An Orphan Esterase ABHD10 Modulates Probenecid Acyl Glucuronidation in Human Liver

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ABSTRACT

Probenecid, a widely used uricosuric agent, is mainly metabolized to probenecid acyl glucuronide (PRAG), which is considered a causal substance of severe allergic or anaphylactoid reactions. PRAG can be hydrolyzed (deglucuronidated) to probenecid. The purpose of this study was to identify enzymes responsible for probenecid acyl glucuronidation and PRAG deglucuronidation in human liver and to examine the effect of deglucuronidation in PRAG formation. In human liver homogenates (HLHs), the intrinsic clearance (CLint) of PRAG deglucuronidation was much greater (497-fold) than that of probenecid glucuronidation. Evaluation of PRAG formation by recombinant UDP-glucuronosyltransferase (UGT) isoforms and an inhibition study using HLHs as an enzyme source demonstrated that multiple UGT isoforms, including UGT1A1, UGT1A9, and UGT2B7, catalyzed probenecid acyl glucuronidation. We found that recombinant α/β hydrolase domain containing 10 (ABHD10) substantially catalyzed PRAG deglucuronidation activity, whereas carboxylesterases did not. Similar inhibitory patterns by chemicals between HLHs and recombinant ABHD10 supported the major contribution of ABHD10 to PRAG deglucuronidation in human liver. Interestingly, it was demonstrated that the CLdata value of probenecid acyl glucuronidation in HLHs was increased by 1.7-fold in the presence of phenylmethylsulfonfonyl fluoride, which potently inhibited ABHD10 activity. In conclusion, we found that PRAG deglucuronidation catalyzed by ABHD10 suppressively regulates PRAG formation via multiple UGT enzymes in human liver. The balance of activities by these enzymes is important for the formation of PRAG, which may be associated with the adverse reactions observed after probenecid administration.

Introduction

Probenecid is used as a uricosuric agent in the treatment of chronic gout and as an adjunct to enhance the blood levels of antibiotics, such as penicillins and cephalosporins. Reported side effects of probenecid include severe allergic, anaphylactoid reactions, and massive liver necrosis associated with the hypersensitivity reaction (Hillecke, 1965). Probenecid is extensively metabolized in humans via acyl glucuronidation and oxidation of alkyl side chains (Israel et al., 1972). Approximately 40% of the dose is eliminated as probenecid acyl glucuronide (PRAG) in urine within 48 hours, and excretion of the other metabolites, including 2-hydroxyprobenecid (7.2–12.5%), 3-hydroxyprobenecid (1.6–3.7%), carboxyprobenecid (6.3–9.2%), and N-depropylprobenecid (4.6–8.0%) as well as unchanged probenecid (<4%), was lower than that of PRAG (Perel et al., 1970) (Fig. 1).

UDP-glucuronosyltransferases (UGTs) catalyze the conjugation of glucuronic acid to compounds possessing a hydroxyl, carboxyl, or amino group to facilitate their elimination via bile and urine. Nineteen human UGT enzymes are classified by evolutionary divergence into three subfamilies, namely UGT1A, UGT2A, and UGT2B (Mackenzie et al., 2005). The UGT1A and UGT2B subfamilies play important roles in the glucuronidation of a variety of endogenous and exogenous compounds (Rowland et al., 2013). The formation of O- or N-glucuronides is generally considered as a detoxification process (Spahn-Langguth and Benet, 1992), whereas the formation of acyl glucuronides has been implicated to be associated with immunogenicity and toxicity due to the ability to form covalent binding to proteins or DNA (Spahn-Langguth and Benet, 1992), although direct evidence is still lacking. Thus, the toxicity of probenecid might be due to the formation of acyl glucuronides, but the UGT isofrom that catalyzes probenecid acyl glucuronidation remains to be identified.

Acyl glucuronides are susceptible to deglucuronidation by esterases in human tissues and plasma. There have been some reports suggesting the involvement of esterases in the deglucuronidation of acyl glucuronides as follows. First, clearance of zomepirac, which is mainly excreted as an acyl glucuronide, was increased by the administration of phenylmethylsultfonyl fluoride (PMSF), a general inhibitor of serine esterases, in guinea pig (Smith et al., 1990). Second, clearance of clofibrate acid, which is also largely excreted as an acyl glucuronide, was increased by the administration of disopropyl fluorophosphate (DFP), a serine esterase inhibitor, in rabbits (Rowe and Meffin, 1984). Third, acylaminoacyl-peptide hydrolase was identified as an enzyme-catalyzing valproic acid acyl glucuronide deglucuronidation from human liver cytosol (Suzuki et al., 2010). Finally, we found that mycophenolic acid acyl glucuronide (AcMPAG) was deglucuronidated by α/β hydrolase domain containing 10 (ABHD10) (Iwamura et al., 2012). When we investigated the substrate specificity of human ABHD10 by screening various acyl glucuronides of drugs, we found that PRAG is a substrate of ABHD10. In addition, the UGT isofroms involved in the formation of PRAG remain unclear. This background prompted us...
to investigate whether human ABHD10 is a major esterase to catalyze
deglucuronidation of PRAG and attenuate the formation of PRAG in
human liver, and to identify the UGT isoforms responsible for the
formation of PRAG. The first purpose of this study was to identify the
enzymes involved in the PRAG formation in human liver, and the second
purpose was to investigate the suppressive effects of ABHD10 on the
PRAG formation in human liver.

Materials and Methods
Alamethicin, bis-p-nitrophenylphosphate (BNPP), D-saccharic acid
1,4-lactone (D-SL), sodium fluoride, and UDP glucuronic acid were obtained from Sigma-
Aldrich (St. Louis, MO). Silver nitrate (AgNO₃), bilirubin, calcium chloride
(CaCl₂), cadmium chloride (CdCl₂), cobaltous chloride, cupric chloride (CuCl₂),
DFP, disulfiram, 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), physostigmine sulfate
(eserine), gemfibrozil, niflumic acid, PMSF, and probenecid were purchased
from Wako Pure Chemical Industries (Osaka, Japan). PRAG was purchased
from Toronto Research Chemicals (Toronto, Ontario, Canada). Human UGT1A1,
UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10,
UGT2B4, UGT2B7, UGT2B10, UGT2B15, and UGT2B17 Supersomes (UGT
enzymes prepared from a baculovirus insect cell system), rabbit anti-human
UGT1A1 antibody, and rabbit anti-human UGT2B7 antibody were purchased
from BD Gentest (Woburn, MA). Mouse anti-human UGT1A9 antibody was
previously prepared in our laboratory (Oda et al., 2012). IRDye680-labeled
goat anti-rabbit secondary antibody and Odyssey Blocking Buffer were ob-
tained from LI-COR Biosciences (Lincoln, NE). Other chemicals were of the
highest commercially available grade.

Preparation of Human Liver Homogenates. The use of human livers was
approved by the Ethics Committee of Kanazawa University (Kanazawa, Japan).
Human liver samples obtained from 20 donors (15 Caucasian, 3 Hispanic, and
2 black donors; 14 males and 6 females) were supplied by the National Disease
Research Interchange (Philadelphia, PA) through the Human and Animal Bridging
Research Organization (Chiba, Japan). Human liver homogenates (HLHs) were
prepared by homogenizing human livers in 10 mM Tris-HCl buffer (pH 7.4)
containing 20% glycerol and 1 mM EDTA. Pooled HLHs were prepared from 10
individuals who were randomlyselected from 20 individuals.

Probenecid Acyl Glucuronidation. The probenecid acyl glucuronidation
was determined as follows. A typical incubation mixture (200 μl of total volume)
containing 50 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 2.5 mg/ml alamethicin,
The temperature was 25°C for 2 minutes, and the reaction was initiated by the addition of UDP glucuronic acid (final concentration 2.5 mM). After incubation at 37°C for 20 minutes, the reaction was terminated by the addition of 20 μl of 60% metaphosphoric acid. After removal of the protein by centrifugation at 15,000 rpm for 5 minutes, a 20-μl aliquot of the supernatant was subjected to liquid chromatography–tandem mass spectrometry (LC-MS/MS). LC-MS/MS analysis was performed using liquid chromatography equipment consisting of an HP1100 system, including a binary pump, an automatic sampler, and a column oven (Agilent Technologies, Santa Clara, CA), which was equipped with a Zorbax SB-C18 column (2.1 x 50 mm, 3.5 μm; Agilent Technologies). The column temperature was 25°C. The mobile phase was 0.1% formic acid (A) and methanol including 0.1% formic acid (B). The conditions for elution were as follows: 20% B (0–2.0 minutes), 20–95% B (2.01–3 minutes), 95% B (3.01–10 minutes), and 20% B (10.01–18 minutes). The flow rate was 0.2 ml/min. The liquid chromatography system was connected to a PE Scien API 2000 tandem mass spectrometer (Applied Biosystems, Foster City, CA), which was operated in the negative electrospray ionization mode. The turbo gas was maintained at 550°C. Nitrogen was used as the nebulizing, turbo, and curtain gas at 70, 40, and 50 psi, respectively. Parent and/or fragment ions were filtered in the first quadrupole and dissociated in the collision cell using nitrogen as the collision gas. The collision energy was 51 V.

Inhibition Analyses of Probenecid Acyl Glucuronidation. Bilirubin and gemfibrozil were used as inhibitors for UGT1A1, UGT1A9, and UGT2B7 (Kato et al., 2012), respectively. Niflumic acid is a selective inhibitor for UGT2B7 (Kato et al., 2012), respectively. Niflumic acid and gemfibrozil were dissolved in methanol. These compounds (1–100 μM) were added to the incubation mixtures as described above to investigate their inhibitory effects on probenecid acyl glucuronidation activities in pooled HLHs at a substrate concentration of 200 μM. In parallel, the effects of the inhibitors on probenecid acyl glucuronidation activities by recombinant UGT1A1, UGT1A9, and UGT2B7 were also confirmed. The final concentration of the organic solvents in the incubation mixture was 1% (v/v). Control incubations contained the same concentration of organic solvent.

PRAG Deglucuronidation. PRAG deglucuronidation activities were determined as follows. A typical incubation mixture (final volume of 200 μl) contained 50 mM Tris-HCl (pH 7.4), and 0.3 mg/ml HLHs, or 0.025 mg/ml recombinant human ABHD10, carboxylesterase (CES) 1, and CES2 as previously prepared (Fukami et al., 2010; Iwamura et al., 2012). PRAG was dissolved in methanol, and the final concentration of the organic solvents in the incubation mixture was 1% (v/v). The reaction was initiated by the addition of PRAG (1–200 μM) after a 2-minute preincubation at 37°C. After incubation at 37°C for 20 minutes, the reaction was terminated by the addition of 20 μl of 60% metaphosphoric acid. After removal of the protein by centrifugation at 15,000 rpm for 5 minutes, a 20-μl aliquot of the supernatant was subjected to LC-MS/MS as described above. Because some part of PRAG is nonenzymatically deglucuronidated (i.e., the peak area of probenecid nonenzymatically hydrolyzed from PRAG was one-tenth or less than that enzymatically hydrolyzed), the content of probenecid in the mixture incubated without enzyme sources was subtracted from that with enzyme sources.

Inhibitory Studies on PRAG Deglucuronidation. The experimental procedure and condition were the same as above. HLHs or recombinant human ABHD10 were used as the enzyme source. The inhibitors used in this study are described below. τ-SL is a β-glucuronidase inhibitor (Levvy, 1952). Organophosphates, such as DFP and BNPP, are known as general CES inhibitors (Heymann and Kirsch, 1967; Yamaori et al., 2006), and PMSF is a general serine hydrolase inhibitor (Johnson and Moore, 2000). We previously found that BNPP and PMSF, but not DFP, showed potent inhibition against ABHD10 (Iwamura et al., 2012). Eserine and sodium fluoride are cholinesterase inhibitors (Iwatsubo, 1965; Preuss and Svensson, 1996). Eserine is also a potent inhibitor of CES2 and arylacetamide deacetylase (Kobayashi et al., 2012). Disulfiram is a monocacylglycerol lipase inhibitor (Labar et al., 2007). DTNB is an aryltransferase inhibitor (Minagawa et al., 1995). Because heavy metals are frequently used for esterase inhibition studies, AgNO3, CdCl2, cobaltous chloride, and CuCl2 were also used. CaCl2 is known to activate paraoxonase activity (Hioki et al., 2011). BNPP, PMSF, disulfiram, and DTNB were dissolved in DMSO such that the final concentration in the incubation mixture was 1.5%. Other inhibitors were dissolved in distilled water. The concentration of inhibitors was obtained according to our previous study (Iwamura et al., 2012). It was confirmed that 1.5% DMSO did not inhibit PRAG deglucuronidation, and the control activity was determined in the presence of 1.5% DMSO. SDS-PAGE and immunoblotting analysis were performed according to our previous study (Fujisawa et al., 2010). HLHs (15 μg for UGT1A1 and UGT1A9, and 5 μg for UGT2B7) were separated on 10% polyacrylamide gels and electrotransferred onto Immobilon-P polyvinylidene difluoride membrane (Millipore, Billerica, MA) for UGT1A1 and UGT2B7 or

<table>
<thead>
<tr>
<th>UGT Isoform</th>
<th>Km (μM)</th>
<th>Vmax (pmol/min per mg protein)</th>
<th>CLint (μl/min per mg protein)</th>
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</thead>
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<tr>
<td>UGT1A1</td>
<td>76.8 ± 3.7</td>
<td>63.6 ± 0.5</td>
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<td>UGT1A3</td>
<td>1177.1 ± 66.8</td>
<td>141.7 ± 9.6</td>
<td>0.12 ± 0.00</td>
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<tr>
<td>UGT1A9</td>
<td>198.3 ± 3.7</td>
<td>59.0 ± 1.0</td>
<td>0.30 ± 0.01</td>
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<tr>
<td>UGT2B7</td>
<td>94.3 ± 0.0</td>
<td>11.3 ± 1.0</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>UGT2B17</td>
<td>24.1 ± 1.8</td>
<td>26.2 ± 2.0</td>
<td>1.09 ± 0.01</td>
</tr>
<tr>
<td>HLH</td>
<td>334.9 ± 0.8</td>
<td>21.3 ± 1.0</td>
<td>0.06 ± 0.00</td>
</tr>
</tbody>
</table>

Table 1. Kinetic parameters of probenecid acyl glucuronidation by recombinant human UGTs and HLHs.

Data are expressed as the mean ± S.D. of triplicate determinations.
Protran nitrocellulose membranes (Whatman GmbH, Dassel, Germany) for UGT1A9. The membranes were probed with polyclonal rabbit anti-human UGT1A1, monoclonal mouse anti-human UGT1A9, or polyclonal rabbit anti-human UGT2B7. The corresponding fluorescent dye–conjugated second antibody and an Odyssey infrared imaging system (LI-COR Biosciences) were used for detection.

**Kinetic Analysis.** Kinetic parameters were estimated from the fitted curves using a KaleidaGraph computer program (Synergy Software, Reading, PA) designed for nonlinear regression analysis. The following equation was applied for Michaelis–Menten kinetics:

\[ V = \frac{V_{\text{max}} \times S}{K_m + S} \]

where \( V \) is the velocity of the reaction, \( S \) is the substrate concentration, \( K_m \) is the Michaelis–Menten constant, and \( V_{\text{max}} \) is the maximum velocity. Data are expressed as the mean ± S.D. of three independent determinations.

**Statistical Analyses.** The statistical significance between kinetic parameters in the presence and absence of PMSF was determined using two-tailed \( t \) tests. Correlation analysis was determined using Pearson’s product moment method. A value of \( P < 0.05 \) was considered statistically significant.

**Results**

**Probenecid Acyl Glucuronidation and PRAG Deglucuronidation in HLHs.** Probenecid acyl glucuronidation in pooled HLHs was determined with varied concentrations of probenecid (Fig. 2A). The kinetics was best fitted to the Michaelis–Menten equation, which was supported by a monophasic Eadie–Hofstee plot. The apparent \( K_m \), \( V_{\text{max}} \), and intrinsic clearance (CL\(_{\text{int}}\)) values were 334.9 ± 0.8 \( \mu \)M, 21.3 ± 1.0 pmol/min per mg protein, and 0.06 ± 0.00 \( \mu \)l/min per mg protein, respectively. PRAG deglucuronidation in the pooled HLH was determined with varied concentrations of PRAG (Fig. 2B). The kinetics was also best fitted to the Michaelis–Menten equation, which was supported by a monophasic Eadie–Hofstee plot. The apparent \( K_m \), \( V_{\text{max}} \), and intrinsic clearance (CL\(_{\text{int}}\)) values were 51.9, 51.1, 7.9, and 7.6 pmol/min per mg protein, respectively. Data are expressed as the mean of duplicate determinations.

**Fig. 4.** Inhibition of probenecid acyl glucuronidation by recombinant human UGT1A1 (A), UGT1A9 (B), UGT2B7 (C), and HLHs (D) by bilirubin, niflumic acid, and gemfibrozil. Control activities in recombinant UGT1A1, UGT1A9, UGT2B7, and HLHs were 51.9, 51.1, 7.9, and 7.6 pmol/min per mg protein, respectively. Data are expressed as the mean of duplicate determinations.

**Fig. 5.** PRAG deglucuronidation by HLHs and recombinant human ABHD10, CES1, and CES2. HLHs (0.3 mg/ml) and recombinant esterases (0.025 mg/ml) were incubated with 10 \( \mu \)M PRAG for 20 minutes. Each column represents the mean ± S.D. of triplicate determinations.
was supported by a monophasic Eadie–Hofstee plot. The apparent $K_{\text{in}}$, $V_{\text{max}}$, and $C_{\text{L0}}$ values were $28.7 \pm 1.2 \mu M$, $85.66 \pm 23.3 \text{ pmol/min per mg protein}$, and $29.8 \pm 2.1 \mu \text{m/min per mg protein}$, respectively. Importantly, the $C_{\text{L0}}$ value of the PRAG deglucuronidation was considerably higher (497-fold) than that of PRAG formation from probenecid.

**Probenecid Acyl Glucuronidation by Recombinant Human UGTs.** To identify UGT isoforms involved in probenecid acyl glucuronidation, recombinant human UGTs were used as enzyme sources for probenecid acyl glucuronidation. As shown in Fig. 3A, UGT1A1, UGT1A3, UGT1A9, UGT2B7, and UGT2B17, which are expressed in human liver, showed probenecid acyl glucuronidation. UGT1A7 and UGT1A8, which are expressed in the intestine but not in the liver, showed relatively little activity. Kinetic analyses of probenecid acyl glucuronidation were performed for the five UGT isoforms, which are expressed in the liver (Fig. 3B), and the calculated parameters are shown in Table 1. The apparent $K_{\text{in}}$ values of the probenecid acyl glucuronidation by UGT1A1, UGT1A9, and UGT2B7 (76.8 $\pm$ 3.7 $\mu M$, 198.3 $\pm$ 3.7 $\mu M$, and 94.3 $\pm$ 0.0 $\mu M$, respectively) were comparable to that in HLHs (334.9 $\pm$ 0.8 $\mu M$). We considered that the contribution of UGT1A3 to probenecid acyl glucuronidation in human liver would be negligible because accumulating evidence revealed that its protein level in human liver is relatively lower than those of other hepatic UGTs (Ohnaka et al., 2012; Fallon et al., 2013; Sato et al., 2014). Therefore, we did not further investigate UGT1A3. We could not directly compare the $V_{\text{max}}$ and $C_{\text{L0}}$ values beyond UGT isoforms because absolute expression levels of UGT protein in each recombinant system are unknown. Thus, to investigate which UGT isoform mainly contributes to probenecid acyl glucuronidation in HLHs, inhibitory studies were subsequently performed.

**Inhibitory Studies of Probenecid Acyl Glucuronidation in HLHs.** First, the inhibitory potencies of bilirubin, niflumic acid, and gemfibrozil were confirmed using recombinant human UGT isoforms. As shown in Fig. 4, A–C, probenecid acyl glucuronidation by recombinant human UGT1A1, UGT1A9, and UGT2B7 was strongly inhibited by bilirubin, niflumic acid, and gemfibrozil, respectively. Niflumic acid inhibited not only UGT1A9 but also UGT1A1 at high concentrations, which was supported by findings obtained by Miners et al. (2011). Next, we examined the effects of these inhibitors on probenecid acyl glucuronidation in HLHs at a substrate concentration of 200 $\mu M$. As shown in Fig. 4D, probenecid acyl glucuronidation by HLHs was moderately inhibited by three inhibitors. The inhibition potency of niflumic acid was stronger than those of bilirubin and gemfibrozil. These results suggested that multiple UGT isoforms, including UGT1A1, UGT1A9, and UGT2B7, contributed to probenecid acyl glucuronidation in HLHs.

**Contribution of Human ABHD10 to PRAG Deglucuronidation in Human Liver.** Our previous study demonstrated that a novel esterase, ABHD10, is responsible for AcMPAG deglucuronidation (Iwamura et al., 2012). It is well known that CES enzymes are involved in the hydrolysis of various drugs in humans (Satoh and Hosokawa, 1998). To investigate which enzymes catalyze PRAG deglucuronidation, the ability of recombinant human ABHD10, CES1, and CES2 toward PRAG deglucuronidation was examined (Fig. 5). At a substrate concentration of 10 $\mu M$, HLHs showed activity with 0.25 nmol/min per mg protein. In recombinant human esterases, ABHD10 showed extremely high activity (7.4 nmol/min per mg protein), whereas CES1 and CES2 showed marginal activities (0.012 and 0.0025 nmol/min per mg protein, respectively). To evaluate the contribution of ABHD10 to PRAG deglucuronidation in human liver, the inhibitory potencies of various inhibitors between recombinant ABHD10 and HLHs were compared (Fig. 6). Activity by recombinant ABHD10 was efficiently inhibited by BNPP, PMSF, disulfiram, DTNB, AgNO$_3$, and CuCl$_2$ but not by d-SL and DFP, which are $\beta$-glucuronidase and CES inhibitors, respectively. The inhibitory pattern in HLHs was similar with that in recombinant ABHD10, indicating that ABHD10 would be the major esterase responsible for PRAG deglucuronidation in human liver.

**Effects of Inhibition of Deglucuronidation on PRAG Formation in Human Liver.** To investigate whether ABHD10-dependent deglucuronidation of PRAG affects PRAG formation by UGTs in HLHs, the kinetics of probenecid acyl glucuronidation in human liver microsomes in the presence of PMSF, which prominently inhibited PRAG deglucuronidation, was determined (Fig. 7). The $K_{\text{in}}$ value of 244.6 $\pm$ 37.9 $\mu M$ was significantly lower than that of the control (Table 2). The $V_{\text{max}}$ of 25.9 $\pm$ 3.5 nmol/min per mg protein was higher than that of the control, although this difference was statistically insignificant. The $C_{\text{L0}}$ value of 106.0 $\pm$ 3.5 nmol/min per mg protein was higher (1.7-fold) compared with the control. Thus, it was demonstrated that the hydrolysis pathway plays a role in suppressing the formation of PRAG in human liver.

Correlation Analysis of UGT1A1, UGT1A9, and UGT2B7 Protein Levels and Probenecid Acyl Glucuronidation in 20 Individual Human Livers. Immunoblotting analyses demonstrated that UGT1A1, UGT1A9, and UGT2B7 protein levels showed 58-fold, 5-fold, and 5-fold variability, respectively, in a panel of 20 human livers. The probenecid acyl glucuronidation activities were from 0.34 to 18.5 pmol/min per mg protein, demonstrating 54-fold variability at a substrate concentration of 200 $\mu M$. These activities were highly correlated ($r = 0.89, P < 0.0001$) with the UGT1A1 protein level (Fig. 8A) and were moderately correlated with UGT1A9 and UGT2B7 ($r = 0.57, P < 0.01$, and $r = 0.49,$
P < 0.05, respectively) (Fig. 8, B and C). With the addition of 1 mM PMSF to the incubation mixture, the activities were increased 1.3- to 2.4-fold (0.48 to 33.4 pmol/min per mg protein, 70-fold variability), and the correlation coefficients in all of the three UGT isoforms were slightly increased. These results suggested that ABHD10 affected the formation of PRAG in human liver.

**Discussion**

It is implicated that acyl glucuronides formed from compounds containing a carboxylic acid covalently bind to protein or DNA to cause immunogenicity and toxicity including anaphylaxis and liver injury (Spahn-Langguth and Benet, 1992), although direct evidence for the toxicity in vivo has not been proven. A major metabolic pathway of probenecid, which contains a carboxylic acid, is acyl glucuronidation in humans (Israelii et al., 1972). Severe allergic or anaphylactoid reactions are known as adverse effects of probenecid (Hillecke, 1965). Some studies have suggested the involvement of PRAG in probenecid-induced toxicity. Using the comet assay, Southwood et al. (2007) found that comet moments after 18 hours of incubation of probenecid with UGT1A3, UGT1A9-, or UGT2B7-expressing HEK293 cells were increased compared with mock cells, demonstrating that PRAG showed genotoxicity. Sawamura et al. (2010) found that the half-life of PRAG in potassium phosphate buffer was relatively short, indicating that PRAG exhibits toxicity because the toxicity of acyl glucuronides was inversely correlated with the chemical stability in buffer. This finding was supported by results obtained by another research group (Jinno et al., 2013). These background studies suggest that severe allergic or anaphylactoid reactions may be due to the formation of PRAG after the administration of probenecid. In this study, we attempted to investigate the balance of activities of the formation and degradation of PRAG.

**TABLE 2**

The kinetic parameters of probenecid acyl glucuronidation in HLHs in the absence or presence of 1 mM PMSF.

<table>
<thead>
<tr>
<th>Group</th>
<th>$K_a$</th>
<th>$V_{max}$ (pmol/min per mg protein)</th>
<th>$C_{int}$ (nM/min per mg protein)</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>334.9 ± 8.0</td>
<td>21.3 ± 1.0</td>
<td>63.8 ± 3.0</td>
</tr>
<tr>
<td>PMSF</td>
<td>244.6 ± 37.9*</td>
<td>25.9 ± 3.5</td>
<td>106.0 ± 3.5**</td>
</tr>
</tbody>
</table>

* $P < 0.01$ and ** $P < 0.001$ compared with control (0.5% DMSO).

First, we examined probenecid acyl glucuronidation in microsomes from human liver, jejunum, and kidney. Activities in human jejunum and renal microsomes were approximately two-thirds and one-sixth, respectively, of that in human liver microsomes (data not shown). The contents of microsomal protein in human liver, intestine, and kidney were 45, 3, and 45 mg/g tissue, respectively (Soars et al., 2002). Considering that the weights of liver, intestine, and kidney were 20, 30, and 4.4 g/kg body weight, respectively, the liver demonstrated the highest capability of probenecid acyl glucuronidation. Thus, we focused on probenecid acyl glucuronidation in the liver. Because UGT enzymes are localized in microsomes, whereas esterases involved in drug hydrolysis are located not only in microsomes but also in the cytosol (Fukami and Yokoi, 2012), we used HLH as an enzyme source to evaluate the reciprocal reaction by UGTs and esterases. The $C_{int}$ of PRAG deglucuronidation was extremely higher than that of probenecid glucuronidation in HLHs (Fig. 2), suggesting that deglucuronidation exhibits a suppressive role toward acyl glucuronidation. Such a phenomenon was previously found in mycophenolic acid acyl glucuronidation (Iwamura et al., 2012). Various human UGT isoforms, including UGT1A1, UGT1A3, UGT1A9, UGT2B4, and UGT2B7, are known to be involved in acyl glucuronidation (Kuehl et al., 2005). In this study, we aimed to identify human UGT isoforms responsible for probenecid acyl glucuronidation. Ten UGT isoforms showed probenecid acyl glucuronidation with varying degrees (Fig. 3), which were supported by the fact that the substrate specificity of UGT isoforms is partially overlapped (Rowland et al., 2013). Although all of UGT1A1, UGT1A9, and UGT2B7 enzymes, which are highly expressed in human liver, showed high activity for PRAG formation, the major UGT enzyme for probenecid acyl glucuronidation could not be determined at this point because of their similar $K_a$ values. Subsequent inhibitory studies revealed that UGT1A1, UGT1A9, and UGT2B7 are all involved in probenecid acyl glucuronidation in the human liver (Fig. 4). Sakaguchi et al. (2004) reported that UGT1A3, UGT1A6, UGT1A9, and UGT2B7 are involved in acyl glucuronidations of valproic acid and gemfibrozil, and UGT1A3, UGT1A7, UGT1A8, UGT1A9, UGT1A10, and UGT2B7 are involved in acyl glucuronidation of clobin in humans. Tachibana et al. (2005) reported that UGT1A1, UGT1A3, UGT1A7, and UGT1A9 are involved in acyl glucuronidation of levofloxacin in humans. Thus, specifically in the case of acyl glucuronidation of drugs, in general, multiple UGT isoforms are involved in the reaction. In addition, probenecid acyl glucuronidation in multiple HLH samples was correlated with three UGT protein levels independent of the presence of PMSF (Fig. 8). Ramírez et al. (2008)
reported a significant correlation of mRNA expression between UGT1A1, UGT1A9, and UGT2B7 in human liver, which is partly supported by the fact that they are commonly regulated by hepatocyte nuclear factor 1α (Bernard et al., 1999; Ishii et al., 2000; Gregory et al., 2004). Thus, the high correlation of the expression between these UGT isoforms results in a significant correlation between probenecid acyl glucuronidation and the expression of three UGT isoforms.

ABHD enzymes have an α/β hydrolase fold, whose domain consists of a predominantly parallel β-sheet structure connected by helical loops of various length. There are 22 isoforms in the human ABHD family (ABHD1–ABHD12, ABHD13, ABHD14A, ABHD14B, ABHD15, ABHD16A, ABHD16B, ABHD17A, ABHD17B, and ABHD17C), but the homology at amino acids between ABHD isoforms is not high. Among the family, ABHD5 was identified as a key co-activator of adipocyte triglyceride lipase (Lass et al., 2006). ABHD6 is an enzymatic regulator of endocannabinoid signaling in the brain (Marrs et al., 2010) and is a key lipase involved in monoacylglycerol and lysophospholipid hydrolysis (Thomas et al., 2013). Before we found that AcMPAG is deglucuronidated by human ABHD10 (Iwamura et al., 2012), there had been no reports about the function of ABHD10. In this study, we found that PRAG deglucuronidation was also catalyzed by recombinant human ABHD10 (Fig. 5). In addition, on the basis of the similarity of the inhibitory pattern by various inhibitors toward PRAG deglucuronidation by recombinant ABHD10 and HLHs, it was suggested that ABHD10 would be the responsible enzyme in HLHs (Fig. 6).

Human serum albumin is known to catalyze the deglucuronidation of acyl glucuronides of fenoprofen, etodolac, ketoprofen, and gemfibrozil (Volland et al., 1991; Smith et al., 1992; Dubois-Presle et al., 1995; Sallustio et al., 1997). However, human serum albumin (purchased from Sigma-Aldrich) did not catalyze the deglucuronidation of PRAG (data not shown). Acylaminoacyl-peptide hydrolase is another enzyme that catalyzes the deglucuronidation of acyl glucuronide (valproic acid acyl glucuronide) but the homology at amino acids between ACAP1 and ABHD10 is not high.

Because PRAG deglucuronidation counteracts probenecid acyl glucuronidation, it is conceivable that inhibition of ABHD10 results in an increase in PRAG deglucuronidation in HLHs. This hypothesis was experimentally proven by data showing that the \( C_{\text{IC}} \) value of probenecid glucuronidation in HLHs was increased in the presence of PMSF (Fig. 7). Interindividual variability in PRAG deglucuronidation activity was not large in a panel of HLH samples (0.11–0.29 mmol/min per mg protein, 2.6-fold variability at 10 \( \mu M \) PRAG) (data not shown), which might reflect the slight increase in correlation coefficients between UGT protein levels and probenecid acyl glucuronidation in the presence of PMSF (Fig. 8). However, there might be drugs or compounds that strongly inhibit ABHD10. When ABHD10 is inhibited by these drugs or compounds, the risk of probenecid-induced toxicity may be increased.

In conclusion, we found that UGT1A1, UGT1A9, and UGT2B7 catalyze probenecid acyl glucuronidation and that ABHD10 counteracts PRAG formation via deglucuronidation in human liver. The balance of activities by these enzymes is important for the formation of PRAG, which may be associated with the adverse reactions observed after probenecid administration.

**Authorship Contributions**

**Participated in research design:** Ito, Fukami, Yokoi, Nakajima.

**Conducted experiments:** Ito, Fukami.

**Contributed new reagents or analytic tools:** Ito, Fukami.


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