Special Section on Transporters in Drug Disposition and Pharmacokinetic Prediction

Organic Anion Transporting Polypeptide 1a4 is Responsible for the Hepatic Uptake of Cardiac Glycosides in Mice

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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 a 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 concentration ratios ($K_{p,liver}$) 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-fold and 1.7-fold higher in *Slco1a4*^{-/-} mice, and $K_{p,liver}$ of ouabain and digoxin were 13.4-fold 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-fold and 4.1-fold lower in *Slco1a4*^{-/-} mice, respectively, accompanied with a marked reduction in their $K_{p,liver}$, 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.

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, Oatp1a4, Oatp1b2, and Oatp2b1 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 3-hydroxy-3-methylglutaryl-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-17 β -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 liver-to-plasma concentration ratio ($K_{p,liver}$) of pravastatin decreased significantly in *Slco1b2*^{-/-} mice (Chen et al., 2008; Zaher et al., 2008). Gong et al. (2011) reported that the plasma area under the curve of the concentration-time profile (AUC) of estradiol-17 β -D-glucuronide was 55% larger in female *Slco1a1*^{-/-} mice than that in wild-type mice, with a 50% reduction in $K_{p,liver}$; however, the knockout of *Slco1a4* did not affect the plasma AUC or $K_{p,liver}$ of estradiol-17 β -D-glucuronide, whereas the plasma AUC of dibromosulfophthalein was 3-fold higher in both *Slco1a1*^{-/-} and *Slco1a4*^{-/-} mice compared with that in wild-type mice. Moreover, several

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ABBREVIATIONS: AUC, area under the curve of the concentration-time profile; BQ-123, cyclo-D-Trp-D-Asp-Pro-D-Val-Leu; CL_{bile}, biliary excretion in an unchanged form; CL_{bile,liver}, biliary excretion clearance with regard to the hepatic concentration; CL_{bile,plasma}, biliary excretion clearance with regard to the plasma concentration; CL_H, hepatic clearance; CL_{H,int}, intrinsic hepatic clearance; C_{liver,ss}, liver concentration at steady state; CL_{met}, metabolism clearance; CL_{tot}, total plasma clearance; C_{p,ss}, plasma concentration at steady state; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; HPLC, high-performance liquid chromatography; K_m, Michaelis constant; K_{p,liver}, liver-to-plasma concentration ratio; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; Oatp/OATP, organic anion-transporting polypeptide; PCR, polymerase chain reaction; PS_{eff}, backflux clearance from hepatocytes to blood; PS_{inf}, hepatic influx; Slco, solute carrier organic anion transporter family.

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%

studies (Iusuf et al., 2012, 2013; Higgins et al., 2014) have reported that the changes in pharmacokinetics and tissue distribution of pravastatin, atorvastatin, simvastatin, rosuvastatin, and carboxydichlorofluorescein in Oatp1a1b cluster knockout mice showed a significant reduction of hepatic uptake because of the lack of major hepatic Oatp isoforms. 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. (2010) demonstrated that the brain-to-blood transport of pitavastatin, rosuvastatin, pravastatin, and taurocholate after microinjection into the cerebral cortex was decreased significantly in *Sclol4*^{-/-} mice compared with wild-type mice. The purpose of the present study was to evaluate the significant involvement of Oatp1a4 in the hepatic uptake of substrate drugs in mice, including digoxin (a selective substrate for Oatp1a4 among Oatp family transporters), with the use of *Sclol4*^{-/-} mice.

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. *Sclol4* knockout (*Sclol4*^{-/-}) mice were obtained from Deltagen (San Carlos, CA) and maintained by Charles River Laboratories (Yokohama, Japan). *Sclol4*^{-/-} 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 *Sclol1*, *Sclol4*, *Sclolb2*, *Sclolb1*, *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 *Sclol4*^{-/-} mice weighing 17.1–29.5 g were used for these experiments. Under pentobarbital anesthesia (30 mg/kg), the jugular vein was cannulated with a PE10 polyethylene catheter (Becton Dickinson, Franklin Lakes, NJ) for drug administration. The mice received a constant intravenous infusion of ouabain (4.8 μg/h), digoxin (125 ng/h), BQ-123 (80 μg/h), fexofenadine (7.0 μg/h), rosuvastatin (1.15 μg/h), pravastatin (8.0 μg/h), nafcillin (34.3 μg/h), or telmisartan (480 ng/h) for 30 minutes (BQ-123), 60 minutes (ouabain), or 120 minutes (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 (10,000g, for 10 minutes at 4°C). The bile duct was cannulated with a polyethylene catheter (model 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 phosphate-buffered saline. The mixture was homogenized using a POLYTRON Homogenizer (Kinematica, Lucerne, Switzerland) in an ice bath. The liver homogenate was centrifuged at 12,000g for 10 minutes 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. An LC-MS/MS system comprising an Alliance 2695 Separation Module (Waters, Milford, MA) equipped with a Quattro Micro API Tandem Mass Spectrometer (Waters) 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 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 + NH₄]⁺. The multiple reaction monitoring transitions of ouabain, digoxin, fexofenadine, rosuvastatin, pravastatin, nafcillin, and telmisartan were mass/charge ratios of 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, and nafcillin samples were separated on an Atlantis T3 column (2.1 × 50 mm, 5 μm; Waters), and for telmisartan an Inertsil ODS-3 column (2.1 × 50 mm, 5 μm; GL Sciences, Tokyo, Japan) was used. The details of the high-performance liquid chromatography (HPLC) conditions are shown in Table 1.

TABLE 2

mRNA expression levels of transporters in the mouse liver

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

Transporter Gene/ <i>Gapdh</i>	Ratio (<i>Sclol4</i> ^{-/-} /Wild-Type)	
	Wild Type	<i>Sclol4</i> ^{-/-}
<i>Sclol1</i>	0.144 ± 0.084	0.0982 ± 0.0675
<i>Sclol4</i>	2.87 ± 0.89	0.0595 ± 0.0386*
<i>Sclolb2</i>	1.29 ± 0.63	1.53 ± 1.21
<i>Sclolb1</i>	1.04 ± 0.35	0.982 ± 0.722
<i>Abcc2</i>	1.31 ± 0.15	1.03 ± 0.35
<i>Abcc3</i>	0.298 ± 0.133	0.355 ± 0.362
<i>Abcb1a</i>	4.61 ± 1.68	6.24 ± 5.42
<i>Abcg2</i>	0.920 ± 0.439	1.34 ± 1.46
<i>Abcb11</i>	0.473 ± 0.292	0.722 ± 0.518

*P < 0.001.

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).

HPLC-Fluorescence Analysis. An HPLC-fluorescence system was used to analyze BQ-123. A diluted sample (100 μ l) was injected into an HPLC system using a YMC-Pack ODS-A Column (4.6 $\times$ 150 mm, 5 μ m; YMC Co., Kyoto, Japan). 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:

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

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

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

where $C_{p,ss}$ is the plasma concentration at steady state, $V_{bile,ss}$ is the biliary excretion rate at steady state, and $C_{liver,ss}$ is the liver concentration at steady state. 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 minutes (BQ-123), 60 minutes

(ouabain), or 120 minutes (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 minutes and from 90 to 120 minutes, respectively. To calculate $CL_{liver,ss}$, the specific gravity of the liver was assumed to be 1. Thus, the amount of compound in 1 g of liver (in nanograms per gram) can be regarded as its liver concentration (in nanograms per milliliter).

Statistical Analysis. The mean and S.D. values 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's two-tailed unpaired *t* test was used to identify significant differences. *P* < 0.05 was considered to be significant.

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 3

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

$C_{p,ss}$ and $C_{liver,ss}$ were estimated as the plasma and liver concentrations of the parent compounds at 30 minutes (BQ-123), 60 minutes (ouabain), or 120 minutes (digoxin, fexofenadine, rosuvastatin, pravastatin, nafcillin, and telmisartan). Values are expressed as the mean $\pm$ S.D.

Compound	$C_{p,ss}$ (ng/mL)		$C_{liver,ss}$ (ng/g liver)		$K_{p,liver}$	
	Wild Type	<i>Slco1a4</i> ^{-/-}	Wild Type	<i>Slco1a4</i> ^{-/-}	Wild Type	<i>Slco1a4</i> ^{-/-}
Ouabain	120 $\pm$ 26	256 $\pm$ 31**	1270 $\pm$ 410	207 $\pm$ 145*	10.4 $\pm$ 1.3	0.774 $\pm$ 0.451***
Digoxin	7.51 $\pm$ 3.36	8.76 $\pm$ 2.26	55.9 $\pm$ 11.6	16.0 $\pm$ 2.8**	8.05 $\pm$ 2.04	1.86 $\pm$ 0.22*
BQ-123	1410 $\pm$ 130	1280 $\pm$ 100	4990 $\pm$ 420	4450 $\pm$ 990	3.52 $\pm$ 0.63	3.46 $\pm$ 0.60
Fexofenadine	185 $\pm$ 11	189 $\pm$ 5	2940 $\pm$ 450	2750 $\pm$ 1150	16.0 $\pm$ 3.4	14.6 $\pm$ 6.5
Rosuvastatin	15.6 $\pm$ 2.3	26.3 $\pm$ 5.8*	43.0 $\pm$ 14.6	58.0 $\pm$ 6.7	2.71 $\pm$ 0.57	2.24 $\pm$ 0.25
Pravastatin	73.3 $\pm$ 11.0	57.6 $\pm$ 9.5	434 $\pm$ 95	323 $\pm$ 140	6.11 $\pm$ 2.04	5.73 $\pm$ 2.81
Nafcillin	897 $\pm$ 361	1050 $\pm$ 110	18,900 $\pm$ 2400	16,100 $\pm$ 4400	23.6 $\pm$ 9.6	15.2 $\pm$ 2.8
Telmisartan	26.4 $\pm$ 3.6	20.2 $\pm$ 1.9	2220 $\pm$ 150	2060 $\pm$ 430	79.4 $\pm$ 14.6	113 $\pm$ 8*

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

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 CL_{tot} calculated by the ratio of infusion rate to $C_{p,ss}$ in wild-type and *Slco1a4*^{-/-} mice is shown in Fig. 2. The CL_{tot} values of ouabain and rosuvastatin were decreased significantly in *Slco1a4*^{-/-} mice, whereas those of other substrates were not different between wild-type and *Slco1a4*^{-/-} mice. The liver concentrations and $K_{p,liver}$ values of substrate drugs at steady state in wild-type and *Slco1a4*^{-/-} mice are shown in Table 3. The liver concentrations and $K_{p,liver}$ values of ouabain and digoxin were decreased significantly in *Slco1a4*^{-/-} mice. By contrast, the $K_{p,liver}$ value of telmisartan was significantly increased in *Slco1a4*^{-/-} mice. The liver concentrations and $K_{p,liver}$

values 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} value of ouabain in *Slco1a4*^{-/-} mice decreased significantly, whereas that of digoxin decreased slightly. Furthermore, after constant intravenous infusion of ouabain and digoxin, both the $V_{bile,ss}$ and the $CL_{bile,plasma}$ values were decreased in *Slco1a4*^{-/-} mice. However, the $CL_{bile,liver}$ values of ouabain and digoxin showed no difference between wild-type and *Slco1a4*^{-/-} mice.

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), the mRNA expression level of Oatp1a4 in the liver was significantly higher in females than in males. Thus, to sensitively characterize the importance of Oatp1a4-mediated transport, we decided to use female mice in the current study. We found that the $K_{p,liver}$ values of ouabain and digoxin were decreased significantly in *Slco1a4*^{-/-} mice.

Fig. 2. 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 (BQ-123), 60 (ouabain), or 120 minutes (digoxin, fexofenadine, rosuvastatin, pravastatin, nafcillin, and telmisartan). Each bar represents the mean $\pm$ S.D. ($n = 3$). ** $P < 0.01$; *** $P < 0.001$.

TABLE 4

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

Values are expressed as the mean ± S.D. (n = 3)

Compounds		CL _{tot}	K _{p,liver}	V _{bile,ss}	CL _{bile,plasma}	CL _{bile,liver}
		mL/min per kilogram		ng/min	mL/min per kilogram	
Ouabain	Wild-type	32.3 ± 6.2	10.4 ± 1.3	22.7 ± 6.7	9.18 ± 3.26	0.916 ± 0.435
	<i>Slco1a4</i> ^{-/-}	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
	<i>Slco1a4</i> ^{-/-}	11.2 ± 2.0	1.86 ± 0.22*	0.0641 ± 0.0295	0.325 ± 0.089*	0.179 ± 0.064

*P < 0.05; **P < 0.01; ***P < 0.001.

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, Michaelis constant (K_m) = 1700–3000 μ M; Oatp1a4, K_m = 470 μ M] (Bossuyt et al., 1996; Noé et al., 1997; Eckhardt et al., 1999). The observed decrease of $K_{p,liver}$ values 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 μ M). In the previous in vitro study, 1 μ M 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 value; 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 per kilogram), so the hepatic clearance of ouabain and digoxin should approximate protein unbound fraction in blood (f_B) × overall intrinsic hepatic clearance (CL_{H,int}). Based on the extended clearance concept, CL_{H,int} consists of multiple intrinsic processes, such as hepatic influx clearance (PS_{inf}), backflux clearance from hepatocytes to blood (PS_{eff}), metabolism clearance (CL_{met}), and biliary excretion clearance in an unchanged form (CL_{bile}). Then, CL_{H,int} and $K_{p,liver}$ can be described theoretically by the following equations:

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

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

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 $K_{p,liver}$ values of ouabain and digoxin were decreased in *Slco1a4*^{-/-} mice, whereas no differences were observed in CL_{bile,liver} values between wild-type and *Slco1a4*^{-/-} mice. The extent of decrease in $K_{p,liver}$ values of ouabain and digoxin in *Slco1a4*^{-/-} mice (92.6% and 76.9% decrease, respectively) was similar to that of CL_{bile,plasma} values in *Slco1a4*^{-/-} mice (95.4% and 75.7% decrease, respectively). Hence, decreases in $K_{p,liver}$ values 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 $K_{p,liver}$ value of

ouabain in *Slco1a4*^{-/-} was decreased to 7.4% of that in wild type. If PS_{eff}, CL_{bile}, and CL_{met} values in wild-type and *Slco1a4*^{-/-} mice were not changed, the CL_H of ouabain in *Slco1a4*^{-/-} mice was decreased to 7.4% on the basis of eqs. 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 actuality, the CL_{bile,plasma} value relative to the CL_{tot} value of ouabain was 28% in wild-type mice, which was higher than that of digoxin (9%). Considering the low metabolic clearance of digoxin in mice (Kawahara et al., 1999), an hepatic elimination is more important for ouabain than digoxin. According to the previous reports, the relative contribution of CL_H to the CL_{tot} of digoxin was 44.2% in C57BL/6 mice (Kawahara et al., 1999), whereas 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 (fraction unbound × glomerular filtration rate) for ouabain and digoxin in mouse, those of ouabain and digoxin are 13.3 and 10.9 mL/min; where the fraction unbound for ouabain and digoxin, and the glomerular filtration rate are 0.95 (rat), 0.78 (mouse) and 14 mL/min per kilogram (Davies and Morris, 1993; Kawahara et al., 1999; Meijer and van Monffoort, 2002). Those values were similar to the CL_{tot} of ouabain and digoxin in *Slco1a4*^{-/-} mice, respectively. Therefore, the change in the difference in the CL_{tot} values of ouabain and digoxin can be explained rationally by the different contribution of hepatic elimination to CL_{tot}.

Considering the hepatic uptake transporters in humans, 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. (2011) reported that digoxin was not a substrate of OATP1A2, OATP1B1, OATP1B3, and OATP2B1 in in vitro experiments. In human hepatocytes, the uptake of digoxin was partly mediated by saturable carriers, although no hepatic OATPs can transport digoxin (Kimoto et al., 2011). Ouabain was recognized by OATP1B3 and OATP1A2 (Kullak-Ublick et al., 2001). Although 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 is 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 because of a lack of genetic and functional correspondence.

The plasma concentration of rosuvastatin was increased significantly in *Slco1a4*^{-/-} mice, and its $K_{p,liver}$ value was decreased slightly. The $K_{p,liver}$ value of rosuvastatin was decreased by approximately 50% in *Slco1b2*^{-/-}

mice (DeGorter et al., 2012). Moreover, the $K_{p,liver}$ value of rosuvastatin after intravenous administration was reduced 5-fold 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 $K_{p,liver}$ value 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 (Cvetkovic et al., 1999; Reichel et al., 1999; Cattori et al., 2001; Ho et al., 2006; Nakakariya et al., 2008). No differences in their $K_{p,liver}$ values were observed between wild-type and *Slco1a4*^{-/-} mice, which suggests that the 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 $K_{p,liver}$ value of pravastatin was reduced 3-fold in *Slco1b2*^{-/-} mice (Zaher et al., 2008) and 10-fold 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 $K_{p,liver}$ value 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 μ M 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 the 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.

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.

Authorship Contributions

Participated in research design: Takano, Maeda, Kusuhara, and Sugiyama.

Conducted experiments: Takano, Maeda, and Sugiyama.

Contributed new reagents or analytic tools: Takano and Maeda.

Performed data analysis: Takano, Maeda, Sugiyama.

Wrote or contributed to the writing of the manuscript: Takano, Maeda, Kusuhara, and Sugiyama.

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