**Activity of lipo-cyclic γ -AApeptides against biofilms of *Staphylococcus epidermidis* and
*Pseudomonas aeruginosa***

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Abstract

Antibiotic resistant bacterial infection is currently a serious public concern. Their ability to form biofilms further complicates the treatment. Herein we investigated the activity of lipo-cyclic γ -AApeptides against both planktonic cells and biofilms of *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*, in comparison to those of the conventional antibiotic ciprofloxacin. Our results suggest that these lipo-cyclic γ -AApeptides exhibit comparable or enhanced performance compared to ciprofloxacin in the prevention of biofilm formation for both Gram-positive and Gram-negative bacteria, providing a potential alternative treatment and prevention for indwelling device-related infections.

Antibiotic resistant bacterial infections are currently a serious public concern. The ability of bacteria to form biofilms further complicates treatment. These biofilms, consist of extra cellular polymeric substance (EPS), render bacteria less susceptible to antimicrobial agents and the host immune response. The EPS, composed of a mix of polysaccharides, nucleic acids, and proteins, serves as a matrix to hold the cells together, and gives rise to matrix-encased multicellular aggregates that can escape engulfment by phagocytic cells. The biofilm, thus, contributes to chronic conditions making bacteria very difficult to eradicate.¹ Among them, the *P.*

aeruginosa (PA) biofilms are responsible for chronic cystic fibrosis (CF) and infections in immunocompromised patients.^{2,3} They are frequently identified in mucus plugs of CF patient lungs, catheters and even contact lens. Similarly, the *Staphylococcus epidermidis* (SE), is also notoriously known to form biofilms on surgical implants and is involved in catheter related urinary tract infections. The formation of *S. epidermidis* biofilms is attributed to their ability to produce polysaccharide intracellular adhesion consisting of β -1,6-linked glycosaminoglycans.⁴ Much effort has been devoted to studying the growth of these bacteria and ways to combat associated biofilms.^{1,5} For instance, human cathelicidin LL-37, a host defense peptide, has recently been reported to be effective against biofilms.^{6,7} In addition, 2-aminobenzimidazole derivatives and peptoids have also shown promising activity against *P. aeruginosa* biofilms.^{8,9} Activity of antibiotics such as linezolid, vancomycin, R-Thanatin and its derivatives against Methicillin-resistant *Staphylococcus aureus* (MRSA) biofilms has also been reported.^{2,3,10} The progress reported so far has encouraged the development of antimicrobial peptidomimetics as a new tool to combat bacterial biofilms.

Natural antibiotics such as host-defense peptides (HDPs) are of considerable interest, and have been studied for a long period of time owing to their broad spectrum activity as well as low probability to induce antibiotic resistance. These HDPs are also a part of host defense systems and act as the first line of defense against pathogens. HDPs are usually short cationic molecules that can segregate into hydrophobic and cationic regions upon interaction with the bacterial membrane, causing membrane disruption. However, one of the concerns for the development of HDPs is that these peptides are usually subject to proteolytic hydrolysis. To overcome the inherent drawbacks, antimicrobial peptidomimetics such as β -peptides, peptoids and others have been developed to increase the stability and potency, while still retain the antimicrobial

mechanism of HDPs.¹¹⁻¹⁸ Based on the chiral peptide nucleic acid (PNA) backbone, we have recently developed a new class of peptidomimetics termed “AApeptides” (Figure 1),^{19,20} which have shown broad-spectrum antimicrobial activities against both Gram-positive and Gram-negative bacteria.²¹⁻²⁴ Among these antimicrobial AApeptides, the latest generation, lipidated cyclic γ -AApeptides, are active against a range of community-acquired multidrug-resistant bacterial pathogens.²³ Furthermore, they appear to mimic host-defense function by suppressing pro-inflammatory cytokines. It is therefore very intriguing to access their anti-biofilm effect so as to further evaluate their potential as a new class of antibiotic agents. As such, herein we report their efficacy in the prevention of biofilm formation and biomass reduction of *PA* and *SE*.

Figure 1. The structures of α -peptides, α -AApeptides, and γ -AApeptides.

We have previously reported AApeptides with potent and broad-spectrum antimicrobial activity.²³ The AApeptides designed by joining amphiphilic AApeptide building blocks together are capable of adopting globally amphipathic structures, which is crucial for the disruption of bacterial membranes.²³ Cyclization or lipidation has proven to effectively increase their potency, as cyclization reduces structure motility and facilitates bacterial membrane disruption, while lipidation encourages their interactions with membranes. Polymyxin and daptomycin that have

been used as the “last-resort” antibiotics to treat infections caused by Gram-negative and Gram-positive bacteria, respectively, are actually lipidated cyclic peptides.^{25,26} We have recently reported a series of lipidated cyclic γ -AApeptides (Figure 2) containing different numbers of amphiphilic γ -AApeptide building blocks.²³ These lipo-cyclic γ -AApeptides have shown more potent and broad-spectrum activity compared with previous versions of γ -AApeptides, and even more intriguingly, they exhibit anti-inflammatory activity by suppressing pro-inflammatory cytokines. As such, we believe it is important to study their activity against biofilms of *PA* and *SE*, because to the best of our knowledge, lipo-cyclic antimicrobial peptidomimetics have not been studied for their activity against biofilms formed by both Gram-positive and Gram-negative bacteria. The study will also further evaluate the potential of lipo-cyclic γ -AApeptides as a new approach for the treatment of bacterial infections such as cystic fibrosis and indwelling device-related infections. As such, we first investigated the activities of a few lipo-cyclic γ -AApeptides (Figure 1) against planktonic cells and biofilms of *PA* and *SE*. The conventional antibiotic ciprofloxacin was included as a control. Their antibacterial activity and hemolytic activities were listed in Table 1.

Figure 2. Structures of the lipo-cyclic γ -AApeptides used in the study.

Table 1. The antimicrobial activity of the γ -AApeptides tested in the experiments.

Compounds	MIC, $\mu\text{g/mL}$ (μM)		Hemolysis HC ₅₀ $\mu\text{g/mL}$ (μM)
	Gram - <i>P. aeruginosa</i>	Gram + <i>S. epidermidis</i>	
YL-1	20 (12)	2 (1)	250 (160)
YL-4	>25 (13)	>25 (13)	250 (130)
YL-12	10 (6)	5 (3)	180 (114)
YL-29	25 (14)	5 (3)	150 (82)
YL-34	10 (6)	1 (0.6)	200 (129)
YL-36	5 (3)	1 (0.6)	100 (65)
Ciprofloxacin ^{27,28}	0.09-1.2	0.12-0.5	

By mimicking lipo-cyclic peptide antibiotics daptomycin and polymyxin B, which contain at least two amino acid residues connecting both lipid tails and ring structures,^{26,29} the lipid tails in these AApeptides were added outside the cyclic rings of γ -AApeptides through an amphipathic γ -AApeptide building block, leading to the sequences of antimicrobial lipo-cyclic γ -

AApeptides. As shown in Table 1, **YL-4** is the least active, **YL-36** is the most active, while **YL-12** possesses the moderate activity. Therefore, **YL-4**, **YL-12** and **YL-36** were next chosen for the time kills studies against *PA* and *SE* in order to understand the relationship between MICs and bacterial killing efficiency. As shown in Figure 3 and Figure 4, consistent to MICs, in the time kill studies of a period of 6 h, **YL-36** still proves to be the most potent in killing planktonic *PA* and *MRSE* cells. **YL-36** lead to a 6- and 8-log decrease in viable cells of *P. aeruginosa* and *MRSE*, respectively. In fact, the activity of **YL-36** against *PA* is comparable to that of ciprofloxacin, and it is even better than ciprofloxacin in killing *MRSE*. **YL-12**, concurrent with the MIC values, displays better time kill bactericidal effects with *MRSE* as compared to *P. aeruginosa*. The **YL-4**, is the least potent AApeptides amongst the three oligomers, show the same trend in the time kill studies. The concentrations used for time kill studies are higher than the effective MIC values because the initial CFU/mL (10^8) count used for the time kill studies was much higher than the CFU/mL (10^6) used for the MIC determination.

Figure 3. Time-kill studies for *P. aeruginosa* challenged with **YL-4**, **YL-12** and **YL-36** (tested at 12.5, 25, 50 $\mu\text{g/mL}$). Values are the mean of three independent tests performed in duplicate.

Figure 4. Time-kill studies for *MRSE* challenged with **YL-4**, **YL-12** and **YL-36** (tested at 12.5, 25, 50 $\mu\text{g/mL}$). Values are the mean of three independent tests performed in duplicate.

To determine if the lipidated cyclic γ -AApeptides can be used prophylactically to prevent the biofilm formation, a crystal violet (CV) staining assay was next conducted.³⁰ Dye binding of bacterial biomass was used to evaluate the efficacy of the lipidated cyclic γ -AApeptides for the prevention of biofilms. **YL-36** shows up to 80% and 70% reduction in the formation of the *P. aeruginosa* and *MRSE* biofilms at concentrations from 6.25-12.56 $\mu\text{g/mL}$ (Figure 5 and 6), respectively. It is surprising that **YL-36** is even more effective than ciprofloxacin in the prevention of biofilm formation, even it is less active in MICs against these bacterial strains. The other lipidated cyclic γ -AApeptides oligomers also show appreciable prevention of biofilms ranging from 60%-80% and 40%-60% for *P. aeruginosa* and *MRSE*, respectively. It suggests

that lipidated antimicrobial peptidomimetics might be more effective in the biofilm prevention than conventional antibiotics, possibly due the existence of lipid tails, which retards the growth of biofilms. It is also interesting that both lipidated cyclic γ -AApeptides and ciprofloxacin are more effective against preventing *P. aeruginosa* biofilms than *MRSE* biofilms. This is consistent to previously reported antimicrobial peptoids, which are active in the prevention of *P. aeruginosa* biofilms, presumably due to the micelle formation of the sequences on the surface of Gram negative biofilms.³⁰

Figure 5. Effect of lipo-cyclic γ -AApeptides on prevention of *P. aeruginosa* biofilm. n=4.

Error bars represent the means $\pm$ standard deviations.

Figure 6. Effect of lipo-cyclic γ -AApeptides on prevention of *MRSE* biofilm formation. n=3.

Error bars represent the means $\pm$ standard deviations.

The lipidated cyclic γ -AApeptides were further tested for their efficacy in destroying preexisting biofilms, which could lead to potential therapeutics for CF patients and to prevent urinary tract infections. In this assay, the biofilms were grown in the absence of antibiotics overnight, and then the biofilm reduction was measured by CV stain assay (Figure 7 and Figure 8). It is consistent to the previous observation that detachment of biofilm is much harder than biofilm prevention.⁹ Ciprofloxacin shows reductions of ~50% and ~30% in *P. aeruginosa* and *MRSE*, respectively. The lipidated cyclic γ -AApeptides show reductions that range from ~50-70% for *P. aeruginosa* biofilms and ~10-40% for *MRSE* biofilms. **YL-1** is observed to reduce the preexisting biofilms of *PA* by ~60%, which is much better than ciprofloxacin. Indeed, almost all lipo-cyclic γ -AApeptides are more effective than ciprofloxacin in the detachment of preexisting *PA* biofilms. **YL-36** at par with ciprofloxacin shows ~30% reduction in preexisting

MRSE biofilms. Overall, in general cases, **YL-36** are more effective, or at least comparable in comparison to ciprofloxacin, to prevent the formation of biofilms and to reduce biofilm biomass.

Figure 7. Effects of γ -AApeptides on detachment of pre-existing *P. aeruginosa* biofilms. n=4.

Error bars represent the means $\pm$ standard deviations.

Figure 8. Effects of γ -AApeptides on detachment of pre existing *MRSE* biofilms . n=3. Error bars represent the means $\pm$ standard deviations.

In conclusion, the lipidated cyclic γ -AApeptides, which exhibit potent and broad-spectrum activity against a multitude of multi-drug resistant Gram-negative and Gram-positive bacteria, have proven to effectively kill planktonic *P. aeruginosa* and *MRSE* cells that could potentially form biofilms. The lipidated cyclic γ -AApeptides can also detach the preexisting biofilms. It is possible that the lipidated cyclic γ -AApeptides form globally amphipathic structures, which align themselves to form cationic micelles upon interaction with the polyanionic exopolysaccharide matrix. This is the first study that demonstrates the activity of lipidated cyclic γ -AApeptides against both *P. aeruginosa* and *MRSE* biofilms. To the best of our knowledge, this is also the first report of lipo-cyclic peptidomimetics for their activity against biofilms of both Gram-positive and Gram-negative bacteria. The findings suggest lipidated cyclic γ -AApeptides are a new class of antibiotics with the ability to prevent biofilm formation and to reduce biofilm biomass in both Gram-positive and Gram-negative bacteria, and may provide a new approach for the treatment of chronic cystic fibrosis as well as indwelling device-related infections.

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Supplementary data

Supplementary data associated with this article can be found, in the online version.

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TOC

Biofilm prevention
Biomass reduction

Lipo-cyclic γ -AApeptides effectively prevent biofilm formation and reduce biomass.