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Short Communication

Evaluation of 24 CYP2D6 Variants on the Metabolism of Nebivolol In Vitro

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ABSTRACT

CYP2D6 is an important cytochrome P450 (P450) enzyme that metabolizes approximately 25% of therapeutic drugs. Its genetic polymorphisms may significantly influence the pharmacokinetics and pharmacodynamics of clinically used drugs. Studying the effects of CYP2D6 on drug metabolism can help reduce adverse drug reactions and therapeutic failure to some extent. This study aimed to investigate the role of CYP2D6 in nebivolol metabolism by evaluating the effect of 24 CYP2D6 variants on the metabolism of nebivolol in vitro. CYP2D6 variants expressed by insect cell systems were incubated with 0.1–80 μM nebivolol for 30 minutes at 37°C and the reaction was terminated by cooling to −80°C immediately. An ultra-performance liquid chromatography–tandem mass spectrometry system was used to analyze nebivolol and its metabolite 4-hydroxy nebivolol. Compared with CYP2D6.1, the intrinsic clearance values of most variants were significantly altered, and most of these variants exhibited either reduced V_{max} and/or increased K_m values. Variant R440C showed much higher intrinsic clearance than the wild type (219.08%). Five variants (CYP2D6.88, CYP2D6.89, R344Q, V342M, and D336N) exhibited no difference from the wild type. CYP2D6.92 and CYP2D6.96 displayed weak or no activity, whereas the intrinsic clearance values of the remaining 16 variants were significantly reduced to various degrees (ranging from 4.07% to 71%). As the first report of 24 CYP2D6 alleles for nebivolol metabolism, these results are valuable to interpreting in vivo studies and may also serve as a reference for rational clinical administration.

Introduction

Hypertension is one of the most important worldwide public health challenges because of its high frequency and concomitant risks of cardiovascular and kidney disease (Xiong et al., 2015). Hypertension was identified as one of the leading causes of the global burden of disease due to leading global risk (64 million disability-adjusted life-years) (Ezzati et al., 2002).

Nebivolol (Fig. 1A), a third-generation, long-acting, and highly selective β1 adrenoreceptor antagonist, was approved by the US Food and Drug Administration in 2007 for the treatment of hypertension (Münzel and Gori, 2009; Lindamood et al., 2011). In previous studies, nebivolol was determined to be efficacious, safe, and well tolerated for treatment of hypertension (Baldwin and Keam, 2009; Hilas and Ezzo, 2009). CYP2D6 is the major pathway for metabolism of nebivolol; CYP2C19 and CYP3A4 contribute to its metabolism to a lesser extent. The major metabolite is 4-hydroxy (4-OH) nebivolol (Fig. 1B).

CYP2D6, as a member of the cytochrome P450 (P450) enzyme superfamily, is involved in the metabolism of many drugs. We have evaluated the enzymatic activities of 24 CYP2D6 variants (CYP2D6.2, CYP2D6.10, CYP2D6.87– CYP2D6.98, R25Q, F164L, E215K, F219S, V327M, D336N, V342M, R344Q, R440C, and R497C) toward numerous CYP2D6 substrates (olanzapine, propafenone, tamoxifen, citalopram, methadone, risperidone, venlafaxine, aripiprazole, bufuralol, and dextromethorphan). The data showed that variants in CYP2D6 not only affect drug metabolism but also have substrate-specific differences.

In this study, we analyzed the efficiency of the 24 variants toward nebivolol. Genetic variation in the CYP2D6 gene may affect nebivolol metabolism in vivo. A previous study s showed that the elimination half-life of nebivolol was prolonged in individuals lacking CYP2D6 activity, which may affect the efficacy and safety of nebivolol, especially in patients with hepatic or renal injury (Fongemie and Felix-Getzik, 2015). Patients with different variants may benefit from personalized medicine. With this study, we aim to provide valuable information about CYP2D6 genetic polymorphisms and provide a reference for the rational administration of clinical drugs.

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Materials and Methods

Chemicals and Reagents. Nebivolol [α,α′-(iminodimethylene)bis-(6-fluoro-2-chromanmethanol)] was purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan), and 4-OH nebivolol (6-fluoro-α-[[[2-(6-fluoro-3,4-dihydro-2*H*-1-benzopyran-2-yl)-2-hydroxyethyl]amino]methyl]-3,4-dihydro-4-hydroxy-2*H*-1-benzopyran-2-methanol) was purchased from Toronto Research Chemicals Inc. (Toronto, Canada). Metoprolol [1-(isopropylamino)-3-[4-(2-methoxyethyl)-phenoxy]propan-2-ol] was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Reduced NADPH was supplied by Sigma-Aldrich (St. Louis, MO). Recombinant human CYP2D6 microsomes and P450 cytochrome b_5 were provided by Beijing Hospital (Beijing, China) (Cai et al., 2016). An Acquity ultra-performance liquid chromatography (UPLC) BEH C18 column (2.1 mm × 50 mm, 1.7 μm) was obtained from Waters (Dublin, Ireland). Other solvents and chemicals were of analytical grade as required.

Enzymatic Activity Analysis and Conditions. The incubation mixtures included phosphate-buffered saline (100 mM, pH 7.4), CYP2D6 recombinant microsomes (5 or 10 pmol), purified cytochrome b_5 (CYP2D6 = 1:1), and 0.1–80 μM nebivolol. After a 5-minute preincubation in a shaking water bath (Shanghai Sumsung Laboratory Instrument Co., Ltd. Shanghai, China), the NADPH regenerating system was added to start the reaction. The final volume was 200 μl. Incubations proceeded at 37°C for 30 minutes. Reactions were terminated by cooling to −80°C. Then 400 μl acetonitrile and 25μl metoprolol (internal standard, 1 μg/ml) were added to the tubes which was taken out of −80°C after

ABBREVIATIONS: 4-OH, 4-hydroxy; *m/z*, mass-to-charge ratio; P450, cytochrome P450; UPLC, ultra-performance liquid chromatography.

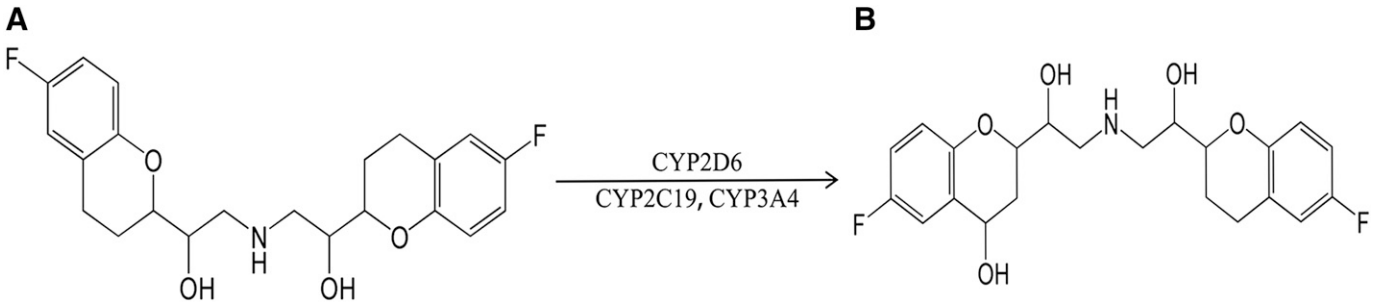

Fig. 1. Structure of the analytes (A) and metabolic pathway of nebivolol (B).

15 minutes. After vortexing, the incubation mixture was centrifuged at 13,000 rpm at 4°C. The supernatant was diluted 1:1 with water and nebivolol and metabolites were measured. Incubations were performed in triplicate and data are presented as means ± S.D.

UPLC–Tandem Mass Spectrometry Instrumentation and Analytical Conditions. An Acquity UPLC system, which consisted of a solvent manager, a sample manager, a column, and a Xevo TQD triple quadrupole mass spectrometer (Waters, Milford, MA), was used to analyze the samples. The mobile phase consisted of acetonitrile (A) and 0.1% formic acid (B). We kept the flow rate at 0.4 ml/min and carried out a gradient elution program as follows: 0–0.3 minutes (40% A), 0.3–0.5 minutes (40%–95% A), 0.5–1.3 minutes (95% A), 1.3–1.5 minutes (95% to 40% A), and 1.5–2.5 minutes (40% A). The total run time was 2.5 minutes. Compounds were separated using an Acquity UPLC BEH C18 column (2.1 mm × 50 mm, 1.7 μm; Waters) maintained at 40°C. Under the above conditions, nebivolol, 4-OH nebivolol, and metoprolol were well separated and their retention times were 1.26, 0.71, and 0.51 minutes, respectively. The lower limit of quantification of 4-OH nebivolol was 1 ng/ml.

The Xevo TQD mass spectrometer was set to positive ion mode. Nitrogen served as the desolvation gas with flow rate of 1000 l/h and the desolvation temperature was maintained at 500°C. The temperature of the ionization source was kept at 150°C while the capillary voltage was set at 2000 V. Multiple reaction monitoring modes (were as follows: mass-to-charge ratio (*m/z*) 406.3 → 151.1, *m/z* 422.3 → 151.1, and *m/z* 268.1 → 115.8 for nebivolol, 4-OH nebivolol, and metoprolol, respectively. The collision energy for nebivolol, 4-OH nebivolol, and metoprolol was set at 35 V, 30 V, and 20 V, respectively; the cone voltage for each was set at 50 V, 30 V, and 45 V, respectively.

Statistical Analysis. Michaelis–Menten curves (substrate versus velocity) and enzyme kinetic parameters (K_m and V_{max}) were obtained by using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA). One-way analysis of variance was used for intergroup comparisons and various variants were considered as factors, whereas V_{max}, K_m, or intrinsic clearance (CL_{int}) values were deemed as dependent variables. SPSS software (version 17.0; SPSS Inc., Chicago, IL) was used to carry out the statistical analysis. $P < 0.05$ was statistically significant.

Results and Discussion

In this study, nebivolol was used as a substrate to evaluate the catalytic activities of the wild type and 24 CYP2D6 variants. Figure 2 demonstrates the Michaelis–Menten kinetics of nebivolol for CYP2D6 variants. Corresponding kinetic parameters are displayed in Table 1. The estimated kinetic parameters K_m, V_{max}, and intrinsic clearance for 4-OH nebivolol of *CYP2D6*1* were 1.8 μM, 1.17 pmol/min per pmol P450, and 0.66 μl/min per nmol P450, respectively.

The 24 alleles displayed considerable differences in K_m, V_{max}, or increased intrinsic clearance values compared with *CYP2D6*1*. R440C, with a higher V_{max} value and lower K_m value, exhibited a significantly increased intrinsic clearance value (2.19-fold) compared with the wild type. Five variants (CYP2D6.88, CYP2D6.89, R344Q, V342M, and D336N) showed no significant difference (1-fold). Sixteen variants exhibited decreased intrinsic clearance values (4.07%–71%, $P < 0.05$), with decreased V_{max} or increased K_m values. CYP2D6.92 and CYP2D6.96 showed no detectable concentration of 4-OH nebivolol, suggesting that they encode nonfunctional protein.

CYP2D6.10 was widely present in Asians (as high as 50% in Korean, Chinese, and Japanese individuals) (Byeon et al., 2015). CYP2D6.10 contains the substitution of S486T and P34S. The latter amino acid substitution has been shown to cause protein instability and reduced substrate affinity (Wang et al., 1999). As reported by previous studies, CYP2D6.10 yields 1.34%–4.57% of the efficiency of *CYP2D6*1* toward bufuralol (1.34%), propranolol (1.41%), risperidone (2.01%), venlafaxine (2.90%), and dextromethorphan (4.57%) in vitro (Liang et al., 2015; Wang et al., 2015; Cai et al., 2016; Zhan et al., 2016). In our study, CYP2D6.10 had a decreased V_{max} (8.71%), an increased K_m (3.62-fold), and a significantly decreased intrinsic clearance (4.07%) of nebivolol compared with CYP2D6.1.

*CYP2D6*2* not only contains the S486T change, but it also possesses the R296C substitution. Cai et al. (2016) found that CYP2D6.2 had lower intrinsic clearance compared with CYP2D6.1 (40.41%) for the probe substrate bufuralol, whereas Liang et al. (2015) found that it exhibited markedly decreased intrinsic clearance (6.93% of the wild type) toward propranolol. Moreover, this variant displayed different intrinsic clearance values for *O*-demethylation (19%) and *N*-demethylation (77%) of dextromethorphan (Yu et al., 2002). Our study showed that the *CYP2D6*2* allele conferred less severe decreased intrinsic clearance (67.83%, $P < 0.01$) for nebivolol, resulting from an obviously decreased V_{max} and a K_m value that was similar to the wild type. This result was consistent with previous observations for bufuralol and dextromethorphan *N*-demethylation but was different from studies on propranolol and dextromethorphan *O*-demethylation. Our results suggest that catalytic activity is substrate specific and that results obtained from one substrate cannot necessarily be extrapolated to other substrates.

*CYP2D6*89* harbored an L142S substitution, which was caused by a T-to-C change in site 1678 of the DNA sequence (425T>C in the cDNA). In a study of dextromethorphan and bufuralol, Dai et al. (2015) suggested that L142S could impair enzymatic activity to some extent, resulting in extremely decreased intrinsic clearance values (<20%) of CYP2D6.1. In other studies, CYP2D6.89 exhibited markedly different catalytic activity toward venlafaxine (71.1%), methadone (74.62%), risperidone (87.56%), and atomoxetine (102.6%) compared with CYP2D6.1 (Wang et al., 2015; Liang et al., 2016; Su et al., 2016; Zhan et al., 2016). Our data revealed that this variant showed no significant difference compared with the wild type but showed a trend of substrate inhibition (Fig. 2).

A higher V_{max} (1.57-fold), a lower K_m (71.05%), and a significantly higher intrinsic clearance (2.19-fold) than CYP2D6.1 also suggested that the R440C amino acid substitution causes substrate inhibition. In contrast, R440C showed impaired activity in the metabolism of bufuralol, propranolol, and methadone (Liang et al., 2015; Cai et al., 2016; Su et al., 2016), indicating that R440 is a functionally important amino acid.

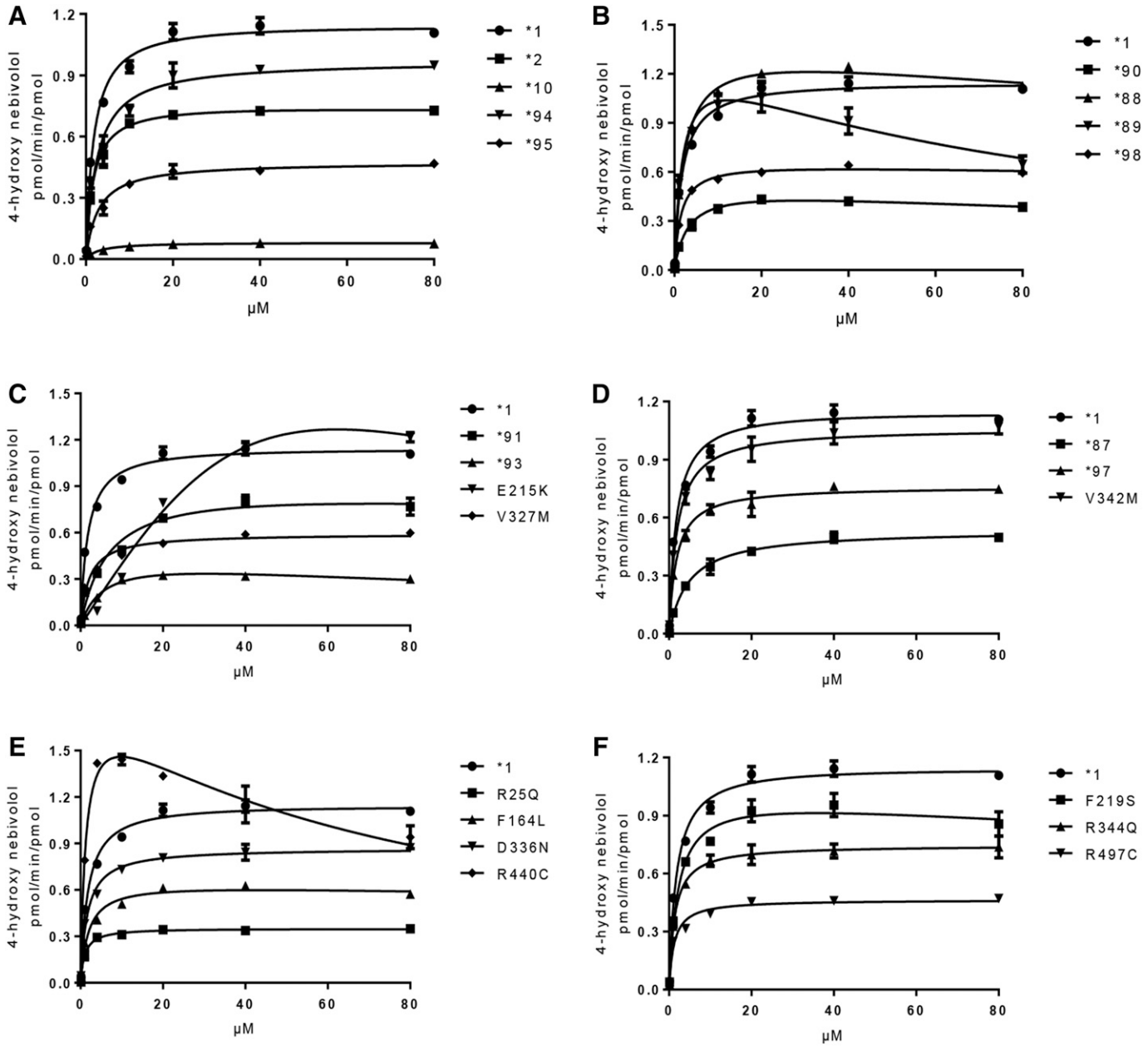

Fig. 2. Michaelis–Menten curves of the enzymatic activity of the wild type and 24 variants toward nebivolol hydroxylation. Each point represents the mean ± S.D. of three parallel experiments. (A–F) The variants with designated allele names were arranged into six groups.

The *E215K* amino acid substation is located within the F helix in the active site of the protein (Dai et al., 2015), affecting catalytic activity. In our study, E215K caused a large K_m value, indicating a reduction in substrate affinity. The intrinsic clearance of E215K (6.15%) was similar to CYP2D6.10 (4.07%) and might be treated as a PM phenotype contingently.

Five allelic isoforms (CYP2D6.87, CYP2D6.90, CYP2D6.91, CYP2D6.93, and CYP2D6.95) exhibited a drastic decrease in enzymatic activity, retaining 12.2%–25.65% of the wild-type activity. CYP2D6.87 had A5V, P34S, and S486T substitutions, which resulted in 16.83% intrinsic clearance compared with CYP2D6.1. Compared with CYP2D6.10, which also carries a P34S amino acid substitution, CYP2D6.87 conveys more activity. CYP2D6.93 (T249P) exhibited decreased metabolic activity (12.2% of wild type), which was in accordance with previous studies. CYP2D6.90 (K147R), CYP2D6.91 (C161S; R296C; splice defect) and CYP2D6.95 (P34S; R388H; S486T) possessed lower V_{max} values and higher K_m values than the wild type, exhibiting significantly decreased intrinsic clearance.

*CYP2D6*92* has one nucleotide deletion at site 1995 and causes a 218 frameshift effect, leading to premature termination of protein synthesis; *CYP2D6*96* contains a single-nucleotide mutation C>T at position 3895 and causes a stop codon (Gln424STOP) (Xu et al., 2016). As a result, CYP2D6.92 and CYP2D6.96 exhibit no activity in nebivolol metabolism, which is consistent with previous studies on different substrates (olanzapine, propafenone, tamoxifen, citalopram, methadone, risperidone, venlafaxine, aripiprazole, bufuralol, and dextromethorphan).

In conclusion, we functionally evaluated the enzymatic activity of 24 CYP2D6 variants on the metabolism of nebivolol. Most of these variants exhibited significant alterations in catalytic activity. As the first

TABLE 1

Kinetic parameters for hydroxylation activities of the wild type and 24 *CYP2D6* allelic variants on nebivolol

Data are presented as means ± S.D.

Variant	V_{max}	K_m	K_i	Intrinsic Clearance (V_{max}/K_m)	Relative Clearance[a]
	pmol/min per pmol P450	*μM*	*μM*	*μl/min per pmol P450*	*%*
*CYP2D6*1*	1.17 ± 0.04	1.80 ± 0.21		0.66 ± 0.06	100.00
CYP2D6*2 (R297C; S486T)	0.77 ± 0.02*	1.77 ± 0.45	2.51 e^3 ± 1.06 e^3	0.45 ± 0.10*	67.83
*CYP2D6*10* (P34S; S486T)	0.09 ± 0.01*	3.62 ± 1.70		0.03 ± 0.01*	4.07
*CYP2D6*87* (A5V)	0.56 ± 0.04*	5.33 ± 1.45*		0.11 ± 0.02*	16.83
*CYP2D6*88* (V104A)	1.38 ± 0.04*	2.18 ± 0.16	531.30 ± 80.47	0.63 ± 0.03	95.79
*CYP2D6*89* (L142S)	1.36 ± 0.09*	1.86 ± 0.32	88.35 ± 34.55	0.74 ± 0.08	111.85
*CYP2D6*90* (K147R)	0.52 ± 0.04*	3.32 ± 1.31	278.37 ± 81.94	0.17 ± 0.05*	25.05
*CYP2D6*91* (C161S)	0.99 ± 0.18*	7.97 ± 3.44*		0.13 ± 0.03*	20.58
*CYP2D6*92* (218 frameshift)	ND	ND		ND	ND
*CYP2D6*93* (T249P)	0.46 ± 0.04*	5.75 ± 0.64*	192.83 ± 116.20	0.08 ± 0.00*	12.20
*CYP2D6*94* (D337G)	0.97 ± 0.00*	2.59 ± 0.68		0.39 ± 0.10*	58.23
*CYP2D6*95* (R388H)	0.48 ± 0.00*	2.87 ± 0.55		0.17 ± 0.03*	25.65
*CYP2D6*96* (424STOP)	ND	ND		ND	ND
*CYP2D6*97* (F457L)	0.77 ± 0.03*	1.83 ± 0.17		0.42 ± 0.02*	64.57
*CYP2D6*98* (H463D)	0.66 ± 0.01*	1.49 ± 0.26		0.45 ± 0.07*	67.97
R25Q	0.36 ± 0.01*	1.04 ± 0.23		0.35 ± 0.08*	52.53
F164L	0.66 ± 0.03*	1.99 ± 0.47	3.05e3 ± 4.14 e^3	0.34 ± 0.06*	51.17
E215K	9.48 e^3 ± 2.43 e^3*	2.31 e^5 ± 5.10 e^4*		0.04 ± 0.00*	6.15
F219S	1.02 ± 0.03*	2.18 ± 0.03	656.43 ± 314.34	0.47 ± 0.01*	71.00
V327M	0.59 ± 0.01*	2.06 ± 0.26		0.29 ± 0.03*	43.77
D336N	0.87 ± 0.01*	1.61 ± 0.13		0.54 ± 0.03	81.61
V342M	1.06 ± 0.02	1.93 ± 0.17		0.55 ± 0.04	83.16
R344Q	0.76 ± 0.03*	1.42 ± 0.20		0.54 ± 0.05	83.28
R440C	1.85 ± 0.10*	1.28 ± 0.12	79.04 ± 24.95	1.44 ± 0.05*	219.08
R497C	0.46 ± 0.01*	1.20 ± 0.09		0.39 ± 0.02*	58.71

ND, not determined.
[a]Data are given as a percentage of the wild type.
*$P < 0.01$ (significantly different from wild-type CYP2D6).

report of all of these alleles with respect to nebivolol metabolism, these data are valuable in interpreting in vivo studies. They may also serve as a reference for rational clinical administration, contributing to the development of personalized medicine.

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