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

Hong Shen, Jun Dai, Tongtong Liu, Yaofeng Cheng, Weiqi Chen, Chris Freeden, Yingru Zhang, W. Griffith Humphreys, Punit Marathe and Yurong Lai

Pharmaceutical Candidate Optimization, Bristol-Myers Squibb Company, 3551 Lawrenceville Road, Princeton, NJ 08540

Running title: Coproporphyrin as endogenous *in vivo* probe for OATP1B

Corresponding author and contact information:

Hong Shen, Ph.D.

Bristol-Myers Squibb Company

F1.3802, Route 206 & Province Line Road

Princeton, NJ 08543-4000

E-mail: hong.shen1@bms.com

Tel: (609) 252-4509

Fax: (609) 252-6802

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Abbreviations: *AUC*, area under the concentration-time curve; CL_R, renal clearance; C_{max}, maximum plasma concentration; cOATP, cynomolgus organic anion transporting polypeptide; CP, coproporphyrin; CsA, cyclosporin A; DDI, drug-drug interaction; DMSO, dimethyl

sulfoxide; ER_R, renal extraction ratio; HEK, human embryonic kidney; hOATP, human organic anion transporting polypeptide; HPLC-FLD, high-performance liquid chromatography-fluorescence detection; IC_{50}, concentration required to inhibit transport by 50%; KO, knockout; LC-MS/MS, liquid chromatography–tandem mass spectrometry; RIF, rifampicin; RS, Rotor syndrome; RSV: rosuvastatin; SEM, standard error of the mean; v/v, volume/volume; WT, wild-type.

ABSTRACT:

Inhibition of organic anion-transporting polypeptide (OATP)1B function can lead to serious clinical drug-drug interactions (DDIs), 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 (CsA; 100 mg/kg, oral) markedly increased the area under the plasma concentration curves (AUCs) of CPs I and III by 2.6- and 5.2-fold, while rifampin (RIF, 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 (1.6- to 4.3-fold) in monkeys. In agreement with this finding, AUC of rosuvastatin (RSV) in cynomolgus monkeys increased when OATP1B inhibitors were co-administered. In Oatp1a/1b gene cluster knockout (Oatp1a/1b$^{-/-}$ KO) mice, CPs in plasma and urine were significantly increased compared to wild-type animals (WT) (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 OATP1B1 (*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 the elimination 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 AUC of ATV was 144% higher in subjects with the 521CC genotype (n = 4) 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 (FDA) and the European Medicines Agency (EMA) require new chemical entities to be characterized for OATP1B inhibition and propose decision trees for assessing OATP1B inhibitors (US FDA 2012; European Medicines Agency 2012). The FDA and EMA recommend using a static mathematical approach to estimate the risk of clinically relevant OATP1B-mediated DDIs by calculating 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 inter-laboratory variability in IC50 assessment using different probe substrate- and incubation conditions (Amundsen et al., 2010, Izumi et al., 2013, Shitara et al., 2013b, Izumi et al., 2015). 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 are made to identify endogenous compounds that could reflect the activities of enzymes and transporters, such as CYP3A4 (Kanebratt et al., 2008, Diczfalusy et al., 2011, Shin et al., 2013, Kasichayanula et al., 2014), CYP2D6 (Tay-Sontheimer et al., 2014), MATEs (Ito et al., 2012, Kato et al., 2014, Muller et al., 2015), 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 byproducts of heme synthesis, and its physiological 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, Aziz et al., 1964b, 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 is relatively limited data available in the literature regarding CP plasma concentrations and how they change in response to physiological alterations because of limited sensitivity of the common assays. Abnormal distribution of CP isomers was reported in the urine of patients with Rotor syndrome (RS), 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 (DBSP) 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 DBSP 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 OATP 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 monkey and Oatp1a/1b$^{-/-}$ KO mouse to demonstrate that CPs I and III are suitable endogenous indicators of hepatic OATP activity in animals.

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MATERIALS AND METHODS

Chemicals and Supplies for *In vitro* and *In vivo* Experiments

Chemicals. Coproporphyrin I dihydrochloride, coproporphyrin III dihydrochloride, and deuteroporphyrin IX dihydrochloride were purchased from Frontier Scientific, Inc. (Logan, UT). RIF, RSV and RSV-d6 were purchased from Toronto Research Chemicals Inc. (North York, Ontario). Estradiol-17β-D-glucuronide (E17βG), cholecystokinin octapeptide (CCK-8), ammonium phosphate, 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). [^{3}H] E17βG (34.3 Ci/mmol) and [^{3}H] CCK-8 (97.5 mCi/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 dimethyl sulfoxide (DMSO) and stored at -70°C.

Cell Culture and Reagents. Hygromycin and penicillin-streptomycin were purchased from Invitrogen (Carlsbad, CA). Other cell culture media and reagents including Dulbecco's modified Eagle's growth medium, fetal bovine serum, non-essential amino acid, L-glutamine, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid, phosphate-buffered saline, and Hanks' balanced salt solution were from Corning (Manassas, VA). Biocoat™ poly-d-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 cOATP1B1, cOATP1B3, hOATP1B1, and hOATP1B3 Transfected HEK 293 Cells. Recombinant cells expressing human and cynomolgus monkey OATP1B transporters were cultured as described before (Shen et al., 2013). Uptake was initiated by the addition of transport buffer containing 3 μM CP isomer and 100 μM RIF or DMSO vehicle after washed cells with transport buffer. In control reactions, CP isomer was replaced with 1 μM [^{3}H] E17βG and 0.1 μM [^{3}H] CCK-8 for OATP1B1 and OATP1B3, respectively, to confirm activity of the transporter. The final concentration of DMSO was 0.5% (v/v) in all incubations. Each test condition was assessed in triplicate. After 5 minutes (min), uptake was terminated by washing cells 3 times with ice-cold transport buffer and the plate was air-dried for at least 30 min 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 for counting radioactivity using a Tri-Carb 3100TR liquid scintillation counter (PerkinElmer Life Sciences, Boston, MA). Protein concentrations in the cell lysate aliquots (20 μL) were measured using the Pierce™ BCA protein assay kit. To measure levels of CP isomers, cells in the dried plate were lysed in 300 μL 3:1 (v/v) ratio of acetonitrile and water at room temperature for 1 h. The lysate was centrifuged through a 96-well filter plate (0.45μm low-binding hydrophilic PTFE) at 3,700 rpm for 15 min. 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 high-performance liquid chromatography coupled fluorescence detection (HPLC–FLD) analysis. Quantification of CPs by the HPLC–FLD method is described below.

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; Baltimore, MD). 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 KH buffer containing CP isomers (1 μM) or RSV (1 μM, positive control) with or without RIF (as 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 300 μL 2:1 (v/v) ratio of acetonitrile and 1N hydrochloric acid containing deuteroporphyrin IX as an internal standard, and 1:1 (v/v) ratio of acetonitrile and water at room temperature, for CP and RSV measurement, respectively. The contents were filtered through a 96-well filter plate (0.45μm low-binding hydrophilic PTFE) and the filtrate was dried under nitrogen. CPs I and III samples were reconstituted in 10% formic acid (v/v) (100 μL) and 10 μL sample was injected for HPLC-FLD analysis as described below. RSV samples were reconstituted in 0.1% formic acid in acetonitrile/water (20:80, v/v) (100 μL) and analyzed by an LC-MS/MS.

The stability of CP isomers in human, monkey and mouse hepatocytes over 4 h and in fresh human plasma and urine over 24 h was examined. A detailed description of cell stability assays is available in the Supplementary Methods and Figures.

In Vivo Studies

Effect of CsA Administration on Disposition of CPs in Monkeys. Plasma and urine samples were obtained from the pharmacokinetic interaction study between CsA (100 mg/kg) and RSV in cynomolgus monkeys conducted previously (Shen et al., 2015). Plasma and urine samples were stored at −70°C until analysis. All animal experiments were approved by the

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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.

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 concentration and urinary excretion of CPs I and III, a 2-way crossover study was performed in 3 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 collected from the saphenous vein at 0 (predose), 0.25, 0.5, 0.75, 1, 2, 3, 5, 7, 24, and 48 h. The plasma samples were obtained followed by centrifugation at 3,000 g for 10 min and stored at -70°C until analysis. Urine samples were collected between 0–7, 7-24, and 24-48 h after dosing.

Baseline levels in the plasma and urine were obtained from 4 normal male monkeys. On Day 1, Day 4 and Day 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 h following vehicle (i.e., saline) administration. On Day 1, Day 4, and Day 7, urine samples were collected between 0–7 and 7-24 h 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 Oatp1a/1b Gene Knockout Mice. Male Oatp1a/1b$^{-/-}$ KO and WT mice (30-40 g) were purchased from Taconic Biosciences (Hudson, NY). Animals were

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placed in metabolism cases and fasted overnight before dosing. Urine was collected in a 0–24 h interval to evaluate baseline urinary excretion of CPs I and III.

Plasma concentrations and urine excretion of CPs I and III were characterized in comparison to the change in plasma concentration of RSV following oral administration of 15 mg/kg RSV in saline to 3 male WT and Oatp1a/1b$^{-/-}$ KO mice. Animals were euthanized at 3 h post-dose by terminal bleeding through cardiac puncture under isoflurane anesthesia, and urine and liver tissues were isolated. The urine samples collected from 3 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 dark and sample processes were carried out by protecting from light as much as possible.

Samples from *in vitro* cell lysate were treated before HPLC-FLD analysis as described above. Urine samples (100 µL) were vortex-mixed with 10 µL concentrated hydrochloric acid and centrifuged at 12,000 rcf for 10 minutes. 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 in centrifuge, 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

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diode array detector, and a 1260 Infinity fluorescence detector with 8 µL bio fluorescence flow cell (Agilent Technologies, Santa Clara, CA). The HPLC sample tray was kept at 4ºC. The fluorescence signal was monitored at 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) Synergi Hydro-RP analytical column (Phenomenex, Torrance, CA) by gradient elution at a flow rate of 0.6mL/min. The gradient HPLC mobile phase A contained 25 mM ammonium phosphate buffer at pH 6, while mobile phase B consisted of 50/50 (v/v) mixture of methanol and acetonitrile. The elution program was adjusted for different matrixes 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 min, linear increase from 20% to 35% B from 6 to 20 min, holding at 95% B from 20 to 25 min. The column was equilibrated under initial conditions (20% B) for 5 min between injections. Column temperature was set at 25ºC.

***Quantification of RSV by LC-MS/MS*.** The cell lysate, plasma and liver concentrations of RSV were determined by LC-MS/MS 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 min, Figure 2). Initial uptake rate (V_{uptake}) was calculated based on the rate of uptake during the linear phase (up to 1.0 or 1.5 min, Figure 2). The uptake clearance (CL_u) and percent active uptake were calculated using the following equations:

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$$CL_{\mathrm{u}} = \frac{V_{\mathrm{uptake}}}{C} \tag{1}$$

$$\text{Active Uptake}\,\% = \frac{CL_{\mathrm{u,\,vehicle}} - CL_{\mathrm{u,100\,\mu M\,RIF}}}{CL_{\mathrm{u,\,vehicle}}}\,\mathrm{X}\,100 \tag{2}$$

where C is the measured concentration of the test compound in the incubation buffer, and $CL_{\mathrm{u,\,vehicle}}$ and $CL_{\mathrm{u,\,100\,\mu M\,RIF}}$ are the uptake clearance values in the absence and presence of inhibitor, respectively.

Pharmacokinetic Analysis. The AUC from time zero to 24 or 48 h [$AUC_{(0\text{-}24\mathrm{h})}$ or $AUC_{(0\text{-}48\mathrm{h})}$] was calculated by mixed log-linear trapezoidal summations using validated software (Kinetica v. 5.0; Thermo Electron, Philadelphia, PA). Maximum plasma concentration (C_{max}) were directly obtained by observation. Renal clearance (CL_{R}) and renal extraction ratio (ER_{R}) were estimated by the following equations:

$$CL_{\mathrm{R}} = \frac{X_{\mathrm{e}}}{AUC} \tag{3}$$

$$ER_{\mathrm{R}} = \frac{CL_{\mathrm{R}}}{f_u \bullet GFR} \tag{4}$$

where X_{e} 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 (i.e., 0.28) (Kaplowitz et al., 1972), and GFR is the glomerular filtration rate in monkeys (i.e., 2.1 mL/min/kg) (Davies and Morris, 1993).

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Statistical Analysis. The *in vitro* data represent 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 2 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 2 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 are presented as the mean ± standard error of the mean (SEM). 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; $p < 0.001$) (Figure 1A-B). OATP1B involvement in the transport of CPs was further confirmed by inhibition of the uptake by co-incubation 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 ([^{3}H] E17βG) and OATP1B3 ([^{3}H] CCK-8). Probe substrate uptake by the HEK cells expressing OATPs were significantly enhanced compared to MOCK-HEK cells and uptake were greatly inhibited by 100 μM RIF, which confirmed the functional activity in cellular uptake of OATP substrates.

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 Figure 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 min 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 hepatocyte, respectively), whereas uptake clearance in monkey hepatocyte was about 2 fold higher than that in human. Approximately 75 to 85% of CP I and III uptake in

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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-h incubation of CPs I and III (1 μM) with the hepatocytes of the 3 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 (Supplementary Figure 2A-C). CPs I and III were also shown to be stable in human plasma and urine samples (Supplementary Figure 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 Figure 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) was observed when co-dosed. Plasma CPs I and III $AUC_{(0–24\ h)}$ 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 (Figure 3A and 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 (Figure 2B-C and Table 1). Consequently, renal clearance of CPs I and III (RSV alone versus the co-dosed) 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 is 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 h, there was no difference in plasma concentration and urinary excretion of CPs I and III beyond

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24 h between groups (Figure 3A-C). As a result, the plasma concentrations at 48 h and urinary excretion during 24-48 h 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 ER_R 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 ER_R 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 ER_R of CP I did not change as expected. Although the ER_R 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 h post-dose 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 $AUC_{(0–48\ h)}$ ($p<0.05$, Table 2) were observed in RIF-treated monkeys compared to the control animals. There is no difference in CP plasma concentration between two group animals before administration of RIF (i.e., at predose).

The amounts of CPs I and III excreted in urine over 48 h were consistently higher in RIF-treated monkeys as compared to the vehicle-treated animals (4.3 ± 1.6- and 3.1 ± 1.5-fold increase, respectively) and the differences were statistically significant ($p < 0.05$; Figure 4B-D and Table 2). However, the renal clearance of CPs I and III, appeared to be unaffected by RIF. In addition, no significant differences were discerned between the RIF and vehicle groups with respect to renal extraction ratios 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. In addition, the ER_R 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_R 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 4 untreated animals, as shown in Figure 5. The mean plasma levels and 24-h 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 1.5-fold difference) (Figure 5). In addition, the degree of intra-individual variability of CPs I and III basal plasma concentrations and urine excretion is low (within 1.9-fold; data not shown).

CPs I and III in Plasma and Urine in the Oatp1a/1b Cluster Genes Knockout Mice. To further understand the effects of OATP transporters on the disposition of CPs, we compared urinary excretion and plasma concentrations of CPs I and III between WT and Oatp1a/1b$^{-/-}$ KO mice, which are functionally deficient in Oatp1a1, Oatp1a4, Oatp1a5, Oatp1a6, and Oatp1b2. Urinary excretion analyses were performed for both 0-24-h and 0-3-h periods with RSV administration. As shown in Figures 6 and 7B, the 24-h and 3-h urinary excretion of CPs I and III were markedly increased in Oatp1a/1b$^{-/-}$ KO mice. In this regard, there was approximately 12.4- and 18.4-fold higher $X_{e\ (0\text{-}24\ h)}$, and 7.1- and 12.1-fold higher $X_{e\ (0\text{-}3\ h)}$ for CPs I and III excretion, respectively, ($p < 0.001$ for 24-h analyses). In addition, Oatp1a/1b$^{-/-}$ KO mice demonstrated 7.4- and 15.2-fold higher CPs I and III plasma levels at 3 h post RSV dose, respectively, compared with WT mice ($p < 0.001$; Figure 7A). Total CP excretion in urine and concentration in plasma of Oatp1a/1b$^{-/-}$ KO mice were 10.8- to 16.7-fold higher than in WT mice ($p < 0.001$; Figure 7A-B). CP III is the predominant form in both plasma and urine (73 to 90%)

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(Figures 6 and 7). Interestingly, a similar increase was observed in plasma RSV concentration at 3 h post-dose when comparing Oatp1a/1b$^{-/-}$ KO mice with WT mice (14.6-fold: 289 ± 62.2 versus 19.8 ± 1.3 nM; $p < 0.001$) (Figure 7C). However, no significant difference was found in the liver concentrations of RSV at 3 h post-dose between knockout and WT mice (Figure 7D).

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 manuscript was in preparation, Bednarczyk and Boiselle assessed the *in vitro* transport of CP by OATP1B1 and OATP1B3 using transporter-overexpressing cells (Bednarczyk and Boiselle, 2015). The authors demonstrated that the *in vitro* transport of CPs I and III by OATP1B1 and OATP1B3 is 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 co-incubation with 100 μM RIF, a potent inhibitor of each of these OATP transporters (Shen et al., 2013) (Figure 1A-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, condition known to deplete active transport by OATP1B1 and OATP1B3 but unlikely by NTCP (IC_{50} = 277 μM) (Prueksaritanont et al., 2014), reduced the uptake of CP by 75 to 85% (Figure 2).

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We have reported that the administration of CsA (100 mg/kg) and RIF (15 mg/kg) to cynomolgus monkeys significantly increased the AUC values of RSV, a well-established exogenous OATP1B probe, by 6.3- and 2.9-fold, respectively, due to OATP inhibition (Shen et al., 2013, Shen et al., 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 line with the increase in RSV exposure, as seen in Tables 1 and 2, CsA increased CPs I and III plasma exposures by 2.7- and 5.2-fold, and RIF increased those by 2.7- and 3.6-fold, respectively. These observations agree with clinical findings that CsA and RIF caused drug-induced porphyria that refers to increased levels of circulating porphyrins including CP in patients (Millar, 1980, Hivnor et al., 2003), likely due to the inhibition of OATP1B1 and OATP1B3. Urinary excretion of CP I was increased to a similar extent compared to the changes in CP I AUC (3.5- versus 2.7-fold and 2.7- versus 4.3-fold by CsA and RIF, respectively). Urinary excretion of CP III, however, was increased to a smaller extent compared to the changes in CP III AUC by CsA but not RIF (1.6- versus 5.2-fold and 3.1- versus 3.6-fold by CsA and RIF, respectively). Apparently transporter-mediated tubular secretion accounts for most of renal clearance of CP III in monkeys as ER_R is significantly greater than unity (approximately 10- to 30-fold) (Tables 1 and 2). CP III constitutes the major form of CP excreted in normal urine in monkeys (Table 3), which has also been observed in humans (Koskelo et al., 1966, Koskelo et al., 1967, Gebril et al., 1990). The active tubular secretion was unlikely inhibited by RIF and slightly reduced by CsA in this study. As a result, the CP III changes in urine in monkeys are small. These findings indicated that inhibition of OATP function could increase plasma CP concentrations and shunt the elimination pathway to renal excretion. The verification of CP as OATP biomarker was then extended in Oatp1a/1b$^{-/-}$ KO mice, where similar observations were

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found for CP plasma levels and urinary excretion. While Oatp1a/1b$^{-/-}$ KO mice showed a significant 7.4- and 15.2-fold increase in plasma concentrations of CPs I and III at 3 h compared with WT animals, respectively, the plasma concentration of RSV at 3 h following the single oral dose of 15 mg/kg RSV increased by 14.6-fold (Figure 7A and 7C). Urinary excretion of CPs I and III over 3 and 24 h 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 $X_{e\ (0\text{-}3\ h)}$ and $X_{e\ (0\text{-}24\ h)}$, 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 OATP1B 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 ER_R 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 CsA and RIF study, 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 (Supplementary Figure 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 mean CL_R and ER_R of CP III in monkeys, while RIF, a relatively specific inhibitor for OATP1B1 and OATP1B3 (Prueksaritanont et al., 2014), did not decrease mean CL_R and ER_R 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 work on characterization of CP excretion in the bile and the 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 OATP1B compared with CP III because CP I is specific to hepatic uptake and is unlikely to be subject to active renal transport.

Urinary CP isomer ratio has been proposed as a biomarker of MRP2 activity although there is no *in vitro* evidence that indicated 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 (mutation in ABCC2 gene) differed from normal subjects. CP III predominated in the urine of normal subjects, whereas the proportion of CP I was higher in the urine of patients with Dubin-Johnson syndrome, accounting for approximately 80% of the CP present. However, in terms of absolute amounts, urinary excretion of CP I was significantly increased compared to normal subjects, while the absolute amounts of CP III were decreased (Koskelo et al., 1967, Ben-Ezzer et al., 1971, Wolkoff et al., 1976, Frank et al., 1990, Toh et al., 1999). The mechanism of these changes has not yet been fully worked out. It is worth noting that the clinical relevance of MRP2 inhibition DDI is minimal in humans, with exposure levels reaching 121% to 234% of those in normal subjects, depending on the drug considered (Chester et al., 2003, Suwannakul et al., 2008, Ieiri et al., 2009, Brennan et al., 2015, Davenport et al., 2015).

Recently, the specificity of CPs I and III transport was studied using cells transfected with various transporters (Bednarczyk and Boiselle, 2015). Besides OATP1B1- and OATP1B3-mediated transport of CPs I and III, OATP2B1 demonstrated significant transport of CP III while NTCP, 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 transfected cells of 2.4 while CP I was not transported by OAT1 (Bednarczyk and Boiselle, 2015). 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 human as CP disposition may not be specific to OATP. Indeed, species-dependent differences in disposition were observed for CP. While CP III predominated in the urine of monkey with the mean CP I/CP III ratio of 0.07 to 0.08, mouse and human excreted relatively more of CP I (0.29 to 0.44 CP I/CP III ratio). Therefore, characterization of CP disposition under in human 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., 2015b). 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 sodium-taurocholate cotransporting polypeptide (NTCP) and bile salt export pump (BSEP). Furthermore, serum bilirubin and bile acids 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 clinic.

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 monkey and mouse. Furthermore, if our findings can indeed be extrapolated to humans, CPs I and III will provide an 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 and Lai

Conducted experiments: Shen, Dai, Liu, Cheng, Chen, and Freeden

Contributed new reagents or analytic tools: Shen, Dai, Liu, and Lai

Performed data analysis: Shen, Dai, and Lai

Wrote or contributed to the writing of the manuscript: Shen, Dai, Cheng, Humphreys, Marathe, and Lai

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Footnotes

Reprint requests: Hong Shen, Room F1.300, Route 206 & Province Line Road., Bristol-Myers Squibb Company, Princeton, NJ 08543. Telephone: (609) 252-4509; Facsimile: (609) 252-6802.

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Figure Legends

Figure 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), [^{3}H]E17βG (1 μM) (C), and [^{3}H]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 ± SEM of 3 separate determinations (***$p < 0.001$ statistically significant difference compared to Mock-HEK cells).

Figure 2. CPs I and III transport by human and monkey hepatocytes. Time course of uptake of CPs (1 μM) and RSV (1 μM) was measured in human (A and B) and monkey hepatocytes (C and D) in the absence and presence of RIF (100 μM). The data are expressed as the mean ± SEM of 3 separate determinations.

Figure 3. Effects of CsA on CP disposition in cynomolgus monkeys. CP plasma concentration-time profiles (A), the 0-7 h, 7-24 h, and 24-48 h urinary excretion rates of CP I (B), CP III (C), and total CP (D) were determined following oral administration of 3 mg/kg RSV alone and 3 mg/kg RSV with 100 mg/kg CsA. CsA was dosed 1 h ahead of RSV. The data are expressed as the mean ± SEM of 3 animals (*$p < 0.05$, statistically significant difference compared to RSV alone group).

Figure 4. Effects of RIF on CP disposition in cynomolgus monkeys. CP plasma concentrations (A), the 0-7 h, 7-24 h, and 24-48 h urinary excretion rates of CPs I (B) and 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 ± SEM of 3 animals (*$p < 0.05$ and **$p < 0.01$ statistically significant difference compared to the vehicle control group).

Figure 5. Mean plasma concentrations (A) and 24-h urinary excretion rates of CPs I and III (B) on Days 1, 4, and 7 in cynomolgus monkeys. The data are expressed as the mean ± SEM of 4 animals. There are no significant differences in CP plasma level and urinary excretion among time points and days ($p > 0.05$).

Figure 6. Effects of Oatp 1a/1b gene deletion on urinary excretion of CP in mice. The 24-h urinary excretion rates of CPs I, III, and total CP were determined in WT and Oatp1a/1b$^{-/-}$ KO mice. The data are expressed as the mean ± SEM of 6 animals (***$p < 0.001$ statistically significant difference compared to wild-type group).

Figure 7. Effects of Oatp 1a/1b gene deletion on CP and RSV disposition in mice. CP plasma concentrations (A), 3-h CP urinary excretion (B), RSV plasma concentrations (C), and RSV liver concentrations (D) at 3 h following oral administration of 15 mg/kg RSV to WT and Oatp1a/1b$^{-/-}$ KO mice were determined. The data are expressed as the mean ± SEM of 3 animals (***$p <$ 0.001 statistically significant difference compared to wild-type group).

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Table 1. Comparison of Pharmacokinetic Parameters of CP I and CP III Following Oral Administration of 3 mg/kg RSV alone and 3 mg/kg RSV with 100 mg/kg CsA in Cynomolgus Monkeys.

Parameter	CP I		CP III	
	RSV alone	**RSV + CsA**	**RSV alone**	**RSV + CsA**
C_{max} **(nM)**	0.93 ± 0.06	3.0 ± 0.29**	0.23 ± 0.06	1.6 ± 0.12***
$AUC_{(0\text{-}24\ h)}$ **(nM*h)**	15.9 ± 2.5	41.5 ± 4.7**	4.1 ± 1.2	20.3 ± 2.8**
$X_{e\ (0\text{-}24\ h)}$ **(nmol)**	1.4 ± 0.61	4.8 ± 1.7*	17.1 ± 6.1	26.6 ± 10.1
CL_R **(mL/min)**	1.5 ± 0.75	1.9 ± 0.55	75.5 ± 40.0	21.5 ± 5.2
ER_R	0.68 ± 0.32	0.85 ± 0.17	33.6 ± 17.0	9.7 ± 1.6

Data are expressed as mean ± SD (n = 3). Oral CsA treatment was given 1 h prior to RSV oral administration.

C_{max}, maximum plasma concentration; AUC, area under concentration-time curve; X_e, amount of compound excreted into urine; CL_R, renal clearance; ER_R, renal extraction ratio.

**p < 0.01, and ***p < 0.001 statistically significant difference compared to RSV alone group.

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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.

Parameter	CP I		CP III	
	Vehicle	RIF	Vehicle	RIF
$AUC_{(0\text{-}48\ h)}$ (nM*h)	14.2 ± 2.2	37.6 ± 8.9*	5.8 ± 0.48	20.6 ± 3.1**
$X_{e\ (0\text{-}48\ h)}$ (nmol)	1.5 ± 0.42	6.2 ± 2.2*	21.5 ± 6.1	61.8 ± 15.0*
CL_R (mL/min)	1.8 ± 0.79	2.8 ± 0.92	62.9 ± 21.8	49.4 ± 5.1
ER_R	0.68 ± 0.34	1.1 ± 0.40	24.1 ± 9.8	18.7 ± 1.7

Data are expressed as mean ± SD (n = 3).

AUC, area under concentration-time curve; X_e, amount of compound excreted into urine; CL_R, renal clearance; ER_R, renal extraction ratio.

*p < 0.05, and **p < 0.01 statistically significant difference compared to vehicle control group.

Table 3. Comparison of Urinary Excretion of CP I and CP III in Cynomolgus Monkeys, Mice, and Humans

Species (n)	$X_{e\ (0\text{-}24\ h)}$ (nmol/kg BW)			% of Total Urinary CP		Urinary CP Ratio
	CP I	CP III	Total CP	CP I	CP III	CP I/CP III
Monkey Experiment 1 (3)	0.37 ± 0.14	4.42 ± 1.23	4.79 ± 1.35	7.6 ± 1.4	92.4 ± 1.4	0.08 ± 0.02
Monkey Experiment 2 (3)	0.15 ± 0.10	2.14 ± 1.43	2.29 ± 1.53	6.7 ± 0.58	93.3 ± 0.58	0.07 ± 0.01
Mouse (3)	0.34 ± 0.06	0.91 ± 0.17	1.25 ± 0.22	27.2 ± 2.2	72.8 ± 2.2	0.37 ± 0.04
Human[a] (30)	0.30 ± 0.09	1.17 ± 0.44	1.46 ± 0.50	ND	78.2 ± 5.8	ND
Human[b] (22)	0.48 ± 0.24	1.13 ± 0.35	1.57 ± 0.46	ND	71.0 ± 10.2	0.44 ± 0.25
Human[c] (86)	0.31	1.10	1.41	22.2	77.8	0.29
	Plasma Concentration (nM)			**% of Total Plasma CP**		**Plasma CP Ratio**
	CP I	CP III	Total CP	CP I	CP III	CP I/CP III
Monkey CsA Inhibition (3)	0.75 ± 0.17	0.19 ± 0.05	0.93 ± 0.20	79.8 ± 4.7	20.2 ± 4.7	4.3 ± 1.6
Monkey RIF Inhibition (3)	0.32 ± 0.08	0.18 ± 0.04	0.50 ± 0.11	64.4 ± 4.3	35.6 ± 4.3	1.8 ± 0.3
Mouse (3)	0.13 ± 0.02	0.58 ± 0.11	0.71 ± 0.12	18.9 ± 1.4	81.1 ± 1.4	0.23 ± 0.02
Human[d] > 30)	ND	ND	< 6	ND	ND	ND
Human[e] (245)	0-10	0-12	ND	ND	ND	ND

BW, body weight; X_e, amount of compound excreted into urine from 0 to 24 h; ND, not determined.

[a]Data on urinary excretion of CP I and CP III in healthy subjects are from a previous publication (Koskelo et al., 1967).

[b]Data on urinary excretion of CP I and CP III in healthy subjects are from a previous publication (Koskelo et al., 1966).

[c]Data on urinary excretion of CP I and CP III in healthy subjects are from a previous publication (Gebril et al., 1990).

[d]Data on plasma concentrations of CP I and CP III in healthy subjects are from a previous publication (Kanayama et al., 1992).

[e]Data on plasma concentrations of CP I and CP III in healthy subjects are from a previous publication (Hindmarsh et al., 1999).

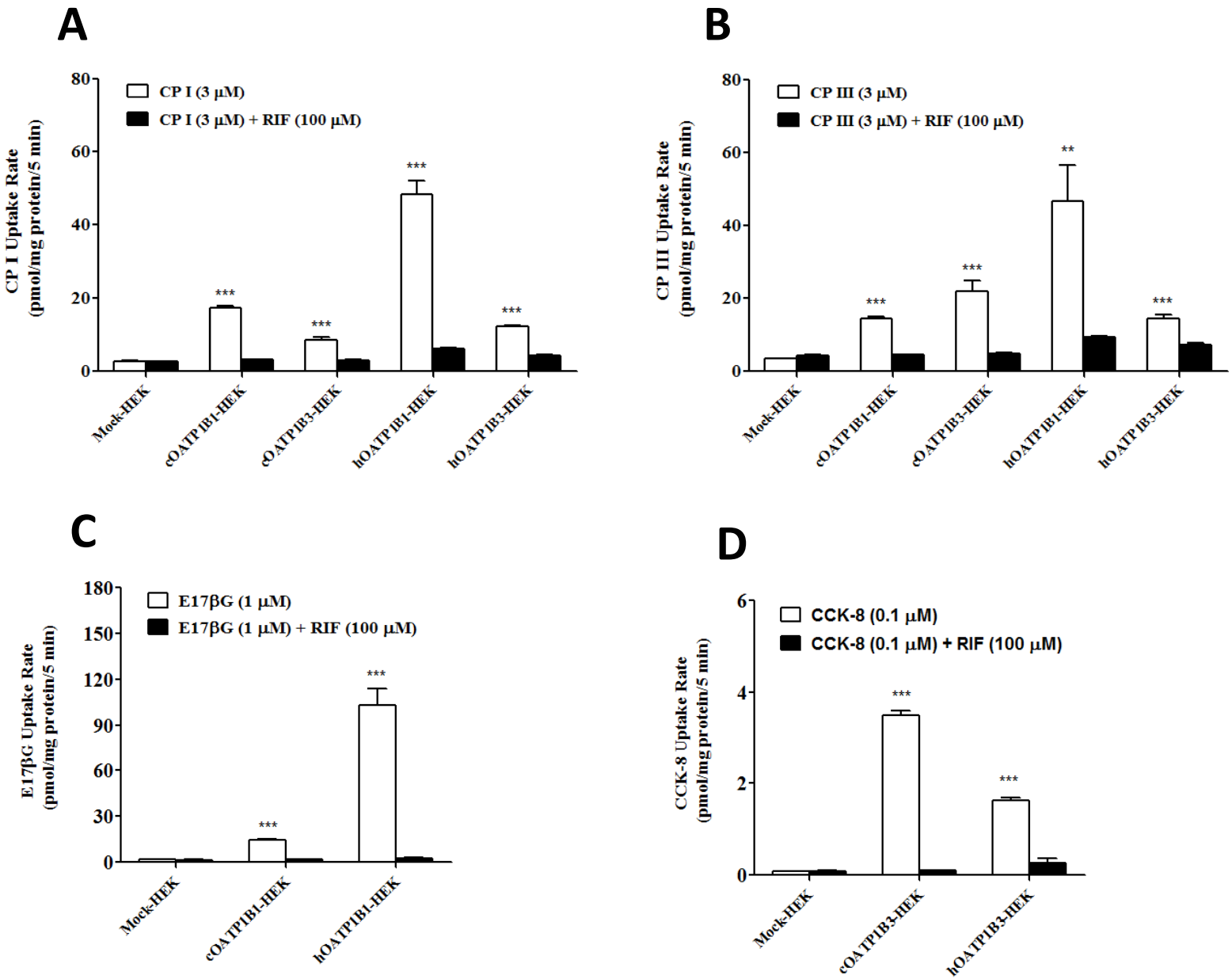
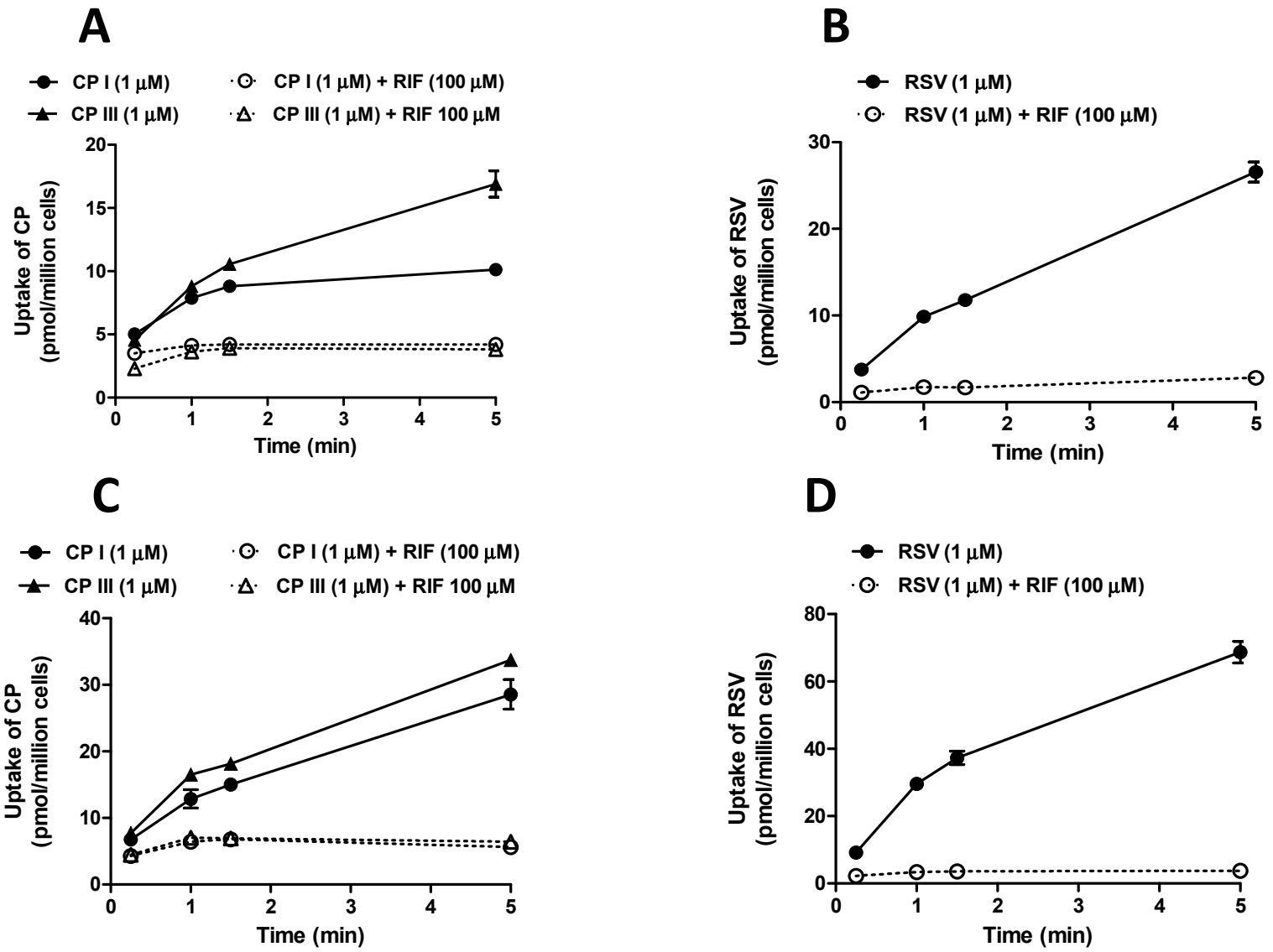


Figure 1



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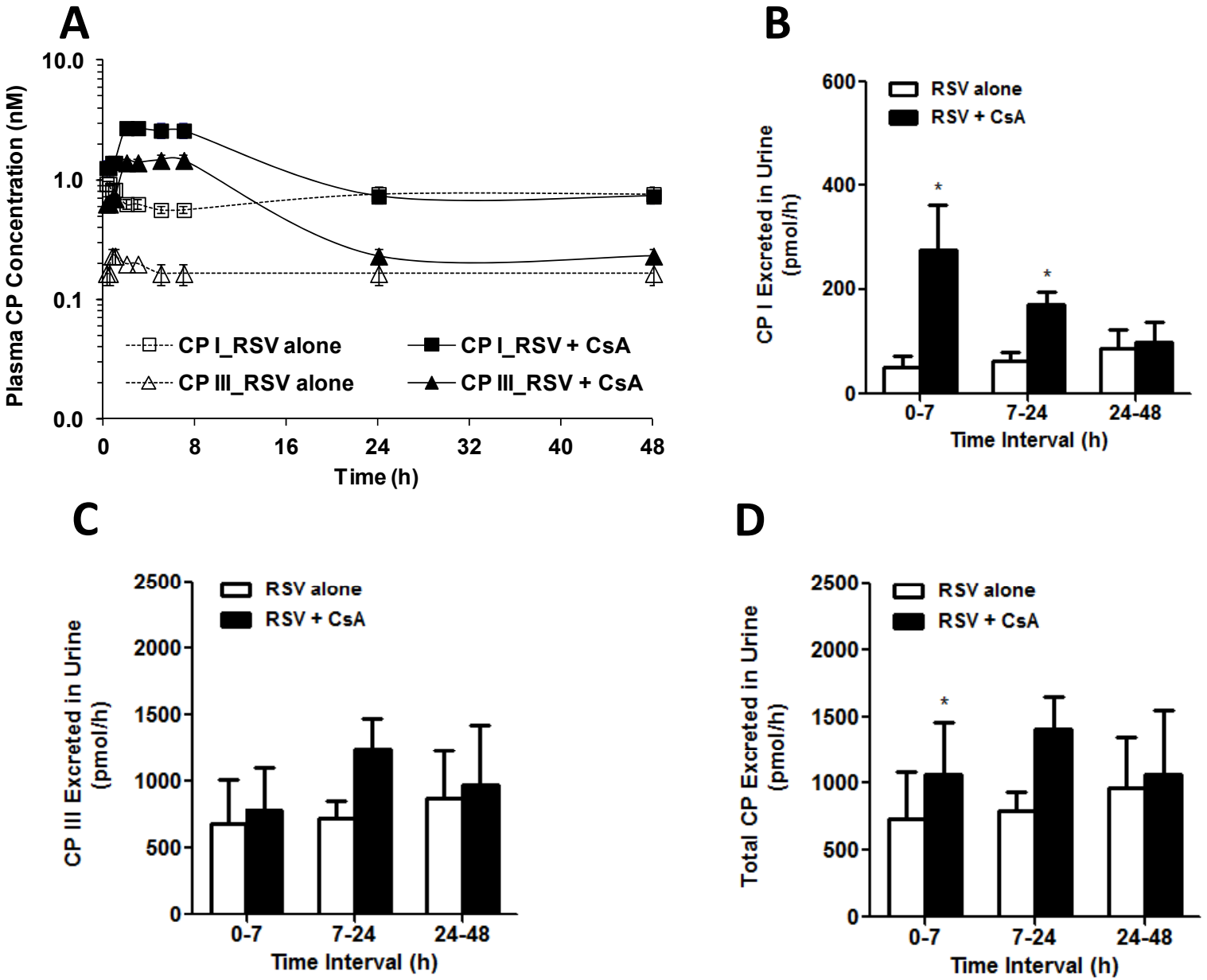

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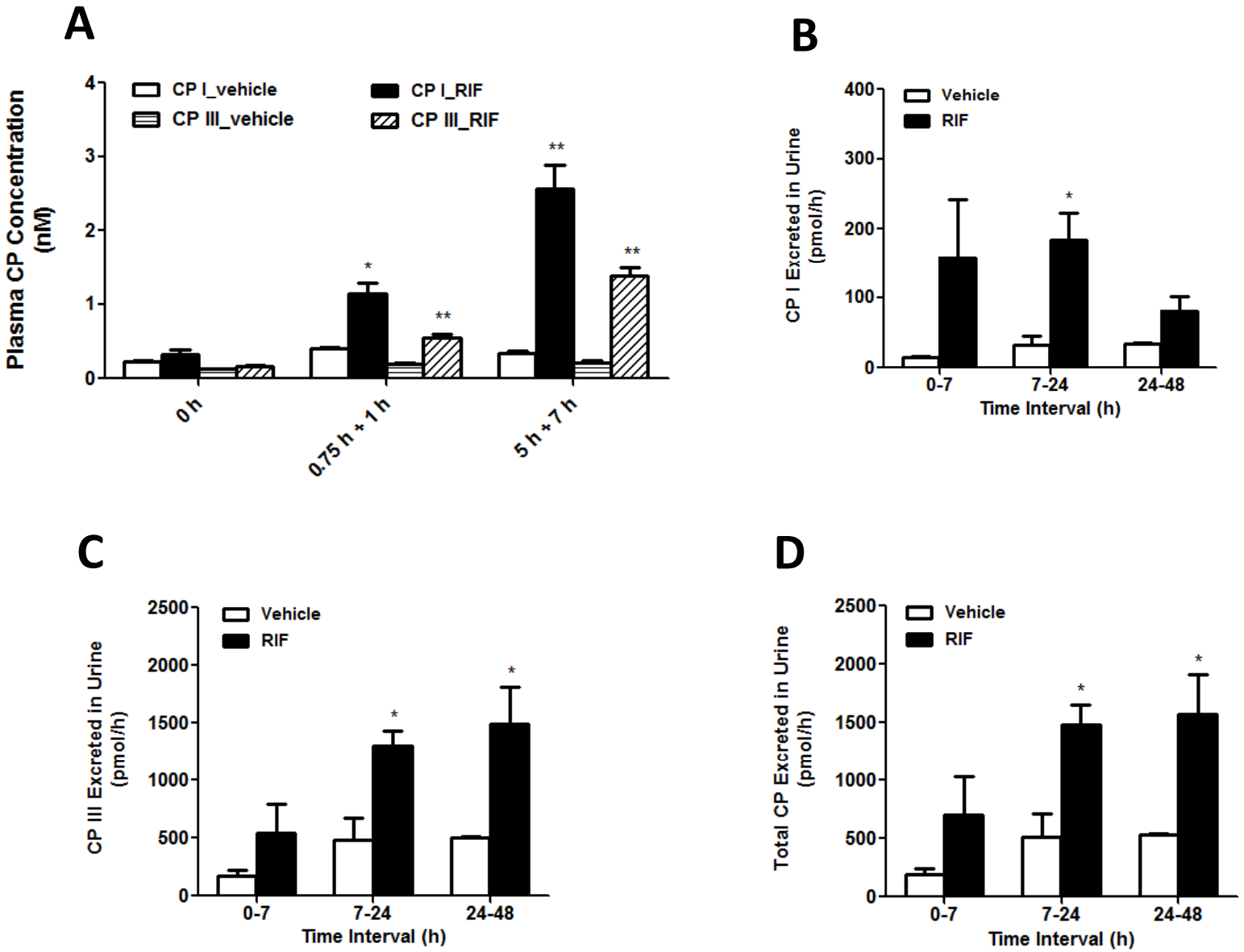

Figure 4

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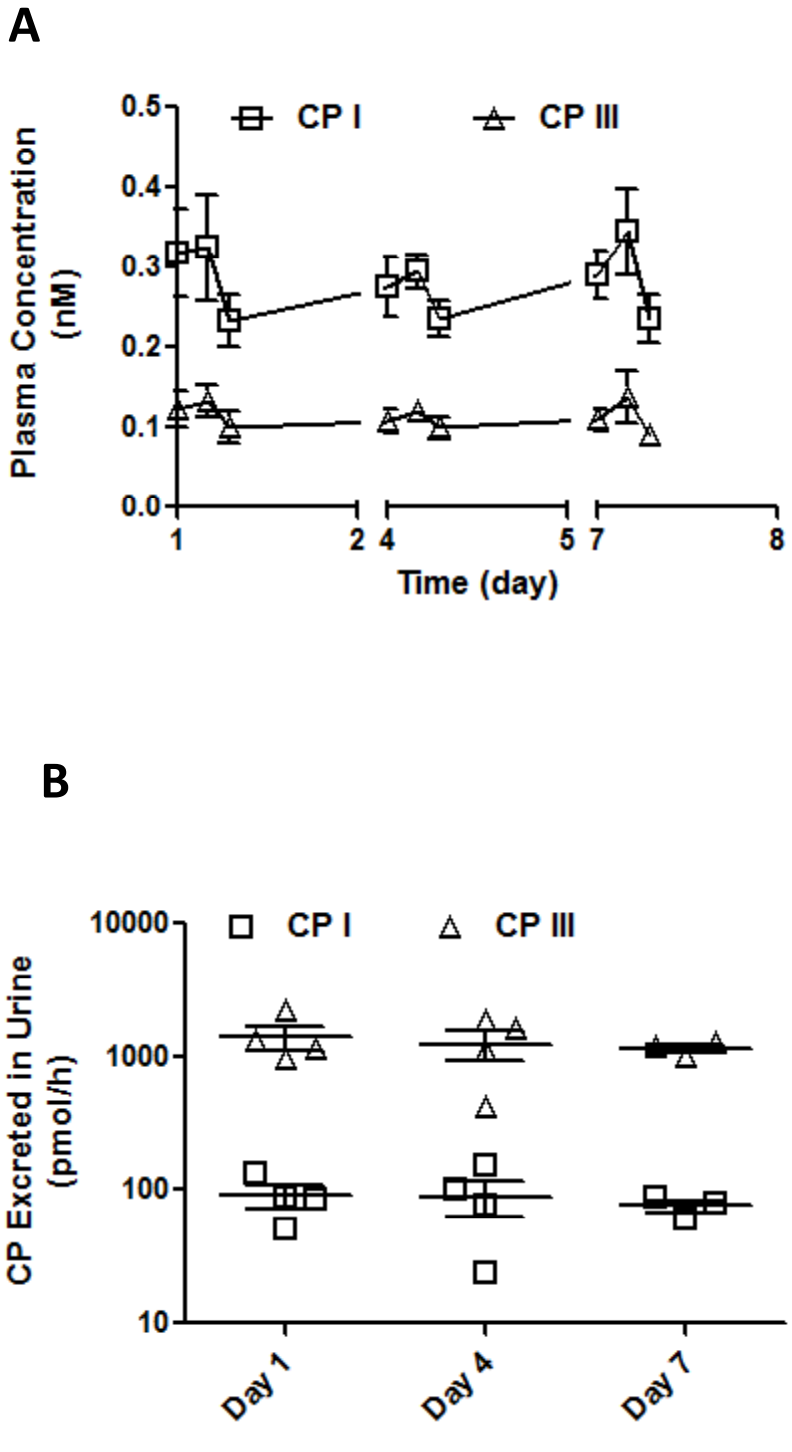

Figure 5

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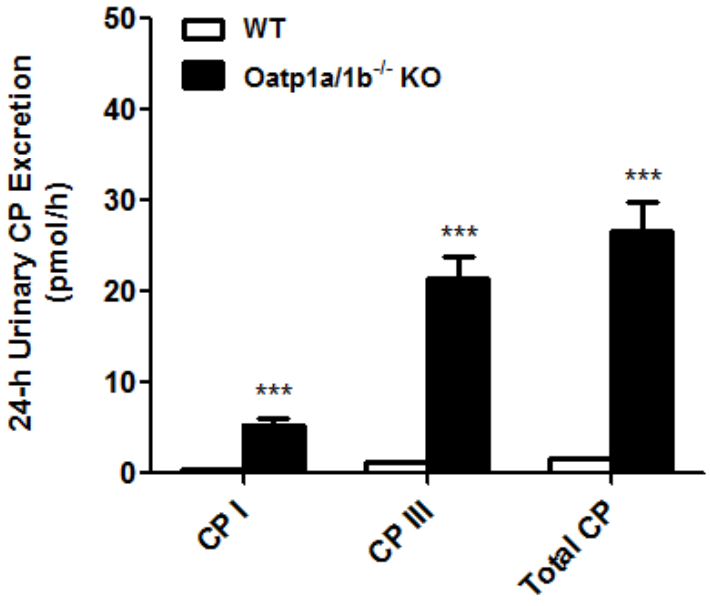

Figure 6

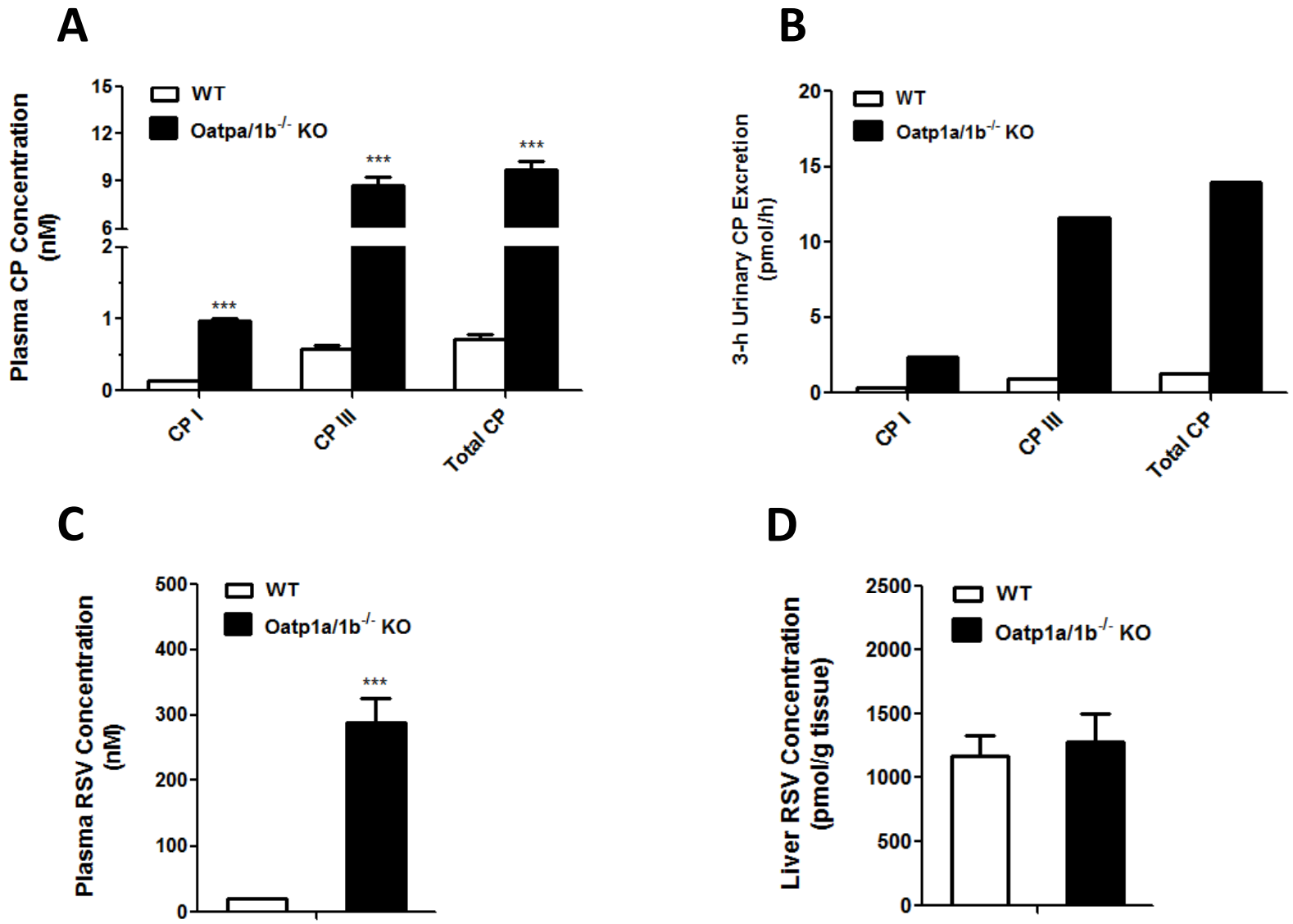

Figure 7