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Fungicidal effect of antimicrobial peptide arenicin-1

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ABSTRACT

Arenicin-1 is a 21-residue peptide which was derived from *Arenicola marina*. In this study, we investigated the antifungal effects and its mechanism of action towards human pathogenic fungi. Arenicin-1 exerted remarkable fungicidal activity with both energy-dependent and salt-insensitive manners. To investigate the fungicidal mechanisms of arenicin-1, the membrane interactions of arenicin-1 were examined. Flow cytometric analysis, using propidium iodide (PI) and bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$], as well as fluorescence analysis, regarding the membrane probe 1,6-diphenyl-1,3,5-hexatriene (DPH), were conducted against *Candida albicans*. The results demonstrated that arenicin-1 was associated with lipid bilayers and induced membrane permeabilization. Additionally, the membrane studies in regard to liposomes resembling the phospholipid bilayer of *C. albicans* confirmed the membrane-disruptive potency of arenicin-1. Therefore, the present study suggests that arenicin-1 exerts its fungicidal effect by disrupting fungal phospholipid membranes.

1. Introduction

Marine invertebrates lack an acquired, memory-type immunity based on T-lymphocyte subsets and clonally derived immunoglobulins. This characteristic differs from that of the vertebrate immune system, which is characterized by somatic gene rearrangement, clonal selection, expansion, and a discriminative ability which includes lymphocytes, among other factors, which impart specificity and memory. The circulating hemolymph in marine invertebrates contains biologically active substances such as complement, lectins, clotting factors, and antimicrobial peptides [1–3]. These factors contribute to a self-defense system in marine invertebrates against invading microorganisms. Above all, antimicrobial peptides, such as tachyplesin from acid extracts of large hemocyte granules of *Tachypleus tridentatus*, clavanin from *Styela clava* and mytilin from *Mytilis edulis*, are major components of the innate immune defense system in marine invertebrates, and also provide an immediate and rapid response to invading microorganisms [4–7].

Arenicin-1 (RWCVYAYVRVRGVLVRYRRCW), a 21-residue antibacterial peptide, is a β-sheet peptide isolated from the coelomocytes of the marine polychaeta lugworm, *Arenicola marina* [8]. This peptide has been reported to exhibit broad antibacterial activity against human pathogenic bacterial strains [9]. The structure of arenicin-1 was found to consist of two stranded anti-parallel β-sheet, constrained by one disulfide bond and connected by a β-turn. As arenicin-1 bears a net positive charge (+6) in combination with a substantial number of hydrophobic amino acid residues, extensive research regarding a membranolytic steps involved in the killing of bacterial strains have been conducted [10]. However, the antifungal activity and its mechanism are still far from being fully answered.

In this study, the antifungal activity of arenicin-1 was investigated, and the mode of action was elucidated by exploring the virtual area from the intact fungal cell membrane to the artificial phospholipid membrane model.

2. Materials and methods

2.1. Peptide synthesis

The peptides were synthesized by the solid-phase method using Fmoc(9-fluorenyl-methoxycarbonyl)-chemistry [11]. The crude peptide was repeatedly washed with diethylether, dried in a vacuum, and purified using a reversed-phase preparative HPLC on a Waters 15-μm Deltapak C_{18} column (19×30 cm). The purity of the peptide was confirmed by an analytical reversed-phase HPLC on an Ultrasphere C_{18} column, 4.6×25 cm (Beckman, U.S.A.). The molecular weights of the synthetic peptides were determined using a matrix-assisted laser desorption ionization MALDI-mass spectrometer [12].

2.2. Fungal strains and antifungal activity assay

Candida albicans (ATCC 90028) and *Candida parapsilosis* (ATCC 22019) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). *Candida albicans* (TIMM 1768) was obtained from the Center for Academic Societies, Osaka, Japan. *Trichophyton rubrum* (KCTC 6345), *Malassezia furfur* (KCTC 7744), and *Trichosporon beigelii* (KCTC 7707) were obtained from the Korean Collection for

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Type Cultures (KCTC) of the Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon, Korea.

The fungal strains were cultured in a YPD medium (Difco), containing yeast extract, peptone, and dextrose (50 g/L), with aeration at 28 °C. *M. furfur* was cultured in a modified Bacto yeast extract/ malt extract (YM) medium (Difco) containing yeast extract, malt extract, peptone, dextrose (21 g/L), and 1% olive oil at 32 °C.

Log-phase fungal cells (2×10^4/ml) were inoculated into a YPD medium and dispensed into 0.1 ml/well microtiter plates. MICs were determined by a serial two-fold dilution of test peptides, following a micro-dilution method and MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay [13,14]. After 48 h of incubation at 28 °C, the minimal peptide concentration which prevented the growth of a given test organism was determined, and was defined as the MIC. The growth was assayed with a microtiter ELISA Reader (Molecular Devices Emax, California, U.S.A.) by monitoring absorption at 580 nm. The MIC values were determined by three independent assays.

2.3. Time-killing kinetics

The kinetics of fungal killing of the peptides were evaluated by using *C. albicans* cells. Log-phase fungal cells (2×10^6/ml YPD) were incubated with peptides at two times the MIC. The culture was obtained and distributed on an YPD agar plate, then the colony forming units (CFUs) were counted after 24 h incubation at 28 °C [15,16]. The results represent the average of triplicate measurements from three independent assays.

2.4. The effect of arenicin-1 on the dimorphic transition in C. albicans

C. albicans cells were maintained by periodic subculturing in a YPD medium. To induce the formation of mycelia, the cultures were directly supplemented with 20% fetal bovine serum (FBS). The dimorphic transition in *C. albicans* was investigated from the cultures in the presence or absence of arenicin-1, and incubated for 48 h at 37 °C [17]. The dimorphic transition to mycelial forms was detected by phase-contrast light microscopy (NIKON, ECLIPSE TE300, Japan).

2.5. The effect of NaN_3 on antifungal activity of arenicin-1

C. albicans cells (2×10^6/ml) were incubated for 1 h with 0.1 mM NaN_3 prior to treatment with the peptide, and then were incubated for 24 h at 28 °C in a YPD medium. The cell population was illustrated by MTT assay [14]. The absorbance of each well was measured at 580 nm by using a Microtiter ELISA Reader (Molecular Devices Emax). The equation of the cell viability percentage is illustrated as follows: [(the absorbance of the compound-treated cells) / (the absorbance of non-treated cells)]×100. The error bars represent the standard deviation (S.D.) values for three independent experiments, performed in triplicate [18].

2.6. Membrane permeabilization assay and salt-sensitivity test of arenicin-1

For the analysis of the permeabilization of fungal membrane after peptide treatment, *C. albicans* cells (2×10^6/ml) were first harvested at the log phase and suspended in a YPD medium. Cells were incubated with peptides at two times the MIC for 2 h at 28 °C with constant shaking (140 rpm). For the salt-sensitivity test, cells were incubated with arenicin-1 at two times the MIC in the presence or absence of various salt conditions (final concentrations; 150 mM NaCl and 3 mM $MgCl_2$) After incubation, cells were harvested by centrifugation and suspended in PBS. Subsequently, cells were treated with PI (9 μM, final concentration) for 5 min. Flow cytometry was performed via a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) [19–21].

2.7. Flow cytometric analysis for the plasma membrane potential

For the analysis of the membrane integrity after peptide treatment, the log-phased cells of *C. albicans* (2×10^6/ml YPD) were harvested and resuspended in a YPD medium, containing 18 μM of arenicin-1 or 5 μM of melittin. After incubation for 3 h, the cells were harvested by centrifugation and suspended in PBS. Subsequently, the cells were treated with 50 μg of bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$; Molecular Probes, Eugene, OR, USA) [22]. Flow cytometric analysis was performed via a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

2.8. Flow cytometric analysis for morphological changes of C. albicans protoplasts

For the preparation of *C. albicans* protoplasts, cells were digested with a 10 mM phosphate buffer (pH 6.0) containing 1 M sorbitol, lysing enzyme (Sigma) (20 mg/ml), and cellulase (Sigma) (20 mg/ml) for 4 h at 28 °C by gentle agitation. The filtrated protoplasts were gathered by centrifugation at 1500 rpm for 10 min. The protoplasts were resuspended in a washing buffer (1 M sorbitol, 0.8 M NaCl, 10 mM $CaCl_2$, and 50 mM Tris–HCl, pH 7.5) and centrifuged [23,24]. The protoplasts, resuspended in YPD medium containing 1 M sorbitol, were treated with arenicin-1 or melittin at two times the MIC and incubated for 2 h at 28 °C with constant shaking (80 rpm). After incubation, the protoplast cells were harvested by centrifugation and suspended in PBS containing 1 M sorbitol. Subsequently, the cells were treated with PI (9 μM, final concentration) for 5 min. Flow cytometry was performed via a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

2.9. Measurement of plasma membrane fluorescence intensity

Fluorescence from the plasma membrane of *C. albicans* cells labeled by 1,6-diphenyl-1,3,5-hexatriene (DPH) (Molecular probes, Eugene, Oregon, U.S.A.) was used to monitor changes in membrane dynamics. Fungal cells (2×10^6/ml YPD) were treated with peptides, and incubated for 2 h at 28 °C. Samples of the fungal culture were fixed by 0.37% formaldehyde, collected and washed with a PBS, and the cells were frozen by liquid nitrogen. For labeling, cells were thawed with a PBS buffer and resuspended in PBS. The suspension was incubated with 0.6 mM DPH for 45 min at 28 °C, and this was followed by washing with a PBS buffer. The fluorescence intensity of DPH was measured by a Spectrofluorometer (Shimadzu RF-5301PC, Shimadzu, Japan) at 350 nm excitation and 425 nm emission wavelengths [25].

2.10. Calcein leakage measurement

Calcein-encapsulated large unilamellar vesicles (LUVs), composed of Phosphatidylcholine (PC)/Phosphatidylethanolamine (PE)/Phosphatidylinositol (PI)/ergosterol (5:4:1:2, w/w/w/w), were prepared by vortexing the dried lipid in a dye buffer solution (70 mM calcein, 10 mM Tris, 150 mM NaCl, and 0.1 mM EDTA, pH 7.4). The suspension was freeze–thawed in liquid nitrogen for 10 cycles and extruded

Table 1
The antifungal activity of arenicin-1.

Fungal strains	MIC (μM)	
	Arenicin-1	Melittin
C. albicans ATCC 90028	9	2.5
C. albicans TIMM 1768	9	2.5
C. parapsilosis ATCC 22019	4.5	2.5
M. furfur KCTC 7744	9	5
T. beigelii KCTC 7707	4.5	5
T. rubrum KCTC 6345	9	5

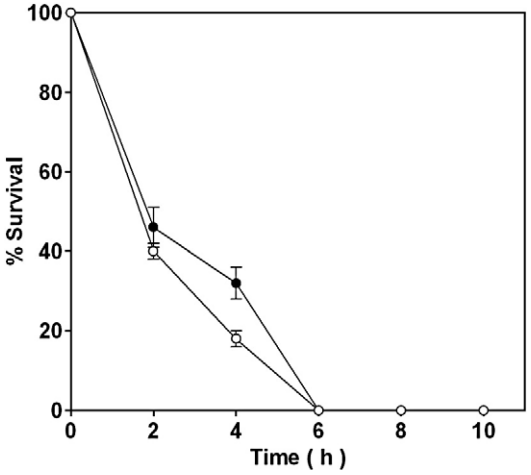

Fig. 1. Time-killing kinetics of arenicin-1 against *C. albicans*. *C. albicans* (2×10^6 cells/ml YPD) was incubated with arenicin-1 at 18 μM (●) or melittin at 9 μM (○). Viability was determined every 2 h by counting colony forming units (CFUs) and expressed as percentage of survivals. The error bars represent the standard deviation (S.D.) for three independent experiments, performed in triplicate.

through polycarbonate filters (two stacked 200 nm pore size filters) by a LiposoFast extruder (Avestin, Inc. Canada). Untrapped calcein was removed by gel filtration on a Sephadex G-50 column. The leakage of calcein from the LUVs was monitored by measuring the fluorescence intensity at an excitation wavelength of 490 nm and an emission wavelength of 520 nm on a Spectrofluorometer (Shimadzu RF-5301PC, Shimadzu, Japan). The measurements were performed at 25 °C. For determination of 100% of dye release, 1% Triton X-100 (30 μl) was added to dissolve the vesicles. The percentage of dye leakage, caused by the peptides, was calculated as follows: Dye leakage $(\%) = 100\times(F-F_0)/(F_t-F_0)$, where F represents the leaked fluorescence intensity achieved 2 min after addition of the peptides, and F_0 and F_t represent the fluorescence intensities without the peptides and with Triton X-100, respectively [26].

2.11. Preparation of GUVs

Giant unilamellar vesicles (GUVs) were prepared by the electroformation method, originally developed by Angelova and Dimitrov [27,28]. The lipid mixture solution (3.75 mg/mL) of PC/1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (18:1 Liss Rhod PE)/PI/ergosterol (5:4:1:2, w/w/w/w) dissolved in chloroform was spread onto a ITO (indium tin oxide) coated glass. The lipid-coated ITO glass was evaporated under a vacuum for 2 h. Both lipid-coated and uncoated glasses were held at a distance of 2 mm by a thin Teflon spacer. After filling the chamber with 10mM HEPES buffer solution (pH 7.4), a sinusoidal 1.7 Vpp (peak-to-peak) and 10 Hz frequency was applied for 2 h at room temperature. To detach the vesicles from the plate, a sinusoidal 4 Vpp and 4 Hz frequency was applied for 10 min. Ten microliters of GUV aliquots solution were observed on an inverted fluorescence phase-contrast microscope (Leica DFC 420C). Peptide solutions were added after the selection of a single GUV.

3. Results and discussion

3.1. Antifungal activity of arenicin-1

Arenicin-1 (RWCVYAYVRVRGVLVRYRRCW) is a 21-residue antibacterial peptide which was isolated from the coelomocytes of the marine polychaeta lugworm, *Arenicola marina* [8,9]. It has been reported to show potent antibacterial activity against bacterial strains [10], while its antifungal effects and mechanisms are poorly understood. To determine whether arenicin-1 possessed antifungal activity, we investigated the effects of the arenicin-1 towards various human pathogenic fungal strains. In this study, melittin (GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2), a 26 residue peptide which is the principal toxic component in the venom of the European honey bee *Apis mellifera*, was used as a reference for comparison with arenicin-1. Its lytic activity is intrinsic and powerful enough to exhibit potent hemolytic and antimicrobial activity [29].

The antifungal activity of arenicin-1 was determined by MTT assay [14]. The result showed that all fungal strains tested were highly susceptible to arenicin-1 with MIC values in 4.5–9 μM range (Table 1). Significantly, arenicin-1 exerted strong inhibitory effects towards the *Candida* species including *C. albicans*. *C. albicans* is the most prevalent systemic fungal pathogen of man and is associated with a range of clinical conditions ranging from irritating superficial infections of the oral and vaginal mucosa to life-threatening systemic disease in immunocompromised patients [30]. Considering these medical importance, *C. albicans* has been selected as a model organism in the following experiments.

In time-killing kinetics, the CFUs of the *C. albicans* cells rapidly decreased after treatment with arenicin-1 and the complete killing of *C. albicans* was observed after 6 h. As showing similar trend with respect to melittin, the killing of fungal cells by arenicin-1 was considerably fast (Fig. 1). These observations suggest that arenicin-1 possesses a significant fungicidal effect and rapid killing mechanism against *C. albicans*. Although the fungicidal effect of arenicin-1 was less potent than that of melittin, these results indicate that arenicin-1 has such a remarkable level of fungicidal effect as to have the potential to be considered as a candidate for drug therapy or as a template for drug design.

To further confirm the fungicidal activity of arenicin-1, the effect of arenicin-1 on the dimorphic transition in *C. albicans* was investigated. With respect to *C. albicans*, the dimorphism plays a crucial role in the pathogenesis, with mycelial shapes being predominantly found during a host tissue invasion [31]. As shown in Fig 2, the serum-

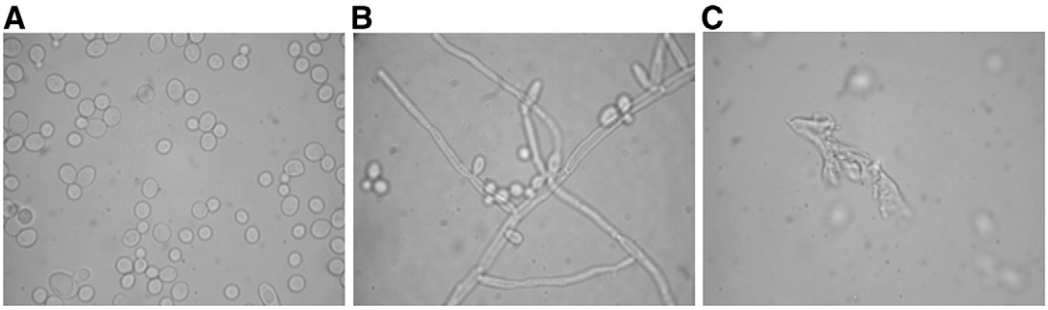

Fig. 2. The effect of arenicin-1 on the dimorphic transition in *C. albicans*. Each culture was incubated for 48 h in YPD medium with 20% fetal bovine serum (FBS). (A) Yeast control without FBS and arenicin-1, (B) Cells treated with only 20% FBS, (C) Cells treated with 20% FBS and 9 μM of arenicin-1.

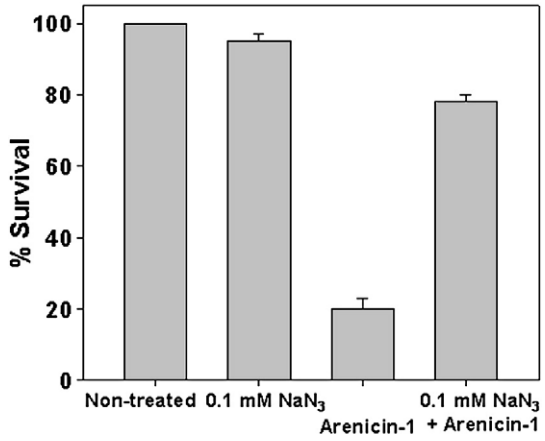

Fig. 3. The effects of NaN_3 on antifungal activity of arenicin-1. *C. albicans* (2×10^6 cells/ml YPD) was incubated with 0.1 mM NaN_3 for 1 h. After incubation, the cells treated with arenicin-1 at 18 μM. The cell population showed by MTT assay. The absorbance of each well was measured at 580 nm by using a Microtiter ELISA Reader (Molecular Devices Emax). The equation of cell viability percentage: [(the absorbance of the compound-treated cells) / (the absorbance of non-treated cells)] × 100. The error bars represent the standard deviation (SD) values for three independent experiments, performed in triplicate.

induced mycelia were significantly inhibited from being extended and destroyed in the presence of arenicin-1 (Fig. 2C), while the mycelia in the absence of arenicin-1 formed normally (Fig. 2B).

3.2. Effect of energy metabolism on antifungal activity of arenicin-1

It was further investigated as to whether the energy charge of *C. albicans* cells influences their susceptibility to arenicin-1. For this purpose, cells were incubated with arenicin-1 in the presence of 0.1 mM sodium azide, which inhibits metabolic ATP synthesis in mitochondria [32]. ATP depletion causes various changes in cell membranes by the regulation of intracellular pH [33]. As illustrated in Fig. 3, in the presence of the metabolic inhibitor which blocks both the conventional as well as the alternative respiratory pathways of *C. albicans* cells, a decrease of the antifungal activity was observed. This result suggests that the change of cellular physiological conditions, which poses a membrane rigidification by energy depletion [34], desensitizes *C. albicans* to arenicin-1. Conversely, this point lead us to speculation that the antifungal action site of arenicin-1 could be fungal plasma membrane, provided that membrane rigidification by energy depletion blocks the membrane-active effect, that is, insertion into and disruption or pore/ion channel formation in cytoplasmic membrane, of arenicin-1.

3.3. Interaction of arenicin-1 with fungal membranes

Naturally occurring antimicrobial peptides are the first step of self-defense against invading pathogenic microorganisms. Antimicrobial peptides take charge of the first-line defense system and possess several features adequate to their function. For rapid killing, the site of action should preferably be the cell surface rather than the cell interior. Also, the development of resistance is considered to be difficult if the target molecules are important components conserved among micro-organisms. To fulfill these requirements, many of the self-defense peptides act on the cytoplasmic membranes of microorganisms [35]. Therefore, initially, the effect of arenicin-1 on the membrane integrity was investigated by monitoring the influx of PI, a DNA-staining fluorescent probe, into *C. albicans* cells [36] (Fig. 4A). PI, a membrane-impermeant dye, only enters membrane compromised cells, after which the fluorescence of this probe is enhanced by 20–30-fold due to its binding to nucleic acids [37]. The treatment of *C. albicans* with arenicin-1 at two times the MIC caused an influx of PI into the fungal cells. This result indicates that arenicin-1 affects fungal cells by injuring their membranes, and thus increasing its permeability.

Based on models regarding the mode of actions of antimicrobial peptides [38,39], their initial interactions with microbial membranes

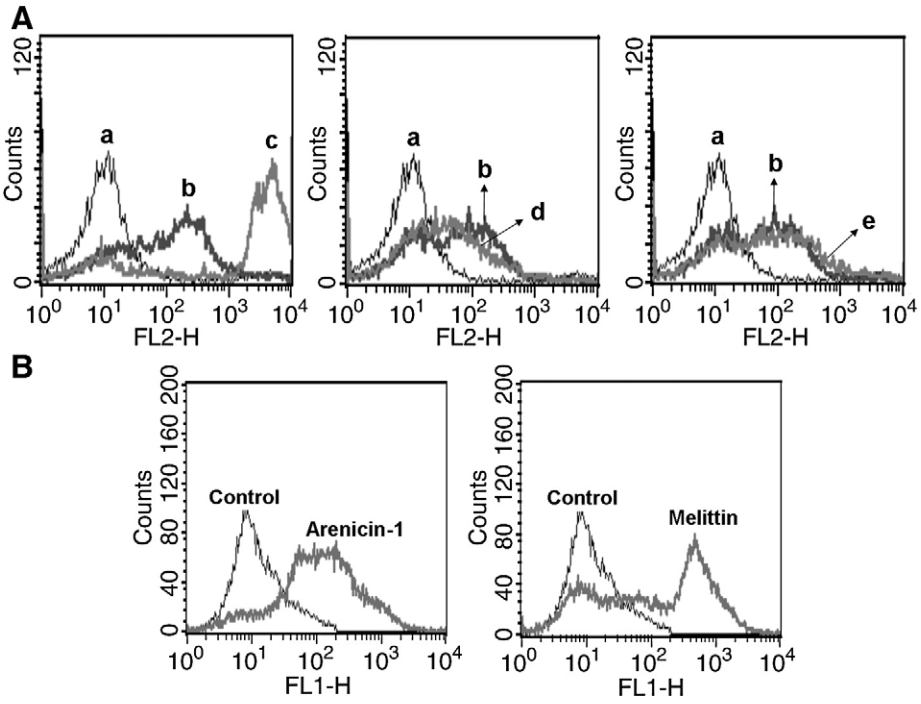

Fig. 4. Flow cytometric analysis of membrane permeablization assay and salt-sensitivity test by PI uptake and of $DiBAC_4(3)$ staining in *C. albicans*. (A) *C. albicans* (2×10^6 cells/ml YPD) was incubated with arenicin-1 or melittin for 2 h at 28 °C. (a) Control, (b) 18 μM arenicin-1, (c) 5 μM melittin, (d) 150 mM NaCl and 18 μM arenicin-1, (e) 3 mM $MgCl_2$ and 18 μM arenicin-1. The distributions of cells according to relative fluorescence intensities are given. (B) *C. albicans* (2×10^6 cells/ml YPD) was incubated for 3 h with arenicin-1 at 18 μM or melittin at 5 μM. The depolarization of the cell membrane was detected by the incubation of peptide-treated cells in $DiBAC_4(3)$. Histograms showed the fluorescence intensity of stained $DiBAC_4(3)$ after *C. albicans* were treated with peptides.

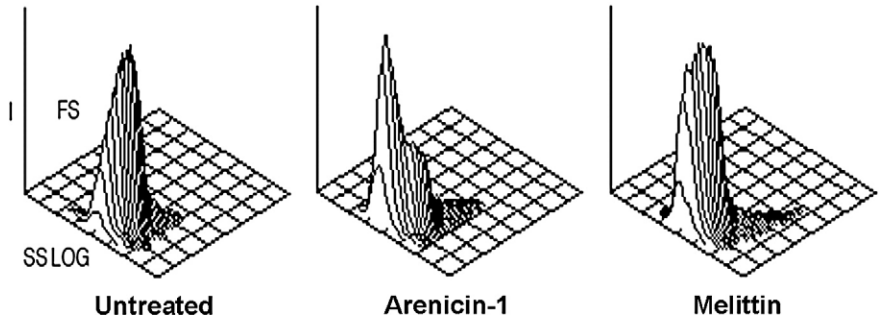

Fig. 5. Three-dimensional flow cytometric contour-plot analysis of *C. albicans* protoplasts treated with arenicin-1 at 18 μM or melittin at 5 μM. FS (*y*-axis) is an indicator of size and SS (90° scattering, SS LOG, *x*-axis) is an indicator of granularity. The *z*-axis represents the cellular population intensity.

are believed to be electrostatic. Thus, an increasing ionic strength under high salt conditions will likely weaken the electrostatic interactions between antimicrobial peptides and microbial membrane, thereby reducing the antimicrobial activity [40]. However, the result showed that the antifungal activity of arenicin-1 against *C. albicans* was still retained at physiological salt concentration and even at higher concentration (150 mM NaCl, 3 mM $MgCl_2$) (Fig. 4A). This result suggests that electrostatic interaction does not have a role in maintaining antifungal activity of arenicin-1. As Tam et al. indicated, the structural characteristics of arenicin-1 containing a monocluster of cationic residues possibly contribute to the retention of membrane-active antifungal activity under high salt condition [41].

To further assess as to whether arenicin-1 can affect the functions of the fungal plasma membrane, its ability to dissipate the membrane potential of *C. albicans* was investigated. In the presence or absence of peptides, the amounts of accumulated $DiBAC_4(3)$ in *C. albicans* cells were analysed with flow cytometry. $DiBAC_4(3)$ has a high voltage sensitivity and only can enter depolarized cells, in which it binds to lipid-rich intracellular components [22]. Cells treated with arenicin-1 caused the accumulation of $DiBAC_4(3)$ with respect to peptide-untreated cells (Fig. 4B), suggesting that arenicin-1 damages the membrane and induces the membrane depolarization of fungal cells.

The morphological effects of arenicin-1 on *C. albicans* were investigated by flow cytometric analysis plotting the forward scatter and side scatter of treated and untreated cells. In particular, the protoplasts of *C. albicans* were utilized to confirm the effect of arenicin-1 at the level of fungal cytoplasmic membrane. As shown in Fig. 5, in the absence of peptides, a homogeneous population of undamaged cells was observed. In the presence of arenicin-1 at two times the MIC, a population of damaged and permeabilized cells became dominant. The results showed that arenicin-1 and melittin permeabilized fungal membrane, leading to a similar necrotic effect, characterized by increased an PI uptake and decreased FS in regards to flow cytometric experiments [42].

The fundamental architecture of biomembranes is the lipid bilayer. Its self-sealing property as well as its fluidity creates an ideal structure for the cellular membrane [43]. To elucidate membrane fluidity by arenicin-1, changes in membrane dynamics were examined with a fluorescent membrane probe, DPH. DPH is a hydrophobic molecule, and this characteristic enables it possible to associate with the hydrocarbon tail region of phospholipids, within the cytoplasmic membrane, without disturbing the structure of the lipid bilayer [44,45]. If the antifungal activity exerted by arenicin-1 on *C. albicans* is at the level of the lipid bilayer, then the DPH, which interacts with an acyl group of the lipid bilayer within the plasma membrane, could not be inserted into the membrane. The results showed that increasing peptide concentrations significantly decreased the plasma membrane DPH fluorescence intensity (Fig. 6). This DPH fluorescence intensity reflects the fungicidal effect of arenicin-1 against *C. albicans* cells, by perturbing the plasma membrane. This also suggests that the fungal plasma membrane is structurally perturbed when exposed to arenicin-1.

An artificial lipid bilayer was utilized to further characterize the capacity of arenicin-1 to disrupt the fungal plasma membrane. To determine whether arenicin-1 could cause the leakage of calcein from

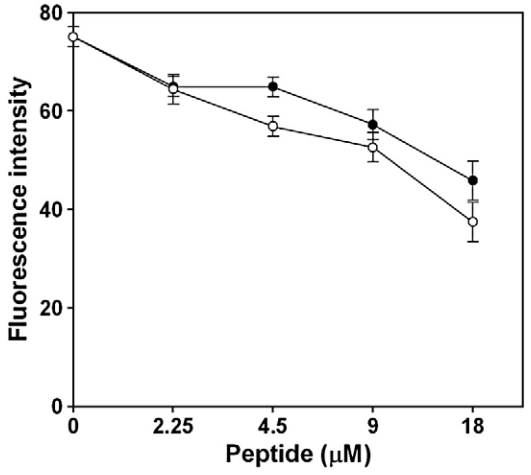

Fig. 6. DPH fluorescence analysis after addition of various concentrations of arenicin-1 (●) or melittin (○). The error bars represent the standard deviation (SD) values for three independent experiments, performed in triplicate.

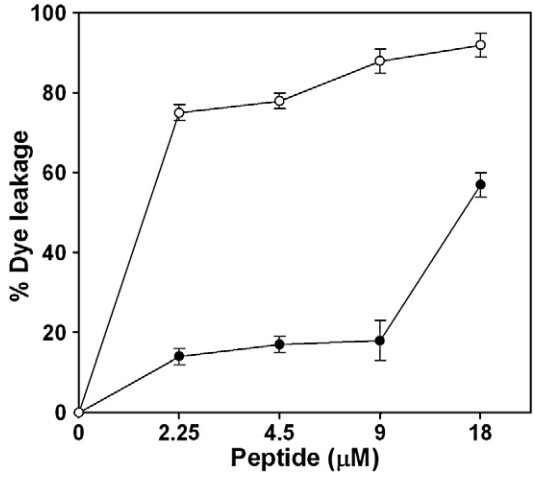

Fig. 7. Percent leakage of fluorescent dye from calcein-entrapped LUVs, composed of PC/PE/PI/ergosterol (5:4:1:2, w/w/w/w), in the presence of arenicin-1 (●) or melittin (○). The percentage of dye leakage caused by the peptides was calculated as follows: Dye leakage (%) = $100 \times (F - F_0)/(F_t - F_0)$, where F is the leaked fluorescence intensity achieved 2 min after addition of the peptides, and F_0 and F_t are the fluorescence intensities without the peptides and with Triton X-100, respectively.

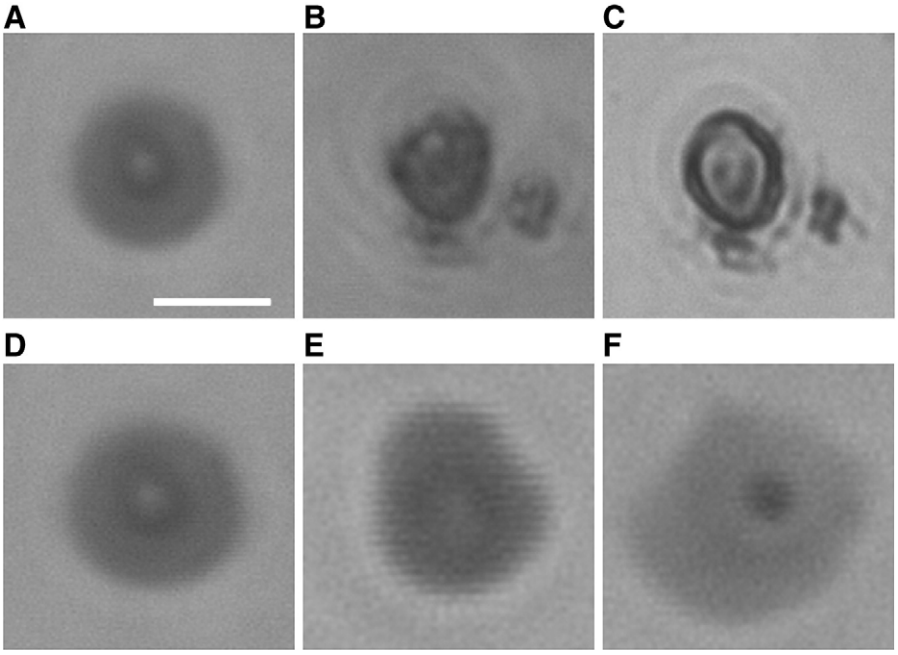

Fig. 8. The response of the single GUV composed of PC/PE/PI/ergosterol (5:4:1:2, w/w/w/w) to the treatment with peptides. For arenicin-1, (A–C) were obtained at 0, 3, and 5 min. respectively. For melittin, (D–F) were obtained at 0, 0.1, and 1 min, respectively. The scale bar represents 10 μm.

model membranes, the membrane study using a suspension of large unilamellar vesicles (LUVs) having a phospholipid composition resembling the bilayer of *C. albicans* composed of 5:4:1:2 (w/w/w/w) PC/PE/PI/ergosterol [46] was conducted. Melittin caused a complete leakage of calcein from the vesicles at 9 μM, whereas 18 μM of arenicin-1 induced a 60% leakage of calcein from LUVs (Fig. 7). The leakage potency of arenicin-1 supports that the lipid bilayer within the plasma membrane is the presumptive fungicidal action site for arenicin-1.

The observation of the morphological changes of a single giant unilamellar vesicle (GUV) responding to peptides offered additional information about the membrane-active mechanism of arenicin-1. Due to their size, the GUVs are a suitable model with respect to cell membranes and, furthermore, permit direct observations of the vesicle–peptide interaction [47–49]. A single yeast size-like GUV which resembles the phospholipid bilayer of *C. albicans* composed of 5:4:1:2 (w/w/w/w) PC/PE/PI/ergosterol [46] was selected and then treated with peptides. The shape of a selected single vesicle was monitored continuously by phase-contrast microscopy. The observed response of each single vesicle to peptides was different (Fig. 8). When treated with melittin, one side of a GUV was rapidly protruded and burst to cause complete lysis and disintegration of membrane, thereby leaving a small lump of lipid membrane (Fig. 8D–F). The lag phase (10 s) was remarkably short and a complete response was concluded within 1 min. On the other hand, a single GUV treated with arenicin-1 slightly crumpled and diminished in size (Fig. 8A–C). After an initial lag phase of 2 min, the single GUV began to shrink and the whole response of it gradually processed for 5 min from start to finish. The lag phase probably indicates that arenicin-1 accumulates on the membrane until a critical concentration is reached, after which the peptides may cooperatively induce membrane destruction [50]. These observations demonstrate that the major target site of arenicin-1 is the fungal membrane and it is thought that arenicin-1 contains membrane-active mechanism(s).

In summary, the fungicidal effect and its mode of action of arenicin-1 were investigated. Although the exact mechanisms(s) at the molecular level are not completely understood, this study suggests that arenicin-1 is likely to exert fungicidal effect by disturbing fungal membrane. Therefore, it can be concluded that understanding fungicidal effect and these modes of action of arenicin-1 may help in the design of more potent as well as more specific antimicrobial peptides.

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