Glucuronidation of drugs and drug-induced toxicity in humanized

UDP-glucuronosyltransferase 1 mice

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Abbreviations: UGT, UDP-glucuronosyltransferase; HPLC, high performance liquid chromatography; hUGT1, humanized UGT1; ALT, alanine amino transferase; ADR, adverse drug reaction; NSAIDs, non-steroidal anti-inflammatory drugs; CL\text{int}, intrinsic clearance; AIC, Akaike's information criterion; C\text{max}, peak plasma concentration; T\text{max}, time to peak concentration; AUC, area under the plasma concentration-time curve; APAP, acetaminophen.
Abstract

UDP-glucuronosyltransferases (UGTs) are phase II drug-metabolizing enzymes that catalyze glucuronidation of various drugs. Although experimental rodents are used in preclinical studies to predict glucuronidation and toxicity of drugs in humans, species differences in glucuronidation and drug-induced toxicity have been reported. Recently, humanized UGT1 mice in which the original Ugt1 locus was disrupted and replaced with the human UGT1 locus (hUGT1 mice) have been developed. In this study, acyl-glucuronidations of etodolac, diclofenac, and ibuprofen in liver microsomes of hUGT1 mice were examined and compared with those of human and regular mice. The kinetics of etodolac, diclofenac, and ibuprofen acyl-glucuronidation in hUGT1 mice were almost comparable to those in humans, rather than in mice. We further investigated hepatotoxicity of ibuprofen in hUGT1 mice and regular mice by measuring serum alanine amino transferase (ALT) levels. As ALT levels were increased at 6 h after dosing in hUGT1 mice and at 24 h after dosing in regular mice, the onset pattern of ibuprofen-induced liver toxicity in hUGT1 mice was different from that in regular mice. These data suggest that hUGT1 mice can be valuable tools for understanding glucuronidations of drugs and drug-induced toxicity in humans.
Introduction

UDP-glucuronosyltransferases (UGTs) that catalyze glucuronidation of compounds by transferring glucuronic acid from a co-substrate, UDP-glucuronic acid, to substrates are important in phase II metabolism reactions (Dutton, 1980). UGTs are super-family enzymes that are mainly classified into two subfamilies, UGT1 and UGT2, on the basis of evolutionary divergence (Mackenzie et al., 2005). The human UGT1 gene is located on chromosome 2q37 and encodes multiple unique exons 1 and common exons 2 to 5, producing nine functional UGT1A isoforms, UGT1A1, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9, and UGT1A10 (Ritter et al., 1992). The UGT2B genes are located on chromosome 4q13, and seven functional UGT2B isoforms are encoded by individual genes (Mackenzie et al., 2005). Each UGT enzyme expresses in a tissue-specific manner and exhibits substrate specificity (Tukey and Strassburg, 2000). UGT1 family enzymes are responsible for more than 50% of the glucuronidation of most prescribed drugs (Williams et al., 2004).

The UGT gene is conserved in mammals such as humans, mice, and rats (Mackenzie et al., 2005). Therefore, to predict glucuronidation of drugs in humans, not only in vitro systems such as human UGTs-expressing cells (Katoh et al., 2007), but also experimental rodents are used in preclinical studies (Deguchi et al., 2011). While most drugs are similarly glucuronidated in humans and rodents, species differences have been reported for some drugs (Tougou et al.,...
Naproxen, diclofenac, and ibuprofen are glucuronidated differently as the $K_m$ values and $V_{\text{max}}$ values were different in liver microsomes of human and rodents.

Adverse drug reactions (ADRs) are a major complication of drug therapy (Pirmohamed et al., 1998 and Lazarou et al., 1998). ADRs may preclude effective drug therapy, and if quite serious, occasionally lead to drug withdrawal (Jefferys et al., 1998). For instance, zomepirac was withdrawn (March 4, 1983) relatively soon after market release (November 1980) due to unexplained fatal anaphylactic reactions (Ross-Degnan et al., 1993). Almost any body systems may be adversely affected by drugs, but the most common serious reactions are those that involve the liver, skin, haemopoietic system, and more generalised toxicities such as systemic anaphylaxis (Park et al., 2001). Non-steroidal anti-inflammatory drugs (NSAIDs) are well-known as the drugs that induced ADRs. NSAIDs that have carboxylic group can be glucuronidated by multiple UGT isoforms (Table 1). It has been postulated that acyl-glucuronides, which are generated by glucuronidation of the carboxylic group of NSAIDs, can bind to proteins and cause idiosyncratic drug toxicity (Pumford et al., 1993), while the conclusive evidence that acyl-glucuronides cause toxicities has not been obtained. In fact, the most serious ADRs of NSAIDs usually occur in liver and kidney, organs associated with high exposure to acyl-glucuronides. To date, both direct toxicity and/or immune-mediated indirect
toxicity of acyl-glucuronides have been suggested as possible mechanisms of idiosyncratic liver injury. With direct toxicity, covalent protein binding via acyl-glucuronide may disrupt the normal physiological function of a critical protein or some critical regulatory pathway which lead to cellular necrosis (Pirmohamed et al., 1996). The inflammation-mediated toxicity was investigated in a recent study, which showed that acyl-glucuronides induce inflammatory toxicity and cytotoxicity against CD14+ cells via p38 MAPK pathway (Miyashita et al., 2013).

In the case of diclofenac, it has been demonstrated that the acyl-glucuronide can either bind directly to protein with displacement of the glucuronide group, or rearrange to form a reactive imine intermediate which binds to proteins (Kretz-Rommel and Boelsterli, 1994). It is assumed that these chemical pathways of activation initiate either direct or immune-mediated indirect hepatotoxicity (Langguth and Benet, 1992).

Species differences in toxicity based on metabolism were reported (O’Brien et al., 1983; Eberhart et al., 1991). To predict and avoid human-specific drug- and metabolite-induced toxicities based on metabolism, species difference in glucuronidation, especially of carboxylic acid drugs, needs to be carefully evaluated in preclinical studies. To overcome the species difference in drug glucuronidation, recently, humanized UGT1 mice in which the original Ugt1 locus was disrupted and replaced with the human UGT1 locus (hUGT1 mice) have been developed (Fujiwara et al., 2010; Fujiwara et al., 2012). In this study, glucuronidations of
etodolac, naproxen, and ibuprofen in hUGT1 mice were examined and compared with those in human and regular mice. Furthermore, we investigated hepatotoxicity of ibuprofen in hUGT1 mice and regular mice.

Materials & Methods

Chemicals and Reagents

UDP-glucuronic acid and alamethicin were purchased from Sigma–Aldrich (St Louis, MO, USA). Etodolac and diclofenac sodium were purchased from Wako Pure Chemical (Osaka, Japan). S-Ibuprofen was purchased from LKT Laboratories (Saint Paul, MN, USA). Diclofenac acyl-glucuronide, ibuprofen acyl-glucuronide, R-ibuprofen, and racemic (rac) ibuprofen were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Human and mouse liver microsomes were obtained from BD Gentest (Woburn, MA, USA). All other chemicals and solvents were of analytical grade or the highest grade commercially available.

Animals and preparation of liver microsomes

Tg(UGT1A1*28)Ugt1−/− (hUGT1) mice were developed previously in a C57BL/6 background (Fujiwara et al., 2010). All animals received food and water ad libitum, and mouse handling and experimental procedures were conducted in accordance with the animal care protocol approved by Kitasato University. For tissue collections, mice were anesthetized by
diethyl ether inhalation, and the liver was perfused with ice-cold 1.15% KCl. The liver was rinsed in cold 1.15% KCl and stored at −80°C. Liver microsomes were prepared using the following procedure. Perfused liver with 1.15% KCl was homogenized in three volumes of homogenization buffer (1.15% KCl/10 mM potassium phosphate buffer, pH 7.4). The homogenate was centrifuged at 10,000 × g for 30 min at 4°C, and the supernatant was collected. The supernatant was centrifuged at 105,000 × g for 60 min at 4°C, and the pellet was suspended in the same buffer and used as the microsomal fraction. Protein concentrations of microsomal fractions were measured by the Bradford method using BSA as a standard (Bradford, 1976).

**Enzyme Assays**

Etodolac and diclofenac acyl-glucuronide formation was determined according to the method of Fujiwara et al. (2007) with slight modifications. Briefly, a typical incubation mixture (200 μl of total volume) contained 100 mM phosphate buffer (pH 7.4), 4 mM MgCl₂, 5 mM UDPGA, 50 μg/ml alamethicin, 0.1 mg/ml liver microsomes and 50 μM to 4 mM etodolac or 10 μM to 1mM diclofenac. The reaction was initiated by the addition of UDPGA after a 3-min preincubation at 37°C. After incubation at 37°C for 30 min, the reaction was terminated by addition of 200 μl of cold acetonitrile. After removal of the protein by centrifugation at 12,000 g for 5 min, a 50-μl portion of the sample was subjected to HPLC.

*S, R,* and *rac*-Ibuprofen acyl-glucuronide formation was determined according to the
method of Fujiwara et al. (2007) with slight modifications. Briefly, a typical incubation mixture (200 μl of total volume) contained 100 mM phosphate buffer (pH 7.4), 4 mM MgCl₂, 5 mM UDPGA, 50 μg/ml alamethicin, 0.2 mg/ml and 50 μM to 3 mM S-ibuprofen, 50 μM to 3 mM R-ibuprofen or 25 μM to 2 mM rac-ibuprofen. The reaction was initiated by the addition of UDPGA after a 3-min preincubation at 37°C. After incubation at 37°C for 60 min, the reaction was terminated by addition of 200 μl of cold methanol. After removal of the protein by centrifugation at 12,000 g for 5 min, a 50-μl portion of the sample was subjected to HPLC.

**HPLC conditions**

Glucuronides were determined by the HPLC system with a LC-10AD pump (Shimadzu, Kyoto, Japan), a FP-2020 fluorescence detector (JASCO, Tokyo Japan), a SIL-10A autosampler (Shimadzu), a SLC-10A system controller (Shimadzu) and a Mightysil RP-18 GP column (4.6 × 150 mm, 5 μm; Kanto Chemical, Tokyo, Japan). The mobile phases were 50% acetonitrile containing 1% aqueous acetic acid for the etodolac glucuronide, 65% methanol containing 0.05 M KH₂PO₄ for diclofenac glucuronide, and 65% ethanol containing pH 3.0 phosphoric acid aqueous solution for ibuprofen glucuronides. The flow rate was 1.0 ml/min. Glucuronides were detected with a fluorescence detector at 276 nm excitation and 667 nm emission for the etodolac glucuronide, at 282 nm excitation, 365 nm emission for the diclofenac glucuronide, and at 263 nm excitation and 288 nm emission for the ibuprofen glucuronides. Quantification of diclofenac
and ibuprofen glucuronides was carried out by comparing the HPLC peak area to that of the authentic standard. Quantification of etodolac glucuronides was carried out by the following procedure. The incubation mixture including 5 µM etodolac and 1 mg/mL human liver microsomes, 100 mM phosphate buffer (pH 7.4), 4 mM MgCl₂, 5 mM UDPGA, and 50 µg/ml alamethicin was incubated at 37°C for 0 h and 3 h. The increase of HPLC peak area of the etodolac glucuronide was compared with decrease of the peak area of the parent compound. As the relationship between the peak area of etodolac glucuronide and the amount of etodolac glucuronide was determined, the etodolac glucuronide in reaction mixture was quantificated by measuring the peak area of glucuronide. The retention times of etodolac glucuronide, diclofenac glucuronide and ibuprofen glucuronides, were 4.0, 6.2, and 5.9 min, respectively.

**Data Analysis**

When kinetics of drug metabolism follows a simple Michaelis-Menten equation, the relationship between substrate concentration and velocity can be described by the following equation (eq. 1):

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

(1)

where \( V \) is the initial velocity of the metabolic reaction, \( S \) is the substrate concentration, \( V_{\text{max}} \) is the maximum rate of metabolism, and \( K_m \) is the Michaelis constant, which is defined as the substrate concentration at 1/2 the maximum velocity. While the clearance is substrate
concentration-dependent, the rate is constant when the substrate concentration is much smaller than $K_m$, providing the parameter, intrinsic clearance ($CL_{int}$) (eq. 2):

$$CL_{int} = \frac{V_{max}}{K_m}$$  \hspace{1cm} (2)

When substrate inhibition was observed, the data were analyzed by eq. 3:

$$V = \frac{V_{max}}{(1 + (K_m / S) + (S / K_{si}))}$$  \hspace{1cm} (3)

where $K_{si}$ is the constant describing the substrate inhibition interaction.

For sigmoidal kinetics, kinetic parameters were obtained by the Hill equation (eq. 4):

$$V = \frac{V_{max} \times S^n}{S_{50}^n + S^n}$$  \hspace{1cm} (4)

where $S_{50}$ is the substrate concentration showing the $1/2$ $V_{max}$ and $n$ is the Hill coefficient.

Kinetic data were also analyzed using Eadie-Hofstee plots. Furthermore, goodness of fit to kinetic models was assessed by calculation of Akaike's Information Criterion (AIC) values. AIC values of each model were compared and the best-fit model was determined.

**Ibuprofen-induced liver injury**

*Rac*-ibuprofen suspended in canola oil containing 10% DMSO was administrated to *hUGT1* mice and regular mice (750 mg/kg, p.o.). Canola oil containing 10% DMSO without *rac*-ibuprofen was also administrated to *hUGT1* mice for a control. Blood was obtained from the submandibular vein immediately before dosing, and at 6 h and 24 h after the dosing. Blood was centrifuged at 9,200 × g for 10 min after incubated at 37°C for 10 min to facilitate the
clotting reaction. The supernatant was collected to obtain serum. Liver damage was assessed by measuring the serum activity of Alanine Transaminase (ALT), which is a sensitive diagnostic indicator of hepatotoxicity (Ozer et al., 2008), using ALT Color Endpoint Assay Kit, IDTox (ID Labs Inc., London, ON, Canada).

Pharmacokinetics study of ibuprofen

Rac-ibuprofen suspended in canola oil was administrated to hUGT1 mice and regular mice (200 mg/kg, p.o.). Blood was collected from the submandibular vein into heparinized tube at 15, 30, 60, 120, 240, 480 min after the administration and immediately centrifuged at 9,200 × g for 10 min to obtain plasma. For removal of protein, plasma was mixed with 2-fold volume of acetonitrile and centrifuged at 12,000 × g for 5 min and the supernatant was filtered through a 0.45μm cellulose acetate membrane filter (Cosmonice Filter S, Nakalai Tesque, Kyoto, Japan).

The plasma concentrations of ibuprofen and ibuprofen acyl-glucuronides were analyzed by HPLC as described above. The peak plasma concentration (C\text{max}) and the time to peak concentration (T\text{max}) were obtained from experimental observation. The area under the plasma concentration-time curve from 0 h to 8 h (AUC\text{0-8h}) was calculated using the trapezoidal integration without extrapolation to infinity.

Statistical analysis

All data were presented as means (± SD). The data of pharmacokinetics study of
ibuprofen were analyzed by student’s t test. The data of ibuprofen-induced liver injury were analyzed by analysis of variance (ANOVA) and Dunnett’s procedure for multiple comparisons. 

\[ P < 0.05 \] was considered significant.

Results

Etodolac glucuronidation in liver microsomes from \textit{hUGT1} mice, human, and regular mice

Etodolac is one of the drugs that are subject to species-different glucuronidation, as the \( K_m \) values and \( V_{\text{max}} \) values were different between liver microsomes of human and rat (Tougou et al., 2004; Brocks, 1991). To examine whether etodolac glucuronidation in \textit{hUGT1} mice is similar to that in humans or not, liver microsomes were prepared from adult \textit{hUGT1} mice and etodolac glucuronidation was determined. The etodolac acyl-glucuronide formation by the liver microsomes from \textit{hUGT1} mice followed a simple Michaelis-Menten equation (Fig. 1A), as Eadie-Hofstee plots were linear (Supplemental Fig. 1A). Obtained parameter values were \( K_m = 483 \ \mu\text{M}, \ V_{\text{max}} = 379 \ \text{pmol/min/mg}, \) and \( CL_{\text{int}} = 0.79 \ \mu\text{L/min/mg} \) (Table 2). In human liver microsomes, Eadie-Hofstee plots were almost linear (Supplemental Fig. 1A). AIC values for a simple Michaelis-Menten equation and the Hill equation were compared and Michaelis-Menten equation gave smaller AIC values (Fig. 1A). The kinetic parameters of etodolac acyl-glucuronide in human liver microsomes were \( K_m = 483 \ \mu\text{M}, \ V_{\text{max}} = 246 \ \text{pmol/min/mg}, \) and
CL_{int} = 0.51 \mu L/min/mg (Table 2). The etodolac acyl-glucuronide formation by the mouse liver microsomes followed a simple Michaelis-Menten equation as Eadie-Hofstee plots were linear (Fig. 1A). Obtained parameter values were $K_m = 1170 \mu M$, $V_{max} = 201 \text{ pmol/min/mg}$, and $CL_{int} = 0.17 \mu L/min/mg$ (Table 2). These data showed that the kinetic parameters obtained in liver microsomes from hUGT1 mice were closer to those in human liver microsomes than to those in mice liver microsomes.

**Diclofenac glucuronidation in liver microsomes from hUGT1 mice, human, and regular mice**

It was reported that diclofenac was glucuronidated differently in human and rodents as the $K_m$ values and $V_{max}$ were different between liver microsomes of human and rat (King et al., 2001). Diclofenac glucuronidation in hUGT1 mice, human, and mice liver microsomes was determined and the kinetic data were analyzed. Although the diclofenac acyl-glucuronide formations by the liver microsomes from hUGT1 mice and regular mice followed a simple Michaelis-Menten equation (Fig.1B) as Eadie-Hofstee plots were linear (Supplemental Fig. 1B), those by liver microsomes from human followed the Michaelis-Menten equation with substrate inhibition. In human liver microsomes, the Eadie-Hofstee plots were not linear at the higher substrate concentration (Supplemental Fig.1B). In the human liver microsomes, the AIC values for a simple Michaelis-Menten equation and Michaelis-Menten equation with a substrate
inhibition were compared and Michaelis-Menten with substrate inhibition model gave a smaller AIC value. The kinetic parameters were as follows: $K_m = 31.8 \, \mu M$, $V_{\text{max}} = 7.05 \, \text{nmol/min/mg}$, and $CL_{\text{int}} = 222 \, \mu L/min/mg$ in $hUGT1$ mice liver microsomes, $K_m = 55.6 \, \mu M$, $V_{\text{max}} = 11.8 \, \text{nmol/min/mg}$, and $CL_{\text{int}} = 211 \, \mu L/min/mg$ in mouse liver microsomes and $K_m = 33.1 \, \mu M$, $V_{\text{max}} = 10.4 \, \text{nmol/min/mg}$, $K_i = 22.5 \, \text{mM}$ and $CL_{\text{int}} = 313 \, \mu L/min/mg$ in human liver microsomes (Table 2). Although $V_{\text{max}}$ value and $CL_{\text{int}}$ in $hUGT1$ mice were not very closer to those in human than those in regular mice, $K_m$ value in $hUGT1$ mice was closer to that in human than that in regular mice.

**Rac-Ibuprofen glucuronidation in liver microsomes from $hUGT1$ mice, human, and regular mice**

Ibuprofen possesses a chiral center in the propionic acid moiety. The pharmacological activity resides mainly in the $S$-ibuprofen. As much of $R$-ibuprofen is converted to the active $S$-form (Hutt et al., 1983), the racemate has been clinically used. It was reported that ibuprofen was glucuronidated differently in human and rodents as the $K_m$ values and $V_{\text{max}}$ were different between liver microsomes of human, rat, and mouse (Magdalou et al., 1990). **Rac-Ibuprofen glucuronide formation in $hUGT1$ mice, human, and mouse liver microsomes was determined and the kinetic data were analyzed.** **Rac-Ibuprofen glucuronidation in liver microsomes of $hUGT1$ mice, human, and regular mice best fitted to a simple Michaelis-Menten equation (Fig.**
1C), as the Eadie-Hofstee plots were linear (Supplemental Fig. 1C). The kinetic parameters were as follows: $K_m = 319$ $\mu$M, $V_{\text{max}} = 4.27$ nmol/min/mg, and $CL_{\text{int}} = 13.4$ $\mu$L/min/mg in hUGT1 mice liver microsomes, $K_m = 651$ $\mu$M, $V_{\text{max}} = 6.44$ nmol/min/mg, and $CL_{\text{int}} = 9.9$ $\mu$L/min/mg in human liver microsomes, $K_m = 364$ $\mu$M, $V_{\text{max}} = 11.2$ nmol/min/mg, and $CL_{\text{int}} = 30.7$ $\mu$L/min/mg in regular mice liver microsomes (Table 2). In racemate glucuronidations, $V_{\text{max}}$ and intrinsic clearance in liver microsomes from hUGT1 mice were similar to those in human liver microsomes than those in mouse liver microsomes. The intrinsic clearance in hUGT1 mice and humans was similar and approximately 2- and 3- fold lower than that in regular mice.

S-Ibuprofen glucuronidation in liver microsomes from hUGT1 mice, human, and regular mice

S-Ibuprofen glucuronide formation in hUGT1 mice, human and mouse liver microsomes was determined and the kinetic data were analyzed. S-Ibuprofen glucuronidation in liver microsomes of hUGT1 mice, human, and regular mice best fitted to a simple Michaelis-Menten equation (Fig. 1D), as the Eadie-Hofstee plots were linear (Supplemental Fig. 1D). The kinetic parameters were as follows: $K_m = 381$ $\mu$M, $V_{\text{max}} = 7.54$ nmol/min/mg, and $CL_{\text{int}} = 19.9$ $\mu$L/min/mg in hUGT1 mice liver microsomes, $K_m = 699$ $\mu$M, $V_{\text{max}} = 8.71$ nmol/min/mg, and $CL_{\text{int}} = 12.5$ $\mu$L/min/mg in human liver microsomes, $K_m = 364$ $\mu$M, $V_{\text{max}} = 16.5$ nmol/min/mg, and $CL_{\text{int}} = 45.1$ $\mu$L/min/mg in mouse liver microsomes (Table 2). In S-ibuprofen
glucuronidations, $V_{\text{max}}$ value and intrinsic clearance in liver microsomes from $hUGT1$ mice were similar to those in human liver microsomes than those in mouse liver microsomes, whereas $K_m$ value in $hUGT1$ mice was not similar to that in human.

**R-Ibuprofen glucuronidation in liver microsomes from $hUGT1$ mice, human, and regular mice**

$R$-Ibuprofen glucuronide formation in $hUGT1$ mice, human and mouse liver microsomes was determined and the kinetic data were analyzed. $R$-Ibuprofen glucuronidation in liver microsomes of $hUGT1$ mice, human, and regular mice best fitted to a simple Michaelis-Menten equation (Fig. 1E), as the Eadie-Hofstee plots were linear (Supplemental Fig. 1E). The kinetic parameters were as follows: $K_m = 320 \, \mu M$, $V_{\text{max}} = 2.18 \, \text{nmol/min/mg}$, and $CL_{\text{int}} = 6.87 \, \mu L/min/mg$ in $hUGT1$ mice liver microsomes, $K_m = 428 \, \mu M$, $V_{\text{max}} = 3.37 \, \text{nmol/min/mg}$, and $CL_{\text{int}} = 7.86 \, \mu L/min/mg$ in human liver microsomes, $K_m = 424 \, \mu M$, $V_{\text{max}} = 8.00 \, \text{nmol/min/mg}$, and $CL_{\text{int}} = 18.9 \, \mu L/min/mg$ in mouse liver microsomes (Table 2). In $R$-ibuprofen glucuronidations, $V_{\text{max}}$ value and intrinsic clearance in liver microsomes from $hUGT1$ mice were similar to those in human liver microsomes than those in mouse liver microsomes, whereas $K_m$ value in $hUGT1$ mice was not similar to that in human.

**Ibuprofen-induced liver injury**
Ibuprofen is one of the most commonly used NSAIDs acting as a cyclooxygenase inhibitor and is most frequently administrated orally as a racemic mixture. Although it is considered that ibuprofen has lower risk of liver injury, several cases of serious liver disease have been reported with ibuprofen (Lapeyre-Mestre et al., 2006; Rubenstein et al., 2004). In this study, rac-ibuprofen-induced liver injury was investigated in hUGT1 mice and regular mice to examine the differences in onset pattern of toxicity between hUGT1 and regular mice. In the control group, there were no statistical differences in ALT levels between before dosing and at 6 h and 24 h after dosing (data not shown). In hUGT1 mice, the serum ALT levels at 6 h after 750 mg/kg rac-ibuprofen administration significantly increased by approximately 2-fold compared with those before dosing (P < 0.01). ALT levels at 24 h after dosing slightly increased compared with those before dosing (Fig. 2A). In contrast, the ALT levels at 6 h after rac-ibuprofen dosing slightly increased compared with those before dosing in regular mice. The ALT levels at 24 h after dosing significantly increased by approximately 3-fold compared with those before dosing in regular mice (P < 0.01) (Fig. 2B). These results suggested that there were differences in the onset pattern of ibuprofen-induced liver injury between hUGT1 mice and regular mice.

**Pharmacokinetics study of ibuprofen**

Pharmacokinetics study was conducted to investigate the concentration of rac-ibuprofen and its glucuronide in hUGT1 mice and wild-type mice. As shown in Fig. 3A, the plasma level
of ibuprofen rapidly increased \( (T_{\text{max}} = 30 \text{ min}) \) in both \( hUGT1 \) mice and regular mice with \( C_{\text{max}} \) of 368 ± 177 μM and 270 ± 60.4 μM, respectively. Furthermore, the \( \text{AUC}_{0-8\text{ h}} \) of ibuprofen in \( hUGT1 \) mice was statistically higher than that of regular mice \( (P < 0.05) \), as \( \text{AUC}_{0-8\text{ h}} \) values of ibuprofen were 1285.3 ± 282.1 μM-h in \( hUGT1 \) mice and 721.1 ± 246.3 μM-h in regular mice.

While the plasma level of ibuprofen acyl-glucuronide rapidly increased in regular mice, plasma level of ibuprofen acyl-glucuronides slowly increased in \( hUGT1 \) mice (Fig. 3B). \( T_{\text{max}} \) of ibuprofen acyl-glucuronides was different between \( hUGT1 \) mice and regular mice as \( T_{\text{max}} \) values were 120 min in \( hUGT1 \) mice and 30 min in regular mice with \( C_{\text{max}} \) values of 96.7 ± 17.7 μM and 114 ± 23.4 μM, respectively. \( \text{AUC}_{0-8\text{ h}} \) of ibuprofen acyl-glucuronides in \( hUGT1 \) mice (634.8 ± 115.4 μM-h) was statistically higher than that in regular mice (452.1 ± 39.6 μM-h).

While ibuprofen acyl-glucuronides were stable in protein-free plasma, they were slightly unstable in mouse blood (data not shown). Therefore, the plasma concentrations of ibuprofen acyl-glucuronides shown in Fig.3 might be slightly lower than actual concentrations.

**Discussion**

There are marked species differences in metabolism (Bogaards et al., 2000). This is especially notable between humans and rodents such as rats and mice, the most commonly used experimental models for studies in pharmacology. It is assumed that metabolic enzymes of
human and experimental animal could have differences in affinity to substrate, expression levels and gene expression profiles. For example, as human \textit{UGT1A4} is functional but rat \textit{UGT1A4} and mouse \textit{Ugt1a4} are pseudogenes, specific probe of UGT1A4 such as trifluoperazine and imipramine are not glucuronidated in mice and rats (Uchaipichat et al., 2006; Shiratani et al., 2008). The species differences of metabolic enzymes are typically assessed by comparing the kinetic parameters such as $K_m$, $V_{\text{max}}$ and $CL_{\text{int}}$. In this study, the method of preparation of liver microsomes was different as the microsomes of human and regular mice were purchased and that of \textit{hUGT1} mice was prepared by our method described above. The difference of the method of microsomes preparation may influence glucuronidation activities. However, in past studies, species differences of glucuronidation activities have been assessed with microsomes prepared by different methods (Cai et al., 2010; Xu et al., 2006). Therefore, it is not critical for assessing the species differences to prepare the microsomes by different methods.

One approach to overcome the gap of species difference is to generate humanized mice by introducing a human gene into the mouse genome (Gonzalez et al., 2003; Henderson et al., 2003). Thus humanized mice offer a better animal model to predict the human drug metabolism and understand the underlying mechanisms. In our recent study, \textit{hUGT1} mice showed comparable glucuronidation activity of furosemide, naproxen, imipramine and trifluoperazine to that of humans (Kutsuno et al., 2013). In this study, we investigated the glucuronidation activity
of etodolac, diclofenac, and ibuprofen metabolized by UGTs. As glucuronidations of a chiral compound can be enantioselective like the several other chiral drugs reported (Silber et al., 1982; Zhang et al., 2000), there are enantiomer-enantiomer interaction between one enantiomer and the other (Mouelhi et al., 1987). In the present study, each kinetic parameters of $S$-ibuprofen, $R$-ibuprofen, and $rac$-ibuprofen were similar in microsomes of human, $hUGT1$ mice and regular mice (Table 2). It is considered that glucuronidation of ibuprofen is not enantioselective. While the metabolic profile of the microsomal glucuronidation activity toward etodolac and ibuprofen in $hUGT1$ mice was similar to that in humans, considering the intrinsic clearance parameters (Table 2), diclofenac glucuronidations in $hUGT1$ mice were not very similar to those in human (Table 2). While it has been reported that etodolac, ibuprofen and diclofenac were glucuronidated by multiple UGT isoforms (Table 1), the major isozyme involved in catalyzing the glucuronidation of diclofenac in human was UGT2B7 because the $K_m$ value for diclofenac glucuronidation in human liver microsomes was similar to that in stably expressed human UGT2B7 (King et al., 2001). In humans, multiple UGT2B genes encode UGT2B4, 2B7, 2B10, 2B11, 2B15, 2B17, and 2B28 proteins. In mice, seven Ugt2b genes include Ugt2b1, 2b5, 2b34, 2b35, 2b36, 2b37, and 2b38. The species difference in the function of human UGT2B and mouse Ugt2b family enzymes has not been fully understood. However, it has been reported that there are species differences in drug glucuronidations which are catalyzed by UGT2B7 (Cai et
Therefore, the differences of diclofenac glucuronidations between \textit{hUGT1} mice and humans might be explained by the functional difference between human UGT2 families and mouse Ugt2 families.

Although rodents such as rats and mice are commonly used as experimental models for studies in toxicology, there are also species differences between rodents and humans in the toxicity based on metabolism of xenobiotics (O’Brien et al., 1983; Eberhart et al., 1991). The humanized mouse models are useful as they offer the knowledge to evaluate and predict the toxicological risk that may aid in the development of safer drugs. For instance, humanized CYP2E1 mice were created to compare the functional differences in response to xenobiotics between human and mouse CYP2E1 (Cheung et al., 2005). Hepatotoxicity resulting from the CYP2E1-mediated activation of acetaminophen (APAP) was investigated in the humanized CYP2E1 mice. After 200 mg/kg APAP dosing, the levels of ALT were unchanged in humanized CYP2E1 mice, but significantly elevated by approximately 12-fold in regular mice. From the result, there was difference in response to APAP between humanized CYP2E1 mice and regular mice (Cheung et al., 2008). In the present study, ibuprofen-induced liver injury and pharmacokinetics of ibuprofen in \textit{hUGT1} and regular mice was investigated. It was shown that there were differences in ibuprofen-induced liver injury between \textit{hUGT1} mice and regular mice (Fig. 2). In \textit{hUGT1} mice, \textit{rac}-ibuprofen increased ALT levels 6 h after dosing. \textit{Rac}-ibuprofen
clearance in hUGT1 mice was much lower than that in regular mice (Table 2) and AUC_{0-8h} of ibuprofen in hUGT1 mice was statistically higher than that in regular mice. These data suggest that hUGT1 mice, which had lower intrinsic clearance of ibuprofen, had higher exposure to ibuprofen than regular mice. In contrast, ALT levels were increased 24 h after dosing in regular mice. Our data indicate that slower intrinsic clearance of ibuprofen in hUGT1 mice still resulted in the higher AUC_{0-8h} of ibuprofen acyl-glucuronides. It is possible that hUGT1 mice have transporters that may be coordinately modulated and thus change exposure of the acyl-glucuronides. The underlying mechanism of different pattern of liver injury expression induced by ibuprofen would be very complicated due to the complexity of acyl-glucuronide disposition with enterohepatic recycling, systemic reversible metabolism and renal clearance of the glucuronide. The differences in the pharmacokinetics of ibuprofen might be associated with the onset of the liver injury in hUGT1 mice and regular mice.

In the present study, we demonstrated that the glucuronidations of etodolac and ibuprofen in hUGT1 mice were almost comparable to those in humans. This indicates that in vitro and in vivo studies utilizing hUGT1 mice are useful for predicting in vivo human glucuronidation of drugs that are mainly catalyzed UGT1As. Furthermore, although only one compound, rac-ibuprofen, was investigated for the toxicity study, the onset pattern of ibuprofen-induced liver toxicity in hUGT1 mice was different from that in regular mice. While further
investigations need to be conducted, hUGT1 mice can also be valuable to understand drug-induced toxicity in humans.

Authorship Contributions:

Participated in research design: Itoh, Tukey, and Fujiwara.

Conducted experiments: Kutsuno and Fujiwara.

Performed data analysis: Kutsuno and Fujiwara.

Wrote or contributed to the writing of the manuscript: Kutsuno, Itoh, Tukey, and Fujiwara.
Reference


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O'Brien K, Moss E, Judah D, and Neal, G. (1983). Metabolic basis of the species difference to aflatoxin B₁ induced hepatotoxicity. *Biochemical and biophysical research*
communications, 114: 813-821.


metabolism and disposition, 34: 1406-1410.


Footnotes

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Figure Legend

Figure 1. Kinetic analyses of etodolac, diclofenac, rac-ibuprofen, S-ibuprofen, and R-ibuprofen acyl-glucuronide formations in liver microsomes.

The substrate concentration-velocity curves of the etodolac (A), diclofenac (B), rac-ibuprofen (C), S-ibuprofen (D), and R-ibuprofen (E) glucuronide formations are shown. Pooled liver microsomes of hUGT1 mice, humans, and regular mice were incubated with each substrate and 5 mM UDP-glucuronic acid at 37°C for 30 min. Data are the means ± S.D. of three independent determinations.

Figure 2. Serum alanine aminotransferase activity in mice treated with rac-ibuprofen.

The ALT activities of hUGT1 mice (A) and those of regular mice (B) treated with 750 mg/kg rac-ibuprofen are shown. The blood was collected before dosing and 6 h and 24 h after oral dosing. The serum was analyzed for ALT activity, and the results are shown as means ± S.D. of the ALT activity (U/L) (n ≥ 3). **, P < 0.01, compared with the group before dosing.

Figure 3. Pharmacokinetics of ibuprofen and its glucuronides in hUGT1 mice and regular mice.

Pharmacokinetics of ibuprofen (A) and ibuprofen acyl-glucuronides (B) in regular mice
(closed circle) and hUGT1 mice (open circle) treated with ibuprofen (200 mg/kg, p.o.) are shown. The results are shown as means ± S.D. of the plasma concentration of ibuprofen or ibuprofen acyl-glucuronides (μM) (n ≥ 3).
Table 1. The recombinant UGT isoforms responsible for acyl-glucuronidations of NSAIDs in human.

<table>
<thead>
<tr>
<th>NSAID</th>
<th>Concentrations</th>
<th>Glucuronidation activity of recombinant UGT isoforms</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diclofenac</td>
<td>500 μM</td>
<td>2B7 &gt; 1A3 &gt; 2B17 &gt; 1A9 &gt; 2B15 &gt; 1A6</td>
<td>Kuehl et al., 2005</td>
</tr>
<tr>
<td>Edotolac</td>
<td>50 μM</td>
<td>1A9 &gt; 1A10 ≧ 2B7</td>
<td>Tougou et al., 2004</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>500 μM</td>
<td>2B7 &gt; 1A3 &gt; 1A9 &gt; 2B4</td>
<td>Kuehl et al., 2005</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>500 μM</td>
<td>2B7 &gt; 2B4 &gt; 1A3 &gt; 1A9</td>
<td>Kuehl et al., 2005</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>500 μM</td>
<td>1A9 &gt; 2B7 &gt; 1A3 &gt; 1A1</td>
<td>Kuehl et al., 2005</td>
</tr>
<tr>
<td>Naproxen</td>
<td>500 μM</td>
<td>2B7 &gt; 1A3 &gt; 1A9 &gt; 2B4</td>
<td>Kuehl et al., 2005</td>
</tr>
</tbody>
</table>
Table 2. Kinetic parameters of diclofenac, etodolac, and ibuprofen glucuronidation in liver microsomes.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Liver microsomes</th>
<th>Equation</th>
<th>(K_m)</th>
<th>(V_{max})</th>
<th>(K_i)</th>
<th>(CL_{int})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etodolac Humanized UGT1 mice</td>
<td>Michaelis-Menten</td>
<td>483 ± 40 µM</td>
<td>379 ± 3 pmol/min/mg</td>
<td>—</td>
<td>0.79 ± 0.06 µL/min/mg</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>Michaelis-Menten</td>
<td>483 ± 50 µM</td>
<td>246 ± 12 pmol/min/mg</td>
<td>—</td>
<td>0.51 ± 0.03 µL/min/mg</td>
<td></td>
</tr>
<tr>
<td>Mice</td>
<td>Michaelis-Menten</td>
<td>1170 ± 287 µM</td>
<td>201 ± 29 pmol/min/mg</td>
<td>—</td>
<td>0.17 ± 0.01 µL/min/mg</td>
<td></td>
</tr>
<tr>
<td>Diclofenac Humanized UGT1 mice</td>
<td>Michaelis-Menten</td>
<td>31.8 ± 1.91 µM</td>
<td>7.05 ± 0.04 nmol/min/mg</td>
<td>—</td>
<td>222 ± 12.2 µL/min/mg</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>Substrate inhibition</td>
<td>33.1 ± 1.70 µM</td>
<td>10.4 ± 0.28 nmol/min/mg</td>
<td>22.5 ± 1.00 mM</td>
<td>313 ± 8.88 µL/min/mg</td>
<td></td>
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<tr>
<td>Mice</td>
<td>Michaelis-Menten</td>
<td>55.6 ± 1.52 µM</td>
<td>11.8 ± 0.10 nmol/min/mg</td>
<td>—</td>
<td>211 ± 5.62 µL/min/mg</td>
<td></td>
</tr>
<tr>
<td>rac-Ibuprofen Humanized UGT1 mice</td>
<td>Michaelis-Menten</td>
<td>319 ± 26 µM</td>
<td>4.27 ± 0.12 nmol/min/mg</td>
<td>—</td>
<td>13.4 ± 0.71 µL/min/mg</td>
<td></td>
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<tr>
<td>Human</td>
<td>Michaelis-Menten</td>
<td>651 ± 8.1 µM</td>
<td>6.44 ± 48 nmol/min/mg</td>
<td>—</td>
<td>9.9 ± 0.05 µL/min/mg</td>
<td></td>
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<tr>
<td>Mice</td>
<td>Michaelis-Menten</td>
<td>364 ± 12 µM</td>
<td>11.2 ± 0.19 nmol/min/mg</td>
<td>—</td>
<td>30.7 ± 0.54 µL/min/mg</td>
<td></td>
</tr>
<tr>
<td>S-Ibuprofen Humanized UGT1 mice</td>
<td>Michaelis-Menten</td>
<td>381 ± 26 µM</td>
<td>7.54 ± 0.16 nmol/min/mg</td>
<td>—</td>
<td>19.9 ± 1.00 µL/min/mg</td>
<td></td>
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<tr>
<td>Human</td>
<td>Michaelis-Menten</td>
<td>699 ± 34 µM</td>
<td>8.71 ± 0.17 nmol/min/mg</td>
<td>—</td>
<td>12.5 ± 0.38 µL/min/mg</td>
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<tr>
<td>Mice</td>
<td>Michaelis-Menten</td>
<td>364 ± 5.0 µM</td>
<td>16.5 ± 0.19 nmol/min/mg</td>
<td>—</td>
<td>45.1 ± 0.50 µL/min/mg</td>
<td></td>
</tr>
<tr>
<td>R-Ibuprofen Humanized UGT1 mice</td>
<td>Michaelis-Menten</td>
<td>320 ± 38 µM</td>
<td>2.18 ± 0.07 nmol/min/mg</td>
<td>—</td>
<td>6.87 ± 0.56 µL/min/mg</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>Michaelis-Menten</td>
<td>428 ± 11 µM</td>
<td>3.37 ± 0.03 nmol/min/mg</td>
<td>—</td>
<td>7.86 ± 0.13 µL/min/mg</td>
<td></td>
</tr>
<tr>
<td>Mice</td>
<td>Michaelis-Menten</td>
<td>424 ± 19 µM</td>
<td>8.00 ± 0.09 nmol/min/mg</td>
<td>—</td>
<td>18.9 ± 0.70 µL/min/mg</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1A: Graphs showing the relationship between Etodolac concentration (mM) and Etodolac glucuronidation (pmol/min/mg) for hUGT1, Human, and Mouse.

Figure 1B: Graphs showing the relationship between Diclofenac concentration (mM) and Diclofenac glucuronidation (nmol/min/mg) for hUGT1, Human, and Mouse.

Figure 1C: Graphs showing the relationship between Rac-Ibuprofen concentration (mM) and Rac-Ibuprofen glucuronidation (nmol/min/mg) for hUGT1, Human, and Mouse.

Figure 1D: Graphs showing the relationship between S-Ibuprofen concentration (mM) and S-Ibuprofen glucuronidation (nmol/min/mg) for hUGT1, Human, and Mouse.

Figure 1E: Graphs showing the relationship between R-Ibuprofen concentration (mM) and R-Ibuprofen glucuronidation (nmol/min/mg) for hUGT1, Human, and Mouse.
Figure 2

A

hUGT1

B

Mouse

ALT activity (U/L)

0 h 6 h 24 h

0 h 6 h 24 h

**

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