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Catalytic activities of human cytochrome P450 2C9*1, 2C9*3 and 2C9*13

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Abstract
Cytochrome P450 2C9 (CYP2C9) is a geneticly polymorphic enzyme responsible for the metabolism of some clinically important drugs. *CYP2C9*13* is an allele identified in a Chinese poor metabolizer of lornoxicam which has a Leu90Pro amino acid substitution. This paper reports on a study aimed at comparing the catalytic properties of CYP2C9*13 with those of the wild-type CYP2C9*1 and mutant CYP2C9*3 (Ile359Leu) in the COS-7 expression system using various substrates. CYP2C9*3 and *13 produced far lower luminescence than CYP2C9*1 in luciferin H metabolism. CYP2C9*13 exhibited an 11-fold increase in K_m but no change in V_{max} with tolbutamide as the substrate, a five-fold increase in K_m and an 88.8% reduction in V_{max} with diclofenac. These data indicate that CYP2C9*13 exhibits reduced metabolic activity toward all studied CYP2C9 substrates. The magnitude of the CYP2C9*13-associated decrease in intrinsic clearance (V_{max}/K_m) is greater than that associated with CYP2C9*3.

Keywords: *Cytochrome P450 2C9, allelic variant, catalytic activity*

Introduction

Cytochrome P450 (CYP) enzymes are products of a supergene family with a major role in the oxidation of both xenobiotic and endogenous compounds. In human, the CYP2C subfamily accounts for approximately 18% of the CYP450 protein content of human liver microsomes and catalyses approximately 20% of drugs in current clinical use (Rendic and Di Carlo 1997; Parkinson et al. 1996). Over the last few years, multiple single nucleotide polymorphisms (SNPs) have been identified in both the coding and the non-coding regions of the *CYP2CP* gene. Currently, at least thirteen *CYP2C9* alleles have been identified as

designated by the human CYP Allele Nomenclature Committee (http://www.imm.ki.se/ CYPalleles/cyp2c9.htm).

Most of the variant alleles are associated with impaired enzyme activity. The most widely studied allele, *CYP2C9*3* (Ile359Leu), is present in approximately 10–15% of Caucasians and is less frequent in the Negro and Asain populations (Lee et al. 2002; Xie et al. 2002). A number of investigations have demonstrated significantly lower intrinsic clearance of certain CYP2C9 substrates in cells expressing the *CYP2C9*3* allele compared with wild-type (Lee et al. 2002). In addition, *in vivo* studies have shown that in homozygous carries of the *3* allele, mean clearances of *S*-warfarin, tolbutamide, glipizide, celecoxid and fluvastatin were 25% below that of the wild type (Kirchheiner and Brockmoller 2005).

The *CYP2C9*13* (Leu90Pro) variant allele occurs is approximately 2% of the Chinese populations (Si et al. 2004). An individual with *CYP2C9*3/*13* genotype was found to have a much lower clearance of lornoxicam (half-life of about 105 h) than those with *CYP2C9*1/*3* and *CYP2C9*1/*1* genotype (half-lives of 5.8–8.1 and 3.2–6.3 h, respectively) (Zhang et al. 2005). Similarly, in three subjects with *CYP2C9*1/*13* genotype, the AUC of lornoxicam was increased 1.9-fold and its CL/F decreased 44% compared with *CYP2C9*1/*1* carriers (unpublished data). To characterize further the catalytic activity of CYP2C9*13 *in vitro*, we separately expressed CYP2C9*1, CYP2C9*3 and CYP2C9*13 proteins in COS-7 cells and compared their functional differences with three CYP2C9 substrates, one of which was luminescent.

Materials and methods

Materials

Diclofenac and 4′-hydroxydiclofenac were purchased from Merck Biosciences (Darmstadt, Germany). Tolbutamide was purchased from Guangzhou Hotspring Pharmaceutical Co., Ltd (Guangzhou, China). Hydroxytolbutamide was provided by the Laboratory of Microorganisms, Shenyang Pharmaceutical University (Shenyang, China). Dulbecco's modified Eagle's medium, pcDNA3.1(+) and Lipofectamine™ 2000 were purchased from Invitrogen (Carlsbad, CA, USA). Foetal bovine serum was purchased from Tianjin, H&Y Bio Co. Ltd (Tianjing, China). *Kpn*I, *Xho*I and *Dpn*I enzymes were purchased from New England Bio Labs (Beverly, MA, USA). *Pfu* DNA polymerase was purchased from Bio Basic, Inc. (Toronto, Canada). A 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 Ltd (Oxford, UK). Alkaline phosphatase-labelled 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 (Basle, Switzerland). COS-7 cells were kindly donated by the Vaccination Center, Jilin Uniersity (Changchun, China). All other reagents were of analytical grade.

Construction of expression plasmids

*CYP2C9*1* cDNA in pREP9 plasmid was subcloned into pcDNA3.1(+) by digestion with *Kpn*I and *Xho*I enzyme. 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 pcDNA3.1(+) plasmids carrying *CYP2C9*1* cDNA as the template for polymerase chain reaction (PCR) 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′-CGA GGT CCA GAG ATA CCT TGA CCT TCT CCC CAC-3′ and 5′-GGA AGC CCT GAT TGA TCC TGG AGA GGA GTT TTC TG-3′, respectively (mutations underlined). After incubation with *Dpn*I enzyme, the original templates were digested but the new amplified PCR products containing substituted base remained and were transformed to *E. coli* Top 10. Clones containing the desired nucleotide change were confirmed by sequencing carried out by Shanghai Sangon Biological Engineering Technology & 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 Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum, $100 \, U \, ml^{-1}$ penicillin and $100 \, \mu g \, ml^{-1}$ streptomycin. When cells were 90–95% confluent, the culture medium was replaced with DMEM without penicillin and streptomycin, and the CYP2C9 expression plasmids ($24 \, \mu g$/flask), purified with QIAGEN plasmid mini kit (Qiagen, Valencia, CA, USA), were transfected into COS-7 cells using $60 \, \mu l$/flask of Lipofectamine 2000 as per the manufacturer's instructions. Forty-eight hours after transfection, cells were scraped from the culture flask and washed twice with Ca^{2+}- and Mg^{2+}- free Hank's solution. The pellets were resuspended in 20 mM potassium phosphate buffer pH 7.4 containing 0.2 mM EDTA, 1 mM dithiothreitol and 20% glycerol, and sonicated with twelve 5-s pulses at 23% power of a SONICS Vibra-Cell sonicator (Sonics & Materials Inc., Newtown, CT, USA). The homogenate was centrifuged at $9000g$, $4°C$ for 20 min and the postmitochondrial supernatant (S9 fraction) collected for assay or storage at $-70°C$. Protein concentrations in S9 were determined by the Bradford method using bovine serum albumin as standard.

Quantification of CYP2C9 protein by Western blotting

S9 fraction ($50 \, \mu g$) was separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore Corp., Billerica, MA, USA). The membrane was incubated with rabbit anti-human cytochrome P450 2C9 antibody for dilution (1:5000) as the primary antibody and then with alkaline phosphatase-labelled anti-rabbit IgG for dilution (1:500) as the secondary antibody. Bands were visualized by incubation with BCIP/NBT and quantified by microsomes from insect cells expressing human CYP2C9 (invitrogen) as a standard with ImageJ software (National Institutes of Health, Bethesda, MA, USA).

Luciferase assay

CYP2C9 activity was measured using luciferin H in the P450^{TM}-Glo assay kit (Promega, Madison, WI, USA). Metabolism was assessed by measurement of luciferase activity according to the manufacturer's instructions. Briefly, luciferin H (100 or $400 \, \mu M$) was preincubated with S9 fraction containing 0.5 pmol CYP2C9s at $37°C$ for 10 min. The reaction was initiated by adding $100 \, \mu M$ NADPH. It was terminated after 90-min incubation at $37°C$ by the addition of reconstituted luciferin detection reagent.

Luminescence was recorded using TECAN GENios (Salzburg, Austria). Results are expressed as relative light units (RLUs) ± SEM of three independent experiments.

Tolbutamide and diclofenac activities

Activities of tolbutamide methylhydroxylase and diclofenac 4′-hydroxylase were determined using HPLC as described (Takanashi et al. 2000). Incubation media contained either tolbutamide (50–800 μM) or diclofenac (1.25–100 μM) as substrate, i.e. S9 fraction of COS-7 cells corresponding to 500 μg protein and 200 μM NADPH in 100 mM Tris buffer (pH 7.5) in a final volume of 200 μl. Incubation was carried out at 37°C for 60 min for tolbutamide, and for 30 min for diclofenac. After terminating reactions by addition of 500 μl methanol, samples were stored overnight at −20°C to allow complete protein precipitation. After centrifugation for 30 min at 12 000 rpm, the supernatant from diclofenac incubation was reduced to 100 μl by warming at 65°C and it was assayed by HPLC. In the case of tolbutamide, a 200-μl aliquot of supernatant was analysed by HPLC.

HPLC analysis was performed on a reversed-phase Waters ZORBAX300SB-C18 column (Agilent, PN880995-902 5 μm, 4.6 × 250 mm). The assay of hydroxytolbutamide involved a mobile phase of 0.05 M potassium phosphate buffer (pH 6.6): acetonitrile (80:20 v/v) at a flow rate of 0.7 ml min^{-1} and UV detection at 230 nm. The assay of 4′-hydroxydiclofenac employed 0.05 M potassium phosphate buffer (pH 7.4): acetonitrile (70:30 v/v) at 0.8 ml min^{-1} with detection at 280 nm. Standard curves for the three assays were prepared using spiked incubation samples.

Data analysis

Michaelis-Menten analysis was performed by non-linear regression using the computer program Prism v4.0 (Graphpad Software Inc., San Diego, CA, USA). Intergroup comparisons were made by Student's t-test. $p < 0.05$ was considered to be statistically significant.

Results

Expression of wild-type and variant CYP2C9s in COS-7 cells

In order to transfect more efficiently the target gene CYP2C9 into COS-7 cells, CYP2C9*1 cDNA from pREP9 vector digested by KpnI and XhoI enzyme was subcloned to pcDNA3.1(+) vector. CYP2C9*3 and CYP2C9*13 were generated by site-directed mutagenesis on the basis of pcDNA3.1-CYP2C9. The gene of wild-type CYP2C9 and two variants was transiently transfected into COS-7 cells. The expression of CYP2C9 protein in S9 fraction was examined by Western blotting (Figure 1). All construct yielded immunodetectable CYP2C9 protein. The expressed protein level of CYP2C9*1 was 7.51 ± 1.2 pmol mg^{-1} S9 protein, and the protein levels of variant CYP2C9*3 or 13 were 63 or 39% of that of CYP2C9*1 in five independent transfections.

Catalytic activities

CYP2C9 activity was measured by luminescence using the P450^{TM}-Glo assay kit (Figure 2). The results showed that CYP2C9*1 enzyme activity is far greater (43 141 ± 1110 RLUs) than CYP2C9*3 andCYP2C9*13 (5000 ± 325 and 1208 ± 257 RULs, respectively) at a
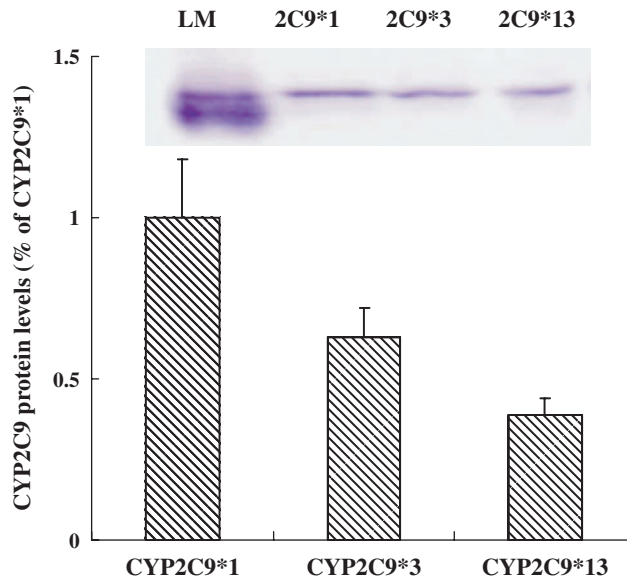
Figure 1. CYP2C9 protein levels in postmitochondrial supernatant (S9) from COS-7 cells expressing wild-type and variant CYP2C9s. S9 fraction (50 µg) isolated from COS-7 cells transfected with the wild-type and variant *CYP2C9* cDNA was used for immunoblotting using anti-human CYP2C9 antibody. Human liver microsome LM (10 µg) was used as a positive control. Results are a percentage of the level of CYP2C9⋆1 by densitometric analysis. Each bar represents the mean ± SEM of five independent experiments.
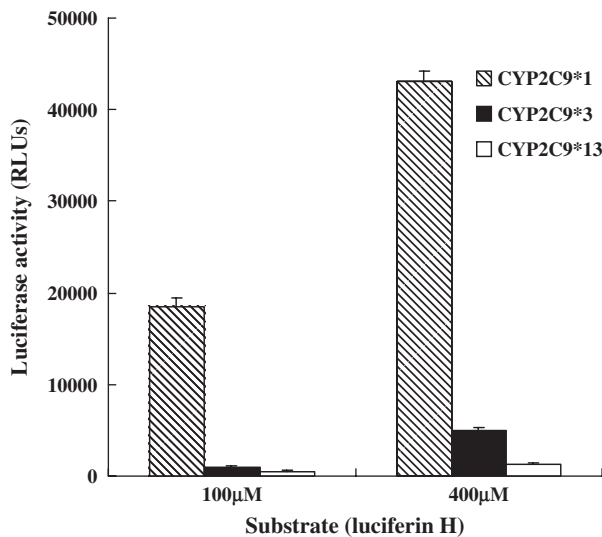

Figure 2. Luciferase assay by postmitochondrial supernatant (S9) from COS-7 cells expressing wild-type and variant CYP2C9s. Catalytic activities of wild-type and variant CYP2C9s were determined using 100 and 400 µM luciferin substrate (luciferin H) as described in the Materials and methods. After 90-min incubation, luciferase activity was assessed. Results are luciferase activity in RLUs and are the mean ± SEM of three independent experiments.
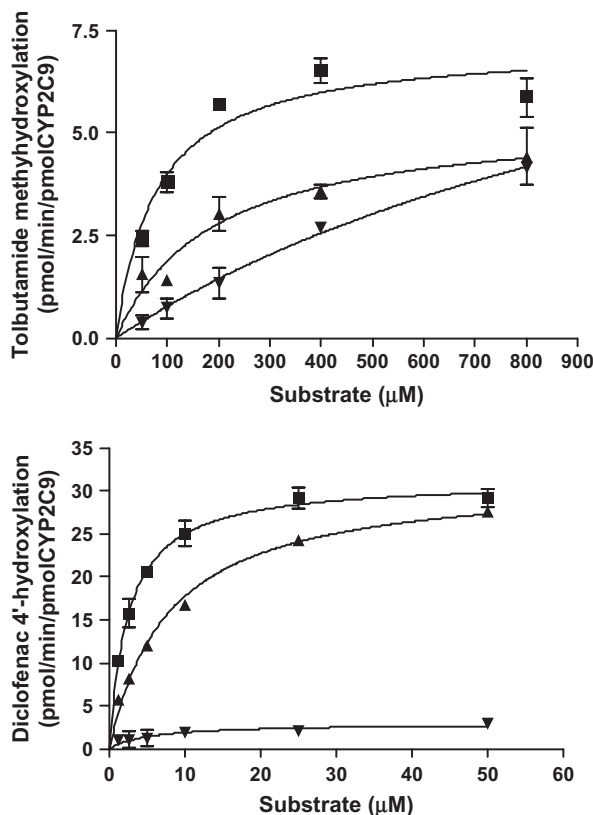
Figure 3. Michaelis–Menten kinetics of tolbutamide, diclofenac and lornoxicam by postmitochondrial supernatant (S9) from COS-7 cells expressing wild-type and variant CYP2C9s. Experimental conditions are described in the Material and methods. Each point is the mean of three independent experiments: ■, CYP2C9*1; ▲, CYP2C9*3; and ▼, CYP2C9*13.

substrate concentration of 400 µM. The same result was also observed at a substrate concentration of 100 µM.

To characterize further the enzyme, tolbutamide and diclofenac were used as substrates to compare catalytic efficiencies of CYP2C9*1, CYP2C9*3 and CYP2C9*13. Michaelis–Menten plots for each substrate are shown in Figure 3, and the calculated K_m, V_{max} and V_{max}/K_m values are summarized in Table I.

Compared with wild-type, K_m values of CYP2C9*3 were four-fold higher for tolbutamide and three-fold higher for diclofenac. Corresponding V_{max} values were unchanged. The reduction in intrinsic clearance (V_{max}/K_m) was 74% for tolbutamide and 63% for diclofenac.

Compared with wild-type, the K_m values of CYP2C9*13 for tolbutamide and diclofenac were increased 11- and five-fold, respectively. Corresponding V_{max} values were unchanged for tolbutamide and were reduced by 11.2% for diclofenac. Intrinsic clearances were decreased by 9.2 and 2.5% for tolbutamide and diclofenac, respectively.

Discussion

CYP2C9 polymorphisms are particularly relevant to the metabolism of drugs with narrow therapeutic indexes and mutant *CYP2C9* alleles are associated with increased risk

Table I. Kinetic parameters for tolbutamide methylhydroxylation and diclofenac 4′-hydroxylation by postmitochondrial supernatant (S9) from COS-7 cells expressing wild-type and variant CYP2C9s.

		K_m (μM)	V_{max} (pmol mol^{-1} pmol^{-1})	V_{max}/K_m (μl min^{-1} pmol^{-1})
Tolbutamide methylhydroxylation	CYP2C9*1	105 ± 0	7.96 ± 0.17	0.076 ± 0.007
	CYP2C9*3	397 ± 20**	8.04 ± 0.07	0.020 ± 0.001**
	CYP2C9*13	1174 ± 188**	7.73 ± 0.15	0.007 ± 0.001**
Diclofenac 4′-hydroxylation	CYP2C9*1	2.33 ± 0.17	27.15 ± 0.94	11.71 ± 0.56
	CYP2C9*3	6.67 ± 0.83**	28.67 ± 3.26	4.34 ± 0.26**
	CYP2C9*13	11.60 ± 2.56**	3.04 ± 0.24**	0.29 ± 0.01**

Each value is the mean $\pm$ SEM of three independent experiments. **$p < 0.01$ versus CYP2C9*1

of adverse events. Thus, individuals carrying *CYP2C9*2* and *CYP2C9*3* alleles have lower warfarin and phenytoin dose requirements and appear more susceptible to adverse events during the initiation of therapy (Lee 2004). Other studies have revealed that a Japanese *CYP2C9*1/*4* individual had a metabolic clearance of phenytoin as slow as that of *CYP2C9*1/*3* individuals (Ieiri et al. 2000) and an African-American individual homozygous for the *CYP2C9*6* null allele experienced severe phenytoin toxicity associated with an elimination half-life of 13 days and a phenytoin clearance only 17% of that seen in the general population (Kidd et al. 2001). These results emphasize the importance of fully characterizing any new *CYP2C9* allele. Previous studies of the *CYP2C9*13* allele show it is associated with low clearance of lornoxicam. In the present study, the enzymatic activities of the variant *CYP2C9*3* and *CYP2C9*13* alleles expressed in COS-7 cells were compared with those expressing wild-type using a luminescent substrate and two routine CYP2C9 substrates.

The COS-7 cell expression system has been widely applied for functional characterization of *CYP* allelic variants (Veronese et al. 1993; Hu et al. 1997; Marcucci et al. 2002; Jinno et al. 2003). Due to the low level of expression in COS-7 cells, spectroscopy cannot be used to determine absolute P450 content. Instead, the expression levels of CYP2C9 protein have been quantified by immunoblotting with microsomes from insect cells expressing human CYP2C9s. This method of quantifying P450 has also been used for the functional characterization of CYP2D6, CYP2B6 and CYP2E1 allelic variants (Marcucci et al. 2002; Hanioka et al. 2003; Jinno et al. 2003).

The protein levels of cDNA-expressed CYP2C9*3 and CYP2C9*13 in S9 fraction from COS-7 cells were lower than from cells expressing wild-type CYP2C9*1. We excluded the possibility that the immunoreactivity of CYP2C9 variants was altered by mutagenesis because polyclonal antibody was used in the immunoblotting assay. Thus, it is likely that reduced protein levels in the CYP2C9 variants contribute to lower transcription or translation efficiency or protein stability.

Our studies clearly show that relative to wild-type, CYP2C9*13 has lower intrinsic clearance of all three investigated substrates. The reductions were the result of increases in K_m and decreases or on changes in V_{max}. The substrate dependence of intrinsic clearance has been previously observed with other *CYP2C9* alleles such as *CYP2C9*3* and *CYP2C9*5* (Takanashi et al. 2000; Dickmann et al. 2001). However, the magnitude of the CYP2C9*3-associated changes in kinetic parameters appears to be dependent on the expression system used. Thus, Takanashi et al. (2002) reported that the tolbutamide K_m was 11-fold higher than wild-type for CYP2C9*3 expressed in yeast cells whereas it is only four-hold higher than wild-type in our COS-7 cells. Similarly, Ieiri et al. (2000) reported that CYP2C9*3

expressed in yeast cells had an eight-fold higher K_m for diclofenac, whereas in our COS-7 cells the K_m was three-fold higher relative to wild-type. The reason for these differences is unclear, but we showed it is not due to insufficient cytochrome P450 reductase in our system (data not shown).

CYP2C9*13 gives rise to lower intrinsic clearances than CYP2C*3 due to greater increases in K_m and greater decreases in V_{max}. The lower affinity and intrinsic clearances for CYP2C9*3 relative to wild-type is because the Ile359Leu substitution is located in the region coding for substrate recognition site 5 of the CYP2C9 protein (Gotoh 1992). However, according to a crystal structure of CYP2C9 published by Williams et al. (2003) and Wester et al. (2004), the Leu90Pro substitution in CYP2C9*13 is located in a non-haeme-binding region far from the substrate binding pocket. Thus, the reason for the CYP2C9*13-mediated reduction in drug-metabolizing capability is unclear. Homology modelling based on the crystal structure of human CYP2C9 is ongoing in our laboratory (Wester et al. 2004).

In conclusion, CYP2C9*13 markedly decreases the intrinsic clearance of substrates relative to wild-type in a substrate-dependent manner consistent with *in vivo* observations. Whether carriers of the *CYP2C9*13* allele are at greater risk of toxicity from CYP2C9 substrate drugs with narrow therapeutic indices remains to be confirmed.

Acknowledgements

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