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Short antimicrobial lipo- α/γ -AA hybrid peptides

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Abstract

The last two decades have seen the rise of antimicrobial peptides (AMPs) to combat emerging antibiotic resistance. Herein we report the solid phase synthesis of short lipidated α/γ -AA hybrid peptides. This family of lipo-chimeric peptidomimetics displays potent and broad-spectrum antimicrobial activity against a range of multi-drug resistant Gram-positive bacteria and Gram-negative bacteria. These lipo- α/γ -AA hybrid peptides also demonstrate high biological specificity, with no hemolytic activity towards red blood cells. Fluorescence microscopy suggests that these lipo- α/γ -AA chimeric peptides can mimic the mode of action of AMPs and kill bacterial pathogens via membrane disintegration. As the composition of these chimeric peptides is simple, therapeutic development may be economically feasible, and amenable for a variety of antimicrobial applications.

Introduction

The omnipresent threat of emerging antibiotic resistance is a serious public health concern. ^[1] World Health Organization (WHO) has recognized that antibiotic resistant pathogens including *E. coli*, *K. pneumoniae* and *S. aureus* as the biggest threats as most conventional antibiotics abolish their efficacy towards these strains. ^[2] Natural cationic antimicrobial peptides (AMPs) have attracted considerable interest due to their ability to kill pathogens by disrupting bacterial membranes. ^[3] AMPs perform their lethal function through hydrophobic interaction, a mechanism that is rooted in the physical properties of the bacteria. ^[4] As this approach lacks a specific cellular target, there is a decreased probability of eliciting antibiotic resistance. ^[5] Meanwhile, AMP selectivity for pathogens is high due to the increased negative charge on bacterial outer lipid membrane leaflets compared to those of mammalian cells. These characteristics have led to rising interest in the development of AMPs and their analogs as novel agents to combat antibiotic resistance. However, AMPs also possess intrinsic drawbacks, including susceptibility to proteolytic degradation, poor

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selectivity, and low-to-moderate activity. To address these issues, one strategy is to develop peptidomimetics that mimic the mechanism of action of AMPs. To date, a range of peptidomimetics have been explored for their antimicrobial activity, including peptoids,^[6] β -peptides,^[7] peptoid-peptide hybrids,^[8] arylamides.^[9] However, most antimicrobial peptidomimetics have fairly large molecular weights (>1000 Da), with structural complexity and the requisite multi-step synthesis posing a challenge for production. As such, a number of groups have started to develop antimicrobial peptidomimetics,^[10] which are smaller in size while still retaining specificity, potency, and broad-spectrum activity.

We have recently developed a new class of peptidomimetics termed “ γ -AApeptides”, as they are oligomers of N-acylated-N-aminoethyl amino acids (Figure 1).^[11] This atypical AApeptide backbone has been shown to be highly resistant to proteolytic degradation, and the variety of possible side chain acylating agents makes for nigh limitless chemical diversity.^[11–12] These features make γ -AApeptides promising peptidomimetics for paralleling the structure and function of bioactive AMPs.^[12a, 13] Indeed, a variety of γ -AApeptides have been developed by our group to mimic the global amphipathic structure of AMPs, and these molecules display potent and broad-spectrum antimicrobial activity against an array of antibiotic resistant Gram-positive and Gram-negative bacteria.^[1, 5, 14] However, most lead γ -AApeptides are long sequences and thus require a number of steps to synthesize. We assert that if short antimicrobial γ -AApeptides can be developed, this would significantly enhance their potential for application.

In order to generate short antimicrobial peptides, we utilized a hybrid backbone of canonical α peptide units fused with γ -AApeptides. Previous work has also shown that lipidation on short cationic peptides or peptoids results in improved activity.^[10a, 15] Increased lipophilicity of molecules with the addition of a hydrophobic tail enhances the interaction between peptides and bacterial membranes. As such, short sequences could become active with the addition of a hydrophobic lipid tail. Moreover, a few research groups have reported the study of peptidomimetic- α hybrid peptidic oligomers for their antimicrobial activity, which revealed unexpected potency and low hemolytic activity.^{[8] [16]} Although the antimicrobial mechanism remains elusive, these results suggest that short lipidated peptidomimetic- α hybrid peptidic oligomers may be active and selective against bacteria.

Results and Discussion

To test our hypothesis that short lipidated peptidomimetic- α hybrid peptidic oligomers may serve as antimicrobials, we synthesized a focused library adapted from our previously reported solid-phase synthesis protocol.^[12b, 14a, 14c] These chimeric peptides contain one or two cationic γ -AApeptide building blocks in addition to one lysine amino acid residue (Figure 2). In addition, each cationic γ -AApeptide building block is composed of either two cationic charges, or one cationic group and one hydrophobic group. As such, their global amphipathic structures mimic those of AMPs. To investigate the impact of hydrophobicity on activity, one or two C16 lipid tails were introduced to the N-terminus or both α - and ϵ -NH₂ groups in the lysine residue. The antimicrobial activity and hemolytic activity of each hybrid peptide were obtained and listed in Table 1.

Demonstrating the efficacy of short peptidomimetics, **1** shows potent activity against an array of pathogens, despite containing just one γ -AApeptide building block and one lysine residue. Critically, **1** is highly selective for various bacteria as it does not exhibit discernable hemolytic activity up to 200 $\mu\text{g/mL}$, and the HC_{50} is greater than 500 $\mu\text{g/mL}$. As a comparison, **1** is superior to the existing drug candidate Pexiganan, an analog of AMP Magainin, in terms of both activity and hemolytic activity. It also attains improved selectivity over previously developed lead lipo- γ -AApeptide **6**.^[14c] When compared to existing antimicrobial drugs, **1** also benefits from a simplified synthetic scheme. These results demonstrate that α/γ -AA hybrid peptides are a class of promising antibiotic agents. Similar to other classes of antimicrobial peptidomimetics, the antimicrobial activity and hemolytic activity of these hybrid peptides can be tuned via the manipulation of charge and hydrophobicity. For example, the selectivity of α/γ -AA hybrid peptide **1** can be further enhanced after another hydrophilic γ -AApeptide building block is included, as seen with **3**. Table 1 shows that with a decreased ratio of hydrophobicity to hydrophilicity, **3** maintains antimicrobial activity, without showing any hemolytic activity up to 350 $\mu\text{g/mL}$. There is an additional trend correlating increases in hydrophobicity to enhancement of antimicrobial activity, as observed with **5**, **7**, **9**, and **11**. These compounds with elevated hydrophobicity have demonstrated improved antimicrobial activity compared to **1** and **3**. Both aromatic phenyl groups and aliphatic isopropyl groups appear to induce similar hydrophobicity, as evidenced by their similar activity. This observation is consistent with the hypothesis that increased hydrophobicity leads to increased antimicrobial activity, though at the price of compromising the selectivity. Indeed, we observe that the more hydrophobic **5**, **7**, **9**, and **11** are more hemolytic than **1** and **3**.

While hydrophobicity appears to correlate with antimicrobial activity, most hybrid peptides containing two C16 alkyl tails are not active against any bacteria. For instance, while peptides **2** and **4** are di-alkyl versions of **1** and **3**, antimicrobial activity is completely abolished. This same phenomenon was also observed for peptides **6**, **8** and **12**. Although **10** displays good activity against limited bacterial strains, its activity is still not as broad-spectrum as mono-alkylated peptides. Initially, these results may appear counterintuitive, since di-alkylation significantly increases the overall hydrophobicity of molecules and so is expected to improve the interactions between hybrid peptides and bacterial membranes. However, it is widely accepted that in order to maximize interactions with bacterial membranes, specific orientations of the peptide backbones are required.^[3a, 18] Since di-alkylated hybrid peptides are very hydrophobic, it is possible that they form stable micelle structures in an aqueous environment.^[19] This strong hydrophobic interaction may prevent micelle dissociation even while interacting with bacterial membranes. As a result, we hypothesize that these peptides are only weakly capable of penetrating bacterial membranes. We believe this result may shed light on the design of future lipo-peptidic antimicrobial agents.

As these lipo- α/γ -AA hybrid peptides were designed to mimic membrane-active AMPs capable of inducing rapid bacterial membrane permeabilization, their bactericidal action should result from a rapid and irreversible disruption of bacterial membranes.^[17b] To this end, we investigated the ability of lipo- α/γ -AA hybrid peptides **3**, **4** and **5** to kill both the

Gram-positive bacteria *MRSA* and the Gram-negative bacteria *E. coli*. Compound **3** was tested at three, six, and twelve times the MIC to generate a dose-response curve. After treatment for up to two hours, cell viability was determined by colony count in agar plates. As shown in Figure 3-a and 3-b, both *MRSA* and *E. coli* were killed in a dose-dependent fashion. In two hours, 25 µg/mL and 50 µg/mL of **3** could completely eradicate bacteria greater than 10⁵ CFU/mL. At 50 µg/mL, **3** eradicated both bacteria completely in just one hour. The data also suggest that *E. coli* is more susceptible to **3** than *MRSA*, since 25 µg/mL of **3** completely abolished *E. coli* in 1 h, and 12.5 µg/mL of **3** reduced *E. coli* survival by 99.9%. Overall, these results show that **3** exerts its bactericidal activity rapidly, mimicking AMP behavior. Similarly to other reported AMP analogues,^[17b] the bactericidal action of **3** is concentration dependent against the exposed strains of bacteria. Compound **4** did not show antimicrobial activity under the tested experiment condition, and as expected, it does have the ability to kill bacteria in time-killing studies (Figure 4-a and 4-b). Compared to **3**, compound **5** display even more efficiency in *MRSA* killing (Figure 5-a), albeit it is less effective in *E. coli* killing (Figure 5-b).

Our results suggest that short lipo-α/γ-AA hybrid peptides mimic AMPs in activity, selectivity, and global amphipathic structures. As such, we further hypothesized that they mimic AMPs by causing membrane disruption to kill bacterial pathogens. To test this hypothesis, fluorescence microscopy studies were carried out to study the impact of peptides **3**, **4** and **5** on the membranes of *S. aureus* (Gram-positive bacterium) and *E. coli* (Gram-negative bacterium). Both bacteria were stained with the dyes 4',6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) (Figure 4). DAPI is a membrane permeable dye that can cross membranes regardless of cell viability, however PI can only stain cells when cell membranes are damaged. As shown in Figure 4, treatment of bacteria with peptide **3** resulted in visibility of red-fluorescent bacteria under PI channel, suggesting the membranes of both *S. aureus* and *E. coli* were damaged. Consistent with our previous results and the established literature, treatment with **3** led to significant aggregation of *S. aureus*, which is believed to arise from the dissipation of membrane potential due to membrane disruption.^[1, 14, 20] Peptide **5** also exhibited very similar behavior to **3** under the same experiment condition, suggesting their antimicrobial mechanism are analogous (Figure S3). However, the inactive peptide **4**, consistent to its weak antimicrobial activity, could not compromise the integrity of bacterial membranes (Figure S4).

Conclusion

In summary, we have developed and identified short lipo-α/γ-AA hybrid peptides that display potent and broad-spectrum activity against medically relevant Gram-positive and Gram-negative bacteria. Compared to previously developed lipo-γ-AApeptides, these short lipo-α/γ-AA chimeric peptides retain activity while benefiting from a more economical synthetic process. Additionally, the lead compounds are more selective for bacteria and show little hemolytic toxicity under the tested conditions. The lipo-α/γ-AA hybrid peptides mimic AMPs by rapidly permeating and damaging bacterial membranes, and kill bacteria rapidly. Due to their simplicity and high selectivity, lipo- α/γ-AA chimeric peptides may have great potential for development as a new class of antibiotic agents to combat drug-

resistance. Additionally, although bacterial membrane disruption is the general mechanism of AMPs, they could have additional modes of actions, including outer and/or inner membrane permeabilization, binding to a membrane or intracellular target, etc).^[3a] Therefore, it is highly possible that lipo- α/γ -AA hybrid peptides could also these mode of actions in addition to bacterial membrane disruption.^[3a] As such, more comprehensive biological studies will be carried out to further probe their antimicrobial mechanisms.^[3a,4]

Experimental Section

General experimental methods

Rink amide MBHA resins (200–400 mesh, 0.7 mmol/g) were obtained from Chem-Impex Int'l Inc. Other chemicals were obtained from either Sigma-Aldrich or Fisher Scientific and used without further purification. The antimicrobial lipo- α/γ -AA hybrid peptides were synthesized in a peptide reaction vessel on a Burrell Wrist-Action shaker, and purified with a Waters HPLC system containing both analytical (C18, 3.5 μ m, 4.6 $\times$ 150 mm) and preparative (C18, 5 μ m, 19 $\times$ 250 mm) functions. HPLC fractions were collected and confirmed using a Bruker AutoFlex MALDI-TOF mass spectrometer. The desired products were dried in a Labcono lyophilizer.

Solid phase synthesis, purification and characterization of lipo- α/γ -AA hybrid peptides.^[14a-c]

Briefly, the lipo- α/γ -AA hybrid peptides sequences were synthesized following the standard solid phase peptide synthesis protocol. For every coupling step, 20% Piperidine in DMF was first used to remove the Fmoc protecting group, then 1.5 equivalent of building blocks/ N^{α} -Fmoc- N^{ϵ} -Boc-L-lysine/palmitic acid, 4 equivalent of HOBT (1-hydroxybenzotriazole monohydrate) and DIC (diisopropylcarbodiimide) in DMF were added to react for 4 h. The assembled sequences were cleaved from the amide resin in 50:48:2 TFA/DCM/TIS (triisopropylsilane) for 2 h. The solvent was removed, and the peptide sequences were purified on a Waters HPLC system monitored at 215 nm (Gradient: Solvent B (acetonitrile) increases to 100% over a 40 min period in Solvent A (water)). The potential fractions were collected and confirmed by Bruker AutoFlex MALDI-TOF mass spectrometer (matrix used: α -Cyano-4-hydroxycinnamic acid) before they were lyophilized.

Antimicrobial assays.^[14d]

To determine the antimicrobial activities of these lipo- α/γ -AA hybrid peptides, MICs (Minimal Inhibitory Concentrations) were measured against five different bacteria strains including Methicillin-resistant *S. aureus* (MRSA, ATCC 33592), Methicillin-resistant *S. epidermidis* (MRSE, RP62A), Vancomycin-resisitant *E. faecalis* (ATCC700802), multi-drug resistant *P. aeruginosa* (ATCC27853), and *E.coli* (ATCC 25922). The highest drug concentration used was 50 μ g/mL. First, a single colony of each bacteria strain was inoculated into 5 mL of Tryptic Soy Broth (TSB) medium to and allowed to grow to the mid-logarithmic phase, and then the bacteria culture was diluted to 1×10^6 CFU/ml suspension. Aliquots of 50 μ L of these bacteria suspension were added into 50 μ L of different concentrations of lipo-AApeptides diluted using the same TSB medium. The mixtures were incubated at 37 $^{\circ}$ C for 12–16 h, and the cell growth was monitored by a

Biotek Synergy HT microtiter plate reader at 600 nm wavelength. MICs are the lowest concentrations of the compounds which can inhibit the bacteria growth. Results were repeated three times with duplicates each time.

Hemolysis assays.^[14d]

The freshly drawn, K2EDTA treated human red blood cells (hRBCs) with PBS buffer was centrifuged at 1000g for 10 min. The procedure was repeated several times until the supernatant was clear. After the clear supernatant was removed, RBCs were diluted into 5% v/v suspension, and mix with serial diluted lipo- α/γ -AA hybrid peptides in a 96-well plate. The mixtures were incubated at 37 °C for 1 h and then centrifuged at 3500 rpm for 10 min. 30 μ L of the supernatant was transferred to 96-well plate and to which 70 μ L of PBS buffer was added. The absorbance was read at 360 nm using a Biotek Synergy TH plate reader. % hemolysis = $(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{PBS}}) / (\text{Abs}_{\text{Triton}} - \text{Abs}_{\text{PBS}}) \times 100\%$. 0% hemolysis (negative control) was determined by mixing blood with PBS and 100% hemolysis (positive control) was determined by mixing blood with Triton X-100 (final concentration 0.1%). The results were repeated at least three times with duplicates for each time.

Fluorescence microscopy.^[14d]

The bacterial membrane integrity of *E. coli* and *MRSA* was assessed by staining with two dyes: DAPI (4',6-diamidino-2-phenylindole dihydrochloride) and PI (propidium iodide), and visualized by fluorescence microscopy. PI can only pass through damaged membranes and therefore it only stains dead cells. However, DAPI can dye any bacterial cells regardless of the viabilities. **3**, **4** and **5** were studied, and the procedure of **3** is detailed below. Bacteria were grown to mid-logarithmic phase and incubated with the lipo- α/γ -AA hybrid peptide **3** at 10 μ g/mL for 2 h. The culture was then centrifuged at 5000g for 15 min. The pellets were washed with PBS, then incubated with PI (5 μ g/mL), followed by PBS washing, and then DAPI (10 μ g/mL) incubation. Each dye incubation was carried out in 15 min at 0 °C in dark. The controls were designated as the bacteria without AApeptides treatment. The stained bacteria were observed under Zeiss Axio Image Zloptical microscope using the 100X oil-immersion objective.

Time kill-Kinetics of bacteria killing for hybrid AApeptides against *MRSA* and *E.coli*.^[9]

MRSA and *E.coli* were grown to mid-logarithmic phase and dilute them into 10^6 CFU/ml suspensions. Incubate the diluted bacteria suspension with different concentrations of lipo- α/γ -AA hybrid peptide **3**, **4** and **5** for 10 min, 30 min, 1h and 2h respectively. After the incubation, the mixture was diluted 10^2 to 10^4 times and then spread on the TSB agar plates. The bacteria number was obtained by colony count after overnight incubation at 37 °C.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

The structure of α -peptides, γ -AApeptides, and α/γ -AA hybrid peptides. A γ -AApeptide is comparable to an α -peptide in unit length, and half of its side chains are introduced through tertiary amide linkage.

Figure 2.

The structures of lipitated α/γ-AA chimeric peptides (in the forms of TFA salts after HPLC). **γ6** is a previously prepared lipitated γ-AA peptide, which is included for comparison.^[14c] Peptides **1**, **3**, **5**, **7**, **9**, and **11** contain one C₁₆ tail, while **2**, **4**, **6**, **8**, **10**, and **12** contain two C₁₆ alkyl tails. Each peptide varies in the number and type of cationic or hydrophobic side chains present, while maintaining a globally amphipathic state.

Figure 3. Time-kill curves of **3**, **4** and **5** for *MRSA* (3-a, 4-a, 5-a) and *E. coli* (3-b, 4-b, 5-b), respectively. The killing activity of α/γ -AA hybrid peptides against those strains was monitored for the first 2 h. The concentrations used in the experiment were 12.5, 25 and 50 $\mu\text{g/mL}$.

Figure 4.

Fluorescence micrographs of *S. aureus* (**A**) and *E. coli* (**B**) that are treated or not treated with 10 $\mu\text{g/mL}$ lipidated α/γ -AA peptide **3** for 2 h. **a1**, control, no treatment, DAPI stained; **a2**, control, no treatment, PI stained; **a3**, control, no treatment, merged. **b1**, treatment with **3**, DAPI stained; **b2**, treatment with **3**, PI stained; **b3**, treatment with **3**, merged. Scale bar for MRSA is 1 μm for control and 2 μm for **3**, and for *E. coli* is 2 μm .

Table 1

The antimicrobial and hemolytic activities of lipo-α/γ-AA hybrid peptides. The microbial organisms used are *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), Methicillin-resistant *S. epidermidis* (MRSE) (RP62A), Vancomycin-resistant *E. faecalis* (ATCC 700802), and Methicillin-resistant *S. aureus* (MRSA) (ATCC 33591). The minimum inhibitory concentration (MIC) is the lowest concentration that completely inhibits microbial growth after 24 h. HC₁₀/HC₅₀ is the concentration causing 10% and 50% hemolysis, respectively. Pexiganan [17] and previously reported lipidated γ-AApeptide γ6^[14c] are included for comparison. Hemolysis activity was not measured for peptidomimetics that did not present antimicrobial activity.

Sequences	MIC (μg/mL)			Hemolysis (HC ₁₀ /HC ₅₀) (μg/mL)		
	MRSA (+)	MRSE (+)	<i>E. faecalis</i> (+)	<i>P. Aeruginosa</i> (-)	<i>E. coli</i> (-)	
1	4	4	5	2	5	200/>500
2	>50	>50	>50	>50	>50	-----
3	4	2	4	2	3	350/>500
4	>50	>50	>50	>50	>50	-----
5	2	2	2	2	4	40/150
6	>50	>50	>50	>50	>50	-----
7	2	2	2	2	4	40/250
8	>50	>50	>50	>50	>50	-----
9	2	2	2	2	2	30/100
10	2	2	2	3	25-50	50/400
11	2	2	2	2	4	45/350
12	>50	>50	>50	>50	>50	-----
γ6	4	4	5	5	3	60/500
Pexiganan	16	8	32	16	8	--/120