Targeted screen for human UDP-glucuronosyltransferases inhibitors and the evaluation of potential drug-drug interactions with zafirlukast

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ABBREVIATIONS: DDI, drug-drug interaction; HIM, human small intestine microsomes; HLM, human liver microsomes; UDPGA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase
Abstract

Inhibition of drug metabolizing enzymes is a major mechanism in drug-drug interactions (DDIs). A number of cases of DDIs via inhibition of UDP-glucuronosyltranseferases (UGTs) have been reported, although the changes in pharmacokinetics are relatively small in comparison to drugs that are metabolized by cytochrome P450s. Most of the past studies have investigated hepatic UGTs, though recent studies have revealed a significant contribution of UGTs in the small intestine to drug clearance. To evaluate potential DDIs caused by inhibition of intestinal UGTs, we assessed inhibitory effects of 578 compounds, including drugs, xenobiotics, and endobiotics, on human UGT1A8 and UGT1A10, which are major contributors to intestinal glucuronidation. We identified 29 inhibitors by monitoring raloxifene glucuronidation with recombinant UGTs. All of the inhibitors potently inhibited UGT1A1 activity, as well. We found that zafirlukast is a potent general inhibitor of UGT1A8s and a moderate inhibitor of UGT2Bs by monitoring 4-methylumbelliferone glucuronidation by recombinant UGTs. However, zafirlukast did not potently inhibit diclofenac glucuronidation, suggesting that the inhibitory effects might be substrate-specific. Inhibitory effects of zafirlukast on some UGT substrates were further investigated in HLM and HIM to evaluate potential DDIs. $R$ values (ratios of intrinsic clearance with and without an inhibitor) revealed that zafirlukast has potential to cause clinical DDIs in the small intestine. Although we could not identify specific UGT1A8 and UGT1A10 inhibitors, zafirlukast was identified as a general inhibitor for UGTs in vitro. The present study suggests that the inhibition of UGT in the small intestine would be an underlying mechanism for DDIs.
Introduction

UDP-glucuronosyltransferases (UGTs) are a family of phase II enzymes that catalyze the transfer of glucuronic acid from the cofactor UDP-glucuronic acid (UDPGA) to a large number of endogenous and exogenous compounds, including pharmaceutical drugs (Ritter, 2000). In humans, there are 19 functional UGT enzymes that are classified into the following three subfamilies: UGT1A, UGT2A, and UGT2B, based on amino acid sequence identity (Mackenzie et al., 2005). In humans, most of the UGT1 and UGT2 enzymes, including UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT2B4, UGT2B7, UGT2B10, and UGT2B15, are expressed in the liver, while UGT1A7, UGT1A8, and UGT1A10 are not expressed (Izukawa et al., 2009). UGT1A1, UGT2B7, UGT2B17, UGT1A8 and UGT1A10 are expressed in the human intestines (Ohno and Nakajin, 2009; Court et al., 2012). Although it has been recognized that hepatic UGTs play an important role in the pharmacokinetics of administered drugs, recent studies have demonstrated the significance of intestinal UGTs (Komura and Iwaki, 2011; Ritter, 2007). The significance of intestinal UGTs is exemplified by raloxifene, a selective estrogen receptor modulator for the treatment of osteoporosis. Orally administered raloxifene is extensively metabolized in the intestines by UGT1A8, UGT1A10, and UGT1A1, which leads to poor bioavailability (2%) (Mizuma, 2009). This raises the possibility that drugs that inhibit UGT1A8, UGT1A10, and/or UGT1A1 may greatly alter the bioavailability of drugs taken orally, such as raloxifene, that are substrates of these UGTs.

The inhibition of drug-metabolizing enzymes by a drug could alter the exposure level of a co-administered drug and thereby cause adverse reactions. The occurrence of drug interactions occasionally leads to restrictions such as warnings, precautions, contradictions, or boxed warnings, or even withdrawal from the market (for example, astemizol, cisapride, terfenadine, cerivastatin, mibebradil, and sorivudine) (Diasio, 1998; Dresser et al., 2000; Shitara et al., 2004). A profound understanding of drug-drug interactions (DDI) via metabolism, in particular by
P450s, which account for the metabolism of 70% of pharmaceutical drugs, has been achieved over the past two decades (Obach et al., 2006). In contrast, UGT enzymes have attracted relatively less attention for their DDI potential, mostly likely because UGTs account for the metabolism of only 15% of pharmaceutical drugs (Williams et al., 2004). Despite the lower contribution of UGTs to the clearance of drugs, drug interactions via inhibition of glucuronidation have been clinically observed as follows: (1) atazanavir induces hyperbilirubinemia in carriers of UGT1A1*28 allele (Zhang et al., 2005); (2) valproic acid dose-dependently increases the area under the curve of lamotrigine up to 2.5-fold and also inhibits zidovudine and lorazepam glucuronidation (Morris et al., 2000; Kiang et al., 2005); and (3) fluconazole significantly alters the pharmacokinetics of zidovudine in HIV patients (Sahai et al., 1994). However, the majority of these studies have focused on hepatic UGTs and the possible contribution of intestinal UGTs to drug interactions has not been considered.

In this study, we sought to identify inhibitors for either UGT1A8 or UGT1A10 to evaluate potential DDIs caused by the inhibition of intestinal UGTs. We assessed the inhibitory effects of 578 compounds, including drugs, dietary components, environmental xenobiotics, and endobiotics, on raloxifene glucuronidation using recombinant UGT1A8 and UGT1A10. We found that zafirlukast is a potent inhibitor of both UGT1A8 and UGT1A10, although it is also a general UGT inhibitor. We further compared the inhibition properties of zafirlukast against glucuronidation in the liver and the small intestine using UGT substrates, including SN-38, furosemide, S-naproxen, etodolac, and diclofenac, and evaluated potential DDIs.
Materials and Methods

Materials.

Zafirlukast and S-naproxen were purchased from Cayman Chemicals (Ann Arbor, MI). 4-Methylumbelliferone (4-MU), UDPGA, alamethicin (from *Trichoderma viride*), and furosemide were purchased from Sigma-Aldrich (St. Louis, MO). SN-38 was purchased from Tokyo Chemical Industry (Tokyo, Japan). Raloxifene, raloxifene 4’- and 6-glucuronides, SN-38 glucuronide, furosemide glucuronide, S-naproxen glucuronide, and diclofenac glucuronide were purchased from Toronto Research Chemicals (Toronto, Canada). Etodolac and diclofenac sodium were purchased from Wako Pure Chemical Industries (Osaka, Japan). The pooled human liver microsomes (HLM) (27 males and 23 females; median age 52 years, range 26-66 years), pooled human small intestine microsomes (HIM) (3 males and 4 females; median age 55 years, range 30-70 years), and recombinant human UGT1A1, UGT1A3, UGT1A6, UGT1A8, UGT1A9, UGT1A10, UGT2B7, UGT2B15, and UGT2B17 proteins expressed in baculovirus-infected insect cells (Supersomes) were purchased from Corning (Corning, NY). Compounds used for the screening of their inhibitory potency and the other chemicals or solvents were of the highest grade commercially available.

Screening compounds for their inhibition of UGT1A8 and UGT1A10 by measuring raloxifene 4’- and 6-glucuronidation.

Five hundred and seventy eight compounds, including drugs, dietary components, environmental xenobiotics, and endobiotics, were screened against recombinant UGT1A1 and UGT1A10 for raloxifene 4’- and 6-glucuronidation inhibition. A typical incubation mixture (100 μl of total volume) contained 50 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 5 mM UDPGA, 25 μg/ml alamethicin, 0.1 mg/ml recombinant UGT1A8 or UGT1A10, 10 μM raloxifene, and a tested compound (20 μM unless otherwise indicated). Raloxifene and all of the tested
compounds were dissolved in DMSO and the final DMSO concentration in the incubation mixture was 2%. The reaction mixture was incubated at 37°C for 10 min and the reaction was terminated by the addition of 50 μl of cold acetonitrile and 20 μL of 0.1 μg/ml haloperidol as an internal standard. After removal of the protein by centrifugation at 12,000 g for 5 min, a 20-μl portion of the sample was subjected to LC-MS/MS.

The LC equipment was comprised 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 × 50 mm, 3.5 μm; Agilent Technologies). The column temperature was set at 25°C and the flow rate was 200 μl/min. The mobile phase was 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). The conditions for elution were as follows: (1) 5% of B (0-0.5 minutes), (2) 5-50% of B (0.5-7 minutes), (3) 50-100% of B (7–8 minutes), (4) 100% of B (8-13 minutes), and (5) 5% of B (13-18 minutes). The LC was connected to a PE Sciex API2000 tandem mass spectrometer (AB Sciex, Framingham, MA) operated in the positive electrospray ionization mode. The turbo gas was maintained at 550°C. Nitrogen was used as the nebulizing, turbo, and curtain gases at 60, 85, and 30 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 mass/charge (m/z) ion transitions were recorded in the multiple reaction monitoring mode with m/z of 650.1 and 474.2 for raloxifene 4’- and 6-glucuronides and an m/z of 376.1 and 132.0 for haloperidol. The analytical data were processed using the Analyst software (version 1.5; Applied Biosystems, Foster City, CA). All of the data were single determination. Quantification of raloxifene 4’- and 6-glucuronides was performed by comparing the peak-height ratios of the glucuronides to haloperidol (as an internal standard) to those of authentic standards. Residual activities were determined as a percentage of the activity in the control sample.
Glucuronidation assays by recombinant UGTs and human liver and intestine microsomes

4-MU glucuronidation by recombinant UGTs (UGT1A1, UGT1A3, UGT1A6, UGT1A8, UGT1A9, UGT1A10, UGT2B7, and UGT2B15) was examined as described previously (Fujiwara et al., 2007) with slight modifications. Briefly, a typical incubation mixture (100 µl of total volume) contained 50 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 2.5 mM UDPGA, 25 µg/ml alamethicin, 0.1 mg/ml UGT Supersomes, 0.5-50 µM zafirlukast, and 4-MU. 4-MU concentrations for each UGT isoform were set at values similar to the reported $K_m$ or $S_{50}$ values shown in Table 1 (Uchaipichat et al., 2004; Fujiwara et al., 2009). $K_m$ is the Michaelis constant in the Michaelis-Menten equation and substrate inhibition model (Houston and Kenworthy, 2000) and $S_{50}$ is the substrate concentration that gives half of the maximum enzyme activity ($V_{max}$) in the Hill equation (Cornish-Bowden, 1995). The reaction mixture was incubated at 37°C for 10 min and then terminated by the addition of 100 µl 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. The formation of 4-MU glucuronide was determined using an L-7100 pump (Hitachi, Tokyo, Japan), an L-7485 FL detector (Hitachi), an L-7200 autosampler (Hitachi), a D-2500 integrator (Hitachi), and a CAPCELL PAK column (4.6 × 150 mm, 5 µm; Shiseido, Tokyo, Japan). The mobile phase was 30% methanol containing 20 mM potassium dihydrogen phosphate, the flow rate was 1.0 ml/min, and the column temperature was 35°C. Detection was accomplished with a fluorescence detector at 315-nm for excitation and 365-nm for emission. Quantification of 4-MU glucuronide was performed by comparing the peak-heights to those of the authentic standards.

Diclofenac glucuronidation by recombinant UGTs (UGT1A3, UGT1A9, UGT2B7, and UGT2B17), HLM, and HIM was examined according to a method reported previously (Kutsuno et al., 2014) 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 HLM or HIM, 0.5-50 μM zafirlukast, and diclofenac. The diclofenac concentration was set at a value similar to the reported \( K_m \) value shown in Table 2 (30 μM diclofenac) (King et al., 2001). The reaction mixture was incubated at 37°C for 30 min and then the reaction was terminated by the addition of 200 μl cold acetonitrile. After removal of the proteins by centrifugation at 12,000 g for 5 min, a 50-μl portion of the sample was subjected to HPLC. The formation of diclofenac glucuronide was determined by the HPLC system with an LC-10AD pump (Shimadzu, Kyoto, Japan), an FP-2020 fluorescence detector (JASCO, Tokyo, Japan), an SIL-10A autosampler (Shimadzu), an 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 65% methanol containing 0.50 M potassium dihydrogen phosphate. Detection was accomplished with a fluorescence detector at 282-nm for excitation and 365-nm for emission. Quantification of diclofenac glucuronide was performed by comparing the peak-heights to those of the authentic standards.

Furosemide glucuronidation in HLM and HIM was examined using a method similar to the method used for diclofenac glucuronidation described above. The furosemide concentration was set at a value similar to the reported \( K_m \) value shown in Table 2 (500 μM furosemide) (Kutsuno et al., 2013). The mobile phases were 30% acetonitrile containing 15 mM sodium dihydrogen phosphate. Detection was accomplished with a fluorescence detector at 345-nm for excitation and 450-nm for emission. Quantification of furosemide glucuronide was performed by comparing the peak-heights to those of the authentic standards.

Etodolac glucuronidation in HLM and HIM was examined using a method similar to the method used for diclofenac glucuronidation described above. The etodolac concentration was set at a value similar to the reported \( K_m \) value shown in Table 2 (400 μM etodolac) (Kutsuno et al., 2013). The mobile phase was 50% acetonitrile containing 1% aqueous acetic acid.
Detection was accomplished with a fluorescence detector at 276-nm for excitation and 667-nm for emission. Quantification of etodolac glucuronide was performed as described previously (Kutsuno et al., 2014).

S-Naproxen glucuronidation in HLM and HIM was examined using a method similar to the method used for diclofenac glucuronidation described above. The S-naproxen concentration was set at a value similar to the reported $K_m$ value shown in Table 2 (300 μM S-naproxen) (Kutsuno et al., 2013). The mobile phases were 35% acetonitrile containing 0.12% acetic acid. Detection was accomplished with a fluorescence detector at 230-nm for excitation and 355-nm for emission. Quantification of S-naproxen glucuronide was performed by comparing the peak-heights to those of the authentic standards.

SN-38 glucuronidation in HLM and HIM was examined as described previously (Hanioka et al., 2001) with slight modifications. Briefly, a typical incubation mixture (100 μl of total volume) contained 50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, UDPGA, 25 μg/ml alamethicin, 0.1 mg/ml HLM or HIM, 0.5-10 μM zafirlukast, and SN-38. When varying concentration of SN-38 (2-100 μM), the UDPGA concentration was set at 3 mM, and when varying concentration of UDPGA (0.3-3 mM), the SN-38 concentration was set at 10 μM that is similar to the reported $K_m$ value (Fujita et al., 2011). The reaction mixture was incubated at 37°C for 10 min and then the reaction was terminated by the addition of 25 μl of 10% (w/v) perchloric acid. After removal of the proteins by centrifugation at 12,000 g for 5 min, a 20-μl portion of the sample was subjected to HPLC. The formation of SN-38 glucuronide was determined using the same HPLC system used for measuring 4-MU glucuronide with a Mightysil RP-18 GP column (4.6 × 150 mm, 5 μm). The mobile phase was 48 mM potassium dihydrogen phosphate containing 2.88 mM sodium 1-octanesulfonate (pH 2.5, adjusted by perchloric acid):acetonitrile:methanol (15:4:1, v/v/v). Detection was accomplished with a fluorescence
detector at 370-nm for excitation and 425-nm for emission. Quantification of SN-38 glucuronide was performed by comparing the peak-heights to those of the authentic standards.

Inhibition kinetics analysis.

The drug concentrations producing a 50% decrease in metabolite formation rate (IC$_{50}$) for 4-MU, diclofenac, furosemide, etodolac, and S-naproxen glucuronidation were determined from a plot of the logarithm of the inhibitor concentration versus the percentage of the enzyme activity relative to the control by non-linear regression analysis according to the Hill equation with GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA). When the assays are performed at a substrate concentration equivalent to the $K_m$ value, inhibition constant ($K_i$) values are calculated using the following equations: (1) $K_i = 1/2 \times IC_{50}$ for competitive inhibition and (2) $K_i = IC_{50}$ for noncompetitive inhibition (Cheng and Prusoff, 1973). Using a competitive inhibition model is considered to be a conservative approach to minimize false-negative predictions because the lowest possible estimate of the $K_i$ is obtained. Accordingly, the present study adopted the former equation.

$K_i$ values for the SN-38 glucuronidation were determined by using various concentrations of SN-38 or UDPGA in the presence or absence of zafirlukast as described above. The type of inhibition was determined from a Dixon-plot analysis of the data. $K_i$ values were calculated by nonlinear regression analysis using the equation for noncompetitive inhibition (eq. 1) with GraphPad Prism 6.

$$V = V_{max} \times S / [(K_m + S) \times (1 + I / K_i)]$$---Eq. 1

where $V$ is the velocity of the reaction; and $S$ and $I$ are the substrate and inhibitor concentrations, respectively.
Calculation of $R$ values.

The degree of inhibition of the human hepatic UGTs was estimated by calculating the $R$ value (eq. 2) (Bjornsson et al., 2003), which represents the ratio of intrinsic clearance of a drug in the presence of the inhibitor to that in its absence:

$$R = 1 + f_u \times \frac{I_{\text{in}, \text{max}}}{K_i}$$

---Eq. 2

where $f_u$ represents the plasma unbound fraction of the inhibitor and $I_{\text{in}, \text{max}}$ represents the estimated maximum inhibitor concentration at the liver inlet. For the estimation of the $R$ value, $I_{\text{in}, \text{max}}$ was calculated using eq. 3 (Ito et al., 1998):

$$I_{\text{in}, \text{max}} = I_{\text{max}} + F_a \times \text{Dose} \times \frac{k_a}{Q_h}$$

---Eq. 3

where $I_{\text{max}}$ represents the reported value for the maximum plasma concentration in the systemic circulation in the clinical situation; $F_a$ represents the absorbed inhibitor fraction; $k_a$ represents the absorption rate constant in the intestine; and $Q_h$ represents the human hepatic blood flow rate (1610 mL/min). To estimate the maximum $I_{\text{in}, \text{max}}$ value, $F_a$ was set at 1; $k_a$ was set at 0.1 min$^{-1}$ (maximum gastric emptying time [10 min]); and the blood-to-plasma concentration ratio was assumed to be 1.

The degree of inhibition of human intestinal UGTs was estimated by calculating the $R$ value (eq. 4):

$$R = 1 + \frac{I_{\text{gut}}}{K_i}$$

---Eq. 4

$$I_{\text{gut}} = \frac{\text{Dose}}{250 \text{ mL}}$$

---Eq. 5

where $I_{\text{gut}}$ represents the estimated inhibitor concentration in the small intestine (Zhang et al., 2008).
Results

Screening of 578 compounds for the inhibition of raloxifene 4’- and 6-glucuronidation by UGT1A8 and UGT1A10.

To find specific inhibitors of either UGT1A8 or UGT1A10, we screened 578 compounds including drugs, dietary components, environmental xenobiotics, and endobiotics for their inhibition of raloxifene 4’- and 6-glucuronidation using recombinant UGT1A8 and UGT1A10 (Supplementary Table 1). The control activities of UGT1A8 were 1304 and 445 pmol/min/mg at 10 µM raloxifene for 4’- and 6-glucuronidation, respectively. For UGT1A10, the control activity for 4’-glucuronidation was 443 pmol/min/mg, while the 6-glucuronidation was negligible, in agreement with a previous report (Kemp et al., 2002). Compounds showing more than 50% inhibition are shown in Supplementary Table 2. Nine compounds inhibited UGT1A8 preferentially to UGT1A10 and another nine compounds inhibited UGT1A10 preferentially to UGT1A8. Eleven compounds strongly inhibited both UGT1A8 and UGT1A10 activities, and zafirlukast showed the most complete inhibition. Because the inhibition potencies of these compounds were similar between UGT1A8 and UGT1A10, these 29 inhibitors were further investigated for the inhibition of UGT1A1. Most of compounds were found to inhibit UGT1A1 activity (Supplementary Table 2), suggesting that these compounds might be non-specific UGT inhibitors. Although we could not find a specific inhibitor for either UGT1A8 or UGT1A10, we subsequently examined the inhibitory potential of zafirlukast on various UGT isoforms.

Inhibitory effects of zafirlukast on 4-MU and diclofenac glucuronidation in recombinant UGT expression systems.

Next, we characterized the inhibitory effects of zafirlukast on various UGT enzymes using 4-MU as a substrate, which is glucuronidated by almost all of the UGT1A and UGT2B isoforms. At substrate concentrations corresponding to the approximate $K_m$ or $S_{50}$ for each isoform,
zafirlukast potently inhibited the enzyme activities of UGT1A1 (IC$_{50}$ values of $0.7 \pm 1.1 \, \mu M$), UGT1A3 ($6.3 \pm 1.0 \, \mu M$), UGT1A6 ($2.0 \pm 1.0 \, \mu M$), UGT1A8 ($1.0 \pm 1.1 \, \mu M$), UGT1A9 ($0.6 \pm 1.1 \, \mu M$), and UGT1A10 ($3.0 \pm 1.0 \, \mu M$), and moderately inhibited the enzyme activities of UGT2B7 ($13.1 \pm 1.0 \, \mu M$) and UGT2B15 ($33.4 \pm 1.0 \, \mu M$) (Fig. 1A and Table 1). Thus, zafirlukast was found to be a UGT general inhibitor with potent and moderate inhibiting properties to UGT1A and UGT2B isoforms, respectively.

To test whether the inhibitory effect of zafirlukast is substrate-dependent, the inhibitory effects of zafirlukast on diclofenac glucuronidation by recombinant UGT1A3, UGT1A9, UGT2B7, and UGT2B17, the responsible isoforms, was determined (Kuehl et al., 2005). Zafirlukast inhibited the activity of UGT2B7 with an IC$_{50}$ value of $40.2 \pm 1.1 \, \mu M$ (Fig. 1B) and slightly inhibited the activities of UGT1A9 and UGT2B17 with IC$_{50}$ values over $50 \, \mu M$. In contrast, zafirlukast did not inhibit the activity of UGT1A3. Taken together, it was demonstrated that the inhibitory effects of zafirlukast on UGT activities were both substrate- and UGT isoform-dependent.

### Inhibitory effects of zafirlukast on furosemide, etodolac, S-naproxen, diclofenac, and SN-38 glucuronidation.

To evaluate possible clinical DDIs of glucuronidation inhibition by zafirlukast, we investigated the inhibitory effects of zafirlukast on glucuronidation of drugs including furosemide, etodolac, S-naproxen, and diclofenac in HLM and HIM (Fig. 2). Furosemide glucuronidation, which is mainly catalyzed by UGT1A1, in HLM and HIM was greatly inhibited by zafirlukast with IC$_{50}$ values of $4.9 \pm 1.1$ and $2.9 \pm 1.1 \, \mu M$, respectively (Fig. 2A and Table 2). Etodolac glucuronidation, which is mainly catalyzed by UGT1A9, in HLM was potently inhibited by zafirlukast with an IC$_{50}$ value of $8.7 \pm 1.3 \, \mu M$ (Fig. 2B and Table 2). Because etodolac and S-naproxen glucuronidation in HIM were marginal, inhibition analyses
were not conducted. S-naproxen glucuronidation, which is mainly catalyzed by UGT2B7, in HLM was moderately inhibited by zafirlukast with an IC$_{50}$ value of 23.2 ± 1.1 (Fig. 2C and Table 2). Diclofenac glucuronidation, which is mainly catalyzed by UGT1A9 and UGT2B7, in HLM and HIM were moderately inhibited by zafirlukast with IC$_{50}$ values of 26.5 ± 1.1 and 16.1 ± 1.2 µM, respectively (Fig. 2D and Table 2).

Inhibitory effects of zafirlukast were further characterized using glucuronidation of SN-38 (an active metabolite of irinotecan), which is mainly catalyzed by UGT1A1. When SN-38 concentration was varied, SN-38 glucuronidation in HLM and HIM was greatly inhibited by zafirlukast in a noncompetitive manner with $K_i$ values of 1.2 ± 0.1 and 1.9 ± 0.1, respectively (Fig. 3A and 3B). When UDPGA concentration was varied, SN-38 glucuronidation in HLM was inhibited by zafirlukast in a noncompetitive manner with $K_i$ value of 0.7 ± 0.0 (Fig. 3C).

Taken together, the inhibitory effects of zafirlukast on glucuronidation are substrate- and UGT isoforms-dependent. A tendency was observed that UGT1A-catalyzing reactions (SN-38 and furosemide glucuronidation) were inhibited by zafirlukast more potently than UGT2B-catalyzing reactions (S-naproxen and diclofenac glucuronidation).

**Prediction of the DDI potential of zafirlukast**

We next evaluated the potential zafirlukast DDIs with SN-38, furosemide, etodolac, S-naproxen, or diclofenac via inhibition of glucuronidation in the liver and small intestine by calculating the $R$ value. As described in Materials and Methods section, $K_i$ values were calculated based on the equation $K_i = 1/2 \times IC_{50}$, assuming competitive inhibition (Table 3) except for SN-38 glucuronidation. The $I_{\text{max}}$ value of zafirlukast was reported to be 0.957 µM after oral administration of 20 mg zafirlukast to healthy subjects (Karonen et al., 2012). Using eq. 3, the $I_{\text{m, max}}$ value was calculated to be 5.3 µM. The $K_i$ values for hepatic glucuronidation of SN-38, furosemide, etodolac, S-naproxen, and diclofenac calculated $R$ values to be 5.4, 3.2, 2.2,
1.5, and 1.4, respectively. When the unbound inhibitor concentration ($f_u = 0.01$) was used, the $R$ value, no interaction was predicted for these drugs (i.e., $R$ value < 1.1) (Table 3). We next predicted the potential clinical DDIs of orally taken drugs via glucuronidation inhibition in the small intestine. SN-38 glucuronidation was not examined because its parental drug irinotecan is only administered intravenously. Using eq. 4, the $I_{\text{gut}}$ value of zafirlukast was calculated to be 277.9 µM. With the $K_i$ value for furosemide and diclofenac glucuronidation in HIM, the $R$ values were calculated to be 192.3 and 35.4, respectively (Table 3). Collectively, it was suggested that concomitantly administered zafirlukast would not cause DDI in the liver, whereas it would cause DDI with furosemide or diclofenac via inhibition of glucuronidation in the small intestine.
Discussion

Glucuronidation is a major phase II metabolic pathway for drugs and xenobiotics that is catalyzed by UGT enzymes. In recent years, much progress has been made in understanding the physiological and clinical roles of UGTs (Court, 2005; Court et al., 2012; Izukawa et al., 2009; Stingl et al., 2014). However, information regarding the inhibitory properties of chemicals against intestinal UGT1A8 and UGT1A10 is limited. Intestinal glucuronidation plays an important role in the first-pass metabolism of some drugs; hence the inhibition of intestinal UGT1A8 or UGT1A10 has a risk to cause DDIs. To evaluate the potential DDIs via inhibition of intestinal UGTs, we initially screened 578 compounds for the inhibition of recombinant UGT1A8 and UGT1A10. Twenty-nine compounds were found to inhibit either UGT1A8 or UGT1A10 or both UGT1A8 and UGT1A10; all of these compounds strongly inhibited UGT1A1 activity. The inhibitory effects observed in three of the UGT isoforms were most likely due to the high degree of amino acid sequence similarity among UGTs (Tukey and Strassburg, 2000). Although specific inhibitors for either UGT1A8 or UGT1A10 were not found, zafirlukast, a selective peptide leukotriene receptor antagonist, showed the most potent inhibitory effect against raloxifene glucuronidation. Because zafirlukast has been reported to cause DDIs with S-warfarin and theophylline via inhibition of CYP2C9 and CYP1A2, respectively (Dekhuijzen and Koopmans, 2002; Suttle et al., 1997), we examined the possibility of DDIs between zafirlukast and clinical drugs that are metabolized by UGTs.

A series of inhibition studies revealed the following characteristics of zafirlukast as an inhibitor of UGTs: (1) zafirlukast is a general inhibitor of UGTs; (2) zafirlukast more potently inhibits glucuronidation of UGT1A substrates than UGT2B substrates; (3) despite its potency against glucuronidation, zafirlukast is unable to inhibit the glucuronidation of diclofenac. (4) Glucuronidation of SN-38 was inhibited by zafirlukast in a noncompetitive manner with respect to both SN-38 and UDPGA. Because zafirlukast is unlikely to be a UGT substrate
(Savidge et al., 1998), it might bind to a sequence common to UGT enzymes, but not affect UDPGA binding. A molecular docking simulation would provide insights into the characteristics of zafirlukast as a UGT inhibitor.

The zafirlukast $K_i$ values for the hepatic glucuronidation of SN-38, furosemide, etodolac, $S$-naproxen, and diclofenac predicted $R$ value of 5.4, 3.2, 2.2, 1.5, and 1.4, respectively, based on the total (unbound + bound) inhibitor concentration. The $[I]$ value should be the inhibitor concentration at the site of the UGT enzymes, which is impossible to measure. The FDA draft guidance recommends the use of a cutoff $R$ value of 1.1, where the maximum total (unbound + bound) systemic concentration of inhibitor is adopted (FDA draft Guidance for Industry, 2012). Based on this, zafirlukast is thought to interact with these five drugs. However, if the unbound inhibitor concentration was used, no interaction was predicted (i.e., $R$ value < 1.1). Nevertheless, we should note that even at the maximum unbound zafirlukast plasma concentration (0.008 µM), which is considerably lower than its $K_i$ values for CYP2C9 and CYP1A2 (3 µM and 18 µM, respectively), the interaction with the CYP2C9 substrate $S$-warfarin or the CYP1A2 substrate theophylline is clinically significant. Therefore, the interaction is feasible even though the predicted $R$ value is below 1.1.

It is well known that inhibition of intestinal drug-metabolizing enzymes leads to clinical DDIs (Kato, 2008). For example, drinking grapefruit juice with midazolam increased the intestinal availability of midazolam but did not affect hepatic availability, suggesting that grapefruit juice inhibited intestinal CYP3A4 but not hepatic CYP3A4 (Kato, 2008). Our study revealed potential interactions between zafirlukast with furosemide and diclofenac in the intestine due to the $R$ values (192.3 and 35.4). To know the extent of the increase in plasma concentration, more precise prediction methods (e.g., a mechanistic static model or a dynamic model) can be alternatively used, or in vivo evaluation would be required.
During the early stages of drug discovery, the metabolic profile of new chemical entities is examined by enzyme reaction phenotyping studies. The contribution of P450 enzymes ($f_{m,P450}$) versus other metabolic enzymes can be assessed in hepatocytes or liver microsomes using non-selective P450 inhibitors such as SKF-525A and 1-aminobenzotriazole (Emoto et al., 2003, 2010). As for UGT enzymes, non-specific inhibitors are limited to borneol, which depletes UDPGA (Watkins and Klaassen, 1983), valproic acid and probenecid. Although the latter two compounds are described in the FDA draft guidance, their inhibitory effects on glucuronidation in vitro were weak. The $IC_{50}$ and $K_i$ values of valproic acid against zidovudine and lamotrigine glucuronidation in HLM are 538 µM and 2465 µM, respectively (Mano et al., 2007; Rowland et al., 2006). The $IC_{50}$ values of probenecid against zidovudine and acetaminophen glucuronidation are 1600 and 1100 µM, respectively (Kiang et al., 2005). In contrast, our study demonstrated that the $IC_{50}$ values of zafirlukast were 0.7 µM to 26.5 µM in HLM or HIM. Therefore, zafirlukast would be a preferable UGT inhibitor to estimate the fraction of given compounds metabolized by glucuronidation ($f_{m,UGT}$) (Kilford et al, 2009), although it should be noted that zafirlukast appeared to be less potent inhibitor for UGT2Bs than for UGT1As.

In conclusion, we found that zafirlukast is a general inhibitor for UGTs. $R$ values calculated from inhibition studies suggest that co-administration of zafirlukast with drugs such as furosemide and diclofenac may cause clinical DDIs via glucuronidation inhibition in the small intestine. Furthermore, in vitro $IC_{50}$ values indicate that zafirlukast has the potential to be used for evaluating the contribution of UGTs in drug metabolism.
Acknowledgments

We would like to acknowledge Mai Shimizu at Kanazawa University preparing the solutions containing the 578 screened compounds.

Authors’ Contributions

Participated in research design: Oda, Fujiwara, Fukami, Itoh, Nakajima, and Yokoi

Conducted experiments: Oda, Fujiwara, and Kutsuno

Contributed new reagents or analytic tools: none

Performed data analysis: Oda, Fujiwara, and Kutsuno

Wrote or contributed to the writing of the manuscript: Oda, Fujiwara, and Nakajima
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Footnotes

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Figure Legends

Figure 1. Inhibitory effects of zafirlukast on the glucuronidation of (A) 4-MU and (B) diclofenac by recombinant UGTs. 4-MU and diclofenac glucuronidation by recombinant UGTs were determined as described in the Materials and Methods section. 4-MU concentrations are shown in Table 1 and the diclofenac concentration was 30 µM. The control activities for 4-MU glucuronidation by each UGT enzyme were as follows: (1) 642 ± 7 (UGT1A1), (2) 496 ± 3 (UGT1A3), (3) 7390 ± 170 (UGT1A6), (4) 1160 ± 20 (UGT1A8), (5) 2660 ± 10 (UGT1A9), (6) 181 ± 3 (UGT1A10), 1660 ± 20 (UGT2B7), and (8) 364 ± 6 (UGT2B15) pmol/min/mg. The control activities for diclofenac glucuronidation by each UGT enzyme were as follows: (1) 1280 ± 290 (UGT1A3), (2) 127 ± 50 (UGT1A9), (3) 2360 ± 100 (UGT2B7), and (4) 1480 ± 46 (UGT2B17) pmol/min/mg. Each column represents the mean ± the SD of triplicate determinations.

Figure 2. Inhibitory effects of zafirlukast on the glucuronidation of (A) furosemide, (B) etodolac, (C) S-naproxen, and (D) diclofenac in HLM or HIM. The control activities were as follows: (1) furosemide glucuronidation, 212 ± 20 (HLM) and 16.8 ± 0.2 (HIM) pmol/min/mg; (2) etodolac glucuronidation, 105 ± 3 (HLM) pmol/min/mg; (3) S-naproxen glucuronidation, 849 ± 6 (HLM) pmol/min/mg; and (4) diclofenac glucuronidation, 4940 ± 200 (HLM) and 102 ± 1 (HIM) pmol/min/mg. Each data point represents the mean ± the SD of triplicate determinations.

Figure 3. Dixon plot analyses for the inhibition of SN-38 glucuronidation by zafirlukast in (A) HLM and (B) HIM under various concentration of SN-38 and in (C) HLM under various concentration of UDPGA. Each data point represents the mean ± SD of triplicate determinations. K_i values are expressed as the mean ± the SE.
Table 1

Reported $K_m$ or $S_{50}$ values, 4-MU concentrations used in this study, and IC$_{50}$ values of zafirlukast for the inhibition of 4-MU glucuronidation by human UGT isoforms.

<table>
<thead>
<tr>
<th>UGT</th>
<th>$K_m$ or $S_{50}$</th>
<th>4-MU conc. µM</th>
<th>IC$_{50}$ µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGT1A1</td>
<td>113 $^a$</td>
<td>100</td>
<td>0.7 ± 1.1</td>
</tr>
<tr>
<td>UGT1A3</td>
<td>1159 $^b$</td>
<td>1000</td>
<td>6.3 ± 1.0</td>
</tr>
<tr>
<td>UGT1A6</td>
<td>109 $^a$</td>
<td>100</td>
<td>2.0 ± 1.0</td>
</tr>
<tr>
<td>UGT1A8</td>
<td>191 $^b$</td>
<td>200</td>
<td>1.0 ± 1.1</td>
</tr>
<tr>
<td>UGT1A9</td>
<td>8.0 $^b$</td>
<td>10</td>
<td>0.6 ± 1.1</td>
</tr>
<tr>
<td>UGT1A10</td>
<td>31 $^a$</td>
<td>100</td>
<td>3.0 ± 1.0</td>
</tr>
<tr>
<td>UGT2B7</td>
<td>335 $^c$</td>
<td>300</td>
<td>13.1 ± 1.0</td>
</tr>
<tr>
<td>UGT2B15</td>
<td>253 $^b$</td>
<td>300</td>
<td>33.4 ± 1.0</td>
</tr>
</tbody>
</table>

4-MU concentrations were similar to the reported $K_m$ values for each UGT enzyme (Uchaipichat et al., 2004; Fujiwara et al., 2009).

$^a$ Michaelis-Menten; $^b$ Substrate inhibition; $^c$ Hill equation.

Data are the mean ± the SE of triplicate determinations.
Table 2
Reported $K_m$ or $S_50$ values, substrate concentrations used in this study, and IC$_{50}$ values of zafirlukast for the inhibition of furosemide, etodolac, S-naproxen, and diclofenac glucuronidation in HLM and HIM.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ or $S_50$</th>
<th>Responsible UGT isoform(s)</th>
<th>Substrate conc.</th>
<th>IC$_{50}$ HLM</th>
<th>IC$_{50}$ HIM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Furosemide</td>
<td>681 $a,d$</td>
<td>1A1</td>
<td>500</td>
<td>4.9 ± 1.1</td>
<td>2.9 ± 1.1</td>
</tr>
<tr>
<td>Etodolac</td>
<td>483 $b,e$</td>
<td>1A9</td>
<td>400</td>
<td>8.7 ± 1.3</td>
<td>ND</td>
</tr>
<tr>
<td>S-Naproxen</td>
<td>308 $a,e$</td>
<td>2B7</td>
<td>300</td>
<td>23.2 ± 1.1</td>
<td>ND</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>20 $c,e$</td>
<td>1A9, 2B7</td>
<td>30</td>
<td>26.5 ± 1.1</td>
<td>16.1 ± 1.2</td>
</tr>
</tbody>
</table>

Substrate concentrations were similar to the reported $K_m$ or $S_50$ values in HLM.

$^a$ Kutsuno et al., 2013; $^b$ Kutsuno et al., 2014; $^c$ King et al., 2001.

$^d$ Hill equation; $^e$ Michaelis-Menten.

Data are the mean ± the SE of triplicate determinations.
Table 3
Prediction of the DDI potential of zafirlukast with UGT substrates by considering the maximum plasma concentration at the inlet to the liver and the concentrations in the small intestine.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Hepatic interaction</th>
<th>Intestinal interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_i$ (µM)</td>
<td>$1 + I_{in, max} / K_i$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SN-38</td>
<td>1.2</td>
<td>5.4</td>
</tr>
<tr>
<td>Furosemide</td>
<td>2.4 $^a$</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Etodolac</td>
<td>4.4 $^a$</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-Naproxen</td>
<td>11.6 $^a$</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diclofenac</td>
<td>13.2 $^a$</td>
<td>1.4</td>
</tr>
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<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ $K_i$ values were calculated using the following equation: $K_i = 1/2 \times IC_{50}$.
Figure 1

A

B
Figure 2

A. Furosemide

- Residual activity (% of control) vs. Zafirlukast (μM)
- IC$_{50}$ = 2.9 ± 1.1

B. Etodolac

- Residual activity (% of control) vs. Zafirlukast (μM)
- IC$_{50}$ = 8.7 ± 1.3

C. S-Naproxen

- Residual activity (% of control) vs. Zafirlukast (μM)
- IC$_{50}$ = 23.2 ± 1.1

D. Diclofenac

- Residual activity (% of control) vs. Zafirlukast (μM)
- IC$_{50}$ = 26.5 ± 1.1
- IC$_{50}$ = 16.1 ± 1.2