However, the minor allele of this SNP has been shown associated with higher enzymatic activity for gemcitbine based on tests using lysates of red blood cells taken from Caucasian cancer patients.[3,4] In line with this, the minor allele is associated with decreased response, shorter time to progression and overall survival and lower frequencies of grade 3 and 4 neutropenia in Caucasian non-small cell lung cancer patients treated with gemcitabine and cisplatin.[4] As for 208G > A (Ala70Thr), the mutant enzyme expressed in yeast has reduced activity for both ara-C and cytidine.[5] Plasma of the patients with the minor allele had reduced activity for gemcitabine and cytidine and 208A was shown associated with reduced clearance of gemcitabine as well as increased frequencies of grade 3 and 4 neutropnia in Japanese cancer patients.[6,7]

Minor allele frequencies (MAFs) of the two SNPs have been reported in a few papers on Japanese and Chinese patients and relatively small numbers of healthy volunteers in Caucasians and Africans. In this study, we determined MAFs of both polymorphisms by newly developed pyrosequencing protocols in 200 healthy volunteers of Koreans. In addition, 206 Japanese, 200 Chinese-Americans, 150 Caucasian-Americans and 150 African-Americans were also genotyped to compare MAFs in order to reveal ethnic differences.

Korean genomic DNA samples from 200 healthy volunteers (189 males and 11 females) with average age of 24.6 years old (ranging from 20 to 53) were collected for genotyping analysis at the INJE pharmacogenomics research center (Inje University College of Medicine, Busan, Korea). DNAs were obtained from Epstein-Barr virus-transformed lymphoblastoid cells prepared from 206 healthy Japanese volunteers at the Tokyo Women's Medical University under the auspices of the Pharma SNP consortium (Tokyo, Japan). DNA from 200 healthy Chinese-Americans was extracted from cord blood samples purchased from AllCells (Emeryville, CA, USA). Peripheral blood samples from healthy Caucasian- and African-American volunteers (150 each) were purchased from the Tennessee Blood Service Corporation (Memphis, TN, USA) and DNA was extracted as described previously.[8] Written informed consent was obtained from all subjects. Ethical review boards of all participating organizations approved this study.

CDA genotypes were determined by pyrosequencing. First, polymerase chain reaction (PCR) was performed to amplify regions containing each target polymorphic site from approximately 25–100 ng of genomic DNA using 0.02 units/μl of *Ex*-Taq (Takara Bio Inc., Shiga, Japan) with 0.2 mM each of dNTP mixtures and 0.2 μM primers as follows: biotin-ATGGCCCAGAAGCGTCCT and CGCCTCTTCCTGTACATCTT for 79A > C and biotin-CCACCTTGTTTGGAGTAACC and TGTGTAAGGAAGATGTTGG for 208G > A. PCR conditions were 94 °C for 5 min, followed by 50 cycles of 94 °C for 30 sec, 55 °C for 45 sec and 72 °C for 20 sec, and then a final extension at 72 °C for 7 min. Generation of the single-stranded fragment and annealing of the sequencing primers were described previously.[9] The sequencing primers used were GGGCAGTAGGCTGACT for 79A > C and ACGGCCTTCTGGAT for 208G > A. Genotypes were determined using the PSQ 96MA (Biostage AB, Uppsala, Sweden) and PSQ 96 SNP reagent set (Biostage AB). The dispensation orders were ATGACTGCT for 79A > C and CAGCTCGTC for 208G > A. The accuracy of genotyping results by pyrosequencing was validated by direct sequencing using at least 5 genomic DNA samples each for wild-type, heterozygote and homozygote of both SNPs (excluding homozygous 208A which was validated by 2 other genomic samples we have). Hardy-Weinberg equilibrium analysis was performed with SNPAlyze version 3.1 (Dynacom Co., Yokohama, Japan). Statistical significance for the differences in MAFs between the Asian populations was analyzed by the Fisher's exact test using Prism 5.0 (La Jolla, CA, USA).

Genotyping of the 79A > C and 208G > A SNPs was successfully performed for all samples from the five populations by pyrosequencing (**Fig. 1**), except for 14 Caucasian-American and 4 African-American samples, which gave small lightning peaks. Genotypes of these samples were clearly determined for confirmation by direct sequencing.[6] All obtained genotypes were in Hardy-Weinberg equilibrium. The genotypes and MAFs of the two non-synonymous SNPs are summarized in **Table 1**.

As for 79A > C (Lys27Gln), MAF was high in Caucasian-Americans (0.327), medium in three Asians (0.153–0.204) and low in African-Americans (0.087). MAF in Korean healthy volunteers (0.153) was slightly lower than that in Japanese (0.204) and comparable to that in Chinese-Americans (0.155). MAF in healthy Japanese was similar to MAFs in our previous report[6] on 256 Japanese cancer patients (MAF = 0.207) suggesting that this polymorphism is not related to cancer-susceptibility. MAF in Chinese-Americans was also similar to that (0.121) in the 286 Chinese patients containing 87 acute leukemia patients.[10] MAF of Caucasian-Americans was comparable to those in previous studies with smaller sample numbers: 0.363 in Europeans (n = 95)[11] and 0.298 in Caucasian-Americans (n = 60).[2] While MAF in African-Americans in the current study was similar to that in the previous report for 60 African-Americans (0.108),[2] our results differed from MAF (0.035) in Kenyans plus Ghanaians (n = 85).[11]

Regarding 208G > A, the minor allele was detected in Koreans and Japanese but not in Chinese-Americans, Caucasian-Americans and African-Americans. Although the difference did not reach significance (p = 0.0640), MAF in the Korean healthy volunteers (0.005) was lower than in the Japanese (0.022), which was slightly lower

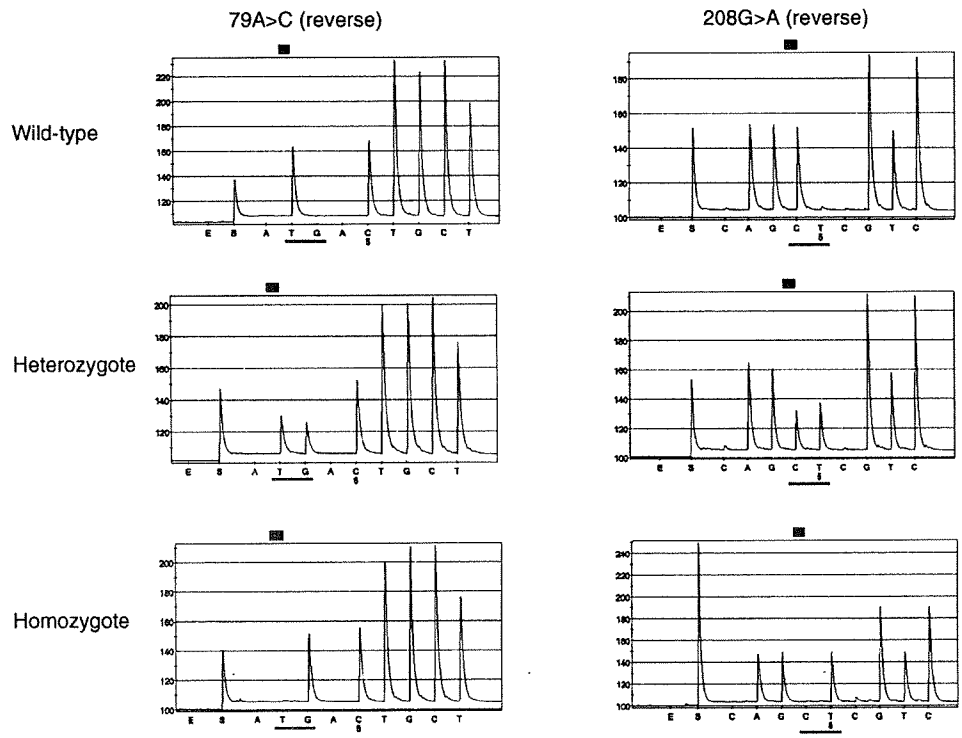

Fig. 1. Representative patterns (pyrograms) of wild-type, heterozygote and homozygote for 79A>C and 208G>A in pyrosequencing were shown

Table 1. Genotypes and minor allele frequencies (MAFs) of the two non-synonymous *CDA* SNPs

Population	N	79A>C (Lys27Gln)					208G>A (Ala70Thr)				
		wild-type	hetero-zygote	homo-zygote	MAF	95% confidence interval	wild-type	hetero-zygote	homo-zygote	MAF	95% confidence interval
Korean	200	144	51	5	0.153	0.118–0.188	198	2	0	0.005	0.000–0.012
Japanese	206	129	70	7	0.204	0.165–0.243	197	9	0	0.022	0.008–0.036
Chinese-American	200	142	54	4	0.155	0.120–0.190	200	0	0	0.000	
Caucasian-American	150	67	68	15	0.327	0.274–0.380	150	0	0	0.000	
African-American	150	126	22	2	0.087	0.055–0.119	150	0	0	0.000	

than in the 256 Japanese cancer patients (0.037).[6] The 208A allele was not detected in Chinese-Americans. By the Fisher's exact test, significant difference in MAF was found between Japanese and Chinese-Americans ($p = 0.0038$) in the current study. However, since the number of subjects with 208A was very small in Koreans ($n = 2$) or none in Chinese-Americans, differences should be confirmed using a larger number of subjects. In the previous paper, the 286 Chinese patients containing 87 acute leukemia patients also had a very low MAF (0.005).[10] The 208G>A was not detected in Caucasian-Americans or African-Americans in this study, as previously reported in relatively small numbers of Caucasians and African-Americans,[2,11] in contrast to Kenyans plus Ghanaians (0.131).[11] This SNP is very important for gemcitabine treatment since 3 out of 4 Japanese patients with gemcitabine-induced life-threatening toxicity had homozygous 208A.[7,12] However, the current study on MAFs suggests that the clinical importance of this SNP is ethnic-dependent, maybe even within the Asian populations.

In conclusion, we determined the genotypes and MAFs of *CDA* non-synonymous SNPs 79A>C (Lys27Gln) and 208G>A (Ala70Thr) in 200 healthy volunteers of Koreans, along with 206 Japanese, 200 Chinese-Americans, 150 Caucasian-Americans and 150 African-Americans in order to demonstrate ethnic differences. Our results suggest that the MAF of 208G>A in Japanese is likely somewhat higher than in Koreans and Chinese-

Americans. These data provide fundamental and useful information for pharmacogenetic studies on cytidine deaminase-catalyzing drugs.

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Substrate-Dependent Functional Alterations of Seven CYP2C9 Variants Found in Japanese Subjects

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ABSTRACT:

CYP2C9 is a polymorphic enzyme that metabolizes a number of clinically important drugs. In this study, catalytic activities of seven alleles found in Japanese individuals, *CYP2C9*3* (I359L), **13* (L90P), **26* (T130R), **28* (Q214L), **30* (A477T), **33* (R132Q), and **34* (R335Q), 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 six variants except for CYP2C9.13. A large part of CYP2C9.13 was present in the apo form P420. 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. 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, the 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 to 75% in CYP2C9.28 and CYP2C9.30. These findings suggest the necessity for careful administration of losartan and glimepiride to patients bearing these six alleles.

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* (R144C) and *CYP2C9*3* (I359L), have been well studied in their associations with reduced catalytic activities toward several substrates such as warfarin, tolbutamide, and losartan, both in vitro and in vivo (Lee et al., 2002; Kirchheiner and Brockmöller, 2005).

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The frequencies of low-activity CYP2C9 alleles differ considerably among different ethnic populations. In whites, the frequencies of *2 and *3 are 0.08 to 0.14 and 0.04 to 0.16, respectively (Schwarz, 2003). In contrast, in East Asian populations, *2 is hardly found, and *3 is present only at 0.01 to 0.04. More recently, a series of novel nonsynonymous variations were identified in several Asian populations (Si et al., 2004; Zhao et al., 2004; Maekawa et al., 2006; Yin et al., 2008). **13* (L90P), an allele originally identified in a Chinese poor metabolizer toward lornoxicam, has been found in Chinese, Korean, and Japanese individuals at allele frequencies of 0.002 to 0.01 (Si et al., 2004; Bae et al., 2005; Maekawa et al., 2006; 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,b). We reported 7 nonsynonymous single nucleotide polymorphisms from 263 Japanese subjects, of which **25* (K118RfsX9) was a null allele and **26* (T130R), **28* (Q214L), and **30* (A477T) were functionally defective toward diclofenac when expressed in COS-1 cells (Maekawa et al., 2006). In addition, two novel variations, **33* (R132Q) and **34* (R335Q), were detected by large-scale direct resequencing of the samples from 724 Japanese individuals. Here, CYP2C9.33 showed a 5-fold lower intrinsic clearance toward diclofenac in vitro (Yin et al., 2008). These results point

ABBREVIATIONS: P450, cytochrome P450; OR, NADPH P450 reductase; ANOVA, analysis of variance; SRS, substrate recognition site.

out that not only **2* and **3* but also many other less-frequent defective alleles could contribute to highly variable interindividual 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, E-3174. For low-frequency alleles, elucidation of the substrate dependencies of their recombinant enzymes is valuable because their functional assessments in vivo are difficult because of the 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 (St. Louis, MO). Losartan and its metabolite, E-3174, were kindly provided by Merck (Whitehouse Station, NJ). Glimepiride was a gift from sanofi-aventis K.K. (Tokyo, Japan). Glibenclamide was obtained from Wako Pure Chemicals (Osaka, Japan). *Spodoptera frugiperda* (*Sf*) 21 insect cells, supplemented Grace's Insect Medium, gentamicin, Pluronic F68, and a Bac-to-Bac Baculovirus Expression System were purchased from Invitrogen (Carlsbad, CA), and fetal bovine serum was from SAFC Biosciences (Manchester, UK). Goat anti-CYP2C6 antiserum, which can cross-react with human CYP2C9, and anti-rat NADPH cytochrome P450 (P450) reductase (OR) antibodies were purchased from Daiichi Pure Chemicals (Tokyo, Japan), horseradish peroxidase-conjugated rabbit anti-goat IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA), and Western Lightning Chemiluminescence Reagent Plus was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). 4′-Hydroxydiclofenac, Baculosomes coexpressing CYP2C9 and OR (lot 63793), Supersomes coexpressing either CYP3A4 (lot 49734) or CYP2C8 (lot 4) with OR and cytochrome b_5, pooled human liver microsomes (lot 32556; 570 pmol of P450/mg of 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 b_5 was purchased from Oxford Biomedical Research (Oxford, UK), and a 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 et al., 2008). The plasmids containing the 1.5-kb full-length CYP2C9 wild-type (pcDNA3.1D/CYP2C9/wild-type) and five variant (pcDNA3.1D/CYP2C9/T130R, pcDNA3.1D/CYP2C9/Q214L, pcDNA3.1D/CYP2C9/A477T, pcDNA3.1D/CYP2C9/R132Q, and pcDNA3.1D/CYP2C9/R335Q) CYP2C9 cDNAs were constructed as described previously (Maekawa et al., 2006; Yin et al., 2008). In addition, two substitutions, 1075A>C (I359L, CYP2C9.3) and 269T>C (L90P, 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 in boldface): 5′-CACGAGGTCCAGAGATACCTTGACCTTCTCCCC-3′ (sense) and 5′-GGGGAGAAGGTCAAGGTATCTCTGGACCTCGTG-3′ (antisense) for pcDNA3.1D/CYP2C9/I359L; and 5′-GGAAGCCCTGATTGATCCTGGAGAGGAGTTTTC-3′ (sense) and 5′-GAAAACTCCTCTCCAGGATCAATCAGGGCTTCC-3′ (antisense) for pcDNA3.1D/CYP2C9/L90P.

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 a 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 of cytochrome *c* reduced/min/nmol of 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 seven 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 to 5 pmol of P450 from insect cell microsomes (2 pmol of P450 for CYP2C9.1 and 5 pmol of P450 for other variants), 4 to 10 pmol of purified cytochrome b_5 (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 of P450 per reaction was used. High-performance liquid chromatography conditions are the same as those 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 the regioselectivity of diclofenac hydroxylation, the concentrations of 5-hydroxydiclofenac 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 of 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 polytetrafluoroethylene membrane filters of 0.2 μm pore size (Millipore Corporation, Billerica, MA), and the aliquots (50 μl) were injected into a Shimadzu Prominence high-performance liquid chromatography system (Shimadzu, Kyoto, Japan) coupled with fluorescence detection (Ritter et al., 1997). Separation was conducted on a Shimpack CLC-ODS (M) column (250 × 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 intraday assay variations were less than 6%.

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

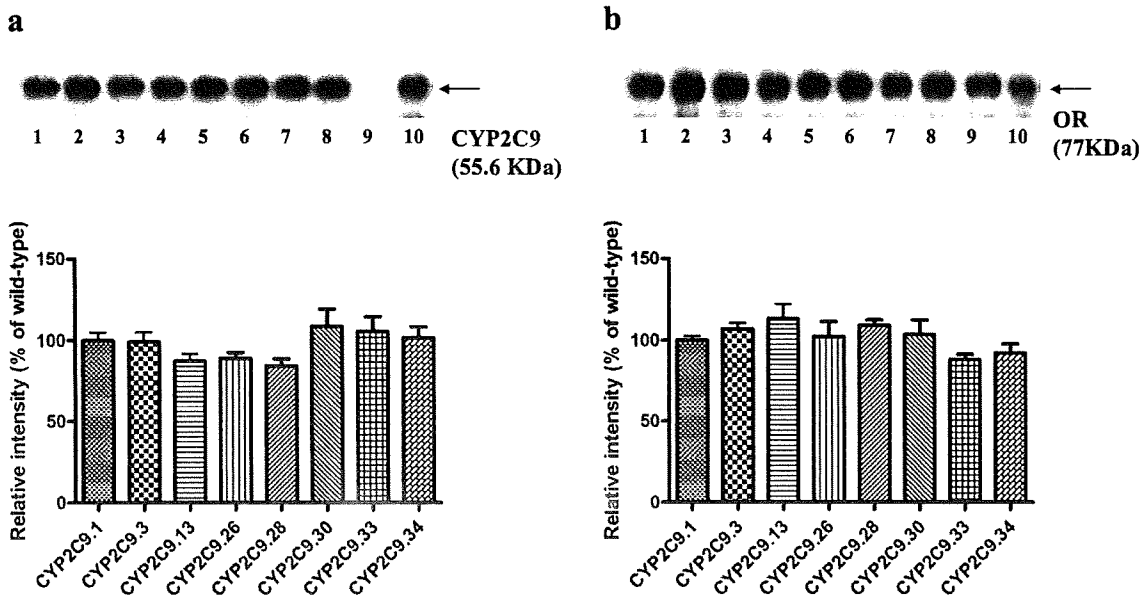

FIG. 1. Expression of wild-type and seven variant CYP2C9s and ORs in insect cell microsomes. Representative Western blots for CYP2C9 (a) and OR (b) proteins (top panel) are shown. Lanes 1 to 8, coexpressed microsomes containing wild type (lane 1), CYP2C9.3 (lane 2), CYP2C9.13 (lane 3), CYP2C9.26 (lane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

methyl 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 glibenclamide as an internal standard. Reaction samples were extracted with 5.0 ml of diethyl ether, 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°C for 20 min and then processed in the same manner as the recombinant enzyme samples. Liquid chromatography-mass spectrometry analysis was performed using an LCMS-2010 Evolution System (Shimadzu). Aliquots of samples (2 μl) were applied onto a Shim-pack FC-ODS column (3.0 μm; 2.0 × 75 mm; Shimadzu) kept at 40°C. 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 atmospheric pressure ionization-electrospray ionization mode under selected ion monitoring conditions as described previously (Suzuki et al., 2006): temperature of the curved desolvation line, 230°C; gas flow rate, 1.5 l/min; and heat block temperature, 200°C. Under these conditions, M-1, glibenclamide, and glimepiride were eluted at 5.8, 10.4, 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 interday 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 nonlinear regression analysis of a hyperbolic Michaelis-Menten equation (Prism version 3.0a; GraphPad Software, San Diego, CA). 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 the 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 a post hoc Dunnett multiple comparisons test.

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 and p = 0.222 for OR by one-way ANOVA) tested. Holoenzyme 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 of P450/mg of microsomal protein. Conversely, CYP2C9.13 was expressed at 22 ± 5 pmol P450/mg of microsomal protein, which was approximately 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 to 4 for the wild type, seven variant enzymes, BD Gentest CYP2C9.1 (commercially available Baculosomes coexpressing CYP2C9.1 and OR), and the pooled human liver microsomes. The ratios (percentages) of intrinsic clearance of the variants to that of the wild type for each substrate are depicted in Fig. 6.

Compared with the pooled human liver microsomes, the recombinant wild-type enzymes produced by the baculovirus-insect cell systems, either in-house or BD 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

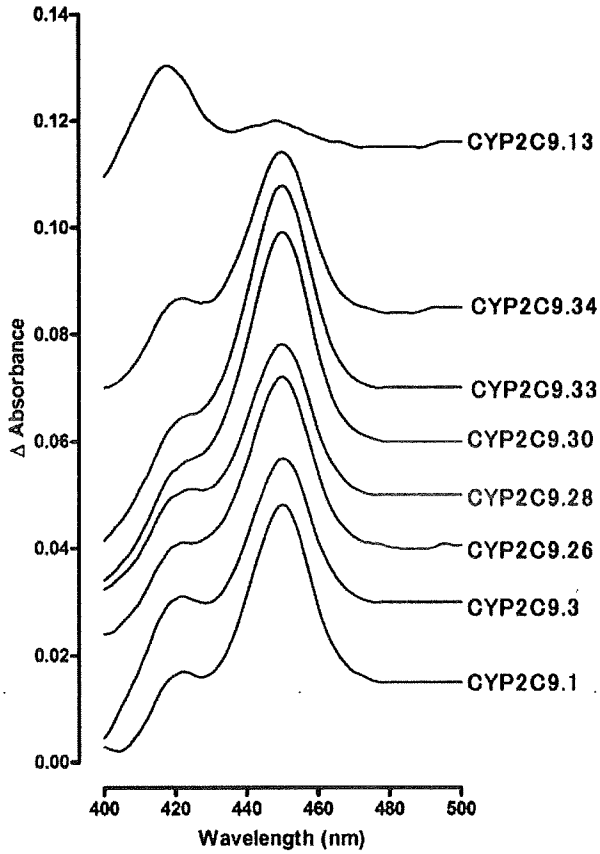

FIG. 2. Representative CO difference spectra of CYP2C9.1 and seven variants. Insect cell microsomes containing 2 mg/ml total protein were used to measure CYP2C9 contents as described under *Materials and Methods*.

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 five variants (CYP2C9.3, CYP2C9.13, CYP2C9.26, CYP2C9.28, and CYP2C9.30). All five 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, whereas 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 five variants was significantly reduced compared with that of 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 seven variants, only CYP2C9.28 exhibited a slight change in regioselectivity for diclofenac hydroxylation, namely in 5-hydroxydiclofenac 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-hydroxydiclofenac to 4′-hydroxydiclofenac by CYP2C9.28 was estimated to be 0.013 ± 0.005, whereas 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. Conversely, all seven 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 versus 4% for losartan), CYP2C9.28 (27% versus 13%), and CYP2C9.33 (18% versus 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 with the wild type, resulting in a 99% decrease in intrinsic clearance. A similar decrease in activity (99%) was observed for CYP2C9.33, which is due mainly to the remarkable decrease in the V_{max} value compared with that for 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).

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

Discussion

In the present study, we focused on the seven 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 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 for 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 those of a previous study using both the yeast expression system and human liver microsomes, in which a 7-fold lower intrinsic clearance of losartan by CYP2C9.3 compared with CYP2C9.1 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 those reported by Suzuki et al. (2006) using insect cells microsomes from BD Gentest. These 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 in subjects with

TABLE 1

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

Data are presented as the mean ± S.D. of three to four different expression experiments.

Recombinant Enzymes (Amino Acid Alteration)	P450 Amount	OR Activity	Molar Ratio (OR/P450)
	pmol of P450/mg of protein	*nmol of cytochrome c reduced/min/mg of protein*	
CYP2C9.1 (wild type)	191 ± 19	686 ± 47	1.21 ± 0.14
CYP2C9.3 (I359L)	190 ± 26	586 ± 77	1.06 ± 0.26
CYP2C9.13 (L90P)	22 ± 5***	608 ± 7	9.63 ± 2.43
CYP2C9.26 (T130R)	158 ± 27	614 ± 205	1.31 ± 0.47
CYP2C9.28 (Q214L)	165 ± 31	674 ± 32	1.40 ± 0.27
CYP2C9.30 (A477T)	201 ± 34	675 ± 86	1.16 ± 0.30
CYP2C9.33 (R132Q)[a]	192 ± 15	758 ± 43	1.32 ± 0.03
CYP2C9.34 (R335Q)[a]	159 ± 5	748 ± 29	1.56 ± 0.03

[a] Data on CYP2C9.33 and CYP2C9.34 was reported previously (Yin et al., 2008).
*** $P < 0.001$ vs. wild type. One-way ANOVA with a post hoc Dunnett multiple comparisons test.

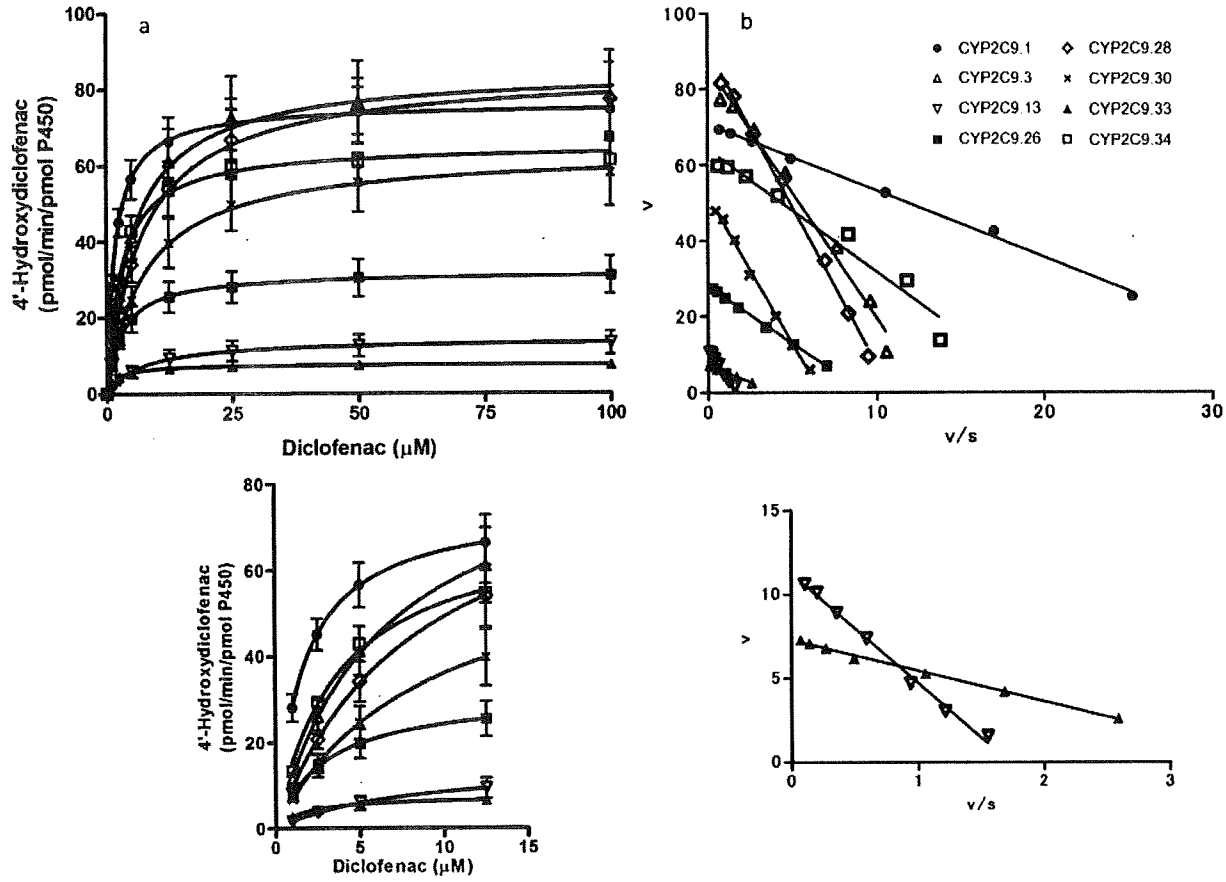

FIG. 3. Kinetic profiles of diclofenac 4'-hydroxylation by the in-house wild type and seven variants. a, Michaelis-Menten plots, in which each point represents the mean ± S.D. of three to four independent preparations derived from different infections. b, Eadie-Hofstee plots of representative preparations. In the bottom panels, the areas near the coordinate origin in the top panels are expanded.

**1/*1* (Yasar et al., 2002; Sekino et al., 2003). On the contrary, there were no significant differences in any diclofenac pharmacokinetic parameters between **1/*1* and **1/*3* genotype groups (Shimamoto et al., 2000). In addition, the **3* heterozygotes showed a 1.3- to 2.5-fold higher mean glimepiride area under the plasma concentration-time curve than that for the wild type (Niemi et al., 2002; Wang et al., 2005; Suzuki et al., 2006), and **3*-bearing patients might have an increased risk of sulfonylurea-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 (allele frequency = 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 forms) 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; Table 1), suggesting that L90P substitution resulted in improper heme in-

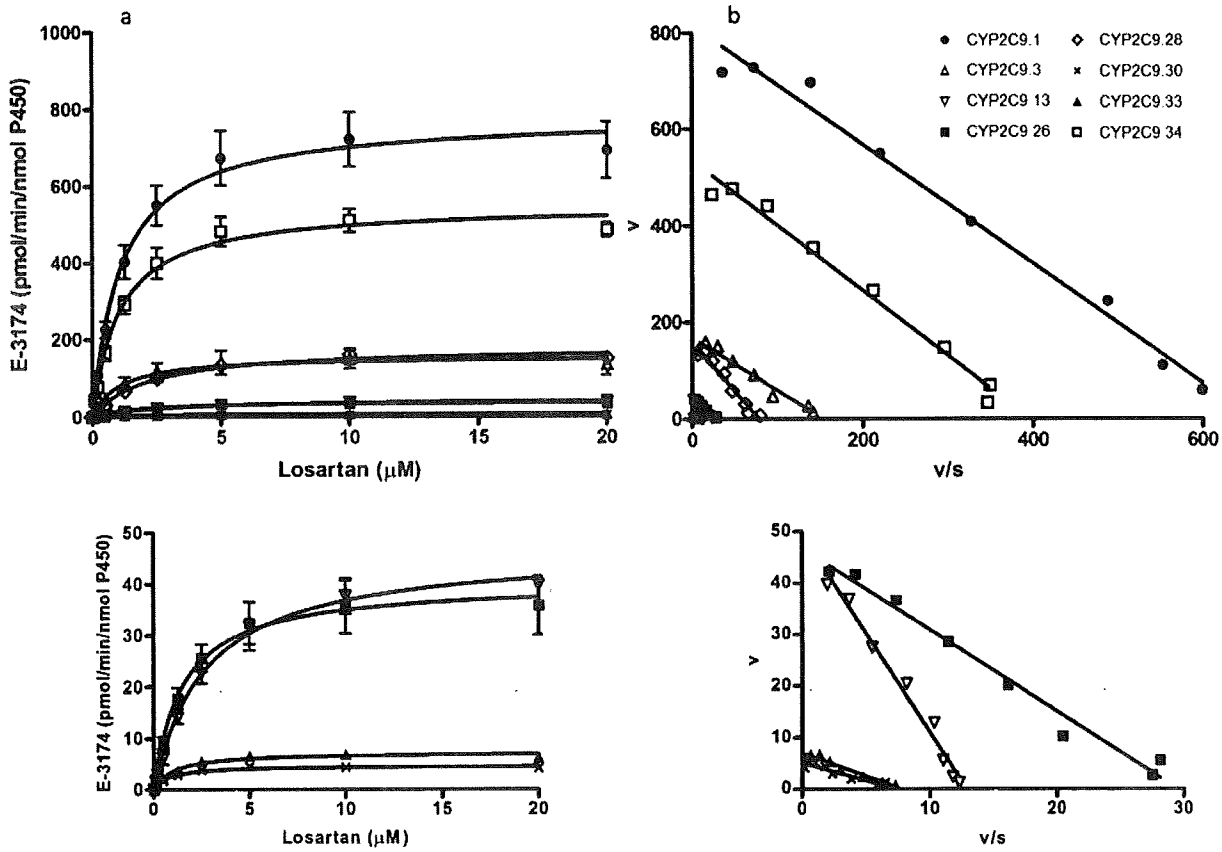

FIG. 4. Kinetic profiles of losartan oxidation by the in-house wild type and seven variants. a, Michaelis-Menten plots, in which each point represents the mean ± S.D. of three to four independent preparations derived from different infections. b, Eadie-Hofstee plots of representative preparations. In the bottom panels, the areas near the coordinate origin in the top panels are expanded.

corporation. 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.

In the in vitro kinetic characterization, Guo et al. (2005a,b) 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 L90P substitution on the residues Ala106 to 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 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 finding 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 cell-produced enzymes for all three variants: that is, 84% (COS-1 cells) versus 76% (insect cells) for CYP2C9.26, 77% versus 73% for CYP2C9.28, and 81% versus 81% for CYP2C9.30.

CYP2C9.26 (T130R), CYP2C9.28 (Q214L), CYP2C9.30 (A477T), and CYP2C9.33 (R132Q) showed a substrate-dependent reduction in activity and changes in the kinetic parameters, whereas CYP2C9.34 (R335Q) held catalytic activities almost similar to those of 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 in a loop region between the C- and D-helices, respectively. As suggested in CYP2C9.2 (R144C) (Crespi and Miller, 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 R132Q 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

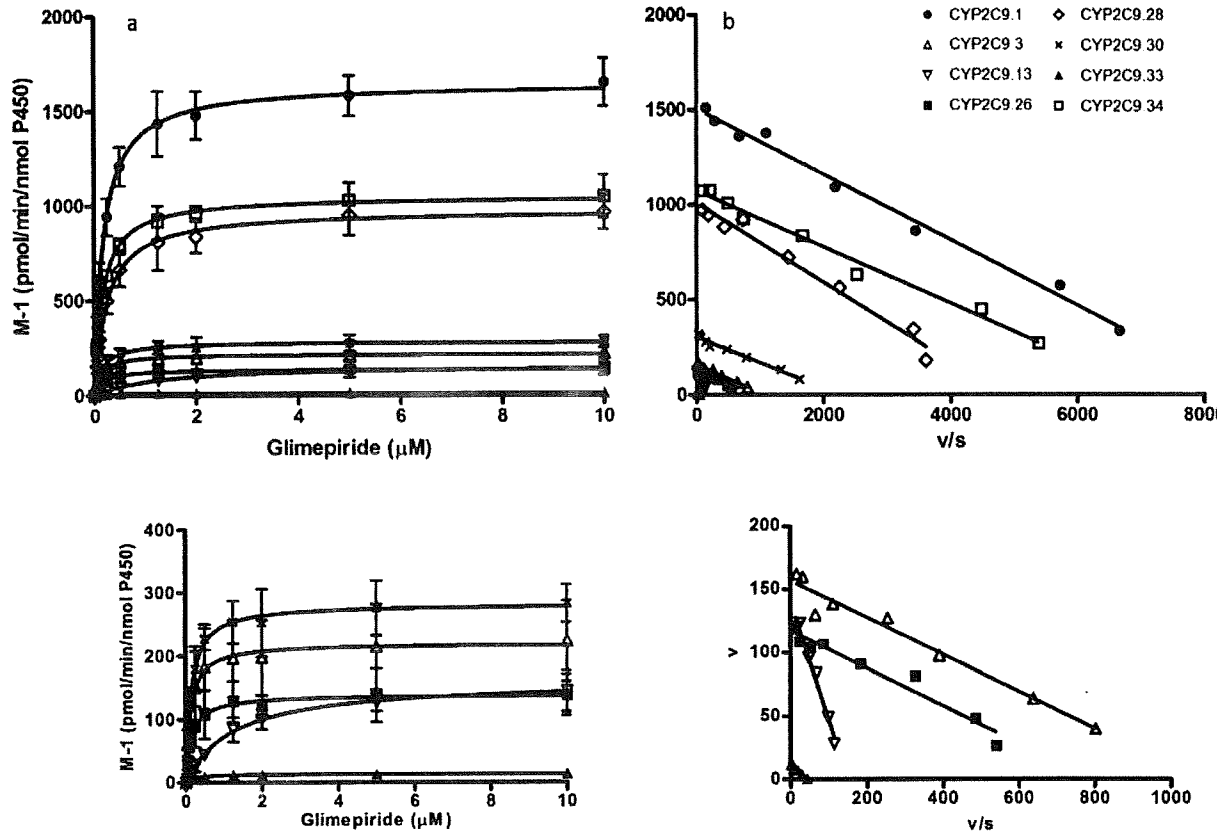

FIG. 5. Kinetic profiles of glimepiride hydroxylation by the in-house wild type and seven variants. a, Michaelis-Menten plots, in which each point represents the mean ± S.D. of three to four independent preparations derived from different infections. b, Eadie-Hofstee plots of representative preparations. In the bottom panels, the areas near the coordinate origin in the top panels are expanded.

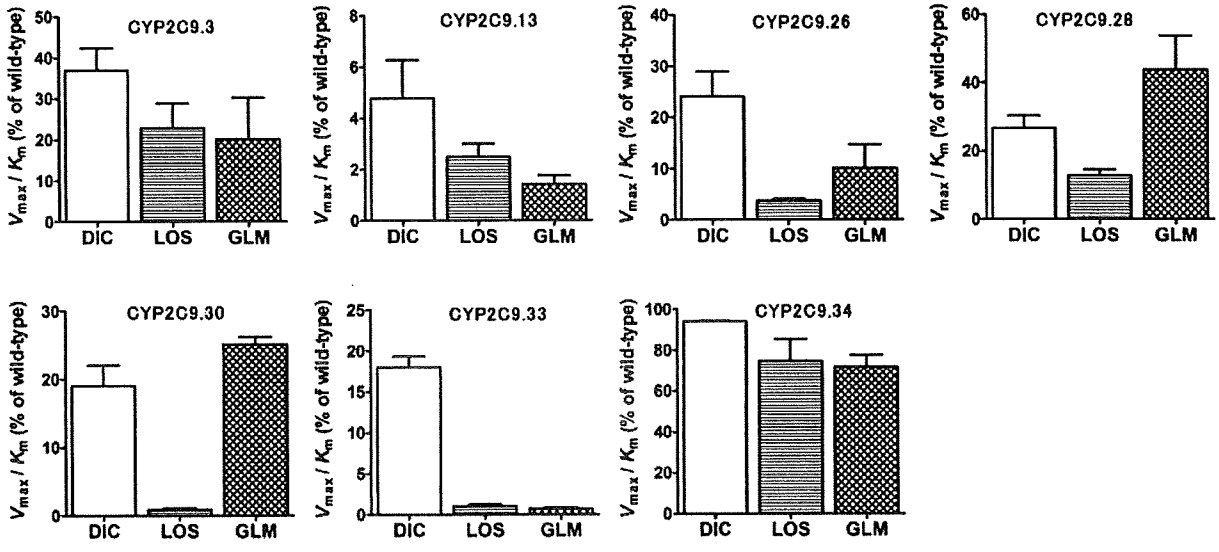

FIG. 6. The ratios (percentages) of intrinsic clearance of the variants to that of the wild type are depicted for each substrate. Diclofenac 4′-hydroxylation by CYP2C9.33 and CYP2C9.34 was performed previously (Yin et al., 2008). DIC, diclofenac; LOS, losartan; GLM, glimepiride.

CYP2C family, is located between the F- and G-helices and is only five amino acids downstream of SRS-2. In the P450 structure, the regions from the 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 (Q214L) 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

TABLE 2

Kinetic parameters for diclofenac hydroxylation activities of wild-type and variant CYP2C9s

Data are presented as the mean ± S.D. of three to four different expression experiments.

Recombinant Enzymes (Amino Acid Alteration)	K_m	V_{max}	Clearance (V_{max}/K_m)
	μM	*pmol/min/pmol P450*	*μl/min/pmol P450*
CYP2C9.1 (wild type)[a]	1.8 ± 0.2	76.2 ± 6.5	43.6 ± 7.2
CYP2C9.3 (I359L)	5.3 ± 0.5***	84.9 ± 12.8	16.1 ± 2.3***
CYP2C9.13 (L90P)	7.0 ± 0.8***	14.3 ± 3.2***	2.1 ± 0.6***
CYP2C9.26 (T130R)	3.1 ± 0.2**	32.0 ± 4.9***	10.5 ± 2.2***
CYP2C9.28 (Q214L)	7.3 ± 0.5***	84.6 ± 10.3	11.6 ± 1.6***
CYP2C9.30 (A477T)	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 (R132Q)[b]	1.8 ± 0.1	7.8 ± 0.4	4.2 ± 0.3
CYP2C9.34 (R335Q)[b]	3.0 ± 0.1	65.4 ± 2.1	22.0 ± 0.1
BD Gentest CYP2C9.1	2.7	30.6	11.5
BD Gentest human liver microsomes	5.3	5.4	1.0

[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).

[b] Previous data on CYP2C9.1, CYP2C9.33, and CYP2C9.34 (Yin et al., 2008) are cited.

** $P < 0.01$ vs. wild type. One-way ANOVA with a post hoc Dunnett multiple comparisons test among CYP2C9.1 and five variants tested in the present study.

*** $P < 0.001$ vs. wild type.

TABLE 3

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

Data are presented as the mean ± S.D. of three to four different expression experiments.

Recombinant Enzymes (Amino Acid Alteration)	K_m	V_{max}	Clearance (V_{max}/K_m)
	μM	*pmol/min/nmol P450*	*μl/min/nmol P450*
CYP2C9.1 (wild type)	1.12 ± 0.13	780 ± 82	704 ± 77
CYP2C9.3 (I359L)	0.99 ± 0.10	157 ± 30***	161 ± 42***
CYP2C9.13 (L90P)	2.76 ± 0.64***	47.0 ± 2.3***	17.6 ± 3.6***
CYP2C9.26 (T130R)	1.50 ± 0.13	40.3 ± 6.2***	26.8 ± 2.0***
CYP2C9.28 (Q214L)	2.03 ± 0.42***	180 ± 18***	90.2 ± 11.7***
CYP2C9.30 (A477T)	0.77 ± 0.12	4.7 ± 0.4***	6.3 ± 1.3***
CYP2C9.33 (R132Q)	1.03 ± 0.21	7.3 ± 0.3***	7.3 ± 1.4***
CYP2C9.34 (R335Q)	1.06 ± 0.11	550 ± 27***	526 ± 75***
BD Gentest CYP2C9.1	1.34	630	470
BD Gentest human liver microsome	2.85	48.2	16.9

*** $P < 0.001$ vs. wild type. One-way ANOVA with a post hoc Dunnett multiple comparisons test.

TABLE 4

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

Data are presented as the mean ± S.D. of three to four different expression experiments.

Recombinant Enzymes (Amino Acid Alteration)	K_m	V_{max}	Clearance (V_{max}/K_m)
	μM	*pmol/min/pmol P450*	*μl/min/pmol P450*
CYP2C9.1 (wild type)	0.18 ± 0.03	1.65 ± 0.11	9.22 ± 1.85
CYP2C9.3 (I359L)	0.13 ± 0.03	0.22 ± 0.06***	1.86 ± 0.94***
CYP2C9.13 (L90P)	1.29 ± 0.37***	0.16 ± 0.03***	0.13 ± 0.03***
CYP2C9.26 (T130R)	0.16 ± 0.05	0.14 ± 0.03***	0.94 ± 0.42***
CYP2C9.28 (Q214L)	0.25 ± 0.04	0.98 ± 0.11***	4.04 ± 0.91***
CYP2C9.30 (A477T)	0.14 ± 0.03	0.28 ± 0.04***	2.32 ± 0.10***
CYP2C9.33 (R132Q)	0.20 ± 0.04	0.013 ± 0.001***	0.07 ± 0.01***
CYP2C9.34 (R335Q)	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

** $P < 0.01$ vs. wild type. One-way ANOVA with a post hoc Dunnett multiple comparisons test.

*** $P < 0.001$ vs. wild type.

of diclofenac, losartan, and glimepiride. However, in contrast with CYP2C9.34, a substitution in the same position, CYP2C9.11 (R335W), 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 holoprotein was expressed at a very low level because of its decreased stability in insect cells (Tai et al., 2005). Therefore, the substituted residues (Trp versus Gln) at this position might influence the stability of protein as well as the catalytic activity differently.

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 π-π 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, the 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 present mainly 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; and 56 to 75% in CYP2C9.28 and CYP2C9.30. Therefore, for the patients bearing these variant alleles, these drugs would have to be administered carefully.

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