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ORIGINAL ARTICLE

Functional characterization of the defective CYP2C9 variant *CYP2C9*18*

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Abstract

Cytochrome P450 2C9 (CYP2C9) is one of the most important drugs metabolizing enzymes and accounts for the metabolism of about 13%–17% of clinical drugs. Like other members in CYP2 family, *CYP2C9* gene exhibits great genetic polymorphism among different races and individuals. *CYP2C9*18* is one *CYP2C9* allelic variant identified in a Southeast Asian population and is estimated to cause the amino acid substitutions of I359L and D397A in CYP2C9 enzyme simultaneously. Limited by the low expression level in bacteria and COS-7 cells, no valuable enzyme kinetics have been reported on this CYP2C9 variant. In this study, the baculovirus-based system was used for the high expression of recombinant CYP2C9 s in insect cells. As a result, together with I359L substitution, D397A could significantly decrease the protein expression of CYP2C9.18 in insect cells, although substitution of D397A alone had no effect on the expression of CYP2C9 in vitro. As compared with that of wild-type enzyme, both CYP2C9.18 variant and D397A variant could decrease more than 80% of the catalytic activity of CYP2C9 enzyme toward three probe substrates, suggesting that caution should be exercised when patients carrying *CYP2C9*18* taking medicines metabolized by CYP2C9 enzyme with a narrow therapeutic window.

KEYWORDS
allelic variant, baculovirus, CYP2C9, metabolic characterization, probe substrate

Abbreviations: BEVS, baculovirus-based protein expression system; CYP2C9, Cytochrome P450 2C9; LC–MS/MS, liquid chromatography tandem-mass spectrometry; PCR, Polymerase Chain Reaction.

Jian Liu and Hao Chen are contributed equally to this work.

1 | INTRODUCTION

Human cytochrome P450 (CYP) is a superfamily of enzymes that play key roles in the oxidative metabolism of a wide range of endogenous and heterogeneous substances. Cytochrome P450 2C (CYP2C) is a large and complex subfamily of the CYP2 family,[1] and contains four members of enzymes: CYP2C8, CYP2C9, CYP2C18, and CYP2C19. Among them, cytochrome P450 2C9 (CYP2C9) is the main isoenzyme of CYP2C subfamily in human liver which accounts for about 20% of hepatic cytochrome P450 protein and is responsible for the metabolism of about 13%–17% of all clinical drugs.[2,3] The *CYP2C9* gene is located in the chromosomal region 10q23.33 and is approximately 55 kb in length with nine exons encoding a protein containing 490 amino acid residues.[4] Similar to other CYP members, such as *CYP2D6*, *CYP2C19*, and *CYP3A4*, human *CYP2C9* gene shows great genetic polymorphism among different races and individuals.[5] At present, totally 62 *CYP2C9* allelic variants are deposited in the Pharmacogene Variation (PharmVar) Consortium (https://www.pharmvar.org/gene/CYP2C9). Most of these *CYP2C9* alleles are single nucleotide polymorphisms (SNPs) that can lead to the single amino acid substitution and affect the catalytic activity of expressed enzyme.[6]

*CYP2C9*2* and *CYP2C9*3* are the most prevalent and widely studied *CYP2C9* variants. They can cause R144C and I359L, respectively, and their frequencies vary greatly among different ethnic groups. *CYP2C9*2* is the most common allele in Caucasian populations with a frequency of approximately 8%–15%, whereas it can be detected in only no more than 1% of Asian and African populations.[5,7,8] In addition to these two alleles, large number of rare alleles have been reported in the past 30 years. Our recent studies detected 25 novel CYP2C9 allelic variants in Han Chinese individuals,[7,9–12] and most of these newly detected variants exhibited reduced catalytic activity compared with that of wild-type enzyme, although their gene frequencies are below 1% in Chinese Han population.[13–15]

*CYP2C9*18* was identified in 2004 with a frequency of 3.8% in Indian populations.[16] It contains two distinct single nucleotide mutations in exons of *CYP2C9*, 1075A>C (resulting in the amino acid substitution of I359L) and 1190A>C (resulting in D397A substitution), indicating that it may be derived from the well-studied allele *CYP2C9*3* that contains the genetic mutation 1075A>C in the exon 7 of *CYP2C9* gene. Considering for the fact that carriers of *CYP2C9*18* require lower warfarin dose than the mean dose of representative Indian people, *CYP2C9*18* was estimated to be a defective variant when it was reported for the first time.[16] Subsequently, DeLozier, T.C. et al. conducted the functional assessment by expressing it in bacteria and found that the variant CYP2C9.18 could not be normally expressed with the bacterial expression system.[17] In 2014, Niinuma et al. used the COS-7 cells to express the variant CYP2C9.18 and tried to evaluate its catalytic activity using S-warfarin and tolbutamide as the probe substrates. However, the protein expression level of CYP2C9.18 was still so low that it was difficult to isolate enough proteins to determine its kinetic parameters.[18]

In this study, we successfully expressed the variant CYP2C9.18 in insect cells with the aid of the baculovirus-based protein expression system (BEVS), and systematically investigated its in vitro kinetic characters with three typical CYP2C9 probe drugs tolbutamide, losartan, and diclofenac. Our data will provide a fundamental direction for the further basic research and clinical practice on the defective variant *CYP2C9*18*.

2 | MATERIALS AND METHODS

The study was conducted in accordance with the BCPT policy for experimental and clinical studies.[19]

2.1 | Chemicals and materials

Spodoptera frugiperda (*Sf*)*21* insect cells, Sf-900™ III SFM insect culture medium, Cellfectin transfection reagent, Grace's Insect Medium, fetal bovine serum, and the Bac-to-Bac Baculovirus Expression System were purchased from Invitrogen. Restriction enzymes were obtained from Takara Bio. Rabbit polyclonal anti-CYP2C9 antibody was obtained from AbD Serotec. The mouse monoclonal anti-OR antibody was from Santa Cruz Biotechnology. Diclofenac and chlorpropamide were obtained from Tokyo Chemical Industry Co., Ltd. Tolbutamide, losartan, and E-3174 were purchased from Sigma-Aldrich. 4-hydroxydiclofenac, 4-hydroxytolbutamide, and telmisartan were purchased from Toronto Research Chemicals Inc. High-performance liquid chromatography-grade solvents were purchased from Fisher Scientific Co. All the other chemicals and solvents were of the highest grade or analytical grade that were commercially available.

2.2 | Construction of the dual-expression vectors and expression of the recombinant CYP2C9 proteins in insect cells

The site-directed mutagenesis of the typical defective *CYP2C9* alleles, *CYP2C9*2* and *CYP2C9*3*, was constructed using the overlap extension PCR amplification method as described previously.[7,13] To introduce the 1190A>C mutation at the predesigned sites, D397A primers (Forward: 5'-GCTACATGcCAACAAAG-3', Reverse: 5'-CTTTGTTGgCATGTAGC-3') were paired with full-length PCR primers (Forward: 5'-GCCT*GAATTC*ATGGATTCTCTTGTGGT-3', Reverse: 5'-GAAC*GTCGAC*TCAGACAGGAATGAAGCA-3'), to amplify the upper and lower part of *CYP2C9* separately, according to the previously reported overlap extension PCR method.[7] Then, the amplicons were gel purified and used for the second round of amplification to get the full length of CYP2C9 variants. After that, the PCR fragments were double-digested with EcoR I/Sal I and ligated into the pFastBac-OR vector to produce the

dual-expression baculovirus vector pFastBac-OR-CYP2C9. Using the expression vectors for wild-type CYP2C9.1 and CYP2C9.3 as the template, expression vectors for variants D397A and CYP2C9.18 (containing substitutions I359L and D397A together) were obtained, respectively. Using the Bac-to-Bac Baculovirus Expression System described previously,[13] these vectors were transfected into DH10B *Escherichia coli* to obtain baculoviruses for the expression of CYP2C9 and OR enzymes simultaneously in insect cell microsomes. Western blot analysis was used to verify the expression of CYP2C9 and OR and to quantitatively analyze the proteins using the commercially available wild-type CYP2C9 insect microsome from BD Gentest (Woburn, MA, USA) as the standard.[13]

2.3 | CYP2C9 catalytic activity analysis

Using our previously reported methods,[9,13,15] the drug metabolic activity of the wild-type and *CYP2C9* variants was analyzed with three typical CYP2C9 probe drugs in vitro: diclofenac, tolbutamide, and losartan. In brief, the reaction mixture contained 5–20 pmol CYP2C9 insect microsomes, 20–40 pmol cytochrome b5 (CYP2C9/b5 = 1:2), and 1–100 μmol/L diclofenac or 10–1000 μmol/L tolbutamide or 0.5–50 μmol/L losartan in a final volume of 200 μL in 100 mmol/L Tris-HCl buffer (pH 7.5). After preincubation for 5 min at 37°C, the NADPH-regeneration system was added to start the reaction at 37 °C water bath and proceed for 20 min (diclofenac), 60 min (tolbutamide), or 30 min (losartan). Then the reaction was terminated by adding 40 μL 0.1 mol/L HCl followed by vortex and extraction with 800 μL acetic ether. The organic layer was evaporated to dryness and reconstituted in 100 μL mobile phase for the separation on XEVO TQD triple quadrupole mass spectrometer. The enzymatic kinetic parameters, K_m and V_{max}, and clearance rate Clint (V_{max}/K_m) were calculated by GraphPad Prism (version 6; GraphPad Software, Inc.). IBM SPSS software (version 16.0, Magneto) was used to evaluate the difference in the enzyme activity between the wild-type and CYP2C9 variant by one-way analysis of variance of Dunnett's test.

2.4 | Homology modeling and structural analysis of CYP2C9.18

The crystal structure of CYP2C9.18 was predicted with the public tools on the SWISS-MODEL website (https://swissmodel.expasy.org/),[20] and performed the homology modeling using the CYP2C9.3 protein (PDB ID 5X24) as a template. PyMOL software (Version 2.4, Schrodinger, LLC) was then used to align the CYP2C9.18 protein model with two crystal structures of human CYP2C9 that bind to losartan (PDB IDs: 5X24, 5XXI) in the Protein Data Bank (PDB), so as to find the differences among them.

3 | RESULTS

3.1 | Expression of CYP2C9.18 and other typical CYP2C9 allelic variants in insect cells

Different from the typical defective variant *CYP2C9*3* (1075A>C), *CYP2C9*18* contains two different mutations, 1075A>C and 1190A>C, in the coding region of *CYP2C9* gene and could express the variant CYP2C9.18 that includes two amino acid changes I359L and D397A. In this study, we constructed the single (1190A>C) and double (1075A>C, 1190A>C) mutated CYP2C9 expression vectors in order to investigate the functional impacts of amino acid substitution of aspartic acid with alanine at position 397. As illustrated in Figure 1, variant D397A showed similar protein expression level to that of wild-type enzyme, whereas CYP2C9.18 exhibited significantly reduced protein expression level when expressed in the insect cells. These data indicated that the substitution of Asp with Ala at position 397 had no much impact on the expression of CYP2C9 enzyme in insect cells, but could significantly influence the protein expression level if combined with the amino acid substitution of I359L.

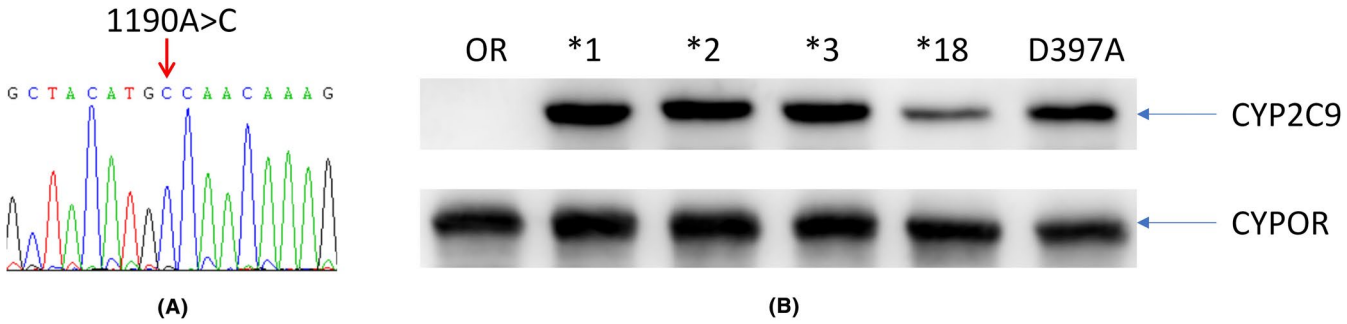

FIGURE 1 Expression of wild-type and CYP2C9 allelic variants in insect cells. A, Electropherogram of expression vector containing 1190A> C mutation. B, Protein expression level detection of recombinant enzymes in insect cells by Western blotting. Lane 1, microsomes expressing only P450 oxidoreductase (OR); lane 2, microsomes co-expressing OR and wild-type CYP2C9.1; lanes 3–6, microsomes co-expressing OR and CYP2C9.2, CYP2C9.3, CYP2C9.18, and D397A variants, respectively

3.2 | CYP2C9 enzyme kinetic analysis in vitro

In order to get the kinetic parameters of variants CYP2C9.18 and D397A, three typical CYP2C9 probe drugs, tolbutamide, losartan, and diclofenac, were used in the catalytic activity analysis in vitro. As shown in Figure 2, relative to those of wild-type protein, the D397A variant exhibited similar intrinsic clearance values to CYP2C9.3, while CYP2C9.18 showed extremely lowest intrinsic clearance values among all tested CYP2C9 variants. Table 1 illustrated the detailed kinetic parameters. It was found that almost all the CYP2C9 allelic variants showed decreased V*max* values and increased K_m values compared with those of the wild-type enzyme CYP2C9.1. As compared with that of wild-type protein, the intrinsic clearance values of variants CYP2C9.18 and D397A decreased more than 80% toward all three CYP2C9 substrates. Specially, no metabolic activity of CYP2C9.18 could be detected toward losartan because of the extremely low concentration of metabolite.

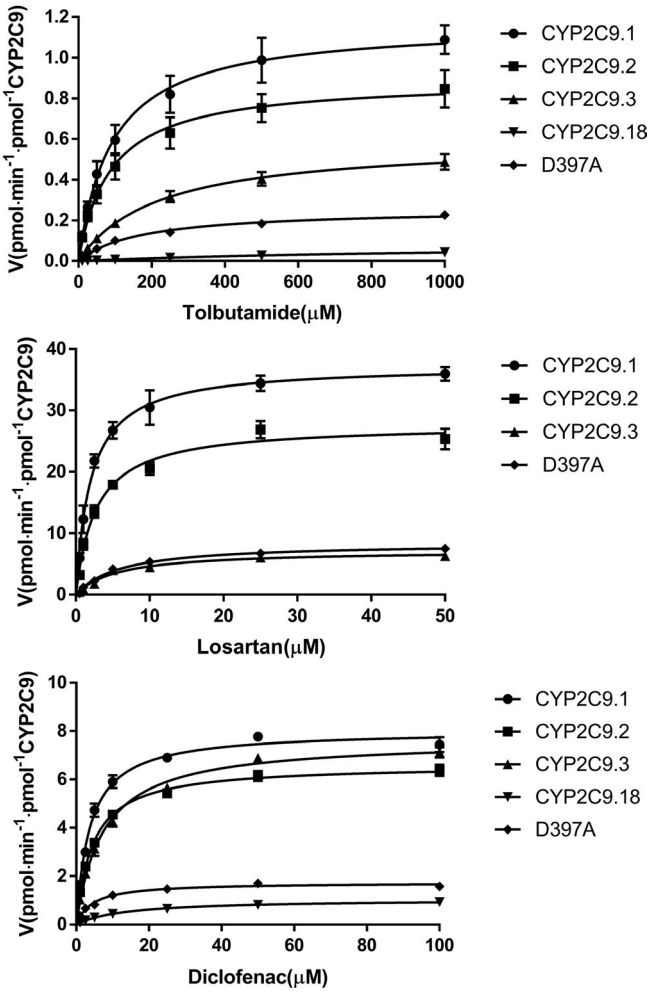

FIGURE 2 Michaelis-Menten curves of enzymatic activity of wild-type and CYP2C9 variants toward tolbutamide, losartan, and diclofenac. Each point represents the mean ± SD of four separate experiments

3.3 | Homology modeling of variant CYP2C9.18

To further explore the reasons for the decreased drug metabolism activity of CYP2C9.18, we predicted the protein structure of CYP2C9.18 and performed the homology modeling using the CYP2C9.3 protein (PDB ID 5X24) as the template. As a result, the amino acid substitution of Asp with Ala at position 397 has little impact on the residues around 397 (Figure S1). However, large differences in substrate recognition site (SRS) 6 could be found between CYP2C9.18 and CYP2C9.3, especially for the residues P464, K465, N466, D468, T469, T470, P471, V473, F476, V479, P480, and F482 (Figure 3). Considering for the function of SRS6, we predict that these changes might have a significant impact on the correct folding and substrate recognition of CYP2C9.18, which in turn causes the decrease in protein stability and metabolic activity.

4 | DISCUSSION

As one of the most important drug-metabolizing enzymes, CYP2C9 exhibits marked genetic polymorphisms that may cause a wide variation in drug-metabolizing capacity within the different races and individuals.[5,21] The *CYP2C9*18* allele was first discovered in Indians and could result in one variant containing two amino acid substitutions D397A and I359L.[16] Limited by its low protein expression capacity, no enzyme kinetic parameters have been reported to date, although two research groups tried to express it in bacteria or COS-7 cells.[17,18]

In the present study, we successfully inserted the main mutation of *CYP2C9*18*, 1190A>C, into the wild-type and *CYP2C9*3* coding regions and transfected them into Sf21 insect cells for the protein expression and drug metabolic activity analysis. Different from the previous reports,[17,18] we used the BEVS for the recombinant CYP2C9 enzyme expression. BEVS was first described in 1989 and was used to express target genes for producing proteins by transfection of insect cells.[19] Unlike bacteria, insect cells can perform proper protein folding like mammalian cells. In addition, it can also normally make protein oligomerization and posttranslational modification.[22] These advantages indicate that BEVS is better than traditional bacterial or yeast expression systems in producing mammalian-derived proteins. Compared with mammalian cells, baculovirus is only restricted in insect cells and the protein production is fast and easy to scale up. Therefore, BEVS is the most widely used protein expression platform for recombinant CYP expression.[23] Using COS-7 cells, one mammalian cell line, Niinuma Y. et al got the signal of expressed CYP2C9.18 variant by immunoblot method. However, limited by its low expression level and small scale, no activities could be detected after incubating the cells with probe drugs tolbutamide and S-warfarin.[18] In contrast, in this study, we characterized the main kinetic parameters of CYP2C9.18 toward CYP2C9 substrates, tolbutamide and diclofenac, for the first time with the aid of the high CYP expression property of baculovirus-based systems.

TABLE 1 Enzyme kinetic values of the recombinant wild-type and CYP2C9 allelic variants

Substrate	Allelic protein	V_{max} (pmol/min/nmol P450)	K_m (μM)	Clearance (V_{max}/K_m)	Relative clearance (/CYP2C9.1)
Tolbutamide	CYP2C9.1	1.17 ± 0.09	93.97 ± 11.18	0.013 ± 0.0023	100%
	CYP2C9.2	0.89 ± 0.09*	88.69 ± 8.25	0.010 ± 0.0017*	80.40%*
	CYP2C9.3	0.59 ± 0.04*	218.33 ± 14.08*	0.0027 ± 0.00032*	21.45%*
	CYP2C9.18	0.09 ± 0.01*	1,113.00 ± 134.30*	0.00008 ± 0.00001*	0.64%*
	D397A	0.26 ± 0.02*	178.83 ± 22.35	0.0015 ± 0.00027*	11.55%*
Losartan	CYP2C9.1	37.33 ± 1.18	2.03 ± 0.20	18.48 ± 1.72	100%
	CYP2C9.2	27.79 ± 1.07*	2.77 ± 0.39*	10.15 ± 1.03*	54.85%*
	CYP2C9.3	7.20 ± 0.24*	5.60 ± 0.54*	1.29 ± 0.13*	7.01%*
	CYP2C9.18	NA	NA	NA	NA
	D397A	8.42 ± 0.38*	6.00 ± 1.01*	1.42 ± 0.16*	7.70%*
Diclofenac	CYP2C9.1	8.03 ± 0.08	3.78v0.32	2.14 ± 0.19	100%
	CYP2C9.2	6.61 ± 0.21*	4.54 ± 0.43*	1.46 ± 0.086*	68.46%*
	CYP2C9.3	7.65 ± 0.16*	7.38 ± 0.91*	1.05 ± 0.11*	48.85%*
	CYP2C9.18	1.04 ± 0.02*	13.99 ± 0.77*	0.07 ± 0.003*	3.50%*
	D397A	1.74 ± 0.15*	4.52 ± 0.66	0.39 ± 0.026*	18.12%*

Data are presented as the mean ± SD of four different experiments.

NA, Not applicable because no signals for metabolite could be detected.

*$p < 0.05$ vs wild-type.

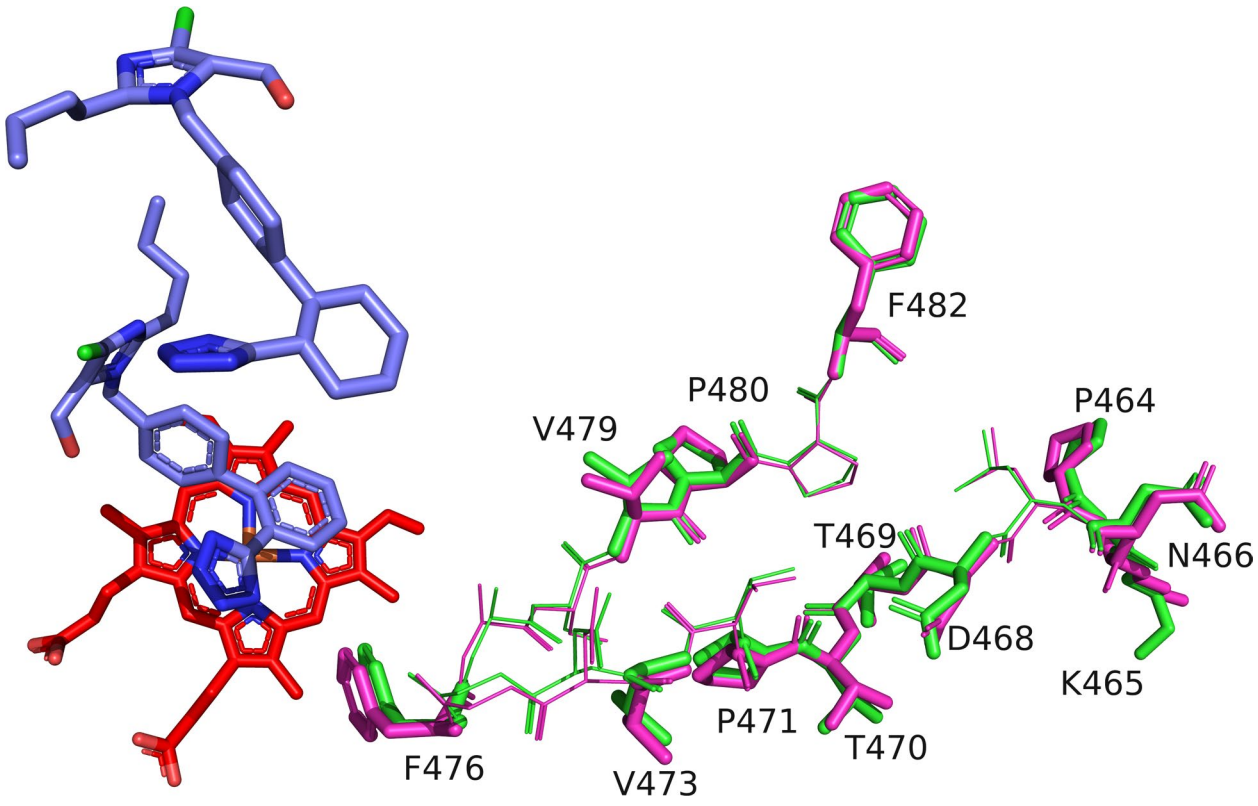

FIGURE 3 Structural overlay of substrate recognition site 6 (SRS6) of CYP2C9.18 (magentas) and typical variant CYP2C9.3 (green). The effects of D397A substitution transduced to the distal residues in SRS6, especially for P464, K465, N466, D468, T469, T470, P471, V473, F476, V479, P480, and F482 residues. The heme is shown in red and losartan is shown in slate

Previously reported studies[17,18] only investigated the synergistic effect of D397A and I359L on CYP2C9 protein. In this study, we found that D397A alone exhibited no effects on protein expression, but could significantly reduce the catalytic activity of CYP2C9. However, combined with the amino acid substitution of I359L, D397A could significantly decrease both protein expression level and catalytic activity of CYP2C9 enzyme (Figures 1 and 2, Table 1). Crystal structure analysis[24] revealed that the substitution of isoleucine with leucine at position 359 (I359L) on the K-helix of CYP2C9 could affect the I-helix residues and thus disturb the position of the *β4* loop. These structure changes lead to the change in orientation of side chains that is vital for substrate interaction, leading to the reduced drug metabolism capacity of defective variant CYP2C9.3.[24,25] To better understand the mechanism behind the poor metabolic activity against losartan, we performed the homology modeling of CYP2C9.18 with CYP2C9*3 (5X24) as the template via the SWISS-MODEL website. Previous reports revealed that the residue Ile 359 locates far from the active sites of CYP2C9, but in the substrate recognition site 5 (SRS5).[26,27] The substitution of isoleucine at position 359 with leucine (I359L) in CYP2C9.3 affects the residues from 301 to 311 on the I helix, leading to altered orientation changes of the residues Y308 and Y476 on the β4 loop. Similar to that of CYP2C9.3 variant, Phe476 in CYP2C9.18 also rotates and extends into the access channel, causing the third losartan binding site to be blocked in CYP2C9 enzyme (Figure S2).[24] In addition, the Phe476 residue position in CYP2C9.18 showed some orientation changes on its surrounding residues, especially on residues P464, K466, D468, and F476 in SRS6 (P464-F482) (Figure 3). It has been reported that SRS6 is mainly involved in the recognition and binding of substrates.[27] We speculated that the changes in the SRS6 of CYP2C9.18 might affect the substrate binding efficiency and cause the lower catalytic activity of the enzyme relative to that of the typical defective variant CYP2C9.3.

In conclusion, the variants D397A and CYP2C9.18 were highly expressed in the baculovirus-based insect cell expression system and the enzyme kinetics had been characterized using three CYP2C9 probe substrates. Our data revealed that amino acid substitution of D397A alone did not affect the expression of CYP2C9 in vitro, but it could cause a decrease in drug metabolic activity. In addition, D397A together with I359L could cause a significant decrease of expression level of protein and inhibit the drug metabolic activity of expressed CYP2C9.18 dramatically, that is much lower than that of typical defective allelic variant CYP2C9.2 or CYP2C9.3. Our data, together with previous reports,[16–18] clearly indicated that *CYP2C9*18* belongs to a defective CYP2C9 variant and caution should be exercised when patients carrying *CYP2C9*18* taking medicines metabolized by CYP2C9 enzyme with a narrow therapeutic window.

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DISCLOSURE

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of the study are available from the corresponding author upon reasonable request.

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REFERENCES

1. Takahashi H, Echizen H. Pharmacogenetics of warfarin elimination and its clinical implications. *Clin Pharmacokinet*. 2001;40(8):587-603. https://doi.org/10.2165/00003088-200140080-00003
2. Zanger UM, Turpeinen M, Klein K, Schwab M. Functional pharmacogenetics/genomics of human cytochromes P450 involved in drug biotransformation. *Anal Bioanal Chem*. 2008;392(6):1093-1108. https://doi.org/10.1007/s00216-008-2291-6
3. Zanger UM, Schwab M. Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacol Ther*. 2013;138(1):103-141. https://doi.org/10.1016/j.pharmthera.2012.12.007
4. Xie HG, Prasad HC, Kim RB, Stein CM. CYP2C9 allelic variants: ethnic distribution and functional significance. *Adv Drug Deliv Rev*. 2002;54(10):1257-1270. https://doi.org/10.1016/s0169-409x(02)00076-5
5. Waring RH. Cytochrome P450: genotype to phenotype. *Xenobiotica*. 2020;50(1):9-18. https://doi.org/10.1080/00498254.2019.1648911
6. Gaedigk A, Ingelman-Sundberg M, Miller NA, et al. The Pharmacogene variation (PharmVar) consortium: incorporation of the human cytochrome P450 (CYP) allele nomenclature database. *Clin Pharmacol Ther*. 2018;103(3):399-401. https://doi.org/10.1002/cpt.910
7. Dai DP, Xu RA, Hu LM, et al. CYP2C9 polymorphism analysis in Han Chinese populations: building the largest allele frequency database. *Pharmacogenomics J*. 2014;14(1):85-92. https://doi.org/10.1038/tpj.2013.2
8. McGraw J, Waller D. Cytochrome P450 variations in different ethnic populations. *Expert Opin Drug Metab Toxicol*. 2012;8(3):371-382. https://doi.org/10.1517/17425255.2012.657626
9. Luo SB, Li CB, Dai DP, et al. Characterization of a novel CYP2C9 mutation (1009C>A) detected in a warfarin-sensitive patient. *J Pharmacol Sci*. 2014;125(2):150-156. https://doi.org/10.1254/jphs.13189fp
10. Dai DP, Wang SH, Li CB, et al. Identification and functional assessment of a new CYP2C9 allelic variant CYP2C9*59. *Drug Metab Dispos*. 2015;43(8):1246-1249. https://doi.org/10.1124/dmd.115.063412
11. Dai DP, Li CB, Wang SH, et al. Identification and characterization of a novel CYP2C9 allelic variant in a warfarin-sensitive patient. *Pharmacogenomics*. 2015;16(13):1475-1486. https://doi.org/10.2217/pgs.15.89

12. Chen H, Dai DP, Zhou S, et al. An identification and functional evaluation of a novel CYP2C9 variant CYP2C9*62. *Chem Biol Interact*. 2020;327:109168. https://doi.org/10.1016/j.cbi.2020.109168
13. Dai DP, Wang YH, Wang SH, et al. In vitro functional characterization of 37 CYP2C9 allelic isoforms found in Chinese Han population. *Acta Pharmacol Sin*. 2013;34(11):1449-1456. https://doi.org/10.1038/aps.2013.123
14. Dai DP, Wang SH, Geng PW, Hu GX, Cai JP. In vitro assessment of 36 CYP2C9 allelic isoforms found in the Chinese population on the metabolism of glimepiride. *Basic Clin Pharmacol Toxicol*. 2014;114(4):305-310. https://doi.org/10.1111/bcpt.12159
15. Wang YH, Pan PP, Dai DP, et al. Effect of 36 CYP2C9 variants found in the Chinese population on losartan metabolism in vitro. *Xenobiotica*. 2014;44(3):270-275. https://doi.org/10.3109/00498254.2013.820007
16. Zhao F, Loke C, Rankin SC, et al. Novel CYP2C9 genetic variants in Asian subjects and their influence on maintenance warfarin dose. *Clin Pharmacol Ther*. 2004;76(3):210-219. https://doi.org/10.1016/j.clpt.2004.05.005
17. DeLozier TC, Lee SC, Coulter SJ, Goh BC, Goldstein JA. Functional characterization of novel allelic variants of CYP2C9 recently discovered in southeast Asians. *J Pharmacol Exp Ther*. 2005;315(3):1085-1090. https://doi.org/10.1124/jpet.105.091181
18. Niinuma Y, Saito T, Takahashi M, et al. Functional characterization of 32 CYP2C9 allelic variants. *Pharmacogenomics J*. 2014;14(2):107-114. https://doi.org/10.1038/tpj.2013.22
19. Tveden-Nyborg P, Bergmann TK, Lykkesfeldt J. Basic & clinical pharmacology & toxicology policy for experimental and clinical studies. *Basic Clin Pharmacol Toxicol*. 2018;123(3):233-235. https://doi.org/10.1111/bcpt.13059
20. Waterhouse A, Bertoni M, Bienert S, et al. SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic Acids Res*. 2018;46(W1):W296-W303. https://doi.org/10.1093/nar/gky427
21. Pratt VM, Cavallari LH, Del Tredici AL, et al. Recommendations for clinical CYP2C9 genotyping allele selection: a joint recommendation of the Association for Molecular Pathology and College of American Pathologists. *J Mol Diagn*. 2019;21(5):746-755. https://doi.org/10.1016/j.jmoldx.2019.04.003
22. Contreras-Gomez A, Sanchez-Miron A, Garcia-Camacho F, Molina-Grima E, Chisti Y. Protein production using the baculovirus-insect cell expression system. *Biotechnol Prog*. 2014;30(1):1-18. https://doi.org/10.1002/btpr.1842
23. Hiratsuka M. In vitro assessment of the allelic variants of cytochrome P450. *Drug Metab Pharmacokinet*. 2012;27(1):68-84. https://doi.org/10.2133/dmpk.dmpk-11-rv-090
24. Maekawa K, Adachi M, Matsuzawa Y, et al. Structural basis of single-nucleotide polymorphisms in cytochrome P450 2C9. *Biochemistry*. 2017;56(41):5476-5480. https://doi.org/10.1021/acs.biochem.7b00795
25. Williams PA, Cosme J, Ward A, Angove HC, Matak Vinkovic D, Jhoti H. Crystal structure of human cytochrome P450 2C9 with bound warfarin. *Nature*. 2003;424(6947):464-468. https://doi.org/10.1038/nature01862
26. Wang JF, Yan JY, Wei DQ, Chou KC. Binding of CYP2C9 with diverse drugs and its implications for metabolic mechanism. *Med Chem*. 2009;5(3):263-270.
27. Zawaira A, Ching LY, Coulson L, Blackburn J, Wei YC. An expanded, unified substrate recognition site map for mammalian cytochrome P450s: analysis of molecular interactions between 15 mammalian CYP450 isoforms and 868 substrates. *Curr Drug Metab*. 2011;12(7):684-700. https://doi.org/10.2174/138920011796504554

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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