Structure-Based Virtual Screening and Biological Evaluation of Non-Azole Antifungal Agent

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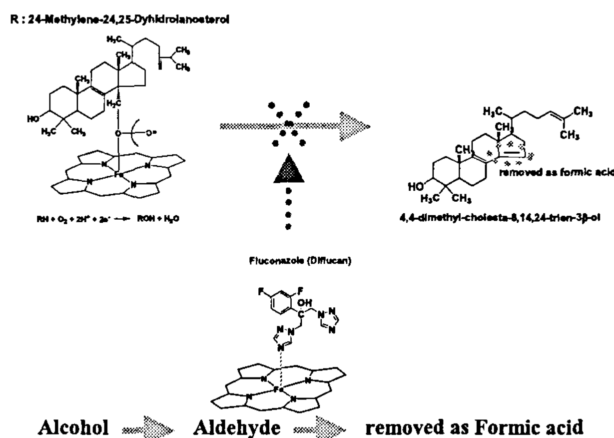
ABSTRACT: Cytochrome P450 14α-sterol demethylase enzyme (CYP51) is the target a of azole type antifungals. The azole blocks the ergosterol synthesis and thereby inhibits fungal growth. A three-dimensional (3D) homology model of CYP51 from *Candida albicans* was constructed based on the X-ray crystal structure of CYP51 from *Mycobacterium tuberculosis*. Using this model, the binding modes for the substrate (24-methylene-24, 25-dihydrolanosterol) and the known inhibitors (fluconazole, voriconazole, oxiconazole, miconazole) were predicted from docking. Virtual screening was performed employing Structure Based Focusing (SBF). In this procedure, the pharmacophore models for database search were generated from the protein-ligands interactions each other. The initial structure-based virtual screening selected 15 compounds from a commercial available 3D database of approximately 50,000 molecule library. Being evaluated by a cell-based assay, 5 compounds were further identified as the potent inhibitors of *Candida albicans* CYP51 (CACYP51) with low minimal inhibitory concentration (MIC) range. BMD-09-01~BMD-09-04 MIC range was 0.5 μg/mℓ and BMD-09-05 was 1 μg/mℓ. These new inhibitors provide a basis for some non-azole antifungal rational design of new, and more efficacious antifungal agents.

1 INTRODUCTION

During the past two decades, the frequency of invasive and systemic fungal infections has increased dramatically in the population with altered immunity [1, 2].

Cytochrome P450 14α-sterol demethylase enzyme (CYP51) catalyze the oxidative removal of the 14α-methyl group of substrate (24-methylene-24, 25-dihydrolanosterol) in ergosterol biosynthesis. The CYP51 in the ergosterol biosynthesis pathway is the specific target for antifungal durgs. The agents of azoles are currently the most widely studied, and fluconazole is the first generation of antifungal inhibitor used for *Candida* infections [3].



Scheme 1. Mechanism of cytochrome P450 14α-sterol demethylation.

Recently, the structure of 14α-sterol demethylase from *Mycobacterium tuberculosis* CYP51 (MTCYP51) was crystallized by X-ray crystallographic study and provides a template for analysis of other CYP51 family. MTCYP51 bound with two azole inhibitors, 4-phenylimidazole (PDB ID : 1E9X) and fluconazole (PDB ID : 1EA1), and their structures at 2.1 and 2.2Å, respectively. MTCYP51 exhibits the P450 fold with the exception of two striking differences which is a bent I helix and an open conformation of BC loop that define and active site access channel running along the heme plane perpendicular to the direction observed for the substrate entry in P450BM3. Although a channel analogous to that in P450BM3 I evident also in MTCYP51, it is not open ate the surface. The presence of two different channels, suggests the possibility of conformationally regulated substrate-in / product-out openings in CYP51 [3]. The N-4 atom of the azole ring of antifungal agents coordinated to the iron atom of heme, and the halogenated phenyl ring of the inhibitors was located in the hydrophobic binding cavity. The X-ray structure of CYP51 from MT-CYP51 exhibits 35-18% sequence identity to plant, 33-35% to animal, and 26-29% to fungal enzymes [4]. This result provide a new strategic opportunity to study the fungal enzyme through structure-based virtual screening.

In the present paper, we report to a 3D homology structure model for *Candida albicans* CYP51 (CACYP51) constructed based on MTCYP51. We also provide an analysis of active site and catalytic protein residues that

interact with ligand by docking. For the identification of potent CACYP51 inhibitors, the reliable pharmacophore models were constructed from known inhibitors. The pharmacophore search performed using the Structure Based Focusing (SBF) program encoded in CERIUS2 [5]. These new hit compounds were evaluated by a cell-based assay and would propose potent non-azole antifungal agents.

2 MATERIALS AND METHODS

2.1 Homology modeling of *Candida albicans* CYP51 (CACYP51)

Template structure was *Mycobacterium tuberculosis* CYP51 (MTCYP51) that complexed with fluconazole. The PDB ID is 1EA1 (http://www.rcsb.org/pdb), the resolution of this structure is 2.2Å [3]. The sequence of *Candida albicans* CYP51 (CACYP51) was used GenBank accession no. CAA31685. Alignment of the MTCYP51 sequence with CACYP51 was performed using CLUSTALW program [6]. The three- dimensional (3D) homology structure of CACYP51 was constructed by using HOMOLOGY module encoded in INSIGHTII [7].

2.2 Docking of azole ligands

The ligand binding pose for protein were used substrate and known inhibitors which are fluconazole, voriconazole, oxiconazole, miconazole. The key binding interactions are analyzed from published x-ray structure that complex with azole inhibitors. The PDB ID is 1EA1 that bound with fluconazole. From binding poses of these inhibitors, we proposed the identification of key enzyme interaction source.

2.3 Virtual screening

The optimized 3D homology model of *Candida albicans* CYP51 (CACYP51) was used as the target for virtual screening on database which was approximately 50,000 commercial available compounds library. The pharmacophore models were generated by Structure Based Focusing (SBF) module encoded in CERIUS2 [5]. The pharmacophore models filtered out the compounds database.

2.4 Biological evaluation

In vitro antifungal activity was measured by means of the minimal inhibitory concentrations (MIC) using the serial dilution method.

The initial structure-based virtual screening selected 15 compounds. Being evaluated MIC range (μg/ml) for *Candida albicans* CYP51 (CACYP51) by a cell-based assay. 4 strains that 90873, MYA-1003, MYA-576, 96901 were tested using 15 compounds. 5 compounds had potent bioactivity.

3 RESULTS AND DISSCUSSION

3.1 3D homology model of *Candida albicans* CYP51 (CACYP51)

PDB ID of the template *Mycobacterium tuberculosis* CYP51 (MTCYP51) structure is 1EA1 (http://www.rcsb.org/pdb), the resolution of this structure is 2.2Å [3]. The sequence of *Candida albicans* CYP51 (CACYP51) was used GenBank accession no. CAA31685. Alignment of the MTCYP51 sequence with CACYP51 was performed using CLUSTALW program [6]. The three-dimensional (3D) homology structure of CACYP51 was constructed by using HOMOLOGY module encoded in INSIGHTII [7].

The sequences alignment of CACYP51 and one of the crystal structure for MTCYP51 in complex with fluconazole were shown in figure 1. The sequence identity between MTCYP51 and CACYP51 is 26% and positive 78%. These structures were high homology.

Figure 1. Sequences alignment of CACYP51 and crystal structure for MTCYP51. ▲ : substrate binding binding residues. ● : inhibitor binding residues.

For the generation of CACYP51 homology structure was superimposed on the crystal structure of MTCYP51 by fitting all the heavy atoms along the backbone, on the basis of sequences alignment. The protein binding site residues of enzyme and inhibitors were very conserved. The active site of CACYP51 was defined by the heme group that these residues aligned to positions belonging to a sphere of centered on fluconazole in the crystallized complex of MTCYP51. The homology structure of CACYP51 and MTCYP51 superimposed were shown in figure 2.

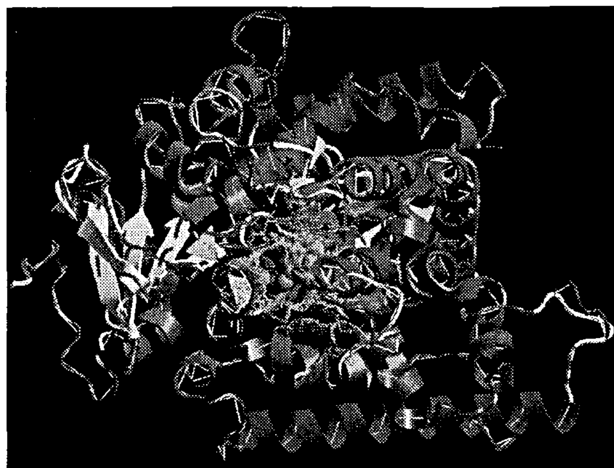


Figure 2. Superimpose of CACYP51 homology structure on crystallized MTCYP51 structure. Red color : crystallzed MTCYP51 (PDB ID : 1EA1) structure. Purple color : homology CACYP51 structure.

3.2 Identification of substrate and potential azole binding sites

The substrate was stabilized in the active site predominantly by hydrophobic interaction and hydrogen bond binding. The distance from the iron atom to the oxidative site C32 of substrate was about 5.0Å. The methyl group of lanosterol was put over the heme iron and an oxygen molecule was set in between. The 17-alkyl side chain of the substrate was deep in a narrow and highly hydrophobic cleft. Three hydrogen bonds were formed between the 3-OH group of substrate and carbonyl and amino group of the main chain and hydroxyl group of the side chain of SER336, which was essential for orienting the substrate to the correct direction in the active site [8]. The residue HIS335, which is unique and absolutely conserved throughout the CYP51 family, is located within 6 Å of 14α-methyl group. The functional role of this histidine in the active site of CYP51 is not known. However, many mutants in rat CYP51 show it to be important for catalytic activity [9]. Some paper proposed that protruding quite deeply into the active site, HIS335 might guide substrate entry by interacting with the OH-group of incoming substrate [10]. All the above analysis was in good agreement with lines of evidence suggesting that the 3-hydroxy group [11], the angular methyl of the β-surface [12], and the 17-side chain [13, 14] of the substrate were the essential structures of substrate for interacting with the fungal CYP51 protein. The docking model of substrate into the active site of *Candida albicans* CYP51 (CACYP51) was shown in figure 3.

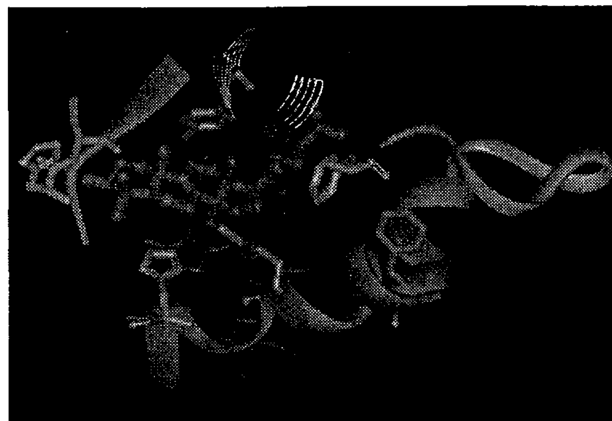


Figure 3. 24-methylene-24,25-dihydrolanosterol docked into the homology CACYP51 structure.

The heme cofactor was extracted from the *Mycobacterium tuberculosis* CYP51 (MTCYP51) structure and merged into the CACYP51 homology model. The ligand binding pose for protein were used known inhibitors which are fluconazole, voriconazole, oxiconazole, miconazole. These 2D structures were shown in figure 4.

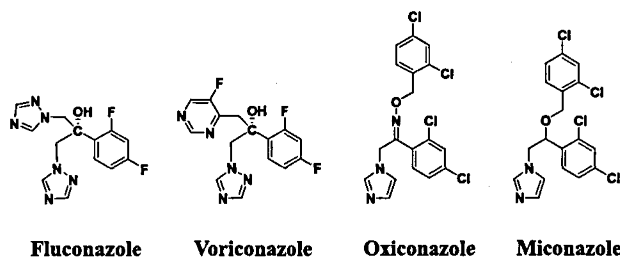


Figure 4. 2D structures of typical azole antifungal agent.

The phenyl group of fluconazole was hydrophobic interaction with TYR76, PHE84, PHE103, MET264. It was difficult that over size of the phenyl ring interacted with protein because of this hole could not enough overcome ring size. Another site of phenyl ring, triazole ring was hydrophobic interaction with LEU334, HIS335, SER336, ILE337. This site was also effected inhibitor size. The binding model of fluconazole was shown in figure 5.

Figure 5. Binding pose of fluconazole into the homology CACYP51 structure.

The azole ring has been demonstrated to be one of the most important key interaction for antifungal activity in extensive structure-activity studies. However, infact, azole antifungal agents are generally toxic and are hampered in the treatment of deep-seated mycoses and life-threatening systemic infections because of their ability to coordinate with the heme of a lot of host cytochrome P450 enzymes [15].

As halogens on the phenyl of the phenyl of the pharmacophoric portion, which are resent as common substituents in almost all of the antifungal azoles [16].

Halogens are very useful to modulate the electronic effects on phenyl rings of drugs. Chlorine has strong inductive electron-attracting effects, while those of fluorine are very weak. Moreover, these atoms may also influence the steric character and the hydrophilic-hydrophobic balance of the molecules [17].

3.3 Pharmacophore filtering

The pharmacophore models were generated by Structure Based Focusing (SBF) that the query to described chemical feature for interaction with protein and ligand. During generate the pharmacophore query, we made a strategy of two-way for combination chemical features. One is that search out T-shape compounds, like fluconazole and the other is searching out Y-shape compounds, like oxiconazole. The pharmacophore query model was shown in figure 6.

For virtual screening, 50,000 commercial available focused molecule library was screening by these key interaction query. Finally, we selected 15 compounds by several modeling method and visual inspection.

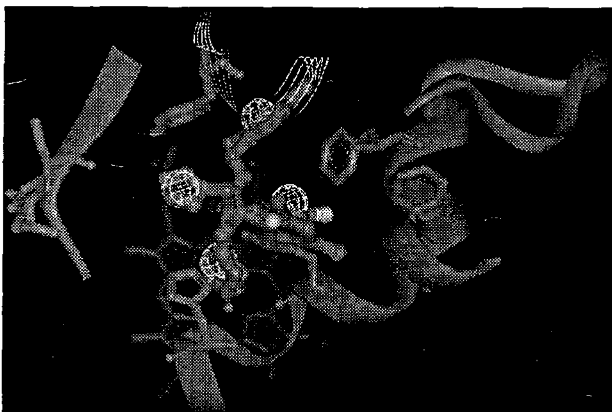


Figure 6. Pharmacophore query model of fluconazole and oxiconazole.

3.4 Cell-based assay of hit compounds

The initial structure-based virtual screening selected 15 *compounds in 50,000 commercial* available compounds library. Being evaluated minimal inhibitory concentration (MIC) range for *Candida albicans* CYP51 (CACYP51) by a cell-based assay. MIC values were defined as the lowest concentration of the antifungal agent that prevents any discernible growth, approximately 90% reduction of growth as compared with drug-free control wells. The 4 strains that 90873, MYA-1003, MYA-576, 96901 were tested. 5 compounds were potent and selective inhibitors for CACYP51. MIC range of BMD-09-01~BMD-09-04 was 0.5 μg/mℓ and BMD-09-05 was 1 μg/mℓ. These results were displayed on the table 1 and binding model for one of the new non-azole hits was shown figure 7.

Table 1. Result of cell-based assay for virtual screening hit compounds

	90873	MYA-1003	MYA-576	96901
BMD-09-01	0.5	>256	>256	>256
BMD-09-02	0.5	>256	>256	>256
BMD-09-03	0.5	>256	>256	>256
BMD-09-04	0.5	>256	>256	>256
BMD-09-05	1	>256	>256	>256
Fluconazole	0.5	128	256	>256
AmphotericinB	0.25	0.5	0.5	0.5

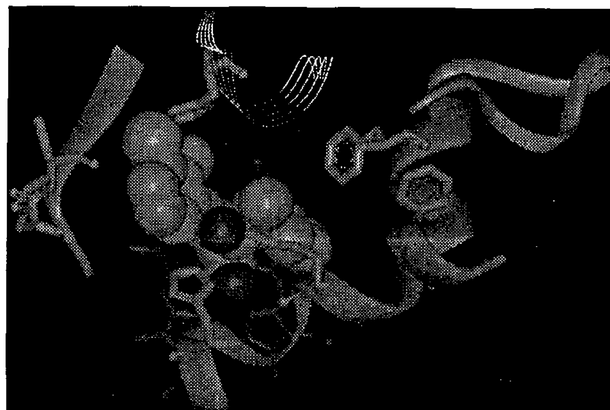


Figure 7. Binding model of new non-azole hits (BMD-09-01).

4 CONCLUSION

In this study, we were able to discover highly potent and selective non-azole inhibitors of *Candida albicans* CYP51 (CACYP51) through structure-based virtual screening. The three-dimensional (3D) homology structure of CACYP51 was constructed based on the *Mycobacterium tuberculosis* CYP51 (MTCYP51) structure for the structure based virtual screening. The active site of CACYP51 was investigated thoroughly and the docking structures of some known inhibitors were predicted for extracting the key binding interactions. After analyzing CACYP51-ligand interactions, we suggested the reliable pharmacophore models include the common binding features. The pharmacophore based virtual screenings were performed for finding the virtual hit compounds. Finally, we purchased 15 compounds from the virtual hits. After biological evaluation, some non-azole antifungal hit compounds were discovered by structure-based de novo design. The non-azole hits of BMD-09-01~BMD-09-05 expressed low minimal inhibitory concentration (MIC) range in vitro cell-based assays.

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