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Fungicidal mechanisms of the antimicrobial peptide Bac8c

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ABSTRACT

Bac8c (RIWVIWRR-NH_2) is an analogue peptide derived through complete substitution analysis of the linear bovine host defense peptide variant Bac2A. In the present study, the antifungal mechanism of Bac8c against pathogenic fungi was investigated, with a particular focus on the effects of Bac8c on the cytoplasmic membrane. We used bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$] staining and 3,3'-dipropylthiacarbocyanine iodide [$DiSC_3(5)$] assays to show that Bac8c induced disturbances in the membrane potential of *Candida albicans*. An increase in membrane permeability and suppression of cell wall regeneration were also observed in Bac8c-treated *C. albicans*. We studied the effects of Bac8c treatment on model membranes to elucidate its antifungal mechanism. Using calcein and FITC-labeled dextran leakage assays from Bac8c-treated large unilamellar vesicles (LUVs) and giant unilamellar vesicles (GUVs), we found that Bac8c has a pore-forming action on fungal membranes, with an estimated pore radius of between 2.3 and 3.3 nm. A membrane-targeted mechanism of action was also supported by the observation of potassium release from the cytosol of Bac8c-treated *C. albicans*. These results indicate that Bac8c is considered as a potential candidate to develop a novel antimicrobial agent because of its low-cost production characteristics and high antimicrobial activity via its ability to induce membrane perturbations in fungi.

1. Introduction

Infectious diseases and antibiotic resistance are now considered the most pressing global healthcare problems [1]. In the search for new treatments, antimicrobial peptides have attracted considerable attention due to their various unique properties [2]. Antimicrobial peptides are important components of the innate immune defense against a variety of microbial infections, and do not easily induce resistance compared to conventional antibiotics [3]. However, a number of antimicrobial peptides are also cytotoxic to mammalian cells, which limits the direct use of these peptides as therapeutics. In addition, native peptides tend to be easily degraded and are expensive to produce [4]. As a result, efforts are focused on modifying the native antimicrobial peptides or designing new synthetic peptides to achieve better specificity against microbial infections, fewer side-effects for the host organism, and a minimal peptide length for inexpensive production [5]. A variety of methods have been applied to design new antimicrobial peptides based on the characteristics of the native peptides, such as replacing some amino acid residues, changing the chirality of peptides, hybridizing different peptide segments to form new chimeric peptides, and *de novo* peptide designs [6–10]. Several studies have found that Bac8c, a positively-charged (net charge +3) 8-mer peptide is modified from Bac2A (RLARIVVIRVAR-NH_2) [11]. The low-cost production and high antimicrobial activity characteristics make Bac8c a potential candidate for development into a novel antimicrobial drug in the clinical setting. However, the effects of Bac8c on pathogenic fungi associated with infectious disease remain largely unknown, limiting the clinical application of Bac8c for the prevention and treatment of fungal infections. In this study, we investigated the antimicrobial activity of Bac8c against pathogenic fungi, the inhibitory effect of Bac8c on *Candida albicans*, and the mechanism of action of Bac8c on lipid membranes. We show that Bac8c has the potential to be an alternative antifungal agent through its actions on fungal cell membranes.

2. Materials and methods

2.1. Solid-phase peptide synthesis

The assembly of peptides consisted of a 60-min cycle for each residue at ambient temperature as follows: (1) the 2-chlorotrityl (or 4-methylbenzhydrylamine amide) resin was charged to a reactor and then washed with dichloromethane (DCM) and N,N-dimethylformamide (DMF), respectively, and (2) a coupling step with vigorous shaking with a 0.14 mM solution of Fmoc-L-amino acids and Fmoc-L-amino acids preactivated for approximately 60 min with a 0.1 mM solution of 0.5 M HOBt/DIC in DMF. Finally, the peptide was cleaved from the resin with a trifluoroacetic acid (TFA) cocktail solution at ambient temperature [12,13]. The purity

Abbreviations: ATCC, American Type Culture Collection; KCTC, Korean Collection for Type Cultures; YPD, yeast extract peptone dextrose; MIC, minimum inhibitory concentration; $DiBAC_4(3)$, bis-(1,3-dibutylbarbituric acid) trimethine oxonol; $DiSC_3(5)$, 3,3'-dipropylthiacarbocyanine iodide; LUV, large unilamellar vesicle; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PI, Phosphatidylinositol; FITC, Fluorescein isothiocyanate; FD, FITC-labeled dextran; GUV, giant unilamellar vesicle

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Table 1
The antifungal activity of Bac8c and melittin.

Fungal strains	MIC (μM)	
	Bac8c	Melittin
C. albicans ATCC 90028	6.3	1.6
C. parapsilosis ATCC 22019	12.5	3.1
M. furfur KCTC 7744	25.0	3.1–6.3
T. beigelii KCTC 7707	12.5	1.6–3.1

of the peptide is determined using high performance liquid chromatography (HPLC), and purity of synthesized peptide is greater than 95% (data not shown). The calculated molecular weight of Bac8c is 1183.46 Da, and observed molecular weight is 1183.50 Da.

2.2. Preparation of the fungal strains

C. albicans (ATCC 90028) and *C. parapsilosis* (ATCC 22019) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). *M. furfur* (KCTC 7744) and *T. beigelii* (KCTC 7707) were obtained from the Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology (KRIBB) (Daejeon, Korea). The fungal strains were cultured in yeast extract peptone dextrose (YPD) broth (BD Pharmigen, San Diego, CA, USA) with aeration at 28 °C, and the *M. furfur* was cultured in a modified yeast malt (YM) broth (BD Pharmigen) containing 1% olive oil at 32 °C.

2.3. Antifungal activity assay

Fungal cells (1×10^6 cells/mL) were inoculated into 0.1 mL wells containing YPD or YM broth, and then dispensed to microtiter plates. The minimum inhibitory concentrations (MICs) were determined using a two-fold serial dilution of the test compounds, based on the Clinical and Laboratory Standards Institute (CLSI) method [14], with the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay used to evaluate fungal cell viability [15]. After 48 h of incubation, the minimal concentration of the test peptides required to prevent the growth of the microorganisms was determined, and was defined as the MIC. Cell growth was measured by optical density at 580 nm with a microtiter ELISA Reader (Molecular Devices Emax; Sunnyvale, CA, USA). The MIC values were determined by three independent assays.

2.4. Hemolytic activity assay

A fresh human blood sample was diluted in PBS (pH 7.4) and centrifuged at 300 ×*g* for 10 min to remove the plasma and buffy coat, and the supernatant was removed. This washing procedure was repeated three times, and the final concentration of the erythrocytes was 8%. The erythrocyte suspension was transferred into 96-well plates and incubated with peptides at 37 °C for 1 h. The plate was centrifuged at 180 ×*g* for 10 min. An aliquot of the supernatant was taken, and then the hemolysis was evaluated by determining the release of hemoglobin from the 8% suspension of human erythrocytes at 414 nm with an ELISA reader. Zero and 100% hemolysis were determined in PBS alone and with 0.1% Triton X-100, respectively. The hemolysis percentage was calculated using the following equation: Hemolysis (%) = [(Abs_{414nm} in the peptide solution − Abs_{414nm} in PBS)/(Abs_{414nm} in 0.1% Triton X-100 − Abs_{414nm} in PBS)] × 100 [16]. Results are based on three independent experiments, performed in triplicate.

2.5. Cell wall regeneration of protoplast

For the protoplast preparation of *C. albicans*, cells (1×10^6 cells/mL YPD) were digested with 10 mM phosphate buffer (pH 6.0) containing 1 M sorbitol, lysing enzyme (20 mg/ml), and cellulose (20 mg/ml) for 4 h at 28 °C by gentle agitation. The digests were filtered through a 3G3 glass filter, and protoplasts in the filtrate were gathered by centrifugation at 700 ×*g* for 10 min. The protoplasts were resuspended in the washing buffer (0.8 M NaCl, 10 mM $CaCl_2$, and 50 mM Tris–HCl, pH 7.5) and centrifuged again. Bac8c or Melittin was added to the protoplasts, then suspended in the washing buffer and incubated for 4 h 28 °C. The protoplasts treated with peptides were then transferred into YPD soft-agar solutions containing 1 M sorbitol and 0.5% agar, and then spread on agar plates of YPD medium containing 1 M sorbitol and 2% agar. The regenerated colonies were counted following incubation of the plates at 28 °C for 3 days [17], and experiments were performed three independent times.

2.6. Membrane depolarization assay

To analyze membrane disturbances due to Bac8c or melittin treatment, *C. albicans* cells (1×10^6 cells/mL YPD) were incubated with Bac8c or melittin at their respective MICs for 4 h at 28 °C; the cells were then harvested by centrifugation and resuspended in 1 mL PBS (pH 7.4). Subsequently, the cells were treated with 5 μg of bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$] (Molecular Probes, Eugene, OR, USA) [18]. Flow cytometry analysis was performed with a FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

C. albicans cells (1×10^6 cells/mL YPD) were washed with Ca^{2+} and Mg^{2+} free PBS, and the collapse of the cytoplasmic membrane potential by peptides was observed with $DiSC_3(5)$ (Sigma Chemical Co., St. Louis, MO, USA). Changes in the fluorescence were continuously monitored with using spectrofluorophotometer at an excitation wavelength of 622 nm and an emission wavelength of 670 nm [19]. All experiments were performed three independent times.

2.7. Propidium iodide influx assay

Fungal membrane permeabilization after treatment with antimicrobial peptides was detected using the propidium iodide influx assay. *C. albicans* cells (1×10^6 cells/mL YPD) were treated with either Bac8c or melittin for 4 h at 28 °C, then were washed in PBS. Cells were harvested by centrifugation and resuspended in PBS. Subsequently, the cells were treated with 6 μM propidium iodide and incubated for 5 min at room temperature. The uptake of propidium iodide into *C. albicans* cells was analyzed with a FACSCalibur flow cytometer, and the results are based on three independent experiments, performed in triplicate.

2.8. Preparation of calcein-loaded liposomes and leakage assay

LUVs, composed of phosphatidylcholine/phosphatidylethanolamine/phosphatidylinositol/ergosterol [PC/PE/PI/ergosterol (5:4:1:2, w/w/w/w)], were prepared by vortexing dried lipids in a dye buffer solution (70 mM calcein, 10 mM Tris, 150 mM NaCl, and 0.1 mM EDTA [pH 7.4]), to obtain large unilamellar vesicles (LUVs) which contained calcein. The suspension was freeze-thawed in liquid nitrogen 13 times and extruded through polycarbonate filters (Avestin Inc., Ottawa, Canada). Any free calcein, which had not been entrapped in the LUVs, was removed using a gel filtration process on a Sephadex G-50 column. For the calcein leakage assay, a suspension of liposomes containing calcein was treated with the putative antifungal peptides. The mixture (1 mL final volume) was stirred for 10 min in the dark and centrifuged at 12,000 ×*g* for

Table 2
Human erythrocyte lysis assay against Bac8c and melittin.

Peptides	Hemolysis (%)						
	100.0 μM	50.0 μM	25.0 μM	12.5 μM	6.3 μM	3.1 μM	1.6 μM
Bac8c	0	0	0	0	0	0	0
Melittin	100	100	98.1	87.4	52.6	22.5	8.6

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