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Antimicrobial peptide protonectin disturbs the membrane integrity and induces ROS production in yeast cells

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ABSTRACT

Candidiasis is often observed in immunocompromised patients and is the 4th most common cause of bloodstream infections. However, antifungals are limited, so novel antifungal agents are urgently needed. Antimicrobial peptides (AMPs) are considered as potential alternatives of conventional antibiotics. In the present study, antimicrobial peptide protonectin was chemically synthesized and its antifungal activity and mode of action were studied. Our results showed that protonectin has potent antifungal activity and fungicidal activity against the tested fungi cells. Its action mode involved the disruption of the membrane integrity and the inducing of the production of cellular ROS. Furthermore, protonectin could inhibit the formation of biofilm and kill the adherent fungi cells. In conclusion, with the increase of fungal infection, protonectin may offer a new strategy and be considered as a potential therapeutic agent against fungal disease.

1. Introduction

Candida is a causal trigger of opportunistic oral and genital infections that frequently coexists in the human oral cavity and gastrointestinal tract. In general, *Candida* is innocuous although overgrowth of *Candida* results in candidiasis. Candidiasis is often observed in immunocompromised patients (e.g., AIDS) and is the 4th most common cause of bloodstream infections, with an estimated mortality rate upwards of 25% [1]. However, treatment against *Candida* infections involving triazoles (i.e. fluconazole and itraconazole) is often inoperative because *Candida* cells could acquire resistance after long periods of treatment, leading to higher susceptibility to fungal infections [2]. The severe adverse effects of antifungals like liver damage, allergic reactions or disorder of estrogen levels also made the traditional antifungals have limited clinical application [3]. In addition, the formation of highly drug-tolerant biofilm by *Candida* cells further exacerbates the antifungal therapeutic challenge [4,5]. So a novel strategy is eagerly needed for the control of fungal infections

It is believed that antimicrobial peptides could be potential alternatives of conventional antifungals [6]. AMPs are an evolutionarily conserved component of the innate immunity and exist among all classes of life, ranging from prokaryotes to plants, insects, amphibians and mammals [7–11]. They have a broad spectrum of antimicrobial activities against bacteria, mycobacteria, enveloped viruses, fungi and even transformed or cancerous cells. So far, lots of AMPs demonstrating antifungal activities have been isolated and characterized. The antifungal action modes of AMPs are diverse, including disrupting fungal cell membrane and inhibiting intracellular targets. So it is believed that the possibility for fungal cells to develop resistance toward AMPs is relatively low [12].

Protonectin was originally isolated from the venom of the neotropical social wasp *Agelaia pallipes pallipes*, with an amino acid sequence of ILGTILGLLKGL-NH_2 (1210.57 Da) [13]. It has potent antimicrobial activity against both gram positive and negative bacteria, but weak hemolytic activity [13,14]. However, the effect of protonectin on fungi cells has not been reported. In this study, protonectin was chemically synthesized and antifungal activity against *Candida* cells was determined. Then, the effects of protonectin on the integrity of fungal cell membrane and some intracellular events were investigated. Our results showed that protonectin had potent antifungal activity against *Candida* cells and the action mode involved membrane disturbance and the inducing of ROS production.

2. Material and methods

2.1. Peptide synthesis

Protonectin (ILGTILGLLKGL-NH_2) was synthesized by a stepwise solid-phase method on rink amide MBHA resin using N-9-fluorenylmethoxycarbonyl (F-moc) chemistry [15]. After cleavage, protonectin was desalted using a Sephadex gel column and purified by reverse-phase high-performance liquid chromatography (RP-HPLC,

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Waters) using a μBondapak C_{18} 19 mm by 300 mm column with gradient elution of 20% to 80% CH_3CN/H_2O with 0.1% trifluoroacetic acid (TFA) at a flow rate of 8 ml/min. The atomic mass of the synthetic peptide was confirmed by electrospray ionization-mass spectrometry (ESI-MS, MaXis 4G, Bruker, Germany). Antimicrobial peptides melittin and magainin-2 also were synthesized and purified. The peptide was dissolved in double-distilled water and stored at −20 °C before use.

2.2. Fungi and materials

Candida albicans (ATCC 14053), *Candida glabrata* (ATCC 2001), *Candida parapsilosis* (ATCC 22019), *Candida tropicalis* (ATCC 750), and *Candida krusei* (ATCC 6258) used in this study were purchased from the American Type Culture Collection (ATCC) and the *C. glabrata* 14-1, *C. albicans* 14-2, *C. albicans* 14-3, and *C. albicans* 1-4 were clinically isolated. Dry powder of *Candida* strains was dissolved in sterile phosphate-buffer solution (PBS), shaken and spread on Sabouraud dextrose broth plate. After overnight incubation at 35 °C, a colony was cultured into Sabouraud dextrose broth medium at 35 °C for 12 h with shaking to get logarithmic phase. The fresh culture was centrifuged, washed and re-suspended in 25% $C_3H_8O_3/H_2O$ and stored at −80 °C before use. Agents used for peptide synthesis were purchased from GL Biochem Ltd (Shanghai, China) with desired purity.

2.3. Antifungal assays

2.3.1. The minimum inhibition concentration assay (MIC)

The antifungal assay was determined according to the EUCAST method with minor modifications [16]. Briefly, cells activated in Sabouraud dextrose broth medium were washed, diluted and inoculated into 96 well flat-bottom plates containing serially two fold dilutions of protonectin. The plates with an inoculum size of 0.5×10^5 to 2×10^5 CFU were statically placed in an incubator at 35 °C overnight. Minimal inhibitory concentration (MIC) of protonectin was defined as the lowest concentration at which no visible turbidity was observed compared with drug-free group. The MICs assay was repeated in triplicate.

2.3.2. The minimum fungicidal concentration assay (MFC)

For minimal fungicidal concentration (MFC) determination, 50 μl cultures at concentration equal to or above MIC were plated on Sabouraud dextrose broth plate for CFU counting. After incubation at 35 °C for 24 h, the MFC was defined as the lowest concentration that resulted in 99.9% killing compared to drug-free group. The MFC assay was repeated in triplicate.

2.3.3. Radial diffusion assay

The fungicidal activity of protonectin was evaluated by a modification of the sensitive radial diffusion assay. Briefly, the fungi cells were cultured as described above. One milliliter of the fungi 10^7 CFU was added to 100 ml of previously autoclaved, warm Sabouraud dextrose agar. After rapid dispersion of the fungi, the agar was poured into an agar plate to form a layer approximately 5 mm deep and was punched with a 3-mm-diameter gel punch to make evenly spaced wells. Following the addition of 25 μl with different concentrations of protonectin to each well, the plates were incubated at 35 °C for 18 to 24 h. 25 μl of sterile water was added as a control.

2.3.4. Time-killing kinetics of protonectin against Candida cells

Time-killing kinetics of protonectin against *Candida* cells were performed according to standard microbiological techniques with minor modifications [17]. Logarithmic cultures diluted to an inoculum size of 10^4–10^5 CFU in fresh Sabouraud dextrose broth medium were incubated with protonectin at the required concentration, and the mixture was incubated at 35 °C with shake. To determine the viabilities of the cultures, the cells were then taken and serially diluted at different intervals after treatment and plated on Sabouraud's dextrose agar plates of 9 cm in diameter for CFU counting. The original number of colonies was confirmed at time zero. The killing activity of protonectin was illustrated by plotting the $\log_{10}$ CFU per milliliter against incubation time. Sabouraud dextrose broth medium was run as negative control.

2.4. Protonectin binding assay with Candida cells

Binding of protonectin to the surface of *Candida* cells was examined by assessing the effect of representative fungal cell wall polysaccharides on killing activity of protonectin described before [18]. In brief, protonectin (final concentration, 256 μM) reacted with increasing concentration of each representative cell wall polysaccharide at 35 °C for 1 h. After incubation, 100 μl of samples was collected directly into tubes containing an equal volume of *Candida* cultures with an inoculum size of 2×10^5 CFU for 1 h and serially diluted in Sabouraud dextrose broth medium. Aliquots of 100 μl were spread onto Sabouraud dextrose broth agar plates and incubated for 48 h at 35 °C. The killing rate % = $100 \times (1 - Ft / Fo)$, in which Fo and Ft represented the CFU of polysaccharide-free group and polysaccharide-treated group. The figure was plotted as killing rate against polysaccharide concentration.

2.5. Localization of FITC-protonectin (FITC-pro) in Candida cells

2.5.1. Confocal laser scanning microscopy

C. glabrata cell cultures at logarithmic phase with an inoculum size of 10^6–10^7 CFU were exposed to 64 μM in Sabouraud dextrose broth medium for timed duration at 35 °C. Microscopic analysis was done with a laser confocal scanning microscope (Zeiss LSM 710 META) after 60 min incubation.

2.5.2. Flow cytometric analysis

For FACS assay, *C. glabrata*cell cultures with an inoculum size of 10^6–10^7 CFU were exposed to FITC-pro in Sabouraud dextrose broth medium at different concentrations for 2 h at 35 °C with shaking. After incubation, the cells were centrifuged, washed and resuspended with PBS. Flow cytometric analysis was conducted by a FACS Calibur flow cytometer (Becton–Dickinson, San Jose, CA, USA).

Table 1
MIC values of protonectin against the tested *Candida* cells.

MIC (μM &μg/ml)[b]									
Peptide	*C. glabrata* ATCC 2001	*C. albicans ATCC 14053*	*C. parapsilosis ATCC 22019*	*C.tropicalis ATCC 750*	*C. krusei ATCC 6258*	*C. glabrata 14-1*	*C. albicans* 14-2	*C. albicans 14-3*	*C. albicans 14-4*
Protonectin[a]	32/38.8[c]	32/38.8[c]	128/155[c]	8/9.7[c]	32/38.8[c]	32/38.8[c]	32/38.8[c]	32/38.8[c]	32/38.8[c]
Melittin	32/91[c]	4/11.4[c]	64/182[c]	1/2.8[c]	16/45.5[c]	32/91[c]	8/22.8[c]	32/91[c]	16/45.5[c]
Magainin2	32/79[c]	32/79[c]	128/315.8[c]	16/39.5[c]	16/39.5[c]	32/79[c]	32/79[c]	64/158[c]	64/158[c]

[a] Initially obtained by Maria Anita Mendes et al. The sequence is ILGTILGLLKGL-amide. The calculated monoisotopic mass value (M_{calc})is 1210.57, while the observed monoisotopic mass value (M_{obs}) is 1211.3. M_{obs} was deduced from the protonated molecule ($M + H^+$).
[b] Both the values in μM &μg/ml were provided.
[c] The left of diagonal mark (/) is the MIC value in μM, while the right is the MIC value in μg/ml.