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

Tomoko Watanabe, Manami Miyake, Toshinobu Shimizu, Miho Kamezawa, Naoya Masutomi, Takesada Shimura, and Rikiya Ohashi

DMPK Research Laboratory, Research Division, Mitsubishi Tanabe Pharma Corporation, Saitama, Japan (T.W., M. K., T. S., R. O.)

Safety Research Laboratory, Research Division, Mitsubishi Tanabe Pharma Corporation, Chiba, Japan (M. M., T. S., N. M.)
Running Title: Biomarkers for inhibition of transporters

Corresponding author: Tomoko Watanabe, Ph. D

Address: DMPK Research Laboratory, Research Division, Mitsubishi Tanabe Pharma Corporation, 2-2-50, Kawagishi, Toda-shi, Saitama 335-8505, Japan

Phone: +81-48-433-8106; Fax: +81-48-433-8170

E-mail: Watanabe.Tomoko@mc.mt-pharma.co.jp

Text pages  45

Tables  3

Figures  4

References  49

Abstract  237 words

Introduction  742 words

Discussion  1480 words

Nonstandard abbreviations list: OATP, organic anion transporting polypeptides; MRP, multidrug resistance-associated protein; NTCP, sodium-taurocholate cotransporting polypeptide; BSEP, bile salt export pump; UGT, UDP-glucuronosyl transferase; E217βG, estradiol-17β-glucuronide; TCA,
taurocholate; DDI, drug-drug interaction; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LC-MS/MS, liquid chromatography-tandem mass spectrometry; SD, Sprague–Dawley
ABSTRACT

It is useful to identify endogenous substrates for the evaluation of drug–drug interactions (DDIs) via transporters. In this study, we investigated the utility of bilirubins, substrates of organic anion transporting polypeptides (OATPs) and multidrug resistance-associated protein 2 (MRP2), and bile acids, substrates of sodium taurocholate cotransporting polypeptide (NTCP) and bile salt export pump (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 h after administration without an elevation of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). This result indicates the transient inhibition of rOatps and/or rMrp2. Although the correlation between free plasma concentrations and IC₅₀ values of rOatps depended on the substrates used in vitro studies, the inhibition of rOatps by rifampicin was confirmed 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 h 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 to evaluate the inhibitory potential for their corresponding transporters.
**Introduction**

Bilirubins and bile acids are commonly used as hepatotoxicity markers in addition to liver enzymes such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT). In hepatobiliary transporters, bilirubins are substrates of organic anion transporting polypeptides (OATPs; Cui et al., 2001; Briz et al., 2003) and multidrug resistance-associated protein (MRP) 2 (Kamisako et al., 1999), whereas bile acids are substrates of sodium taurocholate cotransporting polypeptide (NTCP; Hagenbuch and Meier, 1994; Stieger, 2011) and bile salt export pump (BSEP; Noe et al., 2002; Stieger, 2011). Therefore, when the transporters are inhibited by exogenous substances such as drugs, it appears that plasma levels of bilirubins or bile acids are increased independently of drug-induced hepatotoxicity. We have sometimes observed that the plasma levels of bilirubins and bile acids have increased in a nonclinical toxicity study of candidate compounds without an elevation of hepatic enzymes.

Bilirubin is the degradation product of heme from hemoglobin of senescent erythrocytes and hepatic hemoproteins. Oatp1a1b-knockout mice exhibited markedly increased plasma levels of bilirubin glucuronides and a comparison with wild-type controls has shown the detailed mechanism of the hepatobiliary transport of unconjugated and conjugated bilirubins (van de Steeg et al., 2012). Their report demonstrated that unconjugated bilirubin taken up into the liver by passive diffusion and transporters was conjugated to the bilirubin glucuronide and bilirubin diglucuronide by
UDP-glucuronosyl transferase (UGT) 1A1. These bilirubin glucuronides were partially excreted into the bile by Mrp2 and the remainder may have been secreted back to sinusoidal blood by Mrp3. The bilirubin glucuronides in circulation blood were also taken up by Oatps into the liver and excreted into the bile by Mrp2. In humans, abnormalities in any of the processes for hepatobiliary transport and metabolism of bilirubin result in hyperbilirubinemia as well (Chowdhury et al., 2001; Keppler, 2014). Rotor syndrome and Dubin–Jonson syndrome were characterized as conjugated hyperbilirubinemia and were caused by a complete deficiency of both OATP1B1 and OATP1B3 and by mutations affecting MRP2, respectively. In contrast, Crigler–Najjar syndrome and Gilbert’s syndrome were characterized as unconjugated hyperbilirubinemia because of complete and partial absence of UGT1A1 activity, respectively (Chowdhury et al., 2001). Taken together, these results suggest that conjugated bilirubins and unconjugated bilirubin would probably be predominantly increased in the inhibition of OATPs and/or MRP2 and UGT1A1, respectively. Bile acids are formed in liver from cholesterol, excreted into the bile, and mostly reabsorbed into the intestine. In enterohepatic circulation of bile acids, NTCP and BSEP have important roles in the uptake by the liver and in biliary excretion of bile acids, respectively (Wolkoff and Cohen, 2003), and single nucleotide polymorphisms in the BSEP gene have been responsible for progressive familial intrahepatic cholestasis type 2 (PFIC2), which was characterized by cholestasis and jaundice (Strautnieks et al., 1998; Jansen et al., 1999).
The importance of drug–drug interactions (DDIs) mediated by transporters and metabolic enzymes has been recognized, and the guidelines for DDIs via transporters have been published by the US Food and Drug Administration (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm292362.pdf), European Medicines Agency (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/07/WC500129606.pdf), and Japanese Pharmaceuticals and Medical Devices Agency (http://www.nihs.go.jp/mhlw/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 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 Sandimmune® were purchased from Sigma-Aldrich and Novartis Pharma (Basel, Switzerland), respectively. [3H]Estradiol-17β-glucuronide (E217βG; 34.3 Ci/mmol), [3H]taurocholate (TCA; 5.0 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Unlabeled E217βG, 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-h 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 for investigating 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 (DMA) and diluted two-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, Sandimmune® 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 of 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 h after administration and centrifuged to obtain plasma. In the untreated group, blood collection was performed without administration. The rats were sacrificed at 24 h 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 h 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 ascorbic acid to prevent the oxidation of rifampicin. Liver samples were mixed
with four-fold volumes of phosphate-buffered saline (PBS) and homogenized. All the samples were stored at −20°C until measurement.

In the experiments for investigating 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 DMA and diluted two-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 h 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 h after administration. Liver samples were mixed with four-fold volumes of PBS and homogenized. All the samples were stored at −20°C until measurement.

**Biochemical Parameters Analysis**

AST, ALT, total bilirubin (unconjugated and conjugated), unconjugated bilirubin, and total bile acids 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 four-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 h 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-MSMS) to determine the unbound and the total concentrations, respectively. The liver unbound fractions ($f_t$) were calculated according to the following equation (Maurer et al., 2005).

$$f_t = \frac{1}{D} \left[ \frac{1}{f_{t, \text{diluted}}} - 1 \right] + \frac{1}{D},$$

where $f_{t, \text{diluted}}$ represents the unbound fraction in diluted liver homogenates and D represents the dilution rate.
**Inhibition Study for $[^3H]E217\beta G$ 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 (HBSS; Life Technologies) containing 0.1 μM $[^3H]E217\beta G$ and 0.1 μM $[^3H]TCA$ [tracer condition, much less than the reported Km (Cattori 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 E217βG 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 Sf9 membrane vesicles (Genomembrane, Yokohama, Japan) and the assay reagent kit (Genomembrane) was conducted using a rapid filtration technique, according to the manufacturer’s protocol. Membrane vesicle study was performed as described in the 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 the inhibitor (Mrp2; 0, 1, 3, 10, 30, 100, and 300 μM for rifampicin and 0, 0.1, 0.3, 1, 3, 10, and 30 μM for cyclosporin A, Bsep; 0, 3, 10, 30, and 100 μM for rifampicin and 0, 1, 3 10, and 30 μM for cyclosporin A, 0 μM; solvent control).

**Inhibition Study for Bilirubin Glucuronidation in Rat Hepatic Microsomes**

The inhibition study of bilirubin glucuronidation was conducted at XenTech LLC, according to the reported experimental conditions (Fisher et al., 2000; Walsky and 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-saccharic acid 1,4-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 Km) 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 at 4°C for 10 min. 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 MS 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 min), 95% acetonitrile (0.2–0.7 min), and 2% acetonitrile (0.71–1.0 min). 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 (nitrogen) flow rate of 1200 l/h, and cone gas flow rate of 100 l/h. The mass transition, cone voltage, and collision energy were m/z 821.40 → 397.03, 50 V, and 35 eV for rifampicin and were m/z 423.21 → 321.06, 30 V, and 15 eV for pravastatin, respectively. The mass transition, cone voltage, and collision energy were m/z 436.19 → 235.03, 20 V, and 20 eV for valsartan and were m/z 455.28 → 164.94, 40 V, and 30 eV for verapamil, respectively. For the analysis of cyclosporin A, the LC-MS/MS systems consisted of an 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 condition for elution was 2%–95% acetonitrile (0.0–2.2 min), 95% acetonitrile (2.2–2.7 min), and 2% acetonitrile (2.71–3.0 min). In a positive electrospray mode, molecular ions were formed at a 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 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 were m/z 455.26 → 164.90, 16 V, and 28 eV for verapamil, respectively. The analysis of bilirubin monoglucuronide was performed at XenTech LLC. The LC-MS/MS systems consisted of a Shimadzu HPLC system (Kyoto, Japan) and tandem mass spectrometers (API 5500, AB SCIEX, Framingham, MA). 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 in rat hepatocytes and in rMrp2- and rBsep-expressing membrane vesicles were calculated according to the following equations:

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

In the hepatocytes 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 Corp., Mountain View, CA). The apparent terminal elimination half-life (t1/2) was determined from the terminal elimination rate constant (λz), which was calculated by the log-linear regression of the curves of plasma concentration versus time. The area under the plasma concentration–time curve from time zero to time t (AUC0–t), where t is the last time point with a measurable concentration of compounds, was calculated by the linear trapezoidal method. AUC from time zero to infinity (AUC0–∞) was calculated by the trapezoidal rule and extrapolated to infinity with λz. The total clearance (CLt) was calculated as the dose divided by AUC0–∞. The volume of distribution (Vd) was calculated as the dose divided by the product of λz and AUC0–∞. 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 values <0.05 were considered statistically significant.
Results

Effects of Rifampicin and Cyclosporin A on Plasma Bilirubin and Total Bile Acids Level in SD Rats

Plasma total bilirubin, conjugated bilirubins, unconjugated bilirubin, and total bile acids 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 (Fig. 1 and Fig. 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 h 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 h after rifampicin administration, gradually decreased, and returned to near baseline after 24 h. The plasma level of total bile acids slightly increased but significantly in the rifampicin-treated group. In the cyclosporin A-treated groups at 10 and 30 mg/kg, the plasma levels of total bile acids were elevated compared with those of the control groups from 2 h after administration and persisted for up to 24 h, although there was great variability. In contrast, the bilirubin levels were slightly increased in the group administered 30 mg/kg cyclosporin A and almost returned to the baseline after 24 h. There were no significant differences in the levels of ALT and AST between the inhibitor-treated and control groups.
(Supplemental Fig. 1). This result indicates that the plasma levels of bilirubins and bile acids were increased independently of drug-induced hepatotoxicity and the increase in bilirubins and bile acids was caused by the inhibition of transporters.

In addition to the biochemical parameters, the plasma and liver concentrations of rifampicin and cyclosporin A were measured (Fig. 3). To calculate the free concentrations in the plasma and liver, plasma and liver protein bindings were examined at various concentrations in vivo (Table 1). Cyclosporin A showed a concentration-dependent binding ratio in both plasma and liver homogenates, but rifampicin did not. On the basis of the in vitro binding ratio at a concentration closest to the in vivo concentration, free plasma and liver concentrations were calculated at each time point after cyclosporin A administration (Fig. 3).

**In Vitro Study**

To help understand the mechanism underlying increases in the bilirubin and bile acid levels in the plasma, the inhibitory effects of rifampicin and cyclosporin A on various factors involved in the disposition of bilirubin and bile acid plasma levels were examined in vitro.

**Inhibitory Effects of Rifampicin and Cyclosporin A on rOatps and rNtcp in Rat Hepatocytes**

In primary cultured rat hepatocytes, the inhibitory effects of rifampicin and cyclosporin A on rOatps and rNtcp were examined. [3H]E217βG and [3H]TCA were used as substrates of rOatps and rNtcp,
respectively. The IC₅₀ values of rifampicin and cyclosporin A were 32.7 and 4.55 μM for rOatps, respectively, and >100 and 8.63 μM for rNtcp, respectively (Table 2).

**Inhibitory Effects of Rifampicin and Cyclosporin A on rMrp2 and rBsep in Membrane Vesicles**

In rMrp2- and rBsep-expressing membrane vesicles, the inhibitory effects of rifampicin and cyclosporin A on rMrp2 and rBsep were examined. CDCF and [3H]TCA were used as substrates of rMrp2 and rBsep, respectively. The IC₅₀ 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 IC₅₀ 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 was taken up by Oatps into the liver and predominantly excreted into the bile in the unchanged form by rMrp2 (Yamashiro et al., 2006) and the hepatic uptake was 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\textsubscript{0-t} values of valsartan were increased approximately 8- and 14-fold, respectively, and the clearances of valsartan were reduced to approximately one-ninth and one-fourteenth, respectively, whereas the distribution volume of valsartan decreased to the theoretically minimum value (Fig. 4 and Table 3). In addition, the $K_{p,liver}$ values calculated from the plasma and liver concentrations of valsartan were decreased to similar low values at 1 h after administration in combination with 20 or 80 mg/kg rifampicin. These results indicate that rOatps were substantially inhibited \textit{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 in order 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; in addition, conjugated bilirubins predominantly increased in a manner similar to those in Oatp1a/1b KO 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 h after administration compared with those of the control rats. Because the clearance 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 A compared with those in control rats. Hemolysis may result in an increase in the plasma levels of bilirubins; however, rifampicin and
cyclosporin A did not show a hemolytic effect (in-house data).

Next, the free plasma concentrations of rifampicin and cyclosporin A in rats were compared with the IC₅₀ values of rOatps, rMrp2, rNtcp, rBsep, and rUgt1a1, which are the parameters involved in the disposition of bilirubins and bile acids. The free plasma concentration of rifampicin, even at the maximum dose, did not reach the in vitro IC₅₀ value of rOatps (32.7 μM). To examine the inhibition of rOatps by rifampicin in vivo, valsartan (0.5 mg/kg) was intravenously administered to rats alone and in combination with 20 or 80 mg/kg rifampicin (Fig. 4 and Table 3). The plasma concentrations of valsartan were significantly increased and Kₚ, liver were decreased in the groups receiving valsartan in combination with 20 and 80 mg/kg rifampicin. These results indicate that rOatps were considerably inhibited in vivo after the intravenous administrations of rifampicin at doses of 20 and 80 mg/kg, which was consistent with a previous report in which the hepatic uptake of HMG-CoA reductase inhibitors was inhibited by rifampicin in rats (Imaoka et al., 2013). In this study, [³H]E₂₁₇βG was used as a typical substrate of rOatps for the inhibition study because radiolabeled bilirubin glucuronide could not be commercially obtained. In a report, 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 rOatp1a4 and 1b2 was strong (IC₅₀; <3 μM) but weak on rOatp1a1 (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 E₂₁βG. 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 1h 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 rMrp2. 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 rMrp2 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 IC$_{50}$ 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; Shitara et al., 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 IC$_{50}$ 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 above, it is strongly indicated that the elevation of plasma levels of conjugated bilirubins and bile acids was caused by the inhibition of rOatps and/or rMrp2 and rBsep and/or rNtcp, respectively, although there was a difference between the inhibitory effects of in vitro and the concentration of in vivo.

Our study suggests that bilirubin glucuronides and bile acids in the plasma may be used as biomarkers of OATPs/MRP2 and NTCP/BSEP inhibitions, respectively. The incidence of drug-induced hyperbilirubinemia has been documented since rifamycin SV or cyclosporin A was reported to increase the plasma levels of total bilirubins without an elevation of liver enzyme, such as AST and ALT, in humans (Acocella et al., 1965; List et al., 1993). Recently, many researchers have focused on the mechanism of drug-induced hyperbilirubinemia (Zucker et al., 2001; Campbell...
et al., 2004; Chang et al., 2013; Wlcek et al., 2013; Chiou et al., 2014); however, the exact mechanism of drug-induced hyperbilirubinemia in humans and the relationship between the biomarkers and DDI from a quantitative perspective have not been elucidated. In other cases, previous studies have shown that when the renal transporters were inhibited by drugs, the plasma concentrations of creatinine, N-methylnicotinamide, and 6β-hydroxycortisol increased with the inhibition of OCT2 and/or MATEs, MATEs, and OAT3 by drugs, respectively (Imamura et al., 2011; Ito et al., 2012; Imamura et al., 2014). The utility of an endogenous substrate as a biomarker for transporter inhibition has been a topic of great interest.

In developing drugs and in administering medicines for treatment, 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 prognoses (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 acids, the elevation of plasma level causes generalized itching of skin and follows cholestasis; 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 \textit{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 should be widely recognized as biomarkers for transporter inhibition.
Acknowledgments

The authors thank Naomi Takeba, Tomohiko Tanimoto, Masakatsu Takahashi, Masao Yamanouchi, Takashi Tateoka, Tatsuya Honda, and Yoshimi Inoue for technical assistance.
Authorship Contributions

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

Conducted experiments: Watanabe and Kamezawa

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

Performed data analysis: Watanabe and Miyake

Wrote or contributed to the writing of the manuscript: Watanabe and Ohashi
References


Chang JH, Plise E, Cheong J, Ho Q, and Lin M (2013) Evaluating the in vitro inhibition of UGT1A1,
OATP1B1, OATP1B3, MRP2, and BSEP in predicting drug-induced hyperbilirubinemia. Mol Pharm 10:3067-3075.


metabolism and disposition: the biological fate of chemicals 28:560-566.


Hayashi H and Sugiyama Y (2007) 4-phenylbutyrate enhances the cell surface expression and the
transport capacity of wild-type and mutated bile salt export pumps. Hepatology 45:1506-1516.


Ito S, Kusuhara H, Kumagai Y, Moriyama Y, Inoue K, Kondo T, Nakayama H, Horita S, Tanabe K,

Clinical pharmacology and therapeutics 92:635-641.


Keppler D (2014) The roles of MRP2, MRP3, OATP1B1, and OATP1B3 in conjugated hyperbilirubinemia. Drug metabolism and disposition: the biological fate of chemicals


**Figure legends**

**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, B, and C) at doses of 5 mg/kg (closed diamond), 20 mg/kg (closed triangle), and 80 mg/kg (closed circle) and after the intravenous administration of cyclosporin A (D, E, and F) at doses of 10 mg/kg (closed diamond) and 30 mg/kg (closed circle) in SD rats. The open square and open triangle represent untreated and vehicle-treated rats, respectively. Each point represents the mean ± SD (N = 3). *P < 0.05, **P < 0.01, ***P < 0.001 vs. vehicle-treated rats.

**Fig. 2.** Time profiles of total bile acids (TBA) in plasma after the intravenous administration of rifampicin (A) at doses of 5 mg/kg (closed diamond), 20 mg/kg (closed triangle), and 80 mg/kg (closed circle) and after the intravenous administration of cyclosporin A (B) at doses of 10 mg/kg (closed diamond) and 30 mg/kg (closed circle) in SD rats. The open square and open triangle represent untreated and vehicle-treated rats, respectively. Each point represents the mean ± SD (N = 3). *P < 0.05, **P < 0.01, ***P < 0.001 vs. vehicle-treated rats.

**Fig. 3.** Time profiles of the total plasma and liver concentrations of rifampicin (A) and cyclosporin A (C) and the free plasma and liver concentrations of rifampicin (B) and cyclosporin A (D) in SD rats.
after intravenous administration. Rifampicin was administered to SD rats at a dose of 5 mg/kg (plasma, closed diamond; liver, open diamond), 20 mg/kg (plasma, closed triangle; liver, open triangle), and 80 mg/kg (plasma, closed circle; liver, open circle), and cyclosporin A was administered at doses of 10 mg/kg (plasma, diamond; liver, open diamond) and 30 mg/kg (plasma, closed circle; liver, open circle). Each point represents the mean ± SD (N = 3).

**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 square) and in combination with 20 (closed triangle) or 80 mg/kg (closed circle) of intravenous rifampicin. Each point represents the mean ± S.D. (N = 3 for rifampicin administered groups, N = 6 for no inhibitor group).
Table 1. Unbound fractions of rifampicin and cyclosporin A in rat plasma ($f_{p}$) and rat liver homogenate ($f_{i}$)

The $f_{p}$ and $f_{i}$ values were examined at different concentrations. Each value represents the mean ± S.D. ($N \geq 3$)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Material</th>
<th>Concentration µg/mL</th>
<th>Unbound fraction</th>
</tr>
</thead>
<tbody>
<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>Liver</td>
<td>100</td>
<td>0.110 ± 0.006</td>
</tr>
<tr>
<td></td>
<td></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></td>
<td></td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>Plasma</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 ± 0.00119</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>0.00770 ± 0.00626</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>0.0125 ± 0.0112</td>
</tr>
</tbody>
</table>

ND: not determined (below lower limit of quantification)
Table 2. IC<sub>50</sub> values of rifampicin and cyclosporin A for rOatps, rNtcp, rMrp2, rBsep, and rUgt1a1

IC<sub>50</sub> values were calculated from the data obtained in the inhibition study in triplicate determinations for rOatps, rNtcp, and rUgt1a1 and in duplicate determinations for rMrp2 and rBsep. The average values of the rMrp2 and rBsep IC<sub>50</sub> values were shown in two independent experiments.

<table>
<thead>
<tr>
<th>Transporter /enzyme</th>
<th>Experimental system</th>
<th>Substrate</th>
<th>Rifampicin IC&lt;sub&gt;50&lt;/sub&gt; μM</th>
<th>Cyclosporin A IC&lt;sub&gt;50&lt;/sub&gt; μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>rOatps</td>
<td>Rat hepatocytes</td>
<td>E&lt;sub&gt;217&lt;/sub&gt;βG</td>
<td>32.7</td>
<td>4.55</td>
</tr>
<tr>
<td>rNtcp</td>
<td>Rat hepatocytes</td>
<td>TCA</td>
<td>ND (&gt;100)</td>
<td>8.63</td>
</tr>
<tr>
<td>rMrp2</td>
<td>rMrp2 membrane vesicles</td>
<td>CDCF</td>
<td>34.0</td>
<td>9.20</td>
</tr>
<tr>
<td>rBsep</td>
<td>rBsep membrane vesicles</td>
<td>TCA</td>
<td>67.9</td>
<td>1.75</td>
</tr>
<tr>
<td>rUgt1a1</td>
<td>Rat microsomes</td>
<td>Bilirubin</td>
<td>113</td>
<td>ND (&gt;30)</td>
</tr>
</tbody>
</table>

ND: not determined
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 the control group).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Compound</th>
<th>Dose</th>
<th>AUC₀→₄ (ng·h/mL)</th>
<th>AUC₀→∞ (ng·h/mL)</th>
<th>CLₜₚ (mL/h/kg)</th>
<th>Vₜ (mL/kg)</th>
<th>Kp, liver (1h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>2013 ± 482</td>
<td>2025 ± 547</td>
<td>266 ± 90</td>
<td>359 ± 94</td>
<td>18 ± 10</td>
<td></td>
</tr>
<tr>
<td>Rifampicin</td>
<td>20</td>
<td>16686 ± 330</td>
<td>16781 ± 350</td>
<td>29.8 ± 0.6</td>
<td>96.8 ± 2.5</td>
<td>0.44 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>Rifampicin</td>
<td>80</td>
<td>27428 ± 6358</td>
<td>28208 ± 6952</td>
<td>18.6 ± 5.2</td>
<td>104 ± 11</td>
<td>0.42 ± 0.06</td>
<td></td>
</tr>
</tbody>
</table>

-: control group (only valsartan was administered.)
Fig. 1

(A) Total bilirubin (mg/dL) vs. Time (h)

(B) Conjugated bilirubins (mg/dL) vs. Time (h)

(C) Unconjugated bilirubin (mg/dL) vs. Time (h)

(D) Total bilirubin (mg/dL) vs. Time (h)

(E) Conjugated bilirubins (mg/dL) vs. Time (h)

(F) Unconjugated bilirubin (mg/dL) vs. Time (h)
Fig. 2

(A) The graph shows the concentration of TBA (μM) over time (h) ranging from 0 to 24. The data points are connected by lines, indicating a decrease in TBA concentration over time. Error bars are present to indicate the variability of the data.

(B) This graph displays a similar trend as (A), with TBA concentration measured over the same time period. The error bars suggest a high degree of precision in the measurement.
Fig. 3

(A) Total plasma/liver conc. (ng/mL, ng/g liver) vs. Time (h)
(B) Free plasma/liver conc. (μM, nmol/g liver) vs. Time (h)
(C) Total plasma/liver conc. (ng/mL, ng/g liver) vs. Time (h)
(D) Free plasma/liver conc. (μM, nmol/g liver) vs. Time (h)
Fig. 4

![Graph showing plasma concentration over time in ng/mL.](image-url)