Multiple substrate binding by cytochrome P450 3A4: estimation of the number of bound substrate molecules

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Abbreviations: CYP3A4, cytochrome P450 3A4; 7BQ, 7-benzyloxyquinoline; $n_H$, Hill coefficient; $S_{0.5}$, concentration of substrate giving half maximal rate of catalysis
Abstract

Cytochrome P450 3A4, a major drug metabolising enzyme in man, is well known to show non-Michaelis-Menten steady-state kinetics for a number of substrates, indicating that more than one substrate can bind to the enzyme simultaneously, but it has proved difficult to obtain reliable estimates of exactly how many substrate molecules can bind. We have used a simple method involving studies of the effect of large inhibitors on the Hill coefficient to provide improved estimates of substrate stoichiometry from simple steady-state kinetics. Using a panel of eight inhibitors, we show that at least four molecules of the widely-used CYP3A4 substrate 7-benzyloxyquinoline can bind simultaneously to the enzyme. Computational docking studies show that this is consistent with the recently reported crystal structures of the enzyme. In the case of midazolam, which shows simple Michaelis-Menten kinetics, the inhibitor effects demonstrate that two molecules must bind simultaneously, consistent with earlier evidence, whereas for diltiazem the experiments provide no evidence for the binding of more than one molecule. The consequences of this ‘inhibitor-induced cooperativity’ for the prediction of pharmacokinetics and drug-drug interactions are discussed.
Cytochrome P450 3A4 (CYP3A4) (Guengerich, 2005) has extremely broad substrate specificity and is responsible for the metabolism of ~50% of drugs in current use. It shows complex (non-Michaelis-Menten) steady-state kinetics, including homotropic and heterotropic cooperativity and substrate inhibition (Atkins et al., 2001; Atkins, 2005). These atypical kinetic effects vary considerably from one substrate to another, and the heterotropic effects can be non-reciprocal; for example, nifedipine is a potent inhibitor of testosterone metabolism by CYP3A4, but testosterone has no effect on nifedipine metabolism (Galetin et al., 2003). A substantial number of reports have shown that many of these effects can be rationalised by models involving the simultaneous binding of two (or perhaps three) substrate molecules to the enzyme (for reviews see (Shou et al., 2000; Atkins et al., 2001; Atkins, 2005)). Most discussions of these cooperative kinetic effects propose that the binding of a second molecule within the active site increases turnover by increasing the fraction of the first substrate molecule bound in a productive orientation. However, genuinely ‘allosteric’ models, in which the effector molecule binds at a remote site and produces its effects by inducing a conformational change in the protein, must also be considered.

In the case of CYP3A4, steady-state kinetic experiments support the idea of more than one substrate binding in the active site (for reviews see (Atkins et al., 2001; Atkins, 2005)), as do the effects of site-directed mutagenesis (Harlow and Halpert, 1998), and there is clear evidence for cooperativity in substrate binding as well as in catalytic turnover (Baas et al., 2004; Roberts et al., 2005; Fernando et al., 2006). Furthermore, a number of spectroscopic studies have provided compelling evidence that more than one molecule can bind within the CYP3A4 active site (Dabrowski et al., 2002; Cameron et al., 2005; Roberts et al., 2005; Fernando et al., 2006; Lampe and
Atkins, 2006). The evidence suggests that binding of the second substrate molecule produces most of the change in spin state of the iron (Baas et al., 2004; Roberts et al., 2005; Fernando et al., 2006).

One of the problems in the investigation of the mechanisms underlying non-Michaelis-Menten kinetic behaviour of P450s is the real difficulty in establishing how many molecules of a given substrate can bind simultaneously to the active site. There are practical problems in the use of traditional methods such as equilibrium dialysis (Guengerich, 2005), but very recently good estimates of stoichiometry of ligand binding to CYP3A4 have been obtained by novel optical titration methods (Fernando et al., 2006) and by calorimetry (Isin and Guengerich, 2006). By contrast, kinetic experiments, which are important in understanding the implications of the non-Michaelis-Menten behaviour for drug metabolism, provide much less clear-cut information on stoichiometry. As is well known, the Hill coefficient (describing the degree of sigmoidicity of the curve of velocity as a function of substrate concentration) gives only a lower limit to the number of substrates binding to the enzyme. Reported values of the Hill coefficient for CYP3A4 catalysis generally range from 1.0 to 1.7, consistent with the binding of two substrate molecules, but values of 2.24 for testosterone (Baas et al., 2004) and 3.6 for aflatoxin B1 (Ueng et al., 1997) indicate that higher stoichiometries are possible.

In this report we investigate the use of a simple method, based upon an early “ligand exclusion” theory of allosteric effects (Fisher, 1970) and involving studies of the effect of inhibitors on the Hill coefficient, as a route to a more reliable estimate of substrate stoichiometry from simple steady-state kinetics. We use this to study several
substrates and show that, for example, as many as five molecules of the widely-used 
CYP3A4 substrate 7-benzyloxyquinoline can bind simultaneously to the enzyme, and 
that this is consistent with the crystal structure of the enzyme. The Hill coefficient for 7-
benzyloxyquinoline is only 1.74, emphasising that this parameter can substantially 
underestimate the number of substrate molecules which can bind. Using kinetic 
simulations we demonstrate that such multiple binding can affect drug-drug interactions, 
making multiply binding compounds stronger inhibitors or, as substrates, more resistant 
to inhibition. Our approach will be useful in determining the true stoichiometry of ligand 
binding with CYP3A4, and in improving the prediction of CYP3A4 drug interactions and 
pharmacokinetics.
Materials and Methods

Materials. Glucose-6-phosphate, NADP⁺, bromocriptine, cyclosporine A, erythromycin, troleandomycin, ketoconazole, diltiazem and testosterone were supplied by Sigma (Poole, UK). Glucose 6-phosphate dehydrogenase (type VII) was provided by Roche Molecular Biochemicals (Lewes, UK). 7- Benzyloxyquinoline (7BQ) was obtained from GENTEST Corporation (Woburn, MA). 1′-hydroxy- and 4-hydroxy-midazolam were purchased from Ultra Fine Chemicals (Manchester, UK). Midazolam was a kind gift from Hoffman La Roche. All other chemicals were from BDH (Poole, UK). Individual human liver microsomes (10 mg/ml total protein) were obtained from the UK Human Tissue Bank (Leicester). E. coli membranes expressing human CYP3A4 and human P450 reductase were prepared as described previously (Maréchal et al., 2006).

Fluorimetric assay with 7-benzyloxyquinoline. Reactions were conducted in white 96 well microtiter plates (Thermo Electro Corporation, Vantaa, Finland). 155 μL of 50 mM HEPES buffer, pH 7.4, containing 30 mM MgCl₂, 150 mM KCl and 12.5 pmol CYP3A4 was added to each well, followed by 5 μL of a solution of 7-BQ in acetonitrile, to obtain final concentrations of 10-400 μM substrate. The enzyme-substrate mixture was pre-incubated for 5 min at 37°C and reactions were then initiated by injection of 40 μL of an NADPH regenerating system (NADP⁺, 4mg/ml; glucose-6-phosphate, 7mg/ml; glucose-6-phosphate dehydrogenase, 5U/ml, in HEPES buffer) pre-warmed for 5 min at 37°C. Rates of product formation were measured using a Fluoroskan Ascent FL microtitre plate reader (λₑₓ = 405, λₑᵐ = 530 nm). Accumulation of the product was linear over the reaction time. Reaction rates were calculated using Ascent software v2.1.
For measurements in the presence of inhibitors, compounds were dissolved in methanol and appropriate concentrations included in the reaction mixture; the rate of 7BQ oxidation was measured simultaneously with a methanol only solvent control. Total methanol content in the final reaction mixtures was 1%. For IC\textsubscript{50} measurements, rates were measured for serial dilutions of each inhibitor across eleven wells, in 200 μL final volume of HEPES buffer containing 12.5 pmol CYP3A4 and 34 μM 7BQ. Activity was determined from the linear phase of the reaction plot and expressed as a percentage of the activity determined from the solvent-only control. The total organic solvent concentration (acetonitrile plus methanol) was equal in control incubations and in incubations with the inhibitors. Data from the control incubations (with no inhibitor) resulted in S\textsubscript{50} and Hill coefficient values in good agreement with published data (Stresser et al., 2002), suggesting minimal if any solvent effect. No interference of the inhibitory compounds with the fluorescence of the BQ oxidation product was observed under the reaction conditions. All inhibitors tested were used at high (>10K\textsubscript{i}) concentrations; considering the short reaction times used (< 5min), a negligible consumption of the inhibitors during the reaction was assumed. Data were plotted and IC\textsubscript{50} values were calculated using GraFit software v.5 (Erithacus Software, UK). In experiments with human liver microsomes the final protein concentration was 0.18 mg/ml for samples HLM2 and HLM5, and 0.27 mg/ml for samples HLM3 and HLM4.

Midazolam and Diltiazem assays – 200 μl reactions were performed in 50 mM HEPES buffer, pH 7.4, containing 30 mM MgCl\textsubscript{2}, 150 mM KCl, 10 pmol CYP3A4, an NADPH generating system (as above), and varying substrate (midazolam or diltiazem) concentrations, with erythromycin concentrations of 400 μM and 200 μM for midazolam
and diltiazem respectively. Incubations were carried out at 37°C in opaque Eppendorf tubes and stopped after 4 min (for midazolam) or 6 min (for diltiazem) with 100 μL of ice-cold methanol, incubated on ice for 10 min, then centrifuged at 16,000 g for 10 min to remove particulate matter. 100 μL of the reaction supernatant was assayed for the presence of metabolites by reversed-phase HPLC using an Agilent 1100 Chemstation (Agilent Technologies, UK). Assays included negative control incubations lacking the NADPH regenerating system, as well as solvent only controls for erythromycin. The midazolam metabolites 1'- and 4-hydroxymidazolam were separated using an Agilent Technologies Hypersil ODS column (5 mm pore size; 125 mm x 4 mm) at 25°C under isocratic conditions using 10 mM ammonium acetate (pH 5.0)/methanol/acetonitrile 36/55/9 (v/v/v) with a flow rate of 1 ml/min. 1'- and 4- hydroxymidazolam were detected by UV absorbance at 235 nm and quantified using standard solutions. Midazolam hydroxylation showed good linearity during the reaction time (3 min). The desmethyl diltiazem metabolite was separated using a Agilent C18 BDS (5 mm pore size; 4.6 mm x 250 mm) at 25°C under isocratic conditions using acetonitrile/ammonium acetate (0.1 M, pH 5.8), 35/65 (v/v) and detected by UV absorbance at 238 nm. In the absence of a metabolite standard, the identity of the NADPH-dependent product peak was confirmed to be desmethyldiltiazem by LC-MS/MS. Rates were quantified from the area of the product peak, following background subtraction from a negative control sample. The time course of the product generation was linear for at least 10 min for both the lowest and the highest diltiazem concentrations used.

**Analysis of kinetics of 7BQ turnover.** As discussed in the Results section, the kinetics of 7BQ turnover show both a sigmoidal dependence of rate on substrate
concentration and substrate inhibition at higher substrate concentrations; a number of kinetic models have been used to analyse behaviour of this kind in cytochrome P450s (e.g., (Korzekwa et al., 1998; Shou et al., 2000; Atkins, 2005)). We used three different kinetic models to analyse the data for 7BQ, shown in Schemes 1 and 2.

First we used a model involving the simultaneous binding of two substrate molecules, one in a productive mode, presumed to be near the haem, and the second in a non-productive mode (Scheme 1). The binding of this second molecule may affect $V_{\text{max}}$, by a factor $\alpha$, and/or the interaction between the two substrate molecules may affect the dissociation constants $K_{s1}$ and $K_{s2}$, by a factor $\beta$. The rate of reaction is given by:

$$
\frac{v}{V_{\text{max}}} = \frac{[S] + \frac{\alpha [S]^2}{\beta K_{s2}}}{K_{s1} + [S] \left(1 + \frac{K_{s1}}{K_{s2}}\right) + \frac{[S]^2}{\beta K_{s2}}}
$$

If $\beta < 1$, substrate binding is cooperative, and if $\alpha < 1$, substrate inhibition is observed.

We also considered two models involving the binding of three substrate molecules. The first (scheme 2A) involves the cooperative binding of two substrate molecules, with a cooperativity factor $\beta < 1$ (but no effect on $V_{\text{max}}$), and in addition binding of a third substrate molecule to form a quaternary, non-productive, complex, leading to substrate inhibition. This gives the equation:
\[
\frac{v}{V_{\text{max}}} = \frac{[S]}{K_{s1}} + \frac{[S]^2}{\beta K_{s1}K_{s2}} + \frac{[S]^3}{\beta K_{s1}K_{s2}K_{s3}}
\]

The second ‘three-substrate’ model (scheme 2B) is a more general version of Scheme 2A, in which the quaternary complex can yield product at the rate of \(\alpha V_{\text{max}}\) \((\alpha < 1)\), giving:

\[
\frac{v}{V_{\text{max}}} = \frac{[S]}{K_{s1}} + [S]^3 \left( \frac{1}{\beta K_{s1}K_{s2}K_{s3}} \right) + \frac{\alpha [S]^3}{\beta K_{s1}K_{s2}K_{s3}}
\]

Analysis of kinetic effects of inhibitors. The extension of the two-substrate model of scheme 1 to incorporate an inhibitor which binds competitively with both substrates is shown in scheme 3. The equation describing this model is:

\[
\frac{v}{V_{\text{max}}} = \frac{[S]}{K_{s1}} + \frac{\alpha [S]^2}{\beta K_{s2}} + \frac{[S]}{K_{s1}} + \frac{[K_{s2}]}{[S]} + \frac{[S]^2}{\beta K_{s2}}
\]

It is important to recognise that, as originally pointed out by Fisher (Fisher, 1970), even when there is no interaction between the two substrates \((\alpha = \beta = 1)\), the addition of an inhibitor which competes simultaneously with both substrate molecules leads to
sigmoidicity of the plot of $v$ versus $[S]$ (Fisher, 1970; Segel, 1975) and, correspondingly, to increasing curvature of the Eadie-Hofstee plot (Figure 1A).

The behaviour of enzymes showing kinetic behaviour characteristic of homotropic cooperativity is commonly described empirically by the Hill equation:

$$\frac{v}{V_{\text{max}}} = \frac{[S]^{n_H}}{S_{0.5}^{n_H} + [S]^{n_H}}$$

(5)

where $S_{0.5}$ is the substrate concentration giving $v = V_{\text{max}}/2$ ($= K_M$ if $n_H = 1$) and $n_H$ is the Hill coefficient. As is well known, $n_H$ is less than or equal to $n$, the number of substrate molecules which can bind simultaneously to the enzyme; it is only equal to $n$ if the population of all the enzyme-substrate complexes containing less than $n$ substrate molecules is negligible ('infinite cooperativity'; see (Segel, 1975)). However, addition of sufficient concentrations of an inhibitor which competes for all substrate binding sites (as in scheme 3) leads to $n_H \rightarrow n$, even if there is no cooperativity between the substrates (Segel, 1975); this is illustrated in Figure 1B. Measurements of the Hill coefficient in the presence of saturating concentrations of a large inhibitor molecule therefore provide a method for obtaining an improved estimate of $n$.

In the case of 7-BQ, substrate inhibition is observed in addition to homotropic cooperativity, so that a modification to the simple Hill equation is required. Similar behaviour has been observed for, e.g., *E. coli* aspartate transcarbamylase (Passtrallandis et al., 1978; LiCata and Allewell, 1997) and *M. tuberculosis* D-3-phosphoglycerate dehydrogenase (Burton et al., 2007), and the appropriate modified Hill equations for partial uncompetitive substrate inhibition have been described (Passtrallandis et al., 1978; LiCata and Allewell, 1997).
In the present case we have fitted the data using the equation

\[
\frac{v}{V_{\text{max}}} = \frac{[S]^{n_H}}{S_{0.5}^{n_H} + [S]^{n_H} + \frac{[S]^{n_H+1}}{K_{i,s}}} 
\]

This is a simplified version of the general equation described by LiCata and Allewell (1997) which allows for partial inhibition and for the binding of more than one inhibitory substrate molecule. For CYP3A4, in the absence of any evidence to the contrary, we assume that substrate inhibition is complete (that is, the velocity tends to zero at sufficiently high substrate concentration) and that a single substrate molecule binds to an inhibitory site with an apparent dissociation constant \(K_{i,s}\), leading to equation 6. As shown in the Results section, this equation provides a good empirical description of the data within the substrate concentration range used. In the absence of cooperativity \((n_H = 1)\), equation 6 reduces to the standard equation of uncompetitive substrate inhibition (Cleland, 1979; Segel, 1975):

\[
\frac{v}{V_{\text{max}}} = \frac{[S]}{S_{0.5} + \frac{[S]^2}{K_{i,s}}} = \frac{1}{1 + \left( \frac{K_M}{[S]} \right) + \left( \frac{[S]}{K_i} \right)} 
\]

To establish the appropriate concentration of inhibitor to use, we determined the apparent \(K_i\) value, \(K_{i,\text{app}}\), by measuring the inhibition of 7BQ turnover at 34 \(\mu\)M 7BQ by various concentrations of inhibitor and interpolating to determine its \(IC_{50}\), the
concentration which produced 50% inhibition; under these conditions where the 7BQ concentration is approximately equal to its $S_{0.5}$, $K_{i,app} \sim IC_{50}/2$.

**Molecular Modelling.** The 7BQ structure was downloaded from the ChemIDplus website (http://chem.sis.nlm.nih.gov/chemidplus/) and minimized using the Tripos force field in SYBYL (Molecular Modelling Software Package, Version 6.8, Tripos Associates Inc.). The two ligand free CYP3A4 crystal structures (1tqn (Yano et al., 2004) and 1w0e (Williams et al., 2004)) were downloaded from the Protein Data Bank (PDB) (Berman et al., 2000) and prepared for docking using the Sybyl 6.8 package. Docking calculations were carried out using the program GOLD version 3.0.1 (Verdonk et al., 2003); in each calculation, fifty solutions were generated and only those with the best scores (predicted binding energies) were retained.
Results

Kinetics of de-benzylation of 7-benzyloxyquinoline by CYP3A4. The de-benzylation of 7-benzyloxyquinoline (7BQ) can readily be followed fluorimetrically, since the product 7-hydroxyquinoline is quite strongly fluorescent; this is the basis of the use of 7BQ as a test substrate for CYP3A4 (e.g., (Stresser et al., 2002)). The steady-state kinetics of this reaction have previously been demonstrated to follow a sigmoidal saturation curve with a Hill coefficient in the range of 1.4 – 1.9 (Stresser et al., 2000; Stresser et al., 2002). In our hands, the kinetics showed both sigmoidal (cooperative) behaviour at low substrate concentrations and substrate inhibition at high (>150 μM) substrate concentrations (Figure 2). As described in the Experimental section, we analysed this data using three different mechanistic models (schemes 1 & 2); the results of fitting equations 1-3 to the data in Figure 2 are given in Table 1. Each of the three equations was able to describe the data reasonably well but statistical comparison (Table 1) shows that of these three equation 3, involving the binding of three substrate molecules, one to the catalytic site and two to effector sites (scheme 2B), gives the best fit and this is shown in Figure 2.

The kinetic data thus indicate that at least three molecules of 7BQ can bind simultaneously to the active site of CYP3A4. The same conclusion can be drawn from fitting the data empirically with the modified Hill equation (eq. 6). In the course of the experiments with inhibitors described below, control experiments gave eight estimates of the \( S_{0.5} \) value (apparent \( K_m \)) and Hill coefficient (\( n_H \)) for 7BQ in the absence of inhibitor (Table 2). The average values obtained are \( S_{0.5} = 41 \, \mu M \) and \( n_H = 1.74 \) (the estimated substrate inhibition constant, \( K_{i,s} \), is 890 μM, but because of the limited
substrate concentration range used this is very ill-determined). The $S_{0.5}$ and $n_H$ values are in good agreement with values reported for enzyme expressed in baculovirus-infected insect cells, though substrate inhibition was not reported in this case (Stresser et al., 2000; Stresser et al., 2002). The value of $n_H$ indicates that at least two 7BQ molecules must bind simultaneously, and a third is required to account for the substrate inhibition.

As described in the Experimental section, the effects of an inhibitor which can compete with all the bound substrates can provide additional information. These experiments require an inhibitor which is large enough to compete for all the substrate sites, used at a concentration well above its $K_i$ (see Figure 1B). We thus studied the kinetics of 7BQ debenzylation in the presence of high concentrations ([I] >35 x $K_{i,app}$) of eight typical CYP3A4 substrates of varying size as inhibitors (Figure 3, Table 2). It is clear from Figure 3 that the degree of sigmoidicity ('cooperativity') in the 7BQ kinetics and, correspondingly, the slope of a Hill plot of the data, is indeed increased in the presence of several of the inhibitors, indicating that more than 2-3 7BQ molecules can bind to CYP3A4. It is thus not appropriate to analyse the data in terms of Schemes 1 or 2. While the data could of course be analysed by extending the models shown in schemes 2 and 3 to incorporate a larger number of substrate molecules, this would lead to a substantial increase in the number of parameters, which would not be robustly estimated, and in the complexity of the equations. For the purpose of the present work, to obtain an improved lower limit to the number of substrate molecules which can bind simultaneously, we have fitted the data in Figure 3 empirically by the Hill equation (eq. 5) or, where substrate inhibition is observed, by the modified Hill equation (eq. 6). The values of $S_{0.5}$ and $n_H$ obtained are given in Table 2; as for the data in the absence of
inhibitor, the value of the estimated substrate inhibition constant, $K_{i,s}$, is very ill-determined and is not included in the Table.

The five largest inhibitors tested (cyclosporin A, troleandomycin, erythromycin, bromocriptine and ketoconazole) each led to significant increases in both $S_{0.5}$ and Hill coefficient for 7BQ (Table 2), consistent with the idea that they each compete with all or most of the 7BQ molecules which can bind in the active site of CYP3A4. The largest effect on the Hill coefficient was seen with bromocriptine, which gave an $n_H$ value of 3.7; since $n_H < n$, at least four molecules of 7BQ must be able to bind simultaneously to the active site of CYP3A4, providing an improved lower limit to the stoichiometry of 7BQ binding compared to that from the analysis of 7BQ turnover alone. In the presence of erythromycin, the Hill coefficient for 7BQ is 2.8. However, substrate inhibition by 7BQ is still observed within the same concentration range even in the presence of erythromycin, indicating that that a minimum of four molecules of 7BQ must be able to bind simultaneously to CYP3A4. There is also evidence for substrate inhibition in the presence of bromocriptine (see Figure 3 panel A2), which would indicate the simultaneous binding of a total of five molecules of 7BQ to CYP3A4. The inhibitors troleandomycin, ketoconazole, cyclosporin A, and diltiazem also increase $n_H$ for 7BQ significantly, to 2.1 – 2.7, and substrate inhibition is still observed in the presence of troleandomycin and diltiazem, but not ketoconazole or cyclosporin A.

By contrast to the effects of large inhibitors, the smaller inhibitors midazolam and testosterone lead to a decrease rather than an increase in the Hill coefficient for 7BQ. This behaviour can be accounted for by a two-site model of the kind shown in Scheme 1, in which two molecules of the smaller inhibitors can bind, competing with the
substrate for each site; there is good evidence for the binding of at least two molecules of midazolam ((Khan et al., 2002) and see below) and testosterone ((Roberts et al., 2005) and refs. therein) to CYP3A4. Details of the modelling of this behaviour are presented in the Supplementary Material. It is thus clear that the use of inhibitors to provide improved estimates of the number of substrate molecules which can bind simultaneously requires the use of an inhibitor which can compete with all the bound substrates (see also (Fisher, 1970)).

It is often observed that the extent of ‘cooperativity’ observed in the steady-state kinetics of CYP3A4 depends upon the nature of the enzyme preparation used (e.g., liver microsomes, recombinant enzyme expressed in human or in insect cells (Zhang et al., 2004)). To investigate the general utility of this method for estimating the minimum stoichiometry of substrate binding, we examined the effects of bromocriptine on 7BQ de-benzylation catalysed by four different individual human liver microsome (HLM) preparations (Figure 4, Table 3). The presence of bromocriptine increased both the \( S_{0.5} \) and the Hill coefficient for 7BQ, consistent with the data for the recombinant enzyme in Table 2. However, the values of both these kinetic parameters in the absence of inhibitor were greater in the HLM preparations than for the recombinant enzyme, with \( S_{0.5} \) being about 3-fold greater and \( n_H \) being in the range 2.2 – 3.3 as compared to 1.7 for the recombinant enzyme. It is also notable that substrate inhibition is not apparent in the kinetics of 7BQ de-benzylation by HLM preparations (Figure 4). The differences between recombinant CYP3A4 and HLM in 7BQ kinetics were described by Stresser et al. (2002) who reported that HLM show higher values of both \( S_{0.5} \) and Hill coefficient, as observed here. 7BQ was shown to be oxidised not only by CYP3A4 but also by CYP3A5, CYP1A1 and CYP1A2. CYP3A5 is subject to genetic polymorphism, while
CYP1A1 expression levels can vary >100 times between individuals. Drug and diet history may also be a factor, resulting in other molecules being bound to the active site with consequent changes in $S_{0.5}$ and Hill coefficient for 7BQ.

The effect of bromocriptine on $S_{0.5}$ was similar for each of the four HLM samples, but there were quantitative differences in the Hill coefficient in the presence of the inhibitor, $n_H$ values ranging from 2.8 to 5.0. The higher values seen with some of the HLM samples than for the recombinant enzyme suggest the possibility that as many as five or six 7BQ molecules may be able to bind simultaneously to CYP3A4.

**Molecular docking of 7BQ to the active site of CYP3A4.** In order to establish what is required for several 7BQ molecules to bind simultaneously to the active site of CYP3A4, we carried out docking calculations on the two unbound CYP3A4 crystal structures available in the PDB (1tqn (Yano et al., 2004) and 1w0e (Williams et al., 2004)). It is important to emphasise that these calculations were not intended to model the mechanism of cooperativity, in which the binding of the $(n+1)^{th}$ molecule increases the binding of the $n^{th}$ molecule, by direct interaction and/or by inducing conformational changes in the protein. We retain the approximation that the crystal structures are representative of the most populated conformational state of the free enzyme, and ask whether multiple 7BQ molecules can bind without changes in active site structure. Our approach was to dock one 7BQ molecule at a time, generating 50 solutions each time, and to dock the $(n+1)^{th}$ 7BQ molecule into the structure corresponding to the best solution of the $n^{th}$ run. This approach provides an indication of the number of 7BQ molecules that can bind simultaneously to the active site in the crystal structures of the
free enzyme and provides an understanding of the complementarity between an ensemble of 7BQ molecules and the CYP3A4 binding site.

The results are consistent with the binding of up to five molecules of 7BQ in the active sites of both the 1tqn (Figure 5) and 1w0e structures: 3 molecules located in the core of the site and 2 located in the access channel in more or less solvent exposed positions, with substantial edge-to-face and stacking interactions between the aromatic rings of the successively binding 7BQ molecules. They demonstrate that multiple binding of 7BQ is possible without the need for structural changes in the active site, though they do not of course exclude the possibility of such changes – the recent structures of the ketoconazole and erythromycin complexes of CYP3A4 (Ekroos and Sjogren, 2006) have demonstrated that ligand binding can indeed produce substantial changes in the active site. Calculations using both the 1tqn and 1w0e structures agree in the position of all 7BQ binding sites, although the orientation of the ligand in these sites can differ substantially, in particular for sites B and C (Figure 5). The differences in the predicted binding energies for the successive binding of 7BQ to sites A-E are small (Table 4), suggesting an almost isoenergetic interaction of 7BQ with the different sites within the enzyme active site, so that displacement of one molecule of 7BQ from one site to another should not be energetically difficult.

Kinetics of metabolism of midazolam by CYP3A4. Midazolam, a commonly used benzodiazepine sedative, has proved to be a useful in vivo probe substrate for the prediction of CYP3A activity (Thummel and Wilkinson, 1998). CYP3A4 catalyses both 1′-hydroxylation and 4-hydroxylation of this compound (Khan et al., 2002). The apparent $K_M$ (or $S_{0.5}$) values for the formation of the two products are substantially different.
This observation clearly indicates that two molecules of midazolam must be able to bind to the enzyme simultaneously, and further support for this idea comes from the observation that a peptide inhibitor shows different $K_i$ values for inhibition of the two hydroxylation reactions (Hosea et al., 2000). Notwithstanding this evidence for the binding of more than one molecule of midazolam to CYP3A4, the steady-state kinetics of both 1’-hydroxylation and 4-hydroxylation show simple Michaelis-Menten behaviour (Figure 6; (Khan et al., 2002)), suggesting that there is little or no influence of the presence of one midazolam molecule on the binding of the other. Figure 6 shows that the addition of erythromycin leads, for both reactions, to the conversion of the simple Michaelis-Menten kinetics into ‘cooperative’ behaviour, with a sigmoid $v$ versus $[S]$ plot and Hill coefficients of $\sim 1.5$ (Table 5), demonstrating that at least two midazolam molecules can indeed bind simultaneously to CYP3A4. This shows that the use of a large inhibitor can reveal the existence of multiple substrate binding even when this is not apparent from the steady-state kinetics – though of course in the case of midazolam there was prior evidence for this.

**Kinetics of metabolism of diltiazem by CYP3A4.** The kinetics of the N-demethylation of diltiazem, a benzothiapine calcium-channel blocker used in the treatment of hypertension and angina, by CYP3A4 show essentially Michaelis-Menten kinetics at incubation times selected to avoid any complications from the reported inactivation of the enzyme by diltiazem (Jones et al., 1999) (Figure 7), with a Hill coefficient slightly less than 1.0 (Table 5). Addition of erythromycin at a concentration leading to a $>14$-fold increase in $S_{0.5}$ has no significant effect on the magnitude of the
Hill coefficient (Table 5). This strongly suggests that only one molecule of diltiazem binds to the active site of CYP3A4.
Discussion

The fact that CYP3A4, together with some other drug-metabolising P450s, shows non-Michaelis-Menten kinetics with a variety of substrates has been recognised for a number of years. It has been suggested that this behaviour may confer a selective advantage to the organism in dealing with potential toxins to which it is exposed (Atkins et al., 2002), and it is certainly important in using in vitro data to predict the in vivo pharmacokinetic behaviour of drugs (Houston and Galetin, 2003; Houston and Galetin, 2005). Notwithstanding its importance, the molecular mechanisms of this behaviour remain incompletely understood, and even the stoichiometry of binding to CYP3A4 is not clearly established for the majority of substrates. In this paper we have shown that inhibition by large inhibitor molecules may provide improved estimates of the stoichiometry of substrate binding. This approach simply requires fitting the data in the presence of the inhibitor by the Hill equation (or a modification thereof if substrate inhibition is present) to determine the Hill coefficient. It is important to emphasise that the use of the Hill equation is simply an empirical description of the data, and does not carry any mechanistic implications. The estimates of the number of substrate molecules which can bind simultaneously provided by this approach are still lower limits; however, not only are they significantly better than those provided by the Hill coefficient in the absence of inhibitor but the method can be used to estimate substrate stoichiometry even in the absence of cooperative interactions between the bound substrate molecules.

We have applied this method to the fluorogenic substrate 7BQ, which is widely used as a convenient substrate in CYP3A4 assays (Stresser et al., 2000; Stresser et al.,
We find that 7BQ exhibits sigmoidal kinetics with a Hill coefficient of 1.7 (in agreement with previous work (Stresser et al., 2002)), and shows substrate inhibition at high substrate concentration. However, the addition of any one of a number of large inhibitor molecules led to a significant increase in the Hill coefficient, up to $n_H = 3.7$ with the isolated enzyme and as much as $n_H = 5$ for HLM samples. Taken together with the observation that substrate inhibition is still observed in the presence of many of the inhibitors, this clearly shows that at least five and perhaps six 7BQ molecules can bind simultaneously to the active site of CYP3A4. Our simple molecular modelling studies show that this is consistent with the recently determined crystal structures of CYP3A4.

The increases in $S_{0.5}$ and $n_H$ varied significantly amongst the large inhibitors studied, with no simple relationship to their size; this presumably reflects differences in their precise mode of binding and the extent to which they can compete for all the 7BQ binding sites. It is interesting that cyclosporin A, the largest of the inhibitors tested, does not produce the largest increase in Hill coefficient, even though it was used at a very high concentration ($140 \times K_{i,app}$) and produced a large increase in $S_{0.5}$. A somewhat similar anomalous behaviour of cyclosporin was noted earlier (Fowler et al., 2002) in studies of mutants of CYP3A4 at Leu211, a residue which has been proposed to be involved in cooperativity in substrate binding (Harlow and Halpert, 1997; Harlow and Halpert, 1998). Fowler et al. (Fowler et al., 2002) reported that cyclosporin was an outlier in plots relating inhibition of CYP3A4 Leu211 mutants to the molar volume of the inhibitor, and suggested that this behaviour could be explained if only a part of the very large cyclosporin molecule entered the CYP3A4 active site. A similar explanation could account for the fact that cyclosporin produces a smaller increase in Hill coefficient for 7BQ than does erythromycin or bromocriptine.
By contrast to 7BQ, the work reported here suggests that two molecules of midazolam but only one of diltiazem can bind simultaneously to CYP3A4, and shows that the approach described here can detect the binding of two molecules of midazolam even though this substrate shows Michaelis-Menten kinetics. There is good additional evidence for the simultaneous binding of at least two molecules of midazolam to CYP3A4 (Hosea et al., 2000; Khan et al., 2002). However, only one molecule of the very similar-sized diltiazem appears to be able to bind; it is clear that simple size is not the sole criterion which determines whether more than one molecule of a given substrate can bind to the active site of CYP3A4.

In addition to the usefulness of this method in providing estimates of stoichiometry of substrate binding, the observation that large inhibitors can compete with several substrate molecules has a number of implications for estimates of binding affinity and prediction of pharmacokinetics. (Of course in much work on P450s, ‘inhibitors’ are in fact substrates, whose affinity is estimated by their ability to inhibit the turnover of test substrates such as 7BQ, testosterone or midazolam.) First, if a given inhibitor competes with more than one bound substrate molecule then the sensitivity of the reaction to inhibition will be decreased, since binding of substrate to even the non-productive site(s) will compete off the inhibitor; this is illustrated in Figure 7A for the simple case of two substrate binding sites. This effect may, for example, account for published data on testosterone interaction with erythromycin in human liver microsomes (Wang et al., 1997). In the microsomes tested both substrates showed similar apparent \( K_M \) values, but 1mM erythromycin inhibited hydroxylation of 0.25-0.5mM testosterone by only 20-25%, while 1mM testosterone inhibited N-demethylation of 0.25mM erythromycin by 70-90%. The superior inhibition by testosterone can be explained by
multiple binding: if more than one testosterone molecule can bind (Baas et al., 2004; Denisov et al., 2007) and compete off erythromycin, the sensitivity to inhibition will be increased. More generally, the idea that in P450s one inhibitor can compete with several substrate molecules and vice versa may well contribute to the widespread observation that inhibition parameters such as IC\textsubscript{50} are dependent on the substrate used (e.g. (Stresser et al., 2000; Obach et al., 2006)), since the effect of the inhibitor will depend on the number of molecules of a given substrate bound and the number of these with which the inhibitor competes. In general terms, the use of large test substrates such as bromocriptine or erythromycin rather than small molecules such as 7BQ will increase the sensitivity of the assay to inhibition by compounds which may show multiple binding – as in the testosterone and erythromycin example discussed above.

Houston \textit{et al.} (Houston and Kenworthy, 2000; Houston and Galetin, 2003; Houston and Galetin, 2005) have pointed out that the existence of ‘cooperativity’ ($n_H > 1$) in substrate binding leads to a maximum in the plot of clearance ($v/[S]$) against substrate concentration, and that this must be taken into account in attempts to predict human drug clearance from \textit{in vitro} data. We show here that inhibitors can increase the Hill coefficient for substrates and indeed can lead to the appearance of cooperativity for a substrate which on its own has $n_H = 1$. This provides another way in which drug-drug interactions may be manifest \textit{in vivo} in the way discussed by Houston \textit{et al.}. Thus, even when there is no interaction between two substrates binding to the enzyme, addition of an inhibitor can change the shape of the clearance curve (Figure 7B) in such a way as to lead, in certain ranges of substrate concentration, to faster metabolism than would be predicted by an analysis based on Michaelis-Menten kinetics.
References


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crystallography to 2.05-angstrom resolution. *Journal of Biological Chemistry* **279**:38091-38094.

Footnotes

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Legends for figures

**Figure 1.** Simulations of the effects of an inhibitor which competes with two simultaneously bound substrate molecules. The simulations, based on Equation 4, are shown for the case where there is no interaction between the two substrates (α = β = 1) and $K_{s2}/K_{s1} = 5$. Increasing inhibitor concentrations lead to an increasing curvature of the Eadie-Hofstee plot (A) and an increasing value of the Hill Coefficient (B). Simulations are shown in A for four values of $[I]/K_i$: ——— 0; —— 2.5; ——– 5; ——— 10

**Figure 2.** Kinetics of de-benzylation of 7BQ by recombinant CYP3A4. The rate of de-benzylation is shown as a function of substrate concentration; the curve is the best fit of equation 3 to the data, with the parameters shown in Table 1.

**Figure 3.** Effect of inhibitors on the kinetics of de-benzylation of 7BQ by recombinant CYP3A4. Open circles, no inhibitor; closed circles, with inhibitor, at the concentration given in Table 2. A1-H1: Rate as a function of substrate concentration; A2-H2: Hill plots (eq. 5). Inhibitors: (A) Bromocriptine, (B) Erythromycin, (C) Troleandomycin, (D) Ketoconazole, (E) Cyclosporin A, (F) Midazolam, (G) Testosterone, (H) Diltiazem.

**Figure 4.** Effect of bromocriptine on the kinetics of de-benzylation of 7BQ by human liver microsomes. Open circles, no inhibitor; closed circles, with 5.3μM bromocriptine. A, Rate as a function of substrate concentration; B, Hill plot. Data are shown for one HLM preparation; results for all four preparations studied are given in Table 3.
Figure 5. Multiple modes of 7BQ binding to CYP3A4. Successive 7BQ molecules were docked into the two available crystal structures as described in the text. Two different views of 7BQ bound in the five identified binding sites are shown for structure 1tqn (Yano et al., 2004).

Figure 6. Effect of erythromycin on the metabolism of midazolam and diltiazem by recombinant CYP3A4. Open circles, no inhibitor; closed circles, with erythromycin. A, Midazolam 1′-hydroxylation; B, Midazolam 4-hydroxylation; C, Diltiazem N-demethylation

Figure 7. Modelling the effect of inhibitors on metabolism for the case where two substrate molecules bind simultaneously. The modelling is based on Scheme 3, equation 4, with \( \alpha = \beta = 1 \) (no interaction between the two substrate molecules). A, Comparison of the sensitivity to inhibition of the model in Scheme 3 with that of simple Michaelis-Menten competitive inhibition with the same parameters; B, Comparison of clearance plots for the model in Scheme 3 and simple Michaelis-Menten competitive inhibition with the same parameters. In both parts A & B, the curves are as follows: Michaelis-Menten kinetics, \([I] = 0\) ; Michaelis-Menten kinetics, \([I]/K_i = 10\) ; Scheme 3, \([I] = 0\) ; Scheme 3, \([I]/K_i = 10\).
Tables

Table 1. Analysis of the steady-state kinetics of 7BQ debenzylolation by CYP3A4 in terms of the three models shown in Schemes 1, 2A and 2B.

The equations describing the models (equations 1-3 in Experimental Procedures) were fitted to the experimental data shown in Figure 2. The parameters are defined in the Schemes and in the Experimental Procedures section. Statistical comparison of the fits to the data: eq. 2 versus eq. 1, \( p = 0.02 \); eq. 3 versus eq. 1, \( p = 1.7 \times 10^{-7} \); eq. 3 versus eq. 2, \( p = 0.005 \)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Scheme 1 (eq. 1)</th>
<th>Scheme 2A (eq. 2)</th>
<th>Scheme 2B (eq. 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( V_{max} ) (a.u.)</td>
<td>0.85 ± 0.07</td>
<td>0.167 ± 0.002</td>
<td>0.165 ± 0.005</td>
</tr>
<tr>
<td>( K_{s1} ) (μM)</td>
<td>473 ± 50</td>
<td>267 ± 17</td>
<td>513 ± 77</td>
</tr>
<tr>
<td>( K_{s2} ) (μM)</td>
<td>4600 ± 1900</td>
<td>107 ± 13</td>
<td>195 ± 32</td>
</tr>
<tr>
<td>( K_{s3} ) (μM)</td>
<td>-</td>
<td>739 ± 40</td>
<td>458 ± 103</td>
</tr>
<tr>
<td>( \alpha )</td>
<td>0.004 ± 0.001</td>
<td>0.063 ± 0.005</td>
<td>0.017 ± 0.003</td>
</tr>
<tr>
<td>( \beta )</td>
<td>0.10 ± 0.01</td>
<td>-</td>
<td>0.11 ± 0.04</td>
</tr>
<tr>
<td>( \chi^2 )</td>
<td>5 x 10^{-3}</td>
<td>2 x 10^{-3}</td>
<td>1 x 10^{-4}</td>
</tr>
</tbody>
</table>
Table 2. Effects of inhibitors on the steady-state kinetics of 7BQ debenzylation by CYP3A4

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_{i,app}$, μM</th>
<th>Concentration of inhibitor used, μM</th>
<th>$S_{0.5}$ for 7BQ, μM</th>
<th>Hill coefficient, $n_H$, for 7BQ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>Inhibitor</td>
<td>Control</td>
</tr>
<tr>
<td>Bromocriptine</td>
<td>0.14</td>
<td>40 ± 1.7</td>
<td>172 ± 6</td>
<td>1.85 ± 0.07</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>0.6</td>
<td>43 ± 1.7</td>
<td>159 ± 5</td>
<td>1.7 ± 0.05</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>27</td>
<td>40 ± 1.7</td>
<td>104 ± 8</td>
<td>1.85 ± 0.07</td>
</tr>
<tr>
<td>Troleandomycin</td>
<td>4.9</td>
<td>40 ± 1.7</td>
<td>107 ± 6</td>
<td>1.85 ± 0.07</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>0.02</td>
<td>39 ± 1.6</td>
<td>139 ± 3</td>
<td>1.67 ± 0.05</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>11</td>
<td>39 ± 1.5</td>
<td>95 ± 9</td>
<td>1.73 ± 0.05</td>
</tr>
<tr>
<td>Midazolam</td>
<td>6.4</td>
<td>39 ± 1.6</td>
<td>106 ± 16</td>
<td>1.67 ± 0.05</td>
</tr>
<tr>
<td>Testosterone</td>
<td>n.d. a</td>
<td>50 ± 4</td>
<td>51 ± 7</td>
<td>1.6 ± 0.08</td>
</tr>
</tbody>
</table>

*a not determined due to activation of 7BQ debenzylation seen at some testosterone concentrations
Table 3. Effects of bromocriptine on the steady-state kinetics of 7BQ debenzylation by human liver microsomes

<table>
<thead>
<tr>
<th>HLM sample</th>
<th>$S_{0.5}$ for 7BQ, μM</th>
<th>Hill coefficient, $n_H$, for 7BQ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Bromocriptine</td>
</tr>
<tr>
<td>HLM2</td>
<td>139 ± 7</td>
<td>270 ± 32</td>
</tr>
<tr>
<td>HLM3</td>
<td>145 ± 20</td>
<td>256 ± 76</td>
</tr>
<tr>
<td>HLM4</td>
<td>119 ± 0.5</td>
<td>241 ± 29</td>
</tr>
<tr>
<td>HLM5</td>
<td>114 ± 5</td>
<td>222 ± 6</td>
</tr>
</tbody>
</table>
**Table 4.** Predicted binding energies\(^a\) of 7BQ to the CYP3A4 crystal structures (PDB codes 1tqn and 1w0e.).

<table>
<thead>
<tr>
<th></th>
<th>1tqn</th>
<th>1w0e</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site A</td>
<td>-29.7</td>
<td>-31.9</td>
</tr>
<tr>
<td>Site B</td>
<td>-32.5</td>
<td>-33.7</td>
</tr>
<tr>
<td>Site C</td>
<td>-30.4</td>
<td>-38.6</td>
</tr>
<tr>
<td>Site D</td>
<td>-31.6</td>
<td>-28.2</td>
</tr>
<tr>
<td>Site E</td>
<td>-23.1</td>
<td>-25.8</td>
</tr>
</tbody>
</table>

\(^a\) energies (kJ/mol) obtained from docking calculations using Gold v. 3.0.1
Table 5. Effect of erythromycin on the kinetic parameters for midazolam and diltiazem metabolism by CYP3A4

<table>
<thead>
<tr>
<th>Reaction</th>
<th>$S_{0.5}$, $\mu$M</th>
<th>$V_{max}$, min$^{-1}$</th>
<th>Hill coefficient, $n_H$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>+ inhibitor</td>
<td>Control</td>
</tr>
<tr>
<td>Midazolam 1'-hydroxylation</td>
<td>6.2 ± 1.4</td>
<td>25.9 ± 1.6</td>
<td>3.80 ± 0.39</td>
</tr>
<tr>
<td>Midazolam 4-hydroxylation</td>
<td>23.5 ± 6.3</td>
<td>103 ± 31</td>
<td>2.25 ± 0.25</td>
</tr>
<tr>
<td>Diltiazem N-demethylation</td>
<td>38.2 ± 2.5</td>
<td>552 ± 337</td>
<td>83.8 ± 1.9</td>
</tr>
</tbody>
</table>

¹ As described in the Experimental section, the incubation time for the kinetic experiments was selected to avoid any complications from the reported inactivation of the enzyme by midazolam (Khan et al., 2002) or diltiazem (Jones et al., 1999).
Scheme 1. A simple model for the simultaneous binding of two substrate molecules. One substrate (S₁) binds in a productive mode, and the second (S₂) in a non-productive mode. The binding of this second molecule may affect $k_{cat}$, by a factor $\alpha$, and/or the interaction between the two substrate molecules may affect the dissociation constants $K_{S1}$ and $K_{S2}$, by a factor $\beta$. 
Scheme 2. Two models for the simultaneous binding of three substrate molecules.

Both models involve the cooperative binding of substrate to a productive site (S₁) and a non-productive site (S₂) with a cooperativity factor $\beta < 1$ (but no effect on $k_{\text{cat}}$) and in addition the binding of a third substrate molecule. In A, the third molecule binds to form a non-productive quaternary complex, while in the more general model B the quaternary complex can yield product at the rate of $\alpha k_{\text{cat}}$ ($\alpha < 1$).
Scheme 3. Extension of the model shown in Scheme 1 to incorporate the binding of an inhibitor which binds competitively with both substrate molecules.
Figure 2

[Graph showing the relationship between rate (units/min) and [7BQ] (μM). The graph has a y-axis labeled "rate, units/min" and an x-axis labeled "[7BQ] (μM)."]
Figure 3 – part 2

E1

Rate, units/min

[7BQ], μM

E2

log(v/v_maxυ)

log([7BQ])

F1

Rate, units/min

[7BQ], μM

F2

log(v/v_maxυ)

log([7BQ])

G1

Rate, units/min

[7BQ], μM

G2

log(v/v_maxυ)

log([7BQ])

H1

Rate, units/min

[7BQ], μM

H2

log(v/v_maxυ)

log([7BQ])
Figure 4

A

Rate, units/min

[7BQ], µM

B

log \left( \frac{v}{V_{\text{max}} - v} \right)

log ([7BQ])

log (v / (V_{\text{max}} - v))

log ([7BQ])