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Molecules and Cells

A Novel Antifungal Analog Peptide Derived from Protaetiamycine

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Previously, the 9-mer analog peptides, 9Pbw2 and 9Pbw4, were designed based on a defensin-like peptide, protaetiamycine isolated from *Protaetia brevitarsis*. In this study, antifungal effects of the analog peptides were investigated. The antifungal susceptibility testing exhibited that 9Pbw4 contained more potent antifungal activities than 9Pbw2. A PI influx assay confirmed the effects of the analog peptides and demonstrated that the peptides exerted their activity by a membrane-active mechanism, in an energy-independent manner. As the noteworthy potency of 9Pbw4, the mechanism(s) of 9Pbw4 were further investigated. The membrane studies, using rhodamine-labeled giant unilamellar vesicle (GUV) and fluorescein isothiocyanate (FITC)-dextran loaded liposome, suggested that the membrane-active mechanism of 9Pbw4 could have originated from the pore-forming action and the radii of pores was presumed to be anywhere from 1.8 nm to 3.3 nm. These results were confirmed by 3D-flow cytometric contour-plot analysis. The present study suggests a potential of 9Pbw4 as a novel antifungal peptide.

INTRODUCTION

In the past decade, the frequency of infections provoked by opportunistic fungal strains has increased dramatically. Even though the majority of invasive fungal infections are still due to the *Aspergillus* and *Candida* species, the spectrum of fungal pathogens has changed and diversified (Denning, 1991; Ellis et al., 2000; Odds et al., 2003). Azoles that inhibit sterol formation and polyenes that bind to mature membrane sterols have been the mainstays regarding antifungal therapy for several decades (Kullberg and de Pauw, 1999; Sheehan et al., 1999). However, not only the emergence of fluconazole resistance among different pathogenic strains but also the high toxicity of amphotericin B (Alexander and Perfect, 1997; Mukherjee et al., 2003) have prompted research on new antifungal agents (Kontoyiannis et al., 2003).

Antimicrobial peptides (AMPs) are produced by all species of life and represent essential components in the innate immune system, providing a fast-acting weapon against invading pathogens including bacteria, fungi, and yeast (Boman, 1995; Hancock et al., 2006; Lee et al., 2009a; Selsted and Ouellette, 2005; Zasloff, 2002). Importantly, many AMPs physically and swiftly permeate and destroy the cell plasma membrane, causing damage which is hard to fix, in contrast to conventional antibiotics which target specific intracellular enzymes or DNA. Therefore, microbial resistance may occur with much lower probability than that observed with the antibiotics (Zasloff, 2002).

Insects can produce a wide variety of peptides that contain potent *in vivo* and *in vitro* antimicrobial activity (Hoffmann, 1995). In insects, the defensins especially comprise a large family of cationic cysteine-rich antimicrobial peptides; they are disposed to exert activity against Gram-positive bacteria or filamentous fungi (Taylor et al., 2008; Volkoff et al., 2003). Previously, protaetiamycin, a novel 43-mer insect defensin from the larvae of a beetle *Protaetia brevitarsis*, was identified (Hwang et al., 2008) and four 9-mer analog peptides based on the sequence of protaetiamycin from Ala22 to Gly30 were designed. Especially, 9Pbw2 (RLWLAIKRR-NH_2) and 9Pbw4 (RLWLAWKRR-NH_2) exhibited the highest therapeutic index for the tested bacteria (Shin et al., 2009). However, the antifungal properties of 9Pbw2 and 9Pbw4 have not yet been studied. Therefore, novel antifungal effects of 9Pbw2 and 9Pbw4 were investigated and a potential of 9Pbw4 as a novel antifungal peptide was suggested in this study.

MATERIALS AND METHODS

Peptide synthesis

Peptides were synthesized by the solid-phase method using Fmoc (9-fluorenyl-methoxycarbonyl) chemistry (Merrifield, 1986). The crude peptides were washed with diethylether, dried in a vacuum, and purified using reversed-phase preparative HPLC (high performance liquid chromatography) on a Waters 15-μm Deltapak C_{18} column (19 × 30 cm). The purity of the peptides was checked by analytical reversed-phase HPLC on an Ultrasphere C_{18} column (4.6 × 25 cm; Beckman, USA). The molecular weight of the synthetic peptides was determined using a matrix-assisted laser desorption ionization (MALDI)-mass spectrometer (Jungblut and Thiede, 1997).

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Table 1. Amino acid sequence and physicochemical properties of 9Pbw2 and 9Pbw4

Peptide	Sequence	Molecular mass (Da)	Net charge	Hydrophilicity[a]	Retention time (min)
9Pbw2	RLWLAIKRR-NH_2	1211.16	+5	0.34	17.6
9Pbw4	RLWLAWKRR-NH_2	1283.79	+5	0.12	18.5

[a]Hydrophilicity is the total hydrophilicity (sum of all residue hydrophilicity indices) divided by the number of residues according to the Hopp and Woods index (Hopp and Woods, 1981).

Antifungal susceptibility testing

The fungal strains *Aspergillus flavus* (KCTC 1375), *Candida albicans* (ATCC 90028), *Malassezia furfur* (KCTC 7744) and *Trichosporon beigelii* (KCTC 7707) were used to evaluate the antifungal activity of the peptides. Fungal cells were cultured in YPD media (Difco) with aeration at 28°C. *M. furfur* was cultured in YM media (Difco), containing 1% olive oil, at 32°C. Fungal cells (2×10^4 cells/ml) were inoculated into the media, and 0.1 ml/well of the mixture was dispensed into microtiter plates. The minimum inhibitory concentration (MIC) was determined by means of a serial twofold dilution of the peptides, following a microdilution method and MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay. After 48 h of incubation, the minimal peptide concentration, which prevented the growth of a given test microorganism, was determined, and was defined as the MIC value. The growth was measured at 580 nm by using an ELISA Microplate Reader (Molecular Devices Emax). The MIC values were determined by three independent assays (Lee and Lee, 2009).

Propidium iodide (PI) influx assay

For the analysis regarding the effects of the peptides on fungal membranes and the effects of energy metabolism on the antifungal activity of the peptides, a PI influx assay was conducted. *C. albicans* cells (2×10^6 cells/ml) were harvested at a log phase and suspended in YPD media. Cells were treated with the peptides at 10 times the MIC in the absence or presence of 0.1 mM sodium azide (NaN_3) and were incubated for 2 h at 30°C. After incubation, cells were harvested and resuspended in PBS. Subsequently, cells were treated with 9 μM of PI and incubated for 30 min at 4°C. Flow cytometric analysis was performed by using FACSCalibur flow cytometer (Becton Dickinson, USA) (Lee et al., 2009b; Mangoni et al., 1996; Park et al., 2003; Ramani et al., 1997).

Formation and microscopic observation of giant unilamellar vesicles (GUVs)

GUVs were prepared by using ITO (indium tin oxide) glasses. Lipids [phosphatidylcholine (PC)/rhodamine-conjugated phosphatidylethanolamine (PE)/phosphatidylinositol (PI)/ergosterol (5:4:1:2, w/w/w/w)] were prepared at a concentration of 3.75 mg/ml in chloroform. The lipid solutions (50 μl) were deposited in spin coater (Spin Coater, ACE-1020 Series) and the glass was coated for 5 min. Then the coated ITO glass was evaporated under a vacuum for 2 h. The following procedure was used in succession; a square frame was created from silicon served as a thickness (2 mm) spacer between the lipid-coated glass and normal glass. The chamber was filled with 10 mM HEPES buffer (pH 7.2) through a hole in the silicon spacer. Immediately, the application of 1.7 V (peak-to-peak, sine wave) and 10 Hz to the ITO electrodes was made by using a sweep function generator (Protek, SWEEP FUNCTION GENERATOR 9205C) for 2 h. GUVs from the ITO glass were then detached in conditions of 4 V (peak-to-peak, sine wave) and 4 Hz for 10 min. The peptides (at the MIC) were treated and changes of a single GUV were observed by using an inverted fluorescence phase contrast microscope (Leica, DFC420C) (Lee and Lee, 2009).

Preparation of dextran-loaded liposomes and leakage assay

Fluorescein isothiocyanate (FITC)-labeled dextrans (FD4, FD10 and FD20) were employed to evaluate the extent of membrane damage induced by the peptides. All FDs were purchased from Sigma Chemical Co. (USA). To prepare FD-entrapped liposomes, buffer I (1 ml, 50 mM potassium phosphate, pH 7.4, with 0.1 mM EDTA) containing 2 mg/ml of FD, was sonicated for 30 min with 20 mg/ml of lipid [PC/PE/PI/ergosterol (5:4:1:2, w/w/w/w)] solution in chloroform on ice. Chloroform was removed by using a rotary vacuum evaporator for 2 h at 25°C, resulting first in the formation of a viscous gel, and then a liposome suspension. Buffer I (2 ml) was added and the suspension was evaporated further for the removal of eventual traces of chloroform. The liposome suspension was then centrifuged and washed for several cycles at 13,000 rpm for 30 min to remove unentrapped-FD. For the assay, the peptides (at the MIC) were treated in a suspension of FD-loaded liposomes. The mixture (3 ml, final volume) was stirred for 10 min in the dark and then centrifuged at 13,000 rpm for 20 min. The supernatant was recovered and its fluorescence intensity was recorded by measuring a fluorescent intensity, at wavelengths (λ_{ex} = 494 nm, λ_{em} = 520 nm), with RF-5301PC spectrofluorophotometer (Shimadzu, Japan). Twenty microliters of 10% Triton X-100 was added to vesicles to determine 100% dye leakage (Belokoneva et al., 2004; Park et al., 2008). The percentage of dye leakage was calculated as follows: % dye leakage = $100 \times (F\text{-}F_0) / (F_t\text{-}F_0)$, where F represents the fluorescent intensity achieved by the peptides treatment and F_0 and F_t represent the fluorescent intensities without the peptides treatment and with Triton X-100 treatment, respectively.

3D-flow cytometric contour-plot analysis

C. albicans cells (2×10^6 cells/ml), suspended in YPD media, were treated with the peptides at 10 times the MIC and incubated for 2 h at 30°C. After incubation, cells were harvested and suspended in PBS. Subsequently, cells were treated with 9 μM of PI and incubated for 30 min at 4°C. Flow cytometric analysis was performed by using FACSCalibur flow cytometer (Becton Dickinson, USA).

RESULTS AND DISCUSSION

Antifungal activity of the analog peptides

In order to suggest a potential of 9Pbw2 and 9Pbw4 (Table 1) (Hopp and Woods, 1981; Shin et al., 2009) as novel antifungal agents, the antifungal susceptibility testing was first conducted against human pathogenic fungal strains, including *A. flavus*, *C. albicans*, *M. furfur* and *T. beigelii*, as listed in Table 2. The result showed that the fungal strains were susceptible to 9Pbw2 or 9Pbw4 with MIC values in the 1.25-10 μM range or the 1.25-5 μM range, respectively (Table 2). Melittin, a 26-residue powerful antimicrobial peptide being used as a positive control in this study, showed more potent antifungal activity, with MIC values

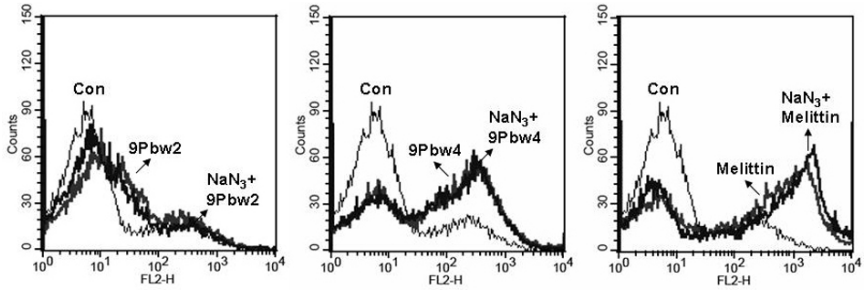

Fig. 1. Flow cytometric analysis of PI staining in *C. albicans*. Histograms showed the fluorescent intensity of stained PI in *C. albicans* after treatment of the peptides.

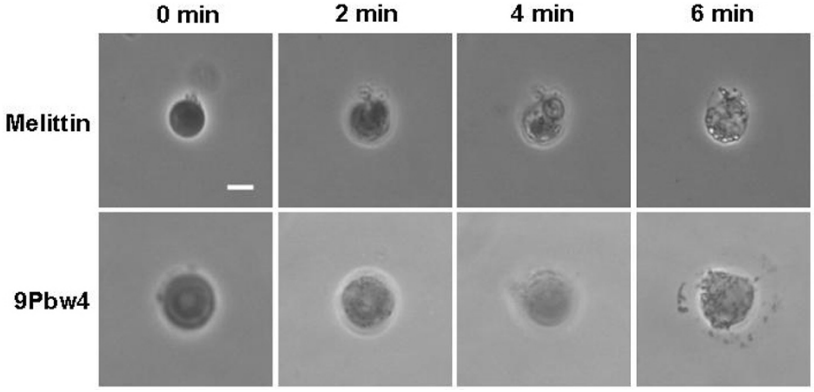

Fig. 2. Phase contrast images of a single GUV, labeled with rhodamine, induced by the peptides. The times above each image show the time after the peptide treatments. The bar corresponds to 20 μm.

Table 2. Antifungal activity of 9Pbw2 and 9Pbw4

Fungal strain	MIC (μM)		
	9Pbw2	9Pbw4	Melittin
A. flavus	10	5	2.5
C. albicans	5	2.5	1.25
M. furfur	1.25	1.25	0.625
T. beigelii	5	5	1.25

in the 0.625-2.5 μM range. Actually, with respect to structure, AMPs are ≈12-50 amino acids long, possess a net positive charge of > 2, and are comprised of ≈50% hydrophobic amino acids (Makovitzki et al., 2006). Both 9Pbw2 and 9Pbw4 are very short peptides consisting of 9 amino acids. When considering the structural property of the peptides, like the length, compared with melittin, these two short peptides are thought to contain very significant antifungal activities. Therefore, 9Pbw2 and 9Pbw4 may be applied as therapeutic agents for treating fungal diseases in humans.

Fungal membrane permeabilization induced by the analog peptides

Many AMPs target on cell membranes. Therefore, in order to investigate the effects of the analog peptides on fungal membranes and the effects of energy metabolism on the antifungal activity of the peptides, *C. albicans* was adopted as a model organism and a PI influx assay was conducted in the absence or presence of 0.1 mM NaN_3. *C. albicans* is both a commensal and opportunistic pathogen of ever-increasing medical importance (Klein et al., 1984). PI can bind to DNA by intercalating between the bases with little sequence preference and with a stoichiometry of one dye per 4-5 base pairs of DNA (Suzuki et al., 1997) and only enters membrane-compromised cells, after which the fluorescence of this dye is enhanced 20- to 30-fold due to its binding to DNA (Pina-Vaz et al., 2001). NaN_3 is known as a metabolic inhibitor blocking ATP synthesis in mitochondria (Gyurko et al., 2001). ATP depletion can cause various changes in cell membranes by the regulation of intracellular pH (Hayashi and Suzuki, 1998). The result showed that all the peptides caused PI influx in *C. albicans*, in an energy-independent manner (Fig. 1). 9Pbw4 especially exerted much more potent influx than 9Pbw2. This result was compatible with the result of MIC test and indicated that the analog peptides affected fungal cell membranes, and thus increased the permeability. It meant that 9Pbw2 and 9Pbw4 exerted their activities by membrane-active mechanism(s) in fungal cells. Moreover, the changes of energy metabolism, such as membrane rigidification by NaN_3 (Veerman et al., 2007), could not affect on the activity of the peptides. After considering the results of a MIC test and a PI influx, 9Pbw4 is thought to be a more attractive model for the antifungal agents in particular, therefore, we focused on 9Pbw4.

Visualization of membrane-active mechanism(s) of 9Pbw4

To visualize and confirm the membrane-active mechanism(s) of 9Pbw4, a single GUV, composed of PC/rhodamine-conjugated PE/PI/ergosterol (5:4:1:2, w/w/w/w), mimicking the membranes of *C. albicans* (Makovitzki and Shai, 2005), was used by employing the electroformation method. GUVs have become a useful model to use when imitating biological membranes. Due to the average diameter of GUVs reaching up to from 10 to 100 μm, they can be easily observed under a fluorescent or confocal microscope provided that the appropriate fluorescent probe was incorporated into the lipid phase during vesicle formation (Wesołowska et al., 2009). It is also a powerful tool to use regarding the investigation of effects of peptides or compounds on model lipid bilayers. The result showed that rhodamine intensity of the GUV gradually decreased after the treatment of 9Pbw4 (Fig. 2). This result indicated that 9Pbw4 disrupted fun-

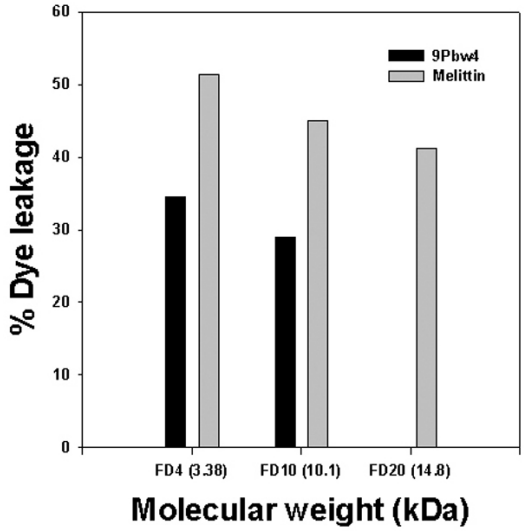

Fig. 3. Percentage of FITC-dextrans (FD) leakage induced by the peptides from PC/PE/PI/ergosterol (5:4:1:2, w/w/w/w) liposome.

gal model membranes. Furthermore, not only the decrease of rhodamine intensity but also the maintenance of the circular shape of single GUV suggested that 9Pbw4 might exert its activity by a pore-forming action, not a lytic action.

Size of the pore formed by 9Pbw4 in fungal model membranes

The leakage assay by employing fluorescent dyes having different molecular weights is a method to determine the pore formation mechanism in the membrane perturbation process (Mancheño et al., 1996; Rex, 1996). In order to elucidate the extent of membrane damage induced by 9Pbw4, the release of FITC-labeled dextran (FD) of various sizes (FD4, FD10 and FD20) from liposomes was measured, composed of PC/PE/PI/ergosterol (5:4:1:2, w/w/w/w), mimicking the membranes of *C. albicans* (Makovitzki and Shai, 2005). The average molecular weight of FD4, FD10 and FD20 is 3.38 kDa, 10.1 kDa and 14.8 kDa, respectively. It is also known that the radii of FD4 and FD20 are 1.8 nm (Bohrer et al., 1979) and 3.3 nm (Laurent and Granath, 1967), respectively. As shown in Fig. 3, 9Pbw4 released, on average, 34.49% of FD4 and 28.93% of FD10 from the liposomes. Even though melittin induced a more potent release of all FDs, 9Pbw4 did not exert the release of FD20 from the liposome at all. These results indicated that 9Pbw4 could make pores in fungal model membranes and suggested that the radii of pores might be anywhere from 1.8 nm to 3.3 nm.

Morphological changes induced by 9Pbw4 in *C. albicans*

The morphological changes induced by 9Pbw4 in *C. albicans* were examined by using flow cytometric methods plotting forward scatter (FSC) and side scatter (SSC). FSC and SSC is an indicator of size and granularity, respectively. The result showed that homogeneous populations of undamaged cells were dominated in the absence of any peptides (Fig. 4A). Conversely, in the presence of 9Pbw4, increased-SSC populations were remarkably observed (Fig. 4B) like the melittin-treated populations (Fig. 4C). The results indicated that 9Pbw4 made the fungal cells permeable, corresponding to a highly granular morphology having a seriously pocked surface (Zelezetsky et al., 2005). Furthermore, the result could confirm the membrane-active mechanism of 9Pbw4, which was suggested in the results of assays regarding the PI influx, the rhodamine-leakage from GUV and the release of FDs.

CONCLUSION

In conclusion, the novel antifungal effects of 9Pbw4 were investigated. 9Pbw4 exhibited antifungal activities by a membrane-disruptive mechanism. Moreover, the membrane studies demonstrated that the mechanism of the peptide in fungal cells could be originated from the pore-forming action. Specifically, it is thought that the discrepant activities of the two analog peptides in fungal cells are due to the degree of hydrophobicity. Since 9Pbw4 has one more tryptophan residue, one of the most hydrophobic amino acids, than 9Pbw2. Finally, it can be concluded that 9Pbw4 may be a template for designing more potent novel antifungal peptides.

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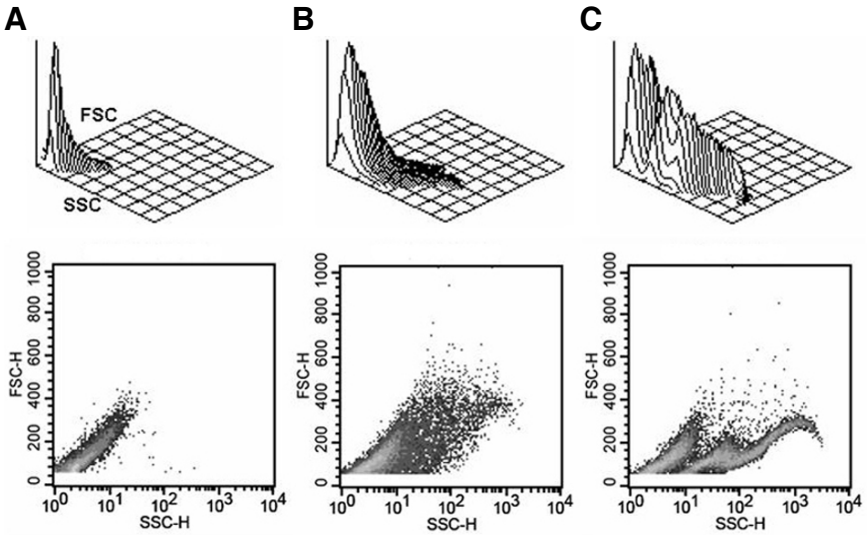

Fig. 4. Three-dimensional (3D)-flow cytometric contour-plot analysis of *C. albicans* treated with the peptides. FSC (y-axis) and SSC (90° scattering, x-axis) is an indicator of size and granularity, respectively. The z-axis represents the cellular population intensity. (A) Control; (B) Cells treated with 9Pbw4; (C) Cells treated with melittin.

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