Helix Stability Confers Salt Resistance upon Helical Antimicrobial Peptides

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Running title: Helix Stability and Salt Resistant Antimicrobial Peptides

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SUMMARY

Salt sensitivity of antimicrobial peptides poses a major obstacle in their development as novel antibiotics. Here we report the use of helix-capping motifs to confer salt-resistance upon helical antimicrobial peptides. The helical content of the template peptide [RLLR]_5 was almost completely destroyed at salt concentrations over 200 mM NaCl, leading to a 8–32-fold decrease in antimicrobial activity. However, the introduction of helix-capping motifs at the helix termini resulted in a structurally stable peptide which retained membrane-permeabilizing and antimicrobial activities upon exposure to salt. Furthermore, the peptide with helix-capping motifs directly inhibited the in vivo growth of Streptococcus pyogenes, which causes localized fasciitis in mice, and prevented the necrosis of the epidermis, dermis, and subcutaneous muscle layers. Results indicate that the adoption of helix-capping motifs into salt-sensitive antimicrobial peptides provides the necessary structural stability for the peptides to permeabilize cell membranes and cause cell death at physiological salt concentrations.
Introduction

The rapid emergence of antibiotic-resistant bacterial pathogens is a serious problem, and extensive efforts have been focused on the development of new classes of antimicrobial agents\(^1\). One major class comprises amphipathic α-helical antimicrobial peptides identified in a variety of organisms, ranging from plants and insects to animals\(^2\). Antimicrobial peptides are often the first line of defense against invading pathogens and play an important role in innate immunity\(^3\). These cationic peptides exhibit antimicrobial activity against a broad range of Gram\(^+\), Gram\(^-\) bacteria and fungi\(^4\). Many antimicrobial peptides appear to act via specific, but not receptor-mediated, permeabilization of microbial membranes. This confers considerable potential for their development as novel therapeutic agents to overcome the resistance problem\(^5\). Numerous studies have focused on discovering novel antimicrobial peptides, elucidating their mechanisms of action, establishing structure-activity relationships and modulating therapeutic properties, with a view to developing these peptides as therapeutic agents\(^5-8\). The salt sensitivity of antimicrobial peptides is a major obstacle in their development as novel agents. As shown by Goldman et al.\(^9\), defensins, which are endogenous antimicrobial peptides in the epithelial surfaces of the lung, become inactive in the presence of high salt concentrations in bronchopulmonary fluids, leading to pulmonary infections in cystic fibrosis patients. Such salt sensitivity is additionally observed in various other antimicrobial peptides, including indolicidins, gramicidins, bactenecins, and magainins\(^10,11\). Although a number of studies report on salt-resistant α-helical antimicrobial peptides\(^11,12\), the factors that determine the salt sensitivity of these peptides remain to be elucidated. Furthermore, to our knowledge, no rationale has been proposed as yet for the systematic design of salt-resistant α-helical antimicrobial peptides peptides without using unnatural amino acids.
The structural features of α-helical antimicrobial peptides have been studied in detail, including helicity, hydrophobic moment, hydrophobicity, net charge, charge distribution and amphipathicity\textsuperscript{5,8}. Interestingly, helix stability is not currently considered a significant parameter in the mechanism of action of these peptides. Since many antimicrobial peptides require an α-helical conformation upon interactions with cell membranes to exert activity, we hypothesize that helix stability plays an important role in the antimicrobial action of these peptides.

An α-helix, by definition, is characterized by consecutive main-chain, $i \rightarrow i+4$ hydrogen bonds between each carbonyl oxygen and an amide hydrogen from the adjacent helical turn\textsuperscript{13}. This pattern is discontinued at the helix termini, since no further turn of helix is present to provide the additional hydrogen bond partners. Helix-capping motifs, which have specific patterns of hydrogen bondings and hydrophobic interactions found at or near the ends of helices in both proteins and peptides\textsuperscript{14}, provide the necessary intramolecular interactions to stabilize α-helices. In this study, we determine the changes in structure, antimicrobial activity and salt sensitivity of α-helical antimicrobial peptides following the introduction of capping motifs at the helix termini.
Experimental procedures

Microorganisms

The microorganisms used in this study were obtained from the American Type Culture Collection (ATCC), and included: *Bacillus subtilis* ATCC 62037, *Staphylococcus aureus* ATCC 15752, *Streptococcus mutans* ATCC 25175, *Escherichia coli* ATCC 27325, *Pseudomonas putida* ATCC 17426, *Salmonella enteritidis* ATCC 13076, *Cryptococcus neoformans* ATCC 34881, *Saccharomyces cerevisiae* ATCC 44774 and *Candida albicans* ATCC 10231. *Streptococcus pyogenes* A77 was a gift from Dr. J. Y. Kong, Korea Research Institute of Chemical Technology.

Peptide preparation

Peptides, either free or biotinylated at the amino termini, were synthesized by solid phase methods, using Fmoc [N-(9-fluorenlymethoxycarbonyl]-protected amino acids on a MilliGen 9050 (Millipore) peptide synthesizer. Synthesized peptides were purified to over 98% homogeneity by preparative reverse phase-high performance liquid chromatography (RP-HPLC) on a C18 column (3.9 X 300 mm, Delta Pak, Millipore), using a linear gradient of 25 to 80% acetonitrile in 0.1% trifluoroacetic acid for 40 min, and assessed for purity by analytical RP-HPLC and matrix-associated laser desorption ionization mass spectroscopy (MALDI II, Kratos Analytical Instruments). The peptide content of lyophilized samples was determined by quantitative amino acid analyses with a Pico-tag system on a Beckman 121 MB amino acid analyzer (Beckman Coulter).
Antimicrobial Activity Assay

The antimicrobial activity of each peptide was determined against nine selected microorganisms using the broth microdilution assay, as described by Steinberg et al.²³ The lowest concentration of peptide that completely inhibited growth was defined as the 'minimal inhibitory concentration' (MIC). MIC values were calculated as an average of independent experiments performed in triplicate. Where required, NaCl was added to each well of the 96-well plate to obtain the desired salt concentration.

Circular Dichroism

Circular dichroism (CD) spectra were obtained using a J-720 spectropolarimeter (Jasco, Tokyo). Each spectrum (190-250 nm) was an average of five scans using a quartz cell with a 1 mm path length at room temperature. The scanning speed was 100 nm/min at a step size of 0.1 nm, a 2 s response time, and 1.0 nm bandwidth. Spectra were measured either in the presence or absence of NaCl in 20 mM sodium dodecylsulfate (SDS). A blank spectrum of a sample containing all components except peptides was subtracted from individual spectra to account for the baseline. The α-helical content was estimated as described previously by Greenfield and Fasman.²⁴

Confocal Laser Scanning Microscopy

Peptide localization in *E. coli* was detected according to the procedure of Park et al.²⁵ *E. coli* cells (10⁵ cfu) in mid-logarithmic phase were incubated with biotinylated peptides (at MIC) in the presence or absence of NaCl in 10 mM NAPB at 37°C for 30 min. Following
incubation, cells were washed with 10 mM NAPB and immobilized on a glass slide. Cells were treated briefly with 0.2% Triton X-100/NAPB. Biotin-labeled peptides were visualized using 20 µg/ml streptavidin-FITC (Boehringer Mannheim) and observed with a Carl Zeiss LSM 410 laser-scanning confocal microscope equipped with a 488 nm bandpass filter.

**Membrane Permeabilization Assays**

The outer membrane permeabilization activity of peptides was determined using the 1-N-phenylnapthylamine (NPN) uptake assay, as described by Falla *et al.* Briefly, an overnight culture of *E. coli* was transferred to fresh LB medium and grown to *A*$_{600}$ of 0.5-0.6. Cells were harvested, washed and resuspended in the same volume of buffer (5 mM HEPES, pH 7.2, 5 mM KCN). To 1 ml cells, 20 µl of 0.5 mM NPN was mixed, and peptide samples (at MIC) were added after 30 sec. The increase in fluorescence as the result of partitioning of NPN into the outer membrane was measured 2 min after the addition of peptides, using a fluorescence spectrophotometer (SLM-Aminco Model 8100). The extent of cytoplasmic membrane permeabilization was determined by measurement of β-galactosidase activity in *E. coli* ML-35 using *o*-nitrophenyl-β-D-galactopyranoside (ONPG), a non-membrane-permeative chromogenic substrate. Mid-logarithmic phase *E. coli* cells were washed in 10 mM NAPB, pH 7.4, and resuspended in the same buffer with 1.5 mM ONPG. The hydrolysis of ONPG to *o*-nitrophenol over time was monitored spectrophotometrically at 420 nm following the addition of peptide samples at MIC. Assays were additionally performed in the presence of 200 mM NaCl.
GAS Infection Model

The *in vivo* antimicrobial activity of the peptides was evaluated by the air pouch GAS infection model\(^{21}\). Groups of 5 4-week-old male ICR mice were anesthesized by injection, chemically depilated of the hair in the backs, and then injected subcutaneously with 1 ml of air to form an air pouch. Bacterial suspension (0.1 ml) containing \(4.5 \times 10^4\) colony forming units (CFUs) of *S. pyogenes* A77 at mid-logarithmic growth phase (100% lethal dose) was inoculated into the air pouch. In peptide inhibition experiments, the mice were given an air pouch injection of the peptide (40 mg/kg-mice) immediately following *S. pyogenes* injection. The animals were observed for a total of 7 days and survival curves were determined. Two days after the inoculation, bacteria in the air pouch were collected and counted, and tissues around the air pouch were excised, fixed in 10% formaldehyde, and embedded in paraffin. The 5-\(\mu\)m-thick tissues were sliced, stained with hematoxylin and eosin, and subjected to histological analysis.
Results

Peptide Design

[RLLR]₅, a peptide consisting of five repeats of RLLR¹⁵, was synthesized as a template peptide because it exhibited strong antimicrobial activity and α-helical structure. To determine the effect of helix stabilization on antimicrobial activity under high salt conditions, analogs were synthesized in which the helix-capping motifs, APKAM (N) and LQKKGI (C)¹⁴, were introduced into either or both the N- and C-terminal ends of [RLLR]ₙ (n=2~5) (data not shown). Analogs with comparable length, charge and activity as [RLLR]₅ were selected to study the effect of helix stabilization on antimicrobial activity (Table 1). Among the numerous helix-capping motifs reported, APKAM and LQKKGI were selected on the basis of short length, net positive charge, and amphipathicity modeled on the Edmundson wheel diagram and Swiss-PdbViewer¹⁶. The N-terminal capping motif, APKAM, designed according to the sequence pattern of an extended capping box, forms a ‘hydrophobic staple’ between the side chains of alanine and methionine¹⁴,¹⁷. The C-terminal helix-capping motif, LQKKGI, which belongs to the Schellman motif family¹⁴, contains a hydrogen bond between the amide group of leucine and the carbonyl group of isoleucine, and between the amide group of glutamine backbone and carbonyl group of glycine. The associated hydrophobic interactions within this motif are between leucine and isoleucine. To determine the effects of either N- or C-terminal capping on helical stability and antimicrobial activity, N-[RLLR]₃ and [RLLR]₃-C were synthesized. In addition, N-[RLLR]₂-C was designed to analyze the combined effect of the N- and C-terminal helix-capping. A truncated analog, TN-[RLLR]₂-TC, similar to N-[RLLR]₂-C except that the residues A1 and I19 were deleted, was also synthesized to study the roles of hydrogen bondings and associated hydrophobic
interactions in the helix-capping motifs. To determine the applicability of the helix capping in natural antimicrobial peptides, an $N$-Mag-C analog was designed where the APKAM and LQKKGI motifs were introduced at the N- and C-terminal ends of the salt-sensitive antimicrobial peptide, magainin 2, respectively.\textsuperscript{11}

Insert [Table 1] here

**Antimicrobial Activity and Structural Stability**

Under standard antimicrobial activity assay conditions, all the peptides designed with RLLR repeats had similar minimal inhibitory concentration (MIC) values against bacteria and fungi in the range of 0.5-4 $\mu$g/ml (Table 2). However, in the presence of 100 or 200 mM NaCl, all peptides except $N$-[RLLR]$_2$-C displayed a substantial decrease in MIC. The template peptide [RLLR]$_5$ exhibited the greatest salt sensitivity, whereby antimicrobial potency was reduced by up to 32-fold in the presence of 200 mM NaCl. The loss of antimicrobial activity of [RLLR]$_5$ in conditions of high salt was accompanied by disruption of the $\alpha$-helical structure, as revealed by the $\alpha$-helical content calculated from CD analyses (Table 3). The $\alpha$-helical content of [RLLR]$_5$ was reduced from 72% in the absence of salt to 13% in the presence of 200 mM NaCl. The adoption of only one helix-capping motif, either at the N- or C-terminus, resulted in slightly less salt-sensitive peptides that displayed a 4~16-fold decrease in antimicrobial activity in salt conditions. Upon the addition of 200 mM NaCl, the antimicrobial activity and $\alpha$-helical content of $N$-[RLLR]$_3$ decreased by 4~8-fold and 43%, respectively, and those of [RLLR]$_3$-C decreased by 8~16-fold and 46%, respectively (Tables 2,3). In contrast to the above peptides, the salt-resistant helix-capping analog $N$-[RLLR]$_2$-C displayed almost identical antimicrobial activity and less than 4%
change in α-helical content at the salt concentrations examined. The MIC values of the above peptides were not determined at or above 300 mM NaCl, due to the slow growth rate of microorganisms at these salt concentrations. However, CD analyses revealed that N-[RLLR]₂-C maintained α-helical content up to 300 mM NaCl, followed by a rapid decrease (data not shown). In addition, the trend of peptides without helix-capping motifs being salt-sensitive and ones with the helix-capping motifs being salt-resistant was also observed with peptides having 3~5 repeats (RLLR) (data not shown).

**Cellular Localization and Mechanism of Action**

To determine the localization site of the analogs in *E. coli*, biotin-labeled peptides were incubated with bacteria and visualized with streptavidin-FITC. N-[RLLR]₂-C localized to the cell membrane, regardless of the salt concentrations. However, [RLLR]₅ was not detected in *E. coli* at 200 mM NaCl (Fig. 1). Peptide localization to the cell membrane suggests that these α-helical antimicrobial peptides exert antimicrobial activity by permeabilization of the cell membranes. To confirm this possibility, an NPN uptake assay was performed to measure the permeabilization of the outer membrane. NPN, a hydrophobic probe that fluoresces weakly in aqueous environments and strongly in hydrophobic environments, partitions only into the permeabilized outer cell membrane¹⁸. The fluorescence emitted as the result of partitioning of NPN into the outer membrane upon addition of [RLLR]₅ and N-[RLLR]₂-C, was similar in intensity in the absence of salt (Fig. 2A). However, in the presence of 200 mM NaCl, the fluorescence level of the [RLLR]₅ mixture decreased by 80%, whereas that of N-[RLLR]₂-C mixtures decreased by only 6%.
This finding implies that $N$-[RLLR]$_2$-C is able to permeabilize the outer cell membrane, regardless of salt concentrations, whereas the permeabilizing activity of [RLLR]$_3$ is severely inhibited in the presence of salt. Next, permeabilization of the cytoplasmic membrane was measured to determine whether this is targeted by the analogs used in this study, which would result in membrane depolarization, dissolution of the electrical potential gradient and eventual cell death. The degree of cytoplasmic membrane permeabilization was analyzed by measuring the hydrolysis of the chromogenic substrate, ONPG, by cytoplasmic $\beta$-galactosidase in *E. coli* ML-35\textsuperscript{19,20}. Both [RLLR]$_3$ and $N$-[RLLR]$_2$-C displayed similar cytoplasmic membrane permeabilizing activity in the absence of salt (Fig. 2B). However, upon the addition of 200 mM NaCl, [RLLR]$_3$ almost completely lost activity (over 91%). $N$-[RLLR]$_2$-C alone displayed salt-independent cytoplasmic membrane permeabilizing activity. Cellular localization and membrane permeabilizing analyses of the analogs collectively revealed that in the presence of salt, sensitive peptides such as [RLLR]$_3$ lost their ability to bind the bacterial cell membrane, and were subsequently unable to permeabilize either the outer or cytoplasmic membranes. In contrast, salt-resistant peptides such as $N$-[RLLR]$_2$-C bound and permeabilized both the outer and cytoplasmic membranes causing cell death even in the presence of high salt concentrations.

Insert [Figure 1] here

**Evaluation and Application of Helix-Capping Motifs**

To assess the helix-stabilizing property of the capping motifs, APKAM and LQKKGI, truncated analog $TN$-[RLLR]$_2$-TC with deleted A1 and I19 residues were designed to destroy the inherent hydrogen bonds and hydrophobic interactions. As shown in Table 2,
the MIC values of $TN$-[RLLR]$_2$-TC against the microorganisms tested ranged between 1 and 4 $\mu$g/ml in the absence of salt. However, the addition of 100 mM and 200 mM NaCl decreased the antimicrobial activity of $TN$-[RLLR]$_2$-TC by up to 16-fold. As observed with [RLLR]$_5$, the decrease in antimicrobial activity of $TN$-[RLLR]$_2$-TC was associated with decreased $\alpha$-helical content (Table 3). In the presence of 200 mM NaCl, the $\alpha$-helical content of $TN$-[RLLR]$_2$-TC was reduced by 49%. In addition, decreased structural stability led to poor membrane binding, and the localization of $TN$-[RLLR]$_2$-TC in $E. coli$ was not detected (Fig. 1). The outer and cytoplasmic membrane-permeabilizing activities of $TN$-[RLLR]$_2$-TC were drastically reduced by 85% and 90%, respectively, when 200 mM NaCl was added (Fig. 2). Helix-capping motifs were introduced into magainin 2, a salt-sensitive antimicrobial peptide isolated from $Xenopus laevis^{11}$, to test their applicability in designing salt-resistant antimicrobial peptides. The resulting peptide, $N$-Mag-C, displayed salt concentration-independent antimicrobial activity and 42~44% $\alpha$-helical content at the salt concentrations tested. In contrast, magainin 2 was inactive against most of the microorganisms tested and lost 36% $\alpha$-helical content upon the addition of 200 mM NaCl to the assay.

In vivo antimicrobial activity

In the air pouch model of group A streptococcus (GAS) infection$^{21}$, inoculation of 4.5 x $10^4$ CFUs of $Streptococcus pyogenes$ A77 (100% lethal dose at 96 h; LD$_{100}$) caused suppuration, bleeding around the air pouch, and partial paralysis before death. The in vivo antimicrobial activity of the peptides was evaluated in GAS-induced infection by observing
the survival rate of the mice after inoculation. *S. pyogenes* caused 60% and 100% mortality at 72 h and 96 h, respectively, in the control group of mice (Fig. 3). Mice treated with [RLLR]₅ after the inoculation showed similar survival rates as the control group. In contrast, mice treated with *N*-[RLLR]₂-C showed 100% survival rate during the observance period. The bacterial count of *S. pyogenes* 48 h after the inoculation showed that the air pouches of mice in the control group and those treated with [RLLR]₅ contained 5~5.5 x 10⁷ CFUs, a 1000-fold increase from the initial inoculum, whereas only 1.2 x 10³ CFUs remained in the air pouches of mice treated with *N*-[RLLR]₂-C. Histological analysis of the skin around the air pouches showed that the inoculation of *S. pyogenes* caused the necrosis of the epidermis, dermis, and subcutaneous muscle layers (Fig. 4B). However, the same tissues remained intact in the air pouches of mice treated with *N*-[RLLR]₂-C (Fig. 4C).

Insert [Figure 3] and [Figure 4] here
Discussion

The role of helix-capping motifs in stabilizing the structure and antimicrobial activity of the \( \alpha \)-helical antimicrobial peptide \([RLLR]_3\) under high salt concentrations was closely examined by synthesizing analogs with these motifs at either the N- or C-terminus or both termini. The motifs, APKAM and LQKKGI, were selected to maximize the net positive charge while maintaining the amphipathicity of the analogs, both of which are important factors in antimicrobial activity.

Analogs containing helix-capping motifs at either the N- or C-terminus, specifically, \( N-\{RLLR\}_3 \) and \([RLLR]_3-C\), exhibited marginal improvement in structural stability and antimicrobial activity in the presence of high salt, compared to \([RLLR]_3\). Fraying of the helix termini, which results in loss of \( \alpha \)-helical content, occurs at both ends of the peptide molecules. Consequently, introducing a helix-capping motif at only one end would not fully prevent the peptide from structural destabilization. Indeed, \( N-\{RLLR\}_2-C\), containing helix-capping motifs at both the N- and C-termini, displayed significantly stronger structural stability upon exposure to 200 mM NaCl and retained antimicrobial activity. \( TN-\{RLLR\}_2-TC\) was examined to confirm the structural features of the helix-capping motifs that confer salt resistance upon \( \alpha \)-helical peptides. Deletion of A1 from the N-terminal motif and I19 from the C-terminal motif caused a dramatic 32-fold decrease in the antimicrobial activity of peptides in the presence of 200 mM NaCl. These results indicate that the helix-stabilizing property of these motifs is responsible for structural integrity of the peptides at high salt concentrations and salt-resistant antimicrobial activity.

The current exponential increase in the number of antibiotic-resistant microorganisms necessitates the development of new antimicrobial agents. Antimicrobial peptides have been extensively studied in recent years as promising candidates, but present
several obstacles in their development as pharmaceutical compounds. One of these major problems is salt sensitivity\textsuperscript{9}. Efficacy is greatly reduced in the presence of high salt, which hinders the development of antimicrobials as systemic agents. Friedrich \textit{et al.}\textsuperscript{12} and others\textsuperscript{11} reported on salt-resistant $\alpha$-helical antimicrobial peptides. To date, no clear rationale has been presented for the observed salt resistance in these peptides. Salt sensitivity may be attributed to the hindering of electrostatic interactions between positively charged peptides and negatively charged membranes. However, this alone does not explain why certain peptides are salt-resistant, while others with a similar net charge are not. Tam \textit{et al.}\textsuperscript{22} suggested that conformational rigidity increases antimicrobial activity under high salt concentrations. The group designed salt-resistant antimicrobial peptides by introducing rigid structural constraints into $\beta$-tile template peptides. Although there are numerous reports on the significance of structure, amphipathicity, positive charges and hydrophobicity for the activity and mechanism of action of antimicrobial peptides\textsuperscript{5,8}, the importance of structural stability of the $\alpha$-helical peptides remains to be clarified.

The ability of a peptide to configure into a well-defined, amphipathic $\alpha$-helix is strongly correlated to antimicrobial activity\textsuperscript{5}. The formation of $\alpha$-helical structures upon interaction with bacterial membranes and subsequent membrane permeabilization are believed to be one of the key steps in the mechanism of action of these $\alpha$-helical antimicrobial peptides. We hypothesize that the decreased antimicrobial activity of peptides under high salt conditions is a result of limitation of this key step of the mechanism. It is possible that imperfect amphipathicity of peptides may cause salt-sensitivity. However, our study showed that, among the peptides with the RLLR repeats, only those with helix-capping motifs at the N- and C-termini show salt-resistance. Furthermore, the salt-resistant peptides reported in other studies\textsuperscript{11,12} also do not have perfect amphipathic structures. Hence, although imperfect amphipathicity may contribute to the salt sensitivity, it is not the major
factor. The results in Tables 2 and 3 clearly demonstrate that salt sensitivity of antimicrobial peptides is a result of the destruction of \( \alpha \)-helical structure, and subsequent loss of membrane binding and permeabilizing activity. This observation may also explain why some peptides are reported to be salt-resistant while others with a similar sequence and net charge are salt-sensitive.

The applicability of the above helix-capping motifs in designing salt-resistant antimicrobial peptides was examined by introducing the motifs at both termini of magainin 2, a salt-sensitive peptide. The resulting peptide, \( N\)-Mag-C, maintained activity and structural stability at the salt concentrations tested. Thus, it is possible that, with consideration to, although not exclusively, net charge and amphipathicity, salt-resistant analogs can be derived from a salt-sensitive antimicrobial peptide by the application of helix-capping motifs. Furthermore, the \textit{in vivo} antimicrobial activity of \( N\)-[RLLR]\(_2\)-C was demonstrated in an air pouch model which mimics localized fasciitis\(^{21}\). \( N\)-[RLLR]\(_2\)-C inhibited the growth of \textit{S. pyogenes} and prevented the necrosis of the epidermis, dermis, and subcutaneous muscle layers, and subsequent death of mice, whereas [RLLR]5 was unable to inhibit the bacterial growth. Thus, the introduction of helix-capping motifs into salt-sensitive antimicrobial peptides can alter the properties of the peptides to become salt-resistant and suitable for \textit{in vivo} applications.

This study was performed to elucidate the mechanism underlying salt sensitivity in antimicrobial peptides and provide a rationale for the systematic design of salt-resistant peptides. The data collectively suggest that structural instability, in addition to the electrostatic interactions, is mainly responsible for salt sensitivity. Furthermore, the implementation of helix-capping motifs into salt-sensitive antimicrobial peptides resulted in analogs efficient in binding and permeabilizing microbial cell membranes at or above physiological salt concentrations.
Acknowledgements

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References


Figure Legends

Figure 1. Confocal fluorescence microscopy images of *E. coli* cells. *E. coli* cells were treated with 4, 1 and 4 μg/ml (MIC values of the peptides against *E. coli*) of biotinylated [RLLR]$_5$, N-[RLLR]$_2$-C, and TN-(RLLR)$_2$-TC in the absence (A, C, E) or presence of 200 mM NaCl (B, D, F), respectively, at 37°C for 30 min and visualized with streptavidin-FITC. The images depict the localization of biotinylated peptides. All three peptides localized to the cell membrane under the standard experimental condition (A, C, E). In the presence of 200 mM NaCl, only N-[RLLR]$_2$-C bound the cell membrane (D), while (RLLR)$_5$ (B) and TN-[RLLR]$_2$-TC (F) did not bind.

Figure 2. Outer and cytoplasmic membrane permeabilization assay. (A) A 20 ml volume of NPN (0.5 mM) was added to 1 ml *E. coli* cells in buffer (5 mM HEPES, pH 7.2, 5 mM KCN). Peptide samples (at MIC) were added 30 sec later. The increase in fluorescence as the result of NPN partitioning into the outer membrane was measured 2 min after addition of the peptides, using a fluorescence spectrophotometer. (B) Mid-logarithmic phase *E. coli* cells were washed in 10 mM NAPB, pH 7.4, and resuspended in the same buffer with 1.5 mM ONPG. The hydrolysis of ONPG to o-nitrophenol over time was monitored spectrophotometrically at 420 nm after the addition of the peptide samples (at MIC). White and grey bars represent the absence and presence of 200 mM NaCl, respectively.
Figure 3. Inhibition of GAS infection. Bacterial suspension (0.1 ml) containing 4.5 x 10^4 CFUs of *S. pyogenes* A77 at mid-logarithmic growth phase was inoculated into the air pouch. In peptide inhibition experiments, the mice were given an air pouch injection of each peptide (40 mg/kg-mice) immediately following *S. pyogenes* injection. The animals were observed for a total of 7 days and survival curves were determined.

Figure 4. Inhibition of GAS-induced tissue necrosis. Tissues around the air pouch were excised 48 h after the inoculation, fixed in 10% formaldehyde, and embedded in paraffin. The 5-µm-thick tissues were sliced, stained with hematoxylin and eosin, and subjected to histological analysis. (A) PBS-inoculated control. (B) Mouse inoculated with *S. pyogenes*. (C) Mouse inoculated with *S. pyogenes* and treated immediately with N-[RLLR]_2-C.
Table 1. Amino acid sequences of helix-capping analogs

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Table 2. Antimicrobial activities of helix-capping analogs

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<td>8</td>
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<tr>
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</table>

* NaCl concentration (mM)
Table 3. α-helical content(A) and circular dichroism spectra (B) of helix-capping analogs.

A)

<table>
<thead>
<tr>
<th>Peptide</th>
<th>% α-helix 0*</th>
<th>% α-helix 200</th>
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<td>[RLLR]₅</td>
<td>72</td>
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<tr>
<td>N-[RLLR]₁</td>
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<td>21</td>
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<tr>
<td>[RLLR]₁-C</td>
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<td>14</td>
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<tr>
<td>N-[RLLR]₂-C</td>
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<td>58</td>
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<tr>
<td>TN-[RLLR]₂-TC</td>
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<td>15</td>
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<tr>
<td>N-Mag-C</td>
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<td>42</td>
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<tr>
<td>Magainin 2</td>
<td>48</td>
<td>12</td>
</tr>
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</table>

* NaCl concentration (mM)

B)
Figure 1. Confocal fluorescence microscopy images of *E. coli* cells.
Figure 2. Outer and cytoplasmic membrane permeabilization assay.
Figure 3. Inhibition of GAS infection.
Figure 4. Inhibition of GAS-induced tissue necrosis.