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Structure–activity relationships of a snake cathelicidin-related peptide, BF-15

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ABSTRACT

Cathelicidin-BF15 (BF-15) is a 15-mer peptide derived from Cathelicidin-BF (BF-30), which is found in the venom of the snake *Bungarus fasciatus* and exhibits broad antimicrobial activity. Since BF-15 retains most part of the antimicrobial activity of BF-30 but has significantly reduced haemolytic activity and a much shorter sequence length (and less cost), it is a particularly attractive template around which to design novel antimicrobial peptides. However, the structure–activity relationship of it is still unknown. We designed and synthesized a series of C-terminal amidated analogs of BF-15 based on its amphipathic α-helix structure. And we characterized their antimicrobial potency and haemolytic activity. We identified the amidated BF-15 (analog B1) with potent antimicrobial activity against several antibiotic-resistant bacteria (MICs between 1 and 64 μg/mL, 2–16-folds higher than BF-30) and much lower haemolytic activity. The subsequent circular dichroism study results showed a typical α-helix pattern of analog B1 and the content of the α-helix structure of it increased significantly comparing with BF-30, which indicates the peptide sequence of BF-15 may provide a major contribution to the α-helix content of the whole BF-30 sequence. The peptide induced chaotic membrane morphology and cell debris as determined by electron microscopy. This suggests that the antimicrobial activity of B1 is based on cytoplasmic membrane permeability. Taken together, our results suggested that peptide B1 should be considered as an excellent candidate for developing therapeutic drugs.

1. Introduction

Due to the abuse of antibiotics clinically [13] and a deficiency of antibiotics with new structures [19], drug-resistant bacteria have developed rapidly in recent years. Multidrug-resistant (MDR) bacterial pathogens have increased worldwide, leading to increasing therapeutic difficulties in infectious diseases. The resistance capacity of bacteria has stimulated the search for novel compounds with activity against bacterial targets and molecules with antimicrobial mechanisms that differ from traditional antibiotics. Antimicrobial peptides (AMPs) are promising possibilities for potential new therapies. AMPs are mainly effective against gram-positive and gram-negative bacteria and also have potent activity against fungi, yeasts, and parasites [2,3,8,17]. Currently, more than 1600 of these peptides have been isolated and described in detail [24]. Cathelicidins and defensins have received the most attention. Cathelicidins are cationic host defense peptides that play an important role in innate immune system. An extensively studied amphibian antimicrobial peptide is magainin-2 which was discovered in 1987 [26]. Pexiganan, a potential magainin-2 analog, was developed and entered clinical trials for topical treatment of diabetic foot ulcers [7,10–12,14,18].

To overcome the emergence of widespread antibiotic resistance, considerable attention has been focused on the design of peptide analogs that have more potent antimicrobial activity than natural peptides but that lack cytotoxicity against mammalian cells. Cathelicidin-BF (BF-30) is a lysine-rich and phenylalanine-rich antimicrobial peptide found in the venom of the snake *Bungarus fasciatus* and exhibits broad antimicrobial activity against bacteria and fungi with the amino acid sequence of KFFRKLKKSVKKRAKEFFKKPRVIGVSIPF. Its antimicrobial activity in vivo and antimicrobial mechanism through interference with cytoplasmic membrane integrity is revealed recently [25,27].

Like most cathelicidins, the secondary structure of BF-30, when investigated by circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopy in the presence of the helicogenic solvent trifluoroethanol (TFE), N-terminal region of BF-30 adopts a typical amphipathic α-helical conformation comprising residues F2–F18 [25]. Cathelicidin-BF15 (BF-15) is a 15-mer peptide derived from BF-30, containing the N-terminal 10 amino acid residues

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(^{162}KFFRKLKKSV^{172} of the precursor) of the peptide and another successive linked 5 amino acid residues (^{157}VKRFK^{161} of the precursor) from the precursor amino acid sequence of BF-30.

C-terminal amidation is common in antimicrobial peptides. Amidating the C-terminal carboxyl is one of the means of enhancing antimicrobial activity of a given peptide, and this is usually attributed to the increased overall positive charge. Often, as in cecropins, clavanins, and protegrins, the net cationicity of antimicrobial peptides is augmented by C-terminal amidation. The positive charge of antimicrobial peptides undoubtedly facilitates their interactions with anionic microbial surface components, such as lipopolysaccharide or various anionic membrane phospholipids [20,22]. Appropriate chain length is also important for antimicrobial activity. Efforts have been done to define the minimum length for bacterial killing and the relationship between length of helix and antimicrobial activity [6,16]. For example, the antimicrobial potency and selectivity of the peptides can be enhanced by increasing peptide length as demonstrated by Vogel et al. [23].

Since BF-15 contains the major part of the N-terminal sequence, we designed several amidated analogs of BF-15 based on the amphipathic structure. These synthetic peptides could retain or increase antimicrobial effectiveness with lower haemolytic activity, making them strong potential candidates for development into broad-spectrum antimicrobial compounds.

In the present work, BF-15 and its amidated analogs were synthesized to investigate their structure–activity relationships. Antimicrobial and hemolytic activities of BF-15 analogs were compared with pexiganan to evaluate their potential clinic application values.

2. Materials and methods

2.1.1. Bacterial strains

A total of ten clinically relevant bacterial strains were used in this study. *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923 and *C. albicans* ATCC 90028 were obtained from the American Type Culture Collection. All other strains, including gentamicin- and ampicillin-resistant microorganisms, were isolates from human clinical specimens collected and identified by the Center of Medical Laboratory of Zhongda Hospital (Southeast University, Nanjing, China). Bacterial strains were prepared by growing overnight in Mueller–Hinton broth (MHB) from Beijing Third Pharmaceutical Technology Co., Ltd. (Beijing, China). The culture was inoculated into fresh medium and incubated at 37 °C with shaking until the bacterial had reached the exponential phase of growth.

2.2. Antimicrobial susceptibility testing

Minimal inhibitory concentrations (MICs) were determined using a standard serial dilution method in appropriate medium for each bacterial strain. Briefly, serial 2-fold dilutions of the test peptides were added to the same number of log-phase bacteria in a 96-well microtiter plate to give a final inoculum of 10^4 colony-forming units/mL. Plates were incubated at 37 °C for 16–24 h and the absorbance at 600 nm was measured. MICs were determined as the lowest peptide concentration that gave no visible growth.

2.3. Peptide synthesis

The synthesis of native BF-30, BF-15 and its analogs were accomplished using a solid-phase methodology on Wang resin (peptides without C-terminal amidation) or Fmoc Rink Amide-MBHA resin (peptides with C-terminal amidation) (GL biochem, Shanghai, China) by a microwave synthesizer (CEM, NC, USA). Fmoc-protected amino acids (GL biochem, Shanghai, China) were used. The general procedure of peptide preparation under microwave irradiation was previously reported [5,21]. After the quantified resin was swelled, deprotected and washed, a mixed solution of Fmoc-protected amino acid and activator (HBTU, HOBT, and DIPEA, all purchased from GL biochem, Shanghai, China) was added. Then the mixture was bubbled with nitrogen under microwave irradiation and washed with DMF (SamSung fine chemicals, Korea). The procedures of deprotection and coupling were repeated with relevant Fmoc-protected amino acids to give peptide-resin complex. Then resin was washed successively with DMF three times. Final peptide was cleaved from the resin by treatment with Reagent K (TFA/thioanisole/water/phenol/EDT, 82.5:5:5:5:2.5) for 1.5 h at room temperature and precipitated with cold ethyl ether. Then ethyl ether was removed through centrifugation. The HPLC analysis was performed on a Shimadzu 2010C HPLC system. Peptides were purified with Shimadzu LC-10 preparative RP-HPLC on a C18 reversed-phase column (5 μm, 340 mm × 28 mm) by a gradient elution using (A) water with 0.1% TFA – (B) acetonitrile (Merck, Darmstadt, Germany) with 0.1% TFA as the mobile phase 0 min (10% B) → 40 min (70% B). The flow rate was 6.0 mL/min, and ultraviolet (UV) detection was at 214 nm. The structures of synthetic peptides were identified by mass spectrometry (Agilent Technologies Series 1100 LC/MSD SL system, Palo Alto, USA). The purity of each synthetic peptide was above 95%.

2.4. Helix-wheel plot

Helix-wheel plot was carried out by software package provided by The Expert Protein Analysis System (ExPASy) proteomics server as described [9]. (http://heliquest.ipmc.cnrs.fr/).

2.5. Circular dichroism

Circular dichroism spectra of the peptides were collected (Jasco J-810 spectropolarimeter). Samples were allowed to equilibrate for 10 min at 25 °C prior to data collection. The spectra were measured between 190 and 250 nm using 0.1 cm path-length cell with 1 nm bandwidth, 1 s response time, and a scan speed of 100 nm/min. Four scans per sample were averaged followed by subtraction of the signal of the solvent. All peptides were analyzed at 200 μg/mL in 10 mM sodium phosphate buffer (pH 7.4) containing 50% TFE, (v/v) or 25 mM sodium dodecyl sulfate (SDS) [15]. The secondary structure elements of the peptides were estimated according to the Yang formula [4].

2.6. Hemolysis assay

Rabbit red blood cells were centrifuged, washed three times with phosphate-buffered saline (PBS; 35 mM phosphate, pH 7.0, 150 mM NaCl) and re-suspended to 4% (v/v) in PBS. Then, 100 μL of peptide solution was added to 100 μL of 4% (v/v) RBCs in sterile 96-well plates, which were incubated for 1 h at 37 °C and centrifuged at 1000 × *g* for 5 min. Aliquots (100 μL) of supernatant were transferred to fresh 96-well plates and haemoglobin release was measured using a Thermo, Waltham, MA Microplate Reader by absorbance at 540 nm. Zero and 100% haemolysis were determined in PBS and 0.1% Triton X-100, respectively. Percent haemolysis was calculated as [(Abs540 nm in the peptide solution – Abs540 nm in PBS)/(Abs540 nm in 0.1% Triton X-100 – Abs540 nm in PBS)] × 100.

2.7. Transmission electron microscope studies of S. aureus ATCC 25923 and P. aeruginosa ATCC 27853 treated with analog B1

Transmission electron microscope was employed to confirm morphological changes, which was performed as described