Effects of Cytochrome P450 2C9 Polymorphism on Bosentan Metabolism

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Received July 29, 2014; accepted August 20, 2014

ABSTRACT

Cytochrome P450 (P450) 2C9 is an important member of the P450 enzyme superfamily, with 58 CYP2C9 allelic variants previously reported. Genetic polymorphisms of CYP2C9 significantly influence the efficacy and safety of some drugs, which might cause adverse effects and therapeutic failure. The aim of this study was to assess the catalytic activities of 38 human CYP2C9 alleles, including 24 novel alleles (*36–*60) found in the Han Chinese population, toward bosentan (BOS) in vitro. Insect microsomes expressing the 38 CYP2C9 alleles were incubated with 10–625 μM bosentan for 30 minutes at 37°C and terminated by cooling to −80°C immediately. BOS and hydroxyl bosentan, the major metabolite of BOS, were analyzed by ultra-performance liquid chromatography–tandem mass spectrometry system. Thirty-eight defective alleles can be classified into three categories according to the relative clearance value compared with wild type: nine alleles exhibited significantly increased intrinsic clearance values (V\text{max}/K\text{m}) compared with the wild type (1.5-fold—4.9-fold relative clearance); nine alleles exhibited significantly reduced intrinsic clearance values compared with the wild type (0.8–28.9% relative clearance). The remaining 20 alleles exhibited no significant difference (1-fold) in enzyme activity compared with the wild type. These findings suggest that more attention should be directed to subjects carrying these infrequent CYP2C9 alleles when administering BOS in the clinic. This is the first report of all these rare alleles for BOS metabolism, providing fundamental data for further clinical studies on CYP2C9 alleles.

Introduction

Genetic polymorphisms of CYP2C9 significantly influence the pharmacokinetics and pharmacodynamics of some drugs, which might result in adverse drug effects and therapeutic failure. CYP2C9 constitutes ~20% of the cytochrome P450 (P450) protein content in human liver microsomes, and it is responsible for clearing a ~15% of drugs that undergo phase I metabolism (Schwarz, 2003; Van Booven et al., 2010). Accumulating evidence indicates that CYP2C9 ranks among the most important drug-metabolizing enzymes in humans. This enzyme hydroxylates weakly acidic or neutral drugs of diverse therapeutic categories, including the hypoglycemic agents tolbutamide and glimepiride, the nonsteroidal anti-inflammatory drugs flurbiprofen and diclofenac, the antihypertensive losartan, the diuretic torsemide, the anticonvulsant phenytoin, and the anticoagulant warfarin (Rettie and Jones, 2005). Genetic polymorphisms of CYP2C9 can lead to wide interindividual variations in drug metabolism (Thakkar et al., 2012). To date, at least 58 allelic variants of CYP2C9 have been reported, and most of these are found in Asian populations (http://www.cypalleles.ki.se/cyp2c9.htm). Among them, CYP2C9 alleles *2 and *3, the most common allelic variants, have been well studied both in vivo and in vitro over the last few years (Tang et al., 1999; Yasar et al., 2001; Ali et al., 2009). Dai et al. have analyzed the CYP2C9 polymorphisms in 2127 people of Han population and discovered 37 kinds of new mutation sites, 21 of which could lead to a change in amino acid coding. Twenty-one kinds of new mutation sites have been nominated as new allele CYP2C9*36–*56 (Dai et al., 2014b). Luo et al. (unpublished data) analyzed the CYP2C9 polymorphisms in the same samples and discovered three kinds of new mutation sites that could lead to a change of amino acid coding (P337T, I434F, L467P); among them, P337T has been submitted to the Human Cytochrome P450 Allele Nomenclature Committee and nominated as new allele CYP2C9*58. The I434F (CYP2C9*59), L467P (CYP2C9*60) are under way.

Bosentan (BOS; Tracleer) is a potent nonpeptide dual-receptor competitive antagonist with high affinity to both endothelin A and endothelin B receptors (Clozel et al., 1994; Weber et al., 1996). It decreases both pulmonary and systemic vascular resistance, thereby increasing cardiac output without increasing heart rate (Rubin et al., 2002; Farber and Loscalzo, 2004). BOS was approved by the US Food and Drug Administration in 2001 and is the first oral drug used for the successful treatment of pulmonary arterial hypertension (Motte et al., 2006). BOS is extensively metabolized in the liver by the cytochrome isoenzymes 2C9 and 3A4 to give three metabolites, namely, hydroxyl bosentan (HYBOS; Ro 48-5033), Ro 47-8634 (4-tert-buty1-N-(6-(2-hydroxy-ethoxy)-5-(2-hydroxy-phenoxy)-2,2′-bipyrimidin-4-yl)-benzenesulfonamide), and Ro 64-1056 (4-(1-hydroxy-2-methylpropan-2-yl)-N-[6-(2-ethoxyethoxy)-5-(2-hydroxyphenoxy)-2-(pyrimidin-2-yl)pyrimidin-4-yl]benzene-1-sulfonamide; UPLC-MS/MS, ultra-performance liquid chromatography–tandem mass spectrometry.
hydroxyethoxy)-5-(2-hydroxyphenoxy)-2-(pyrimidin-2-yl)pyrimidin-4-yl]benzene-1-sulfonamide) (Weber et al., 1999), of which only HYBOS is pharmacologically active (Actelion Pharmaceuticals Ltd., South San Francisco, CA; data on file). Hydroxylation at the β-butyl group by CYP2C9 and CYP3A4 yields metabolite Ro 48-5033, a metabolite that binds to endothelin receptors but with half the affinity of the parent BOS, and its contribution to the pharmacologic activity of BOS is limited to up to 20% (Actelion Pharmaceuticals Ltd., data on file). Ro 47-8634 is formed by oxidative demethylation of the guaiacol ether, catalyzed by CYP3A4, to the corresponding phenol, whereas metabolite Ro 64–1056 is a secondary metabolite that is formed from both Ro 47–8634 and Ro 48–5033, and both CYP3A4 and 2C9 play a role in its formation. The proposed metabolic pathways of BOS in humans, its chemical structure, and those of the metabolites are shown in Fig. 1.


Materials and Methods

Bosentan (purity 98.0%), its metabolite Ro 48-5033 (HYBOS, purity 98.0%), and losartan [internal standard (IS) purity 98.0%] were obtained from Sigma-Aldrich (St. Louis, MO). The NADPH regenerating system was from Sigma-Aldrich (Munich, Germany), and all other reagents used were of analytical grade. Ultrapure water was freshly purified by a Milli-Q A10 System (Millipore, Billerica, MA).

**Instrumentation.** Samples were analyzed by ultra-performance LC–tandem mass spectrometry (UPLC-MS/MS) using a Waters ACQUITY UPLC® I-Class Waters Corp., Milford, MA) and a Waters XEVO TQD triple-quadrupole MS (Waters Corp.) with an electrospray ionization source. Instrument control and data acquisition were performed using Masslynx 4.1 software (Waters Corp.).

**Incubation Conditions.** The incubation mixture consisted of (final assay concentration) recombinant insects’ microsomes containing 5 pmol CYP2C9*1 or 10 pmol of other CYP2C9 mutants, 2.5 to 5 pmol of purified cytochrome b5 (2.5 pmol for CYP2C9*1 or 5 pmol for other CYP2C9 mutants), and 20 μl Tris-HCl buffer (pH 7.4, 1 M), and 1.6 μl of BOS was added to the mixture. BOS was initially prepared in dimethylsulfoxide solution, and the total concentration in the incubation mixture was adjusted to from 10–625 μM. The final concentration of organic solvent (dimethylsulfoxide) in the incubation mixture was less than 1%. The reaction was allowed to preincubate for 5 minutes in a Fisher shaking water bath. Then an NADPH regenerating system (1.3 mmol/liter NADP+, 3.3 mmol/liter glucose 6-phosphate, 3.3 mmol/liter MgCl2, and 0.4 U/ml glucose-6-phosphate dehydrogenase) was added to start the reaction at 37°C in a final volume of 200 μl, and the mixture was incubated at 37°C for 30 minutes. Incubations were performed in individual polystyrene tubes for each time point in triplicate, and the data are presented as the mean ± S.D. from three experiments. Incubations were terminated by cooling to −80°C immediately. Losartan (30 μl of a 1-μg/ml in methanol solution) as an IS was added to the mixture, followed by the addition of 0.4 ml of acetonitrile, which was then vortexed for 2 minutes and centrifuged at 13,000 rpm at 4°C for 10 minutes. The supernatant was 1:1 diluted with water, and 5 μl of the mixture was injected into the UPLC-MS/MS system for analysis.

**Chromatographic Conditions.** The chromatographic separation was carried out using an ACQUITY UPLC-MSMS and performed on a Waters ACQUITY UPLC BEH C18 column (2.1 mm × 50 mm, 1.7-μm particle size, Waters Corp.) and inline 0.2-μm stainless steel frit filter (Waters Corp.). The column temperature was 80°C. The UPLC-MS/MS system was equipped with a 200-μl sample loop, and the injection time was 10 seconds. The mass spectrometer was operated in the multiple-reaction monitoring (LC/MS) mode, and positive ions were used for the analysis. The signal was monitored using the Xevo TQD triple-quadrupole mass spectrometer equipped with an electrospray-ionization source. The details of the product ions used for the determination of each compound are shown in Table 1.
was maintained at 40°C. The chamber temperature in the autosampler was kept at 8°C. The initial mobile phase consisted of solvent A (water containing 0.1% formic acid) and solvent B (acetonitrile) with gradient elution at a flow rate of 0.45 mL/min, injection volume 5 μL. A gradient program was as follows: 20% B (0-0.5 minutes), 20-80% B (0.5-2.5 minutes), maintained at 80% B (2.5-3.5 minutes), 20-80% B (3.5-4 minutes), and maintained at 20% B (4-5 minutes). The total run time of the analytes was 5 minutes. After each injection, the sample manager underwent a needle-wash process, including a strong wash (methanol-water, 50/50, v/v) and a weak wash (methanol-water, 1/10, v/v). Under these conditions, retention times of BOS, HYBOS, and IS were 2.72, 2.16, and 2.23 minutes, respectively. A seven-point standard curve was used to quantify HYBOS.

Mass Spectrometric Conditions. A Waters XEVO TQD triple-quadrupole MS was equipped with an electrospray ionization source that was set to positive electrospray ionization in multiple reaction monitoring mode, with ionization conditions as follows: capillary voltage 3.5 KV, source temperature 150°C, and desolvation temperature 400°C. Nitrogen was used as the desolvation gas (1000 liter/h) and cone gas (50 liter/h). The multiple reaction monitoring transitions were m/z 552.2→202.1, m/z 568.2→202.1, and m/z 423.2→207.2 for BOS, HYBOS, and IS, respectively. The collision energy was set at 35 V for BOS and HYBOS and at 25 V for losartan; the cone voltage was set at 60 V for BOS and HYBOS and 35 V for losartan.

Statistical Analysis. Michaelis-Menten analysis was performed by nonlinear regression curve fitting using the computer program Prism version 5 (GraphPad Software Inc., San Diego, CA). One-way analysis of variance was used for intergroup comparison. Dunnett’s test was used to analyze differences in catalytic activity between CYP2C9*1 and other CYP2C9 mutant enzymes. Statistical analyses were performed with the Statistical Package for the Social Sciences (version 19.0; SPSS Inc., Chicago, IL), with *P < 0.05 considered statistically significant.

Results

Michaelis-Menten kinetics of BOS for wild-type and CYP2C9 mutants are shown in Fig. 2. Corresponding kinetic parameters are summarized in Table 1. Thirty-eight defective alleles can be classified into three categories according to their relative clearance value compared with wild type: nine alleles (CYP2C9*2, *3, *11, *27, *36, *48, *51, *55, and *56) exhibited significantly increased intrinsic clearance values compared with the wild type (1.5-fold–4.9-fold relative clearance); nine alleles (CYP2C9*8, *13, *19, *33, *39, *42, *43, *52, and *59) exhibited significantly reduced intrinsic clearance values compared with the wild type (0.6–2.9% relative clearance). The remaining 20 alleles (CYP2C9*14, *16, *23, *29, *31, *34, *37, *38, *40, *41, *44, *45, *46, *47, *49, *50, *53, *54, *58, and *60) exhibited no significant difference (1-fold) in enzyme activity compared with the wild type. Three of the 38 variants (CYP2C9*41, *47, *50) have no significant difference in value of V\textsubscript{max} compared with the wild type, whereas only two of them (CYP2C9*13, *43) had a significant difference in the value of K\textsubscript{m} compared with the wild type (*P < 0.05). Eighteen of the 38 variants (CYP2C9*2, *3, *8, *11, *13, *19, *27, *33, *36, *39, *42, *43, *48, *51, *52, *55, *56, *59) had a significant difference in the value of intrinsic clearance of BOS (*P < 0.05) compared with the wild type.

CYP2C9*13 and *43 exhibited lower intrinsic clearance values of BOS (*P < 0.05) than did wild-type CYP2C9*1 resulting from greater K\textsubscript{m} values (5.4-fold and 6.3-fold, respectively), and the intrinsic clearance values for variants CYP2C9*13 and *43 separately decreased to 0.92 and 0.55% compared with wild type, respectively. Six had a greater V\textsubscript{max} value than wild type (CYP2C9*27, *36, *48, *51, *55, and *56) and thus showed greater intrinsic clearance values than did the wild type as a result of its value of K\textsubscript{m} having no significant difference (1-fold), but the V\textsubscript{max} value was higher (2.4-fold, 2.0-fold, 1.2-fold, 1.1-fold, 1.3-fold, 1.3-fold). CYP2C9*41, *47, and *50 all have no significant difference in V\textsubscript{max} K\textsubscript{m} and intrinsic clearance. CYP2C9*13 and *43 had significant differences in the corresponding parameters. Variants CYP2C9*8, *13, *19, *33, *39, *42, *43, *52, *59 showed lower intrinsic clearance of BOS (*P < 0.05) than did wild-type CYP2C9*1 as a result of lower V\textsubscript{max} values (*8: 14.3%, *13: 3.6%, *19: 2.1%, *33: 5.6%, *39: 4.7%, *42: 6.0%, *43: 1.2%, *52: 8.9%, *59: 14.8%) compared with the wild type.

Discussion

To understand more clearly the function of the CYP2C9 allele on the metabolism of BOS, we screened the 38 variants with the re combinant insect microsomes. Some of these variants showed decreased enzyme activity, as previous studies reported, but some caused increased or no change in enzyme activity, which is not in accordance with the results of previous research (Dai et al., 2014a; Wang et al., 2014).

Using insect cell expression system, Dai et al. (2013) found that the typical defective allele, CYP2C9*2 (Arg144Cys), exhibited lower intrinsic clearance value for tolbutamide, about 47.2% of wild type. In our study, CYP2C9*2 caused a small decrement in the V\textsubscript{max} (73.6%) and K\textsubscript{m} values for catalysis of BOS, which caused an increase in the clearance rate. The most widely studied allele, CYP2C9*3 (Ile559Leu), is present in approximately 10–15% of the white population and is found less frequently in black and Asian populations (Lee et al., 2002; Xie et al., 2002). Our investigation showed that CYP2C9*3 demonstrated significantly higher intrinsic clearance of BOS compared with wild type. Blaisdell et al. (2004) demonstrated that CYP2C9*8 (R150H) exhibited a significantly increased catalytic activity in tolbutamide metabolism compared with wild-type CYP2C9*1, but Allabi et al. (2005a, 2012) found CYP2C9*8 to be associated with lower clearance for phenytoin and warfarin. In the present study, we found that CYP2C9*8 showed significant decrement in V\textsubscript{max} (14.3%), which caused a lower clearance rate for BOS. Apparent discrepancies regarding CYP2C9*8 might be explained by a substrate-dependent activity, the R150H substitution potentially altering an essential interaction between the cytochrome enzyme and the NADPH-CYP450 reductase in the oxidative metabolism cascade.

The CYP2C9*11 allele is associated with decreased enzyme activity compared with the wild-type variant, as observed in studies on losartan and tolbutamide metabolism (Blaisdell et al., 2004; Allabi et al., 2005b). In a previous study, however, it has been suggested that CYP2C9*11 might not be significantly associated with a slightly lower dose requirement of warfarin (King et al., 2004). In our study, the CYP2C9*11 allele exhibited significantly increased catalytic activity in BOS metabolism compared with wild-type CYP2C9*1. The CYP2C9*13 (Leu90Pro) variant allele occurs in approximately 2% of the Chinese population (Si et al., 2004). CYP2C9*13 also exhibits reduced metabolic activity toward tolbutamide and diclofenac in vitro (Guo et al., 2005b). In the present study, CYP2C9*13 produced a protein with a higher K\textsubscript{m} lower V\textsubscript{max} and lower intrinsic clearance than the CYP2C9*1 allele. However, the Leu90Pro substitution in CYP2C9*13 is located in a non-heme-binding region far from the substrate binding pocket (Guo et al., 2005a). Thus, the reason for the CYP2C9*13-mediated reduction in drug-metabolizing capability is unclear.

CYP2C9*14, CYP2C9*16, and CYP2C9*19 were found in Southeast Asians and reported to exhibit moderately or markedly decreased intrinsic clearance of tolbutamide in vitro (DeLozier et al., 2005). In our study, only CYP2C9*19 markedly decreased the intrinsic clearance of BOS (1.65%) and caused a lower V\textsubscript{max} value (2.1%) relative to wild type, consistent with previous observations.

Although the functional significance of CYP2C9*23 was not determined in the study by Veenstra et al. (2005), our results showed that this allele does not cause a significant change in clearance for BOS. CYP2C9*27 (Arg150Leu) and CYP2C9*29 (Pro279Thr)
showed similar catalytic activities against diclofenac compared with the wild type (Maekawa et al., 2006). In our study, however, CYP2C9*27 caused an increase of 241.32% in the clearance of BOS. CYP2C9*31 was predicted to be a functionally defective allele (Matimba et al., 2009), but our results showed that CYP2C9*31 clearance did not change significantly. Two variations, CYP2C9*33 and CYP2C9*34, were first considered the Michaelis-Menten curves is superposition to CYP2C9*2 in group A.

Fig. 2. Michaelis-Menten curves for BOS hydroxylation from recombinant wild-type and variant CYP2C9. The solid line indicates fitting of the data to the Michaelis-Menten equation by nonlinear regression. Each point represents the mean ± S.D. of three separate experiments. The variants have been manually arranged into eight different groups (A to J) in the order of the designated allele names except CYP2C9*3 consider the Michaelis-Menten curves is superposition to CYP2C9*2 in group A.
detected in 724 Japanese subjects (Yin et al., 2008). CYP2C9*33 showed a lower intrinsic clearance rate of diclofenac in vitro, whereas CYP2C9*34 showed no substantial effect (Yin et al., 2008). In this study, a similar result was obtained in which CYP2C9*33 showed lower intrinsic clearance of BOS (10.84) in vitro, whereas CYP2C9*34 showed no significant changes.

In our study, CYP2C9*36 caused a significant increase in \( V_{\text{max}} \) (2.0-fold) and little or no change in \( K_m \) values for the catalysis of BOS, which caused a significant increase in clearance. Our results showed that R124Q (CYP2C9*42) and R124W (CYP2C9*43) also decreased intrinsic clearance for BOS in vitro, and these data are in accordance with the study performed by Dai et al. (2014b). However, the relative enzymatic activities among these CYP2C9 variants presented a quite different pattern when BOS was used as the substrate. For example, P110S (CYP2C9*40) and S343R (CYP2C9*54) were both predicted to be fast metabolic variants (Dai et al., 2014b), but our results showed no increased activity for either of them. P163L (CYP2C9*47) showed impaired activity in the study by Dai et al. (2014a), but we found that it exhibited no impact on catalytic activity in BOS metabolism compared with wild-type CYP2C9*1. I387V (CYP2C9*56) had no substantial effects on diclofenac, according to previous research (Dai et al., 2014b), but in our study it caused an increase in the intrinsic clearance of BOS compared with the wild type. Of the new-found variants P337T (CYP2C9*58), I434F (CYP2C9*59), and L467P (CYP2C9*60), only I434F significantly decreased the clearance owing to low \( V_{\text{max}} \) (14.8).

In our opinion, the different specificities for these substrates may be the main reason for this inconsistency because previous research 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 (Dai et al., 2014b).

In summary, we screened the enzymatic activity of the 38 variants of CYP2C9 in the metabolism for BOS, including the 24 new coding variants. To our knowledge, this is the first report of all these rare alleles for BOS metabolism. Although the allelic frequencies of these rare alleles in the Chinese population are only about 0.1~1.8%, obtaining relatively precise data for the distribution pattern of other rare alleles in the Chinese population, and then exploit their functional significance, will be important and valuable in clinical practice because more than 1.3 billion people are now living in the mainland of China. Our data provide new information about CYP2C9 genetic polymorphisms and their related biologic impacts; this information can be relevant to personalized medicine in Chinese populations. Further clinical studies are required to determine the clinical importance of the novel CYP2C9 alleles for the metabolism of BOS in vivo. Understanding the pharmacogenetics contributing to the variability in the
BOS dose-response relationship may help in tailoring drug therapy to these patients in a safe, effective manner.

Acknowledgments

The authors thank the members of the Beijing Institute of Geriatrics of the Ministry of Health for their advice and assistance.

Authorship Contributions

Participated in research design: Chen, Pan, L. Wang, X.Q. Wang, Hu. Conducted experiments: Chen, Pan, L. Wang, Zhan. Contributed new reagents or analytic tools: Dai, Cai. Performed data analysis: Chen, Pan, L. Wang. Wrote or contributed to the writing of the manuscript: Chen, Zhang, Pan, Jin, Xia, X.Q. Wang.

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