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Pretreatment with Rifampicin and Tyrosine Kinase Inhibitor Dasatinib Potentiates the Inhibitory Effects toward OATP1B1- and OATP1B3-Mediated Transport without Affecting Plasma Membrane Localization of the Transporters

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Abstract

Current studies determined the effects of pretreatment with rifampicin, an organic anion-transporting polypeptide (OATP) inhibitor, and the tyrosine kinase inhibitor dasatinib on OATP1B1- and OATP1B3-mediated transport, and to evaluate the OATP-mediated drug-drug interaction (DDI) potential of dasatinib using the static R-value and dynamic physiologically-based pharmacokinetic models. Rifampicin and dasatinib pretreatment significantly decreased OATP1B1- and OATP1B3-mediated transport. Rifampicin pretreatment also significantly decreased [^3H]-pitavastatin and [^3H]-CCK-8 accumulation in human sandwich-cultured hepatocytes. Current studies revealed that estrone-3-sulfate is a less sensitive OATP1B1 substrate than estradiol-17 β -glucuronide in assessing rifampicin pretreatment effects. Pretreatment with rifampicin and dasatinib reduced the inhibition constant (K_i) values against OATP1B1 by 3 and 2.1 fold and toward OATP1B3 by 2.4 and 2.1 fold, respectively. The *in vitro* rifampicin K_i values following pre-incubation are comparable to the estimated *in vivo* K_i reported previously. Models predict that dasatinib has a low potential to cause OATP1B1- and OATP1B3-mediated DDIs. Time-lapse confocal microscopy demonstrated that rifampicin and dasatinib pretreatment did not

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Contributed new reagents or analytic tools:

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Wrote or contributed to writing the manuscript: Yue, Pahwa, Farasyn, Neuhoﬀ, Hatley

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affect plasma membrane localization of green-fluorescent protein (GFP)-OATP1B1 and -OATP1B3 in HEK293-GFP-OATP1B1 and -OATP1B3 cells. In summary, we report novel findings that pretreatment with rifampicin and dasatinib potentiates the inhibitory effects toward OATP1B1 and OATP1B3 without affecting plasma membrane levels of the transporters.

Keywords

Drug Transport; Drug interactions; organic anion-transporting polypeptide transporters; hepatocytes; hepatic transport; pharmacokinetics; physiologically based pharmacokinetic modeling

INTRODUCTION

Organic anion transporting polypeptides (OATP) 1B1 and OATP1B3 are localized to the basolateral membrane of hepatocytes and mediate the hepatic uptake of many drugs (*e.g.*, lipid lowering statins, anti-cancer drugs and antibiotics)¹. Co-administration of drugs that are potent OATP inhibitors (*e.g.* rifampicin² and cyclosporine (Cs) A^{3,4}) often resulted in increased systemic exposure of statins and statin-induced myopathy^{5,6}. OATP1B1 and OATP1B3 have been recognized as important determinants for transporter-mediated drug-drug interactions (DDIs)^{7,8}. The static R-value and dynamic physiologically-based pharmacokinetic (PBPK) models are often used to assess the potential for a new molecular entity/drug to cause OATP-mediated DDIs^{9–12}.

It has been reported recently that pre-incubation with some OATP inhibitors (*e.g.* CsA⁴, HIV protease inhibitors saquinavir and ritonavir¹³, and direct-acting antiviral agents simeprevir, asunaprevir and daclatasvir¹⁴) significantly decreases OATP1B1- and 1B3-mediated transport. Currently, the effect of pre-incubation on the IC₅₀/inhibition constant (K_i) of OATP inhibitors has only been reported for CsA^{3,15}, where pre-incubation with CsA results in reduced IC₅₀ values against OATP1B1 and 1B3. Interestingly, a recent publication by Yoshikado *et al.*¹⁶ indicated that the *in vitro* K_i value of CsA against OATP1B1 determined with CsA pre-incubation was comparable to the estimated *in vivo* K_i of CsA¹⁶. This finding supports that the lower *in vitro* K_i values obtained following CsA pre-incubation are likely to be important for the prediction of clinical DDIs. It is plausible to anticipate that the *in vitro* K_i value determined following pretreatment with an OATP inhibitor may be close to the *in vivo* K_i value. However, the pretreatment effects on K_i values against OATP1B1 and OATP1B3 warrants further studies with additional OATP inhibitors.

Rifampicin is a potent inhibitor of OATP1B1 and OATP1B3². Yoshikado *et al.*¹⁶ also reported that the estimated *in vivo* K_i values for rifampicin against OATP substrates are smaller than the reported *in vitro* K_i values of rifampicin determined without rifampicin pre-incubation¹⁶. This apparent discrepancy could be explained by a potential reduction in the K_i value of rifampicin against OATP1B1 following pre-incubation; however, the pre-incubation effect of rifampicin on the K_i values against OATP1B1 and OATP1B3 has not been reported. Shitara *et al.*, however, reported previously that pre-incubation with rifampicin did not affect OATP1B1-mediated transport using estrone-3-sulfate (E₁S) as the substrate¹³. E₁S is a less sensitive substrate than estradiol 17β-D-glucuronide (E₂17βG)

when used for determining the OATP1B1-mediated inhibition without inhibitor pre-incubation¹⁷. It is not known if E₁S is also a less sensitive substrate to assess the pretreatment effects caused by OATP inhibitors. Thus, the pretreatment effects of rifampicin on OATP1B1- and 1B3-mediated transport should be re-evaluated using an additional OATP1B1 probe substrate (*e.g.* E₂17βG) and an OATP drug substrate (*e.g.* pitavastatin).

Tyrosine kinase inhibitors (TKIs) are emerging agents for targeted cancer therapy^{18,19}. Small-molecule TKIs are often administered on a daily basis; therefore, concurrent administration of TKIs with widely prescribed OATP substrates statins is likely. Understanding the OATP-mediated DDI potential of TKIs has clinical significance. Dasatinib is a multi-tyrosine kinase inhibitor used for targeted therapy in certain blood cancers²⁰. Co-administration of dasatinib with simvastatin increases the area under the plasma concentration-time curve (AUC) of simvastatin and simvastatin acid by 20% and 27%, respectively²¹. This DDI was postulated to be due to metabolic inhibition, *i.e.* a weak mechanism based CYP3A4 interaction^{22,23}. Statins are also substrates of OATPs. Currently, only limited information is available regarding the effects of dasatinib on OATP1B1- and OATP1B3-mediated transport. It was reported that co-incubation with 10 μM dasatinib decreased OATP1B1-mediated [³H]-E₂17βG transport by ~70% in an OATP1B1-overexpressing cell line²⁴. The inhibitory potency of dasatinib against OATP1B1 and OATP1B3 has not been characterized, and the OATP-mediated DDI potential of dasatinib has not been assessed.

The current studies were designed to determine the pretreatment effects of rifampicin and dasatinib on OATP1B1- and OATP1B3-mediated transport and on the IC₅₀ values against OATP1B1 and OATP1B3. The effect of rifampicin pretreatment on the accumulation of OATP substrates in physiologically relevant human sandwich-cultured hepatocytes (SCH) was also determined. The OATP-mediated DDI potential of dasatinib was assessed using static R-value and physiologically-based pharmacokinetic (PBPK) models. CsA was used as the calibrator compound to estimate the *in vivo* relevant K_i values of dasatinib against OATPs for DDI prediction using a PBPK model. A confocal microscopy live-cell imaging approach was utilized to determine the effects of rifampicin and dasatinib pretreatment on plasma membrane localization of green-fluorescent protein (GFP)-tagged OATP1B1 and OATP1B3 in HEK293-GFP-OATP1B1 and -OATP1B3 stable cell lines, respectively.

MATERIALS AND METHODS

Materials

[³H]-E₂17βG (specific activity 41.4 Ci/mmol) and [³H]-E₁S (specific activity 54.0 Ci/mmol) were purchased from Perkin Elmer Life Science (Waltham, MA). Dasatinib and CsA were purchased from LC laboratories (Woburn, MA, USA). Rifampicin, unlabeled E₂17βG, Hanks' balanced salt solution (HBSS), dimethyl sulfoxide (DMSO), Triton X-100, Dulbecco's Modified Eagle Medium (DMEM), Dulbecco's phosphate buffered saline (DPBS), trypsin-EDTA solution, and antibiotic antimycotic solution were purchased from Sigma-Aldrich (St. Louis, MO). Poly-L-lysine was purchased from Trevigen Inc. (Gaithersburg, MD). Geneticin[®] (G418), BioCoat[™] culture plates, insulin/transferrin/selenium (ITS⁺), and Matrigel[™] were purchased from BD Biosciences (Bedford, MA). Fetal

bovine serum (FBS) was purchased from Hyclone Laboratories (Logan, Utah). Bio-Safe II liquid scintillation mixture was purchased from Research Products International (Mt. Prospect, IL).

Plasmid Constructs

The pCMV6-AC-GFP-Myc-FLAG-OATP1B1 and -OATP1B3 plasmid expression vectors were constructed by in-frame sub-cloning of the Myc-FLAG tagged open reading frame (ORF) of OATP1B1 and 1B3 expression cassettes from pCMV6-FLAG-OATP1B1²⁵ and pCMV6-FLAG-OATP1B3²⁶ vectors, respectively, into the pCMV6-AC-GFP vector (Origene, Rockville, MD). The ORF of GFP is at the C-terminus of OATP1B1 and OATP1B3. The correctness of constructs was confirmed by sequencing of the full length ORFs.

Cell culture

Human embryonic kidney (HEK) 293 stable cell lines over-expressing OATP1B1 (HEK293–OATP1B1) or OATP1B3 (HEK293–OATP1B3) and the HEK293-Mock cell line were generously provided by Dr. Dietrich Keppler^{27,28}. All HEK293 stable cell lines were cultured in DMEM medium supplemented with fetal bovine serum (10% v/v), 1% antibiotic antimycotic solution, and 600 µg/ml Geneticin® in humidified atmosphere supplemented with 95% O₂ and 5% CO₂ at 37°C.

Human sandwich-cultured hepatocytes (SCH)

Freshly isolated human hepatocytes in suspension were purchased from Life Technologies (Grand Island, NY) and Triangle Research Laboratories, LLC (Research Triangle Park, NC). Sandwich-culture of human hepatocytes was conducted as previously described²⁹. Briefly, the cells were plated at 3.5×10^5 cells per well in 24-well Biocoat™ culture plates in phenol red-free DMEM supplemented with 5% (v/v) FBS, 1% (v/v) MEM NEAA, 2 mM L-glutamine, penicillin-streptomycin (10 U/ml), 4 µg/mL insulin, and 1 µM dexamethasone. When cells were attached (6 h after seeding), cells were overlaid with Matrigel™ (BD Bioscience) at a final concentration of 0.25 mg/mL in phenol red-free DMEM containing 2 mM L-glutamine, 1% (v/v) MEM NEAA, penicillin-streptomycin (10 U/ml), 0.1 µM dexamethasone, and 1% (v/v) ITS⁺ premix. Culture medium was changed every 24 h. Experiments were conducted on day 3 of culture. The demographic characteristics of the human liver donors and representative light microscopic images to evaluate the quality of SCH are shown in supplemental materials (Table S1 and Fig. S1).

Immunoblotting

Immunoblotting was conducted similarly to those described previously^{25,26}. In brief, whole cell lysates (WCL) were prepared by adding ice cold lysis buffer, which contains 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% (v/v) NP-40, 0.5% Na-deoxycholate, and Complete™ protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN), directly onto cells in culture plates after aspirating the culture media and rinsing once with DPBS. WCLs (50 µg) were resolved on a 10% SDS-PAGE gel (Bio-Rad, Hercules, CA). Immunoblots were probed with rabbit polyclonal OATP1B1 and 1B3 antibodies (1:2000

dilution)^{25,26} and with mouse monoclonal anti- β -actin (1:5000) (Sigma-Aldrich, St. Louis, MO) antibody as the loading control. After incubation with HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., Dallas, Texas), signals were detected by Supersignal West Duro (Pierce, Rockford, IL) using a Bio-Rad ChemiDoc XRS imaging system (Bio-Rad Laboratories, Hercules, CA) and densitometry analysis was performed using Image Lab Software (Bio-Rad Laboratories, Hercules, CA).

Transport studies

The IC₅₀ values of rifampicin and dasatinib against OATP1B1 and OATP1B3 were assessed in HEK293-OATP1B1 and -OATP1B3 stable cell lines, similar to what was published previously³⁰. The probe substrate used for the IC₅₀ assessment was [³H]-E₂17 β G (1 μ M, 2 min) for both OATP1B1 and OATP1B3. The probe substrate E₂17 β G concentration (1 μ M) was less than the K_m values for OATP1B1 and OATP1B3 to ensure the IC₅₀ value is approximately the same as the K_i value. [³H]-E₁S (20 nM, 0.5 min) and [³H]-pitavastatin (1 μ M, 0.5 min) were also used as probe substrates for OATP1B1 to determine the pretreatment effect of rifampicin in HEK293-OATP1B1 cells. [³H]-Pitavastatin (1 μ M, 0.5 min) and [³H]-CCK-8 (1 μ M, 3 min) were used as probe substrates in human SCH. Substrate concentrations for E₁S, pitavastatin and CCK-8 used in the current studies are all below the reported K_m values³¹. The uptake duration of all probe substrates were within the linear uptake range as published in previous studies^{25,26,30} or determined in the current studies (data not shown). CsA was used as a positive control inhibitor in the current studies. Experiments were performed in triplicate on three separate occasions as indicated in the figure legends.

HEK293 stable cell lines were seeded at 2×10^5 cells per well in 24-well plates, and allowed to grow for 48 h before the transport studies. Plates for seeding HEK293-OATP1B1 cells were coated with poly-L-lysine. Three scenarios were used to determine the effects of testing drugs on OATP1B1- and -1B3-mediated transport in HEK293-OATP1B1 and -1B3 stable cell lines. In the pre-incubation scenario, the cells were pre-incubated with HBSS buffer (pH 7.4) containing vehicle control (0.1% DMSO v/v) or inhibitors at designated concentrations for the indicated times at 37°C. After washing three times with warm HBSS buffer at 37°C, accumulation of [³H]-E₂17 β G (1 μ M, 2 min), [³H]-E₁S (20 nM, 0.5 min), or [³H]-pitavastatin (1 μ M, 0.5 min) was determined in the absence of inhibitors. In the co-incubation scenario, without any pre-incubation, [³H]-E₂17 β G accumulation (1 μ M, 2 min) was determined in the presence of vehicle control or inhibitors at designated concentrations. In the pre+co-incubation scenario, cells were pre-incubated with HBSS buffer containing vehicle control or inhibitors for 1 h. After washing three times with warm HBSS buffer, cells were incubated with HBSS buffer containing [³H]-E₂17 β G (1 μ M, 2 min) in the presence of vehicle control or inhibitors at the same concentrations used in the pre-incubation. Human SCH were pretreated for 1 h with feeding medium containing vehicle control or rifampicin (10 μ M). After rinsing 3 times with pre-warmed HBSS buffer at 37°C, accumulation of [³H]-pitavastatin (1 μ M, 0.5 min) and [³H]-CCK-8 (1 μ M, 3 min) was determined. Similar to what was previously published^{25,26}, substrate accumulation was determined by scintillation counting and normalized to the protein concentration. The maximal transport velocity (V_{max}) and the affinity constant (K_m) values of OATP1B1- and OATP1B3-mediated

transport of E₂17βG were determined, similarly to published previously, following pretreatment with vehicle control or rifampicin (10 μM, 1 h)²⁵.

Time-lapse confocal fluorescence microscopy

HEK293-GFP-OATP1B1 and -1B3 cells were seeded into 4-compartment 35 × 10mm CELLVIEW cell culture dishes with an integrated glass bottom (Greiner Bio One, Monroe, NC) at a density of 2.5×10^5 cells per compartment. Forty-eight hours after seeding, the culture dish was kept in an Olympus Fluoview FV10i-LIV confocal laser scanning microscope (Olympus, Tokyo, Japan) supplemented with 95% O₂ and 5% CO₂, at a temperature of 37°C. Cells were imaged using a n Olympus UPLSAPO 60x water immersion objective (N/A 1.2) with 473 nm laser excitation and an emission bandpass of 490–590 nm. Confocal Z-series images were acquired using a 1 μm Z-step size with a 1.0 airy disk confocal aperture and 2x Kalman line averaging. Testing drugs or vehicle control were added at time zero to different compartments of the culture dish. Images were taken every 20 min from -19 min to 61 min. This experimental condition has been optimized to make sure that there is minimal photo bleaching in cells without drug treatment (data not shown). Cells from three independent experiments were examined. The images show representative z-stacks of three consecutive optical sections. Contrast/brightness enhancement was done in parallel using Olympus Fluoview ASW v4 software (Olympus, Tokyo, Japan).

Lactate dehydrogenase (LDH) cytotoxicity assay

Experiments were conducted similarly to those published previously³². After treatment with rifampicin, dasatinib or vehicle control, cell culture media was assayed for lactate dehydrogenase activity with a cytotoxicity detection kit (LDH) (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions.

Assessing OATP1B1- and OATP1B3-mediated DDIs using the static R-value model

The extent of substrate accumulation in the presence of test compounds was expressed as a percentage relative to that of the vehicle control. The inhibitory potency of rifampicin and dasatinib against OATP1B1 and OATP1B3 was estimated by nonlinear regression using the three parameter model with GraphPad Prism v.7.0 (GraphPad Software, La Jolla, CA). The following equation was fit to the inhibition-concentration data:

$$E = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + (C / \text{IC}_{50})) \quad \text{Eq. 1}$$

Where E is the remaining OATP1B1- or OATP1B3-mediated substrate transport at a given inhibitor concentration (C). IC₅₀ is the inhibitor concentration eliciting a response half way between the maximal (Top) response and the maximally inhibited (Bottom) response.

The IC₅₀ values against OATP1B1 and OATP1B3 were used to determine C_{max}/IC₅₀ ratios, where C_{max} is the maximum plasma concentration of the inhibitor⁷. The C_{max} value of dasatinib reflecting the maximum therapeutic plasma concentration of dasatinib at steady state⁶ and the C_{max} of rifampicin after single 600 mg oral dose⁷ were used (Supplemental

Table S2). The R-values, which reflect the predicted change in exposure of OATP1B1 and 1B3 substrates, were calculated based on Eq. 2

$$R=1+(f_u \times I_{in,max}/K_i) \quad \text{Eq. 2}$$

$I_{in,max}$ and $I_{u,in,max}$ represent the estimated total and unbound maximal inhibitor concentrations at the inlet to the liver and were calculated based on Eq. 3 and 4, respectively, as described previously³³.

$$I_{in,max}=C_{max}+(k_a \times D \times f_a f_G/Q_h) \quad \text{Eq. 3}$$

$$I_{u,in,max}=I_{in,max} \times f_u \quad \text{Eq. 4}$$

Where the C_{max} and associated dose (D), unbound fraction of the inhibitor in the plasma (f_u) and absorption rate constant of the inhibitor (k_a) are summarized in the supplemental materials (Table S2). The fraction of inhibitor absorbed from the gastrointestinal tract intact ($f_a f_G$) was set at 1³³. To minimize the false negative prediction, R-values are also calculated based on a k_a value of 0.1/min³³, which is the maximal gastric emptying rate constant in humans. The hepatic blood flow (Q_h) was set as 1500 ml/min³⁴

Pharmacokinetic Modeling and Simulations

A PBPK modeling approach was utilized to evaluate the OATP1B1- and OATP1B3-mediated DDI potential of dasatinib against the OATP-substrate pravastatin using the population-based Simcyp simulator (version 15, SimCYP Ltd, Sheffield, UK). A PBPK model of dasatinib was developed as a perpetrator file based on the parameters summarized in Table 2. The default rifampicin (Sim-Rifampicin-SD) and pravastatin (Sim-Pravastatin) PBPK models within the Simcyp simulator library were used and not modified. Simulations were performed for 400 (20 trials $\times$ 20 subjects) virtual subjects (using the default Sim-Healthy Volunteer data library) for dasatinib and rifampicin DDIs against pravastatin. The virtual populations of healthy Caucasian subjects had an age range from 20 to 50 years, and 50% of the population were female. Dose, dosing interval, and dosing duration of dasatinib, rifampicin and pravastatin were similar to those used in the reported clinical studies^{35,36}. CsA was used as a calibrator compound for OATP-mediated DDIs as an *in vitro-to-in vivo* extrapolation of OATP transport in Caucasians could be established³. A sensitivity analysis was conducted to assess the impact of the *in vitro* determined K_i value of dasatinib to the estimated AUCR with pravastatin. Four potential modeling scenarios were assessed: direct use of *in vitro* measured IC_{50} values (1) without and (2) with pre-incubation; (3) assuming saturated *in vitro* conditions K_i values were half of the IC_{50} value with pre-incubation (although the experimental conditions used were already accounting for this, this was only

included as it is often used as ‘worst case’ scenario); calibration of dasatinib OATP1B1 and OATP1B3 K_i values using (4) CsA data according to equation 5.

$$\text{Calibrated } K_{i,\text{OATP,dasatinib}} = \text{in vitro } K_{i,\text{OATP,dasatinib}} * (\text{in vivo } K_{i,\text{OATP,CsA}}) / (\text{in vitro } K_{i,\text{OATP,CsA}})$$

Eq. 5

Where in vivo $K_{i,\text{OATP,CsA}}$ values are the estimated *in vivo* $K_{i,\text{OATP,CsA}}$ values against OATP1B1 (0.019 μM) and OATP1B3 (0.032 μM) that are used in the Simcyp simulator library, based on a previous publication by Gertz et al.³. The *in vitro* $K_{i,\text{OATP,CsA}}$ values are the CsA K_i values following pre-incubation for OATP1B1 and OATP1B3 determined in the current study (Fig. S2).

Data Analysis

For the statistical analysis in Fig. 1A, B, F and G, Fig. 2A and B and Fig. 4B, fold changes and associated standard errors (SE) of substrate accumulation in treatment group(s) *vs.* control were estimated by linear mixed effects models for data in triplicate with a fixed group effect and a random effect (experiment date or hepatocyte donor), adjusting for group-specific variances. In cases of multiple comparisons, p-values were adjusted based on the Bonferroni's method. A two-sided p-value of < 0.05 defines statistical significance. The SAS software (version 9.3, Cary, NC) was used for data analyses.

RESULTS

Effects of rifampicin on OATP1B1- and OATP1B3-mediated transport

To determine the effects of pretreatment with rifampicin on OATP1B1- and OATP1B3-mediated transport, substrate accumulation was determined in HEK293-OATP1B1 and -1B3 cells following pre-incubation with rifampicin or vehicle control (CTL) and subsequent washing. Pre-incubation with rifampicin at concentrations of 1 – 25 μM for 0.5 h and at concentrations of 0.5 – 25 μM for 1 h significantly decreased OATP1B1-mediated [^3H]-E₂17 β G transport (1 μM , 2 min) (Fig. 1A). Pre-incubation with rifampicin (10 μM , 1 h) also significantly decreased OATP1B1-mediated [^3H]-pitavastatin accumulation (1 μM , 0.5 min) (Fig. 1F). Pre-incubation with rifampicin at 0.5 – 25 μM significantly decreased OATP1B3-mediated [^3H]-E₂17 β G accumulation after 0.5 and 1 h pre-incubation (Fig. 1B). As the effects of rifampicin pretreatment on both OATP1B1- (Fig. 1A) and 1B3-mediated transport (Fig. 1B) appear to be similar between 30 min and 1 h, in the current studies, we used a 1 h pretreatment time period to determine the effects of pretreatment on the IC₅₀ values of rifampicin against OATP1B1 and OATP1B3. Pre-incubation with rifampicin at 10 and 25 μM for 1 h significantly decreased OATP1B1-mediated [^3H]-E₁S accumulation (20 nM, 0.5 min) to 0.70 ± 0.16 and 0.23 ± 0.09 fold of control, respectively (Bonferroni adjusted $p < 0.05$ *vs.* CTL, Fig. 1E). Using E₂17 β G as the substrate, rifampicin pretreatment (0.1 – 25 μM) yielded an IC₅₀ value of 1.8 ± 0.2 μM toward OATP1B1 (pretreatment data in Fig. 1C was replotted in 1E for the comparison purpose). Using E₁S as the substrate, rifampicin

pretreatment (0.5 – 25 μM) yielded an IC_{50} value greater than 10 μM against OATP1B1 (Fig. 1E). Similar results were observed for 30 min rifampicin pretreatment (Supplemental results Fig. S3).

Without rifampicin pretreatment, co-incubation with rifampicin (0.01–25 μM) yielded IC_{50} values of 0.74 ± 0.08 and 0.26 ± 0.03 μM against OATP1B1- and 1B3-mediated [^3H]-E₂17 β G transport, respectively. Pre-incubation for 1 h followed by co-incubation (pre+co-incubation) with rifampicin significantly decreased the IC_{50} values to 0.24 ± 0.03 ($p < 0.05$, $n=3$, paired t-test) and 0.10 ± 0.01 μM ($p < 0.05$, $n=3$, paired t-test) against OATP1B1 and OATP1B3, respectively (Fig. 1C and D and Table 1). The effects of CsA pretreatment on the IC_{50} values against OATP1B1 and OATP1B3 are reported in the supplemental materials (Fig. S2).

Accumulation of [^3H]-pitavastatin (1 μM , 0.5 min) and [^3H]-CCK-8 (1 μM , 3 min) was determined in human SCH subjected to 1 h pre-incubation with 10 μM rifampicin or vehicle control (CTL) and subsequent washing. As shown in Fig. 1G, pretreatment with rifampicin significantly decreased accumulation of [^3H]-pitavastatin and [^3H]-CCK-8 to 0.66 ± 0.07 and 0.37 ± 0.17 fold of CTL ($p < 0.05$), respectively.

Effects of dasatinib on OATP1B1- and OATP1B3-mediated transport

Pretreatment with dasatinib at 5 – 20 μM for 0.5 and 1 h significantly decreased OATP1B1-mediated [^3H]-E₂17 β G accumulation (Bonferroni adjusted $p < 0.05$ vs. CTL) (Fig. 2A). Dasatinib pretreatment at 10 and 20 μM for 0.5 h and at 5 – 20 μM for 1 h also significantly decreased OATP1B3-mediated [^3H]-E₂17 β G accumulation (Bonferroni adjusted $p < 0.05$ vs. control) (Fig. 2B).

Without dasatinib pretreatment, co-incubation with dasatinib (0.25 – 20 μM) yielded IC_{50} values of 4.81 ± 1.7 and 5.76 ± 1.1 μM against OATP1B1 and 1B3, respectively. After 1 h dasatinib pretreatment, pre+co-incubation with dasatinib decreased the IC_{50} values to 2.33 ± 0.86 and 2.75 ± 1.17 μM against OATP1B1 and 1B3, respectively (Fig. 2C and D and Table 1). However, such decrease did not reach statistical significance ($p=0.057$ for OATP1B1 and $p=0.059$ for OATP1B3, $n=3$, paired t-test).

Effects of rifampicin and dasatinib on total protein levels of OATP1B1 and OATP1B3

Immunoblot of OATP1B1 and OATP1B3 indicated that the total protein levels of OATP1B1 and OATP1B3 in vehicle control treated HEK293-OATP1B1 and 1B3, respectively, were similar to those in cells treated with rifampicin (Fig. 3A) and dasatinib (Fig. 3B) (both 10 μM , 1 h).

Effects of rifampicin on GFP-OATP1B1 and GFP-OATP1B3 plasma membrane localization

HEK293-GFP-OATP1B1 and -OATP1B3 stable cell lines were established and time-lapse confocal live-cell imaging studies were conducted in these cell lines to determine if treatment with rifampicin affects the plasma membrane localization of GFP-OATP1B1 and -OATP1B3. The GFP-OATP1B1 and -OATP1B3 proteins are also FLAG-tagged as described in the materials and methods. Expression of GFP-OATP1B1 and -OATP1B3 was confirmed

by immunoblotting with FLAG antibody in HEK293-GFP-OATP1B1 and -OATP1B3 stable cell lines, respectively. FLAG immunoblot in HEK293-Mock cells served as the negative control. The FLAG antibody specifically detected the GFP-OATP1B1 and GFP-OATP1B3 in HEK293-GFP-OATP1B1 and -OATP1B3 (Fig. 4A) cells, respectively, but not in the negative control HEK293-Mock cells. The [^3H]-E $_2$ 17 β G accumulation (1 μM , 2 min) in HEK293-GFP-OATP1B1 and -OATP1B3 cells was 390 and ~34 fold higher than in the HEK293-Mock cells (data not shown). Pretreatment with rifampicin (10 μM) for 0.5 and 1 h significantly decreased OATP1B1- and OATP1B3-mediated [^3H]-E $_2$ 17 β G accumulation (Fig. 4B). Time-lapse confocal fluorescent microscopy demonstrated that at each time point determined, following 10 μM rifampicin treatment (1–61 min in Fig. 4Cb–e and g–j), GFP-OATP1B1 and -OATP1B3 are primarily localized at the plasma membrane, similar to the scenario at 20 min prior to rifampicin treatment (–19 min in Fig 4Ca and f).

Effects of rifampicin on the transport kinetics of E $_2$ 17 β G in HEK293-OATP1B1 and HEK293-OATP1B3 cells

As shown in Fig. 5A, the V_{max} values of E $_2$ 17 β G transport mediated by OATP1B1 are 55.2 ± 1.6 vs. 15.5 ± 2.6 pmol/mg protein/min in vehicle control and rifampicin pretreatment (10 μM , 1 h), respectively (mean $\pm$ SD, $n=3$). The K_m values of E $_2$ 17 β G transport mediated by OATP1B1 are 6.2 ± 0.3 and 9.0 ± 1.1 μM in vehicle control and rifampicin pretreatment (10 μM , 1 h), respectively (mean $\pm$ SD, $n=3$). As shown in Fig. 5B, the V_{max} values of E $_2$ 17 β G transport mediated by OATP1B3 are 15.8 ± 1.6 vs. 7.1 ± 1.4 pmol/mg protein/min in vehicle control and rifampicin pretreatment (10 μM , 1 h), respectively (mean $\pm$ SD, $n=3$). The K_m values of E $_2$ 17 β G transport mediated by OATP1B3 are 6.9 ± 1.7 and 24.4 ± 5.7 μM in vehicle control and rifampicin pretreatment (10 μM , 1 h), respectively (mean $\pm$ SD, $n=3$). Each individual experiment results are shown in supplemental Table S3.

LDH cytotoxicity assay

The LDH assay showed negligible toxicity in HEK293-OATP1B1 and -1B3 cells after 1 h treatment with rifampicin (up to 25 μM) and dasatinib (up to 20 μM) (Supplemental materials Fig. S4).

Prediction of *in vivo* OATP-mediated DDIs

The $C_{\text{max}}/\text{IC}_{50}$ and R-values for rifampicin and dasatinib toward the inhibition of OATP1B1 and OATP1B3 are summarized in Table 1.

The PBPK model of dasatinib successfully described the plasma-concentration profile of dasatinib administered to healthy volunteers after a 100 mg dose³⁵ (Fig. 6A), a 50 mg dose twice daily (Fig. 6B)³⁵ and 100 mg dose once daily on day 15 at steady-state (Fig. S5)³⁵. Although it is known that dasatinib has low aqueous solubility, simulations at these doses were adequate without assuming any dissolution considerations in the intestine. The default pravastatin PBPK model within the SimCYP library well describes the reported clinical pravastatin plasma concentrations³⁶ (Fig. 6C). Using the pre+co K_i values of rifampicin against OATP1B1 (0.24 μM) and OATP1B3 (0.11 μM) (Table 1), the simulated AUC and C_{max} ratios of rifampicin against pravastatin in a Caucasian population are 2.74 and 2.82 fold (Fig. 6C), respectively, comparable to previously reported AUCR and C_{max} ratio of 2.33

and 2.7, respectively, in a clinical study³⁷. DDI prediction of dasatinib against pravastatin was determined assuming that dasatinib only affects OATP1B1 and OATP1B3, which is reasonable as pravastatin only undergoes minor metabolism and has been shown to not interact with typical CYP3A inhibitors such as itraconazole or verapamil³⁸. For all 4 scenarios evaluated in the sensitivity analysis of the OATP K_i values, the AUCR is 1 after a single dose co-administration of dasatinib and pravastatin (Figure 6D) or multiple doses of dasatinib and pravastatin at steady-state after 15 days (data not shown).

DISCUSSION

OATP1B1 and OATP1B3 mediate the hepatic uptake of many important drugs. Assessing the OATP-mediated DDI potential of a therapeutic drug is important in order to help reduce adverse drug events in patients. The current study reports novel findings that pre-incubation with rifampicin and dasatinib decreases OATP1B1- and OATP1B3-mediated transport. It also supports published data for the impact of preincubation with Cs A.

The maximum possible unbound concentration of rifampicin at the inlet to the liver ($I_{u,in,max}$) is 10.7 μ M (Supplemental Table S2). Pretreatment with rifampicin at clinically relevant concentrations below the $I_{u,in,max}$, significantly decreased OATP1B1- and 1B3-mediated [³H]-E₂17 β G transport and OATP1B1-mediated [³H]-E₁S and [³H]-pitavastatin transport (Fig. 1A, B, E and F). Pretreatment with 10 μ M rifampicin also significantly decreased accumulation of [³H]-pitavastatin and [³H]-CCK-8 in a physiologically relevant human SCH model (Fig. 1G). These findings suggest that decreased OATP1B1- and 1B3-mediated transport following rifampicin administration is likely to occur under in vivo conditions. [³H]-CCK-8 is a specific substrate of OATP1B3. Pitavastatin is a substrate of multiple OATP transporters, including OATP1B1, OATP1B3 and OATP2B1^{31,39}. OATP1B1 plays a predominant role in the hepatic uptake of pitavastatin⁴⁰. Although one cannot exclude the possibility that other transporters may also be involved in the decreased accumulation of pitavastatin in human SCH following rifampicin pretreatment, OATP1B1 plays a significant role⁴¹.

Two OATP1B1 substrates, E₂17 β G and E₁S, were used to determine the concentration-dependent rifampicin pretreatment effects on OATP1B1-mediated transport (Fig. 1A, C and E). Using E₂17 β G as the substrate, rifampicin pretreatment yielded significant smaller IC₅₀ values (1.8 ± 0.2 μ M) when compared to using E₁S as the substrate (>10 μ M) (Fig. 1E), supporting that E₂17 β G appear to be a more sensitive substrate than E₁S when assessing the pretreatment effects of rifampicin (10 μ M, 1 h) on OATP1B1-mediated transport, at least under the current conditions. This finding has the same trend as data reported in a previous publication which showed that E₂17 β G^{15,17} is a more sensitive substrate than E₁S to assess the inhibition potency against OATP1B1 under a condition without inhibitor pretreatment. Shitara and co-workers reported that pre-incubation with rifampicin at 1–25 μ M did not significantly affect OATP1B1-mediated [³H]-E₁S transport in an OATP1B1-expressing cell line¹³. Our data is consistent with this report in that 1 μ M rifampicin pretreatment (30 min or 1 h) did not affect OATP1B1-mediated [³H]-E₁S transport (Fig. 1E and supplemental Fig. S3). However, in the current study, rifampicin pretreatment at 10 and 25 μ M significantly decreased OATP1B1-mediated [³H]-E₁S transport (Fig. 1E and supplemental Fig. S3).

Noteworthy, the experimental conditions are different between previous¹³ and current studies. In the previous study, OATP1B1-overexpressing cells were pretreated with rifampicin in serum-free media for 30 minutes, washed and stored at 4°C for more than 30 min before the uptake studies. In the current studies, [³H]-E₁S accumulation was determined immediately following pretreatment with rifampicin in HBSS buffer and subsequent washing without the additional 4°C-incubation step. We conducted experiments to compare the rifampicin pretreatment effects on OATP1B1-mediated [³H]-E₁S transport under our current condition and a condition similar to that published previously (pretreatment in serum-free media, followed by washing and a 40 min-incubation step at 4°C in rifampicin-free buffer)¹³. We confirmed that pretreatment with rifampicin at 10 and 25 μM for 30 min significantly decreased OATP1B1-mediated [³H]-E₁S transport (20 nM, 0.5 min), regardless of whether the 4°C rifampicin-free incubation step was included and what pretreatment media was used (HBSS vs. serum-free medium) (supplemental data Fig. S6). The authors in the previous publication did not specify the exact amount of time that the cells were incubated at 4°C prior to the uptake experiment¹³. The potential difference in the time that the cells were incubated in rifampicin-free buffer at 4°C between the current studies (40 min, Fig. S6) and previous studies (> 30 min) may account for the differential pretreatment effects of rifampicin on OATP1B1-mediated [³H]-E₁S transport. Interestingly, a recent report indicated that when CsA or rifampicin were dosed one day prior to pravastatin administration in rats, increased AUC of pravastatin was observed for CsA but not for rifampicin¹³. The inhibitory effects of CsA pretreatment on OATP1B1-mediated transport can last at least up to 18 h *in vitro* after washing off CsA from the incubation media⁴. This *in vivo* study in rats suggests that rifampicin might not have comparable long-lasting inhibitory effects as CsA on Oatp-mediated transport⁴². The current study determined that at least up to 40 min after washing off rifampicin from the pre-incubation media, the inhibitory effects of rifampicin on OATP1B1-mediated [³H]-E₁S uptake can still be observed (supplemental data Fig. S6). Further studies are warranted to characterize if decreased OATP1B1- and OATP1B3-mediated transport following rifampicin pretreatment *in vitro* occurs in a long-lasting manner, similar to that reported for CsA.

In HEK293–OATP1B1 and -OATP1B3 stable cell lines, we determined that pretreatment with CsA for 1 h decreases the IC₅₀ values of CsA against OATP1B1 (0.37 ± 0.03 vs 0.05 ± 0.01 μM, without vs. with CsA pretreatment, mean ± SE, n=3) and OATP1B3 (0.14 ± 0.02 vs. 0.04 ± 0.008 μM, without vs. with CsA pretreatment, mean ± SE, n=3) (supplemental data Fig. S2). These results are similar to previously reported IC₅₀ values of CsA with and without CsA pretreatment^{3,15}. The CsA data further verifies our experimental system in studying the pretreatment effects on OATP1B1- and 1B3-mediated transport. Using this system, we report that pretreatment with rifampicin resulted in ~3 and ~2.6 fold decrease in IC₅₀ values against OATP1B1 and OATP1B3, respectively (Table 1). The pre+co-incubation IC₅₀ values of rifampicin against OATP1B1 (Table 1) are comparable to the previously reported estimated *in vivo* K_i values of rifampicin against OATP substrates^{12,16}. Our findings on rifampicin are consistent with those reported for CsA in that the IC₅₀ values of CsA determined following CsA pre-incubation are comparable to the estimated *in vivo* K_i values¹⁶. Our studies provided additional evidence to support the notion that that the *in vitro* K_i values of OATP following pre-incubation may be close to the *in vivo* relevant K_i values.

Under both the co-incubation and pre+co-incubation scenarios, the $C_{\max}/IC_{50}$ and R-values for rifampicin are all greater than the U.S. FDA recommended cut-off values of 0.1 and 1.25¹¹, respectively (Table 1), suggesting that our *in vitro* experimental system in HEK293-OATP1B1 and -OATP1B3 cell lines can predict *in vivo* OATP-mediated DDIs at least for the positive control rifampicin^{6,43}. For dasatinib, under the co-incubation scenario, the $C_{\max}/IC_{50}$ and R-values of dasatinib against OATP1B1 and 1B3 are less than the US FDA-recommended cut-off values of 0.1 and 1.25, respectively. Using the pre+co-incubation IC_{50} values increased the $C_{\max}/IC_{50}$ value to greater than 0.1 and increased the AUCR up to 1.24 against OATP1B1 and up to 1.20 against OATP1B3 (Table 1). These R-values under both scenarios are less than the US FDA cut-off value of 1.25, while are greater than the European Medicine Agency (EMA) cutoff value of 1.04 for potential *in vivo* inhibition^{9,10}. We further used PBPK modeling to evaluate the OATP-mediated DDI potential of dasatinib against pravastatin with rifampicin as the positive control. The simulated pravastatin AUC and $C_{\max}$ ratios when co-administered with rifampicin are comparable to that reported previously^{37,43}. The prediction from PBPK modelling shows that the AUCR of pravastatin in the presence of dasatinib is 1 even when using the CsA-calibrated K_i values (Fig. 6C and D). Simulations using PBPK modeling also indicate that in order to observe an approximate 2-fold increase in AUC of pravastatin, OATP1B1 and OATP1B3 K_i values below 0.001 μM would be required for dasatinib (data not shown). Our PBPK modeling results support that the potential for dasatinib to cause OATP-mediated DDIs is low. DDI prediction using the static R-values model using the US FDA cut-off values 1.25 is consistent with our PBPK modeling results, while prediction using the more conservative EMA cut-off values of 1.04 appears to over-estimate the OATP-mediated DDI potential of dasatinib.

In an effort to elucidate the potential mechanism underlying the pretreatment effect on OATP1B1- and OATP1B3-mediated transport, we determined whether rifampicin or dasatinib pretreatment affects the total protein levels and surface protein levels of OATP1B1 and OATP1B3. There were no obvious changes on the total protein levels of OATP1B1 and 1B3 following rifampicin and dasatinib treatment (both 10 μM , 1 h) (Fig. 3). Using a time-lapse confocal fluorescent microscopy approach, our results showed that rifampicin treatment did not cause an obvious change on the expression of GFP-OATP1B1 and -OATP1B3 at the plasma membrane (Fig. 4C). Similar results were observed for dasatinib, in that treatment with dasatinib (20 μM) up to at least 1 h did not cause an obvious change in plasma membrane localization of GFP-OATP1B1 and -OATP1B3 (data not shown). Rifampicin pretreatment (10 μM , 1 h) markedly decreased the $V_{\max}$ values of E₂17 β G transport mediated by OATP1B1 and OATP1B3 to ~3.5 and ~2.2 fold of control treatment, respectively (Fig. 5A and B). Rifampicin pretreatment did not apparently affect the K_m values of OATP1B1 (~1.5 fold) (Fig. 5A), however, increased the K_m values of OATP1B3 to ~3.5 fold of control (Fig. 5B). An increase in the K_m values of a transporter following pretreatment with stimuli has been reported previously for OATP1B1 following CsA pretreatment⁴. Decreased $V_{\max}$ of transporters following pretreatment was also reported previously⁴⁴⁻⁴⁷. Decreased $V_{\max}$ of transporters can be associated with decreased plasma membrane levels of the transporters in some studies⁴⁴⁻⁴⁶, while can also be associated with a decreased turn-over rate without altering the localization of the transporter on the membrane⁴⁷. Currently, the underlying mechanism of the decreased transport function of

OATP1B1 and OATP1B3 following pre-incubation with rifampicin or dasatinib remains unknown. The type of inhibition due to pre-incubation with rifampicin or dasatinib has not been characterized. In addition, it is not known whether the same mechanism(s) are involved in the pretreatment effects on OATP1B1- and OATP1B3-mediated transport. For the PBPK simulations, the assumption is that both compounds are competitive inhibitors at the same binding site of the transporter. Our data suggests that a change in total protein levels or plasma membrane localization of OATP1B1 and OATP1B3 does not seem to be the main reason. In addition to being inhibitors of OATP1B1 and OATP1B3, dasatinib and rifampicin are also substrates of OATP1B1⁴⁸ and/or OATP1B3^{48,49}. We further determined the effects of pretreatment with unlabeled E₂17βG, a substrate and competitive inhibitor of OATP1B1 and OATP1B3², on OATP1B1- and OATP1B3-mediated [³H]-E₂17βG uptake. Unlike the results observed for rifampicin and dasatinib, pretreatment with unlabeled E₂17βG (1–30 μM, 0.5 and 1 h) only slightly decreases or has no effect on OATP1B1- and OATP1B3-mediated transport of [³H]-E₂17βG in HEK293-OATP1B1 and –OATP1B3 cells (supplemental results Fig. S7). Thus, the pretreatment effects on OATP1B1- and OATP1B3-mediated transport does not seem to be generalized to E₂17βG, a dual inhibitor and substrate of OATP1B1 and 1B3, at least under the current experimental conditions. We reported previously that decreased OATP1B3-mediated transport following short-term pretreatment with a protein kinase C (PKC) activator is associated with increased phosphorylation of OATP1B3 but not changes in the plasma membrane localization of OATP1B3²⁶. It is also possible that residual inhibitors may be bound to the transporters following pretreatment even after washing. The fact that the effects on OATP1B1- and 1B3 transport is not significant for dasatinib pretreatment may be due to the lower inhibitory potency of dasatinib when compared to rifampicin. Further studies are warranted to characterize the potential mechanism(s) underlying the pre-incubation effects of rifampicin, CsA and dasatinib on OATP1B1- and OATP1B3-mediated transport, including potential cellular signaling pathway(s) that may be involved. This information will ultimately aid in interpreting the clinical relevance of the pre-incubation effects of OATP inhibitors on OATP-mediated transport.

In conclusion, for the first time, we report that pretreatment with rifampicin significantly reduces the IC₅₀ values against OATP1B1 and OATP1B3. Decreased IC₅₀ values were also observed for a weak inhibitor dasatinib following pretreatment, although such decrease did not reach a statistically significant level. In the *in vivo setting*, a perpetrator drug may co-exist with a victim drug in the blood for a longer period of time than what is usually used in co-incubation assays (often a few minutes). Patients may also take the perpetrator drug prior to the victim substrate drug. Thus, adding a pre-incubation step prior to co-incubation when determining the *in vitro* K_i may help to mimic the clinical situation described above, and to yield *in vitro* K_i values close to the *in vivo* K_i values. The current studies predict that dasatinib has a low potential to cause hepatic OATP1B-mediated and clinically significant DDIs. Although adding a pre-incubation step in current studies does not affect the OATP-mediated DDI prediction for dasatinib, a weak OATP inhibitor, this approach may help to mitigate false-negative prediction for compounds with intermediate inhibition potency and warrants further studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AUC	area under the plasma concentration-time curve
BSA	bovine serum albumin
CsA	cyclosporine A
DDI	drug-drug interaction
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
E₁S	estrone-3-sulfate
FBS	fetal bovine serum
HBSS	Hanks' balanced salt solution
IC₅₀	inhibitor concentration to produce 50% inhibition
K_i	inhibition constant
K_{i,u}	unbound concentration-based inhibition constant
K_m	Michaelis constant
OATP	organic anion-transporting polypeptide
TBS-T	Tris-buffered saline containing 0.05% Tween 20

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Fig. 1. Effects of rifampicin on OATP1B1- and OATP1B3-mediated transport in HEK293 stable cell lines and on $[^3\text{H}]$ -pitavastatin and $[^3\text{H}]$ -CCK-8 accumulation in human SCH

Model-estimated fold change and associated SE of $[^3\text{H}]$ -E₂17βG accumulation (1 μM, 2 min) vs. CTL in HEK293-OATP1B1 (A) and HEK293-OATP1B3 (B), and $[^3\text{H}]$ -pitavastatin accumulation (1 μM, 0.5 min) (F) vs. CTL in HEK293-OATP1B1 cells. Linear mixed effects models were fit to the data of (A), (B) and (F) as described in the “Materials and Methods” (n=3 in triplicate). * indicates a statistically significant difference (Bonferroni-adjusted $p < 0.05$) vs. CTL. OATP1B1 (C)- and OATP1B3 (D)-mediated $[^3\text{H}]$ -E₂17βG (1 μM, 2 min) accumulation is expressed as percentage of vehicle control in pre-incubation (open squares), co-incubation (closed circles) and pre+co-incubation (open circles) scenarios. Data represents mean $\pm$ SE (n=3 in triplicate). The IC₅₀ values were determined by fitting dose-response curves to the data by nonlinear regression analysis. Solid (co-incubation) and dashed lines (pre+co-incubation) represent the fitted lines. (E) OATP1B1-mediated accumulation of $[^3\text{H}]$ -E₂17βG (1 μM, 2 min) (open squares) and $[^3\text{H}]$ -E₁S (20 nM, 0.5 min) (open triangles) following 1 h rifampicin pretreatment at indicated concentrations was expressed as percentage of vehicle control treatment. The same 1 h pretreatment data in Fig. 1C is replotted in Fig. 1E for the purpose of comparison between E₂17βG and E₁S. Lines represent the fitted IC₅₀ curves. * indicates a statistically significant difference ($p < 0.05$) vs. respective vehicle CTL pretreatment by one-way ANOVA followed by Dunnett’s test. (G) Model estimated fold change in accumulation of $[^3\text{H}]$ -pitavastatin (1 μM, 0.5 min) and $[^3\text{H}]$ -

CCK-8 (1 μ M, 3 min) in human SCH pre-incubated with rifampicin (10 μ M, 1 h) vs. vehicle control (CTL). For each substrate, a generalized linear mixed model was fit to the data as described in the “Materials and Methods” (n=3 donors in triplicate). * indicates a statistically significant difference ($p<0.05$) vs. CTL.

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Fig. 2. Effects of dasatinib on HEK293-OATP1B1 and -OATP1B3-mediated transport in HEK293 stable cell lines

Model-estimated fold change and associated SE of [^3H]-E $_2$ 17 β G accumulation (1 μM , 2 min) vs. CTL in HEK293-OATP1B1 (A) and HEK293-OATP1B3 (B) cells. Linear mixed effects models were fit to the data of (A) and (B) as described in the “Materials and Methods” (n=3 in triplicate). * indicates a statistically significant difference (Bonferroni-adjusted $p < 0.05$) vs. CTL. OATP1B1 (C)- and OATP1B3 (D)-mediated [^3H]-E $_2$ 17 β G accumulation is expressed as percentage of vehicle control in pre-incubation (open squares), co-incubation (closed circles) and pre+co-incubation (open circles) scenarios. Data represents mean $\pm$ SE (n=3 in triplicate). The IC $_{50}$ values were determined by fitting dose-response curves to the data by nonlinear regression analysis, and are summarized in Table 1. Solid (co-incubation) and dashed (pre+co-incubation) lines represent the fitted lines.

Fig. 3. Immunoblot of OATP1B1 and OATP1B3 in HEK293-OATP1B1 and -OATP1B3 cell lines Immunoblot of OATP1B1 and OATP1B3 in HEK293-OATP1B1 and -OATP1B3 stable cell lines following 1 h treatment with (A) rifampicin (10 μ M) (B) dasatinib (10 μ M) or vehicle control. β -actin was used as loading control. Fold change vs. control (CTL) is expressed as mean $\pm$ SE versus control (n=3 in triplicate). Representative WB images from 3 separate experiments are shown.

Fig. 4. Effects of rifampicin pretreatment on [3H]-E₂17βG accumulation and on plasma membrane localization of GFP-OATP1B1 and -OATP1B3 in HEK293-GFP-OATP1B1 and -OATP1B3 cell lines

(A) Immunoblot of FLAG in HEK293-GFP-OATP1B1 and -OATP1B3 stable cell lines with HEK293-Mock cells as the negative control. β-actin was used as a loading control. (B) Model-estimated fold change and associated SE in [3H]-E₂17βG accumulation (1 μM, 2 min) vs. CTL in HEK293-GFP-OATP1B1 (GFP-OATP1B1) and HEK293-GFP-OATP1B3 (GFP-OATP1B3) cells pre-incubated with rifampicin (10 μM) or vehicle control for 0.5 and 1 h. A generalized linear mixed model was fit to the data as described in the “Materials and Methods” (n=3 in triplicate). * indicates a statistically significant difference (Bonferroni-adjusted *p* < 0.05) vs. CTL. (C) Time-lapse confocal fluorescent live-cell images in HEK293-GFP-OATP1B1 and -OATP1B3 cells. The “zero” time point indicates the addition of rifampicin to the cell culture medium to a final concentration of 10 μM. Representative images are shown from at least three independent experiments.

Fig. 5. Effects of rifampicin on the kinetic parameters of OATP1B1- and OATP1B3-mediated E₂17βG transport

The concentration-dependent accumulation of [³H]-E₂17G (0.1–40 μM, 2 min) was determined in HEK293-OATP1B1, -OATP1B3 and –Mock cells pre-treated with rifampicin (10 μM, 1 h) or vehicle CTL. Values of [³H]-E₂17G accumulation in Mock cells were subtracted from those in HEK293-OATP1B1 and -OATP1B3 cells. Solid and dashed lines represent the best fit lines of the Michaelis–Menten equation to the data of CTL- (black circles) and rifampicin (10 μM, 1 h)-pretreatment (white circles), respectively. Representative graph of three independent experiments in triplicate is shown.

Fig. 6. Simulated versus observed plasma concentration-time profiles of dasatinib and pravastatin in healthy volunteers following oral administration

(A) Dasatinib 100 mg single dose and (B) dasatinib 50 mg twice daily. The grey thin lines represent simulated individual trials (50) of 10 subjects using a population of 500 virtual subjects (10% female, 23–59 years) and 50 trials of 21 male subjects using a population of 1050 virtual subjects (19–47 years) for figure A and B, respectively. The black thin lines represent the upper (95th) and lower (5th) percentiles and the thick black line represents the simulated mean of the healthy volunteers population ($n=500$ for A and $n=1050$ for B). The circles denote mean values from the clinical study by Yago *et al.*, 2014³⁵ and Eley *et al.*, 2009⁵⁰ for figure A and B, respectively. (C) Simulated versus observed plasma concentrations of pravastatin following co-administration of a single dose pravastatin (40 mg) with placebo (black line), rifampicin (600 mg) (black dashed line) or dasatinib (100 mg) (grey dashed line). Lines represent the mean of simulated virtual populations of 400 healthy volunteers (50% female, 20–50 years). Note that the pravastatin placebo simulation and the pravastatin-dasatinib simulation have a significant overlap. (D) Predicted AUCR for the interaction of dasatinib on pravastatin based on a sensitivity analysis of estimated OATP inhibition data. Key from right to left: open triangle (▲) – K_i values based on experimental IC_{50} determined without pre-incubation, closed square (■) – K_i values based on experimental IC_{50} determined with pre-incubation, open square (□) – K_i values based on experimental IC_{50} determined with pre-incubation as $IC_{50}/2$, closed circle (●) – K_i values based on experimental IC_{50} determined with pre-incubation and calibrated using CsA.

Inhibitory effects of rifampicin and dasatinib on OATP1B1- and OATP1B3-mediated uptake of [^3H]-E₂17βG (1 μM, 2 min) in HEK293-OATP1B1 and -OATP1B3 stable cell lines. C_{max}/IC₅₀ and R-values were determined as described in the “Materials and Methods”. IC₅₀ values are expressed as parameter estimates ± parameter S.E. obtained from the output of GraphPad Prism 7.0

Table 1

		Co-Incubation			Pre+Co-Incubation		
		IC ₅₀ (μM)	C _{max} /IC ₅₀	R (range)	IC ₅₀ (μM)	C _{max} /IC ₅₀	R (range)
Rifampicin	OATP1B1	0.74 ± 0.08	25.7	7.6–15.4	0.24 ± 0.03	85.1	21.2 – 45.5
	OATP1B3	0.26 ± 0.03	88.5	19.7–42.1	0.11 ± 0.01	208.5	45.1 – 98.1
Dasatinib	OATP1B1	4.81 ± 1.7	0.06	1.04 – 1.12	2.33 ± 0.86	0.11	1.07 – 1.24
	OATP1B3	5.76 ± 1.1	0.06	1.03 – 1.10	2.75 ± 1.17	0.13	1.06 – 1.20

Range of R represents values obtained using ka values of 0.03⁵¹–0.10⁵²/min and 0.02⁵³–0.1⁵⁴/min for dasatinib and rifampicin, respectively.

Table 2

Parameters values used for the dasatinib PBPK simulations

Dasatinib	Values	Reference/Comments
Dose (mg)	100	
Chemical structure
<i>Physicochemical and blood binding properties</i>		
MW (g/mol)	488.01	
Log P _{O,w}	1.8	Ref. ⁵⁵
Compound type	Diprotic base	
pK _{a1}	6.8	Ref. ⁵⁶
pK _{a2}	10.8	Ref. ⁵⁶
blood-to-plasma ratio	1.8	Experimental value in Ref. ⁵⁷
f _u	0.04	Ref. ²⁰
Main binding protein	Human serum albumin	
<i>Absorption</i>		
f _a	0.781	Meta-analysis, Refs. ^{52,58}
k _a (h ⁻¹)	6	Ref. ⁵²
f _{u,gut}	1	Assumed
Q _{gut} (L/h)	7.71	Predicted by SimCYP
P _{eff,man} (x10 ⁻⁴ cm/s)	1.5	Metoprolol human permeability was used to estimate dasatinib P _{eff,man} in Ref. ⁵⁵
<i>Distribution (minimal PBPK)</i>		
V _{ss} (L/kg)	4.2	Obtained from allometric scaling in Ref. ⁵⁷
<i>Elimination</i>		
CL _{PO} (L/h)	449.4	Observed value in Ref. ³⁵ (CV% 106.5)
CL _R (L/h)	0.4	Observed value in Ref. ⁵⁸

P_{O:w}: neutral species octanol:buffer partition coefficientQ_{gut}: the Q_{gut} value is a drug-dependent flow rate for overall delivery of drug to the gutf_a: fraction available for absorption from dosage formk_a: first-order absorption rate constantf_{u,gut}: unbound fraction of drug in enterocytesP_{eff,man}: human jejunum effective permeability in human 3