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Dimerization of cell-penetrating buforin II enhances antimicrobial properties

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Abstract

Antimicrobial peptides (AMPs) that selectively permeabilize bacterial membranes are promising alternatives to conventional antibiotics. Dimerization of AMP is considered an attractive strategy to enhance antimicrobial and membrane-lytic activity, but it also increases undesired hemolytic and cytotoxic activity. Here, we prepared Lys-linked homodimers of membrane-permeabilizing magainin II and cell-penetrating buforin II. Dimerization did not significantly alter conformational behavior, but it had a substantial impact on antimicrobial properties. We found that while the magainin II dimer showed increased antimicrobial and cytotoxic effects, the buforin II dimer conferred much greater antibacterial potency without exhibiting cytotoxic activity. Interestingly, the buforin II dimer was highly effective against several antibiotic-resistant bacterial isolates. Membrane permeabilization experiments indicated that the magainin II dimer rapidly disrupted both anionic and zwitterionic membranes, whereas the buforin II dimer selectively disrupted anionic membranes. Like the monomeric form, the buforin II dimer was efficiently translocated across lipid bilayers. Therefore, our results suggest that the dimerization of cell-penetrating buforin II not only disrupts the bacterial membrane, but also translocates it across the membrane to target intracellular components, resulting in effective antimicrobial activity. We propose that dimerization of intracellular targeting AMPs may present a superior strategy for therapeutic control of pathogenic bacteria.

Keywords: Antimicrobial peptide, Cell-penetrating peptide, Buforin II, Dimerization, Peptide-membrane interaction

Introduction

Antimicrobial peptides (AMPs) are ubiquitous among unicellular and multicellular organisms and are responsible for first-line host defenses against invading pathogens (Haney et al. 2019; Hilchie et al. 2013; Zhang and Gallo 2016). Resistance to AMP remains at very low levels, making it a promising candidate to overcome antibiotic-resistant bacterial infections (Biswaro et al. 2018; de Breij et al. 2018; Kumar et al. 2018; Lohner 2016; Mahlapuu et al. 2016). AMPs of multicellular organisms have a killing and/or inhibitory effect on a wide range of microorganisms, including gram-positive and gram-negative bacteria. It is widely accepted that most AMPs kill bacterial cells by permeabilizing the negatively charged cytoplasmic membrane (Guha et al. 2019; Matsuzaki 2019; Sani and Separovic 2016; Stone et al. 2019). In contrast to the membrane-lytic mechanism, it has been proposed that some AMPs act intracellularly by binding to DNA or altering enzyme activity (Le et al. 2017; Lee et al. 2019). For example, magainin II is a well-known membrane-permeabilizing antimicrobial peptide, whereas buforin II exerts potent antimicrobial activity without causing membrane lysis (Imura et al. 2008; Takeshima et al. 2003). Indeed, previous confocal fluorescence microscopic studies showed that magainin II is localized to the bacterial surface, whereas buforin II accumulates mainly in the cytoplasm (Park et al. 2000).

The self-association of membrane-active peptides appears to be a crucial parameter for antimicrobial mode of action in either membrane permeabilization or peptide translocation across the membrane to target intracellular components (Petkov et al. 2019; Yang et al. 2006a). Many studies have shown that the dimerization

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of membrane-permeabilizing peptides significantly changes their biological and biophysical properties (Gunasekera et al. 2020; Lorenzon et al. 2019). For example, parallel and antiparallel magainin II dimers have greater biological activity and greater ability to interact with membranes than the monomeric form does (Mukai et al. 2002). The pore formed by the magainin II dimer is characterized by a larger pore diameter and longer lifetime than that of the monomer (Hara et al. 2001). Although dimerization of AMPs may be a promising strategy to improve antimicrobial potency, dimerization of membrane-permeabilizing AMPs increases cytotoxicity against mammalian cells (Gunasekera et al. 2020). These unwanted cytotoxic effects should be minimized or eliminated. On the other hand, although dimerization of cell-penetrating peptides leads to enhanced cellular uptake and drug delivery (Hoyer et al. 2012), little is known about their antimicrobial properties.

In this study, we synthesized membrane-permeabilizing magainin II, cell-penetrating buforin II, and their counterpart homodimeric peptides with a branched Lys core; sequences are listed in Table 1. We tested their antibacterial activities against gram-positive and gram-negative bacteria as well as cytotoxicity against mammalian cells, and we evaluated the roles of dimerization in their antimicrobial properties. Interactions of the peptides with the lipid bilayer were investigated by circular dichroism and fluorescence spectroscopy in membrane mimetic environments. We showed that the Lys-linked buforin II dimer exerted greater antibacterial potency against both gram-positive and gram-negative bacteria, including several antibiotic-resistant bacterial isolates, without inducing cytotoxicity against mammalian cells. Our findings indicated that both membrane-permeabilizing and cell-penetrating abilities of the buforin II dimer seem to influence more efficient and selective killing of pathogenic bacteria.

Materials and methods

Materials and microorganisms

N-α-fluoren-9-yl-methoxycarbonyl (Fmoc) amino acids with orthogonal side chain protecting groups and Fmoc-Lys(Fmoc)-OH (for the dimeric peptides) were purchased from Novabiochem (Läufelfingen, Switzerland).

Table 1 Amino acid sequences and cytotoxic activities of peptides used in this study

Peptides	Amino acid sequences	HC_{50}[a]	LC_{50}[b]
Magainin II	GIGKWLHSAKKFGKAFVGEIMNS	> 200	> 200
K-(magainin II)$_2$	(GIGKWLHSAKKFGKAFVGEIMNS)$_2$K	14.3	2.7
Buforin II	TRSSRAGLQWPVGRVHRLLRK	> 200	> 200
K-(buforin II)$_2$	(TRSSRAGLQWPVGRVHRLLRK)$_2$K	> 200	> 200

[a]Peptide concentration (μM) causing 50% hemolysis
[b]Peptide concentration (μM) causing 50% growth inhibition of RAW 264.7 cells

For peptide synthesis, reagents and solvents were purchased from Applied Biosystems (Foster City, CA, USA). After RP-HPLC purification (above 99% purity), the correct molecular weights were confirmed by MALDI-TOF-MS (Shimadzu, Japan). Phospholipids were obtained from Avanti Polar Lipids (Alabaster, AL, USA). A membrane potential-sensitive fluorescent dye, 3,3′-dipropylthiadicarbocyanine iodide [DiSC3(5)] was purchased from Molecular Probes, Inc. (Eugene, OR, USA). Microorganisms were purchased from the Korean Collection for Type Cultures (Daejon, Korea).

Circular dichroism (CD) spectroscopy

The CD spectra of peptides were collected with a J-715 spectrophotometer (Jasco, Japan) as previously described (Yang et al. 2019). The spectra are expressed as the mean residue ellipticity [θ] versus wavelength. The ellipticity [θ] given in units of deg·cm^2·dmol^{-1} was calculated using the following formula: [θ] = [θ]obs MRW/10lc, where MRW = mean residue molecular weight of the peptide, c is the concentration of the sample, and l is the length of the cell.

Antimicrobial, hemolytic, and cytotoxic activity

The antimicrobial activity of peptides against gram-positive and gram-negative bacteria, including antibiotic-resistant pathogens, was measured by the broth microdilution method, as previously described (Lee et al. 2019). Briefly, single colonies of bacteria cultured on a LB agar plate were inoculated into LB medium and incubated overnight at 37 °C. A 50 μl of this culture was transferred into fresh tube of 10 ml LB medium and grown to mid-log phase. A set of serial dilutions of peptides (100 μl) were added to 100 μl of 2×10^6 CFU/ml in 96-well microtiter plates (Falcon). After incubation at 37 °C for 16 h, the minimal inhibitory concentration (MIC) was defined as the lowest peptide concentration that completely inhibited bacterial growth by measuring optical density (OD) at 600 nm. The MICs were the average of triplicate measurements in three independent assays. The hemolytic activities of peptides were determined by monitoring the release of hemoglobin from human red blood cells, as described previously (Yang et al. 2019). Complete hemolysis was induced by 0.1% Triton X-100. Cytotoxicity of peptides against RAW 264.7 cells was measured with a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, as previously described (Yang et al. 2019). Cell viability was determined as the ratio of the absorbance at 570 nm for peptide-treated cells compared to untreated cells.

Membrane depolarization induced by peptides

The membrane depolarization activities of peptides were determined using a membrane potential-sensitive

fluorescent dye DiSC3(5), as previously described (Yang et al. 2006b). Briefly, *Staphylococcus aureus* cells cultured to mid-log phase were resuspended in 5 mM HEPES buffer. Upon addition of DiSC3(5) to the suspension of *S. aureus*, the fluorescence had quenched and stabilized. Addition of peptides (2 mM) increased the fluorescence intensity due to membrane depolarization. After 5 min, gramicidin D was added to observe the maximum fluorescence intensity due to complete dissipation of the membrane potential. The DiSC3(5) fluorescence change was monitored on a Shimadzu RF-5301 spectrofluorometer at an excitation/emission wavelength of 620/670 nm.

Membrane disruption induced by peptides

Large unilamellar vesicles (LUVs) were generated by the extrusion method, and the membrane disruption induced by peptides was determined using the fluorescent dye calcein, as previously described (Lee et al. 2019). Briefly, peptides were added to calcein-containing LUVs composed of anionic PC/PG (1:1) or zwitterionic PC. Membrane-lytic activity of peptides was defined as the percent leakage from 100 μM LUVs after 10 min incubation with peptides (2 μM for PC/PG (1:1) and 20 μM for PC vesicles). Fluorescence changes due to calcein release from LUVs were monitored on a Shimadzu RF-5301 spectrofluorometer at excitation/emission wavelength = 490/520 nm. Triton X-100 was added in order to show the maximum fluorescence intensity due to complete membrane permeability.

Ability of peptides to translocate into vesicles

LUVs were generated in HEPES buffer (150 mM NaCl, pH 7.4) with chymotrypsin (200 mM), as described previously (Lee et al. 2019). External chymotrypsin was inactivated by the addition of a trypsin-chymotrypsin inhibitor (200 mM). Fluorescence transfer from the Trp residue of peptides to the dansyl group on membranes by peptide translocation into vesicles was monitored on a Shimadzu RF-5301 spectrofluorometer at an excitation/emission wavelength of 280/510 nm.

Results

Circular dichroism (CD) studies

CD spectroscopy was used to investigate structural differences between monomeric and dimeric peptides in aqueous buffer as well as in membrane-mimetic environments (Fig. 1). In aqueous buffer, all peptides had almost completely random coil structures. In contrast, both monomeric and dimeric magainin II assumed typical α-helices in membrane-memetic SDS micelles, as characterized by double minima around 208 and 222 nm. Compared to membrane-permeabilizing magainin II, cell-penetrating buforin II showed a negative peak at 204 nm, indicating a somewhat different structure from a typical α-helix. However, the overall CD spectral patterns of the monomers were similar to those of dimers, suggesting that dimerization did not significantly alter the structure of the peptides.

Antimicrobial and hemolytic activities of the peptides

We next determined the ability of peptides to inhibit bacterial growth by measuring their MIC, which are listed in Table 2. The magainin II dimer and the buforin II dimer showed increased antibacterial activity by 4–8 and 8–16 fold, respectively, compared to their respective monomers. In addition, Table 3 shows that the dimers

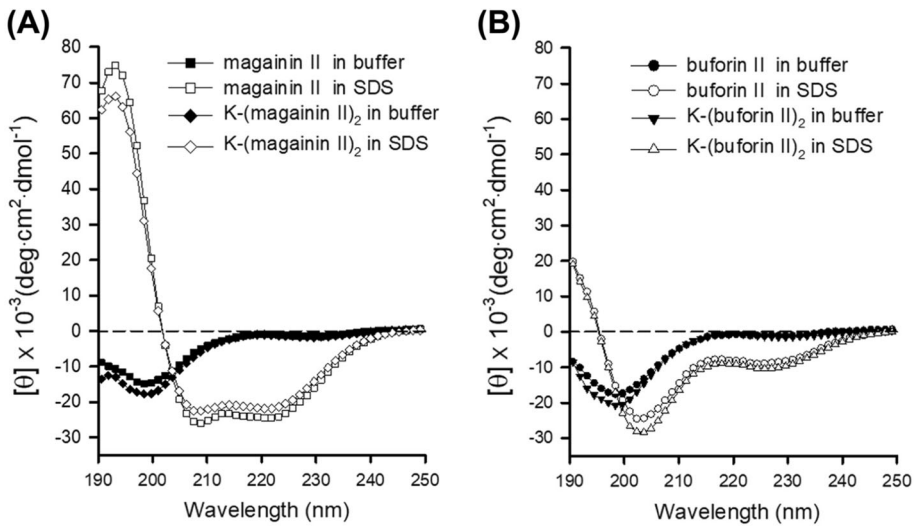

Fig. 1 CD spectra of the peptides. The CD spectra of magainin II and K-(magainin II)$_2$ (**a**) and buforin II and K-(buforin II)$_2$ (**b**) were obtained at 25 °C in 10-mM sodium phosphate buffer (open symbols) or 30 mM SDS micelles (closed symbols). Spectra were taken at peptide concentrations of 20 μM

Table 2 Antimicrobial activities of peptides against gram-positive and gram-negative bacteria

Organism	Antimicrobial activity (MIC: μM)			
	Magainin II	K-(magainin II)$_2$	Buforin II	K-(buforin II)$_2$
Gram-positive bacteria				
Bacillus subtilis	8	2	8	0.5
Staphylococcus aureus	16	2	8	1
Staphylococcus epidermidis	16	4	16	2
Gram-negative bacteria				
Escherichia coli	32	4	8	1
Salmonella Typhimurium	16	2	4	0.5
Pseudomonas aeruginosa	32	4	16	2

had strong antimicrobial activity, in the range of 0.5–4 μM, against antibiotic-resistant pathogens. These results suggest that not only the dimerization of membrane-permeabilizing magainin II, but also the dimerization of cell-penetrating buforin II, is a good way to enhance antimicrobial activity. We also evaluated the hemolytic (HC_{50}) and cytotoxic (LC_{50}) activity against human red blood cells and RAW 264.7 cells, respectively, which are presented in Table 1. Both monomeric magainin II and buforin II showed negligible hemolytic and cytotoxic activities. Interestingly, while magainin II dimer showed strong hemolytic and cytotoxic activity, buforin II dimer was not hemolytic or cytotoxic. These results indicate that dimerization of cell-penetrating buforin II improves selectivity for bacteria over mammalian cells much better than dimerization of membrane-permeabilizing magainin II.

Membrane depolarization by peptides

To determine whether membrane depolarization is related to bacterial death, we next determined the ability of monomeric and dimeric peptides to depolarize the cytoplasmic membrane of *S. aureus* using the membrane potential-sensitive fluorescent dye DiSC3(5). As shown in Fig. 2a, monomeric magainin II effectively depolarized the cytoplasmic membrane of *S. aureus* at the value lower than MIC; however, buforin II did not induced membrane depolarization even at the MIC value. Although there was no direct correlation between cytoplasmic membrane depolarization and MIC values, these results support that magainin II exerts antimicrobial activity by inducing membrane depolarization, whereas the potent antimicrobial activity of buforin II is not related to the membrane depolarization. Both monomeric and dimeric magainin II efficiently dissipated the membrane potential of *S. aureus*, although the magainin II dimer induced more effective membrane depolarization than its monomer. Intriguingly, the buforin II monomer showed negligible membrane depolarization ability, whereas the buforin II dimer dissipated over 50% of membrane potential. These results suggest that the additional ability of buforin II dimer to induce membrane depolarization appears to be associated with potent antimicrobial activity.

Dye leakage from liposomes

To determine whether peptides were able to permeabilize membranes, we examined the ability of peptides to disrupt anionic vesicles composed of L-α-phosphatidylcholine (PC) and L-α-phosphatidylglycerol (PG) (1:1), as well as zwitterionic vesicles composed of PC by monitoring the efflux of fluorescent dye from the vesicles (Fig. 2b). Consistent with their respective abilities to depolarize bacterial membranes, magainin II showed strong calcein release from the PC/PG (1:1) vesicles with 50% leakage at 2 μM, whereas buforin II showed no membrane-lytic activity. These results support the mode of action that membrane disruption represents a major killing event for magainin II, but not for buforin II. When added to PC liposomes, both magainin II and buforin II elicited no calcein release, even at 20 μM, which agrees with results obtained for hemolytic

Table 3 Antimicrobial activities of peptides against antibiotic-resistant bacterial isolates

Antibiotic-resistant bacteria	Antimicrobial activity (MIC: μM)			
	Magainin II	K-(magainin II)$_2$	Buforin II	K-(buforin II)$_2$
Methicillin-resistant *Staphylococcus aureus* (1) [MRSA (1)]	16.0	4.0	8.0	1.0
Methicillin-resistant *Staphylococcus aureus* (2) [MRSA (2)]	16.0	2.0	8.0	0.5
Vancomycin-resistant *Enterococcus faecalis* (1)	32.0	4.0	16.0	1.0
Vancomycin-resistant *Enterococcus faecalis* (2)	16.0	4.0	8.0	1.0

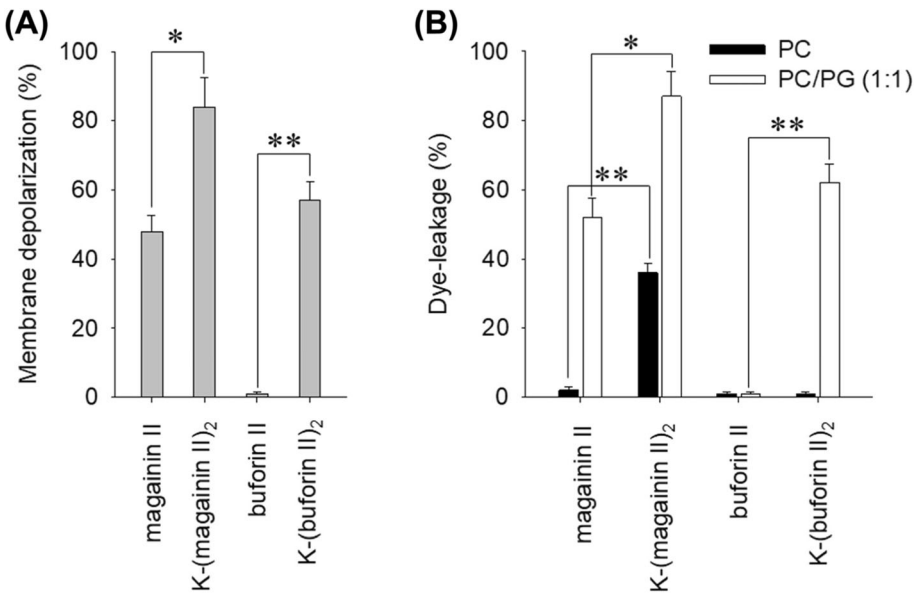

Fig. 2 Ability of peptides to permeabilize membranes. **a** Effect of peptides on membrane potential of intact *S. aureus* cells (OD600 = 0.05). After the fluorescence of DiSC3(5) was stabilized, the peptides (2 μM) were added to *S. aureus*. **b** Release of calcein fluorescent probe from anionic PC/PG (1:1) or zwitterionic PC vesicles. Membrane-lytic activity of peptides was defined as the percent leakage from 100 μM lipid after 10 min incubation with peptides (2 μM for PC/PG (1:1) and 20 μM for PC vesicles). Average ± SD for three independent experiments. Statistically significant ($p < 0.05$) and very significant ($p < 0.01$) differences are represented by (*) and (**), respectively

and cytotoxic activity. Dimerization of magainin II strongly increased the membrane-lytic activity against both anionic and zwitterionic liposomes. Interestingly, the buforin II dimer had relatively strong membrane-lytic activity (~60%) against anionic liposomes, but did not show any dye leakage from PC liposomes. These findings indicate that dimerization of membrane-permeabilizing magainin II non-selectively increases membrane-lytic ability between negatively charged and neutral membranes, whereas cell-penetrating buforin II dimer leads to selective membrane-disrupting activity only for anionic membranes. Although membrane-lytic activity of peptides was related to their respective antimicrobial activity, these variables were not entirely linear.

Ability of peptides to translocate into liposomes

To determine whether a peptide can enter a cell, we examined the ability of peptides to translocate across lipid bilayers (Fig. 3). The extent of peptide translocation across PC/PG/dansyl-PE (50:45:5) vesicles was estimated by monitoring peptide degradation by chymotrypsin encapsulated within vesicles using a fluorescence resonance energy transfer system with a Trp donor and dansyl acceptor. We observed no fluorescence decrease throughout the 500 s with magainin II, indicating a complete lack of membrane translocation. In contrast, buforin II caused a time-dependent decrease in fluorescence intensity, indicating that the peptide effectively crossed the lipid bilayer and entered vesicles. These results are in agreement with previously published translocation data. Similar to monomer results, the buforin II dimer translocated more effectively and rapidly across the membrane than the magainin II dimer. These results suggest that both monomeric and dimeric buforin II can cross the bacterial membrane and target intracellular molecules.

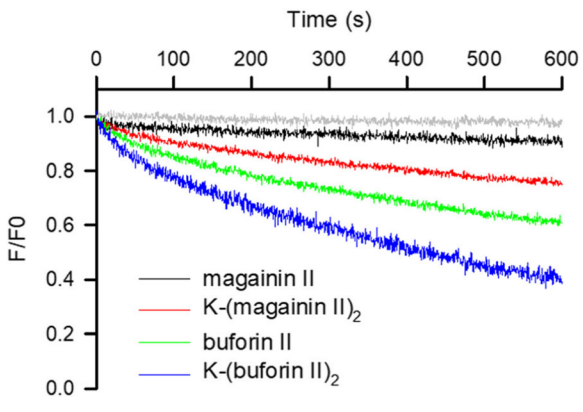

Fig. 3 Ability of peptides to translocate into liposomes. Membrane translocation of peptides was measured by resonance energy transfer from Trp to DNS-PE. Decreased fluorescence means that the peptide has entered the vesicle. Lipid and peptide concentrations were 100 and 1 μM, respectively

Discussion

Because membrane-active molecules such as antimicrobial and cell-penetrating peptides have attracted attention for therapeutic applications, there is currently much work underway in order to understand the basic principles of peptide interaction with target cell membranes (Avci et al. 2018; Henriques et al. 2006). They generally share cationic and amphipathic characteristics, which play important roles in membrane permeabilization and/or cell penetration. Since peptide self-assembly affects membrane permeabilization and cell penetration, several groups have investigated the different structural and biological functions between monomeric and dimeric forms (Hoyer et al. 2012; Lorenzon et al. 2019). It is particularly interesting that dimerization of AMPs leads to greater antibiotic activity against several bacterial species (Koh et al. 2018; Liu et al. 2017; Lorenzon et al. 2018; Panteleev et al. 2017; Thamri et al. 2017). Improved antibacterial activity is correlated with an increase in membrane permeabilization, indicating that bacterial membranes are primarily targeted by dimers. However, dimerization of AMP with membrane-permeabilizing modes of action leads to increased cytotoxicity against mammalian cells, which may hinder therapeutic application (Gunasekera et al. 2020; Lorenzon et al. 2019). Indeed, we showed that membrane-permeabilizing magainin II dimer displayed potent antibacterial activity, as well as strong cytotoxicity against mammalian cells. Our results also demonstrate that the improved antibacterial and cytotoxic activity of the magainin II dimer is associated with strong membrane-lytic activity against anionic and zwitterionic membranes, respectively. In contrast, the membrane-penetrating buforin II dimer had potent broad-spectrum antimicrobial activity, though it was not cytotoxic to mammalian cells. Similar to previously reported results (Park et al. 2000), the buforin II monomer inhibited bacterial cell growth without causing membrane disruption. However, the buforin II dimer has the ability to depolarize bacterial membranes and permeabilize anionic membranes. In addition, the buforin II dimer can translocate across lipid membranes. These results, using both biological and artificial membranes, indicate that the buforin II dimer directly collapses the cytoplasmic membrane potential and also interferes with intracellular targets, which may result in more potent antibacterial activity.

In summary, dimerization was applied to membrane-permeabilizing magainin II and membrane-penetrating buforin II. Magainin II dimer increased both antibacterial and cytotoxic effects, whereas the buforin II dimer enhanced antibacterial activity without cytotoxicity. Interestingly, the buforin II dimer had the ability to depolarize the bacterial membrane and to effectively translocate across lipid bilayers to target intracellular components. Because of the wide range of antimicrobial activity against gram-positive and gram-negative bacteria, including antibiotic-resistant bacterial isolates, the buforin II dimer has promising potential in applications for therapeutic control of multi-resistant pathogenic bacteria. Although more studies are needed to elucidate the exact mode of action of the buforin II dimer, dimerization of intracellular targeting AMPs may be a promising strategy to improve antimicrobial activity while reducing cytotoxic activity against mammalian cells.

Abbreviations

AMP: Antimicrobial peptide; RP-HPLC: Reversed-phase high-performance liquid chromatography; MALDI-TOF: Matrix-assisted laser desorption ionization–time of flight; CD: Circular dichroism; MIC: Minimal inhibitory concentration; LUV: Large unilamellar vesicle; SDS: Sodium dodecyl sulfate; PC: L-α-phosphatidylcholine; PG: L-α-phosphatidylglycerol; PE: L-α-phosphatidylethanolamine

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Authors' contributions

H Lee and S Yang designed research. H Lee performed most experiments. The authors analyzed data and wrote the paper. The authors read and approved the final manuscript.

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Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests.

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