



Research paper

Lipidated α/α -AA heterogeneous peptides as antimicrobial agents

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ABSTRACT

With an increase of resistance in bacteria there is an urgent need for alternative treatment methods that could complement conventional antibiotics. In the past two decades, focus has been drawn to Host Defense Peptides (HDPs) as potential antibiotic agents. Herein we reported our studies on the development of lipidated α/α -AA heterogeneous peptides as a new class of HDP mimetics. These compounds showed potent antimicrobial activity toward both Gram-positive and Gram-negative bacteria, and they also displayed excellent selectivity as they only exhibited limited hemolytic activity. The fluorescence microscopy suggested that the mechanism of action of these heterogeneous peptides is bacterial membrane disruption, which is believed to be the major reason why it is difficult for bacteria to develop resistance. The subsequent time kill studies suggested that these compounds could rapidly eradicate bacteria. Moreover, this class of compounds could also effectively clear biofilms formed by both Gram-positive and Gram-negative bacteria. These findings suggested that lipidated α/α -AA heterogeneous peptides, as a new class of peptidomimetics, are promising antibiotic agents combating antibiotic resistance.

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1. Introduction

Bacterial infections have been the cause of many diseases that could lead to severe conditions including death. For the past seventy years, antibiotics have been used to treat a wide range of bacterial infections. However, due to the prolonged use and frequent misuse of these conventional drugs, many microbes have become resistant to treatment. This problem has led to the loss of effectiveness in the antibiotics to treat infections, resulting in a global health concern [1,2]. Per the Center of Disease Control (CDC), over two million people become infected with antibiotic resistant bacteria every year and of these approximately twenty three thousand die. [3] The World Health Organization (WHO) also reports that microbes are developing new mechanisms of resistance globally and this has caused a threat in the ability to treat common diseases hence leading to prolonged illness, disability, and death [4]. There is an urgent need to search for the alternative approaches combating the emergent resistance in bacteria.

Host Defense Peptides (HDPs), such as cathelicidin and defensins [5,6], are natural antimicrobial peptides which are produced in all classes of life. HDPs work by means of either antimicrobial or by

immunomodulatory functions, or both [7,8]. Briefly, they could directly kill bacteria by compromising bacterial membranes [9]. Meanwhile, HDPs also utilize their immunomodulatory functions to regulate the release of immune cells that affect innate immunization against bacterial infections. For example, monocytes, lymphocytes and granulocytes all activate immune cells to control the rate of inflammation and improve the killing and removal of foreign bodies from the host after infection [10–12]. Although they are diverse in their three-dimensional structures, HDPs generally are short cationic sequences that make up of about no more than fifty amino acids and have both hydrophobic and hydrophilic faces in their structures. Their positive charge allows for selectivity toward bacteria which are all virtually negatively charged on their outer membrane leaflet, whereas the hydrophobic patches of HDPs are critical for penetration and disruption of the bacterial cell membranes [13–15]. Due to their low propensity to develop resistance as they lack specific targets on the bacterial membranes, their high selectivity for bacterial cells over mammalian cells, and their antimicrobial activity for a broad range of bacteria, significant efforts have been devoted to the development of HDPs and their mimics [16,17].

Recently our group has designed a class of peptidomimetics termed α -AApeptides (Fig. 1) whose backbone are derived from α chiral peptide nucleic acids (PNA) [18,19]. The design has been shown to be advantageous as it exhibits enhanced proteolytic

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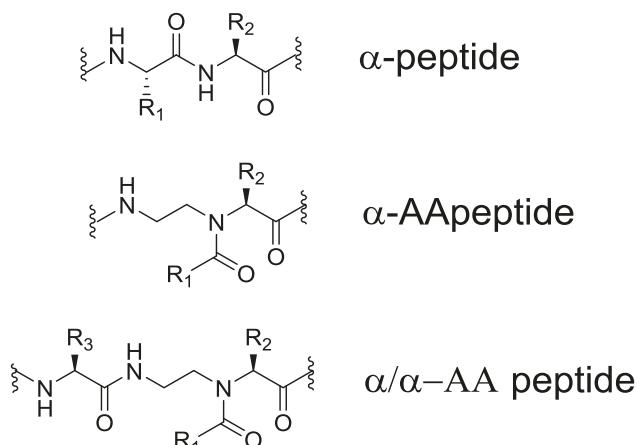


Fig. 1. General structure of an α -peptide, an α -AA peptide, and an α/α -AA heterogenous peptide.

stability, bioavailability, and chemodiversity as the side chains can be diversified with a wide variety of unnatural functional groups to allow design adapted to different circumstances. Our previous studies show that α -AApeptides could be potential antimicrobial agents [20], and their activity could have improved antimicrobial activity upon adding a lipid tail to the building block sequence [21,22]. We reasoned because adding a lipid tail to the sequence makes it more hydrophobic it increases its ability to interact with the lipid structures of the bacterial cell membranes [21]. In addition, we recently show that hybrid peptides could be potent and selective antimicrobial agents [23]. As such, it is envisioned that a new class of lipidated hybrid peptides consisting of both α -AApeptide building blocks and canonical amino acids could yield potent antibiotic agents with simple structures, and thereby more promise of future development for practical use in therapeutics.

2. Results and discussion

Building upon our previous findings [23], our design was aimed for molecular simplicity and strong membrane reactivity. To this end, lysine residue was included to give an overall positive charge, which is expected to attract the sequence to the surface of negatively charged bacterial membranes. A lipid tail was employed to endow hydrophobicity, facilitating sequence insertion into membranes. Furthermore, one or more amphiphilic α -AApeptide building blocks containing a positively charged side chain and a hydrophobic side chain were introduced to fine tune its membrane activity and selectivity (Scheme 1). All the sequences were synthesized on the solid phase using standard solid phase peptide synthesis (SPPS) protocol (Scheme 1 please see supporting information for details) [24], and their structures are shown in Fig. 2.

These sequences were then tested for their antimicrobial activity against five reprehensive strains (Table 1): Gram-negative bacteria *Escherichia coli*, *K. pneumonia* and Gram-positive bacteria Methicillin resistant *Staphylococcus aureus* (MRSA), Methicillin Resistant *S. epidermidis*, and Vancomycin resistant *E. faecalis* [25,26]. We first examined the activity of sequences containing lysine residue, a lipid tail and one α -AA building block ($n = 1$). Interestingly, sequences containing C6, C8, C10 and C12 lipid tails did not show any activity under tested conditions. **C₁₄-K-BB₁**, bearing the C14 lipid tail, started to exhibit weak activity against MRSA and *E. Coli*. With the longer C16 lipid tail, **C₁₆-K-BB₁**

displayed broad spectrum activity against all the tested Gram-positive and Gram-negative strains. The results are indeed consistent to our previous findings that long lipid tails are needed to endow sequences with sufficient hydrophobicity to penetrate lipid bilayers in bacteria. However, to our surprise, **C₁₆-K-BB₁** was highly hemolytic, probably due to its high hydrophobic content.

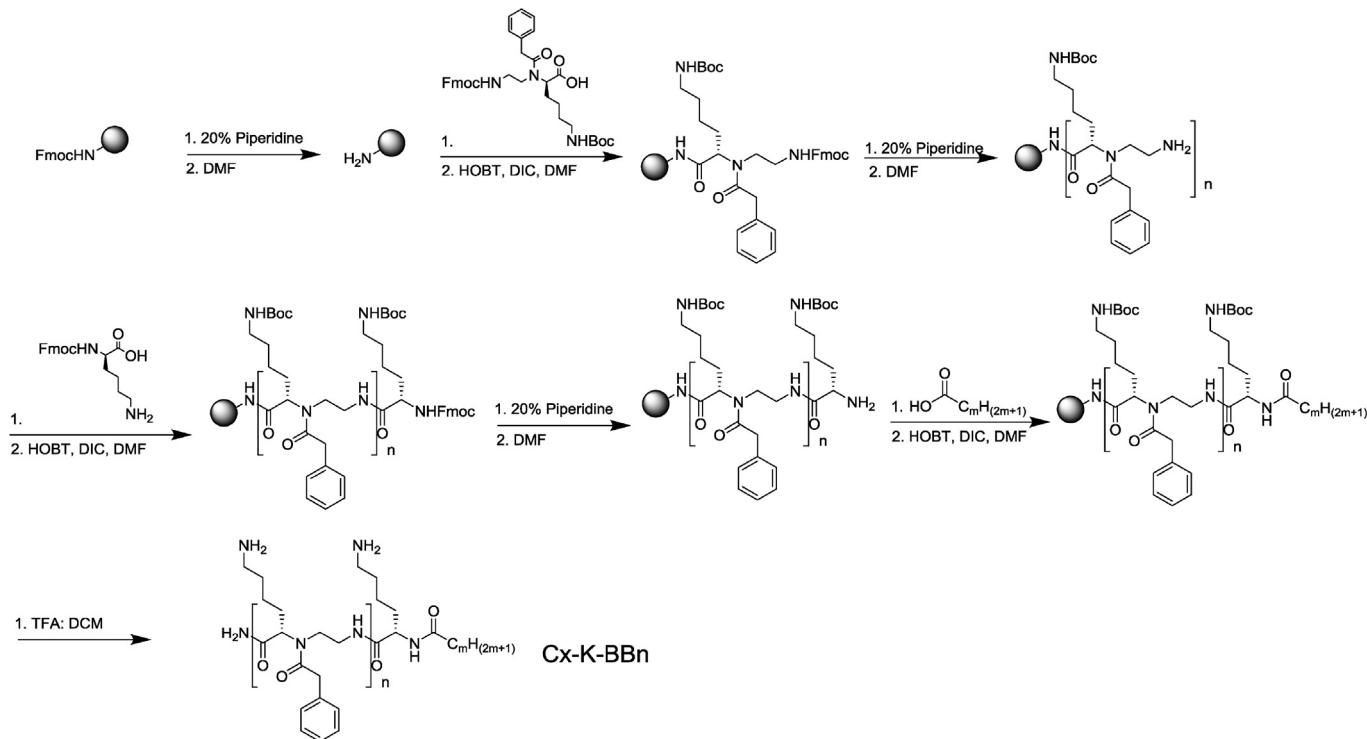
To obtain more potent antimicrobial agents with enhanced selectivity, we introduced more α -AA building blocks ($n = 2, 3; m = 15$). Unfortunately, **C₁₆-K-BB₂**, only showed good activity against *MRSA* and *E. Coli*, but not other stains. Meanwhile, it was still highly hemolytic. However, it was intriguing that **C₁₆-K-BB₃** showed excellent antimicrobial activity towards all tested strains except for *K. pneumoniae*. In addition, it exhibited high selectivity toward *MRSA* over mammalian cells. This demonstrated that proper ratio of hydrophobic groups and positively charged groups could lead to potent and selective antimicrobial α/α -AA hybrid. We next examined the impact of backbone on the antimicrobial activity. As seen in Scheme 1 and Table 1, **C₁₆-BB₁**, which does not contain the lysine residue, lost all the activity compared to **C₁₆-K-BB₁**. Whereas the other sequence **C₁₆-K-F-K**, bearing purely canonical amino acid residue but with similar hydrophobicity and hydrophilicity, also did not show any antimicrobial activity. The results suggested that α/α -AA hybrid backbone are mandatory and unique for antimicrobial development.

We then asked whether the position of the positive charge and the hydrophobic side chains would influence the antimicrobial activity of the compounds. Thus, we synthesized another three sequences bearing a C16 lipid tail, a lysine residue and one or more amphiphilic α -AA building blocks with reversed side chains (Scheme 2) compared to structures in Fig. 2. Surprisingly, all three sequences exhibited broad-spectrum activity against both Gram-positive and Gram-negative bacteria. Among them, **C₁₆-K-RBB₁** is the most potent one which showed excellent activity even toward *K. pneumoniae* whereas displayed limited cell toxicity. Although **C₁₆-K-RBB₂** was not active toward *K. pneumoniae*, its high selectivity toward other bacterial strains including *MRSA* presents itself as a very promising antimicrobial agent. Our results suggest that not only the ratio of hydrophobicity and cationic charge is important for HDP mimetics, the three-dimensional distribution of hydrophobic and cationic groups is also highly critical for bacterial killing. Therefore, based on our above-mentioned findings, both **C₁₆-K-RBB₁** and **C₁₆-K-RBB₂** were chosen for the subsequent mechanistic studies (see Table 2).

As **C₁₆-K-RBB₂** having the best activity and selectivity toward *E. coli* and *MRSA*, we set out to explore its mechanism of action to kill bacterial cells using fluorescence microscopy (Fig. 3). DAPI was used to stain both live and dead bacterial cells while PI was used to stain the dead cells only [27,28]. Upon treatment of *E. coli* and *MRSA* with **C₁₆-K-RBB₂** for 2 h, the bacterial cells showed red fluorescence in the PI channel, indicating that the compound killed the bacterial cells by damaging their membranes.

The Time kill assay was subsequently conducted using the most potent compound **C₁₆-K-RBB₁** to assess bacterial killing kinetics for this class of compounds. As shown in Fig. 4A, it took 120 min for 12.5 $\mu\text{g/mL}$ of **C₁₆-K-RBB₁** to clear the bacteria *E. Coli* while it just took 60 min for 25 $\mu\text{g/mL}$ and 50 $\mu\text{g/mL}$ **C₁₆-K-RBB₁** to eradicate the bacteria. In the case of *MRSA* (Fig. 4B) the compound took 60 min for 12.5 $\mu\text{g/mL}$ and 25 $\mu\text{g/mL}$ whereas only 10 min for 50 $\mu\text{g/mL}$ to completely get rid of the bacteria. The results demonstrated that the compounds could rapidly eradicate bacterial cells with bactericidal function.

One of the challenges faced when treating bacterial infections is their ability to develop biofilm. Bacterial biofilms are formed by the ability of bacteria to irreversibly bind to a surface and form a matrix that is made up of extra-polymer substances. They are complex as



Scheme 1. Reaction scheme for solid phase synthesis of lipidated α/α -AA peptide hybrids.

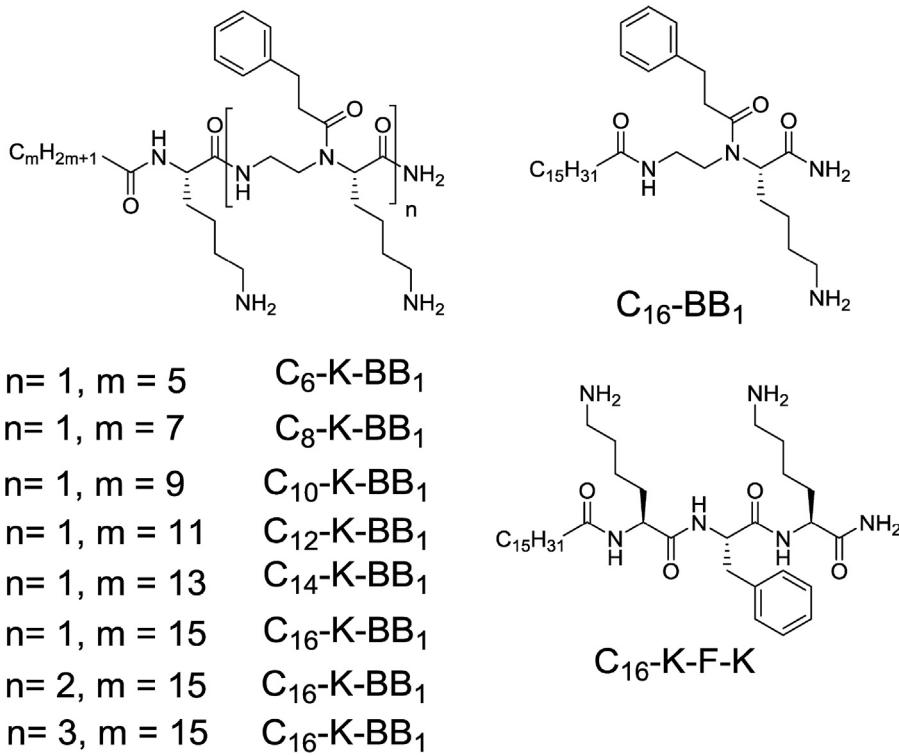


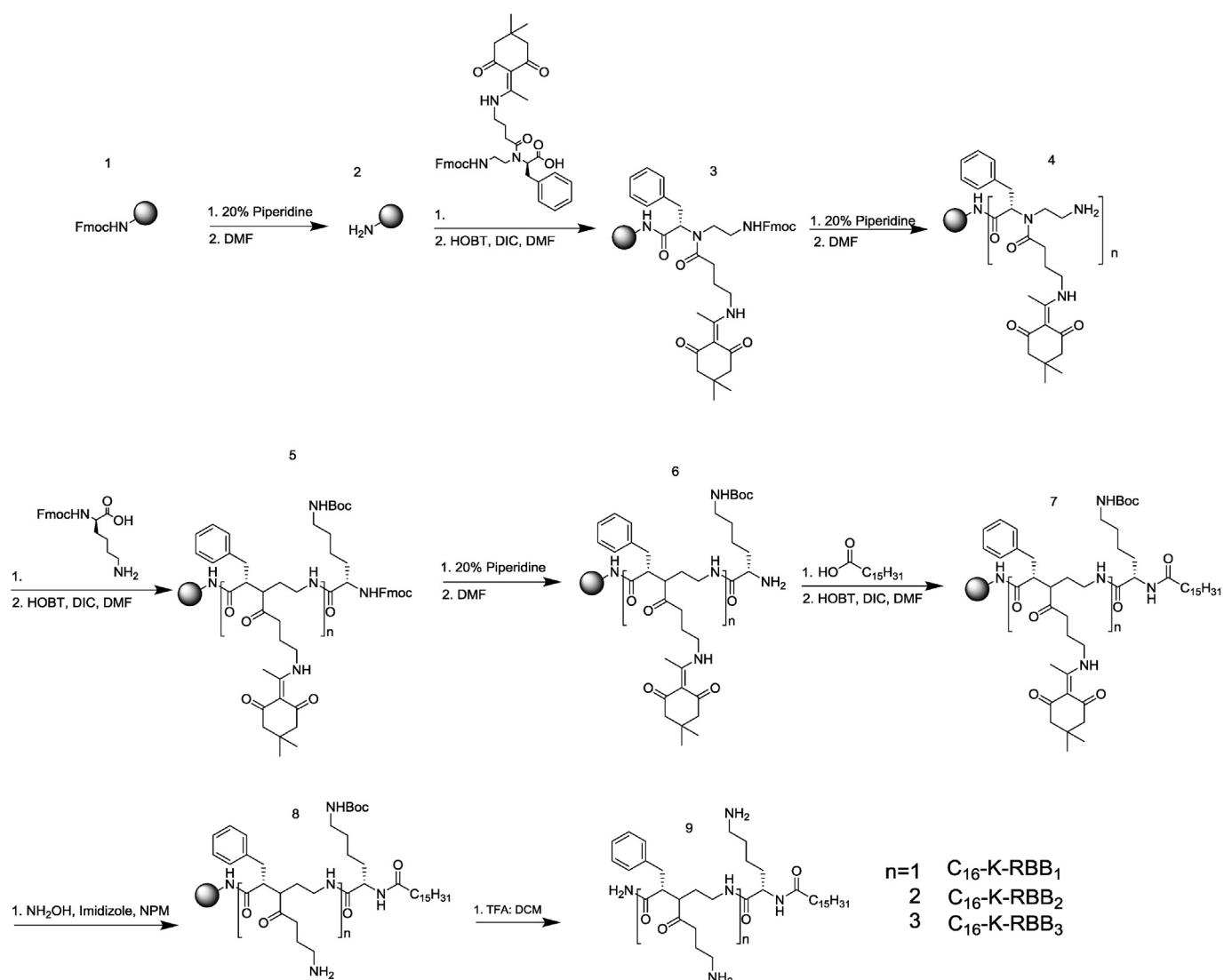
Fig. 2. Structures of sequences synthesized in Scheme 1, with varying lengths of hydrocarbon tail.

they use the environment that they are attached to develop channels that allow for the maintenance of the bacteria that are within the biofilm [29,30]. As such, they are harder to be eradicated compared to planktonic bacterial cells. Due to the ability of bacteria

to bind surfaces such as medical devices and sites of infection, there is an urgent need for the identification of new antimicrobial agents that could inhibit the growth of bacterial biofilms. We thus evaluated the lead compound **C₁₆-K-RBB₂** for its ability to decrease

Table 1The antimicrobial activity and selectivity of α/α -AA peptide hybrid shown in Fig. 2. “—” indicates the activity was not tested.

Compound	MIC ($\mu\text{g/mL}$)		Hemolytic activity HC_{50} ($\mu\text{g/mL}$)				Selectivity ($\text{HC}_{50}/\text{MIC}$ of MRSA)
	Gram +	Gram -	MRSA	<i>E. faecalis</i>	<i>E. coli</i>	<i>K. pneumoniae</i>	
C₆-K-BB₁	>25	>25	>25	>25	>25	—	—
C₈-K-BB₁	>25	>25	>25	>25	>25	—	—
C₁₀-K-BB₁	>25	>25	>25	>25	>25	—	—
C₁₂-K-BB₁	>25	>25	>25	>25	>25	—	—
C₁₄-K-BB₁	12–25	>25	>25	6–12	>25	—	—
C₁₆-BB₁	25	>25	>25	>25	>25	—	—
C₁₆-K-BB₁	1.5–3	3–6	6–12	1.5–3	6–12	31.25	10.4
C₁₆-K-BB₂	<3	>25	>25	1.5–3	>25	31.25	10.4
C₁₆-K-BB₃	1.5–3	3–6	6–12	1.5–3	>25	250	83
C₁₆-K-F-K	>25	>25	>25	>25	>25	—	—

**Scheme 2.** Reaction Scheme for solid phase synthesis of the reverse antimicrobial sequences.

biofilm formation. Intriguingly, this compound was able to significantly reduce biofilm formation in both Gram-negative *E. Coli* (Fig. 5A) and Gram-positive *MRSA* (Fig. 5B) bacteria. The results further strength the potential of lipidated α/α -AA peptide hybrid as a new class of antimicrobial agents.

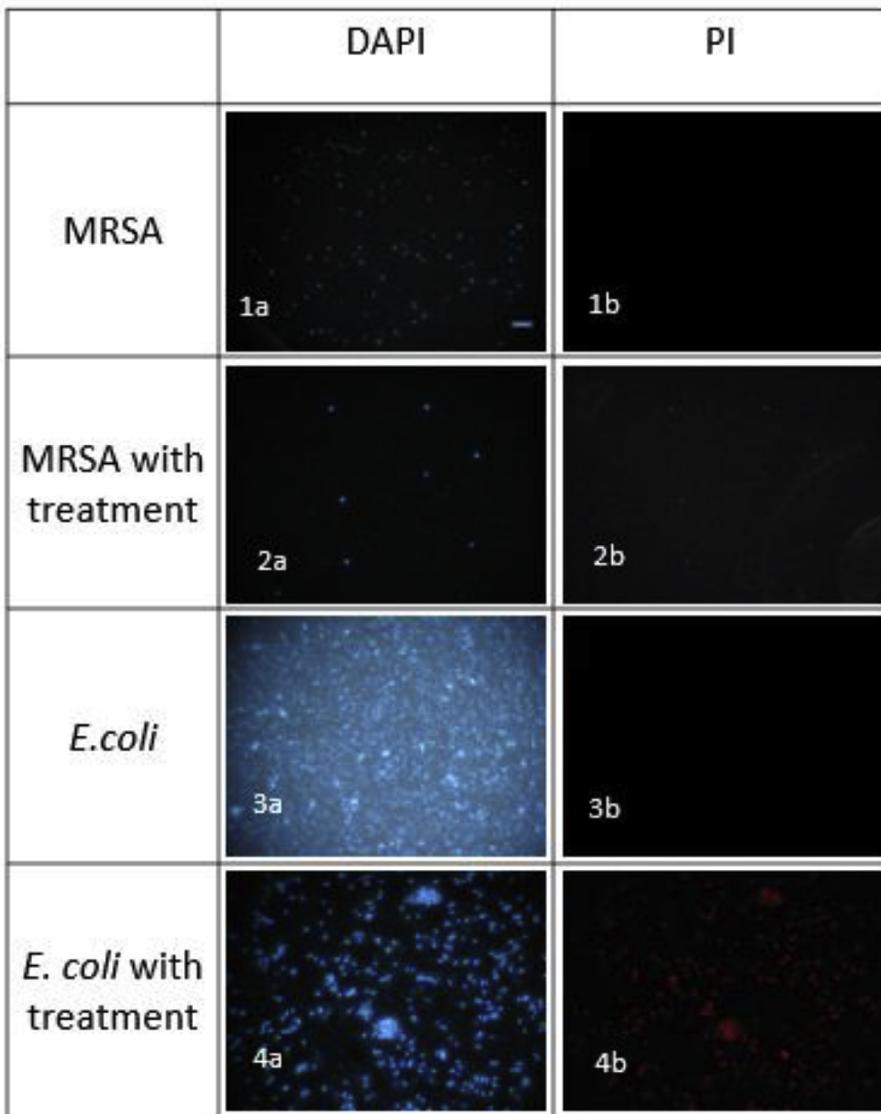
3. Experimental procedure

3.1. General information

Sequences were synthesized using solid phase peptide

Table 2The antimicrobial activity and selectivity of α/α -AA peptide hybrid shown in [Scheme 2](#).

Compound	MIC ($\mu\text{g/mL}$)			Hemolytic activity HC_{50} ($\mu\text{g/mL}$)		Selectivity ($\text{HC}_{50}/\text{MIC}$ of MRSA)	
	Gram +			Gram -			
	MRSA	MRSE	<i>E. faecalis</i>	<i>E. coli</i>	<i>K. pneumonia</i>		
C₁₆-K-RBB₁	1.5	1.5	3	3	3	125	
C₁₆-K-RBB₂	1.5	1.5	3	3	>25	>250	
C₁₆-K-RBB₃	3–6	3–6	6–12	6–12	>25	>42	

**Fig. 3.** Fluorescence Microscopy of bacterial cells treated with C₁₆-K-RBB₂ for 2 h (Scale bar 10 μm). (1a) Control, DAPI stained, no treatment with compound. (1b) Control, PI stained, no treatment with compound. (2a) MRSA, DAPI stained, treated with compound. (2b) MRSA, PI stained, treated with compound. (3a) Control, DAPI stained, no treatment with compound. (3b) Control, PI stained, no treatment with compound. (4a) *E. coli*, DAPI stained, treated with compound. (4b) *E. coli*, PI stained, treated with compound.

synthesis. To do this Rink amide MBHA resin (100–200 mesh, 0.64 mmol/g) from Chem-Impex International, Inc was used. The α -AA peptides were synthesized by using a peptide reaction vessel clamped on a Burrell Wrist-Action Shaker and purified using High performance liquid chromatography (HPLC) equipped with a preparative C18 column (5 μm , 9 \times 250 mm). The molecular weight of the desired fraction was confirmed using MALDI-TOF (Bruker AutoFlex) mass spectrometer, and the product was dried on a lab-cono lyophilizer. All other chemicals were purchased for either

Sigma Aldrich or Fischer unless otherwise stated.

3.2. Solid phase synthesis [24]

The solid phase peptide synthesis was conducted by using 100 mg of Rink amide MBHA resin in a peptide reaction vessel on a shaker. The resin was first treated with 20% piperidine in DMF for 15 min to remove Fmoc group. This step was repeated twice and after each time the beads were washed with 3 mL DMF and DCM

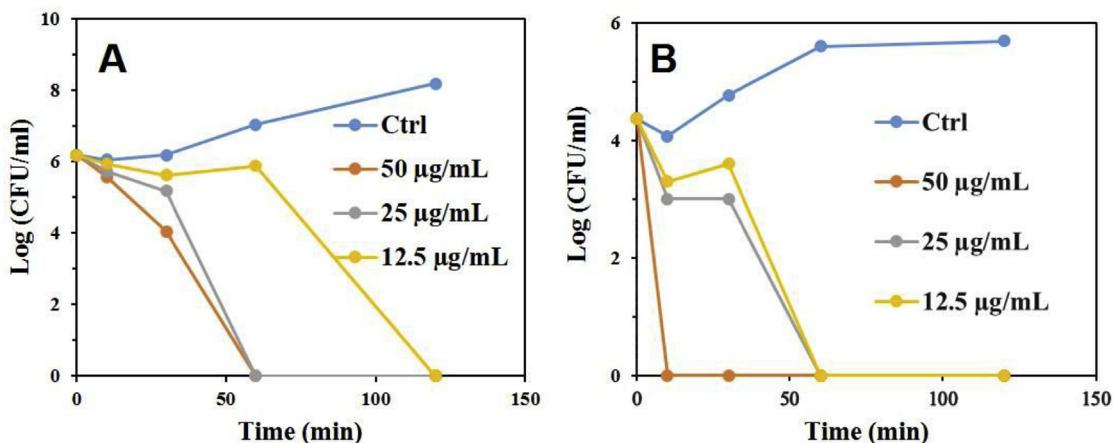


Fig. 4. Time kill assay for gram-negative *E. coli* (A) and MRSAs (B).

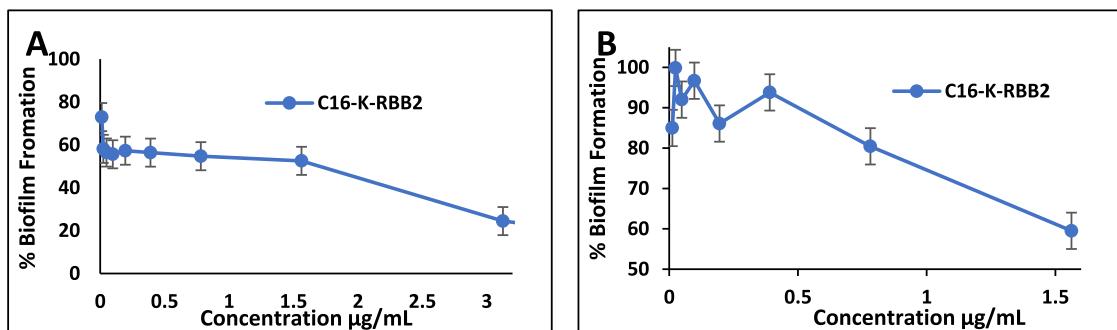


Fig. 5. Biofilm prevention assay for cells treated with C₁₆-K-RBB₂. Error bars based on standard deviation. *E. coli* (A) and MRSAs (B).

alternatively. Subsequently, α -AA build block (2 equiv.), HOBT (6 equiv.) and DIC (6 equiv.) were dissolved in DMF and shaken for 10 min, and the mixture was added to the vessel and left to shake overnight. The beads were washed with DMF and DCM. After Fmoc group was removed, either one more α -AA building block, or the lysine amino acid, or a lipid tail, was coupled onto the beads, respectively depending on the sequences to be synthesized. The synthetic cycle was continued until the desired sequence was completed. The sequence was then cleaved from the beads using a 1:1 ratio of TFA: DCM for 2 h. The solution was collected, dried over the air, and the residue was dissolved in water, which was subsequently purified by HPLC.

3.3. Minimum inhibition concentration (MIC) – antimicrobial assay

[22] MIC is used to determine the minimum compound needed to inhibit both Gram-positive and Gram-negative bacteria. In the case of this experiment *Escherichia coli* (*E. coli*, JM109), and *Klebsiella pneumoniae* (*K. pneumoniae*, ATCC 13383) were used as the Gram-negative specimen, while Methicillin Resistant *Staphylococcus aureus* (MRSA, ATCC 33591), Methicillin Resistant *Staphylococcus epidermidis* (MRSE, RP62A), and Vancomycin Resistant Enterococcus *faecalis* (*E. faecalis*, ATCC 700802) were used as the Gram-positive specimen. To test the inhibition properties, one colony of bacteria was first taken from an agar plate and grown overnight in trypticase soy broth (TBS) media at 37 °C on a shaker. Subsequently, one colony was taken again and allowed to grow to mid-logarithm phase. Of this, 50 µL of 10⁶ CFU/mL bacteria was

added to each well of a 96 well plate along with 50 µL of diluted lipidated α/α -Apeptides (100–0.5 µg/mL). After 18 h of incubation at 37 °C the MIC was determined by using a Biotek microplate reader with optical density of 600 nm. The lowest concentration of peptides to inhibit bacterial growth was considered as the MIC. All experiments were done three times, each time being duplicates. The control was the bacteria not treated with any peptides.

3.4. Hemolytic studies

[26] Freshly obtained human red blood cells were used for measurement of hemolytic activity of compounds. The cells were washed a few times with phosphate buffered saline (PBS) and centrifuged at 1000 g for 10 min. The supernatant was then discarded and the red blood cells were resuspended in PBS to obtain a 5% volume by volume concentration. A 96 well plate was then used to contain a two-fold dilution of C₁₆-K-RBB₂ to make a total of 50 µL in each well. To which 50 µL of 5% volume by volume of red blood cells solution was added. The plate was then stored at for 1 h at 37 °C and centrifuged for 10 min at 3500 rpm. The supernatant was collected and diluted with 100 µL of PBS to detect the hemoglobin using a Biotek microtiter plate reader (Synergy HT) at 540 nm. Controls were used to determine 0% hemolysis by incubation in PBS and 100% hemolysis by treatment with 0.1% Triton-X-100 in PBS. The percent hemolysis was determined by using the formula:

$$\% \text{ Hemolysis} = (\text{Abs}_{\text{sample}} - \text{Abs}_{\text{PBS}}) / (\text{Abs}_{\text{Triton}} - \text{Abs}_{\text{PBS}}) \times 100$$

3.5. Fluorescence microscopy

[25] The double dyes staining method, using 4',6 diamidino-2-phenylindole dihydrochloride (DAPI, Sigma, >98%) and propidium iodide (PI, Sigma), was employed to assess bacterial membrane integrity. DAPI is a cell permeable dye and has a great ability to bind to the A-T rich regions of double stranded DNA which allows for very strong fluorescence [28]. PI on the other hand is an ethidium derivative that are able to penetrate the cell walls of dead cells but not living cells. Therefore combination of both DAPI and PI were used to quantify the bacterial cells that became necrotic due to treatment with **C₁₆-K-RBB₂** [27]. These stains were used to determine the viability of both *E. coli* and MRSA. The bacterial cells were cultured to the mid-logarithmic phase, and then incubated for 4 h at 37 °C with **C₁₆-K-RBB₂** (5 µg/mL). They were then centrifuged at 3000 g for 15 min and the supernatant was removed. The pellets that were collected were washed with PBS then incubated with DAPI (10 µg/mL) for 15 min at 0 °C in the dark. After 15 min the excess DAPI was washed a few times with 1 × PBS. The pellets were also treated with PI (5 µg/mL) and incubated at 0 °C in the dark for 15 min, after which the excess PI was washed off with 1 × PBS. The controls were tested using the same method stated above, however, the cells were not incubated with **C₁₆-K-RBB₂**. The cells were then observed using a Zeiss Axio Imager Z1 optical microscope (100 ×).

3.6. Time kill studies

[26] This assay was done in triplicates to determine the kinetics of the peptide **C₁₆-K-RBB₁** as it relates to termination of bacteria. To determine this, the bacteria were incubated at 0, 10, 20, 30, 60 and 120 min with various concentrations of the peptide. 100 µL of bacterial suspension was then taken from each time interval and further diluted. The diluted suspensions were incubated at 37 °C on agar plates for 20 h and the colonies counted.

3.7. Biofilm assay

[6] This assay was done in duplicates to determine the prevention of biofilm growth. MRSA and *E. coli*, were grown to 1 × 10⁶ CFU/mL in a 96 well plate containing 50 µL trypticase soy media, and 2:1 serial dilution of tested compound in 50 µL of PBS. The plate was then incubated at 37 °C for 24 h and then 10 µL of crystal violet stain was added to each well at a concentration of 0.5% weight by volume. The plates were then washed with TBS to get rid of any cells that did not stick to the plate and 95% ethanol was added to the plates so as to dissolve the stain. A microtiter plate reader was then used to determine the formation of biofilm by taking readings at a wave length of 600 nm. The lesser the optical density at 600 nm is equivalent to a decrease in biofilm formation.

4. Conclusion

In summary, we have developed a new class of lipidated α/α -AA heterogeneous peptides. They show excellent activity against both Gram-positive and Gram-negative bacteria, including clinically relevant bacterial strains. Our structure activity relationship studies suggest that lipidation, hydrophobicity, and positive charge, as well as their spatial arrangement, are of importance to selectively target bacterial cells over mammalian cells. Furthermore, our mechanistic studies show that the compounds could mimic HDPs and are capable of eradicating both Gram-positive and Gram-negative bacteria in a timely manner, while also eliminating the formation of biofilms. Therefore, lipidated α/α -AA heterogeneous peptides could be a new generation of antibiotic agents with potential therapeutic applications.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.ejmech.2018.06.006>.

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