Three-Dimensional Models of Wild-Type and Mutated Forms of Cytochrome P450 14α-Sterol Demethylases from Aspergillus fumigatus and Candida albicans Provide Insights into Posaconazole Binding

Li Xiao,1* Vincent Madison,1 Andrew S. Chau,2 David Loebenberg,2 Robert E. Palermo,2 and Paul M. McNicholas2

Departments of Structural Chemistry1 and Antimicrobial Therapy and Molecular Genetics,2 Schering-Plough Research Institute, Kenilworth, New Jersey 07033

Received 27 August 2003/Returned for modification 3 October 2003/Accepted 24 October 2003

The cytochrome P450 sterol 14α-demethylase enzyme (CYP51) is the target ofazole antifungals. Azoles block ergosterol synthesis, and thereby fungal growth, by binding in the active-site cavity of the enzyme and ligating the iron atom of the heme cofactor through a nitrogen atom of theazole. Mutations in and around the CYP51 active site have resulted inazole resistance. In this work, homology models of the CYP51 enzymes from Aspergillus fumigatus and Candida albicans were constructed based on the X-ray crystal structure of CYP51 from Mycobacterium tuberculosis. Using these models, binding modes for voriconazole (VOR), fluconazole (FLZ), itraconazole (ITZ), and posaconazole (POS) were predicted from docking calculations. Previous work had demonstrated that mutations in the vicinity of the heme cofactor had a greater impact on the binding of FLZ and VOR than on the binding of POS and ITZ. Our modeling data suggest that the long side chains of POS and ITZ occupy a specific channel within CYP51 and that this additional interaction, which is not available to VOR and FLZ, serves to stabilize the binding of these azoles to the mutated CYP51 proteins. The model also predicts that mutations that were previously shown to specifically impact POS susceptibility in A. fumigatus and C. albicans act by interfering with the binding of the long side chain.

For over a decade azoles have been a mainstay of the antifungal armamentarium. Azoles inhibit the synthesis of ergosterol, the bulk sterol in fungal membranes, by binding to the heme cofactor located in the active site of the P450-dependent enzyme lanosterol 14α-demethylase (CYP51, also called Erg11p). Ergosterol depletion, coupled with the accumulation of methylated sterol precursors, has been proposed to affect both membrane integrity and the function of some membrane-bound proteins, including chitin synthase (27). The net result is an inhibition of fungal growth.

Resistance to azoles is a concern, particularly during the long-term treatment of AIDS patients with oropharyngeal candidiasis (25). The two most-prevalent causes of resistance are mutations in the target site, resulting in reducedazole binding to CYP51, and decreasedintracellular drug accumulation, resulting from increased expression of efflux pump genes (recently reviewed in reference 24). To combatazole resistance, and to extend the spectrum of treatable pathogens, more-potent azoles have been developed. One such agent is posaconazole (POS) (SCH56592), a broad-spectrum triazole in phase III trials. Unlike fluconazole (FLZ) and voriconazole (VOR), POS is not effluxed by the pumps encoded by FLU1 and MDR1. However, POS is a substrate for the ATP-dependent pumps encoded by CDR1 and CDR2. In addition, POS appears to be relatively insensitive to amino acid substitutions in CYP51 (D. Sanglard, F. Ischer, and J. Bille, Abstr. 42nd Intersci. Conf. Antimicrob. Agents Chemother., abstr. M-221, 2002). Finally, POS is active against Aspergillus spp. (17).

The CYP51 proteins belong to the cytochrome P450 superfamily. Unlike the soluble bacterial P450s, all the fungal CYP51 proteins characterized to date are integral membrane proteins, making structural and biophysical characterization more challenging. The publication of the X-ray crystal structure of CYP51 from Mycobacterium tuberculosis (MT-CYP51), which has >25% sequence identity to most fungal CYP51s, provided a new opportunity to study the fungal enzymes (18, 19). In this work, we used the MT-CYP51 structure to construct homology models for the CYP51 proteins from Aspergillus fumigatus (AF-CYP51A) and Candida albicans (CA-CYP51). To gain insight into how the various azoles bind these proteins, we combined docking calculations with information gained from studying substitutions that resulted inazole resistance. The resultant binding models help explain why particular CYP51 mutations have more-profound affects on the binding of some azoles than others. They may also provide insights into the design of azoles with improved affinity for their target site.

MATERIALS AND METHODS

Homology modeling of CYP51. The structure of MT-CYP51 complexed with FLZ (PDB code, 1EA1 [18]) was used as the template for the homology models of AF-CYP51A (GenBank accession no. AAK73659) and CA-CYP51 (GenBank accession no. AAY31658). We followed the same naming scheme for secondary structures used by Podust et al. (19). Alignment of the MT-CYP51 sequence with those of AF-CYP51A and CA-CYP51 was performed using ALIGN2D in MODELLER (Accelrys, San Diego, Calif.) (21, 22, 23). From the alignments,
spatial restraints (including distance restraints and torsion angle restraints) were derived and used in the three-dimensional model construction with MODELLER. The models were further optimized (including loop refinement) with the internal optimizer of MODELLER and evaluated via MODELLER and Verify3D scores (INSIGHT II;Accelrys).

Docking of ligands. The torsion angles of several residue side chains around the azole binding cavity in the CYP51 models were manually adjusted and in some cases further minimized using the Tripos force field, Amber charges, and a distance-dependent dielectric model. The three-dimensional structures of all azole ligands were constructed using Sybyl (version 6.9) accessed via CONCORD (Tripos Inc., St. Louis, Mo.). Gasteiger-Huckel charges (Sybyl; Tripos Inc.) were assigned to the azole ligand atoms and heme.

In the MT-CYP51/FLZ X-ray structure, FLZ binds with its triazole ring perpendicular to the heme porphyrin plane and with N-4 of the triazole coordinated to the heme iron. The other azoles were constrained so that their triazole rings overlapped the FLZ triazole. Since VOR is very similar to FLZ, its initial binding conformation was derived by overlap with FLZ in MT-CYP51/FLZ and then overlap of the AF-CYP51A and CA-CYP51 models with MT-CYP51. The program GOLD (version 2.0;CCDC, Cambridge, United Kingdom), an automated docking program (8), was used to dock POS and ITZ into the binding site in AF-CYP51A and CA-CYP51. The active-site radius was set at 15 Å. For the docking calculations, all residues of the protein and heme were fixed, while the ligands were flexible. A covalent-bond distance of 2 Å from N-4 of the triazole ring to the heme iron was set as a constraint. Twenty-one genetic algorithm runs were performed.

RESULTS

Homology model of AF-CYP51. A. fumigatus has two distinct cyp51 genes, cyp51A and cyp51B (14). We focused on cyp51A, which encodes AF-CYP51A, since resistance to VOR, ITZ, and POS has been associated with point mutations in this gene (3, 13, 15; E. K. Manavathu, I. Baskaran, G. J. Alangaden, and P. H. Chandrasekar, Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstr. J-817, 2001). The sequence identity between AF-CYP51A and MT-CYP51 is 28%. An alignment of the two sequences revealed that there were no insertions or deletions located in any of the secondary-structure regions (Fig. 1). However, MT-CYP51 lacks the N-terminal transmembrane domain found in fungal CYP51 proteins. Therefore, the first 29 residues at the N terminus of AF-CYP51A were deleted since they are predicted to form a transmembrane helix and to fold independently from the catalytic domain. The best model generated with MODELLER exhibited the canonical P450 fold (5, 16, 20) (Fig. 2). The Cα root mean square deviation between the template and the model was 0.5 Å over 442 pairs. The largest insertion, a 15-residue loop (residues 427 to 441), was located far from the active site and was not modeled in detail. Instead, an approximate loop model from AF-CYP51A was used in the models; nevertheless, the helix C conformation of MT-CYP51 was well conserved. The local alignment was also reported, as all the other side chain atoms were disordered. The heme structure and surrounding side chain conformations were energy minimized to avoid steric conflicts. Two CA-CYP51 models, based on MT-CYP51, have been recently published (4, 11). In both models, a two-residue insertion that was placed before helix C (Fig. 1) was placed in the middle of the helix. Helix C is in close proximity to the heme and the ligand binding site. In the MT-CYP51/FLZ X-ray structure, helix C exhibited high levels of B-factors along its entire length, indicating thermal motion or multiple conformations. In addition, some side chain atoms of helix C were disordered. Experimental uncertainties in this region resulted in imprecise models; nevertheless, the helix C conformation of MT-CYP51 was retained in our models.

Identification of potential azole binding sites. The heme cofactor was extracted from the MT-CYP51 structure and merged into the AF-CYP51A and CA-CYP51 models. To accommodate the heme, Phe134 in AF-CYP51A and Phe145 in CA-CYP51 were both changed to Ala so that only the Cα side chain atom was retained. Both residues are located in the middle of helix C with their side chains pointing directly at the heme. For the corresponding residue in the MT-CYP51 structure (Leu100) only the Cα atom coordinates of the side chain were reported, as all the other side chain atoms were disordered. The heme structure and surrounding side chain conformations were energy minimized to avoid steric conflicts. Two CA-CYP51 models, based on MT-CYP51, have been recently published (4, 11). In both models, a two-residue insertion that was placed before helix C (Fig. 1) was placed in the middle of the helix. Helix C is in close proximity to the heme and the ligand binding site. In the MT-CYP51/FLZ X-ray structure, helix C exhibited high levels of B-factors along its entire length, indicating thermal motion or multiple conformations. In addition, some side chain atoms of helix C were disordered. Experimental uncertainties in this region resulted in imprecise models; nevertheless, the helix C conformation of MT-CYP51 was retained in our models.
chains of POS and ITZ (Fig. 2 and 3). Channel 1 is parallel to the plane of the heme and is formed by the bent helix I, the open BC loop, and helices B' and G. This channel is apparent in both MT-CYP51 and rabbit P450 2C5 X-ray structures (18, 28). Channel 2, based on bacterial P450BM3 structures (10), is perpendicular to the plane of the heme and is located between the B-sheet and helical domains. The surface entrance of channel 2 in our model is an area surrounded by the FG loop, helix A', and the β turn connecting strands β4-1 and β4-2. We explored both channels in AF-CYP51A and CA-CYP51 for binding of the long POS side chain. Only the docking results which placed POS in channel 2 are consistent with the reported resistance mutations (see below). Although channel 2 is apparent in the MT-CYP51 X-ray structure, it is not open to the surface. In the model of AF-CYP51A, the conformations of the side chains of residues Tyr107, Phe119, and Leu494 were modified to open an entrance to the surface. In addition, the Tyr107 side chain displays an aromatic interaction with the pyrimidine ring of VOR. It is noteworthy that VOR and POS appear to have similar interactions with the protein near the heme.

Similar docking strategies were applied to perform docking calculations on POS, ITZ, and VOR in the CA-CYP51 binding site (Fig. 4C and D). Residues that interact with POS are Ala61, Ala62, Tyr64, Gly65, Leu87, Leu88, Tyr118, Leu121, Thr122, Phe126, Phe145, Phe228, Thr229, Pro230, Ile231, Phe233, Met306, Gly307, His310, Thr311, Leu376, Ser378, Ile379, Phe380, Ser506, Ser507, and Met508. Residues that interact with VOR are Tyr118, Thr122, Phe126, Phe145, Phe228, Met306, Gly307, His310, Thr311, Leu376, Ile379, and Met508.

Locations of substitutions in CYP51 resulting in azole resistance. (i) *A. fumigatus*. Resistance to POS and ITZ in *A. fumigatus* may lead to steric crowding in the region around Met292 and Ala293.

VOR was docked into AF-CYP51A by overlapping with the X-ray structure of FLZ bound to MT-CYP51. The minimized binding conformation, highlighting active-site residues within 5 Å of VOR, is shown in Fig. 4B. Since VOR is a relatively compact molecule, binding does not involve residues from helix A' or the FG loop. Instead, the primary binding determinants are hydrophobic interactions between VOR and residues Phe115, Met292, Ala293, Ile364, Ile367, and Leu494. In addition, the Tyr107 side chain displays an aromatic interaction with the pyrimidine ring of VOR. It is noteworthy that VOR and POS appear to have similar interactions with the protein near the heme.
*A. fumigatus* results from substitutions at Gly54 in AF-CYP51A (3, 13, 15). Gly54 is located on helix A’ next to the triazole-3-one five-membered ring in the long side chain of POS (Fig. 5). This part of the POS molecule fits snugly inside the pocket formed by helix A’, the FG loop, and the β turn between β-strands β4-1 and β4-2. Replacing Gly54 with amino acids that have side chains would create van der Waals (VDW) conflicts between the side chains and POS. The spectrum of substitutions at Gly54 and the resultant changes in MIC appear to support this prediction (Table 1). Replacing Gly54 with either Glu or Arg resulted in a 30-fold increase in the POS MIC. Introduction of Trp, with its large rigid side chain, resulted in a >250-fold MIC increase (13). ITZ occupies the same binding site as POS; it was therefore not surprising that the substitutions at Gly54 also resulted in large increases in the ITZ MIC.

VOR lacks a long side chain that would span channel 2, and therefore substitutions at Gly54 would be predicted to have no impact on VOR binding. This was the case; *A. fumigatus* isolates with substitutions at Gly54 remained susceptible to VOR (Table 1) (12, E. K. Manavathu et al., 41st ICAAC). These two residues are located near the heme cofactor (Fig. 5). Replacing Gly138 with Arg and Gly448 with Ser would be predicted to disturb the

### Table 1. Substitutions in AF-CYP51A resulting inazole resistance in *A. fumigatus* isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>MIC (µg/ml)</th>
<th>Substitution in AF-CYP51A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>POS</td>
<td>ITZ</td>
</tr>
<tr>
<td>ND158*</td>
<td>0.03</td>
<td>0.12</td>
</tr>
<tr>
<td>MS6a</td>
<td>0.5</td>
<td>&gt;16</td>
</tr>
<tr>
<td>R4-1a</td>
<td>1</td>
<td>&gt;16</td>
</tr>
<tr>
<td>R7-1a</td>
<td>4</td>
<td>&gt;16</td>
</tr>
<tr>
<td>F55064b</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>F10b</td>
<td>0.25</td>
<td>2</td>
</tr>
<tr>
<td>F33b</td>
<td>0.25</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* MICs and data on mutations from Mann et al. (13).

b MICs from a personal communication from E. Manavathu; data on mutations as published (Manavathu et al., 41st ICAAC).
heme environment. Specifically, the large charged side chain of Arg at position 138 would clash with one of the heme’s vinyl side chains and with the side chains of neighboring residues. Similarly, substituting serine at position 448 would result in VDW conflicts between the Ser side chain and the adjacent heme pyrole. Interestingly, neither substitution affected the MIC of POS. From our model we predict that the additional contacts between the long side chain of POS and AF-CYP51A protein compensate for the perturbation in the vicinity of the heme.

(ii) C. albicans. An analysis of two series of C. albicans isolates, from two different patients, revealed that stepwise reductions in azole susceptibility were accompanied by the appearance of specific substitutions in CYP51. The first isolate from patient I had two substitutions in CA-CYP51, Tyr257His and Gly464Ser (Fig. 6). Tyr257 is far from the azole binding site and its effect on azole binding is not immediately obvious (Table 2). Gly464 corresponds to Gly448 in AF-CYP51A and is therefore predicted to disturb the heme environment. As seen for the analogous substitution in A. fumigatus, Gly464Ser resulted in a larger decrease in susceptibility to VOR and FLZ than to POS. The second isolate from patient I acquired a third substitution, Gly307Ser. Since Gly307 is located on helix I and contacts both the triazole and phenyl rings, the substitution would be predicted to negatively impact binding of all azoles through steric conflicts between the polar Ser side chain with the triazole and phenyl rings. In practice, the substitution impacted binding of VOR more than POS. The final isolate from patient I acquired a fourth substitution, Ala61Val. Like Gly54 in AF-CYP51A, Ala61 is located on helix A’. Consequently, the additional increase in the MIC of POS most likely resulted from VDW conflicts between the large Val side chain and the end of the POS side chain.

The first isolate from patient II had four substitutions in CA-CYP51 and exhibited a larger decrease in susceptibility to VOR and FLZ than for POS and ITZ (Table 2). The MIC changes can most likely be attributed to the Gly464Ser substitution, as the other three substitutions (Lys128Thr, Asp278Asn, and Tyr132His) are relatively far from the drug binding site (Fig. 6). The next isolate from patient II acquired a fifth substitution, Pro230Leu, which primarily impacted the MICs of POS and ITZ. Pro230 is on the FG loop and is predicted to be in close contact with the POS side chain. The substitution of Leu for Pro at this position would likely affect the binding of POS and ITZ, but not VOR and FLZ, through VDW conflicts between the Leu side chain and POS and ITZ long side chains.

DISCUSSION

The discovery and refinement of azole drugs has relied entirely on whole-cell testing (2). Structure based approaches to the identification of resistance mutations on the molecular level is an important step in understanding the structure-activity relationship (SAR) of azole drugs.
designing more efficacious azoles have been stymied by the fact that the drug target site, CYP51, is an integral membrane protein and has proven difficult to purify and crystallize. Biostructural assessments of the drug-binding determinants have been limited to what could be inferred from more-distant relatives in the P450 superfamily. However, the recent elucidation of the crystal structure of the P450 14α-sterol demethylase from *M. tuberculosis*, coupled with the identification of substitutions in fungal CYP51 enzymes that confer resistance to specific structural classes of azoles, has provided an opportunity to construct homology models that are potentially superior to those based on other bacterial P450 structures (1, 6, 7, 9, 18, 26).

This study provides a model for the binding of azoles with extended side chains such as POZ and ITZ. The key finding from our work is that the long side chains of POS and ITZ appear to be contained within channel 2 and consequently make extensive hydrophobic contacts along their entire lengths. This channel is evident in the X-ray structure of MT-CYP51A as a cavity of 2,600 Å³ (18). The long-chain azoles span channel 2, extending from the heme to the surface of the protein, ending in a region surrounded by helix A’, the β-turn connecting β4-1 and β4-2, and the FG loop (compare Fig. 2 and 4). The model elegantly explains how a substitution, at Gly54 in the A’ helix of AF-CYP51A, confers resistance to POS and ITZ by perturbing the binding of the long side chain in channel 2. Similarly, substitutions in either the A’ helix (at residue Ala61) or the FG loop (at residue Pro230) of CA-CYP51, in combination with substitutions near the heme site, specifically impact resistance to POS by the same mechanism. However, unlike Gly54 in AF-CYP51A, substitutions in CA-CYP51 at Ala61 or Pro230 alone did not confer reduced susceptibility to POS when the corresponding CYP51 allele was expressed in *Saccharomyces cerevisiae* (10a; A. Chau et al., unpublished data).

From the model, it seems probable that the added interactions furnished by the long side chain of POS and ITZ would result in inherently tighter binding affinities compared with azoles lacking such substituents. Although precise biophysical measurements are lacking to prove this hypothesis, the resistance data appear to support this contention. Specifically, mutations near the heme site, which result in significant levels of resistance to FLZ and VOR in *C. albicans* and *A. fumigatus*, have far less impact on the susceptibility of the organisms to POS and ITZ (this study; Sanglard et al., 42nd ICAAC; Manavathu et al. for communicating unpublished data, and Todd Black (SPRI) and Michelle Trietel (SPRI) for editing suggestions).

**ACKNOWLEDGMENTS**

We thank Zhuyan Guo (SPRI) for many helpful discussions, Eduardo Zaborowski (SPRI) for providing computer application software support, Elias Manavathu (Wayne State University, Detroit, Mich.) for communicating unpublished data, and Todd Black (SPRI) and Michelle Trietel (SPRI) for editing suggestions.

**REFERENCES**


