**Multidrug Resistance-Associated Protein 2 Is Primarily Responsible for the Biliary Excretion of Fexofenadine in Mice**

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Received June 20, 2007; accepted October 1, 2007

**ABSTRACT:**

Previous studies implicated P-glycoprotein (P-gp) as the major transport protein responsible for the biliary excretion of fexofenadine (FEX). However, FEX biliary excretion was not impaired in P-gp- or breast cancer resistance protein (Bcrp)-knockout mice or multidrug resistance-associated protein 2 (Mrp2)-deficient rats. The present study tested the hypothesis that species differences exist in the transport protein primarily responsible for FEX biliary excretion between mice and rats. Livers from Mrp2-knockout (Mrp2KO) mice and Mrp2-deficient (TR^-) rats were perfused in a single-pass manner with 0.5 μM FEX. 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 (GF120918) (10 μM) was employed to inhibit P-gp and Bcrp. The biliary excretion rate of FEX was decreased 85% in Mrp2KO relative to wild-type mice (18.4 ± 2.2 versus 122 ± 34 pmol/min/g liver). In mice, more than 50% of FEX unbound intrinsic biliary clearance (CL_{ bile, int} = 3.0 ml/h/g liver) could be attributed to Mrp2 (Mrp2-dependent CL_{ bile, int} = 1.7 ml/h/g liver), with P-gp and Bcrp playing a minor role (P-gp- and Bcrp-dependent CL_{ bile, int} = 0.3 ml/h/g liver). Approximately one third of FEX CL_{ bile, int} was attributed to unidentified mechanisms in mice. In contrast to mice, FEX biliary excretion rate (245 ± 38 and 250 ± 25 pmol/min/g liver) and CL_{ bile, int} (9.72 ± 2.47 and 6.49 ± 0.68 ml/h/g liver) were comparable between TR^- and control Wistar rats, respectively, suggesting that unidentified transport mechanism(s) can completely compensate for the loss of Mrp2 function in rats. Mrp2 clearly plays a major role in FEX biliary excretion in mice. In conclusion, remarkable species differences exist in FEX hepatobiliary transport mechanisms.

FEX is a nonsedating H₁ receptor antagonist used clinically for the treatment of seasonal allergies. FEX is metabolically stable; less than 5% of the dose is eliminated by metabolism in humans (Cvetkovic et al., 1999). FEX is eliminated primarily via biliary excretion. In mice, biliary and renal clearances account for 70% and 10 to 20% of FEX total body clearance, respectively (Tahara et al., 2005). These findings led to the hypothesis that unidentified transport mechanism(s) can completely compensate for the loss of Mrp2 function in rats. In rodents, FEX biliary excretion was not impaired in P-gp- or Bcrp-knockout mice or Mrp2-deficient Eisai hyperbilirubinemic rats (Tahara et al., 2005). These findings led to the hypothesis that one or more unidentified transport protein(s) distinct from P-gp, Mrp2, and Bcrp mediates the biliary excretion of FEX. Recent studies have suggested that species differences exist in transport mechanisms mediating biliary excretion (Zamek-Gliszczynski et al., 2006b). The purpose of this work was to determine whether Mrp2 is responsible for FEX biliary excretion in mice and whether species differences in FEX biliary excretion exist between mice and rats. Furthermore, the involvement of transport mechanisms other than Mrp2, P-gp, and Bcrp in FEX biliary excretion was investigated by using the P-gp and Bcrp inhibitor 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 (GF120918) in Mrp2-knockout mice.

**ABBREVIATIONS:** FEX, fexofenadine; Bcrp, breast cancer resistance protein; B6, wild-type C57BL/6 mouse; CL_{ bile, int} unbound intrinsic biliary clearance; C_{ liver, total} total liver concentration; C_{ liver, unbound} unbound liver concentration; C_{ out, perf} perfusate concentration at steady state; 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; Mrp2, multidrug resistance-associated protein 2; Mrp2KO, Mrp2-knockout; Oatp, organic anion transporting polypeptide; P-gp, P-glycoprotein; TR^-, Mrp2-deficient Wistar rat.
Materials and Methods

Chemicals. FEX, taurocholate, and Krebs-Henseleit buffer packets were purchased from Sigma Chemical Co. (St. Louis, MO). GF120918 was a gift from GlaxoSmithKline (Research Triangle Park, NC). All other chemicals were of reagent grade and were readily available from commercial sources.

Animals. Male C57BL/6 (B6) wild-type and Abcc2 (−/−) (Mrp2KO) mice (25–30 g) were provided by Eli Lilly and Co. Mrp2KO mice were generated as described previously (Nesasa et al., 2006). Male Wistar rats (250–300 g) were purchased from Charles River Laboratories, Inc. (Raleigh, NC); male Mrp2-deficient TR− rats (obtained from Dr. Mary Vore, University of Kentucky, Lexington, KY) were bred in the University of North Carolina School of Pharmacy animal facility. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill.

Single-Pass Liver Perfusion Study. In mice, the gallbladder was cannulated with polyethylene PE-10 tubing, and both the portal vein and inferior vena cava above the liver were cannulated with a 20G catheter (Nesasa et al., 2006). After a 15-min preperfusion period (FEX-free Krebs-Henseleit buffer with 5 μM taurocholate; flow rate = 5 ml/min), single-pass perfusion of the liver was continued for 60 min with buffer containing 0.5 mM FEX and 10 μM GW9156 or vehicle. Perfusate outflow and bile were collected in toto every 10 min. Rat livers were perfused in a similar, single-pass manner with Krebs-Henseleit buffer except that the common bile duct was cannulated with PE-10 tubing and the perfusate flow rate was 30 ml/min.

Analytical Methods. Livers were homogenized in three volumes (v/w) of 0.1 M phosphate buffer (pH 7.4). Homogenates were dialyzed to equilibrium (4.5 h) against buffer using SpectraPor 2 membranes (Spectra Laboratories, Inc., Rancho Dominguez, CA) in a 96-well plate dialysis apparatus (HTDialysis, Gales Ferry, CT) according to the manufacturer’s instructions. Unbound fractions were corrected for dilution (Kalvass and Maurer, 2002).

Bile, outflow perfusate, liver homogenate, and dialysate samples were analyzed by liquid chromatography with tandem mass spectrometry (Applied Biosystems API 4000 triple quadrupole with TurboSpray interface; MDS Sciex, Concord, ON, Canada). FEX and cetirizine (internal standard) were eluted from an Aquasil column (C18, 5 μm, 50 × 2.1 mm; Thermo Electron, Waltham, MA) using a methanol gradient. FEX and cetirizine (internal standard) were eluted from an Aquasil column (C18, 5 μm, 50 × 2.1 mm; Thermo Electron, Waltham, MA) using a methanol gradient. FEX and cetirizine (internal standard) were eluted from an Aquasil column (C18, 5 μm, 50 × 2.1 mm; Thermo Electron, Waltham, MA) using a methanol gradient. FEX and cetirizine (internal standard) were eluted from an Aquasil column (C18, 5 μm, 50 × 2.1 mm; Thermo Electron, Waltham, MA) using a methanol gradient. FEX and cetirizine (internal standard) were eluted from an Aquasil column (C18, 5 μm, 50 × 2.1 mm; Thermo Electron, Waltham, MA) using a methanol gradient. FEX and cetirizine (internal standard) were eluted from an Aquasil column (C18, 5 μm, 50 × 2.1 mm; Thermo Electron, Waltham, MA) using a methanol gradient. FEX and cetirizine (internal standard) were eluted from an Aquasil column (C18, 5 μm, 50 × 2.1 mm; Thermo Electron, Waltham, MA) using a methanol gradient. FEX and cetirizine (internal standard) were eluted from an Aquasil column (C18, 5 μm, 50 × 2.1 mm; Thermo Electron, Waltham, MA) using a methanol gradient. FEX and cetirizine (internal standard) were eluted from an Aquasil column (C18, 5 μm, 50 × 2.1 mm; Thermo Electron, Waltham, MA) using a methanol gradient.

Data Analysis. Based on pilot studies in which outflow FEX concentrations reached a plateau after 50 min of infusion, steady-state FEX concentrations were defined as the average concentration during the 50- to 60-min interval. The hepatic extraction ratio was calculated as the ratio of the difference in steady-state inflow and outflow FEX concentrations and the steady-state inflow FEX concentration (Cliver, unbound). All data are reported as mean ± S.D. (n = 3–4 per group).

Results and Discussion

The biliary excretion rate of FEX was decreased 85% in Mrp2KO mice compared with wild-type B6 mouse livers; GF120918 decreased FEX biliary excretion rate ~45% in B6 and ~40% in Mrp2KO mouse livers compared with vehicle controls (Fig. 1A; Table 1). FEX Clbile, int was significantly decreased in Mrp2KO compared with B6 mouse livers, but was not significantly affected by the coinfusion of 10 μM GF120918 in either B6 or Mrp2KO mouse livers (Table 1).

The contribution of individual canalicular transport proteins to FEX Clbile, int may be estimated as the difference between FEX Clbile, int in wild-type and transport-deficient mouse livers, assuming that other Clbile, int processes in the transport-deficient livers, obtained via genetic knockout or chemical inhibition, are not altered by the loss of functional proteins. Using this approach, the contribution of Mrp2 to FEX biliary excretion rate bile, int was 1.74 ml/h/g liver (the difference between 3.01 and 1.27), consistent with the findings of Tahara et al. (2005). One possible interpretation of these findings is that FEX has affinity for Mrp2 but not rat Mrp2. Alternatively, other transport mechanisms in rat biliary excretion may exist, which accounts for approximately one third of FEX Clbile, int.

Although previous studies indicated that P-gp transports FEX in vitro and that P-gp was the major efflux mechanism for FEX in the mouse intestine and brain (Cvetkovic et al., 1999), this is not the case in mouse liver. As exemplified with FEX, a positive result in vitro in transport assay (Cvetkovic et al., 1999) does not necessarily imply that P-gp is the predominant transport pathway for the molecule in all organs that express P-gp. Tissue- and organ-specific differences in the predominant transport protein for a drug, as reported previously for paeclitaxel (Lagas et al., 2006), may be explained by transport multiplicity, including the affinity of a molecule for multiple transport proteins and the differential expression of these transport proteins in various tissues.

In contrast to mice, the biliary excretion rate of FEX in vitro was comparable between TR− and wild-type Wistar rat livers (Fig. 2A; Table 2), consistent with the findings of Tahara et al. (2005). One interpretation of these findings is that FEX has affinity for mouse Mrp2 but not rat Mrp2. Alternatively, other transport mechanisms in rats may exhibit greater FEX clearance due to greater affinity and/or capacity than Mrp2. The dominant activity of Mrp2 in rats, relative to other preclinical species, has been attributed to high Mrp2 expression (Ninomiya et al., 2005). The biliary excretion of glucuronide and sulfate metabolites is mediated predominantly by Mrp2 in rats.
Mrp2 expression levels. This study demonstrated that impaired biliary canalicular transport are more complex than simple differences in Gliszczynski et al., 2006a,c). Clearly, species differences in hepatic tissue and organ-specific differences within species and interspecies differences in drug transport complicate in vitro-in vivo correlations and allometric scaling for drugs with predominantly transporter-mediated disposition.

Mean steady-state FEX concentrations in outflow perfusate (C_{ss, out}) were significantly higher, consistent with decreased hepatic extraction ratios, in livers from Mrp2KO relative to B6 mice (Fig. 1B; Table 1). Despite the absence of the protein primarily responsible for FEX biliary excretion, FEX liver concentrations and the liver-to-perfusate partition ratio of FEX (C_{liver, unbound}/C_{ss, out}) decreased significantly in Mrp2KO mice (Table 1). This apparent discrepancy reflects the enhanced function of basolateral efflux transport proteins that are responsible for FEX hepatic disposition in Mrp2KO mice. For example, basolateral Mrp3 and Mrp4, which have overlapping substrate specificity with Mrp2, are up-regulated in Mrp2KO mice (Chu et al., 2006; Nezasa et al., 2006; Vlaming et al., 2006). Likewise, FEX liver concentrations were significantly lower in TR- compared with Wistar rats after perfusion (Table 2). The increase in perfusate concentrations during early time points in the livers from TR- relative to Wistar rats (Fig. 2B) may be due to enhanced basolateral efflux of Mrp2 substrates by up-regulated Mrp3 when Mrp2 is deficient.

GF120918 is a more potent inhibitor of P-gp than Bcrp by an order of magnitude, thus concentrations known to inhibit Bcrp in mouse liver perfusions would also have inhibited P-gp transport (de Bruin et al., 1999). The current experiments with GF120918 were designed to inhibit both P-gp and Bcrp based on mouse liver perfusion studies in which 10 \mu M GF120918 extensively impaired Bcrp-mediated biliary excretion of heptically generated 4-methylumbelliferyl sulfate (Zamek-Gliszczynski, 2006a). In the presence of 10 \mu M GF120918, FEX C_{liver, unbound} and partitioning between perfusate and liver were significantly decreased in B6 mice (Table 1). FEX C_{liver, unbound} and partitioning between perfusate and liver tended to be lower in the presence of GF120918 in Mrp2KO mice, although the differences were not statistically significant. A similar trend was observed for the extraction ratios in both B6 and Mrp2KO mice (Table 1). A high concentration of GF120918 (25 \mu M) has been reported to inhibit Oatp2-mediated hepatic uptake of digoxin (Lam and Benet, 2004). Although GF120918 did not significantly alter the CL_{liver, int} of FEX in mouse livers (Table 1), consistent with normal biliary excretion of FEX in P-gp- and Bcrp-knockout mice (Tahara et al., 2005), the potential inhibition of Oatp-mediated hepatic uptake of FEX by GF120918 cannot be ruled out.

In summary, this study demonstrated that FEX biliary excretion is mediated predominantly by Mrp2 in mice; the residual FEX biliary excretion in mice is due to unidentified transport mechanisms and, to

whereas in mice, Bcrp seems to play a more important role (Zamek-Gliszczynski et al., 2006a,c). Clearly, species differences in hepatic canalicular transport are more complex than simple differences in Mrp2 expression levels. This study demonstrated that impaired biliary excretion of a drug in one transporter-deficient animal model does not necessarily translate to impaired biliary excretion in another species deficient in that same transport protein. Overall, tissue- and organ-specific differences within species and interspecies differences in drug transport complicate in vitro-in vivo correlations and allometric scaling for drugs with predominantly transporter-mediated disposition.
a lesser extent, due to Bcrp and P-gp. In rats, the compensatory transport mechanisms responsible for FEX biliary excretion in the absence of Mrp2 remain to be identified. Alterations in liver basolateral transport proteins can have significant impact on the FEX biliary excretion as well. These findings highlight the complexities of in vivo drug transport processes, which are influenced by differences in transporter multiplicity and differential transport protein expression.

Acknowledgments. We sincerely thank Drs. Elaine Leslie and Peijin Zhang for insightful scientific discussion.

References

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