Chapter 26
Plasma Membrane ATPase: Potential Target for Antifungal Drug Therapy

Nikhat Manzoor

Abstract Fungal plasma membrane H^+-ATPase (PM-ATPase) is crucial to cell physiology as it maintains an electrochemical proton gradient across cell membranes required for the uptake of nutrients. It regulates intracellular pH and dimorphic transition that is directly linked with growth and pathogenicity of the fungus. Opportunistic fungal pathogens, mainly *Candida* spp., lead to complications in HIV-infected and other immunocompromised patients. Due to the eukaryotic nature of fungal cells, it is difficult to identify unique antifungal targets not shared with human hosts. Also the currently available drugs have low efficacy and high toxicity and frequently lead to drug resistance. They have undesirable side effects and are ineffective against reemerging fungi. Treatment options for invasive infections are limited and almost always involve the use of nephrotoxic amphotericin B and azoles, which lead to resistance on prolonged use probably due to their fungistatic nature. There is thus a critical need to develop more effective therapies to deal with such infections, and natural products offer a safer alternative. PM-ATPase is unique and crucial to fungal cells and hence is a promising antifungal target. It will help in the development of new mechanism-based drugs. Intracellular pH and glucose-induced H^+ efflux, consequences of PM-ATPase activity, are inhibited by natural compounds to the same extent in both susceptible and resistant *Candida* strains. Several plant essential oil constituents inhibit PM-ATPase activity significantly and hence may be considered as good candidates for designing specific surface active antifungal drugs that target the PM-ATPase and will ultimately help in curbing drug resistance in pathogenic fungi.

Keywords PM-ATPase • *Candida* • Pathogenicity • Reaction kinetics • Natural compounds • Antifungal target

N. Manzoor (✉)
College of Applied Medical Sciences, Taibah University,
Al-Madinah Al-Munnawara, Kingdom of Saudi Arabia

Medical Mycology Lab, Department of Biosciences, Jamia Millia Islamia, New Delhi, India
e-mail: nikhatmanzoor@yahoo.co.in

1 Introduction

Fungal infections have increased tremendously in the past few decades, especially in immunocompromised patients [1, 2] contributing to morbidity and mortality, the main reason being an increase in antimicrobial resistance and limited number of antifungal drugs. Also, most of these conventional drugs have undesirable side effects. *Candida* species are major human fungal pathogens that cause both superficial and systemic infections [1]. *Candida albicans*, the fourth most common cause of hospital-acquired infections, is believed to be an obligate associate of warm-blooded animals. Normally present as a harmless asymptomatic commensal, it can manifest as a pathogen due to its capacity to induce germ tube formation. Besides morphological transition, there are several other virulence factors like adhesion, biofilm formation, and invasion of host responses [3].

The fungal cell membrane possesses an H^+-ATPase (PM-ATPase) that plays a critical role in fungal cell growth and physiology. It is essential for maintaining a proton electrochemical gradient necessary for the secondary transport of nutrients. This membrane protein is also associated with regulation of intracellular pH (pHi 7.0) and dimorphism and pathogenicity of the fungus. Several studies have shown that the inhibition of enzyme activity correlates with cessation of cell growth [4–10] and hence endorse PM-ATPase to be a desirable molecular target for antifungal drug therapy [11, 12].

Several important antifungal drugs in clinical use today are fungistatic. These drugs prevent additional growth of cells but have little effect on existing cell populations. Thus, the immune system is required to clear an infection which is not possible in the case of severely immunocompromised individuals, and large cell populations often remain as potential sources of new infection. It is desirable that antifungal agents be fungicidal and be able to kill existing cells. The PM-ATPase is an essential enzyme that is needed for both new growth and stable cell maintenance in the absence of growth. Due to its slow turnover in the membrane, it is likely that specific inhibitors of this membrane protein will be fungicidal [13].

Medicinal plants and bioactive natural products offer an unlimited source of unique molecules that serve as an unparalleled source of therapeutic agents to treat infectious diseases. Interestingly, around 80 % of all available clinically used antibiotics are directly or indirectly derived from natural products [14]. Several of these molecules have shown binding affinity to other related ATPases [15–17]. There are only a few antifungal drugs available largely due to the eukaryotic nature of fungal cells and hence the difficulty in identifying unique antifungal targets not shared with human hosts. PM-ATPase is unique and crucial to fungal cells and hence is a promising antifungal target. It will help in the development of new mechanism-based drugs, and natural compounds that are able to target this crucial protein will serve a good purpose for the same.

2 PM-ATPase: Structure and Function

The fungal PM-ATPase is a 100 kD single polypeptide constituting ~25 % of the total plasma membrane protein. Like all P-type ATPases, it has both N- and C-terminal domains in the cytoplasm and traverses the lipid bilayer 8–10 times.

This proton pump shares only ~30 % of its sequence identity with P-type ATPase family members from animal cells, while they show a high degree of sequence similarity among diverse fungal PMA gene [18]. The enzyme appears highly asymmetric with nearly 70 % of the mass exposed to the cytoplasm and only ~5 % exposed to the extra-cytoplasmic compartment. The considerable structural conservation within the P-type ATPases occurs most strongly in a set of six sequences found in the cytoplasmic catalytic domains [19] located within the putative transduction and kinase domains which contain the ATP-binding site and site of phosphorylation. The secondary structure and its interaction with the lipid bilayer are strongly conserved. A notable structural difference between the various P-type ATPases with differing cation specificity is the sets of relatively short extracellular turns (~4–20 amino acid residues) [19] that are expected to provide extracellular recognition elements that could help target drugs to cell surface. Therefore, an understanding of the functional properties of the transmembrane loops and the accessibility of their extracellular turns may be invaluable for developing specific targets among the P-type ATPases.

The fungal PM-ATPase is a high-capacity proton pump that plays a critical role in fungal cell physiology by helping to regulate intracellular pH [20, 21] and maintain transmembrane electrochemical proton gradients necessary for nutrient uptake [22, 23]. The gene encoding this enzyme, PMA1, has been cloned from diverse fungi and has been shown to be highly conserved [18]. PM-ATPase plays important roles in the maintenance of cell homeostasis by regulating intracellular pH. This mechanism creates a membrane potential and an electrochemical gradient that allows the uptake of ions and nutrients required for cellular physiology [24–27]. The PM-ATPase is one of the few antifungal targets that have been demonstrated to be essential by gene disruption [28]. In addition to its role in cell growth, it has been implicated in fungal pathogenicity through its effects on dimorphism, nutrient uptake, and medium acidification.

The regulation of PM-ATPase is a complex process that operates at several levels. Apart from transcriptional regulation [29], enzyme activity is autoregulated through the generation of membrane potential [30] and intracellular Ca^{2+} metabolism. The enzyme contains a carboxyl-terminal regulatory domain that includes a phosphorylation site for a calmodulin-dependent multiprotein kinase. This domain governs the response of the enzyme-to-glucose metabolism/starvation. Mutations in the carboxyl-terminal phosphorylation site can retard or even stop fungal cell growth. The carboxyl-terminal domain is a negative regulator that is believed to interact with the ATP-binding region of the enzyme [31].

3   Kinetic Studies on Fungal PM-ATPase

The mechanism of PM-ATPase has been studied in *C. albicans* using fast reaction kinetics in a stopped flow spectrophotometer [32]. A distinct pre-steady-state phase of ATP hydrolysis was recorded on rapid mixing of ATP with ATPase. Around two protons per ATPase molecule were released, of which around ~1.3

were absorbed back. While the rapid mixing of inorganic phosphate and ATPase produced no transient pH changes, the mixing of ADP led to the release of one proton per ATPase molecule. The magnitudes of both proton release and absorption were found to be independent of ATP concentration. The rate of proton release showed ATP dependence, while the rate of proton absorption was independent of ATP concentration. The low rate of proton liberation with ADP in comparison to ATP indicates low affinity of the ATPase for ADP. No change in the difference spectrum was observed with ADP. The stoichiometry of ATP binding to PM-ATPase was found to be unity from UV-difference spectrum studies. The rates of proton release and appearance of a difference spectrum following the addition of ATP were found to be similar beyond an ATP: ATPase ratio of 1:1. The mechanism of ATP hydrolysis is summarized in a four-step kinetic scheme [32].

Similar kinetic studies were done in the presence of some nutrients (glucose, glutamic acid, proline, lysine, arginine) and two glucose analogs (2-deoxy D-GLUCOSE and xylose). In the presence of glucose, proton absorption to release ratio was exceptionally high (0.92) in comparison to other nutrients which was in the range 0.25–0.36 [33]. Although no UV difference spectrum was observed with ADP, mixing of ATP with ATPase led to a large conformational change. Exposure to different nutrients restricted the magnitude of the conformational change; the analogs of glucose were found to be ineffective. This suppression was maximal in the case of glucose (80 %), while with other nutrients, magnitude of suppression was in the range 40–50 %. The rate of H^+ absorption showed positive correlation with suppression of conformational change only in the case of glucose and no other nutrient/analog. Mode of interaction of glucose with PM-ATPase thus appeared to be strikingly distinct in comparison to other nutrients/analogs tested [33].

Mechanism of glucose stimulation of H^+ efflux by PM-ATPase remains a mystery despite extensive research [5, 32–36]. Studies on mechanism of ATP hydrolysis and H^+ efflux including its nutrient regulation by PM-ATPase are crucial for the design of mechanism-based drugs for this vital target. In a study conducted by the same group, the rate of proton efflux and its stimulation by nutrients/analogs was quantitated in cells and spheroplasts of *C. albicans* [5, 36]. Glucose showed a striking stimulation of 7.5-fold in the rate of H^+ efflux, while the rest of the nutrients/analogs were noneffective. Glucose stimulation was not observed much in the case of spheroplasts probably due to slow transport of nutrients and loss of interaction between PM-ATPase and other membrane proteins [5, 34]. Since intracellular ATP remains almost unused in both cells and spheroplasts, there is a possibility that glucose exposure leads to increase in intracellular ATP concentration due to its metabolism, but most of it is consumed by the highly active pump. Therefore, ATP does not appear to be the cause for glucose stimulation of PM-ATPase. A decreased intracellular ATP concentration on exposure to glucose is due to the high utilization of ATP by the stimulated ATPase [36].

4   Fungal PM-ATPase as a Potential Target for Antifungal Therapy

The development of an effective target remains a critical part of the drug discovery process, and fungal PM-ATPase is a highly desirable target for the development of novel antifungal therapeutics. It has well-defined properties that facilitate drug discovery. The enzyme is very crucial to fungal cell physiology, being required for the formation of a large electrochemical proton gradient and maintenance of intracellular pH. Complete inhibition of the proton pump will definitely be lethal; thus, an effective antagonist of the proton pump will be fungicidal, which is an important attribute for a drug being developed to treat opportunistic infections in severely immunocompromised patients. Well-characterized biochemical studies and genetics of this enzyme will facilitate detailed analysis of the interaction of model compounds with the enzyme [12]. Detailed genetic analysis suggests that modification of amino acids in the first two transmembrane segments can either enhance or diminish the drug sensitivity of the PM-ATPase, depending on the nature and location of the amino acid substitution. This region in mammalian P-type enzymes has been implicated in the interaction of cardiac glycosides and reversible gastric pump inhibitors [12]. Hence, it is suggested that this region in the PM-ATPase may be valuable as a potential interaction domain for antifungal agents. Also, a number of primary and secondary screens are available to identify compounds that target the PM-ATPase and affect one or more of its functional properties. These screens assess function of the enzyme and have already yielded promising PM-ATPase-directed antagonists [12]. Like other P-type ATPases, the N- and C-termini and the extracellular surface of the pump show the highest divergence; this contributes to its unique catalytic and regulatory features. PM-ATPase antagonists that are selective for the fungal proton pump over related animal cell ion pumps should display broad-spectrum activity on diverse fungal enzymes because of the high degree of primary sequence similarity found among these enzymes.

It has been observed that inhibition of PM-ATPase leads to intracellular acidification and cell death. Inhibition of cell growth and H$^+$ efflux by certain natural and synthetic compounds suggests that their antifungal properties are related to their inhibitory effects on PM-ATPase. It has been reported that PM-ATPase activity increases in both bud and germ tube forming populations after 135 min of fungal growth. This is the time at which morphological transition is initiated [37]. PM-ATPase regulates dimorphism in *C. albicans*. Its activity is regulated by a large number of environmental factors at both transcriptional and posttranslational levels. Diverse numbers of PM-ATPase genes have been cloned both in fungi and plants [31, 38]. There has been rapid progress on the molecular basis of reaction mechanism and regulation of the proton pump.

Studies with Omeprazole, a sulfhydryl-reactive compound, indicated that PM-ATPase can be inhibited from its extracellular membrane surface [4, 12] and that its inhibition is closely correlated with inhibition of fungal cell growth that is fungicidal [11]. Genetic studies also demonstrated that perturbations of extracellular

protein structure of the PM-ATPase can reduce the catalytic activity of the enzyme [39]. These results were also consistent with the known behavior of antagonists to the Na, K-ATPase and H, K-ATPase which have binding sites on the extracellular surface of the membrane.

The importance of PM-ATPase as an antifungal target has been demonstrated by several studies. *Cryptococcus neoformans* PM-ATPase is an established and viable target for antifungal drug discovery [13]. Ebselen, a nonspecific PM-ATPase antagonist, is also fungicidal and inhibits ATP hydrolysis and medium acidification by whole cells [40, 41]. NC1175, a novel thiol-blocking conjugated styryl ketone, exhibits activity against a wide spectrum of pathogenic fungi. NC1175 inhibited glucose-induced acidification of external medium by *Candida*, *Saccharomyces*, and *Aspergillus* species in a concentration-dependent manner. Vanadate-inhibited ATP hydrolysis by membrane fractions of *C. albicans* was completely inhibited by 50 μM NC1175, suggesting that one of the targets for NC1175 in these fungi may be PM-ATPase [42]. Studies have demonstrated that bafilomycin inhibits ATPase activity with high specificity and potency [43]. Although PMA1 is also present in the human transcriptome, several domains are exclusive to fungi, and gene deletion has been demonstrated to be lethal for some of those microorganisms. As ATPases are promising targets for the development of antimycotics, the differences between fungal and mammalian proteins need to be further investigated [44].

5   Natural Compounds as Potential Inhibitors of PM-ATPase

Natural products play an important role in drug discovery and development [45, 46]. More than 75 % of the drugs used in therapy for infectious diseases are of natural origin [47]. The fungal PM-ATPase is an ideal antifungal target as it is an essential enzyme not found in animals, and an important functional part of the protein is exposed to the cell exterior. Natural plant products have shown potential in having inhibitory effects on this antifungal target. Several of these natural products available may be effective pump inhibitors and should be sought for in plant extracts by screening for their effect on PM-ATPase activity. This enzyme when co-crystallized with identified inhibitors will provide information on inhibitor binding segments in the pump and offer a crucial foundation for the development of novel efficient and specific antifungals.

Plant-derived substances have recently become of great interest owing to their versatile applications [45, 48]. The initial stages of drug development include identification of active principles, meticulously designed biological assays, and dosage formulations, followed by clinical studies to establish safety, efficacy, and pharmacokinetic profile of the new drug [49]. Several studies have shown the antifungal potential of natural plant products and showed that the inhibition of the proton pump activity is correlated with termination of fungal cell growth. A direct relation between intracellular pH (pHi) and functioning of PM-ATPase has been established by several studies [6, 10, 50, 51].

The pathogenicity of *Candida albicans* is due to its capacity to induce germ tube formation [52]. These hyphae penetrate host tissues to extract nutrients required for cell growth. A wide range of biochemical factors have been implicated in a variety of dimorphic fungi as being central to the control of yeast dimorphism [53]. It is however possible to invoke mechanisms which incorporate changes in intracellular pH with the modulation of biochemical activities as a regulatory switch. A rapid but transient increase in pHi has been observed around the time of evagination of germ tubes, and the magnitude of increase in pHi of the population destined to form buds was more sensitive to orthovanadate, an inhibitor of the PM-ATPase [50]. Change in pHi, which has a direct relationship with PM-ATPase, is widely regarded as of crucial importance [6, 50].

High-energy phosphates play an important role in regulating fungal PM-ATPase activity. Phosphocreatine (PCr) is a phosphorylated creatine (Cr) molecule that serves as a rapidly mobilizable backup of high-energy phosphates in skeletal muscles and the brain. It has been shown to influence ATP-dependent enzymes [6] and is found in association with virtually all types of ATPases in vertebrate cells. PCr/Cr ratio in cells is a better reflection of energy status compared to ATP/ADP ratio [50, 52]. Studies report that *Candida* infections are held in check mostly by immunologic factors in healthy human hosts. A number of situations however expose the yeast to other cellular constituents. Our group investigated the effect of PCr on the rate of H⁺ extrusion, pHi, and dimorphism in *C. albicans* [6, 7]. H⁺ efflux by PM-ATPase of *C. albicans* and intracellular pH pattern of cells undergoing morphogenesis were profoundly affected by PCr at concentrations present in vertebrate tissues. In comparison to control cells, PCr-exposed cells showed only 10 % yeast to hyphal transition after 120 min at the same concentration range of 20–40 mM, while the number of hyphae producing cells was not more than 40 %. Exposure to PCr also decreased hyphal length to a large extent [6], and the magnitude of inhibition was comparable to vanadate, a potent inhibitor of PM-ATPase. This indicates that both of them may have binding sites on the ATPase and bring protein conformational changes in a similar manner. Cr alone has no effect on H⁺ extrusion. Since the structure of vanadate is analogous to the structure of phosphate (Fig. 26.1), it may be speculated that PCr may bind to the ATP-binding site via its phosphoryl group. When *Candida* cells were exposed to both vanadate and PCr together, a cumulative effect was produced. Both of them may be having more than one binding sites [7]. Similar studies showed that ATP synthesis and PM-ATPase activity were significantly affected by sodium nitroprusside (SNP), a nitric oxide (NO) donor. A decrease in ATP concentration was observed in SNP-treated cells, the decrease being more in the presence of sugars and amino acids. Hence, NO, not only inhibits mitochondrial electron transport chain but also alters PM-ATPase conformation resulting in a decrease in its activity [54, 55].

Plant essential oils (EOs) possess a broad spectrum of antimicrobial properties due to the presence of bioactive natural molecules. Although several studies demonstrate their antifungal potential [56–59], there are very few reports that clearly reveal their mode of action. The antifungal activity of EOs is basically credited to their ability to cross fungal cell walls and penetrate between fatty acyl chains of the lipid
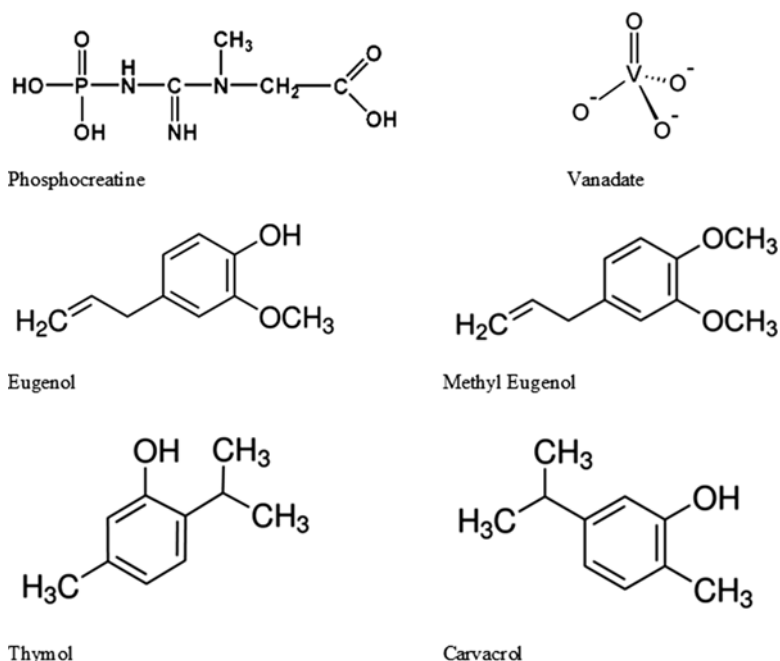
Fig. 26.1 The structure of some antifungal compounds discussed in this chapter that inhibit PM-ATPase activity

bilayer, altering membrane fluidity and permeability and damaging membrane proteins, leading to degradation of the cytoplasmic membrane and to cell death. Loss of cell homeostasis, leakage of cell contents, and lysis are the critical consequences of these induced alterations in membrane structure and function [56]. Most of the studies claim that the antifungal activity of these compounds is due to their ability to destroy the integrity of cell membranes, release cellular components, and drastically inhibit mycelial growth of fungal pathogens [59].

Excellent anti-*Candida* activity has been demonstrated by several studies [58, 60, 61]. PM-ATPase has been explored as a potential antifungal target for several of these natural products. Eugenol, methyl eugenol, thymol, and carvacrol are some of the natural compounds (Fig. 26.1) that showed inhibition of PM-ATPase activity to encouraging levels, i.e., up to 70 % inhibition in both sensitive and resistant *Candida* strains [51] (Table 26.1). Eugenol is a phenylpropanoid present in the essential oil of clove, cinnamon, nutmeg, basil, star anise, and dill. Methyl eugenol, methyl ether of this compound, is also present in various essential oils. Carvacrol and thymol are monoterpene phenols present as major constituents in the essential oils of *Origanum vulgare* and thyme, respectively.

Glucose-induced acidification of the extracellular medium by yeast cells is a convenient measure of PM-ATPase-mediated H^+ pumping [62]. Table 26.1 gives the average percentage inhibition of PM-ATPase-mediated H^+ efflux by *Candida*

Table 26.1 Percentage inhibition of glucose-induced H⁺ efflux by *Candida* spp. at pH 7.0 in the presence of some natural compounds at their respective MICs

Compounds	Percentage inhibition of H⁺ efflux w.r.t. control		
	Standard	Clinical	Resistant
Phosphocreatine (25 mM)	83	–	–
Eugenol (500 μg/ml)	44	46	39
Methyl eugenol (350 μg/ml)	49	42	43
Thymol (100 μg/ml)	60	54	46
Carvacrol (50 μg/ml)	69	61	67
Fluconazole (5 μg/ml)	24	19	25
Amphotericin B (2 μg/ml)	16	16	17
Vanadate (5 mM)	100	91	96

Cells were suspended in 0.1 mM $CaCl_2$ and 0.1 M KCl at 25 °C along with 5 mM glucose
– = not evaluated

species in the presence of some natural compounds at their respective MIC values. The rate of proton extrusion by *Candida* cells was calculated in nmoles min/mg yeast cells by titrating the cell suspension with 0.01 N NaOH [7]. Eugenol, methyl eugenol, thymol, and carvacrol (Fig. 26.1) showed the most significant inhibition of more than 50 % in both clinical and resistant *Candida* stains. Moreover, H⁺ extrusion in every case was inhibited by 91–100 % in the presence of orthovanadate (5 mM), a specific inhibitor of H⁺-ATPase, whereas neither fluconazole nor amphotericin B had any significant effect on the acidification of the extracellular medium (Table 26.1). The intracellular pH (pHi) of yeast cells is maintained between 6.0 and 7.5, and any change in pHi is regarded as of crucial importance as it has a direct relationship with PM-ATPase [6, 50]. A significant decrease in pHi was observed in treated *Candida* cells. In comparison to the control cells (untreated), the decrease in pHi in cells was in the order given: Control > EUG > MEUG > THY > CARV [51].

On exposing *Candida* cells for a short duration, the effect of these bioactive compounds was rapid, irreversible, and lethal which suggests the presence of a cellular target that is accessible to the compounds externally. Since PM-ATPase is present in plasma membranes of pathogenic fungi, there is a possibility that these compounds bind to it externally. Significant inhibition of PM-ATPase-mediated proton pumping activity at MIC values of bioactive compounds suggests that these compounds can be considered as potential ATPase inhibitors. The fact that they have low MIC values and negligible toxicity in comparison to conventional drugs makes them even better candidates. It has been reported that vanadate inhibits H⁺ efflux in *Candida* cells by 91–100 %, while conventional antifungal drugs like FLC and AmB had no significant effect on the PM-ATPase activity [51]. These antifungal drugs are known to interact with the sterol components of the membrane, [63] and there are no reports of their interaction with the proton pumps. Besides these commercially available antifungals have low efficacy and high toxicity and frequently lead to drug resistance. There is thus a critical need to develop more effective therapies to deal with such infection, and natural compounds offer a safer alternative.

6 Conclusion

Fungal infections occur as a result of a complex interaction between the host, pathogen, and the environment. Antibiotics have helped in the treatment of infections to a great extent, but their indiscriminate use has led to the development of drug-resistant pathogens. The emergence of azole resistance in *Candida albicans* and other *Candida* species is a huge crisis today. The antimicrobial activity of plant essential oils and their components is well established against a wide range of microorganisms. Plants and plant products can assist in confronting the issue of infection and provide a better understanding of mechanisms for the designing and development of novel and more effective antimicrobial agents. The discovery of new antifungal therapeutic agents based on natural compounds as scaffolds for molecular targets will help in the management and treatment of fungal and other microbial infections. Fungicidal natural compounds having low MIC values and negligible cytotoxicity have a profound effect on PM-ATPase of *Candida* and other fungal species, suggesting that the PM-ATPase can be explored as a potential surface active antifungal target for these and other potential drugs.

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