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The molecular and enzyme kinetic basis for the diminished activity of the cytochrome P450 2D6.17 (CYP2D6.17) variant Potential implications for CYP2D6 phenotyping studies and the clinical use of CYP2D6 substrate drugs in some African populations

Tashinga E. Bapiro[a], Julia A. Hasler[a], Marianne Ridderström[b], Collen M. Masimirembwa[b,*]

[a]*Department of Biochemistry, University of Zimbabwe, Harare, Zimbabwe*
[b]*DMPK & Bioanalytical Chemistry, AstraZeneca R&D Mölndal, S-431 83 Mölndal, Sweden*

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Abstract

In this study, the basis for the diminished capacity of CYP2D6.17 to metabolise CYP2D6 substrate drugs and the possible implications this might have for CYP2D6 phenotyping studies and clinical use of substrate drugs were investigated *in vitro*. Enzyme kinetic analyses were performed with recombinant CYP2D6.1, CYP2D6.2, CYP2D6.17 and CYP2D6.T107I using bufuralol, debrisoquine, metoprolol and dextromethorphan as substrates. In addition, the intrinsic clearance of 10 CYP2D6 substrate drugs by CYP2D6.1 and CYP2D6.17 was determined by monitoring substrate disappearance. CYP2D6.17 exhibited generally higher K_m values compared to CYP2D6.1. The V_{max} values were generally not different except for metoprolol α-hydroxylation with the V_{max} value for CYP2D6.17 being half that of CYP2D6.1. CYP2D6.1 and CYP2D6.2 displayed similar kinetics with all probe drugs except for dextromethorphan *O*-demethylation with the intrinsic clearance value of CYP2D6.2 being half that of CYP2D6.1. CYP2D6.17 exhibited substrate-dependent reduced clearances for the 10 substrates studied. In a clinical setting, the clearance of some drugs could be affected more than others in individuals with the *CYP2D6*17* variant. The *CYP2D6*17* allele might, therefore, contribute towards the poor correlation of phenotyping results when using different probe drugs in African populations. To investigate effects of *CYP2D6*17* mutations on the structure of the enzyme, a homology model of CYP2D6 was built using the CYP2C5 crystal structure as a template. The results suggest an alteration in position of active-site residues in CYP2D6.17 as a possible explanation for the reduced activity of the enzyme.

Keywords: Cytochrome P450 2D6; *CYP2D6*17*; Dextromethorphan; Bufuralol; Debrisoquine; Metoprolol

1. Introduction

Cytochrome P450 (CYP) 2D6 is a member of the CYP superfamily of enzymes involved in the metabolism of many xenobiotics. Although this mainly hepatic enzyme constitutes only about 1–2% of total CYP protein in the liver [1], it has been shown to contribute significantly towards the metabolism of over 30 prescribed drugs [2]. In addition, the expression of the enzyme has been shown to be polymorphic with clinically significant differences apparent in response to CYP2D6 substrate drugs depending on genotype/phenotype, particularly for drugs with narrow therapeutic indices [3].

The CYP2D6 polymorphism has been extensively studied at both phenotype and genotype levels. Studies have shown that while there is a difference in enzyme activity

* Corresponding author. Tel.: +46-31-776-2201; fax: +46-31-776-3786.
E-mail address: collen.masimirembwa@astrazeneca.com (C.M. Masimirembwa).

Abbreviations: CYP, cytochrome P450; PM, poor metaboliser; EM, extensive metaboliser; MR, metabolic ratio; HLM, human liver microsomes; CL_{int}, intrinsic clearance; RAF, relative activity factor; SRS, substrate recognition site.

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between poor metabolisers (PMs) and extensive metabolisers (EMs) in a given population, there is also variation in enzyme activity between corresponding groups from different populations [4]. Using probe drugs such as debrisoquine, sparteine, dextromethorphan and metoprolol, a clear bimodal distribution of CYP2D6 activity in Caucasians was observed with PMs occurring at a frequency of 5–10% [5]. The distribution in Orientals showed an almost unimodal distribution with less than 1% PMs [6]. Phenotyping in different African populations with the various probe drugs showed either a unimodal distribution or low frequencies of the PM status when using antimodes established in Caucasian populations [7]. A poor correlation of phenotype status when using different probe drugs was also observed in African populations [8,9].

While clinical implications of the PM status are fairly straightforward with respect to the need for dose adjustment of prescribed CYP2D6 substrate drugs, the wide variation in enzyme activities of EMs, however, still presents a challenge. The one extreme of EMs are the ultrarapid metabolisers carrying multiple copies of the *CYP2D6* gene and, therefore, requiring higher than normal doses of some drugs to attain therapeutic levels [10]. On the other extreme are the slow EMs mainly found in Oriental and African populations, and in whom lower doses of CYP2D6 substrate drugs would, therefore, seem to be recommended. Reduced CYP2D6 activity in Caucasians, which accounts for about 10% of the population, is due to the *CYP2D6*41* allele [11]. In Oriental populations, the molecular basis for diminished CYP2D6 activity is a mutant variant, CYP2D6.10 that is unstable and has lower expression levels and lower activity than CYP2D6.1 [12,13]. Pharmacokinetic studies with CYP2D6 substrate drugs in subjects with the *CYP2D6*10* variant compared to those with *CYP2D6*1* clearly demonstrate the clinical relevance of the reduced activity of CYP2D6.10 [14–16]. The high frequency of the *CYP2D6*10* variant in these populations could, therefore, explain the prescription of CYP2D6 substrates (e.g. propanolol, imipramine and amitriptyline) at lower doses compared to those used for their Caucasian counterparts [17].

Among most African populations, both native and those in diaspora, the basis for the diminished CYP2D6 activity is the presence of the *CYP2D6*17* variant, first identified in a Zimbabwean population [18]. This variant bears three nucleotide exchanges 1111C > T, 2938C > T and 4268G > C associated with the amino acid changes T107I, R296C and S486T, respectively. The last two amino acid exchanges R296C and S486T are associated with the *CYP2D6*2* variant. While *CYP2D6*17* occurs in African populations at allele frequencies up to 34% it has, however, not been found in Oriental populations. In Caucasians, *CYP2D6*17* was recently found to occur at an allele frequency of 0.3% [19]. Studies concerning the molecular and enzyme kinetic basis for the diminished activity of CYP2D6.17 and its clinical significance are limited. In one *in vitro* study, the kinetic effects of *CYP2D6*17* mutations introduced in a CYP2D6-methionine variant were investigated using bufuralol and codeine [20], however, the valine and not the methionine variant, has been observed in human populations [21].

The aim of this study was, therefore, to elucidate the enzyme kinetic and molecular basis for the diminished activity of CYP2D6.17 and its impact on phenotyping studies and the clinical use of CYP2D6 substrate drugs. This was achieved by: (a) studying the enzyme kinetics of the metabolism of commonly used phenotyping probe drugs debrisoquine, metoprolol and dextromethorphan and the *in vitro* marker substrate, bufuralol, using recombinant CYP2D6.1 (valine variant) and CYP2D6.17, (b) investigating the capacity of CYP2D6.17 compared to CYP2D6.1 to clear clinically used substrates of CYP2D6, and (c) making a homology model of CYP2D6 and investigating the effects of *CYP2D6*17* mutations on the three-dimensional structure of the enzyme.

2. Materials and methods

2.1. Materials

Yeast nitrogen base without amino acids, yeast extract, bactopeptone and bactoagar were purchased from Difco Laboratories. Glucose, galactose, L-histidine, L-leucine, dextromethorphan, debrisoquine, propafenone, timolol, uracil, reduced NADPH, sodium dithionite and cytochrome *c* were from SIGMA Chemical Co. Bufuralol, 1′-hydroxybufuralol and 4-hydroxydebrisoquine were obtained from Ultrafine.

Metoprolol, the α-hydroxy and demethylated metabolites of metoprolol were from AstraZeneca. Sorbitol and polyethylene glycol 4000 were from Merck. Yeast lytic enzyme and dextrorphan were from ICN Biomedicals Inc. Pefabloc SC and dithiothreitol were from Boehringer Mannheim.

Wizard Plus SV Minipreps DNA Purification System Kits were purchased from Promega. The QuikChange Site-Directed Mutagenesis Kit was obtained from Stratagene. The DNA sequencing kit, BigDye Terminator Cycle Sequencing Ready Reaction Kit was obtained from Applied Biosystems. All other chemicals used were of analytical or HPLC grade.

The *Saccharomyces cerevisiae* strain INVSc1-HR containing the human reductase gene was a gift from the LINK Project (a program of the University of Dundee/Biotechnology and Biology Research Council/Department of Trade and Industry/Pharmaceutical Industry). The galactose-inducible expression vector and cDNA for *CYP2D6*1* (with valine in position 374) were generous gifts from Dr. Magnus Ingelman-Sundberg. Human liver microsomes were prepared from a pooled set of liver pieces obtained from patients of Caucasian origin, undergoing liver resections using the method of [22]. Approval

for use of the liver pieces, was obtained from the Local Ethics Committee, at Salgreska Hospital, Gothenburg, Sweden.

2.2. Methods

2.2.1. Mutagenesis

The three enzyme variants with their characteristic amino acid exchanges, CYP2D6.17 (T107I, R296C and S486T), CYP2D6.2 (R296C and S486T) and CYP2D6.T107I, were produced by site-directed mutagenesis of the *CYP2D6*1* cDNA coding for valine instead of methionine at position 374, using the QuikChange Site-directed Mutagenesis Kit (Stratagene) and mutagenic primers: 5′-CCT GTG CCC ATC ATC CAG ATC CTG GGT-3′, 5′-GAT GAG AAC CTG TGC ATA GTG GTC GCT-3′ and 5′-GCT TTC CTG GTG ACC CCA TCC CCC TAT-3′. Confirmation of the base changes introduced was by sequencing using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Subsequently, the yeast strain INVSc1-HR, bearing the human reductase was transformed with the appropriate cDNA, by the lithium acetate method [23].

2.2.2. Determination of CYP, NADPH–CYP reductase and protein concentration

The culturing of yeast and preparation of microsomes was done as previously described [24]. The CYP content was determined from the reduced carbon monoxide difference spectrum [25] and NADPH–CYP reductase by the reduction of cytochrome *c* [22]. The protein concentration was measured by the modified method of Lowry [26].

2.2.3. Enzyme kinetics

Each reaction mixture consisted of the appropriate concentration of enzyme, substrate (dissolved in 0.1 M phosphate buffer pH 7.4) and 1 mM NADPH, in 0.1 M potassium phosphate buffer pH 7.4 to a final volume of 200 μL. The reactions were started by addition of NADPH after a preincubation of 3 min at 35° and optimised for linearity with respect to time and CYP concentration (data not shown). All reactions were stopped by the addition of 10 μL of perchloric acid (60%). The supernatant (5–20 μL) obtained after centrifugation at 4000 *g* for 10 min was analysed by reversed-phase chromatography. The metabolites formed were quantitated by external standardisation using authentic metabolites. Bufuralol 1′-hydroxylase, debrisoquine 4-hydroxylase and dextromethorphan *O*-demethylase activities were measured according to the method of [27]. All metabolites of metoprolol were assayed by the method of [28].

2.3. Data analysis

Eight data points were used to generate K_m and V_{max} values. These values were determined by non-linear least-squares regression analysis using GraFit version 3.0 (Erithacus Software Limited).

2.3.1. Determination of intrinsic clearance (CL_{int})

For 10 known substrates of CYP2D6 dissolved in water or acetonitrile (1% final concentration), intrinsic clearance studies were performed with human liver microsomes (HLM), recombinant CYP2D6.1, and CYP2D6.17 using the substrate disappearance approach [29]. The typical incubation mixture consisted of 10 pmol of enzyme, 1 μM substrate and 1 mM NADPH, in 0.1 M potassium phosphate buffer pH 7.4 to a final volume of 200 μL. The reactions were pre-incubated for 3 min, started by the addition of NADPH, and incubated for 5, 10, 20, 30 and 40 min. The reactions were stopped by addition of 100 μL ice-cold methanol containing 0.8% formic acid and 0.4 μM verapamil (internal standard). After centrifugation at 4000 *g*, the supernatant was transferred and diluted 1:1 with water and analysed by LC–MS as described below. The intrinsic clearance was determined using the expression:

$$\mathrm{CL_{int}} = \text{volume} \times k$$

where the volume is the total incubation volume (200 μL in this study) and k is the elimination rate constant. Using debrisoquine 4-hydroxylation as a marker reaction, the relative contribution of CYP2D6 in the clearance of the substrate drugs by HLM was evaluated using the relative activity factor (RAF) approach [30]. The RAF was calculated using the expression:

$$\mathrm{RAF_{isoform}} = \frac{\text{velocity for probe substrate with HLM (pmol/(min mg))}}{\text{velocity for probe substrate with recombinant enzyme (pmol/(min pmol))}}$$

The units for RAF are, therefore, pmol CYP/mg protein. The percent contribution towards the clearance of a drug was calculated as follows:

$$\%\,\text{contribution} = \frac{\text{clearance by recombinant enzyme} \times \text{RAF}}{\text{clearance by HLM}} \times 100$$

2.4. Liquid chromatography–mass spectrometry

Supernatant (10 μL) was injected using a CTC HTS autosampler (CTC Analytics) and chromatography was performed on a Zorbax Extend C18 column (2.1 mm × 50 mm, 3.5 μm, Agilent) employing an Agilent 1100 pump with gradient elution at 300 μL/min. The mobile phase consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. The organic modifier content was 5% for B for 0.6 min, then increased linearly from 5 to

95% B over 2 min, kept at 95% B for 1 min, and followed by a step gradient to 5% B for 1.4 min. The total run-time including equilibration amounted to 5.5 min. The pump's mixing chamber was omitted to reduce the system's dwell volume. The front was sent to waste by means of a VICI 6-port two-position switching valve. After 0.6 min, the column effluent was directed towards the mass spectrometer without splitting. Detection was performed with a triple quadrupole mass spectrometer from MDS Sciex API 3000 equipped with a Turbo-Ionspray source. The MS operated at turbogas at 300°, nebuliser gas 11, curtain gas 10, CAD gas 6, dwell time 200 ms, electrospray voltage 4.8 kV. Instrument control, data acquisition and data evaluation were performed with Analyst 1.1 software (Applied Biosystems). Mass spectrometric optimisation for the various analytes and batch acquisition were performed automatically employing Automation Software (Applied Biosystems).

2.5. Homology modelling and docking

The rabbit CYP2C5 and human CYP2D6.1 sequences were obtained from Swissprot database (accession numbers P00179 and P10635, respectively). The first 33 amino acids in the CYP2D6.1 sequence were removed to allow for the absence of the first 29 N-terminal amino acids from the CYP2C5 crystal structure [31]. In addition, the amino acid residue methionine 374 was changed to valine, which is found in the 'wildtype' CYP2D6 protein. The programs: GCG BestFit and Swiss-PdbViewer v 3.7b2 (Glaxo Wellcome Experimental Research) were used to align the CYP2D6.1 sequence to the CYP2C5 sequence with manual adjustments being made to the gap regions.

Subsequent work was done on a Silicon Graphics workstation. The alignment was introduced into the program INSIGHT II version 2000 (Molecular Simulations, Inc. 2000), for modelling. Analysis of the model was done in SYBYL 6.7 (Tripos Associates Inc.) using the Protable module.

The structures of the CYP2D6 substrates were drawn in SYBYL. Atom charges (MMFF94 charges) were added to the structures and energy minimised using the MMFF94s force-field. The docking experiments were done as previously described [32]. In brief, dockings of the substrates were performed using the program GOLD (Genetic Optimisation for Ligand Docking, Dr. Gareth Jones) with active site radii of 10, 12 and 15 Å defined from the heme-bound oxygen, requesting 10 solutions, however, termination was allowed if the root mean square distances (RMSD) were within 1.5 Å. GOLD is an automated ligand docking program that uses a genetic algorithm which explores possible hydrogen-bonding and hydrophobic interactions between the ligand and protein. It indicates the strong interactions it picks during the docking runs [33]. The distances from the heme-bound oxygen to the carbon at the likely sites of oxidation on each substrate were measured.

3. Results

3.1. Enzyme kinetic characterisation

The enzyme kinetic parameters (K_m, V_{max}, and V_{max}/K_m) for the metabolism of bufuralol, dextromethorphan, debrisoquine and metoprolol by the CYP2D6 variants compared to CYP2D6.1 are shown in Table 1. CYP2D6.17 exhibited generally higher K_m values compared to CYP2D6.1. The V_{max} values were generally not different except for metoprolol α-hydroxylation with the V_{max} value for CYP2D6.17 being about half that of CYP2D6.1. The V_{max}/K_m values for CYP2D6.17 were lower than those of CYP2D6.1 with the largest difference apparent with dextromethorphan. The V_{max}/K_m value was affected to a much lesser extent by CYP2D6.17 when debrisoquine was used as substrate. CYP2D6.1 and CYP2D6.2 displayed similar kinetics with all probe drugs except for dextromethorphan *O*-demethylation, with the V_{max}/K_m value of CYP2D6.2 being about half that of CYP2D6.1. The enzyme kinetic profile of the CYP2D6.T107I variant differed from that displayed by CYP2D6.1 for all probe drugs. The effects of the T107I amino acid exchange are, however, of academic interest only, as the amino acid change has not been observed by itself in any subject analysed so far.

The CL_{int} calculated from *in vitro* half-lives for 10 drugs which are known to be substrates of CYP2D6 and the relative contribution (using a RAF of 47 pmol/mg) of this enzyme to their clearance by HLM are shown in Table 2. CYP2D6.17 generally displayed reduced clearance of the CYP2D6 substrates, with the magnitude of the reduction being dependent on the compound. Large differences in clearance between CYP2D6.1 and CYP2D6.17 were observed with bufuralol, dextromethorphan, clomipramine and fluphenazine. Clearance values that were affected to a much lesser extent were observed with metoprolol, debrisoquine, sparteine and thioridazine.

The calculated percent contribution of CYP2D6 in the metabolism of bufuralol, timolol and fluphenazine is, unexpectedly over 100% (Table 2). This may be related to the relative activity factor (RAF), which has been shown to be dependent on the choice of index substrate and a difference in metabolic requirements between index drug and test compound [34]. The RAF depends on two major assumptions: (1) that the factors affecting metabolism of the index drug are the same as those affecting the metabolism of the test compound and (2) that differences between the recombinant system and human liver microsomes in the metabolism of index and test drug are cancelled out. Failure to meet one or both assumptions may result in either, under- or over-estimation of the

Table 1
Kinetic properties of the four variants towards CYP2D6 probe drugs

CYP2D6	Bufuralol 1′OH	Dextromethorphan ODM	Debrisoquine 4OH	Metoprolol ODM	Metoprolol α-OH
1					
K_m[a]	9.65 ± 0.3	1.30 ± 0.2	55.2 ± 17	51 ± 3.2	41 ± 5.3
V_{max}[b]	8.64 ± 2.1	2.21 ± 0.5	0.55 ± 0.1	4.81 ± 1.1	1.47 ± 0.40
V_{max}/K_m[c]	0.89 ± 0.2	1.77 ± 0.6	0.01 ± 0.002	0.095 ± 0.02	0.036 ± 0.009
17					
K_m	21.0 ± 3.6***	7.16 ± 1.7***	59.2 ± 10	75 ± 6.5***	76 ± 7.31***
V_{max}	4.74 ± 2.5	1.68 ± 0.7	0.40 ± 0.1	3.73 ± 0.06	0.90 ± 0.04*
V_{max}/K_m	0.22 ± 0.1***	0.27 ± 0.2***	0.007 ± 0.002	0.05 ± 0.004**	0.012 ± 0.001
2					
K_m	13.0 ± 0.5	2.42 ± 0.5	112 ± 41	57 ± 4.9	47 ± 6.82
V_{max}	8.48 ± 4.1	1.72 ± 0.5	0.79 ± 0.08	5.25 ± 0.37	1.98 ± 0.16*
V_{max}/K_m	0.66 ± 0.3	0.77 ± 0.4*	0.008 ± 0.002	0.093 ± 0.006	0.042 ± 0.003
T107I					
K_m	12.4 ± 2.5	4.08 ± 1.1*	90 ± 51	37 ± 0.73**	42 ± 4.00
V_{max}	6.08 ± 1.4	2.40 ± 0.4	0.51 ± 0.13	3.82 ± 0.31	0.71 ± 0.05**
V_{max}/K_m	0.49 ± 0.1*	0.61 ± 0.2**	0.007 ± 0.002	0.103 ± 0.009	0.017 ± 0.003

ODM: *O*-demethylation; 4OH: 4-hydroxylation; α-OH: alpha-hydroxylation. Values represent mean ± SD of four determinations (twice for each of two separate expressions).

[a] K_m (μM).

[b] V_{max} (pmol product/(min pmol CYP)).

[c] V_{max}/K_m (μL/min pmol CYP).

* Significantly different ($P < 0.05$); ** Significantly different ($P < 0.01$); *** Significantly different ($P < 0.001$) from values for CYP2D6.1.

contribution of a particular CYP isoform towards the metabolism of a test compound.

3.2. CYP2D6 homology model

The rabbit CYP2C5 crystal structure was used as a template for homology modelling of CYP2D6.1. CYP2D6.1 and CYP2C5 have amino acid sequence similarity and identity of 51 and 41%, respectively. This is a higher level of identity compared to that between CYP2D6 and the bacterial CYPs 101, 102 and 108, which have similarities of less than 20%. The sequence alignment between CYP2D6.1 and CYP2C5 is shown in Fig. 1 and the root mean square distance for the polypeptide backbone of the CYP2D6.1 model was 0.92 Å compared to the CYP2C5 crystal structure. The Matchmaker average score was −0.09 and −0.12 kT for the CYP2D6.1 model and CYP2C5 template, respectively. Energy scores below zero are advantageous and values above zero could indicate incorrect regions. The substrate recognition site (SRS) regions in CYP2D6 [35,36] are shown in Fig. 1. The amino acid exchanges in CYP2D6.17 are all located in the SRS regions: T107I in SRS1, R296C in SRS4 and S486T in SRS6.

Table 2
A comparison of the capacity of CYP2D6.1 and CYP2D6.17 to clear CYP2D6 substrates *in vitro*

Compound	CL by HLM (μL/(min mg))	CL by CYP2D6.1 (μL/min pmol CYP)[a]	CL by CYP2D6.17 (μL/min pmol CYP)[a]	% Reduction in Cl[b]	% Contribution by CYP2D6[c]
Bufuralol	37	0.813 ± 0.07	0.306 ± 0.020	62	103
Dextromethorphan	41	0.421 ± 0.02	0.081 ± 0.02	81	48
Metoprolol	6.9	0.069 ± 0.007	0.040 ± 0.004	42	47
Debrisoquine	4.4	0.062 ± 0.003	0.044 ± 0.010	29	66
Propafenone	140	2.771 ± 0.060	1.712 ± 0.020	38	93
Timolol	3.2	0.115 ± 0.050	0.053 ± 0.020	54	169
(−)-Sparteine	ND	0.111	0.097	13	ND
Clomipramine	29	0.422 ± 0.080	0.065 ± 0.006	85	68
Thioridazine	71	0.654 ± 0.140	0.459 ± 0.020	30	43
Fluphenazine	48	1.336	0.451	66	131

HLM: human liver microsomes; RAF: relative activity factor; CL: clearance; ND: not determined.

[a] Values represent mean ± SD of duplicate determinations.

[b] Calculated as follows: [(CL by CYP2D6.1 − CL by CYP2D6.17)/CL by CYP2D6.1] × 100.

[c] Relative contribution of CYP2D6.1 towards clearance of the drug calculated by multiplying the CL_{int} for recombinant CYP2D6.1 by the RAF (47 pmol/mg) and expressing as a percentage of the CL_{int} by HLM.

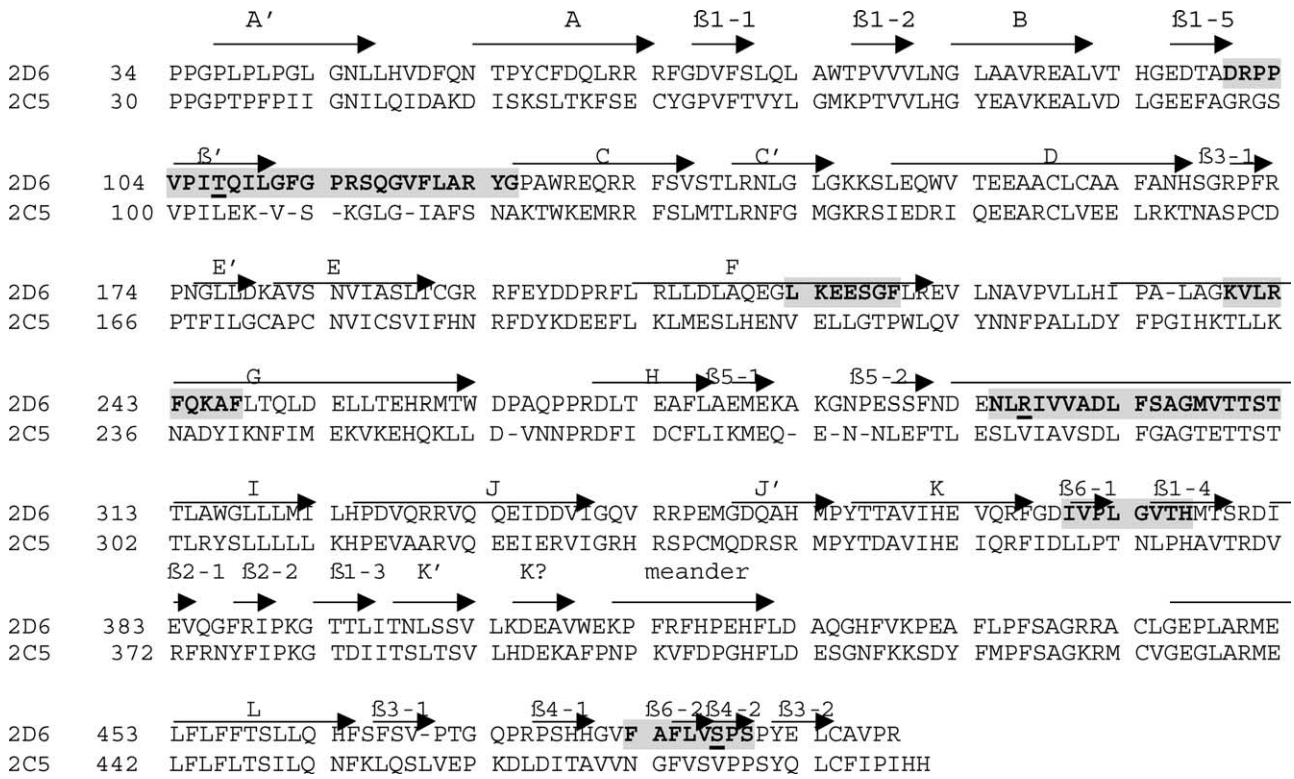


Fig. 1. Alignment between CYP2C5 and CYP2D6.1 showing the amino acids (shaded and bold) in the SRS proposed by [35], from an alignment by [36]. The amino acid exchanges in CYP2D6.17 (underlined) are all located in the SRS regions.

3.3. Docking of CYP2D6 substrate drugs into homology models

Dockings were done using radii of 10, 12 and 15 Å to define the CYP2D6.1 active site. The distance between site of oxidation and the iron-bound oxygen for other CYPs/substrates has been shown to be around 3–6 Å from NMR and crystallography studies [37,38]. The interpretation of Table 3, therefore, took into account the number of solutions favouring known sites of oxidation, and the distance of the closest possible site of oxidation to the heme-bound oxygen. Based on this approach, the results of the docking experiments showed that (1) active site volumes described by 10 or 12 Å, best approximated the experimental data (2) the hydrogen bond interactions of the basic nitrogen in half of the compounds were with Glu 216.

4. Discussion

4.1. In vitro kinetics of mutant variants

The kinetic profiles of CYP2D6.1 and CYP2D6.17 differed in a substrate-dependent manner with CYP2D6.17 displaying a generally reduced capacity to clear the compounds (Tables 1 and 2). The structure of the substrate seemed to be critical, and the largest difference in V_{max}/K_m apparent with dextromethorphan. The V_{max}/K_m data (Table 1) was consistent with CL_{int} data (Table 2) which show that dextromethorphan clearance was affected most by CYP2D6.17, whilst debrisoquine was the least affected. In some cases, the enzyme variants exhibited similar V_{max} values, suggesting a reduction in affinity as a possible explanation for the lower CL_{int} (V_{max}/K_m) values of CYP2D6.17. While CYP2D6.2 displayed similar kinetics to CYP2D6.1 for all probe substrates, except for dextromethorphan, the CYP2D6.T107I variant displayed a different profile for all probe drugs. The kinetic profile exhibited by CYP2D6.17, therefore, probably cannot be attributed to any one single amino acid exchange *per se*, but to the presence of all three amino acid changes. These findings are in general agreement with studies by Oscarson *et al.* [20] and a recent study [39], published when our work was under review. The combination of the amino acid exchanges might change the geometry and/or chemistry of the active site by either being part of the active-site architecture themselves or through long-distance structural effects.

*4.2. Clinical importance of the CYP2D6*17 allele and its implication in the selection of probe drugs for African populations*

The clinical relevance of the CYP2D6 polymorphism has been investigated in several populations. Asians and Orientals have been shown to require lower doses of

antipsychotics compared to their Caucasian counterparts [40]. There is, however, very little biochemical or clinical data on the molecular and phenotypic status of CYP2D6 in Africans. The diminished activity of CYP2D6.17 implies that individuals having the *CYP2D6*17* allele, may have a compromised capacity to clear drugs whose main route of disposition is by this enzyme. The predicted percentage contribution towards the metabolic clearance of CYP2D6 substrate drugs, of at least 43% (Table 2), is generally in agreement with *in vivo* studies, which show differences in plasma concentrations and in some cases, pharmacodynamics between EMs and PMs [41–47]. This significant contribution by CYP2D6 and the observed reduced capacity of CYP2D6.17 to clear the CYP2D6 substrate drugs, points at a potential need for dose-optimising when administering these drugs to African populations, in which the *CYP2D6*17* allele is prevalent.

A study in depressed Tanzanian patients showed that they required lower doses of clomipramine compared to those recommended for Caucasians [48]. The *CYP2D6*17* allele has been found at high frequencies (15–30%) in Tanzanians [49,50]. Our study shows a 7-fold difference in CL_{int} values of clomipramine for CYP2D6.1 and CYP2D6.17, with CYP2D6 predicted to contribute significantly (68%) to its clearance (Table 2). The decreased clearance of the drug by CYP2D6.17 could, therefore, explain the observations *in vivo* in which Tanzanian patients respond to lower than recommended doses of clomipramine.

Studies in African-Americans showed higher plasma concentrations of nortriptyline than in Caucasians after the administration of the same drug dose [51]. The *CYP2D6*17* allele was shown to occur at a frequency of 14.6% in African-Americans [19] and could, therefore, explain these clinical observations. In our study, CYP2D6 is also predicted to contribute significantly to the metabolism of propafenone and timolol. There is a 2–3-fold difference in the clearance of these drugs by CYP2D6.1 and CYP2D6.17 that could be of clinical significance. Our study has, therefore, set the stage for a guided choice of drugs requiring dose optimising studies (clomipramine, propafenone, timolol and fluphenazine) in African populations in which the *CYP2D6*17* allele is common.

The extent of the decrease in CL_{int} of the probe drugs by CYP2D6.17 compared to CYP2D6.1 is dependent on the drug (Tables 1 and 2). Dextromethorphan shows the largest difference, followed by metoprolol, with debrisoquine showing the smallest difference. We would, therefore, expect the increase in MRs (right shift) caused by CYP2D6.17 to be different depending on the probe drug, with dextromethorphan having the largest "right shift." This is consistent with observations made *in vivo* [52], in which the increase in MRs associated with the *CYP2D6*17* allele, was substrate dependent and greatest with dextromethorphan. The *CYP2D6*17* allele, therefore, probably contributes to the poor correlation of MRs between different probe drugs observed in African populations. Data from phenotyping studies [5,10,52] suggest additional factors such as the *CYP2D6*29* allele, tropical diseases, diet, or unknown mutations.

The CL_{int} of debrisoquine and sparteine by CYP2D6.17, although lower compared to that by CYP2D6.1, was not significantly different and this was in contrast to the *in vivo* studies [52–55], in which individuals homozygous for the *CYP2D6*17* allele had a 4–10-fold difference in median MRs when compared to those carrying the *CYP2D6*1* or *CYP2D6*2* alleles. The lack of a significant difference in our study, was also in contrast to recently published *in vitro* studies, in which the CYP2D6 variants were expressed in COS-7 and baculovirus-insect cell systems, with the CL_{int} (V_{max}/K_m) for debrisoquine 4-hydroxylation by CYP2D6.17 being 20% of that by CYP2D6.1 [39]. We currently do not have an explanation for these disparities. Although CYP2D6.17 consistently showed lower CL_{int} (V_{max}/K_m) for CYP2D6 substrates in the study by Marcucci *et al.* [39], it can, however, be noted that kinetic changes (K_m and/or V_{max}) in the metabolism of dextromethorphan, bufuralol and debrisoquine depended on the system used to express the CYP2D6 variants. The use of a yeast expression system in our study could, therefore, in part, explain the above contrasting observations.

Enzyme kinetic results (Table 1) show that dextromethorphan is the only probe drug able to distinguish the three allelic variants. CYP2D6.1 has the highest clearance, followed by CYP2D6.2 whilst CYP2D6.17 presents the lowest clearance. This is consistent with *in vivo* observations showing that the *CYP2D6*1* and *CYP2D6*2* alleles were associated with similar activities for debrisoquine [10], but *CYP2D6*2* had a lower activity than *CYP2D6*1* when dextromethorphan was employed [54]. In a comparative study of the probe drugs in three populations, dextromethorphan was also shown to be the most sensitive in identifying outlying CYP2D6 activity [4]. Dextromethorphan is, therefore, probably the best probe drug for use in resolving individuals carrying the *CYP2D6*1* or *CYP2D6*17* alleles.

4.3. The CYP2D6.1 homology model and the effects of the CYP2D6*17 mutations

Available protein models of CYP2D6 have been based on the crystal structures of bacterial CYPs 101, 102 and 108 [56–59]. Important active-site residues identified by these models include Thr 107, Val 119, Glu 216, Asp 301, Ser 304, Val 374 and Phe 481. The Phe 481 was postulated to make hydrophobic interactions with the aromatic regions of substrates, and the amino acids Asp 301 and Glu 216, predicted to be critical for interactions with the basic nitrogen atoms of substrates. Given the significant structural differences between mammalian CYPs and bacterial ones, the CYP2C5 crystal structure may, therefore, be a better template. In addition, in previous studies, the

Table 3
Results from the docking experiments with CYP2D6.1

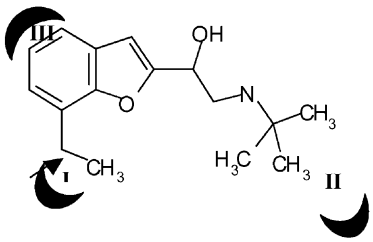
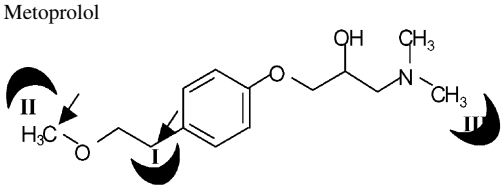
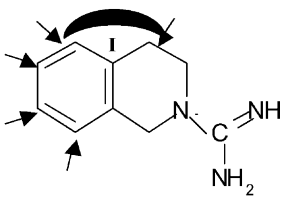
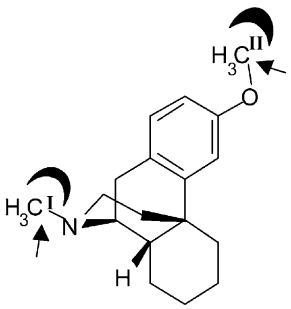
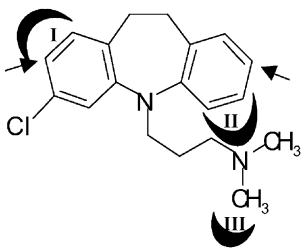
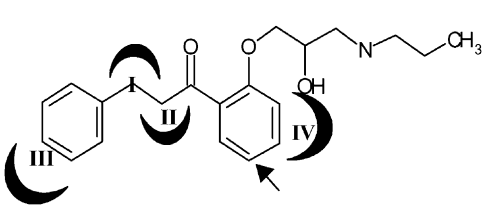
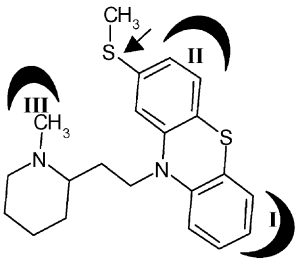
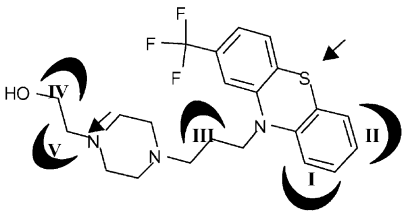
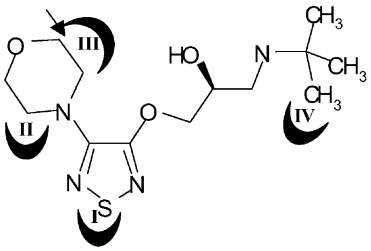
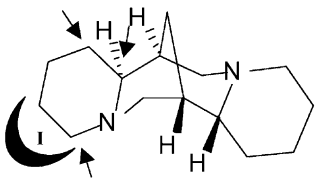
Compound	Active site radius (Å)	Number of solutions	Hits on specified region	Distance (Å)[a]
Bufuralol				
	10	5	5 (**I**)	3.20
	12	3	3 (**I**)	2.78
	15	10	3 (**I**), 5 (**II**), 1 (**III**)	4.85, 2.81, 3.01
Metoprolol				
	10	10	6 (**I**), 4 (**II**)	2.32, 3.68
	12	10	5 (**I**), 4 (**II**), 1 (**III**)	3.36, 4.08, 1.89
	15	10	7 (**I**), 1 (**II**), 2 (**III**)	3.36, 2.20, 2.91
Debrisoquine				
	10	4	4 (**I**)	4.77
	12	3	3 (**I**)	4.33
	15	5	5 (**I**)	4.88
Dextromethorphan				
	10	3	3 (**I**)	2.82
	12	7	6 (**I**), 1 (**II**)	3.32, 7.38
	15	3	3 (**I**)	2.81
Clomipramine				
	10	8	7 (**II**), 1 (**I**)	2.91, 4.11
	12	6	2 (**II**), 1 (**I**), 3 (**III**)	2.88, 3.50, 6.47
	15	6	3 (**III**)	6.64
Propafenone				
	10	10	5 (**I**), 1 (**II**), 3 (**III**), 1 (**IV**)	2.14, 2.90, 2.29, 2.20
	12	8	3 (**I**), 5 (**IV**)	2.52, 3.00
	15	9	1 (**I**), 2 (**III**), 3 (**IV**)	3.59, 2.78, 4.10

Table 3 (*Continued*)

Compound	Active site radius (Å)	Number of solutions	Hits on specified region	Distance (Å)[a]
Thioridazine				
	10	10	5 (**I**), 2 (**II**), 3 (**III**)	3.21, 3.73, 3.98
	12	10	2 (**I**), 7 (**II**), 1 (**III**)	3.95, 3.60, 5.36
	15	6	2 (**I**), 4 (**II**)	3.31, 3.73
Fluphenzine[b]				
	10	10	3 (**I**), 2 (**II**), 2 (**III**), 1 (**IV**), 2 (**V**)	3.89, 3.50, 4.53, 3.20, 2.70
	12	4	1 (**I**), 3 (**IV**)	3.00, 3.59
	15	5	5 (**I**)	3.04
Timolol				
	10	8	3 (**I**), 3 (**II**), 2 (**III**)	2.07, 3.94, 3.80
	12	5	3 (**I**), 2 (**III**)	1.85, 3.53
	15	3	3 (**IV**)	3.02
Sparteine				
	10	3	3 (**I**)	3.01
	12	5	5 (**I**)	3.21
	15	9	9 (**I**)	3.08

The sites of oxidation indicated by arrows are from the references [43,63–67].

[a] Distance from heme-bound oxygen to the carbon on the site of oxidation.

[b] The site of oxidation is from a similar compound perphenazine [68] as that for fluphenazine is not known.

fitting of compounds into the active site was done taking into account the positions of oxidation obtained from experimental data and the amino acid residues postulated to interact with the substrates. In this study, we used the program GOLD to explore the best docking orientations without any constraints on the positions of oxidation or interactions with particular amino acid residues being made.

The docking results from our study (Table 3) show orientations of substrates favouring oxidation in the known positions for most of the compounds. Our homology model was, therefore, able to predict sites of oxidation for some of the compounds, in particular bufuralol, metoprolol, debrisoquine, dextromethorphan and sparteine. In half of the compounds (bufuralol, debrisoquine, metoprolol, timolol and propafenone), the docking program identified strong hydrogen bond interactions of the basic nitrogen of the substrates with the carboxylate group of Glu 216. This is inconsistent with some predictions in which smaller substrates, such as debrisoquine, would interact with Asp 301, whilst the larger ones interact with Glu 216 [60]. The Asp 301 residue in our model, although close to the heme, is not well positioned for interactions optimal for oxidation in the known sites. The orientations adopted by compounds in the active site are also determined by the shape and hydrophobic interactions between substituents of substrates and corresponding regions on the protein [61] thus explaining the orientations assumed by the other half of compounds

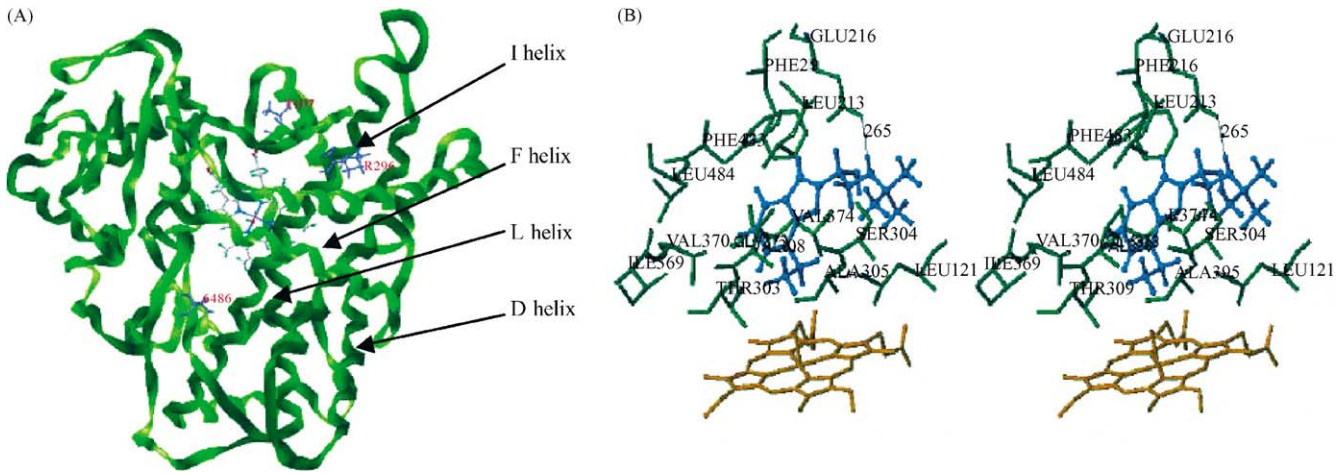


Fig. 2. (A) Ribbon diagram showing CYP2D6.1 model and positions of the amino acid residues exchanged in CYP2D6.17. (B) Stereo view of bufuralol docked in the active site of CYP2D6.1 and the interaction between Glu 216 and the nitrogen in bufuralol.

not involved in interactions with Glu 216 or Asp 301. The aromatic rings of substrates are oriented towards a hydrophobic region in our model consisting of Phe 219, Val 370 and Phe 483, Leu 484. The Thr 107 residue was predicted to be involved in interactions with some substrates [62]. In our model the Thr 107 is, however, not close to the active site and does not interact with any of the substrates.

Homology modelling has a number of limitations and one of them is the difficulty encountered when modelling loop regions due to insertions and deletions (Figs. 1 and 2) that are usually present in these regions [63]. The amino acid exchanges in CYP2D6.17 (T107I, R296C and S486T), are located in the B–C loop, I-helix and β4–2 sheet, respectively. An attempt was made to model the CYP2D6.17 protein and comparison of the overall shapes of CYP2D6.1 and CYP2D6.17 models revealed a difference in the B–C loop, which is close to the T107I and R296C amino acid changes. A closer inspection of the active site residues, revealed a drastic change in their positions, with some residues being totally absent from the CYP2D6.17, which could explain the *in vitro* kinetic results (Tables 1 and 2). It was, however, difficult to attribute the drastic changes in the CYP2D6.17 active site to the amino acid changes *per se* as the changes could, in addition, be due to differences in modelling the B–C loop, that are magnified by the proximity of T107I and R296C.

5. Conclusion

We have shown that the amino acid exchanges in CYP2D6.17 could alter the positions of active site residues causing the reduced activity of the enzyme, with the degree of reduction dependent on the substrate. We also conclude that, in addition to other possible factors such as unknown mutations, diet or unreported medications, the *CYP2D6*17* allele might contribute to the poor correlation of phenotyping results when using different probe drugs. Lastly, our study has highlighted the need for dose optimising studies of some CYP2D6 substrates in African populations.

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