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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.
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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

Table 3
Effect of Bac8c on cell wall regeneration of *C. albicans* protoplast.

Peptides	FR[a]
Control[b]	88.2
Bac8c	4.31
Melittin	3.57

[a] Frequency of regeneration (FR) values were calculated by the formula: FR (%) = [(number of colonies on plate)]/[(number of protoplast used)] × 100.
[b] Control indicates no compound treatment.

10 min. The release of calcein from LUVs was assessed by measuring the fluorescence intensity of the supernatant at 25 °C at an excitation wavelength of 490 nm and an emission wavelength of 520 nm with a spectrofluorophotometer (Shimadzu, RF-5301 PC; Shimadzu, Kyoto, Japan). Twenty microliters of 10% Triton X-100 was added to the vesicles to determine the maximum fluorescence intensity from 100% calcein leakage. The percentage of calcein leakage caused by the peptide compounds was calculated as follows: calcein leakage (%) $= 100 \times (F - F_0)/(F_t - F_0)$, where F represents the fluorescence intensity achieved after addition of the peptides and F_0 and F_t represent the fluorescence intensities without the peptides and with Triton X-100, respectively [20]. The data represent the mean ± standard deviation for three independent experiments.

2.9. Preparation of dextran-loaded liposomes and estimation of the pore size

FITC-labeled dextrans (FD) (FD4, FD10, and FD20) were used to evaluate the membrane-active mechanism of Bac8c. LUVs containing FD were prepared with the reverse-phase evaporation method [21]. To prepare liposomes containing FD, 1 mL buffer I (50 mM potassium phosphate, pH 7.4, with 0.1 mM EDTA), containing 2 mg/ml of FD, was sonicated (JAC 2010; KODO, Korea) 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 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 sonicated, centrifuged, and washed for several cycles at 12,000 ×*g* for 30 min to remove the free FD. For the assay, a suspension of FD-loaded liposomes was treated with 10 μM of the peptides. The molar ratio of peptide:lipid was 1:50. The mixture (1 mL, final volume) was stirred for 10 min in the dark and then centrifuged at 12,000 ×*g* for 20 min. The supernatant was recovered, and its fluorescence intensity was recorded at an excitation wavelength of 494 nm and an emission wavelength of 520 nm with a spectrofluorophotometer. The maximum fluorescence intensity was determined by adding 20 μL of 10% Triton X-100 to the liposome suspension. The percentage of FD leakage was calculated in the same way as the percentage of calcein leakage, above. The data represent the mean ± standard deviation for three independent experiments.

C. albicans cells (1×10^6 cells/mL YPD) were suspended in PBS, treated with either Bac8c or melittin for 4 h at 28 °C and the cells were harvested by centrifugation and resuspended in PBS. Subsequently, the soluble fluorescent molecules FD4, FD10 and FD20 were added to the *C. albicans* cells to final concentration of 0.1 mg/ml, respectively. The influx of the fluorescent molecules was observed with a fluorescent microscope (Nikon eclipse Ti-S; Nikon, Japan) [22].

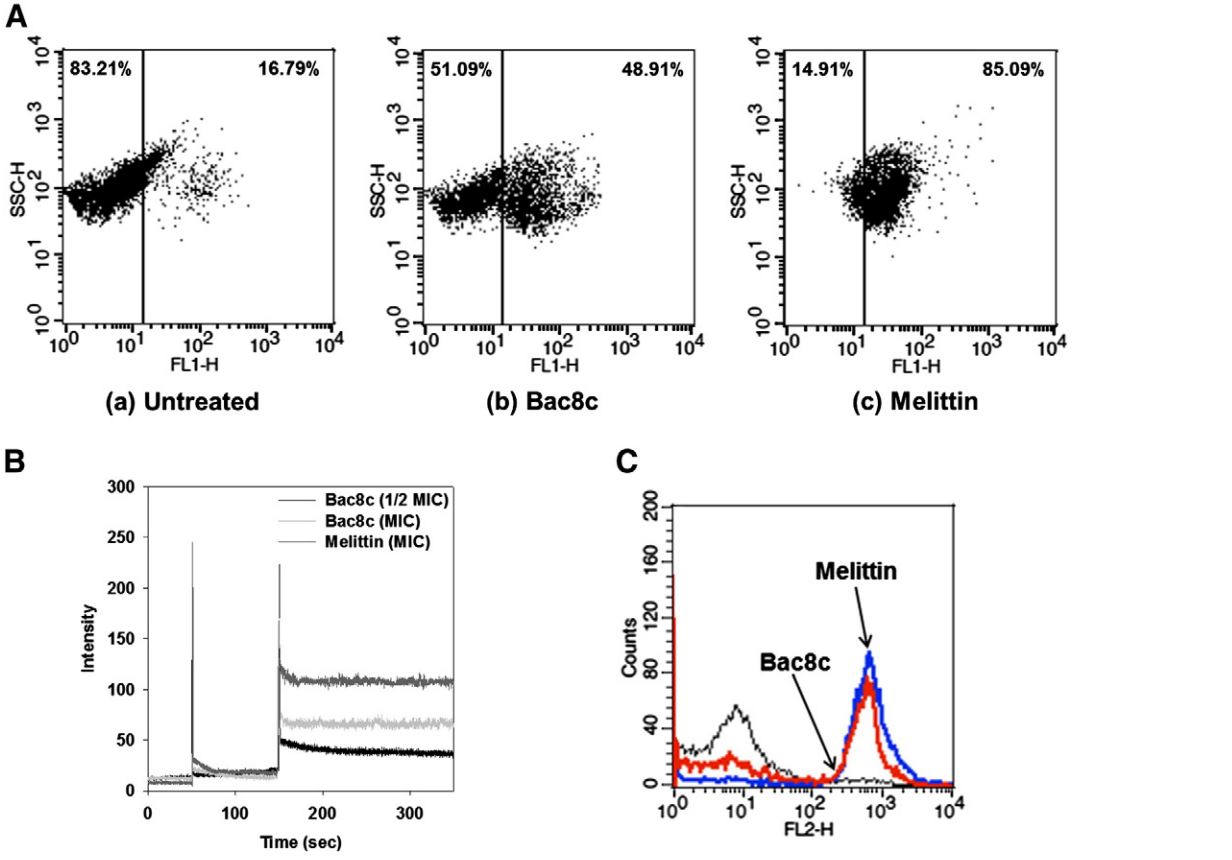

Fig. 1. Membrane damage induced by Bac8c in *C. albicans*. (A) $DiBAC_4(3)$ staining for detection of dissipation of membrane potential in *C. albicans*. (a) Untreated control, (b) cells treated with Bac8c at the MIC, (c) cells treated with melittin at the MIC. (B) Depolarization of the membrane potential was detected by $DiSC_3(5)$. $DiSC_3(5)$ was added at $t = 50$ s, and peptides were added at $t = 150$ s to monitor the changes in fluorescence. (C) Propidium iodide staining for detection of increase of membrane permeability in Bac8c and melittin treated *C. albicans*.

2.10. Potassium release from cytosol assay

The ability peptides to induce pore formation was estimated by measuring efflux of potassium ions from *C. albicans* using an ion-selective electrode (ISE) meter (Orion star A214; Thermo Scientific, Singapore). *C. albicans* cells (1×10^6 cells/mL YPD) were treated with either Bac8c or melittin for 4 h at 28 °C. The incubated cells were centrifuged at 12,000 ×*g* for 5 min to remove the cell debris. Potassium ion concentration in the supernatant was expressed as a percentage of the total free potassium ion, calculated following sonication [23,24].

2.11. Preparation of GUVs

Giant unilamellar vesicles (GUVs) encapsulating calcein were prepared with indium tin oxide (ITO) glasses. Lipids [PC/PE/PI/ergosterol (5:4:1:2, w/w/w/w)] [25] were prepared at a concentration of 3.75 mg/mL in chloroform. The lipid solutions (200 μL) were spread onto ITO-coated glass in a spin coater (Spin coater, ACE-1020 Series) at 700 × *g* for 5 min. The lipid-coated ITO glass was subjected to evaporation under vacuum for 3 h. The lipid-coated and uncoated glasses were placed facing each other with a 2 mm-thick Teflon spacer between them. The chamber was filled with buffer (10 mM HEPES buffer, pH 7.2, 10 μM glucose, 1 mM calcein), and 1.7 V (peak-to-peak, sine wave) and 10 Hz of power was applied to the ITO electrodes using a sweep function generator (Protek, SWEEP FUNCTION GENERATOR 9205C) for 2 h. The voltage across the ITO glass was changed to 4 V (peak-to-peak) at 4 Hz for 10 min. To differentiate the free calcein from GUVs encapsulating calcein, we used gel filtration chromatography on a Sephadex G-50. The GUVs were treated with Bac8c or melittin, and the changes in GUV fluorescence were observed with an inverted fluorescence microscope [26,27].

2.12. Three dimensional (3D) flow cytometric contour plot analysis

C. albicans cells (1×10^6 cells/mL YPD) were treated with either Bac8c or melittin for 4 h at 28 °C. The incubated cells were harvested by centrifugation and resuspended in PBS. The morphological changes were analyzed with a FACSCalibur flow cytometer. Non-stained living cells were evaluated for each sample by excitation with 488 nm light from an argon ion laser by determining their position on a forward scatter (FSC) versus side scatter (SSC) contour plot [28].

3. Results and discussion

3.1. Bac8c has an antimicrobial effect with no hemolytic activity

Bactenecin, a 12-mer peptide isolated from bovine neutrophils, is one of the smallest natural cationic antimicrobial peptides [29]. However, it has been reported that when bactenecin was modified to be linearized, the peptide loses its antimicrobial activity [30]. Bac2A was created by substituting alanine residues for the two cysteine residues of linearized bactenecin. This modified peptide has greater antimicrobial activity than its cyclic or linear forms of bactenecin, with an additional lower agglutinating activity [30]. Furthermore, many researchers found that Bac8c, which is an 8-mer peptide derived from Bac2A, with four favorable amino acid substitutions as determined by a complete substitution analysis of Bac2A [30,31]. Bac8c not only showed improved antimicrobial activity against a range of gram-positive and gram-negative bacteria as well as yeast, but could also be synthesized rapidly and inexpensively, minimizing its production costs [11,30,31]. Most previous studies have explored antibacterial activity, and although there have been previous reports on the antibacterial mechanisms of Bac8c [31–33], the antifungal effect underlying its

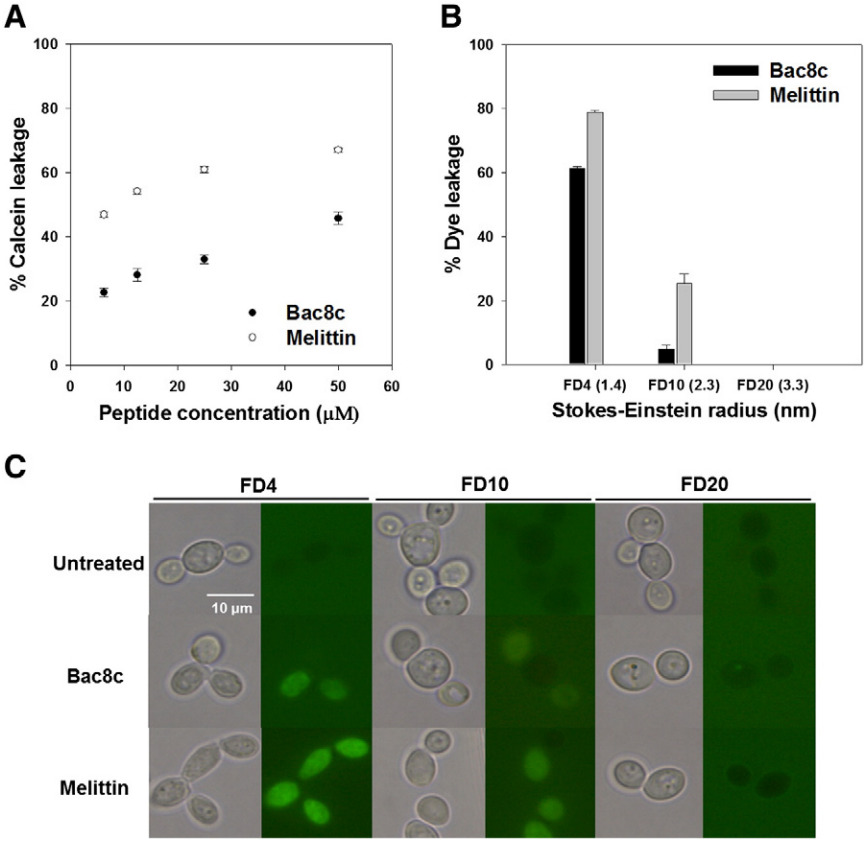

Fig. 2. (A) Percentage of calcein leakage from the LUVs [PC:PE:PI:ergosterol = 5:4:1:2 (w/w/w/w)] was measured after treatment with the peptides for 10 min at various concentrations (MIC, 2 × MIC, 3 × MIC and 4 × MIC). (B) Percentage of FITC-dextrans (FDs) leakage induced by Bac8c and melittin at MIC, respectively, from PC/PE/PI/ergosterol (5:4:1:2, w/w/w/w) liposomes. (C) Observation of fluorescent influx in to viable *C. albicans* cells under fluorescence microscopy.

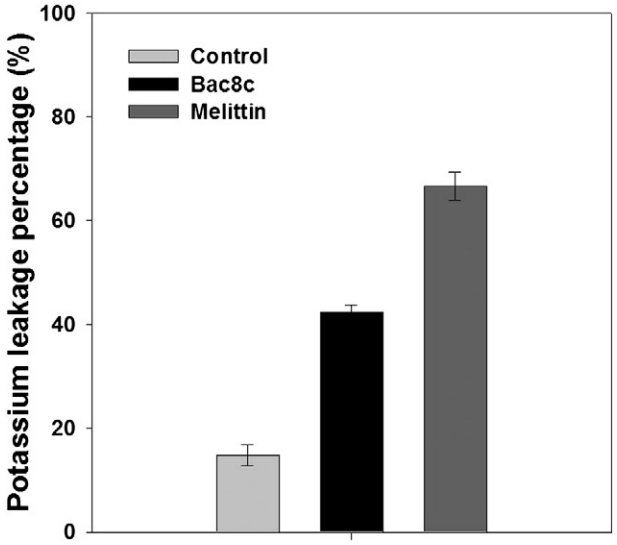

Fig. 3. Potassium leakage after incubation with MIC value of peptides. The error bars represent the standard deviation (SD) for three independent experiments.

mode of action remains unknown. We focused on understanding the mechanism of action of the antimicrobial peptide, Bac8c, in *C. albicans*.

To determine the MIC of Bac8c, we selected pathogenic fungi causing diverse infectious disease in humans: *C. albicans*, *Candida parapsilosis*, *Trichosporon beigelii*, and *Malassezia furfur*. The antimicrobial peptide melittin was used as a positive control peptide for comparing the potency of Bac8c. As shown in Table 1, the fungal strains were susceptible to Bac8c with a MIC range of 6.3–25.0 μM, and to melittin with a MIC range of 1.6–6.3 μM. From these results, we determined that Bac8c shows an inhibitory effect against fungi; we therefore performed further experiments to determine the mechanism of inhibition by Bac8c. Among diverse fungal strains against which the susceptibility to Bac8c was tested, *C. albicans* is selected as a model organism for the experiments in this study because it is the most widespread fungal pathogen causing candidiasis [34,35].

Furthermore, it is essential that the potential toxic effects of the Bac8c are tested. To observe hemolytic effect of Bac8c against human erythrocytes, the release of hemoglobin was estimated. As shown in Table 2, Bac8c has no hemolytic effect at any concentration whereas melittin showed 100% of hemolysis at 50 μM. From these result, we suggests that Bac8c has more activity against fungal cells compared to human erythrocytes and it can be a candidate for further drug development.

3.2. Bac8c induces membrane damage in C. albicans

While antifungal agents can target various cellular structures such as membranes, cell walls, and nuclei, most antifungal agents have a mechanism of action involving the cell membrane [36]. From the investigation regarding an effect of the Bac8c on the cell wall regeneration of protoplast of *C. albicans*, the frequency of regeneration (FR) of Bac8c- or melittin-treated protoplasts was extremely low as compared to the untreated protoplasts (Table 3). This result indicates that the antifungal activity of peptides is due to the interaction between the peptides and the plasma membrane, rather than cell wall [17]. Therefore, we investigated the effect of Bac8c on the membranes of *C. albicans* to elucidate the antifungal mechanism of Bac8c.

To examine whether Bac8c can affect the function of the fungal membrane, changes in membrane potential were detected with the anionic lipophilic dye $DiBAC_4(3)$. Increased fluorescence of $DiBAC_4(3)$ indicates membrane depolarization, as $DiBAC_4(3)$ exhibits high voltage-sensitivity and enters depolarized cells, where it binds to lipid-rich intracellular components [37]. In cells exposed to Bac8c and

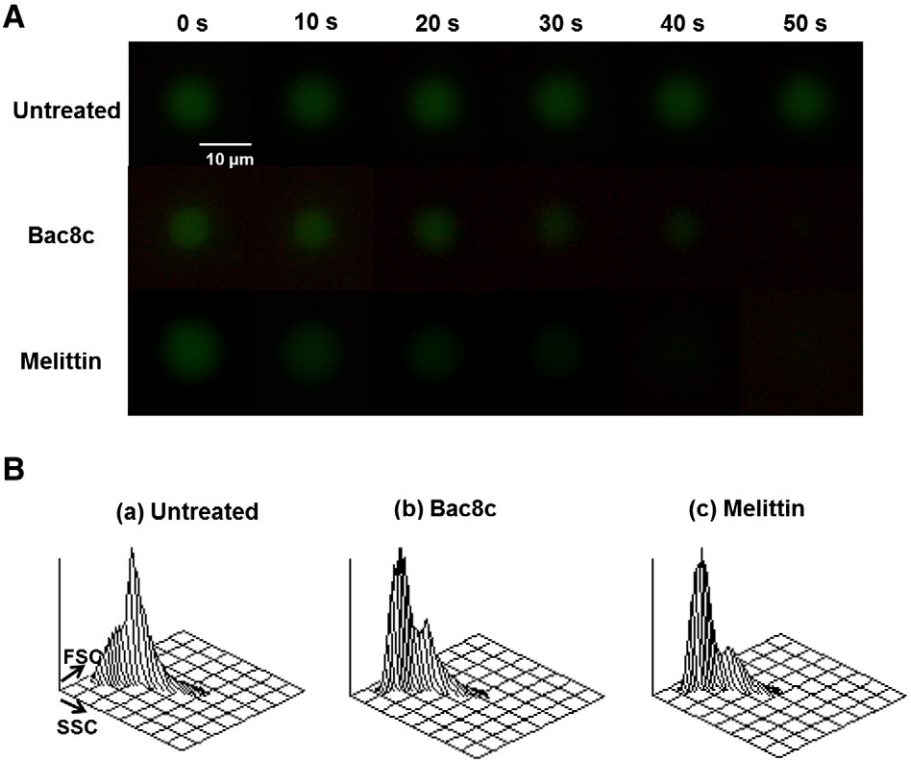

Fig. 4. (A) The response of a single GUV composed of PC/PE/PI/ergosterol (5:4:1:2, w/w/w/w) in the presence of the peptides at 0, 10, 20, 30, 40, and 50 s. The times above each image show the time after the addition of the peptides. (B) Three dimensional (3D)-flow cytometric contour-plot analysis of *C. albicans*. FSC is an indicator of cell size, and peptides at the MIC had increased FSC values in the 3D density plots relative to the main population of untreated cells, indicating shrinkage of *C. albicans*. (a) Untreated control, (b) cells treated with Bac8c at the MIC, (c) cells treated with melittin at the MIC.

melittin, $DiBAC_4(3)$ fluorescence intensity increased by 32.12% and 68.30%, respectively, compared to untreated cells (Fig. 1A). These results indicate that Bac8c induces dissipation of the cytoplasmic membrane potential of *C. albicans*. We also showed that the correlation between concentrations of Bac8c and degree of membrane depolarization using potential sensitive fluorescent probe, $DiSC_3(5)$. After quenching the fluorescence of the $DiSC_3(5)$, treatment with Bac8c (at 150 s) resulted in an increase in the fluorescence intensity and depolarization correlate with dosage of Bac8c (Fig. 1B).

Furthermore, the effect of Bac8c on the integrity of plasma membranes of *C. albicans* was examined by propidium iodide influx assay. Propidium iodide is a membrane-impermeable dye, which only enters cells through damaged membranes [38,39]. As shown in Fig. 1C, the fluorescence of *C. albicans* cells increased by 49.59% when treated with Bac8c at the MIC compared to the negative control, indicating damage to the membrane structure. When *C. albicans* cells were treated with melittin at the MIC, the cell fluorescence increased by 77.34%. These data indicate that Bac8c causes membrane damage, which disturbs the membrane potential and reduces membrane integrity. Although Bac8c has variant structure, this peptide possesses common characteristics of antimicrobial peptides such as cationic charge and amphipathicity [40]. Therefore, we assumed that Bac8c interact with fungal cell membrane with electrostatic and/or hydrophobic interaction, and cause membrane disruption [41]. Based on the results of membrane depolarization and propidium iodide assay, we further examined the antifungal activity of Bac8c on fungal cell membranes.

3.3. Bac8c induces pore formation on the C. albicans membrane

The propidium iodide influx assay can be considered not only indicative of a loss of membrane integrity, but also of pore formation in the cell membrane [42]. Therefore, we hypothesized that the mechanism of action of Bac8c on the cell membrane might be related to pore formation. To elucidate the mechanism of action, and examine the extent of membrane damage induced by the peptides, the leakage of different dyes with various molecular weights from peptide-treated artificial liposomes was measured. The dyes used were calcein (average molecular weight = 623 Da, Stokes-Einstein radius = 0.74 nm) [43] and the FD4 (average molecular weight = 4 kDa, Stokes-Einstein radius = 1.4 nm), FD10 (average molecular weight = 10 kDa, Stokes-Einstein radius = 2.3 nm) and FD20 (average molecular weight = 20 kDa, Stokes-Einstein radius = 3.3 nm) [44]. The artificial liposomes were LUVs composed of PC/PE/PI/ergosterol (5:4:1:2, w/w/w/w), resembling the outer leaflet of the plasma membrane of *C. albicans* [45]. Bac8c induced dose-dependent calcein leakage from LUVs at all the tested concentrations (Fig. 2A). In the FD leakage assay, melittin induced the release of FD4 and FD10 from LUVs, but did not induce release of FD20, suggesting the size of the pore formed by melittin was larger than 2.3 nm. Bac8c also caused the release of FD4 and FD10 from LUVs, but did not induce release of FD20, indicating the size of the pore formed by Bac8c treatment was larger than 2.3 nm (Fig. 2B). This result shows that Bac8c induced the formation of pores with radii between 2.3 nm and 3.3 nm and it is also supported by the observation of the fluorescent influx in to viable *C. albicans* cells using the fluorescent microscope. As shown in Fig. 2C, the influx of FD4 and FD10 were detected, but FD20 did not enter the cytoplasm of the cells after the addition of Bac8c. Taken together, these results showed that Bac8c induced pore formation and increased the permeability of the fungal membrane. Hence, the fungal cells experienced membrane depolarization, which causes a loss of ions, due to Bac8c treatment eventually leading to fungal cell shrinkage and cell death.

3.4. Leakage of intracellular potassium ions

To further validate membrane pore formation in case of Bac8c-treated *C. albicans*, potassium release was measured using a potassium-sensitive electrode. Potassium efflux induced by Bac8c was less efficient than that induced by melittin. In untreated *C. albicans* cells, 14.8% of the potassium ions were released from the cells, while Bac8c-treated cells showed a 42.4% release of potassium ions. In comparison, melittin-treated *C. albicans* showed a 66.7% release of potassium ions (Fig. 3). This result suggests that Bac8c induced leakage of intracellular components, especially potassium ions, from *C. albicans*.

Cellular homeostasis is central to life and in microbial pathogens is attained by controlling the permeability of the cytoplasmic membrane to ions and solutes. The potassium ion gradient is an important determinant of growth and survival through its role in regulating cytoplasmic pH and cell structure [46]. Therefore, a loss of cytoplasmic potassium would lead to cell death in fungi [47]. Leakage of potassium ions can be evaluated to determine membrane-lytic events in fungi and liposomes, since the internal ionic environment in cells is usually potassium-rich [48]. The loss of the potassium gradient across the cell membrane is an outcome of membrane damage, and can lead to compromise of membrane functions and, eventually, cell death.

3.5. Visualization of the fungal membrane-disruptive action of Bac8c

Positively-charged antimicrobial peptides can accumulate on the anionic microbial cell surface containing acidic polymers and contact cytoplasmic membrane [49]. Membrane permeabilization and depolarization by antimicrobial peptides causes disruption of the lipid bilayer, inducing the formation of micelles, transmembrane pores, and toroidal pores [50,51]. To understand the membrane-active mechanisms more accurately, we used GUVs containing calcein. GUVs, composed of PC/PE/PI/ergosterol (5:4:1:2, w/w/w/w) [52] and mimicking the membranes of *C. albicans*, were prepared using the electroformation method [45]. GUVs have become a useful model for imitating biological membranes [53]. Morphological changes in the artificial model membrane allow the study of structural and physical changes during interactions with antimicrobial agents [43,54]. Changes in fluorescence are attributed to structural perturbation of the model membrane after interaction with antimicrobial peptides [55]. Using fluorescence microscopy, we observed changes in fluorescence of the GUVs in response to treatment with peptides. When treated with Bac8c or melittin, the fluorescence of the GUVs was remarkably diminished. This phenomenon demonstrated that the fluorescent calcein probe escaped through peptide-induced pores on the liposome. In addition, the untreated GUVs showed no changes in the fluorescence intensity (Fig. 4A).

Morphological changes in the cells were also characterized using flow cytometry to compare the FSC and SSC profiles of Bac8c-treated cells. In general, FSC is an indicator of cell size and SSC is an indicator of granularity. Fig. 4B shows that the cells treated with Bac8c and melittin had decreased FSC values in the 3D density plots relative to the main population of untreated cells, indicating shrinkage of *C. albicans*. The cell shrinkages demonstrated that membrane permeability increased by pore and cell shrinkage occurred along with the loss of ions or intracellular components [56]. These results clearly suggest that the membrane-targeted mechanism of Bac8c lies in pore formation within the fungal membrane.

In conclusion, Bac8c exhibited potent antifungal effect on various fungal strains via membrane disruption mechanisms involving pore formation. Therefore, we suggest that as Bac8c is considered a potential candidate to develop into a novel antimicrobial agent because of its high antimicrobial activity without cytotoxicity and low production costs.

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References

[1] E.Y. Furuya, F.D. Lowy, Antimicrobial-resistant bacteria in the community setting, Nat. Rev. Microbiol. 4 (2006) 36–45.
[2] A. Som, S. Vemparala, I. Ivanov, G.N. Tew, Synthetic mimics of antimicrobial peptides, Biopolymers 90 (2008) 83–93.
[3] K. Matsuzaki, Control of cell selectivity of antimicrobial peptides, Biochim. Biophys. Acta 1788 (2009) 1687–1692.
[4] L. Zhang, T.J. Falla, Host defense peptides for use as potential therapeutics, Curr. Opin. Investig. Drugs 10 (2009) 164–171.
[5] L. Yu, L. Guo, J.L. Ding, B. Ho, S.S. Feng, J. Popplewell, M. Swann, T. Wohland, Interaction of an artificial antimicrobial peptide with lipid membranes, Biochim. Biophys. Acta 1788 (2009) 333–344.
[6] D.G. Lee, Y. Park, I. Jin, K.S. Hahm, H.H. Lee, Y.H. Moon, E.R. Woo, Structure-antiviral activity relationships of cecropin A-magainin 2 hybrid peptide and its analogues, J. Pept. Sci. 10 (2004) 298–303.
[7] L.H. Kondejewski, S.W. Farmer, D.S. Wishart, C.M. Kay, R.E. Hancock, R.S. Hodges, Modulation of structure and antibacterial and hemolytic activity by ring size in cyclic gramicidin S analogs, J. Biol. Chem. 271 (1996) 25261–25268.
[8] Y. Park, D.G. Lee, K.S. Hahm, HP(2–9)-magainin 2(1–12), a synthetic hybrid peptide, exerts its antifungal effect on *Candida albicans* by damaging the plasma membrane, J. Pept. Sci. 10 (2004) 204–209.
[9] S.A. Muhle, J.P. Tam, Design of Gram-negative selective antimicrobial peptides, Biochemistry 40 (2001) 5777–5785.
[10] C. Landon, F. Barbault, M. Legrain, M. Guenneugues, F. Vovelle, Rational design of peptides active the gram positive bacteria *Staphylococcus aureus*, Proteins 72 (2008) 229–239.
[11] K. Hilpert, R. Volkmer-Engert, T. Walter, R.E. Hancock, High-throughput generation of small antibacterial peptides with improved activity, Nat. Biotechnol. 23 (2005) 1008–1012.
[12] B. Merrifield, Solid phase synthesis, Science 232 (1986) 341–347.
[13] R. Sheppard, The fluorenylmethoxycarbonyl group in solid phase synthesis, J. Pept. Sci. 9 (2003) 545–552.
[14] CLSI, Clinical and Laboratory Standards Institute Performance Standards for Antimicrobial Susceptibility Testing, Fifteenth ed. CLSI, Wayne, PA, 2005.
[15] B. Jahn, E. Martin, A. Studeben, S. Bhakdi, Susceptibility testing of *Candida albicans* and *Aspergillus* species by a simple microtiter menadione-augmented 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphynyl-2H-tetrazolium bromide assay, J. Clin. Microbiol. 33 (1995) 661–667.
[16] J.K. Kim, E. Lee, S. Shin, K.W. Jeong, J.Y. Lee, S.Y. Bae, S.H. Kim, J. Lee, S.R. Kim, D.G. Lee, J.S. Hwang, Y. Kim, Structure and function of papiliocin with antimicrobial and anti-inflammatory activities isolated from the swallowtail butterfly, *Papilio xuthus*, J. Biol. Chem. 286 (2011) 41296–41311.
[17] D.G. Lee, H.N. Kim, Y. Park, H.K. Kim, B.H. Choi, C.H. Choi, K.S. Hahm, Design of novel analogue peptides with potent antibiotic activity based on the antimicrobial peptide, HP (2–20), derived from N-terminus of *Helicobacter pylori* ribosomal protein L1, Biochim. Biophys. Acta 1598 (2002) 185–194.
[18] C. Park, E.R. Woo, D.G. Lee, Antifungal effect with apoptotic mechanism(s) of Styraxjaponoside C, Biochem. Biophys. Res. Commun. 390 (2009) 1256–1259.
[19] E.C. Veerman, M. Valentijn-Benz, K. Nazmi, A.L. Ruissen, E. Walgreen-Weterings, J. van Marle, A.B. Doust, W. van't Hof, J.G. Bolscher, A.V. Amerongen, Energy depletion protects *Candida albicans* against antimicrobial peptides by rigidifying its cell membrane, J. Biol. Chem. 282 (2007) 18831–18841.
[20] S.C. Park, M.H. Kim, M.A. Hossain, S.Y. Shin, Y. Kim, L. Stella, J.D. Wade, Y. Park, K.S. Hahm, Amphipathic α-helical peptide, HP (2–20), and its analogues derived from *Helicobacter pylori*: pore formation mechanism in various lipid compositions, Biochim. Biophys. Acta 1778 (2008) 229–241.
[21] O.S. Belokoneva, H. Satake, E.L. Mal'tseva, N.P. Pal'mina, E. Villegas, T. Nakajima, G. Corzo, Pore formation of phospholipid membrane by the action of two hemolytic arachnid peptides of different size, Biochim. Biophys. Acta 1664 (2004) 182–188.
[22] S.C. Park, J.Y. Kim, C. Jeong, S. Yoo, K.S. Hahm, Y. Park, A plausible mode of action of pseudin-2, an antimidrobial peptide from *Pseudis paradoxa*, Biochim. Biophys. Acta 2011 (1808) 172–182.
[23] S.D. Cox, J.E. Gustafson, C.M. Mann, J.L. Markham, Y.C. Liew, R.P. Hartiland, H.C. Bell, J.R. Warmington, S.G. Wyllie, Tea tree oil causes K^+ leakage and inhibits respiration in *Escherichia coli*, Lett. Appl. Microbiol. 26 (1998) 355–358.
[24] P. Kanmani, S.T. Lim, Synthesis and characterization of pullulan-mediated silver nanoparticles and its activities, Carbohydr. Polym. 97 (2013) 421–428.
[25] A. Makovitzki, D. Avrahami, Y. Shai, Ultrashort antibacterial and antifungal lipopeptides, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 15997–16002.
[26] M.I. Angelova, D.S. Dimitrov, Liposome electroformation, Faraday Discuss. Chem. Soc. 81 (1986) 303–311.
[27] M.I. Angelova, S. Sloeau, P. Meleard, J.F. Faucon, P. Bothorel, Preparation of giant vesicles by external AC electric fields. Kinetics and application, Prog. Colloid Polym. Sci. 89 (1992) 127–131.
[28] S. Herman, N. Zurgil, M. Deutsch, Low dose methotrexate induces apoptosis with reactive oxygen species involvement in T lymphocytic cell lines to a greater extent than in monocytic lines, Inflamm. Res. 54 (2005) 273–280.
[29] D. Romeo, B. Skerlavaj, M. Bolognesi, R. Gennaro, Structure and bactericidal activity of and antibiotic dodecapeptide purified from bovine neutrophils, J. Biol. Chem. 263 (1988) 9573–9575.
[30] M. Wu, R.E. Hancock, Improved derivatives of bactenecin, a cyclic dodecameric antimicrobial cationic peptide, Antimicrob. Agents Chemother. 43 (1999) 1274–1276.
[31] Y. Ding, W. Wang, M. Fan, Z. Tong, R. Kuang, W. Jiang, L. Ni, Antimicrobial and anti-biofilm effect of Bac8c on major bacteria associated with dental caries and *Streptococcus mutans* biofilms, Peptides 52 (2014) 61–67.
[32] E.C. Spindler, J.D. Hale, T.H. Giddings Jr., R.E. Hancock, R.T. Gill, Deciphering the mode of action of the synthetic antimicrobial peptide Bac8c, Antimicrob. Agents Chemother. 55 (2011) 1706–1716.
[33] E.C. Spindler, N.R. Boyle, R.E. Hancock, R.T. Gill, Genome-wide identification of genes conferring energy related resistance to a synthetic antimicrobial peptide (Bac8c), PLoS One 8 (2013) e55052.
[34] R.A. Calderone, W.A. Fonzi, Virulence factors of *Candida albicans*, Trends Microbiol. 9 (2001) 327–335.
[35] E. Lee, K.W. Jeong, J. Lee, A. Shin, K.J. Kim, J. Lee, D.G. Lee, Y. Kim, Structure-activity relationships of cecropin-like peptides and their interactions with phospholipid membrane, BMB Rep. 46 (2013) 282–287.
[36] C. Spampinato, D. Leonardi, *Candida* infections, causes, targets and resistance mechanisms: traditional and alternative antifungal agents, Biomed. Res. Int. 2013 (2013) 204–237.
[37] R.S. Liao, R.P. Rennie, J.A. Talbot, Assessment of the effect of amphotericin B on the vitality of *Candida albicans*, Antimicrob. Agents Chemother. 43 (1999) 1034–1041.
[38] C. Pina-Vaz, F. Sansonetty, A.G. Rodrigues, Cytometric approach for a rapid evaluation of susceptibility of *Candida* strains to antifungals, Clin. Microbiol. Infect. 7 (2001) 609–618.
[39] L. Li, Y. Shi, M.J. Cheserek, G. Su, G. Le, Antibacterial activity and dual mechanisms of peptide analog derived from cell-penetrating peptide against *Salmonella typhimurium* and *Streptococcus pyogenes*, Appl. Microbiol. Biotechnol. 97 (2013) 1711–1723.
[40] L. Zhang, A. Rozek, R.E. Hancock, Interaction of cationic antimicrobial peptides with model membranes, J. Biol. Chem. 276 (2001) 35714–35722.
[41] B.M. Peter, M.E. Shirtliff, M.A. Jabra-Rizk, Antimicrobial peptides: primeval molecules or future drugs? PLoS Pathog. 6 (2010) e1001067.
[42] C.A. Spyr, F. Käsermann, C. Kempf, Identification of the pore forming element of Semliki Forest virus spikes, FEBS Lett. 375 (1995) 134–136.
[43] Y. Tamba, H. Ariyama, V. Levadny, M. Yamazaki, Kinetic pathway of antimicrobial peptide magainin 2-induced pore formation in lipid membranes, J. Phys. Chem. B 114 (2010) 12018–12026.
[44] C.M. Sandoval, B. Salzameda, K. Reyes, T. Williams, V.S. Hohman, L.A. Plesniak, Anti-obesity and anti-tumor pro-apoptotic peptides are sufficient to cause release of cytochrome *c* from vesicles, FEBS Lett. 581 (2007) 5464–5468.
[45] A. Makovitzki, Y. Shai, pH-Dependent antifungal lipopeptides and their plausible mode of action, Biochemistry 44 (2005) 9775–9784.
[46] T.P. Roosild, S. Castronovo, J. Healy, S. Miller, C. Pliotas, T. Rasmussen, W. Bartlett, S.J. Conway, I.R. Booth, Mechanism of ligand-gated potassium efflux in bacterial pathogens, Proc. Natl. Acad. Sci. U. S. A. 102 (2010) 19784–19789.
[47] A. Peña, N.S. Sánchez, M. Calahorra, Effects of chitosan on *Candida albicans*: conditions for its antifungal activity, Biomed. Res. Int. 2013 (2013) 527–549.
[48] D.S. Orlve, T. Nguyen, R.I. Lehrer, Potassium release, a useful tool for studying antimicrobial peptides, J. Microbiol. Methods 49 (2002) 325–328.
[49] R.E. Hancock, H.G. Sahl, Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies, Nat. Biotechnol. 24 (2006) 1551–1557.
[50] K.A. Brogden, Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat. Rev. Microbiol. 3 (2005) 238–250.
[51] M. Wenzel, B. Kohl, D. Münch, N. Raatschen, H.B. Albada, L. Hamoen, N. Metzler-Nolte, H.G. Sahl, J.E. Bandow, Proteomic response of *Bacillus subtilis* to lantibiotics reflects differences in interaction with the cytoplasmic membrane, Antimicrob. Agents Chemother. 56 (2012) 5749–5757.
[52] H. Choi, J. Cho, Q. Jin, E.R. Woo, D.G. Lee, Antifungal property of dihydrodehydrodiconiferyl alcohol 9'-O-β-D-glucoside and its pore-forming action in plasma membrane of *Candida albicans*, Biochim. Biophys. Acta 1818 (2012) 1648–1655.
[53] O. Wesolowska, K. Michalak, J. Maniewska, A.B. Hendrich, Giant unilamellar vesicles – a perfect tool to visualize phase separation and lipid rafts in model systems, Acta Biochim. Pol. 56 (2009) 33–39.
[54] Y.Tamba,T.Tanaka,T.Yahagi,Y.Yamashita,M.Yamazaki,Stabilityofgiantunilamellar vesicles and large unilamellar vesicles of liquid-ordered phase membranes in the presence of Triton X-100, Biochim. Biophys. Acta 1667 (2004) 1–6.
[55] K. Peng, Y. Kong, L. Zhai, X. Wu, P. Jia, J. Liu, H. Yu, Two novel antimicrobial peptides from centipede venoms, Toxicon 55 (2010) 274–279.
[56] J. Cho, H. Choi, J. Lee, M.S. Kim, H.Y. Sohn, D.G. Lee, The antifungal activity and membrane-disruptive action of dioscin extracted from *Dioscorea nipponica*, Biochim. Biophys. Acta 2013 (1828) 1153–1158.