PHARMACOKINETIC INTERACTION OF CYTOCHROME P450 3A-RELATED COMPOUNDS WITH RHODAMINE 123, A P-GLYCOPROTEIN SUBSTRATE, IN RATS PRETREATED WITH DEXAMETHASONE

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ABSTRACT:
The effect of pretreatment with dexamethasone (DEX) on drug-drug interactions between rhodamine 123 (Rho123), a P-glycoprotein (P-gp) substrate, and midazolam, a cytochrome P450 (CYP) 3A substrate, or verapamil, a P-gp/CYP3A substrate, was studied in rats. Rats were pretreated with DEX (100 mg/kg/day, oral) for 2 days. Western blot analysis with a monoclonal antibody for P-gp, C219, revealed that DEX pretreatment increased P-gp level in the intestine 1.9-fold, but not in the liver. In vitro metabolism study of erythromycin in microsomal suspensions indicated the 9.7-fold increase of CYP3A activity in the liver, but not in the intestine, by DEX pretreatment. In an in vivo study, DEX pretreatment increased P-gp-mediated exsorption clearance of Rho123 from blood to the intestinal lumen approximately 2-fold, but not biliary clearances, in good agreement with the results of Western blot analysis. In untreated rats, midazolam (100 μM) or verapamil (30 or 100 μM) added in the intestinal perfusate (single perfusion) decreased the exsorption clearance and biliary clearance of Rho123 by approximately 30 to 50%. In DEX-pretreated rats, however, the inhibitory potency of midazolam in the liver significantly decreased compared with that in untreated rats, although the potency in the intestine did not change. The inhibitory potency of verapamil decreased both in the intestine and liver by DEX pretreatment. In conclusion, it was demonstrated that DEX pretreatment affects not only P-gp-mediated disposition of Rho123 but also pharmacokinetic interactions of P-gp/CYP3A-related compounds with Rho123, probably because concentrations of substrates/inhibitors at target sites such as the intestine and liver are varied.

P-Glycoprotein (P-gp), an ATP-dependent efflux pump, is widely expressed in normal human and rodent tissues (Thiebaut et al., 1987). This protein transports a variety of structurally and pharmacologically unrelated hydrophobic compounds, such as some anticancer agents, steroid hormones, calcium channel blockers, and so on (Wacher et al., 1995; Schuetz et al., 1996). Along with a variety of metabolic enzymes, P-gp is now recognized as an important host defense mechanism for a living body by preventing the influx and/or accumulation of various P-gp substrates in the tissues, including the intestine, brain, and eye (Terao et al., 1996; Tsuji and Tamai, 1996; Kajikawa et al., 1999, 2000).

In humans, many clinically important drugs, such as immunosuppressive agents, antibiotics, calcium channel blockers, and anticancer agents, are metabolized by the cytochrome P450 (CYP) 3A subfamily. This work was supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture in Japan and by a grant from The Naito Foundation.

1 Abbreviations used are: P-gp, P-glycoprotein; CLbile, biliary excretion clearance; CLint, intestine excretion clearance; CLapp, apparent clearance; CLtot, total plasma clearance; Cmax, maximum plasma concentration; CYP, cytochrome P450; DEX, dexamethasone; DMEM, dimethyl sulfoxide; Rho123, rhodamine 123; mdr, multidrug resistance gene; MRP, multidrug resistance-associated protein.

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The major class of phase I drug-metabolizing enzymes (von Molite et al., 1995; Wilkinson, 1996). The CYP3A subfamily, especially CYP3A4 in humans, exists in hepatocytes and small bowel enterocytes and reduces the oral bioavailability of these CYP3A substrates by the first-pass metabolism (Paine et al., 1996, 1997; Wilkinson, 1996). Most of these CYP3A substrates are also recognized as substrates or inhibitors of P-gp because of the strong overlapping substrate specificities between P-gp and CYP3A (Wacher et al., 1995; Schuetz et al., 1996). Such an overlap results in the modulation of the oral bioavailability and subsequent changes in the pharmacodynamics of various P-gp/CYP3A-related drugs, when they are administered concomitantly. Regarding the overlapping, we also demonstrated that the efflux transport of rhodamine 123 (Rho123), a P-gp substrate, across rat intestine and Caco-2 cell monolayers was greatly inhibited by a CYP3A substrate (midazolam) or inhibitors/substrates of both CYP3A and P-gp (erythromycin, ketoconazole, verapamil, and quinidine) (Takano et al., 1998; Yumoto et al., 1999). In addition to the pharmacokinetic drug-drug interactions between P-gp substrates and CYP3A-related compounds, a concomitant induction of P-gp and CYP3A by compounds such as dexamethasone (DEX) is also reported in vivo and in cultured cells (Wacher et al., 1995; Schuetz et al., 1996; Salphati and Benet, 1998; Lin et al., 1999).

The importance of intestinal P-gp, in addition to intestinal CYP3A, in limiting the oral bioavailability of P-gp/CYP3A-related compounds and/or in interpreting the intersubject variation of oral bioavailability is now well recognized (Terao et al., 1996; Tsuji and Tamai, 1996; Wacher et al., 1998). Lown et al. (1997) reported that intestinal P-gp,
rather than the intestinal CYP3A, is an important factor responsible for the interpatient variation in oral bioavailability of cyclosporin, a substrate of both P-gp and CYP3A. Also, there is much literature describing the effect of inducer treatments on the expression of P-gp protein and/or its mRNA (Herzog et al., 1993; Salphati and Benet, 1998; Sérée et al., 1998; Demeule et al., 1999). In contrast, however, studies analyzing the effect of inducers on in vivo P-gp function are quite few. Recently, Hebert et al. (1999) reported that the clearance of tacrolimus, a P-gp/CYP3A substrate, given intravenously increased and its oral bioavailability decreased after rifampin dosing for 18 days in healthy volunteers, probably due to the induction of P-gp and CYP3A in the liver and small intestine. Lin et al. (1999) estimated separately the hepatic and intestinal first-pass metabolism of indinavir, a P-gp/CYP3A substrate, in normal and DEX-pretreated rats and suggested the contribution of increased intestinal P-gp level in the increased intestinal metabolism of indinavir.

In the present study, we pharmacokinetically evaluated the effect of DEX pretreatment on P-gp-mediated intestinal exsorption and biliary clearances of Rho123 and drug-drug interactions of midazolam or verapamil with Rho123 in rats under in vivo conditions, in addition to the analysis of P-gp expression and in vitro CYP3A activity. Rho123 has been widely used as a marker to study the function of P-gp in various multidrug-resistant cells and various normal tissues (Lee et al., 1994; Scala et al., 1997), and the selective binding site of Rho123 has been widely used as a marker to study the function of P-gp in various multidrug-resistant cells and various normal tissues (Lee et al., 1994; Scala et al., 1997), and the selective binding site of Rho123. Because Rho123 is not a substrate of CYP3A, it would be more suitable for evaluating in vivo P-gp function than P-gp/CYP3A substrates.

Experimental Procedures

Materials. Rho123 was obtained from Acros Organics (Geel, Belgium). Cyclosporin A (CsA) and midazolam were kindly supplied by Novartis (Tokyo, Japan) and Nippon Roche (Tokyo, Japan), respectively. DEX and verapamil hydrochloride were purchased from Wako Pure Chemicals (Osaka, Japan). A monoclonal antibody for P-gp, C219, was from Signet Laboratories, Inc. (Dedham, MA), and a secondary antibody, peroxidase-labeled affinity-purified antibody to mouse IgG (H + L), was from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). All other chemicals used were of the highest purity available.

Animals and Treatment. DEX was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 200 mg/ml. Male Wistar rats weighing 230 to 300 g were given DEX at a dose of 100 mg/kg daily for 2 consecutive days by stomach intubation. Untreated (control) rats received an equal volume of vehicle alone (DMSO, 0.5 ml/kg). These rats were subjected to in vivo and in vivo studies 24 h after the last dose of DEX. Experiments with animals were performed in accordance with the Guide for Animal Experimentation from Hiroshima University and the Committee of Research Facilities for Laboratory Animal Sciences, Faculty of Medicine, Hiroshima University (Hiroshima, Japan).

Preparation of Crude Intestinal and Hepatic Membrane Fractions. Crude membrane fractions were prepared from the intestinal mucosa and liver of untreated and DEX-pretreated rats. Briefly, the luminal contents of whole small intestine were thoroughly washed out with a sufficient amount of ice-cold saline, and the intestine was divided into two parts of the same length. The mucosal surface of the lower half of intestine was scraped off with a cover glass. The intestinal mucosa collected and the isolated liver were homogenized in a buffer containing 250 mM sucrose and 5 mM HEPES-Tris (pH 7.4) with a Polytron tissue homogenizer (IKA, Labortechnik, Staufen, Germany). The homogenates were centrifuged at 3,000 rpm for 10 min. The supernatants were then centrifuged at 24,000 rpm for 30 min. The pellets containing the crude membrane fractions were resuspended in 50 mM mannitol and 20 mM HEPES-Tris (pH 7.5). The suspension samples were used immediately after preparation or stored in liquid nitrogen until use.

Determination of P-gp Levels in Crude Intestinal and Hepatic Membrane Fractions. The amount of P-gp in the crude intestinal and hepatic membrane fractions was evaluated by Western blot analysis after SDS-polyacrylamide gel electrophoresis. Briefly, membranes suspended in sample buffer (50 mM mannitol and 20 mM HEPES-Tris, pH 7.5) were diluted to a final protein concentration of 4 mg/ml with loading buffer (62.5 mM Tris-HCl, 2% SDS, 7% glycerol, 0.005% bromophenol blue, pH 6.8) and were loaded at a volume of 20 μl (corresponding to 80 μg of protein) on 7.5% acrylamide-bisacrylamide gels without prior heating. After SDS-polyacrylamide gel electrophoresis, the proteins were transferred electrophoretically onto a 0.45-μm pore size polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). Blots were blocked overnight at 4°C with Tris-buffered saline/ Tween (0.15 M NaCl, 0.05% Tween 20, and 20 mM Tris-HCl, pH 7.5) containing 5 mM sodium azide and 5% (w/v) nonfat powdered milk. The polyvinylidene difluoride membranes were washed three times with Tris-buffered saline/Tween 20, followed by one wash with Tris-buffered saline (0.15 M NaCl and 20 mM Tris-HCl, pH 7.5), and then incubated with C219 (10 μg/ml) for 2 h at 37°C. The antibody C219 was diluted in Tris-buffered saline containing 0.2% (w/v) bovine serum albumin. Peroxidase-labeled affinity-purified antibody to mouse IgG was used as the secondary antibody. Detection was made with enhanced chemiluminescence reagents according to the manufacturer’s instructions (Amersham Pharmacia Biotech, Buckinghamshire, England). The blots were exposed to Hyperfilm enhanced chemiluminescence (Amersham Pharmacia Biotech). The optical densities of immunoreactive protein were estimated by a computer-aided densitometer with NIH Image (the public domain program developed at the U.S. National Institutes of Health, Bethesda, MD). A linear relationship was confirmed between the optical density of immunoreactive protein (P-gp) and the amount of membrane proteins loaded on the gel, at least, in a range from 20 to 100 μg of protein. The P-gp levels observed in the present study were all in this range of a linear relationship. Molecular mass determination was performed over a broad range of protein markers (New England Biolabs, Inc., Beverly, MA).

Determination of CYP3A Activity in Intestinal and Hepatic Microsomes. The CYP3A activity in the intestine and liver was evaluated by measuring metabolism velocity of erythromycin in tissue microsomal suspensions. The preparation of hepatic microsomal suspension was carried out in the same manner as reported previously (Higashikawa et al., 1999b). Briefly, the 105,000g pellet of supernatants obtained after centrifugation of liver homogenates at 18,000g for 15 min was resuspended in solution A (10 mM HEPES-NaOH buffer containing 150 mM KCl, pH 7.4). The preparation of intestinal microsomal suspension was carried out according to the reported method, with a small modification (Weiser, 1973; Bonkovsky et al., 1985). Briefly, the upper half of the intestine was isolated and twice rinsed thoroughly with 50 ml of ice-cold solution B (phosphate buffer containing 96 mM NaCl, 1.5 mM KCl, and 27 mM sodium citrate, pH 7.3) containing 0.5 mM dithiothreitol and 1.5 mM disodium EDTA. Then intestinal lumen was filled with ice-cold solution C (5 mM histidine, 0.25 M sucrose, 40 μM phenylmethylsulfonyl fluoride, 0.5 mM disodium EDTA, pH 7.0). The sac containing solution C was placed on a glass plate on ice and gently tapped with fingers. The luminal fluid was collected, and the procedure was repeated three times. Luminal fluids were all combined and centrifuged at 800g for 10 min. Precipitated cells were washed with 20 ml of solution C by repeating the centrifugation and were resuspended in 5 ml of solution C and homogenized with a loose-fitting Dounce homogenizer by 75 up-and-down hand strokes. The homogenate was centrifuged at 15,000g for 10 min, and the supernatant was collected in a centrifuge tube by decantation. Ice-cold 52 mM CaCl2, was added to the supernatant to make a final concentration of 10 mM, and the tube was allowed to stand on ice for 15 min. It was then centrifuged at 2,000g for 10 min. The pellet was resuspended in solution A.

Microsomal protein suspension (0.2 ml, 2.5–5 mg of protein/ml) was preincubated at 37°C for 30 s. MgCl2 (0.625 mM), NADPH (2.5 mM), and erythromycin (1 mM) were dissolved in solution A. This mixture (0.8 ml), prewarmed at 37°C, was added to microsomal suspension to initiate the metabolic reaction. The enzyme reaction was performed for 5 to 30 min and was terminated by adding 5 M KOH solution (1 ml). Formaldehyde, a product of erythromycin, was measured with a commercially available kit, Formaldehyde-Test Wako (Wako Pure Chemicals).

In Vivo Clearance Study of Rho123. The in vivo clearance study of Rho123 was performed in a manner similar to that in our previous study (Yumoto et al., 1999). Briefly, untreated and DEX-pretreated rats were fasted.
overnight and were anaesthetized with sodium pentobarbital (30 mg/kg, i.p.). Rats were fixed in a supine position on a surface kept at 37°C to maintain normal body temperature. Cannulation (polyethylene tubing, PE-50) was made at a femoral vein for the administration of Rhod123, and at a femoral artery and a bile duct for collecting blood and bile samples, respectively. The proximal and distal ends of whole intestinal lumen were catheterized with a silicone cannula (outer diameter, 5 mm; inner diameter, 3 mm) to perfuse the intestinal lumen with Dulbecco’s phosphate-buffered saline (pH 7.4) containing 25 mM glucose in a single perfusion manner at a rate of 1 ml/min. Rhod123 was dissolved at a concentration of 100 μM in 5% mannitol. The Rhod123 solution was injected via a cannula inserted at a femoral vein as a bolus with a volume of 4.36 ml/kg, followed by constant infusion at a rate of 2 ml/h to attain a steady-state plasma concentration (C_{SS}) of Rhod123. After C_{SS} of Rhod123 was achieved (55 min after the initiation of Rhod123 infusion), the intestinal effluent, blood, and bile were collected at 10-min intervals for 40 min as a control phase. In some cases, rats were sacrificed at the end of control-phase clearance study and the liver excised to measure hepatic Rhod123 concentration. In other cases, after the control-phase clearance study, CsA dissolved in 50% ethanol was injected intravenously at a dose of 30 mg/kg, or the intestinal perfusate used before and after the addition of an inhibitor. In DEX-pretreated rats, erythromycin N-demethylation activity was 1.19 ± 0.05 nmol/min/mg of protein (means ± S.E. of four trials) in the intestine (villus area) and distal ends of whole intestinal lumen remained unchanged (1.20 ± 0.03 nmol/min/mg of protein). In untreated rats, erythromycin N-demethylation activity increased 9.7-fold for untreated rats (4.91 ± 0.32 nmol/min/mg of protein), while the activity in the intestine remained unchanged (1.20 ± 0.03 nmol/min/mg of protein).

P-gp-Mediated Clearance of Rhod123 in Vivo. Under steady-state conditions, CL_{exp} and CL_{bile} of Rhod123 before and after intravenous administration of CsA, a potent P-gp inhibitor, were measured to estimate P-gp-mediated clearance of Rhod123 in untreated and DEX-pretreated rats (Table 1). In DEX-pretreated rats, the CL_{exp} of Rhod123 increased 1.9-fold for untreated rats. In contrast, CL_{bile} of Rhod123 in DEX-pretreated rats did not increase; rather, it decreased by approximately 40%, although the bile flow rate in DEX-pretreated rats was higher than that in untreated rats (65.9 ± 7.3 μl/min/kg in untreated rats; 82.8 ± 6.3 μl/min/kg in DEX-pretreated rats). The increase in the bile flow rate in DEX-pretreated rats may relate to the significant increase of the liver weights (34.9 ± 3.8 g/kg in untreated rats; 59.9 ± 2.4 g/kg in DEX-pretreated rats, P < 0.05). The hepatic concentration of Rhod123 in DEX-pretreated rats was lower than that in untreated rats. Therefore, to normalize the CL_{bile} with hepatic Rhod123 concentrations, a parameter of CL_{bile} of Rhod123, a biliary excretion rate divided by the hepatic Rhod123 concentration, was used. This parameter revealed that the actual P-gp function for Rhod123 biliary excretion was not altered after DEX pretreatment. Thus, the observed decrease in biliary excretion rate or CL_{bile} of Rhod123 in DEX-pretreated rats would be due to the decreased hepatic Rhod123 concentration. CsA given intravenously clearly decreased CL_{exp} and CL_{bile} of Rhod123 in both untreated and DEX-pretreated rats, indicating that P-gp-mediated transport is involved in the disposition of Rhod123. Assuming that CsA suppresses the P-gp function completely, the decreased clearance by CsA corresponds to the P-gp-mediated transport of Rhod123. Based on these considerations, the P-gp-mediated CL_{exp} in DEX-pretreated rats was estimated to be approximately 2-fold higher than that in untreated rats.

Interaction of Rhod123 with CYP3A-Related Compounds in Vivo. The interaction of a CYP3A-related compound, midazolam or...
verapamil, with Rho123 was examined in untreated and DEX-pretreated rats. The time courses of CL<sub>exp</sub> and CL<sub>bile</sub> of Rho123 before and after administration of verapamil (30 μM) into the intestinal lumen are shown in Fig. 2. In untreated rats, verapamil inhibited both CL<sub>exp</sub> and CL<sub>bile</sub> of Rho123 significantly. In contrast, no significant inhibitory effect of verapamil was detected in DEX-pretreated rats. Inhibition potencies of these CYP3A-related compounds including CsA were estimated by comparing the clearance of Rho123 in the absence and presence of an inhibitor (Fig. 3). In untreated rats, midazolam (100 μM) and verapamil (30 or 100 μM) added in the intestinal perfusate decreased CL<sub>exp</sub> of Rho123 by approximately 25 to 50%, and CL<sub>bile</sub> by approximately 30 to 50%. In DEX-pretreated rats, the inhibitory effect of midazolam on CL<sub>bile</sub> of Rho123 was decreased, whereas the effect on CL<sub>exp</sub> of Rho123 remained unchanged. On the other hand, the inhibitory potency of verapamil in DEX-pretreated rats was significantly decreased both in the intestine and in the liver as compared with that in untreated rats. These results indicate that the pharmacokinetic interactions of P-gp/CYP3A-related compounds with Rho123 are modulated under different P-gp/CYP3A activities.

**Discussion**

There is some literature reporting the effect of inducers including DEX on CYP3A/P-gp proteins and/or their mRNA levels in various cell lines and in human and experimental animals (Schuetz et al., 1996; Salphati and Benet, 1998; Sérée et al., 1998; Demeule et al., 1999). However, only few reports have analyzed the effect of inducers on in vivo activity of P-gp. Also, the drug-drug interactions between P-gp substrates and CYP3A-related compounds under modulated CYP3A/P-gp activities are not yet fully understood.

DEX has been recognized as an inducer of CYP3A, and the increase in CYP3A proteins and/or CYP3A-related mRNA concentrations after pretreatment with DEX has been reported for hepatocytes and small bowel enterocytes of rats (Lee and Werlin, 1995; Lin et al., 1999). In the present study, DEX pretreatment increased erythromycin N-demethylation activity 9.7-fold in the liver. An extensive induction of hepatic CYP3A activity by DEX pretreatment has also been observed in our previous study, in which DEX was dissolved in 2% Tween 20 and the solution was injected intraperitoneally at a dose of 50 mg of DEX for 2 days, and CYP3A activity was evaluated by midazolam metabolism (Higashikawa et al., 1999a,b). On the other hand, the intestinal CYP3A activity estimated by erythromycin N-demethylation activity did not alter after DEX pretreatment in the present study. Watkins et al. (1987) reported that DEX pretreatment increased the intestinal CYP3A activity approximately 2-fold in rats as evaluated by erythromycin metabolism, and Lin et al. (1999) found a 3- to 4-fold increase as evaluated by testosterone metabolism, although the estimated CYP3A activity in the intestine was much lower than that in the liver. In the present study, the solution of DEX

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**TABLE 1**

*Effect of CsA on Rho123 clearance under a steady-state plasma concentration of Rho123 in untreated and DEX-pretreated rats*

<table>
<thead>
<tr>
<th>Parameter for Rho123</th>
<th>Untreated Without CsA</th>
<th>Untreated With CsA</th>
<th>DEX-Pretreated Without CsA</th>
<th>DEX-Pretreated With CsA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infusion rate (nmol/min)</td>
<td>3.33</td>
<td>3.33</td>
<td>3.33</td>
<td>3.33</td>
</tr>
<tr>
<td>C&lt;sub&gt;exp&lt;/sub&gt; (μM)</td>
<td>0.22 ± 0.04</td>
<td>0.27 ± 0.04</td>
<td>0.17 ± 0.01</td>
<td>0.23 ± 0.02</td>
</tr>
<tr>
<td>CL&lt;sub&gt;exp&lt;/sub&gt; (ml/min/kg)</td>
<td>68.2 ± 8.6</td>
<td>57.8 ± 8.6</td>
<td>81.0 ± 6.4</td>
<td>64.5 ± 4.6</td>
</tr>
<tr>
<td>Intestinal exsorption rate (nmol/min/kg)</td>
<td>1.06 ± 0.08</td>
<td>0.44 ± 0.10</td>
<td>1.52 ± 0.13</td>
<td>0.59 ± 0.01</td>
</tr>
<tr>
<td>CL&lt;sub&gt;bile&lt;/sub&gt; (ml/min/kg)</td>
<td>4.71 ± 0.28</td>
<td>1.63 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.89 ± 0.77&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.77 ± 0.30&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Biliary excretion rate (nmol/min/kg)</td>
<td>0.56 ± 0.08</td>
<td>0.02 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.30 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.02 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CL&lt;sub&gt;total&lt;/sub&gt; (ml/min/kg)</td>
<td>2.93 ± 0.38</td>
<td>0.06 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.71 ± 0.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.07 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CL&lt;sub&gt;total&lt;/sub&gt;* (g liver/min/kg)</td>
<td>2.31 ± 0.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.13 ± 0.03</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D., not determined.

<sup>a</sup> P < 0.05, significantly different from the values in the absence of CsA.

<sup>b</sup> P < 0.05, significantly different from the values in untreated rats.
Significantly different from that in untreated rats at a level of P < 0.05. 

Fig. 3. Inhibitory potencies of CsA, midazolam (MDZ), and verapamil (VRP) on

**CL**<sub>exp</sub> from the blood to the intestinal lumen (A) and **CL**<sub>bile</sub> (B) of Rho123 in untreated and DEX-pretreated rats.

Open columns are results from untreated and hatched columns from DEX-

pretreated rats. CsA was injected intravenously at a dose of 30 mg/kg. MDZ (100 

μM) or VRP (30 or 100 μM) was perfused into the intestinal lumen at a rate of 1 

ml/min. The inhibitory potencies were estimated by comparing the clearances of 

Rho123 before and after the administration of an inhibitor, and are shown as the 

mean ± S.E. of three trials. a, significantly different from that in untreated rats at a level of P < 0.05. b, significantly different from VRP (30 μM) in untreated rats at a level of P < 0.05.

dissolved in DMSO or DMSO alone (untreated control rats) was administered by stomach intubation. It has been reported that DMSO treatment increased CYP3A protein level in phenobarbital-treated primary cultured rat hepatocytes by decreasing the turnover rate of CYP3A protein (Zanger and Novak, 1998). Therefore, assuming that DMSO increases the intestinal CYP3A level even under in vivo conditions, the effect of DEX on CYP3A level in the intestine may be hidden by the effect of DMSO. Also, the basal erythromycin N-demethylation activity in the intestine was higher than that in the liver in the present study, as reported previously (Watkins et al., 1987, 1989). The intestinal cells isolated by the method used in the present study would be mainly derived from the proximal, villus area, where the concentration of total CYP is higher than the other area cells of the intestine (Bonkovsky et al., 1985; Watkins et al., 1987). Thus, the basal CYP activity in the intestine is considered to be remarkably variable depending on the cell isolation method and preparation method of microsomes, which may also affect the degree of induction by DEX.

DEX pretreatment increased the P-gp level 1.9-fold for untreated rats in the intestine, but not in the liver, as evaluated by Western blot analysis using C219 (Fig. 1). In a preliminary study, the effect of vehicle alone (DMSO) on P-gp expression was examined, since DMSO is reported to affect the P-gp expression in vitro (Fardel et al., 1992; Zhou et al., 1996). However, the P-gp level in DMSO-pretreated rats was 1.17 ± 0.11-fold (mean ± S.E. of three trials) for normal rats (without DMSO) in the intestine and 1.04 ± 0.16-fold in the liver. Thus, the effect of DMSO on P-gp expression in vivo was negligible. Three multidrug resistance (mdr) genes have been reported in rodents: mdr1a (or mdr3), mdr1b (or mdr1), and mdr2, where mdr1a and mdr1b genes encode drug efflux transporter P-gp and mdr2 gene encodes a phospholipid translocator (Devaut and Gros, 1990; Ruetz and Gros, 1994). C219 used as anti-P-gp antibody cross-reacts with products of these three mdr genes. Also, C219 recognizes sister P-gp, a major canalicular bile salt export pump of mammalian liver closely related to the P-gp family (Childs et al., 1995; Gerloff et al., 1998). Like P-gp, sister P-gp is expressed in the intestine by DEX pretreatment in the present study. Lin et al. (1999) reported that DEX pretreatment increased both the intestinal and hepatic P-gp approximately 2- to 3-fold, as evaluated by Western blot analysis using C219. The reason for the absence of effect of DEX pretreatment on hepatic P-gp level in the present study is not clear. However, it has been reported that the effect of DEX on P-gp expression is time- and DEX-concentration-dependent and gene- and cell type-specific (Zhao et al., 1993). Also, the effect of DEX on P-gp expression in vivo varied markedly among different tissues (Sérale et al., 1998; Demeule et al., 1999). Thus, some differences in DEX pretreatment, such as DEX dose, vehicle used, and pretreatment time, and in animal species and strains may affect the degree of P-gp induction.

Using DEX-pretreated rats, first we measured **CL**<sub>exp</sub> and **CL**<sub>bile</sub> of Rho123 in the absence and presence of CsA to estimate P-gp-mediated clearance of Rho123 (Table 1). In our previous study, the renal secretory clearance of Rho123 in untreated rats was almost completely inhibited by CsA given intravenously at a dose of 10 mg/kg (Kunihara et al., 1998). It was suspected, however, that the increased hepatic CYP3A activity in DEX-pretreated rats may decrease the CsA concentration in the liver and intestine by CYP3A-mediated metabolism. Therefore, a higher dose of CsA (30 mg/kg) was used in the present study. In untreated rats, both **CL**<sub>exp</sub> and **CL**<sub>bile</sub> of Rho123 were markedly decreased by CsA (Table 1), suggesting the involvement of P-gp-mediated disposition in the intestine and liver. DEX pretreatment increased CsA-sensitive **CL**<sub>exp</sub> of Rho123 approximately 2-fold, but not **CL**<sub>bile</sub> (Table 1). These in vivo findings were in good agreement with the results of Western blot analysis (Fig. 1). As described already, Rho123 has been used as a typical probe to assess in vitro and in vivo P-gp function (Lee et al., 1994; Scala et al., 1997). Rho123 is also reported as a substrate of multidrug resistance-associated protein (MRP), although the contribution of MRP in Rho123 transport is much less than that of P-gp (Feller et al., 1995; Broxterman et al., 1997). In addition, de Bruin et al. (1999) recently reported that Rho123 is a substrate of mitoxantrone transporter, which is newly identified in human cancers. On the other hand, CsA is known as a potent inhibitor of P-gp, although it also inhibits other transporters, such as taurine transporter, Na<sup>+</sup>-bile salt cotransporter, and canalicular multispecific organic anion transporter (Kim et al., 1998; Kitoshita et al., 1998; Schroder et al., 1998). Therefore, by taking into consideration the expression sites of the transporter and the sensitivity to CsA, the contribution of MRP and/or mitoxantrone transporter in Rho123 clearance was not considered further in the present study.

Because Rho123 is not a substrate of CYP3A, the modulation of CYP3A alone would not affect the pharmacokinetics of Rho123. On
the other hand, Rho123 has an ester moiety in its structure and is extensively metabolized to rhodamine 110 (Rho110, a deacetylated metabolite of Rho123) followed by its glucuronidation in vivo (Sweatman et al., 1990). Since DEX pretreatment also increases total hepatic esterase activity more than 10-fold (Bornheim, 1998), the decrease in \( CL_{\text{hep}} \) of Rho123 in DEX-pretreated rats was considered to be caused by the decreased concentration of Rho123 in the liver by the increased metabolism (Table 1). In fact, the hepatic Rho123 concentration in DEX-pretreated rats was 35% lower than that in untreated rats. To evaluate the biliary excretion of xenobiotics under steady-state conditions, a parameter of \( CL_{\text{hep}} \), is generally used. However, the intracellular unbound concentration of the xenobiotic is actually responsible for the transport from the intracellular compartment to the bile (Yamazaki et al., 1996). In the present study, a parameter of \( CL_{\text{hep}} \) was further used to normalize the change in the hepatic concentration of Rho123 under different conditions. \( CL_{\text{hep}} \) of Rho123 in DEX-pretreated rats was almost the same with that in untreated rats, indicating that \( P-gp \) function for Rho123 biliary excretion is not altered by DEX pretreatment, in good agreement with the results of Western blot analysis (Fig. 1).

Under such modulated \( P-gp \)/CYP activities, the drug-drug interactions between Rho123 and midazolam or verapamil were evaluated (Figs. 2 and 3). The inhibitory effect of midazolam on \( CL_{\text{hep}} \) of Rho123 was significantly decreased in DEX-pretreated rats. Midazolam is a typical substrate of CYP3A, but not of \( P-gp \), although it acts as an inhibitor of \( P-gp \) in Caco-2 cells and in rat intestine (Takano et al., 1998; Yumoto et al., 1999). In DEX-pretreated rats, the hepatic concentration of midazolam would be lower than that in untreated rats due to the increased CYP3A-mediated metabolism, which in turn would decrease the inhibitory effect on \( CL_{\text{hep}} \) of Rho123. In fact, we previously reported that the bioavailability of midazolam administered into the jejunal loop at a dose of 10 μmol was 12% in untreated rats and only 2% in DEX-pretreated rats (Higashikawa et al., 1999a,b). On the other hand, the inhibitory potency of midazolam on \( CL_{\text{exp}} \) of Rho123 remained unchanged by DEX pretreatment. This result could not be ascribed to the presence of DMSO in the intestinal perfusate because DMSO reportedly did not affect CYP3A-mediated metabolism directly when added in the cultured medium, although DMSO pretreatment increased CYP3A levels in primary cultured rat hepatocytes (Zanger and Novak, 1998). Rather, the similar inhibitory potency of midazolam on \( CL_{\text{exp}} \) of Rho123 in DEX-pretreated rats to that in untreated rats may support our finding that the intestinal CYP3A activity was not affected by DEX pretreatment.

In untreated rats, verapamil inhibited \( CL_{\text{exp}} \) and \( CL_{\text{hep}} \) of Rho123 in a dose-dependent manner. In DEX-pretreated rats, the inhibitory potency of verapamil significantly decreased in both the intestine and the liver, different from the case of midazolam (Figs. 2 and 3). Verapamil is a substrate/inhibitor of both CYP3A and \( P-gp \) and is frequently used as an inhibitor of \( P-gp \), as is CsA. Also, some oxidative metabolites of verapamil formed by CYP3A-mediated metabolism are reportedly substrates and/or inhibitors of \( P-gp \) (Pauli-Magnus et al., 2000). Therefore, in the case of verapamil, the effect of increased CYP3A activity in the liver by DEX pretreatment on the inhibitory potency on \( CL_{\text{hep}} \) may be small. In contrast, the increase in functional \( P-gp \) in the intestine by DEX pretreatment would decrease the intestinal absorption of verapamil and therefore the hepatic concentration of verapamil including metabolites, which in turn would decrease the inhibitory effect on \( CL_{\text{exp}} \) and \( CL_{\text{hep}} \) of Rho123.

In conclusion, it was demonstrated that DEX pretreatment affects not only \( P-gp \)-mediated disposition of Rho123 but also pharmacokinetic interactions of \( P-gp \)/CYP3A-related compounds with Rho123, probably because concentrations of substrates/inhibitors at target sites such as the intestine and liver are varied.

References


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