

WHY IS QUINIDINE AN INHIBITOR OF CYTOCHROME P450 2D6? THE ROLE OF KEY ACTIVE SITE RESIDUES IN QUINIDINE BINDING*

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Running Title: Role of E216, D301 and F120 in 2D6-quinidine interactions

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We have previously shown that residues Asp301, Glu216 and Phe120 in the active site of cytochrome P450 2D6 (CYP2D6) play a key role in substrate recognition by this important drug-metabolising enzyme. We have now examined the effect of mutations of these residues on interactions of the enzyme with the prototypical CYP2D6 inhibitor, quinidine. Abolition of the negative charge on either or both residues 216 and 301 decreased quinidine inhibition of bufuralol 1'-hydroxylation and dextromethorphan O-demethylation by at least 100-fold. The apparent dissociation constants (K_d) for quinidine binding to wild type enzyme or to the Glu216Asp and Asp301Glu mutants were 0.25-0.50 μ M. The amide substitutions of Glu216 or Asp301 resulted in 30 to 64-fold increases in K_d for quinidine. The double mutant Glu216Gln/Asp301Gln showed the largest decrease in quinidine affinity with a K_d of 65 μ M. Changes in the mode of quinidine binding were indicated by changes in the optical difference spectra on binding. Alanine substitution of Phe120, Phe481 or Phe483 had only a minor effect on the inhibition of bufuralol 1'-hydroxylation and dextromethorphan O-demethylation, and on binding. In contrast to the wild-type enzyme, a number

of the mutants studied were found to be able to metabolise quinidine. CYP2D6 Asp301Gln and Asp301Asn produced small amounts of 3-hydroxyquinidine, Asp301Ala and Asp301Phe produced O-demethylated quinidine, and Phe120Ala and Glu216Gln/Asp301Gln produced both these metabolites. Homology modelling and molecular docking were used to predict the modes of quinidine binding to wild type and mutant enzymes; these were able to rationalise the experimental observations.

Human cytochrome P450 2D6 (CYP2D6) plays a central role in drug metabolism, metabolising over 30% of the most commonly prescribed drugs (1). The *CYP2D6* gene is highly polymorphic, leading to wide inter-individual and ethnic differences in CYP2D6-mediated drug metabolism (2-4). P450-drug and drug-drug interactions involving CYP2D6 ligands are thus a prime consideration in the development of new drugs, emphasising the importance of a detailed understanding of the factors that govern the substrate specificity of this enzyme.

Quinidine is not metabolised by CYP2D6 and has long been established as a potent competitive inhibitor of the enzyme (5-9). The fact that quinidine is an inhibitor rather than a substrate is intriguing since it produces a classical

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type I binding spectrum with CYP2D6 (10) that is usually associated with the binding of substrate molecules (11). In addition, quinidine possesses a number of features normally associated with CYP2D6 substrates including a basic nitrogen atom, a flat hydrophobic region and a negative molecular electrostatic potential (12). Studies of the relationship between structure and inhibitory activity for quinidine and its (less potent) stereoisomer quinine have been reported (13), but the protein-ligand interactions which are responsible for the fact that quinidine can bind tightly but not in an orientation favourable for catalysis have not hitherto been established.

Recent models of the active site of CYP2D6 (*e.g.*, (14)) suggest that two carboxylate groups, on residues Asp301 and Glu216, may play key roles in the recognition of substrates containing a basic nitrogen, and support for this has come from mutagenesis experiments (15-17). It has also been suggested that the aromatic residues Phe120, Phe481 and Phe483 may have roles in substrate binding through π -interactions with the planar hydrophobic regions common to many CYP2D6 substrates (10,14,18,19). We now describe studies of a series of mutants of these five residues aimed at investigating their role in quinidine binding and in determining whether quinidine is a substrate or an inhibitor of this important drug-metabolising enzyme.

MATERIALS AND METHODS

Materials - Terrific Broth, chloramphenicol, dithiothreitol, glucose 6-phosphate, NADP⁺, phenylmethylsulfonyl fluoride, sodium dithionite, cytochrome *c*, and quinidine were all purchased from Sigma (Poole, UK). Ampicillin was obtained from Beecham Research (Welwyn Garden City, UK), isopropyl β -D-thiogalactopyranoside and δ -aminolevulinic acid, from Melford Laboratories (Ipswich, UK) and glucose 6-phosphate dehydrogenase (type VII) from Roche Molecular Biochemicals (Lewes, UK). HPLC grade solvents were from Rathburn Chemicals (Walkerburn, UK) and HPLC columns from Agilent (Crawford Scientific, UK). DNA modifying enzymes were obtained from Gibco BRL (Paisley, UK) and Promega (Southampton, UK). Bufuralol, 1'-hydroxy bufuralol and (3*S*)-3'-hydroxyquinidine were purchased from Ultra Fine Chemicals (Manchester, UK). Quinidine N-oxide was a kind gift from Merck Sharp and Dohme

(UK). All other chemicals were from BDH (Poole, UK). Library efficient competent *E. coli* JM109 were purchased from Promega.

Mutagenesis and Expression in *E. coli* - The Glu216 and Asp301 mutants of CYP2D6 used in this study were constructed and expressed in *E. coli* along with human P450 reductase as previously described (17). To obtain the remaining mutants, site directed mutagenesis was performed using the single stranded DNA template method (20), using pB81 as a template and the *duf⁻ ung⁻* *E. coli* strain CJ236 along with an appropriate mutagenic oligonucleotide: Phe120Ala 3'ata gcg cgc cag agg cac ccc ttg gga 5'; Phe481Ala 3'cac cag gaa agc agg gac acc atg gtg 5'; Phe483Ala 3'cac cag gaa agc agg gac acc atg gtg 5'. (*Note:* oligonucleotide sequences are reverse complemented.) Once the presence of the desired mutation was confirmed by automated DNA sequencing, the mutants were co-expressed with human P450 reductase as described above.

Quinidine inhibition of bufuralol 1'-hydroxylation and dextromethorphan O-demethylation - Incubations were carried out in triplicate at 37°C with shaking in 300 μ l of 50 mM potassium phosphate, pH 7.4, containing *E. coli* membranes equivalent to 10 pmol CYP2D6 (wild-type or mutant), quinidine (0, 1, 10 or 100 μ M), an NADPH-generating system (comprising 5 mM glucose 6-phosphate, 1 U glucose 6-phosphate dehydrogenase, 1 mM NADP⁺) and bufuralol or dextromethorphan at concentrations equivalent to the K_M of each sample. The specific substrate concentrations used were: **Bufuralol:** CYP2D6 1.1 μ M; Glu216Gln 188 μ M; Glu216Asp 6 μ M; Glu216Phe 117 μ M; Glu216Ala 162 μ M; Glu216Lys 187 μ M; Asp310Glu 2 μ M; Asp301Gln 142 μ M; Asp301Asn 160 μ M; Glu216Gln/Asp301Gln 522 μ M; Phe120Ala 2.7 μ M; Phe481Ala 10 μ M; Phe483Ala 7.1 μ M; **Dextromethorphan:** CYP2D6 2.6 μ M; Glu216Gln 51 μ M; Glu216Asp 13 μ M; Glu216Phe 30 μ M; Glu216Ala 63 μ M; Glu216Lys 312 μ M; Asp301Glu 11 μ M; Asp301Gln 200 μ M; Asp301Asn 3598 μ M; Glu216Gln/Asp301Gln 438 μ M; Phe120Ala 1 μ M; Phe481Ala 11 μ M; Phe483Ala 9.5 μ M. After a 3-minute pre-incubation at 37°C, reactions were initiated by the addition of the NADPH-generating system and were allowed to proceed for 6 minutes before being stopped by the addition of 15 μ l of 60 % perchloric acid. 100 μ l aliquots of the reaction

supernatant were used for HPLC, separating the bufuralol and dextromethorphan metabolites as previously described (17), using a Hewlett Packard 1100 HPLC and Chemstation software.

Quinidine metabolism - To investigate quinidine metabolism, reaction mixtures consisted of 50 mM potassium phosphate, pH 7.4, containing 100 μ M quinidine, *E. coli* membranes equivalent to 10 pmol CYP2D6 (wild-type or mutant) and an NADPH-generating system (as above) in a total volume of 200 μ l. After a 3-minute pre-incubation at 37°C, reactions were initiated by the addition of the NADPH-generating system and incubated for a further 15 minutes before being stopped with 100 μ l of ice-cold methanol. Samples were left on ice for 10 minutes prior to centrifugation at 16,100g for 10 min. Metabolites were separated by HPLC using a Hypersil C18 BDS column (5 μ m; 250 x 4.6 mm) at a flow rate of 1 ml/min. Mobile phases of acetonitrile (A) and sodium perchlorate / perchloric acid (14.05g of sodium perchlorate and 1.6 ml of 60 % PCA dissolved in 5L of dH₂O) (B) were mixed at a constant ratio of 15 % A: 85 % B (v/v) for the first 5 minutes, then a linear gradient was applied over 4 minutes, ending at 31 % A: 69 % B (v/v) which was maintained for a further 7 minutes. The retention times of (3*S*) 3'-hydroxy quinidine and quinidine N-oxide were established using authentic metabolite standards, with fluorescence detection at $\lambda_{\text{ex}} = 252$ nm; $\lambda_{\text{em}} = 302$ nm.

Identification of the novel quinidine metabolite - Further analysis of the novel quinidine metabolite was undertaken by HPLC with mass spectrometric detection. Twenty five μ L of the stopped incubation was separated on a Luna C₁₈ column (3 μ m, 150 x 2 mm, Phenomenex, Cheshire, UK) with a linear gradient of 5 mM ammonium formate, pH 3.5 (A) and acetonitrile (B) delivered by a Waters 2795 separations module (Waters, Elstree, UK). The gradient ran from 5% to 30% A over 20 min at a flow rate of 200 μ L/min before returning to the starting conditions. The eluent was introduced into the source of a Quattro Micro mass spectrometer (Micromass, Manchester, UK) and was ionised by electrospray ionisation in the positive ion mode. The main parameters were: capillary voltage, 3.3 kV; cone voltage, 30 V; source and desolvation temperatures 100 and 300°C, respectively, and cone and desolvation nitrogen gas flows 90 and 300 Lh⁻¹ respectively.

Collision induced dissociation experiments used argon as the collision gas with a collision energy of 30 eV. Data were acquired and analysed by Masslynx software.

Quinidine binding - Quinidine binding was measured by optical difference spectroscopy of *E. coli* membranes containing CYP2D6 and CPR, using a Cary 4000 UV-vis spectrophotometer. *E. coli* membranes containing wild type or mutant CYP2D6 enzymes were diluted in 100 mM potassium phosphate buffer, pH 7.4, to a final concentration of 0.5 μ M P450 and split into two matched black-walled quartz cuvettes. After running a base line, 1 μ l aliquots of quinidine dissolved in deionised water were added to the sample cuvette and equal volumes of water to the reference cuvette. The samples were left for 2 min between additions to equilibrate, and the difference spectrum was then run between 360-460 nm. The final volume of additions was kept to less than 2.5% of the total volume. Changes in absorbance as a function of quinidine concentration, at wavelengths selected on the basis of the spectral characteristics of the individual sample, were used to calculate binding constants using non-linear regression analysis (Prism). Spectral determinations were performed at least twice for each mutant and found to be reproducible with respect to the spectral profile and the position of λ_{max} and λ_{min} .

Modelling and Molecular Docking - The homology model of CYP2D6 was produced as described previously (14). In brief, the model was produced using the comparative modelling program Modeller (21) with five structural templates: P450s cam (22), terp (23), eryF (24), BM3 (25), and 2C5 (26). Model structures for the mutants Glu216Phe, Glu216Gln/Asp301Gln and Phe120Ala were generated by replacing residues within SYBYL (27) and optimising the positions of the new side chains with the rest of the protein held fixed.

Docking studies were performed using the program GOLD v2.0 (28) with the ChemScore fitness function (29,30) to generate 10 possible binding orientations for quinidine in each of the wild type and mutant CYP2D6 models. The orientations were ranked according to the value of the ChemScore fitness function. In addition tethered dockings were performed by applying constraints to the distances between the O-methyl and -CH=CH₂ groups of quinidine and the haem iron. The docked energy of a solution which

positioned the tethered group more than 4.5 Å away from the haem was penalized, the size of the penalty being determined using a harmonic force constant of 5.0 kJ mol⁻¹ Å⁻².

RESULTS

Inhibition of CYP2D6 mutants by quinidine.

We have investigated the effects of mutations of the active site residues Phe120, Glu216, Asp301, Phe481 and Phe483 on the inhibition of CYP2D6 activity by quinidine. The inhibition profile of a panel of 12 mutants was examined by measuring bufuralol 1'-hydroxylation and dextromethorphan O-demethylation in the presence of 1, 10, or 100 µM quinidine (Figure 1). Many of these mutants affect substrate binding so that, in order to isolate the effects of the mutations on quinidine inhibition, the substrate concentrations used were chosen to be equal to the measured K_M for each specific mutant ((17,31); the concentrations used are given in the Materials and Methods section). For the wild-type enzyme under the conditions of this assay, the lowest concentration of quinidine used, 1µM, leads to >95% inhibition of both bufuralol 1'-hydroxylation and dextromethorphan O-demethylation.

It is clear that the negative charges on Glu216 or Asp301 are important for the inhibitory effect of quinidine. The conservative substitutions Glu216Asp and Asp301Glu showed behaviour similar to wild-type, with >90% inhibition by 1µM quinidine, while enzymes with non-conservative replacements were at least 50 % active at 10µM quinidine. The double mutant Glu216Gln/Asp301Gln, with complete removal of the charge but not the polarity, was found to be strikingly insensitive to inhibition by quinidine, retaining 80% of its bufuralol 1'-hydroxylase activity and 85% of its dextromethorphan O-demethylase activity in the presence of 100µM quinidine. By contrast, alanine substitution of the aromatic side-chains of Phe120, Phe481 or Phe483 had only a minor effect on the inhibition of catalytic activity by quinidine. The effects of the mutations were generally similar for quinidine inhibition of both bufuralol 1'-hydroxylase and dextromethorphan O-demethylase activity, although for most of the mutants quinidine was found to be a somewhat better inhibitor with respect to dextromethorphan than with respect to bufuralol. These observations suggest that the

negative charges of Glu216 and Asp301, but not the aromatic rings of the three phenylalanine residues, are important for the binding of quinidine; this is broadly consistent with the effects of mutation of these residues on the K_M values of substrates containing a basic nitrogen (10,16-18,31,32).

Quinidine binding in Glu216 and Asp301 mutants. The effects of the mutations on quinidine binding were determined directly by measuring optical difference spectra on adding quinidine to bacterial membranes expressing P450. Wild-type CYP2D6 showed a 'type I' binding spectrum on quinidine addition (with λ_{max} and λ_{min} of ~420nm and ~390nm respectively; Figure 2A), characteristic of the change from a low spin to high spin state of the ferric iron that is usually associated with the binding of substrate molecules (11). None of the mutants showed evidence of a 'type II' spectrum, characteristic of direct coordination to the haem iron. The majority showed type I difference spectra (Figure 2A) or variations thereof (Figure 2C), but three showed a different form of spectrum with an increase in absorbance at shorter wavelengths (Figure 2B), suggesting a change in the haem environment¹.

The apparent K_d values for quinidine binding, derived from the dependence of the amplitude of the difference spectrum on quinidine concentration, are shown in Table 1. The alanine substitutions of Phe120, Phe481 and Phe483 led to no more than a factor of two decrease in binding affinity. However, removal of the negative charge from either Glu216 or Asp301 produced clear increases in K_d . Wild type CYP2D6 and the two conservative mutants Glu216Asp and Asp301Glu had K_d values in the range 0.4-0.5 µM, whereas the K_d values for the non-conservative substitutions were at least 30-fold higher, in the range 15-65 µM. Consistent with the inhibitory effects on bufuralol and dextromethorphan metabolism (Figure 1), the largest increase in apparent K_d was observed with the double mutant Glu216Gln/Asp301Gln.

Quinidine metabolism. Both the inhibition

¹ The difference spectra reported here for quinidine binding to the Glu216Gln and Glu216Ala mutants appear to be different from those reported by Guengerich *et al.* (16); in the present work, the difference spectra were recorded using *E. coli* membranes expressing CYP2D6, whereas Guengerich *et al.* (16) used detergent-solubilised purified enzyme, and this may account for the difference.

assays and the direct measurements of binding show that removing the negative charge on residues Glu216 and/or Asp301 produces a major quantitative effect on quinidine binding to CYP2D6. Since quinidine binding produces a type I binding difference spectrum, typical of CYP2D6 substrates, but is not metabolised by wild-type CYP2D6 (5,7-9), we carried out experiments to examine the possibility that some of the mutants might be able to metabolise quinidine. Quinidine was incubated for 15 min with 50 nM wild-type or mutant CYP2D6 and the incubation mixture analysed by HPLC. As shown by the chromatograms in Figure 3, quinidine is clearly metabolised by the three mutants Glu216Phe, Glu216Gln/Asp301Gln and Phe120Ala. Two clear metabolite peaks, with retention times 8.2 minutes (M1) and 8.8 minutes (M2) respectively, were observed; both were produced by Glu216Gln/Asp301Gln and by Phe120Ala, while Glu216Phe produced only the metabolite eluting at 8.8 minutes. The peak with a retention time of 8.2 minutes co-migrates with a standard of (3*S*) 3-hydroxy quinidine, demonstrating that, in contrast to wild-type CYP2D6, the Glu216Gln/Asp301Gln and Phe120Ala mutants, alone among the mutants studied, are able to catalyse the 3-hydroxylation of quinidine. The second metabolite, M2 in Figure 3, formed by Glu216Phe, Glu216Gln/Asp301Gln and Phe120Ala, did not co-migrate with either of the quinidine metabolite standards available to us ((3*S*) 3-hydroxy-quinidine and quinidine N-oxide).

Analysis by mass spectrometry (Figure 4) showed that the molecular ion of M2 had a $m/z=310.7$, a reduction of $m/z=14$ compared to quinidine, suggesting the occurrence of a demethylation reaction. The metabolite M2 was not present in the control reaction (upper chromatogram, Figure 4A). Collision induced dissociation generated the spectrum shown in Figure 4B. The daughter ion of $m/z=174.8$ was assigned to the *O*-demethylated fragment, 4-(hydroxymethyl)-quinolin-6-ol, allowing us to identify M2 as *O*-desmethyl-quinidine.

The observation that quinidine is metabolised by three of the mutants studied here but not by the wild-type enzyme clearly implies that the mode of quinidine binding, and not just its affinity, is affected by these mutations. In order to help us understand the structural basis of this, we have carried out computational docking of quinidine into structural models of the active site

of the wild-type and mutant enzymes.

Modelling of quinidine binding to CYP2D6. Computational docking studies were performed using our previously described model of wild type CYP2D6 (14). Ten solutions for quinidine binding were obtained, all of which are in one cluster having quinidine positioned away from the haem; the orientation of quinidine within the active site in the best ranked solution is shown in Figure 5A. The results from these docking studies for the wild-type enzyme are consistent with the experimental data insofar as they produced no solutions for quinidine binding close to the haem in a position appropriate for catalytic turnover.

The predicted binding mode of quinidine in wild-type CYP2D6 appears to be influenced by π -stacking interactions between the aromatic rings of quinidine and the Phe120 side chain. We therefore carried out docking studies with a model of the Phe120Ala mutant. The ten solutions obtained can be divided into two clusters; the best-ranked solutions from each cluster are shown in Figure 5B & C. The first cluster consists of four solutions in which the quinidine molecule is orientated with its *O*-methyl group positioned above the haem, a mode of binding which would be expected to result in the formation of *O*-desmethyl quinidine (metabolite M2). The second cluster consists of six solutions oriented with the quinidine $-\text{CH}=\text{CH}_2$ group positioned above the haem, consistent with the formation of 3-hydroxy quinidine. Thus, the docking studies with this mutant provide results consistent with the experimental observation that it is able to metabolise quinidine to its *O*-desmethyl and 3-hydroxy derivatives.

By contrast, similar docking studies for the mutants Glu216Phe and Glu216Gln/Asp301Gln produced only solutions in which the quinidine molecule was positioned away from the haem, whereas experimentally these mutants are able to metabolise quinidine. To address this apparent discrepancy we carried out 'tethered docking' of quinidine using the structural model of the Glu216Gln/Asp301Gln mutant. In these calculations first the quinidine $-\text{CH}=\text{CH}_2$ group and then the *O*-methyl group of quinidine were in turn constrained to lie within 4.5Å of the haem iron, to produce dockings with the $-\text{CH}=\text{CH}_2$ positioned consistent with formation of 3-hydroxyquinidine and with the *O*-methyl group positioned consistent with formation of *O*-desmethyl quinidine, respectively. The energies of the $-\text{CH}=\text{CH}_2$

'tethered' solutions were only marginally less favourable than the energies of the solutions from the corresponding dockings without distance constraints (Table 2), indicating that they represented plausible modes of binding. Quinidine made good hydrophobic contacts in the models of the complex with the mutant Glu216Gln/Asp301Gln, but in the O-methyl 'tethered' solutions there were some unfavourable contacts – particularly with residues Phe120 and Phe483 – which resulted in slightly poorer binding (Table 2). Similarly, when docking was performed with the O-methyl group constrained to lie close to the haem in the Glu216Phe mutant, the energies of the 'tethered' solutions were only marginally less favourable than the energies of the solutions from the corresponding dockings without distance constraints (Table 2).

DISCUSSION

Quinidine is a well-established and potent competitive inhibitor of CYP2D6 (5,7-9); indeed inhibition by quinidine is often used as a diagnostic for involvement of CYP2D6 in drug metabolism. However, it has not been clear why quinidine is not a substrate, since it has many of the features which are regarded as characteristic of CYP2D6 substrates, including a basic nitrogen atom and a flat hydrophobic region (12). Docking quinidine into our homology-based model of CYP2D6 (Figure 5) leads to a predicted mode of binding in which it fits into the active site but is too far from the haem for catalytic turnover. Quinidine binding to CYP2D6 produces a classical type I optical difference spectrum (*cf.* Figure 2), indicative of the change from a low spin to high spin state of the ferric iron that usually accompanies the binding of substrate molecules (11) and is associated with the displacement of the water molecule bound to the iron in the 'resting' enzyme, converting the iron from six- to five-coordinate. In the proposed mode of binding of quinidine, the inhibitor is too far from the haem iron to displace the bound water molecule directly. However, it is clear, for example from NMR and crystallographic studies of CYP102A (33,34), that the binding of ligands relatively distant (~9Å) from the haem iron of P450s can lead to the displacement of the bound water molecule.

Previous modeling and mutagenesis work had suggested that two carboxylate groups, of

Glu216 and Asp301, and three phenylalanine residues, Phe120, Phe481 and Phe483, play important roles in determining the binding of substrates in the active site of CYP2D6 (*e.g.*, (10,14-19)). The present work shows that several of these residues also play significant roles in binding the inhibitor quinidine. In terms of the binding constants, the two carboxylate groups are clearly the most important; abolition of one or both of these charges increases the K_d for quinidine by 50- to 100-fold. In the best-scoring docked orientation of quinidine in the active site of the wild-type enzyme, the basic quinuclidine nitrogen is closer to Asp301 than to Glu216, although the effects of substituting either residue with the corresponding amide are very similar. The fact that the double mutant Glu216Gln/Asp301Gln shows significantly weaker binding than either single mutant would support the idea that the electrostatic field of both residues is significant for quinidine binding. A recent study (13) of a number of analogues of quinidine found that alkylation of the quinuclidine nitrogen with groups as bulky as naphthyl had no effect on the measured IC_{50} , and it was concluded that the proposed charge-charge interaction with Asp301 does not make a major contribution to binding. This is clearly at variance with our conclusions from the current mutagenesis studies. However, it must be recognised that, particularly for the cytochromes P450, interpretations of the effects of altering either the protein or the ligand are complicated by the possibility – indeed the likelihood – that these changes will lead to an altered mode of binding.

For several of the mutants studied here, it is clear that there is indeed a change in the mode of binding of quinidine, since it becomes a substrate rather than an inhibitor. The Phe120Ala mutant and the double mutant Glu216Gln/Asp301Gln each form both 3-hydroxy-quinidine and O-desmethoxy-quinidine in significant quantities. 3-hydroxy-quinidine is a major product of quinidine metabolism by CYP3A4 (7), and indeed this reaction has been suggested as a specific marker for CYP3A4 in human liver microsomes (35). We showed earlier that this double mutant of CYP2D6 is able to catalyse another characteristic CYP3A4 reaction, the N-oxidation of nifedipine (17) and concluded that Glu216 and Asp301 have central roles in defining the specificity of CYP2D6. We also noted the possibility that the effects of mutation of Asp301 are indirect, since

in our model (14) the side-chain of this residue interacts with the backbone of the B'-C loop, thus helping to position this loop, including Phe120, in the active site – and indeed the Phe120Ala mutant metabolises quinidine to the same two products as the Glu216Gln/Asp301Gln mutant. However, substitution of Asp301 alone is not sufficient to enable the CYP2D6 to metabolise quinidine, and Glu216 clearly plays an important role in determining the mode of binding. This is emphasised by the fact that substitution of this residue by a bulky side-chain, in the Glu216Phe mutant, confers on CYP2D6 the ability to catalyse the O-demethylation of quinidine and also to some extent the 6 β -hydroxylation of testosterone, another characteristic CYP3A4 reaction (17).

To obtain a structural picture of the possible changes in mode of binding of quinidine in the mutants, we have docked quinidine into structural models of the mutants, obtained by simple side-chain substitution in our earlier model of the wild-type enzyme (14). The model of the Phe120Ala mutant immediately gave results consistent with the experimental observations, in that the quinidine was predicted to bind closer to the haem than in the wild-type model, with the most favorable modes of binding predicted to be those corresponding to 3-hydroxylation and O-demethylation, the observed routes of metabolism (Figure 5). Within the limitations of the model, this indicates a direct role of the Phe120 side-chain in determining the unproductive mode of binding of quinidine to CYP2D6.

With the mutants Glu216Gln/Asp301Gln and Glu216Phe, on the other hand, simple docking calculations did not predict a productive mode of binding for quinidine. However, when appropriate distance constraints were introduced into the docking calculations, solutions were obtained which were consistent with the formation of the experimentally observed metabolites. The calculated energies of these solutions were somewhat higher than those of the unconstrained solutions, due largely to unfavourable interactions with the side-chains of Phe120 and Phe483. It is possible that in these mutants quinidine binds most of the time in a non-productive mode, but that productive mode(s) of binding are accessible

and lead to the observed turnover. However, the limitations of the models should be recognised. Residues Phe120 and Phe483, which appear to interact unfavourably with quinidine in the models of these two mutants, are both located in loops, in SRS1 and SRS6 respectively. Loop regions are often highly flexible and therefore difficult to represent with either a single model or a single crystal structure. The docking program we have used (Gold v2.0; (28,36)) allows the ligand full translation and rotational freedom but, like most available docking programs, it treats the protein as rigid. Thus, it may be that the flexibility in these loops is such that the two phenylalanine rings can move away from the bound quinidine, making the productive mode of binding the most favorable. Particularly in P450s, the problem of receptor flexibility is a significant challenge in predicting ligand binding. One approach we are investigating is consensus docking or the use of multiple receptor conformations, which has been applied successfully to the problem of predicting binding modes and binding affinities of inhibitors where multiple crystal structures are available (37).

The results of these mutagenesis experiments clearly show that residues Phe120, Glu216 and Asp301 are important in determining the mode of binding of quinidine to CYP2D6, particularly in determining whether it binds in a non-productive mode, as in the wild-type, and is thus an inhibitor, or whether it binds productively. They also demonstrate that changes in affinity and in mode of binding do not necessarily go hand in hand: in the Phe120Ala mutant a change in mode of binding, indicated by the ability to metabolise quinidine, is not accompanied by any change in K_d , whereas in the Glu216Lys mutant a 70-fold increase in K_d is not accompanied by a change in mode of binding sufficient to allow quinidine to be metabolised. A particularly key role is indicated for Phe120, since substitution of this single residue can allow CYP2D6 to metabolise its 'classical' inhibitor quinidine with no decrease in binding affinity, and the docking calculations strongly suggest that this residue has a direct effect in forcing quinidine to bind in an unproductive mode in CYP2D6.

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TABLE 1

Equilibrium dissociation constants for quinidine binding to wild type and mutant CYP2D6

Enzyme	$K_d(\text{apparent})$ (μM)	λ_{max} (nm)	λ_{min} (nm)	ΔA_{max}
CYP2D6	0.47 ± 0.03	389	419	0.044 ± 0.0003
E216Q	25.72 ± 2.3	403	424	0.008 ± 0.0003
E216D	0.38 ± 0.07	390	422	0.011 ± 0.0003
E216F	15.13 ± 0.9	405	427	0.040 ± 0.0001
E216A	21.76 ± 1.2	403	427	0.051 ± 0.001
E216K	32.31 ± 2.2	404	426	0.010 ± 0.0003
D301E	0.54 ± 0.04	389	424	0.013 ± 0.0003
D301Q	18.0 ± 2.0	404	425	0.015 ± 0.0007
D301N	24.70 ± 1.2	404	427	0.055 ± 0.0002
E216Q/ D301Q	64.94 ± 9.1	402	428	0.037 ± 0.003
F120A	0.8 ± 0.1	390	423	0.037 ± 0.002
F481A	0.4 ± 0.07	390	424	0.012 ± 0.0007
F483A	0.7 ± 0.06	390	424	0.014 ± 0.0005

TABLE 2

ChemScore values of the best solutions for quinidine docking into wild type and mutant CYP2D6 models.

Model	Restrains	ChemScore value of the best ranked docked quinidine solution (kJ/mol)
Wild Type CYP2D6	none	-38.5
F120A	none	-38.4 ^a -33.0 ^b
E216F	none	-37.6
	O-methyl tether	-37.5
E216Q/D301Q	none	-39.9
	-CH=CH ₂ tether	-38.5
	O-methyl tether	-32.7

^a Best ranked quinidine in cluster docked with O-methyl group positioned for formation of O-desmethyl quinidine (metabolite M2).

^b Best ranked quinidine in cluster docked with -CH=CH₂ group positioned for formation of 3-hydroxy quinidine (metabolite M1).

FIGURE LEGENDS

Figure 1. Inhibition of bufuralol 1' hydroxylase and dextromethorphan O-demethylase activity by 1, 10 and 100 μ M quinidine. Samples were run in triplicate under conditions described in "Materials and Methods", at bufuralol and dextromethorphan concentrations equivalent to the K_M for each protein. *A*, Bufuralol 1' hydroxylase; *B*, Dextromethorphan O-demethylase.

Figure 2. Representative optical difference spectra produced by quinidine binding to wild type and mutant CYP2D6. *A*, Type I binding spectra typical of wild type and Glu216Phe, Glu216Ala, Asp301Glu, Asp301Asn, Phe120Ala, Phe481Ala and Phe483Ala. *B*, spectra typical of Glu216Gln, Glu216Lys and Glu216Gln/Asp301Glu. *C*, spectra typical of Glu216Asp and Asp301Gln. Experiments were performed as described in Materials and Methods.

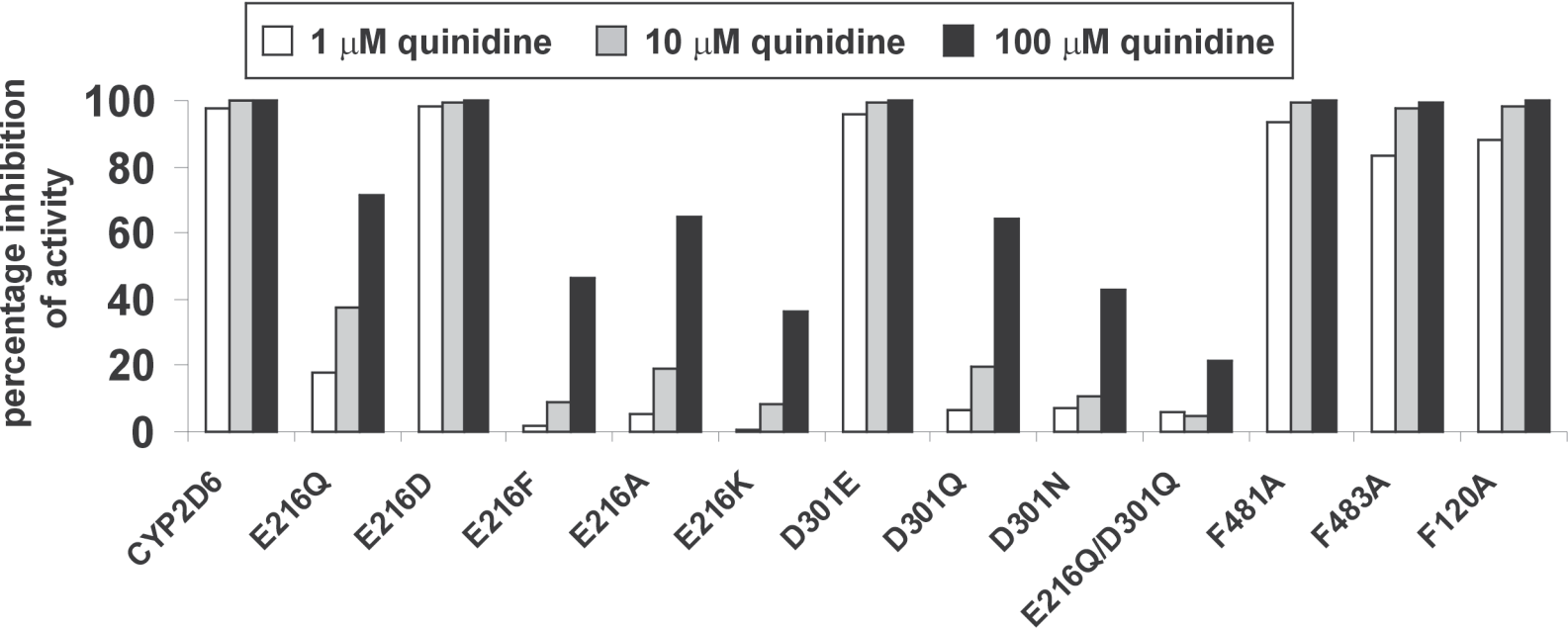
Figure 3. Quinidine metabolism by wild type and mutant CYP2D6. Samples were prepared and analysed as described in "Materials and Methods"; the resulting HPLC chromatograms are shown. Solid lines represent the reaction profiles and the dotted lines are the control reactions (without the NADPH-generating system). Metabolites M1 and M2 discussed in the text are indicated.

Figure 4. Mass spectrometric identification of quinidine metabolite M2. *A*, extracted ion chromatograms ($m/z=311$) of the *O*-desmethyl quinidine metabolite (M2) produced by the Glu216Gln/Asp301Gln mutant enzyme, and *B*, the MS/MS spectra and (inset) structure of the metabolite. The upper chromatogram in *A* is the control reaction in the absence of NADPH. The assignment of the fragment ion $m/z=175$ following collision induced dissociation is shown on the inset structure.

Figure 5. The predicted binding modes of quinidine in wild type and F120A CYP2D6. *A*, the best ranked docking of quinidine in the wild type CYP2D6 model is shown. The haem and residues F120, E216 and ASP301 are highlighted. *B*, the best ranked docking into the F120A CYP2D6 model from the cluster of solutions having an orientation appropriate for formation of *O*-desmethyl quinidine (metabolite M2). *C*, the best ranked docking into the F120A CYP2D6 model from the cluster of solutions having an orientation appropriate for formation of 3-hydroxy quinidine (metabolite M1).

Figure 1

A



B

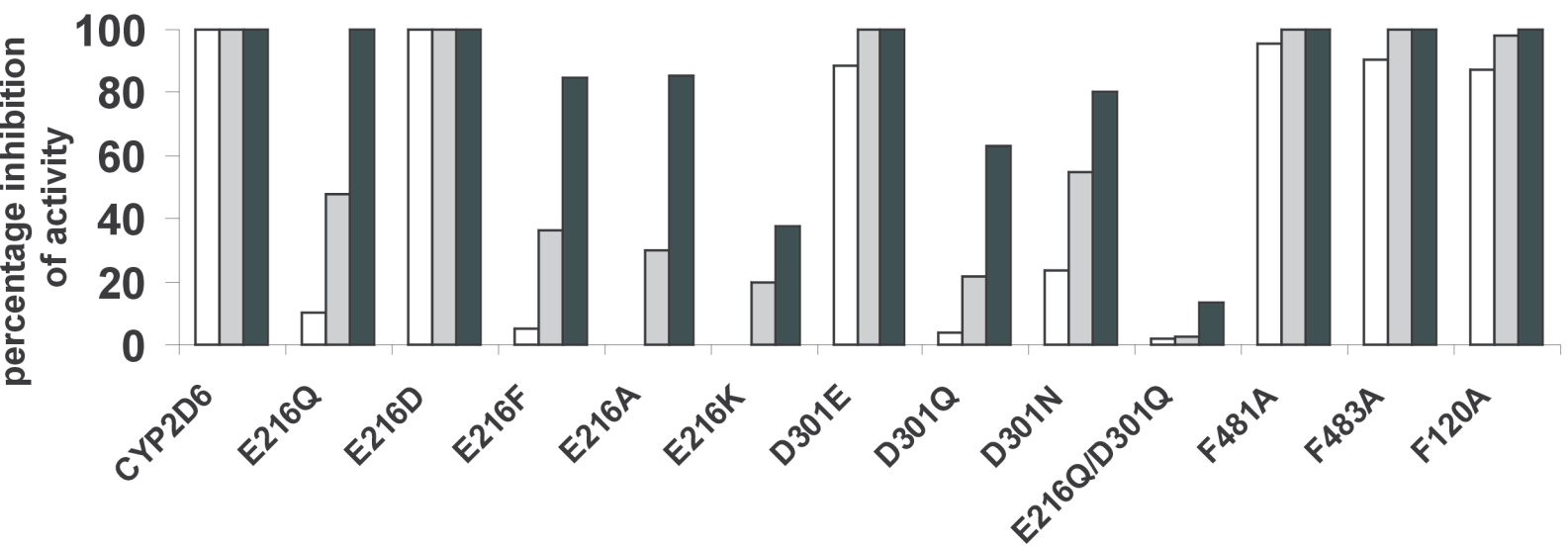


Figure 2

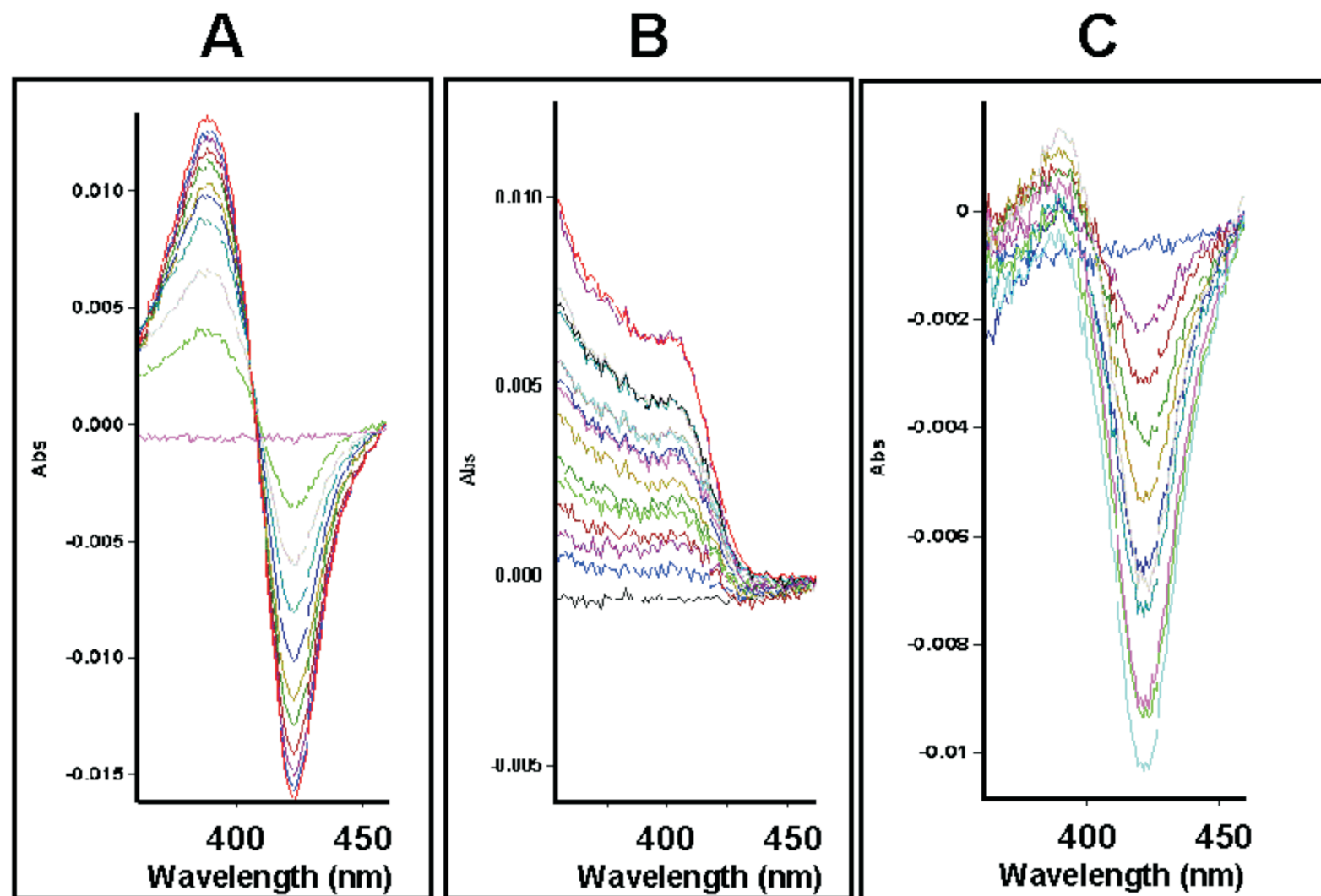


Figure 3

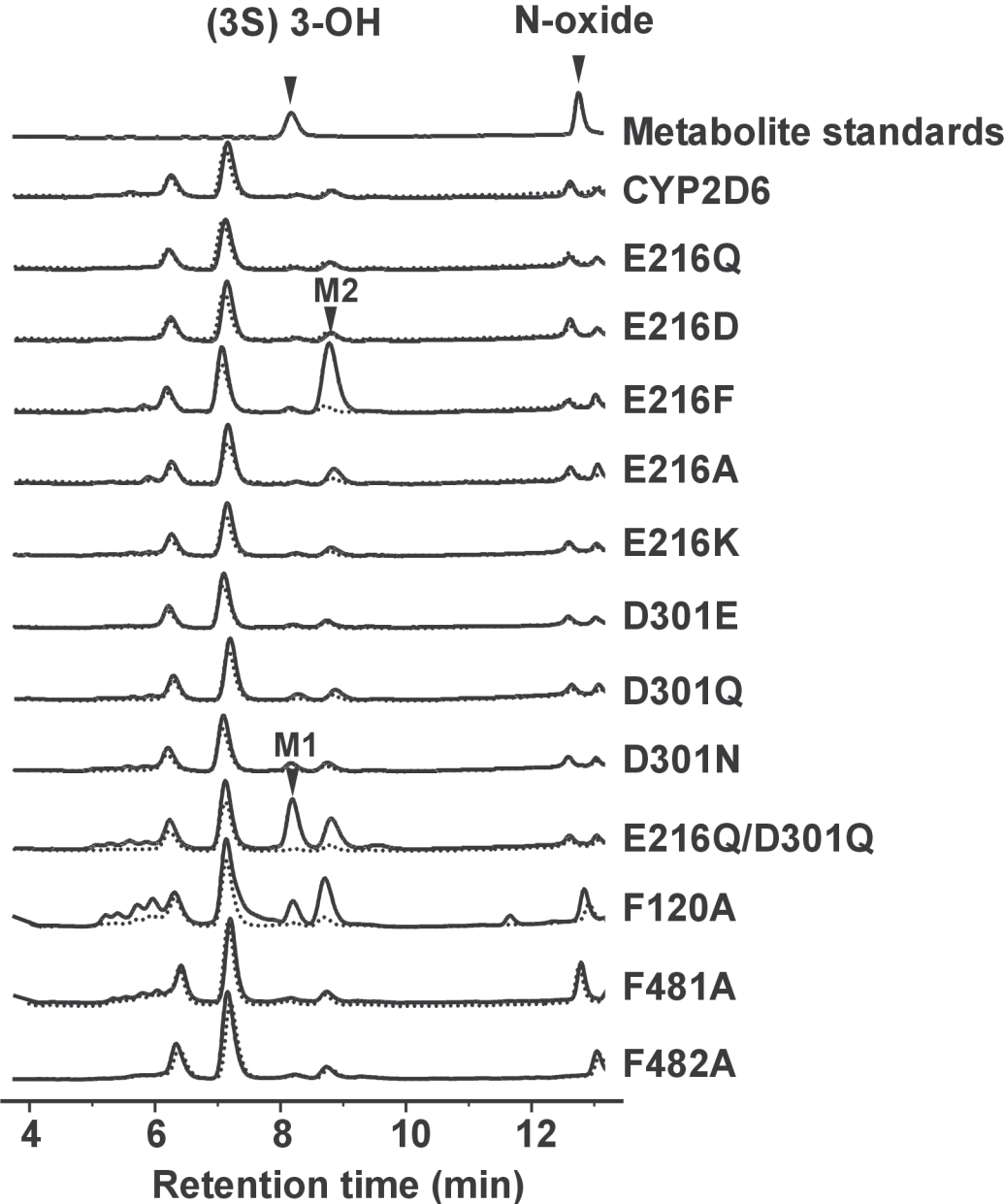


Figure 4

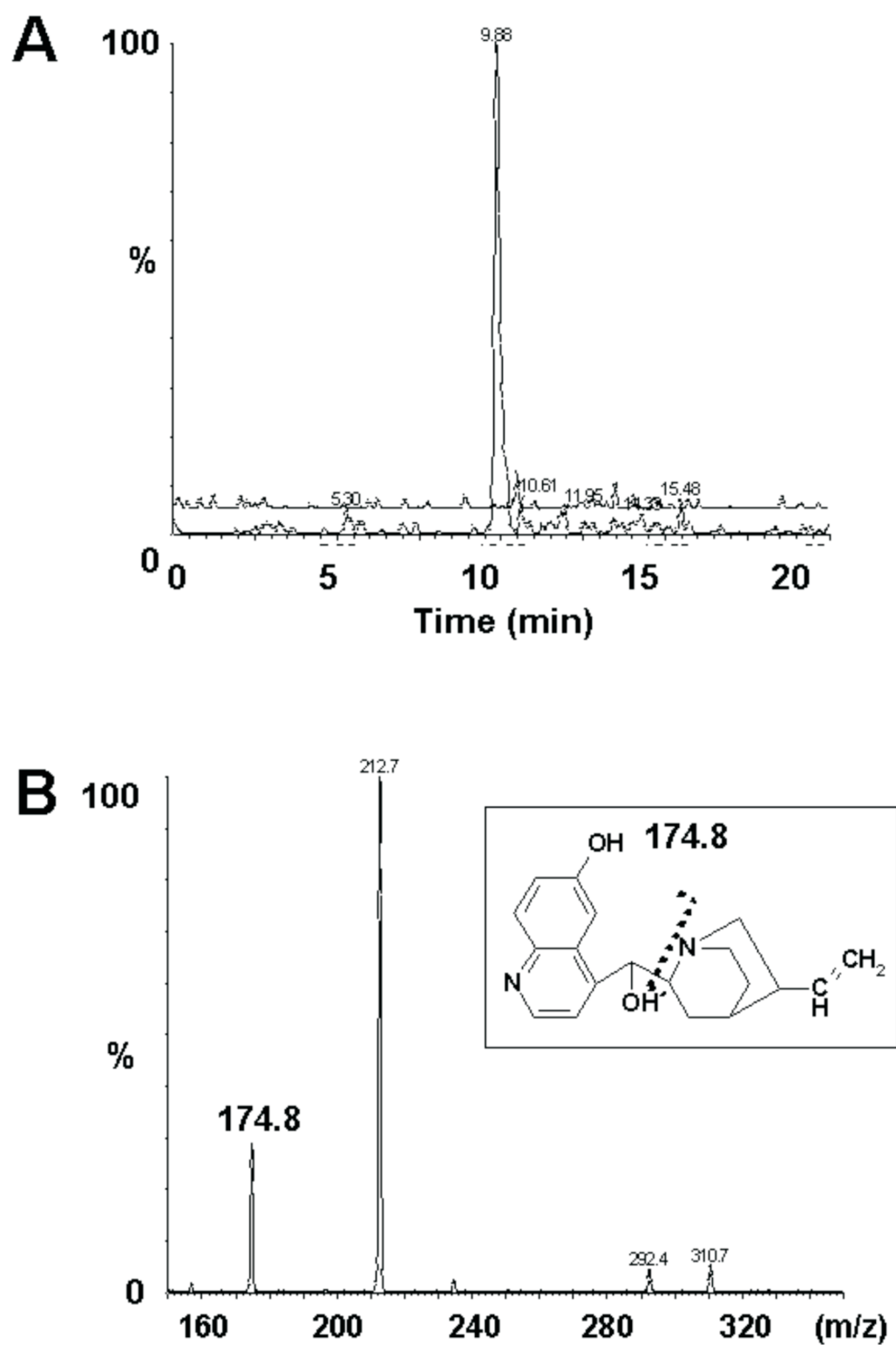
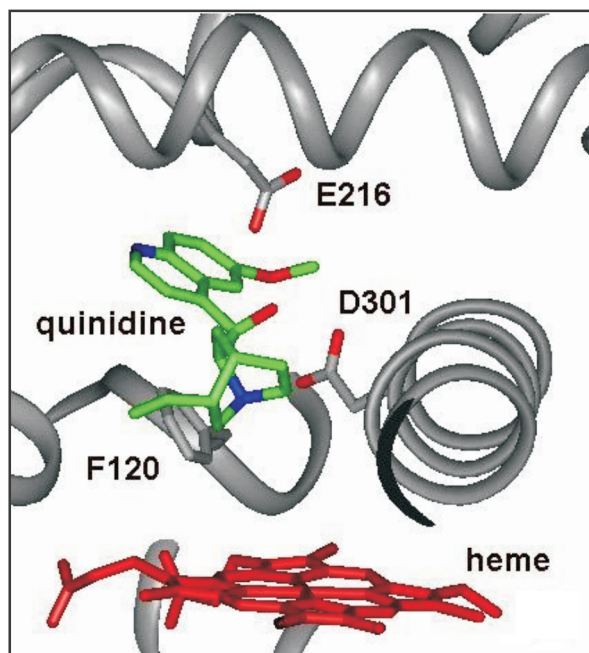
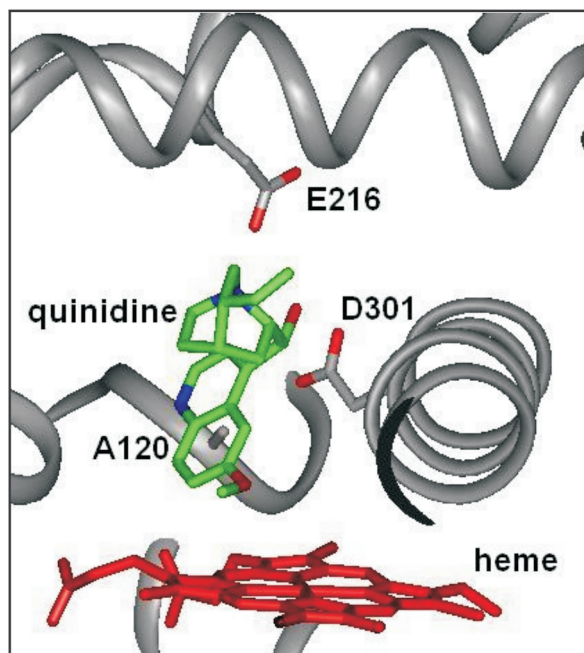


Figure 5

A



B



C

