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Discovery of Short Peptides Exhibiting High Potency against *Cryptococcus neoformans*

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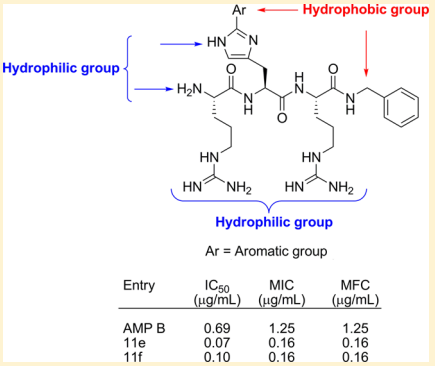
Supporting Information

ABSTRACT: Rapid increase in the emergence of resistance against existing antifungal drugs created a need to discover new structural classes of antifungal agents. In this study we describe the synthesis of a new structural class of short antifungal peptidomimetcis, their activity, and plausible mechanism of action. The results of the study show that peptides **11e** and **11f** are more potent than the control drug amphotericin B, with no cytotoxicity to human cancer cells and noncancerous mammalian kidney cells. The selectivity of peptides to fungus is depicted by transmission electron microscopy studies, and it revealed that **11e** possibly disrupts the model membrane of the fungal pathogen.



Entry	IC_{50} (μg/mL)	MIC (μg/mL)	MFC (μg/mL)
AMP B	0.69	1.25	1.25
11e	0.07	0.16	0.16
11f	0.10	0.16	0.16

KEYWORDS: *Antifungal peptides, microwave, TEM, C. neoformans, arylation*

During the past 20 years, the incidence of invasive fungal infections such as candidiasis, cryptococcosis, and aspergillosis in humans has increased considerably.[1] They are major causes of mortality and morbidity, especially in patients whose immune systems are compromised by AIDS, cancer, or organ transplant.[2] Defense against such infectious agents has relied mainly on the use of three classes of chemotherapeutic agents, including azoles (e.g., fluconazole),[3] macrocyclic polyenes (e.g., amphotericin B),[4] and candins (e.g., micafungin).[5] Each treatment option has several drawbacks, such as drug related toxicity, emergence of resistant strains, nonoptimal pharmacokinetics, poor solubility, and serious drug−drug interactions.[6] Therefore, there is a pressing need to discover new antifungal agents that do not share the same structural scaffold and the same line of mechanism as existing drugs.[7]

The quest for new antifungals is even more critical because recently developed antifungal drugs also are developing resistance against the most prevalent pathogens like *Cryptococcus neoformans* and *Candida albicians*.[8] In that regard, cationic antimicrobial peptides (CAMPs) offer promising opportunities for the development of new antifungal agents.[9] During the past 5−10 years, many CAMPs have been patented for a variety of biological activities, such as antitumor,[10] antiinflammatory,[11] antiviral,[12] and antifungal.[13] Although they are diverse in terms of sequence and structure, they share common features such as a net positive charge and the hydrophobic groups on different sides.[14] The mechanisms of antimicrobial peptides are not completely understood, but in most cases their biological effects are believed to involve membrane disruption of the target cells.[15] CAMPs are generally positively charged and therefore bind preferentially to negatively charged bacterial membranes rather than to mammalian cell membranes, which are neutral.[16] Because of their unique mode of action, the development of resistance against them is considered unlikely, and thus, they can be promising alternatives of traditional known drugs.

As part of our ongoing effort in search of novel peptide and peptidomimetics for infectious diseases,[17,18] we examined the sequence and mode of action of a number of antifungal peptides (AFPs) reported in literature.[19−25] As shown in Table 1, despite having large sequence diversity, these peptides have a number of amino acids in common and sometimes a repeat of a specific sequence. A large number of cationic amino acids such as arginine (R, Arg), lysine (K, Lys), and histidine (H, His) are present in the sequences; while at the same time, hydrophobic amino acids like tryptophan (W, Trp), phenylalanine (F, Phe), leucine (L, Leu), and isoleucine (I, Ile) are also present abundantly. It is already known that a charge to bulk ratio is essential for peptides to show antifungal activity. In the cases of indolicidin[22] and tritrpticin,[23] it is observed that repeated Arg

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Table 1. Sequence and Mode of Action of Natural AFPs

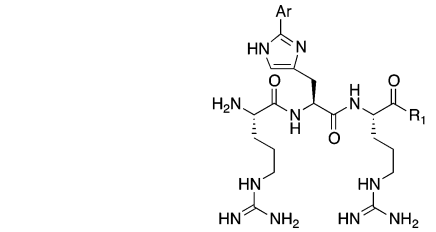
peptide	sequence	target organism/mode of action
defensin NP-1	VVCACRRALCLPAQRRAGFC**RIRGRIH**PLCCRR	*C. neoformans*/Lysis
dermaseptin b	DVL**KKI**GTVALHAGKAALGAVADTISQ	*C. neoformans*/Lysis
histatin-5	DSHA**KRHH**GY**KRK**FHEKHHSHRGY	*C. albicans*/Lysis
indolicidin	ILPWKWPWWP**WRR**-NH_2	*C. neoformans*/Lysis
tritrpticin	VRRFPWWW**PFLRR**	*C. albicans*/Lysis
Sub5	R**RWK**IVVI**RWR**R	*A. nidulans*/Lysis
W3	V**RWR**IRVAVIRA	*C. albicans*/Lysis
Pep15	V**RLR**IRVWVIRA	*C. albicans*/Lysis
Bac2A	**RLAR**IVVIRVAR	*C. albicans*/Lysis
R3	**RLRR**IVVIRVAR	*C. neoformans*/Lysis

units are present on the terminal end, whereas the central core of these peptides contains several Trp residues. It was also noted that tritrpticin exhibits weak antifungal activity as compared to indolicidin, possibly because C-terminus of indolicidin is amidated, whereas C-terminus of tritrpticin is carboxylated.[25]

A curious examination of the sequences of defensin NP-1,[19] Sub5,[24] W3,[24] Pep 15,[24] Bac2A,[24] and R3[24] clearly indicates the presence of Arg residue next to hydrophobic amino acids, confirming the importance of a minimum charge versus bulk ratio in the bioactivity. In specific cases, presence of RIR, RIH (defensin NP-1), KKI (dermaseptin b), KRH, KRK (histatin-5), WRR (indolicidin), RWR (sub5 and W3), and RLR (pep15, Bac2, and R3) motifs containing both hydrophobic and hydrophilic amino acids are observed. Despite having promising activities, these peptides have some disadvantages in terms of high cost of synthesis, proteolytic instability, high cytotoxicity, nonspecific activity, and short circulation lifetime. One method to overcome these obstacles is to synthesize peptides of shorter length, while keeping the presumed bioactive core intact. The truncation of native peptide to smaller fragments is one of the most attractive strategies to discover smaller peptides, which carry the bioactivity of the original sequence.[26] This strategy provides prefabricated structural frameworks for synthetic manipulations that could be successfully optimized for discovering short AFPs.[26] A number of studies based on the truncation approach have been reported, including peptoids,[27] cyclic peptides,[28] and synthetic peptidomimetics.[17,18,29]

Our aim was to develop shorter AFPs with enhanced activity and potency. In this regard, we designed tripeptides by keeping the arginine residue at the end terminals, and the centrally located L-histidine residue was substituted with an aryl moiety at the C-2 position. The presence of the guanidinium side-chains of arginine and imidazole ring of the histidine provide the necessary hydrophilicity. The presence of an aryl moiety at the C-2 position of L-histidine and NHBzl or OMe groups at the C-terminus provides the required bulk and hydrophobicity for the membrane insertion. Furthermore, the introduction of an aryl moiety in the imidazole ring of histidine creates an amino acid with dual hydrophobic—hydrophilic character. The general scaffold of the proposed peptides is shown in Figure 1. A wide variety of lipophilic aryl substituents on the C-2 position of L-histidine were explored. To observe the effect of C-terminus capping on the bioactivity, two series of peptides were synthesized having NHBzl and OMe groups at the C-terminus.

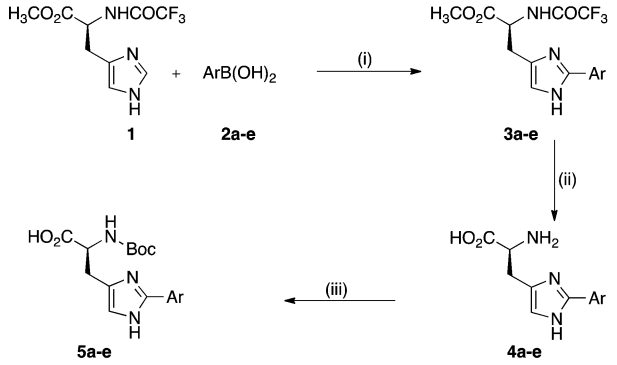
For the synthesis of the target peptides, a series of 2-arylated-L-histidines (**3a−e**) were synthesized using a procedure reported earlier, as depicted in Scheme 1.[30] The removal of protecting groups of **3a−e** was achieved by refluxing in 6 N HCl for 16−24 h. Finally, the synthesis of *N*-α-Boc-2-aryl-L-histidines (**5a−e**) was achieved from 2-aryl-L-histidine dihydrochloride (**4a−e**).



Ar = Aromatic group
Series 1. R_1 = NHBzl; **Series 2.** R_1 = OMe

Figure 1. Representative scaffold of the designed tripeptides.

Scheme 1. Overview of the Synthesis of *N*-α-Boc-2-aryl-L-histidines[a]



Ar = phenyl, 4-methylphenyl, 4-methoxyphenyl, 4-*t*-butylphenyl, biphenyl

[a]Reaction conditions: (i) **1** (1 equiv), **2a−e** (2 equiv), $AgNO_3$ (0.2 equiv), $NH_4(S_2O_8)_2$ (2 equiv), CF_3CO_2H (1.5 equiv), CH_2Cl_2/H_2O (1:1, v/v), 10−18 h, rt; (ii) 6 N HCl, 16−24 h, reflux; (iii) $(Boc)_2O$, 1,4-dioxane/H_2O (1:1), 12 h, rt.

The desired peptides were synthesized by a recently developed, highly efficient and rapid microwave (MW)-assisted peptide synthesis protocol, as depicted in Scheme 2.[31,32] The highlight was the successful synthesis of the desired peptides by using amino acids bearing participating side-chain groups, thereby confirming high reactive functional group tolerance and atom economy of the procedure. In a 10 mL MW vial, equipped with a magnetic stir bar, amino acid (Arg-NHBzl/OMe, dissolved in DMF) and DIEA were added. Boc-2-aryl-His-OH (**5a−e**) was then added, followed by DIC and HONB. The reaction mixture was subjected to MW irradiation with gas cooling for 28 min at 40 W with magnetic stirring and a

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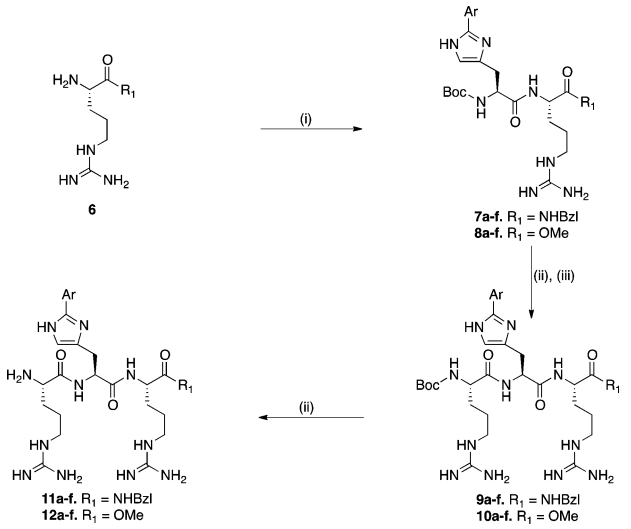
Scheme 2. Synthesis of Arg-His(2-aryl)-Arg-NHBzl (11a−f) and Arg-His(2-aryl)-Arg-OMe (12a−f) under MW Irradiation[a]



[a]Reaction conditions: (i) Boc-His(2-aryl)-OH, DIEA, DIC, HONB, DMF, 50 °C, 28 min, MW; (ii) 3 N HCl in MeOH, 15 min, rt; (iii) Boc-Arg-OH, DIEA, HATU, HOAt, DMF, 50 °C, 28 min, MW.

temperature limit of 50 °C to give Boc-protected peptides **7**−**8** in 45−60% isolated yield.

Boc-His(2-aryl)-Arg-NHBzl/OMe (**7**−**8**) upon reaction with 3 N HCl (5 mL) at 25 °C for 15 min resulted in the removal of Boc group. The salt of dipeptide was neutralized in situ with DIEA and to the resulting NH_2-His(2-aryl)-Arg-NHBzl/OMe in DMF was added Boc-Arg-OH, followed by HATU and HOAt. Mixture was subjected to MW irradiation for 28 min at 40 W with magnetic stirring, and a temperature limit of 50 °C to produce protected peptides **9**−**10** in 40−45% isolated yield. The removal of the Boc group using 3 N HCl afforded the designed peptides **11**−**12**. We have also synthesized Boc-L-His-Arg-OMe, Boc-D-His-Arg-OMe, and Boc-D,L-His-Arg-OMe under MW irradiation and confirmed the extent of racemization using HPLC. As evident from the HPLC chromatograms, purified peptides were free of racemization (see Supporting Information).

The synthesized peptides were evaluated for in vitro activity against fungal (*C. albicians*, *C. glabrata*, *C. krusei*, *Aspergillus fumigates*, and *C. neoformans*) and bacterial (*E. coli*, *S. aureus*, and *MRSA*) strains, and the results are summarized in Tables 2 and 3 for *Cryptococcus*, *S. aureus*, and *MRSA*. All the peptides were found to be inactive against *Candida*, *Aspergillus*, and *E. coli* (results not included). The minimum inhibitory concentration (MIC) was measured using a protocol suggested by the Clinical and Laboratory Standard Institute (previously known

Table 2. In Vitro Antifungal Activities of Peptides

				C. neoformans (μg/mL)[d]			cytotoxicity[e]	selectivity index[f]
peptide	Ar	R_1	R	IC_{50}[a]	MIC[b]	MFC[c]	CTX (μg/mL)	*C. neoformans*
9a	H	NHBzl	Boc	5.35	10.00	10.00	>10	>1.8
9b	C_6H_5	NHBzl	Boc	0.67	1.25	1.25	>10	>14.9
9c	4-CH_3−C_6H_4	NHBzl	Boc	2.15	2.50	2.50	>10	>4.6
9d	4-OCH_3−C_6H_4	NHBzl	Boc	0.68	2.50	5.00	>10	>14.7
9e	4-$C(CH_3)_3$−C_6H_4	NHBzl	Boc	0.18	0.63	0.63	>10	>55.6
9f	4-C_6H_5−C_6H_4	NHBzl	Boc	0.20	0.31	0.31	>10	>50.0
10a	H	OMe	Boc	NA	NA	NA	>10	
10b	C_6H_5	OMe	Boc	10.4	NA	NA	>10	
10c	4-CH_3−C_6H_4	OMe	Boc	9.71	NA	NA	>10	>1.0
10d	4-OCH_3−C_6H_4	OMe	Boc	4.06	5.00	5.00	>10	>2.5
10e	4-$C(CH_3)_3$-C_6H_4	OMe	Boc	0.71	1.25	1.25	>10	>14.1
10f	4-C_6H_5−C_6H_4	OMe	Boc	0.8	1.25	1.25	>10	>12.5
11a	H	NHBzl	H	NA	NA	NA	>10	
11b	C_6H_5	NHBzl	H	0.73	1.25	1.25	>10	>13.7
11c	4-CH_3−C_6H_4	NHBzl	H	0.35	0.63	10.00	>10	>28.6
11d	4-OCH_3−C_6H_4	NHBzl	H	0.43	0.63	0.63	>10	>23.3
11e	4-$C(CH_3)_3$−C_6H_4	NHBzl	H	0.07	0.16	0.16	>10	>142.8
11f	4-C_6H_5−C_6H_4	NHBzl	H	0.10	0.16	0.16	>10	>100.0
12a	H	OMe	H	19.0	NA	NA	>10	
12b	C_6H_5	OMe	H	NA	NA	NA	>10	
12c	4-CH_3−C_6H_4	OMe	H	19.7	NA	NA	>10	
12d	4-OCH_3−C_6H_4	OMe	H	3.93	5.00	5.00	>10	>2.5
12e	4-$C(CH_3)_3$−C_6H_4	OMe	H	0.61	1.25	1.25	>10	>16.3
12f	4-C_6H_5−C_6H_4	OMe	H	0.59	1.25	1.25	>10	>16.9
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]Compounds that did not exhibit activity at the highest tested concentration of 20 μg/mL were considered not active (NA). [e]The in vitro cytotoxicity was determined against four human cancer cell lines (SK-MEL, KB, BT-549, and SK-OV-3) and two noncancerous mammalian kidney cells (VERO and LCC-PK_1) up to a highest tested concentration of 10 μg/mL. [f]Selectivity index was calculated as CTX divided by IC_{50} values for *C. neoformans*.

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Table 3. In Vitro Antibacterial Activity of Peptides[a]

	S. aureus (μg/mL)			*MRSA* (μg/mL)		
peptide	IC_{50}	MIC	MBC	IC_{50}	MIC	MBC
9e	12.51	20.00	NA	11.50	20.00	NA
9f	6.88	20.00	NA	10.77	20.00	20.00
11f	15.41	NA	NA	NA	NA	NA
cipro	0.08	0.25	0.50	0.09	0.25	0.50

[a]MBC (minimum bactericidal concentration) is the lowest test concentration (μg/mL) that kills 100% of the organism. NA, not active.

as the National Committee for Clinical Laboratory Standards, NCCLS).[33] Amphotericin B, which is used clinically for *C. neoformans* infections but is associated with high toxicity toward mammalian cells, served as a positive control in these studies.[34]

To have a better understanding of the structure−activity relationship it was decided to also evaluate Boc-protected intermediate peptides **9a−f** and **10a−f** for their activity, in addition to target peptides **11a−f** and **12a−f** (Table 2). The results from screening of activity against strains fungal and bacterial strains showed that the peptides were in general more selective against the *C. neoformans* than *S. aureus* and *MRSA*. The data shows that the substituents attached to the phenyl ring of the centrally placed amino acid, L-histidine, greatly influenced the overall hydrophobicity and activity of the peptides.

In series 1, peptide **11e** containing a bulky *t*-butyl group at the *para*-position of the phenyl moiety placed at the C-2 position of the imidazole ring was found to be the most potent against *C. neoformans* with an IC_{50} of 0.07 μg/mL as compared to 0.69 μg/mL for amphotericin B. It also showed potent MIC and MFC value of 0.16 μg/mL. Peptide **11f** containing a biphenyl group at the C-2 position of the imidazole ring also showed similar activity. Most noteworthy are the potent MFCs of **9e**, **9f**, and **11d−f**, which are between 2 and 8 times more potent than amphotericin B. Peptides **9e** and **9f** also exhibited weak antibacterial activity against *S. aureus* and *MRSA* with IC_{50} values in the range of 6.88 and 12.51 μg/mL and MIC of 20.00 μg/mL (Table 3).

As discussed above, in series 2 peptides (**12e** and **12f**) having bulky groups at C-2 position of the imidazole ring like *t*-butylphenyl or biphenyl exhibit most potent activity against *C. neoformans* with IC_{50} values of 0.61 and 0.59 μg/mL, respectively. Other peptides of this series **10b−10f**, **12a**, and **12c−d** also showed promising IC_{50} values against *C. neoformans* in the range of 0.71−20 μg/mL. The presence of NHBzl group appeared to be more optimal for antifungal activity compared to OMe group at the C-terminus.

All synthesized peptides were also evaluated for cytotoxicity in a panel of mammalian cell lines to determine their safety profile. The in vitro cytotoxicity 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) by neutral red uptake assay.[35] The results demonstrated that the synthesized peptides were nontoxic up to a concentration of 10.00 μg/mL, which is indicative of a higher selectivity index (>1- to >142-fold) of anticryptococcal activity. The activity of these compounds does not seem to be due to a general cytotoxic effect (Table 2).

In order to examine the impact of lipophilicity on the biological activity of the synthesized peptides, we measured their retention time (t_R) by HPLC using a C-18 column and determined logP values using ACD/LogP software.[36] The results demonstrated a strong correlation between the biological activity and overall lipophilicity of the tripeptides (Table 4). The correlation of activity and lipophilicity of Boc-protected peptides **10a−f** and **9a−f** is provided in Table 4. The data show that the central residue, histidine, when substituted with the bulky *t*-butyl on the para position of phenyl ring shows highest potency.

Table 4. Correlation of Lipophilicity and Activity of Peptides Using RP-HPLC[a]

peptide	HPLC analysis t_R (min)	logP	*C. neoformans* IC_{50} (μg/mL)
10a	10.75	−1.63	NA
10b	11.89	0.41	10.44
10c	14.76	0.87	9.71
10d	13.59	0.57	4.06
10e	20.91	2.09	0.71
10f	18.64	2.05	0.8
9a	16.41	−0.90	5.35
9b	17.63	1.14	0.67
9c	19.41	1.60	2.15
9d	18.93	1.30	0.68
9e	24.41	2.83	0.18
9f	24.04	2.78	0.20

[a]Retention times are given for elution of the respective peptide on a reversed phase analytical HPLC system using a C-18 column. NA, not active. Method: C-18 column (25 cm × 4.6 mm, 5 μM) run for 40 min with a flow of 1 mL/min, using a gradient of 85−5%, where buffer A was 0.1% CF_3CO_2H (TFA) in H_2O and buffer B was 0.1% TFA in CH_3CN and detection at 220 nm.

The peptides **10e** and **9e** exhibited highest t_R and logP of 20.91 min/2.09 and 24.41 min/2.83, respectively. The peptides containing a biphenyl ring, **10f** and **9f** (t_R and logP = 18.64 min/2.05 and 24.04 min/2.78, respectively) are less hydrophobic than **10e** and **9e**, showed the second highest antifungal potency against the *C. neoformans*. The effect on the hydrophobicity is also due to the presence of nonpolar side groups. A closer examination of the results also revealed that peptide **10e** containing an ester group at the C-terminus was less active compared to **9e** containing a more hydrophobic benzylamide group at the C-terminus. The more hydrophobic benzylamide group containing peptides, in general, exhibit higher bioactivity. To conclude, in all the cases, peptides with 4-*t*-butylphenyl and biphenyl substituents at the C-2 position of the histidine residue exert more hydrophobicity than 4-methylphenyl, 4-methoxyphenyl, and phenyl substituted counterparts and thereby exhibit enhanced antifungal activity. The study depicting correlation of lipophilicity and activity of peptides **11a−f** and **12a−f** is provided in the Supporting Information.

For preliminary mechanistic studies, the most potent peptide **11e** was imaged by transmission electron microscopy (TEM) with a view to gain some understanding on its behavior in terms of interactions with the synthetic mimics of membranes.[37,38] The small unilamellar vesicles (SUVs) were prepared using the reported method in the literature.[39] The morphology of the SUVs was monitored in the presence and absence of the peptide **11e** by depositing, on to a carbon coated copper grid, of the treated and untreated SUVs and negatively staining the sample with 2% (w/v) phosphotungstic acid solution.

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The results indicated that the untreated SUVs were uniformly shaped, with intact morphology (Figure 2a,c),

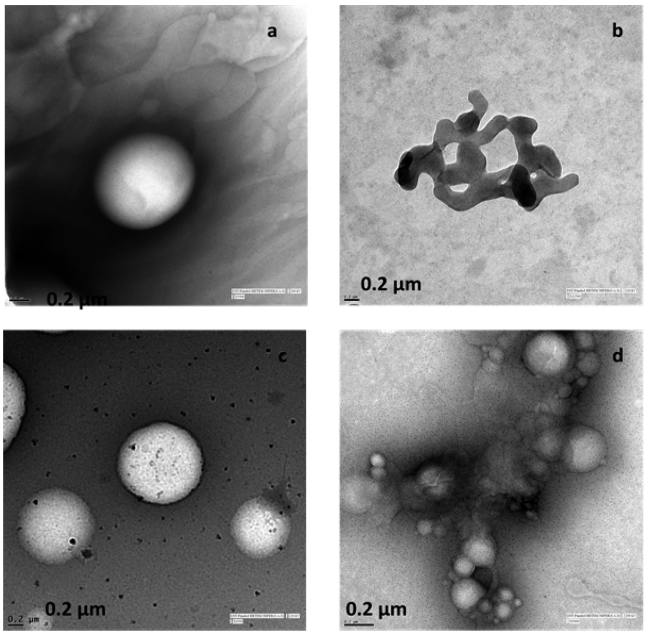

Figure 2. TEM images of untreated and treated SUVs with **11e**. (a) Untreated EYPC/EYPG. (b) EYPC/EYPG treated with **11e**. (c) Untreated EYPC/cholesterol. (d) EYPC/cholesterol treated with **11e**.

whereas SUVs treated with **11e** resulted in the destruction of their cell membrane. In the case of EYPC/EYPG, the integrity of SUVs was disrupted thoroughly when treated with peptide (Figure 2b), whereas in the case of EYPC/cholesterol the integrity of the cell wall is conserved in few cases (Figure 2d). These results indicate that the differential disintegration of the cell membrane of pathogenic fungi and cell membrane of host could be the basis for the preferential activity of these peptides toward *Cryptococcus*. It is presumed that tested peptide being cationic in nature preferably interacts with the negatively charged membrane as compared to zwitterionic membrane thereby exhibiting selectivity to *Cryptococcus*.

In conclusion, we have synthesized a series of novel small peptides exhibiting high selectivity against the fungal pathogen *C. neoformans*. The tripeptides were highly potent against *C. neoformans*, with two of them, **11e** and **11f**, being approximately 8-fold more potent than the standard drug. Most interestingly, peptides containing Boc group at the *N*-terminus also showed very promising antifungal activity. To uncover the plausible mode of selectivity of these peptides, we performed TEM studies. The results from TEM studies revealed that the most potent peptide **11e** possibly kills the fungal pathogen by disrupting the membrane, and thus, this class of peptides may be less susceptible to the common mechanisms of drug-resistance.

■ ASSOCIATED CONTENT

Supporting Information

Detailed synthetic procedures, characterization data, HPLC chromatograms, and details on the biological assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

All authors have given approval to the final version of the manuscript.

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Notes

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■ ABBREVIATIONS

AA, amino acid; AMB B, amphotericin B; DIC, 1,3-diisopropylcarbodiimide; DIEA, *N*,*N*-diisopropylethyl-amine; DMF, *N*,*N*-dimethylformamide; EYPC, egg yolk L-α-phosphatidylcholine; EYPG, egg yolk L-α-phosphatidyl-DL-glycerol; HATU, (1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo-[4,5-*b*]pyridinium 3-oxid hexafluorophosphate); HOAt, 1-hydroxy-7-azabenzotriazole; HONB, *N*-hydroxy-5-norbornene-2,3-dicarboxylic acid imide; IC_{50}, concentration (μg/mL) that affords 50% inhibition of growth; MFC/MBC, minimum fungicidal/bactericidal concentration; MIC, minimum inhibitory concentration; TEM, transmission electron microscopy

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