Relationship between Excretion Clearance of Rhodamine 123 and P-Glycoprotein (Pgp) Expression Induced by Representative Pgp Inducers

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P-Glycoprotein (Pgp) expression induced by representative Pgp inducers rifampicin (RFP), dexamethasone (DEX) and St John’s Wort (SJW) were examined in rat liver, intestine and kidney. After pretreatment with RFP (10 mg/kg/d) for 4 d, DEX (50 mg/kg/d) for 4 d or SJW (15 mg/kg/d) for 7 d orally, the biliary excretion of Rhodamine 123 after intravenous administration (0.2 mg/kg) increased significantly by 40%, 55% and 14%, respectively, and the intestinal excretion increased significantly by 24%, 50% and 27%, respectively, as compared with the controls. In contrast, there were no notable changes in the urinary excretion of Rhodamine 123 among rats that received these inducers. Western blot analysis with a monoclonal antibody for Pgp (C219) showed that Pgp levels in the small intestine and liver in the inducer-treated rats increased markedly as compared with the controls. In addition, there was a significant correlation between the induction levels of Pgp in the liver or small intestine and their clearance ratios (r²=0.7583, p<0.05), but not in the kidney. These observations suggest that the excretion clearances of Rhodamine 123 from blood circulation to the small intestine or to the bile after its intravenous administration are useful indicators to assess the Pgp function in the presence of Pgp inducers.

Key words P-glycoprotein; induction; rhodamine 123; pharmacokinetics

Among various factors that have been identified as being important regulators of oral availability, it has been postulated that the drug transporter P-glycoprotein (Pgp) and cytochrome P450 3A (CYP3A) are functionally linked components of a xenobiotic detoxification cascade, which limits the bioavailability of several drugs. The 170-kDa membrane efflux pump transporter Pgp is expressed constitutively in many normal human tissues, including major organs such as the liver, intestine, kidney, blood-brain barrier and other barrier epithelial tissues, where it plays an important role in the absorption, elimination and distribution of many xenobiotics including a wide range of therapeutic agents. On the other hand, numerous in vitro or in vivo methods using specific protein-overexpressing cell lines such as Caco-2 or LS180V cell lines, or specific Pgp inhibitors or mdr1 knockout mice to assess the contribution of transporters in regulating drug-drug interactions have been described. However, the results obtained using such methods cannot provide a complete understanding of the effects of Pgp on drug disposition in the healthy body, because the results of these methods were obtained under specific conditions and do not reflect the normal in vivo conditions. Recently, it has been noted that several Pgp substrates and/or inhibitors induce Pgp expression in several in vitro studies. However, relationships between the extent of induction effect of Pgp by inducers and the disposition of Pgp substrates were unclear. The fluorescent dye rhodamine 123 (Rho123) has been extensively used as an index of Pgp-mediated transport in rodents and tissue cultured models. In this study, we demonstrate the usefulness of Rho123 as a marker to detect the inducing effect of Pgp by drugs and the relationship between biliary, intestinal or urinary excretion of Rho123 via Pgp and its expression during treatment with the representative Pgp inducers rifampicin (RFP), dexamethasone (DEX) and St John’s Wort (SJW) in rats.

MATERIALS AND METHODS

Materials RFP was extracted from capsules commercially available as Rifadin® (Daiichi Pharmaceutical, Tokyo, Japan) and purified using an HPLC method to 97.6% purity. DEX was purchased from Wako Pure Chemicals (Tokyo, Japan). SJW tablets (contains 4% hyperforin per tablets) (Sato pharmaceuticals, Tokyo, Japan) was commercially available and used as SJW powder by crushing the tablets in a mill. N-Methyl-2-pyrrrolidone (Pharmasolve®) was kindly donated by International Specialty Products (Tokyo, Japan). A surfactant, polyoxyethylene (40) hydrogenated castor oil (HCO-40), was kindly donated by International Specialty Products (Tokyo, Japan). Rhodamine 123 was purchased from Sigma Chemical Co. (Steinheim, Germany). All other reagents were used of analytical grade and were used without further purification.

Animals All animal experiments were performed in accordance with the Guidelines for Animal Experimentation of Kyotanabe Medical College. Male Wistar rats about 10 weeks old (300±20 g) were obtained from Japan Clea Biomedical Co., Ltd. (Hamamatsu, Japan). They were housed individually for at least 3 d under controlled environmental conditions with free access to general food and water.

Preparation of Solutions Standard stock solutions of Rhodamine 123 were prepared by dissolving it in methanol at various concentrations and were stored at 4°C in the dark. Samples for the calibration curve for fluorimetric analysis were prepared by adding a known amount of these standard stock solutions to drug-free plasma in a volume ratio of 1:100. The test solution of RFP for oral administration was prepared
by suspending 100 mg of RFP which was prepared from Rifadin\textsuperscript{8} capsules in 10 ml of 1% (w/v) sodium carboxymethyl cellulose (CMC-Na) solution. The test solution of DEX for oral administration was prepared by suspending 500 mg of DEX in 10 ml of 1% (w/v) CMC-Na solution. The test solution of SJW for oral administration was prepared by suspending 150 mg of SJW in 10 ml of 1% (w/v) CMC-Na solution. The test solution of Rho123 for intravenous administration was dissolved in 2 mg of Rho123 with 10 ml of a mixture containing 5% (v/v) ethanol, 5% (w/v) HCO-40 and 5% (v/v) Pharmasolve\textsuperscript{7} at a final concentration of 0.2 mg/kg.

**Study Design** Rats were divided into four groups for the induction study. All groups were administered vehicle or inducers orally, and the study was carried out for a period of 7 d. Group 1 was administered only vehicle and this group served as the control. Group 2 rats received vehicle for the first 3 d, followed by RFP (10 mg/kg/d) from day 4 to 7; group 3, rats received vehicle for the first 3 d followed by DEX (50 mg/kg/d) from day 4 to 7; and group 4, rats received SJW (15 mg/kg/d) for 7 d. All groups of rats were fasted overnight on day 8 for the induction study and used for intravenous or excretion studies as described in the subsequent section. The doses of RFP and SJW were determined based on clinically used doses from reference data.\textsuperscript{19,20} The dose of DEX was determined based on the previous reports\textsuperscript{10,21} published elsewhere, and used as a positive control.

**Intravenous Administration** Each group of rats fasted overnight for at least 12 h with free access to water received an intravenous dose of Rho123 (0.2 mg/kg) by a bolus injection. Simultaneously, 250 \( \mu \)L aliquots of venous blood samples were collected in heparinized centrifuging tubes at designated times until 120 min. Plasma samples were obtained by centrifuging blood samples at 9000 \( \times g \) for 10 min at 4 °C. All samples were stored at 4 °C after collection.

**Biliary Excretion after Intravenous Administration of Rho123** Each group of rats that had fasted for at least 12 h with free access to water were anesthetized with an intraperitoneal injection of sodium pentobarbital (32 mg/kg). The rats were placed in a supine position on a heating pad under a surgical lamp to maintain normal body temperature. After midline longitudinal abdominal incision, a polyethylene cannula (0.5 mm i.d., 0.8 mm o.d.) was placed in the bile duct. Rho123 (0.2 mg/kg) was intravenously administered by a bolus injection. Then, bile was collected at designated intervals over 120 min. All samples were stored at 4 °C after collection.

**Excretion Study of Rho123 Using in Situ Perfusion Method** Each group of rats fasted overnight for at least 12 h on day 15 was anesthetized with an intraperitoneal injection of sodium pentobarbital (32 mg/kg). They were placed in a supine position on a heating pad under a surgical lamp to maintain constant normal body temperature. The upper jejunum (15 cm) of the intestinal lumen was catheterized with an inlet silicon tube (4.2 mm i.d.). This was perfused with phosphate buffered saline containing 25 mM glucose into the intestinal lumen in a single perfusion manner at a flow rate of 0.5 ml/min. After 30 min of perfusion for stabilization, 0.2 mg/kg of Rho123 was injected via the jugular vein. Simultaneously, the intestinal perfusate was collected every 15 min over 120 min. All samples were stored at 4 °C after collection.

**Assay Methods** The determination of Rho123 concentrations in the samples was performed immediately after the experiment. The perfusate, bile and plasma samples were stored at 4 °C after the collection. For plasma samples, an equal volume of methanol was added to 100 \( \mu \)L aliquots of plasma to precipitate proteins. The mixture was vortexed for 30 s and centrifuged at 9000 \( \times g \) for 10 min at 4 °C. One hundred microliter aliquots of the supernatant were then added to 96-well microplates, and Rho123 concentrations in the samples were analyzed using a Fluoroskan Acent CF (Dainippon-Pharmaceutical Co., Tokyo, Japan) with fluorimetric detection, with an excitation wavelength of 485 nm and an emission wavelength of 527 nm. A aliquots (100 \( \mu \)L) of both perfusate and bile samples were loaded directly on 96-well microplates and Rho123 was analyzed as described above.

**Preparation of Crude Intestinal, Hepatic and Renal Membrane Fractions** Crude membrane fractions were prepared from the intestinal mucosa or liver of untreated or inducer treated rats, according with manufacturer’s instructions with some modifications. Briefly, the luminal contents of the whole small intestine were isolated and flushed with phosphate-buffered saline. The intestine was opened and laid on a chilled glass plate. Intestinal tissue was collected by scraping with a slide glass and homogenized by adding T-PER (Pierce, Rockford, IL, U.S.A.) containing 1 mM phenyl-methylsulfonylfluoride (PMSF) 20 mL/g tissue. For the hepatic crude membranes, 1 g of liver or kidney was homogenized in 20 mL of T-PER containing 1 mM PMSF. These homogenates were centrifuged for 15 min at 3000 \( \times g \), and then the resulting supernatant was centrifuged at 27000 \( \times g \) for 30 min. The pellets were resuspended in buffer containing 300 mM mannitol and 40 \( \mu \)g/mL PMSF (pH 7.5). Protein concentrations in the crude membrane suspensions were determined with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, U.S.A.) with bovine serum albumin as the standard. Samples were normalized to equal protein concentrations, loaded (10 \( \mu \)g/lane, depending on tissue) onto 7.5% SDS-gel, electrophoresed at 200 V for 1 h, and then transferred onto polyvinylidene difluoride membranes at 100 V for 1 h. Blots were blocked for nonspecific binding by overnight incubation (4 °C) on a rotator in Tris-buffered saline buffer containing 0.1% Tween 20 and 5% dry fat milk. The polyvinylidene difluoride membranes were then washed three times with Tris-buffered saline containing 0.1% Tween 20, followed by one washing with Tris-buffered saline and then incubated with C219 (Calbiochemi, CA, U.S.A.) for 1 h at 37 °C. The antibody C219 was diluted in Tris-buffered saline. After incubating with secondary antibody (anti-mouse IgG, Immune-Star HRP chemiluminescent kit, Bio-Rad Lab., U.S.A.) for 45 min, the stained bands were detected and quantified using a Dolphin-chemi (Wealtec Corp., GA, U.S.A.) for 45 min, the stained bands were detected and quantified using a Dolphin-chemi (Wealtec Corp., GA, U.S.A.) to quantify the relative levels of Pgp in each gel.

**Data Analysis** A noncompartmental pharmacokinetic analysis was applied to the plasma concentration vs. time data using the computer program WinHARMONY.\textsuperscript{22} The area under the plasma concentration vs. time curve after intravenous administration of Rho123 was calculated using the linear trapezoidal rule up to the last measured plasma concentration (\( AUC_{t_{\infty}} \)) and extrapolated to infinity (\( AUC_{0-\infty} \)). The terminal elimination half-life (t\(_{1/2}\)) was determined by dividing ln2 by the elimination rate constant at the terminal.
RESULTS

Figure 1 shows the plasma concentration of Rho123 after intravenous administration to the control and inducer-treated rats. The pharmacokinetic parameters of Rho123 are listed in Table 1. DEX-treated rats had significantly decreased plasma concentrations of Rho123 and the \( \text{AUC}_{\text{last}} \) of Rho123 in DEX-treated rats decreased significantly from 53.93 to 41.91 ng·h/ml, while in RFP- or SJW-treated rats, the \( \text{AUC}_{\text{last}} \) showed no changes as compared with the control. There were no significant changes in the value of \( \text{CL}_{\text{tot}} \) in the RFP- and SJW-treated groups. However, the \( \text{CL}_{\text{tot}} \) in the DEX group tended to decrease compared with the control group \((p<0.1)\). Figure 2 shows the biliary, intestinal and urinary excretion of Rho123 after intravenous administration to the control and inducer-treated rats. The excretion clearances of Rho123 are listed in Table 2. The excreted amounts of Rho123 over 120 min in the inducer-treated rats were significantly increased in the bile and intestine. The excreted amounts of Rho123 over 120 min in the bile of the control, RFP-, DEX- and SJW-treated rats were 2.23±0.06 to 3.42±0.09, 2.89±0.10 and 2.66±0.12 µg, respectively (Fig. 2a), and those in the intestine were 0.77±0.03 to 0.89±0.04, 0.91±0.04 and 0.93±0.09 µg, respectively (Fig. 2b). While in the urine, there were no notable changes in the total excreted amounts of Rho123 over 120 min in the control, RFP-, DEX- and SJW-treated rats (Fig. 2c). Figure 3 shows the effects of inducers on the Pgp expression protein levels in the intestine, liver and kidney, as assessed using Western blot analysis. In the intestine, Pgp protein levels after treatment with DEX, RFP and SJW increased by 2.9-, 1.6- and 1.7-fold, respectively (Fig. 3a), and in the liver increased by 1.9-, 1.2- and 1.3-fold, respectively (Fig. 3b). However there were no notable changes in Pgp expression observed in kidney, using any inducer treated rats (Fig. 3c). Figure 4 shows the relationship between clearance ratios of Rho123 and Pgp expression levels during treatment of DEX, RFP or SJW in the liver (Fig. 4a), intestine (Fig. 4b) and kidney. Significant positive correlations were observed between the clearance ra-

Fig. 1. Effects of Repeated Oral Doses of Pgp Inducers on Plasma Rho123 Concentration vs. Time Curves after Intravenous Administration (0.2 mg/kg) to Rats

Each group was pretreated with oral DEX (50 mg/kg/d), RFP (10 mg/kg/d) and SJW (15 mg/kg/d) for indicated days (see the text). Each symbol with a bar represents the mean±S.E. of 8 rats. ○, control; ●, DEX, ▲, RFP, ■, SJW.

Table 1. Pharmacokinetic Parameters of Rho123 after Intravenous Administration to Rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>( \text{AUC}_{\text{last}} ) (ng·h/ml)</th>
<th>( t_{1/2} ) (h)</th>
<th>( \text{CL}_{\text{tot}} ) (ml/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>53.93±0.99</td>
<td>2.32±0.23</td>
<td>536.76±23.97</td>
</tr>
<tr>
<td>RFP</td>
<td>52.06±1.01</td>
<td>2.27±0.13</td>
<td>564.61±22.17</td>
</tr>
<tr>
<td>DEX</td>
<td>41.91±0.98*</td>
<td>3.15±0.25</td>
<td>488.69±32.01</td>
</tr>
<tr>
<td>STJ</td>
<td>51.82±1.99</td>
<td>2.63±0.37</td>
<td>508.52±40.79</td>
</tr>
</tbody>
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** \( p<0.01 \), compared with appropriate control. Each value represents the mean±S.E. of 8 rats.
tios and Pgp expression levels in both tissues. The coefficient of determination ($r^2$) using the results obtained in the liver (Fig. 4a) was 0.961 ($p<0.02, n=4$) and in the intestine (Fig. 4b) 0.969 ($p<0.02, n=4$). Moreover, there was a significant positive correlation between the clearance ratios and induction levels of Pgp using all data obtained both in the liver and intestine ($r^2 = 0.7583$, $p<0.05$). On the other hand, using data obtained from the kidney, there was no correlation between the clearance ratios of Rho123 and induction levels of Pgp.

**DISCUSSION**

The MDR1 (mdr1) gene product Pgp has been demonstrated to be an important determinant of the pharmacokinetics of some lipophilic compounds in various body tissues.\(^2\)\(^-\)\(^5\) The fluorescent dye Rho123 has been extensively used as an index of Pgp-mediated transport in rodents and tissue cultured models.\(^8\)\(^-\)\(^18\) Several transporters other than expressed in the rat intestinal tract, for example, multidrug resistance associated protein (MRP), organic cation transporters (OCTs) and breast cancer resistance protein (BCRP).
Dogan et al. 23) studied the relationship between MRP1 expression and transport of 3 fluorescent dyes, calcine, alizarin and carboxyfluorescein diacetate (CFDA) and Rho123 in combination with the MRP1 modulators cyclosporine A, probenecid and MK571, using 11 cell lines with different levels of MRP1 expression. A good correlation was found between MRP1 expression and calcine-AM or CFDA but not with Rho123. 23) These data indicate that Rho123 makes a minimal contribution to MRP1 in those models. Other transporters, Rho123 is not transported by BCRP, 23 while there is evidence that OCTs carry it. 25) However, the contribution of OCTs in the transport of Rho123 in vivo is unclear. Therefore, to evaluate the usefulness of Rho123 as an indicator to assess the Pgp-inducing conditions in vivo, we focused on the transport of Rho123 via Pgp with several known inducers. A significant relationship between clearance ratios and Pgp expression indicates that Rho123 is a useful indicator to evaluate Pgp function in vivo. In this study, we determined Rho123 levels in samples using a microplate reader for our experimental convenience. It has been well known that Rho123 is metabolized by intestinal esterase, and some metabolites of Rho123 also have fluorescence intensity. However, Tian et al. reported that the measurements of Rho123 using a spectrofluorometer were as same as those using HPLC method in a transport study using the rat everted-sac method. 26) In the everted-sac method, the intestine was washed many times with buffer and the surfaces were in direct contact with incubation buffer containing only Rho123. The in situ perfusion method we used here also has much less effects of intestinal esterase on Rho123 degradation because the intestinal lumen is constantly perfused with buffer solution, and samples were measured immediately. It is, therefore, considered that intestinal esterase has much less effect on the fluorescence intensity of Rho123.

We also determined the relationship between measurements using the microplate reader and those using an HPLC method. There was a significant one-to-one correlation between measurements from the microplate reader and HPLC method when intravenous samples were analyzed. Therefore the results of Rho123 measurements using the microplate reader method did not reflect the effects of Rho123 metabolites. As shown in Fig. 1, plasma concentrations of Rho123 after intravenous administration showed a significant decrease in DEX-treated rats, whereas the values of \( CL_{tot} \) tended to decrease without any significant change compared with the control. Because the oral dose of DEX was much higher than the clinically relevant dose, DEX administration significantly reduced the body weight of rats by 20% compared with the controls. Therefore it may be considered that the volume of distribution of Rho123 in the DEX-treated rats decreased even though the \( AUC \) decreased. Essentially, the pharmacokinetic parameters after intravenous administration reflect mainly the contribution of the liver, and all inducers studied here induce a major liver catalyst, CYP3A. Therefore the lack of any significant change in the \( CL_{tot} \) of Rho123 after intravenous administration in the control and inducer-treated rats suggests that the metabolism of Rho123 via CYP3A can be disregarded.

As shown in Fig. 2, different aspects were found in the portion where Pgp locates. Contrary to the case of the blood circulation system, the biliary and intestinal excretion of Rho123 increased significantly in the inducer-treated groups compared with the controls. It has been reported that the oral administration of digoxin (a well-established Pgp substrate) after concomitant administration of ritonavir (a well-established inhibitor of CYP3A and Pgp) increased the systemic availability of digoxin in healthy volunteers. 27,28 As one possible mechanism, a reduction in the non-renal clearance of digoxin was most likely through the inhibition of hepatic Pgp by ritonavir. 27 On the other hand, the fact that there was no change in the urinary excretion amount of Rho123 indicates that there is a site specific difference in the induction of Pgp by inducers, and that Pgp that locates in the kidney could not reflect an increase in the non-renal clearance of Rho123. These observations are supported by the fact that digoxin is primarily eliminated by passive filtration in the kidney with Pgp-mediated excretion; however, this excretion process has a relatively minor role in the pharmacokinetic alteration. 29) The intestinal and biliary excretion of Rho123 in our study showed inducer-dependent increases without changes in \( CL_{tot} \) suggesting that non-renal clearance of Rho123 via Pgp was increased by inducers.

In this study a Western blot analysis was performed to determine the induction potency of inducers in vivo. One possible method of estimating the induction potency is measuring mRNA levels. However, the relationship between mRNA and protein levels has not been established yet, and using mRNA levels alone may lead to misreading the contribution of Pgp for the pharmacokinetics of drugs in the inductive condition. In our data, both in the intestine and liver Pgp expression was increased upon treatment with inducers, but that in the kidney was not. These results were essentially in accordance with the results of the Rho123 excretion study in vivo. In addition, judging from the induction of Pgp expression found in Western blot analysis, the Pgp expression levels in the intestine were greater than that in the liver or kidney. The reason for these phenomena is that the intestine is exposed with a higher concentration of inducer in comparison with the liver when the inducers are administered orally. Therefore the intestinal Pgp is considered more important to determine the oral bioavailability of substrate drugs mediated by Pgp than the hepatic Pgp. Supporting this, the Pgp inducers investigated here provided no marked alteration in the value of \( CL_{tot} \) of Rho123 after intravenous administration.

In the study on the drug elimination via Pgp, it is important issue to quantitatively grasp the excretion mechanism via Pgp in vivo from the in vitro screening using established cell lines such as Caco-2 cells. However, sometimes the results of in vitro screening, i.e., in the screening of Pgp using the Caco-2 cell monolayer, the culture condition or passage number of cell cultures yielded different results among investigators. Moreover, Hennessy et al. reported that the magnitude of the interaction via Pgp seen in clinical reports is greater than that predicted by in vitro data. 30) Therefore establishing a technique for evaluating Pgp function in vivo would be useful for predicting the degree of drug-drug interaction via Pgp in the living body. From this perspective, we examined the relationship between Pgp expression and excretion clearance ratio of Rho123 using the data obtained from the induction study. As shown in Fig. 4, the relationship between Pgp expression and
excretion clearance ratio of Rho123 using data from the intestinal and hepatic excretion study provided a good correlation. However, when including the data obtained from the urinary excretion of Rho123, that linear relationship was diminished. It is well known that the main elimination route of Rho123 is urinary excretion, and in this study, the amount of Rho123 excreted in the urine was 2- to 9-fold higher than that in the intestine and liver. However, none of the inducers induced Pgp expression in the kidney. Although the reason why Pgp in the kidney was not induced is unclear at present, there seems to be organ-specific expression of Pgp by each inducer. Nevertheless, further experiments are required to elucidate the underlying mechanism of Pgp expression and the contribution of other transporters during treatment with inducers.

In conclusion, measuring the excretion clearance ratios of Rho123 from the blood circulation to the small intestine or to the bile after intravenous administration is useful to assess the Pgp function in the presence of Pgp inducers. This technique enables a quantitative assessment in vivo of the drug–drug interaction between Pgp substrates and inducers.

REFERENCES