Elucidation of Furanone as Ergosterol pathway inhibitor in 
Cryptococcus neoformans

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Abstract

In the era of antiretroviral therapy, the prevalence of Cryptococcal infection among HIV patients in developed countries has decreased considerably. However, *C. neoformans* ranks top among the critical priority pathogen that affects a wide range of immunocompromised individuals. The threat of *C. neoformans* is because of its incredibly multifaceted intracellular survival capabilities. Cell membrane sterols especially ergosterol and enzymes of its biosynthetic pathway are considered fascinating drug targets because of their structural stability. In this study, the ergosterol biosynthetic enzymes were modeled and docked with furanone derivatives. Among the tested ligands Compound 6 has shown a potential interaction with Lanosterol 14 α-demethylase. This best docked protein-ligand complex was taken further to molecular dynamics simulation. In addition, an *in vitro* study was conducted to quantify the ergosterol in Compound 6 treated cells. Altogether the computational and *in vitro* study demonstrates that Compound 6 has anticryptococcal activity by targeting the biosynthetic pathway of ergosterol.

1. Introduction

On average, there are about 6 million species of fungi that are ubiquitously present. Among them, several hundred species of yeast and molds can affect humans in several ways [1]. Globally, Invasive Fungal Infections (IFIs) are recognized as one of the major causes of morbidity and mortality since 1980. [2] [3]. The major predisposing factor of IFIs is immune compression due to COVID-19, cancer, solid organ transplantation, neutropenia, granulocytopenia, AIDS, Diabetes mellitus, congenital immune deficiency, and corticosteroid therapy [4]. For most of the IFIs respiratory tract serves as a primary route of infection, hence patients suffering from asthma, and chronic obstructive pulmonary disease are also susceptible to IFIs [5]. The major invasive fungal diseases are Candidiasis, Aspergillosis, Cryptococcosis, and Mucormycosis. The major etiological agents of these IFIs are *Candida albicans*, *Aspergillus fumigates*, *Cryptococcus neoformans*, *Rhizopus oryzae*, and *Pneumocystis jirovecii* respectively. The endemic dimorphic fungi *Coccidioides* spp., *Histoplasma* spp., *Blastomyces* spp., *Paracoccidioides* spp., and *Sporothrix* spp., are also liable fungal agents distributed worldwide [6].

*Cryptococcus* spp. is a high priority exogenous invasive fungal pathogen, which is a foremost cause of fungal meningitis [7]. Cryptococcal meningitis is of prime concern as it is a defining opportunistic fungal infection where the predominance of HIV is high and it is also elevated among patients with immune suppression by other means such as lymphomas (e.g., Hodgkin's lymphoma), sarcoidosis, liver cirrhosis, long term corticosteroid therapy, Solid Organ Transplantation (SOT), medications to treat rheumatoid arthritis, cancer chemotherapy, and Idiopathic CD4 lymphopenia [8] [7] and [9]. In recent years Cryptococcal infection is also found to affect immunocompetent patients progressively [10]. Besides its high pathogenicity, it is still an underemphasized disease that is challenging to eradicate.

The standard antimycotic drugs for the treatment include azoles, polyenes, echinocandins, allylamines, and pyrimidine analogs drugs which target the cell wall and its components, cytoplasmic membrane and its protein, and nucleic acid synthesis respectively. The latest drug class that has been approved by FDA in 2021 is triterpenoid antifungal or fungerp. The first drug from this class is termed ibrexafungerp (IBX) which has a similar mode of action as echinocandins.

The major antifungal targets are Ergosterol, the major component of the cell membrane (polyene class of drugs), Lanosterol 14 α-demethylase (azole class of drugs), β1, 3 glucan synthase (echinocandins and fungerp), RNA (5-flucytosine a pyrimidine analogue), squalene monooxygenase (terbinafine) and chitin synthase (peptidyl nucleoside analogs) [11] and [12]. The fungerp class shows good activity against a wide range of yeast and mould. But as a
paradox though *C. neoformans* cell wall majorly contains glucans, the fungus showed no activity [13]. This suggests that the cell wall might not be a suitable target for *Cryptococcus* spp.

The crucial part of the antifungal discovery is the similarity of cellular features and functions with mammalians; this has limited the discovery of a sufficient novel class of antifungal drugs [2] and [14]. The limited drug depository for treating fungal infections has led to the prevalence of azole resistance and multidrug resistance [15]. The emergence of polyene resistant strains of *Candida* spp. and *Aspergillus* spp. were also reported in recent studies [16]. The major drug resistant mechanisms include alteration of membrane composition, overexpression of target enzymes, upregulation of multidrug transporters [ATP- Binding Cassette transporters (ABC) Major Facilitator Superfamily (MFS)], exogenous cholesterol import, and biofilms [17].

Amphotericin B is one of the popular and oldest antifungal agents that were been in use to this date. Though the drug showed high efficacy against a broad range of fungal species its usage is limited due to its toxic effects. The long-term use of amphotericin B mainly causes renal dysfunction, liver dysfunction and less commonly causes Hypokalemia (low potassium level in blood) and Hypomagnesemia (low magnesium level in blood) [18] and [19]. Hence there is a desperate need for a new class of antifungal drugs for the management of emerging and re-emerging fungal spp.

Furanones are heterocyclic compounds that have active nuclei made of five-membered rings that make them tremendous therapeutic potential [20]. Furanones originated in a large complex of natural products and it also has a synthetic origin i.e., they can be easily synthesized in lab conditions [21]. Furanones are isolated from various plant, algae and bacterial sources and are classified into 3 classes: 2 (5) H-furanone, 2(3H)-furanone, and 3(2H) - furanone. Furanone and its derivatives have immense and broad spectrum therapeutic potential that serves as an antibacterial, antibiofilm, antiprotozoa, antiulcerative agent, analgesic, anticancer, anti-inflammatory, and it also acts as vasorelaxant [20]. The antifungal potentials of furanone were first explored by our team [22].

The antifungal (E)-5- Benzeneidelene dihydro furan 2(3H) one (Compound 6) belongs to the family of 2(3H) - furanone and are unsaturated gamma lactones that are commonly known as butenolides. In our previous study, Compound 6 has proved to have an immunomodulatory and antifungal activity against *C. neoformans*, *A. fumigatus* and *C. albicans*. The therapeutic potency was displayed with 100% survival of mice in a disseminated cryptococcal infection mice model [22] [Rathore et al., 2017]. In addition, the acute toxicity study displayed the safety profile and bioavailability of Compound 6 in major organs advocates the compound to further investigate the mode of action.

The present study aimed to determine the mode of action of compound 6 and other furanone derivatives against various target proteins of ergosterol biosynthetic pathway (Fig. 1) specifically harbored by *C. neoformans* by an *in silico* approach which was validated by *in vitro* methods.

### 2. Materials And Methods

The integrative methodology is depicted in (Fig. 2)

#### 2.1. Selected furanone compounds

To investigate the mode of action of 5-Benzylidenedihydrofuran-2 (3H)-one (Compound 6), and few more available furanone compounds were selected. The selected compounds include 2(3H) – Furanone, dihydro-3,3- dimethyl-5-methylene (Compound 1), 3-phenyl-2H-furan-5-one (Compound 2), 4,4-dimethyl-5-methylenedihydro-2(3H)-furanone (Compound 3), 5-phenyl-3H-furan-2-one (Compound 5). The positive controls include fluconazole (Lanosterol 14-α
demethylase, Sterol 14-demethylase), fenpromimorph (C-14 sterol reductase) and terbinafine (squalene epoxidase) [23] and [2].

2.2. Selected protein targets

The selected protein targets are included in the ergosterol biosynthetic pathway of *C. neoformans* such as Protein 1, Protein 2, Protein 3, Protein 4 and Protein 5. The protein sequences were retrieved from the UniProt database server in FASTA format. Uniprot IDs of the target proteins are Q9P8P1, J9VN75, E6R000, J9VHT4 and Q5KHK9 respectively.

2.3. 3D structure prediction and validation

The retrieved amino acid sequences were modeled using SWISS and Alpha Fold. The User-defined target-template alignment was used for modeling [24]. Due to the lack of template availability for alcohol phosphotransferase and squalene monoxygenase, the Alpha Fold structure was used. The modeled structures were validated by Ramachandran plot using SAVES 6.0 online server [25].

2.4. Molecular Docking

The modeled protein structures (Fig. 3) and ligands (Table 1) were optimized using the Protein preparation wizard and Lig prep of Maestro-8.4 [Schrodinger, LLC, Newyork, NY, US 2020]. The grid generation was obtained using the Glide grid v8.6 option where the active site residue specification is given after which the grid is generated. The Ligand docking option was launched in a Glide dock with selected ligands and proteins. The conformation with the lowest binding affinity and the lowest RMSD values is considered to be the best docking result [26] and [27]. In PyRx the modeled protein and ligand were preprocessed and saved in PDBQT format [28]. The grid is generated in the active site residue region. The grid box is assigned according to the active site residues predicted using the CASTp server [29]. AutoDock Vina was executed after generating the protein and ligand files in respective format (PDBQT) after which the docking score (Binding energy in kcal/mol) and RMSD values were obtained [28].

2.5. Prime MMGBSA Calculation

The docked complex in PDB format was rescored by using the Molecular Mechanics–Generalized Born Surface Area (MM-GBSA) method. To improve the model quality the TIP3P water model was used [30].

2.6. MD Simulations

MD Simulations were performed for the docked structure to understand the interaction at the molecular level using Desmond v5.6 [31]. The simulation system was prepared using the simulation set-up option where an orthorhombic box with 10 Å distances including periodic boundary conditions was used. The system was solvated using the TIP3P water model and ions are added to neutralize the system to 0.15 M NaCl concentration. The system was relaxed using the default protocol in Desmond. MD simulations were carried out for 100 ns for each system in the NPT ensemble using OPLS 2005 force field parameters. The shape of the box is taken as an orthorhombic shape. Pressure and temperature of 1.01325 bar and 300 K were maintained throughout the simulation. We have selected the apo form of protein with reference ligand and selected ligand to understand the protein-ligand dynamic behavior. The analysis of the simulated trajectories was carried out using the simulation quality analysis and simulation event analysis programs using Maestro in the Schrodinger, LLC, Newyork, NY, US 2020. Trajectories were analyzed for Root Mean Square deviation (RMSD), Root Mean Square Fluctuation (RMSF), Radius of gyration (Rg) and the number of hydrogen bonds and the plots were generated using Qtgrace [32] and [33].

2.7. MMGBSA of Simulated trajectories
Finally, the MMGBSA for the series of simulated trajectory complexes of lanosterol 14-α demethylase bound Compound 6 and fluconazole was calculated using MM GBSA method [34] and [35].

2.8. Experimental validation for ergosterol synthesis inhibition

The ergosterol extraction protocol [36] (Arthington-Skaggs et al., 1999) was used with a slight modification. 10 ml of Potato Dextrose Broth (PDB) was inoculated with C. neoformans. After 48 hours of incubation, 10^6 CFU/ml cells were measured (0.08 O.D) and transferred to fresh conical flasks containing 10 ml of PDB with 64 µg/ ml of Fluconazole (Hi-Media) (drug control), MIC concentration of Compound 6 (treated) and cells alone (Untreated) in three flasks respectively. After 48 hours the treated and untreated cells were recovered by centrifugation at 7200 rpm for 10 minutes. The cell pellet was rinsed with sterile distilled water. The cell pellets were then treated with a 25% alcoholic potassium hydroxide solution. The mixture was subjected to a vigorous vortex for a minute and incubated for an hour at 85°C. After 1 hour, the saponified ergosterol was extracted twice using distilled water and hexane [1:3 (v/v)]. The ergosterol gets separated in the hexane phase. The ergosterol that gets separated in the hexane layer was analyzed using UV-Visible spectrophotometer (Thermofisher Scientific, Evolution 2000 series) and HPLC (Agilent Technologies, 1200 Infinity series). For UV-Visible spectrophotometric analysis the extracted ergosterol was diluted with 1:5 v/v hexane: ethanol and was scanned between 240 to 300 nm. For HPLC analysis, the ergosterol was evaporated to dryness and dissolved in methanol. The mobile phase used for HPLC is Methanol: Acetonitrile (80:20) to run through the C18 (4.6* 280 mm) column, with a flow rate of 1.5ml per minute [37]. The extracted ergosterol peak was compared with the standard ergosterol (AVRA chemicals) at 30 °C and the wavelength of DAD was fixed at 282 nm.

3. Result And Interpretation

3.1. Protein structure prediction and validation

The enzymes, lanosterol 14-α demethylase, sterol 14- demethylase, C-14 sterol reductase, squalene monooxygenase and alcohol phosphotransferase from C. neoformans were considered for the study. Because of the lack of availability of experimentally determined structures of these proteins, we used computational structure prediction approaches. The success of the homology model is correlated to the degrees of amino acid sequence identity between the target and template. The eminence of homology modeling depends on the selection of a suitable template and the optimal sequence alignment. For the enzymes, lanosterol 14-α demethylase, sterol 14- demethylase and C-14 sterol reductase, optimal template structures with almost 90% query coverage and above 40% identity were retrieved from the PDB database. Those templates were used to construct the protein structures using the homology modeling approach. The details of the template used, query coverage, and percentage identity for each protein are given in Supplementary Table 1. The modeled 3D structures were represented in Fig. 3. The 3D structures of squalene monooxygenase and alcohol phosphotransferase were retrieved from the Uniprot database and were reported in Fig. 3. More than 85% of the residues of all the structures were reported to be in the most favored regions of the Ramachandran plot (Supplementary Table 2).

3.2. Molecular docking

The molecular docking was performed using Autodock Vina (v 1.5.7) in PyRx and Glide (v 8.6). The docking scores and the binding energies obtained using AutoDock Vina, Glide and the MM-GBSA approaches are given in Table 2 and Supplementary Tables 3, 4, 5 and 6. The binding affinity and MMGBSA of the protein 1 and ligand complexes indicate that Compound 6 (-6.945 and -41.77 kcal/mol) have almost the same interactions compared to reference ligand (-7.006 and -44.21 kcal/mol). Proteins 2, 3 and 4 have good binding affinity and free energy binding score to the reference ligands than furanone derivatives. Protein 5 also showed a better binding affinity and MMGBSA score with compound 6 (-5.95 and -33.43 kcal/mol)
Table 2: Binding energies and RMSD values of furanone derivatives with Lanosterol 14-α demethylase

<table>
<thead>
<tr>
<th>S.no.</th>
<th>Compounds</th>
<th>Autodock Vina</th>
<th>PyRx</th>
<th>Glide</th>
<th>MM GBSA dG (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Compound 1</td>
<td>-5.1</td>
<td></td>
<td>-4.851</td>
<td>-20.54</td>
</tr>
<tr>
<td>2.</td>
<td>Compound 2</td>
<td>-6.7</td>
<td></td>
<td>-6.152</td>
<td>-31.49</td>
</tr>
<tr>
<td>3.</td>
<td>Compound 3</td>
<td>-5.4</td>
<td></td>
<td>-4.705</td>
<td>-15.94</td>
</tr>
<tr>
<td>4.</td>
<td>Compound 5</td>
<td>-6.5</td>
<td></td>
<td>-4.996</td>
<td>-31.33</td>
</tr>
<tr>
<td>5.</td>
<td>Compound 6</td>
<td>-7.1</td>
<td></td>
<td>-6.945</td>
<td>-41.39</td>
</tr>
<tr>
<td>6.</td>
<td>Fluconazole (Reference ligand)</td>
<td>-7.3</td>
<td></td>
<td>-7.006</td>
<td>-44.21</td>
</tr>
</tbody>
</table>

From the docking of the entire furanone derivatives with the selected target proteins, Compound 6 has shown promising binding affinity with protein 1, a potential target for most fungal species. The results indicate that Compound 6 has an inhibitory role in the ergosterol pathway via Lanosterol 14-α demethylase. Hence it was selected for further structure based studies to understand the binding characteristics of Compound 6 with protein 1 using MD simulations.

### 3.3. Molecular Dynamics simulation

Compound 6 and reference ligand bound conformation of lanosterol 14-α demethylase and the apo form of the protein were subjected to MD simulation studies in an explicit solvent environment to understand the structural stability of the complex and to study the conformational changes.

It gives further perception about the active site binding analysis with the selected ligand. Three separate simulations are done for (1) apo form of the protein, (2) protein 1 with Compound 6 and (3) protein in the presence of the reference ligand. The 100 ns simulation trajectories from each simulation study were subjected to structural analysis to understand the conformational changes in the apoprotein and the protein, ligand interactions.

### 3.3.1. Interaction mode investigation of Compound 6 and reference ligand bound protein 1

To find the interactions between the ligand and protein, the interaction diagram generated after 100 ns simulation was shown in Fig. 4 and Supplementary Figs. 1 and 2 for C6 and reference ligand respectively.

The major interaction of Compound 6 bound protein 1 takes place in residues Tyr 390 (polar, hydrophobic amino acid) and Tyr 131 which contributes to hydrogen bonded interaction and hydrophobic interaction that last for more than 80% in 100ns simulation (Fig. 4a and Supplementary Fig. 1). The Tyr 390 residue forms an interaction with the carbonyl group of the ligand via hydrogen bond contributing to nearly 85% hydrogen bonded interaction. Tyr 131 forms a hydrophobic interaction with the ligand contributing to nearly 80% of hydrophobic interaction in the 100ns simulation and also contributes to water bridge interaction to a lesser extent which is illustrated in Supplementary Fig. 1.

The major interaction of reference ligand (fluconazole) bound protein 1 takes place in residues His 320 (polar amino acid) and Arg 391 (positively charged amino acid). His 320 and Arg 391 contribute to about 80% binding of protein-ligand complex throughout 100 ns simulation. The His 320 (β strand) forms π-π stacking with 2,4- difluoro phenyl
group of fluconazole and Arg 391 (α helix) builds water bridges with 1,2,4 triazole ring. Tyr 131 and Tyr 145 contribute to approximately 50% binding of protein-ligand complex throughout 100 ns simulation. Tyr 131 majorly forms hydrophobic interaction and also builds hydrogen bonds and water bridges. Likewise, Tyr 145 majorly builds water bridges and also forms hydrophobic interaction and hydrogen bonds. Figure 4b and Supplementary Fig. 1.

### 3.3.2. Investigation of structural stability and binding free energies

The average Root Mean Square Deviations (RMSD) values of the apoprotein, protein bound to Compound 6 and reference ligand are 3.6439 Å, 3.4570 Å and 3.2920 Å respectively. From the RMSD profile in Fig. 5, conformational changes in the protein in the presence of reference ligand and Compound 6 were observed. When compared to apoprotein the RMSD deviations are less in both the cases of protein bound to reference ligand and Compound 6. Most of the Root Mean Square Fluctuations (RMSF) are between 0.8 to 2.4 Å. The highest fluctuation is at 6.891 Å (Met 372) for apoprotein and it is the highest fluctuation among all three RMSF graphs. For Compound 6 bound protein the highest fluctuation is at 5.183 Å (His 418) and for Fluconazole bound protein the highest fluctuation is at 5.471 Å (Tyr 412) which is given in Fig. 6. When compared to apoprotein (average fluctuation rate: 1.53) the RMSF fluctuations are low in both the cases of protein bound to Compound 6 (average fluctuation rate: 1.406) and fluconazole (average fluctuation rate: 1.44). The RMSF is very less in places of active site residues of both reference ligand and Compound 6 bound protein. In all three cases, there are some common fluctuations in the residues 1 to 9, 414 to 416 and 418 to 427. All these residues are not from the active site regions.

The radius of Gyration (Rg) shows the compactness of the structure. The average value of Rg for apoprotein, Compound 6 bound and reference ligand bound protein are 24.318 Å, 23.903 Å and 23.773 Å respectively. By comparing the Rg values of the apoprotein (Fig. 7) there is a prominent tendency of compaction in the protein structure in the presence of Compound 6 and reference ligand. In the presence of reference ligand also we could observe slight compaction in the protein. The global analysis of the conformational behavior of the protein indicates that the ligand-induced conformational changes may induce an inhibitory role in biological function.

### 3.4. MMGBSA calculation of simulated trajectory complex

The MMGBSA is an efficient, powerful tool for correct ranking of ligands. The total binding free energy $\Delta G_{\text{MM/GBSA}}$ and the related energy forms of the simulated trajectory complex is reported in Table 3 which delineates the average binding free energy and its different contributing terms. The table shows that the binding energies of complexes fluctuate around a stable value.

<table>
<thead>
<tr>
<th></th>
<th>avg $\Delta G_{\text{bind}}$</th>
<th>avg $\Delta G_{\text{bind}}$</th>
<th>avg $\Delta G_{\text{bind}}$</th>
<th>avg $\Delta G_{\text{bind}}$</th>
<th>avg $\Delta G_{\text{bind}}$</th>
<th>avg $\Delta G_{\text{bind}}$</th>
<th>avg $\Delta G_{\text{bind}}$</th>
<th>avg $\Delta G_{\text{bind}}$</th>
<th>avg $\Delta G_{\text{bind}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coulomb$^1$</td>
<td>Covalent$^2$</td>
<td>Hydrogen bond$^3$</td>
<td>Lipophilic energy$^4$</td>
<td>$\pi-\pi$ packing correction$^5$</td>
<td>Self contact correction$^6$</td>
<td>Generalized Born electrostatic solvation energy$^7$</td>
<td>Van der Waals energy$^8$</td>
<td>Total$^9$</td>
</tr>
<tr>
<td>Compound 6</td>
<td>-9.477</td>
<td>1.158</td>
<td>-0.550</td>
<td>-18.671</td>
<td>-1.430</td>
<td>0</td>
<td>15.534</td>
<td>-28.976</td>
<td>-42.413</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>-3.439</td>
<td>-5.676</td>
<td>0.207</td>
<td>-13.801</td>
<td>-2.768</td>
<td>0.220</td>
<td>12.179</td>
<td>-31.715</td>
<td>-44.793</td>
</tr>
</tbody>
</table>

[Coulomb energy$^1$, Covalent binding energy$^2$, Hydrogen bonding correction$^3$, Lipophilic energy$^4$, $\pi-\pi$ packing correction (Hydrophobic interaction)$^5$, Self contact correction$^6$, Generalized Born electrostatic solvation energy$^7$, Van der Waals energy$^8$, Total$^9$]
Waals energy\textsuperscript{8}, Total binding free energy\textsuperscript{9]}

3.5. Ergosterol estimation for \textit{in vitro} validation

The \textit{in vitro} result confirms that Compound 6 has a killing effect on the fungal cells by targeting ergosterol synthesis. This was evident by analyzing the UV-Vis spectrum and HPLC chromatogram of standard ergosterol, Compound 6 treated and untreated cells. In the UV-Vis spectrum the absence of an ergosterol peak at 282 nm was observed for Compound 6 treated and fluconazole treated samples whereas, in the HPLC chromatogram, a small peak was observed at 282 nm. The untreated sample showed a peak at 282 nm in UV-Visible spectrum and the HPLC chromatogram was comparable with the standard and were shown in Figs. 8 and 9. The chromatogram acquired from the DAD detector of the HPLC instrument showed the reduction of ergosterol when compared to untreated cells. While analyzing the peak area percentage the standard ergosterol and the untreated sample showed 85.09% and 57.07% peak area. The Compound 6 and fluconazole treated cells showed 8.73% and 12.49% peak area, which clearly confirms that Compound 6 has an inhibitory effect on the cell membrane of \textit{C. neoformans}.

Discussion

Pulmonary cryptococcosis and meningitis are caused predominantly by \textit{C. neoformans}\textsuperscript{38}. WHO considered \textit{C. neoformans} as a critical priority pathogen as it infects immunocompetent individuals as well\textsuperscript{39}. Their unique morphogenetic character of titan cell growth in the invivo condition including their capsule size curbs their removal by the immune system, the capsules, melanin production and biofilm formation all as a whole makes the \textit{Cryptococcus spp.}, a highly virulent pathogen\textsuperscript{40} and\textsuperscript{41}.

Amphotericin B, azoles and 5 flucytosine are commonly used to treat Cryptococcal infection\textsuperscript{42}. The increased use of these limited drugs, their insufficiency to treat fungal meningitis and their long term side effects led to the alarming need for new classes of antifungal agents. Our lab has proposed furanone as a new class of antmycotic agent in which (E)-5-Benzylidenedihydrofuran-2 (3H)-one has shown a promising antmycotic property against \textit{Cryptococcus spp.}, \textit{Aspergillus fumigatus} and \textit{Candida albicans}.

Though \textit{Cryptococcus} spp., is considered as one of the significant threats, the protein structures were not still predicted which makes it a hurdle to conduct \textit{in silico} studies. Hence the present study was conducted to predict the 3D transmembrane protein structures (target proteins). Sheng et al., 2009\textsuperscript{43} were the first to model Cryptococcal CYP51 via homology modeling using \textit{Mycobacterium tuberculosis} CYP 51 as the template. His results showed that 83% of atoms of the protein backbone were in the most favored region, 12.3% of atoms were in additionally allowed regions, 2.4% at generously allowed regions and 2.3% of atoms were in the disallowed regions. In our study we used \textit{Candida albicans} CYP51 as the template structure that illustrates 90.9% of atoms of the protein backbone are categorized under most favored regions, 8% atoms are designated under additionally allowed regions, 0.4% atoms come under the group of outlier regions or generously allowed regions and finally, 0.6% of atoms in protein backbone are classified as disallowed regions. Almost 90% of atoms of all the proteins come under the most favored region; the protein structures were validated as good protein models. Through this, it can be stated that the higher the evolutionary relatedness between the template and target protein, the higher will be the chances of getting a validated model. For the other proteins, we are the first to report the structure and its validation.

The docking studies exhibited that the activity of Compound 6 and Fluconazole (reference ligand) was merely similar. Generally, the docking score of a protein-ligand complex, above 5 is considered to be a good docking score. In that case, the docking scores from glide showed that Lanosterol 14-α demethylase (Docking score: -6.945; MMGBSA score: 41.39) and Squalene monooxygenase (Docking score: -5.25; MMGBSA score: -33.437) are having a good binding
affinity with Compound 6. Being the top scorer Lanosterol 14-α demethylase was further validated by simulation studies.

Comparative simulation studies were conducted to study the strength of interaction of Compound 6 docked Lanosterol 14-α demethylase with the apo form of the protein. The interaction studies illustrate that in both cases hydrogen, hydrophobic and water bridge interactions were present but the ratio of occurrence is different. In Compound 6 docked complex nearly 21 residues contribute to the interaction. Among them, Tyr 390 contributes to 85% of hydrogen bonding and 5% of water bridge interaction. In total Tyr 390 come up with 90% interaction in 100ns simulation. The second residue contributing to the highest interaction is Tyr 131 (hydrophobic amino acid) which contributes to approximately 78% hydrophobic interaction and 1% water bridge interaction. As a whole, Tyr 131 contributes to 79% of the interaction in a simulation lasting 100ns. All other residue contributes to less than 50% interaction. Among them, nearly 14 residues contribute to hydrophobic interactions, 1 residue (Leu 134) comes up with a mixture of hydrophobic and water bridge interaction and 1 residue (Ser 388) comes up with a mixture of hydrogen and water bridge interaction. In Fluconazole docked complex nearly 26 residues contribute to the interaction. Among them, His 320 contributes to 95% hydrophobic interaction and 1% water bridge interaction. In total His 320 contributes to 96% interaction in 100ns simulation. Arg 391 comes up with 10% hydrogen and 65% water bridge interaction; on the whole, it contributes to 75% interaction throughout the 100ns simulation. Tyr 131 makes up a mixture of hydrogen bonds (10%), hydrophobic interaction (50%) and water bridge (5%); all together Tyr 131 comes up with approximately 65% interaction rate on 100ns simulation. Tyr 145 contributes to 8% hydrogen bonding, 20% hydrophobic interaction, and 30% water bridge and it sums up to approximately 58% interaction in the scale of 100ns simulation. Less than 50% of interactions are attributed to all other residues. Among them, Leu 134, Phe 139, Val 144, Phe 240, Phe 245, Gly 318 and Ile 389 approximately contribute 8.9% hydrophobic interaction. Other than that Ala 317 makes up with 10% hydrophobic and 1% water bridge interaction. Thr 320, His 387, Ser 388, Pro 482, Gly 484, His 488, Val 491 and Glu 493 all contribute to 8.125% water bridge interaction. Ala 317 contributes to 10% hydrophobic interaction and 1% water bridge interaction, in total Ala 317 contributes to 11% interaction. Ile 386 comes up with 2.25% interaction in 100ns simulation where 22% is hydrophobic interaction and 0.5% is water bridge interaction. Phe 483 contributes to 2% hydrophobic and 2% water bridge interaction. Finally, Cys 490 contributes to 10% hydrogen bond, 13% hydrophobic and 10% water bridge interactions.

The RMSD calculates the difference between the protein backbone's initial structures from its final posture. The Lanosterol 14-α demethylase bound Compound 6 complexes showed an improved overall stability of the protein structure in comparison to the apo form of the protein. The reduction in deviation is due to the interaction of amino acid residues with the ligands. RMSF calculates the average fluctuation of a residue over some time from its original position. The higher the RMSF higher will be the flexibility between the atoms of the residues indicating a reduction in the stability of the structure. The highest fluctuation is seen in the apo form of the protein whereas the Lanosterol 14-α demethylase bound Compound 6 complex showed lower fluctuation and the fluctuations are present in the regions not belonging to the active site residues. The radius of gyration measures the compactness of the protein structure which shows that the Lanosterol 14-α demethylase bound Compound 6 complex is more compact due to the protein-ligand interaction and the compactness is less in the case of apoprotein. From the simulation data, it is concluded that the Lanosterol 14-α demethylase bound Compound 6 is stable when compared to apo form protein.

To calculate the binding affinities of the two compounds, the MMGBSA method was used. The calculated binding free energy for Compound 6 and the reference ligand is not significantly different. Based on the energies of the individual components of binding free energies, vander waals forces, and lipophilic energies influence binding free energies higher than the other individual energy components [34].
Followed by, the *in vitro* studies were conducted to confirm the mode of action of Compound 6. The UV-Vis spectrophotometer and HPLC results of ergosterol estimation confirm that Compound 6 has good inhibitory activity on the ergosterol biosynthetic pathway.

Since the enzyme Lanosterol 14 α demethylase is widely present in many organisms including humans, we have conducted a docking study which resulted that the human Lanosterol 14-α demethylase shares 36.38% identity with Cryptococcal Lanosterol 14 α demethylase and also has a good binding affinity with Compound 6.

**Conclusion**

Both *in silico* and *in vitro* results demonstrated that Compound 6 has fungicidal activity by targeting the biosynthetic pathway of ergosterol. Hence compound 6 is advocated as a potential antifungal compound that belongs to a new class of antifungal drugs.

**Declarations**

**Acknowledgment**

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**Conflict of Interest**

No conflict of interest.

**Author’s contribution**

All authors contributed equally.

**References**


38. World Health Organization, WHO fungal priority pathogens list to guide research, development and public health action, 2022.


**Table 1**

Table 1 is available in the Supplementary Files section.

**Figures**

**Figure 1**

Ergosterol biosynthetic pathway of *C. neoformans*
Figure 2

A flowchart of the integrative methodology applied in the study.
Figure 3

3 Dimensional structures of target proteins

The predicted 3D structure of Lanosterol 14-α-demethylase, Sterol 14-demethylase, C-14 Sterol reductase, Alcohol phosphotransferase and Squalene monooxygenase
Figure 4

2D interaction depiction of Protein- ligand contacts

a) Interactions of Reference ligand (Fluconazole) and b) Compound 6 with protein 1 after 100ns MDS
Figure 5

Root Mean Square Deviation

RMSD of the apoprotein, Reference ligand-bound protein 1 and Compound 6 bound protein 1 complexes over 100 ns MD simulations where the Y-axis represents RMSD value and the X-axis represents the Time (ps)
Figure 6

Root Mean Square Fluctuation (RMSF)

RMSF of the apoprotein, Reference ligand-bound and Compound 6 bound protein 1 complexes over 100 ns MD simulations where the Y-axis represents RMSF value and the X-axis represents the Residue index.
Figure 7

Radius of gyration (Rg)

Rg of the apoprotein, Reference ligand bound and Compound 6 bound to protein 1complexes over 100ns MD simulations where the Y-axis represents Radius of Gyration (Rg) in Angstrom (Å) and X-axis represents the Time (ps)
Quantification of ergosterol in treated and untreated cells by UV-Vis spectrophotometer

Figure 9

Quantification of ergosterol in treated and untreated cells by HPLC


Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.
- Table1.docx
- S1.tiff
- S2.tiff
- S.Table1.docx
- S.Table2.docx
- S.Table3.docx
- S.Table4.docx
- S.Table5.docx
- S.Table6.docx