ROLE OF CYP2C9 AND ITS VARIANTS (CYP2C9*3 AND CYP2C9*13) IN THE METABOLISM OF LORNOXICAM IN HUMANS

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Received January 8, 2005; accepted March 9, 2005

ABSTRACT:

CYP2C9 is an important member of the cytochrome P450 enzyme superfamily with some 12 CYP2C9 alleles (*1-*12) being previously reported. Recently, we identified a new CYP2C9 allele with a Leu90Pro mutation in a Chinese poor metabolizer of lornoxicam [Si D, Guo Y, Zhang Y, Yang L, Zhou H, and Zhong D (2004) Pharmaco- genetics 14:465–469]. The new allele, designated CYP2C9*13, was found to occur in approximately 2% of the Chinese population.

To examine enzymatic activity of the CYP2C9*13 allele, kinetic parameters for lornoxicam 5'-hydroxylation were determined in postmitochondrial supernatant (S9) from transfected cells were lower than those from wild-type CYP2C9*1. Mean values of $K_m$ and $V_{max}$ for CYP2C9*1, *3, and *13 were 1.24, 1.61, and 2.79 μM and 0.83, 0.28, and 0.22 pmol/min/pmol, respectively. Intrinsic clearance values ($V_{max}/K_m$) for variant CYP2C9*3 and CYP2C9*13 on the basis of CYP2C9 protein levels were separately decreased to 28% and 12% compared with wild type. In a subsequent clinical study, the AUC of lornoxicam was increased by 1.9-fold and its oral clearance (CL/F) decreased by 44% in three CYP2C9*1/*13 subjects, compared with CYP2C9*1/*1 individuals. This suggests that the CYP2C9*13 allele is associated with decreased enzymatic activity both in vitro and in vivo.

CYP2C9 constitutes approximately 20% of the cytochrome P450 protein content of human liver microsomes and is responsible for the metabolism of many clinically important drugs. These include drugs with a narrow therapeutic index such as warfarin and phenytoin and other routinely prescribed drugs such as acenocoumarol, tolbutamide, losartan, glipizide, and some nonsteroidal anti-inflammatory drugs (Lee et al., 2002).

The CYP2C9 gene is highly polymorphic. At least 13 CYP2C9 alleles have been identified to date and most of them are associated with reduced CYP2C9 activity. Among them, CYP2C9*3, with an Ile359Leu mutation, has been most widely studied. In vitro studies show that the CYP2C9*3 allele is associated with decreased enzymatic activity both in vitro and in vivo. (Crespi et al., 1997; Aithal et al., 1999; Imai et al., 2000; Dickmann et al., 2001; Kidd et al., 2001; Kircheheiner et al., 2002; Allabi et al., 2004; Blaisdell et al., 2004).

Recently, a new CYP2C9 allele designated CYP2C9*13 has been identified in a Chinese poor metabolizer of lornoxicam. The allele possesses a T269C transversion in exon 2 of CYP2C9 that leads to a Leu90Pro substitution. Frequency analysis shows that approximately 2% of the Chinese populations carry the allele (Si et al., 2004). Genotyping of this poor lornoxicam metabolizer revealed a CYP2C9*9/13 genotype with the two mutations located on separate alleles. His lornoxicam half-life of about 105 h was markedly longer than that of other CYP2C9*1/*3 and CYP2C9*1/*1 carriers (half-lives of 5.8–8.1 and 3.2–6.3 h, respectively; Zhang et al., 2005), suggesting that the CYP2C9*13 allele has a larger effect on CYP2C9 metabolic capability than other alleles.

CYP2C9 has been shown to be the primary enzyme responsible for the biotransformation of the nonsteroidal anti-inflammatory drug lornoxicam to its major metabolite, 5'-hydroxylornoxicam, in human liver microsomes (Bonnabry et al., 1996; Kohl et al., 2000). Recently, it was reported that lornoxicam 5'-hydroxylation by the CYP2C9*3 allele was markedly reduced compared with wild type, both in vitro and in vivo (Iida et al., 2004; Zhang et al., 2005). Thus, lornoxicam is an ideal substrate for the study of CYP2C9 enzyme activity. The purpose of this study was to compare the enzymatic activity of CYP2C9*1, CYP2C9*3, and CYP2C9*13 toward lornoxicam both in vitro and in vivo.
vitro in appropriately transfected COS-7 cells and in vivo in subjects with CYP2C9*1/*3, CYP2C9*1/*13, and CYP2C9*1/*1 genotypes.

Materials and Methods

Materials. Lornoxicam was purchased from Shanghai Xinhui Medical Academy (Beijing, China). 5'-Hydroxylornoxicam was provided by the Laboratory of Microorganisms, Shenyang Pharmaceutical University (Shenyang, China). Dulbecco’s modified Eagle’s medium (DMEM), pDNA3.1 (+), and LipofectAMINE 2000 were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum was purchased from Tianjin, H&Y Bio Co. Ltd. (Tianjing, China). *CYP2C9*1 cDNA and bovine serum was purchased from Tianjin, H&Y Bio Co. Ltd. (Tianjing, China). pREP9 plasmid containing human *CYP2C9*1 cDNA and *E. coli* Top 10 were provided by the Department of Pathophysiology and the Laboratory of Medical Molecular Biology, School of Medicine, Zhejiang University (Zhejiang, China). Rabbit anti-human cytochrome P450 2C9 antibody was purchased from Serotec (Oxford, UK). Alkaline phosphatase-labeled anti-rabbit IgG, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) and bovine serum albumin were purchased from Beijing Dingguo Biotechnology Development Center (Beijing, China). NADPH was purchased from Roche Molecular Biochemicals (Basel, Switzerland). COS-7 cells were kindly donated by the Vaccination Center, Jilin Academy (Beijing, China.) 5'-Hydroxylornoxicam was purchased from Shangdi Xinshiji Medical Materials.

Construction of Expression Plasmids. *CYP2C9*1 cDNA in pREP9 plasmid was subcloned into pDNA3.1 (+) by digestion with KpnI and XhoI enzymes. Site-directed mutagenesis to introduce the A→C transition at position 1075 (*CYP2C9*3) and the T→C transition at position 269 (*CYP2C9*13) was performed using pDNA3.1 (+) plasmids carrying *CYP2C9*1 cDNA as the template for polymerase chain reaction amplification by *Pfu* DNA polymerase. The specific base transition was introduced into the amplification products by a pair of completely complementary primers containing substituted base. The mutagenic primers for *CYP2C9*3 and *13 were 5'-CGAGGTCCAGAGATACCTGACCTTCTCCCAAC-3' and 5'-GGAGGGCCCTGA-TTGATCCTGGAGAGGAGTTTTCTG-3', respectively (mutations underlined). After incubation with DpnI enzyme, the origin templates were digested, but the new amplified polymerase chain reaction products containing substituted base remained and were transformed to *E. coli* Top 10. Clones containing the desired nucleotide change were identified by sequencing carried out by Shanghai Sangon Biological Engineering Technology and Service Co. Ltd. (Shanghai, China).

Transfection of COS-7 Cells and Preparation of Postmitochondrial Supernatant (S9). COS-7 cells were seeded into 10-cm culture flasks in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. When cells were 90 to 95% confluent, the culture medium was replaced with DMEM without penicillin and streptomycin, and the DMEM containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. After centrifugation for 30 min at 12,000 rpm, the supernatants were reduced to 100 µl by warming at 65°C and subjected to high performance liquid chromatography assay. High performance liquid chromatography was carried out on a SB-300A C18 10-µm column (4.6 × 200 mm, Agilent Technologies, Palo Alto, CA) using a mobile phase of 0.1 M NaH2PO4, pH 6.0/acetonic acid (7:3), at a flow rate of 1 ml/min. Detection was by UV absorption at 371 nm. Under these conditions, retention times of 5'-hydroxylornoxicam and lornoxicam were 6.9 and 11.8 min, respectively. A six-point standard curve was used to quantify 5'-hydroxylornoxicam.

In Vivo Lornoxicam Metabolism. The study was approved by the Independent Ethics Committee of the People’s Hospital of Liaoning Province (Shenyang, China). Genotyping of *CYP2C9*3 and *CYP2C9*13 was carried out as described previously (Si et al., 2004; Zhang et al., 2005). Thirteen *CYP2C9*1 homozygotes, 7 *CYP2C9*3 heterozygotes, and 3 *CYP2C9*13 heterozygotes participated in the phenotyping study. All subjects were in good health and were required to refrain from all medication and alcohol prior to the pharmacokinetic study. In vivo lornoxicam metabolism was performed according to the method described previously (Zhang et al., 2005). In brief, after a single oral dose of 8 mg of lornoxicam, blood samples were collected before dosing, and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 13, and 24 h postdose. Plasma concentrations of lornoxicam were determined using a validated liquid chromatography-tandem mass spectrometry method reported elsewhere (Zeng et al., 2004).

Quantification of CYP2C9 Protein by Western Blotting. S9 fraction (50 µg) and human liver microsomes (10 µg) were separated on 10% sodium dodecyl sulfate-polyacrylamide gels and transferred to a polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA). The membrane was incubated with rabbit anti-human cytochrome P450 2C9 antibody as the primary antibody and then with alkaline phosphatase-labeled anti-rabbit IgG as the secondary antibody. Bands were visualized by incubation with BCIP/NBT and quantified by microscans from insect cells expressing human CYP2C9 (Invitrogen) as a standard with ImageJ software (National Institutes of Health, Bethesda, MD).

In Vitro Lornoxicam 5'-Hydroxylation of Recombinant CYP2C9 Protein. Lornoxicam 5'-hydroxylation activity of the recombinant CYP2C9 protein was determined as described previously (Kohl et al., 2000) with minor modifications. S9 fraction containing CYP2C9s was incubated with 100 mM Tris buffer (pH 7.5), 200 µM NADPH, and lornoxicam at 37°C for 1 h in the presence or absence of CPP. The reaction was stopped by addition of 500 µl of methanol and stored overnight at −20°C to allow complete protein precipitation. After centrifugation for 30 min at 12,000 rpm, the supernatants were reduced to 100 µl by warming at 65°C and subjected to high performance liquid chromatography assay. High performance liquid chromatography was carried out on a SB-300A C18 10-µm column (4.6 × 200 mm, Agilent Technologies, Palo Alto, CA) using a mobile phase of 0.1 M NaH2PO4, pH 6.0/acetonic acid (7:3), at a flow rate of 1 ml/min. Detection was by UV absorption at 371 nm. Under these conditions, retention times of 5'-hydroxylornoxicam and lornoxicam were 6.9 and 11.8 min, respectively. A six-point standard curve was used to quantify 5'-hydroxylornoxicam.

Fig. 1. CYP2C9 protein levels in postmitochondrial supernatant (S9) from COS-7 cells expressing wild-type and variant CYP2C9s. A, immunoblot analysis of recombinant human CYP2C9 protein. S9 fraction (50 µg) isolated from COS-7 cells transfected with the wild-type and variant CYP2C9 cDNA was utilized for immunoblotting using anti-human CYP2C9 antibody. Human liver microsome (10 µg) was used as positive control. B, protein levels of CYP2C9 were quantified by densitometric analysis. The results are expressed as a percentage of the level of CYP2C9*1. Each bar represents the mean ± S.E.M. of three independent experiments.
Results

A representative immunoblot of S9 fraction prepared from COS-7 cells expressing CYP2C9*1, CYP2C9*3, and CYP2C9*13 proteins is presented in Fig. 1. All constructs yielded immunodetectable CYP2C9 protein, as did human liver microsomes. The expressed protein level of CYP2C9*1 was 7.51 pmol/mg S9 protein, and the expression levels of variant CYP2C9*3 and *13 were 69.9% and 35.5%, respectively, of that of CYP2C9*1.

The effect of exogenous CPR on lornoxicam 5'-hydroxylation was studied by incubation with recombinant CYP2C9*1. S9 fraction containing 3.75 pmol of CYP2C9*1 was mixed with varying amounts of CPR and 10 µM lornoxicam. When CPR was 0.5, 12.5, or 25 µM, the mean value of $V_{\text{max}}$ for CYP2C9*1 was 0.66, 0.74, 0.68, or 0.68 pmol/min/pmol, respectively. There was no significant alteration in catalytic efficiency of CYP2C9*1 with the increasing CPR concentration, indicating that endogenous reductase is enough for lornoxicam metabolism in the COS-7 expression system. Thus, exogenous CPR was not utilized in the following kinetic study.

Michaels-Menten analysis was performed by nonlinear regression curve fitting using the computer program Prism v4.0 (GraphPad Software Inc., San Diego, CA). Pharmacokinetic parameters were calculated using standard noncompartmental methods. Student’s $t$ test was used for intergroup comparison. A value of $P < 0.05$ was considered to be statistically significant.

Discussion

To investigate the catalytic activity of the CYP2C9*13 allele in vitro, we established a COS cell expression system. This has been widely applied for functional characterization of P450 alleles containing CYP2C9, CYP2D6, CYP2B6, and CYP2E1 (Veronese et al., 1993; Hu et al., 1997; Marcucci et al., 2002; Jinno et al., 2003). However, due to the low levels of expression in COS-7 cells, we failed to quantify CYP2C9 holoenzyme contents by CO-difference spectroscopy. Thus, CYP2C9 proteins were quantified by immunoblotting with microsomes from insect cells expressing human CYP2C9 as standard. This kind of method quantifying P450 was also used for the functional characterization of CYP2D6, CYP2B6, and CYP2E1 allelic variants in the COS expression system (Marcucci et al., 2002; Hanioka et al., 2003; Jinno et al., 2003). Our results show that COS-7 cells can efficiently express active CYP2C9 protein. The protein levels of cDNA-expressed CYP2C9*3 and *13 in S9 fraction from COS-7 cells were lower than those in wild-type CYP2C9*1. The reduced protein levels in the CYP2C9 variants may contribute to

TABLE 1

<table>
<thead>
<tr>
<th>Variant</th>
<th>$K_m$ (µM)</th>
<th>$V_{\text{max}}$ (pmol/min/mg)</th>
<th>$V_{\text{max}}/K_m$ (µmol/min/pmol)</th>
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<tbody>
<tr>
<td></td>
<td>Protein</td>
<td>P450</td>
<td>Protein</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CYP2C9*1</td>
<td>1.24 ± 0.09</td>
<td>6.23 ± 0.14</td>
<td>5.10 ± 0.11</td>
</tr>
<tr>
<td>CYP2C9*3</td>
<td>1.61 ± 0.30</td>
<td>1.47 ± 0.23**</td>
<td>0.90 ± 0.14**</td>
</tr>
<tr>
<td>CYP2C9*13</td>
<td>2.79 ± 0.26**</td>
<td>0.61 ± 0.06**</td>
<td>0.23 ± 0.02**</td>
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</tbody>
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** $P < 0.01$ versus CYP2C9*1.
lower transcription, translation efficiency, and protein stability. Otherwise, although polyclonal antibody was used, the immunoreactivity of mutant CYP2C9s may be altered by maturation. Therefore, the enzymatic activities were assayed in two ways, on the basis of S9 protein level and CYP2C9 protein level. These data obtained using the COS expression system need to be confirmed by a baculovirus system, which is better suited to obtain the quantity of P450 by spectral analysis. These studies are currently under investigation in our laboratory.

In this study, the presence of the CYP2C9*3 allele impairs both intrinsic clearance and systemic clearance of lornoxicam. A recent report shows that CYP2C9*1/*3 individuals have a 55% decrease in CL/F and a 1.9-fold increase in AUC of lornoxicam compared with CYP2C9*1/*1 individuals (Zhang et al., 2005). The magnitude of these changes in pharmacokinetic parameters is consistent with our in vivo results. Iida et al. (2004) reported that CYP2C9*3 expressed in baculovirus-infected insect cells significantly decreased lornoxicam 5'-hydroxylation relative to wild type with a 2.3-fold increase in 5'-hydroxylation relative to wild type with a 2.3-fold increase in AUC of lornoxicam compared with individuals with CYP2C9*3. Interestingly, in our study, individuals with CYP2C9*1/*13 genotype have an impaired clearance of lornoxicam compared with individuals with CYP2C9*1/*1 genotype. Interestingly, in our study, individuals with CYP2C9*1/*1 and CYP2C9*1/*13 genotypes reveal the same extent of reduction in oral clearance of lornoxicam despite the fact that, in vitro, CYP2C9*13 is associated with a lower intrinsic clearance of lornoxicam than is CYP2C9*3.

Given the small number of CYP2C9*1/*13 subjects studied (n = 3), and in the absence of any individuals homozygous for the CYP2C9*13 allele, we recognize that further in vivo studies are required to draw firm conclusions about the role of the CYP2C9*13 allele. According to a crystal structure of CYP2C9 published by Williams et al. (2003) and Wester et al., (2004), Leu90 is located in the B-B loop, which is not the heme-binding region and far from the binding pocket of substrate. Thus, the reason for the increase in Kₘ for lornoxicam 5'-hydroxylation is not clear. Homology modeling based on the crystal structure of human CYP2C9 is ongoing in our laboratory (Wester et al., 2004).

Our results show that the activities of both CYP2C9*3 and CYP2C9*13 toward lornoxicam in vitro are compatible with their activities in vivo, and there is a reasonable correlation between in vitro activity and in vivo metabolic clearance of lornoxicam. Recently, it was reported that individuals carrying the CYP2C9*3 allele are at risk of experiencing drug toxicity, especially of drugs with a narrow therapeutic index such as warfarin and phenytoin (Athyal et al., 1999; Kidd et al., 1999; Ninomiya et al., 2000). By extrapolation, one may speculate that carriers of the CYP2C9*13 allele would experience greater risk from these drugs. Therefore, genotyping for CYP2C9*13 may be important to allow individualization of dosing for CYP2C9 substrate drugs.

In conclusion, the Leu90Pro substitution of CYP2C9*13 markedly decreases the intrinsic clearance of lornoxicam in vitro and in vivo. The reduction in activity due to CYP2C9*13 is greater than that due to CYP2C9*3 in vitro. Whether carriers of the CYP2C9*13 allele may be at greater risk of toxicity from CYP2C9 substrate drugs with a narrow therapeutic index remains to be confirmed by further in vivo studies.

References


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