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Impact of CYP2C9 Polymorphism Found in the Chinese Population on the Metabolism of Propofol *in Vitro*

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The microsomal CYP2C9 alleles involved in the biotransformation of propofol, a widely used anesthetic agent, were investigated *in vitro*. To examine the enzymatic activity of the CYP2C9 alleles, kinetic parameters for propofol 4-hydroxylation were determined in recombinant human P450s CYP2C9 microsomes from *Sf21* insect cells carrying CYP2C9*1 and other variants. Some of the variants showed decreased enzyme activity compared with the wild type, as previously reported. Two variants (CYP2C9*36 and *56) were found substantially to increase intrinsic clearance relative to the wild type variant. Most variants significantly ($p < 0.05$) decreased intrinsic clearance of propofol compared with the wild type, except *11, *47, and *54. This study is the first to report these rare alleles for propofol metabolism, providing fundamental data for further clinical studies on CYP2C9 alleles for propofol metabolism *in vivo*.

Key words propofol; microsome; metabolism; CYP2C9; allelic variant; catalytic character

Propofol is a commonly used anesthetic and sedative because of its short duration of action, rapid onset, and preferable side effects and recovery profiles.¹⁾ These properties are attributable to the rapid and extensive biotransformation of the parent compound, primarily by the liver, to multiple inactive metabolites, which are excreted in the urine.²⁾ The propofol blood concentrations are associated with recovery of consciousness and return of cognitive and psychomotor functioning.³⁾ Significant inter-individual variation in this concentration has been observed.⁴⁾ Various factors have been suggested to be responsible for the pharmacokinetic variability of propofol, for instance, age and body weight.⁴⁾ In humans, more than 50% of an administered dose of propofol is metabolized by glucuronidation and sulfation to yield propofol glucuronide and sulfate, respectively.^{5,6)} Although the glucuronide and sulfate conjugates of propofol appear to be pharmacodynamically inactive, 4-hydroxypropofol is reported to have approximately one third of the hypnotic activity of propofol.⁷⁾ Evidence from *in vitro* studies of propofol hydroxylation in human and rat liver microsomes indicates that this reaction is mediated by CYP.⁸⁾ Court *et al.* concluded that CYP2C9 contributed at least 50% of the oxidation of propofol.⁹⁾

CYP2C9 is a clinically important gene that encodes enzymes involved in the degradation of certain drugs, such as tolbutamide, phenytoin, *S*-warfarin, fluoxetine, amitriptyline, and several nonsteroidal anti-inflammatory drugs.¹⁰⁾ The genetic polymorphisms of CYP2C9 can lead to wide inter-individual variations in drug metabolism.¹¹⁾ Approximately 57 different alleles of the CYP2C9 gene have been identified.¹²⁾

Dai *et al.* have analyzed the CYP2C9 polymorphisms in 2127 people of the Han population and discovered 37 types of new mutation sites, 22 of which could lead to a change in amino acid coding. Twenty-one types of new mutation sites

were nominated as new alleles, CYP2C9*36–*56.¹³⁾ Homozygote carriers of defective alleles of CYP2C9 are poor metabolizers in contrast to the wild type allele (normal gene) carriers, which are extensive metabolizers, while heterozygote carriers of defective alleles show an intermediate phenotype.¹⁴⁾ This situation seems to be the case for propofol, and its pharmacokinetic variability can subsequently be affected.

We aimed to assess the possible impact of frequent CYP2C9 polymorphisms in the corresponding recombinase mutant insect microsomes (*2, *3, *8, *11, *14, *16, *19, *23, *27, *29, *31, *33, *34, *36, *37, *38, *39, *40, *41, *42, *43, *44, *45, *46, *47, *48, *49, *50, *51, *52, *53, *54, *55, and *56) involved in the regulation of drug-metabolism in general, with emphasis on propofol.

MATERIALS AND METHODS

Propofol, 4-hydroxypropofol and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were obtained from Sigma (St. Louis, MO, U.S.A.). P450 Reductase+Cytochrome *b5* microsomes and recombinant human P450s CYP2C9 expressed in the microsomes from *Sf21* insect cells were kind gifts from Beijing Hospital, National Health and Family Planning Commission of the Republic of China (Beijing, China). A reverse-phase SB-C18 column used for HPLC was obtained from Agilent Corp. (U.S.A.). Other reagents and organic solvents were obtained from Chemical Industries (Tianjin, China).

Incubation Conditions The incubation mixture consisted of recombinant microsomes containing 5 pmol of CYP2C9*1 or 10 pmol of other CYP2C9 mutants, 1 mM NADPH, 30 μ L 1 M Tris-HCl and 2.4 μ L propofol (from 25 to 800 μ M) in a total volume of 0.3 mL. The propofol was initially prepared in methanol solution, and the total concentration of methanol was less than 0.4%. The mixture was incubated at 37°C for 15 min.

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The incubations were performed in individual tubes for each time point. The incubations were terminated by the addition of 1 M HClO₄ (15 μ L) and cooled on ice. Thymol (20 μ L of a 10 μ g/mL in methanol solution) was added to the mixture as

an internal standard, which was then vortexed for 2 min and centrifuged at 10000 $\times g$ for 5 min. Thirty microliters of the supernatants was subjected to high performance liquid chromatography assay. The incubations were performed in qua-

Fig. 1. Michaelis-Menten Curves for Propofol 4-Hydroxylation from Recombinant Wild Type and Variant CYP2C9

The solid line indicates the data fitted to the Michaelis-Menten equation via a non-linear regression. Each point represents the mean $\pm$ S.D.

druplicate, and the data are presented as the mean±standard deviation (S.D.) from four experiments. The HPLC system (Agilent 1100) was equipped with a quaternary pump, an online vacuum degasser, auto sampler, column compartment, fluorescence detector and Agilent Chem Station Rev A.10.02. The ratios of mobile phases A (acetonitrile), B (0.1% trifluoroacetic acid (TFA)) and C (water) changed as follows: 0–12.5 min, 45–68% A, 20% B, 55–32% C. The flow rate was maintained at 1.0 mL/min. The Agilent ZORBAX SB-C18 column (4.6 mm×150 mm, i.d. 5 µm) was maintained at 30°C. The excitation and emission wavelengths of the fluorescence detector were 282 and 322 nm, respectively. Under these conditions, the retention times of 4-hydroxypropofol, thymol and propofol were 4.577, 8.301 and 11.981 min, respectively. An eight-point standard curve was used to quantify 4-hydroxypropofol and propofol.

Statistical Analysis Michaelis–Menten analysis was performed *via* nonlinear regression curve fitting using the computer program Prism v 4.0 (GraphPad Software Inc., San

Diego, CA, U.S.A.). One-way ANOVA was used for inter group comparison. Dunnett's test was used to analyze differences in the catalytic activity between CYP2C9*1 and other mutants. Statistical analyses were performed with the SPSS package (version 19.0; SPSS Inc, Chicago, IL, U.S.A.), and $p < 0.05$ was considered statistically significant.

RESULTS

The Michaelis–Menten kinetics of propofol for wild type and mutant CYP2C9 are shown in Fig. 1. The corresponding kinetic parameters are summarized in Table 1. The $V_{\max}$ values of 8 of the 35 variants (*11, *37, *43, *47, *48, *50, *54, and *55) did not significantly differ from that of the wild type, while 4 (*13, *19, *33, and *43) showed significantly different K_m values compared with the wild type ($p < 0.05$). The intrinsic clearance of propofol of most variants significantly differed ($p < 0.05$) from that of the wild type, except for *11, *47, and *54.

Table 1. Michaelis–Menten Kinetics Parameters of Propofol Hydroxylase Activity of Mutant CYP2C9 Alleles in Recombinant Microsomes Compared with That of Wild-Type CYP2C9*1

Variant	K_m (µM)	$V_{\max}$ (pmol/min/pmol CYP2C9)	$V_{\max}/K_m$ (µL/min/pmol CYP2C9)	Clearance/*1
CYP2C9*1	74.915±14.270	69.472±3.856	0.959±0.184	100.0%
CYP2C9*2	88.310±5.711	67.600±1.317	0.765±0.010*	79.7%
CYP2C9*3	110.400±2.515	37.845±0.456*	0.342±0.003*	35.7%
CYP2C9*8	143.650±37.112	28.750±1.017*	0.207±0.038*	21.6%
CYP2C9*11	60.007±11.329	53.357±8.216	0.930±0.298	96.9%
CYP2C9*13	1071.575±187.361*	47.247±5.794*	0.044±0.002*	4.6%
CYP2C9*14	91.670±3.055	20.007±0.172*	0.218±0.006*	22.7%
CYP2C9*16	320.950±44.149	37.812±1.647*	0.118±0.011*	12.3%
CYP2C9*19	3711.750±2703.035*	101.095±62.960*	0.028±0.003*	3.0%
CYP2C9*23	123.725±11.607	25.490±0.866*	0.026±0.013*	21.5%
CYP2C9*27	89.712±8.477	38.307±0.909*	0.429±0.031*	44.7%
CYP2C9*29	87.822±6.730	39.862±1.593*	0.456±0.047*	47.5%
CYP2C9*31	76.865±17.925	28.822±2.479*	0.384±0.056*	40.1%
CYP2C9*33	1025.175±136.926*	37.357±3.275*	0.036±0.001*	3.8%
CYP2C9*34	122.725±2.080	36.262±0.452*	0.295±0.004*	30.7%
CYP2C9*36	232.625±38.351	565.300±33.861*	2.430±0.271*	253.4%
CYP2C9*37	107.620±6.922	53.510±0.614	0.498±0.026*	51.9%
CYP2C9*38	58.262±5.094	42.472±1.465*	0.731±0.038*	76.2%
CYP2C9*39	789.700±94.262	19.445±1.324*	0.024±0.001*	2.5%
CYP2C9*40	56.627±4.614	30.580±1.311*	0.541±0.022*	56.4%
CYP2C9*41	84.357±3.317	47.577±1.062*	0.564±0.009*	58.8%
CYP2C9*42	231.275±21.319	31.522±2.067*	0.136±0.004*	14.2%
CYP2C9*43	4030.750±1057.384*	54.642±11.877	0.013±0.000*	1.4%
CYP2C9*44	86.007±8.036	41.342±1.224*	0.482±0.031*	50.3%
CYP2C9*45	117.675±3.628	24.222±0.202*	0.205±0.008*	21.4%
CYP2C9*46	60.817±2.160	36.127±1.016*	0.594±0.020*	61.9%
CYP2C9*47	71.472±3.364	64.315±0.947	0.900±0.033	93.8%
CYP2C9*48	95.560±4.550	52.867±0.758	0.553±0.019*	57.7%
CYP2C9*49	80.015±1.660	38.565±0.420*	0.482±0.014*	50.2%
CYP2C9*50	174.225±32.502	64.152±2.733	0.375±0.053*	39.1%
CYP2C9*51	55.880±1.714	43.052±0.657*	0.770±0.011*	80.3%
CYP2C9*52	511.300±18.257	19.600±0.314*	0.038±0.000*	3.9%
CYP2C9*53	71.310±7.773	44.712±1.336*	0.631±0.053*	65.7%
CYP2C9*54	59.857±3.575	50.842±0.841	0.851±0.037	88.6%
CYP2C9*55	150.625±9.070	67.580±0.915	0.449±0.027*	46.8%
CYP2C9*56	82.027±9.393	100.44±5.813*	1.230±0.074*	128.2%

Values are mean±S.D. * Significantly different from wild-type CYP2C9, $p < 0.05$.

CYP2C9*13, *19, *33, and *43 exhibited a lower intrinsic clearance of propofol ($p < 0.05$) than wild-type CYP2C9*1, which resulted from higher values of K_m (14.3-fold, 49.5-fold, 13.7-fold and 53.8-fold, respectively). Furthermore, the intrinsic clearance values (V_{max}/K_m) for variants CYP2C9*13, *19, *33, and *43 decreased to 4.6%, 3.0%, 3.8%, and 1.4% of the wild type value, respectively. CYP2C9*43, which showed a higher value of K_m but similar value of V_{max} , showed extremely low intrinsic clearance (1.4%). The V_{max} values of two mutants, CYP2C9*36 and *56, were higher than that of the wild type, but the value of K_m of CYP2C9*36 is much higher (3.10-fold). Thus, the intrinsic clearance of two variants (*36 and *56) were significantly higher than that of the wild type. Both K_m values lacked significant differences with the wild type and the V_{max} values were 8.14-fold for *36 and 1.44-fold for *56 of the wild type with statistical significance. The V_{max} , K_m and intrinsic clearance of CYP2C9*11, *47 and *54 did not significantly differ, while these values did significantly differ in CYP2C9*13, *19, and *33. Variants *2, *3, *8, *14, *16, *23, *27, *29, *31, *34, *38, *39, *40, *41, *42, *44, *45, *46, *51, *52, and *53 showed lower intrinsic clearances of propofol ($p < 0.05$) than the wild-type CYP2C9*1, which resulted from lower values of V_{max} (*2: 97.3%, *3: 54.5%, *8: 41.1%, *14: 28.8%, *16: 54.4%, *23: 36.7%, *27: 55.1%, *29: 57.4%, *31: 41.5%, *34: 52.2%, *38: 61.1%, *39: 27.9%, *40: 44.0%, *41: 68.5%, *42: 45.4%, *44: 59.5%, *45: 34.9%, *46: 52.0%, *51: 62%, *52: 28.2%, and *53: 64.4%) compared with the wild type. Variants *37, *48, *50, and *55 also showed lower intrinsic clearances of propofol ($p < 0.05$), but the values of K_m and V_{max} did not significantly differ.

DISCUSSION

To better understand the effect of the CYP2C9 allele on the metabolism of propofol, we screened the 35 variants with the recombinant insect microsomes. The majority of the mutants showed decreased enzyme activity, in accordance with previous studies.

CYP2C9*2 and CYP2C9*3 are the most studied mutants. In our study, CYP2C9*2 resulted in a small decrease in the V_{max} (0–2.7%) and little or no change in the K_m for the catalysis of propofol, which matches the results of a previous study.¹⁵⁾ The most widely studied allele, CYP2C9*3 (Ile359Leu), is present in approximately 10–15% of Caucasians and is less frequent in the Negro and Asian populations.^{15,16)} CYP2C9*3 demonstrated a significantly lower intrinsic clearance of propofol compared with the wild type, as previously reported.¹⁵⁾

The CYP2C9*13 (Leu90Pro) variant allele occurs in approximately 2% of the Chinese population.¹⁷⁾ An individual with CYP2C9*3/*13 genotype was found to have a much lower clearance of lornoxicam (half-life of approximately 105 h) than those with the CYP2C9*1/*3 and CYP2C9*1/*1 genotype (half-lives of 5.8–8.1 and 3.2–6.3 h, respectively).¹⁸⁾ CYP2C9*13 also exhibited reduced metabolic activity towards tolbutamide and diclofenac *in vitro*.¹⁴⁾ The Leu90Pro substitution in CYP2C9*13, which produces a protein with a higher K_m and lower V_{max} than the wild type, is located in a nonheme-binding region far from the substrate binding pocket.¹⁹⁾ Thus, the reason for the CYP2C9*13-mediated reduction in drug-metabolizing capability remains unclear.

Studies showed that the catalytic activities of CYP2C9*27

(Arg150Leu) and CYP2C9*29 (Pro279Thr) were similar against diclofenac compared with the wild type.²⁰⁾ However, Arg150 is a surface residue of the D-helix, and the different electrostatic status near the substituted residues (His *versus* Leu) might differently influence the substrate-dependent catalytic behavior. This influence was observed in our study; CYP2C9*27 caused a 55.3% decrease in the clearance of propofol. Pro279, located between helices H and I, is not conserved in the CYP2C family and is unlikely to play an important role in the catalytic activity of CYP2C9, which surprisingly exhibited a significant decrease in the clearance of propofol.

In this study, a similar result was obtained in which that G98V variant (CYP2C9*39), R124W variant (CYP2C9*43) and T299R variant (CYP2C9*52) showed significantly decreased enzyme activity toward propofol. The two previously reported variants, T130M (CYP2C9*44) and R132W (CYP2C9*45),¹³⁾ are two similar new coding variants detected in some Chinese populations and exhibited impaired CYP2C9 activity *in vitro*. Our results showed that D49G (CYP2C9*37), G96A (CYP2C9*38), K119R (CYP2C9*41), R124Q (CYP2C9*42), A149T (CYP2C9*46), I207T (CYP2C9*48), P227S (CYP2C9*50), P317S (CYP2C9*53), and L361I (CYP2C9*55) also showed a decreased intrinsic clearance for propofol *in vitro*, and these data were in accordance with the study performed by Dai *et al.*¹³⁾ However, the relative enzymatic activities among these CYP2C9 variants showed a substrate-dependent pattern. For example, F110S (*40) and S343R (*54) are both predicted to be fast metabolic variants,¹³⁾ while our results showed impaired activity for *40 and no change for *54 in the K_m , V_{max} and intrinsic clearance. P163L (CYP2C9*47) showed impaired activity in Dai *et al.*'s study, but we found that its activity was similar to that of the wild type. I222V (CYP2C9*49), I284V (CYP2C9*51) and I387V (CYP2C9*56) were previously reported to not substantially affect diclofenac, while our study revealed that *49 and *51 caused a reduction in the intrinsic clearance of propofol compared with the wild type. Two fast metabolic variant, M1V (CYP2C9*36) and I387V (CYP2C9*56), were identified in our study. These two variants significantly increased the clearance of propofol compared with the wild type. However the protein expression of CYP2C9*36 was substantially impacted with the lowest CYP2C9 protein expression level in COS-7 cells.¹³⁾ The *in vivo* activity of CYP2C9*36 was unclear.

We assumed that the substrate-dependence may explain the inconsistency because previous research has demonstrated that a reduction in the intrinsic clearance of nine different substrates could vary from 3-fold for diclofenac 4-hydroxylation to 27-fold for piroxicam 5-hydroxylation *in vitro* for the typical variant CYP2C9*3.¹³⁾

In summary, we screened the enzymatic activity of the 35 variants of CYP2C9 in the metabolism of propofol, including the 20 new coding variants. To our knowledge, this study is the first to report the propofol metabolism of these rare alleles. Although the frequencies of some alleles tested in our study were very small (0.02–0.19%), the quantity of individuals with a genetic variation of CYP2C9 in the Chinese population is very large because the Han population is currently the largest population in the world (nearly 20%).²¹⁾ Our data provide new information regarding CYP2C9 genetic polymorphisms and their related biological impacts, which could be relevant for

personalized medicine in Chinese populations. Further clinical studies will be required to determine the clinical importance of the novel CYP2C9 alleles to the metabolism of propofol *in vivo*. Understanding the pharmacogenetics that contribute to the variability in the propofol dose–response relationship may help to safely and effectively tailor drug therapy to patients.

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Conflict of Interest The authors declare no conflict of interest.

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