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The effect of thiol functional group incorporation into cationic helical peptides on antimicrobial activities and spectra

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ABSTRACT

Antimicrobial peptides (AMP) have been proposed as blueprints for the development of new antimicrobial agents for the treatment of drug resistant infections. A series of synthetic AMPs capable of forming α-helical structures and containing free-sulfhydryl groups are designed in this study ($(LLKK)_2C$, $C(LLKK)_2C$, $(LLKK)_3C$, $C(LLKK)_3C$). In particular, the AMP with 2 cysteine residues at the terminal ends of the peptide and 2 repeat units of LLKK, i.e., $C(LLKK)_2C$, has been demonstrated to have high selectivity towards a wide range of microbes from Gram-positive *Bacillus subtilis*, Gram-negative *Escherichia coli*, *Pseudomonas aerogenosa*, and yeast *Candida albicans* over red blood cells. At the MIC levels, this peptide does not induce significant hemolysis, and its MIC values occur at the concentration of more than 10 times of their corresponding 50% hemolysis concentrations (HC_{50}). Microscopy studies suggest that this peptide kills microbial cells by inducing pores of ~20–30 nm in size in microbial membrane on a short time scale, which further develops to grossly damaged membrane envelope on a longer time scale. Multiple treatments of microbes with this peptide at sub MIC concentration do not induce resistance, even up to passage 10. However, the same treatment with conventional antibiotics penicillin G or ciprofloxacin easily develop resistance in the treated microbes. In addition, the peptides are shown not to induce secretion of IFN-γ and TNF-α in human monocytes as compared to lipopolysaccharide, which implies additional safety aspects of the peptides to be used as both systemic and topical antimicrobial agents. Therefore, this study provides an excellent basis to develop promising antimicrobial agents that possess a broad range of antimicrobial activities with less susceptibility for development of drug resistance.

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1. Introduction

Peptide-based biomaterials provide excellent avenues to a wide range of bioengineering and biomedical applications [1,2], including regenerative medicines [3,4], biomimetics materials [4,5], therapeutic delivery [6–9], and antimicrobial agents [10–12]. Secreted peptides as antimicrobial agents are abundant in nature, which provide a full-proof mechanism to fight against invasion of various microbial pathogens. Upon meeting potential harmful invasion of microorganisms, multicellular organisms secrete membrane-lytic molecules, often called antimicrobial peptides (AMP). These molecules have been reported to adopt various types of secondary conformations upon interactions with biological membranes [13–15]. On the other hand, they exist in random structures in their native forms, i.e. prior to interactions with biological membranes [15]. Owing to the robust capability to destroy microbial membranes, AMPs have been proposed as important blueprints for new generations of antibiotics in order to overcome multidrug resistant pathogenic microbes [14].

Multidrug resistant pathogens acquire their antibiotics resistance traits through a "natural selection" process in response to antibiotics exposure, which can be perceived as the "environmental pressure". The underlying mechanism of this evolution could involve any one of the following four biochemical processes [16]: (1) drug inactivations/modifications, such as in the case of production of β-lactamase or penicillinase enzyme by methicillin-

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resistant *Staphylococcus aureus* (MRSA) [17]; (2) alteration of antibiotics' inhibitory target site, such as in the case of alteration of penicillin binding protein (PBP) of drug-resistant Gram-Positive bacteria, allowing them to overcome the inhibition of peptidoglycan synthesis, which is an important layer of bacterial membrane integrity [18]; (3) reduced drug accumulation, such as in the case of low antibiotics susceptibility of *Pseudomonas aeruginosa*, and many other Gram-negative bacteria, owing to its low drug permeability of the cellular envelope and multidrug efflux pumps encoded by the drug-resistant genes [19]; and (4) alteration of metabolic pathways, such as in the case of sulfonamide-resistant bacteria, in which bacterial growth is no longer inhibited through the presence of metabolic enzyme-competing sulfonamide drugs [16]. Depending on the nature of the antibiotics, its target sites, and the bacterial species, these biochemical aspects, together with the genetic aspects responsible for the transfer of drug-resistance genes, form the basis for the emergence of multidrug resistant microbes. This, therefore, suggests that there is a great need for the development of alternative antimicrobials that can escape such microbial stress responses.

Macromolecules that attacked on microbial membranes, such as AMPs [13–15,20] or synthetic polymers mimicking AMPs [21–23] have been proposed as one of these alternative anti-infective agents. It is suggested that it may take a significantly much longer "natural selection" process for the microbial cells to totally change their cellular envelope compositions in order to overcome resistance towards AMPs' cell-lytic mechanism of actions [24,25]. Even so, the use of natural AMPs found in human's natural defense system in clinical setting in the long run has been argued to potentially increase dangers to the public's health, especially if these pathogens successfully evolve to become resistant to these natural AMPs [26]. Aware of these, Greg, first proposed a systematic method to generate series of non-natural AMPs through the use of "linguistic model" to identify the commonalities among the reported natural AMPs from various organisms [27]. In our laboratory, we recently developed nanoparticles self-assembled from an amphiphilic peptide as an alternative strategy for combating brain infections caused by Gram-positive bacteria or fungi [10,11]. Jian et al., on the other hand, adopted similar peptide self-assembly approach forming peptide nanotubes to provide alternative antimicrobials with wide antimicrobial spectra [12].

Most recently, we have proposed using protein folding theory as the first principle to design a series of non-natural α-helical AMPs that possess a general primary structure of $(XXYY)_n$, whereby X is a hydrophobic amino acid, Y is a cationic amino acid, and *n* is the number of repeat units varying from 2 to 4. These AMPs are effective in inhibiting microbes belonging to both Gram-positive and yeast families, with AMP having $(LLKK)_3$ sequence that exhibits the highest selectivity towards microbes over mammalian cells [20]. In this study, we attempt to broaden the antimicrobial spectrum of these synthetic α-helical peptides to combat Gram-negative bacteria by providing systematic modification on their primary structure. As highlighted in several earlier studies, the presence of free-sulfhydryl (thiol) group(s) in natural AMPs [28] or in Bismuth-derived organometallic antimicrobials [29] was suggested to significantly increase the potency of the antimicrobials. We hypothesize that by modifying the end-terminal(s) of the peptides with L-cysteine residue, which carries free-thiol functionality on its side group, the antimicrobial spectrum of the previously reported α-helical peptides can be broadened. From the previous series of α-helical peptides, the most optimal amino acid compositions forming α-helical signatures with two and three repeat units, i.e., $(LLKK)_n$, where $n = 2,3$, are used to incorporate thiol groups in this study, owing to their antimicrobial efficacy yet low hemolytic properties. Based on this principle, a new series of peptides are designed: $(LLKK)_2C$, $C(LLKK)_2C$, $(LLKK)_3C$, $C(LLKK)_3C$ (Table 1). To justify the importance of the free-sulfhydryl functionality in this series of peptides, two control peptides with two L-methionine residues (substituting the L-cysteine residues) on the terminal ends of the peptide, $M(LLKK)_2M$ and $M(LLKK)_3M$, are also provided in this study. The antimicrobial properties of these new peptides were studied by bacteriostatic MIC measurement against wider selections of clinically threatening microbes from Gram-positive bacteria: *Bacillus subtilis*, Gram-negative bacteria: *Escheria coli*, *P. aerogenosa*, and yeast: *Candida albicans*. Confocal and scanning electron microscopy techniques were employed to investigate pore formation and membrane destruction mechanism of the peptides. Potential cytotoxic effect of peptides against mammalian cells was characterized by measuring their hemolytic effect on rat's red blood cells (rRBCs). Undesirable immmunogenicity of peptides was also tested *in vitro* by measuring the secretion level of TNF-α and IFN-γ cytokines in human monocytes treated with the peptides. Finally, the capability of the peptide to overcome bacterial resistance was evaluated by repeated treatment of the bacteria with the peptide, in comparison to the conventional β-lactam (penicillin G) and fluoroquinolone (ciprofloxacin) antibiotics.

2. Materials and methods

2.1. Materials

Peptides were purchased from GL Biochem (Shanghai, China), and their molecular weights 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 re-crystallization. Ethanol (analytical grade, 99%) and dimethylsulfoxide (DMSO, synthesis grade, 99%) were purchased from Tee Hai (Singapore). Tryptic soy broth (TSB) powder and yeast mould broth (YMB) powder were purchased from BD Diagnostics (Singapore) and used to prepare the microbial growth media according to the manufacturer's instructions. RPMI growth medium, penicillin-streptomycin solution, and low-endotoxin fetal bovine serum were supplied by Invitrogen and used as received. Sodium dodecyl sulfate (SDS) micelle solution (10% w/v in DI water) was obtained from 1st Base (Malaysia) and used upon dilution to the desirable concentration range. Phosphate-buffered saline solution at 10 × concentration was purchased from 1st Base (Malaysia) and used after dilution to the desired concentration. 100 kDa dextran, 500 kDa dextran, fluorescein isothiocyanate (FITC), and glutaraldehyde (synthetic grade, 50% in H_2O), ciprofloxacin, penicillin G, and sodium tert-butoxide were purchased from Sigma–Aldrich (Singapore) and used as received. *B. subtilis* (ATCC No. 23857), *C. albicans* (ATCC No. 10231), *E. coli* (ATCC No. 25922), and *P. aerogenosa* (ATCC No. 9027), were obtained from ATCC (U.S.A) and reconstituted according to the suggested protocols. Red blood cells (RBCs) used in the experiments were obtained from rats maintained at the Animal Handling Units of

Table 1
α-helical peptide designs with sulfhydryl modification strategy and their molecular weights.

Number of Repeat Units (n)	Notation	Cysteine group	Peptide Sequence	Theoretical M_w	Measured M_w[a]
2	$M(LLKK)_2M$ (negative control 1)	0	MLLKKLLKKM-NH_2	1244.76	1246.39
	$(LLKK)_2C$	1	LLKKLLKKC-NH_2	1085.51	1087.23
	$C(LLKK)_2C$	2	CLLKKLLKKC-NH_2	1188.66	1190.11
3	$M(LLKK)_3M$ (negative control 2)	0	MLLKKLLKKLLK KM-NH_2	1727.43	1729.16
	$(LLKK)_3C$	1	LLKKLLKKLLKKC-NH_2	1568.18	1569.25
	$C(LLKK)_3C$	2	CLLKKLLKKLLKKC-NH_2	1671.32	1672.71

[a] Measured by MALDI-TOF, apparent $M_w = [M_w + H]^+$.

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