Impact of Mrp2 on the Biliary Excretion and Intestinal Absorption of Furosemide, Probenecid, and Methotrexate Using Eisai Hyperbilirubinemic Rats

Cuiping Chen,[1,2] Dennis Scott,[1] Elizabeth Hanson,[1] Judy Franco,[1] Edwin Berryman,[1] Marlo Volberg,[1] and Xingrong Liu[1]

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Purpose. This study assesses the impact of rat multidrug resistance–associated protein 2 (Mrp2) on the biliary excretion and oral absorption of furosemide, probenecid, and methotrexate using Eisai hyperbilirubinemic rats (EHBR).

Methods. To assess Mrp2-mediated biliary excretion, rats received a 2-h intravenous infusion of furosemide, probenecid, or methotrexate. Blood and bile samples were collected at specified intervals. To assess Mrp2's impact on oral absorption, rats received furosemide, probenecid, or methotrexate orally at 5 mg/kg. Jugular and portal blood samples were obtained at timed intervals. All samples were analyzed by LC-MS/MS. Pharmacokinetic parameters were estimated using WinNonlin and standard pharmacokinetic equations.

Results. Thirty seven- and 39-fold reductions in biliary clearance were observed in EHBR as compared to control rats for probenecid and methotrexate, respectively. Biliary clearance was comparable between EHBR and control rats for furosemide. In all cases, no significant difference in absorption was observed between EHBR and control rats.

Conclusions. This study provides the first evidence that Mrp2 mediates the biliary excretion of probenecid but not furosemide. Additionally, Mrp2 apparently has a less profound impact on intestinal absorption than biliary excretion of its substrates. Furthermore, alteration in systemic clearance in EHBR indicates that a potential compensatory mechanism may occur in EHBR.

KEY WORDS: Eisai hyperbilirubinemic rats; biliary excretion; absorption; multidrug resistance–associated protein 2.

INTRODUCTION

Membrane transport proteins play a central role in mediating the cellular uptake and efflux of both endogenous and exogenous substrates. One of the efflux transporters is multidrug resistance–associated protein 2 [rodents, Mrp2; humans, MRP2, ABCC2, or canaliculi multispecific organic anion transporter (cMOAT)]. MRP2/Mrp2 belongs to the ATP-binding cassette (ABC) family and has been found mainly in liver, kidney, and gut (1,2). MRP2/Mrp2 has been shown to be important in the secretion of organic anions from the body (1,2). The range of molecules transported by MRP2/Mrp2 is broad (1–3). Examples of endogenous substrates include bisglucuronosyl bilirubin, monoglucuronosyl bilirubin, and the glutathione S-conjugate leukotriene C_4 (4). Anionic drugs such as furosemide and probenecid (5) have been shown to interact with MRP2/Mrp2 *in vitro*, suggesting that furosemide and probenecid could be potential substrates of MRP2/Mrp2. However, it is not known whether Mrp2 plays any role in the hepatobiliary disposition and oral absorption of these two drugs *in vivo*. Methotrexate (6) and irinotecan (7) are other examples of anionic drugs as substrates of MRP2/Mrp2 in addition to glucuronide conjugates of acetaminophen (8), E3040 (9), telmisaltan (10), and SN-38 (7). Because rat Mrp2 and human MRP2 are orthologs, and sequence identity is reasonably high (70–80%) (2), identification of substrates for MRP2/Mrp2 has extensively relied on the use of Mrp2 mutant rats (6–10).

There are two major Mrp2 mutant rats, i.e., transport-deficient (TR^-, Wistar strain) (11) and Eisai hyperbilirubinemic rats (EHBR, Sprague–Dawley strain) (12). Both EHBR and TR^- rats are deficient in Mrp2 expression and function in liver and intestine (11,13). The discovery of these mutant rats has facilitated not only the identification of MRP2/Mrp2 substrates but also the elucidation of the significance of Mrp2 in hepatobiliary disposition of its potential substrates (6–10,12–14). For example, reduction of biliary clearance and/or biliary recovery (percentage of dose in bile) was observed for methotrexate (6), pravastatin (15), and 17β-estradiol 17β D-glucuronide (16) in EHBR as compared to control rats following intravenous (i.v.) administration. In contrast, limited information is available regarding the role of MRP2/Mrp2 in hindering oral absorption of its substrates *in vivo*. Most studies that have been conducted so far have used *in vitro* systems such as the Ussing chamber, everted sac, and Caco-2 to simply demonstrate the expression and function of MRP2/Mrp2 in the intestine (17,18). For example, Gotoh *et al.* (18) showed that intestinal secretion of 2,4-dinitrophenyl-S-glutathione (DNP-SG), a substrate for MRP2/Mrp2, was reduced in the everted sac preparation from EHBR as compared to the control rats, suggesting that Mrp2 was involved in the secretion of DNP-SG in intestine. However, *in vivo* studies are needed to assess whether DNP-SG secretion by Mrp2 in *in vitro* intestinal preparations alters oral absorption and bioavailability of DNP-SG. One of the challenges in evaluating the role of MRP2/Mrp2 in possibly hindering the oral absorption of DNP-SG or any other conjugated substrates *in vivo* is limited membrane permeability of these conjugated substrates because they are, in general, highly charged and hydrophilic molecules. Consequently, these conjugated molecules will not be readily absorbed after an oral dose *in vivo*. Therefore, unconjugated Mrp2 substrates are more appropriate to serve as model compounds for this purpose. Recently, an *in vivo* study was conducted for a food-derived carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), in TR^- rats (19). The study showed that TR^- rats had approximately a twofold (mean values) increase in both systemic (after oral dose) and portal (after intraduodenal dose) area under the curve (*AUC*) as compared to control rats. Though limited, this study suggested that MRP2/Mrp2 could reduce oral absorption of its substrates, and Mrp2 mutant animals such as

[1] Groton Laboratory, Pfizer Global Research and Development, Pfizer Inc, Eastern Point Road, Groton, Connecticut 06340.

[2] To whom correspondence should be addressed. (e-mail: cuiping_chen@groton.pfizer.com)

ABBREVIATIONS: ABC, ATP-binding cassette; Mrp2/MRP2, multidrug resistance–associated protein 2; EHBR, Eisai hyperbilirubinemic rat; LC-MS, liquid chromatography-mass spectrometry; CL, clearance; CL_p, systemic clearance; $CL_{biliary}$, biliary clearance; AUC, area under the curve; C_{ss}, steady-state concentration; C_{max}, maximum concentration; T_{max}, time to reach C_{max}.

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TR^- rats and EHBR may serve as unique *in vivo* animal models for elucidating the importance of Mrp2 in potentially restricting oral absorption of its substrates. Unfortunately, no studies have been conducted to assess whether Mrp2 plays an important role in limiting oral absorption and bioavailability of any drug substrates *in vivo*.

The objective of the present study, therefore, was to evaluate and compare the role of Mrp2 in mediating biliary excretion and serving as an absorption barrier to three drugs—furosemide, probenecid, and methotrexate—using Mrp2-mutant EHBR. Furosemide, probenecid, and methotrexate were chosen because they represent parent drug molecules as MRP2/Mrp2 substrates (5,6). Additionally, their biliary clearance and oral bioavailability in rats cover a range of low to moderately high (20–24).

METHODS

Materials

Furosemide, probenecid, and methotrexate were obtained from Sigma Chemical Co. (St. Louis, MO). All the other chemicals and reagents were the highest grade available from commercial sources.

Animals

Male Sprague–Dawley (Charles River, NC) and EHBR (Eisai, Japan) rats, 15 weeks of age (385–550 g), were housed in a group of 10 with free access to food and water and were maintained on a 12-h light/dark cycle.

Contribution of Mrp2 to the Hepatobiliary Disposition of Furosemide, Probenecid, and Methotrexate

Before these studies, the jugular vein, femoral vein, and bile duct were cannulated in both EHBR and control rats under general anesthesia with AErrane® (Isoflurane, Baxter Pharmaceutical Products Inc, Deerfield, IL) and oxygen in the IMPAC6® anesthesia system (VetEquip, Inc, Pleasanton, CA). All procedures were approved by the Institutional Animal Care and User Committee at Pfizer. After a 48-h recovery, rats ($n = 4$ per group) received a continuous i.v. infusion of furosemide, probenecid, or methotrexate for 2 h at a rate of 30 μg/min for furosemide and probenecid and 60 μg/min for methotrexate via the femoral vein. Bile samples were collected every 10 min for the first 40 min and every 20 min for up to 80 min and then from 80 to 120 min after a baseline collection. Blood samples were harvested at the midpoint of each bile collection. Plasma samples were obtained by centrifuging the blood samples at 13,000 rpm for 2 min.

Contribution of Mrp2 to the Potential Reduction in Oral Absorption of Furosemide, Probenecid, and Methotrexate

EHBR and control rats were cannulated in jugular and portal veins under general anesthesia. After a 24-h recovery period, rats were fasted overnight, and furosemide, probenecid, or methotrexate was administered to EHBR and control rats (n = 4–5 per group) orally at 5 mg/kg. Blood samples were collected from both jugular and portal veins at timed intervals for up to 6 (methotrexate) or 8 h (probenecid and furosemide). Plasma samples were obtained as described previously. All samples were stored at –20°C before analysis.

Quantitation of Furosemide, Probenecid, and Methotrexate in Plasma and Bile

Sample Pretreatment

For furosemide and probenecid, plasma or bile samples (50 μl) were acidified with acetic acid (1%, 100 μl). After mixing by vortex, ethyl acetate (300 μl) was added to the acidified samples. For methotrexate, an aliquot of plasma or bile (10–100 μl) and internal standard (i.s.) (10 μl of aminopterin) were mixed in 96-well marsh tubes. The samples were precipitated with acetonitrile (200 μl). In all cases, an aliquot of the supernatant was transferred to a new set of tubes after centrifugation (3,000 rpm × 15 min). The supernatant was evaporated to dryness with Evaporex 96 Channels (Apricot Designs Inc., Monrovia, CA) under nitrogen gas and the residue was reconstituted with mobile phase (100 μl).

LC-MS/MS Instrumentation and Conditions

The HPLC-MS consisted of a Hewlett-Packard (HP) 1100 quaternary pump with membrane degasser (Hewlett Packard, Palo Alto, CA), a Gilson 215 liquid handler (Gilson Inc., Middleton, WI), and a PE Sciex API 3000 mass spectrometer with a turbo ion spray interface (PE-Sciex, Thornhill, Ontario, Canada). For furosemide and probenecid, 10 μl of reconstituted solution was injected into a C_{18} column (4.6 × 140 mm) (Phenomenex, Torrance, CA). Furosemide or probenecid was eluted under the following gradient: 0–1 min, 100% solvent A (10 mM ammonium acetate, 0.05% formic acid, and 1% isopropyl alcohol in water); 1–3 min, 100% solvent A to 100% solvent B (0.05% formic acid and 1% isopropyl alcohol in acetonitrile) and return to 100% solvent A within 1 min. For methotrexate, an Xterra RP C_{18} (3.0 i.d. × 150 mm) (Waters Corp., Milford, MS) was used. Methotrexate and aminopterin (10 μl) were eluted under an isocratic condition with a mobile phase composed of 90% solvent A and 10% solvent B. The compositions of solvents A and B were the same as described previously for furosemide and probenecid. The peak areas of all the analytes and i.s. were obtained using MacQuan (PE-Sciex, Thornhill, Ontario, Canada). The limits of quantitation were 1–10 ng/ml for plasma and bile samples. Validation of the analytic procedure was carried out, and the quality control samples provided values within 20% of the added value throughout the calibration range between 1 ng/ml and 10 μg/ml for furosemide and probenecid and between 5 ng/ml to 1 μg/ml for methotrexate.

Data Analysis

For the i.v. infusion study, the steady-state plasma concentration (C_{ss}) for each compound was estimated based on the mean of concentrations that reached steady state (the mean of concentrations from 50 to 100 min for furosemide and probenecid and 50 to 120 min for methotrexate). The systemic (CL_p) and biliary clearance ($CL_{biliary}$) was calculated based on the following equation:

$$CL_p = \frac{infusion\ rate}{C_{ss}}$$

and

$$CL_{biliary} = \frac{biliary\ excretion\ rate\ at\ steady\ state}{C_{ss}}.$$

Percent of dose excretion in bile was estimated based on the cumulative amount in 2 h duration divided by the dose administered. For the oral study, maximum concentration (C_{max}), time to reach C_{max} (T_{max}) and $AUC_{0\text{-tlast}}$ were obtained using WinNonlin (2.1) (SCI, Apex, NC). The percentage of the dose absorbed in gut (F_{gut}) was estimated based on the following equation (25):

$$F_{gut}(\%) = 100 \times \frac{(AUC_{portal} - AUC_{jugular}) \times portal\ plasma\ flow\ rate}{dose}.$$

Student *t*-test was used to compare the difference in parameters between control rats and EHBR, and a p value of less than or equal to 0.05 was considered to be statistically significant.

RESULTS

Contribution of Mrp2 to the Hepatobiliary Disposition of Furosemide, Probenecid, and Methotrexate

The time course of the plasma concentration and biliary recovery, expressed as percentage dose in bile, for furosemide, probenecid, and methotrexate is shown in Fig. 1. The results demonstrated that furosemide, probenecid, and methotrexate reached C_{ss} within the 2-h infusion for both strains of rats (Fig. 1). The C_{ss} was highest for furosemide, followed by methotrexate and probenecid. Consequently, a significantly lower CL_p was observed for furosemide as compared to probenecid or methotrexate (Table I). Comparable systemic plasma concentration–time profiles between EHBR and control rats were observed for probenecid (Fig. 1B) and methotrexate (Fig. 1C). As a result, C_{ss} and CL_p were similar between EHBR and the control rats for methotrexate and probenecid (Table I). In contrast, a significant difference in the plasma concentration–time profile and consequently in C_{ss} and CL_p was observed for furosemide between the two strains of rats (Fig. 1A). The control rats showed approximately a two-fold higher C_{ss} and a two-fold lower CL_p as com-

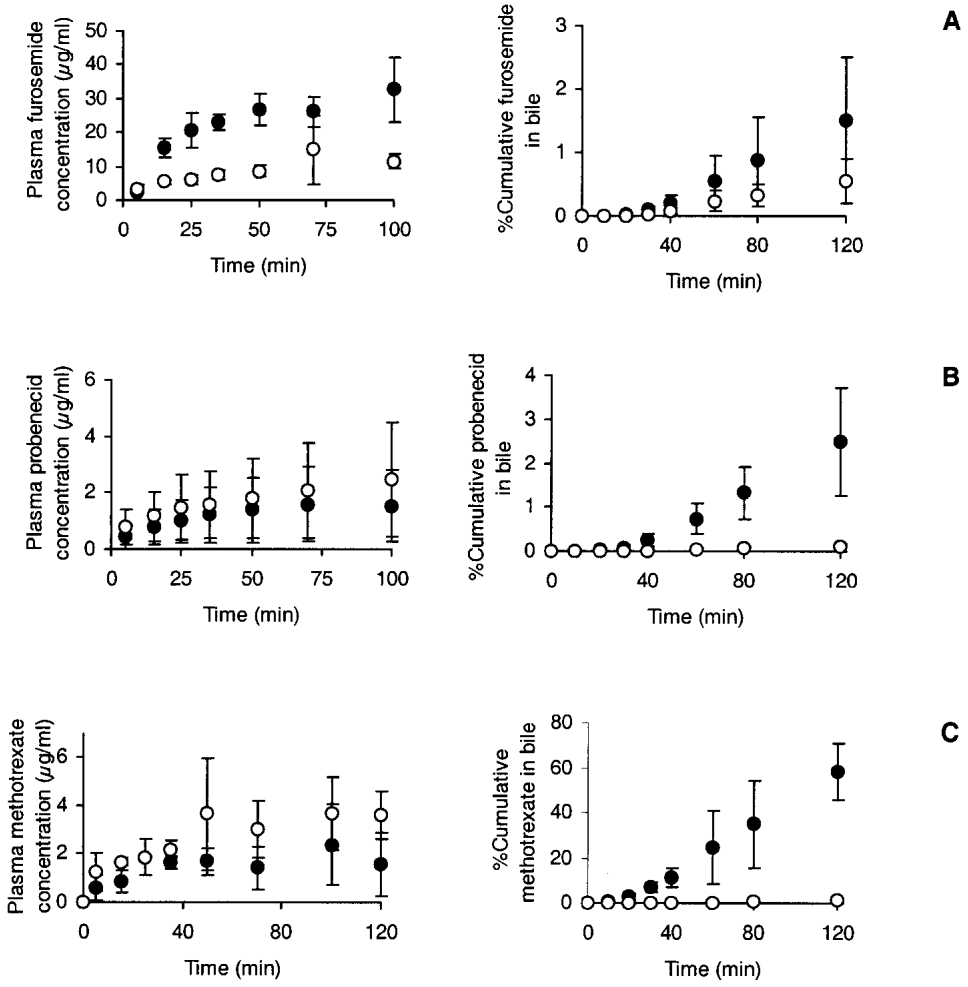

Fig. 1. Plasma concentration–time (left panel) and percentage cumulative dose in bile–time (right panel) profiles of furosemide (A), probenecid (B), and methotrexate (C) after a 2-h i.v. infusion to EHBR *(open symbols)* and control rats *(solid symbols)*. Data are presented as mean ± SD ($n = 4$).

Table I. Pharmacokinetic Parameters of Furosemide, Probenecid, and Methotrexate in Control Rats and EHBR after a 2-h Intravenous Infusion[a]

Pharmacokinetic parameters	Furosemide (30 μg/min)		Probenecid (30 μg/min)		Methotrexate (60 μg/min)	
	Control	EHBR	Control	EHBR	Control	EHBR
C_{ss} (μg/ml)	28.42 ± 4.52	11.56 ± 3.78	1.50 ± 0.81	2.11 ± 0.35	1.91 ± 0.84	3.57 ± 1.47
CL_p (ml/min/kg)	2.74 ± 0.25*	7.32 ± 2.04	63.89 ± 24.61	35.60 ± 6.77	94.64 ± 39.76	46.93 ± 22.10
Percentage cumulative dose in bile over 2 h	1.17 ± 1.06	0.56 ± 0.35	2.48 ± 1.23*	0.09 ± 0.08	58.09 ± 12.61*	1.08 ± 0.85
$CL_{biliary}$ (ml/min/kg)	0.06 ± 0.04	0.08 ± 0.05	28.59 ± 4.66*	0.77 ± 0.53	0.05 ± 0.03*	1.93 ± 0.65

[a] Data are expressed as mean ± S.D. ($n = 4$).
* $p \leq 0.05$ based on Student t test between the control rats and EHBR.

pared to the EHBR (Table I). Unlike plasma C_{ss} and CL_p, the biliary excretion and clearance over the 2-h infusion between EHBR and the control rats were comparable for furosemide. However, both probenecid and methotrexate exhibited drastic reductions in biliary excretion/clearance in EHBR as compared to the control rats (Table I). This reduction in biliary excretion/clearance could not be explained by the reduction in bile flow in EHBR. The bile flow rate–time course profile during an i.v. infusion of methotrexate is shown in Fig. 2.

Contribution of Mrp2 to the Potential Reduction in Oral Absorption of Furosemide, Probenecid, and Methotrexate

The jugular and portal plasma concentration–time profiles are shown in Fig. 3. Probenecid exhibited moderate oral absorption (Table II). In contrast, both furosemide and methotrexate showed low oral absorption in both strains of rats (Table II). No significant difference in either jugular or portal C_{max} or AUC was observed between the two strains of rats for furosemide or probenecid ($p > 0.05$, Table II). As a result, no significant difference in F_{gut} between control rats and EHBR was observed for all compounds tested ($p > 0.05$), although methotrexate showed a significantly higher jugular (1.8-fold) and portal (1.6-fold) C_{max} and jugular AUC (1.9-fold) in EHBR relative to the controls ($p < 0.05$) (Table II).

DISCUSSION

The present study was done to assess the *in vivo* impact of Mrp2 on both biliary excretion and intestinal absorption of furosemide, probenecid, and methotrexate using EHBR. Our results provide the first evidence that Mrp2 mediates the hepatobiliary excretion of probenecid but not furosemide. Additionally, unlike its significant impact on mediating biliary excretion of probenecid and methotrexate, Mrp2 does not appear to limit significantly their oral absorption at the dose investigated (Fig. 3, Table II).

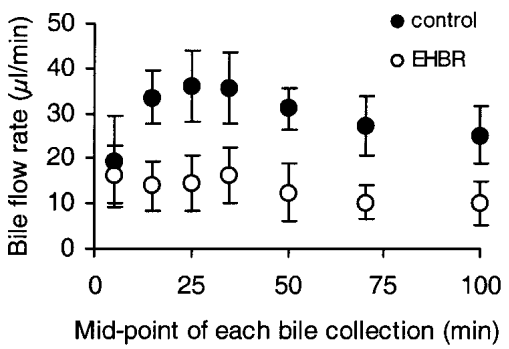

Fig. 2. Bile flow rate–time profile of EHBR *(open symbols)* and control *(solid symbols)* rats that received a 2-h i.v. infusion of methotrexate at 60 μg/min. Data are presented as mean ± SD ($n = 4$).

Furosemide was chosen as a model substrate because it has been shown to stimulate the vanadate-sensitive ATPase activity of MRP2 and, therefore, could be a substrate of MRP2/Mrp2 (5). It has also been speculated that biliary excretion of furosemide in rats, at least in part, is mediated by an active transporter (20), which could be Mrp2. However, the present results showed that a comparable biliary excretion was observed between EHBR and control rats, suggesting that Mrp2 did not contribute to the biliary excretion of furosemide. Consistent with literature, furosemide exhibited a low CL_p, and biliary excretion of unchanged drug was a minor elimination pathway (20,21). The basis of the increase in CL_p and consequent decrease in C_{ss} in EHBR as compared to control rats is not known, and it could be the result of a compensatory mechanism in EHBR. It has been shown that Mrp3 was up-regulated in rat and human liver under cholestatic/hyperbilirubinemic conditions (2). Though Mrp2 and Mrp3 exhibited overlap in substrate specificity for most conjugates and methotrexate (1), it has not been reported that furosemide is a substrate of Mrp3. If we assume that furosemide is an Mrp3 substrate, then the increase in CL_p in EHBR would most likely be secondary to up-regulation of Mrp3 in organs other than liver. This is because no change was observed in biliary clearance of furosemide between the two strains of rats. Further study is warranted to elucidate the exact mechanism that is responsible for the elevation in CL_p of furosemide in EHBR. Nevertheless, the biliary excretion results suggest that furosemide is unlikely to be an Mrp2 substrate. Therefore, we expect to see no difference in the oral absorption of furosemide between the two strains of rats. Indeed C_{max}, AUC, and F_{gut} in control rats were almost identical to the values seen in EHBR ($p > 0.05$, Table II). Additionally, the oral absorption observed in the present study is consistent with literature data (21).

Probenecid has been shown to be a broad-based inhibitor of several organic anion transporters including MRP2/Mrp2 (26,27). Although Bakos *et al.* (5) showed that probenecid interacts with MRP2 through its stimulation of the vanadate-sensitive ATPase activity of MRP2, the contribution of Mrp2 to the biliary excretion and oral absorption of probenecid in rats has not been examined. In rats, the predominant clearance pathway for probenecid is via metabolism; renal and

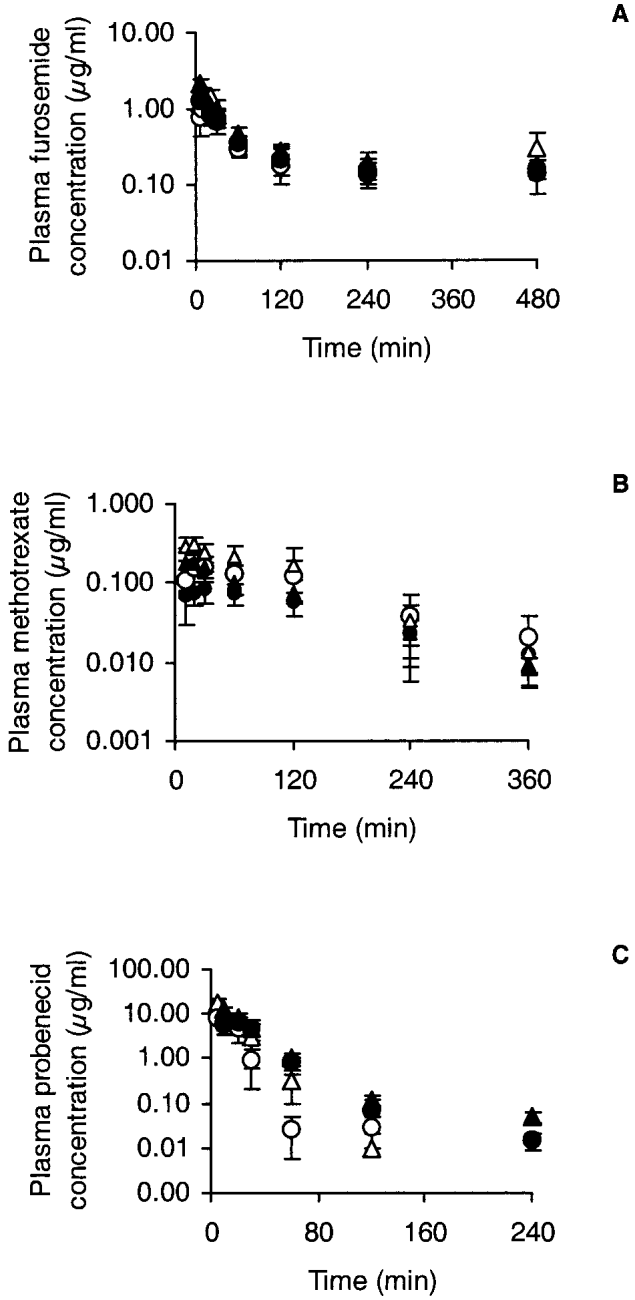

Fig. 3. Jugular *(circles)* and portal *(triangles)* plasma concentration–time profiles of furosemide (A), probenecid (B), and methotrexate (C) after a 5-mg/kg oral administration to EHBR *(open symbols)* and control rats *(solid symbols)*. Data are presented as mean ± SD (n = 4–5).

biliary elimination of the parent molecule account for only 1–10% and <10% of total clearance, respectively (22). This is in good agreement with our observation; only ~2% of the dose was recovered in bile in 2 h. Yet, the contribution of Mrp2 to the biliary excretion of probenecid was obvious, as reflected by approximately 28- and 39-fold reductions ($p < 0.05$) in biliary recovery and biliary clearance, respectively, in EHBR because of their lack of Mrp2 (Table I). This impairment in biliary excretion of probenecid due to lack of Mrp2 in EHBR clearly demonstrates that Mrp2 mediates the biliary excretion of probenecid and that probenecid is a substrate of Mrp2. The drastic decrease in biliary clearance of probenecid led to a trend in, but not statistically significant decrease of, CL_p in EHBR as compared to control rats.

Unlike hepatic Mrp2, intestinal Mrp2 apparently failed to show any significant hindrance to oral absorption of probenecid at a 5 mg/kg dose. The C_{max} and *AUC* in jugular and portal blood and Fgut were all comparable between EHBR and control rats ($p > 0.05$, Table II). Probenecid has been reported to be readily and completely absorbed from the GI tract with an oral bioavailability of ~100% in humans (21). Interestingly, no data are available for rat oral absorption and/or bioavailability. Yet it is very likely that probenecid would have a moderate to high, if not complete, oral absorption in rats, assuming no rate-limiting GI metabolism and/or efflux during the absorption process in rats. Indeed, the F_{gut} was moderate, as shown in the present study, which supports the proceeding assumption.

Methotrexate has been shown to be a substrate of MRP2/Mrp2 (4,5). For example, one study demonstrated that uptake of methotrexate was concentration dependent and saturable in Sf9 cell membrane vesicles expressing MRP2, suggesting that methotrexate is a substrate of MRP2 (5). Furthermore, Masuda *et al.* (6) showed that biliary excretion of methotrexate was significantly reduced in EHBR as compared to the control rats after i.v. bolus administration; EHBR had 1/10 the biliary recovery of controls within 2.5 h following the dose. Consistent with the results from Masuda *et al.* (6), the present study demonstrated that biliary recovery or clearance of methotrexate in EHBR was almost abolished as compared to control rats (Table I). The magnitude of the reduction of biliary recovery and biliary clearance in EHBR for methotrexate definitely cannot be explained by the reduction in bile flow rate (Fig. 2). The bile flow rate in EHBR was approximately 50% of that in control rats, which is in good agreement with literature data (11). Unlike furosemide and probenecid, methotrexate undergoes extensive biliary excretion, and biliary clearance is the major elimination pathway, which concurs with our observation.

The impact of Mrp2 on oral absorption of methotrexate appears more complicated. It is known that methotrexate has a low oral bioavailability, mainly because of limited absorption (24), which agrees with the low F_{gut} value observed in the present study. Although, as for probenecid, no significant strain difference in F_{gut} was seen, both systemic and portal *AUC* values in EHBR are approximately two-fold higher than those in the control rats (Table II). This magnitude of enhancement in intestinal absorption of methotrexate because of the lack of Mrp2 in EHBR is similar to what was observed for [^{3}H]PhIP in TR$^-$ rats (19). Additionally, C_{max} values, both portal and systemic, were significantly higher ($p > 0.05$) in EHBR than control rats (Table II). Overall, the data indicate that Mrp2 might mediate intestinal efflux of methotrexate at the current dose, yet its impact on intestinal absorption (ratio of portal and systemic *AUC* and F_{gut} between EHBR and control rats after oral dose) appears much less significant than biliary excretion (ratio of biliary recovery between EHBR and control rats).

In summary, the present study results provide the first evidence that biliary excretion would be impaired significantly in EHBR as compared to control rats if Mrp2 mediates the biliary excretion of the test compounds, regardless of whether the biliary excretion of the parent compound is (in

Table II. Pharmacokinetic Parameters of Furosemide, Probenecid, and Methotrexate in Control Rats and EHBR after a 5 mg/kg Oral Dose[a]

Compound			Pharmacokinetic parameters			
			C_{max} (μg/ml)	T_{max} (h)	*AUC* (μg/ml × min)	F_{gut} (%)
Furosemide	Control	Jugular	1.44 ± 0.40	0.15 ± 0.04	115 ± 20	13.0 ± 5.7
		Portal	2.22 ± 0.94	0.17 ± 0.00	151 ± 22	
	EHBR	Jugular	1.09 ± 0.32	0.19 ± 0.10	110 ± 15	15.5 ± 10.9
		Portal	1.65 ± 0.21	0.23 ± 0.13	153 ± 36	
Probenecid	Control	Jugular	6.67 ± 1.77	0.33 ± 0.00	270 ± 110	35.2 ± 14.9
		Portal	13.14 ± 4.44	0.21 ± 0.08	368 ± 123	
	EHBR	Jugular	8.28 ± 1.51	0.10 ± 0.04	154 ± 54	41.4 ± 33.9
		Portal	18.40 ± 3.98	0.17 ± 0.00	289 ± 51	
Methotrexate	Control	Jugular	0.10 ± 0.03*	0.53 ± 0.30	17 ± 6*	5.9 ± 4.5
		Portal	0.21 ± 0.08*	0.27 ± 0.15	24 ± 10	
	EHBR	Jugular	0.18 ± 0.02	1.03 ± 0.89	32 ± 13	3.0 ± 2.3
		Portal	0.34 ± 0.07	0.25 ± 0.10	48 ± 25	

[a] Data are presented as mean ± S.D. (n = 4–5).
* $p \leq 0.05$ based on Student t test between the control rats and EHBR.

the case of methotrexate) or is not (in the case of probenecid) the major elimination route. Furthermore, this study indicates that Mrp2 apparently plays a greater role in mediating biliary excretion than hindering intestinal absorption of its substrates such as methotrexate (low absorption) and probenecid (moderate absorption). This may be partially explained by the fact that MRP2/Mrp2 is predominantly expressed in liver and to a much lesser extent in intestine (17). Though the current data suggest that furosemide is unlikely to be an Mrp2 substrate, we cannot definitely rule out the possibility of furosemide as an Mrp2 substrate because of potential up- or down-regulation of other metabolizing enzymes and/or transporters in the mutant animals. A further characterization in these aspects for the mutant animals will shed light on more appropriate interpretation of data that are generated from these animals. Nevertheless, these mutant strains are pivotal in our understanding of the significance of an individual drug transporter in the disposition and elimination of drugs in the absence of specific and potent inhibitors of drug transporters.

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