DMD Fast Forward. Published on August 18, 2016 as DOI: 10.1124/dmd.116.071811
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Title Page

Short Communication

Evaluation of 24 CYP2D6 Variants on the Metabolism of Nebivolol *in vitro*

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Running title page

Running title: Functional impacts of 24 CYP2D6 variants

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Number:

Number of text pages: 14

Number of tables: 1

Number of figures: 2

Number of references: 18

Length of the Abstract: 228 words

Length of the Introduction: 580 words

Length of the Results and Discussion: 885 words

Abbreviations

ADRs, adverse drug reaction

P450, cytochrome P450

4-OH nebivolol, 4-hydroxy nebivolol

UPLC-MS/MS, Ultra-performance liquid chromatography-tandem mass spectrometry system

Abstract

CYP2D6 is an important cytochrome P450 (P450) enzyme that metabolizes about 25% therapeutic drugs. Its genetic polymorphism may significantly influence the pharmacokinetics and pharmacodynamics of clinically used drugs. Studying the effect of CYP2D6 on drug metabolism can avoid adverse drug reactions (ADRs) and therapeutic failure to some extent. This research is aimed at investigating the role of CYP2D6 in nebivolol metabolism, evaluating the effect of 24 CYP2D6 variants on the metabolism of nebivolol *in vitro*. CYP2D6 variants expressed by insect cells system were incubated with 0.1-80 μM nebivolol for 30 min at 37°C and the reaction was terminated by cooling to -80°C immediately. Ultra-performance liquid chromatography-tandem mass spectrometry system (UPLC-MS/MS) was used to analyze nebivolol and its metabolites 4-hydroxy nebivolol. Compared to CYP2D6.1, the intrinsic clearance values of most variants were significantly altered, and most of these variants exhibiting either reduced V_{max} and/or increased K_m values. R440C showed much higher intrinsic clearance than wild type (219.08%); Five variants (CYP2D6.88, CYP2D6.89, R344Q, V342M and D336N) exhibited no difference with wild type; CYP2D6.92 and CYP2D6.96 displayed weak or no activity, while the intrinsic clearance values of remaining 16 variants was significantly reduced in different degrees, ranging from 4.07% to 71%. As first report of 24 CYP2D6 alleles for nebivolol metabolism, these results are valuable to interpret *in vivo* studies and may also serve as a reference for rational administration clinically.

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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 (64 million DALY) was identified as one of the leading cause of global burden of disease (Ezzati et al., 2002).

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

CYP2D6, as a member of 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 variants in CYP2D6 not only affect drug metabolism, but also have substrate-specific differences.

In this research, we analyzed the efficiency of the 24 variants toward nebivolol.

Genetic variation in CYP2D6 gene may affect nebivolol metabolism in vivo. Studies showed that the elimination half-life of nebivolol was prolonged in individuals lacking CYP2D6 activity, which may impact the efficacy and safety of nebivolol, especially in patients with hepatic or renal injury (Fongemie and Felix-Getzik, 2015). Patients with different variants would better to take personalized medicine. We hope our data can provide valuable information about CYP2D6 genetic polymorphisms and provide a reference to rational administration of clinical drugs.

Materials and Methods

Chemicals and Reagents. Nebivolol (alpha,alpha'-(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-2H-1-benzopyran-2-yl)-2-hydroxyethyl]aMino]Methyl]-3,4-dihydro-4-hydroxy-2H-1-benzo pyran-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). The reduced nicotinamide adenine dinucleotide phosphate (NADPH) was supplied by Sigma (St. Louis, MO). Recombinant human P450s CYP2D6 microsomes and P450 Cytochrome b5 were provided by Beijing Hospital (Cai et al., 2016). An Acquity UPLC® BEH C18 column (2.1 mm × 50 mm, 1.7 μm) was obtained from the Waters (Made in Ireland). Other solvents and chemicals were of analytical grade as required.

Enzymatic activity analysis and conditions. The incubation mixtures included PBS buffer (100mmol/L, pH 7.4), CYP2D6 recombinant microsomes (5 or 10 pmol),

purified cytochrome b5 (b5: CYP2D6= 1:1) and 0.1-80 μmol/L nebivolol. After 5 min pre-incubate in a Fisher shaking water bath, NADPH regenerating system was added to start the reaction. The final volume was 200μL. Incubations proceeded at 37°C for 30min. 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 when taking out of -80°C after 15min approximately. After vortexing, the incubation mixture was centrifuged at 13 000 rpm at 4°C. The supernatant was 1:1 diluted with water and nebivolol and metabolites measured. Incubations were performed in triplicates and data presented as mean ± SD.

UPLC-MS/MS instrumentation and analytical conditions. An ACQUITY UPLC System which is consisted of a solvent manager, a sample manager, a column and XEVO TQD triple quadrupole MS (Waters, Milford, MA, USA) was used to analyze samples. The mobile phase consisted of acetonitrile (A) and 0.1% formic acid (B). We kept flow rate at 0.4 mL/min and carried out a gradient elution program as follows: 0-0.3 min (40% A), 0.3- 0.5 min (40- 95% A), 0.5-1.3 min (95% A), 1.3- 1.5 min (95-40% A), and 1.5- 2.5 min (40% A). The total run time was 2.5 min. Compounds were separated using an ACQUITY UPLC® BEH C18 column (2.1 mm × 50 mm, 1.7 μm, Waters, Ireland) maintained at 40°C. Under above conditions, nebivolol, 4-OH nebivolol and metoprolol were well separated and their retention times were 1.26, 0.71 and 0.51 min, respectively. The LLOQ of 4-OH nebivolol was 1ng/ml.

The XEVO TQD MS was set to positive ion mode. Nitrogen was served as desolvation gas with flow rate of 1000 L/h and the desolvation temperature was

maintained at 500°C. The temperature of ionization source was kept at 150°C while capillary voltage was set at 2000 V. The multiple reaction monitoring modes were *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; and the cone voltage for each was set at 50 V, 30 V and 45 V, respectively.

Statistical Analysis. Michaelis-Menten curves (Substrate vs. Velocity) and enzyme kinetic parameters (K_m and V_{max}) were obtained by using GraphPad Prism 5 (GraphPad Software Inc., SanDiego, CA). One-way ANOVA was used for intergroup comparison, and various variants were considered as factor while V_{max}, K_m or CL_{int} values were deemed as dependent list. The Statistical Package for the Social Sciences (version 17.0; SPSS Inc., Chicago, IL) was used to carry out statistical analysis (*P< 0.05 represents statistically significant).

Results and Discussion

In this study, nebivolol was used as a substrate to evaluate the catalytic activities of 24 CYP2D6 variants and wild type. Michaelis-Menten kinetics of nebivolol for CYP2D6 variants are shown in Fig. 2. Corresponding kinetic parameters are displayed in Table **2**. The estimated kinetic parameters K_m, V_{max}, and CL_{int} for 4-OH nebivolol of *CYP2D6*1* were 1.8 μmol/L, 1.17 pmol/min/pmol P450, 0.66 μL/min/nmol P450, respectively.

The 24 alleles displayed considerable differences in K_m, V_{max} or increased intrinsic clearance values when compared to *CYP2D6*1*. R440C, with higher V_{max} value and

lower K_m value, exhibited a significant increased intrinsic clearance value (2.19-fold) compared with 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 they encode nonfunctional protein.

CYP2D6.10 was widely present in Asians, as high as 50% in Korean, Chinese and Japanese (Byeon et al., 2015). It contains the substitution of S486T and P34S. The latter amino acid substitution has been shown to cause protein instability and cause reduced substrate affinity (Wang et al., 1999). As reported by previous studies, CYP2D6.10 yield 1.34 to 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; Zhan et al., 2016; Cai et al., 2016). In our study, CYP2D6.10 had a decreased V_{max} (8.71%), increased K_m (3.62-fold), and significantly decreased intrinsic clearance (4.07%) of nebivolol compared with CYP2D6.1.

*CYP2D6*2* not only contains the S486T change, but also possesses the R296C substitution. Cai *et al.* (2015) found that CYP2D6.2 had lower intrinsic clearance compared to CYP2D6.1(40.41%) for the probe substrate bufuralol, while Liang *et al.* found it exhibited markedly decreased intrinsic clearance (6.93% of wild-type) toward propranolol (Liang et al., 2015; Cai et al., 2016). 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 similar K_m value to wild-type. This result was consistent with previous observations on bufuralol and dextromethorphan N-demethylation, but 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 a L142S substitution, which was caused by a T to C change in site 1678 of DNA sequence (425T>C in the cDNA). In the study of dextromethorphan and bufuralol, Dai *et al*. suggested that L142S could impair enzymatic activity to some extent, resulting in extremely decreased intrinsic clearance values (<20%) of CYP2D6.1 (Dai et al., 2015). 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 this variant showed no significant difference when compared to wild type, but showed a trend of substrate inhibition (Fig. 2.).

Higher Vmax (1.57-fold), lower Km (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 metabolism of bufuralol, propranolol and methadone (Cai et al., 2016; Liang et al.,

2015; Su et al., 2016), indicating that R440 is a functionally important amino acid.

The *E215K* amino acid substation is located within F-helix in the active site of the protein (Dai et al., 2015) impacting 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 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 to 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} value and higher K_m value than 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 substrate (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 report of all these alleles with respect to nebivolol metabolism, these data are valuable to interpret *in vivo* studies. It may also serve as a reference for rational administration clinically, contributing to the development of personalized medicine.

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: X.X. Hu, Lan, Yuan, Zhou, and G.X. Hu

Conducted experiments: X.X. Hu, Yuan, and Zhou

Contributed new reagents or analytic tools: Dai, and Cai

Performed data analysis: X.X. Hu, Lan, and Yuan

Wrote or contributed to the writing of the manuscript: X.X. Hu, Lan, Yuan, Li and Xu

DMD Fast Forward. Published on August 18, 2016 as DOI: 10.1124/dmd.116.071811
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Legends for Figures

Fig. 1. Structure of the analytes and metabolic pathway of nebivolol

Fig. 2. Michaelis-Menten curve 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). The variants with designated allele names have been arranged into 6 groups.

DMD Fast Forward. Published on August 18, 2016 as DOI: 10.1124/dmd.116.071811
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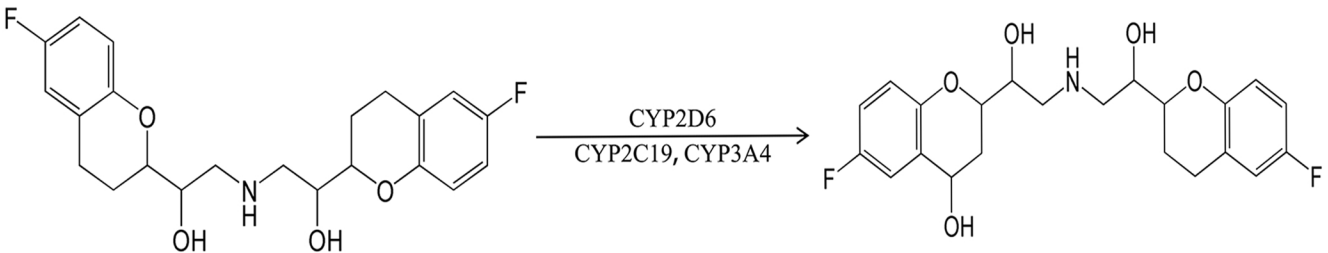
Table 1

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

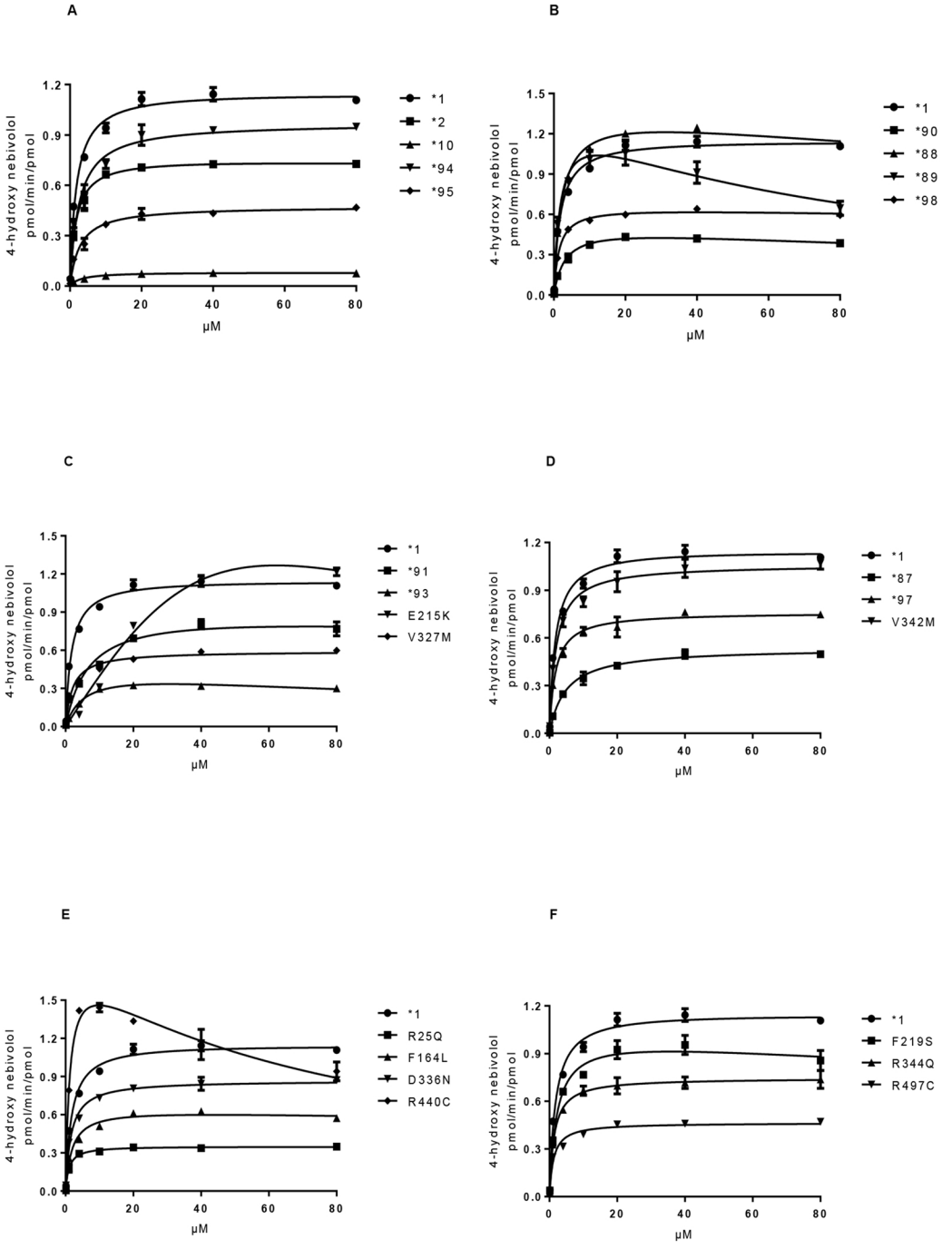
Variants	Vmax (pmol/min/pmol P450)	Km (μM)	Ki (μM)	CLint(Vmax/Km) (μl/min/pmol P450)	Relative clearance (% of wild type)
*CYP2D6*1*	1.17±0.04	1.80±0.21		0.66±0.06	100.00
CYP2D6*2 (R297C; S486T)	0.77±0.02**	1.77±0.45	2.51e3±1.06e3	0.45±0.10**	67.83
*CYP2D6*10* (P34S; S486T)	0.09±0.01**	3.62±1.70		0.03±0.01**	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)	N.D.	N.D.		N.D.	N.D.
*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)	N.D.	N.D.		N.D.	N.D.
*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±4.14e3	0.34±0.06**	51.17
E215K	$9.48e^{3}$±$2.43e^{3}$**	$2.31e^{5}$±$5.10e^{4}$**		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

**Significantly different from wild-type CYP2D6, *P < 0.05, **P < 0.01.*



A B

Figure 1


A
4-hydroxy nebivolol pmol/min/pmol
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E215K
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Figure 2