DMD Fast Forward. Published on July 28, 2020 as DOI: 10.1124/dmd.120.000076
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DMD-AR-2020-000076

Detection of Weak Organic Anion-Transporting Polypeptide (OATP) 1B Inhibition by Probenecid (PROB) with Plasma-Based Coproporphyrin in Humans

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Running title: Use of coproporphyrin to detect weak OATP1B inhibition by PROB

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Number of text pages: 23

Number of tables: 4

Number of figures: 4

Number of references: 57

Number of words:

Abstract: 301

Introduction: 739

Discussion: 1,727

Abbreviations: $AUC_{(0\text{-}24h)}$, area under plasma concentration-time curve from time 0 to 24 hour; C_{max}, maximum plasma concentration; CPI, coproporphyrin I; CPIII, coproporphyrin III; DDI,

drug-drug interaction; FDA, Food and Drug Administration; FSM, furosemide; GCDCA-S, glycochenodeoxycholate-3-sulfate; HDA, hexadecanedioate; HEK293, human embryonic kidney 293 cells; IC_{50}, concentration required to inhibit transport by 50%; LC-MS/MS, liquid chromatography–tandem mass spectrometry; OAT, organic anion transporter; OATP, organic anion-transporting polypeptide; PROB, probenecid; *R*-value: ratio of victim AUC in the presence and absence of perpetrators; SD, standard division; SE, standard error; TDA, tetradecanedioate.

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ABSTRACT

Probenecid (PROB) is a clinical probe inhibitor of renal organic anion transporter (OAT) 1 and OAT3 that inhibits in vitro activity of hepatic drug transporters OATP1B1 and OATP1B3. It was hypothesized that PROB could potentially affect the disposition of OATP1B drug substrates. The plasma levels of the OATP1B endogenous biomarker candidates, including coproporphyrin I (CPI), CPIII, hexadecanedioate (HDA), and tetradecanedioate (TDA), were examined in 14 healthy subjects treated with PROB. After oral administration with 1,000 mg PROB alone and in combination with furosemide (FSM), $AUC_{(0\text{-}24h)}$ values were 1.39 ± 0.21- and 1.57 ± 0.41-fold higher than predose levels for CPI and 1.34 ± 0.16- and 1.45 ± 0.57-fold higher for CPIII. Despite increased systemic exposures, no decreases in CPI and CPIII renal clearance (CL_R) were observed (0.97 ± 0.38- and 1.16 ± 0.51-fold for CPI, and 1.34 ± 0.53- and 1.50 ± 0.69-fold for CPIII, respectively). These results suggest that the increase of CP systemic exposure is caused by OATP1B inhibition. Consistent with this hypothesis, PROB inhibited OATP1B1- and OATP1B3-mediated transport of CPI in a concentration-dependent manner, with IC_{50} values of 167 ± 42.0 and 76.0 ± 17.2 μM, respectively, in transporter overexpressing HEK cell assay. The inhibition potential was further confirmed by CPI and CPIII hepatocyte uptake experiments. In contrast, administration of PROB alone did not change $AUC_{(0\text{-}24h)}$ of HDA and TDA relative to prestudy levels although the administration of PROB in combination with FSM increased HDA and TDA levels compared to FSM alone (1.02 ± 0.18- and 0.90 ± 0.20 versus 1.71 ± 0.43- and 1.62 ± 0.40-fold). Taken together, these findings indicate that PROB displays weak OATP1B inhibitory effects in vivo and CP is a sensitive endogenous probe of OATP1B inhibition. This study provides an explanation for the heretofore unknown mechanism responsible for PROB's interaction with other xenobiotics.

SIGNIFICANCE STATEMENT

This study suggested that PROB is a weak clinical inhibitor of OATP1B based on the totality of evidence from the clinical interaction between PROB and CP, and the in vitro inhibitory effect of PROB on OATP1B-mediated CP uptake. It demonstrates a new methodology of utilizing endogenous biomarkers to evaluate complex DDI, providing explanation for the heretofore unknown mechanism responsible for PROB's inhibition. It provides evidence to strengthen the claim that CP is a sensitive circulating endogenous biomarker of OATP1B inhibition.

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INTRODUCTION

It is well established that membrane-bound transport systems are primarily critical determinants in the cellular traffic and disposition of exogenous and endogenous compounds. Notably, organic anion transporting polypeptide (OATP) 1B1 and OATP1B3 can govern the disposition of drug substrates including the widely used HMG-CoA reductase inhibitors (statins). Moreover, it is increasingly recognized that endogenous biomarkers of OATP1B1 and OATP1B3 such as coproporphyrin I (CPI), CPIII, hexadecanedioate (HDA), and tetradecanedioate (TDA), and glycochenodeoxycholate-3-sulfate (GCDC-S), are useful tools in elucidating the role of OATP1B in complex drug-drug interaction (DDI) in healthy subjects and patients (Lai et al., 2016, Shen et al., 2016, Shen et al., 2017, Shen et al., 2018, Takehara et al., 2018, Barnett et al., 2019, Yee et al., 2019, Jones et al., 2020, Mori et al., 2020).

Probenecid (PROB) is widely used as a uricosuric agent (Cunningham et al., 1981). In addition, PROB has been used as an adjunct to enhance blood levels of antibiotics such as penicillins and cephalosporins due to an inhibitory effect on the renal transporters OAT1 and OAT3. In agreement, most of the drug-drug interactions (DDIs) involving PROB are due to its inhibition on the kidney transport of acidic drugs such as cefaclor (Welling et al., 1979), cefonicid (Pitkin et al., 1981), cefoxitin (Vlasses et al., 1980), cephradine (Welling et al., 1979), dicloxacillin (Beringer et al., 2008), famotidine (Inotsume et al., 1990), and furosemide (Vree et al., 1995, Shen et al., 2019a). As a result, the US Food and Drug Administration (FDA) suggests PROB as an index inhibitor to assess OAT1 and OAT3 in clinical DDI studies (https://www.fda.gov/drugs/drug-interactions-labeling/drug-development-and-drug-interactions-table-substrates-inhibitors-and-inducers). However, knowledge of the transporters involved in PROB inhibition could be incomplete. For example, some studies showed that the action of

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PROB at the renal level was not sufficient to account for several-fold serum level elevations encountered for drugs such as fexofenadine, statins, and methotrexate in humans and animals (Gewirtz et al., 1984, Yasui-Furukori et al., 2005, Liu et al., 2008, Kosa et al., 2018). As an organic acid, probenecid increased the area under plasma concentration-time curve (*AUC*) of fexofenadine approximately 1.5-fold, by interfering with secretion of fexofenadine by the renal tubules in humans (Yasui-Furukori et al., 2005, Liu et al., 2008). The total and renal clearance (CL_R) values of fexofenadine were 16.0 and 6.2 L/h (Lappin et al., 2010). Given the fact that fexofenadine is 60% to 70% bound to plasma proteins (Molimard et al., 2004), the active tubular secretion contributes to approximately half of the CL_R. As a result, inhibition of OAT3-mediated renal secretion clearance alone, which resulted in a decreased amount of dose excreted in urine from 11-12% to 6-8%, cannot explain the observed AUC change (Yasui-Furukori et al., 2005, Liu et al., 2008). Moreover, studies in cynomolgus monkeys and rats have demonstrated that probenecid decreased the clearance of statins and methotrexate by the hepatic route as well (Gewirtz et al., 1984, Kosa et al., 2018), contributing to prolonged elevation of circulating statins and methotrexate levels. The reduction in hepatic uptake of statins in monkey hepatocytes and biliary secretion of methotrexate in rats is thought to arise from inhibition of hepatic uptake mediated by OATP in the presence of PROB. However, a direct interaction between PROB and OATP1B in humans has not been fully tested.

Recent bioanalytical methodologies, especially metabolomics, have identified a number of endogenous biomarker candidates for the transporter proteins expressed in the organs of importance in drug disposition, including liver and kidney (Chu et al., 2018, Muller et al., 2018, Rodrigues et al., 2018, Shen, 2018). Changes in the levels of such endogenous probes were able to phenotype the mechanism of complex DDIs involving multiple elimination pathways. For

example, a clinical DDI study was conducted to assess the inhibition potential of fenebrutinib using midazolam (CYP3A), simvastatin (CYP3A and OATP1B), and rosuvastatin (BCRP and OATP1B) as drug substrates (Jones et al., 2020). Fenebrutinib increased the *AUC* values of all three probes 2- to 3-fold. However, there was no change in the plasma levels of CPI and CPIII, endogenous biomarkers of OATP1B1, suggesting that the DDIs were due to the inhibition of CYP3A and BCRP rather than OATP1B (Jones et al., 2020). The present studies were designed to evaluate the inhibitory effects of PROB on human OATP1B1 and OTP1B3 by analyzing OATP1B biomarker levels in a clinical study and evaluating coproporphyrin uptake in transfected cell lines and hepatocytes in the presence of PROB in order to determine whether PROB modulates OATP1B activity.

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MATERIALS AND METHODS

Reagents and Materials. CPI and CPIII were purchased as dihydrochloride salts from Frontier Scientific (Logan, UT). HDA, TDA, carbamazepine, and high-performance liquid chromatography (HPLC) grade methanol, acetonitrile and water were purchased from Sigma Aldrich (St. Louis, MO). Rosuvastatin (RSV), E17βG, CCK-8, CPI-$^{15}N_4$ sodium bisulfate salt, HDA-d4, TDA-d4 and rifampicin SV (RIF SV) were purchased from Toronto Research Chemicals (North York, ON, Canada). Water-soluble probenecid sodium salt was purchased from Life Technologies (Carlsbad, CA). CPIII-d8 was synthesized at Bristol-Myers Squibb Company (Princeton, NJ). [^{3}H]Estradiol-17β-D-glucuronide (E17βG) (38.8 Ci/mmol) and [^{3}H]Cholecystokinin octapeptide (CCK-8) (92.5mCi/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Charcoal stripped plasma was obtained from Bioreclamation IVT (Westbury, NY). Cryopreserved human hepatocytes from 2 female donors were purchased from Celsis In Vitro Technologies (Baltimore, MD) (Lot NRJ) and Bioreclamation IVT (Westbury, NY) (Lot BXW). Human plasma containing dipotassium EDTA was obtained from Biological Specialty Corporation (Colmar, PA). Cell culture reagents were purchased from Corning (Manassas, VA) and Life Technologies Corporation (Carlsbad, CA). All other reagents and chemicals used were of the highest grade commercially available.

Clinical Drug Interaction Study between PROB and FSM. Plasma and urine samples were collected from an open label, single-dose, three-treatment , three-period clinical DDI study between PROB and FSM reported previously (Shen et al., 2019a). Briefly, fourteen male healthy Indian subjects who had a normal medical history and physical examination participated in the study. Each subject received 1,000 mg PROB alone (period 1), 40 mg FSM alone (period 2), and 40 mg FSM at 1 hour after administration of 1,000 mg PROB (period 3) with a 1-week washout

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between treatments. Subjects fasted the night before and until at least 4 hour (h) after administration of the drugs. PROB and FSM were administered orally with 240 mL of water. Blood samples (3 mL each) for determination of drug and transporter endogenous biomarker concentrations were obtained at predose and at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 12.0, and 24.0 h after dosing. Urine samples were collected during 0–8, 8–16, and 16–24 h postdose. Subjects were housed in a clinical facility 36 h prior to dosing in period 1, and blood and urine samples were also collected over 24 h prior to dosing to obtain the baseline levels of transporter endogenous biomarkers in the absence of drug. Plasma was separated immediately, and the plasma and urine samples were prepared into two aliquots and kept at -70°C until analysis using liquid chromatography–tandem mass spectrometry (LC-MS/MS) (Shen et al., 2019a).

Quantification of CPI and CPIII in Plasma and Urine by LC-MS/MS. Plasma and urine concentrations of CPI and CPIII were quantitated by using the methods developed previously (Kandoussi et al., 2018).

Quantification of HDA and TDA in Plasma by LC-MS/MS. The bioanalytical analyses of HDA and TDA in plasma were performed as described in detail by Santockyte et al. (Santockyte et al., 2018).

Inhibition Studies in OATP1B1- and OATP1B3-Expressing Human Embryonic Kidney (HEK) 293 Cells. The in vitro uptake experiments were repeated in at least two independent experiments. There are three replicates per experiment for each condition (n = 3). The stable OATP1B1- and OATP1B3-transfected and mock cells generated by Bristol Myers Squibb were cultured at 37°C in an atmosphere of 95% air/5% CO2 and subcultured once per week (Han et al., 2010, Shen et al., 2013). OATP1B1- and OATP1B3-expressing cells used in the current study were passaged less than 30 times, to retain consistent transporter expression

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and functional activity. To assess the inhibition potential of PROB toward OATP1B1 and OATP1B3, CPI and radiolabeled probe substrates, as well as testing PROB at various concentrations using a protocol described previously (Shen et al., 2017, Panfen et al., 2019). In brief, transporter expressing cells were grown to confluence in poly-D-lysine-coated 24-well plates (BD Biosciences, San Jose, CA). At confluence, medium was aspirated, cells were rinsed twice with 2 mL of prewarmed Hanks' balanced salt solution (HBSS) (Mediatech, Manassas, VA; Catalog # 21-023-CM) and preincubated with 200 µL of the transport buffer (HBSS with 10 mM HEPES, pH 7.4) containing PROB at concentrations ranging from 1 to 10,000 µM for 30 min at 37°C. Uptake was then initiated by the addition of 200 µL of the prewarmed transport buffer containing CPI (0.2 µM) or radiolabeled probe substrate (1 µM [^{3}H]E17βG and 0.1 µM [^{3}H]CCK-8 for OATP1B1 and OATP1B3, respectively). The probe substrate concentrations are well below the K_m values. Incubation proceeded for 2 min at 37°C to ensure linearity with time (Supplemental figure S1). After 2 min, the uptake buffer was then removed and the cell monolayers were immediately washed three times with 1.5 mL ice-cold HBSS buffer to terminate the uptake process. To analyze the concentrations of radiolabeled compounds, cells in the dried plate were lysed with 300 µL 0.1% Triton X-100. Aliquots of 200 and 20 µL cell lysate were used for radioactivity counting and protein concentration analysis, respectively. After the addition of 5 mL of scintillation cocktail, radioactivity was measured on a dual-channel liquid scintillation counter, Tri-Carb 3100TR liquid scintillation counter (PerkinElmer Life Sciences, Boston, MA). To analyze the concentrations of CPI, cells in the dried plate were lysed in 300 µL 2:1 (vol/vol) ratio of acetonitrile and 1M formic acid with CPI-$^{15}N_4$ (IS). The contents were filtered through a 96-well filter plate (0.45 µm low-binding hydrophilic PTFE) and the filtrate was dried under nitrogen gas. The dried contents were reconstituted in 80 µL of 1 M formic

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acid/acetonitrile (20:80, vol/vol) and the concentration of CPI was measured by LC-MS/MS. Throughout the entire process, appropriate precautions were taken to minimize sample exposure to ambient light. Protein concentration of cell lysates was determined with the bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL). Cellular uptake in OATP1B1- and OATP1B3-HEK cells was normalized based on the protein amount in each well.

Inhibition of CP Uptake in Human Hepatocytes by PROB. To further assess the inhibitory effect of PROB on OATP1B, the uptake of CPI and CPIII in human hepatocytes in the presence of PROB were examined using a protocol described previously, with some modification (Shen et al., 2016, Zhang et al., 2019). Briefly, transporter-qualified cryopreserved human hepatocytes from two female donors were thawed according to the vendor's instructions (Bioreclamation IVT, Westbury, NY). The hepatocytes were then pooled by resuspending the cell pellet in Krebs-Henseleit buffer to give a cell density of 2 million viable cells/mL, which was determined by trypan blue staining (≥ 80% post-thaw viability). After cell suspensions were pre-warmed to 37°C, the uptake of CPI in hepatocytes in the presence and absence of an inhibitor (final concentration of 100, 300 and 1,000 μM PROB, and 200 μM RIF SV) was determined. The uptake study was initiated by adding an equal volume of CP solution to the hepatocyte suspensions at 37°C, resulting in a final cell density of 1 million viable cells/mL and a CP or RSV concentration of 1 μM. There was a 3-min preincubation with the inhibitors or control buffer with hepatocytes at 37°C before the initiation of uptake. At 0.25, 1, 1.5, and 5 min, 100-μL reaction mixtures were removed and overlaid onto prepared 0.4-mL microcentrifuge tubes containing 50 mL 2 M ammonium acetate (bottom layer) and 100 μL filtration oil (top layer; 84.5:15.5 silicon oil-mineral oil mix, final density of 1.015). Samples were centrifuged immediately at 10,000g for 15 seconds using a benchtop centrifuge to pellet the cells. The tubes

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were placed on dry ice, then cut, and the cell pellet was digested in a 2:1 (vol/vol) ratio of acetonitrile and 1M formic acid containing the IS at room temperature. The contents were filtered through a 96-well filter plate (0.45 µm low-binding hydrophilic PTFE) and the filtrate was dried under nitrogen gas. Finally, the dried contents were reconstituted in 80 µL of 1 M formic acid/acetonitrile (20:80, vol/vol) for LC-MS/MS analysis. Throughout the entire process, appropriate precautions were taken to minimize sample exposure to ambient light.

LC-MS/MS Analysis of CPI and CPIII in Cell Lysates. Cell lysate concentrations of CPI, CPIII, and RSV were measured by LC-MS/MS as described previously (Lai et al., 2016, Shen et al., 2016).

Pharmacokinetic and Transport Analyses. The pharmacokinetic parameters of CPI, CPIII, HDA, and TDA were derived using Phoenix WinNonlin, version 8.1 (Certara, Princeton, NJ). Maximum plasma concentration (C_{max}) and area under the concentration-time curve from time zero to 24 h ($AUC_{(0\text{-}24h)}$) were obtained from plasma concentrations versus time data by performing a noncompartmental analysis with mixed log-linear trapezoidal method. Renal clearance (CL_R) was estimated by the following equations:

$$CL_R = \frac{A_{e(0-24h)}}{AUC_{(0-24h)}}$$

where $A_{e(0\text{-}24h)}$ is the cumulative amount excreted in the urine during the time interval from zero to 24 h.

The concentrations required to inhibit transport by 50% (IC_{50}s) of PROB towards OATP1B1- and OATP1B3-mediated uptake of CPI and radiolabeled probes were estimated by fitting the uptake data to the following equation using nonlinear regression approach (Phoenix WinNonlin, Certara; Princeton, NJ).

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$$V = V_0 \times \left(1 - \frac{I^h}{I^h + IC_{50}^h}\right)$$

where V is the mean transporter-mediated uptake of substrate at 2 min observed at the given PROB testing concentration (I), V_0 is the uptake in the absence of PROB, and h is the Hill slope factor.

The hepatic uptake rate in the absence and presence of inhibitor was calculated based on the initial rate of uptake during the linear phase (up to 1.0 or 1.5 min). The uptake clearance (CL_u) was calculated by dividing the uptake rate by the initial substrate concentration. Percent CL_u inhibition was calculated using the following equation:

$$Inhibition\ \% = \frac{CL_u\ in\ the\ absence\ of\ inhibitor - CL_u\ in\ the\ presence\ of\ inhibitor}{CL_u\ in\ the\ absence\ of\ inhibitor} \text{ x } 100$$

Statistical Analyses. The results are expressed as mean ± standard division (SD) in the text and tables. To test for statistically significant differences in C_{max}, $AUC_{(0\text{-}24h)}$, and CL_R among treatments (predose control, PROB, FSM, and PROB with FSM) in the clinical study, repeated measured one-way analysis of variance (ANOVA) was performed. When the F ratio showed that there were significant differences among treatments, the Tukey's method of multiple comparisons was used to determine which treatments differ. All statistical analyses were carried out using GraphPad Prism version 8 (GraphPad Software, San Diego, CA), and a p-value of less than 0.05 was considered statistically significant.

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RESULTS

Effects of Administration of PROB on Plasma Levels of CPI and CPIII. The effect of PROB on the pharmacokinetics of CPI and CPIII, endogenous biomarkers of OATP1B1 and OATP1B3, was evaluated in both plasma and urine after administration of PROB alone, furosemide alone, and probenecid in combination with FSM in 14 subjects. The systemic exposures and CL_R of CPI and CPIII are shown in Figure 1 and Table 1. The table also presents the statistical comparisons for each treatment relative to predose baseline. The administration of 1,000 mg PROB alone and in combination with 40 mg FSM significantly increased C_{max} and $AUC_{(0\text{-}24h)}$ of CPI and CPIII compared to the baseline (1.34- to 1.62-fold) (Table 1) ($p < 0.05$). In contrast, the C_{max} and $AUC_{(0\text{-}24h)}$ values were similar to the basal levels after administration of FSM alone (1.02- to 1.15-fold). These changes were not statistically significant ($p > 0.05$). Moreover, the CL_R values of CPI and CPIII were not significantly changed in the presence of PROB compared to the baseline control although the mean CL_R of CPIII increased by 1.34- to 1.50-fold (Table 1) ($p > 0.05$), indicating no significant inhibition of the CL_R of CPI and CPIII during PROB treatments.

Effects of Administration of PROB on Plasma Levels of HDA and TDA. We also examined the effects of a single dose of 1,000 mg PROB, either alone or in combination with 40 mg FSM on the plasma concentrations of HDA and TDA in healthy subjects. Figure 2 shows the arithmetic mean plasma concentrations ± standard deviation (SD) of HDA and TDA before the first dose of the study (predose) and during the 3 treatment phases. Table 2 shows the arithmetic mean ± SD of C_{max}, $AUC_{(0\text{-}24h)}$, and the ratios of HDA and TDA. While pretreatment with PROB alone did not significantly alter the C_{max} or $AUC_{(0\text{-}24h)}$ of HDA and TDA compared to baseline (0.73- to 1.02-fold) (Table 1) ($p > 0.05$), the coadministration of PROB with FSM significantly

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increased the systemic exposures of HDA and TDA by 1.62- and 2.27-fold compared to FSM alone (Table 2) ($p < 0.05$). In addition, administration of a single oral dose of 40 mg FSM alone resulted in less than a 1% change in the C_{max} and $AUC_{(0\text{-}24h)}$ of HDA compared with the baseline controls ($p > 0.05$). In contrast, administration of FSM alone significantly decreased the C_{max} and $AUC_{(0\text{-}24h)}$ of TDA compared with the baseline (0.61- and 0.76-fold, respectively) (Table 2) ($p < 0.05$). Unfortunately, the amount of HDA and TDA excreted in the urine were not analyzed in this study.

Characterization of PROB as an In Vitro Inhibitor of OATP1B1 and OATP1B3. To evaluate the inhibitory effect of PROB on OATP1B1 and OATP1B3, the uptake of 0.2 μM CPI (OATP1B1 and OATP1B3), 1 μM [^{3}H]E17βG (OATP1B1), and 0.1 μM [^{3}H]CCK-8 (OATP1B3) were measured in the presence of increasing concentrations of PROB in transporter-expressing cells. As shown in Figure 3, PROB inhibited OATP1B1- and OATP1B3-mediated uptake of CPI in a concentration-dependent manner with IC_{50} values of 167 ± 42.0 and 76.0 ± 17.2 μM, respectively (Table 4). PROB showed very similar inhibition on the uptake of [^{3}H]E17βG and [^{3}H]CCK-8 by OATP1B1 and OATP1B3, respectively (IC_{50} values for OATP1B1 and OATP1B3 were 121 ± 7.5 and 147 ± 37.5 μM, respectively).

Effect of PROB on CP Uptake in Hepatocytes. To further demonstrate the inhibition of PROB on the hepatic elimination of CP, the uptake of 1 μM CPI and CPIII in human hepatocytes was assessed. Figure 4 and Table 3 show the uptake of 1 μM CPI and CPIII into human hepatocytes in suspension in the presence of increasing concentrations of PROB. The CPI and CPIII influx was sensitive to the presence of PROB, as a PROB concentration of 100 μM reduced CPI and CPIII uptake by 12% and 33%, respectively, of the control. As the PROB concentration was raised, CPI and CPIII influx was further reduced, so that at 1,000 μM PROB,

uptake has been inhibited by 56% (Table 3). The uptake of the reference substrate RSV (1 μM) also appeared to be inhibited to a similar extent by PROB in hepatocytes (Figure 4 and Table 3).

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DISCUSSION

The role and importance of OATP1B1 and OATP1B3 in the transport of drugs across the basolateral membranes of the hepatocytes are well recognized. It is within the last decade that the inhibition potential of a new molecular entity toward OATP1B transporter proteins has begun to be evaluated during drug discovery and development (International Transporter et al., 2010, Tweedie et al., 2013, Lee et al., 2017). For 34 small molecular drugs approved by the FDA in 2017, OATP1B together with CYP3A4, played a significant role in mediating more than half of the drug interactions with AUC changes ≥ 5-fold (Yu et al., 2019). Moreover, it has become increasingly evident that endogenous biomarkers of OATP1B, for example CPI, can be used to evaluate complex DDIs for better DDI risk assessment and management based on a mechanistic understanding in healthy subjects and patients (Suzuki et al., 2019, Jones et al., 2020, Mori et al., 2020). The described findings suggest that this is the case for PROB. This drug is regarded as a clinically efficient inhibitor of OAT1 and OAT3 since the benzoic acid derivative is known to compete for active renal tubular secretion primarily with acidic drugs (Maeda et al., 2014). Moreover, PROB has recently been identified as a weak inhibitor of the OATP1B-mediated transport of organic anions using a heterologous expression system (Hirano et al., 2006, Matsushima et al., 2008, Izumi et al., 2013, Izumi et al., 2016). Accordingly, the in vivo and in vitro inhibition potential of PROB toward OATP1B1 and OATP1B3 were investigated using the endogenous probes CPI, CPIII, HDA, and TDA.

For the first time, the results of this study showed a significant increase in plasma concentration of CPI and CPIII [C_{max} and $AUC_{(0\text{-}24h)}$], endogenous probes of OATP1B1 and OATP1B3, in humans during PROB treatments compared to baseline levels (1.34- to 1.62-fold)

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(Figure 1 and Table 1). These findings suggest that PROB inhibited the elimination of the endogenous substrates of OATP1B, and this might be explained by an inhibitory effect of PROB on renal and/or hepatic transporters as CPI and CPIII are excreted in not only bile but also the urine (Wolkoff et al., 1976, Lai et al., 2016, Shen et al., 2016). However, the renal clearance of CP was not significantly decreased by PROB (Table 1). When the plasma and urine findings are taken together, this suggests that the interaction occurs in the liver but not in the kidney. This is not unexpected because CPI and CPIII are not substrates for OAT1 and OAT3 (Bednarczyk and Boiselle, 2015, Shen et al., 2017, Kunze et al., 2018), although OAT1 and OAT3 are capable of transporting small anionic endogenous substrates (e.g. *p*-aminohippurate and estrone-3-sulfate) and xenobiotics (e.g. adefovir, benzylpenicillin, and FSM) (Inui et al., 2000, Shen et al., 2019b). By contrast, OATP1B1 and OATP1B3 were found to effectively mediate the uptake of CPI and CPIII, which is consistent with the anionic nature of these endogenous compounds (Bednarczyk and Boiselle, 2015, Shen et al., 2016, Shen et al., 2017). Very recently, Wiebe et al. investigated the effects of four commonly employed drug transporter inhibitors on cocktail drug pharmacokinetics (Wiebe et al., 2020). PROB treatment increased C_{max} and AUC of RSV by 328% and 123%, respectively. Although PROB decreased RSV CL_R by 78%, its inhibition of renal clearance alone could not explain the pronounced AUC increase since approximately 28% of total body clearance of RSV was via the renal route. In line with the clinical data, a monkey transporter-mediated DDI study showed that PROB increased the AUC of RSV and pitavastatin by 2.6- and 2.1-fold, respectively (Kosa et al., 2018). In addition, PROB significantly inhibited the uptake of RSV and pitavastatin in monkey hepatocytes (Kosa et al., 2018). Therefore the interaction between PROB and statin might be attributed to the decreased hepatic uptake mediated clearance by monkey OATP1B. Likewise, PROB had an impact on the OATP1B-

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mediated uptake clearance of fexofenadine in humans since its inhibition of renal organic anion transporter alone could not explain the noted AUC change (Yasui-Furukori et al., 2005, Liu et al., 2008). However, we cannot exclude that the synthesis of CPI and CPIII may be altered by PROB treatment. Interestingly, the mean renal clearance of CPIII but not CPI was increased by PROB although the increase was not statistically significant compared to predose level (1.34- and 1.50-fold) (Table 1) ($p > 0.05$). Previously we reported that CPIII but not CPI was subject to active tubular secretion in the kidney of monkeys and humans (Lai et al., 2016, Shen et al., 2016). One possible explanation for the unexpected increase of CPIII CL_R is that PROB stimulated the function of transporter(s) responsible for the tubular secretion of CPIII. Such stimulation has been observed with many transporters such as MRP2 (Gilibili et al., 2017). In addition, it is possible that CPIII undergoes both renal tubular secretion and reabsorption, and PROB inhibited the renal reabsorption of CPIII, resulting in the increased net renal excretion clearance of CPIII. Future work needs to be done to confirm the hypotheses. Servais et al. previously studied urinary coproporphyrin excretion in rats after inhibition of transporters by PROB and in mutant transport-deficient (TR−) rats, in which MRP2 is lacking (Servais et al., 2006). Total urinary coproporphyrin excretion was similar in TR− rats and in normal rats with or without treatment by PROB, but relative urinary CPI excretion was increased in the mutant rats. Moreover, PROB is a weak inhibitor of MRP2 that is expressed on the apical surface of renal proximal tubule epithelial cells and hepatocytes. CPI and CPIII are substrates for MRP2 (Gilibili et al., 2017, Kunze et al., 2018), and the urinary CPI/(CPI + CPIII) ratio was proposed as a surrogate for MRP2 activity (Benz-de Bretagne et al., 2011, Benz-de Bretagne et al., 2014). Unchanged CL_R values of CPI in the subjects administered with PROB suggested that PROB did not affect MRP2 activity in the kidney in vivo (Table 1). The increased CL_R of CPIII requires

further studies to identify the transporters responsible for the renal disposition of CPIII for better assessment of in vivo inhibition potential of an investigational drug using CPIII. Taken together, administration of PROB at a therapeutic dose can cause clinically relevant OATP1B inhibition.

In agreement with clinical findings, the IC_{50} of PROB in OATP1B1- and OATP1B3-HEK cells using CPI as a substrate is determined to be 167 and 76.0 μM, respectively, in this study (Figure 3). This is consistent with recent reports employing dichlorofluorescein, bromosulfophthalein, E17βG, estrone-3-sulfate, pitavastatin, and fexofenadine as OATPIB1 and OATP1B3 substrates (Hirano et al., 2006, Matsushima et al., 2008, Izumi et al., 2013, Izumi et al., 2016), in which the IC_{50} of PROB ranged from 39.8 to 227 μM. Furthermore, 100, 300, and 1,000 μM PROB reduced the uptake of CPI, CPIII and RSV in human hepatocytes in a concentration-dependent manner with maximum inhibition by 56%, 56%, and 73%, respectively (Figure 4; Table 3). The results suggested that PROB is a weak in vitro inhibitor of OATP1B1 and OATP1B3. However, because the free maximum concentration at the inlet to the liver ($I_{in,max,u}$) following 1,000 mg PROB administration is large [34.6 to 41.5 μM estimated by using the pharmacokinetic parameters reported previously (Shen et al., 2019a)], the predicted *AUC* ratio of a substrate drug of OATP1B in the presence and absence of PROB (*R*-value) is 1.46 to 1.55 using the IC_{50} value of 76.0 μM determined using CPI as a probe substrate (R-value = 1 + $I_{in,max,u}/IC_{50}$), suggesting an in vivo inhibition potential of PROB with OATP1B (Table 4). These *R*-values estimated using the method recommended in the EMA and FDA guidances (https://www.fda.gov/media/134582/download; https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-investigation-drug-interactions-revision-1_en.pdf) are consistent with the observed Cmax and *AUC* fold-changes of endogenous probes CPI and CPIII. It is critical to include sufficient subjects in a clinical study to

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detect weak drug transporter interaction. Barnett et al. demonstrated the sensitivity of CPI to identifying weak OATP1B inhibitors in an adequately powered clinical study using mode-based simulations and power calculations (Barnett et al., 2017). The analysis showed the ability to identify a weak but clinically relevant OATP1B DDI using CPI as a probe (AUCR >1.25 cutoff). Use of criterion of $\alpha = 0.01$ and power of 0.8 required a sample size of 15 subjects (Barnett et al., 2017). This conclusion was further confirmed by CPI clinical data with several weak OATP1B inhibitors (Kunze et al., 2018, Liu et al., 2018). The clinical studies with 14 and 13 subjects indicated that CPI concentration changes were predictive for a weak clinically observed DDI where CPI AUC increases of 1.6- and 1.4-fold were comparable with those observed for statins as victim drugs (Kunze et al., 2018, Liu et al., 2018). Therefore, we believe that our data generated with 14 subjects is sufficient to support the conclusions made in this study.

To quantify the response of HDA and TDA, two other endogenous probes of OATP1B (Yee et al., 2016, Shen et al., 2017, Yee et al., 2019), to PROB pretreatments in vivo, we measured the plasma HDA and TDA concentrations. The variations in plasma concentrations of HDA and TDA were larger than that of CPI and CPIII. The concentration-time profiles for HDA and TDA appear different between the PROB alone and PROB coadministration with FSM groups. While the administration of PROB in combination with FSM significantly increased the $AUC_{(0\text{-}24h)}$ of HDA and TDA by 1.71- and 1.62-fold, respectively, compared to FSM alone, the administration with PROB alone did not significantly alter the $AUC_{(0\text{-}24h)}$ of HDA and TDA compared to prestudy levels (1.02- and 0.90-fold, respectively) (Figure 2; Table 2). It is unclear why the PROB pretreatments show different effects on the plasma HDA and TDA levels. Unfortunately, the CL_R of HDA and TDA could not be determined in the urine. In addition, the treatment with FSM alone significantly decreased the $AUC_{(0\text{-}24h)}$ of TDA compared with the

predose control (0.76-fold) (Table 2). We cannot exclude that the synthesis of the biomarkers may be affected by PROB and/or FSM. These results suggest that plasma HDA and TDA levels may not be good surrogate endogenous probes for weak OATP1B inhibition.

In conclusion, for the first time, our results demonstrate the weak inhibitory effect on OATP1B1 and OATP1B3 by PROB in vivo. Considering the fact that PROB is an index inhibitor for clinical OAT1/3 DDI study, these findings provide an explanation for the heretofore unknown mechanism responsible for the inhibition caused by PROB.

Acknowledgments

The authors acknowledge following scientists for assistance with the clinical and preclinical studies: Susan Lubin and Erika Panfen.

Authorship Contributions

Participated in research design: Zhang, Holenarsipur, Zeng, Mariappan, Sinz, Shen

Conducted experiments: Zhang, Kandoussi

Contributed new reagents or analytic tools: Zhang, Kandoussi, Shen

Performed data analysis: Zhang and Shen

Wrote or contributed to the writing of the manuscript: Zhang, Sinz, Shen

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Footnotes

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- This study is supported by Bristol Myers Squibb Company.

DMD Fast Forward. Published on July 28, 2020 as DOI: 10.1124/dmd.120.000076
This article has not been copyedited and formatted. The final version may differ from this version.

Figure Legends

Figure 1. Effect of 1,000 mg probenecid (PROB) and 40 mg furosemide (FSM) doses on plasma concentration and renal clearance (CL_R) of CP. The plasma concentration-time profiles of CPI (A) and CPIII (B) are shown as the mean and SD values obtained from 14 healthy subjects, before dose (open squares) and after a single oral dose of PROB (open circles), FSM (open triangles), and co-administration of PROB and FSM (open diamonds). The CL_R of CPI (C) and CPIII (D) before and after the indicated treatment.

Figure 2. Effect of 1,000 mg PROB and 40 mg FSM doses on plasma concentration of HDA and TDA. The plasma concentration-time profiles of HDA (A) and TDA (B) are shown as the mean and SD values obtained from 14 healthy subjects, before dose (open squares) and after a single oral dose of PROB (open circles), FSM (open triangles), and co-administration of PROB and FSM (open diamonds).

Figure 3. Inhibitory effect of PROB on OATP1B1- (A) and OATP1B3-mediated transport of CPI and prototypical probe substrates. Uptake of 0.2 μM CP I, 1 μM E17βG (OATP1B1), and 1 M CCK-8 (OATP1B3) was assessed in stably transfected OATP1B1- and OATP1B3-HEK cells over 2 min in the absence (control) or presence of PROB at the indicated concentrations. Uptake changes are normalized to transporter-mediated uptake in the absence of PROB. Results represent the mean and SD from three independent determinations.

Figure 4. Inhibitory effect of PROB on the uptake of CPI (A), CPIII (B), and RSV (C) into human hepatocytes. Open circles, squares, triangles and diamonds, and closed circles represent the uptake of 1 μM substrate alone, with 100 μM PROB, 300 μM PROB, 1,000 μM PROB, and 200 μM RIF SV, respectively. Results represent the mean and SD from three independent determinations.

Table 1. Comparison of pharmacokinetic parameters of CPI and CPIII in healthy subjects following administration of PROB (1,000 mg), FSM (40 mg), and co-administration of PROB and FSM.

Treatment	CPI			CPIII		
	Cmax (nM)	$AUC_{(0\text{-}24h)}$ (nM*h)	CL_R (mL/min)	Cmax (nM)	$AUC_{(0\text{-}24h)}$ (nM*h)	CL_R (mL/min)
Predose	1.34 ± 0.58	27.9 ± 11.5	18.6 ± 7.07	0.19 ± 0.08	3.54 ± 1.38	330 ± 210
PROB alone	1.98 ± 0.74***	41.1 ± 16.4***	16.3 ± 4.77	0.26 ± 0.11***	4.87 ± 2.22***	414 ± 238
Fold-Change[a]	1.54 ± 0.36	1.39 ± 0.21	0.97 ± 0.38	1.41 ± 0.22	1.34 ± 0.16	1.34 ± 0.53
FSM alone	1.44 ± 0.37	28.4 ± 7.27	19.0 ± 7.96	0.21 ± 0.07	3.54 ± 1.28	312 ± 191
Fold-Change[a]	1.15 ± 0.26	1.08 ± 0.27	1.10 ± 0.47	1.15 ± 0.32	1.02 ± 0.23	1.02 ± 0.50
PROB + FSM	1.96 ± 0.47**	41.3 ± 9.75**	21.2 ± 6.67	0.29 ± 0.09***	5.06 ± 2.03*	498 ± 247
Fold-Change[a]	1.60 ± 0.50	1.57 ± 0.41	1.16 ± 0.51	1.62 ± 0.46	1.45 ± 0.37	1.50 ± 0.69
Fold-Change[b]	1.42 ± 0.39	1.58 ± 0.25	1.23 ± 0.65	1.43 ± 0.26	1.34 ± 0.35	1.51 ± 0.41

Data represent the mean and SD from 9 to 14 subjects (n = 9-14); *C*max, maximum plasma concentration; $AUC_{(0\text{-}24h)}$, area under plasma concentration-time curve from time 0 to 24 h; CL_R renal clearance.

[a]The fold-change values of *C*max, $AUC_{(0\text{-}24h)}$, and CL_R are calculated using predose levels

[b]The fold-change values of *C*max, $AUC_{(0\text{-}24h)}$, and CL_R are calculated using FSM alone

*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, statistically significant difference compared to the predose control

Table 2. Comparison of pharmacokinetic parameters of HDA and TDA in healthy subjects following administration of PROB (1,000 mg), FSM (40 mg), and co-administration of PROB and FSM.

Treatment	HDA		TDA	
	Cmax (nM)	***AUC*$_{(0\text{-}24h)}$ (nM*h)**	**Cmax (nM)**	***AUC*$_{(0\text{-}24h)}$ (nM*h)**
Predose	61.2 ± 34.2	884 ± 419	63.6 ± 47.9	787 ± 551
PROB alone	60.1 ± 48.7	891 ± 444	45.4 ± 34.3	693 ± 415
Fold-Change[a]	0.96 ± 0.29	1.02 ± 0.18	0.73 ± 0.25	0.90 ± 0.20
FSM alone	63.2 ± 52.7	890 ± 592	42.7 ± 52.8***	623 ± 585***
Fold-Change[a]	1.00 ± 0.34	0.99 ± 0.21	0.61 ± 0.17	0.76 ± 0.14
PROB + FSM	123 ± 65.7**	1404 ± 540***	72.5 ± 52.9	919 ± 516*
Fold-Change[a]	2.20 ± 1.26	1.66 ± 0.45	1.17 ± 0.49	1.22 ± 0.26
Fold-Change[b]	2.27 ± 1.06	1.71 ± 0.43	1.87 ± 0.86	1.62 ± 0.40

Data represent the mean and SD from 14 subjects (n = 14); *C*max, maximum plasma concentration; *AUC*$_{(0\text{-}24h)}$, area under plasma concentration-time curve from time 0 to 24 h.

[a]The fold-change values of *C*max, and *AUC*$_{(0\text{-}24h)}$ are calculated using predose levels

[b]The fold-change values of *C*max, and *AUC*$_{(0\text{-}24h)}$ are calculated using FSM alone

*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, statistically significant difference compared to the predose control

Table 3. The hepatic uptake clearance of CP incubated in the presence and absence of PROB (100, 300, and 1,000 μM)

Compound	Hepatocyte Uptake Clearance (μL/min/10^6 cells) (%Inhibition)				
	Control	100 μM PROB	300 μM PROB	1,000 μM PROB	200 μM RIF SV
CPI	2.1	1.9 (12%)	1.2 (42%)	0.93 (56%)	0.40 (81%)
CPIII	4.3	2.9 (33%)	2.0 (53%)	1.9 (56%)	0.61 (86%)
RSV	10.8	7.2 (34%)	4.9 (55%)	2.9 (73%)	0.73 (93%)

The hepatic uptake clearance and percent uptake clearance inhibition were determined using the data in Figure 4.

Table 4. Prediction of OATP1B-mediated DDIs for PROB and FSM using *R*-value and endogenous biomarker methods

Treatment	Drug	*C*max (μM)[a]	f_u[a]	OATP1B1		OATP1B3		Observed Index Cmax and AUC Fold-Changes			
				IC_{50} (μM)[b]	*R*-Value	IC_{50} (μM)[b]	*R*-Value	CPI	CPIII	HDA	TDA
PROB alone	1,000 mg PROB	436	0.062	167 ± 42.0	1.25	76.0 ± 17.2	1.55	1.54 (Cmax) 1.39 (AUC)	1.41 (Cmax) 1.34 (AUC)	0.96 (Cmax) 1.02 (AUC)	0.73 (Cmax) 0.90 (AUC)
FSM alone	40 mg FSM	4.1	0.023	30-300	1.00-1.01	>300	1.00	1.15 (Cmax) 1.08 (AUC)	1.15 (Cmax) 1.02 (AUC)	1.00 (Cmax) 0.99 (AUC)	0.61 (Cmax) 0.76 (AUC)
PROB + FSM	1,000 mg PROB	325	0.062	167 ± 42.0	1.21	76.0 ± 17.2	1.46	1.60 (Cmax) 1.42 (AUC)	1.62 (Cmax) 1.45 (AUC)	2.20 (Cmax) 1.66 (AUC)	1.17 (Cmax) 1.22 (AUC)

Cmax, maximum plasma concentration; f_u, unbound fraction; IC_{50}, concentration required to inhibit transport by 50%; *AUC*, area under plasma concentration-time curve from time; *R*-value, ratio of OATP1B substrate AUC in the presence and absence of inhibitor calculated using the FDA method.

[a]Data obtained from (Shen et al., 2019a).

[b]Concentrations required to inhibit OATP1B1- and OATP1B3-mediated transport by 50% obtained from the present study and previously reported (Ebner et al., 2015).

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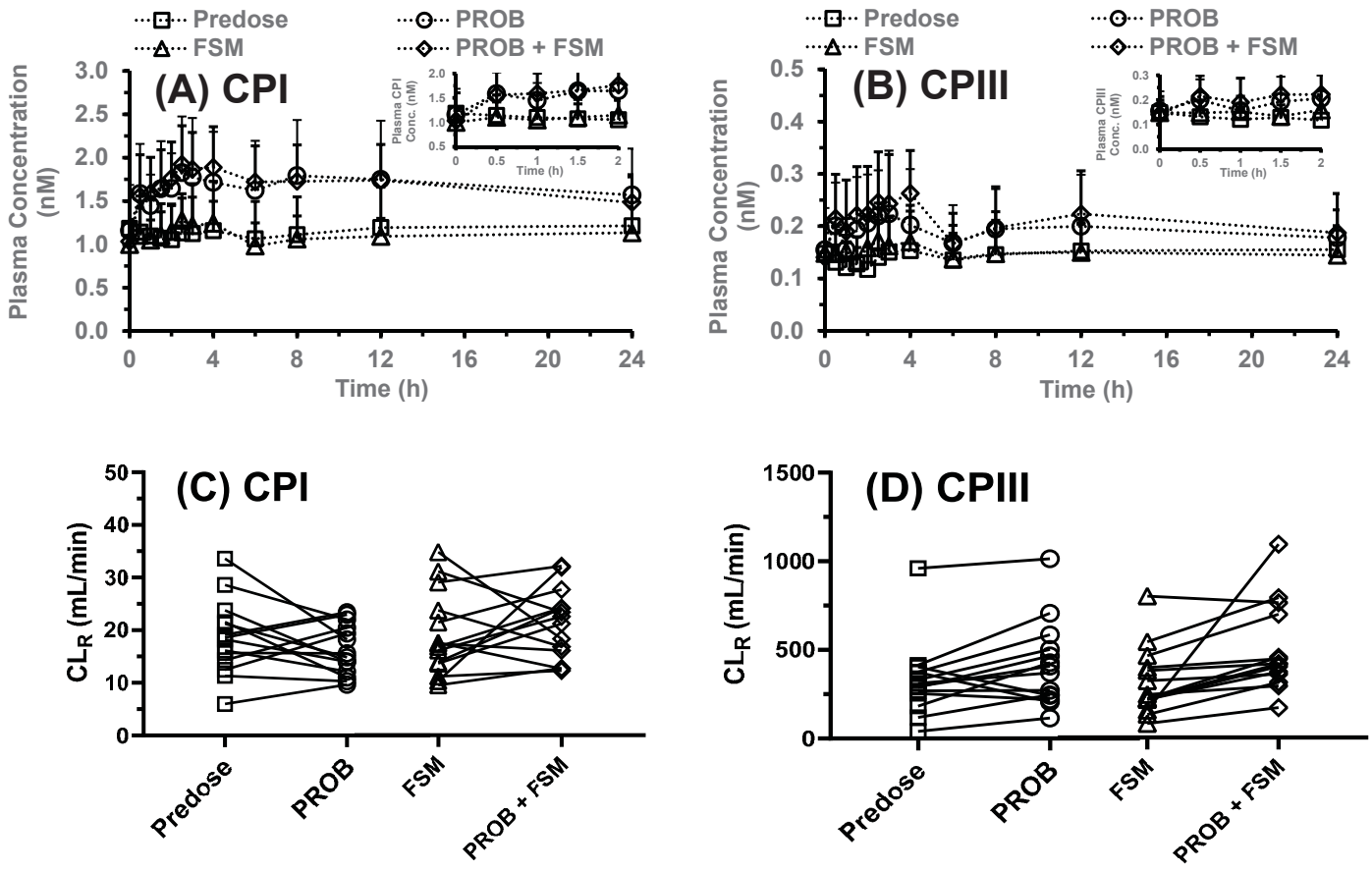

Figure 1

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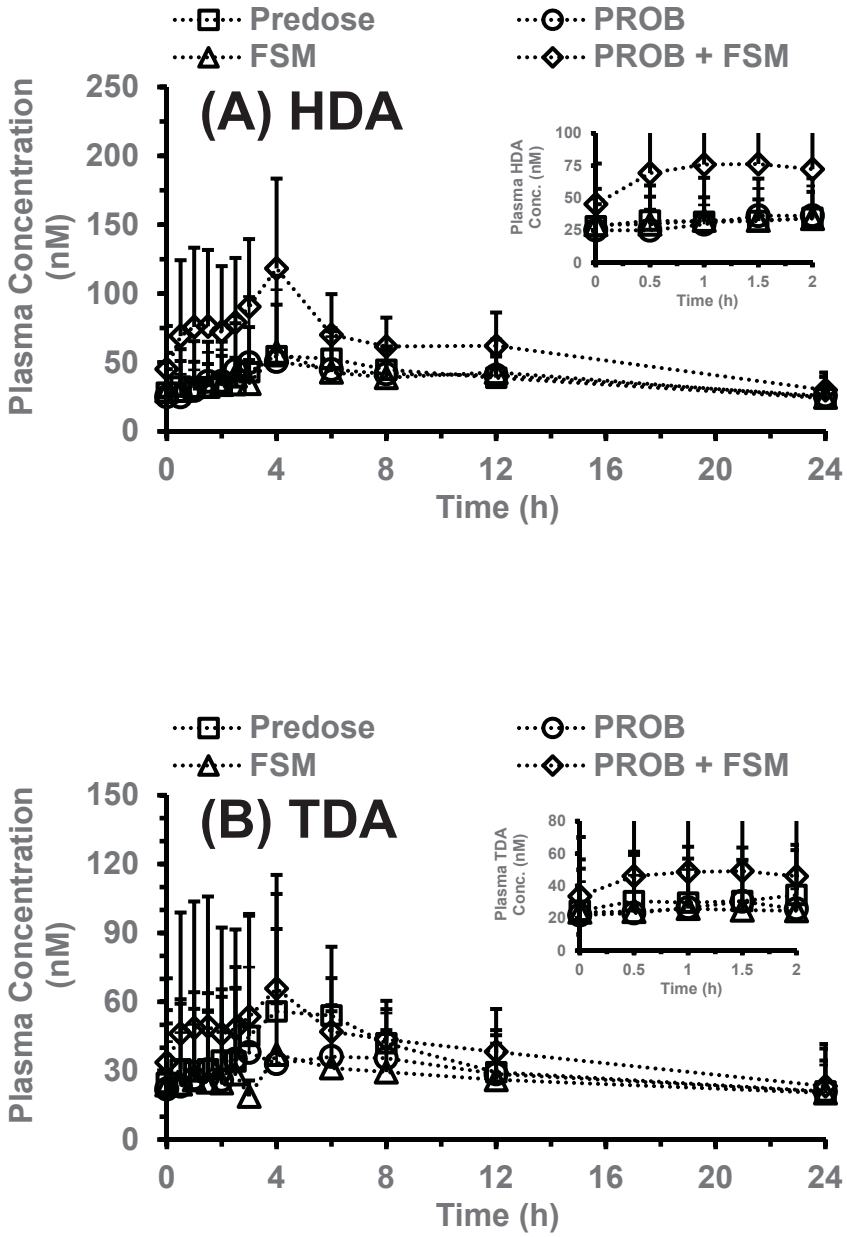

Figure 2

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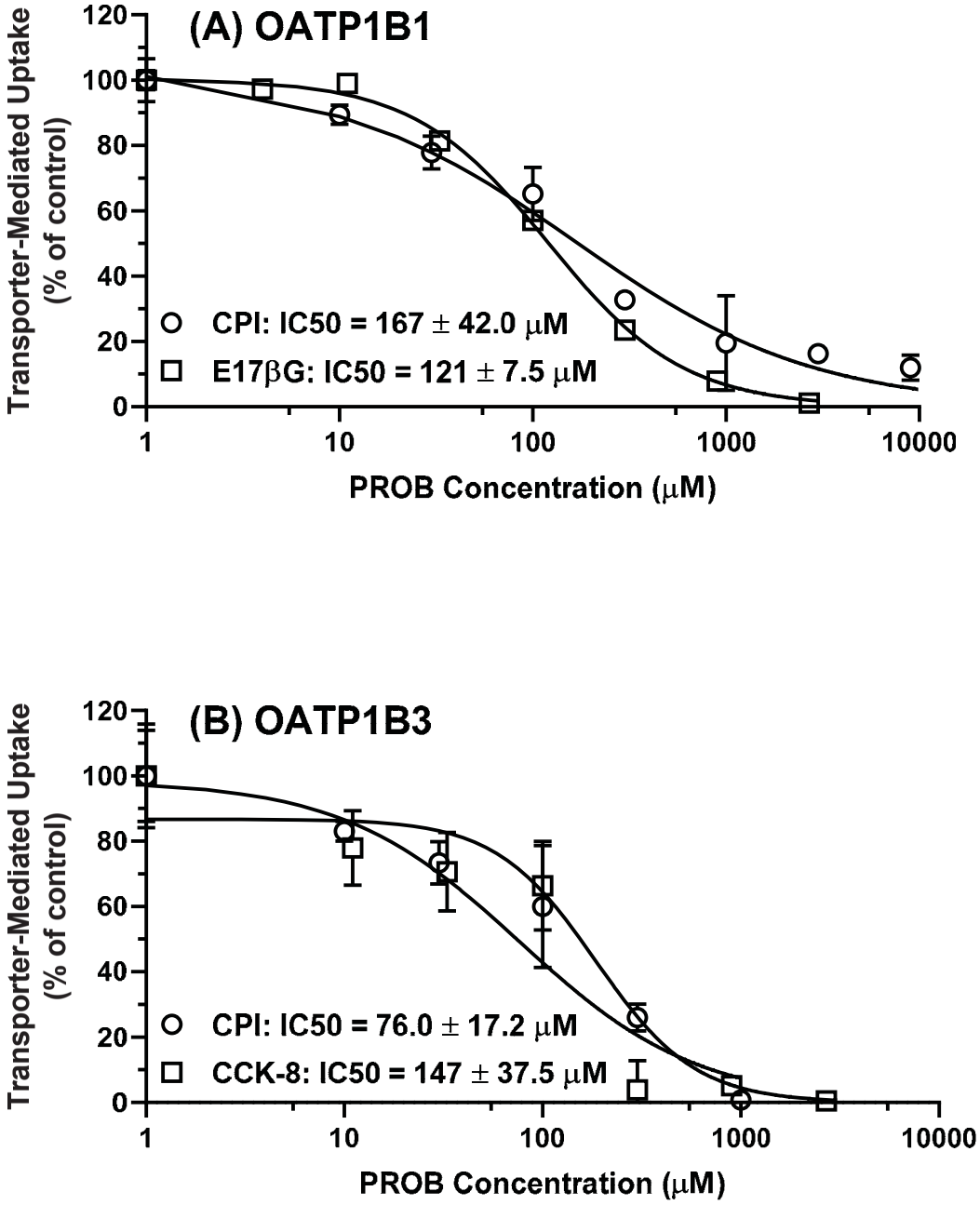

Figure 3

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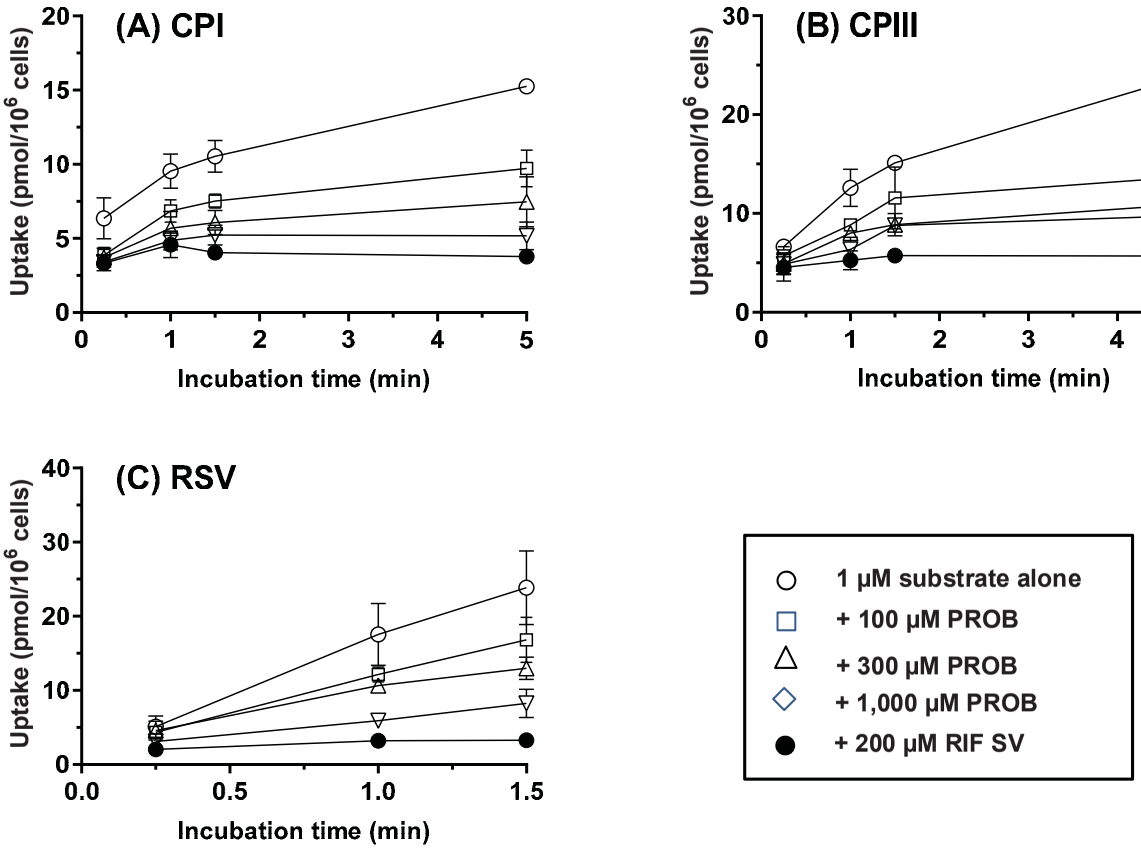

Figure 4