Transport of Rhodamine 123, a P-Glycoprotein Substrate, across Rat Intestine and Caco-2 Cell Monolayers in the Presence of Cytochrome P-450 3A-Related Compounds

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ABSTRACT
Effects of cytochrome P-450 3A- and P-glycoprotein (P-gp)-related compounds, erythromycin, midazolam, ketoconazole, verapamil, and quinidine, on transport of rhodamine 123 (Rho-123), a P-gp substrate, were studied in rat intestine and in Caco-2 cells. Ileum was mainly used in rat studies because this segment showed greater P-gp-mediated Rho-123 transport. In an in vitro everted rat ileum, all the compounds examined significantly inhibited the transport of Rho-123 from serosal to mucosal surfaces across the intestine, with different inhibitory potencies among these compounds. In an in vivo rat study, the exsorption of Rho-123 from blood to the intestinal lumen, which was evaluated as exsorption clearance of Rho-123 under a steady-state plasma concentration of Rho-123, was also inhibited when these compounds were added to the intestinal lumen. Similarly, transepithelial transport of Rho-123 from the basolateral to apical side across Caco-2 cell monolayers was inhibited by these compounds. A linear relationship was observed in their inhibitory potencies on Rho-123 transport between in vitro and in vivo studies using rat ileum and between studies with rat ileum and Caco-2 cells. P-gp-mediated transport across the intestine was found to be inhibited not only by P-gp-related but also by all the cytochrome P-450 3A-related compounds examined. Within experimental error, the relative inhibitory potencies were the same between the studies with rat ileum (in vivo, in vitro) and those with Caco-2 cells. Thus, it is suggested that the function of P-gp and its sensitivity to these drugs may be similar in rat intestine and Caco-2 cells.

It has been demonstrated that the intestinal P-glycoprotein (P-gp), an ATP-dependent multidrug efflux pump, can be an active secretion system or an absorption barrier by transporting some drugs from intestinal cells into the lumen (Terao et al., 1996; Tsuji and Tamai, 1996). Substrates transported by P-gp include a variety of structurally and pharmacologically unrelated, hydrophobic compounds such as some anticancer agents, steroid hormones, calcium channel blockers, immunosuppressing agents, β-blockers, and so on (Hunter et al., 1993; Wils et al., 1994; Terao et al., 1996). P-gp is also expressed in other normal human and rodent tissues, including the adrenal gland, kidney, liver, colon, brain, testis, and eye (Thiebaut et al., 1987; Holash and Stewart, 1993). In these tissues, P-gp is reported to prevent the accumulation of xenobiotics by active efflux.

The P-gp-mediated transport of a substrate is modified not only by other P-gp substrates but also by cytochrome P-450 (CYP) 3A-related compounds because there are some overlapping substrate specificities between CYP3A and P-gp (Wacker et al., 1995; Schuetz et al., 1996a, b). CYP3A enzymes occur in hepatocytes and intestinal epithelial cells (Paine et al., 1996; Thummel et al., 1996) and metabolize many clinically important drugs, such as some immunosuppressing agents, antibiotics, calcium channel blockers, and anticancer agents (Porter and Coon, 1991; Guengerich, 1992; Lown et al., 1994). Some of these CYP3A substrates are recognized as substrates or inhibitors of P-gp. The CYP3A-mediated metabolism is also readily inhibited by the presence of CYP3A substrates and inhibitors, such as erythromycin, ketoconazole, itraconazole, cimetidine, and grapefruit juice, resulting in increased oral bioavailability and changes in the pharmacokinetics of the substrates (Olkkola et al., 1994; Wrighton and Ring, 1994; Kupferschmidt et al., 1995; Ameer and Weintraub, 1997). These complicated drug-drug interactions between P-gp substrates or between a P-gp substrate and a CYP3A substrate are receiving considerable attention in clinical pharmacotherapeutics.

Prediction of these drug-drug interactions preclinically would be valuable information for clinical pharmacotherapeutics.
peptidases. However, there is only a limited number of studies on the in vivo function of P-gp in normal tissues such as the intestine and on the relationship between in vivo and in vitro P-gp function under various experimental conditions. In the present study, we examined and compared the effects of CYP3A- and P-gp-related compounds, erythromycin, midazolam, ketoconazole, verapamil, and quinidine, on the transport of rhodamine 123 (Rho-123), a P-gp substrate, in rat intestine (in vitro and in vivo) and in Caco-2 cell monolayers. Presently, midazolam is categorized as a CYP3A substrate and others are inhibitors/substrates of both CYP3A and P-gp. Rho-123 has been used as a marker to study the function of P-gp in various multidrug-resistant cells and various normal tissues including the intestine (Hsing et al., 1992; Lee et al., 1994). Caco-2, a human colonic adenocarcinoma cell line expressing various functions of differentiated intestinal epithelial cells, is useful in vitro system for studying the function of P-gp in the intestine (Hunter et al., 1993; Wils et al., 1994; Yee, 1997).

Experimental Procedures

Materials. Rho-123 was obtained from Kanto Chemical Co. (Tokyo, Japan). Verapamil hydrochloride and quinidine sulfate dihydrate were from Wako Pure Chemicals (Osaka, Japan). Erythromycin was from Merck (Darmstadt, Germany). Midazolam was from Nippon Roche (Tokyo, Japan). Ketoconazole was from Janssen Pharmaceutica N.V. (Beerse, Belgium). Cell culture medium and reagents were from Gibco Laboratories (Life Technologies Inc., Grand Island, NY). All other chemicals used were of the highest purity available.

In Vivo Exsorption Across Rat Ileum. Rats fasted overnight with free access to water before the experiments. The whole small intestine was flushed with 50 ml of ice-cold saline with the animal under anesthesia with pentobarbital (30 mg/kg i.p. injection). The rat was exsanguinated, and the small intestine isolated was divided into five segments of an equal length. Each segment was everted, and a 10-cm-long everted sac was prepared. Rho-123 was dissolved at a concentration of 5 μM in pH 7.4 isotonic Dulbecco’s PBS (D-PBS) containing 25 mM glucose and different concentrations of DMSO. The Rho-123 solution (1 ml) was introduced into the everted sac (serosal side), and both ends of the sac were ligated tightly. The sac containing Rho-123 solution was immersed into 40 ml of D-PBS containing 25 mM glucose and the same concentration of DMSO as that in the serosal side. The medium was prewarmed at 37°C and preoxygenated with 5% CO2/95% O2 for 15 min. Under bubbling with a CO2/02 mixture gas, the transport of various compounds was added to the mucosal medium to give a designated final concentration (25–200 μM). After a single perfusion for 20 min for stabilization, these biological fluids were further collected periodically for an additional 50 min as an inhibition phase. The perfusate was changed to the buffer containing an inhibitor (25–100 μM). For this calculation, the intestinal effluent, both sides to give a designated final concentration. The concentrations of DMSO in transport media used were 0% for verapamil and quinidine, 0.2% for DMSO for erythromycin, 0.5% for DMSO for midazolam, and 4% for DMSO for ketoconazole. The exsorption from blood to ileal lumen and the biliary and urinary excretions of Rho-123 were expressed as an exsorption clearance (CLexp, ml/min/20 cm ileum) and excretion clearances (CLinj, ml/min), respectively. For this calculation, the intestinal effluent, bile, and urine samples were collected every 10 min, and the sampling of blood was made at the intermediate time of each biological fluid collection. Total plasma clearance (CLpl, ml/min) of Rho-123 was estimated by dividing the infusion rate with a steady-state plasma concentration. It was also started at a rate of 1 ml/min. After a steady-state plasma Rho-123 concentration was achieved (50 min after the initiation of Rho-123 infusion), the intestinal effluent, blood, urine, and bile samples were collected at designated time intervals for 40 min as a control phase. Then, the perfusate was changed to the buffer containing an inhibitor (25–100 μM). After a single perfusion for 20 min for stabilization, these biological fluids were further collected periodically for an additional 50 min as an inhibition phase. The perfusate was changed to the buffer containing an inhibitor (25–100 μM). After a single perfusion for 20 min for stabilization, these biological fluids were further collected periodically for an additional 50 min as an inhibition phase. The perfusate was changed to the buffer containing an inhibitor (25–100 μM). After a single perfusion for 20 min for stabilization, these biological fluids were further collected periodically for an additional 50 min as an inhibition phase.

In Vitro Transepithelial Transport Across Caco-2 Cell Monolayers. The transepithelial transport of Rho-123 across cell monolayers, which were cultured in Transwell chamber (Costar, Cambridge, MA), in the absence or presence of an inhibitor was determined in the similar manner as reported previously (Nagai et al., 1995). Briefly, Caco-2 cells between passages 57 and 77 were cultured in a chamber for 16 to 21 days after seeding. Transepithelial electric resistance (TEER) of Caco-2 cell monolayers was monitored before transport studies using a Millicell ERS testing device (Millipore, Bedford, MA), and monolayers with TEER of more than 300 Ωcm2 were used for the transport studies. Rho-123 was dissolved at a concentration of 5 μM in D-PBS containing 25 mM HEPES, 25 mM glucose, and various concentrations of DMSO (pH 7.4). The drug solution was placed either on the apical (1.5 ml) or basolateral (2.6 ml) side, and the other side was filled with buffer solution containing the same concentration of DMSO. For inhibition study, each inhibitor was added to both sides to give a designated final concentration. The concentrations of DMSO in transport media used were 0% for verapamil and quinidine, 0.05% for erythromycin, 0.1% for midazolam, and 1% for ketoconazole. The transepithelial transport of Rho-123 in the presence or absence of an inhibitor was measured at 37°C periodically for 90 min.

Analysis. Concentrations of Rho-123 in various biological samples obtained from in vivo transport study were determined by HPLC with an instrument equipped with a fluorometric detector using a column of TSKgel ODS-80TM ( Tosoh, Tokyo, Japan). Mobile phase used was a mixture of acetonitrile and 1% acetic acid (40:60, v/v) at a flow rate of 1 ml/min. Detection was made at wavelengths of 485 nm for excitation and 546 nm for emission. Concentrations of Rho-123 in the transport media obtained from in vitro transport studies using everted sac and Caco-2 cells were determined fluorometrically.
with Hitachi fluorescence spectrophotometer F-3000 (Tokyo, Japan) because no metabolism of Rho-123 was observed under these experimental conditions.

Differences among group mean values were assessed by one-way ANOVA and/or Student's t test. A difference of $P < .05$ was considered statistically significant.

## Results

### Regional Differences in Rho-123 Transport Across Everted Rat Intestine In Vitro

The transport of Rho-123 from serosal to mucosal surfaces across everted rat intestine in the absence or presence of verapamil (300 $\mu$M), a potent P-gp inhibitor, was determined at different anatomic regions of rat small intestine. As typical examples, the time courses of Rho-123 transport across the everted duodenum and lower ileum are shown in Fig. 1. The transport of Rho-123 increased with time in a zero-order fashion after a lag time of approximately 10 min in both segments, and a greater transport was observed in the ileum. In both segments, the transport was significantly inhibited by verapamil. Thus, the expression of P-gp in rat intestine was functionally confirmed. The transport rate was calculated from the slope of cumulative Rho-123 amount with time (Fig. 2). Verapamil decreased the transport rate of Rho-123 to nearly the same extent in all regions. The difference in the transport rate between the absence and presence of verapamil can be regarded as P-gp-mediated transport of Rho-123. Thus, a significant regional variation was observed in functional P-gp along the length of rat small intestine.

**Effects of CYP3A-Related Compounds on Rho-123 Transport Across Everted Rat Ileum In Vitro**

As a typical example, the effect of midazolam, a CYP3A substrate, on Rho-123 transport across the everted rat ileum is shown in Fig. 3. The transport of Rho-123 from serosal to mucosal surfaces was inhibited by midazolam depending on its concentration in the mucosal medium. The inhibitory potencies of various inhibitors on Rho-123 transport were studied, and the results were expressed as the percentage of Rho-123 transport in the presence of an inhibitor relative to that in the absence of the inhibitor (Fig. 4). In these studies, various concentrations of DMSO (0–4%), which was used to increase the solubility of inhibitors in the mucosal and serosal media, were used among different inhibitors. However, no significant effect was found of DMSO on Rho-123 transport in the absence of inhibitors (data not shown). The transport of Rho-123 was significantly decreased in a dose-dependent fashion by all the CYP3A-related compounds examined.

### Effects of CYP3A-Related Compounds on Rho-123 Exsorption Across Rat Ileum In Vivo

Under a steady-state plasma concentration of Rho-123, ileum was perfused...
with D-PBS alone, followed by D-PBS containing an inhibitor in a single perfusion, to evaluate the effect of an inhibitor on exsorption from blood to the lumen and on biliary and urinary excretions of Rho-123. As a typical example, the effect of midazolam is shown in Fig. 5, in which each parameter is expressed as a clearance value. In the absence of an inhibitor in the lumen, the CL\textsubscript{total}, CL\textsubscript{exp}, CL\textsubscript{urine}, and CL\textsubscript{bile} of Rho-123 remained constant during the experimental period. By perfusing the ileum with midazolam, the plasma level of Rho-123 increased a little, and the CL\textsubscript{total} of Rho-123 was decreased proportionally. The decreased CL\textsubscript{total} of Rho-123 would be due, at least in part, to the decreased CL\textsubscript{exp}, CL\textsubscript{urine}, and CL\textsubscript{bile}, as shown in Fig. 5. Similar inhibitory effects on Rho-123 clearances were observed in all compounds examined. The effects on CL\textsubscript{exp} of Rho-123 are summarized in Fig. 6. As was the case for the in vitro everted sac study (Fig. 4), all compounds examined significantly reduced the in vivo CL\textsubscript{exp} value of Rho-123. The relationship was examined of their inhibitory potencies on Rho-123 transport between in vivo and in vitro studies. As shown in Fig. 7, a linear relationship was observed between them ($r = 0.947$, $P < .01$).

**Effects of CYP3A-Related Compounds on Rho-123 Transepithelial Transport Across Caco-2 Cell Monolayers In Vitro.** Inhibitory potencies of CYP3A-related compounds on Rho-123 transepithelial transport were evaluated using Caco-2 cells. We first confirmed that the transport of Rho-123 from the basolateral to apical side was significantly higher than that from the apical to basolateral side. In addition, the basolateral-to-apical transport of Rho-123 was reduced and the apical-to-basolateral transport was increased by verapamil and quinidine (100 $\mu$M). The transport of Rho-123 in the presence of these inhibitors was almost the same as was the case for the in vitro everted sac study (Fig. 4).

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**Fig. 4.** Concentration-dependent inhibitory potencies of ketoconazole (○), midazolam (△), erythromycin (□), quinidine (▲), and verapamil (■) on Rho-123 transport across the everted ileum. The concentration of Rho-123 in the serosal side was 5 $\mu$M. Each value represents the mean ± S.E.M. of three to six trials.

**Fig. 5.** Total plasma (CL\textsubscript{total}), exsorption from blood to lumen (CL\textsubscript{exp}), urinary excretion (CL\textsubscript{urine}), and biliary excretion (CL\textsubscript{bile}) clearances of Rho-123 in the absence (○) or presence (■) of luminal midazolam under a steady-state plasma concentration of Rho-123 in rats. Arrows denote the initiation of midazolam perfusion into the jejunal lumen at a concentration of 100 $\mu$M by a single perfusion at a rate of 1 ml/min. The plasma concentration of Rho-123 before perfusion of midazolam was approximately 0.2 $\mu$M. The solid line represents the mean of each clearance of three trials.

**Fig. 6.** Concentration-dependent inhibitory potencies of ketoconazole (○), midazolam (△), erythromycin (□), quinidine (▲), and verapamil (■) on Rho-123 exsorption clearance across rat ileum in vivo under a steady-state plasma concentration of Rho-123. Each inhibitor was perfused into a 20-cm-long ileum at different concentrations by a single perfusion at a rate of 1 ml/min. The plasma concentration of Rho-123 before perfusion of an inhibitor was approximately 0.2 $\mu$M. Each value represents the mean ± S.E.M. of three or four trials.

**Fig. 7.** In vitro versus in vivo inhibitory potencies of various compounds on Rho-123 transport across rat ileum. KTZ, ketoconazole; MDZ, midazolam; EM, erythromycin; QD, quinidine; VRP, verapamil. Each value represents the mean ± S.E.M. of three to five trials. The solid line represents the regression line: $y = 0.816x + 19.3\ (r = 0.947, P < .01, n = 7)$, where $x$ and $y$ denote the in vivo exsorption clearance (percent of control) and the in vitro transport of Rho-123 (percent of control), respectively.
in both directions (data not shown). These data indicate that P-gp is functionally expressed in these Caco-2 cells. We then studied the effect of midazolam on Rho-123 transport (Fig. 8). Midazolam significantly decreased the basolateral-to-apical transport of Rho-123, with an increase in the apical-to-basolateral transport. All other compounds also significantly reduced the basolateral-to-apical transport of Rho-123. Their inhibitory potencies on the basolateral-to-apical transport of Rho-123 were plotted against the concentrations of each inhibitor in the transport medium (Fig. 9). The plotting of their inhibitory potencies observed in rat ileum (in vivo) against those in Caco-2 cells showed a linear relationship (Fig. 10) \( r = 0.895, P < .01 \). Similarly, a linear correlation was also observed between the studies with in vitro rat ileum and those with Caco-2 cells \( r = 0.757, P < .05 \). The rank orders of inhibitory potencies were ketoconazole < erythromycin, midazolam, quinidine < verapamil.

### Discussion

Along the length of rat small intestine, the P-gp-mediated Rho-123 transport showed a regional variation, and the transport rate in the ileum was about 2.5- to 5.0-fold higher than other regions (Fig. 2). Our data are in good agreement with those of Trefzise et al. (1992), in which they reported that the expression of multidrug resistance MDR1 mRNA varies in rat intestine, with moderate expression in the duodenum and the jejunum, maximal expression in the ileum, and then a decrease in expression through the proximal and distal colon. Similar results have also been reported in male CF I mice (Chianale et al., 1995). The transport rate of Rho-123 from serosal to mucosal surfaces in the ileum was double that of the transport rate measured in the jejunum. The level of mdr3 mRNA and P-gp found in the ileum was 6-fold higher than that found in the duodenum.

The transport of Rho-123 in rat intestine was not completely inhibited even by the presence of verapamil at a concentration of 300 \( \mu \)M (Fig. 2), although Chianale et al. (1995) reported that the transport in mice was almost completely inhibited by the presence of verapamil at a concentration of 100 \( \mu \)M. Data similar to ours have been reported by Hsing et al. (1992) using male Sprague-Dawley rats. The mechanism for the transport of Rho-123 in the presence of such a high concentration of verapamil is not clear. However, in rat jejunum, the epithelial cell layers connected by tight junctions are relatively leaky with TEER of approximately 30 ohm-cm\(^2\) (Frömter and Diamond, 1972). Therefore, transport via the paracellular route may be involved in intestinal transport of Rho-123, which should be insensitive to verapamil.

All of the examined CYP3A-related compounds significantly reduced the exsorption of Rho-123 across the rat ileum. Similar inhibitory potencies were also observed in the jejunum in the present study (data not shown). Recently, we examined whether P-gp is involved in the transepithelial...
transport of midazolam, erythromycin, and ketoconazole themselves across Caco-2 cell monolayers. In that study, it was found that P-gp is involved in the transport of erythromycin, at least in part, but not of midazolam and ketoconazole (Takano et al., 1998). Thus, the interaction of such CYP3A-related compounds with P-gp can occur even if the compound is not a P-gp substrate.

In the present study, we measured the effect of various compounds on CL_{exp}, CL_{urine}, and CL_{bile} of Rho-123 in vivo. However, to evaluate the inhibitory potencies of these compounds, including CYP3A substrates, on CL_{urine} and CL_{bile} values of Rho-123 in a quantitative manner, many other factors, such as the absorption rate and the loss of the compound itself in the intestine and/or liver by metabolism (first-pass metabolism), should also be taken into consideration. In addition, as reported by Sweatman et al. (1990), metabolites of Rho-123 such as Rho-110 (a deacetylated metabolite of Rho-123) and/or its glucuronide conjugate were found in biological fluids except for luminal effluents, which makes the analysis more complex. Accordingly, in the present study, the effect of inhibitors on the CL_{exp} value of Rho-123 alone was evaluated as a function of an inhibitor concentration. The inhibitory potencies of various compounds on Rho-123 exsorption in vivo (Fig. 6) were almost the same as those observed in an in vitro study (Fig. 3). A linear relationship between in vitro everted sac and the in vivo studies shown in Fig. 7 suggests that the P-gp-related drug-drug interactions in vivo can be predicted by in vitro everted sac studies.

Recently, we determined the inhibitory effects of erythromycin, midazolam, and ketoconazole on P-gp-mediated transepithelial transport of Rho-123 in Caco-2 cells (Takano et al., 1998). A linear relationship in the inhibitory potencies of CYP3A- and P-gp-related compounds between rat ileum (in vivo, in vitro) and Caco-2 cells (Fig. 10) suggests that the drug-drug interactions related to P-gp-mediated transport in human intestine could be predicted by in vivo or in vitro transport study using rat ileum, as well as by Caco-2 cells. Yee (1997) also suggested that Caco-2 cells can be used to predict in vivo intestinal absorption of various compounds, including P-gp substrates in humans. However, the regression line between rat ileum and Caco-2 cells was not directly proportional; it had an intercept on the y-axis without passing through the origin (Fig. 10). The incomplete inhibition in rat ileum may be due, at least in part, to its leaky epithelia, in which paracellular transport of Rho-123 would occur as discussed previously. In contrast, the monolayers of Caco-2 cells used in the present study were less leaky (with TEER more than 200 ohm cm^{-2}), and transport via a paracellular route should be almost negligible, as reported previously (Harada et al., 1997). Under such conditions, P-gp-mediated transepithelial transport of Rho-123 in Caco-2 cells can be almost completely inhibited by potent P-gp inhibitors like verapamil and quinidine.

The present study analyzed primarily the exsorption or basolateral-to-apical transport of Rho-123 in the presence of inhibitors. However, as shown in Fig. 8, these inhibitors simultaneously increased the transport in the apical-to-basolateral direction of Rho-123 by inhibiting the P-gp function. The increase in the absorption of Rho-123 in the presence of inhibitors was also observed in vivo. For example, in the presence of midazolam at a concentration of 100 μM in the perfusate, the absorption clearance of Rho-123, which was estimated by the difference in Rho-123 concentrations between perfusate and intestinal effluent in a single perfusion, increased to 0.13 ± 0.03 ml/min/20 cm, whereas essentially no absorption of Rho-123 in the absence of an inhibitor was detected. Similarly, other inhibitors such as verapamil also increased the absorption of Rho-123 (data not shown), suggesting the possible enhancement of intestinal absorption of P-gp substrates by the coadministration of CYP3A-related compounds.

In conclusion, P-gp-mediated transport across the intestine was found to be inhibited not only by P-gp-related compounds but also by the CYP3A-related compounds examined. Within experimental error, the relative inhibitory potencies of these compounds were the same between the studies with rat ileum (in vivo, in vitro) and those with Caco-2 cells. Thus, it is suggested that the function of P-gp and its sensitivity to inhibitors may be similar in rat intestine and Caco-2 cells and that these experimental models (rat intestine, Caco-2 cells) may be useful to further study the function of P-gp in human intestine.

References


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