Discovery and Validation of Pyridoxic Acid and Homovanillic Acid as Novel Endogenous Plasma Biomarkers of Organic Anion Transporter (OAT) 1 and OAT3 in Cynomolgus Monkeys

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ABSTRACT

Perturbation of organic anion transporter (OAT) 1- and OAT3-mediated transport can alter the exposure, efficacy, and safety of drugs. Although there have been reports of the endogenous biomarkers for OAT1/3, none of these have all of the characteristics required for a clinical useful biomarker. Cynomolgus monkeys were treated with intravenous probenecid (PROB) at a dose of 40 mg/kg in this study. As expected, PROB increased the area under the plasma concentration-time curve (AUC) of coadministered furosemide, a known substrate of OAT1 and OAT3, by 4.1-fold, consistent with the values reported in humans (3.1- to 3.7-fold). Of the 233 plasma metabolites analyzed using a liquid chromatography–tandem mass spectrometry (LC-MS/MS)–based metabolomics method, 29 metabolites, including pyridoxic acid (PDA) and homovanillic acid (HVA), were significantly increased after either 1 or 3 hours in plasma from the monkeys pretreated with PROB compared with the treated animals. The plasma of animals was then subjected to targeted LC-MS/MS analysis, which confirmed that the PDA and HVA AUCs increased by approximately 2- to 3-fold by PROB pretreatments. PROB also increased the plasma concentrations of hexadecanediolic acid (HDA) and tetradecanediolic acid (TDA), although the increases were not statistically significant. Moreover, transporter profiling assessed using stable cell lines constitutively expressing transporters demonstrated that PDA and HVA are substrates for human OAT1, OAT3, OAT2 (HVA), and OAT4 (PDA), but not OCT2, MATE1, MATE2K, OATP1B1, OATP1B3, and sodium taurocholate cotransporting polypeptide. Collectively, these findings suggest that PDA and HVA might serve as blood-based endogenous probes of cynomolgus monkey OAT1 and OAT3, and investigation of PDA and HVA as circulating endogenous biomarkers of human OAT1 and OAT3 function is warranted.

Introduction

The organic anion transporters (OATs) OAT1 (SLC22A6) and OAT3 (SLC22A8) function as influx transporters that are mainly expressed on the basolateral membrane of renal proximal tubular cells (RPTCs) and mediate cellular uptake of substrates from blood into the cells (Motohashi et al., 2002). OAT1 and OAT3 can serve as the loci of drug-drug interactions (DDIs), and such DDIs can lead to undesired changes in systemic and local exposures of victim drugs and toxins (Morrissey et al., 2013; Nigam et al., 2015). For example, furosemide (FSM) is taken up from the blood in RPTCs via OAT1 and OAT3 (Hasannejad et al., 2004), and probenecid (PROB), an inhibitor of OAT1 and OAT3, causes significant alterations in the pharmacokinetic parameters of FSM in humans through the increased area under the plasma drug concentration-time curve (AUC), decreased total plasma clearance (CL_{TOT}) and renal clearance (CL_{R}), and decreased fraction excreted in the urine (f_{u}) (Chennavasin et al., 1979; Smith et al., 1980; Vree et al., 1995). Moreover, a few uremic toxins recently have been demonstrated to be inhibitors of OAT1 and OAT3, and have the potential to inhibit renal secretion clearance of drug substrates of OAT1 and OAT3 in patients with chronic kidney disease (Hsu et al., 2016). On the other hand, concomitant use of PROB is recommended by the U.S. Food and Drug Administration for reducing the pronounced nephrotoxicity of cidofovir by inhibiting OAT1 and OAT3 to reduce the exposure of cidofovir in RPTCs to an extent that results in an acceptable benefit-risk balance (http://www.accessdata.fda.gov/drugsatfda_docs/label/1999/020638s003lbl.pdf).

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Animal models have often been used to assess preclinical drug-OAT interactions. For example, the clinical famotidine-PROB interaction was reproduced in cynomolgus monkeys recently since PROB caused an approximate 90% reduction in the tubular secretion clearance of famotidine in monkeys (4.58 ± 1.25 vs. 0.38 ± 0.36 ml/min per kilogram) (Tahara et al., 2006). Similarly, PROB significantly decreased the renal tubular secretion clearance of famotidine from 2.80 ± 0.36 to 0.37 ± 0.07 ml/min per kilogram in humans. Furthermore, the protective effect of PROB treatment on the nephrotoxicity of cidofovir in humans was able to be recapitulated in monkeys (Lacy et al., 1998). These results suggest that monkey is the more suitable animal model to predict the clinical DDIs involving OAT1 and OAT3. Recently, endogenous biomarkers have been envisioned as simple, fast, and cost-effective tools to monitor transporter activity in preclinical and clinical settings to facilitate development of a drug candidate (Ito et al., 2012; Müller et al., 2015; Lai et al., 2016; Yee et al., 2016; Bergagnini-Kolev et al., 2017). Therefore, the identification of a sensitive endogenous biomarker for OAT1 and OAT3 would be of great value. In this regard, Imamura et al. (2014) reported that 6β-hydroxy cortisol could be an endogenous probe for OAT3 inhibition evaluation since PROB significantly changed the AUC and CLa of 6β-hydroxy cortisol in healthy subjects. However, 6β-hydroxy cortisol is formed from cortisol by hepatic CYP3A4, and many drugs known to affect this drug-metabolizing enzyme can also change exposure of 6β-hydroxy cortisol (Pen et al., 2011). Very recently, Tsuruya et al. (2016) reported that taurine and glycinecholodeoxycholate sulfates were endogenous biomarkers of OAT1 and OAT3, respectively. However, the plasma levels of taurine and glycinecholodeoxycholate sulfate were not changed significantly by PROB treatment compared with control even though the renal secretion and CLa were significantly decreased (Tsuruya et al., 2016). Reduced activities of blood-facing OAT1 and OAT3 by PROB are supposed to increase plasma concentration of a sensitive and selective endogenous probe, mimicking systemic alterations of probe drugs such as FSM. To our knowledge, all published endogenous biomarkers of OAT1 and OAT3 could not recapitulate plasma drug concentration-time profiles in the presence of OAT inhibitors. Several features including specificity, sensitivity, predictability, reproducibility, acute response, and accessibility have been considered in the identification and validation of ideal endogenous biomarkers for drug transporters (Chu et al., 2017; Mariappan et al., 2017; Rodrigues et al., 2017). Given the aforementioned reasons, there is a need for novel plasma biomarkers of renal OATs. In the present studies we provide direct experimental evidence that several organic anionic compounds, including pyridoxic acid (PDA), homovanillic acid (HVA), hexadecanediolic acid (HDA), and tetradecanediolic acid (TDA) are potential endogenous biomarkers of OAT1 and OAT3 in cynomolgus monkeys. Specifically, an untargeted metabolomics analysis was applied to plasma samples to screen endogenous compounds that were associated with OAT1 and OAT3 inhibition in monkeys. Follow-up quantitative liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis further characterized the time–plasma concentration profiles of selected endogenous compounds (i.e., PDA, HVA, had, and TDA) after PROB administration in cynomolgus monkeys. Moreover, transporter profiling assessed using human embryonic kidney (HEK) 293 cells stably transfected with major human renal and hepatic drug transporters demonstrated that PDA and HVA are OAT1 and OAT3 substrates and thus potential novel plasma endogenous biomarkers of OAT1 and OAT3 inhibition.

Materials and Methods

Materials. [3H]Penciclovir (1.3 Ci/mmol) and [14C]metformin (98 mCi/mmol) were purchased from Moravek Biochemicals, Inc. (Brea, CA), and [3H]paraeaminohippuric acid (4.5 Ci/mmol), [3H]estrone-3-sulfate (44.0 Ci/mmol), [3H]estradiol-17B-D-glucuronide (34.3 Ci/mmol), and [3H]cholecystokinin octapeptide (97.5 mCi/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Nonradiolabeled FSM, PROB, and taurocholic acid, and the corresponding stable isotope-labeled internal standards were purchased from Toronto Research Chemicals Inc. (North York, Ontario, Canada). 4-PDA (≥98%) was purchased from Sigma-Aldrich (St. Louis, MO), and HVA (≥98%) was purchased from Acros Organics (Geel, Belgium). Hexadecanediolic acid-d28, tetradecanedic acid-d24, and malapril maleate-d5 (Malap-d5) were obtained from CDN Isotopes (Pointe-Claire, Quebec, Canada). Other nonradiolabeled compounds were purchased from either Sigma-Aldrich or Toronto Research Chemicals Inc. and were of analytical grade. Cell culture media and reagents were purchased from Invitrogen (Carlsbad, CA) or Mediatech, Inc., A Corning Subsidiary (Manassas, VA).

Cynomolgus Monkeys Pharmacokinetic FSM-PROB Interaction Study Protocol. To identify and verify the endogenous biomarkers of OAT1 and OAT3, we conducted a series of experiments: cynomolgus monkey FSM-PROB interaction, metabolomics, targeted LC tandem MS (LC-MS/MS), and transporter profiling experiments. The experimental workflow is shown in Fig. 1.

A single-dose, three-period crossover intravenous pharmacokinetic DDI study was carried out at the Bristol-Myers Squibb Company, and three male cynomolgus monkeys with body weights ranging from 5.3 to 6.0 kg during the study periods were included in this study. The experiment was performed in accordance with the National Institutes of Health guidelines (https://grants.nih.gov/grants/dlaw/guide-for-the-care-and-use-of-laboratory-animals.pdf) and approved by the Bristol-Myers Squibb Animal Care and Use Committee. The animals were housed in a temperature- and humidity-controlled room with a 12-hour light/dark cycle.

In the first period, 40 mg/kg FSM dissolved in a sodium hydroxide solution (0.1 N) and titrated to neutral pH with hydrochloric acid was intravenously infused via a femoral vein catheter over 5 minutes (5 ml/kg). Venous blood samples (2 ml) were collected before and 0.08, 0.17, 0.25, 0.5, 0.75, 1, 2, 3, 5, 7, and 24 hours after administration in K2-EDTA-containing tubes. In the second period, after a washout period of 7 days, 2 mg/kg FSM dissolved in saline was intravenously infused via a femoral vein over 5 minutes (5 ml/kg), and blood samples were collected before and 0.08, 0.17, 0.25, 0.5, 0.75, 1, 2, 3, 5, 7, and 24 hours after administration. In the third period, 8 days after administrations of the previous dose of FSM, a FSM saline solution (2 mg/kg) was given by femoral vein infusion over 5 minutes to each monkey 30 minutes after PROB administration (40 mg/kg, i.v. infusion for 5 minutes). Blood was sampled before and 0.08, 0.17, 0.25, 0.5, 0.75, 1, 2, 3, 5, 7, and 24 hours after start of the FSM infusion. Blood samples were spun for 5 minutes at 13,000 rpm within 1 hour of collection. Urine samples were collected at 0.08, 0.17, 0.25, 0.5, 0.75, 1, 2, 3, 5, 7, and 7–24 hours after administration, and the volume of urine was recorded. The plasma and urine samples were stored at −80°C until LC-MS/MS and metabolomics analyses were conducted.

LC-MS/MS Analysis of Furosemide and Probenecid. The LC-MS/MS analysis was performed on a SCIEX Triple Quad API-4000 system (AB SCIEX, Framingham, MA) coupled with a Shimadzu Nexera LD-30AD ultra-performance LC system (Shimadzu, Columbia, MD). The chromatographic separation was performed on an Agilent Zorbax RRHD SB-C8 column (2.0 × 100 mm, 1.7 μm) from Agilent Technologies (Santa Clara, CA) using mobile phases of 0.1% formic acid in water and 0.1% formic acid in acetonitrile (ACN). The flow rate was 0.7 ml/min and total run time was 3.7 minutes. The LC column was maintained at 60°C. The analytes were monitored using selected reaction monitoring in negative ionization mode with the optimized nebulizing and desolvation gases. The source temperature was set at 400°C, and the declustering potential and collision energy were optimized. FSM, furosemide-d5, and PROB were detected at the selected reaction monitoring transitions of m/z 329.1 → 285.0, 334.1 → 291.0, and 283.9 → 239.9, respectively.

Before the analysis, urine samples were diluted 100-fold into blank plasma and treated as previously described for plasma. The plasma and diluted urine samples were then extracted using protein precipitation. Specifically, the 100 μl plasma and diluted urine samples were mixed with 100 μl of ACN containing 400 nM of furosemide-d5 (internal standard), followed by vortex mixing on a mixer for 5 minutes at room temperature. The mixed solutions were then filtered using a MultiScreen hydrophilic filtration plate (Billerica, Millipore, MA) by...
centrifugation at 4000g for 10 minutes, and the filtered solution was then injected (5 μl) onto LC-MS/MS. The assay was qualified over the analytical range of 1–5000 nM using a linear 1/x² weighed regression.

**Plasma Metabolomic Profiling.** Frozen plasma samples were thawed and 50 μl aliquots were subjected to protein precipitation by the addition of 600 μl of methanol containing 0.1% formic acid and stable-labeled internal standards. The samples were mixed with a vortex mixer and subjected to centrifugation for 10 minutes at 2700g (4000 rpm). An aliquot (50 μl) of the resulting supernatants was transferred to a 96-well plate for further hydrophilic interaction LC (HILIC) LC-MS analysis and an additional 100 μl was transferred to a separate 96-well plate for reverse phase (RP) LC-MS analysis. Both plates were dried to completeness under a nitrogen stream at room temperature. For RP LC-MS analysis, the dried samples were reconstituted by the addition of 20 μl of methanol, followed by vigorous shaking and the addition of 180 μl of water. The samples were mixed with a vortex mixer and subjected to centrifugation for 10 minutes at 4000 rpm. The resulting supernatants were transferred to a 96-well plate, from which 10 μl was directly injected for analysis.

**HILIC and RP LC-MS analyses were performed on a Nexera ×2 LC-30AD (Shimadzu, Somerset, NJ) ultra-high-performance LC system connected to an Exactive Plus (Thermo Fisher Scientific, Waltham, MA) mass spectrometer. The ultra-high-performance LC column for HILIC analyses was an Acquity BEH-NH₂, 2.1 × 150 mm, 1.7 μ (Waters Corporation, Milford, MA) with mobile phases A (95:5 water:ACN, 10 mM NH₄OAc, 0.05% NH₄OH) and B (ACN, 0.05% NH₄OH) at a flow rate of 300 μl/min with starting conditions of 95% B–37% B at 3.5 minutes, hold for 4 minutes, and down to starting conditions at 7 minutes, for a total run time of 11 minutes. The ultra-high-performance LC column for RP analyses was an Acquity BEH C18, 2.1 × 150 mm, 1.7 μ (Waters Corporation) with mobile phases A (water, 0.1% formic acid) and B (98:2ACN: water, 0.1% formic acid) at a flow rate of 600 μl/min with starting conditions of 100% A, to 80% A at 3 minutes, 40% A at 4 minutes, and 100% B by 7 minutes, and after a 2-minute hold, down to starting conditions at 9 minutes, for a total run time of 11 minutes. Both HILIC and RP LC-MS data were collected in positive and negative polarities (separate injections) at 35,000 resolution and expected mass accuracy of 5 ppm.

LC-MS data analysis was performed using software developed in-house, Expendat Data Mining, as described previously (Hnatyshyn et al., 2013). Direct data input from raw files was performed using the Thermo Fisher Scientific MSFileReader software. The resulting list of components was matched with accurate mass and retention time values using an in-house database of endogenous metabolites stored within Expedent Data Mining. The annotated table of component integrals was exported to Microsoft Excel for further statistical analysis using in-house Visual Basic scripts for Microsoft Excel. For these analyses, mean intensities for each treatment group were compared with the relevant concurrent control. For each component, fold change was calculated by dividing the treatment group value by the control group value and the P values were calculated as a pairwise comparison for two-tailed distribution, using the Student's t test (Excel statistics package; Microsoft; Redmond, Washington).

**Characterization of PDA and HVA Uptake in Stable Cell Lines Constitutively Expressing Major Renal Drug Transporters.** Uptake studies were performed as described previously (Shen et al., 2016b). Uptake of PDA and HVA was first measured at a single concentration for 5-minute incubation with OAT1-, OAT2-, OAT3-, OAT4-, OCT2-, MATE1-, MATE2K-, OATP1B1-, OATP1B3-, and sodium taurocholate cotransporting polypeptide (NTCP)-HEK cells, and then time- and concentration-dependent uptake was measured. The initial concentration used for PDA was 1 μM because the physiologic baseline level ranged from 0.67 to 2.5 μM in cynomolgus monkeys (Fig. 3A). The concentration studied for HVA was 5 μM because of limited bioanalytical sensitivity of this compound in Mock-HEK cells. In addition, the transport Michaelis-Menten constant value (Km) of 274 ± 100 μM (that corresponds to the
Identification of Functional Plasma Markers of OAT1 and OAT3

\[ V = \frac{V_{\text{max}} \times S}{K_m + S} \]
where $V$ is the rate of uptake measured at the given concentration; $V_{\text{max}}$ is the maximal rate of uptake; $K_m$ represents the Michaelis-Menten constant at which the transport rate is half its maximal value; and $[S]$ is the substrate concentration.

Statistical differences between cell lines or treatments were determined by an unpaired two-tailed Student $t$ test (GraphPad Prism version 7; GraphPad Software Inc.). A $P$ value of less than 0.05 was considered to be statistically significant (*$P$, 0.05, **$P$, 0.01, and ***$P$, 0.001).

**Results**

**Effects of PROB on Pharmacokinetics of FSM in Cynomolgus Monkeys.** The mean FSM plasma concentration-time profiles after intravenous administration of 2 mg/kg of FSM in the absence and presence of PROB (40 mg/kg, i.v.) are illustrated in Fig. 2A. The plasma concentrations of FSM were higher in the presence than in the absence of PROB at all time points and in all animals. This statement was supported by the results shown in Table 1.

![Fig. 2](image-url)

**Fig. 2.** (A) Plasma concentration-time curves of FSM administered intravenously alone (2 mg/kg; open circles) or with PROB (40 mg/kg, i.v.; closed circles). (B) Plasma concentration time curves of PROB administered intravenously alone (40 mg/kg; open triangles) or with FSM (2 mg/kg, i.v.; closed triangles). (C) Effects of PROB on urinary excretion rate of FSM administered intravenously alone (2 mg/kg; open bar) or with PROB (40 mg/kg, i.v.; closed bar). Data are shown as mean ± S.D. ($n = 3$). **$P < 0.01$, significantly different from urinary excretion rate in the absence of PROB.

<table>
<thead>
<tr>
<th>Analyte/Parameter</th>
<th>PROB Alone</th>
<th>FSM Alone</th>
<th>FSM with PROB</th>
<th>Ratio (90% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$AUC_{0-24\ h}$ (µM-h)</td>
<td>NA</td>
<td>11.5 ± 0.7</td>
<td>47.7 ± 5.9**</td>
<td>4.1 (3.6–4.8)</td>
</tr>
<tr>
<td>$C_{24\ h}$ (µM)</td>
<td>NA</td>
<td>7.3 ± 5.7</td>
<td>8.9 ± 6.5</td>
<td>1.1 (0.37–3.2)</td>
</tr>
<tr>
<td>$CL_{\text{TOT}}$ (ml/min per kilogram)</td>
<td>NA</td>
<td>0.69 ± 0.12</td>
<td>0.71 ± 0.18</td>
<td>1.0 (0.60–1.7)</td>
</tr>
<tr>
<td>$V_{\text{dSS}}$ (l/kg)</td>
<td>NA</td>
<td>0.20 ± 0.01</td>
<td>0.21 ± 0.02</td>
<td>1.0 (0.85–1.3)</td>
</tr>
<tr>
<td>$T_{\frac{1}{2}}$ (h)</td>
<td>NA</td>
<td>3.7 ± 0.8</td>
<td>3.7 ± 0.9</td>
<td>1.0 (0.87–1.1)</td>
</tr>
</tbody>
</table>

CI, confidence interval; ER$_{\text{R}}$, renal extraction ratio; NA, not applicable; $T_{\frac{1}{2}}$, terminal elimination half-life.

**TABLE 1**

Pharmacokinetic parameters of FSM and PROB in cynomolgus monkeys ($n = 3$) following intravenous administration of PROB alone (40 mg/kg), FSM alone (2 mg/kg), and PROB with FSM.

Pharmacokinetic parameters were determined as described in Materials and Methods. Data are shown as mean ± S.D. ($n = 3$). PROB was intravenously dosed 30 minutes prior to FSM administration in the coadministration treatment group.

*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, when the parameter was compared with that administered with FSM or PROB alone.
Consistently, the CLTOT of FSM was reduced significantly by PROB respectively; not significantly different (7.3 ± 0.6 vs. 2.1 ± 0.03 ml/min per kilogram; P < 0.001). However, the administration of PROB did not cause a significant difference in VdSS and terminal elimination half-life of FSM although the mean values were decreased by PROB pretreatment (0.33 ± 0.11 vs. 0.21 ± 0.03 l/kg and 5.9 ± 1.4 vs. 4.7 ± 0.8 hour, respectively; P > 0.05) (Table 1).

The mean urinary excretion rates of FSM in cynomolgus monkeys are shown in Fig. 2C. A 2.1- to 2.4-fold reduction of FSM urinary excretion in the presence of PROB was observed (P < 0.01). The CLR and extraction ratio of FSM were reduced significantly with PROB pretreatment (3.7 ± 0.6 vs. 0.44 ± 0.12 ml/min per kilogram and 49.5 ± 8.8 vs. 5.9 ± 1.4, respectively; P < 0.01). Furthermore, the CLNR of FSM in the presence of PROB was also reduced markedly (5.0 ± 0.6 vs. 1.7 ± 0.2 ml/min per kilogram, respectively; P < 0.01). Therefore, the reduced CLTOT by PROB pretreatment was not solely due to either decreased CLR or CLNR.

Figure 2B illustrates the PROB concentration in plasma after intravenous PROB alone and concurrently with FSM. The PROB plasma concentrations 24 hours after PROB administration (C24 h) were not significantly different (7.3 ± 5.7 and 8.9 ± 6.5 μM, P > 0.05) (Table 1). Comparing other PROB pharmacokinetic parameters in the absence or presence of FSM indicates that the mean PROB AUC0–24 h, C24 h, CLTOT, and terminal elimination half-life values were almost identical (Table 1).

### Identification of Potential Plasma Endogenous Biomarkers of OAT1 and OAT3 by Metabolomics.

To identify potential endogenous plasma probes of OAT1 and OAT3, LC-MS-based metabolomics was used to determine the alterations in plasma concentrations of endogenous compounds between treatments in cynomolgus monkeys. A total of 233 metabolites of known structural identity by matching accurate mass and retention time values with an in-house database of endogenous metabolites were measured, and the concentration values were used to determine statistical significance using the paired Student’s t test (Supplemental Table 1). Of those metabolites monitored, 29 endogenous molecules were identified to be present at concentrations at least 3-fold higher at 1 and/or 3 hours in PROB pretreatment groups (administered alone or with FSM) (Table 2), suggesting that the changes of endogenous metabolites in the plasma were associated with inhibition of monkey OAT1 and OAT3, with associated reduction of OAT-mediated renal clearance. However, only some metabolites were able to return to baseline at 24 hours, which were included as a criterion for transporter biomarker candidacy.

Based on the structure similarities, the identified metabolites can be categorized into three subset groups. The first subset of metabolites are long-chain dicarboxylic acids and derivatives (Table 2), which include TDA [HOOC(CH2)12COOH] and HDA [HOOC(CH2)14COOH]. The second subset consists of small acids that are produced by gut microflora.

<table>
<thead>
<tr>
<th>Metabolite Type and Name</th>
<th>Ratio of PROB Alone to FSM Alone</th>
<th>Ratio of PROB with FSM to FSM Alone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C1 h</td>
<td>C3 h</td>
</tr>
<tr>
<td>Dicarboxylic acids and derivatives</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18-dicarboxylic acid</td>
<td>5.1</td>
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<td>C18-1 dicarboxylic acid</td>
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<td>C20-1 dicarboxylic acid</td>
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<td>C18-1 dicarboxylic acid</td>
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<td>C16-1 dicarboxylic acid</td>
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<td>TDA</td>
<td>2.3</td>
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<td>C12 dicarboxylic acid</td>
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<td>C12-OH dicarboxylic acid</td>
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<td>C14-OH dicarboxylic acid</td>
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<td>Unsaturated tetradecanoyl carnitine</td>
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<td>Small acids produced by gut microflora</td>
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<td>Indole-3 acetic acid</td>
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<td>Cresoic acid</td>
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The metabolites include indole-3 acetic acid, cresol sulfate, phenyl sulfate, phenylactic acid, indoxyl sulfate, and phenylacetylglycine. The third are amino acids and derivatives, which include hydroxyisovaleric acid, tyrosine, and aspartic acid. The metabolites whose plasma concentrations were significantly increased by PROB pretreatments include PDA, HVA, glucuronic acid, pantothentic acid, xanthurenic acid, and kynurenic acid (Table 2). The administration of PROB caused increases in plasma PDA and HVA concentrations at 1 and 3 hours (approximately 3- to 6-fold), and the concentrations returned to baseline at 24 hours (Table 2). PDA and HVA represent novel types of endogenous biomarkers not known previously to interact with monkey and human OAT1 and OAT3. Therefore, we conducted follow-up experiments to determine the time course of change in plasma PDA and HVA concentrations and transporter-expressing cell uptake to comprehend the sensitivity and specificity of the probes as described subsequently.

**Time-Dependent Effects on Plasma PDA, HVA, HDA, and TDA Levels of PROB Pretreatment.** Administration of 40 mg/kg PROB intravenously caused increases in plasma PDA and HVA concentrations in monkeys. The plasma PDA concentration increased by approximately 3-fold over the first 24 hours (113.5 ± 23.3 and 120.0 ± 17.9 vs. 40.5 ± 2.2 μM·h, *P* < 0.05), and gradually declined to the basal level (1.5 ± 0.3 μM) at 24 hours after PROB pretreatment (Fig. 3A; Table 3). The PDA concentrations were greater at any time point than the control (Fig. 3; Table 3). As shown in Table 3, PROB pretreatment significantly decreased CLR of PDA compared with FSM treatment (*P* < 0.05). The pretreatment also decreased the CLR value of HVA, although the reduction was not statistically significant.

We also detected the effects of PROB on plasma concentrations of TDA and HDA since they are reported as substrates of OAT1 and OAT3. PROB pretreatment increased plasma concentrations of TDA and other long-chain dicarboxylic acids in monkeys (Table 2). Figure 3, C and D show that PROB pretreatments increase plasma concentrations, although the increases were not statistically significant (Table 3).

**Measurement of PDA and HVA Uptake in Stable Cell Lines Constitutively Expressing Renal Transporter.** To determine whether PDA and HVA are substrates for major renal drug transporters, the cellular uptake of the molecules was measured at a single concentration (1 and 5 μM for PDA and HVA, respectively) in human OAT1-, OAT2-, OAT3-, OCT1-, OCT2-, MATE1-, MATE2K-, OATP1B1, OATP1B3-, and NTCP-HEK cells after 5-minute incubation. The cellular uptake of PDA and HVA into the HEK cells stably transfected with the cynomolgus monkey renal OATs was not evaluated because in vitro models are not available in the United States (Tahara et al., 2005). The uptake of PDA in OAT1- and OAT3-HEK cells was approximately 55- and 52-fold higher than that in the control cells (1757 ± 703 vs. 30.6 ± 6.7 and 1548 ± 40.0 vs. 29.8 ± 5.9 pmol/mg after 5 minutes, respectively). In addition, the PDA uptake in OAT1- and OAT3-HEK cells is significantly inhibited by PROB, a potent OAT1 and OAT3 inhibitor (Fig. 4A). Furthermore, PDA is a substrate for human OCT4 because the uptake of PDA in OAT4-HEK cells is significantly greater than that in Mock-HEK cells (1.8-fold; *P* < 0.01) and the uptake is reduced by 1 mM PROB (Fig. 5A). On the other hand, there was no significant OAT2-, OCT2-, MATE1-, and MATE2K-mediated uptake of PDA compared with the control (*P* > 0.05) (Figs. 4A and 5A). Similarly, the uptake of HVA in OAT1- and OAT3-expressing cells was approximately 45- and 6-fold higher than those in the control cells (8.3 ± 0.4 vs. 0.18 ± 0.02 and 1.09 ± 0.09 vs. 0.18 ± 0.02 pmol/mg after 5 minutes, respectively; *P* < 0.001) (Fig. 4B). Additionally, there was also significant OAT2-mediated uptake of HVA compared with the control (5-fold; *P* < 0.001). However, there was no significant OCT2-, MATE1-, and MATE2K-mediated uptake of HVA compared with the control (*P* > 0.05). Moreover, both PDA and HVA are not substrates for human hepatic transporters OATP1B1, OATP1B3, and NTCP because there are no significant differences in the uptake between the transporter-overexpressing cells and mock cells (*P* > 0.05) (Supplemental Fig. 2).

![Figure 3. Effect of PROB on plasma concentration-time curves of PDA (A), HVA (B), TDA (C), and HDA (D) in three cynomolgus monkeys after intravenous administration of PROB alone (40 mg/kg; open squares), FSM alone (2 mg/kg, close triangles), and PROB with FSM (open circles).](image-url)
The affinities of OAT1- and OAT3-mediated transport for PDA and HVA were further determined over a range of PDA and HVA concentrations (0.2–500 μM) in OAT1- and OAT3-HEK cells after 2.5-minute incubation. The incubation time was set by the linear-uptake condition and the lower limit of bioanalytical methods (Supplemental Fig. 1). As shown in Fig. 6, the apparent $K_m$ values were 33.0 ± 6.2, 51.2 ± 2.3, 108 ± 5.3, and 438 ± 63 μM for OAT1- and OAT3-mediated uptake of HVA, respectively, while the apparent $K_m$ values of OAT1-, OAT-2, and OAT3-mediated uptake of PDA, respectively, were 10.8, and 438 μM for OAT1- and OAT3-mediated transport for PDA and HVA in OAT1- and OAT3-HEK cells after 2.5-minute incubation.

Discussion

OAT transporters are mainly expressed in RPTCs and mediate active renal secretion of their substrates. Inhibition of OAT transporter function could lead to decreased renal elimination and increased plasma exposure of xenobiotics and endogenous metabolites that are transported by these transporters. The general approach of using cynomolgus monkey as a model for study of human transporter function has become routine and takes advantage of the similarity and function of drug transporters between humans and monkeys (Tahara et al., 2006; Shen et al., 2013, 2016b). It is recognized that such approaches can identify endogenous biomarker candidates that are selective for a specific transporter (Chu et al., 2015; Shen et al., 2016a; Thakare et al., 2017). To demonstrate that cynomolgus monkey permits quantitative prediction of human OAT-mediated DDIs, we measured the effects of the clinically relevant OAT inhibitor PROB on the pharmacokinetics of the OAT1 and OAT3 probe substrate FSM in monkeys, and compared these results with the changes observed in human subjects. As shown in Fig. 2, the intravenous

![Image](https://dmd.aspetjournals.org/doi/10.1124/dmd.116.075134/fig-4)
administration of PROB generated a plasma total C_{24 h} of 7.3 ± 5.7 μM (Fig. 2; Table 1). Based on the IC_{50} values for both monkey OAT1 and OAT3 (<10 μM) (Tahara et al., 2005), sustained inhibition of OAT1 and OAT3 transport function is anticipated. In agreement with the in vitro inhibition, a pronounced increase in AUC of FSM, a known substrate of OAT1 and OAT3, resulting from PROB pretreatment was evident (Fig. 2; Table 1). At the relevant dose (i.e., 40 mg/kg, i.v.), PROB decreased the FSM CL_R value by 8.3-fold in cynomolgus monkeys. The decreases in the CL_R values of coadministered FSM observed in the clinic were 3.6- to 5.1-fold for PROB (Chennavasin et al., 2014; Smith et al., 1980). Furthermore, PROB decreased the FSM CLR value by 8.3-fold in cynomolgus monkeys pretreated with PROB alone and with FSM (Table 1). In agreement with the in vitro inhibition, a pronounced increase in AUC of FSM, a known substrate of OAT1 and OAT3, resulting from PROB pretreatment was evident. The decreases in the CL_R values of coadministered FSM observed in the clinic were 3.6- to 5.1-fold for PROB (Chennavasin et al., 1979; Smith et al., 1980). Furthermore, PROB decreased FSM CL_R in monkeys and humans by 2.9- and 1.5-fold, respectively, as compared with wild-type animals, suggesting they are substrates for OAT1 and OAT3 (Table 2). LC-MS/MS methods were further optimized for determination of plasma PDA and HVA concentration-time profiles. We found PDA and HVA to be consistently elevated in plasma from cynomolgus monkeys pretreated with PROB alone and with FSM (AUC_{0-24 h} of 2.8- to 2.9-fold and 1.6- to 2.1-fold, respectively), similar to the increase in plasma FSM concentration (4.1-fold) (Figs. 2 and 3; Tables 1 and 3). Furthermore, we demonstrated that PDA and HVA are substrates for human OAT1, OAT3, OAT2 (HVA), and OAT4 (PDA) but not OCT2, MATE1, MATE2K, OATP1B1, OATP1B3, and NTCP (Fig. 5). However, we did not study the transport of PDA and HVA by cynomolgus monkey OAT1 and OAT3 because in vitro cell models are not available in the United States. The species-dependent differences in OAT1- and OAT3-mediated transport of PDA and HVA cannot be excluded, although monkey OAT1 and OAT3 exhibit similar transport kinetics compared with corresponding human orthologs, and cynomolgus monkeys are recommended as more appropriate alternative systems for predicting DDIs involving renal drug transporters in humans (Tahara et al., 2005, 2006).

In the present study, we applied a combination of fit-for-purpose untargeted metabolomics and quantitative multiple reactions monitoring LC-MS/MS methods (Fig. 1) to determine if OAT inhibition is associated with changes in plasma concentrations of endogenous metabolites that are reflective of OAT function. Of the 233 plasma metabolites examined using the metabolomics method, 29 metabolites including PDA and HVA were significantly increased at 1 or 3 hours in plasma from monkeys by PROB. Of those, a number of metabolites identified had not previously been reported as substrates for OAT1 and OAT3 (Table 2). LC-MS/MS methods were further optimized for determination of plasma PDA and HVA concentration-time profiles. We found PDA and HVA to be consistently elevated in plasma from cynomolgus monkeys pretreated with PROB alone and with FSM (AUC_{0-24 h} of 2.8- to 2.9-fold and 1.6- to 2.1-fold, respectively), similar to the increase in plasma FSM concentration (4.1-fold) (Figs. 2 and 3; Tables 1 and 3). Furthermore, we demonstrated that PDA and HVA are substrates for human OAT1, OAT3, OAT2 (HVA), and OAT4 (PDA) but not OCT2, MATE1, MATE2K, OATP1B1, OATP1B3, and NTCP (Fig. 5). However, we did not study the transport of PDA and HVA by cynomolgus monkey OAT1 and OAT3 because in vitro cell models are not available in the United States. The species-dependent differences in OAT1- and OAT3-mediated transport of PDA and HVA cannot be excluded, although monkey OAT1 and OAT3 exhibit similar transport kinetics (i.e., K_m and V_max values) to human orthologs (Tahara et al., 2005). Collectively, the results from the present study demonstrate that circulating PDA and HVA can be potentially used as endogenous biomarkers of OAT1 and OAT3 inhibition.

A few years ago, an investigation was conducted examining the changes of plasma and urine metabolites with murine Oat1 (Slc22a6) deficiency. Using untargeted metabolomics analysis, the researchers identified several physiologically important metabolites, including PDA, that were increased in the plasma from Oat1 knockout mice compared with wild-type animals, suggesting they are substrates for mouse Oat1 (Wikoff et al., 2011). In agreement, PROB increased the plasma HVA levels in rhesus monkey (Bacopoulos et al., 1978). PDA has been shown to undergo active renal secretion in humans, and elevated plasma PDA was observed in patients with renal insufficiency (Coburn et al., 2002). It is worth noting that the present study shows a considerable variation in the urinary excretion and CL_R of PDA and HVA (Fig. 3, B and D; Table 3). In addition, the volumes of urine samples collected in PROB groups during normal diuresis and...
forced diuresis (i.e., administration of PROB alone and with FSM) are significantly different. Despite large variation, PROB pretreatments, either alone or with FSM, significantly reduced the CLR values of PDA in monkeys (ratios of 0.10 and 0.33–0.46, respectively), similar to the decrease in FSM CLR [0.12 (0.11–0.13)] (Tables 1 and 3).

Although the plasma levels of HVA are significantly increased by PROB pretreatments compared with FSM treatment, the CLR value was only slightly reduced by PROB pretreatments (ratios of 0.8 and 0.49–0.50, respectively) and the changes were not statistically significant. It is unclear why PROB pretreatments decreased the CLR of HVA to a lesser extent compared with PDA. However, the following observations may shed some light. Based on the in vitro uptake experiments, the renal basolateral transporters OAT1, OAT2, and OAT3 are involved in the uptake of HVA while PDA is only transported by OAT1 and OAT3 (Figs. 4 and 6). OAT2 is less sensitive to the inhibition by PROB compared with OAT1 and OAT3 (IC50 of 393–766 μM vs. <10 μM) (Enomoto et al., 2002; Jia et al., 2015). In contrast to our findings, previous studies examining expression of human OATs in the choroid plexus and their interactions with neurotransmitter metabolites have reported that HVA is not a substrate for human OATs (OAT1 and OAT3) in mouse cells (second segment of the proximal tubule cells, S2 cells) stably expressing OAT1 or OAT3 (Alebouyeh et al., 2003); the difference in transport profiling is probably due to the use of different host cells (HEK 293 cells vs. S2 cells), and endogenous expression of mouse Oat1, Oat2, and Oat3 in S2 host cells would mask HVA uptake. Although we showed high-affinity uptake of PDA and HVA by basolateral transporters OAT1, OAT3, and/or OAT2 into stably transfected HEK cells (Figs. 4 and 6), the mechanism(s) of the export of PDA and HVA out of the proximal tubule cells into the urine is not fully understood. These molecules may be extruded out of the cell by the efflux transporters MRP2 and MRP4 since most MRP2 and MRP4 substrates are organic anions, which are localized in the apical membrane of RPTCs. In addition, the renal apical transporter OAT4 may play a role in the transport of PDA from RPTCs into the urine since OAT4 can operate in efflux mode in addition to influx fashion (Hagos et al., 2007).

PROB is a selective inhibitor of OAT1 and OAT3 since its IC50 values toward other transporters are greater than 25 μM (Supplemental Table 2), although for other monkey transporters there is still paucity of complete data for PROB inhibition. Therefore, even though the results presented herein showcase PDA and HVA as novel cynomolgus monkey plasma OAT biomarkers, it cannot be assumed that the results translate directly to human subjects. In addition, we cannot exclude that the formation of the biomarkers studied may be affected by PROB. Moreover, PDA is the dead-end catabolite of the B6 vitamins including pyridoxine, pyridoxamine, and pyridoxal (Merrill and Henderson, 1990). Although the plasma PDA baseline level exhibits acceptable interindividual variability in monkeys and humans (1.6 ± 0.4 μM and 43 ± 17 nM) (Coburn et al., 2002), the use of vitamin B6 supplements causes elevation of PDA levels far above the normal physiologic level (Zempleni, 1995; Coburn et al., 2002). Therefore, the use of vitamin B6 supplements should be excluded in a clinical study with the goal of assessing a change in PDA plasma level.

Emerging metabolomics and genome-wide association data revealed that HDA and TDA, two fatty acid dicarboxylates, were potential endogenous biomarkers of human OATP1B1 (Yee et al., 2016). Although HDA and TDA are substrates of OATP1B1, OAT1 and OAT3 were also involved in the disposition of HDA and TDA (Yee et al., 2016). Consistently, we have observed that administration of rifampin, a selective inhibitor of hepatic OATPs over renal OATs, increased the AUCs of HDA and TDA by approximately 2-fold in human subjects (Shen et al., 2017). Although these results indicated that HDA and TDA are endogenous biomarkers of OATP1B1, the in vitro transport profiling suggested the involvement of OAT1 and OAT3 in the disposition of HDA and TDA (Yee et al., 2016). Metabolomics analysis in this study demonstrated the significant elevations in plasma levels of at least 20 dicarboxylic acids and fatty acids in the monkeys pretreated with PROB (Supplemental Table 1; Table 2). A similar trend was more evident for TDA with significant increases in AUC in the LC-MS/MS data set (Fig. 3C; Table 3). A comparable degree of increase has been observed with HDA (Fig. 3D; Table 3). As a result, HDA and TDA may serve as dual hepatic OATP and renal OAT biomarkers although further studies are needed for the relevance of clinical interactions. The concentration-time profiles for TDA and HDA appear different in the PROB-alone and with FSM groups although the concentrations in both groups are greater than those in that of FSM alone (Fig. 3, C and D). This is likely due to the additional inhibitory effect of FSM toward OAT1- and OAT3-mediated transport of TDA and HDA (Hasannejad et al., 2004; Nieskens et al., 2016).

In conclusion, the use of monkey as a transporter-mediated DDI model along with metabolomics has been demonstrated to be a useful approach for transporter biomarker identification. Our investigations showed that PDA and HVA are novel blood biomarkers of monkey renal OAT since the changes in plasma exposures of PDA and HVA are similar to that of a probe substrate in monkeys. Although interspecies differences in the transport and disposition of PDA and HVA need be considered, the information obtained...
in this monkey study on the extent of these endogenous compounds after pretreatment with PROB along with the results from stably transporter-overexpressing cell lines suggest that PDA and HVA are candidate biomarkers of OAT1 and OAT3 for use in clinical settings.

Acknowledgments
We thank Joseph Cantone and Dr. Dieter Drexler for supporting the bioanalysis of PDA and HVA in the OAT1, OATP1B1, OATP1B3, and NTCP uptake experiments.

Authorship Contributions

References
Supplementary Tables and Figures

Discovery and Validation of Novel Endogenous Plasma Biomarkers of Organic Anion Transporter (OAT) 1 and OAT3 in Cynomolgus Monkeys

Hong Shen, David M. Nelson, Regina V. Oliveira, Yueping Zhang, Colleen A. Mcnaney, Xiaomei Gu, Weiqi Chen, Ching Su, Michael D. Reily, Petia A. Shipkova, Jinping Gan, Yurong Lai, Punit Marathe, and W. Griffith Humphreys

Departments of Metabolism and Pharmacokinetics (H.S., Y.Z., X.G., W.C., J.G., Y.L., P.M., W.G.H.), Discovery Toxicology (D.N., C.A.M., M.D.R.), Bioanalytical Research (R.O., P.A.S.), and Pharmaceutical Optimization (C.S.), Bristol-Myers Squibb Research and Development, Princeton, New Jersey 08543, United States
Supplementary Table 1
Comparison of Plasma Concentrations of Selected Endogenous Metabolites between a Single or Combined IV Administration of PROB (40 mg/kg) and FSM with a Single IV Administration of 2 mg/kg FSM in 3 Cynomolgus Monkeys. Each determination was made in samples collected at 1, 3, and 24 h post-dose from 3 cynomolgus monkeys, and P values were determined by Student’s t test.

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<th>Ratio of PROB + FSM to FSM Alone</th>
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</thead>
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<tr>
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<tr>
<td>Nicotinamide</td>
<td></td>
<td></td>
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<tr>
<td>Choline</td>
<td></td>
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<tr>
<td>Bilirubin</td>
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<tr>
<td>N-methyl nicotinamide</td>
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<tr>
<td>Nicotinamide</td>
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</table>
Supplementary Table 2
Comparison of Inhibition Potency of PROB Towards Various Human Transporters. The $IC_{50}$ geometric means of PROB were obtained from University of Washington Metabolism and Transport Drug Interaction database.

<table>
<thead>
<tr>
<th>Transporter</th>
<th>$IC_{50}$ (µM)</th>
<th>geometric mean (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSEP (ABCB11)</td>
<td>564.8</td>
<td></td>
</tr>
<tr>
<td>MATE1 (SLC47A1)</td>
<td>&gt; 300</td>
<td></td>
</tr>
<tr>
<td>MRP2 (ABCC2)</td>
<td>67.8 (35.7 to 182)</td>
<td></td>
</tr>
<tr>
<td>MRP3 (ABCC3)</td>
<td>~ 100</td>
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</tr>
<tr>
<td>MRP4 (ABCC4)</td>
<td>&gt; 50</td>
<td></td>
</tr>
<tr>
<td>OAT1 (SLC22A6)</td>
<td>8.2 (3.6 to 27.4)</td>
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</tr>
<tr>
<td>OAT2 (SLC22A7)</td>
<td>393</td>
<td></td>
</tr>
<tr>
<td>OAT3 (SLC22A8)</td>
<td>5.8 (0.76 to 32)</td>
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<tr>
<td>OAT4 (SLC22A11)</td>
<td>69.3 (56 to 134)</td>
<td></td>
</tr>
<tr>
<td>OATP1B1 (SLCO1B1)</td>
<td>179 (76.2 to 740)</td>
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<tr>
<td>OATP1B3 (SLCO1B3)</td>
<td>130</td>
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<tr>
<td>OATP2B1 (SLCO2B1)</td>
<td>65 to 70% inhibition at 1 mM</td>
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<tr>
<td>OCT2 (SLC22A2)</td>
<td>56% inhibition at 25 uM</td>
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<tr>
<td>P-gp (ABCB1)</td>
<td>74% inhibition at 1.5 mM</td>
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</tr>
</tbody>
</table>
Supplementary Figure 1

Time course of uptake of PDA (1 µM) by OAT1 (A) and OAT3 (B), and HVA (5 µM) by OAT1. Uptake was performed by incubating cells with PDA or HVA dissolved in HBSS buffer supplemented with 10 mM HEPES (pH 7.4) at 37 °C as described under Materials and Methods for different periods of time. Data are expressed as mean ± SD (n = 3 determinations).
Supplementary Figure 2

Profiling of the transport of PDA and HVA by major drug transporters expressed at the basolateral membrane of human hepatocytes. Uptake in the HEK cells stably transfected with the control vector (Mock-HEK), OATP1B1, or OATP1B3 was measured after a 5-min incubation at 37°C with PDA (1 µM) (A), HVA (5 µM) (B), and probe substrate (1 µM [3H]E17βG, [3H]E3S and TCA for OATP1B1, OATP1B3 and NTCP, respectively) (C). Incubations were conducted in the absence and presence of 20 µM CsA to evaluate the effects of these inhibitors on PDA and HVA uptake. Each value represents the mean ± SD (n = 3). ***P < 0.001 statistically significantly different from uptake in Mock-HEK cells, and $$$P < 0.001 statistically significantly different from uptake in the absence of an inhibitor.
B). HVA Uptake by Hepatic Transporters

Fold Uptake of HVA over Mock Cells Treated without Inhibitor

C). Probe Uptake by Hepatic Transporters

Fold Uptake of Probe over Mock Cells Treated without Inhibitor