Synthesis, stability and mechanistic studies of potent anticryptococcal hexapeptides

Kitika Shenmar,^a Krishna K. Sharma,^a Nishima Wangoo,^b Indresh K. Maurya,^c Vinod Kumar,^d Shabana I. Khan,^e Melissa R. Jacob,^e Kulbhushan Tikoo^d and Rahul Jain^{a*}

^a*Department of Medicinal Chemistry, National Institute of Pharmaceutical Education and Research, Sector 67, S. A. S. Nagar, Punjab, 160 062, India*

^b*Department of Applied Sciences, University Institute of Engineering and Technology (U.I.E.T.), Panjab University, Sector-25, Chandigarh-160014, India*

^c*Department of Microbial Biotechnology, Panjab University, Sector-14, Chandigarh-160014, India*

^d*Department of Pharmacology and Toxicology, National Institute of Pharmaceutical Education and Research, Sector 67, S. A. S. Nagar, Punjab, 160 062, India*

^e*National Center for Natural Products Research, School of Pharmacy, The University of Mississippi, University, MS 38677, USA*

Address for correspondence:

Dr. Rahul Jain

Department of Medicinal Chemistry

National Institute of Pharmaceutical Education and Research

Sector 67, S. A. S. Nagar, Punjab, 160 062, India

E-mail: rahuljain@niper.ac.in

Tel.: 91-172-2292024; Fax: 91-172-2214692

Abstract

The growing incidents of *cryptococcosis* in immuno-compromised patients have created a need for novel drug therapies capable of eradicating the disease. The peptide-based drug therapy offers many advantages over the traditional therapeutic agents, which has been exploited in the present study by synthesizing a series of hexapeptides that exhibits promising activity against a panel of Gram-negative and Gram-positive bacteria and various pathogenic fungal strains; the most exemplary activity was observed against *Cryptococcus neoformans*. The peptides **3**, **24**, **32** and **36** displayed potent anticryptococcal activity ($IC_{50} = 0.4 - 0.46 \mu\text{g/mL}$, $MIC = 0.63 - 1.25 \mu\text{g/mL}$, $MFC = 0.63 - 1.25 \mu\text{g/mL}$), and stability under proteolytic conditions. Besides this, several other peptides displayed promising inhibition of pathogenic bacteria. The prominent ones include peptides **18-20**, and **26** that exhibited IC_{50} values ranged between $2.1 - 3.6 \mu\text{g/mL}$, MICs of $5 - 20 \mu\text{g/mL}$ and MBCs of $10 - 20 \mu\text{g/mL}$ against *Staphylococcus aureus* and methicillin-resistant *S. aureus*. The detailed mechanistic study on selected peptides demonstrated absolute selectivity towards the bacterial membranes and fungal cells by causing perturbations in the cell membranes, confirmed by the scanning electron microscopy and transmission electron microscopy studies.

Keywords: Anticryptococcal; Amphiphilic hexapeptides, Solid phase peptide synthesis; Proteolysis; Electron microscopy.

1. Introduction

Immuno-compromised patients are the most susceptible targets for mycosis. Cryptococcosis, a fungal infection, whose etiological agents include, *Cryptococcus neoformans* and *Cryptococcus gatii* affects immuno-compromised and immuno-competent patients. The *C. neoformans* infections manifested in immuno-compromised patients, are the chief reason behind the deaths caused by fatal meningoencephalitis, observed in acquired immune deficiency syndrome (AIDS) patients [1, 2]. Due to growing AIDS pandemic, the simultaneous spread of *C. neoformans* is also expanding creating a need to discover new drugs.

Antimicrobial peptides (AMPs) are part of innate immunity system of the organisms and have been isolated from almost all life forms including plants, animals, microbes and insects [3-9]. AMPs are usually dual cationic and hydrophobic in nature, with a tendency to fold into amphiphilic structures upon coming in close proximity of target membranes. Their unique mode of action involves non-specific membrane interactions leading to its disruption and leakage of the cell contents resulting in cell death [10]. The cell lysis is not peptide sequence dependent, instead physicochemical interactions (amphiphilicity and basicity), which forms the basis of molecular mechanisms are responsible for the targeting and killing of microbial cells [11]. Because of their natural origin, AMPs are predisposed to effectively distinguish between host and pathogen cells. The difference in membrane compositions of host and pathogen cells forms the basis of dissimilarity and results in the selective action of AMPs [12]. A large number of short-peptide based and non-peptide based compounds exhibiting promising antifungal activity, including activity against *C. neoformans* are reported [13-20].

The ability of human body to degrade peptides metabolically poses one of the biggest challenges in the development of peptide-based antibiotics [21]. Other factors that play considerable role are the high cost of production due to long amino acid sequences of AMPs, immunogenic properties, simultaneous action of peptides on multiple targets at once, leading to side effects [22, 23]. Hence, due to all these reasons, traditionally peptide-based drugs have not been explored [24]. The use of unnatural amino acids, amide backbone modification, tethering the peptide core using various linkers, conjugating lipophilic acids, increasing the bulk of the peptide by hydrophobic tagging using tryptophan, and truncation of larger peptides or proteins while retaining the essential bioactive core sequences are some of the techniques employed for the generation of peptide-based therapeutics that retains and enhances the efficacy of native peptides [25-29]. A number of hexapeptides are known to exhibit anti-candidal, anti-staphylococcal and anti-*Escherichia coli* activity with MICs in micro molar range [30, 31]. The growth hormone (GH, somatotropin) regulates body composition in adults and optimal statural growth in children [32]. Ghrelin is the endogenous ligand for the GH secretagogue receptor (GHSR) and stimulates the anterior pituitary gland to release GH [33-38]. Recently, ghrelin has been observed to possess bactericidal activity against Gram-ve *E. coli* and *Pseudomonas aeruginosa* [39].

2. Results and discussion

Several truncated analogues of 191 amino acids containing GH have been synthesized. A study resulted in the identification of a synthetic congener called GHRP-6 (His-D-Trp-Ala-Trp-D-Phe-Lys-NH₂) [40]. This peptide served as the lead for a number of optimization studies resulting in peptides that possessed a variety of pharmacological

activities, ranging from wound healing to antifungal [41, 42]. One such identified peptide, His-D-Trp-D-Phe-Phe-D-Phe-Lys-NH₂ exhibited antifungal activity against five pathogenic fungi in the range of 6.2 – 100 µg/mL. Further structural optimization by bio-screening of synthetic combinatorial libraries resulted in the identification of another antifungal peptide Arg-D-Trp-D-Phe-Ile-D-Phe-His-NH₂ that exhibit IC₅₀ value of 6.8 µM against *Candida albicans* [43, 44]. A detailed investigation by our group, of the above mentioned peptide resulted in several analogues with far superior antimicrobial activity [45]. In the present study, out of the many bioactive peptides, Orn-D-Trp-Cha-Ile-D-Phe-His(1-Bzl)-NH₂ (**1**) and Orn-D-Trp-D-Phe-Phe(4-Me)-D-Phe-His(1-Bzl)-NH₂ (**2**) were chosen for further in-depth structural optimization (Fig. 1). The peptides **1-2** displayed a cross-sectional activity profile with IC₅₀s in the range of 0.07 – 18.9 µg/mL and MICs in the range of 1 – 100 µg/mL against methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-resistant *S. epidermis* (MRSE), *E. coli* and *C. albicans*. In this study, it was assumed that independent replacement to the hydrophilic or hydrophobic core of peptides **1-2** could possibly result in enhancement of biological activity along with selectivity for bacteria or fungi. In peptide **1**, we retained the terminus hydrophilic amino acids while hydrophobic core was replaced with physico-chemically analogous amino acids. In peptide **2**, the hydrophobic core was kept intact, while the terminus hydrophilic amino acids were independently replaced with physico-chemically analogous amino acids (Fig. 1). The overall net charge, facial amphiphicity, and optimal hydrophobic bulk were considered as key factors in selecting replacement amino acids [46-48].

Modifications at positions 2, 4 and 5 of peptide 1

To study the effect of varying the hydrophobicity on the bioactivity of peptide Orn-D-Trp-Cha-Ile-D-Phe-His(1-Bzl)-NH₂ (**1**), constituent amino acids contributing to the hydrophobic core were systematically and independently replaced. First we replaced D-Trp at the position 2 of peptide 1 with bulky proteogenic and non-proteogenic amino acids, resulting in the synthesis of peptides **3-12**. The peptides were synthesized using Fmoc-based solid phase peptide synthesis protocol exemplified by synthesis of peptide **3** (Scheme 1). Isoleucine, a moderately hydrophobic amino acid present at the position 4 of peptide 1 was replaced next with various counterpart amino acids. The chosen replacement amino acids vary from moderately hydrophobic to highly hydrophobic, leading to peptides **13-21**. The amino acid phenylalanine (D-Phe) at provides bulk and considered as a contributing factor in the maintenance of the ideal bulk-to-charge ratio required for exerting antimicrobial activity. The replacement of D-Phe at the position 5 of peptide 1 with non-proteogenic amino acids with enhanced bulk and hydrophobic character resulted in the synthesis of peptides **22-25**. The other hydrophobic amino acid cyclohexylalanine (Cha) considered critical for activity was not replaced.

Modifications at positions 1 and 6 of peptide 2

As discussed above, in the case of antimicrobial peptides a fine balance between amphiphilicity and hydrophobicity enables them to exert activity. The hydrophobic core of peptide Orn-D-Trp-D-Phe-Phe(4-Me)-D-Phe-His(1-Bzl)-NH₂ (**2**) is already composed of hydrophobic unnatural amino acids, and thus we replaced both terminus hydrophilic amino acids [Orn and His(1-Bzl)]. The replacement of ornithine (Orn) at the N-terminus resulted in the synthesis of five peptides **26-30**. The replacement amino acids were aliphatic proteogenic and non-proteogenic amino acids [L-Arg, D-Arg, Lys and 2,4-

diaminobutyric acid (Dab)], besides aromatic 4-aminophenylalanine [Phe(4-NH₂)]. C-Terminus in peptide **2** contains amphiphilic 1-benzylhistidine [His(1-Bzl)] residue that provide charge via imidazole ring and hydrophobicity via benzyl ring. The replacement amino acids used were a combination of both natural and unnatural amino acids [His, D-His, D-Lys, D-Arg, Orn, D-Orn, Dab) and Phe(4-NH₂)], chosen in a way that they would contribute in increasing the overall cationic charge of final peptides **31-38**.

2.1. Antimicrobial activity and cytotoxicity evaluation

Various pathogenic bacteria such as *S. aureus*, *E. coli*, *P. aeruginosa*, *Mycobacterium intracellulare*, and MRSA were used as targets for *in vitro* testing of the peptides. The susceptibility of the fungal strains *C. neoformans*, *C. albicans*, *C. glabrata*, *C. krusei* and *Aspergillus fumigatus* were also evaluated for the synthesized peptides and results are summarized in Tables 1-2.

In general, peptides exhibited promising activity against fungal strains (Table 1). Peptides **13, 17, 18, 19, 20, 21, 24**, and **25-26** produced IC₅₀ values ranged between 6.05 – 19.6 µg/mL, while minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values between 10 – 20 µg/mL were displayed against *C. albicans*. Against *C. glabrata*, peptides **18, 19, 22**, and **24-25** displayed IC₅₀s between 7.56 – 17.9 µg/mL, while MICs and MFCs between 10 – 20 µg/mL were produced. A large number of peptides showed promising inhibition of *C. krusei*. The most promising peptide **25** [Orn-D-Trp-Cha-Ile-Ala(9-anth)-His(1-Bzl)-NH₂] exhibited IC₅₀ of 3.81 µg/mL, MIC of 5 µg/mL and MFC value of 10 µg/mL. The remaining active peptides **3, 10, 13-16, 24, 26-28, 30, 32-33**, and **35-38** produced IC₅₀s between 5.16 – 19.98 µg/mL. The same set of peptides also exhibited MIC and MFC values ranged in 10 – 20 µg/mL against *C.*

krusei. Fungal strain *A. fumigatus* proved to be least susceptible to peptides of the series; the exceptions being peptides **18-20** that produced IC_{50} s ranged between 6.56 – 19.9 $\mu\text{g/mL}$ and MIC and MFC values between 10 – 20 $\mu\text{g/mL}$. In general, peptides exhibited highly potent activity ($IC_{50} = 0.4 - 4.73 \mu\text{g/mL}$, MIC = 0.63 – 5.00 $\mu\text{g/mL}$, MFC = 0.63 – 5.00 $\mu\text{g/mL}$) against *C. neoformans*. Some of the significantly potent peptides of the series are **3** (Orn-Trp-Cha-Ile-D-Phe-His(1-Bzl)-NH₂, $IC_{50} = 0.42 \mu\text{g/mL}$, MIC = MFC = 0.63 $\mu\text{g/mL}$), **24** (Orn-D-Trp-Cha-Ile-Cha-His(1-Bzl)-NH₂, $IC_{50} = 0.46 \mu\text{g/mL}$, MIC = MFC = 1.25 $\mu\text{g/mL}$), **32** (Orn-D-Trp-D-Phe-Phe(4-Me)-D-Phe-Orn-NH₂, $IC_{50} = 0.46 \mu\text{g/mL}$, MIC = MFC = 0.63 $\mu\text{g/mL}$) and **36** (Orn-D-Trp-D-Phe-Phe(4-Me)-D-Phe-D-Lys-NH₂, $IC_{50} = 0.40 \mu\text{g/mL}$, MIC = MFC = 0.63 $\mu\text{g/mL}$). The inhibitory values exhibited by these and a large number of other peptides of the series were superior compared to standard drug amphotericin B ($IC_{50} = 0.69 \mu\text{g/mL}$, MIC = MFC = 1.25 $\mu\text{g/mL}$). The potent anticryptococcal activity was noted when D-Trp at position 2 was replaced with L-Trp residue in peptide **1**, resulting in peptide **3**. Similarly, potent peptide **24** was obtained by replacement of D-Phe⁴ with unnatural residue Cha in peptide **1**. On the other hand, potent peptides **32** and **36** were synthesized by replacing His(1-Bzl)⁶ with unnatural residues ornithine (Orn) and D-lysine (D-Lys), respectively. It may be noted that potent peptides **32** and **36** are composed of only unnatural amino acids, whereas peptides **3** and **24** contains four and five unnatural amino acids in their respective composition (Table 1).

The peptides **18** and **26** exhibited promising antibacterial activity ($IC_{50} = 3.2 \mu\text{g/mL}$, MIC = 10 – 20 $\mu\text{g/mL}$, MBC = 20 $\mu\text{g/mL}$) upon evaluation against pathogenic MRSA strain. The remaining active peptides of the series exhibited IC_{50} values ranged between 5.8 –

18.6 $\mu\text{g/mL}$. In the case of *S. aureus*, peptides **25** produced very promising activity (IC_{50} = 1.61 $\mu\text{g/mL}$, MIC = 5 $\mu\text{g/mL}$, MBC = 10 $\mu\text{g/mL}$), while peptides **18, 19, 20, 26** and **30** exhibited IC_{50} values of 2.4, 2.63, 3.6, 2.1 and 6.0 $\mu\text{g/mL}$, respectively. The peptides were noted to show less potency against *E. coli*. The peptide **37** produced IC_{50} value of 4.86 $\mu\text{g/mL}$ (*E. coli*). In general, peptides did not show promising inhibition of *P. aeruginosa* and *M. intracellulare*; the exception being peptide **26** that exhibited IC_{50} value of 6.5 $\mu\text{g/mL}$ and MIC and MBC value of 10 $\mu\text{g/mL}$ against *M. intracellulare* (Table 2).

The *in vitro* cytotoxic effects of the synthesized peptides was determined against noncancerous VERO cells obtained from ATCC, American Type Culture Collection. None of the tested peptides were cytotoxic up to the highest concentration of 10 $\mu\text{g/mL}$ thereby providing a selective index between >21 – 25 for the potent peptides (**3, 24, 32** and **36**) (Table 1).

2.2. Tryptophan quenching study

Liposomes are microscopic phospholipid bubbles with at least a single lipid-layered membrane structure [49]. When only a single amphiphilic lipid layer is present, liposomes are called small unilamellar vesicles (SUVs). Based on their phospholipid composition, SUVs can be altered to represent either bacterial membrane-mimics or mammalian membrane-mimics. The bacterial cell membranes are composed of more negatively charged lipopolysaccharides and acids, whereas the mammalian cell membranes are zwitterionic in nature. In order to ascertain that synthesized peptides have the ability and specificity to target only bacterial cells over the mammalian cells and cause selective membrane disruption, we used synthetic bacterial and mammalian

membrane mimics for testing five selected peptides (**3**, **24**, **32**, **34** and **36**). Peptides **3**, **24**, **32**, and **36** constitute a group that displayed highest anticryptococcal activity while peptide **34** was the least active of the set. The peptides were read for their fluorimetric emission spectrum and the readings plotted in the form of Stern-Volmer plots (Fig. 2).

The plots depict the difference in the slopes of graphs upon the differential interactions of the peptides in the presence/absence of SUVs. In the case of control (Tris-HCl) graph, the fluorescence of peptides decreases in a concentration dependent manner by addition of acrylamide. But as there were no SUVs in this solution, hence, Trp residue was freely available for quenching by acrylamide resulting in a higher slope of the graph as compared to SUVs containing solutions. On the other hand, the EYPC:EYPG containing negatively charged bacterial membrane mimicking SUVs electrostatically attracted active peptides, resulting in successful burial of peptides within the membrane. As a result, the Trp residue was unavailable for quenching by acrylamide, and the Stern-Volmer plot shows equal Trp-quenching as the slope of the representative graph. In the case of EYPC:cholesterol SUVs, the peptides do not preferentially bind to zwitterionic membrane mimicking SUVs, and thus, the Trp residue is not buried in the SUVs membrane and is available for quenching by acrylamide. The slope of this graph is higher than that of bacterial membrane mimicking SUVs and peptide interactions and lower than that of the control.

2.3. Trypsin digestion study

To determine the proteolytic stability, a trypsin digestion study was performed on the hexapeptides **3**, **32** and **36**. As seen from the HPLC chromatogram, peptide remains stable after 24 h of trypsin treatment. This shows that all three peptides retained its

structural integrity during trypsin digestion, and was not susceptible to proteolytic action of the enzyme (Fig. 3).

2.4. Scanning electron microscopic (SEM) study

In order to gain further insights into the mechanism of action of the designed peptides, two of the most active peptides **3** and **36** along with the least active peptide **34** of the series were treated with real *C. neoformans* fungal biofilms, and analyzed using scanning electron microscopy (SEM). The SEM imaging of the blank sample revealed smooth surfaced fungal cells of the fungal biofilm (Fig 4a). After treatment with the most active peptide **3** and **36**, the cells appeared to have been lysed, in the process, created holes in the fungal biofilm (Fig 4b-c). However, in the case of peptide **34**, the cells appear to be corrugated and perturbations led to the killing of the fungal cells (Fig. 4d).

2.5. Transmission electron microscopy (TEM) study on artificial membranes

Trp-quenching and SEM studies regarding the mechanism of action of the peptides had shed light on their selectivity towards the bacterial cells and the damage caused to the *C. neoformans* fungal biofilms (seen as surface deformities). To gain insights with respect to the internal damage caused to the bacterial cells by the peptides, we undertook transmission electron microscopic (TEM) study. We first analyzed the effect of peptide treatment on the bacterial membrane-mimicking and mammalian-mimicking SUVs. The most active peptide **36** was chosen for the study. The image of bacterial membrane-mimicking liposomes taken in the absence of **36** show smooth and spherical morphology with an average diameter of 20-100 nm (Fig 5a). On the other hand, images of peptide **36** treated liposomes showed lysed and disfigured bacterial membrane-mimicking liposomes (Fig 5b). In the case of mammalian membrane-mimicking liposomes, peptide **36** has

virtually left the liposomes intact proving its selectivity towards the bacterial cells (Figs. 5c-d).

2.6. Transmission electron microscopy (TEM) study on *C. neoformans* biofilms

After gaining information about the interactions of the active peptide **36** with the membrane mimicking SUVs, we finally studied the interactions of peptides **3**, **34** and **36** with *C. neoformans* biofilm. The images obtained in the study describe the events leading to death of the fungal cells. The ultrastructure of the untreated *C. neoformans* cells (Fig. 6a) depicts cells containing a healthy granular cell membrane (as shown by the arrow). Upon treatment of *C. neoformans* biofilm with **3**, it was observed that the cell wall loses its granularity leading to leakage of cytoplasm (Fig. 6b). In the case of peptide **34**, the biofilm show the loss of cell wall as no granular outer layer can be distinguished (Fig. 6c). The TEM image for peptide **36** show the abnormal subcellular morphology with the formation of pits as depicted by the arrows in Fig. 6d. These results demonstrate that peptides exhibit the potent antifungal bioactivity by disruption in the cell wall of the pathogenic fungi.

3. Conclusions

In this study, thirty-six peptides were synthesized by modifying hydrophobic core and hydrophilic C- and N-terminus of peptides **1-2**. All synthesized peptides were subjected to in vitro antimicrobial activity evaluation against a panel of pathogenic bacteria and fungi. Some of the synthesized peptides displayed remarkably high activity against fungi *C. neoformans*. A number of peptides also exhibited high potency against pathogenic bacteria. The study revealed the identity of anticryptococcal hexapeptides having potent efficacy against *C. neoformans* as compared to the standard drug amphotericin B. The

synthesized peptides show selectivity towards the bacterial membranes over their mammalian counterparts as proven by the tryptophan fluorescence quenching study. The trypsin digestion study confirms the stability of peptide **3** and **36** under the proteolytic conditions, thereby providing evidence of its suitability as promising drug candidate. The SEM study provides insights into the damage caused by the active peptides **3** and **36** to fungal biofilm. The TEM study on artificial membranes provided evidence for the damage caused exclusively to the bacterial membrane mimicking SUVs by peptide **3**. The TEM study using fungal biofilm of *C. neoformans* showed the effect of the peptide treatment at the cellular level with the images confirming the effect on the fungal cell wall. In conclusion, the promising peptides composed of mostly non-proteogenic amino acids are a new class of compounds that have potential utility in the treatment of pathogenic opportunistic infections caused by bacteria and fungi.

4. Experimental

4.1. Synthesis

All chemicals were purchased from Sigma-Aldrich, Missouri, U.S.A. and Chem-Impex International, Illinois, U.S.A. and used without any further purification, unless specified. Peptide synthesis was performed on Aapptec fully automated peptide synthesizer using Fmoc synthesis protocol. The high-resolution mass spectra were recorded on a MAXIS-Bruker spectrometer using ESI-TOF method. The peptides were analyzed on a Shimadzu SPD-M20A analytical HPLC system using a supelcosilTM LC-8, 5 μ m (25 cm x 4.6 mm ID) column. The peptides were purified on the Shimadzu HPLC preparative system using a Luna 10 μ M C18 (2) (250 mm x 21.2 mm, 10 μ m) column.

4.1.1. General procedure for solid phase synthesis of peptides

In a reaction vessel, Fmoc-protected Rink amide MBHA resin was first swelled in DMF for fifteen min. A solution of 20% piperidine in DMF was added and mixture shaken mechanically for 15 min resulting in the removal of Fmoc group. The required Fmoc protected amino acids and coupling reagent 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) were placed in amino acid vessels sequentially. DMF was added to the amino acid vessel, which was subsequently added (by positive pressure of N₂) to the reaction vessel containing the resin, followed by addition of *N,N*-diisopropylethylamine (DIEA). After 3 h of mechanical shaking at ambient temperature, the solvent was drained and the resin washed with DMF (3 × 5 min) followed by methanol (2 × 5 mL). The cycles of deprotection and coupling were repeated till the desired peptide chain length was obtained. The resin-bound peptide was transferred to a round bottom flask, and simultaneous removal of resin and protective groups was achieved by using a cocktail combination of TFA:triisopropylsilane (TIPS):H₂O [95:2.5:2.5] for 3 h. The crude peptide was filtered and purified on preparative HPLC system, and analyzed using solvent system of CH₃CN-H₂O-0.1% CF₃COOH in the gradient system: 30 min gradient, 30-100% CH₃CN-H₂O-0.1% CF₃COOH at 215 nm.

4.2. Antimicrobial activity evaluation

The antibacterial activities of synthesized peptides were evaluated against *Staphylococcus aureus* ATCC 29213, methicillin-resistant *S. aureus* ATCC 33591 (MRSA), *Mycobacterium intracellulare* ATCC 23068, *Escherichia coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853. Susceptibility of *S. aureus*, MRSA, *E. coli* and *P. aeruginosa* to test compounds was determined using CLSI methods [50, 51].

Susceptibility of *M. intracellulare* was determined using the modified Alamar Blue procedure of Franzblau *et al.* [52]. The antifungal activities of the target compounds against *Cryptococcus neoformans* ATCC 90113, *C. albicans* ATCC 90028, *C. glabrata* ATCC 90030, *C. krusei* ATCC 6258, and *A. fumigatus* ATCC 90906 were determined according to modified CLSI methods [53].

Test Procedure: Samples are serially-diluted in 20% DMSO/saline and transferred in duplicate to 96 well flat bottom microplates. Inocula are prepared by correcting the OD₆₃₀ of microbe suspensions in incubation broth [RPMI 1640/2% dextrose/0.03% glutamine/MOPS @ pH 6.0 (Cellgro) for *C. albicans*, Sabouraud Dextrose for *C. neoformans*, cation-adjusted Mueller-Hinton (Difco) @ pH 7.3 for MRS, 5% Alamar Blue™ (BioSource International, Camarillo, CA) in Middlebrook 7H9 broth with OADC enrichment, pH = 7.3 for *M. intracellulare*, and 5% Alamar Blue™/RPMI 1640 broth (2% dextrose, 0.03% glutamine, buffered with 0.165M MOPS at pH 7.3) for *A. fumigatus* to afford final target inocula of: *C. albicans*: 1.0×10^4 , *C. neoformans*: 1.0×10^5 , *M. intracellulare*: 2.0×10^6 , MRS, *C. glabrata*, *C. krusei*: 5.0×10^5 CFU/mL, and *A. fumigatus*: 3.0×10^4 CFU/mL. Drug controls [Ciprofloxacin (ICN Biomedicals, Ohio) for bacteria and Amphotericin B (ICN Biomedicals, Ohio) for fungi] are included in each assay. All organisms are read at either 630 nm using the EL-340 Biokinetics Reader (Bio-Tek Instruments, Vermont) or 544ex/590em, (*M. intracellulare*, *A. fumigatus*) using the Polarstar Galaxy Plate Reader (BMG LabTechnologies, Germany) prior to and after incubation: *C. albicans*, MRS, *C. glabrata* and *C. krusei* at 37 °C for 18 – 24 h, *C. neoformans* and *A. fumigatus* at 30 °C for 68 – 72 h, and *M. intracellulare* at 37 °C and 10% CO₂ for 68 – 72 h. The MIC is defined as the lowest test concentration that allows

no detectable growth (for *M. intracellulare* and *A. fumigatus*, no color change from blue to pink). Minimum fungicidal or bactericidal concentrations are determined by removing 5 μ L from each clear (or blue) well, transferring to agar and incubating as previously mentioned. The MFC/MBC is defined as the lowest test concentration that kills the organism (allows no growth on agar).

4.3. Cytotoxicity evaluation

The noncancerous VERO cells obtained from ATCC, American Type Culture Collection, were used to test for the *in vitro* cytotoxic effects of the synthesized peptides by neutral red assay [54].

Test procedure: The assay was performed in 96-well tissue culture-treated microplates and peptides were tested up to a highest concentration of 10.00 μ g/mL. The cells (25000 cells/well) were seeded to the walls of the wells and incubated for 24 h. After sample addition, the plates were again incubated for an additional 48 h. The number of viable cells was counted using a modified procedure for the neutral red assay. The CC₅₀ were computed from the logarithmic graphs of growth inhibition versus concentration of the test peptide. The controls used in this assay were doxorubicin as the positive control and DMSO as the negative or vehicular control.

4.4. Tryptophan quenching assay

The egg yolk L- α -phosphatidylcholine (EYPC), egg yolk L- α -phosphatidyl-D,L-glycerol (EYPG), acrylamide and cholesterol were purchased from Sigma Chemical Company, St. Louis, USA and used without any further purification. All other reagents were analytical grade. The buffers were prepared in double glass-distilled water.

The SUVs were made comprising of lipid mixture EYPC, EYPG and/or cholesterol. The SUVs representative of bacterial (EYPC:EYPG = 7:3) and mammalian cell membranes (EYPC:cholesterol = 10:1) were prepared by dissolving the dry lipids in chloroform and then air drying. The dry lipids were re-suspended in Tris-HCl buffer (pH 7.4) using vortex mixing. The dispersed lipids were sonicated for 10-15 min in an ice-water mixture, till the solution became transparent. The SUVs were incubated with selected peptides **3**, **24**, **32**, **34** and **36**. The concentration of the peptides taken was 0.1 μ M. To investigate the relative extent of peptide burial in the model membranes, a fluorescence quenching experiment was performed using the Cary Eclipse spectrofluorometer at 25 °C and 300 μ L quartz cuvette having 1 cm path length and a magnetically stirred, thermostated cuvette compartment using excitation wavelength of 295 nm. The bandwidth of 5 nm was used for both excitation and emission. To reduce the absorbance by acrylamide, the excitation of Trp at 295 nm, instead at 280 nm was used. The aliquots of 4.0 M solution of the acrylamide were added to the peptide in the absence or presence of liposomes at a peptide-lipid molar ratio of 1:200. The acrylamide concentration in the cuvette varied from 0 to 0.20 M. The scan speed was 500 nm/min with the emission intensities scanned from 300-550 nm. An average of three scans per reading were taken after 5 min of equilibration before recording. The effect of acrylamide on the fluorescence of each peptide was analyzed with a Stern-Volmer equation:

$$F_0/F = 1 + KSV [Q]$$

Where, F_0 and F represent the fluorescence intensities in the absence and the presence of acrylamide, respectively, KSV is the Stern-Volmer quenching constant, and $[Q]$ is the concentration of acrylamide.

4.5. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) studies

SEM sample preparation: The samples were prepared by first culturing *C. neoformans* in overnight growth cultures. The following day, cell suspensions were prepared using RPMI-1640 medium (pH 7.4). This was followed by adding **3**, **34** and **36** (at their MIC concentrations) to fungal cells ($\sim 1 \times 10^6$ CFU/mL) and incubating at 30 °C. Upon treatment, the samples were fixed in glutaraldehyde followed by repetitive rinsing with 0.1 M sodium cacodylate and 0.22 M sucrose solution. Poly-L-lysine hydrobromide was used as the coverslip coating material and the agar films were allowed to settle on the surface. The post-fixation process involved treatment with 2% osmium tetroxide buffered to pH 7.4 with 0.05 M veronal acetate at 4 °C for 1 h. The dehydration process involved a gradual gradient rinsing cycle with progressive ethanol-acetone mixture till the final rinse cycle reached 100% acetone. The sample coverslips were attached to the SEM holder and dried with CO₂ till the critical point. The sample preparation's last step involved sputter coating with gold. The samples were analyzed using SEM (Zeiss EV040) [55, 56].

TEM study using artificial membranes (sample preparation): The appearance of the peptide **36** was monitored by depositing, on to a carbon coated copper grid, a drop of peptide solution (0.5 µg/mL), and negatively staining the sample with 2% (w/v) phosphotungstic acid solution. The interactions were examined using TEM and multiple images were taken for each sample.

TEM study using actual fungal biofilms (sample preparation): The samples were prepared by incubating the fungal biofilm with the peptides **3**, **34** and **36** at their

respective MIC concentrations for 1 h. Usually the samples are negatively stained using 1-2% of phosphotungstic acid, ammonium molybdate or uranyl acetate. The heavy metal staining allows for a better high contrast, which increases the 2D crystal lattices structural clarity under the electron microscope. Ultra-thin section cutting was done with an ultramicrotome (Ultramicrotome Lecia EM UC6). The samples are then spotted on carbon coated copper grids and negatively stained. The transmission electron microscopy was performed on a Hitachi H-7500 microscope at an accelerating voltage of 200 kV. The carbon coated 400 mesh copper grids and the negative stain uranyl acetate was both obtained from the Agar Scientific, USA.

4.6. Trypsin digestion study

To study the stability of the peptides **3**, **32** and **36** for tryptic degradation, peptide along with trypsin were taken in a molar ratio of 0.5:100 (peptide:trypsin) in a solution containing 0.1 M ammonium bicarbonate (NH_4HCO_3) and 0.1 mM calcium chloride (CaCl_2) at pH 8.3. The solution was incubated in a rotary shaker incubator (37 °C, 200 rpm, 24 h). Aliquots of the solution were taken at time intervals of 0, 2, 4, 8, 12, 18 and 24 h. Aliquots were analyzed on a Shimadzu SPD-M20A analytical HPLC system using a Supelcosil TM LC-8, 5 μm (25 cm x 4.6 mm ID) column using CH_3CN (0.1% TFA): H_2O (0.1% TFA) linear gradient system of 30-100% in 30 min with a flow rate of 1 mL/min.

Acknowledgements

Kitika Shenmar thanks National Institute of Pharmaceutical Education and Research (NIPER) to Kitika Shenmar for providing senior research fellowship. Ms Marsh Wright and Mr John Trott are acknowledged for excellent technical support in biological testing at NCNPR. Antimicrobial testing was supported by the NIH, NIAID, Division of AIDS,

Grant No. AI 27094 (antifungal) and the USDA Agricultural Research Service Specific Cooperative Agreement No. 58-6408-1-603 (antibacterial).

References

- [1] K.J. Kwon-Chung, J.A. Fraser, T.L. Doering, Z. Wang, G. Janbon, A. Idnurm, Y.-S. Bahn, *Cryptococcus neoformans* and *Cryptococcus gattii*, the etiologic agents of cryptococcosis, Cold Spring Harb Perspect Med. 4 (2014) a019760.
- [2] C. Coelho, A. Casadevall, Cryptococcal therapies and drug targets: the old, the new and the promising, Cell Microbiol. 18 (2016) 792-799.
- [3] W.F. Broekaert, B.P. Cammue, M.F. De Bolle, K. Thevissen, G.W. De Samblanx, R.W. Osborn, K. Nielson, Antimicrobial peptides from plants. Crit. Rev. Plant Sci. 16 (1997) 297-323.
- [4] W.F. Broekaert, F. Terras, B. Cammue, R.W. Osborn, Plant defensins: novel antimicrobial peptides as components of the host defense system. Plant Physiol. 108 (1995) 1353.
- [5] T. Ganz, Defensins: antimicrobial peptides of innate immunity. Nat. Rev. Immunol. 3 (2003) 710-720.
- [6] T. Ganz, R.I. Lehrer, Antimicrobial peptides of vertebrates. Curr. Opin. Immunol. 10 (1998) 41-44.
- [7] I.F. Nes, H. Holo, Class II antimicrobial peptides from lactic acid bacteria. Pept. Sci. 55 (2000) 50-61.
- [8] P. Bulet, C. Hetru, J.-L. Dimarcq, D. Hoffmann, Antimicrobial peptides in insects; structure and function. Dev. Comp. Immunol. 23 (1999) 329-344.

- [9] P. Bulet, R. Stocklin, Insect antimicrobial peptides: structures, properties and gene regulation. *Protein Pept. Lett.* 12 (2005) 3-11.
- [10] A. Mor, Peptide-based antibiotics: A potential answer to raging antimicrobial resistance. *Drug Dev. Res.* 50 (2000) 440-447.
- [11] Y. Shai, Mode of action of membrane active antimicrobial peptides. *Pept. Sci.* 66 (2002) 236-248.
- [12] S.K. Straus, R.E. Hancock, Mode of action of the new antibiotic for Gram-positive pathogens daptomycin: comparison with cationic antimicrobial peptides and lipopeptides, (BBA)-Biomembranes 1758 (2006) 1215-1223.
- [13] R.K. Sharma, R.P. Reddy, W. Tegge, R. Jain, R. Discovery of Trp-His and His-Arg analogues as new structural classes of short antimicrobial peptides. *J. Med. Chem.* 52 (2009), 7421-7431.
- [14] A. Mahindra, N. Bagra, N. Wangoo, S.I. Khan, M. R. Jacob, R. Jain, Discovery of short peptides exhibiting high potency against *Cryptococcus neoformans*. *ACS Med. Chem. Lett.* 5 (2014), 315-320.
- [15] A. Mahindra, K.K. Sharma, D. Rathore, S.I. Khan, M.R. Jacob, R. Jain, Synthesis and antimicrobial activities of His(2-aryl)-Arg and Trp-His(2-aryl) classes of dipeptidomimetics. *Med. Chem. Commun.* 5 (2014), 671-676.
- [16] A. Mahindra, N. Bagra, N. Wangoo, R. Jain, S.I. Khan, M.R. Jacob, R. Jain, Synthetically modified L-histidine-rich peptidomimetics exhibit potent activity against *Cryptococcus neoformans*. *Bioorg. Med. Chem. Lett.* 24 (2014), 3150-3154.

- [17] S. Mittal, I.K. Maurya, S. Kaur, A. Swami, R. Jain, N. Wangoo, R.K. Sharma, Insights into mechanistic and synergistic aspects of novel synthetic short cationic antibacterial peptides. *ChemistrySelect*, 1 (2016), 5510-5516.
- [18] S. Mittal, S. Kaur, A. Swami, I.K. Maurya, R. Jain, N. Wangoo, R.K. Sharma, Alkylated histidine based short cationic antifungal peptides: synthesis, biological evaluation and mechanistic investigations. *RSC Adv.* 6 (2016), 41951-41961.
- [19] N.P. de Sa, C. I. Lino, N.C. Fonseca, B.M. Borelli, J.P. Ramos, E.M. Souza-Fagundes, C.A. Rosa, D.A. Santos, R.B. de Oliveira, S. Johann, Thiazole compounds with activity against *Cryptococcus gattii* and *Cryptococcus neoformans* *in vitro*. *Eur. J. Med. Chem.* 102 (2015), 233-242.
- [20] W.S. Alwan, R. Karpoomath, M.B. Palkar, H.M. Patel, R.A. Rane, M.S. Shaikh, A. Kajee, K.P. Mlisana, Novel imidazo[2,1-*b*]-1,3,4-thiadiazoles as promising antifungal agents against clinical isolate of *Cryptococcus neoformans*. *Eur. J. Med. Chem.* 95 (2015), 514-525.
- [21] R.I. Mahato, A.S. Narang, L. Thoma, D.D. Miller, Emerging trends in oral delivery of peptide and protein drugs. *Crit. Rev. Ther. Drug Carrier Syst.* 20 (2003) 153-214.
- [22] T. Bruckdorfer, O. Marder, F. Albericio, From production of peptides in milligram amounts for research to multi-tons quantities for drugs of the future. *Curr. Pharm. Biotechnol.* 5 (2004) 29-43.
- [23] A.S. De Groot, D.W. Scott, Immunogenicity of protein therapeutics. *Trends Immunol.* 28 (2007): 482-490.

- [24] B. Findlay, G.G. Zhanel, F. Schweizer, Cationic amphiphiles, a new generation of antimicrobials inspired by the natural antimicrobial peptide scaffold. *Antimicrob. Agents Chemother.* 54 (2010) 4049-4058.
- [25] M. Xiong, M. Chen, J. Zhang, Rational Evolution of Antimicrobial Peptides Containing Unnatural Amino Acids to Combat Burn Wound Infections. *Chem. Biol. Drug Des.* 88 (2016) 404-410.
- [26] Y. Shi, P. Teng, P. Sang, F. She, L. Wei, J. Cai, γ -AApeptides: Design, Structure, and Applications. *Acc. Chem. Res.* 49 (2016) 428-441.
- [27] V. Panduranga, G. Prabhu, R. Kumar, V.V. Sureshbabu, A facile one pot route for the synthesis of imide tethered peptidomimetics. *Org. Biomol. Chem.* 14 (2016) 556-563.
- [28] F. Azmi, A.G. Elliott, N. Marasini, S. Ramu, Z. Ziora, A.M. Kavanagh, M.A. Blaskovich, M.A. Cooper, M. Skwarczynski, I. Toth, Short cationic lipopeptides as effective antibacterial agents: Design, physicochemical properties and biological evaluation. *Biorg. Med. Chem.* 24 (2016) 2235-2241.
- [29] M. Pasupuleti, A. Chalupka, M. Mörgelin, A. Schmidtchen, M. Malmsten, Tryptophan end-tagging of antimicrobial peptides for increased potency against *Pseudomonas aeruginosa*. (BBA)-Gen. Subjects. 1790 (2009) 800-808.
- [30] S.E. Blondelle, E. PeRez-Paya, R.A. Houghten, Synthetic combinatorial libraries: novel discovery strategy for identification of antimicrobial agents, *Antimicrob. Agents Chemother.* 40 (1996) 1067.

- [31] C. Pinilla, J. Appel, S. Blondelle, C. Dooley, B. Dörner, J. Eichler, J. Ostresh, R.A. Houghten, A review of the utility of soluble peptide combinatorial libraries. *Biopolymers*. 37 (1995) 221-240.
- [32] R.S. McDowell, K.A. Elias, M.S. Stanley, D.J. Burdick, J.P. Burnier, K.S. Chan, W.J. Fairbrother, R.G. Hammonds, G.S. Ingle, N.E. Jacobsen, Growth hormone secretagogues: characterization, efficacy, and minimal bioactive conformation. *Proc. Natl. Acad. Sci. U S A* 92 (1995) 11165-11169.
- [33] A. Inui, A. Asakawa, C.Y. Bowers, G. Mantovani, A. Laviano, M.M. Meguid, M. Fujimiya, Ghrelin, appetite, and gastric motility: the emerging role of the stomach as an endocrine organ. *FASEB J.* 18 (2004) 439-456.
- [34] T. Waseem, M. Duxbury, H. Ito, F. Rocha, D. Lautz, E. Whang, S. Ashley, M. Robinson, Ghrelin ameliorates TNF- α induced anti-proliferative and pro-apoptotic effects and promotes intestinal epithelial restitution. *J. Am. Coll. Surg.* 199 (2004) 16.
- [35] T. Waseem, M. Duxbury, H. Ito, S.W. Ashley, M.K. Robinson, Exogenous ghrelin modulates release of pro-inflammatory and anti-inflammatory cytokines in LPS-stimulated macrophages through distinct signaling pathways. *Surgery*. 143 (2008) 334-342.
- [36] A. Stengel, Y. Taché, Ghrelin—a pleiotropic hormone secreted from endocrine X/A-like cells of the stomach. *Front. Neurosci.* 6 (2012) 24.
- [37] M. Lutter, I. Sakata, S. Osborne-Lawrence, S.A. Rovinsky, J.G. Anderson, S. Jung, S. Birnbaum, M. Yanagisawa, J.K. Elmquist, E.J. Nestler, The orexigenic hormone

- ghrelin defends against depressive symptoms of chronic stress. *Nat. Neurosci.* 11 (2008) 752-753.
- [38] D. Cibrián, H. Ajamieh, J. Berlanga, O.S. León, J.S. Alba, M.J.-T. Kim, T. Marchbank, J.J. Boyle, F. Freyre, D.G. Del Barco, Use of growth-hormone-releasing peptide-6 (GHRP-6) for the prevention of multiple organ failure. *Clin. Sci.* 110 (2006) 563-573.
- [39] C. Min, K. Ohta, M. Kajiya, T. Zhu, K. Sharma, J. Shin, H. Mawardi, M. Howait, J. Hirschfeld, L. Bahammam, The antimicrobial activity of the appetite peptide hormone ghrelin. *Peptides*. 36 (2012) 151-156.
- [40] C. Bowers, F. Momany, G. Reynolds, A. Hong, On the *in Vitro* and *in Vivo* activity of a new synthetic hexapeptide that acts on the pituitary to specifically release growth hormone. *Endocrinology*. 114 (1984) 1537-1545.
- [41] B. Kundu, G. Singh, G.K. Jain, A. Shukla, N. Shrivastava, G.K. Patnaik, Wound healing activity of peptides related to growth hormone releasing hexapeptide. *Protein Pept. Lett.* 5 (1998) 83-86.
- [42] B. Kundu, S. Rastogi, S. Batra, S. Raghuwanshi, P. Shukla, Combinatorial approach to lead optimization of a novel hexapeptide with antifungal activity. *Bioorg. Med. Chem. Lett.* 10 (2000) 1779-1781.
- [43] B. Kundu, T. Srinivasan, A. Kesarwani, A. Kavishwar, S. Raghuwanshi, S. Batra, P. Shukla, Identification of novel antifungal nonapeptides through the screening of combinatorial peptide libraries based on a hexapeptide motif. *Bioorg. Med. Chem. Lett.* 12 (2002) 1473-1476.

- [44] M. Kumar, A.K. Chaturvedi, A. Kavishwar, P. Shukla, A. Kesarwani, B. Kundu, Identification of a novel antifungal nonapeptide generated by combinatorial approach, *Int. J. Antimicrob. Agents*. 25 (2005) 313-320.
- [45] R.K. Sharma, S. Sundriyal, N. Wangoo, W. Tegge, R. Jain, New antimicrobial hexapeptides: synthesis, antimicrobial activities, cytotoxicity, and mechanistic studies. *Chem. Med. Chem.* 5 (2010) 86-95.
- [46] T. Kiyota, S. Lee, G. Sugihara, Design and synthesis of amphiphilic α -helical model peptides with systematically varied hydrophobic-hydrophilic balance and their interaction with lipid-and bio-membranes, *Biochemistry*. 35 (1996) 13196-13204.
- [47] S.-Y. Wei, J.-M. Wu, Y.-Y. Kuo, H.-L. Chen, B.-S. Yip, S.-R. Tzeng, J.-W. Cheng, Solution structure of a novel tryptophan-rich peptide with bidirectional antimicrobial activity. *J. Bacteriol.* 188 (2006) 328-334.
- [48] M.A. Schmitt, B. Weisblum, S.H. Gellman, Interplay among folding, sequence, and lipophilicity in the antibacterial and hemolytic activities of α/β -peptides. *J. Am. Chem. Soc.* 129 (2007) 417-428.
- [49] V.P. Torchilin, Recent advances with liposomes as pharmaceutical carriers. *Nat. Rev. Drug Discov.* 4 (2005) 145-160.
- [50] NCCLS, Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically; Approved Standard, Seventh Edition M7-A7. National Committee on Clinical Laboratory Standards, 2006, 26(2).

- [51] NCCLS, Susceptibility Testing of Mycobacteria, Nocardia, and Other Aerobic Actinomycetes; Tentative Standard—Approved Standard, M24-A. National Committee on Clinical Laboratory Standards, 2003, 23(18).
- [52] S.G. Franzblau, R.S. Witzig, J.C. McLaughlin, P. Torres, G. Madico, A. Hernandez, M.T. Degnan, M.B. Cook, V.K. Quenzer, R.M. Ferguson, Rapid, low-technology MIC determination with clinical *Mycobacterium tuberculosis* isolates by using the microplate Alamar Blue assay. J. Clin. Microbiol. 36 (1998) 362-366.
- [53] NCCLS, Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard M27-A2. National Committee on Clinical Laboratory Standards, 2002, 22 (15).
- [54] E. Borenfreund, H. Babich, N. Martin-Alguacil, Rapid chemosensitivity assay with human normal and tumor cells in vitro. In Vitro Cell Dev. Biol. 26 (1990), 1030-1034.
- [55] D. Mares, Electron microscopy of *Microsporium cookei* after ‘*in vitro*’ treatment with protoanemonin: A combined SEM and TEM study. Mycopathologia. 108 (1989) 37-46.
- [56] M. Borgers, M. -A. Van De Ven, J. Van Cutsem, Structural degeneration of *Aspergillus fumigatus* after exposure to saperconazole, J. Med. Veter. Mycol. 27 (1989) 381-389.

Figure Captions

Fig. 1. Structure of lead peptides **1** and **2** indicating the positional replacement with other amino acids resulting in the synthesis of peptides **3-38**. In parentheses is the synthesized peptide number as seen in the tables 1-2.

Fig. 2. Stern-Volmer plots for the quenching of Trp-fluorescence of the peptides **3**, **24**, **32**, **34** and **36** in the presence of acrylamide in Tris-HCl buffer (control), EYPC:cholesterol (mammalian mimicking SUVs) and EYPC:EYPG (bacterial mimicking SUVs).

Fig. 3. Trypsin stability assay depicting the RP-HPLC chromatogram of peptides **3**, **32** and **36** taken at 0, 2, 4, 8, 12, 18 and 24 h after trypsin treatment.

Fig. 4. Surface view of the fungal biofilm cells (a) positive control, (b) upon treatment with the active peptide **36**, (c) upon treatment with the active peptide **3**, (d) upon treatment with the least active peptide **34**.

Fig. 5. TEM images of a) Untreated EYPC:EYPG SUVs (bacterial membrane mimics); b) EYPC: EYPG SUVs (bacterial membrane mimics) treated with **36**; c) Untreated EYPC:cholesterol SUVs (mammalian membrane mimics); d) EYPC: cholesterol SUVs (mammalian membrane mimics) treated with **36**. The peptide concentrations used was 0.5 µg/mL with 2% (w/v) phosphotungstic acid solution being used as the negative stain.

Fig. 6. a) Untreated *C. neoformans* ultrastructure, b) Treated fungal biofilm cells with peptide the active peptide **3**, c) Treated fungal biofilm cells with peptide the least active peptide **34**, d) Treated fungal biofilm cells with the active peptide **36**.

Table 1. Antifungal activities of peptides

Peptide No.	Structure	<i>C. albicans</i> (µg/mL)			<i>C. glabrata</i> (µg/mL)			<i>C. krusei</i> (µg/mL)			<i>A. fumigatus</i> (µg/mL)			<i>C. neoformans</i> (µg/mL)		
		IC ₅₀	MIC	MFC	IC ₅₀	MIC	MFC	IC ₅₀	MIC	MFC	IC ₅₀	MIC	MFC	IC ₅₀	MIC	MFC
3	Orn-Trp-Cha-Ile-D-Phe-His(1-Bzl)-NH ₂	NA	–	–	NA	–	–	7.76	10	10	NA	–	–	0.42	0.63	0.63
4	Orn-Phe-Cha-Ile-D-Phe-His(1-Bzl)-NH ₂	NA	–	–	NA	–	–	NA	–	–	NA	–	–	1.42	2.5	2.5
5	Orn-D-Phe-Cha-Ile-D-Phe-His(1-Bzl)-NH ₂	NA	–	–	NA	–	–	NA	–	–	NA	–	–	0.7	1.25	1.25
6	Orn-Pro-Cha-Ile-D-Phe-His(1-Bzl)-NH ₂	NA	–	–	NA	–	–	NA	–	–	NA	–	–	1.35	2.5	2.5
7	Orn-D-Pro-Cha-Ile-D-Phe-His(1-Bzl)-NH ₂	NA	–	–	NA	–	–	NA	–	–	NA	–	–	1.44	2.5	2.5
8	Orn-Leu-Cha-Ile-D-Phe-His(1-Bzl)-NH ₂	NA	–	–	NA	–	–	NA	–	–	NA	–	–	1.66	2.5	2.5
9	Orn-D-Leu-Cha-Ile-D-Phe-His(1-Bzl)-NH ₂	NA	–	–	NA	–	–	NA	–	–	NA	–	–	0.86	1.25	1.25
10	Orn-Cha-Cha-Ile-D-Phe-His(1-Bzl)-NH ₂	NA	–	–	NA	–	–	17.11	20	20	NA	–	–	1.01	1.25	2.5
11	Orn-Phg-Cha-Ile-D-Phe-His(1-Bzl)-NH ₂	NA	–	–	NA	–	–	NA	–	–	NA	–	–	1.76	2.5	5
12	Orn-Nle-Cha-Ile-D-Phe-His(1-Bzl)-NH ₂	NA	–	–	NA	–	–	NA	–	–	NA	–	–	1.44	2.5	2.5
13	Orn-D-Trp-Cha-D-Ile-D-Phe-His(1-Bzl)-NH ₂	16.82	20	20	NA	–	–	6.44	10	10	NA	–	–	1.41	2.50	2.50
14	Orn-D-Trp-Cha-Leu-D-Phe-His(1-Bzl)-NH ₂	NA	–	–	NA	–	–	15.98	20	20	NA	–	–	3.29	5.00	5.00
15	Orn-D-Trp-Cha-D-Leu-D-Phe-His(1-Bzl)-NH ₂	NA	–	–	NA	–	–	19.98	–	–	NA	–	–	1.29	2.50	2.50
16	Orn-D-Trp-Cha-Val-D-Phe-His(1-Bzl)-NH ₂	NA	–	–	NA	–	–	NA	–	–	NA	–	–	0.62	1.25	1.25
17	Orn-D-Trp-Cha-D-Val-D-Phe-His(1-Bzl)-NH ₂	17.57	20	20	NA	–	–	12.98	20	20	NA	–	–	0.69	1.25	1.25
18	Orn-D-Trp-Cha-Bal-D-Phe-His(1-Bzl)-NH ₂	8.65	10	10	7.56	10	10	6.51	10	10	6.56	10	10	0.68	1.25	1.25
19	Orn-D-Trp-Cha-Nal-D-Phe-His(1-Bzl)-NH ₂	10.50	20	20	17.9	20	20	6.64	10	10	11.5	20	20	1.07	2.50	2.50
20	Orn-D-Trp-Cha-Cha-D-Phe-His(1-Bzl)-NH ₂	6.05	10	10	NA	–	–	6.53	10	10	17.9	20	20	0.70	1.25	1.25
21	Orn-D-Trp-Cha-Phg-D-Phe-His(1-Bzl)-NH ₂	11.79	20	20	NA	–	–	8.91	10	10	NA	–	–	0.63	1.25	2.5
22	Orn-D-Trp-Cha-Ile-Bal-His(1-Bzl)-NH ₂	NA	–	–	10.2	20	20	7.12	20	20	NA	–	–	0.91	1.25	1.25
23	Orn-D-Trp-Cha-Ile-Nal-His(1-Bzl)-NH ₂	NA	–	–	NA	–	–	11.55	10	20	NA	–	–	0.56	1.25	1.25
24	Orn-D-Trp-Cha-Ile-Cha-His(1-Bzl)-NH ₂	17.40	20	20	11.5	20	20	5.76	10	20	NA	–	–	0.46	1.25	1.25
25	Orn-D-Trp-Cha-Ile-Ala(9-anth)-His(1-Bzl)-NH ₂	15.85	20	20	14.4	20	20	3.81	5	10	NA	–	–	0.53	1.25	1.25
26	Arg-D-Trp-D-Phe-Phe(4-Me)-D-Phe-His(1-Bzl)-NH ₂	19.6	20	20	NA	–	–	16.29	20	20	NA	–	–	1.36	2.5	2.5
27	Lys-D-Trp-D-Phe-Phe(4-Me)-D-Phe-His(1-Bzl)-NH ₂	NA	–	–	NA	–	–	13.85	20	20	NA	–	–	1.3	2.5	2.5
28	Dab-D-Trp-D-Phe-Phe(4-Me)-D-Phe-His(1-Bzl)-NH ₂	NA	–	–	NA	–	–	19.0	–	–	NA	–	–	0.8	1.25	1.25
29	Phe(4-NH ₂)-D-Trp-D-Phe-Phe(4-Me)-D-Phe-His(1-Bzl)-NH ₂	NA	–	–	NA	–	–	NA	–	–	NA	–	–	3.74	5.00	5.00
30	D-Arg-D-Trp-D-Phe-Phe(4-Me)-D-Phe-His(1-Bzl)-NH ₂	NA	–	–	NA	–	–	16	20	20	NA	–	–	1.04	2.5	2.5
31	Orn-D-Trp-D-Phe-Phe(4-Me)-D-Phe-His-NH ₂	NA	–	–	NA	–	–	NA	–	–	NA	–	–	0.8	1.25	1.25
32	Orn-D-Trp-D-Phe-Phe(4-Me)-D-Phe-Orn-NH ₂	NA	–	–	NA	–	–	15.49	20	20	NA	–	–	0.46	0.63	0.63
33	Orn-D-Trp-D-Phe-Phe(4-Me)-D-Phe-Dab-NH ₂	NA	–	–	NA	–	–	14.77	20	20	NA	–	–	0.8	1.25	1.25
34	Orn-D-Trp-D-Phe-Phe(4-Me)-D-Phe-Phe(4-NH ₂)-NH ₂	NA	–	–	NA	–	–	NA	–	–	NA	–	–	4.73	5.00	5.00
35	Orn-D-Trp-D-Phe-Phe(4-Me)-D-Phe-D-His-NH ₂	NA	–	–	NA	–	–	5.16	10	10	NA	–	–	0.8	1.25	1.25
36	Orn-D-Trp-D-Phe-Phe(4-Me)-D-Phe-D-Lys-NH ₂	NA	–	–	NA	–	–	12.07	20	20	NA	–	–	0.4	0.63	0.63
37	Orn-D-Trp-D-Phe-Phe(4-Me)-D-Phe-D-Arg-NH ₂	NA	–	–	NA	–	–	13.97	20	20	NA	–	–	0.83	1.25	1.25
38	Orn-D-Trp-D-Phe-Phe(4-Me)-D-Phe-D-Orn-NH ₂	NA	–	–	NA	–	–	9.7	20	20	NA	–	–	0.67	1.25	1.25

IC₅₀ The concentration (µg/mL) that affords 50% inhibition of growth; MIC (Minimum Inhibitory Concentration) is the lowest test concentration (µg/mL) that allows no detectable growth; MFC (Minimum Fungicidal Concentration) is the lowest test concentration (µg/mL) that kills 100% of the organism; Standard used: *C. albicans* (amphotericin B, IC₅₀ = 0.26, MIC = 1.25, MFC = 1.25 µg/mL), *C. glabrata* (amphotericin B, IC₅₀ = 0.34, MIC = 0.63, MFC = 1.25 µg/mL), *C. krusei* (amphotericin B, IC₅₀ = 0.60, MIC = 1.25, MFC = 1.25 µg/mL), *A. fumigatus* (amphotericin B, IC₅₀ = 1.20, MIC = 2.50, MFC = 5.0 µg/mL), *C. neoformans* (amphotericin B, IC₅₀ = 0.69, MIC = 1.25, MFC = 1.25 µg/mL); NA (not active); – (not tested).

All peptides were evaluated for cytotoxicity in the noncancerous Vero cells. None of the peptide showed any cytotoxic effects up to the concentration of 10 µg/mL.

Amino acid abbreviations used: Cha, cyclohexylalanine; Phg, phenylglycine; Nle, norleucine; Bal, 3-benzothienylalanine; Nal, 1-naphthylalanine; Dab, 2,4-diaminobutyric acid.

Table 2. Antibacterial activities of peptides

Peptide No.	Structure	MRSA (µg/mL)			<i>S. aureus</i> (µg/mL)			<i>E. coli</i> (µg/mL)			<i>P. aeruginosa</i> (µg/mL)			<i>M. intracellulare</i> (µg/mL)		
		IC ₅₀	MIC	MBC	IC ₅₀	MIC	MBC	IC ₅₀	MIC	MBC	IC ₅₀	MIC	MBC	IC ₅₀	MIC	MBC
3	Orn-Trp-Cha-Ile-D-Phe-His(1-Bzl)-NH ₂	>20	—	—	>20	—	—	10.6	—	—	NA	—	—	NA	—	—
4	Orn-Phe-Cha-Ile-D-Phe-His(1-Bzl)-NH ₂	>20	—	—	>20	—	—	>20	—	—	NA	—	—	NA	—	—
5	Orn-D-Phe-Cha-Ile-D-Phe-His(1-Bzl)-NH ₂	>20	—	—	>20	—	—	>20	—	—	NA	—	—	18.1	—	—
6	Orn-Pro-Cha-Ile-D-Phe-His(1-Bzl)-NH ₂	>20	—	—	>20	—	—	>20	—	—	NA	—	—	NA	—	—
7	Orn-D-Pro-Cha-Ile-D-Phe-His(1-Bzl)-NH ₂	>20	—	—	>20	—	—	>20	—	—	NA	—	—	NA	—	—
8	Orn-Leu-Cha-Ile-D-Phe-His(1-Bzl)-NH ₂	>20	—	—	>20	—	—	>20	—	—	NA	—	—	NA	—	—
9	Orn-D-Leu-Cha-Ile-D-Phe-His(1-Bzl)-NH ₂	>20	—	—	>20	—	—	>20	—	—	NA	—	—	12.3	20	20
10	Orn-Cha-Cha-Ile-D-Phe-His(1-Bzl)-NH ₂	>20	—	—	15.5	—	—	9	20	20	NA	—	—	NA	—	—
11	Orn-Phg-Cha-Ile-D-Phe-His(1-Bzl)-NH ₂	>20	—	—	>20	—	—	>20	—	—	NA	—	—	NA	—	—
12	Orn-Nle-Cha-Ile-D-Phe-His(1-Bzl)-NH ₂	>20	—	—	>20	—	—	>20	—	—	NA	—	—	NA	—	—
13	Orn-D-Trp-Cha-D-Ile-D-Phe-His(1-Bzl)-NH ₂	18	—	—	7.4	20	20	12.7	20	20	NA	—	—	14.5	20	20
14	Orn-D-Trp-Cha-Leu-D-Phe-His(1-Bzl)-NH ₂	18.6	—	—	11.3	20	20	NA	—	—	NA	—	—	12.8	20	20
15	Orn-D-Trp-Cha-D-Leu-D-Phe-His(1-Bzl)-NH ₂	17.9	—	—	11.4	20	20	NA	—	—	NA	—	—	19.1	—	—
16	Orn-D-Trp-Cha-Val-D-Phe-His(1-Bzl)-NH ₂	>20	—	—	18.7	—	—	NA	—	—	NA	—	—	17.3	—	—
17	Orn-D-Trp-Cha-D-Val-D-Phe-His(1-Bzl)-NH ₂	>20	—	—	17.9	—	—	NA	—	—	NA	—	—	15.3	20	20
18	Orn-D-Trp-Cha-Bal-D-Phe-His(1-Bzl)-NH ₂	3.2	10	20	2.4	5	10	12.2	20	20	15.9	—	—	NA	—	—
19	Orn-D-Trp-Cha-Nal-D-Phe-His(1-Bzl)-NH ₂	5.8	10	20	2.63	10	10	16	—	—	NA	—	—	NA	—	—
20	Orn-D-Trp-Cha-Cha-D-Phe-His(1-Bzl)-NH ₂	5.9	10	20	3.6	10	20	10	20	20	NA	—	—	19.3	—	—
21	Orn-D-Trp-Cha-Phg-D-Phe-His(1-Bzl)-NH ₂	6.6	20	20	6.5	20	20	18.9	—	—	NA	—	—	7.8	10	10
22	Orn-D-Trp-Cha-Ile-Bal-His(1-Bzl)-NH ₂	>20	—	—	20	—	—	NA	—	—	NA	—	—	NA	—	—
23	Orn-D-Trp-Cha-Ile-Nal-His(1-Bzl)-NH ₂	5	10	10	5.9	20	20	NA	—	—	NA	—	—	NA	—	—
24	Orn-D-Trp-Cha-Ile-Cha-His(1-Bzl)-NH ₂	17.6	—	—	17.5	—	—	NA	—	—	NA	—	—	NA	—	—
25	Orn-D-Trp-Cha-Ile-Ala(9-anth)-His(1-Bzl)-NH ₂	5.8	10	10	1.6	5	10	NA	—	—	NA	—	—	NA	—	—
26	Arg-D-Trp-D-Phe-Phe(4-Me)-D-Phe-His(1-Bzl)-NH ₂	3.2	20	20	2.1	5	20	6	10	20	NA	—	—	6.5	10	10
27	Lys-D-Trp-D-Phe-Phe(4-Me)-D-Phe-His(1-Bzl)-NH ₂	9.6	20	20	9.1	20	20	11.5	20	20	NA	—	—	11.9	20	20
28	Dab-D-Trp-D-Phe-Phe(4-Me)-D-Phe-His(1-Bzl)-NH ₂	10.4	20	20	11.7	20	20	NA	—	—	NA	—	—	NA	—	—
29	Phe(4-NH ₂)-D-Trp-D-Phe-Phe(4-Me)-D-Phe-His(1-Bzl)-NH ₂	NA	—	—	10.6	20	20	NA	—	—	NA	—	—	NA	—	—
30	D-Arg-D-Trp-D-Phe-Phe(4-Me)-D-Phe-His(1-Bzl)-NH ₂	7.5	20	20	6.0	20	20	9.9	20	20	NA	—	—	NA	—	—
31	Orn-D-Trp-D-Phe-Phe(4-Me)-D-Phe-His-NH ₂	NA	—	—	NA	—	—	NA	—	—	NA	—	—	NA	—	—
32	Orn-D-Trp-D-Phe-Phe(4-Me)-D-Phe-Orn-NH ₂	NA	—	—	NA	—	—	16.5	—	—	NA	—	—	NA	—	—
33	Orn-D-Trp-D-Phe-Phe(4-Me)-D-Phe-Dab-NH ₂	>20	—	—	16.1	—	—	6	—	—	NA	—	—	NA	—	—
34	Orn-D-Trp-D-Phe-Phe(4-Me)-D-Phe-Phe(4-NH ₂)-NH ₂	>20	—	—	>20	—	—	19.5	—	—	NA	—	—	NA	—	—
35	Orn-D-Trp-D-Phe-Phe(4-Me)-D-Phe-D-His-NH ₂	>20	—	—	18	—	—	12.7	—	—	NA	—	—	NA	—	—
36	Orn-D-Trp-D-Phe-Phe(4-Me)-D-Phe-D-Lys-NH ₂	>20	—	—	>20	—	—	8.2	20	20	20	—	—	NA	—	—
37	Orn-D-Trp-D-Phe-Phe(4-Me)-D-Phe-D-Arg-NH ₂	>20	—	—	13.5	20	20	4.8	10	20	15.7	—	—	NA	—	—
38	Orn-D-Trp-D-Phe-Phe(4-Me)-D-Phe-D-Orn-NH ₂	>20	—	—	>20	—	—	>20	—	—	NA	—	—	NA	—	—

IC₅₀ The concentration (µg/mL) that affords 50% inhibition of growth; MIC (Minimum Inhibitory Concentration) is the lowest test concentration (µg/mL) that allows no detectable growth; MBC (Minimum Bactericidal Concentration) is the lowest test concentration (µg/mL) that kills 100% of the organism; Standard used: *S. aureus* (ciprofloxacin, IC₅₀= 0.13, MIC = 0.5, MBC = 1.00 µg/mL), *MRSA* (ciprofloxacin, IC₅₀= 0.14, MIC = 0.5, MBC = 1.0 µg/mL), *M. intracellulare* (ciprofloxacin, IC₅₀= 0.47, MIC = 1.00, MBC = 1.00 µg/mL), *E. coli* (ciprofloxacin, IC₅₀ = 0.007, MIC = 0.031, MBC = 0.031 µg/mL), *P. aeruginosa* (ciprofloxacin, IC₅₀ = 0.09, MIC = 0.50, MBC = 1.00 µg/mL); NA (not active); — (not tested).

Amino acid abbreviations used: Cha, cyclohexylalanine; Phg, phenylglycine; Nle, norleucine; Bal, 3-benzothienylalanine; Nal, 1-naphthylalanine; Dab, 2,4-diaminobutyric acid.

Fig. 1.

Tris-HCl

EYPC:Cholesterol (10:1)

EYPC:EYPG (7:3)

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.

Scheme 1. Reagents and conditions: (i) 20% piperidine in DMF, 15 min; (ii) Fmoc-His(1-Bzl)-OH, TBTU, DIEA, DMF, 3h; (iii) Fmoc-D-Phe-OH, TBTU, DIEA, DMF, 3h; (iv) Fmoc-Ile-OH, TBTU, DIEA, DMF, 3h; (v) Fmoc-Cha-OH, TBTU, DIEA, DMF, 3h; (vi) Fmoc-Trp-OH, TBTU, DIEA, DMF, 3h; (vii) Fmoc-Orn(Boc)-OH, TBTU, DIEA, DMF, 3h; (viii) TFA:triisopropylsilane:H₂O (95:2.5:2.5), 3h.

Graphical Abstract

Synthesis, stability and mechanistic studies of potent anticryptococcal hexapeptides

Kitika Shenmar, Krishna K. Sharma, Nishima Wangoo, Indresh K. Maurya, Vinod Kumar, Shabana I. Khan, Melissa R. Jacob, Kulbhushan Tikoo and Rahul Jain*

A series of hexapeptides containing non-proteogenic amino acids that exhibit potent inhibition of *C. neoformans* is reported.

TEM image of *C. neoformans* film treated with **36**

SEM image of *C. neoformans* film treated with **36**

C. neoformans:
 IC_{50} = 0.4 mg/mL; MIC = MFC = 0.63 mg/mL
C. krusei:
 IC_{50} = 12.07 mg/mL; MIC = MFC = 20 mg/mL
E. coli:
 IC_{50} = 8.28 mg/mL; MIC = MBC = 20 mg/mL