DMD Fast Forward. Published on May 20, 2016 as DOI: 10.1124/dmd.116.070532
This article has not been copyedited and formatted. The final version may differ from this version.

DMD #70532

SHORT COMMUNICATION

Recommendation to Exclude Bile-Duct-Cannulated Rats with Hyperbilirubinemia for Proper Conduct of Biliary Drug Excretion Studies

Authors: Koji Kato, Yoshitaka Hasegawa, Katsuya Iwata, Takuya Ichikawa, Tohru Yahara, Satoshi Tsuji, Masayuki Sugiura, and Jun-ichi Yamaguchi

Affiliation: Drug Safety and Pharmacokinetics Laboratories, Taisho Pharmaceutical Co. Ltd., Saitama, Japan (K.K., Y.H., K.I., T.I., T.Y., S.T., M.S., J.Y.)

DMD Fast Forward. Published on May 20, 2016 as DOI: 10.1124/dmd.116.070532
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title: Impact of Hyperbilirubinemia Caused by Bile-Duct Cannulation

Correspondence: Koji Kato, Ph.D.

Drug Safety and Pharmacokinetics Laboratories, Taisho Pharmaceutical Co. Ltd.,

1-403, Yoshino-cho, Kita-ku, Saitama-shi, Saitama 331-9530, Japan.

TEL: +81-48-669-3036

FAX: +81-48-652-7254

E-mail: ko-katou@so.taisho.co.jp

Number of text pages: 11 pages

Number of Tables: 2

Number of Figures: 2

Number of References: 23Number of words in Abstract: 250

Number of words in Introduction, 482

Number of words in Results and Discussion, 1383

ABBREVIATIONS

BDC, bile-duct cannulation; CL_{bile}, biliary clearance; CL_{total}, total body clearance with regard to the plasma concentration; EHBR, Eisai hyperbilirubinemic rats; GGT, γ-glutamyltransferase activity; HB, hyperbilirubinemia; MRP, multidrug resistance-associated protein; OATP, organic anion transporting polypeptide; TBIL, total bilirubin concentration

DMD Fast Forward. Published on May 20, 2016 as DOI: 10.1124/dmd.116.070532
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

Hyperbilirubinemia (HB) is sometimes encountered following bile duct cannulation in rats. It possibly originates from the reduced functioning of multidrug resistance-associated protein 2 (Mrp2) and subsequent adaptive alterations in the expression of Mrp3 and the organic anion transporting polypeptides (Oatps). Our aim was to clarify the importance of excluding bile-duct-cannulated (BDC) rats with HB for proper conduct of drug excretion studies. We detected HB (serum total bilirubin concentration, TBIL≥0.20 mg/dL) in 16% of all the BDC rats prepared. The serum activities of aspartate aminotransferase, alanine aminotransferase, leucine aminopeptidase, and alkaline phosphatase were within the respective normal ranges, in the BDC rats with mild HB (TBIL, 0.20 to 0.79 mg/dL), indicating the absence of hepatic failure. In the pharmacokinetics of pravastatin, an Oatps/Mrp2 probe drug in the BDC rats, the apparent volume of distribution and the clearance were smaller in the mild HB group as compared to those in the normal group, suggesting the reduction of apparent hepatic uptake and hepatobiliary elimination. The biliary excretion (% of dose) was significantly reduced by 54%, suggesting that the biliary efflux activity via Mrp2 was reduced greater relative to metabolic activity in hepatocytes. The serum γ-glutamyltransferase (GGT) activity correlated with TBIL and inversely correlated with biliary excretion of pravastatin, a finding which could serve as a clue to uncover the regulatory system involving cooperation between GGT and Mrp2. In conclusion, BDC rats with HB, however mild, should be excluded from drug excretion studies to avoid the risk of underestimation of the biliary excretion of drugs.

DMD Fast Forward. Published on May 20, 2016 as DOI: 10.1124/dmd.116.070532
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Characterization of the elimination pathways of candidate drugs is one of the most important studies in drug development. Rats with bile-duct cannulation (BDC) have been used to obtain valuable information about the biliary excretion of drugs. An extracorporeal BDC bypass loop is implanted into the rats by surgery (Lipsky and Berkley, 1977), in order to collect bile specimens under unrestrained freely moving conditions of the animals (Balabaud et al., 1981). However, the BDC surgery sometimes causes hyperbilirubinemia (HB) (Faure et al., 2006). Decreased hepatobiliary transport of bilirubin and its glucuronides is a possible cause of the HB in such rats. Multidrug resistance-associated protein 2 (MRP2) plays a crucial role in the efflux of endogenous and exogenous anionic compounds from the hepatocytes into bile (van der Schoor et al., 2015). The Eisai hyperbilirubinemic rat (EHBR), an Mrp2-deficient mutant rat, has been reported to show reduced biliary elimination of bilirubin glucuronides (Kato et al, 2012). It would be reasonable to consider that Mrp2 function is reduced by BDC, considering the common knowledge that Mrp2 expression is down-regulated in intrahepatic and obstructive cholestasis (Trauner et al., 1997), and that cholestasis can be induced even by partial obstruction of the bile duct (Rodriguez-Garay, 2004). Under such conditions of Mrp2 deficiency and cholestasis, the expression of Mrp3, a basolateral efflux pump to transport bilirubin glucuronides back into the bloodstream, is strongly upregulated (Kuroda et al., 2004; Soroka et al., 2001) and is responsible for the conjugated HB (Keppler, 2014). As an additional adaptive change against Mrp2 function impairment, the expression of the organic anion transporting polypeptide 1 (Oatp1) and Oatp2 are down-regulated (Kuroda et al., 2004; Dumont et al., 1997). The rat model with partial obstruction of the bile duct has been reported to have the impairment of Oatp1 function along with that of Mrp2 function (Rodriguez-Garay, 2004). Since the Oatps play a role in the uptake of both unconjugated and conjugated bilirubin across the sinusoidal membrane from the bloodstream into the hepatocytes, reduced functioning of these proteins leads to both

DMD Fast Forward. Published on May 20, 2016 as DOI: 10.1124/dmd.116.070532
This article has not been copyedited and formatted. The final version may differ from this version.

unconjugated and conjugated HB (van de Steeg et al., 2010; Chu et al., 2015, Watanabe et al., 2015). Therefore, there is the risk that the use of BDC rats with HB can lead to underestimation of the biliary elimination of Oatps- and/or Mrp2-substrates in drug excretion studies.

In this study, we investigated the frequency distribution of HB, the serum enzyme activities for evaluating hepatobiliary function in BDC rats with HB, and the pharmacokinetics and biliary excretion of pravastatin, an Oatps/Mrp2 probe drug. Our goal is to verify the reduced hepatobiliary elimination of the anionic drugs via the Oatps and/or Mrp2 in the BDC rats with HB and to propose the cutoff value of the serum concentration of total bilirubin (TBIL) to exclude an animal from a drug excretion study. Our findings would serve as useful information for proper conduct of biliary excretion studies during drug development.

Materials and Methods

Animals and Reagents. Eight-week-old male Sprague-Dawley (SD) rats with an extracorporeal BDC bypass loop were purchased from Charles River Laboratories Japan (Yokohama, Japan) and Japan SLC (Hamamatsu, Japan). All of the experimental procedures involving animal handling were approved by the Institutional Animal Care and Use Committee of Taisho Pharmaceutical Co., Ltd. and were in accordance with the Guidelines for the Proper Conduct of Animal Experiments (Science Council of Japan, 2006, http://www.scj.go.jp/en/report/index.html). Pravastatin and pravastatin-d_3 were obtained from Toronto Research Chemicals (North York, Canada). All other chemicals used were of HPLC grade or of the highest purity grade available commercially.

Biochemical Assays. Blood specimens were collected from the tail vein of the BDC rats at 4 to 8 days post-surgery. The serum levels of parameters reflecting hepatobiliary function were assayed using the Hitachi 7180 Auto Analyzer (Hitachi High-Technologies, Tokyo, Japan).

Pharmacokinetics and Biliary Excretion Study. Pravastatin (5 mg/kg) in saline was

DMD Fast Forward. Published on May 20, 2016 as DOI: 10.1124/dmd.116.070532
This article has not been copyedited and formatted. The final version may differ from this version.

injected via the internal jugular vein into the BDC rats. Bile specimens were collected for 1.5 h under freely moving conditions of the animals (Balabaud et al., 1981). Blood specimens were collected through the tail vein at 0.083, 0.25, 0.5, 0.75, 1, and 1.5 h after the administration of pravastatin, transferred into EDTA tubes and then centrifuged to prepare plasma samples. Then, after the animals were sacrificed, their livers were removed, weighed, and homogenized in 4 volumes of ice-cold water. The concentrations of pravastatin in the biological samples were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (See Supplementary Methods for details). The pharmacokinetics of pravastatin was analyzed using the software, Phoenix WinNonlin 6.2 (Pharsight, Mountain View, CA). The total body clearance (CL_{total}) and biliary clearance (CL_{bile}) were calculated by dividing the dose and cumulative biliary excretion amount, respectively, by the area under the plasma concentration–time curve (AUC). The non-biliary clearance ($CL_{non\text{-}bile}$) was calculated by subtracting CL_{bile} from CL_{total}.

Statistical Analysis. Data are presented as the mean ± S.E.M. *P* values < 0.05 were considered to indicate statistical significance. All the statistical analyses were performed using the SAS 9 software (SAS Institute, Cary, NC) (See Supplementary Methods for details).

Results and Discussion

The frequency distribution of the serum concentrations of TBIL was assessed based on the background data (n=94) of the BDC rats prepared for biliary excretion studies of candidate drugs. The median and lower (25th) and upper (75th) quartiles of TBIL were 0.05, 0.01, and 0.11, respectively. Animals with TBIL <0.20, 0.20 to 0.79, 0.80 to 1.99, and ≥2.0 mg/dL were defined as having normal TBIL levels, mild HB, moderate HB, and severe HB, respectively, in this study; the percentages of animals in these 4 categories were 84, 12, 3, and 1%, respectively (Table 1). As might be expected, the frequency distribution probably changes with dependence

DMD Fast Forward. Published on May 20, 2016 as DOI: 10.1124/dmd.116.070532
This article has not been copyedited and formatted. The final version may differ from this version.

on surgical technique. The serum activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were slightly increased in the BDC rats with mild HB as compared to those in the normal BDC rats, and increased by approximately 2-fold in the BDC rats with moderate HB. There were no significant differences in the leucine aminopeptidase (LAP) activity. The alkaline phosphatase (ALP) was found to be rather lower in the normal BDC rats, considering the level of 1543±46 IU/L found in the untreated rats (see Supplemental Table 1). The serum levels of these four enzymes were within 2-fold as compared to the untreated rats (see Supplemental Table 1) in the BDC rats with mild HB as well as in the normal BDC rats, indicating that hepatobiliary failure scarcely developed in these rats.

Both the levels of direct bilirubin (DBIL) and indirect bilirubin (IDBIL) increased with increasing TBIL, although IDBIL to a lesser degree than DBIL (Table 1). The accumulation of IDBIL can be caused by the decreased functioning of the Oatps, and that of DBIL can be caused by the decreased functioning of Mrp2 and/or Oatps, and/or the increased functioning of Mrp3 (van de Steeg et al., 2010; Keppler, 2014). A number of anionic drugs and metabolites are excreted into bile by the OATPs- and/or MRP2-mediated transport (Ieiri et al., 2009). To verify whether these transport activities are reduced in the BDC rats with HB, we conducted a pharmacokinetic and biliary excretion study of pravastatin, an Oatps/Mrp2 probe drug (Watanabe et al., 2009). The mean plasma concentration-time and cumulative biliary excretion-time profiles of pravastatin are shown in Fig. 1. The pharmacokinetic parameters are listed with the biliary flow rate, TBIL, and GGT in Table 2. Scarce difference was observed between the normal (TBIL, 0.00, 0.07, and 0.19 mg/dL) and mild HB (TBIL, 0.33, 0.57, and 0.75 mg/mL) groups with regard to the biliary flow rate. The plasma concentrations and AUC were higher, while the CL_{total} was lower, in the mild HB group as compared to the values in the normal group, although the differences were not statistically significant. The apparent volume of distribution (V_d) and CL_{bile} were significantly reduced by 41% and 72%, respectively, in the mild HB group.

DMD Fast Forward. Published on May 20, 2016 as DOI: 10.1124/dmd.116.070532
This article has not been copyedited and formatted. The final version may differ from this version.

The reduced V_d suggests the reduction of apparent hepatic uptake which catalyzes the marked liver-specific distribution of pravastatin in rats (Komai et al., 1992). It can be led by the decreased functioning of the Oatps (Tokui et al., 1999; Hsiang et al., 1999) and/or the increased functioning of Mrp3, although the involvement of Mrp3 has been little reported in the disposition of pravastatin. The $CL_{non\text{-}bile}$ is considered as the metabolic clearance in the liver, since the renal clearance of pravastatin is negligible in rats (Komai et al., 1992). Thus, the CL_{bile} and the $CL_{non\text{-}bile}$ account for hepatic clearance. The intrinsic hepatic clearance ($CL_{int,h}$) is expressed by the following equation (Kusuhara and Sugiyama, 2009):

$$CL_{int,h} = \frac{PS_{inf}}{PS_{eff} + CL_{int}} \times CL_{int} = \frac{PS_{inf}}{PS_{eff} + (CL_{int,bile} + CL_{int,met})} \times (CL_{int,bile} + CL_{int,met})$$

where PS_{inf} and PS_{eff} represent the intrinsic clearance for cellular uptake (influx) and efflux into the systemic circulation, respectively. CL_{int} represents the the intrinsic clearance based on the unbound concentration in the hepatocytes and consists of two intrinsic clearance for biliary efflux from hepatocytes ($CL_{int,bile}$) and for metabolism in hepatocytes ($CL_{int,met}$). When the CL_{int} is much greater than the PS_{eff}, the $CL_{int,h}$ can be approximated to the PS_{inf}. Indeed, the uptake is rate limiting for the hepatic elimination of pravastatin in normal rats (Kusuhara and Sugiyama, 2009). Therefore, the reduced uptake likely caused the reduction of the CL_{bile} and $CL_{non\text{-}bile}$ in this study. Furthermore, in BDC rats with HB, the uptake may be no longer the only rate limiting for the $CL_{int,h}$, as decreased $CL_{int,bile}$ and increased PS_{eff}, which can be caused by altered functioning of Mrp2 and Mrp3, respectively, progressed. The reduction of CL_{bile} was much greater than that of $CL_{non\text{-}bile}$, supporting that the CL_{bile} was affected by the $CL_{int,bile}$, namely Mrp2 activity, in addition to the hepatic uptake.

The liver concentration and liver-to-plasma concentration ratio at 1.5 h post-dose were greater in the HB group as compared to that in the normal group. Since renal clearance of pravastatin is negligible in rats, the AUC in the liver (AUC_h) is expressed by the following equation (Kusuhara

DMD Fast Forward. Published on May 20, 2016 as DOI: 10.1124/dmd.116.070532
This article has not been copyedited and formatted. The final version may differ from this version.

and Sugiyama, 2009):

$$AUC_h = \frac{Dose}{f_{u,h} \cdot CL_{int}}$$

where $f_{u,h}$ represents the unbound fractions of pravastatin in the hepatocytes. The AUC_h is not influenced by change in PS_{inf} and PS_{eff} for which Oatps and Mrp3, respectively, are responsible. Thus, the elevation of liver concentration suggests the reduced CL_{int}. When renal clearance is negligible, the biliary excretion ($f_{e,bile}$, % of dose) is determined by the following equation:

$$f_{e,bile} = \frac{CL_{bile}}{CL_{bile} + CL_{non-bile}} \times 100 = \frac{CL_{int,bile}}{CL_{int,bile} + CL_{int,met}} \times 100$$

The $f_{e,bile}$ (% of dose) was significantly reduced by 54% in the mild HB group (Table 2), suggesting that the biliary efflux activity via Mrp2 was reduced relative to the metabolic activity in hepatocytes. Given the $CL_{int,met}$ is little affected by BDC, the $f_{e,bile}$ is dependent on the $CL_{int,bile}$ which is controlled by Mrp2 activity.

The down-regulation of mRNA and protein expression of Mrp2 and the internalization of Mrp2 (Sekine et al., 2006) are assumed as candidates of mechanism responsible for the reduced Mrp2 activity. Then, the expression of Oatps and Mrp3 seems to be adaptively altered as observed in rats with the impairment of Mrp2 (Kuroda et al., 2004; Soroka et al., 2001; Dumont et al., 1997), leading to the reduced hepatic uptake. Furthermore, endogenous and food-derived compounds such as phase II metabolites of bilirubin and isoflavonoids, which are accumulated by the impairment of Mrp2 (Kato et al., 2012), might contribute to the reduced hepatic uptake.

The individual values of TBIL were inversely correlated with the $f_{e,bile}$ value of pravastatin, with high correlation coefficients (Fig. 2A). Marked difference in the $f_{e,bile}$ values was observed between the individuals with the minimum and maximum TBIL values of 0.00 and 0.75 mg/dL, respectively (64.4 *versus* 10.8%). Considering from this data, we recommend that the cut-off value of the TBIL is set below 0.20 mg/dL. The purpose of experiment should also be considered for determining the cut-off value. A stricter exclusion criterion is likely adequate in

the GLP-like studies for the new drug application (NDA). On the other hand, in the early drug discovery stage, the experiment including BDC rats with HB may be useful to explore the involvement of transporters in the biliary elimination of anionic candidate drugs.

As unexpected results, the γ-glutamyltransferase (GGT) activity was increased with increasing TBIL in the BDC rats (Table 1). Then, the GGT was inversely-correlated with the $f_{e,bile}$ of pravastatin, which likely reflects the Mrp2 activity (Fig 2B). We can propose two hypotheses about the relationship between GGT and Mrp2 as follows. First, since GGT is present in many tissues and plays an important role in initiating the metabolism of glutathione conjugates to mercapturic acids (Zhang et al., 2005), it may be induced as an adaptive response in the presence of Mrp2 function impairment to prevent the accumulation of glutathione conjugates, which are Mrp2 substrates, by changing the elimination pathway from bile to urine. Second, since both GGT and Mrp2 are responsible for glutathione homeostasis (Zhang et al., 2005; Ballatori and Rebbeor, 1998; Lu et al., 1996), the alterations might be regulatory responses to the negative influence on glutathione homeostasis exerted by the BDC. These hypotheses may serve as clues to find the unrecognized relationship between GGT and Mrp2.

In conclusion, our findings suggest that the use of BDC rats with HB can lead to underestimation of the hepatic uptake and biliary efflux of the anionic drugs via Oatps and Mrp2, respectively. Therefore, such animals with HB, however mild, should be generally excluded by TBIL measurement for proper conduct of drug excretion studies.

Authorship Contributions

Participated in research design: Kato and Sugiura

Conducted experiments: Kato, Hasegawa, Iwata, Ichikawa, Yahara and Tsuji

Performed data analysis: Kato, Hasegawa, and Iwata

Wrote or contributed to the writing of the manuscript: Kato, Hasegawa, Iwata, and Yamaguchi

References

Balabaud C, Saric J, Gonzalez P, and Delphy C (1981) Bile collection in free moving rats. *Lab Anim Sci* **31**:273-275.

Ballatori N and Rebbeor JF (1998) Roles of MRP2 and oatp1 in hepatocellular export of reduced glutathione. *Semin Liver Dis* **18**:377-387.

Chu X, Shih SJ, Shaw R, Hentze H, Chan GH, Owens K, Wang S, Cai X, Newton D, Castro-Perez J, Salituro G, Palamanda J, Fernandis A, Ng CK, Liaw A, Savage MJ, and Evers R (2015) Evaluation of cynomolgus monkeys for the identification of endogenous biomarkers for hepatic transporter inhibition and as a translatable model to predict pharmacokinetic interactions with statins in humans. *Drug Metab Dispos* **43**:851-863.

Dumont M, Jacquemin E, D'Hont C, Descout C, Cresteil D, Haouzi D, Desrochers M, Stieger B, Hadchouel M, and Erlinger S (1997) Expression of the liver Na+-independent organic anion transporting polypeptide (oatp-1) in rats with bile duct ligation. *J Hepatol* **27**:1051-1056.

Faure L, Vignand P, Raynard A, Pasello-Legrand F, and Descotes J (2006) Evaluation of a surgical procedure to measure drug biliary excretion of rats in regulatory safety studies. *Fundam Clin Pharmacol* **20**:587-593.

Hsiang B, Zhu Y, Wang Z, Wu Y, Sasseville V, Yang WP, and Kirchgessner TG (1999) A novel human hepatic organic anion transporting polypeptide (OATP2). Identification of a liver-specific human organic anion transporting polypeptide and identification of rat and human hydroxymethylglutaryl-CoA reductase inhibitor transporters. *J Biol Chem* **274**:37161-37168.

Ieiri I, Higuchi S, Sugiyama Y (2009) Genetic polymorphisms of uptake (OATP1B1, 1B3) and efflux (MRP2, BCRP) transporters: implications for inter-individual differences in the pharmacokinetics and pharmacodynamics of statins and other clinically relevant drugs. *Expert Opin Drug Metab Toxicol* **5**:703-729.

Kato K, Kusuhara H, Kumagai Y, Ieiri I, Mori H, Ito S, Nakai Y, Maeda K, and Sugiyama Y (2012) Association of multidrug resistance-associated protein 2 single nucleotide polymorphism rs12762549 with the basal plasma levels of phase II metabolites of isoflavonoids in healthy Japanese individuals. *Pharmacogenet Genomics* **22**:344-354.

Keppler D (2014) The roles of MRP2, MRP3, OATP1B1, and OATP1B3 in conjugated hyperbilirubinemia. *Drug Metab Dispos* **42**:561-565.

Komai T, Kawai K, Tokui T, Tokui Y, Kuroiwa C, Shigehara E, and Tanaka M (1992) Disposition and metabolism of pravastatin sodium in rats, dogs and monkeys. *Eur J Drug Metab Pharmacokinet* **17**:103-113.

Kuroda M, Kobayashi Y, Tanaka Y, Itani T, Mifuji R, Araki J, Kaito M, and Adachi Y (2004) Increased hepatic and renal expressions of multidrug resistance-associated protein 3 in Eisai hyperbilirubinuria rats. *J Gastroenterol Hepatol* **19**:146-153.

Kusuhara H and Sugiyama Y (2009) In vitro-in vivo extrapolation of transporter-mediated clearance in the liver and kidney. *Drug Metab Pharmacokinet* **24**:37-52.

Lipsky MH and Berkley S (1977) Prolonged biliary fistulization in the rat without interruption of the enterohepatic cycle. *J Surg Res* **22**:65-68.

Lu SC, Cai J, Kuhlenkamp J, Sun WM, Takikawa H, Takenaka O, Horie T, Yi J, and Kaplowitz N (1996) Alterations in glutathione homeostasis in mutant Eisai hyperbilirubinemic rats. *Hepatology* **24**:253-258.

Rodriguez-Garay EA, Rodríguez GP, Pisani G, Taborda M, and Viglianco RA (2004) Reversible cholestasis induced by experimental partial obstruction of the bile duct; Biochemical, morphometric and hepatic transport kinetic studies. *Pathophysiology* **11**:7-15.

Sekine S, Ito K, and Horie T (2006) Oxidative stress and Mrp2 internalization. *Free Radic Biol Med* **40**:2166-2174.

Soroka CJ, Lee JM, Azzaroli F, and Boyer JL (2001) Cellular localization and up-regulation of

multidrug resistance-associated protein 3 in hepatocytes and cholangiocytes during obstructive cholestasis in rat liver. *Hepatology* **33**:783-791.

Tokui T, Nakai D, Nakagomi R, Yawo H, Abe T, and Sugiyama Y (1999) Pravastatin, an HMG-CoA reductase inhibitor, is transported by rat organic anion transporting polypeptide, oatp2. *Pharm Res* **16**:904-908.

Trauner M, Arrese M, Soroka CJ, Ananthanarayanan M, Koeppel TA, Schlosser SF, Suchy FJ, Keppler D, and Boyer JL (1997) The rat canalicular conjugate export pump (Mrp2) is down-regulated in intrahepatic and obstructive cholestasis. *Gastroenterology* **113**:255-264.

van de Steeg E, Wagenaar E, van der Kruijssen CM, Burggraaff JE, de Waart DR, Elferink RP, Kenworthy KE, and Schinkel AH (2010) Organic anion transporting polypeptide 1a/1b-knockout mice provide insights into hepatic handling of bilirubin, bile acids, and drugs. *J Clin Invest* **120**:2942-2952.

van der Schoor LW, Verkade HJ, Kuipers F, and Jonker JW (2015) New insights in the biology of ABC transporters ABCC2 and ABCC3: impact on drug disposition. *Expert Opin Drug Metab Toxicol* **11**:273-293.

Watanabe T, Kusuhara H, Maeda K, Shitara Y, and Sugiyama Y (2009) Physiologically based pharmacokinetic modeling to predict transporter-mediated clearance and distribution of pravastatin in humans. *J Pharmacol Exp Ther* **328**:652-662.

Watanabe T, Miyake M, Shimizu T, Kamezawa M, Masutomi N, Shimura T, and Ohashi R (2015) Utility of bilirubins and bile acids as endogenous biomarkers for the inhibition of hepatic transporters. *Drug Metab Dispos* **43**:459-466.

Zhang H, Forman HJ, and Choi J (2005) Gamma-glutamyl transpeptidase in glutathione biosynthesis. *Methods Enzymol* **401**:468-483.

DMD Fast Forward. Published on May 20, 2016 as DOI: 10.1124/dmd.116.070532
This article has not been copyedited and formatted. The final version may differ from this version.

Figure legends

Fig. 1. (A) Plasma concentration-time and (B) cumulative biliary excretion- time profiles in BDC rats after intravenous administration of pravastatin (5 mg/kg). The concentrations in the normal group (open circles) and mild HB group (closed circles) were measured by LC-MS/MS. The TBIL in the normal and mild HB groups ranged from 0.00 to 0.19 and 0.33 to 0.75 mg/dL, respectively. Each point and vertical bars represents the mean ± S.E.M. of three animals. * $P < 0.05$ *versus* normal BDC rats.

Fig. 2. Relationships between (A) the total bilirubin concentration (TBIL) or (B) γ-glutamyltransferase activity (GGT) and the biliary excretion of the drug (% of dose) in six BDC rats after intravenous administration of pravastatin (5 mg/kg). R, correlation coefficient.

Tables

TABLE 1

Serum biochemistry parameters in the bile-duct cannulated rats

Parameter	Normal BDC rats (TBIL<0.2)	BDC rats with hyperbilirubinemia		
		Mild (TBIL, 0.2 to 0.79)	Moderate (TBIL, 0.8 to 1.99)	Severe (TBIL≥2.0)
n	79	11	3	1
TBIL (mg/dL)	0.05 ± 0.01	0.39 ± 0.03 **	1.19 ± 0.18 **	4.96
DBIL (mg/dL)	0.04 ± 0.005	0.35 ± 0.03 **	1.11 ± 0.17 **	4.39
IDBIL (mg/dL)	0.005 ± 0.001	0.05 ± 0.01 **	0.08 ± 0.02 **	0.57
AST (IU/L)	207 ± 12	236 ± 27	430 ± 50 **	549
ALT (IU/L)	62 ± 3	78 ± 11	135 ± 24 **	211
LAP (IU/L)	85.2 ± 1.4	86.5 ± 4.4	97.4 ± 6.6	95.6
ALP (IU/L)	1196 ± 35	1850 ± 176 **	1460 ± 308	1697
GGT (IU/L)	2.39 ± 0.23	6.06 ± 0.89 **	22.13 ± 11.01 **	50.79

Each data represents the mean ± S.E.M.; ** $P < 0.01$ *versus* normal BDC rats

DMD Fast Forward. Published on May 20, 2016 as DOI: 10.1124/dmd.116.070532
This article has not been copyedited and formatted. The final version may differ from this version.

TABLE 2

Pharmacokinetic parameters after intravenous administration of pravastatin (5 mg/kg) to BDC rats and the serum biochemistry parameters pre-dose.

Parameter	Normal group	Mild HB group
AUC (ng·h/mL)	1180 ± 159	2380 ± 505
CL_{total} (mL/h/kg)	4400 ± 699	2310 ± 521
V_d (mL/kg)	840 ± 69	497 ± 77 *
CL_{bile} (mL/h/kg)	2390 ±247	661 ± 367 *
$CL_{non\text{-}bile}$ (mL/h/kg)	2020 ±505	1650 ± 241
Biliary excretion (% of dose)	55.0 ±4.8	25.5 ± 9.1 *
$C_{plasma,1.5h}$ (ng/mL)	12.9 ± 7.6	17.9 ± 9.5
$C_{liver,1.5h}$ (ng/g)	< 5 (3.39 ± 0.93)†	14.3 ± 4.5
C_{liver}-to-C_{plasma} ratio	< 0.80 ± 0.42 (0.40 ± 0.12) †	1.2 ± 0.4
Biliary flow rate (mL/h/kg)	5.78 ± 0.46	6.89 ± 0.74
TBIL (mg/dL)	0.09 ± 0.06	0.55 ± 0.12 *
GGT (IU/L)	2.20 ± 0.98	7.14 ± 1.35 *

The individual TBIL values in the normal and mild HB groups ranged from 0.00 to 0.19 and 0.33 to 0.75 mg/dL, respectively. The $C_{plasma,1.5h}$ and $C_{liver,1.5h}$ represent the concentrations of plasma and liver, respectively, collected at 1.5 h post-dose. Each data represents the mean ± S.E.M. of three animals.; *P<0.05 *versus* normal group. † The values in parentheses were calculated by extrapolation of standard curves below the lower limit of quantification (LLOQ, 5 ng/g).

Fig. 1

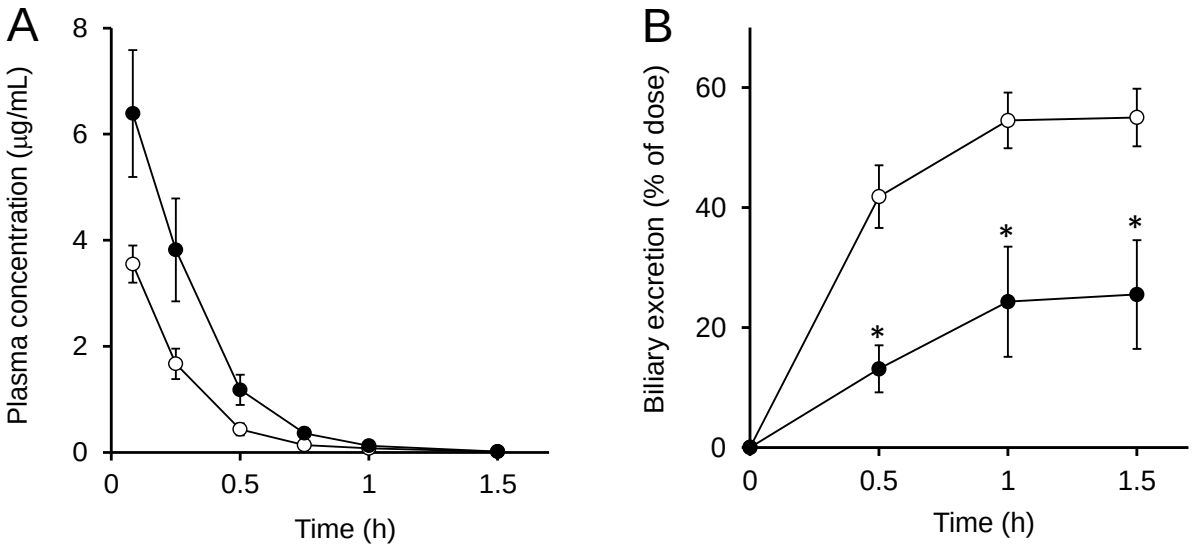
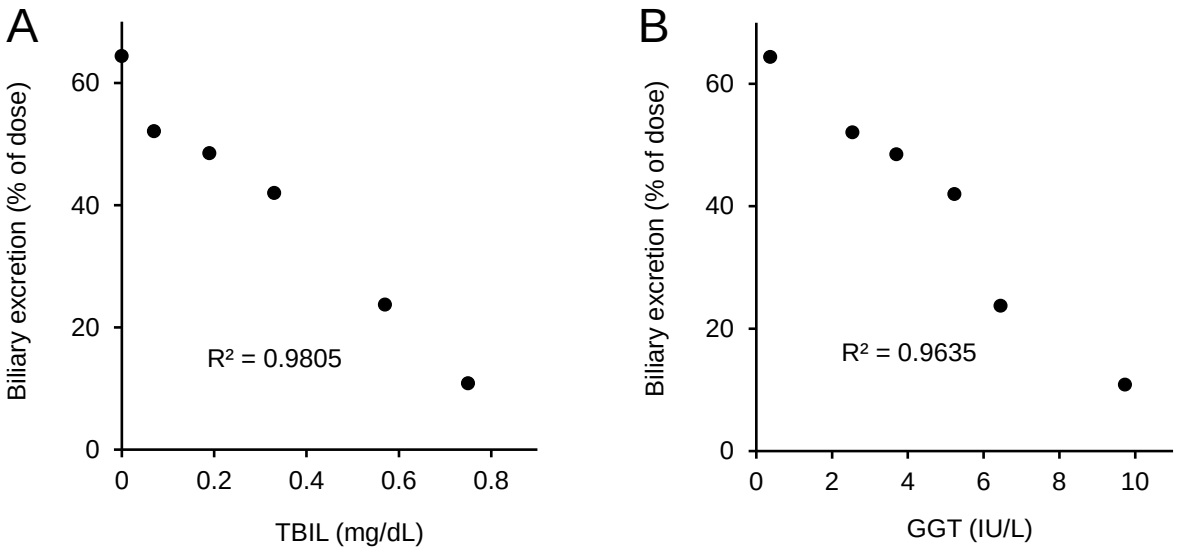

A
Plasma concentration (μg/mL)
8
6
4
2
0
0
0.5
1
1.5
Time (h)
B
Biliary excretion (% of dose)
60
40
20
0
0
0.5
1
1.5
Time (h)
*
*
*

Fig. 2


A
Biliary excretion (% of dose)
60
40
20
0
R² = 0.9805
0
0.2
0.4
0.6
0.8
TBIL (mg/dL)
B
Biliary excretion (% of dose)
60
40
20
0
R² = 0.9635
0
2
4
6
8
10
GGT (IU/L)