Differential Effects of Clotrimazole on X-Ray Crystal Structures of
Human Cytochrome P450 (CYP) 3A5 and 3A4

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Abbreviations.

CYP or P450, a generic term for a cytochrome P450 enzyme, individual P450s are identified using a number-letter-number format based on amino acid sequence relatedness; Fc, calculated structure factor; Fo, observed structure factor; PDB, Protein Data Bank, specific entries are indicated by PDB: followed by 4 alphanumeric symbols; PEG, polyethylene glycol; PMSF, phenylmethylsulfonyl fluoride; SSRL, Stanford Synchrotron Radiation Lightsource.
Abstract.

Cytochrome P450s CYP3A5 and CYP3A4 exhibit differential plasticity that underlies differences in drug metabolism and drug-drug interactions. To extend previous studies, CYP3A4 and CYP3A5 were co-crystallized with clotrimazole, a compact ligand that binds to the heme iron in catalytic center of the active site. Binding studies indicate that clotrimazole exhibits tight binding to CYP3A5 with a Kd of <0.01 µM like that of CYP3A4. A single clotrimazole is bound to the heme iron in CYP3A4 that triggers expansion of active site cavity that reflects a loss of aromatic interactions between phenylalanine sidechains in the distal active site and increased conformational entropy for the F-F’ connector due to reorientation of Phe-304 to accommodate clotrimazole. In contrast to CYP3A4, the CYP3A5 Phe-304 exhibits an induced fit along with Phe-213 to form edge-to-face aromatic interactions with heme bound clotrimazole. These aromatic interactions between aromatic amino acids propagate by induced fits with a second clotrimazole residing in the distal active site and a third clotrimazole bound in an expanded entrance channel as well as between the three clotrimazoles. The large, expanded entrance channel surrounded by the C-terminal loop and the F’ and A’ helices in CYP3A5 suggests conformational selection for the binding of clotrimazole due to its large girth, which may also cause the entrance channel to remain open after the binding of the first clotrimazole to the heme iron. The additional binding sites suggest a path for sequential binding of one molecule to reach and bind to the heme iron.
Significance Statement.

Clotrimazole binds to the heme iron of CYP3A5 and 3A4. In CYP3A5, two clotrimazoles also bind in the distal active site and in an expanded entrance channel. Aromatic interactions between clotrimazoles and phenylalanine sidechains including Phe-304 indicate induced fits for each clotrimazole. In contrast to CYP3A5, displacement of the CYP3A4 Phe-304 rotamer by clotrimazole leads to extensive disruption of phenylalanine interactions that limit the space above the heme, to an expanded active site cavity, and to increased CYP3A4 conformational heterogeneity.
Introduction.

Cytochrome P450s (CYP) 3A4, 3A5 and 3A7 contribute to hepatic, metabolic clearance of a substantial portion of therapeutic drugs, and there is a considerable potential for severe drug-drug interactions for these enzymes. The presence of CYP3A4 is low in the fetal and pediatric populations, whereas CYP3A7 predominates in the fetal liver and diminishes after birth when expression of CYP3A4 increases (Stevens, 2006; Ince et al., 2013). CYP3A5 exhibits genetic variation between ethnic and geographical populations. When expressed, CYP3A5 is present in the fetal, perinatal, and adult periods and in a larger number of organs than CYP3A4 (Aoyama et al., 1989). The substrate free structures of the three proteins differ significantly from each other in portions of their substrate binding cavities (Sevrioukova, 2021).

Clotrimazole is used for skin and vaginal fungal infections as well as an oral troche for treatment of thrush. Clotrimazole is used to inhibit fungal CYP51, which disrupts the synthesis of ergosterol and weakens the pathogen’s cellular membranes. Clotrimazole lozenges exhibit drug-drug interactions with tacrolimus that are exacerbated in organ transplant patients that express CYP3A5 in pediatric (Lalan et al., 2014) and adult (Uno et al., 2018; Uno et al., 2019) populations.

Clotrimazole is a compact molecule with an imidazole moiety and three phenyl groups attached to central carbon, Figure 1. It is a potent inhibitor of 3A P450s (Gibbs et al., 1999; Zhang et al., 2002) as well as a CYP3A4 substrate (Guengerich et al., 2021). The binding affinity (Kd) of clotrimazole to CYP3A4 was reported in two independent studies to be ~0.03 µM (Isin and Guengerich, 2007) and ~0.016 µM (Godamudunage et al., 2018) based on type II spectral shifts that reflect coordinate-covalent binding of
the imidazole lone pair electrons to the open axial site of the heme iron. A Kd of \(~0.010 \mu M\) was reported for the binding of clotrimazole to CYP3A7 (Godamudunage et al., 2018). As reported here, CYP3A5 exhibits a similar Kd of \(0.004\pm0.004 \mu M\) based on the type II spectral shift.

Clotrimazole was targeted for crystallization with CYP3A5 to extend our knowledge of the plasticity of the CYP3A5 active site that contributes to open and closed conformations of the enzyme as well as to induced fits that enhance catalysis and specificity for substrates and the potency of inhibitors. Significant induced fits are not seen in the structure of CYP3A4 with the antifungal fluconazole (Figure 1) and for other small substrates, and these structures are very similar to the ligand free structure of CYP3A4 (Sevrioukova, 2019a). Clotrimazole is smaller than other compounds crystallized with CYP3A5, clobetasol propionate (Wang et al., 2021), azamulin (Hsu and Johnson, 2022) and ritonavir (Hsu et al., 2018), and the clotrimazole complex may not exhibit an induced fit when it is bound to the heme iron in the proximal active site. Additionally, the wide girth of clotrimazole suggests that the entrance channel seen in the substrate free CYP3A5 needs to expand significantly for passage of clotrimazole into the active site. This wide conformational dependence may also underlie the slow binding kinetics observed for binding clotrimazole to the heme iron of CYP3A4 (Isin and Guengerich, 2007; Guengerich et al., 2021).

A 2.8 Å X-ray structure of CYP3A5 crystallized with clotrimazole was obtained with an open structure that accommodates a second clotrimazole in the distal active site cavity and third clotrimazole in an expanded entrance channel. This expansion reflects the large girth and rigidity of clotrimazole that additionally causes modest expansions of
each of the distal and proximal binding sites and an enlarged entry channel. The large expansion of the entrance channel between the helix F-F' connector, helix A' and deflection of the turn in the C-terminal loop is due to the binding of clotrimazole in the entrance channel. This is accompanied by significant changes in amino acid sidechain orientations in the active site relative to the substrate free structure of CYP3A5. Substantial induced fits were also seen for the binding of clotrimazole to the heme iron and in the distal active site. Only modest changes are evident in the protein backbone from the structure of the substrate free enzyme. In contrast a 2.9 Å X-ray crystal structure of CYP3A4 was obtained with a single clotrimazole bound to the heme iron that exhibits an enlarged active site. This is due to reorientation of Phe-304 to accommodate clotrimazole that disrupts aromatic π-HC interactions with Phe-213 and Phe-241 leading to a large expansion of the active site.

**Materials and Methods.**

*Protein Expression and Purification:* The human CYP3A5 was modified for crystallization by deleting the N-terminal trans-membrane helix (amino acids 3-24) and replacement of the five C-terminal amino acids for a 4-histidine tag to enhance and facilitate purification of the protein. The pCW3A5C2dH plasmid was employed to express CYP3A5 in *Escherichia coli* together with the pGro7 plasmid which increased the expression of GroES and GroEL protein chaperones. Methods for bacterial transformation, growth and protein purification are as described earlier (Hsu et al., 2018) and amended (Hsu and Johnson, 2019). Additionally, the expression plasmid pSE3A4dH (Yano et al., 2004) with the pGro7 plasmid was used to express CYP3A4 in
E. coli. The purification protocol for CYP3A4 has been reported previously (Yano et al., 2004).

**Crystallization Conditions for the Clotrimazole Complex with CYP3A5.** A buffer consisting of 50 mM sodium HEPES, pH 7.4, 50 mM potassium acetate, pH 7.4, 20% glycerol, 0.5 mM EDTA, and 10 mM 2-mercaptoethanol was used for the P450. A 60 mM solution of clotrimazole in DMSO was added to 0.95 mM solution of CYP3A5 at a 3-fold molar ratio and incubated for 7 min on a rotator at room temperature followed by centrifugation for 1 min to remove particulates. A spectrum of the diluted protein indicated that the wavelength maximum of the Soret band was at 425 nm, which indicates the complexation of the azole nitrogen of clotrimazole to the ferric heme iron of CYP3A5.

A 0.43 mM solution of the CYP3A5 clotrimazole complex was combined with an 0.1% (v/v) aqueous solution of the detergent C12E9 and with the precipitant solution in a volume ratio of 0.5:0.1:0.5 for crystallization using sitting drop vapor diffusion with 0.4 ml of precipitant solution in the well at 23° C. The precipitant solution comprised 0.2 M sodium malonate, pH 6.5, and 30% PEG 3350 (w/v). The crystal was flash frozen in liquid nitrogen for data collection after a 24 sec soak in Paratone-N oil for cryoprotection. Data from a single crystal at 100 K were collected on beamline 12-1 at the Stanford Synchrotron Radiation Lightsource using Blu-Ice. The data were indexed using XDS (Kabsch, 2010) in space group P 1 21 1, and the data were reduced using Aimless (Evans and Murshudov, 2013). Supplemental Table S1 provides the results and statistics for data refinement.
Molecular replacement using Phaser (McCoy et al., 2007) with the ligand free CYP3A5 6mjm structure as probe indicated eight protein molecules were present in the asymmetric unit with scores for LLG: 16238 and TFA: 71.6. Adjustments to the model were made using Coot (Emsley et al., 2010), and Phenix (Liebschner et al., 2019) was used for reciprocal space refinement against the data to 2.8 Å. The model is complete for residues 26-496 except for residues 260-269 in the loop connecting helices G to H and 280-287 connecting helices H to I that could not be modeled due to configurational heterogeneity. The residue numbers correspond to the native protein. Additionally, chain H is missing residues 419-425 in an external loop. In some chains, an additional 1 to 3 residues were not defined at the N-terminus, and the terminal residue 496 was not defined in chain C. Loops between helices G-H and H-I were disordered in all chains. Three clotrimazoles (CL6) were modeled in each protein chain, and a molecule of the detergent C12E9 (CE9) was fit on the outer surface of each chain except H. The Grade server (Smart et al., 2011) was used to generate restraints for the ligands using numbering for CL6 and CE9 in Protein Databank (PDB) Ligand Expo. Statistics for model refinement are summarized in Supplemental Table S1. Coordinates and structure factors were deposited with the PDB ID:8sg5 (pdbdoi: 10.2210/pdb8sg5/pdb).

**Crystallization Conditions for the Clotrimazole Complex with CYP3A4.** The purified CYP3A4 protein was used in a buffer of 100mM potassium phosphate (pH 7.4), 100mM NaCl, 20% glycerol (v/v), 0.5 mM EDTA, and 0.2 mM dithiothreitol. A 30 mM solution of clotrimazole in acetonitrile was added to 1.43 mM solution of CYP3A4dH at a 3:1 molar ratio. After centrifugation to remove sediment, a spectrum of the diluted protein indicated that the wavelength maximum of the Soret band was shifted to 425 nm.
indicating saturation of the protein with clotrimazole. For crystallization, a 0.5 mM solution of the CYP3A4 clotrimazole complex was combined with an aqueous solution of the detergent CHAPSO (80 mM) and with the precipitant solution in a volume ratio of 0.8:0.2:1 for crystallization using sitting drop vapor diffusion with 0.4 ml of precipitant solution in the well. The precipitant solution comprised 0.2M HEPES, pH 7.0, 9.9 mM LiSO₄, and 15% PEG 3350 (w/v). The crystal was harvested after addition of 4µl 80 mM HEPES, pH 7.0, 12% glycerol, 3.96 mM LiSO₄, 6% PEG3350, 40mM potassium phosphate, pH 7.4, 40mM NaCl, 0.2mM EDTA, 0.08mM dithiothreitol to the drop. The crystal was flash frozen in liquid nitrogen for data collection after a 19 sec soak in Paratone-N oil for cryoprotection. Data from a single crystal at 100 K were collected on beamline 12-1 at the Stanford Synchrotron Radiation Lightsource using Blu-Ice. The data were indexed using XDS (Kabsch, 2010) in space group I222 and reduced using Aimless (Evans and Murshudov, 2013).

Molecular replacement using chain A of the CYP3A4 erythromycin complex [PDB:2j0d] indicated one molecule of CYP3A4 in the asymmetric unit and confirmed the space group as I222. Electron density maps confirmed the binding of clotrimazole to the heme. The model was refined and modified as described earlier for the CYP3A5 clotrimazole complex. Supplemental Table S2 provides the results and statistics for data refinement and structure modelling. The coordinates and X-ray reflections were deposited in the PDB with the ID: 8spd (pdbdoi: 10.2210/pdb8spd/pdb).

*Ligand binding studies.* Clotrimazole binding to CYP3A5 and CYP3A4 was characterized by the formation of the coordinate covalent bond of the proximal clotrimazole nitrogen to the heme iron. This interaction causes the absorption of the
prominent Soret band to shift to a higher wavelength (~417nm to ~425 nm). This shift was used to determine concentration dependence of clotrimazole to form the type II complex with the heme iron using UV/Visible spectroscopy. For this purpose, the spectrum of the protein was recorded at 1 nm increments for 1 sec between 800 nm to 250 nm and following each administration of clotrimazole after a 3 min equilibration period. The digital spectrum of the protein before addition of clotrimazole was subtracted from the spectra obtained after clotrimazole addition to create difference spectra using Microsoft Excel. The trough to peak differences were determined at different concentrations of clotrimazole. The initial P450 concentrations were estimated from the height of the Soret peak using an extinction coefficient of 0.115 (µM x cm)\(^{-1}\) (Dawson et al., 1982).

Acetonitrile solutions of clotrimazole were used with a final acetonitrile concentration of 0.7% (v/v) at the end of the titration. CYP3A5 was diluted in 50 mM sodium HEPES, 50 mM potassium acetate (pH 7.4), 20 % glycerol, and 0.5 mM EDTA to ~1 µM. The CYP3A4 stock was diluted in 100 mM potassium phosphate, pH 7.4, 100 mM NaCl, 0.5 mM EDTA, and 20% glycerol to a concentration of ~1 µM.

A one-site binding equation that exhibited tight binding was used to fit the data points. This required correction for free substrate concentration due to significant depletion of the free ligand by protein binding using the quadratic equation, Eq 1, with non-linear least squares regression using the program SlideWrite. L is the total concentration of the ligand and P is concentration of the P450. Kd is the dissociation constant, ΔA is the trough to peak difference observed at each concentration of the ligand, and ΔAmax is the maximum change of absorbance at saturation.
Eq 1: \[ \Delta A = \Delta A_{\text{max}} \left( \frac{(P+L+K_d)-\sqrt{(P+L+K_d)^2-4PL}}{2P} \right) \]

Structural Comparisons: PyMol (DeLano, 2005) was used to generate figures for molecular structures. Superposition of structures were generated using the “Align” command in PyMol with default settings. The default uses 5 cycles for all atoms with rejection of outliers in each cycle. These results reflect most atoms that are in the core of the protein. RMS comparisons between two structures were computed using the align function for Cα-atoms without culling outliers. Distances between heavy atoms that are H-bonded were determined using the Pymol distance command and reported in Angstroms. Bond lengths and angles for axial bonds of clotrimazole N and Cys-442 SG were determined using Coot (Emsley et al., 2010) for the CYP3A5 and CYP3A4 clotrimazole complexes and previously released structures of clotrimazole complexes of other P450s, PDB Codes: 6UW2, 6HIT, 2XFH, and 4XE3. Means and standard deviations were computed using Microsoft Excel.

Results.

Clotrimazole Binding Isotherms. The isotherm for the binding of clotrimazole to ~1.3 µM CYP3A4 is shown in Figure 2A. The linear rise and sharp transition to \( \Delta A_{\text{max}} \) at ~1.2 µM clotrimazole is consistent with a 1:1 stoichiometry for equal concentrations of ligand and protein at the saturation of binding and indicates that the CYP3A4 protein used for crystallization retains a high affinity for binding clotrimazole. Spectra of the protein at saturation indicated that the peak of the Soret band had shifted to between 424 and 425. The isotherm was best fit when the protein concentration P was estimated along with \( \Delta A_{\text{max}} \) and Kd because the fit is very sensitive to small errors in
concentrations of the ligand and/or protein. The fit exhibited an $r^2$ coefficient of 0.999 with Std Error of 0.001. The protein concentration was estimated to be 1.2 µM with Std Error of 0.02 µM. The Kd was estimated to be $0.01 \pm 0.002$ µM and a ΔAmax of $0.09 \pm 0.002$ change in absorption. The average Kd and standard deviation for three replicates was $0.005 \pm 0.003$ µM, which is similar to the Kd values reported for the binding of clotrimazole to CYP3A4 0.03 µM (Isin and Guengerich, 2007), for CYP3A4 0.016 µM (Godamudunage et al., 2018) and for CYP3A7 0.010 µM (Godamudunage et al., 2018).

The binding of clotrimazole to ~1.1 µM CYP3A5 also exhibited linear rise with a sharp transition at a concentration of clotrimazole that was equivalent to the concentration of protein indicating 1:1 stoichiometry at saturation (Figure 2B) with a ΔAmax of 0.095 with a standard error of 0.001 for the change in absorption, a Kd for binding estimated at 0.01 µM with a standard error of 0.003 µM, and predicted value for P of 1.05 µM with a standard error of 0.015 µM. The overall fit was characterized by an $r^2$ coefficient of 0.999 with a standard error of 0.001. The average Kd and standard deviation for three replicates was $0.004 \pm 0.004$ µM. The spectra for the protein at saturation indicated that the Soret peak had shifted to between 424 and 425 nm.

**Structure of the CYP3A5 Clotrimazole Complex.** The X-ray crystal structure of CYP3A5 clotrimazole complex revealed eight protein chains in the asymmetric unit. There were two exterior loops between helices G-H and H-I as well as residues at the N-terminus and C-terminus that could not be defined. Each protein chain exhibited three clotrimazoles with the aromatic nitrogen of imidazole moiety of one clotrimazole coordinate-covalently bonded to the heme iron. The orientation and placement of the three clotrimazoles were well-defined by a 2m|Fo|-D|Fc| composite omit map (Figure 3).
The orientation of the clotrimazole molecules was also evident by additional electron density that encompasses the chlorine atom on one phenyl ring and the smaller size of the imidazole moiety. It is not clear that the binding of additional clotrimazoles would alter the type II shift because of the strong interaction of the nitrogen with the heme iron unless the presence of the second clotrimazole displaces the iron bound clotrimazole and significantly affects the bond length and angles. The bond lengths seen in this structure exhibit nominal axial bond lengths of ~2.0 Å and ~2.3 Å for the iron-nitrogen and iron-sulfur bonds, respectively, and the N-Fe-S bond angle is ~173° for eight chains (Supplemental Table S3). The apparent 1:1 stoichiometry of clotrimazole observed in the 10⁻⁶ M range suggests that the higher concentrations used for crystallization, 10⁻⁴ M, may contribute to binding of additional clotrimazoles to CYP3A5.

Additionally, a C12E9 detergent molecule was bound in the interstitial space between the protein chains in crystal lattice (Supplemental Figure S1). The interstitial spaces differ between chains of the asymmetric unit with sparse contacts with C12E9 leading to incomplete 2m|Fo|-D|Fc| difference maps, particularly for the ends of the linear C12E9 molecule and were truncated from the models. There was insufficient density to determine the presence of the detergent near chain H.

In most aspects, the Cα traces match the trace of substrate free structure of CYP3A5 (PDB 6mjm) with rms values between 0.55Å and 0.75Å. A notable exception leads to a large displacement of the helix F-F' connector coupled with a change in the axis of F’ helix that widens the opening between the F’ and A’ helices (Fig. 4). Moreover, this displacement exhibits an induced fit of phenylalanine residues to make favorable edge to face aromatic interactions with the clotrimazoles (Fig.3). Additionally,
an altered turn in the C-terminal loop expands the proximal active site cavity, as well as an expansion of the entrance channel at the top of the cavity (Fig. 4). A smaller shift for the upper portion of the helix B-B’ loop leads to additional space to accommodate the clotrimazoles. There is a small translation and tilt of the heme that may reflect the coordinate covalent binding of clotrimazole to the heme iron and steric restraints on the clotrimazole. The eight chains did not exhibit significant differences in the Cα traces for pair wise comparison to chain A having root mean square values <0.6 Å with the exception deviations for the C-terminal loop and a small portion of the F-F’ connector near helix F of chains D and H (Figure 4).

A more detailed examination of each of the three clotrimazole binding sites for chain A with substrate-free CYP3A5 indicates that significant changes in amino acid rotamers enlarge the cavity and engage in favorable edge-to-face aromatic interactions with aromatic rings of the clotrimazoles. These interactions include edge-to-face aromatic groups between adjacent clotrimazoles as well as between aromatic amino acid side chains and the clotrimazoles as shown for the clotrimazole (bright orange carbons) bound to the heme in Figure 5A. These interactions are thought to occur when the centroids of the aromatic rings are between 4.5 to 7.0 Å and most often exhibit dihedral angle near 90° (Burley and Petsko, 1985). The figure shows amino acid residues residing within 4.5Å of the clotrimazole for chain A with yellow carbons. The corresponding amino acid residues of the superimposed substrate free structure of CYP3A5 (PDB: 6mjm) are shown with gray carbons. The two other clotrimazoles are also within 4.5Å of heme bound clotrimazole and with each other.
The phenyl bound to the central carbon in the same plane as the imidazole group contacts Arg-105 at the edge of the heme, which may contribute to the minor differences seen for Arg-105 relative to the 6mjm structure. Other amino acid rotamers in this stratum are like those in the 6mjm structure except for Ser-119, which donates an H-bond to imidazole of the clotrimazole bound in distal active site. The other clotrimazole phenyl group extends upward toward Leu-481 in the turn of C-terminal loop where it pushes Leu-481 outward and which adopts a different rotamer. The chlorophenyl group is oriented toward helix I where it forces Phe-304 on helix I rotate upward, and in turn, forcing Phe-213 to adopt a new rotamer and that shifts the position of Phe-213. In this process, Phe-213 and Phe-304 retain the aromatic interaction and interact with aromatic sidechains of the clotrimazoles as well as cause an expansion of the proximal active site. Additionally, the repositioning of Phe-304 enables π-π stacking interaction with Phe-210.

A second clotrimazole (purple carbons) binds in the distal active site between the first clotrimazole and the β-sheet and salt-bridges at the end of the distal active site. The imidazole group of the second clotrimazole accepts hydrogen bond from Ser-119 which may underlie the differences for Ser-119 and Leu-120 from their positions in the 6mjm structure (Figure 5B). Arg-106 is part of the salt bridges in beta-sheet region that forms the boundary of the distal cavity, and it is pushed outward by the change in the position of Arg-105 (Figure 5A) as well as contacts of the second clotrimazole with Ser-107 and Arg-106, which are on the B-B’ loop. Additionally, the aromatic rings of the second clotrimazole exhibit edge-to-face aromatic interactions with Phe-304, Phe-213, and Phe-220 to enhance the binding of the second clotrimazole in the distal active site.
A third clotrimazole (brown carbons) is bound in the enlarged entry channel relative to the partial opening seen in the 6mjm structure. Occupation by the rigid clotrimazole causes an expansion of the cavity to accommodate the wide girth of propeller-like clotrimazole molecule (Figure 5C). The orientation of the imidazole moiety can receive a hydrogen bond from Asp-217 nitrogen. Additionally, changes in the orientation of the Phe-215 and Phe-220 optimize the binding stability of third clotrimazole in the entry channel.

Structure of the CYP3A4 Clotrimazole Complex. There is only one protein chain in the asymmetric unit of the CYP3A4 clotrimazole complex. Six and eleven amino acid residues not defined for N-terminus and C-terminus, respectively, and two gaps reflect the disordered exterior loops between helices G-H and H-I. In contrast to CYP3A5, the structure of the CYP3A4 clotrimazole complex prepared with the same ligand stoichiometry at similar concentrations of P450 and clotrimazole led to a crystal structure with a single clotrimazole molecule evident in the active site as defined by the 2m|Fo|-D|Fc| composite omit map (Figure 6A). The iron-nitrogen bond length is 1.4 Å, which is in the range seen for CYP3A5 and other P450 complexes with clotrimazole. However, the 2.2 Å iron-sulfur bond length is shorter than that seen for CYP3A5 but within the range seen for other P450 clotrimazole complexes. The deviation of N-Fe-S bond angle from 180° is larger for CYP3A4 at ~156° than seen for CYP3A5 at ~173°. As the coordinate error and B-values are higher for the CYP3A4 structure, these values are less accurate than the values for other clotrimazole complexes (Supplemental Table S3). The interactions of clotrimazole with CYP3A4 amino acid sidechains (≤4.5Å) are sparse and confined to amino acids that reside near the heme surface with exception.
for Leu-482 on the turn in the C-terminal loop and Phe-304 on the upper side of helix I (Figure 6A). Comparison of the structure of CYP3A4 clotrimazole complex to that of CYP3A4 fluconazole (ruby red carbons) complex (PDB:6maj) is shown in Figure 6B. Fluconazole is another antifungal drug with different moieties on the central carbon than those of clotrimazole (Figure 1). The chlorophenyl group of clotrimazole is replaced by a small hydroxyl group oriented toward Phe-304. Additionally, the azole bound to the heme iron is linked to the central carbon by a methylene link as is the second azole that is oriented toward B-B’ loop. The methylene links provide additional flexibility to allow the large side chain to bind to the heme iron. The difluoro-phenyl group is directed at the helix F-F’ connector. The overall structure of the fluconazole complex is like the substrate free structure of CYP3A4, but Arg-212 is displaced from the active site by fluconazole (Sevrioukova, 2019a). Phe-304 interacts with edge-to-face interactions with Phe-241 and Phe-213, which also interact with Phe-215, Phe-220 and Phe-108 that lie close above the fluconazole.

The chlorophenyl group of clotrimazole clashes with the CYP3A4 Phe-304 rotamer seen in the fluconazole complex. This causes Phe-304 to adopt a different rotamer in CYP3A4 clotrimazole complex (Figure 6B), and the alternate rotamer is accommodated by a substantial change in the trajectory of helix F and F’ helices as seen for the large displacement of Arg-212 Cα by 8.9 Å as well as neighboring amino acids such as Phe-213 and Phe-215 on the helix F-F’ connector. Additionally, there is a 2.9 Å translation of Phe-241 Cα on the helix G’-G connector. Reorientation of the Phe-304 disrupts aromatic interactions between phenylalanine sidechains seen above the fluconazole as well as interactions with Phe-108 and Phe-220 seen for fluconazole complex (Figure
6C). The loss of amino acid interactions between the helix F-F' connector with other parts of the structure increases disorder that prevents the modeling of large sidechains beyond Cβ in helix F-G region and for Phe-108 of CYP3A4-clotrimazole structure. This expansion allows the turn in C-terminal loop to move inward toward the helix I and Leu-482 to contact a clotrimazole phenyl group. These changes lead to an expansion of the upper portion of the active site cavity seen in CYP3A4 clotrimazole complex and may contribute to the high B-factors for this structure.

Clotrimazole binds to the heme iron in CYP3A4 like that seen for CYP3A5, but the Phe-304 rotamers differ between the two complexes (Figure 7A). In the CYP3A5 complex, Phe-304 adopts a rotamer that provides face-to-face interaction with Phe-210 and enables face to edge (π-HC) interactions with chlorophenyl group of clotrimazole and Phe-241. Additionally, the longer F helix of CYP3A5 stabilizes these interactions and places Phe-213 in a place to propagate these aromatic interactions with the other two clotrimazoles. Moreover, the longer F helix of CYP3A5 leads to a lower and wider trajectory for the helix F-F' connector than seen for the CYP3A4 clotrimazole complex (Figure 7A). Although the substantial change in the trajectory helix F and the helix F-F' connector expands active site cavity above the clotrimazole relative to substrate free structure of CYP3A4, the cavity is smaller than that of CYP3A5, and there is not sufficient room to accommodate additional clotrimazoles in the CYP3A4 structure (Figure 7B). The conformation of CYP3A4 B-B' loop impinges on the binding site for clotrimazole in the CYP3A5 distal cavity (Figures 6C and 7B) and the CYP3A4 helix F-G region does not expand to accommodate clotrimazole in the entrance channel seen for CYP3A5 (Figure 5C) which is outside the CYP3A4 helix F'-F connector (Figure 7B).
Discussion.

A main finding in this study is that although clotrimazole binds to the heme iron of CYP3A4 and CYP3A5 in the same way with only minor effects on protein residues near the heme surface, CYP3A4 and CYP3A5 exhibit divergent outcomes for repositioning of Phe-304 on the I helix to accommodate the clotrimazole bound to heme iron. The displacement of Phe-304 in CYP3A5 leads to a favorable edge-to-face aromatic interaction with chlorophenyl group of clotrimazole and face to face aromatic interaction with opposite face Phe-304 with Phe-210 with minor effects relative to the substrate free structure of CYP3A5.

In contrast, the repositioning Phe-304 in CYP3A4 disrupted the extensive aromatic interactions that are seen in structures for substrate free CYP3A4 and small ligand complexes. These changes were accompanied by an increase in heterogeneity for the helix F-F' connector. Phe-304 has unique role in the formation of these aromatic interactions in CYP3A4 because it interacts with Phe-213 on the helix F-F' connector and Phe-241 on the helix G'-G connector which draws the two connectors together to promote additional phenylalanines to extend the aromatic interactions. In the absence of these interactions, the volume of the cavity expands and helix F-F' connector becomes more disordered. Conformational changes that increase the entrance channel relative to the conformations of apo or substrate-bound structures have been reported for structures of type II complexes of clotrimazole with the eukaryotic enzyme CYP51A1 of *Acanthamoeba Castellanii* (Nienhaus et al., 2022) and with the prokaryotic enzymes CYP102A, a fatty acid hydroxylase (Jeffreys et al., 2019), CYP107D1, a fatty acid
epoxidase (Montemiglio et al., 2021), and CYP113A1, the erythromycin B/D 12-hydroxylase (Montemiglio et al., 2010).

This structural difference in the behavior of helix F-F’ connector between CYP3A4 and CYP3A5 is also seen when azamulin binds to the enzymes. The binding of azamulin to form the type I complex is similar for both CYP3A4 and CYP3A5 with the pleuromutilin macrocycle bound near the heme iron and with terminus of thioester group directed out of the cavity under the helix F-F’ connector that was well defined in CYP3A5 (Hsu and Johnson, 2022) but was undefined in the structure of CYP3A4 azamulin complex (Sevrioukova, 2019b). The helix F-F’ connectors are incomplete in other CYP3A4 structures in the PDB and/or exhibit alternative rotamers for Phe-304 from t80 rotamer seen in the fluconazole complex. Interestingly, an accelerated molecular-dynamics experiment captured the trajectory of testosterone into the lipid bilayer, entry into the active site to reside in an intermediate binding site near Phe-304 and Phe-213 in the upper cavity before sequestration of testosterone above heme surface, near the heme iron below the interacting phenylalanines in the upper cavity (Hackett, 2018). These observations suggest that disruption of the aromatic interactions occurs during substrate access and in the case of clotrimazole, Phe-304 is not able to adopt an orientation that contributes the restoring aromatic interactions or to engage with chlorophenyl group of clotrimazole like that seen for CYP3A5.

These aromatic π-HC interactions seen for CYP3A4 are not seen in substrate-free CYP3A5 (Hsu et al., 2018; Hsu and Johnson, 2019) or CYP3A7 (Sevrioukova, 2021) due to differences in secondary structures and evolutionary amino acid substitutions in helix F to helix G region. The F helix of CYP3A5 is longer than that of CYP3A4 with
different trajectory, and only a minor deviation from the substrate free structure was needed to optimize the edge-to-face aromatic interaction of Phe-213 near the end of helix F in order for the phenyl group to engage the heme iron bound clotrimazole. Additionally, the Phe-304 rotamer CYP3A5 facilitates an induced fit with two additional phenylalanines 210 and 241.

A second major finding is the extent of the expansion needed to accommodate entry of clotrimazole into the active site due to its girth and rigidity. Conformational selection is thought to modulate binding rates of substrates and inhibitors for CYP3A4 and other P450s (Guengerich, 2022; Guengerich et al., 2021; Redhair and Atkins, 2021; Guengerich et al., 2019; Mast et al., 2019; Estrada et al., 2014). This entrance channel is partially open in the 6mjm structure of ligand-free CYP3A5 (Hsu and Johnson, 2019). The expansion of the entrance channel to accommodate clotrimazole involves an outward movement of helix F’ from helix A’ that changes the trajectory of the helix F-F’ connector and a modest change in the path of turn in C-terminal loop (Figure 4). Additionally, amino acid rotamers that restricted entrance in the 6mjm structure in other CYP3A4 structures in the PDB adopt orientations that open the entrance channel. As a result, the clotrimazole in the CYP3A5 entrance channel remains exposed to the exterior solvent.

Furthermore, the structure of CYP3A5 with three clotrimazoles illustrates potential steps for a sequential binding pathway for clotrimazole from the initial entry site, occupation of distal substrate binding cavity and final binding in the proximal cavity near the heme iron where catalysis occurs. This is similar to the stepwise path to active site to form the testosterone complex, that was documented for accelerated molecular-
dynamics study for testosterone entry into CYP3A4 active site (Hackett, 2018). The hydrogen bonding of each of the peripheral clotrimazoles with the protein suggests that clotrimazole might reside in similar positions enroute to the proximal active site. Alternatively, the cavity would need to open further for a direct descent to the proximal active site and the heme iron. The edge-to-face interactions suggest that following the binding of the heme bound clotrimazole, the binding of second and third molecules would be cooperative but may not affect the binding isotherm for the type II complex for heme bound clotrimazole.

In CYP3A5, the binding of the clotrimazole in the distal active site propagates induced fits to enhance aromatic interactions between clotrimazoles and phenylalanines, Figure 5B, with only minor effects on protein sidechains to expand the cavity. The type II binding of clotrimazole to CYP3A5 shows high affinity binding that reflects the formation of a strong coordinate covalent binding to the heme iron that may not be affected by the binding of 1 or 2 additional clotrimazoles. As the bond lengths of heme iron bound clotrimazole are like those for the CYP3A4 clotrimazole complex, it suggests that the binding of two additional clotrimazoles does not significantly disturb the binding of clotrimazole to heme iron. There is a possibility that the additional clotrimazole could hinder dissociation as seen in the molecular-dynamics studies (Wang et al., 2014) for the two clotrimazoles bound in the structure of CYP113A1, an erythromycin B/D 12-hydroxylase (Montemiglio et al., 2010). As the apparent 1:1 stoichiometry in the $10^{-6}$ M range for the tight binding isotherm indicates that second and third molecules bind with lower binding affinity, however this is unlikely to occur at concentrations used for in vitro or in vivo inhibition.
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Data Availability Statement.

Structures and structure factors were deposited in the Protein Data Base with accession identities 8spd (pdbdoi:10.2210/pdb8spd/pdb) and 8sg5 (pdbdoi:10.2210/pdb8sg5/pdb) for CYP3A4 and CYP3A5, respectively. Other data are described in the manuscript or supplement information available from the publisher’s website.

Authorship Contributions.

Participated in research design: Hsu and Johnson

Conducted experiments: Hsu.

Performed data analysis: Hsu and Johnson

Wrote or contributed the writing of the manuscript: Hsu and Johnson
References.


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Footnote

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Figure Legends.

Figure 1. Atomic structures of clotrimazole and fluconazole. Carbons are colored gray, hydrogens white, oxygen red, nitrogen blue, chlorine green, fluorine lime green. The images were generated using PubChem®.

Figure 2. Spectral binding studies for clotrimazole with (A) CYP3A4 and (B) CYP3A5 revealed tight binding with saturation at a 1:1 ratio of the concentrations of clotrimazole and the protein for both enzymes.

Figure 3. X-ray crystal structure of CYP3A5 clotrimazole complex revealed one clotrimazole (yellow-orange carbons) with the aromatic nitrogen (blue) of imidazole moiety of clotrimazole coordinate-covalently bonded to the heme iron (chocolate carbons) with the iron shown as an orange sphere. Oxygen and chlorine atoms are colored red and green, respectively. A second clotrimazole (purple carbons) is bound in the distal active site near the β-sheet domain, and a third clotrimazole (brown carbons) resides in an expanded entrance channel. The orientation and placement of the three clotrimazoles are well defined by a 2m|Fo|-D|Fc| composite omit map (gray mesh) contoured at 2σ. Helices are designated by letters and beta sheets by numbers. Hydrogen bonds (2.5 ≤ and ≥ 3.5Å) are depicted by dashed lines. Units for numbered distances are Ångstroms.
Figure 4. Superposition of the Cα-traces of the eight chains of CYP3A5 clotrimazole complexes in the asymmetric unit with carbons from yellow for chain A to dark red for chain H. The overlaid structure of substrate free CYP3A5 (PDB: 6mjm) depicted as Cα-trace with gray carbons.

Figure 5. Amino acid sidechains (yellow carbons) are displayed within 4.5Å of (A) the clotrimazole bound to the heme with yellow-orange carbons, (B) the clotrimazole with purple carbons in the distal active site, and (C) the clotrimazole with brown carbons bound in the entrance channel. The amino acid sidechains of the overlayed 6mjm structure of the ligand free structure of CYP3A5 are shown with gray carbons. The clotrimazoles are also within 4.5Å of each other with edge-to-face interactions between their aromatic rings. Additionally, Phe-304 exhibits a face-to-face interaction with Phe-210 in panel A.

Figure 6. (A) The binding of clotrimazole (slate blue carbons) in the CYP3A4 (cyan carbons) active site is defined by 2m|Fo|-D|Fc| composite omit map contoured at 1σ (gray mesh). Sidechains within 4.5 Å from clotrimazole are depicted as stick figures. (B) Superposition of the structure (PDB:6ma7) of the CYP3A4 complex (lime green carbons) with fluconazole (ruby red carbons, pale green fluorine) on the structure of the CYP3A4 clotrimazole complex (cyan carbons). Amino acid side chains that are within 4.5 Å of Phe-304 and/or clotrimazole in CYP3A4 clotrimazole complex are shown for both structures together with Phe-108, Phe-215 and Phe-220. Phe-304 adopts a different rotamer (m-83) than seen in fluconazole structure (t-80) to accommodate the
chlorophenyl group of clotrimazole. Additionally, there is a large change in trajectory of
the helix F-F', as seen for the 9.6 Å (dotted line) distance for Arg-212 Cα. This is also
evident for G'-G helix regions as for 3.0 Å differences between Phe-241 Cβ for the two
complexes. Note that the side chains of Arg-212, Phe-213, Phe-215 and Phe-241 are
truncated after Cβ due to disorder in CYP3A4 clotrimazole complex. (C) The upper
cavity of the CYP3A4 fluconazole complex is filled with phenylalanine sidechains that
interact with side to face interactions between > 3.5 Å and < 5.0 Å that provides a low
ceiling above the heme. These interactions are disrupted by the change in the
trajectory of helix F through helix G region seen for clotrimazole complex and causes
several phenylalanine sidechains to become disordered.

Figure 7. Superposition of the CYP3A4 (cyan carbons) clotrimazole (slate carbons)
complex on chain A of the CYP3A5 structure (yellow carbons) clotrimazole (bright
orange carbons) complex. (A) The Phe-304 rotamer differs between the CYP3A4 and
CYP3A5 clotrimazole complexes. CYP3A5 Phe-304 adopts an orientation that provides
a face to edge (π-HC) interaction with the clotrimazole chlorophenyl group and with
Phe-241. In addition, a face-to-face (π-π) interaction of CYP3A5 Phe-304 occurs with
Phe-210. The longer CYP3A5 F helix and its trajectory confers greater stability and
positions Phe-213 to enable an induced fit of Phe-304. (B) The trajectory of the CYP3A5
helix F-F' connector also expands the helix F-G region to accommodate the second
clotrimazole (purple carbons) and turns upward sharply to stabilize the open entrance
channel occupied by third clotrimazole (brown carbons).
Clotrimazole

Fluconazole

Fig. 1
Fig. 7