Interaction of Novel Platelet-Increasing Agent Eltrombopag with Rosuvastatin via Breast Cancer Resistance Protein in Humans

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ABSTRACT

Eltrombopag (ELT), an orally available thrombopoietin receptor agonist, is a substrate of organic anion transporting polypeptide 1B1 (OATP1B1), and coadministration of ELT increases the plasma concentration of rosuvastatin in humans. Since the pharmacokinetic mechanism(s) of the interaction is unknown, the present study aimed to clarify the drug interaction potential of ELT at transporters. The OATP1B1-mediated uptake of ELT was inhibited by several therapeutic agents used to treat lifestyle diseases. Among them, rosuvastatin was a potent inhibitor with an IC50 of 0.05 μM, which corresponds to one-seventh of the calculated maximum unbound rosuvastatin concentration at the inlet to the liver. Nevertheless, a simulation study using a physiologically based pharmacokinetic model predicted that the effect of rosuvastatin on the pharmacokinetic profile of ELT in vivo would be minimal. On the other hand, ELT potently inhibited uptake of rosuvastatin by OATP1B1 and human hepatocytes, with an IC50 of 0.1 μM. However, the results of the simulation study indicated that inhibition of OATP1B1 by ELT can only partially explain the clinically observed interaction with rosuvastatin. ELT also inhibited transcellular transport of rosuvastatin in MDCKII cells stably expressing breast cancer resistance protein (BCRP), and was found to be a substrate of BCRP. The interaction of ELT with rosuvastatin can be almost quantitatively explained on the assumption that intestinal secretion of rosuvastatin is essentially completely inhibited by ELT. These results suggest that BCRP in small intestine may be the major target for interaction between ELT and rosuvastatin in humans.

Introduction

Eltrombopag (ELT) is an orally available, small-molecular, nonpeptide thrombopoietin receptor agonist (Erickson-Miller et al., 2004; Sellers et al., 2004; Bussel et al., 2007; Jenkins et al., 2007) that has been approved worldwide (Promacta/Revolade) for the treatment of idiopathic thrombocytopenic purpura (ITP). ELT interacts with the transmembrane domain of the thrombopoietin receptor (Erickson-Miller et al., 2009) and activates intracellular signal transduction pathways, leading to stimulation of the proliferation and differentiation of megakaryocytes and progenitor cells in bone marrow, thereby resulting in an increase of platelets in the circulating blood. Further clinical trials of ELT are ongoing for treatment of cancer chemotherapy-induced thrombocytopenia and hepatitis C-induced thrombocytopenia. Thus, the potential clinical importance of ELT is substantial.

Due to the decrease in platelets, ITP induces severe symptoms, including intracerebral bleeding. ELT is expected to prevent such bleeding symptoms by inducing a recovery of platelet numbers. On the other hand, an overdose of ELT may lead to activation of the blood coagulation system, which in turn may promote thromboembolism (Cheng et al., 2011). Therefore, it is important to maintain the systemic plasma concentration of ELT at the optimum level. In addition, pharmacotherapy with ITP requires long-term treatment (Stasi and Provan, 2004). This may increase the likelihood that ELT will be coadministered with other therapeutic agents. Thus, it is very important to consider potential drug interactions that might unexpectedly increase or decrease the systemic concentration of ELT. Nevertheless, only limited information is available on pharmacokinetic mechanism(s) of ELT and the potential for interaction with other drugs.

ELT is minimally excreted into urine after oral administration in humans, suggesting that the liver is the major clearance organ (Bauman et al., 2011). According to our previous study, hepatic uptake could be the rate-limiting process in the overall elimination of
ELT (Takeuchi et al., 2011). Hepatic uptake of ELT is mediated at least in part by organic anion transporting peptide 1B1 (OATP1B1) (Takeuchi et al., 2011), although the contribution of this transporter to hepatic ELT uptake remains to be precisely clarified. Nevertheless, certain drugs such as gemfibrozil, rifampicin, and cyclosporin A are known to interact in a clinically significant way with OATP1B1-mediated hepatic uptake of therapeutic agents in humans (Kalliokoski et al., 2010). OATP1B1 recognizes a wide range of substrate drugs, including antihyperlipidemic agents, such as hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase inhibitors and antidiabetic agents such as regaplinide and nateglinide (Kalliokoski et al., 2008; Kitamura et al., 2008). Therefore, these drugs may also inhibit OATP1B1-mediated hepatic uptake of other substrate drugs, including ELT. It should be noted that these drugs are commonly used for the treatment of lifestyle diseases and thus are quite likely to be coadministered with ELT in the clinical situation. Thus, it is important to quantitatively assess the interaction potential of these drugs with hepatic uptake of ELT via transporters.

Interaction potential of ELT as a perpetrator drug with other therapeutic agents has already been clinically reported. Allred et al. (2011) reported that the plasma concentration of rosuvastatin was increased by concomitant administration of ELT, whereas rosuvastatin did not affect the plasma concentration of ELT. ELT can act as an inhibitor of OATP1B1, at least in gene-transfected cell lines in vitro (Takeuchi et al., 2011). Because rosuvastatin is mainly eliminated from the liver (Martin et al., 2003), and its hepatic uptake process is primarily mediated by OATP1B1 (Kitamura et al., 2008), the interaction by ELT is considered to involve the OATP1B1-mediated hepatic uptake process, although the details remain unclear.

Drug interactions could thus be a critical issue in clinical use of ELT. Quantitative assessment of possible interactions between ELT and concomitantly administered drugs is urgently required to ensure safe administration of ELT to patients. In the present study, we first investigated the inhibitory effect of various therapeutic agents, including statins, on OATP1B1-mediated uptake of ELT. Physiologically based pharmacokinetic (PBPK) analysis was performed to quantitatively assess the interaction via OATP1B1 in humans. We focused particularly on the clinically reported interaction of ELT with rosuvastatin (Allred et al., 2011). However, although ELT potently inhibits OATP1B1-mediated uptake of rosuvastatin in human hepatocytes in vitro, PBPK analysis indicated that such an interaction is unlikely to be significant in vivo. Instead, ELT was found to be a potent inhibitor of breast cancer resistance protein (BCRP), an ATP binding cassette (ABC) efflux transporter. Our results indicate that BCRP in the small intestine could be the major site of interaction by ELT.

Materials and Methods

ELT was synthesized by Nissan Chemical Industries, Ltd. (Tokyo, Japan). Rosuvastatin was purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada). Rhodamine 123 was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Cryopreserved human hepatocytes (H1000.H15T, #694, from a 29-year-old male Caucasian; XenoTech, Lenexa, KS) were obtained from Sekisui Medical (Tokyo, Japan). A cryopreserved hepatocyte purification kit (454500) was purchased from Becton Dickinson (Franklin Lakes, NJ). All other chemicals and reagents were of analytical grade and were obtained from commercial sources.

Animals. Six- to 9-week-old male CD1/Ltk−/− and FVB mice were purchased from Taconic (Germantown, NY) and CLEA Japan Inc. (Tokyo, Japan), respectively. The mice were kept in a temperature- and light-controlled environment with standard food and tap water provided ad libitum. Animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals at the Takara-machi Campus of Kanazawa University.

Pharmacokinetic Studies in Mice. Mice were fasted overnight with free access to water and anesthetized with diethyl ether during drug administration and blood sampling. ELT was dissolved in saline containing 5 μM human serum albumin (HSA) to obtain a final concentration of 0.4 mg/ml and was orally administered by gavage (2 mg/kg body weight). This concentration of albumin fully suppresses nonspecific adsorption of ELT on the experimental apparatus (Takeuchi et al., 2011). Rosuvastatin was prepared in 0.5% hydroxypropyl methylcellulose oral administration by gavage (10 mg/5 ml per kilogram body weight) and in saline containing 5 μM HSA for intravenous administration via the tail vein (4 mg/2 ml per kilogram body weight). At various intervals after administration, blood samples were collected through the caudal vein. All blood samples were immediately centrifuged to obtain plasma, which was used for quantitation.

In the closed loop study, mice were fasted for approximately 6 hours with free access to water and were anesthetized with an intraperitoneal injection of pentobarbital. The abdomen was opened, and a 10-cm closed loop from just below the duodenal papilla was prepared by ligating both ends of the gut. ELT (2 mg/l ml per kilogram body weight) or water (1 ml/kg body weight) was injected into the intestinal loop. At 1 minute after the administration, rosuvastatin (10 mg/5 ml per kilogram body weight) was also injected into the intestinal loop. The intestinal loop was returned to the abdominal cavity, and the abdomen was closed with sutures. At various intervals after rosuvastatin administration, blood samples were collected through the jugular vein, followed by immediate centrifugation to obtain plasma. The body temperature was maintained by placing the animals on a thermostated heating pad.

Cell Culture and Transport Studies in HEK293 Cells Expressing OATP1B1 and Organic Cation Transporter 1. HEK293 cells stably expressing full-length OATP1B1 (HEK293/OATP1B1 cells) were previously constructed (Fujita et al., 2014) and HEK293/organic cation transporter 1 (OCT1) cells were constructed in the present study by transfecting HEK293 cells with pcDNA3 vector (Invitrogen, Carlsbad, CA) into which the full-length human OCT1 gene had been subcloned, using the calcium phosphate precipitation method. HEK293/OCT1 cells were then selected by adding geneticin (1 mg/ml; Wako Pure Chemical Industries, Ltd., Osaka, Japan) to the medium and were grown in Dulbecco’s modified Eagle’s medium without l-glutamine or phenol red (Wako Pure Chemical Industries, Ltd.) containing 10% fetal bovine serum, penicillin, streptomycin, and geneticin in a humidified incubator at 37°C under an atmosphere of 5% CO2 in air. After the HEK293/OCT1 cells had reached confluence, they were harvested and suspended in ice-cold transport buffer (pH 7.4; Sugita et al., 2010). The uptake experiment for ELT in HEK293/OATP1B1 was performed in the presence of 5 μM HSA, and ELT uptake was measured according to the silicone oil layer method (Sugita et al., 2010). Free fractions of ELT, rosuvastatin, and atorvastatin in the transport buffer containing 5 μM HSA were determined by equilibrium dialysis method using the BD Gentest Serum Binding System (Becton Dickinson). The uptake experiment for rosuvastatin was performed in the absence of HSA, and the ELT concentration in the medium was directly measured by liquid chromatography–tandem mass spectrometry (LC-MS/MS). In the inhibition study, the inhibitor was added to cell suspension simultaneously with the substrate. In the case of HEK293/OCT1 cells, they were cultured in poly (l-lysine)-coated 12-well plates and were directly used for the uptake study (Takeuchi et al., 2011). The cellular protein content was determined according to the Bradford method using a protein assay kit (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as the standard.

Transport Studies in Human Cryopreserved Hepatocytes. Cryopreserved human hepatocytes were prepared using a cryopreserved hepatocyte purification kit. The hepatocytes were resuspended in the transport buffer to give a cell density of 1.0 × 106 cells/ml. We checked cell viability by means of a trypan blue exclusion test and used hepatocytes showing more than 90% viability. The uptake experiment was then performed according to the silicone oil layer method (Takeuchi et al., 2011) with some modifications. In brief, 350 μl cell suspension was preincubated for 5 minutes at 37°C, and the reaction was then started by mixing the suspension with an equal volume of prewarmed transport buffer containing rosuvastatin with or without ELT. At appropriate times, 200-μl aliquots of the mixture were withdrawn and quickly centrifuged through a silicone oil layer (density, 1.015) to separate the cells from the transport buffer. Then, 50-μl aliquots of the supernatant (upper layer) were immediately removed and mixed with an equal volume of the transport buffer containing 0.5% (v/v)
Twee 80 to avoid nonspecific adsorption of ELT. The lower layer was incubated overnight in alkali (0.75 N KOH) to dissolve the hepatocytes. Both the upper and lower layers were stored at −30°C until LC-MS/MS analysis for determination of rosuvastatin uptake and medium concentration of ELT.

Transport Studies with MDCKII/BCRP/PDZK1 Cells. MDCKII cells stably expressing both BCRP and PDZK1 (MDCKII/BCRP/PDZK1 cells) and those stably expressing PDZK1 alone (MDCKII/mock/PDZK1) were previously obtained (Shinnizu et al., 2011), and were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, penicillin, streptomycin, 1 mg/ml geneticin, and 0.2 mg/ml zeocin in a humidified incubator at 37°C under an atmosphere of 5% CO2 in air. MDCKII/BCRP/PDZK1 and MDCKII/mock/PDZK1 cells were seeded in Transwell polycarbonate inserts (3 μm pore size, 12 mm diameter; Corning Life Sciences, Edison, NJ) at a density of 3 × 105 cells/well. After 3 days of culture, the cell monolayers were washed twice with the transport buffer including 5 μM HSA. The same buffer also containing ELT or rosuvastatin with or without inhibitors was added to the basal (BL) or apical (AP) chamber. At the designated times, a 100-μl aliquot was sampled from the opposite side and replaced with an equal volume of prewarmed fresh buffer.

The efflux ratio (ER) was calculated as the ratio of the apparent permeability coefficient (Papp) in the BL-to-AP direction to that in the AP-to-BL direction, where Papp was calculated as the slope of the regression line in the transport-normalized by both cellular protein amount and substrate concentration in the lysed with 0.2 N NaOH, and rosuvastatin in the cell lysate and medium was 490 and 530 nm, respectively. To determine uptake of rosuvastatin, cells were assayed with 0.25 mg/ml cimetidine and ranitidine for 6 hours.

Uptake Studies with LLC-PK1 and LLC-GA5-COL150 Cells. LLC-PK1 cells and LLC-GA5-COL150 cells stably expressing P-glycoprotein (Tanigawara et al., 1992, Ueda et al., 1992) were obtained from Riken Cell Bank (Tsukuba, Japan), and were cultured and grown in medium 199 containing 10% fetal calf serum, penicillin, and streptomycin in a humidified incubator at 37°C under an atmosphere of 5% CO2 in air. For LLC-GA5-COL150 cells, 150 mg/ml colchicine was added to ensure stable expression of P-glycoprotein. LLC-PK1 cells and LLC-GA5-COL150 cells were seeded on 24-well plates at densities of 0.5 × 105 cells/well and 5.7 × 105 cells/well, respectively. After 3 days of culture, LLC-GA5-COL150 cells were cultured without colchicine for 6 hours. LLC-PK1 cells and LLC-GA5-COL150 cells were then washed twice with the transport buffer and preincubated with the same buffer for 30 minutes. After the preincubation, the transport buffer containing substrate and inhibitor with 5 μM HSA was added and incubation was continued for 90 minutes. Cells were then washed twice with ice-cold buffer. To determine uptake of rhodamine 123, cells were lysed with 10 mM KH2PO4 buffer (pH 7.4) containing 0.1% Triton HSA. The same buffer also containing ELT was added. Cells were then incubated for 90 minutes. Cells were then solubilized with 0.2 N NaOH, and ELT and rosuvastatin in the cell lysate and medium was 490 and 530 nm, respectively. To determine uptake of rosuvastatin, cells were assayed with 0.25 mg/ml cimetidine and ranitidine for 6 hours.

Pharmacokinetic parameters for ELT and rosuvastatin

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ELT</th>
<th>Rosuvastatin</th>
</tr>
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<tbody>
<tr>
<td>Fb – Fa</td>
<td>0.56</td>
<td>0.543</td>
</tr>
<tr>
<td>kfb (min⁻¹)</td>
<td>0.0084</td>
<td>0.0046</td>
</tr>
<tr>
<td>Rb</td>
<td>0.718</td>
<td>0.69</td>
</tr>
<tr>
<td>fA</td>
<td>0.002</td>
<td>0.12</td>
</tr>
<tr>
<td>Vd (liters)</td>
<td>2.94</td>
<td>245</td>
</tr>
<tr>
<td>PSun (ml/min)</td>
<td>3250</td>
<td>10,100</td>
</tr>
<tr>
<td>Cmax (ml/min)</td>
<td>0</td>
<td>330</td>
</tr>
<tr>
<td>Qb (ml/min)</td>
<td>1450</td>
<td></td>
</tr>
<tr>
<td>Vnumax (ml)</td>
<td>469</td>
<td></td>
</tr>
<tr>
<td>Vnumax (ml)</td>
<td>1690</td>
<td></td>
</tr>
</tbody>
</table>

Cited from Martin et al. (2003).

Estimated by fitting in the present study.

Cited from Davies and Morris (1993).

Cited from Watanabe et al. (2009).
since both compounds are primarily distributed to the liver (Martin et al., 2003; Bauman et al., 2011); 2) hepatic elimination is primarily mediated by the influx process without back flux from the liver to extravascular space, since hepatic uptake is rate-limiting for both compounds (Takeuchi et al., 2011; Jones et al., 2012); and 3) the plasma concentration profile is evaluated over a sufficiently short time period that enterohepatic circulation can be neglected. The fraction orally absorbed (F<sub>p</sub>, F<sub>i</sub>), blood-to-plasma partition coefficient (R<sub>b</sub>), plasma unbound fraction, and nonhepatic clearance of rosuvastatin and ELT were obtained from the literature (Martin et al., 2003; U.S. Food and Drug Administration [http://www.accessdata.fda.gov/drugsatfda_docs/nda/2008/022291s000_TOC.cfm]) (Table 1). The volume of extracellular space in liver and volume of liver were also taken from the literature (Davies and Morris, 1993; Watanabe et al., 2009) (Table 1). The k<sub>ur</sub>, distribution volume (V<sub>r</sub>), and intrinsic clearance for hepatic uptake (P<sub>Sinf</sub>) were estimated in the present study by simultaneous nonlinear least-squares fitting of the model to the blood concentration profiles of each compound (without coadministered drugs) reported previously (Allred et al., 2011; Deng et al., 2011) using the Napp nonlinear regression analysis program (version 2.3.1 for Macintosh OS-X; The University of Tokyo Hospital, Tokyo, Japan), where the blood concentration was obtained as the product of plasma concentration and R<sub>b</sub>.

To simulate plasma concentration profile in the presence of coadministered drug, the apparent intrinsic clearance for hepatic uptake (P<sub>Sinf,app</sub>) was defined according to eq. 4:

\[
P_{\text{Sinf,app}} = \frac{P_{\text{Sinf}}}{(1 + f_u \cdot C_{\text{ur}},R_b)}
\]

where f<sub>u</sub> represents the plasma unbound fraction and K<sub>i</sub> represents the inhibition constant and. In deriving eq. 4, we assumed that hepatic uptake is primarily mediated by OATP1B1, which is inhibited by the coadministered drug. Therefore, K<sub>i</sub> was set to be the IC<sub>50</sub> obtained from the uptake study in HEK293/OATP1B1 cells. When we simulated the inhibition of intestinal secretion by coadministered drug, F<sub>i</sub> - F<sub>p</sub> was assumed to be close to unity. The simulation was performed using the Napp program.

Results

Effects of Therapeutic Agents on OATP1B1-Mediated ELT Uptake. The inhibitory effects of various types of drugs, including those for ITP and lifestyle diseases, on ELT uptake by OATP1B1 were examined in HEK293/OATP1B1 cells. OATP1B1-mediated uptake of ELT was decreased in the presence of rosuvastatin, atorvastatin, repaglinide, nateglinide, glibenclamide, and chlorothromycin (Fig. 1). Inhibition by rosuvastatin and atorvastatin was particularly potent, and they had the lowest IC<sub>50</sub> values (Table 2). Inhibitory effects of repaglinide, nateglinide, glibenclamide, and chlorothromycin were less potent, but appeared to be concentration dependent (Fig. 1). Note that the IC<sub>50</sub> values could be apparent values since the inhibition study was performed in the presence of 5 μM HSA (see Materials and Methods), which is essential to assess OATP1B1-mediated uptake of ELT to minimize nonspecific adsorption (Takeuchi et al., 2011). Therefore, the unbound fraction of rosuvastatin and atorvastatin in the transport buffer containing HSA was measured by equilibrium dialysis, and the IC<sub>50</sub> in terms of the unbound concentration was also determined. The obtained IC<sub>50</sub> values for rosuvastatin and atorvastatin were 47 and 28 nM, respectively (Table 2).

Assessment of Drug Interaction Potential by Therapeutic Agents. We next attempted to assess possible inhibition of OATP1B1-mediated ELT uptake by these therapeutic agents in vivo, using both static and dynamic (PBPK) models. In the static model, the IC<sub>50</sub> obtained in the present study was compared with C<sub>max,sys</sub> and C<sub>max,pv</sub> (Table 2). For all drugs except rosuvastatin and atorvastatin, both C<sub>max,sys</sub> and C<sub>max,pv</sub> were much lower than the IC<sub>50</sub> value (Table 2). On the other hand, unbound C<sub>max,pv</sub> of rosuvastatin and atorvastatin was higher than the IC<sub>50</sub> value defined in terms of the unbound concentration (Table 2), indicating possible inhibition of OATP1B1-mediated hepatic uptake of ELT in vivo. However, this was in conflict with the clinical finding that the plasma concentration of ELT was not affected by rosuvastatin (Allred et al., 2011). Therefore, to estimate drug interaction potential more quantitatively, we simulated plasma concentration-time profiles based on the PBPK model (Supplemental Fig. 1). First, the PBPK model was fitted to plasma concentrations after oral administration of ELT alone (75 mg; Deng et al., 2011) to estimate k<sub>ur</sub>, V<sub>r</sub>, and P<sub>Sinf</sub> of ELT (shown in Table 1). The fitted line was almost superimposed on the observed data (Fig. 2A, solid line). Similarly, the PBPK model was fitted to the plasma concentration profile after oral administration of rosuvastatin alone (10 mg; Allred et al., 2011) to estimate k<sub>ur</sub>, V<sub>r</sub>, and P<sub>Sinf</sub> of rosuvastatin (Table 1). The fitted line thus obtained was in good agreement with the observed data (Fig. 2B, solid line). Next, using PS<sub>Sinf,app</sub> calculated according to eq. 4, we simulated the plasma concentration of ELT when rosuvastatin was simultaneously administered. The simulated plasma concentration-time profile of ELT was not greatly changed from the control (ELT alone; Fig. 2A). Thus, the present simulation was in agreement with the clinical findings (Allred et al., 2011). We also performed a sensitivity analysis, simulating the plasma ELT profile for the case that the IC<sub>50</sub> in vivo was one-third or one-tenth of that obtained in vitro; again, the simulated plasma concentration was not remarkably different from the control (Fig. 2A).

Effect of ELT on Hepatic Uptake of Therapeutic Agents. We next focused on the clinically observed interaction of ELT with rosuvastatin (Allred et al., 2011). Since rosuvastatin is excreted mainly from the liver (Martin et al., 2003), the effect of ELT on hepatic uptake of rosuvastatin was first examined using cryopreserved human hepatocytes. Rosuvastatin uptake by human hepatocytes was inhibited by ELT in a concentration-dependent manner (IC<sub>50</sub> of approximately 0.11 μM; Fig. 3A). Since rosuvastatin and ELT are OATP1B1 substrates, we also investigated the effect of ELT on uptake of rosuvastatin in HEK293/OATP1B1 cells. OATP1B1-mediated uptake of rosuvastatin was also inhibited by ELT in a concentration-dependent manner (Fig. 3B), with the obtained IC<sub>50</sub> being close to that obtained in human hepatocytes (Table 2). These experiments were performed in the absence of HSA. Therefore, to eliminate the influence of nonspecific adsorption of ELT on the experimental apparatus, we directly measured the medium concentration of ELT in both human hepatocytes and

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**Fig. 1.** Effect of therapeutic agents on ELT uptake by HEK293/OATP1B1 cells. HEK293/OATP1B1 cells were incubated with ELT (10 μM) for 30 minutes at 37°C in the presence or absence of the following various therapeutic agents for ITP (A) or for lifestyle diseases (B). (A) Therapeutic agents for ITP included prednisolone (♦), chlorothromycin (♀), amoxicillin (△), omeprazole (●), rabeprazole (○), lansoprazole (○), atazanavir (■), and cyclophosphamide (□). (B) Therapeutic agents for lifestyle diseases included rosuvastatin (♦), atorvastatin (♀), pravastatin (△), repaglinide (○), glibenclamide (○), and nateglinide (■). Transport buffer containing 5 μM HSA was used to prevent nonspecific adsorption of ELT. OATP1B1-mediated uptake was calculated by subtraction of the uptake observed in HEK293/mock cells from that in HEK293/OATP1B1 cells, and normalized by the uptake in the absence of inhibitors. Each value represents the mean ± S.E.M. (n = 6–15).
HEK293/OATP1B1 cells; the IC50 values for ELT shown in Table 2 were defined in terms of observed ELT concentration in the medium. Alternatively, if we used ELT concentration added to the medium, the calculated IC50 was 2.0 μM, which leads to approximately 20-fold underestimation of its inhibitory potency. Thus, care is needed in assessing potential inhibition by highly adsorbed compounds such as ELT.

ELT is also a substrate and inhibitor of OCT1, so the inhibitory effect of ELT on uptake of OCT1 substrate drugs, metformin, ranitidine, and cimetidine (Bourdet et al., 2005; Kimura et al., 2005), was examined in HEK293/OCT1 cells. OCT1-mediated uptake of ranitidine, and cimetidine (Bourdet et al., 2005; Kimura et al., 2005), was inhibited in a concentration-dependent manner, with an IC50 value of 39 nM, which leads to one-tenth of that obtained in vitro (Fig. 2B). This explains only a part of the clinically reported interaction (the increase amounted to 55 and 103% for AUC and Cmax, respectively, compared with those after administration of rosuvastatin alone (Fig. 2B). According to this simulation, the increase of the area under the curve (AUC) and Cmax for rosuvastatin caused by coadministration of ELT amounted to just 24 and 21%, respectively, compared with those after administration of rosuvastatin alone (Fig. 2B). This explains only a part of the clinically reported interaction (the increase amounted to 55 and 103% for AUC and Cmax, respectively; Allred et al., 2011). The simulated plasma concentration became more consistent with the clinical data only if we assumed that IC50 in vivo was one-third or one-tenth of that obtained in vitro (Fig. 2B).

Interaction of ELT with ABC Transporters in Small Intestine. We next focused on the secretion process of rosuvastatin as a possible target for the clinically observed interaction with ELT. In the liver,

<table>
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<th>Substrate</th>
<th>Therapeutic Agent</th>
<th>IC50 a</th>
<th>Cmax,sim b</th>
<th>Cmax,sys c</th>
<th>Cmax,pv d</th>
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<tr>
<td>ELT</td>
<td>Rosuvastatin</td>
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<td>0.046 (0.0055)</td>
<td>2.9 (0.35)</td>
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<td></td>
<td>Atorvastin</td>
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<td>0.049 (0.0021)</td>
<td>5.0 (0.22)</td>
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<td>Pravastatin</td>
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<td>Rosuvastatin</td>
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<td>0.090</td>
<td>29 (0.029)</td>
<td>41 (0.041)</td>
<td></td>
</tr>
</tbody>
</table>

a fp, unbound fraction in plasma.

b Maximum plasma concentration cited from the interview forms. Values in parentheses represent the unbound concentration, which was calculated as fp × Cmax,sim.

c The maximum concentration at the inlet to liver was calculated using eq. 3. Values in parentheses represent the unbound concentration, which was calculated as fp × Cmax,sys.

d Protein binding in the uptake study for these compounds was experimentally determined, and the IC50 values in parentheses were defined in terms of unbound concentration.

e For these compounds, IC50, Cmax,sys, and Cmax,pv were defined in terms of total concentration.

To quantitatively explain the drug interaction between ELT and rosuvastatin in vivo in humans, the plasma concentration of rosuvastatin coadministered with ELT was simulated based on the PBPK model using PSinf,app estimated according to eq. 4. The simulated plasma concentration profile of rosuvastatin was not much different from that after oral administration of rosuvastatin alone (Fig. 2B). The simulated plasma concentration profile of rosuvastatin caused by coadministration of ELT amounted to just 24 and 21%, respectively, compared with those after administration of rosuvastatin alone (Fig. 2B). This explains only a part of the clinically reported interaction (the increase amounted to 55 and 103% for AUC and Cmax, respectively; Allred et al., 2011). The simulated plasma concentration became more consistent with the clinical data only if we assumed that IC50 in vivo was one-third or one-tenth of that obtained in vitro (Fig. 2B).

Fig. 2. Fitting and simulation of plasma concentration profile of ELT (A) and rosuvastatin (B and C) when these drugs are administered alone or together. The fitted circles represent clinical data previously obtained when ELT (75 mg) or rosuvastatin (10 mg) was orally administered alone, whereas dotted lines represent those previously obtained when both drugs were simultaneously administered (Allred et al., 2011). Solid lines in (A) and (B) represent fitted ones to the PBPK model shown in Supplemental Fig. 1. Broken lines in (A) and (B) represent simulated profiles after coadministration of both drugs when we considered the interaction at OATP1B1 for the cases in which Ki was set to be the same as the IC50 obtained in HEK293/OATP1B1 cells, or one-third and one-tenth of that value. Broken lines in (C) represent the simulated profiles after coadministration of both drugs when we consider the interaction at BCRP in the small intestine, and Fp2 is assumed to be 0.9. Note that the broken line (IC50) in (A) is unclear because it is almost superimposed on the solid line.
rosuvastatin is secreted into the bile via multiple ABC transporters (Kitamura et al., 2008). Intestinal secretion of rosuvastatin may also be governed by ABC transporters (Keskitalo et al., 2009). Therefore, as the first step to study possible involvement of ABC transporters in the interaction between ELT and rosuvastatin, the pharmacokinetic studies using \(\text{mdr1a/1b/bcrp}^{+/+}\) mice were conducted to simultaneously evaluate the effect of ELT on the two ABC transporters, BCRP and P-glycoprotein. Involvement of BCRP and/or P-glycoprotein in intestinal secretion of rosuvastatin was supported by the present finding that the plasma concentration profile of rosuvastatin after oral administration in \(\text{mdr1a/1b/bcrp}^{+/+}\) mice was much higher than that in wild-type mice (Fig. 4A), whereas the difference in plasma concentration profile between the two strains was not so marked after intravenous administration of rosuvastatin (Fig. 4B). Plasma concentration of ELT after oral administration in \(\text{mdr1a/1b/bcrp}^{+/+}\) mice was also much higher than that in wild-type mice (Fig. 4C), suggesting that ELT also interacts with these ABC transporters.

To examine the possible interaction of ELT with intestinal absorption of rosuvastatin, the plasma concentration-time profile of rosuvastatin was examined after injection of rosuvastatin into an intestinal loop with or without ELT. The plasma concentration of rosuvastatin after coadministration with ELT was higher than that after injection of rosuvastatin alone (Fig. 5). The present study was limited to pharmacokinetic interaction, but the interaction in terms of clinical endpoints was not analyzed because ELT is able to increase the platelet count only in humans and chimpanzees (Erckson-Miller et al., 2009) whereas HMG-CoA reductase inhibitors do not show the low-density lipoprotein-lowering effect in rodents (Fujioka et al., 1995).

**Interaction of ELT with BCRP, But Not P-Glycoprotein.** Since the interaction of ELT with rosuvastatin cannot be fully explained by the interaction at the hepatic uptake process (Fig. 2B), we speculated that interaction might also occur at BCRP or P-glycoprotein. To clarify the inhibitory effect of ELT on BCRP-mediated transport of rosuvastatin, we examined transcellular transport of rosuvastatin across MDCKII/BCRP/PDZK1 cells. Transport of rosuvastatin in the AP-to-BL direction across MDCKII/BCRP/PDZK1 cells was lower than that across MDCKII/mock/PDZK1 cells (Fig. 6A). In the presence of \(10 \mu M\) ELT, transport of rosuvastatin in the AP-to-BL direction across MDCKII/BCRP/PDZK1 cells was elevated and became similar to that across MDCKII/mock/PDZK1 cells (Fig. 6A). Next, bidirectional transport of rosuvastatin was measured in the presence of various concentrations of ELT in both cells to determine the net flux ratio of rosuvastatin. We found that the net flux ratio was decreased by ELT in a concentration-dependent manner (Fig. 6B).

To further support the interaction of ELT with BCRP, transcellular transport of ELT by BCRP was also examined. Transport of ELT in the AP-to-BL direction across MDCKII/BCRP/PDZK1 cells was lower than that across MDCKII/mock/PDZK1 cells (Fig. 7A). In the presence of \(1 \mu M\) Ko143, an inhibitor of BCRP, transport of ELT in

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**Fig. 3.** Effect of ELT on uptake of rosuvastatin by human hepatocytes (A) and OATP1B1 (B) and effect of ELT on OCT1-mediated drug uptake (C). (A) Human hepatocytes were incubated with rosuvastatin (1 \(\mu M\)) for 10 minutes at 37°C in the absence or presence of ELT. (B) HEK293/OATP1B1 cells were incubated with rosuvastatin (1 \(\mu M\)) for 5 minutes at 37°C in the absence or presence of ELT. OATP1B1-mediated uptake was calculated by subtraction of the uptake in HEK293/mock cells from that in HEK293/OATP1B1 cells. In (A) and (B), the uptake was examined in the absence of HSA, and the abscissa represents the ELT concentration experimentally measured. Each value represents the mean ± S.E.M. \((n = 3–6)\). (C) HEK293/OCT1 cells were incubated with the transport buffer containing 5 \(\mu M\) HSA and metformin (3 mM, 30 minutes; ●), ranitidine (30 \(\mu M\), 15 minutes; ○), or cimetidine (30 \(\mu M\), 15 minutes; ▲) at 37°C in the absence or presence of ELT. OCT1-mediated uptake was calculated by subtraction of the uptake in HEK293/mock cells from that in HEK293/OCT1 cells. Each value represents the mean ± S.E.M. \((n = 6–12)\).

**Fig. 4.** Involvement of two ABC transporters in gastrointestinal absorption of rosuvastatin and ELT in mice. (A and B) Plasma concentration-time profiles of rosuvastatin were measured after oral administration (10 mg/kg) (A) and intravenous bolus injection (4 mg/kg) (B) in wild-type mice (C) and \(\text{mdr1a/1b/bcrp}^{+/+}\) mice (●). Each value represents the mean ± S.E.M. \((n = 3)\). (C) Plasma concentration-time profiles of ELT were measured after oral administration of ELT (1 mg/kg) in wild-type mice (C) and \(\text{mdr1a/1b/bcrp}^{+/+}\) mice (●). Each value represents the mean ± S.E.M. \((n = 5–6)\).
Fa
trosuvastatin was simulated using the PBPK model for the case that
of rosuvastatin by ELT in humans, the plasma concentration of
significant difference in uptake between the two cell lines (data not
(3
m
ELT (2 mg/kg). Each value represents the mean
increased by 30
Uptake of rhodamine 123 in LLC-GA5-COL150 cells was slightly
was minimally affected by ELT up to 10
strong, since uptake of rhodamine 123 in LLC-GA5-COL150 cells
increase only by 23% at most when the \( K_i \) was set to be one-tenth of
the IC50 experimentally obtained in the present study (Fig. 2A). Thus, it
is considered that inhibition of OATP1B1-mediated hepatic uptake of
ELT by rosuvastatin would be minor.

Discussion

Uptake of ELT by OATP1B1 was inhibited by several drugs in a
concentration-dependent manner, and rosuvastatin and atorvastatin
were the most potent inhibitors (Fig. 1). IC50 values defined in terms
of unbound concentrations of rosuvastatin and atorvastatin were
approximately one-seven to one-eighth of the respective unbound
\( C_{max,pv} \) (Table 2). Therefore, a possible interaction between ELT and
these statins cannot be ruled out. However, this seems incompatible
with the clinical observation that there was no change in the plasma
concentration of ELT upon concomitant administration of rosuvastatin
(Alfred et al., 2011). Prediction of a drug interaction using \( C_{max,pv} \)
based on a so-called static model sometimes overestimates the
interaction, yielding a false-positive prediction. Therefore, we focused
on a more quantitative analysis using the PBPK model. This dynamic
model (Supplemental Fig. 1) predicted that the plasma concentration
interaction. In fact, in the present study, ELT inhibited uptake of
rosuvastatin in both HEK293/OATP1B1 cells and human hepatocytes,
with the IC50 values being almost the same in both cases (Table 2).
These results suggest that ELT potentially inhibits hepatic uptake of
rosuvastatin via OATP1B1. However, quantitative simulation using the
PBPK model indicated that the plasma concentration of rosuvastatin
in humans was only modestly affected by coadministration of ELT
(Fig. 2B). Thus, inhibition of OATP1B1-mediated hepatic uptake of
rosuvastatin by ELT can account for only a part of the drug interaction.
Sensitivity analysis was also performed by changing the \( K_i \) value (Fig.

Fig. 5. Interaction of ELT with rosuvastatin in small intestine in mice. Plasma
concentration-time profiles of rosuvastatin were measured in wild-type mice after
injection into an intestinal loop of rosuvastatin (10 mg/kg) with \( \bullet \) or without \( \circ \)
ELT (2 mg/kg). Each value represents the mean ± S.E.M. (n = 3–8).

Fig. 6. Inhibition by ELT of BCRP-mediated transport of rosuvastatin (A and B), but minimal
effect of ELT on P-glycoprotein (C). (A) Transcellular transport of rosuvastatin (5 \( \mu \)M) in the
apical-to-basal direction was measured across MDCKII/BCRP/PDZK1 (closed symbols) and
MDCKII/mock/PDZK1 cells (open symbols) in the presence (triangles) or absence (circles) of
ELT (10 \( \mu \)M). Each value represents the mean ± S.E.M. (n = 3–12). (B) Transcellular transport of
rosuvastatin was measured in the absence or presence of various concentrations of ELT in both
MDCKII/BCRP/PDZK1 and MDCKII/mock/PDZK1 cells, and the net flux ratio of rosuvastatin was
calculated. Each value represents the mean ± S.E.M. (n = 3–6). (C) Uptake of rhodamine 123 (5 \( \mu \)M)
was measured in the absence or presence of ELT or P-glycoprotein inhibitor verapamil (VER) in LLC-
PK1 (open bar) and LLC-GA5-COL150 (closed bar). The uptake was then normalized by medium
cellular volume (n = 7–12).

To evaluate the possible inhibition of BCRP-mediated secretion of
rosuvastatin by ELT in humans, the plasma concentration of
rosuvastatin was simulated using the PBPK model for the case that
\( F_p \cdot F_o \) of rosuvastatin was elevated to a value close to unity due to the
inhibition of intestinal BCRP by ELT. On the basis of this assumption,
the simulated plasma concentration of rosuvastatin was in good ac-
cordance with the clinical findings (Fig. 2C).

the AP-to-BL direction across MDCKII/BCRP/PDZK1 cells was
elevated and became similar to that across MDCKII/mock/PDZK1 cells
(Fig. 7A). Bidirectional transport of ELT was then measured at
various concentrations of ELT. The net flux ratio of ELT decreased as
the ELT concentration was increased (Fig. 7B).

On the other hand, interaction of ELT with P-glycoprotein was not
strong, since uptake of rhodamine 123 in LLC-GA5-COL150 cells
was minimally affected by ELT up to 10 \( \mu \)M, but was increased in the
presence of a typical P-glycoprotein inhibitor, verapamil (Fig. 6C).
Uptake of rhodamine 123 in LLC-GA5-COL150 cells was slightly
increased by 30 \( \mu \)M ELT, but such an increase was also observed in
LLC-PK1 cells (Fig. 6C). Uptake of rosuvastatin (5 \( \mu \)M) and ELT
(3 \( \mu \)M) was also evaluated in the present study, but there was no
significant difference in uptake between the two cell lines (data not
shown).

To evaluate the possible inhibition of BCRP-mediated secretion of
rosuvastatin by ELT in humans, the plasma concentration of
rosuvastatin was simulated using the PBPK model for the case that
\( F_p \cdot F_o \) of rosuvastatin was elevated to a value close to unity due to the
inhibition of intestinal BCRP by ELT. On the basis of this assumption,
the simulated plasma concentration of rosuvastatin was in good ac-
cordance with the clinical findings (Fig. 2C).
2B). The increase in AUC or $C_{\text{max}}$ of rosuvastatin could be largely explained only if we assume that the $K_f$ in vivo is one-third to one-tenth of the IC$_{50}$ experimentally obtained in vitro. However, if we assume such potent inhibition of OATP1B1 by ELT, the time of maximum plasma concentration ($T_{\text{max}}$) was simulated to be prolonged, probably due to the inhibition of systemic clearance via OATP1B1 (Fig. 2B). This is not in agreement with the clinical observation that $T_{\text{max}}$ was minimally changed (Fig. 2B; Allred et al., 2011).

Another possible target of ELT–rosuvastatin interaction could be an efflux transporter(s) for rosuvastatin in the liver and/or small intestine. Rosuvastatin is a substrate of BCRP, P-glycoprotein, and multidrug resistance-associated protein 2 (Huang et al., 2006; Kitamura et al., 2008). Considering the minimal change in the terminal phase of the rosuvastatin profile caused by ELT (Fig. 2B; Allred et al., 2011), a plausible interaction mechanism could be inhibition of a small-intestinal efflux transporter(s) for rosuvastatin by ELT. This hypothesis was supported by the present finding that triple gene knockout of mdrla/b/bcrp in rodents had a greater effect on the plasma rosuvastatin profile after oral administration (Fig. 4A) than after intravenous administration (Fig. 4B). It was also demonstrated in the mouse intestinal loop that coadministration of ELT delayed absorption of rosuvastatin (Fig. 5). Therefore, in the present study, possible inhibition by ELT of small-intestinal efflux transporters BCRP and P-glycoprotein was examined. ELT inhibited BCRP-mediated rosuvastatin transport in MDCK/BCRP/PDZK1 cells, but had no effect in MDCK/mock/PDZK1 cells (Fig. 6A), and the net flux ratio of rosuvastatin was decreased by 10 $\mu$M ELT (Fig. 6B). In addition, ELT is transported by BCRP, and BCRP-mediated transport of ELT was saturated at approximately 10 $\mu$M ELT (Fig. 7B). Tachibana et al. (2009) proposed the drug interaction number as an index of potential for inhibition of CYP3A4 and P-glycoprotein in the small intestine. In the present study, we attempted to apply this theory to BCRP by using the IC$_{50}$ for BCRP (Fig. 6B) and the clinical dose of ELT. The apparent IC$_{50}$ for BCRP was approximately 10 $\mu$M (Fig. 6B), but this inhibitory effect of ELT was estimated in the presence of HSA. Therefore, we further considered the unbound fraction of ELT in the transport buffer containing HSA (approximately 0.0072), and the IC$_{50}$ defined in terms of unbound ELT concentration was estimated to be 0.07 $\mu$M. If we use this IC$_{50}$ and the ELT dose (75 mg oral), the drug-interaction number is calculated to be more than 2000 liters. According to the criteria proposed by Tachibana et al. (2009), the risk of ELT–rosuvastatin interaction is therefore considered to be high, supporting the idea that the drug interaction is mediated by BCRP inhibition. Finally, the rosuvastatin concentration in plasma was simulated based on the assumption that $F_2 - F_1$ is increased to a level close to unity by ELT-mediated inhibition of BCRP in the small intestine. The simulation indicated that the rosuvastatin concentration would be elevated to a level comparable with the clinical observation after coadministration with ELT (Fig. 2C). This supports the conclusion that BCRP in the intestinal tract plays a key role in the interaction between rosuvastatin and ELT. It was reported that the plasma concentration of rosuvastatin after oral administration is increased in patients with a genetic polymorphism (421C->A) of ABCG2 (Keskitalo et al., 2009). BCRP in the small intestine is considered to be primarily important for absorption of rosuvastatin since the AUC, but not the elimination half-life, of rosuvastatin is increased by the gene polymorphism (Keskitalo et al., 2009). Interestingly, the AUC of rosuvastatin after oral administration in patients with 421C->A ABCG2 was approximately twice that in individuals with the wild-type genotype (Keskitalo et al., 2009). So, if we consider that the genetic polymorphism (421C->A) of ABCG2 results in substantial loss of function of BCRP, the assumption in the present study (that $F_2 - F_1$ is increased from 0.5 to 0.9) is likely to be compatible with substantial inhibition of BCRP by ELT. However, such a simple assumption may not be applied for other BCRP substrates since concentration of the inhibitor drug in gastrointestinal tract should not be constantly high after its oral administration. In contrast with BCRP, the inhibitory effect of ELT on P-glycoprotein could be less potent (Fig. 6C). However, the inhibition study was performed up to 30 $\mu$M of ELT using rhodamine 123, but not rosuvastatin, as a substrate of P-glycoprotein. Thus, there could be limitations to this in vitro study that would underpredict the role of P-glycoprotein in vivo.

In the present study, we investigated the effect of drugs on OATP1B1-mediated ELT transport. The IC$_{50}$ values of those drugs, other than rosuvastatin and atorvastatin, were higher than the $C_{\text{max, pv}}$ values (Table 2). One possible issue in the present study is that the transport buffer used to measure OATP1B1-mediated uptake of ELT contains HSA, and thus the IC$_{50}$ values for these compounds were defined based on the total concentration in the presence of HSA. Nevertheless, the HSA concentration (5 $\mu$M) used in the transport study was less than the physiologic albumin concentration (approximately 600 $\mu$M), so the unbound fraction in the transport study could be equal to or higher than that in human plasma. If the IC$_{50}$ value is higher than the $C_{\text{max, pv}}$ value even under this condition, interaction of these drugs with OATP1B1-mediated hepatic uptake of ELT may be unlikely in vivo.

It has been reported that the plasma concentration profile of ELT is affected by a genetic polymorphism (421C->A) of ABCG2 (Allred et al., 2011). In the present study, we demonstrated that ELT is a high-affinity substrate for BCRP, with saturation being observed at around 10 $\mu$M (Fig. 7B). Thus, BCRP could be an important determinant of the pharmacokinetics of ELT. It is noteworthy that inhibition of BCRP by ELT occurred at the clinical dose, implying that ELT may at least partially saturate small-intestinal BCRP. Therefore, further studies seem necessary to evaluate the possible interaction of ELT with other orally administered BCRP substrate drugs.

The IC$_{50}$ values for rosuvastatin and atorvastatin for OATP1B1-mediated ELT uptake (0.05 and 0.03 $\mu$M, respectively; Table 1) were much smaller than the $K_m$ values for the uptake of these drugs by
OATP1B1 (0.802 and 12.4 μM, respectively; Kameyama et al., 2005; Kitamura et al., 2008). This may imply that the substrate recognition site of OATP1B1 for ELT does not completely overlap with that for these typical OATP1B1 substrates, and the inhibition potential of other drugs for ELT uptake cannot be precisely predicted from uptake studies using substrates other than ELT itself. Therefore, drug interaction potential at OATP1B1 should be further examined to confirm the safety of pharmacotherapy using ELT.

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Authorship Contributions
Participated in research design: Takeuchi, Sugiura, Horikawa, Nakamichi, Ishiwata, Kato. Conducted experiments: Takeuchi, Matsubara, Sato, Shimizu. Performed data analysis: Takeuchi, Sugiura, Matsubara. Wrote or contributed to the writing of the manuscript: Takeuchi, Sugiura, Masuo, Kato.

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