Pergamon

Bioorganic & Medicinal Chemistry Letters 10 (2000) 1779–1781

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Combinatorial Approach to Lead Optimization of a Novel Hexapeptide with Antifungal Activity†

B. Kundu,[a,*] S. K. Rastogi,[a] S. Batra,[a] S. K. Raghuwanshi[b] and P. K. Shukla[b]

[a]*Division of Medicinal Chemistry, Central Drug Research Institute, Lucknow 226 001, India*
[b]*Division of Medical Mycology, Central Drug Research Institute, Lucknow 226 001, India*

Received 29 March 2000; accepted 24 May 2000

Abstract—Three sets of sublibraries of an antifungal lead peptide His-D-Trp-D-Phe-Phe-D-Phe-Lys-NH_2 **(I)** have been prepared by introducing variations at positions 1, 4 and 6. They were screened for their antifungal activity against *C. albicans* and *C. neoformans* in order to quantify inhibition at each step of the hexapeptide sublibrary iteration. The studies led to the identification of Arg-D-Trp-D-Phe-Ile-D-Phe-His-NH_2 as a novel hexapeptide with potent antifungal activity against both *C. albicans* and *C. neoformans*.

The incidence of fungal infections continues to grow and is generally associated with host immunodeficiency. Current agents are inadequate in patients whose immune systems are debilitated due to AIDS, ARC organ transplant and cancer therapy. *Candida albicans* and *Cryptococcus neoformans* are two of the most common opportunistic fungi responsible for infections.[1] Out of these *Candida albicans* infections may become problematic in severely immunocompromised patients and may induce oral candiasis, esophageal candiasis, and vaginal candiasis.[2] On the contrary *Cryptococcus neoformans* is the causative agent of cryptococcosis, which is the leading cause of morbidity and mortality due to fungi in patients with AIDS.[3] Thus, there is urgent need for more effective and novel antifungal therapies.

In our laboratory we initiated screening of a variety of structurally diverse small synthetic peptides with the view to identify a lead peptide with a broad spectrum of antifungal activity. The studies led to the identification of a hexapeptide His-D-Trp-D-Phe-Phe-D-Phe-Lys-NH_2 **(I)** related to growth hormone releasing hexapeptide (GHRP-6) as a lead molecule with antifungal activity against five pathogenic fungi in the range of 6.2–100 μg/mL[4]. The D-amino acids present at position 2, 3 and 5 were found to be essential for antifungal activity whereas amino acids at position 1, 4 and 6 could be varied without loss in the activity. Thus the lead peptide provides an interesting pharmacophore for the design of more potent antifungal agents. This prompted us to apply combinatorial approach to create mixture based library of the lead peptide **I** with the view to identify congeners with a higher antifungal activity. The libraries were designed in a manner wherein positions 1, 4 and 6 were subject to randomization whereas D-amino acids at positions 2, 3 and 5 have been retained. Mixture based synthetic combinatorial libraries of peptides have been successfully used in many areas of biomedical research by others[5] and us[6] for the optimization of lead molecule. In this paper we describe the synthesis and screening of hexapeptide libraries based on pharmacophore **I**.

Synthesis

The libraries were prepared on Rink Amide AM resin using premix method.[7] In the first instance 19 sublibraries of pharmacophore **I** represented by general formula $O^1wfX^4fX^6$-NH_2 were synthesized in which position 1 has been individually defined with each of the 19 amino acids (excluding Cys) at a time whereas X^4 and X^6 are equimolar mixture of 19 amino acids. The peptide mixtures were cleaved from the resin using TFA:phenol:ethanedithiol:thioanisole:water mixture and precipitated with the addition of 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.[7]

*Corresponding author. Tel.: 212411-18; fax: +91-0522-223405
†CDRI communication No. 6044.

0960-894X/00/$ - see front matter
PII: S0960-894X(00)00343-7

Amino acid analysis of libraries confirmed ES–MS results and the expected equimolarity with the accuracy of such analysis (+/−10%).

Antifungal Activity

The libraries were tested for their antifungal activity by twofold microbroth dilution technique.[8] Serial dilutions of the libraries from mM to nM range were used to determine IC_{50} and MIC values. The fungal inoculum were prepared from fresh slant growth (at 28 °C) of *C. albicans* (24 h) and *C. neoformans* (48 h) in sterile normal saline and the colony forming units (cfu) determined by dilution and plating technique. To each well of a sterile 96-well flat bottom microtitre plate, 150 μL Sabouraud's dextrose broth was added except the fourth column where 270 μL broth was used. To this fourth column 30 μL of the test compound was added and diluted two fold from 4th through to the 11th column. Appropriate growth control, test compound control and blank were also taken. Finally 20 μL of the inoculum was added to each well (final cfu $1\times10^{3-4}$). To the first row 5 μL of 4% formaldehyde was added which served as blank. The plates were incubated at 28 °C in a moist chamber for 24–48 h. The optical density was recorded on an ELISA reader at 492 nM. The MIC was defined as the lowest concentration of compound that substantially inhibited the growth of *C. albicans* (*Ca*) after 24 h and *C. neoformans* (*Cn*) after 48 h in accordance to NCCLS standards. 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 twofold 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 have been summarized in Table 1. As is evident out of the 19 pools, one of the sublibraries, $RwfX^4fX^6\text{-}NH_2$ with Arg at position 1 exhibited best inhibition in comparison to other sublibraries with IC_{50} of 84 μM against *Ca* and MIC of 28 μM against *Cn*. $RwfX^4fX^6\text{-}NH_2$ sublibrary was therefore selected for further iteration in order to define the remaining two mixture positions. Beside this iteration of $HwfX^4fX^6\text{-}NH_2$ ($IC_{50}=114$ μM against *Ca* and MIC = 57 μM against *Cn*) was also pursued for the purpose of comparison, as His is present at position 1 in the lead molecule. Thus in the next step two set of sublibraries each comprising of 19 pools (each pool comprising of 19 peptides) and represented by the general formula: $HwfO^4fX^6\text{-}NH_2$ and $RwfO^4fX^6\text{-}NH_2$ were synthesized and evaluated for their antifungal activity.

From these 2×19 pools, the best inhibition was observed for Ile ($IC_{50}=15.78$ μM against *Ca* and MIC = 14.09 μM against *Cn*) and Leu ($IC_{50}=18.04$ μM against *Ca* and MIC = 14.09 μM against *Cn*) with Arg at position 1. In contrast $HwfFfX^6\text{-}NH_2$ ($IC_{50}=30.72$ μM against *Ca* and MIC = 27.72 μM against *Cn;* data not shown) corresponding to the lead molecule **I**, and also the best inhibitor among the 19 pools with His at position 1 exhibited higher IC_{50} and MIC values. Therefore iteration for His^1 sublibrary was dropped and deconvolution for $RwfIfX^6\text{-}NH_2$ was continued to define the third and final mixture position in order to complete the iterative process. Deconvolution of $RwfLfX^6\text{-}NH_2$ with IC_{50} and MIC values close to $RwfIfX^6\text{-}NH_2$ may be also of interest and will be taken up later.

Table 1. IC_{50} and MIC values for iteration carried out on $O^1wfX^4fX^6\text{-}NH_2$[a,b,c]

Iteration 1	*Ca* IC_{50} [μM]	*Cn* MIC [μM]	Iteration 2	*Ca* IC_{50} [μM]	*Cn* MIC [μM]	Iteration 3	*Ca* IC_{50} [μM]	*Cn* MIC [μM]
$AwfX^4fX^6\text{-}NH_2$	495	247	$RwfAfX^6\text{-}NH_2$	86.43	59.2	$RwfIfA\text{-}NH_2$	>14.91	14.91
$DwfX^4fX^6\text{-}NH_2$	>469	>469	$RwfDfX^6\text{-}NH_2$	150.95	112.65	$RwfIfD\text{-}NH_2$	>14.91	113.37
$EwfX^4fX^6\text{-}NH_2$	>461	>461	$RwfEfX^6\text{-}NH_2$	>221.57	221.57	$RwfIfE\text{-}NH_2$	>28.4	55.79
$FwfX^4fX^6\text{-}NH_2$	243	113	$RwfFfX^6\text{-}NH_2$	31.49	27.15	$RwfIfF\text{-}NH_2$	13.67	13.67
$GwfX^4fX^6\text{-}NH_2$	406	251	$RwfGfX^6\text{-}NH_2$	81.87	60.2	$RwfIfG\text{-}NH_2$	33.85	15.16
$HwfX^4fX^6\text{-}NH_2$	114	57	$RwfHfX^6\text{-}NH_2$	38.43	27.45	**$RwfIfH\text{-}NH_2$**	**6.85**	**6.85**
$IwfX^4fX^6\text{-}NH_2$	>447	223	**$RwfIfX^6\text{-}NH_2$**	**15.78**	**14.09**	$RwfIfI\text{-}NH_2$	45.56	28.40
$KwfX^4fX^6\text{-}NH_2$	107	57	$RwfKfX^6\text{-}NH_2$	53.45	27.72	$RwfIfK\text{-}NH_2$	14.41	**6.92**
$LwfX^4fX^6\text{-}NH_2$	357	235	**$RwfLfX^6\text{-}NH_2$**	**18.04**	**14.09**	$RwfIfL\text{-}NH_2$	14.91	**7.04**
$MwfX^4fX^6\text{-}NH_2$	305	230	$RwfMfX^6\text{-}NH_2$	30.94	27.63	$RwfIfM\text{-}NH_2$	24.49	27.83
$NwfX^4fX^6\text{-}NH_2$	163	117	$RwfNfX^6\text{-}NH_2$	77.73	28.16	$RwfIfN\text{-}NH_2$	>14.91	14.18
$PwfX^4fX^6\text{-}NH_2$	408	479	$RwfPfX^6\text{-}NH_2$	87.29	57.43	$RwfIfP\text{-}NH_2$	51.38	28.93
$QwfX^4fX^6\text{-}NH_2$	>462	462	$RwfQfX^6\text{-}NH_2$	197.42	110.91	$RwfIfQ\text{-}NH_2$	30.16	55.86
$RwfX^4fX^6\text{-}NH_2$	**84**	**28**	$RwfRfX^6\text{-}NH_2$	52.7	26.89	$RwfIfR\text{-}NH_2$	>12.5	**6.71**
$SwfX^4fX^6\text{-}NH_2$	265	242	$RwfSfX^6\text{-}NH_2$	126.66	58.1	$RwfIfS\text{-}NH_2$	>14.91	14.63
$TwfX^4fX^6\text{-}NH_2$	434	238	$RwfTfX^6\text{-}NH_2$	139.49	57.16	$RwfIfT\text{-}NH_2$	>14.91	14.39
$VwfX^4fX^6\text{-}NH_2$	364	239	$RwfVfX^6\text{-}NH_2$	28.64	28.64	$RwfIfV\text{-}NH_2$	>28.4	14.43
$WwfX^4fX^6\text{-}NH_2$	205	108	$RwfWfX^6\text{-}NH_2$	25	**15.01**	$RwfIfW\text{-}NH_2$	25.38	13.11
$YwfX^4fX^6\text{-}NH_2$	186	222	$RwfYfX^6\text{-}NH_2$	183.62	53.38	$RwfIfY\text{-}NH_2$	15.91	13.43
$HwfFfK\text{-}NH_2$ (Lead peptide)	29.6	6.81	$HwfFfK\text{-}NH_2$ (Lead peptide)	28.65	6.81	$HwfFfK\text{-}NH_2$ (Lead peptide)	28.56	6.81

[a]All natural amino acids have been represented by capital letters.
[b]D-Amino acids have been denoted by small letters.
[c]Libraries with maximal inhibition have been shown in bold.

The final iteration of RwfIfX^6-NH_2 resulted in the synthesis of 19 single peptides with all the positions defined. The purity and identity of all individual peptides were characterized by RPHPLC (purities ranging from 85 to 95%) and ES-MS. Peptides with less than 95% purities were purified to more than 95% homogeneity for final IC_{50} and MIC determination. After assaying for their antifungal activity, it was found that RwfIfH-NH_2 was the most effective peptide inhibitor against *Ca* with IC_{50} value of 6.85 μM and was at least four times more potent than the lead molecule **I** with IC_{50} value of 28.56 μM. However, against *Cn* it was observed that His (MIC = 6.85 μM), Lys (MIC = 6.92 μM), Leu (MIC = 7.04 μM) and Arg (MIC = 6.71 μM) were the most acceptable ones at position 6 and their MIC values were more or less equal to the lead molecule **I** (MIC = 6.81 μM). Indeed out of these only RwfIfH-NH_2 exhibited potent inhibitory effect against *Ca* as well.

Our studies thus led to the identification of a novel hexapeptide RwfIfH-NH_2, as the most effective peptide inhibitor against *Ca* and *Cn*. Although this confirms the validity of combinatorial approach to identify inhibitors, we did not observe drastic gains in the potency from 2nd to 3rd iteration as described earlier by us[6] and others.[5] Further modifications are nevertheless necessary to optimize our lead structure in order to enhance its inhibitory capacity.

References

1. Hazen, K. C. *Clin. Microbiol. Rev.* **1995**, *8*, 462.
2. Diamond, R. D. *Rev. Infect. Dis.* **1991**, *13*, 480.
3. Powderly, W. G. *Clin. Infect. Dis.* **1993**, *17*, 837.
4. Kundu, B.; Khare, S. K.; Raghuwanshi, S. K.; Shukla, P. K. *Antimicrob. Agents Chemother.* **2000** (submitted for publication).
5. Houghten, R. A.; Pinilla, C.; Appel, J. R.; Blondelle, S. E.; Dooley, C. T.; Eichler, J.; Nefzi, A.; Ostresh, J. M. *J. Med. Chem.* **1999**, *42*, 3743.
6. Kundu, B.; Bauser, M.; Betschinger, J.; Kraas, W.; Jung, G. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1669.
7. In *Combinatorial Peptide and Nonpeptide Libraries*; Jung, G.; Ed.; VCH: Germany.
8. Iwata, K.; Bossche, H. V.; *In Vitro and In Vivo Evaluation of Antifungal Agents*; Elesevier Science: Amsterdam, 1986; pp 31–34.