Two Hits Are Better than One: Membrane Active and DNA Binding Related Double Action

Mechanism of NK-18, a Novel Antimicrobial Peptide Derived from Mammalian NK-lys

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Abstract:
The extensive use and misuse of antibiotics in medicine has resulted in the emergence of multi-drug resistant bacteria, which make an urgent need for developing new chemotherapeutic agents. Nowadays, antimicrobial peptides are widely recognized as one class of promising candidate for withstanding the multi-drug resistant bacteria. NK-18 was a truncated peptide derived from NK-lysin, an effector of cytotoxic T cells and natural killer cells. In this study, we studied the antibacterial action mechanism of NK-18. The results revealed that NK-18 has potent antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. According to our finding, NK-18 is membrane active and its action target is not only the bacterial membrane but also the DNA in the cytoplasm. The double action targets of NK-18 make it difficult for bacteria to generate resistance, which may present a new strategy to defend multi-drug resistant bacteria and provide a new lead in the design of potent antimicrobial peptides with therapeutic application with the increasing resistance to conventional antibiotics.

Keywords: NK-18, antimicrobial peptide, antimicrobial activity, membrane permeabilization, DNA binding.

Abbreviations:
- F-moc: N-9-fluorenylmethoxycarbonyl
- FITC: fluorescein isothiocyanate
- ESI-MS: electrospray ionization mass spectrometry
- CFU: colony formation unit
- PI: propidium iodide
- OM: outer membrane
- NPN: 1-N-phenylnaphthylamine
- IM: inner membrane
- LUVs: large liposome vesicles
- ONPG: o-nitrophenyl-β-d-galactoside
- EYPE: egg yolk l-α-phosphatidylethanolamine
- EYPG: egg yolk l-α-phosphatidyl-dl-glycerol
- TEM: transmission electron microscopy
Introduction

For recent decades, the misuse of conventional antibiotics has resulted in the emergence of many multi-drug resistant strains (1, 13, 32). With the increasing severity of this phenomenon, it has been a serious menace to human health and quality of life. Therefore, developing of a new class of antibiotics with different mechanism compared with conventional antibiotics is becoming more and more urgent and critical. The outbreak of "Superbugs" in several countries in the world in 2011 emphasizes once again the need for the search and development of new antimicrobial agents or resources. Nowadays, it is widely recognized that antimicrobial peptides (AMPs) could play a promising role in fighting the multi-drug resistant strains and AMPs are considered as a new class of “antibiotic”, with characteristics including an ability to kill target cells rapidly, an unusually broad spectrum of activity and activity against some of the more serious antibiotic-resistant pathogens in clinic, as well as the relative difficulty in selecting resistant mutants in vitro (6, 22, 43).

AMPs are widespread in nature, including microorganisms, insects, invertebrates, amphibians, plants, birds, and mammals (19, 26). It comprises a wide range of short, cationic peptides which constitute the first line of innate immune defense against infectious agents (4, 11, 27, 38). To date, more than 1,800 AMPs have been purified from a wide range of organisms (39) or chemo-synthesized based on the sequence of purified peptides. NK-18 is a derivative of a mammalian protein NK-lysin. It derives from the core region of NK-lysin(39-56) and possess potent antitumor activity against prostate and bladder tumors with a membrane active action mode (42). The amino acid sequence of NK-18 is KILRGVCKKIMRTFLRRI-NH₂. Compared with NK-lysin, NK-18 has the potential to be used clinically for its shorter amino acid sequence and lower cost of chemosynthesis.

In the present study, the antibacterial activity of NK-18 was studied by 2-fold dilution method and the radial diffusion assay. In order to get a better understanding of its antibacterial mechanism, the OM/IM permeability assay and confocal laser scanning microscopy assay were employed to examine the effect of NK-18 on the integrity of bacterial membrane. The effect of NK-18 on the morphology of bacteria was observed under the scanning microscopic view. To investigate the interaction between
NK-18 and the DNA, DNA retardation assay and DNA absorption assay, as well as atomic force microscopy (AFM) imaging were employed in this study. Furthermore, in order to exclude the influence of other non-lipid components on the activity exhibition of NK-18, we also examined the effect of NK-18 on the model membrane LUVs composed of EYPE/EYPG.

Materials and Methods

Peptide synthesis and purification

NK-18 was synthesized by stepwise solid-phase method using N-9-fluorenylmethoxycarbonyl (F-moc) chemistry that has been reported previously (8). The FITC-labeled NK-18 was synthesized as described by Wender (40). All the peptides were purified by sephadex gel column and reverse-phase HPLC (Waters) using μ-BONDAPAKTM C18 19mm×300mm column with gradient elution of 20%-75% CH3CN/H2O with 0.1%TFA at a flow rate of 8ml/min. The atomic masses of these peptides were confirmed by electrospray ionization mass spectrometry (ESI-MS).

Bacteria and reagents

Bacterial strains used in this study were obtained from Institute of Microbiology, Lanzhou University. Escherichia coli (CMCC44102) and Staphylococcus aureus (CMCC26003) were cultured in LB-broth. Prior to assays, bacteria were grown overnight to the stationary phase at 37 in 5 ml LB-broth. A 1/50 dilution of the overnight culture was suspended in 5 ml fresh LB-broth and grown for an additional hours at 37 to obtain a mid-log phase culture and then the optical density was determined using a spectrophotometer to obtain a certain cell population. 1-N-phenylnaphthylamine (NPN) was purchased from J&K Scientific Ltd. o-nitrophenyl-β-D-galactoside (ONPG) was obtained from Beyotime Institute of Biotechnology (Shanghai, China). Egg yolk 1-α-phosphatidylethanolamine (EYPE) and egg yolk 1-α-phosphatidyl-DL-glycerol (EYPG) were purchased from Avanti Polar. Plasmid DNA (pBR322) was purchased from Fermentas. Propidium iodide (PI) was purchased from Invitrogen.

Radial diffusion assay

The antibacterial activity of NK-18 was evaluated by the radial diffusion assay, a modification of the sensitive assay described by Takemura(33). Briefly, the bacteria were cultured as described above and the optical density of the aliquot at 600 nm reached 0.5. 1ml of the bacteria was added to 100 ml of
previously autoclaved, warm LB-agar. After a rapid dispersion of the bacteria, the agar was poured into
an agar plate to form a layer approximately 5 mm deep and was punched with a 3-mm-diameter gel
punch to make evenly spaced wells. Following the addition of 20 µl of NK-18 with different
concentrations to each well, the plates were incubated at 37°C for 18 to 24 h. Then the size of the clear
zone surrounding each well was measured. 20 µl of sterile water was also added as the control.

Minimum inhibitory concentration (MIC) determinations

The MICs were measured using methods described previously by (20) and the CLSI (formerly NCCLS)
broth microdilution method (14) with some modification. Briefly, for the MICs determination, two-fold
serial dilutions of NK-18 were prepared in LB medium. 100 µl aliquots of the bacterial suspension were
dispensed into each well of a 96-well microtiter plate (Costar 3599; Corning) and then 100 µl of peptide
solution was added. PBS buffer was used as a negative control. The antibacterial activity of NK-18 was
evaluated by visible turbidity in each well after 18 h of incubation at 37°C. The MICs were expressed as
the minimum concentration of each sample required for a visible inhibition of growth. All MIC
determinations were made in triplicate and if results were within two doubling dilutions of each other
the highest reading were recorded for analysis.

Killing kinetics

Bacterial suspension was prepared as described before. The killing kinetics assay was measured
according to the method with a little modification (29). Bacteria were incubated with different
concentrations of peptides determined by the MIC assay. The viable count was monitored up to 24 h.
Aliquots were taken at defined intervals and diluted appropriately in phosphate saline buffer (pH 7.4),
and then 100 µl of the dilutions were plated in triplicate on LB agar. The plates were incubated at 37°C
and the colony forming units (CFU) were counted after 24 h.

Flow cytometric analysis

The integrity of the bacterial membrane after NK-18 treatment was determined by FACScan analysis
via nuclear staining with propidium iodide (PI) as described by Yeaman et al (17). The test strains in
the mid-log phase (OD600=0.5) were mixed with different concentrations of NK-18, and then incubated
for 60 min at 37°C. The peptide-treated cells were stained with PI solution (20 µg/ml) for 25 min at
room temperature in the dark. After incubation, the unbound dye was removed via the excessive washing with PBS. Flow cytometric measurements were performed on a flow cytometry (Beckman Coulter, USA) with computer-assisted evaluation of data (CellQuest software).

**Outer membrane (OM) permeability**

The OM permeabilization of *E. coli* by NK-18 was evaluated using the hydrophobic NPN (1-N-phenylnaphthylamine) fluorescent probe as described before with a little modification (36). *E. coli* cultures with an OD_{600} value of 0.5±0.02 were centrifuged for 10 min at 1000 g and the cells were suspended in half volume of 5mM HEPES (pH7.2). 100μl *E. coli* and 50μl NK-18 were mixed with 50μl NPN (final concentration 10μM). The final concentration of NK-18 was 12.5μg/ml, 25μg/ml, 50μg/ml and 100μg/ml, respectively. Control was carried out with 0.5% NaCl alone. An increase in fluorescence due to partitioning of NPN into the OM was recorded as a function of time until no further increase in intensity was observed. The excitation and emission wavelengths were set at 350 and 420 nm, respectively. Each assay was performed at least three times by a Pro Multimode Reader (Tecan Infinite M200).

**Inner-membrane (IM) permeability**

IM permeabilization was determined by measuring the release of cytoplasmic β-galactosidase activity from *E. coli* into the culture medium using ONPG as the substrate (18). *E. coli* cultured with LB-broth containing 5% lactose were harvested, washed and resuspended in 0.5% NaCl solution to get an absorbance of 1.2 at 420 nm, then resuspended in half volume of 0.5% NaCl solution. The concentrations of peptide were the same as described in the previous section. 100μl of *E. coli* and 90μl of the peptide were mixed with 10μl of ONPG (30mM) in a 96-well plate. Negative control was carried out with 0.5% NaCl and positive control was TritonX-100, respectively. The production of o-nitrophenol over time was determined by monitoring the changes in absorbance at 420nm using Tecan Infinite M200 Pro Multi Reader.

**Examination of bacterial membrane damage by scanning electron microscopy (SEM)**

The test strain of *E. coli* was grown to mid-logarithmic phase in LB broth. Suspension of *E. coli* (~10^5 CFU/ml) was incubated with NK-18 (100μg/ml) at 37 °C for 2h, and then centrifuged for 5 min
at 10000 rpm. The resulting pellet was washed with PBS gently. 1ml of 3% glutaraldehyde solution was added into the tube subsequently to fix these cells. After fixation with glutaraldehyde, the precipitations were impregnated in 2.5% tannic acid (Sigma) for 2 days. Counter fixation in 2% osmium tetroxide (Sigma) for 1h was followed by dehydration in ethanol and drying in a freeze-drying device (JEOL-JFD-310, Japan). Cells were coated with gold and analyzed by scanning electron microscope (JSM-6380Lv, Japan).

Calcein release from large unilamellar liposomes (LUVs)

In order to assess the ability of NK-18 to cause leakage of liposomal content, LUVs composed of bacterial mimic membrane EYPE/EYPG (7/3, w/w) were used. LUVs were prepared by dissolving required amounts of dry lipids in chloroform, respectively (30, 44). The solvents were removed by rotary evaporation to form a thin lipid film. After being dried under vacuum overnight, the lipid was hydrated in dye buffer solution (70 mM calcein, 10 mM Tris, 150 mM NaCl, and 1 mM EDTA, pH 7.4) (20). The suspensions were frozen in liquid nitrogen and thawed for ten cycles and then successively extruded through polycarbonate filters (100 nm pore size filter, 21 times) by Avanti mini-extruder. Untrapped calcein was removed by gel filtration chromatography on a Sephadex G-50 column (10 mm × 150 mm). The release of calcein from the LUVs with or without NK-18 was monitored on a Tecan Infinite M200 plate reader by measuring the fluorescence intensity at an excitation wavelength of 490 nm and an emission wavelength of 520 nm. For determination of 100% dye release, 2% TritonX-100 in Tris-buffer was added to dissolve the vesicles. The percentage of dye-leakage caused by the peptides was calculated as follows:

\[
dye\text{ leakage} \% = 100 \times \left( \frac{F - F_0}{F_t - F_0} \right)
\]

Where F is the fluorescence intensity after addition of the peptide, F₀ and Fₜ are fluorescence intensities without the peptides and with Triton X-100, respectively.

Transmission electron microscopy (TEM)

LUVs for TEM analysis were prepared as described before but hydrated with Tris-EDTA buffer (10 mM Tris, 150 mM NaCl, and 1 mM EDTA, pH 7.4). LUVs in the presence or absence of 100μg/ml of NK-18 were deposited on carbon-coated copper grids and dried naturally. The grids were negative...
stained with (2% (w/v)) phosphotungstic acid for 1 min. Then the additional phosphotungstic acid was soaked up by filter paper and electron micrographs were then recorded at nominal magnification (>20,000–40,000) with an accelerating voltage of 120 kV (JEM-1230, Japan).

Confocal laser scanning microscopy

_E.coli_ cells in mid-log phase were prepared and washed with PBS buffer. After incubation with 100μg/ml FITC-labeled NK-18 at 37°C for 1h, the cells were washed with the same buffer for three times and immobilized on a glass slide. The accumulation of the FITC-labeled peptides in the bacteria was observed with a confocal laser scanning microscope (Leica SP2, German).

DNA binding assay

Gel retardation experiments were performed for NK-18 as described previously (3). Briefly, 300 ng of the plasmid DNA (pBR322) was mixed with increasing amounts of peptide in 30 μl of buffer (10mM Tris-HCl, 1mM EDTA buffer, pH 8.0). Reaction mixtures were incubated at room temperature for 30min. Subsequently, native loading buffer was added (10% Ficoll 400, 10 mM Tris–HCl, pH 7.5, 50 mM EDTA, 0.25% bromophenol blue, and 0.25% xylene cyanol), and a 20μl aliquot was subjected to 1.5% agarose gel electrophoresis. The migration of DNA was detected by the fluorescence of ethidium bromide (Bio-Rad).

Absorption spectroscopy

Extraction of genomic DNA from _E. coli_ was performed according to a procedure described previously with a little modification (35). The purity and concentration of the extracted DNA was evaluated by Nanodrop 2000 Spectrophotometer (Thermo Scientific) which was coincidental with the request of experiment (2) (34). DNA and NK-18 were dissolved in 10 mM Tris-HCl buffer (pH 8.0) and then mixed to obtain various DNA/NK-18 samples with constant DNA concentration (82.0 ng/μl) and increasing NK-18 concentrations (0, 10, 50, 100 and 200μg/ml). Reaction mixtures were incubated for 30min. Absorbance of the mixed solutions was measured in the range of wavelengths 220–320 nm (Nanodrop 2000 Spectrophotometer). All measurements were performed at room temperature.

Atomic force microscopy (AFM) imaging
DNA and NK-18 were dissolved in 10 mM Tris-HCl buffer (pH 8.0) and then mixed to obtain DNA/NK-18 complex with 92.8 ng/μl of DNA and 100 μg/ml of NK-18 for 30 min at room temperature. Prior to AFM measurements, 50 μl of the suspension was deposited onto a freshly cleaved mica substrate. Samples were imaged after water evaporation completely. Experiments were performed with a multimode nanoscope atomic force microscopy (Agilent, 5400) operating in contact mode (23, 34). All images were recorded in air at room temperature at a scan speed of 0.8 Hz.

Results

Antimicrobial activity of NK-18

To investigate the antimicrobial activity of NK-18, it was synthesized by stepwise solid-phase assay, purified by HPLC and confirmed by ESI-MS. The primary sequence of NK-18 is KILRGVCKIMRTFLRRI-NH2. The antimicrobial activities of NK-18 against *E. coli* and *S. aureus* were screened by radial diffusion assay and determined by the broth microdilution method. The zones of inhibition corresponded to the antibacterial effect of NK-18 after incubation at 37 °C for 18-24h. As shown in Fig. 1, NK-18 showed remarkable antibacterial activity. It could induce significantly larger zones of clearance than the control group of sterile water. Furthermore, the diameter of inhibition zone produced by NK-18 was increasing with the peptide concentration increasing, which indicates that the antimicrobial activity of NK-18 was in a concentration dependent manner. According to the broth microdilution method, NK-18 displayed very impressive antimicrobial activity both against *E. coli* and *S. aureus*. The MICs of NK-18 against *E. coli* and *S. aureus* were 12.5 μg/mL and 6.25 μg/mL, respectively.

Time killing kinetics of NK-18

Although the MICs assay could reflect the antimicrobial activity of NK-18, it was determined by the overnight effect and the detailed information about NK-18 killing rates might be obscured in this assay. In order to understand the detail of NK-18 action, time killing kinetics assay were performed against *E. coli* and *S. aureus*. As shown in Figure 2, NK-18 acted on a different rate against *E. coli* and *S. aureus*, respectively. For the targeted bacterium *S. aureus*, NK-18 showed a very rapid killing rate that about 3 times log killing and 7 times log killing were observed at 2.5× MIC and 5× MIC within 5 minute after treatment, respectively. As for *E. coli*, NK-18 acted a little slowly compared with *S. aureus*. 
PI uptake assay

In order to characterize the effects of NK-18 on the integrity of E.coli membranes, PI, a DNA-staining fluorescent probe, was employed. Figure 3 showed the results of the intracellular PI measurements. The majority of the E.coli cells were labeled fluorescently after 60 min of incubation with NK-18, thereby indicating that NK-18 induced the influx of PI into the cells. That is to say NK-18 may destabilize or disturb the integrity of bacteria membrane and induce the uptake of PI.

OM/IM permeability

In order to further confirm the effect of NK-18 on the integrity of the membrane of bacteria, OM/IM permeability was measured respectively. The OM permeability was detected by NPN, which is a hydrophobic fluorescent probe. The fluorescence of NPN is weak in an aqueous environment and strong in the hydrophobic environment, e.g. the hydrophobic core of the bacterial membrane (24). As shown in Figure 4, NK-18 could induce NPN incorporate into the hydrophobic core of bacterial cell membrane in a dose-dependent manner and the relative fluorescence increased to a maximum in 5 min. Apart from this, the integrity of the inner membrane of E. coli was also evaluated by ONPG assay. As we know, the cytoplasmic β-galactosidase could only be released as a consequence of alteration/compromise in inner membrane of E. coli. β-galactosidase could react with ONPG and yield orth-nirophe nol, which is absorbed at 420 nm (7). As shown in Figure 5, after the treatment of different concentrations of NK-18, β-galactosidase released from E. coli cells from a lag time of about 5min till 90min to reach a steady state. Meanwhile, the release of β-galactosidase is in a dose dependent manner.

Examination of bacterial membrane change by SEM

In order to gain a more direct insight into the interaction between NK-18 and E. coli, the morphological changes of bacterial membrane were also examined by SEM in order to confirm these results got before. As shown in Figure 6: untreated E.coli had a normal smooth surface (Fig. 6A); in contrast, cells treated with NK-18 for 1h showed clear morphological changes. The changes caused by NK-18 appeared to involve the formation of blebs on the cell surface and the surface seem like been disrupted (Fig. 6B). The SEM observations provided a directly morphological evidence of the strong influence of NK-18 on bacterial membrane.
Peptide-lipid interaction using model liposome mimicking bacterial membrane

Because the component of bacterial membrane was complicated and sometimes it's hard to investigate the interaction between AMPs and bacterial membrane under different influential factors, most studies of the interaction of cationic AMPs with membranes have been performed using model liposome with compositions chosen to reflect those of the bacterial cytoplasmic membrane (9). To determine whether the antimicrobial activity of NK-18 depends on its capacity to permeate bacterial membranes, its ability of inducing calcein leakage from negatively charged EYPE/EYPG (7:3, w/w) LUVs mimicking the composition of \textit{E. coli} membrane was also measured. For this study, calcein encapsulated LUVs were employed at a concentration of 70mM calcein, a concentration at which fluorescence of calcein will be self-quenched. If the peptide is able to break the liposome membrane after treatment, the calcein encapsulated LUVs will burst and release calcein into the aqueous environment. Meanwhile the dye starts fluorescing and thus the fluorescence values increase. The fluorescence intensity curves of calcein release induced by NK-18 were shown in Figure 7. NK-18 induced a sharp increase in leakage of calcein and this ability was concentration-dependent. At the concentration of 6.25μg/ml and 12.5μg/ml, the fluorescence intensity induced by NK-18 was similar with that of positive control (Fig. 7A). Furthermore, NK-18 interacted with LUVs as soon as addition into the specimen, and caused maximum leakage of calcein dye up to 90% at the concentration of 6.25μg/ml (Fig. 7B). Knowing the peptides are acting on the surface of bacterial membrane, TEM studies were conducted with NK-18 with LUVs composed of bacterial mimic membranes. TEM studies were conducted with NK-18 at a concentration of 100μg/ml to monitor the morphologic changes of LUVs and the results showed surface alterations of the liposome. As shown in Figure 8, the membranes of untreated LUVs were smooth and intact. On the contrary, the surface of LUVs treated with NK-18 changed thoroughly. Its membrane showed incomplete and the fringe of it was vague. This was strong evidence that NK-18 could act against the model liposome mimicking bacterial membrane and this result was consistent with that of calcein leakage assay.

Internalization of FITC-labeled NK-18 in \textit{E.coli} cells

For the determination of site of action of NK-18, FITC-labeled NK-18 (100μg/ml) was incubated with log phase \textit{E. coli} cells for 1h and their localization was visualized by confocal laser-scanning.
microscopy. Based on the observed phenomenon, the localization of this peptide was almost in the cytoplasm of the bacteria cells (shown in Figure 9). These pictures illustrated that the FITC-labeled NK-18 could penetrate the cell membrane of *E. coli* and accumulate in the cytoplasm.

**Interaction between NK-18 and plasmid DNA and the genomic DNA of *E. coli***

Although NK-18 showed membrane activity both against model liposomes and bacterial membrane as described before, it still couldn’t conclude that the membrane was the only target for NK-18 exerting its antibacterial activity basing on the finding of the location of FITC-labeled NK-18 in bacteria. On this account, the DNA interacting ability of NK-18 was also studied by monitoring the electrophoretic mobility of plasmid DNA on agarose gel. Different concentrations of NK-18 were mixed with a fixed amount of plasmid DNA, after which the mixture were electrophoresised on agarose gel. Figure 10 indicated that NK-18 could interact with the plasmid DNA and retard its migration in the gels in a concentration-dependent manner. At a peptide concentration of 12μg/ml, a part of the plasmid DNA was still able to migrate into the gel as non-treated DNA, whereas, at a concentration of 16μg/ml, almost the entire DNA remained at the origin. At higher concentrations, complete retardation of the DNA was observed, showing that the DNA was aggregated by NK-18. To compare its mechanism of action with that of a membrane-active antimicrobial peptide, the same experiment was performed with magainin 2. By contrast, magainin 2 didn’t show any DNA interacting ability even at a concentration up to 128μg/ml which was absolutely different from NK-18. These results showed that NK-18 has intrinsic DNA-binding ability.

Now that NK-18 could interact with the plasmid DNA of bacteria and retard DNA migration in agarose gel, the relationship between this peptide and the genomic DNA of *E. coli* was also evaluated by electronic absorption spectroscopy. As shown in Figure 11, in the absence of NK-18, the genomic DNA exhibited a fluorescence emission maximum at 257 nm, and addition of NK-18 caused the fluorescence intensity to decrease and the emission maximum to shift from 257 to 261 nm, showing that NK-18 could bind with the genomic DNA of *E. coli*. The results of this experiment were in agreement with our previous gel retardation assay.

**AFM imaging**

The above gel retardation and fluorescence spectra data indicated that NK-18 interacted with DNA. To
further perceive the interaction between DNA and peptide more directly, the genomic DNA of *E. coli* was immobilized and AFM imaging was employed in this study. Representative AFM images of free DNA and DNA after treatment were shown in Figure 12. The images showed that DNA in the absence of peptide displayed as circles. However, most geometrical structures of DNA was influenced in the presence of NK-18 accompany by an increased height of peaks. This phenomenon indicated that most of DNA molecules could interact with NK-18 which proved the results of gel retardation assay and absorption spectroscopy.

**Discussion**

AMPs as one of the primary defense or attack strategies are common in the nature. Nowadays, with the emergency of the burst of multi-drug resistant bacteria, AMPs has attacked great interesting for its potential antimicrobial activity and different action mechanisms compared with conventional antibiotics. Although extensive researches have been made (12, 28, 37, 41), the exact mode of action of antimicrobial peptide is still controversial till now. It is generally accepted that AMPs produce effects on bacterial function via changes in cell wall synthesis and composition, inhibition of proteases and/or enzyme activity, inhibition of nucleic acid and/or protein synthesis, binding to DNA, and transmembrane pore formation (5, 21).

NK-18 was a derivative of mammalian natural killer cell effector NK-lysin. It has a much shorter sequence than NK-lysin and has more desirable features to be used clinically. NK-18 exhibits its antitumor activity through a membrane active action mode, which is superiority compared with conventional chemotherapeutic agents (42). However, the effects and the action mode of NK-18 against bacteria are unknown. With the increasing of multidrug resistant bacteria, the development of membrane active antibiotics should be a new strategy. So the aim of this study was attempted to investigate the antimicrobial activity and to clarify the antimicrobial mechanism of NK-18 with the purpose of developing novel antibiotic template for the current urgent status. Our results showed that NK-18 could inhibit the growth of *E. coli* and *S. aureus* in a dose dependent manner. It could induce PI, a membrane impermeable fluorescent dye, entering into the *E. coli* bacteria cells and binding with DNA. To our knowledge, the cell membrane of Gram stain bacteria is absolutely distinct (http://micro.digitalproteus.com/morphology2.php). In order to further verify the effect of NK-18 on the membrane of bacteria, the OM/IM permeability of *E. coli* were examined. Our results showed that
integrity of both outer membrane and inner membrane was disturbed after the treatment of NK-18.

Finally, the morphologic changes of the bacteria under SEM view provide intuitionist evidence that NK-18 have effect on the *E.coli* cell membrane. To exclude the influence of non-lipid components in the membrane on the activity of NK-18 and further confirm the ability of NK-18 affecting microbial plasma membrane, the effect of NK-18 on integrity of artificial liposome LUVs was investigated, which was composed of EYPE/EYPG (7:3, w/w) as the model bacterial plasma membrane. The results showed that NK-18 could disrupt the integrity of LUVs, which implied that NK-18 is membrane active against both the bacterial plasma membrane and the artificial mimicking pure membrane.

In addition of these results, we also found that the FITC-labeled NK-18 was visible on the cell membrane and in the cytoplasm of *E.coli* under the confocal laser microscopy. This result indicated that NK-18 could penetrate both the cell wall and cell membrane and accumulate inside the bacterial cells. This result may imply that the peptides were not only associated with the plasma membrane but also were internalized. This prompted us to look for some intracellular targeting mode of action for this peptide. With this aim we compared the relative affinity of NK-18 to bind plasmid DNA. In accordance with our confocal microscopy data, in DNA binding assay a DNA binding affinity of NK-18 was observed which was reflected as retardation in the movement of plasmid DNA. For magainin 2, an antimicrobial peptide studied more thoroughly, no retardation was observed even at a concentration of 128μg/ml which was accordance with the studies carried by Park et al (31). Afterward, the absorption spectroscopy and AFM was employed to investigate the interaction between NK-18 and bacterial genome DNA. It showed that NK-18 could not only induce the change of maximum absorption intensity, but also the shift of maximum absorption wave length (from 257nm to 261nm) of the genome DNA of bacteria.

So, taking all the data into account, we could have a certain understanding of the whole antibacterial activity mechanism of NK-18. It could be reasonably assumed that the cationic, hydrophilic characteristic of NK-18 initiates electrostatic interaction with the negatively charged components of the membrane of microbes firstly (10, 25). Then the peptide leads to permeabilization and disturbance of the outer and inner membranes, as well as the cytoplasm membrane of bacteria, allowing the free exchange of intra- and extra-cellular ions, resulting in the leakage of cytoplasmic components. The changes of membrane permeability further reflect the transport of the peptide across the membrane. Because DNA appeared as a polyion with considerable negative charges, alkalescent
amino acids (e.g. Lys and Arg) in NK-18 could bind into the phosphate fragments of DNA through electrostatic interaction spontaneously. So after the perturbation and transfer to the cytoplasm, NK-18 then interacts with its intracellular target DNA. As the barrier function of cell membrane has been disrupted and the cellular functions are also inhibited (such as DNA repair function) owing to the binding with peptide, the bacteria is killed in a rapid manner without the exploitation of a specific receptor. As with any new class of antimicrobial therapeutics, one key point is whether resistance can be provoked. The fact that NK-18 has double targets related with the cell membrane and intracellular DNA means that the chances of resistance are slim, as this would need the complete alteration of the cell membrane or bypassing of several biochemical pathways (15, 16).

In conclusion, our results revealed that NK-18 could effectively kill bacteria by disturbing the bacterial membrane and DNA binding in a rapid manner which is not affected by the known resistance mechanism. Although ongoing work on other properties of NK-18 will be necessary to obtain a systematic understanding of its application in the future, knowledge of the mechanism of NK-18 could no doubt provide a new lead in the design of potent antimicrobial peptides with therapeutic application with the increasing resistance to conventional antibiotics.

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References


**Figure Legends:**

**Figure 1.** NK-18 exhibited antimicrobial activity against different bacterial strains. The direct antimicrobial activity of NK-18 (0, 5, 10, 20, 30 and 40µg/well)) against *E. coli* and *S. aureus* was determined in a radial diffusion assay. NK-18 both gave rise to clear, bacteria-free zones around the wells of *E. coli* and *S. aureus.*

**Figure 2.** Bacterial killing kinetics of NK-18 at different concentrations. (A) Bacterial killing kinetics of NK-18 against *S. aureus.* (B) Bacterial killing kinetics of NK-18 against *E. coli.* Control was samples treated with PBS.

**Figure 3.** Effect of NK-18 on membrane integrity of *E. coli.* Bacteria (OD₆₀₀=0.5) were incubated for...
60 min with NK-18 at 25μg/ml and 50μg/ml. The percentage of PI-fluorescent cells after treatment is shown. One of the duplicated tests was presented here.

**Figure 4.** The uptake of 1-N-phenylnaphthylamine (measured as fluorescence intensity in arbitrary units) by *E. coli* with addition of different concentrations of NK-18. Data are the mean ± SEM (n = 3).

**Figure 5.** Release of cytoplasmic β-galactosidase activity (measured by absorbance at 420 nm) of *E. coli* cells treated by NK-18. C− represented the bacterial cells treated with 0.5% NaCl and C+ represented the bacterial cells treated with 2% Triton-X 100. Data are the mean ± SEM (n = 3).

**Figure 6.** Effect of NK-18 on the bacterial membrane of *E. coli* by scanning electron microscopy. Typical example of untreated *E. coli* (A) showed a normal smooth surface, while treated with 100μg/ml NK-18 revealed an incomplete pattern and bubbled on the outer face (B).

**Figure 7.** Fluorescent dye calcein released from large unilamellar vesicles (LUVs) composed of EYPE/EYPG (7/3) induced by NK-18. (A) Change of fluorescence intensity of the samples treated with NK-18 for 10min; (B) Concentration-dependent leakage of calcein from LUVs measured after 1min, 5min and 10min of incubation with different peptide concentrations.

**Figure 8.** Transmission electron microscopy images of LUVs composed of bacterial mimicking membrane. (A) Untreated liposomes; (B) Liposomes treated with 100μg/ml NK-18 for 3min.

**Figure 9.** Localization of FITC-labeled peptide in *E. coli* cells by confocal laser scanning microscopy. The upper panel was untreated *E. coli* cells and the lower panel was cells treated with FITC-labeled NK-18. For each treatment, fluorescence (A and D) and DIC (B and E) images, as well as merged images have been presented (C and F).

**Figure 10.** DNA binding activity of NK-18. Binding was assayed by the inhibitory effect of peptides on the migration of DNA bands. Various amounts of peptides were incubated with 300 ng of plasmid DNA at room temperature for 30min and the reaction mixtures were applied to a 1.5% agarose gel electrophoresis. The gel was visualized after ethidium bromide staining and UV irradiation. The numbers below represented the concentration of NK-18 and magainin 2 (μg/ml). C represented control which was plasmid DNA only. These results shown were representative of three experiments.

**Figure 11.** Ultraviolet spectra of *E. coli* genomic DNA in the presence of different concentrations of NK-18. A fixed concentration (82.0 ng/μl) of *E. coli* genomic DNA was treated with increasing amounts of NK-18 for 30min. Ultraviolet spectra were measured in the range of wavelengths 220-320nm. All measurements were recorded at room temperature.
Figure 12. AFM images of genomic DNA of *E. coli* in the absence or presence of NK-18. (A) The DNA in the absence of NK-18; (B) the DNA treated with 100μg/ml of NK-18 for 30min.