ORIGINAL ARTICLE

Investigation of the role of tryptophan residues in cationic antimicrobial peptides to determine the mechanism of antimicrobial action

X. Bi¹, C. Wang², L. Ma², Y. Sun¹ and D. Shang¹,²

¹ Faculty of Life Science, Liaoning Normal University, Dalian, China
² Liaoning Provincial Key Laboratory of Biotechnology and Drug Discovery, Dalian, China

Keywords
antimicrobial activities, calcein-loaded liposomes, helicity, membrane, temporins, tryptophan.

Correspondence
Dejing Shang, Liaoning Provincial Key Laboratory of Biotechnology and Drug Discovery, Liaoning Normal University, Dalian 116081, China.
E-mail: djshang@lnnu.edu.cn

2013/2013-0319: received 18 February 2013, revised 4 May 2013 and accepted 18 May 2013
doi:10.1111/jam.12262

Abstract
Aims: To understand the effects of Trp residues in linear antimicrobial peptides with α-helical conformations on cell permeation ability and membrane transduction efficacy.

Methods and Results: A series of L-K6 analogues were designed and synthesized by replacing Ile or Leu with Trp at different positions on the hydrophobic face of L-K6. The antimicrobial and haemolytic activity and secondary structure of the designed Trp-containing peptides were assessed. In addition, the role of Trp in membrane disruption for these designed peptides was investigated. I1W, I4W and L5W demonstrated stronger activity than the other peptides against both Gram-positive and Gram-negative bacteria. All of the tested peptides preferentially interacted with negatively charged vesicles composed of phosphatidylglycerol (PG)/cardiolipin (CL) or PG/CL/phosphatidylethanolamine, and, to a lesser extent, with zwitterionic vesicles. I1W, I4W and L5W caused calcein release at 2.5 μmol l⁻¹.

Conclusions: The position of Trp, rather than the number of Trp residues, in these peptides was an important factor in the antimicrobial activity. Trp residues were deeply inserted into negatively charged membranes but were largely exposed in aqueous buffer solution.

Significance and Impact of the Study: These Trp-containing peptides may represent good candidates for new antibiotic agents and for use in new therapeutic approaches.

Introduction

Rapidly increasing antibiotic resistance represents a serious challenge to human health and medicine, and there is therefore a clear need to search for new types of therapeutic agents (Goates et al. 2002; Furuya and Lowy 2006). Antimicrobial peptides (AMPs) are typically relatively short, positively charged, amphipathic and important for the innate immune response in organisms (Hancock and Lehrer 1988; Boman 1995; Zasloff 2002). To date, hundreds of such peptides have been identified (Hancock and Chapple 1999; Wang and Wang 2004) that show broad-spectrum antimicrobial activity. Therefore, AMPs are of particular interest as candidates for the development of new antimicrobial agents due to their widespread action and low level of associated induced resistance (Gordon et al. 2005).

There are a number of different classes of AMPs, which achieve their bactericidal effects in a number of different ways. Many of these peptides rapidly kill invading pathogens through initial interactions with the outer and/or inner membranes of bacteria that cause membrane permeabilization and disruption (Epand and Vogel 1999; Hancock and Rozek 2002). Numerous factors are believed to be important for the high antimicrobial activity of AMPs, including cationicity (which mediates the interaction with the negatively charged microbial membrane), hydrophobicity (which facilitates the permeabilization...
and disruption of the membrane), amphipathicity (the segregation of hydrophilic and hydrophobic residues in an amphipathic α-helical AMP) and the secondary structure of many cationic AMPs (Lequin et al. 2006; Liu et al. 2007). In addition, particular amino acids also help to improve antimicrobial activity. Cationic residues (Arg and Lys) are thought to mediate the initial electrostatic attraction to the negatively charged bacterial cell surface (Eisenberg et al. 1982). Tryptophan (Trp) residues reportedly exhibit the unique property of being able to interact with the interfacial region of a membrane, thereby anchoring the peptide to the bilayer surface. This anchoring occurs because the aromatic side chain of tryptophan is able to form hydrogen bonds, with a dipole moment of ~2.1 D (Khandelia and Kaznessis 2007). The special features of Trp residues in Trp-containing peptides make Trp a very interesting molecule for designing short peptide antibiotics (Williams and Deber 1991; Schiffer et al. 1992; Rex 2000; Lu et al. 2001). To understand whether Trp residues in linear AMPs with α-helical conformations are unique in their cell permeation ability and whether selective Trp residue modifications may be able to increase membrane transduction efficacy or modify the cell entry mode of the peptide, a series of Trp-containing peptides were synthesized, designated I1W, I4W, L5W, L11W, L12W, L5WL11W, L5WL12W, L11WL12W and L5WL11WL12W. These peptides were all based on the structure of L-K6, a peptide derived from the frog skin peptide temporin-1C Eb (Shang et al. 2009, 2012). L-K6 is comprised of only 13 amino acids. Although L-K6 exhibits high antimicrobial activity against Gram-positive bacteria, it shows no obvious activity towards most Gram-negative bacteria (Shang et al. 2012). We designed the nine analogues by replacing Ile or Leu residues with 1–3 Trp residues at different positions on the hydrophobic face of L-K6. Our results showed that some of these Trp-containing peptides, such as I1W and I4W, exhibited desirable characteristics, including strong antibacterial activity towards Gram-negative bacteria, together with negligible haemolytic activity. We found that the Trp residues showed a strong membrane-disruptive activity, and this property endows Trp-containing peptides with the unique ability to interact with the surface of bacterial cell membranes. Moreover, the position of Trp residues in these peptides is an important factor for antibacterial activity.

Materials and methods

Bacterial strains

The following bacterial strains were acquired from the China General Microbiological Culture Collection Centre: *Escherichia coli* (AS 1.349), *Pseudomonas aeruginosa* (CGMCC 1.860), *Enterobacter cloacae* (CGMCC 1.58), *Klebsiella pneumoniae* subsp. *Pneumoniae* (CGMCC1.176), *Staphylococcus aureus* (AS 1.72), *Bacillus cereus* (AS 1.126), *Enterococcus faecalis* (CGMCC 1.595) and *Enterococcus faecium* (CGMCC 1.2334). The bacterial strains *Proteus mirabilis* (CICC 22931) and *Staphylococcus epidermidis* (CICC 23664) were acquired from the China Centre for Industrial Culture Collection.

Peptide synthesis

Peptides were synthesized in crude form using standard Fmoc solid-phase peptide synthesis protocols by GL Biochemistry (Shanghai, China). The peptides were purified to near-homogeneity (>95%) via reverse-phase HPLC on a 2:2 × 25-cm Vydac 218TP1022 (C-18) column equilibrated with aqueous acetonitrile/trifluoroacetic acid.

Antimicrobial assay

The minimum inhibitory concentrations (MICs) for the peptides against the bacteria were measured using a standard microdilution method with 96-well microtitre plates (Pál et al. 2006). Briefly, bacterial cells were grown overnight at 37°C in brain heart infusion (BHI) medium and were diluted in the same medium. Serial dilutions of the peptides, at final concentrations between 1·56 and 200 μmol l⁻¹, were added to the microtitre plates in a volume of 50 μl, followed by the addition of 50 μl of bacteria, for a final inoculum of 10⁶ CFU ml⁻¹. The plates were then incubated at 37°C for 24 h, and the OD₅₆₀ was measured using a microtitre plate reader for each sample. Four replicates were tested for each condition. The MICs were determined as the lowest concentration of peptides where no visible growth was observed.

Haemolysis assay

The haemolytic activity of every peptide was determined using a previously reported method (Lequin et al. 2006). Briefly, 2 × 10⁶ erythrocytes from healthy human blood were washed three times with 0·9% (w/v) NaCl and incubated with peptides (100–500 μmol l⁻¹ final concentration) at 37°C for 3 h. After centrifugation, the OD₅₄₀ was measured in each resuspended pellet solution. Zero and 100% haemolysis were determined in 0·9% (w/v) NaCl and water, respectively. The HC₅₀ was defined as the mean peptide concentration from three independent experiments that produced 50% haemolysis.
Circular dichroism spectroscopy

The CD spectra of the peptides were recorded with a J-810 spectropolarimeter (JASCO, Victoria, BC, Canada). The spectra were measured between 190 and 250 nm at 0.1 nm intervals at 25°C with a scan rate of 20 nm min⁻¹. Peptides at a constant concentration of 0.3 mg ml⁻¹ were prepared in three different solvents [water, 30 mmol l⁻¹ SDS, 50% trifluoroethanol (TFE)], and a quartz cuvette with a path length of 1 mm was used in the measurements. The spectra of three consecutive scans were averaged, and the CD spectra of the appropriate solvent were subtracted from each corresponding peptide spectrum. The percentage of helicity was estimated with the program CDNN.

Preparation of liposomes

Phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), cholesterol and cardiolipin (CL) were purchased from Sigma (Shanghai, China). Small unilamellar vesicles (SUVs) were prepared as follows: the Staph. aureus type had a PG/CL molar ratio of 3 : 1, the E. coli type had a PG/CL/PE molar ratio of 2 : 7 : 1, and the human erythrocyte type had a PC/cholesterol molar ratio of 10 : 1 (Ishibashi et al. 1999; Epand et al. 2006; Chen et al. 2007). Briefly, the phospholipids were dissolved in chloroform at each of the aforementioned ratios and dried via rotatory evaporation to form a multilamellar lipid film. The lipid film was then dried under vacuum overnight and then suspended in 3 ml of 5 mmol l⁻¹ N-Tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES) buffer (pH 7.4) containing 0.1 mol l⁻¹ NaCl via vortex mixing. The suspension was sonicated under nitrogen in an ice bath for 8 min using a probe-type sonicator. The generated liposomes were immediately used for titration measurements. Large unilamellar vesicles (LUVs) with encapsulated calcein were prepared according to previously reported protocols (Epand et al. 2006; Kim et al. 2010; Coccia et al. 2011), with slight modifications. Briefly, the phospholipids were dissolved in chloroform at each of the previously described ratios. After vacuum evaporation and overnight drying, a dye solution (90 mmol l⁻¹ calcein, 20 mmol l⁻¹ TES, 100 mmol l⁻¹ NaCl, pH 7.4) was added to each sample. The mixed solution was frozen and thawed in liquid nitrogen for 10 cycles and then extruded 10 times through a 200-nm polycarbonate membrane. After extrusion, untrapped calcein was removed from the LUVs with encapsulated calcein via gel filtration through a Sephadex G-50 column and eluted using TES buffer. The calcein-loaded liposomes (90 μmol l⁻¹ final concentration) were used for leakage measurements.

Trp fluorescence and quenching via acrylamide and KI

Measurement of Trp fluorescence was performed in quartz cuvettes with a path length of 1 cm. Fluorescence spectra were recorded on a Varioskan Flash spectrophotometer (Thermo Scientific, Beijing, China) with both excitation and emission band passes set at 5 nm. Spectroscopic titration of the peptides with liposomes was performed as described by Park et al. (1992). Appropriate liposomes (5 mmol l⁻¹) were successively added to a solution of peptides (10 μmol l⁻¹) in 5 mmol l⁻¹ TES buffer (pH 7.4). The mixture was then incubated at 25°C for 10 min to achieve equilibration. The Trp residues in each peptide were excited at 280 nm, and the emission spectra were recorded in the range of 300–400 nm. Spectra were recorded as a function of the lipid/peptide molar ratio and corrected for the contribution of light scattering in the presence of liposomes. Blue shifts were calculated as the differences in wavelength of the maxima in emission spectra of lipid/peptide and peptide-only samples. The quenching of peptide Trp fluorescence was measured via titration with a water-soluble quencher (either acrylamide or KI) for the peptides in the absence or presence of liposomes (Park et al. 1992). The lipid/peptide molar ratio was 100 : 1. The quenched samples were excited at 295 nm, and emission was recorded at 350 nm. The effect of the quencher on the fluorescence of the peptide was analyzed using the following Stern–Volmer equation: 

\[ F_0/F = 1 + K_{sv}[Q] \]

where \( F_0 \) and \( F \) are the fluorescence intensities in the absence or presence of a quencher at a concentration of \([Q]\), respectively, and \( K_{sv} \) is the Stern–Volmer quenching constant.

Leakage of calcein from liposomes

To address the interactions of Trp-containing AMPs with model membranes, we measured the ability of peptides to induce calcein leakage from three types of LUVs (Kim et al. 2010; Coccia et al. 2011). The fluorescence intensity of calcein released from the LUVs was recorded at 530 nm with an excitation wavelength of 493 nm. To achieve complete release of calcein, 10% (v/v), TritonX-100 (final concentration 0-1%) was added to dissolve the vesicles. Measurements were repeated three times under each condition. The percentage of dye leakage caused by the peptides was calculated as follows: 

\[ \text{dye leakage} = 100 \times \left( F - F_0 \right)/\left( F_1 - F_0 \right) \]

where \( F_0 \) is the fluorescence intensity in the absence of a peptide; \( F_1 \) is the initial fluorescence intensity after the addition of TritonX-100; and \( F \) is the fluorescence intensity after treatment with the peptides.
Results

Design of Trp-containing antimicrobial peptides

To investigate the relationship between the Trp residues in Trp-containing peptides and antimicrobial activity, five Trp-containing peptides were designed and synthesized by substituting Trp residues for Ile, Ile, Leu, and Leu in L-K6. The mean hydrophobicity values of the peptides used in this study were calculated using hydrophobicity scales (Mant et al. 2009) and were determined as follows: Trp, 32.4; Phe, 29.1; Leu, 23.3; Ile, 21.4; Met, 15.7; Tyr, 14.7; Val, 13.4; Pro, 9.0; Cys, 7.6; Lys, 2.8; Glu, 2.8; Ala, 2.8; Thr, 2.3; Asp, 1.6; Arg, 0.6; Gln, 0.6; His, 0.6; Ser, 0.6; Gly, 0.6; and Asn, −0.6. Amphiphilicity values were calculated for the peptides based on the hydrophobic moment (Eisenberg et al. 1982) using the software package Jemboss version 1.5 (Carver and Bleasby 2003). The amino acid sequences, calculated molecular weights, net charges, mean hydrophobicity values (H) and amphiphilicity values of the peptides are shown in Table 1. The amount of positive charge and number of amino acids for each of these designed Trp-containing peptides were the same as in the model peptide L-K6, but the H values were increased. The H values of the peptides varied from 12.45 (L-K6) to 14.85 (L5WL11-WL12W). The amphiphilicity values of the nine designed peptides were not significantly changed compared with L-K6.

Antimicrobial and haemolytic activities of the peptides

The MICs of the peptides against the examined bacteria are listed in Table 2. In addition to L-K6, all of the peptides exhibited antimicrobial activity against Gram-positive bacteria, E. coli and Ps. aeruginosa, suggesting that the Leu and Ile residues of L-K6 can be replaced with bulky aliphatic amino acids. In comparison with L-K6, I1W, I4W and L5W exhibited stronger activity against Gram-positive bacteria (the geometric means for these MICs for Gram-positive bacteria were 3.06, 1.74 and 2.93 μmol l⁻¹, respectively). Additionally, I1W and I4W exhibited significant activity against all of the tested Gram-negative bacteria. However, six of the peptides, L5W, L11W, L12W, L5WL11W, L11WL12W and L5WL11WL12W, were inactive against the Gram-negative bacteria Ent. cloacae, Kl. pneumonia (subsp.) and Pr. mirabilis at the tested concentrations. The antimicrobial activity against Gram-positive and Gram-negative bacteria did not increase when the Leu residue at position 12 or 11 was substituted with a Trp residue. L12W exhibited the lowest activities against both Gram-positive and Gram-negative bacteria, although its hydrophobicity was higher than that of L-K6, I1W and I4W. Moreover, when Leu 5 and Leu 11, Leu 5 and Leu 12, Leu 11 and Leu 12, or all three residues was substituted with Trp, antimicrobial activity decreased. Therefore, the antimicrobial activity of these peptides did not increase as their hydrophobicity increased. However, the position of the Trp residues in the designed peptides is an important factor in their antimicrobial activity. The effect of the peptides on erythrocyte haemolysis is shown in Table 2. Despite their increased hydrophobicity, none of the designed peptides showed haemolytic activity at the tested concentrations.

Circular dichroism spectra

The secondary structures of the designed Trp-containing peptides in water, 30 mmol l⁻¹ SDS or 50% (v/v) TFE/water, which mimicked the hydrophobic environment of membrane, were assessed via CD spectroscopy. The results showed that all of the peptides presented a random coil structure in water (Fig. 1a) but adopted a significantly ordered structure in 30 mmol l⁻¹ SDS and 50% (v/v) TFE/water (Fig. 1b,c) that exhibited negative double-minimum peaks at 208 and 222 nm, indicating that the peptides formed α-helical structure. Based on the molar ellipticity at 222 nm, the calculated helical contents for these peptides were 33–99% to 75% in TFE or SDS solution. I4W showed the highest α-helical percentage (75%), while L12W exhibited the lowest helical conformation.

Leakage of calcein from liposomes

The percentage of calcein leakage after the addition of the peptides was used as a measure of membrane permeability. Five of the designed peptides were tested for their

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Amphiphatic*</th>
<th>H†</th>
<th>MW</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-K6</td>
<td>IKKLSIKKKLLK</td>
<td>0.83</td>
<td>12.45</td>
<td>16630</td>
</tr>
<tr>
<td>I1W</td>
<td>WIKILSIKKKLLK</td>
<td>0.82</td>
<td>12.57</td>
<td>16261</td>
</tr>
<tr>
<td>I4W</td>
<td>IKKWLISIKKKLLK</td>
<td>0.83</td>
<td>12.57</td>
<td>16261</td>
</tr>
<tr>
<td>L5W</td>
<td>IKKWSIKKKKLK</td>
<td>0.83</td>
<td>13.33</td>
<td>16261</td>
</tr>
<tr>
<td>L11W</td>
<td>IKKLSIKKKWLK</td>
<td>0.82</td>
<td>13.33</td>
<td>16261</td>
</tr>
<tr>
<td>L12W</td>
<td>IKKLSIKKKWK</td>
<td>0.83</td>
<td>13.33</td>
<td>16261</td>
</tr>
<tr>
<td>L5WL11W</td>
<td>IKKWSIKKKWLK</td>
<td>0.83</td>
<td>14.09</td>
<td>16992</td>
</tr>
<tr>
<td>L5WL12W</td>
<td>IKKWSIKKKWK</td>
<td>0.83</td>
<td>14.09</td>
<td>16992</td>
</tr>
<tr>
<td>L11WL12W</td>
<td>IKKLSIKKKWWK</td>
<td>0.82</td>
<td>14.09</td>
<td>16992</td>
</tr>
<tr>
<td>L5WL11</td>
<td>IKKWSIKKKW</td>
<td>0.83</td>
<td>14.85</td>
<td>17722</td>
</tr>
</tbody>
</table>

*Amphiphaticity was determined by the calculation of hydrophobic moment (Eisenberg et al. 1982; Carver and Bleasby 2003).
†The mean hydrophobicity values (H) of the peptides calculated using the hydrophobicity scales (Mant et al. 2009) were the total hydrophobicity (sum of all residue hydrophobicity indices) divided by the number of residues. Net charges of all peptides are +6, pl values are 10.7. **Bold indicates the position of tryptophan W.
Table 2  Minimum inhibitory concentrations of the peptides against representative Gram-positive and Gram-negative bacteria and their haemolytic activities

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>G⁺ bacteria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>5.00</td>
<td>3.13</td>
<td>1.56</td>
<td>2.50</td>
<td>5.00</td>
<td>12.5</td>
<td>2.50</td>
<td>2.50</td>
<td>12.5</td>
<td>2.50</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>3.13</td>
<td>2.50</td>
<td>2.50</td>
<td>1.56</td>
<td>2.50</td>
<td>5.00</td>
<td>2.50</td>
<td>2.50</td>
<td>10.0</td>
<td>5.00</td>
</tr>
<tr>
<td>Enterococcus faecium</td>
<td>5.00</td>
<td>1.56</td>
<td>1.56</td>
<td>2.50</td>
<td>5.00</td>
<td>10.0</td>
<td>5.00</td>
<td>5.00</td>
<td>12.5</td>
<td>12.5</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>6.25</td>
<td>5.00</td>
<td>1.56</td>
<td>5.00</td>
<td>12.5</td>
<td>18.75</td>
<td>25.0</td>
<td>20.0</td>
<td>6.25</td>
<td></td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>4.69</td>
<td>3.13</td>
<td>1.56</td>
<td>3.13</td>
<td>12.5</td>
<td>2.50</td>
<td>5.00</td>
<td>6.25</td>
<td>2.50</td>
<td></td>
</tr>
<tr>
<td><strong>G⁻ bacteria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>3.13</td>
<td>3.13</td>
<td>6.25</td>
<td>2.50</td>
<td>2.50</td>
<td>9.37</td>
<td>2.50</td>
<td>3.13</td>
<td>10.0</td>
<td>2.50</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>6.25</td>
<td>3.13</td>
<td>3.13</td>
<td>9.73</td>
<td>4.69</td>
<td>18.75</td>
<td>15.62</td>
<td>5.00</td>
<td>10.0</td>
<td>4.69</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>&gt;100</td>
<td>25.0</td>
<td>50.0</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td>&gt;100</td>
<td>12.5</td>
<td>12.5</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>&gt;100</td>
<td>6.25</td>
<td>12.5</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>HCSO (µmol l⁻¹)</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
</tr>
</tbody>
</table>

MIC: minimal peptide concentration required for total inhibition of microbe growth in liquid medium. The values are the means of three independent experiments performed in four replicates.

Figure 1  CD spectra of the designed Trp-containing peptides in water (a), 50% TFE/water (b) and 30 mmol l⁻¹ SDS (c). All of the peptides are unstructured in water alone but adopt an α-helical structure in the presence of trifluoroethanol or SDS micelles, which mimic a membrane environment. (●) I1W; (●) I4W; (▲) L5W; (●) L11W and (●) L12W.

ability to induce the release of the self-quenching fluorescent dye calcein from calcein-encapsulated LUVs. The results showed that all of the tested peptides caused calcein release from negatively charged LUVs composed of PG/CL or PG/CL/PE, but the leakage-inducing ability of L12W was weaker than that of the other peptides, as I1W, I4W, L5W and L11W caused total release of calcein at a concentration of 5 µmol l⁻¹ (Fig. 2a,b). The leakage-inducing ability of the peptides at 2.5 µmol l⁻¹ from the PG/CL or PG/CL/PE liposomes is indicated as follows: I4W (100%) > L5W (96.21%) > I1W (90.58%) > L11W (83.28%) > L12W (70.69%), and L5W (95.11%) > L11W (90.26%) > I1W (89.71%) > I4W (82.47%) > L12W (68.53%). Conversely, when the peptides were added to zwitterionic LUVs composed of PC/cholesterol lipids, there was little calcein released from the LUVs (Fig. 2c). This demonstrated that these five peptides did not disrupt bilayer organization in neutral liposomes.

Fluorescence spectra

The fluorescence emission characteristics of the Trp indole group are sensitive to the immediate environment and are often used to monitor the binding of peptides.
to membranes. We examined the fluorescence of five of the designed peptides in aqueous buffer and in three types of liposomes. In aqueous buffer, the maximum wavelengths of the tested peptides were approximately 340 nm, meaning that the Trp residues were fully exposed to the aqueous environment (Rex 2000). However, a large blue shift in the fluorescence emission maximum occurred when the peptides were titrated with increasing concentrations of liposomes mimicking Gram-positive (Staph. aureus) and Gram-negative (E. coli) bacteria, indicating that these Trp-containing peptides bind to bacterial membranes. In the PG/CL liposomes, the blue shifts of I1W, I4W, L11W and L12W in the Trp fluorescence emission maximum all occurred around a lipid/peptide molar ratio of 60 : 1, but the blue shift of L5W occurred at a lipid/peptide molar ratio of 20 : 1, and I1W and I4W exhibited the maximum blue shift (24 nm) (Fig. 3). However, in the presence of PG/CL/PE liposomes, the blue shifts of all of the peptides, except L12W, were saturated at a lipid/peptide molar ratio of 80 : 1, and the maximum shifts for L5W and L11W were the highest among the examined peptides. The results showed that I1W and I4W presented the highest membrane affinity in the PG/CL liposomes, but in the presence of PG/CL/PE liposomes, L5W and L11W exhibited the highest membrane affinity.

Figure 2 The release of calcein from liposomes composed of (a) phosphatidylglycerol (PG) and cardiolipin (CL) (3 : 1); (b) PG, CL and phosphatidylethanolamine (2 : 1 : 7); (c) phosphatidylcholine and cholesterol (10 : 1). The fluorescence intensity is shown as a function of the time after the addition of a series of concentrations of the tested peptides. The lipid concentration was 70 µmol l⁻¹. Each data point represents an average of six independent experiments. (♦) I1W; (■) I4W; (▲) L5W; (×) L11W and (□) L12W.

Figure 3 Blue shifts in Trp emission maxima of peptides in (a) phosphatidylglycerol (PG) and cardiolipin (CL) (3 : 1); (b) PG, CL and phosphatidylethanolamine (2 : 1 : 7); (c) phosphatidylcholine and cholesterol (10 : 1) liposomes. Each data point is the average of six independent experiments ± the standard error of the mean. (©) L12W; (○) L11W; (●) L5W; (■) I4W and (♦) I1W.
Quenching of Trp fluorescence by acrylamide and KI

Fluorescence-quenching experiments were next performed for the five peptides using the quencher acrylamide or KI to further investigate the membrane-integrated state of the Trp fluorophores in various membrane environments. If a Trp side chain inserts into the hydrophobic core of a bilayer, it becomes less accessible to soluble acrylamide or KI quenching. The quenching of Trp fluorescence is directly related to the concentration of the quencher, so titration with neutral acrylamide or KI allows for the calculation of the Stern–Volmer constant ($K_{sv}$), which quantitatively measures the accessibility of the Trp residues in various lipid environments. The fluorescence of Trp residues decreased significantly upon the addition of either acrylamide (Fig. S1a–e) or KI (Fig. S2a–e) to the peptide solution in the presence of liposomes compared with that in the absence of liposomes. The slopes of the Stern–Volmer plots of all of the tested peptides showed a 15- to 60-fold decrease in $K_{sv}$ with KI quenching and a 6- to 40-fold decrease in $K_{sv}$ with acrylamide quenching in PC/PG and PG/CL/PE liposomes, compared with that in aqueous buffer solution (Fig. 4a,b). In the PG/CL liposomes, the values for $K_{sv}$ quenching with both KI and acrylamide decrease in the following order: L12W = L11W > L5W > I1W > I4W, indicating that the Trp residue of I4W ($K_{sv} = 0.6$ and 0.8 with KI and acrylamide quenching, respectively) was more deeply buried in the membrane than those of the other peptides. In PG/CL/PE-type liposomes, L11W showed the lowest $K_{sv}$ value associated with KI quenching ($K_{sv} = 0.6$), but I4W presented the lowest $K_{sv}$ value associated with acrylamide quenching ($K_{sv} = 2.8$) out of all of the tested peptides. The results indicated that the Trp residues were less accessible to quenchers in the presence of liposomes and were inserted into the membrane. As shown in Fig. 4a,b, all the tested peptides showed much lower $K_{sv}$ in KI quencher compared with those in acrylamide quencher.

Discussion

Tryptophan residues show a strong preference for the interfacial region of biological membranes, and this property endows Trp-containing peptides with the unique ability to interact with the surface of bacterial cell membranes (Rex 2000; Lu et al. 2001; Khandelia and Kaznessis 2007). By designing several active membrane-associated Trp-rich peptides, we attempted to reveal a complex relationship between the structural factors that contribute to antimicrobial activity, and the features that determine haemolytic activity related to Trp residues. Thus, a series of Trp-containing peptides were designed and synthesized. Most of the designed peptides showed good antimicrobial activity against the tested bacterial strains, without cytotoxicity. I1W, I4W and L5W demonstrated stronger activity than the other peptides against both Gram-positive and Gram-negative bacteria, and the other peptides exhibited antimicrobial activities that were...
weaker than that of the model peptide, L-K6. Increasing the number of Trp residues in the peptides to two or three did not enhance their antimicrobial potency, suggesting that the number of Trp residues in the peptide is not a critical factor for cell lysis, at least in this case. However, the position of the Trp residues in the peptide is an important factor in its antimicrobial activity. We found that the fourth and first Trp residues (I4W and I1W) at the amino terminus of the peptides played important roles in increasing their antimicrobial activities, whereas the first and second Trp residues (L11W and L12W) at the carboxyl terminus of the peptides inhibited activity. The data supported the view that strong hydrophobic amino acid residues at the N-terminus and cationic residues at the C-terminus are required for peptide antimicrobial activity (Campanga et al. 2007; Chi et al. 2007). Hydrophobicity allows a peptide to insert more deeply into the hydrophobic core of the lipid bilayer, thereby affecting pore size and peptide stability via the ‘barrel stave’ mechanism (Conlon et al. 2007). However, in the present study, increasing peptide hydrophobicity did not increase the antimicrobial activity of the designed Trp-containing peptides, suggested that these peptides did not act via the barrel stave mechanism. Haemolysis was not observed at peptide concentrations lower than 500 μmol l⁻¹, indicating that the designed peptides, like their parent peptide, were not particularly cytotoxic.

At this point, it is unclear why I4W and I1W acted as more potent antimicrobials than the other Trp-substituted peptides. We determined the secondary structures of the designed Trp-containing peptides based on their CD spectra and investigated the interactions between the peptides and phospholipid membranes by monitoring the permeation kinetics across the liposome lipid bilayer and the fluorescence emission of the tryptophan residues in peptides. Permeation of the peptides across a bacterial mimic membrane and mammalian mimic membrane was indirectly measured using a calcein release assay. Calcein release from LUVs reflects the membrane perturbation and selectivity of peptide-membrane interaction with model membrane (Dos et al. 2004). All tested peptides caused calcein release from negatively charged LUVs composed of PG/CL or PG/CL/PE, I1W, I4W, and L5W caused calcein release at 2.5 μmol l⁻¹. Conversely, when the peptides were added to zwitterionic LUVs composed of PC/cholesterol lipids, there was little calcein released from the LUVs. This demonstrates that these five peptides do not disrupt bilayer organization in neutral liposomes. All of the tested Trp-containing peptides adopted an α-helical conformation in TFE or SDS micelles, which mimic a hydrophobic environment of membrane, and I4W and I1W presented the highest helical content (75%), whereas that of L12W was lowest (30%).
et al. 2006; Park et al. 2011). Moreover, membranes saturation can account for the fluorescence intensity of Trp residues. A deviation in the fluorescence intensity vs lipid concentration relationships was observed at low lipid concentrations for the most anionic systems (Melo and Castanho 2007). The data from the Trp quenching titration experiments using acrylamide or KI also demonstrated that the Trp residues in 1, 4 and 5 were deeply embedded in negatively charged membranes. 1, 4 and 5 showed the lowest \( K_{sv} \) values associated with acrylamide and KI quenching in the presence of PG/PG liposomes, and 5 and 11 showed the lowest \( K_{sv} \) values associated with acrylamide and KI quenching in the presence of PG/CL/PE liposomes out of all of the tested peptides. Interestingly, all the tested peptides showed much lower \( K_{sv} \) in KI quencher compared with those in acrylamide quencher. Acrylamide, a neutral quencher, is very sensitive to the degree of accessibility of tryptophan to the solvent containing the acrylamide and presents an advantage in that no electrostatic interactions take place with the negatively charged phospholipids (Zhao and Kinnunen 2002; Rezanso et al. 2005; Stobiecka 2005). But KI, an ionic quencher, the electrostatic interaction of I\( ^{-} \) with positively charged residues of peptide, as well as neutralization of repulsion with acidic headgroups in the liposomes might affect the quenching of KI. The results indicated that the Trp residues were less accessible to the quencher in the presence of liposomes and were inserted into the membrane. Overall, our findings suggest that Trp residues exhibit a strong membrane-disruptive activity, and this property endows Trp-containing AMPs with a unique ability to interact with the surface of bacterial cell membranes, possibly improving antimicrobial activity. The Trp-containing peptides designed in this study may be good candidates for new antibiotic agents and for developing new therapeutic approaches.

**Acknowledgements**

This work was supported by the National Natural Science Foundation of China (Grant No. 31272314 and No. 81202448), the Natural Science Foundation of Liaoning (Grant No. 201202121) and the Program for Liaoning Innovative Research Team in University (LT2012019).

**References**


### Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1** Stern–Volmer plots of peptide L5W (a), L11W (b), L12W (c), I1W (d) and I4W (e) for the designed peptides in TES buffer, PC/cholesterol (10 : 1), PG/CL (3 : 1) and PG/PE/CL (2 : 7 : 1) liposomes.

**Figure S2** Stern–Volmer plots of peptide L5W (a), L11W (b), L12W (c), I1W (d) and I4W (e) for the designed peptides in TES buffer, PC/cholesterol (10 : 1), PG/CL (3 : 1) and PG/PE/CL (2 : 7 : 1) liposomes.

---

Journal of Applied Microbiology © 2013 The Society for Applied Microbiology