In Vivo Assessment of the Impact of Efflux Transporter on Oral Drug Absorption Using Portal Vein–Cannulated Rats

Yoshiki Matsuda, Yoshihiro Konno, Takashi Hashimoto, Mika Nagai, Takayuki Taguchi, Masahiro Satsukawa, and Shinji Yamashita

Pharmacokinetics and Safety Research Department, Central Research Laboratories, Kaken Pharmaceutical Co., Ltd., Kyoto, Japan (Y.M., Y.K., T.H., M.N., T.T., M.S.); and Faculty of Pharmaceutical Sciences, Setsunan University, Osaka, Japan (S.Y.)

ABSTRACT

The purpose of this study was to evaluate the impact of intestinal efflux transporters on the in vivo oral absorption process. Three model drugs—fexofenadine (FEX), sulfasalazine (SASP), and topotecan (TPT)—were selected as P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), and P-gp and BCRP substrates, respectively. The drugs were orally administered to portal vein–cannulated rats after pretreatment with zosuquidar (ZSQ), P-gp inhibitor, and/or Ko143, BCRP inhibitor. Intestinal availability (Fa) of the drugs was calculated from the difference between portal and systemic plasma concentrations. When rats were orally pretreated with ZSQ, Fa of FEX increased 4-fold and systemic clearance decreased significantly. These data clearly show that the method presented herein using portal vein–cannulated rats can evaluate the effects of intestinal transporters on Fa of drugs independently of variable systemic clearance. In addition, it was revealed that 71% of FEX taken up into enterocytes underwent selective efflux via P-gp to the apical surface, while 79% of SASP was effluxed by Bcrp. In conclusion, the use of portal vein–cannulated rats enabled the assessment of the impact of efflux transporters on intestinal absorption of model drugs. This experimental system is useful for clarifying the cause of low bioavailability of various drugs.

Introduction

P-glycoprotein (P-gp/ABCB1) and breast cancer resistance protein (BCRP/ABCG2), both members of the ATP-binding cassette (ABC) transporter family, are expressed at the apical membrane of various polarized cells such as intestinal enterocytes, hepatocytes, renal epithelial cells, as well as the blood-brain barrier. Both P-gp and BCRP exhibit broad substrate specificity, potentially resulting in limited gastrointestinal absorption or brain penetration and increased renal or hepatic excretion of various drugs via their transport back to the apical surface (Thiebaut et al., 1987; Schinkel and Jonker, 2003). In the drug discovery stage, new chemical entities (NCEs) often suffer from poor systemic exposure due to the limited gastrointestinal absorption via these efflux transporters. Whether NCEs are subject to active efflux by one or both transporters can be evaluated by using Caco-2 cell lines and/or Madin-Darby canine kidney (MDCK) cell lines transfected with individual efflux transporter genes (Troutman and Thakker, 2003). However, it is difficult to predict the intestinal availability (Fa) of NCEs from those in vitro experiments because the in vivo absorption process from the gastrointestinal tract is restricted not only by intestinal efflux transporters but also by other factors, including solubility, membrane permeability, or metabolism. In addition, a method to quantitatively assess the impact of these transporters on the in vivo absorption process has not been fully established.

To investigate the effects of intestinal efflux transporters on oral drug absorption, coadministration studies with transporter inhibitors are often carried out in vivo using experimental animals (Bardelmeijer et al., 2004; Takeuchi et al., 2008). In this approach, to calculate oral bioavailability (F), not only oral administration but also intravenous injection study should be performed. Then, Fa is obtained by dividing oral F by hepatic availability (Fh) (Kato et al., 2003), where the renal clearance should be estimated to calculate Fh. If the systemic clearance of the test compound is significantly affected by oral pretreatment with the transporter inhibitor, intravenous studies should be conducted at each inhibitor dose.

Transporter gene knockout mice and rats are also used to assess the effects of transporters (Chen et al., 2009; Zamek-Gliszczynski et al., 2012); however, a general concern with the use of knockout animals for pharmacokinetic studies is the potential compensatory effects from up- or down-regulation of other transporters and drug metabolism-related genes. Alteration of mRNA levels of several transporter and metabolism-related genes were reported in Abcg1- and Abcg2-knockout mice and rats (Cistermino et al., 2004; Chu et al., 2012). On the other hand, Agarwal et al. (2012) have reported there was no significant difference in the expression of P-gp between the wild-type and Abcg2-knockout mice in the quantitative proteomics. To use gene knockout animals for pharmacokinetic study, this kind of quantitative

ABBREVIATIONS: AUC, area under the concentration-time curve; BCRP, breast cancer resistance protein; Fa, intestinal availability; FEX, fexofenadine; Fg, hepatic availability; LC, liquid chromatography; MDCK, Madin-Darby canine kidney; MS/MS, tandem mass spectrometry; NCE, new chemical entity; P-gp, P-glycoprotein; Rb, blood/plasma concentration ratio; SASP, sulfasalazine; TPT, topotecan; ZSQ, zosuquidar.
analysis on the expression of other transporters or enzymes should be necessary.

In our previous report, we demonstrated the usefulness of portal vein–cannulated rats in evaluating Fa/Fg of orally administered drugs. The use of portal vein–cannulated rats, and monitoring both portal and systemic blood concentrations of the drug, enables calculation of Fa/Fg from a single oral dosing study without the need for intravenous injection (Matsuda et al., 2012). Calculation of Fa/Fg using portal vein–cannulated rats shows less variability than conducting typical kinetic analyses, because our calculation method is less sensitive to interindividual fluctuation in portal blood flow. In addition, since systemic clearance, renal clearance, and Fh are not necessary to calculate Fa/Fg, our method is considered to be highly applicable to transporter inhibition studies.

In this study, the impact of intestinal efflux transporters on oral absorption of three model drugs, fexofenadine (FEX), sulfasalazine (SASP), and topotecan (TPT), was evaluated. FEX, a nonnading histamine H1 receptor antagonist, is well-known as a substrate for OATP1A2 and OATP2B1 as well as P-gp (Cvetkovic et al., 1999). Following oral administration of FEX, a majority of the dose is recovered in the urine and feces in an unchanged form. Kalugutkar et al. (2009) reported that systemic exposure of FEX after oral dosing was dramatically increased by P-gp inhibition in rats. SASP, an anti-inflammatory drug, shows low intestinal absorption due to low solubility and permeability. The bioavailability of SASP in Abcg2-deficient mice and rats was 9- and 17-fold higher, respectively, than that of wild-type (Zaher et al., 2006; Huang et al., 2012). In addition, coadministration of SASP and curcumin, a BCRP inhibitor, increased SASP exposure 3-fold in humans (Kusuhara et al., 2012), suggesting that BCRP-mediated efflux limits intestinal absorption of SASP. The anticancer drug TPT is reported to be a good substrate for BCRP and a weaker substrate for P-gp (Hendricks et al., 1992; Maliepaard et al., 1999). Uptake of [11C]TPT in the brains of Mdr1a/b−/−/Bcrp1−/− mice was about two times higher than in wild-type mice. Similarly, brain penetration of [11C]TPT increased in mice by treatment with elacridar, a P-gp and Bcrp dual inhibitor (Yamasaki et al., 2011).

Zosuquidar (ZSQ) and Ko143 are used as selective inhibitors of P-gp and BCRP. ZSQ is reported to be an extremely potent P-gp inhibitor and does not modulate BCRP-mediated resistance (Shepard et al., 2003), while Ko143 is also well-known as a potent BCRP inhibitor (Allen et al., 2002).

Materials and Methods

Materials. Topotecan was purchased from LKT Laboratories (St. Paul, MN), and ketocnoazole, fexofenadine, and sulfasalazine were purchased from Sigma-Aldrich (St. Louis, MO). Zosuquidar was purchased from Diverchim (Montantaine, France), and Ko143 was purchased from Enzo Life Sciences (Farmingdale, NY). All other chemicals used were reagent grade or better.

Animals. All animal procedures were conducted under protocols approved by the Kaken Institutional Animal Care and Use Committee. Cannulated male Sprague-Dawley rats (8-weeks-old, 260–300 g body weight) were purchased from Charles River Laboratory Japan (Yokohama, Japan) and were kept in an experimental animal room with an ambient temperature of 22–24°C and a 12-hour light-dark cycle for 6 days before use. The cannulated rats were shipped to our laboratory from Charles River Laboratory Japan 2 days after the surgical procedure and arrived the next day. The oral and intravenous administration studies were conducted on the 9th day after the surgery. On the 9th day, no significant differences were observed in the physiologic condition of cannulated and untreated rats (Matsuda et al., 2012).

Surgical Procedure for Portal Vein–Cannulation. The surgical procedure for catheter insertion was reported previously (Matsuda et al., 2012). Animals were implanted with catheters in the portal vein as follows. Rats were anesthetized with ketamine (42.9 mg/kg) and xylazine (8.2 mg/kg) administered intraperitoneally. A midline incision 1–2 cm was made in the abdominal cavity and the portal vein was detached near the liver. To prevent bleeding, the portal vein was ligated temporally as the catheter was inserted. The catheter (3.5-Fr polyurethane tube; Ash Access Technology, Inc., Lafayette, IN) was inserted immediately and fixed by a purse-string suture on the portal vein. The time to reperfusion was about 1 minute after intercepted blood flow. This method for insertion of catheter can avoid the occlusion of the vessel. In addition, a catheter with trumpet-shaped opening was used to prevent the catheter from slipping out of the vessel and minimize the effect on blood flow. Another end of the catheter was passed subcutaneously to the dorsal base of the neck and the laparotomy was closed in two layers, with a 4/0 silk blade to the muscle, and a surgical clip to close the skin. Surgical procedures were approved by the Institutional Animal Care and Use Committee of Charles River Laboratory Japan. This surgical procedure allows the collection of blood samples without the necessity of restraints and anesthesia.

Blood/Plasma Concentration Ratio. The blood/plasma concentration ratio (Rb) was determined in vitro after incubation of 2 μl of methanol solution of test compounds with 2 ml of fresh pooled blood including heparin. Pooled blood was taken from four cannulated rats. Blood was preincubated at 37°C in a water bath, and spiked with the test compounds at 100 ng/ml. The blood samples were incubated at 37°C for 15 minutes. After centrifugation at 14,000g for 10 minutes, the plasma samples were transferred into 4 volumes of methanol containing ketocnoazole [internal standard (IS)] and then centrifuged. The concentrations of test compounds in the supernatant were determined by liquid chromatography–tandem mass spectrometry (LC-MS/MS).

Preparation of Drug Solution. For oral administration studies, each of the substrates and inhibitors was suspended in aqueous 0.5% methyl cellulose as follows: FEX, 1 mg/ml; SASP, 1 mg/ml; TPT, 0.02, 0.06, 0.2, and 0.6 mg/ml; ZSQ, 0.2, 0.6, 2, and 6 mg/ml; and Ko143, 0.2, 0.6, and 2 mg/ml. For intravenous administration study, FEX or ZSQ was dissolved in dimethyl sulfoxide (DMSO), then mixed with a solution containing ethanol, cremophor EL, and saline (DMSO:ethanol:cremophor:saline = 1:2.5:2.5:94). Final concentration of the drug was adjusted to 0.5 mg/ml for FEX and 1 mg/ml for ZSQ.

Study Design and Drug Administration. Three substrates (FEX, SASP, and TPT) and two inhibitors (ZSQ and Ko143) were used for the pharmacokinetic studies. In the oral administration study, substrates and inhibitors were administered directly into stomach using gastric tube without anesthesia. Each substrate was orally administered to the fasted rats (FEX, 5 mg/kg; SASP, 5 mg/kg; and TPT, 0.3 mg/kg) at 40 minutes after oral administration of vehicle or inhibitors (ZSQ, 30 mg/kg and/or Ko143, 10 mg/kg). For dosing studies of TPT, TPT was orally administered to fasted rats at doses of 0.1, 0.3, 1, and 3 mg/kg. For dosing studies of ZSQ and Ko143, ZSQ (1, 3, 10, and 30 mg/kg) or Ko143 (1, 3, and 10 mg/kg) was orally administered to the rats 40 minutes prior to oral administration of TPT (0.3 mg/kg). For the pharmacokinetic studies of FEX, FEX was orally administered to the rats (5 mg/kg) 40 minutes after oral administration (30 mg/kg) or 5 minutes after intravenous administration (2 mg/kg) of ZSQ. FEX (1 mg/kg) was intravenously administered to the rats 40 minutes after oral administration of ZSQ (30 mg/kg). FEX (1 mg/kg) and ZSQ (2 mg/kg) were coadministered intravenously. Following administration, blood samples were taken from the portal and caudal veins of the unanesthetized rats at 0.083, 0.25, 0.5, 1, 2, 4, 6, and 8 hours under unrestricted conditions. The plasma samples were separated by centrifugation at 14,000g for 10 minutes at 4°C and stored at −30°C until use. The main working concentrations in the plasma were quantified using LC-MS/MS.

LC-MS/MS Analysis. The LC-MS/MS system consisted of a HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland), Accela HPLC, and TSQ Ultra mass spectrometer (Thermo Fisher Scientific, San Jose, CA). LC conditions were as follows: column, CAPCELL PAK C18 ACR (1.5 mm I.D. × 35 mm, 3 μm; Shiseido, Tokyo, Japan); YMC-Triart C18 (2.0 mm I.D. × 30 mm, 3 μm; YMC, Kyoto, Japan); column temperature, 40°C; gradient elution at 0.3 ml/min with methanol/aqueous 0.1% formic acid or methanol/aqueous 1 mM ammonium acetate; and injection volume, 15 μl. The main working parameters for mass spectrometers were as follows: ion mode, electrospray ionization, positive; spray voltage, 4000 V; sheath gas pressure, 30 arbitrary units (Arb); auxiliary gas pressure, 35 Arb; capillary temperature, 300°C;...
multireaction monitoring method with transitions of m/z 502.3 → 466.3 for FEX, m/z 397.1 → 197.1 for SASP, m/z 422.2 → 377.2 for TPT, and m/z 531.3 → 243.9 for ketoconazole (IS). The lower limit of determination was 0.2 or 1 ng/ml and the linear detection range was up to 500 ng/ml.

**Pharmacokinetic Analysis.** Noncompartmental pharmacokinetics were calculated using Phoenix WinNonlin 6.1 (Pharsight, Mountain View, CA) for individual animals, and reported as the mean ± standard deviation (S.D.) of the group. Intestinal availability (Fa Fg) was calculated using eq. (1):

\[
Fa \cdot Fg = \frac{Q_{por} \times Rb \times (\text{AUC}_{por} – \text{AUC}_{sys})}{\text{Dose}}
\]

where \(Q_{por}, Rb, \text{AUC}_{por}, \) and \(\text{AUC}_{sys} \) were the portal blood flow, the blood/plasma concentration ratio, AUC (area under the concentration-time curve) calculated from plasma concentration in the portal vein and the systemic circulation, respectively. As \(Q_{por} \), the value of 32.9 ml/min/kg was used, as calculated in our previous report by assuming that Fa Fg of antipyrine was 1 (Matsuda et al., 2012).

**Statistics.** The presented values were all mean ± S.D. For comparison of control, ZSQ, Ko143, and ZSQ+Ko143 groups, the statistical significance of the difference between mean values was calculated using analysis of variance (ANOVA) with Tukey-Kramer test used for multiple comparisons. For comparison between with and without inhibitors in other inhibition tests, Dunnet’s test was used. Differences with a P value of less than 0.05 were considered to be statistically significant.

**Results**

**Effect of P-gp Inhibition on Pharmacokinetics of FEX After Oral or Intravenous Administration.** To evaluate the effect of P-gp inhibition on systemic clearance of FEX, pharmacokinetic studies on FEX were conducted. First, to observe the effect of P-gp inhibition on the systemic clearance of FEX, FEX (1 mg/kg) was intravenously injected with or without oral preadministration of ZSQ (30 mg/kg, at 40 minutes before FEX injection). As shown in Table 1, the systemic clearance of FEX was significantly lowered by ZSQ oral pretreatment (39.3 ± 4.0 ml/min/kg in control versus 28.6 ± 3.0 ml/min/kg in ZSQ p.o.). Systemic clearance of FEX following intravenous coadministration of ZSQ (2 mg/kg) also decreased significantly (29.3 ± 1.9 ml/min/kg) compared with control. Although the systemic clearance of FEX after oral or intravenous pretreatment with ZSQ was nearly identical, Fig. 1 shows that Fa Fg of FEX increased 4-fold after oral, but not intravenous pretreatment with ZSQ. These data indicate that the use of portal vein–cannulated rats enables the assessment of intestinal availability in the oral absorption process independently of variable systemic clearance.

**Intestinal Availability of TPT in Dose-Dependent Studies.** Portal vein–cannulated rats were orally administered TPT in a dose-dependent manner (0.1, 0.3, 1, and 3 mg/kg). As shown in Fig. 2 and Table 2, the AUCsys and AUCpor of TPT increased proportionally with dose, and a constant value for Fa Fg was obtained at doses of 0.1, 0.3, 1, and 3 mg/kg. Intestinal absorption of TPT was found to follow linear kinetics, and intestinal efflux transport involved in TPT absorption was unsaturated at these doses. For the following study, TPT at a dose of 0.3 mg/kg was orally administered to portal vein–cannulated rats 40 minutes after pretreatment with transporter inhibitors. The concentration of TPT in the drug solution was set at 0.06 mg/ml, the same dose concentration in clinical use. Because the recommended dose of TPT is 2.3 mg/m² per day in humans, the intestinal concentration of TPT (dose/250 ml) is estimated at approximately 0.02 mg/ml.

**Effects of P-gp and Bcrp Inhibition on Intestinal Availability of TPT.** As shown in Fig. 3, A and B, pretreatment of rats with a single oral dose of ZSQ (30 mg/kg) or Ko143 (10 mg/kg) 40 minutes prior to oral administration of TPT (0.3 mg/kg) resulted in a significant increase in Fa Fg of TPT compared with vehicle-pretreated rats. These data indicate that both P-gp- and Bcrp-mediated active effluxes were involved in the intestinal absorption of TPT. In addition, both transporters in the intestine were inhibited almost completely by ZSQ (30 mg/kg) and Ko143 (10 mg/kg) since the Fa Fg of TPT did not increase further when higher doses of inhibitors were administered.

**Assessment of the Contributions of Efflux Transporters in the Oral Absorption Process.** To assess the impact of P-gp- and Bcrp-mediated efflux on the intestinal absorption of three model drugs (FEX, a P-gp substrate; SASP, a Bcrp substrate; and TPT, a P-gp and Bcrp substrate), each drug was orally administered to portal vein–cannulated rats with or without pretreatment with inhibitors. As shown in Figs. 4 and 5 and Table 3, systemic and portal plasma concentrations of FEX after oral pretreatment with ZSQ were higher than those of vehicle-pretreated rats. The Fa Fg of FEX (0.22 ± 0.18) increased 4-fold with ZSQ (0.84 ± 0.10), but not with Ko143

---

**TABLE 1**  
Pharmacokinetic parameters of FEX (1 mg/kg) after oral (30 mg/kg) and intravenous (2 mg/kg) administration of ZSQ

<table>
<thead>
<tr>
<th></th>
<th>AUC</th>
<th>t1/2</th>
<th>Clint</th>
<th>Vdss</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ng/ml</td>
<td>h</td>
<td>ml/min/kg</td>
<td>ml/kg</td>
</tr>
<tr>
<td>Control</td>
<td>428 ± 41</td>
<td>2.1 ± 0.2</td>
<td>39.3 ± 4.0</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>ZSQ p.o.</td>
<td>587 ± 58**</td>
<td>1.4 ± 0.1***</td>
<td>28.6 ± 3.0**</td>
<td>1.4 ± 0.2*</td>
</tr>
<tr>
<td>ZSQ i.v.</td>
<td>570 ± 34**</td>
<td>1.4 ± 0.1***</td>
<td>29.3 ± 1.9**</td>
<td>1.1 ± 0.2**</td>
</tr>
</tbody>
</table>

ZSQ p.o., ZSQ was orally administered 40 minutes before FEX was intravenously administered. ZSQ i.v., ZSQ, and FEX were intravenously coadministered. Values represent the mean ± S.D. for three to four rats.

---

**TABLE 2**  
The systemic AUCs, portal AUCs, and Fa Fg of TPT following oral administration of increasing doses in portal vein–cannulated rats

<table>
<thead>
<tr>
<th>TPT Dose</th>
<th>AUCsys</th>
<th>AUCpor</th>
<th>Rb</th>
<th>Fa Fg</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/kg</td>
<td>ng/ml</td>
<td>ng/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>6.0 ± 0.9</td>
<td>11.2 ± 2.1</td>
<td>1.26</td>
<td>0.13 ± 0.03</td>
</tr>
<tr>
<td>0.3</td>
<td>14.1 ± 0.3</td>
<td>25.8 ± 2.2</td>
<td>0.10 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>50.1 ± 7.4</td>
<td>102 ± 12</td>
<td>0.13 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>172 ± 2</td>
<td>313 ± 22</td>
<td>0.12 ± 0.02</td>
<td></td>
</tr>
</tbody>
</table>

Values represent the mean ± S.D. for three rats.
pretreatment (0.20 ± 0.05). In addition, no further increase in Fa·Fg was seen with ZSQ+Ko143 pretreatment compared with pretreatment with only ZSQ, suggesting that intestinal absorption of FEX was highly restricted by P-gp but not by Bcrp-mediated efflux. In the case of SASP, the Fa·Fg without added inhibitor was quite low (0.03 ± 0.01), and increased to 0.14 ± 0.07 with Ko143 pretreatment. Pretreatment with ZSQ showed no effect on the intestinal absorption of SASP (0.02 ± 0.01). This result suggests that the Fa·Fg of SASP was restricted only by Bcrp-mediated efflux. In contrast, the Fa·Fg of TPT was 0.11 ± 0.03 in the absence of inhibitors, and increased to 0.23 ± 0.07 by ZSQ pretreatment, although this difference was not significant. The Fa·Fg of TPT after pretreatment with Ko143 increased significantly to 0.42 ± 0.10, and further increased to 0.64 ± 0.20 following pretreatment with both inhibitors. The difference in the Fa·Fg between Ko143 and ZSQ+Ko143 pretreatment was not significant, as the impact of P-gp was likely small.

As shown in Fig. 6, the Fa·Fg after pretreatment with ZSQ+Ko143 was regarded as a fraction of the dose influxed into enterocytes since all three drugs were reported to be hardly metabolized (Das et al., 1979; Herben et al., 1997; and Strelevitz et al., 2006). In FEX and SASP, a fraction of the efflux by P-gp or Bcrp was calculated as the difference between the Fa·Fg in ZSQ+Ko143-pretreated rats and that of control rats. Thus, the fractions of influx, efflux, and Fa·Fg in control rats were calculated as 0.77, 0.55, and 0.22 for FEX, and 0.14, 0.11, and 0.03 for SASP, respectively. In the case of TPT, the contribution of each transporter was calculated by eqs. (2) to (4).

When pretreatment was with ZSQ, the Fa·Fg and Bcrp efflux of TPT were

$$\text{Fa} \cdot \text{Fg : Bcrp efflux} = 0.23 : 0.41 (= 0.64 - 0.23) \quad (2)$$

When pretreatment was with Ko143, the Fa·Fg and P-gp efflux of TPT were

$$\text{Fa} \cdot \text{Fg : P-gp efflux} = 0.42 : 0.22 (= 0.64 - 0.42) \quad (3)$$

Taken together, when both inhibitors were absent, the Fa·Fg, P-gp, and Bcrp efflux of TPT were

$$\text{Fa} \cdot \text{Fg : P-gp efflux : Bcrp efflux} = 30 : 16 : 54 \quad (4)$$

Influx fraction was separated by eq. (4). Thus, as shown in Fig. 6, the fractions of P-gp and Bcrp efflux were calculated as 0.10 and 0.35, respectively. These data suggest that Bcrp was the dominant efflux transporter that restricted TPT absorption in rats.

**Discussion**

Recent studies on intestinal transporters have revealed important roles for various transporters in regulating the absorption of drugs...
from the intestinal tract, such as the case for solute carrier, SLC, transporters (PEPT1, OATPs) to facilitate absorption, and for ATP-binding cassette, ABC, transporters (P-gp, BCRP, and MRP2) to limit absorption. In the process of drug development and also for clinical use, it is highly beneficial to determine the contribution of these transporters to overall absorption because this information enables prediction of the change in the rate and amount of drug absorption when the functions of transporters are altered by genetic polymorphisms, disease states, or drug-drug interactions.

Typically, to assess the effect of intestinal transporters on drug absorption in vivo, test compounds are orally coadministered with an inhibitor specific to each transporter, then the Fa-Fg and Fh are estimated from pharmacokinetic (PK) analysis of intravenous and oral administration following eqs. (5) and (6)

\[ F_h = 1 - \frac{(CL_{tot} - CL_r)}{Q_h} \]  

\[ Fa \cdot Fg = F / Fh \]  

where CL_{tot} and CL_r are systemic and renal clearance after intravenous administration, respectively, and Q_h is hepatic blood flow. Using these equations, if systemic or renal clearance of the test compound is altered by oral coadministration of a transporter inhibitor, an intravenous study is required for each inhibitor dose. The present study showed

![Graphs showing plasma concentration-time profiles for different treatments](image-url)

**Fig. 4.** Systemic and portal plasma concentration-time profile of FEX (5 mg/kg), SASP (5 mg/kg), and TPT (0.3 mg/kg) after pretreatment with ZSQ (30 mg/kg) and/or Ko143 (10 mg/kg) in the portal vein-cannulated rats. The systemic plasma concentration-time profile of FEX (A), SASP (C), and TPT (E) after pretreatment with ZSQ and/or Ko143. The portal plasma concentration-time profile of FEX (B), SASP (D), and TPT (F) after pretreatment with ZSQ and/or Ko143. Each symbol represents the mean ± S.D. for three to five rats.
that pretreatment with ZSQ (both by i.v. and p.o.) significantly decreased the systemic clearance of FEX (Table 1). Similarly, Adane et al. (2012) reported alteration of the systemic AUC of a camptothecin analog in oral and intravenous administration studies following oral pretreatment with ZSQ or elacridar, a dual P-gp and Bcrp inhibitor. In these cases, to consider the effect of transporters on oral absorption of FEX or camptothecin, not only systemic but also renal clearance should be estimated since P-gp and Bcrp are expressed in the renal tubule.

In this study using portal vein–cannulated rats, changes in FaFg of FEX after oral and intravenous pretreatment with ZSQ were estimated (Fig. 1). Oral administration of ZSQ was considered to inhibit both systemic and intestinal P-gp, while intravenous administration of ZSQ inhibited only systemic P-gp expression in liver, kidney, and other organs, except for intestine. That is, FaFg of FEX after oral administration of ZSQ was 4-fold higher than control but was almost the same as control after intravenous administration despite a significant change in systemic clearance. The change in systemic clearance is negligible because the absorbed amount is calculated from the difference between the systemic and portal amount in eq. (1). These results clearly show that our method using portal vein–cannulated rats enables the evaluation of the effects of selective inhibitors on oral absorption of substrate drugs independently of variable systemic clearance, without the requirement of an intravenous administration study.

As shown in Fig. 3, FaFg of TPT did not increase at higher inhibitor doses, which might be attributed to the complete inhibition of P-gp- and Bcrp-mediated intestinal efflux by ZSQ (30 mg/kg) and Ko143 (10 mg/kg). Poller et al. (2011) reported that TPT transport in a double-transfected MDCKII-ABCB1/ABCG2 cell line treated with 1.6 and 500 μM TPT was completely blocked in the presence of ZSQ (5 μM) and Ko143 (1 μM). Chemical knockdown with ZSQ and Ko143 was therefore assumed to almost completely inhibit the intestinal efflux transporter. In addition, ZSQ was reported to have a much lower affinity for CYP3A than for P-gp (Dantziger et al., 1999).

In our preliminary study, FaFg of felodipine, a drug easily metabolized at the intestine (Wang et al., 1989), did not change after pretreatment with ZSQ or Ko143 (data not shown), suggesting that intestinal metabolism was not affected by ZSQ (30 mg/kg) and Ko143 (10 mg/kg), although FEX, SASP, and TPT were resistant to oxidative metabolism.

To understand the impact of P-gp and Bcrp quantitatively, the contributions of enterocyte efflux transporters to overall absorption were represented as dose fraction, shown in Fig. 6. In the case of FEX, 71% of the amount taken up into enterocytes was effluxed to the apical surface by P-gp in rats. This result is in good agreement with results using Caco-2 cell monolayers reported by Petri et al. (2004) in which P-gp attenuated the permeability of FEX to 30%. Also, our results indicated that 77% of orally administered FEX was taken up into enterocytes despite its large polar surface area and high molecular weight. Qiang et al. (2009) reported that pretreatment with fluvastatin, a substrate of OATP1B1, OATP2B1, and OATP1B3 (Noé et al., 2007), decreased the bioavailability of FEX 0.45-fold in rats due to metabolism.

SASP was found not to be a substrate of P-gp; however, 79% of that taken up by enterocytes was effluxed to the apical surface by Bcrp, resulting in a very low FaFg of only 0.03 in the control study. Additionally, low solubility and low permeability contributed to the low oral absorption of SASP. While the solubility of SASP is 0.0024 mg/ml in water (Benet et al., 2011), the concentration of SASP in the drug solution was 1 mg/ml, meaning that the majority of SASP remained insoluble in the gastrointestinal tract.

De Vries et al. (2012) and Tang et al. (2012) have reported that single gene disruption of Abcb1a/1b or Abcg2 in mice has little or even no detectable effect on the accumulation of erlotinib or sunitinib in the brain, whereas simultaneous disruption of both transporters results in a dramatic increase. This unexpected effect has led to postulation of a synergistic role for P-gp and Bcrp (Polli et al., 2009). However, using a blood-brain barrier kinetic model, Kodaira et al. (2010) analyzed brain uptake of these drugs and proposed a kinetic concept for this apparent synergism. They demonstrated that P-gp involved in the efflux of these drugs together with Bcrp attenuates the
The systemic AUCs, portal AUCs, and FaFg of FEX (5 mg/kg), SASP (5 mg/kg), and TPT (0.3 mg/kg) after pretreatment with ZSQ (30 mg/kg) and/or Ko143 (10 mg/kg) in the portal vein–cannulated rats

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Pretreatment</th>
<th>AUCsys</th>
<th>AUCport</th>
<th>Rb</th>
<th>FaFg</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEX</td>
<td>Control</td>
<td>59.9 ± 40.9</td>
<td>617 ± 490</td>
<td>0.99</td>
<td>0.22 ± 0.18</td>
</tr>
<tr>
<td>ZSQ</td>
<td>Control</td>
<td>431 ± 63***</td>
<td>2563 ± 286***</td>
<td>0.84</td>
<td>0.10***</td>
</tr>
<tr>
<td>Ko143</td>
<td>Control</td>
<td>55.0 ± 19.7***</td>
<td>565 ± 118***</td>
<td>0.20</td>
<td>0.05***</td>
</tr>
<tr>
<td>ZSQ + Ko143</td>
<td>Control</td>
<td>284 ± 39***</td>
<td>2243 ± 330***</td>
<td>0.77</td>
<td>0.13***</td>
</tr>
<tr>
<td>SASP</td>
<td>Control</td>
<td>366 ± 37</td>
<td>499 ± 42</td>
<td>0.58</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>ZSQ</td>
<td>Control</td>
<td>339 ± 29</td>
<td>443 ± 32</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>Ko143</td>
<td>Control</td>
<td>2881 ± 646***</td>
<td>3492 ± 716***</td>
<td>0.14</td>
<td>0.07‡</td>
</tr>
<tr>
<td>ZSQ + Ko143</td>
<td>Control</td>
<td>2529 ± 540***</td>
<td>3146 ± 411***</td>
<td>0.14</td>
<td>0.04‡</td>
</tr>
<tr>
<td>TPT</td>
<td>Control</td>
<td>18.3 ± 1.6</td>
<td>30.9 ± 1.5</td>
<td>1.26</td>
<td>0.11 ± 0.03</td>
</tr>
<tr>
<td>ZSQ</td>
<td>Control</td>
<td>36.2 ± 13.7</td>
<td>63.8 ± 19.4</td>
<td>0.23</td>
<td>0.07</td>
</tr>
<tr>
<td>Ko143</td>
<td>Control</td>
<td>69.0 ± 17.5***</td>
<td>119 ± 18***</td>
<td>0.42</td>
<td>0.10*</td>
</tr>
<tr>
<td>ZSQ + Ko143</td>
<td>Control</td>
<td>122 ± 15***</td>
<td>200 ± 35***</td>
<td>0.64</td>
<td>0.20***</td>
</tr>
</tbody>
</table>

Values represent the mean ± S.D. for three to five rats. Statistically significant differences: *P < 0.05, **P < 0.01, ***P < 0.001, control versus ZSQ, Ko143, or ZSQ+Ko143; †P < 0.05, ††P < 0.01, †††P < 0.001, ZSQ versus Ko143; ‡P < 0.05, ‡‡P < 0.01, ‡‡‡P < 0.001, ZSQ versus ZSQ+Ko143; †††P < 0.001, ZSQ versus ZSQ+Ko143; †††P < 0.001, Ko143 versus ZSQ+Ko143.

Fig. 6. Schematic diagram of the impact of P-gp and Bcrp on intestinal absorption of FEX (A), SASP (B), and TPT (C). Values represent the fractions of influx, efflux, and FaFg when the orally administered amount was regarded as 1, and values given in parentheses represent the each fraction when influx into enterocytes was regarded as 100.

The effect of Bcrp impairment on drug concentrations in the brain. That is, two efflux transporters act as a safety net that prevents brain penetration of common substrates when another efflux transporter is dysfunctional. Intestinal absorption of TPT was also restricted by both P-gp and Bcrp. As shown in Fig. 6, 70% of TPT taken up into enterocytes was effluxed to the apical surface by P-gp and Bcrp, and Bcrp-mediated efflux was 3.5-fold higher than that of P-gp. Li et al. (2008) assessed the involvement of efflux transporters on TPT transport using engineered MDCK cells that overexpress P-gp (MDCKII/P-gp) or BCRP (MDCKII/BCRP). The intrinsic clearance (CLint) calculated by dividing Vmax by Km in MDCKII/BCRP cells was found to be higher than that in MDCKII/P-gp cells (0.43 ± 0.04 μl/cm2 per minute in MDCKII/P-gp and 0.60 ± 0.06 μl/cm2 per minute in MDCKII/BCRP), which also supports our results.

In conclusion, the use of portal vein–cannulated rats enables quantitative assessment of the contributions of intestinal efflux transporters to the oral absorption of three model drugs and to estimate the effect of selective inhibitors independently of variable systemic clearance. This experimental system is useful at the drug discovery stage for clarifying the cause of low bioavailability of new drug candidates.

Acknowledgments
The authors thank Kunihiro Morisaki (Charles River Laboratories Japan), Dr. Jiro Kuze (Taiho Pharmaceutical Co. Ltd.), and Dr. Toshiyuki Kume (Mitsubishi Tanabe Pharma Corporation) for useful discussions.

Authorship Contributions
 Participated in research design: Matsuda, Konno, Satsukawa, Yamashita.
 Conducted experiments: Matsuda, Konno, Hashimoto, Nagai, Taguchi.
 Performed data analysis: Matsuda.
 Wrote or contributed to the writing of the manuscript: Matsuda, Yamashita.

References
Agarwal S, Uchida Y, Mitapalli RK, Sane R, Terasaki T, and Elmquist WF (2012) Quantitative proteomics of transporter expression in brain capillary endothelial cells isolated from P-glycoprotein (P-gp), breast cancer resistance protein (Bcrp), and P-gp/Bcrp knockout mice. Drug Metab Dispos 40:1164–1169.
Assessment of the Impact of Intestinal Efflux Transporter


Address correspondence to: Dr. Yoshiki Matsuda, Pharmacokinetics and Safety Research Department, Central Research Laboratories, Kaken Pharmaceutical Co., Ltd., 14, Shinomiya Minamigawara-cho, Yamashina-ku, Kyoto, 607-8042, Japan.

E-mail: matsuda_yoshiki@kaken.co.jp