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ORIGINAL PAPER

Repurposing benzbromarone as antifolate to develop novel antifungal therapy for *Candida albicans*

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Abstract

Fungal infections in humans are responsible for mild to severe infections resulting in systemic effects that cause a large amount of mortality. Invasive fungal infections are having similar symptomatic effects to those of COVID-19. The COVID-19 patients are immunocompromised in nature and have a high probability of developing severe fungal infections, resulting in the development of further complications. The existing antifungal therapy has associated problems related to the development of drug resistance, being sub-potent in nature, and the presence of undesirable toxic effects. The fungal dihydrofolate reductase is an essential enzyme involved in the absorption of dietary folic acid and its conversion into tetrahydrofolate, which is a coenzyme required for the biosynthesis of the fungal nucleotides. Thus, in the current study, an attempt has been made to identify potential folate inhibitors of *Candida albicans* by a computational drug repurposing approach. Based upon the molecular docking simulation-based virtual screening followed by the molecular dynamic simulation of the macromolecular complex, benzbromarone has been identified as a potential anti-folate agent for the development of a novel therapy for the treatment of candidiasis.

Keywords Benzbromarone · Antifungal · Candida albicans · DHFR · Repurposing · White-fungus · COVID-19

Introduction

Fungal infections in humans range from mild skin diseases causing rashes and itching to fungal pneumonia, meningitis, and bloodstream infections responsible for causing a large number of fatalities. [1] The fungal infection responsible for systemic effects is considered an invasive fungal infection (IFI). Some of the common IFI diseases, like coccidioidomycosis, blastomycosis, and histoplasmosis, have symptoms like cough, cold, fever, and breathing difficulties, similar to those of COVID-19. The fungi responsible for causing such types of symptomatic effects are highly communicable to humans through the air. IFI pneumonia is one of the probable causes of respiratory illness in COVID-19–negative patients. [2]

Patients suffering from COVID-19 infections are supposed to have compromised immunity and are also at a high risk of healthcare-associated infections like candidemia and other systemic fungal infections caused by *Candida* [3, 4]. Candidemia and other fungal pneumonias can also show synergistic effects with the SARS-CoV-2 virus, making the infection more complicated and responsible for increasing the mortality rate. It has been reported that the fungal co-infections in the COVID-19 patients are showing resistance to the existing antifungal therapy. [5]

Azoles are the most widely used antifungal drugs to inhibit pathogenic ergosterol biosynthesis. They nowadays have limited clinical utility because of the development of pathogenic resistance. The polyene compound amphotericin-B has broad spectrum antifungal activity through the disruption of the fungal membrane by interacting with ergosterol, but its systemic use has serious toxic effects, limiting its medicinal utility [6]. Echinocandins are recently approved antifungal agents effective only against *Candida* species by targeting their cell wall biosynthesis via intravenous administration. [7, 8]

It is a matter of high concern that the mediocre efficacy and limited clinical utility of the available three major classes of antifungal agents are responsible for increasing

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mortality rates in patients having IFI, with a high rate of transmission of the infection among humans. More than one-third of the patients with Candida infections resisted treatment with azoles like fluconazole, voriconazole, and isavuconazole. [9] The desired therapeutic effects were also not achieved by using echinocandin and amphotericin-B against candidiasis.

Therefore, to counter the resistant fungal pathogen, there is an urgent requirement to develop a novel antifungal agent with amended therapeutic efficacy, patient safety, and broad-spectrum of activity to counter the development of drug resistance by the fungal pathogen against the existing antifungal therapy and control the complications caused by fungal co-infection in COVID-19 patients.

Tetrahydrofolate is a vital coenzyme acting as a carrier for the transfer of a carbon unit for various cellular enzymes during their interconversion between various oxidative states required for the biosynthesis of important metabolites like methionine and purines. In the absence of necessary enzymes, mammals are incompetent for the biosynthesis of folate and depend only upon the dietary source in the form of folic acid. Cellular uptake of folic acid is regulated through a specialized transport mechanism followed by its conversion by dihydrofolate reductase (DHFR) to activated tetrahydro folate (THF). While the prokaryotes and the majority of the microbial eukaryotes lack this mammalian transport system, it is required for the cellular uptake of folic acid. Thus, they have to synthesize de novo folic acid by themselves to satisfy their biological needs. Targeting the folate biosynthetic pathway of pathogenic microbes by developing a suitable antifolate is a highly successful approach in developing different types of antimicrobial therapies for humans. [7, 10]

Drug repurposing is a trending approach to the identification of a newer pharmacological role for an existing approved drug, whereas establishing the newer mechanism of an existing approved drug by identifying its potent binding affinity towards an alternate drug target is called drug repositioning. The high cost and the slow pace involved in the traditional drug development process are becoming barriers in the combat against the new diseases affecting mankind. [11, 12] Thus, computational repurposing and repositioning of existing approved drugs are an emerging trend for developing newer therapies to counter upcoming human diseases. Drug repurposing is the preferred approach for developing novel therapies because it saves a lot of time as only the binding interaction-based affinity of the molecule against the specific macromolecular drug target has to be established, as the existing approved drugs have an established pharmacokinetics and toxicity profile. Data-driven computational approaches applied to the repurposing of existing drugs are a low-budget approach having a higher rate of success in a short span of developmental time. [11, 13, 14]

Thus, in the current study, we are trying to develop and reposition the existing FDA-approved drugs against the DHFR of *C. albicans* by using molecular docking simulation-based computational repurposing molecular dynamics for their further validation.

Methods

The computational drug repurposing of existing approved drugs against the DHFR enzyme of *C. albicans* was performed by using a computer system having a 10th generation i7 processor with 16 GB of random-access memory and 4 GB of graphic memory card with the help of various molecular modeling tools including AutoDock, Desmond, PyMol, and Chimera.

The molecular docking simulation-based virtual screening of a ligand library consisting of 2880 FDA-approved drugs against the fungal DHFR enzyme of *C. albicans* was performed by underwriting procedures.

Molecular docking simulation

The three-dimensional structure model of the DHFR enzyme of *C. albicans* complexed with NADPH and e meta-heterobiaryl propargyl-linked (18G) antifolate ligand was procured from the RCSB protein data bank (pdb id-4HOE). [15, 16] The complex antifolate ligand 18G was separated from the macromolecular complex by using the software Chimera. [17] The macromolecular target was prepared for molecular docking simulation by addition of polar hydrogens and Gasteiger charge to the amino acid residues with even distribution [12, 14, 18]. Ligand 18G was prepared for the docking simulation by assigning nonrotatable, rotatable and unrotatable bonds. The active ligand binding site of the fungal DHFR receptor was confirmed by observing the active binding interaction of the bioactive complex ligand 18G by using the Discovery Studio Visualizer. [19] The revealed binding site of the macromolecular target was further utilized for finalizing the size and position of the grid-box required to perform the docking simulations. The grid-box was prepared by centering the complex bioactive ligand to cover its each and every extended conformation as well as the binding residues actively interacting with it. [20, 21] The molecular docking simulation process for the fungal DHFR is validated by re-docking the separated bioactive ligand by considering the overlay and chemical resemblance of the docked conformation of the ligand with respect to its bioactive crystallized conformation [14, 18, 22–26].

Virtual screening of the drug library

The validated docking parameters were further utilized for performing the in silico screening of a ligand library consisting of 2880 FDA approved drug molecules against the fungal DHFR receptor [23, 24]. The virtual screening against the fungal DHFR receptor is performed with the intent of identifying potential leads having high affinity for the antifungal drug target [11, 12, 27].

Molecular dynamic simulation

To analyze the binding stability and binding patterns of the potential hits obtained after virtual screening, molecular dynamic simulations for 10 ns were performed using the Desmond module of Schrödinger for the macromolecular complex of DHFR with all the shortlisted leads [28, 29]. Based on the stability of the ligands within the macromolecular complex observed in the dynamics simulation for a shorter duration of 10 ns, the dynamics simulation was amplified to 100 ns for the most stable ligand. An orthorhombic simulation box was prepared and a minimum distance of 10 Å was set between the box wall and the ligand–protein complex [21, 27, 30, 31]. A TIP3P explicit water model was used. The system was neutralized by the addition of counter ions and an isosmotic environment was created by providing 0.15 M NaCl. For system energy minimization, 2000 iterations were performed with a convergence criterion of 1 kcal/mol^{-1}/. This energy-minimizing complex system was then subjected to 100 ns molecular dynamic simulations. A constant temperature of 300 K and a constant atmospheric pressure of 1.013 bars were maintained during the simulation. An energy interval of 1.2 ps and a trajectory path were set at 9.6. In the end, the trajectories were utilized to generate the simulation interaction diagrams [32–34].

In vitro *antifungal activity*

For testing of *Candida albicans*, YPD (yeast extract:peptone:dextrose, 10 g:20 g:20 g per liter) medium was used for growing fungal strains at 28 °C. Fungal cells were seeded in wells inside YPD media at density 2*103 cells. Test compound was serially diluted (100, 200, 300, 400, 500, 600, 700, 800 900, 1000 µg/ml) and mix 10 µl with cell suspension then incubated at 28 °C for 24 h. Furthermore, 10 µl solution of a 3-(4,5- dimethyl-2-thiazolyl)- 2,5-diphenyl-2H-terrazolium bromide (MTT) was added to respective wells and incubated at 37 °C. In order to dissolve the formazan crystals, 0.02 M HCl was taken 30 µl of 20% (w/v) and added to plates further incubating it at 37 °C for 16 h. ELISA microplate-reader was utilized to measure the turbidity of each well at 570 nm [35, 36].

Results

Molecular docking simulation

The fungal DHFR macromolecular complex obtained from the RCSB database protein data bank is made up of two polypeptide chains of 192 amino acids. Chain A was retained to perform docking simulations while chain B was removed. The processed monomeric macromolecular structure is represented in Fig. 1. The ligand has 16 aromatic carbons, and all six flexible bonds were kept rotatable in the current docking protocol. Bioactive complex ligand 18G has major interactions with Pro63, Ile62, Thr58, Ile112, Phe36, Ile9, Ala11, Ile33, and Glu32 amino acids within the active ligand binding site of the fungal DHFR receptor. An imaginary three-dimensional grid-box was prepared by wrapping all the macromolecular binding residues interacting with the ligand. The grid-box is shown in Fig. 2 and their coordinates are tabulated in Table 1.

The docking parameter file consisting of various docking parameters for the present studies was utilized by Autodock software for performing docking simulations of the 18G ligand against the prepared fungal DHFR protein. The results obtained after the docking study of the bound ligand 18G with the DHFR protein are shown in Table 2. The three-dimensional binding confirmation ligand 18G is shown in Fig. 3.

The parameters used in the current docking protocol of 18G ligand against the fungal DHFR receptor were

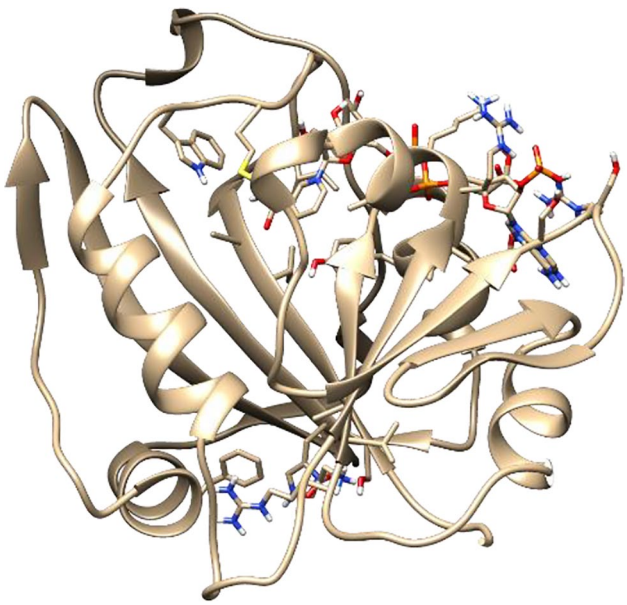


Fig. 1 Monomeric processed macromolecular structure of fungal DHFR procured from the RCSB database protein data bank

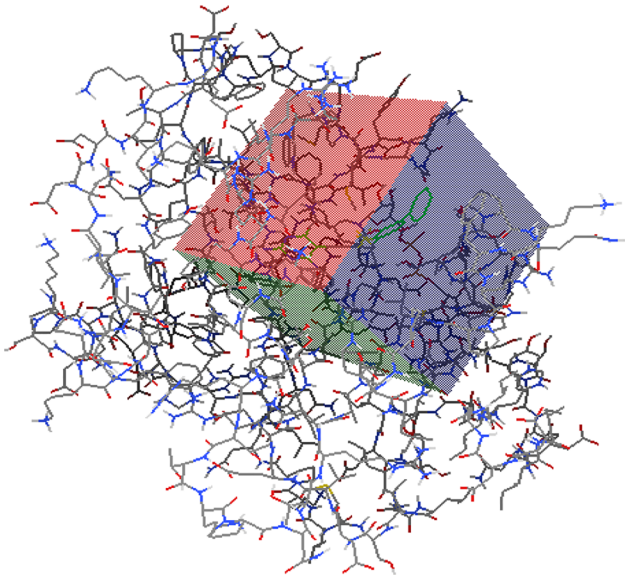


Fig. 2 Three-dimensional imaginary grid-box was prepared by wrapping all the macromolecular binding residues interacting with the ligand

successfully validated by considering the overlay and chemical resemblance of the docked conformation of the ligand with respect to its bioactive crystallized conformation. The docked conformation of the ligand 18G was perfectly overlaid over the bioactive conformation of the ligand present in the macromolecular complex obtained from the RCSB protein data bank with a root mean square deviation (RMSD) value of 0.6. The perfect overlapping of the docked conformation of 18G with respect to its crystallized conformation successfully validates the molecular docking simulation process and parameters utilized for performing the same. The docked ligand 18G has similar binding interactions that were present in the bioactive conformation obtained from the crystallized enzyme complex.

Virtual screening of the drug library

From a ligand library containing 2880 FDA-approved drugs, drug molecules having potential binding affinity for the fungal DHFR receptor were selected on the basis of the lowest binding energy in the predefined range of − 5 to − 15 kcal/mol, as well as the observed chemical interactions between the ligand and the target receptor. The binding energy obtained for the top ten drug molecules after performing molecular docking simulation-based virtual screening is given in Table 3.

Molecular dynamic simulation

The benzbromarone was found to beS the most stabilized ligand after performing dynamic simulation for a short duration of time, and thus the same procedure has been amplified for a longer duration of 100 ns for identification of the stability of the complex ligand benzbromarone within the macromolecular target.

The RMSD protocol measures the stability and conformational changes in the protein backbone during the simulation time. Based on MD results, we revealed that docked complex trajectories were found to be stable throughout the simulation with a mean RMSD value ranging from 4 Å (Fig. 4), showing stable behavior with less fluctuation.

We also calculated the RMSF values by using the Cα atoms of enzymes from the stable trajectory to get insight into the structural fluctuations of active amino acid residues in the enzyme. The lower the fluctuations, the lesser the mobility of amino acids in the active site, and vice versa. In the RMSF plot, the amino acid residues are shown in the x-axis and their RMSF value in the y-axis. The active amino acid residues were evaluated and were observed to show a lower RMSF value for the complex system. The average fluctuation range of the benzbromarone was observed to be 1.2–1.8 Å. Thus, RMSF analysis of selected complexes showed comparable fluctuations in active site residues that are important for molecular interactions.

The establishment of hydrogen bonds, hydrophobic interactions, and ionic interactions between the protein ligand

Table 1 The grid coordinates for the fungal DHFR protein

Proteins	x-D	y-D	z-D	Spacing (Å)	x center	y center	z center
4HOE	40	40	40	0.408	0.679	5.441	32.464

Table 2 Docking results of 18G ligand against the fungal DHFR protein

Proteins	Ligand	Interacting residues	RMSD	Binding energy (kcal/mol)	Binding affinity (nM)
4HOE	18G	Pro63, Ile62, Thr58, Ile112, Phe36, Ile9, Ala11, Ile33, and Glu32	0.6	− 9.93	52.84

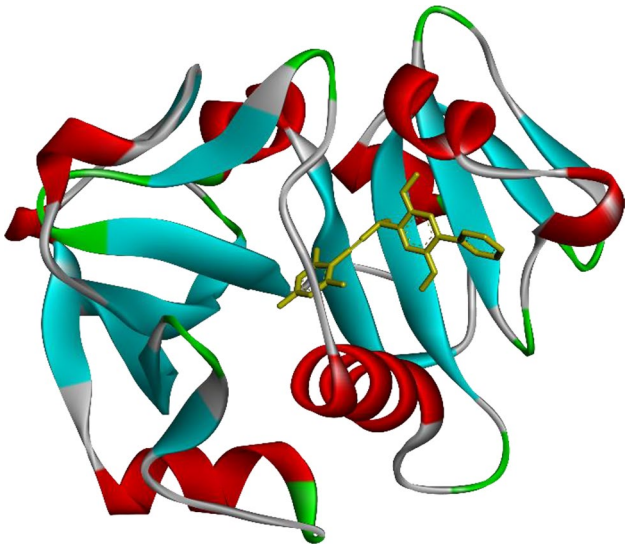


Fig. 3 The three-dimensional binding confirmation ligand 18G against the target receptor fungal DHFR

complexes during the MD simulation is responsible for their stability. Thus, we also evaluated the strength of these interactions to quantify the stability of our screened compounds against the DHFR enzymes. The interaction analysis was performed using the simulation interaction analysis module of Desmond in the Maestro. The interaction analysis of benzbromarone with DHFR reveals that hydroxy molecules interact with PHE-36 amino acid by hydrophobic interaction (56%), and amino acid LYS-37 forms a cationic bridge (36%) with the benzene nucleus (Fig. 5).

In vitro *antifungal activity*

Antifungal activity of drug benzbromarone was determined against *C. albicans* by MTT assay. Benzbromarone diluted different concentration (100 to 1000) µg/ml and voriconazole taken as standard (Fig. 6). The in vitro results suggested that benzbromarone has antifungal activity and the formation of formazan crystals directly proportional to the concentration of the drug. Benzbromarone 600 µg/ml showed comparable efficacy to standard 500 µg/ml. An incremental antifungal activity is seen if test compound concentration increases.

Discussion

Fungal infections are a well-described phenomenon in critically ill, instinctively ventilated COVID-19 patients. In an earlier report, four fungal pathogens, namely, Candida albicans, Candida glabrata, Aspergillus flavus, and Aspergillus fumigatus, were attributed to central line sources for infection. The mortality rate of COVID-19 patients was 38%, but it increased drastically, up to 51%, in fungal species as co-pathogens. The enhanced life expectancy in patients receiving antifungal therapy suggests that the drug dose regimen is appropriate [37, 38].

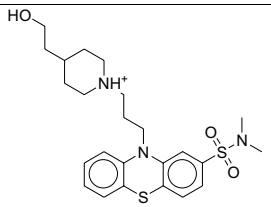
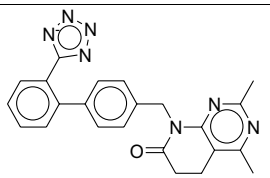
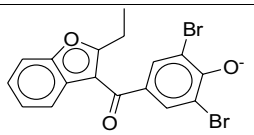
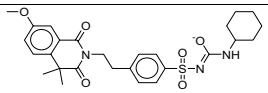
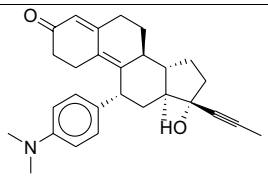
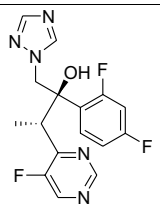
The available therapeutics for antifungals have a number of limitations. A particular strong antifungal-resistance strain observed against azole derivatives and amphotericin-B in all the formulations showed nephrotoxicity [39]. According to the literature, 15% of COVID-19 patients are also fungally infected; thus, to meet this criterion, a new, significant, and effective antifungal drug with the least adverse effect that is resistant to a growing pool of resistant pathogenic organisms must be developed [40, 41].

The folate metabolic pathway is vital for the generation of essential precursors for physiological metabolism for cells (prokaryotes and eukaryotes). It has been demonstrated that the DHFR enzyme is a target for the longest-acting antibiotics, antiprotozoal, and anti-cancerous agents (sulfonamide, pyrimethamine, and methotrexate) [42, 43]. Therefore, computational studies designed to assess the pharmacology of the folate metabolic pathway lead to the exploration of the molecule with desired pharmacological outcomes. Repurposing of drugs is a new approach in which the identification of new pharmacological uses of already approved drugs is done for a different activity. Furthermore, the advantages of this approach include a known complete profile of drugs, and the cost of drug development is reduced exponentially. The computational outcome was successfully validated by comparative analysis of benzbromarone with the standard antifungal drug voriconazole via MTT assay–based in vitro analysis.

Conclusions

Fungal infections are responsible for both local as well as systemic effects in humans. The invasive fungal infection involves different organ systems, including the lungs and results in pneumonia-like symptoms characterized by difficulty in breathing. These breathing problems have a symptomatic similarity to COVID-19 infections. COVID-19 patients who are immunocompromised are highly prone to getting fungal infections. The increasing number of fungal infections among the COVID-19 patients worsens the scenario by increasing the complications in these patients, resulting in multiorgan failure leading to death. The existing antifungal therapy seems to be inefficient to counter the fungal infections among the COVID-19 patients because of the development of drug resistance by the pathogen and co-infection among the COVID-19 patients. Fungal DHFR was explored as a potential antifungal drug target involved in the biosynthesis of the pathogenic nuclear material. Thus, in the

Table 3 The binding energy of the top ten drug molecules obtained after Autodock-based virtual screening of ligand library containing 2890 FDA-approved drugs

S. No.	Name of Compound	Structure	Binding Energy (kcal/mol)	Interacting Residues
1	Pipotiazine		-10.15	Glu120, Ser78, Arg79, Arg56, Lys57, Ile117, Thr58, Gly20, Ile19, Ala11, Met25, Ala115, Glu116, Leu77, Ser94
2	Tasosartan		-10.08	Glu120, Ser78, Arg79, Arg56, Lys57, Ile117, Thr58, Gly23, Gly20, Ile19, Ala11, Met25, Gly113, Gly114, Ala115, Leu77, Ser94
3	Benzbromarone		-10.07	Glu120, Ser78, Arg79, Arg56, Lys57, Ile117, Thr58, Gly20, Ile19, Ala11, Met25, Gly113, Gly114, Ala115, Glu116
4	Gliquidone		-10.07	Glu120, Ser78, Arg79, Arg56, Lys57, Ile117, Thr58, Gly20, Ile19, Ala11, Met25, Ala115, Glu116, Leu77, Ser94
5	Mifepristone		-9.99	Glu120, Ser78, Arg79, Arg56, Lys57, Ile117, Thr58, Gly20, Ile19, Ala11, Met25, Gly113, Gly114, Ala115, Glu116, Leu77, Ser94
6	Voriconazole		-5.42	Glu120, Ser78, Arg79, Arg56, Lys57, Ile117, Thr58, Gly20, Ile19, Ala11, Met25, Gly113, Gly114, Ala115, Glu116, Leu77, Ser94

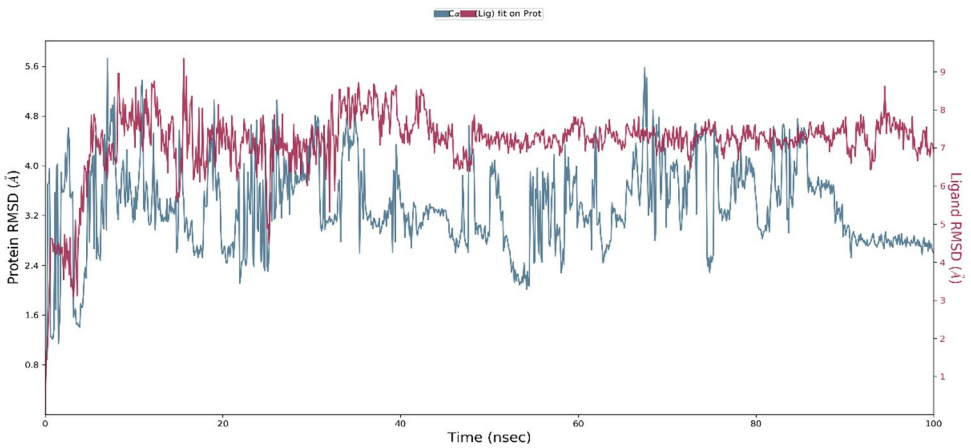


Fig. 4 RMSD trajectory for the macromolecular target as well as ligand obtained after performing molecular dynamics simulation

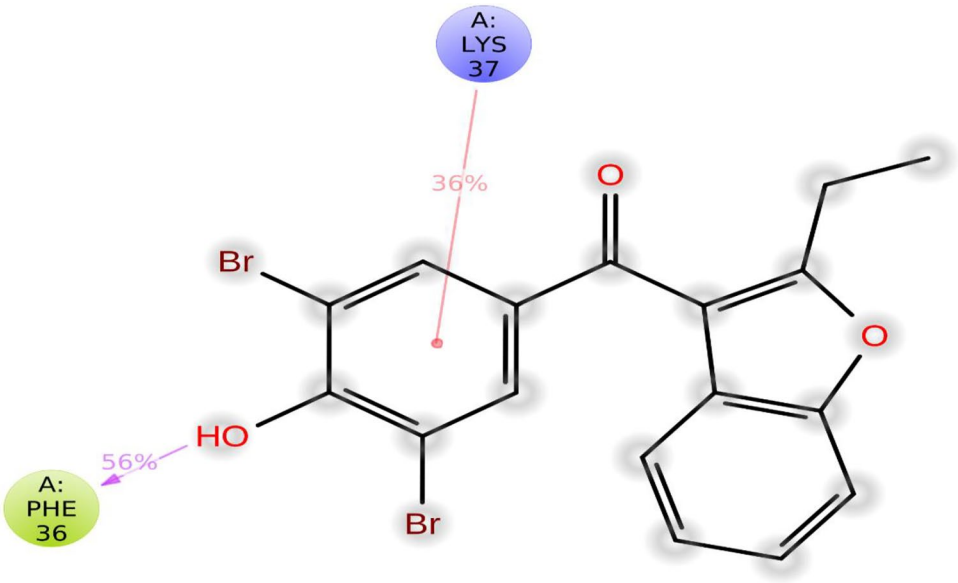


Fig. 5 Interaction analysis of benzbromarone against fungal DHFR

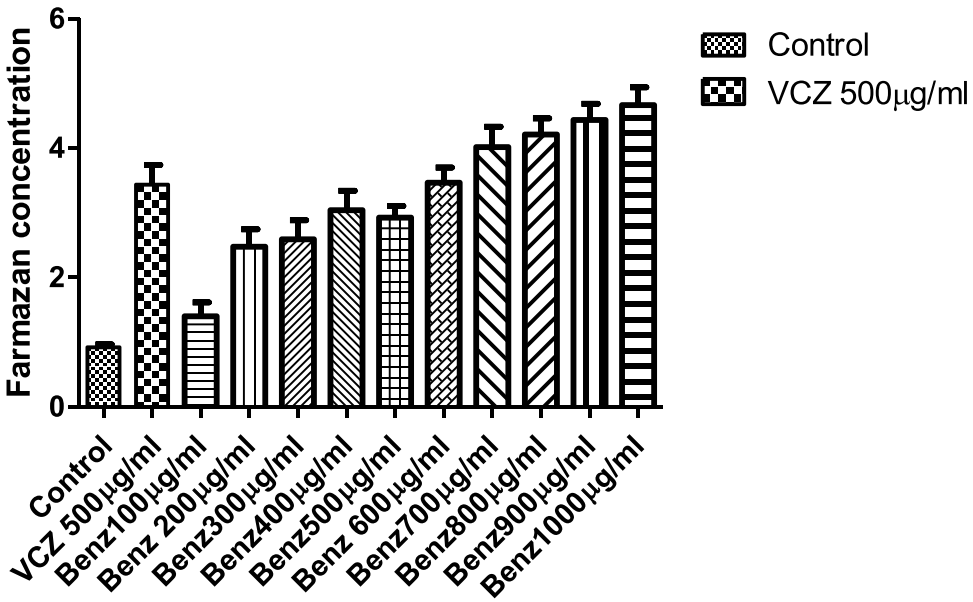


Fig. 6 MTT assay: Formazan was extracted with acid and determined as A570 nm ± standard deviation (SD). All assays were run as triplicates ($n = 3$)

current research, a computational repurposing approach has been employed to identify potential inhibitors of the fungal DHFR to develop a new antifungal therapy. Benzbromarone was identified as a potent inhibitor of fungal DHFR by molecular docking simulation-based screening of a ligand library of existing approved drugs, followed by the confirmation of the stability with respect to time by molecular dynamics simulations. Antifungal potential of benzbromarone was further validated by using MTT assay–based in-vitro analysis in comparison to the standard drug voriconazole.

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Author contribution Both the authors have contributed equally for the execution of current research. The framework is designed and executed by SM while the validation of the research outcomes and drafting of the manuscript were done by AT.

Availability of data and materials Not applicable.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare no competing interests.

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