Pharmacokinetics and Hepatic Uptake of Eltrombopag, A Novel Platelet-Increasing Agent

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Hepatic Uptake Governs Overall Elimination of Eltrombopag

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Abbreviations: ELT, eltrombopag; OATP, organic anion-transporting polypeptide; OCT, organic cation transporter; HEK, human embryonic kidney; ITP, idiopathic thrombocytopenic purpura; TPO, thrombopoietin; RIF, rifampicin; TPeA, tetrapentylammonium
ABSTRACT

Eltrombopag (ELT) is a novel thrombopoietin receptor agonist for the treatment of idiopathic thrombocytopenic purpura. Previous reports indicate that ELT is mainly eliminated in the liver, although its pharmacokinetic profile has not yet been clarified in detail. The purpose of the present study is to investigate the overall elimination mechanism of ELT. After intravenous administration of ELT to rats, approximately 40% of unchanged ELT was excreted into the bile in 72 hr, whereas less than 0.02% of the dose was excreted in urine, indicating that liver is the major elimination organ for ELT. The total clearance was much lower than the hepatic blood flow rate and comparable with hepatic uptake clearance obtained from integration plot analysis. Coadministration of rifampicin, an organic anion transporter inhibitor, reduced both total clearance and hepatic uptake clearance of ELT. These results suggest that hepatic uptake is the rate-limiting process in the overall elimination of ELT. To further characterize the uptake mechanism, uptake of ELT by freshly isolated mouse hepatocytes was examined. The ELT uptake showed concentration- and energy-dependence, and was inhibited by various compounds, including not only organic anions, but also organic cations. Hepatic uptake clearance in vivo was reduced by coadministration of an organic cation, tetrampentylammonium. Finally, uptake of ELT was observed in HEK293 cells transfected with human hepatic transporters OATP1B1, OATP2B1 and OCT1. These results suggest that multiple transporters, including organic anion transporters and organic cation transporters, are involved in hepatic ELT uptake.
INTRODUCTION

Thrombocytopenia is characterized by an abnormally low circulating platelet count (< 150,000 cells/mL) and is seen in chronic diseases such as idiopathic thrombocytopenic purpura (ITP) (Kuter et al., 2002), as well as being induced by medical treatments such as chemotherapy for cancer (Elting et al., 2001; Kaushansky, 1996) and interferon therapy for hepatitis C (Curry et al., 2005; Ong et al., 2004). ITP is an autoimmune disease in which antibodies to circulating platelets are produced, and stimulate the destruction of platelets, resulting in a decrease in the platelet count in the circulating blood, leading to symptoms such as hemorrhage, prolonged bleeding and purpura. Thrombopoietin (TPO) is the dominant cytokine involved in thrombopoiesis. Recombinant human TPO and its peptide mimetics have been developed for treatment of ITP. Although recombinant TPO is effective for the treatment of thrombocytopenia in some clinical settings (Kuter et al., 2002), it was found that neutralizing antibodies to TPO may be induced. Peptide mimetics of TPO are also effective for ITP patients (Wang et al., 2004; Kuter et al., 2006), but they have to be administered intravenously or subcutaneously because of their low stability in the body. Therefore, in the development of second-generation drugs for the treatment of thrombocytopenia, researchers have focused on weakly immunogenic peptides or non-peptide TPO receptor agonists.

Eltrombopag (Promacta®, ELT, Supplementary Figure S1), 3’-{N’-[1-(3,4-dimethylphenyl)-3-methyl-5-oxo-1,5-dihydropyrazol-4-ylidene]hydrazine}-2’-
hydroxybiphenyl-3-carboxylic acid, is a novel, small-molecular, non-peptide TPO receptor agonist, which can be orally administered (Erickson-Miller et al., 2004; Sellers et al., 2004; Jenkins et al., 2007; Bussel et al., 2007). ELT interacts with the transmembrane domain of the TPO receptor (Erickson-Miller et al., 2008), and thereby activates intracellular signal transduction pathways leading to stimulation of the proliferation and differentiation of megakaryocytes and progenitor cells in bone marrow, resulting in an increase of platelets in the circulating blood. ELT has already been approved for the treatment of ITP in several countries.

In a clinical study, ELT was found to be metabolized to oxidized metabolites and conjugated metabolites, such as glucuronide, in the liver; 59 and 31% of the dose of ELT were recovered in feces and urine, respectively. In particular, unchanged ELT was excreted in feces (20% of the dose), but was not detectable in urine (Bauman et al., 2010). In a phase 1 clinical trial, the pharmacokinetics of ELT after oral administration in healthy volunteers was dose-proportional up to 75 mg (Jenkins et al., 2007). On the other hand, when ELT was administered to patients with mild, moderate, or severe hepatic impairment, the area under the curve (AUC) of ELT was increased by 41, 93 or 80%, respectively, compared with healthy subjects. (Bauman et al., 2010). Although these results imply that overall elimination of ELT is likely to be mediated by the liver, its pharmacokinetic profile has not yet been clarified in detail.
Possible interaction of xenobiotic transporters with ELT has also been proposed: in vitro studies indicated that ELT is an inhibitor of organic anion transporting peptide (OATP) 1B1 and breast cancer resistance protein (Huang et al., 2010). In addition, the plasma concentration of rosuvastatin, a substrate of OATP1B1, was increased by repeated daily coadministration of ELT in a clinical study (Danish et al., 2010). However, there is no evidence as to whether ELT is transported by these transporters or not. Information on the mechanism of overall elimination and hepatic handling of ELT would be important for appropriate treatment in order to avoid unfavorable pharmacokinetic and/or pharmacodynamic drug-drug interactions, or drug-induced toxicity. Therefore, the aim of the present study was to investigate the pharmacokinetic properties of ELT in detail.
MATERIALS AND METHODS

Materials

ELT was synthesized by Nissan Chemical Industries, Ltd. (Tokyo, Japan). [3H]Estrone 3-sulfate (57.3 Ci/mmol) was purchased from PerkinElmer (Boston, MA). [3H]Estradiol 17β-glucuronide (41.8 Ci/mmol), [3H]carnitine (85 Ci/mmol) and [14C]tetraethylammonium (55 Ci/mol) were obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Rifampicin (RIF) and tetrapentylammonium (TPeA) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Sigma-Aldrich Japan (Tokyo, Japan), respectively. All other chemicals and reagents were of analytical grade and were obtained from commercial sources.

Animals

Intact male Sprague-Dawley rats (7 to 9 weeks old) and those with the jugular vein and bile duct precannulated (8 to 9 weeks old) were purchased from Charles River Japan (Yokohama, Japan). Male mice were used at 7 to 9 weeks old. The transporter adaptor PDZK1 gene knockout (pdzk1−/−) mice were previously constructed (Lan and Silver, 2005) and bred in the Institute for Experimental Animals, Kanazawa University as described previously (Sugiura et al., 2008). All animals were maintained under standard conditions with a reversed light/dark cycle and were treated humanely. Food and water were available ad libitum. The
studies were carried out in accordance with the guidelines of the Institutional Animal Care Committee of Kanazawa University, and Biological Research Laboratories, Nissan Chemical Industries, Ltd.

**Pharmacokinetic Studies in Rats**

Rats with the jugular vein and bile duct precannulated were kept in Bollman cages with free access to food and water. ELT was dissolved in distilled water with sonication to obtain a concentration of 1 mg/ml and injected via the tail vein (1 mg/kg). RIF was injected via the penile vein at a dose of 20 mg/kg at 5 min before ELT administration (Lau et al., 2006). Blood and bile samples were collected at designated time intervals via the jugular vein cannula and bile duct cannula, respectively. Blood samples were collected using a heparinized syringe and centrifuged to obtain plasma. Total clearance (CL<sub>total</sub>), distribution volume to the central compartment (V<sub>0</sub>) and that at steady-state (V<sub>ss</sub>) were estimated by means of moment analysis using the WinNonLin software package (Professional version 5.2; Pharsight, Mountain View, CA). The biliary clearance defined with respect to the plasma concentration (CL<sub>bile,p</sub>) was calculated as the amount of ELT excreted into the bile divided by calculated AUC for the corresponding time period. The biliary clearance defined with respect to the hepatic concentration (CL<sub>bile,h</sub>) was calculated as the biliary excretion rate obtained at the final sampling period divided by the hepatic concentration at the corresponding time.
Integration Plot Analysis in Rats and Mice

Rats were anesthetized with pentobarbital, and ELT (1 mg/kg) was injected via the tail vein. Blood samples were collected from the jugular vein at 0.5, 1, 3, 10, 20 min after administration, and a small amount of liver was also obtained by biopsy at 1 and 10 min. After 20 min, the rats were sacrificed, and the liver was excised. In combination experiments with RIF, RIF was injected via the penile vein at a dose of 20 mg/kg at 5 min before the administration of ELT.

Mice were anesthetized with pentobarbital. ELT (1 mg/kg) was injected via the left jugular vein. Blood samples were collected from the right jugular vein at 30, 60, 90, 110 sec after administration and centrifuged to obtain plasma. After 120 sec, the mice were sacrificed, and liver and kidney were collected. In combination experiments with TPeA and RIF, TPeA and RIF were also injected via the jugular vein at doses of 30 µmol/kg and 20 mg/kg, respectively, according to previous reports (Lau et al., 2006; Choi et al., 2007) at 1 and 5 min, respectively, before the administration of ELT.

The concentrations of ELT in plasma, liver and kidney were measured as described below. In rats, efflux from the liver soon after intravenous administration was assumed to be negligible, and the tissue uptake clearance (CLuptake) was calculated with the following equation:
\[
\frac{X(t)}{C_p(t)} = CL_{\text{uptake}} \cdot \frac{AUC}{C_p(t)} + V_e
\]

where \(X(t)\) and \(C_p(t)\) are the amounts in tissue and plasma concentration at time \(t\), respectively. \(V_e\) is the volume of distribution, within which a rapid equilibrium with the plasma compartment is assumed. When \(\frac{X(t)}{C_p(t)}\) is plotted against \(\frac{AUC}{C_p(t)}\), the slope represents the value of \(CL_{\text{uptake}}\). In mice, \(CL_{\text{uptake}}\) was approximately estimated as \(\frac{X(t)}{AUC_p(0-t)}\).

**Isolation and Transport Experiments in Mouse Hepatocytes**

Hepatocytes were isolated from mice by the collagenase perfusion method with some modifications as described previously (Sugiura et al., 2010). We checked cell viability by means of a trypan blue exclusion test and used hepatocytes showing more than 90% viability. The isolated cells were resuspended in ice-cold transport buffer (125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM CaCl\(_2\), 1.2 mM KH\(_2\)PO\(_4\), 1.2 mM MgSO\(_4\), and 25 mM HEPES, pH 7.4). The uptake experiment was then performed according to the silicone oil layer method (Sugiura et al., 2010) with some modifications to avoid nonspecific adsorption of ELT. Briefly, 50 µL of the cell suspension was pre-incubated for 5 min at 37 °C, followed by start of the reaction by mixing the suspension with 50 µL of pre-warmed transport buffer, which contained ELT and 10 µM human serum albumin (fatty acid free). In the inhibition study, the pre-incubation was performed in the absence of inhibitors except for rotenone and FCCP which was also included in the pre-incubation mixture. The reaction was stopped by adding...
600 µL of ice-cold transport buffer, which also contained 0.1% BSA, and the mixture was then quickly centrifuged through a silicone oil layer.

**Transport Studies in HEK293 Cells Transfected with Human Hepatic Transporters**

HEK293/PDZK1 cells were routinely grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, penicillin, streptomycin and 1 mg/mL G418 in a humidified incubator at 37 °C and 5% CO₂. Plasmid DNA encoding human OATP1B1, OATP1B3, OATP2B1, organic cation transporter (OCT) 1 or OCTN2, all of which had been subcloned in pcDNA3 vector, was transiently transfected into HEK293/PDZK1 cells according to the calcium phosphate precipitation method. This HEK293/PDZK1 cell line was used since the PDZ adaptor protein PDZK1 increases the transport activity of various xenobiotic transporters in vitro (Sugiura et al., 2006) and regulates expression of basolateral membrane transporter in hepatocytes in vivo (Wang et al., 2005) although possible interaction of PDZK1 with OATP1B1, OATP1B3, OATP2B1 or OCT1 has not yet been examined. At 48 h after transfection, the cells were harvested and suspended in transport buffer, and uptake studies were performed by the silicon oil layer method as described above, using isolated hepatocytes. For the transfection of OCT1, cells were cultured in poly-L-lysine-coated 12-well plates, and directly used for uptake study. The associated radioactivity was measured with a liquid scintillation counter (Aloka, Tokyo, Japan) with Clearsol I (Nacalai Tesque, Inc.,
Kyoto, Japan) as the scintillation fluid, after neutralization with HCl of the cell lysate obtained by solubilization with KOH. Cellular protein content was determined according to the method of Bradford using a protein assay kit (Bio-Rad), with bovine serum albumin as the standard (Bradford, 1976). Concentration of ELT in the transport buffer and cell lysate was measured as described below.

Liquid Chromatography

Quantification of ELT was performed using a triple quadrupole mass spectrometer with electrospray ionization (ESI) (Quattro Premier XE, Waters Corporation, Milford, MA) coupled to a liquid chromatography system (ACQUITY UPLC, Waters Corporation, Milford, MA). Chromatography was performed by means of step-gradient elution (flow rate, 0.5 mL/min) as follows: 0 to 0.4 min, 65% A/ 35% B; 0.4 to 3.6 min, 65% A/ 35% B to 5% A/95% B; 3.6 to 4.6 min, 5% A/ 95% B; 4.6 to 5.5 min, 65% A/ 35% B (A, 0.1% formic acid; B, acetonitrile/methanol (3:2) containing 0.1% formic acid) using an ACQUITY UPLC BEH Shield RP18 (1.7 µm particle size, 2.1 mm I.D. × 50 mm; Waters Corporation, Milford, MA) at 45°C. The multiple reaction monitor was set at 443.2 to 228.6 m/z for ELT, and 295.9 to 214.7 m/z for I.S. (diclofenac). The quantitation limit for ELT was 10 ng/mL or 20 nM. Determination of metformin was performed using an HPLC system consisting of a model LC-10AD VP pump and a model SPD-10A VP UV monitor (Shimadzu, Tokyo, Japan) with a
COSMOSIL 5C_{18}-AR-II column (4.6 mm I.D.×150 mm; Nacalai Tesque, Kyoto, Japan). The mobile phase consisted of 20 mM ammonium acetate containing 2.5 mM 1-octanesulfonic acid sodium salt / methanol (85:15) at the flow rate of 1 ml/min. The wavelength of the UV detection was at 236 nm.

Data Analysis

Statistical analysis was performed by using Student’s *t* test. A difference between means was considered to be significant when *p* < 0.05. Kinetic parameters for ELT uptake were obtained using nonlinear least-squares regression analysis based on the following equations:

\[ v = \frac{V_{\text{max}} \cdot s}{(K_m + s)} + K_{\text{ns}} \cdot s \]  
\[ v = \frac{V_{\text{max}} \cdot s}{(K_m \cdot (1 + I/K_i) + s)} + K_{\text{ns}} \cdot s \]

where *v*, *s*, *I*, *V_{\text{max}}*, *K_m*, *K_i* and *K_{\text{ns}}* represent the initial uptake velocity, substrate concentration, inhibitor concentration, maximum uptake velocity, Michaelis constant, inhibitory constant and non-saturable clearance, respectively. The selection of the equation was based on Akaike's Information Criterion.
RESULTS

Pharmacokinetic Profile of ELT

Following intravenous injection of ELT (1 mg/kg) into rats, ELT in plasma slowly disappeared and was detectable up to 72 hr after dosing (Figure 1A). Approximately 40% of the dose was excreted into the bile in unchanged form up to 72 hr (Figure 1B), whereas the amount detected in the urine was less than 0.02% of the dose (Table 1). The CL<sub>tot</sub> (Table 1) was much lower than the hepatic blood flow rate (3.31x10<sup>3</sup> mL/hr/kg; Davies and Morris 1993). The values of V<sub>0</sub> and V<sub>des</sub> (Table 1) were close to the plasma volume and extracellular fluid space (31.2 and 297 mL/kg, respectively; Davies and Morris 1993), respectively. Thus, ELT exhibits quite low clearance and low distribution characteristics. ELT was distributed to the liver and kidney, whereas distribution to other organs was much lower (Figure 1C).

The effect of RIF, a known potent inhibitor of OATPs on plasma concentration-time course, biliary excretion and hepatic uptake of ELT was examined. When RIF was coadministered, plasma concentration of ELT was higher than that in the control (Figure 2A), with a concomitant decrease in biliary excretion of ELT (Figure 2B). Coadministration of RIF also reduced hepatic uptake of ELT, which was directly estimated by means of integration plot analysis (Figure 2C). The CL<sub>uptake</sub> assessed from the slope of the integration plot (Figure 2C) was decreased to 51% of the control by the coadministration of RIF (Table 2). Similar reduction by RIF (to 59% of the control) was also observed for the CL<sub>tot</sub> assessed from the
plasma concentration-time profile (Figure 2A, Table 2). In addition, the absolute values of 
CL_{uptake} in both groups were comparable to those of CL_{tot} (Table 2). On the other hand, CL_{bile,h},
defined as the biliary excretion rate divided by the hepatic concentration of ELT, was not very
different between the two groups (Table 2).

Characterization of ELT Uptake by Freshly Isolated Hepatocytes

To characterize the hepatic uptake mechanism of ELT, we prepared freshly isolated
mouse hepatocytes, and performed several uptake studies (Figures 3 and 4). The reason for
using hepatocytes isolated from mice rather than rats is gene knockout animals (pdzk1^{−/−} mice)
available to examine possible involvement of a certain transporters in hepatic uptake \textit{in vivo}.
ELT uptake at 37°C by the isolated hepatocytes linearly increased up to 5 min, whereas that at
4°C was minimal (Figure 3A). Based on this result, the initial uptake of ELT was determined
at 5 min at 37°C in subsequent experiments. The initial uptake of ELT was saturable at higher
concentration of ELT (Figure 3B), but the Km value for ELT uptake was not evaluated due to
the limit of solubility for ELT (~200 µM). ELT uptake and was slightly lower at acidic pH
compared with that at pH 7.4 (Figure 3C). On the other hand, the ELT uptake was almost
unchanged in the absence of Na^{+} or Cl^{−} (Figure 3D).

To further characterize the ELT uptake, the inhibitory effects of various compounds
on the initial uptake were determined (Figure 4). The uptake of ELT was decreased in the
presence of ATP depletors, such as rotenone and FCCP, suggesting an energy-dependent uptake. ELT is an anionic compound, and the ELT uptake was inhibited by OATP inhibitors, such as taurocholate and RIF (Figure 4). On the other hand, cationic compounds such as tetraethylammonium, TPeA, quinidine and verapamil also inhibited ELT uptake (Figure 4). Both GABA and phenylalanine significantly reduced the ELT uptake, although the effect was not large (Figure 4). Thus, hepatic uptake of ELT was inhibited by not only organic anions, but also organic cations.

Effect of TPeA on Hepatic Uptake of ELT in vivo

To determine whether or not ELT uptake is inhibited by an organic cation in vivo, we next examined the effect of TPeA on hepatic uptake of ELT (Figure 5A, 5B). TPeA has previously been used as an OCT inhibitor for in vivo studies (Choi et al., 2007). When ELT (1 mg/kg) was injected into mice, the maximum plasma concentration of ELT was at most 40 µM (data not shown), and at this concentration, hepatic uptake of ELT was almost linear (Figure 3B), implying that hepatic uptake process of ELT is not saturated under this condition. The CLuptake of ELT in the liver was significantly decreased by coadministration of TPeA (Figure 5A). Inhibition by RIF of hepatic ELT uptake was also confirmed in this experiment (Figure 5A). On the other hand, CLuptake in kidney was not affected by TPeA or RIF (Figure 5B). As a positive control, we examined the effect of coadministration of TPeA on tissue
uptake of metformin (30 µmol/kg), a substrate of OCT1 and OCT2 (Figure 5C). The CL$_{uptake}$ in both liver and kidney for metformin was decreased in the presence of TPeA (Figure 5C), confirming the inhibition of OCTs by TPeA in vivo. To demonstrate the involvement of transporter(s), systemic elimination of ELT was examined in $p$dzk1$^{-/-}$ mice. The CL$_{tot}$ of ELT in $p$dzk1$^{-/-}$ mice was not completely reduced, but significantly lower than that in wild-type mice ($77.3 \pm 4.9$ and $55.2 \pm 3.9$ in wild-type and $p$dzk1$^{-/-}$ mice, respectively; n = 4 for each).

PDZK1 regulates expression and/or localization of various transporters in vivo (Wang et al., 2005; Sugiura et al., 2008). Especially, Oatp1a1 is down-regulated in $p$dzk1$^{-/-}$ mice (Wang et al., 2005). Therefore, the partial reduction in CL$_{tot}$ of ELT in $p$dzk1$^{-/-}$ mice is possibly due to the reduction in uptake by a certain transporters interacting with PDZK1. All these results indicate that hepatic uptake of ELT in vivo is mediated by multiple mechanisms, including not only organic anion transporter, but also organic cation transporter.

**Recognition of ELT by Human Hepatic Uptake Transporters**

To further investigate the possible involvement of multiple transporters in hepatic ELT uptake, possible interaction of ELT with human hepatic uptake transporters OATP1B1, OATP1B3, OATP2B1, OCT1 and OCTN2 was examined (Figure 6). ELT showed inhibitory effects on OATP1B1- and OATP2B1-mediated uptake of $[^3$H]estrone-3-sulfate, OATP1B3-mediated uptake of $[^3$H]estradiol 17β-glucuronide and OCT1-mediated uptake of
[14C]tetraethylammonium in the concentration range of 1 - 50 µM, whereas the inhibitory effect of ELT on OCTN2-mediated uptake of [3H]carnitine was minimal (Figure 6). In the control study, typical substrates or inhibitors for each transporter (bromosulfophthalein for OATP1B1, OATP1B3 and OATP2B1, tetraethylammonium for OCT1 and carnitine for OCTN2) reduced the uptake of radiolabeled substrate by the corresponding transporter (Figure 6). The Ki values for ELT estimated according to Eq. 3 were 14.9 ± 1.2, 25.6 ± 7.3, 8.48 ± 1.62 and 103 ± 9 µM for OATP1B1, OATP1B3, OATP2B1 and OCT1, respectively.

To evaluate the uptake of ELT by these transporters, uptake studies in HEK293/PDZK1 cells transfected with human transporter were conducted (Figure 7). The ELT uptake in HEK293/PDZK1 cells transfected with OATP1B1, OATP2B1 and OCT1 was higher than that in vector-transfected cells. Heterologous transfection of OATP1B3 did not increase the uptake of ELT in HEK293/PDZK1 cells (Figure 7), but increased the uptake of [3H]estradiol 17β-glucuronide.
DISCUSSION

The present study has established that the liver is the major distribution and elimination organ for ELT (Figure 1, Table 1). In addition, hepatic uptake could be the rate-limiting process in the overall elimination of ELT based on the following findings. First, the CL$_{\text{tot}}$ of ELT was close to CL$_{\text{uptake}}$ in the liver, which was obtained from integration plot analysis (Tables 1, 2). Second, the inhibition of hepatic ELT uptake by coadministration of RIF decreased both CL$_{\text{tot}}$ and CL$_{\text{uptake}}$ of ELT in the liver, whereas CL$_{\text{bile,clh}}$ was not affected (Figure 2, Table 2). Metabolism of ELT has already been proposed to be important in the hepatic disposition of ELT (Jenkins et al., 2007; Bauman et al., 2010). In addition, biliary excretion accounted for ~40% of the overall elimination of ELT (Figure 1, Table 1). Thus, ELT is eliminated both by metabolism and excretion. Nevertheless, hepatic uptake could also be a key contributor to the pharmacokinetics of ELT. These findings may be of importance for the design of clinical studies of ELT. For example, a clinical trial of ELT in patients with chronic liver disease or hepatitis C related liver disease has recently been conducted, since thrombocytopenia is often found in conditions involving liver injury. However, the plasma concentration of ELT is greatly increased in patients with such hepatic impairments (Bauman et al., 2010), probably because of reduced hepatic disposition of ELT due to malfunction in the major clearance organ. Hepatic uptake of ELT is temperature- and energy-dependent, saturable and reduced by various types of transporter inhibitors (Figure 3, 4), suggesting the
involvement of carrier-mediated mechanisms. These results may imply that systemic exposure to ELT can be affected by changes in hepatic uptake transporters due to drug-drug interaction and/or genetic polymorphisms, as in cases of other therapeutic agents, including HMG-CoA reductase inhibitors (Simonson et al., 2004).

ELT contains a carboxyl group, and is an anionic compound at physiological pH. Various types of such anionic therapeutic agents are taken up by hepatic uptake transporters, and both OATP1B1 and OATP1B3 play important roles in their hepatic disposition (Watanabe et al., 2010; Kalliokoski et al., 2010). Nevertheless, ELT has been thought to be a non-substrate of OATP1B1 (Gibiansky et al., 2010). On the other hand, ELT was found to be a substrate of OATP1B1 in the present study, since the uptake of ELT by HEK293/PDZK1 cells transfected with OATP1B1 was higher than that in vector-transfected cells (Figure 7). In the present study, nonspecific adsorption of ELT onto experimental apparatus and/or plasma membranes due to its hydrophobic character greatly hindered measurement of the real uptake by the cells. This might have led to difficulty in identification of transporters for this compound in previous studies. It might be a unique property of ELT to be recognized by multiple transporters: ELT inhibits not only organic anion transporters (OATP1B1, OATP1B3 and OATP2B1), but also organic cation transporter (OCT1) (Figure 6). In addition, ELT is taken up by anion (OATP1B1 and OATP2B1) and cation (OCT1) transporters (Figure 7). This may be compatible with the inhibition of hepatic ELT uptake by various compounds
with a broad range of structures (Figures 4 and 5). In particular, both organic anions such as RIF and organic cations such as TPeA inhibited hepatic uptake of ELT both *in vitro* and *in vivo* (Figures 4 and 5). This is the first report of an anionic compound being transported by organic cation transporter OCT1. Regarding organic anion transporters which also belong to solute carrier 22A family as for OCT1, Ahn *et al.* (2009) reported the transport of organic cations by the anion transporter. According to our calculations of partial electric charge in the ELT molecule, the carbon of the carboxyl group and the carbon of the carbonyl group in the pyrazolone ring have partial positive charge (Supplementary Figure S1). OCT1 might recognize these partial structures of ELT, although further studies will be needed to clarify the structural requirement for the transporter-mediated recognition. Interestingly, coadministration of a polyvalent cation-containing antacid, such as aluminium hydroxide, magnesium carbonate or sodium alginate, altered the plasma concentration of ELT in humans (Williams *et al.*, 2009). Although this drug-drug interaction may be explained by chelate reaction between ELT and positive ionic metals (Williams *et al.*, 2009), another possibility may be that polyvalent cation-containing antacid inhibits the membrane permeation of ELT mediated by unknown cation transporters. In the present study the recognition of ELT by human hepatic uptake transporters was demonstrated in gene transfected cell lines (Figures 6 and 7). However, to understand the design of clinical use of ELT, further experiments using human hepatocytes are required to investigate the contribution of each transporter to hepatic
uptake of ELT in human.

In the present study ELT inhibited OATP1B1-mediated transport (Figure 6A). This would be compatible with the clinically observed drug-drug interaction: administration of a single dose of rosuvastatin following repeated daily ELT dosing increased AUC of rosuvastatin by 55% and C\text{max} by 103% (Danish et al., 2010). Considering the fundamental role of OATP1B1 in overall elimination of rosuvastatin (Kitamura et al., 2008), such interaction could occur via inhibition of OATP1B1 by ELT. The inhibition constant of ELT for OATP1B1 (14.9 µM, Figure 6A) was close to the clinical concentration of ELT (C\text{max} = 14 µM; Daphne et al., 2008). However, because ELT is highly bound to plasma protein (> 99.9%; Bauman et al., 2010), the unbound concentration of ELT could be much lower in the clinical context. In addition, the maximum unbound concentration in the portal vein after oral administration of ELT was calculated to be at most ~0.026 µM according to the equation proposed by Ito et al (1998). Thus, the interaction between ELT and rosuvastatin cannot be quantitatively explained if we assume simple competition of these compounds at OATP1B1. Other possible explanations may include the hypothesis that bound form of ELT can also inhibit OATP1B1-mediated transport. This may be supported by the previous finding that OATP1B1-mediated transport is relatively insensitive to albumin compared with OATP1B3-mediated transport (Cui et al., 2001; Cui and Walter 2003). Another possibility is that the inhibitory effect of ELT may be substrate-dependent. Because OATP1B1 has
multiple transport sites (Tamai et al., 2001), substrate recognition sites in OATP1B1 could be different between estrone-3-sulfate and rosuvastatin. OATP1B1 is also involved in disposition of certain anticancer drugs, such as SN-38, the active metabolite of irinotecan, and methorexate (Nozawa et al., 2005; van de Steeg et al., 2009). Since thrombocytopenia is often induced by cancer chemotherapy, and ELT has recently been used also for patients receiving anticancer drugs, it is necessary to consider the possible interaction of ELT with OATP1B1-mediated transport of anticancer drugs.

In humans with renal impairment, AUC of ELT was 32~60% lower than in healthy subjects (Bauman et al., 2010). In the present study, we found that ELT is also highly distributed to the kidney (Figure 1), but is not detectable in urine (Table 1). This could indicate minimal secretion and/or efficient reabsorption of ELT in renal tubules. Considering the major contribution of the liver to overall ELT elimination (Figure 1, Table 1), it may be difficult to speculate the association of renal function with ELT disposition. In renal impairment, levels of uremic toxins and other unwanted substances are elevated in plasma (Lim et al. 1993; Sakai et al. 1996; Fujita et al., 2010). Because protein binding of some kinds of uremic toxins is very high (Takamura et al., 1997), uremic toxins may saturate plasma protein binding. This in turn might lead to a higher unbound concentration of ELT and more efficient hepatic elimination, resulting in a lower ELT concentration in the circulation.

In summary, our present findings suggest that hepatic uptake is the rate-limiting
process in the elimination of ELT, and that ELT is taken up by multiple hepatic transporters including organic anion transporter OATP1B1 and organic cation transporter OCT1. Further examination of the mechanisms of hepatic uptake of ELT would be helpful both for optimizing administration of ELT to patients and for development of new therapeutic agents.
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AUTHORSHIP CONTRIBUTIONS

Participated in research design:  Sugiura, Horikawa, Kato

Conducted experiments:  Takeuchi, Sugiura, Umeda, Matsubara

Contributed new reagents or analytic tools:  Takeuchi, Ishiwata

Performed data analysis:  Takeuchi, Umeda, Matsubara, Nakamichi

Wrote or contributed to the writing of the manuscript:  Sugiura, Kato

Other (provided $pdzk1^{-/-}$ mice and discussed the experiments):  Silver
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relevance of first-pass effect from hepatic uptake transporters, and intestinal and hepatic metabolism. *Drug Metab Dispos* **34**: 1175-1181.


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LEGENDS FOR FIGURES

Figure 1

**Plasma concentration-time course (A), cumulative biliary excretion (B) and tissue distribution (C) of ELT after intravenous administration.**

ELT was intravenously injected into bile-duct-cannulated rats at 1 mg/kg. Serial plasma (A) and bile (B) samples were collected at designated time intervals. (C) Tissue-to-plasma concentration ratio (Kp) of ELT was obtained 8 hr after administration. Each value represents the mean ± SEM (n = 4).

Figure 2

**Plasma concentration-time course (A), cumulative biliary excretion (B) and integration plot (C) of ELT after intravenous administration with or without rifampicin.**

ELT was intravenously injected into bile-duct-cannulated rats at 1 mg/kg with (○) or without (●) RIF given as a bolus dose (20 mg/kg). Serial plasma (A) and bile (B) samples were collected at designated time intervals. In panel (C), liver and plasma concentrations were determined up to 20 min after ELT administration to analyze the initial phase of hepatic distribution. The CL_uptake was obtained from the slopes of the regression lines (showed as solid lines). Each value represents the mean ± SEM (n = 4). *, Significant difference from the control (p < 0.05).
Figure 3

**Time course (A), concentration dependence (B), pH dependence (C) and Na\(^+\) or Cl\(^-\) dependence (D) of the uptake of ELT by isolated mouse hepatocytes.**

(A) Uptake of ELT by mouse hepatocytes was measured over 10 min at 37 (●) or 4 °C (○) and pH 7.4. (B) Uptake of ELT was measured at 37°C and pH 7.4 for 5 min in the concentration range of 3 to 200 μM. (C) Uptake of ELT was measured at 37°C and pH 5.5, 6.5 and 7.4 for 5 min. (D) Uptake of ELT was measured at 37°C and pH 7.4 for 5 min with (control) or without extracellular Na\(^+\) or Cl\(^-\) (Na\(^+\)(-): Na\(^+\) was replaced with Li\(^+\); Cl\(^-\)(-): Cl\(^-\) was replaced with gluconate). In panels (A), (C) and (D), the results are shown as cell-to-medium ratio (A, C, D). Each value represents the mean ± SEM (n = 3).

Figure 4

**Inhibitory effects of various compounds on ELT uptake by isolated mouse hepatocytes.**

Uptake of ELT by freshly isolated mouse hepatocytes was measured at 37°C and pH 7.4 for 5 min in the absence (open column) and presence of various compounds (closed columns). Results are shown as percent of control. Each value represents the mean ± SEM (n = 3 - 6). *, Significant difference from the control (p < 0.05).
Figure 5

Effect of TPeA or RIF on CLuptake of ELT (A, B) and metformin (C)

ELT (A, B; 1 mg/kg) or metformin (C; 30 µmol/kg) was intravenously injected into mice. TPeA (30 µmol/kg) or RIF (20 mg/kg) was injected before the administration of ELT or metformin. Plasma concentrations were determined up to 2 min after the administration of ELT or metformin, and the liver and kidney were excised. CLuptake was estimated as the ratio of the tissue amount to AUC. Each value represents the mean ± SEM (n = 4). *, Significant difference from the control (p < 0.05).

Figure 6

Effects of ELT on the uptake of typical substrates by human hepatic uptake transporters

HEK293/PDZK1 cells transfected with OATP1B1 (A), OATP1B3 (B), OATP2B1 (C), OCT1 (D) or OCTN2 (E) gene were incubated with [3H]estrone 3-sulfate (OATP1B1 and 2B1) for 3 min, [3H]estradiol 17beta-glucuronide (OATP1B3) for 5 min, [14C]tetraethylammounium (OCT1) for 15 min or [3H]carnitine (OCTN2) for 3 min at 37°C in absence or presence of ELT (●) or typical substrates or inhibitors (bromosulfophthalein for OATP1B1, OATP1B3 and OATP2B1, tetraethylammonium for OCT1 and carnitine for OCTN2, ○) at the designated concentrations. Uptake of labeled compound was measured, and transporter-mediated uptake was calculated by subtraction of the uptake observed in
vector-transfected HEK293/PDZK1 cells from that in transporter gene-transfected HEK293/PDZK1 cells. Each value represents the mean ± SEM (n = 3-4).

Figure 7
Uptake of ELT by HEK293/PDZK1 cells transfected with OATP1B1 (A), OATP1B3 (B), OATP2B1 (C) and OCT1 (D).

HEK293/PDZK1 cells transfected with the transporter gene (●) or vector alone (○) were incubated with ELT for 30 min at 37°C. The results are shown as cell-to-medium ratio. Each value represents the mean ± SEM (n = 3). *, Significant difference from the vector alone (p < 0.05).
### Table 1. Pharmacokinetic parameters for ELT (1 mg/kg) in rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$CL_{tot}$ (mL/hr/kg)</td>
<td>33.0 ± 5.2</td>
</tr>
<tr>
<td>$CL_{bile,p}$ (mL/hr/kg)</td>
<td>13.6 ± 2.9</td>
</tr>
<tr>
<td>$CL_{urine}$ (mL/hr/kg)</td>
<td>&lt; 0.07</td>
</tr>
<tr>
<td>$V_0$ (mL/kg)</td>
<td>41.1 ± 5.3</td>
</tr>
<tr>
<td>$V_{dss}$ (mL/kg)</td>
<td>287 ± 38</td>
</tr>
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</table>

a) Each value represents the mean ± SEM (n = 4).

b) Calculated as the amount of ELT excreted into the bile for 72 hr divided by AUC for the same time period.

c) Under the detection limit.
Table 2. Pharmacokinetic parameters for ELT (1 mg/kg) in rats with or without RIF (20 mg/kg)\textsuperscript{a)}

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>With RIF</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL\textsubscript{tot} (mL/hr/kg)\textsuperscript{b)}</td>
<td>34.3 ± 3.9</td>
<td>20.1 ± 2.1 *</td>
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<tr>
<td>CL\textsubscript{bile,h} (mL/hr/kg)\textsuperscript{b,c)}</td>
<td>2.38 ± 0.37</td>
<td>1.95 ± 0.20</td>
</tr>
<tr>
<td>CL\textsubscript{uptake} (mL/hr/kg)\textsuperscript{d)}</td>
<td>44.8 ± 4.4</td>
<td>22.8 ± 3.3 *</td>
</tr>
</tbody>
</table>

a) Each value represents the mean ± SEM (n = 4).
b) Obtained in bile-duct-cannulated rats.
c) Calculated as the excretion rate of ELT into the bile for 6 to 8 hr divided by the hepatic concentration at 8 hr.
d) Obtained for the liver in the integration plot analysis.
* Significantly different from control (p < 0.05).
Figure 2

(A) Plasma Conc. (µg/mL) vs. Time (hr)

(B) Cumulative Amount (% of Dose) vs. Time (hr)

(C) $X_h(t)/C_p(t)$ (mL/g liver) vs. $\text{AUC}_{0-t}/C_p(t)$ (min)
Figure 3

(A) Uptake (μL/mg protein) vs. Time (min)

(B) Uptake Velocity (pmol/min/mg protein) vs. ELT Conc. (μM)

(C) Uptake (μL/mg protein) at different pH levels: 5.5, 6.5, 7.4

(D) Uptake (μL/mg protein) in control and with Na⁺ (-) and Cl⁻ (-)
### Figure 4

<table>
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<tr>
<th>Compound</th>
<th>Concentration (mM)</th>
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<tr>
<td>Taurocholic acid (TCA)</td>
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<td></td>
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<td>Lactic acid</td>
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<tr>
<td></td>
<td>0.1</td>
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<tr>
<td>Tetraethylammonium (TEA)</td>
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Figure 5

(A) CL_{uptake} (mL/min/kg)

<table>
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<th>+RIF</th>
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(B) CL_{uptake} (mL/min/kg)

<table>
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(C) CL_{uptake} (mL/min/kg)

<table>
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<tr>
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<tr>
<td>Kidney</td>
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</tbody>
</table>
Figure 6

(A) Uptake (% of Control)

(B) Uptake (% of Control)

(C) Uptake (% of Control)

(D) Uptake (% of Control)

(E) Uptake (% of Control)

Inhibitor Conc. (µM)
Figure 7

(A) and (B) show the uptake of a substance over time (in minutes) with different conditions. (A) demonstrates a significant increase in uptake starting from 10 minutes onwards, while (B) shows a similar trend but with a slight lag compared to (A). (C) and (D) also depict similar trends, with (C) showing a more pronounced effect from 20 minutes and (D) showing a delayed effect starting from 15 minutes.