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Anti-biofilm peptides as a new weapon in antimicrobial warfare

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Abstract

Microorganisms growing in a biofilm state are very resilient in the face of treatment by many antimicrobial agents. Biofilm infections are a significant problem in chronic and long-term infections, including those colonizing medical devices and implants. Anti-biofilm peptides represent a very promising approach to treat biofilm-related infections and have an extraordinary ability to interfere with various stages of the biofilm growth mode. Anti-biofilm peptides possess promising broad-spectrum activity in killing both Gram-positive and Gram-negative bacteria in biofilms, show strong synergy with conventional antibiotics, and act by targeting a universal stringent stress response. Understanding downstream processes at the molecular level will help to develop and design peptides with increased activity. Anti-biofilm peptides represent a novel, exciting approach to treating recalcitrant bacterial infections.

Keywords

biofilm; synergy; c-di-GMP; ppGpp; stringent response

Introduction

Biofilms are multicellular, three-dimensional aggregates that form on surfaces in both nature and the clinic. They are difficult to treat since biofilms are adaptively resistant to antibiotics (up to 1000-fold) as compared to their free-swimming, planktonic counterparts [1]. Biofilms can form on a variety of tissues and implanted devices, and are implicated in diverse diseases such as cystic fibrosis, wounds, otitis media, pneumonia, and osteomyelitis [2]. Bacterial aggregates that form on medical implants, such as catheters, valves, stents and shunts are difficult to remove except by surgery [2]. The annual cost to the U.S. health care system is on the order of billions [3]. Therefore new therapeutic options are urgently needed. The treatment of biofilm-related infections is very challenging and scientific attention has recently turned to developing agents with specific anti-biofilm activity [4,5]. In particular,

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this review will focus on anti-biofilm peptides, their activity in combination with other antimicrobial agents, and their mechanism of action.

Antimicrobial peptides with potential to fight biofilm-related infections

Antimicrobial peptides (AMPs), characterized here as peptides with activity vs. planktonic bacteria, possess broad-spectrum antibiotic activity against most bacterial pathogens. They are a subset of the host defence peptides, named due to their frequent anti-infective immunomodulatory activity, and are an important part of human innate immunity [6]. Importantly, AMPs do not necessarily affect biofilms. For example, numerous peptides have been developed over the past few years, but comparatively few show anti-biofilm activity below their minimal inhibitory concentration (MIC). A few examples of recently described peptides with anti-biofilm properties are shown in Table 1 and described below.

The human cathelicidin peptide LL-37 has very weak AMP (planktonic antibiotic) activity under physiological conditions [7]. A breakthrough was achieved when it was demonstrated that LL-37 inhibited biofilm formation at concentrations 16-fold below its MIC against planktonic bacteria [8]. LL-37 was subsequently shown to possess anti-biofilm activity against urinary tract isolates of *Staphylococcus aureus* and *Escherichia coli* at 1/32 to 1/2 MIC [9]. Recently, synthetic cathelicidin-derived anti-biofilm peptides (such as innate defense regulator 1018, DJK-5, and DJK-6) were developed, which exhibited broad-spectrum activity against multidrug resistant organisms [10,11].

Aside from cathelicidins, novel discoveries also draw from the diversity of AMPs found in nearly all domains of life [12]. For example, Anunthawan *et al.* demonstrated that the two tryptophan-rich cationic antimicrobial peptides KT2 and RT2 showed anti-biofilm activity at sub-MIC levels against the multidrug-resistant, enterohemorrhagic *E. coli* O157:H7 strain and were able to prevent biofilm formation and eradicate mature biofilms at a concentration of 1 μM [13]. Both peptides interacted with and bound to negatively-charged LPS molecules to enable self-promoted uptake (without forming pores or aggregates) across the outer membrane and subsequently interacted with cytoplasmic membrane phospholipids [13].

Two classes of peptides with unusual structure were also recently developed. The first, SB056, a semi-synthetic peptide with a dendrimeric (dimeric) scaffold was active against planktonic *E. coli* and *S. aureus* and showed anti-biofilm activity against *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* at concentrations half or less the MICs [14]. Remarkably, an optimized linear form (with enhanced amphiphilic profile) of SB056 as well as the dimeric dendrimer were even more active against *S. epidermidis* biofilms. These peptides showed strong affinity for bacterial membranes and the authors postulated that the distribution of hydrophobic and charged residues within the peptide sequence play a role in peptide-lipid interaction [14]. Secondly, Bionda *et al.* [15] used a positional-scanning combinatorial method to screen a cyclic lipopeptide library (peptides derived from fusaricidin/LI-F) against multidrug resistant pathogens. The lead peptide from this study showed activity against all ESKAPE pathogens at 110 μM. Intriguingly, at much lower concentrations (22–28 μM), antibacterial activity was observed against *Enterococcus faecium*, *S. aureus*, *Acinetobacter baumannii*, and *P. aeurginosa.* Furthermore, at a

concentration as low as 4.4 μM, the lead peptide inhibited biofilm formation and eradicated mature biofilms of both *P. aeruginosa* and *S. aureus*. A screen revealed that improved potency depended on hydrophobic as well as positively charged amino acids. These examples highlight the importance of studying and understanding peptide scaffolds, structural order of amino acids within a sequence, peptide activity, and interaction with bacterial membranes.

Peptides can also be active against fungal biofilms. De Brucker *et al*. [16] showed that the cathelicidin-derived peptide AS10 had specific anti-biofilm activity at a concentration of only 0.22 μM (~1 μg/ml) against fungal *Candida albicans* biofilms. This concentration was more than 200-fold less than that needed to inhibit planktonic growth. AS10 also inhibited biofilm formation in a mixed *C. albicans* and *S. epidermidis* population and was active against Gram-negative pathogens including *E. coli*, *P. aeruginosa*, and *Porphyromonas gingivalis*.

Peptides enhance the activity of other antimicrobial agents

In the past few years, many peptides were identified that show strong action against microbial biofilms. Recent studies have also demonstrated that peptides can be used in conjunction with antibiotics, antifungals, or other antimicrobial compounds, which leads to enhanced activity (i.e. synergistic effects) [16–18]. Lowering antibiotic concentrations helps to reduce expenses, toxic side effects, and the spread of antimicrobial resistance. Synergy with peptides can also enhance the activity of antibiotics against multidrug resistant strains [17]. This is highly relevant because biofilm-related infections often result in chronic diseases that fail to be eradicated by antibiotics alone [4].

The synthetic peptides IDR-1018, DJK5, and DJK6 acted synergistically against several Gram-negative pathogens with one or more of the major conventional antibiotics ceftazidime, ciprofloxacin, imipenem and tobramycin [11,19], lowering their effective concentrations up to 64-fold. IDR-1018 also showed synergy with the antiseptic agent chlorhexidine against multispecies oral biofilm [20] and DJK-6 enhanced the activity of the carbapenem imipenem against plasmid-mediated carbapenemase-producing *Klebsiella pneumoniae* [17], highlighting how peptides can be used to repurpose antibiotics.

Not only do peptides show synergy with antibiotics, they also enhance the activity of antifungal drugs. The combination of the lipopeptide bacillomycin D and the antifungal drug amphotericin B was strongly synergistic against *C. albicans* biofilms [18]. Moreover, peptide AS10 was able to act synergistically with the antifungal drugs caspofungin and amphotericin B against *C. albicans* biofilms [16]. The concentration of antifungal required to eradicate a biofilm was reduced 5- to 8-fold in the presence of 0.39–1.56 μM AS10 [16].

In addition to their synergy with antimicrobial agents, peptides can also be used to extend the spectrum of antibiotics. Recently, Mishra *et al*. [21] conjugated CRAMP (murine cathelicidin) to vancomycin and found that the resultant compound was active against both Gram-positive and Gram-negative species. The activity of the conjugate was strongly improved compared to an equimolar mixture of CRAMP and vancomycin, demonstrating

the benefit of covalent linkage. The authors postulated that CRAMP helped translocate vancomycin into the periplasm of Gram-negative bacteria, showing that peptides can help to repurpose and extend the spectrum of antibiotics.

Controlling biofilm infections: Interference with small signaling molecules

Although much attention has been given to AMPs and the broad-spectrum activity of their anti-biofilm counterparts against various bacterial biofilms, the treatment of chronic infections is still very challenging. Critically, the above-described disparity between antibiotic (vs. planktonic cells) and anti-biofilm activity (see also [22]) indicates clear mechanistic differences. Therefore, novel approaches that show how anti-biofilm peptides work on a molecular level in terms of preventing biofilm formation and/or eradicating pre-existing biofilms is the next step in anti-biofilm peptide research.

Intracellular signaling systems are ubiquitous in that the regulatory mechanisms are found in many Gram-positive and Gram-negative pathogens. In this context, nucleotide signaling is an important mechanism that allows microorganisms to control several key processes required for bacterial colonization and adaptation (including quorum sensing), host-microbe interaction [23], and biofilm formation [24]. Second messenger nucleotides include guanosine tetraphosphate (ppGpp), cyclic adenosine/guanosine monophosphate (cAMP, cGMP), and cyclic di-adenosine/di-guanosine (c-di-AMP, c-di-GMP) [4].

Microorganisms must cope with stressors encountered in their environment. The stringent response is mediated by rapid accumulation of pppGpp that is quickly processed to the second messenger ppGpp. Synthesis of these molecules occurs through the global stress response regulator enzymes RelA and SpoT in most Gram-negative bacteria, while a single bifunctional enzyme Rsh is present in Gram-positive bacteria. Amino acid starvation triggers the production of the cellular alarmone ppGpp through RelA. RelA binds to the ribosome, which is blocked by uncharged tRNA molecules, hence catalyzing the synthesis of ppGpp (Figure 1) [25]. Conversely, SpoT promotes ppGpp synthesis under phosphorus, fatty acid or iron starvation [26]. SpoT is additionally a bifunctional enzyme that is able to hydrolyze ppGpp. Rapidly accumulating intracellular ppGpp triggers a switch from cell growth to survival [25]. Recent studies have demonstrated that ppGpp signaling plays a pivotal role in antibiotic resistance, virulence, and biofilm formation. Stringent response signaling acts through several mechanisms such as interaction with RNA polymerase (thereby affecting transcription or influencing the binding of the sigma factor) [27], interaction with other proteins involved in translation, replication and RNA turnover, crosstalk with other second messengers such as c-di-GMP, and by regulation of cellular processes (such as cell-to-cell communication) [26].

Recently, de la Fuente-Nunez *et al.* [10,11] reported that the anti-biofilm peptides IDR-1018, DJK-5, and DJK-6 are able to bind to and trigger degradation of ppGpp, thus preventing intracellular accumulation of this second messenger and thereby preventing biofilm formation in multiple Gram-negative and Gram-positive pathogens. These results show promise that anti-biofilm peptides can be used as broad-spectrum biofilm inhibitors. Of

further interest, the stringent stress response also controls the development of so-called persisters that are able to avoid the action of antibiotics [28].

Second messenger c-di-GMP has emerged as an important universal bacterial second messenger molecule that regulates lifestyle changes in many bacteria. As such it is recognized to play a role in the establishment of multicellular communities (i.e. switch from motile to sessile state), biofilm dispersal, motility, adaptations from the virulent state of acute infections to less virulent (but more resilient) states of chronic infections, cell differentiation and other processes [4,23,24]. Interfering with c-di-GMP signaling pathways potentially constitutes a novel approach of controlling biofilm formation and dispersal, especially since these molecules are absent in mammalian organisms [24].

Biofilm-related infections are often associated with elevated c-di-GMP levels in bacterial pathogens [29], which show reduced motility and increased expression of extracellular matrix components (such as exopolysaccharides, pili formation, adhesins, extracellular DNA) [24,27]. Low intracellular c-di-GMP levels promote dispersal from biofilms and increase bacterial motility [24]. Microorganisms respond to various environmental stimuli by quickly adjusting intracellular c-di-GMP concentrations through the enzymes diguanylate cyclase (catalyzes the formation of c-di-GMP from two GTP) and phosphodiesterase (degrades c-di-GMP into pGpG) (Figure 2) [23,24,27]. It has been shown in *P. aeruginosa* that cells with low c-di-GMP levels are more resistant to the lipopeptide antibiotic colistin, indicating that c-di-GMP signaling also plays an important role in antimicrobial peptide resistance [30]. However, many downstream processes and molecular mechanisms of this regulation involving c-di-GMP have yet to be identified.

Concluding remarks and future directions

Currently, biofilm-related infections are one of the most recalcitrant diseases that make treatment with conventional antibiotics a major challenge in clinics. Understanding the nature of microbial biofilms will help combat biofilm infections. Consequently, research towards the development of novel therapeutic strategies is urgently needed and anti-biofilm peptides appear to be a very promising approach. Single antibiotic administration is often inadequate to overcome bacterial invaders, high antibiotic concentrations are toxic, and multiple antibiotic resistant strains are emerging. Co-administration of antibiotics with anti-biofilm peptides offers a novel strategy that will enhance human health and medicine. Anti-biofilm peptides that can kill multiple species in biofilms or inhibit developed biofilms are promising since they allow administration of lower antibiotic concentrations and therefore present a hopeful alternative treatment with conventional antibiotics. Anti-biofilm peptides interfere with second messenger molecules that control global signaling pathways in both Gram-positive and Gram-negative bacteria, indicative of their broad-spectrum activity. Interrupting complex regulatory systems without killing bacteria should help to circumvent the emergence of drug resistant populations through synergy with existing antibiotics. Future directions will lead to understanding the downstream processes of anti-biofilm peptides and this will help to optimize peptides and enable them to be developed as antibiotic adjuvants as well as stand-alone anti-biofilm therapies.

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Highlights

- To date no antibiotics have been specifically developed for biofilm infections
- Anti-biofilm peptides directly address chronic multi-resistant bacterial infections
- They demonstrate broad spectrum activity
- They uniquely target the stringent stress response required for biofilm growth
- They are synergistic with common antibiotics even against antibiotic resistant bacteria

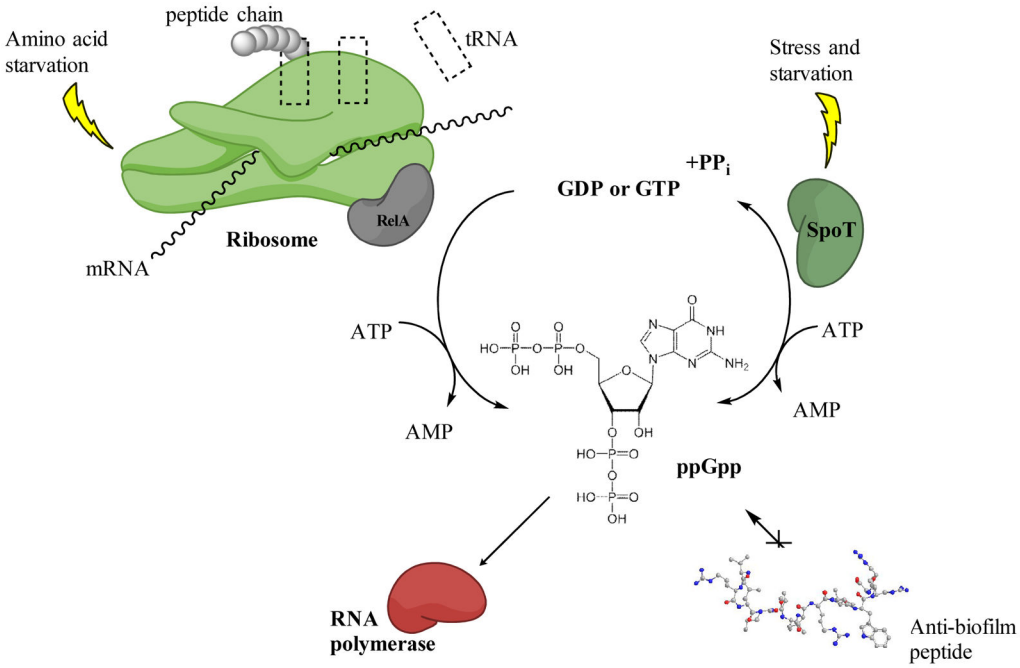

Figure 1. The stringent response

In response to amino acid starvation (uncharged tRNA molecules bind to the ribosome) RelA binds to the ribosome and triggers the production of cellular alarmones ppGpp. Under other stresses such as iron starvation, SpoT triggers the production of ppGpp. ppGpp signalling molecules bind to the RNA polymerase thereby affecting transcription. Rapid accumulation of ppGpp in the cell cause a switch from cell growth to survival. Anti-biofilm peptides are able to block intracellular accumulation of ppGpp. Figure modified from [32].

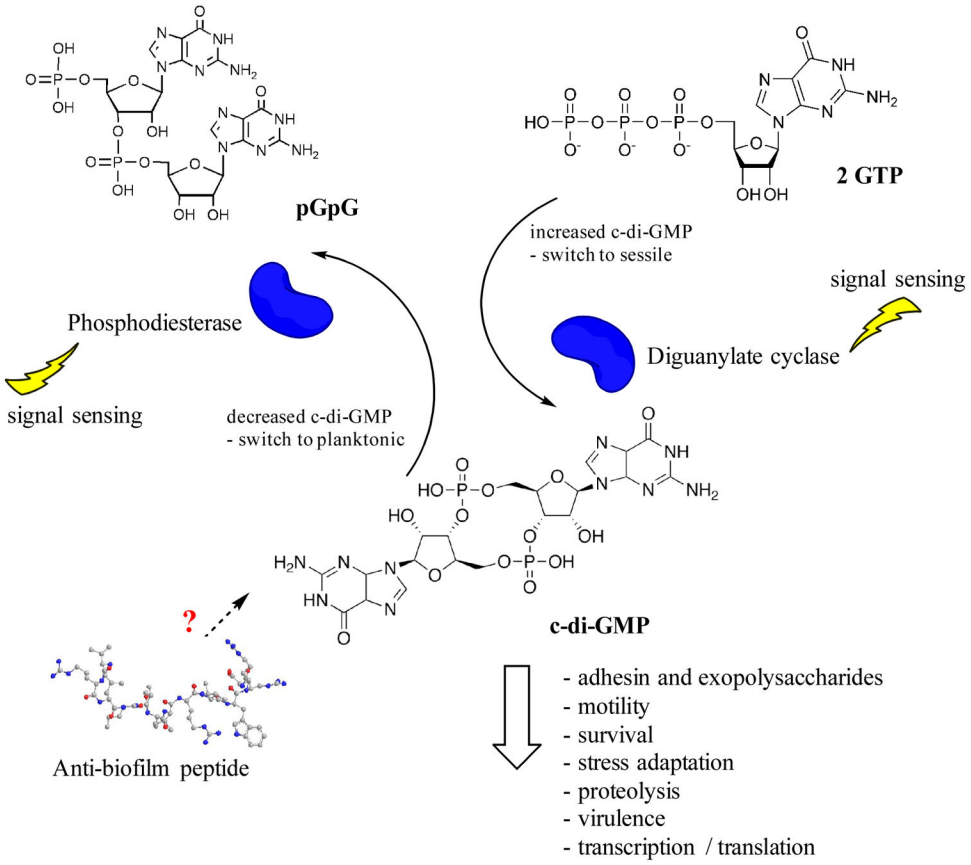

Figure 2. Cyclic-di-GMP levels are controlled by the enzymes diguanylate cyclase (increasing) and phosphodiesterase (decreasing)

Thereby, they regulate the switch from planktonic growth to sessile and vice versa. High c-di-GMP levels stimulate biofilm formation and other factors such as stress adaptation, virulence, etc. The role of anti-biofilm peptides in the c-di-GMP pathway has yet to be identified.

Table 1

Recent studies on peptides with specific anti-biofilm activity.

Peptide	Minimal Inhibitory Concentration	Active biofilm concentration	Active against	Reference
AS10	50 μM[a]	0.22 μM[b]	*C. albicans*	[16]
KT2 and RT2	5 – 18 μM	1 μM[c]	*E. coli*	[13]
SB056 and derivatives	10 – >40 μM	5–20 μM[d]	*S. epidermidis*, *P. aeruginosa*	[14]
Cyclic lipopetide **3**	22 –55 μM	4 μM[c]	*S. aureus*, *P. aeruginosa*	[15]
LL-37 and derivatives	32 μg/ml	1–16 μg/ml[d]	*S. aureus*, *E. coli*	[9,31]
(IDR-)1018	8 – 128 μg/ml	2–8 μg/ml[b]	*A. baumannii, E. coli, K. pneumoniae, P. aeruginosa, S. enterica, S. aureus*	[10]
DJK-5	1.6 – 16 μg/ml	0.8 – 4 μg/ml[b]	as for IDR-1018	[11]
DJK-6	4 – 16 μg/ml	0.5 – 8 μg/ml[b]	as for IDR-1018	[11]

[a]Minimal fungicidal concentration

[b]Minimal biofilm inhibitory concentration

[c]Active concentration in flow cells

[d]Concentrations showing biofilm inhibition