LIGAND BINDING TO CYTOCHROME P450 3A4 IN PHOSPHOLIPID BILAYER NANODISCS: THE EFFECT OF MODEL MEMBRANES

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The membrane-bound protein Cytochrome P450 3A4 (CYP3A4) is a major drug-metabolizing enzyme. Most studies of ligand binding by CYP3A4 are currently carried out in solution, in the absence of a model membrane. Therefore, there is little information concerning membrane effects on CYP3A4 ligand binding behavior. Phospholipid bilayer Nanodiscs are a novel model membrane system derived from high-density lipoprotein particles, whose stability, monodispersity and consistency are ensured by their self-assembly. We explore the energetics of four ligands: (6-(p-toluidino)-2-naphthalenesulfonic acid, TNS; α-naphthoflavone, ANF; miconazole; and bromocriptine) binding to CYP3A4 incorporated into Nanodiscs. Ligand binding to Nanodiscs was monitored by a combination of environment-sensitive ligand fluorescence and ligand-induced shifts in the fluorescence of tryptophan residues present in the Nanodiscs’ scaffold proteins; binding to the CYP3A4 active site was monitored by ligand-induced shifts in the heme Soret band absorbance. The dissociation constants for binding to the active site in CYP3A4-Nanodiscs were 4.0 μM for TNS, 5.8 μM for ANF, 0.45 μM for miconazole and 0.45 μM for bromocriptine. These values are for CYP3A4 incorporated into a lipid bilayer, and are therefore presumably more biologically relevant that those measured using CYP3A4 in solution. In some cases, affinity measurements using CYP3A4 in Nanodiscs differ significantly from solution values. We also studied the equilibrium between ligand binding to CYP3A4 and to the membrane. TNS showed no marked preference for either environment, ANF preferentially bound to the membrane, and miconazole and bromocriptine preferentially bound to the CYP3A4 active site.

Cytochrome P450 3A4 (CYP3A4) is a major drug-metabolizing enzyme, and its ligand binding and catalysis are therefore of wide interest. CYP3A4 is an integral membrane protein, interacting with the membrane via an embedded N-terminal helix and other hydrophobic surface regions; however, most investigations of ligand binding are currently carried out in solution using recombinant protein with a partially truncated helical anchor, in the absence of any model membrane. There is, therefore, a critical lack of understanding of how the membrane environment affects ligand binding to CYP3A4. Membrane effects may be especially important when in vitro binding or kinetic data are extrapolated to predictions of in vivo pharmacokinetics.

Model membranes can profoundly alter the ligand binding behavior of membrane proteins. For example, the choice of model membranes can shift the apparent affinity of a spider venom toxin for voltage-dependent K+ channels more than four orders of magnitude (1). Apart from the structural and dynamic effects of incorporation into a model membrane, competition for ligand binding between a model membrane and an incorporated protein can alter the apparent affinity and stoichiometry of ligand binding by the protein in equilibrium and kinetic experiments.

Parry et al. (2) presented separate analytical equations describing ligand binding to a membrane protein with a solvent-exposed active site, or with a membrane-exposed active site. Heirwegh et al. (3) extended this approach to account for amphilic binding sites and multiphasic lipid systems. Both these models assume that only the ligand concentration accessible to the protein is relevant: for solvent-exposed active sites, only the free ligand concentration in bulk solvent need be taken into account; for lipid-facing sites, only the substrate concentration in the membrane is relevant. This implies that the free ligand
approximation holds for both the bulk solvent and lipid phases (i.e., ligand binding to the protein does not significantly deplete ligand from the membrane or from solution), requiring that the protein concentration is lower than the true \( K_D \) of the ligand in question, and that the model membrane be present in substantial excess to the protein. This approximation also causes the binding equations to vary depending on the route of ligand access to the active site. A thermodynamically accurate and general equilibrium binding model would predict identical ligand binding behavior regardless of the route or kinetics of ligand binding to a protein.

Previous attempts to quantify the effect of the membrane phase on ligand binding and turnover by cytochrome P450s have generally involved measuring binding \( K_D \) (4,5) or turnover \( K_{TV} \) values (6) for protein incorporated into liposomes, over a range of lipid concentrations. Extrapolation to a lipid-free system provides qualitative information as to how the membrane affects the apparent affinity of a membrane protein for a substrate. Of particular interest is Kühl-Velten’s analysis (5) of progesterone binding to CYP17 in liposomes using a combination of absorbance spectroscopy, ultracentrifugation and equilibrium dialysis to measure the concentration of ligand bound to protein, bound to the membrane and free in solution. The apparent \( K_D \) was some three orders of magnitude lower than the true \( K_D \) corrected for the free progesterone concentration.

It should be noted that the lipid mixes widely used in CYP3A4 turnover studies (7-9) are useful empirical functional tools in terms of solubilizing CYP3A4 and enhancing activity of recombinant CYP3A4 in vitro, but they are not suitable model membranes for investigations of ligand binding. They contain detergents such as cholic acid or CHAPS well below their critical micellar concentrations (10,11), along with smaller amounts of phospholipids. These mixtures can not form canonical mixed micelles with the phospholipids (12,13), but are unlikely to exist as liposomes because the detergent concentrations are much higher than the phospholipid concentrations (14). The consequent uncertainty about the structure and composition of the lipid phase in reconstituted systems greatly complicates attempts to describe small-molecule binding to these systems, making a rigorous analysis of their effect on ligand binding to CYP3A4 intractably complex.

Phospholipid bilayer Nanodiscs (15-26), promising new model membranes, may clarify our understanding of ligand binding to CYP3A4. Nanodiscs resemble nascent discoidal high-density lipoprotein particles (see Fig. 1a), and consist of a POPC bilayer about 10 nm in diameter surrounded by two monomers of a 23 kDa Membrane Scaffold Protein (MSP). The protein coat ensures that Nanodiscs are monodisperse, relatively stable, and consistent between preparations; it also allows the determination of the affinity and stoichiometry with which small molecules bind to the Nanodisc membrane. As an additional benefit, incorporation of CYP3A4 into Nanodiscs prevents its oligomerization, which can affect its reduction kinetics (21) and substrate turnover (27).

Here, we study the binding of four small-molecule ligands (TNS, ANF, miconazole and bromocriptine; structures in Fig. 1b) to CYP3A4-Nanodiscs, to determine the true affinity of these compounds to CYP3A4 incorporated into a membrane, and to determine how bound ligand partitions between the CYP3A4 active site and a lipid bilayer. TNS is a well-characterized fluorescent probe of protein structure which fluoresces in non-polar environments but is quenched and red-shifted in solution. ANF is a CYP3A4 substrate and effector of testosterone metabolism, which fluoresces in solution and is quenched and blue-shifted in non-polar environments. Miconazole is an azole drug that is a known inhibitor of CYP3A4. Bromocriptine, an ergot alkaloid used as a dopamine receptor agonist, is also a substrate (28) and potent inhibitor of CYP3A4 (29).

**EXPERIMENTAL PROCEDURES**

Chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

**Protein Expression & Purification:** The CYP3A4 NF14 construct was purified and expressed essentially as described (30,31), except that 2% ANAPOE C12E10 (Anatrace, Maumee, OH) was used to solubilize the membrane fraction instead of Emulgen 911. The NF-14 construct lacks residues 3-11 of wild-type CYP3A4, which constitute an N-terminal helical
membrane anchor. CYP3A4 was stored in 100 mM phosphate (pH 7.4) with 20% glycerol at -80°C, but was dialyzed into 10 mM Tris HCl pH 7.4 with 100 mM NaCl for all subsequent experiments. MSP1D1(-) was purified and expressed as described previously (17,26).

**Nanodisc and CYP3A4-Nanodisc Preparation:**
Nanodiscs and CYP3A4-Nanodiscs were prepared essentially as previously described (17); see Fig. 2 for schematic. To make Nanodiscs, MSP and POPC were mixed in a 1:65 molar ratio with 50 mM sodium cholate and incubated on a shaker for 1 hour at 4°C. In order to remove the detergent and initiate Nanodisc self-assembly, washed Amberlite XAD-2 (a hydrophobic adsorbent resin) was added at 0.5 g/mL, and the mixture was incubated for 4 hours on ice. The resin was removed using filter columns under gravity flow, and the Nanodiscs were concentrated and purified by size-exclusion chromatography on a Superdex 200 10/300 GL column (GE Healthcare, Piscataway, NJ).

To make CYP3A4-Nanodiscs, MSP, POPC and CYP3A4 were mixed in a 1:63:0.1 molar ratio with 50 mM sodium cholate and incubated on a shaker for 1 hour at 4°C. After incubation with 0.8 g/mL Amberlite XAD-2 for 4 hours on ice, nickel column chromatography was used to separate CYP3A4-Nanodiscs (which posses a His-tag at the CYP3A4 C-terminus) from excess Nanodiscs. The five-fold excess of Nanodiscs to CYP3A4 ensures that only one monomer of CYP3A4 is incorporated into a given Nanodisc. Size-exclusion was used to purify the CYP3A4-Nanodiscs and verify stable and monomeric incorporation. The absorbance spectrum of CYP3A4 did not change significantly upon incorporation into Nanodiscs (data not shown). Unless otherwise stated, the concentration of CYP3A4-Nanodiscs in experiments was 1.4 μM.

**Ligand binding to Nanodiscs monitored by fluorescence spectroscopy:** An SLM-Aminco AB/2 fluorimeter (SLM-Aminco, Urbana, IL) was used for all experiments, and the concentration of Nanodiscs used for titrations was 1.0 μM unless otherwise stated. Data were corrected for the primary and secondary inner-filter effects by the method of Lakowicz (32), and normalized for the change in volume over the course of a titration.

TNS fluorescence (λ_{ex} = 320 nm, λ_{em} = 440 nm) increases upon binding to Nanodiscs. To determine the affinity of the first mode of TNS binding to Nanodiscs, TNS fluorescence was monitored while Nanodiscs were titrated into 0.5 μM TNS (Fig. 3a). Data were fit to the hyperbolic binding equation. The fluorescence of tryptophan residues (λ_{ex} = 295 nm) in MSP is quenched upon TNS binding, via a Forster resonance energy transfer to TNS. Relative quenching of the MSP Trp fluorescence at 340 nm upon addition of TNS to Nanodiscs was used to monitor binding (Fig. 3b). (There are two Trp residues in each MSP monomer, and we assume that they are quenched equally upon ligand binding.) Data were fit to kinetic simulations (vide infra) of a three-site sequential binding model.

ANF fluorescence is quenched upon binding to Nanodiscs. 0.5 μM ANF was mixed with varying concentrations of Nanodiscs between 0 μM and 12.5 μM, and excited at 320 nm. The relative quenching of ANF emission between 400 nm and 500 nm (Fig. 4a) was fit to the quadratic binding equation, since the apparent K_{D} was close to the concentration of ANF. ANF binding also quenches MSP Trp fluorescence; ANF was titrated into 0.5 μM Nanodiscs, and the resulting quenching of Trp fluorescence at 340 nm (Fig. 4b) was fit to the multi-site quadratic binding to determine the stoichiometry of ANF binding to Nanodiscs.

The affinity of miconazole for Nanodiscs was measured using a competitive binding assay with ANF. 1.0 μM Nanodiscs were mixed with 20 μM ANF, and excited at 320 nm. The recovery of ANF fluorescence at 440 nm was monitored as aliquots of miconazole were added (Fig. 5a). Data were fit to the hyperbolic binding equation.

Bromocriptine also quenches MSP Trp fluorescence upon binding to Nanodiscs. Bromocriptine was separately titrated into 0.83 μM, 1.7 μM and 3.3 μM Nanodiscs, and the three binding curves (Fig. 6a) were globally fit to kinetic simulations of a five-site sequential binding model.

**Ligand binding to CYP3A4:** Ligand binding was monitored by absolute absorbance spectroscopy of the heme Soret band. Data were collected on a Cary 3E spectrophotometer (Varian, Cary, NC). Unless otherwise stated, the sample cuvette contained 1.4 μM total CYP3A4-Nanodiscs, while the reference cuvette contained an equal concentration of Nanodiscs. Equal
 aliquots of ligand were added to both cuvettes, and absorbance was recorded from 350 nm to 600 nm. Absolute absorbance spectroscopy minimizes interference from any ligand absorbance that overlaps with the heme. This is especially useful for ligands that display environment-sensitive absorbance, such as ANF and TNS.

All of the absorbance scans from a titration were smoothed over a 4 nm window, baseline-corrected, corrected for the effect of solvent, and normalized for the change in volume. Difference spectra were calculated by subtracting the absorbance signal for CYP3A4 alone from each scan in the titration, and the peak-to-trough ΔAbsorbance was plotted against ligand concentration.

Data analysis was performed using scripts written in the Python (http://python.org) programming language, and curve fitting was performed using Gepasi (33) for kinetic simulations and gnuplot (http://www.gnuplot.info) for analytical equations. When fitting to kinetic simulations, Gepasi was used to model the relevant binding scheme. All bimolecular on-rates were set to 1 μM⁻¹s⁻¹, and suitable selected off-rates and scaling factors were varied so as to minimize the χ² between steady-state metabolite concentrations and experimental data, using Levenburg-Marquadt, evolutionary programming and Nelder & Mead fitting algorithms. Since the data are from equilibrium binding experiments, the time scales and the absolute values of rate constants in these simulations are arbitrary. Equilibrium binding constants for any given step are given by the ratios of relevant rate constants. This procedure obviates the need to derive analytical binding equations for complex binding schemes, making it especially useful when the free ligand approximation is inapplicable due to high-affinity binding to CYP3A4 or to Nanodiscs.

RESULTS

The affinity of an amphiphile binding to a lipid bilayer is traditionally described by a membrane/solvent partition coefficient $K_{p}$, but equilibrium association ($K_{a}$) or dissociation constants ($K_{D}$) can be used instead when the molar volume ($V_{memb}$ with units of concentration⁻¹) of the model membrane can be estimated using the equations $K_{p} = K_{a} / V_{memb} = 1 / (V_{memb} K_{D})$ (34-38). We describe multiple small molecules binding to the Nanodisc membrane as a series of sequential binding modes, each with an associated $K_{p}$.

Scheme 1 lays out the framework we use to describe ligand binding to CYP3A4 in Nanodiscs, and the competition between binding to the membrane and to CYP3A4. $K_{i}$ is the dissociation constant of ligand binding to the membrane. $K_{i}^{'}$ is the dissociation constant of ligand binding to the protein, and can be directly compared to $K_{D}$ measurements made using other model membranes, or in the absence of model membranes, in as much as all represent the affinity of drug for the active site under those specific conditions. $K_{s} = K_{i}^{'} / K_{i}$ is a unitless equilibrium constant describing the ratio of ligand bound to the membrane and to the protein. In cases where multiple ligands can bind the Nanodisc membrane, a different $K_{s}$ is defined for each occupancy state of the CYP3A4-Nanodisc complex.

Steady-state fluorescence spectroscopy could not adequately distinguish ligand bound to the Nanodisc bilayer from ligand bound to CYP3A4 for any of the small molecules under consideration. Therefore, the same general strategy was used to obtain models for each of the various ligands binding to CYP3A4 in Nanodiscs. Ligand binding to free Nanodiscs (Figures 3a, 3b, 4a, 4b, 5a and 6a) was used to determine $K_{i}$. Binding to CYP3A4 in Nanodiscs (Figures 3c, 4c, 5b and 6b) was monitored via ligand-induced changes in the absorbance of the heme Soret band. These data were fit to kinetic simulations of suitable binding models, to recover $K_{i}^{'}$. $K_{s}$ is given by the ratio between $K_{i}^{'}$ and $K_{i}$.

TNS: The affinity with which a single TNS molecule bound to a Nanodisc was determined by titrating Nanodiscs into a fixed concentration of TNS, and monitoring the increase in TNS fluorescence at 440 nm as it entered a non-polar environment (Fig. 3a). A fit to the hyperbolic binding equation yielded a $K_{i}$ for this binding mode of 2.9 ± 0.07 μM. In the complementary titration, TNS binding to a fixed concentration of Nanodiscs was monitored via the quenching of Trp fluorescence at 340 nm (Fig. 3b). These data were fit to a model with two additional binding modes, each with a $K_{i}$ of 6.1 ± 0.1 μM, and with each bound TNS molecule causing one third of the
total quenching. In other words, at least three TNS molecules bind to each Nanodisc, with apparent negative binding cooperativity – the binding of the first TNS molecule altered the properties of the bilayer so as to disfavor subsequent binding events. The overall membrane partition coefficient \( K_F \) can be calculated from these \( K_I \) values by using the equation \( K_F = \frac{1}{(V_{\text{memb}} K_I)} \) for each of the three binding modes, and summing the resultant individual partition coefficients. Assuming a molar volume for POPC of 1263 Å³ (39) and 130 POPC molecules per Nanodisc, the volume of a Nanodisc is \( 1.64 \times 10^{-22} \) L, and therefore the molar volume \( V_{\text{memb}} \) is 98.9 L/mol. Consequently, the overall \( K_F \) for TNS is \( 6.8 \times 10^{3} \). This closely matches the \( K_F \) for TNS binding phospholipid vesicles (= \( 6.5 \times 10^{3} \)) (37,40,41), implying that Nanodiscs are suitable model membranes for the study of small-molecule binding to lipid bilayers.

TNS binding to CYP3A4 in Nanodiscs caused a Type-II spectral change, with an increase in absorbance at 440 nm and a decrease at 414 nm (Fig. 3c). Data from the optical titration were fit to the model in Scheme 2, incorporating the \( K_I \) values obtained from the experiments with Nanodiscs alone. The fit yielded a \( K_{I'} \) value of 4.0 ± 0.4 μM – this is the dissociation constant of TNS binding to the active site of CYP3A4 in Nanodiscs. The equivalent value measured for CYP3A4 in solution is 25.4 μM (42). This apparent difference in affinity may be due to structural changes in CYP3A4 caused by incorporation into a lipid bilayer. The \( K_{I'} \) values calculated from these \( K_I \) and \( K_{I'} \) values vary between 1.4 ± 0.1 (for the singly-occupied CYP3A4-Nanodisc complex) and 0.66 ± 0.07 (for doubly- and triply-occupied complexes). Values of \( K_2 \) close to 1 indicate that TNS does not have a strong thermodynamic preference for either the Nanodisc bilayer or the CYP3A4 active site.

ANF: As for TNS, the affinity with which a single molecule of ANF bound to Nanodiscs was determined by titrating Nanodiscs into a fixed concentration of ANF and monitoring the decrease in integrated ANF fluorescence between 400 nm and 500 nm (Fig. 4a). Fitting the resulting curve to the quadratic binding equation yielded a \( K_F \) of 0.81 ± 0.20 μM. In the converse titration, ANF binding to Nanodiscs was monitored by Trp fluorescence quenching at 340 nm (Fig. 4b) and fit to the multi-site quadratic binding equation:

\[
Q = \frac{Q_{\text{max}}}{2 \cdot [M]} \left( \frac{K_1 + n \cdot [M] + [ANF]}{\sqrt{(K_1 + n \cdot [M] + [ANF])^2 - 4 \cdot n \cdot [M] \cdot [ANF]}} \right)
\]

Here \([M]\) is the Nanodisc concentration and \( n \) is the stoichiometry of ANF binding to Nanodiscs. The recovered value of \( n \) was 14.3 ± 0.6, indicating that about fourteen molecules of ANF bind to each Nanodisc. This value was confirmed by kinetic simulations. Assuming 14 molecules of ANF bind to a Nanodisc, each with a \( K_I \) of 0.81 μM, the overall membrane partition coefficient is \( 1.7 \times 10^{5} \).

ANF binding to CYP3A4 in Nanodiscs caused a high-spin (Type I) spectral change, with an increase in absorbance at 388 nm and a decrease at 417 nm (Fig. 4c). Data from this titration were fit to the model in Scheme 3, and the recovered \( K_{I'} \) value was 5.8 ± 0.4 μM. ANF binding to CYP3A4 in solution has been studied extensively (43-48), and is thought to be cooperative in nature with at least two distinct ANF binding sites in each CYP3A4 monomer. This interaction has been described frequently (43,46,48) in terms of the Hill equation, with \( n \) values around 1.8 and \( S_{50} \) values around 5 μM, but the latter are not \( K_D \) values and can not be directly compared to \( K_{I'} \) values. More recently, Roberts and Atkins (47) proposed a two-site model for ANF binding to CYP3A4: a high-affinity (~1 μM) peripheral site that weakly perturbs the heme, and a lower-affinity site close to the heme with a \( K_D \) of 55 μM. The current work can not prove or disprove the existence or effect of the high-affinity peripheral site when CYP3A4 is incorporated into a bilayer, since it has not been possible to resolve ANF bound in the putative peripheral site from ANF bound to the bilayer. Importantly, the results in the Nanodiscs should not be taken as evidence against multiple binding of ANF. Rather, the multiple binding observed in other experimental models is likely to be masked by the membrane binding here. As with TNS, the difference between a \( K_{I'} \) of 5.8 μM and a \( K_D \) of 55 μM for the active site may reflect differences between CYP3A4 structure or active-site environment in solution and in Nanodiscs.
The calculated $K_2$ value was 7.2 ± 1.8, indicating that ANF preferentially binds to the Nanodisc bilayer. The free energy difference between binding to the membrane and to the CYP3A4 active site is $RT \ln(K_2) = 1.2$ kcal/mol. Note that the free energy values reported in this paper refer specifically to the CYP3A4-Nanodisc system described here, and since they are ultimately calculated using $K_I$ values, they depend on the dimensions of the Nanodiscs used. Therefore, they are not strictly equivalent to free energy values for an idealized “infinite” lipid bilayer.

**Miconazole:** Miconazole displaces ANF from the Nanodisc bilayer, resulting in an increase in ANF fluorescence (Fig. 5a). Fitting the resulting curve to the hyperbolic binding equation yielded a $K_I$ value of 9.0 ± 0.4 μM (and hence a membrane partition coefficient of $1.1 \times 10^3$). Miconazole binding to CYP3A4 in Nanodiscs causes a Type-II spectral change, with an increase in absorbance at 430 nm and a decrease at 408 nm (Fig. 5b). These data were fit to the model in Scheme 4, and resulted in a $K_{I'}$ value of 0.45 μM. (For CYP3A4 in solution, the equivalent $K_D$ is about 0.04 μM (Fig. 5c).) The calculated $K_2$ value was 0.05 ± 0.003, indicating that miconazole prefers binding to CYP3A4 active site by a ΔG of -1.8 kcal/mol.

**Bromocriptine:** Like ANF and TNS, bromocriptine quenches Trp fluorescence upon binding to Nanodiscs. However bromocriptine’s fluorescence itself is not sufficiently altered upon binding to allow for an accurate observation of the first binding mode, as was performed for ANF and TNS. Therefore, in order to arrive at a complete picture of bromocriptine binding, the ligand was titrated into three different concentrations of Nanodiscs and the resulting quenching data (Fig. 6a) were globally fit to a variety of binding models. The best fit was obtained using a model where five molecules of bromocriptine bound to each Nanodisc, each accounting for one-fifth of the total quenching, with two initial high-affinity binding modes (each with a $K_I$ of 5.2 ± 0.3 μM) and three subsequent low-affinity modes (each with a $K_I$ of 15 ± 0.9 μM). Consequently, the overall membrane partition coefficient for bromocriptine is $5.9 \times 10^3$.

Bromocriptine binding to CYP3A4 in Nanodiscs caused a high-spin (Type-I) spectral change, with an increase in absorbance at 388 nm and a decrease at 417 nm (Fig. 6b). These data were fit to the model in Scheme 5, and resulted in a $K_{I'}$ value of 0.45 ± 0.04μM. The equivalent $K_D$ measured for CYP3A4 in solution is 0.31 μM. The resultant $K_2$ values for bromocriptine varied between 0.09 ± 0.009 (for singly- and doubly-occupied CYP3A4-Nanodisc complexes) and 0.03 ± 0.003 (for higher occupancy states). Thermodynamically, bromocriptine prefers binding to the CYP3A4 active site by a ΔG between -1.4 kcal/mol and -2.1 kcal/mol.

**DISCUSSION**

Qualitatively, our results agree well with previous experimental studies of the membrane effect on CYP ligand binding (4-6). The presence of model membranes can significantly alter the apparent affinity with which a ligand binds; the extent of this effect varies depending on the ligand. Kominami et al. (4) reported that while the apparent $K_{D,s}$ of progesterone and 17α-hydroxyprogesterone binding to CYPs from bovine adrenocortical microsomes vary between about 25 nM and 0.4 μM as the membrane content of the system is increased, the corrected $K_{D,s}$ were in the 30 μM – 90 μM range (assuming these substrates access CYP active sites via the membrane). Kuhn-Velten’s (5) extensive analysis showing a similar effect on progesterone binding to CYP17 in liposomes has already been mentioned. Margolis and Obach (6) studied membrane effects on the inhibition of bufuralol 1’-hydroxylation by CYP2D6 by four drugs: the more lipophilic the drug, the more the apparent $K_I$ values increased with the amount of lipid; ezlopitant and fluoxetine showed 330-fold and 40-fold increases respectively in apparent $K_{D,s}$ at the highest lipid concentration tested. Fortunately, advances in model membranes and analytical techniques have allowed us to perform a more quantitative and precise analysis of these effects than was previously possible.

The equilibrium constant $K_2$ is a useful and concise descriptor of this competition for ligand binding between the protein and the membrane: if $K_2 > 1$, the ligand will preferentially bind to the membrane, while if $K_2 < 1$ binding to the protein is more favorable. For example, consider Scheme 2, our proposed model for TNS binding to the CYP3A4-Nanodisc complex. The various $K_2$
values describe the equilibrium between TNS bound at the CYP3A4 active site and in the Nanodisc bilayer at different occupancies of the complex as a whole. For the singly-occupied complex, a $K_2$ of 1.4, being greater than 1, indicates that TNS slightly favors binding to the bilayer. For the doubly- and triply-occupied complexes, $K_2 = 0.66 < 1$ indicates that TNS now slightly favors binding to the CYP3A4 active site. $K_2$ values for bromocriptine and miconazole in the 0.03 to 0.09 range indicate that both these ligands greatly prefer the CYP3A4 active site; a $K_2$ of 7.2 for ANF means that it binds more favorably to the Nanodisc bilayer. Fig. 7 provides a summary of these equilibria, expressed as free energy values. This representation amplifies the qualitative differences in ligand binding energetics for four representative CYP3A4 ligands, wherein some ligands can preferentially remain in the membrane, others have a significant preference for the CYP3A4 active site, and some have negligible preference for either location.

The affinities with which some ligands bind to CYP3A4 in solution differ considerably from the values of $K_2$ – the dissociation constant for binding to the CYP3A4 active site – obtained in this study. For TNS, $K_D$ in solution is 25 μM, while $K_D$ is 4.0 μM; for ANF, $K_D$ in solution is 55 μM while $K_D$ is 5.8 μM; for miconazole, $K_D$ is 0.04 μM while $K_D$ is 0.45 μM. These altered affinities may be caused by changes in CYP3A4 structure and/or dynamics or the environment of the active site upon incorporation into the Nanodisc bilayer, and presumably better reflect the true in vivo affinities than data from experiments with CYP3A4 in solution. Therefore, extrapolation of in vitro data to predictions of in vivo pharmacokinetics would be better served by using $K_2$ values than by solution $K_D$ values. Ideally, any turnover $K_M$ values for the membrane proteins involved in a drug’s metabolism would also be measured for proteins incorporated into lipid bilayers, while taking drug partitioning into the membrane into account. If experimental considerations make this difficult, prediction accuracy may be improved by scaling $K_M$ by $K_2/K_D$ to reflect how CYP structure and dynamics are affected by incorporation into a membrane.

This work also illustrates the importance of characterizing ligand binding to the model membrane. A titration with protein incorporated into a model membrane does not provide enough information by itself to arrive at an accurate binding model. For example, a simple fit of the sigmoidal binding curve in Fig. 4c to the Hill binding equation results in an $S_0$ of 19 μM and an $n$ of 3.3, implying a much more cooperative and lower-affinity interaction than we propose. These artificial conclusions would result from a failure to properly analyze the effect of the membrane phase. Understanding ligand binding to the membrane is a necessary prerequisite to obtaining accurate models of binding to a membrane protein. An attempt to fit a more complex binding model (such as one that accounted for ligand binding to the membrane) to the same single binding curve would be grossly overparametrized, and recovered values would not be meaningful. Combining data from Nanodiscs alone with data from CYP3A4-Nanodiscs ensures that none of the fits to data were overparametrized, and in fact no more than two fitting parameters were obtained from any given binding curve.

We recognize that this approach relies on the assumption that the presence of the membrane protein does not significantly alter the thermodynamics of ligand binding to the model membrane. Ideally, $K_2$ would be measured by simultaneously measuring occupancy of the active site and membrane phase of the CYP3A4-Nanodisc complex. Unfortunately, with steady-state fluorescence spectroscopy we have not been able to resolve CYP3A4-bound ligand from membrane-bound ligand for the small molecules in this study. CYP3A4 adds three additional Trp residues to the complex, each of which may respond differently to ligand (TNS, ANF or bromocriptine) binding; TNS and ANF fluoresce with differing intensities in the membrane and in the CYP active site. These additional variables mean that while steady-state fluorescence titrations with CYP3A4-Nanodiscs are consistent with the models presented here, some uncertainty may be introduced due to CYP-dependent effects on ligand-Nanodisc interactions.

More advanced techniques, such as time-resolved fluorescence, fluorescence anisotropy, or NMR T1 relaxation experiments, may allow us to simultaneously measure occupancy of the protein’s binding site and the membrane for these or other CYP3A4 ligands. This would provide a more rigorous measurement of model parameters,
as well as insight into how incorporated proteins affect amphiphile binding to lipid bilayers. It is difficult to predict what this effect would be a priori; although the presence of an incorporated protein might be expected to decrease the membrane area available for ligand binding (and thereby decrease a ligand’s affinity), the ordering effect (49-51) of a membrane protein on nearby lipids could either promote or hinder ligand binding. Taking these factors into account, the presented strategy is a reasonable compromise that is conveniently applied to a broad range of ligands and provides valuable insight into membrane effects on ligand binding.

The approach presented here improves upon earlier attempts (2,3) to theoretically describe ligand binding to cytochrome P450s for two reasons. The first is that the monodisperse and consistent size of the lipid phase in Nanodiscs leads to a well-defined lipid volume competing with the CYP3A4 active site for ligand binding, allowing for the determination of specific equilibrium constants (K_s) for ligand partitioning between the active site and the membrane. The second is that fitting experimental data to kinetic simulations of binding models (33) removes the need to derive analytical binding equations for each of the various binding models considered. These analytical equations can become intractably complex when the free ligand approximation is not applicable (as when lipid is not present in great excess relative to the protein, or when a ligand binds with high affinity to either protein or membrane). Independence from the free ligand approximation means that the binding models presented here do not depend on the route of substrate entry into the CYP3A4 active site, and so are true equilibrium binding models.

This work demonstrates how a well-defined, consistent model membrane such as phospholipid bilayer Nanodiscs can be used to understand the details of ligand binding to a membrane protein. It also illustrates how the presence of a model membrane can alter the apparent ligand binding behavior of incorporated proteins, by competing with them for ligand binding.

REFERENCES


**FOOTNOTES**

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Abbreviations used are: CYP3A4, Cytochrome P450 3A4; POPC, palmitoyloleoylphosphatidylcholine; MSP, membrane scaffold protein; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; ANF, α-naphthoflavone; TNS, 6-(p-toluidino)-2-naphthalenesulfonic acid.

**FIGURE LEGENDS**

**Fig. 1:** *A*, Model of Nanodisc structure, based on a model of discoidal high-density lipoprotein particles (52). Two MSP monomers (blue) form an amphipathic helical ring around a DPPC bilayer (white surface). Nanodiscs are about 10 nm in diameter and 4 nm thick. *B*, structures of TNS, ANF, miconazole and bromocriptine.

**Fig. 2:** *A*, Outline of Nanodisc and CYP3A4-Nanodisc preparation. Mixing MSP (blue) and POPC in a 1:65 ratio (for Nanodiscs), or MSP, POPC and CYP3A4 (red prism) in a 1:63:0.1 ratio (for CYP3A4-Nanodiscs) in the presence of 50 mM cholate (green) results in the formation of mixed micelles. When the cholate is removed by Amberlite XAD-2 resin, Nanodiscs and CYP3A4-Nanodiscs self-assemble. CYP3A4-Nanodiscs can be purified using a nickel affinity resin, since CYP3A4 bears a His tag but MSP does not.

**Fig. 3:** *A*, TNS binding to Nanodiscs. TNS fluorescence ($\lambda_{ex} = 320$ nm, $\lambda_{em} = 440$ nm) increases upon binding to Nanodiscs. A fit to the hyperbolic binding equation returned a $K_I$ of 2.9 µM for a single TNS molecule binding Nanodiscs. Inset: TNS fluorescence as a function of Nanodisc concentration. *B*, The relative quenching of MSP tryptophan fluorescence ($\lambda_{ex} = 295$ nm, $\lambda_{em} = 340$ nm) was monitored as a function of TNS concentration. Data were fit to a three-site sequential binding model, with $K_i$s of 6.1 µM for the second and third binding events. Inset: MSP Trp fluorescence is quenched in response to TNS addition (indicated by arrow). *C*, TNS binding to 1.4 µM CYP3A4-Nanodiscs. The difference between ΔA at 440 nm and 414 nm was plotted against TNS concentration. Data were fit to kinetic simulations of the binding model in Scheme 2, with a $K_I'$ of 4.0 µM. Inset: the Type-II calculated difference spectra induced by TNS binding to CYP3A4 in Nanodiscs.

**Fig. 4:** *A*, ANF binding to Nanodiscs. The integrated fluorescence of 0.5 µM ANF ($\lambda_{ex} = 320$ nm) from 400 nm to 500 nm was monitored, and relative quenching was plotted against Nanodisc
concentration. Data were fit to the quadratic binding equation, with a $K_f$ of 0.81 μM. Inset: ANF fluorescence is quenched as a function of Nanodisc concentration. $B$, Trp fluorescence quenching ($\lambda_{ex} = 295$ nm, $\lambda_{em} = 340$ nm) of 0.5 μM Nanodiscs was monitored as a function of ANF concentration. Data was fit to the multi-site quadratic binding equation to determine that about 14 ANF molecules bind to each Nanodisc. Inset: Inset: MSP Trp fluorescence is quenched in response to ANF addition (indicated by arrow). $C$, ANF binding to 1 μM CYP3A4-Nanodiscs. The difference between ΔA at 388 nm and 417 nm was plotted against ANF concentration. Data were fit to kinetic simulations of the binding model in Scheme 3, with $K_f$ of 5.8 μM. Inset: the Type-I calculated difference spectra induced by ANF binding to CYP3A4 in Nanodiscs.

Fig. 5: $A$, Miconazole binding to Nanodiscs. Miconazole was titrated into a mixture of 1 μM Nanodiscs and 20 μM ANF, and a recovery of ANF fluorescence ($\lambda_{ex} = 320$ nm, $\lambda_{em} = 440$ nm) was monitored as a function of miconazole concentration. Data were fit to the hyperbolic binding equation, with a $K_f$ of 9.0 μM. Inset: ANF fluorescence increases in response to increased miconazole concentration (indicated by arrow). $B$, Miconazole binding to 1.4 μM CYP3A4-Nanodiscs. The difference between miconazole-induced heme absorbance changes at 430 nm and 408 nm was plotted against miconazole concentration. Data were fit to kinetic simulations of the binding model in Scheme 4, with a $K_f$ of 0.45 μM. Inset: the Type-II calculated difference spectra induced by miconazole binding to CYP3A4 in solution. $C$, Miconazole binding to 1.4 μM CYP3A4 in solution. The difference between ΔA at 430 nm and 408 nm was plotted against miconazole concentration. Data were fit to the quadratic binding equation, with a $K_D$ of 0.04 μM. Inset: the Type-II calculated difference spectra induced by miconazole binding to CYP3A4 in solution.

Fig. 6: $A$, Bromocriptine binding to Nanodiscs. Trp fluorescence quenching ($\lambda_{ex} = 295$ nm, $\lambda_{em} = 340$ nm) for three different concentrations of Nanodiscs (circles, 0.83 μM; triangles, 1.7 μM; squares, 3.3 μM) was plotted as a function of bromocriptine concentration. The three binding curves were globally fit to a five-site sequential model with $K_n$ of 5.2 μM for the first two binding modes and 15 μM for all subsequent binding events. Inset: Trp fluorescence of 1.7 μM Nanodiscs is quenched in response to increasing bromocriptine concentrations. $B$, Bromocriptine binding to 1.4 μM CYP3A4-Nanodiscs. The difference between ΔA at 388 nm and 417 nm was plotted against bromocriptine concentration. Data were fit to kinetic simulations of the binding model in Scheme 5, with $K_f$ of 0.45 μM. Inset: the Type-I calculated difference spectra induced by bromocriptine binding to CYP3A4 in Nanodiscs.

Fig. 7: Summary of membrane/CYP3A4 equilibria. The Gibbs free energy difference between one ligand molecule bound to the Nanodisc membrane and to the CYP3A4 active site is plotted on the ordinate, for all four ligands used in this study. The red prism represents CYP3A4, the disc represents the Nanodisc, and yellow hexagons represent molecules of ligand. ANF preferentially binds to the membrane, and its $K_2$ is therefore greater than 1. TNS binds with almost equal affinity to either site, and so its $K_2$ is about 1. Miconazole and bromocriptine preferentially bind to CYP3A4, giving them $K_2$ values less than 1.

Scheme 1: Generic model of a ligand $L$ (yellow hexagon) binding to a membrane protein $E$ (red prism) incorporated into a model membrane $M$ (white disc). $L\cdot ME$ represents a single ligand molecule bound to the model membrane, and $ME\cdot L$ represents ligand bound to the protein. $K_f$ is the dissociation constant of ligand binding to the membrane (related to the membrane/solvent partition coefficient of the ligand), and $K_f'$ is the dissociation constant of ligand binding to the protein. $K_2 = K_f'K_f$ is a unitless equilibrium constant describing the relative amounts of ligand bound to the membrane and to the protein.

Scheme 2: Model of TNS binding to CYP3A4 in Nanodiscs. $L$ (yellow hexagon) represents TNS, $ME$ (red prism on white disc) represents the CYP3A4-Nanodisc complex, $L\cdot ME$ represents a single TNS molecule bound to the Nanodisc bilayer, $ME\cdot L$ represents TNS bound in the CYP3A4 active site, $L_n\cdot ME$ represents $n$ TNS molecules bound to the Nanodisc bilayer, and $L_n\cdot ME\cdot L$ represents one TNS bound in the
CYP3A4 active site and n bound to the bilayer. Three TNS molecules bind sequentially to the Nanodisc bilayer, with $K_f$ values of 2.9 μM for the first binding event and 6.1 μM for the second and third binding modes (indicating apparent negative cooperativity). $K_{i'}$ (4.0 μM) is the dissociation constant of TNS binding to CYP3A4 in Nanodiscs, compared to the solution $K_D$ of 25.4 μM. $K_f$ values between 1.4 (for singly-occupied complexes) and 0.66 (for doubly- and triply-occupied complexes) indicate TNS does not have a strong thermodynamic preference for either the Nanodisc bilayer or the CYP3A4 active site.

**Scheme 3:** Model of ANF binding to CYP3A4 in Nanodiscs. L (yellow hexagon) represents ANF, ME (red prism on white disc) represents the CYP3A4-Nanodisc complex, $L_n$:ME represents n ANF molecules bound to the Nanodisc bilayer, and $L_n$:ME·L represents one ANF bound in the CYP3A4 active site and n bound to the bilayer. In this model, 14 ANF molecules bind to the Nanodisc bilayer with a dissociation constant of 0.81 μM. $K_{i'}$ is 5.8 μM for CYP3A4 in Nanodiscs, compared to a $K_D$ of 55 μM for CYP3A4 in solution. $K_f$ (7.2) indicates that ANF preferentially binds the bilayer, with a ΔG between the two states of 1.2 kcal/mol.

**Scheme 4:** Model of miconazole binding to CYP3A4 in Nanodiscs. L (yellow hexagon) represents miconazole, ME (red prism on white disc) represents the CYP3A4-Nanodisc complex, ME·L represents a miconazole molecule bound to the Nanodisc bilayer, and ME·L represents miconazole bound in the CYP3A4 active site. Miconazole binds to the Nanodisc bilayer with a $K_f$ of 9.0 μM and to CYP3A4 with a $K_{i'}$ of 0.45 μM (compared to a solution $K_D$ of 0.04 μM). The value of $K_f$ (0.05) indicates that miconazole preferentially binds the CYP3A4 active site, with a ΔG of -1.8 kcal/mol.

**Scheme 5:** Model of bromocriptine binding to CYP3A4 in Nanodiscs. L (yellow hexagon) represents bromocriptine, ME (red prism on white disc) represents the CYP3A4-Nanodisc complex, $L_n$:ME represents n bromocriptine molecules bound to the Nanodisc bilayer, and $L_n$:ME·L represents one bromocriptine bound in the CYP3A4 active site and n bound to the bilayer. Five bromocriptine molecules bind to the Nanodisc bilayer, with $K_f$ values of 5.2 μM for the first two binding events and 15 μM for the last three events. $K_{i'}$ is 0.45 μM, compared to a solution $K_D$ of 0.18 μM. $K_f$ varies between 0.09 (for singly- and doubly-occupied complexes) and 0.03 (for higher occupancies), indicating a ΔG for the CYP3A4 active site between -1.4 kcal/mol and -2.1 kcal/mol.
MSP (1:65)

Cholate (50 mM)

Mixed micelles

Amberlite XAD-2

Nanodiscs

MSP (1:63:0.1)

Cholate (50 mM)

Mixed micelles

Amberlite XAD-2

Nanodiscs + CYP3A4-Nanodiscs

Nickel column

CYP3A4-Nanodiscs
Membrane is preferred

ΔG (kcal/mol)

TNS
K₂ ≈ 1

ANF
K₂ > 1

Protein is preferred

Miconazole
K₂ < 1

Bromocriptine
K₂ < 1
Ligand binding to cytochrome P450 3A4 in phospholipid nanodiscs: The effect of model membranes

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