Influence of phenylalanine-481 substitutions on the catalytic activity of cytochrome P450 2D6

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Homology models of the active site of cytochrome P450 2D6 (CYP2D6) have identified phenylalanine 481 (Phe^{481}) as a putative ligand-binding residue, its aromatic side chain being potentially capable of participating in π–π interactions with the benzene ring of ligands. We have tested this hypothesis by replacing Phe^{481} with tyrosine ($Phe^{481} \rightarrow Tyr$), a conservative substitution, and with leucine ($Phe^{481} \rightarrow Leu$) or glycine ($Phe^{481} \rightarrow Gly$), two non-aromatic residues, and have compared the properties of the wild-type and mutant enzymes in microsomes prepared from yeast cells expressing the appropriate cDNA-derived protein. The $Phe^{481} \rightarrow Tyr$ substitution did not alter the kinetics [K_m (μM) and V_{max} (pmol/min per pmol) respectively] of oxidation of *S*-metoprolol (27; 4.60), debrisoquine (46; 2.46) or dextromethorphan (2; 8.43) relative to the respective wild-type values [*S*-metoprolol (26; 3.48), debrisoquine (51; 3.20) and dextromethorphan (2; 8.16)]. The binding capacities [K_s (μM)] of a range of CYP2D6 ligands to the $Phe^{481} \rightarrow Tyr$ enzyme (*S*-metoprolol, 22.8; debrisoquine, 12.5; dextromethorphan, 2.3; quinidine, 0.13) were also similar to those for the wild-type enzyme (*S*-metoprolol, 10.9; debrisoquine, 8.9; dextromethorphan, 3.1; quinidine, 0.10). In contrast, the $Phe^{481} \rightarrow Leu$ and $Phe^{481} \rightarrow Gly$ substitutions increased significantly (3–16-fold) the K_m values of oxidation of the three substrates [*S*-metoprolol (120–124 μM), debrisoquine (152–184 μM) and dextromethorphan (20–31 μM)]. Similarly, the K_s values of the ligands to $Phe^{481} \rightarrow Leu$ and $Phe^{481} \rightarrow Gly$ mutants were also increased 3 to 10-fold (*S*-metoprolol, 33.2–41.9 μM; debrisoquine, 85–90 μM; dextromethorphan, 15.7–18.8 μM; quinidine 0.35–0.53 μM). However, contrary to a recent proposal that Phe^{481} has the dominant role in the binding of substrates that undergo CYP2D6-mediated N-dealkylation routes of metabolism, the $Phe^{481} \rightarrow Gly$ substitution did not substantially decrease the capacity of the enzyme to N-deisopropylate metoprolol (wild-type, 1.12 pmol/min per pmol of P450; $Phe^{481} \rightarrow Gly$, 0.71), whereas an $Asp^{301} \rightarrow Gly$ substitution decreased the N-dealkylation reaction by 95% of the wild-type rate. Overall, our results are consistent with the proposal that Phe^{481} is a ligand-binding residue in the active site of CYP2D6 and that the residue interacts with ligands via a π–π interaction between its phenyl ring and the aromatic moiety of the ligand. However, the relative importance of Phe^{481} in binding is ligand-dependent; furthermore, its importance is secondary to that of Asp^{301}. Finally, contrary to predictions of a recent homology model, Phe^{481} does not seem to have a primary role in CYP2D6-mediated N-dealkylation.

Key words: active site, homology models, ligand binding, N-dealkylation, site-directed mutagenesis.

INTRODUCTION

Cytochrome P450 2D6 (CYP2D6), a member of the P450 superfamily of enzymes, is responsible for the metabolism in humans of many clinically prescribed drugs, especially compounds acting within the central nervous system [1]. The *CYP2D6* gene is highly polymorphic: more than 50 alleles have been described so far [2], several of which result in either absent, decreased or enhanced enzyme activity [3]. Because of the prominence of CYP2D6 in human drug metabolism, and the potential clinical consequence of its genetic polymorphism, there is much interest in the development of a predictive model of the active site of this enzyme [4–6]. Such a model would aid in the identification of substrates and inhibitors of CYP2D6 at an early stage in the discovery and development of new drugs [7]. However, the development of a predictive model is currently hampered by the lack of accurate structural information, due principally to technical difficulties in obtaining good-quality crystals of eukaryotic P450s for X-ray crystallographic studies, although a recently published crystal structure of rabbit CYP2C5 [8] could aid progress in this area. The alternative approach of homology modelling, using the crystal structures of soluble bacterial P450s as templates [9–12], is currently being used to delineate the topography of the active site of several human P450s [4], including CYP2D6 [13–18]. As a continuing programme of work to validate published homology models of CYP2D6, we are conducting site-directed mutagenesis studies to probe the role of putative active-site residues, identified from homology models, in ligand recognition and binding and in enzyme activity.

With this approach we have previously confirmed the central role of Asp^{301} in CYP2D6 ligand binding [19]. This negatively charged residue, identified in most published homology models of the active site of CYP2D6 [14–18], participates in an electrostatic interaction with a basic nitrogen present in all CYP2D6 ligands and aids in the correct orientation of ligands in the active site. The site(s) of oxidation in substrates that interact with Asp^{301} occur at a distance of 5 or 7 Å from the basic nitrogen of the substrate [18]. Some homology models also nominate Glu^{216} as an alternative negatively charged residue that interacts with the basic nitrogen of certain large CYP2D6 substrates, such as tamoxifen and ondansetron [17,18,20], in which the site of

Abbreviations used: CYP or P450, cytochrome P450; CYP2D6, cytochrome P450 2D6; K_s, binding coefficient.

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oxidation occurs at 10–12 Å from the basic nitrogen. However, this proposal still needs to be verified experimentally by site-directed mutagenesis studies. Other homology models also implicate Ser^{304} in ligand selectivity [14,17,21,22] via a hydrogen-bonding interaction; however, a recent publication refutes its significance as a ligand-contact residue [23].

Although an acidic residue appears to be the principal determinant of ligand binding, pharmacophore and homology models of CYP2D6 also suggest a ligand-binding role for one, or possibly two, aromatic residues within the active site of the enzyme [24]. Such residues are capable of π–π interactions with the benzene ring(s) of aromatic ligands, a chemical moiety found in all CYP2D6 ligands. Several homology models have identified Phe^{481} as such a putative ligand-binding residue [6,14,18], although other models propose that Phe^{483}, not Phe^{481}, is associated with ligand binding [16]. A recent paper has also implicated Phe^{481} as the key ligand-binding residue for substrates that undergo N-dealkylation reactions by CYP2D6, furthermore indicating that binding with Asp^{301} is not necessary in this metabolic route [25].

A preliminary account of the role of Phe^{481} has been published in abstract form only [26]. To clarify further the role of Phe^{481} as a substrate-contact residue, the preliminary work has been extended to include the influence of Phe^{481} substitutions on the kinetics of oxidation of debrisoquine and dextromethorphan, in addition to *S*-metoprolol. The effects of the substitutions on ligand binding, product and substrate selectivity, and the inhibitory potency of the diastereoisomer pair quinine/quinidine on enzyme activity, were also studied. Finally, the proposed role of Phe^{481} in CYP2D6-mediated N-dealkylation reactions was also investigated. These findings should help to validate and refine existing homology models of CYP2D6 and aid in the development of a unified, predictive model of the active site of this important drug-metabolizing enzyme.

EXPERIMENTAL

Materials

Escherichia coli strains DH5α, CJ236 and TG1 and *Saccharomyces cerevisiae* strain AH22 have been described previously [19,27]. Human CYP2D6 cDNA, representing the wild-type *CYP2D6*1A* sequence [3], and the yeast expression vector pMA91 have been reported elsewhere [19]. Debrisoquine hemisulphate and its 4-hydroxy metabolite were gifts from Roche Products Ltd (Welwyn Garden City, Herts., U.K.). Metoprolol tartrate (racemate), O-demethylmetoprolol, α-hydroxymetoprolol, N-deisopropylmetoprolol hydroxybenzoate metabolites were gifts from Astra Hassle (Mölndal, Sweden). The hydrochloride salts of the *R*-(+)- and *S*-(−) enantiomers of metoprolol were gifts from Ciba Geigy (Basle, Switzerland). Dextromethorphan and dextrorphan, its O-demethylated metabolite, were obtained from Roche (Basle, Switzerland). Quinidine, quinine, cinchonine and cinchonidine hydrochloride were purchased from Sigma (Poole, Dorset, U.K.). All other chemicals were obtained commercially and were of the highest grade of purity.

Recombinant DNA manipulation and generation of mutant cDNA

Recombinant DNA procedures were performed with standard protocols as described by Sambrook et al. [27]. Three CYP2D6 cDNA species with point mutations at (1) bp 1442 (T → A) and 1443 (T → C), encoding a Phe^{481} → Tyr substitution, (2) bp 1443 (T → A), encoding a Phe^{481} → Leu substitution, and (3) bp 1141 (T → G), 1442 (T → G) and 1443 (T → C), encoding a Phe^{481} → Gly substitution, were generated with the Sculptor™ *in vitro* mutagenesis system (Amersham International, Little Chalfont, Bucks., U.K.). An additional CYP2D6 cDNA with a point mutation at bp 902 (A → G), encoding an Asp^{301} → Gly substitution, was prepared by the method of Kunkel et al. [28], as described previously [19]. The wild-type and mutated cDNA species were ligated into the *Bgl*II cloning site of the expression vector pMA91 as described previously [19]. All constructs were sequenced (Sequenase 2.0 kit; Amersham International) before and after subcloning in pMA91 to confirm the presence of the desired mutation(s) and the lack of any additional changes in the CYP2D6 sequence.

Yeast culture conditions and microsome preparation

Transformation of *S. cerevisiae* AH22 cells with the pMA91 constructs was by electroporation [29]. Co-transfection of AH22 cells with NADPH:P450 reductase cDNA was unnecessary because the yeast endogenous reductase was sufficient to effect metabolite formation. Transformants were grown for 42 h in batch culture in 1 litre of selective synthetic medium [0.67 % yeast minimal medium without amino acids/0.04 % histidine/3 % (w/v) glucose] at 200 rev./min and 30 °C. After being harvested by centrifugation, the cells were washed and resuspended in 20 ml of ice-cold microsomal buffer (0.1 M potassium phosphate buffer, pH 7.4, containing 0.65 M sorbitol and 0.1 mM EDTA). The yeast cells were disrupted mechanically with glass beads (0.45–0.50 mm diameter) at 4000 rev./min for 40 s with liquid CO_2 in an MSK Cell Homogeniser (B. Braun Medical Limited, Aylesbury, Bucks., U.K.). The microsomal fraction was prepared by ultracentrifugation of the homogenate; pellets were resuspended in microsomal buffer and stored at −80 °C before use. All of these procedures have been described in detail previously [14].

Immunoblotting and spectrophotometric measurements

The relative CYP2D6 apoprotein content of microsomes was estimated by SDS/PAGE [10 % (w/v) gel] and electrophoretic transfer to Hybond™ ECL® nitrocellulose membrane (Amersham International). Blots were probed with rabbit anti-human CYP2D6 polyclonal antibodies and revealed by enhanced chemiluminescence with a streptavidin–horseradish-peroxidase conjugate and luminol (Amersham International). The CYP2D6 holoprotein content of microsomes was quantified by carbon monoxide difference spectroscopy [30] and total protein was determined by the Folin reaction [31]. Binding constants (apparent K_s) were determined from ligand-induced difference spectra [32].

Assay of metoprolol, debrisoquine and dextromethorphan metabolites

The incubation conditions for the oxidation of *R*-(+)- and *S*-(−)-metoprolol (40 μM) and the kinetic analyses of *S*-(−)-metoprolol, debrisoquine and dextromethorphan oxidation over a substrate concentration range of 10–2000 μM were as described previously [14,33]. In brief, incubations were started by the addition of 10–50 pmol CYP2D6 microsomal preparation to substrates dissolved in 1.15 % (w/v) KCl and an NADPH-generating system comprising 0.4 μmol of NADP, 4 μmol of glucose 6-phosphate, 2 μmol of $MgCl_2$ and 0.4 unit of glucose-6-phosphate dehydrogenase dissolved in 0.2 M potassium phosphate buffer, pH 7.4. Incubations (0.5 ml final volume) were performed at 37 °C for 10 min in a shaking water bath. Preliminary experiments confirmed that the rates of formation of

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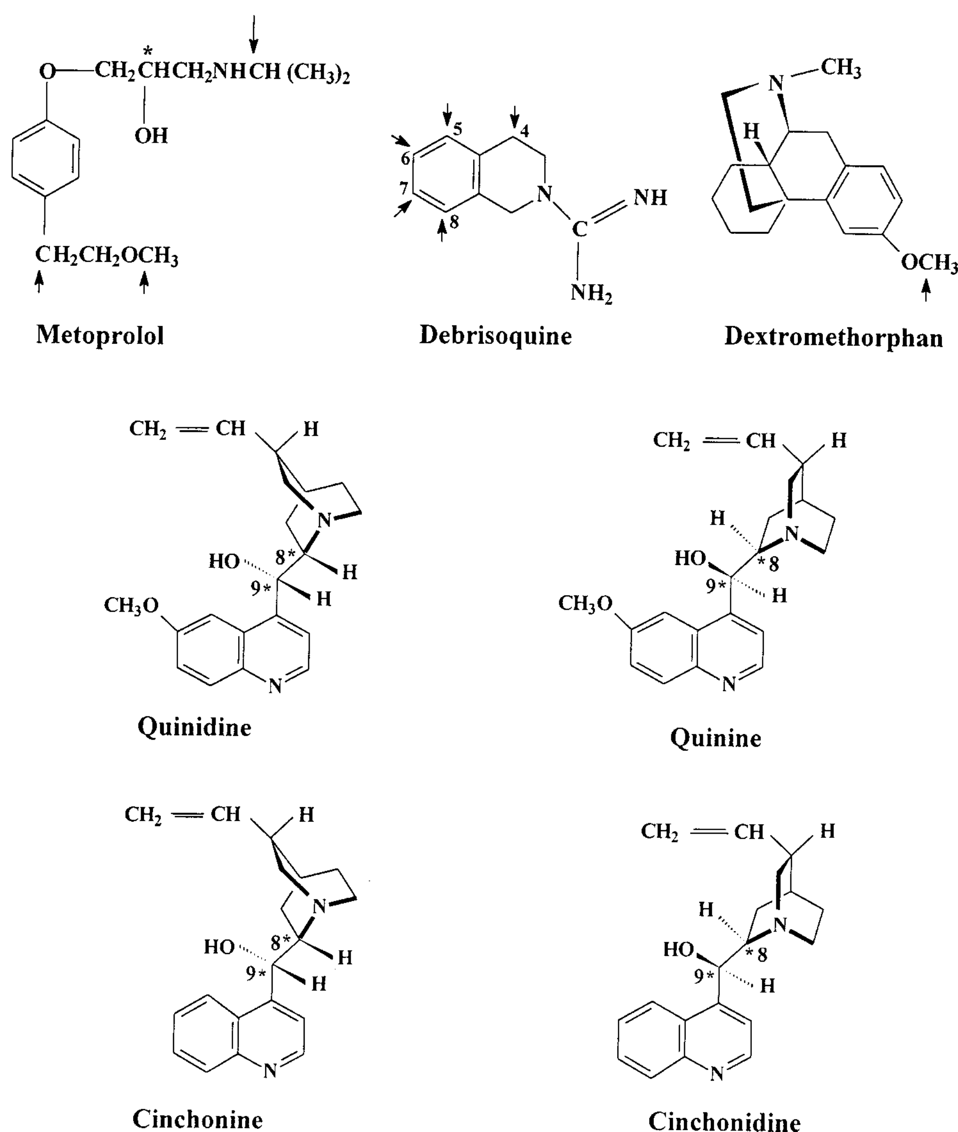

Figure 1 Chemical structures of the compounds studied, showing sites of oxidation (↑) and chiral centres (*)

products were linear under these conditions. Control incubations, containing the equivalent amount of microsomal protein derived from yeast cells transformed with plasmid lacking CYP2D6 cDNA, were conducted in parallel.

The O-demethyl, α-hydroxy and N-deisopropyl metabolites of metoprolol were assayed with a modification of the method of Otton et al. [34], as described previously [14]. The identity of the N-deisopropyl metabolite of metoprolol was confirmed by on-line HPLC–electrospray ionization MS [23]. 4-Hydroxydebrisoquine and dextrorphan were assayed by the methods of Lightfoot et al. [33] and Chen et al. [35] respectively.

Inhibition of CYP2D6 activity by quinine, quinidine, cinchonine and cinchonidine

The inhibition of racemic metoprolol O-demethylase activity by quinine, quinidine, cinchonine and cinchonidine (Figure 1) was evaluated by the method of Otton et al. [34]. IC_{50} values were determined at a substrate concentration of 40 μM over a range of inhibitor concentrations (0.001–100 μM). The inhibitors were not preincubated with the microsomes owing to the lability of the recombinant enzymes. Instead, the reactions were started by the addition of microsomes. Product (O-demethylmetoprolol) formation was determined after a 10 min incubation period.

N-dealkylation of metoprolol by Phe^{481} and Asp^{301} mutant forms of CYP2D6

The influence of $Phe^{481} \rightarrow Gly$, $Phe^{481} \rightarrow Tyr$, and $Asp^{301} \rightarrow Gly$ substitutions on the N-dealkylation of racemic metoprolol was investigated at low (40 μM) and high (400 μM) substrate concentrations under incubation conditions identical with those described earlier for the assay of the O-demethyl and α-hydroxy metabolites of metoprolol.

Data analysis

Data from kinetic experiments were analysed by Grafit, version 3 (Erithacus Software Ltd), an iterative weighted least-squares-fitting program. Kinetic parameters were treated statistically

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with ANOVA and the Tukey post hoc test for multiple parameters.

RESULTS

Expression of Phe[481] and Asp[301] mutant forms of CYP2D6

The relative CYP2D6 apoprotein contents, as determined by Western immunoblotting, of microsomes prepared from yeast cells expressing wild-type and Phe[481] and Asp[301] mutant forms of the enzyme were similar. A single immunodetectable band, corresponding to a molecular mass of 50 kDa, was observed with each microsomal preparation (results not shown). The P450 holoprotein content of the microsomes, as determined by CO difference spectroscopy, was also similar (wild-type, 52 pmol of P450/mg of microsomal protein; Phe[481] mutants, 30–63 pmol of P450/mg of microsomal protein; Asp[301] → Gly mutant, 40 pmol of P450/mg of microsomal protein). Each P450 spectrum exhibited a Soret absorption maximum at 448–450 nm and, with the exception of the Phe[481] → Leu mutant, no P420 peak was detectable.

Kinetics of oxidation of *S*-(−)-metoprolol, debrisoquine and dextromethorphan by Phe[481] mutants

Data on the influence of the Phe[481] substitutions on the kinetics of oxidation of *S*-(−)-metoprolol, debrisoquine and dextromethorphan are shown in Table 1. Replacement of the Phe[481] aromatic residue with the aliphatic residue leucine (Phe[481] → Leu) or glycine (Phe[481] → Gly) resulted in a significant ($P < 0.001$) increase in the K_m values for *S*-metoprolol O-demethylation (4–5-fold), debrisoquine 4-hydroxylation (3–4-fold) and dextromethorphan O-demethylation (10–16-fold) (Table 1). The influence of these substitutions on the V_{max} values was variable; the rates of metoprolol O-demethylation and debrisoquine 4-hydroxylation were slightly lower than wild-type values, whereas the V_{max} values for dextromethorphan O-demethylation were 2–4-fold greater than the wild-type value. In contrast with the non-conservative Phe[481] → Leu and Phe[481] → Gly substitutions, the substitution of Phe[481] with tyrosine (Phe[481] → Tyr), a polar residue retaining an aromatic moiety, had no significant effect on the kinetics of oxidation of the three substrates, the respective K_m and V_{max} values being similar to those obtained with the wild-type enzyme (Table 1). Accordingly, the specificity constant (V_{max}/K_m) values, a measure of intrinsic clearance *in vivo*, of the wild-type and Phe[481] → Tyr mutant enzymes were similar, whereas these values for the Phe[481] → Leu and Phe[481] → Gly mutant enzymes were 5–10-fold lower (Table 1).

Influence of Phe[481] mutants on product regioselectivity and substrate enantioselectivity

The regioselective oxidation (O-demethylation/α-hydroxylation ratio) of *R*-(+)- and *S*-(−)-metoprolol was not altered markedly by any of the Phe[481] substitutions; the O-demethylation reaction remained the preferred route of oxidation with each form of the enzyme (Table 2). With regard to substrate enantioselectivity (*R*-to-*S* ratio), the wild-type and Phe[481] → Tyr mutant showed a similar preference for the *R*-(+)-enantiomer with regard to the O-demethylation reaction (*R*-to-*S* ratio 1.5), whereas the Phe[481] → Leu mutant showed no enantioselectivity (*R*-to-*S* ratio 1.0) and the Phe[481] → Gly mutant showed a preference for the *S*-(−)-enantiomer (*R*-to-*S* ratio 0.7). With regard to the α-hydroxylation reaction, each form of the enzyme preferred the *S*-(−)-enantiomer of metoprolol, although this preference was slightly greater with the Phe[481] → Leu and Phe[481] → Gly mutants (*R*-to-*S* ratio 0.4) relative to the wild-type and the Phe[481] → Tyr mutant (*R*-to-*S* ratio 0.6) (Table 2).

Table 1 Estimates of the apparent Michaelis–Menten constants for *S*-metoprolol O-demethylation, debrisoquine 4-hydroxylation and dextromethorphan O-demethylation by microsomes prepared from yeast cells expressing wild-type, Phe[481] → Tyr, Phe[481] → Leu and Phe[481] → Gly mutant forms of CYP2D6

K_m (μM) and V_{max} (pmol of product/min per pmol of P450) values are the means ± S.D. from three experiments, each experiment comprising two replicates. V_{max}/K_m values (μl/min per nmol of P450 for metoprolol O-demethylation and debrisoquine hydroxylation, and μl/min per pmol of P450 for dextromethorphan O-demethylation) were derived from the mean K_m and V_{max} values.

	S-(−)-metoprolol O-demethylation			Debrisoquine 4-hydroxylation			Dextromethorphan O-demethylation		
Enzyme	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m
Wild-type	26 ± 8	3.48 ± 0.17	133.9	51 ± 16	3.20 ± 0.40	62.7	2 ± 0.3	8.16 ± 0.74	4.1
Phe[481] → Tyr	27 ± 10	4.60 ± 0.58	170.4	46 ± 9	2.46 ± 0.34	53.5	2 ± 0.2	8.43 ± 0.86	4.2
Phe[481] → Leu	120 ± 34	2.85 ± 0.66	23.8	152 ± 10	1.00 ± 0.52	6.6	31 ± 3	30.30 ± 3.61	1.0
Phe[481] → Gly	124 ± 5	2.32 ± 0.39	18.7	184 ± 49	1.17 ± 0.29	6.4	20 ± 2	18.35 ± 2.10	0.9

Table 2 Oxidation of *R*-(+)- and *S*-(−)-metoprolol (40 μM) by microsomes prepared from yeast cells expressing wild-type and Phe[481] mutant forms of CYP2D6

Results are means ± S.D. for four experiments, each comprising three replicates. Abbreviations: ODM, O-demethylation; αOH, α-hydroxylation; *R/S*, *R*-(+)- to *S*-(−)-metoprolol ratio.

	Catalytic activity (pmol of product/min per pmol of P450)							
	R-(+)-Metoprolol		*S*-(−)-Metoprolol		Regioselectivity (ODM/αOH)		Enantioselectivity (*R/S*)	
Enzyme	ODM	αOH	ODM	αOH	*R*-(+)-metoprolol	*S*-(−)-metoprolol	ODM	αOH
Wild-type	3.42 ± 0.27	0.57 ± 0.01	2.35 ± 0.16	0.97 ± 0.20	5.9 ± 0.6	2.5 ± 0.4	1.5 ± 0.2	0.6 ± 0.1
Phe[481] → Tyr	3.09 ± 0.72	0.40 ± 0.13	2.01 ± 0.49	0.67 ± 0.29	7.8 ± 0.8	3.2 ± 0.8	1.6 ± 0.1	0.6 ± 0.1
Phe[481] → Leu	0.49 ± 0.01	0.05 ± 0.02	0.50 ± 0.04	0.13 ± 0.03	10.7 ± 4.1	3.9 ± 0.6	1.0 ± 0.1	0.4 ± 0.1
Phe[481] → Gly	0.29 ± 0.03	0.06 ± 0.03	0.42 ± 0.06	0.16 ± 0.05	5.8 ± 2.6	2.8 ± 0.7	0.7 ± 0.1	0.4 ± 0.1

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Table 3 Type I binding coefficients (K_s) of known ligands of CYP2D6 determined with microsomes prepared from yeast cells expressing wild-type and Phe^{481} mutant forms of CYP2D6

Values are means for two determinations.

		K_s (μM)			
Enzyme	Ligand ...	*S*-(−)-Metoprolol	Debrisoquine	Dextromethorphan	Quinidine
Wild-type		10.9	8.9	3.1	0.10
Phe^{481} → Tyr		22.8	12.5	2.3	0.13
Phe^{481} → Leu		33.2	85	18.8	0.35
Phe^{481} → Gly		41.9	90	15.7	0.53

Table 4 N-deisopropylation and O-demethylation of racemic metoprolol (40 and 400 μM) by wild-type, Phe^{481} and Asp^{301} mutant forms of CYP2D6

Results are presented as pmol of product/min per pmol of P450 and are means ± S.D. for three experiments, each comprising three replicates.

		N-deiosopropylation (pmol/min per pmol)		O-demethylation (pmol/min per pmol)	
Enzyme	[Metaprolol] (μM) ...	40	400	40	400
Wild-type		1.12 ± 0.40	2.46 ± 1.13	1.85 ± 0.33	3.56 ± 1.01
Phe^{481} → Tyr		0.90 ± 0.26	1.85 ± 0.44	1.68 ± 0.11	3.03 ± 0.14
Phe^{481} → Gly		0.71 ± 0.26	2.17 ± 0.48	0.23 ± 0.02	0.80 ± 0.06
Asp^{301} → Gly		0.05 ± 0.03	0.15 ± 0.04	0.03 ± 0.01	0.22 ± 0.03

Influence of Phe^{481} mutations on ligand binding

The effect of the Phe^{481} substitutions on ligand binding, as assessed by K_s determinations, is shown in Table 3. The Phe^{481} → Tyr substitution did not alter the binding capacity of any of the tested ligands; in contrast, the Phe^{481} → Leu and Phe^{481} → Gly substitutions decreased the binding of *S*-(−)-metoprolol 3-fold, that of debrisoquine 10-fold and that of dextromethorphan 5-fold. The binding of quinidine, a potent inhibitor of CYP2D6, to the Phe^{481} → Leu and Phe^{481} → Gly mutants was decreased 3–5-fold relative to the wild-type and the Phe^{481} → Tyr mutant (Table 3).

Influence of Phe^{481} → Gly substitution on the potency of quinine/quinidine inhibition

The Phe^{481} → Gly substitution (other Phe^{481} mutants were not tested) did not alter the inhibitory potency of quinine or quinidine on CYP2D6-mediated metoprolol O-demethylation. Thus quinidine (IC_{50} 0.1 μM) was 100-fold more potent than quinine (IC_{50} 10 μM) in inhibiting the activity of the wild-type and mutant enzymes (Figure 2). Furthermore, cinchonine and cinchonidine, the respective desmethoxy analogues of quinidine and quinine (Figure 1), each had a inhibitory potency similar to that of quinine (10 μM); this was not altered by the Phe^{481} → Gly substitution (Figure 2).

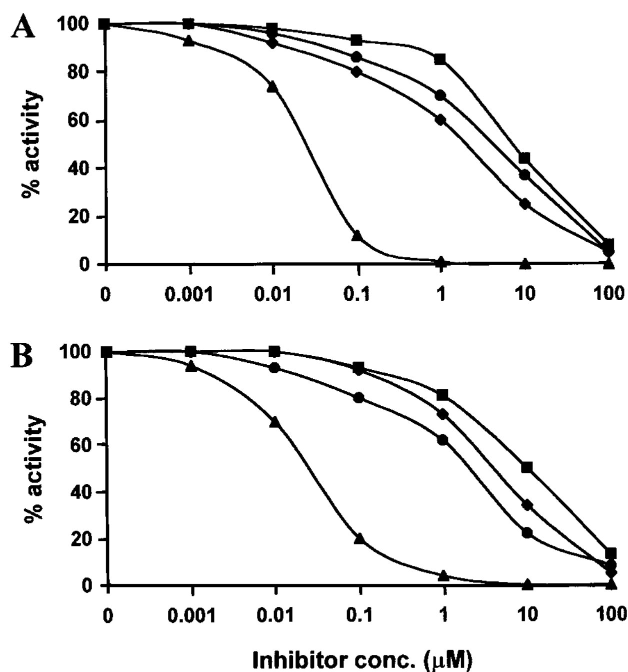

Figure 2 Inhibition of wild-type (A) and Phe^{481} → Gly mutant (B) metoprolol O-demethylase activity by quinidine (▲), quinine (●), cinchonine (◆) and cinchonidine (■)

IC_{50} values for quinidine of approx. 0.1 μM and for quinine, cinchonine and cinchonidine of approx. 10 μM were obtained with both the wild-type enzyme and Phe^{481} → Gly mutant enzyme.

Influence of Phe^{481} substitutions on the N-dealkylation of metoprolol

The rate of N-deisopropylation of racemic metoprolol by the Phe^{481} → Gly mutant, both at low (40 μM) and high (400 μM) substrate concentrations, was not markedly different from that observed with the wild-type and Phe^{481} → Tyr mutant forms of CYP2D6. In contrast, the O-demethylation reaction was substantially decreased (80–90 %) by the Phe^{481} → Gly substitution. However, the Asp^{301} → Gly substitution decreased the rate of both the N-dealkylation and O-dealkylation routes of metabolism by 94–99 % of wild-type activities (Table 4).

DISCUSSION

The purpose of this study was to confirm the importance of Phe^{481} as a ligand-binding residue in the active site of CYP2D6. The enzyme kinetic data show clearly that replacing Phe^{481} with a non-aromatic residue, such as leucine (Phe^{481} → Leu) or glycine (Phe^{481} → Gly), significantly increased the K_m values of the enzyme with regard to the oxidation of metoprolol, debrisoquine and dextromethorphan, three probe substrates of CYP2D6. In contrast, replacing Phe^{481} with tyrosine (Phe^{481} → Tyr), a conservative substitution, did not alter the K_m values of oxidation of any of the substrates. Although K_m is not necessarily a measure of the affinity of the enzyme for the substrate, the ligand binding data (K_s values) confirmed that the binding of debrisoquine and dextromethorphan, and, to a smaller extent, *S*-metoprolol, were decreased by the Phe^{481} → Leu and Phe^{481} → Gly substitutions. Again, there was no significant difference in the binding capacity of the substrates to the Phe^{481} → Tyr mutant. Overall, these results support the proposition that Phe^{481}, a residue identified as a putative CYP2D6 active-site residue by a number of homology models [6,14,18], is a ligand-binding residue, and that the likely contact is via a π–π interaction, in either a co-planar or orthogonal manner, between the phenyl ring of the residue and the aromatic moiety of the ligand.

The extent of the role of Phe^{481} in binding seems to be dependent on the ligand. Of the ligands tested, debrisoquine, a relatively planar, rigid molecule possessing a guanidinium moiety

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and a single aromatic moiety adjacent to an alicyclic ring (Figure 1), exhibited the greatest increase in K_m and K_s values when Phe[481] was replaced with Leu or Gly. This might reflect the possibility that the binding of debrisoquine in the active site of CYP2D6 is due predominantly to an interaction with two residues only, namely Asp[301], through an ionic interaction with the substrate's guanidinium basic nitrogen [19], and Phe[481], via a π–π interaction with the aromatic ring of the substrate. However, the binding to Asp[301] seems to be the major interaction [19], the π–π interaction with Phe[481] providing an additional stabilizing interaction. In contrast, the binding of *S*-metoprolol or quinidine, the latter being a potent specific inhibitor of CYP2D6, was not as markedly altered by the Phe[481] → Leu or Phe[481] → Gly substitutions, suggesting that these ligands might interact with one or more other unidentified residues in the active site, in addition to Asp[301] and Phe[481]. These ligands possess a hydroxy group that is potentially capable of hydrogen-bonding with an active-site residue(s), although it has recently been shown that serine 304, a putative active-site residue, is not involved in such an interaction [23]. Interestingly, however, the observation that cinchonine, the desmethoxy analogue of quinidine, was 100-fold less potent than quinidine in inhibiting CYP2D6 activity, but similar in potency to quinine (IC_{50}, 10 μM), suggests that the methoxy group of quinidine might enhance the inhibitory potency of this ligand by fitting into a cleft in the active site. The interaction between the methoxy group and cleft could be either hydrophobic or hydrophilic, the lone pair of electrons of the methoxy oxygen atom being capable of hydrogen-bonding with active-site residue(s) that form the cleft. The presence of such a cleft in the active site of CYP2D6 has not been considered by published homology models, although the presence of a cleft was suggested in an early pharmacophore model of CYP2D6 inhibitors [36]. The confirmation, through homology modelling, of the occurrence of a cleft in the active site of CYP2D6, a cleft that might accommodate the methoxy group of quinidine, could be a fruitful area of investigation, particularly in relation to defining the factors that determine the potency of CYP2D6 inhibitors.

A recent combined homology/pharmacophore model of CYP2D6 has implicated the involvement of Phe[481] in the less common N-dealkylation route of metabolism mediated by this enzyme [25]. The model specifically implies that Phe[481] is the primary substrate-contact residue in the active site that determines the N-dealkylation route of oxidation and furthermore that binding to Asp[301] is not involved. However, the results presented here refute this proposal unambiguously; the replacement of Phe[481] with glycine (Phe[481] → Gly) had only a modest effect on the rate of the N-deisopropylation of metoprolol, whereas the Asp[301] → Gly substitution abolished the reaction almost completely, in an identical manner to the O-demethylation (and α-hydroxylation; results not shown) route of metabolism. Accordingly, homology models that predict a dominant role for Phe[481] in the binding of substrates that undergo N-dealkylation by CYP2D6 need to be re-evaluated.

Although the results presented here support a role for Phe[481] as a ligand-contact residue, other models [16] propose that Phe[483], not Phe[481], is associated with ligand binding. However, the few published results on the influence of Phe[483] substitutions on CYP2D6 activity do not support this proposal; replacement of Phe[483] with isoleucine, a non-aromatic residue, did not alter the kinetics of 1-hydroxylation of bufuralol, a classical probe substrate of CYP2D6 activity [37]. Nevertheless, the Phe[483] → Ile substitution did alter the substrate specificity of CYP2D6, enabling the mutated enzyme to metabolize testosterone, a non-CYP2D6 substrate [37]. Smith et al. [37] suggested that the altered substrate specificity is a consequence of the greater access of bulky substrates into the active site of the mutant enzyme. However, whereas these results suggest that Phe[483] might be located in the active-site cavity, the role of this residue in the binding and orientation of typical CYP2D6 substrates needs to be investigated further.

In summary, our results confirm the proposal of homology models that Phe[481] is an active-site residue of CYP2D6 and that the residue interacts with ligands via a π–π interaction between its phenyl ring and the aromatic moiety of the ligand. However, the relative importance of Phe[481] in binding is dependent on the ligand; furthermore, its importance is secondary to that of Asp[301]. Finally, contrary to predictions of a recent homology model [25], Phe[481] does not seem to have a dominant role in N-dealkylation routes of metabolism by CYP2D6.

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