BPB Reports

Regular Article

Changes in Bile Acid Concentrations in Chimeric Mice Transplanted with Different Replacement Indexes of Human Hepatocytes

Chieri Fujino,[a] Seigo Sanoh,[*,a] Yuka Tamura,[a] Yuji Ishida,[b,c] Chise Tateno,[b,c] Shigeru Ohta,[a,d] and Yaichiro Kotake[a]

[a]*Graduate School of Biomedical and Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima City, Hiroshima 734-8553, Japan;* [b]*R&D Dept., PhoenixBio, Co., Ltd., 3-4-1 Kagamiyama, Higashi-Hiroshima City, Hiroshima 739-0046, Japan;* [c]*Research Center for Hepatology and Gastroenterology, Hiroshima University, Kagamiyama, Higashi-Hiroshima City, Hiroshima 739-8511, Japan;* [d]*Wakayama Medical University, 811-1 Kimiidera, Wakayama City, Wakayama 641-8509, Japan*

Received March 28, 2019; Accepted April 17, 2019

Chimeric mice with humanized liver that are repopulated with human hepatocytes are useful to study hepatitis B and C viruses, predict drug metabolism and pharmacokinetics (PK), and evaluate hepatotoxicity. Understanding the characteristics of chimeric mice is important for making predictions in humans. In general, chimeric mice with more than 70% of replacement indexes (RIs), a value representing the occupancy ratio of the region of the human liver to that of the mouse liver, are used. However, chimeric mice with RIs less than 70% are also useful in understanding the species differences between mice and humans. In this study, to elucidate the effects of proliferating human hepatocytes and remaining mouse hepatocytes on bile acid concentrations in detail, we investigated the differences in the total concentrations of bile acids and their compositions in chimeric mice with different RIs. The total concentrations of bile acids in their sera increased as the RIs increased. The ratios of primary to secondary bile acids, percentages of glycine conjugates, and hydrophobicity indexes, obtained upon classifying bile acids based on their compositions in the serum and comparing them with those in normal mice and humans, were found to approach the values observed in humans as the RIs increased. The percentages of taurine conjugates were high in chimeric mice with high RIs, although their values were close to those in humans. These results could be fundamental in providing knowledge to accurately predict human PK and toxicity in chimeric mice with humanized liver.

Key words chimeric mice with humanized liver, bile acids, replacement index, human hepatocytes

INTRODUCTION

Chimeric mice with humanized liver are produced by the transplantation of human hepatocytes into host chimeric mice with liver injuries and immunodeficiencies. Several models of the host mice such as the urokinase-type plasminogen activator (uPA)/severe combined immunodeficient (SCID) mouse, Fah$^{-/-}$, Rag2$^{-/-}$, Il2r$^{-/-}$ (FRG) mouse and a NOG mouse expressing a thymidine kinase transgene (TK-NOG) were developed to establish chimeric mice.[1,2] The transplanted human hepatocytes proliferate and within two months constitute more than 70% of the liver of the chimeric mouse.[3] Chimeric mice with humanized liver are considered to be useful animal models for conducting studies on the virology of the hepatitis B virus (HBV) and hepatitis C virus (HCV), prediction of drug metabolism and pharmacokinetics (PK), and hepatotoxicity.[4-8]

The replacement index (RI) of human hepatocytes in chimeric mice represents the occupancy ratio of the region of the human liver to that of the mouse liver. These values can be estimated by calculating the concentration of human albumin in the blood.[3] In general, chimeric mice with humanized liver having a high RI (>70%) are often used. However, those with RI lower than 70% are also found to be useful. For example, it is known that HBV infects chimeric mice with both low and high RIs; however, HCV does not infect chimeric mice with a low RI.[6] To elucidate the effects of species differences in the metabolic profiles of mice and humans, Sanoh *et al*.[9] used mass spectrometry imaging to report changes occurring in the phosphatidylcholines localized in the human and mouse regions in the liver of chimeric mice with a medium RI. By using chimeric mice with low, medium and high RIs respectively, Kitamura *et al*.[10] and Sanoh *et al*.[7] demonstrated the effects of aldehyde oxidase and cytochrome P450 (CYP) metabolism on the remaining mouse hepatocytes. Furthermore, Kamimura *et al*.[11] predicted the clearance of the partial glucokinase activator, PF-04937319, in humans by stepwise plotting each clearance value obtained in the chimeric mice with a wide range of RIs and then extrapolating these results for the chimeric mice with RI of 100%. Using chimeric mice with RIs increasing gradually from low to high enables us to understand the effect of species differences in the metabolism of mice and humans in detail, and reliable prediction becomes possible.

Recently, remarkably high concentrations of bile acid were reported in the bile, plasma and livers of the chimeric mice

*To whom correspondence should be addressed. e-mail: sanoh@hiroshima-u.ac.jp

with RI greater than 80% as compared to those of the host mice, FRG on non-obese diabetic strain background (FRGN) mice.[12] Furthermore, Chow *et al*.[12] suggested that the reason for this difference in the concentration of bile acids was due to the enzyme responsible for the biosynthesis of bile acids, CYP7A1. According to them, CYP7A1 was not regulated because the mouse fibroblast growth factor (FGF)15 derived from the intestine of the mouse cannot recognize a human fibroblast growth factor receptor 4 contributing to the negative regulation of CYP7A1 in the human livers in chimeric mice.

Perturbations in the homeostasis of bile acids may affect the predictions of drug metabolism, pharmacokinetics, and hepatotoxicity. In this study, we investigated the differences in the concentrations of bile acids in the sera of the host chimeric mice, cDNA urokinase-type plasminogen activator-transgenic/severe combined immunodeficient (cDNA-uPA$^{wild/+}$/SCID) mice, with low to high RIs to elucidate the effects of both, proliferating human hepatocytes and remaining mouse hepatocytes, on the bile acid concentrations in detail. The findings of the current study could be fundamental in providing the knowledge for the use of chimeric mice with humanized liver with a wide range of RIs.

MATERIALS AND METHODS

Chemicals Cholic acid (CA) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA), lithocholic acid (LCA), tauroursodeoxycholic acid dihydrate (TUDCA), sodium glycocholate hydrate (GCA), and sodium glycodeoxycholate (GDCA) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Sodium deoxycholate (DCA) and sodium taurocholate (TCA) were purchased from FUJIFILM Wako Pure Chemical Corporation). Taurochenodeoxycholic acid (sodium salt) (TCDCA) and taurodeoxycholic acid (sodium salt) (TDCA) were purchased from Merck KGaA (Darmstadt, Germany). Tauro-β-muricholic acid (sodium salt) (TMCA), sodium glycochenodeoxycholate (GCDCA), and nor-desoxycholic acid (Nor-DCA) were purchased from Cayman Chemical (MI, USA), Sigma-Aldrich Co. (St. Louis, MO, USA), and Toronto Research Chemicals Inc. (Ontario, Canada) respectively.

Animals We used 17-18 weeks old, male chimeric mice with humanized liver (PXB mouse®; PhoenixBio Co., Ltd., Hiroshima, Japan), which were produced by transplanting commercially available human hepatocytes (BD195; Hispanic female two years old girl; Corning Japan KK, Tokyo, Japan) into urokinase-type plasminogen activator cDNA transgenic/severe combined immunodeficient (c-DNA-uPA$^{wild/+}$/SCID) mice. Their RIs were calculated as the ratio of the area occupied by human cytokeratin 8/18 (hCK8/18)-positive human hepatocytes to the entire area examined on immunohistochemical liver frozen sections stained with anti-hCK8/18 mouse monoclonal antibodies (Cappel Laboratory, Cochranville, PA) as described previously[3]. All experiments were approved by the Animal Ethics Committee at PhoenixBio Co., Ltd. and the Hiroshima University.

LC-MS/MS The serum samples were mixed with methanol containing Nor-DCA as an internal standard. After centrifugation for 5 min at 14,000 × *g*, the supernatants were diluted with 10 mM ammonium acetate in the ratio of 1:1. Then, aliquots were injected into an LC-MS/MS. A Nexera high-performance liquid chromatography system (Shimadzu, Kyoto, Japan) with an Inertsil ODS-3 column (5 μm, 2.1 × 100 mm; GL Sciences Inc., Tokyo, Japan) was used for the LC separation. The mobile phases used in the study were 10 mM ammonium acetate (A) and acetonitrile (B). The gradient condition (A:B) at the flow rate of 200 μL/min was as follows: 0 min (75:25), 12 min (60:40), 16–20 min (5:95) and 20.1–23 min (75:25). The MS/MS measurements were performed using an LCMS-8050 system (Shimadzu). The precursor ion and product ions (m/z) of bile acids and the internal standard under negative-ion mode are described as follows: CA (m/z, precursor ion: 407.25→product ion: 407.25), CDCA (m/z, 391.25→391.25), DCA (m/z, 391.25→391.25), UDCA (m/z, 391.25→391.25), LCA (m/z, 375.25→375.25), TCA (m/z, 514.30→80.05), TCDCA (m/z, 498.25→80.10), TDCA (m/z, 498.20→80.05), TUDCA (m/z, 498.30→80.05), TMCA (m/z, 514.20→80.05), GCA (m/z, 464.30→74.20), GCDCA (m/z, 448.25→74.20), GDCA (m/z, 448.30→74.20), and Nor-DCA (m/z, 377.20→377.20). α+β forms of tauromuricholic acid were measured and the β-form was used to calculate the calibration curve.

RESULTS AND DISCUSSION

Serum concentrations of 13 bile acids including principal non-conjugates and amino acid conjugates in chimeric mice with RI values of 30.2%, 48.7%, 57.8%, 79.7%, 79.9%, 84.4%, 89.5%, 92.3% and 93.8%, and in cDNA-uPA/SCID mice as non-transplanted host mice (RI, 0%) were measured using LC-MS/MS. The total concentrations of 13 bile acids in the chimeric mice with humanized liver were found to increase with a stepwise elevation in the RIs (Fig. 1A). The total concentration of bile acids in the chimeric mice with the highest RI (93.8%) was approximately 20-fold higher than that in the host mice (RI, 0%). The concentrations of bile acids in the chimeric mice with RIs ranging from 48.7%–93.8% were remarkably higher than those in C57BL/6 mice (3.1 μM), as reported by Thakare *et al*.[13], as well as those in the host mice. Furthermore, these concentrations were also higher than those found in humans (3.9 μM).[13] These findings suggest that the extent of transplantation of human hepatocytes in mice influenced the bile acid concentrations in their sera. In a previous study by Chow *et al*.[12], concentrations of bile acids in the plasma were compared among non-transplanted host mice (FRGN mice), mouse hepatocyte-transplanted mice and chimeric mice with high RIs >80%. Although the host mice used by them were different from those involved in our study, it was noticed that our results were similar to their findings because Chow *et al*.[12] had reported that the total bile acids in the plasma of chimeric mice with high RIs (>80%) was also 24- and 49-fold higher than those occurring in the mouse hepatocyte-transplanted mice and non-transplanted host mice, respectively. Chow *et al*.[12] suggested that the biosynthesis of bile acids by CYP7A1 is excessively promoted in the livers of chimeric mice. Moreover, when the nuclear receptor, farnesoid X receptor, is excessively activated by an increase in the concentration of bile acids, the forkhead box protein m1b is remarkably induced and abnormal hepatocyte proliferation occurs. Therefore, high concentrations of bile acids in the chimeric mice with humanized liver are believed to increase the liver weight excessively.[14] In fact, ratios of the liver-to-body weights in the chimeric mice with humanized liver are 1.5 to 2-fold higher than those

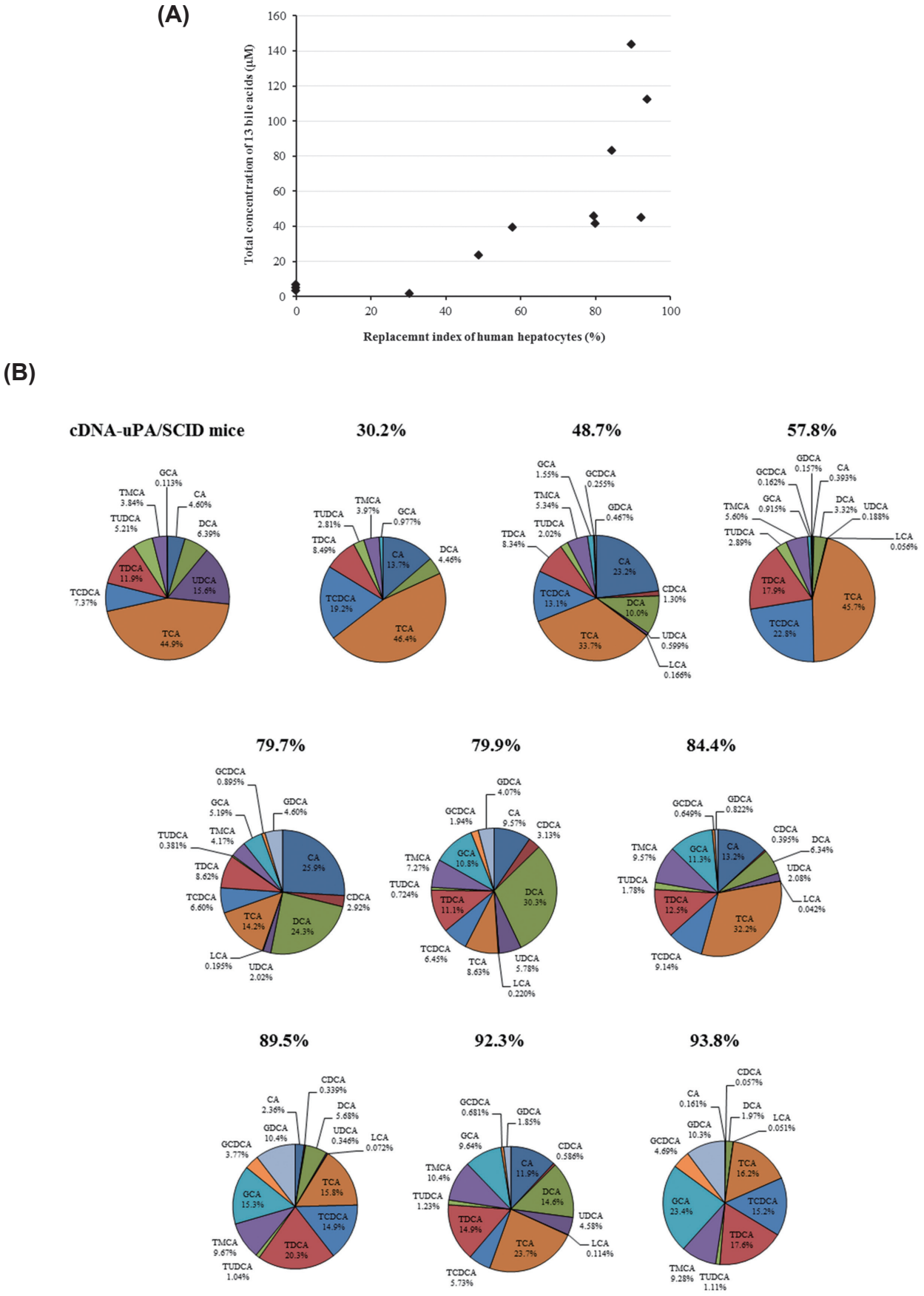

Fig. 1. Total Concentration of 13 Bile Acids (A) and the Bile Acid Composition (B) in Chimeric Mice with Different RIs of Human Hepatocytes

The concentration of individual bile acids in the serum was measured by LC-MS/MS. The bile acid composition in cDNA-uPA/SCID mice was shown as the average (n=3). The bile acid concentrations and compositions below the quantification limit (< 0.01 μM) were set to zero.

of the normal mice.[15] Also, in the current study, ratios of the liver-to-body weights in chimeric mice with high RIs were observed to be greater than those with low RIs. Furthermore, these profiles could be correlated with their bile acid concentrations (Fig.1A, Table 1). Therefore, it can be suggested that the expression of CYP7A1 can also increase with a stepwise elevation of the RIs.

Fig. 1B shows the composition of each bile acid in the sera of the chimeric mice with humanized liver (RI, 30.2%-93.8%) and the cDNA-uPA/SCID mice. With reference to the study by Thakare *et al*.[13] (Table 2), these composition profiles were largely classified into five indexes based on the following parameters: the number of hydroxyl groups in their structures, primary and secondary bile acids, glycine and taurine conjugates, CA/CDCA ratio and the hydrophobicity index (HI).

First, bile acids were classified based on the number of hydroxyl groups contained in their chemical structures and the percentage of each with respect to the total bile acid concentration was calculated (Table 2). The bile acid with one hydroxyl group (Mono-OH BA), which is corresponding to LCA in this study, was detected in the chimeric mice with RIs ranging from 48.7%-93.8%. The ratio (%Mono-OH BA) was similar to that of the C57BL/6 mouse but was found to be lower than the ratio in humans. It is known that the ratios of bile acids with two hydroxyl groups (%Di-OH BA), which correspond to CDCA, DCA, UDCA, TCDCA, TDCA, TUDCA, GCDCA and GDCA in C57BL/6 mice were lower than those in humans. On the other hand, the ratios of bile acids with three hydroxyl groups (%Tri-OH BA), which are corresponding to CA, TCA, TMCA and GCA in C57BL/6 mice were found to be higher in humans (Table 2).[13] However, the percentages of the bile acids with two and three hydroxyl groups in chimeric mice with humanized liver did not significantly reflect the differences between mice and humans. It is necessary to consider the effects of liver injury characterized in the host mice and of the bile acid metabolism in the livers of chimeric mice. Muricholic acid (MCA), a bile acid specific to rodents, is biosynthesized by Cyp2c70 from CDCA and UDCA. Cyp2c70 is not expressed in humans and the homologue of Cyp2c70 in humans is also not known.[16] However, in Fig. 1B, the percentages of TMCA, the taurine conjugate of MCA, were high even in the chimeric mice with high RIs. Cyp2c70 expressed in the remaining mouse liver may be contributing to the biosynthesis of MCA.

Second, the bile acids were classified into primary bile acids, which are biosynthesized in the livers, and secondary bile acids, which are biosynthesized by the intestinal bacteria. Percentages of primary and secondary bile acids to the total bile acid concentrations and the ratios of primary to secondary bile acids were calculated. In C57BL/6 mice, the percentages of primary bile acids were higher than those in humans, while those of secondary bile acids were found to be lower (Table 2).[13] Although the percentage ranges of primary and secondary bile acids in chimeric mice with different RIs were not significantly clear, the ratios of primary to secondary bile acids were seen to decrease as the RIs increased. The ratios of primary to secondary bile acids in C57BL/6 mice were higher than those in humans. The profiles of chimeric mice reflected the differences between mice and humans (Table 2). However, it is necessary to consider the effects of intestinal bacterial flora on the profiles of secondary bile acids in the intestines of chimeric mice.

The percentages of glycine and taurine conjugations to the total bile acid concentrations were calculated. It is known that the percentages of glycine conjugations in C57BL/6 mice are lower than those in humans. As the RIs in chimeric mice were higher, the percentages of glycine conjugations were inclined to increase (Table 2). Conversely, the percentages of taurine conjugations are higher in the C57BL/6 mice than those in humans. As the RIs were higher, the percentages of taurine conjugations were inclined to be on the lower side. However, the values of taurine conjugations in the chimeric mice with high RIs were still higher as compared to those in humans (Table 2). It is known that, in humans, the glycine conjugations are biosynthesized more than the taurine conjugations whereas the opposite takes place in C57BL/6 mice.[13] The enzymatic activity of the bile acid-CoA: amino acid *N*-acyltransferase, which catalyzes the amidation of bile acids for the taurine and glycine conjugations is comparable in human.[17] However, it has been suggested that there is a species difference in the intra-peroxisomal concentrations of the amino acids when utilized as sources of amidation because of a species difference in the synthesis of the amino acids.[17,18] Considering these reports, it can be suggested that in the chimeric mice with humanized liver, taurine may have been supplied by the remaining mouse liver cells or other tissues, which led to an increase in the taurine conjugates.

The ratios of CA to CDCA as a probe of the sterol 12-α-hydroxylase, CYP8B1, were calculated. [13] This ratio in humans is markedly lower than that in the C57BL/6 mice. The CA metabolic pathway is regarded as a dominant pathway in mice. As the RIs in the chimeric mice with humanized liver were higher, the CA/CDCA ratios were likely to be lower. However, the values in the chimeric mice with high RIs were still high when compared to those in humans. It can be suggested that CYP8B1 in the remaining mouse liver might be promoting CA biosynthesis.

Finally, the HI was calculated according to the method by Heuman.[19] This index is a sum of the multiplications of the individual bile acids with the total concentration ratio and

Table 1. Information Regarding Each cDNA-uPA/SCID Mouse and Chimeric Mouse Used in This Study

	cDNA-uPA/SCID mouse			Chimeric mouse with humanized liver (PXB mouse®)								
Replacement index (%)	0	0	0	30.2	48.7	57.8	79.7	79.9	84.4	89.5	92.3	93.8
Donor	None	None	None	BD195	BD195	BD195	BD195	BD195	BD195	BD195	BD195	BD195
Sexuality	M	M	M	M	M	M	M	M	M	M	M	M
Days after transplantation	–	–	–	98	98	98	98	98	98	98	98	98
Body weight (g)	23.8	23.8	24.1	24.1	24.2	22.5	23.3	25.1	23.2	22.2	22.4	20.4
Liver weight (g)	1.31	1.30	1.27	1.45	1.78	1.69	2.15	2.80	2.22	2.60	2.13	2.58
Liver-to-body weight ratio (%)	5.51	5.48	5.27	6.02	7.36	7.52	9.22	11.1	9.54	11.7	9.52	12.7
Human albumin (mg/mL)	–	–	–	1.54	4.64	5.15	9.21	11.6	8.47	12.9	11.5	14.6

Table 2. Classification of Bile Acid Profiles in Mice, Humans and Chimeric Mice with Humanized Liver

	C57BL/6 mouse	cDNA-uPA/ SCID mouse	Chimeric mouse with humanized liver (PXB mouse®)									Human
	Thakare *et al*.[13]	Replacement index (%)										Thakare *et al*.[13]
		0	30.2	48.7	57.8	79.7	79.9	84.4	89.5	92.3	93.8	
	plasma	serum (n=3)	serum	serum	serum	serum	serum	serum	serum	serum	serum	plasma
%Mono-OH BA	0.1 ± 0.1	N.D.	N.D.	0.166	0.056	0.195	0.220	0.042	0.072	0.114	0.051	12 ± 9
%Di-OH BA	23 ± 7.9	47.6 ± 6.58	35.0	36.1	47.3	50.3	63.5	33.7	56.8	44.2	50.9	74 ± 9
%Tri-OH BA	77 ± 8.1	52.4 ± 6.58	65.0	63.7	52.6	49.5	36.3	66.3	43.1	55.7	49.0	12 ± 7
%Primary BA	80 ± 8	59.7 ± 6.52	84.2	78.4	75.5	59.9	47.8	76.4	62.1	62.7	69.0	45 ± 16
%Secondary BA	20 ± 8	40.3 ± 6.52	15.8	21.6	24.5	40.1	52.2	23.6	37.9	37.3	31.0	55 ± 16
P/S	4.5 ± 2.0	1.53 ± 0.444	5.35	3.63	3.09	1.50	0.916	3.25	1.64	1.68	2.22	1.1 ± 1.3
%G-conjugation	0.3 ± 0.05	0.084 ± 0.146	0.977	2.27	1.24	10.7	16.8	12.8	29.5	12.2	38.3	54 ± 13
%T-conjugation	71 ± 18	70.4 ± 13.8	80.9	62.5	94.8	34.0	34.2	65.2	61.7	56.0	59.4	12 ± 7.3
CA/CDCA	14 ± 9.0	6.59 ± 0.992	3.18	3.98	2.05	4.35	2.52	5.56	1.77	6.47	1.99	0.34 ± 0.22
HI	-0.09 ± 0.03	0.056 ± 0.012	0.145	0.172	0.179	0.304	0.310	0.108	0.251	0.159	0.218	0.45 ± 0.07

N.D.: not detected (below the quantification limit, < 0.01 μM), BA: bile acid.
% Mono-OH BA: the percentages of mono-OH BA (LCA), % Di-OH BA: the percentages of di-OH BAs (CDCA, DCA, UDCA, TCDCA, TDCA, TUDCA, GCDCA, GDCA), % Tri-OH BA: the percentages of tri-OH BAs (CA, TCA, TMCA, GCA), % Primary BA: the percentage of primary BAs (CA, CDCA, TCA, TCDCA, TMCA, GCA, GCDCA), % Secondary BA: the percentage of secondary BAs (DCA, UDCA, LCA, TDCA, TUDCA, GDCA), P/S: the ratio of primary to secondary BAs, % G-conjugation: the percentage of glycine conjugates (GCA, GCDCA,GDCA), % T-conjugation: the percentage of taurine conjugates (TCA, TDCA, TCDCA, TUDCA, TMCA), G/T: the ratio of G-conjugation to T-conjugation, CA/CDCA: the ratio of CA (CA, TCA, GCA) to CDCA (CDCA, TCDCA, GCDCA), HI: hydrophobicity index calculated according to the previous report as per the following equation [19]: HI=∑constant of individual hydrophilic hydrophobic balance×(ratio of individual bile acid to the total concentration), (The constant of individual hydrophilic hydrophobic balance of LCA was not available.) The values of C57BL/6 mice and humans used are the values described previously.[13]

the constants of the hydrophilic-hydrophobic balance in the individual bile acids, as estimated from the retention time in HPLC. In the C57BL/6 mice, negative values of the HI are observed, which indicate that the hydrophobicity of the bile acids is low, whereas a positive value observed in humans indicates a high hydrophobicity of the bile acids (Table 2).[13] Positive values were observed in chimeric mice with humanized liver. In general, it is known that the high hydrophobicity of bile acids is the cause of potent cytotoxicity.[20,21] Bosentan, an endoserine receptor antagonist for pulmonary hypertension, was reported to cause liver injury specific to humans.[22,23] When bosentan was repeatedly administered to chimeric mice with humanized liver whose host mice were TK-NOG, the concentrations of alanine transaminase and total bile acids increased in the plasma. It can be assumed that a cholestatic liver injury might have occurred in the chimeric mice with humanized liver.[24] The mechanism of liver injury by bosentan in different species is still unclear and evaluation of the change in the HI may reveal the mechanism underlying bosentan-induced cholestasis.

In conclusion, here we studied the varying concentrations of bile acids in the sera of chimeric mice with different RIs. The concentration of total bile acids increased as the RIs were elevated and their values exceeded the actual concentrations observed in humans. The indexes obtained upon evaluating the compositions of bile acids, primary and secondary bile acid ratios, amino acid conjugates, and the HI were found to approach the human profiles. However, the composition of each bile acid does not correspond to that of humans. It is necessary to consider the contribution of bile acid metabolism in the remaining mouse hepatocytes, primary to secondary bile acid metabolism by intestinal flora and susceptibility of mouse FGF15 in chimeric mice with humanized liver. Chimeric mice expressing the gene coding for human FGF19 could be utilized in previous study.[14] The findings of the current study could be fundamental in providing knowledge on the use chimeric mice with humanized liver with a wide range of RIs to study drug metabolism, PK, and hepatotoxicity.

Acknowledgments This research was supported by JSPS KAKENHI Grant Numbers 18K06750 and PhoenixBio Co., Ltd. We would like to thank Prof. Eiso Hiyama, the representative of Hiroshima University and Dr. Takanori Harada of the Nuclear and Molecular Micro-Imaging Platform, for the facilitating the use of LC-MS/MS.

Conflict of interest The present study was conducted in collaboration with the PhoenixBio Co. Ltd.

REFERENCES

1) Grompe M, Strom S. Mice with human livers. *Gastroenterology*, **145**, 1209–1214 (2013).
2) Scheer N, Wilson ID. A comparison between genetically humanized and chimeric liver humanized mouse models for studies in drug metabolism and toxicity. *Drug Discov. Today*, **21**, 250–263 (2016).
3) Tateno C, Kawase Y, Tobita Y, Hamamura S, Ohshita H, Yokomichi H, Sanada H, Kakuni M, Shiota A, Kojima Y, Ishida Y, Shitara H, Wada NA, Tateishi H, Sudoh M, Nagatsuka S, Jishage K, Kohara M. Generation of novel chimeric mice with humanized livers by using hemizygous cDNA-uPA/SCID mice. *PLoS One*, **10**, e0142145 (2015).
4) Foster JR, Jacobsen M, Kenna G, Schulz-Utermoehl T, Morikawa Y, Salmu J, Wilson ID. Differential effect of troglitazone on the human bile acid transporters, MRP2 and BSEP, in the PXB hepatic chimeric mouse. *Toxicol. Pathol.*, **40**, 1106–1116 (2012).
5) Sanoh S, Horiguchi A, Sugihara K, Kotake Y, Tayama Y, Uramaru N, Ohshita H, Tateno C, Horie T, Kitamura S, Ohta S. Predictability of metabolism of ibuprofen and naproxen using chimeric mice with human hepatocytes. *Drug Metab. Dispos.*, **40**, 2267–2272 (2012).
6) Yoshizato K, Tateno C. A mouse with humanized liver as an animal model for predicting drug effects and for studying hepatic viral infection: where to next? *Expert Opin. Drug Metab. Toxicol.*, **9**, 1419–1435 (2013).
7) Sanoh S, Naritomi Y, Fujimoto M, Sato K, Kawamura A, Horiguchi A, Sugihara K, Kotake Y, Ohshita H, Tateno C, Horie T, Kitamura S, Ohta S. Predictability of plasma concentration-time curves in humans using single-species allometric scaling of chimeric mice with humanized liver. *Xenobiotica*, **45**, 605–614 (2015).

8) Uchida T, Imamura M, Kan H, Hiraga N, Hayes CN, Tsuge M, Abe-Chayama H, Aikata H, Makokha GN, Miki D, Ochi H, Ishida Y, Tateno C, Chayama K. Usefulness of humanized cDNA-uPA/SCID mice for the study of hepatitis B virus and hepatitis C virus virology. *J. Gen. Virol.*, **98**, 1040–1047 (2017).
9) Sanoh S, Yamachika Y, Tamura Y, Kotake Y, Yoshizane Y, Ishida Y, Tateno C, Ohta S. Assessment of amiodarone-induced phospholipidosis in chimeric mice with a humanized liver. *J. Toxicol. Sci.*, **42**, 589–596 (2017).
10) Kitamura S, Nitta K, Tayama Y, Tanoue C, Sugihara K, Inoue T, Horie T, Ohta S. Aldehyde oxidase-catalyzed metabolism of N1-methylnicotinamide *in vivo* and *in vitro* in chimeric mice with humanized liver. *Drug Metab. Dispos.*, **36**, 1202–1205 (2008).
11) Kamimura H, Ito S, Chijiwa H, Okuzono T, Ishiguro T, Yamamoto Y, Nishinoaki S, Ninomiya SI, Mitsui M, Kalgutkar AS, Yamazaki H, Suemizu H. Simulation of human plasma concentration-time profiles of the partial glucokinase activator PF-04937319 and its disproportionate N-demethylated metabolite using humanized chimeric mice and semi-physiological pharmacokinetic modeling. *Xenobiotica*, **47**, 382–393 (2017).
12) Chow EC, Quach HP, Zhang Y, Wang JZ, Evans DC, Li AP, Silva J, Tirona RG, Lai Y, Pang KS. Disrupted murine gut-to-human liver signaling alters bile acid homeostasis in humanized mouse liver models. *J. Pharmacol. Exp. Ther.*, **360**, 174–191 (2017).
13) Thakare R, Alamoudi JA, Gautam N, Rodrigues AD, Alnouti Y. Species differences in bile acids I. Plasma and urine bile acid composition. *J. Appl. Toxicol.*, **38**, 1323–1335 (2018).
14) Naugler WE, Tarlow BD, Fedorov LM, Taylor M, Pelz C, Li B, Darnell J, Grompe M. Fibroblast growth factor signaling controls liver size in mice with humanized livers. *Gastroenterology*, **149**, 728–740 (2015).
15) Utoh R, Tateno C, Kataoka M, Tachibana A, Masumoto N, Yamasaki C, Shimada T, Itamoto T, Asahara T, Yoshizato K. Hepatic hyperplasia associated with discordant xenogeneic parenchymal-nonparenchymal interactions in human hepatocyte-repopulated mice. *Am. J. Pathol.*, **177**, 654–665 (2010).
16) Takahashi S, Fukami T, Masuo Y, Brocker CN, Xie C, Krausz KW, Wolf CR, Henderson CJ, Gonzalez FJ. Cyp2c70 is responsible for the species difference in bile acid metabolism between mice and humans. *J. Lipid Res.*, **57**, 2130–2137 (2016).
17) Solaas K, Ulvestad A, Söreide O, Kase BF. Subcellular organization of bile acid amidation in human liver: a key issue in regulating the biosynthesis of bile salts. *J. Lipid Res.*, **41**, 1154–1162 (2000).
18) Thakare R, Alamoudi JA, Gautam N, Rodrigues AD, Alnouti Y. Species differences in bile acids II. Bile acid metabolism. *J. Appl. Toxicol.*, **38**, 1336–1352 (2018).
19) Heuman DM. Quantitative estimation of the hydrophilic-hydrophobic balance of mixed bile salt solutions. *J. Lipid Res.*, **30**, 719–730 (1989).
20) Ashby K, Navarro Almario EE, Tong W, Borlak J, Mehta R, Chen M. Review article: therapeutic bile acids and the risks for hepatotoxicity. *Aliment. Pharmacol. Ther.*, **47**, 1623–1638 (2018).
21) Perez MJ, Britz O. Bile-acid-induced cell injury and protection. *World J. Gastroenterol.*, **15**, 1677–1689 (2009).
22) Fattinger K, Funk C, Pantze M, Weber C, Reichen J, Stieger B, Meier PJ. The endothelin antagonist bosentan inhibits the canalicular bile salt export pump: a potential mechanism for hepatic adverse reactions. *Clin. Pharmacol. Ther.*, **69**, 223–231 (2001).
23) Humbert M, Segal ES, Kiely DG, Carlsen J, Schwierin B, Hoeper MM. Results of European post-marketing surveillance of bosentan in pulmonary hypertension. *Eur. Respir. J.*, **30**, 338–344 (2007).
24) Xu D, Wu M, Nishimura S, Nishimura T, Michie SA, Zheng M, Yang Z, Yates AJ, Day JS, Hillgren KM, Takeda ST, Guan Y, Guo Y, Peltz G. Chimeric TK-NOG mice: a predictive model for cholestatic human liver toxicity. *J. Pharmacol. Exp. Ther.*, **352**, 274–280 (2015).