Role of Breast Cancer Resistance Protein (Bcrp1/Abcg2) in the Extrusion of Glucuronide and Sulfate Conjugates from Enteroocytes to Intestinal Lumen

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ABSTRACT

The purpose of this study is to examine the significance of efflux transporters in the small intestine to extrude glucuronide (G) and sulfate (S) conjugates into the intestinal lumen. From this standpoint, we performed in situ intestinal perfusion experiments by using Eisai hyperbilirubinemic rats (EHBRs) in which the multidrug resistance protein 2 (Mrp2/Abcc2) is hereditarily defective and breast cancer resistance protein (Bcrp1/Abcg2) knockout mice. The intestinal lumen of EHBRs and Bcrp1 (−/−) mice was perfused with medium containing 4-methylumbellif erone (4MU) and E3040 [6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridilmethyl) benzothiazole] to determine the efflux of metabolites into the outflow. The efflux of E3040-glucuronide (G) in EHBRs was significantly lower compared with that in normal rats. However, no significant difference was observed for the efflux of 4MU-G, 4MU-sulfate (S), and E3040-S between EHBRs and normal rats. In contrast, the efflux of intracellularly formed 4MU-G, 4MU-S, and E3040-G in Bcrp1 (−/−) mice was significantly lower than that in normal mice. Therefore, Bcrp1 has an important role in extruding glucuronide and sulfate conjugates formed in enterocytes into the intestinal lumen, whereas Mrp2 is responsible for the efflux of some glucuronide conjugates.

It has been recognized that metabolism and active efflux in the small intestine act synergistically to reduce the oral bioavailability of substrate drugs. For example, cytochrome P450 CYP3A4 (Prueksaritanont et al., 1996; Zhang et al., 1999; Doherty and Charman, 2002; Kaminsky and Zhang, 2003) and P-glycoprotein/MDR1/ABCB1 (Wacher et al., 1998; Benet et al., 1999; Suzuki and Sugiyama, 2000; Kusuhara and Sugiyama, 2002) are expressed in the enterocytes and play an important role in reducing the drug absorption because of their similar substrate specificity. In addition, the synergistic action of conjugation and cellular extrusion into the intestinal lumen is also highlighted as a possible mechanism to account for the lower oral bioavailability of substrate drugs; substrate drugs are metabolized to their conjugates in enterocytes and then excreted into the lumen via efflux transporters (Suzuki and Sugiyama, 2000; Kusuhara and Sugiyama, 2002). Among the conjugating enzymes, it is known that UDP-glucuronosyl transferases and sulfotransferases are highly expressed in the gastrointestinal tract (Doherty and Charman, 2002; Kaminsky and Zhang, 2003). For the extrusion of glucuronide and sulfate conjugates formed in enterocytes, possible candidates are multidrug resistance protein 2 (MRP2/ABCC2) and/or breast cancer resistance protein (BCRP/ABCG2) (Suzuki and Sugiyama, 2000; Kusuhara and Sugiyama, 2002). In the human intestine, reverse transcription-polymerase chain reaction analysis revealed that MRP2 is more highly expressed in enterocytes than MDR1, an efflux transporter for neutral and cationic compounds (Taipalensuu et al., 2001).

The purpose of the present study is to examine the role of the efflux transporters Mrp2 and Bcrp1 in the small intestine in the extrusion of glucuronide and sulfate conjugates into the intestinal lumen. From this standpoint, we performed in situ intestinal perfusional experiments in Eisai hyperbilirubinemic rats (EHBRs) and corresponding normal rats and mice, respectively. As test substances, we chose 4-methyl-umbelliferone (4MU) and E3040 which are...
metabolized in enterocytes by UDP-glucuronosyl transferases and sulfotransferases to yield conjugated metabolites (Mulder et al., 1985; Zimmerman et al., 1991; Takenaka et al., 1995a,b; Chen and Pang, 1997).

Materials and Methods

Chemicals. 4MU, 4MU-G, and 4-MU-S were purchased from Sigma-Aldrich (St. Louis, MO). E3040, E3040-G, and E3040-S were kindly donated by Eisai Co., Ltd. (Tsukuba, Japan). All other chemicals were products of Sigma-Aldrich.

Animals. EHBRs and normal rats were purchased from Clea Japan (Tokyo, Japan). The rats were housed in groups (3–5/cage) under controlled conditions (23°C, 55% air humidity, 12-h light cycle). Bcrp1 (−/−) mice were prepared as described previously (Jonker et al., 2002). FVB mice (control mice) were purchased from Clea Japan. All mice were housed individually in cages with paper bedding (α-dry, Shepherd Specialty Papers, Inc., Kalamazoo, MI) under controlled conditions as described above. The rats and mice were acclimatized for at least 1 week before carrying out experiments and had unrestricted access to water and rodent pellet food (MF, Oriental Yeast Co. Ltd., Tokyo, Japan).

Jejunum Perfusion Experiments. The perfusion experiments were performed according to methods described previously (Loria et al., 1976; Yuasa et al., 1993; Barthe et al., 1999; Adachi et al., 2003). Rats (240–280 g) and mice (23–28 g) were fasted overnight before the perfusion experiment with access to tap water only. Anesthesia was induced by ether for rats and by i.v. injection of nembutal (pentobarbital sodium, 50 mg/kg) for mice. The animals were placed on a heating pad to maintain body temperature at 37°C. The abdomen was opened by a midline longitudinal incision and an 8- to 10-cm jejunal segment was isolated and cannulated at both ends with plastic tubing. The segment was rinsed with phosphate-buffered saline, pH 6.4. Saline was dropped onto the surgical area, which was then covered with a paper sheet to avoid loss of fluid. The experiment was initiated by filling the segment with a 1-ml bolus of the perfusion solution followed by perfusion at 0.2 ml/min for rats and 0.1 ml/min for mice using an infusion pump (syringe infusion pump; Harvard Apparatus Inc., Holliston, MA). The perfusion solution consisted of 20.1 mM Na2HPO4, 47.0 mM KH2PO4, and 101 mM NaCl, pH 6.4, and contained 10 µM 4MU or E3040 with a tracer concentration of 14C-labeled inulin as a nonabsorbable marker (Yuasa et al., 1993; Adachi et al., 2003). The outflow perfusate was collected at 10-min intervals for 30 min. The length of the segment was measured at the end of experiments. Scintillation cocktail (Hionic-fluor; PerkinElmer Life and Analytical Sciences, Boston, MA) was added to the aliquots from the outflow samples to measure the radioactivities in a liquid scintillation counter (model 2700 TR; PerkinElmer Life and Analytical Sciences).

Data Analysis. The absorbed fraction (Fa) of each ligand was estimated according to the following equation, which corrects for the volume change using [14C]inulin as a nonabsorbable marker (Loria et al., 1976; Yuasa et al., 1993; Barthe et al., 1999; Adachi et al., 2003):

$$F_a = 1 - \frac{C_{in,1}}{C_{out,1}} \times \frac{C_{out}}{C_{in}}$$

(1)

where $C_{in,1}$ and $C_{out,1}$ represent the concentration of [14C]inulin in the inflow and outflow solutions, respectively, and $C_{in}$ and $C_{out}$ represent the 4MU and E3040 concentration in the inflow and outflow solutions, respectively.

The apparent membrane permeability clearance for the unit length of intestinal segments was calculated as follows (Yuasa et al., 1993; Adachi et al., 2003):

$$CL_{a,app} = \frac{-Q}{L} \times \ln(1 - F_a)$$

(2)

where $CL_{a,app}$ is the apparent membrane permeability clearance, $Q$ is the perfusion rate (0.1 and 0.2 ml/min), and $L$ is the length of perfused segments.

The efflux rate of metabolites was calculated as follows (Chen and Pang, 1997):

$$Efflux \ rate = C_{out,M} \times Q$$

(3)

where $C_{out,M}$ is the concentration of 4MU and E3040 metabolites, and $Q$ is the perfusion rate (0.1 and 0.2 ml/min).

Analytical Methods. For the determination of 4MU, 4MU-G, and 4-MU-S, two methods were used. In the rat experiments, high-performance liquid chromatography equipped with triple stage mass spectrometer analysis was performed on a model API4000 (MDS Sciex, Concord, ON, Canada) equipped with a G1312A pump (Agilent Technologies, Palo Alto, CA). After addition of umbelliferone as an internal standard (IS) solution into perfusate, the samples were injected and chromatographic separation was performed on a column (Xterra C18; 4.6 mm i.d. × 50 mm in length) (Waters, Milford, MA). Mobile phase, consisting of 0.1% acetic acid and methanol, was delivered at a flow rate of 0.5 ml/min in a gradient (methanol concentration) of 20% at 0 min, 20% at 2 min, 75% at 2.1 min, 75% at 5 min, 20% at 5.1 min, and 20% at 10 min. 4MU, 4MU-G, 4-MU-S, and IS were detected as ion pairs at m/z 175.2/132.9, 351.4/175.3, 254.7/175.1, and 161.3/132.9, respectively. For mouse experiments, high-performance liquid chromatography analysis was carried out using an LC-10Avp pump equipped with an SPD-10Avp detector (Shimadzu, Kyoto, Japan). Perfusion was directly injected onto a column (Inertsil ODS-3; 4.6 mm i.d. × 150 mm long) (GL Science, Tokyo, Japan). Chromatography was achieved using a gradient from 25% (0 min) to 50% (20 min) methanol in 50 mM KH2PO4 at a flow rate of 1 ml/min, and UV detection was performed at 313 nm.

For the determination of E3040, E3040-G, and E3040-S, high-performance liquid chromatography equipped with triple stage mass spectrometer analysis was used. After addition of phenacetin as an IS solution into perfusate the samples were injected onto the column (Xterra C18; 4.6 mm i.d. × 50 mm long) (Waters). Mobile phase, consisting of 10 mM ammonium acetate and acetonitrile, was delivered at a flow rate of 0.5 ml/min in a gradient (acetonitrile concentration) of 10% at 0 min, 10% at 4 min, 60% at 4.5 min, 60% at 6.5 min, 10% at 6.6 min, and 10% at 10 min. E3040, E3040-G, E3040-S, and IS were detected as ion pairs at m/z 299.9/221.1, 475.6/300.2, 380.5/300.1, and 180.1/110.4, respectively.

Results

Perfusion Experiment in Rats. To examine the role of Mrp2 in the efflux of conjugated metabolites, we performed perfusion of the intestinal lumen of EHBRs in which Mrp2 expression is hereditarily defective using medium containing 4MU and E3040. Although no significant difference was observed between the two rat strains for the efflux of 4MU-G, 4MU-S, and E3040-S (Figs. 1, A and B, and 2B), the efflux rate of E3040-G in EHBRs was significantly lower than that in normal rats (Fig. 2A). The concentrations of conjugated metabolites of 4MU and E3040 in intestinal tissue were almost the same at the end of experiments between the two strains (Table 1). The mass balance after perfusion experiments is summarized in Fig. 3. In addition, there was no significant difference in the extent of absorption of 4MU and E3040 between the normal rats and EHBRs (Figs. 1C and 2C).

Perfusion Experiment in Mice. To examine the role of Bcrp1 in the efflux of conjugated metabolites formed in enterocytes, the intestinal lumen of Bcrp1 (−/−) and normal mice was perfused with medium containing 4MU and E3040.
Efflux rate of intracellularly formed 4MU-G, 4MU-S, and E3040-G into the lumen was calculated from the concentration of these conjugates in the outflow perfusate. In Bcrp1 (−/−) mice, the efflux rate of 4MU-G, 4MU-S, and E3040-G was significantly lower than that in normal mice (Figs. 4, A and B, and 5A). In particular, 4MU-S was not detectable in the outflow collected from Bcrp1 (−/−) mice (Fig. 4B). It was also found that the amount of 4MU-G, 4MU-S, and E3040-G associated with the intestinal tissue were almost the same at the end of experiments between two strains (Table 1). In contrast to 4MU-S formation, the sulfate conjugate of E3040 was not detectable in either the outflow or the intestinal tissue in either Bcrp1 (−/−) or normal mice. The mass balance of 4MU and E3040 after perfusion experiments is summarized in Fig. 6.

In addition, the apparent membrane permeability clearance (CLa,app) of 4MU and E3040 was also calculated. The time profile for the CLa,app of 4MU and E3040 indicated that there is no significant difference in the extent of absorption of parent compounds between two strains (Figs. 4C and 5B).

**Discussion**

It has been suggested that conjugating enzymes and efflux transporters may play an important role synergistically in extruding organic anions into the intestinal lumen (Suzuki and Sugiyama, 2000; Kusuhara and Sugiyama, 2002). In the present study, we have focused on the function of Mrp2 and Bcrp1, apically located efflux transporters. It has been reported that both transporters are responsible for the cellular

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**TABLE 1**

Mucosal concentration of glucuronide and sulfate conjugates remaining in the intestine

Amount of glucuronide and sulfate conjugates of 4MU and E3040 remaining in intestine was determined at the end of experiments. Results are given as the mean ± S.E. of three independent experiments. Statistical difference between Bcrp1 (−/−) mice and normal mice or EHBR and normal rats were compared by two-sided Student's t test with p < 0.05 as the limit of significance.

<table>
<thead>
<tr>
<th></th>
<th>4MU-G</th>
<th>4MU-S</th>
<th>E3040-G</th>
<th>E3040-S</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EHBR</strong></td>
<td>917 ± 263</td>
<td>1.25 ± 0.38</td>
<td>861 ± 25</td>
<td>2.26 ± 0.44</td>
</tr>
<tr>
<td>Normal rats</td>
<td>989 ± 363</td>
<td>0.697 ± 0.092</td>
<td>778 ± 47</td>
<td>1.79 ± 0.32</td>
</tr>
<tr>
<td>Bcrp1 (−/−) mice</td>
<td>768 ± 148</td>
<td>73.4 ± 16.1</td>
<td>114 ± 9</td>
<td>N.D.</td>
</tr>
<tr>
<td>Normal mice</td>
<td>751 ± 158</td>
<td>56.5 ± 34.0</td>
<td>90.0 ± 17.0</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D., not detected.
extrusion of glucuronide and sulfate conjugates (Suzuki and Sugiyama, 2000; Nakatomi et al., 2001, Suzuki, 2003) and that sulfate conjugates are much preferred substrates compared with glucuronides for Bcrp (Suzuki et al., 2003). In addition, the expression level of MRP2 and BCRP in human small intestine was found to be much higher than MDR1 at
the mRNA level (Taipalensuu et al., 2001). Based on these results, we focused on the above-mentioned two transporters as candidates involved in intestinal efflux of glucuronide and sulfate conjugates. In the present study, we have quantitatively determined the contribution of Mrp2 and Bcrp1 to the secretion of glucuronide and/or sulfate conjugates into the intestinal lumen by using an in situ intestinal perfusion technique in EHBRS, Bcrp1 (−/−) mice, and corresponding control animals. To clarify which efflux transporters are responsible for the efflux of conjugates, we also performed kinetic analyses. We previously demonstrated that the function of the Mdr1 product can be quantitatively estimated by this perfusion method (Adachi et al., 2003).

The appearance of 4MU-S and 4MU-G in the outflow was almost the same between normal rats and EHBRS, suggesting that Mrp2 is not responsible for the intestinal excretion of these conjugates (Fig. 1, A and B). It was also found that the intestinal excretion of E3040-G, but not that of E3040-S, was decreased in EHBRS. On the whole, although glucuronide conjugates are substrates for Mrp2, the functional significance of Mrp2 in intestinal glucuronide excretion depends on the substrates. From this standpoint, the functional significance of Mrp2 in intestinal excretion of conjugated metabolites should also be discussed. In our previous investigation, it has been demonstrated that the efflux of intracellularly formed 2,4-dinitrophenyl-S-glutathione, an Mrp2 substrate, into the intestinal lumen after intravenous administration of its precursor, 1-chloro-2,4-dinitrobenzene, was significantly lower in EHBRS than that in normal rats (Gotoh et al., 2000). This in vivo observation was further confirmed using Ussing chamber and everted intestinal sack studies. We have demonstrated that transport in the serosal-to-mucosal direction was 2-fold higher in Sprague-Dawley rats compared with EHBRS (Gotoh et al., 2000). In addition, the serosal-to-mucosal transport was greater than that in the opposite direction, and unidirectional transport disappeared in EHBRS (Gotoh et al., 2000). These results indicate the significance of Mrp2 in the intestinal extrusion of 2,4-dinitrophenyl-S-glutathione.

In contrast, it was shown that efflux rates of glucuronide and sulfate conjugates were significantly decreased in Bcrp1 (−/−) mice (Figs. 4, A and B, and 5A). In particular, the efflux of 4MU-S, a Bcrp substrate (Suzuki et al., 2003), decreased to an undetectable level in Bcrp1 (−/−) mice. This observation indicates that Bcrp1 has an important role in extruding sulfamyl conjugates. In addition, extrusion of 4MU-G and E3040-G was reduced to one fifth and one half that of normal mice, respectively. There is no significant difference in each conjugate concentration in intestinal mucosa between Bcrp1 (−/−) and normal mice. It was thus demonstrated that the efflux activity for these conjugates across the apical membrane of enterocytes was reduced in Bcrp1 (−/−) mice. Although the extent of oral absorption of 4MU and E3040 was almost the same between normal and Bcrp1 (−/−) mice (Figs. 4C and 5B), the results of the present study indicate the synergistic role of conjugating enzymes and Bcrp.

The significance of Bcrp1 in reducing the oral absorption and renal secretion of substrate has also been demonstrated. Jonker et al. (2000) demonstrated that the oral absorption of topotecan, a Bcrp1 substrate, was enhanced by the simulta-

Fig. 6. Mass balance of 4MU and E3040 absorption in mouse intestinal perfusion experiments. The intestinal disposition of 4MU and E3040 and their metabolites determined at the end of experiments was summarized. Absorption was defined by subtracting recovered amount from input amount. Data were taken from Figs. 4 and 5. A, 4MU in normal mice. B, 4MU in Bcrp1 (−/−) mice. C, E3040 in normal mice. D, E3040 in Bcrp1 (−/−) mice. Statistical difference between Bcrp1 (−/−) mice and normal mice was compared by two-sided Student’s t test with p < 0.05 as the limit of significance (+, p < 0.05; ++, p < 0.01; ++++, p < 0.001).
neous administration of GF120918, a potent inhibitor of Bcrp1, even in mdr1a/1b knockout mice (Jonker et al., 2000). In addition, the oral bioavailability of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, a food carcinogen, was higher in Bcrp1 (−/−) mice compared with normal mice (van Herwaarden et al., 2003). Jonker et al. (2002) also demonstrated that mice lacking Bcrp1 become extremely sensitive to the dietary chlorophyll-breakdown product phaeophorbid a, resulting in severe lethal phototoxic lesion on light-exposed skin. Bcrp1 transports phaeophorbid a and is highly efficient in limiting its uptake from ingested food (Jonker et al., 2002). These observations strongly suggest that Bcrp1 restricts the absorption of toxic substrates in intestine. Furthermore, the fact that the renal clearance of E3040-S administered by i.v. infusion is 2.4-fold lower in Bcrp1 (−/−) mice compared with normal mice suggests that Bcrp1 also has a significant role in renal secretion (Mizuno et al., 2004). It is unfortunate that we cannot compare the results between kidney and intestine, because E3040-S was not significantly formed in enterocytes (Fig. 5). The function of single-nucleotide polymorphism-type Bcrp1 proteins has been extensively studied to explain the interindividual difference of pharmacokinetic profiles of substrates (Honjo et al., 2001, Iida et al., 2002, Zamber et al., 2003). Investigations are now underway to determine whether pharmacokinetics is influenced by BCRP genotypes in humans.

In conclusion, we have quantified the contribution of Bcrp1 and Mrp2 to restricting the intestinal absorption of its substrate drugs. It was clearly demonstrated by in situ intestinal perfusion experiments that Bcrp1 has an important role in extruding glucuronide and sulfo-conjugates produced in enterocytes to intestinal lumen, although Mrp2 may be responsible for the intestinal excretion of some glucuronide conjugates. These results suggest the synergistic role of conjugating enzymes and efflux transporters in extruding xenobiotics.

References


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