Biomaterials 35 (2014) 1315–1325

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

# Effect of stereochemistry, chain length and sequence pattern on antimicrobial properties of short synthetic β-sheet forming peptide amphiphiles

Zhan Yuin Ong [a], Junchi Cheng [b], Yuan Huang [b], Kaijin Xu [c], Zhongkang Ji [c], Weimin Fan [b], Yi Yan Yang [a,*]

[a] Institute of Bioengineering and Nanotechnology, 31 Biopolis Way, The Nanos, Singapore 138669, Singapore
[b] Program of Innovative Therapeutics, The First Affiliated Hospital, Zhejiang University College of Medicine, 79 Qingchun Road, Hangzhou 310003, China
[c] State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, Zhejiang University College of Medicine, 79 Qingchun Road, Hangzhou 310003, China

ARTICLE INFO

*Article history:*
Received 27 September 2013
Accepted 19 October 2013
Available online 7 November 2013

*Keywords:*
Antimicrobial peptide (AMP)
β-Sheet
Stereochemistry
Protease stability
Antibiotics resistance
Broad spectrum

ABSTRACT

In the face of mounting global antibiotics resistance, the identification and development of membrane-active antimicrobial peptides (AMPs) as an alternative class of antimicrobial agent have gained significant attention. The physical perturbation and disruption of microbial membranes by the AMPs have been proposed to be an effective means to overcome conventional mechanisms of drug resistance. Recently, we have reported the design of a series of short synthetic β-sheet folding peptide amphiphiles comprised of recurring $(X_1Y_1X_2Y_2)_n$-$NH_2$ sequences where X: hydrophobic amino acids, Y: cationic amino acids and *n*: number of repeat units. In efforts to investigate the effects of key parameters including stereochemistry, chain length and sequence pattern on antimicrobial effects, systematic D-amino acid substitutions of the lead peptides $(IRIK)_2$-$NH_2$ (IK8-all L) and $(IRVK)_3$-$NH_2$ (IK12-all L) were performed. It was found that the corresponding D-enantiomers exhibited stronger antimicrobial activities with minimal or no change in hemolytic activities, hence translating very high selectivity indices of 407.0 and >>9.8 for IK8-all D and IK12-all D respectively. IK8-all D was also demonstrated to be stable to degradation by broad spectrum proteases trypsin and proteinase K. The membrane disrupting bactericidal properties of IK8-all D effectively prevented drug resistance development and inhibited the growth of various clinically isolated MRSA, VRE, *Acinetobacter baumanni*, *Pseudomonas aeruginosa*, *Cryptococcus. neoformans* and *Mycobacterium tuberculosis*. Significant reduction in intracellular bacteria counts was also observed following treatment with IK8-all D in the *Staphylococcus. aureus* infected mouse macrophage cell line RAW264.7 ($P < 0.01$). These results suggest that the D-amino acids substituted β-sheet forming peptide IK8-all D with its enhanced antimicrobial activities and improved protease stability, is a promising therapeutic candidate with potential to combat antibiotics resistance in various clinical applications.



## 1. Introduction

The rapid emergence of antibiotics-resistant bacteria and fungi in both the nosocomial and community settings has created a significant strain on healthcare systems around the world [1–3]. While global incidences of antibiotics-resistant pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enteroccoci* (VRE) and multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter* spp. have reached epidemic levels [1,2,4], the number of new antibiotics entering the clinical development pipeline has been dismal; with only three new structural classes of antibiotics including the oxazolidinones (linezolid), lipopeptides (daptomycin) and pleuromutilins (retapamulin) entering the market since 2000 [5]. This development is especially alarming given that pathogenic bacteria such as *S. aureus*, *Enterobacter* and *P. aeruginosa* are developing resistance to potent antibiotics such as vancomycin and carbapenems which are traditionally reserved as the last line of defense for vulnerable patients in hospitals [3]. With the ongoing dearth in small molecular antibiotics development, the design and identification of alternative classes of antimicrobial agents with new modes of action that can effectively overcome drug resistance mechanisms is more pressing than ever.

* Corresponding author. Tel.: +65 6824 7106; fax: +65 6478 9084.
*E-mail address:* yyyang@ibn.a-star.edu.sg (Y.Y. Yang).

0142-9612/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2013.10.053

In the last two decades, antimicrobial peptides (AMPs) have gained considerable attention as a novel class of antibiotics with exceptionally broad spectrum antimicrobial activities [6,7]. Most AMPs share common features such as an overall cationic charge and amphipaticity; with the ability to adopt secondary structures in microbial membranes, leading to membrane perturbation once a critical concentration is reached. Due to their rapid and direct membrane lytic mechanisms, AMPs are believed to possess an inherent advantage in overcoming conventional mechanisms of antibiotics resistance such as the increased expression of drug efflux pumps on microbial membranes, production of drug degradation enzymes or alteration to drug interaction sites acquired by microbes against small molecular antibiotics targeting specific biosynthetic pathways [8]. Although it was previously thought that AMPs interact with microbial membranes in an achiral manner through hydrophobic interactions due to the lack of significant differences in antimicrobial and hemolytic activities observed between enantiomeric pairs of cecropin, magainin and melittin [9,10], the divergent effects between other types of stereoisomers reported in numerous other subsequent studies have since revealed the complex role of peptide stereochemistry in structural and biological activities [11–14]. For example, the D-enantiomer of an oligodendrimeric M33 peptide identified from a phage-display peptide library against *Escherichia coli* was found to have stronger activity against Gram-positive *S. aureus* and *Staphylococcus epidermidis* while retaining strong activities against Gram-negative bacteria owing at least partly to enhanced bacterial proteases stability [11]. In another instance, incorporation of D-amino acids into a 12 amino acids long α-helical folding peptide led to the retention of antimicrobial activities with reduced hemolytic potentials despite the loss of secondary structures in the diastereomers [15]. Taken together, the varied results of these studies illustrate that stereochemical effects are highly dependent on individual peptide sequences and that the modification of AMPs with D-amino acids which are not recognized by human and microbial proteases is an useful strategy to prevent premature proteolytic degradation that warrants further investigation in the development of new AMP systems.

Significant barriers limiting the successful clinical translation of AMPs, include high systemic toxicities as a result of poor microbial membrane selectivities and susceptibility to degradation by proteases present in biological fluids such as blood serum, wound exudates or lacrimal fluids. Recently, we have reported the rational design of a series of short synthetic β-sheet folding peptide amphiphiles comprised of recurring $(X_1Y_1X_2Y_2)n$-$NH_2$ sequences where X: hydrophobic amino acids, Y: cationic amino acids and $n$: number of repeat units based upon the common occurrence of amphipatic dyads in membrane spanning proteins [16]. The designed peptides readily assembled into β-sheets in microbial membrane mimicking conditions and were found to possess broad spectrum and highly selective antimicrobial activities. Additionally, optimized β-sheet forming peptides effectively inhibited *S. aureus* biofilm growth and neutralized bacterial endotoxins without inducing overt cytotoxicities. In efforts to investigate effects of key parameters such as stereochemistry, chain length and sequence pattern variations on the antimicrobial properties of the synthetic β-sheet forming AMPs, we performed systematic substitutions of lead peptide sequences $(IRIK)_2$-$NH_2$ and $(IRVK)_3$-$NH_2$ of $n = 2$ and 3 repeat units, respectively, with D-amino acids. Herein, the β-sheet forming propensities of the peptide stereoisomers were investigated in relation to their antimicrobial activities and selectivities. The stability of the designed peptide to degradation by broad spectrum proteases (trypsin and proteinase K) was evaluated. The ability of the peptide stereoisomer to prevent drug resistance development was investigated using a stimulation study in which *E. coli* and *S. aureus* were treated repeatedly with sub-lethal doses of peptide and conventional antibiotics, and monitored for changes in minimum inhibitory concentrations over time. Antimicrobial activities of the designed peptide were also studied in various clinically isolated drug resistant microorganisms including MRSA, VRE, multidrug resistant *Acinetobacter baumanni*, *P. aeruginosa*, *Cryptococcus neoformans* and *Mycobacterium tuberculosis*. Lastly, the ability of the designed peptide to kill bacteria present inside a *S. aureus*-infected mouse macrophage cell line was examined.

## 2. Materials and methods

### 2.1. Materials

Peptides used in this study were synthesized by GL Biochem (Shanghai, China) and purified to more than 95% using analytical reverse phase (RP)-HPLC. The molecular weights of the peptides were further confirmed using 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 (4-HCCA) as matrix. 4-HCCA was purchased from Sigma–Aldrich (Singapore) and used in saturated acetonitrile/water (1:1 volume ratio) after re-crystallization. Phosphate-buffered saline (10× PBS) was purchased from 1st Base (Singapore) and diluted to the intended concentration before use. Cation-adjusted Mueller-Hinton broth II (MHB II) and yeast mould broth (YMB) powders were purchased from BD Diagnostics (Singapore) and reconstituted according to the manufacturer's instructions. *S. epidermidis* (ATCC No. 12228), *S. aureus* (ATCC No. 29737), *E. coli* (ATCC No. 25922), *P. aeruginosa* (ATCC No. 9027) and yeast *Candida albicans* (ATCC No. 10231) were obtained from ATCC (U.S.A.) and cultured according to the recommended protocols. Ciprofloxacin, gentamicin sulfate and penicillin G were obtained from Sigma–Aldrich.

### 2.2. Circular dichroism (CD) spectroscopy

Each peptide was first dissolved at 0.5 mg $mL^{-1}$ in deionized (DI) water alone or DI water containing 25 mM of SDS surfactant. The CD spectra were recorded at room temperature with a CD spectropolarimeter (JASCO Corp. J-810), using a quartz cell with 1.0 mm path length. CD spectra were acquired with solvent subtraction from 190 to 240 nm at 10 nm $min^{-1}$ scanning speed, and were averaged from 5 runs per peptide sample. The acquired CD spectra were converted to mean residue ellipticity using the following equation:

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

where $\theta_M$ refers to the mean residue ellipticity (deg $cm^2$ $dmol^{-1}$), $\theta_{obs}$ is the observed ellipticity corrected for DI water at a given wavelength (mdeg), $M_{RW}$ is the residue molecular weight ($M_w$ · number of amino acid $residues^{-1}$), $c$ is the peptide concentration (mg $mL^{-1}$), and $l$ is the path length (cm).

### 2.3. Minimal inhibitory concentration (MIC) measurements

The antimicrobial activities of the β-sheet forming peptides were investigated against *S. epidermidis* and *S. aureus* (Gram-positive), *E. coli* and *P. aeruginosa* (Gram-negative), and *C. albicans* (yeast) using the broth microdilution method. Prior to the experiment, bacteria cells were cultivated in MHB II at 37 °C and yeast cells were grown in YMB at room temperature under constant shaking at 300 rpm overnight to reach mid-logarithmic growth phase. The microbial suspensions were diluted with the appropriate broths and adjusted to give an initial optical density (O.D.) reading of approximately 0.07 at a wavelength of 600 nm on a microplate reader (TECAN, Switzerland). The O.D. reading corresponds to McFarland Standard No. 1 (approximately $3 \times 10^8$ CFU $mL^{-1}$). The peptides were dissolved in HPLC grade water and subjected to a series of two-fold dilutions using the appropriate broths. Subsequently, 100 μL of microorganism suspension with an initial loading level of $3 \times 10^5$ CFU $mL^{-1}$ was added to an equal volume (100 μL) of polymer solutions to achieve final polymer concentrations ranging from 3.9 to 500 mg $L^{-1}$ and with water content fixed at 10% (by volume) in each well of a 96-well plate. After 18 h (for bacteria) and 42 h (for fungi) incubation with shaking at 37 °C or room temperature, the MIC was taken as the lowest polymer concentration at which no microbial growth was observed visually and with no change in O.D. readings from 0 h. Microbial cells in broth containing 10% (by volume) water as well as pure broth alone were used as the negative controls. To ensure aseptic handling, wells containing pure broth without microbes were included in each experiment. Each test was performed in 6 replicates on at least 2 independent occasions.

### 2.4. Killing efficiency testing

After 18 h treatment of bacteria and 42 h treatment of fungi with various concentrations of peptide (0.5 × MIC, MIC and 2 × MIC), the respective samples were subjected to a series of ten-fold dilutions and plated onto LB agar plates. The plates were then incubated overnight and counted for colony-forming units. A sample