Substrate-Dependent Inhibition of Organic Anion Transporting Polypeptide 1B1: Comparative Analysis with Prototypical Probe Substrates Estradiol-17β-Glucuronide, Estrone-3-Sulfate, and Sulfofobromophthalein

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ABSTRACT

Organic anion transporting polypeptide (OATP) 1B1 plays an important role in the hepatic uptake of many drugs, and the evaluation of OATP1B1-mediated drug-drug interactions (DDIs) is emphasized in the latest DDI (draft) guidance documents from U.S. and E.U. regulatory agencies. It has been suggested that some OATP1B1 inhibitors show a discrepancy in their inhibitory potential, depending on the substrates used in the cell-based assay. In this study, inhibitory effects of 14 compounds on the OATP1B1-mediated uptake of the prototypical substrates [3H]estradiol-17β-glucuronide (E2G), [3H]estrone-3-sulfate (E1S), and [3H]sulfobromophthalein (BSP) were studied in OATP1B1-transfected cells. Inhibitory potencies of tested compounds varied depending on the substrates. Ritonavir, gemfibrozil, and erythromycin caused remarkable substrate-dependent inhibition with up to 117-, 14-, and 13-fold difference in their IC50 values, respectively. Also, the clinically relevant OATP inhibitors rifampin and cyclosporin A exhibited up to 12- and 6-fold variation in their IC50 values, respectively. Regardless of the inhibitors tested, the most potent OATP1B1 inhibition was observed when [3H]E2G was used as a substrate. Mutual inhibition studies of OATP1B1 indicated that E2G and E1S competitively inhibited each other, whereas BSP noncompetitively inhibited E2G uptake. In addition, BSP inhibited E1S in a competitive manner, but E1S caused an atypical kinetics on BSP uptake. This study showed substrate-dependent inhibition of OATP1B1 and demonstrated that E2G was the most sensitive in vitro OATP1B1 probe substrate among three substrates tested. This will give us an insight into the assessment of clinically relevant OATP1B1-mediated DDI in vitro with minimum potential of false-negative prediction.

Introduction

Pharmacokinetic drug-drug interactions (DDIs) involve altered pharmacokinetics of a drug by a concomitant medication, and consequently affect pharmacologic efficacy and/or adverse effects. As DDIs can cause life-threatening adverse events, particularly when the therapeutic range of a substrate drug is narrow, evaluation of the DDI potential of new chemical entities is an integral part of drug development from the viewpoint of clinical safety. During this decade, research on membrane transporters has also achieved significant progress and revealed a major, clinically important role of membrane transporters in the pharmacokinetics of a broad range of drugs (Mizuno et al., 2003). Along with this, inhibition of membrane transporters has been recognized as one of the underlying mechanisms of pharmacokinetic DDIs (Shitara et al., 2005). In 2010, International Transporter Consortium published a so-called “transporter white paper,” in which a systematic approach to transporter-mediated DDIs during drug development was proposed (Giacomini et al., 2010). Furthermore, the importance of the evaluation of transporter-mediated DDI potential of an investigational drug has been emphasized in the latest DDI draft guidance materials and guidelines released by the U.S. Food and Drug Administration and the European Medicines Agency, respectively (Center for Drug Evaluation and Research, 2012; Committee for Human Medicinal Products, 2012).

Potential risk evaluation of DDIs involving transporters as well as drug-metabolizing enzymes at nonclinical levels has become a crucial task for scientists in pharmaceutical industries as they seek to prioritize and develop drug candidates. Two models have been proposed for quantitative prediction of transporter-mediated DDIs: a static model and a dynamic (physiologically-based pharmacokinetic) model. The static model assumes a constant concentration of inhibitors (Hirano et al., 2006; Sharma et al., 2012), whereas the time-course of inhibitor concentration is considered in the physiologically-based pharmacokinetic model.
results suggested the usefulness of E2G as a most likely sensitive in vitro substrates on OATP1B1, mutual inhibition studies were performed. Our commercially available.

IL) and used with purification. All other chemicals were of analytical grade and Paul, MN), and ritonavir was obtained from Abbott Laboratories (Abbott Park, Osaka, Japan). Tacrolimus was purchased from LKT Laboratories, Inc., (St.

doing the hepatic uptake of a wide range of drugs, including 3-hydroxyethylglutaryl-CoA reductase inhibitors (statins), angiotensin II receptor blockers, and endothelin receptor antagonists (Abe et al., 1999; Hsiang et al., 1999; Katz et al., 2006; Yamashiro et al., 2006; van Giersbergen et al., 2007; Yamada et al., 2007; Sharma et al., 2012). It has been reported that genetic polymorphisms of OATP1B1 are associated with interindividual variation in plasma concentration of various statins (Shitara and Sugiyama, 2006; Niemi et al., 2011) and adverse event rates of statin therapy (Link et al., 2008). Meanwhile, clinically relevant DDDS of OATP1B1 substrate drugs (e.g., statins) with cyclosporin A, a single dose of rifampin, and other OATP inhibitors are thought to be attributable to OATP1B1 inhibition (Shitara et al., 2003; Maeda et al., 2011; Niemi et al., 2011). These cumulative data suggest the clinical importance of OATP1B1 in the drug disposition, and consequently OATP1B1 has been recognized as an important transporter in the International Transporter Consortium white paper and regulatory DDIs guidance and guidelines (Giacomini et al., 2010; Center for Drug Evaluation and Research, 2012; Committee for Human Medicinal Products, 2012).

When evaluating inhibitory potency of compounds of interest on OATP1B1, in vitro assay with OATP1B1-transfected cells using an appropriate probe substrate has been widely conducted. Meanwhile, Noé et al. (2007) reported that inhibitory potency of gemfibrozil on OATP1B1 depends on the substrates employed in their in vitro studies. This suggests that inhibition of OATP1B1 can be over- or under-estimated depending on the substrates selected, potentially resulting in false-negative prediction of OATP1B1-mediated DDIs in the worst-case scenario. Thus, we should pay much attention to the OATP1B1 probe substrate for in vitro inhibition assays, as in the case of CYP3A4 (Kenworthy et al., 1999; Obach et al., 2006), OATP2B1 (Shirasaka et al., 2012), and OCT2 (Zolk et al., 2009). However, limited information is available on the substrate-dependent inhibition of OATP1B1.

Therefore, this study compared the inhibition profiles of 14 compounds for OATP1B1 using prototypical OATP1B1 probe substrates: estraadiol-17β-glucuronide (E2G), estrone-3-sulfate (E1S), and sulfobromophthalein (BSP) that have been widely used for in vitro studies. Furthermore, to provide insight into the binding sites of the three substrates on OATP1B1, mutual inhibition studies were performed. Our results suggested the usefulness of E2G as a most likely sensitive in vitro OATP1B1 probe substrate.

Materials and Methods

Materials

[3H]Estradiol-17β-glucuronide (50.3 Ci/mmmol) and [3H]estrone-3-sulfate (54.3 Ci/mmmol) were purchased from PerkinElmer Life Sciences (Boston, MA). [3H]Sulfobromophthalein (5.5 Ci/mmmol) was synthesized by Hartmann Analytic GmbH (Braunschweig, Germany). Unlabeled E2G, E1S, BSP, rifampin, taurocholate (TCA), ketocazole, gemfibrozil, verapamil, and probenecid were purchased from Sigma-Aldrich (St. Louis, MO). Cyclosporin A (CSA), erythromycin, and cimetidine were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Taurocholic was purchased from LKT Laboratories, Inc. (St. Paul, MN), and ritonavir was obtained from Abbott Laboratories (Abbott Park, IL) and used with purification. All other chemicals were of analytical grade and commercially available.

Uptake and Inhibition Studies Using Transfected Cells

The OATP1B1 (SLCO1B1) open reading frame was cloned into the Kpnl/ Xhol sites of expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA), and the inserted sequence was confirmed by DNA sequencing. The expression vector with or without OATP1B1 cDNA was transfected into human embryonic kidney 293 cells using lipofectamin 2000 (Invitrogen) according to the manufacturer’s protocol. The stably OATP1B1-transfected cells and their control cells were established and maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) 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% CO2 gas. The cells were trypsinized and uniformly suspended in the desired volume of the culture medium to provide 4 × 105 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 transport study was carried out as described previously (Hirano et al., 2004). Cell culture medium was replaced with prewarmed 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 preincubated for 5 minutes at 37°C. The preincubation buffer was aspirated, and the uptake reaction was initiated by addition of 250 μl of a prewarmed KH buffer containing a radiolabeled substrate with or without a test compound. For inhibition studies with CsA, BSP, ritonavir, rifampin, tacrolimus, E2G, and gemfibrozil, each of the dimethylsulfoxide (DMSO) original solutions was prepared, serially diluted with DMSO, and 1,000-fold diluted with KH buffer containing a radiolabeled substrate (final DMSO concentration 0.1%). KH buffer containing a radiolabeled substrate and 0.1% DMSO without any inhibitors was used as the solvent control. For E1S, erythromycin, TCA, probenecid, and cimetidine, each of the compounds was directly dissolved in KH buffer containing a radiolabeled substrate and serially diluted with KH buffer containing the radiolabeled substrate. For ketocazone and verapamil, each of the compounds was completely dissolved in KH buffer containing a radiolabeled substrate at acidic pH to prepare the original solution, which was serially diluted with KH buffer containing the radiolabeled substrate to prepare the working solutions. The pH of the working solutions was adjusted at 7.4 before the inhibition assays. KH buffer containing a radiolabeled substrate without any inhibitors was used as the control for inhibition studies with E1S, erythromycin, TCA, ketocazone, verapamil, probenecid, and cimetidine. The uptake was terminated at a designated time point by adding ice-cold KH buffer immediately after removal of the incubation buffer. The cells were washed twice with 1 ml of ice-cold KH buffer and lysed with 0.5 ml of 0.1 N NaOH overnight at room temperature.

The resulting cell lysate was neutralized with 50 μl of 1N HCl. A 400-μl sample of the aliquot was mixed with 4 ml of scintillation fluid (Hionic-Flour; PerkinElmer Life Sciences), and the radioactivities associated with the cells and incubation buffer were measured with a liquid scintillation counter (Tri-Carb 3100TR; PerkinElmer Life Sciences). Remaining neutralized cell lysate samples were used to quantify protein concentrations (BCA Protein Assay Kit; Thermo Fisher Scientific, Waltham, MA).

Determination of Kinetic Parameters

Uptake of a radiolabeled substrate was expressed as the uptake volume (μl/mg protein), which was given as the radioactivity associated with the cells (dpm/well) divided by the concentration in the incubation buffer (dpm/μl) and the protein amount (mg protein/well). OATP1B1-mediated uptake was obtained by subtracting the uptake into control cells from the uptake into OATP1B1-transfected cells.

Half maximal inhibitory concentration (IC50) of an inhibitor was estimated by examining the inhibitory effect on the uptake of a radiolabeled substrate (% of control) using the following equation:

\[
\text{uptake (of % control)} = \frac{CL_i}{CL} \times 100 = \frac{P}{(1 + \frac{IC50}{P})}
\]

where CL and CLi represent the uptake clearance in the absence and presence of an inhibitor, respectively, and I (μM) is the concentration of the inhibitor. P was set as a free parameter to achieve the best fit in the nonlinear iterative
least squares regression analysis. To the inhibitor which showed biphasic inhibition for OATP1B1, the following equation was applied:

\[
\text{uptake (\% of control)} = \frac{CL_1}{CL} \times \frac{P_1}{1 + (\frac{S}{IC_{50,1}})} + \frac{P_2}{1 + (\frac{S}{IC_{50,2}})}
\]  

(2)

where IC_{50,1} and IC_{50,2} represent IC_{50} values for high- and low-affinity components, respectively. P_i and P_2 are parameters for high- and low-affinity components, and P_i / (P_1 + P_2) and P_2 / (P_1 + P_2) represent the contribution of high- and low-affinity components, respectively. Substrate concentrations employed in this study were sufficiently lower than their K_m values, where IC_{50} theoretically approximates the K_i regardless of inhibition mechanisms, except in the case of uncompetitive inhibition (Ito et al., 1998). However, as some tested compounds showed not only inhibition, but also stimulation of a substrate uptake, the term IC_{50} was used throughout this study.

Concentration dependence of the uptake of a substrate mediated by OATP1B1 was analyzed using the following Michaelis-Menten equation:

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

(3)

where v, S, K_m, and V_{\text{max}} represent uptake velocity of the substrate (pmol/min/mg protein), Michaelis constant (\mu M), and the maximum uptake rate (pmol/min/mg protein), respectively. When nonsaturable component was observed, the following equation was used for the analysis:

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

(4)

where P_{\text{dif}} represents nonsaturable uptake clearance (\mu l/min/mg protein). Fitting was performed by a nonlinear least-squares regression method using the MULTI program.

**Statistical Analysis**

The data are presented as mean ± S.D. One-way analysis of variance followed by Dunnett’s post hoc test was used to identify significant differences between groups where appropriate. P < 0.05 was considered significant.

**Results**

**Uptake of Prototypical OATP1B1 Substrates by OATP1B1-Transfected Cells**

Time profiles of the uptake of three prototypical OATP1B1 substrates: [\textsuperscript{3}H]E2G, [\textsuperscript{3}H]E1S, and [\textsuperscript{3}H]BSP by OATP1B1-transfected cells and control cells are shown in Fig. 1. The OATP1B1-transfected cells showed higher transport activities for all three substrates compared with the control cells, and the uptake of [\textsuperscript{3}H]E2G and [\textsuperscript{3}H]BSP was linear over the first 10 minutes. Thus, an incubation time of 5 minutes was selected for [\textsuperscript{3}H]E2G uptake in OATP1B1-transfected cells, mean ± SD, 112 ± 2 \mu l/mg protein; uptake in control cells, 1.93 ± 0.14 \mu l/mg protein) and [\textsuperscript{3}H]BSP uptake (OATP1B1, 503 ± 52 \mu l/mg protein; control, 113 ± 11 \mu l/mg protein) in the following inhibition and concentration-dependent uptake studies. As shown in Fig. 1B, uptake of [\textsuperscript{3}H]E_{1S} reached steady-state at 10 minutes in OATP1B1-transfected cells. The uptake of [\textsuperscript{3}H]E_{2S} was determined at 1 minute, the practically earliest sampling point for subsequent analysis (OATP1B1, 132 ± 9 \mu l/mg protein; control, 3.97 ± 0.78 \mu l/mg protein).

**Inhibitory Effects of 14 Compounds on the OATP1B1-Mediated Uptake of [\textsuperscript{3}H]E2G, [\textsuperscript{3}H]E1S, and [\textsuperscript{3}H]BSP**

Inhibitory effects of unlabeled E1S, CsA, BSP, ritonavir, rifampin, tacrolimus, erythromycin, E2G, TCA, ketoconazole, gemfibrozil, verapamil, probenecid, and cimetidine on the OATP1B1-mediated uptake of [\textsuperscript{3}H]E2G, [\textsuperscript{3}H]E1S, and [\textsuperscript{3}H]BSP were investigated (Fig. 2). All tested compounds except for cimetidine inhibited uptake of [\textsuperscript{3}H]E2G, [\textsuperscript{3}H]E1S, and [\textsuperscript{3}H]BSP in a concentration-dependent manner. Although almost all the inhibition curves were well fitted to the equation for monophasic inhibition (Equation 1), the inhibition of [\textsuperscript{3}H]BSP uptake by unlabeled E2S was well described as a biphasic inhibition (Equation 2), with IC_{50} values of 0.429 \mu M and 611 \mu M.

Several inhibitors showed a biphasic effect on OATP1B1-mediated uptake; stimulation at low concentrations followed by inhibition along with increasing inhibitor concentrations. Only lower concentrations of E1S, CsA, ritonavir, rifampin, E2G, TCA, ketoconazole, verapamil, and probenecid caused a significant stimulation of [\textsuperscript{3}H]BSP uptake, while cimetidine significantly stimulated [\textsuperscript{3}H]BSP uptake over the concentration range from 3 \mu M to 1,000 \mu M (Fig. 2 and Supplemental Table 3). Similarly, [\textsuperscript{3}H]E2G and [\textsuperscript{3}H]E1S uptake were significantly stimulated by some inhibitors at lower concentrations (Supplemental Tables 1 and 2), but the extent of stimulation was less than occurred with [\textsuperscript{3}H]BSP uptake.

IC_{50} values of inhibitors tested are summarized in Table 1, and comparisons between IC_{50} values for [\textsuperscript{3}H]E2G, [\textsuperscript{3}H]E1S, and [\textsuperscript{3}H]BSP uptake are shown in Fig. 3. As shown in Fig. 3, A and B, the most potent inhibition was observed when [\textsuperscript{3}H]E2G was used as a substrate, and the IC_{50} values of all inhibitors tested were lower than those for
\[^{3}H\]E\textsubscript{1}S and \[^{3}H\]BSP uptake. Inhibitory potencies on OATP\textsubscript{1B1} varied depending on the substrates used, and ritonavir, gemfibrozil, and erythromycin showed a remarkable substrate-dependency. IC\textsubscript{50} values (\(\mu M\)) of ritonavir (0.397, 46.4, and 3.38 for \[^{3}H\]E\textsubscript{2}G, \[^{3}H\]E\textsubscript{1}S, and \[^{3}H\]BSP uptake, respectively), gemfibrozil (26.4, 381, and 173, respectively), and erythromycin (4.88, 13.4, and 63.3, respectively) showed up to 117-, 14-, and 13-fold variation depending on the substrates, respectively. Additionally, rifampin and CsA, which are frequently used as OATP inhibitors in clinical DDI studies, showed up to 12-fold (IC\textsubscript{50} [\(\mu M\]); 0.585 for \[^{3}H\]E\textsubscript{2}G versus 6.96 for \[^{3}H\]E\textsubscript{1}S)
and 6-fold (0.118 for \[^{3}H\]E2G versus 0.732 for \[^{3}H\]E1S) variation in the IC\textsubscript{50} values, respectively. Other inhibitors also showed 2-fold (BSP) to 9-fold (probenecid) discrepancy in the IC\textsubscript{50} values depending on the substrates selected, and \[^{3}H\]E2G provided the lowest IC\textsubscript{50} regardless of the inhibitors tested.

### Mutual Inhibition of OATP1B1-Mediated Uptake of Prototypical Substrates

Concentration dependence of OATP1B1-mediated uptake of \[^{3}H\]E2G, \[^{3}H\]E1S, and \[^{3}H\]BSP were investigated in the presence or absence of unlabeled E2G, E1S, and BSP to see the mode of inhibition between prototypical substrates tested. Representative data are given in Fig. 4, and obtained kinetic parameters are summarized in Table 2.

Uptake of \[^{3}H\]E2G consisted of one saturable component, and the Km values significantly increased from 8.17 \(\mu\)M to 18.7 \(\mu\)M in the presence of unlabeled E1S at 0.1 \(\mu\)M with minimal effect on V\text{max}. In the presence of unlabeled BSP at 0.3 \(\mu\)M, the V\text{max} value significantly decreased to less than 22\% of that in the absence of BSP with minimal effect on Km. These results suggested that E1S and BSP inhibited \[^{3}H\]E2G uptake by OATP1B1 in competitive and noncompetitive manners, respectively.

Uptake of \[^{3}H\]E1S showed a saturable component, and the Km (0.286 \(\mu\)M) significantly increased to 0.488 \(\mu\)M and 0.677 \(\mu\)M in the presence of unlabeled E2G (10 \(\mu\)M) and BSP (0.3 \(\mu\)M), respectively, without affecting V\text{max}. Thus, E2G and BSP competitively inhibited OATP1B1-mediated uptake of \[^{3}H\]E1S.

\[^{3}H\]BSP uptake via OATP1B1 also consisted of one saturable component with a Km of 0.280 \(\mu\)M. Unlabeled E2G (10 \(\mu\)M) showed a trend to increase and decrease the Km and V\text{max}, respectively, but without a statistically significant difference. Unlabeled E1S at 10 \(\mu\)M showed an atypical inhibition profile for \[^{3}H\]BSP uptake, in which E1S significantly increased both Km and V\text{max} of \[^{3}H\]BSP uptake by 17- and 5-fold, respectively.

### Discussion

It has been demonstrated that membrane transporters play significant roles in drug disposition, therapeutic efficacy, and adverse events, and clinical evidence showing the importance of transporter-mediated DDIs is increasingly accumulating (Giacomini et al., 2010; Yoshida et al., 2013). OATP1B1 is one of the major human hepatic uptake transporters, and clinically observed DDIs involving OATP1B1 have been reported (Shitara et al., 2003; Maeda et al., 2011; Niemi et al., 2011; Shitara et al., 2013). Regulatory agencies also consider OATP1B1 as one of the important transporters (Committee for Human Medicinal Products, 2012; Center for Drug Evaluation and Research, 2012). Selection of the OATP1B1 probe substrate for in vitro study is important for predicting the DDI risk.

E2G and E1S are prototypical OATP1B1 probe substrates and are widely used in cellular uptake studies with OATP1B1-transfected cells and human hepatocytes (Hirano et al., 2004; Sharma et al., 2010). OATP1B1-transfected cells used in this study also showed significantly

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**Table 1**

<table>
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<tr>
<th>Inhibitors</th>
<th>[^{3}H]E2G</th>
<th>[^{3}H]E1S</th>
<th>[^{3}H]BSP</th>
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<tr>
<td>E1S</td>
<td>0.095 ± 0.015</td>
<td>0.271 ± 0.013 (^{b})</td>
<td>0.429 ± 0.175 (^{a})</td>
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<tr>
<td>CsA</td>
<td>0.118 ± 0.015</td>
<td>0.732 ± 0.224</td>
<td>0.694 ± 0.149</td>
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<tr>
<td>BSP</td>
<td>0.131 ± 0.010</td>
<td>0.215 ± 0.058</td>
<td>0.327 ± 0.033 (^{a})</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>0.397 ± 0.023</td>
<td>46.4 ± 9.8</td>
<td>3.38 ± 0.66</td>
</tr>
<tr>
<td>Rifampin</td>
<td>0.585 ± 0.074</td>
<td>6.96 ± 13.1</td>
<td>2.75 ± 0.62</td>
</tr>
<tr>
<td>Tacrolimus</td>
<td>0.668 ± 0.156</td>
<td>1.78 ± 0.34</td>
<td>3.57 ± 0.43</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>4.88 ± 0.65</td>
<td>13.4 ± 4.0</td>
<td>63.3 ± 11.5</td>
</tr>
<tr>
<td>E2G</td>
<td>7.04 ± 0.53 (^{b})</td>
<td>16.6 ± 2.4</td>
<td>39.3 ± 9.0</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>9.90 ± 2.40</td>
<td>15.4 ± 3.7</td>
<td>60.9 ± 26.1</td>
</tr>
<tr>
<td>TCA</td>
<td>19.0 ± 1.0</td>
<td>50.0 ± 4.8</td>
<td>161 ± 35</td>
</tr>
<tr>
<td>Verapamil</td>
<td>22.3 ± 4.2</td>
<td>44.0 ± 7.3</td>
<td>84.3 ± 30.1</td>
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<tr>
<td>Gemfibrozil</td>
<td>26.4 ± 2.1</td>
<td>381 ± 60</td>
<td>173 ± 34</td>
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<tr>
<td>Probenecid</td>
<td>79.4 ± 5.8</td>
<td>227 ± 69</td>
<td>740 ± 181</td>
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\(^{a}\)E1S showed biphasic inhibition of \[^{3}H\]BSP uptake, and the IC\textsubscript{50} values for high- and low-affinity components are presented.

\(^{b}\)Km values.

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**Fig. 3.** Comparison of IC\textsubscript{50} values between \[^{3}H\]E2G and \[^{3}H\]E1S (A), \[^{3}H\]E2G and \[^{3}H\]BSP (B), and \[^{3}H\]E1S and \[^{3}H\]BSP (C). IC\textsubscript{50} values are taken from Table 1, and each point represents the mean ± S.D. The solid line and the dashed lines represent the line of unity and the lines of 1:10 and 10:1 correlations, respectively. 1, E1S; 2, CsA; 3, BSP; 4, ritonavir; 5, rifampin; 6, tacrolimus; 7, erythromycin; 8, E2G; 9, ketoconazole; 10, TCA; 11, verapamil; 12, gemfibrozil; and 13, probenecid. IC\textsubscript{50} values of E1S for \[^{3}H\]BSP uptake were eliminated from panels (B) and (C) because E1S showed biphasic inhibition for \[^{3}H\]BSP uptake. The correlation coefficients were 0.870 (A), 0.987 (B), and 0.868 (C).
higher uptake of \[^{3}H\]E2G and \[^{3}H\]E1S compared with the control cells (Fig. 1). To investigate substrate-dependent interaction with OATP1B1, IC50 values of inhibitors calculated against \[^{3}H\]E2G and \[^{3}H\]E1S uptake are compared in Fig. 3A. These comparisons clearly demonstrate that the IC50 values of all inhibitors tested were higher for \[^{3}H\]E1S uptake than for \[^{3}H\]E2G uptake. Notably, ritonavir, gemfibrozil, and rifampin showed 117-, 14-, and 12-fold higher IC50 values when \[^{3}H\]E1S was used as a substrate, respectively, and a similar trend was also reported previously (Noé et al., 2007; Soars et al., 2012). Also, to a lesser extent, CsA produced a 6-fold greater IC50 for \[^{3}H\]E1S uptake compared with \[^{3}H\]E2G uptake. Other inhibitors showed higher IC50 values for \[^{3}H\]E1S uptake than \[^{3}H\]E2G uptake, but the difference was within 3-fold. These substrate-dependent differences in IC50, particularly for rifampin and CsA, are used clinically as OATP inhibitors, should be taken into account in accurately estimating the impact of OATP1B1-mediated DDIs.

BSP is also a typical OATP1B1 substrate (Cui et al., 2001; Kindla et al., 2011) and showed significantly higher uptake in OATP1B1-transfected cells compared with the control cells (Fig. 1). As observed in the relationship of IC50 values between \[^{3}H\]E2G and \[^{3}H\]E1S, IC50 values of all inhibitors for \[^{3}H\]BSP uptake were higher than those for \[^{3}H\]E2G (Fig. 3B). Among the inhibitors tested, erythromycin showed the most remarkable difference, and the IC50 for \[^{3}H\]BSP uptake was 13-fold higher than that for \[^{3}H\]E2G, although the difference between \[^{3}H\]E1S and \[^{3}H\]E2G was less than 3-fold. Similarly, BSP, ritonavir, tacrolimus, E2G, ketoconazole, TCA, gemfibrozil, and probenecid also showed more than 5-fold higher IC50 values for \[^{3}H\]BSP uptake compared with \[^{3}H\]E2G.

These results clearly indicate substrate-dependent interaction with OATP1B1, and some inhibitors (ritonavir, gemfibrozil, erythromycin, and rifampin) caused more than 10-fold differences in the IC50 values. This means OATP1B1-mediated DDI risk can be more than 10-fold underestimated if the substrate is not appropriately selected, which could result in false-negative DDI prediction and potentially serious clinical safety issues. Thus, careful attention will be needed when selecting an OATP1B1 probe substrate for in vitro inhibition assay, as in the case of CYP3A4 (Kenworthy et al., 1999; Obach et al., 2006), OATP2B1 (Shirasaka et al., 2012), and OCT2 (Zolk et al., 2009). Although it would be ideal to evaluate DDI potentials for all OATP1B1 substrate-inhibitor combinations anticipated in clinical settings, this

### TABLE 2

<table>
<thead>
<tr>
<th>Substrates</th>
<th>[^{3}H]E2G</th>
<th>[^{3}H]E1S</th>
<th>[^{3}H]BSP</th>
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<td>0.667 ± 0.642</td>
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<td>BSP</td>
<td>58.0 ± 2.53</td>
<td>0.677 ± 0.112</td>
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NA, not applicable.

*Concentration-dependent uptake of \[^{3}H\]E2G (0.003–10 \(\mu\)M) for 5 min was examined in the absence and presence of unlabeled E2G (0.1 \(\mu\)M) or BSP (0.3 \(\mu\)M). A concentration of 0.007–10 \(\mu\)M for 5 min was examined in the absence and presence of unlabeled E2G (10 \(\mu\)M) or BSP (0.3 \(\mu\)M).

*P < 0.05, **P < 0.01 compared with parameters determined without any inhibitors.
approach will not be practical in drug-development processes, particularly at the early stage, where a large number of newly synthesized compounds are evaluated in a high-throughput manner. Among the substrates tested in this study, [3H]E2G was the OATP1B1 probe substrate that could most sensitively detect inhibitory potential on OATP1B1, because IC50 values of all inhibitors tested for [3H]E2G uptake were lower than those for [3H]E1S and [3H]BSP uptake (Fig. 3). Therefore, this finding of E2G as a sensitive OATP1B1 probe substrate will be helpful in achieving this goal.

Previously, Hirano et al. (2006) comprehensively evaluated the IC50 values for [3H]pitavastatin uptake via OATP1B1 and estimated the extent of in vivo DDIs using a static model. Although assay conditions were not identical, we have compared the IC50 values reported by Hirano et al. (2006) with those for [3H]E2G uptake obtained in this study. This comparison clarified that IC50 values for [3H]pitavastatin uptake were within 2.5-fold of those for [3H]E2G uptake in this study, suggesting that E2G and pitavastatin behave similarly in terms of their susceptibility to OATP1B1 inhibitors. Most recently, Sharma et al. (2012) reported that the extent of inhibition of [3H]E2G by rifampicin SV, gemfibrozil, and a proprietary compound was similar to that observed for the inhibition of statins, and they suggested that E2G could be a surrogate probe for statins when assessing OATP1B1 inhibition. These findings indicate that E2G could be used not only as a sensitive substrate to mitigate the risk of false-negative DDI prediction, but also potentially as a surrogate probe for statins. In addition to statins, however, a broad range of drugs including angiotensin II receptor blockers (Yamashiro et al., 2006; Yamada et al., 2007), endothelin receptor antagonists (Katz et al., 2006; van Giersbergen et al., 2007), anti-diabetics (Niemi et al., 2005; Zhang et al., 2006), and a diuretic (Vormfelde et al., 2008) are reported to be OATP1B1 substrates. Further studies will be needed to elucidate if E2G could be a universal surrogate probe of OATP1B1 by comparing IC50 values between [3H]E2G and OATP1B1 substrate drugs including statins and other classes of drugs.

Substrate-dependent inhibition with OATP1B1 suggests that OATP1B1 has multiple allosteric binding sites for the substrates. To gain an insight into the binding sites, mutual inhibition studies were performed (Fig. 4). [3H]E2G and [3H]BSP showed monophasic uptake for OATP1B1, which was consistent with previous reports (Cui et al., 2001; Hirano et al., 2004; Sharma et al., 2010). As for [3H]E1S uptake by OATP1B1-transfected cells, biphasic uptake was demonstrated by some groups (Tamai et al., 2001; Noé et al., 2007; Sharma et al., 2012) and monophasic uptake by others (Hirano et al., 2004), and the latter held true for this study. Although we do not have a good explanation for the discrepancy, experimental conditions (e.g., cells, cell culture conditions, assay procedures) might be among the underlying reasons. Through the mutual inhibition studies, it was elucidated that OATP1B1-mediated uptake of [3H]E2G and [3H]E1S competitively inhibited each other, suggesting that both substrates could share the same binding site. However, some inhibitors, such as ritonavir, gemfibrozil, and rifampin showed notably different IC50 (i.e., affinity) for [3H]E2G and [3H]E1S uptake, and thus it would be difficult to conclude that both substrates completely share the same binding site. Moreover, BSP noncompetitively inhibited [3H]E2G uptake by OATP1B1, which suggests the presence of a different binding site than that of [3H]E1S. We also analyzed the relationship between [3H]E2G and [3H]BSP uptake in the same manner, and BSP competitively inhibited [3H]E2G uptake, although E2G showed atypical kinetics on [3H]BSP uptake, where the Km and Vmax of [3H]BSP uptake increased by 17- and 5-fold, respectively. These results suggest more than one binding site on OATP1B1, which might be an underlying mechanism of substrate-dependent inhibition of OATP1B1. Further kinetic analysis with molecular biology techniques as reported previously (Miyagawa et al., 2009) will be needed to identify each binding site of E2G, E1S, and BSP, and clarify the complex mutual interactions among them on OATP1B1.

In the inhibition studies, some compounds, such as E2S, ritonavir, and E1G, caused not only inhibition at higher concentrations, but also statistically significant stimulation of the substrate uptake at lower concentrations (Supplemental Tables 1, 2, and 3), and this behavior was more evident in [3H]BSP uptake (Fig. 2). Cimetidine was also found to significantly stimulate [3H]BSP uptake, but the stimulatory effect was sustained over the concentration range from 3 μM to 1,000 μM (Supplemental Table 3). Although the underlying mechanism of the observed stimulation remains to be clarified, the inhibitors at lower concentrations might have dominantly interacted with the allosteric site, resulting in stimulation of [3H]BSP uptake, while the inhibitors at higher concentrations, except cimetidine, might have not only interacted with the allosteric site, but also inhibited the BSP binding site, resulting in net inhibition of [3H]BSP uptake. Sustained stimulation of OATP1B1-mediated [3H]BSP uptake by cimetidine over the concentration range from 3–1,000 μM might be explainable in terms of the preferential affinity of cimetidine for an allosteric site relative to BSP binding site. Although further studies will be needed to draw more definite conclusions, the significant stimulatory effect observed in this study and another study (Kindll et al., 2011) suggests the presence of multiple binding sites on OATP1B1.

In conclusion, inhibition potentials of 14 compounds on OATP1B1 were comparatively analyzed using the prototypical OATP1B1 probe substrates E2G, E1S, and BSP, and OATP1B1 showed substrate-dependent inhibition. Some inhibitors showed more than 10-fold differences in the IC50 values depending on the substrate used in the in vitro inhibition studies. Among the three substrates tested, E2G was the OATP1B1 probe substrate that could most sensitively detect inhibitory potential on OATP1B1. E2G could be used as a sensitive probe substrate when evaluating OATP1B1 inhibition, which will help mitigate the risk of false-negative DDI prediction.

Authorship Contributions
Participated in research design: Izumi, Nozaki, Komori, Maeda, Takenaka, Sugiyama.
Conducted experiments: Izumi.
Contributed new reagents or analytic tools: Maeda.
Performed data analysis: Izumi, Nozaki, Komori, Maeda.
Wrote or contributed to the writing of the manuscript: Izumi, Nozaki, Komori, Maeda, Takenaka, Kusano, Yoshimura, Kusuhara, Sugiyama.

References


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Drug Metabolism and Disposition

Supplemental Fig. 1. Individual data of mutual inhibition of OATP1B1-mediated uptake of [3H]E2G, [3H]E1S, and [3H]BSP. (A-1 to A-3) concentration-dependent uptake of [3H]E2G (0.003 – 100 μM) for 5 min in the absence (closed circles) and presence of unlabeled E1S (0.1 μM, open triangles) or BSP (0.3 μM, open squares). (B-1 to B-3) concentration-dependent uptake of [3H]E1S (0.003 – 10 μM) for 1 min in the absence (closed circles) and presence of unlabeled E2G (10 μM, open triangles) or BSP (0.3 μM, open squares). (C-1 to C-3) concentration-dependent uptake of [3H]BSP (0.007 – 10 μM) for 5 min in the absence (closed circles) and presence of unlabeled E2G (10 μM, open triangles) or E1S (10 μM, open squares). Data are shown as Eadie-Hofstee plot, and each point represents mean ± SD (n=3). Individual data from three independent experiments are given in this supplemental figure.
**Supplemental Table 1**

Statistical analysis of stimulatory effect of tested compounds on OATP1B1-mediated uptake of $[^3H]E_2G$

<table>
<thead>
<tr>
<th>Substrate $[^3H]E_2G$</th>
<th>E$_1$S</th>
<th>CsA</th>
<th>BSP</th>
<th>ritonavir</th>
<th>rifampin</th>
<th>tacrolimus</th>
<th>erythromycin</th>
<th>E$_2G$</th>
<th>TCA</th>
<th>ketoconazole</th>
<th>gemfibrozil</th>
<th>verapamil</th>
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Inhibitor concentrations, at which OATP1B1-mediated uptake of $[^3H]E_2G$ (0.1 μM) was significantly stimulated in inhibition studies (Fig. 2), are highlighted in bold. *$P<0.05$, **$P<0.01$, ***$P<0.001$, significantly different from OATP1B1-mediated uptake of $[^3H]E_2G$ without any inhibitors. E$_1$S, estrone-3-sulfate; CsA, cyclosporin A; BSP, sulfobromophthalein; E$_2G$, estradiol-17ß-glucuronide; TCA, taurocholate.
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[Journal] Drug Metabolism and Disposition

### Supplemental Table 2
Statistical analysis of stimulatory effect of tested compounds on OATP1B1-mediated uptake of [3H]E1S

<table>
<thead>
<tr>
<th>Substrate</th>
<th>E1S</th>
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<th>BSP</th>
<th>ritonavir</th>
<th>rifampin</th>
<th>tacrolimus</th>
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Inhibitor concentrations, at which OATP1B1-mediated uptake of [3H]E1S (0.01 μM) was significantly stimulated in inhibition studies (Fig. 2), are highlighted in bold. *P<0.05, **P<0.01, ***P<0.001, significantly different from OATP1B1-mediated uptake of [3H]E1S without any inhibitors. E1S, estrone-3-sulfate; CsA, cyclosporin A; BSP, sulfobromophthalein; E2G, estradiol-17β-glucuronide; TCA, taurocholate.
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Drug Metabolism and Disposition

**Supplemental Table 3**

Statistical analysis of stimulatory effect of tested compounds on OATP1B1-mediated uptake of [3H]BSP

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<th>Substrate</th>
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Inhibitor concentrations, at which OATP1B1-mediated uptake of [3H]BSP (0.01 μM) was significantly stimulated in inhibition studies (Fig. 2), are highlighted in bold. *P < 0.05, **P < 0.01, ***P < 0.001, significantly different from OATP1B1-mediated uptake of [3H]BSP without any inhibitors. E1S, estrone-3-sulfate; CsA, cyclosporin A; BSP, sulfobromophthalein; E2G, estradiol-17β-glucuronide; TCA, taurocholate.