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Effect of central PxxP motif in amphipathic alpha-helical peptides on antimicrobial activity and mode of action

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Abstract

Amphipathic α-helical peptides (AHPs) have shown potential as a therapeutic approach against multi-drug-resistant bacterial infections due to their broad-spectrum antimicrobial activity by disrupting bacterial membranes. However, their nonspecific interactions with membranes often result in cytotoxicity toward mammalian cells. Previous studies have shown that a PxxP motif near the middle of cathelicidin-derived antimicrobial peptides contributes to potent and selective antibacterial activity. In this study, we compared KL18 with KL-PxxP to examine the effects of the central PxxP motif in AHPs on their structure, antibiotic activity, and mode of action. In a membrane-mimetic environment, we observed that KL18 had a much higher helical content compared to KL-PxxP. In aqueous buffer, KL18 adopted a highly ordered α-helical conformation, while KL-PxxP exhibited a disordered conformation. We found that KL-PxxP exhibited 4–16 times higher antibacterial activity than KL18 and significantly reduced the hemolytic activity. These findings suggest that the dynamic conformational behaviors caused by the central PxxP motif conferred the antibacterial selectivity of AHPs. Additionally, KL-PxxP showed strong binding to anionic liposomes and weak binding to zwitterionic liposomes, explaining its selectivity for bacteria over mammalian cells. Despite having a low ability to dissipate the bacterial membrane potential, KL-PxxP translocated efficiently across lipid membranes. Therefore, we propose that the central PxxP motif in AHPs provides dynamic conformational behavior in aqueous and membrane-mimetic environments, enhances binding to anionic membranes, and facilitates translocation across lipid bilayers, resulting in improved antibacterial potency and selectivity. Understanding the unique structural characteristics and functional roles of the PxxP motif in the antimicrobial mechanism of action holds great potential for advancing the development of novel peptide antibiotics.

Keywords Amphipathic alpha-helical peptides, Antimicrobial activity, PXXP motif, Helix-hinge-helix, Peptide-membrane interaction

Introduction

The proliferation of antibiotic-resistant microorganisms has led to global health issues, prompting significant efforts to develop novel antimicrobial agents to combat these infections (Haney et al. 2019; Molton et al. 2013; Zaman et al. 2017). Among the major structural types of antimicrobial peptides, amphipathic α-helical peptides (AHPs) have emerged as a promising class of antimicrobial agents due to their effective activity against a broad range of bacterial pathogens (Mant et al. 2019; Matos et al. 2023; Tossi et al. 2000; Zhang and Gallo

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2016; Zhang et al. 2016). AHPs possess a cationic nature that enables them to bind to bacterial membranes that are negatively charged. Once bound, the hydrophobic face of the α-helix structure inserts into the hydrophobic region of the lipid bilayers, causing membrane disruption and ultimately leading to the death of the bacterial cell (Muller et al. 2020; Park et al. 2022; Sato and Felix 2006; Shai 1999; Yokoo et al. 2021). AHPs work by disrupting bacterial cell membranes, resulting in a lower potential to induce bacterial resistance compared to conventional antibiotics that target specific cellular components (Biswaro et al. 2018; de Breij et al. 2018; Kumar et al. 2018; Lohner 2016; Moravej et al. 2018). However, their nonspecific interactions with membranes can often result in cytotoxicity toward mammalian cells, thereby limiting their clinical potential (Hadianamrei et al. 2022; Juretic and Simunic 2019; Ma et al. 2016).

Several AHPs have been utilized to examine the relationships between molecular structure and function and to identify the factors that regulate cell selectivity (Chen et al. 2015; Xu et al. 2014; Yokoo et al. 2021). Importantly, the antimicrobial selectivity and potency of AHPs are influenced by their structural flexibility and local backbone distortion (Amos et al. 2018; Joodaki et al. 2021; Liu et al. 2013; Tuerkova et al. 2020). In particular, AHPs with helix-destabilizing amino acids have been found to exhibit strong antibacterial activity without causing cytotoxicity compared to stable AHPs (Lu et al. 2021; Nam et al. 2020; Yang et al. 2006b). Earlier studies have shown that the central hinge, which is the flexible segment connecting the N- and C-terminal helices in AHPs, is essential for conferring potent and selective antimicrobial activity (Yang et al. 2019, 2006a, 2021). This region often contains one or more proline residues, which impart flexibility to the peptide backbone, allowing it to undergo conformational changes critical for the peptide to bind to and disrupt the microbial membrane.

The central PxxP motif, which consists of a proline (P) followed by any two amino acids (x) and another proline (P), induces a hinge that significantly affects the antimicrobial activity of some AHPs. The PxxP motif is often found in cathelicidin-derived antimicrobial peptides, including BMAP-27 and PMAP-23 (Yang et al. 2019, 2006a). Previous studies have shown that the central PxxP motif in these peptides leads to increased flexibility and mobility of the peptide chain, allowing the peptides to more effectively interact with the lipid bilayer of microbial cell membranes (Yang et al. 2006b, 2021). Our findings demonstrate that the central PxxP motif plays a crucial role in making PMAP-23 amphipathic, promoting selective antimicrobial activity by facilitating preferential interaction with the negatively charged membrane. This motif allows the N-terminal helix to electrostatically bind to the negatively charged head group of the membrane and enables the C-terminal helix to insert into the hydrophobic region. Additionally, we have shown that the PxxP motif is indispensable for the translocation of PMAP-23 across the membrane (Yang et al. 2006b, 2021). Despite the crucial roles of the PxxP motif in the antimicrobial activity and the interaction of AHPs with membranes, the details of how the motif influences the structural behavior and antimicrobial mechanism of action are not yet well understood.

In this context, we prepared PxxP-free AHP (KL18) and PxxP-containing AHP (KL-PxxP) to investigate the effects of the PxxP motif in AHPs on the structural behaviors, biological activities, and antimicrobial modes of action. KL18 had a much more ordered α-helical structure in a membrane-mimetic environment than KL-PxxP. Interestingly, KL18 formed a helix even in an aqueous buffer, but KL-PxxP was unordered, indicating that the PxxP motif provides structural dynamics and prevents the peptide self-association in the buffer. We found that KL-PxxP had 4–16 times higher antibacterial activity than KL18 and almost no hemolytic activity. We also observed that KL-PxxP bound strongly to anionic liposomes and poorly to zwitterionic liposomes. Importantly, despite its low ability to dissipate the bacterial membrane potential, KL-PxxP was able to efficiently cross lipid membranes. Our results demonstrated that the dynamic conformational behaviors caused by the central PxxP motif confer the antibacterial selectivity of AHPs. It is proposed that comprehending the distinctive structural aspect of the PxxP motif and its functions in the antimicrobial mechanism of action would facilitate the development of new antibiotic agents.

Materials and methods

Materials and microorganisms

Solvents and reagents for peptide synthesis were supplied from Applied Biosystems (Foster City, CA, USA), and Fmoc (fluoren-9-yl-methoxycarbonyl) amino acids were purchased from Novabiochem (Läufelfingen, Switzerland). DiSC3(5) and SYTOX green were purchased from Molecular Probes (Eugene, OR, USA) and Invitrogen (Carlsbad, CA), respectively. Phospholipids were supplied from Avanti Polar Lipids (Alabaster, AL). Microorganisms (*Pseudomonas aeruginosa* [KCTC 1637], *Salmonella typhimurium* [KCTC 1926], *Escherichia coli* [KCTC 1682], *Bacillus subtilis* [KCTC 3068], *Staphylococcus aureus* [KCTC 1621], and *Staphylococcus epidermidis* [KCTC 1917]) were obtained from the Korean Collection for Type Cultures of the Korea Research Institute of Bioscience and Biotechnology (Daejon, Korea).

Peptide synthesis

The peptides were synthesized using solid-phase peptide synthesis methods with Fmoc chemistry and purified by reversed-phase preparative HPLC, as previously described (Lee et al. 2019). We verified the purity and hydrophobicity of the peptides by performing reversed-phase analytical HPLC on a C18 column (4.6 mm × 250 mm; Vydac). Additionally, their correct molecular masses were confirmed using electrospray ionization-mass spectrometry (ESI–MS).

Circular dichroism (CD) spectroscopy

The CD spectra of peptides were recorded on a J-715 spectrophotometer (Jasco, Japan), and the α-helicity was estimated, as previously described (Yang et al. 2019, 2021). Briefly, the peptides were solubilized in sodium phosphate buffer (10 mM) and sodium dodecyl sulfate (SDS) micelle (30 mM). Subsequently, they were introduced into a quartz cell with a thickness of 0.1 cm. A wavelength scan was conducted from 190 to 250 nm at a rate of 20 nm/min, with a bandwidth of 1 nm and a resolution of 0.1 nm. The spectra are presented as the mean residue ellipticity [θ] (deg cm^2 $dmol^{-1}$) as a function of wavelength.

Antimicrobial activity

The standard microdilution methods recommended by the Clinical and Laboratory Standard Institute (CLSI) were utilized to determine the antimicrobial efficacy of the peptides against both Gram-positive and Gram-negative bacteria, as previously described. (Lee et al. 2019). In brief, a bacterial colony was cultivated in Mueller–Hinton (MH) broth within a shaking incubator at 37 °C for 24 h. A 50 μL sample of the culture was grown in 5 mL of fresh MH medium until mid-logarithmic phase was reached and then, diluted in Mueller–Hinton broth (MHB). Subsequently, 100 μL of the diluted bacteria (2×10^6 CFU/mL) were introduced into the wells of a 96-well microtiter plate, which already contained serial dilutions of the peptides. Peptides in the concentration range from 0 to 64 μM were on the plates. The plate was then placed in an incubator set at 37 °C for 24 h. The MIC (μM) was determined as the minimum concentration of the antimicrobial that prevented visible bacterial growth by measuring the optical density (OD600). Experiments were performed in triplicate and repeated three times.

Hemolytic activity

The measurement of hemoglobin released as a result of sheep red blood cell (sRBC) lysis was used to evaluate the hemolytic activity of the peptides. (Lee and Yang 2021). Briefly, the sRBCs were collected via centrifugation (1000 × *g*) for 10 min, washed twice with PBS (pH 7.2). 4% blood solution was prepared in PBS and added to 96-well plate containing peptides in the concentration range from 0 to 256 μM, and the mixture was incubated at 37 °C for one hour. The plates were subsequently centrifuged at 1000 × *g* for 5 min, and the OD414 of the supernatant was measured using an ELISA microplate reader at 414 nm. PBS was taken as a negative control, and 0.1% triton-X100 was taken a positive control. PBS and 0.1% triton-X100 were taken as a negative and a positive control, respectively. Experiments were performed in triplicate and repeated three times.

Membrane depolarization induced by peptides

The fluorescent dye DiSC3(5) was used to evaluate the membrane depolarization activities of the peptides, as previously described (Yang et al. 2006c). Briefly, the logarithmic growth phase of *S. aureus* was diluted to an OD600 of 0.05 in 5 mM HEPES buffer (pH 7.4) containing 20 mM glucose. The cell suspension was then treated with DiSC3(5). After fluorescence stabilized and there was no further reduction in fluorescence, it was mixed with peptides in the concentration range from 0 to 64 μM. The fluorescence intensity was measured at 670 nm (with excitation at 622 nm) using an RF-5301 spectrofluorometer (Shimadzu, Tokyo, Japan). The addition of gramicidin D used as a positive control completely dissipated the membrane potential of *S. aureus*. Experiments were repeated three times.

Membrane disruption induced by peptides

The extent of membrane disruption induced by the peptides was assessed by quantifying the fluorescence intensity of the calcein dye (Lee et al. 2019). Briefly, the lipid film was hydrated with 20 mM HEPES buffer supplemented with 70 mM calcein, and subsequently, the large unilamellar vesicles (LUVs) were formed using the extrusion method. The suspension was extruded through a 100 nm polycarbonate filter, and any unencapsulated calcein was eliminated by passing the vesicles through a Sephadex G-50 column. The effect of peptides on membrane disruption was measured by detecting the release of calcein from the purified vesicles, and the fluorescence intensity was quantified at 520 nm (excited at 490 nm) using a Shimadzu RF-5301 spectrofluorometer. The percentage of leakage was calculated using the formula: Leakage (%) = 100 × (F − F0)/(Ft − F0), where F and Ft denote the fluorescence intensities after exposure to the peptide and 0.1% Triton X-100, respectively, and F0 represents the initial fluorescence intensity in the buffer. Experiments were repeated three times.

SYTOX Green uptake

SYTOX Green, which is a membrane-impermeable dye, was used to assess the membrane integrity of *S. aureus*. The disruption of plasma membranes of bacterial cells results in a significant rise in fluorescence as the dye enters the cell and binds to DNA. To perform SYTOX Green assays, the bacterial cells were cultured until they reached mid-logarithmic growth phase, washed, and suspended to a concentration of 2×10^6 cells/mL in PBS. The cell suspensions were then treated with 1 μM SYTOX Green for 15 min in the dark, followed by incubation with the peptides (8 μM). The SYTOX Green fluorescence was recorded on a Shimadzu RF-5301 spectrofluorometer at an emission wavelength of 520 nm (excitation wavelength of 485 nm). Triton X-100 was utilized to obtain the maximum level of fluorescence. Experiments were repeated three times.

Translocation of peptides across lipid bilayers

LUVs were prepared in HEPES buffer (150 mM NaCl, pH 7.4) containing chymotrypsin (200 mM), as described previously (Lee et al. 2019). The activity of external chymotrypsin was abolished by the addition of trypsin-chymotrypsin inhibitor (200 mM). Fluorescence transfer from Trp to dansyl groups was performed on a Shimadzu RF-5301 spectrofluorometer at an emission wavelength of 510 nm (excitation wavelength of 280 nm) for 10 min. Experiments were repeated three times.

Binding of peptides to membranes

The binding of the peptide to the membrane was estimated by measuring the tryptophan (Trp) fluorescence, as previously described (Yang et al. 2006c). Briefly, peptides are loaded into small unilamellar vesicles (SUVs) generated by sonication at a peptide/lipid molar ratio of 1:100. After a 10-min incubation period, the Trp emission spectra of the peptides were measured in the 300–400 nm range (excitation wavelength of 280 nm). Experiments were repeated three times.

Results

Peptide design and secondary structure

We synthesized KL18 and KL-PxxP. The structural diagrams and characteristics of the peptides are shown in Table 1 and Fig. 1. KL18 consists of hydrophobic Leu and basic Lys residues in order to form an α-helical structure that is perfectly amphipathic. The model amphiphilic α-helical peptides have been extensively employed in studies aimed at identifying critical factors governing cell selectivity through structure–activity relationships. Moreover, they have contributed significantly to the comprehension of their antibacterial mode of action by analyzing the interactions between the peptides and cellular membranes (Kabelka and Vacha 2021). KL-PxxP contains a PxxP motif with two Pro residues, which are substituted for one Leu and one Lys residue. The PxxP motif appears to act as a hinge at a central location connecting

Table 1 Amino acid sequences and cytotoxic activities of the peptides used in this study

Peptides	Amino acid sequences	Rt[a] (min)	Net charge	HM[b] (μH)	Helicity (%)
KL18	KWLKKLLKLLKKLLKKLK	30.80	+9	0.876	58
KL-PxxP	KWLKKLLKPLKPLLKKLK	21.68	+8	0.794	31

[a] Retention time (Rt) was measured using a C18 reversed-phase analytical HPLC column

[b] Hydrophobic moment (HM) was calculated online (http://heliquest.ipmc.cnrs.fr/cgi-bin/ComputParams.py)

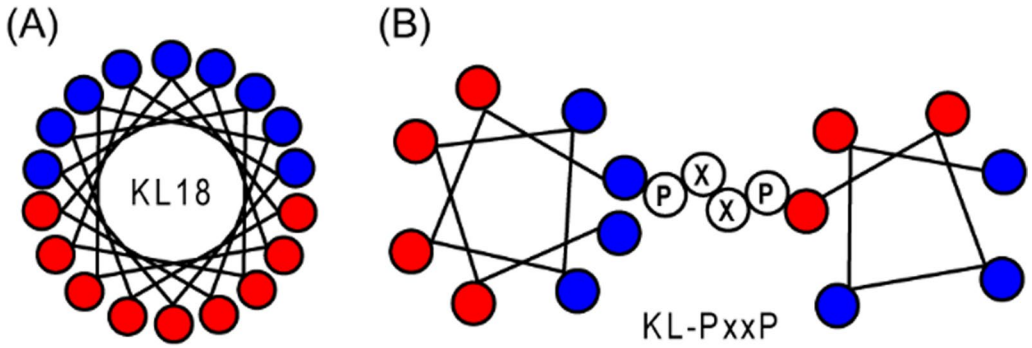

Fig. 1 Helical wheel diagram of AHPs. **A** KL18 is composed of repeats of hydrophobic and cationic residues to create perfect amphipathic α-helical structure. **B** KL-PxxP contains a PxxP motif connecting N- and C-terminal helices. Red circles refer to hydrophobic residues, blue circles to cationic amino acids. The helical wheel diagram was prepared using the pepwheel program (online at: https://www.bioinformatics.nl/cgi-bin/emboss/pepwheel)

the N- and C-terminal helices. To enable the fluorescent determination of their interactions with lipid membranes, a single Trp was introduced at position 2 of the peptides. The secondary structure of the peptides was estimated using circular dichroism (CD) spectroscopy. The CD spectra of the peptides were obtained in either phosphate buffer (20 mM) or membrane-mimetic SDS (30 mM) micelles (Fig. 2). In the presence of SDS micelles (Fig. 2A), the CD spectra of the peptides revealed the presence of α-helix structures, but there was a significant variation in the helical contents among the peptides. As expected, the lower helicity of KL-PxxP compared to KL18 suggests that it has a partially distorted helical structure in a membrane mimic environment. Interestingly, under aqueous buffer conditions (Fig. 2B), the CD spectra of KL18 showed characteristic α-helical patterns, with molar lower mean residue ellipticity at 208 nm and 222 nm (Fig. 2B). In contrast, KL-PxxP exhibited a negative band below 200 nm in its CD spectra, which suggests the absence of any ordered structure.

Antimicrobial and hemolytic activities of the peptides

Table 2 displays the antibacterial activity of the peptides designed derivatives as minimal inhibitory concentration (MIC) against Gram-positive bacteria, including *B. subtilis*, *S. aureus*, and *S. epidermidis* as well as Gram-negative bacteria, including *E. coli*, *S. typhimurium*, and *P. aeruginosa*. KL18 exhibited activity with MIC values in the 16–32 μM range against *B. subtilis*, *S. aureus*, *E. coli*, and *S. typhimurium* but was inactive against *S. epidermidis* and *P. aeruginosa*. Importantly, KL-PxxP was found to be more potent against both Gram-positive and Gram-negative bacteria than KL18, with MIC values in the 2 to 4 μM range against tested all strains. To evaluate the cell-selectivity of the peptides, we measured hemolytic activity against sheep erythrocytes. As shown in Fig. 3, KL18 showed relatively strong hemolytic activities (25% at 32 μM), but KL-PxxP was almost no hemolytic (~1% at 32 μM). These results indicate that the central PxxP motif in AHPs are critical for antimicrobial potency and selectivity.

Membrane depolarization caused by the peptides

To investigate whether the peptides kill bacteria by depolarizing the cytoplasmic membrane, we used DiSC3(5), a membrane potential-sensitive fluorescent dye, to determine their ability to depolarize the cytoplasmic membrane of *S. aureus*. Many membrane-active antimicrobial peptides are believed to pass through the peptidoglycan layer and then, interact with and permeabilize the cytoplasmic membrane to kill the target microorganism. As depicted in Fig. 4A, KL18 effectively dissipated the membrane potential of *S. aureus*, whereas KL-PxxP caused only a slight reduction in the membrane potential. It is important to note that this observation did not match the results of antimicrobial activity. To further investigate the peptides' ability to induce membrane permeabilization, we measured the release of entrapped calcein from the liposomes into an aqueous buffer (Fig. 4B). KL18 strongly induced calcein release from the PC/PG (1:1) vesicles,

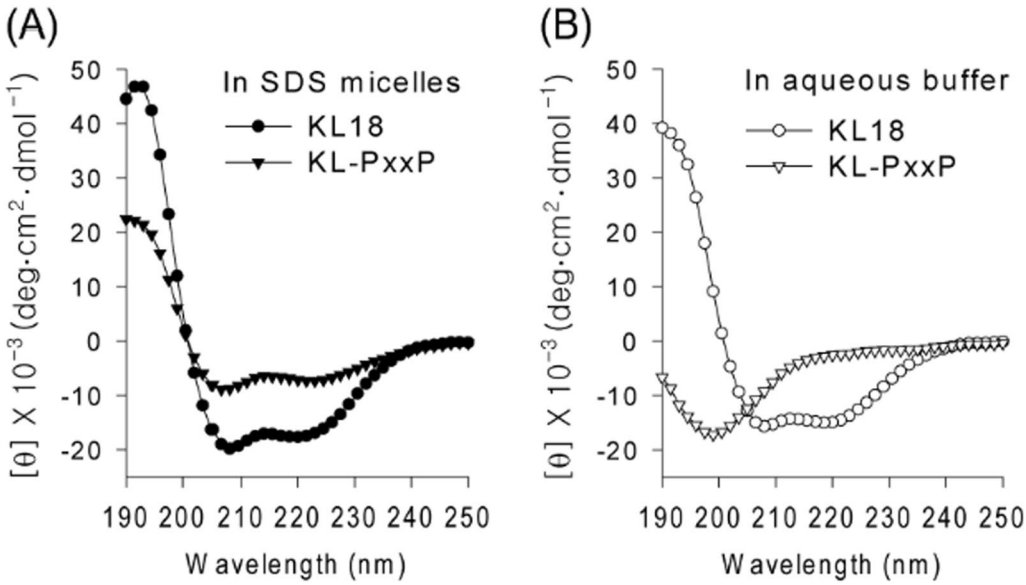

Fig. 2 CD spectra of the peptides. The CD spectra were obtained at 25 °C in **A** aqueous buffer and **B** 30 mM SDS micelles. Spectra were taken at peptide concentrations of 25 μM. The values from three scans were averaged per sample

Table 2 Antibacterial activities of KL18 and KL-PxxP against Gram-positive and Gram-negative bacteria

Peptides	Antimicrobial activity (MIC: μM)[a]					
	B. subtilis	*S. aureus*	*S. epidermidis*	*E. coli*	*S. typhimurium*	*P. aeruginosa*
KL18	16	16	> 64	32	32	> 64
KL-PxxP	4	4	4	4	2	4

[a] MIC (minimum inhibitory concentration) was determined as the lowest concentration of peptide that inhibits visible growth of bacteria

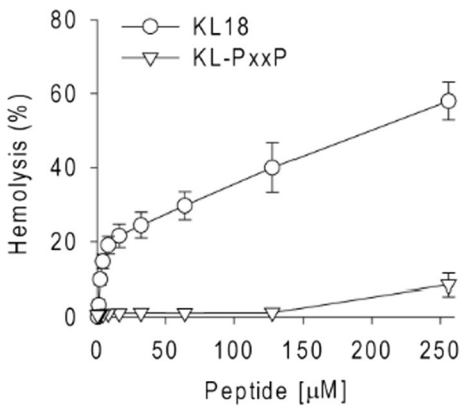

Fig. 3 Dose-dependent curves of hemolytic activity caused by the peptides toward sheep erythrocytes. Assay were carried out as described in the experimental section. Data are shown as mean ± SD (n = 3)

causing 67% leakage at 2 μM, whereas KL-PxxP exhibited weak membrane-lytic activity, causing 17% leakage at 2 μM. It is worth mentioning that KL-PxxP had a stronger antimicrobial effect than KL18, and while the membrane-lytic activity of the peptides correlated with their respective antimicrobial activities, the correlation was not entirely linear. The absence of a relationship between membrane depolarization and antimicrobial activity implies that the primary bactericidal mechanism of KL-PxxP is not related to membrane potential loss.

SYTOX Green uptake in bacterial cells caused by the peptides

To further investigate the impact of the peptides on the membrane integrity of live microbial cells, we utilized SYTOX Green fluorescence, a DNA-binding dye that cannot permeate intact membranes. When a membrane is permeabilized, the dye can enter and result in increased fluorescence. In Fig. 5A, we monitored SYTOX Green fluorescence after adding 8 μM peptide to *S. aureus* cells suspended in PBS buffer. We observed that KL18 significantly induced SYTOX Green influx, while KL-PxxP had no effect on the influx of SYTOX Green into the cells.

Ability of the peptides to translocate into liposomes

To determine if the peptides have the ability to enter cells, we evaluated their capacity to translocate across lipid bilayers (Fig. 5B). The lipid membranes were composed of PC/PG/dansyl-PE (50:45:5), and chymotrypsin

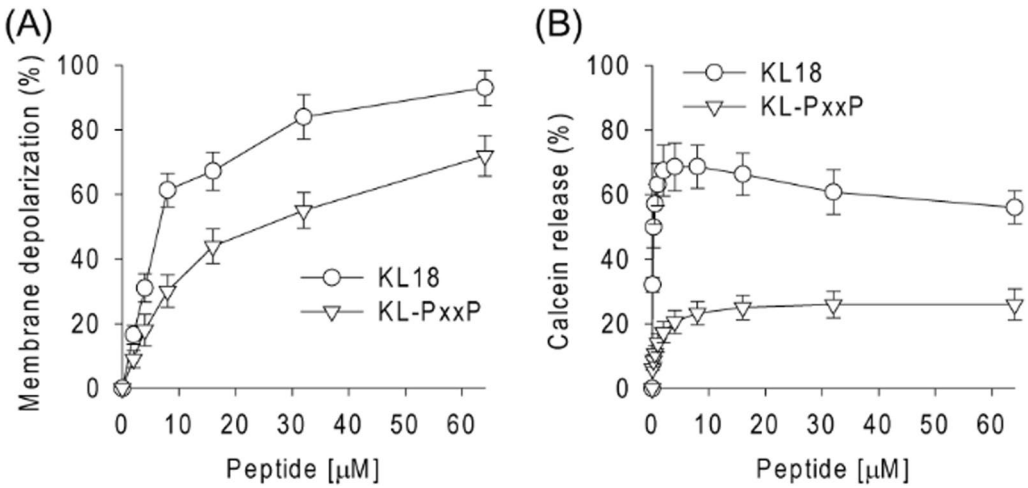

Fig. 4 Membrane permeabilization caused by the peptides. **A** Peptide-induced membrane depolarization of *S. aureus*. Complete depolarization was induced by adding gramicidin D. **B** Membrane disruption induced by peptides. Peptide-induced membrane disruption is defined as the percent calcein leakage from PC/PG (1:1). after 10 min incubation with peptides

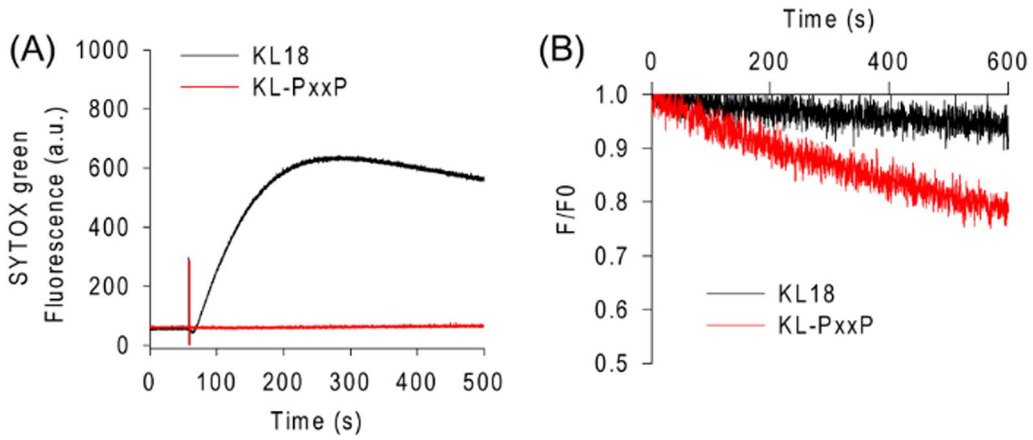

Fig. 5 Membrane penetration of SYTOX green and time-dependent peptide translocation. **A** SYTOX green uptake of *S. aureus* caused by the peptides. Fluorescence was measured at the indicated times. **B** Ability of peptides to translocate into liposomes. A decrease in fluorescence intensity after addition of a peptide is indicative of digestion of the internalized peptide. Membrane translocation of the peptides was measured by resonance energy transfer from Trp to DNS-PE. The lipid and peptide concentrations were 100 and 1 μM, respectively

was encapsulated within the vesicles. Using a fluorescence resonance energy transfer system with a Trp donor and a dansyl acceptor, we estimated the extent of peptide translocation across the lipid membranes by monitoring peptide degradation by chymotrypsin. KL18 did not cause any fluorescence decrease during the 600 s, indicating a lack of membrane translocation. In contrast, KL-PxxP led to a time-dependent reduction in fluorescence intensity, suggesting successful crossing of the lipid bilayer and entry into vesicles. The ability of peptides to translocate across the lipid bilayer corresponds with their respective antibacterial activity, implying that the cell-penetrating efficiency might play a role in determining the antimicrobial efficacy of KL-PxxP. Our results align with previously published translocation data and suggest that KL-PxxP could potentially target intracellular molecules.

Trp fluorescence analysis for the binding of the peptides to membranes

As the intensity and emission maximum (λ_{max}) of Trp fluorescence can be significantly altered by changes in the environment surrounding Trp residues, we used these properties to assess the partitioning of peptides into lipid bilayers. Figure 6 displays the Trp fluorescence emission spectra and λ_{max} of the peptides in buffer and in the presence of liposomes consisting of PC/PG (1:1) or PC at a molar ratio of peptide to lipid of 1/100. The λ_{max} value

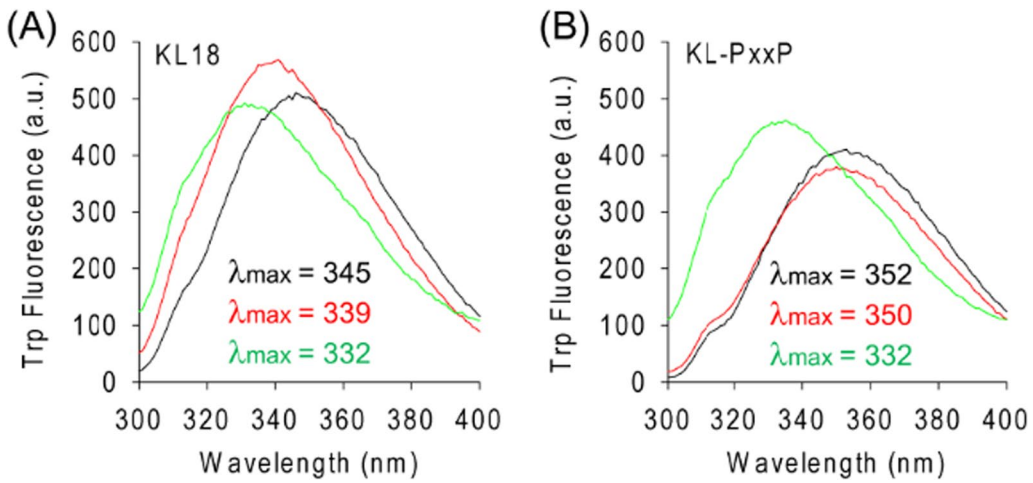

Fig. 6 Trp fluorescence spectra and emission maxima (λmax) of the peptides. **A** KL18 and **B** KL-PxxP were added in buffer (black) and in the presence of vesicles composed of PC/PG (green), or PC (red) at a peptide/lipid molar ratio of 1:100

of KL-PxxP in aqueous buffer was about 352 nm, indicating that Trp residues were exposed to a hydrophilic environment. In contrast, the λ_{max} range of KL18 was 343–345 nm, indicating that the peptides are self-assembled in buffer. The α-Helix structure of KL18 obtained from CD measurements in buffer appears to be accompanied by self-association. In the presence of PC/PG (1:1) vesicles, both peptides caused a significant blue shift in the emission maximum, indicating their binding to negatively charged membranes. However, while KL18 exhibited an intermediate blue shift, KL-PxxP caused only a minor shift (0–2 nm) in Trp emission maxima upon addition to PC liposomes. Based on these observations, it appears that peptides containing a PxxP motif selectively target microbes by exploiting the abundance of negatively charged phospholipids in prokaryotic membranes, as opposed to eukaryotic membranes that are mainly composed of zwitterionic phospholipids. These results, obtained through the investigation of Trp λ_{max}, are well correlated with our previous findings regarding the self-assembly of Pro-free AHPs in buffer and the selective binding of Pro-containing AHPs to negatively charged membranes (Yang et al. 2006b).

Discussion

Antimicrobial peptides including AHPs have garnered considerable interest as potential substitutes for traditional antibiotics due to their broad-spectrum antimicrobial activity and reduced likelihood of inducing bacterial resistance (Tossi et al. 2000; Zhang et al. 2021). AHPs primarily exert their antimicrobial activity by interacting with and disrupting the membranes of target cells via electrostatic and hydrophobic interactions (Sato and Felix 2006; Shai 1999). However, these peptides can also interact with host cell membranes in a non-specific manner, resulting in toxicity toward mammalian cells. Therefore, there is a need to design AHPs with enhanced antibacterial potency and selectivity while minimizing their toxicity toward host cells. Previous studies have indicated that the hinge PxxP sequence in AHPs is crucial for bacterial selectivity and efficient interaction with negatively charged membranes. Therefore, this study aimed to compare the antimicrobial modes of action between KL18, a single amphipathic α-helical peptide, and KL-PxxP, a helix-hinge-helix peptide.

Circular dichroism (CD) spectroscopy confirmed that KL18 had a higher α-helical content than KL-PxxP in membrane-mimetic environments, indicating that KL18 forms a stable α-helical structure, while KL-PxxP forms a flexible α-helical structure. Our study found that KL-PxxP demonstrated a 4–16-fold increase in antibacterial activity while exhibiting almost no hemolytic activity compared to KL18. The enhanced antibacterial potency of KL-PxxP was attributed to the dynamic conformational behaviors conferred by the central PxxP motif. Trp fluorescence measurements revealed that KL18 interacted non-selectively with both anionic and zwitterionic liposomes, while KL-PxxP bound more strongly to anionic liposomes than zwitterionic liposomes. This selective binding to negatively charged phospholipids in bacterial membranes could account for the specificity of KL-PxxP toward bacteria rather than red blood cells, as zwitterionic phospholipids are primarily located in the outer leaflet of mammalian cell membranes. Moreover, CD and fluorescence spectra of the peptides in buffer showed that KL-PxxP had an unordered structure, while KL18 exhibited a typical α-helical structure due to self-association. The aggregation of KL18 in buffer correlated with its ability to bind to zwitterionic liposomes and cause lysis of sheep erythrocytes. These results provide further evidence that the central PxxP motif, with its unique structural features, confers high selectivity for bacterial cells.

Although KL-PxxP exhibited stronger antibacterial activity than KL18, our study found no correlation between the peptides' ability to permeabilize bacterial membranes and their antimicrobial activity. We observed that KL-PxxP caused less depolarization of bacterial membranes than KL18 and had a lower ability to disrupt artificial membranes. Additionally, KL-PxxP had no effect on the influx of SYTOX Green into *S. aureus*, while KL18-induced significant SYTOX Green influx. However, we found that KL-PxxP readily translocated across the lipid bilayer, while KL18 did not enter the liposomes. The ability of peptides to cross the lipid bilayer aligns with the improved antibacterial activity of KL-PxxP, highlighting the critical role of cell-penetrating efficiency in determining their antimicrobial potency. As suggested by Sansom and Weinstein (Sansom and Weinstein 2000), the structural dynamics, such as twisting and kinking of helix-hinge-helix peptides, may facilitate N- or C-terminus penetration into the core of the lipid bilayer. Our recent study demonstrated that the PxxP-induced central hinge promotes conformational flexibility in amphipathic α-helical structures, which could drive AHP migration from the outer to the inner leaflet of lipid bilayers. Thus, the conformational flexibility caused by the central PxxP motif may be a prerequisite for importing AHPs into membranes or the cytosol. Since membrane disruption and/or cell-penetrating ability are crucial for the antibacterial mechanism of AHPs (Avci et al. 2018; Henriques et al. 2006), the PxxP motif with unique structural and functional properties serves as a key factor for developing AHPs with enhanced antibacterial efficacy and selectivity. The distinctive physicochemical property of the central PxxP motif in the translocation of AHPs across artificial lipid membranes was demonstrated by substituting the two

Pro residues with α-helix-forming Ala or α-helix-breaking Gly (Yang et al. 2021). While the antimicrobial effectiveness of AHPs is influenced by its multifaceted mode of action beyond mere membrane permeability, comprehending the molecular-level interaction between AHPs and lipid bilayers holds significant importance for the development of antimicrobial drugs.

In summary, our study found that KL-PxxP has significantly enhanced antibacterial activity compared to KL18, while also displaying reduced hemolytic activity. KL-PxxP had a lower helical content than KL18 in a membrane-mimetic environment. We observed that KL-PxxP is distributed randomly while KL18 is self-associated in buffer. Importantly, KL-PxxP had a strong affinity for anionic liposomes and efficiently translocated across lipid bilayers despite its low ability to dissipate the bacterial membrane potential. Therefore, our findings suggest that the central PxxP motif in AHPs provides dynamic conformational behavior in both aqueous and membrane-mimetic environments, enhancing binding to anionic membranes and translocation across lipid bilayers, resulting in improved antibacterial potency and selectivity. This study sheds light on the structural and functional aspects of the central PxxP motif in AHPs, which holds promise as an approach to enhance antimicrobial efficacy while mitigating the cytotoxic effects against mammalian cells. Furthermore, we propose that this motif could be applied to other antimicrobial peptides to facilitate the development of peptides with intracellular targets and the creation of multifunctional antimicrobial peptides.

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Author contributions
HL and SY performed experiments: All authors designed research, analyzed data, and wrote the paper.

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Availability of data and materials
Not applicable.

Declarations

Competing interests
The authors declare no competing financial interests.

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