Evaluation of the selectivity of several OATP1B biomarkers using relative activity factor method

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Nonstandard abbreviations used:

AUC, area under the plasma concentration–time curve; AUCR, area under the curve ratio; CPI and CPIII, coproporphyrins I and III; CCK-8, cholecystokinin; CsA, cyclosporine A; CL_{int}, hepatocytes, intrinsic uptake clearance in hepatocytes; CL_{int, cell}, intrinsic uptake clearance in transfected cells mediated by the transporter; DDI, drug-drug interaction; EPP, estropipate; GCDCA-S, glycochenodeoxycholic acid sulfate; GDCA-S, glycodeoxycholic acid sulfate; TCDCA-S, taurochenodeoxycholic acid sulfate; HEK293, human embryonic kidney 293; f_t, fraction transported; LC-MS/MS, liquid chromatography–tandem mass spectrometry; PK, pharmacokinetic(s); NTCP, sodiumtaurocholate co-transporting polypeptide; OATP1B, organic anion transporting polypeptide 1B; OAT3, organic anion transporter 3; RAF, relative activity factor; R3G, resveratrol-3-O-glucuronide; TCA, taurocholic acid.
Abstract

In recent years, some endogenous substrates of organic anion transporting polypeptide 1B (OATP1B) have been identified and characterized as potential biomarkers to assess OATP1B-mediated clinical drug-drug interactions (DDIs). However, quantitative determination of their selectivity to OATP1B are still limited. In this study, we developed a relative activity factor (RAF) method to determine the relative contribution of hepatic uptake transporters OATP1B1, OATP1B3, OATP2B1, and sodium-taurocholate co-transporting polypeptide (NTCP) on hepatic uptake of several OATP1B biomarkers, including coproporphyrins I (CPI), CPIII, sulfate conjugates of bile acids: glycochenodeoxycholic acid sulfate (GCDCA-S), glycodeloxycholic acid sulfate (GDCA-S), and taurochenodeoxycholic acid sulfate (TCDCA-S). RAF values for OATP1B1, OATP1B3, OATP2B1, and NTCP were determined in cryopreserved human hepatocytes and transporter transfected cells using pitavastatin, cholecystokinin, resveratrol-3-O-β-D-glucuronide, and taurocholic acid (TCA) as reference compounds, respectively. OATP1B1-specific pitavastatin uptake in hepatocytes was measured in the absence and presence of 1 µM estropipate, while NTCP-specific TCA uptake was measured in the presence of 10 µM rifampin. Our studies suggested that CPI was a more selective biomarker for OATP1B1 than CPIII, while GCDCA-S and TCDCA-S were more selective to OATP1B3. OATP1B1 and OATP1B3 equally contributed to hepatic uptake of GDCA-S. The mechanistic static model, incorporating the fraction transported (f_t) of CPI/III estimated by RAF and in vivo elimination data, predicted several perpetrator interactions with CPI/III. Overall, RAF method combined with pharmacogenomic and DDI studies is a useful tool to determine the selectivity of transporter biomarkers and facilitate the selection of appropriate biomarkers for DDI evaluation.
Significance Statement (80/80 words)

We developed a new RAF method to quantitatively determine the contribution of hepatic uptake transporters OATP1B1, OATP1B3, OATP2B1, and NTCP on several OATP1B biomarkers (CPI, CPIII, GCDCA-S, GDCA-S, and TCDCA-S) and evaluated their predictivity on perpetrator-biomarker interactions. Our studies suggest that RAF method is a useful tool to determine the selectivity of transporter biomarkers. This method combined with pharmacogenomic and DDI studies will facilitate mechanistic interpretation and modeling of biomarker data and the selection of appropriate biomarkers for DDI evaluation.
Introduction

Inhibition of organic anion transporting polypeptide (OATP) 1B1 and OATP1B3 (OATP1B), the major hepatic uptake transporters for many clinically used drugs, has been associated with serious adverse effects, including statin-induced myopathy and rhabdomyolysis (Neuvonen et al., 2006; Gupta et al., 2016; Hougaard Christensen et al., 2019). Given the importance of transporters in drug interactions, regulatory agencies have recommended evaluating transporter-mediated drug-drug interaction (DDI) for new molecular entities (NMEs) (EMA, 2012; FDA, 2020). However, there are still significant challenges to use in vitro data to predict transporter-related perpetrator DDIs due to the limitations of in vitro transporter inhibition assays and gaps in in vitro to in vivo extrapolation (IVIVE) (Chu et al., 2017). Therefore, in recent years, there has been a tremendous interest in identifying, characterizing, and applying endogenous biomarkers to access DDI potential for hepatic and renal transporters in early clinical trials (Chu et al., 2018; Rodrigues et al., 2018; Mochizuki et al., 2021; Arya et al., 2022).

Among all, several classes of endogenous substrates of OATP1B demonstrated the potential to serve as surrogate clinical probes to assess DDIs. For instance, the suitability of coproporphyrins I and III (CPI and CPIII), the by-products of heme synthesis, as OATP1B biomarkers have been broadly evaluated in vitro and in vivo. Majority of CPs are synthesized in the liver (Yoshikado et al., 2018). They are metabolically stable and eliminated intact into bile and urine (Shen et al., 2016). CPI/III are substrates of OATP1B1, OATP1B3, multidrug resistance proteins (MRP) 2/3/4, and CPIII is also a substrate of OATP2B1 (Bednarczyk and Boiselle, 2016; Kunze et al., 2018; Chatterjee et al., 2021). In collective clinical studies, CPI has exhibited higher sensitivity to detect potent, moderate, and weak OATP1B inhibition (Kunze et al., 2018; Mori et al., 2020b; Zhang et al., 2020; Mochizuki et al., 2022; Tess et al., 2022). Recently, CPI model-based approaches have been developed and applied to quantitatively predict the magnitude of DDIs for OATP1B clinical substrates (Yoshikado et al., 2018; Takita et al., 2021; Kimoto et al., 2022; Tess et al., 2022).

Other than CPs, conjugated bile acids (BA) could also be sensitive biomarkers for OATP1B. Bile acids are first synthesized in liver from cholesterol, conjugated with sulfate or glucuronide, then actively secreted into bile, and eventually reabsorbed into blood circulation. In several
clinical DDI studies, the area under plasma concentration-time profile (AUC) of glycochenodeoxycholic acid sulfate (GCDCA-S), a substrate of OATP1B1, OATP1B3, sodium-taurocholate co-transporting polypeptide (NTCP) and MRP2, were significantly increased following the administration of rifampin (600 mg, SD, PO), a potent OATP1B inhibitor (Takehara et al., 2017; Takehara et al., 2018; Tatosian et al., 2021). In fact, the response of GCDCA-S (the ratio of AUC (AUCR)–10–20-fold) after rifampin administration was much greater than other biomarkers (e.g. CPI). Likewise, a recent clinical pharmacogenomic study suggested that glycochenodeoxycholate and glycodeoxycholate 3-O-glucuronides (GCDCA-3G and GDCA-3G) could also be sensitive and specific OATP1B1 biomarkers (Neuvonen et al., 2021).

As multiple transporters are involved in hepatic elimination of OATP1B biomarkers, identifying the biomarkers with higher selectivity to the transporter(s) of the interest will enable mechanistic interpretation of biomarker data and quantitative prediction of DDIs (Chu et al., 2018). The selectivity of transporter biomarkers can be determined in vitro and in vivo. In vitro relative activity factor (RAF) method has been established to quantitatively determine relative contribution of multiple uptake transporters to hepatic uptake of drugs (Hirano et al., 2004; Kunze et al., 2014; Izumi et al., 2018; Izumi et al., 2020). It can be explored to study the selectivity of OATP1B biomarkers. Using this approach, functional uptake of a “relatively” selective probe of specific transporter in hepatocyte over that in transporter transfected cells can be measured to obtain the RAF value. However, previous RAF methods may over or underestimate the contribution of selective uptake transporter(s), due to the lack of selective probe drugs.

The objectives of this work are to evaluate the in vitro selectivity of CPI, CPIII, GCDCA-S, glycodeoxycholic acid sulfate (GDCA-S), taurochenodeoxycholic acid sulfate (TCDCA-S) by determining relative contribution of major hepatic uptake transporters (OATP1B1, OATP1B3, OATP2B1, and NTCP) on uptake of these biomarkers. In this study, we have developed a new RAF method to determine transporter specific uptake by employing selective probe substrates and inhibitors, which will improve the accuracy of determining in vitro selectivity of OATP1B biomarkers. The data generated will be useful to improve quantitatively predict OATP1B-
mediated DDIs using biomarkers. It is worth noting that organic anion transporter OAT2 and organic cation transporter OCT1 may also contribute to hepatic uptake of endogenous compounds and substrate drugs. Since OATP1B biomarkers we studied are not substrates of these transporters (Table 1), RAF values of OAT2 and OCT1 are not evaluated.
Materials and Methods

Chemicals and Reagents

$[^3]H$Pitavastatin and unlabeled pitavastatin were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). $[^3]H$Estradiol-17β-D-glucuronide ($E_2^{17\beta}G$), $[^3]H$cholecystokinin (CCK8), $[^3]H$estrone 3-sulfate (ES), and $[^3]H$taurocholic acid (TCA) were purchased from PerkinElmer Life Sciences (Boston, MA). CPI, $E_2^{17\beta}G$, ES, cyclosporine A (CsA), rifampin, rifamycin SV, quinidine, sodium taurochenodeoxycholate (TCDC), estropipate (EPP), and labetalol were purchased from Millipore Sigma (St. Louis, MO). CPIII was purchased from Chemscene (Monmouth Junction, NJ). GCDCA-S, GDCA-S, and TCDCA-S, unlabeled, as well as the deuterium labeled version (GDCA-d4-S, GCDCA-d4-S and TCDCA-d4-S), were purchased from Isosciences LLC (Ambler, PA). Resveratrol-3-O-β-D-glucuronide (R3G) was purchased from Cayman Chemical (Ann Arbor, Michigan). Bromosulfophthalein (BSP) were purchased from MP Biomedicals (Solon, OH).

Cell Culture and Reagents

Human Embryonic Kidney 293 (HEK293) cells, HEK293 cells stably expressing OATP1B1 ($SLCO1B1$) or OATP1B3 ($SLCO1B3$) (HEK293-OATP1B1 or HEK293-OATP1B3, respectively) were provided by Solvo Biotechnology (Budapest, Hungary) and were used under license agreement; HEK293, HEK293-OATP1B1 and HEK293-OATP1B3 cells were cultured and cryopreserved by Evotec (Hamburg, Germany) and used for OATP1B1 and OATP1B3 experiments.

HEK293 cells transiently expressing OATP2B1 ($SLCO2B1$) or NTCP ($SLC10A1$) (HEK293-OATP2B1 or HEK293-NTCP, respectively), and control HECK293 cells were purchased from Corning Life Sciences (Manassas, VA).

Cryopreserved human hepatocytes, mixed gender, lot HCE (20 donors) and lot MMN (10 donors) were purchased from BioIVT (Baltimore, MD). All cell culture media and reagents were obtained from Thermo Fisher Scientific (Carlsbad, CA). All other reagents were commercially obtained with the highest analytical purity grade.
In vitro biomarker uptake and inhibition studies in OATP1B1-, OATP1B3-, OATP2B1-, and NTCP-transfected cells

Uptake and inhibition studies of several OATP1B biomarkers were conducted in OATP1B1 or OATP1B3 stably transfected HEK293 cells, as well as OATP2B1 or NTCP transiently transfected HEK293 cells. Briefly, cryopreserved HEK293, HEK293-OATP1B1, or HEK293-OATP1B3 aliquots were thawed at 37°C, recovered in Dulbecco Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), and washed with Hanks balanced salt solution (HBSS) with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4. Cells were then re-suspended in 96-well glass coated plates at a density of 0.125 × 10⁶ cells/well. For cryopreserved HEK293, HEK293-OATP2B1 or HEK293-NTCP aliquots, cells were thawed at 37°C, recovered in DMEM containing 10% FBS and cultured for 24 hours. For NTCP-transfected cells, cells were treated with 2 mM sodium butyrate (Millipore Sigma, Burlington, MA) for 24 hours to increase transporter expression prior to the experiment and uptake/inhibition studies were conducted at a cell density of 0.2 × 10⁶ cells/well.

For uptake time course studies, uptake was initiated by the addition of biomarker or positive control substrates (0.1 µM [³H]E₂17βG, 5 nM [³H]CCK8, 0.1 µM [³H]ES and 1 µM [³H]TCA for OATP1B1, OATP1B3, OATP2B1 and NTCP respectively), with or without positive control inhibitor (10 µM CsA for OATP1B1, OATP1B3, NTCP and 100 µM BSP for OATP2B1), into control or transporter transfected cells at different time points.

For uptake kinetic studies, uptake was initiated by the addition of various concentrations of the biomarker at indicated time point based on the linear uptake of the biomarker in the respective transporter transfected cell lines.

Inhibitory effects of several compounds on uptake of biomarkers were also determined in transporter transfected and control cells. For inhibition studies in OATP1B1-, OATP1B3- and OATP2B1- transfected cells, cells were pre-incubated with various concentration of inhibitors for 30 minutes at 37°C, uptake then was initiated by the addition of the biomarker and incubated at 37°C for indicated time. For inhibition studies in NTCP transfected cells, uptake was initiated by the addition of the biomarker containing various concentration of inhibitors into cells and co-incubated at 37°C for indicated time.
For all studies, uptake was stopped by the addition of ice-cold phosphate-buffered saline (PBS), followed by immediate centrifugation (Model 5180R; Eppendorf, Hamburg, Germany) for 1 minute at 3,000 rpm at 4°C and washing of the cell pellets with PBS three times.

For radiolabeled positive control samples, cell pellets were resuspended in 50% acetonitrile, and scintillation fluid (Ultima Gold; PerkinElmer Inc., Waltham, MA) was added. Radioactivity was then determined by liquid scintillation counting in a Tri-Carb 4910TR liquid scintillation counter (PerkinElmer, Waltham, MA).

For non-radiolabeled biomarker samples, cell pellets were resuspended in 80% acetonitrile with 0.2 µM internal standard labetolol, followed by immediate centrifugation for 5 minutes at 3000 rpm at 4°C. The supernatant was then transferred to 96 well plate and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

**Determination of RAF of OATP1B1, OATP1B3, OATP2B1, and NTCP using reference compounds**

To estimate the contribution of individual transporter to hepatic uptake of biomarker, RAF of OATP1B1, OATP1B3, OATP2B1, and NTCP was measured by determining transporter-specific uptake kinetics in transporter transfected cells and hepatocytes using reference compounds. In this study, pitavastatin, CCK8, R3G and TCA were selected as reference compounds for OATP1B1, OATP1B3, OATP2B1 and NTCP, respectively.

Uptake time course and kinetic studies of pitavastatin, CCK8, R3G and TCA were conducted in HEK293-OATP1B1, HEK293-OATP1B3, HEK293-OATP2B1 and HEK293-NTCP respectively, following the procedure described above.

Except for CCK-8 (specific substrate of OATP1B3; (Gui and Hagenbuch, 2008)) and R3G (specific substrate of OATP2B1; (Bi et al., 2019)) (Supplemental Figure 1), pitavastatin (substrate of OATP1B1, OATP1B3, OATP2B1 and NTCP; (Prueksaritanont et al., 2014)) and TCA (substrate of OATP1B1, OATP1B3, and NTCP; (Maeda et al., 2006)) were not selective probe substrate for OATP1B1 and NTCP, respectively. For pitavastatin, OATP2B1 and NTCP was reported to only exhibit low to negligible roles on its hepatic uptake (Hirano et al., 2006; Vildhede et al., 2016; Bi et al., 2017; Bi et al., 2019). To measure OATP1B1 (pitavastatin) and NTCP (TCA)-specific uptake kinetics of these reference compounds in human hepatocytes, we
therefore evaluated differential inhibitory effects of EPP (reported selective OATP1B1 inhibitor; (Zhang et al., 2019)) and rifampin (not a potent inhibitor of NTCP; (Prueksaritanont et al., 2014)) in transporter transfected cells. The results will help in selecting an appropriate inhibitor and inhibitor concentration to selectively inhibit relevant transporters (see below).

Inhibitory effect of EPP on OATP1B1-mediated pitavastatin uptake and OATP1B3-mediated CCK8 uptake

To determine the concentration of EPP that can only inhibit OATP1B1 in human hepatocytes, inhibitory effect of EPP on OATP1B1-mediated pitavastatin uptake and OATP1B3-mediated CCK8 uptake were assessed in transporter transfected cells, respectively. In these studies, we did not preincubate EPP with OATP1B1- and OATP1B3- transfected cells, as EPP will be used in uptake kinetic studies of pitavastatin in suspended human hepatocytes, where 30 minutes preincubation of hepatocytes at 37°C will reduce cell viability and subsequently the transport activity of substrate drugs (unpublished observation). Uptake was initiated by the addition of pitavastatin or CCK8 containing various concentration of EPP into control and OATP1B1- or OATP1B3-transfected cells and incubated at 37°C for indicated time. Other procedures were conducted as described above. Due to very low transport activity of pitavastatin by OATP2B1 (data not shown), we are not able to estimate inhibitory effect of EPP on OATP2B1-mediated pitavastatin uptake.

Inhibitory effect of rifampin on OATP1B1-, OATP1B3-, NTCP-mediated TCA uptake

TCA is a known substrate of OATP1B1, OATP1B3, and NTCP (Maeda et al., 2006). To determine the concentration of rifampin to selectively inhibit OATP1B1 and OATP1B3, but not NTCP, in human hepatocytes, inhibitory effect of rifampin on OATP1B1-, OATP1B3-, NTCP-mediated TCA uptake were assessed. The studies were conducted without preincubation of rifampin with transfected cells based on the reasons discussed above for EPP inhibition study, and the lack of pre-incubation-dependent inhibitory effects of rifampin to these transporters (unpublished observations). Uptake was initiated by the addition of TCA containing various concentration of rifampin into OATP1B1-, OATP1B3- or NTCP-transfected and control cells and incubated at 37°C for indicated time. Other procedures were conducted as described above.

Uptake kinetic studies of reference compounds in cryopreserved human hepatocytes
Uptake kinetic studies of reference compounds were conducted in two lots of cryopreserved human hepatocyte (lots HCE and MMN). Briefly, hepatocytes were thawed and resuspended in Krebs-Henseleit modified buffer (KHB) (Sigma-Aldrich, St. Louis, MO) (pH 7.4) at a density of $0.5 \times 10^6$ cells/tube. After 3 minutes of preincubation at 37°C, uptake was initiated by the addition of various concentrations of reference compound into hepatocytes and incubated at 37°C at 0.5 and 1.0 or 1.5 min (within the linear range of uptake time course, data not shown) under the following conditions: 1) pitavastatin in the absence and presence of EPP (1 µM); 2) CCK8; 3) R3G; 4) TCA in the presence of rifampin (10 µM). The reaction was terminated by spinning down the hepatocyte suspension through an oil layer (a mixture of silicone oil and mineral oil, d=1.008) at 13,000 rpm for 1 minute. The supernatant (top portion above the oil layer) was collected for recovery determination. After aspirating the oil layer, the hepatocyte pellet was then lysed in 50% acetonitrile and mixed well. The radioactivity of pitavastatin, CCK8 and TCA in both supernatant and cell pellets was then determined by Tri-Carb liquid scintillation counter. For R3G (non-radioactive) samples, the hepatocyte pellets were resuspended in 80% acetonitrile with 0.2 µM internal standard labetolol, followed by centrifugation for 1 minute at 13,000 rpm. The supernatant was then transferred to 96 well plate and analyzed by LC-MS/MS.

**Uptake of several OATP1B biomarkers into cryopreserved human hepatocytes**

To assess the contribution of active hepatic uptake vs. passive diffusion of several OATP1B biomarkers, uptake of unlabeled CPI (0.01 µM) and CPIII (0.05 µM), and of deuterium (d4) labeled GCDC-S (0.1µM), GDCA-S (0.1µM) and TCDCA-S (0.1µM), were conducted in cryopreserved human hepatocytes (lot HCE) at 0.5 and 1.5 minutes in absence and presence of a transporter inhibitor cocktail (CsA 10 µM, rifamycin SV 10 µM, rifampin 100 µM, quinidine 50 µM and TCDC 50 µM). CsA and TCDC are OATP1B and NTCP inhibitors (Schroeder et al., 1998), whereas rifampin and rifamycin SV are OATP1B and OATP2B1 inhibitors and quinidine is organic cation transporter 1 (OCT1) inhibitor. This transporter inhibitor cocktail was known to fully inhibit transporter-mediated uptake in human hepatocytes by OATP1B1, OATP1B3, OATP2B1, NTCP, and OCT1 (Houle et al., manuscript under preparation). The reaction was stopped according to the method described above and samples were then analyzed by LC-MS/MS.

**LC-MS/MS quantification of biomarkers and R3G in in vitro transport studies**
The sample analysis was conducted on a SCIEX 4500 tandem mass spectrometer (Applied Biosystems/MDS SCIEX, Toronto, Canada) coupled to a UPLC (Ultra High Performance Liquid Chromatography) Dionex Ultimate 3000 RS pump and an HTS PAL autosampler (CTC Analytics, Zwingen, Switzerland). Samples (5 µL) were injected onto an Acquity UPLC HSS T3 2.1 50 mm UHPLC column, particle size 1.7 µm (Waters, Ireland). For quantification of biomarkers, the samples were eluted by a gradient program of 5% B, held for 15 seconds, 5% B to 95% B in 75 seconds, 95% B for 25 seconds, and held 95% B for 60 seconds. For quantification of R3G, the samples were eluted by a gradient program of 5% B, held for 15 seconds, 5% B to 95% B in 90 seconds, 95% B for 25 seconds, and held 95% B for 60 seconds. The mobile phase was a mixture of 0.1% formic acid in water (A) and acetonitrile containing 0.1% formic acid (B). The column temperature was maintained at ambient, and the flow rate was 0.75 mL/min. The spectrometry and multiple reaction monitoring (MRM) precursor/product ion transitions of each biomarker were referenced in Supplemental Table 1.

Data analysis

Inhibition studies

Transporter-mediated uptake is calculated by subtracting the uptake rate in control cells from that in transporter-expressing cells (within the linear range of uptake time course of a probe substrate). Data are normalized to percent control (% control) and the IC$_{50}$ values for inhibition of transporter-mediated uptake were obtained by fitting the data to the formula below by nonlinear regression analysis.

\[
\% \text{ Control} = \frac{100}{1 + \left(\frac{I}{IC_{50}}\right)^\gamma}
\]

Where “% Control” represents transporter-mediated uptake measured in the presence of various concentrations of inhibitor to that in the absence of inhibitor; “I” represents nominal inhibitor concentration (µM) and “γ” represents the Hill Slope. The IC$_{50}$ is calculated using GraphPad Prism (San Diego, CA) and reported as best-fit value ± standard error from three independent measurements.
Uptake kinetic analysis of biomarkers and reference compounds in transporter transfected cell lines

The initial uptake rate, determined within the linear uptake range, for transporter-mediated uptake of biomarkers or reference compounds was obtained by subtracting the uptake rate in control cells from that in the transporter-transfected cells, and the kinetic parameters were estimated by fitting the data to eq. 2 or 3 (wherever appropriate) using nonlinear least squares method by GraphPad Prism.

\[
v = \frac{V_{\text{max}} \times S}{K_m + S} + C_{L_{\text{ns}}} \times S \quad \text{Eq. 2}
\]

\[
v = \frac{V_{\text{max}} \times S}{K_m + S} \quad \text{Eq. 3}
\]

where \(v\) is the initial uptake rate of test compound (pmol/min/10^6 cells), \(S\) is substrate concentration (\(\mu\)M), \(K_m\) is the Michaelis-Menten constant (\(\mu\)M), \(V_{\text{max}}\) is the maximum uptake rate (pmol/min/10^6 cells), and \(C_{L_{\text{ns}}}\) is the nonsaturable uptake clearance via passive diffusion or other mechanisms (\(\mu\)l/min/10^6 cells).

\(C_{L_{\text{int,cells}}}\), intrinsic uptake clearance in transfected cells mediated by the transporter, is the sum of \(V_{\text{max}}/K_m\) and \(C_{L_{\text{ns}}}\).

Uptake kinetic analysis of reference compounds in human hepatocytes

Pitavastatin

To estimate the hepatic uptake clearance of pitavastatin mediated by OATP1B1 in human hepatocytes, the initial uptake rate of pitavastatin (0.03-30 \(\mu\)M) in the absence or presence of EPP (1 \(\mu\)M) was first determined using the eq. 4 and 5 respectively. The OATP1B1-mediated hepatic clearance of pitavastatin was then obtained by subtracting the uptake rate of pitavastain in the presence of EPP from that in the absence of EPP (eq. 6) and the kinetic parameters were obtained by fitting the data to eq. 2 or 3.

\[
v_{pita} = \frac{\text{uptake at 1.5 min} - \text{uptake at 0.5 min}}{1.5 \text{ min} - 0.5 \text{ min}} \quad \text{Eq. 4}
\]

\[
v_{pita+EPP} = \frac{\text{uptake at 1.5 min} - \text{uptake at 0.5 min}}{1.5 \text{ min} - 0.5 \text{ min}} \quad \text{Eq. 5}
\]
\[ v_{\text{pita,OATP1B1}} = v_{\text{pita}} - v_{\text{pita+EPP}} \quad \text{Eq. 6} \]

where \( v_{\text{pita}} \), \( v_{\text{pita+EPP}} \) and \( v_{\text{pita,OATP1B1}} \) represent the initial uptake rate of pitavastatin in the absence of EPP (1 \( \mu \)M) (uptake by OATP1B1 and OATP1B3), the initial uptake rate of pitavastatin in the presence of EPP (1 \( \mu \)M) (uptake only by OATP1B3, as 1 \( \mu \)M EPP inhibited OATP1B1-mediated uptake), and the OATP1B1-mediated hepatic uptake clearance of pitavastatin respectively.

**CCK8**

To estimate the hepatic uptake clearance of CCK8 mediated by OATP1B3 in human hepatocytes, the initial uptake rate of CCK8 (0.005 – 15 \( \mu \)M) was determined according to eq. 7 and the kinetic parameters were estimated by fitting the data to eq. 2 or 3.

\[ v_{\text{CCK8,OATP1B3}} = \frac{\text{uptake at 1.5 min} - \text{uptake at 0.5 min}}{1.5 \text{ min} - 0.5 \text{ min}} \quad \text{Eq. 7} \]

**R3G**

To estimate the hepatic uptake clearance of R3G mediated by OATP2B1 in human hepatocytes, the initial uptake rate of R3G (0.1 – 15 \( \mu \)M) was determined according to eq.8 and the kinetic parameters were estimated by fitting the data to eq 2 or 3.

\[ v_{\text{R3G,OATP2B1}} = \frac{\text{uptake at 1.5 min} - \text{uptake at 0.33 min}}{1.5 \text{ min} - 0.33 \text{ min}} \quad \text{Eq. 8} \]

**TCA**

To estimate the hepatic uptake clearance of TCA mediated by NTCP in human hepatocytes, the initial uptake rate of TCA (0.1 – 50 \( \mu \)M) in the presence of rifampin (10 \( \mu \)M) (NTCP uptake only, as 10 \( \mu \)M rifampin only inhibited OATP1B-mediated TCA uptake without impacting NTCP-mediated uptake) was determined according to eq. 9 and the kinetic parameters were estimated by fitting the data to eq 2 or 3.

\[ v_{\text{TCA+rif,NTCP}} = \frac{\text{uptake at 1.0 min} - \text{uptake at 0.5 min}}{1.0 \text{ min} - 0.5 \text{ min}} \quad \text{Eq. 9} \]
Total and active uptake clearance of reference compounds were subsequently estimated as follows:

\[ \text{CL}_{\text{total,hepatocytes}} = \frac{v_{\text{max}}}{K_m} + \text{CL}_{\text{ns}} \]  \hspace{1cm} \text{Eq. 10} \\
\[ \text{CL}_{\text{int,hepatocytes}} = \frac{v_{\text{max}}}{K_m} \]  \hspace{1cm} \text{Eq. 11}

**Determination of RAF**

RAF is defined as the ratio of active and transporter specific uptake clearance of respective reference compound in hepatocyte to that in transporter transfected cells and is determined as below.

\[ \text{RAF} = \frac{\text{CL}_{\text{int,hepatocytes}}}{\text{CL}_{\text{int,cells}}} \]  \hspace{1cm} \text{Eq.12}

**Estimation of hepatic uptake clearance and relative contribution of transporters to hepatic uptake of biomarkers**

**Predicted active hepatic uptake clearance of biomarkers (CPI, CPIII, GCDCA-S, GDCA-S and TCDCA-S)**

\[ \text{CL}_{\text{hep}} = \sum \text{uptake clearance of biomarker mediated by individual transporter calibrated by corresponding RAF} \]  \hspace{1cm} \text{Eq. 13}

*For CPI:*

\[ \text{CL}_{\text{hep}} = \text{CL}_{\text{int,1B1}} \times \text{RAF}_{1B1} + \text{CL}_{\text{int,1B3}} \times \text{RAF}_{1B3} \]  \hspace{1cm} \text{Eq. 14}

*For CPIII:*

\[ \text{CL}_{\text{hep}} = \text{CL}_{\text{int,1B1}} \times \text{RAF}_{1B1} + \text{CL}_{\text{int,1B3}} \times \text{RAF}_{1B3} + \text{CL}_{\text{int,2B1}} \times \text{RAF}_{2B1} \]  \hspace{1cm} \text{Eq. 15}

*For GCDCA-S, GDCA-S and TCDCA-S:*

\[ \text{CL}_{\text{hep}} = \text{CL}_{\text{int,1B1}} \times \text{RAF}_{1B1} + \text{CL}_{\text{int,1B3}} \times \text{RAF}_{1B3} + \text{CL}_{\text{int,NTCP}} \times \text{RAF}_{NTCP} \]  \hspace{1cm} \text{Eq. 16}
% contribution by specific transporter = \frac{CL_{int,transporter} \times RAF_{specific transporter}}{CL_{hep}} \times 100 

Eq. 17

Determination of the fraction transported (f_{th}) of biomarkers to human hepatocytes in vitro

The f_{th} of the biomarkers by in vitro active hepatic uptake (f_{th,active}) and passive diffusion (f_{th,passive}) was estimated by uptake studies of the biomarker in the absence and present of transporter inhibitor cocktails and calculated using eqs 18-21. The fraction transported by specific transporter (f_{th, specific transporter}) was calculated by eq. 22.

\begin{align*}
v_{biomarker, no inhibitor} &= \frac{uptake \ at \ 1.5 \ min \ - \ uptake \ at \ 0.5 \ min}{1.5 \ min \ - \ 0.5 \ min} \quad \text{Eq. 18} \\
v_{biomarker, cocktail inhibitor} &= \frac{uptake \ at \ 1.5 \ min \ - \ uptake \ at \ 0.5 \ min}{1.5 \ min \ - \ 0.5 \ min} \\
\end{align*}

Uptake clearance = V/S, where S is the substrate concentration of the biomarker.

\begin{align*}
f_{th,passive} &= \frac{uptake \ clearance, cocktail \ inhibitor}{uptake \ clearance, no \ inhibitor} \quad \text{Eq. 20} \\
f_{th,active} &= 1 - \frac{uptake \ clearance, cocktail \ inhibitor}{uptake \ clearance, no \ inhibitor} \quad \text{Eq. 21} \\
f_{th, specific transporter} &= % \ contribution \ by \ specific \ transporter \ (from \ RAF \ prediction) \times f_{th, active} \quad \text{eq. 22}
\end{align*}

Estimation of the fraction transported (f_{i}) to overall elimination of several biomarkers and prediction of perpetrator-biomarker interactions using mechanistic static models

Estimation of f_{i} using clinical pharmacogenomic and DDI data

f_{i} of a biomarker by a transporter-mediated pathway (e.g., OATP1B1) can be calculated by eq. 23 or eq. 24.

\begin{align*}
f_{t_{OATP1B1}} &= 1 - \frac{AUC_{SLC01B1T/T \ or \ 1b/1b}}{AUC_{SLC01B1C\ or \ 15/15}} \quad \text{Eq. 23} \\
f_{t_{transporter}} &= 1 - \frac{AUC_{control}}{AUC_{inhibitor}} \quad \text{Eq. 24}
\end{align*}
$AUC_{SLCO1B1 \, T/T \, or \, *1b/*1b}$ and $AUC_{SLCO1B1 \, C/C \, or \, *15/*15}$ represent plasma AUC of a biomarker in the subjects carry $SLCO1B1 \, T/T \, or \, *1b/*1b$ genotype/haplotype (wild type) and $SLCO1B1 \, C/C \, or \, *15/*15$ (poor OATP1B1 function), respectively (Cooper-DeHoff et al., 2022). $AUC_{control}$ and $AUC_{inhibitor}$ represent plasma AUC of a biomarker in the absence and presence of relatively selective and potent inhibitor to the transporter of the interest.

*Estimation of $f_t$ for several biomarkers and prediction of perpetrator-biomarker interaction using mechanistic static models*

**CPI**

CPI is ~85% and ~15% eliminated via liver and kidney, respectively with minimal metabolism (Barnett et al., 2018; Takita et al., 2022). As OATP1B1 and OATP1B3 are major transporters for its hepatic uptake. The fold change of AUC (AUCR) for CPI with/without an OATP1B (OATP1B1 and OATP1B3) inhibitor drug can be calculated by eq. 25 (Chu et al., 2022):

$$AUCR = \frac{1}{\sum f_{t, \text{hepatic uptake}} + (1 - \sum f_{t, \text{hepatic uptake}})}$$

Eq. 25

the fraction transported by active hepatic uptake ($f_{t, \text{active hepatic uptake}}$) of CPI, including the fraction transported by OATP1B1 ($f_{t, \text{OATP1B1}}$) and OATP1B3 ($f_{t, \text{OATP1B3}}$), were estimated to be 0.57 and 0.17, respectively, based on the fact that CPI was ~85% eliminated via liver, and in vitro $f_{t, h, OATP1B1}$ and $f_{t, h, OATP1B3}$ were 0.67 and 0.20 (Table 4). It was assumed that OATP1B-mediated hepatic uptake was the rate-determining step for hepatic elimination of CPI and inhibition of hepatic efflux transporters (e.g., MRP2) has limited effect on its AUCR (Yoshikado et al., 2018). It was also assumed that OATP1B inhibitor drugs did not inhibit renal elimination and synthesis.
of CPI. $I_{\text{in, max, } u}$ represents unbound inhibitor concentration at the inlet to the liver and was calculated via eq. 26 (FDA, 2020).

$$I_{\text{in, max, } u} = f_u \times \{I_{\text{max}} + [(F_a x F_g x \text{dose} x k_a) / Q_H/R_b]\} \quad \text{Eq. 26}$$

where $f_u$ represents the plasma unbound fraction of the inhibitor drug, $I_{\text{max}}$ is the maximum plasma concentration of the inhibitor drug, $F_a$, fraction absorbed; $F_g$, the fraction escaping intestinal metabolism, $k_a$, the absorption rate constant of the inhibitor; $Q_H$, the hepatic blood flow rate in humans (1,500 ml/min) and $R_b$, the blood-to-plasma concentration ratio. To estimate the $I_{\text{in, max, } u}$ value, $F_a$ and $F_g$ were set at 1, $k_a$ was set at 0.1 min$^{-1}$ as the worst-case scenario and $R_b$ was assumed to be 1; $f_u$ was set at 0.01 if $f_u$ of the inhibitor was determined to be less than 0.01 (FDA, 2020).

**CPIII**

Likewise, AUCR of CPIII with/without OATP1B (OATP1B1, OATP1B3) and OATP2B1 inhibitor drug can be calculated by eq. 25. CPIII was also eliminated via hepatic and renal elimination. Based on the amount of CPIII and CPIII excreted into urine and the ratio of CPIII/CPI measured in feces in human (Kuhnel et al., 1999; Feng et al., 2021), as well as the fraction eliminated by liver and kidney for CPI (see above), the fraction of CPIII eliminated via liver and kidney were estimated to be 0.46, and 0.54, respectively. Given that CPIII in vitro $f_{t, h, OATP1B1}$, $f_{t, h, OATP1B3}$ and $f_{t, h, OATP2B1}$ were 0.45, 0.22 and 0.07 (averaged from hepatocyte lots HCE and MMN) (Table 4), the fraction transported of CPIII by OATP1B1 ($f_{t, OATP1B1}$), and OATP1B3 ($f_{t, OATP1B3}$), and OATP2B1 ($f_{t, OATP2B1}$) were estimated to be 0.21, 0.10, and 0.03, respectively. Again, it was under the assumption that OATP1B inhibitor drugs did not inhibit renal elimination and synthesis of CPIII.
Results

Uptake time course and kinetics of several OATP1B biomarkers in transporter transfected cells.

Uptake time course of CPI, CPIII, GCDCA-S, GDCA-S, and TCDCA-S were conducted in OATP1B1-, OATP1B3- (Figure 1), OATP2B1- (CPIII only), and NTCP- (GCDCA-S, GDCA-S, and TCDCA-S only) transfected and control cells (Figure 2). Uptake of these biomarkers in transporter transfected cells were time-dependent and much higher than that in control cells, indicating that these biomarkers are substrates of OATP1B1 and OATP1B3. In addition, CPIII was transported by OATP2B1, while BA sulfate conjugates GCDCA-S, GDCA-S, and TCDCA-S were transported by NTCP. Uptake of positive control substrates (E\textsubscript{2}17\textbeta G, CCK8, ES, and TCA for OATP1B1, OATP1B3, OATP2B1, and NTCP, respectively) were significantly higher in transporter transfected cells than control cells and were completely inhibited by respective positive control inhibitors, confirming the functionality of the assays (Figures 1-2).

Transporter-mediated uptake kinetics of CPI, CPIII, GCDCA-S, GDCA-S, and TCDCA-S were conducted in OATP1B1-, OATP1B3-, OATP2B1- (CPIII), and NTCP- (GCDCA-S, GDCA-S, and TCDCA-S) transfected and control cells. Concentration-, and transporter-mediated uptake kinetic data were shown in Figure 3, and corresponding kinetic parameters were summarized in Table 1.

Determination of RAF values for OATP1B1, OATP1B3, OATP2B1, and NTCP

Transporter-mediated uptake of reference compounds in transfected cells.

Pitavastatin, CCK-8, R3G, and TCA were selected as the reference compounds to determine RAF value for OATP1B1, OATP1B3, OATP2B1, and NTCP, respectively. Uptake time course and transporter-mediated uptake kinetics of pitavastatin, CCK-8, R3G, and TCA were conducted in OATP1B1-, OATP1B3-, OATP2B1-, and NTCP- transfected and control cells and the results were shown in Figure 4, and corresponding kinetic parameters were summarized in Table 2.

Selection of in vitro inhibitor test concentrations to determine OATP1B1- and NTCP-specific uptake of pitavastatin and TCA in human hepatocytes.
Unlike CCK8 and R3G (OATP1B3 and OATP2B1 selective substrate, respectively (Gui and Hagenbuch, 2008; Bi et al., 2019) and Supplemental Figure 1), pitavastatin is not a OATP1B1 specific probe substrate, as OATP1B3 also contributed to its hepatic uptake. To determine OATP1B1-specific uptake of pitavastatin, we evaluated the inhibitory effect of EPP, a relatively selective in vitro inhibitor of OATP1B1 (Zhang et al., 2019), on OATP1B1- and OATP1B3-mediated pitavastatin uptake in OATP1B1- and OATP1B3-transfected cells, respectively (Figure 5A). EPP demonstrated differential inhibitory effects on OATP1B1- and OATP1B3-mediated pitavastatin uptake with obtained IC$_{50}$ value of 0.08 ±0.02 µM, and 12.32 ±1.54 µM, respectively. As such, 1µM EPP, which inhibits OATP1B1, but not OATP1B3, was selected in subsequent uptake kinetic studies of pitavastatin to determine OATP1B1-specific uptake in human hepatocytes. Likewise, TCA is not a selective in vitro probe for NTCP, as it is also transported by OATP1B1 and OATP1B3 (Maeda et al., 2006). To determine NTCP-specific uptake of TCA, we measured inhibitory effects of rifampin on NTCP-, OATP1B1-, and OATP1B3-mediated TCA uptake in corresponding transporter transfected cells (Figure 5B). Rifampin inhibited OATP1B1-, OATP1B3-, and NTCP-mediated TCA uptake with obtained IC$_{50}$ value of 0.40 ± 0.10 µM, 0.15 ± 0.02 µM, and 140.3 ± 34.1 µM, respectively. Therefore, 10 µM rifampin, which completely inhibits OATP1B1- and OATP1B3-mediated TCA uptake without inhibiting NTCP-mediated uptake, was used in subsequent TCA uptake kinetic study to determine NTCP-specific TCA uptake in human hepatocytes.

**Kinetics of OATP1B1-, OATP1B3- OATP2B1-, and NTCP-specific uptake of the reference compound in cryopreserved human hepatocytes and RAF values.**

Kinetics of OATP1B1-, OATP1B3- OATP2B1-, and NTCP-specific uptake of pitavastatin, CCK8, R3G, and TCA was evaluated in two batches of cryopreserved human hepatocytes, lots MMN and HCE, respectively (Figure 6). Concentration-dependent initial uptake rate of pitavastatin was measured in the absence and presence of 1µM EPP. OATP1B1-specific pitavastatin uptake kinetics was obtained by subtracting its initial uptake rate in the presence of 1 µM EPP from that in its absence. NTCP-specific uptake kinetics of TCA was measured in the presence of 10 µM rifampin., OATP1B3-specific uptake kinetics of CCK8 and OATP2B1-specific uptake kinetics of R3G were measured directly as CCK8 and R3G are selective substrates of these transporters. Obtained kinetic parameters were summarized in Table 2. The
RAF values for OATP1B1, OATP1B3, OATP2B1, and NTCP were 0.15, 0.50, 0.31, and 0.02 for hepatocytes lot HCE, and 0.28, 0.84, 0.37, and 0.01 for lot MMN, respectively (Table 2).

**Relative contribution of OATP1B1, OATP1B3, OATP2B1, NTCP, and passive diffusion to hepatic uptake of several OATP1B biomarkers**

*Prediction of relative contribution of each transporter to active hepatic uptake of several OATP1B biomarkers using RAF method*

RAF-based uptake clearance of several OATP1B biomarkers mediated by OATP1B1 (RAF$_{1B1}$ × $\text{CL}_{\text{uptake,1B1}}$), OATP1B3 (RAF$_{1B3}$ × $\text{CL}_{\text{uptake,1B3}}$), OATP2B1 (RAF$_{2B1}$ × $\text{CL}_{\text{uptake,2B1}}$), and NTCP (RAF$_{\text{NTCP}}$ × $\text{CL}_{\text{uptake,NTCP}}$) was summarized in Table 3. The relative contribution of these transporters to active hepatic uptake of CPI (OATP1B1 vs. OATP1B3), CPIII (OATP1B1 vs. OATP1B3 vs. OATP2B1), GCDCA-G, GDCA-S, and TCDCA-S (OATP1B1 vs. OATP1B3 vs. NTCP) was determined and the results were also shown in Table 3. OATP1B1 demonstrated higher contribution to active uptake of CPI (76.2–78.6% in lot HCE and MMN) than that for CPIII (58.8–63.7%), while OATP1B3 was a primary transporter for active hepatic uptake of GCDCA-S (94.3–94.8%) and TCDCA-S (96.2–96.5%). OATP1B1 and OATP1B3 equally contributed to hepatic uptake of GDCA-S (46.9–50.5% for OATP1B1 and 49.3–52.4% for OATP1B3). OATP2B1 only contributed to 7.5–10.9% of CPIII active uptake. NTCP contributed negligibly to active hepatic uptake of GCDCA-S, GDCA-S and TCDCA-S (0.1–1.6%). Under the conditions tested, hepatocyte inter-lot variability between HCE and MMN was low.

*Prediction of the fraction transported for hepatic uptake of several OATP1B biomarkers via uptake transporters and passive diffusion*

To understand relative contribution of passive diffusion and active hepatic uptake, uptake rate of CPI, CPIII, GCDCA-S, GDCA-S, and TCDCA-S were measured in human hepatocytes (lot HCE) in the absence and presence of transporter inhibitor cocktails, which can fully inhibit active uptake by major human hepatic uptake transporters (OATP1B1, OATP1B3, OATP2B1, NTCP, and OCT1) (Houle et al., manuscript under preparation). Fraction transported for hepatic uptake by passive diffusion and active uptake by individual transporters was summarized in Table 4 and relative contribution (%) of each pathway on hepatic uptake of these biomarkers was shown in Figure 7. Passive diffusion contributed to 13.4%, 27.4%, 10.8%, 22.7%, and
34.1% of hepatic uptake of CPI, CPIII, GCDCA-S, GDCA-S, and TCDCA-G, respectively. In terms of transporter-mediated uptake, OATP1B1 contributed to 66-68%, 42.7–46.2%, 3.6–4.1%, 36.3–39%, and 2.1–2.4%, while OATP1B3 contributed to 18.6–20.6%, 20.9–22%, 84.2–84.6%, 38.1–40.5%, and 63.4–63.6% of hepatic uptake of CPI, CPIII, GCDCA-S, GDCA-S, and TCDCA-G, respectively. In addition, OATP2B1 contributed to 5.4–7.9% of CPIII uptake and NTCP only accounted for 0.5–1.5%, 0.2–0.5%, 0.04–0.1% of hepatic uptake of GCDCA-S, GDCA-S, and TCDCA-S, respectively.

**Inhibitory effects of CsA, rifampin, simeprevir and itraconazole on transporter-mediated uptake of CPI, CPIII, and GCDCA-S.**

Inhibitory effects of CsA, rifampin, simeprevir and itraconazole on transporter-mediated uptake of CPI (OATP1B1, OATP1B3), CPIII (OATP1B1, OATP1B3, OATP2B1), and GCDCA-S (OATP1B1, OATP1B3, NTCP) were conducted in transporter transfected cells, respectively. Obtained IC$_{50}$ values were summarized in Table 5 and inhibitory profiles were shown in Supplemental Figure 2. CsA and simeprevir showed potent inhibition (IC$_{50}$ <1 µM) to uptake of CPI, CPIII, and GCDCA-S for all transporters tested. As expected, rifampin inhibited OATP1B1- and OATP1B3-mediated CPI, CPIII, and GCDCA-S uptake, but showed moderate and weak inhibition to OATP2B1-mediated uptake of CPIII and NTCP-mediated uptake of GCDCA-S, respectively. Interestingly, inhibitory effects of itraconazole to OATP1B1 and OATP1B3 were substrate-dependent. It showed more potent inhibition to GCDCA-S (<1 µM) than CPI and CPIII (3-48 µM) (Table 5).

**Prediction of perpetrator-biomarker interaction of CPI and CPIII with several OATP1B inhibitor and non-inhibitor drugs using mechanistic static model.**

To further understand the translation of in vitro RAF data on predicting perpetrator-biomarker interaction, we estimated f$_i$ values of CPI (OATP1B1, OATP1B3) and CPIII (OATP1B1, OATP1B3, and OATP2B1) based on their in vivo elimination and in vitro relative contribution to hepatic uptake. Using mechanistic static modeling, we predicted AUCR of CPI and CPIII following the administration of several perpetrator drugs for which in vitro inhibition data using CPI and CPIII as probe substrates were determined in this study or reported in literature (Table 6). The predicted AUCRs were compared with those observed AUCRs of CPI and CPIII, as well as pitavastatin (relatively selective clinical probe drug for OATP1B) (Supplemental Figure 3).
Based on this limited dataset, mechanistic static models combined with $f_i$ values estimated by RAF method as well as in vitro inhibition data provided a reasonably good prediction of the perpetrator interaction with CPI and CPIII (predicted vs. observed AUCR <2-fold, except for one DDI study with 600 mg rifampin SD PO for CPIII: 2.3-fold). Furthermore, CPI AUCR estimated by $f_i$ and RAF method showed a good prediction of AUCR for pitavastatin (predicted vs. observed AUCR <2-fold). In contrast, predicted and observed AUCR of CPIII were less sensitive to detect weak to moderate clinical DDIs of pitavastatin (Supplemental Figure 3).

The $f_i$, OATP1B1 of CPI, CPIII, and GCDCA-S was also estimated based on clinical pharmacogenomic data in healthy subjects carrying variant $SLCO1B1$ c.521C/C or $SLCO1B1*$15/15 genotypes, which have a poor but not complete deficiency of OATP1B1 function, as compared to those carrying wild $SLCO1B1$ c.521T/T or $SLCO1B1*$1b/1b (Supplemental Table 2). The estimated $f_i$, OATP1B1 was 0.48 (geometric mean of 4 independent studies), 0.26 (geometric mean of 2 independent studies), and 0.41 (one study) for CPI, CPIII, and GCDCA-S, respectively. The $f_i$, OATP1B1 of CPI and CPIII based on pharmacogenomic data is comparable to those estimated by RAF method (0.57 and 0.21 for CPI and CPIII).

Because of the lack of quantitative data on overall elimination of GCDCA-S via liver and kidney, the $f_i$ values of OATP1B1, OATP1B3, and NTCP could not be determined using RAF approach. Despite that, our studies indicate that OATP1B3 is a major contributor than OATP1B1 and NTCP on hepatic uptake of GCDCA-S. Assuming that rifampin (600 mg S.D., P.O.) completely inhibited OATP1B (OATP1B1 + OATP1B3)-mediated GCDCA-S uptake without impacting the uptake by NTCP, $f_{OATP1B1+1B3}$ was estimated to be 0.92 based on GCDCA-S AUCR of 13.2 (geometric mean of 5 rifampin clinical DDI studies) (Supplemental Table 3). As such, estimated $f_i$, OATP1B1, $f_i$, OATP1B3, and $f_i$, NTCP was 0.04, 0.87, and 0.01, respectively, in which, $f_i$, OATP1B1 was much lower that the value estimated by clinical pharmacogenomic study (0.04 vs. 0.41).
Discussion

RAF method has been developed to determine relative contribution of OATP1B1 and OATP1B3 to hepatic uptake of several OATP1B substrate drugs (Hirano et al., 2004; Kunze et al., 2014; Izumi et al., 2020). In these studies, ES and CCK8 were selected as OATP1B1 and OATP1B3 specific substrates. However, ES is not specific to OATP1B1, it is also transported by OATP1B3 and OATP2B1. Furthermore, the contribution of other hepatic uptake transporters, including OATP2B1 and NTCP, were not evaluated. As such, over/under-estimation of the contribution of each pathway and their hepatic uptake clearance is possible (Izumi et al., 2018; Izumi et al., 2020). In our studies, we developed a new RAF method to determine transporter specific uptake of OATP1B1 (pitavastatin without-with 1 µM EPP), OATP1B3 (CCK8), OATP2B1 (R3G), and NTCP (TCA with 10 µM rifampin). This method has overcome the limitations of overlapping substrate specificity of reference compounds and provided a more accurate estimation of relative contribution of these transporters to hepatic uptake of transporter substrates. For the first time, we applied this approach to quantitatively determine the contribution of hepatic uptake transporters on several OATP1B biomarkers (CPI, CPIII, GCDCA-S, GDCA-S, and TCDCA-S) and evaluated their predictivity on perpetrator-biomarker interactions.

Our studies have indicated that CPI is a more selective biomarker for OATP1B1 than CPIII (f_t, OATP1B1 was 0.57 and 0.21 for CPI and CPIII, respectively). Estimated f_t, OATP1B1 values were generally in line with those obtained from pharmacogenomic studies (CPI 0.48, CPIII 0.26, Supplemental Table 2). To further understand the predictivity of RAF method, we predicted AUCR of CPI/III with/without the administration of several OATP1B inhibitor or non-inhibitor drugs. Within this limited dataset, predicted AUCR was within 2-fold of observed values for CPI, with one outlier for CPIII, suggesting that f_t values and in vitro inhibition data generally predicted in vivo interaction with CPI/III (Table 6). Interestingly, predicted and observed AUCR of CPI was comparable to AUCR of pitavastatin, a relatively selective OATP1B substrate (Chu et al., 2018). However, CPIII trended towards underpredicting pitavastatin DDIs. CPIII has higher renal and passive hepatic diffusion clearance than CPI. This may explain the poor predictive performance of CPIII on pitavastatin DDIs.
In a clinical DDI study, several sulfated BAs demonstrated differential responses to rifampin (600 mg S.D., P.O.) (Tatosian et al., 2021), suggesting that these biomarkers may have different selectivity to OATP1B. In fact, our studies suggest that OATP1B3 is a major contributor for hepatic uptake of GCDCA-S (84%) and TCDCA-S (64%), while OATP1B1 (38%) and OATP1B3 (39%) equally contribute to hepatic uptake of GDCA-S. Furthermore, % contribution of passive hepatic diffusion clearance varied among these biomarkers (GCDCA-S 11%, GDCA-S 22%, and TCDCA-S 34%). These data highlight the need to quantitatively determine the selectivity of biomarkers to mechanistically interpret and translate observed data. Among these biomarkers, GCDCA-S is the most well investigated. Besides being the substrate of OATP1B1, OATP1B3, NTCP, and MRP2, GCDCA-S undergoes active renal secretion via OAT3 (Tsuruya et al., 2016). Probenecid, a known inhibitor of organic anion transporters, decreased renal clearance of GCDCA-S dose-dependently with only minimal to weak increase of plasma AUC of GCDCA-S, which is attributed to inhibition of renal OAT3 (Tsuruya et al., 2016; Willemin et al., 2021). As such, GCDCA-S has also been considered as a sensitive biomarker for OAT3. It is worth noting that probenecid at high doses (1000 mg P.O.) weakly inhibited OATP1B (Zhang et al., 2020). Consequently, monitoring both renal and plasma exposure of GCDCA-S is recommended to deconvolute the inhibition between OATP1B and OAT3.

Unlike CPI/III, quantitative contribution of hepatic and renal clearance to elimination of GCDCA-S in human remains unknown, so we were unable to estimate ft values of respective transporters, despite our data suggesting that OATP1B3, not OATP1B1 and NTCP, is a primary transporter for hepatic uptake of GCDCA-S. Following rifampin administration (600 mg S.D., P.O.), AUCR of GCDCA-S in healthy subjects was ~13-fold in 5 independent studies, which was much higher than that for CPI/III, and some OATP1B probe substrates, e.g., pitavastatin, measured in the same study (Table 6, Supplemental Tables 3-5). Assuming that rifampin (600 mg S.D. P.O.) completely inhibits OATP1B in vivo without affecting NTCP and OAT3 (IC50, NTCP=48 µM, Table 5; IC50, OAT3>300 µM (Prueksaritanont et al., 2017)), ft, OATP1B1+1B3 was 0.92. Based on our RAF data, ft, OATP1B1, ft, OATP1B3 was estimated to be 0.04 and 0.77, respectively. In contrast, ft, OATP1B1 estimated by a clinical pharmacogenomic study (Supplemental Table 2) was 0.41 (AUCR SLC01B1*15/15/*1b/*1b was 1.7) (Mori et al., 2019), which was 10-fold higher than the value obtained by rifampin DDI and RAF studies. It is worth noting that the reported
A pharmacogenomic study was based only on two subjects carrying OATP1B1*15/*15. Additional studies are needed to confirm this finding. Despite that, high AUCR of GCDCA-S following rifampin treatment cannot be fully explained by inhibition of OATP1B1/1B3 (predicted AUCR based on RAF and in vitro inhibition data was only 4.5). The possible explanations for such discrepancy are 1) rifampin inhibition of GCDCA-S is largely attributed to OATP1B3 and in vitro IC\textsubscript{50} value of OATP1B3 were higher than in vivo K\textsubscript{i}; 2) rifampin inhibited other elimination mechanisms of GCDCA-S, e.g., canalicular efflux by MRP2 (Takashima et al., 2012); and 3) rifampin increased synthesis rate of GCDCA-S from bile acid (Mori et al., 2019).

In this study, we observed a substrate-dependent inhibition of itraconazole to OATP1B biomarkers (Table 5). Consistent with clinical data (Sane et al., 2021), our studies indicated that itraconazole, at clinically relevant exposure, did not inhibit CPI/III uptake by OATP1B1/1B3. However, it showed potent in vitro inhibition to OATP1B1/1B3 by GCDCA-S. Future itraconazole clinical studies with GCDCA-S will confirm this in vitro finding. Nevertheless, caution is required to translate in vitro biomarker inhibition data from one to another.

Characterization of biomarker selectivity is an important element for DDI risk assessment. Currently, the selectivity of OATP1B biomarkers is investigated via 1) in vitro transporter phenotyping studies; 2) clinical DDI studies with potent OATP1B inhibitors, e.g., rifampin; 3) pharmacogenomic/pharmacokinetic studies in subjects with poor transporter function (e.g., \textit{SLCO1B1} c.521C/C or \textit{SLCO1B1}*15/15). Although these approaches have greatly improved our understanding on biomarker selectivity, they have some limitations. In vitro transporter phenotyping screening can only identify substrate specificity of biomarkers without quantitatively determine their contribution to disposition/elimination of biomarkers. Clinical DDIs with rifampin have provided a useful dataset to identify OATP1B biomarkers, but rifampin is neither a selective inhibitor to OATP1B nor being able to differentiate the inhibition between OATP1B1 and OATP1B3. Pharmacogenomic studies are powerful tools to identify OATP1B1 specific biomarkers, but these studies require large sample size and recruitment of the subjects with certain genotypes (e.g., \textit{SLCO1B1} C521T>C), which is resource and time consuming. Importantly, despite a poor function (\textit{SLCO1B1} C/C or *15/*15), OATP1B1 expression/activity is not completely abolished. As such, contribution of OATP1B1 may be under-estimated. In
this regard, RAF method has the advantage of quantitatively determine biomarker selectivity in vitro. Alternatively, relative expression factor (REF) method based on quantification of transporter expression in human hepatocytes and transfected cells using LC-MS/MS, is another useful tool to study relative contribution of multiple transporters (Kumar et al., 2021). However, the data could be confounded if transporter protein abundance did not correlate with functional activity. Future studies are needed to directly compare RAF and REF approach to study biomarker selectivity.

Although physiologically based pharmacokinetic (PBPK) models are promising tools to predict transporter-mediated DDIs, underprediction of DDIs have been reported when in vitro transporter inhibition data are directly used in PBPK modeling (Taskar et al., 2020). To improve DDI prediction, Yoshikado et al. has proposed to use biomarkers as a translational tool to bridge in vitro and in vivo biomarker/probe drugs transporter inhibition. They successfully predicted several perpetrator DDIs with statins using CPI biomarker data and PBPK modeling (Yoshikado et al., 2018; Kimoto et al., 2022). However, in vivo Kᵢ obtained from this approach is still an apparent parameter without deconvoluting the effects of perpetrator drugs to different OATP1B isoforms and other transporters. Understanding the contribution of transporters on disposition/elimination of biomarkers may help to accurately determine in vivo Kᵢs and improve DDI prediction. Furthermore, the RAF method developed in this study can be extended to predict relative contribution of multiple hepatic uptake transporters on uptake of substrate drugs, and therefore may improve DDI prediction for drugs that their hepatic uptake involves multiple transporters, especially for inhibitors with differential inhibition potency to multiple transporters, e.g., paclitaxel (Mori et al., 2020a).

It is worth noting that current RAF method can only determine relative contribution of hepatic uptake transporters, but not the impact of canalicular (MRP2), and sinusoidal (MRP3/4) efflux transporters, or the net effects of all these transporters on hepatic disposition/elimination of OATP1B biomarkers. Although inhibition of hepatic uptake transporters will predominately impact plasma exposure of OATP1B biomarkers. Inhibition of MRP2, to a less extent, may also increase plasma AUC of OATP1B biomarkers (Yoshikado et al., 2018). The roles of MRP2/3/4 on the disposition of these OATP1B biomarkers need further evaluation. Moreover,
pharmacokinetic characterization of biomarker elimination clearance is required to extrapolate RAF data to in vivo.

In summary, we have developed a RAF method to quantitatively determine the selectivity of several OATP1B biomarkers. Our studies suggest that CPI is a more selective biomarker for OATP1B1 than CPIII, while GCDCA-S and TCDCA-S are more selective to OATP1B3. OATP1B1 and OATP1B3 equally contribute to hepatic uptake of GDC-A-S. RAF combined with clinical pharmacogenomic and DDI studies will help to determine the selectivity of transporter biomarkers and provide valuable information on selection of appropriate biomarkers for DDI evaluation.
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Authorship Contributions

Participated in research design: Chan, Houle, Zhang, Chu

Conducted experiments: Chan, Houle, Zhang, and Katwaru

Contributed new reagents or analytic tools: NA

Performed data analysis: Chan, Houle, Zhang, Li, Chu

Wrote or contributed to the writing of the manuscript: Chan and Chu
References


Footnotes

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Figure Legends

Figure 1. Uptake time course of several OATP1B biomarkers into human OATP1B1 and OATP1B3 stably transfected cells.
Panels A-F: Time-dependent uptake of CPI (0.01 µM) (A), CPIII (0.1 µM) (B), GCDCA-S (0.1 µM) (C), GDCA-S (0.1 µM) (D), and TCDCA-S (0.1 µM) (E) into HEK293 and HEK293-OATP1B1 cells. (F) Positive control; uptake of [3H]E217βG (0.1 µM) into HEK293 and HEK293-OATP1B1 cells at 5 minutes in the presence or absence of CsA (10 µM).
Panels G-L: Time-dependent uptake of CPI (0.01 µM) (G), CPIII (0.1 µM) (H), GCDCA-S (0.1 µM) (I), GDCA-S (0.1 µM) (J), and TCDCA-S (0.1 µM) (K) into HEK293 and HEK293-OATP1B3 cells. (L) Positive control; uptake of [3H]CCK8 (5 nM) into HEK293 and HEK293-OATP1B3 cells at 5 minutes in the presence or absence of CsA (10 µM). Open and closed squares/bars represent HEK293 and HEK293-OATP1B1 or HEK-OATP1B3, respectively. Each point represents the mean ± the standard deviation (S.D.) of experiments performed in triplicate (n=3).

Figure 2. Uptake time course of several OATP1B biomarkers into human NTCP and OATP2B1 stably transfected cells.
Panels A-D: Time-dependent uptake of GCDCA-S (0.1 µM) (A), GDCA-S (0.1 µM) (B), and TCDCA-S (0.1 µM) (C) into HEK293 and HEK293-NTCP cells. (D) Positive control; uptake of [3H]TCA (1 µM) into HEK293 and HEK293-NTCP cells at 5 minutes in the presence or absence of CsA (10 µM).
Panels E and F: Time-dependent uptake of CPIII (0.1 µM) (E) into HEK293 and HEK293-OATP2B1 cells. (F) Positive control; uptake of [3H]ES (0.1 µM) into HEK293 and HEK293-OATP2B1 at 10 minutes in the presence or absence of BSP (100 µM). Open and closed squares/bars represent HEK293 and transporter (NTCP or OATP2B1) transfected HEK293 cells, respectively. Each point represents the mean ± the standard deviation (S.D.) of experiments performed in triplicate (n=3).

Figure 3. Concentration-dependent uptake of several OATP1B biomarkers by OATP1B1 (A-E), OATP1B3 (F-J), NTCP (K-M) and OATP2B1 (N). OATP1B1-mediated uptake of
various concentrations of biomarkers at 37°C: CPI at 5 minutes (A), CPIII at 5 minutes (B), GCDCA-S at 5 minutes (C), GDCA-S at 2 minutes (D) and TCDCA-S at 5 minutes (E); OATP1B3-mediated uptake of various concentrations of biomarkers at 37°C: CPI at 5 minutes (F), CPIII at 5 minutes (G), GCDCA-S at 2 minutes (H), GDCA-S at 2 minutes (I) and TCDCA-S at 5 minutes (J); NTCP-mediated uptake of various concentrations of biomarkers at 37°C: GCDCA-S at 5 minutes (K), GDCA-S at 5 minutes (L) and TCDCA-S at 5 minutes (M); OATP2B1-mediated uptake of various concentrations of CPIII at 37°C at 5 minutes (N). Each point represents the mean ± the standard deviation (S.D.) of experiments performed in triplicate (n=3). The solid line depicts the fitted line obtained by nonlinear regression analysis as described in Materials and Methods.

Figure 4. Uptake time course and kinetics of transporter reference compounds into OATP1B1, OATP1B3, OATP2B1 and NTCP stably transfected cells.

Panels A, D, G, and J are time-dependent uptake of pitavastatin (0.1 μM) (A), CCK8 (5 nM) (D), resveratrol-3-O-β-D-glucuronide (R3G, 1 μM) (G), and TCA (0.1 μM) (J) into HEK293 and HEK293-OATP1B1, HEK293-OATP1B3, HEK293-OATP2B1 or HEK293-NTCP cells respectively. Panels B, E, H, K represent transporter-mediated uptake kinetics of pitavastatin (OATP1B1, panel B), CCK8 (OATP1B3, panel E), R3G (OATP2B1, panel H), TCA (NTCP, panel K), respectively. The solid line in these panels depicts the fitted line obtained by nonlinear regression analysis as described in Materials and Methods. All uptake rate was measured within linear range of transporter-mediated uptake. Eadie-Hofstee plots for the compounds which show bi-phasic kinetic profiles are also inserted. Panels C, F, I, L represent uptake of pitavastatin, CCK8, R3G and TCA at 5 minutes in the presence or absence of CsA (10 μM) for OATP1B1 (C), OATP1B3 (F), and NTCP (L) or Rifamycin SV (20 μM) for OATP2B1 (I). Open and closed squares/bars represent parental HEK293 cells and transporter transfected HEK293 cells, respectively. Each point represents the mean ± the standard deviation (S.D.) of experiments performed in triplicate (n=3).

Figure 5. Effects of estropipate (EPP) and rifampin on OATP1B1- and OATP1B3-mediated uptake (EPP and rifampin) and NTCP-mediated uptake (rifampin) in transporter transfected cells.
Panel A: The inhibitory effect of EPP on OATP1B1-mediated pitavastatin (0.1 µM) uptake (open square), and OATP1B3-mediated CCK8 (5 nM) uptake (closed square) was conducted in HEK293 cells and HEK293-OATP1B1 or HEK293-OATP1B3 cells at 2 minutes respectively. Obtained IC₅₀ values for OATP1B1 and OATP1B3 was 0.08 ± 0.02 µM, and 12.32 ± 1.54 µM, respectively. Panel B: The inhibitory effect of rifampin on OATP1B1-, OATP1B3-, and NTCP-mediated TCA uptake. The inhibitory effect of rifampin on OATP1B1-mediated (open square), OATP1B3-mediated (closed square), and NTCP-mediated (closed triangle) TCA (1 µM) uptake was conducted in HEK293 cells and HEK293-OATP1B1 at 4 minutes, HEK293-OATP1B3 at 2 minutes or HEK293-NTCP cells at 2 minutes, respectively. Obtained IC₅₀ values for OATP1B1, OATP1B3, and NTCP was 0.40 ± 0.10 µM, 0.15 ± 0.02 µM, and 140.3 ± 34.1 µM, respectively. Transporter-mediated uptake was determined by subtracting the uptake rate in control cells from that in transporter-transfected cells. Each point represents the mean ± the standard deviation (S.D.) of experiments performed in triplicate (n=3). The solid line depicts the fitted line obtained by nonlinear regression analysis as described in Materials and Methods.

Figure 6. Uptake kinetics of reference compounds in cryopreserved human hepatocytes lot MMN (A-D) and lot HCE (E-H).

Pitavastatin, CCK8, R3G and TCA are reference compounds for OATP1B1, OATP1B3, OATP2B1 and NTCP, respectively. To estimate the hepatic uptake clearance of reference compounds mediated by specific transporter, uptake rate was determined as follows: for OATP1B1 specific uptake clearance of pitavastatin (A, E), OATP1B1-specific initial uptake rate of pitavastatin was calculated by subtracting the initial uptake rate of pitavastatin in the presence of estropipate (EPP, 1 µM) from that in its absence over a range of pitavastatin concentration at 0.5 minute and 1.5 minute; for OATP1B3 specific uptake clearance of CCK8 (B, F), initial uptake rate of CCK8 was obtained by measuring uptake of CCK8 over a range of concentration at 0.5 minute and 1.5 minute; for OATP2B1-specific uptake clearance of R3G (C, G), initial uptake rate of R3G was obtained by measuring uptake of R3G over a range of concentration at 0.5 minute and 1.5 minute; for NTCP-specific uptake clearance of TCA (D, H), initial uptake rate of TCA was obtained by the uptake rate of TCA in the presence of rifampin (10 µM) over a range of TCA concentration at 0.5 minute and 1 minute. Eadie-Hofstee plots for the compounds which show bi-phasic kinetic profiles are also inserted. Each point represents the mean ± the standard deviation.
deviation (S.D.) of experiments performed in triplicate (n=3). The solid line depicts the fitted line obtained by nonlinear regression analysis as described in Materials and Methods.

Figure 7. Relative contribution of multiple hepatic uptake transporters and passive diffusion on hepatic uptake of several OATP1B biomarkers in cryopreserved human hepatocytes
Relative contribution of hepatic uptake transporters OATP1B1 (red bar), OATP1B3 (blue bar), OATP2B1 (yellow bar), NTCP (green bar), and passive diffusion (gray bar) on hepatic uptake of CPI, CPIII, GCDCA-S, GDCA-S, and TCDCA-S were determined using RAF method in cryopreserved human hepatocytes Lot MMN (A) and Lot HCE (B).
Table 1. Kinetic parameters of OATP1B1-, OATP1B3-, OATP2B1-, and NTCP-mediated uptake of several OATP1B biomarkers.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>OATP1B1</th>
<th>OATP1B3</th>
<th>OATP2B1</th>
<th>NTCP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (µM)</td>
<td>$V_{max}$ (pmol/10^6 cells/min)</td>
<td>$CL_{int,cells}$ (µl/µg/min)</td>
<td>$K_m$ (µM)</td>
</tr>
<tr>
<td>CPI</td>
<td>0.05±0.02</td>
<td>1.46±0.11</td>
<td>N/A</td>
<td>0.139</td>
</tr>
<tr>
<td>CPHII</td>
<td>0.30±0.03</td>
<td>5.11±0.23</td>
<td>N/A</td>
<td>0.075</td>
</tr>
<tr>
<td>GCDCA-S</td>
<td>2.31±0.58</td>
<td>0.84±0.18</td>
<td>0.04±0.01</td>
<td>0.002</td>
</tr>
<tr>
<td>GDCA-S</td>
<td>0.99±0.10</td>
<td>15.9±0.7</td>
<td>N/A</td>
<td>0.071</td>
</tr>
<tr>
<td>TCDDA-S</td>
<td>1.65±0.44</td>
<td>7.58±1.21</td>
<td>N/A</td>
<td>0.020</td>
</tr>
</tbody>
</table>

Each value represents the best-fit value ± standard error.

*: $CL_{int,cells}$ is reported as µl/µg/min based on measured total protein amount of 226.7 µg, 211.2 µg, 398.8 µg and 398.8 µg per million of OATP1B1, OATP1B3, OATP2B1 and NTCP cells, respectively, using BCA assay (unpublished in-house observation).

N/A: not applicable.

NS: not a substrate (Bednarczyk and Boiselle, 2016; Shen et al., 2017; Yee et al., 2019).

CPI, CPHII, and GCDCA-S are not substrates of human organic anion transporter OAT2 (Shen et al., 2017; Willemin et al., 2021) and organic cation transporter OCT1 (Bednarczyk and Boiselle, 2016; Kunze et al., 2018).
Table 2. Kinetic parameters of reference compounds in transporter-transfected cells and human hepatocytes and their respective RAF values

<table>
<thead>
<tr>
<th>Reference compound</th>
<th>Transporter</th>
<th>Transporter-transfected cell lines</th>
<th>Human hepatocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Km (µM)</td>
<td>Vmax (pmole/10^6 cells/min)</td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>OATP1B1</td>
<td>0.21±0.03</td>
<td>23.58±1.63</td>
</tr>
<tr>
<td>CCK8</td>
<td>OATP1B3</td>
<td>3.46±0.52</td>
<td>21.21±2.70</td>
</tr>
<tr>
<td>R3G</td>
<td>OATP2B1</td>
<td>1.36±0.66</td>
<td>4.98±0.98</td>
</tr>
<tr>
<td>TCA</td>
<td>NTCP</td>
<td>3.75±0.22</td>
<td>1765±59.35</td>
</tr>
</tbody>
</table>

Each value represents the mean ± standard error (S.E.).

N/A: not applicable.

*a*: CLint, cells is reported as µl/µg/min based on measured total protein amount of 226.7 µg, 211.2 µg, 398.8 µg and 398.8 µg per million of OATP1B1, OATP1B3, OATP2B1 and NTCP cells, respectively using BCA assay (unpublished in-house observation).

*b*: CLint, hep is reported as µl/µg/min based on total protein amount of 823.9 µg and 985.1 µg per million of human hepatocyte lot HCE and MMN, respectively using BCA assay (unpublished in-house observation).
<table>
<thead>
<tr>
<th>Human hepatocytes</th>
<th>Biomarkers</th>
<th>$\text{CL}<em>{\text{uptake,1B1}} \times \text{RAF}</em>{1B1}$ ($\mu l/\mu g/min$)</th>
<th>% contribution by OATP1B1</th>
<th>$\text{CL}<em>{\text{uptake,1B3}} \times \text{RAF}</em>{1B3}$ ($\mu l/\mu g/min$)</th>
<th>% contribution by OATP1B3</th>
<th>$\text{CL}<em>{\text{uptake,2B1}} \times \text{RAF}</em>{2B1}$ ($\mu l/\mu g/min$)</th>
<th>% contribution by OATP2B1</th>
<th>$\text{CL}<em>{\text{uptake,NTCP}} \times \text{RAF}</em>{\text{NTCP}}$ ($\mu l/\mu g/min$)</th>
<th>% contribution by NTCP</th>
</tr>
</thead>
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<td><strong>Lot HCE</strong></td>
<td>CPI</td>
<td>0.0203</td>
<td>76.2</td>
<td>0.0063</td>
<td>23.8</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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<tr>
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<td>CPIII</td>
<td>0.0110</td>
<td>58.8</td>
<td>0.0057</td>
<td>30.3</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td></td>
<td>GCDCA-S</td>
<td>0.0003</td>
<td>4.0</td>
<td>0.0060</td>
<td>94.3</td>
<td>NS</td>
<td>NS</td>
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<td>1.6</td>
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<td>GDCA-S</td>
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<td>46.9</td>
<td>0.0115</td>
<td>52.4</td>
<td>NS</td>
<td>NS</td>
<td>0.0002</td>
<td>0.7</td>
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<td>TCDDA-S</td>
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<td>3.3</td>
<td>0.0874</td>
<td>96.5</td>
<td>NS</td>
<td>NS</td>
<td>0.0002</td>
<td>0.2</td>
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<tr>
<td><strong>Lot MMN</strong></td>
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<td>78.6</td>
<td>0.0106</td>
<td>21.4</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td></td>
<td>CPIII</td>
<td>0.0211</td>
<td>63.7</td>
<td>0.0095</td>
<td>28.8</td>
<td>0.0025</td>
<td>7.5</td>
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<td>NS</td>
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<tr>
<td></td>
<td>GCDCA-S</td>
<td>0.0005</td>
<td>4.6</td>
<td>0.0101</td>
<td>94.8</td>
<td>NS</td>
<td>NS</td>
<td>0.0001</td>
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<tr>
<td></td>
<td>GDCA-S</td>
<td>0.0198</td>
<td>50.5</td>
<td>0.0194</td>
<td>49.3</td>
<td>NS</td>
<td>NS</td>
<td>0.0001</td>
<td>0.2</td>
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<tr>
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<td>TCDDA-S</td>
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<td>3.7</td>
<td>0.1470</td>
<td>96.2</td>
<td>NS</td>
<td>NS</td>
<td>0.0001</td>
<td>0.1</td>
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NS: not a substrate.
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<tr>
<th>Biomarkers</th>
<th>f_{h,\text{passive}}</th>
<th>f_{h,OATP1B1}</th>
<th>f_{h,OATP1B3}</th>
<th>f_{h,OATP2B1}</th>
<th>f_{h,\text{NTCP}}</th>
<th>f_{h,OATP1B1}</th>
<th>f_{h,OATP1B3}</th>
<th>f_{h,OATP2B1}</th>
<th>f_{h,\text{NTCP}}</th>
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<tr>
<td>CPI</td>
<td>0.134</td>
<td>0.660</td>
<td>0.206</td>
<td></td>
<td></td>
<td>0.680</td>
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<tr>
<td>CPIII</td>
<td>0.274</td>
<td>0.427</td>
<td>0.220</td>
<td>0.079</td>
<td></td>
<td>0.463</td>
<td>0.209</td>
<td>0.054</td>
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<tr>
<td>GCDCA-S</td>
<td>0.108</td>
<td>0.036</td>
<td>0.842</td>
<td></td>
<td>0.015</td>
<td>0.041</td>
<td>0.846</td>
<td>0.005</td>
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<tr>
<td>GDCA-S</td>
<td>0.227</td>
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<td>0.005</td>
<td>0.390</td>
<td>0.381</td>
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<tr>
<td>TCDCA-S</td>
<td>0.341</td>
<td>0.021</td>
<td>0.636</td>
<td></td>
<td>0.001</td>
<td>0.024</td>
<td>0.634</td>
<td>0.0004</td>
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Table 5. Summary of IC50 values of CsA, rifampin, simeprevir, and itraconazole on transporter-mediated uptake of several OATP1B biomarkers.

<table>
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<tr>
<th>Inhibitor</th>
<th>IC50 (µM)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>CPI (0.01 µM)</td>
</tr>
<tr>
<td></td>
<td>OATP1B1 OATP1B3</td>
</tr>
<tr>
<td>CsA</td>
<td>0.073±0.010 0.043±0.015</td>
</tr>
<tr>
<td>Rifampin</td>
<td>0.609±0.102 0.567±0.209</td>
</tr>
<tr>
<td>Simeprevir</td>
<td>0.090±0.019 0.027±0.005</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>3.291±1.073 6.494±2.460</td>
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</table>

Each value represents the best fit value ± standard error. N/A: not applicable.
Table 6. Prediction of several perpetrator interaction with CPI and CPIII using mechanistic static model and comparison with pitavastatin DDIs

<table>
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<tr>
<th>Perpetrator drugs</th>
<th>Perpetrator dose regimen</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (µM)</th>
<th>f&lt;sub&gt;u&lt;/sub&gt;</th>
<th>I&lt;sub&gt;in,max,u&lt;/sub&gt; (µM)</th>
<th>Predicted CPI AUCR</th>
<th>Observed CPI AUCR</th>
<th>Predicted CPIII AUCR</th>
<th>Observed CPIII AUCR</th>
<th>Observed Pitavastatin AUCR (90% CI)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CsA</td>
<td>20 mg SD, PO</td>
<td>0.12</td>
<td>0.07</td>
<td>0.053</td>
<td>1.50</td>
<td>1.23</td>
<td>1.15</td>
<td>1.12</td>
<td>1.64 (1.55, 1.73)</td>
<td>(Mochizuki et al., 2022)</td>
</tr>
<tr>
<td></td>
<td>75 mg SD, PO</td>
<td>0.45</td>
<td></td>
<td>0.198</td>
<td>2.25</td>
<td>1.98</td>
<td>1.28</td>
<td>1.61</td>
<td>3.46 (2.91, 4.12)</td>
<td>(Mochizuki et al., 2022)</td>
</tr>
<tr>
<td></td>
<td>100 mg</td>
<td>0.6</td>
<td>0.07</td>
<td>0.427</td>
<td>2.79</td>
<td>1.71</td>
<td>1.37</td>
<td>1.3</td>
<td>3.71&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(Yee et al., 2019)</td>
</tr>
<tr>
<td>Rifampin</td>
<td>150 mg SD, PO</td>
<td>4.78</td>
<td></td>
<td>2.540</td>
<td>2.49</td>
<td>1.54</td>
<td>1.31</td>
<td>NA</td>
<td>2.45 (2.11, 2.85)</td>
<td>(Mori et al., 2020b)</td>
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<tr>
<td></td>
<td>300 mg SD, PO</td>
<td>12.4</td>
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<td>5.505</td>
<td>3.01</td>
<td>2.33, 2.3</td>
<td>1.37</td>
<td>NA</td>
<td>3.36 (2.90, 3.90)</td>
<td>(Takehara et al., 2018; Mori et al., 2020b)</td>
</tr>
<tr>
<td>Simeprevir</td>
<td>150 mg</td>
<td>7.13</td>
<td>0.01</td>
<td>0.205</td>
<td>2.20</td>
<td>NA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.30</td>
<td>NA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>(Kakuda et al., 2018; Kunze et al., 2018)</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>200 mg QD for 9 days</td>
<td>1.9</td>
<td>0.036</td>
<td>0.749</td>
<td>1.14</td>
<td>0.89</td>
<td>1.02</td>
<td>0.95</td>
<td>0.8</td>
<td>(Sane et al., 2021)</td>
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<td></td>
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<td></td>
<td>10.711</td>
<td>3.34</td>
<td>3.67, 3.5</td>
<td>1.43</td>
<td>2.74, 3.3</td>
<td>4.01 (3.45, 4.65)</td>
<td>(Takehara et al., 2018; Mori et al., 2020b)</td>
</tr>
<tr>
<td>Probenecid&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1000 mg SD</td>
<td>381</td>
<td>0.09</td>
<td>55.32</td>
<td>1.27</td>
<td>1.39</td>
<td>NA&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1.34</td>
<td>2.23&lt;sup&gt;e&lt;/sup&gt;</td>
<td>(Wiebe et al., 2020; Zhang et al., 2020)</td>
</tr>
</tbody>
</table>

C<sub>max</sub> and f<sub>u</sub> values of perpetrator drugs and observed clinical perpetrator-biomarker AUCR and clinical DDIs with pitavastatin (the ratio of AUC with/without perpetrator drug; AUCR) were obtained from the University of Washington DDI database (http://https://www.druginteractioninfo.org) or cited references. CI, confident interval. I<sub>in,max,u</sub>, and predicted CPI and CPIII AUCR using mechanistic static model were obtained as described in the Materials and Methods.

<sup>a</sup>: pravastatin (substrate of OATP1B) was used as a clinical probe drug.
<sup>b</sup>: NA, not available; in 120 plasma samples obtained from 54 hepatitis C virus-infected patients, CPI and CPIII plasma concentrations increased when simeprevir plasma concentrations exceeded 3 µM (Kunze et al., 2018).
<sup>c</sup>: Odalasvir (substrate of OATP1B) was used as a clinical probe drug.
<sup>d</sup>: Prediction of CPI AUCR was conducted based on IC₅₀ values of probenecid for OATP1B1 (167 µM) and OATP1B3 (76 µM) using CPI as an in vitro probe substrate (Zhang et al., 2020).
<sup>e</sup>: Rosuvastatin was used as a clinical probe drug. The reduction of rosuvastatin renal clearance by 78% by probenecid due to inhibition of renal uptake transporter OAT3 (Wiebe et al., 2020).
<sup>f</sup>: NA: not available due to the lack of IC₅₀ values of Respective transporters using CPIII as an in vitro probe substrate.
Figure 2

A. GCDCA-S uptake (pmole/10^6 cells) over time (min).
B. GDCA-S uptake (pmole/10^6 cells) over time (min).
C. TCDCA-S uptake (pmole/10^6 cells) over time (min).
D. Positive Control uptake (pmole/10^6 cells) at various CsA (μM) concentrations.
E. CPIII uptake (pmole/10^6 cells) over time (min).
F. [3H]ES uptake (pmole/10^6 cells/10 min) at various BSP (μM) concentrations.
Figure 3

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Figure 4

A. Pitavastatin-OATP1B1

B. Pitavastatin uptake

C. HEK293

D. CCK8-OATP1B3

E. CCK8 uptake

F. HEK293

G. R3G-OATP2B1

H. R3G uptake

I. HEK293

J. TCA-NTCP

K. TCA uptake

L. HEK293
Figure 5

A. Efflux of Estropipate (μM) from cells expressing OATP1B3 or OATP1B1. The graph shows the percentage of control over the concentration of Estropipate.

B. Efflux of Rifampin (μM) from cells expressing NTCP, OATP1B3, or OATP1B1. The graph also shows the percentage of control over the concentration of Rifampin.
Figure 6

Human hepatocyte Lot MMN

Pitavastatin

CCK8

R3G

TCA

Human hepatocyte Lot HCE

Vo/S (µl/min/10^6 cells)

Vo (pmol/min/10^6 cells)

Vo/S (µl/min/10^6 cells)

Vo (pmol/min/10^6 cells)
Figure 7

A. MMN

B. HCE

- NTCP
- OATP2B1
- OATP1B3
- OATP1B1
- Passive