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In Vitro Activity of Synthetic Antimicrobial Peptides Against *Candida*

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Abstract

Yeast-like fungi are the most common cause of fungal infections in humans. Actually, in the age of opportunistic infections and increasing resistance, development of modern antifungal agents becomes a very important challenge. This paper describes synthesis and antimicrobial assay of four naturally occurring peptide antibiotics (aurein 1.2, citropin 1.1, temporin A, uperin 3.6) and three chemically engineered analogues actually passing clinical trials (iseganan, pexiganan, omiganan) against *Candida* strains isolated from patients with infections of the oral cavity or respiratory tract. The peptides were synthesized using solid-phase method and purified by high-performance liquid chromatography. Biological tests were performed using the broth microdilution method. The antifungal activity of the peptide antibiotics was compared to that of nystatin and amphotericin B. We found synthetic peptides to be generally less potent than amphotericin B or nystatin. However, some of the naturally occurring peptides still retained reasonable antifungal activities which were higher than these of iseganan, pexiganan or omiganan. We think that the naturally occurring peptide antibiotics included in our study can be a good matrix for development of novel antifungal compounds.

Key words: antimicrobial activity, antimicrobial peptides; fungi

Introduction

Nowadays superficial fungal infections emerge as an important problem. Many fungal species, among others *Candida*, reside either in oral flora or on the skin and mucous membranes. However, these commensal fungi may become pathogenic under certain circumstances, such as immunity disorders in the presence of a particular host factor (Zuber *et al.*, 2000). Neutropenia, cancer, chemotherapy and HIV are additional risk factors for opportunistic infections (Karabinis *et al.*, 1988; Punzon *et al.*, 2002). It is estimated that the yeast-like fungi *Candida* account for approximately 15% of all nosocomial infections (Vazquez, 2003). The unfavourable efficacy/toxicity ratio of some antifungals (*e.g.*, amphotericin B), limitations in usage (*e.g.*, azoles in the case of neutropenia) and appearance of resistant strains represent a major challenge for developing new methods of treatment (Marchetti *et al.*, 2003). Moreover a comparison of the susceptibility of fungi in these days to that recorded earlier shows a dramatic increase of resistant strains (Nierebinska *et al.*, 1992). New mechanisms of resistance are developed and are propagated rapidly among pathogenic strains. Accumulating resistance factors lead to a multidrug resistance phenotype (MDR), affecting an increasing number of strains (Vanden Bossche *et al.*, 1998; Prasad *et al.*, 2002). Since fungi belong to Eukarya domain, their mechanism of gene expression, replication and some structural features are similar to those occurring in human cells. This is the reason why developing of new, highly specific antifungals may be

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difficult. Therefore, researchers look for new agents with different chemical structures and different mechanisms of action to treat fungal infections.

Recently much attention has been devoted to natural antimicriobial peptides which are produced by living organisms and act as a very ancient line of innate immunity. Herein we present the results of our studies on anticandidal activity of some naturally occurring antimicrobial peptides: aurein 1.2, citropin 1.1, temporin A and uperin 3.6 (Chia *et al.*, 1999; Rozek *et al.*, 2000; Wegener *et al.*, 1999; Conlon *et al.*, 2004), and peptides actually passing clinical trials: iseganan, omiganan and pexiganan (Kamysz, 2005). Their fungicidal activities are compared to those of two commonly used antifungal drugs, amphotericin B and nystatin.

Experimental

Materials and Methods

Fungal strains The yeasts were isolated from patients with infections of the oral cavity and respiratory tract (Medical University of Gdańsk). The material was inoculated onto the Sabouraud agar (Becton-Dickinson) and incubated under aerobic conditions at room temperature for 72 hours. All *Candida* strains included in the study were identified according to the classification proposed by de Paiva Martins *et al.* (2002). We identified 18 strains of *Candida albicans*, 3 strains of *Candida tropicalis*, 2 strains of *Candida kefyr*, 2 strains of *Candida krusei*, 5 strians of *Candida parapsilosis* and 4 strains of *Candida glabrata*.

Antibacterial compounds. All the peptides included in the study were synthesized manually by the solid-phase method on Polystyrene AM-RAM resin (0.66 mmol/g; Rapp Polymere, Germany) using the 9-fluorenylmethoxycarbonyl (Fmoc) chemistry (Fields and Noble, 1990). The peptides were synthesized by the following procedure: (i) 5 and 15 min deprotection steps using 20% piperidine in dimethylformamide (DMF) in the presence of 1% Triton; (ii) the coupling reactions carried out with the protected amino acid (Fmoc-AA) diluted in a DMF/N-methyl-2-pyrrolidone (NMP) (1:1, v/v) mixture in the presence of 1% Triton using diisopropylcarbodiimide (DIC) as the coupling reagent in the presence of 1-hydroxybenzotriazole (HOBt) (Fmoc-AA/DIC/HOBt, 1:1:1) for 2 h. The completeness of each coupling reaction was monitored by the chloranil test (Christensen, 1979). The peptides were cleaved from the solid support by trifluoroacetic acid (TFA) in the presence of water (2.5%), and triisopropylsilane (2.5%) as scavengers. The cleaved peptides were precipitated with diethyl ether. Iseganan was cyclized by air oxidation as described in (Chen *et al.*, 2000). The peptides were purified by high-performance liquid chromatography (HPLC) on a Knauer two-pump system with a Kromasil C8 column 10×250 mm (5 µm particle diameter, 100 Å pore size) with a flow rate of 5 ml/min, absorbance at 226 nm. The resulting fractions of purity greater than 95–98% were tested by HPLC. The peptides were analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF). Sequences, net charges and molecular masses of particular peptides are shown in Table I.

Table I
Amino acid sequences of antimicrobial peptides

Peptide	Sequence	Molecular mass	Net charage of peptide
Naturally occurring antimicrobial peptides			
Aurein 1.2	GLFDIIKKIAESF-NH_2	1 479.8 Da	+1
Citropin 1.1	GLFDVIKKVASVIGGL-NH_2	1 614.9 Da	+2
Temporin A	FLPLIGRVLSGIL-NH_2	1 396.7 Da	+2
Uperin 3.6	GVIDAAKKVVNVLKNLF-NH_2	1 827.2 Da	+3
Chemically engineered peptides			
Omiganan	ILRWPWWPWRRK-NH_2	1 779.2 Da	+5
Iseganan	RGGLCYCRGRFCVCVGR-NH_2	1 904.3 Da	+5
Pexiganan	GIGKFLKKAKKFGKAFVKILKK-NH_2	2 477.2 Da	+10

Nystatin and amphotericin were obtained from Sigma-Aldrich.

Antifungal susceptibility testing. The yeasts were tested by the broth microdilution method, performed according to the CLSI (M27-A2) standard guidelines (CLSI guideline, 2002). The antimicrobial peptides, nystatin and amphotericin B, were used to obtain final drug concentrations ranging from 0.25 to 512 µg/ml. A standard inoculum of *Candida* was diluted to a final concentration of $0.5–1.5\times10^3$ CFU/ml in microtiter plates. The broth microdilution testing was performed in sterile flat-bottomed 96-well microplates which contained 100 µl of each tested drug (concentration 1 mg/ml). At the beginning of experiment 100 µl of a suspension containing a final concentration of fungi was dispensed into wells of each row containing diluted antifungal agents. Drug-free purity controls and growth controls were included for each experiment. The plates were incubated at 35°C for 48 h. The MIC assumed as the lowest drug concentration which inhibited the growth of tested fungi. Experiments were performed in triplicates.

Results

The antibiotics used commonly in antifungal therapies; nystatin and amphotericin B, tested against our clinical strains were several times more active than the peptide antibiotics included in the study, and their MIC values varied between 0.125 and 8 μg/ml. In some cases, the activity of the peptide antibiotics was found to be promising. In particular, uperin 3.6 and aurein 1.2 exhibited relatively high antifungal activity against *C. albicans* (Table II). Similarly, the antifungal activity of uperin 3.6, much higher than that of other peptide antibiotics, was noticed for non *C. albicans* strains (Table III). For 2 out of 3 strains of *C. tropicalis,*

Table II
The activity of antimicrobial peptides and conventional antifungals against *C. albicans* strains

Agent	Minimal inhibitory concentration (MIC) (μg/ml) *Candida albicans* (18)		
	Range	50%	90%
Aurein 1.2	16–64	16	32
Citropin 1.1	8–256	64	128
Temporin A	16–256	64	128
Uperin 3.6	4–128	16	32
Iseganan	2–128	64	128
Omiganan	32–128	64	128
Pexiganan	16–512	128	256
Amphotericin	0.125–4	0.5	1
Nystatin	0.25–8	2	4

Table III
The activity of antimicrobial peptides and conventional antifungals against fungi strains other than *C. albicans*

Agent	Minimal inhibitory concentration (MIC) (μg/ml)				
	Candida tropicalis (3) Range	*Candida kefyr* (2) Range	*Candida krusei* (2) Range	*Candida parapsilosis* (5) Range	*Candida glabrata* (4) Range
Aurein 1.2	16–64	32–256	32–128	16–128	16–64
Citropin 1.1	16–64	64–256	8–64	16–128	2–64
Temporin A	32–256	64–256	64–256	32–512	32–128
Uperin 3.6	2–32	64–256	16–64	8–64	4–32
Iseganan	32–128	64–256	32–128	2–64	8–128
Omiganan	32–128	128–512	32–128	64–128	64–128
Pexiganan	32–128	128–512	32–128	8–128	16–64
Amphotericin	0.25–0.5	0.5	0.5–1	0.25–2	0.25–1
Nystatin	2–8	1–2	1–2	1–4	2–8

the MIC value of uperin 3.6, it was below 16 mg/ml, whereas that for 90% or 75% strains of *C. albicans* and *C. glabrata* respectively was below 16 μg/ml. The peptide antibiotics currently under clinical trials exhibited generally lower activities than the naturally occurring sequences. The MIC range of tested substances for particular strains are presented in Tables II and III.

Discussion

Summing up our results, it can be stated that generally the peptide antibiotics are several times less active against fungi than the conventional drugs tested, nystatin and amphotericin B. Among the peptides tested, uperin 3.6 was the most effective one towards all *Candida* strains except for *C. kefyr*, the finding that

cannot be considered as a coincidence. Thus, this 17-residue peptide originally isolated from Australian toadlet, *Uperoleia mjobergii*, appears to be a promising antifungal compound. Interestingly, the remaining naturally occurring peptides exhibit higher anticandida activities than those currently under clinical trials (omiganan, pexiganan and iseganan) as drugs for the treatment of oral mucositis, lung infections, infected diabetic ulcers and catheter infections (Kamysz, 2005). This shows that the peptide antibiotics possess a broad spectrum of activity and even those peptides not previously considered as being worth attention can turn out to be useful in the case of particular pathogens. Fungal infections caused by *Candida* spp. are especially difficult in treatment. *Candida* species reside on mucosal surfaces of about 60% of human population. However, in certain conditions, they can cause superficial mucosal infections such as vaginitis,thrush, and esophagitis. At present, an increasing number of *Candida* and *Candida*-like infections can be observed. On the other hand, also the number of fungal pathogens resistant to common therapeutic antifungals does increase (Branchini *et al.*, 1998; Barchiesi *et al.*, 1998; Wong-Beringera *et al.*, 2001). Such a situation becomes dangerous, especially in the age of opportunistic infections. For instance, almost a half of AIDS patients develop oral candidiasis and most of responsible strains are drug-resistant (Law *et al.*, 1994; Georgopapadakou, 1998). A similar problem concerns patients undergoing cancer chemotherapy and immunosupression (de Paiva Martins *et al.*, 2002). These immunocompromised patients are also susceptible to severe systemic infections. Taken together, candidiasis is an emerging problem and new effective and safe antifungals are absolutely needed. The naturally occurring peptides studied herein seem to be a good matrix for development of novel antifungal compounds. Modification of their structure by substitution of particular amino acid residues, introduction of D-amino acids or non-peptide entities, coupling of fatty acid chain or shortening the sequence without affecting cytocidal properties could lead to activity enhancement, improvement of pharmacological parameters and finally cost-effectiveness.

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