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Novel short antibacterial and antifungal peptides with low cytotoxicity: Efficacy and action mechanisms

Xiaobao Qi [a], Chuncai Zhou [a], Peng Li [a], Weixin Xu [b], Ye Cao [a], Hua Ling [a], Wei Ning Chen [a], Chang Ming Li [a], Rong Xu [a], Mouad Lamrani [c], Yuguang Mu [b,*], Susanna Leong [a], Matthew Wook Chang [a,**], Mary B. Chan-Park [a,***]

[a] School of Chemical and Biomedical Engineering, Nanyang Technological University, 62 Nanyang Drive, 637459 Singapore, Singapore
[b] School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, 637551 Singapore, Singapore
[c] Menicon Co., France

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ABSTRACT

Short antimicrobial peptides with nine and eleven residues were developed against several clinically important bacterial and fungal pathogens (specifically *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Candida albicans*, and *Fusarium solani*). Twelve analogues of previously reported peptides BP76 (KKLFKKILKFL) and Pac-525 (KWRRWVRWI) were designed, synthesized, and tested for their antimicrobial activities. Two of our eleven amino acid peptides, P11-5 (GKLFKKILKIL) and P11-6 (KKLIKKILKIL), have very low MICs of 3.1–12.5 μg ml^{-1} against all five pathogens. The MICs of these two peptides against *S. aureus*, *C. albicans* and *F. solani* are four to ten times lower than the corresponding MICs of the reference peptide BP76. P9-4 (KWRRWIRWL), our newly designed nine-amino acid analogue, also has particularly low MICs of 3.1–6.2 μg ml^{-1} against four of the tested pathogens; these MICs are two to eight times lower than those reported for Pac-525 (6.2–50 μg ml^{-1}).These new peptides (P11-5, P11-6 and P9-4) also exhibit improved stability in the presence of salts, and have low cytotoxicity as shown by the hemolysis and MTT assays. From the results of field-emission scanning electron microscopy, membrane depolarization and dye-leakage assays, we propose that these peptides exert their action by disrupting membrane lipids. Molecular dynamics simulation studies confirm that P11-6 peptide maintains relatively stable helical structure and exerts more perturbation action on the order of acyl tail of lipid bilayer.

1. Introduction

The emergence of antibiotic-resistant pathogens has led to enormous efforts to develop new classes of antibiotics [1,2]. As a potential new class, antimicrobial peptides (AMPs) possess attractive properties in comparison with conventional antibiotics, including limited propensity to induce resistance in pathogens, diverse compositions, broad spectrum activity and rapid onset of killing [3]. Despite these properties, the use of peptides in antimicrobial applications is presently limited by their inadequate efficacy, excessive cytotoxicity, and high cost of synthesis [4]. The latter is attributed to the fact that AMPs are typically up to thirty amino acids in length. Short AMPs are particularly attractive since their synthesis cost would be low [5]. However, few short AMPs have been reported [6,7] and they are usually cytotoxic. Here we report short peptides, containing nine or eleven amino acids, which are highly effective against clinically important bacterial and fungal pathogens, specifically *Escherichia coli* (ATCC8739), *Pseudomonas aeruginosa* (ATCC9027), *Staphylococcus aureus* (ATCC6538), *Candida albicans* (ATCC10231), and *Fusarium solani* (ATCC36031). We designed 12 peptide analogues of Pac-525 and BP76, which have nine and eleven amino acids, respectively, by substituting different amino acids at specific sites (Table 1) to achieve specific properties in the resulting peptides [8,9].

2. Materials and methods

2.1. Antimicrobial peptides

All peptides listed in Table 1 were synthesized using the solid-phase method and standard 9-fluorenyl methoxy carbonyl chemistry and purified to >95% purity using reverse-phase high-pressure

* Corresponding author.
** Corresponding author. Fax: +65 6794 7553.
*** Corresponding author.
E-mail addresses: ygmu@ntu.edu.sg (Y. Mu), matthewchang@ntu.edu.sg (M. Wook Chang), mbechan@ntu.edu.sg (M.B. Chan-Park).

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Table 1
Peptide properties: MICs (μg ml^{-1}) for five pathogens, retention time of RP-HPLC, α-helix percentage and H_{50} (μg ml^{-1}) values.

Peptide name	Peptide sequence[a]	*E. coli*	*P. aeruginosa*	*S. aeurus*	*C. albicans*	*F. solani*	$t_{RP\text{-}HPLC}$ (min)[b]	α-Helix (%)	H_{50}
P9-0 (Pac-525)	KWRRWVRWI	12.5	50	25	6.2	25	18.157	4.0	400
P9-1	K**L**RRWVRWI	25	50	50	6.2	25	19.374	17.6	
P9-2	KWRRWVRW**L**	12.5	25	12.5	3.1	12.5	18.684	64.5	
P9-3	K**L**RRWVRW**L**	50	50	25	3.1	25	18.361	11.9	
P9-4	KWRRW**I**RW**L**	6.2	6.2	3.1	3.1	25	18.689	2.3	400
P9-5	KWRRW**I**RW**W**	12.5	12.5	6.2	3.1	25	18.430	37.7	
P9-6	K**L**RR**L**i**R**L**L**	12.5	25	50	6.2	12.5	18.829	98.3	
P11-0 (BP76)	KKLFKKILKFL	8	16	62.3	25	50	18.002	20.9	400
P11-1	KKLFKKI**K**KFL	16	8	500	50	100	16.719	18.3	
P11-2	KKLFKK**A**LKFL	16	62.3	500	100	>200	17.547	25.4	
P11-3	KKLFKK**G**LKFL	125	500	500	100	>200	17.258	53.7	
P11-4	**G**KLFKKILKFL	8	16	31.3	25	50	19.158	36.2	
P11-5	**G**KLFKKILK**I**L	3.1	12.5	12.5	3.1	12.5	18.862	90.7	400
P11-6	KKL**I**KKILK**I**L	3.1	6.25	6.25	3.1	12.5	18.585	99.8	400

[a] The bold amino acids in the sequences are the substituted amino acids.
[b] Retention time of the reverse phase HPLC.

liquid chromatography at the Peptide Synthesis Core Facility of the School of Biological Sciences at Nanyang Technological University. Mass spectrometry was used to confirm the peptide identity.

2.2. Minimum inhibitory concentrations (MICs)

Bacteria were grown to a mid-log phase in Mueller–Hinton broth and diluted to 10^5 CFU ml^{-1} inoculum sizes. The synthetic peptides were diluted to obtain final concentrations of 100, 50, 25, 12.5, 6.25, 3.1 μg ml^{-1}. Positive controls contained no antimicrobial peptides, and negative controls contained no bacterial cells. Microbial growth was determined by optical density measurement at 600 nm. The experiments were independently repeated four times with two technical replicates each. The MIC was defined as the lowest peptide concentration that resulted in no bacterial growth at 37 °C after 18 h [10]. To determine the resistance of the antimicrobial peptides against salts, NaCl and $MgCl_2$ were added to the samples.

2.3. Peptide hydrophobicity

Peptide hydrophobicity was evaluated by retention time of the reverse phase HPLC (Phenomenex®, 4.6 by 250 cm column; 4-μm particle size) with a C_{18} reverse-phase column. The retention time was taken as the elapsed time between the time of injection and the time of elution of the peak maximum of the peptide.

2.4. Hemolytic activity

The hemolytic activity of the peptides was evaluated by testing hemoglobin release from erythrocyte suspensions of fresh human blood (5%, vol:vol). Hemolysis was measured as the absorbance at 540 nm using a microplate reader (Bio-Rad Inc. USA). Complete hemolysis was determined in PBS buffer plus 0.1% Triton X-100 as a positive control. The percentage of hemolysis (H) was calculated using the relation: $H = 100 \times [(O_p - O_b)/(O_T - O_b)]$, where O_p is the absorbance for a given peptide concentration, O_b is the absorbance for the PBS buffer, and O_T is the absorbance for the positive control [11].

2.5. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay

The MTT assay was conducted as previously described [12]. Briefly, after keratinocyte cells (10^5 cells ml^{-1}) were exposed to each of the AMPs at a range of concentrations for 24 h, MTT solution (5 mg ml^{-1} in PBS) sterilized through 0.2 μm filter was added into 96-well plates with the a culture medium. The plates were incubated for 4 h at 37 °C in a humidified incubator with 5% CO_2. The medium was removed and DMSO was added to dissolve crystals, followed by gentle shaking for 30 min. The assay was conducted with two biological replicates. The absorbance at 490 nm was measured using a microplate reader (Bio-Rad Laboratories).

2.6. CD spectroscopy

CD spectra were determined using a Chirascan Circular Dichroism Spectrometer (Applied Photophysics Limited, UK). The spectra were sampled over a wavelength range of 190–250 nm at 0.1 nm intervals, with 50 nm min^{-1} speed, 0.5-s response time, and 1-nm bandwidth. A baseline correction spectrum was measured with 50% (vol:vol) trifluoroethanol in PBS buffer. Six scans were averaged to produce each measured spectrum. Data were expressed in terms of mean residue ellipticity [θ] (degrees × cm^2 dmol^{-1}). The percent helicity of the peptide was calculated as α-helix (%) $=([\theta]_{222} - [\theta]^0_{222})/[\theta]^{100}_{222}$, where $[\theta]_{222}$ is the experimentally observed absolute mean residue ellipticity at 222 nm. Values for $[\theta]^0_{222}$ and $[\theta]^{100}_{222}$, corresponding to 0% and 100% helix content at 222 nm, were estimated to be −2000 and −30,000 (degrees × cm^2 dmol^{-1}), respectively [9].

2.7. Field-emission scanning electron microscopy (FESEM)

The pathogens were incubated at the respective MICs for 15 min, after which the morphology of the cell membrane was visualized. Bacterial cells were fixed with 4% gluteraldehyde in 0.15 M sodium phosphate buffer (pH 7.4). The slides were rinsed with 0.15 M sodium phosphate buffer (pH 7.4) and dehydrated through a graded ethanol series (30–100%). After dehydration with ethanol, the slides were dried at 60 °C for 5mins. The slides were coated with platinum metal for 80 s at 20 mA condition and transferred to FESEM (JEOL field electron microscope, Jsm-6700F, Japan) for imaging.

2.8. Analysis of bactericidal activity

The bactericidal activity of the peptides was determined by counting CFUs. *E. coli*, *P. aeruginosa*, and *S. aureus* were inoculated at 3×10^7 CFU ml^{-1} and incubated with the peptides. Aliquots of 500 μl were removed at 5, 10, 15, 30, 45 and 60 min and diluted 10-fold. The dilutions were plated on LB agar plates [13]. The CFUs were counted after 48-h incubation at 35 °C. For the determination of antimicrobial activity against *F. solani*, the spores were collected

after YM agar culture for 5 days and diluted to 5×10^6 CFU ml^{-1} with Sabouraud Dextrose broth. The dilutions were plated on YM agar plates for 5 days.

2.9. Membrane depolarization, dye leakage, and molecular dynamics simulations

Detailed methodology is described in Supplementary information.

3. Results and discussion

The reference peptides BP76 and Pac-525 are hereafter denoted as P11-0 and P9-0, respectively. Fig. 1 shows the two distinct hydrophobic and positively charged faces of these reference peptides and our AMPs; these distinct faces each affects antimicrobial activity and may be adjusted or tuned to modulate that activity. The hydrophobic face facilitates penetration into the membrane, disturbing the bilayer curvature [7,14–16]; higher hydrophobicity has frequently been observed to lead to higher antimicrobial activity. One of our design strategies involves optimizing the AMP hydrophobicity through substitution with hydrophobic amino acids including leucine (L) and isoleucine (I) [17]. As shown in Table 1 and Fig. 1, we substituted leucine for tryptophan (W, P9-1), leucine for isoleucine (P9-2), two leucines for tryptophan and isoleucine (P9-3), isoleucine and leucine for valine (V) and isoleucine, respectively (P9-4), and two isoleucines for two phenylalanines (F, P11-6). We also increased the number of tryptophan, isoleucine and leucine (P9-5 and P9-6). The efficacy of AMPs has also been observed to vary with the net positive charge, which enables binding to the negatively charged membrane by electrostatic effects [18]. Our second strategy involves increasing the net positive charge beyond that of P11-0 by substituting lysine (K) for leucine at the interface of positive and hydrophobic groups (P11-1) (Fig. 1(B)). Third, we enhanced the α-helix structure of P11-0 by replacing the amino acids at the positions 7 (P11-2 and P11-3) and 1 (P11-4 and P11-5) with small and flexible amino acids such as alanine (A) and glycine (G) (Fig. 1(B)); the positions 1 and 7 are regarded as key positions in the formation of α-helix structure [14,19].

Table 1 shows the antimicrobial activities of the reference peptides and the 12 analogues. Compared to P11-0 and P9-0, three of our designed peptides (P9-4, P11-5 and P11-6) exhibited significantly smaller minimum inhibitory concentrations (MICs) against the tested pathogens. In particular, P9-4 has particularly low MICs of 6.2 μg ml^{-1}, 6.2 μg ml^{-1}, 3.1 μg ml^{-1} and 3.1 μg ml^{-1} against *E. coli*, *P. aeruginosa*, *S. aureus* and *C. albicans*. These MICs are two to eight times lower than those of P9-0; there was no improvement against *F. solani* though the MIC remains low (25 μg ml^{-1}). P11-5 and P11-6 have very low MICs (3.1 μg ml^{-1} to 12.5 μg ml^{-1}) against all five tested pathogens. The MICs of P11-5 and P11-6 against *S. aureus, C. albicans*, and *F. solani* are four to ten times lower than those of P11-0. To further investigate the antimicrobial activity of P9-4, P11-5 and P11-6, we compared the time courses to kill mid-log-phase culture suspensions. Fig. S1 (Supplementary information) shows the survival kinetics of the pathogens in the presence of P9-4, P11-5 and P11-6. Treatment for 15 min at the respective MICs resulted in three to five log reductions in viable cell numbers.

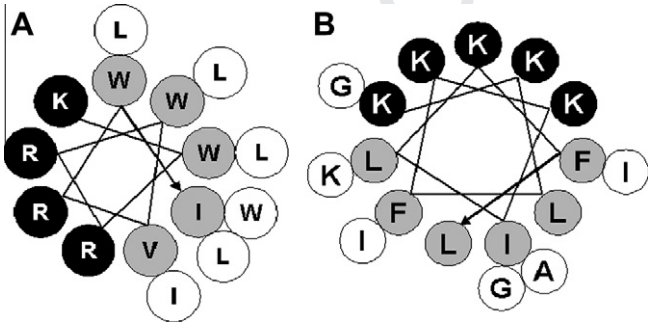

Fig. 1. Schematic diagram illustrating rational design of peptides based on the Edmundson wheel projection (10). Gray and black amino acids in the template peptides represent, respectively, hydrophobic amino acids (Leu, Iso, Phe, and Trp) and positively charged amino acids (Lys and Arg) in the template peptides. The white amino acids are substitutions to yield analogues. The peptide sequences are shown in Table 1. (A) Nine amino acid analogues and (B) eleven amino acid analogues.

To estimate the antimicrobial activities under physiological conditions, which contain salts, the MICs of P9-4, P11-5 and P11-6 were determined in the presence of NaCl and $MgCl_2$ (Table S1 in Supplementary information). All three new peptides generally have low MICs (100 μg ml^{-1} or lower) in various salt solutions against the five pathogens. The longer reference peptide P11-0 seems less resistant to salts than P9-0 as several of its MICs are greater than 100 μg ml^{-1}. P11-5 and P11-6 possessed significantly improved resistance to salts, compared to P11-0, with MICs generally less than or equal to 100 μg ml^{-1} in the presence of NaCl and $MgCl_2$ (except against *P. aeruginosa* in 5 mM $MgCl_2$). P9-4 also has improved pathogen resistance in NaCl and $MgCl_2$ solutions and has low MICs (50 μg ml^{-1} or less) in various salt solutions compared to the reference P9-0. Early research has shown that the influence of salts on MICs is partly due to damage to the secondary peptide structure [20,21]. However, the cause of the variation of the effect of salts for different pathogens remains to be elucidated.

One of the most desired properties of AMPs is low toxicity to eukaryotic cells. To study the cytotoxicity of the newly designed peptides, we determined their ability to lyse human red blood cells (RBS) and inhibit the proliferation of human keratinocytes. First, the hemolytic activity of the AMPs was determined using 0.1% Triton X-100 as a standard, in terms of the quantity H_{50} which is defined as the minimum peptide concentration that produces 50% hemolysis. The H_{50} values of our best three designed peptides (P9-4, P11-5 and P11-6) remains the same as those of unmodified P11-0 and P9-0 (i.e. 400 μg ml^{-1}) (Table 1) despite the improved antimicrobial activity, which reportedly is correlated with increased hemolytic activity [22]. Note that the H_{50} of mellitin, a widely used AMP, has been determined by us to be 8 μg ml^{-1}, suggesting that P9-4, P11-5 and P11-6 have relatively low cytotoxicities. Second, the cytotoxicity of the AMPs was also examined using the MTT assay with human keratinocytes. Fig. S2 in Supplementary information shows that keratinocytes survived well against the newly designed peptides (P9-4, P11-5 and P11-6). In particular, the cell viability remained over 95%, compared to the control cells, even after 24-h incubation with P11-5 and P11-6 at 25μg ml^{-1}. Upon exposure to P9-4, the viability of human keratinocytes remained around 90% against up to 12.5μg ml^{-1}. Given that the MICs against most target pathogens are in a range of 3.1 and 12.5μg ml^{-1} (Table 1), this outcome implies that the cytotoxicity of the newly designed peptides is generally low, while the eleven amino acid peptides (i.e. P11-5, and P11-6) possess lower cytotoxicity than the nine amino acid peptides (i.e. P9-4).

To determine the secondary structures of the peptides, we measured the circular dichroism (CD) of 50% (vol:vol) peptide/trifluoroethanol solutions in PBS buffer. One of the typical characteristics of α-helix conformation is the presence of two negative minimum bands at 208 nm and 222 nm (Fig. 2). The extent of α-helix conformation was determined as a mean residue ellipticity $[\Theta]_{222}$ (degrees × cm^2 $dmol^{-1}$) (refer to the Experimental section). As shown in Table 1, the helicity percentages of P9-0 and its six analogues (P9-1 to P9-6) are in the range of 2.3 to 98.3 with P9-4 having

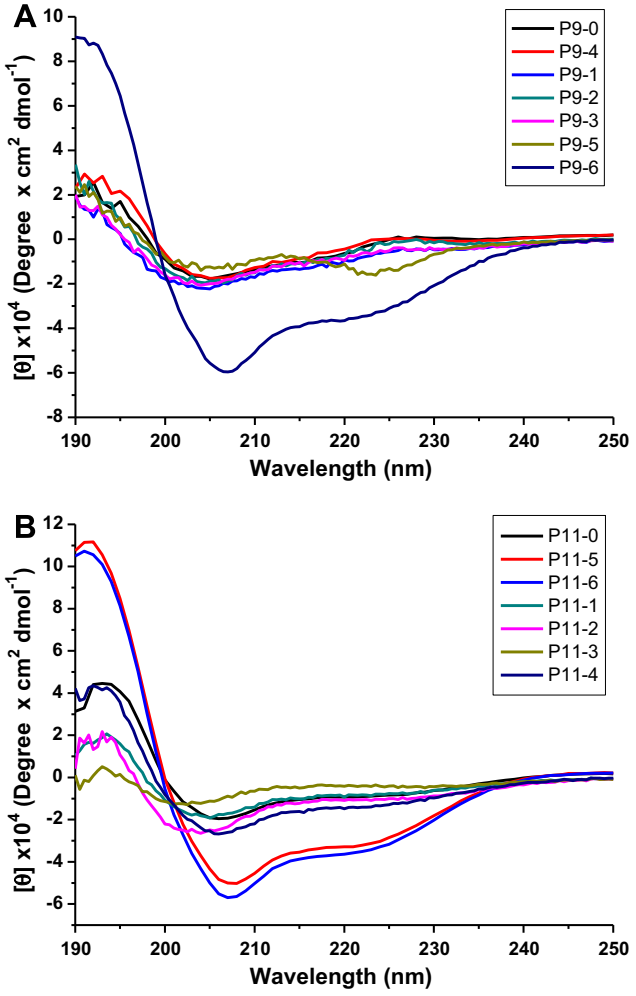

Fig. 2. CD spectra of peptides with 50% (vol:vol) trifluoroethanol and 50%PBS buffer at pH 7.4. The peptide concentration was 900 μg ml^{-1}. θ is mean molar ellipticity (degree × cm^2 dmol^{-1}). (A) CD spectra of 9-amino acid peptides and (B) CD spectra of eleven amino acid peptides.

the lowest value. Among the six analogues of P11-0, P11-5 and P11-6 have the highest helicity percentages (90.7% and 99.8%). The position 7 substitutions (P11-2 and P11-3) did not enhance the α-helix conformation but the position 10 change (P11-5 and P11-6) led to a significant improvement in α-helix structure. The finding that P11-6 has the highest helicity may be due to the replacement of two bulky phenylalanines with isoleucines.

The reversed phase high pressure liquid chromatography (RP-HPLC) retention times ($t_{RP\text{-}HPLC}$) for all the peptides [23] including P11-0 and P9-0 were measured to evaluate the hydrophobicity of the peptides. As may be noted in Table 1, some of our designed peptides are more hydrophobic as indicated by the higher $t_{RP\text{-}HPLC}$; examples of this are P9-4 (18.689 min), P11-5 (18.862 min) and P11-6 (18.585 min) which have more hydrophobic residues than P9-0 (18.157 min) and P11-0 (18.002 min). The short retention time of P11-1 (16.719 min) is attributed to its increased positively charged residues ratio.

The effects of the peptides on pathogen morphology were examined by field-emission scanning electronmicroscopy (FESEM) measurements. We treated *E. coli, P. aeruginosa, S. aureus,* and *C. albicans* with P9-4, P11-5 and P11-6 for 15 min at the respective MICs, while *F. solani* spores were treated for 1 h. Fig. 3 shows the FESEM images of the treated and untreated pathogens. The surfaces of the treated pathogens underwent obvious morphological changes compared with the untreated controls. The membrane surfaces of the untreated bacteria and fungi appeared smooth and rounded, whereas the treated bacteria and fungi exhibited puckered and withered surfaces. The membrane surface of the treated *F. solani* spores was coarse with a number of spikes.

To assess membrane depolarization effects of the peptides, we used $DiSC_3(5)$, a potential-sensitive dye [24]. This cationic dye aggregates in the cytoplasmic membrane by membrane potential, leading to self-quenching of fluorescence. Upon disruption of the membrane, the dye dissociates into the buffer, resulting in an increase in fluorescence. The depolarization was monitored over a period of 1500s. As shown in Fig. S3 in Supplementary information, P9-4, P11-5 and P11-6 completely depolarized the cytoplasmic membrane of representative *S. aureus* and *C. albicans* at 10 μg ml^{-1}. This result suggests that the antimicrobial mechanism of P9-4, P11-5 and P11-6 includes depolarization and/or disruption of the cytoplasmic membrane. In order to further explore the membrane-permeabilizing mechanism of the peptides, we also monitored the peptide ability to induce the release of calcein, a fluorescent dye, from artificial phospholipid vesicles [25]. Upon addition of P9-4, P11-5 and P11-6 to large unilamellar vesicles (LUVs), the entrapped calcein was released into the buffer due to membrane lysis or disruption. The leakage percentages of the three peptides approached 100% at a very low concentration (3 μg ml^{-1}, Fig. S4 in Supplementary information). This result verifies that P9-4, P11-5 and P11-6 can strongly disrupt the lipid bilayer.

The detailed interaction between the short AMPs and lipid bilayer was explored by all-atomic molecular dynamics simulation studies. Two peptides, P11-3 and P11-6, were selected for study. Fig. 4(A and B) illustrates the positions of P11-3 and P11-6 relative to the lipid bilayer. Both peptides lie on the interface between water and lipid with hydrophobic side chains facing the lipid tail groups and charged side chains (lysine residues) interacting with head groups and solvent. The helical structure is well maintained for P11-6 peptide; for P11-3 peptide the helical segment is shorter than that of P11-6 (Fig. 4(C)). The perturbation effects of peptides on lipid are characterized by the deuterium lipid order parameters $|S_{CD}|$ [26] (where S is a tensor, C is carbon and D is deuterium) of the two carbon tails which are shown in Fig. 4(D and E). The general profiles of the $|S_{CD}|$ are similar to that of the lipid-only system [27]. The orders of lipid tail atoms in the presence of P11-6 are less than those of lipid with P11-3, which indicates that P11-6 may exert stronger perturbative effects on lipid stability than P11-3 does.

The action mechanism of our short AMPs is thought to be disintegration of the membrane by the "carpet-like" model because they are shorter than the 20–30 amino acids needed to span the entire plasma membrane [9,15]. Our P9-4, P11-5 and P11-6 have strong membrane-disrupting activity as shown by the FESEM, membrane depolarization and dye-leakage assays. First, the FESEM images of the target pathogens with the three new peptides showed pronounced membrane wrinkling and surface blebbing. The cytoplasmic membranes were disintegrated, and fibrous and cellular materials were dispersed outside cells as a result of leakage and cell lysis. Second, the results of the membrane depolarization assay suggest that P9-4, P11-5 and P11-6 have nearly the same membrane-disrupting activity as gramicidin D, which causes complete membrane depolarization [11]. Third, the dye leakage results suggest that P9-4, P11-5 and P11-6 induce 100% dye leakage from the entrapped calcein artificial membrane vesicles which mimic the cytoplasmic membrane, even at 3 μg ml^{-1}.

Of particular interest is the potential correlation between α-helix structure and antimicrobial activity of the peptides with eleven amino acids peptides. When positively charged peptides bind onto negatively charged target membrane, the peptides reorient themselves such that the hydrophobic face is embedded deeply within the lipid membrane while the hydrophilic face electrostatically

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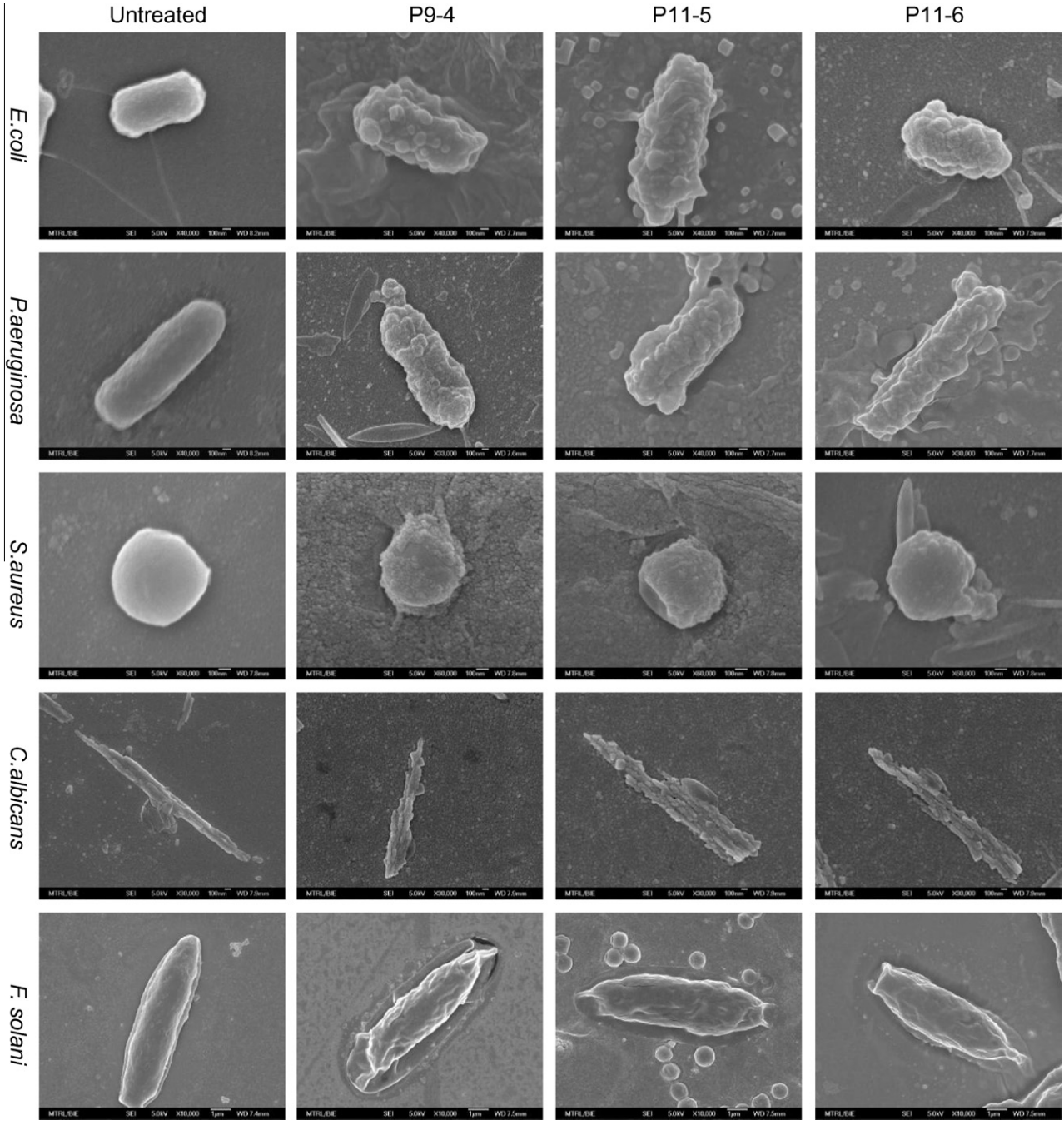

Fig. 3. SEM images of bacteria or fungi untreated and treated with the peptides at their MICs for 15 min.

interacts with the phospholipids head groups. Stable α-helical conformation increases the efficiency of self-reorientation [28,29] and peptide assembly and hence, lipid penetration [7]. Our observation suggests that a pronounced α-helical structure may favor the antimicrobial potency of the eleven amino acid peptides. This correlation was not apparent for the peptides with nine amino acids, which may be too short to form two independent faces of hydrophobic and positively charged groups according to the ideal α-helical structure (Fig. 1(A)).

In conclusion, we have designed a series of short peptides, with nine and eleven amino acids, of which three, P9-4 and P11-5 and P11-6, are highly effective against clinically significant pathogens of both bacterial and fungal character. P11-5 and P11-6 have very low MICs (3.1 μg ml^{-1} to 12.5 μg ml^{-1}) against all five tested pathogens. P9-4 has particularly low MIC of 3.1–6.2 μg ml^{-1} against four tested pathogens (all except *F. solani*); there was no improvement compared to P9-0 against *F. solani* though the MIC remains low (25 μg ml^{-1}). These peptides also possess low susceptibility to salts and surprisingly low cytotoxicities, compared to previously reported peptides. Note that as shown in Table S2 in Supplementary Information, our P9-4 and P11-5 and P11-6 have superior selectivity, a ratio of H_{50} to MIC, compared to the previously reported short AMPs. Membrane depolarization, dye-leakage, and FESEM assays confirm that the newly designed peptides interact strongly with the membranes of bacteria and fungi, indicating that the mechanism of killing is disruption of lipids in the

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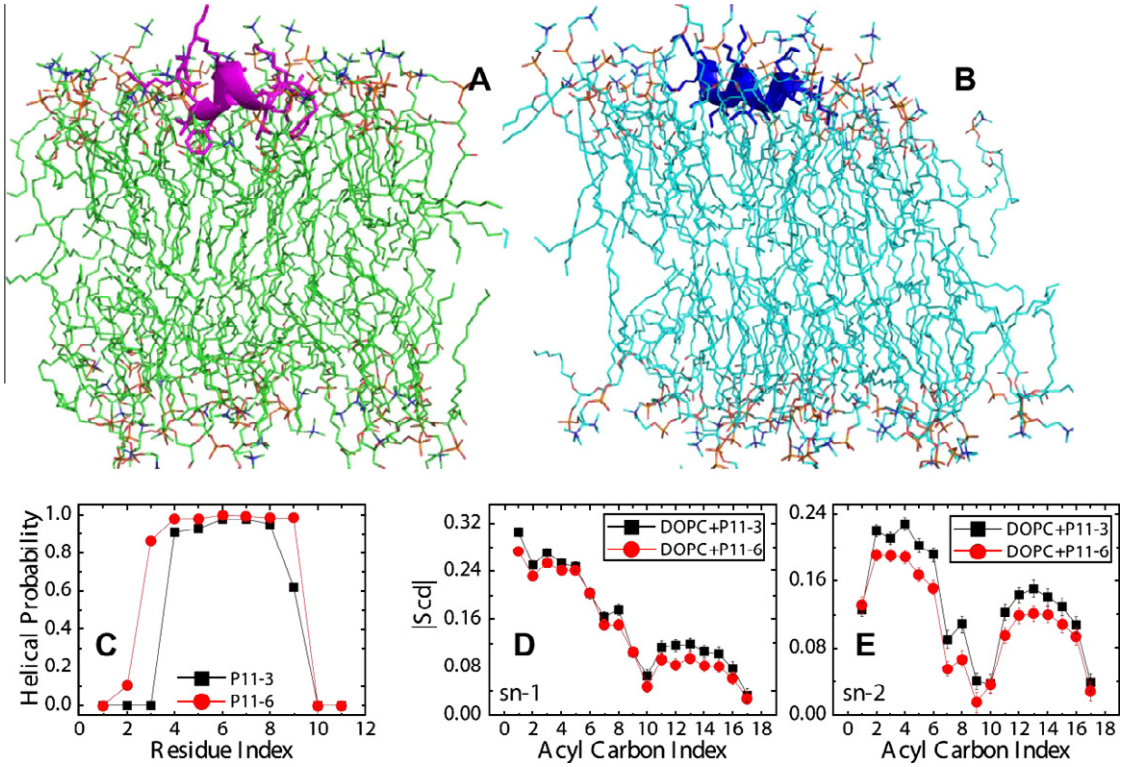

Fig. 4. Molecular dynamics simulation snapshots of peptides (A) P11-3 and (B) P11-6, interacting with DOPC (dioleoyl-phosphatidylcholine) lipid bilayer; (C) probability of helical conformation of peptides in the presence of lipid bilayer; (D,E) deuterium order parameters, $|S_{CD}|$, for the two acyl chains of DOPC lipids. Data are taken from 10 to 20 ns simulation trajectories; and the error bars are standard deviations.

membrane. Our P11-5 and P11-6 show high helicity, implying that pronounced α-helix structure may contribute to antimicrobial activity for the eleven amino acid peptides. Molecular dynamics simulation study confirms the correlation of lipid perturbation and helicity for P11-6. This study suggests that these short peptides (P9-4, P11-5 and P11-6) are new highly effectively antimicrobial agents against both fungal and bacterial pathogens. Considering their low cytotoxicity, relativity low cost and their high broad-spectrum efficacies against clinically important Gram-negative and Gram-positive bacteria and fungi, especially in salt solutions, our new peptides have great potential as topical agents for treatment of superficial fungal or bacterial infections.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.06.131.

References

[1] R.E. Hancock, Cationic peptides: effectors in innate immunity and novel antimicrobials, Lancet Infect. Dis. 1 (2001) 156–164.
[2] A. Coates, Y. Hu, R. Bax, C. Page, The future challenges facing the development of new antimicrobial drugs, Nat. Rev. Drug Discov. 1 (2002) 895–910.
[3] R.E.W. Hancock, H.G. Sahl, Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies, Nat. Biotechnol. 24 (2006) 1551–1557.
[4] R.E. Hancock, M.G. Scott, The role of antimicrobial peptides in animal defenses, Proc. Natl Acad. Sci. USA 97 (2000) 8856–8861.
[5] 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.
[6] A. Makovitzki, D. Avrahami, Y. Shai, Ultrashort antibacterial and antifungal lipopeptides, Proc. Natl Acad. Sci. USA 103 (2006) 15997–16002.
[7] H. Leontiadou, A.E. Mark, S.J. Marrink, Antimicrobial peptides in action, J. Am. Chem. Soc. 128 (2006) 12156–12161.
[8] S.Y. Wei, J.M. Wu, Y.Y. Kuo, H.L. Chen, B.S. Yip, S.R. Tzeng, J.W. Cheng, Solution structure of a novel tryptophan-rich peptide with bidirectional antimicrobial activity, J. Bacteriol. 188 (2006) 328–334.
[9] R. Ferre, E. Badosa, L. Feliu, M. Planas, E. Montesinos, E. Bardaji, Inhibition of plant-pathogenic bacteria by short synthetic cecropin A-melittin hybrid peptides, Appl. Environ. Microbiol. 72 (2006) 3302–3308.
[10] Clinical and Laboratory Standards Institute, I. Clinical and Laboratory Standards, Performance standards for antimicrobial susceptibility testing, CLSI document M100-S16, vol. 188, Clinical and Laboratory Standards Institute, Wayne, PA, 2005, pp. 328–334.
[11] K.H. Park, Y. Park, I.S. Park, K.S. Hahm, S.Y. Shin, Bacterial selectivity and plausible mode of antibacterial action of designed Pro-rich short model antimicrobial peptides, J. Pept. Sci. 14 (2008) 876–882.
[12] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.
[13] Z. Zhao, Y. Ma, C. Dai, R. Zhao, S. Li, Y. Wu, Z. Cao, W. Li, Imcroporin, a new cationic antimicrobial peptide from the venom of the scorpion Isometrus maculates, Antimicrob. Agents Chemother. 53 (2009) 3472–3477.
[14] I. Zelezetsky, A. Tossi, Alpha-helical antimicrobial peptides – using a sequence template to guide structure–activity relationship studies, Biochim. Biophys. Acta – Biomembr. 1758 (2006) 1436–1449.
[15] M.N. Melo, R. Ferre, M.A. Castanho, Antimicrobial peptides: linking partition, activity and high membrane-bound concentrations, Nat. Rev. Microbiol. 7 (2009) 245–250.
[16] R. Rathinakumar, W.C. Wimley, Biomolecular engineering by combinatorial design and high-throughput screening: small, soluble peptides that permeabilize membranes, J. Am. Chem. Soc. 130 (2008) 9849–9858.
[17] R. Mackinnon, Structural biology. Membrane protein insertion and stability, Science 307 (2005) 1425–1426.
[18] T.L. Raguse, E.A. Porter, B. Weisblum, S.H. Gellman, Structure–activity studies of 14-helical antimicrobial beta-peptides: probing the relationship between conformational stability and antimicrobial potency, J. Am. Chem. Soc. 124 (2002) 12774–12785.

[19] I. Zelezetsky, U. Pag, H.G. Sahl, A. Tossi, Tuning the biological properties of amphipathic alpha-helical antimicrobial peptides: rational use of minimal amino acid substitutions, Peptides 26 (2005) 2368–2376.
[20] M.J. Goldman, G.M. Anderson, E.D. Stolzenberg, U.P. Kari, M. Zasloff, J.M. Wilson, Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis, Cell 88 (1997) 553–560.
[21] I.Y. Park, J.H. Cho, K.S. Kim, Y.B. Kim, M.S. Kim, S.C. Kim, Helix stability confers salt resistance upon helical antimicrobial peptides, J. Biol. Chem. 279 (2004) 13896–13901.
[22] Y. Chen, M.T. Guarnieri, A.I. Vasil, M.L. Vasil, C.T. Mant, R.S. Hodges, Role of peptide hydrophobicity in the mechanism of action of alpha-helical antimicrobial peptides, Antimicrob. Agents Chemother. 51 (2007) 1398–1406.
[23] H. Meng, K. Kumar, Antimicrobial activity and protease stability of peptides containing fluorinated amino acids, J. Am. Chem. Soc. 129 (2007) 15615–15622.
[24] S. Ohkuma, Y. Moriyama, T. Takano, Electrogenic nature of lysosomal proton pump as revealed with a cyanine dye, J. Biochem. 94 (1983) 1935–1943.
[25] D.H. Liu, S. Choi, B. Chen, R.J. Doerksen, D.J. Clements, J.D. Winkler, M.L. Klein, W.F. DeGrado, Nontoxic membrane-active antimicrobial arylamide oligomers, Angew. Chem. Int. Edit. 43 (2004) 1158–1162.
[26] D.P. Tieleman, S.J. Marrink, H.J.C. Berendsen, A computer perspective of membranes: molecular dynamics studies of lipid bilayer systems, Biochim. Biophys. Acta (BBA) Rev. Biomembr. 1331 (1997) 235–270.
[27] S.W.I. Siu, R. Vacha, P. Jungwirth, R.A. Bockmann, Biomolecular simulations of membranes: physical properties from different force fields, J. Chem. Phys. 128 (2008) 125103–125112.
[28] M. Dathe, T. Wieprecht, Structural features of helical antimicrobial peptides: their potential to modulate activity on model membranes and biological cells, Biochim. Biophys. Acta 1462 (1999) 71–87.
[29] Y. Shai, Mode of action of membrane active antimicrobial peptides, Biopolymers 66 (2002) 236–248.

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