Effects of CYP2C9*3 and CYP2C9*13 on Diclofenac Metabolism and Inhibition-based Drug-Drug Interactions

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Summary: Cytochrome P450 2C9 (CYP2C9) is a polymorphic enzyme responsible for the metabolism of many important drugs, including diclofenac. CYP2C9*3 and CYP2C9*13 are the principal variant alleles found in the Chinese population. CYP2C9*3 has been reported to reduce the metabolism of diclofenac and alter the extent of drug-drug interactions (DDIs). The effects of CYP2C9*13 on diclofenac metabolism are not well studied, and the influences of CYP2C9*13 on DDIs between diclofenac and clinical drugs are still unknown. In this study, CYP2C9.1 (the wildtype enzyme), CYP2C9.3 and CYP2C9.13 were expressed in yeast, and their metabolic kinetics for diclofenac 4'-hydroxylation were examined. From the in vitro data, we predicted a decrease in the ratio of diclofenac oral clearance (the ratio of oral clearance in subjects with variant CYP2C9 alleles to that in wildtype subjects (CLoralR)) in subjects carrying CYP2C9*3 or CYP2C9*13 alleles. Furthermore, we investigated the effects of these two alleles on diclofenac-drug interactions. The potentials of nine clinically used drugs to inhibit diclofenac 4'-hydroxylation catalyzed by the alleles were compared. Our results indicated that CYP2C9.3 and CYP2C9.13 can alter the CYP-inhibitory potencies of some tested drugs. In particular, CYP2C9.13 significantly weakened the inhibitory potencies of sulfaphenazole, fluvastatin, fluvoxamine and tranylcypromine. These data provide helpful guidelines for co-administration of diclofenac with other drugs in individuals carrying CYP2C9*3 and CYP2C9*13 alleles.

Keywords: CYP2C9 genetic polymorphism; diclofenac; enzyme kinetics; intrinsic clearance; IC₅₀

Introduction

It is well known that much of the observed variation in drug efficacy and safety has a hereditary basis, essentially arising from genetic polymorphisms in drug-metabolizing enzymes. Cytochrome P450 2C9 (CYP2C9) is a major isoform of the CYP2C subfamily that constitutes approximately 20% of the CYP protein content in human liver microsomes (HLMs). CYP2C9 is involved in the metabolism of about 10% of clinically important drugs, particularly nonsteroidal anti-inflammatory drugs, antidepressants, antidiabetics, lipid-lowering drugs and oral anticoagulants.¹,² There is much inter-individual variation in CYP2C9 activity in the population, due in large part to non-synonymous single-nucleotide polymorphisms (SNPs) in CYP2C9. Such SNPs are crucial genetic elements responsible for individual differences in drug metabolism and drug responses.³,⁴

So far, 31 human CYP2C9 alleles that encode amino acid substitutions have been discovered.⁵ The most studied variant alleles are CYP2C9 *2 (R144C) and CYP2C9 *3 (I359L), which result in decreased enzyme activity. In the Chinese population, the frequency of the main variant allele, CYP2C9*3, is approximately 3.3%, while CYP2C9*2...
is seldom found.\textsuperscript{6-9} The \textit{CYP2C9}*3 allele has been identified in the Chinese population with a frequency of approximately 1.02%.\textsuperscript{9} This allele has a T269C transversion in exon 2 that leads to the expression of a variant \textit{CYP2C9} protein. This change results in reduced metabolism of lornoxicam and losartan in humans.\textsuperscript{10,11} Unlike \textit{CYP2C9}*3, which has been extensively studied in humans, clinical studies of \textit{CYP2C9}*13 have been limited by the difficulty in finding subjects carrying this low-frequency allele. Therefore, it would be very helpful to be able to predict the pharmacokinetic changes caused by \textit{CYP2C9}*13 from \textit{in vitro} data. Methods to predict the effects of \textit{CYP2C9} polymorphic alleles on the pharmacokinetics of \textit{CYP2C9}-metabolized drugs from \textit{in vitro} data have been successfully established.\textsuperscript{13} However, there is little \textit{in vitro} data currently available for predicting the effects of \textit{CYP2C9}*13 on \textit{in vivo} drug metabolism.

Diclofenac is a commonly used drug substrate for studies of \textit{CYP2C9} metabolic activity because diclofenac \textit{4'-hydroxylation} is primarily catalyzed by \textit{CYP2C9}.\textsuperscript{13} This drug is classified as a nonsteroidal anti-inflammatory drug and is frequently prescribed for the treatment of musculoskeletal complaints, especially arthritis and ankylosing spondylitis. Diclofenac is also often used to treat chronic pain associated with cancer, particularly if inflammation is present.\textsuperscript{14,15} The therapeutic use of diclofenac is associated with adverse events such as depression, cardiovascular disease, gastrointestinal hemorrhage and hepatotoxicity.\textsuperscript{16,17} Therefore, the co-administration of diclofenac with antidepressants or cardiovascular drugs is sometimes recommended in patients. However, most of these drugs are also metabolized by \textit{CYP2C9}, thus increasing the chances of drug-drug interactions (DDIs) that may result in adverse drug responses.

Studies of \textit{CYP2C9} genetic polymorphisms increasingly indicate that the degree of \textit{CYP2C9}-mediated DDIs can be extensively influenced by variant alleles. For instance, the \textit{CYP2C9}*3 allelic protein can alter the inhibitory potencies of many drugs toward \textit{CYP2C9}-mediated metabolism \textit{in vitro},\textsuperscript{18} and the expression of the \textit{CYP2C9}*13 allele can also alter the degree of DDIs between flurbiprofen and fluconazole in humans.\textsuperscript{19} To date, no studies have reported the effect of \textit{CYP2C9}*13 on the interaction between diclofenac and other drugs. Understanding the effects of variant \textit{CYP2C9} alleles on DDIs can help prevent adverse drug responses in individuals with different \textit{CYP2C9} genotypes when co-administering diclofenac with other drugs.

The aims of this study were to evaluate the effects of \textit{CYP2C9}*3 and \textit{CYP2C9}*13 on diclofenac metabolism and DDIs. We determined the kinetic parameters of diclofenac metabolism using yeast-expressed \textit{CYP2C9} variant enzymes. Using these \textit{in vitro} data, we predicted the ratio of diclofenac oral clearance (\textit{CL}_{oralR}) in subjects carrying \textit{CYP2C9}*3 or \textit{CYP2C9}*13 alleles to that in subjects carrying wild-type alleles. Furthermore, to provide information for avoiding DDIs in patients carrying variant \textit{CYP2C9} alleles, we investigated the effects of \textit{CYP2C9}*3 and \textit{CYP2C9}*13 on the potencies of nine drugs for inhibiting \textit{CYP2C9}-mediated diclofenac metabolism. These nine drugs are commonly co-administered with diclofenac in clinical practice, or are known to have the potential for DDIs with diclofenac. Our results provide information that may improve the selection of drug combinations in the clinical setting in order to lower the risk of adverse diclofenac-drug interactions.

**Materials and Methods**

**Chemicals and reagents:** Diclofenac sodium, \textit{4'-hydroxydiclofenac}, sulfaphenazole, ketoconazole, troglitazone, sertraline, fluvoxamine, tranylcypromine, fluoxetine, \(\beta\)-nicotinamide adenine dinucleotide phosphate (\(\beta\)-NADP), glucose 6-phosphate (G-6-P), and glucose-6-phosphate dehydrogenase (G6PDH) were purchased from Sigma (St. Louis, MO). Fluvatatin and simvastatin were supplied by Prof. C. F. Chen (Xuzhou Normal University, Xuzhou, Jiangsu). \textit{E. coli} TOP 10 was obtained from Tiangen Biotech (Beijing, China) and the prostate-deficient \textit{Saccharomyces cerevisiae} strain BJ5457 was purchased from ATCC (Manassas, VA). The galactose-inducible expression vector pYES2/CT and anti-V5 antibody were obtained from Invitrogen (Carlsbad, CA). Other chemicals and reagents were of the highest quality commercially available.

**Construction of expression plasmids:** The coding region of wildtype \textit{CYP2C9} was amplified by polymerase chain reaction (PCR). \textit{Kpn} I and \textit{Xho} I restriction sites were engineered into the forward and reverse primers, respectively, to facilitate downstream subcloning. The PCR product and the pYES2/CT vector were digested with \textit{Kpn} I and \textit{Xho} I and then the two gel-purified fragments were ligated together using T4 DNA ligase at 4\textdegree C overnight. Eliminating the stop codon in the reverse primer permitted the V5 epitope gene located downstream of the multiple cloning site of the vector to be translated. The fusion proteins were detected by Western blotting using anti-V5 antibody. Vectors containing the \textit{CYP2C9} cDNA constructs were transformed into \textit{E. coli} and selected by ampicillin resistance. Positive transformants were confirmed by sequence analysis using a 3730 DNA Analyzer (Applied Biosystems; Foster City,
CA). CYP2C9*3 and CYP2C9*13 cDNAs were constructed by site-directed mutagenesis using PCR. Each full-length SNP cDNA fragment was sub-cloned into the pYES2/CT vector in the same way as for the wildtype cDNA. The SNPs were confirmed by DNA sequencing before expression.

**Enzyme expression and microsomal preparation:** To co-express the CYP2C9 enzymes with human NADPH-cytochrome P-450 reductase (CPR), the cDNA of CPR was first integrated into the yeast chromosome by homologous recombination, and then the expression vectors containing CYP2C9 cDNAs were electroporated into the CPR-integrated yeast for expression. Enzyme expression was initiated by switching the carbon source in the media from glucose to galactose, since the CYP2C9 cDNAs were sub-cloned downstream of the GAL1 promoter. Yeast microsomes were prepared by differential ultracentrifugation according to a previously described method. The microsomal protein concentration was measured according to the Bradford method. The CYP2C9 concentration was determined spectrophotometrically from the CO-reduced difference spectra.

**Kinetic analysis of functional allelic enzymes:** Preliminary experiments were performed to identify the initial rate conditions using different enzyme concentrations and incubation times. In kinetic studies, the substrate was two-fold serially diluted from 160 to 0.25 μM with deionized water. Each concentration of substrate was added separately into 200 μl of reaction mixture containing 1 M Tris-HCl (pH 7.5) and NADPH-regenerating buffer (1.3 mM NADP, 3.3 mM G-6-P, 3.3 mM MgCl₂, and 0.4 U/ml G6PDH). After 5 min of pre-incubation at 37°C, the reactions were initiated by adding CYP2C9 enzymes; final kinetic studies were conducted with CYP2C9.1 and CYP2C9.3 at 0.5 mg/ml and CYP2C9.13 at 1.0 mg/ml, with incubation times for CYP2C9.1, CYP2C9.3 and CYP2C9.13 of 15, 10 and 5 min, respectively. All experiments used enzyme concentrations and incubation times that were within the linear range of metabolite formation in order to obtain accurate kinetic parameters. Reactions were stopped by adding 100 μl of a 94% acetonitrile and 6% acetic acid glacial solution. Subsequently, protein was sedimented by centrifugation at 13000 g for 6 min. Supernatants (50 μL) were subjected to HPLC analysis on a HC-C18 column (5 μm, 4.6 × 250 mm, Agilent Technologies). The mobile phase consisted of 20% acetonitrile (with 0.1% perchloric acid) and methanol (70:30) at a flow rate of 0.8 ml/min, and the column temperature was set at 40°C. The samples were detected by UV280 nm. The retention times of 4' hydroxydiclofenac and diclofenac were 14.5 and 18.5 min, respectively. Calibration curves were generated from 0.125 to 80 μM.

**Prediction of diclofenac CLoralR for CYP2C9 variants:** The prediction of the effects on oral clearance (CLoral) of CYP2C9 variants was performed using established methods. The expression levels of CYP2C9.3 in HLMs were obtained from the literature. The ratio of the expression levels of CYP2C9*13/*13 to that of the wildtype (ExpR*13/*13) was calculated from the heterologous expression systems (COS-7 cells and insect cells) since the values in HLMs are currently unavailable. These predictions were based on the assumption that the two alleles would be expressed equally and independently, so the ratio of the expression level of CYP2C9*1/*13 to that of wildtype (ExpR*1/*13) could be estimated using the following equation:

$$\text{ExpR}_{1/13} = (1 + \text{ExpR}_{*13/13})/2$$

The intrinsic hepatic clearance ratio (the ratio of variant enzyme intrinsic hepatic clearance to that of wildtype (CLh,int,2C9)) was calculated using the following equation:

$$\text{CL}_{h,int,2C9} = (\text{ActR}_{2C9-1} \times \text{ExpR}_{2C9-1} + \text{ActR}_{2C9-2} \times \text{ExpR}_{2C9-2}) \times \frac{1}{2}$$

$$\text{CL}_{h,int,2C9}$$, variant enzyme intrinsic hepatic clearance; ActR_{2C9}, the ratio of variant enzyme intrinsic clearance (CLint) in recombinant microsomes to that of wildtype (the diclofenac CLint of CYP2C9 wildtype and variants were obtained in our study from the ratio of Vmax/Km); ExpR_{2C9}, the ratio of variant enzymes expression level in HLMs or heterologous expression system to that of wildtype.

If a substrate exhibits low CLint, such as diclofenac, the predicted CLoralR value was calculated using the following equation:

$$\frac{\text{CL}_{oral}'}{\text{CL}_{oral}} = \left( \frac{f_h \times f_m \times \text{CL}_{h,int,2C9}}{\text{CL}_{h,int,2C9-wt}} + (1 - f_h \times f_m) \right) \times \frac{F_g}{F_g'}$$

$$\text{CL}_{oral}'$$, variant enzyme oral clearance; $$\text{CL}_{oral}$$, wildtype enzyme oral clearance; $$f_h$$, the contribution of non-CYP clearance to total drug clearance; $$f_m$$, the contribution of CYP2C9 to the total hepatic intrinsic clearance in wildtype subjects; $$F_g$$ and $$F_g'$$ are the intestinal availability in CYP2C9 wildtype and variants, respectively. The values of $$f_m$$, $$f_h$$, $$F_g$$ and $$F_g'$$ were obtained from the literature.

**Inhibition assay:** The inhibitors were two-fold serially diluted from 128 to 0.0625 μM before addition to the incubation mixtures. All reactions were performed at a substrate concentration equivalent to the Km of the CYP2C9 reaction. The other components of the incubation mixture and the reaction conditions were the same as described above. The formation of 4' hydroxydiclofenac was monitored by HPLC. The CYP2C9-specific inhibitor sulphonazole acted as positive control for the inhibition of diclofenac hydroxylation.

**Data analysis:** Km and Vmax were determined by
nonlinear regression curve fitting using the computer program Prism version 5.0 (GraphPad Software, Inc., San Diego, CA). CLint was determined as the ratio of $V_{\text{max}}/K_m$. The concentration of inhibitor producing 50% inhibition (IC$_{50}$) values were calculated by linear interpolation with Microsoft Excel (Microsoft Corp., Redmond, WA). Statistical significance was determined by one-way analysis of variance (ANOVA) with a post-hoc Dunnett multiple comparisons test. A value of $p < 0.05$ was considered to be statistically significant.

**Results**

**Expression of CYP2C9 enzymes:** The expression of CYP2C9.1, CYP2C9.3 and CYP2C9.13 proteins in yeast cells was examined by Western blotting. All constructs yielded immuno-detectable CYP2C9 protein. The cytochrome P450 (P450) contents of yeast expressing CYP2C9.1, CYP2C9.3 and CYP2C9.13 were 48, 54 and 42 pmol/mg, respectively.

**Kinetics of recombinant human CYP2C9 enzymes:** Prior to our kinetic assays, we determined that the linear ranges of enzyme concentrations for CYP2C9.1, CYP2C9.3 and CYP2C9.13 microsomes are 0.25–1.0, 0.25–1.0 and 0.5–2.0 mg/ml, respectively, and the linear ranges of incubation times are 5–30, 5–20 and 1–5 min, respectively. The kinetic parameters of diclofenac 4'-hydroxylation catalyzed by CYP2C9.3 and CYP2C9.13 were tested and compared with those of the wildtype (Table 1). The diclofenac CL$_{int}$ values catalyzed by CYP2C9.3 and CYP2C9.13 were significantly lower than that of wildtype enzyme (35% and 6% of wildtype, respectively). The $K_m$ values of CYP2C9.3 and CYP2C9.13 were 2.4-fold and 4.4-fold higher, respectively, than that of wildtype enzyme (35% and 6% of wildtype, respectively). The $V_{\text{max}}$ value of CYP2C9.13 was not significantly different from that of wildtype, whereas the $V_{\text{max}}$ value of CYP2C9.3 was not significantly different from that of the wildtype, whereas the $V_{\text{max}}$ value of CYP2C9.13 was significantly lower ($n = 4$, $p < 0.01$) (Fig. 1). These results indicate that diclofenac metabolism catalyzed by both variant enzymes was significantly decreased.

**Prediction of the diclofenac CL$_{oralR}$ in CYP2C9 variants:** Using the in vitro CL$_{int}$ values, we predicted the diclofenac CL$_{oralR}$ values in subjects carrying CYP2C9*3 and CYP2C9*13 alleles (Table 2). The diclofenac CL$_{oralR}$ values predicted in individuals with *1/*3 and *3/*3 genotypes were 0.81 and 0.61, respectively, which were comparable to the values predicted in a published report and to the in vivo data. These results suggest that the diclofenac CL$_{oralR}$ values predicted in our study are reliable. Furthermore, the diclofenac CL$_{oral}$ predicted in subjects with *1/*13 and *13/*13 genotypes is 77% and 54% of that in subjects with the *1/*1 genotype (CL$_{oral}$ of 0.77 and 0.54, respectively). These results suggest that the diclofenac dosage should be decreased in patients carrying CYP2C9*13 alleles.

**Table 1. Kinetic parameters for diclofenac 4'-hydroxylation by recombinant CYP2C9*1, CYP2C9*3 and CYP2C9*13**

<table>
<thead>
<tr>
<th>Alleles</th>
<th>$K_m$ (μM)</th>
<th>$V_{\text{max}}$ (pmol/min/μmol)</th>
<th>CL$_{int}$ (μl/min/μmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2C9*1</td>
<td>5.5±0.4</td>
<td>37.2±3.7</td>
<td>6.9±0.8</td>
</tr>
<tr>
<td>CYP2C9*3</td>
<td>13.1±0.5*</td>
<td>31.4±1.6</td>
<td>2.4±0.1**</td>
</tr>
<tr>
<td>CYP2C9*13</td>
<td>24.1±4.1**</td>
<td>9.9±1.5**</td>
<td>0.4±0.05**</td>
</tr>
</tbody>
</table>

Data are shown as the mean ± S.E. of at least four independent experiments. $K_m$, apparent Michaelis-Menten constant; $V_{\text{max}}$, maximum velocity of the reaction; CL$_{int}$, microsomal intrinsic clearance values. *$p < 0.05$, **$p < 0.01$ vs. CYP2C9*1.

**Table 2. Diclofenac CL$_{oralR}$ values (as a ratio with *1/*1) predicted from in vitro in vitro experiments using recombinant enzymes**

<table>
<thead>
<tr>
<th>CYP2C9 genotype</th>
<th>ActR$_{2C9}$</th>
<th>ExpR$_{2C9}$</th>
<th>CL$_{oralR}$</th>
<th>Predict CL$_{oralR}$ in vitro</th>
<th>CL$_{oralR}$ in vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td>*1/*3</td>
<td>/</td>
<td>0.74*</td>
<td>0.58</td>
<td>0.81</td>
<td>0.95</td>
</tr>
<tr>
<td>*3/*3</td>
<td>0.35</td>
<td>0.48*</td>
<td>0.17</td>
<td>0.61</td>
<td>0.86</td>
</tr>
<tr>
<td>*1/*13</td>
<td>/</td>
<td>0.65</td>
<td>0.51</td>
<td>0.77</td>
<td>/</td>
</tr>
<tr>
<td>*13/*13</td>
<td>0.058</td>
<td>0.29*</td>
<td>0.02</td>
<td>0.54</td>
<td>/</td>
</tr>
</tbody>
</table>

aData were calculated from our assay; bdata were obtained from Kirchheiner and Brockmoller; c ratio of expression level were obtained from HLMs; dvalue was calculated as described in the Materials and Methods; ereaction level was calculated from the heterologous expression systems.

**Inhibitory potentials of drugs with respect to CYP2C9 enzymes:** To study the effects of CYP2C9.3 and CYP2C9.13 on the potentials of drugs to inhibit CYP2C9-mediated diclofenac 4'-hydroxylation, the IC$_{50}$ values of nine drugs for the reaction catalyzed by CYP2C9 enzymes were established (Table 3). The CYP2C9 wildtype enzyme, CYP2C9.1, was studied first.
The CYP2C9-specific inhibitor sulphinphazone was employed to validate the inhibitory assay. The IC$_{50}$ value of sulfaphenazole was 0.7 μM, which was in good agreement with previous reports. Two tested compounds, fluvastatin and fluvoxamine, showed strong inhibitory effects on diclofenac 4'-hydroxylation catalyzed by CYP2C9.1 (IC$_{50}$ values were 4.26 and 7.11 μM, respectively). Four compounds (ketocconazole, troglitazone, tranylcypromine and fluoxetine) exhibited moderate inhibitory effects with IC$_{50}$ values between 10–50 μM. The other two compounds (simvastatin and sertraline) had weak inhibitory effects on diclofenac hydroxylation. These results are consistent with previous reports.

When compared with CYP2C9.1, minor changes were observed in the potential of tested drugs to inhibit diclofenac 4'-hydroxylation catalyzed by CYP2C9.3. The IC$_{50}$ values of tested drugs with respect to CYP2C9.3 and CYP2C9.1 were not significantly different, except for two drugs, fluvastatin and tranylcypromine, whose IC$_{50}$ values to CYP2C9.3 were 8-fold higher (n = 3, p < 0.01) and 2-fold lower (n = 3, p < 0.05), respectively, than to CYP2C9.1. In total, four drugs, sulfaphenazole, fluvastatin, fluvoxamine and tranylcypromine, exhibited significantly higher IC$_{50}$ values to CYP2C9.13 than to CYP2C9.1 (n = 3, p < 0.01). The IC$_{50}$ values of ketocconazole, troglitazone, simvastatin, fluoxetine and sertraline to all three CYP2C9 enzymes showed no statistically significant difference. These results suggest that the inhibitory potencies of drugs to both CYP2C9.3 and CYP2C9.13 may be altered relative to CYP2C9.1 in a drug-dependent manner. These results also suggest guidelines for co-administration of diclofenac with the tested drugs in individuals carrying CYP2C9*3 or CYP2C9*13 alleles.

### Table 3. IC$_{50}$ values of drugs for the metabolism of diclofenac by CYP2C9.1, CYP2C9.3 and CYP2C9.13 expressed in yeast

<table>
<thead>
<tr>
<th>Drugs</th>
<th>CYP2C9.1</th>
<th>CYP2C9.3</th>
<th>CYP2C9.13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfaphenazole</td>
<td>0.70±0.08</td>
<td>1.39±0.14</td>
<td>4.70±0.79***</td>
</tr>
<tr>
<td>Ketocconazole</td>
<td>42.30±4.99</td>
<td>41.37±5.78</td>
<td>44.43±6.9</td>
</tr>
<tr>
<td>Troglitazone</td>
<td>16.33±0.90</td>
<td>21.55±3.97</td>
<td>20.80±1.67</td>
</tr>
<tr>
<td>Fluvastatin</td>
<td>4.26±0.38</td>
<td>32.96±3.53**</td>
<td>34.92±5.95**</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>92.72±5.83</td>
<td>114.70±1.49</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Fluvoxamine</td>
<td>7.11±0.59</td>
<td>14.20±0.42</td>
<td>75.08±5.81**</td>
</tr>
<tr>
<td>Tranylcypromine</td>
<td>27.25±0.39</td>
<td>14.40±0.48*</td>
<td>111.60±3.79**</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>42.12±3.28</td>
<td>61.78±4.82</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Sertraline</td>
<td>110.10±0.65</td>
<td>&gt;128</td>
<td>&gt;128</td>
</tr>
</tbody>
</table>

IC$_{50}$ values (μM) calculated for the mean±S.E. of three independent experiments. Drugs were categorized as having extremely strong inhibition (IC$_{50}$ < 1 μM); strong inhibition (1 μM < IC$_{50}$ < 10 μM); moderate inhibition (10 μM < IC$_{50}$ < 50 μM); weak inhibition (50 μM < IC$_{50}$ < 128 μM); or no inhibition (IC$_{50}$ > 128 μM). *p < 0.05, **p < 0.01 vs. CYP2C9.1.

### Table 4. Comparison of the CYP2C9 genotype-specific enzyme kinetics for diclofenac 4'-hydroxylation with literature values

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>CYP2C9.1</th>
<th></th>
<th></th>
<th>CYP2C9.3</th>
<th></th>
<th></th>
<th>CYP2C9.13</th>
<th></th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K$_m$</td>
<td>V$_{max}$</td>
<td>CL$_{int}$</td>
<td>K$_m$</td>
<td>V$_{max}$</td>
<td>CL$_{int}$</td>
<td>K$_m$</td>
<td>V$_{max}$</td>
<td>CL$_{int}$</td>
</tr>
<tr>
<td>Yeast</td>
<td>5.5</td>
<td>37.2</td>
<td>6.9</td>
<td>13.1</td>
<td>31.4</td>
<td>2.4</td>
<td>24.1</td>
<td>9.9</td>
<td>0.4</td>
</tr>
<tr>
<td>Yeast</td>
<td>3.9</td>
<td>35.6</td>
<td>9.16</td>
<td>12.6</td>
<td>33.3</td>
<td>2.69</td>
<td></td>
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</tr>
<tr>
<td>Yeast</td>
<td>2.03</td>
<td>12.4</td>
<td>6.23</td>
<td>16.5</td>
<td>17.9</td>
<td>1.09</td>
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<tr>
<td>Yeast</td>
<td>1.8</td>
<td>12.5</td>
<td>6.9</td>
<td>11.1</td>
<td>8.1</td>
<td>0.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeast</td>
<td>(7.0–7.7)</td>
<td>(25.1–26.5)</td>
<td>(3.4–3.6)</td>
<td>(22.1–26.4)</td>
<td>(25.2–28.7)</td>
<td>(1.1–1.1)</td>
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<tr>
<td>Yeast</td>
<td>25.9</td>
<td>57.9</td>
<td>2.2</td>
<td>46.9</td>
<td>138.1</td>
<td>3.0</td>
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<tr>
<td>Baculovirus/insect</td>
<td>4.8</td>
<td>29</td>
<td>6.04</td>
<td>17</td>
<td>13</td>
<td>0.76</td>
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<td>Baculovirus/insect</td>
<td>1.8</td>
<td>76.2</td>
<td>43.6</td>
<td>5.3</td>
<td>84.9</td>
<td>16.1</td>
<td>7.0</td>
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<td>COS-7 cell</td>
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<td>27.15</td>
<td>11.71</td>
<td>6.67</td>
<td>28.67</td>
<td>4.34</td>
<td>11.6</td>
<td>3.04</td>
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<td>HLMs$^{a,b}$</td>
<td>(6–40)</td>
<td>(339–1423)</td>
<td>(14–96.3)</td>
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<td>HLMs$^{c}$</td>
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<td>215</td>
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</table>

K$_m$, apparent Michaelis-Menten constant, measured in μM; V$_{max}$, maximum velocity of the reaction, measured in pmol/min/pmol; CL$_{int}$, intrinsic clearance value, measured in μl/min/pmol. *Data from our laboratory; **range in parentheses; °V$_{max}$ measured in pmol/min/mg protein, CL$_{int}$ measured in μl/min/mg protein.

### Discussion

The present study suggests that the CYP2C9.3 and CYP2C9.13 variants, relative to CYP2C9.1, significantly decrease the intrinsic clearance of diclofenac. The kinetic parameters for CYP2C9.1, CYP2C9.3 and CYP2C9.13 obtained in our yeast system were generally comparable with values available in the literature established using yeast and other recombinant cell systems (Table 4). The slight discrepancies between the observed kinetic parameters of CYP2C9.13 and those reported using other recombinant systems might be caused by the different methods of enzyme preparation and experimental conditions. Furthermore, K$_m$ for CYP2C9.1 observed in our study also correlated well with that reported in HLMs (Table 4). These comparisons suggest that the results obtained from our yeast system are valid for predicting drug metabolism.

Previous studies on the structure of CYP2C9.13 and...
CYP2C9.3 suggested the reasons why the diclofenac-metabolizing activities of these two variant enzymes are reduced. The backbone of residues 106–108 in CYP2C9.13 turns over and their side chains block the active site of CYP2C9.13 so that lipophilic compounds (such as diclofenac) cannot readily approach the active site. In addition, the affinity of diclofenac for CYP2C9.13 may be affected by the decrease in the interaction energy between heme and diclofenac caused by the longer distance.13) The Ile359Leu substitution in CYP2C9.3 slightly decreases the affinity and metabolic capacity for diclofenac hydroxylation, most likely because the substitution takes place in putative substrate recognition site 5 (SRS 5) of CYP2C9, which is close to SRS 4, the most important site to diclofenac specificity.44,45

Considering the complexity of clinical studies and the difficulty of finding subjects carrying low-frequency CYP alleles, quantitatively predicting the oral clearance of drugs from in vitro metabolism data would be very helpful in guiding the use of drugs in patients carrying rare CYP alleles. In this study, the predicted diclofenac CLoral values in subjects with *1/*3 and *3/*3 genotypes were 81% and 61%, respectively, of that in subjects with the *1/*1 genotype. These values were similar to the values predicted in a previous report (79% and 59%),12 but slightly lower than the in vivo data (95% and 86%).26) The lower predicted values suggest that the significance of the influence of CYP2C9*3 on diclofenac CLoral is overestimated in the in vitro studies. This is probably because the predicted diclofenac CLoral values were calculated from data using individual CYP2C9 enzymes, thus ignoring the contribution of other CYPs to the total clearance of diclofenac in humans. The predicted Cloral values in subjects carrying CYP2C9*3 alleles were, however, comparable to the in vivo values, because this calculation takes into account non-CYP metabolic clearance pathways, which improve the accuracy of the prediction. Therefore, we concluded that our in vitro data are reliable for predicting the Cloral in individuals carrying variant CYP2C9 alleles.

We predicted the diclofenac CloralRs in subjects with *1/*13 and *13/*13 genotypes to be 0.77 and 0.54, respectively. These results indicate that a reduction in diclofenac dosage may be needed in individuals carrying the CYP2C9*13 allele. In the present prediction, we used the value of ExpRCloral obtained from the heterologous expression systems to calculate the diclofenac Cloral values in subjects with *1/*13 and *13/*13 alleles, because the ExpRCloral value for HLMs is currently unavailable. It is unlikely, however, that using this calculated value would have much influence on the predicted diclofenac CloralR value, because for the other variant studied, the predicted CloralR values in subjects carrying CYP2C9*3 were similar whether the ExpRCloral value was calculated from the heterologous expression system or obtained from HLMs (data not shown).

Genetic polymorphisms in the CYP2C9 gene can alter the inhibitory potencies of some drugs toward the CYP2C9 enzyme, resulting in unexpected DDIs in individuals carrying polymorphic genotypes. Our results showed that the inhibitory potency of fluvastatin toward diclofenac metabolism is significantly reduced by CYP2C9.3 relative to CYP2C9.1 (Table 3), indicating a lower risk of DDIs between fluvastatin and diclofenac in individuals carrying a CYP2C9*3 allele. It is known that the CYP2C9.3 allelic protein can also alter the inhibitory potencies of some other drugs in vitro.18) This alteration may also have clinical consequences.19

To our knowledge, no other studies have explored the effect of the CYP2C9*13 allele on drug interactions with diclofenac. In this study, we first demonstrated that the inhibitory potencies of four drugs, sulfaphenazole, fluvastatin, fluvoxamine and tranylcypromine, toward diclofenac metabolism were significantly reduced for CYP2C9.13, compared with CYP2C9.1 (Table 3). These results indicate that co-administration of these four drugs with diclofenac may cause less risk of DDIs in patients carrying CYP2C9*13 than in those carrying the wildtype allele.

Co-administration of diclofenac with drugs that inhibit CYP2C9 potentially leads to a higher, and possibly unsafe, diclofenac plasma concentration in humans. Our results provide important information for avoiding unexpected DDIs in patients carrying different CYP2C9 alleles. For example, when co-administering cardiovascular drugs with diclofenac, patients carrying CYP2C9 wildtype alleles should take simvastatin rather than fluvastatin, because the inhibitory potency of simvastatin to diclofenac metabolism is about 22-fold weaker than that of fluvastatin. Likewise, for the treatment of depression in patients taking diclofenac, fluvoxamine may be recommended for patients carrying CYP2C9*13, but not for those carrying the CYP2C9*1 or CYP2C9*3 alleles, since the inhibitory potency of fluvoxamine to diclofenac metabolism is significantly reduced for CYP2C9.13 (Table 3). Our results therefore provide a rationale for the appropriate clinical use of diclofenac in combination with nine other drugs in patients carrying wild-type and variant CYP2C9 alleles.

In conclusion, we expressed CYP2C9.1, CYP2C9.3 and CYP2C9.13 in yeast cells and studied the effects of the two variant enzymes on diclofenac metabolism and DDIs. Using the in vitro kinetic parameters for diclofenac metabolism, we predicted the diclofenac CloralR values in individuals carrying CYP2C9*3 and CYP2C9*13 alleles, which suggested lower diclofenac Cloral values in individuals carrying these two alleles than in individuals carrying the wildtype alleles. We further studied the inhibitory potencies of nine drugs toward diclofenac metabolism catalyzed by CYP2C9.3 and CYP2C9.13, relative to CYP2C9.1. We found that the inhibitory poten-
cies of some drugs toward CYP2C9-mediated diclofenac metabolism were weakened by the two allelic variants. These results provide useful information for rational co-administration of diclofenac with other drugs in individuals carrying variant CYP2C9 alleles.

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References


