Title: Quantitative prediction of OATP-mediated disposition and biliary clearance using human liver chimeric mice

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AUC, area under the curve;
CL_{bile}, biliary clearance;
CL_{h}, hepatic clearance;
CL_{h,int}, hepatic intrinsic clearance;
CYP, cytochrome P450;
DDI, drug-drug interaction;
ECCS, Extended Clearance Classification System;
fup, free fraction in plasma;
NME, new molecular entity;
OATP, organic-anion-transporting polypeptide;
PBPK, physiologically based pharmacokinetic;
P-gp, P-glycoprotein;
\( R_b \), ratio of blood to plasma concentration

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Abstract

Drug biliary clearance (CL\textsubscript{bile}) in vivo is among the most difficult pharmacokinetic parameters to predict accurately and quantitatively because biliary excretion is influenced by metabolic enzymes, transporters, and passive diffusion across hepatocyte membranes. The purpose of this study is to demonstrate the use of Hu-FRG\textsuperscript{TM} mice (Fah\textsuperscript{−/−}/Rag2\textsuperscript{−/−}/Il2rg\textsuperscript{−/−} [FRG] mice transplanted with human-derived hepatocytes) to quantitatively predict human organic-anion-transporting polypeptide (OATP)-mediated drug disposition and CL\textsubscript{bile}. To predict OATP-mediated disposition, six OATP substrates (atorvastatin, fexofenadine, glibenclamide, pitavastatin, pravastatin, and rosuvastatin) were administered intravenously to Hu-FRG\textsuperscript{TM} and Mu-FRG\textsuperscript{TM} mice (FRG mice transplanted with mouse hepatocytes) with or without rifampicin as an OATP inhibitor. We calculated the hepatic intrinsic clearance (CL\textsubscript{h,int}) and the change of hepatic clearance (CL\textsubscript{h}) caused by rifampicin (CL\textsubscript{h} ratio). We compared the CL\textsubscript{h,int} of humans with that of Hu-FRG\textsuperscript{TM} mice, and the CL\textsubscript{h} ratio of humans with that of Hu-FRG\textsuperscript{TM} and Mu-FRG\textsuperscript{TM} mice. For predicting CL\textsubscript{bile}, twenty compounds (two cassette doses of ten compounds) were administered intravenously to gallbladder-cannulated Hu-FRG\textsuperscript{TM} and Mu-FRG\textsuperscript{TM} mice. We evaluated the CL\textsubscript{bile} and investigated the correlation of human CL\textsubscript{bile} with that of Hu-FRG and Mu-FRG mice. We found good correlations between humans and Hu-FRG\textsuperscript{TM} mice in CL\textsubscript{h,int} (100% within 3-fold) and CL\textsubscript{h} ratio (R\textsuperscript{2} = 0.94). Moreover, we observed a much better relationship between humans and Hu-FRG\textsuperscript{TM} mice in CL\textsubscript{bile} (75% within 3-fold). Our results suggest that OATP-mediated disposition and CL\textsubscript{bile} can be predicted using Hu-FRG\textsuperscript{TM} mice, making them a useful in vivo drug discovery tool for quantitatively predicting human liver disposition.
Significance Statement

OATP-mediated disposition and biliary clearance of drugs are likely quantitatively predictable using Hu-FRG™ mice. The findings can enable the selection of better drug candidates and the development of more effective strategies for managing OATP-mediated DDI in clinical studies.
1 Introduction

The quantitative prediction of clearance based on pharmacokinetic mechanisms is very important for the success of drug discovery and development. The construction of physiologically based pharmacokinetic (PBPK) models which can accurately predict target concentration profiles and assess the risk of drug-drug interactions (DDIs) ahead of clinical trials.

Organic-anion-transporting polypeptides (OATPs) in the liver are one of the most important pharmacokinetic regulators of clearance among drug transporters. The European Medicines Agency (EMA), the US Food and Drug Administration (FDA), and the Japan Ministry of Health, Labour and Welfare (MHLW) recommend having OATP substrate or inhibition studies in the preclinical stages of drug development (EMA, 2012; FDA, 2020; MHLW, 2018). However, using only in vitro data, it is still challenging to quantitatively predict human OATP-mediated clearance or DDIs for new molecular entities (NMEs) (Nozaki and Izumi, 2020).

Drug biliary clearance (CL\text{bile}) in vivo is one of the most difficult pharmacokinetic parameters to predict accurately and quantitatively because it is composed of various elements; metabolic enzymes, transporters, and passive diffusion across hepatocyte membranes all influence the biliary excretion of drugs. Sandwich-cultured human hepatocytes (SCHHs) are commonly used to assess in vitro CL\text{bile}. The SCHH system has been reported to successfully predict human hepatobiliary clearance using an empirical scaling factor (Kimoto et al., 2017). In that report, clearance of all assayed compounds with active uptake was underpredicted without a scaling factor. That means that SCHH usually requires scaling factors to make up for the lack of dynamic bile and blood flow, as well as the time-dependent alterations in the protein concentration of influx and efflux transporters (Li et al., 2010; Swift et al., 2010; Zou et al., 2013). Moreover, it is difficult to evaluate low CL\text{bile} compounds using the SCHH system due to its calculation method. In contrast, a bile duct-cannulated pre-clinical animal model is
often used for in vivo assessments of the elimination pathway and \( \text{CL}_{\text{bile}} \). Monkeys have shown biliary excretion comparable to humans (Kimoto et al., 2017). Moreover, allometric scaling can satisfactorily predict human \( \text{CL}_{\text{bile}} \) with multiple-species data (Morris et al., 2012). However, there are numerous differences between humans and pre-clinical animals in the expression or function of metabolic enzymes and transporters (Bleasby et al., 2006; Martignoni et al., 2006; Wang et al., 2015). That indicates \( \text{CL}_{\text{bile}} \) in pre-clinical animals cannot always be used to construct human PBPK models of NMEs in the late stage of drug discovery.

The extended clearance concept, which incorporates processes of hepatic uptake, metabolism, passive diffusion, and biliary secretion, was adapted to determine how much each process contributes to total \( \text{CL} \) and DDIs (Liang and Lai, 2021). Quantitative human prediction of liver disposition for NMEs requires reliable parameters for all processes. Current in vitro systems have not achieved that goal, which will be even more difficult to achieve for compounds with poor physical characteristics such as molecules beyond the rule of five.

Molecular biology and gene/chromosome engineering have been used to develop several unique humanized animal models for predicting pharmacokinetics (Satoh et al., 2018; Scheer and Wilson, 2016). Genetically and chromosomally humanized mouse models can successfully predict a specific metabolic enzyme or specific transporter-mediated drug disposition (Mitsui et al., 2014; Miyake et al., 2021). Human liver chimeric mice are liver-specific humanized models transplanted with human hepatocytes and are reported to be good at predicting human drug metabolism and pharmacokinetics (Naritomi et al., 2018). There are many reports on the metabolism of the chimeric mice, but not many on their transporters (Feng et al., 2021; Sanoh et al., 2020; Takehara et al., 2019; Uchida et al., 2018). In particular, there are few complete and mechanism-based human extrapolation studies on drug transporters that can function as platforms for drug discovery and development. Therefore, we hypothesized that human liver chimeric mice can be used to predict transporter-mediated drug
disposition, including clearance, the target transporter’s contribution to total clearance (CL\text{total}), and CL\text{bile}. This is the first comprehensive and mechanism-based study to demonstrate that human OATP-mediated clearance and DDI can be quantitatively predicted using one of the human liver chimeric mice, Hu-FRG\textsuperscript{TM} mice (Fah\textsuperscript{-/-} /Rag2\textsuperscript{-/-} /Il2rg\textsuperscript{-/-} [FRG] mice transplanted with human hepatocytes). Moreover, this is the first study to evaluate whether human CL\text{bile} can be predicted using the humanized mice.
2 Materials and Methods

2.1 Animals

All experiments involving animals were conducted in accordance with the guidelines for the Care and Use of Laboratory Animals adopted by the Committee on Animal Research at Chugai Pharmaceutical Co. Ltd. as well as the Principles of Laboratory Animal Care adopted by the National Institutes of Health.

Male FRG on C57BL/6 background with human hepatocyte repopulated (Hu-FRG™) mice and male FRG on C57BL/6 background with C57BL/6 hepatocyte repopulated (Mu-FRG™) mice were purchased from the Yecuris Corporation (Tualatin, OR, USA). Information on the donors of human hepatocytes transplanted to Hu-FRG™ mice are shown in Table S1. These liver chimeric mice were 6 to 10 months old and weighed 25–35 g. The animals were given free access to food and water and housed in an environmentally controlled room with a temperature maintained at 23°C ± 3°C, humidity at 50% ± 20%, and a 12-hour light/dark cycle (light from 07:00–19:00). They were cared for in a pathogen-free animal facility. The Hu-FRG™ and Mu-FRG™ mouse models were first reported by Azuma et al. (Azuma et al., 2007) and commercially produced by the Yecuris Corporation. The animals are widely used in preclinical research. According to the Yecuris protocol, FRG mice were cycled on 2-(2-nitro-4-trifluoro-methyl-benzoyl)-1,3 cyclohexanedione (NTBC) once a month for 4 days at 8 mg mL⁻¹ in drinking water to maintain health. Animals were not given NTBC from three weeks before and during the studies.

We set the sample size based on our previous study using humanized mouse models (Mitsui et al., 2014). Normally, evaluating human pharmacokinetic predictability requires a minimum of three animals per group. In the present study, more than three animals per group were basically used.
Animals were randomly assigned for the study. No blinding method was applied because of bioanalysis by LC–MS/MS quantification.

2.2 Donor hepatocytes selection

Metabolic activity of human hepatocyte donors transplanted to FRG mice was obtained from Yecuris (Table S2). FRG mice can be transplanted with hepatocytes from various donors (Strom et al., 2010). Moreover, even a few mice that have undergone primary transplantation can be used to produce large numbers of humanized mice through serial transplantation for large-scale studies (Strom et al., 2010). When using human liver chimeric mice for pharmaceutical research, it is important to select appropriate donor hepatocytes for pharmacokinetic evaluation and secure a certain number of donor hepatocytes for consistent evaluation.

Donor information for hepatocytes transplanted to the human liver chimeric mice is needed to correctly interpret the results obtained from the humanized mice. This is because genomic DNA from the liver of a chimeric mouse and that from the liver of a donor can exhibit the similar genotype (Katoh et al., 2004). In the present study, we used female and male donor hepatocytes for OATP-mediated drug disposition and biliary clearance, respectively. Sex-related pharmacokinetic differences are recognized for many drugs, but they are generally minimal (Meibohm et al., 2002; Schwartz, 2003; Tanaka, 1999). Differences between male and female subjects in the expression levels of OATP transporters were not considered significant (Badée et al., 2015). Sex differences may have affected the present bile excretion study, but the magnitude of the effect is unknown.

2.2 Substrate selection
For predicting OATP-mediated disposition, we used the following six established OATP substrates: atorvastatin, fexofenadine, glibenclamide, pitavastatin, pravastatin, and rosuvastatin. We selected these OATP substrates and rifampicin as an OATP inhibitor because their OATP-related clinical DDI information is available in the University of Washington Metabolism and Transport Drug Interaction Database (DIDB, accessed in Dec 2022). We selected substrates with a wide range of DDI magnitude to clarify correlations between human and mouse models. Table S3 shows pharmacokinetic characteristics of the six OATP substrates and rifampicin. The extended clearance classification system (ECCS) can be used to predict the primary rate-determining clearance mechanism based on basic drug properties (i.e., ionization, permeability, and molecular weight) (Varma et al., 2015). As shown in Table S3, the six substrates can be classified as ECCS class 1B (ionization: acid/zwitterion, high permeability, MW > 400 Da) or ECCS class 3B (ionization: acid/zwitterion, low permeability, MW > 400 Da). The hepatic uptake of ECCS class 1B and 3B compounds often involves OATP1B1 and OATP1B3 (El-Kattan and Varma, 2018).

For predicting CL bile, we used the following twenty substrates: cyclosporine A, epirubicin, erythromycin, fexofenadine, indocyanine green, paclitaxel, pitavastatin, pravastatin, rosuvastatin, valsartan, cefazolin, cefixime, cefoperazone, cefotetan, cefpiramide, ceftriaxone, ciprofloxacin, diclofenac, irinotecan, and ranitidine. We categorized the first 10 compounds, including those with relatively high human CL bile, into Group 1 and the latter 10 compounds, including those with relatively low human CL bile into Group 2. The pharmacokinetic characteristics and human CL bile of the substrates are shown in Table 1. In the table, ECCS class 3B compounds tend to be mainly eliminated in bile as unaltered parents (Kimoto et al., 2017).

2.3 Pharmacokinetic studies to predict OATP-mediated disposition

2.3.1 In vivo study
We administered six OATP substrates (atorvastatin, fexofenadine, glibenclamide, pitavastatin, pravastatin, and rosuvastatin) separately and intravenously to the Hu-FRG™ (donor: HHF13023) and Mu-FRG™ mice with or without rifampicin (n = 4 per group). Rifampicin as a OATP inhibitor was orally administered at a dose of 100 mg kg\(^{-1}\) one hour before substrate dosing. The dosage of rifampicin was determined by body surface area conversion based on the clinical human dose referenced in the DDI study (FDA, 2005). The dosage regimen for rifampicin was decided by referring to the previous report (Imaoka et al., 2013). To calculate the urinary excretion, we intravenously administered the six substrates in two cassette doses of three compounds. Details of the study design are shown in Table S4. Blood samples were collected from the jugular vein using a heparinized syringe at 0.033, 0.167, 0.5, 1.5, 4, 7, and 24 hours after substrate administration. Each time, 20 μL of blood was collected. Plasma was prepared by centrifuging the blood at 4°C at 13000 × g for 5 minutes and freezing at –30°C until analysis. Urine samples were collected in a metabolic cage for 8 hours after intravenous administration and volume was recorded.

### 2.3.2 Determination of hepatic intrinsic clearance

An outline of the analytical procedure in the OATP mediated drug disposition study is shown in Figure S1. Hepatic intrinsic clearance (CL\(_{h,int}\)) of the six substrates in Hu-FRG™ and Mu-FRG™ mice and humans was determined using the following equations and dispersion model:

\[
\text{CL}_{h,p}(b) = \text{CL}_{\text{total},p}(b) - \text{CL}_{\text{renal},p}(b)
\]

\[
\text{CL}_{\text{renal},p}(b) = \text{CL}_{\text{total},p}(b) \times \text{urinary excretion (\% of injected dose)}
\]

\[
F_h = 1 - \left(\frac{\text{CL}_{h,p}/R_B}{Q_h}\right)
\]

\[
f_B = \frac{f_{u_p}}{R_B}
\]
Where CL_{h,p} (b) is hepatic plasma (blood) clearance, CL_{total,p} (b) is total systemic plasma (blood) clearance, CL_{renal,p} (b) is renal plasma (blood) clearance, F_h is hepatic availability, R_B is the ratio of blood to plasma concentration, f_{up} is free fraction in plasma, Q_h is the rate of hepatic blood flow, and f_B is blood unbound fraction. The Q_h was set at 90 ml min^{-1} kg^{-1} for mice (Davies and Morris, 1993) and 25.5 ml min^{-1} kg^{-1} for humans (Wynne et al., 1989). The human liver mass was set at 25.7 g kg^{-1} body weight (Davies and Morris, 1993) and that of Hu-FRG™ and Mu-FRG™ mice were calculated using the observed values (Table S7).

Dispersion model (Roberts and Rowland, 1986):

\[
F_h = \frac{4a}{(1 + a)^2 \cdot \exp\left[\frac{(a - 1)}{2D_N} \right] - (1 - a)^2 \cdot \exp\left[-\frac{(a + 1)}{2D_N} \right]} \]

\[
a = \left(1 + 4R_N \cdot 2D_N\right)^{1/2}
\]

\[
R_N = f_B \cdot CL_{h,int} / Q_h
\]

\[
D_N = 0.17
\]

D_N is the dispersion number and R_N is the efficiency number. The R_B and f_{up} of the six substrates in humans were derived from published reports or in-house data and those of Hu-FRG™ and Mu-FRG™ mice were from in-house data. In-house data was obtained using a previously reported method (Mitsui et al., 2014) (Table S7).

2.3.3 Determination of hepatic clearance ratio as a quantitative index of OATP-mediated DDI

The dose-normalized area under the curve ratios (AUCR_{IV}) of the six substrates after intravenous dosing in Hu-FRG™ and Mu-FRG™ mice were calculated using the following equation:
\[
AUCR_{IV,FRG} = \frac{AUC_{FRG \text{ with rifampicin}}/\text{dose}}{AUC_{FRG \text{ without rifampicin}}/\text{dose}}
\]

Moreover, the \( \text{CL} \text{h} \) ratio of these six substrates in \( \text{Hu-FRG}^\text{TM} \) and \( \text{Mu-FRG}^\text{TM} \) mice were calculated using the following equation:

\[
\text{CL} \text{h ratio}_{FRG} = \frac{\text{CL}_{h,FRG \text{ without rifampicin}}}{\text{CL}_{h,FRG \text{ with rifampicin}}}
\]

We gathered clinical information on OATP inhibition using DIDB. Then we calculated changes to the human dose-normalized AUC caused by OATP inhibition by rifampicin after oral dosing (\( AUCR_{po} \)) using the following equation:

\[
AUCR_{po, human} = 1 + \frac{\% \text{change in } AUC \text{ human by rifampicin}}{100}
\]

Then, \( AUCR_{po} \) is also expressed by the following equation:

\[
AUCR_{po, human} = \frac{\frac{\text{CL}_{\text{total,b}}}{\text{F}}}{\frac{\text{CL}_{\text{total, rifa,b}}}{\text{F}_{\text{rifa}}}} = \frac{\frac{\text{CL}_{h,b}}{\text{F}} + \frac{\text{CL}_{\text{renal,b}}}{\text{F}}}{\frac{\text{CL}_{h, rifa,b}}{\text{F}_{\text{rifa}}} + \frac{\text{CL}_{\text{renal, rifa,b}}}{\text{F}_{\text{rifa}}}} = \frac{\frac{\text{CL}_{h,b}}{\text{F}_{\text{h}} \cdot \text{F}_{a} \cdot \text{F}_{g}} + \frac{\text{CL}_{\text{renal,b}}}{\text{F}_{\text{h}} \cdot \text{F}_{a} \cdot \text{F}_{g}}}{\frac{\text{CL}_{h, rifa,b}}{\text{F}_{\text{rifa}} \cdot \text{F}_{g}} + \frac{\text{CL}_{\text{renal, rifa,b}}}{\text{F}_{\text{rifa}} \cdot \text{F}_{g}}}
\]

Where \( \text{F} \) is absolute bioavailability, \( \text{F}_{a} \) is fraction absorbed, \( \text{F}_{g} \) is intestinal availability, and \( \text{F}_{h} \) is hepatic availability. Assuming \( \text{F}_{a} \text{F}_{g} \) does not change in the presence of rifampicin, the following equations are derived:
In order to properly investigate the correlations between human and the model mice (Kirby and Unadkat, 2010), we estimated the human CL\textsubscript{h} ratio of the six substrates using the following equation:

\[
\frac{\text{CL}_{\text{h}, \text{ratio}}}{\text{CL}_{\text{h}, \text{ratio}}} = \frac{\text{AUCR}_{\text{PO,human}}}{\text{AUCR}_{\text{PO,human}}} \cdot \frac{\text{Fh}}{\text{Qh}} = \frac{\text{CL}_{\text{h,b}}}{\text{CL}_{\text{h,b}}} \cdot \frac{\text{Fh}}{\text{Fh}} + \frac{\text{CL}_{\text{renal,b}}}{\text{CL}_{\text{renal,b}}} \cdot \frac{(1 - \text{Fh})Qh}{Qh} + \frac{\text{CL}_{\text{renal,rifa,b}}}{\text{CL}_{\text{renal,rifa,b}}} \cdot \frac{\text{Fh}_{\text{rifa}}}{\text{Fh}_{\text{rifa}}}
\]

\[
\text{Fh}_{\text{rifa}} = \frac{\text{AUCR}_{\text{PO,human}} \cdot \text{Fh} \cdot (\text{Qh} + \text{CL}_{\text{renal,rifa,b}})}{\text{AUCR}_{\text{PO,human}} \cdot \text{Fh} \cdot \text{Qh} + \text{CL}_{\text{h,b}} + \text{CL}_{\text{renal,b}}}
\]

We used pharmacokinetic data from healthy volunteers treated with a single high dose of rifampicin (600 mg) to calculate AUCR\textsubscript{PO,human} for comparison with the mouse study results. Moreover, we also used data from a human pharmacokinetic study showing CL\textsubscript{renal} with or without rifampicin to calculate the human CL\textsubscript{h} ratio for fexofenadine, pravastatin, and rosuvastatin. CL\textsubscript{renal,p} of atorvastatin, glibenclamide, and pitavastatin with or without rifampicin was set to 0 because of their low CL\textsubscript{renal,p} (Table S7). The human body weight was set at 70 kg\textsuperscript{-1}.

### 2.4 Pharmacokinetic studies to predict biliary clearance

#### 2.4.1 Gallbladder cannulation

Gallbladder cannulations were performed aseptically under isoflurane anesthesia. After subcutaneously injecting flunixin meglumine (2.5 mg kg\textsuperscript{-1}) for analgesia, the abdomen of each mouse was opened, the common bile duct was ligated, and the wall of the gallbladder was opened using micro scissors. A polyurethane catheter (ID 0.2 mm × OD 0.4 mm, length 60 cm) was inserted into the incised
gallbladder and fixed to gallbladder. The other end of the catheter traveled through subcutaneous tissue and exited an incision on the back of the mouse. The incised area at the back and abdomen was closed, 0.25% of bupivacaine was applied directly to the incised area and the mouse was hydrated with 500 μL of warm saline subcutaneous injection. The catheter was passed through the tethers and integrated with a harness on each mouse. Lastly, the catheter was fixed to bile collection tubes. Mice were used an hour or more after awakening to avoid blood contamination in bile resulting from the surgery. Mice that secreted 25 or more microliters of bile for 30 minutes after the surgery were assigned to the study. Bile samples were collected from conscious mice every 30 minutes for 6 hours after dose administration.

2.4.2 In vivo study

We intravenously administered twenty substrates in two cassette doses of ten compounds each intravenously to the gallbladder-cannulated Hu-FRG™ (donor: HHM18029) and Mu-FRG™ mice. In the Hu-FRG™ mouse study, Group 1 consisted n = 6 because it was the most important group for this evaluation. The other groups consisted of n = 4. Cyclosporine A, epirubicin, erythromycin, fexofenadine, indocyanine green, paclitaxel, pitavastatin, pravastatin, rosuvastatin, and valsartan are in Group 1. Cefazolin, cefixime, cefoperazone, cefotetan, cefpiramide, ceftriaxone, ciprofloxacin, diclofenac, irinotecan, and ranitidine are in Group 2. The study design details are shown in Table S5. Blood samples were collected from the orbital sinus under anesthesia or the jugular vein (6 hours only) in a heparinized capillary tube at 0.033, 0.167, 0.5, 1, 2, 4, and 6 hours after dose administration. Blood was collected at a volume of 20 μL each time. Plasma was prepared by centrifuging the blood at 4°C at 13000 × g for 5 minutes and freezing at –30°C until analysis. Bile samples were collected every 30 minutes for 6 hours after dose administration and the volume was recorded.
2.4.3 Determination of biliary clearance

We calculated the $\text{CL}_{\text{bile}}$ of the twenty substrates in Hu-FRG™ and Mu-FRG™ mice using the following equation:

$$\text{CL}_{\text{bile}} = \text{CL}_{\text{total,p}} \times \text{biliary excretion (\% of injected dose)}$$

2.5 Bioanalysis

Plasma (5 μL), urine (5 μL), and bile (5 μL) samples were analyzed using LC-MS/MS after protein precipitation with acetonitrile (110 μL) containing an internal standard. The LC-MS/MS conditions were as follows. The instrument was an ACQUITY UPLC I-Class System (Waters Corp., Milford, MA, USA) equipped with a Xevo TQ-XS system (Waters Corp., Milford, MA, USA). The column was an InertSustain C18 HP (2.1 mm I.D. × 50 mm, 5 μm, GL Sciences, Inc., Tokyo, Japan) at a column temperature of 40°C. The mobile phase was operated under gradient conditions and consisted of 10 mmol L$^{-1}$ ammonium acetate in water (A) and methanol (B). Mass spectrometry was performed using positive or negative ionization electrospray. The selective reaction monitoring mode was used to monitor ions as follows (m/z: precursor ion/production): atorvastatin (559.35/440.17), cefazolin (455.03/323.00), cefixime (454.04/126.00), cefoperazone (646.14/143.00), cefotetan (576.00/432.20), cefpiramide (613.12/257.00), ceftriaxone (555.04/396.00), ciprofloxacin (332.23/314.20), cyclosporine A (1202.9/156.00), diclofenac (296.20/214.00), epirubicin (544.27/130.10), erythromycin (734.58/158.20), fexofenadine (502.35/171.14), indocyanine green (753.40/330.22), irinotecan (587.28/124.00), paclitaxel (854.50/286.20), pitavastatin (422.21/274.10), pravastatin (423.40/321.20), ranitidine (315.14/176.00), rifampicin (823.36/791.11), rosuvastatin (482.26/258.15), valsartan (436.20/235.20) and an internal standard niflumic acid (283.06/245.07) in the positive ionization mode. glibenclamide (492.14/169.97), pravastatin (423.40/320.90) and an
internal standard niflumic acid (280.75/236.87) in the negative ionization mode. The calibration curves (ranging from 0.1 to 100 ng mL\(^{-1}\) or from 1 to 1000 ng mL\(^{-1}\)) were fitted to a 1/x\(^2\)-weighted linear regression model. The plasma, urine, and bile concentrations met the criteria for inclusion in the present study (Table S6).

2.6 Data and statistical analysis

A pharmacokinetic noncompartmental analysis was conducted using Phoenix WinNonlin version 8.2.0 software (Pharsight Corp., Mountain View, CA, USA). AUC was calculated using the linear-log trapezoidal rule. The group size was set as the number of independent values. Statistical analysis was performed with these independent values. In the animal pharmacokinetics study, data are presented as means ± S.D. or geometric means (CL\(_{\text{bile}}\) only). We calculated the correlation in CL\(_{\text{h,int}}\) based on non-linear regression analysis and the correlations in AUCR, CL\(_{\text{h}}\) ratio, and CL\(_{\text{bile}}\) based on the linear regression analysis. We determined the prediction accuracy of CL\(_{\text{h,int}}\), CL\(_{\text{h}}\) ratio, and CL\(_{\text{bile}}\) based on the number of predictable compounds using regression analysis. All statistical analysis used GraphPad Prism version 9.40 software (GraphPad Software, Inc., San Diego, CA, USA).

2.7 Materials

Atorvastatin calcium salt trihydrate was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Cefazolin, cefixime, ceftriaxone, ciprofloxacin, cyclosporine A, diclofenac, glibenclamide, irinotecan, paclitaxel, pitavastatin calcium, pravastatin sodium salt, rifampicin, and rosuvastatin calcium were purchased from the FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Cefoperazone, epirubicin, ranitidine, and valsartan were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Cefotetan was purchased from Toronto Research Chemicals, Inc. (North York, ON, Canada). Cefpiramide, erythromycin, and indocyanine green were purchased from the United States
Pharmacopeia (North Bethesda, MD, USA). Fexofenadine hydrochloride was purchased from LKT Laboratories, Inc. (St. Paul, MN, USA). All other chemicals were of reagent grade and were available from commercial sources.
Results

Pharmacokinetic studies to predict OATP-mediated disposition

The plasma concentration–time profiles of the OATP substrates in Hu-FRG™ and Mu-FRG™ mice are summarized in Figure 1. The pharmacokinetic parameters obtained in the study are summarized in Table 2. The plasma concentration–time profiles of rifampicin is also shown in Figure S2. The CL\textsubscript{total,\text{p}} of each substrate in Hu-FRG™ mice was lower than in Mu-FRG™ mice. High rifampicin exposure was observed in both Hu-FRG™ and Mu-FRG™ mice although the terminal half-life in Hu-FRG™ mice was shorter than in Mu-FRG™ mice. The rifampicin plasma concentrations in Hu-FRG™ and Mu-FRG™ mice up to 7 hours after substrate administration were 50 times higher than rifampicin’s previously reported in vitro IC\textsubscript{50} for OATP1B1 (0.88 µM) and OATP1B3 (0.31 µM) (Durmus et al., 2015). Rifampicin increased the AUC of all six substrates in both Hu-FRG™ and Mu-FRG™ mice.

Except for pravastatin, the AUC\textsubscript{RIV} in Mu-FRG™ mice tended to be higher than in Hu-FRG™ mice. The CL\textsubscript{h} ratio for pravastatin in Hu-FRG™ mice was higher than that of AUC\textsubscript{RIV} because of the relatively large contribution of the CL\textsubscript{renal} to the CL\textsubscript{total}. The rifampicin DDI resulted in the decrease of V\textsubscript{ss} for atorvastatin, pitavastatin, pravastatin, and rosuvastatin, but not for fexofenadine and glibenclamide in Hu-FRG™ mice. On the other hand, the DDI resulted in the decrease of V\textsubscript{ss} for all except glibenclamide in Mu-FRG™ mice. Pharmacokinetic properties of the substrates used to calculate CL\textsubscript{h,int} are shown in Table S7. In Mu-FRG™ mice, the CL\textsubscript{h,int} of substrates other than fexofenadine and glibenclamide were uncalculatable because their CL\textsubscript{h,b} was above the hepatic blood flow. The pharmacokinetic correlations in CL\textsubscript{h,int} of the OATP substrates with or without rifampicin are shown in Figure 2. They clearly show a much stronger correlation in CL\textsubscript{h,int} between Hu-FRG™ mice and humans than that between Mu-FRG™ mice and humans. Moreover, the change of CL\textsubscript{h,int}
caused by rifampicin was calculated in Figure S3. The figure indicates that the rate of CLh,int change between Hu-FRG™ mice and humans were comparable.

The clinical OATP-mediated DDI studies on the six substrates are shown in Table S8. The correlations of human AUCRpo with Hu-FRG™ mouse AUCRiv and Mu-FRG™ mouse AUCRiv, and the correlations of the CLh ratio in humans with those of Hu-FRG™ mice and Mu-FRG™ mice are shown in Figure 3. The correlations between AUCRpo and AUCRiv were exploratively evaluated and are not suitable for compounds with high first-pass extraction (Kirby and Unadkat, 2010). A better correlation between Hu-FRG™ mice and humans was observed for CLh ratio (R² = 0.94) than AUCR (R² = 0.44). Moreover, the figure clearly shows a much stronger correlation in CLh ratio between Hu-FRG™ mice and humans than between Mu-FRG™ mice and humans (R² = 0.028).

Pharmacokinetic studies to predict biliary clearance

The plasma concentration–time profiles and biliary excretion rate–time profiles are summarized in Figure 4 and Figure 5. The pharmacokinetic parameters obtained in the study are also summarized in Table 3. The number of Hu-FRG™ mice in Group 2 was reduced to three because the condition of one gallbladder-cannulated mouse deteriorated. The amount of bile recovered during the 6-hour experiment was 615 ± 160 µL for Hu-FRG™ mice and 733 ± 152 µL for Mu-FRG™ mice. Rosuvastatin showed the highest biliary excretion rate in both Hu-FRG™ and Mu-FRG™ mice. Furthermore, epirubicin, erythromycin, pravastatin, and rosuvastatin showed a relatively higher CLbile in Hu-FRG™ mice. There was an over 2-fold difference in CLbile between Hu-FRG™ and Mu-FRG™ mice for 17 out of 20 compounds. Table S9 shows the relationship between the average free plasma concentration (Cave,free) of cyclosporine A in Hu-FRG™ mice and cyclosporine A’s in vitro IC50. In the present study, the Cave,free of cyclosporine A was more than 300 times higher than the in vitro IC50 for each transporter and enzyme.
The correlations in CL\textsubscript{bile} between Hu-FRG\textsuperscript{TM} mice and humans and between Mu-FRG\textsuperscript{TM} mice and humans are shown in Figure 6. The regression line for Hu-FRG\textsuperscript{TM} mice was $y = 0.963x$ and the line for Mu-FRG\textsuperscript{TM} mice was $y = 0.411x$. Hu-FRG\textsuperscript{TM} mice showed a much better CL\textsubscript{bile} correlation ($R^2 = 0.563$, RMSE = 0.887) than Mu-FRG\textsuperscript{TM} mice ($R^2 = 0.027$, RMSE = 2.741).

**Prediction accuracy of human pharmacokinetics**

Figure 7 shows the prediction accuracy for human CL\textsubscript{h,int}, CL\textsubscript{h} ratio, and CL\textsubscript{bile}. We compared our results with widely used prediction methods. Human CL\textsubscript{h,int} prediction for OATP substrates using human hepatocytes has been reported (Izumi et al., 2017), but our results were more accurate (100% within 3-fold, Figure 7a). Furthermore, there have been many reports predicting OATP-mediated DDI using human in vitro systems (Bi et al., 2019; Yoshida et al., 2012) and, of these, our results also showed the highest prediction accuracy (100% within \(\pm 30\%\), Figure 7b). Human CL\textsubscript{bile} prediction using SCHHs has been reported (Kimoto et al., 2017), but our results were more accurate than that without scaling factors (75% within 3-fold, Figure 7c). Additionally, prediction of human CL\textsubscript{bile} for relatively high clearance drugs using monkey single-species scaling has been reported (SSS) (Kimoto et al., 2017), but our results were also more accurate (90% within 3-fold, Group1 in Figure 7c). Although we would ideally need to compare prediction accuracy using the same compounds, use of Hu-FRG\textsuperscript{TM} mice appears to be one of the best methods.

The Pharmaceutical Research and Manufacturers of America initiative assessed the predictability of pharmacokinetics in humans using preclinical data and compared available prediction methods (Jones et al., 2011; Poulin et al., 2011a; Poulin et al., 2011b; Ring et al., 2011; Vuppugalla et al., 2011). Across methods, the highest success rates for predicting CL of the 19 drugs in humans were 78%, 94%, and 100%, and of the observed CL within an error margin of 2-fold, 3-fold, and 10-fold, respectively (Ring.
et al., 2011). Despite the difficulty of predicting human CL bile, our success rate in predicting human CL bile using Hu-FRG™ mice (75% within 3-fold and 95% within 4-fold, Figure 7c) is comparable to the highest. A prediction accuracy within the range of ±3-fold is not high enough. The large inter-individual differences in protein expression in humans (Ohtsuki et al., 2012) make it more difficult to predict clearance with accuracy.
Discussion

The main goal of this study is to demonstrate that human liver chimeric mice can be used to successfully predict transporter-mediated drug disposition. We expect this model to be especially effective for predicting complex pharmacokinetic parameters such as xenobiotic contribution and CL_{bile}. These parameters are difficult to predict using genetically or chromosomally single humanized mouse models. We selected OATP-mediated disposition as the initial prediction target because OATP-mediated DDI is the most frequent hepatic transporter-mediated DDI in clinical settings and can be easily detected by measuring plasma concentrations. Then, we proceeded to predict CL_{bile} as an output of hepatic uptake, passive diffusion, metabolism, and biliary excretion.

The pharmacokinetic studies using OATP substrate demonstrated that human OATP-mediated disposition is much better captured by Hu-FRG™ mice than by Mu-FRG™ mice. The good correlations in CL_{h,int} between Hu-FRG™ mice and humans indicate that OATP-mediated clearance can be predicted using Hu-FRG™ mice (Figure 2). Moreover, the even better correlation in CL_{h} ratio between Hu-FRG™ mice and humans indicates that the contribution of OATP-mediated CL_{h} to substrate CL_{h} in Hu-FRG™ mice corresponds well with that in humans (Figure 3). These findings suggest that human OATP in Hu-FRG™ mouse liver functions similarly in humans without being greatly affected by the remaining murine hepatocytes.

In contrast, we observed a weak correlation in CL_{h} ratio between Mu-FRG™ mice and humans (or Hu-FRG™ mice). This result can be explained by two related factors: species differences in OATPs/Oatps activity and other contributors to total CL_{h}. According to ECCS framework, class 3B compounds (fexofenadine, pravastatin, and rosvuastatin) can be greatly influenced by the former factor whereas class 1B compounds (atorvastatin, glibenclamide, and pitavastatin) can be influenced by both the factors. Regarding OATPs/Oatps activity, OATP family transporters are known to be poorly
conserved, and for some, no orthologs have been found in species such as rodents, humans, and dogs (Chu et al., 2013). Actually, OATP1B1, OATP1B3 and OATP2B1 are expressed in the hepatocyte sinusoidal membrane in humans whereas Oatp1a1, Oatp1a4, Oatp1b2, and Oatp2b1 are expressed in that of mice (Iusuf et al., 2012). In the present study, the much higher fexofenadine CLh ratio in Mu-FRG™ mice than those in humans and Hu-FRG™ mice can be explained by the finding that the decrease rate in CLh,int caused by rifampicin in Mu-FRG™ mice showed higher that in humans and Hu-FRG™ mice (Figure S3). Moreover, in vitro hepatocyte uptake clearance (CLint,uptake) shows the relation of the CLh ratio in Mu-FRG™ mice to the that in Hu-FRG™ mice is similar to the relation of the CLint,uptake in Mu-FRG™ mice to the that in Hu-FRG™ mice in the class 3B compounds (Figure S4). This means the poor correlation in CLh ratio between Mu-FRG™ mice and humans (or Hu-FRG™ mice) in the class 3B may be explained mainly by species differences in OATPs/Oatps activity in accordance with the ECCS framework. Regarding other contributors to total CLh, OATPs/Oatps expressed in the hepatic sinusoids are the most abundant drug transporters in humans, dogs, monkeys, and rats. However, there are significant species differences in the levels of the other transporters (Wang et al., 2015). For instance, the second most highly expressed transporter in humans was OCT1, whereas it was Mrp2 in Wistar rats and Ntcp in Sprague-Dawley rats (Wang et al., 2015). There can be interspecies differences in the relative amount of drug transporters and metabolic enzymes between humans and mice. This hypothesis is supported by a previous report that Oatp1a/1b-deficient mouse models could not capture the clinical interaction (Chang et al., 2014). In our study, the relation of the CLh ratio in Mu-FRG™ mice to the that in Hu-FRG™ mice is not similar to the relation of the CLuptake in Mu-FRG™ mice to that in Hu-FRG™ mice for the class 1B compounds (Figure S4). This means the poor class 1B correlation in CLh ratio between Mu-FRG™ mice and humans may be explained by species differences in other contributors to total CLh. Considering the characteristics of the OATP substrates, P-gp, BCRP, MRP2, CYP3A4, and CYP2C9 may be involved in the remaining clearance.
Rifampicin decreased the $V_{ss}$ of many OATP substrates in Hu-FRG™ and Mu-FRG™ mice (Table 2). The effects of drug transporters on $V_{ss}$ have been comprehensively reported (Grover and Benet, 2009). The decreased $V_{ss}$ of OATP substrates is clearly explained by the fact that rifampicin prevents liver entry of the substrates. In contrast, the little change in the Hu-FRG™ mouse $V_{ss}$ of fexofenadine and glibenclamide and the Mu-FRG™ mouse $V_{ss}$ of glibenclamide can be explained by the relatively small $CL_h$ ratio and rifampicin inhibition effect for efflux transporters such as MRP2 (Lau et al., 2006) and/or P-gp (Reitman et al., 2011). The pharmacokinetics of rifampicin is also important for evaluating its DDI (Figure S2). The gastrointestinal absorption of rifampicin is supposed to be the same in Hu-FRG™ and Mu-FRG™ mice. The shorter plasma half-life in Hu-FRG mice could be explained the failure of rodent arylacetamide deacetylase (AADAC) to catalyze rifampicin hydrolase activity (Kobayashi et al., 2012). Interestingly, this result may indicate that human AADAC functions well in Hu-FRG™ mice.

Our subsequent pharmacokinetic study demonstrated that human $CL_{bile}$ can be predicted in Hu-FRG™ mice. In the study, several compounds showed relatively higher $CL_{bile}$ in Hu-FRG™ mice than that in Mu-FRG™ mice. Possible reasons for this based on their pharmacokinetic characteristics (Table 1) are as follows: epirubicin is an in vivo substrate for P-gp, erythromycin is an in vivo substrate for OATPs and P-gp, pravastatin is an in vivo substrate for P-gp, BCRP, MRP2, OATPs, and OAT3, and rosuvastatin is an in vivo substrate for OATPs and BCRP. These results indicate that liver efflux transporters, which are essential for drug biliary excretion, functioned in the Hu-FRG™ mice. In addition to strongly indicating that human drug transporters and metabolic enzymes in the humanized mice functioned properly as expected, the good correlation of $CL_{bile}$ between Hu-FRG™ mice and humans also showed that the balance of protein expression in the Hu-FRG™ mice remained the same as that in humans. Moreover, the correlation formula for $CL_{bile}$ between Hu-FRG™ mice and humans
(y = 0.963x) indicates that human CL\textsubscript{bile} can be predicted earlier, without the need for a formula to correlate CL\textsubscript{bile} between species (Figure 7c).

A limitation of the study for evaluating CL\textsubscript{bile} is that multiple compounds were delivered in cassette doses, although in low dosage. Table S9 shows the inhibition potency of cyclosporine A against major transporters and enzymes. Cyclosporine A does not seem to have a significant effect on the pharmacokinetics of other compounds, suggesting that it would have little effect on their CL\textsubscript{bile} in the present study. In fact, the four OATP substrates were not significantly affected by the cassette administration and the gallbladder cannulation surgery (Figure S5). If coproporphyrins I and III as markers of OATP inhibition could be measured with a high enough sensitivity, the degree of the inhibition in mice could be easily assessed at the same time (Shen et al., 2016; Takehara et al., 2019). However, it is hard to confirm whether the drugs in this study could potentially affect each other’s CL\textsubscript{bile}. This is a common problem with cassette dosing, which is why it is important to minimize the potential for DDIs (White and Manitpisitkul, 2001).

Understanding of liver physiological parameters and human xenobiotic protein expression in human liver chimeric mice is also necessary for quantitative extrapolation to humans. Hepatic flow patterns in human repopulated non-obese diabetic FRG (hFRGN) mice remain intact and hFRGN mice produce a lot of human-specific bile acid (Chow et al., 2016). Protein expression of MRP2 and SULT1A1 was similar in hFRGN and human liver tissue, but that of UGT1A1 was slightly higher in hFRGN mouse livers (Chow et al., 2016). Another report showed that the protein expression levels of the quantified metabolic enzymes and transporters in human liver chimeric PXB mice closely reflected the levels in human donor liver (Ohtsuki et al., 2014). In addition, humanized TK-NOG livers also expressed many human mRNAs, including phase I/II enzymes, drug transporters, and nuclear receptors (Hasegawa et al., 2011). Considering these previous reports, most human metabolic enzymes and drug transporters in the chimeric mouse livers appear to be maintained at levels comparable to human donors. These
facts are consistent with our results. Moreover, pharmacokinetic regulators involved in the hepatic uptake function in Hu-FRG™ mice appear to be maintained according to our in vitro results (Figure S4). Even so, the effects of the remaining murine hepatocytes in the humanized mice must be considered for their use. Specifically, comparing the results in the humanized mice and control mice is important. Moreover, it may be necessary to correct the predictive correlation between hepatocyte donors using representative compounds when implementing these humanized models in the pharmaceutical industry.

In conclusion, our findings suggest that both OATP-mediated disposition and CL_{bile} can be predicted using Hu-FRG™ mice. This is the first mechanism-based in vivo approach to accurately predict OATP-mediated DDI and CL_{bile}. We believe this new platform using humanized mouse models will accelerate the progress of drug discovery and development.
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Data Availability Statement

The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data.

Authorship Contributions

Participated in research design: Miyake, Tsutsui, and Hirabayashi.

Conducted experiments: Miyake, Tsutsui, and Hirabayashi.

Contributed new reagents or analytic tools: Tachibana.

Performed data analysis: Miyake, Tsutsui, and Tachibana.

Wrote or contributed to the writing of the manuscript: Miyake, Tsutsui, Hirabayashi, and Tachibana.
References


liver model: protein quantification of transporters, cytochromes P450, and UDP-glucuronosyltransferases by LC-MS/MS. *Drug Metab Dispos* **42**:1039-1043.


Footnotes

No author has an actual or perceived conflict of interest with the contents of this article. This work received no external funding.
Figure Legends

Figure 1. Plasma concentration–time profiles of the six substrates in Hu-FRG™ and Mu-FRG™ mice after intravenous administration without treatment or pretreated with rifampicin (oral 100 mg kg⁻¹)

Circles represent plasma concentrations in Hu-FRG™ mice with (closed) or without rifampicin (open). Inverted triangles represent plasma concentrations in Mu-FRG™ mice with (closed) or without rifampicin (open). Mice (n = 4) were intravenously administered atorvastatin (1 mg kg⁻¹, a), fexofenadine (1 mg kg⁻¹, b), glibenclamide [1 or 0.5 (Hu-FRG™ mice with rifampicin only) mg kg⁻¹, c], pitavastatin (1 mg kg⁻¹, d), pravastatin (1 mg kg⁻¹, e), and rosuvastatin (1 mg kg⁻¹, f). Data are shown as means ± S.D. of 4 mice.

Figure 2. Pharmacokinetic correlations in CLh,int of the OATP substrates with or without rifampicin

(a and b) Correlations of human CLh,int with Hu-FRG™ mouse CLh,int (R² = 0.82, p = 0.013, a) and Mu-FRG™ mouse CLh,int (R² and p values were not calculated, b). (c and d) Correlations of human CLh,int with Hu-FRG™ mouse CLh,int (R² = 0.96, p = 0.0008, c) and Mu-FRG™ mouse CLh,int (R² = 0.45, p = 0.14, d) when used in combination with rifampicin. Human CLh,int,rifa was calculated using Fhrifa value as shown in Methods. The solid line represents a nonlinear regression line empirically (power) fitted to the data for all substrates, and the dotted lines represent 3-fold or 1/3-fold the regression value (a). R² and p values were calculated from correlation analysis.
Figure 3. Pharmacokinetic correlations of the OATP substrates in the DDI study

(a and b) Correlations of human AUC<sub>po</sub> with Hu-FRG™ mouse AUC<sub>iv</sub> (R<sup>2</sup> = 0.44, p = 0.15, a) and Mu-FRG™ mouse AUC<sub>iv</sub> (R<sup>2</sup> = 0.0032, p = 0.92, b). (c and d) Correlations in CL<sub>h</sub> ratio between humans and Hu-FRG™ mice (R<sup>2</sup> = 0.94, p = 0.0013, c) and humans and Mu-FRG™ mice (R<sup>2</sup> = 0.028, p = 0.75, d). The solid lines represent simple linear regression.

Figure 4. Plasma concentration–time profiles of the twenty substrates in Hu-FRG™ and Mu-FRG™ mice after intravenous administration

Gallbladder-cannulated mice were intravenously administered Group 1 (compounds 1-10, 0.5 mg kg<sup>-1</sup>) or Group 2 (compounds 11-20, 0.5 mg kg<sup>-1</sup>). The Hu-FRG™ mouse data are shown as means ± S.D. of 6 mice (Group 1) and 3 mice (Group 2). The Mu-FRG™ mouse data are shown as means ± S.D. of 4 mice (Group 1 and Group 2). Cyclosporine A, epirubicin, erythromycin, fexofenadine, indocyanine green, paclitaxel, pravastatin, rosvastatin, and valsartan are in Group1. Cefazolin, cefixime, cefoperazone, cefotetan, cefpiramide, ceftriaxone, ciprofloxacin, diclofenac, irinotecan, and ranitidine are in Group2.

Figure 5. Biliary excretion rate–time profiles of the twenty substrates in Hu-FRG™ and Mu-FRG™ mice after intravenous administration

Gallbladder-cannulated mice were intravenously administered Group 1 (compounds 1-10, 0.5 mg kg<sup>-1</sup>) or Group 2 (compounds 11-20, 0.5 mg kg<sup>-1</sup>). The Hu-FRG™ mouse data are shown as means ± S.D. of 6 mice (Group 1) and 3 mice (Group 2). The Mu-FRG™ mouse data are shown as means ± S.D. of 4 mice (Group 1 and Group 2). Cyclosporine A, epirubicin, erythromycin, fexofenadine, indocyanine green, paclitaxel, pravastatin, rosuvastatin, and valsartan are in Group1. Cefazolin, cefixime, cefoperazone, cefotetan, cefpiramide, ceftriaxone, ciprofloxacin, diclofenac, irinotecan, and ranitidine are in Group2.
green, paclitaxel, pitavastatin, pravastatin, rosuvastatin, and valsartan are in Group1. Cefazolin, cefixime, cefoperazone, cefotetan, cefpiramide, ceftriaxone, ciprofloxacin, diclofenac, irinotecan, and ranitidine are in Group2.

**Figure 6. Correlation in CL bile**

(a and b) Correlation in CL bile between Hu-FRG™ mice and humans (R² = 0.563, RMSE = 0.887, a), and between Mu-FRG™ mice and humans (R² = 0.027, RMSE = 2.741, b). Black circles represent Group 1 (compounds 1-10) and gray circles represent Group 2 (compounds 11-20). The Hu-FRG™ mouse data are shown as geometric means of 6 mice (Group 1) and 3 mice (Group 2). The Mu-FRG™ mouse data are shown as geometric means of 4 mice (Group 1 and Group 2). The solid lines represent linear regression, and the dotted lines represent 3 or 4-fold or 1/3 or 1/4-fold the regression value.

**Figure 7. Prediction accuracy of human pharmacokinetics using Hu-FRG™ mice**

(a) Predicted and observed CLh,int. Predicted CLh,int was converted by the correlation equation in Figure 2a. Solid and dotted line represents unity and ±3-fold range, respectively. (b) Predicted and observed CLh ratio. Predicted CLh ratio was converted by the correlation equation in Figure 3c. Solid and dotted line represents unity and ±30% range, respectively. (c) Predicted and observed CL bile. Predicted CL bile was converted by the correlation equation in Figure 6a. Solid, dotted, and dashed line represents unity, ±3-fold range, and ±4-fold range, respectively. 1, Cyclosporine A; 2, Epirubicin; 3, Erythromycin; 4, Fexofenadine; 5, Indocyanine green; 6, Paclitaxel; 7, Pitavastatin; 8, Pravastatin; 9, Rosuvastatin; 10, Valsartan; 11, Cefazolin; 12, Cefixime; 13, Cefoperazone; 14, Cefotetan; 15, Cefpiramide; 16, Ceftriaxone; 17, Ciprofloxacin; 18, Diclofenac; 19, Irinotecan; 20, Ranitidine.
Tables

Table 1. Pharmacokinetic characteristics and Human CL\textsubscript{bile} of the substrates. The pharmacokinetic characteristics of the compounds were collected using DIDB.

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<tr>
<th>Compound</th>
<th>ECCS\textsuperscript{a}</th>
<th>Uptake transporters</th>
<th>Efflux transporters</th>
<th>Metabolic enzymes</th>
<th>Human CL\textsubscript{bile} (mL min\textsuperscript{-1} kg\textsuperscript{-1}) \textsuperscript{b}</th>
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<tr>
<td>Ceftriaxone</td>
<td>OATP1B3, OAT</td>
<td>-</td>
<td>-</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>OATP1A2, OAT1, OAT3, OCT</td>
<td>P-gp, BCRP, MRP4</td>
<td>SULT2A1</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Diclofenac</td>
<td>OATP1B3, OAT2</td>
<td>BCRP</td>
<td>CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP3A4, UGT1A3, UGT1A6, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B10, UGT2B17</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Irinotecan</td>
<td>OAT2</td>
<td>P-gp, BCRP, MRP2</td>
<td>BuChE, CES1, CES2, CYP3A4, CYP3A5, UGT1A1</td>
<td>0.94</td>
<td></td>
</tr>
<tr>
<td>Ranitidine</td>
<td>OAT1, OAT2, OAT3, OCT1, OCT2, OCT3, (OCT), NTCP</td>
<td>P-gp</td>
<td>CYP1A2, CYP2C19, CYP2D6, FMOs</td>
<td>0.15</td>
<td></td>
</tr>
</tbody>
</table>

Bold transporters and enzymes represent in vivo substrates.

\(^a\) ECCS classification is based on previous reports (Kimoto et al., 2017; Steyn and Varma, 2020; Varma et al., 2015).

\(^b\) Human CL\(_{\text{bile}}\) other than that for pitavastatin are referenced from previous reports (Grime and Paine, 2013; Kimoto et al., 2017). T-tube method (in most cases) or duodenal perfusion method were used for bile collection.

\(^c\) Pitavastatin CL\(_{\text{bile}}\) was calculated using the following equation: 

\[
\text{CL}_{\text{bile}} = \text{CL}_{\text{total},p} \times [\text{Fecal excretion rate} - (1 - \text{FaFg})] 
\]

(FDA drug approval package for pitavastatin, NDA #022363, Kowa Company, Ltd.).

\(^d\) ‘-‘ means no report in DIBD.
Table 2. Pharmacokinetic parameters of the six OATP substrates in Hu-FRG™ and Mu-FRG™ mice

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Parameter</th>
<th>Hu-FRG™ (pretreated with rifampicin)</th>
<th>Hu-FRG™</th>
<th>Mu-FRG™ (pretreated with rifampicin)</th>
<th>Mu-FRG™</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atorvastatin (i.v.)</td>
<td>Dose (mg kg⁻¹)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>AUC (ng hr mL⁻¹)</td>
<td>832 ± 141</td>
<td>3670 ± 530</td>
<td>202 ± 36</td>
<td>993 ± 199</td>
</tr>
<tr>
<td></td>
<td>CL_total,p (mL min⁻¹ kg⁻¹)</td>
<td>20.5 ± 3.5</td>
<td>4.62 ± 0.76***</td>
<td>84.4 ± 15.1</td>
<td>17.2 ± 2.9***</td>
</tr>
<tr>
<td></td>
<td>Vss (L kg⁻¹)</td>
<td>0.939 ± 0.156</td>
<td>0.277 ± 0.023***</td>
<td>0.627 ± 0.205</td>
<td>0.208 ± 0.023***</td>
</tr>
<tr>
<td></td>
<td>T₁/₂ (hr)</td>
<td>2.37 ± 0.47</td>
<td>1.49 ± 0.78</td>
<td>0.641 ± 0.0396</td>
<td>0.517 ± 0.358</td>
</tr>
<tr>
<td></td>
<td>Urinary excretion (%)</td>
<td>0.347 ± 0.152</td>
<td>1.68 ± 0.287</td>
<td>0.0113 ± 0.0159</td>
<td>0.0776 ± 0.116</td>
</tr>
<tr>
<td></td>
<td>AUCR_IV,FRG</td>
<td>4.41</td>
<td>4.92</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CL_h ratio</td>
<td>4.50</td>
<td>4.91</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fexofenadine (i.v.)</td>
<td>Dose (mg kg⁻¹)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>AUC (ng hr mL⁻¹)</td>
<td>806 ± 87</td>
<td>2100 ± 390</td>
<td>341 ± 34</td>
<td>3920 ± 950</td>
</tr>
<tr>
<td></td>
<td>CL_total,p (mL min⁻¹ kg⁻¹)</td>
<td>20.9 ± 2.4</td>
<td>8.12 ± 1.35****</td>
<td>49.2 ± 4.7</td>
<td>4.44 ± 1.06****</td>
</tr>
<tr>
<td></td>
<td>Vss (L kg⁻¹)</td>
<td>0.732 ± 0.08</td>
<td>0.801 ± 0.248</td>
<td>1.96 ± 0.23</td>
<td>0.544 ± 0.158****</td>
</tr>
<tr>
<td></td>
<td>T₁/₂ (hr)</td>
<td>1.24 ± 0.09</td>
<td>3.02 ± 0.31****</td>
<td>1.33 ± 0.17</td>
<td>2.14 ± 0.46*</td>
</tr>
<tr>
<td></td>
<td>Urinary excretion (%)</td>
<td>21.6 ± 4.99</td>
<td>13.4 ± 1.64</td>
<td>9.28 ± 4.41</td>
<td>9.59 ± 3.03</td>
</tr>
<tr>
<td></td>
<td>AUCR_IV,FRG</td>
<td>2.61</td>
<td>11.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CL_h ratio</td>
<td>2.33</td>
<td>11.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glibenclamide (i.v.)</td>
<td>Dose (mg kg⁻¹)</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>AUC (ng hr mL⁻¹)</td>
<td>6710 ± 1840</td>
<td>5800 ± 1190</td>
<td>1780 ± 390</td>
<td>7270 ± 3580</td>
</tr>
<tr>
<td></td>
<td>CL_total,p (mL min⁻¹ kg⁻¹)</td>
<td>2.63 ± 0.72</td>
<td>1.49 ± 0.34*</td>
<td>9.71 ± 1.99</td>
<td>2.64 ± 0.96***</td>
</tr>
<tr>
<td></td>
<td>Vss (L kg⁻¹)</td>
<td>0.0922 ± 0.0433</td>
<td>0.141 ± 0.028</td>
<td>0.172 ± 0.035</td>
<td>0.19 ± 0.064</td>
</tr>
<tr>
<td></td>
<td>T₁/₂ (hr)</td>
<td>1.00 ± 0.04</td>
<td>1.55 ± 0.7</td>
<td>0.89 ± 0.073</td>
<td>1.26 ± 0.46</td>
</tr>
<tr>
<td></td>
<td>Urinary excretion (%)</td>
<td>0.039 ± 0.0399</td>
<td>0.049 ± 0.00472</td>
<td>0.00943 ± 0.00572</td>
<td>0.0439 ± 0.0277</td>
</tr>
<tr>
<td></td>
<td>AUCR_IV,FRG</td>
<td>1.73</td>
<td>4.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CL_h ratio</td>
<td>1.77</td>
<td>3.68</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pitavastatin (i.v.)</td>
<td>Dose (mg kg⁻¹)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>AUC (ng hr mL⁻¹)</td>
<td>450 ± 61</td>
<td>490 ± 71</td>
<td>430 ± 61</td>
<td>490 ± 71</td>
</tr>
<tr>
<td></td>
<td>CL_total,p (mL min⁻¹ kg⁻¹)</td>
<td>2.13 ± 0.34</td>
<td>1.52 ± 0.240.34</td>
<td>1.97 ± 0.23</td>
<td>1.46 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>Vss (L kg⁻¹)</td>
<td>0.109 ± 0.023</td>
<td>0.121 ± 0.028</td>
<td>0.108 ± 0.035</td>
<td>0.112 ± 0.035</td>
</tr>
<tr>
<td></td>
<td>T₁/₂ (hr)</td>
<td>0.89 ± 0.04</td>
<td>1.55 ± 0.7</td>
<td>0.89 ± 0.073</td>
<td>1.26 ± 0.46</td>
</tr>
<tr>
<td></td>
<td>Urinary excretion (%)</td>
<td>0.019 ± 0.018</td>
<td>0.039 ± 0.00472</td>
<td>0.00943 ± 0.00572</td>
<td>0.0439 ± 0.0277</td>
</tr>
<tr>
<td></td>
<td>AUCR_IV,FRG</td>
<td>1.73</td>
<td>4.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CL_h ratio</td>
<td>1.77</td>
<td>3.68</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Data are shown as means ± S.D. of 4 mice. Urinary excretion (%) was obtained from another study at a dose of 0.2 mg kg⁻¹. 

CL_{total,p}, V_{ss}, and T_{1/2} were statistically compared to the non-treatment of rifampicin group (*p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001).
Table 3. Pharmacokinetic parameters of the twenty compounds in gallbladder-cannulated Hu-FRG™ and Mu-FRG™ mice

<table>
<thead>
<tr>
<th>Compound (Group 1)</th>
<th>Parameter</th>
<th>Hu-FRG™</th>
<th>Mu-FRG™</th>
<th>Compound (Group 2)</th>
<th>Parameter</th>
<th>Hu-FRG™</th>
<th>Mu-FRG™</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclosporine A</td>
<td>AUC (ng h mL⁻¹)</td>
<td>549 ± 192</td>
<td>786 ± 314</td>
<td>Cefazolin</td>
<td>AUC (ng h mL⁻¹)</td>
<td>3330 ± 1670</td>
<td>2020 ± 650</td>
</tr>
<tr>
<td></td>
<td>CL&lt;sub&gt;total,p&lt;/sub&gt; (mL min⁻¹ kg⁻¹)</td>
<td>16.8 ± 5.7</td>
<td>12 ± 4.9</td>
<td></td>
<td>CL&lt;sub&gt;total,p&lt;/sub&gt; (mL min⁻¹ kg⁻¹)</td>
<td>2.9 ± 1.24</td>
<td>4.44 ± 1.28</td>
</tr>
<tr>
<td></td>
<td>V&lt;sub&gt;s&lt;/sub&gt; (L kg⁻¹)</td>
<td>4.63 ± 0.6</td>
<td>2.78 ± 0.73</td>
<td></td>
<td>V&lt;sub&gt;s&lt;/sub&gt; (L kg⁻¹)</td>
<td>0.273 ± 0.116</td>
<td>0.129 ± 0.015</td>
</tr>
<tr>
<td>Biliary excretion</td>
<td>(%)</td>
<td>0.993 ± 0.618</td>
<td>0.0971 ± 0.0781</td>
<td></td>
<td>Biliary excretion</td>
<td>1.83 ± 0.988</td>
<td>2.01 ± 1.02</td>
</tr>
<tr>
<td></td>
<td>CL&lt;sub&gt;bile&lt;/sub&gt; (mL min⁻¹ kg⁻¹)</td>
<td>0.162 ± 0.103</td>
<td>0.0139 ± 0.0133</td>
<td></td>
<td>CL&lt;sub&gt;bile&lt;/sub&gt; (mL min⁻¹ kg⁻¹)</td>
<td>0.044 ± 0.00662</td>
<td>0.0803 ± 0.028</td>
</tr>
<tr>
<td>Epirubicin</td>
<td>AUC (ng h mL⁻¹)</td>
<td>202 ± 124</td>
<td>259 ± 115</td>
<td>Cefixime</td>
<td>AUC (ng h mL⁻¹)</td>
<td>7700 ± 6490</td>
<td>3800 ± 1520</td>
</tr>
<tr>
<td></td>
<td>CL&lt;sub&gt;total,p&lt;/sub&gt; (mL min⁻¹ kg⁻¹)</td>
<td>56.4 ± 31.1</td>
<td>37.3 ± 16.3</td>
<td></td>
<td>CL&lt;sub&gt;total,p&lt;/sub&gt; (mL min⁻¹ kg⁻¹)</td>
<td>1.61 ± 0.97</td>
<td>2.43 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>V&lt;sub&gt;s&lt;/sub&gt; (L kg⁻¹)</td>
<td>7.41 ± 3.53</td>
<td>7.16 ± 5.24</td>
<td></td>
<td>V&lt;sub&gt;s&lt;/sub&gt; (L kg⁻¹)</td>
<td>0.361 ± 0.166</td>
<td>0.158 ± 0.033</td>
</tr>
<tr>
<td>Biliary excretion</td>
<td>(%)</td>
<td>12.9 ± 4.05</td>
<td>9.7 ± 3.97</td>
<td></td>
<td>Biliary excretion</td>
<td>3.12 ± 1.16</td>
<td>10.3 ± 6.24</td>
</tr>
<tr>
<td></td>
<td>CL&lt;sub&gt;bile&lt;/sub&gt; (mL min⁻¹ kg⁻¹)</td>
<td>8.05 ± 5.77</td>
<td>3.62 ± 1.68</td>
<td></td>
<td>CL&lt;sub&gt;bile&lt;/sub&gt; (mL min⁻¹ kg⁻¹)</td>
<td>0.047 ± 0.0306</td>
<td>0.219 ± 0.0901</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>AUC (ng h mL⁻¹)</td>
<td>334 ± 219</td>
<td>282 ± 241</td>
<td>Cefoperazone</td>
<td>AUC (ng h mL⁻¹)</td>
<td>1840 ± 670</td>
<td>1170 ± 280</td>
</tr>
<tr>
<td></td>
<td>CL&lt;sub&gt;total,p&lt;/sub&gt; (mL min⁻¹ kg⁻¹)</td>
<td>34.9 ± 20.1</td>
<td>46.4 ± 30.7</td>
<td></td>
<td>CL&lt;sub&gt;total,p&lt;/sub&gt; (mL min⁻¹ kg⁻¹)</td>
<td>4.93 ± 1.71</td>
<td>7.39 ± 1.65</td>
</tr>
<tr>
<td></td>
<td>( V_{ss} ) (L kg(^{-1}))</td>
<td>Biliary excretion (%)</td>
<td>( CL_{ss} ) (mL min(^{-1}) kg(^{-1}))</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Fexofenadine</td>
<td>1.54 ± 0.59</td>
<td>10.6 ± 1.7</td>
<td>3.71 ± 2.09</td>
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<tr>
<td></td>
<td>1.38 ± 0.53</td>
<td>1.23 ± 0.73</td>
<td>0.452 ± 0.236</td>
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<td></td>
</tr>
<tr>
<td>Cefotetan</td>
<td>0.476 ± 0.178</td>
<td>9.63 ± 4.67</td>
<td>0.437 ± 0.163</td>
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<tr>
<td></td>
<td>0.14 ± 0.019</td>
<td>30.1 ± 10.8</td>
<td>2.11 ± 0.498</td>
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<tr>
<td>Indocyanine</td>
<td>0.794 ± 0.163</td>
<td>8.33 ± 2.08</td>
<td>0.761 ± 0.381</td>
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</tr>
<tr>
<td>green</td>
<td>0.749 ± 0.16</td>
<td>17 ± 2.12</td>
<td>1.72 ± 0.494</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Cefpiramide</td>
<td>0.464 ± 0.278</td>
<td>5.82 ± 2.94</td>
<td>0.153 ± 0.0747</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>0.185 ± 0.044</td>
<td>3.55 ± 1.76</td>
<td>0.188 ± 0.0766</td>
<td></td>
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</tr>
<tr>
<td>Paclitaxel</td>
<td>0.14 ± 0.038</td>
<td>25.2 ± 8.43</td>
<td>1.38 ± 1.04</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>0.0492 ± 0.0061</td>
<td>20.8 ± 3.95</td>
<td>0.259 ± 0.0751</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>0.442 ± 0.22</td>
<td>12.7 ± 5.52</td>
<td>0.189 ± 0.134</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.17 ± 0.02</td>
<td>36.3 ± 16.6</td>
<td>1.67 ± 0.523</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( V_{ss} \) (L kg\(^{-1}\)) and Biliary excretion (%) for different drugs are presented above.
<table>
<thead>
<tr>
<th></th>
<th>Pitavastatin</th>
<th>Ciprofloxacin</th>
<th>Pravastatin</th>
<th>Diclofenac</th>
<th>Rosuvastatin</th>
<th>Irinotecan</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC (ng h mL⁻¹)</td>
<td>299 ± 111</td>
<td>1330 ± 680</td>
<td>961 ± 311</td>
<td>515 ± 299</td>
<td>1330 ± 680</td>
<td>515 ± 299</td>
</tr>
<tr>
<td>CLslop (mL min⁻¹ kg⁻¹)</td>
<td>31.8 ± 13.4</td>
<td>1710 ± 420</td>
<td>96 ± 311</td>
<td>641 ± 213</td>
<td>1330 ± 680</td>
<td>515 ± 299</td>
</tr>
<tr>
<td>Vₚ (L kg⁻¹)</td>
<td>0.656 ± 0.079</td>
<td>0.641 ± 0.213</td>
<td>0.641 ± 0.213</td>
<td>0.641 ± 0.213</td>
<td>0.656 ± 0.079</td>
<td>0.641 ± 0.213</td>
</tr>
<tr>
<td>Biliary excretion (%)</td>
<td>25.2 ± 10.8</td>
<td>60.7 ± 26.6</td>
<td>44.9 ± 3.72</td>
<td>44.9 ± 3.72</td>
<td>25.2 ± 10.8</td>
<td>44.9 ± 3.72</td>
</tr>
</tbody>
</table>

**Vss (L kg⁻¹)**
- Pitavastatin: 2.14 ± 0.56, 0.255 ± 0.057
- Ciprofloxacin: 0.209 ± 0.03
- Pravastatin: 0.37 ± 0.053, 0.37 ± 0.053
- Diclofenac: 0.209 ± 0.03
- Rosuvastatin: 0.118 ± 0.016
- Irinotecan: 0.118 ± 0.016

**Biliary excretion (%)**
- Pitavastatin: 0.624 ± 0.354, 0.255 ± 0.057
- Ciprofloxacin: 13.5 ± 2.58
- Pravastatin: 13.5 ± 2.58
- Diclofenac: 13.5 ± 2.58
- Rosuvastatin: 0.624 ± 0.354, 0.255 ± 0.057
- Irinotecan: 13.5 ± 2.58

**CLslop (mL min⁻¹ kg⁻¹)**
- Pitavastatin: 0.0774 ± 0.0417, 0.036 ± 0.0287
- Ciprofloxacin: 0.036 ± 0.0287
- Pravastatin: 0.0774 ± 0.0417, 0.036 ± 0.0287
- Diclofenac: 0.036 ± 0.0287
- Rosuvastatin: 0.0774 ± 0.0417, 0.036 ± 0.0287
- Irinotecan: 0.0774 ± 0.0417, 0.036 ± 0.0287
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value 1</th>
<th>± S.D. 1</th>
<th>Value 2</th>
<th>± S.D. 2</th>
<th>Value 3</th>
<th>± S.D. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{ss}$ (L kg$^{-1}$)</td>
<td>0.392 ± 0.043</td>
<td>0.254 ± 0.031</td>
<td>2.96 ± 1.41</td>
<td>10.7 ± 2.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biliary excretion (%)</td>
<td>48.8 ± 18.4</td>
<td>67.7 ± 7.79</td>
<td>2.89 ± 1.29</td>
<td>0.297 ± 0.0382</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL$_{bile}$ (mL min$^{-1}$ kg$^{-1}$)</td>
<td>3.79 ± 2.24</td>
<td>15.3 ± 5.65</td>
<td>0.544 ± 0.264</td>
<td>0.292 ± 0.0618</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Hu-FRG™ data are shown as means ± S.D. of 6 mice (Group 1) and 3 mice (Group 2). Mu-FRG™ data are shown as means ± S.D. of 4 mice (Group 1 and Group 2).
Figure 1

(a) Atorvastatin
(b) Fexofenadine
(c) Glibenclamide
(d) Pitavastatin
(e) Pravastatin
(f) Rosuvastatin

Legend:
- Humanized
- Humanized + Rifampicin
- Murinized
- Murinized + Rifampicin

Data points and error bars represent mean ± standard deviation.
Figure 2
Figure 3
Figure 4
Figure 6

Figure 7