Utility of Bilirubins and Bile Acids as Endogenous Biomarkers for the Inhibition of Hepatic Transporters

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ABSTRACT

It is useful to identify endogenous substrates for the evaluation of drug-drug interactions via transporters. In this study, we investigated the utility of bilirubins, substrates of OATPs and MRP2, and bile acids and substrates of NTCP and BSEP, as biomarkers for the inhibition of transporters. In rats administered 20 and 80 mg/kg rifampicin, the plasma levels of bilirubin glucuronides were elevated, gradually decreased, and almost returned to the baseline level at 24 hours after administration without an elevation of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). This result indicates the transient inhibition of rOatps and/or Mrp2. Although the correlation between free plasma concentrations and IC₅₀ values of rOatps depended on the substrates used in the in vitro studies, the inhibition of rOatps by rifampicin was confirmed in the in vivo study using valsartan as a substrate of rOatps. In rats administered 10 and 30 mg/kg cyclosporin A, the plasma levels of bile acids were elevated and persisted for up to 24 hours after administration without an elevation of ALT and AST. This result indicates the continuous inhibition of rNTcp and/or rBsep, although there were differences between the free plasma or liver concentrations and IC₅₀ values of rNTcp or rBsep, respectively. This study suggests that the monitoring of bilirubins and bile acids in plasma is useful in evaluating the inhibitory potential of their corresponding transporters.

Introduction

Bilirubins and bile acids are commonly used as hepatotoxicity markers in addition to liver enzymes such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST). In hepatobiliary transporters, bilirubins are substrates of OATPs and MRP2, and bile acids and substrates of NTCP and BSEP, as biomarkers for the inhibition of transporters. In rats administered 20 and 80 mg/kg rifampicin, the plasma levels of bilirubin glucuronides were elevated, gradually decreased, and almost returned to the baseline level at 24 hours after administration without an elevation of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). This result indicates the transient inhibition of rOatps and/or Mrp2. Although the correlation between free plasma concentrations and IC₅₀ values of rOatps depended on the substrates used in the in vitro studies, the inhibition of rOatps by rifampicin was confirmed in the in vivo study using valsartan as a substrate of rOatps. In rats administered 10 and 30 mg/kg cyclosporin A, the plasma levels of bile acids were elevated and persisted for up to 24 hours after administration without an elevation of ALT and AST. This result indicates the continuous inhibition of rNTcp and/or rBsep, although there were differences between the free plasma or liver concentrations and IC₅₀ values of rNTcp or rBsep, respectively. This study suggests that the monitoring of bilirubins and bile acids in plasma is useful in evaluating the inhibitory potential of their corresponding transporters.

Abstract

It is useful to identify endogenous substrates for the evaluation of drug-drug interactions via transporters. In this study, we investigated the utility of bilirubins, substrates of OATPs and MRP2, and bile acids and substrates of NTCP and BSEP, as biomarkers for the inhibition of transporters. In rats administered 20 and 80 mg/kg rifampicin, the plasma levels of bilirubin glucuronides were elevated, gradually decreased, and almost returned to the baseline level at 24 hours after administration without an elevation of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). This result indicates the transient inhibition of rOatps and/or Mrp2. Although the correlation between free plasma concentrations and IC₅₀ values of rOatps depended on the substrates used in the in vitro studies, the inhibition of rOatps by rifampicin was confirmed in the in vivo study using valsartan as a substrate of rOatps. In rats administered 10 and 30 mg/kg cyclosporin A, the plasma levels of bile acids were elevated and persisted for up to 24 hours after administration without an elevation of ALT and AST. This result indicates the continuous inhibition of rNTcp and/or rBsep, although there were differences between the free plasma or liver concentrations and IC₅₀ values of rNTcp or rBsep, respectively. This study suggests that the monitoring of bilirubins and bile acids in plasma is useful in evaluating the inhibitory potential of their corresponding transporters.
and the Japanese Pharmaceuticals and Medical Devices Agency (http://www.nihs.go.jp/mlhw/20131488.pdf). Therefore, in developing new drugs, we have focused on transporter-mediated DDIs. Among transporters, OATPs are involved in the hepatic uptake of various kinds of anionic drugs, such as HMG-CoA reductase inhibitors (Hirano et al., 2004; Kitamura et al., 2008) and angiotensin II receptor antagonists (Nakagomi-Hagihara et al., 2006; Yamashiro et al., 2006), and they affect the pharmacokinetics of their substrates (Yoshida et al., 2012; Prueksaritanont et al., 2014). Significant DDIs mediated by OATPs have been reported in coadministration of inhibitors, such as rifampicin and cyclosporin A, and many of their substrates, such as pravastatin, atorvastatin, and repaglinide (Kajosaari et al., 2005; Lau et al., 2007; Maeda et al., 2011). In addition, it has been shown that a severe adverse effect of simvastatin was caused by a genetic polymorphism of OATP1B1 (Link et al., 2008).

In the present study, to investigate the potential of plasma conjugated bilirubins and bile acids as biomarkers for the inhibition of transporters, we examined the changes in plasma levels of bilirubins and bile acids in rats administered transporter inhibitors. Rifampicin and cyclosporin A were used as model compounds because rifampicin is a potent and relatively selective inhibitor of OATPs and cyclosporin A is a nonselective inhibitor of multiple transporters (Yoshida et al., 2012). If endogenous substances can be used as biomarkers for the inhibition of transporters, the inhibitory potential for transporter-mediated DDIs of a compound in vivo can be predicted by only monitoring certain biochemical parameters.

### Materials and Methods

#### Reagents.
Rifampicin was purchased from Sigma-Aldrich (St. Louis, MO), and cyclosporin A in powder form and Sandimmun were purchased from Sigma-Aldrich and Novartis Pharma (Basel, Switzerland), respectively. [1H]estradiol-17β-glucuronide (E17βG) (34.3 Ci/mmol) and [1H]taurocholate (TCA) (5.0 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Unlabeled E17βG, TCA, and (5-6)-carboxy-2′,7-dichlorofluorescein (CDCF) were purchased from Sigma-Aldrich. Bilirubin was purchased from Frontier Scientific, Inc. (Logan, UT) and bilirubin glucuronide was enzymatically generated in Xenotech, LLC (Lenexa, KS). Rat pooled liver microsomes were prepared by XenoTech. All other chemicals were commercially available and of reagent grade.

#### Animals.
All procedures for the animal experiments were approved by the Animal Ethics Committee of Mitsubishi Tanabe Pharma Corporation. Six-week-old male Sprague-Dawley (SD) rats were obtained from Charles River Japan (Yokohama, Japan). The rats were housed in a temperature- and humidity-controlled room with a 12 hour light/dark cycle and acclimatized for at least 7 days before experiments. Food and water were provided ad libitum.

#### In Vivo Study.
In the experiments to investigate the effects of rifampicin and cyclosporin A on endogenous bilirubins and bile acids, SD rats were divided into eight experimental groups: four groups for rifampicin, three for cyclosporin A, and one for untreated. Rifampicin was dissolved in N,N-dimethylacetamide and diluted 2-fold with saline. SD rats received a bolus intravenous injection of 5, 20, or 80 mg/kg rifampicin or vehicle alone (1 ml/kg) into the tail vein. For the dosing solution of cyclosporin A, sandimmun was directly used or diluted with saline. SD rats received a bolus intravenous injection of 10 or 30 mg/kg cyclosporin A (0.5 or 0.6 ml/kg, respectively) or vehicle alone (0.6 ml/kg) into the tail vein. As a vehicle control for cyclosporin A, a solution containing 61.9% Cremophor EL and 38.1% ethanol was prepared and administered to the rats. Blood samples were collected from the jugular vein at 0.17, 0.5, 1, 2, 6, and 24 hours after administration and centrifuged to obtain plasma. In the untreated group, blood collection was performed without administration. The rats were sacrificed at 24 hours after administration and their livers were immediately removed. To measure the concentrations of rifampicin and cyclosporin A in the liver at an earlier time, additional rats were intravenously administered rifampicin or cyclosporin A and sacrificed at 1 or 2 hours when the plasma levels of bilirubins or bile acids reached their maximums, respectively. Plasma samples for rifampicin quantification were mixed with an equal volume of 200 μg/ml acetic acid to prevent the oxidation of rifampicin. Liver samples were mixed with 4-fold volumes of phosphate-buffered saline (PBS) and homogenized. All the samples were stored at −20°C until measurement.

In the experiments to investigate the effects of rifampicin on the hepatic uptake of valsartan, valsartan was intravenously administered to SD rats alone and in combination with rifampicin. Valsartan and rifampicin were dissolved in N,N-dimethylacetamide and diluted 2-fold with saline. SD rats received a bolus intravenous injection of 0.5 mg/kg valsartan with and without 20 or 80 mg/kg rifampicin (1 ml/kg) into the tail vein. Blood samples were collected from the jugular vein at 0.083, 0.17, 0.5, 1, 2, 4, 6, 8, and 24 hours after administration and centrifuged to obtain plasma. To measure the concentrations of valsartan in the liver at an earlier time, additional rats were intravenously administered valsartan alone and in combination with rifampicin and sacrificed at 1 hour after administration. Liver samples were mixed with 4-fold volumes of PBS and homogenized. All the samples were stored at −20°C until measurement.

#### Analysis of Biochemical Parameters.
ALT, AST, total bilirubin (unconjugated and conjugated), unconjugated bilirubin, and total bile acids (TBAs) were measured using an automatic blood chemistry analyzer (Hitachi Model 7180, Hitachi High-Technologies Corporation). Because the UV absorption of rifampicin affects the quantification of bilirubins, the bilirubin concentrations after administration of rifampicin were calculated by subtracting the UV absorption of the rifampicin concentration itself from that in bilirubin quantification. The concentrations of unconjugated bilirubin were calculated by subtracting the concentration of conjugated bilirubin from that of total bilirubin.

#### Plasma Protein Binding and Liver Tissue Binding.
The unbound fractions in rat plasma and liver were determined using an ultracentrifugation method (Barre et al., 1985). Plasma and liver samples were collected from drug untreated male SD rats, and liver samples were mixed with 4-fold volumes of PBS and homogenized. Rifampicin and cyclosporin A were individually added to the plasma and liver homogenates at designated final concentrations. The mixtures were centrifuged at 100,000g for 4 hours at 37°C. The concentrations of the drug in the upper fraction of the centrifuged sample and in the mixture before the centrifugation were measured using liquid chromatography–tandem mass spectrometry (LC-MS/MS) to determine the unbound and total concentrations, respectively. The liver unbound fractions (ft) were calculated according to the following equation (Maurer et al., 2005):

\[
ft = \frac{(1/D)}{\left(\frac{1}{f_{\text{tissue}}} - 1\right) + 1/D}
\]

where f_{tissue} represents the unbound fraction in diluted liver homogenates and D represents the dilution rate.

#### Inhibition Study for [1H]E17βG and [3H]TCA Uptake in Primary Cultured Rat Hepatocytes.
The isolation of hepatocytes was performed using a liver perfusion medium (Life Technologies, Rockville, MD) and liver digest medium (Life Technologies) according to the manufacturer’s protocol. Rat hepatocytes were cultured and the uptake study was performed as described in previous reports (Shitara et al., 2009). In brief, the uptake process was initiated by replacing the Hank’s balanced salt solution (Life Technologies) containing 0.1 μM [1H]E17βG or 0.1 μM [3H]TCA [tracer condition, much less than the reported Km (Carotti et al., 2001; Hata et al., 2003)] and an inhibitor (0, 0.3, 1, 3, 10, 30, and 100 μM for rifampicin and 0, 0.03, 0.1, 0.3, 1, 3, and 10 μM for cyclosporin A, 0 μM; solvent control) or 100 μM E17βG or 1000 μM TCA (excess condition, much higher than the reported Km).

#### Inhibition Study for CDCF and [3H]TCA Transport in Rat Mrp2- and Bsep-Expressing Membrane Vesicles.
The vesicular transport study using rat Mrp2- and Bsep-expressing S9 membrane vesicles (Genomembrane, Yokohama, Japan) and an assay reagent kit (Genomembrane) was conducted using a rapid filtration technique according to the manufacturer’s protocol. The membrane vesicle study was performed as described in previous reports (Fukuda et al., 2010). In brief, the vesicular transport reaction was initiated by addition of transport buffer containing the substrate [0.1 μM CDCF for Mrp2 or 0.54 μM [3H]TCA for Bsep, much less than the reported Km (Gerloff et al., 1998; Colombo et al., 2012)] and an inhibitor (Mrp2; 0, 0.1, 3, 10, 30, and 100 μM for rifampicin and 0.03, 0.1, 0.3, 1, 3, and 10 μM for cyclosporin A; 0 μM; solvent control) or 100 μM E17βG or 1000 μM TCA (excess condition, much higher than the reported Km).

#### Inhibition Study for [3H]E17βG and [3H]TCA Transport in Rat Mrp2- and Bsep-Expressing Membrane Vesicles.
This study focused on transporter-mediated DDIs, and the Japanese Pharmaceuticals and Medical Devices Agency (http://www.nihs.go.jp/mlhw/20131488.pdf) approved the study. Therefore, in developing new drugs, we have focused on transporter-mediated DDIs. Among transporters, OATPs are involved in the hepatic uptake of various kinds of anionic drugs, such as HMG-CoA reductase inhibitors (Hirano et al., 2004; Kitamura et al., 2008) and angiotensin II receptor antagonists (Nakagomi-Hagihara et al., 2006; Yamashiro et al., 2006), and they affect the pharmacokinetics of their substrates (Yoshida et al., 2012; Prueksaritanont et al., 2014). Significant DDIs mediated by OATPs have been reported in coadministration of inhibitors, such as rifampicin and cyclosporin A, and many of their substrates, such as pravastatin, atorvastatin, and repaglinide (Kajosaari et al., 2005; Lau et al., 2007; Maeda et al., 2011). In addition, it has been shown that a severe adverse effect of simvastatin was caused by a genetic polymorphism of OATP1B1 (Link et al., 2008).
Obach, 2004; Ogilvie et al., 2006). In brief, rat pooled liver microsomes were diluted with the incubation buffer (100 mM Tris-HCl, 10 mM MgCl₂, 1 mM EDTA, and 2.5 mM d-tartaric acid/L-lactone, pH 7.7) to 50 μg protein/ml. After adding the stock solution of an inhibitor (final concentration: 0, 0.03, 0.1, 0.3, 1, 3, 10, and 30 μM for cyclosporin A; 0, 0.1, 0.3, 1, 3, 10, 30, and 300 μM for rifampicin; 0 μM for the solvent control) and bilirubin (final concentration: 0.5 μM, much less than the reported Kᵢₑ) to the diluted microsomes, reactions were initiated by the addition of uridine diphosphate glucuronic acid. The reaction was terminated by the addition of a stop reagent (50:50 v/v methanol:acetonitrile with 100 mM ascorbic acid containing an internal standard).

**LC-MS/MS Analysis.** The plasma and liver homogenized samples were deproteinized in acetonitrile and centrifuged at 3,100 g for 10 minutes. The supernatants were subjected to LC-MS/MS analysis. The appropriate standard curves were prepared in the equivalent blank matrix and used for each analysis. For the analysis of rifampicin and valsartan, the LC-MS/MS systems consisted of an Acquity UPLC system (Waters, Milford, MA) and tandem mass spectrometers (TQ-S Xevo, Waters). Rifampicin and pravastatin (internal standard) or valsartan and verapamil (internal standard) were separated on an Acquity UPLC BEH C₁₈ column (1.7 μm, 2.1 × 30 mm, Waters) at a flow rate of 0.5 ml/min for mobile phases consisting of 0.1% formic acid and acetonitrile. The gradient condition for elution was 2–95% acetonitrile (0.0–0.2 minutes), 95% acetonitrile (0.2–0.7 minutes), and 2% acetonitrile (0.71–1.0 minutes). Using negative (for rifampicin) and positive (for valsartan) electrospray mode, molecular ions were formed at a capillary voltage energy of 0.5 kV, source temperature of 150°C, desolvation gas temperature of 600°C, desolvation gas of nitrogen, flow rate of 1000 l/h, and cone gas flow energy were m/z 436.19, 437.03, 20 V, and 20 eV for rifampicin, and m/z 821.40 → 397.03, 50 V, and 35 eV for verapamil, respectively. For the analysis of cyclosporin A, the LC-MS/MS systems consisted of the Acquity UPLC system (Waters) and tandem mass spectrometers (TQ-S Xevo, Waters). Cyclosporin A and verapamil (internal standard) were separated on an Acquity UPLC BEH C₁₈ column (1.7 μm, 2.1 × 30 mm, Waters) at a flow rate of 0.5 ml/min for mobile phases consisting of 0.025% formic acid and acetonitrile. The gradient conditions for elution were 2–95% acetonitrile (0.0–2.2 minutes), 95% acetonitrile (2.2–2.7 minutes), and 2% acetonitrile (2.71–3.0 minutes). In the positive electrospray mode, molecular ions were formed at capillary voltage energy of 3.0 kV, source temperature of 150°C, desolvation gas temperature of 600°C, desolvation gas of nitrogen, flow rate of 1000 l/h, and cone gas flow

**Fig. 1.** Time profiles of total bilirubin (A, D), conjugated bilirubin (B, E), and unconjugated bilirubin (C, F) in plasma after the intravenous administration of rifampicin (A–C) at doses of 5 mg/kg (closed diamonds), 20 mg/kg (closed triangles), and 80 mg/kg (closed circles) and after the intravenous administration of cyclosporin A (D–F) at doses of 10 mg/kg (closed diamonds) and 30 mg/kg (closed circles) in SD rats. Open squares and open triangles represent untreated and vehicle-treated rats, respectively. Each point represents the mean ± S.D. (N = 3). *P < 0.05, **P < 0.01; ***P < 0.001 versus vehicle-treated rats.

**Fig. 2.** Time profiles of TBAs in plasma after the intravenous administration of rifampicin (A) at doses of 5 mg/kg (closed diamonds), 20 mg/kg (closed triangles), and 80 mg/kg (closed circles), and after the intravenous administration of cyclosporin (B) at doses of 10 mg/kg (closed diamonds) and 30 mg/kg (closed circles) in SD rats. Open squares and open triangles represent untreated and vehicle-treated rats, respectively. Each point represents the mean ± S.D. (N = 3). *P < 0.05, **P < 0.01; ***P < 0.001 versus vehicle-treated rats.
rate of 150 l/h. The mass transition, cone voltage, and collision energy were m/z 1203.81 → 156.09, 50 V, and 46 eV for cyclosporin A and m/z 455.26 → 164.90, 16 V, and 28 eV for verapamil, respectively. The analysis of bilirubin monoglucuronide and fulvestrant 3-glucuronide (internal standard) were separated on a Phenomenex Kinetex column (2.6 μm, 2.1 × 50 mm, Waters).

Data Analysis. The uptake clearances (CLs) in rat hepatocytes and in rMrp2- and rBsep-expressing membrane vesicles were calculated according to the following equation:

\[
\text{Uptake CL (μL/mg protein)} = \frac{\text{uptake amount (dpm/well)}}{\text{protein amount (mg/protein/well)} \times \text{incubation buffer (dpm/μL)}}
\]

In the vesicle study, transporter-mediated uptake was calculated by subtracting the uptake under excess condition from that under tracer condition. In the vesicle study, ATP-dependent transport was calculated by subtracting the uptake in the presence of AMP from that in the presence or ATP. The IC50 values were determined by nonlinear regression analysis determined using the Prism software package (version 5; GraphPad Software Inc., La Jolla, CA).

Pharmacokinetic parameters were calculated using noncompartmental methods (WinNonlin software package; Pharsight Corporation, Mountain View, CA). The apparent terminal elimination half-life (t1/2) was determined from the terminal values were determined by nonlinear regression analysis.

\[
\text{CL} = \frac{\text{dose}}{\text{AUC}_{0→\infty}}
\]

The volume of distribution (Vd) was calculated as the dose divided by AUC0→∞. The liver/plasma concentration ratio (Kp,liver) was calculated as the liver concentration divided by the plasma concentration.

Statistical Analysis. The significance of the differences in results for chemical parameters after rifampicin or cyclosporin A administration was determined by using a one-way analysis of variance followed by Dunnett’s multiple comparison test. The Prism software package (version 5; GraphPad Software Inc.) was used to perform statistical analyses. P < 0.05 values were considered statistically significant.

Results

Effects of Rifampicin and Cyclosporin A on Plasma Levels of Bilirubins and TBAs in SD Rats. Plasma total bilirubin, conjugated bilirubins, unconjugated bilirubin, and TBAs were measured after intravenous administration of rifampicin at doses of 5, 20, or 80 mg/kg or cyclosporin A at doses of 10 or 30 mg/kg to SD rats (Figs. 1 and 2).

In the rifampicin-treated groups at 20 and 80 mg/kg, the plasma levels of total bilirubin and conjugated bilirubins were significantly and dose-dependently elevated compared with those in the control groups (untreated and vehicle-treated rats). The increases of 62.8% ± 12.2% and 78.3% ± 16.0% of total bilirubin at 1 hour after administration of 20 and 80 mg/kg rifampicin, respectively, were due to conjugated bilirubins. The plasma levels of total and conjugated bilirubin reached maximums at 1 hour after rifampicin administration, gradually decreased, and returned to near baseline after 24 hours. The plasma level of TBAs increased slightly, but significantly, in the rifampicin-treated groups. In the cyclosporin A–treated groups at 10 and 30 mg/kg, the
The inhibitory effects of rifampicin and cyclosporin A on rOatps and rNtcp in Rat Hepatocytes were examined in vitro. 

Inhibitory Effects of Rifampicin and Cyclosporin A on rMrp2 and rBsep in Membrane Vesicles. In Mrp2- and Bsep-expressing membrane vesicles, the inhibitory effects of rifampicin and cyclosporin A on rMrp2 and rBsep were examined. CDCF and [1H]TCA were used as substrates of rMrp2 and rBsep, respectively. The IC_{50} values of rifampicin and cyclosporin A were 34.0 and 9.20 μM for rMrp2, respectively, and 67.9 and 1.75 μM for rBsep, respectively (Table 2).

Inhibitory Effects of Rifampicin and Cyclosporin A on rUGT1A1 in Rat Microsomes. In rat liver microsomes, the inhibitory effects of rifampicin and cyclosporin A on bilirubin glucuronidation mediated by Ugt1a1 were examined. The IC_{50} values of rifampicin and cyclosporin A for rUGT1A1 were 113 and >30 μM, respectively (Table 1).

Inhibitory Effects of Rifampicin on the Pharmacokinetics of Valsartan in SD Rats. It has been reported that valsartan is taken up by Oatps into the liver and predominantly excreted into the bile in an unchanged form by rMrp2 (Yamashiro et al., 2006) and that the hepatic uptake is a rate-limiting process in hepatic elimination (Watanabe et al., 2009). Therefore, to investigate the inhibitory effects of rifampicin on the hepatic uptake in vivo, valsartan was used as a sensitive substrate of Oatps. After 0.5 mg/kg valsartan was intravenously administered to SD rats in combination with 20 or 80 mg/kg intravenous rifampicin, the plasma concentrations of valsartan significantly increased compared with those after intravenous administration of valsartan alone. The AUC_{0-6} values of valsartan were increased approximately eight- and 14-fold, respectively, and the CL_{s} of valsartan were reduced to approximately one-ninth and one-fourteenth, respectively, whereas V_{d} of valsartan decreased to the theoretically minimum value (Fig. 4; Table 3). In addition, K_{p,liver} values calculated from K_{p,liver} of valsartan were decreased to similar low values at 1 hour after administration in combination with 20 or 80 mg/kg rifampicin. These results indicate that Oatps were substantially inhibited in vivo after the intravenous administration of rifampicin at doses of 20 and 80 mg/kg.

**Discussion**

In this study, the plasma levels of the biochemical parameters were evaluated in rats after intravenous administration of rifampicin or cyclosporin A to examine the potential of plasma conjugated bilirubins and plasma bile acids as biomarkers for the inhibition of transporters. In the groups administered 20 and 80 mg/kg rifampicin, the plasma levels of bilirubins were significantly elevated compared with those in the control rats. As shown in Fig. 1, the plasma levels of bilirubins quickly responded to the changes in the plasma concentrations of rifampicin and cyclosporin A.

**TABLE 1**

Unbound fractions of rifampicin and cyclosporin A in rat plasma (f_p) and rat liver homogenate (f_l).

The f_p and f_l values were examined at different concentrations. Each value represents the mean ± S.D. (N ≥ 3).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Material</th>
<th>Concentration</th>
<th>Unbound Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>μg/ml</td>
<td></td>
</tr>
<tr>
<td>Rifampicin</td>
<td>Plasma</td>
<td>1</td>
<td>0.144 ± 0.082</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>0.0864 ± 0.0037</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>0.110 ± 0.006</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>1</td>
<td>0.0626 ± 0.0039</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>0.0496 ± 0.0009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>0.0538 ± 0.0007</td>
</tr>
<tr>
<td>Cyclosporin</td>
<td>Plasma</td>
<td>1</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>0.0143 ± 0.0002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>0.0364 ± 0.0024</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>3</td>
<td>0.00207 ± 0.0040</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>0.000770 ± 0.00626</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>0.0125 ± 0.0112</td>
</tr>
</tbody>
</table>

N.D., not determined (below the lower limit of quantification).

**TABLE 2**

IC_{50} values of rifampicin and cyclosporin A for Oatps, Ntcp, Mrp2, Bsep, and Ugt1a1

The IC_{50} values were calculated from the data obtained in the inhibition study in triplicate determinations for Oatps, Ntcp, and Ugt1a1 and in duplicate determinations for Mrp2 and Bsep. The average values of the IC_{50} values were shown in two independent experiments.

<table>
<thead>
<tr>
<th>Transporter/Enzyme</th>
<th>Experimental System</th>
<th>Substrate</th>
<th>Rifampicin IC_{50}</th>
<th>Cyclosporin A IC_{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oatps</td>
<td>Rat hepatocytes</td>
<td>E_{17}βG</td>
<td>32.7</td>
<td>4.55</td>
</tr>
<tr>
<td>Ntcp</td>
<td>Rat hepatocytes</td>
<td>TCA</td>
<td>N.D. (&gt;100)</td>
<td>8.63</td>
</tr>
<tr>
<td>Mrp2</td>
<td>Mrp2 membrane vesicles</td>
<td>CDCF</td>
<td>34.0</td>
<td>9.20</td>
</tr>
<tr>
<td>Bsep</td>
<td>Bsep membrane vesicles</td>
<td>TCA</td>
<td>67.9</td>
<td>1.75</td>
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<tr>
<td>Ugt1a1</td>
<td>Rat microsomes</td>
<td>Bilirubin</td>
<td>113</td>
<td>N.D. (&gt;30)</td>
</tr>
</tbody>
</table>

N.D., not determined.
rifampicin; in addition, conjugated bilirubins predominantly increased in a manner similar to those in Oatp1a1/1b knockout mice (van de Steeg et al., 2012) or Eisai hyperbilirubinuria rats (Kawaguchi et al., 1994). This result indicates that rOatps and/or Mrp2 were transiently inhibited during the first few hours after administration. In the groups administered 10 and 30 mg/kg cyclosporin A, the plasma levels of bile acids were elevated significantly from 2 hours after administration compared with those of the control rats. Because the CL of $[^3]$H]TCA has been reported to be sufficiently large in rats (29.4 ml/min/kg) (Hayashi and Sugiyama, 2007), transient increases in bile acids could be immediately cleared. Therefore, the long-lasting elevation of bile acids in the plasma was possibly induced by the continuous inhibition of rNtcp and/or rBsep. In addition, the ALT and AST were not significantly changed in rats administered rifampicin and cyclosporin because of the elevation of bile acids. The free plasma concentration of bilirubin glucuronide could not be commercially obtained. When pravastatin was used as a substrate for rOatps, the uptake was decreased to 50% by 1 µM rifampicin in a rat hepatocytes study (Shingaki et al., 2013). The discrepancy in the inhibitory effects between the reported results and our results may be due to the differences in the contribution of Oatp molecules to the rat hepatic uptake of E217βG and pravastatin. Furthermore, another report stated that the inhibitory effect of rifampicin on rOatpl1a4 and 1b2 was strong (IC50 < 3 µM) but weak on rOatpl1a1 (IC50 > 100 µM) (Fattinger et al., 2000; Lau et al., 2006). Because the reported in vitro result with pravastatin as a substrate of rOatps was in accordance with our in vivo results after administration of rifampicin, it is possible that the contribution of Oatp molecules on the rat hepatic uptake of bilirubin glucuronides is similar to that of pravastatin, and not E217βG.

With pravastatin as a substrate, the ratios of in vitro IC50 values to the free plasma concentrations at time zero (C0, extrapolated initial drug concentration) were 0.46, 1.9, and 9.2 in rats administered 5, 20, and 80 mg/kg rifampicin, respectively (Supplemental Table 1) and conjugated bilirubins were increased 2.75-, 10.5-, and 12.0-fold at 1 hour after administration compared with the vehicle-treated rats, respectively (Fig. 1). As for rMrp2, the free liver concentration of rifampicin did not reach the IC50 value of Mrp2 (34.0 µM), which was obtained using CDCF as a typical substrate for Mrp2. Although CDCF was used as a substrate for rMrp2 in the present study, the reported IC50 value of cyclosporin A using bilirubin monoglucuronide as an Mrp2 substrate (Kamisako et al., 1999) was almost consistent with our determined IC50 value of cyclosporin A. In addition, inhibition studies of rifampicin for rNtcp, rBsep, and rUgt1a1 were conducted but the inhibitory effects were low. On the other hand, after cyclosporin A administration, it was suggested that rNtcp and/or rBsep were inhibited because of the elevation of bile acids. The free plasma concentration even at C0 after administration of 30 mg/kg cyclosporin A was approximately 10 times lower than the rNtcp IC50 value (8.63 µM). As for rBsep, the initial free concentration of the liver would approximately reach the rBsep IC50 value (1.75 µM) in the 30 mg/kg cyclosporin A group but not in the 10 mg/kg cyclosporin A group. The inhibitory effects of cyclosporin A on rat and human Oatps/OATPs were enhanced by preincubation with cyclosporin A in vitro and the inhibitory effects lasted ≥3 days in rats (Shitara et al., 2009, 2012; Gertz et al., 2013). The in vivo result can be explained by the hypothesis that cyclosporin A has a long-lasting inhibitory effect on rBsep and rNtcp. In addition, the IC50 of cyclosporin A values for rOatps and rMrp2 were 4.55 and 9.20 µM, respectively. In the group administered 30 mg/kg cyclosporin A, the small transient increase in plasma bilirubin levels was presumably due to the temporal inhibition of rOatps and/or rMrp2. Considering the aforementioned results, this strongly indicates that the

**TABLE 3**

<table>
<thead>
<tr>
<th>Inhibitor Compound</th>
<th>Inhibitor Dose</th>
<th>AUIC0→1</th>
<th>AUIC0→6</th>
<th>CLt</th>
<th>Vt</th>
<th>Ke,low</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampicin</td>
<td>20 mg/kg</td>
<td>16,686 ± 330</td>
<td>16,781 ± 350</td>
<td>29.8 ± 0.6</td>
<td>96.8 ± 2.5</td>
<td>0.44 ± 0.07</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>80 mg/kg</td>
<td>27,428 ± 6358</td>
<td>28,208 ± 6952</td>
<td>18.6 ± 5.2</td>
<td>104 ± 11</td>
<td>0.42 ± 0.06</td>
</tr>
</tbody>
</table>

Pharmacokinetic parameters of valsartan after intravenous administration with and without rifampicin in SD rats

Valsartan was intravenously administered to SD rats at a dose of 0.5 mg/kg alone and in combination with 20 or 80 mg/kg intravenous rifampicin. Each value represents the mean ± S.D. (N = 3 for the rifampicin-administered groups; N = 6 for no inhibitor group).

**Fig. 4.** Time profiles of the plasma concentrations of valsartan. A dose of 0.5 mg/kg valsartan was intravenously administered to SD rats alone (open squares) or in combination with 20 (closed triangles) or 80 mg/kg (closed circles) of intravenous rifampicin. Each point represents the mean ± S.D. (N = 3 for rifampicin administered groups; N = 6 for no inhibitor group).

**TABLE 3** Pharmacokinetic parameters of valsartan after intravenous administration with and without rifampicin in SD rats

Valsartan was intravenously administered to SD rats at a dose of 0.5 mg/kg alone and in combination with 20 or 80 mg/kg intravenous rifampicin. Each value represents the mean ± S.D. (N = 3 for the rifampicin-administered groups; N = 6 for no inhibitor group).
elucidated. In other cases, previous studies have shown that when the renal biomarkers and DDI from a quantitative perspective have not been

Chiou et al., 2014); however, the exact mechanism of drug-induced

sporin A was reported to increase the plasma levels of total bilirubins

BSEP inhibitions, respectively. The incidence of drug-induced

plasma may be used as biomarkers of OATPs/MRP2 and NTCP/

as evidenced in vitro and the concentration in vivo.

respectively, although there was a difference between the inhibitory
elevation of plasma levels of conjugated bilirubins and bile acids was

skin and follows cholestasis; therefore, drugs that cause the inhibition

bile acids, the elevation of plasma level causes generalized itching of

prognoses (Chowdhury et al., 2001; Keppler, 2014), which suggests

great care is required to avoid hepatotoxicity. However, in the case of

hyperbilirubinemia caused by the inhibition of transporters, the risk of hepatotoxicity appears to be less of a concern in drug development and treatment. Patients with Rotor syndrome and Dubin-Johnson syndrome who complain of conjugated hyperbilirubinemia generally have benign
genes (Chowdhury et al., 2001; Keppler, 2014), which suggests a low toxicity of conjugated bilirubinemia. In contrast, patients with

Crigler-Najjar syndrome type 1 who have unconjugated hyperbilirubinemia often develop kernicterus and die in infancy or childhood (Chowdhury et al., 2001); therefore, great care is required to determine if a marked elevation of plasma unconjugated bilirubin levels occurs. Regarding bile acid, the elevation of plasma level causes generalized itching of skin and follows choleresis; therefore, drugs that cause the inhibition of NTCP and/or BSEP should be avoided, particularly in the case of chronic administration.

Bilirubins and bile acids are commonly used as hepatotoxicity markers, but our study shows that these compounds were elevated by the inhibition of transporters, regardless of hepatotoxicity. The use of conjugated bilirubins and bile acids as biomarkers may help to efficiently elucidate the inhibition of transporters in vivo. In the nonclinical stage, biomarkers for the inhibition of transporters could be a powerful tool for drug candidate selection. In addition, in the clinical stage, particularly in phase

1, the prediction of DDIs through transporters could be performed only by examining the plasma levels of bilirubins, bile acids, and liver enzyme without an additional clinical DDI study using a typical transporter substrate. We hope that the monitoring of bilirubins and bile acids will be

widely recognized as biomarkers for transporter inhibition.

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Authorship Contributions

Participated in research design: Watanabe, Ohashi, Miyake, Shimizu, Masutomi, Shimura.

Conducted experiments: Watanabe, Kamezawa.

Contributed new reagents or analytic tools: Watanabe, Miyake, Kamezawa.

References


