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Characterizing the effect of UDP-glucuronosyltransferase (UGT) 2B7 and UGT1A9 genetic polymorphisms on enantioselective glucuronidation of flurbiprofen

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ABSTRACT

Flurbiprofen (FPF), available commercially as a racemic mixture, is a propionic acid derivative of non-steroidal anti-inflammatory drugs (NSAIDs) with known stereoselective glucuronidation. The major enzyme catalyzing this conjugation reaction is UDP-glucuronosyltransferase (UGT) 2B7, with minor contributions by UGT1A9. This study examines the role of the genetic variants of UGT2B7 and 1A9 enzymes involved in the formation of acyl glucuronides (FPFGs). Variants caused by three single nucleotide polymorphisms (SNPs) (A71S, 211G>T; H268Y, 802C>T; and D398N, 1192G>A) in UGT2B7 and three SNPs (C3Y, 8G>A; M33T, 98T>C; D256N, 766G>A) in UGT1A9 showed activity changes toward different substrates. However the functional impacts of these SNPs on chiral substrates were not examined. Upon stable expression in Bac-to-Bac system, UGT2B7*71S ($A^{71}S$), UGT2B7*2 ($H^{268}Y$) and UGT2B7*5 ($D^{398}N$) were all associated with a decrease in the formation of FPFGs. Compared with UGT2B7*1 (wild-type), UGT2B7*71S exhibited a >2-fold lower intrinsic clearance mainly by altered capacities (V_{max}). Furthermore, a >14-fold decreased intrinsic clearance of the *1 protein was produced by UGT2B7*2 and UGT2B7*5. However, no significantly stereoselective difference for the formation of (R)- and (S)-FPFG was found among these UGT2B7 allozymes. UGT1A9*2 ($C^{3}Y$) exhibited a higher V_{max} (3.2-fold), K_m (2.1-fold) and intrinsic clearance (1.6-fold) toward (S)-FPF than UGT1A9*1 (wild-type). UGT1A9*3 ($M^{33}T$) almost lost the catalytic activity to FPF. A significantly stereoselective difference on the glucuronidation of rac-FPF was seen between the two variants compared with the wild type of UGT1A9.

1. Introduction

Flurbiprofen (FPF), [(±)-(R,S)-2-(2-fluoro-4-biphenyl)propionic acid], is a derivative of 2-arylpropionic acids (2-APA), an important group of nonsteroidal anti-inflammatory drugs (NSAIDs) which exists as a chiral compound with a stereoselective disposition in humans [1,2]. Approximately 60–70% of the administered dose is eliminated as acyl glucuronides of FPF, 4′-hydroxyFPF and 3′-hydroxy-4′-methoxyFPF [2,3]. After oral administration of racemic FPF (100 mg/kg), 8.4 and 7.3% of the dose was excreted into the urine as the acyl glucuronide of (R)- and (S)-FPF, respectively [2], which indicated that glucuronidation constituted some portion of the metabolic pathway of FPF. The major UGT isoforms involved in FPF glucuronidation is UGT2B7 and with minor contributions by UGT1A1, UGT1A3, UGT1A9 and UGT2B4 [4].

Genetic polymorphisms have been reported in virtually every UGT family member [5]. The functional significance and genotype–phenotype correlation of these UGT polymorphisms is an ongoing area of research. Distinct polymorphisms in one major isoform UGT2B7 and one minor isoform UGT1A9 involved in the glucuronidation of FPF are of interest in this study. UGT2B7 polymorphisms include three nonsynonymous coding single nucleotide polymorphisms (SNPs) found in exon 1-211G>T, exon 2-802C>T, and exon 5 1192G>A-which encode the amino acid changes of A71S (UGT2B7*71S), H268Y (UGT2B7*2), and D398N (UGT2B7*5) and yield four UGT2B7 alleles. SNP 211G>T (A71S) was reported in the Japanese with a frequency of 0.185 [6]. Codon 71 is located within the N-terminal (substrate binding) domain, and A71S causes a change from a lipophilic side chain to a hydrophilic one. Although some studies have suggested that the polymorphism of UGT2B7 at codon 268 is probably not associated with altered enzyme activities [7,8], Sawyer et al. [9] reported that homozygous mutant genotype T/T802 (Y/Y268) carriers displayed the strongest catalyzing abilities toward morphine. For SNP 1192G>A (UGT2B7*5), D398 is located in the latter half (UDP–glucuronic acid binding) domain. The alteration from an acidic amino acid (D) to a neutral amino acid (N) might influence the

Abbreviations: RP-HPLC, reverse phase high performance liquid chromatography; UGT, uridine 5-diphosphoglucuronosyl transferases; UDPGA, uridine 5-diphosphoglucuronic acid; FBS, fetal bovine serum; FPF, flurbiprofen; FPFG, flurbiprofen glucuronide; ZPF, zaltoprofen.

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binding of UDP–glucuronic acid. Saeki et al. [10] have found that the variant enzyme with the UGT2B7*398N haplotype has reduced glucuronidation activity compared to the wild-type enzyme (UGT2B7*1) toward 7-hydroxy-4-trifluoromethylcoumarin (50 μM) in vitro. UGT1A9 is composed of a unique first exon and 4 common exons 2–5 [11]. Three nonsynonymous SNPs 8G>A (C3Y, UGT1A9*2), 98T>C (M33T, UGT1A9*3), and 766G>A (D256N, UGT1A9*5) have been reported in exon 1 of this gene (http://galien.pha.ulaval.ca/labocg/alleles/UGT1A/UGT1A9.htm). The functional impact of these three polymorphisms on various UGT1A9 substrates has been studied [12–14].

Racemic FPF is stereoselectivly glucuronidated by UGT isozymes. UGT2B7 formed the (R)-glucuronide at a rate 2.8-fold higher than that for (S)-glucuronide [4]. Since glucuronidation is an essential pathway for the elimination of FPF, genetic polymorphisms of UGTs may be potentially of toxicological and physiological importance on the pharmacokinetics, pharmacologic effects, and toxicity of FPF. To our knowledge, UGT genotype-dependent FPF glucuronidation has not been evaluated to date. In the present study, wild-type and variant human UGT2B7s and UGT1A9s were expressed in Bac-to-Bac baculovirus expression system. FPF was used as substrate to characterize their glucuronidation capacity. Enantioselectivity in FPF glucuronidation by these UGT variants was also observed. Our study adds evidence to the body of work by serving as a foundation for future studies on effects of genetic polymorphisms on NSAIDs.

2. Materials and methods

2.1. Materials

Polymerase chain reaction (PCR) primers were synthesized by Sangon (Shanghai, China). pGEM-T plasmid and pUC18 plasmid were obtained from Promega (Madison, WI). Restriction endonucleases, DNA molecular marker, and T4 ligase were obtained from MBI Fermentas (Amherst, NY). A MutanBest kit was obtained from Takara (Tokyo, Japan). Cellfectin reagent, pFastBac1 vector, DH10Bac-competent cells, and Grace's medium were purchased from Invitrogen (Calsbad, CA). *Spodoptera frugiperda* Sf9 insect cells were obtained from China Center for Type Culture Collection (Wuhan, China). Fetal bovine serum was purchased from Hyclone (Logan, UT). The primary anti-UGT1A antibody (sc-25847), anti-UGT2B antibody (sc-50386) and Peroxidase-conjugated anti-rabbit secondary antibody were all purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The polyvinylidene fluoride (PVDF) membrane was acquired from AMRESCO (Solon, OH).

Rac-FPF (S)-FPF and (R)-FPF (chemical purity >99.0%) was purchased from National Institutes for Food and Drug Control (Beijing, China), zaltoprofen (ZPF, chemical purity >99.0%) was obtained from Yikang Pharm. Co. Ltd. (Tengzhou, Shandong, China). Uridine 5-diphosphoglucuronic acid (UDPGA), alamethicin and β-D-glucuronidase were purchased from Sigma Chemical Co. (St Louis, MO, USA). Chloroform, anhydrous sodium sulfate, trifluoroacetic acid (TFA), ammonium acetate, acetic acid, formic acid, acetonitrile, $MgCl_2$, Tris–HCl and other chemicals and solvents were analytical reagents obtained from Sinopharm Chemical Regent Co. (Beijing, China) or chromatographic grade obtained from Tedia, Co. (Fairfield, OH, USA).

2.2. Construction of allelic variants of UGT2B7s and UGT1A9s

UGT2B7*1, UGT2B7*71S, UGT2B7*2, UGT2B7*5 alleles had been obtained and expressed successfully in Bac-to-Bac system in our laboratory [15]. In this study, using pUC18-UGT1A9*1 as the template, UGT1A9 alleles were generated by site-directed mutagenesis following the instructions of MutanBest kit.

Table 1
Oligonucleotide primers and mutant sites for site-directed mutagenesis using pUC18-UGT1A9*1 as template.

SNPs	Primers
C3Y	Up: 5′-AGCCCCCTTCCTCTATGTGT-3′
	Down: 5′-GGTCCACCCTGTG(*T*)AAGCCAT-3′
M33T	Up: 5′-GTAGTGCCCA(*C*)GGATGGGAGCCACTGGTTCACC-3′
	Down: 5′-CAGTAGCTTCCCTGCCTCGG-3′
D256N	Up: 5′-GTTGTTGCGAACG(*A*)ACTTTGTTTTGGAC-3′
	Down:5′-CAAATTGATGTGTGGCTGTAGAGATCATACTCC-3′

Mutant nucleotides are indicated in italic.

Oligonucleotide primers for introducing nucleotide transversions are shown in Table 1. To ensure the accuracy during the mutagenesis, all PCR products were verified by DNA sequencing.

Expression of UGT1A9s was also performed in Bac-to-Bac system according to the method established by Chen et al. [16]. Briefly, the UGT1A9 gene was subcloned into the pFastBac^{TM}1 vector, and transformed into *Escherichia coli* DH10BacTM. Recombinant bacmid-UGT1A9 was isolated and analyzed by PCR using the M13 forward and M13 reverse primers to confirm the transposition of UGT1A9 into the bacmid. Sf9 cells were usually grown at 27 °C in Grace's medium containing 10% FBS. The minipreparation bacmid-UGT1A9 was transfected into Sf9 cells. High titre baculovirus stocks were used to express UGT2B7 and UGT1A9 enzymes. After 72 h, the first passage of baculovirus was harvested from supernatant and rinsed twice with phosphate-balanced solution (PBS). Infection conditions were optimized for subsequent amplification or expression. Infected cells were collected by centrifugation and disrupted by sonication. Transfection with an untransposed bacmid was performed as a negative control. All the four UGT1A9 enzymes were expressed in the same condition.

2.3. Western blot analyses

The cell homogenates of UGT2B7s and UGT1A9s were subjected to Western blot analysis for determination of relative levels of expressed UGT protein. The same loading amounts of each UGT2B7 and each UGT1A9 supersomes were electrophoresed on 10% SDS–polyacrylamide gels and transferred onto PVDF membranes. The membranes were blocked in 5% fat-free dry milk in TBST wash buffer (20 mM Tris–HCl, pH 7.5, 500 mM NaCl, and 0.05% Tween 20) for 1.5 h at room temperature, and followed by incubation with 1:1000 anti-UGT2B and anti-UGT1A primary antibody overnight. Membranes were washed three times with TTBS, followed by incubation with 1:4000 horseradish peroxidase-conjugated anti-rabbit secondary antibody for 2 h at room temperature and washed three times before detection. The membranes were subsequently developed using ECL (FuJiFilm, Japan) reagent (Applygen Technologies Inc. China) and exposed to film according to the manufacturer's protocol. The relative expressions of UGT2B7s and UGT1A9s were analyzed with Gel-Pro Analyzer. To optimize the loading amount, the linear ranges of Western Quantification were determined by assaying UGT2B7*1 homogenates and UGT1A9*1 (wild-type) at different concentrations.

2.4. Calibration of FPF glucuronide

Because pure standards of the FPF glucuronides (FPFGs) were not commercially available, they were biosynthesized by the method proposed by us (not published). In brief (R)-FPF was incubated with rat hepatic microsomes and UDPGA (final concentration: 5 mM) for 2 h, respectively. The two-fold volume of $CHCl_3$ and 4 mL of 10% formic acid were added. After the mixture

was vortexed, the protein was precipitated by centrifugation and the remaining FPF was extracted with $CHCl_3$. The aqueous fraction was transferred into a clean tube and the two-fold volume of $CHCl_3$ was added again. The procedure was repeated to completely detach the remaining FPF, which was confirmed with HPLC. The aqueous layer after extraction was loaded onto Agilent SampliQ C18 cartridges (500 mg, 3 mL). Each cartridge was then washed with 2 mL of water–TFA (90:10). Glucuronides were eluted with 2 mL of acetonitrile. The final elution solution was evaporated to dryness under N_2 at 37 °C, and the residue was reconstituted with deionized water which was then used as the standard stock solution for the assay of FPFG.

Content of the stock standard solution was determined as follow. A 4 μL aliquot of the FPFG stock solution and 4 μL of the β-glucuronidase solution (20 U/μL) were added into 192 μL of PBS (pH 5.0). After incubation at 37 °C for 24 h, the FPFG was completely hydrolyzed to FPF. Three replicate samples and three control samples without β-glucuronidase were examined. The contents of FPF in hydrolysates were analyzed accurately by HPLC. The calibration standards of FPF were prepared as followed: Working solutions of FPF in the concentration range from 1 to 100 μM and internal standard (zaltoprofen, ZPF, 50 μM) (IS) were prepared by diluting the stock solution with PBS (pH 5.0). Four microliters of β-glucuronidase solution (20 U/μL) was added into 196 μL of the above working solutions. After incubation at 37 °C for 24 h, 40 μL of 50 μM IS and 160 μL of acetonitrile were added to terminate the reaction. The mixture was vortexed for 3 min and centrifuged at 13,000 rpm for 15 min. 20 μL of the supernatant was injected into HPLC system. The peak area of FPF in hydrolytes was compared with that of the reference standard of FPF. The concentration of FPFG stock standard solution was 0.72 mM. FPFG stock solution was found to be stable in water at 4 °C.

2.5. FPF glucuronidation assays

Racemic FPF glucuronidation activities were determined in recombinant UGT2B7s and UGT1A9s. The conditions for linearity with respect to time and protein concentration were optimized in preliminary studies. The incubation mixture (100 μL of total volume) including 100 mM (final concentration) Tris–HCl buffer (pH 7.4), 10 mM (final concentration) $MgCl_2$, enzyme cell homogenate (final concentration, 1 mg/mL protein), 15 μg/mL alamethicin, the substrate FPF dissolved in DMSO was preincubated for 5 min in a shaking water bath at 37 °C. An appropriate volume of the cofactor UDPGA (final concentration, 2 mM) was then added to start the reaction. Concentrations of racemic FPF in the incubation mixtures ranged from 1.0 μM to 401.2 μM for UGT2B7s and ranged from 4.0 μM to 600.9 μM for UGT1A9s. The tubes were incubated for 50 min at 37 °C. The enzymatic reaction was terminated by adding 40 μl of acetonitrile and 10 μl of internal standard (ZPF, 50 μM), followed by centrifugation at 13,000 rpm for 15 min to obtain the supernatant. A 20 μl of the supernatant was analyzed by RP-HPLC with an ultraviolet detector. For each enzyme fraction, appropriate negative control experiments were performed under the same conditions but without UDPGA or substrate. For each concentration, three samples in parallel were determined.

2.6. HPLC analysis

Cell homogenates generated for each of the four UGT2B7 allozymes and the four UGT1A9 allozymes were characterized for catalytic activity toward the substrates racemic and enantiomeric FPF by a sensitive RP-HPLC assay using methods modified from those described by Mano et al. [17]. The HPLC system (HP 1200 series; Agilent Technologies, Palo Alto, CA) consisted of an autoinjector, binary pump, UV absorbance detector set at a wavelength of 240 nm, and a LUNA C_{18} column (4.6 mm × 150 mm, 5 μm particle size; Phenomenex, Torrance, CA, USA) with a 4.0 mm × 3.0 mm Security Guard C_{18} (5 μm) guard column (Phenomenex). The HPLC separation was carried out at 20 °C using a mobile phase consisted of 10 mM, pH 5.0 ammonium acetate in water (solution A) and acetonitrile (solution B). The solvent program consisted of an initial isocratic mobile phase mix (29% solution B) for 8 min, followed by a linear gradient from 29% to 39% solution B over 1 min at a flow rate of 1 ml/min. Analysis was carried out at ambient temperature. The areas of the glucuronide peaks formed were normalized to that of the IS.

The calibration standards of (R)-FPFG in the concentration range from 0.48 to 192.42 μM were prepared by spiking a series of stock solution of (R)-FPFG with blank recombinant cell homogenates incubates respectively. The mixture was mixed with 40 μL of acetonitrile and 10 μL of 50 μM ZPF. After removal of the protein by centrifugation at 13,000 rpm for 15 min, a 20 μL aliquot of supernatant was injected into HPLC for analysis. The analytical method was validated with respect to specificity, accuracy, precision and limit of quantification. Intra-assay variability was determined by analyzing five parallel samples, and inter-assay variability was determined by analyzing samples on five separate days.

2.7. Data analysis for enzyme kinetics

The kinetic constants for racemic FPF by recombinant UGT2B7s and UGT1A9s in the glucuronidation were obtained from the fitted curves using the GraphPad Prism version 5.0 software (GraphPad Software Inc., San Diego, CA). The following equation was applied, assuming a Michaelis–Menten equation: $V = V_{max} \times [S]/(K_m + [S])$, where V is the rate of reaction, V_{max} is the maximum velocity, K_m is the substrate concentration at which the rate of metabolism is 50% of V_{max}, and $[S]$ is the substrate concentration. The two-sided Student's test was used for statistical comparison. A value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Expression of allelic variants of UGT2B7s and UGT1A9s

In the present study, four UGT2B7 sequences and four UGT1A9 sequences were obtained by mutagenesis. To characterize the function of the UGT2B7 variants and the UGT1A9 variants in vitro, these UGT alleles were expressed in Bac-to-Bac insect expression system, which had been applied in our laboratory [16,18,19]. The expression levels of UGT2B7 protein in the four allozymes and UGT1A9 protein in the four allozymes were determined via immunoblotting techniques, and a representative Western blot is shown in Fig. 1. As shown in Fig. 1, expression level of UGT2B7 variants (A) and UGT1A9 variants (B) was 1.277–2.199 times and 2.047–2.660 times as many as that of the wild type, respectively. The protein expression levels relative to the corresponding UGT*1 allele derived from western blots were used for the normalization of glucuronidation activity.

3.2. Validation of analytical method

In this study, a modified RP-HPLC method was developed to determine (R)-FPFG and (S)-FPFG simultaneously. To determine the concentration of the stock solution of (R)-FPFG, the calibration curve of FPF was constructed by plotting the peak area ratios (y) of the FPF to the IS against the concentration (x) of FPF firstly. The standard curve was linear between 0.5 and 100 μM, and the regression equation of the calibration curve of FPF was

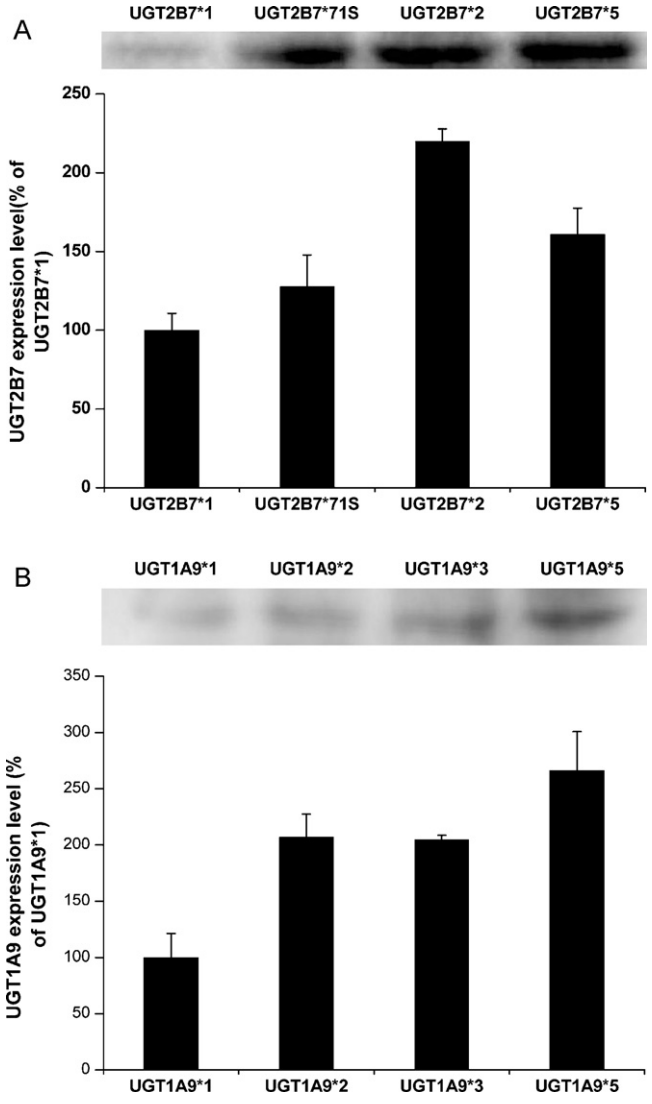

Fig. 1. Western blots of UGT2B7 recombinant enzymes (A) and UGT 1A9 recombinant enzymes (B) expressed in Sf9 cells. The results are expressed as a relative percentage compared with UGT2B7*1 and UGT1A9*1, respectively. Each bar represents the scanning analysis result of the expression level of the above UGT enzymes. The results are indicated as mean ± S.D. of three independent experiments.

$y = 0.0137x - 0.018$ ($r^2 = 0.9998$, $n = 5$). Typical chromatograms of the incubation mixture are shown in Fig. 2 without interferential peaks being found in the position of (R)-FPFG or (S)-FPFG. The calibration curve of (R)-FPFG was constructed by plotting the peak

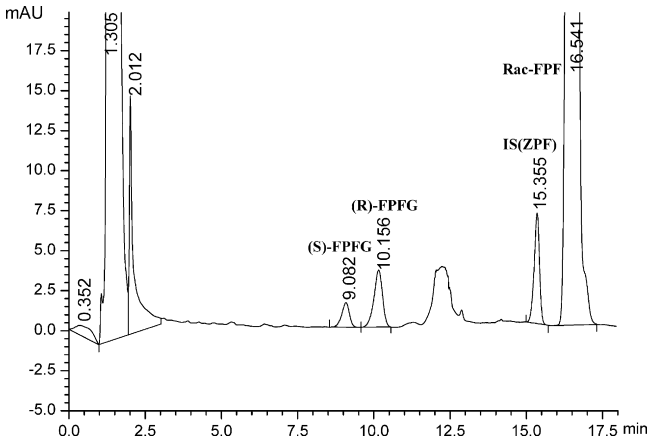

Fig. 2. Representative chromatogram of HPLC analysis of the (S)- and (R)-ZPFG formation from rac-FPF in recombinant enzymes.

area ratios (y) of the (+)- and (−)-ZPFG to the IS against the concentration (x) of (R)-FPFG. The regression equations of the calibration curve of (R)-ZPFG was $y = 0.0248x - 0.0047$ ($r^2 = 0.9996$, $n = 5$). The limit of detection (LOD) of FPFG was 0.08 μM and the limit of quantitation (LOQ) of FPFG was established at 0.48 μM (RSD < 9%, $n = 5$). The accuracy of (R)-FPFG was in the range from 91.6% to 105.6% at three different concentrations (data not shown). The relative standard deviation of the intra and inter day repeatability were all less than 10.8% at three different concentrations (data not shown). Thus, the assay method we modified was simple, sensitive, and direct for the determinations of (R)-FPFG and (S)-FPFG. The experimental results indicated that the UV spectra for (R)-FPFG and (S)-FPFG were identical at the same concentration. So, the concentration of (S)-FPFG produced in the incubation also can be determined according to the established calibration standard curve of (R)-FPFG.

3.3. Glucuronidation of rac-FPF by UGT2B7s and UGT1A9s

UGT2B7 and UGT1A9 both catalyzed the glucuronidation of both (R)-FPF and (S)-FPF, with UGT2B7 having the higher activity [4]. To conduct pharmacogenetic studies on rac-FPF, the glucuronidation velocities of UGT2B7s and UGT1A9s were normalized with UGT2B7*1 and UGT1A9*1, respectively. As shown in Figs. 3 and 4, the formation of (R)-FPFG and (S)-FPFG from racemic FPF yielded Michaelis–Menten kinetics for both UGT2B7s and UGT1A9s. The corresponding enzyme kinetic parameters are listed in Tables 2 and 3. Glucuronidation activities of 100, 42, 6% to (S)-FPF and 100, 39, 7% to (R)-FPF were observed for UGT2B7*1, UGT2B7*71S and UGT2B7*2 when Rac-FPF was used as the

Table 2
Enzymatic kinetic parameters of recombinant UGT2B7 enzymes to rac-FPF.

FPFG	UGT2B7*1		UGT2B7*71S		UGT2B7*2	
	S-FPFG	R-FPFG	S-FPFG	R-FPFG	S-FPFG	R-FPFG
K_m (μM)	50.21 ± 3.413	34.55 ± 1.680[a]	52.45 ± 5.482	47.73 ± 2.769[*]	46.64 ± 2.711	38.46 ± 3.708
V_{max} (pmol min^{-1} mg^{-1})	119.7 ± 2.510	169.8 ± 2.306[a]	52.12±2.181[*]	85.51 ± 4.413[a*]	6.75 ± 0.258[*]	12.10 ± 0.726[a*]
V_{max}/K_m (μL min^{-1} mg^{-1} protein)	2.39 ± 0.237	4.92 ± 0.701[a]	1.00±0.119[*]	1.80 ± 0.338[a*]	0.15 ± 0.001[*]	0.32 ± 0.003[a*]
Percentage of UGT2B7*1 (%)	100	100	42	39	6	7
R/S	2.1		1.8		2.2	
R^2	0.9906	0.9919	0.9807	0.9870	0.9915	0.9742

Assay were performed for 60 min at 37 °C in the presence of 5 mM UDPGA and increasing concentration of rac-FPF (1–400 μM) in 100 mM Tris–HCl buffer (pH 7.4). Data are mean ± SD of three independent determinations. The V_{max} values were normalized with the relative UGT expression levels.
R/S ratio of the enzymatic efficacies of (R)-FPF to (S)-FPF.
[a] Values significantly different from the corresponding value of the enantiomorph ($P < 0.01$).
[*] Values significantly different from the corresponding value of UGT2B7*1 ($p < 0.01$).

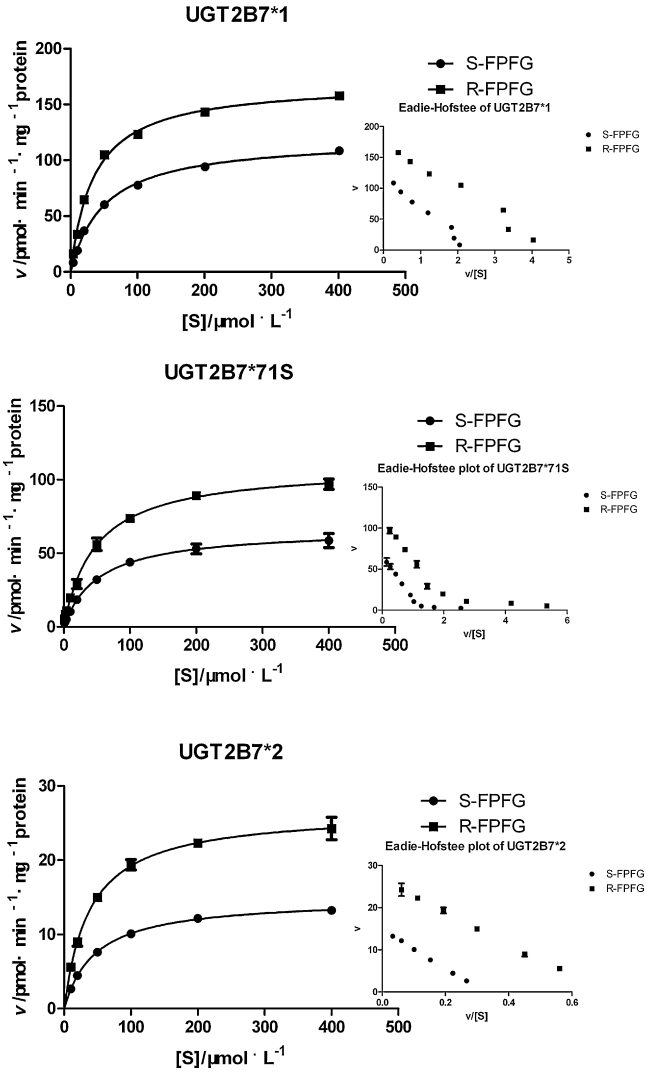

Fig. 3. Kinetics for the formation of (S)- and (R)-flurbiprofen glucuronide by incubation of racemic flurbiprofen with the recombinant UGT2B7s. Racemic flurbiprofen (1–400 μM) was incubated with protein concentrations of 1.0 mg of protein/mL for 60 min. Data are depicted as mean ± S.E.M., $n = 3$. The Eadie-Hofstee plot is presented in the inset.

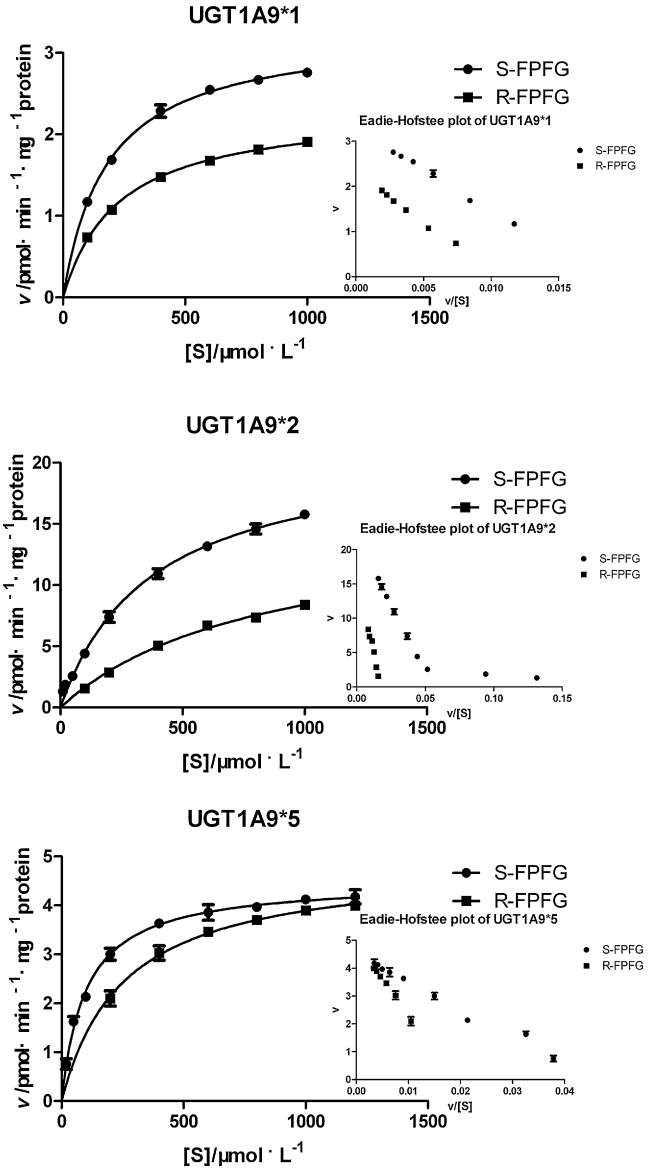

Fig. 4. Kinetics for the formation of (S)- and (R)-flurbiprofen glucuronide by incubation of racemic flurbiprofen with the recombinant UGT1A9s. Racemic flurbiprofen (10–1200 μM) was incubated with protein concentrations of 1.0 mg of protein/mL for 60 min. Data are depicted as mean ± S.E.M., $n = 3$. The Eadie-Hofstee plot is presented in the inset.

substrate. For UGT2B7*5, the glucuronidation velocity to Rac-FPF is even less than 5 pmol min^{-1} mg^{-1} (data not shown). For (S)-FPFG formation by UGT2B7*1, V_{max} was 119.7 ± 2.5 pmol min^{-1} mg^{-1}, and for (R)-FPFG formation, V_{max} was 169.8 ± 2.3 pmol min^{-1} mg^{-1} (Table 2). Thus, the relative intrinsic clearance (V_{max}/K_m) of (R)-FPFG formation (4.92 μL min^{-1} mg^{-1} protein) was 2.1-fold higher than that of (S)-FPFG formation (2.39 μL min^{-1} mg^{-1} protein). UGT2B7*2 and UGT2B7*5 led to a considerable decrease of glucuronidation, whereas UGT2B7*71S shows a relatively mild decrease of glucuronidation for Rac-FPF. There were no significant differences among the UGT2B7 allozymes on the values of R/S ratio.

UGT1A9 and UGT2B7 present opposite stereoselectivity. The four UGT2B7 allozymes are all predominantly active toward R-enantiomer. The four UGT1A9 allozymes are all predominantly active toward S-enantiomer. For UGT1A9*1, the (S)- and (R)-glucuronidation velocity values were 3.286 ± 0.056 pmol min^{-1} mg^{-1} and 2.324 ± 0.045, respectively. The relative intrinsic clearance (V_{max}/K_m) values were 18 and 10 (μL min^{-1} mg^{-1} protein × 1000) for the formation of (S)- and (R)-glucuronide, respectively. As shown in Table 3, Glucuronidation activities of 100, 155, 94% to (S)-FPF and 100, 90, 70% to (R)-FPF were observed for UGT1A9*1, UGT1A9*2 and UGT1A9*5. The glucuronidation of UGT1A9*3 to Rac-FPF was not detectable under the determined conditions. Especially, for the formation of (S)-FPFG, UGT1A9*2 improved the function in glucuronidation of rac-FPF, with a relative intrinsic clearance (V_{max}/K_m) approximately 1.6 times that of UGT1A9*1. UGT1A9*5 showed only a slight decrease in glucuronidation efficiency. The respective ratios of (R)-glucuronide to (S)-glucuronide were 0.6, 0.3 and 0.4 for UGT1A9*1, UGT1A9*2, and UGT1A9*5.

4. Discussion

Glucuronidation is a very important metabolic pathway of FPF. UGT2B7 has been identified as the main enzyme involved in the FPF glucuronidation, and the recombinant human UGT1A9 also shows slight glucuronidation activity [4]. Some UGT2B7 variants and UGT1A9 variants show significantly different activities in many drug metabolisms. However, their activities toward chiral

Table 3
Enzymatic kinetic parameters of recombinant UGT1A9 enzymes to rac-FPF.

FPFG	UGT1A9*1		UGT1A9*2		UGT1A9*5	
	S-FPFG	R-FPFG	S-FPFG	R-FPFG	S-FPFG	R-FPFG
K_m (μM)	182.2 ± 10.83	226.5 ± 13.94[a]	373.8 ± 31.27[*]	827.5 ± 82.91[b*]	99.64 ± 6.525[*]	254.4 ± 25.64[b*]
V_{max} (pmol min^{-1} mg^{-1})	3.286 ± 0.056	2.324 ± 0.045[b]	10.37 ± 0.725[*]	7.414 ± 0.847[b*]	1.693 ± 0.067[*]	1.835 ± 0.141[a*]
V_{max}/K_m (μL min^{-1} mg^{-1} protein × 1000)	18 ± 1.021	10 ± 0.633[b]	28 ± 1.336[*]	9 ± 0.023[b]	17 ± 0.901	7 ± 0.315[b]
Percentage of UGT1A9*1 (%)	100	100	155	90	94	70
R/S	0.6		0.3		0.4	
R^2	0.9863	0.9874	0.9904	0.9907	0.9835	0.9538

Assay were performed for 60 min at 37 °C in the presence of 5 mM UDPGA and increasing concentration of rac-FPF (10–1000 μM) in 100 mM Tris–HCl buffer (pH 7.4). Data are mean ± SD of three independent determinations. The V_{max} values were normalized with the relative UGT expression levels.
R/S ratio of the enzymatic efficacies of (R)-FPF to (S)-FPF.
[a] Values significantly different from the corresponding value of the enantiomorph ($P < 0.05$).
[b] Values significantly different from the corresponding value of the enantiomorph ($P < 0.01$).
[*] Values significantly different from the corresponding value of UGT1A9*1 ($p < 0.01$).

NSAIDs and the possible effects on the stereoselectivity have not been determined. In the present study, flurbiprofen was used as the substrate for glucuronidation to increase the understanding of UGT2B7 and UGT1A9 variants.

Statistically significant differences in catalytic activities of UGT2B7s to FPF were observed in the present study, which are inconsistent as observations reported for mycophenolic acid [20] and morphine metabolism [9]. The UGT2B7 variants clearly showed different alteration in activity, depending on the substrates. UGT2B7*71S and UGT2B7*2 exhibited a decreased activity in the metabolism of FPF to approximately 50% and less than 10%, respectively. For UGT2B7*5, the formation of FPFGs was almost undetectable under the determined conditions, indicating a missing glucuronidation activity, which provided further evidence that the alteration from an acidic amino acid (D) to a neutral amino acid (N) in the C-terminal domain would directly influence the binding of UDP–glucuronic acid. It is generally accepted that the C-terminal domain conserved in UGT1 and UGT2 families is responsible for UDPGA binding, whereas the N-terminal domain is responsible for the substrate binding [21]. Codon 71 and 268 are all located within the substrate binding domain. These results indicate that amino acid residues at positions 71 and 268 may be in a key region of UGT2B7 protein. The formation of FPFGs by UGT1A9*3 (M33T) was drastically decreased almost to be undetectable. Although the values of K_m and V_{max} of UGT1A9*2 and UGT1A9*5 allozymes were significantly different from those of UGT1A9*1, the differences in the resulting relative efficiencies did not reach statistical significance.

Previous researches reported that some UGT enzymes including UGT1A9, UGT1A10, UGT2B7, UGT2B15, and UGT2B17 stereoselectively catalyzed glucuronidation [22–26]. In the present study, we characterized the effect of human UGT2B7 and UGT1A9 polymorphisms on the stereoselective glucuronidation using racemic FPF. We found that UGT2B7s dominantly formed (R)-FPF glucuronide, but UGT1A9s all dominantly formed (S)-FPF glucuronide. There was no significant difference on the values of R/S ratio of relative efficiencies (V_{max}/K_m) among the UGT2B7 allozymes, which indicated minimal effect on the stereoselective glucuronidation. However, compared with the UGT1A9*1, the relative intrinsic clearance of the UGT1A9*2 allele to (S)-FPF was almost 1.6-fold higher than that of the UGT1A9*1 protein and similarly intrinsic clearance to (R)-FPF, resulting in a more significant difference in the stereoselective glucuronidation. For UGT1A9 allozymes, the ratio of (R)-glucuronide to (S)-glucuronide was 0.6, 0.3 and 0.4 for UGT1A9*1, UGT1A9*2, and UGT1A9*5, respectively. The two variants all exhibited stronger stereoselectivity to Rac-FPF compared with the wild-type.

Concerning stereoselectivity, the question raised whether the preference for the (R)- and (S)-enantiomers is determined by the binding affinity of substrates or by the rate of transfer of glucuronic acid to the already-bound substrate. Kinetic analyses for UGT2B7s activities revealed that the K_m values for the two enantiomers differed much less than the corresponding V_{max} values (Table 2). On this basis, it appears that the differences in the rate of glucuronic acid transfer to the aglycone are the major determinant of the stereoselectivity [22]. In the case of UGT1A9s, both differences in the affinity of the enzyme for FPF enantiomers and in the rate of glucuronic acid transfer to the aglycone may be involved (Table 3). We are interested in the more significantly stereoselective glucuronidation produced by the variants of UGT1A9. Because of the minor contribution of UGT1A9 to FPF, further studies on other chiral substrates will be worth further examination.

In conclusion, comparisons of the variants and the wild type yielded significant differences in catalytic activities for UGT2B7 and UGT1A9 to FPF for the first time, respectively. Furthermore, it was demonstrated that polymorphisms of UGT1A9 may affect the stereoselectivie glucuronidation to FPF. The clinical relevances of these genetic variants of UGT2B7 and UGT1A9 remain to be assessed.

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