in skimmed milk with a 1000-fold dilution of anti-OATP1B1 polyclonal antibody, a 200-fold dilution of monoclonal antibody against MRP2 (M1116), a 1000-fold dilution of anti-MRP3 rabbit serum, and a 200-fold dilution of anti-MRP4 monoclonal antibody (M4-10). For the detection of each transporter, the membrane was incubated with a 2500-fold diluted donkey anti-rabbit (OATP1B1 and MRPs), anti-mouse IgG (MRP2), and anti-rat IgG (MRP4) conjugated with horseradish peroxidase (GE Healthcare, Little Chalfont, Buckinghamshire, UK) for 1 h in skimmed milk. The immunoreactive band was detected with an ECL Plus Western blotting starter kit (GE Healthcare), and its intensity was quantified in a luminescent image analyzer (LAS-3000mini; Fuji Film Corp., Tokyo, Japan).

Immunohistochemical Staining of MRP3 and MRP4. For the immunohistochemical staining, MDCK II cells were grown in 12-well dishes. Recombinant adenoviruses containing MRP3 (150 MOI) or MRP4 (150 MOI) were infected 48 h before the experiments were started. After fixation with ice-cold methanol for 10 min and permeabilization in 1% Triton X-100 in phosphate-buffered saline for 10 min, cells were incubated with a 40-fold dilution of monoclonal antibody against MRP2 (M1116), a 200-fold dilution of anti-MRP3 rabbit serum, or a 50-fold dilution of monoclonal antibody against MRP4 (M4-10) for 1 h at room temperature. Then cells were washed three times with phosphate-buffered saline and incubated for 1 h at room temperature with a 250-fold dilution of goat anti-mouse IgG Alexa 568 (Invitrogen, Carlsbad, CA) for MRP2, goat anti-rabbit IgG Alexa 488 for MRP3, and goat anti-rat IgG Alexa 488 for MRP4. Nuclei were stained with 250-fold diluted TO-PRO-3 iodide (Invitrogen). The localization of each protein was visualized by confocal laser microscopy (Zeiss LSM-510; Carl Zeiss Inc., Thornwood, NY).
vivo relevant probes for Mrp3, in vivo experiments using Mice after Oral Administration of the Parent Compounds. Wild-type and ratios are shown in Table 1.

Micromass, Manchester, UK). Detailed LC conditions and mass-to-charge ratios are shown in Table 1.

Statistical Analysis. Statistical analysis was performed by Student’s t test to identify significant differences between pairs of data sets.

Results

Plasma Concentrations of Glucuronide Conjugates in Mrp3(−/−) Mice after Oral Administration of the Parent Compounds. To find in vivo relevant probes for Mrp3, in vivo experiments using Mrp3(−/−) mice were performed. Wild-type and Mrp3(−/−) mice were given gemfibrozil, E3040, troglitazone, bisphenol A, and 4-methylumbelliferon orally, and the plasma concentrations of their glucuronide conjugates were determined (Fig. 2). Mrp3 dysfunction resulted in a much lower plasma concentration of all glucuronide conjugates compared with that in wild-type mice (Fig. 2), whereas the area under the plasma concentration-time curve of the parent drugs tested was not statistically different between wild-type and Mrp3(−/−) mice except for bisphenol A and 4-methylumbelliferon (Table 2). Plasma concentrations of bisphenol A and 4-methylumbelliferon were below the limit of detection both in wild-type and Mrp3(−/−) mice.

ATP-Dependent Uptake of Glucuronide Conjugates in Membrane Vesicles. To confirm the transport activities of Gem-G, E3040G, and TGZ-G by MRp2, MRp3, and MRp4, the uptake of the glucuronide conjugates was determined in mock and MRp2-, MRp3-, or MRp4-expressing membrane vesicles in the presence of ATP and AMP by the rapid filtration method. E217βG was used as a positive control because this compound is well known to be a common substrate for MRp2, MRp3, and MRp4 (Suzuki and Sugiyma, 1998; Chen et al., 2001; Akita et al., 2002a).

The ATP-dependent uptake of all of the glucuronide conjugates at 2 min was significantly higher in MRp2-expressing membrane vesicles (Fig. 3). The ATP-dependent uptake of all the glucuronide conjugates at 2 min was also significantly higher in MRp3-expressing membrane vesicles than in control membrane vesicles (Fig. 3). The ATP-dependent uptake of all the glucuronide conjugates at 2 min was also significantly higher in MRp3-expressing membrane vesicles than in control membrane vesicles (Fig. 3), whereas only Gem-G and E217βG exhibited statistical significance in ATP-dependent uptake by MRp4-expressing membrane vesicles compared with that in control vesicles.

Expression and Membrane Localization of MRp3 and MRp4 in MDCK II Cells. The expression levels of OATP1B1, MRp2, and MRp3 or MRp4 proteins were confirmed by Western blotting (Fig. 4). OATP1B1 and MRp2 proteins were detected in the OATP1B1/MRP2 double and OATP1B1/MRP2/MRP3 and OATP1B1/MRP2/MRP4 triple transfectants. Semiquantitative analysis of the results showed that the expression levels of OATP1B1 and MRp2 in the triple-transfected MDCK II cells were approximately the same as those in the OATP1B1/MRP2 double transfectants. The signals for MRp3 or MRp4 could only be detected in the triple-transfected MDCK II cells, showing the specificity of the antibody.

The expression levels of MRp3 and MRp4 protein in the triple transfectants were compared with those in human liver by Western blotting (Fig. 4). Three bands were detected in human liver by anti-MRP3 antibody for an unknown reason (Fig. 4a), and the band density of the major band (the middle band) was compared with that in the triple transfectants in terms of the intensity and molecular weight. Semiquantitative analysis showed that the band densities were comparable between human liver and the MRp3 triple transfectant. In the case of MRp4, the frontline of the bands detected by anti-MRP4 antibody was similar between human liver and the OATP1B1/MRP2/MRP4 triple transfecant. However, the band density was markedly lower in the human liver than that in the MRp4 triple transfecant (Fig. 4b).

Double immunostaining of MRp3 or MRp4 with MRp2 was performed to show membrane localization of MRp3 and MRp4 in

<table>
<thead>
<tr>
<th>Compound</th>
<th>Column</th>
<th>Mobile Phase</th>
<th>Gradient Condition</th>
<th>Flow Rate</th>
<th>Desolvation Temperature</th>
<th>Capillary Voltage</th>
<th>Cone Voltage</th>
<th>m/z</th>
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<td>L-Column ODS, 5 μm, 2.1 × 150 mm</td>
<td>0.05% Formic acid</td>
<td>Acetonitrile</td>
<td>0</td>
<td>85/15</td>
<td>0.3</td>
<td>350</td>
<td>3.2</td>
<td>25</td>
</tr>
<tr>
<td>4-MUS</td>
<td>CAPCELL PAK C18</td>
<td>0.01 M Ammonium acetate (pH 6)</td>
<td>Acetonitrile</td>
<td>0</td>
<td>85/15</td>
<td>0.4</td>
<td>350</td>
<td>3.2</td>
<td>40</td>
</tr>
<tr>
<td>4-MUG</td>
<td>CAPCELL PAK C18</td>
<td>0.01 M Ammonium acetate (pH 6)</td>
<td>Acetonitrile</td>
<td>0</td>
<td>85/15</td>
<td>0.4</td>
<td>350</td>
<td>3.2</td>
<td>40</td>
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<tr>
<td>Bis-AG</td>
<td>CAPCELL PAK C18</td>
<td>0.01 M Ammonium acetate (pH 6)</td>
<td>Acetonitrile</td>
<td>0</td>
<td>85/15</td>
<td>0.4</td>
<td>350</td>
<td>3.2</td>
<td>40</td>
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<tr>
<td>E3040</td>
<td>CAPCELL PAK C18</td>
<td>0.01 M Ammonium acetate (pH 6)</td>
<td>Acetonitrile</td>
<td>0</td>
<td>85/15</td>
<td>0.4</td>
<td>350</td>
<td>3.2</td>
<td>40</td>
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<tr>
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<td>CAPCELL PAK C18</td>
<td>0.01 M Ammonium acetate (pH 6)</td>
<td>Acetonitrile</td>
<td>0</td>
<td>85/15</td>
<td>0.4</td>
<td>350</td>
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<td>0.01 M Ammonium acetate (pH 6)</td>
<td>Acetonitrile</td>
<td>0</td>
<td>85/15</td>
<td>0.4</td>
<td>350</td>
<td>3.2</td>
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4-MU, 4-methylumbelliferone; 4-MUS, 4-MU sulfate; E3040S, E3040 sulfate; TGZ-S, troglitazone sulfate.
MDCK II cells. MRP2 is predominantly localized in the apical membrane of MDCK II cells as reported previously (Evers et al., 1999; Cui et al., 1999; Sasaki et al., 2002). The signals associated with MRP3 were detected both in the cytoplasmic area and basal membrane but did not show any overlap with those associated with MRP2 (Fig. 5a). The signals associated with MRP4 were predominantly localized on the basolateral membrane of the double-transfected MDCK II cells and did not show any overlap with MRP2 signals (Fig. 5b). The membrane localization of MRP3 and MRP4 was consistent with previous reports (König et al., 1999b; Lai and Tan, 2002).

Transcellular Transport of the Glucuronide Conjugates across MDCK II Monolayers. Basal-to-apical and apical-to-basal transcellular transport of Gem-G, E\(_{217}\)G, E3040G, and TGZ-G across the monolayers was determined in mock control, OATP1B1/MRP2 double transfectants, and OATP1B1/MRP2/MRP3 triple transfectants. However, the basal-to-apical flux of E\(_{217}\)G and Gem-G in the OATP1B1/MRP2/MRP3 triple transfectants was significantly lower than that in OATP1B1/MRP2 double transfectants (69, 77, 66, and 43% of the values in the double transfectants, respectively). The directional transport of the glucuronide conjugates in the basal-to-apical direction was also maintained in the OATP1B1/MRP2/MRP4 triple transfectants. However, the basal-to-apical flux was significantly lower in the OATP1B1/MRP2/MRP4 triple transfectants only for E\(_{217}\)G and Gem-G compared with that in OATP1B1/MRP2 double transfectants (78 and 64% of the values in the double transfectants, respectively). Expression of MRP4 did not affect the basal-to-apical flux of E3040G and TGZ-G (Fig. 6, c and d). Because the apical-to-basal transport of the glucuronide conjugates was similar between the double and triple transfectants, adenovirus infection and butylate treatment did not induce nonspecific transport.

Discussion

MRP3 and MRP4 mediate the efflux of drugs and their metabolites into the blood circulation in the sinusoidal membrane of hepatocytes and compete with canalicullar efflux and metabolism in the liver for their common substrates. In the present study, we constructed triple-transfected MDCK II cells that express OATP1B1, MRP2, and either MRP3 or MRP4 as in vitro model for hepatobiliary transport of organic anions in humans and examined the effect of the expression of MRP3 or MRP4 on the directional transcellular transport of their substrate compounds in the basal-to-apical direction by OATP1B1 and MRP2.

Mrp3\((-/-)\) mice have provided information about the role of hepatic Mrp3 in the disposition of several glucuronide conjugates and methotrexate (Manautou et al., 2005; Zelcer et al., 2005, 2006; Zamek-Gliszczynski et al., 2006). To identify some probe compounds for sinusoidal efflux analysis, we examined the in vivo disposition of five glucuronide conjugates, namely Gem-G, E3040G, TGZ-G, Bis-AG, and 4-MUG in wild-type and Mrp3\((-/-)\) mice. Mrp3 dysfunction caused a marked reduction in the plasma concentration of these glucuronide conjugates after oral administration of their parent compounds, suggesting that Mrp3 plays a key role in the efflux of these glucuronide conjugates into the systemic circulation. This finding is in

Table 2

Area under the curve of the plasma concentration time profiles of parent drugs and their glucuronide conjugates in wild-type and Mrp3\((-/-)\) mice

<table>
<thead>
<tr>
<th>Drug</th>
<th>Wild-type</th>
<th>Mrp3((-/-))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gemfibrozil</td>
<td>3330 ± 90</td>
<td>3020 ± 140</td>
</tr>
<tr>
<td>E3040</td>
<td>1500 ± 200</td>
<td>431 ± 13**</td>
</tr>
<tr>
<td>Troglitazone</td>
<td>174 ± 0.030</td>
<td>0.384 ± 0.110</td>
</tr>
<tr>
<td>E3040G</td>
<td>11.7 ± 5.3</td>
<td>0.500 ± 0.120**</td>
</tr>
<tr>
<td>TGZ-G</td>
<td>323 ± 60</td>
<td>687 ± 181</td>
</tr>
<tr>
<td>Bis-AG</td>
<td>246 ± 6</td>
<td>N.D.</td>
</tr>
<tr>
<td>Bisphenol A</td>
<td>620 ± 133</td>
<td>61.7 ± 10.0**</td>
</tr>
<tr>
<td>4-MUG</td>
<td>753 ± 118**</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

AUC, area under the plasma concentration-time curve; 4-MU, 4-methylumbelliferone; N.D., not detected.

**p < 0.01.
a good agreement with previous findings (Manautou et al., 2005; Zelcer et al., 2005, 2006; Zamek-Gliszczynski et al., 2006). It should be noted that Mrp3 in the enterocytes could contribute to the differences in plasma levels of the glucuronide conjugates determined in addition to Mrp3 in the liver because the parent drugs were given orally, and glucuronidation also occurs in the small intestine (Grams et al., 2000) where Mrp3 is expressed in the basolateral membrane (Rost et al., 2002; Shoji et al., 2004; Zelcer et al., 2006).

An in vivo study using Mrp3(−/−) mice showed that the glucuronide conjugates are in vivo relevant probes for Mrp3. To examine species differences in the substrate recognition/transport activities between mouse and human MRP3, in vitro transport studies using membrane vesicles were performed to confirm that the glucuronide conjugates are substrates of MRP2, MRP3, and MRP4. Both MRP2- and MRP3-expressing membrane vesicles exhibited a significantly higher ATP-dependent accumulation of Gem-G, E3040G, and TGZ-G as well as E217βG than the mock control (Fig. 3). Mrp3 is deeply involved in the in vitro expression of the glucuronide conjugates (Fig. 2), which are MRP3 substrates, excluding a remarkable species difference in the substrate recognition/transport activity by mouse and human MRP3. Unlike MRP2 and MRP3, only E217βG and Gem-G exhibited a significant ATP-dependent uptake in MRP4-expressing membrane vesicles that was statistically significant, indicating that E3040G and TGZ-G are poor MRP4 substrates. It should be noted that, for quantitative interpretation of the results, the uptake under linear conditions must be compared. For E3040G, which exhibited a striking difference between MRP3 and MRP4, the Kᵢ value for MRP3 was reported to be 5.6 μM (Akita et al., 2002a) and that for MRP4 was 27.9 μM (K. Takeuchi, H. Kusuhara, and Y. Sugiyama, unpublished data). These values were considerably greater than the concentration in the incubating buffer (0.5 μM). The absence of significant ATP-dependent uptake of E3040G in MRP4-expressing membrane vesicles compared with control vesicles is not due to saturation.

We previously constructed MDCK II cells that express both human OATP1B1 and MRP2, which greatly enhance the transcellular transport of their common substrates, generally amphipathic anionic drugs in the basal-to-apical direction (Sasaki et al., 2002). In the present study, we have constructed the triple transfectant expressing either MRP3 or MRP4 by infection of adenovirus containing MRP3 or MRP4 cDNA to the OATP1B1/MRP2 double transfectants. The expression of OATP1B1 and MRP2 proteins was maintained in the OATP1B1/MRP2/MRP3 and OATP1B1/MRP2/MPR4 triple transfectants (Fig. 4). Immunoﬂuorescence analysis by confocal laser microscopy showed that MRP3 was localized at the basolateral membrane as well as the cytoplasmic region in MDCK II cells (Fig. 5a), whereas MRP4 was also localized at the basal membrane (Fig. 5b). The membrane localization of MRP2, MRP3, and MRP4 is consistent with the results of previous studies (Evers et al., 1998; Cui et al., 1999; König et al., 1999b; Lai and Tan, 2002; Sasaki et al., 2002). Therefore, the basal-to-apical transport in the triple transfectants will become lower than that in the double transfectants when MRP3- or MRP4-mediated basolateral efflux is comparable to or greater than the
apical efflux mediated by MRP2. The basal-to-apical flux of Gem-G, E$_2$17βG, E3040G, and TGZ-G in the OATP1B1/MRP2/MRP3 triple transfectant was significantly reduced compared with that in the OATP1B1/MRP2 double transfectant (Fig. 6). In the OATP1B1/MRP2/MRP4 triple transfectant, only Gem-G and E$_2$17βG showed a significant reduction in the basal-to-apical flux compared with the OATP1B1/MRP2 double transfectant. MRP4 expression had no effect on the basal-to-apical transport of E3040G and TGZ-G. This is reasonable considering that E3040G and TGZ-G are poor MRP4 substrates (Fig. 3). Therefore, the triple transfectants allow screening of MRP3 and MRP4 substrates with significant transport activities. Expression of MRP3 and MRP4 protein was compared between human liver specimens and the triple transfectants. Expression of MRP3 protein was similar between the human liver and OATP1B1/MRP2/MRP3 triple transfectants (Fig. 4b). However, considering the cytosolic distribution of MRP3 as well as plasma membrane in MDCK II cells (Fig. 5a), the actual amount of MRP3 protein involved in the efflux will be smaller in the triple transfectants compared with that in human liver. On the other hand, expression of MRP4 protein is considerably higher in the OATP1B1/MRP2/MRP4 triple transfectants than in the human liver (Fig. 4b), indicating that the impact of MRP4 is overestimated in the triple transfectants.

The triple transfectants have an advantage in the application of existing high throughput screening systems compared with membrane vesicles for the first screening. We have already constructed all combinations of the uptake and canalicular efflux transporters using MDCK II cells (Matsushima et al., 2005; Ishiguro et al., 2008). Infection of adenovirus containing MRP3 or MRP4 cDNA to other double transfectants will easily allow examination of any overlap of substrate specificities between sinusoidal and canalicular efflux transporters. However, it is evident that the triple transfectants cannot quantitatively predict the in vivo impact of MRP3 and MRP4 on the hepatic elimination because of the difference in protein expression between human liver and triple transfectants and overlapped substrate specificities of MRP3 and MRP4. Actually, the impact of Mrp4 dysfunction on the plasma concentrations of the glucuronide conjugates of acetaminophen, 4-methylumbelliferone, and harmol was negligible in mice, whereas Mrp3 dysfunction showed a great impact (Zamek-Gliszczynski et al., 2006). Further studies are necessary to establish a more quantitative method to predict the impact of MRP3 and MRP4-mediated sinusoidal efflux in the hepatobiliary transport.

The hepatobiliary elimination of amphipathic anionic drugs involves three membrane transport processes: uptake and sinusoidal and canalicular efflux. The rate-determining step in the hepatic elimination must be taken into consideration to predict the effect of functional changes in the metabolism and canalicular efflux caused by drug-drug interactions and genetic polymorphisms on the systemic exposure of drugs (Kusuhara and Sugiyama, 2009). When the sinusoidal efflux is markedly lower than the hepatic metabolism and canalicular efflux, the uptake process becomes the rate-determining step of the net hepatic elimination from the systemic circulation. Under this condition, alteration of activities of hepatic metabolism and canalicular efflux hardly affects the systemic exposure of drugs, whereas it greatly affects the liver concentration. Whether drugs are substrates of MRP3 and/or MRP4 with the transport activities comparable with metabolism and canalicular efflux caused by drug-drug interactions and genetic polymorphisms on the systemic exposure of drugs, it greatly affects the liver concentration. Whether drugs are substrates of MRP3 and/or MRP4 with the transport activities comparable with metabolism and canalicular efflux caused by drug-drug interactions and genetic polymorphisms on the systemic exposure of drugs, it greatly affects the liver concentration. Whether drugs are substrates of MRP3 and/or MRP4 with the transport activities comparable with metabolism and canalicular efflux caused by drug-drug interactions and genetic polymorphisms on the systemic exposure of drugs, it greatly affects the liver concentration.
and simple experimental system for predicting the significance of sinusoidal efflux in hepatobiliary transport.

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References


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