Journal of Microbiology (2013) Vol. 51, No. 2, pp. 200–206
DOI 10.1007/s12275-013-2441-1

The Intracellular Mechanism of Action on *Escherichia coli* of BF2-A/C, Two Analogues of the Antimicrobial Peptide Buforin 2

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(Received August 21, 2012 / Accepted December 10, 2012)

In the present study, the antimicrobial peptides BF2-A and BF2-C, two analogues of Buforin 2, were chemically synthesized and the activities were assayed. To elucidate the bactericidal mechanism of BF2-A/C and their different antimicrobial activities, the influence of peptides to *E. coli* cell membrane and targets of intracellular action were researched. Obviously, BF2-A and BF2-C did not induce the influx of PI into the *E. coli* cells, indicating nonmemebrane permeabilizing killing action. The FITC-labeled BF2-A/C could penetrate the *E. coli* cell membrane and BF2-C penetrated the cells more efficiently. Furthermore, BF2-A/C could bind to DNA and RNA respectively, and the affinity of BF2-C to DNA was powerful at least over 4 times than that of BF2-A. The present results implied that BF2-A and BF2-C inhibited the cellular functions by binding to DNA and RNA of cells after penetrating the cell membranes, resulting in the rapid cell death. The structure-activity relationship analysis of BF2-A/C revealed that the cell-penetrating efficiency and the affinity ability to DNA were critical factors for determining the antimicrobial potency of both peptides. The more efficient cell-penetrating and stronger affinity to DNA caused that BF2-C displayed more excellent antimicrobial activity and rapid killing kinetics than BF2-A.

Keywords: antimicrobial peptide, Buforin 2, analogue, DNA action, mechanism

Introduction

The studies about antimicrobial peptides have already lasting for several decades, since Boman isolated Cecropins from *Hyatophoya cecropia* in the 1970's (Boman *et al*., 1972; Hultmark *et al*., 1980). Many researchers aimed the bacterial cell membrane during the study of action mechanism, because most cationic peptides have been considered to kill bacteria by permeabilizing negatively charged bacterial membranes (Shai, 1999; Zhu *et al*., 2006). In contrast to these membrane-active peptides, other classes of antimicrobial peptides, such as pyrrhocoricin (Kragol *et al*., 2001) and indolicidin (Subbalakshmi and Sitaram, 1998), were suggested to have multiple targets including intracellular components other than membranes. A 21-aa peptide, Buforin 2, that was isolated from the stomach tissue of an Asian toad, *Bufo bufo gargarizans*, was hypothesized to kill bacteria by entering cells and binding nucleic acids, and showed stronger antimicrobial activities against a broad spectrum of microorganisms compared with other cationic polypeptides (Park *et al*., 1996, 1998; Uyterhoeven *et al*., 2008).

Recently, a 17-aa peptide, named BF2-A (RAGLQFPVGRV HRLLRK), was designed out based on the structure-activity analysis of Buforin 2 (TRSSRAGLQFPVGRVHRLLRK). The BF2-A was an N-terminally truncated analog of Buforin 2 corresponding to residues 5 to 21 (Park *et al*., 2000). Depended on the previous research, according to the structure-activity relationship of Buforin 2, we had designed and synthesized a novel 21-aa antimicrobial peptide, named BF2-C (RAGLQ FPLGRLLRRLLRRLLR). The BF2-C contained the N-terminal residues 5 to 13 and three repeats of the C-terminal regular α-helical motif RLLR of Buforin 2, and was a substitution of residue Val by Leu at position 12 on N-terminal of Buforin 2, which caused the augment of amphipathic α-helix content and hydrophobicity on C-terminal of BF2-C. In previous study, the interaction of BF2-A with cell membrane were investigated in detail. The results indicated that BF2-A couldn't cause significant bacterial membrane permeabilization, and hardly caused the leakage of intracellular macromolecules (Hao *et al*., 2009). And BF2-C owned more net positive charge and more remarkable hydrophobic C-terminal, compared with BF2-A.

In this study, the *E. coli* was chosen as model microorganism to research the action mechanism of BF2-A/C to bacteria. The influence of peptides to *E. coli* cell membrane and the target molecular of intracellular action were investigated by use of FACS analysis and AFM imaging. The results indicated that BF2-A/C inhibited the cellular functions by binding to DNA and RNA of cells after penetrating the cell membranes, resulting in the rapid cell death. The structure-activity relationship analysis of BF2-A/C revealed that the cell-penetrating efficiency and the affinity ability to DNA were critical factors for determining the antimicrobial potency of both peptides.

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Materials and methods

Materials

Trifluoroethanol (Fluka, China); Fluorescein isothiocyanate (Promega, China); Propidium iodide (Sigma, China); Ethidium bromide (Amersco, China); D0063 Spin Column Miniprep Kit (Beyotime, China); Diethyl pyrocarbonate (Invitrogen, China)

Peptide synthesis and purification

All peptides were synthesized by the Fmoc (9-fluorenylmethoxycarbonyl)-chemistry in solid phase peptide synthesis by using a model 432A peptide synthesizer (Fields and Noble, 1990). The synthetic peptides were purified by C_{18} reverse-phase HPLC (Waters, China) and the purity was confirmed by amino acid analysis and matrix associated laser desorption ionization (MALDI) mass spectroscopy (Applied Biosystems, China).

The calculated molecular weight, calculated isoelectric point and charge number of antimicrobial peptides were analyzed by bioinformatics software - ProtParam (http://us.expasy.org/tools/protparam.html).

Antimicrobial effect assay

Antimicrobial activity was expressed as the minimal inhibitory concentration (MIC), which was defined as the lowest concentration of peptides that completely inhibited microbial growth (Hao *et al*., 2008). Antimicrobial activity of the peptides against 8 selected organisms, including Gram-positive and Gram-negative bacteria, was determined by the broth microdilution assay. Briefly, single colonies of bacteria were inoculated in nutrition broth and cultured overnight at 37°C. An aliquot of each was transferred to 10 ml of fresh culture medium and incubated for additional 3–5 h at 37°C to obtain mid-logarithmic phase organisms. Then, 50 μl of a set of two-fold serial dilutions of peptides in 1% bactopeptone was added to 100 μl of bacterial suspension (1.0×10^6 CFU/ml) in 96-well microtiter plates and the plates were incubated at 37°C for 12 h. After incubation, microbial growth was determined by measuring the increase in the turbidity of each well at 630 nm using a microplate reader (Thermo, China).

Circular Dichroism (CD)

CD experiments were performed by using a Jasco 720 spectropolarimeter (Jasco, China) to determine the secondary structure of BF2-A/C. The spectra were measured between 190 and 250 nm either in the presence or absence of 50% (v/v) trifluoroethanol in 50 mM NAPB. Five consecutive scans per sample were performed in a 1-mm cell at 25°C. The helicity of the peptides was determined from the mean residue helicity at 222 nm.

FITC-labeling of peptides

Fluorescein isothiocyanate (FITC) was freshly dissolved in 0.02 M Na_2CO_3-$NaHCO_3$ buffer (pH 9.1, containing 0.02 M NaCl) to 1 mg/ml, and 700 μl of FITC solution was added to 100 μl of a solution of peptides BF2-A/C (2 mg/ml) in 0.02 M Na_2CO_3-$NaHCO_3$ buffer to give a final concentration of 25 μg/ml (Park *et al*., 1998). After incubation for 16 h in the dark at 4°C, 500 μl of 500 mM NH_4Cl was added to inactive the residual FITC. The solution was incubated in the dark for an additional 2 h at 4°C and stored in aliquots at -20°C. The FITC-labeled peptides were purified by reverse-phase HPLC on a C_{18} column (3.9×300 nm, Amersham Pharmacia, China) to give final products that were ≥95% pure. The purified FITC-labeled peptides were dried under vacuum and then resuspended in the PBS.

FACScan analysis

The integrity of the *E. coli* membranes after peptide treatment was determined by FACScan analysis, via nuclear staining with propidium iodide (PI). In brief, *E. coli* cells were first harvested at log phase, and then washed twice with PBS. The washed cells (1.0×10^6 CFU/ml) were mixed with peptides at MIC in PBS, and then incubated with PI solution (50 μg/ml final concentration) for 10 min at 4°C. After incubation, the unbound dye was removed via the excessive washing of cells with PBS (Jang *et al*., 2006; Sung *et al*., 2008). Flow cytometric analysis was performed via a FACScan flow cytometer (Becton Dickinson, China).

The cell-penetrating efficiency of both peptides was also investigated by FACScan analysis, via the influx of FITC-labeled peptides into bacterial cells. *E. coli* cells were first harvested at log phase, and then washed twice with PBS. The washed cells (1.0×10^6 CFU/ml) were mixed with FITC-labeled peptides at final concentration 5 μg/ml (Lee *et al*., 2002). The solution was incubated in the dark for 20 min at 37°C. After incubation, *E. coli* cells were harvested via centrifugation and then washed once with PBS. Then the treated *E. coli* cells were resuspended in the PBS. Flow cytometric analysis was performed via a FACScan flow cytometer.

DNA/RNA binding assay

Gel retardation experiments were performed by mixing 100 ng of the *E. coli* genomic DNA or 2 μg of the *E. coli* total RNA with increasing the amount of peptides in 10 μl of binding buffer (5% glycerol, 10 mM Tris-HCl; pH 8.0, 1 mM EDTA, 1 mM DTT, 20 mM KCl, and 50 μg/ml BSA). The reaction mixtures were incubated at room temperature for 10 min. Subsequently, for DNA-peptides mixtures, 2 μl of native loading buffer was added (10% Ficoll 400, 10 mM Tris-HCl; pH 7.5, 50 mM EDTA, 0.25% bromophenol blue) and then the reaction solution was applied to a 0.8% agarose gel electrophoresis with 2 μg/ml ethidium bromide (final concentration) in 0.5× Tris borate-EDTA buffer (Zhang *et al*., 1999; Ulvatne *et al*., 2004). The genomic DNA used in this experiment was purified by D0063 Spin Column Miniprep Kit.

E. coli total RNA was prepared by the bacterial RNA preparation method and resuspended in diethyl pyrocarbonate (DEPC) - treated water (Park *et al*., 1998). The RNA-peptides mixtures were added 6 μl of formamide, 2 μl of loading buffer (5% glycerol, 50 mM EDTA, 0.25% bromophenol blue, and 0.25% xylene cyanol) and 2 μl of 37% formaldehyde. And then a 1% agarose gel electrophoresis was performed in 1× MOPS NaAc-EDTA buffer with 2 μg/ml ethidium bromide and 2.2 mol/L formaldehyde (final concentration).

Table 1. **The analysis results of physical-chemical properties of BF2-A/C**

Antimicrobial peptides	Observed molecular weight	Calculated molecular weight	Calculated isoelectric point	Purify	Charge number	Retention time (min)
BF2-A	2003.8	2003.4	12.48	≥97%	+6	11.292
BF2-C	2560.4	2560.2	12.78	≥97%	+7	14.892

Atomic force microscopy (AFM) imaging

E. coli genomic DNA and peptides were dissolved in 10 mM Tris-HCl buffer (pH 7.2) and then mixed to obtain DNA-peptide complex with 0.5 mg/ml DNA and different concentration of peptides for 10 min at room temperature. Prior to AFM measurements, the complex suspension was diluted 10-times with purified water and 10 µl of the resulting solution was then deposited onto a freshly cleaved mica substrate (Volcke *et al.*, 2006). Samples were imaged after water evaporation. Experiments were performed with a multimode nanoscope atomic force microscopy (Benyuan, China) operating in tapping mode (TM). All images were recorded in air at room temperature, at a scan speed of 1.0 Hz.

Results and Discussion

Peptide synthesis and purification

The analysis results of physical-chemical properties of antimicrobial peptides BF2-A/C was shown in Table 1. The observed molecular weight and calculated molecular weight of both peptides were very close.

Antimicrobial activities

Both peptides displayed very impressive antimicrobial activities against Gram-positive and Gram-negative bacteria tested, and stronger activities than their parent peptides, Buforin 2 (Table 2). Furthermore, the antimicrobial activity of BF2-C was 2 to 4-fold greater than that of BF2-A, except *Salmonella typhimurium*. These results suggested that the α-helical content might directly correlate with the enhanced antibacterial activity (Park *et al.*, 2000).

Table 2. **Antimicrobial activities of BF2-A, BF2-C and their parent peptide, Buforin2**

Microorganism	MIC (µg/ml)		
	Buforin2	BF2-A	BF2-C
Gram-negative bacteria			
Escherichia coli	4	2	1
Shigella dysenteriae	8	4	1
Salmonella typhimurium	1	1	1
Serratia marcescens	4	2	1
Gram-positive bacteria			
Streptococcus pneumoniae	4	2	1
Staphylococcus aureus	4	2	1
Listeria monocytogene	4	2	1
Streptococcus hemolyticus	2	1	0.5

Minimal inhibitory concentrations were the average values obtained in triplicates on three independent measurements.

Structural analysis of peptides by CD measurements

The secondary structure of linear antibacterial peptides in lipid membranes rather than in phosphate buffer must correlate well with the activity. In order to investigate the secondary structure of the peptides in lipid membranes, CD spectrum of the peptides was measured in phosphate buffer and a membrane mimic condition-in the 50% TFE solution. Both peptides showed a random coil structure in an aqueous solution, while displaying a typical α-helical spectrum with two minimum peaks at 208 and 222 nm in 50% TFE solution (Fig. 1). According to the α-helicity calculated from the molar ellipticity at 222 nm, BF2-C showed higher α-helical contents, 34%, than BF2-A, 28%, in 50% TFE solution.

Effects of BF2-A/C on the integrity of the *E. coli* membrane

As many antimicrobial peptides have been determined to exert their effects via the induction of permeability changes in the membranes of microbes, we attempted to characterize the effects of BF2-A/C on the integrity of *E. coli* membranes by recording the uptake of PI, a DNA-staining fluorescent probe, into the *E. coli* cells. Detection of internal PI in single cells was analyzed by flow cytometry. The results of the intracellular PI measurements showed that almost untreated normal *E. coli* cells showed no signal with respect to the fluorescence activity of PI and the majority of the *E. coli* cells treated with MIC of BF2-A/C were not labeled fluorescently (Fig. 2). Meanwhile Figs. 2B–2C showed trivial upward light scatters (vertical axis) along PI fluorescence whereby the dots indicated individual cells, which about 4.98% of cells treated by BF2-A and 5.93% of cells treated by BF2-C were found to move to the upper left area. The results indicated that both peptides slightly disturbed *E. coli* cell membrane

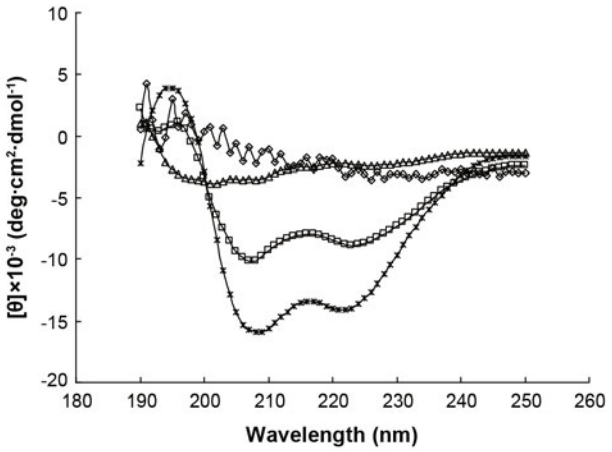

Fig. 1. **CD spectra of BF2-A in the absence (◇), or presence (□) of 50% (v/v) TFE; BF2-C in the absence (△), or presence (*) of 50% (v/v) TFE.**

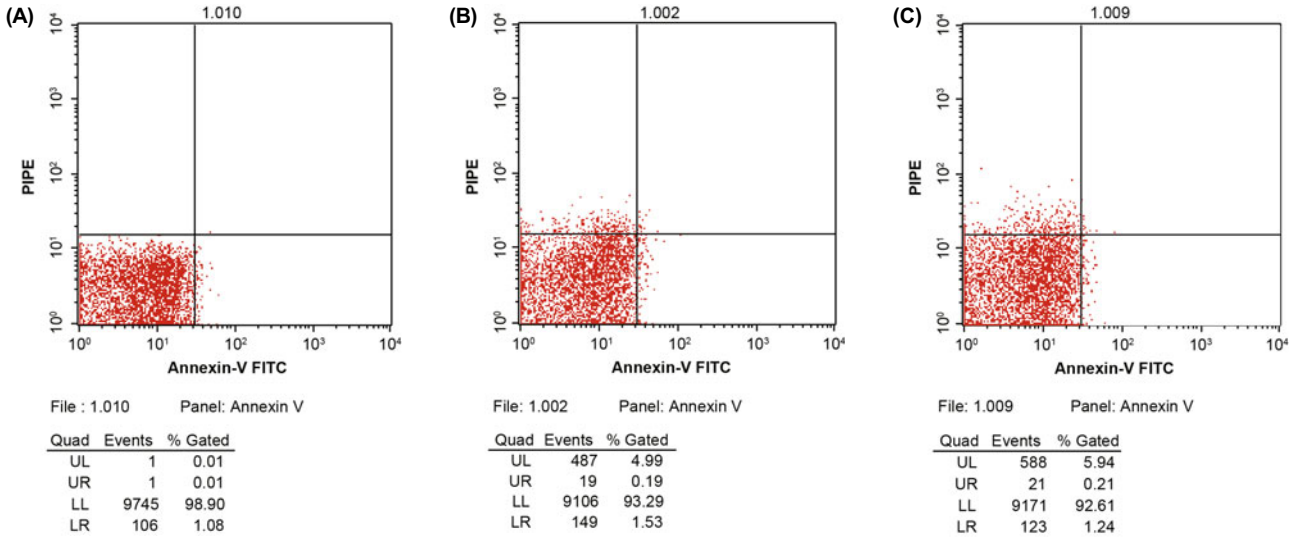

Fig. 2. FACScan analysis of PI labeling of *E. coli* cells treated with BF2-A and BF2-C. *E. coli* cells (1.0×10^6 CFU/ml) were mixed with peptides at MIC in PBS, and then incubated with PI solution (50 μg/ml final concentration) for 10 min at 4°C. Histograms show the fluorescence intensity of internalized PI after peptide treatment of *E. coli*. (A) control staining without any peptide treatment, (B) staining of cells treated with BF2-A, and (C) staining of cells treated with BF2-C.

during crossing membrane, resulting in the influx of micro PI, and the disturbance of BF2-C to cell membrane was slightly higher than that of BF2-A. These rates of positive cells stained by fluorescent probe were still remarkably low, taking into account the interference of background. Thereby, the results of FACScan analysis implied that BF2-C did not induce the influx of PI into the *E. coli* cells, indicating non-memebrane permeabilizing killing action.

BF2-A/C cell-penetrating efficiency

To evaluate the cell-penetrating efficiency of both peptides, we labeled the peptides with FITC and examined their influx into *E. coli* cells by using FACS analysis. Figure 3 showed the results of the FACS analysis of *E. coli* cells incubated with FITC-labeled BF2-A and BF2-C. The evident forward light scatters (horizontal axis) along FITC fluorescence were visualized in Figs. 3B–3C whereby the dots indicated individual cells, which about 21.29% of cells treated by BF2-A and 29.23% of cells treated by BF2-C were found to move to the lower right area. When *E. coli* cells (1.0×10^6 CFU/ml) were treated with each of the FITC-labeled peptides (5 μg/ml each) for 20 min, the rates of positive cells stained by FITC-labeled BF2-C was higher than FITC-labeled BF2-A did, that was, BF2-C penetrated the cells more efficiently, resulting in higher antimicrobial activity. This result implied that the

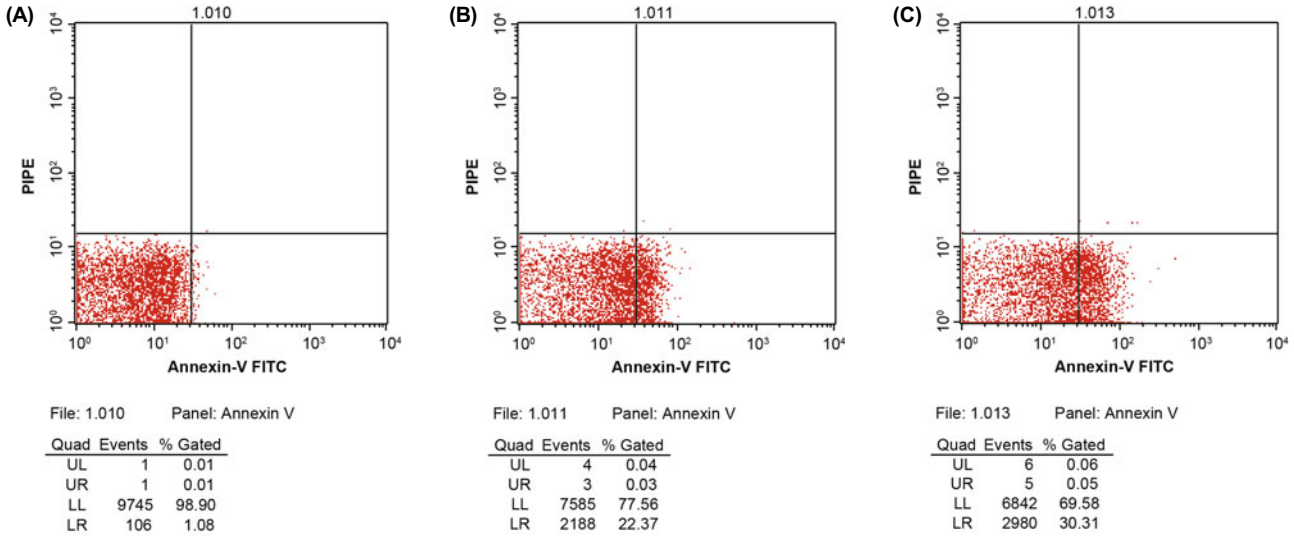

Fig. 3. FACS analysis of *E. coli* cells treated with FITC-labeled BF2-A/C. *E. coli* cells in log phase were washed with PBS and resuspended in the same buffer. *E. coli* cells (1.0×10^6 CFU/ml) were incubated in the dark for 20 min at 37°C with PBS (control) (A), 5 μg/ml of FITC-BF2-A (B), 5 μg/ml of FITC-BF2-C (C).

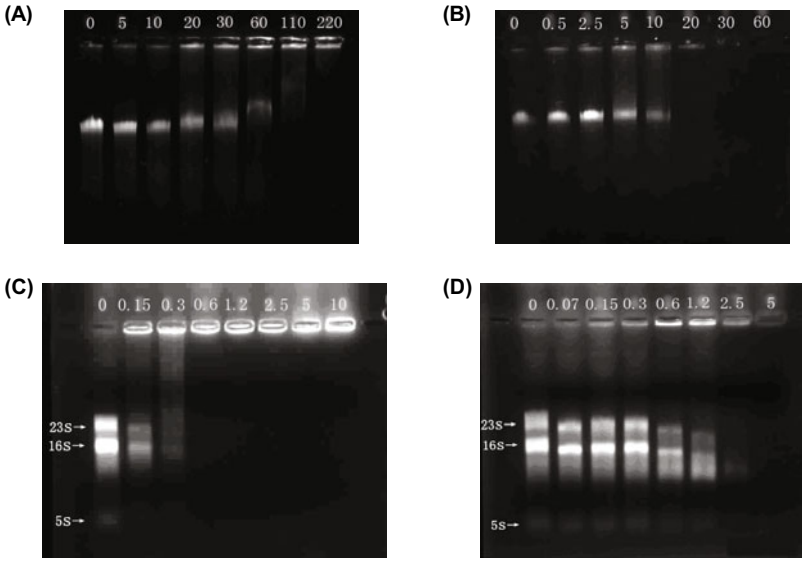

Fig. 4. Interaction of *E. coli* genomic DNA or total RNA with peptides. DNA was incubated with various amounts of BF2-A (A) and BF2-C (B) in binding buffer at room temperature for 10 min. RNA was incubated with various amounts of BF2-A (C) and BF2-C (D) under the same condition. The reaction mixtures were applied to a 0.8% (DNA) or 1% (RNA) agarose gel electrophoresis, respectively. The 23S, 16S and 5S rRNA of *E. coli* ribosomal RNA were clearly observed in picture (C) and (D). The weight ratio (peptide: DNA or peptide: RNA) was indicated above each lane.

cell-penetrating efficiency of the peptides determined their antimicrobial activity.

DNA/RNA binding activity

Since there was no clear evidence that the peptides studied here worked by a membrane permeabilization mechanism, we investigated the possibility of other intracellular targets. In an attempt to clarify the molecular mechanism of action, the binding properties exerted by BF2-A and BF2-C on DNA or RNA were examined by analyzing the electrophoretic mobility of DNA or RNA bands at the various weight ratios of peptides to DNA or RNA on an agarose gel (0.8% or 1%, respectively). The DNA or RNA binding activities of BF2-A and BF2-C were shown in Fig. 4. Both peptides bound to DNA or RNA within 2 min and caused retardation of DNA mobility, with minor differences. BF2-A inhibited the migration of DNA and RNA above the weight ratio of 10 (Fig. 4A) and 0.15 (Fig. 4C), respectively, and BF2-C suppressed the migration of DNA and RNA above the weight ratio of 2.5 (Fig. 4B) and 0.6 (Fig. 4D), respectively. These results indicated that BF2-C bound to DNA at least over 4 times tightly than BF2-A, on the other hand, BF2-A bound to RNA at least over 4 times tightly than BF2-C. Interestingly, BF2-C could reduce the fluorescence-emission of DNA-EB (ethidium bromide) mixtures above the weight ratio of 60 (Fig.

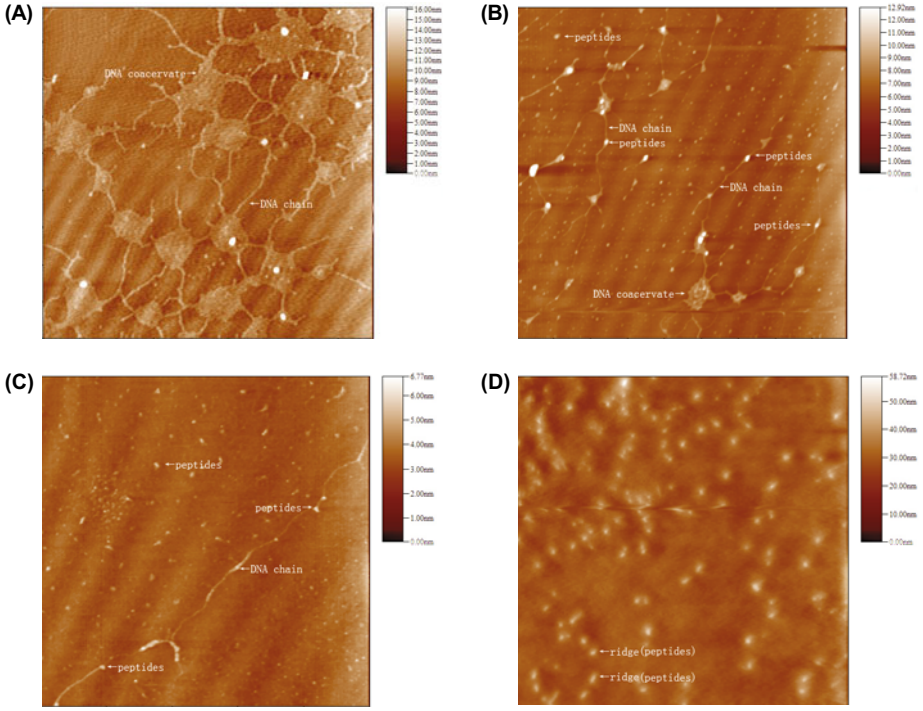

Fig. 5. AFM images of *E. coli* genomic DNA with peptides. DNA was mixed without peptides (control) (A), with 400 μg/ml of BF2-A (B), 10 μg/ml of BF2-C (C), 400 μg/ml of BF2-C (D) in Tris-HCl buffer (pH 7.2) for 10 min at room temperature. The complex suspension was then deposited onto a freshly cleaved mica substrate. The bar charts besides pictures were nano-scale.

4A). This result implied that the binding of BF2-C and DNA might cause relaxation of the DNA double helix structure, resulting in EB could not tightly insert into the base pair of DNA. The binding mode of both peptides with DNA was further supported by following AFM, which might directly illustrate their antimicrobial mechanism.

AFM imaging

Immobilizing DNA was one way to know the interaction with other material, and the AFM imaging was widely applied to see the immobilized DNA (Tang *et al*., 2009). To investigate binding of *E. coli* genomic DNA to BF2-A and BF2-C, high-resolution images of DNA in the presence of both peptides were obtained by using AFM. We performed AFM imaging in the tapping mode (TM-AFM). Representative images of free DNA and AFM images of peptides-DNA complexes were shown in Fig. 5. The image showed that DNA in the absence of peptides displayed a closed geometry, and many DNA chains twisted together to form coacervate (Fig. 5A). In the presence of 400 μg/ml of BF2-A (Fig. 5B) or 10 μg/ml of BF2-C (Fig. 5C), an increase in the number and size of peaks (peptides) associated along the backbone of DNA could be clearly observed. This phenomenon indicated that most of DNA chains could be adsorbed by the molecules of BF2-A or BF2-C. Interestingly, at a concentration of 400 μg/ml of BF2-C (Fig. 5D), owing to the intense binding action between peptides and DNA, so many BF2-C molecules were adsorbed on the DNA chains, resulted in the formation of gibbous ridge (white light dot), that nano-probe of tapping mode could not sweep the valley (DNA chains). Consequently, only these gibbous light dots along DNA chains, not DNA chains, could be observed (Tang *et al*., 2009). These results proved that the binding action of BF2-C and DNA was stronger than that of BF2-A, in accordance to the results of gel retardation assay.

The mechanism of action of the antimicrobial peptides BF2-A and BF2-C, the analogues of Buforin 2, was studied against *E. coli* in this study to understand the difference of antimicrobial activities of both peptides. Structurally BF2-A/C belonged to one major group of antimicrobial peptides: a linear amphipathic α-helical peptide without cysteine. The members of this group included cecropins (Qu *et al*., 1982), magainins (Matsuzaki, 1999), and dermaseptin (Dagan *et al*., 2002). Recent studies on several antimicrobial peptides of this family suggested that the peptides killed microorganisms either by forming pores and increasing the permeability or by disintegration of the cell membrane. However, the mechanism of killing action of BF2-A/C was quite different from that of other linear α-helical peptides. Our earlier studies illustrated that BF2-A didn't cause significant membrane permeabilization, and hardly caused the leakage of intracellular macromolecules (Hao *et al*., 2009). In present experiment, FACS analysis showed that MIC of BF2-A and BF2-C did not induce the influx of PI into the *E. coli* cells, indicating nonmemebrane permeabilizing killing action, however, BF2-C slightly increased *E. coli* cell membrane permeability during crossing membrane. Meanwhile, FACS analysis revealed that FITC-labeled BF2-A and BF2-C could penetrate the *E. coli* cell membrane at a concentration of 5 μg/ml. Subsequently, the observation of confocal microscopy confirmed that FITC-labeled BF2-A and BF2-C penetrated the cell membrane and accumulated in the cytoplasm of the cell (data not shown). Matsuzaki (1998) reported that magainin translocated across lipid bilayes as a model membrane system coupled with pore formation and lipid flip-flop. However, the magainin remained associated with the inner leaflet of the lipid bilayer after translocation of the artificial membrane. Magainin tended to remain on cell membranes because its translocation efficiency, as well as its affinity for DNA and RNA, was very low. Interestingly, the both peptides didn't induce membrane permeabilization on translocation of the membrane, indicating that they had a different translocation mechanism. Hereby, the translocation mechanism of BF2-A/C was proposed to the similar to that of their parent peptide, Buforin 2 (Kobayashi *et al*., 2004).

Thus it seemed that the target site of both peptides was the cytoplasm of the cell. What did then both peptides do inside the *E. coli* cell? Recent studies suggested that several antimicrobial peptides such as PR-39 (Boman *et al*., 1993) and tachyplesin 1 (Yonezawa *et al*., 1992) bound tightly to DNA or RNA and inhibited the macromolecular synthesis of the cell. PR-39, the proline-arginine-rich peptide, was known to stop the synthesis of protein or DNA after penetrating the cytoplasm of the cell. BF2-A and BF2-C might kill *E. coli* in a way similar to that of PR-39. In the gel retardation experiment, it was proved that BF2-A and BF2-C bound to DNA and RNA with different affinity ability, respectively. Furthermore, the intense binding between both peptides and DNA was clearly demonstrated by AFM imaging with the tapping mode. And the different binding ability of the BF2-A/C and DNA was also observed by AFM imaging. Although a detailed experiment on the interaction between both peptides and nucleic acid *in vivo* was needed, the present results leads us to believe that BF2-A and BF2-C inhibited the cellular functions by binding to DNA and RNA of cells after penetrating the cell membranes, without destroying it, resulting in the rapid cell death.

Although BF2-A and BF2-C, two analogues of Buforin 2, possessed similar structure and action mechanism against *E. coli* cell, their antimicrobial activities were quite different. Both peptides showed stronger antimicrobial activities against a broad spectrum of microorganisms than their parent peptide. In addition, the antimicrobial activity of BF2-C was 2 to 4-fold greater than that of BF2-A, except *Salmonella typhimurium*. The results of FACS analysis of the flux of FITC-labeled peptides into *E. coli* cells revealed that BF2-C penetrated the cells more efficiently, probably owing to higher α-helical content. In other words, BF2-C with higher α-helical content penetrated the cells more efficiently, resulting in higher antimicrobial activity. It was proposed that α-helical content of the peptides determined the cell-penetrating efficiency, a key factor determining the antimicrobial activity.

Although BF2-A and BF2-C were cationic antimicrobial peptides, BF2-C owned more net positive charge, resulting in stronger electrostatic interaction between BF2-C and DNA. Furthermore, the more remarkable hydrophobic C-terminal of BF2-C, compared with BF2-A, promoted hydrophobic interaction between BF2-C and the groove of DNA double helix. It seemed that the net positive charge and hydrophobicity played an important role in the peptides binding

to DNA. Therefore, that the affinity of BF2-C to DNA was more powerful than that of BF2-A, revealing in gel retardation assay and AFM imaging, contributed a portion of higher antimicrobial activity of BF2-C. Interestingly, BF2-A bound to RNA seemed more tightly than BF2-C. It was supposed that the structure of single chain RNA without strong hydrophobic core appeared promiscuous, relative to compact regular DNA double helix, which profited the electrostatic interaction between RNA and BF2-A with shorter peptide chain and better flexibility structure.

In summary, our results demonstrated clearly that BF2-A and BF2-C target intracellular substances, exactly nucleic acids, without significantly permeabilizing the cell membrane. The structure-activity relationship analysis of BF2-A/C revealed that the cell-penetrating efficiency and the affinity ability to DNA were critical factors for determining the antimicrobial potency of both peptides. Our studies provided important information in designing potent new antimicrobial peptides with different mechanisms.

Acknowledgements

This work was supported by the grant from National Natural Science Foundation of China (NSFC, NO.30871805 and 31172214), and the Fundamental Research Funds for the Central Universities, Southwest University for Nationalities (11NZYQN30) and 2010RC04, and 12th Five-Year Plan for Science and Technology Development (No. 2012BAD33B05).

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