Murine Oatp1a/1b uptake transporters control rosuvastatin systemic exposure without affecting its apparent liver exposure

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Abstract:

Organic anion-transporting polypeptides (OATPs) mediate the liver uptake and hence plasma clearance of a broad range of drugs. For rosuvastatin, a cholesterol-lowering drug and OATP1A/1B substrate, the liver represents both its main therapeutic target and its primary clearance organ. Here we studied the impact of Oatp1a/1b uptake transporters on the pharmacokinetics of rosuvastatin using wild-type and Oatp1a/1b-null mice. After oral administration (15 mg/kg), intestinal absorption of rosuvastatin was not impaired in Oatp1a/1b-null mice, but systemic exposure (AUC) was 8-fold higher in these mice compared with wild-type. Although liver exposure was comparable between the two mouse strains (despite the increased blood exposure), the liver-to-blood ratios were markedly decreased (>10-fold) in the absence of Oatp1a/1b transporters. After intravenous administration (5 mg/kg), systemic exposure was 3-fold higher in Oatp1a/1b-null mice than in the wild-type mice. Liver, small intestinal and kidney exposure were slightly, but not significantly, increased in Oatp1a/1b-null mice. The biliary excretion of rosuvastatin was very fast, with 60% of the dose eliminated within 15 minutes after intravenous administration, and also not significantly altered in Oatp1a/1b-null mice. Rosuvastatin renal clearance, although still minor, was ~15-fold increased in Oatp1a/1b-null males, suggesting a role of Oatp1a1 in the renal re-absorption of rosuvastatin.

Conclusion: Absence of Oatp1a/1b uptake transporters increases the systemic exposure of rosuvastatin by reducing its hepatic extraction ratio. However, liver concentrations are not significantly affected, most likely due to the compensatory activity of high-capacity, low-affinity alternative uptake transporters at higher systemic rosuvastatin levels, and the absence of efficient alternative rosuvastatin clearance mechanisms.
Introduction

Organic anion transporting polypeptides (human: OATP, gene: \textit{SLCO}; rodents: Oatp, gene: \textit{Slco}) form a superfamily of transmembrane transporters which mediate the cellular uptake of structurally diverse endogenous and exogenous compounds (Hagenbuch and Meier, 2004). With wide and overlapping substrate specificities and expressed in tissues important for pharmacokinetics (liver, small intestine and kidney), the OATP1A/1B subfamilies are thought to have an important role in drug absorption, distribution and elimination. Based on tissue distribution and amino acid sequence, there are no straightforward orthologues between mouse and human members of these subfamilies. OATP1A/1B subfamilies contain 3 human members (OATP1A2, -1B1, and -1B3) but at least 5 mouse members (Oatp1a1, -1a4, -1a5, -1a6 and -1b2) (Hagenbuch and Meier, 2003). Human OATP1B1 and OATP1B3 are predominantly expressed in the hepatic sinusoidal membrane and thought to play a key role in the hepatic uptake and plasma clearance of drugs. Several low-activity polymorphic variants of human OATP1B1 have been associated with decreased transport activity and increased plasma levels and hence toxicity of statins (cholesterol-lowering drugs) (reviewed in (Kalliokoski and Niemi, 2009)). In addition, a previous study revealed that Rotor syndrome is caused by a complete simultaneous deficiency in the OATP1B1 and OATP1B3 genes (van de Steeg et al., 2012a). While Rotor syndrome is very rare (~1 in 10^6 individuals), individuals with complete deficiencies in either OATP1B1 or OATP1B3 alone likely exist at a much higher frequency in various populations.

Rosuvastatin is one of the most efficacious 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins), and widely used in the treatment of hypercholesterolemia. Its high potency in inhibiting cholesterol synthesis is mainly due to liver-selective distribution of rosuvastatin (Nezasa et al., 2002b; Olsson et al., 2002). Rosuvastatin has very low passive membrane permeability and with limited metabolism, its disposition is mediated almost entirely by uptake and efflux transporters. Rosuvastatin can be transported \textit{in vitro} by multiple hepatic uptake transporters, e.g. OATP1B1, OATP1B3 and OATP2B1 and a bile acid-uptake transporter in the liver, the sodium-taurocholate co-transporting polypeptide (NTCP) (Choi et al., 2011a; Ho et al., 2006e; Kitamura et al., 2008e). In human hepatocytes OATP1B1, OATP1B3 and NTCP are the predominant uptake transporters, with OATP1B1 and/or OATP1B3 accounting for ~55% of the rosuvastatin uptake, both having a high affinity and high capacity, while
NTCP accounts for ~35%, having high capacity but lower affinity for rosuvastatin (Ho et al., 2006d). The efflux transporters ABCG2 and ABCC2 are responsible for the biliary excretion of rosuvastatin in humans, as demonstrated by *in vitro* and *in vivo* studies (Hu et al., 2010; Jemnitz et al., 2010; Kitamura et al., 2008d).

Patients carrying polymorphic variants of *SLCO1B1* exhibit increased rosuvastatin plasma concentrations (Choi et al., 2008a; Hua et al., 2011a; Lee et al., 2005a; Pasanen et al., 2007a), but evidence regarding a correlation between *SLCO1B1* genotype and therapeutic response is equivocal. Some studies find no correlation between polymorphic variants of *SLCO1B1* and cholesterol lowering efficacy of rosuvastatin (Romaine et al., 2010; Sirtori et al., 2011), while others do observe an association between these factors (Chasman et al., 2012). These findings raise the question how reduced hepatic uptake by OATP proteins affects systemic and liver concentrations of rosuvastatin.

Several single (Oatp1b2, Oatp1a1, Oatp1a4) and combined knockout mouse models (Oatp1a/1b knockout mice) are available and have proved very useful in elucidating the *in vivo* physiological and pharmacological functions of OATP1A/1B (reviewed in (Iusuf et al., 2012d)). First, Ose *et al* showed that Oatp1a4 can transport rosuvastatin across the blood brain barrier, but only upon *in situ* injection into the brain (Ose et al., 2010). Using Oatp1b2 knockout mice, a small-scale study showed that mouse Oatp1b2 might contribute to the liver uptake of rosuvastatin after intravenous administration, although only the liver-to-plasma ratio was significantly decreased in comparison with wild-type mice, whereas the plasma or liver concentrations were not significantly affected (Degorter et al., 2011a).

In the present study, we aimed to obtain an in depth understanding of the *in vivo* role of Oatp1a/1b transporters in the oral absorption and hepatic uptake of rosuvastatin. For this we used the Oatp1a/1b knockout mouse model (*Slco1a1b*−/− mice, lacking all Oatp1a and -1b transporters) (van de Steeg et al., 2010a). We compared the disposition of rosuvastatin in Oatp1a/1b knockout and wild-type mice after oral and intravenous administration.
Materials and methods

Animals

Animals were housed in small groups in a temperature-controlled environment with a 12-hour light/12-hour dark cycle. They received a standard diet (AM-II; Hope Farms) and acidified water \textit{ad libitum}. All mouse experiments were approved by the Animal Experiments Review Board of the Netherlands Cancer Institute (Amsterdam), complying with Dutch legislation and in accordance with European Directive 86/609/EEC and the GSK Policy on the Care, Welfare and Treatment of Animals. Male or female wild-type and \textit{Slco1a/1b}–/– (Oatp1a/1b knockout) mice (van de Steeg et al., 2010b) of comparable genetic background (>99% FVB) between 9 and 14 weeks of age were used as indicated.

Chemicals and reagents

Rosuvastatin calcium salt was from Sequoia Research Products (Pangbourne, UK), and other chemicals were from Sigma (St. Louis, USA), isoflurane (Forane) from Abbott Laboratories (Queenborough, Kent, UK) and disodiumEDTA (Ethylenediaminetetraacetic acid) from LeoPharma BV (Breda, The Netherlands).

Drug analysis

Concentrations of rosuvastatin in blood, organs (homogenized in 1:10 volumes of ice-cold 4% (w/v) BSA) and bile (diluted 100 times with human blank plasma) were determined by LC-MS/MS analysis as described (Hobbs et al., 2012).

Blood and tissue pharmacokinetic experiments

Rosuvastatin was dissolved in dimethylsulfoxide (DMSO) and diluted with saline (to 1 mg/mL or 1.5 mg/mL) for administration of dose levels of 5 mg/kg i.v. or 15 mg/kg oral to mice. The maximum concentration of DMSO in the final solution was 2%. Ten μL/g body weight were administered via oral gavage (n = 5 - 7 for each group), and 5 μL/g body weight were used for administration in the tail vein of mice. At different time points, EDTA-blood (via cardiac puncture) was sampled under isoflurane anesthesia. Mice were then sacrificed by cervical dislocation and tissues (liver without gall bladder) were isolated. After 15 mg/kg oral administration to male mice, portal vein blood samples
were taken prior to cardiac puncture. Blood samples were diluted 1:1 with water and then stored at -20°C until analysis.

**Biliary excretion of rosuvastatin**

Gall bladder cannulations and collection of bile in male wild-type and Slco1a/1b−/− mice (n = 7) were performed as described (van Herwaarden et al., 2003). At the end of the experiment, blood and tissue samples were isolated and treated as described above.

**Urinary and fecal excretion of rosuvastatin**

A mass balance study was performed with Rucco Type M/1 stainless steel metabolic cages (Valkenswaard, The Netherlands). Mice (n = 5) received rosuvastatin orally (15 mg/kg) or intravenously (5 mg/kg). Urine and feces were collected in a 0-24 hour fraction after the drug administration, followed by isolation of blood and tissue samples as described above. For female mice, rosuvastatin was only given orally (15 mg/kg) and at different times after administration (7.5, 15, 30, 60 and 120 minutes) blood samples were isolated from the tail vein. After collecting the urine and feces for 24 hours, mice were sacrificed as described above.

**RNA isolation, cDNA synthesis and RT-PCR**

RNA isolation from mouse kidney and subsequent cDNA synthesis and RT-PCR were performed as described (van Waterschoot et al., 2008). Specific primers (QIAGEN, Hilden, Germany) were used to detect expression levels of the following mouse Oatp1a genes: Oatp1a1, Oatp1a4 and Oatp1a6.

**Pharmacokinetic calculations and statistical analysis**

When variances were not homogeneous, the data were log-transformed in order to obtain normal distribution and equal variances. The two-sided unpaired Student’s t-test was used throughout the study to assess the statistical significance of differences between two sets of data. Results are presented as the means ± S.D. Differences were considered to be statistically significant when P < 0.05. Averaged blood concentrations for each time point were used to calculate the area under the blood concentration versus time curve (AUC) from t = 0 to the last sampling time point by the linear trapezoidal rule; S.E. was calculated by the law of propagation of errors (Bardelmeijer et al., 2000). Results of the AUC measurements are presented as means ± S.E.M. We calculated the
ratio between plasma exposure after i.v. and oral administration, corrected for dose levels: \( \frac{AUC_{\text{oral}}}{AUC_{\text{i.v.}}} \cdot \frac{\text{Dose}_{\text{i.v.}}}{\text{Dose}_{\text{oral}}} \). The apparent hepatic extraction ratio was calculated as \( E = \left[ 1 - \frac{AUC_{\text{oral systemic}}}{AUC_{\text{oral portal vein}}} \right] \) (Gridelli et al., 1986).

**Pharmacokinetic modeling**

The modeling software Phoenix WinNonlin 6.1 (Phoenix™ WinNonlin® Copyright ©1998-2009, Tripos L.P.) was used. Non-compartmental analysis was performed using blood data from intravenously and orally dosed mice from both strains. As the study design involved composite sampling, the ‘sparse’ sampling function was used to maximize the contribution of the data from each mouse at each sample time. After oral administration, we calculated the renal clearance based on the amount of rosuvastatin recovered in the urine over 24 hours after oral administration corrected for AUC\(_{\text{systemic}}\) extrapolated to infinity (AUC\(_{\text{0-inf}}\)) and for individual mouse body weight. After intravenous administration, we calculated both renal and non-renal clearance based on the amount of rosuvastatin recovered in the urine or the feces corrected for AUC\(_{\text{systemic}}\) extrapolated to infinity (AUC\(_{\text{0-inf}}\)) and for individual mouse body weight.
Results

Oatp1a/1b transporters are not essential for the intestinal absorption of rosuvastatin

Rosuvastatin is administered orally to patients, but not much is known about the transporters which facilitate its intestinal absorption. In wild-type mice, Oatp1a4 and Oatp1a5 are expressed in the small intestine (van de Steeg et al., 2012b), where they might theoretically facilitate the absorption of various drugs. Therefore, we first investigated a possible role of Oatp1a uptake transporters in absorption of rosuvastatin across the intestinal wall using wild-type and Oatp1a/1b knockout mice. We measured rosuvastatin portal vein blood concentrations at various time points after oral administration (15 mg/kg). Oral absorption was very rapid, with the highest blood rosuvastatin concentrations observed at the earliest technically feasible time point, 5 minutes after dosing (Figure 1A). The portal vein blood concentrations were modestly increased in Oatp1a/1b knockout mice (Figure 1A, Table 1), indicating that Oatp1a transporters are not essential for the intestinal absorption of rosuvastatin. The modest increase in the portal vein blood concentrations in Oatp1a/1b knockout mice likely reflects the higher systemic blood concentrations (see below, Figure 1B, C).

Increased systemic exposure of rosuvastatin in Oatp1a/1b-null mice after oral administration

We also determined the systemic blood concentrations after oral administration of 15 mg/kg rosuvastatin to wild-type and Oatp1a/1b knockout mice. Rosuvastatin blood concentrations were markedly increased in Oatp1a/1b knockout mice in comparison with wild-type mice (Figure 1B, C), with an 8.2-fold higher blood AUC(5-240) (Table 1). Although liver concentrations were not significantly different between the mouse strains (Figure 1D, E), liver-to-blood ratios were at least 10-fold decreased in Oatp1a/1b-null mice at most time points, indicating a partially impaired liver uptake in the absence of Oatp1a/1b transporters (Figure 1F).

The liver represents both the therapeutic target and the main clearance organ for rosuvastatin. Therefore, the extraction capacity of the liver is an important parameter to assess. Assuming that portal vein blood concentrations represent the amount of rosuvastatin before entering the liver, and the systemic blood concentrations represent the amount of drug escaping the uptake in the liver, we calculated the apparent hepatic extraction ratio $E = \frac{1}{1} - \left( \frac{\text{AUC}_{\text{oral systemic}}}{\text{AUC}_{\text{oral portal vein}}} \right)$ (Table 1). This approach assumes...
there is little alternative clearance (e.g., metabolic or renal) and tissue distribution of rosuvastatin outside of the liver, as was previously described for the rat (Nezasa et al., 2002a). In wild-type mice, rosuvastatin distributes almost exclusively to the liver after oral administration, with a very high apparent hepatic extraction ratio (0.93). Interestingly, in the absence of Oatp1a/1b uptake transporters this ratio dropped to 0.72, indicating a diminished efficacy in hepatic uptake in Oatp1a/1b knockout mice.

**Increased systemic exposure of rosuvastatin in Oatp1a/1b-null mice after intravenous administration**

To further increase our understanding of how Oatp1a/1b transporters modulate the liver uptake of rosuvastatin we performed a pharmacokinetic study upon intravenous administration of rosuvastatin (5 mg/kg). Similar to the oral administration experiment, the systemic exposure of rosuvastatin was markedly higher in Oatp1a/1b knockout mice in comparison with wild-type mice (Figure 2A, B), with a 3.1-fold higher blood AUC\(_{(5-240)}\) (Table 1). Again, liver concentrations were not significantly different between the two mouse strains (Figure 2C, D), whereas liver-to-blood ratios were significantly and substantially reduced (5- to 10-fold) at most time points from 15 min on in Oatp1a/1b knockout mice (Figure 2E), indicating a partially impaired hepatic uptake.

For the small intestinal wall (tissue) and small intestinal content concentrations and tissue-to-blood ratios of rosuvastatin we observed very similar results as for the liver (Figure 3A-D). These results would be in line with the liver concentrations and liver-to-blood ratios: The substantial % of dose of rosuvastatin (~15%) found in the small intestinal wall early after administration (Figure 3A), may reflect extensive entero-hepatic circulation of rosuvastatin, assuming rapid hepatobiliary excretion in the intestine (see below). The kidney concentrations of rosuvastatin were also increased in Oatp1a/1b knockout mice, most likely reflecting the increased systemic exposure. There were no significant differences in kidney-to-blood ratios between the strains, suggesting that there is no important role of Oatp1a/1b transporters in the uptake of rosuvastatin into the kidney (Figure 4A-C).

**Effect of Oatp1a/1b transporters on the biliary excretion of rosuvastatin**

We investigated the effect of Oatp1a/1b deficiency on biliary elimination of rosuvastatin after intravenous administration (5 mg/kg) to mice with a cannulated gall bladder and ligated common bile duct. The bile flow was not different between the two
mouse strains (~1.5 μL/min/g of liver). Biliary excretion of rosuvastatin was very rapid in both strains, with ~60 % of the dose being excreted in the first 15 minutes (Figure 5A). In the first 30 minutes there was no significant difference between the two strains of mice, and only from 30 minutes on there was a slightly higher biliary output of rosuvastatin in Oatp1a/1b knockout mice (Figure 5A), possibly reflecting slightly higher liver concentrations (e.g. Figure 2C). Additionally, from 30 minutes after dosing, biliary excretion of rosuvastatin was much slower in both strains than in the first 15 minutes after intravenous administration (Figure 5B). In this experiment enterohepatic circulation of rosuvastatin is interrupted due to ligation of the common bile duct, blocking possible recharging of the liver with rosuvastatin reabsorbed from the intestinal lumen, and thus continued biliary excretion. Note that the mRNA expression of Abcc2, one of the canalicular efflux transporters responsible for the biliary excretion of rosuvastatin, is somewhat lower in the Oatp1a/1b knockout mice, while expression of Abcg2 is not changed (van de Steeg et al., 2012c).

There were no significant differences in the blood, liver and small intestinal tissue concentrations in gall bladder-cannulated wild-type and Oatp1a/1b knockout mice 60 minutes after dosing (Supplemental Figure 1). In the small intestinal content we observed significantly higher levels of rosuvastatin in Oatp1a/1b knockout mice compared to wild-type (1.1 ± 0.8 versus 0.4 ± 0.2 % of dose, \( P < 0.05 \), Supplemental Figure 1). It is notable that only a small fraction of rosuvastatin was found back in the small intestinal wall and lumen. In the context of a ligated common bile duct, rosuvastatin can only reach the small intestinal lumen via direct intestinal excretion from the blood possibly mediated by Abcg2 or Abcc2, whose mRNA expression levels in the small intestine are similar in both strains (van de Steeg et al., 2012d). Note that the amount of rosuvastatin directly excreted from the blood (Supplemental Figure 1B, C) is far lower than that excreted via the bile and probably reabsorbed via the small intestinal wall (Figure 3A). Taken together these data suggest that rosuvastatin undergoes extensive enterohepatic circulation.

**Effect of Oatp1a/1b transporters on the urinary and fecal excretion of rosuvastatin**

Next, we performed a mass-balance experiment over 24 hours after intravenous (5 mg/kg) or oral (15 mg/kg) administration of rosuvastatin to male wild-type and Oatp1a/1b knockout mice. In line with the similar and high % of dose excreted in the bile after intravenous administration (Figure 5A), the dose recovered in the feces was nearly
60% in the wild-type mice and slightly, albeit significantly, lower in the Oatp1a/1b-null mice (57.3 ± 6.7 versus 49 ± 4.1 % of dose, *P* < 0.05) (Figure 6A). The amount of rosuvastatin recovered in the urine was 3-fold higher in the Oatp1a/1b knockout mice (20.4 ± 3 versus 5.9 ± 2.5 % of dose) (Figure 6A), probably reflecting the 3-fold higher systemic exposure of rosuvastatin after intravenous administration (Table 1) and the diminished renal reabsorption of rosuvastatin in the Oatp1a/1b-null mice (see below and Table 2). The recovery after intravenous administration was ~70%, possibly because upon intravenous administration rosuvastatin can distribute more extensively to other compartments in the body, from which rosuvastatin may be released only after 24 hours after administration.

After oral administration to male mice, the total rosuvastatin recovery was close to 100% of the dose (Figure 6B), indicating very limited metabolism of this drug in mice. In line with the high apparent hepatic extraction ratio after oral administration (Table 1), the % of dose recovered in the feces in wild-type mice was very high (~100%), while it was reduced to ~86% in Oatp1a/1b knockout mice, albeit not significantly (Figure 6B). The amount of rosuvastatin recovered in the urine of wild-type mice was very low (0.10 ± 0.04 % of dose), while in the absence of Oatp1a/1b transporters it was about 100-fold higher (10.7 ± 4.7) (Figure 6B). As a consequence, the renal clearance of rosuvastatin was 15.5-fold increased, from 1.3 ± 0.6 to 20.1 ± 9.0 ml/min/kg (*P* < 0.01) in the male Oatp1a/1b knockout mice (Table 2).

**Role of Oatp1a/1b transporters in the renal and non-renal clearance of rosuvastatin**

The increased renal clearance of rosuvastatin in the Oatp1a/1b knockout mice might be explained if one or more of the Oatp1a/1b proteins in the kidney played a role in the tubular reabsorption of glomerularly filtrated or otherwise renally secreted rosuvastatin. Cheng et al. (2005) demonstrated that only Oatp1a1 and Oatp1a6, and to a lesser extent Oatp1a4, are significantly expressed in the male kidney. Interestingly, Oatp1a1 was hardly expressed in female kidney. If Oatp1a1 would be primarily responsible for renal rosuvastatin reabsorption, the renal clearance in female wild-type mice should be higher than in male wild-type mice, and female Oatp1a/1b knockouts should show little increase in clearance. To test whether this was the case, we performed an oral systemic exposure and mass balance study with 15 mg/kg rosuvastatin in female mice (Figure 7). Similar to results obtained in male mice (Figure
systemic blood concentrations were highly increased in female Oatp1a/1b knockout mice (Figure 7A). Most of the rosuvastatin was recovered in the feces with similar levels in wild-type and Oatp1a/1b-null mice (~75 % of dose) (Figure 7B), while the amount in the urine was 19-fold higher in the female Oatp1a/1b knockout mice in comparison with wild-type controls (18.9 ± 3 versus 1 ± 0.3 % of dose). Importantly, when comparing the male versus female mice, we observed that the amount of rosuvastatin in the urine of female wild-type mice was 9-fold higher than in the male wild-type mice (Figure 7C), and 1.7-fold higher in the female Oatp1a/1b-null mice than in the male Oatp1a/1b-null mice (Figure 7C).

Subsequent calculation of the renal clearances (Table 2) showed that renal clearance in wild-type females was 15 times higher than that in wild-type males, but not different from that in Oatp1a/1b knockout males. Moreover, the renal clearance was not significantly increased in female Oatp1a/1b knockout compared to wild-type female mice (Table 2). These results are consistent with a renal rosuvastatin reabsorption role of Oatp1a1 in male mice. RT-PCR analysis of Oatp1a1, Oatp1a4, and Oatp1a6 expression in kidney of our FVB strain wild-type mice (Supplemental Figure 2) confirmed that Oatp1a1 was far more highly expressed in male than in female kidney (about 5000-fold), whereas Oatp1a4 was not differentially expressed, and Oatp1a6 only slightly (about 2-fold) more in male mice than in female mice. Collectively, the data suggest that Oatp1a1 plays a role in the renal reabsorption of rosuvastatin, and thus diminishes its renal clearance.

After intravenous administration, the renal clearance was only 2-fold increased in Oatp1a/1b knockout male mice in comparison with wild-type mice and the non-renal clearance was 2-fold decreased (Table 2). This reflects the decreased renal reabsorption of rosuvastatin in the male Oatp1a/1b knockout mice (see above, Table 2). The renal clearance accounted for ~10% of the total clearance in the male wild-type mice and for ~30% in the Oatp1a/1b knockout mice (Table 2).

**Pharmacokinetic modeling**

We further performed a limited non-compartmental modeling of the pharmacokinetic data using the sparse sampling function. The results are presented in Table 3. It is noteworthy that values for blood AUC after intravenous administration calculated using the pharmacokinetic software are much higher than the AUC values observed from t = 5 until t = 240 min, calculated using the linear trapezoidal rule (Table 1
versus Table 3). This discrepancy is mainly due to the extrapolation of the blood concentration data to $t = 0$ min. Below we discuss only the data from Table 3.

The ratio between blood exposure after oral versus i.v. administration of rosuvastatin was increased in the Oatp1a/1b knockout mice (from 0.011 to 0.058), most likely as a consequence of the impaired first-pass uptake in the liver of these mice after oral administration (Table 3).

After intravenous administration we observed a decrease, albeit modest, in the total clearance (from 20.3 to 11.8 mL/min/kg). Finally, the half-life of rosuvastatin after intravenous administration was almost 2-fold higher (26.2 in wild-type mice versus 49.9 minutes in Oatp1a/1b knockout mice) (Table 3).

The pharmacokinetic parameters (total clearance and exposure) we obtained after oral and intravenous administration of the wild-type mice were in general agreement with previous studies, although there were some differences in half-life values, probably due to different genetic background of the mice and the dosages used (Peng et al., 2009).

**Discussion**

Here we show that Oatp1a/1b uptake transporters are not essential for the intestinal absorption of rosuvastatin after oral administration, but that they strongly affect rosuvastatin systemic exposure after oral and intravenous administration. Interestingly, the strong increase (8-fold) in systemic exposure in Oatp1a/1b-null mice is not accompanied by a significant decrease in liver exposure, or in biliary excretion of rosuvastatin after intravenous administration, but the hepatic extraction ratio is markedly decreased in the absence of Oatp1a/1b transporters. The major pharmacokinetic impact of Oatp1a/1b transporters on rosuvastatin therefore occurs through their hepatic uptake activity. We also show that renal clearance of rosuvastatin, while small compared to hepatic clearance, is gender-dependent and might be affected by the different expression levels of Oatp1a1 in the kidney of male versus female wild-type mice.

It has been proposed that OATP1A/Oatp1a transporters can mediate the intestinal absorption of many drugs, including statins. Despite being quite polar, rosuvastatin was very rapidly and efficiently absorbed, with both portal vein and systemic blood concentrations highest at the earliest feasible sampling time point (5 minutes) in both wild-type and Oatp1a/1b knockout mice. Although Oatp1a/1b uptake transporters are clearly not essential for the intestinal uptake of rosuvastatin, given its polarity (logP
1.92) it is almost certain that other uptake transporters must be involved. One candidate could be mouse Oatp2b1, since several studies have shown that its human orthologue OATP2B1 can transport rosuvastatin *in vitro* (Ho et al., 2006c; Kitamura et al., 2008c; Varma et al., 2011b). The contribution of OATPs in the oral absorption of rosuvastatin was also investigated in an *in vivo* study in pigs, where gemfibrozil (an OATP inhibitor) was co-administered with rosuvastatin (Bergman et al., 2009a). However, despite high concentrations of gemfibrozil, enough to efficiently inhibit OATP1A2 and OATP2B1 in the small intestine, the intestinal absorption of rosuvastatin was not affected (Bergman et al., 2009b). Additional studies are therefore required to establish the transporters responsible for the intestinal uptake of rosuvastatin.

Interestingly, our results suggest that Oatp1a1 in the kidney might play a role in the renal reabsorption of rosuvastatin, although the contribution of renal clearance to the systemic clearance of rosuvastatin is small in wild-type mice. We observed a gender-dependent difference in the renal clearance of rosuvastatin, which has also been described in rats for perfluorooctanoic acid, a potentially toxic chemical and substrate of Oatp1a1, which is mainly eliminated renally (Yang et al., 2010). Therefore, besides their predominant role in mediating hepatic clearance of drugs, Oatp1a transporters might affect the renal clearance of drugs as well.

Systemic exposure of rosuvastatin after oral administration was 7- to 14-fold higher in the absence of Oatp1a/1b transporters. Oatp1a1, Oatp1a4 and Oatp1b2, present in the basolateral membrane of hepatocytes in mice, likely mediate the same function(s) as human OATP1B1 and OATP1B3. Therefore, our results are in line with data from patients carrying low-activity genetic polymorphic variants of OATP1B1. These variants are associated with increased plasma levels of rosuvastatin (reviewed in (Hua et al., 2011b)), but it seems that the magnitude of effects varies between different ethnic groups. For example, Korean individuals with the low activity variant *15/*15 (two copies of the 521T>C allele) had 1.7-fold higher rosuvastatin AUCs than the control group (Choi et al., 2008b). In a study comparing white and Asian subjects, the variant *15/*15 was associated with higher rosuvastatin AUC only in the white subjects, and not in the Asian ones (Lee et al., 2005b). Similarly, in a Finnish population, a slightly higher systemic exposure to rosuvastatin was observed in carriers of the OATP1B1 521T>C variant (Pasanen et al., 2007b). In addition, documented drug-drug interactions between rosuvastatin and OATP1B inhibitors further support the importance of OATP1B1 in the systemic exposure of rosuvastatin. In humans, it was shown that after repeated
administrations of oral gemfibrozil (at plasma concentrations which mainly inhibit OATP1B1) the plasma AUC of rosuvastatin was 1.8-fold higher (Schneck et al., 2004). Co-administration with cyclosporine led to 7-10-fold higher rosuvastatin AUCs (Simonson et al., 2004). The net effect of OATP1B inhibition by cyclosporine is difficult to estimate as cyclosporine can also inhibit various other influx and efflux transporters involved in the pharmacokinetics of rosuvastatin, e.g. NTCP and/or ABCC2 and ABCG2. A similar pronounced effect of co-administration of cyclosporine and rosuvastatin was seen in a study with pigs (Bergman et al., 2009c).

Despite the markedly increased systemic exposure of rosuvastatin in Oatp1a/1b knockout mice, the liver concentrations were not significantly reduced in these mice. However, the liver-to-blood ratios were markedly decreased, indicating an impaired liver uptake. A recent small-scale study in Oatp1b2-null mice showed that at 30 minutes after intravenous administration, liver-to-plasma ratios of rosuvastatin were 2.7-fold lower than in wild-type mice, while the plasma concentrations were not significantly different between the two mouse strains (Degorter et al., 2011b). We observed 10-fold lower liver-to-blood ratios in Oatp1a/1b-null mice at the same time point, and 7-fold increased blood concentrations. This indicates that, in addition to Oatp1b2, hepatic Oatp1a1 and/or Oatp1a4 also play an important role in liver uptake of rosuvastatin.

As previously mentioned, liver (and bile) concentrations of rosuvastatin were mostly not significantly altered by the absence of Oatp1a/1b transporters, in spite of the strongly increased blood exposure. This surprising finding can be explained by the intrinsic properties of rosuvastatin. While our data show a very high hepatic extraction ratio (0.93) of rosuvastatin in wild-type mice after oral administration, this dropped only to 0.72 in Oatp1a/1b knockout mice (Table 1), indicating that in the knockouts there is still a very substantial hepatic uptake of rosuvastatin. Considering the high and similar amount of rosuvastatin taken up in wild-type and knockout liver (Figure 1E), the modest decrease in hepatic extraction is sufficiently offset by the higher portal vein concentrations. Small differences in liver concentration can be easily lost in the experimental variation and thus not become obvious. However, any small decrease in liver exposure theoretically translates into a much larger (4-fold) increase in the fraction of rosuvastatin which “escapes” the liver (from 0.07 to 0.28 (1 - apparent hepatic extraction ratio), Table 1), and thus ends up in the systemic circulation. Note that the estimated total amount of rosuvastatin in the systemic circulation represents less than 0.5% of the dose (at 1 μg/mL, Figure 1B), an amount that is negligible compared to the
~20% of dose found in the liver over the first 15-60 min (Figure 1E). It is therefore not surprising that a relatively big change in systemic blood concentrations can occur with little impact on the liver concentrations. This idea is also supported by a physiologically based pharmacokinetic (PBPK) model described for pravastatin (Watanabe et al., 2009). This model predicts that, for a predominantly hepatically cleared drug, a diminished hepatic uptake (like in the absence of Oatp1a/1b transporters) leads to a substantial increase in the systemic exposure, while the liver exposure is not so much affected, especially for drugs which have a negligible renal clearance. In our study renal clearance of rosuvastatin accounts for maximally 1% of total clearance in the wild-type mice, versus 25% in the Oatp1a/1b-null mice after oral administration (Table 2). This is in line with data from humans (Martin et al., 2003b) and rats (Nezasa et al., 2002c), which also exhibit a low renal clearance of rosuvastatin.

The still substantial hepatic uptake of rosuvastatin in Oatp1a/1b knockout mice, albeit at higher blood concentrations, indicates that alternative transporters for rosuvastatin can partially compensate for the loss of Oatp1a/1b transporters. We hypothesize that already shortly after administration, reduced liver uptake due to the absence of Oatp1a/1b transporters results in a substantial increase in rosuvastatin blood concentrations. This high blood concentration allows continued substantial uptake of rosuvastatin into the liver via low-affinity, but high-capacity alternative transporters. Nevertheless, the rescue provided by these alternative transporters is partial as the systemic blood concentrations remain markedly increased in the Oatp1a/1b-null mice over at least 4 hours after administration. The most obvious candidate as an alternative transporter in mouse or human, but not rat, would be NTCP/Ntcp, which has been described to facilitate cellular uptake of rosuvastatin in vitro (Ho et al., 2006b; Kitamura et al., 2008a). Using double expressing-oocytes of wild-type and/or polymorphic variants of OATP1B1 and NTCP, it was shown that reduced rosuvastatin uptake by OATP1B1*15 can be masked in the presence of NTCP, suggesting that NTCP can rescue OATP1B1 loss of function in vitro (Choi et al., 2011b). Another transporter which might compensate for the loss of Oatp1a/1b function is OATP2B1 (human) or Oatp2b1 (mouse/rat), which is also present in the basolateral membrane of hepatocytes and can mediate rosuvastatin transport in vitro (Ho et al., 2006a; Kitamura et al., 2008b; Varma et al., 2011a).

Previously, we showed that mouse Oatp1a/1b uptake transporters control the hepatic uptake of pravastatin (Iusuf et al., 2012a). Similar to rosuvastatin, absence of
Oatp1a/1b resulted in a substantial increase in systemic exposure of pravastatin both after oral and intravenous administration. However, in contrast to rosuvastatin, pravastatin liver exposure was 2-fold reduced in the Oatp1a/1b-null mice, and the impact of Oatp1a/1b transporters was very obvious in the 8-fold decreased biliary excretion of pravastatin after intravenous administration (Iusuf et al., 2012b). Although similar in hydrophilicity, (pravastatin, logP 1.65 and rosuvastatin, logP 1.92), rosuvastatin has a higher affinity to distribute to the liver than pravastatin (Nezasa et al., 2003; Nezasa et al., 2002a). Indeed, rosuvastatin has a higher apparent hepatic extraction ratio (0.93 in wild-type versus 0.72 in Oatp1a/1b-null mice, Table 1) than pravastatin (0.88 in wild-type versus 0.52 in Oatp1a/1b-null mice) (Iusuf et al., 2012c). In addition, rosuvastatin appears to be more substantially transported by alternative transporters such as Ntcp and/or Oatp2b1 when compared to pravastatin, resulting in a somewhat less pronounced increase in the systemic exposure in the Oatp1a/1b knockout model. Nevertheless, hepatic uptake of both compounds is still substantial in the absence of Oatp1a/1b transporters, indicating that the uptake transporters involved have an appreciable redundancy and are capable of relatively efficient clearance even when the main disposition mechanism has been compromised.

In conclusion, Oatp1a/1b uptake transporters determine the systemic exposure of rosuvastatin, without substantially affecting its liver exposure after bolus administration. Whether this is also true after chronic administration of rosuvastatin in patients remains to be seen. Our findings are clinically relevant for individuals with low-activity polymorphic variants of OATP1B1, and heterozygous carriers of the various full-deficiency of OATP1B1 and/or OATP1B3 (Pasanen et al., 2008; van de Steeg et al., 2012e). These individuals, when treated with rosuvastatin, might be at risk of developing myopathy, the major systemic side effect of statins. On the other hand, the therapeutic effect of rosuvastatin in the liver might be less affected.
Acknowledgments: We thank Marion Ludwig for technical assistance.

Authorship contribution:
Participated in research design: DI, KEK, AHS.
Conducted experiments: DI, MH, AvE, EW.
Contributed new reagents or analytic tools: MT, EW.
Performed data analysis: DI, MH.
Wrote or contributed to the writing of the manuscript: DI, MH, EvdS, KEK, AHS.
Reference List


MOL #81927


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Legends for figures:

**Figure 1.** Oatp1a/1b uptake transporters control systemic exposure of rosuvastatin, but not intestinal uptake after oral administration (15 mg/kg) to male wild-type and Slco1a/1b−/− mice. (A) Rosuvastatin portal vein blood concentrations. (B) Rosuvastatin systemic blood concentrations and semi-log plot of data (C). Rosuvastatin liver levels in (D) μg/g and (E) % of dose. (F) Liver-to-systemic blood ratios (semi-log plot). Averaged liver-to-systemic blood ratios were calculated from individual mouse data. Data are presented as mean ± SD (n = 5-6, *, P < 0.05; **, P < 0.01; ***, P < 0.001 when compared with wild-type).

**Figure 2.** Oatp1a/1b transporters control systemic exposure of rosuvastatin after intravenous administration (5 mg/kg) to male wild-type and Slco1a/1b−/− mice. (A) Rosuvastatin systemic blood concentrations and semi-log plot (B). Rosuvastatin liver levels in (C) μg/g and (D) as % of dose. (E) Liver-to-systemic blood concentrations (semi-log plot). Data are presented as mean ± SD (n = 3-5, *, P < 0.05; **, P < 0.01; ***, P < 0.001 when compared with wild-type).

**Figure 3.** Role of Oatp1a/1b in the intestinal disposition of rosuvastatin after intravenous administration (5 mg/kg) to male wild-type and Slco1a/1b−/− mice. Small intestinal tissue levels as (A) % of dose and (B) small intestinal tissue-to-systemic blood ratios (semi-log plot). (C) Small intestinal content levels as % of dose and (D) small intestinal content-to-systemic blood ratios (semi-log plot). Data are presented as mean ± SD (n = 3-5, *, P < 0.05; **, P < 0.01; ***, P < 0.001 when compared with wild-type).

**Figure 4.** Role of Oatp1a/1b in the kidney disposition of rosuvastatin after intravenous administration (5 mg/kg) to wild-type and Slco1a/1b−/− mice. Kidney levels in (A) μg/g and (B) % of dose. (C) Kidney-to-systemic blood ratios (semi-log plot). Data are presented as mean ± SD (n = 3-5, *, P < 0.05; **, P < 0.01; ***, P < 0.001 when compared with wild-type).

**Figure 5.** Role of Oatp1a/1b in the biliary excretion of rosuvastatin in gall bladder-cannulated mice after intravenous administration (5 mg/kg) to wild-type and Slco1a/1b−/− mice. (A) Rosuvastatin cumulative biliary excretion (in % of dose) and (B) rosuvastatin bile concentration (μg/mL). Data are presented as mean ± SD (n = 6-7, *, P < 0.05; **, P < 0.01; ***, P < 0.001 when compared with wild-type).

**Figure 6.** Role of Oatp1a/1b transporters in the urinary and fecal excretion of rosuvastatin in male mice. Rosuvastatin (% of dose) recovered in the urine, feces and urine plus feces combined after (A) 15 mg/kg oral and (B) 5 mg/kg intravenous administration of rosuvastatin. Data are presented as mean ± SD (n = 5, *, P < 0.05; **, P < 0.01; ***, P < 0.001 when compared with wild-type).

**Figure 7.** Role of Oatp1a/1b transporters in the urinary and fecal excretion of rosuvastatin after 15 mg/kg oral rosuvastatin administration. (A) Blood rosuvastatin concentrations (μg/mL) versus time (semi-log plot) and (B) rosuvastatin (% of dose) recovered in the urine, feces and urine plus feces combined, in female mice. (C) Comparison between rosuvastatin (% of dose) recovered in urine in male versus female wild-type and Oatp1a/1b knockout mice. Data are presented as mean ± SD (n = 5, *, P < 0.05; **, P < 0.01; ***, P < 0.001 when compared with wild-type).
Tables:

Table 1. Pharmacokinetic parameters after rosuvastatin administration to wild-type and Oatp1a/1b knockout mice.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Slco1a/1b-/—</th>
<th>Fold difference (KO / WT)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>15 mg/kg oral male</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood</td>
<td>AUC(5-240) (μg/mL·min)</td>
<td>11.3 ± 2.4</td>
<td>93 ± 11.7**</td>
</tr>
<tr>
<td></td>
<td>Systemic blood</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Portal vein</td>
<td>169.8 ± 17.9</td>
<td>330.9 ± 39.8*</td>
</tr>
<tr>
<td></td>
<td>Apparent hepatic extraction ratio</td>
<td>0.93</td>
<td>0.72</td>
</tr>
<tr>
<td><strong>5 mg/kg i.v male</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systemic blood</td>
<td>AUC(5-240) (μg/mL·min)</td>
<td>13.9 ± 2.5</td>
<td>43.2 ± 4.0**</td>
</tr>
</tbody>
</table>

Data presented as mean ± S.E.M. *, P < 0.05; **, P < 0.01 when compared with wild-type mice.
Table 2. Renal and non-renal clearance (calculated based on urinary and fecal output) after rosvastatin administration to wild-type and Oatp1a/1b knockout mice.

<table>
<thead>
<tr>
<th></th>
<th>Renal clearance (ml/min/kg)</th>
<th>Non-renal clearance (ml/min/kg)</th>
<th>Total clearance (ml/min/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>15 mg/kg oral</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT male</td>
<td>1.3 ± 0.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Slco1a/1b/- male</td>
<td>20.1 ± 9.0 **</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>WT female</td>
<td>20.1 ± 6.4 ##</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Slco1a/1b/- female</td>
<td>27.4 ± 4.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>5 mg/kg i.v.</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT male</td>
<td>1.2 ± 0.6</td>
<td>11.6 ± 1.3</td>
<td>12.8 ± 1.6</td>
</tr>
<tr>
<td>Slco1a/1b/- male</td>
<td>2.4 ± 0.3**</td>
<td>5.8 ± 0.5**</td>
<td>8.2 ± 0.3</td>
</tr>
</tbody>
</table>

Data presented as mean ± S.E.M. **, P < 0.01; ###, P < 0.001 when compared with wild-type mice of the same gender, ##, P < 0.01; ###, P < 0.001 when compared with male mice from the same genotype (wild-type or knockout). "-" : non-renal clearance could not be directly calculated for oral administration in the absence of reliable oral bioavailability data.
<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Slco1a/1b(^{-})</th>
<th>Fold difference (KO / WT)</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td><strong>15 mg/kg oral male</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systemic blood</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(c_{\text{max}}) ((\mu g/\text{mL}))</td>
<td>0.4</td>
<td>1.2</td>
<td>3.0</td>
</tr>
<tr>
<td>(t_{\text{max}}) (min)</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>(AUC_{\text{oral}}/AUC_{\text{i.v.}})</td>
<td>11.3</td>
<td>80.1</td>
<td>7.1</td>
</tr>
<tr>
<td>(Dose_{\text{i.v.}}/Dose_{\text{oral}})</td>
<td>0.011</td>
<td>0.058</td>
<td>5.2</td>
</tr>
<tr>
<td>Half-life (min)</td>
<td>89</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>Portal blood</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(AUC_{\text{oral}}) (min*(\mu g/\text{mL}))</td>
<td>174</td>
<td>296</td>
<td>1.7</td>
</tr>
<tr>
<td><strong>15 mg/kg oral female</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systemic blood</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(AUC_{\text{oral}}) (min*(\mu g/\text{mL}))</td>
<td>7.3</td>
<td>103.3</td>
<td>14.2</td>
</tr>
<tr>
<td>Half-life (min)</td>
<td>67.4</td>
<td>42.7</td>
<td>0.6</td>
</tr>
<tr>
<td><strong>5 mg/kg i.v male.</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systemic blood</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clearance (mL/min/kg)</td>
<td>20.3</td>
<td>11.8</td>
<td>0.58</td>
</tr>
<tr>
<td>Half-life (min)</td>
<td>26.2</td>
<td>49.9</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1

A. Portal vein blood rosuvastatin (μg/mL) over time for Wild-type (closed circles) and Slco1a1b/- (open circles) mice.

B. Systemic blood rosuvastatin (μg/mL) over time.

C. Systemic blood rosuvastatin (μg/mL) over time.

D. Liver rosuvastatin (μg/g) over time.

E. Liver rosuvastatin (% of dose) over time.

F. Ratio of liver to blood rosuvastatin over time.
Figure 2

A. Systemic blood rosvastatin (µg/mL) over time for Wild-type and Slco1a1b-/- mice.

B. Systemic blood rosvastatin (µg/mL) over time for LLQ.

C. Liver rosvastatin (µg/g) over time.

D. Liver rosvastatin (% of dose) over time.

E. Ratio of liver: blood rosvastatin over time.
Figure 3

A

Small intestinal tissue rosvastatin (% of dose)

B

Ratio small intestinal tissue: blood rosvastatin

C

Small intestinal content rosvastatin (% of dose)

D

Ratio small intestinal content: blood rosvastatin

Wild-type

Slco1a/1b-/-

0 15 30 60 120 200 240

Time (min)

0 15 30 60 120 200 240

Time (min)

0 15 30 60 120 200 240

Time (min)

0 15 30 60 120 200 240

Time (min)
Figure 4

A. Kidney rosvastatin (µg/g) for wild-type and Slco1a/1b-/- mice. 

B. Kidney rosvastatin (% of dose) over time for wild-type mice. 

C. Ratio of kidney to blood rosvastatin over time for wild-type and Slco1a/1b-/- mice.
Figure 5

A

Bile rosuvastatin (% of dose)

0  15  30  45  60

Time (min)

- Wild-type
- Slco1a/1b-/-

B

Bile rosuvastatin (µg/mL)

0  15  30  45  60

Time (min)

*
Figure 6

Panel A: The graph shows the distribution of Rpsuvastatin (% of dose) in urine, feces, urine + feces for male mice administered 5 mg/kg i.v. The graph includes bars for each 24-hour period.

Panel B: The graph demonstrates the distribution of Rpsuvastatin (% of dose) in urine, feces, urine + feces for male mice administered 15 mg/kg oral. The graph includes bars for each 24-hour period.
Figure 7

A

![Graph showing systemic blood rosvastatin (µg/mL) over time for wild-type and Slco1a1/1b-/- mice.](image)

B

![Bar graph showing rosvastatin (% of dose) in urine and feces for wild-type and Slco1a1/1b-/- mice, with female 15 mg/kg oral dose.](image)

C

![Bar graph showing rosvastatin urine (% of dose) for male and female wild-type and Slco1a1/1b-/- mice, with 15 mg/kg oral dose.](image)
Supplemental Figure 1. Role of Oatp1a/1b in the disposition of rosuvastatin in gall bladder-cannulated mice after intravenous administration (5 mg/kg) to wild-type and Slco1a/1b<sup>-/-</sup> mice. (A) Rosuvastatin blood concentrations (µg/mL). Liver, small intestinal wall (SI) and content (SIC) in (B) (µg/g) and (C) % of dose. (D) Liver, small intestinal wall, and small intestinal content to blood ratios. Data are presented as mean ± SD (n = 6-7, *, P < 0.05; **, P < 0.01; ***, P < 0.001 when compared with wild-type).
Supplemental Figure 2. Kidney Oatp1a expression in male and female wild-type mice. (A) ΔCt values of the RT-PCR analysis. Analysis of the results was done by the comparative Ct method. Quantification of the target cDNAs in all samples was normalized against the endogenous control Gapdh (Ct\text{target} - Ct\text{Gapdh} = ΔCt). Accordingly, the lower the ΔCt value, the higher the expression level. Note that ΔCt values between different genes/primer sets cannot be used to assess relative expression levels between different genes. (B) Fold difference in mRNA expression levels in male wild-type mice relative to values in female wild-type. Data are presented as mean ± S.D (n = 3) (*, \(P<0.05\); ***, \(P<0.001\) when compared with female wild-type values).