DMD Fast Forward. Published on January 22, 2015 as DOI: 10.1124/dmd.114.061200
This article has not been copyedited and formatted. The final version may differ from this version.

In vitro and in vivo characterization of 13 CYP2C9 allelic variants found in Chinese Han population

Guo-Xin Hu, Pei-Pei Pan, Zeng-Shou Wang, Li-Ping Yang, Da-Peng Dai, Shuang-Hu Wang, Guang-Hui Zhu, Xiang-Jun Qiu, Tao Xu, Jun Luo, Qing-Quan Lian, Ren-Shan Ge, Jian-Ping Cai

Testing and Analysis Laboratory for Phase I Clinical Trails, Wenzhou Medical University, Wenzhou, P.R.China.(G.H., P.P., S.W., T.X., J.L.)

The Second Affiliated Hospital and Yuying Children's Hospital, Wenzhou Medical University, Wenzhou, P.R.China.(Z.W., G.Z., Q.L.,R.G.)

Department of Pharmacy, Beijing Hospital, Ministry of Health, Beijing, P.R.China(L.Y.)

The Key Laboratory of Geriatrics, Beijing Hospital and Beijing Institute of Geriatrics, Ministry of Health, Beijing, P.R.China.(D.D., J.C.)

Medical College of Henan University of Science and Technology, Luoyang, 471003, P.R.China.(X.Q.)

Running title：**Study for Novel *CYP2C9* variants**

Address correspondence to: Dr. Jian-ping Cai, The Key Laboratory of Geriatrics, Beijing Hospital & Beijing Institute of Geriatrics, Ministry of Health, No. 1, Dahua Road, Dongdan, Beijing 100730, P.R.China.

Telephone: +86-10-58115039

Fax numbers: +86-10-65237929

E-mail: caijp61@vip.sina.com.

Address co-correspondence to : Dr. Renshan Ge.

E-mail: r_ge@yahoo.com

Authors Guo-Xin Hu and Pei-Pei Pan contributed equally to this work.

The number of text pages: 15

The number of tables: 4

The number of figures: 7

The number of references: 31

The number of words in the Abstract: 228

The number of words in the Introduction: 589

The number of words in the Discussion: 1347

Abbreviations

CYP, Cytochromes P450; AUC, the area under the plasma concentration–time curve; $t_{1/2}$, the half-life; PCR, the polymerase chain reaction; DMEM, Dulbecco's modified eagle medium; CL/F, oral clearance; EM, extensive metabolizer; PM, poor metabolizer.

DMD Fast Forward. Published on January 22, 2015 as DOI: 10.1124/dmd.114.061200
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

Our previous study detected totally 35 *CYP2C9* allelic variants in 2127 Chinese subjects, of whom 21 novel alleles were reported for the first time in Chinese populations. The aim of present study was to characterize the 13 *CYP2C9* allelic variants both *in vitro* and *in vivo*. Different types of *CYP2C9* variants were highly expressed in COS-7 cells and 50 μM of tolbutamide was added as the probing substrate to evaluate their metabolic abilities *in vitro.* Subsequently, the concentrations of tolbutamide and its metabolite in the plasma and urine within individuals with different types of genotypes were determined by HPLC to evaluate the catalytic activity of the thirteen mutant CYP2C9 proteins *in vivo*. Our results showed that compared with **1/*1* wild-type subjects, subjects with **1/*40* genotype showed increased oral clearance (CL/F), whereas individuals with **1/*3, *1/*13, *3/*3, *3/*13, *1/*16, *1/*19, *1/*34, *1/*42, *1/*45, *1/*46* and **1/*48* genotype exhibited significantly decreased CL/F, and those with **1/*27, *1/*29, *1/*40* and **1/*41* genotype presented similar CL/F value. When expressed in COS-7 cells, the *CYP2C9* variants showed similar pattern to the results in clinical study. The study suggests that besides two typical defective alleles **3* and **13,* seven *CYP2C9* allelic variants (**16, *19, *34, *42, *45, *46* and **48*) cause defective effects on the enzymatic activities both *in vitro* and *in vivo*. In clinic, patients with these defective alleles should be paid close attention to.

Introduction

The cytochrome P450 superfamily, officially abbreviated as CYP, is a group of enzymes mainly localized in the liver and intestinal tract, and responsible for the metabolism of many endogenous hormones and xenobiotic substances including drugs and toxins. CYP2C9, making up about 20% CYP proteins in the human liver, is a major member of the CYP subfamily and is responsible for the metabolism of approximately 15% clinically used drugs (Rendic, 2002; Van Booven et al., 2010).

Human *CYP2C9* has been shown to exhibit genetic polymorphism since the discovery of the first allelic variant in 1970s. To date, more than 58 non-synonymous variants and a series of sub-variants of *CYP2C9* have been reported (see http://www.imm.ki.se/CYPalleles; access date: Nov.2014). *CYP2C9*2* (430T>C Arg144Cys) and *CYP2C9*3* (1075A>C Ile359Leu) are the most commonly investigated defective alleles, including the characterization of enzymatic activity *in vivo* and *in vitro* and population distribution data (García-Martín et al., 2001; Takahashi et al., 2003; Scordo et al., 2004; Scott et al., 2007; Silan et al., 2012; Krajciova et al., 2014). Different population had different frequencies of *CYP2C9*2* and *CYP2C9*3*. For instance, in Chinese Han population, *CYP2C9*1*, *CYP2C9*2* and *CYP2C9*3* had a frequency of 0.963, 0.001 and 0.036 (Yang et al., 2003), while in Italian and Ethiopian population, the corresponding frequencies were 0.8, 0.11, 0.09 and 0.94, 0.04, 0.02 (Scordo et al., 2001), respectively. *CYP2C9*13* was firstly discovered in Chinese Han population by Si et al, and reported to have a frequency of 0.02% in Chinese (Si et al., 2004). Thereafter, a series of investigations into the catalytic activity of *CYP2C9*13 in vivo* and *in vitro* were subsequently performed using tolbutamide, diclofenac and lornoxicam as the probing substrates (Guo et al., 2005a; Guo et al., 2005b). Moreover, genetic and clinic investigations in Korea had

suggested that the presence of *CYP2C9*13* allele statistically resulted in the poor metabolism of losartan and irbesartan after a single oral dose (Li et al., 2009; Choi et al., 2012), and also influenced the pharmacokinetics and pharmacodynamics of meloxicam (Bae et al., 2011).

*CYP2C9*16*(T299A) and *CYP2C9*19*(Q454H) were discovered after studying 125 Asians from Singapore (Zhao et al., 2004). *In vitro* study showed that *CYP2C9*16* exhibited 80 to 90% lower catalytic activity of tolbutamide, while *CYP2C9*19* exhibited 30 to 40% lower catalytic activity of tolbutamide, when compared with wild-type *CYP2C9*1*, *CYP2C9*27* and *CYP2C9*29* were discovered in 263 Japanese individuals (DeLozier et al., 2005). Michaelis–Menten curves for diclofenac 4-hydroxylation from recombinant microsomes showed the identical Km and Vmax values of *CYP2C9*27*and *CYP2C9*29* compared to the wild-type(Maekawa et al., 2006). *CYP2C9*34* (R335Q) was discovered after studying 724 Japanese individuals, including 39 hypertensive patients under treatment with losartan when compared to the wild-type(Maekawa et al., 2006). When expressed in a baculovirus-insect cell system, *CYP2C9*34* had no substantial effect. A study on substrate-dependent functional alteration of *CYP2C9* demonstrated that *CYP2C9*34* exhibited catalytic activities almost similar to those of the wild-type for diclofenac, losartan, and glimepiride (Maekawa et al., 2009).

In our previous study, we sequenced all 9 exons of *CYP2C9* in 2127 non-related individuals, and found 21 novel *CYP2C9* allelic variants *(CYP2C9*36-*56*) besides 14 previously reported allelic variants, of whom allelic variants (*CYP2C9*3*, *CYP2C9*13*, *CYP2C9*16*, *CYP2C9*19*, *CYP2C9*27*, *CYP2C9*29* and *CYP2C9*34*) had highest gene frequencies in Chinese population (Dai et al., 2014b). In this study, we comprehensively characterized the catalytic activities of above 7 reported *CYP2C9*

alleles and 6 newly found allelic variants (*CYP2C9*40*, *CYP2C9*41, CYP2C9*42*, *CYP2C9*45*, *CYP2C9*46* and *CYP2C9*48*) both *in vitro* and *in vivo*, aiming at confirming the consistency of *in vitro and in vivo* study as well.

Materials and Methods

Pharmaceutical preparation and reagents

Tolbutamide and 4-hydroxytolbutamide were purchased from Sigma (St. Louis, MO, USA). Carboxytolbutamide was purchased from Toronto Research Chemicals (North York, Canada). Carbamazepine was purchased from the National Institute for Food and Drug Control (Beijing, China). Tolbutamide tablets were purchased from the Second Affiliated Hospital of Wenzhou Medical School. All other reagents were analytical grade.

Construction of dual-expression plasmid

Construction of dual-expression plasmid was performed as described previously (Dai et al., 2014b). In brief, the intact Gaussia princeps luciferase (Gluc) open reading frame (ORF) was amplified from the pGluc-Basic plasmid (New England Biolabs) using the Gluc-XF (introducing one XbaI site) and Gluc-NR (introducing one NotI site) primers. The amplicon was purified and doubly digested with the XbaI and NotI enzymes and ligated to the XbaI/NotI site of the pIRES vector (Clontech, Mountain View, CA, USA) to obtain recombinant receptor plasmid pIRES-Gluc. To introduce one nucleotide mutation at the expected site, overlap extension PCR amplification was performed using the wild-type *CYP2C9* plasmid (a kind gift from Professor Shu-feng Zhou at University of South Florida) as a template, except for the starting coding mutation *CYP2C9*36*. The PCR products were then gel purified, pooled and used as the template for the second round amplification using the *2C9-XF* and *2C9-ER* primer pairs to obtain the complete ORF region of mutated *CYP2C9*. For

amplification of the entire ORF regions of the wild-type *1 or novel allele *36, only one round of PCR was needed, using *2C9-XF* or SCMF as the forward primer and *2C9-ER* as the reverse primer. Detailed information on all primers used in the construction process was presented in our previous study (Dai et al., 2014b). The entire cDNA ORF regions were purified and double digested with the XhoI and EcoRI enzymes and inserted into the XhoI/EcoRI site of the receptor plasmid pIRES-Gluc to obtain the final dual expression plasmid *pIRES-Gluc-2C9*.

Plasmid transfection

Approximately 0.7×10^5 cells per well were plated on a 6-well plate 1 day before transfection, and cells were grown to 80% confluency at the time of transfection. The next day, 400 ng of individual dual-expression plasmids was transfected into the COS-7 cells using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions.

In vitro catalytic activities of recombinant CYP2C9 proteins

Forty-eight hours after transfection, cells were digested with trypsinogen and resuspended in 300 μL of DMEM culture medium supplemented with 10% fetal bovine serum, 10000 U/mL penicillin, 10000 μg/mL streptomycin and 50 μM tolbutamide (this concentration was selected based on its metabolic Km by the human liver microsomes (Tang et al., 2002)). The samples of various genotypes were cultured in suspension on a shaking table at 800 r.p.m. in a CO_2 incubator for 1 h. The reactions were stopped by addition of 100 μL of 0.1 M HCl, and 50 μL 20 μg/mL carbamazepine in the methanol as an internal standard was added into each tube. 1 mL ethyl acetate was added for extraction. After centrifugation for 30 min at 13,000 r.p.m., the supernatant of organic phase was separated, and the organic phase was transferred to another tube and evaporated to dryness at 50 °C under a gentle stream of nitrogen.

The residue was dissolved in 100 μL of mobile phase for analysis.

Protein expression quantification by western blotting

Cells in each well were lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.6), 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1% NP-40, 0.01 mg/ml aprotinin and 1 mM phenylmethanesulfonyl fluoride. Twenty micrograms of the total cellar lysates were applied to 12% SDS–PAGE and electrotransferred onto PVDF membranes. Rabbit polyclonal anti-CYP2C9 antibody (AbD Serotec, Puchheim, Germany), rabbit polyclonal anti-Gluc antibody (New England Biolabs) or rabbit monoclonal anti-GAPDH antibody (Abmart, Shanghai, China) was used as the primary antibody at 1:5000. After incubation at 4^{o}C overnight, goat anti-rabbit IgG horseradish peroxidase-conjugated antibody at 1:2000 was applied as the secondary antibody, and the materials were incubated at room temperature for 1 h. The Super Signal West Pico Trial Kit (Thermo, Rockford, IL, USA) was used to visualize the blot signals.

Subjects and sample collecting

28 healthy subjects with different genotypes, consisting of seven *CYP2C9*1* homozygotes, seven *CYP2C9*3* heterozygotes, one *CYP2C9*3* homozygotes, seven subjects with identified variants and six subjects with novel variants, aged 21~22 years, were enrolled in this study for identifying *CYP2C9* enzymatic activities *in vivo*. The details were presented in Table 1. All subjects were in good health and required to refrain from all medication and alcohol prior to the pharmacokinetic study. The study was approved by the Ethics Committee of the Second Affiliated Hospital of Wenzhou Medical School and all subjects signed informed consents before participating in the investigation.

The 28 healthy subjects received an oral administration of 500 mg tolbutamide after fasting for 12 hours. Blood samples were collected before dosage and 0.5, 1, 1.5, 2.5,

4.5, 6, 9, 12, 24, and 36 h at least, followed by immediate centrifugation. The plasma was extracted to 1.5 mL tube and stored at -80℃ till analysis. The 36-hour concentrations of tolbutamide in plasma samples were analyzed immediately. Subjects with high tolbutamide concentrations were required to continue sample collecting till the concentration level fell below the level of 6 μg/ml. 75 g glucose was administered to subjects 0.5 h post administration of the drug. Plasma at the time points of 0-4.5 h was monitored for glucose concentration as well. Urine samples of the subjects at periods of 0~6 h, 6~12 h and 12~24 h were collected in individuals for determination of 4-hydroxytolbutamide and carboxytolbutamide.

Sample analysis

The concentrations of substrate and product in plasma were determined by high performance liquid chromatography according to the method as described previously (Lin et al., 2012). The urinary concentrations were determined as above except the mobile phrase. The first 8 min, Solvent A reduced from 60% to 48%, while Solvent B increased from 20% to 32%, the next 7min, Solvent A reduced from 48% to 16%, while Solvent B increased from 32% to 64%. Solvent C maintained to be 20%. All compounds were detected at an optimum wavelength of 230 nm. Under these conditions, the retention times of 4-hydroxytolbutamide, carboxytolbutamide, and tolbutamide were 7.73, 9.114, and 14.15 min, respectively.

Data analysis

Percentage of enzymatic activity matching the wild type was calculated to compare the catalytic activity of novel variants with that of *CYP2C9*1/*1*. Dunnett's test was used to analyze differences in catalytic activity in the mutant *CYP2C9* group to a single control group (*CYP2C9*1*) for *in vitro* study. The plasma concentration–time profiles of tolbutamide and 4'-hydroxytolbutamide were evaluated by

non-compartmental analysis. The area under the plasma concentration–time curve from time zero to infinity [$AUC_{(0-\infty)}$], $AUC_{(0-t)}$, the half-life ($t_{1/2}$), C_{max}, T_{max} were calculated by DAS3.0 (Mathematical Pharmacology Professional Committee of China, Shanghai, China). The sum of amounts of metabolites excreted in urine over 24 hours post administration was calculated. The Student t test was used to analyze pharmacokinetic and pharmacodynamic differences in catalytic activity between the *CYP2C9*1/*1* and *CYP2C9*1/*3* subjects. Statistical analyses were performed with the SPSS package (version 19.0; SPSS Inc, Chicago, Ill), with $P < 0.05$ considered to be statistically significant. The correlation between in vitro enzymatic activity and in vivo activity (half-life) was analyzed using a linear regression analysis program (GraphPad Software, San Diego, CA).

Results

In vitro study of novel alleles

When expressed in COS-7 cells, besides two typical defective alleles *CYP2C9*3* and *CYP2C9*13*, three allelic variants (*CYP2C9*19, CYP2C9*34* and *CYP2C9*46*) exhibited significantly lower expression levels as compared with that of wild-type protein CYP2C9*1. Consequently, their corresponding catalytic activities significantly were impaired when tolbutamide was used as the probing substrate in our analytical system. In addition, five other CYP2C9 isoforms (*CYP2C9*16*, *CYP2C9*42*, *CYP2C9*45*, *CYP2C9*46* and *CYP2C9*48*) exhibited impaired enzymatic activities compared with wild-type protein although their protein expression levels were slightly influenced or comparable to that of wild-type enzyme. Remaining four variants had similar protein expression levels but two variants (*CYP2C9*29* and *CYP2C9*40*) showed higher catalytic activities towards tobutamide and the other two isoforms (*CYP2C9*27* and *CYP2C9*41*) had similar characters to

DMD Fast Forward. Published on January 22, 2015 as DOI: 10.1124/dmd.114.061200
This article has not been copyedited and formatted. The final version may differ from this version.

wild-type (Fig.1). These data infer that most of the mutations in *CYP2C9* genes could significantly affect the enzyme's catalytic activities towards tolbutamide *in vitro* (Fig 1 and Table2).

The in vivo effects of novel alleles on concentrations of tolbutamide and its hydroxyl metabolites in plasma

The pharmacokinetic parameters of $AUC_{(0-\infty)}$, $AUC_{(0-t)}$, C_{max}, $t_{1/2}$ and CL/F were calculated to functionally characterize the catalytic activities of *CYP2C9* alleles, and the data were shown in Table 3 and 4, the concentration-time curve of tolbutamide and 4'-hydroxytolbutamide were shown in Figures 2-4.

In the present study, subjects with the genotype of *CYP2C9*1/*3* and *CYP 2C9*1/*13* had 1.78-fold and 2.81-fold increases in $AUC_{0\rightarrow t}$ as well as 1.69-fold and 2.08-fold increases in $t_{1/2}$, respectively, while their CL/F values were decreased by 43% and 65%, respectively, when compared with subjects with genotype of *CYP2C9*1/*1*. $AUC_{0\rightarrow t}$ and $t_{1/2}$ of the individual with the genotype of *CYP2C9*3/*3* were increased by 5.84 fold and 5.48 fold, respectively, while its CL/F was decreased by 86%. $AUC_{0\rightarrow t}$ and $t_{1/2}$ of the individual with the genotype of *CYP2C9*3/*13* were increased by 8.06 fold and 6.07 fold respectively, while its CL/F was decreased by 89%.

*CYP2C9*16, CYP2C9*19, CYP2C9*27, CYP2C9*29 and CYP2C9*34* were five rare variants that had already been identified in Chinese Han population. Compared with the wild type, subjects with genotype of *CYP2C9*1/*16*, *CYP2C9*1/*19* and *CYP2C9*34* showed a 1.36-fold, 1.50-fold and 1.90-fold increases in $AUC_{0\rightarrow t}$ and 1.51-fold, 1.31-fold, and 1.76-fold increases in $t_{1/2}$, while the CL/F values were decreased by 28%, 35% and 49%, respectively. The individual with the genotype of *CYP2C9*1/*27* had 12% and 10% decrease in $AUC_{0\rightarrow t}$ and $t_{1/2}$, respectively, while the

CL/F was increased by 11%. The individual with the genotype of *CYP2C9*1/*29* had a very slight (1.05- and 1.02-fold) increase in $AUC_{0\rightarrow t}$ and $t_{1/2}$, and the CL/F was also decreased slightly (7%). The data showed a serious impact on *CYP2C9*34* enzymatic activity, while in the study of Tong YIN (Yin et al., 2008), a slight diminution in intrinsic clearance (6%) was observed for the *CYP2C9*34* (Arg335Gln) protein with slightly decreased Km and Vmax values in in-vitro investigation.

*CYP2C9*40*, *CYP2C9**41, *CYP2C9**42, *CYP2C9**45, *CYP2C9**46 and *CYP2C9**48 were the six novel variants found in our previous study (Dai et al., 2014b). Compared with the wild type, the individual with the genotype of *CYP2C9*1/*40* had a 22% and 19% decrease in $AUC_{0\rightarrow t}$ and $t_{1/2}$, while the CL/F was increased by 25%; the individual with the genotype of *CYP2C9*1/*41* had 7% increase in $AUC_{0\rightarrow t}$, while the $t_{1/2}$ and CL/F was decreased by 6% and 9%, showing a slight decrease in enzymatic activity (Table 3). The individuals with the genotype of *CYP2C9*1/*42*, **1/*45*, **1/*46* and **1/*48* had a 2.16-fold, 1.70-fold, 1.87-fold and 3.29-fold increases in $AUC_{0\rightarrow t}$ and 1.95-fold, 1.65-fold, 1.47-fold and 2.89-fold increases in $t_{1/2}$ while the CL/F was decreased by 55%, 43%, 48% and 70%, respectively. The results of 4'-hydroxytolbutamide (as listed in Table.4) showed greater variability than tolbutamide in plasma.

In vitro-in vivo correlation analysis

Combined the *in vitro* and *in vivo* data together, good correlation (Figure 7) was found between *in vitro* enzymatic activity and half-life of tolbutamide *in vivo* when excluding the data of CYP2C9*48 (r_2=0.8031). These data infer that most of newly found mutated sites in *CYP2C9* can significantly decrease the metabolic activity of CYPs and result in the longer half-life time of tolbutamide for individuals carrying these novel alleles.

DMD Fast Forward. Published on January 22, 2015 as DOI: 10.1124/dmd.114.061200
This article has not been copyedited and formatted. The final version may differ from this version.

Concentration of tolbutamide and its metabolites in urine

The 0-24h urinary excretions of both metabolites were calculated and were shown in Fig 5. Significant decrease in urinary excretion of tolbutamide metabolites in the 0 to 24-hour interval were observed in individuals with the genotype of *CYP2C9 *1/*3*, compared with subjects expressing the *CYP2C9*1/*1*.

Pharmacodynamic study

Before tolbutamide administration, there were no significant differences in serum glucose concentrations between the *CYP2C9*1/*1* and *CYP2C9*1/*3* subjects. The time course of serum glucose levels and delta glucose levels after oral administration of 75g dextrose was presented in Fig 6. After tolbutamide administration, statistical significance of delta glucose concentration was only observed at time points of 0.5h and 4.5h.

Discussion

The present investigation provides comprehensive data on functional characters of 13 CYP2C9 allelic variants both *in vitro* and *in vivo*. The previous effort on the large-scale direct sequencing of *CYP2C9* gene allowed us to discover 36 genetic variations in 2127 Chinese Han individuals. Volunteers in the present study included 16 different types of genotypes: *CYP2C9*1/*1*, *CYP2C9*1/*3*, *CYP2C9*3/*3*, *CYP2C9*1/*13*, *CYP2C9*3/*13*, *CYP2C9*1/*16*, *CYP2C9*1/*19*, *CYP2C9*1/*27*, *CYP2C9*1/*29*, *CYP2C9*1/*34*, *CYP2C9*1/*40*, *CYP2C9*1/*41*, *CYP2C9*1/*42*, *CYP2C9*1/*45*, *CYP2C9*1/*46* and *CYP2C9*1/*48*.

In this study, tolbutamide, a first-generation sulphonylurea hypoglycemic drug, was selected as the probe substrate for determining CYP2C9 activity *in vitro* and *in vivo*. Tolbutamide is commonly chosen as the typical probing substrate for CYP2C9 enzyme both *in vitro* and *in vivo*, since it is primarily (78-93%) metabolized by

hepatic CYP2C9 to 4'-OH-tolbutamide, which is further oxidized to carboxytolbutamide by alcohol dehydrogenase and excreted by kidney (Wester et al., 2000). Though CYP2C19 also participated in tolbutamide metabolism, it has been stated that no pharmacokinetic and pharmacodynamic differences were found between CYP2C19EM and PM genotype subjects and tolbutamide was a selective probe for measuring CYP2C9 activity in humans (Shon et al., 2002b).

*CYP2C9*3* and *CYP2C9*13* are two defective alleles studied most in the Chinese population. Our data showed that both *CYP2C9*3* and *CYP2C9*13* alleles dramatically impaired catalytic activities of their CYP2C9 proteins. *CYP2C9*3* carriers exhibited statistical significance on AUC, $t_{1/2}$ and CL/F of tolbutamide and C_{max} of 4'-hydroxytolbutamide. Due to the fact that *CYP2C9* alleles on two parallel chromosomes are both responsible for the expression of enzymes *in vivo*, thus affection of the enzymatic deficiency on function in homozygote is far beyond that in heterozygous individual. In our study, concentrations of 4'-hydroxytolbutamide of the *CYP2C9*3/*3* and *CYP2C9*3/*13* subjects were lower than those of the *CYP2C9*1/*1*, *CYP2C9*1/*3* and *CYP2C9*1/*13* subjects. Dayong Si et al reported that one individual with the genotype of *CYP2C9*3/*13* had a 108 h half-life of tolbutamide, which was much longer than that (half-life of 56.9 h) discovered in our study (Si et al., 2004). We believe that the longer monitoring time of drug concentration in our present study makes our result more precise than the previous report. Our *in vitro* and *in vivo* data indicate that individual carrying *CYP2C9*13* allele exhibits a more defective catalytic activity than that of *CYP2C9*3* allele carriers, which is well in accord with the previous report (Choi et al., 2012).

Previously reported *CYP2C9*16, *19, *27, *29 and *34* alleles showed different impact on enzymatic activities. *CYP2C9*16 and *19* had apparently defective effects

in tolbutamide metabolism both *in vitro* and *in vivo*, while *CYP2C9*27* and **29* exhibited a slight increase in catalytic activities toward tolbutamide when expressed in COS-7 cells and heterozygous carriers of *CYP2C9*27* and **29* showed similar kinetic parameters as wild type. We have previously reported that five alleles had relatively higher frequencies in Chinese population, which includes *CYP2C9*2* (0.0014), *CYP2C9*3* (0.0294), *CYP2C9*13* (0.0016), *CYP2C9*16*(0.0019), *CYP2C9*27*(0.0012) *and CYP2C9*29*(0.0019) (Dai et al., 2014b). Combined the functional analysis data, our present data suggested that *CYP2C9*16* was another important defective *CYP2C9* allele for Chinese Han individuals except for the commonly studied alleles **2*, **3* and **13*. Keiko Maekawa et al reported that recombinant microsome of *CYP2C9*34* had no substantial effect on the metabolism of diclofenac, losartan, and glimepiride (Maekawa et al., 2009). However, in the present study, *CYP2C9*34* exhibited decreased catalytic activity for tolbutamide in COS-7 cells, which is in consistent with activity *in vivo*. Western blotting result indicated that expression of *CYP2C9*34* in COS-7 cells lowered and this may partially explain its defective effect on metabolic activity of enzyme. When expressed in insect microsomes, variant *CYP2C9*34* also exhibited significantly decreased enzymatic activities towards tolbutamide(Dai et al., 2013), glimepiride(Dai et al., 2014a) and losartan (Wang et al., 2014). Our *in vitro* and *in vivo* data inferred that *CYP2C9*34* can be regarded as one defective allele.

For the newly found allelic variants, *CYP2C9*42,*45,*46, and *48* showed defects in enzymatic activities. As shown in Fig 1B and Table 2, *CYP2C9*42* and **45* were the most defective in metabolizing tolbutamide in COS-7 cells, with 99.18 and 99.68% decreases in clearance, respectively. Similarly, heterozygotes of *CYP2C9*42* and *45 had apparent deficiency in metabolizing tolbutamide *in vivo* with a much

longer half-life and decreased clearance values compared with wild-type. *In vitro* data showed that *CYP2C9*46* and **48* had modest deficiency in metabolizing tolbutamide in COS-7 cells. Heterozygote of *CYP2C9*46* also had modest decrease in metabolizing tolbutamide whereas heterozygote of *CYP2C9*48*, as shown in Table 3, exhibited the most defective effect in all tested heterozygotes. The *in vivo* outcome of CYP2C9*48 was confusing and the reason of the discrepancy remained unknown. The analysis showed that *in vivo* and *in vitro* catalysis was well correlated, except CYP2C9*48.

In this study, *CYP2C9*40* and **41* showed apparently increased enzymatic activities in COS-7 cells toward tolbutamide, which is in accordance with those observed for diclofenac (Dai et al., 2014b). However, when expressed in insect microsomes, *CYP2C9*40* exhibited decreased activity toward tolbutamide and losartan while showing similar activity to the second generation sulphonylurea-type antidiabetic drug glimepiride. *CYP2C9*41* exhibited lower metabolic activities toward all these three substrates (Dai et al., 2013; Dai et al., 2014a; Wang et al., 2014). Thus, heterozygotes of *CYP2C9*40* and *CYP2C9*41* had equivalent activities toward tolbutamide *in vivo*. We believe that different protein expression system expressed CYPs with different protein conformation characteristics, thus exhibited different catalytic activities towards same substrate. Comparing with insect microsome expression system, mammalian cells have adequate endogenous NADPH-CYP oxidoreductase (OR) and cytochrome b5 levels to support CYP activities and are much closer to the native state of the CYP protein (Hiratsuka, 2012; Dai et al., 2014b). Correlation analysis also confirmed this, in which data obtained in COS-7 cells is much closer to that observed *in vivo*.

Drawn from above, CYP2C9 enzymes expressed in COS-7 cells, to a great extent,

DMD Fast Forward. Published on January 22, 2015 as DOI: 10.1124/dmd.114.061200
This article has not been copyedited and formatted. The final version may differ from this version.

represented its activity *in vivo*. But the expression of double chromosomes would undermine its effect and the complexity of *in vivo* ADME brought variables into *in vitro/in viv*o prediction.

The urinary measurement of tolbutamide metabolism was controversial (Veronese et al., 1993; Lee et al., 2002). It has been demonstrated that the variability observed in urinary excretion of tolbutamide was much greater than that of two metabolites since urinary concentrations of two metabolites were much higher than those of unchanged tolbutamide (Lee et al., 2002), which made the amount of urinary 4'-OH-tolbutamide and carboxytolbutamide less variable than tolbutamide metabolic ratio (the amount of the metabolites in urine/the amount of tolbutamide in urine). In our investigation, urinary excretion of two metabolites of tolbutamide in 0 to 24-hour interval was chosen and estimated. As shown in Fig 5, significant decrease was observed between the *CYP2C9*1/*1* and *CYP2C9*1/*3* subjects. For other subjects, no statistical results were obtained since only one or two subjects were involved for one genotype. These data demonstrated that poor metabolizers (PMs) tended to excrete less tolbutamide metabolites than extensive metabolizers (EMs).

The gene-response effect of tolbutamide was controversial as well (Kirchheiner et al., 2002; Shon et al., 2002a; Chen et al., 2005), where positive or negative results were reported. In our investigation, statistically significant differences of delta blood glucose were only observed at the time points of 0.5 h and 4.5 h, indicating that the gene-response effect of tolbutamide, to some degree, may be more related to fasting blood glucose.

In summary, we identified 13 variations of *CYP2C9* in its catalytic activities both *in vitro* and *in vivo* using tolbutamide as probe drug, including 7 previously reported variations and 6 newly found variations in Chinese population. Our data indicate that

most of these mutations in *CYP2C9* gene could significantly impair the catalytic activity of enzyme both *in vitro* and *in vivo*. Enzymes expressed in COS-7 cells, to an extent, well presented the enzymatic activity *in vivo*. Our data provide fundamental information on these allelic variants and could be relevant for the future personalized medicine in China. However, further clinical studies must be carried out in the future to precisely evaluate the effects of variations on *CYP2C9* enzymatic activities *in vivo* because only one heterozygous carrier for each novel allele was included in the current pharmacokinetics study.

Acknowledgements

The authors thank the members of the Department of Pharmacology in Wenzhou Medical University and the Institute of Geriatrics of the Ministry of Health for their technical advice and helpful discussion.

Authorship Contributions

Participated in research design: Guo-Xin Hu, Jian-Ping Cai, Li-Ping Yang

Conducted experiments: Guo-Xin Hu, Pei-Pei Pan, Zeng-Shou Wang, Li-Ping Yang, Da-Peng Dai, Shuang-Hu Wang, Guang-Hui Zhu, Xiang-Jun Qiu, Tao Xu, Jun Luo, Qing-Quan Lian

Contributed new reagents or analytic tools: Pei-Pei Pan, Zeng-Shou Wang, Da-Peng Dai, Shuang-Hu Wang, Guang-Hui Zhu, Tao Xu, Jun Luo, Qing-Quan Lian

Performed data analysis: Pei-Pei Pan, Ren-Shan Ge, Jian-Ping Cai

Wrote or contributed to the writing of the manuscript: Guo-Xin Hu, Pei-Pei Pan, Ren-Shan Ge, Jian-Ping Cai

References

Bae JW, Choi CI, Jang CG, and Lee SY (2011) Effects of CYP2C9*1/*13 on the pharmacokinetics and pharmacodynamics of meloxicam. ***British journal of clinical pharmacology*** **71:550-555.**

Chen K, Wang R, Wen SY, Li J, and Wang SQ (2005) Relationship of P450 2C9 genetic polymorphisms in Chinese and the pharmacokinetics of tolbutamide. ***Journal of clinical pharmacy and therapeutics*** **30:241-249.**

Choi C-I, Kim M-J, Chung E-K, Lee H-I, Jang C-G, Bae J-W, and Lee S-Y (2012) CYP2C9* 3 and* 13 alleles significantly affect the pharmacokinetics of irbesartan in healthy Korean subjects. ***European journal of clinical pharmacology*** **68:149-154.**

Dai DP, Wang SH, Geng PW, Hu GX, and Cai JP (2014a) In vitro assessment of 36 CYP2C9 allelic isoforms found in the Chinese population on the metabolism of glimepiride. ***Basic & clinical pharmacology & toxicology*** **114:305-310.**

Dai DP, Wang YH, Wang SH, Geng PW, Hu LM, Hu GX, and Cai JP (2013) In vitro functional characterization of 37 CYP2C9 allelic isoforms found in Chinese Han population. ***Acta pharmacologica Sinica*** **34:1449-1456.**

Dai DP, Xu RA, Hu LM, Wang SH, Geng PW, Yang JF, Yang LP, Qian JC, Wang ZS, Zhu GH, Zhang XH, Ge RS, Hu GX, and Cai JP (2014b) CYP2C9 polymorphism analysis in Han Chinese populations: building the largest allele frequency database. ***Pharmacogenomics J*** **14:85-92.**

DeLozier TC, Lee SC, Coulter SJ, Goh BC, and Goldstein JA (2005) Functional characterization of novel allelic variants of CYP2C9 recently discovered in southeast Asians. ***The Journal of pharmacology and experimental therapeutics*** **315:1085-1090.**

García-Martín E, Martínez C, Ladero JM, Gamito FJ, and Agúndez JA (2001) High frequency of mutations related to impaired CYP2C9 metabolism in a Caucasian population. ***European journal of clinical pharmacology*** **57:47-49.**

Guo Y, Wang Y, Si D, Fawcett PJ, Zhong D, and Zhou H (2005a) Catalytic activities of human cytochrome P450 2C9*1, 2C9*3 and 2C9*13. ***Xenobiotica; the fate of foreign compounds in biological systems*** **35:853-861.**

Guo Y, Zhang Y, Wang Y, Chen X, Si D, Zhong D, Fawcett JP, and Zhou H (2005b) Role of

CYP2C9 and its variants (CYP2C9* 3 and CYP2C9* 13) in the metabolism of lornoxicam in humans. ***Drug metabolism and disposition*** **33:749-753.**

Hiratsuka M (2012) In vitro assessment of the allelic variants of cytochrome P450. ***Drug metabolism and pharmacokinetics*** **27:68-84.**

Kirchheiner J, Bauer S, Meineke I, Rohde W, Prang V, Meisel C, Roots I, and Brockmöller J (2002) Impact of CYP2C9 and CYP2C19 polymorphisms on tolbutamide kinetics and the insulin and glucose response in healthy volunteers. ***Pharmacogenetics and genomics*** **12:101-109.**

Krajciova L, Petrovic R, Deziova L, Chandoga J, and Turcani P (2014) Frequency of selected single nucleotide polymorphisms influencing the warfarin pharmacogenetics in Slovak population. ***Eur J Haematol*** **93:320-328.**

Lee CR, Pieper JA, Hinderliter AL, Blaisdell JA, and Goldstein JA (2002) Evaluation of cytochrome P4502C9 metabolic activity with tolbutamide in CYP2C91 heterozygotes. ***Clinical pharmacology and therapeutics*** **72:562-571.**

Li Z, Wang G, Wang L-S, Zhang W, Tan Z-R, Fan L, Chen B-L, Li Q, Liu J, and Tu J-H (2009) Effects of the CYP2C9* 13 allele on the pharmacokinetics of losartan in healthy male subjects. ***Xenobiotica; the fate of foreign compounds in biological systems*** **39:788-793.**

Lin D, Wang Z, PAN P, XIA M, ZHANG X, WANG M, KAN X, and HU G (2012) Determination of Tolbutamide and its Metabolite in Human Plasma by High Performance Liquid Chromatography and its Application to Pharmacokinetics. ***Lat Am J Pharm*** **31:1464-1468.**

Maekawa K, Fukushima-Uesaka H, Tohkin M, Hasegawa R, Kajio H, Kuzuya N, Yasuda K, Kawamoto M, Kamatani N, and Suzuki K (2006) Four novel defective alleles and comprehensive haplotype analysis of CYP2C9 in Japanese. ***Pharmacogenet Genomics*** **16:497-514.**

Maekawa K, Harakawa N, Sugiyama E, Tohkin M, Kim SR, Kaniwa N, Katori N, Hasegawa R, Yasuda K, Kamide K, Miyata T, Saito Y, and Sawada J (2009) Substrate-dependent functional alterations of seven CYP2C9 variants found in Japanese subjects. ***Drug metabolism and disposition: the biological fate of chemicals*** **37:1895-1903.**

Rendic S (2002) Summary of information on human CYP enzymes: human P450 metabolism

data. *Drug metabolism reviews* 34:83-448.

Scordo MG, Aklillu E, Yasar U, Dahl ML, Spina E, and Ingelman-Sundberg M (2001) Genetic polymorphism of cytochrome P450 2C9 in a Caucasian and a black African population. *British journal of clinical pharmacology* 52:447-450.

Scordo MG, Caputi AP, D'Arrigo C, Fava G, and Spina E (2004) Allele and genotype frequencies of CYP2C9, CYP2C19 and CYP2D6 in an Italian population. *Pharmacological research : the official journal of the Italian Pharmacological Society* 50:195-200.

Scott SA, Edelmann L, Kornreich R, Erazo M, and Desnick RJ (2007) CYP2C9, CYP2C19 and CYP2D6 allele frequencies in the Ashkenazi Jewish population. *Pharmacogenomics* 8:721-730.

Shon J-H, Yoon Y-R, Kim K-A, Lim Y-C, Lee K-J, Park J-Y, Cha I-J, Flockhart DA, and Shin J-G (2002a) Effects of CYP2C19 and CYP2C9 genetic polymorphisms on the disposition of and blood glucose lowering response to tolbutamide in humans. *Pharmacogenetics and genomics* 12:111-119.

Shon JH, Yoon YR, Kim KA, Lim YC, Lee KJ, Park JY, Cha IJ, Flockhart DA, and Shin JG (2002b) Effects of CYP2C19 and CYP2C9 genetic polymorphisms on the disposition of and blood glucose lowering response to tolbutamide in humans. *Pharmacogenetics* 12:111-119.

Si D, Guo Y, Zhang Y, Yang L, Zhou H, and Zhong D (2004) Identification of a novel variant CYP2C9 allele in Chinese. *Pharmacogenetics* 14:465-469.

Silan C, Dogan OT, Silan F, Kukulguven FM, Asgun HF, Ozdemir S, Uludag A, Atik S, Gungor B, Akdur S, Aksulu HE, and Ozdemir O (2012) The prevalence of VKORC1 1639 G>A and CYP2C9*2*3 genotypes in patients that requiring anticoagulant therapy in Turkish population. *Mol Biol Rep* 39:11017-11022.

Takahashi H, Wilkinson GR, Caraco Y, Muszkat M, Kim RB, Kashima T, Kimura S, and Echizen H (2003) Population differences in S-warfarin metabolism between CYP2C9 genotype-matched Caucasian and Japanese patients&ast. *Clinical Pharmacology & Therapeutics* 73:253-263.

Tang C, Lin Y, Rodrigues AD, and Lin JH (2002) Effect of albumin on phenytoin and tolbutamide metabolism in human liver microsomes: an impact more than protein

binding. ***Drug metabolism and disposition: the biological fate of chemicals*** **30:648-654.**

Van Booven D, Marsh S, McLeod H, Carrillo MW, Sangkuhl K, Klein TE, and Altman RB (2010) Cytochrome P450 2C9-CYP2C9. ***Pharmacogenetics and genomics*** **20:277-281.**

Veronese ME, Miners JO, Rees DL, and Birkett DJ (1993) Tolbutamide hydroxylation in humans: lack of bimodality in 106 healthy subjects. ***Pharmacogenetics and genomics*** **3:86-93.**

Wang YH, Pan PP, Dai DP, Wang SH, Geng PW, Cai JP, and Hu GX (2014) Effect of 36 CYP2C9 variants found in the Chinese population on losartan metabolism in vitro. ***Xenobiotica; the fate of foreign compounds in biological systems*** **44:270-275.**

Wester MR, Lasker JM, Johnson EF, and Raucy JL (2000) CYP2C19 participates in tolbutamide hydroxylation by human liver microsomes. ***Drug metabolism and disposition*** **28:354-359.**

Yang JQ, Morin S, Verstuyft C, Fan LA, Zhang Y, Xu CD, Barbu V, Jaillon P, and Becquemont L (2003) Frequency of cytochrome P450 2C9 allelic variants in the Chinese and French populations. ***Fundamental & clinical pharmacology*** **17:373-376.**

Yin T, Maekawa K, Kamide K, Saito Y, Hanada H, Miyashita K, Kokubo Y, Akaiwa Y, Otsubo R, and Nagatsuka K (2008) Genetic variations of CYP2C9 in 724 Japanese individuals and their impact on the antihypertensive effects of losartan. ***Hypertension research*** **31:1549-1557.**

Zhao F, Loke C, Rankin SC, Guo JY, Lee HS, Wu TS, Tan T, Liu TC, Lu WL, Lim YT, Zhang Q, Goh BC, and Lee SC (2004) Novel CYP2C9 genetic variants in Asian subjects and their influence on maintenance warfarin dose. ***Clinical pharmacology and therapeutics*** **76:210-219.**

Footnotes

This work was supported by the Ministry of Health of the People's Republic of China [Grant 201302008]; National Key Project for New Drug Investigations [Grant 2012ZX09303008] founded by the Ministry of Science and Technology of the People's Republic of China; and the National Natural Science Foundation of China [Grant 31371280].

DMD Fast Forward. Published on January 22, 2015 as DOI: 10.1124/dmd.114.061200
This article has not been copyedited and formatted. The final version may differ from this version.

Legends for Figures

Fig.1 Expression efficacies of *CYPs* and their activities to hydroxylize tolbutamide. Panel A, Expression efficacies evaluated by western blotting. The first line (COS-7) was transfected by the mock agent, and the expression efficacy was adjusted by the internal transfection control protein (Gaussia princeps luciferase, Glu). GAPDH is a house-keeping protein; Panel B, the relative tolbutamide hydroxylase activities of mutant *CYP2C9* alleles in COS-7 cells were compared with that of wild-type *CYP2C9*1* (as 100%). *, **, significantly different from wild-type *CYP2C9*, $P < 0.05$ and 0.01, respectively.

Fig.2 A. Plasma concentration-time curves of tolbutamide in healthy subjects with genotype of *CYP2C9*1/*1* (■) (n=7), *CYP2C9*1/*3* (●) (n=7), *CYP2C9*1/*13* (▲) (n=2), *CYP2C9*3/*3* (*), and *CYP2C9*3/*13* (□) after a single oral dose of 500 mg of tolbutamide. B. Plasma concentration-time curves of 4-hydroxytolbutamide in healthy subjects with the corresponding genotypes.

Fig.3 A. Plasma concentration-time curves of tolbutamide in healthy subjects with genotypes of *CYP2C9*1/*1* (■) (n=7), *CYP2C9*1/*16* (●) (n=2), *CYP2C9*1/*19* (▲), *CYP2C9*1/*27* (□), *CYP2C9*1/*29* (○), and *CYP2C9*1/*34* (*) after a single oral dose of 500mg of tolbutamide. B. Plasma concentration-time curves of 4-hydroxytolbutamide in healthy subjects with the corresponding genotypes.

Fig.4 A. Plasma concentration-time curves of tolbutamide in healthy subjects with genotype *CYP2C9*1/*1* (■) (n=7), *CYP2C9*1/*40* (●) and *CYP2C9*1/*41* (▲) after a single oral dose of 500 mg of tolbutamide. B. Plasma concentration-time curves of

4-hydroxytolbutamide in healthy subjects with the corresponding genotypes C. Plasma concentration-time curves of tolbutamide in healthy subjects with genotype *CYP2C9*1/*1* (■) (n=7), *CYP2C9*1/*42* (●), *CYP2C9*1/*45* (▲), *CYP2C9*1/*46* (*) and *CYP2C9*1/*48* (□) after a single oral dose of 500 mg of tolbutamide. D. Plasma concentration-time curves of 4-hydroxytolbutamide in healthy subjects with the corresponding genotypes.

Fig.5 The amount of urinary 4'-OH-tolbutamide and carboxytolbutamide of *2C9* variants excreted over 24 hours compared with that of wild type *2C9*1/*1*.

Fig.6 (A) Average serum glucose concentration-time profiles after single oral administration of 75g dextrose in seven normal healthy subjects with CYP2C9*1/*1(■) (n=7) genotypes and seven subjects with CYP2C9*1/*3(●) (n=7) genotypes. (B) Average delta serum glucose level from the baseline measured before oral intake of 75g dextrose in subjects with CYP2C9*1/*1(■) (n=7) and CYP2C9*1/*3(●) (n=7) genotypes.

Fig.7 Correlations between *in vitro* enzymatic activity and *in vivo* activity (half-life of tolbutamide).

DMD Fast Forward. Published on January 22, 2015 as DOI: 10.1124/dmd.114.061200
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1. Demographic characteristics and *CYP2C9* genotypes of 30 subjects aged 21~22 years old.

Genotype	Number	Gender		Body Weight (kg)	BMI
		Male	Female		
*CYP2C9*1/*1*	7	3	4	57.9±13.3	21.31±4.47
*CYP2C9*1/*3*	7	4	3	53.3±10.3	19.71±2.92
*CYP2C9*1/*13*	1		1	44	18.31
*CYP2C9*3/*3*	1	1		68	22.56
*CYP2C9*3/*13*	1		1	53	20.20
*CYP2C9*1/*16*	1	1		80	27.68
*CYP2C9*1/*19*	1	1		52.5	19.40
*CYP2C9*1/*27*	1	1		65	20.52
*CYP2C9*1/*29*	1		1	50	19.05
*CYP2C9*1/*34*	1	1		65	22.49
*CYP2C9*1/*40*	1		1	47	19.07
*CYP2C9*1/*41*	1	1		54	18.47
*CYP2C9*1/*42*	1		1	43	19.15
*CYP2C9*1/*45*	1		1	65	20.02
*CYP2C9*1/*46*	1	1		60	21.37
*CYP2C9*1/*48*	1	1		50	21.16

DMD Fast Forward. Published on January 22, 2015 as DOI: 10.1124/dmd.114.061200
This article has not been copyedited and formatted. The final version may differ from this version.

Table.2 Tolbutamide hydroxylase activities of mutant *CYP2C9* alleles in COS-7 cells compared with that of wild-type *CYP2C9*1*

Genotype	Percentage of matching with wild type	Genotype	Percentage of matching with wild type
*CYP2C9*1*	100.00% ± 10.18%	*CYP2C9*34*	24.34% ± 0.39%**
*CYP2C9*3*	10.73% ± 1.90%**	*CYP2C9*40*	125.78% ± 2.60%**
*CYP2C9*13*	1.28% ± 0.48%**	*CYP2C9*41*	97.62% ± 6.11%
*CYP2C9*16*	3.45% ± 0.48%**	*CYP2C9*42*	0.32% ± 0.24%**
*CYP2C9*19*	31.11% ± 7.43%**	*CYP2C9*45*	0.82% ± 0.01%**
*CYP2C9*27*	114.99% ± 5.38%**	*CYP2C9*46*	29.06% ± 1.35%**
*CYP2C9*29*	109.14% ± 2.60%	*CYP2C9*48*	38.22% ± 1.27%**

Values are mean ± SD. *,** indicate significant difference from wild-type *CYP2C9*,at $P < 0.05$, and 0.01, respectively.

DMD Fast Forward. Published on January 22, 2015 as DOI: 10.1124/dmd.114.061200
This article has not been copyedited and formatted. The final version may differ from this version.

Table 3. Pharmacokinetic parameters of tolbutamide in healthy subjects.

Genotype	$AUC_{0\rightarrow\infty}$	$AUC_{0\rightarrow t}$	C_{max}	$t_{1/2}$	CL/F
	mg/L*h	mg/L*h	mg/L	h	L/h
*CYP2C9*1/*1*	970.61±152.14	933.90±149.02	65.94±11.68	8.14±1.24	0.528±0.094
*CYP 2C9*1/*3*	1722.88±379.50**	1666.49±377.69**	77.34±11.95	13.73±1.80**	0.301±0.058**
*CYP 2C9*1/*13*	2636.63	2729.30	95.42	16.92	0.183
*CYP 2C9*3/*3*	6639.49	6393.57	77.02	52.71	0.075
*CYP 2C9*3/*13*	8792.82	8462.01	103.72	57.53	0.057
*CYP 2C9*1/*16*	1269.659	1322.83	71.53	12.30	0.378
*CYP 2C9*1/*19*	1451.51	1375.93	86.33	10.66	0.344
*CYP 2C9*1/*27*	853.5	814.95	70.72	7.30	0.586
*CYP 2C9*1/*29*	1015.49	992.06	71.58	8.29	0.492
*CYP 2C9*1/*34*	1846.61	1808.14	75.62	14.34	0.271
*CYP 2C9*1/*40*	756.18	729.97	42.13	6.60	0.661
*CYP 2C9*1/*41*	1038.72	985.34	65.99	7.69	0.481
*CYP 2C9*1/*42*	2094.06	1919.56	87.16	15.85	0.239
*CYP 2C9*1/*45*	1654.64	1563.04	71.12	13.41	0.302
*CYP 2C9*1/*46*	1813.07	1754.54	94.98	12.00	0.276
*CYP 2C9*1/*48*	3197.57	3168.09	79.45	23.54	0.156

Values are mean ± SD. *,** indicate significant difference from wild-type *CYP2C9*,at $P < 0.05$, and 0.01, respectively.

DMD Fast Forward. Published on January 22, 2015 as DOI: 10.1124/dmd.114.061200
This article has not been copyedited and formatted. The final version may differ from this version.

Table 4. Pharmacokinetic parameters of 4'-hydroxy tolbutamide in healthy subjects.

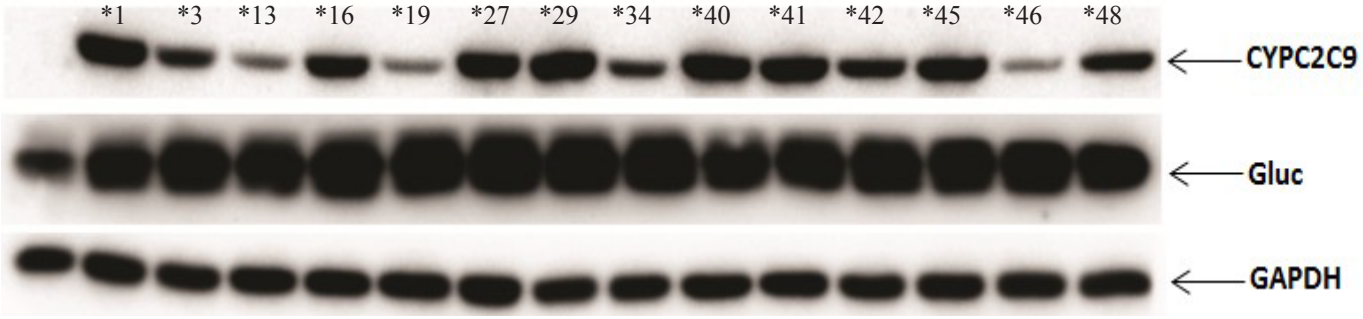
Genotype	$AUC_{0\rightarrow\infty}$	$AUC_{0\rightarrow t}$	C_{max}	$t_{1/2}$
	mg/L*h	mg/L*h	mg/L	h
*CYP2C9*1/*1*	5.812±1.219	5.579±1.253	0.359±0.090	7.642±2.285
*CYP 2C9*1/*3*	5.176±0.904	5.042±0.858	0.252±0.035*	9.237±3.052
*CYP 2C9*1/*13*	7.608	7.426	0.301	10.025
*CYP 2C9*3/*3*	2.653	2.441	0.065	211.759
*CYP 2C9*3/*13*	3.976	3.908	0.072	43.997
*CYP 2C9*1/*16*	3.551	3.542	0.177	5.553
*CYP 2C9*1/*19*	7.981	6.749	0.360	16.716
*CYP 2C9*1/*27*	7.506	6.414	0.396	11.635
*CYP 2C9*1/*29*	9.643	9.151	0.514	10.175
*CYP 2C9*1/*34*	5.947	5.860	0.252	8.453
*CYP 2C9*1/*40*	5.47	4.746	0.389	7.092
*CYP 2C9*1/*41*	7.534	7.013	0.409	8.351
*CYP 2C9*1/*42*	5.938	5.839	0.242	10.033
*CYP 2C9*1/*45*	5.975	5.901	0.221	7.773
*CYP 2C9*1/*46*	6.605	6.597	0.298	4.812
*CYP 2C9*1/*48*	7.402	7.268	0.217	19.589

Values are mean ± SD. *,** indicate significant difference from wild-type *CYP2C9*,at $P < 0.05$, and 0.01, respectively.

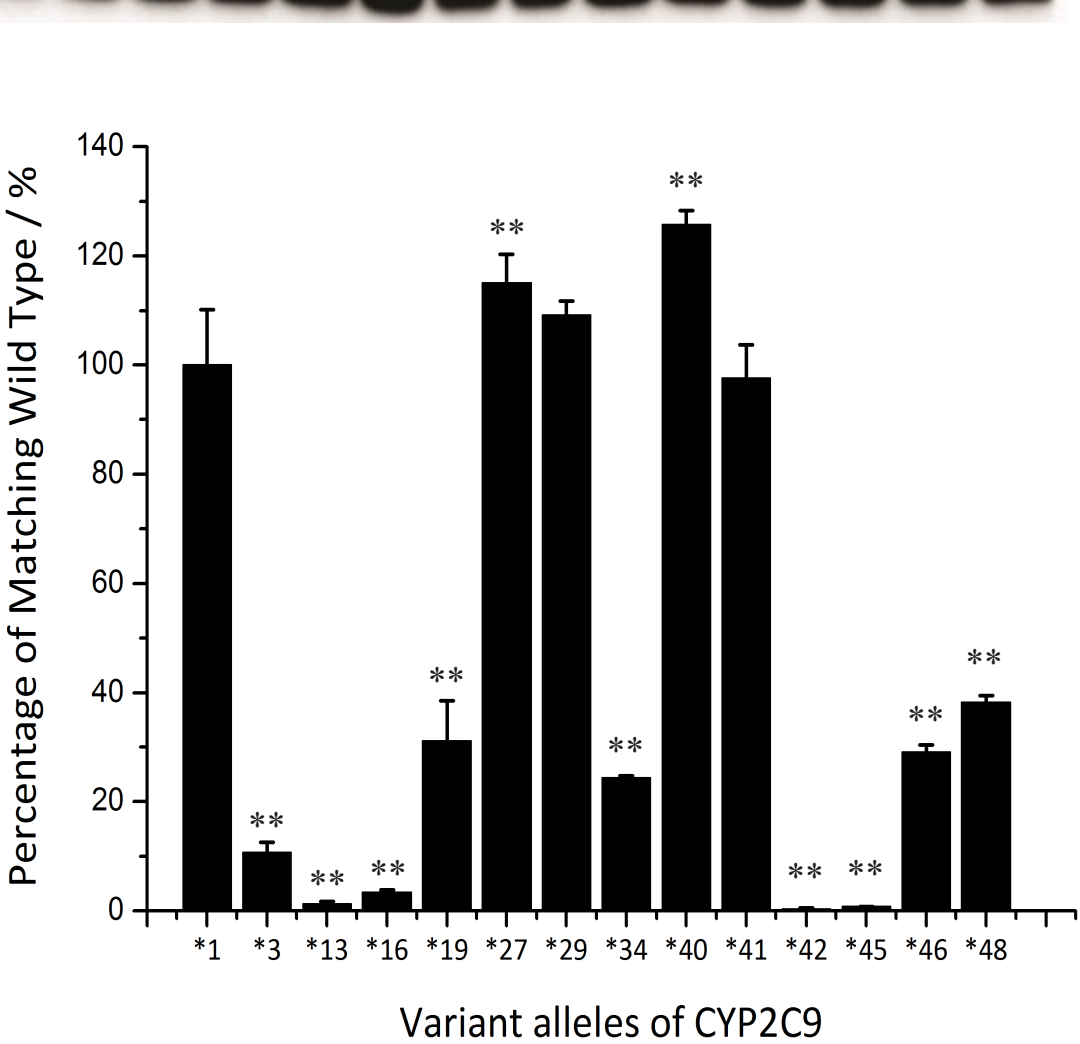
DMD Fast Forward. Published on January 22, 2015 as DOI: 10.1124/dmd.114.061200
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 1

(A)

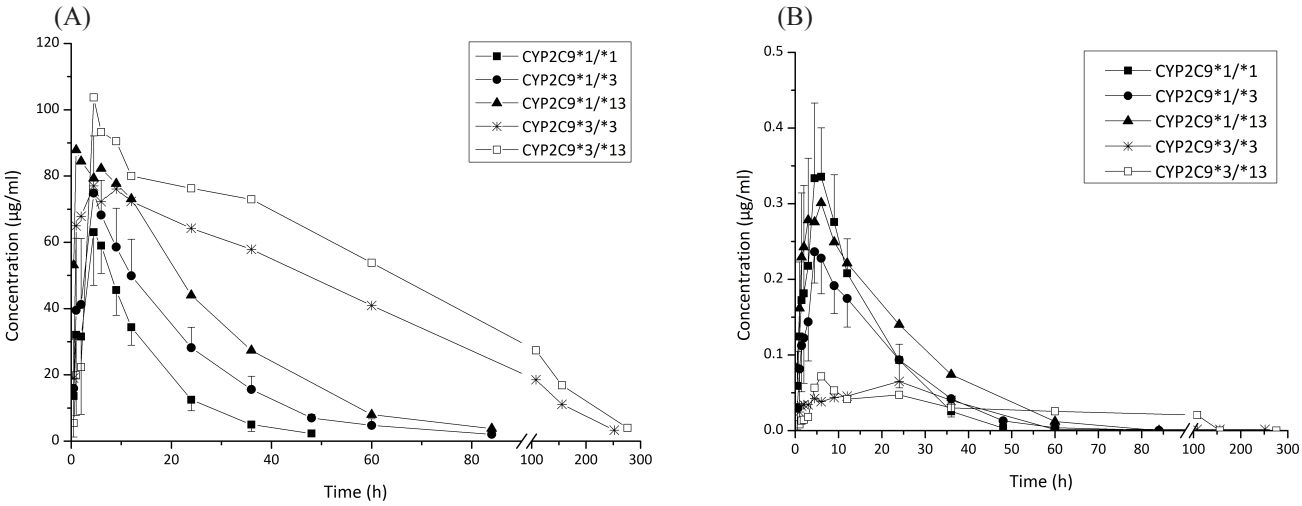


(B)



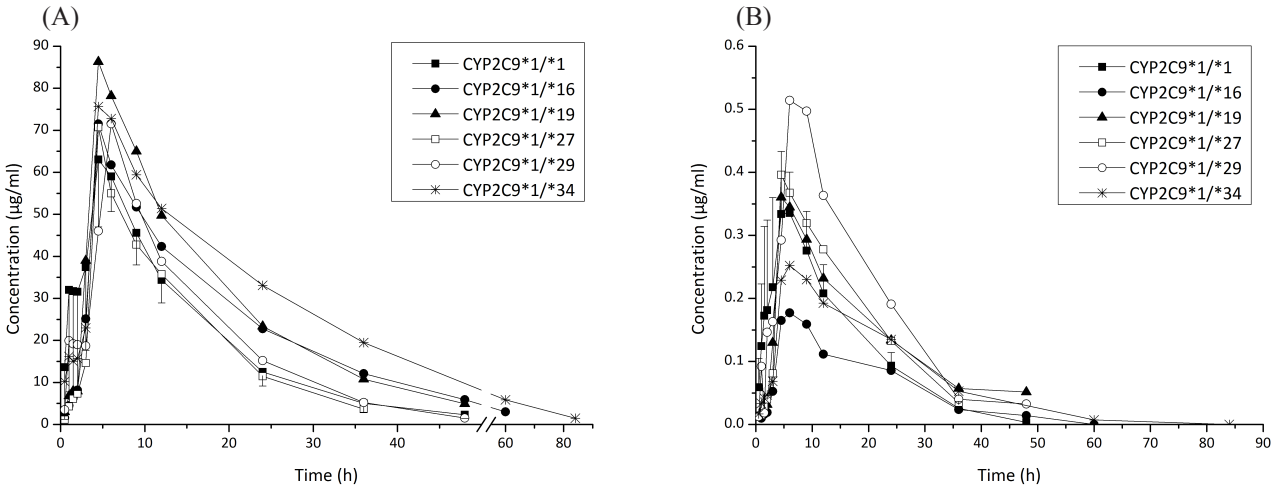
DMD Fast Forward. Published on January 22, 2015 as DOI: 10.1124/dmd.114.061200
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 2



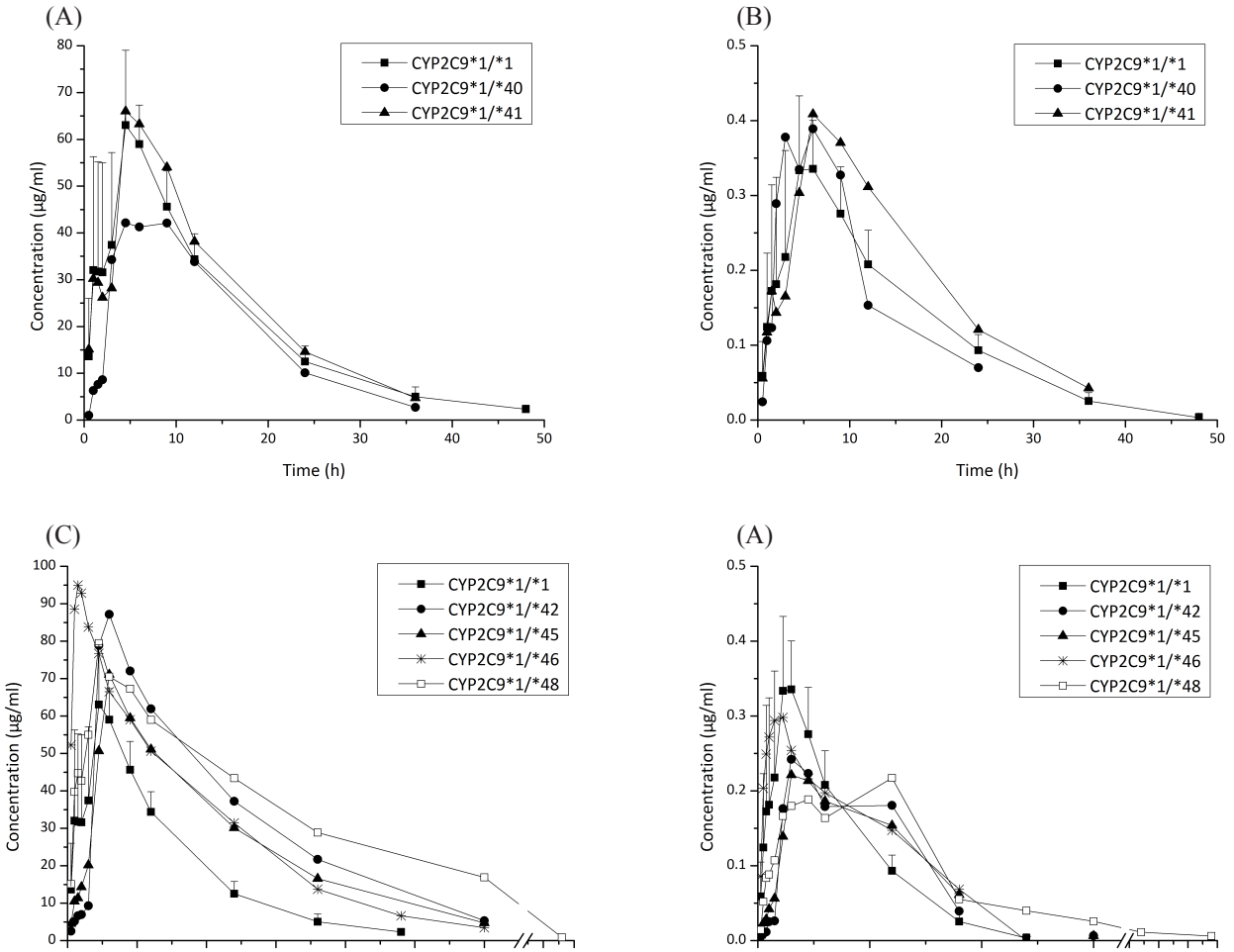
DMD Fast Forward. Published on January 22, 2015 as DOI: 10.1124/dmd.114.061200
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 3



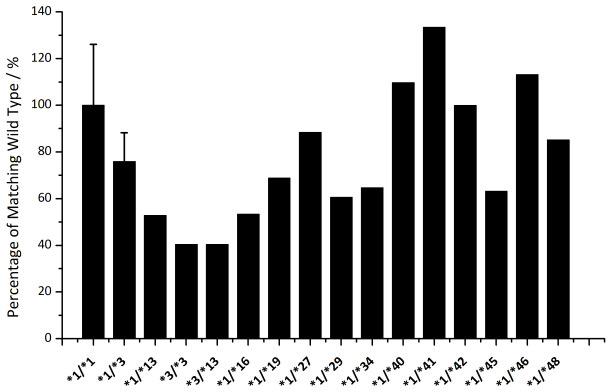
DMD Fast Forward. Published on January 22, 2015 as DOI: 10.1124/dmd.114.061200
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 4



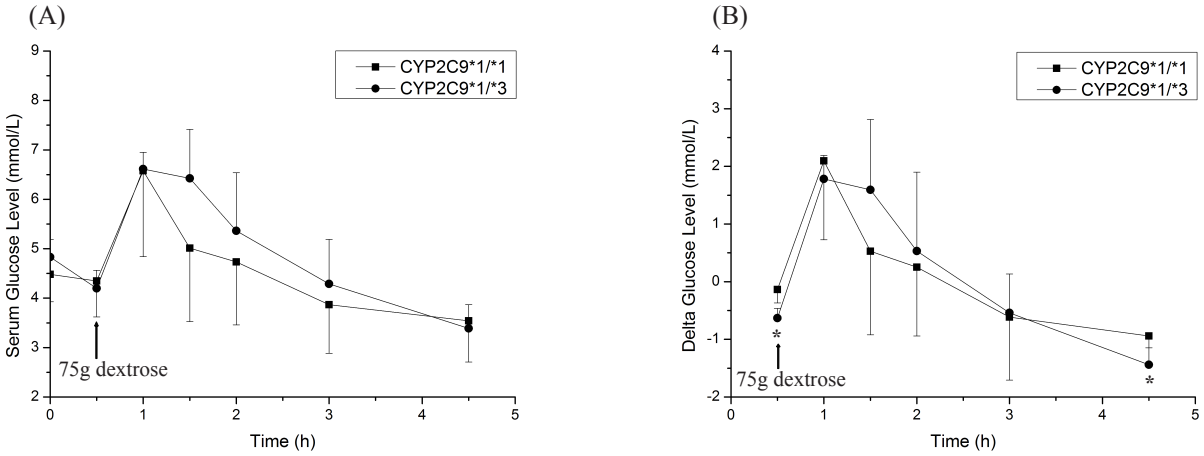
DMD Fast Forward. Published on January 22, 2015 as DOI: 10.1124/dmd.114.061200
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 5



DMD Fast Forward. Published on January 22, 2015 as DOI: 10.1124/dmd.114.061200
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 6



DMD Fast Forward. Published on January 22, 2015 as DOI: 10.1124/dmd.114.061200

Figure 7