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Organic anion transporting polypeptide 1a4 is responsible for the hepatic uptake of cardiac glycosides in mice

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Running title: Oatp1a4-mediated hepatic uptake of cardiac glycosides

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Abbreviations:

BQ-123, cyclo-D-Trp-D-Asp-Pro-D-Val-Leu; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; HPLC, high performance liquid chromatography; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; MRM, multiple reaction monitoring; Oatp/OATP, organic anion transporting polypeptide; PCR, polymerase chain reaction; Slco, solute carrier organic anion transporter family

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Abstract:

Among organic anion transporting polypeptide (Oatp) family transporters expressed in the rodent liver, such as Oatp1a1, Oatp1a4, Oatp1b2, and Oatp2b1, Oatp1a4 has a unique character to recognize neutral cardiac glycosides as substrate in addition to organic anions. The relative contribution of Oatp1a4 to the substrate uptake into hepatocytes has not been clarified. In this study, we investigated the importance of Oatp1a4 in the hepatic uptake of its substrate drugs using *Slco1a4*$^{-/-}$ mice. The hepatic mRNA expression of *Slco1a4* was decreased significantly in *Slco1a4*$^{-/-}$ mice, whereas no differences were seen in other hepatic transporters between wild-type and *Slco1a4*$^{-/-}$ mice. We determined the plasma concentrations, and liver-to-plasma ratio of Oatp1a4 substrates including ouabain, digoxin, BQ-123, fexofenadine, rosuvastatin, pravastatin, nafcillin, and telmisartan, after continuous intravenous infusion. The plasma concentrations of ouabain and rosuvastatin were 2.1 and 1.7-fold higher in *Slco1a4*$^{-/-}$ mice, and the liver-to-plasma concentration ratios of ouabain and digoxin were 13.4 and 4.3-fold lower in *Slco1a4*$^{-/-}$ mice, respectively. Furthermore, the biliary clearance of ouabain and digoxin with regard to plasma concentration were 21.9 and 4.1-fold lower in *Slco1a4*$^{-/-}$ mice, respectively, accompanied with a marked reduction in their liver-to-plasma ratios, whereas the systemic clearance of ouabain, but not digoxin, was reduced significantly in *Slco1a4*$^{-/-}$ mice. These results suggest that Oatp1a4 plays a major role in the hepatic accumulation of cardiac glycosides in mice.

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Introduction

Transporters, expressed in several tissues, including liver and kidney, are important proteins governing the pharmacokinetics of many drugs. Transporter-mediated uptake from the portal vein into hepatocytes is the first step in their hepatic elimination. Thus, the changes in hepatic uptake clearance can directly affect overall intrinsic hepatic clearance. Organic anion transporting polypeptide (Oatp) family transporters are involved in the efficient uptake of several organic anions into the liver. In rodents, multiple Oatp isoforms such as Oatp1a1, 1a4, 1b2, and 2b1 are expressed on the sinusoidal membrane in hepatocytes (Cheng *et al.*, 2005). Among them, Oatp1a4 encoded by *Slco1a4* (solute carrier organic anion transporter family, member 1a4) is predominantly expressed in the liver and brain. A number of studies for Oatp1a4 in vitro have demonstrated that it accepts not only anionic compounds, such as HMG-CoA reductase inhibitors (pitavastatin, pravastatin, and rosuvastatin), and β-lactam antibiotics (nafcillin, cefmetazole, and cefazolin), but also neutral cardiac glycosides (digoxin and ouabain) and zwitterionic compounds such as fexofenadine (Noé *et al.*, 1997; Cvetkovic *et al.*, 1999; Reichel *et al.*, 1999; Tokui *et al.*, 1999; Ho *et al.*, 2006; Nakakariya *et al.*, 2008; Ose *et al.*, 2010). Oatp1a4 has at least two recognition sites, one for digoxin (neutral compound) and taurocholate (organic anion), and the other for estradiol-17β-D-glucuronide (Sugiyama *et al.*, 2002). In particular, among rodent Oatp family transporters, digoxin is selectively recognized by Oatp1a4 (Noé *et al.*, 1997; Reichel *et al.*, 1999; Cattori *et al.*, 2000, 2001). Several groups have generated knockout mice for Oatp isoforms and investigated their roles in the hepatic uptake and subsequent pharmacokinetics of substrates. The $K_{p,liver}$ of pravastatin decreased

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significantly in *Slco1b2*$^{-/-}$ mice (Chen *et al.*, 2008; Zaher *et al.*, 2008). Gong et al. reported that the plasma area under the curve of the concentration–time profile (AUC) of estradiol-17β-D-glucuronide was 55% larger in female *Slco1a1*$^{-/-}$ mice than that in wild-type mice, with a 50% reduction in $K_{p,liver}$; however, knockout of *Slco1a4* did not affect the plasma AUC or $K_{p,liver}$ of estradiol-17β-D-glucuronide, while the plasma AUC of dibromosulfophthalein was 3-fold higher in both *Slco1a1*$^{-/-}$ and *Slco1a4*$^{-/-}$ mice compared with that in wild-type mice (Gong *et al.*, 2011). Moreover, several studies have reported that the changes in pharmacokinetics and tissue distribution of pravastatin, atorvastatin, simvastatin, rosuvastatin, and carboxydichlorofluorescein in Oatp1a/1b cluster knockout mice showed a significant reduction of hepatic uptake because of the lack of major hepatic Oatp isoforms (Iusuf *et al.*, 2012, 2013; Higgins *et al.*, 2014). However, to date, the quantitative contribution of Oatp1a4 to the drug disposition and subsequent pharmacokinetics of substrate drugs in rodents has not been clarified. Ose et al. demonstrated that the brain-to-blood transport of pitavastatin, rosuvastatin, pravastatin, and taurocholate after microinjection into the cerebral cortex was decreased significantly in *Slco1a4*$^{-/-}$ mice compared with wild-type mice (Ose *et al.*, 2010). The purpose of the present study was to evaluate the significant involvement of Oatp1a4 in the hepatic uptake in mice of substrate drugs, including digoxin (a selective substrate for Oatp1a4 among Oatp family transporters), with the use of *Slco1a4*$^{-/-}$ mice.

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Materials and Methods

Chemicals

Ouabain, digoxin, BQ-123, rosuvastatin calcium salt, pravastatin sodium salt, and nafcillin sodium salt were purchased from Wako Pure Chemical Industries (Osaka, Japan). Fexofenadine hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO). Telmisartan was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). All other chemicals were commercially available, of reagent grade, and used without further purification.

Animals

Slco1a4 knockout (*Slco1a4*$^{-/-}$) mice were obtained from Deltagen (San Carlos, CA) and maintained by Charles River Laboratories (Yokohama, Japan). *Slco1a4*$^{-/-}$ mice were fertile and exhibited no obvious abnormalities. Wild-type (C57BL/6J) mice were supplied by Oriental Yeast Co. (Tokyo, Japan). All animal experiments were performed with female mice. All mice (10–12 weeks old) were maintained under standard conditions with a reverse dark–light cycle. Food and water were available *ad libitum*. All animal experiments in the present study were performed according to the guidelines provided by the Institutional Animal Care Committee of the Graduate School of Pharmaceutical Sciences, The University of Tokyo (Tokyo, Japan).

Quantification of mRNA expression of various transporters in the murine liver

The mRNA levels of *Slco1a1*, *Slco1a4*, *Slco1b2*, *Slco2b1*, *Abcc2*, *Abcc3*, *Abcb1a*, *Abcg2*,

Abcb11, and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) were quantified by real-time polymerase chain reaction (PCR). Total RNA was isolated from the liver excised from each of four mice using an RNeasy Mini Kit (QIAGEN, Hilden, Germany). Real-time PCR was performed using a Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan) and an Applied Biosystems 7500 Fast Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA). An external standard curve was generated by dilution of the mouse liver total RNA (Agilent Technologies, Santa Clara, CA).

In vivo infusion study

Female wild-type and *Slco1a4*$^{-/-}$ mice weighing 17.1 to 29.5 g were used for these experiments. Under pentobarbital anesthesia (30 mg/kg), the jugular vein was cannulated with a polyethylene catheter PE10 (Becton, Dickinson and Company, Franklin Lakes, NJ) for drug administration. The mice received a constant intravenous infusion of ouabain (4.8 μg/hr), digoxin (125 ng/hr), BQ-123 (80 μg/hr), fexofenadine (7.0 μg/hr), rosuvastatin (1.15 μg/hr), pravastatin (8.0 μg/hr), nafcillin (34.3 μg/hr) or telmisartan (480 ng/hr) for 30 min (BQ-123), 60 min (ouabain), and 120 min (digoxin, fexofenadine, rosuvastatin, pravastatin, nafcillin, and telmisartan). Blood samples were collected from the jugular vein, and plasma samples were obtained by centrifugation of the blood samples (10,000*g*, 10 min, 4°C). The bile duct was cannulated with a polyethylene catheter UT-03 (Unique Medical, Tokyo, Japan) for the bile collection to evaluate the biliary excretion of ouabain and digoxin. The mice were humanely killed after constant infusion, and the entire liver was resected immediately. The liver was weighed and minced, and then mixed with 4-fold volume of

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PBS. The mixture was homogenized using a polytron homogenizer (Kinematica, Lucerne, Switzerland) in an ice bath. The liver homogenate was centrifuged at 12,000*g* for 10 min at 4°C. The plasma and liver concentrations of ouabain, digoxin, fexofenadine, rosuvastatin, pravastatin, nafcillin, and telmisartan were determined using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis.

LC-MS/MS analysis

A LC-MS/MS system comprising an Alliance 2695 separation module (Waters, Milford, MA) equipped with a Quattro Micro API tandem mass spectrometer was used for the analysis of ouabain, digoxin, fexofenadine, rosuvastatin, pravastatin, nafcillin, and telmisartan. The mass spectrometer was operated in a positive- and negative-ion electrospray ionization and multiple reaction monitoring (MRM) mode to detect eluting compounds. Ouabain, fexofenadine, and telmisartan were detected as $[M+H]^+$. Rosuvastatin, pravastatin, and nafcillin were detected as $[M-H]^-$. Digoxin was detected as $[M+NH_4]^+$. The MRM transitions of ouabain, digoxin, fexofenadine, rosuvastatin, pravastatin, nafcillin, and telmisartan were m/z 585, 798/651, 502/466, 480/418, 423/321, 333/192 and 515/276, respectively. The collected supernatants were diluted with mobile phase. The diluted samples (10 μL) were injected into the LC-MS/MS system. Samples containing ouabain, digoxin, fexofenadine, rosuvastatin, pravastatin, nafcillin samples were separated on an Atlantis T3 column (2.1 × 50 mm, 5 μm, Waters), and for telmisartan, a Inertsil ODS-3 column (2.1 × 50 mm, 5 μm, GL Sciences) was used. The details of the high performance liquid chromatography (HPLC) conditions are shown in Table 1.

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HPLC-fluorescence analysis

An HPLC-fluorescence system was used to analyze BQ-123. A diluted sample (100 μL) was injected into an HPLC system using a YMC-Pack ODS-A column (4.6 × 150 mm, 5 μm, YMC Co.). The column was eluted with water–acetonitrile (65:35, v/v) containing 0.1% trifluoroacetic acid at 0.8 mL/min. BQ-123 was detected by fluorescence (excitation 287 nm, emission 348 nm).

Pharmacokinetic analysis

Total clearance (CL_{tot}), biliary excretion clearance with regard to plasma concentration ($CL_{bile,plasma}$), and biliary excretion clearance with regard to liver concentration ($CL_{bile,liver}$) were calculated using the following equations:

$$CL_{tot} = \text{Infusion rate}/C_{p,ss} \quad (1)$$

$$CL_{bile,\ plasma} = V_{bile}/C_{p,ss} \quad (2)$$

$$CL_{bile,\ liver} = V_{bile}/C_{liver,ss} \quad (3)$$

where $C_{p,ss}$, $V_{bile,ss}$, and $C_{liver,ss}$ represent the plasma concentration, biliary excretion rate, and liver concentration at steady state, respectively. In the present study, $C_{p,ss}$ and $C_{liver,ss}$ were defined as the plasma and liver concentrations of the parent compounds at 30 min (BQ-123), 60 min (ouabain), or 120 min (digoxin, fexofenadine, rosuvastatin, pravastatin, nafcillin, and telmisartan), judging from their time courses of plasma concentration. $V_{bile,ss}$ was calculated as the biliary excretion rate of ouabain and digoxin from 40 to 60 min and from 90 to 120 min, respectively. To calculate $C_{liver,ss}$, the specific gravity of the liver was

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assumed to be 1. Thus, the amount of compound in one gram of liver (ng/g) can be regarded as its liver concentration (ng/mL).

Statistical analysis

The means and standard deviations of mRNA expression level, drug concentration in the plasma and liver, and pharmacokinetic parameters were calculated for both wild-type and *Slco1a4*$^{-/-}$ mice. A Student two-tailed unpaired *t* test was used to identify significant differences. $P < 0.05$ was considered significant.

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Results

Analysis of mRNA expression levels of hepatic transporters in wild-type and *Slco1a4*$^{-/-}$ mice

The mRNA expression levels of hepatic transporters in wild-type and *Slco1a4*$^{-/-}$ mice are compared in Table 2. As expected, *Slco1a4* mRNA showed a significantly lower expression in *Slco1a4*$^{-/-}$ mice, but no significant difference was observed in mRNA expression levels of other tested hepatic transporters between wild-type and *Slco1a4*$^{-/-}$ mice.

Time profiles for plasma concentrations of substrate drugs in wild-type and *Slco1a4*$^{-/-}$ mice after constant intravenous infusion

The plasma concentrations of ouabain, digoxin, BQ-123, fexofenadine, rosuvastatin, pravastatin, nafcillin, and telmisartan after constant intravenous infusion into wild-type and *Slco1a4*$^{-/-}$ mice are shown in Fig. 1 and Table 3. The plasma concentrations of ouabain and rosuvastatin at steady state were significantly increased in *Slco1a4*$^{-/-}$ mice, whereas the plasma concentrations of pravastatin and telmisartan were slightly decreased in *Slco1a4*$^{-/-}$ mice. The plasma concentrations of other substrates were not different between wild-type and *Slco1a4*$^{-/-}$ mice. The total clearance calculated by the ratio of infusion rate to $C_{p,ss}$ (CL_{tot}) in wild-type and *Slco1a4*$^{-/-}$ mice is shown in Fig. 2. The CL_{tot} of ouabain and rosuvastatin was decreased significantly in *Slco1a4*$^{-/-}$ mice, whereas that of other substrates were not different between wild-type and *Slco1a4*$^{-/-}$ mice. The liver concentrations and liver-to-plasma concentration ratio ($K_{p,liver}$) of substrate drugs at steady

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state in wild-type and *Slco1a4*$^{-/-}$ mice are shown in Table 3. The liver concentrations and $K_{p,liver}$ of ouabain and digoxin were decreased significantly in *Slco1a4*$^{-/-}$ mice. By contrast, the $K_{p,liver}$ of telmisartan was significantly increased in *Slco1a4*$^{-/-}$ mice. The liver concentrations and $K_{p,liver}$ of other substrates were not different between wild-type and *Slco1a4*$^{-/-}$ mice.

Pharmacokinetic parameters of ouabain and digoxin after constant intravenous infusion into wild-type and *Slco1a4*$^{-/-}$ mice

The pharmacokinetic parameters of ouabain and digoxin in wild-type and *Slco1a4*$^{-/-}$ mice are shown in Table 4. Comparing the pharmacokinetic parameters between wild-type and *Slco1a4*$^{-/-}$ mice, the CL_{tot} of ouabain in *Slco1a4*$^{-/-}$ mice decreased significantly, while that of digoxin decreased slightly. Furthermore, after constant intravenous infusion of ouabain and digoxin, both the biliary excretion rate at steady state (V_{bile}) and the biliary excretion clearance with regard to the plasma concentration ($CL_{bile,\ plasma}$) were decreased in *Slco1a4*$^{-/-}$ mice. However, the biliary excretion clearance with regard to the hepatic concentration ($CL_{bile,liver}$) of ouabain and digoxin showed no difference between wild-type and *Slco1a4*$^{-/-}$ mice.

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Discussion

To elucidate the role of Oatp1a4 in the hepatic uptake of substrate drugs, we determined the hepatic clearance and distribution of Oatp1a4 substrates using *Slco1a4*$^{-/-}$ mice. According to the previous report (Cheng *et al.*, 2005), mRNA expression level of Oatp1a4 in the liver was significantly higher in female than male. Thus, to sensitively characterize the importance of Oatp1a4-mediated transport, we decided to use female mice in the current study. We found that the $K_{p,liver}$ of ouabain and digoxin were decreased significantly in *Slco1a4*$^{-/-}$ mice. In experiments using *Xenopus* oocytes expressing rodent Oatp transporters, digoxin was found to be transported selectively via Oatp1a4 (Noé *et al.*, 1997; Reichel *et al.*, 1999; Cattori *et al.*, 2000, 2001). By contrast, ouabain was recognized by Oatp1a1 and Oatp1a4 with higher affinity to Oatp1a4 (Oatp1a1; K_m = 1700–3000 μM, Oatp1a4; K_m = 470 μM (Bossuyt *et al.*, 1996; Eckhardt *et al.*, 1999; Noé *et al.*, 1997)). The observed decrease of $K_{p,liver}$ of these compounds in *Slco1a4*$^{-/-}$ mice can be explained by the relatively higher contribution of Oatp1a4 to their overall uptake. By contrast, the identical uptake of digoxin by primary cultured hepatocytes isolated from wild-type and *Slco1a4*$^{-/-}$ mice was reported (Gong *et al.*, 2011). This apparent discrepancy can be explained, at least in part, by the high affinity of digoxin for rat Oatp1a4 (K_m = 0.24 μM). In the previous in vitro study, 1 μM of digoxin was used in the incubation medium, which may partially saturate Oatp1a4-mediated transport. Conversely, in our study, the plasma unbound concentration of digoxin was far below its K_m; therefore, Oatp1a4-mediated hepatic uptake was expected to be clearly observed. The CL_{tot} of ouabain in *Slco1a4*$^{-/-}$ mice decreased significantly, but that of digoxin decreased only slightly. The CL_{tot} of ouabain and digoxin is

much lower than the liver blood flow rate in mice (90 mL/min/kg), so the hepatic clearance of ouabain and digoxin should approximate $f_B x CL_{H,int}$. Based on the extended clearance concept, overall intrinsic hepatic clearance ($CL_{H,int}$) consists of multiple intrinsic processes, such as hepatic influx (PS_{inf}), backflux from cells to blood (PS_{eff}), metabolism (CL_{met}), and biliary excretion in an unchanged form (CL_{bile}). Then, $CL_{H,int}$ and $K_{p,liver}$ can be described theoretically by the following equations:

$$CL_{H,\,int} = PS_{inf} \times \frac{CL_{bile}+CL_{met}}{PS_{eff}+CL_{bile}+CL_{met}} \qquad (4)$$

$$K_{p,\,liver} = \frac{f_B}{f_T} \times \frac{PS_{inf}}{PS_{eff}+CL_{bile}+CL_{met}} \qquad (5)$$

where f_B and f_T represent the protein unbound fraction in blood and tissue, respectively.

Based on these equations, the decrease in the transport activity of Oatp1a4-mediated hepatic uptake results in a decrease in PS_{inf}, and subsequently, $CL_{H,int}$ and $K_{p,liver}$ should be decreased. Actually, the $K_{p,liver}$ of ouabain and digoxin were decreased in *Slco1a4*$^{-/-}$ mice, whereas no differences were observed in the $CL_{bile,liver}$ between wild-type and *Slco1a4*$^{-/-}$ mice. The extent of decrease in $K_{p,liver}$ of ouabain and digoxin in *Slco1a4*$^{-/-}$ mice (92.6% and 76.9% decrease, respectively) was similar to that of $CL_{bile,plasma}$ in *Slco1a4*$^{-/-}$ mice (95.4% and 75.7% decrease, respectively). Hence, decreases in $K_{p,liver}$ can be explained by functional deficiency of Oatp1a4-mediated hepatic uptake. On the other hand, the CL_{tot} of ouabain in *Slco1a4*$^{-/-}$ mice decreased significantly to 45.8% of wild-type mice, whereas that of digoxin slightly decreased to 76.7% (Table 4). The change in CL_{tot} in *Slco1a4*$^{-/-}$ mice depends on the relative contribution of CL_H to CL_{tot} in wild-type mice. The $K_{p,liver}$ of ouabain in *Slco1a4*$^{-/-}$ was decreased to 7.4% of that in wild-type. If PS_{eff}, CL_{bile}, and CL_{met}

between wild-type and *Slco1a4*$^{-/-}$ mice were not changed, CL_H of ouabain in *Slco1a4*$^{-/-}$ mice was decreased to 7.4% on the basis of equations (4) and (5). Thus, the relative contribution of CL_H to CL_{tot}, which can quantitatively explain the observed CL_{tot} change (46% of wild-type) in *Slco1a4*$^{-/-}$ mice, should be calculated as 58.5% in wild-type mice (58.5*0.074+41=45.8). In the case of digoxin, its CL_H in *Slco1a4*$^{-/-}$ mice was calculated to be decreased to 23.1%. Thus, the change in the CL_{tot} of digoxin can be explained if CL_H is 30% of CL_{tot} in wild-type mice. In actual, the relative $CL_{bile,plasma}$ to CL_{tot} of ouabain was 28% in wild-type mice, which was higher than that of digoxin (9%). Considering low metabolic clearance of digoxin in mice (Kawahara *et al.*, 1999), a hepatic elimination is more important for ouabain than digoxin. According to the previous reports, the relative contribution of CL_H to CL_{tot} of digoxin was 44.2% in C57BL/6 mice (Kawahara *et al.*, 1999), while that of ouabain was more than 74% in rats, although no data were available for mice (Meijer and van Monffoort, 2002). In addition, the urinary recovery of ouabain in humans was 37% (Selden and Smith, 1972), whereas 80% of digoxin was excreted into urine in an unchanged form (Aronson, 1980). These results suggest that more digoxin than ouabain tends to be excreted into the urine. Calculating the glomerular filtration clearance of unbound drug (fu*GFR) for ouabain and digoxin in mouse, those of ouabain and digoxin are 13.3 and 10.9 mL/min; where fu for ouabain and digoxin, and GFR are 0.95 (rat), 0.78 (mouse) and 14 mL/min/kg (Davies and Morris, 1993; Kawahara *et al.*, 1999; Meijer and van Monffoort, 2002). Those were similar to the CL_{tot} of ouabain and digoxin in *Slco1a4*$^{-/-}$ mice, respectively. Therefore, the different change in the CL_{tot} of ouabain and digoxin can be explained rationally by the different contribution of hepatic elimination to total clearance.

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Considering the hepatic uptake transporters in human, digoxin was selectively recognized by OATP1B3 despite only 43% amino acid sequence identity with mouse Oatp1a4 (Kullak-Ublick *et al.*, 2001). By contrast, Taub et al. reported that digoxin was not a substrate of OATP1A2, OATP1B1, OATP1B3, and OATP2B1 in in vitro experiments (Taub *et al.*, 2011). In human hepatocytes, uptake of digoxin was partly mediated by saturable carrier(s), though no hepatic OATPs can transport digoxin (Kimoto *et al.*, 2011). Ouabain was recognized by OATP1B3 and OATP1A2 (Kullak-Ublick *et al.*, 2001). Though OATP1A2 shows the highest identity with mouse Oatp1a4 (73%) among human OATP isoforms, its protein expression was very low in human liver (Wegler *et al.*, 2017) and mainly expressed in cholangiocytes (Lee *et al.*, 2005). Therefore, the species differences in transporter–mediated uptake of cardiac glycosides between humans and rodents need to be carefully discussed due to a lack of genetic and functional correspondence.

The plasma concentration of rosuvastatin was increased significantly in *Slco1a4*$^{-/-}$ mice, and its $K_{p,liver}$ was decreased slightly. The $K_{p,liver}$ of rosuvastatin was decreased by approximately 50% in *Slco1b2*$^{-/-}$ mice (DeGorter *et al.*, 2012). Moreover, $K_{p,liver}$ of rosuvastatin after intravenous administration was reduced 5- to 10-fold in Oatp1a/1b cluster knockout mice (Iusuf *et al.*, 2013). This reduction suggested that the hepatic uptake of rosuvastatin is mediated by Oatp1a/1b transporters including Oatp1b2, with a minor contribution of Oatp1a4.

By contrast, the $K_{p,liver}$ of BQ-123, fexofenadine, pravastatin, and nafcillin were not different between wild-type and *Slco1a4*$^{-/-}$ mice. BQ-123, fexofenadine, and nafcillin are transported via multiple Oatp isoforms, Oatp1a1 and Oatp1b2, in addition to Oatp1a4

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(Cvetkovic *et al.*, 1999; Reichel *et al.*, 1999; Cattori *et al.*, 2001; Ho *et al.*, 2006; Nakakariya *et al.*, 2008). No differences in their $K_{p,liver}$ were observed between wild-type and *Slco1a4*$^{-/-}$ mice, which suggests that contribution of Oatp1a4 to their hepatic uptake is not dominant. Pravastatin can be transported by Oatp1a1, Oatp1a4, and Oatp1b2 (Hsiang *et al.*, 1999; Tokui *et al.*, 1999; Sasaki *et al.*, 2004). The $K_{p,liver}$ of pravastatin was reduced 3-fold in *Slco1b2*$^{-/-}$ mice (Zaher *et al.*, 2008) and 10- to 200-fold in Oatp1a/1b cluster knockout mice (Iusuf *et al.*, 2012; Higgins *et al.*, 2014). These results suggest that Oatp1a/1b transporters, at least Oatp1b2, contribute to the hepatic uptake of pravastatin. About 90% of nafcillin uptake into isolated rat hepatocytes is mediated by Oatp1a4 based on a relative activity factor method (Nakakariya *et al.*, 2008). Although the exact reason for that discrepancy is unknown, it might be the result of species difference in the relative contribution of Oatp isoforms.

Unexpectedly, we found that the $K_{p,liver}$ of telmisartan was significantly increased in *Slco1a4*$^{-/-}$ mice. The uptake of telmisartan in isolated rat hepatocytes is inhibited by digoxin in a concentration-dependent manner (Ishiguro *et al.*, 2006). Oatp1a4-mediated transport is completely inhibited by 100 μM digoxin in rats, whereas Oatp1a1-mediated transport is not (Sugiyama *et al.*, 2002). Therefore, rat Oatp1a4 is considered to be partly involved in the hepatic uptake of telmisartan. Although the exact mechanism remains unknown, it is implied that hepatic influx clearance mediated by other transporters is increased and/or that the hepatic efflux clearance and metabolic clearance of telmisartan is decreased by knocking out the *Slco1a4* gene, even though the mRNA expression levels of several typical hepatic transporters are unchanged in *Slco1a4*$^{-/-}$ mice.

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In conclusion, we clarified that the hepatic distribution of ouabain and digoxin is dominated by Oatp1a4 in mice, and that systemic clearance of ouabain is significantly reduced in *Slco1a4*$^{-/-}$ mice because of the large contribution of its hepatic elimination. These findings suggest that Oatp1a4 plays an important role in the hepatic uptake of neutral cardiac glycosides in mice.

Author Contributions

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Figure legends

Fig. 1. Time profiles of the plasma concentration of substrate drugs in wild-type and *Slco1a4*$^{-/-}$ mice. The plasma concentrations of ouabain (A), digoxin (B), BQ-123 (C), fexofenadine (D), rosuvastatin (E), pravastatin (F), nafcillin (G), and telmisartan (H) after constant intravenous infusion into wild-type (○) and *Slco1a4*$^{-/-}$ mice (●) are shown. Each point represents the mean ± S.D. (n = 3).

Fig. 2. Total clearance (CL_{tot}) in wild-type and *Slco1a4*$^{-/-}$ mice. CL_{tot} was calculated as the ratio of infusion rate to $C_{p,ss.}$ $C_{p,ss}$ was estimated as the plasma concentration of the parent compounds at 30 min (BQ-123), 60 min (ouabain), or 120 min (digoxin, fexofenadine, rosuvastatin, pravastatin, nafcillin and telmisartan). Each bar represents the mean ± S.D. (n = 3).

Table 1. Analytical conditions for HPLC

Compound	Mobile phase		Gradient condition (B concentration %)
	A	B	
Ouabain	5 mM ammonium formate	Acetonitrile	1 min, 40%→2 min, 80%→5 min, 80%→5.01 min, 40%→8 min, 40%
Digoxin	10 mM ammonium formate	Acetonitrile	0.5 min, 60%→1 min, 95%→3 min, 95%→3.05 min, 60%→5 min, 60%
Fexofenadine	0.1% Formic acid	Acetonitrile	0.5 min, 18%→1.5 min, 60%→4 min, 60%→4.05 min, 60%→6 min, 18%
Rosuvastatin	0.1% Formic acid	Acetonitrile	1 min, 5%→3 min, 45%→3.5 min, 45%→3.55 min, 5%→6.5 min, 5%
Pravastatin	0.1% Formic acid	Acetonitrile	0.5 min, 25%→1.5 min, 80%→4 min, 80%→4.01 min, 25%→6 min, 25%
Nafcillin	0.1% Formic acid	Acetonitrile	1 min, 15%→2 min, 45%→4 min, 45%→4.1 min, 15%→6 min, 15%
Telmisartan	10 mM ammonium acetate	Methanol	0.5 min, 55%→1.5 min, 80%→4 min, 80%→4.01 min, 25%→6 min, 25%

Table 2. mRNA expression levels of transporters in the mouse liver

	Transporter gene/*Gapdh*		Ratio ($Slco1a4^{-/-}$/wild-type)
	Wild-type	$Slco1a4^{-/-}$	
Slco1a1	0.144 ± 0.084	0.0982 ± 0.0675	0.68
Slco1a4	2.87 ± 0.89	0.0595 ± 0.0386***	0.021
Slco1b2	1.29 ± 0.63	1.53 ± 1.21	1.2
Slco2b1	1.04 ± 0.35	0.982 ± 0.722	0.95
Abcc2	1.31 ± 0.15	1.03 ± 0.35	0.79
Abcc3	0.298 ± 0.133	0.355 ± 0.362	1.2
Abcb1a	4.61 ± 1.68	6.24 ± 5.42	1.4
Abcg2	0.920 ± 0.439	1.34 ± 1.46	1.5
Abcb11	0.473 ± 0.292	0.722 ± 0.518	1.5

Values are normalized by expression of *Gapdh* and expressed as mean ± S.D. (n = 4).

****P* < 0.001

Table 3. $C_{p,ss}$, $C_{liver,ss}$, and $K_{p,liver}$ of substrate drugs in wild-type and *Slco1a4*$^{-/-}$ mice

Compound	$C_{p,ss}$ (ng/mL)		$C_{liver,ss}$ (ng/g liver)		$K_{p,liver}$	
	Wild-type	*Slco1a4*$^{-/-}$	Wild-type	*Slco1a4*$^{-/-}$	Wild-type	*Slco1a4*$^{-/-}$
Ouabain	120 ± 26	256 ± 31**	1270 ± 410	207 ± 145*	10.4 ± 1.3	0.774± 0.451***
Digoxin	7.51 ± 3.36	8.76 ± 2.26	55.9 ± 11.6	16.0 ± 2.8**	8.05 ± 2.04	1.86 ± 0.22 *
BQ-123	1410 ± 130	1280 ± 100	4990 ± 420	4450 ± 990	3.52 ± 0.63	3.46 ± 0.60
Fexofenadine	185 ± 11	189 ± 5	2940 ± 450	2750 ± 1150	16.0 ± 3.4	14.6 ± 6.5
Rosuvastatin	15.6 ± 2.3	26.3 ± 5.8*	43.0 ± 14.6	58.0 ± 6.7	2.71 ±0.57	2.24 ± 0.25
Pravastatin	73.3 ± 11.0	57.6 ± 9.5	434 ± 95	323 ± 140	6.11 ± 2.04	5.73 ± 2.81
Nafcillin	897 ± 361	1050 ± 110	18900±2400	16100±4400	23.6±9.6	15.2±2.8
Telmisartan	26.4 ± 3.6	20.2 ± 1.9	2220 ± 150	2060 ± 430	79.4 ± 14.6	113 ± 8*

$C_{p,ss}$ and $C_{liver,ss}$ were estimated as the plasma and liver concentrations of the parent compounds at 30 min (BQ-123), 60 min (ouabain), or 120 min (digoxin, fexofenadine, rosuvastatin, pravastatin, nafcillin and telmisartan).

*$P < 0.05$

**$P < 0.01$

***$P < 0.001$

Table 4. Pharmacokinetic parameters of ouabain and digoxin after constant intravenous infusion in wild-type and *Slco1a4*$^{-/-}$ mice

Compounds		CL_{tot} (mL/min/kg)	$K_{p,liver}$	V_{bile} (ng/min)	$CL_{bile,plasma}$ (mL/min/kg)	$CL_{bile,liver}$ (mL/min/kg)
Ouabain	Wild-type	32.3 ± 6.2	10.4 ± 1.3	22.7 ± 6.7	9.18 ± 3.26	0.916 ± 0.435
	Slco1a4$^{-/-}$	14.8 ± 1.9**	0.773 ± 0.451***	2.30 ± 0.91**	0.420 ± 0.164**	0.642 ± 0.408
Digoxin	Wild-type	14.6 ± 6.8	8.05 ± 2.04	0.206 ± 0.100	1.34 ± 0.59	0.165 ± 0.63
	Slco1a4$^{-/-}$	11.2 ± 2.0	1.86 ± 0.22*	0.0641 ± 0.0295	0.325 ± 0.089*	0.179 ± 0.064

CL_{tot}: total plasma clearance, $K_{p,liver}$: liver-to-plasma concentration ratio at steady state, V_{bile}: biliary excretion rate at steady state, $CL_{bile,plasma}$: biliary excretion clearance with regard to the plasma concentration, $CL_{bile,liver}$: biliary excretion clearance with regard to the hepatic concentration.

*$P < 0.05$

**$P < 0.01$

***$P < 0.001$

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Figure 1

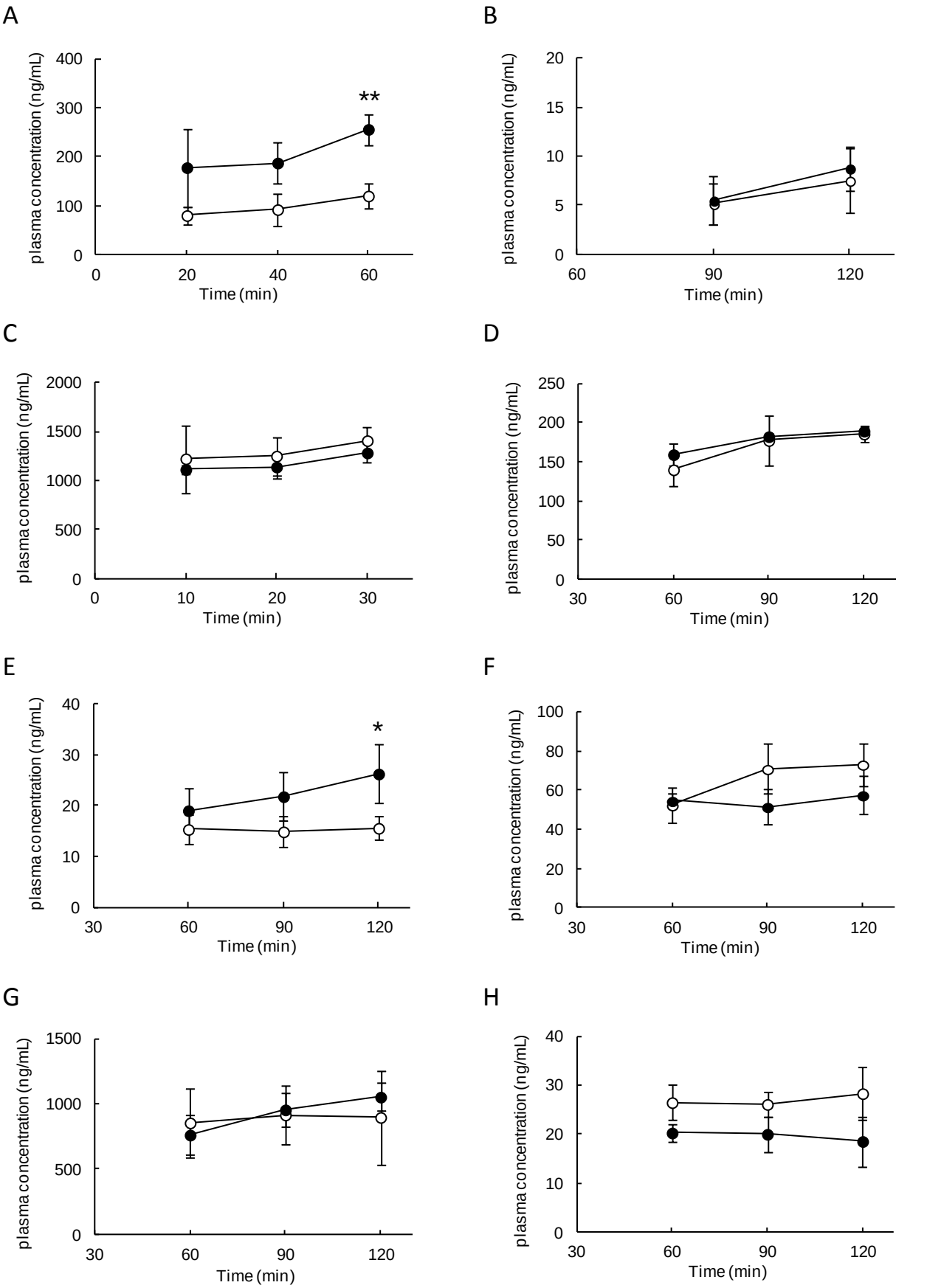
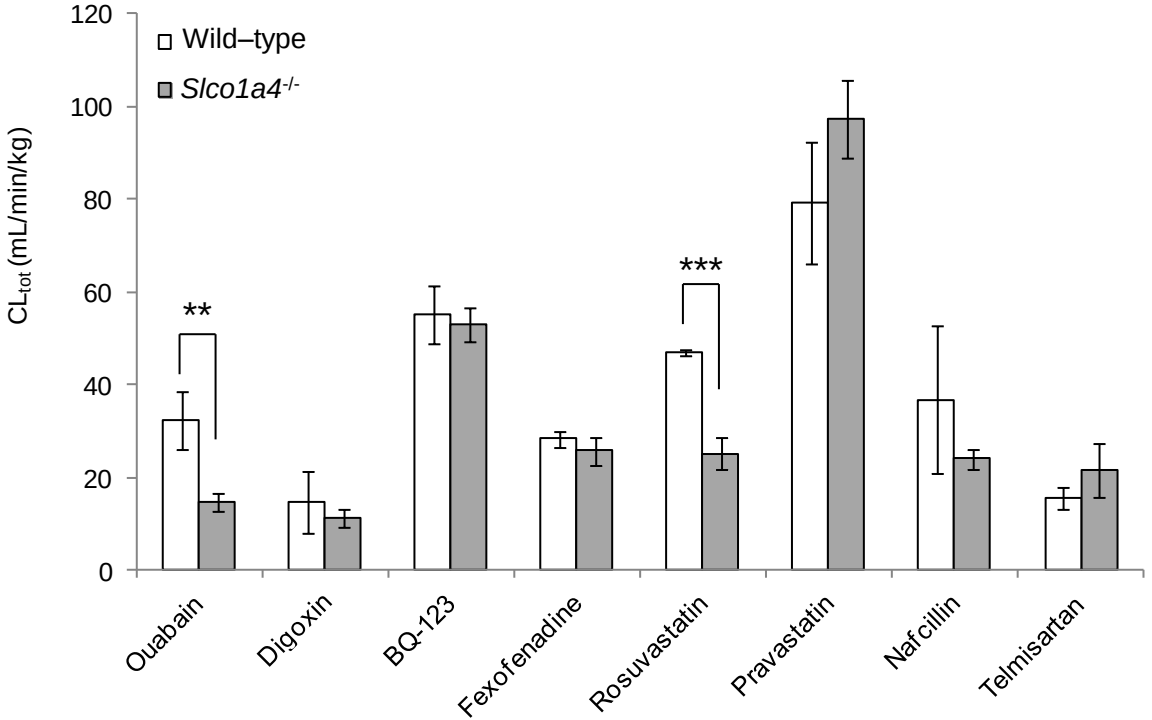


Figure 2


120
100
80
60
40
20
0
CLtot (mL/min/kg)
Wild–type
Slco1a4-/-
**
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Ouabain
Digoxin
BQ-123
Fexofenadine
Rosuvastatin
Pravastatin
Nafcillin
Telmisartan