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ORIGINAL ARTICLE

Design and synthesis of cationic antibacterial peptide based on Leucrocin I sequence, antibacterial peptide from crocodile (*Crocodylus siamensis*) white blood cell extracts

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Leucrocin I is an antibacterial peptide isolated from crocodile (*Crocodylus siamensis*) white blood cell extracts. Based on Leucrocin I sequence, cationic peptide, NY15, was designed, synthesized and evaluated for antibacterial activity against *Bacillus sphaericus* TISTR 678, *Bacillus megaterium* (clinical isolate), *Vibrio cholerae* (clinical isolate), *Salmonella typhi* (clinical isolate), *Salmonella typhi* ATCC 5784 and *Escherichia coli* 0157:H7. The efficacy of the peptide made from all L-amino acids was also compared with all D-amino acids. The peptide made from all D-amino acids was more active than the corresponding L-enantiomer. In our detailed study, the interaction between peptides and the cell membrane of *Vibrio cholerae* as part of their killing mechanism was studied by fluorescence and electron microscopy. The results show that the membrane was the target of action of the peptides. Finally, the cytotoxicity assays revealed that both L-NY15 and D-NY15 peptides are non-toxic to mammalian cells at bacteriolytic concentrations.

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INTRODUCTION

Antimicrobial peptides (AMPs) have an important role in the innate immune system of most living organisms. These peptides are small, cationic and amphipathic molecules. Currently, >900 AMPs have been isolated from a wide variety of organisms.[1] Evidence has suggested that AMPs are of greatest potential to represent a new class of antibiotics.[2,3] Cationic AMPs belong to the innate immune system and host defense mechanism of a wide range of living organisms.[4,5] Many AMPs appear to act via specific, but not receptor-mediated, permeabilization of microbial membranes and have selective targets in their specificity of killing.[6] All of them cause lysis by two-step mechanisms, which consists of (i) binding to a negatively charged membrane with a cationic nature and (ii) permeabilization of the microbial membrane.[7] Nevertheless, these peptides use many structures and mechanisms to destroy micro-organisms, including binding to an intracellular target such as DNA, RNA and/or protein, and interacting with enzymes causing inhibition of metabolic processes.[8] These properties confer considerable potential for the development of these agents as novel therapeutic agents to overcome the resistance problem.[9]

In a previous study, Pata *et al.*[10] purified Leucrocin I, II, III and IV from crocodile (*Crocodylus siamensis*) white blood cell extracts. Leucrocin I exhibits strong antibacterial activity toward *Staphylococcus epidermidis*, *Salmonella typhi* and *Vibrio cholerae* but the MIC values of synthetic peptide Leucrocin I seem higher than native peptide. Hence, in this study, we have taken Leucrocin I peptide as sequence template for the design and synthesis the novel antibacterial peptides with low MIC and low hemolytic activity.

Generally, synthetic AMPs have focused on variation of the natural peptide sequences or development of novel peptides from large synthetic combinatorial libraries,[11] which represents a major advance in the discovery of new compounds for drug development.[12–14] Furthermore, discrimination between eukaryotic and prokaryotic cell membranes[15] provides a better understanding of the mechanisms underlying the lytic specificity of peptides. One important class of membrane-interacting AMPs assumes an amphipathic, α-helical conformation that permits insertion of a well-defined hydrophobic sector into the lipid bilayer.[16]

Tossi *et al.*[11] reported an alternative methodology for the design of α-helical AMPs, which is based on the comparison of known

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sequences. The sequence templates were filled out with appropriate residues, so as to ensure a high helix-forming potential and to maintain the highest possible amphipathicity. Other potentially important features, such as length and cationicity were also considered. Two types of amino-acid side chains have important roles in antimicrobial activity of peptides, namely, (i) the cationic side chains arginine (R), lysine (K) and histidine (H) mediate peptide interactions with negatively charged membranes or cell walls of bacteria, and (ii) bulky nonpolar side chains, for instance proline (P), phenylalanine (F), tryptophan (W) and Trp show a distinct preference for the interfacial region of lipid bilayers.[17]

Based on Leucrocin I sequence, NGVQPKY, the peptide, NY15, $N^1K^2K^3A^4G^5L^6F^7V^8V^9Q^{10}F^{11}P^{12}K^{13}K^{14}Y^{15}$, was designed by using the Antimicrobial Peptide Database (APD: http://aps.unmc.edu/AP/main.html).[18] This web tool offers a research interface to select peptides with search criteria such as length, structure, hydrophobic percentage, net charge and the target organism and provides statistical information on peptide sequence, structure and function, average peptide length, average net charge per peptide and frequency of each amino acid. Hydrophobic amino acids (A^4, L^6, F^7, V^8, F^{11}) were added to the central part of Leucrocin I sequence to construct a hydrophobic rich domain. As we wished to increase the positive change in the peptides structure, the amino acids lysine (K) were added to N-terminus and C-terminus (hydrophilic lysine domain) (K^2, K^3, K^{13}) of this sequence. In this study, we chose lysine (K) as a positive charge in the peptide structure instead of arginine (R) because previous results[19] have shown that arginine (R)-containing peptides cause relatively strong calcein leakage from zwitterionic 1-palmitoyl-2-oleoylphosphatidylcholine, eukaryotic mimic membrane, whereas lysine (K)-substituted analogues showed less membrane lytic activity. Therefore, the design of the NY15 structure is based on characteristics of naturally occurring AMPs: short, preferably cationic and with >30% of hydrophobic residues. The synthesis of NY15 was made from all L-amino acids (L-NY15) and all D-amino acids (D-NY15). Further comparisons were made of efficacies and the killing mechanism of both enantiomers peptides. Here we report that both L- and D-NY15 appeared to be potent antibacterial peptides against Gram-positive and Gram-negative bacteria without any toxicity for mammalian cells at their bacteriolytic concentrations. We also found that the peptide made from all D-amino acids was more active than the corresponding L-enantiomer.

MATERIALS AND METHODS

Peptide design and synthesis

The NY15 was constructed using Antimicrobial Peptide Database (APD: http://aps.unmc.edu/AP/ main.html) based on the Leucrocin I (NGVQPKY) sequence. The sequence of Leucrocin I was submitted to APD2: Antimicrobial Peptide Designer to improved antimicrobial property depends on length, structure, hydrophobic percentage and net charge. Hydrophobic amino acids (A^4, L^6, F^7, V^8, F^{11}) and positive change amino acids (K^2, K^3, K^{13}) were added to Leucrocin I sequence. NY15 (NKKAGLFVVQFPKKY) sequence was predicted antimicrobial potent by using APD2: Antimicrobial Peptide Calculator and Predictor. The prediction showed that this peptide form alpha helices and it may have at least three residues on the same hydrophobic surface, and may interact with membranes and has a chance to be an AMP.

Peptides were synthesized using solid-phase methodology with Fmoc-protected amino acids (GL Biochem, Shanghai, China). Purification by preparative reversed phase-HPLC gave final products that were >95% pure. Peptide structures were characterized by electrospray ionization-MS (Table 1).

Table 1 Sequences, masses, net positive charge and % hydrophobicity of peptides

Peptide	Amino-acid sequence	Molecular mass (Da)		Net positive charge	% Hydro-phobic
		Calculated[a]	Measured[b]		
Leucrocin I	NGVQPKY	804.90	804.91	1	11
NY15 (L-and D-form)	NKKAGLFVVQFPKKY	1767.15	1767.16	4	40

[a]Mass of each peptide was determined by electrospray ionization (ESI)—MS.
[b]Mass of each peptide was calculated by PeptideMass (http://web.expasy.org/peptide_mass/).

CD spectroscopy

The secondary structures of peptides were examined by CD spectroscopy. Each peptide was dissolved in 10 mM sodium phosphate buffer pH 7.2 or 50% 2,2,2-Trifluoroethanol (TFE) to a final concentration of 0.1 mg ml^{-1}. The molar ellipticities of peptides were determined using a Jasco J-715 spectropolarimeter (Jasco, Easton, MD, USA). Each spectrum was the average of five scans over the range 190–260 nm using a quartz cell of 1 mm optical path length at room temperature.[20,21] The scanning speed was 20 nm min^{-1} at an interval of 0.1 nm, 1-s response time and 1.0 nm bandwidth. Before calculation of the final ellipticity, all spectra were smoothed and corrected for buffer blanks.

Antibacterial activity

Antibacterial activity against *Bacillus sphaericus* TISTR 678, *Bacillus megaterium* (clinical isolate), *V. cholerae* (clinical isolate), *S. typhi* (clinical isolate), *S. typhi* ATCC 5784 and *Escherichia coli* 0157:H7 was measured using liquid growth inhibition assays[22] using a broad spectrum AMP Magainin2 as the positive control. Briefly, 10 µl of peptide solution were incubated in microtiter plates with 100 µl of a suspension of bacteria at a starting OD of $OD_{600} = 0.001$ in nutrient broth. Bacterial growth was assayed by measurement of OD at A_{550} after 16- to 20-h incubation at 37 °C. The MIC of each peptide was defined as the lowest peptide concentration that completely inhibited growth.[23]

Enzymatic degradation

The peptide (L-NY15 or D-NY15) was dissolved in 0.1 M Tris–HCl, pH 8.0 and digested with trypsin (1/50, w/w, TR-TPCK, Cooper Biomedical Co., Malvern, PA, USA) at 37 °C, for 20 min. After the incubation, digested peptides were determined for the antibacterial activity against *V. cholerae* using liquid growth inhibition assay.

Bactericidal activity

The bactericidal activity of peptides was assayed against *V. cholerae* as described by Boman *et al.*[24] *V. cholerae* was grown in nutrient broth and harvested at the logarithmic phase of growth by centrifugation at 3000 *g* for 5 min, washed three times with 10 mM sodium phosphate buffer pH 7.2. The bacterial cells were re-suspended with the same buffer to a final concentration of 1×10^8 CFU ml^{-1}. One hundred microliters of *V. cholerae* cells were mixed with peptides solution containing fivefold of MIC (5 × MIC) value of each peptide, and incubated at 37 °C. Aliquots (5 µl) were withdrawn at time intervals, adequately diluted, plated on a nutrient agar plate and then the colonies were counted after incubation overnight at 37 °C.

Hemolytic activity

The hemolytic activity of peptides was determined based on hemolysis of human red blood cells. The human red blood cells were centrifuged, washed three times with saline (0.85% NaCl) and re-suspended to 2% (v/v) in phosphate-buffered saline (35 mM phosphate, pH 7.0, 150 mM NaCl). One hundred microliters of human red blood cells solution were incubated with 10 µl of different peptide concentrations for 1 h at 37 °C. The samples were centrifuged for 5 min and hemolysis was determined by measuring the OD of the supernatant at 415 nm. Zero hemolysis (blank) and 100% hemolysis were

determined in phosphate-buffered saline buffer and 0.1% Triton X-100, respectively.

Cytotoxicity

The colorimetric 3-(4 5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide assay was used to determine the cytotoxicity of peptides on Vero cell. Briefly, 1×10^5 cells per well in Dulbecco's modified Eagle's medium-F12 medium supplemented with 10% fetal bovine serum were placed into 96-well plates. After incubation for 24 h under a fully humidified atmosphere of 95% room air and 5% CO_2 at 37 °C, peptides were added to cell cultures at a final concentration of MIC and 5 × MIC values. Toxicity was evaluated after 24 h of incubation by measuring the OD of the culture at 570 nm using 3-(4 5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide dye reduction assay based on conversion of the yellow tetrazolium salt 3-(4 5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide into purple formazan crystals by metabolically active cells.

Outer membrane permeability

The outer membrane assay was performed to determine the ability of peptides to permeabilize the bacterial outer membrane by measuring the uptake of the normally impermeable fluorophor 1-*N*-phenylnapthylamine (NPN). For assays, overnight cultures of *V. cholerae* were transferred to fresh nutrient broth medium and grown to OD_{600} values of 0.5–0.6. Cells were harvested, washed with 5 mM HEPES buffer pH 7.24 and resuspended in 5 mM KCN in HEPES buffer pH 7.24 (OD_{600} around 0.5). To 3 ml of cells, 60 µl of 500 µM NPN was added, followed by the peptide samples after 30 s. Excitation and emission wavelengths were set at 315 and 400 nm, respectively. After the addition of peptides, the increase in fluorescence as a result of partitioning of NPN into the interior outer membrane was measured for 5 min by using a fluorescence spectrophotometer. Polymyxin B sulfate, an antibiotic that can alter bacterial outer membrane permeability, was used as a positive control.

Inner membrane permeability

Inner membrane permeability was determined by measuring SYTOX Green influx assay. The experiment was performed according to Li *et al.*[25] with some modifications. *V. cholerae* cells were grown to mid logarithmic phase at 37 °C, washed two times and suspended in phosphate-buffered saline buffer (1×10^8 cells ml^{-1}). Aliquots of 100 µl of this cell suspension were deposited in each well of a standard 96-well plate. Ten microliters of the peptide solution (final concentration at MIC value) containing 5 µM SYTOX Green was added to the wells. The time-dependent increase in fluorescence excited by binding of the cationic dye to intracellular DNA was monitored using a SpectraMax M5 fluorescence microplate reader (Sunnyvale, CA, USA). The excitation and emission wavelengths were 504 and 523 nm, respectively.

DNA-binding assay

A gel retardation experiment was carried out to observe the influence of peptides on the migration of DNA in agarose gels.[26] Bacterial DNA used for this test was λDNA/*Eco*RI (Promega US G1721, Madison, WI, USA), which had been cut with restriction enzyme *Eco*RI into fragments sized 21.2, 7.4, 5.8, 5.6, 4.8 and 3.5 kbp. The DNA was mixed with 10X binding buffer consisting of 100 mM Tris, 200 mM KCl, 10 mM EDTA, 10 mM DTT and 50% (v/v) glycerol, water and peptides at increasing peptide to DNA ratios. The solutions were incubated at room temperature for an hour. To separate the DNA fragments, 2 µl of loading buffer was added before the samples were loaded onto a 0.75% agarose/Tris-acetate-EDTA gel and run at 70 mA for 1.5 h. The gel was then stained with ethidium bromide and photographed using the Gel Doc XR System (Bio-Rad Laboratories, Hercules, CA, USA). Buforin II, an AMP that kills bacteria by entering cells and binding nucleic acids, was used as a positive control.

Scanning electron microscopy (SEM)

SEM was performed according to Lau *et al.*[27] with slight modifications. *V. cholerae* was grown in nutrient broth and harvested at the logarithmic phase of growth by centrifugation at 3000 *g* for 5 min. The bacterial cells were then washed twice with 10 mM sodium phosphate buffer pH 7.2 and re-suspended with the same buffer to a final concentration of 1×10^8 CFU ml^{-1} ($OD_{600} =$ 0.1). Aliquots of suspensions of *V. cholerae* were individually incubated with MIC and 5 × MIC of peptides at 37 °C for 1 h. The 100 µl solutions of the bacterial cells were carefully pipetted and applied to a 0.2 µm cellulose acetate membrane filter (Sartorius AG, Göttingen, Germany) for 30 min whereupon cells were fixed with 300 µl of 2.5% (v/v) glutaraldehyde (Sigma, St Louis, MO, USA) for 1 h. The fixed material was dehydrated by rinsing (for 15 min) repeatedly with a series of ethanol solutions containing 30, 50, 70, 90 and finally 100% ethanol. Dry materials were coated with a sputter coater (SC7620, Polaron, Hertfordshire, UK) with gold palladium and examined by SEM (LEO1450VP, LEO Electron Microscopy, Cambridge, UK) operating at 12–20 kV.

Transmission electron microscopy

Transmission electron microscopy was performed according to Hao *et al.*[28] with slight modifications. *V. cholerae* was grown in nutrient broth and harvested at the logarithmic phase of growth by centrifugation at 3000 *g* for 5 min. The bacterial cells were washed twice with 10 mM sodium phosphate buffer pH 7.2 and re-suspended with the same buffer to a final concentration of 1×10^8 CFU ml^{-1}. The bacterial cells were treated with peptides at MIC and 5 × MIC, for 1 h at 37 °C. After treatment, the bacterial pellets were fixed with fixative reagent (5% glutaraldehyde and 10% formaldehyde in distilled water) for 10 min. Ten microliters of bacterial cells were dropped on copper grids and then washed with distilled water. The copper grids were dried in a desiccator. Microscopy was performed with a Tecnai 20 Twin microscope (Hillsboro, OR, USA) under standard operating conditions.

RESULTS AND DISCUSSION

Structural analysis of peptides by CD measurements

The secondary structure of linear antibacterial peptides in lipid membranes rather than in phosphate buffer should correlate well with the activity.[28] To investigate the secondary structure of the peptides, CD spectra of Leucrocin I, L- and D-NY15 peptides were measured in phosphate buffer and membrane mimic conditions in the presence of 50% TFE solution. Structural analysis showed that the CD spectra of D-NY15 in an aqueous solution and 50% TFE are a mirror image of that of L-NY15. All peptides showed a random coil structure in an aqueous solution, whereas secondary structures of L- and D-NY15 tend to be more ordered when they interacted with bacterial mimic membrane (50% TFE; Figure 1). However, they did

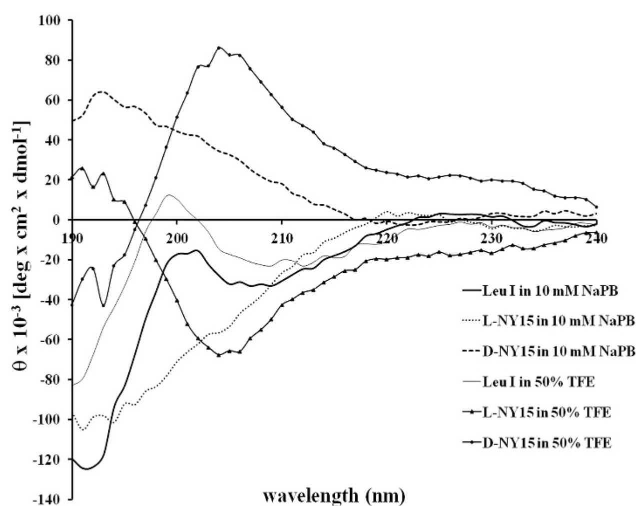

Figure 1 CD spectrum of Leucrocin I (Leu I), L-NY15 and D-NY15 dissolved in 10 mM sodium phosphate buffer (NaPB) and a membrane mimic condition in the presence of 50% TFE solution.

not have their maximum α-helical content when interacted with bacterial membranes. The deconvolution of spectrum was determined by using K2D2 program.[29] The results showed that the α-helical content in Leucrocin I and NY15 peptides (in 50% TFE) were 8.2% and 87.6%, respectively (Table 2). These results implied that NY15 adopts amphipathic α-helical structure on the membrane. This characteristic is similar to linear AMPs, such as the silk moth's cecropin and the African clawed frog's magainin, which adopt this organization only when they enter a membrane, whereupon they assume an amphipathic α-helical secondary structure.[30]

Antibacterial activity assays

The MICs of synthetic peptides obtained for *B. sphaericus* TISTR 678, *B. megaterium* (clinical isolate), *V. cholerae* (clinical isolate), *S. typhi* (clinical isolate), *S. typhi* ATCC 5784 and *E. coli* 0157:H7 compared with Magainin2 are shown in Table 3. The results show that synthetic Leucrocin I showed less activity than L- and D-NY15. A comparison of antibacterial peptides MICs indicates that the lowest MIC was found in NY15 made from D-amino acids. This result is similar to the antibacterial effects of lactoferricin B,[31] the lactoferricin B peptide made of all D-amino acids was more active than the corresponding L-enantiomer. However, Wade *et al.*[32] have indicated that enantiomers of AMPs are equally active. Peptides with equally active enantiomers, cannot have stereo-specific targets like protein receptors. Alternatively, the results might indicate that the L-enantiomer is being enzymatically degraded before reaching the target, as most proteolytic enzymes have greatly reduced activity against D-aminoacid residues.[31] To test this hypothesis, L-NY15 and D-NY15 were digested with trypsin and subsequently evaluated for the antibacterial activity against *V. cholerae* using liquid growth inhibition assays. The results showed that L-NY15 was very sensitive to trypsin degradation, leading to lower antimicrobial activity (14% inhibition). In contrast, all D-NY15 were very resistant, after trypsin treatment they still rendered relatively high activity (84% inhibition; Supplementary Figure S1).

Table 2 The percentage content in α-helix and β-sheet of a peptide

	% Of content in 10 mM NaPB[a]		*% Of content in 50% TFE*[a]	
Peptide	*α-Helix*	*β-Sheet*	*α-Helix*	*β-Sheet*
Leucrocin I	8.0	22.1	8.2	24.1
NY15	8.0	22.1	87.6	0.5

Abbreviation: NaPB, sodium phosphate buffer.
[a]The percentage content in alpha helix and beta sheet of a peptide in 10 mM NaPB or 50% TFE was estimated by the K2D2 method from website: http://www.ogic.ca/projects/k2d2/, the estimated maximum error was >0.32.

Table 3 Antibacterial activities of peptides against Gram-positive and Gram-negative bacteria

	MIC (μM)					
Peptide	*B. megaterium (clinical isolate)*	*B. sphaericus TISTR 678*	*V. cholerae (clinical isolate)*	*E. coli 0157:H7*	*S. typhi (clinical isolate)*	*S. typhi ATCC 5784*
Leucrocin I	>64	>64	>64	>64	>64	>64
L-NY15[a]	28.3	64	52	>64	>64	>64
D-NY15[b]	5.1	15.4	15.4	41.2	52	52
Magainin2[c]	3.7	18.4	7.4	36.9	11.1	14.7

[a]The peptide made from all L-amino acids.
[b]The peptide made from all D-amino acids.
[c]Used as the positive control.

Bactericidal activity

The bactericidal activity of L- and D-NY15 was assayed against *V. cholerae*, and the same experiment was performed with Magainin2. The results showed that when *V. cholerae* cells were treated with 5 × MIC that is, 260.1 μM of L-NY15 and 77 μM of D-NY15, the number of viable cells decreased by seven orders of magnitude (Figure 2). The results indicate that L- and D-NY15 showed rapid killing of the bacteria. However, when the cells were treated with 5 × MIC of Magainin2 the number of viable cells decreased by eight orders of magnitude.

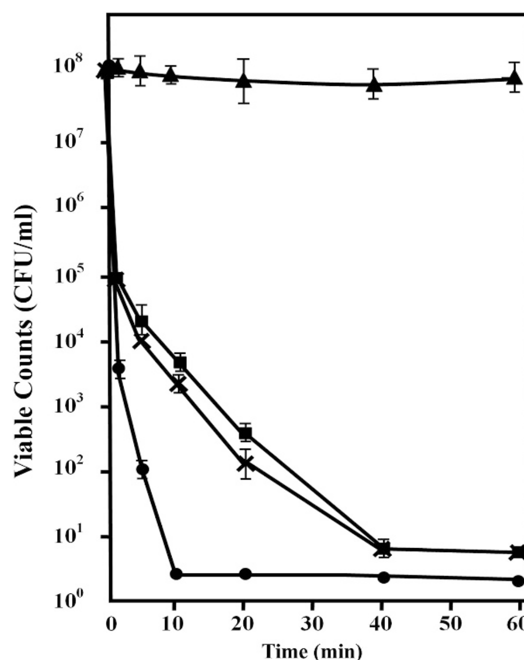

Figure 2 The bactericidal activity of NY15 peptides against *Vibrio cholerae;* ▲ = 0.01% acetic acid, ■ = L-NY15 (L-form) 260.05 μM, × = D-NY15 (D-form) 77.15 μM, ● = Magainin2 36.85 μM.

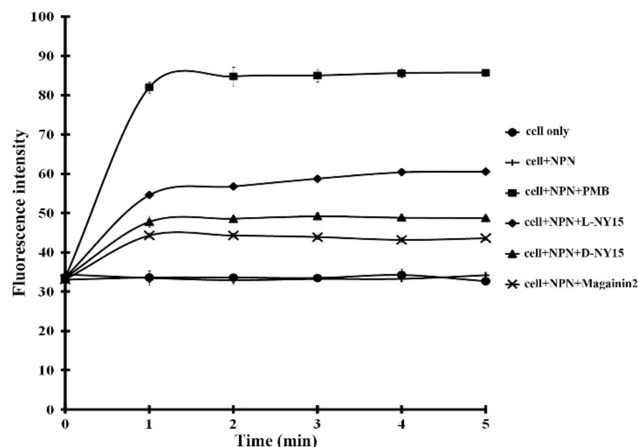

Figure 3 1-*N*-phenylnapthylamine (NPN) uptake assay of peptides using *Vibrio cholerae* (clinical isolated).

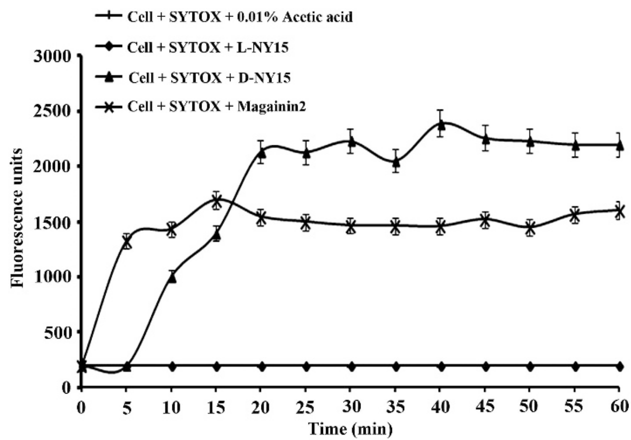

Figure 4 Cytoplasmic membrane permeabilization of *Vibrio cholerae* monitored by entry of the DNA fluorescent probe SYTOX Green at $\lambda_{excitation} = 504$ nm and $\lambda_{emission} = 523$ nm.

Hemolytic activity and cytotoxicity assay

The peptides were tested for hemolytic activity against human red blood cells and cytotoxicity against Vero cells at MIC and $5 \times$ MIC values. No significant hemolytic activity and cytotoxic activity were observed. These results indicate that L- and D-NY15 may be a good candidate for antimicrobial drug development.

Outer membrane permeability

Gram-negative bacteria have two envelope membranes, an outer and an inner membrane. In the case of Gram-negative bacteria, the outer membrane has a role as a drug barrier.[14] To assay the mode of action of the antibacterial peptides on bacterial outer membrane, the NPN, a hydrophobic fluorescence probe, was used.[33,34] Normally, NPN fluoresces weakly in an aqueous environment and strongly when it enters a hydrophobic environment such as the interior of a membrane.[34] The addition of L- and D-NY15 to *V. cholerae* suspensions in the presence of NPN caused increase in the fluorescence intensity from ~33 to ~50 within 1 min when

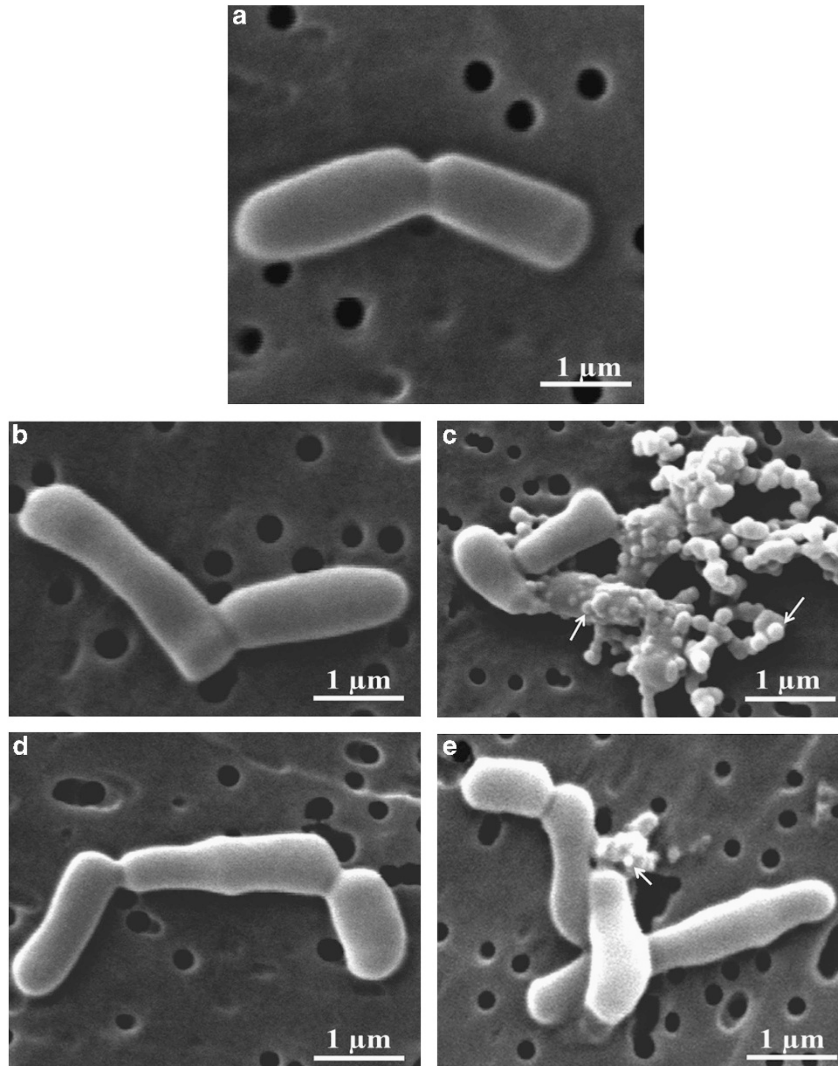

Figure 5 Scanning electron micrographs of *Vibrio cholerae* treated with peptides. (**a**) Control bacteria after treatment with 0.01% acetic acid for 1 h. (**b**, **c**) Bacteria after treatment with L-NY15 at MIC and $5 \times$ MIC for 1 h. (**d**, **e**) Bacteria after treatment with D-NY15 at MIC and $5 \times$ MIC for 1 h.

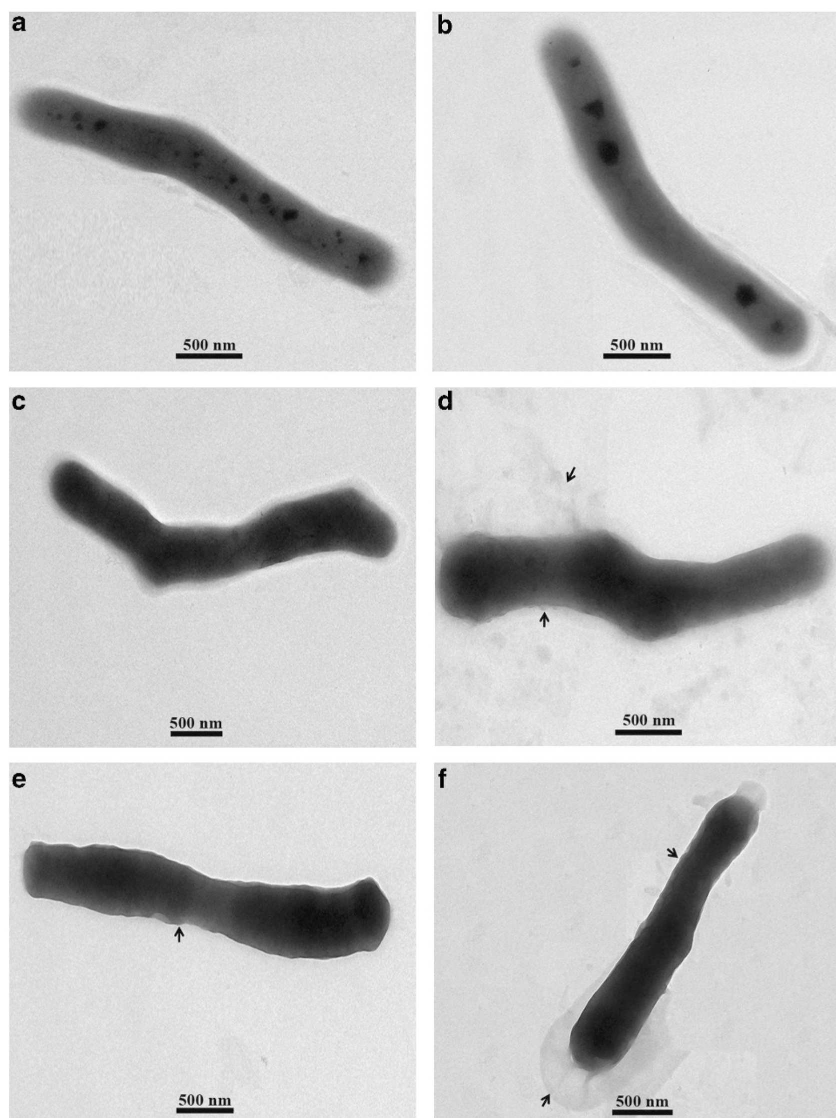

Figure 6 Transmission electron micrographs of *Vibrio cholerae* treated with peptides. (**a**, **b**) Control bacteria after treatment with 0.01% acetic acid only 1 h. (**c**, **d**) Bacteria after treatment with L-NY15 at MIC and 5 × MIC for 1 h. (**e**, **f**) Bacteria after treatment with D-NY15 at MIC and 5 × MIC for 1 h. See the Results and Discussion section for other experimental details and descriptions of the images.

compared with cells in buffer only and cells with NPN only that acted as the negative control (Figure 3). After 1 min, all of the tested samples except the negative control seemed to stable intensity of fluorescence until the end of the experiment (5 min). A higher increase in fluorescence of *V. cholerae* treated with L-NY15 peptide was observed when compared with D-NY15 peptide. However, they were lower than the fluorescence of *V. cholerae* treated with the positive control, polymyxin B sulfate. Therefore, our results indicated that both L- and D-NY15 peptides have permeabilized the outer membrane of the intact cells similarly to polymyxin B sulfate and other cationic AMPs including SMAP-29,[35] Indolicidin[33] and Temporin-L.[36]

Inner membrane permeability

We subsequently evaluated the ability of synthetic L- and D-NY15 to permeate the *V. cholerae* inner membrane. SYTOX Green can penetrate cells with a compromised plasma membrane but will not cross the membranes of live cells.[37] Fluorescence of SYTOX Green is enhanced when bound to intracellular nucleic acids. SYTOX Green was used to analyze the effect of L- and D-NY15 on membranes of living *V. cholerae* cells. The addition of D-NY15 at MIC to *V. cholerae* suspensions in the presence of SYTOX Green caused increased fluorescence after 5 min (Figure 4) when compared with cells with 0.01% acetic acid that acted as the negative control. However, L-NY15 did not cause an increase in fluorescence. This suggested that the influx of SYTOX Green into *V. cholerae* cells increased over time with the increase in plasma membrane permeability induced by D-NY15. It might be L-enantiomer is being enzymatically degraded before reaching the target, as most proteolytic enzymes have greatly reduced activity against D-aminoacid residues.[31] Magainin2, which kills bacteria by targeting membranes and forming a pore, was used as a positive control. Compared with D-NY15, Magainin2 induced a rapid fluorescence increase and quickly reached a maximum fluorescence state after 17 min, however, D-NY15 showed an increase in fluorescence intensity, which was greater than that by Magainin2. The SYTOX

Green influx analysis demonstrated that only D-NY15 (final concentration at MIC value) could increase living *V. cholerae* cell plasma membrane permeability.

DNA-binding assay

However, it has been reported that some cationic AMPs, such as Buforin II are able to transverse the cytoplasmic membrane and enter the cytoplasm where they can act on polyanionic molecules, such as DNA.[38,39] Therefore, a DNA gel electrophoresis separation was carried out to determine whether L- and D-NY15 were able to interact with bacterial DNA. Peptides that are able to bind to λDNA will inhibit the migration of the DNA on the gel. When the peptides bind to DNA, they formed larger molecules, hence blocking their migration through the gel.[40] We found that both L- and D-NY15 peptides could not inhibit the migration of the DNA on the gel at various weight ratios of peptides to DNA (0.5:1, 1:1, 2:1 and 4:1; Supplementary Figure S2). Buforin II, which was used as positive control, was effective in binding to DNA with all DNA fragments bound at a DNA: peptide ratio of 1:4. Some AMPs, such as Magainin2, require high concentrations to bind to DNA and RNA.[38] As the direct killing mechanism of Magainin2 is to kill bacteria by targeting membranes and forming a pore, the result is leakage of small cellular molecules. From this result, the direct targeting site of both L- and D-NY15 action may be the cell membrane.

Scanning electron microscopy

SEM was chosen to measure the effects of both L- and D-NY15 peptides on the cell membranes of *V. cholerae*. The results show that untreated bacteria displayed a smooth bright surface with no apparent cellular debris (Figure 5a). Obvious abnormality of cell membrane was observed in cells treated with $5\times$ MIC of L- and D-NY15 peptides. The cell surfaces of *V. cholerae* showed blebs and then a collapsed cell structure (Figures 5c and e). The SEMs results at $5\times$ MIC clearly demonstrate that the bacterial cells are drastically physical damaged.

Transmission electron microscopy

Owing to *V. cholerae* is a Gram-negative bacterium; Gram-negative bacteria cell walls are more complex than Gram-positive cell walls. Structurally, a Gram-negative cell wall contains two layers external to the cytoplasmic membrane and some bacteria (Gram-positive or Gram-negative) are closely surrounded by loose polysaccharide or protein layers called capsules. Therefore, transmission electron microscopy observations of cells have been used to reveal the presence of capsule on *V. cholerae* and to observe the bacterial membrane. The bacteria were treated with MIC and $5\times$ MIC of L- and D-NY15 for 1 h before fixing. These electron micrographs showed that the untreated cells exhibited normal, smooth surfaces as shown by visualization of the capsule and the capsular material cells of *V. cholerae* (black spot on the cell in Figures 6a and b). In contrast, peptide-exposed cells exhibited a wide range of significant abnormalities (Figures 6c–f). The peptide-exposed cells at MIC value were found to have a disorder of capsular materials (Figures 6c and e). Moreover, the cell membrane of *V. cholerae* treated with D-NY15 at $5\times$ MIC value showed deep roughening. When the concentration of peptides was increased to $5\times$ MIC value, a membrane permeabilizing effect became evident, releasing capsular material surrounding cells and some apparent leakage of cytoplasmic components (Figures 6d and f). Patrzykat *et al.*[41] have shown similar concentration-dependent behavior with some derivatives of pleurocidin, an AMP isolated from winter flounder. At the MIC concentration, these peptides had little capacity to damage the cell membranes of *E. coli*, whereas, at 10-fold their MIC value, all these peptides permeabilized the *E. coli* cell membranes. These results indicate that L- and D-NY15 have active target sites at the cell surface.

In summary, we report the design, synthesis and antibacterial activity of novel antibacterial peptides L- and D-NY15. The peptide made from all D-amino acids was more active than the corresponding L-enantiomer. We presume that both L- and D-NY15 kill bacteria by perturbing the permeability of the bacterial cell membrane. The cytotoxicity assays revealed that both L- and D-NY15 are non-toxic to mammalian cells at bacteriolytic concentrations.

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