Investigation of the Impact of Substrate Selection on In Vitro Organic Anion Transporting Polypeptide 1B1 Inhibition Profiles for the Prediction of Drug-Drug Interactions

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Running title: In vitro substrate-dependent inhibition of OATP1B1

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Abbreviations:
AUC, area under the plasma concentration-time curve; BSP, sulfobromophthalein; CsA, cyclosporine A; CYP, cytochrome P450; DDI, drug-drug interaction; DMSO, dimethylsulfoxide; E2G, estradiol-17β-glucuronide; E1S, estrone-3-sulfate; EMA, European Medicines Agency; FDA, Food and Drug Administration; OATP, organic anion transporting
Abstract

The risk assessment of organic anion transporting polypeptide (OATP) 1B1-mediated drug-drug interactions (DDIs) is an indispensable part of drug development. We previously reported that in vitro inhibitory potencies of several inhibitors on OATP1B1 depend on the substrates when prototypical substrates, estradiol-17β-glucuronide (E₂G), estrone-3-sulfate, and sulfobromophthalein were used as test substrates. The purpose of this study was to comprehensively investigate this substrate-dependent inhibition of OATP1B1 using clinically-relevant OATP1B1 inhibitors and substrate drugs. Effects of cyclosporine A (CsA), rifampin, and gemfibrozil on OATP1B1-mediated uptake of twelve substrate drugs were examined in OATP1B1-expressing HEK293 cells. The $K_i$ values ($\mu$M) for CsA varied from 0.0771 to 0.486 (6.3-fold), for rifampin from 0.358 to 1.23 (3.4-fold), and for gemfibrozil from 9.65 to 252 (26-fold). Except for the inhibition of torasemide uptake by CsA and that of nateglinide uptake by gemfibrozil, the $K_i$ values were within 2.8-fold of those obtained using E₂G as a substrate. Pre-incubation potentiated the inhibitory effect of CsA on OATP1B1 with similar magnitude regardless of the substrates. R-values calculated based on a static model showed some variations depending on the $K_i$ values determined with various substrates, and such variability could have an impact on the DDI predictions particularly for a weak-to-moderate inhibitor (gemfibrozil). OATP1B1 substrate drugs except for torasemide and nateglinide, or E₂G as a surrogate, is recommended as an in vitro probe in the inhibition experiments, which will help mitigate the risk of false-negative DDI predictions potentially caused by substrate-dependent $K_i$ variation.
**Introduction**

Pharmacokinetic drug-drug interaction (DDI) is a concern in clinical practice. Since alteration of pharmacokinetics by a concomitant medication can sometimes significantly affect pharmacological and/or adverse effects of the victim drug, accurate evaluation of DDI potentials of drug candidates during drug development is indispensable from the viewpoint of clinical safety. Recent scientific progress revealed that membrane transporters as well as drug metabolizing enzymes play an important role in the pharmacokinetics of many drugs and thus, they can also serve as the sites of DDIs (Mizuno et al., 2003; Shitara et al., 2005; Yoshida et al., 2013). After publication of the “transporter whitepaper” by International Transporter Consortium (Giacomini et al., 2010), DDI guidelines or draft guidance materials were released by European Medicines Agency (EMA), U.S. Food and Drug Administration (FDA), and Japanese Ministry of Health, Labour and Welfare (MHLW), in which systematic approaches to the evaluation of transporter-mediated DDIs are given (Center for Drug Evaluation and Research, 2012; Committee for Human Medicinal Products, 2012; Ministry of Health, Labour and Welfare, 2014).

Organic anion transporting polypeptide (OATP) 1B1 (encoded by *SLCO1B1*) is expressed on the sinusoidal membrane of hepatocytes and mediates the uptake of a wide range of anionic drugs (Niemi et al., 2011). The genetic polymorphisms of *SLCO1B1* are associated with inter-individual variation in the plasma concentrations of 3-hydroxymethylglutaryl-CoA reductase inhibitors (statins) (Shitara and Sugiyama, 2006) and adverse event rate during simvastatin therapy (Link et al., 2008). In clinical situations, DDIs involving OATP substrate drugs and cyclosporine A (CsA) or a single dose of rifampin are considered to be attributable to the inhibition of OATP1B1 and its closely-related isoform, OATP1B3 (Shitara et al., 2003; Maeda et al., 2011; Takashima et al., 2012). Based on the accumulated evidence, OATP1B1 has been widely recognized as a clinically important transporter, and the evaluation of OATP1B1-mediated DDI potential of drug candidates is crucial in the drug development.
For the evaluation of transporter-mediated DDIs, static and dynamic (physiologically-based pharmacokinetic) models have been used. The static model that assumes a constant inhibitor concentration is suitable in the early stage of drug development due to its simplicity (Yoshida et al., 2012), while allowing for false-positive prediction. The dynamic model allows more accurate prediction by taking time-course of the inhibitor concentration into account (Imamura et al., 2011; Kudo et al., 2013). In both models, inhibition constant (\( K_i \)) is an essential parameter to evaluate the clinical relevance of the DDIs; therefore, its accurate estimation for the target transporter is a key step to achieve their purposes.

To determine \( K_i \) values of drug candidates for OATP1B1, in vitro cell-based inhibition assays using representative probe substrates are commonly conducted. However, it was reported that in vitro inhibitory potencies of gemfibrozil and other inhibitors on OATP1B1 depend on the probe substrates (Noé et al., 2007; Izumi et al., 2013). Therefore, care is needed in probe substrate selection for in vitro OATP1B1 inhibition assays to avoid the false-negative DDI predictions. We previously examined in vitro inhibitory effects of 14 compounds on OATP1B1 using three prototypical probe substrates; estradiol-17\( \beta \)-glucuronide (E2G), estrone-3-sulfate (E1S), and sulfobromophthalein (BSP). Among them, E2G provided the lowest \( K_i \) value for all inhibitors examined, and thus, we proposed that the use of E2G as an in vitro probe substrate may help mitigate the risk of false-negative DDI prediction (Izumi et al, 2013). Currently, various clinically-used drugs including statins (Sharma et al., 2012), angiotensin II receptor blockers (Yamashiro et al., 2006), endothelin receptor antagonists (Treiber et al., 2007), antidiabetics (Takanohashi et al., 2011), and diuretics (Werner et al., 2010) are known as OATP1B1 substrates. These clinically-used OATP1B1 substrate drugs, in addition to prototypical substrates, could also serve as in vitro probe substrates because of their clinical relevance. However, even using the OATP1B1 substrate drugs, substrate-dependence in the in vitro inhibitory potencies of OATP1B1 inhibitors is a topic of great concern.

Therefore, this study evaluated the inhibitory effects of CsA, rifampin, and gemfibrozil
on OATP1B1-mediated uptake of 12 clinically-used substrate drugs including statins (pitavastatin, atorvastatin, fluvastatin, rosuvastatin, and pravastatin), antidiabetics (repaglinide, nateglinide, and glibenclamide), and other classes of drugs (bosentan, valsartan, torasemide, and fexofenadine) and compared the $K_i$ values with those we previously reported with E$_2$G, E$_1$S, and BSP as substrates. In addition, the impact of probe substrate selection on the prediction of OATP1B1-mediated DDIs was evaluated based on a static model.
Materials and Methods

Materials

$[^3]H$E$_2$G (48.9 Ci/mmol) and $[^3]H$E$_1$S (45.6 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA). $[^3]H$BSP (5.5 Ci/mmol) was synthesized in HARTMANN ANALYTIC GmbH (Braunschweig, Germany). Unlabeled E$_2$G, rifampin, repaglinide, and gemfibrozil were purchased from Sigma-Aldrich (St. Louis, MO). CsA, pitavastatin calcium, pravastatin sodium, and glibenclamide (glyburide) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Atorvastatin calcium trihydrate, rosuvastatin calcium, and valsartan were purchased from LKT Laboratories (St. Paul, MN), and fluvastatin sodium, fexofenadine hydrochloride, and bosentan were from Toronto Research Chemicals (Ontario, Canada). Torasemide and nateglinide were obtained from Tokyo Chemical Industries (Tokyo, Japan) and Tocris Bioscience (Minneapolis, NM), respectively. All other chemicals were of analytical grade and commercially available.

Uptake and Inhibition Studies Using OATP1B1-Expressing Cells

The stably OATP1B1-expressing HEK293 cells and the corresponding control HEK293 cells established previously (Izumi et al., 2013) were used in this study. The cells were maintained in Dulbecco’s Modified Eagle Medium (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% (v/v) of fetal bovine serum, penicillin (final concentration, 100 units/mL), streptomycin (100 µg/mL), and hygromycin B (80 µg/mL) in a humidified incubator containing 5% CO$_2$ gas at 37 °C. For cell seeding, the cells were trypsinized and uniformly suspended in the designated volume of the culture medium to provide $4 \times 10^5$ cells/mL. One mL of the cell suspension was added to each well of a poly-D-lysine-coated 24-well plate (BD Biosciences, San Jose, CA), and the cells were further cultured in the incubator for 48 hours.

The uptake and inhibition studies were carried out as described previously (Izumi et al., 2013). Briefly, cell culture medium was replaced with pre-warmed Krebs Henseleit (KH) buffer...
(118 mM NaCl, 23.1 mM NaHCO₃, 4.83 mM KCl, 0.96 mM KH₂PO₄, 1.20 mM MgSO₄, 12.5 mM HEPES, 5.0 mM glucose, and 1.53 mM CaCl₂, pH 7.4), and the cells were pre-incubated for 5 minutes at 37 °C. After aspirating the pre-incubation buffer, the uptake reaction was initiated by addition of 250 µL of a pre-warmed KH buffer containing a substrate (pitavastatin, atorvastatin, fluvastatin, rosuvastatin, pravastatin, repaglinide, nateglinide, glibenclamide, bosentan, valsartan, torasemide, or fexofenadine) in the presence or absence of an inhibitor (CsA, rifampin, or gemfibrozil). Concentrations of the substrates and inhibitors used are given in Figure legends. At the designated time, the buffer was aspirated, and the cells were washed twice with ice-cold KH buffer.

Effect of Pre-incubation with CsA on the Inhibition of OATP1B1-mediated Uptake of [³H]E₂G, [³H]E₁S, [³H]BSP, Pitavastatin, and Atorvastatin

Effect of pre-incubation with CsA on the OATP1B1 inhibition was examined according to the previous reports with some modifications (Amundsen et al., 2010; Shitara et al., 2013). After 48-hour cell culture, cell culture medium was replaced with pre-warmed KH buffer. The KH buffer was aspirated, and the OATP1B1-expressing cells and control cells were pre-incubated for 1 hour at 37 °C with KH buffer containing or not containing CsA (or directly co-incubated with a substrate ([³H]E₂G, [³H]E₁S, [³H]BSP, pitavastatin, or atorvastatin) and CsA as no pre-incubation condition). After the 1-hour pre-incubation, the pre-incubation buffer was aspirated, and the cells were co-incubated with a substrate and CsA to initiate uptake reaction. At the designated time (1 minute, [³H]E₁S and atorvastatin; 2 minutes, pitavastatin; 5 minutes, [³H]E₂G and [³H]BSP), the buffer was aspirated, and the cells were washed twice with ice-cold KH buffer.

Quantification of Test Compounds Taken up by the Cells.

For the cellular uptake studies with radiolabeled substrates ([³H]E₂G, [³H]E₁S, and
[\(^3\)H]BSP), the cells were lysed with 0.5 mL of 0.1 N NaOH over night at room temperature. Resulting cell lysate was neutralized with 50 µL of 1N HCl. Four-hundred µL of the aliquot was mixed with 4 mL of scintillation fluid (Hionic-Fluor; Perkin Elmer Life Sciences), and the radioactivity associated with the cells were measured with a liquid scintillation counter (Tri-Carb 3100TR; Perkin Elmer Life Sciences). Remaining neutralized cell lysate samples were used to quantify protein concentrations (BCA Protein Assay Kit; Thermo Fisher Scientific, Waltham, MA). Radioactivities of the radiolabeled substrates in the incubation buffer were also measured by the liquid scintillation counting.

For the cellular uptake studies with unlabeled substrates (pitavastatin, atorvastatin, fluvastatin, rosuvastatin, pravastatin, repaglinide, nateglinide, glibenclamide, bosentan, valsartan, torsemide, and fexofenadine), the cells were vigorously mixed and deproteinized with 250 µL of methanol containing an appropriate internal standard, which was followed by centrifugation. The obtained supernatant was filtrated, and the resulting filtrates were subjected to high-performance liquid chromatography with tandem mass spectrometry analysis. Chromatography was performed using an Atlantis T3 column (3.0 µm, 2.1 mm i.d., 50 mm, Waters, Milford, MA) at a flow rate of 0.3 mL/min. Distilled water containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B) were used as the mobile phases. The initial condition was 100% solvent A, and the percentage of solvent B was linearly increased to 80% over 3 minutes, to 100% over the next 0.01 minutes and was maintained at this level for 1 minute. The column was equilibrated with the initial mobile phase before each injection (injection volume, 10 µL). A Quattro Premier mass spectrometer (Waters) was used for the detection. Analytes were ionized by electrospray ionization in positive or negative (only for pravastatin) ion mode, and the selected ion monitoring transitions were: 422.2\textgreater 274.0 for pitavastatin, 559.3\textgreater 249.9 for atorvastatin, 412.2\textgreater 265.9 for fluvastatin, 482.4\textgreater 258.0 for rosuvastatin, 423.3\textgreater 320.9 for pravastatin, 453.3\textgreater 230.0 for repaglinide, 318.3\textgreater 68.7 for nateglinide, 494.3\textgreater 368.9 for glibenclamide, 552.3\textgreater 201.8 for bosentan,
436.4 > 180.0 for valsartan, 349.3 > 263.9 for torasemide, and 502.7 > 466.3 for fexofenadine.

In the studies with unlabeled substrates, extra cells were prepared to quantify the protein amount per well. The cells were lysed with 0.5 mL of 0.1 N NaOH over night at room temperature, and the resulting cell lysate was neutralized with 50 µL of 1N HCl. The neutralized cell lysate samples were used to quantify the protein concentrations by BCA protein assay (Thermo Fisher Scientific).

**Determination of Kinetic Parameters**

Uptake of a substrate was expressed as the uptake volume (µL/mg protein), which was given as the amount of the substrate associated with the cells (dpm/well or pmol/well) divided by the concentration in the incubation buffer (dpm/µL or pmol/µL) and the protein amount (mg protein/well). OATP1B1-mediated net uptake was obtained by subtracting the uptake into control cells from the uptake into OATP1B1-expressing cells. Concentration dependence of the uptake of a substrate mediated by OATP1B1 was analyzed using the following Michaelis-Menten equation:

\[
  v = \frac{V_{\text{max}} \times S}{K_m + S}
\]  

(1)

where \(v\), \(S\), \(K_m\), and \(V_{\text{max}}\) represent uptake velocity of the substrate (pmol/min/mg protein), the substrate concentration in the incubation buffer (µM), Michaelis constant (µM), and the maximum uptake rate (pmol/min/mg protein), respectively. When an additional component was observed at higher substrate concentrations, the following equation was used for the analysis:

\[
  v = \frac{V_{\text{max}} \times S}{K_m + S} + P_{\text{dif}} \times S
\]  

(2)

where \(P_{\text{dif}}\) represents nonsaturable uptake clearance (µL/min/mg protein). Fitting was performed by a nonlinear least-squares regression method using MULTI program (Yamaoka et al., 1981).

Half maximal inhibitory concentration (IC\(_{50}\)) of an inhibitor was estimated by examining
the inhibitory effect on the OATP1B1-mediated uptake of a substrate (% of control) using the following equation as reported previously (Izumi et al., 2013):

\[
\text{uptake (% of control)} = \frac{\text{CL}_i}{\text{CL}} \times 100 = \frac{P}{1 + \frac{I}{I_{50}}}
\]

(3)

where \(\text{CL}\) and \(\text{CL}_i\) represent the uptake clearance in the absence and presence of an inhibitor, respectively, and \(I\) is the concentration of the inhibitor. \(P\) (initial value = 100) was set as a free parameter to achieve the best fit in the nonlinear iterative least squares regression analysis. The parameters were estimated by a nonlinear least-squares regression method using the MULTI program. With the assumption of competitive inhibition, the inhibition constant, \(K_i\) was estimated by Eq. 4 (Amundsen et al., 2010):

\[
K_i = \frac{I_{50}}{1 + \frac{S'}{K_m}}
\]

(4)

where \(S'\) and \(K_m\) represent the substrate concentration in the incubation buffer used for inhibition studies and Michaelis constant of the substrate for OATP1B1, respectively.

**Prediction of OATP1B1-mediated Drug-drug Interactions with a Static Model**

The degree of inhibition of OATP1B1-mediated hepatic uptake (R-value) was calculated for 3 inhibitors (CsA, rifampin, and gemfibrozil) based on the DDI guidelines and draft guidance materials released by regulatory agencies in U.S. (Center for Drug Evaluation and Research, 2012), E.U. (Committee for Human Medicinal Products, 2012), and Japan (Ministry of Health, Labour and Welfare, 2014). The decision tree of U.S. FDA for the inhibition of hepatic uptake transporters including OATP1B1 consists of 2 steps and recommends to evaluate the inhibition potential of an investigational drug on OATP1B1 using the following R-value as the first step:

\[
R = 1 + \frac{C_{\text{max}}}{K_i}
\]

(5)
where $C_{\text{max}}$ represents the maximum systemic total (bound + unbound) plasma concentration of inhibitor. U.S. FDA (the second step), EMA, and MHLW recommend to evaluate the inhibitory potential of an investigational drug on OATP1B1 using the following R-value:

$$R = 1 + \frac{[I]_{u,\text{inlet, max}}}{K_i}$$

(6)

where $[I]_{u,\text{inlet, max}}$ represents the estimated maximum unbound inhibitor concentration at the inlet to the liver and is defined as follows (Ito et al., 1998):

$$[I]_{u,\text{inlet, max}} = f_u \times \left( I_{\text{max}} + \frac{k_a \times F_a \times F_g \times \text{Dose}}{Q_h} \right)$$

(7)

where $f_u$ is the unbound fraction of an inhibitor in blood, which was calculated from the unbound fraction in plasma assuming the blood to plasma concentration ratio to be unity in this study, $I_{\text{max}}$ is the maximum circulating blood concentration of an inhibitor, $k_a$ is the absorption rate constant of the inhibitor, $F_a$ is the fraction of the inhibitor dose absorbed, $F_g$ is the fraction of the absorbed inhibitor dose escaping gut wall extraction, Dose is the inhibitor dose, and $Q_h$ is the hepatic blood flow rate (97 L/h) (Committee for Human Medicinal Products, 2012; Ministry of Health, Labour and Welfare, 2014). To minimize the risk of false-negative DDI prediction, $k_a = 0.1 \text{ min}^{-1}$ and $F_a\times F_g = 1$ were applied to the $[I]_{u,\text{inlet, max}}$ calculations in this study. Dose, $f_u$, $C_{\text{max}}$, and $[I]_{u,\text{inlet, max}}$ of CsA, rifampin, and gemfibrozil are given in supplemental table 1.
Results

Uptake of Clinically-Used OATP1B1 Substrate Drugs in OATP1B1-Expressing Cells

Time profiles of the uptake of 12 clinically-used OATP1B1 substrate drugs; pitavastatin, atorvastatin, fluvastatin, rosuvastatin, pravastatin, repaglinide, nateglinide, glibenclamide, bosentan, valsartan, torasemide, and fexofenadine in OATP1B1-expressing cells and control cells are shown in Fig. 1. Single representative substrate concentrations that were equivalent to or lower than reported $K_m$ values for OATP1B1 were selected for respective substrate drugs based on the detection sensitivities in this time-course study. For substrates whose $K_m$ values were not available, lower concentrations (0.1 µM, repaglinide and glibenclamide; 1 µM, nateglinide and fexofenadine) were selected based on the detection sensitivities. Our subsequent experiments confirmed that substrate concentrations selected in this time-course study were lower than respective $K_m$ values we examined (Table 1). OATP1B1-expressing cells showed higher transport activities for all compounds examined compared with the control cells, indicating that they are substrates of OATP1B1. On the basis of the time profiles and detection sensitivities, the following incubation times were selected for subsequent in vitro studies: 0.5 minute for rosuvastatin, pravastatin, glibenclamide, bosentan, and torasemide; 1 minute for atorvastatin; 2 minutes for pitavastatin and nateglinide; 5 minutes for fluvastatin, repaglinide, and valsartan; 10 minutes for fexofenadine.

Saturation Kinetics of OATP1B1-Mediated Uptake of Clinically-Used Substrate Drugs

Concentration dependence of the uptake of the 12 substrate drugs via OATP1B1 was examined to determine the saturation kinetic parameters. Representative Eadie-Hofstee plots of the uptake are shown in Fig. 2, and obtained kinetic parameters ($K_m$, $V_{max}$, and $P_{dif}$) are summarized in Table 1. OATP1B1-mediated uptake of compounds examined consisted of one-saturable component with or without minor nonsaturable component, and the $K_m$ values ranged from 0.761 (atorvastatin) to 61.6 µM (fexofenadine). The $K_m$ values of pitavastatin (2.48 µM),
atorvastatin (0.761 µM), fluvastatin (4.80 µM), rosuvastatin (9.31 µM), pravastatin (27.0 µM), valsartan (7.48 µM), and torasemide (20.9 µM) determined in this study were within the range of those previously reported using OATP1B1-expressing mammalian cells (Table 1). To our knowledge, this is the first report to examine $K_m$ values of repaglinide (1.36 µM), nateglinide (36.4 µM), glibenclamide (1.24 µM), and fexofenadine (61.6 µM) for OATP1B1 using the OATP1B1-expressing mammalian cells.

**Inhibitory Effects of CsA, Rifampin, and Gemfibrozil on OATP1B1-Mediated Uptake of Substrate Drugs**

Inhibitory effects of CsA, rifampin, and gemfibrozil on OATP1B1-mediated uptake of the 12 substrate drugs were investigated without any pre-incubation of the cells in the presence of inhibitors (Fig. 3). CsA, rifampin, and gemfibrozil inhibited the uptake of all substrates examined in a concentration-dependent manner, and all the inhibition curves were well described by the equation for monophasic inhibition (Eq. 3). Since the majority of OATP1B1-mediated uptake of clinically-used substrate drugs consisted of one-saturable component at the selected substrate concentrations in this inhibition assay, the $K_i$ values of CsA, rifampin, and gemfibrozil for the OATP1B1-mediated uptake of the substrate drugs were estimated by applying respective $IC_{50}$ values (supplemental table 2), substrate concentrations, and the $K_m$ values (Table 1) into Eq. 4, and are summarized in Table 2. For comparison, $K_i$ values of CsA, rifampin, and gemfibrozil on OATP1B1-mediated uptake of prototypical in vitro probe substrates (E$_2$G, E$_1$S, and BSP) that we examined under the same experimental conditions and reported previously (Izumi et al., 2013) are also listed in Table 2. In addition, reported $K_i$ or $IC_{50}$ values of CsA, rifampin, and gemfibrozil for the OATP1B1-mediated uptake of the prototypical substrates and substrate drugs are summarized in supplemental table 3.

The $K_i$ values of CsA showed 6.3-fold difference depending on the substrates selected, ranging from 0.0771 (fexofenadine as a substrate) to 0.486 µM (torasemide). Except for the $K_i$
value for torasemide uptake (0.486 µM), the $K_i$ values (range: 0.0771 to 0.301 µM) were within 2.6-fold of that obtained using E2G as a substrate (0.118 µM), but were overestimated by those given by E1S and BSP as substrates (0.732 and 0.694 µM, respectively).

The $K_i$ values of rifampin showed 3.4-fold difference depending on the substrate selected, ranging from 0.358 (nateglinide) to 1.23 µM (torasemide). These $K_i$ values were within 2.1-fold of that obtained using E2G as a substrate (0.585 µM), but were overestimated by those given by E1S and BSP as substrates (6.96 and 2.75 µM, respectively).

The $K_i$ values of gemfibrozil showed 26-fold difference depending on the substrates selected, ranging from 9.65 (pravastatin) to 252 µM (nateglinide). Except for the $K_i$ value for nateglinide uptake (252 µM), the $K_i$ values (range: 9.65 to 72.7 µM) were within 2.8-fold of that obtained using E2G as a substrate (26.4 µM), but were overestimated by those given by E1S and BSP as substrates (381 and 173 µM, respectively).

**Impact of Substrate Selection on Prediction of Clinical OATP1B1-Mediated DDIs Using a Static Model**

In order to evaluate the impact of substrate selection on the prediction of OATP1B1-mediated DDIs by a static model, R-values of CsA, rifampin, and gemfibrozil were calculated for the 12 clinically-used OATP1B1 substrate drugs and 3 typical prototypical substrates (E2G, E1S, and BSP) using the $K_i$ values estimated for each substrate (Table 2). Based on the DDI guidelines or draft guidance materials released by U.S. FDA, EMA, and MHLW, the maximum systemic total plasma concentration of inhibitor ($C_{\text{max}}$) or the estimated maximum unbound inhibitor concentration at the inlet to the liver ($[I]_{\text{u,inlet,max}}$) was used as the inhibitor concentration, $[I]$. $C_{\text{max}}$ and $[I]_{\text{u,inlet,max}}$ of CsA, rifampin, and gemfibrozil used for the R-value calculations are given in supplemental table 1. The calculated R-values and observed AUCR that is the ratio of the area under the plasma concentration-time curve (AUC) of a substrate drug with an inhibitor to that without the inhibitor in clinical DDI studies are summarized in Table 3.
R-values of CsA calculated with the [I]_{u,inlet,max} were slightly higher than those using the C_{max} as the [I]. When C_{max} was used, R-values of CsA for the 12 clinically-used substrate drugs ranged from 2.95 (torasemide as substrate) to 13.3 (fexofenadine), and those for E_{2}G, E_{1}S, and BSP were 9.05, 2.30, and 2.37, respectively. When [I]_{u,inlet,max} was used as [I], R-values of CsA for the clinically-used substrate drugs ranged from 3.47 (torasemide) to 16.6 (fexofenadine), and those for E_{2}G, E_{1}S, and BSP were 11.2, 2.64, and 2.73, respectively. Therefore, R-values of CsA for clinically-used substrate drugs were within 2.2-fold of that for E_{2}G under the both [I] conditions except for torasemide that yielded 3.1- to 3.2-fold greater K_{i} values than E_{2}G but the R-values for E_{1}S and BSP uptake were equal to or less than the lower limit of R-values obtained for the clinically-used substrate drugs. Although R-values of CsA showed some variations depending on the K_{i} values determined with various substrates, all clinically-used substrate drugs and prototypical substrates provided the R-values greater than the corresponding cut-off values recommended by regulatory agencies in both C_{max} (R \geq 1.1, U.S. FDA) and [I]_{u,inlet,max} (R \geq 1.25, U.S. FDA and MHLW; R \geq 1.04, EMA) conditions.

R-values of rifampin calculated with the [I]_{u,inlet,max} were lower than those using C_{max} as the [I]. When C_{max} was used, R-values of rifampin for the clinically-used substrate drugs ranged from 19.7 (torasemide) to 65.2 (nateglinide), and those for E_{2}G, E_{1}S, and BSP were 40.3, 4.30, and 9.36, respectively. When [I]_{u,inlet,max} was used as [I], R-values of rifampin for the clinically-used substrate drugs ranged from 9.13 (torasemide) to 28.9 (nateglinide), and those for E_{2}G, E_{1}S, and BSP were 18.1, 2.44, and 4.64, respectively. Therefore, R-values of rifampin for clinically-used substrate drugs were within 2.0-fold of that for E_{2}G under the both [I] conditions, but the R-values for E_{1}S and BSP uptake were less than the lower limit of R-values obtained for clinically-relevant substrate drugs. Although R-values of rifampin showed some variations depending on the K_{i} values determined with various substrates, all clinically-used substrate drugs and prototypical substrates provided the R-values greater than the corresponding cut-off values in both [I] conditions.
R-values of gemfibrozil calculated with the [I]_{u,inlet,max} were lower than those using C_{max} as the [I]. When C_{max} was used, R-values of gemfibrozil for the clinically-used substrate drugs ranged from 1.40 (nateglinide) to 11.4 (pravastatin), and those for E_{2}G, E_{1}S, and BSP were 4.79, 1.26, and 1.58, respectively. Therefore, the R-values for clinically-used substrate drugs were within 2.4-fold of that for E_{2}G except for nateglinide that yielded 3.4-fold greater K_{i} values than E_{2}G, but the R-values for E_{1}S and BSP uptake (1.26 and 1.58, respectively) were equal to or less than the lower limit of R-values obtained for clinically-used substrate drugs. Although R-values of gemfibrozil showed some variations depending on the K_{i} values determined with various substrates, all clinically-used substrate drugs and prototypical substrates provided R-values greater than the cut-off value (R \geq 1.1).

When [I]_{u,inlet,max} was used as [I], R-values of gemfibrozil for the clinically-used substrate drugs ranged from 1.01 (nateglinide) to 1.26 (pravastatin), and those for E_{2}G, E_{1}S, and BSP were 1.09, 1.01, and 1.01, respectively. Based on the cut-off value recommended by U.S. FDA and MHLW (R \geq 1.25), only pravastatin (R = 1.26) showed the R-value greater than the threshold. Based on the cut-off value recommended by EMA (R \geq 1.04), R-values of 11 out of 15 substrates examined were greater than the threshold.

Effect of Pre-Incubation With CsA on the Inhibition of OATP1B1-Mediated Uptake of \[^{3}H\]E_{2}G, \[^{3}H\]E_{1}S, \[^{3}H\]BSP, Pitavastatin, and Atorvastatin

Amundsen et al. (2010) and Shitara et al. (2013a) reported that the inhibitory effect of CsA on OATP1B1 was potentiated by pre-incubating OATP1B1-expressing cells with CsA before co-incubation with a substrate and CsA, but whether the effect depends on the substrate selected remained to be clarified. To address this, the OATP1B1-mediated uptake of \[^{3}H\]E_{2}G (0.1 \mu M), \[^{3}H\]E_{1}S (0.01 \mu M), \[^{3}H\]BSP (0.01 \mu M), pitavastatin (0.1 \mu M), and atorvastatin (0.1 \mu M) was examined in the presence of CsA after 1-hour pre-incubation with or without CsA. For comparison, co-incubation of CsA and each of the substrates without the 1-hour pre-
incubation process was also conducted as no pre-incubation condition.

IC\textsubscript{50} values of CsA for the 5 substrates after 1-hour pre-incubation without CsA were comparable to or slightly lower than those under a reference, no pre-incubation condition (Fig. 4 and Table 4). One-hour pre-incubation with CsA enhanced its inhibitory effect on OATP1B1 compared to 1-hour pre-incubation without CsA (Fig. 4), and the IC\textsubscript{50} values of CsA for E\textsubscript{2G}, E\textsubscript{1S}, BSP, pitavastatin, and atorvastatin uptake decreased by 3.29-, 5.08-, 3.15-, 3.88-, and 4.32-fold, respectively (Table 4). The OATP1B1 inhibitory effect potentiated by pre-incubation with CsA resulted in higher R-values for the substrates examined (Table 4).
Discussion

We previously reported that in vitro inhibitory effects of several OATP1B1 inhibitors showed remarkable substrate-dependence using prototypical substrates, E2G, E1S, and BSP (Izumi et al., 2013). In addition to the prototypical substrates, clinically-used OATP1B1 substrate drugs could also serve as in vitro OATP1B1 probe substrates, for which the potential substrate-dependent inhibition has not been comprehensively evaluated. In order to identify representative in vitro OATP1B1 probe substrates that could mitigate the risk of false-negative DDI prediction, this study investigated the impact of in vitro substrate selection on OATP1B1 inhibition and the subsequent DDI prediction for 12 clinically-used OATP1B1 substrate drugs comparing with the prototypical probe substrates.

Twelve OATP1B1 substrate drugs, including statins (pitavastatin, atorvastatin, fluvastatin, rosuvastatin, and pravastatin), antidiabetics (repaglinide, nateglinide, and glibenclamide), a dual endothelin receptor antagonist (bosentan), an angiotensin II receptor blocker (valsartan), a loop diuretic (torasemide), and a histamine H1 receptor antagonist (fexofenadine), were selected in this study because accumulated evidence suggests the involvement of OATP1B1 in their hepatic uptake in vivo. As expected, all drugs were confirmed to be substrates of OATP1B1 (Fig. 1), and their OATP1B1-mediated uptake showed saturation kinetics (Fig. 2). Although the majority of the obtained Km values were within the range of reported values (Table 1), the Km value of bosentan (4.27 µM) was, for unknown reasons, approximately 10-fold lower than the reported value (44 µM) (Treiber et al., 2007).

In order to investigate substrate-dependence in the inhibitory potency against OATP1B1, in vitro inhibitory effects of CsA, rifampin, and gemfibrozil on the OATP1B1-mediated uptake of the clinically-used substrate drugs were examined (Fig. 3) and compared with those on E2G, E1S, and BSP uptake (Table 2). CsA, rifampin, and gemfibrozil were used as the strong (CsA and rifampin) or weak (gemfibrozil) OATP1B1 inhibitors in this study because they showed substrate-dependent OATP1B1 inhibition with 6- to 14-fold difference in the Ki values when
E2G, E1S, and BSP were used as in vitro test probes (Izumi et al., 2013), and administration of
the inhibitors has caused clinical DDIs with OATP1B1 substrate drugs (Shitara et al., 2013b).
In fact, inhibition of the hepatic uptake by rifampin was demonstrated in healthy subjects by
positron emission tomography using 15R-[11C]TIC as an OATP substrate (Takashima et al.,
2012). The K_i values of CsA, rifampin, and gemfibrozil for the clinically-used substrate drug
uptake showed 6.3-, 3.4-, and 26-fold variations depending on the substrates, respectively
(Table 2). This large variation was mainly attributable to the lower inhibitory effect of CsA on
torasemide uptake and that of gemfibrozil on nateglinide uptake. Except for these two cases,
the variation decreased to 3.9- and 7.5-fold for CsA and gemfibrozil, respectively. Notably, the
K_i values for the clinically-used substrate drugs, except for the two cases, were within 2.8-fold
of those given by E2G as a substrate. In contrast, E1S and BSP yielded higher K_i values of all 3
inhibitors compared to the clinically-used substrate drugs. Therefore, E2G behaved similarly to
the clinically-used substrate drugs in terms of the susceptibility to OATP1B1 inhibition in
general.

To evaluate the impact of substrate selection on OATP1B1-mediated DDI prediction, R-
values of CsA, rifampin, and gemfibrozil were calculated based on a static model using the K_i
values determined for each substrate (Table 3). According to the regulatory DDI guidelines and
draft guidance materials, R-values were calculated using C_max (U.S. FDA) or [I]_inlet,max (U.S.
FDA, EMA, and MHLW) as the [I] and compared with the corresponding cut-off values. CsA is
a clinically-relevant inhibitor of OATPs, as well as CYP3A and other transporters in the liver
and intestine (e.g., BCRP and P-gp) (Yoshida et al., 2012), and showed the observed AUCRs for
OATP1B1 substrate drugs ranging from 2.0 (bosentan) to 23 (pravastatin). Although R-values
of CsA showed some variations depending on the K_i values determined with various substrates,
the variation had no impact on the DDI prediction for CsA, and CsA was classified as a
potential in vivo OATP1B1 inhibitor regardless of substrates due to its strong inhibitory potency
against OATP1B1 (Table 3). A single dose of rifampin is also used as a clinical OATP inhibitor
(Maeda et al., 2011) with the observed AUCRs for OATP1B1 substrate drugs ranging from 2.2 (glibenclamide) to 12 (atorvastatin). As in the case of CsA, rifampin was classified as a potential in vivo inhibitor regardless of substrates due to its strong inhibitory potency against OATP1B1 (Table 3). Gemfibrozil is a weak OATP1B1 inhibitor, and the observed AUCRs of statins and nateglinide ranged from 1.1 (fluvastatin) to 2.0 (pravastatin). Although observed AUCRs of repaglinide were 7.0 to 8.2, this is thought to be mainly attributable to the mechanism-based inhibition of CYP2C8-mediated metabolism of repaglinide by gemfibrozil-1-O-glucuronide (Tornio et al., 2008). R-values of gemfibrozil calculated with the $C_{\text{max}}$ showed some variations, but were greater than the cut-off value (1.1) regardless of substrates. However, R-values calculated with $[I]_{\text{u, inlet, max}}$ suggested that substrate selection as well as regulatory standards applied could affect the decision as to need for the clinical DDI studies. Indeed, gemfibrozil was considered as a potential in vivo OATP1B1 inhibitor when E2G and several clinically-used substrate drugs (e.g., pravastatin, valsartan) were used as in vitro probes under the regulations in EU ($R \geq 1.04$), or pravastatin was used under the regulations in U.S. and Japan ($R \geq 1.25$) (Table 3). Therefore, care should be exercised in in vitro OATP1B1 probe substrate selection particularly for weak-to-moderate inhibitors, because decision as to need for the clinical DDI studies is more susceptible to substrate-dependent $K_i$ variation. Based on the susceptibility to OATP1B1 inhibition in vitro (Table 2), clinically-used substrate drugs except for torasemide and nateglinide, or E2G as a surrogate could be used as an in vitro probe to mitigate the risk of the false-negative prediction.

Although the static model is widely used for DDI prediction due to its simplicity, we need to understand the limitation. In vivo DDIs can involve multiple elimination pathways of a victim drug, but the R-value calculation based on the regulatory guidelines or draft guidance materials considers only a single pathway (e.g., 100% contribution of OATP1B1 to the overall hepatic uptake of drugs in this study). In addition, not only an unchanged drug, but also its metabolite(s) could serve as an inhibitor in vivo, but our R-value calculation considers only the
inhibitory effects of unchanged drugs on OATP1B1. Regarding clinical DDIs involving gemfibrozil, it is suggested that gemfibrozil-1-O-glucuronide as well as unchanged gemfibrozil has an inhibitory effect on OATP1B1 and partly contributes to the clinical DDIs (Shitara et al., 2004). Indeed, Yoshida et al. (2012) demonstrated that a static model with $[I]_{u,inlet,max}$ provided the R-values greater than the cut-off values ($R \geq 1.25$) for clinical DDIs between gemfibrozil and statins (e.g., atorvastatin, fluvastatin, pitavastatin, pravastatin, and rosuvastatin) by considering both gemfibrozil and its glucuronide as OATP1B1/1B3 inhibitors. Whereas, R-values considering $[I]_{u,inlet,max}$ of only unchanged gemfibrozil resulted in less than 1.25 for all substrates except for pravastatin in this study (Table 3). Since quantitative contribution of gemfibrozil and its glucuronide to the clinical DDIs with OATP1B1 substrate drugs remains to be fully elucidated, we can not judge whether R-values of unchanged gemfibrozil less than the cut-off value in this study should be considered as false negatives or true negatives. However, this may highlight the importance of considering not only unchanged drug, but also its metabolite(s) as an inhibitor in DDI prediction with a static model when the metabolite(s) has a potential to inhibit OATP1B1.

Amundsen et al. (2010) and Shitara et al. (2013a) reported that the inhibitory effect of CsA on OATP1B1 was potentiated by pre-incubating OATP1B1-expressing cells with CsA before co-incubation of a probe substrate and CsA. As reported previously, the inhibitory effect of CsA was 3- to 5-fold potentiated by the 1-hour pre-incubation in this study, resulting in higher R-values (Table 4). However, remarkable substrate-dependence was not observed in the degree of the potentiation. Therefore, even if inhibitory effect of an inhibitor on OATP1B1 is potentiated after the pre-incubation, E2G or clinically-used substrate drugs such as pitavastatin and atorvastatin could sensitively detect the inhibition potential as in the case of no pre-incubation condition. However, this effect has been observed only for a limited number of inhibitors (CsA, saquinavir, and ritonavir) (Shitara et al., 2013a), and it remains to be elucidated whether lower $K_i$ values obtained by inhibitor pre-incubation is clinically relevant or not.
Further evaluation will be needed for OATP1B1 inhibitors in the light of the pre-incubation effect to see if this type of experiment is useful to further mitigate the risk of false-negative DDI predictions.

This study demonstrated that E₂G and clinically-used OATP1B1 substrate drugs except for torasemide and nateglinide behaved similarly in terms of the susceptibility to the OATP1B1 inhibition by CsA, rifampin, and gemfibrozil. Therefore, it is recommended that the clinically-used OATP1B1 substrate drugs or E₂G as a surrogate should be used as an in vitro probe for OATP1B1 inhibition assays, which will help mitigate the risk of false-negative DDI prediction potentially caused by substrate-dependent Kᵢ variation. These findings will contribute to the establishment of optimal in vitro evaluation systems for clinically-relevant OATP1B1-mediated DDIs.
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Authorship Contributions

*Participated in research design:* Izumi, Nozaki, Komori, Maeda, Takenaka, and Sugiyama.

*Conducted experiments:* Izumi.

*Performed data analysis:* Izumi, Nozaki, Komori, and Maeda.

*Wrote or contributed to the writing of the manuscript:* Izumi, Nozaki, Komori, Maeda, Takenaka, Kusuhara, and Sugiyama.
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Footnotes

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Legends for Figures

**Fig. 1.** Time profiles of the uptake of clinically-used OATP1B1 substrate drugs in OATP1B1-expressing cells and the control cells. Uptake of (A) pitavastatin (0.1 µM), (B) atorvastatin (0.1 µM), (C) fluvastatin (1 µM), (D) rosuvastatin (1 µM), (E) pravastatin (10 µM), (F) repaglinide (0.1 µM), (G) nateglinide (1 µM), (H) glibenclamide (0.1 µM), (I) bosentan (0.1 µM), (J) valsartan (1 µM), (K) torasemide (1 µM), and (L) fexofenadine (1 µM) in OATP1B1-expressing cells (closed circles) and the control cells (open circles) was examined over a period of 2 (rosuvastatin, pravastatin, glibenclamide, and bosentan), 10 (pitavastatin, atorvastatin, fluvastatin, nateglinide, valsartan, and torasemide), or 30 minutes (repaglinide and fexofenadine) at 37 °C. Each point represents the mean ± S.D. (n = 3).

**Fig. 2.** Concentration dependence of the OATP1B1-mediated uptake of (A) pitavastatin (0.01-100 µM), (B) atorvastatin (0.01-10 µM), (C) fluvastatin (0.03-30 µM), (D) rosuvastatin (0.1-100 µM), (E) pravastatin (1-100 µM), (F) repaglinide (0.01-100 µM), (G) nateglinide (0.1-100 µM), (H) glibenclamide (0.01-10 µM), (I) bosentan (0.03-10 µM), (J) valsartan (0.3-1000 µM), (K) torasemide (0.1-100 µM), and (L) fexofenadine (0.1-1000 µM). The uptake was determined for 0.5 (rosuvastatin, pravastatin, glibenclamide, bosentan, and torasemide), 1 (atorvastatin), 2 (pitavastatin and nateglinide), 5 (fluvastatin, repaglinide, and valsartan), or 10 minutes (fexofenadine) at 37 °C. Representative data from 2 to 4 independent experiments are shown as Eadie-Hofstee plots, and each point represents the mean ± S.D. (n = 3). Fitted lines that were obtained by a nonlinear least-squares regression analysis based on Eq. 1 or 2 are also presented as solid lines.

**Fig. 3.** Inhibitory effects of CsA, rifampin, and gemfibrozil on OATP1B1-mediated uptake of clinically-used substrate drugs. Uptake of (A) pitavastatin (0.1 µM), (B) atorvastatin (0.1 µM), (C) fluvastatin (1 µM), (D) rosuvastatin (1 µM), (E) pravastatin (10 µM), (F) repaglinide (0.1 µM), (G) nateglinide (1 µM), (H) glibenclamide (0.1 µM), (I) bosentan (0.1 µM), (J) valsartan (1 µM), (K) torasemide (1 µM), and (L) fexofenadine (1 µM) in OATP1B1-expressing cells (closed circles) and the control cells (open circles) was examined over a period of 2 (rosuvastatin, pravastatin, glibenclamide, and bosentan), 10 (pitavastatin, atorvastatin, fluvastatin, nateglinide, valsartan, and torasemide), or 30 minutes (repaglinide and fexofenadine) at 37 °C. Each point represents the mean ± S.D. (n = 3).
µM), (G) nateglinide (1 µM), (H) glibenclamide (0.1 µM), (I) bosentan (0.1 µM), (J) valsartan (1 µM), (K) torasemide (1 µM), and (L) fexofenadine (1 µM) were investigated for 0.5 (rosuvastatin, pravastatin, glibenclamide, bosentan, and torasemide), 1 (atorvastatin), 2 (pitavastatin and nateglinide), 5 (fluvastatin, repaglinide, and valsartan), or 10 minutes (fexofenadine) at 37 °C in the presence and absence of CsA (closed circles), rifampin (open triangles), or gemfibrozil (open squares). The data are shown as percent of control. Thick, thin, and dashed lines represent fitted lines for the inhibition by CsA, rifampin, and gemfibrozil, respectively, which were obtained by a nonlinear least-squares regression analysis based on Eq. 3, and the estimated IC₅₀ values were summarized in supplemental table 2. Each point represents the mean ± S.D. (n = 6 or 9).

Fig. 4. Effect of pre-incubation with CsA on the inhibition of OATP1B1-mediated uptake of (A) [³H]E₂G (0.1 µM), (B) [³H]E₁S (0.01 µM), (C) [³H]BSP (0.01 µM), (D) pitavastatin (0.1 µM), and (E) atorvastatin (0.1 µM). Cells were co-incubated with a substrate and CsA for 1 ([³H]E₁S and atorvastatin), 2 (pitavastatin), or 5 minutes ([³H]E₂G and [³H]BSP) after 1-hour pre-incubation with (open squares) or without (open triangles) CsA. As a reference, cells were co-incubated with a substrate and CsA without the 1-hour pre-incubation step (closed circles). Representative data from 3 independent experiments are shown as percent of control. Thick, thin, and dashed lines represent fitted lines for co-incubation of a substrate and CsA without pre-incubation, co-incubation after 1-hour pre-incubation without CsA, and co-incubation after 1-hour pre-incubation with CsA, respectively that were obtained by a nonlinear least-squares regression analysis based on Eq. 3. Each point represents the mean ± S.D. (n = 3).
Table 1. Saturation kinetics of OATP1B1-mediated uptake of clinically-used OATP1B1 substrate drugs

Kinetic parameters were estimated by nonlinear least-squares regression analysis based on the Eq. 1 or 2 as described under Materials and Methods, and are shown as mean of 2 independent experiments or mean ± S.D. (n = 3 or 4).

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<th>Substrates</th>
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<th>$P_{diff}$</th>
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<td>Pitavastatin</td>
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<td>0.62&lt;sup&gt;b&lt;/sup&gt;, 0.77&lt;sup&gt;e&lt;/sup&gt;, 0.93&lt;sup&gt;f&lt;/sup&gt;, 12&lt;sup&gt;g&lt;/sup&gt;, 19&lt;sup&gt;h&lt;/sup&gt;</td>
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<td>Fluvastatin&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.80 (4.98, 4.63)</td>
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<td>2.5&lt;sup&gt;i&lt;/sup&gt;, 12&lt;sup&gt;j&lt;/sup&gt;</td>
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<td>Rosuvastatin&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.31 (9.15, 9.48)</td>
<td>103 (90.6, 116)</td>
<td>0.624 (0.588, 0.660)</td>
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<td>Pravastatin</td>
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<td>Glibenclamide</td>
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<td>Bosentan&lt;sup&gt;o&lt;/sup&gt;</td>
<td>4.27 (4.98, 3.55)</td>
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<td>44&lt;sup&gt;p&lt;/sup&gt;</td>
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-, not reported.

NA, not applicable.

<sup>a</sup>Kinetic parameters are presented as mean with individual values in parenthesis from 2 independent experiments.

<sup>b</sup>Sharma et al. (2012); <sup>c</sup>Hirano et al. (2004); <sup>d</sup>Soars et al. (2012); <sup>e</sup>Karlgren et al. (2012); <sup>f</sup>Amundsen et al. (2010); <sup>g</sup>Kameyama et al. (2005); <sup>h</sup>Lau et al. (2007); <sup>i</sup>Noé et al. (2007); <sup>j</sup>Kitamura et al. (2008); <sup>k</sup>Ho et al. (2006); <sup>l</sup>van de Steeg et al. (2013); <sup>m</sup>Shen et al. (2013); <sup>n</sup>Hsiang et al. (1999); <sup>o</sup>Treiber et al. (2007); <sup>p</sup>Yamashiro et al. (2006); <sup>q</sup>Poirier et al. (2009); <sup>r</sup>Werner et al. (2010).
Table 2. $K_i$ values of CsA, rifampin, and gemfibrozil for OATP1B1-mediated uptake of clinically-used OATP1B1 substrate drugs

OATP1B1-mediated uptake of 12 clinically-used OATP1B1 substrate drugs was examined in the presence and absence of CsA, rifampin, or gemfibrozil as shown in Fig. 3. The IC$_{50}$ values were estimated by a nonlinear least-squares regression analysis. The $K_i$ values (parameter estimate ± parameter S.D.) were estimated using substrate concentrations, $K_m$, and IC$_{50}$ values by Eq. 4 (n = 6 or 9). $K_i$ values of CsA, rifampin, and gemfibrozil for the OATP1B1-mediated uptake of prototypical substrates (E$_2$G, E$_1$S, and BSP) were cited from our previous study (Izumi et al., 2013).

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Inhibitors</th>
<th>$K_i$, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CsA$^a$</td>
<td>rifampin</td>
</tr>
<tr>
<td>In vitro prototypical probe substrates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E$_2$G$^b$</td>
<td>0.118 ± 0.015</td>
<td>0.585 ± 0.074</td>
</tr>
<tr>
<td>E$_1$S$^b$</td>
<td>0.732 ± 0.224</td>
<td>6.96 ± 1.31</td>
</tr>
<tr>
<td>BSP$^b$</td>
<td>0.694 ± 0.149</td>
<td>2.75 ± 0.62</td>
</tr>
<tr>
<td>Clinically-used substrate drugs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>0.228 ± 0.027</td>
<td>1.07 ± 0.08</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>0.160 ± 0.016</td>
<td>0.922 ± 0.122</td>
</tr>
<tr>
<td>Fluvastatin</td>
<td>0.157 ± 0.016</td>
<td>1.05 ± 0.19</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>0.301 ± 0.031</td>
<td>0.952 ± 0.098</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>0.184 ± 0.046</td>
<td>0.653 ± 0.117</td>
</tr>
<tr>
<td>Repaglinide</td>
<td>0.0857 ± 0.0330</td>
<td>0.598 ± 0.198</td>
</tr>
<tr>
<td>Nateglinide</td>
<td>0.244 ± 0.038</td>
<td>0.358 ± 0.079</td>
</tr>
<tr>
<td>Glibenclamide</td>
<td>0.102 ± 0.005</td>
<td>0.442 ± 0.102</td>
</tr>
<tr>
<td>Bosentan</td>
<td>0.206 ± 0.056</td>
<td>0.694 ± 0.211</td>
</tr>
<tr>
<td>Valsartan</td>
<td>0.138 ± 0.017</td>
<td>0.377 ± 0.022</td>
</tr>
<tr>
<td>Torasemide</td>
<td>0.486 ± 0.112</td>
<td>1.23 ± 0.30</td>
</tr>
<tr>
<td>Fexofenadine</td>
<td>0.0771 ± 0.0100</td>
<td>0.423 ± 0.032</td>
</tr>
</tbody>
</table>

$^a$ $K_i$ values of CsA listed in the table were obtained by co-incubation of a substrate and CsA without CsA pre-incubation.

$^b$Izumi et al., 2013
Table 3  Prediction of OATP1B1-mediated DDIs with a static model

R-values of CsA, rifampin, and gemfibrozil were determined using Ki values obtained from each probe substrate and [I] (C_max or [I]_{u,inlet,max}) based on Eqs. 5 and 6. The Ki values were taken from Table 2. C_max and [I]_{u,inlet,max} of the inhibitors are given in supplemental Table 1.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Ci</th>
<th>Observed AUCR^a</th>
<th>Inhibitors</th>
<th>R-value (= 1+[I]/Ki)</th>
<th>Observed AUCR^b</th>
<th>Inhibitors</th>
<th>R-value (= 1+[I]/Ki)</th>
<th>Observed AUCR^c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(µM)</td>
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<td></td>
</tr>
<tr>
<td>In vitro prototypical probe substrates</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2G</td>
<td>0.118</td>
<td>9.05 (4.05–13.7)</td>
<td>11.2 (4.81-17.1)</td>
<td>NA</td>
<td>0.585</td>
<td>40.3</td>
<td>18.1</td>
<td>NA</td>
</tr>
<tr>
<td>E1S</td>
<td>0.732</td>
<td>2.30 (1.49-3.05)</td>
<td>2.64 (1.61-3.60)</td>
<td>NA</td>
<td>6.96</td>
<td>4.30</td>
<td>2.44</td>
<td>NA</td>
</tr>
<tr>
<td>BSP</td>
<td>0.694</td>
<td>2.37 (1.52-3.16)</td>
<td>2.73 (1.65-3.74)</td>
<td>NA</td>
<td>2.75</td>
<td>9.36</td>
<td>4.64</td>
<td>NA</td>
</tr>
<tr>
<td>Clinically-used substrate drugs</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>0.228</td>
<td>5.17 (2.58-7.58)</td>
<td>6.26 (2.97-9.33)</td>
<td>4.6^d</td>
<td>1.07</td>
<td>22.5</td>
<td>10.3</td>
<td>5.7^e</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>0.160</td>
<td>6.94 (3.5-10.4)</td>
<td>8.50 (3.81-12.9)</td>
<td>9.0^d, 15^d</td>
<td>0.922</td>
<td>25.9</td>
<td>11.8</td>
<td>7.3^d, 8.5^d, 12^d</td>
</tr>
<tr>
<td>Fluvastatin</td>
<td>0.157</td>
<td>7.05 (3.29-10.6)</td>
<td>8.64 (3.87-13.1)</td>
<td>3.5^d</td>
<td>1.05</td>
<td>22.9</td>
<td>10.5</td>
<td>-</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>0.301</td>
<td>4.16 (2.20-5.98)</td>
<td>4.99 (2.50-7.31)</td>
<td>7.1^d</td>
<td>0.952</td>
<td>25.2</td>
<td>11.5</td>
<td>4.4^d</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>0.184</td>
<td>6.16 (2.96-9.15)</td>
<td>7.52 (3.45-11.3)</td>
<td>12^d, 23^d</td>
<td>0.653</td>
<td>36.2</td>
<td>16.3</td>
<td>2.6^d, 4.6^d</td>
</tr>
<tr>
<td>Repaglinide</td>
<td>0.0857</td>
<td>12.1 (5.20-18.5)</td>
<td>15.0 (6.25-23.2)</td>
<td>2.4^d</td>
<td>0.598</td>
<td>39.5</td>
<td>17.7</td>
<td>-</td>
</tr>
<tr>
<td>Nateglinide</td>
<td>0.244</td>
<td>4.89 (2.48-7.15)</td>
<td>5.92 (2.84-8.79)</td>
<td>-</td>
<td>0.358</td>
<td>65.2</td>
<td>28.9</td>
<td>-</td>
</tr>
<tr>
<td>Glibenclamide</td>
<td>0.102</td>
<td>10.3 (4.53-15.7)</td>
<td>12.8 (5.41-19.6)</td>
<td>-</td>
<td>0.442</td>
<td>53.0</td>
<td>23.6</td>
<td>2.2^d, 2.2^d</td>
</tr>
<tr>
<td>Bosentan</td>
<td>0.206</td>
<td>5.61 (2.75-8.28)</td>
<td>6.83 (3.18-10.2)</td>
<td>2.0^f</td>
<td>0.694</td>
<td>34.1</td>
<td>15.4</td>
<td>5^d, 5^d</td>
</tr>
<tr>
<td>Valsartan</td>
<td>0.138</td>
<td>7.88 (3.61-11.9)</td>
<td>9.70 (4.26-14.8)</td>
<td>-</td>
<td>0.377</td>
<td>62.0</td>
<td>27.5</td>
<td>-</td>
</tr>
<tr>
<td>Torasemide</td>
<td>0.486</td>
<td>2.95 (1.74-4.09)</td>
<td>3.47 (1.93-4.91)</td>
<td>-</td>
<td>1.23</td>
<td>19.7</td>
<td>9.13</td>
<td>-</td>
</tr>
<tr>
<td>Fexofenadine</td>
<td>0.0771</td>
<td>13.3 (5.67-20.5)</td>
<td>16.6 (6.84-25.6)</td>
<td>-</td>
<td>0.423</td>
<td>55.4</td>
<td>24.6</td>
<td>3.9-4.6^d</td>
</tr>
</tbody>
</table>

NA: Not applicable.
- Not reported.

* Dose of CsA ranged from 75 to 322 mg in clinical DDI studies. R-value is presented as the representative value that was calculated based on 200-mg dose of CsA with range in parenthesis. R-value range that corresponds to the clinical dose range (75 to 322 mg) was calculated based on the parameters of CsA given in supplemental Table 1 assuming the linear pharmacokinetics.

b Dose of rifampin was 600 mg in clinical DDI studies and R-value calculation.

c Dose of gemfibrozil was 600 mg in clinical DDI studies and R-value calculation.

d Yoshida et al., 2012

e Simonson et al., 2004

f Binet et al., 2000

Prueksaritanont et al., 2014

van Giersbergen et al., 2007

Kusuhara et al., 2013

The inhibitor was given as a single intravenous dose.

k Fold increase in the trough concentration on Day 2.
Table 4 Effect of pre-incubation with CsA on the inhibition of OATP1B1-mediated uptake of $[^3H]$E$_2$G, $[^3H]$E$_1$S, $[^3H]$BSP, pitavastatin, and atorvastatin

Inhibitory effect of CsA on OATP1B1-mediated uptake of $[^3H]$E$_2$G (0.1 µM), $[^3H]$E$_1$S (0.01 µM), $[^3H]$BSP (0.01 µM), pitavastatin (0.1 µM), and atorvastatin (0.1 µM) was examined in the absence and presence of 1-hour pre-incubation with or without CsA as shown in Fig. 4. IC$_{50}$ values were estimated by a nonlinear least-squares regression analysis (Mean ± S.D., n=3). R-values of CsA were calculated by Eqs. 5 and 6 with the exception that IC$_{50}$ values instead of K$_i$ values were used for the calculations because applicability of Eq. 4 to pre-incubation-dependent inhibition by CsA remains to be established.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>No pre-incubation$^a$</th>
<th>After 1-hour pre-incubation without CsA$^b$</th>
<th>After 1-hour pre-incubation with CsA$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC$_{50}$ of CsA (µM)</td>
<td>R-value ($= 1 + [I]/IC_{50}$)</td>
<td>IC$_{50}$ of CsA (µM)</td>
</tr>
<tr>
<td>E$_2$G</td>
<td>0.0620 ± 0.0142</td>
<td>16.3</td>
<td>20.4</td>
</tr>
<tr>
<td>E$_1$S</td>
<td>0.253 ± 0.088</td>
<td>4.75</td>
<td>5.74</td>
</tr>
<tr>
<td>BSP</td>
<td>0.283 ± 0.070</td>
<td>4.36</td>
<td>5.24</td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>0.144 ± 0.015</td>
<td>7.60</td>
<td>9.33</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>0.176 ± 0.018</td>
<td>6.40</td>
<td>7.82</td>
</tr>
</tbody>
</table>

$^a$Cells were co-incubated with a substrate and CsA (0.01 to 10 µM) for 1 ($[^3H]$E$_1$S and atorvastatin), 2 (pitavastatin), or 5 minutes ($[^3H]$E$_2$G and $[^3H]$BSP).

$^b$Cells were co-incubated with a substrate and CsA (0.01 to 10 µM) for 1 ($[^3H]$E$_1$S and atorvastatin), 2 (pitavastatin), or 5 minutes ($[^3H]$E$_2$G and $[^3H]$BSP) after 1-hour pre-incubation with KH buffer without CsA.
cCells were co-incubated with a substrate and CsA (0.001 to 1 µM) for 1 ([3H]E1S and atorvastatin), 2 (pitavastatin), or 5 minutes ([3H]E2G and [3H]BSP) after 1-hour pre-incubation with KH buffer containing CsA (0.001 to 1 µM).

dR-values that were calculated based on 200-mg dose of CsA were presented.
A. Pitavastatin

B. Atorvastatin

C. Fluvastatin

D. Rosuvastatin

E. Pravastatin

F. Repaglinide

G. Nateglinide

H. Glibenclamide

I. Bosentan

J. Valsartan

K. Torasemide

L. Fexofenadine

Fig1
Fig 2
Fig 4