Original Article

Two Small Molecule Lead Compounds as New Antifungal Agents Effective against *Candida albicans* and *Saccharomyces cerevisiae*

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Abstract

Background: Antifungal drug resistance and few numbers of available drugs limit therapeutic options against fungal infections. The present study was designed to discover new antifungal drugs.

Materials and Methods: This study was carried out in two separate steps, that is, in silico lead identification and in vitro assaying of antifungal potential. A structural data file of a ternary complex of fusicuccin (legend), C terminus of H^+-ATPase and 14-3-3 regulatory protein (1o9F.pdb file) was used as a model. Computational screening of a virtual 3D database of drug-like molecules was performed and selected small molecules, resembling the functional part of the ligand performing ligand docking, were tested using ArgusLab (4.0.1). Two lead compounds, 3-Cyclohexan propionic acid (CXP) and 4-phenyl butyric acid (PBA) were selected according to their ligation scores. Standard Strains of *Candida albicans* and *Saccharomyces cerevisiae* were used to measure the antifungal potential of the two identified lead compounds against the fungi using micro-well plate dilution assay.

Results: Ligation scores for CXP and PBA were -9.33744 and -10.7259 kcal/mol, respectively, and MIC and MFC of CXP and PBA against the two yeasts were promising.

Conclusion: The evidence from the present study suggests that CXP and PBA possess potentially antifungals properties.

Keywords: Computer aided drug design, Fungicide, Fusicoccin, H^+-ATPase

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Introduction

Despite traditional approaches to drug discovery, which is predicated upon laboratory practices through stepwise synthesis and screening methods, rational drug deigning using calculating power of a new generation of powerful computers affords new opportunities to in silico drug designing and evaluation. The rational approaches to drug discovery are based upon lead discovery using known information with respect to the structure of a drug receptor or one of its natural ligands[1]. The information may incorporate three-dimensional structure data of

protein, ligand, or their combination determined by X-ray crystallography or nuclear magnetic resonance (NMR) spectroscopy, which is usually registered as PDB file in easy accessible protein data bank. According to the available information, one can search databases for structures resembling identified lead and select the desired compounds to evaluate their efficacy by a favorite bioassay[2].

The lead, which is a small molecule, performs a desired biological or pharmacological activity. Structural modification of an identified lead is often a prerequisite for production of an effective drug. To inhibit or accelerate their activities, drugs usually interact with target molecules (receptors). Moreover, a lead is directed to its target molecule and selected based upon its interaction with the target. The lead is proposed predicated upon a known ligand with binding specificity to its target molecule with a rational design. A lead is commonly made by a small part of the ligand with specificity to the binding moiety on the target molecule[3,4].

The powerful tools of molecular biology have been utilized in fungi and plants over the last decade and made rapid progress in the molecular basis of the fungal and plant proton pumps. The cells are to actively extrude protons across the plasma membrane mediated by an ATP-driven proton pump. The proton pump (H^+-ATPase), which is an integral membrane protein, keeps high rate of identity among plants and fungi; nevertheless, the pump in animal cell membrane is related to a different protein family[5]. The plasma membrane H^+-ATPase (PMA) of fungi is a single polypeptide, which has a molecular mass of approximately 100 kDa, comprised of 10 transmembrane alpha helices. Both N- and C-terminals of the helices are at the cytoplasmic surface of the membrane. H^+-ATPase pumps protons across the plasma membrane control membrane potential, intracellular ion concentrations and provide the driving force for nutrient uptake by consuming ATP as an energy source[6]. Preliminary studies into both plant and yeast materials indicated that C-terminal region of the molecule exerted an autoinhibitory role in its ATPase activity and penultimate threonine residue phosphorylation is essential for subsequent association of the C-end with 14-3-3 regulatory proteins and thus for activation of the pump. The phosphopeptide end of the molecule occupies the central binding groove of 14-3-3 protein. The eukaryotic 14-3-3 families of molecules refer to dimeric highly conserved regulatory proteins bound to their numerous target proteins in a sequence-specific and phosphorylation-dependent manner. The 14-3-3 binding motif at the C-terminus of the H^+-ATPase includes the amino acid sequences of QQXYpT (948) V[7-9].

Fusicoccin (FC), a phytotoxin, is made by the fungus *Fusicoccin amygdali* (figure 1)[10]. Isolated FC exerts wilting impact upon higher plants and plasma membrane H+-ATPase (PMA) is its molecular target. Preceding research demonstrated that FC stabilized the interaction between the C-terminus of the plant PMA, and 14-3-3 proteins, and brout about permanent activation of the proton pump and wilting of plants by formation of an irreversible stomatal pore[11]. It was suggested that the toxin alone binds weakly to 14-3-3 regulatory protein and peptide-toxin association mutually hightens the binding affinity by 93-fold. The fungal toxin fills a solvent-exposed cavity in the 14-3-3 complex, and hence increases the affinity of the complex by roughly two orders of magnitude[12].

The formation of an H^+-ATPase-14-3-3 complex in the absence of FC absolutely depends on the phosphorylation of penultimate threonine residue (Thr-948), however, binding of 14-3-3 in the presence of FC happens independent of phosphorylation embodies the C-terminal motif YTV. Effectively, substitution of Thr-948 in the plant H^+-ATPase for alanine is lethal inasmuch as this mutant fails to functionally replace the yeast H^+-ATPase[13].

The crystal structure of the ternary complex between 14-3-3 protein, Fusicoccin and a phosphopeptide obtained from the C-terminus of the H^+-ATPase was first introduced by Martin Wurtele in 2003. For elucidation of the contributory mechanisms to the Fusicoccin action, comparisons were drawn between the ternary structure and the corresponding binary complexes and showed no major conformational change in the protein by Fusicoccin; FC fills a cavity in the protein-phosphopeptide interaction surface and stabilizes this interaction[14]. The PMA-binding peptide QSYpTV-COOH differs from the binding motifs of a polyoma middle-T peptide (RSXpS/TXP) and a library-derived peptide (RXXXpS/TXP), the two letters are recognized by all

mammalian 14-3-3 isoforms and are not situated at the very C-terminal end of the interaction partner[14,15].

Antifungal drug resistance and low number of available drugs curtail therapeutic options against fungal infections. Therefore, further research may be required to discover and introduce new antifungal agents. Should be plasma membrane H^+-ATPase a selective target, which its structure are quite different between fungal and mammalian cells, it can be considered as a promising new drug target. The crystal structure of the ternary complex between 14-3-3 protein, Fusicoccin and C-terminus of the pump is accessible through searching protein data banks (pdb entry: 1o9F). The present study set out to find new lead compounds having antifungal property by means of silico methods with required structural data.

Methods

This study was carried out in two separate steps as follows: 1) in silico lead identification 2) assaying the antifungal potential of purchased two lead compounds using standard microdilution method.

In silico lead identification: Structural data files related to Fusicoccin, as a ligand for lead identification, C terminus of H^+-ATPase and 14-3-3 regulatory protein are available through online data banks (1o9F.pdb file). The functional part of the ligand, Fusicoccin that comes into interactions between C end of ATPase and binding moiety of 14-3-3 protein was introduced in the study of Martin Wurtele[12]. To identify candidate therapeutics, we performed computational screening of a virtual 3D database of drug-like molecules. Also, we tested computationally selected small molecules, which resemble the functional part of the ligand performing ligand docking or geometry optimization, using Argus Lab application (4.0.1). Several small molecules, as lead compounds, were identified; however, the two molecules that showed higher ligation scores and were commercially available were selected and purchased.

Antifungal bioassay: Standard strains of *Candida albicans* (ATCC10231), as a pathogen yeast, and *Saccharomyces cerevisiae* (PTCC 5052), as a nonpathogenic yeast, were applied to evaluate antifungal potential of the two identified lead compounds using micro-well plate dilution assay based upon the NCCLS reference method. The two lead compounds included 4-phenyl butyric acid (PBA, Merck: 820986) and 3-Cyclohexan propionic acid (CXP, Merck: 818673). Malt Extract Broth (Oxoid) was used to subculture the yeasts and for serial dilutions. DMSO was used as solvent for dissolving the compounds. Minimum inhibitory concentrations (MIC) were carried out by using serial dilution technique in 96 well microtiter plates. The yeasts were subcultured in MEB media at 35°C for 24 h. Prior to inoculation, the yeast strains were adjusted to 0.5 McFarland standards turbidity and diluted 1:1000 in MEB. The two lead compounds were first diluted in DMSO to the highest concentration of 10 mg/ml. Moreover, the concentration of 3.2 mg/ml of the antifungal agent amphotericin B (Sigma, Germany) in dimethyl sulfoxide was utilized as medium positive control.

Two-fold serial dilutions of the leads were produced in the eight consecutive wells. One hundred µl of yeast suspensions were added to 100 µl of diluted compound for all wells. The plates were incubated at 30°C for 48 h and assessed by a light microscope. All the tests were done in broth and repeated three times in separate testing. Addition of culture media and inoculum to the wells of these plates diluted the final tested compounds and amphotericin B concentrations at 1000 - 1.9 and 32 - 0.06 µg/ml, respectively. Microdilution trays were thereafter incubated at 35°C and examined after 24-48 hr to determine MIC (Minimum inhibitory concentration) and MFC (Minimum fungicidal concentration) values. It is worth noting that the MIC was defined as the lowest concentration of the compounds to inhibit the growth of yeast. Minimum

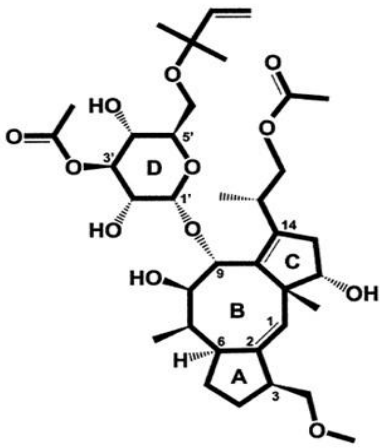


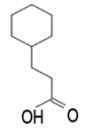
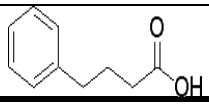
Figure 1. Fusicoccin structure

fungicidal concentration (MFC) was determined by plating samples from clear wells onto Malt Extract Agar. More to the point, MFC was defined as the lowest concentration yielding negative subculture.

Results

The best ligation scores obtained from ArgusLab pertained to Cyclohexan propionic acid (-11.810379 kcal/mol) and 4-phenyl butyric acid (-14.641133 kcal/mol). The more characteristics of the two lead compounds identified through in silico screening methods are illustrated in table 1. Antifungal activities of the two compounds were compared with Miconazole and are shown in table 2. Our results showed that *C. albicans* (ATCC10231) and *S. cerevisiae* (PTCC 5052) isolates were partly susceptible to these compounds. Two lead compounds, cyclohexane propionic acid and 4-phenyl butyric acid, exerted antifungal properties in low concentrations. This makes these molecules suitable candidates for further optimization. The MIC and MFC obtained for the two compounds are depicted in table 2.

Table 1: Characteristics of the two identified lead compounds

Chemical Structure	Molecular Formula	Name & Cat. No.
	C9H16O2	3-Cyclohexan propionic acid (CXP) Merck: 818673 Drug bank ID: DB02242 PDB entry: 2ay2 CAS Number: 701-97-3
	$C_{10}H_{12}O_2$	4-phenyl butyric acid (PBA) Merck: 820986 CAS Number: 1821-12-1

The scores calculated by the Argus Lab application for the compounds were as follows:
Cyclohexan propionic acid: -11.810379 kcal/mol
4-phenyl butyric acid: -14.641133 kcal/mol

Table 2: MIC (µg/ml) and MFC (µg/ml) calculated for the two leads

	S. cerevisiae		Candida albicans	
Drug	**MIC***	**MFC***	**MIC***	**MFC***
CXP	50	150	100	200
PBA	100	200	100	250
Miconazole	1.5	1.5	2	2.5

* Results obtained from 48 h broth cultures
Highly-susceptible: MICs < 8 µg/ml
Susceptible: 8< MICs <100 µg/ml
Semi-susceptible: MICs 100< MICs< 1000 µg/ml

Discussion

Fungi are important agent for human disease. The increasing incidence of opportunistic fungal infections and the limited number of existing antifungal drugs and the emergence of drug resistance in fungal pathogens make serious health problems owing to an augmented incidence of treatment failures. Yeast species, which are the most crucial fungal pathogens, belong to the genus Candida. These species can cause a wide range of human diseases, namely life-threatening invasive infections in immunocompromised individuals. *Candida albicans* exists as a normal flora on the mucus membranes and in the intestinal tract of some healthy individuals; nevertheless, it is frequently found in certain patients and may result in serious opportunistic infections. Furthermore, *Saccharomyces cerevisiae* is nonpathogenic yeast. Both species of *candida albicans* and *Saccharomyces cerevisiae* have membrane-bound H^+-ATPases highly similar to the plant H^+-ATPase[16].

Plasma membrane H^+-ATPases produce a chemiosmotic H^+-gradient across the plant plasma membrane. This

gradient is used to drive solute uptake through H^+-coupled transporters. The proton gradient generated by the pump energizes many important transport systems of the cells[17]. Yeast plasma membrane ATPase is fundamental for growth [18]. Therefore, any amino acid replacements that fail functionality of yeast H^+-ATPases are associated with lethality [7]. Plant and animal cells use entirely different ion pumps. For instance, plants use an H^+-ATPase in lieu of a Na^+/K^+-ATPase to energize the plasma membrane with an electrochemical gradient[17]. In spite of animal cells using Na^+ ions, protons are exclusively used as the coupling ion in plant cells[5]. It was demonstrated that the amino acid sequence of the plant H^+-ATPase is more closely related to fungi and protozoan H^+-ATPase than bacterial K^+-ATPases or animal (Na^+/K^+) , (H^+/K^+)-ATPases[19]. The necessity of membrane H^+-ATPase for yeast cell growth and distinction of the pump between yeast and animal cells makes the molecule a promising target for drug design. Computer modeling has recently been used to discover and design new pharmaceuticals. A number of easy accessible species specific genomic, proteomic and metabolomics database have been established to offer novel and clinically important targets for screening. For lead identification, structural and functional information concerning target molecule and the nature of its interaction with known ligand is used. Virtual screening adopts an alternative approach and applies computer-based methods to screen large chemical libraries targeted on a biological receptor. Such a task is greatly facilitated by the advent of high performance computing environments, data management software and the internet to take the advantage of delivering new drug candidates more quickly and with lower costs[20,21]. The major roles of computation in drug discovery are virtual screening and de novo design, evaluation of drug likeness, and advanced methods for determining protein-ligand binding[22,23]. In the current study, two lead compounds were identified and used for targeting the pumps. Our findings indicate that stable activation of the membrane H+-ATPase by the compounds in candida albicans and *Saccharomyces cerevisiae* is accompanied by the cell death. The results from the present study indicate that the two identified lead compounds, cyclohexane propionic acid and 4-phenyl butyric acid, act potentially against yeast cells and demonstrate proper potentials for further optimization to fulfill the needs for safe and effective drug design.

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