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## Graphical Abstract

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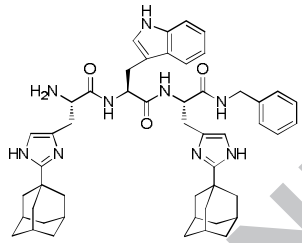

**6f.** $IC_{50}$ = 0.60 μg/mL; MIC = MFC = 0.63 μg/mL

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# Synthetically Modified L-Histidine-Rich Peptidomimetics Exhibit Potent Activity Against *Cryptococcus neoformans*

Amit Mahindra,[a] Nitin Bagra,[b] Nishima Wangoo,[b] Rohan Jain,[a] Shabana. I. Khan,[c] Melissa R. Jacob[c] 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]*Center for Nanoscience and Nanotechnology, Panjab University, Sector 14, Chandigarh 160 014, India*

[c]*National Center for Natural Products Research, School of Pharmacy, The University of Mississippi, University, Mississippi 38677, USA*

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ABSTRACT

We describe the synthesis and antimicrobial evaluation of structurally new peptidomimetics, rich in synthetically modified L-histidine. Two series of tripeptidomimetics were synthesized by varying lipophilicity at the C-2 position of L-histidine and at the *N*- and *C*-terminus. The data indicates that peptides (**5f**, **6f**, **9f** and **10f**) possessing highly lipophilic adamantan-1-yl group displayed strong inhibition of *C. neoformans*. Peptide **6f** is the most potent of all with $IC_{50}$ and MFC values of 0.60 µg/mL and 0.63 µg/mL, respectively, compared to the commercial drug amphotericin B ($IC_{50}$= 0.69 and MFC = 1.25 µg/mL). The selectivity of these peptides to microbial pathogen was examined by a tryptophan fluorescence quenching study and transmission electron microscopy. These studies indicate that the peptides plausibly interact with the mimic membrane of pathogen by direct insertion, and results in disruption of membrane of pathogen.



Invasive fungal infections represent a growing threat, and over the past two decades the incidence and diversity of fungal infections has increased enormously. As secondary infections, fungal pathogens are global threat for mankind, especially in resource limited countries.[1] The invasive mycoses of *Candida*, *Aspergillus* and *Cryptococcus* origin have drastically decreased the life expectancy of HIV/AIDS, cancer and immuno-compromised patients.[2] In addition to *Candida* and *Aspergillus* spp., *Cryptococcus neoformans* is becoming a growing danger to human health.[3] Opportunistic fungi like *C. neoformans* is long known to cause fungal meningitis and encephalitis.[4] There are over one million new cases each year and 625,000 deaths due to this facultative pathogen.[5] In regions like sub-Saharan Africa and Southeast Asia, the annual mortality due to fungus has exceeded tuberculosis.[6] The recommended treatment of fungal infections by the World Health Organization (WHO) is amphotericin B (intravenous) and flucytosine or fluconazole for two to four weeks (induction therapy).[7] The side effects of amphotericin B in the form of severe toxicity to kidney, central nervous system and gastrointestinal intolerance have been observed.[8] Since flucytosine is commonly combined with amphotericin B, the renal impairment caused by amphotericin B further increases flucytosine hepatotoxicity. Also flucytosine is losing effectiveness and reliability due to emergence of resistant strains.[9] Thus, there is currently an urgent need for the discovery and development of new antifungal agents.

Relatively new antifungal echinocandins (e.g. capsofungin, and micafungin) represent the most recent class of antifungal drugs that reached the market in the last decade, and act on a novel target.[10] They are semi-synthetic derivatives of the natural fermentation products and their total synthesis is difficult, due to the complex structure and presence of large number of reactive functional groups.[11] Moreover, they are not active towards *C. neoformans* and this is a limitation for their use.[12]

The other promising future candidates with potent antifungal activity are cationic and amphipathic peptides, isolated from diverse sources like plants, bacteria, fungi and animals.[13] Selective targeting of microbes is a key feature with these compounds, and multiple modes of action offers minimal or no chance of resistance to occur.[14] There are a number of reports, which describe various classes of antifungal peptides (AFPs) and their mode of action.[15-16] One group of peptides, that act by lysis of cell wall, which occurs by binding to the membrane surface and disrupting it, includes maganin, lactoferricin, dermaseptin etc.[17] Lipodepsipeptide syringomycin E, and iturins; defensins isolated from rabbit granulocytes NP-1; and mammalian origin HNP-1, HNP-2, HNP-3 are potent fungicidal agents against *C. albicans* and *C. neoformans*, which act by selective aggregation and form variable size aqueous pores on the cell wall, resulting in the passage of ions and solute through them, and finally cell death.[18] Peptides of another group act by interfering with the biosynthesis of chitin and glucan mainly by inhibiting membrane integrase enzyme (1,3)-β-D-glucan and examples include,

aculeacins, echinocandin, mulundocandin, and pneumocandin A (it should be noted that echinocandins are not active against *C. neoformans*).[19]

Over the past few years, our research focuses on the discovery of short peptide based drugs for infectious diseases.[20-21] In literature there are an increasing number of peptides, both natural and synthetic, which exhibit antifungal activity.[22] These peptides vary in length and amino acid sequences, but most of them have a large number of common amino acids. The lack of sequence or structural homology makes it challenging to design potent synthetic antifungal peptides with the desired activities. One particular class of peptides that exhibit strong antifungal activity against various strains have histidine- and tryptophan-rich sequences.[23-24] A large number of natural and synthetic antifungal peptides, rich in histidine and tryptophan containing are shown in Table 1.[25-30]

**Table 1.** Sequence of natural and synthetic histidine-rich and tryptophan containing AFPs

| Peptide | Sequence | No. of AAs |
|---|---|---|
| Histatin-1 | DSHEKRHHGYRRKFHEKHHSHREFPFYG DYGSNYLYDN | 38 |
| Histatin-3 | DSHAKRHHGYKRKFHEKHHSHRGYRSN YLYDN | 32 |
| Histatin-5 | DSHAKRHHGYKRKFHEKHHSHRGY | 24 |
| P-113 | AKRHHGYKRKFH | 12 |
| P-113 amide | AKRHHGYKRKFH-$NH_2$ | 12 |
| Dh5 | KRKFHEKHHSHRGY | 14 |
| H2K | KHKHHKHHKHHKHHKHHKHK | 20 |
| LfcinB | FKC$_1$RRWQWRMKKLGAPSITC$_1$VRRAF* | 25 |
| LfcinB4–14 | RRWQWRMKKLG | 11 |
| LfcinB4–9 | RRWQWR-$NH_2$ | 6 |
| PAF-26 | RRKWFW | 6 |

*Disulfide bonded cysteines are denoted by subscript numbers

Histatins are a distinct group of linear cationic peptides that are isolated from human saliva and have potent and specific biological activity against fungi.[25] Histatin-1, -3, and -5 are homologous and contain 38, 32, and 24 amino acids, respectively. Histatin-5, a proteolytic fragment of histatin-3, is potently fungicidal against several strains of fungi.[26] There are numerous examples of truncation of peptides or proteins to smaller fragments that retain the activity or whose activities even exceed that of the native peptide. P-113, a 12-mer fragment of histatin-5, is the smallest peptide that retains fully original anticandidal activity.[27] P-113 when amidated on its *C*-terminus has shown promising activity in comparison to its unamidated form, thereby confirming the importance of capping of *C*-terminus. A class of synthetic histidine-rich peptides, exemplified by H2K (with 60% histidines in sequence) are potent inhibitors of several *Candida* species in vitro.[28] In addition to histidine-rich peptides, other peptides such as LfcinB have several tryptophan residues in the sequence.[29] Smaller peptide sequences such as LfcinB4–9 and PAF-26 containing tryptophan are also reported with potent antifungal activity.[30] Despite having promising activities, these peptides have disadvantages in terms of high cost, proteolytic instability, and cytotoxicity. One possible method to overcome these shortcomings of larger peptides is to synthesize peptides with shorter sequences keeping the bioactive core intact.

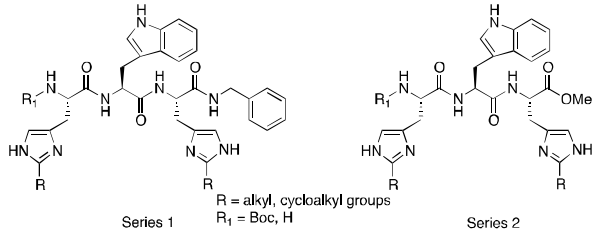
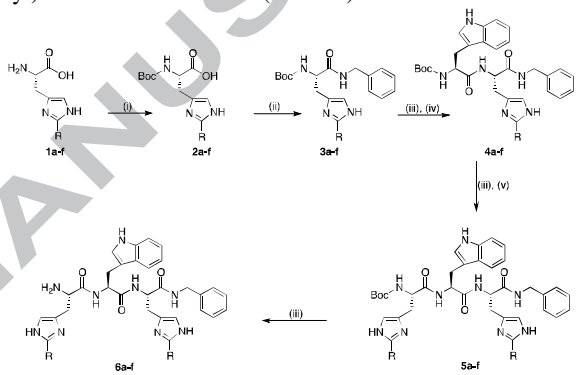



**Figure 1**.General structures of synthesized tripeptides (series 1 and 2).

In this study, we first synthesized 2-alkylated-L-histidines and later incorporated them in the designed peptide, with the intention of increasing the lateral amphipathicity of the peptides, and evaluated the effect on the antifungal and antibacterial activity. In this direction, we designed two series of histidine-rich tripeptidomimetics by keeping 2-alkyl-L-histidine residues at the end terminals and tryptophan residue at the center. The rationale behind these substitutions is that histidine is itself a cationic amino acid, and the placement of bulkier groups at the 2-position of the imidazole ring would impart hydrophobicity. On the other hand, tryptophan has the unique property of binding in the interfacial region of a membrane, thereby anchoring the peptide to the bilayer surface. Another important factor is the extensive π–electron system of the aromatic side chain that provides significant quadrupole moment.[31] The *C*-terminus in the designed peptides is converted to an ester or an amide linkage to further increase the overall lipophilicity. At the same time, lipophilicity at the *N*-terminus was modulated by a free amino group or by keeping Boc group intact (Fig. 1).

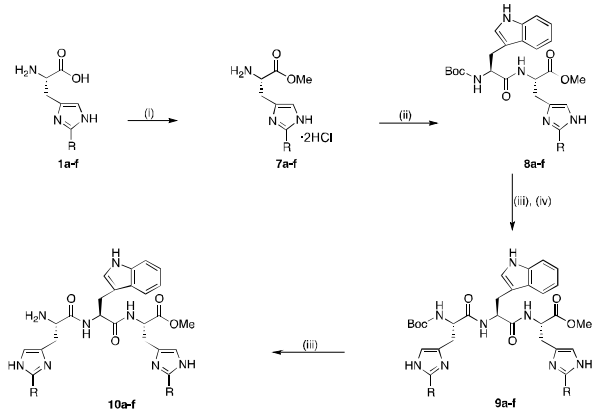
**Scheme 1.** MW-assisted synthesis of His(2-alkyl)-Trp-His(2-alkyl)-NHBzl **6a-f** in water (series 1).




Reagents and conditions: (i) $(Boc)_2O$, NaOH, dioxane-$H_2O$ (1:4), 24 h; (ii) $C_6H_5CH_2NH_2$, DIC, HONB, 60 °C, 30 min, MW; (iii) 3N HCl, rt, 15 min; (iv) DIEA, Boc-Trp-OH,TBTU, HOBt, $H_2O$, 60 °C, 30 min, MW; (v) DIEA, Boc-His(R)-OH, TBTU, HOBt, $H_2O$, 60 °C, 30 min.

For the synthesis of target peptides, a series of 2-alkylated-L-histidine was synthesized using earlier reported methods.[32-33]The peptides were synthesized by a recently developed environmentally benign microwave (MW)-assisted peptide synthesis protocol in neat water.[34-35]

**Scheme 2.** MW-assisted synthesis of His(2-alkyl)-Trp-His(2-alkyl)-OMe **10a-f** in water (series 2).



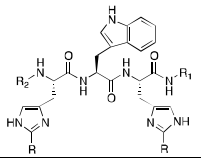

Reagents and conditions: (i) MeOH, HCl gas, 4 °C, 2 h; (ii) DIEA, Boc-Trp-OH, TBTU, HOBt, $H_2O$, 60 °C, 30 min, MW; (iii) 3N HCl, rt, 15 min; (iv) DIEA, Boc-His(R)-OH, TBTU, HOBt, $H_2O$, 60 °C, 30 min.

Using this protocol, synthesis of peptides of series 1 and 2 is achieved as depicted in scheme 1 and 2. Key features of this original synthetic protocol are the replacement of commonly used toxic solvent like DMF, short reaction time, the use of side-chain

unprotected histidine, and racemization-free peptide synthesis, in high purity and yield. We also undertook the synthesis of Boc-L-Trp-His-OMe, Boc-D-Trp-His-OMe, and Boc-D,L-Trp-His-OMe under MW irradiation in water. The purified peptides upon HPLC analysis confirmed the racemization-free synthesis of peptide (see Supporting Information).

The synthesized peptides (**5**-**6** and **9**-**10**) were evaluated for in vitro activity against fungal *C. albicans, C. glabrata, C. krusei, A. fumigatus* and *C. neoformans* and bacterial (*E. coli, S. aureus, MRSA, M. intracellulare and P. aeruginosa)* strains, and the results are summarized in Table 2 and Table 3. All the peptides were found to be inactive against *Candida*, *Aspergillus*, *E. coli*, *M. intracellulare and P. aeruginosa* (data not shown). The minimum inhibitory concentration (MIC) was measured using a protocol suggested by the Clinical and Laboratory Standard Institute (previously known as the National Committee for Clinical Laboratory Standards, NCCLS).[36] Amphotericin B, served as a positive control in these studies.[37]

**Table 2.** In vitro antifungal activities of peptides



| Peptide | R | $R_1$ | $R_2$ | *C. neoformans*[d] (µg/mL) | | | |
|---|---|---|---|---|---|---|---|
| | | | | $IC_{50}$[a] | MIC[b] | MFC[c] | CTX[e] (µg/mL) |
| **5a** | H | NHBzl | Boc | 0.80 | 1.25 | 1.25 | >10 |
| **5b** | $CH(CH_3)_2$ | NHBzl | Boc | NA | NA | NA | >10 |
| **5c** | $C(CH_3)_3$ | NHBzl | Boc | NA | NA | NA | >10 |
| **5d** | *c*-$C_5H_9$ | NHBzl | Boc | 20 | NA | NA | >10 |
| **5e** | *c*-$C_6H_{11}$ | NHBzl | Boc | NA | NA | NA | >10 |
| **5f** | adamantan-1-yl | NHBzl | Boc | 2.12 | 2.5 | 5 | >10 |
| **6a** | H | NHBzl | H | 8.1 | 10 | 10 | >10 |
| **6b** | $CH(CH_3)_2$ | NHBzl | H | 6.84 | 20 | 20 | >10 |
| **6c** | $C(CH_3)_3$ | NHBzl | H | 11.63 | 20 | 20 | >10 |
| **6d** | *c*-$C_5H_9$ | NHBzl | H | NA | NA | NA | >10 |
| **6e** | *c*-$C_6H_{11}$ | NHBzl | H | 1.02 | 1.25 | 1.25 | >10 |
| **6f** | adamantan-1-yl | NHBzl | H | 0.60 | 0.63 | 0.63 | >10 |
| **9a** | H | OMe | Boc | NA | NA | NA | >10 |
| **9b** | $CH(CH_3)_2$ | OMe | Boc | 8 | NA | NA | >10 |
| **9c** | $C(CH_3)_3$ | OMe | Boc | 8 | NA | NA | >10 |
| **9d** | *c*-$C_5H_9$ | OMe | Boc | 8.24 | NA | NA | >10 |
| **9e** | *c*-$C_6H_{11}$ | OMe | Boc | 5.78 | 10 | 10 | >10 |
| **9f** | adamantan-1-yl | OMe | Boc | 2.44 | 5 | 5 | >10 |
| **10a** | H | OMe | H | 9.81 | 20 | 20 | >10 |
| **10b** | $CH(CH_3)_2$ | OMe | H | 11.34 | NA | NA | >10 |
| **10c** | $C(CH_3)_3$ | OMe | H | 16.14 | NA | NA | >10 |
| **10d** | *c*-$C_5H_9$ | OMe | H | 5 | 10 | 10 | >10 |
| **10e** | *c*-$C_6H_{11}$ | OMe | H | 2.28 | 5 | 5 | >10 |
| **10f** | adamantan-1-yl | OMe | H | 0.68 | 1.25 | 1.25 | >10 |
| **Amphotericin B** | | | | 0.69 | 1.25 | 1.25 | |

[a]$IC_{50}$ is the concentration (µg/mL) that affords 50% inhibition of growth; [b]MIC (Minimum Inhibitory Concentration) is the lowest test concentration (µg/mL) that allows no detectable growth; [c]MFC (Minimum Fungicidal Concentration) is the lowest test concentration (µg/mL) that kills 100% of the organism; [d]Highest tested concentration was 20 µg/mL; [e]CTX (Cytotoxicity) Highest tested concentration was 10 µg/mL. NA, not active.

In general, the alkyl group placed at the C-2-position of the imidazole ring greatly influenced the overall antifungal activity of the peptides. Also, the nature of groups present at the *N*- and *C*-terminus played a significant role in activity. Peptide **5a** with Boc and NHBzl groups at end terminus was equipotent to standard drug as antifungal. Thus it appeared that a combination of Boc and NHBzl group at the end terminus along with an alkyl group at the C-2 position is detrimental for activity (peptides (**5b-f**). In series 1, peptide **6f** (R = adamantan-1-yl, $R_1$ = NHBzl, $R_2$ = H) was the most potent fungicidal compound against *C. neoformans*, with an $IC_{50}$ and MFC value of 0.60 and 0.63 µg/mL as compared to 0.69 and 1.25 µg/mL, respectively for standard drug AMB. Peptide **6e** (R = *c*-$C_6H_{11}$, $R_1$ = NHBzl, $R_2$ = H) also showed potent activity with an $IC_{50}$ and MFC value of 1.02 and 1.25 µg/mL, respectively. At the same time, peptides **6a**, **6b** and **6c** produced modest antifungal activities with $IC_{50s}$in the range of 9.81-16.14 µg/mL. Peptides **5f** and **6f** also exhibited weak antibacterial activity against *S. aureus* and *MRSA* with $IC_{50}$values in the range of 3.45-4.94 and 5.55-8.81 µg/mL and MIC of 20.00 µg/mL (Table 3). In series 2, a similar trend is observed with peptides (**9f** and **10f**) having adamantan-1-yl group at the C-2 position.

Peptides (**9f** and **10f**) exhibit most potent activity against *C. neoformans* with $IC_{50}$ values of 2.44 and 0.68 µg/mL, respectively. Other peptides of this series **9b**-**9e** and **10a**-**10e** also showed promising $IC_{50}$ values against *C. neoformans* in the range of 2-16 µg/mL. These results clearly demonstrate the selectivity of the designed peptides for *Cryptococcus*.

**Table 3.** In vitro antibacterial activity of peptides

| Peptide | *S. aureus* (µg/mL) | | | *MRSA* (µg/mL) | | |
|---|---|---|---|---|---|---|
| | $IC_{50}$ | MIC | MBC | $IC_{50}$ | MIC | MBC |
| **5f** | 4.94 | 20.00 | 20.00 | 8.81 | 20.00 | 20.00 |
| **6f** | 3.45 | 5.00 | 5.00 | 5.55 | 10.00 | 10.00 |
| **9f** | 4.22 | 10.00 | 20.00 | 11.28 | NA | NA |
| **10f** | 8.76 | 20.00 | NA | 16.04 | NA | NA |
| **Cipro** | 0.08 | 0.25 | 0.50 | 0.09 | 0.25 | 0.50 |

MBC (Minimum Bactericidal Concentration) is the lowest test concentration (µg/mL) that kills 100% of the organism. NA, not active.

All synthesized peptides were also evaluated for cytotoxicity in a panel of mammalian cell lines to determine their safety profile.

The *in vitro* cytotoxicity of analogs was determined against four human cancer cell lines (SK-MEL, KB, BT-549, and SK-OV-3) and two noncancerous mammalian cells (VERO and LLC-PK1) using an earlier reported protocol.[38] The results demonstrated that the synthesized peptides were non-toxic up to a concentration of 10.00 µg/mL indicating a higher selectivity index of anticryptococcal activity (Table 2).

A plausible correlation between lipophilicity and activity was obtained for all compounds. To verify whether lipophilicity and therefore cell penetration of peptides (**6a-f** and **10a-f**) is correlated to the antifungal activity, the degree of lipophilicity was expressed as the Clog*P* value and shown in Fig. 2. The Clog*P* values were calculated using the ACD/CLog*P* software and are indicated as Clog*P*. The values of ClogP are an indicator of cell penetration potential of the synthesized inhibitors and the number increases with an increase in hydrophobicity.

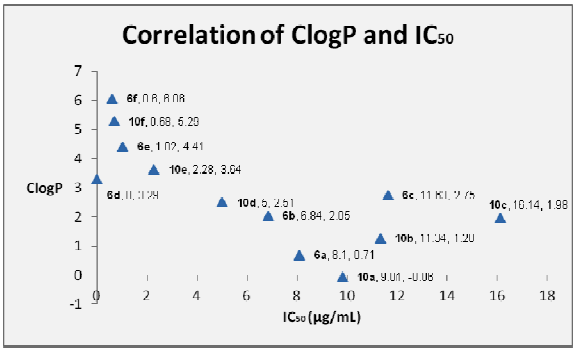
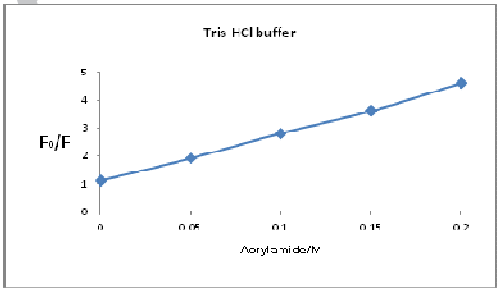



**Figure 2.** Correlation between lipophilicity and biological activity of peptides 6a-f and 10a-f (Δ represents peptide, $IC_{50}$ and ClogP values respectively)

Peptide **6f**, most potent against *C. neoformans* having most hydrophobic substituent adamantan-1-yl showed Clog*P* and $IC_{50}$ values of 6.06 and 0.60 µg/mL, which are in agreement. The correlation of lipophilicity with Clog*P* for peptides **5a-f** and **9a-f** is included in the supporting information.

To elucidate the interaction of peptides with synthetic mimics of membrane, we performed tryptophan fluorescence quenching and transmission electron microscopy studies on analogs that may be of future interest.[39] This requires the synthesis of the small unilamellar vesicles (SUVs), which were prepared using the reported method in the literature.[40] We selected three most promising peptides **6e**, **6f** and **10f** for the tryptophan fluorescence quenching study. To investigate whether the selectivity of peptides (**6e**, **6f** and **10f**) towards microbial pathogen is related to the differences in the interaction with the outer monolayer of membranes of microbial and mammalian cells, tryptophan fluorescence quenching study was performed in the presence of negatively charged (EYPC/EYPG) and zwitterionic model membranes (EYPC/cholesterol), respectively. The Stern-Volmer plots of the tryptophan quenching study, in the absence and presence of lipid vesicles are shown in Figure 3.



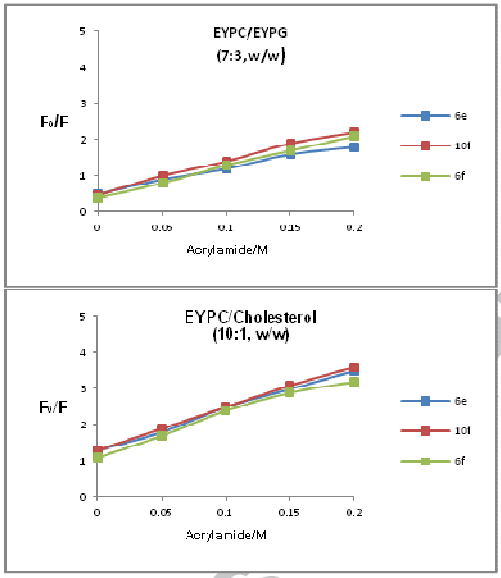




**Figure 3.** Stern-Volmer plots for the quenching of Trp fluorescence of the peptides in the presence of acrylamide.

The fluorescence of tryptophan decreased in a concentration-dependent manner by the addition of acrylamide to the peptide solution both in the absence and presence of liposomes, without any other effects. Compared to the measurements in the absence of liposomes (Tris HCl buffer, *K*sv = 18.0), the values for the Stern-Volmer quenching constant (*K*sv) were decreased in the presence of EYPC/EYPG (7:3, w/w *K*sv = 11.3 for **6f**) and EYPC/cholesterol (10:1, w/w *K*sv = 14.0 for **6f**), suggesting that the tryptophan was buried in the bilayers, thereby becoming inaccessible for quenching by acrylamide. The comparison of *K*sv values for EYPG containing pure EYPC liposomes indicates that the binding of peptide to liposomes is enhanced by EYPG. The data thus suggest that affinity of peptide for liposomes increased in the order EYPC/cholesterol<EYPC/EYPG. This indicates that the peptide effectively embedded the negatively charged membrane, but not the zwitterionic membrane, suggesting that the selectivity of the tested peptide towards mimics of microbial cell membrane is associated with a preferential interaction with the negatively charged phospholipids.

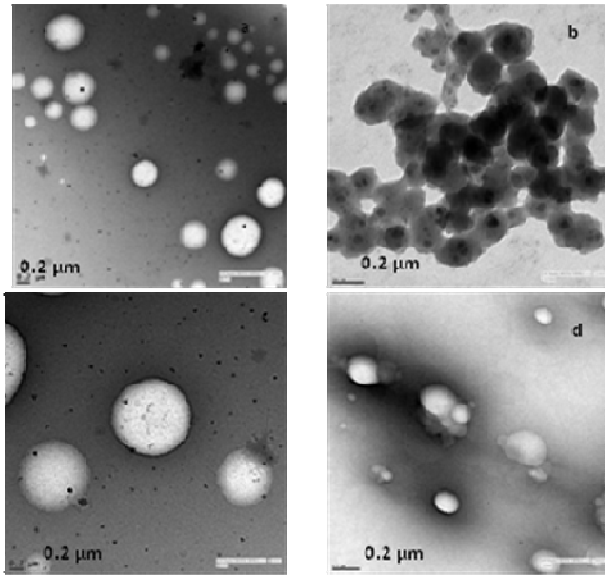


**Figure 4.** TEM images of SUVs with **6f**; (a) Untreated EYPC/EYPG; (b) EYPC/EYPG treated with **6f**; (c) Untreated EYPC/cholesterol; (d) EYPC/cholesterol treated with **6f.** The peptide was used at 10 µg/mL concentrations.

From TEM study, we observed the morphology of the SUVs in the presence and absence of the peptide **6f** by depositing a sample of the treated and untreated SUVs, on to a carbon coated copper grid, and negatively staining the sample with 2% (w/v) phosphotungstic acid solution. The results indicated that the untreated SUVs were uniformly shaped, with intact morphology (Fig. 4a) whereas SUVs treated with **6f** resulted in the destruction of morphology (Fig. 4b), in case of EYPC/EYPG. Whereas in case of EYPC/cholesterol, the SUVs treated with **6f** retains the integrity of membrane (Fig. 4d). From these studies, we conclude that the peptide does not lyse mammalian membranes at the concentrations, at which it destroys mimic of the pathogenic membranes further confirming the results of cytotoxicity experiments.

In summary, two series of synthetic histidine-rich tripeptidomimetics with varying lipophilicity were synthesized and evaluated in vitro against fungal and bacterial strains. Peptides **6f** and **10f** substituted with a bulky adamantan-1-yl group at the C-2-position of the imidazole ring were found to be the most active against *C. neoformans*. To study the interaction of these peptides, tryptophan fluorescence quenching and TEM studies were performed using synthetic mimics of membrane. The results indicate that tested peptides possibly interact with membrane, followed by the non-specific disruption of the cell membrane.

## Corresponding Author

*E-mail: rahuljain@niper.ac.in; Fax: +91 (172) 2214692; Tel: +91 (172) 2292024.

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## Abbreviations

AA, amino acid; Boc, *tert*-Butoxycarbonyl; DIC, 1,3-Diisopropylcarbodiimide; DIEA, *N,N*-Diisopropylethylamine; EYPC, Egg yolk L-a-phosphatidylcholine; DMF, *N,N*-Dimethylformamide; EYPG, Egg yolk L-a-phosphatidyl-DL-glycerol; TBTU, *O*-(Benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate; HOBt, 1-Hydroxybenzotriazole; HONB, *N-endo*-Hydroxy-5-norbornene-2,3-dicarboximide; $IC_{50}$, The concentration (µg/mL) that affords 50% inhibition of growth; MeOH, Methanol; MFC/MBC, Minimum Fungicidal/Bactericidal Concentration; MIC, Minimum Inhibitory Concentration; TEM, Transmission electron microscopy; Trp, Tryptophan.

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## Supplementary Material

Detailed synthetic procedures, characterization data, HPLC chromatograms and details on the biological assays are available in supporting information.