Evidence that serine 304 is not a key ligand-binding residue in the active site of cytochrome P450 2D6

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Homology models of cytochrome P450 2D6 (CYP2D6) have identified serine 304 as an active-site residue and implicated a putative role for this residue in substrate enantioselectivity and the differential inhibition of enzyme activity by the diastereoisomers quinine and quinidine. The role of serine 304 in selectivity is thought to be achieved through a preferential hydrogen-bond interaction between the hydroxyl group of the residue and one of the stereoisomers of each ligand. We have tested this hypothesis by substituting serine 304 with alanine, a non-hydrogen-bonding residue, and compared the properties of the wild-type and mutant enzymes in microsomes prepared from yeast cells expressing the appropriate cDNA-derived enzyme. The Ser³⁰⁴Ala substitution did not alter the enantioselective oxidation of metoprolol; the O-demethylation reaction remained *R*-(+)-enantioselective (wild-type, *R*/*S*, 1.7; mutant, *R*/*S*, 1.6), whereas α -hydroxylation remained *S*-(-)-enantioselective (wild-type and mutant, *R*/*S*, 0.7). Similarly, the selective oxidation of the *R*-(+) and *S*-(-) enantiomers of propranolol to the major 4-hydroxy metabolite was identical with both wild-type and mutant forms of the enzyme (*R*/*S* 0.9), although the formation of minor metabolites (5-hydroxy and deisopropylpropranolol) did show some slight alteration in enantioselectivity. The differential inhibition of enzyme activity by quinine and quinidine was also identical with both forms of CYP2D6, the IC₅₀ values for each enzyme being approx. 10 μ M and 0.1 μ M for quinine and quinidine, respec-

tively. The kinetics of formation of α -hydroxymetoprolol and 4-hydroxydebrisoquine by wild-type and the Ser³⁰⁴Ala mutant was also very similar. However, modest changes in the regioselective oxidation of metoprolol and debrisoquine were observed with the Ser³⁰⁴Ala mutant. The regio- and enantioselective oxidation of an analogue of metoprolol, in which the hydroxyl group attached to the chiral carbon was replaced by a methyl moiety, was again identical with both wild-type and Ser³⁰⁴Ala mutant. However, the observed selectivity was the reverse of that observed with metoprolol. Collectively, these data indicate that Ser³⁰⁴ is unlikely to be a key ligand-binding residue, although the residue may indeed be located in the active-site cavity. The reversal of selectivity with the methyl analogue of metoprolol indicates that the hydroxyl group attached to the chiral centre of ligands, such as metoprolol, is important in defining the enzyme's selective properties, and that a hydrogen-bonding residue, other than Ser³⁰⁴, may be involved in this interaction. Current homology models of the active site of CYP2D6 that predict a hydrogen-bond interaction between Ser³⁰⁴ and specific ligands will need to be re-evaluated, and other candidate residues capable of such an interaction nominated and tested by site-directed mutagenesis studies.

Key words: enantioselectivity, hydrogen bonding, metoprolol, quinidine, quinine.

INTRODUCTION

Cytochrome P450 2D6 (CYP2D6) is a polymorphic human drug-metabolizing enzyme responsible for the oxidation of many commonly prescribed drugs including cardiac anti-arrhythmics, β -adrenergic antagonists, anti-depressants, neuroleptics and analgesics [1]. Because of its prominent role in human drug metabolism and the implications of the genetic polymorphism for clinical drug use, there is considerable interest within academia and the pharmaceutical industry to develop a predictive model of the active site of CYP2D6. However, the absence of a crystal structure of a eukaryotic cytochrome P450 (P450), let alone a human drug-metabolizing P450, makes the development of a predictive model problematic owing to the lack of accurate structural information. Nevertheless, through the use of multiple sequence alignment and homology modelling with reference to known three-dimensional structures of a limited number of

prokaryotic P450s [2–5], the delineation of the active site of some of the human P450s ([6], and references therein), including CYP2D6 [7–11], has been attempted. However, such computer-derived homology models need to be validated experimentally using a variety of approaches, such as pharmacophore docking, chemical probe analysis, antibody-binding-site studies and site-directed mutagenesis.

Using the technique of site-directed mutagenesis in conjunction with heterologous expression in yeast, we have previously identified Asp³⁰¹, indicated initially by homology modelling [7,12,13], as a key ligand-contact residue in the active site of CYP2D6 [14]. This negatively charged residue is believed to ion-pair with the basic nitrogen of CYP2D6 substrates and inhibitors, and facilitates the binding and orientation of ligands in the active site [14]. Whereas all known CYP2D6 ligands possess a basic nitrogen [15], a number of substrates (e.g. β -blockers such as metoprolol and propranolol) and inhibitors (quinidine and quinine; Figure

Abbreviations used: CYP2D6, cytochrome P450 2D6; P450, cytochrome P450; HD, hydroxydebrisoquine; ODM, O-demethylation; α OH, α -hydroxylation; MAM, methyl analogue of metoprolol.

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

1) also possess a hydroxyl group, which could provide a second important interaction by donating or accepting a hydrogen bond to or from an active-site residue. In this context, most homology models of CYP2D6 locate Ser³⁰⁴, a neutral, polar residue capable of hydrogen bonding, in the active site of the enzyme [7–11]. Furthermore, the position and orientation of Ser³⁰⁴, relative to Asp³⁰¹, in the central region of the spatially conserved I-helix of the P450 core [16], is compatible with the atomic distance between the hydroxyl group and the basic nitrogen of ligands such as metoprolol and quinidine. The hydroxyl group of these ligands is also attached to a chiral carbon centre (Figure 1). Consequently, the modest enantioselectivity exhibited by CYP2D6 towards substrates such as metoprolol, propranolol and bunitrolol [14,17,18] could be due to a more favourable hydrogen-bond interaction between Ser³⁰⁴ and the hydroxyl group of one of the enantiomers [8,18]. Similarly, it has been proposed that the differential potency in inhibition of CYP2D6 activity by the diastereoisomers quinidine and quinine [17] is due to the orientation of the hydroxyl group attached to a chiral carbon atom (C-9) of each inhibitor (Figure 1) which, in the case of quinidine, would selectively facilitate hydrogen bonding with Ser³⁰⁴, but which is sterically less favourable with quinine [11,13].

We have investigated, through site-directed mutagenesis and heterologous yeast expression, the structural role of Ser³⁰⁴ as a ligand-binding residue in the active site of CYP2D6 by determining its influence on the oxidation of three probe substrates, debrisoquine, metoprolol and propranolol. Specifically, the effect of substituting Ser³⁰⁴ with alanine (Ser³⁰⁴Ala), a non-polar

residue, on ligand binding, catalytic activity, substrate enantioselectivity and/or product regioselectivity, alone and in combination with an Asp³⁰¹Gly substitution, was investigated. The influence of the Ser³⁰⁴Ala substitution on the metabolism of an analogue of metoprolol, in which the hydroxyl group attached to the chiral carbon centre was replaced with a methyl group, was also analysed. The effect of the Ser³⁰⁴Ala substitution on the differential potency of inhibition of CYP2D6 by quinidine and quinine was also studied.

EXPERIMENTAL

Materials

Escherichia coli strains DH5 α , CJ236 and TG1 and *Saccharomyces cerevisiae* strain AH22 have been described elsewhere [14,19]. Human CYP2D6 cDNA representing the wild-type CYP2D6*1 sequence [20], and the yeast expression vector pMA91 have been reported previously [14]. Debrisoquine hemisulphate and its 4-hydroxy metabolite [4-hydroxydebrisoquine (4-HD)] were gifts from Roche Products (Welwyn Garden City, Herts, U.K.). Authentic standards of 5-, 6-, 7- and 8-HD metabolites were provided generously by Professor R. L. Smith, Department of Pharmacology and Toxicology, Imperial College School of Medicine, London, U.K. Metoprolol tartrate (racemate), and *O*-demethylmetoprolol and α -hydroxymetoprolol hydroxybenzoate metabolites were gifts from Astra Hassle (Mölndal, Sweden). The hydrochloride salts of *R*-(+)- and *S*-(-)-metoprolol were gifts from Ciba Geigy (Basle, Switzerland). The hydrochloride

Figure 2 Synthesis of MAM

*, Refers to the chiral centre. See text for details.

salts of *R*-(+)- and *S*-(-)-propranolol and the 4-hydroxy (4-hydroxypropranolol), 5-hydroxy (5-hydroxypropranolol) and *N*-deisopropyl (*N*-deisopropylpropranolol) metabolites were obtained from Zeneca Pharmaceuticals (Macclesfield, Cheshire, U.K.). Quinidine and quinine sulphate were obtained from Sigma (Poole, Dorset, U.K.). All other chemicals were obtained commercially and were of the highest grade of purity.

Synthesis of the methyl analogue of metoprolol (MAM)

Oxalate salts of the (*R*)-(-) and (*S*)-(+) enantiomers of MAM, (*R*)-(-) and (*S*)-(+)-3-(*N*-isopropylamino)-2-methyl-1-[4-(2-methoxyethyl)phenoxy]propane (Figure 1), were synthesized as shown in Figure 2. 4-(2-Methoxyethyl)-phenol (1.5 g, 9.87 mmol) was dissolved in acetonitrile (20 ml) at room temperature and (*R*)-(-)-3-bromo-2-methyl-1-propanol or (*S*)-(+)-3-bromo-2-methyl-1-propanol (1.24 ml, 11.84 mmol; Figure 2) were added to this solution with K₂CO₃ (2.72 g, 19.72 mmol). The mixture was refluxed for 48 h, filtered and the solvent evaporated *in vacuo*. The residue was purified by column chromatography (toluene/ethyl acetate, 8:1, v/v; yield, 1.7 g, 7.73 mmol, 78 %). The alcohol product, (*R*)-(-) or (*S*)-(+)-3-[4-(2-methoxyethyl)phenoxy]-2-methylpropanol (890 mg, 3.97 mmol; compound 1, Figure 2), was dissolved in dry pyridine (20 ml) and treated with triphenylphosphine (2.08 g, 7.94 mmol) and CBr₄ (1.34 g, 4.05 mmol) at room temperature overnight. The solvent was evaporated, and the residue co-evaporated twice with toluene and purified by column chromatography (toluene/ethyl acetate, 20:1, v/v; yield, 898 mg, 3.12 mmol, 79 %).

The bromoether product, (*R*)-(-) or (*S*)-(+)-3-bromo-2-methyl-1-[4-(2-methoxyethyl)phenoxy]propane (360 mg, 1.25 mmol; compound 2, Figure 2), was dissolved in dry acetonitrile (10 ml) and stirred at room temperature. *N*-Isopropylbenzylamine (1.05 ml, 6.27 mmol) was added to this solution and, after refluxing overnight, the yellowish mixture was cooled at room temperature and the solvent evaporated *in vacuo*. The residue was redissolved in ether and twice extracted with HCl (2 M, 10 ml). The aqueous layers were combined and basified with solid sodium carbonate. The mixture was extracted with ether (3 × 15 ml), the combined ether layers dried over MgSO₄ and filtered. Solvent was evaporated and the colourless oil was purified by column chromatography (toluene/ethyl acetate, 20:1, v/v; yield, 402 mg, 1.1 mmol, 88 %). The amine product, (*R*)-(-) or (*S*)-(+)-3-(*N*-benzyl-*N*-isopropylamino)-2-methyl-1-[4-(2-methoxyethyl)phenoxy]propane (161 mg, 0.45 mmol; compound 3, Figure 2) was dissolved in ethanol, palladium hydroxide (45 mg, 20 %, w/w) adsorbed on carbon added and the resulting mixture stirred at room temperature under hydrogen overnight. The mixture was filtered through Celite and the solvent evaporated. The resulting colourless oil was co-evaporated three times with dichloromethane and required no further purification (yield, 118 mg, 0.44 mmol, 98 %).

Oxalate salts of the final product, (*R*)-(-) or (*S*)-(+)-3-(*N*-isopropyl-amino)-2-methyl-1-[4-(2-methoxyethyl)phenoxy]propane (MAM; compound 4, Figure 2), were synthesized by dissolving the oil in dry ether and a saturated solution of oxalic acid in dry ether added dropwise to give a precipitate, which was filtered and dried under vacuum (yield, 139 mg, 0.36 mmol, 81 %). The identity of each intermediate in the synthesis of each enantiomer of MAM was verified by NMR (¹H and ¹³C) and high-resolution MS analyses.

Recombinant DNA manipulation and generation of mutant cDNA

Recombinant DNA procedures were performed according to standard protocols, as described by Sambrook et al. [19]. A

CYP2D6 cDNA with a point mutation T → G at bp 910, encoding a protein with a Ser³⁰⁴Ala substitution, was generated by site-directed mutagenesis using the *dut⁻ ung⁻* *E. coli* strain CJ236 according to the method of Kunkel et al. [21]. Two additional CYP2D6 cDNAs, one with point mutations A → G and T → G at bp 902 and 910, respectively, and the other with point mutations G → T, A → C, T → G and C → A at bp 901, 902, 910 and 911, respectively, were generated using the Sculptor[™] *in vitro* mutagenesis system (Amersham, Little Chalfont, Bucks, U.K.). These latter cDNAs encoded CYP2D6 proteins with Asp³⁰¹Gly/Ser³⁰⁴Ala and Asp³⁰¹Ser/Ser³⁰⁴Asp double substitutions, respectively. The wild-type and mutated cDNAs were ligated into the *Bgl*/II cloning site of the expression vector pMA91 as described previously [14]. All constructs were sequenced (Sequenase 2.0 kit, Amersham) before and after sub-cloning 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 microsomal preparation

S. cerevisiae AH22 cells were transformed with the pMA91 constructs by electroporation [22]. Co-transfection of AH22 cells with NADPH-P450 reductase cDNA was unnecessary since the yeast's endogenous reductase was sufficient to effect metabolite formation. Transformants were grown in batch culture in 1 litre of selective synthetic medium [0.67 % (w/v) yeast minimal medium without amino acids/0.04 % (w/v) histidine/3 % (w/v) glucose] at 200 revs./min and 30 °C for 42 h. Cells were harvested by centrifugation, washed and resuspended in 20 ml of ice-cold 0.1 M potassium phosphate buffer, pH 7.4, containing 0.65 M sorbitol and 0.1 mM EDTA (microsomal buffer) and disrupted mechanically with an MSK Cell Homogeniser (B. Braun Medical Limited, Aylesbury, Bucks, U.K.). The microsomal fraction was prepared by centrifugation of the homogenate and pellets resuspended in microsomal buffer and stored at -80 °C prior to use. All these procedures have been described in detail previously [8].

Immunoblotting and spectrophotometric measurements

The relative CYP2D6 apoprotein content of microsomes was estimated by separation on SDS/polyacrylamide (10 %) gel and electrophoretic transfer on to Hybond[™] ECL nitrocellulose membrane (Amersham). Blots were probed with rabbit anti-human CYP2D6 polyclonal antibodies and visualized by ECL using a streptavidin-horseradish peroxidase conjugate and luminol (Amersham). CYP2D6 holoprotein content of microsomes was quantified by carbon monoxide-difference spectroscopy [23] and total protein determined by the Folin reaction [24]. Binding constants (apparent *K_s*) were determined from ligand-induced difference spectra [25].

Assay of metoprolol, metoprolol analogue, propranolol and debrisoquine metabolites

The incubation conditions for the oxidation of metoprolol [40 μM racemate, 40 μM *R*-(+) or *S*-(-) enantiomers], MAM [40 μM *R*-(-) or *S*-(+) enantiomers], propranolol [10 μM *R*-(+) or *S*-(-) enantiomers] and debrisoquine (20 μM) at single-substrate concentrations, and the kinetic analyses of rac-metoprolol and debrisoquine oxidation over a substrate concentration range of 10–2000 μM were as described previously [8,26,27]. Briefly, incubations were initiated by the addition of 10–50 pmol of CYP2D6 microsomal preparation to substrates dissolved in 1.15 % (w/v) KCl and an NADPH-generating

system comprising 0.4 μ mol of NADP, 4 μ mol of glucose 6-phosphate, 2 μ mol of MgCl_2 and 0.4 unit of glucose-6-phosphate dehydrogenase dissolved in 0.2 M potassium phosphate buffer (pH 7.4). Incubations were carried out at 37 °C for 10 min in a shaking water bath. Preliminary experiments confirmed that the rates of formation of 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 and α -hydroxy metabolites of metoprolol and its methyl analogue (MAM) were assayed according to a modification of the method of Otton et al. [17] as described previously [8]. The metabolites of propranolol and debrisoquine were assayed as described previously [26,27]. The identities of the *O*-demethyl and α -hydroxy metabolites of MAM were confirmed by on-line HPLC-electrospray ionization MS. A Varian 9012 solvent-delivery system (Varian, Walton-on-Thames, Oxon, U.K.) coupled to a VG Quattro BQ tandem quadrupole mass spectrometer (VG Organic, Manchester, U.K.) fitted with an atmospheric-pressure ionization electrospray source was used. Sample injection was by a Rheodyne 7125 injector (Cotati, CA, U.S.A.) fitted with a 200- μ l loop. The separation was carried out on a 5- μ m particle size Hypersil BDS C_{18} reversed-phase column (250 mm $\times$ 4.6 mm inner diameter) from Hypersil (Runcorn, Cheshire, U.K.) with 0.1% (w/v) trifluoroacetic acid (A) and acetonitrile (B) as the gradient mobile-phase mixture. Elution was initially isocratic at 10% B/90% A for 20 min, followed by a linear-gradient elution from 10% to 40% B for a further 20 min. The flow rate was 1 ml/min. The HPLC effluent was split in the ratio of 1:6 before entering the mass spectrometer. The electron spray ionization capillary and high-voltage electrode potentials were 0.38 and 3.78 kV, respectively. The source temperature was 150 °C and the cone and lens 2 voltages were set at 80–85 V. All spectra were acquired in the positive-ion mode, over a mass range of m/z 150–500, at a rate of one scan every 2 s. The peaks identified from mass chromatograms were confirmed as α -hydroxy-MAM (m/z 282; 31 min), *O*-demethyl-MAM (m/z 252; 32 min) and MAM (m/z 266; 40 min).

RESULTS

Influence of Ser³⁰⁴Ala, Asp³⁰¹Gly/Ser³⁰⁴Ala and Asp³⁰¹Ser/Ser³⁰⁴Asp substitutions on the expression and stability of CYP2D6

Yeast cells transformed with wild-type, Ser³⁰⁴Ala mutant, Asp³⁰¹Gly/Ser³⁰⁴Ala or Asp³⁰¹Ser/Ser³⁰⁴Asp double mutant forms of

Figure 3 Regiospecific hydroxylation of debrisoquine (20 μ M) by wild-type CYP2D6 and the Ser³⁰⁴Ala mutant

Values are the means $\pm$ S.D. from three experiments, each comprising three replicates.

CYP2D6 cDNA expressed similar amounts of CYP2D6 apo-protein, as determined by Western immunoblotting (results not shown). The immunoreactive proteins migrated with apparent molecular masses consistent with the known size of CYP2D6 (50 kDa) and coincident with native CYP2D6 in microsomes prepared from the human liver of an extensive metabolizer with respect to CYP2D6 activity. Similar quantities of CYP2D6 holoprotein, quantified by carbon monoxide-difference spectroscopy, were detected in microsomes prepared from yeast cells expressing the wild-type [52 ± 11 pmol/mg of protein (mean $\pm$ S.D.), $n = 6$] and the Ser³⁰⁴Ala mutant (48 ± 11 , $n = 6$). The holoprotein content of microsomes prepared from the Asp³⁰¹Gly/Ser³⁰⁴Ala double mutant (20 ± 8 , $n = 6$) was significantly lower than of microsomes prepared from the wild-type and the Ser³⁰⁴Ala mutant ($P < 0.05$). Moreover, the Soret absorption maximum observed with this double mutant was slightly shifted from 448 to 450 nm and a shoulder was apparent in the spectrum at 420 nm, indicative of the presence of P420, the inactive form of P450. No holoprotein was detected in microsomes prepared from yeast cells transformed with the Asp³⁰¹Ser/Ser³⁰⁴Asp double mutant, although a peak at 420 nm was apparent (results not shown).

Table 1 Estimates of the apparent Michaelis–Menten constants for debrisoquine 4-hydroxylation and metoprolol α OH by microsomes prepared from yeast cells expressing wild-type, Ser³⁰⁴Ala, Asp³⁰¹Gly and Asp³⁰¹Gly/Ser³⁰⁴Ala mutant forms of CYP2D6

K_m (μ M) and V_{max} (pmol of product/min per pmol of P450) values are the means $\pm$ S.D. from three (or two, *) experiments, each experiment comprising three replicates. V_{max}/K_m (μ l/min per nmol of P450) values are derived from the mean K_m and V_{max} values.

	Debrisoquine 4-hydroxylation			Metoprolol α OH		
	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m
Wild-type	15 $\pm$ 3	1.48 $\pm$ 0.15	98.6	30 $\pm$ 0.3	1.47 $\pm$ 0.16	49.0
Ser ³⁰⁴ Ala	19 $\pm$ 3	1.28 $\pm$ 0.07	67.4	18 $\pm$ 4	0.85 $\pm$ 0.16	47.2
Asp ³⁰¹ Gly	229 $\pm$ 77	0.49 $\pm$ 0.07	2.1	884 $\pm$ 47	0.21 $\pm$ 0.03	0.3
Asp ³⁰¹ Gly/Ser ³⁰⁴ Ala	2175 $\pm$ 290	0.34 $\pm$ 0.11	0.2	2346*	0.95*	0.4

Table 2 Oxidation of *R*-(+)- and *S*-(-)-metoprolol (40 μ M) and *R*-(+)- and *S*-(-)-MAM (40 μ M) by microsomes prepared from yeast cells expressing wild-type and Ser³⁰⁴Ala mutant forms of CYP2D6

The values shown represent the means $\pm$ S.D. from three separate experiments, each experiment comprising three replicates. Absolute rates of formation of the metabolites of MAM could not be determined due to the unavailability of standards. For direct comparison, regio- and enantio-selectivity values are based on metabolite to internal standard (guanosine) peak height ratios for both metoprolol and MAM.

	Catalytic activity (pmol of product/min per pmol of P450)				Selectivity			
	<i>R</i> -(+)-		<i>S</i> -(-)-		Regio- (ODM/ α OH)		Enantio- [<i>R</i> -(+)-/ <i>S</i> -(-)-]	
	ODM	α OH	ODM	α OH	<i>R</i> -(+)-	<i>S</i> -(-)-	ODM	α OH
Metoprolol								
Wild-type	2.24 $\pm$ 0.20	0.40 $\pm$ 0.03	1.50 $\pm$ 0.18	0.57 $\pm$ 0.07	5.4 $\pm$ 0.2	2.4 $\pm$ 0.2	1.7 $\pm$ 0.2	0.7 $\pm$ 0.2
Ser ³⁰⁴ Ala	3.15 $\pm$ 0.43	0.38 $\pm$ 0.04	1.93 $\pm$ 0.29	0.53 $\pm$ 0.07	8.1 $\pm$ 0.2	3.5 $\pm$ 0.3	1.6 $\pm$ 0.3	0.7 $\pm$ 0.1
MAM								
Wild-type	—	—	—	—	2.5 $\pm$ 0.1	4.9 $\pm$ 0.1	0.8 $\pm$ 0.1	1.5 $\pm$ 0.1
Ser ³⁰⁴ Ala	—	—	—	—	4.5 $\pm$ 0.1	7.4 $\pm$ 0.2	0.9 $\pm$ 0.2	1.6 $\pm$ 0.1

Table 3 Oxidation of *R*-(+)- and *S*-(-)-propranolol (10 μ M) by microsomes prepared from yeast cells expressing wild-type and Ser³⁰⁴Ala mutant form of CYP2D6

Values represent the means $\pm$ S.D. from four experiments, each experiment comprising three replicates. Values in parentheses represent relative rates of formation of metabolites as a percentage of the total. 4-HP, 4-hydroxypropranolol; 5-HP, 5-hydroxypropranolol; DIP, *N*-deisopropylpropranolol.

	Catalytic activity (pmol of product/min per pmol of P450)						Enantioselectivity [<i>R</i> -(+)-/ <i>S</i> -(-)-]		
	<i>R</i> -(+)-			<i>S</i> -(-)-					
	4-HP	5-HP	DIP	4-HP	5-HP	DIP	4-HP	5-HP	DIP
Wild-type	3.42 $\pm$ 0.30 (87%)	0.43 $\pm$ 0.03 (11%)	0.10 $\pm$ 0.01 (3%)	4.03 $\pm$ 0.35 (84%)	0.53 $\pm$ 0.06 (11%)	0.23 $\pm$ 0.02 (5%)	0.9 $\pm$ 0.1	0.8 $\pm$ 0.1	0.5 $\pm$ 0.1
Ser ³⁰⁴ Ala	2.31 $\pm$ 0.12 (82%)	0.35 $\pm$ 0.01 (12%)	0.15 $\pm$ 0.01 (5%)	2.60 $\pm$ 0.08 (85%)	0.25 $\pm$ 0.01 (8%)	0.19 $\pm$ 0.01 (6%)	0.9 $\pm$ 0.1	1.4 $\pm$ 0.1	0.8 $\pm$ 0.1

Influence of Ser³⁰⁴Ala and Asp³⁰¹Gly/Ser³⁰⁴Ala substitutions on the kinetics of debrisoquine 4-hydroxylation and metoprolol α -hydroxylation (α OH)

The Ser³⁰⁴Ala substitution did not markedly alter the apparent Michaelis–Menten constants (K_m and V_{max}) for the 4-hydroxylation of debrisoquine or α OH of rac-metoprolol (Table 1). Consequently, the specificity constant (V_{max}/K_m) for the oxidation of each substrate by the Ser³⁰⁴Ala mutant was similar to that observed with the wild-type enzyme (Table 1). When the Ser³⁰⁴Ala substitution was combined with the Asp³⁰¹Gly mutation (Asp³⁰¹Gly/Ser³⁰⁴Ala), the apparent K_m value for each substrate oxidation was substantially greater than the K_m value obtained with the Asp³⁰¹Gly and Ser³⁰⁴Ala substitutions alone (Table 1). The influence of the Asp³⁰¹Gly/Ser³⁰⁴Ala double substitution on V_{max} values was inconsistent, reducing debrisoquine 4-hydroxylation by 75 % relative to Ser³⁰⁴Ala, whereas the V_{max} value for metoprolol α OH was not affected (Table 1).

Influence of Ser³⁰⁴Ala substitution on the regio- and/or enantioselective oxidation of debrisoquine, metoprolol and propranolol

The effect of the Ser³⁰⁴Ala substitution on the regioselective oxidation of debrisoquine (Figure 3), metoprolol (Table 2) and propranolol (Table 3) was minimal. With debrisoquine, the alicyclic 4-hydroxy metabolite was the major product formed by both the wild-type and Ser³⁰⁴Ala mutant forms of the enzyme.

However, the rates of formation of the phenolic 6- and 7-hydroxy products by the Ser³⁰⁴Ala mutant were reduced relative to wild-type such that the overall regioselectivity was shifted slightly from 4-HD > 7-HD > 6-HD > 8-HD > 5-HD with the wild-type enzyme to 4-HD > 8-HD > 7-HD > 6-HD > 5-HD with the Ser³⁰⁴Ala mutant (Figure 3). The regioselective oxidation of metoprolol by wild-type and Ser³⁰⁴Ala mutant was similar in that the O-demethylation (ODM) reaction was the preferred route of oxidation with each enantiomer [wild-type ODM/ α OH ratio, *R*-(+)-, 5.4; *S*-(-)-, 2.4; Ser³⁰⁴Ala ODM/ α OH ratio, *R*-(+)-, 8.1; *S*-(-)-, 3.5; Table 2]. The modest increase in the ODM/ α OH ratio with the *R*-(+) enantiomer by the Ser³⁰⁴Ala mutant was a consequence of an increased preference for the ODM reaction (Table 2). The rates of formation of 4-hydroxy, 5-hydroxy and deisopropylpropranolol by wild-type and Ser³⁰⁴Ala mutant CYP2D6 were also similar, the relative rates of formation of each metabolite expressed as a percentage of total metabolite formation being almost identical. The 4-hydroxy metabolite was the most prominent product formed by each form of CYP2D6 (Table 3).

The enantioselective oxidation of metoprolol and propranolol was not altered by the Ser³⁰⁴Ala substitution. ODM of metoprolol remained distinctly *R*-enantioselective (*R*/*S*, 1.6) while the α OH reaction showed a slight preference for the *S*-(-) enantiomer (*R*/*S*, 0.7), similar to the selectivity observed with the wild-type enzyme (Table 2). With propranolol, the formation of the major 4-hydroxy product was not significantly enantioselective (*R*/*S*,

Figure 4 Inhibition of metoprolol O-demethylase activity by quinidine (□, ○) and quinine (■, ●) with wild-type (■, □) and Ser³⁰⁴Ala mutant (●, ○) forms of CYP2D6

Values are the means of two independent experiments.

0.9) with both wild-type and Ser³⁰⁴Ala (Table 3). However, formation of the minor 5-hydroxy product showed an apparent shift from being *S*-(−)-enantioselective with the wild-type (*R*/*S*, 0.8) to being *R*-(+)-enantioselective with the Ser³⁰⁴Ala mutant (*R*/*S*, 1.4). The formation of the deisopropyl product remained *S*-(−)-enantioselective with both the wild-type and Ser³⁰⁴Ala mutant (Table 3).

Regio- and enantioselective oxidation of MAM

In terms of regioselectivity, the ODM reaction was the preferred route of oxidation of MAM by both the wild-type and Ser³⁰⁴Ala mutant forms of CYP2D6. However, the preference for the ODM reaction was greater with the *S*-(−) enantiomer (ODM/ α OH, wild-type, 4.9; mutant, 7.4) than with the *R*-(+) enantiomer (ODM/ α OH, wild-type, 2.5; mutant, 4.5; Table 2). This is in contrast to the ODM of metoprolol in that, although the ODM reaction was again the preferred route of oxidation by both forms of CYP2D6, the preference was greater with the *R*-(+) enantiomer (ODM/ α OH, wild-type, 5.4; mutant, 8.1) than with the *S*-(−) enantiomer (ODM/ α OH, wild-type, 2.4; mutant, 3.5; Table 2).

In terms of enantioselectivity, the wild-type and the Ser³⁰⁴Ala mutant forms of CYP2D6 oxidized MAM in an identical manner, the ODM reaction being slightly *S*-enantioselective (*R*/*S*, wild-type, 0.8; mutant, 0.9), whereas α OH was distinctly *R*-enantioselective (*R*/*S*, wild-type, 1.5; mutant, 1.6; Table 2). However, as with regioselectivity, the pattern of enantioselectivity with MAM was the reverse of that observed with metoprolol (ODM, *R*/*S*, 1.6–1.7; α OH, *R*/*S*, 0.7; Table 2).

Influence of Ser³⁰⁴Ala on ligand binding and the differential potency of inhibition of CYP2D6 activity by quinidine and quinine

Quinidine, a potent specific inhibitor of CYP2D6, induced a type-I difference spectrum with microsomes prepared from yeast cells expressing wild-type and Ser³⁰⁴Ala forms of the enzyme, the binding constant (K_s) being identical (0.18 μ M) with each form of CYP2D6. Debrisoquine concentrations in excess of 100 μ M,

however, failed to induce a type-I difference spectrum with the Ser³⁰⁴Ala mutant, despite a clear type-I spectrum (K_s , 12.8 μ M) being obtained with the wild-type enzyme.

The Ser³⁰⁴Ala substitution did not markedly alter the differential potency of inhibition of CYP2D6 activity by quinidine and quinine (Figure 4). With both wild-type and Ser³⁰⁴Ala mutant, quinidine was 100-fold more potent than quinine in inhibiting metoprolol α -hydroxylase and O-demethylase activities, the IC₅₀ values for each reaction being approx. 0.1 μ M with quinidine and 10 μ M with quinine (Figure 4).

DISCUSSION

Current homology models of CYP2D6 locate Ser³⁰⁴ in the active-site cleft [7–11]. Furthermore, its position in the I-helix relative to Asp³⁰¹, a key substrate-binding residue that interacts with the basic nitrogen of CYP2D6 ligands [14], suggests that Ser³⁰⁴ is an ideal candidate for a possible hydrogen-bond interaction with the hydroxyl groups of certain CYP2D6 ligands, such as the β -blockers metoprolol and propranolol, and the inhibitors quinidine and quinine. Such a hydrogen-bond interaction has been proposed to account for the enzyme's modest substrate enantioselectivity with β -blockers [8,18] and the differential potency in inhibition of enzyme activity by the diastereoisomers, quinidine and quinine [11,13]. However, the data presented in this study demonstrate conclusively that the substitution of Ser³⁰⁴ with alanine, a non-polar residue incapable of hydrogen bonding, does not alter CYP2D6 substrate enantioselectivity, at least not towards metoprolol and propranolol, and neither does the substitution affect the differential inhibitory potency of quinidine and quinine towards CYP2D6. The identical quinidine-binding coefficients with the wild-type and Ser³⁰⁴Ala further support the latter conclusion. Thus contrary to predictions from current homology models of the active site of CYP2D6, the hydrogen-bond capacity of Ser³⁰⁴ is not a key determinant of substrate enantioselectivity or quinidine/quinine inhibitory potency.

Whereas the data indicate that Ser³⁰⁴ does not play a major role in ligand selectivity, the observation that the regio- and enantioselective oxidation of MAM by both the wild-type and Ser³⁰⁴Ala mutant is the reverse of that observed with metoprolol indicates that the hydroxyl group attached to the chiral centre of the ligands is important in defining the enzyme's selective properties. This result suggests the possible involvement of a hydrogen-bond interaction between the ligand and an as-yet-unidentified active-site residue, other than Ser³⁰⁴.

Although the data suggest that Ser³⁰⁴ is not a key ligand-binding residue, the residue may nevertheless be located in the active-site cleft of the enzyme. Indeed, the modest influence of the Ser³⁰⁴Ala substitution on metoprolol and debrisoquine regioselectivity, together with the greater influence of the Asp³⁰¹Gly/Ser³⁰⁴Ala double mutant on the K_m values of formation of debrisoquine and metoprolol metabolites compared with the single Asp³⁰¹Gly and Ser³⁰⁴Ala mutants, indicates that Ser³⁰⁴ may be located in the active site. Furthermore, the lack of detectable holoenzyme with the Asp³⁰¹Ser/Ser³⁰⁴Asp double mutant, even though apoprotein was expressed at a similar level to wild-type enzyme, indicates that the interaction of these two amino acids in the I-helix is critical for the creation of the correct environment in the active site for protein folding, haem incorporation and/or the spin state of the Fe in the haem moiety. It would appear that the transposition of Asp and Ser at positions 301 and 304 is sufficient to disturb this critical environment and supports further the location of Ser³⁰⁴ in the vicinity of the active site of CYP2D6.

In conclusion, the data demonstrate conclusively that Ser³⁰⁴ is not a key ligand-binding residue in the active site of CYP2D6, although, due to its close proximity to Asp³⁰¹, a critical substrate-contact residue, it could still be located within the active-site cavity. Current homology models of the active site of CYP2D6 that predict a hydrogen-bonding interaction between Ser³⁰⁴ and certain ligands will need to be re-evaluated and other candidate residues nominated and tested by site-directed mutagenesis studies.

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