Analysis of the bile acid composition in a fibroblast growth factor 19-expressing liver-humanized mouse model and its use for CYP3A4-mediated drug-drug interaction studies


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Nonstandard abbreviations used:
AUC, Area under the concentration time curve; AUC/D, Dose normalized AUC; AUC_{0-t}, Area under the concentration time curve from time 0 to timepoint t; AUC_{0-t/D}, AUC_{0-t} devided by the dose; BA, bile acid; CA, cholic acid (G-CA, glycine conjugated CA; T-CA, taurine conjugated CA); CDCA, chenodeoxycholic acid; C_{max}, Peak concentration; C_{max/D}, Dose normalized C_{max}; CYP, Cytochrome P450; DCA, deoxycholic acid; DDI, drug-drug interaction; FAH, fumarylacetoacetate hydrolase; FGF15, murine fibroblast growth factor-15; FGF19, human fibroblast growth factor-19; FRGN, Fah^{-/-}, Rag2^{-/-}, Il2rg^{-/-}, NOD mouse model; FRGN19, FRGN mouse model expressing human FGF19; huFRGN/huFRGN19,
chimeric FRGN/FRGN19 mice repopulated with human hepatocytes; huFRGN19SD, huFRGN19 mice receiving standard diet; huFRGN19YF, huFRGN19 mice receiving YF-10 mouse chow; LCA, lithocholic acid; LC-HRMS, liquid chromatography high resolution mass spectrometry; MCA, muricholic acid; NTBC, 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione; P/M, Parent/ Metabolite ratio; P/M C\text{max}, Parent/ Metabolite ratio over C\text{max}; P/M AUC\text{0-t}, Parent/ Metabolite ratio over AUC\text{0-t}; PXR, pregnane X receptor; qRT-PCR, quantitative reverse transcription real-time PCR; RIF, rifampicin; T\text{max}, Time to peak concentration; TRZ, triazolam
Abstract

Numerous biomedical applications have been described for liver-humanized mouse models, such as in drug metabolism or drug-drug interaction (DDI) studies. However, the strong enlargement of the bile acid (BA) pool due to lack of recognition of murine intestine-derived fibroblast growth factor-15 (FGF15) by human hepatocytes and a resulting up-regulation in the rate-controlling enzyme for BA synthesis, Cytochrome P450 (CYP) 7A1, may pose a challenge in interpreting the results obtained from such mice. To address this challenge, the human FGF19 gene was inserted into the Fah<sup>−/−</sup>, Rag2<sup>−/−</sup>, Il2rg<sup>−/−</sup>, NOD (FRGN) mouse model, allowing repopulation with human hepatocytes capable of responding to FGF19. While a decrease in CYP7A1 expression in human hepatocytes from humanized FRGN19 mice (huFRGN19) and a concomitant reduction in BA production was previously shown, a detailed analysis of the BA pool in these animals has not been elucidated. Furthermore, there is sparse data on the use of this model to assess potential clinical DDI. In the present work, the change in BA composition in huFRGN19 compared to huFRGN control animals was systematically evaluated, and the ability of the model to recapitulate a clinically described CYP3A4-mediated DDI was assessed. In addition to a massive reduction in the total amount of BA, FGF19 expression in huFRGN19 mice resulted in significant changes in the profile of various primary, secondary, and sulfated BAs in serum and feces. Moreover, as observed clinically, administration of the pregnane X receptor agonist rifampicin reduced the oral exposure of the CYP3A4 substrate triazolam.
Significance statement:

Transgenic expression of FGF19 normalizes the unphysiologically high level of bile acids in a chimeric liver humanized mouse model and leads to massive changes in bile acid composition. These adaptations could overcome one of the potential impediments in the use of these mouse models for drug-drug interaction studies.
Introduction

Liver-humanized mouse models in which mouse hepatocytes are largely replaced by transplanted human liver cells have potential utility in testing compounds intended for use in humans (Grompe and Strom, 2013; Peltz, 2013; Scheer and Wilson, 2016; Naritomi et al., 2018; Tateno and Kojima, 2020). Different types of liver-humanized mouse models have been described, such as uPA-SCID (Tateno et al., 2004), TK-NOG (Hasegawa et al., 2011b) and FRG (Azuma et al., 2007). Common traits of these models are that they are immune-compromised and allow the deletion of mouse hepatocytes, albeit in different ways. Both features enable an efficient replacement of murine hepatocytes with transplanted human hepatocytes. Such liver-humanized mouse models have a variety of potential applications, for example in studies of human-liver infectious diseases (Dandrieu et al., 2001; Mercer et al., 2001; Kosaka et al., 2013; Sayed et al., 2017; Tyagi et al., 2018), the testing of gene therapy vectors (Kakuni et al., 2013; Lisowski et al., 2014) or of other therapeutic agents. With regard to the latter, there are numerous studies in which these models were used, for example, in drug metabolism (Sanoh et al., 2015; Nakada et al., 2016; Dickie et al., 2017; Wilson et al., 2018; Uehara et al., 2022b), hepatotoxicity (Xu et al., 2014; Xu et al., 2015b; Xu et al., 2015a; Nihira et al., 2019; Eguchi et al., 2021) or DDI studies (Hasegawa et al., 2012; Yamazaki et al., 2013; Uchida et al., 2018; Uehara et al., 2022a).

A challenge in the use of these chimeric mouse models is the possible disruption of communication between the murine and human cells, in that species-specific signaling molecules might not be recognized by the cells of the respective other species. A possible consequence of this is physiological abnormalities, which can significantly complicate the interpretation of the results obtained in these models. In this context, a dysregulation of hepatocyte proliferation and bile acid (BA) homeostasis in huFRGN livers and corresponding hepatotoxicity, gallbladder distension, and liver deformity have been reported (Chow et al.,
A key factor leading to dysregulation of BA homeostasis is the inability of human hepatocytes to recognize murine FGF15, which is released by mouse intestinal cells for inhibiting BA synthesis in the liver (Ellis et al., 2013). This unresponsiveness to FGF15 leads to an upregulation of the rate-controlling enzyme for BA synthesis, CYP7A1, in the human hepatocytes, explaining the increased amount of BAs and significant enlargement of the liver in these mice (Naugler et al., 2015). It should be noted that the aberrant liver-intestine BA signaling is presumably an inherent feature of all liver-humanized mouse models and not specific to the huFRGN mice.

To overcome the aforementioned problem, a bacterial artificial chromosome containing the genomic sequence for the human FGF19 gene including its regulatory sequences was stably inserted into the genome of FRGN mice (Naugler et al., 2015). A physiological regulation of FGF19 expression was demonstrated in these FRGN19 mice. Importantly, when huFRGN19 mice were generated by transplantation of human hepatocytes, a normalization of liver to body weight ratio, CYP7A1 expression and BA pool was observed, indicating that the human liver cells were capable of responding to FGF19. However, a systematic analysis of individual BAs, such as recently described for various species (Sangaraju et al., 2021), and a comparison to the situation in huFRGN mice has not yet been performed. Furthermore, while DDI studies particularly around the induction of the human drug metabolizing enzyme CYP3A4 with the human-specific pregnane x receptor (PXR)-agonist rifampicin (RIF), were reported in uPA-SCID (Hasegawa et al., 2012) and TK-NOG (Uehara et al., 2022a) mice, such data are currently lacking in huFRGN or huFRGN19 mice.

In the present work, a comprehensive quantification of various BA types in serum and feces of huFRGN and huFRGN19 mice using liquid chromatography high resolution mass spectrometry (LC-HRMS) was performed. Furthermore, the utility of both mouse models in recapitulating the effect of RIF on the pharmacokinetics of the CYP3A4 substrate triazolam.
(TRZ) was evaluated. Key observations were a massive reduction of total BA levels in huFRGN19 compared to huFRGN mice, a corresponding decrease of individual BAs, including several toxic species such as deoxycholic acid (DCA) and its conjugates, and a similar lowering of TRZ exposure following RIF treatment in both models.
Methods

Animals, diet and housing conditions.

For the drug-drug interaction study, 6-10 week old female Fah<sup>−/−</sup>; Rag2<sup>−/−</sup>; Il2rg<sup>−/−</sup> mice on a NOD background (FRGN, Yecuris) and FRGN mice carrying a BAC including the human FGF19 locus (Naugler et al., 2015) (FRGN19; Yecuris) were preconditioned with adenoviral urokinase plasminogen activator and repopulated with cryopreserved primary human hepatocytes from donor HHF13022 (Yecuris) using 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC, Yecuris) cycling as previously described (Azuma et al., 2007). For comparisons of bile acids in blood and feces, FRGN and FRGN19 mice were repopulated with male donor HHM18029 as described above. Characteristics of both human donors are presented in Supplemental Table 1. Mice were maintained on Pico Lab High Energy Mouse diet 5LJ5 (LabDiet, Inc) for 10-12 weeks post-transplant. Subsequently, all mice used for drug-drug interaction studies and a subset of mice used for bile acid studies were maintained on a synthetic, modified Baker synthetic rodent diet (Hirakawa et al., 1984) (YF-10, Yecuris) with amino acid levels optimized for sustaining liver-humanized FRGN mice. Liver humanization was determined by measurement of human albumin (hALB) in whole blood using a human-specific albumin antibody ELISA (Bethyl Laboratories Inc, Cat. # E80-129). Mice with hALB of > 4mg/mL (equivalent to > 70% human hepatocyte repopulation) were enrolled. Treatment of mice with NTBC or antibiotics was discontinued for at least 21 days prior to start of the study. All in vivo experiments were conducted at Yecuris corporation following an approved institutional animal use protocol.

Serum and feces collection for bile acid analysis.

Serum was prepared from blood collected from animals that were fasted overnight. Blood was collected into uncoated tubes, coagulated at room temperature for 1-2 hours and then centrifuged at 1200 g for 20 minutes at 4°C. To collect feces, fasted mice were placed in
individual caging without bedding for 1-2 hours. Fecal pellets were collected from each cage and snap frozen. All serum and feces were stored at -80°C or liquid nitrogen until processed for BA analysis.

**Bile acid analysis**

Bile acids (BAs) were quantified as described previously by liquid chromatography-high resolution mass spectrometry (LC-HRMS) analysis (Sangaraju et al., 2021). Briefly, fecal samples were homogenized in 80:20 methanol:water (10-fold dilution) and 25 µL of homogenate or 10 µL of serum was precipitated with acetonitrile containing 20 stable isotope labeled internal standards of BAs. Samples were then mixed and centrifuged at 3600 g for 10 min at 4°C followed by evaporation of supernatants under nitrogen flow. Dried supernatants were reconstituted in LC buffer A and subjected to LC-HRMS analysis as described previously (Sangaraju et al., 2021). Sample analysis also included surrogate matrix calibration curves and quality control samples, enabling the determination of absolute concentrations of >50 different BAs and evaluating batch performance. BA data analysis was also performed as described previously (Sangaraju et al., 2021). Briefly, individual BA concentration data was used for the calculation of various classes of total BAs (e.g. total DCA, total HCA etc.) followed by calculation of BA indices (percentages or ratios: e.g. %DCA, %HCA etc.) by normalizing to the overall total BA concentration within each sample. Detailed formulae used for calculations of various total BAs and BA indices were described previously (Sangaraju et al., 2021). BA data was plotted either as donut plots (Supplemental Figure 2) to show the BA distribution (% mono-hydroxy (1-OH), % di-hydroxy (2-OH), % tri-hydroxy (3-OH) and % oxo-iso-nor-dehydro (OIND) BAs) or their trends among different groups as described previously (Sangaraju et al., 2021) or as box plots (Figures 1A, 3 and 4). A pairwise statistical comparison of the box plot mean values was performed using the Wilcoxon signed-rank test (wilcox.test) in R. Mean +/- SD is shown for each of the groups within the boxplots.
Animal treatment for pharmacokinetic study and qRT-PCR analysis.

A 10mg/mL solution of Rifampicin (EMD Millipore) was prepared immediately prior to dosing in 10% DMSO. A 0.5mg/mL Triazolam (Lipomed) solution was prepared in cold 0.5% methylcellulose (400cp viscosity, Sigma-Aldrich) in 1X PBS. Liver humanized FRGN (huFRGN) and huFRGN19 mice were dosed once daily for 6 days p.o. 5mL/kg rifampicin (50 mg/kg) or 10% DMSO vehicle. Thirty minutes after the final rifampicin or vehicle dose, all mice were then dosed with 6mL/kg triazolam (3 mg/kg) p.o. Blood collection was subsequently performed on all animals at 15 minutes, 30 minutes, 1 h, 2 h, 4 h and 6 h post triazolam dosing. For plasma processing, blood was collected into EDTA coated collection tubes on ice and then centrifuged at 1200 g for 15 minutes at 4°C. Plasma was then removed and stored at -80°C or in liquid nitrogen until analysis. Six to eight hours after triazolam dosing, animals were terminally harvested following anesthesia with a ketamine cocktail. Livers were removed from the body cavity, rinsed briefly in ice cold 1X PBS, dabbed dry and then snap frozen in liquid nitrogen. Liver samples were stored at -80°C or liquid nitrogen until processed for RNA extraction.

qRT-PCR analysis.

RNA was isolated from manually homogenized frozen liver using the Invitrogen Purelink RNA mini kit. Homogenates made in kit-provided lysis buffer were passed through a 23 gauge needle 5 times, then processed per kit instructions and eluted in 100 µl RNase-free water. Total RNA was further purified and concentrated by ethanol precipitation in 0.3 M sodium acetate (pH 5.2). Next, 10 µg of RNA was treated with ThermoFisher RapidOut DNA removal kit at 37°C for 30 min per manufacturer’s instructions and then 1.5 µg of purified total RNA was reverse transcribed using an Applied Biosystems High-Capacity cDNA synthesis kit following the manufacturer’s instructions. qPCR was performed using PowerUp SYBR Green Master mix (Invitrogen) according to manufacturer’s instructions. Samples were
run in triplicate for genes of interest and duplicate for housekeeping genes in 10 µl reactions containing 15 ng cDNA and 500nM of forward and reverse gene-specific primers. For each test sample, all genes of interest and housekeeping genes were run on the same qPCR plate. Cycling conditions were 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Melt curve analysis was done when validating qPCR conditions and no alternate amplification products were detected. The sequences for qPCR primers used are provided in **Supplemental Table 2.**

**Pharmacokinetic analysis**

**Bioanalysis:** An intermediate solution was prepared from compound stock solution using 50 % acetonitrile (ACN). Calibrators S1 to S11 were prepared from intermediate solution using 50 % ACN. 50 µL of each calibrator was transferred to tubes containing 10 µL blank plasma, and 400 µL internal standard (dexamethasone, 40 nM, or d3-alpha-OH-triazolam, 550 nM, IS) dissolved in acetonitrile was added. To 10 µL of plasma sample, 50 µL of 50 % ACN was added. Then, 400 µL of IS solution was added. Following sample preparation, microplates were tightly closed and shaken. Subsequently, microplates were centrifuged for 10 min at 3000 g and 4°C. 125 µL of supernatant was transferred into a microplate and 125 µL of 0.1 % formic acid was added.

Triazolam, alpha-OH-Triazolam and 4-OH-Triazolam and spiked IS were extracted from samples (plasma) by protein precipitation with ACN. Chromatography of the extracted sample supernatant was conducted using a Waters Acquity BEH C18, 2.1x50 mm 1.7 µm column running a mobile phase gradient with a constant flow rate of 0.6 mL/min (mobile phase A: 95 % 10 mM ammonium acetate in water, 4 % methanol and 1 % acetonitrile, mobile phase B: 10 % 10 mM ammonium acetate in water, 80 % methanol and 10 % acetonitrile). For rifampicin, chromatography was performed using a Waters Acquity HSS T3, 2.1x50 mm 1.8 µm column running a mobile phase gradient with 0.7 mL/min (mobile phase
A: 0.1 % formic acid in water, mobile phase B: 0.1 % formic acid in Acetonitrile).

Chromatography of the extracted sample supernatant for detection of rifampicin was conducted using a Waters Acquity HSST3 C18, 2.1x50 mm 1.8 µm column running a mobile phase gradient with a constant flow rate of 0.7 mL/min (mobile phase A: 0.1 % formic acid in water, mobile phase B: 0.1 % formic acid in acetonitrile). Detection was performed on a Sciex 5500 Triple Quad mass spectrometer using electro spray ionization in positive mode. The following mass transitions were used: Triazolam 343.1>>308.1, internal standard Dexamethasone 393.2>>373.2, 4-OH-triazolam 359.03>>75, alpha-OH-triazolam 359>>330.9, internal standard d4-alpha-OH-triazolam 363.1>>243.05, rifampicin 824.5>>792.3 and its appropriate global internal standard 261.1>>95. Thereby, to improve the chromatographic range for co-eluting IS, a novel IS mix was created containing sixteen compounds structurally like tolbutamide, by incrementing each compound with one additional carbon. It was applied at a concentration of 200 nM. Analyte and IS peak areas were determined using Sciex Analyst version 1.7.1. The concentration of each sample was calculated by least squares regression analysis of the analyte/IS ratio using a calibration curve spiked with defined concentrations of analyte in equivalent matrix. The limits of quantification for triazolam were 0.97 ng/mL (lower limit of quantification; LLOQ) - 3705 ng/mL (upper limit of quantification; ULOQ), for 4-OH-triazolam 0.925 - 1415 ng/mL, for alpha-OH-triazolam 2.315 - 1415 ng/mL and for rifampicin 14.3 - 54500 ng/mL.

**PK Data Analysis:** The plasma concentration data were submitted to noncompartmental analysis using WinNonlin (WinNonlin-Professional®, Version 5.2.1, Pharsight Corporation, Mountain View, CA) and Pharmkin (PharmKin is a Macintosh Microsoft Excel add-in macro file which contains several custom functions). The area under the concentration-time curve from 0 to 6 hours after dosing (AUC\(_{0-6h}\)) was calculated using the linear trapezoidal rule for the concentration-time profiles. Maximum plasma concentration (C\(_{max}\)) is defined as the
maximum drug concentration observed in plasma over all PK sample concentrations and it was obtained from the $C_{\text{max}}$ parameter calculated by WinNonlin®. Time of maximum plasma concentration ($T_{\text{max}}$) is defined as the time at which the $C_{\text{max}}$ occurs, and it was obtained from the $T_{\text{max}}$ parameter calculated by WinNonlin®. The apparent terminal elimination half-life ($t_{1/2}$) is defined as the time required for the drug concentration to decrease by a factor of one-half in the terminal phase. $t_{1/2}$ can be estimated as $\ln(2) / K_e$ and it was obtained from the $HL_{\text{-Lambda}_z}$ parameter calculated by WinNonlin®.

**Statistical analysis:** Differences between groups of interest were assessed using parametric and non-parametric methods, such as two samples t-test, Welch test, chi-squared test and Wilcoxon/Kruskal-Wallis test. When there was evidence of non-homogeneity of variances (Levene’s test for homogeneity of variances), skewed data were log-transformed before testing. All p-values are uncorrected for multiplicity. JMP 14.1.0 (SAS institute), R studio Version 1.1.453 with R V4.0.5, and GraphPad Prism V.9.1.0 (GraphPad Software, Inc., San Diego, CA) were used for statistical analysis and data presentation. Significant differences are highlighted, with * = p < 0.05; ** = p < 0.01, and *** p < 0.001.
Results

The liver to body weight ratio is decreased in huFRGN19 compared to huFRGN mice.

Highly repopulated liver-humanized FRGN (huFRGN) and FRGN19 (huFRGN19) mice for the present study were generated by transplantation of FAH+/+ human hepatocytes from the same donor (Supplemental Table 1). It has been previously reported that the liver to body weight ratio is higher in huFRGN compared to wild type mice, and that this imbalance was largely corrected in huFRGN19 mice (Naugler et al., 2015). Therefore, we wanted to determine the effect of FGF19 expression on the liver to body weight ratio in the animals used in our DDI studies, which were treated with TRZ and either vehicle or RIF (see below).

The liver to body weight ratio in vehicle-treated huFRGN19 mice (8.52 %; n = 7) was significantly (p = 0.026) lower than in huFRGN mice (9.59 %; n = 7) (Supplemental Figure 1). The same trend was observed in the RIF-treated animals, but the reduction in the huFRGN19 (7.83 %; n = 7) relative to huFRGN mice (8.85 %; n = 7) was not significant (p = 0.056) in this case. No significant difference was observed between the RIF-treated huFRGN or huFRGN19 animals compared to the respective vehicle controls of the same genotype. It can therefore be concluded that the expression of FGF19 in the huFRGN19 mice leads to a slight decrease in the liver to body weight ratio, whereas the administered dose of RIF does not seem to have any effect on this ratio.

The total bile acid amount and the level of human CYP7A1 mRNA is lower in huFRGN19 compared to huFRGN mice.

Next, we investigated whether FGF19 expression leads to a reduction in total BA levels in huFRGN19 mice as previously described (Naugler et al., 2015). For this purpose, we collected serum and feces from untreated huFRGN and huFRGN19 mice and determined the total BA amount in these samples by LC-HRMS analysis. In the case of huFRGN19 mice, two different groups were included in this study, one receiving a standard diet (huFRGN19^SD)
and another on a synthetic, low tyrosine mouse chow (YF-10; huFRGN19\textsuperscript{VF}), whereas the huFRGN mice were kept on a standard diet. With this design, we intended to gain additional information about a possible influence of diet on BA levels in the huFRGN19 mice. The total mean BA amount in feces of huFRGN mice was 1077 ± 159 µM (n = 4), which was significantly decreased by 36-fold in huFRGN19\textsuperscript{SD} (30 ± 13 µM; n = 10) and 8-fold in huFRGN19\textsuperscript{VF} mice (136 ± 144 µM; n = 9) (Figure 1a). BA levels in the serum were significantly lower in FRGN19 mice on both diets, with a 4.3-fold decrease in total BA levels in huFRGN19\textsuperscript{SD} (25 ± 33 µM; n = 10) and an 18-fold decrease in huFRGN19\textsuperscript{VF} (6 ± 2 µM; n = 10) compared to huFRGN mice (108 ± 62 µM; n = 8). Although sulfated bile acids were observed in the fecal samples (data not shown), their levels were not considered for the interpretation of results as contamination of urine with feces was suspected. In general, BA sulfation metabolic capability in mice was previously reported to be very low (Alnouti, 2009). Since it has been shown that the reduction of BA levels in huFRGN19 mice is associated with a lower expression of the rate-limiting enzyme of BA synthesis, CYP7A1, in the human hepatocytes (Naugler et al., 2015), we compared the expression of human \textit{CYP7A1} mRNA in the livers of huFRGN19 mice with that in huFRGN mice. As no livers from mice used for the BA analysis were available for this purpose, \textit{CYP7A1} expression analysis was assessed in liver samples from the mice from the DDI study (see below). In vehicle-treated huFRGN19 mice (n = 7) there was a non-significant (p = 0.072) trend towards a 10-fold lower \textit{CYP7A1} mRNA expression than in vehicle-treated huFRGN mice (n = 7) (Figure 1b). We also assessed the effect of transgenic FGF19 expression on mouse \textit{Cyp7a1} mRNA levels. A trend similar to that described for human \textit{CYP7A1} was observed, i.e. lower murine \textit{Cyp7a1} expression in huFRGN19 compared to huFRGN mice, but with a statistically significant (p = 0.044) 3.3-fold reduction in vehicle-treated mice this effect was less pronounced than for human \textit{CYP7A1} (Figure 1b).
Taken together, these data show that (1) the expression of FGF19 leads to a very strong reduction of BA levels in the huFRGN19 mice, (2) the diets used in this study for huFRGN19 groups have relatively less effect on the BA quantities as compared to observed differences between huFRGN and huFRGN19 groups, and (3) the CYP7A1 mRNA expression in human hepatocytes and to a lesser extent the Cyp7a1 mRNA expression in mouse hepatocytes of the huFRGN19 mice is lower than in huFRGN mice.

Profound differences in the individual bile acid amounts and composition can be observed between huFRGN and huFRGN19 mice.

To gain more detailed insights into the changes of specific BAs in the huFRGN19 mice, the quantitative BA profile of a variety of individual BAs was compared between the huFRGN and huFRGN19 mice using a recently described LC-HRMS-based comprehensive targeted metabolomics method (Sangaraju et al., 2021). As above, huFRGN19 mice on the two different diets were again included in the analysis. The upper panel of Figure 2 shows the feces and the lower panel the serum BA pool chemical diversity for the three mouse groups analyzed in this study (see Supplemental Figure 2 for a more detailed breakdown of the individual BAs). It is notable that in both the feces and the serum of the two huFRGN19 groups studied, there were significantly more tri-hydroxy BAs but fewer di-hydroxy BAs than in the huFRGN mice. While di-hydroxy BAs accounted for 52 % of the total BAs in the feces (n = 4) and 38% in the serum (n = 8) of the huFRGN mice, this was only 14 % (n = 10) and 19 % (n = 9) in the feces and 5 % (n = 10) and 9 % (n = 10) in the serum of huFRGN19SD or huFRGN19YF mice, respectively. In contrast, huFRGN mice exhibited only 17 % tri-hydroxy BAs in feces and 55 % in serum, compared to 60 % and 63 % in feces and 87 % and 86 % in serum of huFRGN19SD or huFRGN19YF mice, respectively. No major difference was observed for the BA pools of the huFRGN19 mice receiving the two different diets.
A comprehensive description of all the BAs examined in the course of the present work is beyond the scope of this report, so in the following we focus on a few selected examples. For the interested reader, a complete list of the results is appended as a database (Supplemental Figure 3). A striking observation was the very strong reduction of the highly toxic BA deoxycholic acid (DCA) in the huFRGN19 relative to the huFRGN mice. The total amount of this BA equaled 500.23 ± 82.72 µM in the feces and 40.03 ± 32.46 µM in the serum of the huFRGN mice, but only 2.64 ± 3.38 µM and 17.91 ± 14.04 µM in the feces and 0.16 ± 0.25 µM and 0.04 ± 0.03 µM in the serum of the huFRGN19SD and huFRGN19VF mice, respectively (Figure 3a). This substantial reduction is also reflected in the relative contribution of total DCA to the amount of all BAs, which was 46.6 ± 6.0 % in the feces and 34.6 ± 18.4 % in the serum of the FRGN mice, but only 9.8 ± 12.4 % and 5.5 ± 6.8 % in the feces and 0.6 ± 0.8 % and 0.7 ± 0.5 % in the serum of the two groups of huFRGN19 mice, respectively (Figure 3b). The reduction of this BA in huFRGN19 mice affects both the unconjugated as well as the conjugated forms of DCA. The amount of unconjugated DCA (Figure 3c), glycine conjugated DCA (Figure 3d) and taurine conjugated (Figure 3e) was 484.288 ± 74.189 µM, 12.771 ± 13.417 µM and 3.170 ± 1.711 µM in feces and 6.627 ± 7.345 µM, 15.394 ± 16.123 µM and 16.538 ± 16.132 µM in serum of the huFRGN mice. In the huFRGN19SD mice the respective values were 2.620 ± 3.381 µM, 0.011 ± 0.0001 µM and 0.010 ± 0.0001 µM in feces and 0.023 ± 0.030 µM, 0.003 ± 0.005 µM and 0.040 ± 0.073 µM in serum, which was comparable to the amounts in feces (17.662 ± 13.807 µM, 0.078 ± 0.072 µM and 0.170 ± 0.222 µM) and serum (0.009 ± 0.007 µM, 0.002 ± 0.002 µM and 0.010 ± 0.012 µM) of the huFRGN19VF mice.

Another BA that warrants mention is muricholic acid (MCA), which is produced by mice but not humans (Li and Dawson, 2019). As with DCA, the total level of MCA in the huFRGN19 animals was lower than in the huFRGN mice. While the amount of MCA was 161.5 ± 27.2 µM in the feces and 14.2 ± 6.8 µM in the serum of the huFRGN mice, the corresponding
values were only 6.8 ± 3.8 µM and 3.6 ± 3.4 µM in the huFRGN19SD mice (Figure 4a). However, in contrast to DCA, the relative proportion of MCA in the total BA content did not decline in the huFRGN19 compared to the huFRGN mice, but actually slightly increased. In the huFRGN mice this proportion was 15.0 ± 1.8 % in the feces and 14.5 ± 4.2 % in the serum, compared to 22.6 ± 9.2 % or 19.9 ± 8.0 % in the feces and 17.7 ± 5.4 % or 37.6 ± 8.4 % in the serum of huFRGNSD and huFRGNYF mice, respectively (Figure 4b).

Furthermore, lithocholic acid (LCA) was of interest for the DDI part of our studies (see below), because LCA is a known endogenous PXR activator (Staudinger et al., 2001; Goodwin and Kliewer, 2002). Therefore, differences in the amount of LCA between huFRGN and huFRGN19 mice could have a potential effect on PXR-dependent DDI s. The LCA amount in the feces of huFRGN mice was 42.4 ± 22.8 µM, which was significantly higher than the 0.5 ± 0.4 µM or 2.2 ± 1.5 µM detected in huFRGN19SD and huFRGNYF mice, respectively (Figure 4c). The serum the concentration of this BA was very low, with slightly higher levels in the huFRGN19SD (1.0 ± 0.7 µM) relative to the huFRGN (0.2 ± 0.1 µM) and huFRGNYF (0.2 ± 0.1 µM) mice.

Chenodeoxycholic acid (CDCA) is more abundant in human plasma compared to other species (Sangaraju et al., 2021). The total amount of CDCA was higher in feces (2.59 ± 1.29 µM) and serum (0.46 ± 0.45 µM) of huFRGN animals compared to huFRGN19SD (0.15 ± 0.21 µM and 0.02 ± 0.02 µM) and huFRGNYF (1.02 ± 1.18 µM and 0.01 ± 0.01 µM) mice, respectively (Supplemental Tables and Figures, Supplemental Figure 3, page 86). In contrast, the relative proportion of this BA slightly increased in the feces and serum of the latter two groups in comparison to the huFRGN mice (Supplemental Tables and Figures, Supplemental Figure 3, page 8).

Finally, we were interested in the ratio of taurine conjugated cholic acid (T-CA) and glycine conjugate cholic acid (G-CA) in these mice. Generally, whereas mice conjugate BAs with
taurine (Inoue et al., 2004), humans conjugate BAs with both glycine and taurine (SJOVALL, 1959), but produce a higher proportion of glycine-conjugated BAs (Haekenscheid and Hector, 1975). A previous study found high amounts of G-CA in gallbladder bile of FRG mice highly repopulated with human hepatocytes, but only T-CA in non-repopulated controls (Ellis et al., 2013). In the samples analyzed in the present study, the amounts of T-CA and G-CA in feces were below limit of quantification in all three mouse lines. In contrast, both BAs were readily detectable in the serum of these mice, where T-CA accounted for 39.2% of the total CA amount in the huFRGN, 76.3% in the huFRGN19^SD^ and 83.5% in the huFRGN19^YF^ mice, while G-CA contributed 28.1%, 5.1% and 3.4%, of the total CA amount respectively (Figure 4d). This translates into T-CA to G-CA ratios of 1.4, 14.9 and 24.4 in the respective mouse group.

In summary, the huFRGN19^SD^ and huFRGN19^YF^ mice differ from the huFRGN mice by a shift from predominately di- to tri-hydroxy BAs, a profound reduction of the highly toxic BA DCA and its conjugates, lower total but higher relative levels of MCA, a decrease of the LCA amounts in feces but not in serum, and increased T-CA to G-CA ratios. In contrast, the differences in these parameters between the two huFRGN19 groups receiving different diets are only minor.

*Rifampicin induces the expression of hepatic CYP3A4 mRNA in huFRGN and huFRGN19 mice.*

As the use of huFRGN and huFRGN19 mice for CYP3A4-mediated DDI studies has not been described so far, we wanted to assess the utility of these mice for this purpose. In particular, we were interested in whether the altered BA composition in the two mouse lines might have an impact on the results of such a DDI study, since the expression of CYP3A4 could potentially be modified by different BAs. For these analyses, mice of both genotypes on YF-10 diet received daily oral doses of either RIF or vehicle for six days, followed by a single
oral dose of TRZ administered after the final dose of RIF. Six hours after the last dose the animals were sacrificed, livers were harvested, and RNA was extracted for quantitative real-time PCR (qRT-PCR) analysis. Given the lower levels of BAs in the huFRGN19 animals (Figure 1a), including LCA (Figure 4c), an agonist of the CYP3A4 regulator PXR, we anticipated that FGF19 would reduce basal hepatic CYP3A4 expression. There was indeed a non-significant (p = 0.08) trend of 2-fold lower CYP3A4 mRNA levels in the livers of vehicle-treated huFRGN19 mice (n=7) compared to the vehicle-treated huFRGN (n=7) controls (Figure 5a). Treatment with RIF led to a strong increase in CYP3A4 expression in both mouse groups. A 39.9-fold increase was observed in the huFRGN mice (n = 7, p = 0.0005) and an 18.5-fold increase in the huFRGN19 animals (n=7, p = 3.8e-05) relative to the vehicle-treated huFRGN mice. CYP3A4 expression increased 39.8-fold in RIF-treated huFRGN19 mice relative to the vehicle-treated huFRGN19 controls. In addition, the expression of Cyp3a11, the murine homolog of CYP3A4, was also examined to assess the extent of induction of this gene in the remaining mouse hepatocytes by RIF. The basal Cyp3a11 mRNA expression in the liver of vehicle-treated huFRGN was slightly (1.7-fold) lower than in vehicle-treated huFRGN19 mice, though this was not statistically significant (p = 0.254). RIF treatment resulted in a modest, but significant 3.0-fold increase in huFRGN (p = 0.0045) and a 2.9-fold increase in huFRGN19 (p = 0.0003) mice compared to the vehicle treated huFRGN controls (Figure 5b). The increase in Cyp3a11 expression in the huFRGN19 mice was 4.9-fold relative to the vehicle treated huFRGN19 animals (p = 0.0003). Therefore, we conclude that the basal CYP3A4 mRNA expression in the human hepatocytes and Cyp3a11 mRNA in the mouse hepatocytes is slightly lower in vehicle treated huFRGN19 compared to huFRGN control mice and that RIF-treatment leads to a comparable modest Cyp3a11 induction and a strong CYP3A4 induction in both mouse lines.

*The exposure of triazolam decreases in rifampicin-treated huFRGN19 and huFRGN mice.*
The RIF or vehicle and TRZ treated animals used for qRT-PCR (n=7 per group; see above) were also subjected to serial blood sampling (15 min, 30 min, 1 h, 2 h, 4 h and 6 h post TRZ-treatment) for pharmacokinetic analysis. The samples were used to assess the TRZ plasma concentration at the different time points and to determine the $C_{\text{max}}$, $T_{\text{max}}$ and AUC values of the parent compound. In addition, the pharmacokinetic parameters for the two main TRZ metabolites, alpha-OH-TRZ and 4-OH-TRZ, were measured in this manner. The TRZ $C_{\text{max}}$ and AUC significantly decreased in both the huFRGN and huFRGN19 mice treated with RIF as compared to their vehicle treated controls (Figure 6a; Table 1). The $C_{\text{max}}$ in the huFRGN mice was 4.4-fold lower ($p < 0.0001$) in the RIF-treated (17.9 ± 4.8 ng/ml) than in the vehicle-treated huFRGN mice (78.1 ± 6.3 ng/ml). The corresponding decrease in the RIF-treated huFRGN19 mice (17.7 ± 5.2 ng/ml) was 2.9-fold ($p = 0.0080$) relative to their controls (treated hFRGN19 mice, 50.8 ± 8.6 ng/ml). The AUC in the animals receiving RIF (huFRGN = 75.4 ± 21.7 ng*h/ml; huFRGN19 = 58.4 ± 17.9 ng*h/ml) was also 4.0- ($p = 0.001$) and 3.1-fold ($p = 0.0077$) lower, respectively, than in the corresponding vehicle controls (huFRGN = 298.2 ± 34.1 ng*h/ml; huFRGN19 = 183.7 ± 35.6 ng*h/ml). The calculated $T_{\text{max}}$ did not significantly differ between vehicle and RIF-treated huFRGN mice, but there was a significant ($p = 0.025$) difference between the vehicle (1.57 ± 0.2 h) and RIF-treated (0.86 ± 0.21 h) huFRGN19 mice.

The reduction in $C_{\text{max}}$ and AUC for the TRZ parent compound was accompanied by a slight, 1.2- ($p = 0.9009$) and 1.4-fold ($p = 0.625$) respectively, increase in the corresponding parameters for the alpha-OH-TRZ metabolite in the RIF- compared to the vehicle-treated huFRGN mice, although these changes were not significant (Figure 6b; Table 1). Specifically, the $C_{\text{max}}$ and AUC in the RIF-treated huFRGN mice were determined as 31.2 ± 9.1 ng/ml and 127.3 ± 45.8 ng*h/ml and in the vehicle controls as 26.5 ± 3.0 ng/ml and 93.4 ± 13.6 ng*h/ml, respectively. The changes for this metabolite were stronger, but still not statistically significant, for the RIF-treated huFRGN19 mice relative to their vehicle controls.
(1.9-fold higher $C_{\text{max}}$ and 1.8-fold higher $AUC$; $p = 0.133$ for $C_{\text{max}}$ and $p = 0.13$ for $AUC$), with an increase from $16.6 \pm 1.5$ ng/ml ($C_{\text{max}}$) and $57.6 \pm 9.7$ ng*h/ml ($AUC$) in the vehicle-treated to $31.5 \pm 8.5$ ng/ml and $105.2 \pm 30.8$ ng*h/ml, respectively, in the RIF-treated group. However, $T_{\text{max}}$ was significantly ($p = 0.013$) earlier in the RIF-treated huFRGN19 group (0.67 ± 0.11 h) in comparison to the vehicle treated controls 1.14 ± 0.14 h). Due to the opposite trends for the parent compound and the alpha-OH-TRZ metabolite in the RIF-treated compared to the control animals, namely a decrease of TRZ and an increase of alpha-OH-TRZ, there was a significant 5.2-fold (huFRGN; $p < 0.0001$) and 5.0-fold (huFRGN19; $p < 0.0001$) decrease in the TRZ to alpha-OH-TRZ $C_{\text{max}}$ ratio in the RIF-treated mice relative to their vehicle-treated controls (Table 1). A similar and significant decrease was observed for the TRZ to alpha-OH-TRZ $AUC$ ratio in the RIF-treated groups compared to their vehicle-treated controls (not shown).

The other metabolite analyzed in our study, 4-OH-TRZ, showed the opposite trend compared to alpha-OH-TRZ, i.e. the $C_{\text{max}}$ and $AUC$ for this metabolite was consistently lower in the RIF-treated than in the vehicle-control animals (Figure 6c; Table 1). The 4-OH-TRZ $C_{\text{max}}$ was significantly decreased from $123.6 \pm 19.7$ ng/mL in the vehicle treated huFRGN mice to $51.5 \pm 18.0$ ng/mL in the corresponding RIF-treated animals (2.4-fold; $p = 0.0063$) and non-significantly from $99.7 \pm 6.3$ ng/mL in the control huFRGN19 mice to $53.9 \pm 17.6$ ng/mL in the animals receiving RIF (1.8-fold; $p = 0.1014$). At the same time, the $AUC$ was 2.3-fold ($p = 0.0089$) higher in the huFRGN control animals ($541.5 \pm 80.7$ ng*h/ml) than in the RIF group ($237.9 \pm 85.5$ ng*h/ml) and 1.9-fold ($p = 0.0686$) higher in the respective huFRGN19 animals ($437.4 \pm 74.5$ ng*h/ml vs $226.6 \pm 74.8$ ng*h/ml).

In summary, the presented data suggest that treatment of huFRGN and FRGN19 mice with the PXR agonist RIF leads to a lower TRZ exposure, that this effect is accompanied by corresponding changes in the TRZ metabolite profile and that the effects achieved in the two mouse lines with this treatment are largely comparable.
Discussion

In the present study, the BA profile in the feces and serum of chimeric liver-humanized mice that do (huFRGN19) and do not (huFRGN) express human FGF19 was systematically investigated. In addition, the principal utility of both models to assess a RIF-mediated DDI was demonstrated.

Consistent with previous observations showing that the expression of FGF19 normalizes the liver size in chimeric liver-humanized mice (Naugler et al., 2015), we found a lower liver-to-body weight ratio in huFRGN19 compared to huFRGN mice. However, whereas Naugler et al. observed a 40% reduction of the liver-to-body weight ratio in huFRGN19 mice, only a 12% reduction in liver-to-body weight ratio was observed in the present study. One potential contributor to these deviations is the different housing conditions for the animals used in the studies or small differences in the degree of humanization. The fact that RIF did not cause liver enlargement, in contrast to some other reports (Huang et al., 2016; Kim et al., 2017), is probably due to the lower dose and/or shorter administration period in the present study. Ectopic FGF19 has been shown to have mitogenic activity (Wu et al., 2010), but an assessment of a potential effect on hepatocyte proliferation was not subject of the current investigation. However, hepatocyte proliferation was previously shown to be significantly decreased in huFRGN19 mice compared to huFRGN controls (Naugler et al., 2015), so it can be inferred that the physiological expression of FGF19 under control of its natural human promotor is not associated with aberrant liver cell proliferation.

Although the effect of transgenic FGF19 on the liver-to-body weight ratio was relatively small, we detected large reductions in total BA in the feces and serum of huFRGN19 mice. Specifically, a 36-fold decrease in feces and a 4.3-fold decrease in serum of huFRGN19 mice receiving the same (standard) diet as the huFRGN controls was observed. These findings are consistent with previously reported reductions in total BA in different compartments.
(intestine, portal vein, systemic, hepatic) of the huFRGN19 mice (Naugler et al., 2015),
ranging from a ~1.7-fold decrease in the intestine to a ~2.8-fold decrease in the portal vein of
the huFRGN19 relative to the huFRGN mice.

While hepatic human CYP7A1 mRNA levels were decreased in huFRGN19 compared to
huFRGN mice by 10-fold, this effect was not significant and not as strong as the 70-fold
difference previously reported (Naugler et al., 2015). The stronger reduction in total BA
amounts we observed in huFRGN19 animals together with a moderate reduction in CYP7A1
expression suggest that external factors, such as diet and housing conditions, may exert a
varying degree of influence on these two parameters in FRGN mice. In huFRGN19 mice,
mouse hepatic Cyp7a1 mRNA expression was only ~3.5-fold lower than in huFRGN mice,
indicating that FGF19 has a much smaller impact on BA metabolism of the mouse
hepatocytes relative to the human hepatocytes in huFRGN19 mice.

Examination of individual BAs using a LC-HRMS-based comprehensive targeted
metabolomics method (Sangaraju et al., 2021) expanded the knowledge of the effects of
FGF19 expression in these chimeric liver-humanized mice. Of interest in this context was the
effect of FGF19 expression on taurine vs. glycine-conjugated BAs in huFRGN19 compared to
the huFRGN mice. In a previous study, it was observed that the T-CA to G-CA ratio in
gallbladder bile from huFRG mice with a high degree of human hepatocyte repopulation (88-
94 %) was significantly lower (T-CA/G-CA = 10-58) than in non-chimeric FRG control mice
(587-903), thus indicating the expected shift towards increased glycine-conjugation in the
humanized mouse model (Ellis et al., 2013). In the present study, the T-CA to G-CA ratios
increased from 1.4 in serum of the huFRGN to 14.9 in the huFRGN19 mice receiving the
standard diet. As mouse hepatocytes conjugate BAs almost exclusively with taurine whereas
humans conjugate with both glycine and taurine (Hafkenscheid and Hectors, 1975; Inoue et
al., 2004; Ellis et al., 2013), the increase in T-CA relative to G-CA in huFRGN19 compared
to huFRGN mice observed in our study might indicate a higher contribution of the remaining
mouse hepatocytes to BA synthesis in huFRGN19 mice. Since mice with a high degree of repopulation (≥70%) with human hepatocytes were used for these studies, a likely explanation is a stronger inhibitory effect of human FGF19 on BA synthesis in the human hepatocytes than in the remaining mouse hepatocytes, which are still responsive to mouse FGF15. This would also be consistent with the differential effect of FGF19 on human CYP7A1 compared with murine Cyp7a1 mRNA expression described above as well as our measurements of the rodent-specific bile acid MCA. Specifically, although the total amount of MCA was decreased by 24- and 4-fold in feces and serum, respectively, of huFRGN19SD mice compared to the huFRGN controls, the proportion of MCA in the total BA content in these mice slightly increased by 1.5-fold in feces and 1.2-fold in serum.

Highly toxic bile acid DCA and its taurine and glycine conjugated forms have been reported to be 220-fold higher in plasma of huFRGN mice compared to non-humanized FRGN controls (Chow et al., 2017), which may compromise the use of such liver humanized mouse models for drug metabolism studies. Notably, we found that the total concentration of DCA including its conjugates was 189-fold lower in the feces and 250-fold lower in the serum of the huFRGN19 mice receiving standard diet compared to the huFRGN mice. Thus, the levels of DCA in huFRGN19 mice would be comparable to that in the non-humanized FRGN animals, in which the communication between the gut and liver should function normally.

Analysis of the chemical diversity of the BA pool revealed that huFRGN19 mice have more tri-hydroxy BAs whereas huFRGN mice have more di-hydroxy BAs. This profound difference might be explained by the very high levels of BAs in huFRGN mice, which are initially produced by the liver as tri-hydroxy BAs (CA, MCA) and then further converted to di-hydroxy BAs (eg: DCA, HDCA) by their gut microbiome due to its increased metabolic activity to consume tri-hydroxy BAs in order to maintain local gut homeostasis. In contrast, huFRGN19 mice produced significantly lower levels of BAs compared to huFRGN mice, including tri-hydroxy BAs by the liver, thereby leading to reduced exposure or normal
metabolic activity by the microbiome which are involved in the metabolism of tri-hydroxy BAs (CA, MCA) to di-hydroxy BAs (DCA, UDCA, HDCA) and mono-hydroxy BAs (LCA) (Russell, 2009; Chiang, 2013; Rudling, 2016). Another possible explanation might be a different gut microbiome composition and thus an altered metabolic biotransformation of tri-hydroxy BAs to di-hydroxy BAs in the two mouse lines, however this has not been experimentally confirmed.

The second major objective of our studies was to examine the possible utility of liver-humanized FRG mice in PXR/CYP3A4-mediated DDI studies. The effect of treatment with the PXR-agonist RIF on the expression of human CYP3A4 and mouse Cyp3a11 mRNA in hepatocytes of huFRGN and huFRGN19 mice was determined. Though the high levels of BAs in the huFRGN mice could have altered the basal expression of these genes because some BAs (e.g. LCA) are PXR agonists and therefore Cyp3a11/CYP3A4 inducers (Staudinger et al., 2001), only minor differences in the basal hepatic CYP3A4 and Cyp3a11 mRNA levels were observed in the vehicle-treated huFRGN19 compared to the huFRGN mice.

Treatment with RIF resulted in a profound ~40-fold increase of CYP3A4 mRNA levels in the humanized mice compared to their respective vehicle controls. Cyp3a11 mRNAs were also increased in the RIF-treatment groups, but not to the same extent (3.0-fold in huFRGN and 4.9-fold in huFRGN19 animals relative to their untreated controls). The observed differences between CYP3A4 and Cyp3a11 induction are consistent with RIF being a significantly stronger agonist of human compared with murine PXR (Xie et al., 2000) and the strong induction of CYP3A4 confirms the principle usability of the liver-humanized FRGN mice for DDI studies depending on PXR-mediated induction of CYP3A4.

This notion was further verified by the results from our pharmacokinetic analysis, in that the exposure of the co-administered CYP3A4 substrate TRZ was significantly reduced in the RIF-treated huFRGN and huFRGN19 mice compared to their vehicle treated controls. The decrease in TRZ C\text{max} (4.4-fold in huFRGN and 2.9-fold in huFRGN19 mice) and AUC (4.0-
fold in huFRGN and 3.1-fold in huFRGN19 mice) appeared to be slightly stronger in the huFRGN than in the huFRGN19 mice. On the other hand, for the alpha-OH TRZ metabolite, the increase in $C_{\text{max}}$ (1.9- vs 1.2-fold) and the AUC (1.8- vs 1.4-fold) was stronger in RIF treated huFRGN19 compared to huFRGN mice, reflecting an increased conversion of the parent compound into this metabolite in the huFRGN19 model. However, the RIF effect on alpha-OH TRZ was not significant in either mouse strain. Taken together, the differences between the two mouse models were small, consistent with the comparable induction of CYP3A4 in these mice by RIF. In contrast to alpha-OH TRZ, the $C_{\text{max}}$ and AUC of the 4-OH TRZ metabolite decreased in both RIF-treated mouse models. Similar observations were made in other studies (Kim et al., 2010; Hasegawa et al., 2011a) and a likely explanation is the expected induction of other drug metabolizing (for example phase II) enzymes and transporters which may contribute to a fast elimination of this metabolite.

In a previous study the TRZ AUC was decreased by 3.5-fold and the $C_{\text{max}}$ by 1.6-fold in chimeric liver-humanized uPA-SCID mice treated for 4 days with 50 mg/kg RIF compared to vehicle controls (Hasegawa et al., 2012). Taking into account the different housing conditions, the use of different human hepatocytes for transplantation and other differences in study design, the effects obtained in both studies are similar. In a clinical trial with healthy volunteers receiving 600 mg of RIF once daily for 5 days the $C_{\text{max}}$ and AUC of co-administered TRZ decreased to 12.4 % (8.1-fold) and 5.1 % (19.6-fold) of the placebo group, respectively (Villikka et al., 1997). Since the mg/kg RIF dose in the mouse studies is higher compared to that in humans, a less pronounced reduction in TRZ exposure is achieved in the liver-humanized mouse models than under clinical conditions. Possible reasons for this could be, for example, the incomplete humanization of the liver in the chimeric mice, differences in physiological hepatic blood flow, plasma protein binding differences, or species differences in the influence of the intestine on TRZ exposure. The latter rationale is corroborated by previous work with CYP3A4 transgenic humanized mouse models that demonstrated a greater
impact of intestinal than hepatic CYP3A4 on oral TRZ systemic exposure (van Waterschoot et al., 2009). We conclude that accurate quantitative predictions of clinical CYP3A4-mediated DDI in chimeric liver-humanized mouse models may be difficult. Nevertheless, the present work suggests that huFRGN/huFRGN19 mice may have utility in ranking the relative CYP3A4-induction potential of different PXR agonists under in vivo conditions, as has been shown in other liver-humanized mouse models, such as uPA-SCID (Hasegawa et al., 2012).

In summary, this report confirms that the expression of FGF19 in liver-humanized FRGN mice normalizes the amount of total BAs in the feces and serum. The present study systematically analyzed how the profile of individual BAs is affected by physiological FGF19 expression and it demonstrates that the levels of certain toxic BAs such as DCA and its conjugates are greatly reduced. Thus, demonstrates that transgenic FGF19 expression in liver-humanized mice could be beneficial for certain study areas, and here we provide a broad and relevant database to be considered for the design of such studies. Furthermore, the presented data show for the first time a potential utility of the huFRGN and huFRGN19 mice for DDI studies based on PXR-mediated induction of CYP3A4. Finally, it should be taken into account that while the miscommunication between mouse intestinal cells and human hepatocytes based on the lack of recognition of murine FGF15 is restored in the FGF19 transgenic mice, the contribution of the remaining mouse hepatocytes is clearly visible and that other communication signals between murine and human cells might still be dysfunctional.
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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: Scheer, Jones, Mezler, Goldman, Hoffmann, Heikkinen, Chang, Mannila, Foquet, Pusalkar, Clothe

Conducted experiments: Sangaraju, Goldman, Foquet

Contributed new reagents or analytic tools: Sangaraju

Performed data analysis: Scheer, Sangaraju, Jones, Mezler, Goldman, Pusalkar, Clothe

Wrote or contributed to the writing of the manuscript: Scheer, Jones, Sangaraju, Chang, Mezler, Goldman, Hoffmann, Heikkinen

Disclosures

M.M., M.H., S.P., P.C., D.C.G., L.F., J.M., A.T.H., R.S.J., D.S., J.H.C. contributed to the overall study design and planning as well as data analysis. The in vivo portion of the studies and qRT-PCR analysis were performed by Yecuris. D.C.G. and L.F. are employees of Yecuris. Yecuris provided materials and in-kind support to the research, which was performed within the context of a consortium. AbbVie, BMS, Takeda, Genentech and Admescope in part sponsored and funded the study, contributed to the design; participated in the collection, analysis and interpretation of the data, in writing, reviewing and approval of the final version. M.H. is an employee of BMS and may own BMS stock, M.M. is an employee of AbbVie and may own AbbVie stock, S.P. is a current employee of Servier Bioinnovation, LLC and may own stock in Takeda Pharmaceuticals as a former employee. P.C. is a current employee of AstraZeneca. As a former employee PC holds common stock in Takeda Development Center Americas, Inc. J.M. is an employee and A.T.H. was an employees of Symeres Finland Oy, Oulu Finland (operating under Admescope brand). N.S. received consultancy fees from Yecuris.
References


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Footnotes

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Figure Legends

Fig. 1: Total bile acid amount and relative hepatic murine Cyp7a and human CYP7A mRNA expression in huFRGN and huFRGN19 mice. (A) Total BA amount in feces (left) and serum (right) of huFRGN (n=4 for feces and n=8 for serum), huFRGN19<sup>SD</sup> (n=10 for feces and serum) and huFRGN19<sup>YF</sup> (n=9 for feces and n=10 for serum) mice. Values represent mean ± SD. Group means were compared to one another using pairwise non-parametric wilcoxon t-test with p-values indicated above the respective boxplots. (B) Relative human CYP7A (upper panel) and murine Cyp7a (lower panel) mRNA expression levels in liver of vehicle treated huFRGN and huFRGN19 mice, respectively (n=7 per mouse line and treatment). mRNA expression was analyzed by qRT-PCR and normalized to murine Gapdh in case of mCyp7a and human GAPDH for hCYP7A. Relative mRNA levels were assessed by a comparative (2<sup>-ΔΔCT</sup>) approach. Values represent the mean expression level ± SEM with mean expression of murine Cyp7a or human CYP7A mRNA, respectively, in vehicle treated huFRGN mice arbitrarily set as 1. A Student’s t-test (2-tailed) was performed on the results for each study group vs. other study groups as indicated by the crossbars, * and ** statistically different from comparative mice at p<0.05 and <0.01 and, respectively.

Fig. 2: Chemical diversity of bile acid pool differences as %BAs in feces and serum groups are represented as donut plots. % mono hydroxy BAs(1-OH): % LCA; % di-hydroxy BAs (2-OH): Sum of % CDCA, % DCA, % UDCA, % HDCA, %MDCA; % tri-hydroxy BAs (3-OH): Sum of % CA, % MCA, % HCA. % Oxo-isoo-nor-dehydro BAs (OINDBAs): Sum of % 12-oxo-CDCA, % 12-oxo-LCA, % 7-oxo-LCA, % 6-oxo-LCA, % Iso-LCA, % Iso-DCA, % Nor-DCA, and % 3-dehydro-CA BAs.
**Fig. 3:** Amount of deoxycholic acid and its conjugates in feces and serum of huFRGN and huFRGN19 mice. (A) Total DCA amount and (B) relative contribution of total DCA to all BAs in feces and serum of huFRGN, huFRGN19SD and huFRGN19YF mice. Total amount of unconjugated DCA (C), glycine conjugated DCA (D) and taurine conjugated DCA (E) in feces and serum of these mice. All values represent mean ± SD, with n=4 for feces and n=8 for serum for huFRGN mice, n=10 for feces and serum for huFRGN19SD mice and n=9 for feces and n=10 for serum huFRGN19YF mice. Group means were compared to one another using pairwise non-parametric wilcoxon t-test with p-values indicated above the respective boxplots.

**Fig. 4:** Amount of muricholic acid, lithocholic acid and ratio of taurine and glycine conjugated cholic acid in huFRGN and huFRGN19 mice. (A) Total MCA amount, (B) relative contribution of total MCA to all BAs and (C) total LCA amount in feces and serum of huFRGN, huFRGN19SD and huFRGN19YF mice. All values in (A)-(C) represent mean ± SD, with n=4 for feces and n=8 for serum for huFRGN mice, n=10 for feces and serum for huFRGN19SD mice and n=9 for feces and n=10 for serum huFRGN19YF mice. Group means were compared to one another using pairwise non-parametric wilcoxon t-test with p-values indicated above the respective boxplots. (D) Percentages of taurine conjugated cholic acid (T-CA) and glycine conjugate cholic acid (G-CA) to total cholic acid (sum of T-CA, G-CA, CA and their sulfates) in serum of huFRGN (n=8), huFRGN19SD (n=10) and huFRGN19YF (n=10) mice and the derived T-CA to G-CA ratios.

**Fig. 5:** Human CYP3A4 and murine Cyp3a11 mRNA expression in liver of vehicle and rifampicin treated huFRGN and huFRGN19 mice. (A) Relative human CYP3A4 mRNA and (B) relative murine Cyp3a11 mRNA expression levels in livers of vehicle or rifampicin
treated huFRGN and huFRGN19 mice, respectively (n=7 per mouse line and treatment). mRNA expression was analyzed by qRT-PCR and normalized to human GAPDH in the case of hCYP3A4 and murine Gapdh for mCyp3a11. Relative mRNA levels were assessed by a comparative (2^ΔΔCT) approach. Values represent the mean expression level ± SEM with mean expression of human CYP3A4 or murine Cyp3a11 mRNA, respectively, in vehicle treated huFRGN mice arbitrarily set as 1. A Student’s t-test (2-tailed) was performed on the results for each study group vs. other study groups as indicated by the crossbars, with *, ** and *** statistically different from comparative mice at p<0.05, <0.01 and <0.001, respectively.

**Fig. 6:** Pharmacokinetics of triazolam and its metabolites in vehicle and rifampicin treated huFRN and huFRGN19 mice. (A) Triazolam, (B) Alpha-OH-triazolam and (C) 4-OH-triazolam concentration versus time dependencies in vehicle treated huFRGN mice (red line), vehicle treated huFRGN19 mice (green line), rifampicin treated huFRGN mice (blue line), and rifampicin treated huFRGN19 mice (black line), with n=7 for each study group.
Table 1 Pharmacokinetic parameters of triazolam and its metabolites in vehicle or rifampicin treated huFRGN and huFRGN19 mice

<table>
<thead>
<tr>
<th>Group</th>
<th>t₁/₂ [h]</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; [ng/ml]</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; [h]</th>
<th>AUC&lt;sub&gt;0-6&lt;/sub&gt; [ng*hr/mL]</th>
<th>(P/M) AUC&lt;sub&gt;0-6&lt;/sub&gt;</th>
<th>(P/M)C&lt;sub&gt;max&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TRZ</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>huFRGN vehicle</td>
<td>1.7</td>
<td>78.1 ± 6.3</td>
<td>1.57 ± 0.2</td>
<td>298.2 ± 34.1</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>huFRGN RIF</td>
<td>2.6</td>
<td>17.9 ± 4.8***</td>
<td>1.36 ± 0.24</td>
<td>75.4 ± 21.7***</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>huFRGN19 vehicle</td>
<td>2.0</td>
<td>50.8 ± 8.6</td>
<td>1.57 ± 0.2</td>
<td>183.7 ± 35.6</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>huFRGN19 RIF</td>
<td>1.9</td>
<td>17.7 ± 5.2**</td>
<td>0.86 ± 0.21*</td>
<td>58.4 ± 17.9**</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td><strong>Alpha-OH-TRZ</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>huFRGN vehicle</td>
<td>2.0</td>
<td>26.5 ± 3.0</td>
<td>1.21 ± 0.21</td>
<td>93.4 ± 13.6</td>
<td>3.3 ± 1.03</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td>huFRGN RIF</td>
<td>2.8</td>
<td>31.2 ± 9.1</td>
<td>1.57 ± 0.47</td>
<td>127.3 ± 45.8</td>
<td>0.6 ± 0.05***</td>
<td>0.6 ± 0.1***</td>
</tr>
<tr>
<td>huFRGN19 vehicle</td>
<td>2.4</td>
<td>16.6 ± 1.5</td>
<td>1.14 ± 0.14</td>
<td>57.6 ± 9.7</td>
<td>3.2 ± 0.3</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>huFRGN19 RIF</td>
<td>2.5</td>
<td>31.5 ± 8.5</td>
<td>0.67 ± 0.11</td>
<td>105.2 ± 30.8</td>
<td>0.5 ± 0.04***</td>
<td>0.6 ± 0.1***</td>
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<tr>
<td><strong>4-OH-TRZ</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>huFRGN vehicle</td>
<td>4.7</td>
<td>123.6 ± 19.7</td>
<td>2.86 ± 0.4</td>
<td>541.5 ± 80.7</td>
<td>0.6 ± 0.1</td>
<td>0.7 ± 0.1</td>
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<tr>
<td>huFRGN RIF</td>
<td>3.5</td>
<td>51.5 ± 18.0**</td>
<td>3.43 ± 0.37</td>
<td>237.9 ± 85.5**</td>
<td>0.4 ± 0.04**</td>
<td>0.4 ± 0.032**</td>
</tr>
<tr>
<td>huFRGN19 vehicle</td>
<td>7.5</td>
<td>99.7 ± 6.3</td>
<td>3.14 ± 0.4</td>
<td>437.4 ± 74.5</td>
<td>0.4 ± 0.3</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>huFRGN19 RIF</td>
<td>4.5</td>
<td>53.9 ± 17.6</td>
<td>2.86 ± 0.6</td>
<td>226.6 ± 74.8</td>
<td>0.4 ± 0.12</td>
<td>0.4 ± 0.1</td>
</tr>
</tbody>
</table>

Significant differences following treatment with rifampicin in comparison with the respective vehicle treated controls are highlighted, with * = p < 0.05, ** = p < 0.01, and *** p < 0.001.
Figure 1

A) 

B) 

hCYP7A

p = 0.072

0.1 ± 0.01

mCyp7a

p = 0.044

1.0 ± 0.3

0.3 ± 0.1
Figure 2

% 1OH-BAs: % Mono-hydroxy BAs is % Lithocholic acid (% LCA).
% 2OH-BAs: % Di-hydroxy BAs is sum of % Chenodeoxycholic acid (% CDCA), % Deoxycholic acid (% DCA), % Ursodeoxycholic acid (% UDCA), % Hyodeoxycholic acid (% HDCA) and % Murideoxycholic acid (% MDCA).
% 3OH-BAs: % Tri-hydroxy BAs is sum of % Cholic acid (%CA), % Muncholic acid (%MCA) and %Hyocholic acid (% HCA)
% OIND-BAs: % Oxo-iso-nor-dehydro BAs is sum of % 12-oxo-CDCA, % 12-oxo-LCA, % 7-oxo-LCA, % 6-oxo-LCA, % Iso-LCA, % Iso-DCA, % Nor-DCA, and % 3-dehydro-CA BAs.
Percentages of above bile acids were calculated as described previously in Sangarie et al., 2021.
Figure 3

A) 

B) 

C) 

D) 

E)
Figure 4

<table>
<thead>
<tr>
<th></th>
<th>% T-CA</th>
<th>% G-CA</th>
<th>Ratio T-CA/G-CA</th>
</tr>
</thead>
<tbody>
<tr>
<td>huFRGN</td>
<td>39.2</td>
<td>28.1</td>
<td>1.4</td>
</tr>
<tr>
<td>huFRGN1950</td>
<td>76.3</td>
<td>5.1</td>
<td>14.9</td>
</tr>
<tr>
<td>huFRGN1977</td>
<td>83.5</td>
<td>3.4</td>
<td>24.4</td>
</tr>
</tbody>
</table>
Figure 5

A) hCYP3A4

- **3.8x10^5***
- **0.005***
- **0.082***

- **39.9±5.8**
- **3.8x10^5***

- **huFRGN**
- **huFRGN19**

B) mCyp3a11

- **0.0006***
- **0.0254***
- **0.0045**
- **0.0003***

- **1.0±0.1**
- **3.0±0.5**
- **2.9±0.3**

- **huFRGN**
- **huFRGN19**
Figure 6

A) TRZ

B) Alpha-OH-TRZ

C) 4-OH-TRZ

Concentration (ng/mL) vs. Hours After Dose

- □□□ huFRGN vehicle
- □▪ huFRGN RIF
- □▪ huFRGN19 vehicle
- □▪ huFRGN19 RIF