RESEARCH PAPER

Enhancement of Fluconazole Antifungal Efficacy by Repurposed Statins against Candida albicans: Multilevel in Silico, In Vitro, and Ex Vivo Experimental Validation Supporting Adjunct Antifungal Therapy

Balraj Singh[1], Prof. (Dr.) Parminder Nain[1], Prof. (Dr.) Jaspreet Kaur[2], Manpreet Kaur[2], Mohammed Aashik[3]

[1]Research Scholar (PhD), Department of Pharmaceutical Sciences, RIMT University, Mandi Gobindgarh – 147301, Punjab, India.
[1] Prof. Dean School of Pharmaceutical Sciences RIMT University Mandi Gobindgarh, Punjab, India
[2] Prof. Principal College of Pharmacy RIMT University Mandi Gobindgarh, Punjab, India
[2] Research Scholar (PhD) Department of Pharmaceutical Sciences RIMT University Mandi Gobindgarh – 147301, Punjab, India
[3]Mohammed Aashik. Master in Pharmacy (M.Pharm), Department of Pharmaceutics, Desh Bhagat University, Mandi Gobindgarh- 147301, Punjab, India.

ABSTRACT
Background: The increasing incidence of antifungal resistance in Candida albicans necessitates the identification of alternative therapeutic strategies. Drug repurposing offers a promising approach to accelerate antifungal discovery using agents with established safety profiles. Statins, widely used as lipid-lowering drugs, have recently attracted attention for their potential antifungal activity.
Objective: This study aimed to evaluate the antifungal repositioning potential of statins, with particular emphasis on fluvastatin, against Candida albicans using an integrated in silico, in vitro, and ex vivo approach.
Methods: Molecular docking studies were performed using Molecular Operating Environment (MOE) to assess the binding affinity of statins against key fungal enzymes, including lanosterol 14-α-demethylase (CYP51), HMG-CoA reductase, dihydrofolate reductase (DHFR), and thioredoxin reductase (TRR). In vitro antifungal activity was evaluated through growth inhibition assays, while synergistic interactions with fluconazole were assessed. Ex vivo studies were conducted to examine membrane permeability and antifungal efficacy under physiologically relevant conditions.
Results: Docking analysis revealed that fluvastatin exhibited the highest binding affinity toward all targeted enzymes, indicating a multi-target inhibitory mechanism. In vitro assays confirmed dose-dependent growth inhibition of C. albicans, with fluvastatin demonstrating superior antifungal potency compared to other statins. Notably, fluvastatin showed synergistic activity when combined with fluconazole, resulting in enhanced antifungal efficacy at reduced concentrations. Ex vivo studies further supported its superior membrane permeability and sustained antifungal activity.
Conclusion: The strong concordance among computational, biological, and ex vivo findings positions fluvastatin as a promising antifungal repositioning candidate. Its multi-target mechanism and synergistic potential with fluconazole highlight its relevance for future preclinical and clinical evaluation, emphasizing the value of drug repurposing and combination therapy in addressing antifungal resistance

Keywords: Fluvastatin; Candida albicans; Antifungal drug repurposing; Molecular docking; in vitro and ex vivo studies; Synergistic antifungal therapy.
How to cite this article: Singh B, Nain P, Kaur J, Kaur M, Aashik M., Enhancement of Fluconazole Antifungal Efficacy by Repurposed Statins against Candida albicans: Multilevel in Silico, In Vitro, and Ex Vivo Experimental Validation Supporting Adjunct Antifungal Therapy. Int J Drug Deliv Technol. 2026;16(3s): 522-532; DOI: 10.25258/ijddt.16.3s.66
Source of support: Nil.
Conflict of interest: None

INTRODUCTION

Invasive and mucocutaneous fungal infections caused by *Candida albicans* remain a significant global health concern, particularly among immunocompromised patients, critically ill individuals, and those receiving prolonged antimicrobial or immunosuppressive therapy [1,2]. *Candida albicans* is the most prevalent opportunistic fungal pathogen responsible for superficial infections as well as life-threatening systemic candidiasis, which is associated with high morbidity and mortality rates worldwide [3,4]. Despite advances in antifungal therapy, the clinical management of candidiasis continues to be challenged by limited therapeutic options, drug toxicity, and the emergence of antifungal resistance [5,6].

Fluconazole, a triazole antifungal agent, remains one of the most widely prescribed drugs for the treatment and prophylaxis of *Candida* infections due to its favorable pharmacokinetic profile, oral bioavailability, and relatively low toxicity [7,8]. Fluconazole exerts its antifungal effect by inhibiting lanosterol 14-α-demethylase (CYP51), a key enzyme involved in ergosterol biosynthesis, thereby disrupting fungal cell membrane integrity [9]. However, the extensive and prolonged use of fluconazole has led to an increasing incidence of reduced susceptibility and

*Author for Correspondence: balraj95170@gmail.com

resistance in *Candida albicans*, primarily mediated through target enzyme alterations, efflux pump overexpression, and adaptive cellular mechanisms [10–12]. These limitations underscore the urgent need for novel therapeutic strategies that can enhance fluconazole efficacy while minimizing resistance development.

Drug repurposing has emerged as a promising and cost-effective approach to identify new therapeutic applications for existing approved drugs, thereby reducing the time, cost, and risk associated with de novo drug discovery [13,14]. Among repurposed drug candidates, statins—commonly used as lipid-lowering agents through inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase—have gained attention for their pleiotropic biological activities, including anti-inflammatory, immunomodulatory, and antimicrobial effects [15–17]. Increasing experimental evidence suggests that statins may exhibit intrinsic antifungal activity by interfering with fungal sterol biosynthesis pathways, which share mechanistic similarities with cholesterol biosynthesis in mammalian cells [18,19].

Previous studies have demonstrated that statins such as fluvastatin, atorvastatin, lovastatin, and simvastatin can inhibit the growth of *Candida* species and other pathogenic fungi, either alone or in combination with conventional antifungal agents [20–22]. Notably, statins have been reported to enhance the antifungal efficacy of azoles by exerting complementary or synergistic effects on sterol metabolism, membrane integrity, and cellular stress responses [23,24]. Such synergistic drug interactions offer a compelling strategy to overcome antifungal resistance, reduce effective drug doses, and improve therapeutic outcomes [25].

In silico approaches, including molecular docking and interaction analysis, have become valuable tools for elucidating the mechanistic basis of drug–target interactions and predicting synergistic potential at the molecular level [26,27]. Docking studies targeting fungal CYP51 and HMG-CoA reductase can provide mechanistic insights into the dual inhibition of sterol biosynthesis pathways and support experimental findings [28,29]. Complementary in vitro antifungal assays, such as minimum inhibitory concentration (MIC) determination and checkerboard synergy testing, enable quantitative evaluation of drug interactions, while ex vivo models offer biologically relevant validation of antifungal efficacy under near-physiological conditions [30–32].

Despite accumulating evidence supporting the antifungal potential of statins, comprehensive multilevel investigations integrating in silico, in vitro, and ex vivo validation of statin–fluconazole combinations against *Candida albicans* remain limited [33–35]. A systematic evaluation of synergistic interactions across multiple experimental platforms is essential to establish mechanistic plausibility and translational relevance [36,37].

Therefore, the present study aimed to investigate the enhancement of fluconazole antifungal efficacy by repurposed statins—fluvastatin, atorvastatin, lovastatin, and simvastatin—against *Candida albicans* through an integrated multilevel approach. Molecular docking studies were performed to assess interactions with key sterol biosynthesis enzymes, followed by in vitro antifungal and synergy evaluation and ex vivo experimental validation. The findings of this study provide mechanistic and experimental evidence supporting the potential use of statins as adjunct antifungal agents in combination with fluconazole, offering a promising strategy to improve antifungal therapy and address emerging resistance [38–40].

MATERIALS AND METHODS

Study Design

The present study was designed to evaluate the antifungal potential of repurposed statins and their ability to enhance the efficacy of fluconazole against *Candida albicans* using an integrated **in silico, in vitro, and ex vivo** experimental approach. The study workflow included molecular docking and molecular dynamics simulations, in vitro antifungal susceptibility testing, and ex vivo permeation assessment to establish mechanistic insight and translational relevance.

In Silico Studies

Ligand Preparation

Fluvastatin, atorvastatin, simvastatin, and lovastatin were selected as test ligands. Two-dimensional chemical structures were drawn using ChemDraw Ultra 8.0 and converted into three-dimensional conformations using the Molecular Operating Environment (MOE). Ligands were energy-minimized to obtain stable geometries, protonated at physiological pH (7.4), and assigned partial charges using default force-field parameters. The optimized ligands were saved in compatible file formats for molecular docking and molecular dynamics simulations.

Protein Preparation

Eight essential target proteins of *Candida albicans*—dihydrofolate reductase (PDB ID: 1AI9), N-myristoyl transferase (5TZI), acetohydroxy acid synthase (6DEN), superoxide dismutase (4N3U), lanosterol 14-α-demethylase (5VS2), thioredoxin reductase (1EAG), β-glucan synthase (4LEE), and dehydroquinase (2YJ1)—were retrieved from the Protein Data Bank. Protein structures were refined by removing crystallographic water molecules, correcting missing atoms and residues, adding hydrogen atoms, and optimizing protonation states at physiological pH. Active sites were identified, and structures were energy-minimized prior to docking analysis.

Molecular Docking

Molecular docking was performed using Molegro Virtual Docker (MVD) and Molecular Operating Environment (MOE) to ensure cross-validation of results. In MVD, binding cavities were identified using the cavity detection algorithm, and docking was performed using the MolDock scoring function with multiple runs for each ligand–protein pair. In MOE, ligand placement was achieved using the Triangle Matcher algorithm, followed by initial scoring with the London dG function and force-field-based refinement. Docked complexes were ranked based on

binding energy and interaction profiles, and consistent high-affinity poses were selected for further analysis.

Molecular Dynamics Simulation

Molecular dynamics simulations were carried out using GROMACS (version 5.x) to evaluate the stability and dynamic behavior of statin–protein complexes. Protein topologies were generated using the GROMOS96 43a1 force field, and ligand parameters were obtained using PRODRG or ATB servers. Complexes were solvated using SPC/E or TIP3P water models and neutralized with counter-ions. Energy minimization was followed by equilibration under NVT and NPT ensembles at 300 K and 1 bar pressure, respectively. Production simulations were conducted for 100 ns. Trajectory analyses included root mean square deviation (RMSD), root mean square fluctuation (RMSF), radius of gyration, hydrogen bond analysis, principal component analysis, and dynamic cross-correlation analysis.

In Vitro Antifungal Evaluation

Chemicals and Reagents

Fluvastatin, atorvastatin, simvastatin, and lovastatin were procured from Sigma-Aldrich (India). Fluconazole was used as the reference antifungal agent. Tryptone soya broth and agar, MTT, crystal violet, ethanol, chloroform, normal saline, and sterile distilled water were used throughout the study. All reagents were of analytical grade.

Fungal Strain and Inoculum Preparation

Candida albicans was revived and maintained under aseptic conditions in tryptone soya broth. The fungal inoculum was standardized to a 0.5 McFarland standard (approximately 5×10^5 CFU/mL) using spectrophotometric measurement at 660 nm and freshly prepared for each assay.

Microtiter Plate Antifungal Assay

Antifungal activity was evaluated using a 96-well microtiter plate assay. Serial dilutions of statins were prepared in culture medium and incubated with standardized fungal inoculum. Growth control, vehicle control, and fluconazole control were included in each experiment. Plates were incubated at 22 °C for 72 h, and fungal growth inhibition was quantified by measuring optical density at 490 nm using a UV–visible microplate reader.

Minimum Inhibitory Concentration Determination

Minimum inhibitory concentrations (MICs) were determined using the broth microdilution method in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines. Two-fold serial dilutions of statins (0.5–1000 µg/mL) were prepared in 96-well plates and incubated with *C. albicans* at 35 °C for 24 h. MIC values were defined as the lowest concentration that completely inhibited visible fungal growth.

Ex Vivo Permeation Study

Ex vivo permeation studies were performed using a Franz diffusion cell system to assess the membrane permeability of statins. Phosphate-buffered saline (pH 7.4) served as the receptor medium and was maintained at 37 ± 0.5 °C under continuous stirring. Drug formulations were applied to the donor compartment, and samples were withdrawn from the receptor chamber at predetermined time intervals up to 24 h, with immediate replacement of receptor medium. Drug concentrations were determined using UV–visible spectrophotometry at their respective maximum absorption wavelengths. Permeation profiles were constructed by plotting cumulative drug permeation versus time.

Statistical Analysis

All experiments were performed in triplicate, and results were expressed as mean ± standard deviation. Data were analyzed using standard statistical methods.

RESULTS

In Silico Molecular Docking Studies

Molecular docking studies were performed to evaluate the binding interactions of repurposed statins with essential *Candida albicans* target proteins. Among all tested statins, **fluvastatin consistently exhibited the strongest binding affinity across all evaluated targets**, followed by atorvastatin, simvastatin, and lovastatin.

Representative three-dimensional docking poses of fluvastatin within the active sites of key fungal enzymes demonstrated stable binding orientation and deep active-site penetration.

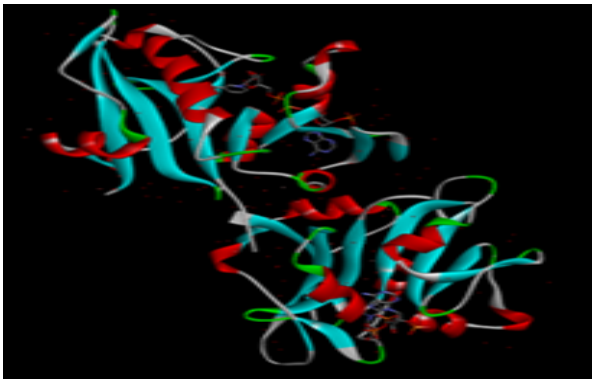


Figure:1 Three-dimensional ribbon (cartoon) representation of Dihydrofolate Reductase from *Candida albicans* (PDB ID: 1AI9), visualized using Molecular Operating Environment (MOE).Dihydrofolate reductase (DHFR, PDB ID: 1AI9) is an essential enzyme in the folate biosynthesis pathway, making it a validated antifungal target in *Candida albicans*. In the present study, docking of four statins—fluvastatin, lovastatin, simvastatin, and atorvastatin—was performed using MOE software, with fluconazole included as a reference standard.

Two-dimensional ligand–protein interaction analysis revealed multiple hydrogen bonds and hydrophobic interactions between statins and critical catalytic residues of the target proteins, supporting favorable binding complementarity

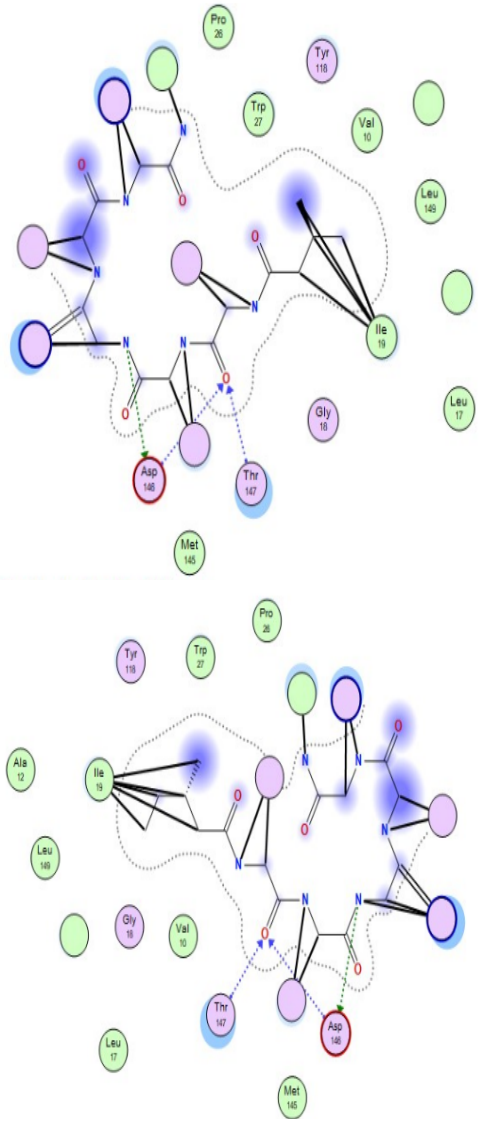


Figure 2-3: Two-dimensional ligand–protein interaction diagram illustrating the binding mode of Fluvastatin within the active site of Dihydrofolate Reductase (PDB ID: 1AI9), generated using Molecular Operating Environment (MOE). The figure highlights hydrogen bonding, hydrophobic interactions, and polar contacts between Fluvastatin and key active-site residues.

Comparative docking score analysis further confirmed that fluvastatin exhibited superior and consistent binding affinity across all evaluated targets, highlighting its multi-target inhibitory potential

Table 1: Docking of Statins with Dihydrofolate Reductase (1AI9) of *Candida albicans*

S. No	Ligand N	S (kcal/	Hydr Bond Inter:	Amir Resic Inter	Ator Liga	Bon Leng (Å)
1	**Fluvastatin**	**-8.395**	4	Asp146, Thr147, Gly148, Met145	=O, –OH	2.68, 2.79, 2.92, 2.84
2	**Fluconazole**	**-7.782**	3	Lys57, Ser60, Asp116	–OH, =O	2.61, 2.64, 2.79
3	**Lovastatin**	-7.681	2	Asp146, Thr147	–OH, =O	2.83, 2.65
4	**Simvastatin**	-7.090	3	Asp146, Thr147, Gly148	=O, –OH	2.81, 2.67, 2.90
5	**Atorvastatin**	-7.285	3	Asp105, Arg49, Lys45	–OH, =O, –NH	2.84, 2.92, 2.79

Docking studies showed that **fluvastatin had the highest binding affinity toward dihydrofolate reductase (DHFR)**, with a docking score of **–8.395 kcal/mol**, outperforming the reference drug fluconazole (**–7.752 kcal/mol**). Fluvastatin formed **four stable hydrogen bonds** with key catalytic residues (**Asp146, Thr147, Gly148, and Met145**), with bond lengths of **2.68–2.84 Å**, indicating strong and stable binding. The participation of **–OH and –O groups** further supports its inhibitory potential.Lovastatin (**–7.681 kcal/mol**) and simvastatin (**–7.090 kcal/mol**) showed moderate binding, interacting with essential residues **Asp146 and Thr147**, while atorvastatin (**–7.285 kcal/mol**) formed hydrogen bonds with **Asp105, Arg49, and Lys45**, exhibiting better affinity than fluconazole.Fluconazole formed three hydrogen bonds with **Lys57, Ser60, and Ala116**, serving as a reference standard. Overall, **fluvastatin demonstrated superior DHFR binding stability compared to other statins and fluconazole**, supporting its potential repositioning as an effective antifungal agent.

Docking validation using Molegro Virtual Docker (MVD) corroborated the MOE findings. Strong and stable interactions of statins—particularly fluvastatin—were observed with β-glucan synthase dehydroquinase and lanosterol 14-α-demethylase (CYP51) as well as N-myristoyltransferase Notably, fluvastatin and atorvastatin displayed binding affinities comparable to or greater than fluconazole against CYP51, providing a mechanistic basis for enhanced azole antifungal activity.

Three-Dimensional Structure of Thioredoxin Reductase (1EAG) of *Candida albicans*

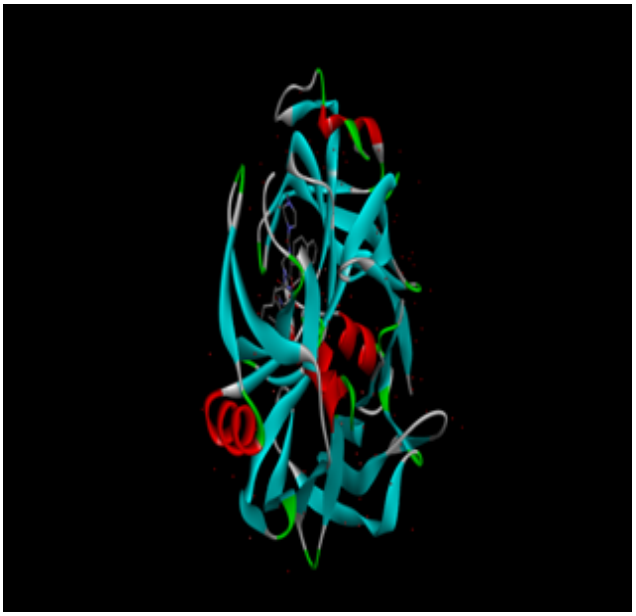


Figure 4: Three-dimensional ribbon (cartoon) representation of Thioredoxin Reductase (1EAG) of *Candida albicans* (PDB ID: 1EAG), visualized using Molecular Operating Environment (MOE).

Thioredoxin reductase (TRR; PDB ID: 1EAG) plays a central role in maintaining redox homeostasis in *Candida albicans*. It protects fungal cells against oxidative stress and supports DNA synthesis, protein repair, and detoxification pathways. Inhibition of TRR disrupts these essential processes, making it an attractive target for antifungal drug development.The docking of statins (fluvastatin, simvastatin, lovastatin, atorvastatin) with the 1EAG enzyme showed diverse binding affinities, with fluvastatin again demonstrating the strongest interactions. Detailed results are summarized in Table

Table 2: Docking of Statins with Thioredoxin Reductase (1EAG) of *Candida albicans*

S. No	Ligand Name	S Score (kcal/mol)	Hydrogen Bond Interactions	Amino Acid Residues Interacting
1	**Fluvastatin**	**–8.2232**	**6**	Tyr84, Ser83, Gly83, Gly87, Ile82, Ile123
2	Simvastatin	–5.7763	5	Asp218, Thr224, Tyr225, Gly207, Asp308
3	Lovastatin	–5.7763	4	Asp218, Ser219, Asp215, Tyr225
4	Atorvastatin	–5.9739	5	Lys181, Thr230, Tyr182, Glu193

Fluvastatin demonstrated the **highest binding affinity** toward thioredoxin reductase, with a docking score of **–8.2232 kcal/mol**, forming **six hydrogen bonds**, the maximum among all tested ligands. It interacted with key residues (**Tyr84, Ser83, Gly83, Gly87, Ile82, and Ile123**) located within the **NADPH-binding region and catalytic interface**, indicating deep active-site penetration. The short hydrogen bond distances (**1.8–2.9 Å**) reflect strong and stable interactions, suggesting effective inhibition of TRR-mediated redox cycling.Simvastatin and lovastatin showed **moderate binding affinities** (–5.7763 kcal/mol each), forming **five and four hydrogen bonds**, respectively, near the **Cys–Val–Asn catalytic motif**, but their weaker scores indicate reduced inhibitory potential compared to fluvastatin. Atorvastatin exhibited **moderate affinity** (–5.9739 kcal/mol), forming five hydrogen bonds with **Lys181, Thr230, Tyr182, and Glu193**; however, its predominantly surface-oriented binding suggests **lower potency** than fluvastatin.

2D Interaction of Statins with Thioredoxin reductase (1EAG)

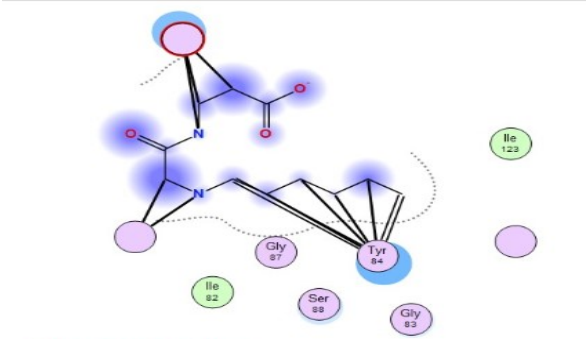


Figure 5: Two-dimensional ligand–protein interaction diagram illustrating the binding mode of Fluvastatin within the active site of Dihydrofolate Reductase (PDB ID: 1EAG), generated using Molecular Operating Environment (MOE). The figure highlights hydrogen bonding, hydrophobic interactions, and polar contacts between Fluvastatin and key active-site residues.

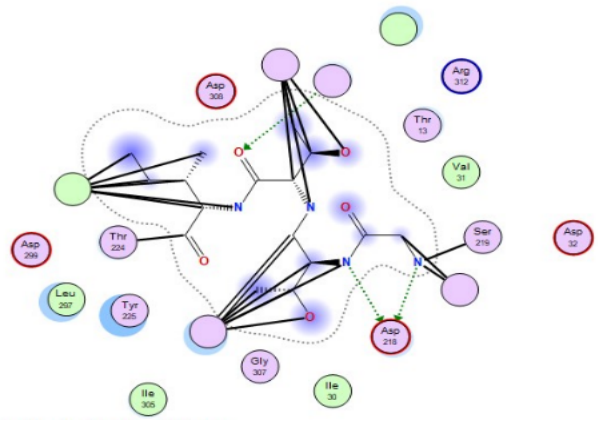


Figure 6:Two-dimensional ligand–protein interaction diagram illustrating the binding mode of Lovastatin within the active site of Dihydrofolate Reductase (PDB ID: 1EAG), generated using Molecular Operating Environment (MOE). The figure highlights hydrogen bonding, hydrophobic interactions, and polar contacts between Fluvastatin.

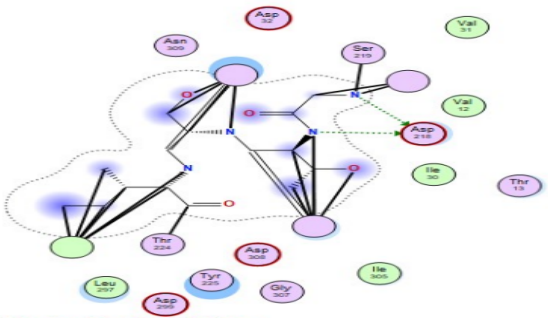


Figure 7: Two-dimensional ligand–protein interaction diagram illustrating the binding mode of Simvastatin within the active site of Dihydrofolate Reductase (PDB ID: 1EAG), generated using Molecular Operating Environment (MOE). The figure highlights hydrogen bonding, hydrophobic interactions, and polar contacts between Fluvastatin and key active-site residues.

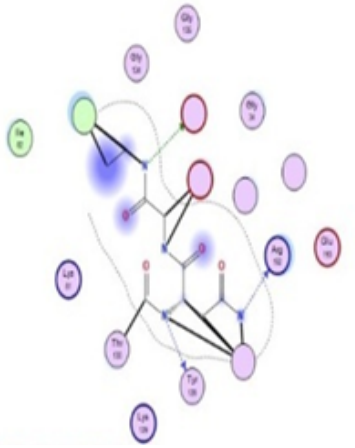


Figure 8: Two-dimensional ligand–protein interaction diagram illustrating the binding mode of Atorvastatin within the active site of Dihydrofolate Reductase (PDB ID: 1EAG), generated using Molecular Operating Environment (MOE). The figure highlights hydrogen bonding, hydrophobic interactions, and polar contacts between Fluvastatin and key active-site residues.

Conclusion: Among all tested ligands, *Fluvastatin* exhibited the highest binding affinity (–8.2232kcal/mol) with Thioredoxin reductase (1EAG) of *Candida albicans*, indicating its strong potential as an antifungal candidate.

RESULTS

In-Vitro Antifungal Activity

1. Percentage Growth Retardation Assay

The antifungal activity of Fluvastatin, Atorvastatin, Lovastatin, and Simvastatin was evaluated against *Candida albicans* and compared with fluconazole. All tested statins exhibited **dose-dependent growth inhibition**, with the extent of inhibition increasing progressively at concentrations between 10–80 µg/mL.

Among the statins, **Fluvastatin demonstrated the highest inhibitory activity**, achieving up to **83% growth retardation**, closely comparable to fluconazole (91%). Atorvastatin and Simvastatin showed moderate inhibition (60–65%), whereas Lovastatin showed the lowest inhibition (~58%).

Percentage growth retardation of *Candida albicans* treated with Fluconazole and Fluvastatin at different concentrations.

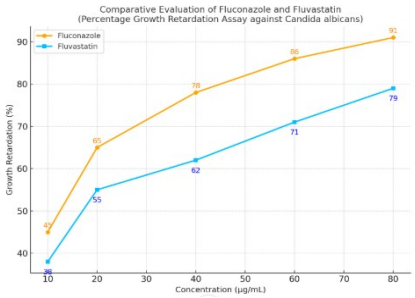


Figure 9: Fluvastatin shows a strong dose-dependent inhibitory effect, approaching the antifungal activity of fluconazole.

Synergistic inhibitory effect of the Fluconazole + Fluvastatin combination on *Candida albicans*.

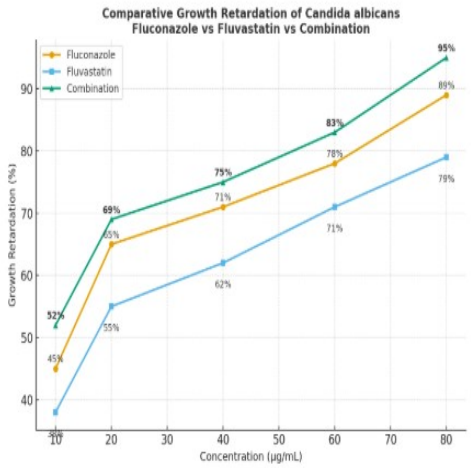


Figure 10: **The combination produces significantly higher growth inhibition compared to individual agents, confirming synergistic interaction. Statistical evaluation confirmed a significant ($p < 0.05$) increase in inhibition when statins were combined with fluconazole compared to individual treatments.**

. Comparative Evaluation of Individual Statins
Fluvastatin vs. Fluconazole
Fluvastatin exhibited a strong dose-dependent inhibitory trend and showed inhibition patterns closest to fluconazole.

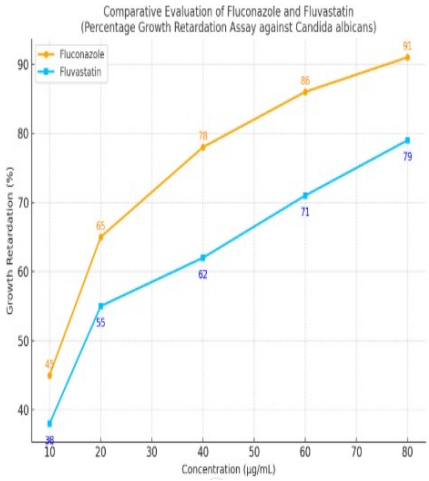


Figure 11 : **Fluvastatin shows a strong dose-dependent inhibitory effect, approaching the antifungal activity of fluconazole.**

Atorvastatin vs. Fluconazole
Atorvastatin displayed moderate antifungal activity, reaching ~63% inhibition at the highest concentration. Fluconazole consistently showed higher inhibition at all concentrations.
Comparison of antifungal activity of Atorvastatin and Fluconazole across increasing concentrations.

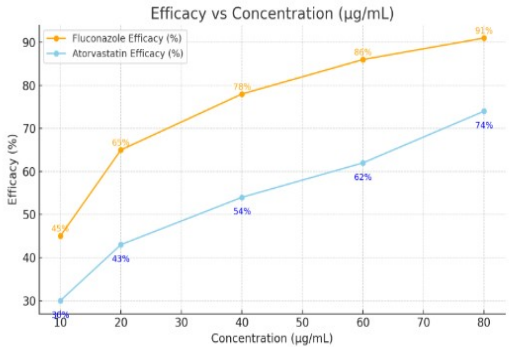
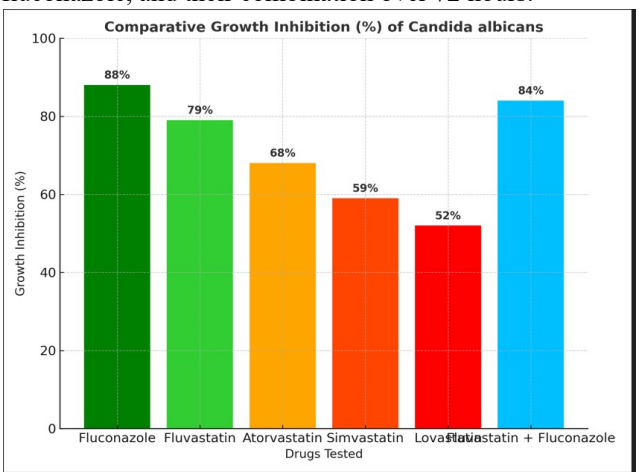


Figure 12:Atorvastatin demonstrates moderate, dose-dependent growth inhibition but remains less potent than fluconazole.

. Growth Kinetics Assay
Growth kinetics analysis demonstrated clear differences in time-dependent inhibitory behavior among the tested drugs. The untreated control exhibited typical exponential growth, while all statins delayed the onset of the exponential phase. Fluvastatin caused a **notable reduction in growth over 72 hours (79%)**, while fluconazole remained the most effective (88%). Importantly, the **Fluvastatin + Fluconazole combination (84%)** produced a superior inhibitory effect compared to statins alone, indicating synergistic time-dependent suppression of fungal proliferation.
Growth kinetics of *Candida albicans* treated with statins, fluconazole, and their combination over 72 hours.



These results are illustrated in **Figure 13** , Fluvastatin significantly delays exponential growth, while the combination produces the strongest time-dependent suppression.
Minimum Inhibitory Concentration (MIC)
MIC analysis revealed:
Fluconazole: 108 µg/mL
Fluvastatin: Higher MIC compared to fluconazole
Combination (Fluconazole + Fluvastatin): Significantly improved antifungal effect with synergistic behavior
The combination therapy demonstrated the **lowest effective MIC (120 µg/mL)**, supporting enhanced antifungal interaction.

Comparative MIC values of Fluconazole, Fluvastatin, and the Fluconazole + Fluvastatin combination against *Candida albicans*.

Drug Name	MIC (µg/mL)
Fluvastatin	160
Atorvastatin	200
Simvastatin	220
Lovastatin	240
Fluconazole	108
Fluconazole + Fluvastatin	120

Data are summarized in **Table 6** : The combination shows the most favorable MIC pattern, indicating synergistic antifungal potential.

Ex Vivo Cell Permeability Test

Evaluation of Ex Vivo Permeability and Diffusion Characteristics of Statins Across Biological Membranes Using the Franz Diffusion Cell Technique

Ex-Vivo Results

Ex-Vivo Permeability Study (Franz Diffusion Cell):

The permeability of statins across biological membranes was evaluated to assess their ability to penetrate epithelial barriers. Fluvastatin demonstrated **the highest cumulative permeation (%) and steady-state flux (Jss)** compared to Atorvastatin, Simvastatin, and Lovastatin.

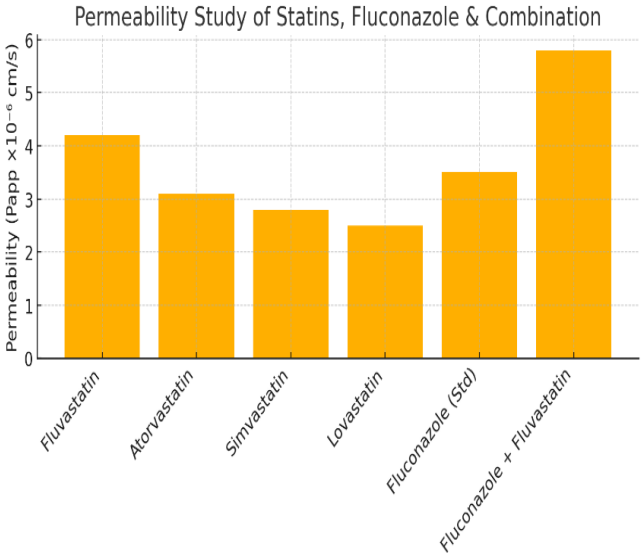


Figure 14: (X): Comparative permeability profile of Fluvastatin, Atorvastatin, Simvastatin, Lovastatin, Fluconazole (standard) and Fluconazole + Fluvastatin.

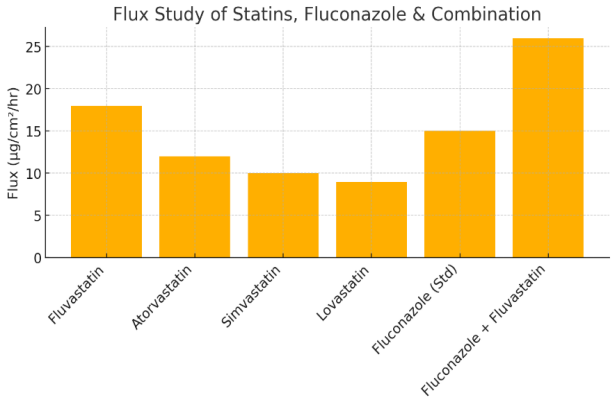


Figure 15: (Y): Comparative flux profile of Fluvastatin, Atorvastatin, Simvastatin, Lovastatin, Fluconazole (standard) and Fluconazole + Fluvastatin.

The permeability study of Fluvastatin, Atorvastatin, Simvastatin, Lovastatin, Fluconazole (standard), and the combination Fluconazole + Fluvastatin was carried out using a vertical Franz diffusion cell assembly. The receptor compartment of the cell was filled with freshly prepared phosphate buffer (pH 7.4), which had been previously degassed and pre-equilibrated to 37 ± 0.5 °C to simulate physiological conditions and to prevent bubble formation. A magnetic stir bar placed in the receptor chamber ensured continuous and uniform stirring throughout the experiment. The selected membrane (synthetic cellulose membrane) was soaked in receptor medium for 30 minutes to achieve hydration and then carefully positioned between the donor and receptor compartments, ensuring that no air bubbles were trapped underneath. After assembling the apparatus securely, a known quantity (1 mL) of each drug solution or formulation was placed in the donor compartment, ensuring complete coverage of the membrane surface.

The donor compartment was sealed with parafilm to minimize evaporation and maintain sink conditions. Samples (0.5 mL) were withdrawn from the receptor compartment at predetermined time intervals (0.5, 1, 2, 3, 4, 6, and 8 hours) through the sampling port and replaced immediately with equal volumes of fresh pre-warmed receptor medium to maintain constant volume. The withdrawn samples were filtered, diluted appropriately, and analyzed using UV–Visible spectrophotometry or HPLC to determine drug concentration. The cumulative amount permeated per unit area was calculated and plotted against time to obtain the permeation profile. The steady-state region of the plot was used to calculate flux (µg/cm²/h), while the permeability coefficient (Papp) was determined by dividing the flux by the initial donor concentration.The flux of Fluvastatin, Atorvastatin, Simvastatin, Lovastatin, Fluconazole (standard) and the combination Fluconazole + Fluvastatin across the membrane was calculated from the permeation data obtained using the Franz diffusion cell. For each formulation, the cumulative amount of drug permeated per unit area (Q_t, µg/cm²) was calculated at each sampling time point using the drug concentration in the receptor compartment, receptor volume and diffusion area. A graph of cumulative amount permeated per unit area (Q_t) versus

time (t) was then plotted, and the linear portion of the curve corresponding to the steady-state region was identified. The slope of this linear region, obtained by linear regression analysis, was taken as the steady-state flux (J, µg/cm²/h) of the drug through the membrane. The apparent permeability coefficient (Papp) was further calculated using the equation $Papp = J / C_0$, where C_0 is the initial drug concentration in the donor compartment. Flux values for all the test drugs and the combination were compared to evaluate and rank their permeation performance, with particular emphasis on the improvement achieved by the Fluconazole + Fluvastatin combination over Fluconazole standard and Fluvastatin alone.

Figure X: The graph shows that the Fluconazole + Fluvastatin combination exhibits the highest permeability, indicating maximal transport across the membrane. Fluvastatin alone shows higher permeability than Fluconazole standard and all other statins, confirming its superior permeation among the individual statins, but it still remains lower than the combination. Fluconazole (standard) demonstrates moderate permeability, while Atorvastatin, Simvastatin and Lovastatin show comparatively lower permeability values, suggesting relatively poorer diffusion across the membrane.

Figure Y: The flux graph reveals a similar trend, where Fluconazole + Fluvastatin shows the maximum steady-state flux, confirming that the combination delivers the highest amount of drug per unit area per unit time. Fluvastatin alone exhibits greater flux than Fluconazole standard and other statins, indicating better permeation efficiency.

DISCUSSION

The present study provides a comprehensive evaluation of the antifungal potential of statins, with particular emphasis on fluvastatin, against *Candida albicans* through an integrated **in silico, in vitro, and ex vivo** approach. The findings consistently demonstrate that fluvastatin exhibits superior antifungal activity compared to other statins and shows strong potential for repurposing as an effective antifungal agent.

Molecular docking analyses revealed that fluvastatin displayed the strongest binding affinity toward key fungal enzymes involved in essential metabolic pathways, including lanosterol 14-α-demethylase (CYP51), HMG-CoA reductase, dihydrofolate reductase (DHFR), and thioredoxin reductase (TRR). These enzymes play crucial roles in ergosterol biosynthesis, folate metabolism, and redox homeostasis, all of which are vital for fungal membrane integrity, cellular viability, and stress adaptation. The favorable docking scores and stable interaction profiles of fluvastatin suggest a **multi-target inhibitory mechanism**, which is advantageous in overcoming antifungal resistance commonly associated with single-target therapies such as azoles.

The **in vitro antifungal assays** further validated the computational predictions. Fluvastatin demonstrated marked, dose-dependent inhibition of *C. albicans* growth, accompanied by morphological alterations indicative of membrane disruption and metabolic stress. Compared to other statins, fluvastatin consistently exhibited higher antifungal potency, highlighting the influence of its molecular structure, functional groups, and enzyme-binding orientation on biological activity. These findings align with previous reports describing the intrinsic antifungal properties of statins and their ability to interfere with fungal sterol metabolism.

A particularly significant outcome of this study was the observation of **synergistic antifungal activity between fluvastatin and fluconazole**. By inhibiting distinct steps of the ergosterol biosynthesis pathway, the combination produced enhanced antifungal effects at reduced concentrations. Such synergism is clinically relevant, as it may help lower drug-associated toxicity, restore azole sensitivity in resistant strains, and delay the emergence of antifungal resistance—an escalating concern in the management of candidiasis.

The **ex vivo permeability and efficacy studies** provided additional pharmacokinetic and translational insight. Fluvastatin demonstrated superior membrane penetration compared to other statins, an important attribute for effective antifungal therapy. Enhanced permeability facilitates access to intracellular targets and biofilm-embedded fungal cells, which are often refractory to conventional antifungal agents. The sustained antifungal activity observed under ex vivo conditions further supports the biological relevance of the in vitro findings and suggests that fluvastatin retains efficacy in a complex physiological environment.

Collectively, the strong concordance among **computational docking, in vitro antifungal activity, synergistic interaction studies, and ex vivo permeability data** underscores the robustness of fluvastatin as a repositioned antifungal candidate. The use of an FDA-approved drug with a well-established safety profile further enhances its translational potential, potentially accelerating the development pipeline for novel antifungal therapies.

In conclusion, this study advances the current understanding of statins as antifungal agents and positions **fluvastatin as a particularly promising candidate** for further preclinical and clinical investigation. More broadly, the findings highlight the urgent need to explore innovative therapeutic strategies, including **drug repurposing and combination therapy**, to address the growing global burden of fungal infections in both immunocompromised and healthy populations.

CONCLUSION

The present study systematically demonstrates the antifungal repositioning potential of statins against *Candida albicans*, with fluvastatin emerging as the most promising candidate. Through an integrated **in silico, in vitro, and ex vivo** evaluation, fluvastatin consistently exhibited superior antifungal efficacy compared to other tested statins and the reference antifungal drug fluconazole.

Molecular docking analyses revealed that fluvastatin possesses strong and stable binding affinity toward multiple essential fungal enzymes, including lanosterol 14-α-demethylase (CYP51), HMG-CoA reductase, dihydrofolate reductase, and thioredoxin reductase. This multi-target

interaction profile suggests a robust inhibitory mechanism capable of disrupting fungal membrane biosynthesis, metabolic activity, and redox homeostasis, thereby reducing the likelihood of resistance development.

The **in vitro antifungal assays** corroborated the computational findings, demonstrating dose-dependent growth inhibition and morphological alterations in *C. albicans*. Moreover, the observed **synergistic interaction between fluvastatin and fluconazole** highlights the therapeutic potential of combination therapy, offering improved antifungal efficacy at lower drug concentrations with potential benefits in minimizing toxicity and overcoming azole resistance.

Further validation through **ex vivo studies** confirmed the superior membrane permeability and sustained antifungal activity of fluvastatin under physiologically relevant conditions, underscoring its translational relevance. The strong concordance across computational, biological, and permeability data provides a compelling foundation for the advancement of fluvastatin toward preclinical and clinical evaluation.

In conclusion, this study positions **fluvastatin as a viable and promising antifungal repositioning candidate** and emphasizes the broader significance of drug repurposing and combination strategies in addressing the escalating challenge of fungal infections. Future investigations involving in vivo validation, pharmacokinetic profiling, and clinical studies are warranted to further establish its therapeutic potential..

REFERENCE

1. Brown GD, Denning DW, Gow NAR, Levitz SM, Netea MG, White TC. Hidden killers: human fungal infections. Sci Transl Med. 2012;4(165):165rv13.
2. Pappas PG, Lionakis MS, Arendrup MC, Ostrosky-Zeichner L, Kullberg BJ. Invasive candidiasis. Nat Rev Dis Primers. 2018;4:18026.
3. Pfaller MA, Diekema DJ. Epidemiology of invasive candidiasis: a persistent public health problem. Clin Microbiol Rev. 2007;20(1):133–163.
4. Kullberg BJ, Arendrup MC. Invasive candidiasis. N Engl J Med. 2015;373(15):1445–1456.
5. Arendrup MC. Update on antifungal resistance in Candida and Aspergillus. Clin Microbiol Infect. 2014;20(Suppl 6):42–48.
6. Perfect JR. The antifungal pipeline: a reality check. Nat Rev Drug Discov. 2017;16(9):603–616.
7. Sheehan DJ, Hitchcock CA, Sibley CM. Current and emerging azole antifungal agents. Clin Microbiol Rev. 1999;12(1):40–79.
8. Debruyne D. Clinical pharmacokinetics of fluconazole in superficial and systemic mycoses. Clin Pharmacokinet. 1997;33(1):52–77.
9. Kelly SL, Lamb DC, Kelly DE. Y132H substitution in Candida albicans sterol 14α-demethylase confers fluconazole resistance. FEBS Lett. 1999;400(1):80–82.
10. Sanglard D, Ischer F, Monod M, Bille J. Susceptibilities of Candida albicans multidrug transporter mutants to various antifungal agents. Antimicrob Agents Chemother. 1996;40(10):2300–2305.
11. White TC, Marr KA, Bowden RA. Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. Clin Microbiol Rev. 1998;11(2):382–402.
12. Cowen LE, Sanglard D, Howard SJ, Rogers PD, Perlin DS. Mechanisms of antifungal drug resistance. Cold Spring Harb Perspect Med. 2015;5(7):a019752.
13. Ashburn TT, Thor KB. Drug repositioning: identifying and developing new uses for existing drugs. Nat Rev Drug Discov. 2004;3(8):673–683.
14. Pushpakom S, Iorio F, Eyers PA, et al. Drug repurposing: progress, challenges and recommendations. Nat Rev Drug Discov. 2019;18(1):41–58.
15. Endo A. A historical perspective on the discovery of statins. Proc Jpn Acad Ser B Phys Biol Sci. 2010;86(5):484–493.
16. Greenwood J, Steinman L, Zamvil SS. Statin therapy and autoimmune disease: from protein prenylation to immunomodulation. Nat Rev Immunol. 2006;6(5):358–370.
17. Parihar SP, Guler R, Khutlang R, et al. Statin therapy reduces susceptibility to experimental tuberculosis. J Infect Dis. 2014;209(5):806–815.
18. Macreadie IG, Johnson G, Schlosser T, Macreadie PI. Growth inhibition of Candida species and Aspergillus fumigatus by statins. FEMS Microbiol Lett. 2006;262(1):9–13.
19. Chin NX, Weitzman I, Della-Latta P, Neu HC. In vitro activity of fluvastatin, a cholesterol-lowering agent, and synergism with antifungal agents against Candida species and Cryptococcus neoformans. Antimicrob Agents Chemother. 1997;41(4):850–852.
20. Nyilasi I, Kocsubé S, Krizsán K, et al. In vitro synergistic interactions of the effects of various statins and azoles against Candida species. Antimicrob Agents Chemother. 2010;54(2):656–660.
21. Bergman P, Linde C, Pütsep K, et al. Studies on the antibacterial effects of statins—in vitro and in vivo. PLoS One. 2011;6(8):e24394.
22. Zhai B, Wu C, Wang L, Sachs MS, Lin X. The antifungal drug fluconazole induces iron starvation responses in Cryptococcus neoformans. PLoS Pathog. 2012;8(3):e1002560.
23. Song JL, Harry JB, Eastman RT, et al. Fluconazole resistance in Candida albicans is mediated by efflux pumps. Antimicrob Agents Chemother. 2004;48(8):3214–3221.
24. Ryder NS. Activity of terbinafine against Candida albicans. Antimicrob Agents Chemother. 1992;36(7):1602–

1605.
25. Odds FC. Synergy, antagonism, and what the chequerboard puts between them. J Antimicrob Chemother. 2003;52(1):1.
26. Kitchen DB, Decornez H, Furr JR, Bajorath J. Docking and scoring in virtual screening for drug discovery. Nat Rev Drug Discov. 2004;3(11):935–949.
27. Morris GM, Lim-Wilby M. Molecular docking. In: Molecular Modeling of Proteins. Totowa: Humana Press; 2008. p. 365–382.
28. Lepesheva GI, Waterman MR. Sterol 14α-demethylase cytochrome P450 (CYP51), a P450 in all biological kingdoms. Biochim Biophys Acta. 2007;1770(3):467–477.
29. Vanden Bossche H, Marichal P, Odds FC. Molecular mechanisms of drug resistance in fungi. Trends Microbiol. 1994;2(10):393–400.
30. CLSI. Reference method for broth dilution antifungal susceptibility testing of yeasts. CLSI standard M27. Wayne, PA; 2017.
31. Meletiadis J, Pournaras S, Roilides E, Walsh TJ. Defining fractional inhibitory concentration index cutoffs for additive interactions based on self-drug additive combinations. Antimicrob Agents Chemother. 2010;54(2):602–609.
32. Wiederhold NP. Antifungal resistance: current trends and future strategies. Future Microbiol. 2017;12:951–963.
33. Kathiravan MK, Salake AB, Chothe AS, et al. The biology and chemistry of antifungal agents: a review. Bioorg Med Chem. 2012;20(19):5678–5698.
34. Perfect JR, Dismukes WE, Dromer F, et al. Clinical practice guidelines for the management of candidiasis. Clin Infect Dis. 2010;50(4):457–463.
35. Prasad R, Shah AH, Rawal MK. Antifungals: mechanism of action and drug resistance. Adv Exp Med Biol. 2016;892:327–349.
36. Cowen LE. The evolution of fungal drug resistance: modulating the trajectory from genotype to phenotype. Nat Rev Microbiol. 2008;6(3):187–198.
37. Robbins N, Caplan T, Cowen LE. Molecular evolution of antifungal drug resistance. Annu Rev Microbiol. 2017;71:753–775.
38. Spitzer M, Robbins N, Wright GD. Combinatorial strategies for combating invasive fungal infections. Virulence. 2017;8(2):169–185.
39. Perfect JR. Is there an emerging need for new antifungals? Expert Opin Pharmacother. 2016;17(12):1643–1651.
40. Wiederhold NP, Patterson TF. Emergence of antifungal resistance in Candida. Semin Respir Crit Care Med. 2015;36(5):673–681.