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A peptide fragment derived from the T-cell antigen receptor protein α-chain adopts β-sheet structure and shows potent antimicrobial activity

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ABSTRACT

A 9-residue peptide, CP-1 (GLRILLLKV-NH_2), is synthesized by solid-phase synthesis method. CP-1 is a C-terminal amidated derivative of a hydrophobic transmembrane segment (CP) of the T-cell antigen receptor (TCR) α-chain. CP-1 shows broad-spectrum antimicrobial activities against Gram-positive and Gram-negative bacteria with the minimal inhibitory concentration (MIC) values between 3 and 77 μM. Circular dichroism (CD) spectral data shows that CP-1 adopts a well-defined β-sheet structure in membrane-mimicking environments. CP-1 kills *E. coli* without lysing the cell membrane or forming transmembrane pores. However, CP-1 can penetrate the bacterial cell membranes and accumulate in the cytoplasm in both Gram-positive *S. aureus* and Gram-negative *E. coli*. Moreover CP-1 shows binding affinity for plasmid DNA. These results indicate that the killing mechanism of CP-1 likely involves the penetration into the cytoplasm and binding to intracellular components such as DNA.

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1. Introduction

In recent years, studies of new classes of antibiotics have been paid more attention because of the emergence of serious antibiotic resistance. Host defense peptides known as antimicrobial peptides discovered in a wide range of species with the common characteristics of net positive charges and the ability to form amphipathic structures have been considered as a novel class of antibiotics [5,9,10,29]. These peptides play significant roles in host defense against invading pathogenic microorganisms. Ideally, these new antibiotics should possess both novel modes of action as well as different cellular targets with existing antibiotics to decrease the cross-resistance [4]. On the basis of amino acid composition and three-dimensional structure, antimicrobial peptides can be divided into three families: (1) linear α-helical peptides such as cecropins, magainins and melittin, which adopt a random structure in dilute aqueous solution and form α-helices in organic solvents and upon contact with cell membrane phospholipids; (2) β-sheet peptides such as defensins, tachyplesin, and protegrin, which contain cysteine residues linked by disulfide bridges and adopt either a β-sheet or β-hairpin fold; (3) peptides rich in specific amino acids such as proline, glycine, tryptophan, arginine or histidine, most of which adopt an extended α-helical structure. Despite these very diverse structural motifs, most of these antimicrobial peptides are membrane-active agents. The net positive charges facilitate initial binding to the negatively charged bacterial membrane through an electrostatic interaction, then the hydrophobic portion of the peptide inserts into bacterial membrane to form pores, leading to membrane permeabilization [6,21]. There are also several peptides having the mechanisms of action other than membrane permeabilization. For example buforin 2 kills bacteria without inducing membrane lysis and has strong affinity with DNA and RNA [14,22]. PR-39 penetrates cells to inhibit the activity of specific molecular material essential to bacterial growth [1]. Among these antimicrobial peptides, short and linear peptides, which are accessible to chemical synthesis, have been considered as good candidates for the development of potential antimicrobial therapeutic agents [21,27,28]. Almost all the short linear natural antimicrobial peptides or the segments of natural proteins with potent antimicrobial activity adopt α-helical structure in membrane mimetic environments. Only a few synthetic short linear peptides with antimicrobial activity adopt β-sheet structures [3,12,23,24]. Here we report that a hydrophobic transmembrane peptide CP with amidated C-terminus (termed CP-1) shows potent antimicrobial activity and adopts β-sheet structure in membrane mimetic environments. CP (core peptide) is a 9-amino acid fragment derived from the T-cell antigen receptor (TCR) α-chain transmembrane sequence and shows inhibition of T-cell antigen specific activation *in vitro* and *in vivo* [19].

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2. Materials and methods

2.1. Materials

Rink amide-MBHA resin was purchased from Tianjin Nankai Hecheng Co. (Tianjin, China). 9-Fluorenylmethoxycarbonyl (Fmoc) amino acids, N-hydroxybenzotriazole hydrate (HOBt) and diisopropyl carbodiimide (DIC) were obtained from Beijing Bo Mai Jie Technology Co. (Beijing, China). Trifluoroacetic acid (TFA) and thioanisole were purchased from ACROS (New Jersey, USA). 1,8-Diazabicyclo[5,4,0]undec-7-ene (DBU) was purchased from Nanjing Tianzun Chemicals (Nanjing, China). Piperidine and phenol were from Tianjin Guangfu Chemical Research Institute (Tianjin, China). Ciprofloxacin was from The Central Pharmaceutical Co., Ltd. (Tianjin, China) and roxithromycin was from Guangzhou Baiyunshan Pharmaceutical Co., Ltd. (Guangzhou, China). *o*-Nitrophenyl-β-D-galactoside (ONPG) was obtained from Beyotime Institute of Biotechnology (Shanghai, China). Egg yolk L-α-phosphatidylcholine (EYPC) and egg yolk L-α-phosphatidyl-DL-glycerol (EYPG) were purchased from Sigma Chemical Co. (St. Louis, USA). Fluorescein isothiocyanate (FITC) was obtained from Beijing Newprobe Biotechnology Co. Ltd. (Beijing, China). Plasmid DNA (pUC18) was purchased from Novagen (Madison, WI, USA). Melittin was isolated and purified from bee venom according to literature [17].

2.2. Peptide synthesis and purification

CP-1 was synthesized by solid-phase peptide synthesis with manual Fmoc/*t*Bu strategy on Rink amide-MBHA resin. The Fmoc group on the starting resin was removed by treatment with 20% piperidine/*N*,*N*-dimethylformamide (DMF). The deprotected resin was suspended in Fmoc-protected amino acid (3 equiv.), DIC (3 equiv.) and HOBt (3 equiv.) in DMF/dichloromethane (DCM) (1/1, volume ratio) and the mixture was stirred until a negative Kaiser test [13]. A second coupling reaction may be required to achieve quantitative conversion. Before the next coupling reaction, the Fmoc group was removed by treatment with 4% DBU in DMF/DCM (1/1, volume ratio). After the incorporation of all amino acids, the peptide was cleaved with a mixture of TFA/phenol/H_2O/thioanisole (95/2/2/1, volume ratio). The resulting cleavage solution was precipitated with cold diethyl ether to give crude peptide. The peptide was purified by semi-preparative reversed-phase HPLC (Spherigel NC-3015-06213-C18, 15 mm × 300 mm, Spherigel). The purified peptides, which was shown to be homogeneous (>95%) by analytical HPLC (Agilent ZORBAX SB-C18, 4.6 mm × 150 mm, Agilent), was subjected to mass spectrometry assay.

2.3. Synthesis of FITC labeled CP-1

Resin-bound CP-1 prepared as shown above before cleavage was treated with 4% DBU in DMF/DCM (1/1, volume ratio) to remove the N-terminal Fmoc protection. Then the peptide-bound resin was treated with FITC in DMSO in the presence of *N*,*N*-diisopropylethylamine at room temperature in the dark for 6 h. The FITC-labeled CP-1 was cleaved from the resins via the same strategy for CP-1 and purified by semi preparative RP-HPLC.

2.4. Antimicrobial activity

The antimicrobial activities of the peptides were assayed in nutrient broth (10 g of peptone, 3 g of beef powder and 5 g of NaCl per liter of medium) under aerobic conditions using microdilution method. Different concentrations of peptides or antibiotics were added to 1 mL of medium containing the inocula of the test organism (~10^6 CFU) in mid-logarithmic phase of growth. Growth inhibition was determined by measuring the OD_{600} following incubation for 24 h at 37 °C. The antibacterial activity is expressed as minimal inhibitory concentration (MIC). The microorganisms used were Gram-positive bacteria, *S. aureus*, *B. subtilis*, *B. thuringiensis* and *arthrobacter*, and Gram-negative bacteria *E. coli* and *Pseudomonas* sp. *ADP*.

2.5. Preparation of small unilamellar vesicles (SUVs)

Small unilamellar vesicles (SUVs) were produced for circular dichroism (CD) measurement. A chloroform/methanol (2/1, v/v) solution of lipids, either EYPC or EYPG, was dried under nitrogen until a thin film was formed. The film was then lyophilized for 12 h. The lipids were dispersed in 10 mM sodium phosphate buffer (pH 7.4), and the suspension was sonicated until the solution became transparent. The concentration of the lipid was determined according to literature [2].

2.6. Circular dichroism (CD) spectroscopy

CD spectra were recorded on a JASCO-J-715 spectropolarimeter under nitrogen flush in a 1 cm path length cell at 25 °C. The average of three recordings was taken for each assay.

2.7. Scanning electron microscopy (SEM) observation

The test strains of *E. coli* and *S. aureus* were individually grown to mid-logarithmic phase in nutrient broth. Suspensions of *E. coli* and *S. aureus* (~10^6 CFU/mL) were incubated with peptide (78 μM/mL) in a water shaker (37 °C) for 30 min, and then centrifuged for 5 min at 1700 rpm. The resulting pellet was washed in phosphate buffer and then fixed under 2.5% glutaraldehyde and dehydration in 20%, 40%, 60%, 80%, 95% and 100% ethanol sequentially. The samples were observed with a scanning electron microscope (Hitachi S-3500N, Japan).

2.8. Inner-membrane permeability

Inner-membrane permeability in the presence of the CP-1 was assayed by the ability of the peptide to unmask β-galactosidase activity using ONPG as a substrate [15,16]. β-Galactosidase can hydrolyze the colorless substrate ONPG to yellow *o*-nitrophenol. *E. coli* ML-35, which produces the enzyme β-galactosidase, was grown to mid-logarithmic phase in nutrient broth. The cells were centrifuged to remove nutrient broth and then washed three times with sodium phosphate buffer (10 mM, pH 7.4) containing 100 mM NaCl. The cells were resuspended in the same buffer and adjusted to a concentration of 1×10^8 CFU/mL. The bacterium suspension was mixed with the peptide and ONPG solutions in the same buffer. The final concentration of *E. coli* ML-35, ONPG and CP-1 were 1×10^7 CFU/mL, 1.5 mM and 78 μM, respectively. The bacteria solution was then incubated at 37 °C. The production of *o*-nitrophenol was measured by UV absorbance at 420 nm at different time intervals. The control without peptide was taken as the blank. Activity obtained from cells incubated with SDS (1%, w/v) was taken as 100% permeabilization.

2.9. Dye leakage assay

Large unilamellar vesicles (LUVs) composed of EYPC/EYPG (7/3, w/w) were prepared by dissolving required amounts of dry lipids in chloroform. 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). The

suspensions were frozen in liquid nitrogen and thawed for ten cycles and then successively extruded through polycarbonate filters (100 nm pore size filter, 20 times). The lipid concentration was determined by phosphorus analysis. Untrapped calcein was removed by gel filtration chromatography on a Sephadex G-50 column (10 mm × 1500 mm). The release of calcein from the LUVs was monitored on a Hitachi F-4500 spectrophotometer (Japan) by measuring the fluorescence intensity at an excitation wavelength of 490 nm and an emission wavelength of 520 nm. The fluorescence intensity of the vesicles in Tris buffer with 10% Triton X-100 was taken as 100% dye leakage. The apparent percent leakage value was calculated as follows:

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

where F is the fluorescence intensity after addition of the peptide, F_0 is the fluorescence intensity of intact vesicles and F_t is fluorescence intensity upon 100% dye leakage.

2.10. Confocal laser-scanning microscopy

Gram-negative *E. coli* and Gram-positive *S. aureus* cells in mid-logarithmic phase were prepared and washed with Tris–HCl buffer (pH 7.4). After incubation with FITC-labeled CP-1 (50 μg/mL) at 37 °C for 30 min, the cells were washed with same buffer and immobilized on a glass slide. The accumulation of the FITC-labeled peptides in the cells was observed with an Olympus FV1000 confocal laser-scanning microscope (Japan).

2.11. DNA binding assay

The DNA (50 ng, in 10 mM Tris, 1 mM EDTA buffer, pH 8.0) was mixed with different amounts of peptides in 30 μL for 20 min. After adding 3.3 μL of loading buffer (10× Loading dye, Takara, Japan), the sample was subjected to electrophoresis using 1.5% agarose gel in Tris acetate-EDTA buffer (40 mM Tris–acetate and 1 mM EDTA, pH 8.0) and the migration of DNA was detected by the fluorescence of ethidium bromide.

3. Results

3.1. Antimicrobial activities

The antimicrobial activity of CP-1 (GLRILLLKV-NH_2) against bacterial strains, including four Gram-positive species (*S. aureus*, *B. subtilis*, *B. thuringiensis* and *arthrobacter*) and two Gram-negative species (*E. coli* and *Pseudomonas* sp. *ADP*), were investigated by serial dilution microbroth. The minimal inhibitory concentrations of CP-1 against these bacterial stains are summarized in Table 1. These results showed that CP-1 shows broad-spectrum antimicrobial activities against Gram-positive and Gram-negative bacteria with MIC values between 3 and 77 μM. For comparison, the antimicrobial activities of two clinically used antibiotic compounds ciprofloxacin and roxithromycin against Gram-positive species *S. aureus* and Gram-negative species *E. coli* were assayed, as shown in Table 1.

Table 1
Antimicrobial activities of CP-1 and two clinically used antibiotic compounds.

Bacteria		MIC (μM)		
		CP-1	Ciprofloxacin	Roxithromycin
Gram-positive bacteria	*S. aureus*	3.0	1.7	3.2
	B. subtilis	12.4		
	B. thuringiensis	30.8		
	Arthrobacter	46.3		
Gram-negative bacteria	*E. coli*	77.2	54.4	25.0
	Pseudomonas sp. *ADP*	15.4		

3.2. Secondary structure of CP-1

CD was used to characterize the secondary structure of the peptide. The CD spectra of CP-1 (15 μM) were measured in physiological pH environment (10 mM Tris–HCl buffer, pH 7.4) and membrane mimicking environments, 30 mM SDS, 0.5 mM EYPC and 0.3 mM EYPG, respectively. Fig. 1A shows that CP-1 at concentration of 15 mM in Tris–HCl buffer (pH 7.4) exhibited typical random coil structure. However, the CD spectra of the CP-1 at the same concentration in 30 mM SDS, 0.5 mM EYPC and 0.3 mM EYPG, respectively, showed negative bands at ~216 nm (Fig. 1A), indicating that CP-1 adopted β-sheet structure in the membrane mimicking environments. The CD spectra of CP-1 at higher concentrations in Tris–HCl buffer were also assayed (Fig. 1B). It can be seen from Fig. 1B that, at concentrations of 60 and 75 μM, the negative band moved to higher wavelength, indicating that a small ratio of CP-1 probably adopted β-sheet structure.

3.3. Morphology changes of bacteria after treating by CP-1

The effect of CP-1 on the bacterial plasma membrane was visualized by SEM, as shown in Fig. 2. Fig. 2 shows that no lysed bacteria were observed after incubation with CP-1 even at concentrations higher than MIC. Almost no change in the morphology of *S. aureus* after incubation was observed. The

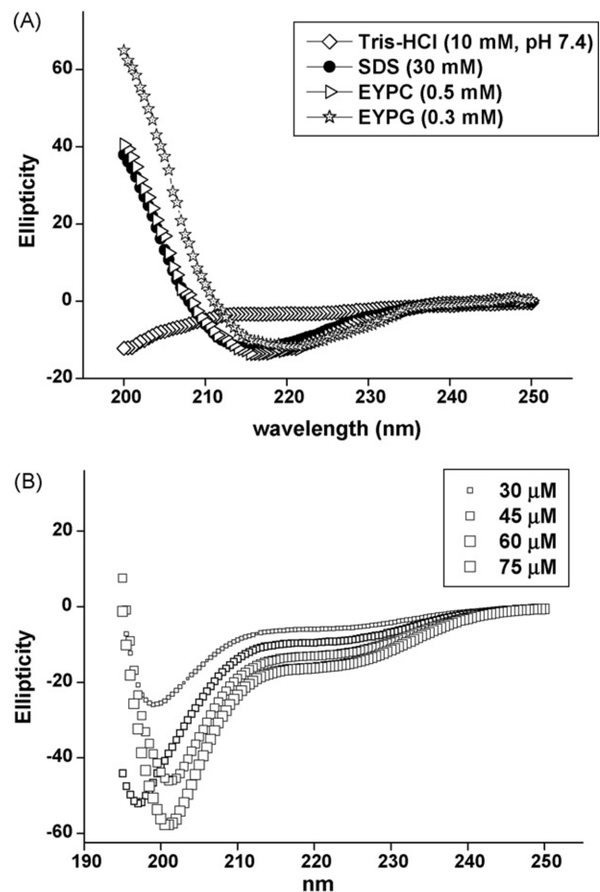

Fig. 1. CD spectra of CP-1. (A) 1.5×10^{-5} M of CP-1 in Tris–HCl buffer (pH 7.4), 30 mM of SDS, 0.5 mM of EYPC and 0.3 mM of EYPG, respectively; (B), CP-1 at different concentrations in Tris–HCl buffer (pH 7.4).

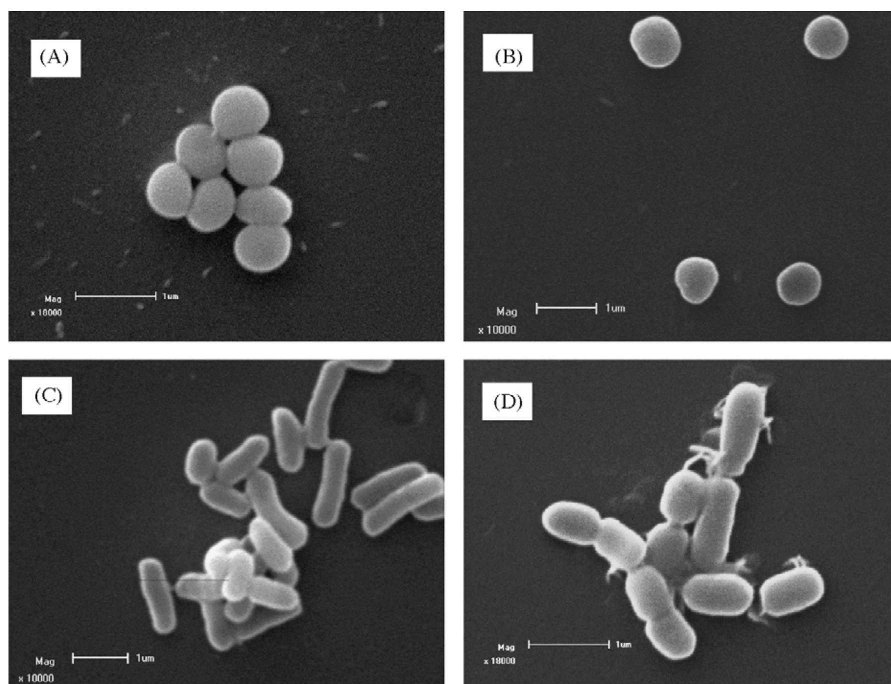

Fig. 2. SEM images of *E. coli* and *S. aureus* untreated and treated with CP-1 (78 μM). (A) Untreated *S. aureus*; (B) *S. aureus* after treating with CP-1; (C) untreated *E. coli*; (D) *E. coli* after treating with CP-1.

morphology of *E. coli* after incubation changed slightly. These results indicated that the killing mechanism of CP-1 was not lysis of bacterial membranes.

3.4. Inner-membrane permeability

To further investigate the interaction of the peptide with the cell membranes, we assessed the ability of CP-1 to permeabilize the cytoplasmic membranes of *E. coli* ML-35. *E. coli* ML-35 produces the enzyme β-galactosidase. If the peptide induces permeability of the inner membranes, ONPG can be hydrolyzed by β-galactosidase leaked to out side of the cell, producing yellow *o*-nitrophenol [7,11,15,16]. An ideal hydropathic α-helical antimicrobial peptide GLK (GGLLKLLGKLLKLLLK-NH_2) that kills bacteria by destabilization of bacterial membrane (unpublished result) was used as control. As shown in Fig. 3, at the concentration of MIC, CP-1 did not exhibit inner-membrane permeabilization. In contrast, GLK, used as a positive control, rapidly permeabilized the inner membrane at the concentration of its MIC (7 μM). This result implies that CP-1 did not form transmembrane pores in killing *E. coli*.

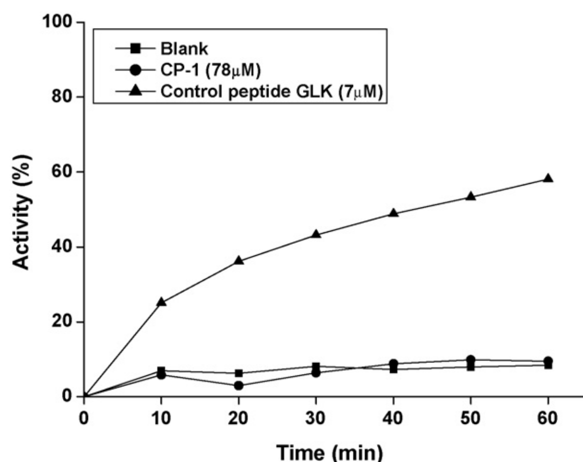

Fig. 3. Kinetics of hydrolysis of ONPG due to inner-membrane permeabilization of *E. coli* ML-35 by peptide CP-1 and control peptide GLK. The blank is the assay without peptide.

3.5. Peptide-induced leakage of dye from negatively charged LUVs

The ability of the peptide to release the fluorescent marker calcein from bacterial cell membrane mimicking negatively charged EYPC/EYPG (7:3, w/w) LUVs was assessed. The result was shown in Fig. 4. Consistent with the ability to permeabilize the inner membrane, CP-1 induced little or no calcein release from EYPC/EYPG LUVs (Fig. 4A). Even at a concentration of 43 μM, CP-1 exhibited more than 10-fold weaker leakage activity compared with the control peptide GLK (Fig. 4B). These results further confirm that CP-1 inhibits bacteria in some other way other than destabilize membrane permeability.

3.6. Confocal laser-scanning microscopy

The Gram-negative *E. coli* and Gram-positive *S. aureus were incubated* with FITC-labeled CP-1 for 30 min and then visualized by confocal laser-scanning microscopy (Fig. 5). It can be seen from the figure that the FITC-labeled CP-1 can penetrate both Gram-negative *E. coli* and Gram-positive *S. aureus* membranes and accumulate in the cytoplasm. This finding confirmed that the cytoplasm may be the major site for killing the bacteria by CP-1.

3.7. DNA binding assay

The DNA binding ability of the CP-1 was observed by gel retardation assay (Fig. 6). The results showed that CP-1 possessed DNA binding affinity. As a contrast, melittin, a typical membrane-active antimicrobial peptide, possessed no binding affinity for DNA in the ratio range studied.

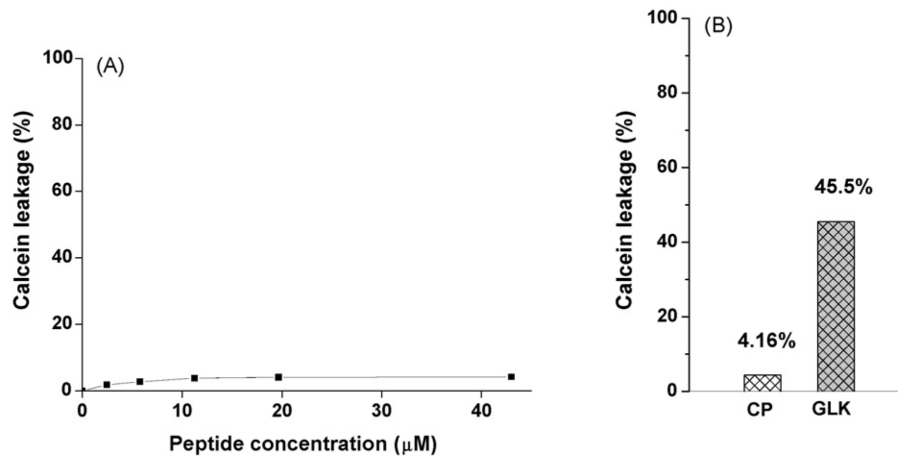

Fig. 4. (A) Dose-dependent percent leakage of calcein from negatively charged EYPC/EYPG (7:3, w/w) LUVs after incubation with CP-1 for 10 min at pH 7.4. (B) Percent leakage of calcein from negatively charged EYPC/EYPG (7:3, w/w) LUVs in the presence of CP-1 and control peptide at concentration of 43 μM at pH 7.4.

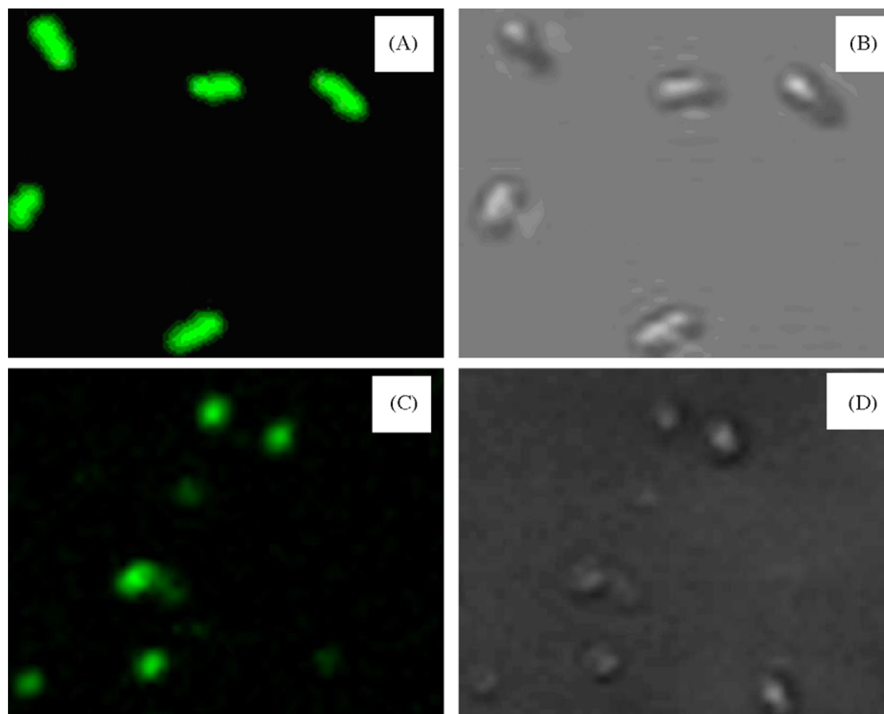

Fig. 5. Confocal laser-scanning microscopy images of Gram-negative *E. coli* (A) and Gram-positive *S. aureus* (C) treated with FITC-labeled CP-1. (B and D) were the bright field images corresponding (A and C), respectively.

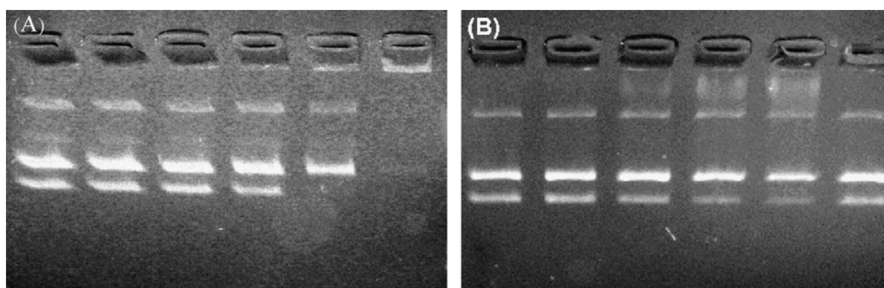

Fig. 6. Gel retardation assay. CP-1 (A) and melittin (B) were incubated with 50 ng of plasmid DNA pUC18, and the DNA binding affinity was assessed by the inhibition of the electrophoretic migration of DNA by the peptides. The peptide-to-DNA weight ratios for the different lanes from left to right are 0:1, 10:1, 25:1, 50:1,100:1,150:1, respectively.

4. Discussion

The T-cell antigen receptor is a critical component of the immune system able to recognize foreign antigens and to initiate the immune response. A 9-amino acid fragment (GLRILLLKV), termed core peptide (CP), derived from the T-cell antigen receptor α-chain transmembrane sequence, can suppress the immune response in animal models of T cell-mediated inflammation [19]. CP contains 2 basic amino acids and 6 hydrophobic amino acids, which is the structural features of antimicrobial peptides. The net positive charge at neutral pH is prerequisite for antimicrobial peptides. The net charge of CP-1, the C-terminal amidation derivative of CP, is +3. Indeed, CP-1 exhibits broad antimicrobial activities against both Gram-positive and Gram-negative bacteria (Table 1). The antimicrobial activities of CP-1 are comparable with those of the most of synthetic linear β-sheet antimicrobial peptides [3,12,24] such as KIGAKI with MIC value of 8 μg/mL against both *S. aureus* and *E. coli* [3] and the most of naturally occurring antimicrobial peptides such as magainins with MIC values ranging from 10 to 256 μg/mL [3,18,20]. What's more CP-1 held the similar antimicrobial activities against Gram-positive species *S. aureus* and Gram-negative species *E. coli* with clinically used antibiotics such as ciprofloxacin and roxithromycin (Table 1).

Most of antimicrobial peptides target the cell membranes of bacteria. One of the killing mechanisms for antimicrobial peptides is disruption of the outer and cytoplasmic membranes of bacteria by forming transmembrane pores or by "carpet mechanism", leading to lysis of bacterial cells [26,29]. SEM images of *E. coli* and *S. aureus* incubated with CP-1 indicated that no lysis of the bacteria was observed. Morphology of the bacteria after treating changed little. Only slightly perturbation of the outer membrane of *E. coli* occurred. Therefore, membrane lysis seems not to be the killing mechanism of CP-1. Another killing mechanism of antimicrobial peptides is the formation of pores across the cytoplasmic membrane of bacteria without causing extensive damage to host membranes [18]. The transmembrane pores lead to the enhanced permeability of the membrane and the loss of cell contents (e.g., ATP), causing the death of the bacteria. Such transmembrane pore forming ability of CP-1 was studied by the hydrolysis of ONPG assay and dye leakage observation from LUVs. The assay of the hydrolysis of ONPG (Fig. 3) shows that CP-1 did not cause any leakage of β-galactosidase, which can hydrolyze ONPG, through the inner membrane of *E. coli* at the concentration of 78 μM in 30 min, indicating no transmembrane pores formed. Phosphatidylglycerol-containing lipid bilayers are a common model to mimick bacterial membranes and can be used to investigate the antimicrobial mechanism of this class of peptides [8,14,25]. The ability of CP-1 causing leakage of the fluorescent marker calcein entrapped within EYPC/EYPG (7:3, w/w) LUVs was assayed. CP-1 induced little leakage of calcein from LUVs at the concentration below 43 μM when incubated with CP-1 for 10 min (shown in Fig. 4A). At concentration of 43 μM, CP-1 induced 4.16% calcein leakage, 10-fold weaker leakage activity compared with the control peptide GLK. These results indicated that the transmembrane pore formation might not be the killing mechanism of CP-1. It has been reported that CP has the ability to enter cells and reach the cell nucleus [19]. Here we examined the site in bacteria where the CP-1 might take effect by confocal laser-scanning microscopy. It was confirmed that the CP-1 can penetrate the bacterial membranes and accumulated in the cytoplasm. Moreover the DNA binding observation showed that the peptide CP-1 held the DNA binding affinity. So CP-1 might kill bacteria by targeting intracellular components such as DNA after entering the cells and reaching the cell nucleus.

CD spectral data suggested that CP-1 adopts a well-defined β-sheet structure in membrane mimetic environments (30 mM SDS micelles, 3 mM PC or 3 mM PG liposomes). Great structural diversity is a typical characteristic of antimicrobial peptides. More than 1000 antimicrobial peptides have been reported so far in the literature (http://www.bbcm.univ.trieste.it/~tossi/pag1.htm). Almost all the naturally occurring linear antimicrobial peptides or the segments of natural proteins with potent antimicrobial activity adopt α-helical structure in membrane mimetic environments. Naturally occurring antimicrobial peptides with β-sheet structure generally contain cysteine residues linked by disulfide bridges. Few naturally occurring linear antimicrobial peptides adopt β-sheet structure. Only a few synthetic short linear peptides with antimicrobial activity adopt β-sheet structure [3,12,23,24]. CP-1 might be the first short linear fragment from naturally occurring proteins which adopts β-sheet structure and shows potent antimicrobial activity.

5. Conclusions

In summary, we found a novel 9-residue peptide CP-1, which adopted β-sheet structure in membrane mimicking environments, had high antimicrobial activities. Unlike most membrane-active peptides, CP-1 did not induce any influx of ONPG through the inner membrane of *E. coli*. It also caused very little dye leakage from EYPC/EYPG (7:3, w/w) LUVs which mimic the bacterial membranes and morphology of the *E. coli* and *S. aureus* after treating with CP-1 changed little. However CP-1 can penetrate the membrane of the bacteria and held the DNA binding ability. All these results indicated that CP-1 had a novel mechanism of killing bacteria rather than disrupting the cytoplasmic membranes or forming peptide-lipid supramolecular pores but killing bacteria after permeating the membrane by targeting intracellular components, probably DNA, in the cell nucleus.

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