Effective antimicrobial activity of Cbf-14, derived from a cathelin-like domain, against penicillin-resistant bacteria

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ABSTRACT

Cbf-14, a cationic peptide derived from a cathelin-like domain, was designed by inserting the highly α-helical sequence RLLR into an antibacterial sequence and deleting the inactive amino acids in Cbf-K16. Clinical penicillin-resistant isolates as well as NDM-1-carrying Escherichia coli and a correspondingly infected mice model were employed to evaluate Cbf-14 antibacterial activity. The results showed that Cbf-14 possessed potent antimicrobial effects with an MIC of 8–64 μg/ml, and killed almost all bacteria within 240 min. Cbf-14-treated mice achieved an 80% survival rate and approximate 2.5 log unit reduction in CFU in tissues; additionally, this peptide significantly suppressed the production of pro-inflammatory cytokines by the disaggregation of lipopolysaccharide (LPS), suggesting its anti-inflammatory effects. Furthermore, Cbf-14, concentration higher than 2 × MIC value, increased membrane uptake to NPN and PI dye by 96.2% and 63.7%, respectively, neutralised the negative zeta potential of LPS and bacteria surface, and induced 100% leakage of liposome-entrapped calcein and cytoplasmic membrane disruption of E. coli, indicating obvious membrane permeation. Finally, it bound to DNA and respectively evoked 85.0% and 63.3% inhibition of gene replication and protein expression of NDM-1 at sub-MIC concentration in E. coli BL21 (DE3)-NDM-1. These data indicated that Cbf-14 possessed effective antimicrobial activity against penicillin-resistant bacteria in vitro/vivo through membrane disruption, DNA binding, down-regulating NDM-1 expression by plasmid replication inhibition, and anti-inflammatory activity by LPS disaggregation, suggesting a potential anti-infective clinical agent.

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1. Introduction

Microbial resistance, the cause of invalidation of major antimicrobial drugs in clinical settings, has become a worldwide health problem in recent years [1]. The “superbugs”, such as methicillin-resistant Staphylococcus aureus, vancomycin-resistant enterococci and extended-spectrum β-lactamase-producing bacteria, exhibit multdrug resistance to antibiotics as a result of carrying several antibiotic genes, especially New Delhi metallo-β-lactamase-1 (NDM-1), which was found in patients in South Asia and first reported in the Lancet in 2010 [2]. NDM-1, mainly expressed in Escherichia coli (E. coli) and Klebsiella pneumonia, is a novel broad spectrum carbapenemase with a high ability to hydrolyse and inactivate almost all carbapenems, which are used as a last resort for the treatment of multidrug-resistant bacterial infections [3]. Therefore, there is an urgent need for novel, broad-spectrum compounds with rapid antimicrobial effects, new mechanisms of action and the ability to limit the induction of antimicrobial resistance in “superbugs”.

Cathelicidins form a family of antimicrobial peptides (AMPs) that harbour a highly conserved cathelin-like domain. They are effective against a broad range of microorganisms, including gram-positive and gram-negative bacteria, fungi, and viruses, some of which are clinical drug-resistant strains [4,5]. Cathelicidin-BF (BF-
30) is a 30 amino acids cathelicidin-like polypeptide from the non-mammalian vertebrate Bungarus fasciatus. Its precursor has a cathelin-like domain at the N-terminus and the mature cathelicidin-BF at the C-terminus [6]. Our previous study indicated that BF-30 could destabilize the lipid head groups and produce pores in the cell membrane, resulting in the leakage of cytoplasmic content [7]. Despite their incomparable advantages compared with conventional antibiotics, source and biological stability limit the widespread clinical application of natural AMPs. In a previous report, we introduced a single amino acid mutation, Cbf-K16, by replacing Glu16 with Lys16 in BF-30 to increase the cationic charge [8]. Consequently, the cathelicidin-BF Lys16 mutant, Cbf-K16, has a higher net positive charge (+13) at neutral pH than BF-30, and this mutant can permeate bacterial membrane and interfere with the function of specific macromolecular, such as DNA and protein, to induce bacterial cell death [8].

However, it remained unclear whether the mutated Cbf-K16 was competent in terms of bacterial activity and whether the improvement of its permeation ability enhanced its antibacterial activity against penicillin-resistant gram-negative strains. Thus, we designed a modified small peptide, Cbf-14, by inserting a highly $\alpha$-helical sequence RLLR into the N-terminal region of Cbf-K16. Consequently, the cost of Cbf-14 was significantly reduced because we retained the antibacterial sequence (KFFRKLKKSV) but deleted the inactive amino acids of Cbf-K16 (Fig. 1A and B). Cbf-14, with the sequence RLLRKKFRKLKKSV, has a higher fat-soluble index than Cbf-K16 [104.29 vs 71.33] because of the high proportion of leucine residues. Furthermore, its $\alpha$-helical content in a bio-mimetic membrane, 0.2 DPPG/DPPE + DPPC molar ratio system, was increased from 7.1% to 57.0% when compared to the 50% TFE/water system (Fig. 1C).

In the present study, we aimed to utilize penicillin-resistant isolates and mice model induced by NDM-1-carrying E. coli to elucidate the antimicrobial activity and potential mode-of-action of Cbf-14 against penicillin-resistant bacteria in vitro and in vivo.

2. Materials & methods

2.1. Materials

Cbf-14 (≥98%) and FITC-Cbf-14 were synthesized using standard Fmoc solid-phase synthesis protocols (GL Biochem Co., Ltd, Shanghai, China). The following reagents and kits were utilized: IPTG (Amresco, Canada, US); TNF-$\alpha$, IL-6, IL-10 and IL-4 ELISA Kits (MultiSciences Biotech Co., Ltd, Hangzhou, Zhejiang, China); bacterial genomic DNA extraction kit (Sangon Biotech Co., Ltd, Shanghai, China); tigecycline (Xianhang Medical Technology Co., Ltd, Nanjing, Jiangsu, China); ampicillin (Sunshine Biotechnology Co., Ltd, Nanjing, Jiangsu, China); penicillin V (Sangon Biotech Co., Ltd, Shanghai, China); caspase 1 polyclonal antibody and NF-$\kappa$B p65 polyclonal antibody (Abclonal Biotechnology Co., Ltd, Wuhan, Hubei, China); LIVE/DEAD BacLightTM Bacterial Viability kit L7012 (Molecular Probes Inc., Eugene, OR, US); FITC-conjugated LPS (Sigma–Aldrich, St. Louis, MO, US); DPPC and DPPE (CordenPharma International, Planckstadt, Germany). Other analytical grade chemical reagents were acquired from commercial sources. All drugs were determined using the E-TOXATE kit. Test samples used in the study did not show detectable levels of endotoxin within the sensitivity limit of kit (0.1–1.0 EU/ml).

2.2. Bacterial strains

The NDM-1-carrying bacteria, recombinant E. coli BL21 (DE3)-NDM-1, was constructed by inserting the Klebsiella pneumoniae NDM-1 gene (GenBank accession no. HQ328085) into a pET-28(a) vector and transforming it into E. coli BL21 (DE3) cells to obtain high NDM-1 activity [9]. E. coli ATCC25922, Pseudomonas aeruginosa ATCC27853, S. aureus ATCC25923 were obtained from American Type Culture Collection; K. pneumoniae, P. aeruginosa, E. coli, S. epidermidis, and S. aureus were penicillin-resistant strains isolated from clinical specimens that had been identified by Zhongda Hospital Southeast University (Nanjing, Jiangsu, China).

2.3. Antimicrobial activity assay in vitro

The minimal inhibitory concentration (MIC) of Cbf-14 was measured using a standard micro-dilution method [10]. The minimal bactericidal concentration (MBC) was determined as previously described [11]. Bacterial cells were diluted to achieve log (CFU/ml) values in the range of 5–6 and incubated with Cbf-14 or other drugs for 0, 10, 30, 60, 240, or 480 min. At each time point, samples (50 μl/time point) were plated onto agar plates at appropriate diluted concentration. After overnight culture, the surviving colonies were counted, and an untreated inoculum group was used as a negative control.

2.4. The establishment of the E. coli BL21 (DE3)-NDM-1 infected model in vivo

Mice were intravenously injected with 0.6 g/kg IPTG once or multiple times, $n = 6$ mice per group. Before injection and at 2 h intervals after injection, blood samples were collected to determine the serum IPTG levels using HPLC analysis. Column: Agilent, Hedaer NH$_2$ 4.6 × 250 mm, 5 μm (Hanbon Sci. & Tech. Co., Ltd., Jiangsu, China).

After the injection of 1.8 g/kg IPTG, mice were inoculated intraperitoneally with $1 \times 10^9$ CFU/ml (0.5 ml/mice) of E. coli BL21 (DE3)-NDM-1 in the presence (n = 15 mice per group, 10 animals for survival study and other 5 for NDM-1 activity measurement) or absence (n = 10 mice per group) of additional IPTG and then treated with Cbf-14 (10 mg/kg, i.p.), ampicillin (80 mg/kg, i.v.), tigecycline (16 mg/kg, i.v.) or the same amount of saline (Fig. 2C). Survival was monitored over seven days, mice liver and lung samples were collected 24 h after infection, homogenized and centrifuged. The tissue pellets were washed, re-suspended and ultrasonicated on ice; then, the sonicated lysates were centrifuged to harvest the crude enzymes in the supernatant. NDM-1 activity in bacterial-infected liver or lung was determined as previously described [12]. The metallo-$\beta$-lactamase producing bacteria in mice liver or lung were analysed by the imipenem-EDTA disk method as previously reported [13]. Briefly, imipenem disks containing 10 μg imipenem were incubated with or without 4.5 μl of a 0.5 M EDTA solution (pH 7.8). After drying, the imipenem disks (−/− EDTA) were placed onto the agar surface evenly coated with 100 μl of liver or lung homogenate. The inhibition zone diameter (IZD) of each disk was determined and imaged after incubation for 18 h.

Manipulations of animals were performed in accordance with the standards set by Science and Technology Department of Jiangsu Province (SYXK 2012-0035). In this experiment, a total of 137 male ICR mice were used to study the E. coli BL21 (DE3)-NDM-1 infected model in vivo.

2.5. Antimicrobial and anti-inflammatory activity assay of Cbf-14 in E. coli BL21 (DE3)-NDM-1-infected mice

Mice (n = 10 mice per group) were infected, induced and treated as described above (Fig. 2C). After 12 h of treatment, blood samples were obtained from the mice to determine the serum levels of interleukin-10 (IL-10), interleukin-4 (IL-4), interleukin-6 (IL-6) and tumour necrosis factor (TNF-α). The lung was bluntly isolated and
photographed, and the liver, kidney, lung and spleen were all collected to determine the bacterial titres in tissues at 24 h post-infection. Next, the left lobe of the lung was fixed in 10% formaldehyde and embedded in paraffin for haematoxylin and eosin (H&E) staining and immunohistochemical analysis. Furthermore, the right lobe of the lung was weighed, sliced into pieces and homogenized to determine caspase-1 or NF-κB/p65 expression by routine western blot. The study was conducted in duplicate, and data from one representative experiment are shown.

In this experiment, a total of 100 male ICR mice were used to evaluate the antimicrobial and anti-inflammatory activity of Cbf-14 against E. coli BL21 (DE3)-NDM-1.

2.6. The haemolytic activity and toxicity of Cbf-14 against mammalian cells

Haemolytic activity was assessed using the sheep erythrocyte (SRBC) pellets according to previous methods [8]. Briefly, the SRBC cell suspension was mixed with a serial of diluted-peptides (64, 128, 256, 512, 1024 μg/ml) (1:1, v:v) and centrifuged to collect the supernatants for the measurement of absorbance at 540 nm after 4 h incubation at 37 °C. For calibration, 100% haemolysis was achieved by incubating samples with 0.1% Triton X-100, while no-haemolysis samples were incubated with only PBS. The cytotoxicity of Cbf-14 to mouse spleen cells was evaluated using the CCK-8.
method. Briefly, the spleen cells were obtained steriley from a healthy mice, seeded in wells of 96-well plates (1 × 10^7 cells/well) and cultured in RPMI-1640 medium containing 10% FBS with Cbf-14 at the final concentrations of 20, 100, 200, 400, 800, 1600 mg/ml for 48 h. Each measurement was performed in duplicate.

2.7. Cbf-14 effects on LPS-induced cytokines production and LPS aggregates disruption

2.7.1. Cbf-14 effects on LPS-induced cytokines production

For the determination of cytokines generation by peptide-LPS interactions in vivo, ICR mice were induced by intraperitoneal (i.p.) injection of E. coli LPS (18 mg/kg), followed by i.p. injection of 10 mg/kg Cbf-14 or normal saline (n = 10 mice per group) in 30 min later. Cytokine levels at 12 h after LPS injection in mice serum were assayed by ELISA kit.

2.7.2. Cbf-14 effects on LPS aggregates disruption

The ability of this peptide to dissociate LPS micelles was determined by a Zeta Potential & Particle Size Analyser (Brookhaven Instruments Corporation, Austin, Texas USA). Cbf-14 and the buffer solutions were filtered through 0.45 mm filters before starting the test. LPS (0.1 mg/ml) was incubated with or without Cbf-14 (4–32 μg/ml) for 30 min and analysed using the dynamic light scattering software supplied with the instrument. In addition, the zeta potential of LPS aggregates in the absence and presence of peptide (4–32 μg/ml) were also studied by phase analysis light scattering at the LPS concentration of 0.1 mM. Furthermore, FITC-conjugated LPS (5 μg/ml) was excited at 480 nm and the fluorescence change in the emission of FITC at 515 nm was monitored with different concentrations of peptide (0.01–100 μg/ml) dissolved in PBS in a Synergy™ HT Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA). PBS was used as control.
2.8. Cbf-14 induced membrane rupture

2.8.1. Zeta-potential measurements

The zeta-potential of the bacteria, in the absence and presence of different Cbf-14 concentrations (0.25, 1, 4 × MIC), was determined at 25 °C from the mean of 15 measurements and obtained by phase analysis light scattering in a Zeta Potential & Particle Size Analyser as described before [14]. Bacterial concentrations were fixed at 3 × 10^7 CFU/ml, in order to acquire high enough count rates.

2.8.2. Flow cytometry assay of Cbf-14 affinity to the bacterial cell membrane

E. coli BL21 (DE3)-NDM-1 and S. aureus were suspended at 1 × 10^7 CFU/ml in PBS and incubated in separate suspensions with FITC-Cbf-14 (32 µg/ml) for 30 min at 37 °C. The efficiency of Cbf-14 adherence to the bacterial cell surface was quantified by flow cytometry.

2.8.3. TEM of Cbf-14-treated bacteria

Bacteria from mid-exponential phase cultures (OD_{600} = 0.5) were incubated with Cbf-14 for 4 h to determine the morphological changes using TEM as previously described [15]. The sliced samples were double stained with uranylacetate and lead citrate, observed and imaged to investigate the changes in the bacterial cell membrane.

2.8.4. Outer membrane permeability

Peptide-induced outer membrane permeability was measured as previously described [16]. Briefly, log-phase cultures of E. coli BL21 (DE3)-NDM-1 cells were collected at different time points (0, 4, 8 and 16 h) to count the bacteria. The time-dependent effect of Cbf-14 on NPN fluorescence was measured at the excitation/emission wavelengths of 350/420 nm, respectively.

2.8.5. Inner membrane permeability

The ability of Cbf-14 to permeate the inner membrane of E. coli BL21 (DE3)-NDM-1 was assessed using LIVE/DEAD BacLight™ Bacterial Viability kit L7012. Briefly, mid-log phase E. coli cells were harvested and re-suspended in 0.85% (w/v) NaCl to yield 1 × 10^7 CFU/ml. Each sample was incubated with Cbf-14 (0.25, 0.5, 1, 2, 4 × MIC) at 37 °C for 1 h, then washed and re-suspended in 0.85% (w/v) NaCl. Following the addition of SYTO 9/PI dye mixture, the samples were incubated for 15 min at room temperature in dark and then analysed by a flow cytometer. The additional bacterial samples for staining with SYTO 9 alone and with PI alone were prepared. A bacterial suspension without peptide treatment was served as the negative control.

2.8.6. Calcein release assay

Cbf-14 at final concentrations of 0.25×, 0.5×, 1×, 2× and 4 × MIC was adding to calcein-loaded liposomes, which were prepared by thin film hydration as described previously with DPPE/ DPPG at a ratio of 0.8:0.2 (mol: mol) [17, 18]. Real-time fluorescence intensity was monitored at 530 nm (excitation at 490 nm). The extent of calcein efflux was calculated as (F_t – F_0)/(F_{max} – F_0), where F_t is the fluorescence of a peptide liposome/calcein solution at time t, and F_0 and F_{max} are the initial fluorescence and the fluorescence after complete liposomal disruption by the detergent Triton X-100 (final concentration, 10% w/w), respectively.

2.9. Cbf-14 effects on genomic DNA and plasmid-based replication and expression of NDM-1

2.9.1. Growth curve of E. coli BL21 (DE3)-NDM-1 in response to sub-MIC Cbf-14

The assay was conducted according to Pal’s method with minor modifications [19]. Bacterial suspensions (50 µl; OD_{600} = 0.5) were inoculated into a series of duplicate tubes and cultured with constant shaking in the absence and presence of different concentrations of Cbf-14 (1, 2, or 4 µg/ml). Bacterial suspensions (500 µl) were collected at different time points (0, 4, 8 and 16 h) to count the bacteria. The experiment was performed in triplicate, and the results were averaged.

2.9.2. Confocal laser scanning microscopy

The localization of sub-MIC Cbf-14 on E. coli BL21 (DE3)-NDM-1 was determined by confocal laser scanning microscopy. Bacteria were cultured as described for electron microscopy and respectively incubated with FITC-Cbf-14 (4 or 8 µg/ml) for 15, 30 and 60 min, washed twice with PBS and fixed in 4% polyformaldehyde/PBS for 10 min at room temperature. Followed by the centrifugation at 8000 rpm for 3 min, the sediment was re-suspended in DAPI/PBS (DAPI 0.1 µg/ml) and stained for 10 min in dark. After washing DAPI dye, bacteria pellets were re-suspended in 0.1 ml PBS and analysed with a LSM 700 confocal laser scanning microscope (Carl Zeiss Meditec, Oberkochen, Germany).

2.9.3. Cbf-14 DNA binding assay

The DNA binding assay was performed by agarose gel electrophoresis. E. coli BL21 (DE3)-NDM-1 cells were incubated at 37 °C for 8 h, with a 4 h induction with 1.0 mM IPTG; then, the cells were obtained by centrifugation. Bacterial plasmid DNA was extracted using a SanPrep Column Plasmid Mini-Prep Kit and quantified with an ELISA reader at 280 nm. Then, the DNA samples were incubated with an equal volume of Cbf-14 (8, 16, 32, or 64 µg/ml) at 25 °C for 10 min. DNA migration was detected on a 1% agarose gel [20].

2.9.4. In vivo replication assay

Mid-log-phase E. coli BL21 (DE3)-NDM-1 bearing plasmid pET-28(a) which constructed NDM-1 gene was centrifuged, washed and re-suspended in PBS supplemented with 1% TS, adjusted to OD_{600} = 0.2, and supplemented with 170 µg/ml chloramphenicol. Each sample was respectively treated with peptide or kept untreated, and then taken out at the 0, 60, 120, 180, 240 min of co-incubation. Meanwhile, the bacteria pellets treated with different concentrations of peptide (1, 2, 4, 8 µg/ml) were also collected after 60 min co-incubation. Before the subsequent analysis of PCR, the

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<td>The antimicrobial activity of Cbf-14 <em>in vitro</em></td>
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<th>Bacterial strain</th>
<th>MIC (µg/ml)</th>
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<td>S. epidermidis*</td>
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*NDM-1 carrying E. coli BL21 (DE3) with IPTG (1.0 mM) induction for 4 h. |
samples were heated at 100 °C for 30 min and immediately froze at −80 °C for 1 h, and so repeated three times. The supernatant (1 μl) was used as template in a 25 μl reaction solution including 2.5 mM dNTPs mixture, 10 pM each primer and 0.25 U Ex Taq or Pfu DNA polymerase for 30 or 35 cycles. The sequences of the primer pair for the NDM-1 gene were 5′-CGCGGTACCATGGAATTGCCCAA-TATTAGCGACCCGG-3′ (forward) and 5′-CCGGAATTCATGGCACCCTTGTCGGCCAT-3′ (reverse) with an annealing temperature of 56 °C. The plasmid replication of pET-28(a) was monitored over time with the primer as 5′-TAATAGACTCATATAAGG-3′ (T7 promoter) and 5′-TGCTAGTCCTGGTCAAGG-3′ (T7 terminator). As control for stalled chromosomal DNA replication, the 16S rRNA gene was amplified. The PCR samples were subsequently loaded on a 1.5% agarose gel containing 0.5 M Tris-Cl, 25 mM EDTA, 15% (v/v) glycerol, and 0.25 μg/ml ethidium bromide and run in a 1 × TAE buffer system. Bands were visualized under UV light.

2.9.5. NDM-1 expression of E. coli BL21 (DE3)-NDM-1 in response to sub-MIC Cbf-14

After an overnight culture, a 3 ml suspension was removed as the normal control, and IPTG at a final concentration of 1.0 mM was added to the residual culture. After adding Cbf-14 at a final concentration of 1, 2, or 4 μg/ml, the mixtures were shaken continuously for 4 h. Protein was obtained by sonicating the bacterial cells after centrifugation. SDS-PAGE was performed with a 12% separating gel and a 5% stacking gel to investigate NDM-1 expression in E. coli BL21 (DE3)-NDM-1 cells treated with Cbf-14.

3. Results

3.1. Effective in vitro antimicrobial activity of Cbf-14, derived from a cathelin-like domain, against penicillin-resistant and NDM-1-carrying bacteria

Cbf-14 was designed and synthesized based on its parent peptide, Cbf-K6, in which a highly α-helical sequence RLLR was introduced in the N-terminus, and inactive amino acids in the C-terminus were deleted (Fig. 1A and B). Its amino acids sequence was H-Arg-Leu-Leu-Arg-Lys-Phe-Phe-Arg-Lys-Lys-Ser-Val-OH and its terminal mature peptides with antimicrobial activities, like dCATH, a α-helical peptide identified from ducks, has a strong antimicrobial activity against a broad range of bacteria in vitro [21].

3.2. NDM-1 expression in E. coli BL21 (DE3)-NDM-1-infected mice after IPTG induction

To establish an NDM-1-carrying bacterial infection model to evaluate the antimicrobial activity of Cbf-14 in vivo, we first determined the metabolic parameters of IPTG and NDM-1 expression in the mice. IPTG was quickly eliminated from the blood, with t1/2 values of 5 min and 203.6 min, respectively (Fig. 2A). According to the metabolic parameters after a single injection, Phoenix WinNonlin 6.0 calculated that if the IPTG induction dose was increased to 1.8 g/kg, blood IPTG levels would be maintained at >1.0 mM for longer than 2 h. Thus, in a follow-up experiment, mice were injected with IPTG for six times (1.8 g/kg each time) at an interval of 2 h. As the drug concentration–time curve of the multiple injections shows in Fig. 2B, blood levels of IPTG could be maintained at an efficient induction concentration, which was consistent with in vitro reports, and no symptoms of toxicity were observed in the mice. Therefore, 1.8 g/kg IPTG was an efficient and safe induction dose.

After culturing agar plates coated with liver or lung homogenate for 18 h, there was no obvious bacteriostatic ring around the imipenem disks, but a significant IZD surrounded the imipenem disks containing EDTA (Fig. 2D). Compared with the control group, the liver and lung NDM-1 activity in the infected mice were elevated 12.6- and 16.2-fold, respectively (Fig. 2E).

These data indicated that our induction method could successfully establish an NDM-1-carrying bacterial-infected mice model.

3.3. Effective antimicrobial activity of Cbf-14 in E. coli BL21 (DE3)-NDM-1-infected mice

The in vivo antimicrobial activity was mainly determined based on animal survival, tissue bacterial titres and attenuation of lung injury. The administration of Cbf-14 (10 mg/kg) in both the IPTG-induced and non-IPTG-induced groups significantly improved the survival rate, from 20% to 0% in the saline-control groups to 90% and 80%, respectively. However, ampicillin (80 mg/kg) was only effective in mice without IPTG induction, not in induced mice (Fig. 3A and B). According to the bacteria enumeration results, the survival improvements were associated with decreased bacterial titres in the tissues (liver, lung, spleen, and kidney) of infected mice. Averages, there was more than a 2.5 log unit reduction of CFU in the liver, lung, spleen and kidney in Cbf-14-treated mice (Fig. 3C–F).

Meanwhile, the gross appearance revealed obvious pathological damage with abscesses and blood extravasation in infected mice lung. Furthermore, alveolar interstitial congestion and oedema, pulmonary blood vessel dilatation and substantial inflammatory cell infiltration were also observed in the mice model, as evidenced by H&E staining. The administration of Cbf-14 could alleviate these lesions to some degree, but no clear improvement was observed in the mice model, as evidenced by H&E staining. The administration of Cbf-14 could alleviate these lesions to some degree, but no clear improvement was observed in the mice model, as evidenced by H&E staining. The administration of Cbf-14 could alleviate these lesions to some degree, but no clear improvement was observed in the mice model, as evidenced by H&E staining.
this peptide has no apparent toxicity against mammalian cells.

3.4. Effective anti-inflammatory activity of Cbf-14 in E. coli BL21 (DE3)-NDM-1 -infected mice

The anti-inflammatory effect of Cbf-14 was evaluated by determining the serum levels of IL-6, TNF-α, IL-4 and IL-10 and the expression of caspase-1 and NF-kB p65. IL-6 and TNF-α level in the model group significantly increased at 12 h post-infection, but Cbf-14 treatment decreased the levels of these two pro-inflammatory factors, especially those of TNF-α, by approximately 50%. Ampicillin had no obvious effect on IL-6 or TNF-α production. Interestingly, this peptide also increased serum IL-4 and IL-10 levels to a greater extent than did ampicillin (Fig. 4A–D). Actually, similar findings were obtained by Lee JK et al. for HPA3P2, a helix-PXXP-helix peptide [23] and by Paula et al. for LL-37 [22]. Chae et al. have demonstrated that caspase-1 activation is regulated by Pyrin. Pyrin is cleaved by caspase-1 and the cleaved N-terminal fragment translocates to nucleus and enhances caspase-recruitment domain-independent NF-kB activation through interactions with p65 NF-kB and IκB-α [24], NF-kB is of central importance in immune and inflammatory responses, and its activation related to Pyrin regulated-caspase-1 activation [25]. Our results indicated that caspase-1 and NF-kB p65 immunohistochemistry on model mice lungs showed strong positive staining, but this staining was significantly reduced by Cbf-14 (Fig. 4E). Furthermore, the expression of caspase-1 and NF-kB p65 as detected by western blotting (Fig. 4F) was consistent with the immunohistochemistry results. Our results demonstrated that Cbf-14 possessed effective anti-inflammatory activity in NDM-1-carrying E. coli-infected mice.

3.5. Cbf-14 suppressed LPS-induced inflammation and disassociated LPS aggregation

LPS aggregation has been found to be important for inflammation triggering [26]. Peptide-induced LPS aggregate disruption can potentially be coupled to anti-inflammatory effects. Here, ELISA method was used to confirm the effect of Cbf-14 on the production of pro-inflammatory cytokines induced by LPS. As shown in Fig. 5A, LPS (18 mg/kg, i.p.) alone induced the production of TNF-α and IL-6 for about 948 and 360 ng/L, respectively. But in Cbf-14 (10 mg/kg) treated group, mice serum levels of IL-6 and TNF-α significantly decreased, while IL-10 and IL-4 were much higher than that of LPS alone group, which was consistent with the anti-inflammatory activity of Cbf-14 against NDM-1-carrying E. coli infection model. Similarly, Artur Schmidtchen et al. also found that pro-inflammatory cytokines IL-6, TNF-α, and MCP-1 were reduced in KYE28-treated mice, whereas the anti-inflammatory IL-10 was transiently increased [27]. Hu X et al. demonstrated that TRAP derived decoy peptide (TR6) significantly inhibited LPS-induced production of TNF-α, IL-1β and IL-6 [28].

LPS is a major component of E. coli outer membrane and contribute to the negative zeta potential of bacterial surface. In our present study, the results of zeta potential measurements indicated that pure LPS solution and the suspension of E. coli cells exhibited average negative zeta potential of −28.0 mV and −33.2 mV (Fig. 5C), respectively, but the potential shifted towards neutrality were observed as the addition of Cbf-14 (16 μg/mL), suggesting this peptide effectively neutralizes the negative charge of LPS and bacterial surface. As we know, LPS forms micellar aggregates in water and FITC fluorescence is highly quenched in FITC-LPS micelles [29]. Interactions of some AMPs with LPS may cause an enhancement of the FITC fluorescence because of dequenching, which indicates the dissociation of large LPS aggregates into smaller sizes [30]. Here, the dynamic-light-scattering studies showed that LPS alone produced micellar aggregates with a diameter centred at 10360 nm, but a dramatic shift of the size of LPS toward a lower value (175 nm) was observed in the presence of Cbf-14 (16 μg/mL), and the aggregation centred at 10360 nm totally disappeared (Fig. 5B). Additionally, Cbf-14 had a strong effect on the FITC-conjugated LPS aggregates, and the addition of peptide caused a dose-dependent increase of FITC-conjugated LPS fluorescence. When the concentration of Cbf-14 was higher than 10 μg/mL, the fluorescence intensity increased more than 60% after 1 h incubation, as shown in Fig. 5D. Similar findings were also obtained by Sun Y et al. for chensinin-1β [17] and by Bhunia et al. for fowlciclin-1 fragments [31], the fluorescence intensity was increased as the addition of these peptides in a dose-dependent manner.

The data presented above demonstrated that Cbf-14 exerted anti-inflammatory by inducing the LPS aggregate disintegration.

3.6. Cbf-14 effectively enhanced the membrane permeation of penicillin-resistant bacteria

Zeta-potential was used to test the interaction of Cbf-14 with E. coli which presents a negative charge (−33.15 mV). By adding peptide, there was a concentration-dependent zeta-potential increase from −19.49 to −2.49 mV (Fig. 6A), suggesting Cbf-14 could effectively neutralize the negative charge of gram-negative bacterial surface.

Affinity plays a vital role in peptide-membrane interactions that enable membrane disruption [32]. The affinity of Cbf-14 for the membranes of penicillin-resistant E. coli and S. aureus was evaluated using FITC-labelled Cbf-14. Cbf-14 (1 × MIC) had a high affinity for the bacterial cell membrane after 30 min of co-incubation; the adherence rates were 97.9% and 91.1% for E. coli and S. aureus, respectively (Fig. 6B).

To further explore whether Cbf-14 could interfere with bacterial cell membrane integrity, morphological changes in E. coli and S. aureus isolates were determined by TEM 4 h after treatment with Cbf-14 (80 μg/mL). As shown in Fig. 6C, compared with the smooth, clearly visible structure and continuous double membrane of the untreated bacteria, the cytoplasmic membrane of Cbf-14-treated cells was almost completely ruptured. Nevertheless, the cell wall backbone remained recognizable. Simultaneously, a large amount of cellular debris was observed in the visual field, and numerous bleb-like structures were present, indicating that the outer walls of the bacterial cells had been separated layer-by-layer from the cell.
The directly morphological changes of bacteria surface could often be observed by some cationic peptide-treated bacterial cells [21,9]. As shown in Fig. 6D, the addition of Cbf-14 induced a dramatic increase of fluorescence intensity of NPN in a concentration-dependent manner as this hydrophobic fluorescent probe was...
taken up by the outer membrane of *E. coli*. After 2 min, uptake gradually increased to more than 40%, while it increased to approximately 90% in 30 min later when the peptide concentration was higher than 0.5 MIC, which was similar to the result treated by 0.1% Triton X-100 (positive control). The influx of SYTO 9 and PI was monitored to analyse the ability of Cbf-14 to disrupt plasma

Fig. 5. Disaggregation of LPS by Cbf-14. (A) Cbf-14 reduced the production of pro-inflammatory cytokines induced by i.p. injection of *E. coli* LPS (18 mg/kg) in ICR mice, but increased the levels of IL-10 and IL-4 (**p < 0.01), n = 6 mice per group. (B) Size distribution of LPS in the absence and presence of Cbf-14 (16 μg/ml). The figure showed one representative result of three independent experiments. (C) The zeta potential of LPS aggregates in the absence and presence of peptide from 15 measurements. (D) Fluorescence intensity changes of FITC-conjugated LPS in the presence of different concentrations of peptide over indicated time periods. This data showed the mean of twice experiments, and three parallel samples were prepared for each peptide concentration.

Fig. 6. Cbf-14 induced membrane-destabilizing effects. (A) The zeta-potential of *E. coli* BL21 (DE3)-NDM-1 treated with 0.25, 1 and 2 × MIC peptide, respectively. Bars represented the standard deviation from three independent experiments, each one with 15 measurements. (B) Flow cytometry assay of Cbf-14 adhering to the cell membrane of penicillin-resistant *E. coli* and *S. aureus* strains. (C) TEM of Cbf-14-induced bacterial membrane disruption. (D) Outer membrane permeability induced by Cbf-14 as detected by NPN uptake in *E. coli* BL21 (DE3)-NDM-1. Enhanced uptake of NPN was measured by an increased fluorescence of NPN. The data represented the mean of three independent measurements. (E) Inner membrane permeability induced by peptide was detected by the influx of SYTO/PI in *E. coli* BL21 (DE3)-NDM-1 with 0.5, 1, and 2 × MIC peptide concentration. Control: 0.85% NaCl. (F) Calcein release from Cbf-14-treated liposomes. Each value represented the mean of twice independent experiments, which three parallel samples for each peptide concentration.
membranes of intact E. coli cells. The data demonstrated that the permeability of bacterial inner membrane was increased by Cbf-14 in a concentration-dependent manner as shown in Fig. 6E. Peptide (0.5 × MIC) could lower the ratio of SYTO/PI by about 27.5% compared to the control. As the increase of peptide concentration, an obvious decrease ratio of SYTO/PI was observed, suggesting the plasma membrane structures are significantly altered and membranes integrity are damaged. As Gopal et al. and Sun Y et al. reported, myxinidin and chensinin-1b, two charge-enriched peptides, could increase NPN and PI dye uptake through bacteria membrane, respectively [16,17].

Additionally, membrane disruption by Cbf-14 was detected by measuring calcein release from negatively charged liposomes. As shown in Fig. 6F, Cbf-14 (4 × MIC) rapidly induced 90% leakage of liposome-entrapped calcein in 1 min, and the leakage rate reached 100% in 15 min. Meanwhile, Cbf-14 (1 × MIC) induced 42.2% leakage in 15 min, suggesting that the gram-negative bacterial membrane is very sensitive to Cbf-14. When exposed to Cbf-14, calcein leaked from liposomes in a time- and concentration-dependent manner. This suggested that Cbf-14 increased membrane permeation and damaged the membrane integrity of penicillin-resistant bacteria.

3.7. Cbf-14 bound genomic DNA and inhibited a plasmid-based gene replication and protein expression of NDM-1 in E. coli BL21 (DE3)-NDM-1

DNA binding, in vivo replication and SDS-PAGE analysis of NDM-1 gene replication and its expression were employed to identify the non-membrane-disrupting antibacterial properties of Cbf-14. We found that the growth curve showed no obvious growth retardation of E. coli BL21 (DE3)-NDM-1 after exposure to Cbf-14 (1, 2, and 4 μg/ml) (Fig. 7A). This result was consistent with the calcein release assay data; at 4 μg/ml, Cbf-14 only caused 8.2% leakage,
which represented the maximum percentage of calcein leakage at this concentration (Fig. 6E). Furthermore, confocal laser scanning microscopy was used to investigate the location of sub-MIC Cbf-14 within bacterial cells. As shown in Fig. 7B, Cbf-14 (4 μg/ml) was able to permeate bacterial membrane and then bind nuclear acid after 1 h co-incubation. In addition, the electrophoretic gel mobility shift assay showed that Cbf-14 bound genomic DNA in E. coli BL21 (DE3)-NDM-1, and the electrophoretic movement of DNA was almost completely inhibited by Cbf-14 (8 μg/ml). Compared with the non-complexed DNA control, an obvious retardation was observed, and the DNA migration at different concentrations of Cbf-14 was similar (Fig. 7C). This DNA binding effect, like that of Tachyplesin and Buforin, could interfere with the biological functions of bacteria [33]. Furthermore, a plasmid-based replication assay was developed to test the effect of Cbf-14 on bacterial replication in vivo. As Fig. 7D showed, in bacteria treated solely with chloramphenicol, plasmid concentration increased over time. But addition of Cbf-14 at concentration of its sub-MIC value could impede plasmid replication. Meanwhile, we found that the NDM-1 gene replication was also significantly blocked by Cbf-14 over indicated time periods compared to the bacteria treated solely with chloramphenicol, and this inhibitory in a dose-dependent manner (Fig. 7E–F). Similarly, Hansmann et al. found that FLG2, a skin-derived C-terminal Filaggrin-2 Fragments, interfered with the bacterial replication in vitro and in vivo [34]. Notably, NDM-1 expression in E. coli BL21 (DE3)-NDM-1 induced with IPTG was inhibited by different concentrations of Cbf-14 (1, 2, and 4 μg/ml) in a concentration-dependent manner after 4 h exposure, as shown in Fig. 7G.

Specially, Cbf-14 (4 μg/ml) evoked a greater than 64% reduction in NDM-1 expression. These suggested that at low concentrations (sub-MIC), Cbf-14 achieved antibacterial effects against NDM-1-carrying bacteria mainly by binding the genomic DNA, inhibiting plasmid replication and finally down-regulating NDM-1 expression instead of disrupting the bacterial membrane.

4. Discussion

Antimicrobial resistance represents a general public health concern; certain multi-resistant organisms not only exhibit resistance to a single agent but are frequently resistant to most available antimicrobial agents [35]. Cathelicidin-like peptides exert potent antimicrobial activity against clinical drug-resistant strains. In the present study, a cationic peptide, Cbf-14, was designed based on the structure-activity analysis of an analogue of the cathelicidin-like polypeptide BF-30, Cbf-K16. Cbf-14 contains 14 amino acids, antimicrobial residues 1 to 10 of Cbf-K16 and an RLLR α-helical motif, and exhibits a high helical-content, hydrophobicity, and α-helical content in hydrophobic environments. As CD spectroscopy shown, its α-helical content was increased sharply from 0% in water to 57.0% in 500 μM MLVs, It has reported that an increased antimicrobial activity of a linear α-helical peptide is close correlated with an increase of α-helical secondary structure [36]. To investigate whether this design was benefit to its antimicrobial activity, clinical penicillin-resistant strains as well as NDM-1-carrying E. coli BL21 (DE3)-NDM-1 cells and a correspondingly infected mouse model was used. The in vitro antimicrobial activity assay showed that Cbf-14 displays potent antimicrobial activity against penicillin-resistant bacteria with an MIC of 8–64 μg/ml; and kills almost all bacteria within 240 min. Furthermore, Cbf-14-treated mice achieved an 80% survival rate, a greater than 2.5 log unit reduction in CFU in tissues and a 43.8% reduction in lung injury, suggesting that Cbf-14 exerts effective antimicrobial activity in vivo, especially against NDM-1-carrying bacteria. It also indicates that Cbf-14, which combines the antibacterial sequence of Cbf-K16 and the RLLR sequence, has powerful and broad-spectrum antibacterial activity against penicillin-resistant strains. Meanwhile, this peptide exhibits low haemolytic activity and cytotoxicity toward mammalian cells.

However, the antimicrobial activity of Cbf-14 against gram negative bacteria was higher than gram positive bacteria (Table 1), such as S. aureus, suggesting its more competitive potentials. Similar results also appeared from the membrane disruption data (Fig. 6C). As previously reported [7,9], AMPs of cathelicidins family have direct antimicrobial effects against a broad spectrum of microbes, but exhibits a more prominent activity against gram-negative bacteria than gram-positive bacteria, such as Cbf-K16 and BF-30, which range of MICs for gram-negative bacteria and gram-positive bacteria were 2–32 μg/ml and 16–32 μg/ml, respectively.

In our previous study, NDM-1-carrying E. coli with high NDM-1 activity were generated by inserting the K. pneumoniae NDM-1 gene (GenBank accession no. HQ328085) into a pET28a vector and transforming it into E. coli BL21 (DE3) cells, because the robust reproductible activity, rapid transmission speed and limited source make it difficult to perform numerous experiments by NDM-1-carrying isolates. Excitingly, this recombinant E. coli BL21 (DE3)-NDM-1 cell has been utilized in in vivo studies with IPTG induction at the final concentration of 1.0 mM for 4 h [39]. Therefore, in this study, we attempted to use this strain to duplicate a safe and stable infection model in mice to investigate the antimicrobial activity of Cbf-14 in vivo. Herein, mice were injected with IPTG for six times (1.8 g/kg each time) at an interval of 2 h to induce the in vivo expression of NDM-1. As a result, higher NDM-1 expression and a lower survival rate were observed in IPTG-induced mice. This suggests that the mice model induced by NDM-1-carrying E. coli was duplicated successfully and it could be widely and safely used for the screening of antibacterial agents.

In many cases, the current therapy for infection involves antibacterial and anti-inflammatory drugs [37]. Here, the antimicrobial and anti-inflammatory activities of Cbf-14 were evaluated by this mode. We found that this peptide significantly improved the survival rate to approximately 80% and alleviated the lung injury in E. coli infected mice, and this protective effect was similar to LL-37, which could elevate the bacterial clearance in tissues and enhance host defence against pulmonary infection [22]. Meanwhile, these results are consistent with the in vitro bacterial susceptibility assays, and they demonstrate that Cbf-14 exerts strong antibacterial activity against penicillin-resistant bacteria.

LPS plays a crucial role in the pathophysiology of inflammation sepsis and shock caused by gram-negative bacteria [38]. Once released into the blood system, it will cause monocytes and phagocytic cells to produce large amounts of cytokines such as TNF-α, IL-6, and interleukin-8 (IL-8). Because of a growing number of studies on peptide-LPS interaction, we investigated the consequence of their interaction for cytokine generation in this study. Bezzerri et al. [39] and Liu Y et al. [40] have reported that the peptide TB-KKG6A and the synthetic hybrid peptides LB-PG/CA-PG can down-regulate the cytokines IL-8, IL-6 and TNF-α and inhibit the expression of LPS-induced proinflammatory cytokines. Similarly, our peptide also reduced serum IL-6 and TNF-α levels but increased IL-4 and IL-10 levels in not only bacterial infected mice but also LPS alone induced-mice, suggesting Cbf-14 exerts anti-inflammatory activity through interfering with peptide-LPS interaction. As LPS aggregation has been found to be important for inflammation triggering, we investigated whether Cbf-14 could induce LPS aggregate disruption, and demonstrated that this peptide, similar with anti-inflammatory peptide LL-37, could disassociate LPS aggregates. Meanwhile, a down-regulation of NF-κB activation was observed in our study, but the anti-inflammatory mechanism involved in NF-κB pathway deserves future.
investigation. For AMPs, which can bind to negatively charged anionic phospholipids on LPS of gram-negative bacteria [41], damages the cell membrane and kills bacteria by membrane-lytic effects after achieving a threshold concentration on bacteria surface [42]. Here, Cbf-14 accumulated on bacteria surface, and neutralised the negative charges, thereby increased the permeability of cell membrane. Consequently, the bacterial membrane structure was altered; pores were formed as the concentrations increase, leading Cbf-14 combined an anti-inflammatory assay with antibacterial activity in mice infected with LPS-1 carrying bacteria, and these activities are responsible for the peptide induced LPS aggregates disruption. For its antimicrobial mechanism, high concentration of Cbf-14 (≥MIC) could directly increase the permeability and even disrupt the membrane of bacterial cells; Cbf-14 at concentration of sub-MIC potentially by binding genomic DNA, inhibiting plasmid replication and finally down-regulating the plasmid-based gene replication and protein expression of drug-resistant genes.

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References


