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Lipo-γ-AApeptides as a New Class of Potent and Broad-Spectrum Antimicrobial Agents

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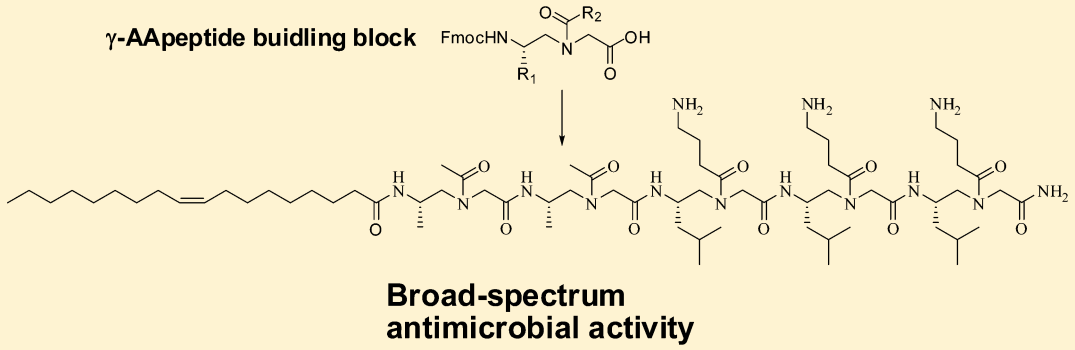
Supporting Information



ABSTRACT: There is increasing demand to develop antimicrobial peptides (AMPs) as next generation antibiotic agents, as they have the potential to circumvent emerging drug resistance against conventional antibiotic treatments. Non-natural antimicrobial peptidomimetics are an ideal example of this, as they have significant potency and in vivo stability. Here we report for the first time the design of lipidated γ-AApeptides as antimicrobial agents. These lipo-γ-AApeptides show potent broad-spectrum activities against fungi and a series of Gram-positive and Gram-negative bacteria, including clinically relevant pathogens that are resistant to most antibiotics. We have analyzed their structure−function relationship and antimicrobial mechanisms using membrane depolarization and fluorescent microscopy assays. Introduction of unsaturated lipid chain significantly decreases hemolytic activity and thereby increases the selectivity. Furthermore, a representative lipo-γ-AApeptide did not induce drug resistance in *S. aureus*, even after 17 rounds of passaging. These results suggest that the lipo-γ-AApeptides have bactericidal mechanisms analogous to those of AMPs and have strong potential as a new class of novel antibiotic therapeutics.

INTRODUCTION

Antimicrobial peptides (AMPs) are naturally occurring, short cationic amphiphilic peptides that are virtually ubiquitous and form a vitally important defense against infection as part of the innate immune system.[1,2] It is thought that antimicrobial peptides are unlikely to elicit extensive resistance observed for conventional antibiotic treatments, as they have bactericidal mechanism of membrane disruption, and that is probably why they have been deployed against invading pathogens by a variety of organisms for millions of years. Conventional antibiotics target specific metabolic processes in bacteria,[3] while antimicrobial peptides are believed to adopt amphipathic conformations on negatively charged bacterial membranes, leading to killing through a variety of mechanisms.[4] It would be difficult for microbes to counter AMPs multiple mechanisms of cell killing all at once. The negatively charged membranes of bacteria cells are distinct from zwitterionic mammalian cell membranes, facilitating the high selectivity of AMPs toward prokaryotes.[5] Because of the emergence of bacterial resistance to conventional antibiotics, there has been significant demand for the development of antimicrobial peptides as therapeutic treatments.[1,2] Since the initial step of killing by AMPs is bacterial membrane binding and disruption, followed by aggregation, poration, and carpet-like formation,[6] it is extremely difficult for bacteria to develop resistance against antimicrobial peptides.[1] Another advantage of AMPs is that they normally exhibit broad-spectrum activity against both Gram-negative and Gram-positive bacteria, as well as toward fungi and viruses.[1,2] As such, there is strong motivation for the development of antimicrobial peptides into antibiotic therapeutics to supplement, or even replace, existing conventional antibiotics treatments.[4]

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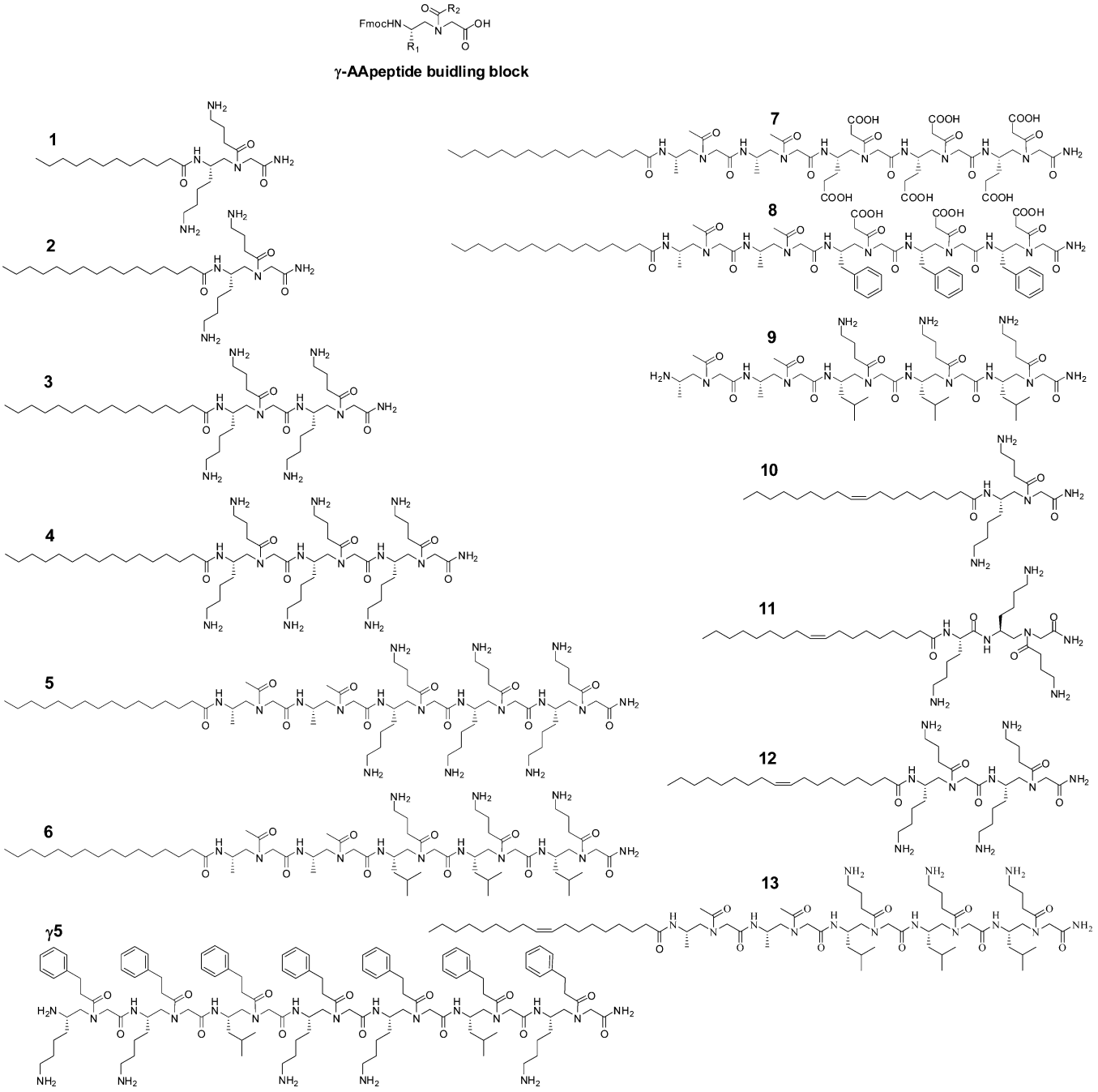

Figure 1. γ-AApeptides used in antimicrobial assays. The first structure shows the general structure of γ-AApeptide building blocks.

Naturally occurring[7−9] lipopeptides are another class of AMPs; however, they are different from naturally occurring host-defense peptides. They are produced as metabolites in bacteria and fungi in the presence of various carbon sources[10,11] and are cationic,[9,12,13] neutral,[14] or anionic.[7,8] Therefore, their mode of action is sometimes quite different from that of natural cationic AMPs. As such, many of these natural lipoantibiotics are not broad-spectrum in their activity. For example, daptomycin is only active against Gram-positive bacteria,[7,8] polymyxin B is active only toward Gram-negative bacteria,[9,12,13] while echinocandins[14] function only as antifungal drugs, as they noncompetitively inhibit β-1,3-D-glucan synthase. Although their antimicrobial properties are different, the lipid chain in these natural lipopeptides has been found to be critical for activity, as it endows lipophilicity, which facilitates bacterial membrane interaction.[10,11,15−17] Such recognition has led to the development of cationic lipopeptides that can mimic AMP killing of bacteria through membrane disruption.[10,11,18,19] Of note, they are generally composed of aliphatic acids attached to the N-terminus of short cationic peptides and are broadly active against both Gram-positive and Gram-negative bacteria, as well as fungi. Non-natural antimicrobial peptidomimetics such as arylamides,[6,20,21] peptoids,[4,22] β-peptides,[23−28] and oligourea[29] have been developed in recent years because they can mimic AMP activity while proving more effective as a result of their resistance to proteolytic hydrolysis.[30] However, the development and investigation of lipidated peptidomimetics are rare.[31]

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Table 1. Antimicrobial Activities of γ-AApeptides[a]

	MIC (μg/mL)									
	Gram-negative			Gram-positive				fungi	hemolysis	
oligomers	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*B. subtilis*	*S. epidermidis*	*E. faecalis*	*S. aureus*	*C. albicans*	HC_{10}/HC_{50}	selectivity[b]
1	>50	>50	>50	>50	>50	>50	>50	>50	400/>500	
2	2.5	35	10	1.5	2	2.5	2.5	2	25/40	16
3	30	20	20	1.5	2.5	8	5	2	40/300	60
4	20	>50	15	1.5	2	10	5	2	300/>500	>100
5	2.5	15	15	2.5	10	10	10	7.5	200/>500	>50
6	2.5	5	5	2.5	4	5	4	5	60/>500	>125
7	>100	>100	>100	>100	>100	>100	>100	>100	250/>500	
8	>100	>100	>100	>100	>100	>100	>100	>100	200/>500	
9	>100	>100	>100	>100	>100	>100	>100	>100	120/>500	
10	2.5	35	3	2	2	2.5	4	2	40/100	25
11	10	40	15	2	2	2.5	5	5	80/>500	>100
12	10	15	3	2.5	4	5	5	2	250/>500	>100
13	3	3	3	3	3	4	3	3	100/>500	>167
γ5	3	5	>50	2	5	5	5	8	75/300	60

[a] HC_{10} and HC_{50} are the concentrations of γ-AApeptides at which 10% or 50% hemolysis was observed. The two most potent and broad-spectrum lipo-γ-AApeptides, **6** and **13**, are shaded in grey. [b] Selectivity is calculated based on H_{50}/the MIC of *S. aureus*.

Herein, we report lipo-γ-AApeptides as a new class of antimicrobial peptidomimetics that mimic the bactericidal functions of AMPs. γ-AApeptides were recently developed by our group as a new class of peptidomimetic.[32−34] The solid phase synthesis of γ-AApeptides is straightforward, and their potential diversification is large by the introduction of a wide variety of functional groups.[32−34] Preliminary studies have demonstrated the ability of γ-AApeptide to modulate protein−protein and protein−RNA interactions[33,34] and their resistance to proteolytic degradation.[34] Recently, we have designed and identified a linear γ-AApeptide, **γ5**,[32] that has potent, broad-spectrum activity toward a range of bacteria and fungi. In this report, we demonstrate that lipo-γ-AApeptides, containing hydrophobic alkyl tails and short cationic γ-AApeptide sequences, display more potent, broad-spectrum, and highly selective activities against fungi and a series of clinically related Gram-positive and Gram-negative bacteria. Our mechanistic studies indicate that the mechanism of action for lipo-γ-AApeptides is analogous to that of AMPs, functioning via disruption of bacterial membranes. Additionally, lipo-γ-AApeptide does not elicit drug resistance in *S. aureus*, even after 17 rounds of passaging. These results demonstrate the strong potential of lipo-γ-AApeptide as a new class of novel antibiotic therapeutics.

RESULTS AND DISCUSSION

The lipo-γ-AApeptides were designed based on the widely accepted understanding that globally amphipathic structures are important for bacterial membrane disruption, which can be easily achieved by attaching hydrophobic alkyl tails to cationic γ-AApeptides. The synthesis of lipo-γ-AApeptides was carried out on solid phase (Figure S1) and purified by HPLC adapted from previously reported protocols (see Supporting Information for details).[32−34] Briefly, the necessary γ-AApeptide building blocks were synthesized and assembled on the solid support. Next, the fatty acids, in our experiments lauric acid, palmitic acid, or oleic acid, were coupled to the N-terminus of γ-AApeptide sequences. Lastly, sequences were cleaved from the resin and purified by HPLC to give the desired lipidated γ-AApeptides (Figure 1). Sequences **1**−**6** are cationic lipo-γ-AApeptides with saturated lauric acid or palmitic acid tails. Sequences **7** and **8** are anionic lipo-γ-AApeptides that were prepared as negative controls. Sequence **9** contains the same γ-AApeptide sequence as **6**, yet lacks an alkyl tail. Sequences **10**−**13** are cationic γ-AApeptides alkylated with unsaturated oleic acid. These sequences were tested for their antimicrobial activity toward a range of Gram-positive and Gram-negative bacteria, as well as the fungus *C. albicans*. Their selectivity was also evaluated via hemolytic assays. The results of these investigations are shown in Table 1, with **γ5**, a linear γ-AApeptide reported previously,[32] included for comparison.

Our results demonstrate that lipo-γ-AApeptides appear to be an effective and novel class of antimicrobial agents. As shown in Table 1, almost all the cationic lipo-γ-AApeptides display broad-spectrum antimicrobial activity against an array of Gram-negative and Gram-positive bacteria, as well as toward the fungus *C. albicans*. Sequences **7**, **8**, and **9**, however, are not active at all under these experimental conditions. This is likely because, with a positively charged surface post-self-assembly, cationic lipo-γ-AApeptides selectively target bacteria that have negatively charged membranes. Sequences **7** and **8**, which are negatively charged lipo-γ-AApeptides, should not, and do not, present any activity because of electrostatic repulsion with bacterial membranes. Linear sequence **9**, although positively charged, cannot strongly interact with bacterial membranes because of lack of lipid tail. In order to shed light on the further development of lipo-γ-AApeptide based antimicrobial agents, the structure−function relationship of these compounds was investigated. Sequence **1**, bearing one cationic γ-AApeptide building block and a lauric acid alkyl tail, is not active toward any microorganism. However, simply changing this tail to a palmitic alkyl chain renders **2** a potent antimicrobial agents toward most bacteria, as well as the fungus *C. albicans*. This indicates that lipophilicity of the alkyl tail is highly important for interaction with cell membranes and that increased cationic charge does not necessarily lead to more potent antimicrobial agents. Sequences **3** and **4**, which have many more cationic charges than **2**, are seemingly less active toward bacteria and fungus. As such, the balance of hydrophobicity and cationic charge appears to be critical for antimicrobial activity. This led

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to the discovery of **5** and **6**, with two more hydrophobic γ-AApeptide building blocks, which are more broadly active. Sequence **6**, which is a lipidated version of **9**, effectively arrests the growth of all tested bacteria and fungi. However, as lipophilicity increases, the hemolytic activity also increases, compromising selectivity. To enhance selectivity, sequences **10**−**13**, which contain similar or identical sequences to **2**−**6** but with unsaturated oleic tails, were prepared. We hypothesized that unsaturated tail would have less propensity for aggregation but still possess the same hydrophobicity compared to their saturated counterparts. Surprisingly, a number of even more potent and broad-spectrum-active lipo-γ-AApeptides were obtained, some of which are much less hemolytic, as seen with **10**, **12**, and **13**. For example, **3** and **12** only differ in their alkyl tails, yet **3** is somewhat hemolytic, while **12** is virtually nonhemolytic, and is generally more active toward bacteria. Sequence **13**, being the most potent and broad-spectrum sequence, has similar and better antimicrobial activity than **6** toward all microorganisms, while it is again much less hemolytic. Such increased potency and enhanced selectivity may indicate that bacterial membranes are more sensitive to unsaturated alkyl tails than mammalian cells. This is an important finding and would be of considerable significance for the development of lipoantibiotics in the future. It is noticeable that both **13** and **6** are more potent and broad-spectrum than the previously reported linear sequence **γ5**,[32] particularly toward the Gram-negative bacterium *P. aeruginosa* and fungus *C. albicans*, although they contain shorter lengths of γ-AApeptide fragments. Furthermore, the liposequence **13** is less hemolytic than **γ5**. These results may indicate that bacteria and fungi are more sensitive and susceptible to hydrophobic lipid chains, which augments the potential of lipo-γ-AApeptides as novel antibiotic agents.

To probe the antimicrobial mechanism of lipo-γ-AApeptides activity, we first performed fluorescence microscopy to assess the ability of our two most potent sequences **13** (Figure 2) and **6** (Figure S3) to cause membrane leakage, since membrane disruption is a general function of AMPs. A double staining method with DAPI and PI was used, where DAPI stains all bacterial cells irrespective of their viability and PI only stains injured or dead cells with compromised membranes.[19,35] Parts a1 and b1 of Figure 2 show that, without treatment by **13**, both *E. coli* and *B. subtilis* stain with DAPI but not PI, indicating that their membranes are intact. When cells were treated with **13** for 2 h, however, both *E. coli* and *B. subtilis* strongly stain with both DAPI (parts a3 and b3 of Figure 2) and PI (parts a4 and b4 of Figure 2), demonstrating membrane disruption. The aggregation of dead cells is also observed because of the loss of membrane ζ potential, which is consistent with the findings of previous studies on antimicrobial peptide amphiphiles.[19] Similar membrane disruption was also seen with the treatment of **6** (Figure S3).

The antimicrobial mechanism of membrane disruption for lipo-γ-AApeptides was further investigated using a membrane depolarization assay. MRSA, a clinically relevant and widely drug resistant bacterial pathogen, was selected for this study using the membrane potential-sensitive dye $DiSC_3$ to test membrane integrity.[21] In this assay, fluorescence intensity increases dramatically if there is significant membrane disruption or permeation, resulting from a loss in membrane potential.[21] Consistent with previous reports,[21] our analysis shows that the concentrations of lipo-γ-AApeptides required for complete depolarization are much higher than their MICs, and

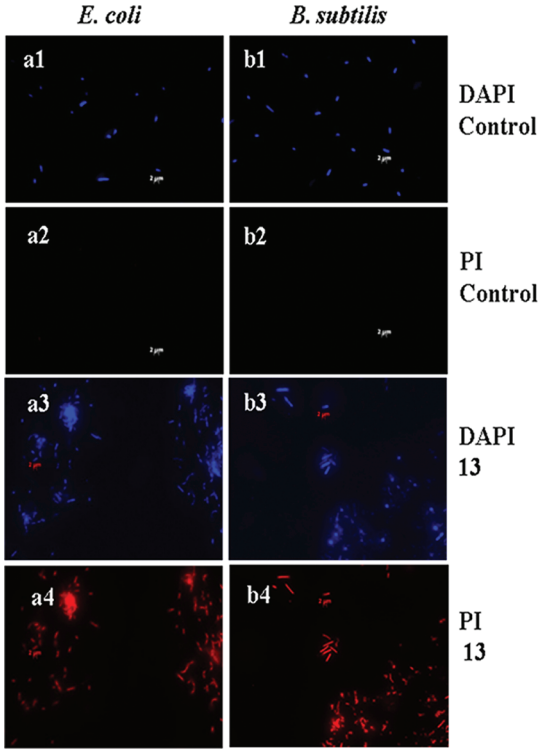

Figure 2. Fluorescence micrographs of *E. coli* and *B. subtilis* treated with 10 μg/mL **13** for 2 h: (a1−a4) *E. coli*; (a1) control, no treatment, DAPI stained; (a2) control, no treatment, PI stained; (a3) **13** treatment, DAPI stained; (a4) **13** treatment, PI stained; (b1−b4) *B. subtilis*; (b1) control, no treatment, DAPI stained; (b2) control, no treatment, PI stained; (b3) **13** treatment, DAPI stained; (b4) **13** treatment, PI stained.

there is no perfect relationship between the MIC and the capability for depolarization (Figure 3). However, the treatment of MRSA with lipo-γ-AApeptides led to dramatically increased fluorescence, which was maximal after 10 min (Figure 3). Meanwhile, there is a general trend that lipo-γ-AApeptides with higher MICs require higher concentrations to cause the

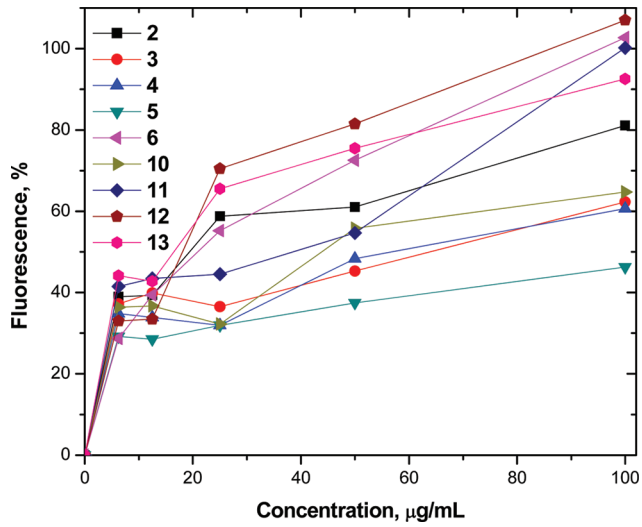

Figure 3. Depolarization of the membrane of *S. aureus*. The fluorescence intensity of membrane potential-sensitive dye DiSC3 was used as the positive control.

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same degree of depolarization than those with lower MICs. Taken together, these data further suggest that lipo-γ-AApeptides kill bacteria via membrane disruption.

The emergence of drug resistance in bacteria for conventional antibiotic treatments is a major public-health concern. To investigate the potential of lipo-γ-AApeptides to select for drug resistant isolates, meticillin-resistant *S. aureus* was serially passaged on half-MIC concentrations of **6**, with new MIC values determined every 24 h. Sequence **6** was chosen as a representative sequence as a result of its broad spectrum of activity against test microorganisms. As a positive control, parallel cultures were exposed to 2-fold dilutions of the antibiotic norfloxacin (Figure 4).[21] We determined that, while

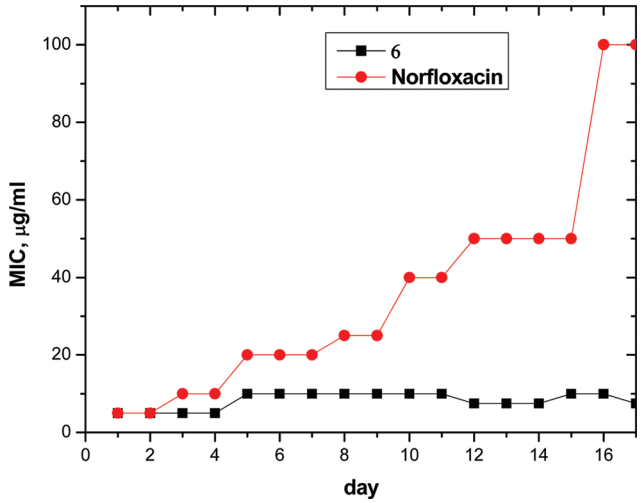

Figure 4. Development of resistance by *S. aureus* ATCC 33592 toward **6** and norfloxacin.

there are almost no changes in the MIC for **6** after 17 days with 17 passages, an increase in MIC for norfloxacin was found after just three passages, with a more than 20-fold increase in MIC observed after 17 days. This is yet further support that lipo-γ-AApeptides do not readily permit the development of drug resistance.

In order to further assess the potential of γ-AApeptide amphiphiles as novel antibiotics, we also evaluated the toxicity of **6** toward mammalian cells using a MTT assay (Figure 5). At concentrations up to 50 μg/mL, almost no toxicity was observed, while at 100 μg/mL, only around 50% of N2a/APP cell viability was compromised. These results show selectivity that is 10- to 50-fold lower than for bacteria, further

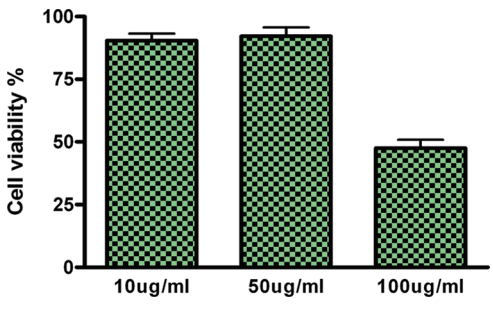

Figure 5. MTT assay of N2a/APP cells treated with different concentrations of **6**.

demonstrating the feasibility of lipo-γ-AApeptides for use as antimicrobial therapeutics.

CONCLUSION

In summary, we have successfully designed and synthesized lipo-γ-AApeptides, revealing them to be a new and important class of antimicrobial agents. These lipo-γ-AApeptides show very potent, broad-spectrum activity against fungi and a series of Gram-positive and Gram-negative bacteria, including clinically relevant pathogens that are resistant to most antibiotics. More importantly, analogue **6**, a representative potent example, does not induce drug resistance, possibly because of its membrane disruption properties. Our results also show that unsaturated lipid chain can decrease the hemolytic activity, thereby enhancing the selectivity of lipo-γ-AApeptides. With the additional advantage of their resistance to proteolytic degradation and limitless potential for derivatization, lipo-γ-AApeptides may lead to a new generation of antibiotic agents to circumvent emerging drug resistance. Further optimization of lipo-γ-AApeptides and in vivo studies with lead compounds **6** and **13** in mouse models are currently under investigation.

EXPERIMENTAL SECTION

Solid Phase Synthesis, Purification, and Characterization of Lipidated γ-AApeptides.[32−34] Lipo-γ-AApeptides were prepared on the Rink amide resin in peptide synthesis vessels on a Burrell wrist-action shaker following the standard Fmoc chemistry of solid phase peptide synthesis protocol. Each coupling cycle included an Fmoc deprotection using 20% piperidine in DMF and 4 h coupling of 1.5 equiv of γ-AApeptide building blocks[32−34] onto resin in the presence 2 equiv of DIC (diisopropylcarbodiimide)/DhBtOH (3-hydroxy-1,2,3-benzotriazin-4(3*H*)-one) in DMF. The lipidation was accomplished by reacting lauric acid, palmitic acid, or oleic acid with the N-terminus of γ-AApeptides using DIC/DhBtOH as activation agents on the solid phase. After the desired sequences were assembled, the resin was transferred into a 4 mL vial and the sequences were cleaved from solid support in 50:45:5 TFA/CH_2Cl_2/triisopropylsilane overnight. The solvent was evaporated and the residues were analyzed and purified on a Waters HPLC instrument installed with both analytic module (1 mL/min) and preparative module (20 mL/min). Both modules had the same methods, which were using 5−100% linear gradient of solvent B (0.1% TFA in acetonitrile) in solvent A (0.1% TFA in water) over 40 min, followed by 100% solvent B over 10 min. The desired fractions were generally over 70% in crude (determined by HPLC) and eluted as single peaks at >95% purity. They were collected and lyophilized. The molecular weights of lipo-γ-AApeptides were obtained on a Bruker AutoFlex MALDI-TOF mass spectrometer using α-cyano-4-hydroxycinnamic acid as the matrix.

Antimicrobial Assays.[32] The bacterial strains used in the experiment were *E. coli* (JM109), *B. subtilis* (BR151), multidrug resistant *S. epidermidis* (RP62A), vancomycin-resistant *E. faecalis* (ATCC 700802), methicillin-resistant *S. aureus* (ATCC 33592), *K. pneumoniae* (ATCC 13383), and multidrug resistant *P. aeruginosa* ATCC 27853. The fungal strain used was *C. albicans* (ATCC 10231). The antimicrobial activities of the lipo- γ-AApeptides were determined in sterile 96-well plates by broth microdilution method. Bacterial cells[36] and fungi[37] were grown overnight at 37 °C in 5 mL of medium, after which a bacterial suspension (approximately 10^6 CFU/ml) or fungal suspension *Candida albicans* (ATCC 10231) (approximately 10^3 CFU/ml) in Luria broth or trypticase soy was prepared. Aliquots of 50 μL of bacterial or fungal suspension were added to 50 μL of medium containing the γ-AApeptides for a total volume of 100 μL in each well. The γ-AApeptides were prepared in PBS buffer in 2-fold serial dilutions, with a final concentration range of 0.5−100 μg/mL. Plates were then incubated at 37 °C for 24 h (for bacteria) or 48 h (for *Candida albicans* (ATCC 10231). The lowest concentration at which the complete inhibition of bacterial growth (determined by a lack of

turbidity) is observed throughout the incubation time is defined as the minimum inhibitory concentration (MIC). The experiments were carried out independently three times in duplicate.

Hemolytic Assay.[32] Freshly drawn human red blood cells (hRBCs) with additive K_2 EDTA (spray-dried) were washed with PBS buffer several times and centrifuged at 1000*g* for 10 min until a clear supernatant was observed. The hRBCs were resuspended in 1× PBS to make a 5% v/v suspension. Two-fold serial dilutions of lipo-γ-AApeptides dissolved in 1× PBS from 1 mg/mL through 6.3 μg/mL were added to a sterile 96-well plate to make up a total volume of 50 μL in each well. Then 50 μL of 5% v/v hRBC solution was added to make up a total volume of 100 μL in each well. The 0% hemolysis point and 100% hemolysis point were determined in 1× PBS and 0.2% Triton-X-100, respectively.[36] The plate was then incubated at 37 °C for 1 h and centrifuged at 3500 rpm for 10 min. The supernatant (30 μL) was diluted with 100 μL of 1× PBS, and absorption was detected by measuring the optical density at 360 nm by Biotek Synergy HT microtiter plate reader. The percent hemolysis was determined by the following equation:

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

Fluorescence Microscopy.[32] A double staining method with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride, Sigma, >98%) and PI (propidium iodide, Sigma) as fluorophores was used to visualize and differentiate the viable from the dead *E. coli* or *B. subtilis* cells. DAPI as a double stranded DNA binding dye stains all bacterial cells irrespective of their viability. Propidium iodide (PI) is capable of passing through only damaged cell membranes and intercalates with the nucleic acids of injured and dead cells to form a bright red fluorescent complex.[35] The cells were first stained with PI and then with DAPI. Bacterial cells were grown until they reached mid-logarithmic phase, and then they (~2×10^6 cells) were incubated with the lipo-γ-AApeptides at a concentration of 2 MIC (10 μg/mL) for 2 h. Then the cells were pelleted by centrifugation at 3000*g* for 15 min in an Eppendorf microcentrifuge. The supernatant was decanted, and the cells were washed with 1× PBS several times and then incubated with PI (5 μg/mL) in the dark for 15 min at 0 °C. The excess PI was removed by washing the cells with 1× PBS several times. Then the cells were incubated with DAPI (10 μg/mL in water) for 15 min in the dark at 0 °C. The DAPI solution was removed, and cells were washed with 1× PBS several times. Controls were performed following the exact same procedure for bacteria without the addition of γ-AApeptides. The bacterial cells were then examined by using the Zeiss Axio imager Z1 optical microscope with an oil-immersion objective (100×).[38]

Lipid Depolarization.[21,39,40] The lipid depolarization of the bacterial cell membrane was conducted using the membrane potential sensitive dye 3,5′-dipropylthiacarbocyanine iodide ($DiSC_3$-5) that distributes between the cells and the medium depending on the membrane potential gradient. *S. aureus* (ATCC 33592) cells were grown in Trypticase soy broth medium to reach a mid-logarithmic phase (OD_{600} = 0.5−0.6). The bacterial cells were then collected by centrifugation at 3000 rpm for 10 min and then washed once with buffer (5 mM HEPES and 5 mM glucose, pH 7.2). The cells were resuspended to OD_{600} = 0.05 with 100 mM KCl, 2 μM $DiSC_3$-5, 5 mM HEPES, and 5 mM glucose and were incubated for 30 min at 37 °C for maximal dye uptake and fluorescence self-quenching. This bacterial suspension (50 μL) and 50 μL of lipo-γ-AApeptide stock solution or control drug solution were added to white flat bottomed polypropylene 96-well plate (Costar). The fluorescence reading was recorded every 2 min for 30 min using the microplate reader (Biotek) at an excitation wavelength of 622 nm and an emission wavelength of 670 nm. Valinomycin (final concentration 250 μg/mL) was used as a positive control, and the blank with only cells and dye was used as the background.

Drug Resistance Study.[21,32] The initial MIC of lipo-γ-AApeptide **6** and control antibiotics norfloxacin against *S. aureus* was obtained as described above. Bacteria from duplicate wells at a concentration of one-half MIC were then used to prepare the bacterial dilution (approximately 10^6 CFU/mL) for the next experiment. These bacterial suspensions were then incubated with **6** and norfloxacin, respectively. After incubation at 37 °C for 24 h, the new MIC was determined. The experiment was repeated each day for 17 passages.

MTT Assay. N2a APP cells were used to access the cell viability after treatment of **6**, daptomycin, and polymyxin B (see Supporting Information). Typically, stock concentration of lipo-γ-AApeptide **6** (1 mg/mL) was diluted in medium in a 96-well plate to make different concentrations and then incubated at 37 °C. In another 96-well plate, N2a APP cells were seeded to 1×10^4 cells/well, each of which contained 100 μL of medium. After incubation for 12 h, an amount of 100 μL of different concentrations of lipo-γ-AApeptide **6** was added and the plate was incubated for another 36 h. At 1 h before time is due, MTT reagent (Roche) was incubated at 37 °C degree water bath. The medium in the 96-well plate was removed and washed with fresh medium once, followed by adding 110 μL of MTT reagent, and then incubated for another 4 h, after which 100 μL of prewarmed solubilization solution was added. The plate was then incubated at 37 °C for 12 h before absorbance at 550 nm was read. Percentage of cell viability was calculated based on the following equation:

$$\%\,\text{cell viability} = (A/A_{\text{control}}) \times 100$$

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures, representative HPLC traces, NMR spectra, and MALDI-TOF MS results. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AMP, antimicrobial peptide; MIC, minimum inhibitory concentration; APP, amyloid precursor protein

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