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Influence of *CYP2C8* polymorphisms on the hydroxylation metabolism of paclitaxel, repaglinide and ibuprofen enantiomers *in vitro*

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ABSTRACT: CYP2C8 plays an important role in the metabolism of various drugs, such as paclitaxel, repaglinide and ibuprofen. Polymorphisms in the *CYP2C8* gene were shown to influence interindividual differences in the pharmacokinetics of paclitaxel, repaglinide and ibuprofen enantiomers. In this study, three CYP2C8 allelic variants (CYP2C8.2, CYP2C8.3 and CYP2C8.4) and wild-type CYP2C8 (CYP2C8.1) were co-expressed for the first time with human cytochrome P450 oxidoreductase (POR) and cytochrome *b5* by using a baculovirus-assisted insect cell expression system. Further, the effects of genotype–phenotype correlations of *CYP2C8* alleles on the metabolism of paclitaxel, repaglinide and ibuprofen enantiomers were evaluated. The CL_{int} values of CYP2C8.2, CYP2C8.3 and CYP2C8.4 for paclitaxel were 47.7%, 64.3% and 30.2% of that of CYP2C8.1 ($p < 0.01$). The CL_{int} values of CYP2C8.2 and CYP2C8.4 for repaglinide were 77.9% and 80.2% of that of CYP2C8.1 ($p < 0.05$), respectively, while the CL_{int} value of CYP2C8.3 was 1.31-fold higher than that of CYP2C8.1 ($p < 0.05$). The relative CL_{int} values of CYP2C8.2, CYP2C8.3 and CYP2C8.4 were 110.5%, 72.3% and 49.7% of that of CYP2C8.1 and were 124.6%, 83.4% and 47.4% of that of CYP2C8.1 for *R*-ibuprofen and *S*-ibuprofen, respectively. Comparing hydroxylation by CYP2C8.1 and CYP2C8.3 resulted in higher and lower intrinsic clearance of repaglinide and ibuprofen enantiomers, respectively. These *in vitro* findings were consistent with the pharmacokinetics in volunteers who were heterozygous or homozygous carriers of *CYP2C8*3*. The results of this study provide useful information for predicting CYP2C8 phenotypes and may contribute to individualized drug therapy in the future. Copyright © 2013 John Wiley & Sons, Ltd.

Key words: CYP2C8; genetic polymorphisms; paclitaxel; repaglinde; ibuprofen enantiomers

Introduction

Cytochrome P450 2C8 (CYP2C8) comprises 7% of the total hepatic CYP content and plays an important role in the metabolism of many exogenous and endogenous compounds [1]. CYP2C8 is mainly expressed in the liver but is also present in the gastrointestinal tract, kidney, adrenal gland and tonsils.

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Fourteen *CYP2C8* alleles, from *CYP2C8*2* to *CYP2C8*14*, have been identified to date. *CYP2C8*2*, *CYP2C8*3* and *CYP2C8*4* are the three most common alleles. CYP2C8 and some of its variants have been recombinantly expressed using *Escherichia coli* (2C8.1 (R139K), 2C8.1 (K399R), 2C8.3 and 2C8.4) [2] and yeast expression systems (CYP2C8.2, 2C8.3 and 2C8.4) [3]. Baculovirus-assisted insect cell expression system is also used widely and offers many advantages, such as eukaryotic post-translational modifications without complication [4], proper protein folding and S–S bond formation, and high expression levels [5].

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Cytochrome P450 2C8 is involved in the metabolism of many clinically available drugs, such as paclitaxel, repaglinide and ibuprofen [6]. Paclitaxel is a probe drug for measuring CYP2C8 activity *in vitro*; however, the fraction of paclitaxel eliminated by CYP2C8 was estimated to be only 36% [7]. Clinical studies have reported no association between *CYP2C8* polymorphisms and paclitaxel pharmacokinetics in breast cancer patients [8]. Repaglinide, an insulin secretagogue used in the treatment of type 2 diabetes, is almost metabolized by CYP2C8 and CYP3A4 which have a comparable *in vitro* contribution [9]. When a single dose of repaglinide was administered to healthy volunteers, the mean area under the plasma concentration–time curve (*AUC*) and the maximum plasma concentration (C_{max}) were both lower in the subjects with the *CYP2C8*1/*3* genotype than in the wild-type homozygotes [10–12]. However, the effect of genotype–phenotype correlation of *CYP2C8* polymorphisms on repaglinide metabolism has not been evaluated *in vitro*.

Although it is known that *CYP2C8* genetic polymorphisms are associated with variable disposition of certain drugs, their influence on the metabolism of chiral drugs and the possible effects on the stereoselectivity has not been determined *in vitro*. To better understand the stereoselective metabolism by CYP2C8 variants, ibuprofen enantiomers were used as substrates in this study. Ibuprofen, a widely used chiral nonsteroidal anti-inflammatory drug (NSAID), is clinically administered in the racemic form. *R*-Ibuprofen is converted unidirectionally to *S*-ibuprofen *in vivo*, and mainly the *S*-enantiomer contributes to the pharmacological activity [13]. Ibuprofen is stereoselectively metabolized to hydroxylated metabolites by CYP2Cs, and CYP2C8 plays a role in *R*-ibuprofen (< 10%) and *S*-ibuprofen (< 13%) clearance [14]. The presence of *CYP2C8* alleles may influence the pharmacokinetics of *R*-ibuprofen, which can be converted to *S*-ibuprofen, resulting in different dispositions of both enantiomers. To our knowledge, the effect of *CYP2C8* polymorphisms on the stereoselective hydroxylation of ibuprofen enantiomers has not been investigated to date. Meanwhile, data regarding the association between *CYP2C8* polymorphisms and ibuprofen pharmacokinetics are conflicting and inconclusive [15]. Potential explanations for the discrepancies include inherent factors in *in vivo* research, such as a correlation between *CYP2C8*3* and *CYP2C9*2* polymorphisms, differences in the extent of conversion from *R*-ibuprofen to *S*-ibuprofen *in vivo*, and few *CYP2C8*3* homozygotes. Considering this, the construction of the recombinant CYP2C8 variants *in vitro* may be useful for estimating the functional consequence of genetic variations in the *CYP2C8* gene on ibuprofen metabolism.

In the present study, wild-type CYP2C8 and its three variants, CYP2C8.2, CYP2C8.3 and CYP2C8.4, were expressed in the baculovirus-assisted insect cell expression system to evaluate the impact of *CYP2C8* genetic polymorphisms on the metabolism of repaglinide and ibuprofen. Paclitaxel 6α-hydroxylation was used as the marker reaction. Stereoselective hydroxylation of ibuprofen by CYP2C8 variants was also investigated.

Materials and Methods

Materials

Polymerase chain reaction (PCR) primers were synthesized by Sangon (Shanghai, China). The pMD19-T vector was purchased from TaKaRa Biotechnology Co. (Dalian, China). The donor plasmid pFastBac^{TM}1 vector, DH10 Bac^{TM} competent cells and TRIzol® reagent were purchased from Invitrogen (Carlsbad, CA, USA). The primary anti-histidine antibody was purchased from Merck (Darmstadt, Germany). Paclitaxel, nateglinide, 7-hydroxycoumarin (chemical purity > 99.0%) were purchased from the National Institutes for Food and Drug Control (Beijing, China). *R*-Ibuprofen and *S*-ibuprofen were obtained from Biomol Research Laboratories Inc. (Plymouth Meeting, PA, USA). 4-Androstene-3,17-dione, repaglinide and 6α-hydroxy paclitaxel were purchased from Sigma Chemical Co. (St Louis, MO, USA). All other solvents and chemicals were obtained from commercial sources.

Construction of human CYP2C8 variant cDNAs

Human liver total RNA was obtained from human liver tissue by TRIzol reagent. This RNA was utilized as a template to obtain *CYP2C8*1* cDNA by reverse transcription-PCR using the forward primer 5′-CCCAAGCTTATGGAACCTTTT

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GTGGTCCTG-3′ and the reverse primer 5′-CCGCTCGAGTCAATGATGATGATGATGATG GACAGGGATGAAGCAGATC-3′. cDNA encoding *CYP2C8*1* tagged with histidine (His6) was subcloned to pMD19-T vector. Using pMD19-T/*CYP2C8*1* vector as the template, *CYP2C8* alleles were generated by site-directed mutagenesis PCR with the following 5′-phosphorylated primers: 5′-TATCGATTGCTTCCTG**t**TCAAAATGGAGCA GGA-3′ for *CYP2C8*2*, 5′-TTGGGATGGGGA AGA**a**GAGCATTGAGGACCG-3′ and 5′-GTG CTACATGATGACA**g**AGAATTTCCTAATCCAA A-3′ for *CYP2C8*3*, 5′-ATCCTCGGGACTTTA T**g**GATTGCTTCCTGATCA-3′ for *CYP2C8*4*. The sites of the mutated bases are shown in lowercase bold. To ensure accuracy during the mutagenesis, all PCR products were verified by DNA sequencing.

Recombinant expression of CYP2C8 and its variants in insect cells (Sf9 cells)

Recombinant expression of CYP2C8 and its variants was performed in the Bac-to-Bac® baculovirus expression system using the modified procedure described by Chen *et al*. [16]. The Sf9 cells infected with recombinant baculovirus were harvested after 96 h. The cells were lysed in lysis buffer (20% glycerol, 0.1 M K_3PO_4, 1 mM EDTA and 0.1 mM PMSF, pH 7.4) by ultrasonication for 30 min. The whole-cell lysate (S9) was prepared by centrifugation at $9000 \times g$ for 10 min at 4 °C, and then stored at −80 °C until analysis. The protein concentrations were measured by the Bradford method. The functional P450 content was recorded by the reduced CO difference spectral method described by Gonzalez *et al*. [17].

Western blot analysis

Total CYP2C8 protein levels in the Sf9 whole-cell lysate were determined by western blot analysis. S9 (25 mg/lane) was separated by 8% sodium dodecyl sulfate–polyacrylamide gels and transferred onto a polyvinylidene difluoride membrane (Bio-Rad, Mississauga, ON, Canada). The membranes were incubated overnight at 4 °C with monoclonal antibody against histidine as the primary antibody (1:2000 dilution), and then with a secondary antibody (goat anti-mouse IgG-HRP, 1:5000 dilution) for 1 h at room temperature. Immunoreactive proteins were visualized with enhanced chemiluminescence-plus (ECL) reagents (Amersham Biosciences, USA). The relative expressions of CYP2C8s were analysed using Quantity One analysis software (Bio-Rad).

Enzymatic assay of CYP2C8 toward paclitaxel, regaglinide and ibuprofen enantiomers

Kinetic parameters for the hydroxylation of paclitaxel, repaglinide and ibuprofen enantiomers were determined in recombinant CYP2C8.1, CYP2C8.2, CYP2C8.3 and CYP2C8.4. The incubation mixture contained the S9 expressing CYP2C8s (0.25–1.0 mg/ml), the substrate as paclitaxel (1–30 μM) or repaglinide (0.5–30 μM) or ibuprofen enantiomers (16–2000 μM), $MgCl_2$ (15 mM) and NADPH generating system made up to a final volume (100 μl) with phosphate buffer solution (0.1 M, pH 7.4). Each sample was pre-incubated for 5 min at 37 °C, initiated by the addition of NADPH/$NADP^+$ and terminated with 400 μl of internal standard in ice-cold methanol. Internal standards for paclitaxel, repaglinide and ibuprofen enantiomers were 4-androstene-3,17-dione, nateglinide and 7-hydroxycoumarin, respectively. The reaction mixture was then centrifuged at $16000 \times g$ for 20 min. 5 μl of the supernatant was directly analysed by ultra high performance liquid chromatography/tandem mass spectrometry (UPLC-MS).

Analytical methods

Quantification of 6α-hydroxy paclitaxel was carried out by an Agilent 1200 series HPLC system (Agilent Technologies, Palo Alto, CA, USA). Separation was performed on a Diamonsil™ C18 column (4.6 mm 200 mm, 5 μm) (Dikma Technologies, Beijing, China) with methanol–water as the mobile phase. The elution was performed with a linear gradient from 60% to 70% methanol (v/v) in 20 min at a flow rate of 1.0 ml/min. The detection wavelength was set at 230 nm.

A Waters UPLC-TQD system (Waters Acquity, Waters, Milford, MA) was used to establish an LC-MS/MS method for quantitation of the hydroxy metabolites of repaglinide and ibuprofen. Chromatographic separation of 3′-hydroxy repaglinide and 2-hydroxy ibuprofen was achieved on a Hypersil C18 column (50 mm × 2.1 mm, 3 μm) (Elite, Dalian, China) and a Waters Acquity UPLC™ HSS T3 column (100 mm × 2.1 mm, 1.8 μm) (Waters), respectively. Data were acquired by using Masslynx software (version 4.1, Waters) in the multiple

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reaction monitoring mode for the following transitions: 469.2 *m/z* to 246.3 *m/z* for 3′-hydroxy repaglinide, 318.1 *m/z* to 166.0 *m/z* for nateglinide, 221.5 *m/z* to 177.2 *m/z* for 2-hydroxy ibuprofen, and 161 *m/z* to 133 *m/z* for 7-hydroxycoumarin. 3′-Hydroxy repaglinide and 2-hydroxy ibuprofen concentrations were given in arbitrary units (AU) relative to the peak area ratio of each metabolite to that of the internal standard in the chromatogram [10].

Data analysis

The kinetic constants, K_m and V_{max}, except for the metabolism of ibuprofen by CYP2C8.4 were estimated using nonlinear regression analysis of Michaelis-Menten model by GraphPad Prism version 5.0 software (GraphPad Software Inc., San Diego, CA). The kinetic constants of ibuprofen catalysed by CYP2C8.4 were estimated using the Hill equation as follows:

$$v = \frac{V_{max} \times S^n}{S_{50}{}^n + S^n}$$

where the substrate concentration resulting in 50% of V_{max} (S_{50}) is analogous to the K_m parameter, and n is the Hill coefficient. Intrinsic clearance (CL_{int}) values were determined as the ratio of V_{max}/K_m. All values are expressed as the mean ± SD of three separate experiments derived from independent preparations. The two-sided Student's *t*-test was used for statistical comparison. A probability value of less than 0.05 ($p < 0.05$) was accepted as a significant difference.

Results

Expression of recombinant CYP2C8s in Sf9 cells

CYP2C8.1, CYP2C8.2, CYP2C8.3 and CYP2C8.4 were heterologously co-expressed with human cytochrome P450 oxidoreductase (POR) and cytochrome *b5* in a Bac-to-Bac insect expression system. The co-expression conditions were optimized to have a ratio of 3:3:1 (CYPs:POR:*b5*) in the preliminary experiment. The expression levels of functional P450 content and CYP2C8 holoproteins in Sf9 cells were examined using the reduced carbon monoxide (CO) difference spectra and western blot analysis, respectively. The CO difference spectrum of recombinant CYP2C8s showed a Soret peak at approximately 450 nm (Figure 1). The average P450 content of recombinant CYP2C8.1, CYP2C8.2, CYP2C8.3 and CYP2C8.4 was 35.6 ± 2.1, 30.82 ± 1.5, 31.72 ± 1.6 and 25.40 ± 1.9 pmol/mg protein, respectively.

Western blot results showed a single band at approximately 55 kDa, consistent with the previously reported molecular mass of CYP2C8 [18] (Figure 2A). Negligible levels of non-specific proteins were detected in the negative control group. As shown in Figure 2B, no significant difference was observed between the protein expression level of CYP2C8.1 and that of the three CYP2C8 variants, indicating that all CYP2C8 proteins were expressed uniformly.

Enzymatic activities of CYP2C8s toward paclitaxel

Paclitaxel is the preferred substrate for CYP2C8 *in vitro*, and its 6α-hydroxylation has been accepted widely as a marker reaction [18]. Non-linear regression curves of Michaelis-Menten kinetics are shown in Figure 3A and the kinetic parameters are summarized in Table 1. All CYP2C8s exhibited typical Michaelis-Menten behaviour. CYP2C8.3 had a 1.9-fold higher K_m value than that of CYP2C8.1 ($p < 0.01$) and a slightly higher V_{max} value than that of CYP2C8.1. CYP2C8.2 and CYP2C8.4 had slightly higher K_m values than that of CYP2C8.1 and significantly lower V_{max} values than that of CYP2C8.1 ($p < 0.01$). The CL_{int} values of CYP2C8.2, CYP2C8.3 and CYP2C8.4 were 47.7%, 64.3% and 30.2% of that of CYP2C8.1 ($p < 0.01$), respectively.

Enzymatic activities of CYP2C8s toward repaglinide

3′-Hydroxy repaglinide is the major metabolite of repaglinide formed after metabolism by CYP2C8 *in vitro*. The Michaelis-Menten plots for repaglinide hydroxylation by recombinant CYP2C8 variants are shown in Figure 3B and the K_m values and the V_{max} and CL_{int} values in arbitrary units are given in Table 2. The K_m values of CYP2C8.2 and CYP2C8.4 were comparable to that of CYP2C8.1, while the V_{max} values were significantly lower than that of CYP2C8.1, resulting in CL_{int} values of CYP2C8.2 and CYP2C8.4 that were 77.9% and 80.2% of that of CYP2C8.1 ($p < 0.05$), respectively.

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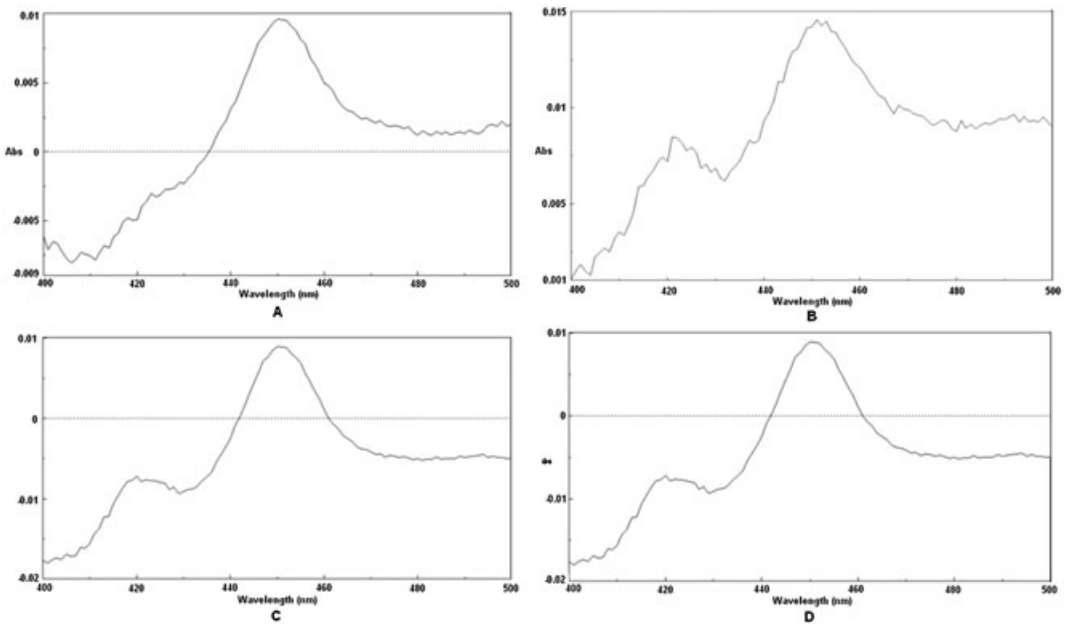

Figure 1. Carbon monoxide difference spectra of recombinant proteins. (A) CYP2C8.1; (B) CYP2C8.2; (C) CYP2C8.3; (D) CYP2C8.4

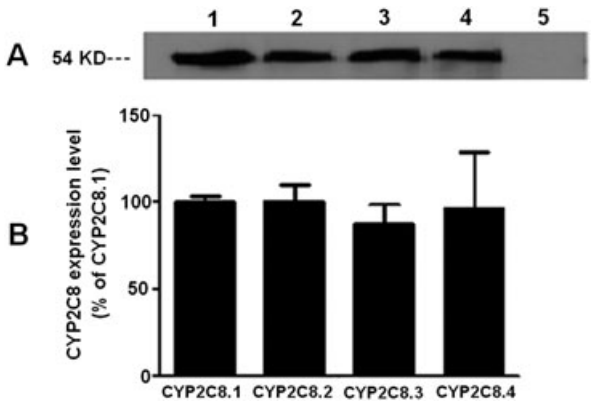

Figure 2. Western blot analysis of recombinant protein expression in sf9 insect cells after infection. (A) Lane 1/2/3/4, Sf9 cell lysates co-infected with v-*CYP2C8**1/*2/*3/*4, v-POR and v-Cyt b5; lane 5, Sf9 cell lysates co-infected with v-POR and v-Cyt b5 as control. (B) The results were expressed as a relative percentage compared with CYP2C8.1. Each bar represents the scanning analysis result of the expression level of the above CYP enzymes. The results are indicated as mean ± SD of three independent experiments

CYP2C8.3 and CYP2C8.1 had significantly different K_m ($p < 0.01$), V_{max} ($p < 0.01$) and CL_{int} values ($p < 0.05$). The CL_{int} value of CYP2C8.3 was 1.31-fold higher than that of CYP2C8.1 ($p < 0.05$). These results indicated that metabolism by CYP2C8.2 and CYP2C8.4 decreased repaglinide hydroxylation, whereas metabolism by CYP2C8.3 significantly increased repaglinide hydroxylation.

Enzymatic activities of CYP2C8s toward ibuprofen enantiomers

The kinetic plots of 2-hydroxylation of *R*- and *S*-ibuprofen are shown in Figure 3C, and the corresponding enzyme kinetic parameters are listed in Table 3. The relative CL_{int} values of CYP2C8.2, CYP2C8.3 and CYP2C8.4 were 110.5%, 72.3% and 49.7% of that of CYP2C8.1 and were 124.6%, 83.4% and 47.4% of that of CYP2C8.1 for *R*-ibuprofen and *S*-ibuprofen, respectively. The Hill coefficients of *R*-ibuprofen and *S*-ibuprofen catalysed by CYP2C8.4 were 1.32 and 1.02, respectively. For both *R*-ibuprofen and *S*-ibuprofen, the amino acid substitutions present in CYP2C8.2 increased the metabolic ability, whereas CYP2C8.3 and CYP2C8.4 had a significantly reduced metabolic ability. The four CYP2C8 allozymes were all predominantly active toward *R*- ibuprofen. The CL_{int} values for *R*-ibuprofen were over 1.5-fold higher than those for *S*-ibuprofen.

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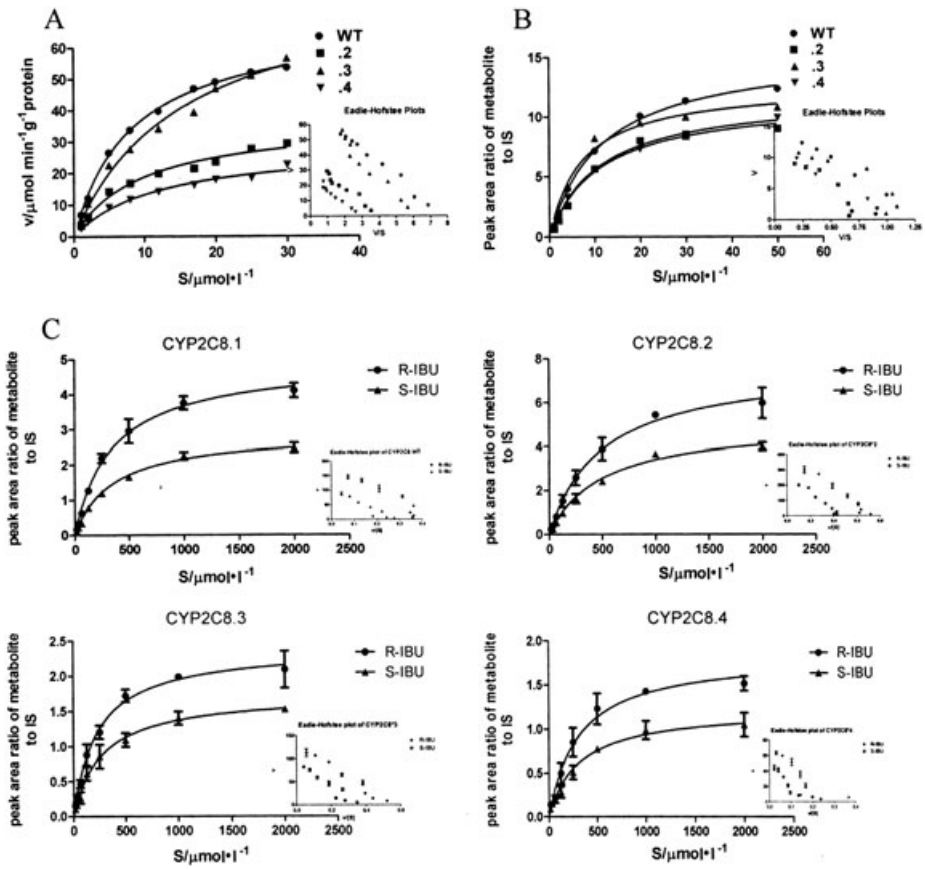

Figure 3. CYP2C8 activity comparisons for paclitaxel, repaglinide and ibuprofen (IBU) enantiomers hydroxylation among CYP2C8.1/.2/.3/.4 ($n = 3$). (A) paclitaxel; (B) repaglinide; (C) ibuprofen enantiomers

Discussion

In this study, recombinant CYP2C8.1, CYP2C8.2, CYP2C8.3 and CYP2C8.4 were systematically co-expressed with POR and cytochrome *b5* using the Bac-to-Bac insect expression system. To ensure that the four CYP2C8s were uniformly expressed, the viral titres of baculovirus (*CYP2C8*1*, *CYP2C8*2*, *CYP2C8*3*, *CYP2C8*4*, *POR* and cytochrome *b5*) were determined by real-time PCR (data not shown). The expression level of CYP2C8 holoprotein in CYP2C8.2, CYP2C8.3 and CYP2C8.4 was comparable to that in CYP2C8.1 (Figure 2), whereas the functional P450 content in CYP2C8.4 was significantly lower than that in CYP2C8.1. As shown in Figure 1D, CYP2C8.4 exhibited a large peak at 420 nm in addition to the Soret peak at 450 nm. However, the peaks at 420 nm were relatively small compared with that at 450 nm in CYP2C8.2 and CYP2C8.3. There was no obvious peak at 420 nm in CYP2C8.1. The presence of a large peak at 420 nm has been shown to indicate the altered stability of the proteins or the formation of an incorrectly folded enzyme resulting in a significantly lower functional P450 content [19].

The amino acid substitution caused by *CYP2C8*4* polymorphism was I264M in helix H located on the protein surface, away from regions involved in heme and substrate binding [20]. As a highly conserved amino acid among the CYP2C subfamily of enzymes, Ile264 is important for heme insertion and protein folding. Because of the weak iron–thiolate interaction between the heme and coordinated Cys435, the secondary and tertiary structures of CYP2C8.4 changed significantly resulting in the loss of stability. CYP2C8.4 was thermally unstable and more sensitive to proteinase K digestion [21,22], which may explain the remarkably poor catalytic activity of

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Table 1. Metabolism kinetics of CYP2C8s for paclitaxel hydroxylation ($n = 3$)

Protein	R^2	K_m(μmol·l^{-1})	V_{max} (pmol·min^{-1}·pmol^{-1} protein)	CL_{int} (μl·min^{-1}·pmol^{-1}protein)/(% of CYP2C8.1)
CYP2C8.1	0.9980	8.65 ± 0.25	69.83 ± 0.84	8.09 ± 0.14 (100)
CYP2C8.2	0.9794	9.65 ± 0.93	36.95 ± 1.30[b]	3.86 ± 0.23[b] (47.7)
CYP2C8.3	0. 9875	16.25 ± 1.45[b]	84.15 ± 3.47	5.20 ± 0.25[b] (64.3)
CYP2C8.4	0.9745	11.81 ± 1.35[a]	28.90 ± 1.18[b]	2.44 ± 0.20[b] (30.2)

Significant difference between the marked data and the data for CYP2C8.1,
[a]$p < 0.05$,
[b]$p < 0.01$.

Table 2. Metabolism kinetics of CYP2C8s for repaglinide hydroxylation ($n = 3$)

Protein	R^2	K_m (μmol·l^{-1})	V_{max} (AU)	CL_{int} (AU)/(% of CYP2C8.1)
CYP2C8.1	0.9985	12.01 ± 1.63	15.69 ± 0.93	1.31 ± 0.09 (100)
CYP2C8.2	0.9868	11.34 ± 1.61	11.56 ± 0.58[b]	1.02 ± 0.10[a] (77.9)
CYP2C8.3	0.9833	7.43 ± 0.84[b]	12.74 ± 0.44[b]	1.71 ± 0.13[a] (130.5)
CYP2C8.4	0.9974	11.38 ± 1.00	11.91 ± 0.35[b]	1.05 ± 0.06[a] (80.2)

Significant difference between the marked data and the data for CYP2C8.1,
[a]$p < 0.05$,
[b]$p < 0.01$.

Table 3. Metabolism kinetics of CYP2C8s for *R*- /*S*- ibuprofen (IBU) hydroxylation ($n = 3$)

Protein		R^2	K_m (μmol·l^{-1})	V_{max} (AU)	CL_{int} (AU)/(% of CYP2C8.1)	R/S of CL_{int}
CYP2C8.1	*R*-IBU	0.9861	341.3 ± 25.2	4.92 ± 0.13	14.42 ± 0.70 (100)	1.86
	S-IBU	0.9917	388.8 ± 26.1	3.02 ± 0.07[d]	7.76 ± 0.33[d] (100)	
CYP2C8.2	*R*-IBU	0.9782	479.2 ± 55.5[a]	7.63 ± 0.08[b]	15.93 ± 1.16 (110.5)	1.65
	S-IBU	0.9901	544.9 ± 43.6[b]	5.27 ± 0.17[b,d]	9.67 ± 0.51[b,d] (124.6)	
CYP2C8.3	*R*-IBU	0.9775	230.1 ± 22.9[a]	2.40 ± 0.07[b]	10.43 ± 0.72[b] (72.3)	1.61
	S-IBU	0.9750	269.5 ± 29.2[b]	1.74 ± 0.06[b,c]	6.47 ± 0.48[b,d] (83.4)	
CYP2C8.4	*R*-IBU	0.9726	226.1 ± 27.8	1.62 ± 0.08[b]	7.16 ± 0.33[b] (49.7)	1.95
	S-IBU	0.9638	342.5 ± 83.3	1.26 ± 0.12[b,c]	3.68 ± 0.34[b,d] (47.4)	

Significant difference between CYP2C8.1 group and its variants group
[a]$p < 0.05$,
[b]$p < 0.01$.
Significant difference between *R*-IBU group and *S*-IBU group
[c]$p < 0.05$,
[d]$p < 0.01$.

CYP2C8.4 toward paclitaxel, repaglinide and ibuprofen in the present study (Tables 1–3).

The catalytic activity of CYP2C8.3 in paclitaxel 6α-hydroxylation was 64.3% of that of CYP2C8.1 ($p < 0.01$), which was roughly similar to the enzyme activity measured in the microsomes of yeast cells or HepG2 cells (50%–75%) [10,19]. Furthermore, in the human liver microsomes homozygous for *CYP2C8*3*, the paclitaxel 6α-hydroxylation activities represented 31%–84% of the corresponding median activities in the microsomes homozygous for *CYP2C8*1* [22], similar to the values obtained in the present study. However, CYP2C8.3 showed 145% higher activity than that of CYP2C8.1 in the *E. coli* expression system [23].

To date, no studies have been performed to investigate the influence of *CYP2C8* genetic polymorphisms on the hydroxylation metabolism of repaglinide *in vitro*. CYP2C8.2 and CYP2C8.4 had significantly lower repaglinide hydroxylation activities than CYP2C8.1 ($p < 0.05$), whereas CYP2C8.3 showed 130.5% activity of that of CYP2C8.1 (Table 2). The mean *AUC* and C_{max} of repaglinide were significantly lower in subjects with the *CYP2C8*1/*3* genotype than in those with the *CYP2C8*1/*1* genotype after being

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administered a single dose (0.25 mg) of repaglinide [11,12]. The result further validated that *in vitro* CYP2C8.3 played a major role in lowering the *AUC* and C_{max} of repaglinide in subjects with the *CYP2C8*1/*3* genotype.

For the stereoselective metabolism of ibuprofen, our results showed that the four CYP2C8 allozymes mainly formed 2-hydroxy *R*-ibuprofen, which was consistent with the *in vivo* data [24]. The K_m values of the two enantiomers differed as much as the corresponding V_{max} values (Table 3). Additionally, both the increased rate of *R*-ibuprofen hydroxylation and the higher affinity of the CYP2C8s to *R*-ibuprofen, confirmed the preference for *R*-ibuprofen metabolism. Interestingly, Eadie-Hofstee plots in Figure 3C for CYP2C8.4 indicated that atypical kinetics existed. Therefore, the Hill equation was used to estimate the kinetic parameters. Furthermore, no significant difference was found in the *R/S*-ibuprofen CL_{int} ratio among the CYP2C8 allozymes, indicating that the *CYP2C8* genetic polymorphisms may have minimal influence on the stereoselective metabolism of ibuprofen enantiomers.

Some clinical studies have shown lower clearance of paclitaxel in ovarian cancer patients heterozygous or homozygous for *CYP2C8*3* [25] and lower clearance of ibuprofen in healthy volunteers heterozygous or homozygous for *CYP2C8*3* [26], while the *CYP2C8*3* allele was associated with an increased clearance of repaglinide [11,12]. In healthy white subjects, CYP2C8*3 and the two novel haplotypes significantly influenced repaglinide pharmacokinetics in SLCO1B1c.521 T/C heterozygous individuals: haplotype B was associated with a reduced and haplotype C with an increased repaglinide *AUC* (0–∞) [27]. Thus, the *CYP2C8*3* polymorphism influenced the *in vivo* drug disposition in a substrate-dependent manner. The results of this study were consistent with the data *in vivo*. The catalytic activities of CYP2C8 allozymes for paclitaxel 6α-hydroxylation were in the order CYP2C8.4 CYP2C8.2 < CYP2C8.3 < CYP2C8.1, while the order for repaglinide metabolism was CYP2C8.2 ≤ CYP2C8.4 < CYP2C8.1 < CYP2C8.3. For the metabolism of ibuprofen enantiomers, the order was CYP2C8.4 < CYP2C8.3 < CYP2C8.1 < CYP2C8.2. For CYP2C8.2 and CYP2C8.3, the significant differences in enzymatic activities toward paclitaxel were inconsistent with the corresponding differences toward repaglinide and ibuprofen enantiomers, suggesting that CYP2C8.2 and CYP2C8.3 had substrate-dependent activities *in vitro*.

The substrate-dependent activities of CYP2C8.2 and CYP2C8.3 can be partly interpreted by evaluating the structure–function relationship. Several significant substrate recognition sites (SRSs) and amino acid residues have been identified within the relatively large substrate-binding cavity of CYP2C8 [21,28]. The large size of the binding site should permit more substrate mobility and allow more interactions with the related SRSs. The amino acid substitutions present in the CYP2C8 variants may result in structural changes in some specific SRSs, followed by a changed affinity to related substrates, while the affinity to unrelated substrates may remain unchanged. Therefore, the influence of *CYP2C8* polymorphisms on substrate affinity should be expected to be substrate-dependent. It has been reported that SRS-1 and SRS-5 are important for binding to paclitaxel [29]. However, further studies are required to determine the SRSs responsible for binding to repaglinide and ibuprofen. Furthermore, the substrate-dependent activities of CYP2C8s may be associated with POR and cytochrome *b*5, as the modulatory effects of POR and cytochrome *b*5 on CYPs were both substrate- and CYP-dependent [30]. For CYP2C8.3, both amino acid mutations (R139K and K399R) were located on the proximal side of the heme-binding region of cytochrome *b*5 and POR. Recent studies have shown that drug metabolism by CYP2C8.3 was directed by substrate-dependent interactions with POR and cytochrome *b*5 [23].

In summary, the clearance profiles of paclitaxel, repaglinide and ibuprofen mediated by CYP2C8.3 *in vitro* were consistent with the pharmacokinetics in volunteers who were heterozygous or homozygous for *CYP2C8*3*. The results of this study provide useful information for predicting *CYP2C8* phenotypes and may contribute to individualized drug therapy in the future.

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Conflict of Interest

There are no conflicts of interest.

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