Utility of Oatp1a/1b-Knockout and OATP1B1/3-Humanized Mice in the Study of OATP-Mediated Pharmacokinetics and Tissue Distribution: Case Studies with Pravastatin, Atorvastatin, Simvastatin, and Carboxydichlorofluorescein

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ABSTRACT
Although organic anion transporting polypeptide (OATP)-mediated hepatic uptake is generally conserved between rodents and humans at a gross pharmacokinetic level, the presence of three major hepatic OATPs with broad overlap in substrate and inhibitor affinity, and absence of rodent-human orthologs preclude clinical translation of single-gene knockout/knockin findings. At present, changes in pharmacokinetics and tissue distribution of pravastatin, atorvastatin, simvastatin, and carboxydichlorofluorescein were studied in oatp1a/1b-knockout mice lacking the three major hepatic oatp isoforms, and in knockout mice with liver-specific knockin of human OATP1B1 or OATP1B3. Relative to wild-type controls, oatp1a/1b-knockout mice exhibited 1.6- to 19-fold increased intrahepatic and 2.1- to 119-fold increased oral drug exposure, due to 33%-75% decreased clearance, 14%-60% decreased volume of distribution, and ≤74-fold increased oral bioavailability, with the magnitude of change depending on the contribution of oatp1a/1b to pharmacokinetics. Hepatic drug distribution was 4.2- to 196-fold lower in oatp1a/1b-knockout mice; distributional attenuation was less notable in kidney, brain, cardiac, and skeletal muscle. Knockin of OATP1B1 or OATP1B3 partially restored control clearance, volume, and bioavailability values (24%-142% increase, ≤47% increase, and ≤77% decrease vs. knockout, respectively), such that knockin pharmacokinetic profiles were positioned between knockout and wild-type mice. Consistent with liver-specific humanization, only hepatic drug distribution was partially restored (1.3- to 6.5-fold increase vs. knockout). Exposure and liver distribution changes in OATP1B1-humanized versus knockout mice predicted the clinical impact of OATP1B1 on oral exposure and contribution to human hepatic uptake of statins within 1.7-fold, but only after correcting for human/humanized mouse liver relative protein expression factor (OATP1B1 = 2.2, OATP1B3 = 0.30).

SUPPLEMENTAL MATERIAL

Introduction
Organic anion transporting polypeptides (OATPs) are uptake transporters for a variety of organic amphiphiles of all charges, including small-molecule drugs (Niemi, 2007; Shitara et al., 2013). Although OATPs are expressed in all tissues relevant to drug disposition, including intestine, kidney, and brain, hepatic OATPs have by far the greatest impact on drug pharmacokinetics (Giacomini et al., 2010; Iusuf et al., 2012b). Clinical inhibition of hepatic OATPs can elicit high-magnitude drug interactions (>5-fold increase in systemic exposure), in contrast to other drug transporters, whose inhibition generally poses a low risk (≤2-fold increase in exposure) (Giacomini et al., 2010). Further highlighting the pharmacokinetic importance of OATPs, humans carrying the OATP1B1 521T polymorphism consistently exhibit increased exposure to substrate drugs with appreciable clearance via this mechanism (Giacomini et al., 2013).

Clinical drug interactions with hepatic OATPs are especially relevant to “statin” drugs due to the potential for altered efficacy and toxicity (Neuvonen et al., 2006). Inhibition of hepatic uptake can markedly impair drug distribution to the liver, the site of pharmacological activity, while elevating systemic exposure, which increases the potential for myotoxicity. OATP hepatic uptake influences pharmacokinetics of even the statins ultimately eliminated by metabolism and/or biliary excretion, because uptake is the rate-determining step in removal of these drugs from circulation (Elsby et al., 2012; Shitara et al., 2013).

Although human hepatic OATPs (1B1, 1B3, 2B1) have no direct rodent orthologs (major: 1a1, 1a4, 1b2; minor: 2b1), most substrates and inhibitors are nonspecific and interact with multiple isoforms in each species (van Montfoort et al., 2003; Iusuf et al., 2012b; Zamek-Gliszczynski et al., 2012b). Total OATP hepatic uptake is conserved between rodents and humans at a gross kinetic level, with generally good translation of overall apparent substrate or inhibitor affinity and collective impact of OATPs on pharmacokinetics (Badolo et al., 2010; Iusuf et al., 2012b).
Absence of direct rodent-human OATP orthologs precludes isoform-specific mechanistic extrapolation from rodents to humans (Iusuf et al., 2012b). Knockout models lacking a single murine oatp isoform and OATP-humanized wild-type mice, where a single human isoform is added to the full complement of rodent oatps, have virtually no predictive utility (Chen et al., 2008; Zaher et al., 2008; van de Steeg et al., 2009). Oatplb2-knockout mice exhibited increased systemic exposure to some drugs taken up into the liver by OATPs [rifamycin SV (3- to 6-fold), rifampin (≤2-fold)] but not other such drugs (cerivastatin, lovastatin, simvastatin) (Chen et al., 2008; Zaher et al., 2008). These hit-and-miss findings cannot be explained by compensatory changes in oatp1a1/1a4/2b1, which were not observed (Zaher et al., 2008). Instead, these results are kinetically consistent with hepatic uptake by multiple OATP isoforms (van Montfoort et al., 2003; Iusuf et al., 2012b; Zamek-Gliszczynski et al., 2013). When the fraction transported by the knockout-aided OATP is not sufficiently large (>50%), exposure will not be markedly (>2-fold) increased (Zamek-Gliszczynski et al., 2009, 2013). Oatplb2 mediates ~50% and 67%–83% of rifamipin and rifamycin SV systemic clearance, respectively; however, for the statins, Oatplb2 contribution was too low (<50%) to be detected within the variability of a mouse study (Zamek-Gliszczynski et al., 2009, 2013). Likewise, knockin of human OATP1B1 into wild-type mice elicited only a 17% decrease in oral methotrexate exposure (van de Steeg et al., 2009). Adding OATP1B1 to a liver already containing the full complement of murine oatps is expected to have little impact on drug pharmacokinetics. Not only are exposure changes in OATP-humanized wild-type mice small and difficult to discern, but clinical translation of these data is virtually impossible.

In contrast, oatp1a1/1b gene cluster knockout mice lack the three major liver isoforms and are essentially devoid of hepatic oatp function (van de Steeg et al., 2010). Although these mice still express oatp2b1, which is important in the intestine, the pharmacokinetic importance of rodent hepatic oatp2b1 is negligible (van de Steeg et al., 2010; Iusuf et al., 2012a,b; Shitara et al., 2013). These knockout mice are a powerful in vivo model to investigate if, and to what extent, collective OATP hepatic uptake impacts pharmacokinetics. Furthermore, the knockout mice are the appropriate background for liver-specific knockin of human OATPs due to the effective absence of competing murine hepatic oatp activity (van de Steeg et al., 2012). Thus, at present, systemic pharmacokinetics and tissue distribution of pravastatin, atorvastatin, simvastatin, and carboxyldichlorofluorescein were compared across wild-type, oatp1a1/1b-knockout, OATP1B1-humanized, and OATP1B3-humanized mice to determine the impact of hepatic OATPs on pharmacokinetics, as well as to explore the clinical translation of humanized mouse findings.

### Materials and Methods

#### Chemicals.
5-((and 6-carboxy-2′,7′-dichlorofluorescein was purchased from Sigma-Aldrich (St. Louis, MO). Pravastatin, atorvastatin, simvastatin lactone, and simvastatin acid were purchased from Toronto Research Chemicals (North York, Ontario, Canada). All other chemicals were of reagent grade and were readily available from commercial sources.

#### Animals.
Age-matched Oatplb1/1b cluster-knockout, OATP1B1- or OATP1B3-knockin mice humanized on the Oatp1a1/1b-knockout background, and wild-type FVB male mice were purchased from Taconic (Hudson, NY). Mice were between 8 and 10 weeks of age (22–34 g) at the time of study. Mice were delivered to Covance (Greenfield, IN), where they were acclimated for at least 3 days prior to study initiation. The Institutional Animal Care and Use Committee at Covance approved all animal procedures. In animal studies, carboxyldichlorofluorescein intravenous dose levels were selected based on body surface area scaling of rat doses (Zamek-Gliszczynski et al., 2012a). Statin dose levels were selected to reflect the high end of human exposure in wild-type mice [University of Washington Drug Interaction database; control human pharmacokinetic (PK) values]. Pravastatin exposures were comparable to the high end of human values; atorvastatin and simvastatin exposures were an order of magnitude higher in wild-type mice. The higher murine atorvastatin and simvastatin concentrations are not expected to affect translation of PK findings based on accurate (within 1.5- to 1.7-fold) clinical translation of these data with respect to OATP1B1 (see Discussion).

#### Human Liver Procurement.
The human liver tissue samples for transporter protein quantification were sourced from the Eli Lilly and Company liver bank, which is composed of ethically sourced livers obtained under protocols approved by the appropriate committees for the conduct of human research at the Medical College of Wisconsin (Milwaukee, WI), the Medical College of Virginia (Richmond, VA), Indiana University School of Medicine (Indianapolis, IN) and University of Pittsburgh (Pittsburgh, PA). The panel of livers analyzed contained five healthy male donors ranging in age from 18 to 45 years, and three healthy female donors ranging in age from 35 to 77 years.

#### Carboxyldichlorofluorescein i.v. PK and Liver Distribution.
Carboxyldichlorofluorescein is a preclinical OATP/multidrug resistance-associated protein (MRP) probe, which is metabolically stable and cleared approximately equally by biliary urinary excretion; hepatopatibiliary disposition is transporter-mediated with OATP uptake, MRP3 sinusoidal, and MRP2 canalicular excretion (Zamek-Gliszczynski et al., 2003, 2012a). At present, carboxyldichlorofluorescein intravenous pharmacokinetics were studied to demonstrate expected alterations with a molecule that exhibits simple pharmacokinetic properties. Blood concentration-time course of carboxyldichlorofluorescein was determined in mice over 6 hours following tail vein injection (10 mg/kg; 5 ml/kg; 20% Captisol; Ligand La Jolla, CA) in 25 mM phosphate buffer, pH = 8). Blood spots were collected onto dried blood spot (DBS) cards (226 Bioanalytic Card; PerkinElmer, Greenville, SC) via tail bleeds at 0.08, 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, and 6 hours postdose, and livers were excised and frozen at 6 hours.

#### Pravastatin and Atorvastatin PK and Tissue Distribution.
Blood concentration-time courses of the two drugs were determined in two separate intravenous/oral crossover studies with a 3-day washout period between drug administrations. Pravastatin was administered on day 0 by tail vein injection (10 mg/kg; 2 ml/kg of 20% Captisol in 25 mM phosphate buffer, pH = 8) and on day 3 by oral gavage (100 mg/kg; 10 ml/kg of 1% hydroxyethylcellulose, 0.25% polysorbate-80, 0.05% antifoam in water). In a separate study, atorvastatin was administered on day 0 by tail vein injection (10 mg/kg; 5 ml/kg of 20% Captisol, 15% ethanol in 25 mM phosphate buffer, pH = 8) and on day 3 by oral gavage (300 mg/kg; 10 ml/kg of 1% hydroxyethylcellulose, 0.25% polysorbate-80, 0.05% antifoam in water). Blood spots were collected via tail bleeds at 0.08, 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, and 6 hours postdose; 6 hours post–oral drug administration, livers, right kidneys, brains, hearts, and right quadriceps were collected and frozen.

#### Simvastatin Composite PK and Tissue Distribution.
Simvastatin PK was determined differently from the other analytes to describe PK of both the lactone and acid forms, which requires plasma sampling with immediate acidification (Yang et al., 2005). Composite plasma concentration-time course of simvastatin lactone and acid was determined over 6 hours. Simvastatin lactone was administered by tail vein injection (10 mg/kg; 1 ml/kg of 25% dimethylacetamide, 15% ethanol, 1% propylene glycol, 25% 2-pyrrolidone, 25% water) or by oral gavage (100 mg/kg; 10 ml/kg of 1% hydroxyethylcellulose, 0.25% polysorbate-80, 0.05% antifoam in water). Plasma samples were collected at 0.08, 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, and 6 hours postdose from two groups of mice, which were sampled at alternating time points, with the first four samples collected via retro-orbital bleeds and the final blood draw by cardiac puncture. All plasma samples were immediately acidified by addition of equal volume of 21% phosphoric acid to maintain pH ~4.5 to prevent ex vivo simvastatin lactone hydrolysis to the acid (Yang et al., 2005). Livers, right kidneys, brains, hearts, and right quadriceps were collected and frozen at the time of final blood sampling (5 or 6 hours post–oral drug administration).

#### Bioanalysis.
Carboxyldichlorofluorescein, pravastatin, atorvastatin, and simvastatin lactone and acid concentrations in relevant matrices [blood spots (3-mm punch), acidified plasma, and tissue homogenates] were quantified by liquid chromatography–tandem mass spectrometry (LC-MS/MS). All samples were mixed with an organic internal standard solution to precipitate protein, centrifuged, and the resulting supernatants were directly analyzed. Analytes were separated using reverse-phase chromatography with gradient elution and detected in negative or positive ion mode using selected reaction monitoring (Sciex API 4000 triple quadrupole mass spectrometer equipped with a TurboIonSpray interface; Applied Biosystems/MDS, Foster City, CA): carboxyldichlorofluorescein, [M-H] m/z...
samples (approximately 100 mg), prepared in duplicate, were homogenized in cold hypotonic buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 0.15 mM MgCl2) with 1.5 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (Sigma) with 2 ml of buffer per 100 mg of tissue, then allowed to swell for 30 minutes on ice. The samples were then homogenized again and centrifuged at 10,000 × g for 10 minutes at 4°C. The supernatant was transferred to a tube and centrifuged at 100,000 × g for 60 minutes at 4°C. The supernatant was discarded, then the pellet was suspended in 0.2 ml phosphate-buffered saline and stored at −80°C until later measurement for protein content and analysis for OATP expression by targeted quantitative proteomics with LC-MS/MS.

OATPs 1B1 and 1B3 were quantified in the hepatocyte membrane fractions by modification of a previously published targeted quantitative proteomic method (Fallon et al., 2013) and with guidance from similar methods published by others (Ohitsu et al., 2011; Balogh et al., 2012; Ji et al., 2012). Methods development followed procedures previously outlined by Picotti et al. (2010). Stable isotope-labeled proteotypic peptides, purchased from JPT (Acton, MA), were used as standards in each sample. Briefly, the membrane samples (50 μg of total membrane protein) were solubilized in 1% sodium deoxycholate, denatured with heat, reduced with dithiothreitol, and carbamidomethylated with iodoacetamide. Stable isotope-labeled peptides were added, and samples were digested overnight with trypsin (20:1 protein-to-trypsin ratio), the optimum digestion time having been determined during assay qualification. A liver membrane sample previously prepared and analyzed in our laboratory was used as a control between batches, all samples for the study being analyzed in two batches. Following digestion, the reaction was stopped by addition of 10% trifluoroacetic acid, the volume added being 10% of the total reaction volume. Following centrifugation at 10,000 rpm for 5 minutes to remove most deoxycholate, the supernatant was treated with solid-phase extraction. The eluate was evaporated to dryness under vacuum and reconstituted in 50 μl of modified mobile phase A (water/acetonic acid/formic acid 98/2/0.1; i.e., 2% acetonic acid) for analysis by nano UPLC-MS/MS (selected/multiple reaction monitoring mode). Injection volume was 2 μl (4% of the sample). The analytical instrumentation and chromatographic conditions used were as previously described (nanoAcquity (Waters, Milford, MA), BEH130 C18 column (Waters), AB Sciex QTRAP 5500 (AB Sciex, Framingham, MA) (Fallon et al., 2013)). The stable isotope-labeled peptides and multiple reaction monitoring (MRM) transitions used in the final analyses are shown in Supplemental Table 1.

Data Analysis. Noncompartmental pharmacokinetic parameters were calculated using Watson version 7.4 (Thermo Scientific, Waltham, MA). The tissue distribution data were analyzed using a one-way analysis of variance (ANOVA) model on the log10 transformed responses to test for differences among the mouse groups by compound and tissue. The PK parameters for pravastatin and atorvastatin were analyzed using a one-way ANOVA model; simvastatin PK parameters are point estimates based on composite PK curves, and are not amenable to significance testing. For PK parameters that are best compared as a difference [ex. bioavailability (F)], time of maximum concentration (Tmax), clearance (CL)], no data transformation was performed. For PK parameters that are compared as a ratio [ex. area under the curve (AUC), Cmax)], the response data were transformed using the log10 transformation prior to analysis. The PK parameters for carboxydichlorofluorescein were analyzed with a mixed-effect ANOVA model to combine the data from the two studies. Study was treated as a random effect and mouse group was treated as a fixed effect. All post-hoc t tests for prespecified contrasts of mouse groups were conducted using Fisher’s least significant difference method. In those instances where the overall model was not statistically significant, the Bonferroni adjustment was used. Comparisons of OATPB1 and OATPB3 protein levels between humanized mouse and human livers were conducted using an unequal variance t test.

Data are reported as the mean ± S.D. In all cases, P < 0.05 was considered significant.

Results

Carboxydichlorofluorescein intravenous pharmacokinetics are summarized in Fig. 1 and Table 1. Systemic exposure was, on average, 2.6-fold higher in oatpl1α/1b-knockout mice as a result of 43% decreased clearance and 55% lower volume. Knockin of OATPIB3 decreased exposure to levels comparable with wild type (2.6-fold decrease relative to knockouts), but the decrease was modest in OATPIB1-knockin mice (1.6-fold decrease vs. knockouts). Knockin of OATPIB3 increased clearance and volume 41% and 24% relative to knockout mouse, respectively, whereas OATPIB1 knockin did not markedly increase these parameters.

Carboxydichlorofluorescein biliary excretion accounts for approximately half of systemic clearance (Zamek-Gliszczynski et al., 2012a), thus reduced clearance and volume in oatpl1α/1b-knockout mice are consistent with impaired hepatic uptake. Indeed, liver distribution was 37-fold decreased in oatpl1α/1b-knockout mice (Fig. 1C), and was partially restituted by OATPIB3 knockin (6.5-fold increase vs. knockout), but was only modestly increased in OATPIB1-knockin mice (2.4-fold higher than knockout).

Pravastatin pharmacokinetics are summarized in Fig. 2 and Table 1. In oatpl1α/1b-knockout mice, clearance was decreased 33% and volume was 21% lower. Conceptually consistent with both decreased clearance and volume, pravastatin systemic exposure following intravenous administration was, on average, 4.3-fold higher in oatpl1α/1b-knockout mice. OATPB1 knockin decreased intravenous exposure to levels comparable with wild type (4.6-fold decrease relative to knockouts), whereas knockin of OATPIB3 decreased exposure only slightly (15% decrease vs. knockouts). Knockin of OATPB1 increased clearance and volume 24% and 8% relative to knockout mice, respectively, but OATPB3 knockin did not similarly increase these parameters. Following oral pravastatin administration, exposure and Cmax were 115- and 213-fold increased in oatpl1α/1b-knockout mice, a change driven primarily by 74-fold increased oral bioavailability, and secondly by the relatively minor decreases in clearance and volume. Time of Cmax (Tmax) was shifted 3.1-fold earlier in the knockouts. OATPB1 and OATPB3 knockin modestly decreased oral drug exposure relative to the knockout background (AUC 45%–58%, Cmax 67%–88%), as a result of 10%–48% decreased bioavailability, in addition to the decreases in clearance and volume.

Atorvastatin pharmacokinetics are summarized in Fig. 3 and Table 1. Intravenous drug exposure was, on average, 19-fold increased in oatpl1α/1b-knockout mice, due to 4.0-fold reduced clearance and 2.5-fold reduced volume of distribution. In OATPB1- and OATPB1-humanized mice, intravenous drug exposure was reduced 57- and 12-fold relative to the knockout background, respectively, as a result of both 2.2- to 2.4-fold increased clearance and 25%–47% higher volume. Oral drug exposure was increased 2.9-fold in oatpl1α/1b-knockout mice, but was partially attenuated by knockin of OATPB1 (28%) or OATPB3 (33%). Likewise, Cmax was 4.1-fold higher in knockout mice, but was decreased 23%–27% in the humanized mice. Overall, oral bioavailability was comparable across mouse groups.

Simvastatin lactone composite pharmacokinetics are summarized in Fig. 4 and Table 1. Intravenous exposure was, on average, increased 1.6-fold in oatpl1α/1b-knockout mice as a result of both 39% reduction in clearance and 14% reduction in volume of distribution. In OATPB3-knockin mice, exposure was reduced 27% relative to knockouts, due to 37% increase in clearance but with no change in volume. Intravenous exposure was not decreased by knockin of OATPB1, and decreases in clearance or volume were not observed. Oral exposure was increased 2.1-fold in oatpl1α/1b-knockout mice, consistent with 31% increased bioavailability, as well as reduced clearance and volume. Relative to the
knockout background, OATP1B1 and OATP1B3 knockin decreased oral exposure 2.9- and 1.9-fold due to 3.4- and 1.4-fold decreased bioavailability, respectively, as well as increased clearance in OATP1B3-knockin mice. Simvastatin acid composite pharmacokinetics following intravenous and oral administration of simvastatin lactone are summarized in Fig. 5 and Table 1. Intravenous exposure was, on average, 1.8-fold increased in oatp1a/1b-knockout mice. Although clearance of the acid was not measured, this finding is consistent with 42% reduced total (acid + lactone) simvastatin clearance. Knockin of OATP1B3 reduced exposure 29% relative to the knockout background, and increased total simvastatin clearance 38%. Knockin of OATP1B1 did not reduce simvastatin acid exposure or increase total simvastatin clearance. Simvastatin acid oral exposure was 3.2-fold greater in oatp1a/1b-knockout animals. Although bioavailability of the acid was not measured, this finding is consistent with 52% increased total simvastatin clearance. Knockin of OATP1B1 reduced oral exposure and total simvastatin oral bioavailability 4.3-fold; however, knockin of OATP1B3 did not reduce either parameter.

Statin tissue distribution is summarized in Fig. 6. In oatp1a/1b-knockout mice, liver tissue-to-blood/plasma concentration ratio (Kp) of pravastatin, atorvastatin, and total simvastatin was on average decreased 196, 6.8, and 4.2 fold, respectively. Knockin of OATP1B1 partially restored hepatic distribution (2.5-, 1.2-, and 1.2-fold increase vs. knockout), as did knockin of OATP1B3 (5.7-, 1.3-, and 1.6-fold increase vs. knockout) for pravastatin, atorvastatin, and total simvastatin, respectively. Atorvastatin and simvastatin renal tissue distribution was decreased 1.4- to 2.9-fold in knockout and knockin mice relative to wild type. In contrast, pravastatin kidney distribution was surprisingly increased 76-fold in oatp1a/1b-knockout mice, an effect which was 5.2- and 13-fold attenuated by knockin of OATP1B1 and OATP1B3, respectively. Brain distribution was decreased in all knockout and knockin mice: 6.3- to 11-fold, 1.3- to 1.4-fold, and 3.0- to 4.2-fold for pravastatin, atorvastatin, and simvastatin, respectively. Cardiac and skeletal muscle distribution of pravastatin was inconsistently attenuated (<2.9-fold decreased), whereas atorvastatin and simvastatin distribution was 1.5- to 2.6-fold and 1.8- to 2.9-fold decreased in knockout and knockin mice.

Hepatic OATP1B1 and OATP1B3 protein levels are presented in Fig. 7 (expression of all quantified transporters is summarized in Supplemental Table 2). As expected, human OATP protein was not knocked out in control mice, and OATP1B1 and OATP1B3 knockin decreased oral exposure 2.9- and 1.9-fold due to 3.4- and 1.4-fold decreased bioavailability, respectively, as well as increased clearance in OATP1B3-knockin mice. Simvastatin acid composite pharmacokinetics following intravenous and oral administration of simvastatin lactone are summarized in Fig. 5 and Table 1. Intravenous exposure was, on average, 1.8-fold increased in oatp1a/1b-knockout mice. Although clearance of the acid was not measured, this finding is consistent with 42% reduced total (acid + lactone) simvastatin clearance. Knockin of OATP1B3 reduced exposure 29% relative to the knockout background, and increased total simvastatin clearance 38%. Knockin of OATP1B1 did not reduce simvastatin acid exposure or increase total simvastatin clearance. Simvastatin acid oral exposure was 3.2-fold greater in oatp1a/1b-knockout animals. Although bioavailability of the acid was not measured, this finding is consistent with 52% increased total simvastatin clearance. Knockin of OATP1B1 reduced oral exposure and total simvastatin oral bioavailability 4.3-fold; however, knockin of OATP1B3 did not reduce either parameter.

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detected in wild-type mice, nor in oatp1a/1b-knockout mice. In OATP1B1-humanized mice, hepatic expression of OATP1B1 was, on average, 55% lower than in human, whereas OATP1B3 protein was not detected. In contrast, OATP1B3 hepatic protein expression was, on average, 3.3-fold higher in OATP1B3-humanized mice than in human livers. As such, the relative abundance of OATP1B1 versus OATP1B3 in these humanized mice (4.4-fold higher OATP1B3) is essentially reversed relative to the human liver, where OATP1B1 expression is higher: 1.7-fold in the present study and, on average, 2.7-fold in human liver, impaired distribution to extrahepatic tissues was not restored—loss of endogenous murine oatp function. It merits noting that, unlike OATP1B1 or 1B3 partially restored hepatic drug distribution by up to three human OATPs does not fully compensate for nearly complete loss of endogenous murine oatp function in wild-type mice. As such, knockin pharmacokinetic profiles were positioned between knockout and wild-type mice.

## Discussion

The present study evaluated changes in pharmacokinetics and tissue distribution of pravastatin, atorvastatin, simvastatin (lactone and acid forms), and carboxydichlorofluorescein in oatp1a/1b−/− mice, which effectively lack hepatic oatp function (van de Steeg et al., 2010), as well as oatp1a/1b-knockout mice with liver-specific knockin of human OATP1B1 or 1B3 (van de Steeg et al., 2012). Relative to wild-type mice, systemic exposure of five different OATP substrates was markedly increased in knockout mice due to reduced clearance, volume, and/or increased bioavailability. Liver-specific knockin of human OATP1B1 or 1B3 decreased drug exposure, but not to the levels observed in wild-type mice. As such, knockin pharmacokinetic profiles were conceptually consistent with knockout of all three major murine oatps but replacement with only one of three human OATPs (Iusuf et al., 2012b).

Since the three predominant hepatic marine oatps are absent in oatp1a/1b gene cluster knockout mice, liver distribution of substrate drugs is expected to be strikingly impaired (van de Steeg et al., 2010). In fact, liver distribution was 1–2 orders of magnitude decreased across the tested OATP substrates. Liver-specific knockin of human OATP1B1 or 1B3 partially restored hepatic drug distribution by up to 1 order of magnitude. This incomplete restoration of liver distribution in the humanized mice again emphasizes that knockin of only one of three human OATPs does not fully compensate for nearly complete loss of endogenous murine oatp function. It merits noting that, unlike the liver, impaired distribution to extrahepatic tissues was not restored.

### TABLE 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild Type</th>
<th>oatp1a/1b KO</th>
<th>OATP1B1 KI</th>
<th>OATP1B3 KI</th>
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<tr>
<td>AUC (μg/h/ml)*</td>
<td>22.2 ± 17.6</td>
<td>57.0 ± 64.0*</td>
<td>35.8 ± 24.7</td>
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<td>CL_{blood} (ml/min/kg)</td>
<td>10.1 ± 4.0</td>
<td>5.8 ± 5.6*</td>
<td>5.8 ± 2.0*</td>
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<td>V_{SS} (ml/kg)</td>
<td>583 ± 419</td>
<td>264 ± 144</td>
<td>237 ± 97</td>
<td>327 ± 78</td>
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<td>Pravastatin (10 mg/kg i.v.; mean ± S.D., n = 4)</td>
<td>13.7 ± 9.6</td>
<td>58.8 ± 92.8</td>
<td>12.8 ± 1.8</td>
<td>50.2 ± 56.5</td>
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<td>AUC (μg/h/ml)</td>
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<td>24.1 ± 4.8*</td>
<td>16.6 ± 1.4*</td>
<td>15.3 ± 2.7*</td>
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<td>C_{max} (ng/ml)</td>
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<td>10,300 ± 6170*</td>
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<td>T_{max} (h)</td>
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<td>0.6 ± 0.3</td>
<td>0.6 ± 0.3</td>
<td>6.3 ± 0.3</td>
</tr>
<tr>
<td>F (%)</td>
<td>0.2 ± 0.1</td>
<td>14.7 ± 10.9*</td>
<td>13.2 ± 2.5*</td>
<td>7.6 ± 6.1*</td>
</tr>
<tr>
<td>Atorvastatin (10 mg/kg p.o.; mean ± S.D., n = 6)</td>
<td>5.2 ± 3.4</td>
<td>89.1 ± 161.1*</td>
<td>16.7 ± 28.5</td>
<td>7.9 ± 4.2</td>
</tr>
<tr>
<td>AUC (μg/h/ml)</td>
<td>49.9 ± 8.2</td>
<td>14.4 ± 6.4*</td>
<td>10.4 ± 4.4*</td>
<td>96.4 ± 5.9*</td>
</tr>
<tr>
<td>C_{max} (ng/ml)</td>
<td>2160 ± 3110</td>
<td>8778 ± 4763*</td>
<td>6762 ± 3140*</td>
<td>6407 ± 3973*</td>
</tr>
<tr>
<td>T_{max} (h)</td>
<td>3.0 ± 2.3</td>
<td>0.6 ± 0.3*</td>
<td>0.8 ± 0.3*</td>
<td>1.5 ± 2.2</td>
</tr>
<tr>
<td>F (%)</td>
<td>3.2 ± 3.4</td>
<td>2.1 ± 2.0</td>
<td>6.8 ± 3.4</td>
<td>4.3 ± 3.3</td>
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<tr>
<td>Simvastatin (10 mg/kg i.v.; composite PK mean, n = 5–6 mice per time point)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactone AUC (μg/h/ml)</td>
<td>2.04</td>
<td>3.32</td>
<td>3.86</td>
<td>2.43</td>
</tr>
<tr>
<td>Acid AUC (μg/h/ml)</td>
<td>1.55</td>
<td>2.87</td>
<td>3.91</td>
<td>2.04</td>
</tr>
<tr>
<td>Lactone CL (ml/min/kg)</td>
<td>81.7</td>
<td>50.2</td>
<td>43.2</td>
<td>68.6</td>
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<tr>
<td>Total CL (ml/min/kg)</td>
<td>47.3</td>
<td>27.5</td>
<td>21.9</td>
<td>38.0</td>
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<tr>
<td>V_{SS} (ml/kg)</td>
<td>13,900</td>
<td>11,900</td>
<td>12,500</td>
<td>12,500</td>
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<tr>
<td>Simvastatin (10 mg/kg p.o.; composite PK mean, n = 5–6 mice per time point)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactone AUC (μg/h/ml)</td>
<td>3.87</td>
<td>8.24</td>
<td>2.85</td>
<td>4.32</td>
</tr>
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<td>Acid AUC (μg/h/ml)</td>
<td>3.17</td>
<td>10.3</td>
<td>2.58</td>
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<tr>
<td>Lactone C_{max} (ng/ml)</td>
<td>1640</td>
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<td>517</td>
<td>767</td>
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<tr>
<td>Acid C_{max} (ng/ml)</td>
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<td>679</td>
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<td>Lactone T_{max} (h)</td>
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<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Acid T_{max} (h)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Lactone F (%)</td>
<td>19.0</td>
<td>24.8</td>
<td>7.4</td>
<td>17.8</td>
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<tr>
<td>Total F (%)</td>
<td>19.6</td>
<td>29.8</td>
<td>7.0</td>
<td>34.8</td>
</tr>
</tbody>
</table>

*CL, clearance; CL_{blood}, blood clearance; F, bioavailability; T_{max}, time of C_{max}; V_{SS}, volume of distribution.

*P < 0.05, knockout or knockin versus wild-type mice.
in humanized mice, functionally confirming that the knockin is liver-specific (van de Steeg et al., 2012).

Despite qualitative consistency in pharmacokinetic alterations in oatp1a/1b-knockout mice, the magnitude of change differed between the five analytes studied, because the disposition of these molecules is dependent on hepatic OATPs to a varying extent (Zamek-Gliszczynski et al., 2003, 2012a; Elsby et al., 2012; Shitara et al., 2013). Specifically, the relatively high magnitude of change in oral PK and liver distribution of pravastatin in oatp1a/1b-knockout mice resulted in a wide range of quantitative PK alterations. Pravastatin (logD<sub>7.4</sub> = 2.1) is considerably more hydrophilic (lower passive permeability) than simvastatin and atorvastatin (logD<sub>7.4</sub> =+ 1.0–1.8), thus the impact of hepatic oatp knockout on pravastatin is more pronounced. Furthermore, pravastatin exhibited the lowest oral bioavailability in wild-type mice, resulting in the greatest margin for a large-magnitude increase in oral exposure for this drug. Unlike simvastatin and atorvastatin, where intestinal metabolism is a limiting factor in oral exposure (Gertz et al., 2011) that is not subject to change by genetic OATP modification, pravastatin is not metabolized in the intestine (Varma et al., 2012). As such, pravastatin was more sensitive to the absence of hepatic OATP function than the other compounds studied.

To date, a total of five studies of OATP substrate disposition in these knockout and transgenic models have been published, which are conceptually consistent with the current findings of altered systemic pharmacokinetics and hepatic drug distribution (van de Steeg et al., 2010, 2011, 2012; Iusuf et al., 2012a, 2013). Specifically, pravastatin, rosuvastatin, and fexofenadine were studied in oatp1a/1b-knockout mice, whereas paclitaxel and methotrexate disposition was examined across both knockout and OATP-humanized mice. Previously reported alterations in pravastatin pharmacokinetics in oatp1a/1b<sup>2/-</sup> mice are quantitatively in good agreement with the present findings: intravenous exposure was increased 4-fold, oral exposure 30-fold, and liver distribution was 10- to 100-fold decreased (Iusuf et al., 2012a); in the present study, these changes were 4-, 115-, and 196-fold, respectively. Rosuvastatin intravenous exposure was 3-fold increased, oral exposure 8-fold increased, and liver distribution was decreased 1 order of magnitude (Iusuf et al., 2013); similarly, fexofenadine intravenous exposure was 3-fold increased and hepatic distribution 10-fold decreased (van de Steeg et al., 2010). Methotrexate intravenous exposure was 3- to 5-fold increased and hepatic distribution was 69- to 131-fold decreased (van de Steeg et al., 2010, 2011, 2013); knockin of individual human OATP isoforms partially restored methotrexate exposure (~2-fold decrease) and increased liver distribution 6- to 15-fold (van de Steeg et al., 2013). Paclitaxel intravenous exposure was ~2-fold increased, whereas liver distribution was 2- to 4-fold decreased (van de Steeg et al., 2011, 2013); knockin of human OATPs resulted in low and inconsistent restoration of pharmacokinetics and liver distribution, due to the relatively low overall impact of OATP on paclitaxel disposition (van de Steeg et al., 2013).
Notably, murine oatps from the 1a and 1b subfamilies are also expressed in other organs relevant to drug disposition, and therefore, attenuated statin distribution was expected in these tissues (Iusuf et al., 2012b). Evidence exists for expression of the oatp1a subfamily in the renal proximal tubule (Iusuf et al., 2012b), and this was supported at a functional level in the present study by the modest decreases (1.4- to 2.9-fold) in atorvastatin and simvastatin kidney distribution. Quantitatively, these findings are consistent with the previously reported 3-fold decrease in rosuvastatin and 2-fold decrease in fexofenadine kidney distribution in oatp1a/1b-knockout mice (van de Steeg et al., 2010; Iusuf et al., 2013). Surprisingly, pravastatin renal tissue distribution was markedly increased 2 orders of magnitude in oatp1a/1b-knockout mice, and this increase was partially attenuated in humanized mice. Pravastatin clearance is partially renal (Singhvi et al., 1990); therefore, one possible explanation for this unexpected increase is that the kidneys were compensating for the effective absence of hepatic clearance in knockout mice. This hypothesis is supported by attenuation of the increase in knockin mice with partially restored hepatic clearance. Oatp1a4 is an important isoform in the murine blood-brain barrier, where it contributes to brain uptake of exogenous and endogenous substrates (Kalvass et al., 2013). Previously, initial uptake of four different OATP-substrate drugs was shown to be 1.3- to 3.8-fold impaired in oatp1a4-knockout mice during 1-minute brain perfusions (Ose et al., 2010), whereas fexofenadine and methotrexate in vivo brain distributions were 2-fold decreased (van de Steeg et al., 2010). In the present study, brain distribution of atorvastatin and simvastatin was impaired to an extent comparable with previous observations, but the magnitude of impairment in pravastatin central nervous system distribution (6- to 11-fold) was notably larger. The extent to which pravastatin appears to be taken up into the brain by OATP is greater than previously reported for a drug, and nearly approached the 19-fold enhancement in taurocholate brain uptake by Oatp1a4, the largest-magnitude functional example to date (Ose et al., 2010). The observed increase in pravastatin brain distribution does not contradict the findings of Ose et al. (2010), because 1) Ose et al. measured initial uptake in brain perfusions, where the magnitude of change is lower (Kalvass et al., 2013), and 2) Ose et al. used oatp1a4-knockout as opposed to oatp1a/1b gene cluster knockout mice, in which, for example, oatp1a5 is also absent. Finally, pravastatin is far more hydrophilic than simvastatin and atorvastatin, so its lower passive central nervous system distribution makes the oatp1a/1b-knockout effect more apparent.

Cardiac and skeletal muscle express OATPs (ex. rodent Oatp1a4 and human OATP2B1) capable of transporting statin drugs in vitro (Grube et al., 2006; Sakamoto et al., 2008). Statin distribution to the heart and quadriceps was up to 2.9-fold decreased in mice lacking...
OATP1a/1b subfamilies. This trend of impaired statin muscle distribution was observed in both oatp1a/1b-knockout and OATP1B1- or OATP1B3-knockin mice, in which the humanization is liver-specific, such that these transgenic mice exhibit the oatp1a/1b phenotype in extrahepatic tissues (van de Steeg et al., 2012, 2013). The present study provides the first in vivo evidence of statin uptake into muscle by the Oatp1a/1b subfamilies. These data suggest that OATP uptake into muscle may play a role in statin myotoxicity (Neuvonen et al., 2006).

Oatp1a/1b-knockout mice are useful in qualitatively demonstrating the impact of OATPs on pharmacokinetics. The ability to quickly determine whether hepatic OATP uptake affects in vivo drug disposition is a major advancement in its own right. However, can these murine knockout and humanized transgenic models be used for more quantitative clinical predictions? Although it is premature to claim that a preclinical-to-clinical correlation has been established based on three statins, a quantitative translational approach is proposed later and appears to be in good agreement with clinical data.

Shitara et al. (2013) estimated the fractional contribution of OATP1B1 to human hepatic uptake to be 0.83 for pravastatin and 0.47 for atorvastatin based on pharmacokinetic changes in human carriers of OATP1B1 521T>C polymorphism(s); estimates for simvastatin are not available. Using the current preclinical data, the fractional contribution of human OATP1B1 to hepatic uptake can be estimated from the following relationship (Hirano et al., 2004; Zamek-Gliszczynski et al., 2009, 2013):

\[
\text{OATP1B1 Hepatic Uptake Fraction} = \frac{1}{1 + \left[\frac{\text{OATP1B1 KI Liver } Kp}{\text{oatp1a/1b KO Liver } Kp} - 1\right] \times \text{REF}} + 1
\]

where OATP1B1 relative expression factor (REF) is 2.2, and the knockin (KI)/knockout (KO) liver Kp ratio is 2.5 for pravastatin and 1.2 for atorvastatin. The fraction of hepatic uptake mediated by human OATP1B1 is thus estimated to be 0.77 for pravastatin and 0.31 for atorvastatin, which is within 1.5-fold of estimates based on human pharmacokinetics (Shitara et al., 2013).

Elsby et al. (2012) estimated the maximal increase in statin oral exposure in the theoretical scenario of complete OATP1B1 inhibition in humans. Based on the projected maximal increases in exposure to pravastatin, atorvastatin, and simvastatin of 2.0-, 3.2-, and 4.8-fold (Elsby et al., 2012), the fractional contributions of OATP1B1 to overall clearance are 0.50, 0.69, and 0.79, respectively (Zamek-Gliszczynski et al., 2009). Using the current preclinical data, the

![Fig. 4](image_url) Simvastatin lactone composite concentration-time profiles following administration of a 10 mg/kg intravenous bolus dose (A and B) or a 100 mg/kg oral dose (C and D) to wild-type (closed circles), oatp1a/1b-knockout (open circles), OATP1B1-knockin (red triangles), or OATP1B3-knockin (red squares) mice. Comparison of oatp1a/1b-knockout to wild-type mice is presented in (A) and (C). OATP1B1- and OATP1B3-knockin mice are displayed in red relative to mean pharmacokinetic profiles for wild-type (black solid line) and oatp1a/1b-knockout (black dashed line) mice in (B) and (D). Mean + S.D., n = 5–6 mice per time point.
fractional contribution of human OATP1B1 to oral systemic drug clearance can be estimated from the following relationship (Hirano et al., 2004; Zamek-Gliszczynski et al., 2009, 2013):

\[
OATP1B1 \text{ Oral Clearance Fraction} = 1 - \left( \frac{\text{OATP1B1 KI AUCpo}}{\text{OATP1B1 REF AUCpo}} - 1 \right) \times \text{REF} + 1
\]

where OATP1B1 REF is 2.2, and the KO/KI oral exposure (AUCpo) ratios are 1.45, 1.39, and 3.6 for pravastatin, atorvastatin, and simvastatin, respectively. Using these data, the fractional contributions of human OATP1B1 to oral systemic drug clearance are estimated to be 0.50, 0.46, and 0.85 for pravastatin, atorvastatin, and simvastatin, respectively, which are within 1.5-fold of human values (Elsby et al., 2012).

The proposed translational approach is only applicable to OATP hepatic uptake and its influences on systemic drug pharmacokinetics. The present study does not provide mechanistic information on translation of downstream hepatic clearance of drugs. However, the observed changes in systemic pharmacokinetics and liver distribution are consistent with OATP hepatic uptake being the rate-determining step in the removal of these drugs from circulation in mice as in humans at a gross kinetic level (Elsby et al., 2012; Shitara et al., 2013).

Furthermore, accurate estimation (within 1.5-fold) of the fraction of hepatic uptake mediated by human OATP1B1 calculated based on changes in liver Kp in these mice indicates that, on a gross kinetic level, the relative rate of downstream hepatic clearance is consistent with humans. Specifically, the Kp parameter is a function of uptake (modulated parameter), clearance by metabolism and/or excretion, as well as the extent of plasma and tissue binding, which are generally conserved between species (Kalvass et al., 2013). Thus, accurate prediction of the fraction of hepatic uptake mediated by human OATP1B1 calculated from changes in liver Kp in knockout...
versus knockin mice supports comparable relative rate of downstream clearance between mice and humans. Taken together, these two kinetic observations (OATP uptake as the rate-determining step and accurate translation of liver $K_p$ values) support the relevance of these murine models for the study of hepatic OATP uptake. However, the present and previous studies conducted in these mice provide no mechanistic characterization of downstream hepatic clearance via metabolism and/or excretion, and further validation would be needed to support studies of these processes.

In conclusion, oatp1a/1b-knockout mice are useful in determining whether hepatic OATP uptake influences systemic pharmacokinetics and hepatic distribution. In addition, they provide insight into OATP involvement in brain, kidney, and muscle drug distribution. Liver-specific knockin of OATP1B1 or OATP1B3 into these knockout mice may be used to predict their impact on clinical pharmacokinetics, but only after correcting for the differences in hepatic protein expression in knockin mice versus humans.

Authorship Contributions

Participated in research design: Higgins, Bao, Smith, Zamek-Gliszczynski.
Conducted experiments: Bao, Fallon.
Contributed new reagents or analytic tools: Fallon, Smith.
Performed data analysis: Higgins, Bao, Ke, Manro, Fallon, Smith, Zamek-Gliszczynski.
Wrote or contributed to the writing of the manuscript: Higgins, Bao, Ke, Manro, Fallon, Smith, Zamek-Gliszczynski.

References


Address correspondence to: Dr. Maciej J. Zamek-Gliszczynski, Lilly Corporate Center, Indianapolis, IN 46285. E-mail: m_zamek-gliszczynski@illy.com
Utility of oatp1a/1b-knockout and OATP1B1/3-humanized mice in the study of OATP-mediated pharmacokinetics and tissue distribution: case studies with pravastatin, atorvastatin, simvastatin, and carboxydichlorofluorescein.

J. William Higgins, Jing Q. Bao, Alice B. Ke, Jason R. Manro, John K. Fallon, Philip C. Smith, and Maciej J. Zamek-Gliszczynski
Supplemental Table 1. Transporter proteotypic heavy labeled tryptic peptides standards purchased (JPT) and MRMs acquired. C-terminus R and K amino acids are $^{13}$C and $^{15}$N heavy labeled (shown in bold). The mass differences between labeled (shown) and unlabeled (not shown) R and K are 10 and 8 respectively. Mass shift for transition ions, between labeled and unlabeled, also depends on the charge state. The product ion (all y ions) for each heavy labeled peptide MRM contains the heavy label. The peptides used to report concentrations are noted, with the second peptide for each protein providing confirmation of protein in the matrix.

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>Transporter (Gene)</th>
<th>Peptide Sequence</th>
<th>MRMs 1 and 2 (product ion)$^a$ [mass spectrometer specific]</th>
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<tbody>
<tr>
<td>Q9Y6L6</td>
<td>OATP1B1 (SLCO1B1)</td>
<td>N$<em>{321}$VTGFFQSFK$</em>{330}$$^{b,c}$</td>
<td>591.81/969.50 (y8), 591.81/868.46 (y7)</td>
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<td></td>
<td></td>
<td>L$<em>{402}$NTVGIAK$</em>{409}$$^d$</td>
<td>412.27/596.40 (y6), 412.27/710.44 (y7)</td>
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<td>Q9NPD5</td>
<td>OATP1B3 (SLCO1B3)</td>
<td>L$<em>{402}$SLVGIAK$</em>{409}$</td>
<td>404.78/608.43 (y6), 404.78/695.46 (y7)</td>
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<tr>
<td></td>
<td></td>
<td>I$<em>{615}$YNSVFFGR$</em>{623}$$^{b,c,d,e}$</td>
<td>556.79/836.43 (y7), 556.79/722.34 (y6)</td>
</tr>
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$^a$MRMs are in order of highest intensity

$^b$Peptide used to report transporter concentration

$^c$Peptides used by Balogh et al. (2012) for quantification

$^d$Peptides used by Ohtsuki et al. (2011) for quantification

$^e$1$^{st}$ MRM used to report concentration. 2$^{nd}$ MRM in some human samples <LOD
### Supplemental Table 2: Hepatic Human Transporter Protein Expression

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Human Liver</th>
<th>Wild Type</th>
<th>oatp1a/1b KO</th>
<th>OATP1B1 KI</th>
<th>OATP1B3 KI</th>
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<td>P-gp</td>
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<tr>
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Mean ± S.D., n = 4 (mouse) or 8 (human)
Limit of detection (LOD) = 0.1 pmol/mg protein
BLOD = below limit of detection