Conjugation of Cell-Penetrating Peptides to Antimicrobial Peptides Enhances Antibacterial Activity

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ABSTRACT: Antimicrobial peptides (AMPs), essential elements in host innate immune defenses against numerous pathogens, have received considerable attention as potential alternatives to conventional antibiotics. Most AMPs exert broad-spectrum antimicrobial activity through depolarization and permeabilization of the bacterial cytoplasmic membrane. Here, we introduce a new approach for enhancing the antibiotic activity of AMPs by conjugation of a cationic cell-penetrating peptide (CPP). Interestingly, CPP-conjugated AMPs elicited only a 2- to 4-fold increase in antimicrobial activity against Gram-positive bacteria, but showed a 4- to 16-fold increase in antimicrobial activity against Gram-negative bacteria. Although CPP–AMP conjugates did not significantly increase membrane permeability, they efficiently translocated across a lipid bilayer. Indeed, confocal microscopy showed that, while AMPs were localized mainly in the membrane of Escherichia coli, the conjugates readily penetrated bacterial cells. In addition, the conjugates exhibited a higher affinity for DNA than unconjugated AMPs. Collectively, we demonstrate that CPP–AMP conjugates possess multiple functional properties, including membrane permeabilization, membrane translocation, and DNA binding, which are involved in their enhanced antibacterial activity against Gram-negative bacteria. We propose that conjugation of CPPs to AMPs may present an effective approach for the development of novel antimicrobials against Gram-negative bacteria.

INTRODUCTION

Antimicrobial peptides (AMPs) play crucial roles in non-specific host defenses and innate immunity and are known to have a wide range of activity against both Gram-positive and -negative bacteria.1–3 AMPs exhibit considerable potential as novel antimicrobials and have been studied extensively as a result of the increased tolerance to currently available antimicrobial agents.4–12 Because of their cationic nature, AMPs are initially attracted to negatively charged molecules on microbial surfaces, such as lipopolysaccharides (LPS) in Gram-negative and teichoic acids in Gram-positive bacteria. Although the precise mechanisms are still not completely understood, it is widely accepted that their mode of antibiotic action involves depolarization and/or permeabilization of the bacterial cell membrane.13–20 Some AMPs have also been suggested to cross the membrane without eliciting significant membrane permeabilization, following which they inhibit varied intracellular functions, including the nucleic acid and protein synthesis.21–24

Cationic cell-penetrating peptides (CPPs) can cross plasma membranes and facilitate cellular uptake of numerous molecules.25,26 Consequently, arginine-rich CPPs, like R9, have been widely employed as vectors for intracellular delivery of membrane-impermeable biomacromolecules.25,27 In this study, we utilized R9 to enhance the antimicrobial activity of AMPs through a dual mode of action, namely, membrane integrity disruption and intracellular activity inhibition. We conjugated R9 to AMPs (magainin and M15), the sequences of which are listed in Table 1. Here, we evaluated the antimicrobial activity and cytotoxicity of magainin, M15, and CPP–AMP conjugates by measuring the minimum inhibitory concentration (MIC) and the levels of hemolysis, respectively. To investigate the mechanism of action of the conjugates, we conducted membrane depolarization and permeabilization assays and visualized the site of action by confocal laser-scanning microscopy. We found that, compared to AMPs, CPP–AMP conjugates showed significantly enhanced antimicrobial activity against Gram-negative bacteria, likely because of membrane integrity disruption coupled to secondary intracellular targeting. This is among the first to show CPP–AMP conjugation as a novel strategy for the design of peptide antibiotics with potent antibacterial activity against Gram-negative bacteria. We propose that the multiple functions of CPP–AMP conjugates make it less likely that microorganisms will develop antibiotic resistance against them.

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that CPP conjugation to AMPs greatly improves antimicrobial activity, increasing the MICs of magainin and M15. Together, these data suggest that R9. At a concentration of 16 μM, R9 had a limited effect on the MICs of magainin and M15. Together, these data suggest that CPP conjugation to AMPs greatly improves antimicrobial activity, especially against Gram-negative bacteria.

### Table 2. Antibacterial Activities of the AMPs and CPP–AMP Conjugates

| Organism               | antimicrobial activity (MIC: μM) |
|------------------------|----------------------------------|
|                        | magainin (−R9/+R9) | R9-magainin | M15 (−R9/+R9) | R9-M15 |
| **Gram-Positive Bacteria** |                     |             |             |        |
| B. subtilis            | 8/8                  | 2–4         | 8–16/8–16   | 4–8    |
| S. aureus              | 16–32/16            | 8           | 8/8         | 4      |
| E. faecalis            | 16/16               | 4           | 16/8        | 4–8    |
| S. epidermidis         | 16/8–16             | 4–8         | 16–32/16    | 8      |
| **Gram-Negative Bacteria** |                     |             |             |        |
| E. coli                | 32/32               | 2–4         | 16–32/16    | 2–4    |
| S. typhimurium         | 16–32/16–32         | 1–2         | 32/32       | 2      |
| P. aeruginosa          | 32/32               | 4           | 32/32       | 4      |
| P. vulgaris            | 32–64/32            | 4–8         | 32/32       | 4–8    |

Antimicrobial activity tested in the presence (+R9) or absence (−R9) of 16 μM R9. Alone, R9 did not exhibit antimicrobial activity, even at the highest tested concentration of 128 μM.

### RESULTS

**Effect of CPP–AMP Conjugation on Antibacterial and Hemolytic Activities.** The magainin and M15 used in this study are cationic and amphipathic α-helical peptides, which is believed that the peptides exert their activity by permeabilizing cytoplasmic membranes. R9 belongs to the class of arginine-rich CPPs, which have the ability to cross cell membranes. First, AMPs (magainin and M15) and CPP-conjugated AMPs (R9-magainin and R9-M15) were tested for their cytotoxicity against human erythrocytes (Table 1) and their ability to kill bacteria (Table 2). Magainin and M15 both showed weak hemolytic activity, even at high concentrations. Moreover, only a small difference in hemolytic activity was observed between AMPs and CPP–AMP conjugates, indicating that CPP conjugation to AMPs does not elicit cytotoxicity. Magainin and M15 showed moderate antibacterial activity against all Gram-positive and -negative bacteria tested, with MIC values in the 8–32 μM range. Alone, R9 did not exhibit antimicrobial activity against any of the bacterial strains, even at the highest tested concentration of 128 μM, but there was a 2- to 4-fold increase in the antimicrobial activity of CPP–AMP conjugates against Gram-positive bacteria; interestingly, however, CPP–AMP conjugates exerted more improved antimicrobial activity (4- to 16-fold) against Gram-negative bacteria, with MIC values in the 1–4 μM range. We also evaluated the antimicrobial activity of unconjugated AMPs in the presence of R9. At a concentration of 16 μM, R9 had a limited effect on the MICs of magainin and M15. Together, these data suggest that CPP conjugation to AMPs greatly improves antimicrobial activity, especially against Gram-negative bacteria.

### Structural Characterization

We next analyzed the secondary structure of magainin, M15, and their R9 conjugates (R9-magainin and R9-M15) in aqueous buffer and in membrane-mimicking environments using circular dichroism (CD) spectroscopy (Figure 1). All the peptides exhibited negative bands at approximately 200 nm in aqueous buffer, indicating that their structures are random. However, the peptides also displayed two negative bands at 208 and 222 nm and a positive band at 195 nm in 30 mM sodium dodecyl sulfate (SDS) micelles, suggesting that the peptides adopt an α-helical structure in membrane environments. Compared to magainin and M15, the R9 conjugates showed a relatively strong negative band at 205 nm, which is very different from a typical α-helix, likely because of the flexible R9 sequences. However, the overall CD spectral patterns were similar between AMPs and CPP–AMP conjugates, suggesting that CPP conjugation did not significantly affect the amphipathic α-helical structure of the AMPs.

### Membrane Permeabilization and Depolarization

To determine the extent to which membrane permeabilization of Gram-negative bacteria contributed to the antimicrobial activity of the peptides, we first examined the ability of the peptides to increase outer membrane permeability by measuring the incorporation of the 1-N-phenylnaphthylamine (NPN) fluorescent probe into the outer membrane of *Escherichia coli* (Figure 2A). The NPN probe is excluded from an intact outer membrane of *E. coli*, but membrane destabilization allows entry of NPN into the phospholipid layer, resulting in prominent fluorescence. The peptides induced NPN uptake in a dose-dependent manner, indicative of their ability to disrupt the outer membrane barrier. However, the AMPs and CPP-conjugated AMPs both showed similar permeabilizing activity on the *E. coli* outer membrane...
and could not, therefore, explain the enhanced antimicrobial activity of the CPP-AMP conjugates. To complete membrane permeabilization, peptides must reach and permeabilize the inner membrane. We next investigated the permeability of inner membranes induced by the peptides using E. coli ML-35 cells that lack the lactose permease enzyme necessary for o-nitrophenyl-β-galactosidase (ONPG) uptake. If the peptides induced inner membrane permeabilization, ONPG would enter the cytoplasm and be cleaved to o-nitrophenol (ONP) by cytoplasmic β-galactosidase. Released ONP was determined spectrophotometrically. Although all the peptides induced E. coli inner membrane permeability in a concentration-dependent manner (Figure 2B), there were only marginal differences in the degrees of inner membrane permeabilization between the AMPs and CPP-conjugated AMPs. Therefore, permeabilization of the inner membrane does not seem to correlate with the antimicrobial potency of the conjugates.

Because Gram-negative bacteria were more sensitive to CPP-AMP conjugates, we then compared membrane depolarization between Staphylococcus aureus and E. coli as representatives of Gram-positive and Gram-negative bacteria, respectively (Figure 2C). The ability of the peptides to depolarize the membrane was assessed using the membrane potential-sensitive fluorescent dye 3,3’-dipropylthiacarbocyanine (DiSC3(5)). This dye inserts into the cytoplasmic membrane and its fluorescence is influenced by the membrane potential gradient. As expected, magainin and M15 depolarized the cytoplasmic membrane of both S. aureus and E. coli, even below their MIC values; however, the CPP-AMP conjugates also elicited effective membrane depolarization, similar to that observed for the unconjugated AMPs. Therefore, there was no direct correlation between cytoplasmic membrane depolarization and MIC values, indicating that other mechanisms likely exist through which CPP-conjugated AMPs exert their potent antimicrobial activity against Gram-negative bacteria.

We also evaluated the ability of the peptides to induce membrane permeabilization by examining calcine leakage from negatively charged PC/PG (1:1) and zwitterionic PC liposomes (Figure 2D). Following the addition of the peptides to liposomes encapsulating calcine, the release of calcine from the liposomes was measured. All the peptides showed a relatively weak ability to disrupt zwitterionic PC liposomes, agreeing well with the results obtained for hemolytic activity. In the negatively charged liposomes, AMPs and CPP-conjugated AMPs both showed similarly strong membrane-lytic activity, consistent with their respective capacities to depolarize bacterial cell membranes. However, the similar membrane-lytic activities could not explain the enhanced antimicrobial activity of the CPP-AMP conjugates. While the disruption of membrane integrity represents a major killing event for AMPs, other targets may be involved in the bactericidal effect of CPP-conjugated AMPs against Gram-negative bacteria.

### LPS Neutralization by Peptides

It is assumed that AMPs initially interact with the surfaces of Gram-negative bacteria composed of LPS; moreover, the permeability of the outer membrane appears to be a key determinant of their antimicrobial activity. Indeed, some AMPs have been shown to exhibit effective bactericidal and anti-inflammatory activities against Gram-negative bacteria by binding to LPS and neutralizing it. To examine whether the improved antimicrobial activity of CPP-AMP conjugates is correlated with their ability to bind LPS, we next assessed the LPS neutralization capacity of the peptides using a limulus amebocyte lysate (LAL) assay (Figure 3A). All the peptides neutralized LPS activity in a dose-dependent manner. Interestingly, CPP-AMP conjugates showed stronger LPS-neutralizing activity than unconjugated AMPs. These results suggest that the increased LPS-neutralizing activity of CPP-conjugated AMPs may correlate with their antimicrobial activity against Gram-negative bacteria.

LPS induces inflammatory pathways, leading to cytokine production. To further investigate the ability of the peptides to neutralize LPS, we measured their effects on LPS-stimulated tumor necrosis factor-alpha (TNF-α) release and nitric oxide (NO) production in RAW264.7 macrophage cells (Figure 3B). When the cells were treated with 20 ng/mL LPS, TNF-α and NO were released. Compared to magainin and M15 alone, the R9 conjugates (R9-magainin and R9-M15) significantly
AMPs across PC/PG (1:1) liposomes labeled with DNS-PE peptides to the dansyl group of DNS-PE incorporated into the liposomes. Once peptides are translocated into liposomes, the internalized peptides are degraded by α-chymotrypsin trapped in the liposome, which eventually reduces fluorescence. The peptide and lipid concentrations were 2 and 200 μM, respectively.

Figure 4. Peptide translocation across lipid bilayers. Peptide translocation across the membrane was monitored by measuring the resonance energy transfer from the Trp residues of the peptides to the dansyl group of DNS-PE incorporated into the liposomes. Once peptides are translocated into liposomes, the internalized peptides are degraded by α-chymotrypsin trapped in the liposome, which eventually reduces fluorescence. The peptide and lipid concentrations were 2 and 200 μM, respectively.

Figure 3. LPS neutralization by the peptides. (A) LPS-neutralizing activity of the peptides as determined by the LAL assay. LPS was incubated with different peptide concentrations for 30 min. (B) Inhibitory effect of peptides on TNF-α release and NO production from LPS-stimulated RAW264.7 cells. The cells (5 x 10⁶ cells/mL) were treated with 20 ng/mL LPS in the presence or absence of 2 μM of each peptide. The error bars represent standard deviations of the mean determined from three independent experiments.

Inhibited TNF-α release and NO production in LPS-stimulated RAW264.7 cells, indicating that the conjugates bind LPS more efficiently than unconjugated AMPs. The efficient binding of CPP–AMP conjugates to LPS could partially explain their enhanced antimicrobial activity against Gram-negative bacteria. These results also suggest that CPP conjugation to AMPs can be an effective approach for the development of anti-inflammatory agents.

Ability of CPP–AMP Conjugates to Translocate into Liposomes. Several studies recently showed that several peptides show strong bactericidal activity without causing severe disruption of membrane integrity.21,23,32,33 These peptides are thought to kill bacteria by interfering with essential cellular processes by blocking synthesis of DNA or protein. Because the antimicrobial activity of AMPs may be coupled to intracellular targets, we investigated the capacity of the peptides to translocate across membranes by measuring the resonance energy transfer from the Trp residues of the peptides to the dansyl group of DNS-PE incorporated into the membrane (Figure 4). The translocation of Trp-containing AMPs across PC/PG (1:1) liposomes labeled with DNS-PE was investigated by monitoring the degradation of the peptides by chymotrypsin confined in the liposomes. Trypsin inhibitors were used to prevent peptide degradation by extraliposomal chymotrypsin. When a peptide solution (2 μM) was added to liposomes (200 μM), binding of the peptide to the membrane increased fluorescence intensity by resonance energy transfer from the Trp residues to dansyl group. Following translocation, the internalized peptides should be digested by liposome-entrapped chymotrypsin, resulting in reduced fluorescence intensity. We observed only a small change in fluorescence with magainin and M15, indicating an absence of membrane translocation. In contrast, R9-magainin and R9-M15 both evoked a time-dependent reduction in fluorescence intensity, indicating that the peptides effectively translocated across the lipid bilayer.

Confocal Laser-Scanning Microscopy. To further examine the entry of CPP–AMP conjugates into bacterial cells, we incubated FITC-labeled peptides with E. coli and visualized their localization by confocal laser-scanning microscopy (Figure 5). As expected, FITC-labeled magainin was associated with the surface of E. coli cells, indicating that its major site of action is the bacterial membrane. In contrast, FITC-labeled R9-magainin penetrated the E. coli cell membranes and accumulated in the cytoplasm. These results suggest that CPP-conjugated AMPs have a secondary intracellular target.

Interaction of the Peptides with Plasmid DNA. We next examined the DNA-binding properties of the peptides because some AMPs have the ability to target intracellular molecules after passing through cell membranes. Peptide interaction with plasmid DNA was assessed by a 1% agarose gel electrophoresis (Figure 6). The plasmid (DNA 200 ng) was mixed with various concentrations of the peptides to form complexes at room temperature for 30 min and the DNA-binding abilities of the peptides were evaluated by measuring the retardation of plasmid DNA migration on the agarose gel. Magainin had little effect on DNA migration even at concentrations up to 128 μg/mL. In contrast, R9-magainin completely inhibited DNA migration at a concentration of 2 μg/mL, indicating that the R9-magainin conjugate bound DNA efficiently.

Figure 5. Localization of the peptides in E. coli cells. E. coli were incubated with FITC-labeled magainin (A) or FITC-labeled R9-magainin conjugate (B) for 30 min at 37 °C and imaged by confocal laser-scanning microscopy.
molecules in cells. Therefore, the increased antimicrobial activity observed for the CPP-conjugated AMPs could be explained by membrane disruption coupled to secondary intracellular targeting.

Numerous bacteria have developed resistance to existing antibiotics, and this resistance is a growing threat to public health.49,50 Gram-negative bacteria are of particular concern because they possess a protective outer membrane consisting of LPS.51,52 The first step in the interaction between AMPs and bacterial membranes is thought to be the binding of positively charged peptides to the negatively charged LPS on the outer bacterial surface. Because the bacterial outer membrane functions as a permeability barrier, Gram-negative bacteria appear to be protected from the lytic action of some antimicrobial proteases.53 To be effective against Gram-negative bacteria, the outer membrane permeability barrier must first be overcome. The conjugates also showed stronger anti-inflammatory activity than the AMPs alone in LPS-stimulated macrophages through neutralization of LPS. Binding to LPS may facilitate conjugate delivery to its site of action, thus strengthening antimicrobial activity against Gram-negative bacteria. In addition, these results also indicate that CPP conjugation to AMPs can be an effective approach for the development of anti-inflammatory agents.

To the best of our knowledge, this is the first study to investigate the effects of CPP–AMP conjugates on antimicrobial activity as well as their mode of action. Our results suggest that CPP conjugation to AMPs may be useful for increasing antimicrobial activity and selectivity against Gram-negative bacteria. Our data also suggest that CPP conjugation to AMPs may bestow multiple additional functions on AMPs, including LPS-binding, translocation across membranes, and DNA binding, factors that are key for enhanced antimicrobial activity. The present design of CPP–AMP conjugation may be a promising strategy for the development of new types of antimicrobial drugs against multidrug-resistant bacteria.

**EXPERIMENTAL SECTION**

**Materials.** All peptides were synthesized using the standard Fmoc-based solid-phase method on Rink amide MBHA resin. Nα-Fmoc (fluoren-9-yl-methoxycarbonyl) amino acids with orthogonal side-chain-protecting groups were purchased from Novabiochem (Laufelfingen, Switzerland). The reagents and solvents (highest commercially available purity) for peptide synthesis were obtained from Applied Biosystems (Foster City, CA, USA). For the FITC-labeled peptides, the Fmoc-ε-Ahx-OH was added to the N-terminus of the protected peptide using standard coupling conditions and it was confirmed that FITC-labeled peptides exhibited similar antimicrobial activity to their respective unlabeled peptides. The purity of the synthesized peptides was confirmed by analytical reverse-phase high-performance liquid chromatography (above 98% pure). The correct molecular mass of the purified peptides was confirmed by MALDI-TOF-MS (Shimadzu, Japan). The phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA). A membrane potential-sensitive probe, DiSC3(5), was obtained from Molecular Probes (Eugene, OR, USA). All other reagents were of analytical grade.

**Circular Dichroism Spectroscopy.** The CD spectra of the peptides were recorded using a Jasco J-715 CD spectrophotometer (Tokyo, Japan) in wavelengths ranging from 190 to 250 nm, with a scanning speed of 50 nm/min, a step resolution of 0.1 nm, a response time of 0.5 s, and a
bandwidth of 1 nm. The CD spectra of the peptides were collected and averaged over four scans in 10 mM sodium phosphate buffer (pH 7.2) or 30 mM SDS micelles, at 25 °C.

**Antimicrobial and Hemolytic Activities.** Antimicrobial activity against Gram-positive and Gram-negative bacteria (2 × 10^6 CFU/mL) was determined by measuring the MIC using a broth microdilution method, as previously described. Four types of Gram-positive bacterial strains, namely, *Bacillus subtilis* (KCTC 3068), *Staphylococcus epidermidis* (KCTC 1917), *Enterococcus faecalis* (KCTC 2011), and *S. aureus* (KCTC 1621); and four types of Gram-negative bacterial strains, namely, *E. coli* (KCTC 1682), *Pseudomonas aeruginosa* (KCTC 1637), *Proteus vulgaris* (KCTC 2433) and *Salmonella typhimurium* (KCTC 1926), were procured from the Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology (Daejon, Korea). Hemolytic activity was tested against human red blood cells (1 × 10^8 cells/mL) as previously described. Zero and one hundred percent hemolysis were determined in phosphate-buffered saline and 0.1% Triton X-100, respectively.

**Inner and Outer Membrane Permeability.** The inner membrane permeabilizing potential of the peptides was investigated using the fluorescent dye N-phenyl-1-naphthylamine (NPN), as previously described. Briefly, *E. coli* cells grown to the midlogarithmic phase were resuspended in 5 mM N- (2-hydroxyethyl)piperazine-N’-ethanesulfonic acid (HEPES) buffer (pH 7.2) containing 5 mM KCN and diluted to an OD600 of 0.05. The NPN dye (10 μM) was added to the cell suspension in a quartz cuvette and aliquots of the peptides were added to the cuvette. The fluorescence was recorded as a function of time at 420 nm (excitation at 350 nm) using an RF-5301PC spectrofluorophotometer (Shimadzu, Japan) until no further increase in fluorescence was observed. The NPN incorporation into the membrane after peptide addition increases fluorescence intensity. Percent NPN uptake was calculated via the following equation: NPN uptake (%) = \[(0D_{blank} - 0D_{peptide})/0D_{peptide}\] × 100, where 0D_{obs} represents the fluorescence observed at a given peptide concentration, 0D_{0} represents the initial fluorescence in the absence of peptide, and 0D_{100} represents the fluorescence upon addition of polymyxin B (10 μg/mL). The inner membrane permeabilizing ability of the peptides was determined by measurement of β-galactosidase activity in *E. coli* ML-35 cells using the normally impermeable, chromogenic substrate o-nitrophenyl-β-D-galactoside (ONPG), as described previously. Briefly, *E. coli* ML-35 cells were washed with 5 mM HEPES buffer (pH 7.2) containing 20 mM HEPES and resuspended in the buffer containing 1.5 mM ONPG and adjusted to an OD600 of 0.05. The rate of inner membrane permeability was assessed by the hydrolysis of ONPG to ONP determined by reading the absorbance at 405 nm.

**Membrane Depolarization and Disruption.** Membrane depolarization was detected using a membrane potential-sensitive probe, DiSC3(5), as described previously. Briefly, *S. aureus* and *E. coli* grown to the midlogarithmic phase in LB were harvested by centrifugation (3500 rpm, 7 min) and washed with 5 mM HEPES buffer (100 mM KCl, pH 7.2) containing 20 mM glucose and resuspended in buffer to an OD600 of 0.05. The fluorescence changes resulting from the dissipation of the cytoplasmic membrane potential by peptides was monitored at excitation/emission wavelengths = 622 nm/670 nm on the RF-5301 spectrofluorometer (Shimadzu). The peptides were added to the cells when the fluorescence intensity had stabilized because of maximal dye uptake by the bacterial membranes. The complete collapse of membrane potential was achieved by addition of Gramicidin D (0.25 nM). Membrane disruption was determined by the release of entrapped calcein from LUVs (large unilamellar vesicles), as previously described. The fluorescence intensity of the calcein released from the liposomes following peptide addition was monitored at excitation/emission wavelengths = 490 nm/520 nm on a Jasco FP-750 spectrophotometer (Tokyo, Japan). Complete dye release was obtained after treatment with 0.1% Triton X-100.

**LPS-Neutralizing Activity.** LPS neutralization by peptides was assessed using a commercially available LAL assay kit (Kinetic-QCL 1000; BioWhittaker Inc., Walkersville, MD, USA) following the manufacturer’s instructions. In Gram-negative bacteria, LPS activates a proenzyme in LAL that catalytically releases a colored product, paranitroanilide (pNA), from the colorless Ac-Ile-Glu-Ala-Arg-pNA substrate, which is detected spectrophotometrically at OD_{490}. The peptides were prepared in the pyrogen-free water and adjusted to pH 7.0 with 1 M HCl or 1 M NaOH. Increasing concentrations of the peptides were incubated for 30 min at 37 °C with one endotoxin unit. Approximately, 50 μL of LAL reagent was added and incubated for 10 min. After addition of 100 μL of substrate and further incubation of the reaction for 6 min, the release of the colored product was recorded at OD_{490}. Percent LPS neutralization was calculated via the following equation: LPS neutralization (%) = \[(0D_{blank} - 0D_{peptide})/0D_{blank}\] × 100. Water was used as a negative control that could not neutralize LPS (blank).

**TNF-α Release and NO Production in LPS-Stimulated Cells.** RAW264.7 cells were grown in 96-well plates (5 × 10^5 cells/well) in Dulbecco’s modified Eagle’s medium culture media. After one day, the medium was removed and fresh media was added to each well. The cells were stimulated with 20 ng/mL LPS as a positive control, or medium alone as a negative control. The levels of LPS-induced TNF-α were determined using a mouse TNF-α ELISA kit (R&D Systems, Minneapolis, USA), according to the manufacturer’s protocol. The levels of NO production was also estimated by quantifying the nitrite accumulation, using Griess reagent (1% sulfanilic acid, 0.1% N-1-naphthylethylenediamine dihydrochloride, and 5% phosphoric acid) after incubating for 24 h.

**Peptide Translocation.** The ability of the AMPs and CPP–AMP conjugates to translocate across lipid bilayers was assessed by fluorescence transfer from Trp to a dansyl group, as previously described. Briefly, UVs composed of POPC/POPG/DNS-PE (50:45:5) with entrapped 200 μM chymotrypsin in buffer (20 mM HEPES, 150 mM NaCl, pH 7.2) were prepared by extrusion. The extraliposomal enzyme was inactivated by adding a trypsin-chymotrypsin inhibitor to the suspension. The peptides (2 μM) were added to the suspension and fluorescence resonance energy transfer from the Trp residues of the peptides to the dansyl moiety in DNS-PE was employed to investigate peptide translocation across lipid bilayers. The fluorescence transfer was monitored at 510 nm (excitation at 280 nm) on using a Shimadzu RF 5301 PC spectrofluorometer. Internalized peptides into liposomes can be digested by the enzyme within the liposomes, thus reducing fluorescence transfer.

**Confocal Laser-Scanning Microscopy.** *E. coli* (KCTC 1682) cells grown to the midlogarithmic phase were harvested by centrifugation and washed 3 times with phosphate-buffered
saline. E. coli cells (1 × 10⁷ CFU/mL) were pretreated with the FITC-labeled peptides (0.2 μM) for 30 min at 37 °C. After washing with 10 mM sodium phosphate buffer, the bacterial cells were immobilized on a glass slide and visualized by an Olympus IX 70 confocal laser-scanning microscope (Tokyo, Japan) with a 488 nm band-pass filter for FITC excitation.

**DNA Binding Assay.** After purification of plasmid DNA (pBluescript II SK+) by CsCl density-gradient ultracentrifugation, gel retardation experiments were performed by mixing 300 ng of the plasmid DNA with increasing peptide concentrations. The mixture in 20 μL of binding buffer [10 mM Tris-HCl, 20 mM KCl, pH 7.5, 5% glycerol, 1 mM dithiothreitol, 0.25% xylene cyanol, and 1 mM ethylenediaminetetraacetic acid (EDTA)] was incubated at room temperature for 50 min. After addition of 4 μL of native loading buffer (10 mM Tris-HCl, pH 7.5, 10% Ficoll 400, 0.25% bromophenol blue, 0.25% xylene cyanol, and 50 mM EDTA), an aliquot (20 μL) was subjected to 1% agarose gel electrophoresis in Tris-borate–EDTA buffer (45 mM Trisborate, pH 8.0, and 1 mM EDTA). Migration of DNA was detected by ethidium bromide fluorescence.

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H.H.L. and S.Y. performed experiments and all authors evaluated the data. H.H.L. and S.Y. designed the experiments and wrote the paper.

**Notes**
The authors declare no competing financial interest.

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