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Effects and mechanisms of the secondary structure on the antimicrobial activity and specificity of antimicrobial peptides‡

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A 15-mer cationic α-helical antimicrobial peptide HPRP-A1 was used as the parent peptide to study the effects of peptide secondary structure on the biophysical properties and biological activities. Without changing the amino acid composition of HPRP-A1, we designed two α-helical peptides with either higher or lower helicity compared with the parent peptide, a β-sheet peptide and a random coiled peptide using *de novo* design approach. The secondary structures were confirmed by circular dichroism spectroscopy. The three α-helical peptides exhibited comparable antibacterial activities, but their hemolytic activity varied from extreme hemolysis to no hemolysis, which is correlated with their helicity. The β-sheet peptide shows poor antibacterial and strong hemolytic activities. More interestingly, the random coil peptide shows no antibacterial activity against Gram-negative bacteria, weak antibacterial activity against Gram-positive bacteria, and extremely weak hemolytic activity. Bacterial membrane permeabilization was also testified on peptides with different secondary structures. Tryptophan fluorescence experiment revealed that the peptide binding preference to the lipid vesicles for mimicking the prokaryotic or eukaryotic membranes was consistent with their biological activities. With the *de novo* design approach, we proved that it is important to maintain certain contents of amphipathic secondary structure for a desirable biological activity. We believe that the *de novo* design approach of relocation of the amino acids within a template sequence could be an effective approach in optimizing the specificity of an antimicrobial peptide. Copyright © 2015 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antimicrobial peptide; secondary structure; specificity; mechanism of action

Introduction

In recent years, the growing problem of antibiotic resistance due to the antibiotic abuse prompts new classes of antibiotics [1,2]. Antimicrobial peptides (AMPs) attract many studies to develop new antibiotics, due to their extraordinary properties [3,4]. The most attractive feature of AMPs comes from the potency against multidrug-resistant bacteria, especially those fatal strains that invalided the current defenders [1,5]. Basically, the action mechanism of most AMPs is targeting on the membrane system and disrupting the integrity of membrane, resulting in the loss of barrier function. Other kinds of AMPs may transfer into the cytoplasm and target to more specific molecules, causing fatal intercellular interference [4,6]. Despite their various mechanisms of action to cause cell death, the first step of AMPs is to interact with the cell membrane. Moreover, for most AMPs, the membrane is their primary target [7,8]. Thus, the potency of AMPs to disrupt the eukaryotic cell membranes (toxicity to host cells) must be evaluated. The structure–activity relationship should be clarified for obtaining high antimicrobial activity yet low cytotoxicity against eukaryotic cells [9,10].

The lipid compositions of prokaryotic and eukaryotic cell membranes are different, which lead to different interaction modes of AMPs toward a specific membrane and thus provide chances to find or design AMPs with desirable activity. Prokaryotic membranes are predominantly composed of phosphatidylglycerol (PG), cardiolipin, or phosphatidylserine resulting in a negatively charged membrane surface. While mammalian membranes contain the zwitterionic phospholipids, which are neutral on net charge, such as phosphatidylethanolamine, phosphatidylcholine (PC), or sphingomyelin [6,11]. Moreover, cholesterol exists only in the mammalian cell membrane, which can dramatically reduce the activity of AMPs as reported previously [12]. Despite of the different lipid compositions, the membrane asymmetry and transmembrane potential also provide alternatives for AMPs [13].

As mentioned earlier, to design AMPs with desirable activity, researchers have made great efforts on the structure–activity relationship, and quite a few physiochemical parameters have been presented and well-studied, such as charge, helicity, conformation,

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amphipathicity, hydrophobicity, hydrophobic/hydrophilic angle, and self-association [14,15]. These parameters have been studied alone or in a combined manner to elucidate the structure–function relationships of AMPs [16,17]. The secondary structures of AMPs include four major classes, α-helix, β-sheet, extended helices (polyprohelices), and loop structures [3,4]. In this study, we intended to study three kinds of conformation, i.e. α-helical, β-sheet, and random coiled structure, with a definite amino acid composition by the relocation of amino acids along the peptide chain, in order to illustrate the effect of secondary structures on peptide biological activities. An α-helical AMP, named HPRP-A1, was obtained based on the sequence of peptide HPA3NT1 from the *N*-terminus of the *Helicobacter pylori* ribosomal protein L1 [18]. HPRP-A1 exhibited promising antibacterial activity and weak hemolytic activity, which is suitable as a parent peptide for the evaluation of the effect of secondary structures. The sequence of HPRP-A1 was shuffled to design four peptides as α-helical structure, β-sheet structure, or random coil structure, respectively. The biophysical and biological properties of the analogs were determined, and the specificity mechanism was further investigated.

Materials and Methods

Materials

Rink amide 4-methylbenzhydrylamine resin (0.8 mmol/g) and all *N*-α-Fmoc-protected amino acids were purchased from GL Biochem (Shanghai, China). The coupling reagents for peptide synthesis *O*-benzotriazole-1-yl-*N*,*N*,*N*′,*N*′-tetramethyl-uronium HBTU, HOBt, *N*,*N*′-DIEA, TFA, 1-*N*-phenylnaphthylamine (NPN) were purchased from Sigma (St. Louis, MO, USA). Acetonitrile (HPLC grade) was obtained from Fisher (Hampton, NH, USA). L-α-phosphatidyl-DL-glycerol (PG), egg yolk L-α-phosphatidylcholine (PC), and cholesterol were obtained from Avanti Polar Lipids (Alabaster, AL, USA). DCM, *N*,*N*-DMF, piperidine, and 2,2,2-TFE were analytical grade and purchased from a local supplier (Jintai Chemicals, Changchun, China).

Peptide Synthesis and Purification

Peptides were synthesized by solid phase synthesis approach using Fmoc (9-fluorenyl-methoxycarbonyl) chemistry as described previously [17]. The crude peptides were purified on a Shimadzu LC-6A preparative reversed phase-HPLC (RP-HPLC) using a Zorbax 300 SB-C_8 column (250 × 9.4 mm inner diameter, 6.5-μm particle size, 300-Å pore size; Agilent Technologies, Santa Clare, CA, USA) with a linear AB gradient at a flow rate of 2 ml/min. Mobile phase A was 0.1% aqueous TFA and B was 0.1% TFA in acetonitrile. The purity of the peptide was verified by analytical RP-HPLC as described in the succeeding text. The purified peptides were further characterized by mass spectrometry and amino acid analysis.

Analytical RP-HPLC of Peptides

Peptides were analyzed on a Shimadzu LC-20A HPLC using a Zorbax 300 SB-C_8 column (150 × 4.6 mm inner diameter, 5-μm particle size, 300-Å pore size) from Agilent Technologies using a linear AB gradient and a flow rate of 1 ml/min, where solvent A was 0.1% aqueous TFA and solvent B was 0.1% TFA in acetonitrile. Absorbance signals of peptides were detected at 210 nm.

CD Spectroscopy

Circular dichroism spectra were measured with a 0.02-cm path-length quartz cuvette on a Jasco J-810 spectropolarimeter (Jasco, Easton, MD, USA) at 25 °C. Data were collected from 190 to 250 nm at a sensitivity of 100 millidegrees, response time of 1 s, bandwidth of 1.0 nm, and a scan speed of 100 nm/min. Peptides were measured at a concentration of 75 μM in a benign buffer (50 mM KH_2PO_4/K_2HPO_4, 100-mM KCl, pH 7) or the benign buffer in the presence of 50% 2,2,2-TFE. The mean residue molar ellipticities were calculated using the equation $[\theta] = \theta/10\,lcMn$ [19], where θ is the ellipticity in millidegrees, l is the optical path length of the cuvette in centimeters, cM is the peptide concentration in mole/liter, and n is the number of residues in the peptide.

Measurement of Antimicrobial Activity

MICs were determined using a broth dilution method [20]. Briefly, bacteria were grown overnight at 37 °C in Mueller–Hinton broth, diluted in the same medium, and transferred into 96-well microtiter plates (90 μl/well). Peptides were serially diluted using 0.2% bovine serum albumin containing 0.01% acetic acid and added to the microtiter plates in a volume of 10 μl per well to give a final concentration of 5×10^5 CFU/ml. MICs were defined as the lowest peptide concentration that inhibited bacterial growth after incubation for 24 h at 37 °C. Experiments were performed in triplicates.

Measurement of Hemolytic Activity

Peptide samples were serially diluted using phosphate-buffered saline (PBS) in 96-well plates (round-bottom) to give a volume of 60 μl of the sample solution in each well. Human erythrocytes that had been anticoagulated using $EDTAK_2$ were collected by centrifugation (1000× g) for 5 min, washed twice with PBS, and then diluted to a concentration of 2% in PBS. The erythrocytes (60 μl of 2% solution) were added to each well to achieve a final concentration of 1% human erythrocytes per well, and the reactions were incubated at 37 °C for 4 h. The plates were then centrifuged for 10 min at 3000 rpm, and the supernatant (80 μl) was transferred to a 96-well plate (flat-bottom). The release of hemoglobin was determined by measuring the absorbance of the supernatant at 540 nm. Peptide samples were diluted in a twofold series to determine the minimum concentration at which hemolysis occurred. Erythrocytes in PBS and distilled water were used as controls of 0% and 100% hemolysis, respectively. Experiments were performed in triplicates.

Calculation of Therapeutic Index (MHC/MIC Ratio)

Therapeutic index values were determined by the ratio of minimal hemolytic concentration (MHC)/MIC, indicating the specificity of peptides against bacterial and eukaryotic cells, respectively.

Permeabilization of Bacterial Outer Membranes

The NPN uptake assay was used to detect disturbances in the outer membrane of Gram-negative bacteria by the peptides [21]. An overnight culture of *Pseudomonas aeruginosa* (1 ml, ATCC 27853) was transferred into 50 ml of Mueller–Hinton broth. After incubation for 2–3 h, middle mid-log phase cells ($OD_{600} = 0.4 - 0.6$) were washed once and resuspended to an OD_{600} of 0.5 (8×10^8 CFU/ml) with 5-mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer containing 5 mM NaN_3 (pH 7.4). Fluorescence measurements were run on a Shimadzu RF-5301 spectrofluorometer

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using a 3-ml quartz cuvette with an excitation wavelength of 350 nm and an emission wavelength of 420 nm at 25 °C. The cell suspension (2 ml) in the cuvette was first measured for 0.5 min, then 20 μl of NPN solution in acetone (0.25 mM) was added, and the results were recorded for about 15 s. Immediately following this step, 20 μl of peptide solution in water was added to the cuvette, and the reaction cuvette was monitored for 10 min. A negative control was carried out with the buffer (5-mM HEPES, pH 7.4, 5-mM NaN_3), and a 1 μg/ml polymyxin B sulfate (final concentration) was used as a positive control.

Preparation of Lipid Vesicles

Unilamellar vesicles were prepared by a standard procedure with either PC/PG (7 : 3, w/w) or PC/cholesterol (8 : 1, w/w) as described previously [22]. Briefly, the desired mixtures of lipids were dissolved in a chloroform/methanol mixture (2 : 1, v/v), dried under nitrogen, and then lyophilized for 24 h to remove traces of organic solvent. Dry lipid films were suspended in 10-mM HEPES buffer containing 150-mM NaCl (pH 7.4) by vortex mixing. After five freeze-thawing cycles, the suspension was extruded 15 times through double polycarbonate membranes with 0.1-μm-diameter pores on an Avanti mini-extruder apparatus. The lipid concentration was determined by phosphorus analysis as previously reported [23].

Fluorescence Spectroscopy

Fluorescence emission spectra of peptide tryptophan residue were monitored by a Shimadzu RF-5301 spectrofluorometer (Shimadzu, Tokyo, Japan). The excitation of wavelength was set as 280 nm, and the emission spectra of each peptide in HEPES buffer, with or without lipid vesicles, were recorded between 300 and 450 nm. Thus, three curves were recorded for each peptide, in HEPES buffer or with either PC/PG vesicles or PC/cholesterol vesicles. The blue shifts of Trp residues in vesicles were recorded. The fluorescence spectrum of each peptide with liposomes was subtracted from the spectrum of liposome alone.

Results

Peptide Design

In order to demonstrate the effect of secondary structures of AMPs on their cytotoxic activity and antimicrobial specificity, peptides with different secondary structures were *de novo* designed by shuffling the peptide sequence while keeping the same amino acid composition to eliminate the influence of other properties, such as hydrophobicity, charge, and peptide length. The sequences of peptide analogs were shown in Table 1. For all peptide analogs used in this study, the *N*-terminus is acetylated and the *C*-terminus

Table 1. Sequences of the peptides and their biophysical properties

Peptides	Amino acid sequence[a]	t_R (min)[b]	Benign[c]		50% TFE[d]	
			$[\theta]_{222}$	Helix[e] (%)	$[\theta]_{222}$	Helix[e] (%)
HPRP-A1	Ac-FKKLKKLFSKLWNWK-amide	36.0	−2550	16.2	−13 380	84.8
HPRP-A3	Ac-FKKLKKLFSKLKNWW-amide	36.6	−6450	40.9	−15 770	100.0
HPRP-A4	Ac-KFLKLKWLKSWKNFK-amide	29.3	−1960	12.4	−11 020	69.9
HPRP-B	Ac-FKLKLKFSNKLKWKW-amide	29.9	—[f]	—	—	—
HPRP-C	Ac-LFKKNKLWFKSKKWL-amide	28.1	—	—	—	—

[a]One-letter codes are used for the amino acid residues; Ac, N^{α}-acetyl; amide, *C*-terminal amide. All amino acids are L-amino acids.
[b]t_R(min) denotes the retention times during the RP-HPLC at 25 °C.
[c]The mean residue molar ellipticities, $[\theta]_{222}$(degree cm^2 $dmol^{-1}$) at wavelength 222 nm were measured at 25 °C in KP buffer.
[d]The mean residue molar ellipticities, $[\theta]_{222}$(degree cm^2 $dmol^{-1}$) at wavelength 222 nm were measured at 25 °C in KP buffer diluted 1 : 1 (v/v) with TFE.
[e]The helical content (in percentage) of a peptide relative to the molar ellipticity value of peptide HPRP-A3 in 50% TFE.
[f]Because peptides HPRP-B and HPRP-C are not in α-helical structure, thus, the data were not been determined.

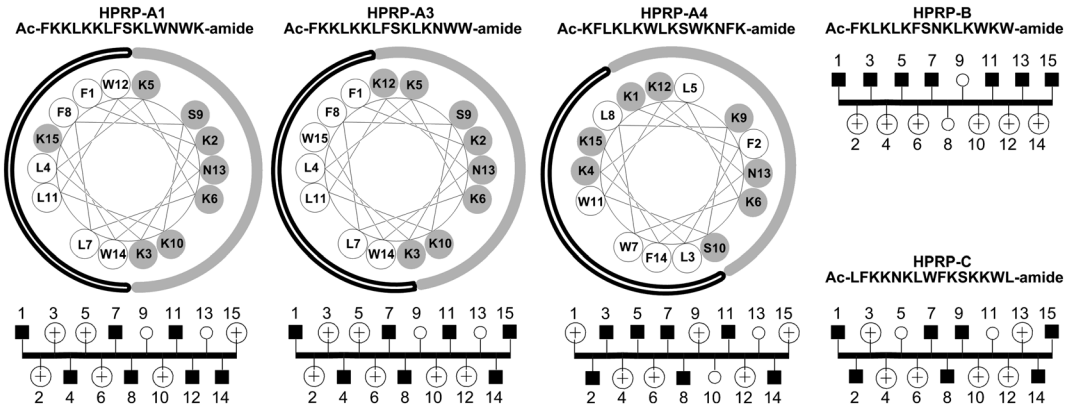

Figure 1. Amino acid sequence, helical wheel representations, and β-strand diagrams showing the distribution of amino acid side chains. The hydrophilic amino acid residues are in gray background in helical wheel representations. The hydrophobic amino acids are shown as solid squares in the β-strand diagrams; the hydrophilic and charged amino acids are shown as circles in the β-strand diagrams.

is amidated. The purity of the peptides is at least 95% as analyzed by the analytical RP-HPLC.

As shown in Figure 1, different secondary structures in α-helical wheel and/or β-strand diagrams are for comparison purpose only of amino acid locations, which do not reflect their corresponding structures. The parent peptide HPRP-A1 is an amphipathic α-helical peptide with a non-polar face and a polar face.

Biophysical Properties

The conformations of the peptide analogs were determined by CD spectroscopy both in benign buffer and in a hydrophobic environment. The CD data are shown in Table 1, and the CD spectra are shown in Figure 2. The helicity of peptide analogs relative to that of peptide HPRP-A3 in the presence of 50% TFE ($[\theta]_{222}$ −15 770), which is the greatest value among all analogs, was also summarized in Table 1. Because peptides HPRP-B and HPRP-C are not in α-helical structure, their helicity was not determined in this study.

In benign buffer, all peptides, except peptide HPPR, exhibited random coil structures as evidenced by the minimum peaks at around 195 to 200 nm. In contrast, peptides HPRP-A1, HPRP-A3, and HPRP-A4 exhibited different degrees of α-helical structure in the presence of 50% TFE. As a polarity moderating reagent, TFE is largely used as the mimic of membrane by decreasing the polarity of the solvent [24]. For most of amphipathic α-helical AMPs, the secondary structure would undergo a transformation to present the polar face and non-polar face under this condition. The double minimums at 208 and 222 nm represent the increased or decreased helicity for peptides HPRP-A3 and HPRP-A4, respectively, compared with the parent peptide HPRP-A1. The peptide helicity follows this order: HPRP-A3 > HPRP-A1 > HPRP-A4. For peptide HPRP-B, single minimum peak at around 215 nm shows the β-sheet contents in the presence of hydrophobic environment. Peptide HPRP-C exhibited as a random coil structure in the absence and presence of 50% TFE. The CD spectra results confirmed the desired design about the peptide secondary structures without changing the amino acid composition.

As mentioned earlier, all peptides contain the same composition of amino acids, thus, the intrinsic hydrophobicity of all analogs is identical. However, the retention times of these analogs on RP-HPLC vary in a wide range as shown in Table 1. HPRP-A3 with a defined amphipathic α-helical structure possessed the longest elution time. In contrast, the random coiled peptide HPRP-C exhibited the shortest retention time. It is interesting to see that the order of retention times of α-helical peptides HPRP-A1, HPRP-A3 and HPRP-A4 is exactly consistent with the helicity order, that is HPRP-A3 > HPRP-A1 > HPRP-A4.

Biological Activities and Therapeutic Index

The antimicrobial activity of the peptide analogs against both Gram-negative and Gram-positive bacteria was determined as the minimum inhibitory concentrations (MIC) against Gram-negative bacteria (Table 2) and against Gram-positive bacteria (Table 3). The overall evaluation of peptide antimicrobial activity was determined by the geometric mean MIC values from three microbial strains in each table. For peptide HPRP-A1 and HPRP-A3, their MICs against Gram-negative and Gram-positive bacteria were roughly at the same level of 1.6-6.4 μM, while peptide HPRP-A4 and HPRP-B exhibited poor activities. Peptide HPRP-C with a random coil structure both in aqueous and hydrophobic conditions exhibited the

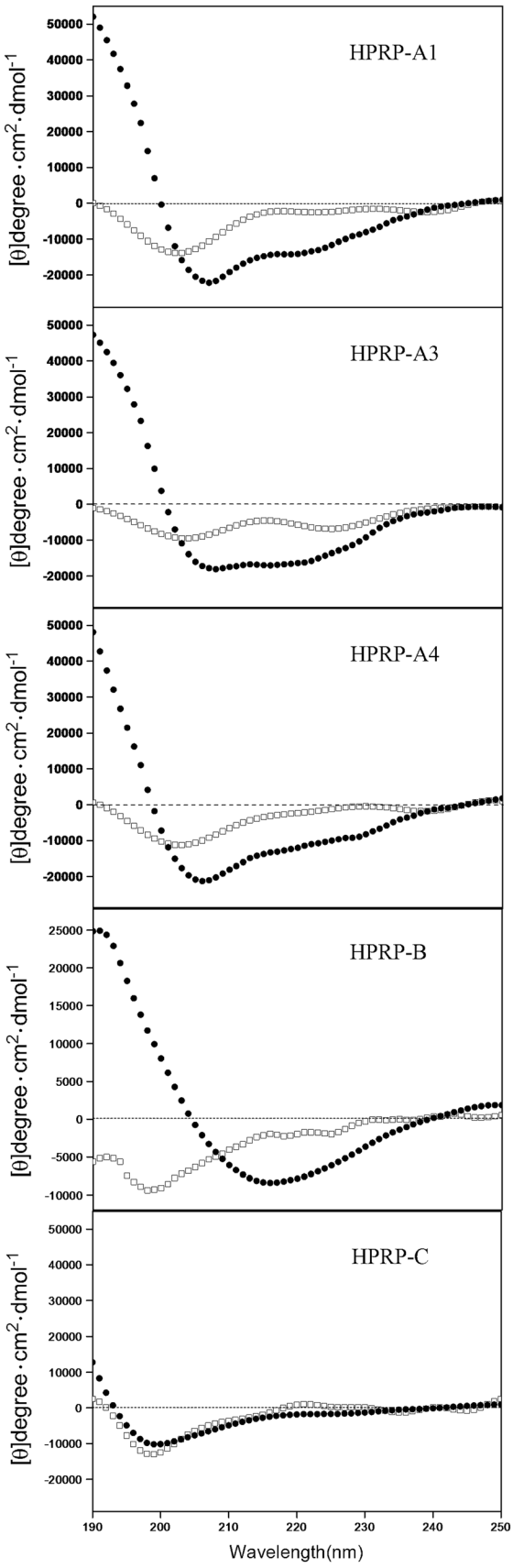

Figure 2. CD spectra of peptide analogs at pH 7 and 25 °C in 50 mM KH_2PO_4/K_2HPO_4 containing 100-mM KCl. Open symbols denote the CD spectra of peptide analogs in benign buffer without TFE, and solid symbols denote CD spectra obtained in the presence of 50% TFE.

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Table 2. Antimicrobial (MIC) and hemolytic (MHC) activities of peptide analogs against Gram-negative bacteria and hRBCs

Peptides	MIC[a] (μM)				MHC[b] hRBC (μM)	Therapeutic index[c]
	Escherichia coli ATCC25922	*Pseudomonas aeruginosa* ATCC27853	*Klebsiella pneumoniae* ATCC 700603	*GM*[d]		
HPRP-A1	1.6	3.2	3.2	2.5	32.0	12.8
HPRP-A3	1.6	3.2	3.2	2.5	16.0	6.4
HPRP-A4	12.5	12.5	25.0	15.7	250.0	15.9
HPRP-B	12.5	12.5	25.0	15.7	64.0	4.1
HPRP-C	50.0	50.0	50.0	50.0	500.0	10.0

MHC, minimal hemolytic concentration.
[a]Antimicrobial activity (MIC) was determined as the minimal concentration of peptide to inhibit microbial growth.
[b]Hemolytic activity (MHC) was determined on human red blood cells (hRBCs).
[c]Therapeutic index = MHC (μM)/geometric mean of MIC (μM). Larger values indicate greater antimicrobial specificity.
[d]GM denotes the geometric mean of MIC values from all four microbial strains in this table.

Table 3. Antimicrobial (MIC) and hemolytic (MHC) activities of peptide analogs against Gram-positive bacteria and hRBCs

Peptides	MIC[a](μM)				MHC[b] hRBC (μM)	Therapeutic index[c]
	Staphylococcus aureus ATCC25923	*Bacillus subtilis* ATCC6633	*Staphylococcus epidermidis* ATCC12228	*GM*[d]		
HPRP-A1	6.4	1.6	1.6	2.5	32.0	12.8
HPRP-A3	3.2	1.6	1.6	2.0	16.0	8.0
HPRP-A4	50.0	6.4	12.5	15.9	250.0	15.7
HPRP-B	25.0	3.2	12.5	10.0	64.0	6.4
HPRP-C	50.0	6.4	25.0	20.0	500.0	25.0

MHC, minimal hemolytic concentration.
[a]Antimicrobial activity (MIC) was determined as the minimal concentration of peptide to inhibit microbial growth.
[b]Hemolytic activity (MHC) was determined on hRBCs.
[c]Therapeutic index = MHC (μM)/geometric mean of MIC (μM). Larger values indicate greater antimicrobial specificity.
[d]GM denotes the geometric mean of MIC values from all four microbial strains in this table.

weakest antibacterial activity. This result indicated that the secondary structures strongly affected the antibacterial activity.

The hemolysis of the peptides was determined against human red blood cells and defined as the MHC (Tables 2 and 3). Peptide HPRP-A3 with the greatest α-helical structure showed the strongest hemolytic activity. The parent peptide HPRP-A1 exhibited a moderate hemolytic activity. Peptides HPRP-A4 and HPRP-C, with the lowest α-helical structure and no defined secondary structure, showed the weakest hemolytic activity. Peptide HPRP-B, with β-sheet structure, showed a slightly weaker hemolytic activity than peptide HPRP-A1.

Therapeutic index (TI) is used to represent the specificity of antimicrobial reagents. Larger values of TI indicate greater antimicrobial specificity. The TI data in Tables 2 and 3 show that peptide HPRP-B possesses the poorest specificity with regard to both Gram-negative bacteria and Gram-positive bacteria, while peptide HPRP-A4 possesses the highest specificity among the three α-helical peptides due to its optimized hemolytic activity. Although peptide HPRP-C exhibited the weakest hemolytic activity, its poor antimicrobial activity makes it a weak peptide on the antimicrobial application against bacteria.

Permeabilization of the Outer Membrane of Gram-negative Bacteria

It is well documented that the outer membrane of Gram-negative bacteria protected the cell from the chemical molecules to access. We used *P. aeruginosa* (ATCC 27853) as a model strain. The NPN uptake assay was employed to investigate the peptides ability to disturb bacterial outer membranes. NPN is a small hydrophobic molecule, which will fluoresce weakly in a hydrophilic environment (aqueous solution) but strongly in a hydrophobic environment (biomembrane) [25]. Thus, it is widely used to reflect the integrity of the outer membrane of Gram-negative bacteria [26,27]. Figure 3 shows the perturbation of the outer membrane caused by the peptide analogs at the different concentrations of 4, 2 or 1 μM. It is clear to see that the peptide permeabilization ability is concentration-dependent and all analogs showed the outer membrane disturbing activity at the indicated concentrations. The tremendous low fluorescence level induced by peptide HPRP-C indicated that this peptide possesses the weakest outer membrane perturbing activity. This result demonstrates that the outer membrane of Gram-negative bacteria constraints the bactericidal activity of peptide HPRP-C, which is consistent with the specificity of Gram-positive bacteria of this peptide.

Binding Preference of Lipid Vesicles

The binding preference of the peptide to cytoplasmic membrane was assessed by testing the interaction of peptide with lipid vesicles composed of PC/PG (7 : 3, w/w) for mimicking the prokaryotic membrane or PC/cholesterol (8 : 1, w/w) for mimicking the eukaryotic membrane. The interaction of peptide with liposomes

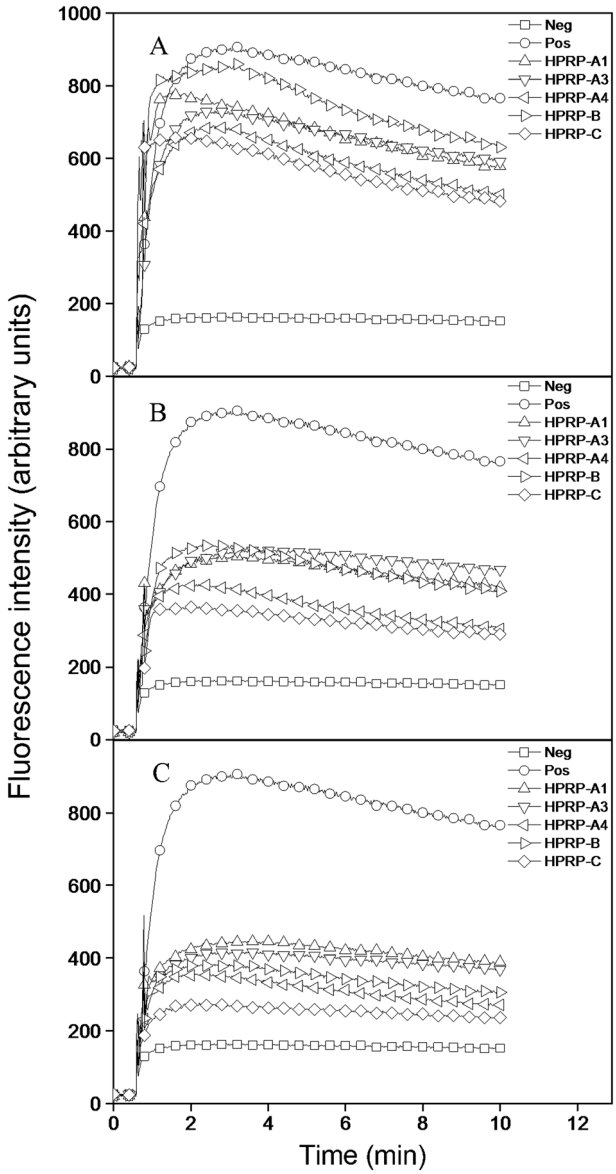

Figure 3. Outer membrane permeabilization induced by peptides as detected by NPN uptake in *Pseudomona aeruginosa* ATCC 27853. The peptide concentrations were 4, 2, and 1 μM in panels A, B, and C, respectively. The negative and positive controls were indicated as Neg and Pos, respectively.

were monitored by the fluorescence emission of the tryptophan residue. The fluorescence emission maxima of Trp residue would undergo a blue shift and emission intensity would increase when it entered into a hydrophobic environment [28]. Thus, the binding of peptides with cytoplasmic membrane would alter the environment of tryptophan residues. In the presence of negatively charged lipid vesicles (PC/PG), the blue shift of Trp residues for all peptide analogs are around of 10-14 nm (Table 4), which indicated the participation of Trp residue in the hydrophobic moiety of membrane. In contrast, only peptide HPRP-A1 and HPRP-A3 exhibited obvious blue shift in the presence of neutral vesicles (PC/cholesterol) (Table 5), showing that there is a strong membrane preference of these peptides. The data demonstrate that all the peptides exhibited stronger interaction on prokaryotic membrane than eukaryotic membrane.

Table 4. Tryptophan fluorescence emission maxima of the representative peptides in HEPES buffer or in the presence of PC/PG (7 : 3) liposomes

Peptides	HEPES[a] (nm)	Liposomes[b] (nm)	Blue shift[c] (nm)	Intensity[d]
HPRP-A1	350	340	10	426.4
HPRP-A3	351	338	13	549.9
HPRP-A4	351	337	14	251.4
HPRP-B	351	341	10	217.0
HPRP-C	351	340	11	210.9

[a]Emission maxima in HEPES buffer.
[b]Emission maxima in liposome buffer.
[c]Blue shift in emission maxima.
[d]Relative fluorescence intensity increases.

Table 5. Tryptophan fluorescence emission maxima of the representative peptides in HEPES buffer or in the presence of PC/cholesterol (8 : 1) liposomes

Peptides	HEPES[a] (nm)	Liposomes[b] (nm)	Blue shift[c] (nm)	Intensity[d]
HPRP-A1	350	343	7	225.6
HPRP-A3	351	341	10	407.4
HPRP-A4	351	350	1	11.9
HPRP-B	351	350	1	−14.5
HPRP-C	351	350	1	28.3

[a]Emission maxima in HEPES buffer.
[b]Emission maxima in liposome buffer.
[c]Blue shift in emission maxima.
[d]Relative fluorescence intensity increases.

Discussion

Most of the AMPs present amphipathic structures in either α-helical or β-sheet conformation, especially in the state of membrane-binding. Still, there is amount of AMPs that present no specific secondary structure, i.e. random coil structure [3,4]. To elucidate the conformational effects on peptide biological activities, three α-helical peptides with the different helicity (HPRP-A3 > HPRP-A1 > HPRP-A4), one β-sheet peptide HPRP-B and one coiled peptide HPRP-C peptides were designed by shuffling the amino acid sequence but maintaining the same amino acid composition. This designing approach was to eliminate other biophysical properties such as hydrophobicity, charge and peptide length, which may influence peptide biological activities as well. However, due to the preferred binding domain [29], the retention times of the peptides in RP-HPLC are closely correlated with the peptide conformational changes, as determined in this study that the retention time follows the order of HPRP-A3 > HPRP-A1 > HPRP-A4 > HPRP-B > HPRP-C.

The conformational styles of the peptides are also strongly correlated with their hemolytic activity toward erythrocytes. It seems that peptides with more organized secondary structure showed stronger hemolytic activity, that is, peptides HPRP-A1, HPRP-A3 and HPRP-B exhibited stronger hemolysis than HPRP-A4 and HPRP-C. In contrast, the antimicrobial results demonstrated that it is important to maintain certain α-helical contents for the desirable antimicrobial activity. The random coiled HPRP-C showed the weakest antimicrobial activity. In the previous studies, D-amino

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acids were used to alter the conformation of α-helical peptides and showed that α-helical content was more correlated with the cytotoxicity than the antimicrobial activity [9,30,31]. Hence, for *de novo* design of AMP with high specificity against bacteria, certain secondary structure would be crucial but within a certain range.

The outer membrane disturbing abilities of the peptides in this study were also consistent with their conformational integrity. The notably low disturbing activity of HPRP-C may explain its specificity against Gram-positive bacteria. It is largely accepted that cationic peptides take the place of the Mg^{2+} or Ca^{2+}, which functions as the stabilizer of LPS – the main component of outer member and thus causes the disturbance of the outer membrane and results in more peptide molecules to access to and permeate into the cytoplasmic membrane [3].

The binding preference of the peptides to lipid vesicles for mimicking the prokaryotic and eukaryotic membranes revealed by the Trp fluorescence assay was consistent to their biological activities. The amino acid relocations did not affect the binding to anionic lipid vesicles, but significantly influence the binding to neutral lipid vesicles, which is consistent with the conformational integrity. That is, the conformational integrity is strongly correlated with the biophysical and biological properties of helical AMP. In this study, the peptide analogs with α-helical structure exhibited stronger antimicrobial activity than β-sheet and random coil structure, which is consistent with the fact that α-helical AMPs represent the largest type of AMP in the nature [13,14].

In summary, the effects of secondary structures on the biophysical properties and biological activities of AMPs were studied without changing the amino acid composition. The peptide analogs with α-helical structure exhibited stronger antimicrobial activity against both Gram-negative and Gram-positive bacteria than β-sheet and random coil structure. In order to design an effective AMP with low cytotoxicity, it is important to maintain certain contents of amphipathic secondary structure for a desirable biological activity. We believe that the *de novo* design approach of relocation of the amino acids within a template sequence could be an effective approach in optimizing the specificity of an AMP.

Conflict of Interests

The authors declare that there is no conflict of interest.

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