Organic Anion–Transporting Polypeptide (OATP)–Mediated Drug–Drug Interaction Study between Rosuvastatin and Cyclosporine A in Chimeric Mice with Humanized Liver^S

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ABSTRACT

The influence of transporters on the pharmacokinetics of drugs is being increasingly recognized, and DDIs via transporters may be a risk factor for adverse events. Cyclosporine A, a strong OATP inhibitor, has been reported to increase the systemic exposure of rosuvastatin, an OATP substrate, by 7.1-fold in clinical studies. PXB mice are chimeric mice with humanized livers that are highly repopulated with human hepatocytes and have been widely used for drug discovery in drug metabolism and pharmacokinetics studies. In the present study, we examined in vivo and in vitro DDIs between rosuvastatin and cyclosporine A in PXB mice and fresh human hepatocytes (PXB cells) obtained from PXB mice. We initially investigated the active transport of rosuvastatin into PXB cells, and found concentration-dependent uptake with a Michaelis–Menten constant value of 4.0 $\mu\text{mol/l}$ and a V_{max} value of 4.63 pmol/min per 10^6 cells. Cyclosporine A inhibited the uptake of rosuvastatin with an

IC_{50} value of 0.21 $\mu\text{mol/l}$. We then examined in vivo DDIs, and the exposure of orally administered rosuvastatin increased by 3.3-fold and 11-fold in PXB mice pretreated with 10 and 50 mg/kg cyclosporine A, whereas it increased by 2.5-fold and 6.2-fold when rosuvastatin was administered intravenously, in studies that were conducted for considering gastrointestinal DDIs. The liver-to-blood concentration ratio of rosuvastatin was dose-dependently decreased by pretreatment with cyclosporine A in PXB mice and SCID mice. Observed DDIs in vivo were considered to be reasonable based on the estimated concentrations of cyclosporine A at the inlet to the liver and in the liver tissues of both mice. In conclusion, our results indicate that PXB mice might be a useful tool for predicting human OATP-mediated DDIs in drug discovery, and its limitation due to the differences of gastrointestinal condition from human should also be considered.

Introduction

The influence of transporters on the pharmacokinetics of drug is being increasingly recognized. Drug–drug interactions (DDIs) mediated by transporters may be a risk factor for adverse events, and recent studies have demonstrated increased systemic exposure of victims by combination dosing with inhibitors of various transporters. Therefore, guidelines in the United States, Europe, and Japan now request safety assessments of DDIs in drug development by large-scale clinical trials (European Medicines Agency and Committee for Human Medicinal Products, 2012; US Department of Health and Human Services et al., 2012; Pharmaceuticals and Medical Devices Agency, 2014).

Organic anion–transporting polypeptide (OATP) 1B1 and OATP1B3 are mainly expressed in the sinusoidal membrane of human hepatocytes

and solute carrier (SLC) transporters, which are playing an important role in the hepatic uptake of drugs. Clinical drug interactions via hepatic OATPs are particularly relevant to statins (Neuvonen et al., 2006; Giacomini et al., 2010). The systemic exposure of rosuvastatin, a 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor that is widely used in the treatment of hypercholesterolemia, was significantly increased by concomitant dosing with cyclosporine A (Simonson et al., 2004), and commonly a lower dosage of rosuvastatin is therefore required when administered with cyclosporine A in a clinical setting. Rosuvastatin has low passive membrane permeability, and its systemic clearance is mostly mediated by hepatic uptake and biliary efflux transporters (Martin et al., 2003a,b). Multiple hepatic uptake transporters (e.g., OATP1B1, OATP1B3 and OATP2B1) and efflux transporters [e.g., breast cancer resistance protein (BCRP) and multi-drug resistance protein 2 (MRP2)] are involved in its transport (Ho et al., 2006; Huang et al., 2006; Ellis et al., 2013). However, hepatic uptake of rosuvastatin via OATPs significantly influences its pharmacokinetics rather than biliary efflux of rosuvastatin via other transporters, because the uptake process is the

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ABBREVIATIONS: AUC_{0–4h}, area under the concentration–time curve in blood from initial administration to 4 hours after its administration; AUCR, area under the blood concentration–time curve ratio; BCRP, breast cancer resistance protein; DDI, drug–drug interaction; F_a , fraction of the inhibitor dose absorbed; F_g , fraction of the absorbed inhibitor dose escaping gut wall extraction; $f_{u,b}$, fraction unbound in blood; HBSS, Hanks’ balanced salt solution; $I_{n,\text{max},u}$, maximum protein-unbound concentration of cyclosporine A at the inlet to the liver; k_a , absorption rate constant of the inhibitor; K_m , Michaelis–Menten constant; LC, liquid chromatography; MRP2, multidrug resistance protein 2; MS/MS, tandem mass spectrometry; OATP, organic anion–transporting polypeptide; Q_h , hepatic blood flow rate in mice; SCID, severe combined immune deficiency; SLC, solute carrier; UGT, UDP-glucuronosyltransferase.

rate-limiting step in its disposition from the systemic circulation (Shitara and Sugiyama, 2006; Maeda et al., 2011; Shitara et al., 2013).

In vivo DDI studies in experimental animals have been conducted to predict DDIs in human (Lau et al., 2006; Imaoka et al., 2013; Shen et al., 2013; Takahashi et al., 2013; Chu et al., 2015), but the predictability of traditional preclinical animal models may be limited by species differences (Giacomini et al., 2010; Chu et al., 2015). Orthologous human *OATP* genes have not been identified for many rat/mouse *Oatp* genes, indicating the absence of a strict one-to-one relationship between rodent *Oatps* and human *OATPs* (Hagenbuch and Meier, 2003). Knockout models lacking murine *oatp* isoforms and OATP-humanized transgenic mice have been developed to estimate the pharmacokinetics of drugs and interactions with concomitants via human OATPs (van de Steeg et al., 2013; Zimmerman et al., 2013; Higgins et al., 2014; Salphati et al., 2014). However, the drug disposition of some drugs may be affected by other murine transporters (Iusuf et al., 2013; Chang et al., 2014; Salphati et al., 2014).

PXB mice are chimeric mice with humanized livers that are highly repopulated with human hepatocytes (Tateno et al., 2004, 2015). The expression levels (both mRNA and protein) of metabolic enzymes and transporters in human hepatocytes of PXB mice were previously reported to be similar to those in humans (Tateno et al., 2004; Nishimura et al., 2005; Okumura et al., 2007; Ohtsuki et al., 2014), and PXB mice have been characterized in detail and widely used in drug discovery to predict human-specific metabolite identification (Samuelsson et al., 2012; Bateman et al., 2014), induction and inhibition of drug metabolic enzymes (Katoh et al., 2005; Katoh and Yokoi, 2007; Hasegawa et al., 2012; Kakuni et al., 2013), human pharmacokinetics (Sanoh et al., 2012, 2015; Miyamoto et al., 2017), and secretion into bile (Okumura et al., 2007).

In the present study, we examined the utility of PXB mice to predict human OATP-mediated DDIs between rosuvastatin, a typical OATP substrate, and cyclosporine A, a strong OATP inhibitor. Rosuvastatin is more hydrophilic than other statins and is mainly excreted into bile in its unchanged form with negligible metabolism (Martin et al., 2003a,b); however, two metabolites, the lactone form (a metabolite via uridine diphosphate glucuronosyltransferase 1A, UGT1A) and the *N*-desmethyl form (a metabolite via CYP2C) have been identified (Prueksaritanont et al., 2002; Martin et al., 2003b). In the present study, these metabolites were also monitored to identify the replacement of human livers in PXB mice as well as the inhibitory effects of cyclosporine A on the metabolism of rosuvastatin.

Materials and Methods

Chemicals

Rosuvastatin and cyclosporine A were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). *d*6-rosuvastatin, rosuvastatin lactone, *N*-desmethyl rosuvastatin, and cyclosporine AM1 were obtained from Toronto Research Chemicals Inc. (North York, ON, Canada). All other reagents and solvents were commercial products of the highest available grade or analytical grade.

Animals

The present study was approved by the Ethics Committees of PhoenixBio Co., Ltd., and Toray Industries, Inc., and animal experiments were conducted according to the Guidelines for Animal Experiments of PhoenixBio Co., Ltd. and Toray Industries, Inc. To generate chimeric mice with humanized livers, cryopreserved human hepatocytes (a 2-year-old Hispanic female), which had been donated with informed consent, were purchased from BD Biosciences (Woburn, MA). Chimeric mice with humanized livers (PXB mice) were generated using a previously reported method (Tateno et al., 2015). Briefly, 1.0×10^6 human hepatocytes were injected into the inferior splenic pole of 2- to 4-week-old male

cDNA-uPA^{wild/+}/SCID mice. Mice were used in experiments between 17 and 19 weeks of age and with a body weight of 16.0–23.4 g. The replacement index or extent of repopulation of the chimeric mouse liver with human hepatocytes was 92–99%, as assessed by the measurement of human albumin levels in blood (Tateno et al., 2015). Native SCID mice were used as controls. SCID mice were obtained from Charles River Laboratories Japan Inc. (Yokohama, Japan).

Human Hepatocytes Isolated from Chimeric Mice, PXB Cells

Approximately $1.5\text{--}1.7 \times 10^8$ human hepatocytes (PXB cells) were obtained from a PXB mouse (repopulation ratio, 89–91%) using the collagenase perfusion method (Yamasaki et al., 2010). Fresh human hepatocytes attached to 24-well cell plates confluent at a density of 2.1×10^5 cells/cm² (4.0×10^5 cells/well) and were maintained for 2–4 weeks with d-HCGM medium consisting of Dulbecco's modified Eagle's medium (Yamasaki et al., 2006).

In Vitro Time- and Concentration-Dependent Uptake of Rosuvastatin in PXB Cells

Prior to the assay, cells were rinsed twice with prewarmed phosphate-buffered saline (37°C) and equilibrated in HBSS for 2 minutes. Uptake studies were initiated by removing the equilibrating buffer and adding 0.50 ml of HBSS containing 0.1–30 $\mu\text{mol/l}$ rosuvastatin. Hepatocytes were then incubated at 37°C in a humidified 5% CO₂ atmosphere. At each of the designed time points, the incubation was terminated by removing the transport buffer, and cells were immediately washed with ice-cold phosphate-buffered saline three times. After adding 0.5 ml of acetonitrile containing 0.1% formic acid to each well, cells were scraped and recovered to another tube using a pipette. The concentrations of rosuvastatin were measured by LC-MS/MS. To assess the passive uptake of rosuvastatin in the hepatocytes, the same experiment was conducted on ice.

In Vitro DDI for the Uptake of Rosuvastatin with Cyclosporine A in PXB Cells

The in vitro inhibitory effects of cyclosporine A on the uptake of rosuvastatin in PXB cells were examined using a method similar to that used in the uptake studies on rosuvastatin described above. Uptake studies were initiated by removing the equilibrating buffer and adding 0.50 ml of HBSS containing between 0.01 and 3 $\mu\text{mol/l}$ cyclosporine A and 1 $\mu\text{mol/l}$ rosuvastatin. Cells were incubated at 37°C for 10 minutes.

In Vivo DDI for the Pharmacokinetics of Rosuvastatin with Cyclosporine A in PXB Mice

Cyclosporine A was dissolved and formed a clean yellow-colored solution with ethanol and olive oil (1:9, v/v). Cyclosporine A or vehicle (5 ml/kg) was administered orally to mice at a dose of 10 or 50 mg/kg 4 hours before the oral administration of rosuvastatin. Rosuvastatin was dissolved in saline containing 1% dimethylsulfoxide for its intravenous administration and suspended in 0.3% hydroxypropyl methylcellulose for its oral administration. Rosuvastatin (5 ml/kg) was administered intravenously to mice at a dose of 1 mg/kg or orally at a dose of 2 mg/kg. In the pharmacokinetics analysis, 30 μl of blood was taken at the designated time points, 0.25, 0.5, 1, 2, and 4 hours postdose (after oral administration) and 0.083, 0.25, 1, 2, and 4 hours postdose (after intravenous administration), from the orbital vein, mixed with an equal volume of 20 mmol/l ice-cold sodium acetate buffer (pH 4.0), and immediately placed on ice to prevent the degradation of the lactone form of rosuvastatin. At termination (4 hours after the oral administration of rosuvastatin), all animals were euthanized by bleeding via the heart under isoflurane anesthesia, and each liver was collected to measure the tissue concentrations of rosuvastatin and cyclosporine A in the liver. Collected livers were rinsed, weighed, and homogenized in a 4-fold volume of 20 mmol/l ice-cold sodium acetate buffer (pH 4.0) by the homogenizer, Shake Master Neo (BMS-M10N21; Biomedical Sciences, Tokyo, Japan). Treated blood and liver homogenates were stored at -20°C or lower for later bioanalyses.

Fraction Unbound of Cyclosporine A in Blood

Because we could not obtain fresh blood of PXB mice due to transport issue from the supplier to us, we used ICR mice blood instead. Heparinized fresh blood was collected from ICR mice (11 years old, male; Charles River Laboratories). Ten microliters of cyclosporine A (10, 100 $\mu\text{mol/l}$) was added to 490 μl of the

fresh blood (final concentration, 0.2, 2 $\mu\text{mol/l}$), mixed, and incubated for 30 minutes in a water bath (NTS-4000BM; Tokyo Rikakikai, Manila, Philippines) set at 37°C. Samples were centrifuged at 1800g for 10 minutes (S700RF; Kubota, Osaka, Japan). The obtained plasma was analyzed by LC-MS/MS described below. Blood concentration (C_{blood}) to plasma concentration (C_{plasma}) ratio (Rb) was calculated from the equation: $C_{\text{blood}}/C_{\text{plasma}}$. Five microliters of cyclosporine A (100, 1000 $\mu\text{mol/l}$) was added to 2495 μl of heparinized plasma of PXB mice (final concentration, 0.2, 2 $\mu\text{mol/l}$), mixed, and incubated for 10 minutes in a water bath set at 37°C. Samples were centrifuged at 608,000g for 120 minutes at 25°C (CS120GXL; Hitachi, Tokyo, Japan). The clear middle layer of obtained plasma, the protein-free fraction, was collected and analyzed by LC-MS/MS, as described below. The fraction unbound in blood, $f_{u,b}$, in PXB mice was calculated in the following equation: $1 - [(C_{\text{plasma}} - C_{\text{unbound}})/C_{\text{unbound}}]/\text{Rb}$, where C_{unbound} is the unbound concentration in plasma.

Bioanalysis by LC-MS/MS

Analytes in pretreated blood and liver homogenates were extracted by adding acetonitrile containing 0.1% formic acid. After vortexing the extraction mixture and centrifuging, each supernatant was filtrated and diluted with 0.1% acetic acid, and analytes were quantified by LC-MS/MS with the Nexera LC system (Shimadzu Corporation, Kyoto, Japan) and the mass spectrometer QTRAP5500 (SCIEX, Concord, ON, Canada). Chromatographic separation was performed with CAPCELLPAK C18 MGIII, 50 $\times$ 2.0 mm, 5 μm (Shiseido Co., Ltd., Tokyo, Japan). Mobile phase A consisted of distilled water containing 0.1% formic acid, whereas mobile phase B consisted of acetonitrile. The initial percentage of phase B was 10%. A linear gradient was applied from 10% to 70% phase B over 3 minutes, from 70% to 95% phase B over 2 minutes, maintained at 95% phase B for 1 minute, and decreased to 10% phase B for equilibration over 3 minutes. The total flow rate of the mobile phases was 0.4 ml/min. The mass spectrometer spray voltage was set at 5.5 kV, whereas the probe temperature was 600°C with positive ion polarity. Rosuvastatin, its lactone form, its *N*-desmethyl form, cyclosporine A, its hydroxy form AM1, and *d6*-rosuvastatin, which was the internal standard, were measured by multiple reaction monitoring. Each precursor to product ion transitions was *m/z* 482 to 258 for rosuvastatin, *m/z* 464 to 270 for rosuvastatin lactone, *m/z* 468 to 258 for *N*-desmethyl rosuvastatin, *m/z* 1203 to 425 for cyclosporine A, *m/z* 1219 to 425 for cyclosporine AM1, and *m/z* 488 to 264 for *d6*-rosuvastatin.

Data Processing

In Vitro Transport Activity. The in vitro uptake rates of rosuvastatin in PXB cells were calculated using eq. 1:

$$\text{Uptake Rate (pmol/min/well)} = \frac{\text{Uptake Amount of Rosuvastatin in PXB-cells per well (pmol/well)}}{\text{Incubation Time (min)}} \quad (1)$$

A kinetic analysis of the transporter-mediated uptake of rosuvastatin in PXB cells was performed with the extended Michaelis-Menten equation, and K_m and V_{max} were calculated using eq. 2:

$$\text{Uptake Rate} = \frac{V_{\text{max}} \times S}{K_m + S} + P_{\text{diff}} \times S \quad (2)$$

where S is the substrate concentration of rosuvastatin in the incubation buffer (in micromoles per liter), K_m is the Michaelis-Menten constant (in micromoles per liter), and V_{max} values are reported in picomoles per minute per 10^6 cells. P_{diff} represents nonsaturable uptake clearance via passive diffusion (in microliters per minute per 10^6 cells). Fitting was performed by a nonlinear least-squares regression method using Phoenix WinNonlin version 6.2 (Certara Inc., Princeton, NJ). In addition, the uptake rate of rosuvastatin in cells (as the y-axis) was plotted against the common logarithm of the concentration of cyclosporine A (as the x-axis), and a regression analysis was performed using the equation “log (inhibitor) versus response variable slope (four parameters)” of GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA) to obtain the IC_{50} . The equation is a four-parameter logistic regression as follows:

$$Y = A(\infty) + \frac{A(0) - A(\infty)}{1 + 10^{((\text{LogIC}_{50} - X) \times \text{Hillslope})}} \quad (3)$$

Where $A(\infty)$ is the maximum inhibitory effect observed by an inhibitor, and $A(0)$ is the minimum inhibitory effect in the absence of an inhibitor, which should be the maximum uptake rate of rosuvastatin in the study.

Pharmacokinetics Analysis. Pharmacokinetic parameters were calculated by a noncompartmental method using Phoenix WinNonlin version 6.2.

Estimation of the Maximum Protein-Unbound Concentration of Cyclosporine A at the Inlet to the Liver, and Observed Concentrations of Cyclosporine A in Blood and Liver Tissue. To assess the relationship between inhibitor concentrations and inhibitory effects against each transporter, we estimated the maximum unbound concentration of cyclosporine A at the inlet to the liver as an effective concentration of the inhibitor and compared it with the reported IC_{50} values as the inhibitory effect against each transporter. The maximum unbound concentration of cyclosporine A at the inlet to the liver was calculated based on its systemic concentration in blood using eq. 4:

$$I_{\text{in,max,u}} = f_{u,b} \times (I_{\text{max,b}} + F_a F_g \times k_a \times \text{Dose} / Q_h) \quad (4)$$

where $I_{\text{max,b}}$ is the maximum circulating blood concentration of the inhibitor, which is equal to C_{max} ; k_a is the absorption rate constant of the inhibitor; F_a is the fraction of the inhibitor dose absorbed; F_g is the fraction of the absorbed inhibitor dose escaping gut wall extraction; Dose is the inhibitor dose; and Q_h is the hepatic blood flow rate in mice. In addition, the concentrations of cyclosporine A in blood at the same sampling points as rosuvastatin and the liver at the terminal point, 8 hours after the oral administration of cyclosporine A (at the same time as 4 hours after the oral administration of rosuvastatin), were measured in PXB mice and SCID mice.

Statistical Analysis

The Student's *t* test ($P < 0.05$) was applied to assess the statistical significance of each parameter and value in the in vivo DDI study between rosuvastatin and cyclosporine A.

Results

In Vitro Time- and Concentration-Dependent Uptake of Rosuvastatin in PXB Cells. Rosuvastatin was incubated with PXB cells at a concentration of 1 $\mu\text{mol/l}$ and temperature of 37°C, and uptake into cells was observed in a time-dependent manner almost linearly up to 10 minutes. The transport rate was 1.38 pmol/min per well (Fig. 1a). On the other hand, the passive uptake of rosuvastatin into cells was assessed with an incubation on ice, and the transport rate was 0.0693 pmol/min per well, which was 20-fold lower than that at 37°C (Fig. 1a). Furthermore, to assess the concentration-dependent kinetics of the active uptake of rosuvastatin in PXB cells, rosuvastatin was incubated at 37°C and on ice at different concentrations between 0.1 and 30 $\mu\text{mol/l}$. The active uptake amount of rosuvastatin was calculated by subtracting each value obtained on ice from the corresponding value at 37°C. The Michaelis-Menten equation showed that K_m and V_{max} were 4.0 $\mu\text{mol/l}$ and 4.63 pmol/min per 10^6 cells (1.85 pmol/min per well), respectively (Fig. 1b).

In Vitro DDI for the Uptake of Rosuvastatin with Cyclosporine A in PXB Cells. The inhibitory effects of cyclosporine A on the uptake of rosuvastatin in PXB cells were examined. The uptake of rosuvastatin was inhibited in the concentration-dependent manner of cyclosporine A, and the IC_{50} was 0.21 $\mu\text{mol/l}$ (Fig. 1c).

In Vivo DDI between Rosuvastatin and Cyclosporine A in PXB Mice. To investigate the effects of cyclosporine A on the pharmacokinetics of rosuvastatin, systemic concentrations of rosuvastatin were measured with or without cyclosporine A in PXB mice and SCID mice as the control. When only rosuvastatin was administered orally at a dose of 2 mg/kg to PXB mice, the C_{max} in blood and area under the concentration-time curve in blood from initial administration to 4 hours after its administration ($\text{AUC}_{0-4\text{h}}$) were 46.7 ng/ml and 105 ng·h/ml, respectively (Fig. 2a; Table 1). However, when cyclosporine A was administered orally at doses of 10 and 50 mg/kg 4 hours before the

Fig. 1. In vitro uptake studies of rosuvastatin. (a) Time-dependent uptake of rosuvastatin in PXB cells. Rosuvastatin was incubated with cells at the concentration of 1 $\mu\text{mol/l}$ both at 37°C and on ice for up to 10 minutes. (b) Concentration-dependent uptake of rosuvastatin in PXB cells. Rosuvastatin was incubated with cells at the concentration from 0.1 to 30 $\mu\text{mol/l}$ both at 37°C and on ice for 10 minutes. By subtracting each value obtained on ice from corresponding value at 37°C, an active uptake amount of rosuvastatin was calculated at each time point. (c) Concentration-dependent inhibition of cyclosporine A against the uptake of rosuvastatin in PXB cells. Rosuvastatin was incubated with cells at the concentration of 1 $\mu\text{mol/l}$ at 37°C for 10 minutes with or without the inhibitor, cyclosporine A. The concentrations of the inhibitor were from 0.01 to 3 $\mu\text{mol/l}$. Values shown are the mean $\pm$ S.D. for experiments performed in triplicate. Conc., concentration; CsA, cyclosporine A.

administration of rosuvastatin, the systemic exposure, AUCs, of the orally administered rosuvastatin increased significantly by 3.3-fold and 11-fold, respectively (Fig. 2a; Table 1). On the other hand, rosuvastatin was also administered to SCID mice to assess the control pharmacokinetic profile of rosuvastatin in mice, and the C_{max} and $\text{AUC}_{0-4\text{h}}$ were 3.31 ng/ml and 5.14 ng·h/ml, respectively, after the oral administration of rosuvastatin at a dose of 2 mg/kg without cyclosporine A (Fig. 2b; Table 1). These results showed that the systemic exposure of rosuvastatin in SCID mice was approximately 20-fold lower than that in PXB mice. Furthermore, the effects of cyclosporine A on the pharmacokinetics of rosuvastatin in SCID mice were investigated, and the AUCs of rosuvastatin increased by 13-fold with the oral coadministration of 10 mg/kg cyclosporine A 4 hours before the administration of rosuvastatin, and further increased by 78-fold with 50 mg/kg cyclosporine A (Fig. 2b; Table 1). To assess the uptake of rosuvastatin into cells in liver tissue and the inhibitory effects of cyclosporine A on uptake in PXB mice and SCID mice, liver concentrations of rosuvastatin 4 hours after its oral administration with or without cyclosporine A were measured. The liver partition coefficients ($K_{\text{p,liver}}$) value was calculated using the concentration ratio of rosuvastatin in the liver over that in blood. Its $K_{\text{p,liver}}$ value was higher in SCID mice (336) than in PXB mice (44) and decreased with the administration of cyclosporine A in a dose-dependent manner in PXB mice and SCID mice (Fig. 3).

In the present study, the major metabolites of rosuvastatin reported in humans, which are the lactone and *N*-desmethyl forms, were also measured in blood and liver tissue; however, the lactone form of rosuvastatin was not detected in the blood of PXB mice or SCID mice (lower than 3 ng/ml) but was present in liver tissues. The concentration ratios of the lactone form against rosuvastatin in the liver exhibited a strain difference: 37–50% in PXB mice, but only 1% in SCID mice. On the other hand, the *N*-desmethyl form was detected only in PXB mice. The exposure ratios of the *N*-desmethyl form against rosuvastatin were

4–6% in blood and 2% in liver tissue. The metabolism of these two metabolites of rosuvastatin was not affected by the coadministration of cyclosporine A in PXB mice and SCID mice.

As described above, blood concentrations of rosuvastatin administered orally were lower in SCID mice than in PXB mice, and thus an intravenous administration study of rosuvastatin was conducted to assess the systemic clearance of rosuvastatin in SCID mice and PXB mice. Systemic exposure, $\text{AUC}_{0-4\text{h}}$, after an intravenous dose of 1 mg/kg rosuvastatin was higher in PXB mice (280 ng/h per milliliter) than in SCID mice (26.2 ng/h per milliliter), and similar results were obtained after the oral administration of rosuvastatin. The total systemic clearance of rosuvastatin was 3.82 l/h per kilogram in PXB mice and 38.1 l/h per kilogram in SCID mice (Table 1).

Estimation of the Maximum Protein-Unbound Concentration of Cyclosporine A at the Inlet to the Liver, and Observed Concentrations of Cyclosporine A in Blood and Liver Tissue. The systemic exposure of cyclosporine A was measured from 4 to 8 hours after its oral administration at the same sampling time points as those for rosuvastatin. The results obtained revealed that the systemic exposure of cyclosporine A increased in a dose-dependent manner at doses of 10 and 50 mg/kg cyclosporine A in PXB mice and SCID mice (Table 3). Based on the systemic exposure of cyclosporine A, the maximum protein-unbound concentration of cyclosporine A at the inlet to the liver, $I_{\text{in,max,u}}$ was estimated to clarify the relationship between cyclosporine A concentrations and its inhibitory effects on each transporter in the liver. Several pharmacokinetics and physiologic parameters were referred from previous studies to calculate $I_{\text{in,max,u}}$ in the present study: $f_{\text{u,b}}$ was 10% in humans (Yoshida et al., 2012) and 10% in mice obtained in the study (Supplemental Table 2). Q_{h} was 1.8 ml/min per 0.02 kg (Davies and Morris, 1993). $F_{\text{a}}F_{\text{g}}$ was regarded as 0.3–1.0 (Kato, 2008; Yoshida et al., 2012; Kadono et al., 2014), and k_{a} was regarded as 0.1 minute⁻¹ to minimize the risk of the false-negative

Fig. 2. Blood concentration-time profiles of rosuvastatin after the oral administration of rosuvastatin at a dose of 2 mg/kg to male PXB mice (a) and SCID mice (b), with oral preadministration of doses of 10 and 50 mg/kg cyclosporine A or vehicle. Open circles represent the group receiving vehicle (control); solid circles represent the group receiving 50 mg/kg cyclosporine A; solid triangles represent the group receiving 10 mg/kg cyclosporine A. Each point is represented as the mean $\pm$ S.D. ($n = 3-4$).

prediction of DDI (European Medicines Agency and Committee for Human Medicinal Products, 2012; US Department of Health and Human Services et al., 2012; Yoshida et al., 2012; Pharmaceuticals and Medical Devices Agency, 2014; Izumi et al., 2015). We consequently estimated that the $I_{in,max,u}$ values of cyclosporine A in PXB mice were 0.29–0.94 and 1.4–4.7 $\mu\text{mol/l}$ at doses of 10 and 50 mg/kg cyclosporine A, respectively, and 0.36–0.96 and 1.5–4.8 $\mu\text{mol/l}$ at doses of 10 and 50 mg/kg, respectively, in SCID mice (Table 2). In the present study, the concentrations of cyclosporine A in the blood at the same sampling points as rosuvastatin and in the liver 8 hours after the oral administration of cyclosporine A (the same time as 4 hours after the oral administration of rosuvastatin) were measured in PXB mice and SCID mice to assess not only its exposure at the inlet to the liver, but also the systemic and liver exposures. Liver concentrations were 1.07 and 5.89 $\mu\text{mol/kg}$ tissue at doses of 10 and 50 mg/kg, respectively, in PXB mice, and were 4.95 and 30.0 $\mu\text{mol/kg}$ tissue at doses of 10 and 50 mg/kg, respectively, in SCID mice (Table 3). The reported $I_{in,max,u}$ concentration of cyclosporine A was 1.2–1.3 $\mu\text{mol/l}$ (Yoshida et al., 2012; Izumi et al., 2015) when a 7.1-fold increase in the systemic exposure of rosuvastatin was observed in a clinical study (Simonson et al., 2004), and the IC_{50} values of cyclosporine A to OATP1B1, OATP1B3, MRP2, BCRP, and multidrug resistance protein 1 were 0.15, 0.68, 9.3, 1.5, and 1.7 $\mu\text{mol/l}$, respectively (Table 3) (Yoshida et al., 2012).

Discussion

In the present study, we demonstrated *in vitro* and *in vivo* DDIs between rosuvastatin, a substrate of OATPs, and cyclosporine A, a strong

inhibitor of OATPs, in PXB cells and PXB mice. Okumura et al. (2007) investigated the gene expression of human transporters in human hepatocytes of PXB mice and reported that the expression of OATP1B1/1B3 was strong, whereas that of oatp1b2 was weak. They also reported that the mRNA expression levels of hepatic transporters in the PXB mice and donor hepatocytes, and the levels of OATP1B1/1B3 in PXB mice were almost same as, 0.58–1.58 higher than, those in donor hepatocytes, which would indicate that the intestinal-liver axis does not affect expressions of OATPs in the mouse model compared with the other (Chow et al., 2017). Ohtsuki et al. (2014) also conducted the protein qualification of transporters using an LC-MS/MS technique and showed similar expression levels for human transporters between human hepatocytes of PXB mice and 17 human liver biopsy samples, with no significant difference of the protein expression of OATP1B1/1B3 among three different donors (2, 5, and 10 years old) of hepatocytes of PXB mice. Unfortunately, we do not have any genotype information of the SLCO1B1 in PXB mice we used in the study. PXB cells are fresh human hepatocytes isolated from PXB mice and have been used in *in vitro* drug metabolism and pharmacokinetics studies (Yamasaki et al., 2010; Kakuni et al., 2013).

We evaluated the transporting activity of rosuvastatin as a probe for OATPs and the *in vitro* DDI between rosuvastatin and cyclosporine A in PXB cells, and the results obtained showed time- and temperature-dependent uptake. The transport of rosuvastatin at 1 $\mu\text{mol/l}$ into PXB cells was strongly controlled by active uptake via transporters, and the contribution ratio of active uptake to total uptake was calculated to be 95%, similar to the reported values of 84–93% (Bi et al., 2012). The active transport of rosuvastatin occurred in a concentration-dependent

TABLE 1

Pharmacokinetic parameters of rosuvastatin after oral (2 mg/kg) and intravenous (1 mg/kg) administrations of rosuvastatin to male PXB mice and SCID mice, and the effect of oral preadministration of cyclosporine A on systemic exposure of rosuvastatin in the both mice

Data are presented as the mean $\pm$ S.D. from three or four animals.

	PXB Mice			SCID Mice		
Cyclosporine A (mg/kg)	50	10	0 (vehicle)	50	10	0 (vehicle)
C_{max} (ng/ml)	506 $\pm$ 126*	196 $\pm$ 26*	46.7 $\pm$ 22.2	237 $\pm$ 68*	40.5 $\pm$ 15.9*	3.31 $\pm$ 1.23
$AUC_{0-4h,oral}$ (ng·h/ml)	1162 $\pm$ 315*	344 $\pm$ 84*	105 $\pm$ 43	402 $\pm$ 167*	69.2 $\pm$ 20.3*	5.14 $\pm$ 1.50
$C_{0,iv}$ (ng/ml)			699 $\pm$ 426			133 $\pm$ 22
$AUC_{0-4h,iv}$ (ng·h/ml)			280 $\pm$ 169			26.2 $\pm$ 2.8
CL_{tot} (l/h/kg)			3.82 $\pm$ 1.56			38.1 $\pm$ 4.5
V_d (l/kg)			1.76 $\pm$ 0.75			7.66 $\pm$ 1.36
$V_{d,ss}$ (l/kg)			5.31 $\pm$ 2.25			10.0 $\pm$ 4.1
AUCR	11	3.3		78	13	

$AUC_{0-4h,iv}$, AUC_{0-4h} after i.v. administration; $AUC_{0-4h,oral}$, AUC_{0-4h} after oral administration; AUCR, $AUC_{0-4h,oral}$ with pretreatment of cyclosporine A/ $AUC_{0-4h,oral}$ with pretreatment of vehicle (control); CL_{tot} , total clearance; V_d , dose/ $C_{0,iv}$; $V_{d,ss}$, V_d at steady state; $C_{0,iv}$, concentration just after i.v. administration (0 hr).

* $P < 0.05$, statistically significant compared with control (vehicle) group.

Fig. 3. Liver-to-blood concentration ratios of rosuvastatin at 4 hours after oral administration of 2 mg/kg rosuvastatin to male PXB mice (a) or SCID mice (b) with oral preadministration of doses of 10 and 50 mg/kg of cyclosporine A. Each point is represented as the mean $\pm$ S.D. ($n = 3-4$). * $P < 0.05$, statistically significant compared with control (vehicle) group. CsA, cyclosporine A.

manner with a K_m value of $4.0 \mu\text{mol/l}$ and a $V_{\max}$ value of $4.63 \text{ pmol/min per } 10^6 \text{ cells}$, whereas the reported K_m value was $10-11 \mu\text{mol/l}$ and the $V_{\max}$ value was $16.3-114 \text{ pmol/min per } 10^6 \text{ cells}$ in human hepatocytes (Menochet et al., 2012; Shen et al., 2013). In the study, we performed the conventional approach of parallel experiments at 37°C and 4°C based on an extended Michaelis-Menten equation that contains passive clearance but does not consider the bidirectional nature of the passive process and intracellular binding (Zamek-Gliszczynski et al., 2013). Those processes would be especially carefully considered to estimate the precise quantitative relationship between in vitro and in vivo. In the in vitro DDI experiment, the uptake of rosuvastatin in PXB cells was inhibited by cyclosporine A in a concentration-dependent manner, and the observed IC_{50} value was $0.21 \mu\text{mol/l}$, whereas the reported IC_{50} values of cyclosporine A to OATP1B1/1B3 were $0.2-0.9 \mu\text{mol/l}$ in OATP1B1- and OATP1B3-expressing cells (Hirano et al., 2006; Treiber et al., 2007; Karlgren et al., 2012; Shen et al., 2013). Therefore, we concluded that the observed uptakes of rosuvastatin in PXB cells were via OATPs and cyclosporine A-inhibited OATPs, as reported previously. In cyclosporine A and other potent inhibitors of OATP1B1 and OATP1B3, preincubation enhances its inhibitory effects on these transporters in vitro (Shitara et al., 2013; Shitara and Sugiyama, 2017), and Gertz et al. (2013) reported that the inhibitory potency of cyclosporine A against OATP1B1 and OATP1B3 is $0.019-0.093 \mu\text{mol/l}$ with preincubation, and $0.198-0.411 \mu\text{mol/l}$ without preincubation using transporter-expressing HEK293 cells, whereas observed value in the study using PXB mice was $0.21 \mu\text{mol/l}$. When conducting a quantitative prediction, such as in vitro-in vivo extrapolation, it would be more suitable to conduct the in vitro DDI study with preincubation via typical transporters like OATPs. In terms of

the species difference between mouse and human, the gene homology of oatps between mice and rats is quite high (Meier-Abt et al., 2005). In addition, regarding in vitro DDIs, there are a few reports (Kamisako et al., 1999; Shitara et al., 2002; Yasuda et al., 2002) about the inhibition of rodent transporters by cyclosporine A in cells, and the IC_{50} values of cyclosporine A against rodent Oatps, Mrp2, and Mdr1 were similar or slightly higher than those in humans. We therefore think that the species difference observed in the IC_{50} values for these transporters might not cause the different DDI potentials between PXB mice and SCID mice.

We then investigated in vivo DDIs in PXB mice. Prior to the interaction study, the pharmacokinetic characteristics of cyclosporine A and rosuvastatin were confirmed in mice. The blood concentration of cyclosporine A reached its maximum rapidly after oral administration, and the blood concentration-time curve was almost constant from 1 to 8 hours after the oral dose in PXB mice and SCID mice. The systemic and liver tissue exposures of cyclosporine A were 3-fold to 5-fold higher in SCID mice than in PXB mice. Cyclosporine A is mainly metabolized in the liver by CYP3A (Turgeon et al., 1992). This may be one of the reasons for the difference in its exposure between SCID mice and PXB mice; however, we cannot reach a concrete conclusion based on the results obtained in the present study. The systemic exposure of rosuvastatin after its oral administration was markedly lower in SCID mice than in PXB mice. Furthermore, we assessed the total systemic clearance of rosuvastatin after its intravenous administration; its total clearance was markedly higher in SCID mice than in PXB mice, and its blood concentrations just after its intravenous administration were approximately 5-fold lower in SCID mice than in PXB mice. Therefore, the lower systemic exposure of rosuvastatin in SCID mice may be

TABLE 2

Estimation of maximum protein-unbound concentration of cyclosporine A at the inlet to the liver, and observed AUCRs in DDI studies

	Rosuvastatin Observed AUCR	Cyclosporine A						Reference
		Dose	$F_u F_g$	k_a	Q_h	$f_{u,b}$	$I_{in,max,u}$	
		mg/kg		min^{-1}	ml/min	%	$\mu\text{mol/l}$	
PXB mice	3.3	10	0.3–1.0	0.1	1.8	10	0.29–0.94	Yoshida et al. (2012) Izumi et al. (2015)
	11	50	0.3–1.0	0.1	1.8	10	1.4–4.7	
SCID mice	13	10	0.3–1.0	0.1	1.8	10	0.36–0.96	
	78	50	0.3–1.0	0.1	1.8	10	1.5–4.8	
Patients	7.1	200 ^a	1.0	0.1	1450	10	1.3	
					1620	11	1.2	

^aMilligrams per body.

TABLE 3

Blood and tissue concentrations in the liver of cyclosporine A after oral administration of doses of 10 and 50 mg/kg cyclosporine A in PXB mice and SCID mice. Data are presented as the mean from three or four animals.

	Dose	Systemic Exposure		Liver Tissue Exposure	Inlet to Liver	Reported IC ₅₀					Reference
		C _{max}	AUC			C _{sh}	I _{in,max,u}	OATP1B1	OATP1B3	MRP2	
		mg/kg	μmol/l	μmol·h/l	μmol/kg tissue	μmol/l	μmol/l				
PXB mice	10	0.131	0.292	1.07	0.29–0.94	0.15	0.68	9.3	1.5	1.7	Yoshida et al. (2012)
	50	0.349	1.02	5.89	1.4–4.7						
SCID mice	10	0.402	1.20	4.95	0.36–0.96						
	50	1.58	5.25	30.0	1.5–4.8						

C_{8h}, corresponding to liver concentrations 8 hours after oral administration of rosuvastatin; MDR1, multidrug resistance protein 1.

caused by the higher distribution volume of rosuvastatin rather than the higher elimination rate. Iusuf et al. (2013) reported that normal mice had low systemic concentrations of rosuvastatin, mainly due to rapid and high liver distribution. In the present study, the liver-to-blood concentration ratio of rosuvastatin was markedly higher in SCID mice than in PXB mice, which indicates a higher amount of uptake in SCID mice than in PXB mice. Previous studies also reported that rodent hepatocytes exhibit higher uptake activities for substrates via oatps/OATPs than human hepatocytes (Menochet et al., 2012; Grime and Paine, 2013), and that human OATPs-transgenic mice have a weaker uptake potency for statins than control mice (Higgins et al., 2014; Salphati et al., 2014). Based on the characteristics of cyclosporine A and rosuvastatin, we investigated in vivo DDI and showed that the systemic exposure of rosuvastatin in PXB mice increased by 3.3-fold and 11-fold with the oral preadministration of cyclosporine A at doses of 10 and 50 mg/kg 4 hours before the administration of 2 mg/kg rosuvastatin. Furthermore, 13-fold and 78-fold increases in the systemic exposure of rosuvastatin were observed in SCID mice with the same conditions in the treatment with cyclosporine A. In a clinical study, a 7.1-fold increase in the systemic exposure of rosuvastatin was observed with treatment with 75–200 mg of cyclosporine in heart transplant patients (Simonson et al., 2004).

The mechanisms for the inhibitory process of an inhibitor against the uptake transporters, OATPs, in the liver have been elucidated in detail. The $I_{in,max,u}$ may directly affect its potency to inhibit transporters expressed on the sinusoidal membrane of the liver. Yoshida et al. (2012) reported that predicted AUCRs based on $I_{in,max,u}$ were similar to clinically observed AUCRs within a 2-fold to 3-fold error margin in 44 of 52 studies. In the present study, we calculated the $I_{in,max,u}$ in each dosing case in PXB mice and SCID mice to evaluate comparability between calculated $I_{in,max,u}$ values and observed AUCRs. With the preadministration of cyclosporine A at a dose of 10 mg/kg, 3.3-fold and 13-fold increases in the systemic exposure of rosuvastatin were observed, with $I_{in,max,u}$ values of 0.29–0.94 and 0.36–0.96 μmol/l cyclosporine A in PXB mice and SCID mice, respectively. When the dosage of cyclosporine A increased to 50 mg/kg, AUCRs were 11 and

78 with $I_{in,max,u}$ values of 1.4–4.7 and 1.5–4.8 μmol/l cyclosporine A in PXB mice and SCID mice, respectively. Although the observed AUCRs markedly differed between PXB mice and SCID mice at the same dose level of cyclosporine A, both $I_{in,max,u}$ values were close to each other. Clinical findings showed that when a 7.1-fold increase was observed, the estimated $I_{in,max,u}$ value was 1.2–1.3 μmol/l (Yoshida et al., 2012; Izumi et al., 2015). Therefore, the observed AUCRs of rosuvastatin in PXB mice in the present study may be reasonable and in accordance with the estimated $I_{in,max,u}$ values of cyclosporine A. On the other hand, the 78-fold increase in the systemic exposure of rosuvastatin in SCID mice with 50 mg/kg cyclosporine A requires scientific and biologic explanations, and we found and focused on the high concentrations of cyclosporine A in the liver. Eight hours after the oral administration of 50 mg/kg cyclosporine A (at the same time as 4 hours after the oral administration of rosuvastatin), liver concentrations were 30.0 μmol/kg tissue in SCID mice and 5.89 μmol/kg tissue in PXB mice. Rosuvastatin is known to be excreted into bile via transporters expressed on the apical membrane of hepatocytes (e.g., MRP2 and BCRP) (Huang et al., 2006; Ellis et al., 2013), and the reported IC₅₀ values of cyclosporine A compared with MRP2 and BCRP were 9.3 and 1.5 μmol/l, respectively (Yoshida et al., 2012). The mechanisms by which an inhibitor acts toward transporters expressed on the apical membrane inside hepatocytes have not been elucidated; however, the results of the present study indicate that high concentrations of cyclosporine A in the liver affect the pharmacokinetics of rosuvastatin and increase its systemic exposure by 78-fold in SCID mice (Fig. 4). In addition, we have additionally investigated concentrations of the hydroxy form of cyclosporine A, a potential inhibitor of OATPs, and the concentrations of the metabolite in both blood and liver were very high, and were at the same levels as the unchanged form (Supplemental Table 3). Since the inhibitory potency in vitro of the metabolite cyclosporine AM1 against OATPs has been reported at the same level as the unchanged form (Gertz et al., 2013), the inhibitory potency in vivo of cyclosporine AM1 should be noticed to estimate the quantitative relationship between concentrations of those inhibitors and AUCRs of victim rosuvastatin observed. To assess

Fig. 4. (a) Estimated mechanism of in vivo DDI between rosuvastatin and cyclosporine A via transporters (absorbed rosuvastatin is taken up by multiple transporters expressed in the sinusoidal membrane of human hepatocytes, e.g., OATPs, and excreted via other transporters expressed on the apical membrane inside hepatocytes, e.g., BCRP and MRP2). (b) The uptake and efflux of rosuvastatin were inhibited by the coadministered cyclosporine A, and subsequently the systemic exposure of rosuvastatin would increase.

intestinal DDIs, we additionally conducted *in vivo* DDI experiments by intravenous administration of rosuvastatin with pretreatment using 10 and 50 mg/kg cyclosporine A in PXB mice, which yielded observed AUCRs of 2.5 and 6.2, respectively (Supplemental Table 1), and the AUCRs were 3.3 and 11 with the oral administration of rosuvastatin. Therefore, the AUCRs for the intestinal DDIs would be considered to be 1.3 at a dose of 10 mg/kg cyclosporine A and 1.8 at a dose of 50 mg/kg cyclosporine A. We think that the magnitude of the hepatic portion of total DDIs is still major, but there was some level of intestinal DDIs that occurred between rosuvastatin and cyclosporine A, especially at the higher cyclosporine A dose. Takahashi et al. (2013) reported the possibility of the intestinal DDIs between pitavastatin and cyclosporine A in cynomolgus monkeys. As described above, cyclosporine A is known to inhibit not only OATPs but also BCRP and MRP2, which are the transporters that rosuvastatin is taken by (Fig. 4). The intestinal DDIs might be one of the limiting factors for the usage of the chimeric mice with humanized liver to estimate human DDIs accurately because of the lack of the human gastrointestinal condition. Explorations of the dose dependency of an inhibitor and its magnitude in relation to gastrointestinal transporters should be conducted when using the chimeric mice for hepatic DDI studies.

In the present study, we also measured the metabolites of rosuvastatin to identify the replacement of human livers in PXB mice and to assess the inhibitory effects of cyclosporine A on the metabolism of rosuvastatin. Rosuvastatin is known to be absorbed (~50%) and largely excreted in its unchanged form into bile (Martin et al., 2003a,b). In addition, the lactone form (UGT1A metabolite) and the *N*-desmethyl form (CYP2C metabolite) have also been reported as the metabolites of rosuvastatin in clinical studies (Prueksaritanont et al., 2002; Martin et al., 2003b). In the present study, these two metabolites were monitored, and the lactone form was detected in the livers of PXB mice, whereas the *N*-desmethyl form was also detected in the plasma and livers of PXB mice. However, these metabolites were hardly detected in SCID mice. These results suggest that these metabolites may be human-specific or human-abundant metabolites. In the *in vivo* DDI experiments of the present study, because the exposure of these two metabolites in PXB mice was markedly lower than the increased AUCs of the unchanged form of rosuvastatin, and there were almost no effects on the concentrations of these metabolites with or without the administration of cyclosporine A, we estimate that the *in vivo* DDIs observed in the present study were considered to be mainly due to a transporter-mediated phenomenon, not a metabolism-mediated one. In conclusion, the results in this study indicate that PXB mice might be a useful tool for predicting human OATP-mediated DDIs, and its limitations due to differences in gastrointestinal conditions from humans should also be considered. We observe that the mice would be quite useful when we are estimating the potency of a drug candidate as an inhibitor on any DDI via human hepatic transporters; however, a probe compound (victim) should be administered not only orally but also intravenously to estimate the effect of the inhibitory magnitude of a candidate drug on gastrointestinal transports. In addition, it would be better for us to conduct those studies with different dose levels of a candidate drug to learn the linearity/proportionality of the *in vivo* DDIs.

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Authorship Contributions

Participation in research design: Uchida and Tajima.

Conducted experiments: Uchida, Tajima, Kakuni, Kageyama, Okada, and Sakurada.

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