Organic cation transporter 1 is responsible for hepatocellular uptake of the tyrosine kinase inhibitor pazopanib

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Abbreviations: ABT, 1-aminobenzotriazole; AUC, area under the curve; CL\text{uptake}, hepatic uptake clearance; C_{\text{max}}, maximum concentration; CMZ, cefmetazole; CsA, cyclosporin A; CYP, cytochrome P450; E3S, Estrone-3-sulfate; HNF, hepatocyte nuclear factor; HSA, human serum albumin; K_p, tissue-to-plasma concentration ratio; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; PBD, probenecid; RIF, rifampicin; t_{1/2}, half-life at the terminal phase; TKI, tyrosine kinase inhibitor; TPeA, tetrapentylammonium; UGT, UDP-glucuronosyltransferase; USF, upstream stimulating factor; V_0, distribution volume to the central compartment
ABSTRACT

Pazopanib is an orally active tyrosine kinase inhibitor that exhibits hepatotoxicity in some patients. Despite the clinical importance of its hepatic distribution, the transporter(s) responsible for hepatic uptake of pazopanib in humans remain undetermined. In order to characterize its hepatic uptake mechanism, we screened the effects of several transporter inhibitors, including tetrapentylammonium (TPeA) for organic cation transporters (OCTs) and cyclosporin A (CsA) for organic anion-transporting polypeptides (OATPs), on both plasma disappearance and hepatic distribution of pazopanib in mice after its intravenous administration. Among the inhibitors, TPeA largely reduced hepatic distribution and plasma clearance of pazopanib, whereas CsA showed only partial reduction. Pazopanib uptake by isolated mouse hepatocytes was similarly reduced by these inhibitors, suggesting that OCTs play a major role in the overall hepatic uptake of pazopanib in mice. In HEK293 cells stably transfected with human OCT1, pazopanib uptake was significantly higher than that in vector-transfected cells. Moreover, pazopanib uptake by OCT1 became saturated and was inhibited by TPeA, but not by CsA, confirming that pazopanib is also a substrate of human OCT1. Importantly, OCT1-mediated uptake of a typical OCT1 substrate metformin was inhibited by pazopanib with an IC$_{50}$ value of 0.253 μM, indicating that
pazopanib has the potential for clinically relevant inhibition of human OCT1. Finally, pazopanib was taken up by cryopreserved human pooled hepatocytes in a time-dependent manner, and this uptake was largely reduced by TPeA, but only partially reduced by CsA. Thus, the present findings suggest that OCT1 is responsible for hepatocellular uptake of pazopanib.
Introduction

Pazopanib is an orally active, second-generation tyrosine kinase inhibitor (TKI) that targets multiple proteins including vascular endothelial growth factor receptors 1, 2, and 3, platelet derived growth factor receptor-α and β, and stem cell factor (Ward and Stadler, 2010). Pazopanib was first approved in 2009 by the United States Food and Drug Administration for the treatment of metastatic renal cell cancer after it was found to prolong progression-free survival in a phase III trial (Sternberg et al., 2010; Sternberg et al., 2013). It was further approved in 2012 for salvage treatment of metastatic soft tissue sarcoma (van der Graaf et al., 2012). During clinical treatment, a large interindividual variability in the pharmacokinetics of pazopanib was observed, with 80–200% and 70–180% of variation in area under the plasma concentration-time curve (AUC) and maximum concentration (C_{max}), respectively (Hurwitz et al., 2009). Because pazopanib is known to be metabolized in the liver and primarily eliminated via feces with minimal renal elimination (Keisner and Shah 2011), elucidation of its hepatic uptake, metabolism, and excretion is essential to comprehensively understand the overall disposition of this TKI.

Pazopanib undergoes hepatic metabolism largely via cytochrome P450 (CYP) 3A4 and to minor extent by CYP1A2 and CYP2C8 (Keisner and Shah 2011). In fact,
coadministration with the CYP3A4 inhibitor ketoconazole increased both the AUC and $C_{\text{max}}$ of pazopanib by 66 and 45%, respectively, in cancer patients (Tan et al., 2013), suggesting that this enzyme has a primary role in systemic elimination of pazopanib. In addition, coadministration of pazopanib and midazolam resulted in a small increase in systemic exposure to midazolam relative to that observed with administration of midazolam alone, suggesting that pazopanib is a clinically relevant inhibitor of CYP3A (Goh et al., 2010). Regarding its potential for interaction with hepatic transporters, pazopanib can inhibit the hepatic uptake transporter organic anion-transporting polypeptide (OATP) 1B1, but not OATP1B3 (Khurana et al., 2014b). Indeed, coadministration with pazopanib at 400 and 800 mg/day increases the AUC of SN-38, an active metabolite of irinotecan by 38 and 89%, respectively (Bennouna et al., 2015), probably because SN-38 is primarily taken up by OATP1B1 (Fujita et al., 2014).

Pazopanib administration also resulted in an elevation in plasma bilirubin concentration, which could be attributed to its inhibition of both UDP-glucuronosyltransferase 1A1 and OATP1B1 (Xu et al., 2010). The increase in the AUC of docetaxel by coadministration with pazopanib might be caused by its inhibition of CYP3A and OATP1B1 (Hamberg et al., 2015).

Pazopanib treatment is associated with hepatotoxicity, and approximately 41%
of the patients exhibit an increase in alanine aminotransferase levels (Kapadia et al., 2013). Therefore, clarification of its hepatic uptake mechanism in humans may be helpful for understanding the risk of such hepatotoxicity. Despite numerous studies on its interaction with drug-metabolizing enzymes and transporters in the liver, the mechanism responsible for hepatic uptake of pazopanib itself in humans is still largely unknown. Only in vitro experimental data obtained in cell lines overexpressing transporters are available, demonstrating that uptake of pazopanib in HEK293 cells transfected with OATP1B1 and OATP1B3 is higher than in vector-transfected HEK293 cells (Zimmerman et al., 2013). Conversely, Khurana et al. (2014a) reported that pazopanib uptake in CHO cells transfected with OATP1B1 and OATP1B3 is similar to that in vector-transfected cells. Additionally there has been no direct evidence in either pharmacogenomic or drug interaction studies that demonstrates a role for OATPs in hepatic uptake of pazopanib in humans.

In the present study, we sought to determine the transporter(s) responsible for hepatocellular uptake of pazopanib. The hepatic uptake mechanism was first characterized in mice by coadministering pazopanib with various inhibitors of hepatic transporters. The potential hepatic uptake transporters were then confirmed in HEK293 cells stably transfected with human transporter genes. Uptake studies in cryopreserved
pooled human hepatocytes were further performed to examine the contribution of each transporter using transporter inhibitors. We also examined the possible inhibitory potential of pazopanib on the uptake transporters.
Materials and Methods

Materials

Pazopanib was purchased from AdooQ Bioscience (Irvine, CA). 1-Aminobenzotriazole (ABT) was purchased from Tokyo Chemical Industries (Tokyo, Japan). Rifampicin (RIF), cyclosporin A (CsA) and probenecid (PBD) were purchased from Wako Pure Chemical Industries (Tokyo, Japan). Tetrabutylammonium (TPeA), human serum albumin (HSA, fatty acid free), silicone oil, and mineral oil were purchased from Sigma-Aldrich (St. Louis, MO). Cefmetazole (CMZ) was purchased from Daiichi-Sankyo (Tokyo, Japan). Metformin was obtained from LKT Laboratories Inc. (St. Paul, MN). [\(^{3}H\)]Estrone-3-sulfate (E3S, 57.3 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). All the other chemicals and reagents were commercial products of analytical grade.

Animals

Male ICR mice (7–9 weeks old) were purchased from Japan SLC (Hamamatsu, Japan) and housed in an experimental animal room with standard food and tap water provided ad libitum, with an ambient temperature range of 21–25°C, 30–70% humidity, and a 12 h light/dark cycle for at least 1 week before use. Animal experiments were
performed in accordance with the Guidelines for the Care and Use of Laboratory Animals in Kanazawa University, with efforts to minimize the number of animals used and their suffering. All protocols were approved by the Institutional Animal Care and Use Committee of Kanazawa University.

Time profile of hepatic distribution of pazopanib in mice

Pazopanib was dissolved in dimethyl sulfoxide to yield a 10 mM stock solution, and then diluted with 1% (v/v) Tween 80 dissolved in saline. Four groups of mice were used. All mice received pazopanib (0.02 mg/kg) through the jugular vein, and blood samples were taken from the caudal veins of non-anesthetized mice at 1, 5, 10, 30, and 60 min, 1, 30, 60, and 120 min, 1, 60, 120, and 240 min, and 1, 60, 120, 240, and 480 min in each group. Plasma was collected by centrifugation at 3,913 g for 5 min at 4 °C. At the last time point, mice were euthanized, and whole liver was immediately collected and stored with plasma samples at -80 °C until further processing. The concentration of pazopanib in plasma and liver tissue was quantified using liquid chromatography/tandem mass spectrometry (LC-MS/MS). Efflux from the liver soon after intravenous administration was assumed to be negligible, and the hepatic uptake clearance (CL\textsubscript{uptake}) was calculated with the following equation (Takeuchi et al., 2011):
where $X_{\text{liver}(t)}/C_{\text{p}(t)}$, and AUC$_{(0-t)}$ represent hepatic concentration, plasma concentration, and area under the curve, respectively. The $X_{\text{liver}(t)}/C_{\text{p}(t)}$ is regarded as tissue-to-plasma concentration ratio ($K_{\text{p}(t)}$). According to Eq. (1), the CL$_{\text{uptake}}$ was estimated as initial slope of a plot of $K_{\text{p}(t)}$ vs AUC$_{(0-t)}/C_{\text{p}(t)}$. The AUC$_{(0-t)}$ and distribution volume to the central compartment ($V_0$) were estimated from the plasma concentration-time profile with extrapolation to time zero by application of the trapezoidal rule. The half-life at the terminal phase ($t_{1/2}$) was estimated from the last three points in plasma concentration-time profile.

**Effect of transporter inhibitors on pazopanib disposition in mice**

CsA and RIF were dissolved in a mixture of dimethyl sulfoxide, ethanol, Kolliphor ELP, and 0.9% saline (2:2.5:2.5:93) (Taguchi et al., 2016). PBD was dissolved in a mixture of 2 N NaOH, 1 M Tris, and PBS (3:17:80), and pH was adjusted to 7.3 with 1 N HCl (Qi et al., 2015). ABT, CMZ, and TPeA were dissolved in saline. The inhibitors, TPeA (11.3 mg/kg, i.v.), RIF (25 mg/kg, i.v.), CMZ (200 mg/kg, i.v.), PBD (150 mg/kg, i.p.), ABT (50 mg/kg, i.v.), and CsA (5 mg/kg, i.v.) were injected at 1, 5, 15, 30, 120 min, and 1 day, respectively, prior to intravenous administration of pazopanib.
according to previous reports (Akanuma et al., 2011; Balani et al., 2004; Choi et al., 2007; Lau et al., 2006; Sani et al., 2010; Taguchi et al., 2016; Takeuchi et al., 2011). The selection of times and doses chosen for administration of each inhibitor was not optimized in the present study, but based on the previous reports. Because of the differences in vehicle and the timing of treatment between each inhibitor, we performed control experiments using the corresponding vehicle alone for each inhibitor. Blood samples were taken from the caudal veins of non-anesthetized mice at 1, 30, 60, 120, 240, and 480 min after pazopanib injection under unrestricted conditions. At the last time point, mice were euthanized, and whole liver was immediately collected and stored with plasma samples at -80 °C until further processing. The apparent CL\text{uptake} value (CL_{\text{uptake,app}}) was approximately estimated as \( X_{\text{liver}(8\ h)}/AUC(0-8\ h) \).

**Transport studies in isolated mouse hepatocytes**

Hepatocytes were isolated from mice by the collagenase perfusion method (Takeuchi et al., 2011). Viability was assessed by trypan blue exclusion, and hepatocyte preparations with more than 90% viability were used. The isolated hepatocytes were resuspended at a final cell density of \( 1.0 \times 10^6 \) viable cells/mL in ice-cold transport buffer (123 mM NaCl, 4.8 mM KCl, 1.2 mM K\textsubscript{2}PO\textsubscript{4}, 1.2 mM MgSO\textsubscript{4}, 1.2 mM CaCl\textsubscript{2}, 5.6 mM...
glucose, 4.17 mM NaHCO₃, and 20 mM HEPES, pH 7.4), and stored on ice before the experiment. The uptake experiment was performed according to the silicone oil layer method (Sugiura et al., 2010). In brief, 50 µL of the cell suspension was preincubated for 10 min at 37 °C, and the reaction was initiated by mixing the suspension with 50 µL of prewarmed transport buffer containing pazopanib and 0.3% (w/v, final concentration) of HSA, in order to minimize nonspecific adsorption of pazopanib. In the inhibition experiments, the cell suspension was preincubated as described above, and inhibitors were added in the incubation mixture containing pazopanib. The reaction was stopped by adding 600 µL of ice-cold transport buffer containing 0.3% HSA, and 200 µL of the mixture was then immediately added to a 0.4 mL centrifuge tube containing 50 µL of 0.75 M KOH under a layer of 100 µL of oil mixture (a mixture of silicone and mineral oil at a density of 1.015), and the sample tubes were subsequently centrifuged for 60 sec at 6,700g. After an overnight incubation to dissolve the hepatocytes in alkali, the tubes were frozen at -30°C and cut at the oil layer. The bottom layer of the tubes containing hepatocytes was transferred to 1.5 mL tubes, centrifuged, and stored at -30 °C until LC-MS/MS analysis after neutralization with 50 µL of 1 N HCl.

Uptake study in HEK293 cells transfected with hepatic transporters
HEK293 cells stably expressing OCT1 or OATP1B1 (HEK293/OCT1 and HEK293/OATP1B1, respectively) were previously established (Fujita et al., 2014; Takeuchi et al., 2014). HEK293 cells stably expressing an empty vector (HEK293/mock) were also used as a control. These cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Sigma-Aldrich), 1% sodium pyruvate, 100 U/mL penicillin, 100 mg/mL streptomycin, and 1 mg/mL G418 in a humidified incubator at 37 °C and 5% CO₂. To perform uptake studies, cells were seeded on 24-well plates coated with 0.1 mg/mL poly-D-lysine at a density of 1.0, 0.73 and 0.5×10⁵ cells/well for HEK293/OATP1B1, HEK293/OCT1, and HEK293/mock cells, respectively. Culture medium was replaced with fresh medium supplemented with 5 mM sodium butyrate 1 day before the uptake studies to induce the expression of proteins. Three days after seeding, cells were washed with transport buffer. The transport buffer contained 0.3% HSA when the uptake of pazopanib and [³H]E3S was measured; however, the buffer did not contain HSA when metformin uptake was measured, since the uptake of metformin was clearly observed in the absence of HSA. After incubating for the designated times, the medium was collected to determine pazopanib concentration, and the cells were washed three times with ice-cold transport buffer. Cells were lysed with 300 µL of pure water and recovered using a cell scraper. The amount of cellular protein was determined.
according to the Bradford method (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as the standard. The concentrations of pazopanib and metformin in the medium and cells was determined by LC-MS/MS. Concentration-dependent data for OCT1-mediated uptake of pazopanib were fitted to the Michaelis-Menten equation to obtain $K_m$ and $V_{max}$ values, whereas concentration-dependent inhibition data of metformin uptake by pazopanib were fitted to the following equation to estimate the $IC_{50}$ values:

$$\frac{V_{inhibitor}}{V_{control}} = \frac{IC_{50}^g}{(I^g + IC_{50}^g)}$$

(2)

where $V_{inhibitor}$ and $V_{control}$ represent OCT1-mediated uptake of metformin by pazopanib-treated and control cells, respectively, and $I$ is the medium pazopanib concentration, which was directly measured by LC-MS/MS. The Damping Gauss-Newton Method algorithm was used with a MULTI program to perform nonlinear least-squares data fitting (Taguchi et al., 2016).

**Transport studies in cryopreserved human hepatocytes**

Cryopreserved human pooled hepatocytes (GIBCO Lot#HUE50-J, Thermo Fisher Scientific, Malvern, PA) were thawed and purified using Hepatocyte Thaw Medium (Thermo Fisher Scientific) according to the manufacturer’s instructions. The
cells were resuspended at a density of 2 mg protein/mL in the transport buffer (pH 7.4) containing 0.3% HSA. The uptake experiment was then performed according to the silicone oil layer method (Fujita et al., 2014). In brief, 20 µL of the cell suspension was preincubated for 5 min at 37˚C, and then the reaction was initiated by combining the suspension with 80 µL of prewarmed transport buffer containing pazopanib with or without inhibitors. The reaction was stopped by the addition of 600 µL of ice-cold transport buffer, and the mixture was then quickly centrifuged through a layer of oil mixture to separate the cells from the medium. Hepatocytes were then lysed overnight with alkali solution (3N KOH), and the lysed hepatocytes and medium were stored at -30˚C until LC-MS/MS analysis.

**Liquid Chromatography for determination of pazopanib and metformin**

Liver samples were mixed with 3 mL/g liver of PBS and homogenized using a hand-held sonicator (Ultrasonic Disruptor UR-20P, Tomy Seiko, Tokyo, Japan). Plasma, the homogenized liver, and cell lysates were deproteinized with a 50% methanol solution including 1 µM sorafenib and cimetidine as internal standards for pazopanib and metformin, respectively, and then centrifuged at 25,000g for 10 min. The supernatants were analyzed by LC-MS/MS. All drugs were quantified using a triple quadrupole mass
spectrometer with electrospray ionization (LCMS-8040; Shimadzu, Kyoto, Japan) coupled to a liquid chromatography system (Nexera, Shimadzu). Chromatography was performed by means of step-gradient elution (flow rate, 0.4 mL/min) as follows: 0 to 0.5 min, 90% A/10% B; 0.5 to 3.0 min, 90% A/10% B to 10% A/90% B; 3.0 to 4.0 min, 10% A/90% B; 4.0 to 4.1 min, 90% A/10% B to 10% A/90% B; 4.1 to 5.5 min, 90% A/10% B; (A, water containing 0.1% formic acid; B, acetonitrile containing 0.1% formic acid) on a Cosmosil C18-MS-II packed column (3 µm particle size, 2.0 mm I.D. × 50 mm; Nacalai Tesque, Kyoto, Japan) at 50°C for pazopanib. Multiple reaction monitoring in positive ion mode was set at 438.10 to 357.10 for pazopanib and 465.30 to 252.00 for sorafenib. Chromatography was performed by means of gradient elution (flow rate: 0.4 mL/min) as follows: 0 to 0.5 min, 5% A/95% B; 0.5 to 2.5 min, 5% A/95% B to 40% A/60% B; 2.5 to 3.0 min, 40% A/60% B to 60% A/40% B; 3.0 to 4.0 min, 60% A/40% B 4.0 to 4.1 min, 60% A/40% B to 5% A/95% B; 4.1 to 7.0 min, 5% A/95% B; (A, water containing 0.1% formic acid; B, acetonitrile containing 0.1% formic acid) using an ACQUITY UPLC BEH Amide Column (1.7 µm particle size, 2.1 mm I.D. × 100 mm; Waters, Milford, MA) at 40°C for metformin. Multiple reaction monitoring in positive ion mode was set at 131.1 to 71.0 for metformin and 253.1 to 159.1 for cimetidine. The method was linear over the range of 1 - 3,000 and 6 – 1,800 fmol/injection of pazopanib
and metformin, respectively.

**Statistical Analysis**

All values are presented as the mean ± SEM. The statistical significance of differences was determined using Student’s *t*-test or ANOVA with Dunnett’s test. Differences with a *P* value of less than 0.05 were considered to be statistically significant.
Results

Effect of transporter inhibitors on hepatic distribution of pazopanib in mice

To characterize the hepatic uptake mechanism, we first screened the effects of several transporter inhibitors on hepatic distribution of pazopanib after intravenous administration in mice. The inhibitors included those of OATPs (CsA and RIF), OCTs (TPeA), and organic anion transporters and multidrug resistance associated proteins (PBD and CMZ). The plasma concentration profile of pazopanib after intravenous injection was first examined. Administration of TPeA prior to pazopanib injection largely increased the plasma concentration of pazopanib (Fig. 1C), whereas that of CsA exhibited only a modest effect (Fig. 1A). Administration of other inhibitors minimally affected the plasma concentration profile of pazopanib (Fig. 1B, 1D, 1E), whereas administration of TPeA and CsA combined delayed the elimination of pazopanib (Fig. 1F). Since pazopanib is metabolized by CYPs (Keisner and Shah 2011), the effect of a nonspecific CYP inhibitor ABT was examined as a positive control. Indeed, administration of ABT prior to pazopanib injection also delayed the disappearance of pazopanib (Fig. 1G). Additionally, administration of both TPeA and ABT delayed the systemic elimination of pazopanib even further (Fig. 1H).

To directly investigate the hepatic uptake process of pazopanib, time profile of
plasma and hepatic concentration of pazopanib was next examined until 8 h after intravenous administration, and the data were expressed as a plot of $K_p(t)$ vs $\text{AUC}(0-t)/C_p(t)$ (Fig. 2A) based on Eq. (1). As a result, the $y$-intercept seemed to be close to extracellular volume in the liver (~0.2 mL/g liver), and gradual increase in the $K_p(t)$ value was seen with the $\text{CL}_{\text{uptake}}$ value of 5.40 µL/h/g liver (Fig. 2A). Thus, hepatic uptake of pazopanib was slow, and this may hinder the analysis of hepatic distribution within shorter period (~4 h). Therefore, time profile of plasma concentration until 8 h and hepatic concentration at 8 h after the intravenous injection of pazopanib was next examined, and the $\text{CL}_{\text{uptake,app}}$ value was estimated to examine the effect of each inhibitor on hepatic uptake by minimizing the number of experimental animals. Note that the $\text{CL}_{\text{uptake,app}}$ would be affected by $y$-intercept in Fig. 2A and represents rough approximation of $\text{CL}_{\text{uptake}}$. TPeA and TPeA plus CsA reduced the $\text{CL}_{\text{uptake,app}}$ value by 70 and 87%, respectively, whereas CsA reduced the $\text{CL}_{\text{uptake,app}}$ value by 17 % (Fig. 2B). Hepatic concentration and other pharmacokinetic parameters such as $\text{AUC}(0-8 \text{ h})$, $K_p(8 \text{ h})$, $V_0$, and $t_{1/2}$ were shown in supplementary Table S1. Both $\text{AUC}(0-8 \text{ h})$ and $t_{1/2}$ were significantly increased whereas both $X_{\text{liver}}(8 \text{ h})$ and $K_p(8 \text{ h})$ were reduced by administration of TPeA prior to pazopanib injection (Table S1).
Uptake of pazopanib by isolated mouse hepatocytes

To confirm the hepatic uptake of pazopanib and its inhibition by TPeA, pazopanib uptake was examined in isolated mouse hepatocytes. Time-dependent uptake of pazopanib at two different concentrations revealed an almost linear increase within 90 sec (Fig. 3A). Uptake of pazopanib for 1 min was then examined in the presence of CsA and/or TPeA. Pazopanib uptake decreased in the presence of TPeA (1-100 µM), and further decreased in the presence of both TPeA (100 µM) and CsA (10 µM) (Fig. 3B). Conversely, uptake pazopanib uptake was not clearly affected by the presence of CsA alone (0.1-10 µM), although a decreasing trend was observed (Fig. 3B).

Transport of pazopanib by human OCT1

The inhibitory effect of TPeA on the hepatic distribution of pazopanib in mice (Fig. 2) and uptake of pazopanib in isolated mouse hepatocytes (Fig. 3B) suggests a possible involvement of OCTs in the hepatic uptake of pazopanib. To examine the role of OCT1 in humans, pazopanib uptake was evaluated in HEK293/OCT1 cells and compared with that in HEK293/mock cells. Pazopanib was efficiently taken up in HEK293/OCT1 cells in a time-dependent manner, whereas the uptake of pazopanib was lower in HEK293/OATP1B1, although uptake by both cell lines was significantly higher than that
in HEK293/mock cells (Fig. 4A). To further characterize the transport of pazopanib by human OCT1, concentration dependency was also examined. OCT1-mediated uptake of pazopanib became saturated as the concentration increased (Fig. 4B). The apparent Km and Vmax values were estimated to be 3.47 µM and 530 pmol/min/mg protein, respectively, although it should be noted that these values were obtained in the presence of 0.3% HSA. The OCT1-mediated uptake of pazopanib decreased in the presence of TPeA, but CsA had a minimal effect (Fig. 4C). The effects of these two inhibitors on OATP1B1 were also examined. Since OATP1B1-mediated uptake of pazopanib was quite low (Fig. 4A), [³H]ES was used as a substrate of OATP1B1, and transport buffer also contained 0.3% HSA. OATP1B1-mediated uptake of [³H]ES decreased in the presence of CsA, but not with TPeA (Fig. 4D).

**Inhibition of human OCT1 by pazopanib**

The potential for pazopanib to inhibit OCT1 was further examined by evaluating the uptake of metformin, a typical OCT1 substrate, in HEK293/OCT1 cells. The uptake of metformin in HEK293/OCT1 cells was much higher than that in HEK293/mock cells (Fig. 5A), and OCT1-mediated metformin uptake decreased in the presence of pazopanib (Fig. 5B). In this analysis, pazopanib concentrations in the medium were measured and
shown in abscissa (Fig. 5B). The IC$_{50}$ value for pazopanib obtained based on Eq. 1 was estimated to be 0.253 µM.

**Uptake of pazopanib by human hepatocytes**

The uptake of pazopanib was next examined in cryopreserved human pooled hepatocytes. Time-dependent uptake of pazopanib was observed at two different concentrations, 1 and 10 µM (Fig. 6A). Pazopanib uptake decreased in the presence of TPeA (1-100 µM), and further decreased in the presence of both TPeA (100 µM) and CsA (10 µM) (Fig. 6B). Conversely, CsA (0.1–10 µM) had no effect on pazopanib uptake, although a decreasing trend was observed (Fig. 6B).


**Discussion**

The present study demonstrated that OCT1 is responsible for hepatocellular uptake of pazopanib, based on the following findings: (i) pazopanib uptake in HEK293/OCT1 cells was significantly higher than that in HEK293/mock cells (Fig. 4A), and OCT1-mediated uptake of pazopanib exhibited saturation (Fig. 4B); (ii) pazopanib uptake by human pooled hepatocytes (Fig. 6A) largely decreased in the presence of an OCT inhibitor TPeA (Fig. 6B), which also inhibited pazopanib uptake in OCT1-transfected HEK293 cells (Fig. 4C); and (iii) systemic elimination and hepatic distribution of pazopanib in mice were reduced by administration of TPeA prior to pazopanib injection (Figs. 1 and 2), and pazopanib uptake by isolated mouse hepatocytes also decreased in the presence of TPeA. Thus far, no other report has suggested that pazopanib is transported by OCT1, and hepatic uptake mechanism for pazopanib has remained undetermined. The present report is thus the first to identify OCT1 as the transporter responsible for hepatocellular uptake of pazopanib.

The involvement of OCT1 in pazopanib uptake by hepatocytes suggests that the intrinsic transport activity and/or expression level of OCT1 could affect the hepatic distribution of pazopanib in cancer patients, which might be associated with pazopanib-induced hepatotoxicity. *SLC22A1* gene polymorphisms have been identified that result in
various amino acid substitutions in the OCT1 gene product and potentially cause a loss of function (Seitz et al., 2015). Interestingly, strong global differences in the frequency of loss of OCT1 function have been observed: while most East Asian and Oceanian individuals had completely functional OCT1, 80% of native South American Indians lacked functional OCT1 alleles. Europeans also had alleles associated with reduced or lack of OCT1 function. These findings propose a possible ethnic difference between White and Japanese patients in the pharmacokinetics and/or hepatotoxicity of pazopanib.

Indeed, a subset analysis of a phase III study of pazopanib in patients with soft tissue sarcoma has demonstrated a higher incidence of grade 3 alanine transaminase elevation in Japanese patients compared to the global population (Kawai et al., 2016). This suggests a difference in hepatic exposure to pazopanib between Japanese and White patients, which can be explained in part by the difference in SLC22A1 polymorphisms. Reduced OCT1-mediated uptake of another OCT1 substrate, sumatriptan, was also reported to be caused by the loss-of-function OCT1 polymorphisms, leading to significantly increased plasma concentrations of sumatriptan in German subjects (Matthaei et al., 2016). Nevertheless, phase 1 studies for pazopanib with small numbers of patients did not show a difference in its systemic exposure between Japanese and White patients (Inada-Inoue et al., 2014; Hurwitz et al., 2009), probably due to large interindividual variability and/or
low systemic clearance via the liver (Di Gion et al., 2011). Thus, various factors other than \textit{SLC22A1} gene polymorphisms could also affect the pharmacokinetics and toxicity of pazopanib in humans.

OCT1 is a multispecific transporter responsible for the hepatic uptake of various drugs, including metformin. Therefore, an interaction of pazopanib with this transporter may lead to inhibition of the uptake of OCT1 substrate drugs. In fact, OCT1-mediated uptake of metformin was inhibited by pazopanib with an $IC_{50}$ value of 0.253 $\mu$M (Fig. 5B). The $C_{\text{max}}$ of pazopanib after single and repeated clinical dosing is 40-100 $\mu$M (Inada-Inoue et al., 2014), and the unbound fraction of pazopanib in human plasma is approximately 0.01 (Imbs et al., 2016), indicating that the unbound plasma concentration of pazopanib would be 0.4–1 $\mu$M, which is slightly higher than the $IC_{50}$ value (Fig. 5B). This suggests a clinically relevant inhibition of OCT1-mediated uptake of metformin by pazopanib. Sauzay et al. (2016) have recently reported that pazopanib inhibits renal transporter OCT2 with an $IC_{50}$ value of 3.5 $\mu$M, an approximately ten-fold higher difference than that for OCT1. This may indicate a difference in the inhibitory potential of pazopanib between these two OCT homologs. However, experimental conditions were also different between the two reports: the previous study by Sauzay et al. (2016) performed preincubation with pazopanib for 1 h before the uptake experiment, whereas
both pazopanib and metformin were simultaneously added in the present study (Fig. 5B).

Sprowl et al. (2016) have recently revealed posttranslational regulation of OCTs by inhibition of tyrosine kinase; therefore, preincubation may affect the apparent inhibitory potential of pazopanib. The difference in substrates used between the two reports, e.g., 4-(4-[dimethylamino]styryl)-N-methylpyridinium iodide by Sauzay et al. (2016) and metformin in the present study, may also affect IC₅₀ values. More practically, the previous report by Sauzay et al. (2016) did not mention direct measurement of pazopanib concentration in medium, whereas in the present study, its medium concentration was directly measured and found to be much lower than the initial concentration.

Since OCT1 is expressed in Ewing’s sarcoma cells (Huber et al., 2015), pazopanib may be taken up into a certain cancer cells not only by passive diffusion but also through OCT1-mediated transport, resulting in its interaction with the kinase domain of target receptors such as PDGFR-α and -β located inside the cells and subsequent inhibition of tyrosine kinases. Therefore, the OCT1-mediated uptake of pazopanib may be related to the antitumor activity of pazopanib, although further studies are needed to clarify this hypothesis.

CsA inhibits OATP1B1 and OATP1B3 in humans (Hirano et al., 2006; Amundsen et al., 2010; Shitara et al., 2012; Wang et al., 2017), and it inhibited the uptake
of pazopanib by human hepatocytes in the present study, although the inhibition potency was lower than that of TPeA (Fig. 6B). Since OCT1-mediated uptake of pazopanib was only minimally inhibited by CsA (Fig. 4C), the inhibition of pazopanib uptake by CsA in human pooled hepatocytes (Fig. 6B) indicates that OCT1 is not the sole transporter responsible for pazopanib uptake. Indeed, combined treatment with CsA and TPeA resulted in significantly more inhibition of pazopanib uptake compared to TPeA alone in human pooled hepatocytes (Fig. 6B), further supporting the involvement of transporter(s) other than OCT1. Zimmerman et al. (2013) proposed that pazopanib is transported by OATP1B1 and OATP1B3, and the present finding (Fig. 4A) supports this result. However, the apparent OATP1B1-mediated uptake of pazopanib is low in both the present (Fig. 4A) and previous (Zimmerman et al., 2013) studies, hindering further characterization of this transporter-mediated pazopanib transport. Thus, the possible involvement of these OATPs in the uptake of pazopanib by human hepatocytes should be clarified by further analyses. Transporter inhibition study in mice in vivo also has limitation since experimental condition was not optimized in the present study. Effect of administration of RIF and TPeA on disposition of eltrombopag, which is a substrate of both OATP1B1 and OCT1, was previously confirmed in our laboratory (Takeuchi et al., 2011), whereas that of other inhibitors was not. Therefore, minimal effect of CMZ or PBD on CL$_{uptake,app}$
of pazopanib (Fig. 2) does not necessarily exclude possible contribution of MRPs or OATs in mice.

In conclusion, OCT1 is responsible for hepatocellular uptake of pazopanib, and pazopanib exhibits potential inhibition of OCT1 at clinically relevant concentrations. The present findings may aid in optimizing cancer chemotherapy using pazopanib, with an aim to minimizing hepatotoxicity and interindividual variability.
Authorship Contributions

Participated in research design: Fujita and Kato

Conducted experiments: Ellawatty, Yamazaki

Performed data analysis: Ellawatty, Masuo

Discussion regarding clinical relevance: Fujita, Ishida, and Sasaki

Wrote or contributed to the writing of the manuscript: Ellawatty, Fujita, Masuo, Yamazaki, Arakawa, Nakamichi, Abdelwahed, and Kato
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*Genome Med* 7: 56.


*Eur J Cancer* **49**: 1287-1296.


Drug Metab Dispos 42: 726-734.


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Figure Legends

Figure 1: Effect of transporter inhibitors on plasma concentration/time profiles of pazopanib in mice. Transporter inhibitors (●) or vehicle alone (○) were administered prior to intravenous injection of pazopanib (0.02 mg/kg) in mice, and plasma concentration of pazopanib was measured for (A) CsA, (B) RIF, (C) TPeA, (D) PBD, (E) CMZ, (F) CsA+TPeA, (G) ABT, or (H) ABT+TPeA. Each point represents the mean ± SEM (n = 3). *, Significant difference from vehicle-treated mice (p<0.05).

Figure 2: Effect of transporter inhibitors on hepatic uptake of pazopanib in mice.

(A) Pazopanib (0.02 mg/kg) was intravenously administered in mice, and time course of plasma and hepatic concentration was examined until 8 h. Data were expressed as a plot of $K_p(t)$ vs $AUC_{(0-t)}/C_p(t)$ based on Eq. (1). Each value represents the mean ± SEM (n = 4 or 5). (B) Each inhibitor (■) or vehicle alone (□) was administered prior to intravenous injection of pazopanib (0.02 mg/kg) in mice, and $CL_{uptake,app}$ values were estimated based on both plasma concentration profile until 8 h and hepatic concentration of pazopanib at 8 h after the injection. Each value represents the mean ± SEM (n = 3). *, Significant difference from vehicle-treated mice (p<0.05).
**Figure 3: Effect of CsA and TPeA on uptake of pazopanib in isolated mouse hepatocytes.** (A) Time course of the uptake of 1 (●) and 10 (◆) µM pazopanib was examined in isolated mouse hepatocytes at 37°C in the presence of 0.3% HSA. (B) Mouse hepatocytes were incubated with pazopanib (1 µM) for 1 min at 37°C in the presence 0.3% HSA and various concentrations of CsA and TPeA, and uptake of pazopanib was measured. Each point represents the mean ± SEM (n = 3). *, Significant difference from control (p<0.05); **, significant difference from control (p<0.01); ##, significant difference from CsA (10 µM) group (p<0.01); †, significant difference from TPeA (100 µM) group (p<0.05).

**Figure 4: Uptake of pazopanib by human OCT1 and OATP1B1.** (A) Time course of the uptake of pazopanib (1 µM) was evaluated in the presence of 0.3% HSA in HEK293/OCT1 (●), HEK293/OATP1B1 (◆), and HEK293/mock cells (○). Each point represents the mean ± SEM (n = 6). (B) Uptake of pazopanib at various concentrations (3, 6, 10, 30, 60, and 100 µM) in the presence of 0.3% HSA was measured at 5 min in both HEK293/OCT1 and HEK293/mock cells. OCT1-mediated uptake of pazopanib (●) was then calculated by subtracting the uptake in HEK293/mock from that in HEK293/OCT1 cells. The straight line represents the fitted one. Each point represents
the mean ± SEM (n = 3). (C) Uptake of pazopanib (1 µM) in the presence of 0.3% HSA and various concentrations of CsA (●) and TPeA (◆) was measured at 5 min in both HEK293/OCT1 and HEK293/mock cells. OCT1-mediated uptake of pazopanib was then calculated by subtracting the uptake in HEK293/mock from that in HEK293/OCT1 cells. Each point represents the mean ± SEM (n = 3). (D) Uptake of [3H]E3S in the presence of 0.3% HSA and various concentrations of CsA (●) and TPeA (◆) was measured at 4 min in both HEK293/OATP1B1 and HEK293/mock cells. OATP1B1-mediated uptake of [3H]E3S was then calculated by subtracting the uptake in HEK293/mock from that in HEK293/OATP1B1 cells. Each point represents the mean ± SEM (n = 3). *, Significant difference from control (p<0.05); **, significant difference from control (p<0.01)

Figure 5: Inhibition of OCT1-mediated uptake of metformin by pazopanib. (A) Time course of the uptake of metformin (250 µM) was evaluated in HEK293/OCT1 (●) and HEK293/mock cells (○). (B) HEK293/OCT1 and HEK293/mock cells were incubated with metformin (250 µM) for 15 min in the presence of various concentrations (0, 1, 3, 10, and 30 µM) of pazopanib. OCT1-mediated uptake of metformin was then calculated by subtracting the uptake in HEK293/mock from that in HEK293/OCT1 cells. The abscissa represents mean pazopanib concentration in the medium at the end of
experiments, measured by LC-MS/MS. Each point represents the mean ± SEM (n = 3).

*, Significant difference from mock cells (p<0.05); **, significant difference from mock cells (p<0.01).

**Figure 6: Uptake of pazopanib in cryopreserved human pooled hepatocytes.** (A) Time course of the uptake of 1 (●) and 10 (◆) µM pazopanib was evaluated in cryopreserved human pooled hepatocytes at 37 °C in the presence of 0.3% HSA. (B) Human hepatocytes were incubated with pazopanib (1 µM) for 1 min at 37 °C in the presence 0.3% HSA and various concentrations of CsA and TPeA, and uptake of pazopanib was measured. Each point represents the mean ± SEM (n = 3). *, Significant difference from control (p<0.05); **, significant difference from control (p<0.01); #, significant difference from CsA (10 µM) group (p<0.05); †, significant difference from TPeA (100 µM) group (p<0.05).
Figure 1

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Figure 2
Figure 3

**A**

Pazopanib Uptake [pmol/mg protein] over time for 10 µM and 1 µM concentrations.

**B**

Pazopanib Uptake [pmol/min/mg protein] in the presence of CsA, TPeA, and various concentrations of inhibitors.
Figure 4
Figure 5

A

**Metformin Uptake [pmol/mg protein]**

Time [min]

B

**Metformin Uptake [% of control]**

Pazopanib [µM]
Figure 6
Supplemental Table

Drug Metabolism and Disposition

Organic cation transporter 1 is responsible for hepatocellular uptake of the tyrosine kinase inhibitor pazopanib

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Table S1: Pharmacokinetic parameters and hepatic concentration of pazopanib\textsuperscript{a}

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>AUC\textsubscript{(0-8 h)}\textsuperscript{b} [\mu g/h/ml]</th>
<th>X\textsubscript{liver(8 h)}\textsuperscript{c} [ng/g liver]</th>
<th>Kp\textsubscript{(8h)}\textsuperscript{d}</th>
<th>V\textsubscript{o}\textsuperscript{e} [mL/kg]</th>
<th>t\textsubscript{1/2}\textsuperscript{f} [h]</th>
</tr>
</thead>
<tbody>
<tr>
<td>CsA (-)</td>
<td>1.10 ± 0.27 20.1 ± 1.0</td>
<td>0.619 ± 0.041</td>
<td>77.5 ± 10.5</td>
<td>4.07 ± 0.65</td>
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</tr>
<tr>
<td>(+)</td>
<td>1.34 ± 0.16 17.2 ± 1.5</td>
<td>0.369 ± 0.045</td>
<td>80.1 ± 28.4</td>
<td>4.51 ± 0.70</td>
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<tr>
<td>RIF (-)</td>
<td>1.08 ± 0.19 21.4 ± 1.3</td>
<td>0.501 ± 0.012</td>
<td>86.3 ± 13.2</td>
<td>4.25 ± 0.95</td>
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<tr>
<td>(+)</td>
<td>1.13 ± 0.24 17.5 ± 2.0</td>
<td>0.293 ± 0.040</td>
<td>77.6 ± 24.1</td>
<td>5.84 ± 0.79</td>
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<td>TPeA (-)</td>
<td>1.59 ± 0.36 26.6 ± 2.4</td>
<td>0.728 ± 0.082</td>
<td>58.1 ± 33.8</td>
<td>2.01 ± 0.86</td>
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<tr>
<td>(+)</td>
<td>2.36 ± 0.24* 11.8 ± 1.8*</td>
<td>0.153 ± 0.054*</td>
<td>58.3 ± 14.5</td>
<td>5.85 ± 0.72*</td>
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<tr>
<td>PBD (-)</td>
<td>1.25 ± 0.13 24.5 ± 2.0</td>
<td>0.544 ± 0.073</td>
<td>94.3 ± 42.6</td>
<td>3.90 ± 0.24</td>
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<tr>
<td>(+)</td>
<td>1.26 ± 0.09 21.4 ± 1.1</td>
<td>0.358 ± 0.054</td>
<td>91.3 ± 28.5</td>
<td>4.16 ± 0.38</td>
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<td>CMZ (-)</td>
<td>1.15 ± 0.16 20.2 ± 2.2</td>
<td>0.331 ± 0.070</td>
<td>47.6 ± 21.7</td>
<td>4.16 ± 0.66</td>
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<tr>
<td>(+)</td>
<td>1.19 ± 0.19 21.5 ± 9.9</td>
<td>0.252 ± 0.034</td>
<td>30.2 ± 13.4</td>
<td>4.43 ± 0.85</td>
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<td>CsA (+)</td>
<td>1.49 ± 0.17 27.9 ± 4.0</td>
<td>0.758 ± 0.091</td>
<td>66.1 ± 32.9</td>
<td>2.09 ± 0.74</td>
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<tr>
<td>+TPeA (+)</td>
<td>2.67 ± 0.25* 9.59 ± 1.81*</td>
<td>0.104 ± 0.043*</td>
<td>65.1 ± 21.5</td>
<td>5.67 ± 0.55*</td>
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<tr>
<td>ABT (-)</td>
<td>0.849 ± 0.018 16.7 ± 1.4</td>
<td>0.547 ± 0.062</td>
<td>78.3 ± 13.7</td>
<td>3.75 ± 0.69</td>
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<tr>
<td>(+)</td>
<td>1.16 ± 0.15* 14.9 ± 1.3</td>
<td>0.178 ± 0.036*</td>
<td>78.5 ± 39.2</td>
<td>5.87 ± 0.80*</td>
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<td>ABT (+)</td>
<td>1.57 ± 0.09 28.2 ± 3.2</td>
<td>0.796 ± 0.089</td>
<td>65.3 ± 23.6</td>
<td>2.07 ± 0.47</td>
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<td>+TPeA (+)</td>
<td>2.27 ± 0.24* 12.6 ± 2.4*</td>
<td>0.151 ± 0.028*</td>
<td>65.1 ± 34.1</td>
<td>6.51 ± 0.58*</td>
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</tr>
</tbody>
</table>

\textsuperscript{a} Each inhibitor (+) or vehicle alone (-) was administered prior to intravenous injection of pazopanib (0.02 mg/kg) in mice. Plasma concentration of pazopanib was measured for 8 h, and hepatic concentration of pazopanib was measured at 8 h after the injection. Data represent the mean ± SEM (n = 3).

\textsuperscript{b} Area under the curve from 0 to 8 h.

\textsuperscript{c} Hepatic concentration at 8 h.

\textsuperscript{d} Liver-to-plasma concentration ratio at 8h.

\textsuperscript{e} Distribution volume to the central compartment.

\textsuperscript{f} Half-life in terminal phase.

*, Significant difference from vehicle-treated mice (p<0.05).