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Synthetic cationic amphiphilic α-helical peptides as antimicrobial agents

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ABSTRACT

Antimicrobial peptides (AMPs) secreted by the innate immune system are prevalent as the effective first-line of defense to overcome recurring microbial invasions. They have been widely accepted as the blueprints for the development of new antimicrobial agents for the treatment of drug resistant infections. However, there is also a growing concern that AMPs with a sequence that is too close to the host organism's AMP may inevitably compromise its own natural defense. In this study, we design a series of synthetic (non-natural) short α-helical AMPs to expand the arsenal of the AMP families and to gain further insights on their antimicrobial activities. These cationic and amphiphilic peptides have a general sequence of $(XXYY)_n$ (X: hydrophobic residue, Y: cationic residue, and n: the number of repeat units), and are designed to mimic the folding behavior of the naturally-occurring α-helical AMPs. The synthetic α-helical AMPs with 3 repeat units, $(FFRR)_3$, $(LLRR)_3$, and $(LLKK)_3$, are found to be more selective towards microbial cells than rat red blood cells, with minimum inhibitory concentration (MIC) values that are more than 10 times lower than their 50% hemolytic concentrations (HC_{50}). They are effective against Gram-positive *B. subtilis* and yeast *C. albicans*; and the studies using scanning electron microscopy (SEM) have elucidated that these peptides possess membrane-lytic activities against microbial cells. Furthermore, non-specific immune stimulation assays of a typical peptide shows negligible IFN-α, IFN-γ, and TNF-α inductions in human peripheral blood mononuclear cells, which implies additional safety aspects of the peptide for both systemic and topical use. Therefore, the peptides designed in this study can be promising antimicrobial agents against the frequently-encountered Gram-positive bacteria- or yeast-induced infections.

1. Introduction

Antimicrobial peptides (AMPs) secreted by innate immune system of various organisms have been reported since three decades ago as the first form of natural defense against environmental parasitic infections [1]. To date, the primary structure of these peptides is so diverse that more than 1000 AMP sequences have been reported and documented in the AMP database [2]. Most of these peptides were derived from larger precursors [1], often empirically "optimized" by means of chemical modifications, such as glycosylation [3], fluorination [4], cyclization [5], or introduction of point amino acids mutations [6,7]. In addition, some peptides were also derived from a larger protein sequence through a proteolysis, such as lactoferricin α-helical AMP from lactoferrin [8]. These derived AMPs often adopt different conformational structures in aqueous or membrane-like environments; however, they exhibit a certain degree of generalities, such as net cationic charge, amphiphilicity, primarily targeting the microbial cell membrane, and possessing antimicrobial activities through disruption of the microbial cell membrane [9–13]. Since most of these peptides targeted to disrupt bacterial cell membranes in exerting their antimicrobial actions, it suggests that AMPs can potentially escape the mechanisms involved in multidrug resistance, which is an increasingly difficult phenomenon faced as a result of repeated treatments with small molecular antibiotics [14]. Indeed, more recent works of AMPs have shown that these antimicrobial peptides are less susceptible to microbial resistance as compared to small molecular antimicrobial agents [15]. In particular, A. Mor et al., reported that repeated exposure of Gram-positive bacteria towards different AMPs at sub-minimum inhibitory concentrations (MIC) did not alter the MIC value significantly for up to more than 10–15 passes of the bacterial passage [16]. With such great

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potential in overcoming microbial resistance, the topical applications of these peptides have gained rising attentions as there are less toxic implications in comparison to systemic applications [17,18].

Even though natural AMPs have shown to be superior in overcoming multidrug resistance, the development of natural AMPs has been lacking of design principles and not systematic. It mostly begins with a known natural peptide/short protein sequence, followed by modification and/or "optimization" to obtain improved antimicrobial effects, while reducing the undesirable cytotoxic effects towards mammalian cells. As such, this process becomes "random" involving "black box trial-and-error" procedures. Moreover, there is an increasing argument recently that clinical use of AMPs with sequences that are too close to those of human AMPs would inevitably compromise own natural defenses, possibly posing threat to public health [19]. In this view, non-natural (or synthetic) peptides were developed to provide wider arsenals of AMPs. One of the first attempts to design a synthetic class of AMP was demonstrated by G. Stephanopoulos et al., by introducing rules analogous to "grammatical rules" in linguistics to describe certain commonalities observed from the reported AMP database. These rules were used as algorithms to produce permutations of different non-natural AMP primary sequences [20]. Following that, antimicrobial synthetic peptides were developed in our laboratory in the form of self-assembled core-shell nanoparticles that were effective against a wide spectrum of microbes [21], and were capable of crossing the blood–brain barrier for the treatment of brain infections in a rabbit meningitis model [22]. More recently, another self-assembled non-natural peptide was also shown to have antimicrobial activity in the form of peptide nanotubes [23].

In this study, we would like to expand the arsenals of synthetic AMPs by using material design principles to mimic the naturally-occurring α-helical AMPs. A series of cationic peptide amphiphiles were designed based on the α-helical protein folding principles, whereby the peptide carbonyl O atom and amide proton between the ith and (i + 4)th amino acid positions form a paired hydrogen bonding, resulting in a folded structure with a regular turn every 3.6 amino acids [24,25]. Even though such conformation is stabilized through paired hydrogen bonding within the backbone of the peptide molecules, theoretical framework has shown that hydrophobic interactions between the amino acid side groups contribute significantly towards the nucleation of this helical conformation [26,27]. By garnering hydrophobic interactions between the side groups of the adjacent ith and (i + 4)th amino acids, we hypothesize that α-helical folding can be enhanced if the amino acids are not selected from the helical breakers category [28]. Therefore, to maintain the α-helical periodicity, a repeat primary sequence containing 4 amino acids is adopted in designing the AMPs. Furthermore, by providing amino acids of the same charge at the (i + 2)th and (i + 3)th positions of the peptide, cationic repulsive forces would unfold the peptide molecules in solution, thereby mimicking the α-helical folding characteristics of natural α-helical AMPs, which occurs upon interaction with bacterial membranes [11]. This mimicry is ensured by keeping the cationic and hydrophobic content within the repeat units to be balanced, so that the long-range repulsive forces can overcome the short-range hydrophobic interactions. As such, the resulting primary peptide structure can be simplified as repeat of XXYY amino acid sequence (i.e. $(XXYY)_n$), whereby X is a hydrophobic amino acid, and Y is a cationic amino acid. As it was reported that the presence of zwitterionic moieties in antimicrobial agents reduced its effectiveness [29], the C-terminal of the peptides designed in this study was amidated to maintain the high net positive charges.

The peptides were first characterized for their secondary conformation in solution and simulated membrane environment. The hemolytic properties of the peptides were then characterized and the effectiveness of the designed peptides in killing microbial cells was tested against the model microbes such as *B. subtilis* (Gram-positive) and *C. albicans* (Yeast). The mechanism of actions was examined against *B. subtilis* bacterial membrane using SEM technique. Meanwhile, kinetics of antibacterial effect was also observed. In addition, non-specific immune stimulation *via* cytokines pathways was examined by measuring the *in vitro* secretion level of IFN-α, IFN-γ, and TNF-α by treated human peripheral blood mononuclear cells (PBMCs).

2. Materials and methods

2.1. Materials

Peptides were obtained from GL Biochem (Shanghai, China), and their fidelity was further confirmed *via* matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS, Model Autoflex II, Bruker Daltonics Inc., U.S.A.), using α-cyano-4-hydroxycinnamic acid as matrix. The purity of the peptides was also tested to be more than 95% with analytical reverse phase (RP)-HPLC. α-Cyano-4-hydroxycinnamic acid (HCCA) was purchased from Sigma–Aldrich (Singapore) and used in saturated acetonitrile/water (1:1 volume ratio) after being re-crystallized. Sodium dodecyl sulphate (SDS) micelle solution (10% w/v in DI water) was obtained from 1st Base (Malaysia) and used upon dilution to the desirable concentration range. Tryptic soy broth (TSB) powder and yeast mould broth (YMB) powder were purchased from BD Diagnostics (Singapore) and used to prepare the microbial broths according to the manufacturer's instructions. Phosphate-buffered saline solution at 10× concentration was purchased from 1st Base (Malaysia) and used after dilution to the desired concentration. Ethanol (analytical grade, 99%) and glutaraldehyde (synthetic grade, 50% in H_2O) were purchased from Sigma–Aldrich (Singapore) and used as received. *B. subtilis* (ATCC No. 23857) and *C. albicans* (ATCC No. 10231) were obtained from ATCC (U.S.A) and re-constituted according to the suggested protocols. Red blood cells used in the experiments were obtained from rats maintained at the Animal Handling Units of the Biomedical Research Centers (Singapore). Human PBMCs were extracted from healthy blood donors and maintained with RPMI medium supplemented with 10% low-endotoxin fetal bovine serum (FBS) and 1% penicillin-streptomycin. Granulocyte macrophage-colony stimulating factor (GM-CSF) and lipopolysaccharide were purchased from Sigma–Aldrich and used as received. Enzyme-linked immuno-sorbent assay (ELISA) kit for human interferons alpha (IFN-α, Cat No. 41100-1) and gamma (IFN-γ, Cat No. 41500-1) detection were purchased from PBL Interferon Source (U.S.A.), while that for the detection of human tumor necrosis factor alpha (TNF-α, Cat No. BMS223INST) was purchased from Bender MedSystem (Austria). These kits were used according to the manufacturer's protocols.

2.2. Peptide characterization

The peptides designed in this study were synthesized by the standard Fmoc-solid phase peptide synthesis protocol at GL Biochem (Shanghai, P. R. China). The fidelity of the synthesis was confirmed *via* MALDI-TOF MS and RP-HPLC techniques. An equal volume of peptide aqueous solution (0.5 mg/mL) and α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution (saturated in acetonitrile/water mixture at 1:1 volume ratio) were pre-mixed and spotted onto the MALDI ground-steel target plate to measure the molecular weight of the peptide. The same peptide solution was also run through an RP-HPLC (with C-18 as the stationary phase, and the mixture of acetonitrile and water as the mobile phase with a gradient being varied from 5% to 20% acetonitrile from 0 to 20 min). From the area of the chromatograms obtained, the purity of the peptides was estimated to be more than 95%.

2.3. Circular dichroism (CD) spectroscopy

Peptide solutions were prepared to contain 0.5 mg/mL peptides dissolved in deionised (DI) water or 25 mM SDS surfactant. The CD spectra of the peptide solutions were recorded at room temperature with a CD spectropolarimeter (JASCO, J-810), using a quartz cell having 1.0 mm path length. The spectra were obtained from 190 to 240 nm with solvent subtracted at 10 nm/min scanning speed, and averaged from 3 runs of each sample. The acquired CD signal spectra were then converted to mean residue ellipticity by using the following equation:

$$\theta_M = \frac{\theta_{obs}}{10} \cdot \frac{M_{RW}}{c \cdot l}$$

where θ_M is residue ellipticity [deg. $M^{-1}.m^{-1}$], θ_{obs} is the observed ellipticity corrected for the buffer at a given wavelength [mdeg], M_{RW} is residue molecular weight (M_w/number amino acids), c is peptide concentration [mg/mL], and l is the path length [cm].