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Identification of Novel Antifungal Nonapeptides Through the Screening of Combinatorial Peptide Libraries Based on a Hexapeptide Motif †

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Abstract—Four sets of mixture based nonapeptide libraries derived from an antifungal hexapeptide pharmacophore Arg-D-Trp-D-Phe-Ile-D-Phe-His-NH_2 (**II**) have been synthesized. The three C-terminal positions 7, 8 and 9 were subject to randomization using 19 genetically coded amino acids. They were then screened for their antifungal activity against *Candida albicans* and *Cryptococcus neoformans* in order to quantify inhibition at each step of the nonapeptide sublibrary deconvolution. The studies led to the identification of several novel nonapeptides with potent antifungal activity. Two of the nonapeptides exhibited approximately 17-fold increase in the activity in comparison to the lead hexapeptide motif His-D-Trp-D-Phe-Phe-D-Phe-Lys-NH_2 (**I**) against *C. albicans*.

The frequency of serious fungal infections is rising, and this trend has been attributed to advances in therapeutic technology as well as the AIDS epidemic. There has been a constant effort to develop more effective and safe antifungal drug to combat these infections. Most of the current antifungal drugs simply reduce the level of growth of fungi. Amphotericin B, a drug of ultimate choice for the treatment of most of the systemic mycoses, is a potent antifungal agent, but is extremely toxic to kidney and to the central nervous system.[1,2] The development of resistant fungal strains in response to the widespread use of current antifungal drugs is likely to cause a serious problem in future.[3] Therefore, there is a need to develop novel antifungal drugs with a new mechanism of action. The development of novel antifungal agents from peptides has been the center of attraction owing to its unique property of perturbing the cell membrane of the pathogen thereby resulting in cell death.

Recently using combinatorial approach we optimized antifungal activity of a lead peptide His-D-Trp-D-Phe-Phe-D-Phe-Lys-NH_2 (**I**; IC_{50} 28.65 μM against *Candida albicans* and MIC 6.81 μM against *Cryptococcus neoformans*) that culminated in the identification of Arg-D-Trp-D-Phe-Ile-D-Phe-His-NH_2 (**II**), as a novel hexapeptide with potent antifungal activity against *Candida albicans* (IC_{50}; 6.8 μM) and *Cryptococcus neoformans* (MIC; 6.8 μM).[4] However, during our iteration studies with the hexapeptide libraries we could observe only four-fold increase in the antifungal activity of **II** over that of **I** against *C. albicans* whereas against *C. neoformans* there was no gain in the activity. This led us to choose **II** as a motif in a new library of nonapeptides with the view to identify congeners with higher antifungal activity. We propose to examine the effect of increasing the chain length to nonapeptides on the biological activity as they are known to adopt more ordered conformations than hexapeptides. The libraries have been designed in a manner wherein positions 7, 8, 9 were subject to randomization whereas amino acids from positions 1 to 6 were retained. Synthetic combinatorial libraries of peptides have been extensively used in many areas of biomedical research for the optimization of lead molecules resulting in several-fold increase in the activity.[5,6] In this paper we describe the synthesis and screening of nonapeptide libraries based on hexapeptide motif **II**.

Synthesis

Libraries were prepared on Rink amide AM (0.63 mmol/gm) resin using premix method.[7] In the first

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instance 19 nonapeptide sublibraries involving hexapeptide motif **II** represented by a general formula RwfIfHO^{7}X^{8}X^9-NH$_2$ have been synthesized. The position 7 of this library has been individually defined with each of the 19 genetically coded amino acids (excluding Cys) at a time whereas X^8 and X^9 contain equimolar mixture of 19 genetically coded amino acids. The peptide mixtures were cleaved from the resin using reagent K[8] (TFA: phenol: ethanedithiol: thioanisole: water mixture) and precipitated with the addition of dry ether. The precipitates were washed with ether and lyophilized from *t*-BuOH: H_2O (4:1). The libraries were characterized using ES–MS and the observed mass distribution for different libraries showed good correlation with the simulated mass distribution. Amino acid analysis of libraries confirmed ES–MS results and the expected equimolarity with the accuracy of such analysis ($\pm 10\%$).

Antifungal Activity

The libraries were tested for their antifungal activity by two-fold microbroth dilution technique.[4,9] Serial dilutions of the libraries from mM to μM range were used to determine IC_{50} and MIC values as described earlier. The optical density was recorded on an microplate reader at 492 nm. The MIC was defined as the lowest concentration of compound that substantially inhibited the growth of *C. albicans* (*Ca*, SKF) after 24 h and *C. neoformans* (*Cn*-17) after 48 h. The inhibition of *Ca* by libraries has been expressed as IC_{50} whereas for *Cn* it has been expressed as MIC. In case of *Cn* it was not possible to determine IC_{50} values using two-fold dilution technique protocol due to sudden drop in inhibition from one dilution to other.

Results and Discussion

The results for the antifungal assay of the first set of 19 pools of sublibraries are summarized in Table 1. As is evident out of the 19 pools, one of the sublibraries RwfIfHK^{7}X^{8}X^9-NH$_2$ exhibited best inhibitions in comparison to other sublibraries with IC_{50} value of 3.7 μM against *Ca* and MIC value of 2.4 μM against *Cn*. Two other peptide mixtures with His (IC_{50} 7.6 μM against *Ca* and MIC 2.4 μM against *Cn*) and Arg (IC_{50} 8.5 μM against *Ca* and MIC 4.8 μM against *Cn*) at position 7 had activity within two-fold of the most active peptide mixture. RwfIfHKX^{8}X^9-NH$_2$ sublibrary was therefore selected for further deconvolution in order to define the remaining two mixture positions. Thus, in the next step a second set of sublibraries comprising of 19 pools (each pool comprising 19 equimolar peptides) and represented by general formula RwfIfHKO^{8}X^9-NH$_2$ were synthesized and evaluated for their antifungal activity (Table 2). From these pools, the best inhibition was observed for the sublibrary RwfIfHKK^{8}X^9-NH$_2$ with IC_{50} value of 3.5 μM against *Ca* and MIC value of 1.22 μM against *Cn*. Surprisingly from the second set of sublibraries we could identify a mixture with only marginal gain in the antifungal activity against *Ca* in comparison to the parent sublibrary RwfIfHKX^{8}X^9-NH$_2$, however, against *Cn* a gain of two-fold in the antifungal activity was observed. The only other mixture close to RwfIfHKK^{8}X^9-NH$_2$, in terms of activity was sublibrary RwfIfHKR^{8}X^9-NH$_2$ with Arg at position 8 ($IC_{50} = 4.15$ μM and MIC = 2.39 μM).

Lys was therefore selected for the eight position however; iteration of RwfIfHKR^{8}X^9-NH$_2$ was also pursued for the purpose of comparison. Thus, in the next step, two sets of sublibraries: RwfIfHKK^{8}O^9-NH$_2$ and RwfIfHKR^{8}O^9-NH$_2$ were synthesized and evaluated for their antifungal activity. The final iteration resulted in the synthesis of two sets of 19 single peptides with all the position defined. The purity and identity of all individual

Table 1. Antifungal data for RwfIfHO^{7}X^{8}X^9-NH$_2$

Iteration 1	*Ca* IC_{50} (μM)	*Cn* MIC (μM)
RwfIfHAXX-NH$_2$	11.1	5.1
RwfIfHDXX-NH$_2$	24.6	19.8
RwfIfHEXX-NH$_2$	39.5	19.6
RwfIfHFXX-NH$_2$	31.3	19.3
RwfIfHGXX-NH$_2$	24.6	5.2
RwfIfHHXX-NH$_2$	7.6	2.4
RwfIfHIXX-NH$_2$	22.2	9.9
RwfIfHKXX-NH$_2$	**3.7**	**2.4**
RwfIfHLXX-NH$_2$	9.5	4.9
RwfIfHMXX-NH$_2$	13.4	9.8
RwfIfHNXX-NH$_2$	14.7	9.9
RwfIfHPXX-NH$_2$	17.5	5.0
RwfIfHQXX-NH$_2$	16.4	4.9
RwfIfHRXX-NH$_2$	8.5	4.8
RwfIfHSXX-NH$_2$	12.4	5.0
RwfIfHTXX-NH$_2$	11.5	5.0
RwfIfHVXX-NH$_2$	11.5	5.0
RwfIfHWXX-NH$_2$	22.4	9.4
RwfIfHYXX-NH$_2$	11.7	4.7
HwfFfK-NH$_2$ (**I**)	28.65	6.81
RwfIfH-NH$_2$ (**I**)	6.85	6.86

All L-amino acids have been denoted by capital single letter code whereas for D-amino acids following single letter code has been used: w = D-Trp, f = D-Phe.

Table 2. Antifungal data for RwfIfHKO^{8}X^9-NH$_2$

Iteration 2	*Ca* IC_{50} (μM)	*Cn* MIC (μM)
RwfIfHKAX-NH$_2$	10.54	2.55
RwfIfHKDX-NH$_2$	19.7	4.94
RwfIfHKEX-NH$_2$	24.07	4.88
RwfIfHKFX-NH$_2$	7.4	2.40
RwfIfHKGX-NH$_2$	7.41	1.29
RwfIfHKHX-NH$_2$	> 20.0	> 5.0
RwfIfHKIX-NH$_2$	6.61	1.23
RwfIfHKKX-NH$_2$	**3.5**	**1.22**
RwfIfHKLX-NH$_2$	5.54	2.47
RwfIfHKMX-NH$_2$	7.07	2.43
RwfIfHKNX-NH$_2$	7.34	2.47
RwfIfHKPX-NH$_2$	8.43	2.51
RwfIfHKQX-NH$_2$	6.92	2.44
RwfIfHKRX-NH$_2$	4.15	2.39
RwfIfHKSX-NH$_2$	7.15	2.52
RwfIfHKTX-NH$_2$	6.12	2.49
RwfIfHKVX-NH$_2$	15.86	2.45
RwfIfHKWX-NH$_2$	6.94	2.33
RwfIfHKYX-NH$_2$	11.09	2.37
HwfFfK-NH$_2$ (**I**)	28.65	6.81
RwfIfH-NH$_2$ (**I**)	6.85	6.86

All L-amino acids have been denoted by capital single letter code whereas for D-amino acids following single letter code has been used: w = D-Trp, f = D-Phe.

Table 3. Antifungal data for RwfIfHKKO^9-NH_2

Iterative 3		*Ca* IC_{50} (μM)	*Cn* MIC (μM)	Iterative 3		*Ca* IC_{50} (μM)	*Cn* MIC (μM)
RwfIfHKKA-NH_2	(A)	3.17	2.48	RwfIfHKKN-NH_2	(N)	3.35	2.40
RwfIfHKKD-NH_2	(D)	3.52	4.80	RwfIfHKKP-NH_2	(P)	3.89	2.43
RwfIfHKKE-NH_2	(E)	3.57	2.37	RwfIfHKKQ-NH_2	(Q)	2.58	2.37
RwfIfHKKF-NH_2	(F)	>7.0	>7.0	RwfIfHKKR-NH_2	(R)	1.64	2.32
RwfIfHKKG-NH_2	(G)	2.77	2.51	RwfIfHKKS-NH_2	(S)	1.77	2.45
RwfIfHKKH-NH_2	(H)	1.90	2.36	RwfIfHKKT-NH_2	(T)	2.38	2.44
RwfIfHKKI-NH_2	(I)	1.65	2.40	RwfIfHKKV-NH_2	(V)	3.08	2.43
RwfIfHKKK-NH_2	(K)	1.83	2.37	RwfIfHKKW-NH_2	(W)	2.61	**1.14**
RwfIfHKKL-NH_2	(L)	2.18	2.40	RwfIfHKKY-NH_2	(Y)	2.74	**1.16**
RwfIfHKKM-NH_2	(M)	2.62	2.37	HwfFfK-NH_2	(**I**)	28.65	6.81
RwfIfH-NH_2	(**II**)	6.85	6.86				

Table 4. Antifungal data for RwfIfHKRO^9-NH_2

Iterative 3		*Ca* IC_{50} (μM)	*Cn* MIC (μM)	Iterative 3		*Ca* IC_{50} (μM)	*Cn* MIC (μM)
RwfIfHKRA-NH_2	(A)	5.99	4.96	RwfIfHKRN-NH_2	(N)	4.64	2.39
RwfIfHKRD-NH_2	(D)	7.71	4.79	RwfIfHKRP-NH_2	(P)	4.70	2.42
RwfIfHKRE-NH_2	(E)	4.23	4.74	RwfIfHKRQ-NH_2	(Q)	3.92	4.74
RwfIfHKRF-NH_2	(F)	5.50	4.67	RwfIfHKRR-NH_2	(R)	4.15	4.64
RwfIfHKRG-NH_2	(G)	4.48	2.50	RwfIfHKRS-NH_2	(S)	4.38	4.89
RwfIfHKRH-NH_2	(H)	4.11	2.35	RwfIfHKRT-NH_2	(T)	5.97	4.84
RwfIfHKRI-NH_2	(I)	4.64	2.39	RwfIfHKRV-NH_2	(V)	4.88	4.85
RwfIfHKRK-NH_2	(K)	**3.34**	**2.36**	RwfIfHKRW-NH_2	(W)	6.35	4.54
RwfIfHKRL-NH_2	(L)	4.37	2.39	RwfIfHKRY-NH_2	(Y)	7.08	4.62
RwfIfHKRM-NH_2	(M)	4.62	2.36	HwfFfK-NH_2	(**I**)	28.65	6.81
RwfIfH-NH_2	(**II**)	6.85	6.86				

peptides were characterized by RPHPLC (purities ranging from 80 to 93%) and ES–MS. Peptides with less than 90% homogeneity were purified for final IC_{50} and MIC determination. After assaying the antifungal activity, a number of peptides exhibited high order of antifungal activity at this iterative step (Tables 3 and 4). From these sublibraries, best inhibition against *Ca* was observed for Arg (IC_{50} = 1.64 μM) and Ile (IC_{50} = 1.65 μM) nonapeptides with Lys at position 8. Interestingly, against *Cn* though several nonapeptides were found to exhibit equipotent activities with MIC's in the range of 2.32 to 2.51 μM, the most active peptides were RwfIf HKKW-NH_2 and RwfIfHKKY-NH_2 with MIC's 1.14 and 1.16 μM, respectively. In contrast best inhibition in the second set of 19 peptides with Arg at position 8 was observed for RwfIfHKRK-NH_2 with IC_{50} value of 3.34 against *Ca* and MIC of 2.36 μM against *Cn*. In order to establish the differences in the antifungal activity exhibited by the two sets of 19 single peptides, graphical representations of the inverse of IC_{50} values for *Ca* and MIC values for *Cn* are shown in Figures 1 and 2. It is clearly evident that nonapeptides with Lys at position 8 resulted in more active compounds than nonapeptides with Arg at position 8.

Our studies thus led to the identification of several nonapeptides with potent antifungal activity against *Ca* and *Cn*. Though the most active peptides RwfIfHKKR-NH_2, RwfIfHKKI-NH_2, RwfIfHKKW-NH_2 and RwfIfH KKY-NH_2 exhibited approximately 4- to 5-fold increase in the antifungal activity over that of the hexapeptide **II**, RwfIfHKKR-NH_2 (IC_{50} 1.64 μM) and RwfIfHKKI-

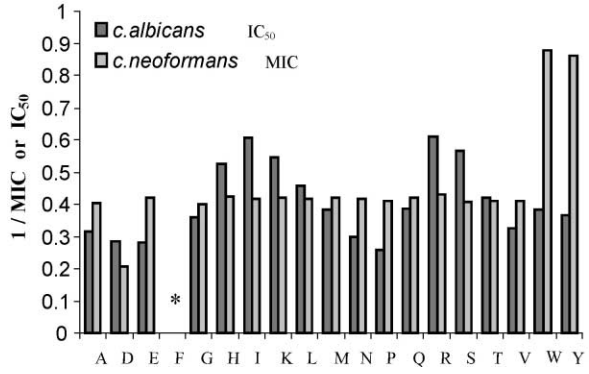

Figure 1. Graphical representation of antifungal activity of 19 single peptides with Lys at position 8 (**n.d*).

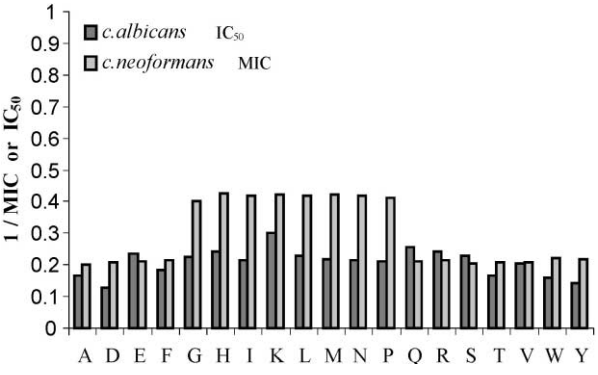

Figure 2. Graphical representation of antifungal activity of 19 single peptides with Arg at position 8.

NH_2 (IC_{50} 1.65 μM) were found to be approximately 17 times more active than the lead peptide I (IC_{50} 28.65 μM) against *Ca*. Thereby suggesting that extending the chain length in motif **II** from hexapeptide to nonapeptides with randomization at the C-terminal region had a favorable affect on the biological activity. Encouraged by the significant gains in the activity further studies have been initiated to evaluate the affect on the antifungal activity when the chain length of the hexapeptide motif **II** is increased by introducing randomization either at the N-terminal region only or on both sides simultaneously.

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