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In vitro metabolism of phenytoin in 36 CYP2C9 variants found in the Chinese population

Lian-Guo Chen [a, 1], Zhe Wang [b, 1], Yuan Zhu [a], Jian-Hua Xiong [a], Li-Rong Sun [a], Da-Peng Dai [c], Jian-Ping Cai [c, **], Guo-Xin Hu [d, *]

[a] Wenzhou People's Hospital, Wenzhou, Zhejiang, 325000, China
[b] Department of Pharmacy, The Second Affiliated Hospital & Yuying Children's Hospital of Wenzhou Medical University, Wenzhou, 325027, China
[c] The Key Laboratory of Geriatrics, Beijing Hospital & Beijing Institute of Geriatrics, Ministry of Health, Beijing, 100730, China
[d] Department of Pharmacology, Wenzhou Medical University, Wenzhou, Zhejiang, 325035, China

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ABSTRACT

Cytochrome P450 2C9 (CYP2C9) is an important member of the cytochrome P450 enzyme superfamily, with 57 CYP2C9 allelic variants being previously reported. Recently, we identified 22 novel alleles (*36 -*56 and N418T) in the Han Chinese population. This study aims to assess the catalytic activities of wild-type (CYP2C9*1) and 36 CYP2C9 allelic variants found in the Chinese population toward phenytoin (PHT) *in vitro*. Insect microsomes expressing CYP2C9*1 and 36 CYP2C9 variants were incubated with 1–200 μM phenytoin for 30 min at 37 °C. Then, these products were extracted and the signal detection was performed by HPLC-MS/MS. The intrinsic clearance (V_{max}/K_m) values of all variants, with the exception of CYP2C9*2, CYP2C9*11, CYP2C9*23, CYP2C9*29, CYP2C9*34, CYP2C9*38, CYP2C9*44, CYP2C9*46 and CYP2C9*48, were significantly different from CYP2C9*1. CYP2C9*27, *40, *41, *47, *49, *51, *53, *54, *56 and N418T variant exhibited markedly larger values than CYP2C9*1 (>152.8%), whereas 17 variants exhibited smaller values (from 48.6% to 99.9%) due to larger K_m and/or smaller V_{max} values than CYP2C9*1. The findings suggest that more attention should be paid on subjects carrying these infrequent CYP2C9 alleles when administering phenytoin in clinic.

1. Introduction

Genetic polymorphisms of drug-metabolizing enzymes often result in large inter-individual variations in the rate and pathways of drug metabolism. Using kinetic data *in vitro* to predict drug metabolic stability has attracted widespread interests. Cytochrome P450 2C9 (CYP2C9), as a major member of the human CYP2C subfamily, constitutes about 20% of the CYP protein content in human liver microsomes [1]. This enzyme mediates the oxidative metabolism of approximately 10% of drugs, some of which are characterized by a narrow therapeutic index, including anticonvulsants phenytoin (PHT), anticoagulants warfarin, hypoglycemic agents glimepiride, antihypertensives losartan, non-steroidal anti-inflammatory drugs flurbiprofen and piroxicam and diuretics torsemide [2,3]. Human CYP2C9 exhibits genetic polymorphisms as at least 57 allelic variants have been reported (http://www.cypalleles.ki.se/cyp2c9.htm). *CYP2C9*2* (Arg144Cys) and *CYP2C9*3* (Ile359Leu) are the most common allelic variants and have been widely studied in both *vitro* and *vivo* [4]. DeLozier and her colleagues found that defective alleles of CYP2C9 were more common than previously reported in various Asian populations [5].

Anti-epileptic drug phenytoin (PHT) has been used as an effective anticonvulsant agent for over 60 years, although its clinical use is complicated by nonlinear pharmacokinetics, metabolically based drug-drug interactions, and a narrow therapeutic index. With high efficacy and low cost, phenytoin is still widely used for the treatment of complex partial seizures, secondarily generalized tonic-clonic seizures, and acute seizures in clinic in China [5,6]. In Cuttle's report, he found that some of the toxic effects may result from primary and secondary metabolites, rather than the parent drug [7]. PHT is a substrate of CYP2C9, and- primarily metabolized to 5-(-4-hydroxyphenyl)-5- phenylhydantoin (HPPH) [8–10]. CYP2C9

* Corresponding author.
** Corresponding author.
E-mail addresses: caijp61@vip.sina.com (J.-P. Cai), wzhhgx@aliyun.com (G.-X. Hu).
[1] These authors contributed equally to the manuscript.

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appears to be responsible for PHT metabolism for up to 90% [11], suggesting that CYP2C9 is the primary enzyme responsible for the formation of HPPH and the rate-limiting step in PHT clearance [12]. HPPH is excreted in either the urine or the bile principally as a glucuronic acid conjugate, estimated to account for approximately 80% of phenytoin clearance [13,14]. Additionally, CYP2C9 has been proved to be the catalysts of PHT metabolism to HPPH in human liver microsomes [15]. Previous reports documented that defective alleles of CYP2C9, especially CYP2C9*2 and *3, affect the metabolism and clinical toxicity of PHT [14,16]. However, it is still not clear that the 22 novel identified CYP2C9 variants found in the Chinese population affect the metabolism and clinical toxicity of PHT. In this study, we systematically analyzed the catalytic activities of CYP2C9*1 and 36 CYP2C9 allelic variants (*2, *3, *8, *11, *13, *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, *56 and NT418), including the 22 novel identified variants found in the Chinese population, toward PHT in insect cell microsomes expressing corresponding CYP2C9 allelic variants. We hope this study can provide valuable information for further studies on CYP2C9 alleles for PHT metabolism and personalized oral dosing in clinic.

2. Materials and methods

2.1. Chemicals and materials

Baculosomes co-expressing human CYP2C9 and NADPH-cytochrome P450 oxidoreductase (OR) or cytochrome b5 and OR were purchased from BD Gentest (Woburn, MA, USA). PHT was purchased from Toronto Research Chemicals (Canada), HPPH was purchased from Sigma-Aldrich (USA). Diazepam was purchased from Sigma-Aldrich (St. Louis, MO, USA). The NADPH-regenerating system was from Promega (Madison, WI, USA). High-pressure liquid chromatography-grade solvents were purchased from Fisher Scientific Co. (Fair Lawn, New Jersey, USA). All of the other chemicals and solvents used were of analytical grade or the highest commercially available grade.

2.2. Conditions for enzymatic activity analysis

Insect microsomes expressing 36 CYP2C9 allelic variants were obtained according to the previously reported method [17,18]. In brief, cDNAs encoding CYP2C9 allelic variants were obtained by overlap extension amplification method and fused to the dual-expression baculovirus vector pFastBac -OR so as to obtain the ultimate expression vector pFastBac−OR-CYP2C9. To express CYP2C9 and OR enzymes simultaneously, pFastBac−OR-CYP2C9 plasmid was transfected into DH10B to get the plasmid and used for the baculovirus generation. The *Sf21* insect cells were then infected with these viruses and used for the microsome preparation. The CYP2C9 or OR content in the insect cell microsomes was quantified using a commercially available microsomal protein as a standard after the western blotting analysis.

Reaction mixtures contained the following: 5–10 pmol of P450 from insect microsomes (5 pmol for CYP2C9*1 or 10 pmol for variants), 20–40 pmol of purified cytochrome b5 (P450/b5 = 1:4) and 1–200 μM PHT in 100 mM Tris-HCl buffer (pH 7.4). The reaction need to be pre-incubated for 5 min. Then an NADPH-regeneration system (1.3 mmol/L NADP+, 3.3 mmol/L glucose 6-phosphate, 3.3 mmol/L $MgCl_2$ and 0.4 unit/mL glucose-6-phosphate dehydrogenase) was added to start the reaction at 37 °C in a final volume of 200 μL. The reaction was allowed to proceed for 30 min. Incubation was terminated by the addition of 40 μL HCl (0.1 mol/L) and 10 μL diazepam (20 pg/μL) as an internal standard, and 0.8 mL acetic ether for the extraction. After overtaxing for 2 min, the incubation mixture was centrifuged at 13000 rpm for 5 min. The organic phase was transferred into a clean tube, and dried under a nitrogen stream at 45 °C. The resulting residue was dissolved in 100 μL of the mobile phase. And a 2 μL aliquot was injected into the LC–MS/MS system for the following measurement of HPPT. The incubations were performed in triplicate. The data are presented as the mean ± standard deviation (SD).

Chromatography was performed on an Agilent ZORBAX SB-C18 chromatography column (5 μm, 4.6 × 150 mm) column, with the mobile phase consisting of acetonitrile (solvent A) and 0.1% formic acid in water (solvent B). The mobile phase consisted of an isocratic flow rate of 0.8 mL/min with the following gradient: 70% solvent A plus 30% solvent B for 4 min.

An Agilent 1290 Rapid Resolution LC (Agilent, USA) with an Agilent 6430 triple-quadrupole mass spectrometer equipped with a Turbo IonSpray source was used for the LC–MS/MS analysis. The electro -spray ionization source was applied and the detection was performed in a positive ion model with multiple reaction monitoring (MRM) served as the scanner mode. The detected ions were m/z 253.1 → 182 (PHT), m/z 269 → 198 (HPPH) and m/z 316 → 270 (internal standard, diazepam). Nitrogen was used as the nebulizing gas with the optimum values set at 50 psi. The temperature of the vaporizer was set at 350 °C and the capillary was adjusted to 4500 V. The gas flow was set as 12 L/min.

Prior to the experiments, the LC–MS/MS method was validated. The calibration curves were linear in the range of 0.1–250 μg/mL for HPPH; the linear regression equation was $y = 0.098x + 0.0096$ ($r^2 = 0.9999$) for HPPH. The relative standard deviations (RSDs) of intra-day and inter-day precisions were both below 10%. The accuracy was from 97% to 113%, and the recovery was above 78%.

2.3. Statistical analysis

The kinetic parameters (K_m and V_{max}) were estimated by using a software program designed for the non-linear regression analysis of a hyperbolic Michaelis-Menten equation (Prism version 5, GraphPad Software, San Diego, USA). Kinetic data for each variant are presented as the mean ± SD of three microsomal preparations derived from separate transfections. The one-way ANOVA was used for inter groupcomparison. Dunnett's test was used to analyze the differences in catalytic activity between CYP2C9*1 and 36 mutants. Statistical analyses were all performed with the SPSS package (version 19.0; SPSSInc., Chicago, IL), with $p < 0.05$ considered to be statistically significant.

3. Results

In the present study, the catalytic activities of wild-type CYP2C9 and 36 allelic variants were assessed by using PHT as the probe substrate. Michaelis- Menten plots for each of the CYP2C9 variants are shown in Fig. 1, and the corresponding kinetic parameters and relative clearance value compared with wild type (% relative clearance) are summarized in Table 1. Similar to the results of previous *in vitro* metabolic ability assessment on tolbutamide [17], bosentan [19] and carvedilol [20] (summarized in Table 2), most of the tested CYP2C9 isoforms in the present study exhibited significantly different intrinsic clearance value towards PHT from CYP2C9*1. As shown in Table 1, twelve of the 36 variants (CYP2C9*27, *29, *40, *41, *47, *48, *49, *51, *53, *54, *56 and N418T) exhibited significantly larger V_{max} values compared with CYP2C9*1 ($P < 0.05$), and twenty-two of the 36 variants exhibited significantly smaller values ($P < 0.05$). CYP2C9*2 and *11 have no significant difference to CYP2C9*1. Meanwhile, four of the 36 variants (CYP2C9*33, *36, *42, and *52) exhibited significantly larger K_m