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ROYAL SOCIETY OF CHEMISTRY

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View Article Online
DOI: 10.1039/C6RA05883C

Journal Name

ARTICLE

Alkylated histidine based short cationic antifungal peptides: synthesis, biological evaluation and mechanistic investigations

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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Current clinically used antifungal agents suffer from several drawbacks that have urgently necessitated the development of new antifungal agents with unusual mechanisms of action. In this context, antifungal peptides (AFPs) open up new perspectives in drug design by providing an entire range of highly selective and nontoxic pharmaceuticals. Here, we report the development of novel short AFPs with the synthesis of two series of tripeptide based compounds named as His(2-alkyl)-Arg-Lys (Series I) and His(2-alkyl)-Arg-Arg (Series II). The series II peptides were found to be selectively active against *Cryptococcus neoformans* whereas some peptides displayed encouraging activities against other fungal strains such as *Candida albicans, Candida kyfer, Aspergillus niger and Neurospora crassa*. The cytotoxic experiments were performed on active compounds using Hek-293 and HeLa cells which exhibited negligible cytotoxic effect up to the highest test concentration. Further, the most potent peptide was subjected to mechanistic studies using TEM analysis. Two sets of SUVs mimicking microbial membrane and mammalian membrane were treated with the most potent peptide. The results of this study were found to be perfectly in corroboration with the antifungal activity in relation to the differences between microbial and mammalian cell membrane composition, thereby, indicating that the reported peptides may also be less susceptible to the common mechanisms of drug resistance.

Introduction

Over the past few decades, systemic fungal infections have emerged as a major cause of morbidity and mortality in immunocompromised individuals such as organ recipients, cancer patients, and human immunodeficiency virus (HIV)-infected patients.[1-3] Candida species, Aspergillus species and *Cryptococcus neoformans (C. neoformans)* have been found to be the most common aetiological agents of fungal infections among various fungal species.[4] In recent years, even though new antifungal agents including third-generation broader-spectrum azoles, echinocandins (acting on fungal cell wall) and lipid formulations of Amphotericin B have emerged, invasive fungal infections continue to be associated with high mortality rates.[5] On top of it, earlier reports have suggested that approximately 17% of Candida isolates have become resistance to azoles.[6] Moreover, many clinically used antifungal agents suffer with drawbacks ranging from high toxicity, resistance development during therapy, intrinsic resistance to antifungal treatment, non-optimal pharmacokinetics, poor solubility, efficacy, high cost and their frequent use that has led to the emergence of resistant strains.[7,8] Thus, it has urgently necessitated the development of new antifungal agents with unusual mechanisms of action. The demand for novel antifungal agents belonging to wide range of structural classes and acting selectively on novel targets with fewer side effects is very much the need of the present anti-infective search. In the search of more effective chemotherapeutic approaches for treating invasive fungal infections, antimicrobial peptides (AMPs) open up new perspectives in drug design by potentially providing an entire range of highly selective and nontoxic pharmaceuticals. In past few decades, a number of naturally occurring and synthetic peptides have been discovered, out of which some have entered into clinical trials with majority of these peptides displaying anti-bacterial activity.[9] The selectivity of AMPs towards microbes is mainly due to the fundamental differences between microbial and mammalian cells. As far as anti-fungal activity is concerned, the presence of ergosterol in fungal cell membrane differentiates them from the mammalian cells and acts as a potential target for AMP.[10] Apart from membrane composition, other structural features such as fungal cell wall, trans-membrane potential and

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

membrane polarization play a crucial role in imparting selectivity.[11]

The classification of anti-fungal peptides (AFPs) can be made on the basis of their mode of action and other parameters like amino acid sequence and their characteristic features. One group of peptides which is positively charged and having hydrophobic regions, acts by binding to the membrane surface and disrupting it without traversing the membrane.[12] Another class of amphiphilic peptides forms pores in membrane allowing the passage of ions and other solutes by aggregating in a selective manner.[13] Naturally occurring peptides have been generally found to exhibit broad spectrum of antimicrobial action whereas the ones with exclusive antifungal properties are less ubiquitous.[14] The evolutionary reason for such an existence is that the nature requires formation of peptides that could protect all forms of life from a variety of infectious pathogens. Therefore, a vast majority of small natural peptides are antimicrobial in nature thereby targeting bacteria, fungi and viruses.[15] However, few peptides also exist in nature that exhibit exclusive antifungal activity.[16] Therapeutically, peptides specific for fungi have a potential advantage over peptides having broad antimicrobial activity. The AFPs also differ in terms of mechanism of action as these act on specific targets that may be intracellular or on the cell membrane/cell wall whereas broad-spectrum peptides generally induce membrane lysis. AFPs can be employed in both transgenic plant and human pharmaceutical applications. Moreover, it has been widely reported that many short AMPs consisting of Lys, Arg and Trp exhibit antifungal activity.[17,18] Recent investigations have reported the antifungal effects of small KW and RW series of peptides against plant pathogens such as *Fusarium solani* and *Fusarium oxysporum*.[19] Similarly, the synthesis along with biological evaluation and conformational study of a new series of short peptides as antifungal agents have been recently reported in which conformational analysis using molecular electrostatic potentials (MEPs) have been performed.[20] Specifically, three peptides containing 9-11 amino acids were synthesized and tested on the basis of the theoretical predictions. These peptides exhibited significant antifungal activity with MIC value of 25 μM against human pathogenic strains including *Candida albicans (C. albicans)* and *C. neoformans*.

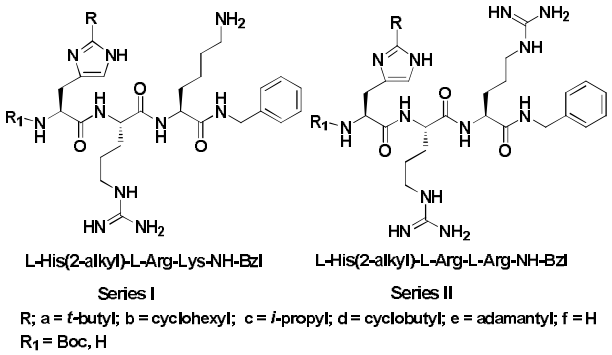
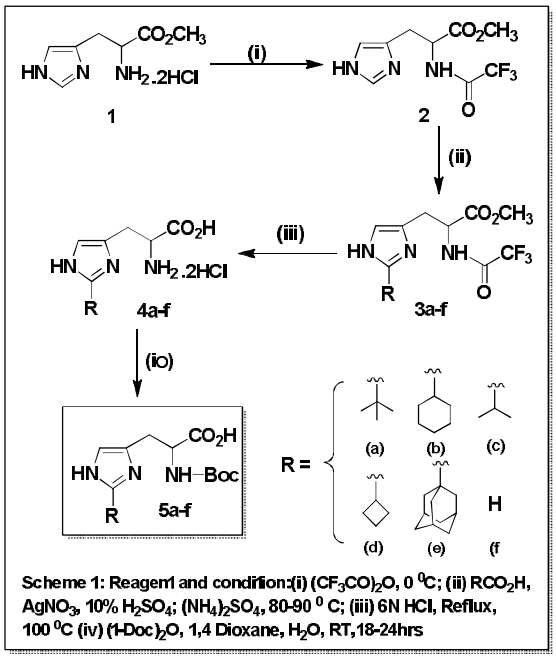


Fig 1. General structure of the compounds belonging to series I and II

Further, a series of tripeptides has been synthesized and screened for antifungal activity against *Candida* strains.[21] In particular, the tripeptide FAR displayed low anti-fungal activity with minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of 171.25 μg/ml and 685 μg/ml, respectively against *Candida krusei*. These peptides were found to be non-toxic to human cells with well acceptable therapeutic index. The SEM analysis of these peptides exhibited effective disruption of cell wall and bleb-like surface changes upon their application. On careful observation of sequence and mechanism of a number of AFPs as reported in literature, it can be safely stated that these peptides have a number of amino acids in common with repeat of a specific sequence in some peptides.[14,22,23] In general, these peptides contain cationic amino acids such as arginine, lysine, and histidine in their sequences; while at the same time, hydrophobic amino acids like tryptophan, phenylalanine, leucine, and isoleucine are also abundantly present. Also, it is quite evident that charge to bulk ratio plays a crucial role in antifungal activity.

Taking cue from the above stated observations, we report the development of short AFPs with focus on enhanced activity and potency. In this regard, we earlier reported the synthesis of two series of dipeptide based compounds labeled as Trp-His and His-Arg classes of AMPs exhibiting potential antimicrobial activities.[24] Basically, the histidine residue was substituted with bulkier alkyl groups like *i*-propyl, *t*-butyl, cyclobutyl, cyclohexyl, and adamantan-1-yl at the second position of the imidazole to evaluate the effect of magnitude and relative position of positively ionizable (PI) and hydrophobic (HYD) features on antimicrobial activity. The synthesized dipeptides were evaluated for antimicrobial activities against different bacterial and fungal strains resulting in encouraging activities. Further, to study the effect of increased cationicity on antifungal activity, we propose to insert an extra PI feature in form of the cationic amino acids lysine and arginine in the reported His-Arg classes of peptides thereby proposing two new series of compounds as His-Arg-Lys (Series I) and His-Arg-Arg (Series II) in the present study (**Fig. 1**). These structural



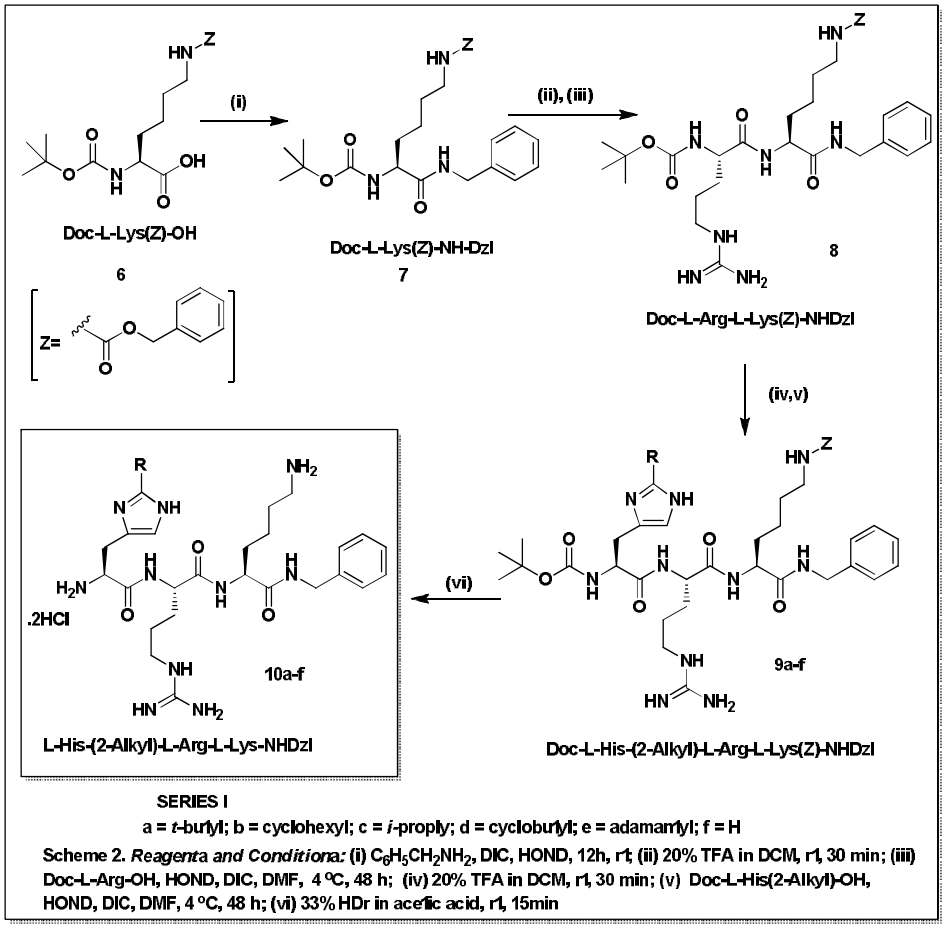
manipulations have the potential to vary the charge to bulk ratio and thereby may have a profound effect on activity of the compounds. Moreover, such a study can draw importance of relative position of cationicity and hydrophobicity needed for small tripeptide based compounds for being effective antimicrobial agents.

Results and discussion

Chemistry

The chemical synthetic procedure adopted to synthesize proposed peptides, having both natural and unnatural amino acid residues, has been described (Schemes 1-3). [25-29] Firstly, the synthesis of *N*-αBoc-2-alkyl-L-histidines (**5a-f**) was initiated by protection of amino group of L-His-OMe dihydrochloride (**1**) using trifluroacetic anhydride (Scheme 1). The resulting compound (**2**) was made to undergo regiospecfic alkylation reaction at C-2 position of the imidazole ring of histidine in a Minisci like reaction,[25-27] which follows free radical mechanism.[28,29] The key step leading to direct C-2 alkylation in this reaction involves nucleophilic addition of an alkyl radical, which is generated *in situ* from silver catalyzed oxidative decarboxylation of alkylcarboxylic acid using ammonium persulfate, to protonated imidazole ring followed by rearomatization. The method was found to be applicable for variety of substituent as the direct introduction of isopropyl, *tert*-butyl, cyclobutyl, cyclohexyl and adamantan-1-yl was made possible onto the imidazole ring of histidine. Further, the trifluroacetyl protected L-His(2-alkyl)-OMe (**3a-f**) was subjected to acidolysis for the deprotection of methyl ester and trifluroacetyl group in one pot reaction to obtain the corresponding L-His(2-alkyl)-OH.2HCl (**4a-f**). Finally, the amino group of compounds **4a-f** was protected with *tert*-Boc group to obtain the desired *N*-*α*-Boc-L-His(2-alkyl)-OH (**5a-f**).

For the synthesis of peptides belonging to the series I, commercially available Boc-L-Lys(Z)-OH (**6**) was firstly coupled with benzylamine in the presence of *N*-hydroxy-5-norborene-*endo*-2,3-dicarboxamide (HONB), 1,3-diisopropylcarbodiimide (DIC) using dimethyl formamide (DMF) as solvent to afford Boc-L-Lys(Z)-NHBzl (**7**) (Scheme 2). The compound **7** was then subjected to acidolysis to obtain L-Lys(Z)-NHBzl in salt form which was neutralized with methanolic ammonia followed by coupling with Boc-L-Arg-OH to obtain Boc-L-Arg-L-Lys(Z)-NHBzl (**8**). Further, the deprotection of Boc group was accomplished followed by coupling with Boc-L-His(2-alkyl)-OH (**5a-f**) to afford Boc-L-His(2-alkyl)-L-Arg-Lys(Z)-NHBzl **(9a-f)**. Finally, the Boc group and orthogonal protections were removed using 33% HBr in acetic acid to afford the desired peptides L-His(2-alkyl)-L-Arg-Lys-NHBzl (**10a-f**). Similarly, the synthesis of peptides belonging to series II was initiated with the benzyaltion of Boc-L-Arg-OH (**11**) to obtain Boc-L-Arg-NHBzl (**12**). After Boc group removal, it was coupled with Boc-Arg-OH to form dipeptide Boc-L-Arg-Arg-NHBzl (**13**) (Scheme 3). After removal of Boc group and neutralization step, compound **13** was further



SERIES I

a = *t*-butyl; b = cyclohexyl; c = *i*-proply; d = cyclobutyl; e = adamantyl; f = H

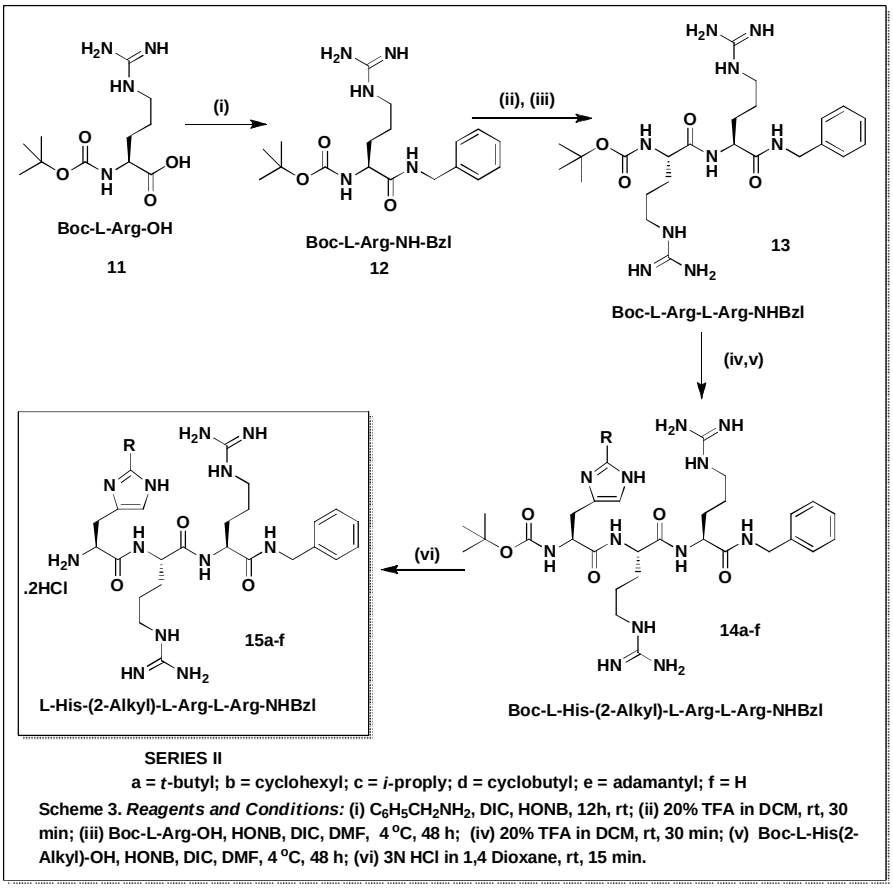
Scheme 2. *Reagents and Conditions:* (i) $C_6H_5CH_2NH_2$, DIC, HOND, 12h, rt; (ii) 20% TFA in DCM, rt, 30 min; (iii) Doc-L-Arg-OH, HOND, DIC, DMF, 4 °C, 48 h; (iv) 20% TFA in DCM, rt, 30 min; (v) Doc-L-His(2-Alkyl)-OH, HOND, DIC, DMF, 4 °C, 48 h; (vi) 33% HDr in acetic acid, rt, 15min

coupled with Boc-L-His(2-alkyl)-OH (**5a-f**) to obtain desired Boc protected tripeptides Boc-L-His(2-alkyl)-Arg-Arg-NHBzl (**14a-f**). Finally, the removal of Boc group was carried out to obtain desired tripeptides L-His(2-alkyl)-Arg-Arg-NHBzl (**15a-f**).

Antifungal activity

The synthesized peptides belonging to both series were evaluated for *in vitro* activity against variety of fungal strains such as *C. albicians*, *Candida kyfer (C. kyfer)*, *Aspergillus niger (A. nigar)*, *Neurospora crassa (N. crassa)* and *C. neoformans* and the results have been summarized in Table 1. Additionally, in order to have a better understanding of the structure-activity relationship with respect to biological activity, Boc-protected intermediate peptides were also evaluated (**9a-f**; **14a-f**) for their antifungal activity. Antifungal assay of all synthesized compounds was replicated three times and standard deviation values have been mentioned separately (Table S1).

In general, it was observed that the synthesized tripeptides were more active against *C. neoformans* than other fungal strains irrespective of whether their N-terminal was protected or not. This result was consistent with the already reported tripeptides of similar nature but different structure which also exhibited selective activity against *C. neoformans.*[30,31] In particular, the compounds belonging to series II [His(2-alkyl)-Arg-Arg] were found to more active than the series I [(His(2-alkyl)-Arg-Lys] compounds (Table 1). This observation led to the inference that the additional positively ionisable feature in the form of guanidinium group provided by arginine in series II is essential for antifungal activity especially against *C. neoformans*. Specifically, in series II, peptide **15b** possessing bulkier cyclohexyl group at the C-2 position of the imidazole ring of histidine was found to be the most potent against *C. neoformans* with MIC value of 1.25 µg/mL (Table 1). On the other hand, the peptides **14c**, **14d** and **15a** containing *i*-propylcyclobutyl and *t*-butyl substituent, respectively at the C-2 position of the imidazole ring also showed potent activity with MIC value of 10.9, 11.1 and 9.6 µg/mL against *C. neoformans* respectively. The above mentioned peptides were, however, not active against other tested fungal strains but the peptide **14b** (R=cyclohexyl; R_1=Boc) was found to be more active against *C. albicans* than *C. neoformans* with observed MIC value of 18.5 µg/mL against the former and 46.2 µg/mL against the latter. Similarly, peptide **14e** (R=adamantyl; R_1=Boc) showed activity against variety of fungal strains such as *N. crassa*, *C. neoformans*, *C. albicans* and *A. niger* with MIC values of 19.8 µg/mL, 24.7 µg/mL, 98.9 µg/mL and 98.9 µg/mL respectively. Against *C. neoformans*, other synthesized analogs such as peptides **14a** (R=*t*-butyl; R_1=Boc) and **15f** (R=H; R_1=H) displayed MIC value of 44.6 and 34.8 µg/mL respectively whereas as peptides **14f** (R=H; R_1=Boc), **15c** (R=i-propyl; R_1=H), **15d** (R=cyclobutyl; R_1=H) and **15e** (R=adamantyl; R_1=H) showed modest activity with MIC of 82.1, 74.8 and 76.4 µg/mL (Table 2). Peptide **15e**, in particular, exhibited modest activity with MIC of 86.3 µg/mL against four tested fungal strains namely *C. albicans*, *C. kyfer*, *C. neoformans*, and *A. niger*.



SERIES II

a = *t*-butyl; b = cyclohexyl; c = *i*-proply; d = cyclobutyl; e = adamantyl; f = H

Scheme 3. ***Reagents and Conditions:*** **(i) $C_6H_5CH_2NH_2$, DIC, HONB, 12h, rt; (ii) 20% TFA in DCM, rt, 30 min; (iii) Boc-L-Arg-OH, HONB, DIC, DMF, 4 °C, 48 h; (iv) 20% TFA in DCM, rt, 30 min; (v) Boc-L-His(2-Alkyl)-OH, HONB, DIC, DMF, 4 °C, 48 h; (vi) 3N HCl in 1,4 Dioxane, rt, 15 min.**

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As stated earlier, the series I peptides [(His(2-alkyl)-Arg-Lys] were relatively inactive against the tested fungal strains. However peptide **10d** (R=cyclobutyl; R_1=H) exhibited significant activity against *C. neoformans* with MIC value of 18.2 µg/mL whereas peptide with **9e** (R=adamantyl; R_1=Boc), with bulkier substituents at C-2 position of imidazole ring and N-terminus, exhibited activity with MIC value of 112.2 µg/mL against *C. neoformans* and *N. crassa*. In general it was observed that series I tripeptides were less active against *C. albicans* in comparison to the earlier reported dipeptide series [(His(2-alkyl)-Arg)].[32] This observation indicates that the addition of an extra positively ionisable feature in form of amino group provided by lysine in this particular series of compounds is not helping in augmenting the antifungal activity especially against *C. albicans*. However, this inference may not be possible to apply to other fungal strains as the earlier

Table 1. Antifungal activity of synthesized tripeptides (series I and II)

No.	R[a]	R_1[a]	*C. albicans* MIC[b]	*C. kyfer* MIC	*C. neoformans* MIC	*A. niger* MIC	*N. crassa* MIC
			(Series I)				
9a	t-butyl	Boc	>250	>250	>250	>250	>250
9b	Cyclohexyl	Boc	>250	>250	>250	>250	>250
9c	i-propyl	Boc	>250	>250	>250	>250	>250
9d	Cyclobutyl	Boc	>250	>250	>250	>250	>250
9e	Adamantyl	Boc	>250	>250	**112.2 (125)**	>250	**112.2 (125)**
9f	H	Boc	>250	>250	>250	>250	>250
10a	t-butyl	H	>250	>250	>250	>250	250
10b	Cyclohexyl	H	>250	>250	>250	>250	>250
10c	i-propyl	H	>250	>250	>250	>250	>250
10d	Cyclobutyl	H	>250	>250	**18.2 (31.25)**	>250	>250
10e	Adamantyl	H	>250	>250	>250	>250	>250
10f	H	H	>250	>250	>250	>250	>250
			(Series II)				
14a	t-butyl	Boc	>250	>250	**44.6 (62.5)**	>250	>250
14b	Cyclohexyl	Boc	**18.5 (25)**	>250	**46.2 (62.5)**	>250	>250
14c	i-propyl	Boc	>250	>250	**10.9 (15.62)**	>250	>250
14d	Cyclobutyl	Boc	>250	>250	**11.1 (15.62)**	**177.8 (250)**	**177.8 (250)**
14e	Adamantyl	Boc	**98.9 (125)**	>250	**24.7 (31.25)**	**98.9 (125)**	**19.8 (25)**
14f	H	Boc	>250	>250	**82.1 (125)**	>250	>250
15a	t-butyl	H	>250	>250	**9.6 (15.62)**	>250	>250
15b	Cyclohexyl	H	>250	>250	**1.25 (1.95)**	>250	>250
15c	i-propyl	H	>250	>250	**74.8 (125)**	>250	>250
15d	Cyclobutyl	H	>250	>250	**76.4 (125)**	>250	>250
15e	Adamantyl	H	**86.3 (125)**	**86.3 (125)**	**86.3 (125)**	**86.3 (125)**	>250
15f	H	H	>250	>250	**34.8 (62.5)**	>250	>250
Amphotericin B		Control	0.72 (0.78)	11.55 (12.5)	<0.18 (0.20)	0.36 (0.39)	0.36 (0.39)

[a] Refer Figure 1; [b] MIC: Minimum Inhibitory Concentration [in µg/mL (µM)]

reported dipeptides were not tested against fungal strains other than *C. albicans*. As compared to the already reported long chain peptides which displayed MIC value between 12.5 to 25 μg/mL, the present relatively much shorter peptides exhibited better and more selective antifungal activities.[32] Moreover, these active tripeptides have been able to display more or less equivalent activity as reported for cathelicidin peptides which exhibited fungicidal activity of MIC value in the range of 0.5 to 32 μg/mL.[33] Similarly, previously reported hexapeptides from our group were also found to be in the same fungicidal range as some of the active tripeptides of the presently reported series.[34]

Cytotoxicity studies

To determine the effect of synthesized peptides on mammalian cells, toxicity of few active compounds was evaluated to determine their safety profile. On the basis of the results from the antifungal activity, Hek-293 and HeLa cells were treated with the most active peptides. These cells were incubated with test samples for 24h in RPMI-1640 and the untreated cells served as control. The ratio of the OD_{570} for compounds-treated cells to the OD_{570} for untreated cells was used to calculate the percent viability of the cells and standard deviations from the three observations are plotted. As it is evident from the **Fig. 2**, the tested compounds (**14a**, **14b**, **14c**, **14d**, **14e**, **14f**, **15a**, **15b**, **15c**, **15d**, **15e**, **15f**) displayed significant lesser cytotoxic behavior up to the highest test concentration of 250 μg/mL. It was observed that these peptides displayed significantly much lesser cytotoxicity as compared to naturally occurring AMP, Melittin.[35]

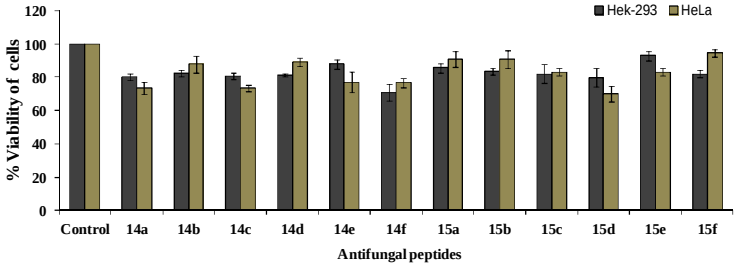

Fig 2. Mammalian cell cytotoxicity studies of selective compounds at 250 μg/ml against Hek-293 and HeLa cells

Mechanistic studies

Though cell wall may or may not play a role in the initial interaction of the peptide with microbial membranes,[11] the selectivity of active peptides toward microbial cells may be attributed to differences in their interaction with the outer membrane monolayers of microbial versus mammalian cells. With the aim of observing directly, the effect of synthesized active peptide on microbial cell membrane, we chose small unilamellar vesicles (SUVs) as model membranes.[36] As we know that phosphotidyl choline (PC) and phosphotidyl glycerol (PG) are the major components of prokaryotic membranes, we selected SUVs prepared using egg yolk L-α-phosphatidylcholine EYPC and egg yolk L-α-phosphatidyl-d,l-glycerol EYPG (7:3) as a relevant model of microbial cell membrane and EYPC/cholesterol (10:1) as that of mammalian cell membrane. The small unilamellar vesicles (SUVs) of EYPC/EYPG and EYPC/cholesterol were synthesized using standard protocol as reported in the literature.[37-39] The morphology of untreated and treated SUVs with **15b** were monitored by TEM. From TEM images (**Fig. 3**), it was revealed that the SUVs treated with selected peptide **15b** resulted in the disruption of the integrity of the SUVs after a short time exposure. In the case of EYPC/EYPG, the outer layer of the SUVs is deformed after a short time of incubation with peptide and complete disruption of their integrity occurs within 2-3 hrs of incubation. However, in the case of EYPC/cholesterol, the smooth regular surface of SUVs becomes loose and fragmented, but the integrity of the SUVs is conserved. This clearly indicates that the peptides have a preferential interaction with negatively charged phospholipids (EYPC/EYPG) rather than zwitter ionic membrane (EYPC/cholesterol). Although specialized studies based on surface plasmon resonance phenomenon are required to ascertain the affinity of the peptide for the different membranes, yet the results presented in this work strongly support the above statement. Therefore, it can be safely interpreted that the observations made from the mechanistic studies using TEM analysis with the selected active peptide are in line with the differences between microbial (negatively charged phospholipids) and mammalian (cholesterol and zwitterionic phospholipids) cell membrane composition.

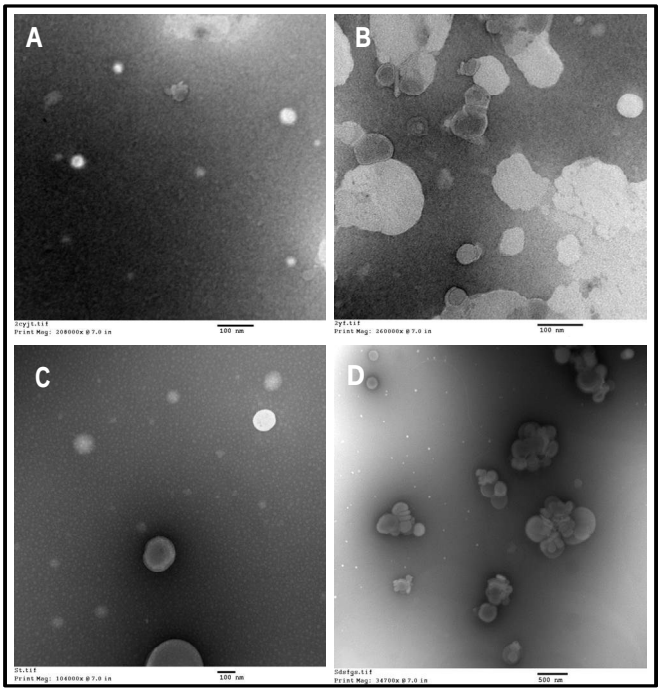

Fig 3. TEM images of untreated and treated SUVs with compound **15b**: untreated EYPC/EYPG (A); EYPC/EYPG treated with **15b** after 1-2 hrs (B);Untreated EYPC/Cholesterol (C) and EYPC/Cholesterol treated with **15b** after 1-2 hrs (D).

Conclusions

Based on the already reported His-Arg class of AMPs, a series of novel small peptides were synthesized which exhibited a broad spectrum of activities against the tested fungal strains. However, high selectivity was observed against the fungal pathogen *C. neoformans*. Out of all the synthesized tripeptides, series II [His(2-alkyl)-Arg-Arg] peptides were found to more active than the series I [(His(2-alkyl)-Arg-Lys] peptides. Peptide **15b** (series II) possessing bulkier cyclohexyl group on the imidazole ring of histidine was found to be the most potent against *C. neoformans* (MIC = 1.25 µg/mL). In general, it was found that the additional positively ionisable feature in the form of guanidinium group provided by arginine in series II is essential for antifungal activity especially against *C. neoformans*. Among series I peptides, compound **10d** with cyclobutyl substituent on imidazole ring, exhibited modest activity with MIC value of 18.2 µg/mL against *C. neoformans*. Interestingly, peptides having Boc group at the N-terminus also displayed promising antifungal activities. Further, the cytotoxicity studies of synthesized compounds showing antifungal activity was performed against Hek-293 and HeLa cells to determine their safety profile. These experiments revealed that the tested compounds exhibited none or relatively acceptable cytotoxicity profile. To understand the plausible mode of selectivity of reported peptides, TEM analysis of SUVs of EYPC/(EYPG) (7:3 w/w) mimicking the microbial cell membrane and SUVs of EYPC/cholesterol (10:1 w/w) mimicking the mammalian membrane was performed. It was observed that the SUVs treated with most potent peptide **15b** resulted in the complete disruption of SUVs within 1-2 hrs of incubation in the case of EYPC/EYPG. However, the integrity of EYPC/cholesterol SUVs is relatively conserved under same treatment. Based on the general observation that the effect of the interaction with EYPC/EYPG is different from that with EYPC/cholesterol, it can be safely interpret that the tested peptides have a higher affinity for the EYPC/EYPG based membrane as compared to EYPC/cholesterol based membrane. The preferential interaction of most potent peptide with negatively charged membrane over the neutral mammalian membrane based on mechanistic studies may also indicate that the synthesized active peptides might be engaged in disruption of the cell membrane leading to probable disruption of the microbial cell. Therefore, owing to the clear disruption of cell membrane in a random manner, the general observation is that the reported class of peptides may also be less susceptible to the common mechanisms of drug resistance.

Experimental section

Chemistry

All the starting chemical compounds were purchased from Sigma-Aldrich and Chem-Impex. All these compounds were used further without any additional purification. Analytical thin-layer chromatography (TLC) was performed using aluminum plates precoated with silica gel (0.25 mm, 60 A pore-size) impregnated with a fluorescent indicator (254 nm). Visualization on TLC was achieved by the use of UV light (254 nm), stained with iodine vapors. Column chromatography was done on silica gel (60-120 and 100-200 mesh). Proton nuclear magnetic resonance spectra (^{1}H NMR) were recorded on AVANCE III 400 Bruker (400 MHz). Proton chemical shifts are expressed in parts per million (ppm, δ scale) and are referenced to residual proton in the NMR solvent (CD_3OD). The following abbreviations were used to describe peak patterns where appropriate: br = broad, s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet. Coupling constants, *J*, were reported in Hertz unit (Hz). Carbon 13 nuclear magnetic resonance spectroscopy (^{13}C NMR) was recorded on AVANCE III 400 Bruker (400 MHz) and was fully decoupled by broad band decoupling. Chemical shifts were reported in ppm referenced to the centre line at a 49.0 ppm of CD_3OD. Mass spectra were taken with MAXIS-Bruker using APCI-TOF method. HPLC analysis was performed on the SHIMADZU-prominence using Supelcosil TM LC-18 column (25 cm × 4.6 mm, 5mm) run for 30 min with a flow of 1 mL/min, using a gradient of 90-0% (A:B) where buffer A was 0.1% TFA in H_2O and buffer B was 0.1% TFA in CH_3CN and detection at 220 nm.

Synthesis of *N*-α-Boc-2-alkyl-L-histidines (5a-e)

To a solution of *N*-α-trifluoroacetyl-L-histidine methyl ester (**2**, 7.54 mmol, 1 equiv) in acetonitrile (CH_3CN, 5 mL), a solution of silver nitrate (4.52 mmol, 0.6 equiv) in water (5 mL) was added followed by the addition of corresponding alkyl carboxylic acid (3 equiv). The reaction mixture was then stirred with immediate dropwise addition of 10% sulfuric acid (H_2SO_4, 10 mL), freshly prepared solution of ammonium persulfate [$(NH_4)_2S_2O_8$, 3.0 equiv] at 78-80^{o}C temperature for 15 min. After evolution of CO_2, the reaction mixture was poured to ice and the solution was made alkaline by augmenting the pH value to 12 by the addition of aqueous ammonia solution. This was followed by the extraction of the reaction mixture with three cycles of ethyl acetate (30 mL) and the organic layer was washed with brine solution. The resulting organic solution was dried and the residue was purified by using column chromatography to provide desired compounds (**3a-f**). Further, L-His(2-alkyl)-OH (**4a-f**) were synthesized by refluxing the solution of compounds **3a-f** in 6 N HCl for 24 h. For Boc protection, the compounds **4a-f** were dissolved in water-dioxane (2:3) mixture followed by addition of 4N NaOH so that the pH of the reaction mixture adjusts to 12. Then, *di-tert*-butyl dicarbonate (3 equiv) was added to the reaction mixture in one portion. After 10 min, pH was again adjusted to 12 and *di-tert*-butyl dicarbonate (2 mmol) was added in second portion. Reaction was allowed to stir at ambient temperature for 12 h followed by removal of solvent under reduced pressure and addition of methanol (10 mL) to the viscous residue. The resulting solution was stirred at ambient temperature for additional 12 h. The complete removal of solvent followed by treatment with saturated aqueous solution of $KHSO_4$ to adjust the pH at 3-4 generated the free *N*-α-Boc-L-His(2-alkyl)-OH (**5a-f**). The solvent was removed under reduced pressure and the resulting residue was extracted with

t-butanol (4 × 50 mL) followed by removal of solvent under vacuum afforded relatively purified **5a-f**.

Synthesis of and Boc-L-His(2-alkyl)-L-Arg-Lys-NHBzl (9a-f) and L-His(2-alkyl)-L-Arg-Lys-NHBzl (10a-f)

To a solution of Boc-L-Lys(Z)-OH (**6**, 1 mmol) in DMF (3 mL), benzyl amine (1.3 mmol), DIC (1.3 mmol) and HONB (1.3 mmol) was added. The reaction mixture was stirred for 48 hrs at room temp followed by removal of solvent under vacuum. The reaction mixture was subjected to column chromatography yielding desired Boc-L-Lys(Z)-NHBzl (**7**). The obtained compound was then subjected to acidolysis for 15 mins to afford L-Lys(Z)-NHBzl which was further coupled with Boc-L-Arg-OH (1.3 mmol) in the presence of DIC (1.3 mmol), HONB (1.3 mmol) and DMF(3ml). The reaction mixture was stirred for 48 hrs at room temp followed by purification using column chromatography which yielded desired Boc-Arg-Lys(Z)-NHBzl (**8**). The Boc group was further removed by using 25%TFA in DCM (5 mL) at 25 ˚C for 15 min followed by neutralization with methanolic ammonia (5 ml). Finally, the resulting L-Arg-Lys(Z)-NHBzl (1 mmol) was coupled with Boc-His(2-alkyl)-OH (1.3 mmol) in the presence of HONB (1.3 mmol), DIC (1.3 mmol) under constant stirring for 48 hrs at room temp and purification using column chromatography to afford desired Boc-His(2-alkyl)-Arg-Lys(Z)-NHBzl (**9a-f**). The Boc and other orthogonal protection were removed using 33% HBr in acetic acid (5 mL) at 25 ˚C for 15 min resulting in the formation of desired His(2-alkyl)-Arg-Lys-NHBzl (**10a-f**).

4.1.2.1. **Boc-His(2-t-butyl)-Arg-Lys(Z)-NHBzl (9a):** Yield: 60%, off white solid, mp 187-189 ^{o}C, ^{1}H NMR (CD_3OD): δ 1.28-1.30 (m, 2H, CH_2), 1.35 (s, 9H, 3xCH_3), 1.38 (s, 9H, 3xCH_3), 1.56-1.58 (m, 4H, 2xCH_2), 1.61-1.63 (m, 4H, 2xCH_2), 2.71 (t, 2H, *J*=7.1 Hz, CH_2), 3.19-3.21 (m, 2H, CH_2), 3.22 (t, 2H, *J*=7.3 Hz, 2xCH_2), 4.42 (s, 2H, CH_2), 4.56-4.58 (m, 2H, 2xCH), 4.90-4.92 (m, 1H, CH), 7.23-7.30 (m, 5H, Ar-H), 7.34-7.36 (m, 1H, Im-H), 7.38-7.45 (m, 5H, Ar-H). ^{13}C NMR (100.6 MHz, CD_3OD): δ 23.3, 24.2, 28.4, 28.5, 31.0, 42.1, 43.3, 53.8, 57.2, 118.3, 126.3, 126.9, 128.0, 137.2. MS (APCI): m/z 819.00 $[MH]^+$. $[\alpha]_D^{25}$ = -12.5^{o} (c =1.3, CH_3OH)

4.1.2.2. **Boc-His(2-cyclohexyl)-Arg-Lys(Z)-NHBzl (9b):** Yield: 55%, off white solid, mp 195-197 ^{o}C, ^{1}H NMR (CD_3OD): δ 1.25-1.27 (m, 2H, CH_2), 1.38 (s, 9H, 3xCH_3), 1.45-1.47 (m, 2H, CH_2), 1.49-1.51 (m, 4H, 2xCH_2), 1.54-1.56 (m, 4H, 2xCH_2), 1.74-1.77 (m, 4H, 2xCH_2), 1.85-1.87 (m, 4H, 2xCH_2), 2.87 (t, 2H, *J*=6.8 Hz, CH_2), 3.00 (m, 1H, CH), 3.20 (t, 2H, *J*=7.0 Hz, CH_2), 3.23-3.25 (m, 2H, CH_2), 4.40 (s, 2H, CH_2), 4.57-4.59 (m, 2H, 2xCH), 4.92-4.94 (m, 1H, CH), 5.05 (s, 2H, CH_2), 7.23-7.29 (m, 5H, Ar-H), 7.38-7.40 (m, 1H, Im-H), 7.40-7.48 (m, 5H, Ar-H). ^{13}C NMR (100.6 MHz, CD_3OD): δ 11.8, 15.9, 17.4, 24.9, 25.1, 27.2, 27.4, 29.4, 30.3, 30.4, 35.7, 42.4, 53.2, 53.5, 54.4, 79.4, 116.0, 129.3, 151.2. MS (APCI): m/z 846.23 $[MH]^+$. $[\alpha]_D^{25}$ = -18.2^{o} (c =1.1, CH_3OH)

4.1.2.3. **Boc-His(2-i-propyl)-Arg-Lys(Z)-NHBzl (9c):** Yield: 39%, off white solid, mp 183-185 ^{o}C ^{1}H NMR (CD_3OD): δ 1.22-1.24 (m, 6H, 2xCH_3), 1.28-1.30 (m, 2H, CH_2), 1.40 (s, 9H, 3xCH_3), 1.44-1.46 (m, 4H, 2xCH_2), 1.64-1.66 (m, 4H, 2xCH_2), 2.71 (t, 2H, *J*=6.8 Hz, CH_2), 3.09-3.11 (m, 2H, CH_2), 3.18 (t, 2H, *J*=6.9 Hz, CH_2), 4.35 (s, 2H, CH_2), 4.53-4.55 (m, 2H, 2xCH), 4.84-4.86 (m, 1H, CH), 5.07 (s, 2H, CH_2), 7.21-7.27 (m, 5H, Ph-H), 7.29-7.35 (m, 5H, Ph-H), 7.38-7.40 (m, 1H, Im-H). ^{13}C NMR (100.6 MHz, CD_3OD): δ 20.5, 24.0, 24.2, 26.9, 28.3, 29.2, 29.9, 30.3, 42.5, 43.3, 58.6, 116.9, 126.2, 127.0, 128.2, 132.0. MS (APCI): m/z 805.39$[MH]^+$. $[\alpha]_D^{25}$ = -8.3^{o} (c =1.0, CH_3OH)

4.1.2.4. **Boc-His(2-cyclobutyl)-Arg-Lys(Z)-NHBzl (9d):** Yield: 41%, off white solid, mp 190-192 ^{o}C ^{1}H NMR (CD_3OD): δ 1.23-1.25 (m, 2H, CH_2), 1.38 (s, 9H, 3xCH_3), 1.47-1.49 (m, 4H, 2xCH_2), 1.69-1.71 (m, 4H, 2xCH_2), 1.88-1.90 (m, 2H, CH_2), 2.36-2.38 (m, 4H, 2xCH_2), 2.61 (t, 2H, *J*=7.2 Hz, CH_2), 3.11-3.13 (m, 2H, CH_2), 3.18 (t, 2H, *J*=6.9 Hz, CH_2), 3.24-3.26 (m, 1H, CH), 4.38 (s, 2H, CH_2), 4.55-4.57 (m, 2H, 2xCH), 4.83-4.85 (m, 1H, CH), 5.10 (s, 2H, CH_2), 7.21-7.28 (m, 5H, Ph-H), 7.41-7.43 (m, 1H, Im-H), 7.43-7.48 (m, 5H, Ph-H) . ^{13}C NMR (100.6 MHz, CD_3OD): δ 18.19, 23.9, 25.0, 25.9, 27.0, 29.9, 31.8, 35.1, 41.9, 43.1, 53.2, 118.4, 119.9, 126.9, 127.6, 128.1, 131.5. MS (APCI): m/z 817.21$[MH]^+$. $[\alpha]_D^{25}$ = -14.0^{o} (c =1.6, CH_3OH)

4.1.2.5. **Boc-His(2-adamantyl)-Arg-Lys(Z)-NHBzl (9e):** Yield: 43%, off white solid, mp 205-207 ^{o}C ^{1}H NMR (CD_3OD): δ 1.26-1.28 (m, 2H, CH_2), 1.38 (s, 9H, 3xCH_3), 1.66-1.68 (m, 4H, 2xCH_2), 1.74-1.76 (m, 6H, 3xCH_2), 1.84-1.86 (m, 4H, 2xCH_2), 2.02-2.04 (m, 6H, 3xCH_2), 2.12-2.14 (m, 6H, 3xCH_2), 2.71 (t, 2H, *J*=7.2 Hz, CH_2), 3.18 (m, 2H, CH_2), 3.21 (t, 2H, *J*=6.9 Hz, CH_2), 4.39 (s, 2H, CH_2), 4.48-4.50 (m, 2H, 2xCH), 4.93-4.95 (m, 1H, CH), 5.07 (s, 2H, CH_2), 7.23-7.30 (m, 5H, Ph-H), 7.34-7.36 (m, 1H, Im-H), 7.41-7.45 (m, 5H, Ph-H). ^{13}C NMR (100.6 MHz, CD_3OD): δ 21.4, 22.9, 26.8, 29.2, 31.7, 32.0, 36.2, 41.9, 42.6, 43.2, 58.9, 66.2, 79.1, 119.1, 126.2, 126.4, 128.5, 137.5, 155.6. MS (APCI): m/z 898.21$[MH]^+$. $[\alpha]_D^{25}$ = -6.9^{o} (c =1.2, CH_3OH)

4.1.2.6. **Boc-His-Arg-Lys(Z)-NHBzl (9f):** Yield: 52%, off white solid, mp 179-181 ^{o}C, ^{1}H NMR (CD_3OD): δ 1.29-1.31 (m, 2H, CH_2), 1.39 (s, 9H, 3xCH_3), 1.54-1.56 (m, 4H, 2xCH_2), 1.74-1.76 (m, 4H, 2xCH_2), 2.87 (t, 2H, *J*=7.1 Hz, CH_2), 3.09-3.11 (m, 2H, CH_2), 3.18 (t, 2H, *J*=7.3 Hz, CH_2), 4.36 (s, 2H, CH_2), 4.61-4.63 (m, 2H, 2xCH), 4.89-4.91 (m, 1H, CH), 7.25-7.31 (m, 5H, Ar-H), 7.38-7.40 (m, 1H, Im-H), 7.42-7.48 (m, 5H, Ar-H). ^{13}C NMR (100.6 MHz, CD_3OD): δ 27.3, 112.4, 115.3, 118.2, 121.1, 127.1, 127.3, 127.6, 128.1, 161.3, 161.6, 161.9. MS (APCI): m/z 763.32 $[MH]^+$. $[\alpha]_D^{25}$ = -10.4^{o} (c =1.1, CH_3OH)

4.1.2.7. **His(2-t-butyl)-Arg-Lys-NHBzl (10a):** Yield: 68%, off white solid, mp 235-237 ^{o}C, ^{1}H NMR (CD_3OD): δ 1.22-1.24 (m, 2H, CH_2), 1.36 (s, 9H, 3xCH_3), 1.58-1.60 (m, 4H, 2xCH_2), 1.71-1.73 (m, 4H, 2xCH_2), 2.61 (t, 4H, *J*=7.1 Hz, 2xCH_2), 3.17-3.19 (m, 2H, CH_2), 3.86-3.88 (m, 1H, CH), 4.40 (s, 2H, CH_2), 4.56-4.58 (m, 2H, 2xCH), 7.21-7.29 (m, 5H, Ar-H), 7.37-7.39 (m, 1H, Im-H). ^{13}C NMR (100.6 MHz, CD_3OD): δ 22.9, 24.3, 27.9, 29.3, 30.0, 41.9, 42.7, 54.0, 58.2, 117.3, 126.4, 127.5, 128.1, 138.0. MS (APCI): m/z 585.76 $[MH]^+$. $[\alpha]_D^{25}$ = -20.0^{o} (c =1.0, CH_3OH)

4.1.2.8. **His(2-cyclohexyl)-Arg-Lys-NHBzl (10b):** Yield: 70%, off white solid, mp 243-245 ^{o}C, ^{1}H NMR (CD_3OD): δ 1.25-1.27 (m, 2H, CH_2), 1.48-1.50 (m, 2H, CH_2), 1.51-1.53 (m, 4H, 2xCH_2), 1.57-1.59 (m, 4H, 2xCH_2), 1.71-1.73 (m, 4H, 2xCH_2), 1.83-1.85 (m, 4H, 2xCH_2), 2.68 (t, 4H, *J*=6.7 Hz, 2xCH_2), 3.03-3.05 (m, 1H, CH), 3.30-3.32 (m, 2H, CH_2), 3.86-3.88 (m, 1H, CH), 4.45 (s, 2H, CH_2), 4.52-4.54 (m, 2H, 2xCH), 7.21-7.28 (m, 5H, Ar-H), 7.34-7.36 (m, 1H, Im-H). ^{13}C NMR (100.6 MHz, CD_3OD): δ 20.9, 24.2,

25.9, 26.0, 28.5, 28.9, 29.1, 31.5, 33.0, 41.6, 43.9, 58.2, 118.9, 126.9, 127.4, 128.1, 138.0. MS (APCI): m/z 611.69 $[MH]^+$. $[\alpha]_D^{25}$ = -15.1^o (c =1.4, CH_3OH)

4.1.2.9. **His(2-i-propyl)-Arg-Lys-NHBzl (10c):** Yield: 75%, off white solid, mp 229-231 ^oC, ^1H NMR (CD_3OD): δ 1.21-1.23 (m, 6H, 2xCH_3), 1.27-1.29 (m, 2H, CH_2), 1.53-1.55 (m, 4H, 2xCH_2), 1.74-1.76 (m, 4H, 2xCH_2), 2.81 (t, 4H, *J*=6.8 Hz, 2xCH_2), 3.17-3.19 (m, 2H, CH_2), 3.82-3.84 (m, 1H, CH), 4.45 (s, 2H, CH_2), 4.62-4.64 (m, 2H, 2xCH), 7.22-7.28 (m, 5H, Ph-H), 7.41-7.43 (m, 1H, Im-H). ^{13}C NMR (100.6 MHz, CD_3OD): δ 21.9, 23.4, 25.2, 28.5, 29.3, 30.2, 32.5, 41.5, 43.4, 58.2, 119.3, 126.7, 126.9, 128.5, 134.5. MS (APCI): m/z 571.93$[MH]^+$. $[\alpha]_D^{25}$ = -11.3^o (c =1.2, CH_3OH)

4.1.2.10. **His(2-cyclobutyl)-Arg-Lys-NHBzl (10d):** Yield: 71%, off white solid, mp 238-240 ^oC, ^1H NMR (CD_3OD): δ 1.25-1.27 (m, 2H, CH_2), 1.52-1.54 (m, 4H, 2xCH_2), 1.74-1.76 (m, 4H, 2xCH_2), 1.90-1.92 (m, 2H, CH_2), 2.33-2.35 (m, 4H, 2xCH_2), 2.65 (t, 4H, *J*=7.2 Hz, 2xCH_2), 3.08-3.10 (m, 2H, CH_2), 3.22-3.24 (m, 1H, CH), 3.93-3.95 (m, 1H, CH), 4.40 (s, 2H, CH_2), 4.60-4.62 (m, 2H, 2xCH), 7.25-7.33 (m, 5H, Ph-H), 7.43-7.45 (m, 1H, Im-H). ^{13}C NMR (100.6 MHz, CD_3OD): δ 17.9, 23.9, 24.4, 26.1, 31.1, 31.5, 35.1, 42.5, 44.5, 54.9, 119.1, 126.2, 126.9, 128.2, 131.4. MS (APCI): m/z 583.92 $[MH]^+$. $[\alpha]_D^{25}$ = -14.5^o (c =1.5, CH_3OH)

4.1.2.11. **His(2-adamantyl)-Arg-Lys-NHBzl (10e):** Yield: 76%, off white solid, mp 249-251 ^oC, ^1H NMR (CD_3OD): δ 1.22-1.24 (m, 2H, CH_2), 1.42-1.44 (m, 4H, 2xCH_2), 1.78-1.80 (m, 6H, 3xCH_2), 1.83-1.85 (m, 4H, 2xCH_2), 2.04-2.06 (m, 6H, 3xCH_2), 2.10-2.12 (m, 6H, 3xCH_2), 2.74 (t, 4H, *J*=7.2 Hz, 2xCH_2), 3.18-3.20 (m, 2H, CH_2), 3.77-3.79 (m, 1H, CH), 4.38 (s, 2H, CH_2), 4.56-4.58 (m, 2H, 2xCH), 7.26-7.31 (m, 5H, Ph-H), 7.37-7.39 (m, 1H, Im-H). ^{13}C NMR (100.6 MHz, CD_3OD): δ 21.3, 24.1, 28.5, 30.1, 35.2, 41.6, 42.9, 58.5, 116.9, 126.2. 126.6, 128.3, 137.1, 156.4. MS (APCI): m/z 663.96 $[MH]^+$. $[\alpha]_D^{25}$ = -7.6^o (c =1.4, CH_3OH)

4.1.2.12. **His-Arg-Lys-NHBzl (10f):** Yield: 85%, yellow solid, mp 210-212 ^oC, ^1H NMR (CD_3OD): δ 1.25-1.27 (m, 2H, CH_2), 1.57-1.59 (m, 4H, 2xCH_2), 1.68-1.70 (m, 4H, 2xCH_2), 2.74 (t, 4H, *J*=7.1 Hz, 2xCH_2), 3.17-3.19 (m, 2H, CH_2), 3.91-3.93 (m, 1H, CH), 4.38 (s, 2H, CH_2), 4.45-4.47 (m, 2H, 2xCH), 7.25-7.31 (m, 5H, Ar-H), 7.38-7.40 (m, 1H, Im-H). ^{13}C NMR (100.6 MHz, CD_3OD): δ 22.7, 24.2, 28.9, 31.3, 41.9, 43.2, 53.5, 57.2, 117.4, 126.6, 126.9, 128.5, 136.2. MS (APCI): m/z 529.38 $[MH]^+$. $[\alpha]_D^{25}$ = -12.1^o (c =1.3, CH_3OH)

Synthesis of Boc-L-His(2-alkyl)-Arg-Arg-NHBzl (14a-f) and L-His(2-alkyl)-Arg-Arg-NHBzl (15a-f)

To a solution of Boc-L-Arg-OH (1 mmol) in DMF (3 mL), benzyl amine (1.3 mmol), DIC (1.3 mmol) and HONB (1.3 mmol) was added. The reaction mixture was stirred for 48 hrs at room temp followed by removal of solvent under vacuum. The reaction mixture was subjected to column chromatography yielding desired Boc-L-Arg-NHBzl (**12**). Further, compound **12** was subjected to acidolysis for 15 min to yield L-Arg-NHBzl. The obtained compound was (1mmol) coupled with Boc-L-Arg-OH (1.3 mmol) in the presence of DIC (1.3 mmol) and HONB (1.3 mmol). After stirring of 48 hrs at room temp, the obtained crude product was purified by column chromatography using CH_2Cl_2: MeOH (8:2) to afford Boc-Arg-Arg-NHBzl (**13**).Further compound **13** was treated with 25%TFA in DCM (5 mL) at 25 ˚C for 15 min. and neutralized to obtain L-Arg-Arg-NHBzl .The resulting compound was coupled with Boc-His(2-alkyl)-OH (**5a-e**,1.3 mmol) in the presence of HONB (1.3 mmol) and DIC (1.3 mmol). The reaction mixture was purified to afford Boc-His(2-alkyl)-Arg-Arg-NHBzl (**14a-f**). For the removal of Boc group, **14a-f** was treated with 3N HCl in MeOH (5 mL) at 25 ˚C for 15 min resulted in the desired L-His(2-alkyl)-Arg-Arg-NHBzl (**15a-f**).

4.1.3.1. Boc-His(2-t-butyl)-Arg-Arg-NHBzl (14a). Yield: 56%, off white solid, mp 175-177 ^oC, ^1H NMR (CD_3OD): δ 1.33 (s, 9H, 3xCH_3), 1.39 (s, 9H, 3xCH_3), 1.55-1.57 (m, 4H, 2xCH_2), 1.65-1.67 (m, 4H, 2xCH_2), 2.67 (t, 4H, *J*=6.7 Hz, 2xCH_2), 3.09-3.11 (m, 2H, CH_2), 4.38-4.40 (m, 2H, CH_2), 4.49-4.51 (m, 2H, CH), 4.73-4.75 (m, 1H, CH), 7.20-7.27 (m, 5H, Ar-H), 7.37-7.39 (m, 1H, Im-H). ^{13}C NMR (100.6 MHz, CD_3OD): δ 25.9, 26.4, 28.7, 29.9, 30.3, 32.9, 41.9, 42.2, 44.1, 54.5, 55.3, 116.7, 119.7, 128.3, 128.5, 129.6, 139.7, 158.7, 163.0, 163.4, 174.0. MS (APCI): m/z 713.64 $[MH]^+$. $[\alpha]_D^{25}$ = -12.6^o (c =1.4, CH_3OH)

4.1.3.2. Boc-His(2-cyclohexyl)-Arg-Arg-NHBzl (14b). Yield: 45%, off white solid, mp 184-186 ^oC, ^1H NMR (CD_3OD): δ 1.39 (s, 9H, 3xCH_3), 1.43-1.45 (m, 4H, 2xCH_2), 1.51-1.53 (m, 4H, 2xCH_2), 1.54-1.56 (m, 4H, 2xCH_2), 1.72-1.74 (m, 4H, 2xCH_2), 1.84-1.86 (m, 4H, 2xCH_2), 2.70 (t, 4H, *J*=6.9 Hz, 2xCH_2), 2.91-2.93 (m, 1H, CH), 3.18 (t, 2H, *J*=7.3 Hz, CH_2), 4.42 (s, 2H, CH_2), 4.57-4.59 (m, 2H, 2xCH), 4.88-4.90 (m, 1H, CH), 7.23-7.28 (m, 5H, Ar-H), 7.31-7.33 (m, 1H, Im-H). ^{13}C NMR (100.6 MHz, CD_3OD): δ 21.4, 24.8, 25.0, 25.6, 25.8, 27.3, 28.8, 31.7, 40.6, 42.7, 53.1, 126.9, 127.1, 128.2, 138.3, 157.2, 172.4. MS (APCI): m/z 739.52 $[MH]^+$. $[\alpha]_D^{25}$ = -8.0^o (c =1.3, CH_3OH)

4.1.3.3. Boc-His(2-i-propyl)-Arg-Arg-NHBzl (14c). Yield: 37%, off white solid, mp 170-172 ^oC, ^1H NMR (CD_3OD): δ 1.28-1.30 (m, 6H, 2xCH_3), 1.39 (s, 9H, 3xCH_3), 1.62-1.64 (m, 4H, 2xCH_2), 1.74-1.76 (m, 4H, 2xCH_2), 2.73 (t, 4H, *J*=6.8 Hz, 2xCH_2), 3.17-3.19 (m, 2H, CH_2), 4.41 (s, 2H, CH_2), 4.48 (t, 2H, *J*=7.3 Hz, 2xCH), 4.93-4.95 (m, 1H, CH), 7.24-7.31 (m, 5H, Ar-H), 7.37-7.39 (m, 1H, Im-H). ^{13}C NMR (100.6 MHz, CD_3OD): δ 20.9, 23.9, 27.9, 28.9, 32.6, 41.2, 43.7, 53.7, 126.8, 127.1, 129.0, 157.9. MS (APCI): m/z 699.42 $[MH]^+$. $[\alpha]_D^{25}$ = -27.3^o (c =1.6, CH_3OH)

4.1.3.4. Boc-His(2-cyclobutyl)-Arg-Arg-NHBzl (14d). Yield: 40%, off white solid, mp 180-182 ^oC, ^1H NMR (CD_3OD): δ 1.39 (s, 9H, 3xCH_3), 1.45-1.47 (m, 4H, 2xCH_2), 1.64-1.66 (m, 4H, 2xCH_2), 1.91-1.93 (m, 2H, CH_2), 2.31-2.33 (m, 4H, 2xCH_2), 2.69 (t, 4H, *J*=6.7 Hz, 2xCH_2), 3.17-3.19 (m, 2H, CH_2), 3.21-3.23 (m, 1H, CH), 4.40 (s, 2H, CH_2), 4.53 (t, 2H, *J*=7.1 Hz, 2xCH), 4.91-4.93 (m, 1H, CH), 7.24-7.30 (m, 5H, Ar-H), 7.37-7.39 (m, 1H, Im-H). ^{13}C NMR (100.6 MHz, CD_3OD): δ 18.9, 24.9, 28.4, 30.6, 34.3, 41.2, 43.6, 58.2, 79.5, 126.8, 128.3, 128.6, 129.4, 157.8. MS (APCI): m/z 711.42 $[MH]^+$. $[\alpha]_D^{25}$ = -21.8^o (c =1.7, CH_3OH)

4.1.3.5. Boc-His(2-adamantyl)-Arg-Arg-NHBzl (14e). Yield: 40%, off white solid; mp 199-201 ^oC, ^1H NMR (CD_3OD): δ 1.39 (s, 9H, 3xCH_3), 1.44-1.46 (m, 4H, 2xCH_2), 1.64-1.66 (m, 4H, 2xCH_2), 1.75-1.77 (m, 6H, 3xCH_2), 1.96-1.98 (m, 6H, 3xCH_2), 2.06-2.08 (m, 6H, 3xCH_2), 2.85 (t, 2H, *J*=6.9 Hz, 2xCH_2), 3.20-3.22 (m, 2H, CH_2), 4.40 (s, 2H, CH_2), 4.48-4.50 (m, 2H, 2xCH), 4.94-4.96 (m, 1H, CH), 7.24-7.31 (m, 5H, Ar-H), 7.34-7.36 (m,

1H, Im-H). ^{13}C NMR (100.6 MHz, CD_3OD): δ 19.5, 24.6, 25.0, 27.3, 27.8, 28.4, 28.6, 28.8, 34.5, 36.3, 38.2, 40.5, 40.6, 41.1, 42.7, 52.9, 53.1, 55.1, 79.5, 126.9, 127.1, 128.2, 138.3, 156.1, 156.6, 157.2, 172.4, 173.5. MS (APCI): m/z 791.82 $[MH]^+$. $[\alpha]_D^{25}$ = -19.6° (c =1.1, CH_3OH)

4.1.3.6. Boc-His-Arg-Arg-NHBzl (14f). Yield: 60%, off white solid, mp 169-171 °C, ^{1}H NMR (CD_3OD): δ 1.38 (s, 9H, 3xCH_3), 1.54-1.56 (m, 4H, 2xCH_2), 1.73-1.75 (m, 4H, 2xCH_2), 2.51 (t, 4H, *J*=7.1 Hz, 2xCH_2), 3.16-3.18 (m, 2H, CH_2), 4.31 (s, 2H, CH_2), 4.51-4.53 (m, 2H, 2xCH), 4.86-4.88 (m, 1H, CH), 7.21-7.32 (m, 5H, Ar-H), 7.42-7.44 (m, 1H, Im-H). ^{13}C NMR (100.6 MHz, CD_3OD): δ 24.9, 28.3, 29.4, 40.2, 43.7, 57.5, 79.5, 126.9, 128.5, 136.9, 157.1. MS (APCI): m/z 657.04 $[MH]^+$. $[\alpha]_D^{25}$ = -15.5° (c =1.5, CH_3OH)

4.1.3.7. His(2-t-butyl)-Arg-Arg-NHBzl (15a). Yield: 76%, off white solid, mp 217-219 °C, ^{1}H NMR (CD_3OD): δ 1.39 (s, 9H, 3xCH_3), 1.57-1.59 (m, 4H, 2xCH_2), 1.78-1.80 (m, 4H, 2xCH_2), 2.70 (t, 2H, *J* = 7.0 Hz, CH_2), 3.10-3.12 (m, 2H, CH_2), 3.93-3.95 (m, 1H, CH), 4.35 (s, 2H, CH_2), 4.49-4.51 (m, 2H, 2xCH), 7.21-7.26 (m, 5H, Ar-H), 7.36-7.38 (m, 1H, Im-H). ^{13}C NMR (100.6 MHz, CD_3OD): δ 23.6, 24.8, 25.1, 25.2, 26.2, 27.4, 27.5, 28.2, 28.4, 28.7, 29.3, 32.8, 40.4, 40.6, 42.7, 52.3, 53.6, 126.9, 127.1, 127.2, 128.2, 138.3, 138.4, 157.2, 168.7, 172.3, 172.5. MS (APCI): m/z 613.91 $[MH]^+$. $[\alpha]_D^{25}$ = -13.4° (c =1.6, CH_3OH)

4.1.3.8. His(2-cyclohexyl)-Arg-Arg-NHBzl (15b). Yield: 67%, off white solid, mp 227-229 °C, ^{1}H NMR (CD_3OD): δ 1.40-1.42 (m, 4H, 2xCH_2), 1.45-1.47 (m, 4H, 2xCH_2), 1.53-1.55 (m, 4H, 2xCH_2), 1.68-1.70 (m, 4H, 2xCH_2), 1.87-1.89 (m, 4H, 2xCH_2), 2.65 (t, 4H, *J*=6.9 Hz, 2xCH_2), 2.84-2.86 (m, 1H, CH), 3.22-3.24 (m, 2H, CH_2), 3.89-3.91 (m, 1H, CH), 4.40 (s, 2H, CH_2), 4.47-4.49 (m, 2H, 2xCH), 7.25-7.31 (m, 5H, Ar-H), 7.40-7.42 (m, 1H, Im-H). ^{13}C NMR (100.6 MHz, CD_3OD): δ 23.8, 25.2, 29.3, 32.9, 39.2, 40.6, 53.3, 57.8, 126.9, 127.1, 128.2, 157.2. MS (APCI): m/z 639.96 $[MH]^+$. $[\alpha]_D^{25}$ = -11.6° (c =1.2, CH_3OH)

4.1.3.9. His(2-i-propyl)-Arg-Arg-NHBzl (15c). Yield: 70%, off white solid, mp 209-211 °C, ^{1}H NMR (CD_3OD): δ 1.25-1.27 (m, 6H, 2xCH_3), 1.66-1.68 (m, 4H, 2xCH_2), 1.80-1.82 (m, 4H, 2xCH_2), 2.68 (t, 4H, *J*=6.9 Hz, 2xCH_2), 3.20 (t, 2H, *J*=7.1 Hz, CH_2), 3.96-3.98 (m, 1H, CH), 4.40 (s, 2H, CH_2), 4.51-4.53 (m, 2H, 2xCH), 7.25-7.32 (m, 5H, Ar-H), 7.35-7.37 (m, 1H, Im-H). ^{13}C NMR (100.6 MHz, CD_3OD): δ 23.5, 25.0, 28.2, 28.8, 40.4, 40.6, 42.8, 52.4, 53.5, 126.9, 127.1, 128.2, 138.3, 157.2, 168.7, 172.3. MS (APCI): m/z 599.98 $[MH]^+$. $[\alpha]_D^{25}$ = -10.8° (c =1.0, CH_3OH)

4.1.3.10. His(2-cyclobutyl)-Arg-Arg-NHBzl (15d). Yield: 80%, off white solid, mp 215-217 °C, ^{1}H NMR (CD_3OD): δ 1.55-1.57 (m, 4H, 2xCH_2), 1.76-1.78 (m, 4H, 2xCH_2), 1.97-1.99 (m, 2H, CH_2), 2.24-2.26 (m, 4H, 2xCH_2), 2.64 (t, 4H, *J*=6.7 Hz, 2xCH_2), 3.11-3.13 (m, 2H, CH_2), 3.84-3.86 (m, 1H, CH), 4.35 (s, 2H, CH_2), 4.44-4.46 (m, 2H, 2xCH), 4.80-4.82 (m, 1H, CH), 7.21-7.27 (m, 5H, Ar-H), 7.39-7.41 (m, 1H, Im-H). ^{13}C NMR (100.6 MHz, CD_3OD): δ 19.5, 26.2, 26.6, 28.0, 28.7, 28.8, 29.9, 30.2, 30.8, 32.2, 42.1, 44.2, 53.1, 55.1, 55.2, 119.6, 128.3, 128.6, 129.6, 139.8, 152.7, 158.6, 169.1. MS (APCI): m/z 611.78 $[MH]^+$. $[\alpha]_D^{25}$ = -23.4° (c =1.2, CH_3OH)

4.1.3.11. His(2-adamantyl)-Arg-Arg-NHBzl (15e). Yield: 80%, off white solid; mp 230-232 °C, ^{1}H NMR (CD_3OD): δ 1.44-1.46 (m, 4H, 2xCH_2), 1.57-1.59 (m, 4H, 2xCH_2), 1.83-1.85 (m, 6H, 3xCH_2), 1.95-1.97 (m, 6H, 3xCH_2), 2.12-2.14 (m, 6H, 3xCH_2), 2.73 (t, 2H, *J*=6.9 Hz, 2xCH_2), 3.20-3.22 (m, 2H, CH_2), 3.84-3.86 (m, 1H, CH), 4.42 (s, 2H, CH_2), 4.45-4.47 (m, 2H, 2xCH), 7.24-7.29 (m, 5H, Ar-H), 7.39-7.41 (m, 1H, Im-H). ^{13}C NMR (100.6 MHz, CD_3OD): δ 23.5, 26.7, 29.2, 30.3, 37.1, 41.2, 42.3, 55.1, 128.4, 128.6, 129.7, 139.8, 157.9. MS (APCI): m/z 691.02 $[MH]^+$. $[\alpha]_D^{25}$ = -7.4° (c =1.3, CH_3OH)

4.1.3.12. His-Arg-Arg-NHBzl (15f). Yield: 87%, yellow solid, mp 204-206 °C, ^{1}H NMR (CD_3OD): δ 1.62-1.64 (m, 4H, 2xCH_2), 1.74-1.76 (m, 4H, 2xCH_2), 2.67 (t, 4H, *J* = 6.7 Hz, 2xCH_2), 3.20-3.22 (m, 2H, CH_2), 3.92-3.94 (m, 1H, CH), 4.38 (s, 2H, CH_2), 4.55-4.57 (m, 2H, 2xCH), 7.23-7.27 (m, 5H, Ar-H), 7.42-7.44 (m, 1H, Im-H). ^{13}C NMR (100.6 MHz, CD_3OD): δ 25.2, 28.7, 40.9, 42.9, 56.5, 126.9, 127.3, 128.3, 157.1. MS (APCI): m/z 557.20 $[MH]^+$. $[\alpha]_D^{25}$ = -20.1° (c =1.9, CH_3OH)

Antifungal activity

The fungal strains used in this study were received from Microbial Type Culture Collection, Institute of Microbial Technology (MTCC-IMTECH), Chandigarh, India and National Collection of Pathogenic Fungi (NCPF), Post-Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India. *C. albicans* NCPF 400034, *C. kyfer* NCPF 410004 and *C. neoformans* NCPF 250316 were cultured in yeast extract-peptone-dextrose (YEPD broth, HiMedia, India) and RPMI -1640 media (HiMedia, India). *A. niger* MTCC 2546 was grown on potato dextrose broth, whereas M2 medium was used for *N. crassa* MTCC 1876. For agar plates, 2.5% (w/v) bactoagar (HiMedia, Mumbai) was added to the medium. All strains were stored with 15% glycerol at -80°C as frozen stocks. The cells were freshly revived on respective agar plates from the stock before each experiment.[40]

The *in vitro* antifungal activity was performed according to the Clinical and Laboratory Standards Institute (CLSI, formerly NCCL) M27-A3 and M-38-A2 in RPMI-1640 medium by broth microdilution methods.[41] Final concentrations of synthesized compounds ranged between 1.95 µM and 250 µM. The microtiter plates were incubated without shaking at 30°C for 48 h. Both visual observation and determination of absorbance at 492 nm using a microplate reader (BioRed, Model 680) were used for growth inhibition. The MIC is defined as the lowest concentration of antifungal drug which resulted in >99% inhibition of growth compared to that of the drug free control. Amphotericin B, a known antifungal drug is used as a positive control.

Cytotoxicity Studies

MTT (3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was used for the mammalian toxicity of syntheiszed compounds against Hek-293 (normal human embryonic kidney cells) and HeLa (cervical cancer cells). Briefly, cells (5 X 10^4 /well) were cultured in RPMI-1640 medium supplemented with 10% fetal bovin serum in 96-well microtiter plate at 37° C for overnight. Synthesized peptides were added in the concentration of 250 µM, 125 µM, 62.5 µM, 31.5 µM, 15.75 µM, 07.37 µM and 03.68 µM to the cells in separate wells and incubated at 37°C for 18 hours. The cells

were further incubated at 37ºC for 3 to 4 h in 20µl of MTT solution (5 mg/ml) in PBS. The supernatant (120µl) was removed, 100µl DMSO was added, and the resulting suspension was mixed to dissolve the formazan crystals. The percent viability of cells was calculated by the ratio of OD_{570} of treated cells to the OD_{570} of untreated cells. Untreated cells and 10% dimethyl sulfoxide (DMSO) were taken as negative and positive control respectively.

Mechanistic studies

EYPC/EYPG (7:3 w/w) and EYPC/cholesterol (10:1w/w), were dissolved in chloroform in a glass vessel. Solvent was evaporated by air drying to form a thin film on the wall of a glass vessel. Then re-suspension of dried thin film was carried out in Tris-HCl buffer by vortexing and solution was sonicated in an ice/H_2O mixture for 10–20 mins with an ultrasonicator bath until the solution became clear. Further, to observe the effect of active peptide 15b on the synthesized SUVs, 10 µM of SUVs were incubated with 200 µM of peptide for 1-2 hrs. The morphological changes in untreated and treated SUVs were monitored by TEM. The samples for TEM analysis were prepared by depositing the solution on to a carbon coated copper grid and negatively staining the sample with 2% (w/v) phosphotungstic acid solution of pH 7.

Acknowledgements

This work was supported by the Science Engineering & Research Board (SERB), India grant no. SB/SO/BB/0040/2013, University Grants Commision (UGC), India grant no. 20-10 (12)/2012 (BSR) and Department of Science and Technology (DST-INSPIRE), India grant no. IFA12-CH-52. Sherry Mittal and Sarabjit Kaur thank the University Grants Commission (UGC), New Delhi and Council of Scientific and Industrial Research (CSIR), New Delhi, respectively for the award of Senior Research Fellowship.

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View Article Online
DOI: 10.1039/C6RA05883C

Graphical Abstract