Coproporphyrins I and III as Functional Markers of OATP1B Activity: In Vitro and In Vivo Evaluation in Preclinical Species

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ABSTRACT

Inhibition of organic anion-transporting polypeptide (OATP)1B function can lead to serious clinical drug-drug interactions, thus a thorough evaluation of the potential for this type of interaction must be completed during drug development. Therefore, sensitive and specific biomarkers for OATP function that could be used in conjunction with clinical studies are currently in demand. In the present study, preclinical evaluations were conducted to characterize the suitability of coproporphyrins (CPs) I and III as markers of hepatic OATP functional activity. Active uptake of CPs I and III was observed in human embryonic kidney (HEK) 293 cells singly expressing human OATP1B1 (hOATP1B1), hOATP1B3, cynomolgus monkey OATP1B1 (cOATP1B1), or cOATP1B3, as well as human and monkey hepatocytes. Cyclosporin A (100 mg/kg, oral) markedly increased the area under the curve (AUC) plasma concentrations of CPs I and III by 2.6- and 5.2-fold, while rifampicin (15 mg/kg, oral) increased the AUCs by 2.7- and 3.6-fold, respectively. As the systemic exposure increased, the excretion of both isomers in urine rose from 1.6- to 4.3-fold in monkeys. In agreement with this finding, the AUC of rosuvastatin (RSV) in cynomolgus monkeys increased when OATP1B inhibitors were coadministered. In Oatp1a/1b gene cluster knockout mice (Oatp1a/1b−/−), CPs in plasma and urine were significantly increased compared with wild-type animals (7.1- to 18.4-fold; P < 0.001), which were also in agreement with the changes in plasma RSV exposure (14.6-fold increase). We conclude that CPs I and III in plasma and urine are novel endogenous biomarkers reflecting hepatic OATP function, and the measurements have the potential to be incorporated into the design of early clinical evaluation.

Introduction

Human organic anion-transporting polypeptide (OATP)1B1 (SLCO1B1) and OATP1B3 (SLCO1B3) are expressed on the basolateral membrane of hepatocytes and are responsible for the hepatic uptake of numerous drugs and endogenous compounds. Transporter-mediated hepatic uptake could be a rate-determining process in elimination of both metabolically stable and unstable drugs (Kalliokoski and Niemi, 2009; Niemi et al., 2011; Yoshida et al., 2012; Shitara et al., 2013a). Hepatic uptake has clearly been demonstrated to be a potential source of pharmacokinetic variability for drugs such as rosuvastatin (RSV) and atorvastatin (ATV). For example, in 32 healthy volunteers with different SLCO1B1 genotypes dosed with 20 mg ATV, the mean area under the curve (AUC) of ATV was 144% higher in subjects with the 521CC genotype (reference genotype; n = 5) than in subjects with the 521TT genotype (reference genotype; n = 16) (Pasanen et al., 2007), although ATV is mainly eliminated via metabolism mediated by cytochrome P450 enzymes (Neuvonen et al., 2006).

Currently, both the Food and Drug Administration (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM292302.pdf) and the European Medicines Agency (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/07/WC500129606.pdf) require new chemical entities to be characterized for OATP1B inhibition and propose decision trees for assessing OATP1B inhibitors. The Food and Drug Administration and European Medicines Agency recommend using a static mathematical approach to estimate the risk of clinically relevant OATP1B-mediated drug-drug interactions (DDIs) by calculating the ratio of unbound maximum portal vein drug concentration in vivo against in vitro IC50. However, this approach is solely based on in vitro-to-in vivo extrapolation without any in vivo calibration. In addition, the use of this in vitro-to-in vivo extrapolation approach is challenged by the uncertain impact of protein binding–measured, drug absorption, and interlaboratory variability in IC50 assessment using different probe substrate and incubation conditions (Amundsen et al., 2010; Izumi et al., 2013, 2015; Shitara et al., 2013b). Therefore, the...
availability of endogenous biomarkers to assess transporter activities during early drug development would have substantial benefits for the pharmaceutical industry in order to avoid expensive clinical trials and also minimize the risk of late-stage failures and even drug withdrawal. In this regard, many attempts have been made to identify endogenous compounds that could reflect the activities of enzymes and transporters, such as CYP3A4 (Kanebratt et al., 2008; Diczfalussy et al., 2011; Shin et al., 2013; Kasichayana et al., 2014), CYP2D6 (Kay-Sontheimer et al., 2014), MATEs (Ito et al., 2012; Kato et al., 2014; Müller et al., 2015), and OAT3 (Imamura et al., 2014) to improve prediction of DDI. However, as far as we know, no validated in vivo clinical endogenous probe of OATP1B has been identified.

Coproporphyrin (CP) is one of the by-products of heme synthesis, and its physiologic role is unclear. CPs I and III are not enzymatically altered in the liver (Kaplowitz et al., 1972), but instead are removed from the body via the bile and urine as intact molecules, albeit unequally. While CP III predominates over CP I in urine, the reverse is encountered in bile (Aziz et al., 1964a,b; French and Thonger, 1966; Koskelo et al., 1966; Koskelo and Toivonen, 1966; Koskelo et al., 1967; Aziz and Watson, 1969; Ben-Ezzer et al., 1971). There are relatively limited data available in the literature regarding CP plasma concentrations and how they change in response to physiologic alterations because of limited sensitivity of the common assays (Table 4). Abnormal distribution of CP isomers was reported in the urine of patients with Rotor syndrome, i.e., a marked preponderance of CP I over CP III (Ben-Ezzer et al., 1971; Wolkoff et al., 1976). In addition, administration of ethinylestradiol and phenodibromophthalein disulfonate was shown to result in a reduction in biliary CP excretion and an increase in urinary CP excretion in rats (Kaplowitz et al., 1972). Ethinylestradiol was later found to be a potent inhibitor of OATPs (De Bruyn et al., 2013) and phenodibromophthalein disulfonate also appears to be an OATP inhibitor (Milne et al., 2000). The genetic basis of Rotor’s syndrome was recently identified to be linked to the mutations that cause complete and simultaneous deficiencies of OATP1B1 and OATP1B3 (van de Steeg et al., 2012). Taken together, these observations suggest that CPs I and III are possible substrates for hepatic OAT transporters and can potentially be used as endogenous biomarkers to assess OATP1B1 and OATP1B3 activities. However, direct evidence of in vivo activity is still lacking. In the present study, we describe a combination of in vitro and in vivo investigations in cynomolgus monkeys and Oatp1a1/1b−/− knockout (KO) mice to demonstrate that CPs I and III are suitable endogenous indicators of hepatic OAT activity in animals.

Materials and Methods

Chemicals and Supplies for In vitro and In vivo Experiments

Chemicals. CP I dihydrochloride, CP III dihydrochloride, and deuteroporphyrin IX dihydrochloride were purchased from Frontier Scientific, Inc. (Logan, UT). Rifampicin (RIF), RSV, and RSV-d6 were purchased from Toronto Research Chemicals Inc. (North York, Ontario, Canada). Estradiol-17β-D-glucuronide (E17βG), cholecystokinin octapeptide (CCK-8), ammonium phosphate, and high-performance liquid chromatography (HPLC) grade methanol and acetonitrile were purchased from Sigma Aldrich (St. Louis, MO). HPLC water was obtained from a Barnstead Nanopure deionizing system (Thermo Scientific, Waltham, MA). Formic acid and concentrated hydrochloric acid (12 N) were obtained from EMD Chemicals Inc. (Gibbstown, NJ). [3H]E17βG (34.3 Ci/mmol) and [3H]CCK-8 (97.5 Ci/mmol) were purchased from Perkin Elmer Life and Analytical Sciences (Waltham, MA). All compounds were of analytical grade (≥95% purity). Stock solutions of CPs and RIF were prepared in dimethylsulfoxide and stored at −70°C.

Cell Culture and Reagents. Hygromycin and penicillin-streptomycin were purchased from Invitrogen (Carlsbad, CA). Other chemicals and media reagents including Dulbecco’s modified Eagle’s growth medium, fetal bovine serum, nonessential amino acid, L-glutamine, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, phosphate-buffered saline, and Hanks’ balanced salt solution were purchased from Corning (Manassas, VA). Biocoat poly-l-lysine 24-well plates were purchased from BD Biosciences (San Jose, CA). Cryopreserved mouse, cynomolgus monkey, and human hepatocytes were purchased from BioreclamationIVT (Baltimore, MD). Pierce BCA protein assay kit was purchased from Thermo Scientific (Rockford, IL).

In vitro Studies

CP Uptake and Inhibition Studies in Cynomolgus Organic Anion-Transporting Polypeptide (cOATP)1B1, cOATP1B3, Human Organic Anion-Transporting Polypeptide (hOATP)1B1, and hOATP1B3 Transfected Human Embryonic Kidney (HEK) 293 Cells. Recombinant cells expressing human and cynomolgus monkey OATP1B transporters were cultured as described previously (Shen et al., 2010). Uptake was initiated by the addition of transport buffer containing 3 μM CP isomers and 100 μM RIF or dimethylsulfoxide vehicle after washing cells with transport buffer. In control reactions, CP isomer was replaced with 1 μM [3H]E17βG and 0.1 μM [3H]CCK-8 for all incubations. Each test condition was assessed in triplicate. After 5 minutes, uptake was terminated by washing cells 3 times with ice-cold transport buffer and the plate was air dried for at least 30 minutes in the fume hood. To measure levels of tritium-labeled probe substrates, cells in the dried plate were lysed with 300 μl 0.1% Triton-X 100. Aliquots of cell lysates (200 μl) were used to count radioactivity using a Tri-Carb 3100TR liquid scintillation counter (PerkinElmer Life Sciences, Boston). Protein concentrations in the cell lysate aliquots (20 μl) were measured using the Pierce BCA protein assay kit (Thermo Scientific). To measure levels of CP isomers, cells in the dried plate were lysed in a 300 μl 3:1 (v/v) ratio of acetonitrile and water at room temperature for 1 hour. The lysate was centrifuged through a 96-well filter plate (0.45 μm low-binding hydrophilic polystyrene/fluorocarbon) at 3700 rpm for 15 minutes. The filtrate was then diluted with 900 μl 4N hydrochloric acid containing deuteroporphyrin IX as an internal standard, and 50 μl injection was used for HPLC fluorescence detection (FLD) analysis. Quantification of CP by the HPLC-FLD method is described subsequently.

CP Uptake in Human and Monkey Hepatocytes. Cryopreserved human (Lot NRJ) and cynomolgus monkey hepatocytes (Lot DQA) were processed in a stepwise manner, according to the instructions provided by the manufacturer (BioreclamationIVT). The uptake studies of CPs I and III were conducted as described previously (Morse et al., 2015). In brief, hepatocytes were incubated with the pre-prepared Krebs-Henseleit buffer containing CP isomers (1 μM) or RSV (1 μM, positive control) with or without RIF (as the OATP inhibitor, 100 μM, respectively). At designated time points, the cells were spun down through the oil layer and the hepatocyte pellet was then lysed in a 300 μl 2:1 (v/v) ratio of acetonitrile and 1N hydrochloric acid containing deuteroporphyrin IX as an internal standard, and a 1:1 (v/v) ratio of acetonitrile and water at room temperature, for CP and RSV measurements, respectively. The contents were filtered through a 96-well filter plate (0.45 μm of low-binding hydrophilic polystyrene/fluorocarbon) and the filtrate was dried under nitrogen. The CPs I and III samples were reconstituted in 10% formic acid (v/v) and a 10 μl sample was injected for HPLC-FLD analysis as described.
were frozen on dry ice and stored at −70°C until analysis. Aliquots (10 ml) of urine samples collected from each period were frozen on dry ice and stored at −70°C until analysis. All animal experiments were approved by the Bristol-Myers Squibb Institutional Animal Care and Use Committee and were performed under the standards recommended by the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011).

Effect of RIF Administration on Disposition of CPs I and III in Monkeys. To assess the effect of a single dose of RIF on plasma and urinary excretion of CPs I and III, a 2-way crossover study was performed in three male cynomolgus monkeys. Male cynomolgus monkeys (4–8 kg) were purchased from Charles River Laboratories (Wilmington, MA), and placed in stainless steel metabolic cages prior to the experiments. The animals were administered with vehicle (i.e., saline) after an overnight fast on day 1, followed by a single oral dose of 15 mg/kg RIF dissolved in saline on day 8 via oral gavage. Serial blood samples (1 ml) were obtained, followed by centrifugation at 3,000 g for 10 minutes, and stored at −70°C until analysis. Urine samples were collected 0–7, 7–24, and 24–48 hours after dosing.

Baseline levels in the plasma and urine were obtained from four normal male monkeys. On days 1, 4, and 7, the animals were placed in metabolic cages and serial blood samples (1 ml) were collected from the saphenous vein at 0, 4, and 7 hours following vehicle (i.e., saline) administration. On days 1, 4, and 7, urine samples were collected 0–7 and 7–24 hours after vehicle dosing. The total volumes of urine samples collected during different periods were measured and recorded. Aliquots (10 ml) of urine samples collected from each period were frozen on dry ice and stored at −70°C until analysis.

Disposition of CPs I and III in Oatpl1a/b−/− Gene KO Mice. Male Oatpl1a/b−/− KO and wild-type (WT) mice (30–40 g) were purchased from Taconic Biosciences (Hudson, NY). Animals were placed in metabolism cages and fasted overnight before dosing. Urine was collected in a 0–24 hour interval to evaluate baseline urinary excretion of CPs I and III.

Plasma concentrations and urinary excretion of CPs I and III were characterized in comparison with the change in plasma concentration of RSV following oral administration of 15 mg/kg RSV in saline to three male WT and Oatpl1a/b−/− KO mice. Animals were euthanized at 3 hours postdose by terminal bleeding through cardiac puncture under isoflurane anesthesia, and urine and liver tissues were isolated. The urine samples collected from three animals in each group were pooled and preserved at −70°C until analysis. The liver was dissected and rinsed with phosphate-buffered saline, weighted, and stored at −70°C until analysis.

Analytical Assays

Quantification of CPs I and III by HPLC-FLD. All samples were kept in the dark and sample processes were carried out by protecting them from light as much as possible. Samples from in vitro cell lysate were treated before HPLC-FLD analysis as described previously. Urine samples (100 μl) were vortex mixed with 10 μl concentrated hydrochloric acid and centrifuged at 12,000 g for 10 minutes. A total of 10 μl of the supernatant was injected for HPLC-FLD analysis. Plasma samples (100 μl) were first acidified with 10 μl concentrated hydrochloric acid, followed by vortex mixing with 250 μl acetonitrile containing deuteroporphyrin IX as an internal standard. After centrifuging at 12,000 g for 10 minutes, the supernatant was dried under nitrogen. Samples were then reconstituted in 50 μl 10% formic acid and 25 μl of the resulting solution was injected for HPLC-FLD analysis.

Chromatographic separations were carried out using an Agilent 1260 Infinity Quaternary LC system equipped with a quaternary pump, an autosampler, a column temperature controller, a diode array detector, and a 1260 Infinity fluorescence detector with an 8 μl biofluorescence flow cell (Agilent Technologies, Santa Clara, CA). The HPLC sample tray was kept at 4°C. The fluorescence signal was monitored at an excitation wavelength of 391 nm and emission wavelength of 621 nm. Data were collected and processed using Agilent ChemStation software. Chromatographic separation was conducted on a 100 mm × 3 mm (particle size 2.5 μm) Synergy Hydro-RP analytical column (Phenomenex, Torrance, CA) by gradient elution at a flow rate of 0.6 ml/min. The gradient HPLC mobile phase A contained 25 mM ammonium phosphate buffer at pH 6, while mobile phase B consisted of a 50/50 (v/v) mixture of methanol and acetonitrile. The elution program was adjusted for different matrices to optimize separation and reduce analysis time. For example, analyses of mouse plasma samples were conducted with an elution program of isocratic at 20% B from 0 to 6 minutes, linear increase from 20% to 55% B from 6 to 20 minutes, and holding at 55% B from 20 to 25 minutes. The column was equilibrated under initial conditions (20% B) for 5 minutes between injections. Column temperature was set at 25°C.

Quantification of RSV by Liquid Chromatography–Tandem Mass Spectrometry. The cell lysate, plasma, and liver concentrations of RSV were determined by liquid chromatography–tandem mass spectrometry analysis as described previously (Shen et al., 2015).

Data Analysis

Hepatocyte Uptake Data Analysis. The calculated amounts of compound taken into hepatocytes in the absence and presence of RIF were graphed with mean data (up to 5.0 minutes) (Fig. 2). The initial uptake rate (V uptake) was calculated based on the rate of uptake during the linear phase (up to 1 or 1.5 minutes) (Fig. 2). The uptake clearance (Clu) and percent active uptake were calculated using the following equations:

$$\text{Cl}_u = \frac{V_{\text{uptake}}}{C}$$

(1)

where C is the measured concentration of the test compound in the incubation buffer, and $\text{Cl}_u$ is the uptake clearance in the absence or presence of inhibitor, respectively.

Pharmacokinetic Analysis. The AUC from time zero to 24 or 48 hours (AUC(0–24 h) or AUC(0–48 h)) was calculated by mixed log-linear trapezoidal summations using validated software (Kinetics version 5.0; Thermo Electron, Philadelphia). Maximum plasma concentrations (Cmax) were directly obtained by observation. The renal clearance (ClR) and renal extraction ratio (ER) were estimated by the following equations:

$$\text{Cl}_R = \frac{X_c}{AUC}$$

(3)

$$\text{ER} = \frac{\text{Cl}_R}{f_u \times \text{GFR}}$$

(4)

where $X_c$ is the cumulative amount of CP I or CP III excreted in urine; $f_u$ is the fraction of CP unbound in human plasma reported.
Statistical Analysis. The in vitro data represented the results of triplicates run in one experiment with a minimum of two experiments. The two-tailed unpaired Student’s t test was used to evaluate the statistical significance of differences between two sets of data generated from in vitro studies. The one-sided paired Student’s t test was used to assess the statistical significance of differences between two sets of data in monkey studies because of the crossover design, while the one-sided unpaired Student’s t test was used for mouse studies. To test for statistically significant differences among multiple time points in different days for CP plasma level in monkeys, two-way analysis of variance was performed. To test for statistically significant differences among multiple days for CP urinary excretion in monkeys, one-way analysis of variance was performed. When the F ratio showed that there were significant differences among days, the Dunnett method of multiple comparisons was used to determine which treatments differ. All statistical analyses were performed using Prism version 5.0 (GraphPad Software, Inc., San Diego, CA). Results were presented as the mean ± S.E.M. Differences were considered statistically significant when P < 0.05.

Results
Transport of CPs I and III in HEK 293 Cells Stably Transfected with Monkey and Human OATP1B1 and OATP1B3
A significant increase in the uptake rate of CPs I and III (3 μM) was observed in cOATP1B1-HEK, cOATP1B3-HEK, hOATP1B1-HEK, and hOATP1B3-HEK cells, relative to parental HEK 293 cells (Mock-HEK) (3.1- to 17.4-fold; P < 0.001) (Fig. 1, A and B). OATP1B involvement in the transport of CP was further confirmed by inhibition of the uptake by coincubation of the cells with 100 μM RIF, a potent inhibitor of each of these OATP transporters (Shen et al., 2013, Chu et al., 2015). The validity of the OATP assays was confirmed with commonly used positive controls for monkey and human OATP1B1 ([3H]E17βG) and OATP1B3 ([3H]CCK-8).

Fig. 1. CPs I and III transport by HEK 293 cells expressing cOATP1B1, cOATP1B3, hOATP1B1, or hOATP1B3. Uptake of CP I (3 μM) (A), CP III (3 μM) (B), [3H]E17βG (1 μM) (C), and [3H]CCK-8 (0.1 μM) (D) was measured in HEK 293 cells stably transfected with control vector (Mock-HEK) and individual transporter in the absence and presence of RIF (100 μM). The data are expressed as the mean ± S.E.M. of three separate determinations (**P < 0.01, ***P < 0.001, statistically significant difference compared with Mock-HEK cells).
Transport of CPs I and III in Human and Monkey Hepatocytes

To understand hepatic transport of CP, the uptake study with 1 μM CPs I and III was conducted in human and cynomolgus monkey hepatocytes at 37°C in the presence or absence of 100 μM RIF. As shown in Fig. 2, CPs I and III exhibited saturable and time-dependent uptake by human and monkey hepatocytes. The initial linear phase for uptake was maintained for at least 1 minute of incubation in both human and monkey hepatocytes. The initial uptake clearance was comparable for CPs I and III for each species (3.8 versus 5.6 μl/million cells and 8.2 versus 11.7 μl/million cells for human and monkey hepatocytes, respectively), whereas uptake clearance in monkey hepatocyte was about 2-fold higher than that in human. Approximately 75%–85% of CPs I and III uptake in human and monkey hepatocytes was inhibited by RIF, indicating that the entry of CPs into hepatocyte is mainly via active processes mediated by OATP.

After a 4-hour incubation of CPs I and III (1 μM) with the hepatocytes of the three different species, greater than 97% of CPs I and III remained unchanged. The results suggested that CPs I and III are metabolically stable in human, monkey, and mouse hepatocytes (Supplemental Fig. 2, A–C). CPs I and III were also shown to be stable in human plasma and urine samples (Supplemental Fig. 2D).

CPs I and III Levels in Monkey Plasma and Urine Following the Administration of CsA. Mean CPs I and III plasma concentrations over time with RSV (3 mg/kg) alone and in combination with CsA (100 mg/kg) in cynomolgus monkeys are shown in Fig. 3A. A significant 3.3-fold increase in CP I C max (P = 0.0045) and a 6.9-fold increase in CP III C max (P = 0.0003) (Table 1) were observed when codosed. Plasma CPs I and III AUC(0–24 h) values were significantly increased by 2.6-fold from 15.9 ± 2.5 to 41.5 ± 4.7 nM·h and by 5.2-fold from 4.1 ± 1.2 to 20.3 ± 2.8 nM·h (Fig. 3A; Table 1), respectively, whereas the amounts of CPs I and III excreted in urine X e(0–24 h) were increased by 3.5-fold from 1.4 ± 0.61 to 4.8 ± 1.7 nmol and by 1.6-fold from 17.1 ± 6.1 to 26.6 ± 10.1 nmol (Fig. 2, B and C; Table 1). Consequently, renal clearance of CPs I and III (RSV alone versus the codosed) was not significantly different (1.5 ± 0.75 versus 1.9 ± 0.55 ml/min and 75.5 ± 40.0 versus 21.5 ± 5.2 ml/min, respectively). CP III was the dominant form in urine in both RSV alone and combination groups (92.4% ± 1.4% and 84.5% ± 4.4%, respectively), while CP I was the dominant form in monkey plasma for both groups (79.8% ± 4.7% and 67.7% ± 5.0%, respectively). Although plasma and urine samples were...
collected over 48 hours, there was no difference in plasma concentration and urinary excretion of CPs I and III beyond 24 hours between groups (Fig. 3, A–C). As a result, the plasma concentrations at 48 hours and urinary excretion during 24–48 hours were not included in the pharmacokinetic analysis.

When CPs I and III renal clearance was normalized for fraction unbound and functional nephron mass (i.e., GFR), the ERR of CP I in monkeys treated with RSV alone was 0.68 ± 0.32 and was not significantly different from unity, suggesting no involvement of active renal secretion and reabsorption. In contrast, the ERR of CP III was substantially greater in monkeys treated with RSV alone (33.6 ± 17.0), indicating a net secretory mechanism for renal clearance. In the presence of CsA, the ERR of CP I did not change as expected. Although

**TABLE 1**

Comparison of pharmacokinetic parameters of CPs I and III following oral administration of 3 mg/kg RSV alone and 3 mg/kg RSV with 100 mg/kg CsA in cynomolgus monkeys

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CP I</th>
<th>CP III</th>
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<tr>
<td></td>
<td>RSV Alone</td>
<td>RSV + CsA</td>
</tr>
<tr>
<td>$C_{max}$ (nM)</td>
<td>0.93 ± 0.06</td>
<td>3.0 ± 2.9**</td>
</tr>
<tr>
<td>AUC(0–24 h) (nM·h)</td>
<td>15.9 ± 2.5</td>
<td>41.5 ± 4.7**</td>
</tr>
<tr>
<td>$X_{eq}$ (nmol)</td>
<td>1.4 ± 0.61</td>
<td>4.8 ± 1.7*</td>
</tr>
<tr>
<td>$C_{Lp}$ (ml/min)</td>
<td>1.5 ± 0.75</td>
<td>1.9 ± 0.55</td>
</tr>
<tr>
<td>$\text{ERR}$</td>
<td>0.68 ± 0.32</td>
<td>0.85 ± 0.17</td>
</tr>
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</table>

Oral CsA treatment was given 1 hour prior to RSV oral administration. Data are expressed as mean ± S.D. (n = 3); *P < 0.05, **P < 0.01, and ***P < 0.001, statistically significant difference compared to the RSV alone group.
the ERR of CP III was reduced from 33.6 ± 17.0 to 9.7 ± 1.6, the reduction was not statistically different (Table 1).

**CPs I and III Levels in Monkey Plasma and Urine Following Administration of RIF**

Plasma samples for the determination of CPs I and III concentrations were collected from predose until 48 hours postdose during both vehicle and RIF (15 mg/kg) treatment periods. A significant 2.7 ± 0.75-fold increase in CP I AUC(_0–48 h_) (P < 0.05) and a 3.6 ± 0.74-fold increase in CP III AUC(_0–48 h_) (P < 0.01) (Table 2) were observed in RIF-treated monkeys compared with the control animals. There was no difference in CP plasma concentration between the two group animals before administration of RIF (i.e., at predose).

The amounts of CPs I and III excreted in urine over 48 hours were consistently higher in RIF-treated monkeys compared with the vehicle-treated animals (fold increases of 4.3 ± 1.6 and 3.1 ± 1.5, respectively), and the differences were statistically significant (P < 0.05) (Fig. 4, B–D; Table 2). However, the renal clearance of CPs I and III appeared to be unaffected.

**TABLE 2**

Comparison of pharmacokinetic parameters of CP I and CP III following oral administration of vehicle and 15 mg/kg RIF in cynomolgus monkeys

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CP I</th>
<th>CP III</th>
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<tr>
<td></td>
<td>Vehicle</td>
<td>RIF</td>
</tr>
<tr>
<td>AUC(<em>0–48 h</em>) (nM·h)</td>
<td>14.2 ± 2.2</td>
<td>37.6 ± 8.9*</td>
</tr>
<tr>
<td>X(0–48 h) (nmol)</td>
<td>1.5 ± 0.42</td>
<td>6.2 ± 2.2*</td>
</tr>
<tr>
<td>C_{Lk} (ml/min)</td>
<td>1.8 ± 0.79</td>
<td>2.8 ± 0.92</td>
</tr>
<tr>
<td>ERR</td>
<td>0.68 ± 0.34</td>
<td>1.1 ± 0.40</td>
</tr>
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</table>

Data are expressed as mean ± S.D. (n = 3); *P < 0.05 and **P < 0.01, statistically significant difference compared to the vehicle control group.

Fig. 4. Effects of RIF on CP disposition in cynomolgus monkeys. CP plasma concentrations (A), the 0–7-hour, 7–24-hour, and 24–48-hour urinary excretion rates of CP I (B) and CP III (C), and total CP (D) were determined following oral administration of vehicle and 15 mg/kg RIF. The data are expressed as the mean ± S.E.M. of three animals (*P < 0.05 and **P < 0.01, statistically significant difference compared with the vehicle control group).
by RIF. In addition, no significant differences were discerned between the RIF and vehicle groups with respect to the ER values of CPs I and III (P > 0.05) (Table 2), indicating that the presence of RIF did not interfere with the tubular disposition of CPs. Furthermore, the ER values of CP I in both treatments did not significantly differ from unity (0.68 ± 0.34 and 1.1 ± 0.40, respectively). On the other hand, the ER values for CP III were significantly greater than unity, presenting further evidence that this isomer undergoes active tubular secretion in agreement with CsA experiments.

The basal plasma concentrations and urinary excretion of CPs I and III were studied over a 7-day period and the levels were found to be stable throughout the experiment in four untreated animals, as shown in Fig. 5. The mean plasma levels and 24-hour urine excretion of CPs I and III on day 1 did not statistically differ from those on days 4 and 7 (P > 0.05; within a 1.5-fold difference) (Fig. 5). In addition, the degree of intraindividual variability of CPs I and III basal plasma concentrations and urine excretion is low (within 1.9-fold; data not shown).

**Discussion**

Although CPs have been suggested as putative endogenous markers of OATP1B activity (Benz-de Bretagne et al., 2011; van de Steeg et al., 2012), no studies to date have reported their in vivo disposition, in comparison with known OATP1B probe substrates, after administration of a potent OATP...
inhibitor in animal models and humans. In vitro interaction of several porphyrins including CPs I and III with OATP1B1 has been reported (Campbell et al., 2009; Li et al., 2015). In addition, while this paper was in preparation, Bednarczyk and Boiselle (2016) assessed the in vitro transport of CP by OATP1B1 and OATP1B3 using transporter-overexpressing cells. These authors demonstrated that the in vitro transport of CPs I and III by OATP1B1 and OATP1B3 was time dependent, and can be saturated and inhibited. However, it is not entirely clear if there is temporal association between the potential biomarkers and OATP-based DDI in vivo. In addition, the driving force of OATP1B-mediated CP transport has not been investigated in the report and remains unclear in the literature (Hagenbuch and Stieger, 2013). In the present study, the uptake of CPs I and III by hOATP1B1-, hOATP1B3-, cOATP1B1-, and cOATP1B3-HEK cells was inhibited by coincubation with 100 μM RIF, a potent inhibitor of each of these OATP transporters (Shen et al., 2013) (Fig. 1, Fig. 7.

**TABLE 3**

Comparison of urinary excretion of CPs I and III in cynomolgus monkeys, mice, and humans

<table>
<thead>
<tr>
<th>Species (n)</th>
<th>$X_e$ (0-24 h) (Body Weight)</th>
<th>Total Urinary CP</th>
<th>Urinary CP Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CP I gmol/kg</td>
<td>CP III gmol/kg</td>
<td>Total CP gmol/kg</td>
</tr>
<tr>
<td>Monkey experiment 1 (3)</td>
<td>0.37 ± 0.14</td>
<td>4.42 ± 1.23</td>
<td>4.79 ± 1.35</td>
</tr>
<tr>
<td>Monkey experiment 2 (3)</td>
<td>0.15 ± 0.10</td>
<td>2.14 ± 1.43</td>
<td>2.29 ± 1.53</td>
</tr>
<tr>
<td>Mouse (3)</td>
<td>0.34 ± 0.06</td>
<td>0.91 ± 0.17</td>
<td>1.25 ± 0.22</td>
</tr>
<tr>
<td>Human$^a$ (30)</td>
<td>0.30 ± 0.09</td>
<td>1.17 ± 0.44</td>
<td>1.46 ± 0.50</td>
</tr>
<tr>
<td>Human$^b$ (22)</td>
<td>0.48 ± 0.24</td>
<td>1.13 ± 0.35</td>
<td>1.57 ± 0.46</td>
</tr>
<tr>
<td>Human$^c$ (86)</td>
<td>0.31</td>
<td>1.10</td>
<td>1.41</td>
</tr>
</tbody>
</table>

N.D., not determined.

$^a$Data on urinary excretion of CPs I and III in healthy subjects are from Koskelo et al. (1967).

$^b$Data on urinary excretion of CPs I and III in healthy subjects are from Koskelo et al. (1966).

$^c$Data on urinary excretion of CPs I and III in healthy subjects are from Gebril et al. (1990).
A and B). Additionally, OATP involvement was confirmed by demonstration of RIF-sensitive uptake in cryopreserved human and monkey hepatocytes. Incubation in the presence of 100 μM RIF, a condition known to deplete active transport by OATPB1 and OATPB3 but unlikely by sodium-taurocholate cotransporting polypeptide (IC50 = 277 μM) (Prueksaritanont et al., 2014), reduced the uptake of CP by 75% versus 3.6-fold by CsA and RIF, respectively. Apparently, inhibition of OATP1B1 and OATP1B3 but unlikely by sodium-taurocholate cotransporting polypeptide (IC50 = 277 μM) (Prueksaritanont et al., 2014), reduced the uptake of CP by 75% versus 3.6-fold by CsA and RIF, respectively. However, urinary excretion of CP was increased to a similar extent compared with the changes in CP III AUC by CsA but not RIF (1.6- versus 5.2-fold and 3.1- versus 2.9-fold, respectively) (Simonson et al., 2004; Prueksaritanont et al., 2014). In line with the increase in RSV exposure, as seen in Tables 1 and 2, CsA increased CPs I and III plasma exposures by 6.3- and 2.9-fold, respectively, due to OATP inhibition (Shen et al., 2013, 2015). The changes in AUC are comparable to those observed in humans (i.e., 7.1- and 4.4-fold, respectively) (Simonson et al., 2004; Prueksaritanont et al., 2014). In addition, OATP involvement was confirmed by absolute transport by OATP1B1 and OATP1B3 but unlikely by sodium-taurocholate cotransporting polypeptide (IC50 = 277 μM) (Prueksaritanont et al., 2014), reduced the uptake of CP by 75% versus 3.6-fold by CsA and RIF, respectively. These findings suggest that the renal elimination of CP I is likely mediated by transporter-mediated tubular secretion accounts for most of the renal clearance of CP III in monkeys since the ERR value is significantly greater than unity (approximately 10- to 30-fold) (Prueksaritanont et al., 2014, 2015). The verification of CP as an OATP bio-marker was then extended in Oatp1a/1b cluster gene deletion in Oatp1b KO mice, where similar observations were found for CP plasma levels and urinary excretion. While Oatp1a/1b KO mice showed significant 7.4- and 15.2-fold increases in plasma concentrations of CPs I and III at 3 hours compared with WT animals, respectively, the plasma concentration of RSV at 3 hours following the single oral dose of 15 mg/kg RSV increased by 14.6-fold (Fig. 7, A and C). Urinary excretion values of CPs I and III over 24 hours are also increased by the disruption of Oatp1a and Oatp1b transporters. However, the effect of Oatp1a and 1b cluster gene deletion on urinary excretion of CP I was less than CP III (7.1- versus 12.1-fold and 12.4- versus 18.4-fold for X0 (0–3 h) and X0 (0–24 h), respectively). Taken together, these results demonstrate a validated assessment of CPs I and III activity using plasma CP concentrations and urinary CP excretion values either with administration of potent OATPIB inhibitor or genetic mutation of OATP genes relative to the well-established exogenous probe. Additionally, the time course of CP plasma concentration and urinary excretion in animal experiments displayed the temporal changes, allowing an assessment of the utility of these indices in studies of varying duration.

When the CP renal clearance was corrected by protein binding and renal function (i.e., GFR), the ERK value of CP I approximates unity, whereas that of CP III is substantially greater than unity in untreated monkeys (0.68 ± 0.32 versus 33.6 ± 17.0 and 0.68 ± 0.34 versus 24.1 ± 9.8 for the CsA and RIF studies, respectively) (Tables 1 and 2). These findings suggest that the renal elimination of CP I is likely mediated by glomerular filtration only; in contrast, active tubular secretion plays an important role in CP III renal clearance, although the isomers differ only in the position of the methyl and propionic acid groups in the porphyrin molecule (Supplemental Fig. 1). This observation agrees with other CP disposition studies in which CP III favors urinary excretion over biliary excretion in both humans and animals (Aziz et al., 1964a; Koskelo et al., 1967; Kaplowitz et al., 1972). In addition, CsA, an inhibitor of multiple transporters, reduced the mean CLR and ERK values of CP III in monkeys, while RIF, a relatively specific inhibitor for OATPB1 and OATPB3 (Prueksaritanont et al., 2014), did not decrease the mean CLR and ERK values of CP III compared with the vehicle control (62.9 ± 21.8 versus 49.4 ± 5.1 ml/min and 24.1 ± 9.8 versus 18.7 ± 1.7, respectively) (Table 2). Nevertheless, further studies on the characterization of CP excretion in the bile and elucidation of active CP III renal secretion are required. These results suggest that CP I may have better value in vivo as a biomarker of OATPB1 compared with CP III because CP I is specific to hepatic uptake and is unlikely to be subject to active renal transport.

The urinary CP isomer ratio has been proposed as a biomarker of MRP2 activity, although there is no in vitro evidence indicating CPs I and III are substrates for MRP2 (Benz-de Bretagne et al., 2011). The proportion of each CP isomer in urine of patients with Dubin-Johnson syndrome

### Table 4

**Comparison of plasma profiles of CPs I and III in cynomolgus monkeys, mice, and humans**

<table>
<thead>
<tr>
<th>Species (n)</th>
<th>Plasma Concentration</th>
<th>Percentage of Plasma CP</th>
<th>Plasma CP Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CP I</td>
<td>CP III</td>
<td>Total CP</td>
</tr>
<tr>
<td>Monkey (3)</td>
<td>0.75 ± 0.17</td>
<td>0.19 ± 0.05</td>
<td>0.93 ± 0.20</td>
</tr>
<tr>
<td>Mouse (3)</td>
<td>0.32 ± 0.08</td>
<td>0.18 ± 0.04</td>
<td>0.50 ± 0.11</td>
</tr>
<tr>
<td>Human (30)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>&lt;6</td>
</tr>
<tr>
<td>Human (245)</td>
<td>0–10</td>
<td>0–12</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D., not determined.

Data on plasma concentrations of CPs I and III in healthy subjects are from Kanayama et al. (1992). Data on plasma concentrations of CPs I and III in healthy subjects are from Hindmarsh et al. (1999).
Recent studies of CPs I and III transport was studied using cells transfected with various transporters (Bednarzyk and Boiselle, 2016). Besides OATP1B1- and OATP1B3-mediated transport of CPs I and III, OATP2B1 demonstrated significant transport of CP III, while sodium-taurocholate cotransporting polypeptide, OCT1, OCT2, OAT1, and OAT3 were negative for CP transport. However, CP III appeared to be transported by OAT1 with the ratio of uptake rate of transporter over vector transected cells of 2.4, while CP I was not transported by OAT1 (Bednarzyk and Boiselle, 2016). Therefore, it is important to reexamine whether renal organic anion transporters OAT1, OAT3, and OATP4C1 contribute to the renal secretion of CP III if a clinical study confirms apparent renal tubular secretion of CP III. Such a study will provide comprehensive understanding of CP disposition and dissect OATP-mediated DDI from other mechanisms underlying CP excretion. Furthermore, the in vivo CP findings from animals need to be carefully translated to humans since CP disposition may not be specific to OATP. Indeed, species-dependent differences in disposition were observed for CP (Tables 3 and 4). While CP III predominated in the urine of monkeys with a mean CP I/CP III ratio of 0.07–0.08, mice and humans excreted relatively more CP I (0.29–0.44 CP I/CP III ratio); therefore, characterization of CP disposition in humans is critical for the use of CP biomarkers for OATP functional inhibition, and the report of clinical studies is forthcoming.

Bilirubin and bile acids are proposed to be biomarkers for hepatic OATP inhibition as suggested by their increased plasma levels in cynomolgus monkeys and rats after administration of RIF (Chu et al., 2015; Watanabe et al., 2015). However, in addition to the biochemical defect leading to reduced hepatic uptake of conjugated and unconjugated bilirubin, other factors such as impaired efflux transporters (MRP2 and MRP3) and enzyme activity (UGT1A1) may also result in the change in bilirubin plasma concentration. Bile acids are associated with other intestinal and hepatic transporters, including the sodium-taurocholate cotransporting polypeptide and bile salt export pump. Furthermore, serum bilirubin and bile acid levels, along with other liver enzymes, are complicated with drug-induced liver injuries (Ozer et al., 2008). In this regard, our results indicated that the CP levels in plasma and urine are independent of changes in liver function; therefore, they are suitable biomarkers for inhibition of hepatic OATP1B used in the clinical setting.

Based on the results presented herein, it is concluded that CP I and CP III can be used as endogenous probes to assess hepatic OATP activity in cynomolgus monkeys and mice. Furthermore, if our findings can indeed be extrapolated to humans, CPs I and III will provide a unique tool for predicting and explaining OATP-based DDI.

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Authorship Contributions
Participated in research design: Shen, Dai, Cheng, Zhang, Humphreys, Marathe, Lai.
Contributed new reagents or analytic tools: Shen, Dai, Liu, Lai.
Performed data analysis: Shen, Dai, Lai.
Wrote or contributed to the writing of the manuscript: Shen, Dai, Cheng, Humphreys, Marathe, Lai.

References
Brennan BJ, Poirier A, Moreira S, Morcos PN, Goelerz P, Portmann R, Asthappan J, Funk C, and Smith PF (2015) Characterization of the transmembrane transport mechanisms underlying CP excretion. Furthermore, the in vivo CP findings from animals need to be carefully translated to humans since CP disposition may not be specific to OATP. Indeed, species-dependent differences in disposition were observed for CP (Tables 3 and 4). While CP III predominated in the urine of monkeys with a mean CP I/CP III ratio of 0.07–0.08, mice and humans excreted relatively more CP I (0.29–0.44 CP I/CP III ratio); therefore, characterization of CP disposition in humans is critical for the use of CP biomarkers for OATP functional inhibition, and the report of clinical studies is forthcoming.

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