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

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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 h, 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, tetrapentylammonium. Finally, uptake of ELT was observed in human embryonic kidney 293 cells transfected with human hepatic transporters or- ganic anion-transporting polypeptide; RIF, rifampicin; TPeA, tetrapentylammonium; PDZ, postsynaptic density 95/disc-large/zona occludens; HEK, human embryonic kidney; OCT, organic cation transporter; SN-38, 7-ethyl-10-hydroxycamptothecin.

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 and Begley, 2002), as well as being induced by medical treatments such as chemotherapy for cancer (Kaushansky, 1996; Elting et al., 2001) and interferon therapy for hepatitis C (Ong and Younossi, 2004; Curry and Afdhal, 2005). ITP is an autoimmune disease in which antibodies to TPO are also effective for patients with ITP (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 nonpeptide TPO receptor agonists.

Eltrombopag (Promacta, 3'-{N-[1-(3,4-dimethylphenyl)-3-methyl-5-oxo-1,5-dihydropyrazol-4-ylidene]hydrazine}-2'-hydroxybiphenyl-3-carboxylic acid) (Supplemental Fig. S1), is a novel, small-molecule, nonpeptide TPO receptor agonist, which can be orally administered (Erickson-Miller et al., 2004; Sellers et al., 2004; Bussel et al., 2007; Jenkins 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, which results in an increase in 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 was 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 that for 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. Information on the mechanism of overall elimination and hepatic handling of ELT would be important for appropriate treatment 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 Life and Analytical Sciences (Waltham, MA). [3H]Estradiol 17β-glucuronide (41.8 Ci/mmole), [3H]carcinine (85 Ci/mmole), and [3H]tetra-ethylammonium (55 Ci/mmol) were obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Rifampicin (RIF) and tetratetrapentylammonium (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 (7–9 weeks old) and rats with the jugular vein and bile duct precannulated (8–9 weeks old) were purchased from Charles River Japan (Yokohama, Japan). Male mice were used at 7 to 9 weeks of age. Transporter adaptor PDZK1 gene knockout (−/−) mice were constructed previously (Sugiura et al., 2010) with some modifications to avoid nonspecific 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 CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, and 25 mM HEPES, pH 7.4).

The uptake experiment was then performed according to the silicone oil layer method (Sugiuara et al., 2010) with some modifications to avoid nonspecific adsorption of ELT. In brief, 50 μl of the cell suspension was preincubated for 5 min at 37°C, followed by start of the reaction by mixing the suspension with 50 μl of prewarmed transport buffer, which contained ELT and 10 μM human serum albumin (fatty acid free). In the inhibition study, the preincubation was performed in the absence of inhibitors except for rotenone and carboxyl cyanide-p-trifluoromethoxyphenylhydrazone, which was also included in the preincubation mixture. The reaction was stopped by addition of 600 μl of ice-cold transport buffer, which also contained 0.1% bovine serum albumin, and the mixture was then quickly centrifuged through a silicone oil layer.

Transport 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 the administration of ELT. After 120 s, 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 eq. 1:

$$\frac{X(t)}{C_p(t)} \times \text{AUC}_{\text{uptake}} + V_c \frac{C_p(t)}{C_p(0)} \times t = C_p(t)$$

where $X(t)$ and $C_p(t)$ are the amounts in tissue and plasma concentration at time $t$, respectively. $V_c$ is the volume of distribution, within which a rapid equilibrium with the plasma compartment is assumed. When $X(t)/C_p(t)$ is plotted against AUC/Cp(t), the slope represents the value of CLuptake. In mice, CLuptake was approximately estimated as $X(t)/AUC_{pl}(0 - t)$.

Isolation and Transport Experiments in Mouse Hepatocytes. Hepatocytes were isolated from mice by the collagenase perfusion method with some modifications as described previously (Sugiuara 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 CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, and 25 mM HEPES, pH 7.4).

Isolation and Transport Experiments 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 (OCT1) 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 because 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 a 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 the uptake study. The associated radioactivity was measured with a liquid scintillation counter (Aloka, Tokyo, Japan) with Cleasol I (Nacalai Tesque, Kyoto, Japan).
Japan) as the scintillation fluid, after neutralization with HCl of the cell lysate obtained by solubilization with KOH. The cellular protein content was determined according to the method of Bradford (1976) using a protein assay kit (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as the standard. The 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 (Quattro Premier XE; Waters, Milford, MA) coupled to a liquid chromatography system (ACQUITY UPLC, Waters). 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; and 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 column (1.7-µm particle size, 2.1 mm i.d. × 50 mm; Waters) 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 the internal standard (diclofenac). The quantitation limit for ELT was 10 ng/ml or 20 nM. Determination of metformin was performed using a high-performance liquid chromatography system consisting of a model LC-10AD VP pump and a model SPD-10A VP UV monitor (Shimadzu, Tokyo, Japan) with a COSMOSIL 5C18-AR-II column (4.6 mm i.d. × 150 mm; Nacalai Tesque). The mobile phase consisted of 20 mM ammonium acetate containing 2.5 mM 1-octanesulfonic acid sodium salt/acetate buffer (pH 5.0) 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 on the basis of eqs. 2 and 3:

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

(2)

where v, s, I, V_{\text{max}}, K_m, K_{ns}, and K_{ns} represent the initial uptake velocity, substrate concentration, inhibitor concentration, maximal uptake velocity, Michaelis constant, inhibitory constant, and nonsaturable clearance, respectively. The selection of the equation was based on the Akaike information criterion.

Results

Pharmacokinetic Profile of ELT. After intravenous injection of ELT (1 mg/kg) into rats, ELT in plasma slowly disappeared and was detectable up to 72 h after dosing (Fig. 1A). Approximately 40% of the dose was excreted into the bile in unchanged form up to 72 h (Fig. 1B), whereas the amount detected in the urine was less than 0.02% of the dose (Table 1). The CL_{bile} (Table 1) was much lower than the hepatic blood flow rate (3.31 ± 0.7 ml/min). The cumulative biliary excretion (B), and tissue distribution (C) of ELT after intravenous administration. ELT was injected intravenously into bile duct-cannulated rats at 1 mg/kg. Serial plasma (A) and bile (B) samples were collected at designated time intervals. C, the tissue/plasma concentration ratio (K_p) of ELT was obtained 8 h after administration. Each value represents the mean ± S.E.M. (n = 4).

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)

Fig. 1. Plasma concentration-time course (A), cumulative biliary excretion (B), and tissue distribution (C) of ELT after intravenous administration. ELT was injected intravenously into bile duct-cannulated rats at 1 mg/kg. Serial plasma (A) and bile (B) samples were collected at designated time intervals. C, the tissue/plasma concentration ratio (K_p) of ELT was obtained 8 h after administration. Each value represents the mean ± S.E.M. (n = 4).

### Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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<tr>
<td>CL_{bile} (ml·h^{-1}·kg^{-1})</td>
<td>33.0 ± 5.2</td>
</tr>
<tr>
<td>CL_{urine} (ml·h^{-1}·kg^{-1})</td>
<td>13.6 ± 2.9*</td>
</tr>
<tr>
<td>V_{bile} (ml/kg)</td>
<td>&lt;0.07b</td>
</tr>
<tr>
<td>V_{urine} (ml/kg)</td>
<td>41.1 ± 5.3</td>
</tr>
<tr>
<td>V_{bile} (ml/kg)</td>
<td>287 ± 38</td>
</tr>
</tbody>
</table>

* Calculated as the amount of ELT excreted into the bile for 72 h divided by AUC for the same time period.

* Under the detection limit.
concentration at 8 h.

CLuptake assessed from the slope of the integration plot (Fig. 2C) was lower (Fig. 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, the plasma concentration of ELT was higher than that in the control (Fig. 2A), with a concomitant decrease in biliary excretion of ELT (Fig. 2B). Coadministration of RIF also reduced hepatic uptake of ELT, which was directly estimated by means of integration plot analysis (Fig. 2C). The CLuptake assessed from the slope of the integration plot (Fig. 2C) was decreased to 51% of the control by the coadministration of RIF (Table 2). A similar reduction by RIF (to 59% of the control) was also observed for the CLtot assessed from the plasma concentration-time profile (Fig. 2A; Table 2). In addition, the absolute values of CLuptake in both groups were comparable to those of CLtot (Table 2). On the other hand, CLbile, 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 (Figs. 3 and 4). The reason for using hepatocytes isolated from mice rather than rats is that gene knockout animals [pdxk(−/−) mice] are available to examine possible involvement of specific transporters in hepatic uptake in vivo. ELT uptake at 37°C by the isolated hepatocytes linearly increased up to 5 min, whereas that at 4°C was minimal (Fig. 3A). On the basis of 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 a higher concentration of ELT (Fig. 3B), but the Km value for ELT uptake was not evaluated because of the limit of solubility for ELT (~200 μM). ELT uptake was slightly lower at acidic pH than at pH 7.4 (Fig. 3C). On the other hand, the ELT uptake was almost unchanged in the absence of Na+ or Cl− (Fig. 3D).

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

**TABLE 2**

Pharmacokinetic parameters for ELT (1 mg/kg) in rats with or without RIF (20 mg/kg)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>With RIF</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLtot (ml·h⁻¹·kg⁻¹)</td>
<td>34.3 ± 3.9</td>
<td>20.1 ± 2.1*</td>
</tr>
<tr>
<td>CLbile, h (ml·h⁻¹·kg⁻¹)</td>
<td>2.38 ± 0.37</td>
<td>1.95 ± 0.20</td>
</tr>
<tr>
<td>CLuptake (ml·h⁻¹·kg⁻¹)</td>
<td>44.8 ± 4.4</td>
<td>22.8 ± 3.3*</td>
</tr>
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</table>

* Significantly different from control (p < 0.05).
Effect of TPeA on Hepatic Uptake of ELT In Vivo. To determine whether ELT uptake is inhibited by an organic cation in vivo, we next examined the effect of TPeA on hepatic uptake of ELT (Fig. 5, A and B). 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 maximal plasma concentration of ELT was at most 40 μM (data not shown), and at this concentration, hepatic uptake of ELT was almost linear (Fig. 3B), implying that the hepatic uptake process of ELT was not saturated under this condition. The CLuptake of ELT in the liver was significantly decreased by coadministration of TPeA (Fig. 5A). Inhibition by RIF of hepatic ELT uptake was also confirmed in this experiment (Fig. 5A). On the other hand, CLuptake in kidney was not affected by TPeA or RIF (Fig. 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 (Fig. 5C). The CLuptake in both liver and kidney for metformin was decreased in the presence of TPeA (Fig. 5C), confirming the inhibition of OCTs by TPeA in vivo. To demonstrate the involvement of transporter(s), systemic elimination of ELT was examined in pdzk1(−/−) mice. The CLuptake of ELT in pdzk1(−/−) mice was not completely reduced but was significantly lower than that in wild-type mice (77.3 ± 4.9 and 55.2 ± 3.9 in wild-type and pdzk1(−/−) mice, respectively; n = 4 for each). PDZK1 regulates expression and/or localization of various transporters in vivo (Wang et al., 2005; Sugihara et al., 2008). In particular, Oatp1a1 is down-regulated in pdzk1(−/−) mice (Wang et al., 2005). Therefore, the partial reduction in CLuptake of ELT in pdzk1(−/−) mice is possibly due to the reduction in uptake by a certain transporter 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, a potential interaction of ELT with human hepatic uptake transporters OATP1B1, OATP1B3, OATP2B1, OCT1, and OCTN2 was examined (Fig. 6). ELT showed inhibitory effects on OATP1B1- and OATP2B1-mediated uptake of [3H]estradiol 17β-glucuronide, and OCT1-mediated uptake of [14C]tetraethylammonium in the concentration range of 1 to 50 μM, whereas the inhibitory effect of ELT on OCTN2-mediated uptake of [3H]carnitine was minimal (Fig. 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 (Fig. 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 (Fig. 7). 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 (Fig. 7) but increased the uptake of [3H]estradiol 17β-glucuronide.
Control results may imply that systemic exposure to ELT can be affected by reduced by various types of transporter inhibitors (Figs. 3 and 4), uptake of ELT is temperature- and energy-dependent, saturable, and incorporation of ELT due to malfunction in the major clearance organ. Hepatic (Bauman et al., 2010), probably because of reduced hepatic disposition of ELT in patients with such hepatic impairments of ELT. For example, a clinical trial of ELT in patients with ELT. These findings may be of importance for the design of clinical studies of ELT. 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 (Figs. 4 and 5). This is the first report of an anionic compound being uptake is coadministration of RIF decreased both CLtot and CLuptake of ELT in the liver, whereas CLnhep,h was not affected (Fig. 2; Table 2). The 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, bile excretion accounted for ~40% of the overall elimination of ELT (Fig. 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, because 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 (Figs. 3 and 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 for 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 (Kallikoski et al., 2010; Watanabe et al., 2010). Nevertheless, ELT has been thought to be a nonsubstrate of OATP1B1 (Gibiansky et al., 2010). On the other hand, ELT was found to be a substrate of OATP1B1 in the present study, because the uptake of ELT by HEK293/PDZK1 cells transfected with OATP1B1 was higher than that in vector-transfected cells (Fig. 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, which 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 an organic cation transporter (OCT1) (Fig. 6). In addition, ELT is taken up by anion (OATP1B1 and OATP2B1) and cation (OCT1) transporters (Fig. 7). This uptake may be compatible with the inhibition of hepatic ELT uptake by various compounds with a broad range of structures (Figs. 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 (Figs. 4 and 5). This is the first report of an anionic compound being uptake by freshly 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 and presence of various compounds. Results are shown as percentage control. Each value represents the mean ± S.E.M. (n = 3–6). * significant difference from the control (p < 0.05). GlySar, glycyl-sarcosine; Phe, phenylalanine; FCCP, carbonyl cyanide-p-trifluoromethoxyphenyl-hydrazone.

**Discussion**

The present study has established that the liver is the major distribution and elimination organ for ELT (Fig. 1; Table 1). In addition, hepatic uptake could be the rate-limiting process in the overall elimination of ELT on the basis of the following findings. First, the CLtot of ELT was close to CLuptake in the liver, which was obtained from integration plot analysis (Tables 1 and 2). Second, the inhibition of hepatic ELT uptake by coadministration of RIF decreased both CLtot and CLuptake of ELT in the liver, whereas CLnhep,h was not affected (Fig. 2; Table 2). The 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, bile excretion accounted for ~40% of the overall elimination of ELT (Fig. 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, because 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 (Figs. 3 and 4), suggesting the involvement of carrier-mediated mechanisms. These results may imply that systemic exposure to ELT can be affected by

![Figure 4](image1.png) 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 and presence of various compounds. Results are shown as percentage control. Each value represents the mean ± S.E.M. (n = 3–6). * significant difference from the control (p < 0.05). GlySar, glycyl-sarcosine; Phe, phenylalanine; FCCP, carbonyl cyanide-p-trifluoromethoxyphenyl-hydrazone.

![Figure 5](image2.png) Effect of TPeA or RIF on CLuptake of ELT (A and B) and metformin (C).
transported by organic cation transporter OCT1. Regarding organic anion transporters that also belong to solute carrier 22A family as does 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 a partial positive charge (Supplemental 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. Of interest, coadministration of a polyvalent cation-containing antacid, such as aluminum 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 a chelate reaction between ELT and positive ionic metals (Williams et al., 2009), another possibility is that a 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 (Figs. 6 and 7). However, to understand the design of the clinical use of ELT, further experiments using human hepatocytes are required to investigate the contribution of each transporter to hepatic uptake of ELT in humans.

In the present study, ELT inhibited OATP1B1-mediated transport (Fig. 6A). This result would be compatible with the clinically observed drug-drug interaction: administration of a single dose of rosuvastatin after repeated daily ELT dosing increased the AUC of rosuvastatin by 55% and maximal plasma concentration (Cmax) by 103% (Danish et al., 2010). In consideration of the fundamental role of OATP1B1 in overall elimination of rosuvastatin (Kitamura et al., 2008), such an interaction could occur via inhibition of OATP1B1 by ELT. The inhibition constant of ELT for OATP1B1 (14.9 \mu M) (Fig. 6A) was close to the clinical concentration of ELT (Cmax = 14 \mu M) (Williams et al., 2009). 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 maximal unbound concentration in the portal vein after oral administration of ELT was calculated to be at most ~0.026 \mu M, 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 include the hypothesis that the 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 7-ethyl-10-hydroxy camptothecin (SN-38), the active metabolite of irinotecan, and methotrexate (Nozawa et al., 2005; van de Steeg et al., 2009). Because 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, the AUC of ELT was 32 to 60% lower than that in healthy subjects (Bauman et al., 2010). In the present study, we found that ELT is also highly distributed to the kidney (Fig. 1) but is not detectable in urine (Table 1). This finding could indicate minimal secretion and/or efficient reabsorption of ELT in renal tubules. In consideration of the major contribution of the liver to overall ELT elimination (Fig. 1; Table 1), it may be difficult to speculate on 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., 2011). 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 transporters OATP1B1 and organic cation transporter OCT1. Further examination of the mechanisms of hepatic uptake of ELT would be helpful both for optimizing the administration of ELT to patients and for development of new therapeutic agents.

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Authorship Contributions

Participated in research design: Sugiuira, Horikawa, and Kato.

Conducted experiments: Takeuchi, Sugiuira, Umeda, and Matsubara.

Contributed new reagents or analytic tools: Takeuchi and Ishiwata.

Performed data analysis: Takeuchi, Umeda, Matsubara, and Nakamichi.

References


Silver provided

Wrote or contributed to the writing of the manuscript: Sugiuira and Kato.

Other: Silver provided pkfki (−/−) mice and discussed the experiments.

Fig. 7. Uptake of ELT by HEK293/PKDZ1 cells transfected with OATP1B1 (A), OATP1B3 (B), OATP2B1 (C), and OCT1 (D). HEK293/PKDZ1 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/medium ratio. Each value represents the mean ± S.E.M. (n = 3), * significant difference from the vector alone (p < 0.05).


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