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Mechanism of action of novel synthetic dodecapeptides against *Candida albicans*

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ABSTRACT

Background: Three de novo designed low molecular weight cationic peptides (IJ2, IJ3 and IJ4) containing an unnatural amino acid α,β-didehydrophenylalanine (ΔPhe) exhibited potent antifungal activity against fluconazole (FLC) sensitive and resistant clinical isolates of *Candida albicans* as well as non-*albicans* and other yeast and filamentous pathogenic fungi. In the present study, their synthesis, susceptibility of different fungi and the mechanism of anti-candidal action have been elucidated.

Methods: The antimicrobial peptides (AMPs) were synthesized by solid-phase method and checked for antifungal activity against different yeasts and fungi by broth microdilution method. Anti-candidal mode of action of the peptides was investigated through detecting membrane permeabilization by confocal microscopy, Reactive Oxygen Species (ROS) generation by fluorometry, apoptosis and necrosis by flow cytometry and cell wall damage using Scanning and Transmission Electron Microscopy.

Results and conclusions: The MIC of the peptides against *C. albicans* and other yeast and filamentous fungal pathogens ranged between 3.91 and 250 μM. All three peptides exhibited effect on multiple targets in *C. albicans* including disruption of cell wall structures, compromised cell membrane permeability leading to their enhanced entry into the cells, accumulation of ROS and induction of apoptosis. The peptides also showed synergistic effect when used in combination with fluconazole (FLC) and caspofungin (CAS) against *C. albicans*.

General significance: The study suggests that the AMPs alone or in combination with conventional antifungals hold promise for the control of fungal pathogens, and need to be further explored for treatment of fungal infections.

1. Introduction

Fungal infections are becoming an ever-growing medical problem resulting in an enormous economical burden for healthcare throughout the world. The major fungal pathogens include *Candida* spp. (*Candida albicans*, *Candida glabrata*, *Candida tropicalis* and *Candida parapsilosis* and others) accounting for ~75% of all infections, and represent the 4th leading cause of nosocomial diseases. Further, filamentous *Aspergillus* spp. (e.g. *A. fumigatus*), yeast *Cryptococcus neoformans*, and rare emerging fungal pathogens cause up to 25% of disseminated fungal infections. *C. albicans* is normally a commensal organism in humans, but when the host is unable to mount an adequate immune response, it results in mucosal, cutaneous or invasive mycosis. The mortality of ~40% just for candidemias exceeds that of all Gram-negative bacterial septicemias, emphasizing the medical importance of fungal diseases. In disparity to the steep rise in number of fungal infection cases, only few drugs have been developed over the past 5–6 decades. Currently, five classes of compounds, namely polyenes, azoles, allylamines, fluoropyrimidines and echinocandins, are used for the treatment of fungal infections. Along with the limited repertoire of clinically available antifungals, the drawbacks associated with them such as development of resistance in the pathogen, acute or chronic side-effects (e.g. Amphotericin B), limited clinical efficacy (e.g. Terbinafine) owing to poor pharmacokinetics, lack of potency, complex drug interactions with the host cells, and poor bioavailability, demand for the development of new antifungal agents

Abbreviations: ABC, ATP-Binding Cassette; MFS, Major Facilitator Superfamily; MDR, Multidrug Resistance; SEM, Scanning Electron Microscopy; TEM, Transmission Electron Microscopy; FICI, Fraction Inhibitory Concentration Index; ROS, Reactive Oxygen Species

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[1]. Further, the new antifungal compounds should have broad-spectrum activity, target specificity, different mechanisms of action and no cross-resistance to the currently available antifungal drugs [2].

Host defense peptides or cationic antimicrobial peptides (AMPs) are emerging as potential therapeutic antifungal agents, primarily because the development of resistance to them is very low [3]. Antifungal peptides with varied structures have been identified in plants, mammals, and microorganisms. Based on these scaffolds, superior semisynthetic and fully synthetic analogs have also been prepared [4]. Naturally occurring AMPs are generally 12 to 50 amino acids in length and are folded into several structural groups, including helix, sheet extended and looped structures, having two common features, namely, a net positive charge that facilitates interaction with negatively charged microbial surface and the ability to form an amphipathic secondary structure that permits incorporation into the lipid bilayer of microbial membranes [5,6]. Interaction of the peptides with membranes resulting in formation of pores in the membrane by 'barrel-stave', 'carpet' or 'toroidal-pore' mechanisms is an important requirement for most, if not all, antimicrobial peptides [7,8]. Though it has been well established that AMPs alter the cell membrane function through increased permeability, it is not a sole mode of action and more subtle mechanisms determine the specificity and toxicity of the peptides [9]. Other suggested mechanisms include alteration in cytoplasmic membrane, septum formation, and inhibition of cell-wall, nucleic acid and protein synthesis as well as some enzyme activities [8].

A major concern with peptide based inhibitor/drug is the fact that they are highly susceptible to the enzymatic degradation and thus have relatively short half-life under in vivo conditions. Introduction of conformational constraining, non-protein amino acids is one strategy to overcome this hurdle. We have earlier shown that introduction of α,β-dehydro acids not only can help in stabilization of secondary structure like turns, β-turns and helices, but can also enhance stability of enzymatic degradation [10,11]. We have earlier designed and synthesized eleven helical AMPs containing α,β-didehydrophenylalanine (ΔPhe) residues and found that they not only showed significant antimicrobial activity, but also exhibited hemolytic activity [12]. Here, we have modified these eleven ΔPhe containing AMPs and tested for antifungal activity.

2. Materials and methods

2.1. Chemicals

Amino acid derivatives and resin for peptide synthesis were of Novabiochem from Merck Millipore (Darmstadt, Germany). *N*,*N*′-diisopropylcarbodiimide (DIC), piperidine, dimethylformamide (DMF), dichloromethane (DCM), dimethylsulfoxide (DMSO), *N*-hydroxybenzotriazole (HOBt), trifluoroacetic acid (TFA), triisopropylsilane (TIS), polyethylene glycol (PEG), 2,7-dichlorofluorescein diacetate (DCFHDA), ascorbic acid (AA), fluorescein 5-isocyanate (FITC), and fetal bovine serum (FBS) were from Invitrogen (Carlsbad, CA). Sodium chloride and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). RPMI-1640 and horse serum were from HiMedia (Mumbai, India). Propidium iodide (PI) and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Molecular Probes (Eugene, OR). An FITC-labeled Annexin V apoptosis detection kit was obtained from BD biosciences (San Jose, CA).

2.2. Peptide synthesis, purification and mass spectrometry

Peptides were synthesized by the solid-phase methods using Fmoc (9-fluorenyl-methoxycarbonyl) chemistry on Rinkamide-MBHA (4-methylbenzhydrylamine hydrochloride salt) resin in the manual mode using DIC and HOBt as coupling reagents [13]. Removal of Fmoc protecting groups was carried out with 20% piperidine in DMF. Both, the coupling of amino acids and Fmoc deprotection were monitored by Kaiser test [14]. Peptides were cleaved off from the resin by TFA (95%), water (2.5%), and TIS (2.5%). The crude peptides were purified by reverse-phase high-performance liquid chromatography on a C_{18} Phenomenex column (2.1 × 150 mm, flow rate 5 ml/min; Shimadzu, Kyoto, Japan); using a water–acetonitrile gradient of 5% to 65% acetonitrile and 0.1% TFA for 70 min and detection at 214 nm and 280 nm. Purity of peptides was confirmed by an analytical reverse-phase HPLC (analytical C_{18} Phenomenex column). Molecular weights of synthetic peptides were determined using a MALDI-TOF mass spectrometer (Autoflex II, Bruker Daltonics, Billerica, MA).

2.3. Synthesis, purification and mass spectrometry of FITC-labeled peptides

To the free amino terminus of peptides IJ2, IJ3 and IJ4 on resins, Fmoc-aminohexanoic (Ahx) acid-OH was coupled using HOBt and DIC in DMF. Fmoc was removed by treatment with 20% piperidine in DMF. The resin was washed with DMF and equilibrated in pyridine–DMF–DCM (12:7:5). A 1.1 equivalent of FITC in pyridine–DMF–DCM (12:7:5) was added to the resin and left overnight for the completion of coupling. Development of orange color on the resin and negative Kaiser test indicated completion of coupling [14].

Peptides were cleaved by stirring the resin in a cleavage mixture (95% TFA, 2.5% water, and 2.5% TIS) at room temperature for 2 h. The resulting suspension was filtered under suction using sintered glass funnel. The TFA from the filtrate was removed under vacuum using rotary evaporator and peptide was precipitated by adding cold dry ether. The ether was filtered through a sintered glass funnel; the peptide retained above was dissolved in 10% acetic acid and lyophilized.

Crude peptides were purified by reverse-phase high-performance liquid chromatography (RP-HPLC) on a DeltaPak C_{18} column (19 × 300 mm; Waters, Milford, MA) using acetonitrile (0.1% TFA)–water (0.1% TFA) linear gradient of 5 to 65% acetonitrile with a flow rate of 5 ml/min for 60 min, and detection at 214 nm and 280 nm. The purified peptides were re-injected on an analytical reversed-phase C_{18} column (4.6 × 250 mm, Phenomenex) using acetonitrile (0.1% TFA)–water (0.1% TFA) linear gradient of 5% to 65% acetonitrile at a flow rate of 1 ml/min over 60 min. The identity of the purified (>98% pure) peptides was confirmed by electrospray ionization mass spectrometry (SYNAPT G2-S HDMS; Waters, Milford, MA).

2.4. Fungal strains and growth media

Various fungal strains used and their source are listed in Supplementary Table S1. *C. albicans*, non-*albicans* species of *Candida* (*C. krusei*, *C. glabrata*, *C. kefyr*, *C. dubliniensis*, *C. tropicalis*, *C. parapsilosis*), and *C. neoformans* were cultured in yeast extract–peptone–dextrose (YEPD) broth (BIO101, Vista, CA) and RPMI-1640 media (Gibco BRL, Gaithersburg, MD). *Aspergillus niger*, *Aspergillus flavus*, *A. fumigatus*,

Table 1
Amino acid sequences of IJ2, IJ3 and IJ4 peptides.

Peptides	Sequences	Length	Net charge	ΔPhe	Trp	Ala	Mw	Retention time (min)
IJ2	Ac-K-W-ΔF-W-K-ΔF-V-K-ΔF-A-K-NH_2	11	+4	3	2	1	1552.1	20
IJ3	Ac-K-A-ΔF-W-K-ΔF-V-K-ΔF-A-K-NH_2	11	+4	3	1	2	1434.8	20
IJ4	Ac-K-W-ΔF-W-K-ΔF-A-K-ΔF-A-K-NH_2	11	+4	3	2	2	1521.7	17