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Small lipopeptides possess anti-biofilm capability comparable to daptomycin and vancomycin

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Antibiotic resistance, to a large extent, is related to the formation of bacterial biofilms. Thus, compounds with anti-biofilm capability are of practical importance. Inspired by the recent discovery of two amino acid lipopeptides from marine bacteria, we constructed a family of small lipopeptides with 2-3 amino acids. While no antimicrobial activity was found for anionic lipopeptides, cationic candidates are potent against Staphylococcus strains, such as methicillin-resistant *Staphylococcus aureus* (MRSA) USA200, USA300, USA400, UAMS-1, Newman, and Mu50. In the simplest design, two lysines (C14-KK) or three arginines (C14-RRR) attached to an acyl chain of 14 carbons were sufficient to make the compounds antimicrobial. These simple lipopeptides are inherently stable towards *S. aureus* V8 proteinase and fungal proteinase K, more soluble in water, and more selective than other lipopeptides containing a mixture of hydrophobic and cationic amino acids. Furthermore, the activity of C14-RRR was not compromised by salts, serum, or a change in pH. Live cell experiments revealed that these lipopeptides, with a detergent-like structure, killed bacteria rapidly by targeting cell membranes. Importantly, these compounds were also able to inhibit biofilm formation and could even disrupt preformed biofilms of clinically relevant MRSA strains with an in vitro efficacy comparable to daptomycin and vancomycin. These results indicate that small lipopeptides are potentially useful candidates for preventing or eliminating bacterial biofilms alone or in combination with daptomycin or vancomycin.

Introduction

In the United States, 9% of patients acquire a nosocomial infection[1] that is generally related to infection in surgical sites, bloodstream, and medical devices[2, 3], which may be further complicated by the formation of bacterial biofilms.[4] In particular, the hospital acquired infections caused by Gram-positive cocci (GPC) such as the methicillin resistant *Staphylococcus aureus* (MRSA) are significant[5] and have surpassed the total deaths caused by AIDS.[6] However, the pace in developing new antibiotics is relatively slow.

The situation is worsened by the fact that the currently used antibiotics, such as tobramycin, tetracycline and norfloxacin, could induce biofilm formation when the level is below the minimal inhibitory concentrations (MICs).[7, 8] The severity of biofilm related infection has been raised in recent years. It is estimated that up to 80% of chronic infections are biofilm related.[3] These infections are not only difficult to eradicate but impose a serious threat of drug resistance.[9] Usually, 10-1000 times the amount of antibiotics are needed to eradicate biofilms compared to planktonic bacteria.[10] At present, the majority of biofilm related infections involve Staphylococcus and pseudomonas species.[11] For a variety of GPC infections, daptomycin and vancomycin are currently in use.[12, 13] While daptomycin itself is a lipopeptide, vancomycin is a glycopeptide. Talavancin, a recent drug approved by FDA in 2009, is a semi-synthetic lipidated vancomycin used for complicated skin and skin structure infections caused by GPC.[14] Although these are the current drugs used for biofilm related infections, there are serious concerns. Daptomycin, which acts on the bacterial membrane in a calcium dependent manner, has been shown to have reduced susceptibility to a variety of MRSA strains and is also proved ineffective in biofilm-related and deep seated infections.[13, 15] Likewise, bacterial development of resistance to vancomycin is notable due to the emergence of vancomycin intermediate *S. aureus* (VISA) and vancomycin-resistant Enterococci (VRE) strains.[16] Moreover, a combined pharmacodynamic qualitative and quantitative *S. aureus* biofilm model shows the failure of vancomycin against mature MRSA biofilms.[17] In addition, vancomycin was also ineffective after one day against biofilm formed by catheter associated MRSA.[18] Several therapeutic procedures have been recorded failure for daptomycin/vancomycin therapy in MRSA and susceptible *S. aureus* clinical isolates that are not susceptible to daptomycin.[13] In addition, the newest antibiotics talavancin shows renal failure and teratogenic effects.[19] All these facts imply that we are running short of potent antimicrobials. Thus, it is urgent to search for new options.

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Antimicrobial peptides (AMPs) are host defence molecules (less than 100 amino acids) that have remained potent for millions of years. Therefore, they are considered useful templates for developing future antibiotics.[20, 21] According to the antimicrobial peptide database (APD), over 2,500 AMPs were found from all the kingdoms of life as of May 2015.[22-24] These peptides can be positively charged, neutral, or negatively charged. However, most of them bear a net positive charge, has broad spectrum activity and share a common membrane target.[25, 26] As cationic AMPs are fast killers, the development of bacterial resistance is more difficult.[20] Importantly, AMPs are also active against multidrug resistant superbugs and can prevent or disrupt bacterial biofilms.[27-29]

Our search for novel AMPs has reached previously unexplored organisms deep in the sea. Recently, short lipopeptides with merely a few amino acids have been discovered from marine bacteria.[30] One of the shortest peptides is composed of only leucine and glutamic acid followed by a unique fatty acid chain.[31] The simplicity in the design of these short peptides is attractive compared to lipidated peptides currently in use: polymyxin B for Gram-negative infections, daptomycin for GPC and echinocandins (acting as non competitive inhibitor of β-1,3-D-glucan synthase) for fungi.[32] Shai and colleagues had previously reported antimicrobial activity for cationic ultra-short lipopeptides.[33] Other colleagues investigated antibiofilm capabilities of some lipopeptides.[34-36] However, a detailed evaluation of the anti-biofilm activities against commonly associated MRSA strains is lacking. Here we synthesized a panel of even smaller lipopeptides and systematically evaluated their anti-biofilm capability against clinically relevant MRSA strains, including *S. aureus* USA200, USA300, USA400, Mu50 and UAMS-1. For comparison, we also evaluated anti-biofilm activity of daptomycin and vancomycin. In addition, we also performed synergistic studies between the best lipopeptide and these current-in-use antibiotics. Importantly, our results reveal inherent stability of these peptides to select proteases, as well as tolerance of their activity to salts, serum, and pH, making them potentially useful templates for designing new antibiofilm agents.

Results and discussion

Peptide design

To design lipopeptides, we followed the basic design principles for AMPs,[21, 37] which usually consist of cationic and hydrophobic amino acids. However, we also considered anionic peptides based on the recent peptide discovery from marine bacteria.[31] Thus, three groups of lipopeptides were designed in this study. The first group is composed of peptides with a string of basic amino acids attached to a fatty acid chain. The second group contains peptides with mixed hydrophobic and cationic amino acids. The third group consists of anionic lipopeptides that contain a mixture of acidic and hydrophobic amino acids. To simplify the lipid portion, an acyl chain with 14 carbons (C14) is attached to the N-terminus of all the peptides designed in Table 1. The amino acid sequence for each peptide is directly included in the name. In addition, these small lipopeptides, consisting of 2-3 amino acids, were amidated at the C-terminus.

Peptide activity

Most of the cationic peptides showed antibacterial activity against Gram-positive bacteria. Consistent with our previous finding,[37] small lipopeptides with a single lysine or arginine (C14-RW, C14-WR, C14-WWK, C14-KWI and C14-LKW) were very active against *S. aureus* USA300. Because of the dominant C14 fatty chain, select lipopeptides remained active after further increase in basic residues (C14-KK, C14-LKK and C14-RRR). Of note, the MIC value (1.56 μM) of C14-LK, C14-LKK, and C14-RRR is approaching that of daptomycin and vancomycin (0.78 μM). Surprisingly, C14-LLK showed no activity against *S. aureus, E. coli, P. aeruginosa*, and *K. pneumonia*, while C14-LKK killed three of the four bacteria (Table 1), indicating additional charge is helpful. Although the exact reason is unclear, it might be due to the unfavorable combination of a pair of leucines directly attached to a long hydrophobic fatty acid chain, making the initial membrane binding unfavorable. Interestingly, an attachment of a C14 fatty acid chain to a pair of lysines (C14-KK) or three arginines (C14-RRR) led to anti-staphylococcal peptides.

In general, these lipopeptides are poor in inhibiting Gram-negative bacteria. However, one peptide named C14-LK inhibited the growth of *K. pneumonia* at an MIC in the range of 6.2-12.5 μM. In the case of *P. aeruginosa*, only C14-LKK was active (MIC 3.1 μM). The requirements for anti-*E. coli* activity seemed less stringent. Four cationic lipopeptides were active against *E. coli* ATCC 25922 with an MIC of 3.1 μM (Table 1). These four compounds comprise only basic amino acids (i.e., C14-KK and C14-RRR) or a combination of basic and hydrophobic amino acids (i.e., K and L). Trp-rich peptides are known to be active against both Gram-positive and Gram-negative bacteria.[38] However, Trp-containing lipopeptides designed in Table 1 only inhibited *S. aureus* but not *E. coli*. It appeared that the sequence order did not matter since we obtained identical MICs for C14-RW and C14-WR peptides against *S. aureus* USA300. Basic residues are clearly important for peptide activity because we found no activity for anionic lipopeptides even in the presence of 2 mM Ca^{2+} (Table 1).

Because the cationic lipopeptides designed here are active against community isolate *S. aureus* USA300, additional *S. epidermidis* and *S. aureus* strains were also tested (Table 2). These include the clinical isolate *S. aureus* USA200 and another community isolate *S. aureus* USA400 lineage. In addition, *S. aureus* UAMS-1 is a clinical osteomyelitis isolate involved in hyaluronidase expression and biofilm formation.[39] While *S. aureus* Mu50 is a MRSA strain with vancomycin resistance isolated in 1997.[40] The Newman subtype, a robust virulent strain isolated form human infections, is responsible for pathogenesis.[41] With few exceptions, all the cationic lipopeptides were found to be active against these clinical strains. C14-LKK and C14-RRR were found to be the most active in this group and their activity ranged from 1.56-3.1 μM.

Table 1 Net charge, minimal inhibitory concentration (MIC), 50% hemolytic concentration, and cell selectivity index of small lipopeptides

Group	Peptide	Net Charge	MIC (μM)				HL_{50}[#]	CSI*
			SA[§]	*PA*	*EC*	*KP*		
I	C_{14}-KK	2	6.25	50	3.1	>50	50	8
	C_{14}-RRR	3	1.56	>50	3.1	>50	42	27
II	C_{14}-LK	1	1.56	>50	3.1	6.2-12.5	<<12.5	<<8
	C_{14}-RW	1	3.1	>50	>50	>50	<<12.5	<<4
	C_{14}-WR	1	3.1	>50	>50	>50	19	6.1
	C_{14}-WWK	1	25	>50	>50	>50	<<12.5	<<0.5
	C_{14}-KWI	1	3.1	>50	>50	>50	<<12.5	<<4
	C_{14}-LKW	1	6.25	> 50	> 50	> 50	<<12.5	<<2
	C_{14}-LKK	2	1.56	3.1	3.1	>50	<<12.5	<<8
	C_{14}-LLK	1	>50	>50	>50	>50	ND	ND
III	C_{14}-EE	-2	>50	>50	>50	>50	>>100	ND
	C_{14}-LE	-1	>50	>50	>50	>50	>>100	ND
	C_{14}-WE	-1	>50	>50	>50	>50	>>100	ND
	C_{14}-EW	-1	>50	>50	>50	>50	>>100	ND
	C_{14}-LWE	-1	> 50	> 50	> 50	> 50	>>100	ND
Antibiotics	Daptomycin	-3	0.78	ND	ND	ND	ND	ND
	Vancomycin	0	0.78	> 25	> 25	> 25	ND	ND

[§]SA: *Staphylococcus aureus* USA300; PA: *Pseudomonas aeruginosa* PAO1; EC: *Escherichia coli* 25922; KP: *Klebsiella pneumoniae*

[#]HL_{50} is the hemolytic concentration of the peptide required to lyse 50% of hRBCs (2% v/V); << indicates over 80 % of the hemolysis at this concentration or lower; >> indicates less than 20 % of hemolysis at this concentration or higher

* CSI refers to the cell selectivity index; calculated using the ratio of HL_{50}/MIC for *S. aureus* USA300. The antibacterial assay of the group III anionic peptides was done in the presence or absence of 2 mM Ca^{2+}.

ND: not determined.

We also tested the antifungal ability of these lipopeptides (supporting Table S1). Overall, they showed poor antifungal activities against *C. albicans*. All the anionic lipopeptides were inactive even in the presence of calcium. Among the cationic peptides, only C14-LK was active against *C. albicans* at 6.25 μM. This KL peptide may be of a broad activity spectrum against yeasts since it also inhibited the growth of *C. glabrata* and *C. tropicalis*. C14-LKK, another LK peptide, also displayed a good activity against these two yeasts (MIC 1.56 - 6.25 μM). Previous studies have shown that lipopeptides with four amino acids are often antifungal, indicating a slightly longer peptide sequence may be required to inhibit candida.[33]

Salt, pH and serum effects

Factors like salt, pH and serum may influence peptide activity. To evaluate this, we tested the activity of C14-RRR against *S. aureus* USA300 in the presence of physiologically relevant salts. As shown in Table 3, the MIC of C14-RRR remained constant at 1.56 μM in the presence of 150 mM NaCl or 2 mM Ca^{2+}. Furthermore, we also compared the peptide activity at three different pH values: 6.8, 7.4, and 8.0. Interestingly, we did not observe an increase in the MIC in this pH range (Table 3). Finally, we also evaluated the effect of human serum. Again, serum did not compromise the antimicrobial activity of C14-RRR up to 10%. Thus, small lipopeptides appeared to have a robust activity in the presence of salts, serum or upon change in pH. In contrast, salt and serum had clear effects on the activity of other AMPs such as human beta defensin 3 (hBD-3).[42, 43]

Hemolytic activity

Hemolytic activities of the lipopeptides were performed using 2% (v/v) human red blood cells (Fig. 1A). 100% lysis was observed for all cationic lipopeptides coupled to a hydrophobic residue at merely 12.5 μM. However, the two lipopeptides bearing only the charged amino acids showed a HL_{50} value of 50 μM for C14-KK and 42 μM for C14-RRR, respectively. The anionic lipopeptides are neither antimicrobial nor hemolytic.

The cell selectivity index (CSI) is a selectivity parameter between bacteria and mammalian cells when toxic effects of AMPs are observed.[27, 44] CSI is defined as a ratio of the HL_{50}/MIC of the particular antimicrobial compound. C14-RRR was found as the most selective lipopeptide with a CSI of 27 followed by C14-KK with a CSI of 8. This means 50% hemolysis for C14-KK and C14-RRR only when 8 fold and 27 fold the MIC is applied, respectively. Therefore, no toxicity to mammalian cells is anticipated when treated at the MIC of each peptide. Because these two peptides are highly soluble and more

Table 2 Minimal inhibitory concentration (MIC) of selected lipopeptides against various *Staphylococcus aureus* strains

Peptide name	MIC (μM)					
	USA200	*USA400*	*UAMS-1*	*Mu50*	*Newman*	*S. epidermidis*
C14-KK	6.25	6.25	6.25	12.5	6.25	6.25
C14-RRR	1.56	1.56	3.1	3.1	3.1	1.56
C14-LK	1.56	1.56	3.1	1.56	1.56	1.56
C14-RW	25	3.12	> 12.5	> 12.5	6.2	6.25
C14-WR	6.25	6.25	> 12.5	3.1	3.1-6.2	6.25
C14-KWI	25	25	1.56	12.5	6.2-12.5	12.5
C14-LKK	3.1	3.1	3.1	3.1	1.56	1.56
Daptomycin	0.78	0.78	> 12.5	3.1	ND	ND
Vancomycin	0.35	0.78	> 12.5	1.56	ND	ND

ND; not determined

selective, additional experiments were conducted using C14-KK and C14-RRR.

Cytotoxicity assessment

To provide additional insight into potential cell toxicity, we also evaluated the effects of C14-KK and C14-RRR on HeLa cells. While C14-KK caused 50% of the cell death at 50 μM, no cell death occurred when treated with the same concentration of C14-RRR (Fig. 1, B and C). Consistent with haemolytic experiments (Fig. 1A), both the peptides became toxic at a high concentration of 100 μM. However, it should be pointed out that such a concentration is 16-62 fold higher than the MIC values of the peptide (1.6 to 6.2 μM) needed to kill most of the *S. aureus* strains (Tables 1 and 2). As shown below, these two peptides can even disrupt bacterial biofilms at 6.2 μM, further verifying their potential use.

Mechanism of action

To uncover the possible mechanism of action of small lipopeptides, we conducted killing kinetics, growth inhibition, and dye incorporation into live cell studies (Fig. 2). The killing kinetics of *S. aureus* USA300 by C14-KK and C14-RRR is displayed in Fig. 2A. At 6.25 μM, C14-KK showed a slow killing, while C14-RRR killed the bacteria within 90 min, indicating membrane disruption.[25,26,45] To further compare these two peptides, we also presented the growth inhibition curves in Fig. 2 (B and C). A dose-dependent growth inhibition was evident for both C14-KK and C14-RRR. Nevertheless, a more rapid inhibition was observed for C14-RRR (Fig. 2C). Similar trends were found for other AMPs acting on membranes.[26]

To provide insight into the membrane anchoring role of the acyl chain portion of lipopeptides, we also synthesized a series of C14-KK analogs with varying chain lengths at C6, C8, C10, and C12. We observed a drastic reduction in peptide activity with decrease in acyl chain length (Supporting Table S2). While C12-KK only inhibited the growth of *S. aureus, E. coli*, and *B. subtilis* at 100 μM, peptides with even shorter acyl chains (i.e., C6 to C10) chains were ineffective at 200 μM. We conclude that the C14 chain is the minimal requirement to attach to KK in order to retain potent activity.

To further verify membrane targeting, we also conducted the FITC entrance assay using live cells (Fig. 2D).[33] This fluorescent probe (green) can only enter the cells if the membrane integrity is compromised. When bacteria were incubated with the dye and C14-RRR we observed a time dependent increment of fluorescence inside the cytoplasm of bacteria. We could observe the entrance of the dye into the cytoplasm in 150 s, suggesting that the membrane had been compromised by the action of C14-RRR and thereby the dye enters. Our results are consistent with previous observations using ultra-short lipopeptides.[33] These authors also observed detergent-like property based on micelle formation at higher concentrations. Such observations are in line with the carpet model[33] where cationic AMPs may act like detergents[46] to disintegrate bacterial membranes into small particles, leading to a clear solution due to bacteria lysis.[47]

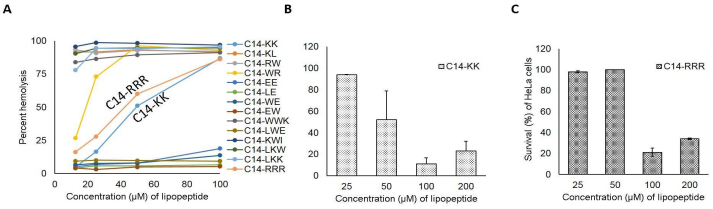

Fig. 1 Cytotoxicity of small lipopeptides. (A) Hemolysis of 2% hRBCs by lipopeptides in table 1 and survival assays of HeLa CCL-2 cells after treatment with (B) C14-KK and (C) C14-RRR.

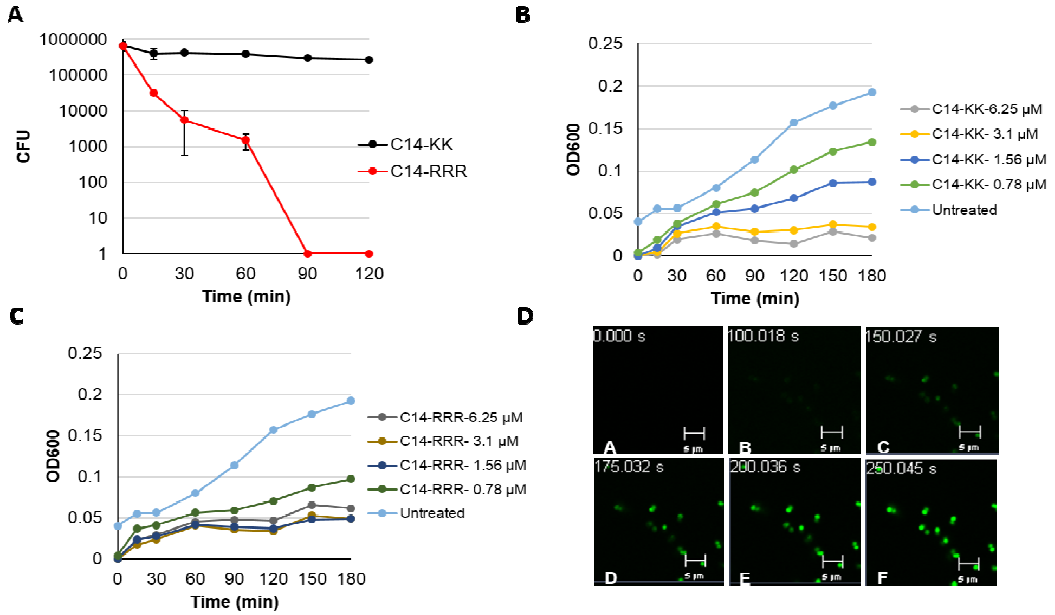

Fig. 2 Shown are (A) time dependent kinetic killing of *S. aureus* USA300 by C14-KK and C14-RRR at 6.25 μM, (B and C) growth inhibition by C14-KK and C14-RRR, and (D) live cell imaging of *S. aureus* USA300 incubated with C14-RRR and FITC. A time-dependent increment of the FITC fluorescence is indicative of the membrane permeation by the peptide.

Based on the results above, we may summarize the mechanism of action of small lipopeptides. The basic amino acid portion is important for recognition of anionic bacteria via electrostatic interactions since all the anionic lipopeptides synthesized here were inactive. The acyl chain acts as a membrane anchor that interdigitates into the hydrophobic core of the membranes. Indeed, we previous detected direct interactions between cationic AMPs and anionic phosphatidylglycerols by NMR spectroscopy.[47,48] In terms of acyl chain length, our results establish that C14-KK is the best lipopeptide. While an increase in chain length makes the peptide more hemolytic, chain-shortened analogs are ineffective (Supporting Table S2). These peptides resemble daptomycin that primarily targets membranes, but differs from vancomycin that inhibits cell wall synthesis.

Table 3 Effects of physiologically relevant salts, pH and serum on the anti-staphylococcal activity of the lipopeptide C14-RRR

Factor	MIC (μM)					
Salt	No Ca^{2+} or NaCl	1 mM Ca^{2+}	2 mM Ca^{2+}	50 mM NaCl	100 mM NaCl	200 mM NaCl
	1.56	1.56	1.56	1.56	1.56	1.56
pH	6.8	7.4		8.0		
	0.78-1.56	1.56		1.56		
Serum	0 %	5%		10%		
	1.56	≤ 0.78		≤ 0.78		

Stability to proteases

Molecular stability has always been of tremendous concern in the process of drug development. The use of modified amino acids (AA), such as α-AA, β-AA, cyclic-AA and D-AA, has been reported to provide enzymatic stability to the molecule of interest.[21] In our current study, we have also tested the effects of important proteases on peptide stability. These include chymotrypsin and trypsin from mammalian cells, the *S. aureus* V8 protease and fungal proteinase K from pathogens. To ensure rapid degradation in hours, we incubated the lipopeptides (C14-KK and C14-RRR) with the proteases at a molar ratio of 40:1 (peptide:protease). Interestingly, both the lipopeptides were found to have intrinsic stability towards *S. aureus* V8 and proteinase K after incubation even for six days (Fig. 3). In addition, both peptides showed partial stability to chymotrypsin and trypsin.

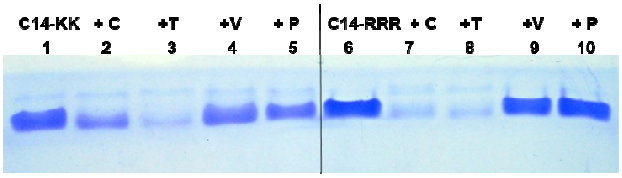

Fig. 3 Stability of C14-KK and C14-RRR in the presence of four proteases.[27, 37] Lanes 1-5 are peptide C14-KK, and lanes 6-10 are C14-RRR. Lanes 1 and 6 are peptide controls without any protease treatment, lanes 2-5 (or 7-10) were treated with chymotrypsin (+C), trypsin (+T), *S. aureus* V8 protease (+V), and proteinase K (+P), respectively.

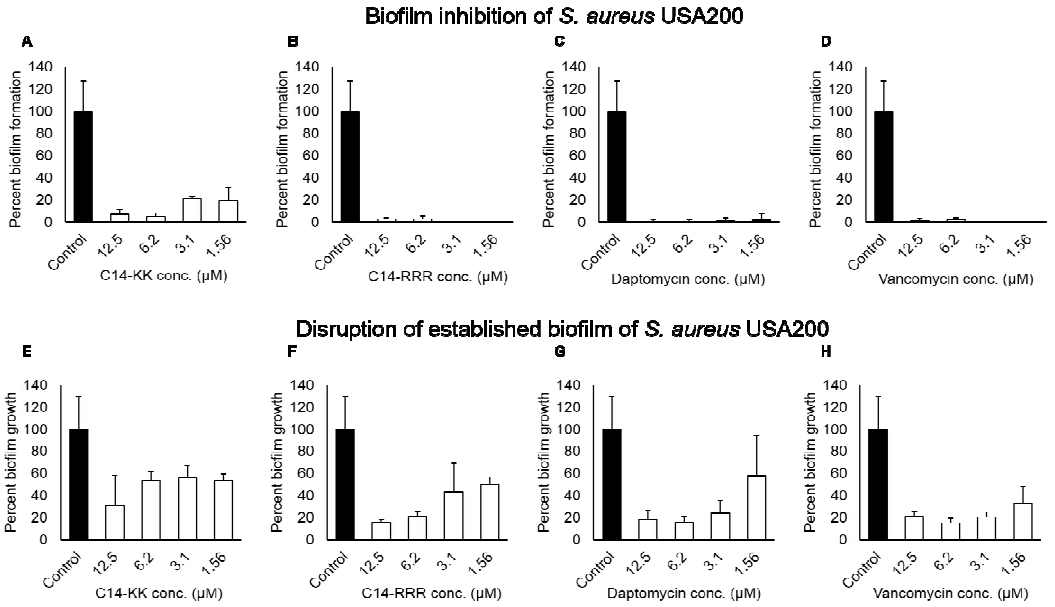

Fig. 4 *S. aureus* USA200 biofilms. C14-KK, C14-RRR daptomycin and vancomycin not only can inhibit biofilm formation (panels A to D) but also disrupt the 24 h biofilms of *S. aureus* USA200 (panels E to H).

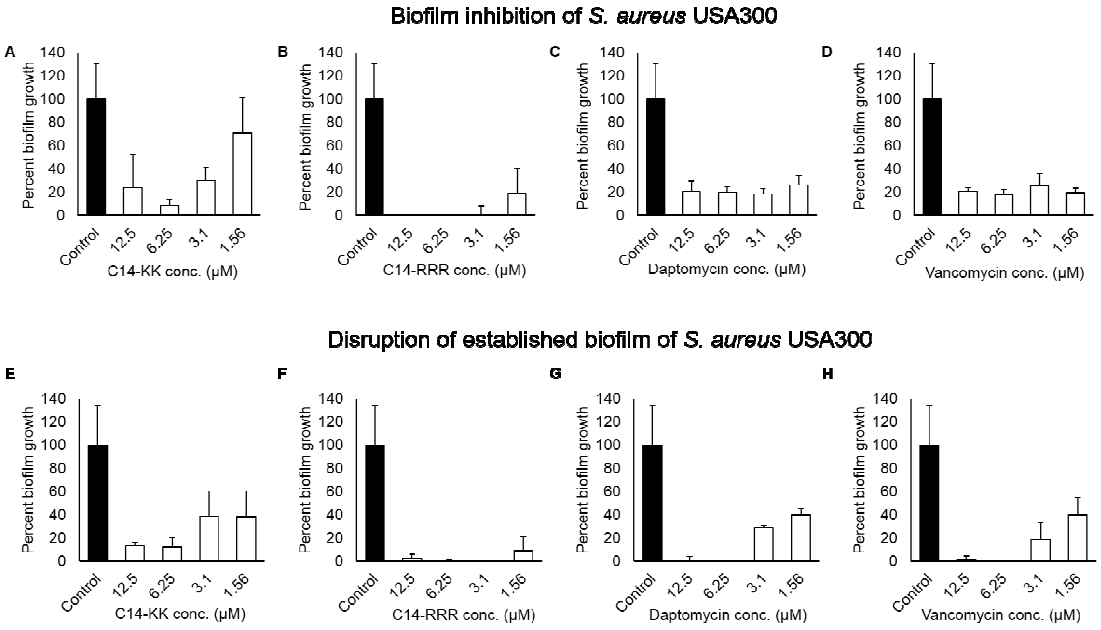

Fig. 5 *S. aureus* USA300 biofilms. C14-KK, C14-RRR, daptomycin and vancomycin can not only inhibit biofilm formation (panels A to D) but also disrupt the 24 h established biofilms of *S. aureus* USA300 (panels E to H).

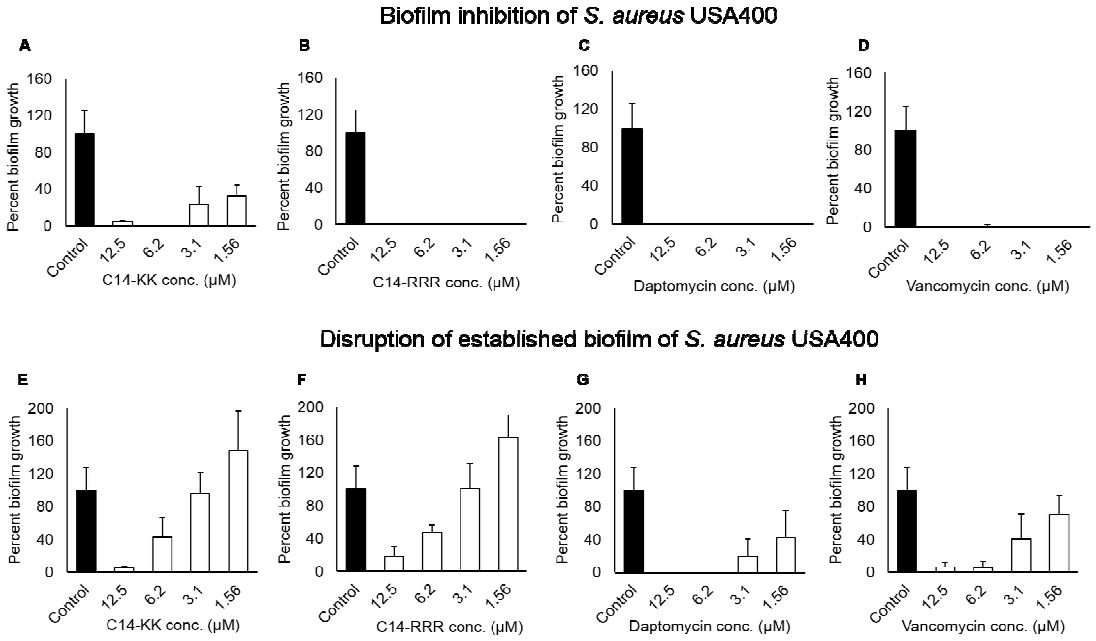

Fig. 6 *S. aureus* USA400 biofilms. C14-KK, C14-RRR, daptomycin and vancomycin can not only inhibit biofilm formation (panels A to D) but also disrupt the 24 h established biofilms of *S. aureus* USA400 (panels E to H).

Anti-biofilm activity of C14-KK and C14-RRR against clinical strains

The involvement of the GPCs in biofilm formation has been increasing, leading to higher doses of antibiotics and increased bacterial resistance. It, too, increases the mortality and morbidity of infectious patients. Currently used daptomycin was reported to have anti-biofilm activity at 5 mg/mL and lower concentrations were found to be ineffective.[49] Meanwhile, vancomycin has been shown to have very limited anti-biofilm properties.[50] Moreover, both antibiotics suffer from the development of resistance phenotype.[13] As a consequence, we also tested the anti-biofilm ability of both C14-KK and C14-RRR against relevant *S. aureus* strains. The USA300 and USA400 clades are community associated methicillin resistance strains. The USA300 are more commonly seen in infections related to the skin and soft tissue while the USA400 is related to lethal necrotizing pneumonia.[51] In contrast, the USA200 is related to severe surgical wound infection, infective endocarditis, sepsis and problems related to its colonization in the mucus layers. All these strains release α-toxins in an order: USA300 (150-500 μg/ mL) > USA400 (50 μg/ mL) > USA200 (Less than 5 μg/ mL).[51] Additionally, the Mu50 is vancomycin resistant MRSA and UAMS-1 is responsible for hyaluronidase production and biofilm formation.[39] Hence, these life threating bacterial strains are included in the anti-biofilm studies. Both lipopeptides were found to possess anti-biofilm properties, although in general C14-RRR is better than C14-KK. In case of USA200 (Fig. 4), C14-RRR was found to be as good as daptomycin and vancomycin in inhibiting biofilm formation. In addition, 80% of the established biofilms of the same bacterium were disrupted at 6.25 μM by these three compounds. Although C14-KK was not as effective in biofilm disruption, it was able to inhibit biofilm formation (Fig. 4). To evaluate the impact of the type of microplate, we also did the same experiment using polypropylene plates and found similar results (data not shown). Hence, the rest of the biofilms assays were conducted using polystyrene microplates. For *S. aureus* USA300 (Fig. 5), C14-KK completely inhibited the biofilm growth between 3.1 and 12.5 μM, while daptomycin and vancomycin showed similar effects (80% inhibition). At 6.25 μM, C14-KK was better than either daptomycin or vancomycin. Although it was not as efficient in inhibiting the formation of biofilms at low concentrations Likewise, C14-RRR is also best in disrupting biofilms of USA300, while C14-KK is comparable to daptomycin and vancomycin. C14-RRR, daptomycin and vancomycin are equally good in inhibiting the biofilm formation of *S. aureus* USA400 and achieved 100% inhibition at all the tested concentrations from 1.56 to 12.5 μM. We found that the established biofilms of *S. aureus* USA400 in microplate wells appeared to be thicker than those of USA200 or 300 strains. However, the biofilms of USA400 could be disrupted by both daptomycin and vancomycin at 6.2-12.5 μM (Fig. 6). At 12.5 μM, the majority of the biofilms were destroyed by the two lipopeptides, C14-KK and C14-RRR, although the effects were reduced at lower peptide concentrations.

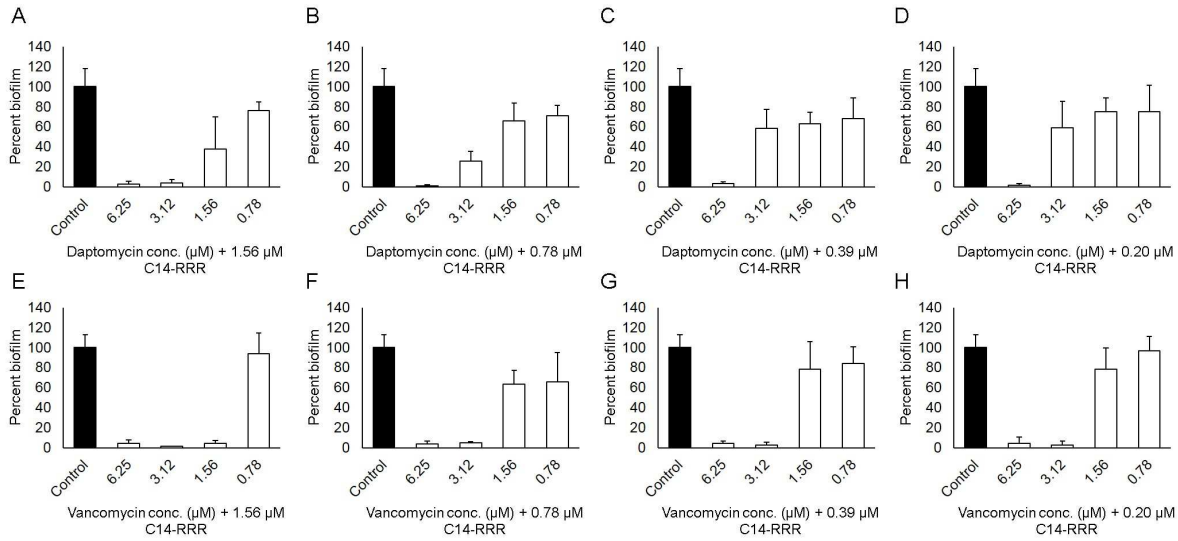

Fig. 7 Synergistic effects between C14-RRR and daptomycin (panels A to D) or between C14-RRR and vancomycin (panels E to H) against *S. aureus* USA300 biofilms. From left to right, the concentration of C14-RRR ranges from 1.56 to 0.20 μM, while the concentration of daptomycin or vancomycin varied from 6.25 to 0.78 μM.

To get a better understanding of the anti-biofilm activity of these lipopeptides, we also included two additional *S. aureus* strains: UAMS-1 and Mu50. We found tremendous biofilm disruption of these two strains by C14-KK and C14-RRR (supporting Figure S1). For *S. aureus* UAMS- 1, C14-RRR was able to disrupt all established biofilms at 6.25 μM. At the same concentration, C14-KK eliminated 93%, whereas daptomycin and vancomycin disrupted ~70% of the biofilms. Although all the compounds were less potent at lower concentrations, they showed a similar dose-dependent effect on the established biofilms of *S. aureus* Mu50 with nearly complete disruption at 6.25 μM. In line with our findings, Dawgul and colleagues found that palmitic and lauric acid based lipopeptides are also active against clinical *S. aureus* biofilms.[36]

We also investigated a possible additive antibiofilm effect between C14-RRR and vancomycin or daptomycin (Fig. 7). For a 24 h matured *S. aureus* USA300 biofilm, C14-RRR was indeed more potent in the presence of either daptomycin or vancomycin. A combination of 3.12 μM daptomycin with 1.56 μM C14-RRR could eradicate ~95% of the biofilm. Likewise, a combination of 1.56 to 3.1 μM vancomycin with 1.56 μM C14-RRR achieved a better effect. Remarkably, vancomycin at 3.1 μM was unable to completely disrupt the biofilm by itself but became sufficient to disrupt nearly all the biofilms with the aid of even 0.20 μM of C14-RRR. Our observation is in agreement with others who observed synergistic effects between other AMPs and daptomycin or vancomycin.[52]

To confirm biofilm disruption, we obtained fluorescence images for the 24 h matured biofilm of *S. aureus* USA300 using confocal microscopy (Fig. 8). In these images, live cells appeared green due to the incorporation of the SYTO-9 dye, while the dead cells looked red due to propidium iodide (PI) intercalation into DNA. At 12.5 μM, there are clear red patches of the dead cells in the C14-RRR-treated biofilms, indicating disruption of the biofilms (Fig. 8B), but not the biofilms in the control (Fig. 8A) treated with water.

Conclusions

Inspired by the recent discovery of small lipopeptides from marine bacteria,[30,31] we designed a panel of lipopeptides with two to three amino acids. While anionic lipopeptides were inactive, cationic peptides were antimicrobial, primarily against Gram-positive staphylococcal bacteria. In particular, we demonstrated the potential of simple lipopeptides candidates as anti-biofilm agents against various resistant strains of *S. aureus* isolated from communities (USA300 and 400) or clinically (UAMS-1 and USA200). For the first time, the anti-biofilm capabilities of novel small lipopeptides were evaluated together with daptomycin and vancomycin under the same platform. C14-RRR, consisting of a fatty acid chain and three arginines, was found to be most potent among the designed peptides (Table 1). Its anti-biofilm activity was comparable to daptomycin and vancomycin, and even better in certain cases at clinically feasible concentrations. While, both the antibiotics showed synergistic antibiofilm properties with C14-RRR, vancomycin was found to be more efficient when used in combinations. The membrane penetration and fast killing abilities of these peptides are beneficial to reduce the likelihood of bacterial resistance development. Because of the simplicity in molecular design, cost effective chemical synthesis, high solubility in water, inherent stability to select pathogen proteinases, and antimicrobial activity tolerance to salts, pH, and serum, the small lipopeptides reported herein provide excellent starting templates for developing alternative anti-biofilm agents.

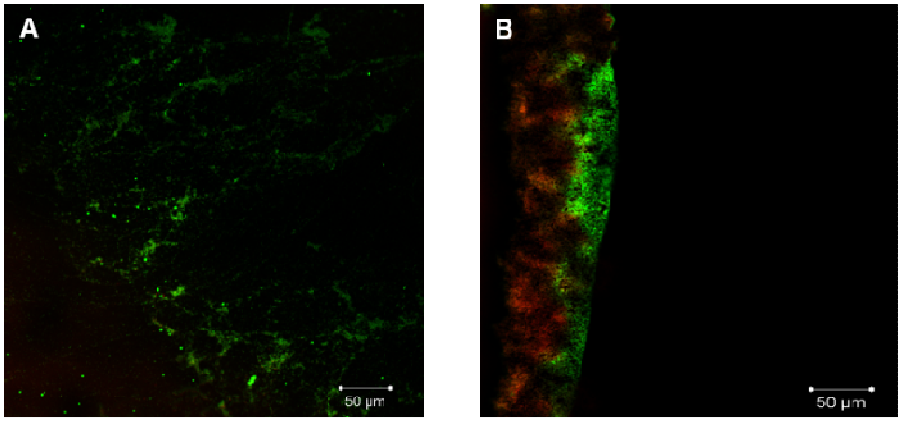

Fig. 8 Anti-biofilm activities of C14-RRR against established biofilms (24 h) of *S. aureus* USA300. Live (green) and dead (red) cells are viewed by confocal laser scanning microscopy after staining with SYTO-9 and propidium iodide. Shown are untreated biofilms (A) and biofilms treated with C14-RRR (B). For more details, refer to the text.

Experimental

Strains and media

The bacterial strains used in this study include *Staphylococcus epidermidis, Staphylococcus aureus* USA200, USA300, USA400, Mu50, Newman, and UAMS-1, *Psudomonas aeruginosa* PAO1, *Escherichia coli* ATCC 25922 and *Klebsiella pneumonia*. While the fungal strains used are *Candida albicans* ATCC 10231, *C. glabrata* ATCC 2001and *C. tropicalis* ATCC 13803. Tryptic soy broth (TSB) growth medium for bacterial growth was obtained from BD Bioscience MD, USA and Remel Dex broth (Thermo Fisher Scientific, KS, USA) for fungal culture. Daptomycin and vancomycin were obtained from Sigma, USA. In all the assays for daptomycin, the medium was supplemented with 2 mM Ca^{2+}. The lipopeptides used were synthesized chemically and purified to >95% (GeneMed, TX).

Measurement of the minimal inhibitory concentration (MIC)

The assay was performed as described previously.[48] In brief, the bacterial strains were inoculated overnight. These cultures were then freshly inoculated and allowed to reach the exponential growth phase. The cultures were diluted accordingly to reach a count of 10^6 CFU/mL and 90 µL of this solution was added to a 96 well microplate (Costar, Corning, NY) containing 10 µL of serially diluted lipopeptides or antibiotics solutions and incubated overnight at 37°C for 20 h. The growth as a function of absorbance was read with a CHROMATE microplate reader at 630 nm. The wells containing sterilized water instead of peptide served as the positive control and the uninoculated media was treated as the negative control. Additionally, lipopeptides activity in the presence of various salts were done in the same way, instead the media was supplemented with the corresponding salts. For anticandidal activity, the above protocol was modified. The inoculum was 10^7 CFU/mL and plates were incubated for 48 h before final reading.

Measurement of peptide hemolytic concentrations

The experiment was conducted similar to the MIC determination assay. Shortly, blood was obtained from the UNMC Blood Bank and washed three times (800 g, 10 min) with normal saline to remove plasma. A final of 2% hRBC solution was then prepared in normal saline and used for the assay. 90 µL of this solution was added to 10 µL of serially diluted lipopeptide solutions and was incubated at 37ºC for one hour. It was then centrifuged at 13,000 rpm, 5 min on an Eppendorf bench-top centrifuge 5415D. Aliquots of the supernatant was transferred to a fresh 96 well microplate (Costar, Corning, NY) and absorbance was read at 545 nm to detect the amount of hemoglobin released. Percent lysis was calculated based on the extent of hemoglobin released, where 100% release is assumed due to 1% Triton X-100 and 0% release is assumed in saline.

Calculation of the cell selective index (CSI)

As a measure of the cell selectivity of the lipopeptide towards the bacterial and own human cells the CSI value was calculated using the MIC and the HL_{50} values from earlier experiments. Numerically it is the ratio of HL_{50} to MIC and larger values correlates more specificity towards bacterial cells.

Cellular cytotoxicity assessment

HeLa CCL-2 cells from American Type Culture Collection (ATCC) were maintained in DMEM High Glucose media with 4mM L-Glutamine (NyClone) and 100 U/mL penicillin, 100 µg/mL streptomycin (pen/strep) (Life Technologies), and 10% (v/v) inactivated fetal bovine serum (FBS) (NyClone). Cells were grown in 5% CO2 at 37°C and were detached from culturing dish at 80% confluency using 0.025% trypsin-EDTA (NyClone) treatment. The lipopeptide influence on the cell viability was estimated by using the MTS assay according to manufacturer's

protocol (MTS, CellTiter96 AQ One Solution Cell Proliferation Assay, Promega) with minor modifications. In short, cells were cultured in flat bottomed 96 well microtiter plates (Corning Life Science) at a seeding density of 10,000 cells/well. At confluence 80–90% achieved after 20–24 h of cultivation, the cells were washed twice with 100 µL Dulbecco's Phosphate Buffered Saline (Life Technologies). Further, 90 µL of DMEM media with 10% FBS, pen/step was added before exposure to 10 µL of the peptide solution in the concentration range 12.5-100 µM. Plates were incubated at 37°C. After 1 h 50 µl of media was removed and 10 µL MTS, CellTiter96 was added. Plates were further incubated for another 2 h at 37°C and finally, the absorbance was measured on ChroMate reader (Awareness Technology) at 492 nm. Culture medium and 0.2% SDS were used as negative and positive controls, respectively.

Growth inhibition experiments

The experiment was performed as described previously.[53] Serially diluted lipopeptides at fixed concentrations were incubated with bacteria with a final OD600 ~0.1 with continuous shaking at 100 rpm, 37°C. The plates were read every 30 minutes for a total duration of 3 h. Experiments were conducted in duplicates and the averaged values were reported.

Killing kinetics

Killing kinetics experiments were conducted similar to antibacterial assays described above with the following additions. Aliquots of cultures (10^5 CFU) treated with lipopeptides were taken at 15, 30, 50, 90, and 120 min, diluted 100-fold, and plated on Luria-Bertani agar plates. Colonies were counted after overnight incubation at 37°C.

Live cells dye permeation assay

S. aureus USA300 was grown to the exponential phase from overnight culture. The cells were then washed twice with fresh saline and final cell density was adjusted to 10^8 CFU/mL. 1.5 mL of the culture was added to the cuvette chambers (Borosilicate cover glass systems, Nunc Cat. No: 155380) and was treated with C14-RRR and FITC with a concentrations of 12.5 µM for the lipopeptide and 6.25 µM of the dye. The samples were examined with a confocal laser scanning microscope (Zeiss 710) with live time series of picture taken every 5 seconds for 5 min and the data were processed using Zen 2010 software.

Peptide stability to proteases

Enzymatic stability assessment of the lysine and arginine-containing lipopeptide was done using functionally relevant proteases including the mammalian trypsin, chymotrypsin, pathogen *S. aureus* V8 protease and the fungal Proteinase K. Experimentally, a solution (100 µL) of peptide/protease molar ratio, 40:1 was made in 10 mM PBS buffer (pH 8.0) and was incubated at 37°C. Aliquots (10 µL) of the reaction solutions were taken at 3 h, 1 day and 6 day and immediately mixed with 20 µl of 2× SDS loading buffer and boiled in a water bath to stop the reaction. For the SDS gel analysis, 10 µl of each sample was loaded to the well of a 5% stacking/18% resolving tricine gel and run at a constant current of 35 mA.

Inhibition of biofilm formation

The potency of the small lipopeptides to inhibit the formation of biofilms was evaluated by following an established protocol with modifications.[54] In short, *S. aureus* USA300 cells were inoculated in TSB overnight. From these, freshly inoculated cells were allowed to attain exponential phase of growth. A bacterial density of 10^5 CFU/mL was prepared and 180 µL of it was delivered to flat bottom, 96 wells, polystyrene microtiter plates (Corning Costar Cat No. 3595) or polypropylene (Evergreen, CA, USA) containing 20 µL of serially diluted 10X lipopeptides and antibiotics solution. Media containing bacteria and water is treated as positive control while un-inoculated media with water served as the negative control. The plates were then incubated at 37°C for 24 h. Media was than pipetted out and the wells were washed with normal saline to remove the non-adherent planktonic cells. Calorimetric quantitation of the inhibition of biofilms was done by XTT [2,3-bis(2-methyloxy-4-nitro-5-sulfophenyl)-2*H*-tertazolium-5-carboxanilide] assay following manufacture instructions with minor adjustments. 180 µL of fresh TSB and 20 µL of XTT solution were added to each well and the plates were again incubated for 2 h at 37°C. Absorbance at 450 nm (only media with XTT containing wells served as the blank) was obtained using a Chromate™ microtiter plate reader. Percentage of biofilm growth for the peptide was plotted assuming 100% biofilm growth is achieved in the bacterial wells without peptide treatment.

Disruption of established biofilms

A cell density of *S. aureus* USA300 (10^5 CFU/mL) was made from logarithmic growth phase. 200 µL was placed into each well of the 96 well microtiter plates. The plates were incubated at 37°C for 24 h to allow biofilm formation. Media containing bacteria and water is treated as positive control while un-inoculated media with water served as the negative control. Media was then pipetted out and the biofilms were washed with normal saline to remove the planktonic cells. 10X lipopeptide solution (alone or in combination with antibiotics) followed by 180 µL of fresh TSB media was then added to each well and the pates were further incubated at 37°C for another 24 h. Quantification of the disruption of the biofilm by the lipopeptide was done using XTT and followed the same methods described earlier.

Live and dead staining assays of established biofilms using confocal laser scanning microscopy

S. aureus USA300 (10^5 CFU/mL) was made form exponential phase bacteria. 2 mL of the culture was added to the chambers of cuvette (Borosilicate cover glass systems, Nunc Cat. No: 155380) and was incubated for 37°C, 24 h for establishment of biofilm. Media was than pipetted out and chambers were washed with normal saline to remove non-adhered cells. To disrupt the established biofilms, 200 µL of 10X (125 µM) stocks of the lipopeptide was added followed by

1.8 mL TSB. Control cuvettes contained water instead of peptide. The cuvettes were again incubated for another 24 h at 37°C. Chambers were than cleaned with normal saline washings. For evaluation under confocal laser scanning microscope, the remaining established biofilms were stained with 10 µL of LIVE/DEAD kit (Invitrogen Molecular Probes, USA) according to the manufacturer's instructions. The samples were examined with a confocal microscope (Zeiss 710) and the data were processed using Zen 2010 software.

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