Short Communication

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

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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 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, 0.20–0.79 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 with the normal group, suggesting the reduction of apparent hepatic uptake and hepatobiliary elimination. The biliary excretion (percentage of dose) was significantly reduced by 54%, suggesting that the biliary efflux activity via Mrp2 was reduced to a greater extent 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.

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) to collect bile specimens from the animals under unrestrained freely moving conditions (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, 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 downregulated 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 et al., 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 (Soroka et al., 2001; Kuroda et al., 2004) 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 is downregulated (Dumont et al., 1997; Kuroda et al., 2004). 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 et al., 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 unconjugated and conjugated HB (van de Steeg et al., 2010; Chu et al., 2015; Watanabe et al., 2015). Therefore, there is a risk that the use of BDC rats with HB can lead to underestimation of the biliary elimination of Oatp 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 rats with an extracorporeal BDC bypass loop were purchased from Charles River Laboratories (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 (http://www.scj.go.jp/en/report/index.html). Pravastatin and pravastatin-d_{4} were obtained from Toronto Research Chemicals (North York, Canada). All other chemicals used were of high-performance liquid chromatography grade or of the highest purity grade available commercially.

Biochemical Assays. Blood specimens were collected from the tail vein of the BDC rats at 4–8 days postsurgery. 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 injected via the internal jugular vein into the BDC rats. Bile specimens were collected for 1.5 hours with the animals under freely moving conditions (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 hours 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 biologic samples were measured by liquid chromatography–tandem mass spectrometry (see Supplemental 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_{tota}) 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 nonbiliary clearance (CL_{non-bile}) was calculated by subtracting CL_{bile} from CL_{tota}.

Statistical Analysis. Data are presented as the mean ± S.E.M. P values <0.05 were considered to indicate statistical significance. All statistical analyses were performed using the SAS 9 software (SAS Institute, Cary, NC) (see Supplemental 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, 0.20–0.79, 0.80–1.99, 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 four categories were 84, 12, 3, and 1%, respectively (Table 1). As might be expected, the frequency distribution probably changes with dependence on surgical technique. The serum activities of aspartate aminotransferase and alanine aminotransferase were slightly increased in the BDC rats with mild HB as compared with those in the normal BDC rats, and were increased by approximately 2-fold in the BDC rats with moderate HB. There were no significant differences in the leucine aminopeptidase activity. The alkaline phosphatase was found to be lower in the normal BDC rats, considering the significance of the leucine aminopeptidase activity. The alkaline phosphatase was found to be lower in the normal BDC rats, considering the significant differences in the leucine aminopeptidase activity. The alkaline phosphatase was found to be lower in the normal BDC rats, considering the significant differences in the leucine aminopeptidase activity. The alkaline phosphatase was found to be lower in the normal BDC rats, considering the significant differences in the leucine aminopeptidase activity.

The levels of both direct bilirubin (DBIL) and indirect bilirubin (IDBIL) increased with increasing TBIL, although IDBIL to a lesser degree than

### Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal BDC Rats (TBIL &lt; 0.2)</th>
<th>BDC Rats with Hyperbilirubinemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>79</td>
<td>11</td>
</tr>
<tr>
<td>TBIL (mg/dl)</td>
<td>0.05 ± 0.01</td>
<td>0.39 ± 0.03**</td>
</tr>
<tr>
<td>DBIL (mg/dl)</td>
<td>0.04 ± 0.005</td>
<td>0.35 ± 0.03**</td>
</tr>
<tr>
<td>IDBIL (mg/dl)</td>
<td>0.005 ± 0.001</td>
<td>0.05 ± 0.01**</td>
</tr>
<tr>
<td>AST (IU/l)</td>
<td>207 ± 12</td>
<td>236 ± 27</td>
</tr>
<tr>
<td>ALT (IU/l)</td>
<td>62 ± 3</td>
<td>78 ± 11</td>
</tr>
<tr>
<td>LAP (IU/l)</td>
<td>85.2 ± 1.4</td>
<td>86.5 ± 4.4</td>
</tr>
<tr>
<td>ALP (IU/l)</td>
<td>1196 ± 35</td>
<td>1850 ± 176**</td>
</tr>
<tr>
<td>GGT (IU/l)</td>
<td>2.39 ± 0.23</td>
<td>6.06 ± 0.89**</td>
</tr>
</tbody>
</table>

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LAP, leucine aminopeptidase.

**P < 0.01 versus normal BDC rats.

### Figure 1

Plasma concentration–time (A) and cumulative biliary excretion–time (B) 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 liquid chromatography–tandem mass spectrometry. 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 represent the mean ± S.E.M. of three animals. *P < 0.05 versus normal BDC rats.
higher, whereas the CL\text{total} was lower, in the mild HB group as compared to the biliary flow rate. The plasma concentrations and AUC were statistically significant. The apparent volume of distribution (Vd) and CL\text{bile} were significantly reduced by 41 and 72%, respectively, in the mild HB group. The reduced Vd suggests the reduction of apparent hepatic uptake, which catalyzes the marked liver-specific distribution of pravastatin in rats (Komi et al., 1992). The reduction of apparent hepatic uptake can be led by the decreased functioning of the Oatps (Hsiang et al., 1999; Tokui et al., 1992). The reduction of apparent hepatic uptake can be led by the decreased functioning of Mrp2 and/or Oatps, and/or the increased functioning of Mrp3, although the involvement of Mrp3 has been little reported in the disposition of pravastatin. The CL\text{non-bile} is considered as the metabolic clearance in the liver, since the renal clearance of pravastatin is negligible in rats (Komi et al., 1992). Thus, the CL\text{bile} and the CL\text{non-bile} account for hepatic clearance. The intrinsic hepatic clearance (CL\text{int,h}) is expressed by the following equation (Kusuhara and Sugiyama, 2009):

\[
\text{CL}_{\text{int,h}} = \frac{\text{PS}_{\text{inf}} \times \text{CL}_{\text{int}}}{\text{PS}_{\text{eff}} + \text{CL}_{\text{int}} + \text{PS}_{\text{inf}} + (\text{CL}_{\text{int,bile}} + \text{CL}_{\text{int,met}})}
\]

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

The liver concentration and liver-to-plasma concentration ratio at 1.5 hours postdose were greater in the HB group as compared with that in the normal group. Since renal clearance of pravastatin is negligible in rats, the AUC in the liver (AUC\text{l}) is expressed by the following equation (Kusuhara and Sugiyama, 2009):

\[
\text{AUC}_{\text{l}} = \frac{\text{Dose}}{f_{\text{u,h}} \times \text{CL}_{\text{int}}} \times 100
\]

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

\[
f_{\text{e,bile}} = \frac{\text{CL}_{\text{bile}}}{\text{CL}_{\text{bile}} + \text{CL}_{\text{non-bile}}} \times 100 = \frac{\text{CL}_{\text{int,bile}}}{\text{CL}_{\text{int,bile}} + \text{CL}_{\text{int,met}}} \times 100
\]

The f_{\text{e,bile}} (percentage 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_{bile} is dependent on the CL_{int,bile}, which is controlled by Mrp2 activity.

The downregulation of mRNA, reduced protein expression, and 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 impairment of Mrp2 (Dumont et al., 1997; Soroka et al., 2001; Kuroda et al., 2004), leading to the reduced hepatic uptake. Furthermore, endogenous and food-derived compounds, such as bilirubin glucuronides and phase II metabolites of 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 $f_{bile}$ value of pravastatin, with high correlation coefficients (Fig. 2A). Marked differences in the $f_{bile}$ values were observed between the individuals with the minimum and maximum TBIL values of 0.00 and 0.75 mg/dl, respectively (64.4 vs. 10.8%). Considering these data, we recommend the minimum and maximum TBIL values of 0.00 and 0.75 mg/dl, respectively (64.4 vs. 10.8%). Considering these data, we recommend that the experiment including BDC rats with HB should be useful to explore the involvement of transporters in the biliary elimination of anionic candidate drugs.

As an unexpected result, the GGT activity increased with increasing TBIL in the BDC rats (Table 1). Then, the GGT activity was inversely correlated with the $f_{bile}$ value 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 (Lu et al., 1996; Ballatori and Rebbeor, 1998; Zhang et al., 2005), 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.

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