Glucuronidation of Drugs and Drug-Induced Toxicity in Humanized *UDP-Glucuronosyltransferase 1* Mice^S

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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. Humanized *UGT1* mice in which the original *Ugt1* locus was disrupted and replaced with the human *UGT1* locus (*hUGT1* mice) were recently developed. In this study, acyl-glucuronidations of etodolac, diclofenac, and ibuprofen in liver microsomes of *hUGT1* mice were examined and compared with those of humans 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 the hepatotoxicity of ibuprofen in *hUGT1* mice and regular mice by measuring serum alanine amino transferase (ALT) levels. Because ALT levels were increased at 6 hours after dosing in *hUGT1* mice and at 24 hours 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 cosubstrate (UDP-glucuronic acid) to substrates are important in phase II metabolism reactions (Dutton, 1980). UGTs are superfamily 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 2–5, producing nine functional UGT1A isoforms (UGT1A1 and UGT1A3–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, preclinical studies use not only in vitro systems such as human UGTs-expressing cells (Katoh et al., 2007) but also experimental rodents (Deguchi et al., 2011). Although most drugs are

similarly glucuronidated in humans and rodents, species differences have been reported for some drugs (Magdalou et al., 1990; Brocks and Jamali, 1991; King et al., 2001; Tougou et al., 2004). Naproxen, diclofenac, and ibuprofen are glucuronidated differently because the K_m values and V_{max} values are different in liver microsomes of human and rodents.

Adverse drug reactions (ADRs) are a major complication of drug therapy (Lazarou et al., 1998; Pirmohamed et al., 1998). ADRs may preclude effective drug therapy. If ADRs are quite serious, they may occasionally lead to drug withdrawal (Jefferys et al., 1998). For instance, zomepirac was withdrawn on March 4, 1983, relatively soon after its November 1980 market release, due to unexplained fatal anaphylactic reactions (Ross-Degnan et al., 1993). Almost all body systems may be adversely affected by drugs, but the most common serious reactions are those that involve the liver, skin, hemopoietic system, and more generalized toxicities such as systemic anaphylaxis (Park et al., 2001). Nonsteroidal anti-inflammatory drugs (NSAIDs) are well known as drugs that induce ADRs. NSAIDs that have a 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), although the conclusive evidence that acyl-glucuronides cause toxicities has not been obtained. In fact, the most serious ADRs of NSAIDs usually occur in the 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

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ABBREVIATIONS: ADR, adverse drug reaction; AIC, Akaike information criterion; ALT, alanine amino transferase; APAP, acetaminophen; AUC, area under the plasma concentration-time curve; CL_{int} , intrinsic clearance; HPLC, high-performance liquid chromatography; *hUGT1*, humanized *UGT1*; NSAID, nonsteroidal anti-inflammatory drug; UDPGA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase.

TABLE 1
The recombinant UGT isoforms responsible for acyl-glucuronidations of NSAIDs in humans

NSAID	Concentration μM	Glucuronidation Activity of Recombinant UGT Isoforms	Reference
Diclofenac	500	2B7 > 1A3 > 2B17 > 1A9 > 2B15 > 1A6	Kuehl et al., 2005
Etodolac	50	1A9 > 1A10 $\geq$ 2B7	Tougou et al., 2004
Ibuprofen	500	2B7 > 1A3 > 1A9 > 2B4	Kuehl et al., 2005
Ketoprofen	500	2B7 > 2B4 > 1A3 > 1A9	Kuehl et al., 2005
Indomethacin	500	1A9 > 2B7 > 1A3 > 1A1	Kuehl et al., 2005
Naproxen	500	2B7 > 1A3 > 1A9 > 2B4	Kuehl et al., 2005

disrupt the normal physiologic function of a critical protein or some critical regulatory pathway that leads to cellular necrosis (Pirmohamed et al., 1996). Inflammation-mediated toxicity was investigated in a recent study, which showed that acyl-glucuronides induce inflammatory toxicity and cytotoxicity against CD14⁺ cells via the p38 mitogen-activated protein kinase pathway (Miyashita et al., 2014). In the case of diclofenac, it was previously demonstrated that the acyl-glucuronide can either bind directly to protein with displacement of the glucuronide group, or can rearrange to form a reactive imine intermediate that 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 (Spahn-Languth and Benet, 1992).

Species differences in toxicity based on metabolism were previously reported (O'Brien et al., 1983; Eberhart et al., 1991). To predict and avoid human-specific drug- and metabolite-induced toxicities based on metabolism, the 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, humanized *UGT1* mice in which the original *Ugt1* locus was disrupted and replaced with the human *UGT1* locus (*hUGT1* mice) were recently developed (Fujiwara et al., 2010, 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 the hepatotoxicity of ibuprofen in *hUGT1* mice and regular mice.

Materials and Methods

Chemicals and Reagents. UDP-glucuronic acid (UDPGA) and alamethicin were purchased from Sigma-Aldrich (St. Louis, MO). Etodolac and diclofenac sodium were purchased from Wako Pure Chemical (Osaka, Japan). *S*-Ibuprofen was purchased from LKT Laboratories (Saint Paul, MN). 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). All other chemicals and solvents were of analytical grade or the highest grade commercially available.

Animals and Preparation of Liver Microsomes. *Tg(UGT1^{A1*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 (Tokyo, Japan). 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 $10,000 \times g$ for 30 minutes at 4°C , and the supernatant was collected. The supernatant was centrifuged at $105,000 \times g$ for 60 minutes 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 (1976) method using bovine serum albumin.

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 MgCl_2 , 5 mM UDPGA, 50 $\mu\text{g}/\text{ml}$ alamethicin, 0.1 mg/ml liver microsomes, and 50 μM to 4 mM etodolac or 10 μM to 1 mM diclofenac. The reaction was initiated by the addition of UDPGA after a 3-minute preincubation at 37°C . After incubation at 37°C for 30 minutes, the reaction was terminated by the addition of 200 μl cold acetonitrile. After removal of the protein by centrifugation at $12,000 \times g$ for 5 minutes, a 50- μl portion of the sample was subjected to high-performance liquid chromatography (HPLC).

S-Ibuprofen, *R*-ibuprofen, 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 MgCl_2 , 5 mM UDPGA, 50 $\mu\text{g}/\text{ml}$ 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-minute preincubation at 37°C . After incubation at 37°C for 60 minutes, the reaction was terminated by the addition of 200 μl cold methanol. After removal of the protein by centrifugation at $12,000 \times g$ for 5 minutes, 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 KH_2PO_4 for diclofenac glucuronide, and 65% methanol 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 and 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 with that of the authentic standard. Quantification of etodolac glucuronides was conducted 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 MgCl_2 , 5 mM UDPGA, and 50 $\mu\text{g}/\text{ml}$ alamethicin was incubated at 37°C for 0 hours and 3 hours. The increase of HPLC peak area of the etodolac glucuronide was compared with the 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 the reaction mixture was quantified 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 minutes, 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):

$$V = \frac{V_{\max} \times S}{K_m + S} \quad (1)$$

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

$$CL_{\text{int}} = \frac{V_{\max}}{K_m} \quad (2)$$

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

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

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

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

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

where S_{50} is the substrate concentration showing the one-half $V_{\max}$ 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 the Akaike 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% dimethylsulfoxide was administered to *hUGT1* mice and regular mice (750 mg/kg, p.o.). Canola oil containing 10% dimethylsulfoxide without *rac*-ibuprofen was also administered to *hUGT1* mice for a control. Blood was obtained from the submandibular vein immediately before dosing and at 6 hours and 24 hours after the dosing. Blood was centrifuged at $9200 \times g$ for 10 minutes after incubation at 37°C for 10 minutes to facilitate the clotting reaction. The supernatant was collected to obtain serum. Liver damage was assessed by measuring the serum activity of alanine amino transaminase (ALT), which is a sensitive diagnostic indicator of hepatotoxicity (Ozer et al., 2008), using the IDTox ALT Color Endpoint Assay Kit (ID Laboratories Inc., London, ON, Canada).

Pharmacokinetics Study of Ibuprofen. *Rac*-ibuprofen suspended in canola oil was administered to *hUGT1* mice and regular mice (200 mg/kg, p.o.). Blood was collected from the submandibular vein into heparinized tubes at 15, 30, 60, 120, 240, and 480 minutes after the administration and immediately centrifuged at $9200 \times g$ for 10 minutes to obtain plasma. For removal of protein, plasma was mixed with 2-fold volume of acetonitrile and centrifuged at $12,000 \times g$ for 5 minutes and the supernatant was filtered through a $0.45\text{-}\mu\text{m}$ 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 ($C_{\max}$) and the time to peak concentration ($T_{\max}$) were obtained from experimental observation. The area under the plasma concentration-time curve from 0 hours to 8 hours ($AUC_{0-8\text{ h}}$) was calculated using the trapezoidal integration without extrapolation to infinity.

Statistical Analysis. All data were presented as means $\pm$ S.D. The t test was used to analyze data from the pharmacokinetics study of ibuprofen. Analysis of variance and Dunnett's procedure for multiple comparisons were used to analyze data on ibuprofen-induced liver injury. $P < 0.05$ was considered significant.

Results

Etodolac Glucuronidation in Liver Microsomes from *hUGT1* Mice, Humans, and Regular Mice. Etodolac is one of the drugs that are subject to species-different glucuronidation because the K_m values and $V_{\max}$ values are different between liver microsomes of humans and rats (Brocks and Jamali, 1991; Tougou et al., 2004). To examine

whether etodolac glucuronidation in *hUGT1* mice is similar to that in humans, liver microsomes were prepared from adult *hUGT1* mice and etodolac glucuronidation was determined. The etodolac acyl-glucuronide formation by the liver microsomes from *hUGT1* mice followed a simple Michaelis–Menten equation (Fig. 1A) because Eadie–Hofstee plots were linear (Supplemental Fig. 1A). Obtained parameter values were as follows: $K_m = 483\text{ }\mu\text{M}$, $V_{\max} = 379\text{ pmol/min per mg}$, and $CL_{\text{int}} = 0.79\text{ }\mu\text{l/min per mg}$ (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 the Michaelis–Menten equation gave smaller AIC values (Fig. 1A). The kinetic parameters of etodolac acyl-glucuronide in human liver microsomes were as follows: $K_m = 483\text{ }\mu\text{M}$, $V_{\max} = 246\text{ pmol/min per mg}$, and $CL_{\text{int}} = 0.51\text{ }\mu\text{l/min per mg}$ (Table 2). The etodolac acyl-glucuronide formation by the mouse liver microsomes followed a simple Michaelis–Menten equation because Eadie–Hofstee plots were linear (Fig. 1A). Obtained parameter values were as follows: $K_m = 1170\text{ }\mu\text{M}$, $V_{\max} = 201\text{ pmol/min per mg}$, and $CL_{\text{int}} = 0.17\text{ }\mu\text{l/min per mg}$ (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 those in mice liver microsomes.

Diclofenac Glucuronidation in Liver Microsomes from *hUGT1* Mice, Humans, and Regular Mice. Previous reports indicate that diclofenac is glucuronidated differently in humans and rodents because the K_m values and $V_{\max}$ are different between liver microsomes of humans and rats (King et al., 2001). Diclofenac glucuronidation in *hUGT1* mice, humans, 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) because Eadie–Hofstee plots were linear (Supplemental Fig. 1B), those by liver microsomes from humans 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 a Michaelis–Menten equation with a substrate inhibition were compared and the Michaelis–Menten with substrate inhibition model gave a smaller AIC value. The kinetic parameters were as follows: $K_m = 31.8\text{ }\mu\text{M}$, $V_{\max} = 7.05\text{ nmol/min per mg}$, and $CL_{\text{int}} = 222\text{ }\mu\text{l/min per mg}$ in *hUGT1* mice liver microsomes; $K_m = 55.6\text{ }\mu\text{M}$, $V_{\max} = 11.8\text{ nmol/min per mg}$, and $CL_{\text{int}} = 211\text{ }\mu\text{l/min per mg}$ in mouse liver microsomes; and $K_m = 33.1\text{ }\mu\text{M}$, $V_{\max} = 10.4\text{ nmol/min per mg}$, $K_i = 22.5\text{ mM}$, and $CL_{\text{int}} = 313\text{ }\mu\text{l/min per mg}$ in human liver microsomes (Table 2). Although the $V_{\max}$ and CL_{int} values in *hUGT1* mice were not very close to those in humans compared with in regular mice, the K_m value in *hUGT1* mice was closer to that in humans than those in regular mice.

***Rac*-Ibuprofen Glucuronidation in Liver Microsomes from *hUGT1* Mice, Humans, and Regular Mice.** Ibuprofen possesses a chiral center in the propionic acid moiety. The pharmacological activity resides mainly in the *S*-ibuprofen. Because much of *R*-ibuprofen is converted to the active *S*-form (Hutt and Caldwell, 1983), the racemate has clinically been used. A previous report indicated that ibuprofen was glucuronidated differently in humans and rodents because the K_m values and $V_{\max}$ were different between liver microsomes of humans, rats, and mice (Magdalou et al., 1990). *Rac*-Ibuprofen glucuronide formation in *hUGT1* mice, humans, and mouse liver microsomes was determined and the kinetic data were analyzed. *Rac*-Ibuprofen glucuronidation in liver microsomes of *hUGT1* mice, humans, and regular mice best fit to a simple

Fig. 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 minutes. Data are the means $\pm$ S.D. of three independent determinations.

Michaelis–Menten equation (Fig. 1C) because the Eadie–Hofstee plots were linear (Supplemental Fig. 1C). The kinetic parameters were as follows: $K_m = 319 \mu\text{M}$, $V_{\max} = 4.27 \text{ nmol/min per mg}$, and $\text{CL}_{\text{int}} = 13.4 \mu\text{l/min per mg}$ in *hUGT1* mice liver microsomes;

$K_m = 651 \mu\text{M}$, $V_{\max} = 6.44 \text{ nmol/min per mg}$, and $\text{CL}_{\text{int}} = 9.9 \mu\text{l/min per mg}$ in human liver microsomes; and $K_m = 364 \mu\text{M}$, $V_{\max} = 11.2 \text{ nmol/min per mg}$, and $\text{CL}_{\text{int}} = 30.7 \mu\text{l/min per mg}$ in regular mice liver microsomes (Table 2). In racemate glucuronidations, $V_{\max}$ and

TABLE 2
Kinetic parameters of diclofenac, etodolac, and ibuprofen glucuronidation in liver microsomes

Compound	Liver Microsome	Kinetic Parameter			
		K_m	V_{max}	K_i	CL_{int}
		μM	$nmol/min \text{ per } mg$	mM	$\mu l/min \text{ per } mg$
Etodolac	Humanized <i>UGT1</i> mice ^a	483 ± 40	0.379 ± 0.003	—	0.79 ± 0.06
	Humans ^a	483 ± 50	0.246 ± 0.012	—	0.51 ± 0.03
	Mice ^a	1170 ± 287	0.201 ± 0.029	—	0.17 ± 0.01
Diclofenac	Humanized <i>UGT1</i> mice ^a	31.8 ± 1.91	7.05 ± 0.04	—	222 ± 12.2
	Humans ^b	33.1 ± 1.70	10.4 ± 0.28	22.5 ± 1.00	313 ± 8.88
	Mice ^a	55.6 ± 1.52	11.8 ± 0.10	—	211 ± 5.62
<i>rac</i> -Ibuprofen	Humanized <i>UGT1</i> mice ^a	319 ± 26	4.27 ± 0.12	—	13.4 ± 0.71
	Humans ^a	651 ± 8.1	6.44 ± 0.05	—	9.9 ± 0.05
	Mice ^a	364 ± 12	11.2 ± 0.19	—	30.7 ± 0.54
<i>S</i> -Ibuprofen	Humanized <i>UGT1</i> mice ^a	381 ± 26	7.54 ± 0.16	—	19.9 ± 1.00
	Humans ^a	699 ± 34	8.71 ± 0.17	—	12.5 ± 0.38
	Mice ^a	364 ± 5.0	16.5 ± 0.19	—	45.1 ± 0.50
<i>R</i> -Ibuprofen	Humanized <i>UGT1</i> mice ^a	320 ± 38	2.18 ± 0.07	—	6.87 ± 0.56
	Humans ^a	428 ± 11	3.37 ± 0.03	—	7.86 ± 0.13
	Mice ^a	424 ± 19	8.00 ± 0.09	—	18.9 ± 0.70

^aCalculated using the Michaelis–Menten equation.

^bCalculated using substrate inhibition.

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, Humans, and Regular Mice.** *S*-Ibuprofen glucuronide formation in *hUGT1* mice, humans, and mouse liver microsomes was determined and the kinetic data were analyzed. *S*-Ibuprofen glucuronidation in liver microsomes of *hUGT1* mice, humans, and regular mice best fit to a simple Michaelis–Menten equation (Fig. 1D) because the Eadie–Hofstee plots were linear (Supplemental Fig. 1D). The kinetic parameters were as follows: $K_m = 381 \mu M$, $V_{max} = 7.54 \text{ nmol/min per mg}$, and $CL_{int} = 19.9 \mu l/min \text{ per mg}$ in *hUGT1* mice liver microsomes; $K_m = 699 \mu M$, $V_{max} = 8.71 \text{ nmol/min per mg}$, and $CL_{int} = 12.5 \mu l/min \text{ per mg}$ in human liver microsomes; and $K_m = 364 \mu M$, $V_{max} = 16.5 \text{ nmol/min per mg}$, and $CL_{int} = 45.1 \mu l/min \text{ per mg}$ in mouse liver microsomes (Table 2). In *S*-ibuprofen glucuronidations, V_{max} values 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 values in *hUGT1* mice were not similar to that in humans.

***R*-Ibuprofen Glucuronidation in Liver Microsomes from *hUGT1* Mice, Humans, and Regular Mice.** *R*-Ibuprofen glucuronide formation in *hUGT1* mice, humans, and mouse liver microsomes was determined and the kinetic data were analyzed. *R*-Ibuprofen glucuronidation in liver microsomes of *hUGT1* mice, humans, and regular mice best fit to a simple Michaelis–Menten equation (Fig. 1E) because the Eadie–Hofstee plots were linear (Supplemental Fig. 1E). The kinetic parameters were as follows: $K_m = 320 \mu M$, $V_{max} = 2.18 \text{ nmol/min per mg}$, and $CL_{int} = 6.87 \mu l/min \text{ per mg}$ in *hUGT1* mice liver microsomes; $K_m = 428 \mu M$, $V_{max} = 3.37 \text{ nmol/min per mg}$, and $CL_{int} = 7.86 \mu l/min \text{ per mg}$ in human liver microsomes; and $K_m = 424 \mu M$, $V_{max} = 8.00 \text{ nmol/min per mg}$, and $CL_{int} = 18.9 \mu l/min \text{ per mg}$ in mouse liver microsomes (Table 2). In *R*-ibuprofen glucuronidations, V_{max} values 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 values in *hUGT1* mice were not similar to that in humans.

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

Fig. 2. Serum ALT activity in mice treated with *rac*-ibuprofen. ALT activities of *hUGT1* mice (A) and those of regular mice (B) treated with 750 mg/kg *rac*-ibuprofen are shown. 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 (units per liter) ($n \geq 3$). ** $P < 0.01$, compared with the group before dosing.

suggested that there were differences in the onset pattern of ibuprofen-induced liver injury between *hUGT1* mice and regular mice.

Pharmacokinetics Study of Ibuprofen. A 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 ($T_{\max} = 30$ minutes) in both *hUGT1* mice and regular mice with $C_{\max}$ values of $368 \pm 177 \mu\text{M}$ and $270 \pm 60.4 \mu\text{M}$, respectively. Furthermore, the $\text{AUC}_{0-8 \text{ h}}$ values of ibuprofen in *hUGT1* mice was statistically higher than that of regular mice ($P < 0.05$), because the $\text{AUC}_{0-8 \text{ h}}$ values of ibuprofen were $1285.3 \pm 282.1 \mu\text{M}\cdot\text{h}$ in *hUGT1* mice and $721.1 \pm 246.3 \mu\text{M}\cdot\text{h}$ in regular mice. Whereas the plasma level of ibuprofen acyl-glucuronide rapidly increased in regular mice, the plasma level of ibuprofen acyl-glucuronides slowly increased in *hUGT1* mice (Fig. 3B). The $T_{\max}$ value of ibuprofen acyl-glucuronides was different between *hUGT1* mice and regular mice because $T_{\max}$ values were 120 minutes in *hUGT1* mice and 30 minutes in regular mice, with $C_{\max}$ values of $96.7 \pm 17.7 \mu\text{M}$ and $114 \pm 23.4 \mu\text{M}$, respectively. $\text{AUC}_{0-8 \text{ h}}$ values of ibuprofen acyl-glucuronides in *hUGT1* mice ($634.8 \pm 115.4 \mu\text{M}\cdot\text{h}$) was statistically higher than that in regular mice ($452.1 \pm 39.6 \mu\text{M}\cdot\text{h}$). Although 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 humans and experimental animals could have differences in affinity to substrate, expression levels, and gene expression profiles. For example, because human *UGT1A4* is functional but rat *UGT1A4* and mouse *Ugt1a4* are pseudogenes, specific probes of *UGT1A4* such as trifluoperazine and imipramine are not glucuronidated in mice or rats (Uchaipichat et al., 2006; Shiratani et al., 2008). The species differences of metabolic enzymes are typically assessed by comparing kinetic parameters such as K_m , $V_{\max}$, and CL_{int} . In this study, the method of preparation of liver microsomes was different because the microsomes of humans and regular mice were purchased and microsomes of *hUGT1* mice were prepared by our above-described method. The difference in

the method of microsome preparation may influence glucuronidation activities. However, in past studies, species differences of glucuronidation activities have been assessed with microsomes prepared by different methods (Xu et al., 2006; Cai et al., 2010).

One approach to overcome the gap of species difference is to generate humanized mice by introducing a human gene into the mouse genome (Gonzalez, 2003; Henderson and Wolf, 2003). Thus, humanized mice offer a better animal model to predict the human drug metabolism and understand the underlying mechanisms. In our recent study, *hUGT1* mice showed glucuronidation activity of furosemide, naproxen, imipramine, and trifluoperazine that was comparable to that of humans (Kutsuno et al., 2013). In this study, we investigated the glucuronidation activity of etodolac, diclofenac, and ibuprofen metabolized by UGTs. Because 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 interactions between one enantiomer and the other (el Mouelhi et al., 1987). In this study, kinetic parameters of *S*-ibuprofen, *R*-ibuprofen, and *rac*-ibuprofen were similar in microsomes of humans, *hUGT1* mice, and regular mice (Table 2). Therefore, we believe that glucuronidation of ibuprofen is not enantioselective. Although 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 humans (Table 2). Although etodolac, ibuprofen, and diclofenac were glucuronidated by multiple UGT isoforms (Table 1), the major isozyme involved in catalyzing the glucuronidation of diclofenac in humans 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, UGT2B7, UGT2B10, UGT2B11, UGT2B15, UGT2B17, and UGT2B28 proteins. In mice, seven *Ugt2b* genes include *Ugt2b1*, *Ugt2b5*, *Ugt2b34*, *Ugt2b35*, *Ugt2b36*, *Ugt2b37*, and *Ugt2b38*. The species difference in the function of human UGT2B and mouse *Ugt2b* family enzymes has not been fully understood. However, it was previously reported that there are species differences in drug glucuronidations that are catalyzed by UGT2B7 (Cai et al., 2010). Therefore, the differences of diclofenac glucuronidations between *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 because they offer the knowledge to evaluate and predict the toxicological risk, which 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 were significantly elevated by approximately 12-fold in regular mice. These results showed that there was a difference in response to APAP between humanized CYP2E1 mice and regular mice (Cheung and Gonzalez, 2008). This study investigated ibuprofen-induced liver injury and pharmacokinetics of ibuprofen in *hUGT1* and regular mice. Our results showed that there were differences in ibuprofen-induced liver injury between *hUGT1* mice and regular mice (Fig. 2). In *hUGT1*

Fig. 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 circles) and *hUGT1* mice (open circles) treated with ibuprofen (200 mg/kg, p.o.) are shown. Results are shown as means $\pm$ S.D. of the plasma concentration of ibuprofen or ibuprofen acyl-glucuronides (in micromoles) ($n \geq 3$).

mice, *rac*-ibuprofen increased ALT levels 6 hours after dosing. *Rac*-ibuprofen clearance in *hUGT1* mice was much lower than that in regular mice (Table 2) and AUC_{0–8 h} values of ibuprofen in *hUGT1* mice were statistically higher than those in regular mice. These data suggest that *hUGT1* mice, which had lower intrinsic clearance of ibuprofen, had higher exposure to ibuprofen than regular mice. By contrast, ALT levels were increased 24 hours after dosing in regular mice. Our data indicate that slower intrinsic clearance of ibuprofen in *hUGT1* mice still resulted in the higher AUC_{0–8 h} values 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 patterns 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.

This study demonstrated that the glucuronidations of etodolac and ibuprofen in *hUGT1* mice were almost comparable to those in humans. These findings indicate that in vitro and in vivo studies utilizing *hUGT1* mice are useful for predicting in vivo human glucuronidation of drugs that are mainly catalyzed by 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. Although 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, Fujiwara.

Conducted experiments: Kutsuno, Fujiwara.

Performed data analysis: Kutsuno, Fujiwara.

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

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