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The role of CYP2C9 genetic polymorphisms in the oxidative metabolism of diclofenac *in vitro*

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Received May 22, 2014, accepted June 27, 2014

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Pharmazie 69: 898–903 (2014) *doi: 10.1691/ph.2014.4669*

CYP2C9 is one of four known members of the human cytochrome P450 CYP2C superfamily, with at least 57 CYP2C9 alleles being previously identified. Genetic polymorphisms of CYP2C9 significantly influence the efficacy and safety of some drugs, which might cause adverse effects and therapeutic failure. The purpose of the present study was to clarify the role of 36 CYP2C9 alleles, 21 novel alleles (*36-*56) found in the Chinese population, in the oxidative metabolism of diclofenac *in vitro*. Insect microsomes expressing the 36 human CYP2C9 alleles were incubated with 2-100 μM diclofenac for 30 min at 37 °C and terminated by the addition of 30 μL 0.1 M HCl. Diclofenac and 4'-hydroxyl (OH)-diclofenac, the major diclofenac metabolite, were analyzed by high-performance liquid chromatography (HPLC). Compared with wild-type CYP2C9*1, most variants showed significantly altered values in V_{max}, K_m and intrinsic clearance (V_{max}/K_m). Only one variant exhibited markedly increased intrinsic clearance value, whereas 31 variants exhibited significantly decreased values. Thus, this study demonstrated that more attention should be given to subjects carrying these CYP2C9 alleles when administering diclofena.

1. Introduction

Human cytochrome P450 2C9 (CYP2C9) accounts for ∼20% of total hepatic CYP protein content (Zhou et al. 2010). As a major member of the CYP2C subfamily, CYP2C9 is responsible for the metobolism of approximately 15% clinically used drugs, including the antidiabetic agents tolbutamide, glimepiride and glibenclamide, the anticonvulsant phenytoin, the antihypertensive drugs losartan and irbesartan, the anticoagulants warfarin, the diuretic torasemide and most of nonsteroidal anti-inflammatory drugs such as piroxicam, ibuprofen, tenoxicam and diclofenac (Schwarz 2003; Zhou et al. 2009). The human CYP2C9 gene is highly polymorphic, and its polymorphisms are relevant for the adverse effects and efficacy of numerous nonsteroidal anti-inflammatory agents, including diclofenac (Wang et al. 2009). Up to date, there are at least 57 variants of CYP2C9 (*1-*57) being reported and identified offically (see http://www.cypalleles.ki.se). Among them, CYP2C9 alleles *2 and *3, the most common allelic variants, have been well studied both *in vivo* and *in vitro* over the last few years (Ali et al. 2009; Tang et al. 2001; Yasar et al. 2001).

Diclofenac is a nonsteroidal anti-inflammatory drug (NSAID) used to reduce inflammation and as an analgesic reducing pain in conditions such asgout attacks, arthritis, mild to moderate post-operative or post-traumatic pain and menstrual pain (Wang et al. 2009). In humans, diclofenac is extensively metabolized to a number of hydroxylated metabolites, such as 4'-hydroxy-, 3'-hydroxy-, 5-hydroxy- and 4', 5-dihydroxy-diclofenac. 4'-hydroxy (OH)-diclofenac, the major metabolite both *in vivo* and *in vitro*, is mainly formed by the hepatic cytochrome P450 2C9 (Shen et al. 1999; Tang et al. 1999; Yasar et al. 2001). Furthermore, diclofenac (4'-hydroxylation) have been commonly used as a probe substrate for CYP2C9 (Kumar et al. 2006). Previous reports revealed that the diclofenac/4'-hydroxyl (OH)-diclofenac ratios were associated with CYP2C9 genotype *in vivo*, and the ratio was significantly higher among subjects with CYP2C9*1/*3 and CYP2C9*2/*3 genotypes compared to CYP2C9*1/*1 (Dorado et al. 2003). Moreover, absence of clinically significant effects in the CYP2C9 variants-mediated metabolism of diclofenac has been reported in several *in vivo* studies. However, the *in vitro* effects of CYP2C9 variants on the extent of the reduction in 4'-hydroxyl (OH)-diclofenac were less pronounced than those observed with other CYP2C9 substrates such as tolbutamide, phenytoin and warfarin (Takanashi et al. 2000). Taking all the factors into consideration, further CYP2C9 *in vitro* studies in a larger number of variants are required.

In a recent study, the CYP2C9 polymorphisms were analyzed in 2127 people of Han population, and 21 novel alleles were discovered and named CYP2C9*36 ∼ CYP2C9*56 (Dai et al. 2013). In the present study, we intend to assess the catalytic activities of 36 CYP2C9 alleles, including wild-type CYP2C9*1 and the 21 novel identified variants, toward diclofenac in insect cell microsomes expressing corresponding CYP2C9 allelic variants, thus providing valuable information for further studies on CYP2C9 alleles for diclofenac metabolism.

2. Investigations and results

In this study, the catalytic activities of 36 CYP2C9 alleles were detected using diclofenac as the probe substrate. Michaelis-Menten kinetics of diclofenac for wild-type CYP2C9 and 35

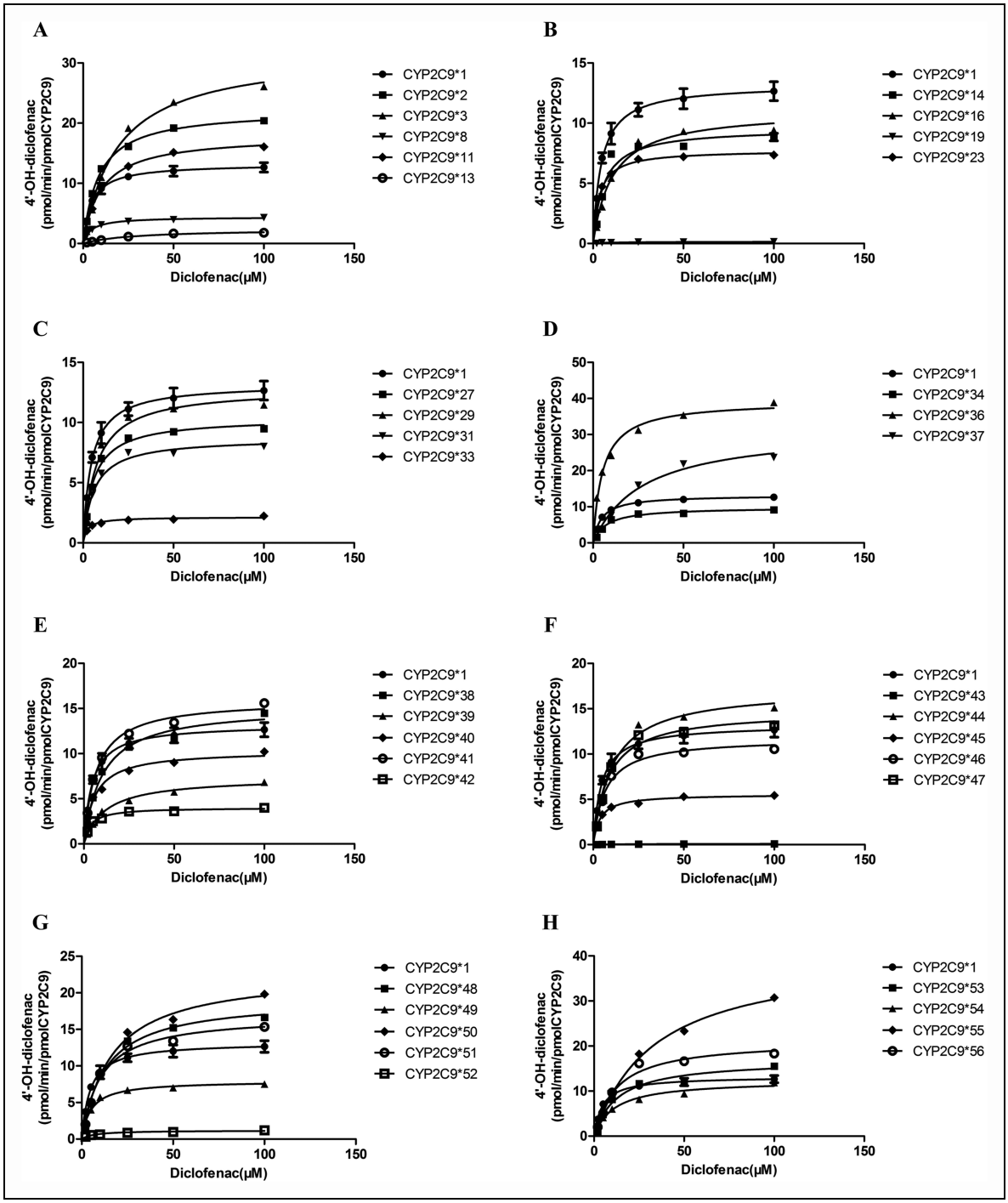

Fig. 1: Michaelis–Menten curves of the enzymatic activity of the recombinant wild-type CYP2C9*1 and 35 variants toward diclofenac.

allelic variants are presented in Fig. 1, and the corresponding kinetic parameters are summarized in the Table.

As shown in Table 1, most variants showed significantly altered values in V_{max}, K_m and intrinsic clearance (V_{max}/K_m) compared with wild-type CYP2C9*1. Only one variant exhibited markedly increased intrinsic clearance value, whereas 31 variants exhibited significantly decreased values. CYP2C9*2, *23 and *41 had no significant difference in intrinsic clearance of diclofenac compared with wild-type protein. CYP2C9*13, *19, *43 and *52 showed significantly decreased V_{max} and increased K_m values, as a result, the the intrinsic clearance values for these variants were decreased to 2.92%, 0.49%, 0.21% and 5.97% compared with wild-type protein, respectively. In our study, the V_{max} value for variant CYP2C9*33 was among the lowest. One variant, CYP2C9*36, exhibited a similar K_m value as wild-type protein but displayed a 3-fold increase in the V_{max} value. Therefore, the intrinsic clearance of this variant increased to 256% of wild-type CYP2C9*1.

According to the relative clearance values compared with wild-type protein, the 31 defective isoforms could be manually clarified into 3 categories: 5 variants showed 50-80% relative values to wild-type CYP2C9*1 and were regarded as the mild defective group which included the defective variants CYP2C9*11, *27, *40, *46 and *47. 22 variants (CYP2C9*3, *8, *14, *16, *29, *31, *33, *34, *37, *38, *39, *42, *44, *45, *48, *49, *50, *51, *53, *54, *55 and *56) exhibited 20-50% relative intrinsic clearance values and could be grouped into the moderate defective group. The remaining 4 variants

Table 1: Kinetic parameters of the enzymatic activity of the recombinant wild-type CYP2C9*1 and 35 variants toward diclofenac

Allelic protein	V_{max} (pmol/min/pmol of P450)	K_m (μM)	Clearance V_{max}/K_m	Relative clearance/*1 (%)
CYP2C9*1	13.210 ± 0.5079	4.574 ± 0.7576	2.9027 ± 0.6203	100.00
CYP2C9*2	22.230 ± 0.2799**	8.562 ± 0.3919**	2.5972 ± 0.1010	89.48
CYP2C9*3	31.910 ± 0.6869**	19.090 ± 1.1950**	1.6715 ± 0.0315**	57.58**
CYP2C9*8	4.400 ± 0.04916**	4.405 ± 0.2143	1.0102 ± 0.1261**	34.80**
CYP2C9*11	17.970 ± 0.2429**	10.090 ± 0.4746**	1.7808 ± 0.0175**	61.35**
CYP2C9*13	2.390 ± 0.1008**	28.250 ± 3.0570**	0.0849 ± 0.0086**	2.92**
CYP2C9*14	9.557 ± 0.3572**	5.725 ± 0.8654	1.6691 ± 0.0672**	57.50**
CYP2C9*16	11.050 ± 0.2854**	10.670 ± 0.9443**	1.0357 ± 0.0269**	35.68**
CYP2C9*19	0.154 ± 0.0085**	10.840 ± 2.0350**	0.0143 ± 0.0004**	0.49**
CYP2C9*23	7.758 ± 0.0838**	3.437 ± 0.1742**	2.2616 ± 0.1241	77.91
CYP2C9*27	10.360 ± 0.1597**	5.820 ± 0.3613	1.7802 ± 0.0319**	61.33**
CYP2C9*29	12.840 ± 0.3067	7.399 ± 0.6674**	1.7353 ± 0.0561**	59.78**
CYP2C9*31	8.696 ± 0.2048**	6.032 ± 0.5664**	1.4442 ± 0.1075**	49.76**
CYP2C9*33	2.135 ± 0.0420**	2.425 ± 0.2519**	0.8821 ± 0.0977**	30.39**
CYP2C9*34	9.809 ± 0.2563**	7.134 ± 0.7109**	1.3774 ± 0.0812**	47.45**
CYP2C9*36	39.300 ± 0.6893**	5.293 ± 0.3837	7.4201 ± 0.1235**	255.63**
CYP2C9*37	31.610 ± 1.2950**	27.370 ± 2.9080**	1.1562 ± 0.0254**	39.83**
CYP2C9*38	15.200 ± 0.3812	10.210 ± 0.8879**	1.4947 ± 0.2091**	51.49**
CYP2C9*39	7.361 ± 0.2095**	11.820 ± 1.1220**	0.6251 ± 0.0039**	21.53**
CYP2C9*40	10.320 ± 0.1923**	5.896 ± 0.4409	1.7513 ± 0.1351**	60.33**
CYP2C9*41	15.950 ± 0.2678**	6.840 ± 0.4427**	2.3326 ± 0.0252	80.36
CYP2C9*42	4.029 ± 0.05953**	3.650 ± 0.2484	1.103 ± 0.0672**	38.00**
CYP2C9*43	0.0987 ± 0.0061**	17.020 ± 3.1730	0.006 ± 0.0029**	0.21**
CYP2C9*44	17.200 ± 0.4041**	10.280 ± 0.8364**	1.6733 ± 0.0139**	57.65**
CYP2C9*45	5.554 ± 0.0940**	3.654 ± 0.2847	1.5230 ± 0.1735**	52.47**
CYP2C9*46	11.720 ± 0.2794	6.579 ± 0.6111**	1.7832 ± 0.0211**	61.43**
CYP2C9*47	14.760 ± 0.3621	8.361 ± 0.7502**	1.7644 ± 0.1084**	60.79**
CYP2C9*48	19.220 ± 0.4410**	12.900 ± 0.9632**	1.4927 ± 0.0661**	51.42**
CYP2C9*49	7.945 ± 0.1634**	5.056 ± 0.4352	1.5717 ± 0.0675**	54.14**
CYP2C9*50	22.800 ± 0.5315**	16.360 ± 1.1610**	1.3948 ± 0.0449**	48.05**
CYP2C9*51	16.950 ± 0.4312**	10.400 ± 0.9130**	1.6311 ± 0.0327**	56.19**
CYP2C9*52	1.181 ± 0.0417**	6.886 ± 0.9352**	0.1732 ± 0.0267**	5.97**
CYP2C9*53	16.940 ± 0.6288**	12.980 ± 1.5660**	1.3080 ± 0.0288**	45.06**
CYP2C9*54	12.400 ± 0.3115	11.340 ± 0.9607**	1.0929 ± 0.0463**	37.65**
CYP2C9*55	40.550 ± 0.9189**	33.510 ± 1.8410**	1.2093 ± 0.0525**	41.66**
CYP2C9*56	21.260 ± 0.7608**	12.330 ± 1.4540**	1.7248 ± 0.0531**	59.42**

Data are presented as the mean mean ± SD from four different experiments. **Represents $P < 0.05$ versus wild type.

(CYP2C9*13, *19, *43 and *52) exhibited severely reduced intrinsic clearance values (< 20%) compared with wild-type protein toward diclofenac and could be regarded as the severe defective group.

3. Discussion

Like other P450 CYP2C members, CYP2C9 is highly polymorphic. Up to date, there are at least 57 variants of CYP2C9 (*1-*57) being identified offically (see http://www.cypalleles.ki.se). Previous studies have shown that the frequency of CYP2C9 alleles varies among populations according to the race and ethnic background (Grant and Hakonarson 2007). The two commonly variant alleles, CYP2C9*2 and *3, have been widely investigated in various populations. They were reported to be the most common variants in Caucasian populations with allelic frequencies of 8%–14% and 4%–16%, respectively (Sullivan-Klose et al. 1996). However, the two alleles are found more frequently in Caucasian than in Black populations. It was observed that African-Americans have a significantly lower rate of CYP2C9*2 and *3 than Caucasian populations, with 2.5% and 1.25% frequency, respectively (Wang et al. 2009). In contrast, the CYP2C9*2 and CYP2C9*3 alleles were not observed in the Tarahumara, Purepecha and Huichol populations (Castelan-Martinez et al. 2013). Numerous clinical studies have revealed that the CYP2C9 polymorphisms are relevant for the efficacy and adverse effects of various agents, and polymorphisms in CYP2C9 should be considered in drug therapy (Zhou et al. 2009). In the present study, we systematically assessed the catalytic activities of wild-type CYP2C9 and 35 allelic variants found in the Chinese populations (CYP2C9*1, *2, *3, *8, *11, *13, *14, *16, *19, *23, *27, *29, *31, *33, *34, *36-*56) toward diclofenac *in vitro* in insect cell microsomes expressing corresponding CYP2C9 allelic variants.

Diclofenac is extensively metabolized to a number of hydroxylated metabolites and the main metabolite in urine and plasma is 4'-hydroxy-diclofenac (Davies and Anderson 1997). Various studies have indicated that the 4'-hydroxylation of diclofenac is primarily catalyzed by CYP2C9. In fact, diclofenac has been suggested as a possible phenotyping probe *in vivo* for CYP2C9 activity because single doses of diclofenac are widely used and relatively well tolerated in the clinic (Kumar et al. 2002). It has also been used as an *in vitro* probe for CYP2C9 activity. Up to now, the enzymatic characteristics of CYP2C9*2, *3 and *8 toward diclofenac have been well studied both *in vivo* and *in vitro*, and it was proposed that these defective

alleles displayed decreased or similar relative metabolic clearance toward diclofenac compared with wild-type CYP2C9*1 (Dorado et al. 2003; Llerena et al. 2014; Yasar et al. 2001). Zi et al. (2010) reported that the kinetic parameters of diclofenac 4'-hydroxylation catalyzed by CYP2C9*3 and CYP2C9*13 were tested and compared with wild-type. The results of the study showed that the diclofenac intrinsic clearance values catalyzed by CYP2C9*3 and CYP2C9*13 were significantly lower than that of wild-type enzyme (35% and 6% of wild-type, respectively). In a recent study, Dai et al. analyzed the metabolism characteristics of 21 novel CYP2C9 alleles (CYP2C9*36, *37, *38, *39, *40, *41, *42, *43, *44, *45, *46, *47, *48, *49, *50, *51, *52, *53, *54, *55 and *56) that are present in the Chinese Han population toward the typical CYP2C9 phenotyping probe substrate diclofenac (100 μM diclofenac in a final volume of 300 μL DMEM culture medium) in transfected COS-7 cells, and 17 of the 21 novel variants exhibited altered catalytic activities. Among them, CYP2C9*40 and CYP2C9*54 showed higher metabolic activities toward diclofenac than the wild type (Dai et al. 2013). Although mammalian cells have sufficient endogenous cytochrome b5 and NADPH-CYP oxidoreductase to support CYP activities and are closer to the native state of the CYP proteins, it is difficult to obtain enough protein to precisely assess CYP catalytic activity using mammalian cell expression system.

In the present study, the enzymatic activities of the 35 variants were analyzed and compared with wild-type CYP2C9*1 in insect cell microsomes using diclofenac as substrate. Our study clearly shows that relative to wild-type protein, only one variant showed increased catalytic activity, while most of the CYP2C9 variants exhibited decreased intrinsic clearance values toward diclofenac. In our study, CYP2C9*3 and CYP2C9*8 showed significantly decreased intrinsic clearance values (42.4% and 65.2%, respectively) relative to wild-type as the previous studies reported. CYP2C9*2 exhibited significantly increased K_m and V_{max} values, therefore, CYP2C9*2 had no significant difference in intrinsic clearance of diclofenac compared with wild-type protein. The CYP2C9*36 variant showed similar K_m value but much higher V_{max} value toward diclofenac, compared with that of the wild-type CYP2C9*1. As a result, CYP2C9*36 allele exhibited the highest relative clearance value. The discrepancy between this and Dai's reports might be because of the different degradation rates of improperly folded proteins between mammalian and insect cell expression systems, as reported by Maekawa et al. 2009).

When compared with Wang et al.'s study (Wang et al. 2013), most of the tested CYP2C9 variants exhibited similar catalytic activities to both diclofenac and losartan, as shown in Fig. 2. Only 4 allelic variants showed different enzymatic activities toward diclofenac and losartan hydroxylation which included the variants CYP2C9*2, *23, *41 and *56. The CYP2C9*2, *23 and *41 variants showed similar metabolic activities toward the typical CYP2C9-probing substrate diclofenac while exhibiting significantly decreased catalytic activities toward losartan, compared with wild-type CYP2C9*1. The remaining variants, CYP2C9*56, exhibited decreased relative enzymatic activity toward diclofenac, but value analogous to the wild-type value was obtained when losartan was used as the substrate (Fig. 2). In addition, for most of the tested variants, the increasing or decreasing trend is quite similar for both diclofenac and losartan.

In conclusion, the purpose of the present study was to clarify the role of the 36 CYP2C9 alleles that exist in Chinese populations in the metabolism of diclofenac *in vitro* in insect microsomes. To the best of our knowledge, this is the first report of all these rare alleles for diclofenac metabolism in insect microsomes. Our result suggest that only one variant (CYP2C9*36) exhibited increased catalytic activity toward diclofenac compared with the wild type, whereas 31 allelic variants exhibited significantly decreased intrinsic clearance values compared to CYP2C9*1. Therefore there seems to be a relationship between the CYP2C9 variant alleles and diclofenac metabolism. Our data provide information regarding CYP2C9 genetic polymorphisms and individual variation in diclofenac efficacy, which could be relevant for personalized medicine in clinical practice. Further work will be required in order to better understand the basis for CYP2C9 genetic polymorphisms in the metabolism of diclofenac *in vivo*.

4. Experimental

4.1. Chemicals and reagents

Diclofenac and 4'-hydroxy (OH)-diclofenac were purchased from Sigma-Aldrich (St. Louis, MO, USA). Diazepam was from the second Affiliated Hospital of Wen Zhou Medical University (Wenzhou, China). 0.1 M Hydrochloric acid was purchased from Aladdin-Reagent (Shanghai, China). Cytochrome b5 and insect microsomes expressing 36 human CYP2C9 allelic variants were kindly gifts from the Key Laboratory of Geriatrics, Beijing Hospital (Beijing, China). Nicotinamide Adenine Dinucleotide Phosphate (NADPH) was purchased from Roche Molecular Biochemicals (Basle, Switzerland). Other reagents and organic solvents were of analytical grade.

4.2. Incubation of diclofenac with recombinant P450 CYP2C9

In vitro incubations were carried out at 37 °C for 30 min in a Fisher shaking water bath. The incubation mixture consisted of (final assay concentration) 0.1 M Tris-HCL, 1.0 mM NADPH, 5 to 10 pmol of P450 from insect microsomes (5 pmol for CYP2C9*1 or 10 pmol for other variants), 20 to 40 pmol of purified cytochrome b5 (P450/b5 = 1:4) and 2 to 100 μM diclofenac dissolved in 50 mM Tris-HCl (pH 7.4). The total incubation volume was 0.30 mL. The reaction was started by the addition of the NADPH-regeneration system (1.3 mM $NADP^+$, 3.3 mM glucose 6-phosphate, 3.3 mM $MgCl_2$ and 0.4 unit/mL glucose-6-phosphate dehydrogenase) after a 5-min preincubation and was allowed to proceed for 30 min. The reaction was terminated with 30 μL 0.1 M HCl and the internal standard, diazepam (50 μL of a 10 μg/ml stock), was added to the sample. Then 1 mL acetic ether was added for the extraction. After overtaxing for 2 min, the incubation mixture was centrifuged at 13 000 rpm for 5 min, the organic phase was transferred into a clean tube, and it was dried under a nitrogen stream at 45 °C. The resulting residue was reconstituted in 100 μL of the mobile phase and used for HPLC analysis. Under these conditions, the rate of 4'-hydroxy (OH)-diclofenac formation was linear with respect to protein concentration and time of incubation. The incubations were performed in quadruplicate, and the data are presented as the mean mean ± standard deviation (SD) from four experiments.

4.3. HPLC-DAD Analysis

Diclofenac and its metabolite were separated on an Agilent ZORBAX SB-C18 column (150 mm × 4.6 mm, id 5 μm, Agilent Technologies, Palo Alto, CA) using an Agilent HPLC system. The mobile phase consisted of water (solvent A), acetonitrile (solvent B) and 0.1% trifluoroacetic acid in water (solvent C) at an isocratic flow rate of 1 mL/min with the following gradient: solvent A decreased from 35% to 30% while solvent B increased from 45% to 50% for the first 6 min; solvent A decreased from 30% to 12% while solvent B increased from 50% to 68% for the following 10 min. The Agilent ZORBAX SB-C18 column (150 mm × 4.6 mm, id 5 μm) was kept at 40 °C. Detection was performed at Photodiode Array Detector (DAD) at excitation and emission wavelengths of 250 and 410 nm, respectively. The retention times of 4'-hydroxy (OH)-diclofenac, diazepam and diclofenac were 4.2, 4.8 and 9.5 min, respectively, under these conditions. The standard curves for diclofenac and 4'-hydroxy (OH)-diclofenac were prepared using spiked incubation samples. Concentrations of diclofenac 4'-hydroxylation formed in the incubations were determined by comparison of the peak area ratios of the analyte to the internal standard to those in the standard curve samples.

4.4. Statistical analysis

The kinetic parameters (K_m and V_{max}) for diclofenac 4'-hydroxylation by recombinant CYP2C9 were estimated using a computer program (Prism version 5; GraphPad Software Inc., San Diego, CA) designed for nonlinear regression analysis of a hyperbolic Michaelis-Menten equation. *In vitro* intrinsic clearance for the metabolism of diclofenac to 4'-hydroxy diclofenac in insect cell microsomes was calculated as the V_{max}/K_m ratio. Kinetic data for each variant are presented as the mean ± SD of four microsomal prepa-

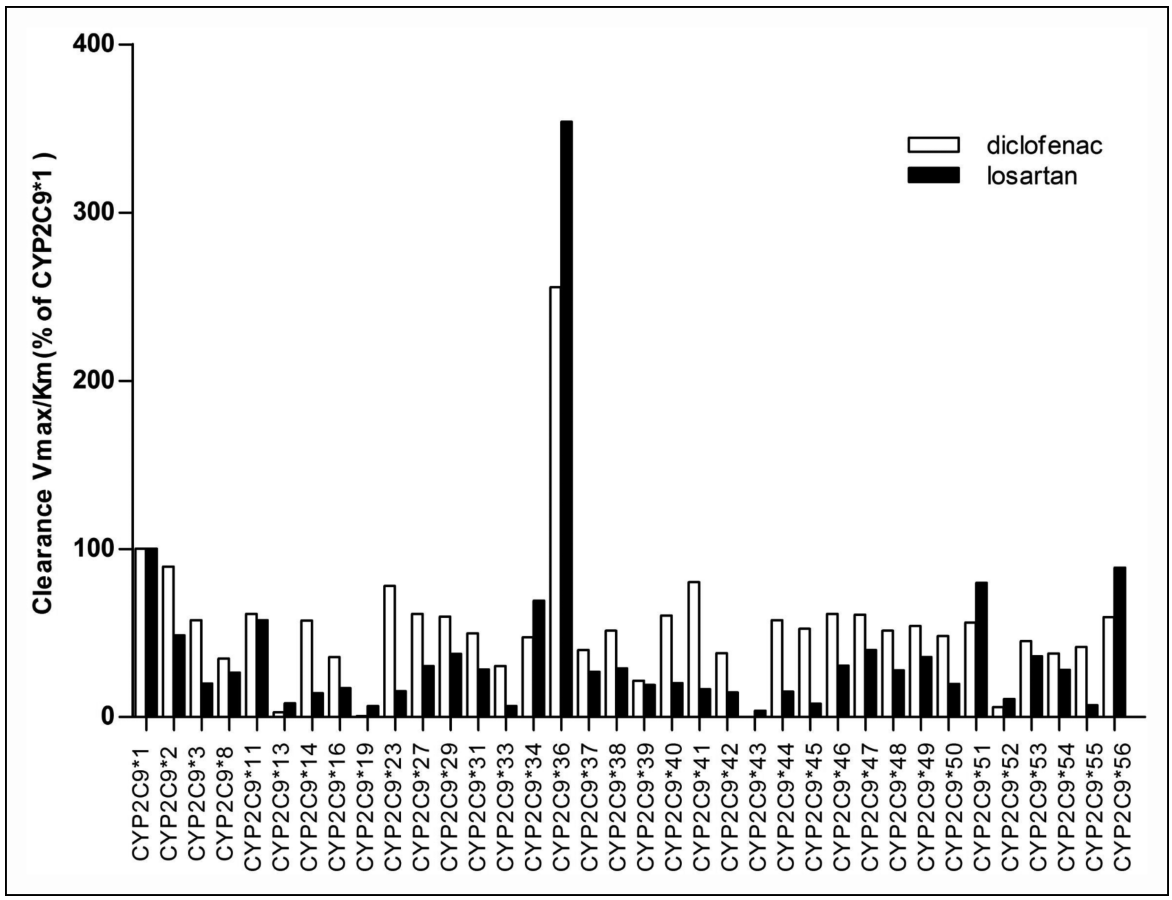

Fig. 2: Enzymatic activity of the recombinant wild-type CYP2C9*1 and 35 variants toward diclofenac (closed bar) and losartan (open bar) oxidation.

rations derived from separate transfections. The results were analyzed using one-way analysis of the variance, with $P < 0.05$ considered to be statistically significant.

Conflict of interest: The authors declare that there is no conflict of interest associated with this study.

Acknowledgements: This work was supported by the National Department Public Benefit Research Foundation by Ministry of Health of China (201302008) and National Natural Science Foundation of China (31371280). The authors are indebted to the teachers Guoxin Hu and Jianping CAI, for their technical advice and helpful assistance.

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