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Substrate-dependent functional alterations of seven CYP2C9 variants found in Japanese subjects

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Abbreviations: CYP2C9, cytochrome P450 2C9; PM, poor metabolizer; Sf21, Spodoptera frugiperda 21; OR, NADPH P450 reductase; LC-MS, liquid chromatography-mass spectrometry; IS, internal standard; API-ES, atmospheric pressure ionization-electrospray ionization; AUC, area under the plasma concentration-time curve; SRSs, substrate recognition sites

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Abstract

Cytochrome P450 2C9 (CYP2C9) is a polymorphic enzyme which metabolizes a number of clinically important drugs. In this study, catalytic activities of seven alleles found in Japanese individuals, *CYP2C9*3* (Ile359Leu), **13* (Leu90Pro), **26* (Thr130Arg), **28* (Gln214Leu), **30* (Ala477Thr), **33* (Arg132Gln) and **34* (Arg335Gln), were assessed using three substrates, diclofenac, losartan and glimepiride. When expressed in a baculovirus-insect cell system, the holo- and total (apo- and holo-) CYP2C9 protein expression levels were similar among the wild-type (CYP2C9.1) and 6 variants except for CYP2C9.13. A large part of CYP2C9.13 was present in the apo-form P420. As compared with CYP2C9.1, all variants except for CYP2C9.34 exhibited substrate-dependent changes in K_m, V_{max} and intrinsic clearance (V_{max}/K_m). For diclofenac 4'-hydroxylation, the intrinsic clearance was decreased markedly (by >80%) in CYP2C9.13, CYP2C9.30 and CYP2C9.33 and variably (63 – 76%) in CYP2C9.3, CYP2C9.26 and CYP2C9.28 due to increased K_m and/or decreased V_{max} values. As for losartan oxidation, CYP2C9.13 and CYP2C9.28 showed 2.5- and 1.8-fold higher K_m values, respectively, and all variants except for CYP2C9.34 showed >77% lower V_{max} and intrinsic clearance values. For glimepiride hydroxylation, K_m of CYP2C9.13 was increased 7-fold, and the V_{max} values of all variants significantly decreased, resulting in

reductions in the intrinsic clearance by >80% in CYP2C9.3, CYP2C9.13, CYP2C9.26 and CYP2C9.33 and by 56 - 75% in CYP2C9.28 and CYP2C9.30. These findings suggest the necessity for careful administration of losartan and glimepiride to patients bearing these 6 alleles.

Introduction

Cytochrome P450 2C9 (CYP2C9) is a polymorphic enzyme responsible for the oxidative metabolism of up to 15% of the drugs that undergo phase I metabolism (Miners and Birkett, 1998). This enzyme hydroxylates weakly acidic or neutral drugs of diverse therapeutic categories, including the hypoglycemic agents tolbutamide and glimepiride, the nonsteroidal anti-inflammatory drugs flurbiprofen and diclofenac, the antihypertensive losartan, the diuretic torsemide, the anticonvulsant phenytoin, and the anticoagulant warfarin (Rettie and Jones, 2005). To date, 34 *CYP2C9* alleles located in the coding region have been reported (http://www.cypalleles.ki.se/cyp2c9.htm). Some of these alleles, particularly *CYP2C9*2* (Arg144Cys) and *CYP2C9*3* (Ile359Leu), have been well studied in their associations with reduced catalytic activities towards several substrates such as warfarin, tolbutamide, and losartan, both *in vitro* and *in vivo* (Lee et al., 2002; Kirchheiner and Brockmoller 2005).

The frequencies of low-activity CYP2C9 alleles differ considerably among different ethnic populations. In Caucasians, the frequencies of **2* and **3* are 0.08-0.14 and 0.04-0.16, respectively (Schwarz, 2003). In contrast, in East Asian populations, **2* is hardly found, and **3* is present only at 0.01-0.04. Recently, a series of novel

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non-synonymous variations were identified in several Asian populations (Si et al., 2004; Zhao et al., 2004; Maekawa et al., 2006; Yin et al., 2008). **13* (Leu90Pro), an allele originally identified in a Chinese poor metabolizer (PM) toward lornoxicam, has been found in Chinese, Korean and Japanese individuals at allele frequencies of 0.002-0.01 (Si et al., 2004; Maekawa et al., 2006; Bae *et al.* 2005; Yin et al., 2008). CYP2C9.13 was reported to show decreased enzymatic activity toward lornoxicam, tolbutamide and diclofenac *in vivo* and/or *in vitro* (Guo et al., 2005a; Guo et al., 2005b). We reported 7 non-synonymous SNPs from 263 Japanese subjects, of which **25* (Lys118ArgfsX9) was a null allele, and **26* (Thr130Arg), **28* (Gln214Leu) and **30* (Ala477Thr) were functionally defective toward diclofenac when expressed in COS-1 cells (Maekawa et al., 2006). Additionally, two novel variations, **33* (Arg132Gln) and **34* (Arg335Gln), were detected by large-scale direct re-sequencing of the samples from 724 Japanese individuals. Here, CYP2C9.33 showed a five-fold lower intrinsic clearance toward diclofenac *in vitro* (Yin et al., 2008). These results point out that not only **2* and **3* but many other less-frequent defective alleles could contribute to highly variable inter-individual and ethnic differences in the pharmacokinetics and pharmacodynamics of CYP2C9 substrate drugs.

The defective *CYP2C9* alleles, **3*, **5* and **13* are known to exhibit substrate-dependent changes in their kinetic parameters (Takanashi et al., 2000; Dickmann et al., 2001; Guo et al., 2005a). In our previous study, losartan showed no antihypertensive effects in two **30* heterozygotes (Yin et al., 2008). This finding suggested that **30* might be inactive for the conversion of losartan to its active metabolite, E3174. For low-frequency alleles, elucidation of the substrate-dependencies of their recombinant enzymes is valuable because their functional assessments *in vivo* are difficult due to scarcity of patients with these alleles. In the present study, we focused on the low-activity alleles found in Japanese populations, **3*, **13*, **26*, **28*, **30*, **33* and **34* and characterized their functional alterations using three CYP2C9 substrates, diclofenac, losartan and glimepiride.

Materials and Methods

Chemicals and Materials

Diclofenac, δ-Aminolevulinic acid and ferric citrate were purchased from Sigma-Aldrich Co. (St. Louis, MO). Losartan and its metabolite, E-3174, were kindly provided by Merck & Co., Inc. (Whitehouse Station, NJ). Glimepiride was gifts from Sanofi-Aventis K.K. (Tokyo, Japan). Glibenclamide was obtained from Wako Pure Chemical Ind. (Osaka, Japan). *Spodoptera frugiperda* (*Sf*) 21 insect cells, supplemented Grace's Insect medium, gentamycin, Pluronic F68, and Bac-to-Bac Baculovirus Expression system were purchased from Invitrogen (Carlsbad, CA), while fetal bovine serum (FBS) was from SAFC Biosciences (Manchester, UK). Goat anti-CYP2C6 antiserum, which can cross-react with human CYP2C9, and anti-rat NADPH P450 reductase (OR) antibodies were purchased from Daiichi Pure Chemicals (Tokyo, Japan); horseradish peroxidase-conjugated rabbit anti-goat IgG from Jackson ImmunoResearch Laboratories (West Grove, PA); and Western Lightning Chemiluminescence Reagent Plus from PerkinElmer Life Sciences (Boston, MA). 4'-hydroxydiclofenac, Baculosomes co-expressing CYP2C9 and OR (Lot63793), Supersomes coexpressing either CYP3A4 (Lot49734) or CYP2C8 (Lot4) with OR and cytochrome b5, pooled human liver microsomes (Lot32556, 570 pmol P450/mg protein),

and an NADPH generation 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) were obtained from BD Gentest (Woburn, MA). Purified human cytochrome b5 was purchased from Oxford Biomedical Research (Oxford, UK), and Protein Assay Kit was purchased from Bio-Rad Laboratories (Hercules, CA). All other chemicals and solvents used were of the highest grade or analytical grade commercially available.

Expression of recombinant wild-type and variant CYP2C9 proteins

A full-length human OR cDNA was isolated as described previously (pcDNA3.1D/OR) (Yin T et al., 2008). The plasmids containing the 1.5 kb-full length CYP2C9 wild-type (pcDNA3.1D/CYP2C9/wild-type) and 5 variants (pcDNA3.1D/CYP2C9/Thr130Arg, pcDNA3.1D/CYP2C9/Gln214Leu, pcDNA3.1D/CYP2C9/Ala477Thr, pcDNA3.1D/CYP2C9/Arg132Gln and pcDNA3.1D/CYP2C9/Arg335Gln) CYP2C9 cDNAs were constructed as described previously (Maekawa et al., 2006; Yin T et al., 2008). Additionally, two substitutions, 1075A>C (Ile359Leu, CYP2C9.3) and 269T>C (Leu90Pro, CYP2C9.13), were introduced into the wild-type plasmid (pcDNA3.1D/CYP2C9/wild-type) using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The primer sequences used for the construction of variant plasmids were as follows (the position of the altered nucleotide is underlined in

boldface): 5'- CACGAGGTCCAGAGATAC**C**TTGACCTTCTCCCC -3' (sense) and 5'- GGGGAGAAGGTCAA**G**GTATCTCTGGACCTCGTG -3' (antisense) for pcDNA3.1D/CYP2C9/Ile359Leu.

5'- GGAAGCCCTGATTGATC**C**TGGAGAGGAGTTTTC -3' (sense) and 5'- GAAAACTCCTCTCCA**G**GATCAATCAGGGCTTCC -3' (antisense) for pcDNA3.1D/CYP2C9/Leu90Pro.

To ensure that no errors had been introduced during amplification, the entire cDNA regions were confirmed by sequencing the plasmid constructs. Then, both OR and CYP2C9 wild-type or variant cDNAs were inserted into the baculovirus transfer vector, pFastBac Dual (Invitrogen), at the downstream cloning sites of the P10 promoter and the polyhedron promoter, respectively (pFastBac Dual/P10.OR/polh.CYP2C9). Recombinant baculoviruses carrying both CYP2C9 and OR cDNAs were produced according to the protocol recommended for the Bac-to-Bac Baculovirus Expression system. The recombinant proteins were expressed in *Sf*21 insect cells, and microsomal fractions were prepared as described previously (Yin et al., 2008).

Determination of protein expression levels

The cytochrome P450 content in insect cell microsomes was measured by reduced CO-spectrum using the method of Omura and Sato (1964). The microsomal OR activity was measured using cytochrome c as a substrate as described by Phillips and Langdon (1962). The molar amount of OR was calculated based on an assumed specific activity of 3.0 μmol cytochrome c reduced/min/nmol purified human OR (Yamazaki et al. 1999). Western blotting of CYP2C9 and OR was performed using 2 μg of the microsomal proteins from insect cells as described previously (Yin et al, 2008).

Assays for CYP2C9 enzymatic activity

To compare alterations in kinetic parameters among substrates, the same enzyme preparations of the wild-type and 7 variants were consistently used for all kinetic studies.

Diclofenac 4'-hydroxylation activities of the wild type (CYP2C9.1) and seven variant proteins were assessed as described previously (Yin et al, 2008). In brief, the mixture (0.5 ml) containing diclofenac (1.0-100 μM), 2-5 pmol of P450 from insect cell microsomes (2 pmol P450 for CYP2C9.1 and 5 pmol P450 for other variants), 4-10 pmol of purified cytochrome b5 (P450: b_5 = 1:2) and an NADPH regenerating system were incubated at 37 °C for 10 min. For pooled human liver microsomes, 25 pmol P450 per reaction was used. HPLC conditions are the same as described previously, and the

retention times of 4'-hydroxydiclofenac, 5-hydroxydiclofenac, and diclofenac were 14.2, 14.7 and 19.6 min, respectively. For experiments on regioselectivity of diclofenac hydroxylation, the concentrations of 5-hydroxy diclofenac were estimated using a calibration curve for 4'-hydroxydiclofenac under the assumption that both have similar extinction coefficients.

Kinetic analysis for losartan oxidation was performed as described previously (Yasar et al., 2001) with minor modifications. Insect cell microsomes and purified cytochrome b_5 (P450: b_5 = 1:2) were incubated with eight different concentrations of losartan (0.1 – 20 µM) in the presence of an NADPH regenerating system at 37 °C for 10 min in 100 mM Tris-HCl buffer (pH 7.5) in a final volume of 500 µl. The amount of P450 used per incubation varied depending on the variants (10 pmol P450 for CYP2C9.1 and CYP2C9.34, 20 pmol for CYP2C9.3, CYP2C9.13, and CYP2C9.28, 50 pmol for CYP2C9.26, and 100 pmol for CYP2C9.30 and CYP2C9.33) because of the large differences in activities among the wild-type and variants. We confirmed that differences in microsomal protein concentrations between the wild-type and variants did not affect the measurements of kinetic parameters by adjusting the protein concentrations with control (uninfected) microsomes. All reactions were within linear

ranges of the metabolite formation with respect to P450 concentrations and incubation time. For pooled human liver microsomes, 100 pmol of P450 was incubated with various concentrations of losartan at 37 °C for 20 min. Reactions were terminated by the addition of 50 µl of 5 M *ortho*-phosphoric acid, followed by centrifugation at 3,000 g for 10 min at 4 °C. The supernatants were filtered through polytetra-fluoroethylene membrane filters of 0.2 µm pore size (Millipore, Bedford, MA), and the aliquots (50 µl) were injected into a Shimadzu Prominence HPLC system (Shimadzu, Kyoto, Japan) coupled with fluorescence detection (Ritter et al., 1997). Separation was conducted on a Shim-pack CLC-ODS (M) (250 x 4.6 mm i.d., Shimadzu) according to the conditions described by Kobayashi et al (2008). Elution was performed isocratically with 10 mM phosphate buffer (pH 2.3) / acetonitrile (60:40, v/v) at a flow rate of 1.0 ml/min. The retention times of losartan and E-3174 were 9.0 and 15.6 min, respectively. The lower limit of E-3174 quantification was 5 nM and inter- and intra-day assay variations were less than 6%.

Hydroxylated glimepiride (M-1) was measured by a liquid chromatography-mass spectrometry (LC-MS) according to a previously reported method (Suzuki et al., 2006). Reactions mixtures contained 10-50 pmol of P450, 20-100 pmol of purified cytochrome b_5 (P450: b_5 = 1:2), 0.05 – 10 µM of glimepiride, and NADPH regenerating system in a

final volume of 2.5 ml in 100 mM Tris-HCl buffer (pH 7.5). Glimepiride was dissolved in methanol/dimethyl sulfoxide (50:50, v/v). The final concentration of organic solvent (methanol and dimethyl sulfoxide) in the incubation mixture was 0.5%. The reactions were allowed to proceed for 10 min and terminated by addition of 1.0 ml of 0.05 M KCl (adjusted with HCl to pH=1.0) and 25 µl of 5.0 µg/ml glybenclamide as an internal standard (IS). Reaction samples were extracted with 5.0 ml of diethylether and the organic layer was then evaporated to dryness. The residue was reconstituted in 200 µl of acetonitrile. For pooled human liver microsomes, a sample containing 100 pmol of P450 was incubated with various concentrations of glimepiride at 37 ℃ for 20 min and then processed in the same manner as the recombinant enzyme samples. LC-MS analysis was performed using Shimadzu LCMS-2010 Evolution systems. Aliquots of samples (2 µl) were applied onto a Shim-pack FC-ODS column (3.0 µm; 2.0 x 75 mm, Shimadzu) kept at 40 ℃. The initial mobile phase was 80% of 10 mM ammonium acetate and 20% of acetonitrile, and the proportion of acetonitrile was linearly increased to 45% up to 7 min and then increased to 70% for the next 6 min with the flow rate of 0.25 ml/min. The quadrupole mass spectrometer was operated in the positive API-ES (atmospheric pressure ionization- electrospray ionization) mode under selected ion monitoring conditions as described previously (Suzuki et al., 2006): temperature of

CDL, 230 °C; gas flow rate, 1.5 L/min; and heat block temperature, 200 °C. Under these conditions, M-1, glybenclamide (IS) and glimepiride were eluted at 5.8 min, 10.4 min, and 11.0 min, respectively. The control microsomes were used to prepare the samples for generation of a standard curve in the same manner as that of the incubation samples. The lower limit of detection for M-1 was 0.5 pmol/assay. Intra- and inter-day variation coefficients did not exceed 10% in any assay.

The kinetic parameters such as K_m, V_{max}, and intrinsic clearance (V_{max}/K_m) were estimated using a computer program designed for non-linear regression analysis of a hyperbolic Michaelis-Menten equation (Prism v.3.0a, GraphPad Software, San Diego, CA, USA). Because the substrate consumption at the two lowest substrate concentrations (1 and 2.5 μM) was greater than 20% in diclofenac 4'-hydroxylation by the in-house CYP2C9.1, these two points were omitted from the kinetic parameter estimation. Data are presented as mean ± S.D. for three to four microsomal preparations derived from separate infections for in-house wild-type and variant CYP2C9s. Statistical significance was determined by one-way analysis of variance (ANOVA) with post-hoc Dunnett multiple comparisons test.

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Results

Expression of wild-type and seven variant CYP2C9s in insect cell microsomes

Immunoblot analysis was performed using the insect cell microsomes coexpressing CYP2C9 and OR, and representative data are shown in Fig. 1. Neither CYP2C9 nor OR protein expression levels were significantly different among the wild-type and seven variants (p= 0.138 for CYP2C9, p= 0.222 for OR by one-way ANOVA) tested. Holo-enzyme contents in the wild-type and variant CYP2C9 microsomes were measured by CO-difference spectra. Typical spectra with a maximum absorbance at 450 nm were observed for both wild-type and variant proteins except for CYP2C9.13, which exhibited a large peak at 420 nm, indicating the presence of the apo-form cytochrome P420 (Fig. 2). As shown in Table 1, except for CYP2C9.13, the mean P450 contents in the wild-type and the six variants were in the range of 158 to 201 pmol P450 / mg microsomal protein. On the other hand, CYP2C9.13 was expressed at 22 ± 5 pmol P450 /mg microsomal protein, which was about 12% of the mean P450 content of the wild-type. OR activities varied among the preparations but were not significantly different between the wild-type and all variants (p= 0.201 by one-way ANOVA, Table 1).

Functional activities of wild-type and seven variant CYP2C9s

Catalytic activities of the wild-type and seven variants were compared using diclofenac, losartan and glimepiride as substrates. Michaelis-Menten and Eadie-Hofstee plots for each substrate are shown in Figs. 3 to 5. The kinetic parameters are summarized in Tables 2-4 for the wild-type, 7 variant enzymes, Gentest CYP2C9.1 (commercially available baculosomes coexpressing CYP2C9.1 and OR) and the pooled human liver microsomes. The ratios (%) of intrinsic clearance of the variants to that of the wild-type for each substrate are depicted in Fig. 6.

When compared with the pooled human liver microsomes, the recombinant wild-type enzymes produced by the baculovirus-insect cells systems, either in-house or Gentest preparations, exhibited 1.5- to 4-fold lower K_m values and 6- to 17- fold higher V_{max} values, regardless of the substrates tested (Tables 2-4). Kinetic parameters of diclofenac 4'-hydroxylation are summarized in Table 2. In an earlier study, we already compared the diclofenac 4'-hydroxylation activities of CYP2C9.33 and CYP2C9.34 with that of CYP2C9.1 (Yin et al., 2008), and the previous data are also shown in Table 2. In this study, kinetic parameters were analyzed for CYP2C9.1 and 5 variants (CYP2C9.3, CYP2C9.13, CYP2C9.26, CYP2C9.28 and CYP2C9.30). All 5 variants exhibited 1.7-

to 4.3-fold higher K_m values than the wild-type CYP2C9.1. The V_{max} values of CY2C9.13 and CYP2C9.26 were significantly decreased by 81% and 58%, respectively, while those of CYP2C9.3, CYP2C9.28 and CYP2C9.30 were not significantly different from that of CYP2C9.1. As a result, intrinsic clearance (V_{max}/K_m) of 5 variants was significantly reduced compared to CYP2C9.1 in the following order: CYP2C9.13 (95%), CYP2C9.30 (81%), CYP2C9.26 (76%), CYP2C9.28 (73%) and CYP2C9.3 (63%). In addition, as reported previously, CYP2C9.33 showed 82% lower intrinsic clearance than CYP2C9.1.

Of the 7 variants, only CYP2C9.28 exhibited a slight change in regioselectivity for diclofenac hydroxylation, namely in 5-hydroxy diclofenac formation. In the presence of 100 μM substrate, diclofenac 5-hydroxylation activity was 1.02 ± 0.31 pmol/min/pmol P450. The formation ratio of 5-hydroxy diclofenac to 4'-hydroxy diclofenac by CYP2C9.28 was estimated to be 0.013 ± 0.005, while that by commercially available CYP2C8 and CYP3A4 was 3.9 and 22.7, respectively (data not shown).

The kinetic parameters of losartan oxidation are summarized in Table 3. K_m values of two variants, CYP2C9.13 and CYP2C9.28, were 2.5- and 1.8-fold higher than that of

CYP2C9.1, respectively. On the other hand, all 7 variants showed significantly decreased V_{max} and intrinsic clearance values. The reductions in intrinsic clearance values were more than 96% in CYP2C9.13, CYP2C9.26, CYP2C9.30 and CYP2C9.33, 87% in CYP2C9.28, 77% in CYP2C9.3, and 25% in CYP2C9.34. It should be noted that CYP2C9.30 had a very low activity for losartan oxidation (1% of the wild-type), which is in contrast to its moderate activity (19% of the wild-type) for diclofenac hydroxylation (Fig. 6). Such substrate-dependent differences between diclofenac and losartan were also observed in CYP2C9.26 (the ratios of intrinsic clearance of the variants to that of the wild-type was 24% for diclofenac vs. 4% for losartan), CYP2C9.28 (27% vs. 13%) and CYP2C9.33 (18% vs. 1%) (Fig. 6).

As for the hydroxylation of glimepiride (Table 4), CYP2C9.13 exhibited a 7-fold higher K_m value and a 10-fold lower V_{max} value compared to the wild-type, resulting in a 99% decrease in intrinsic clearance. A similar decrease in activity (99%) was observed for CYP2C9.33, which is mainly due to the remarkable decrease in V_{max} value compared to the wild-type. The V_{max} values of the other variants were also significantly decreased, resulting in reduced intrinsic clearance values (10% of the wild-type in CYP2C9.26, 20% in CYP2C9.3, 25% in CYP2C9.30, 44% in CYP2C9.28 and 72% in CYP2C9.34).

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As shown in Fig. 6, the percent reductions in intrinsic clearances were comparable between glimepiride and diclofenac for CYP2C9.3 (37% for diclofenac vs. 20% for glimepiride), CYP2C9.28 (27% for diclofenac vs. 44% for glimepiride) and CYP2C9.30 (19% for diclofenac vs. 25% for glimepiride) although CYP2C9.30 exhibited substantial decrease in activity of losartan oxidation. In contrast, CYP2C9.26 and CYP2C9.33 showed large difference in the intrinsic clearance ratio between diclofenac and glimepiride, as between diclofenac and losartan: CYP2C9.26 (24% for diclofenac vs. 10% for glimepiride) and CYP2C9.33 (18% vs. 1%).

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Discussion

In the present study, we focused on the 7 alleles found in Japanese subjects, **3*, **13*, **26*, **28*, **30*, **33 and **34*, and performed a functional characterization of these alleles using diclofenac, losartan and glimepiride as substrates.

The commonly found defective allele, **3*, exhibited the substrate-dependent changes in kinetic parameters for the three substrates, leading to lower intrinsic clearance values for diclofenac hydroxylation (63%), losartan oxidation (77%) and glimepiride hydroxylation (80%) than the wild-type. The reduction in intrinsic clearance was a result of the increase in K_m without significant changes in V_{max} for diclofenac hydroxylation and the decreases in V_{max} without significant changes in K_m for both losartan oxidation and glimepiride hydroxylation. Our results are in good agreement with a previous study using both the yeast expression system and the human liver microsomes, where a 7-fold lower intrinsic clearance of losartan by CYP2C9.3 compared to CYP2C9.1 was resulted from a 5-fold lower V_{max} without large differences in K_m (Yasar et al., 2001). As for glimepiride hydroxylation, our results were consistent with that reported by Suzuki *et al* (2006) using insect cells microsomes from Gentest. The authors showed that CYP2C9.3 had unchanged K_m values and 3.3-fold lower V_{max}

values than CYP2C9.1. Similar changes in CYP2C9.3 with unaltered K_m and lowered V_{max} were reported for piroxicam 5'-hydroxylation (Takanashi et al., 2000; Tracy et al., 2002). However, for many other substrates such as diclofenac, *S*-warfarin and tolbutamide, CYP2C9.3 shows altered K_m with or without changes in V_{max} (Takanashi et al., 2000; Lee et al., 2002).

The substrate-specific effects of *CYP2C9*3* on pharmacokinetics were also reported. Plasma losartan/E-3174 ratios in subjects with **1/*3* were reported to be 2-fold higher than those with **1/*1* (Yasar et al., 2002; Sekino et al, 2003). On the contrary, there was no significant differences in any diclofenac pharmacokinetic parameters between **1/*1* and **1/*3* genotype groups (Shimamoto et al, 2000). Also the **3* heterozygotes showed a 1.3- to 2.5-fold higher mean glimepiride area under the plasma concentration-time curve (AUC) than the wild-type (Niemi et al., 2002; Wang et al., 2005; Suzuki et al, 2006), and **3*-bearing patients might be at an increased risk of sulphonylurea-associated severe hypoglycemia (Holstein et al, 2005).

**13* was first identified in a Chinese individual who showed a poor metabolizer phenotype for both lornoxicam and tolbutamide (Si et al., 2004). Thereafter, this allele

was also found in Koreans (allele frequency = 0.006) (Bae et al., 2005) and Japanese (0.0014 – 0.002) (Maekawa et al., 2006, Yin et al., 2008), indicating that it is a relatively common allele among East Asians. In our baculovirus-insect cell system, total (apo- and holo-) CYP2C9 expression levels determined by Western blotting were not significantly different between CYP2C9.1 and CYP2C9.13 (Fig.1). On the other hand, CO different spectra demonstrated that the CYP2C9.13 preparations contained a small amount of holo-form P450 (12% of the wild-type) and a large amount of inactive apo-form P420 (Fig.2 and Table 1), suggesting that Leu90Pro substitution resulted in improper heme incorporation. Guo et al. (2005a) reported that the protein expression level of CYP2C9.13 was 39% of that of CYP2C9.1 in the COS-1 expression system by Western blotting. The discrepancy in total protein levels between their study and ours might be due to different rates of degradation of improperly folded proteins between the two expression systems, as observed for CYP2C9.24 between yeast and mammalian systems (Herman et al., 2007). The mammalian cell system seems to be more relevant to assess the potential effects of *CYP2C9*13* on its protein expression *in vivo* although accurate estimation of its holoprotein levels might be difficult in this system because of the low expression levels.

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In the *in vitro* kinetic characterization, Guo et al. (2005a and 2005b) reported that CYP2C9.13 was less active in catalyzing diclofenac, lornoxicam and tolbutamide with increased K_m and decreased or unaltered V_{max} depending on the substrates. In their study, reductions in intrinsic clearance were by 88.2% for lornoxicam, 97.5% for diclofenac and 90.8% for tolbutamide. In our experiments, CYP2C9.13 influenced both K_m and V_{max} values for three substrates, resulting in decreases in intrinsic clearance by 95.2% for diclofenac, 97.5% for losartan and 98.6% for glimepiride. Using the three-dimensional structure models, Zhou et al. (2006) proposed a long-range effect of the Leu90Pro substitution on the residues Ala106 - Arg108, a part of substrate entrance constitution. Further pharmacodynamic studies are necessary to confirm whether **13* is associated with altered responses to and increased risks of toxicities of CYP2C9 substrate drugs.

**26*, **28* and **30*, detected recently in a Japanese population, were functionally defective alleles toward diclofenac when expressed in COS-1 cells (Maekawa et al, 2006). In the present study, we used a baculovirus-insect cell system as a recombinant enzyme source because high expression levels enabled the precise measurements of holoenzyme contents and catalytic activities using several substrates. Between our

mammalian and baculovirus-insect cell systems, consistent results were obtained showing that the total CYP2C9 levels of these variants (through Western blot analysis) were not significantly different from that of the wild-type. This suggests that these enzymes are stably expressed. The reductions in intrinsic clearance of diclofenac 4'-hydroxylation activity were comparable between the COS-1-produced enzymes and the insect cells-produced enzymes for all three variants: that is, 84% (COS-1 cells) vs. 76% (insect cells) for CYP2C9.26, 77% vs. 73% for CYP2C9.28, and 81% vs. 81% for CYP2C9.30.

CYP2C9.26 (Thr130Arg), CYP2C9.28 (Gln214Leu), CYP2C9.30 (Ala477Thr) and CYP2C9.33 (Arg132Gln) showed a substrate-dependent reduction in activity and changes in the kinetic parameters, whereas CYP2C9.34 (Arg335Gln) held catalytic activities almost similar to the wild-type for all three substrates. Thr130 and Arg132, highly conserved residues in the CYP2C family, are not within the substrate recognition sites (SRSs), but are on the surface of the protein, in the C-helix and a loop region between the C- and D-helices, respectively. As suggested in CYP2C9.2 (Arg144Cys) (Crespi et al., 1997; Wei et al., 2007), alternations in OR binding, electron transfer, or the P450 catalytic cycle (coupling and uncoupling) might be responsible for reduced

function of CYP2C9.26 and CYP2C9.33. It should be noted that the substitution Arg132Gln also occurs in *CYP2C19*6* (395G>A), and that this allele has negligible catalytic activity toward tolbutamide *in vitro* (Ibeanu et al,. 1998). Gln214, which is also conserved in the CYP2C family, is located between the F- and G-helices and only 5 amino acids downstream of SRS-2. In the P450 structure, the regions from F- to G-helices are conformationally flexible, indicative of an adaptive fit to the various substrates with different sizes, polarity, and stereochemical features. Therefore, this substitution (Gln214Leu) could affect substrate access and binding in a substrate-dependent manner. In addition, it is noteworthy that a slight change in regioselectivity in diclofenac metabolism (from 4'-hydroxylation to 5-hydroxylation) was observed in CYP2C9.28.

Arg335 is located on the exterior of the protein and in a loop region between the J- and J'-helices. Its location may support our findings showing that CYP2C9.34 had no substantial effect on the metabolism of diclofenac, losartan and glimepiride. However, in contrast to CYP2C9.34, a substitution in the same position, CYP2C9.11 (Arg335Trp), was reported to exhibit decreased catalytic activity for tolbutamide when expressed in a bacterial cDNA expression system (Blaisdell et al., 2004). In addition, catalytically

active CYP2C9.11 holo protein was expressed at a very low level due to its decreased stability in insect cells (Tai et al., 2005). Therefore, the substituted residues (Trp versus Gln) at this position might quite differently influence the stability of protein as well as the catalytic activity.

Ala477 is within SRS-6 and forms the β4-2 sheet. In a previous study, the systolic blood pressure in two patients with *CYP2C9*1/*30* was not lowered after 3 months of losartan treatment (Yin et al., 2008). The functional data obtained here are consistent with the *in vivo* study and clearly demonstrate the important role of **30* in the metabolism of losartan, and also to some extent, diclofenac and glimepiride. Substitution of the small Ala477 residue with the more bulky and nucleophilic Thr residue might lead to changes in protein conformation, substrate access, or affinity (the pi-pi interaction between substrates and Phe476 adjacent to Ala477) (Melet et al., 2003). Diminished activity of CYP2C9.30 for losartan oxidation suggests that increased dosage of losartan or alternative treatments should be considered for hypertensive patients with **30*.

In summary, catalytic activities of CYP2C9.3, CYP2C9.13, CYP2C9.26, CYP2C9.28, CYP2C9.30, CYP2C9.33 and CYP2C9.34 were assessed for diclofenac, losartan and

glimepiride as substrates. The variants except for CYP2C9.34 exhibited substrate-dependent changes in their activities for the three substrates examined. CYP2C9.13 was mainly present in the inactive form, P420, suggesting that **13* is an inactive allele toward a broad spectrum of CYP2C9 substrate drugs. The intrinsic clearance (V_{max}/K_m) for losartan oxidation was markedly decreased (> 77%) in all variations except for CYP2C9.34. On the other hand, reductions in the intrinsic clearance of glimepiride hydroxylation were rather variable: more than 80% in CYP2C9.3, CYP2C9.13, CYP2C9.26 and CYP2C9.33; 56 -75% in CYP2C9.28 and CYP2C9.30. Therefore, for the patients bearing these variant alleles, careful administrations of these drugs would be needed.

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Footnotes

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Figure legends

Fig. 1. Expression of wild-type and 7 variant CYP2C9s and ORs in insect cell microsomes. Representative Western blots for CYP2C9 (a) and OR (b) proteins (upper panel) are shown. Lane 1-8, co-expressed microsomes containing wild-type (lane 1), CYP2C9.3 (lane 2), CYP2C9.13 (lane 3), CYP2C9.26 (lane 4), CYP2C9.28 (lane 5), CYP2C9.30 (lane 6), CYP2C9.33 (lane 7) CYP2C9.34 (lane 8); lane 9, microsomes containing solely OR, lane 10, commercially available co-expressed baculosome containing CYP2C9.1 and OR (BD Gentest). Relative intensities of immunoreactive CYP2C9 (a) and OR (b) protein are shown in the lower panels. Each bar represents the mean ± S.D. of three separate experiments.

Fig.2. Representative CO difference spectra of CYP2C9.1 and 7 variants. Insect cell microsomes containing 2 mg/ml of total protein were used to measure CYP2C9 contents as described in Materials and Methods.

Fig. 3. Kinetic profiles of diclofenac 4'-hydroxylation by in-house wild-type and 7 variants. (a) Michaelis-Menten plots, in which each point represents the mean ± S.D. of 3 to 4 independent preparations derived from different infections. (b) Eadie-Hofstee

plots of representative preparations. In the lower panels, the areas near the coordinate origin in the upper panels are expanded.

Fig. 4. Kinetic profiles of losartan oxidation by in-house wild-type and 7 variants. (a) Michaelis-Menten plots, in which each point represents the mean ± S.D. of 3 to 4 independent preparations derived from different infections. (b) Eadie-Hofstee plots of representative preparations. In the lower panels, the areas near the coordinate origin in the upper panels are expanded

Fig. 5. Kinetic profiles of glimepiride hydroxylation by in-house wild-type and 7 variants. (a) Michaelis-Menten plots, in which each point represents the mean ± S.D. of 3 to 4 independent preparations derived from different infections. (b) Eadie-Hofstee plots of representative preparations. In the lower panels, the areas near the coordinate origin in the upper panels are expanded

Fig. 6. The ratios (%) of intrinsic clearance of the variants to that of the wild-type are depicted for each substrate. DIC, diclofenac; LOS, losartan; GLM, glimepiride. Diclofenac 4'-hydoxylation by CYP2C9.33 and CYP2C9.34 was performed previously

(Yin et al., 2005).

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Table 1 Characterization of insect cell microsomes coexpressing CYP2C9 and NADPH-cytochrome P450 oxidoreductase

Recombinant enzymes (Amino acid alteration)	P450 amount (pmol P450/mg protein)	OR activity (nmol cytochrome c reduced /min/mg protein)	Molar ratio (OR/P450)
CYP2C9.1 (Wild-type)	191 ± 19	686 ± 47	1.21 ± 0.14
CYP2C9.3 (Ile359Leu)	190 ± 26	586 ± 77	1.06 ± 0.26
CYP2C9.13 (Leu90Pro)	22 ± 5 ***	608 ± 7	9.63 ± 2.43
CYP2C9.26 (Thr130Arg)	158 ± 27	614 ± 205	1.31 ± 0.47
CYP2C9.28 (Gln214Leu)	165 ± 31	674 ± 32	1.40 ± 0.27
CYP2C9.30 (Ala477Thr)	201 ± 34	675 ± 86	1.16 ± 0.30
CYP2C9.33 (Arg132Gln)[a]	192 ± 15	758 ± 43	1.32 ± 0.03
CYP2C9.34 (Arg335Gln)[a]	159 ± 5	748 ± 29	1.56 ± 0.03

Data is represented by mean ± S.D. of 3-4 different expression experiments.

[a]Data on CYP2C9.33 and CYP2C9.34 was reported previously (Yin et al., 2008).

**$p<0.01$, ***$p<0.001$ vs. wild type. One-way ANOVA with post-hoc Dunnett multiple comparisons test.

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Table 2 Kinetic parameters for diclofenac hydroxylation Activities of wild-type and variant CYP2C9s

Recombinant enzymes (Amino acid alteration)	*K*m (μM)	*V*max (pmol/min/pmol P450)	Clearance (*V*max/*K*m) (μl/min/pmol P450)
CYP2C9.1 (Wild-type) [a]	1.8 ± 0.2	76.2 ± 6.5	43.6 ± 7.2
CYP2C9.3 (Ile359Leu)	5.3 ± 0.5 ***	84.9 ± 12.8	16.1 ± 2.3 ***
CYP2C9.13 (Leu90Pro)	7.0 ± 0.8 ***	14.3 ± 3.2 ***	2.1 ± 0.6 ***
CYP2C9.26 (Thr130Arg)	3.1 ± 0.2 **	32.0 ± 4.9 ***	10.5 ± 2.2 ***
CYP2C9.28 (Gln214Leu)	7.3 ± 0.5 ***	84.6 ± 10.3	11.6 ± 1.6 ***
CYP2C9.30 (Ala477Thr)	7.7 ± 0.3 ***	63.7 ± 9.1	8.3 ± 1.3 ***
CYP2C9.1 (Wild-type) [b]	3.4 ± 0.2	79.8 ± 6.6	23.4 ± 0.8
CYP2C9.33 (Arg132Gln) [b]	1.8 ± 0.1	7.8 ± 0.4	4.2 ± 0.3
CYP2C9.34 (Arg335Gln) [b]	3.0 ± 0.1	65.4 ± 2.1	22.0 ± 0.1
Gentest CYP2C9.1	2.7	30.6	11.5
Gentest human liver microsome	5.3	5.4	1.0

Data is represented by mean ± S.D. of 3-4 different expression experiments.

[a] Because the substrate consumption at the two lowest substrate concentrations (1 and 2.5 μM) was greater than 20%, these two points were omitted from the kinetic parameter estimation. However, this had no effect on the estimate of V_{max} and a very minor effect on the derived K_m (1.7 vs. 1.8 μM).

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[b] The previous data on CYP2C9.1, CYP2C9.33 and CYP2C9.34 (Yin et al., 2008) were cited.

$^{**}p<0.01$, $^{***}p<0.001$ vs. wild type. One-way ANOVA with post-hoc Dunnett multiple comparisons test among CYP2C9.1 and 5 variants tested in the present study.

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Table 3 Kinetic parameters for losartan oxidation activities of wild-type and variant CYP2C9s

Recombinant enzymes (Amino acid alteration)	*K*m (μM)	*V*max (pmol/min/nmol P450)	Clearance (*V*max/*K*m) (μl/min/nmol P450)
CYP2C9.1 (Wild-type)	1.12 ± 0.13	780 ± 82	704 ± 77
CYP2C9.3 (Ile359Leu)	0.99 ± 0.10	157 ± 30 ***	161 ± 42 ***
CYP2C9.13 (Leu90Pro)	2.76 ± 0.64 ***	47.0 ± 2.3 ***	17.6 ± 3.6 ***
CYP2C9.26 (Thr130Arg)	1.50 ± 0.13	40.3 ± 6.2 ***	26.8 ± 2.0 ***
CYP2C9.28 (Gln214Leu)	2.03 ± 0.42 ***	180 ± 18***	90.2 ± 11.7 ***
CYP2C9.30 (Ala477Thr)	0.77 ± 0.12	4.7 ± 0.4 ***	6.3 ± 1.3 ***
CYP2C9.33 (Arg132Gln)	1.03 ± 0.21	7.3 ± 0.3 ***	7.3 ± 1.4 ***
CYP2C9.34 (Arg335Gln)	1.06 ± 0.11	550 ± 27***	526 ± 75 ***
Gentest CYP2C9.1	1.34	630	470
Gentest human liver microsome	2.85	48.2	16.9

Data is represented by mean ± S.D. of 3-4 different expression experiments.

** $p<0.01$, *** $p<0.001$ vs. wild type. One-way ANOVA with post-hoc Dunnett multiple comparisons test.

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Table 4 Kinetic parameters for glimepiride hydroxylation activities of wild-type and variant CYP2C9s

Recombinant enzymes (Amino acid alteration)	*K*m (μM)	*V*max (pmol/min/pmol P450)	Clearance (*V*max/*K*m) (μl/min/pmol P450)
CYP2C9.1 (Wild-type)	0.18 ± 0.03	1.65 ± 0.11	9.22 ± 1.85
CYP2C9.3 (Ile359Leu)	0.13 ± 0.03	0.22 ± 0.06 ***	1.86 ± 0.94 ***
CYP2C9.13 (Leu90Pro)	1.29 ± 0.37 ***	0.16 ± 0.03 ***	0.13 ± 0.03 ***
CYP2C9.26 (Thr130Arg)	0.16 ± 0.05	0.14 ± 0.03 ***	0.94 ± 0.42 ***
CYP2C9.28 (Gln214Leu)	0.25 ± 0.04	0.98± 0.11 ***	4.04 ± 0.91 ***
CYP2C9.30 (Ala477Thr)	0.14 ± 0.03	0.28 ± 0.04 ***	2.32 ± 0.10 ***
CYP2C9.33 (Arg132Gln)	0.20 ± 0.04	0.013 ± 0.001 ***	0.07 ± 0.01 ***
CYP2C9.34 (Arg335Gln)	0.16 ± 0.03	1.05 ± 0.09 ***	6.62 ± 0.55 **
Gentest CYP2C9.1	0.14	0.88	6.40
Gentest human liver microsome	0.56	0.10	0.19

Data is represented by mean ± S.D. of 3-4 different expression experiments.

** $p<0.01$, *** $p<0.001$ vs. wild type. One-way ANOVA with post-hoc Dunnett multiple comparisons test.

Fig. 1

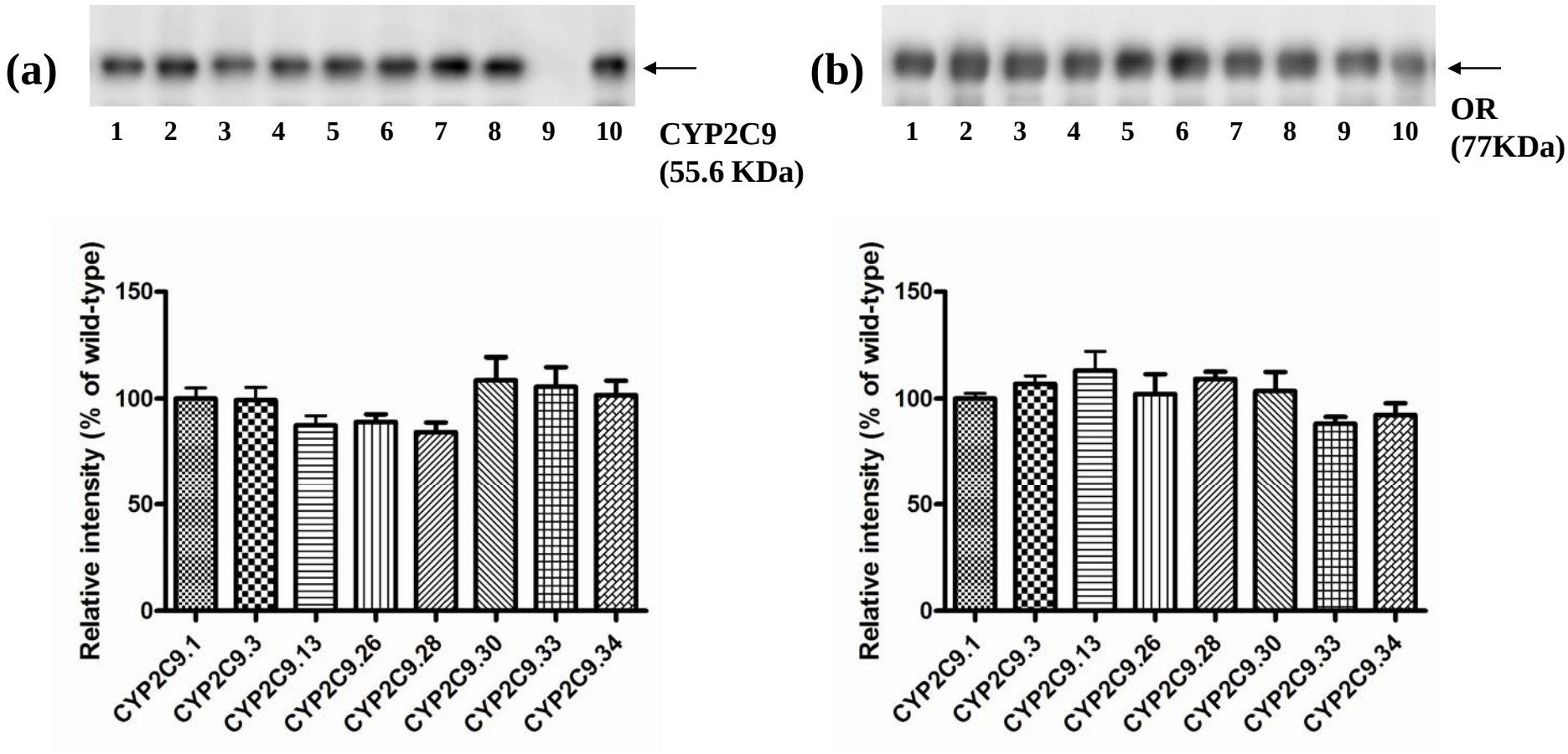

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Fig.2

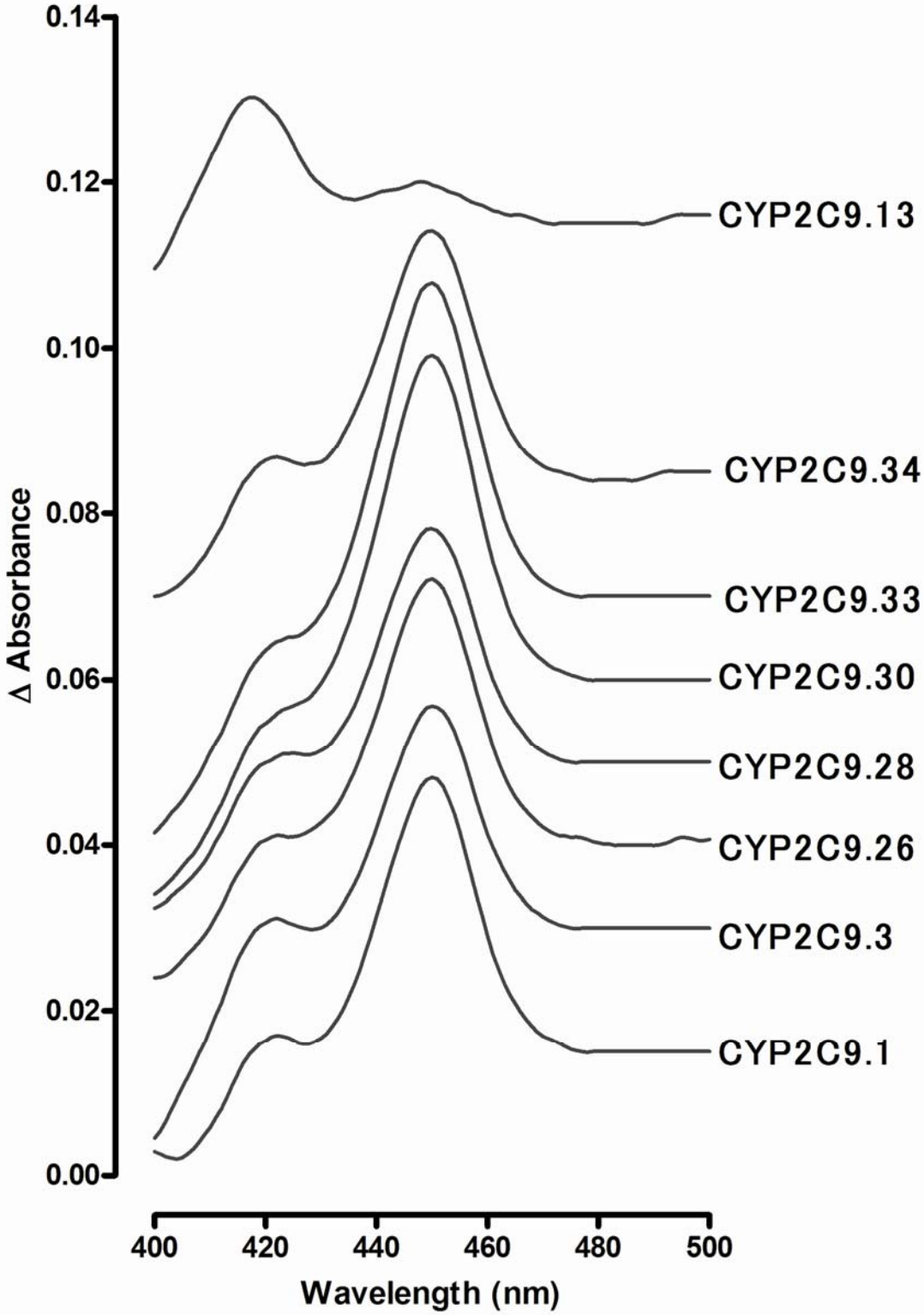

Fig. 3

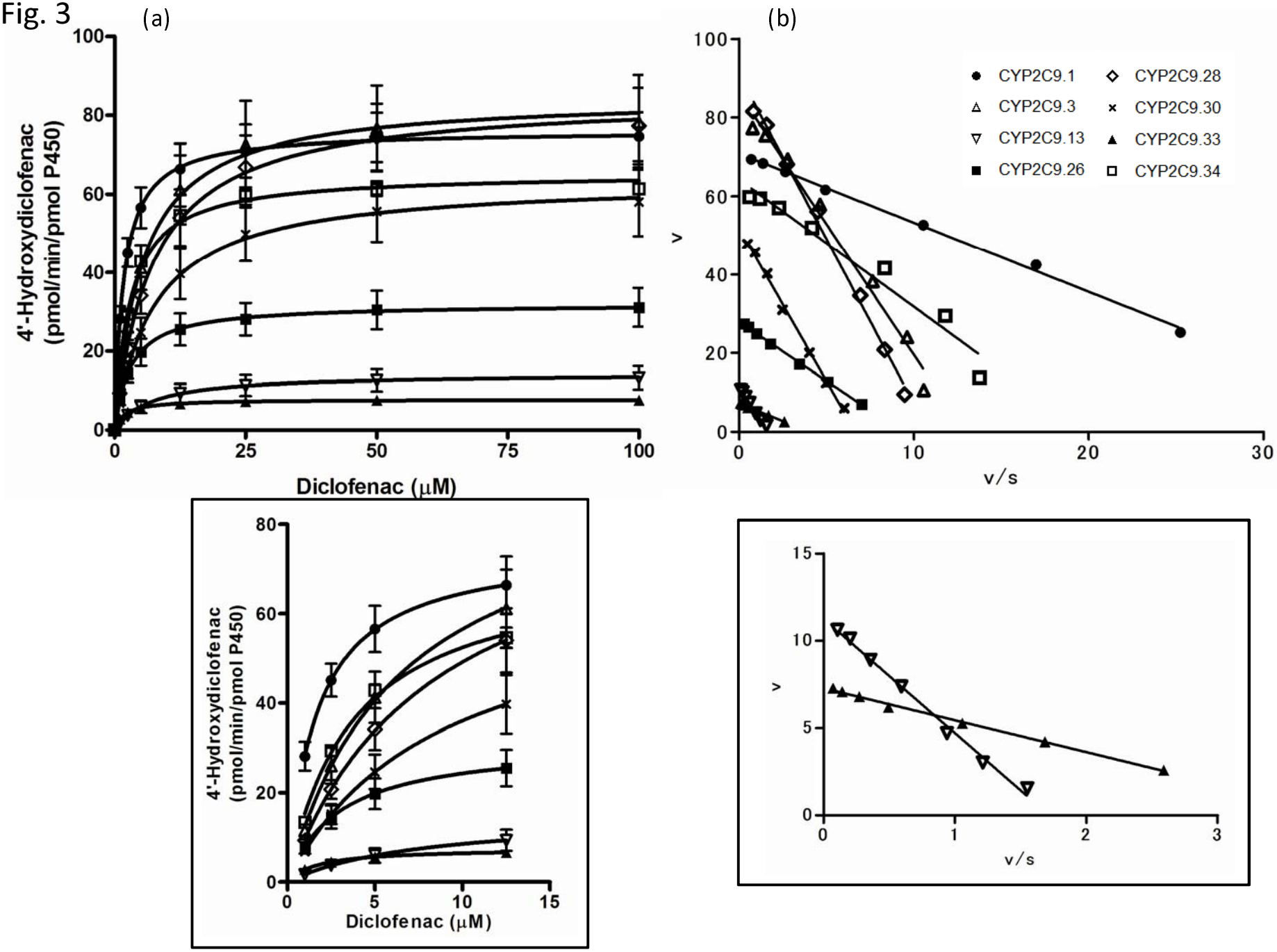

Fig. 4

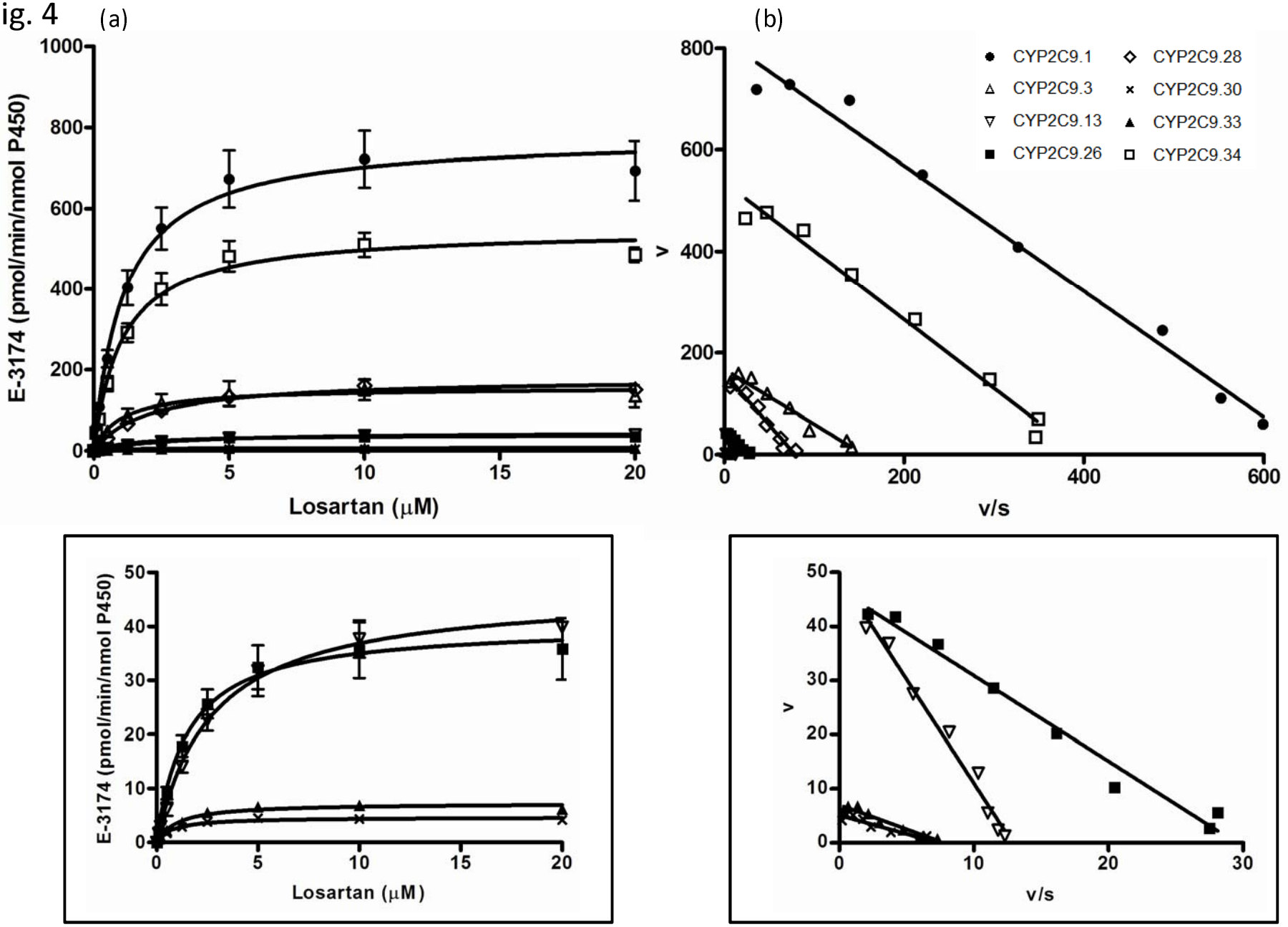

Fig. 5

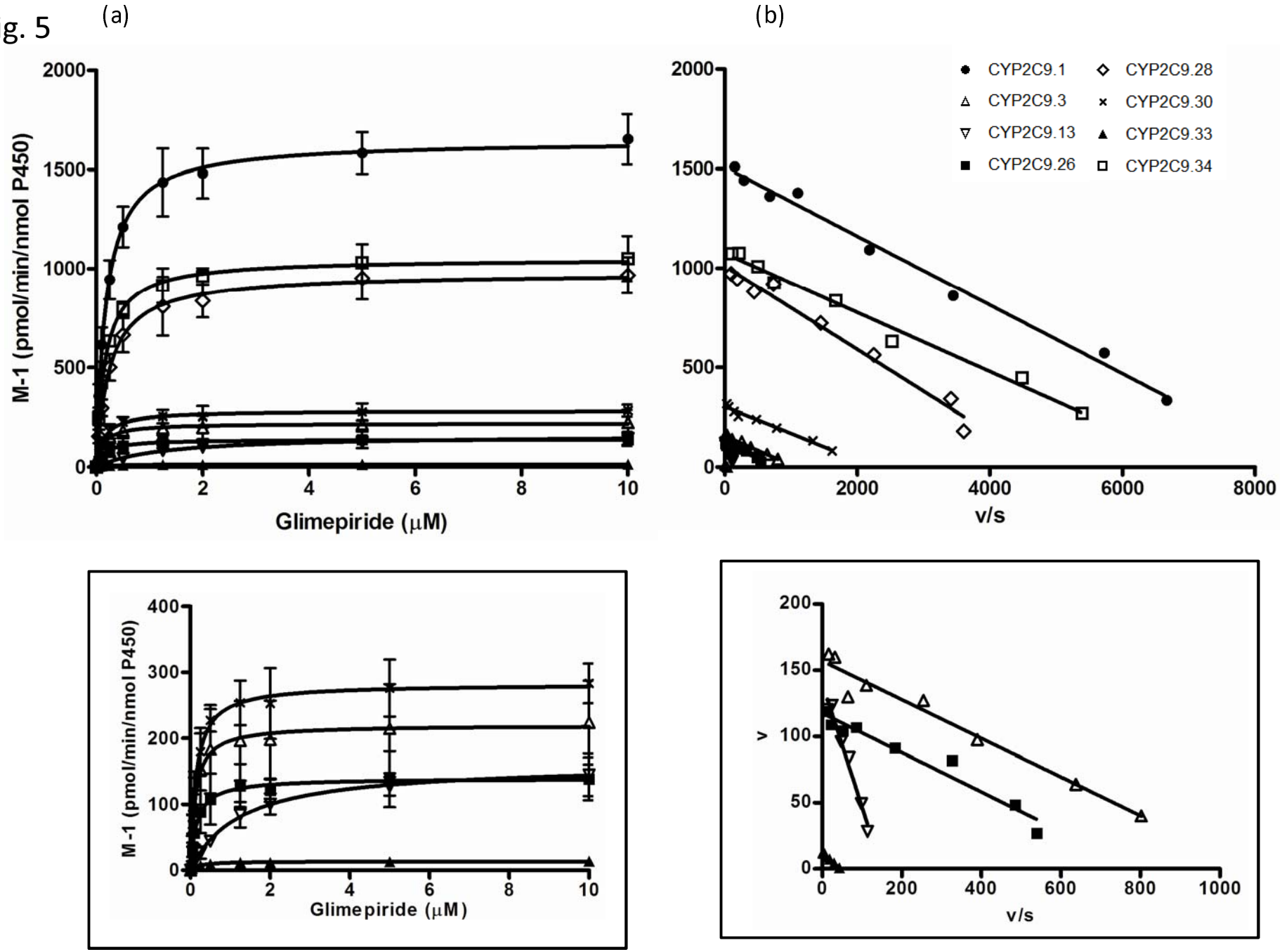

Fig. 6