Selective cytotoxicity following Arg-to-Lys substitution in tritrpticin adopting a unique amphipathic turn structure

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Abstract  In antimicrobial peptides, the cationic property due to basic amino acids has been widely recognized as an important factor to promote electrostatic interaction with negatively charged phospholipids. However, little is known about the differences between two basic residues, Arg and Lys, in membrane binding affinity. Tritrpticin is an Arg- or Trp-rich antimicrobial peptide with a broad spectrum of antibacterial and antifungal activity. To investigate the structural and functional differences between Arg and Lys residues, here we designed and synthesized Arg-containing peptides, tritrpticin and SYM11, and their counterpart Lys-substituted peptides, TRK and SYM11KK, respectively. Although there were no remarkable conformational differences between Arg-containing and Lys-substituted peptides, TRK and SYM11KK exhibited almost two-fold enhanced antibacterial activity but significantly reduced hemolytic activity as compared to tritrpticin and SYM11, respectively. Furthermore, Arg-containing peptides showed strong binding affinity to both zwitterionic and anionic liposomes, whereas Lys-substituted peptides interacted weakly with zwitterionic liposomes but strongly with anionic liposomes. These results suggest that the primary amine of Lys interacts less electrostatically with zwitterionic phospholipids than the guanidinium group of Arg. Our results obtained in this study may be helpful in the design of drugs that target negatively charged phospholipids.

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Key words: Antimicrobial peptide; Tritrpticin; Arg-to-Lys substitution; Cell selectivity

1. Introduction

Natural antimicrobial peptides are produced by a wide variety of insects, amphibians and mammals, including humans, and their structures and functions have been characterized over recent decades [1–3]. Although antimicrobial peptides show great structural diversity, a common feature is their cationic nature due to multiple Arg and/or Lys residues [4–6]. These cationic properties are very important for binding to negatively charged surfaces in lipid membranes of bacteria [7]. Although the exact mechanism by which peptides kill pathogens is not fully understood, most natural antimicrobial peptides appear to act by damaging membranes [8].

Recent approaches have aimed to produce peptides which are more potent towards bacteria than native peptides, but are not toxic to mammalian cells. For example, the incorporation into lytic peptides of \(\alpha\)-amino acids [9], central hinges induced by Pro residues [10,11], or reduced amide bonds [12] contributed to improved selective cytolytic activity. Furthermore, while Trp residues in several antimicrobial peptides are involved in hemolytic activity, Trp→Leu substitutions in indolicidin and melittin significantly reduced hemolytic activity but maintained antibacterial activity [13,14]. Structure-function studies of antimicrobial peptides indicate that a number of parameters modulate antibiotic activity, including net positive charge, overall hydrophobicity, hydrophobic moment and helical propensity [15–18]. However, despite extensive studies little is known about the molecular basis underlying the selectivity of peptides for bacterial rather than mammalian cells.

Tritrpticin, a member of the cathelicidin family, is an Arg- or Trp-rich antimicrobial peptide first identified following screening of a porcine bone marrow cDNA library [19]. It was found to have broad spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria and fungi. The primary structure of tritrpticin is remarkable because of its high content of Arg and Trp residues, which are very important in electrostatic and hydrophobic interactions with phospholipids, respectively. It forms a unique amphipathic turn structure when bound to sodium dodecyl sulfate (SDS) micelles with the Trp side chains separated from the Arg residues [20]. In previous studies, we investigated the effects of aromatic Trp residues on hydrophobic interaction with lipid acyl chains. The presence of the multiple Trp residues was essential for hemolytic activity and membrane-disrupting activity [21]. In this report, we compare the differences between Arg-containing and Lys-substituted analogs based on tritrpticin in antibiotic activity and secondary structure. In addition, we describe the functional differences of two basic amino acids in the interaction with zwitterionic and anionic phospholipids by dye release experiments and tryptophan fluorescence shift measurements.

Abbreviations: CD, circular dichroism; hRBC, human red blood cells; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MIC, minimal inhibitory concentration; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol; RP-HPLC, reverse-phase high-performance liquid chromatography; SDS, sodium dodecyl sulfate; SUVs, small unilamellar vesicles

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2. Materials and methods

2.1. Microorganisms
Escherichia coli KCTC 1682, Salmonella typhimurium KCTC 1926, Pseudomonas aeruginosa KCTC 1637, Bacillus subtilis KCTC 3068, Staphylococcus epidermidis KCTC 1917, Staphylococcus aureus KCTC 1621 and Candida albicans KCTC 7965 were purchased from the Korean Collection for Type Cultures (KCTC), Korea Research Institute of Bioscience and Biotechnology (Taejon, Korea).

2.2. Peptide synthesis, purification and characterization
Peptides were synthesized using solid-phase methodology with fluorescein-9-yl-methoxycarbonyl-protected amino acids. Purification by preparative reversed-phase high-performance liquid chromatography (RP-HPLC) gave final products >95% pure by analytical RP-HPLC. Peptides were characterized by Kratos Kompact matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Shimadzu, Japan) (Table 1).

2.3. Antimicrobial activity
The antimicrobial activity of peptides against a range of microorganisms was determined by broth microdilution assay. Briefly, single colonies of bacteria were inoculated into culture medium (LB broth) and cultured overnight at 37°C. An aliquot of this culture was transferred to 10 ml of fresh culture medium and incubated for an additional 3–5 h at 37°C to obtain mid-logarithmic phase organisms. A two-fold dilution series of peptides in 1% peptone was prepared. A set of peptide serial dilutions (100 μl) was added to bacteria (2×10^6 CFU/ml; 100 μl) in 96 well microtiter plates (Falcon), and plates were incubated at 37°C for 16 h. After incubation each well was scanned for the absorbance at 620 nm. The lowest peptide concentration that completely inhibited growth of the organisms was defined as the minimal inhibitory concentration (MIC). The recorded MICs were the average of triplicate measurements from three independent assays.

2.4. Hemolytic activity
Hyman red blood cells (hRBCs) were centrifuged and washed three times with phosphate-buffered saline (PBS; 35 mM phosphate, pH 7.0, 150 mM NaCl). One hundred microliters of 4% (v/v) hRBC suspended in PBS was dispensed into sterile 96-well plates, and 70 μl of freshly prepared peptide solutions was added to each well. Plates were incubated for 1 h at 37°C, then centrifuged at 1000g for 5 min. Aliquots (100 μl) of supernatant were transferred to 96 well plates, where hemoglobin release was monitored using an ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA) by measuring absorbance at 414 nm. Zero and 100% hemolysis were determined in PBS and 0.1% Triton X-100, respectively.

2.5. Circular dichroism (CD) spectroscopy
The CD spectra of peptides were recorded using a Jasco J-715 CD spectrophotometer (Tokyo, Japan) with a 1 mm path length cell. Wavelengths from 190 to 250 nm were measured, with 0.1 nm step resolution, 50 nm/min speed, 0.5 s response time, and 1 nm bandwidth. CD spectra of peptides were collected and averaged over four scans in 10 mM sodium phosphate buffer (pH 7.0) or 30 mM SDS micelles, at 25°C.

2.6. Preparation of liposomes
Small unilamellar vesicles (SUVs) were prepared for dye leakage and tryptophan fluorescence experiments as follows. Phospholipid (7.5 mg) was dissolved in chloroform and dried with a stream of nitrogen to form a thin lipid film on the wall of a glass tube. The lipid film was dried under vacuum overnight and then hydrated with 2 ml Tris–HCl buffer (10 mM Tris–HCl pH 7.4, 154 mM NaCl, 0.1 mM EDTA). The suspension was sonicated under nitrogen in an ice bath for 30 min using a titanium tip sonicator. The lipid concentration was 0.5 mM. Calcein-entrapped liposomes for dye leakage experiments were prepared as follows. Dried lipid was hydrated with 2 ml Tris–HCl buffer containing 70 mM calcein, after which the suspension was vortex-mixed for 10 min. The resultant lipid dispersions were sonicated in ice water for 20–30 min with a titanium-tipped sonicator until clear. Calcein-entrapped vesicles were separated from free calcein by gel filtration chromatography on a Sephadex G-50 column using Tris–HCl buffer.

3. Results

3.1. Peptide synthesis and characterization
In order to investigate the structural and functional differences between Arg and Lys amino acids, we synthesized the Arg-containing peptides, tritrpticin and SYM11, and their counterpart Lys-substituted peptides, TRK and SYM11KK, respectively. The symmetric tritrpticin analogs (SYM11 and SYM11KK) were designed previously by Nagpal et al. [22]. The amino acid sequences of peptides used in this study are summarized in Table 1. The correct molecular weights of the synthetic peptides were confirmed by MALDI-TOF MS.

3.2. Antimicrobial and hemolytic activities
Peptides were tested for their ability to kill bacteria and to lyse hRBC. The MICs of peptides for bacteria are listed in Table 2, and a dose–response curve for hemolytic activities is presented in Fig. 1. We recently reported that tritrpticin has antibacterial and hemolytic properties comparable to those of indolcinid [21]. Compared to tritrpticin and SYM11 (Arg-containing peptides), TRK and SYM11KK (Lys-substituted peptides) showed approximately two-fold greater antibacterial activity, respectively. Interestingly, while tritrpticin and SYM11KK (Table 2) of supernatant were transferred to 96 well plates, where hemoglobin release was monitored using an ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA) by measuring absorbance at 414 nm. Zero and 100% hemolysis were determined in PBS and 0.1% Triton X-100, respectively.

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3.7. Dye leakage
Tris–HCl buffer (pH 7.4) (2 ml) was added to vesicles (20 μl) containing 70 mM calcein in a cuvette to give a vesicle solution with a final concentration of 70 μM lipid. The fluorescence intensities of calcein released from liposomes were monitored at 520 nm (excited at 490 nm) on a Jasco FP-750 spectrofluorometer (Tokyo, Japan) and measured 2 min after the addition of peptides. Fluorescence from liposomes lysed with Triton X-100 was used as an indicator of 100% leakage.

3.8. Tryptophan fluorescence
Tryptophan fluorescence measurements were made on a Jasco FP-750 spectrofluorometer (Tokyo, Japan). Each peptide was added to 1 ml 10 mM Tris–HCl buffer (pH 7.4) containing 0.5 mM liposomes, and the peptide/liposome mixture was allowed to interact at 25°C for 10 min. Fluorescence was excited at 280 nm, and the emission scanned from 300 to 400 nm. The fluorescence spectrum of each peptide with liposomes was subtracted from the spectrum of the liposomes alone.

Table 1
Amino acid sequences and molecular weights of tritrpticin and synthetic analogs

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Mass Calculated</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tritrpticin</td>
<td>VRKFPHWLPFFRR</td>
<td>1902.4</td>
<td>1903.3</td>
</tr>
<tr>
<td>TRK</td>
<td>VKKFPHWLPFFKK</td>
<td>1790.4</td>
<td>1791.0</td>
</tr>
<tr>
<td>SYM11</td>
<td>RKKFPWLPFFRR</td>
<td>1690.1</td>
<td>1690.9</td>
</tr>
<tr>
<td>SYM11KK</td>
<td>KKFPWLPFFKK</td>
<td>1578.1</td>
<td>1578.9</td>
</tr>
</tbody>
</table>

*Mass from MALDI-TOF MS.

Table 2
MIC (μg/ml) values for the peptides

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Peptide</th>
<th>Tritrpticin</th>
<th>TRK</th>
<th>SYM11</th>
<th>SYM11KK</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>32</td>
<td>16</td>
<td>16</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>S. typhimurium</td>
<td>32</td>
<td>16</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>32</td>
<td>16</td>
<td>16</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>B. subtilis</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>S. aureus</td>
<td>16</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>8</td>
<td>4</td>
<td>2</td>
<td>1</td>
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</tr>
</tbody>
</table>
SYM11 were relatively strongly hemolytic (37% and 24% at 100 μg/ml, respectively), TRK and SYM11KK displayed negligible hemolytic activity up to 200 μg/ml. These data suggest Arg→Lys substitutions result in improved peptide selectivity between bacterial and mammalian cells.

3.3. CD studies

To examine conformational differences between Arg-containing and Lys-substituted peptides, the secondary structures of peptides were estimated by CD spectroscopy in aqueous environments as well as in SDS micelles. The CD spectra of peptides are presented in Fig. 2. A negative band appeared at about 225 nm in both buffer and SDS micelles, which may be due to tryptophan side chains in peptides. A positive band at 212 nm suggests characteristic β-turn conformation [22]. CD spectrum patterns of Arg-containing peptides were similar to those of Lys-substituted peptides in both buffer and SDS micelles. Similar behaviors were also observed for the peptides in 1-palmitoyl-2-oleoylphosphatidyl-DL-glycerol (POPG) SUVs (data not shown). These data suggest that Arg→Lys substitution has no effect on its structural conversion.

3.4. Dye leakage from the liposomes

The membrane-lytic abilities of peptides were investigated by examining calcine dye release from zwitterionic 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and anionic POPG liposomes. The Arg-containing peptides tritrpticin and SYM11 caused relatively strong calcine leakage from POPC liposomes, with 34% and 23% leakage at 32 μg/ml, respec-

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Fig. 1. Hemolytic activity dose-response curves of the peptides against human erythrocytes. The assay was performed as described in Section 2. Designations are as follows: tritrpticin (●); TRK (○); SYM11 (▼); SYM11KK (▼).

Fig. 2. CD spectra of tritrpticin (A), TRK (B), SYM11 (C) and SYM11KK (D) in 10 mM sodium phosphate buffer, pH 7.2 (open circles), or 30 mM SDS micelles (closed circles). Spectra were taken at peptide concentrations of 50 μg/ml.

Fig. 3. Release of calcine fluorescent probe from POPC (A) or POPG (B) liposomes. Leakage is defined as the percent leakage from 70 μM lipid after 2 min incubation with peptides. Symbols: tritrpticin (●); TRK (○); SYM11 (▼); SYM11KK (▼).
tively, whereas the Lys-substituted analogs TRK and SYM11KK showed less membrane-lytic activity, with 10% and 9% leakage at 32 μg/ml, respectively (Fig. 3). In contrast, Lys-substituted analogs showed similar or slightly increased potency towards anionic POPG liposomes, compared to Arg-containing peptides. These results indicate that Arg-to-Lys substitution in tritrpticin leads to selective membrane-disrupting ability between POPC and POPG liposomes.

3.5. Binding of peptides to liposomes

The fluorescence emission characteristics of Trp residues are sensitive to their environment, and were used to monitor the binding of peptides to lipid vesicles. All peptides listed in Table 1 have three Trp residues in the central sequence region. We monitored fluorescence changes of Trp residues of peptides in buffer as well as in the presence of lipid vesicles (Table 3). In Tris buffer, the wavelength maximum range of peptides was 346–347 nm, indicating that Trp residues were hydrophic in nature. Upon adding peptides to POPC and POPG liposomes, the Trp fluorescent maxima shifted to a shorter wavelength. TRK and SYM11KK showed a small blue shift (5 nm) upon interaction with POPC liposomes, whereas tritrpticin and SYM11 displayed a large blue shift (10 nm). In contrast, both Arg-containing and Lys-substituted peptides exhibited similar fluorescent maxima in the presence of POPG liposomes (335–336 nm). These results suggest Lys-substituted peptides interact weakly with zwitterionic phospholipids, but strongly with anionic phospholipids.

4. Discussion

The non-specific binding affinity of antimicrobial peptides to negatively charged bacterial membranes depends upon electrostatic interactions. In spite of the diversity of amino acid sequences in antimicrobial peptides, most have a high net positive charge due to multiple Arg and/or Lys residues. Recent studies have investigated the effect of different basic residues on antibacterial peptide activity and cell selectivity. Muhle and Tam [23] found that Arg-to-Lys substitution in a cyclic dicysteine-stabilized β-stranded peptide decreased activity against Gram-negative bacteria. Other studies showed that for lactoferricin B and bactenecin 5, which have no hemolytic activity, the replacement of Arg for Lys reduced antibacterial activity [24]. Conversely, for protegrin-1, the substitution of Arg with Lys or ornithine resulted in a two- or four-fold increase in activity against Gram-negative bacteria [25]. Thus, in the studies cited above, in which only antibacterial activity was investigated, the effect of Arg for Lys substitution on antimicrobial activity varied, with both enhancement and reduction observed for different peptides.

In the present study, we investigated the differences between Arg-containing and Lys-substituted peptides based on tritrpticin sequence, with respect to biological activity, secondary structure and membrane interaction. We found that while CD measurements indicated there were no remarkable conformational differences between Arg-containing and Lys-substituted peptides, the Arg-containing peptides (tritrpticin and SYM11) were both antibacterial and hemolytic, whereas the Lys-substituted analogs (TRK and SYM11KK) exhibited only antibacterial activity. In addition, tryptophan fluorescence shift measurements suggested that the four Arg residues strongly interacted with both zwitterionic and anionic phospholipids, whereas the four Lys residues interacted weakly with zwitterionic phospholipids, but strongly with anionic phospholipids. The selective membrane interaction with negatively charged phospholipids following Arg-to-Lys substitution in tritrpticin might explain the selective antibacterial activity. Peptides that promote increased membrane curvature tend to be lytic to membranes in a non-specific manner [26]. Tryptophan is known to promote positive membrane curvature [27] but substitution of Arg with Lys will reduce the positive curvature [28] and may cause the peptide to be less lytic to erythrocytes.

Like tritrpticin, indolicidin (ILPWKWPPWRR-NH₂) is unusually rich in Trp, Pro and Arg amino acids, and the peptide structure is extended with two half-turns following the binding to zwitterionic dodecylphosphocholine [29]. Interestingly, our preliminary results indicate that Arg→Lys substitution in indolicidin caused a significant decrease in hemolytic activity, but did not affect antibacterial activity (data not shown). Further study of the biological and biophysical differences between Arg and Lys residues in other antimicrobial peptides is in progress. Although both Arg and Lys residues have +1 charge in neutral buffer, Arg residue has a more dispersed positive charge due to its guanidinium side chain group. The primary amine of Lys and the guanidinium group of Arg seem to interact differently with phospholipids, and this may explain differences in antibiotic activity between Arg- and Lys-containing peptides.

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References


