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Potential Application of Antimicrobial Peptides in the Treatment of Bacterial Biofilm Infections

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Abstract: The increasing prevalence of persistent biofilm infections, such as wound infections, chronic lung infections or medical device-related infections, which usually tolerate conventional antibiotic treatment, calls for the development of new therapeutic strategies. To date, antimicrobial peptides (AMPs) are considered as promising agents in the fight against multidrug-resistant bacterial biofilm infections, since many of them have been shown to prevent biofilm formation or even kill preexisting, mature biofilms of several Gram-positive and Gram-negative bacteria in addition to their bactericidal actions to planktonic cells. In this mini-review, we summarize *in vitro* and *in vivo* antibiofilm properties of natural and synthetic cationic AMPs against clinically relevant bacterial pathogens. Furthermore, the benefits and challenges in the use of AMPs for the medical treatment of bacterial biofilm infections are discussed.

Keywords: Antimicrobial peptide, cationic peptides, biofilm infection, host defense peptide, multidrug-resistant bacteria, chronic infection, antibiofilm.

INTRODUCTION

Biofilm formation is the critical factor and ultimate cause of persistence in chronic bacterial infections such as implant-associated osteomyelitis [1], heart valves endocarditis [2], chronic wound infections [3], catheter and ventilator tube infections [4-6] as well as chronic lung infections in cystic fibrosis patients [7], among many others. Bacteria grown in biofilms are imbedded in a self-produced, extracellular polymeric matrix consisting of proteins, lipids, nucleic acids and polysaccharides [8]. Once established, these sessile communities are protected from the host immune response and are extremely difficult to eradicate due to their high intrinsic resistance against various antibiotic agents [9-12]. Thus, biofilm growing bacteria can possess a 10 – 1000 fold higher tolerance to antibiotics and disinfectants based on conventional resistance mechanisms, for example efflux pump expression, modification of antibiotic targets or enzymatic cleavage of antibiotics [13], as well as specific properties of biofilms [14]. These properties comprise the stationary-phase physiology, extremely slow growth rates of bacteria in the center of the biofilm, possible matrix mediated binding of antimicrobial agents and diffusion retardation. Furthermore high concentrations of extracellular enzymes capable of degrading antibiotics have been detected in biofilms [12, 14, 15]. In general, biofilms cause more than 80 % of all bacterial infections [16] and are responsible for considerable morbidity and significantly contribute to the escalation in the cost of health care [17]. In a cohort study by Whitehouse *et al*., orthopedic surgical site infections increased hospitalization costs by 300 % compared to non-infected orthopedic surgery cases ($24,344 vs. $6,636 per patient) [18]. Accordingly, annual costs for medical treatment of an infected diabetic ulcer have been estimated to $17,000 per patient compared to $8000 for the treatment of a single ulcer [17].

AMPs are an abundant and diverse group of molecules which are produced by a wide range of organisms as part of their first line defense. They are typically relatively short consisting of 12 – 100 amino acids, are positively charged with a net charge of +2 to +9 (Table **1**), are amphiphilic and have been isolated from single cell microorganisms to plants, amphibians, birds, fish and mammals including humans [19]. Until now, more than 1000 AMPs have been isolated or predicted by computational programs and have been divided into different sub-groups based on their structure and amino acid composition [20-22]. Some cationic peptides exhibit a rather weak antimicrobial activity under physiological conditions and therefore their ability to modulate the immune response through a variety of different mechanisms may be of more importance [23, 24]. Human LL-37, for example, has been shown to induce the expression of monocyte- and lymphocyte-derived chemokines and cytokines [25-27] and to stimulate the differentiation of dendritic and bone forming-like cells [24, 28]. Nevertheless several cationic peptides possess strong antimicrobial properties against a broad spectrum of Gram-positive and Gram-negative bacteria (Fig. **1**) [24, 29]. In addition to intracellular targets like inhibition of protein synthesis, binding of nucleic acids, blocking of DNA replication and interference with cell-wall synthesis (Fig. **1**) [20, 24, 30, 31], many AMPs target the bacterial cell membrane leading to membrane disruption and subsequent cell death. This mode of action is an appealing strategy to combat especially dormant, non-growing bacteria in persistent biofilm infections [32]. It is mainly based on the attachment and integration of AMPs into the bacterial cell membrane for which several models have been proposed. Most prominent models including the barrel-stave model, the carpet model, the aggregate model and the toroidal model of AMP-induced killing have been reviewed previously in more detail elsewhere and will not be discussed in this mini-review [20, 31-34]. Since their mechanism of action has been shown to address multiple targets within the bacterial cell resulting in low resistance development, cationic AMPs are considered as a promising class of new antimicrobial agents against many highly resistant human pathogens including multidrug-resistant *Pseudomonas aeruginosa (P. aeruginosa)*, methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecalis* and *Enterococcus faecium* (VRE) or extended spectrum ß-lactamase producing *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae (K. pneumoniae)* [35].

Antibiotic activities of AMPs are dependent on different peptide properties including a positive net charge, hydrophobicity, amphipathicity, secondary structures (α-helix or β-sheet) and the presence of aromatic amino acid residues (especially tryptophan) [20, 36, 37]. As mentioned previously, most AMPs possess an overall positive net charge due to a high content of lysine and arginine residues which facilitates the interaction with negatively

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Table 1. Properties of selected AMPs exhibiting an antibiofilm activity.

Peptide	Amino acid sequence	No. of amino acids	Molecular weight (g/mol)[1]	Net charge[1; 2]
Citropin 1.1	GLFDVIKKVASVIGGL-NH_2 [204]	16	1615	2
G10KHc	KKHRKHRKHRKH-GGSGGS-KNLRRIIRKGIHIIKKYG [99]	36	4267.12	15.4
HBD-3	GIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKK [205]	45	5161.24	10.7
Indolicidin	ILPWKWPWWPWRR-NH_2 [206]	13	1906.32	4
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES [58]	37	4493.33	6
RIP	YSPWTNF-NH_2 [170]	7	913	1
Tachyplesin III	KWCFRVCYRGICYRKCR-NH_2 [74]	17	2239.8	6.8
1037	KRFRIRVRV [16]	9	1229.54	5

[1]Calculation by http: //www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp
[2]Net charge at pH 7

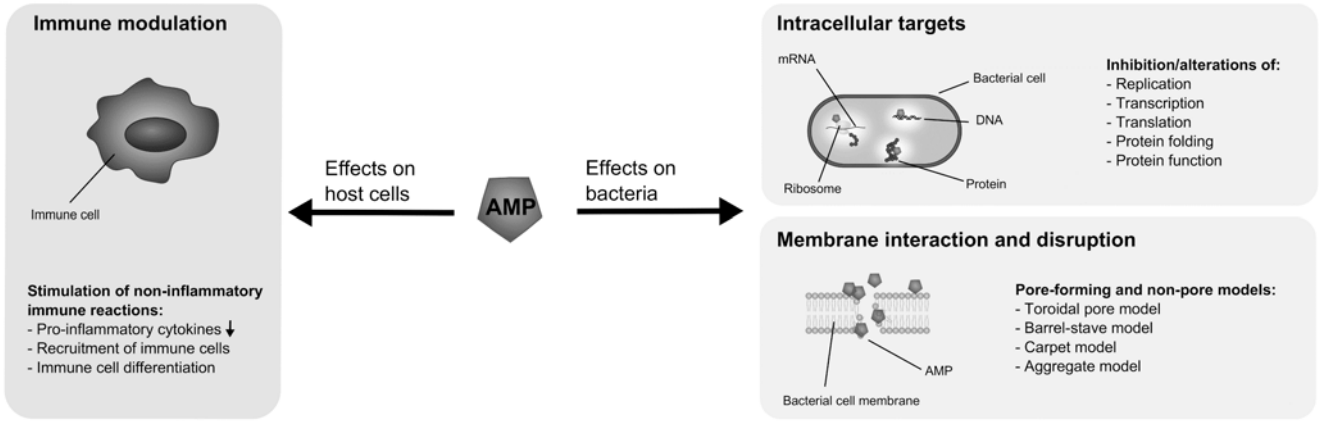

Fig. (1). Antibacterial activities of AMPs against planktonic bacteria. Bactericidal actions of AMPs include direct killing of bacteria by cell membrane disruption and inhibition of cellular processes, such as DNA-replication, transcription, protein biosynthesis and folding or impairment of protein functions and immunomodulatory effects leading to bacterial clearance by stimulation of non-inflammatory host immune responses. ↓ inhibition.

charged bacterial membranes [37]. Several studies demonstrated a clear correlation between cationicity and antimicrobial activity [38-40] with an optimum charge between +3 and +5 [40]. A further enhancement in cationicity, however, impaired cell selectivity and therefore enhanced cytotoxicity against mammalian cells, whereas the antimicrobial activity was decreased [40]. Strøm *et al.* investigated the minimum antibacterial motif of a series of short peptides by systematic alterations of either net charge, the content of bulky residues or hydrophobicity [41]. They figured out that a net charge of +2 and the presence of not more than two bulky moieties were required for anti-staphylococcal activity of the tested peptides. In case of *E. coli*, at least three bulky and two cationic residues were necessary and the antibacterial activity of AMPs was furthermore enhanced by the addition of charged or bulky moieties [41]. The hydrophobicity of natural AMPs typically ranges between 40 and 60 % [36] and an excess in hydrophobicity has been described dramatically lower the antimicrobial activity and enhance the hemolytic potential of several AMPs [42-44] including synthetic lipopeptides [45, 46]. Similar observations have been made for unusual high degrees of amphipathicity, i.e. the spatial separation of clusters of hydrophobic and polar residues [47]. In general, moderate levels of mentioned features favor the interaction between AMPs and bacterial membranes and thus promote peptide integration and membrane disruption [37].

Besides immune modulation and direct killing of planktonic bacteria (Fig. **1**), AMPs have been increasingly recognized in the last years as potential agents to combat chronic bacterial biofilms. Here we give an overview about reported *in vitro* and *in vivo* antibiofilm properties of natural and synthetic AMPs against different Gram-positive and Gram-negative bacteria, which are known to cause persistent biofilm infections (summarized in Table **2** and Fig. **2**). The use of AMPs against oral biofilms as well as properties and therapeutic potential of anionic AMPs have been extensively discussed in recent publications [48-52] and will not be part of this review.

CATHELICIDINS AND DERIVATIVES

Cathelicidins represent one major class of cationic AMPs in vertebrates, containing a highly conserved N-terminal cathelin region and a C-terminal domain, which exhibits a strong intra- and interspecies diversity. During immune reaction, mature peptides are cleaved from inactive precursor proteins by neutrophil proteases

Table 2. ***In vitro / in vivo*** **antibiofilm activities of selected AMPs.**

Peptide	Origin/Description	Structure	*in vitro* antibiofilm activity			*in vivo* anti-biofilm activity
			Prevention	Killing	Dispersal	
Cathelicidins of human and animal origin (and derivatives)						
LL-37	Human cathelicidin	α-helical, cationic, linear	*P. aeruginosa* [57-59], *E. coli* [60], *S. epidermidis* [62], *S. aureus* [63], *F. novicida* [61]	*P. aeruginosa* [58], *B. pseu-domallei* [207]	*P. aeruginosa* [57, 59]	
D-LL-37	D-enantiomer of human LL-37		*P. aeruginosa* [59], *S. aureus* [63]		*P. aeruginosa* [59]	
LL-19, LL13-31, LL7-25, LL7-37, LL-31, LL7-31	LL-37 fragments		*P. aeruginosa* [58]	*P. aeruginosa* [58], *B. pseu-domallei* [207]	*P. aeruginosa* [58]	*P. aeruginosa* [157]
NA-CATH	Chinese cobra (*Naja atra*) cathelicidin		*S. aureus* [63]			
NA-CATH: ATRA1-ATRA1	NA-CATH derivative		*S. aureus* [63]			
SMAP-29	Sheep cathelididin		*P. aeruginosa, S. maltophilia* [65]	*S. aureus, P. aeruginosa, S. maltophilia* [65]		
Novispirin G10	Synthetic, based on SMAP-29					*P. aeruginosa* [69, 71], *S. aureus* [70]
BMAP-27	Bovine cathelicidin		*P. aeruginosa, S. maltophilia* [65, 66], *S. aureus* [66]	*P. aeruginosa* [65, 66], *S. aureus, S. maltophilia* [65]		
BMAP-28	Bovine cathelicidin		*S. aureus* [65-67], *P. aeruginosa , S. maltophilia* [65, 66]	*P. aeruginosa* [65, 66], *S. aureus, S. maltophilia* [65]		*S. aureus* [67]
$F_{2, 5, 12}W$	Short variant of chicken cathelicidin-2		*S. epidermidis* [208]	*S. epidermidis* [208]		
Indolicidin	Bovine cathelicidin	Extended α-helix structure, high tryptophane content	*P. aeruginosa* [57], *S. aureus* [68]			

(Table 2) Contd….

Peptide	Origin/Description	Structure	*in vitro* antibiofilm activity			*in vivo* anti-biofilm activity
			Prevention	Killing	Dispersal	
Non-Cathelicidins of human and animal origin						
Lactoferrin	Bovine/human iron binding protein	Glycoprotein, 692 amino acids	*P. aeruginosa* [209-211], *E. coli* [212, 213], *B. cenocepacia*, *B. multivorans*, *B. dolosa* [214]	*P. aeruginosa* [210]	*B. cenocepacia*, *B. multivorans*, *B. dolosa* [214], *P. aeruginosa* [211]	
HBD-3	Human β-defensin	Cationic, linear, cysteine-rich, β-sheet structure	*S. aureus* [81], *S. epidermidis* [81]	*S. aureus* [81, 82], *S. epidermidis* [81]	*S. aureus* [81, 82], *S. epidermidis* [81]	
Coprisin	Defensin-like peptide from *Copris tripartitus* (dung beetle)	Cationic, linear, 43 amino acids, β-sheet and α-helix structures [215]			*P. aeruginosa, S. aureus, E. coli, E. faecium* [73]	
Citropin 1.1	Major AMP of *Litoria citropa* (green tree frog)	α-helical, cationic, linear		*S. aureus* [76]		*S. aureus* [76]
Tachyplesin III	Southeast Asian horseshoe crab *Tachypleus gigas*	β-sheet, circular, cationic, 17 amino acids		*P. aeruginosa* [74]		*P. aeruginosa* [74]
Aurein 2.5	Australian Bell Frogs *Litoria aurea* and *Litoria raniformis*	Cationic , linear C-terminally amidated		*E. coli, B. subtilis* [78]		
PSN-1	Phylloseptin from *Phyllomedusa sauvagei* (waxy monkey frog) skin	Cationic, linear C-terminally amidated 19 amino acids		*S. aureus* [77]		
5-CC	*Paracentrotus lividus* (sea urchin) β -thymosin fragment	5-kDa peptide	*S. epidermidis, S. aureus* [75]		*S. epidermidis, S. aureus* [75]	
Pleurocidin	*Pleuronectes americanus* (winter flounder)	α-helical, cationic, linear			*P. aeruginosa, P. acnes, S. aureus, E. coli, E. faecium* [79]	
Bacterial origin						
Polymyxin B	*P. polymyxa* polypeptide antibiotic	Lipopeptide, circular, cationic, branched		*P. aeruginosa* [216, 217], *S. aureus* [217, 218]	*P. aeruginosa* [216]	

(Table 2) Contd….

Peptide	Origin/Description	Structure	*in vitro* antibiofilm activity			*in vivo* anti-biofilm activity
			Prevention	Killing	Dispersal	
Polymyxin E (Colistin)				*P. aeruginosa* [88, 89], *S. maltophilia* [90], *A. baumanii* [91]	*A. baumanii* [91]	*P. aeruginosa* [88, 219] *A. baumanii* [92]
Nisin	*L. lactis* lantibiotic	Polycyclic, lanthionine-containing	*S. aureus* [68]			
Bacitracin	*B. subtilis*	Circular, cationic				*S. aureus* [158]
Synthetic AMPs						
1037	Synthetic AMP based on bovine cathelicidin derivative Bac2a	Cationic, linear, 9 amino acids	*P. aeruginosa, B. cenocepacia, L. monocytogenes* [16]	*P. aeruginosa* [16]		
P19(9/B)	Synthetic cathelicidin	α-helical, cationic, linear	*P. aeruginosa, S. maltophilia, S. aureus* [66]	*P. aeruginosa* [66]		
$(RW)_3$-NH_2	Hexameric peptide	Linear, cationic, various arginine and tryptophane repeats	*E. coli* [97]	*E. coli* [97, 98]	*E. coli* [97]	
$(RW)_4$-NH_2	Octameric peptide		*E. coli* [97]	*E. coli* [97, 98]	*E. coli* [97, 98]	
$(RW)_{4D}$	Dendrimeric peptide	Circular, arginine and tryptophane-rich	*E. coli* [96]	*E. coli* [96, 98]		
Ltx5, Ltx9, Ltx10	Synthetic tripeptides	Cationic, 2 arginine-residues, 700-800 Da		*S. aureus, S. epidermidis, S. haemolyticus* [220]		
PTP-7	Lytic peptide, analogue of Gaegurin 5 from *Glandirana emeljanovi* (Korean frog)	α-helical, cationic, linear	*S. aureus* [221]	*S. aureus* [221]	*S. aureus* [221]	
DASamP1	STAMP	Linear, cationic, no cysteine residues				*S. aureus* [101]
G10KHc	STAMP, Novispirin G10 derivative	Chimeric peptide: novispirin G10 + targeted peptide domain for *Pseudomonas spp*.		*P. aeruginosa* [99]		
β6-20-G_3K_6	STAMP	Cationic polylysine peptide		*S. epidermidis* [100]	*S. epidermidis* [100]	
Peptidomimetics						
D2S	Disubstituted dexamethasone-spermine	Cationic corticosteroid derivative		*P. aeruginosa* [222]	*P. aeruginosa* [222]	

(Table 2) Contd….

Peptide	Origin/Description	Structure	*in vitro* antibiofilm activity			*in vivo* anti-biofilm activity
			Prevention	Killing	Dispersal	
Ceragenin (CSA-13)	Peptidomimetic	Amphiphilic steroide conjugate	*P. aeruginosa* [107]	*P. aeruginosa* [108, 109], *E. faecalis, S. aureus, H. pylori, M. catarrhalis* [108]	*P. aeruginosa* [108]	
Peptoid 1	Peptoid	Oligo-*N*-substituted glycine containing peptides		*P. aeruginosa* [110]	*P. aeruginosa* [110]	
Peptoids 1-C13$_{4mer}$, 1-achiral, 1-Pro$_9$	Peptoid 1 derivatives		*P. aeruginosa* [110]	*P. aeruginosa* [110]	*P. aeruginosa* [110]	
FD2	Glycopeptide dendrimers	Fucosylated branchened peptide	*P. aeruginosa* [152, 223]		*P. aeruginosa* [152, 223]	
GalAG2, GalBG2		Galactosylated branchened peptides	*P. aeruginosa* [152, 223]			
VAN, 1d, 2b, 3b, 4c, 4d	Peptidomimetics	β-peptoid-peptide hybrid oligomers	*S. epidermidis* [111]	*S. epidermidis* [111]	*S. epidermidis* [111]	

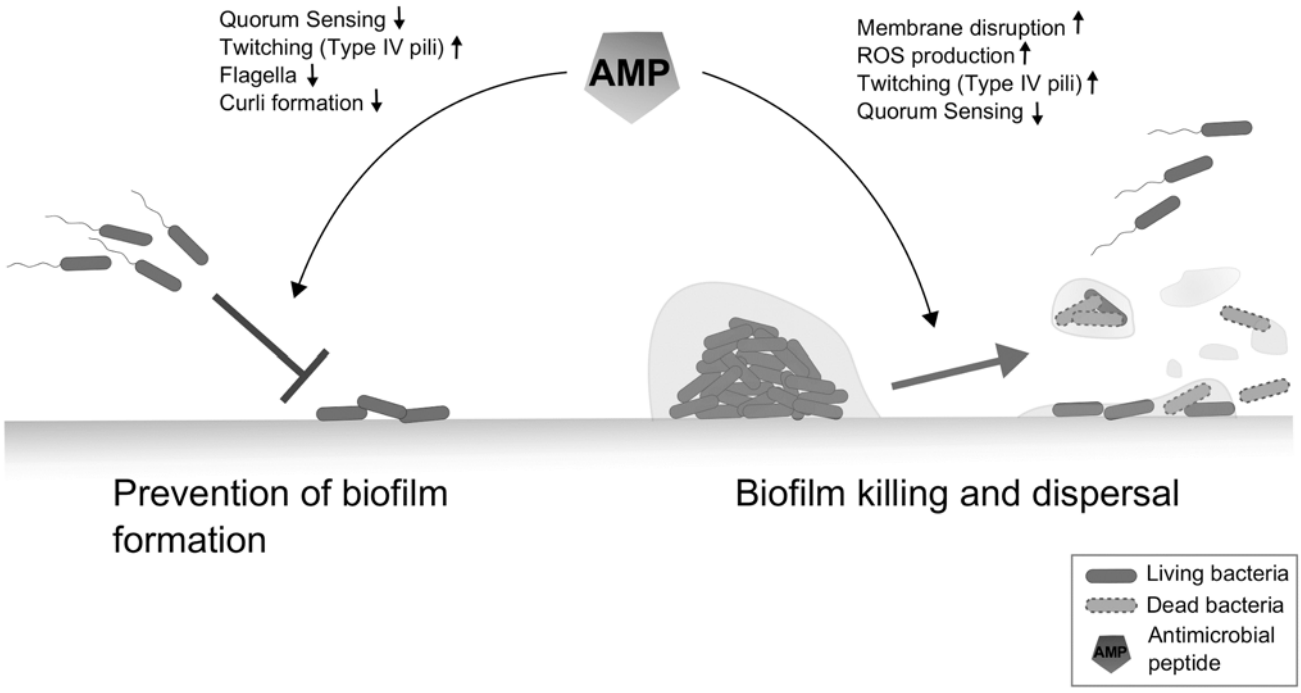

Fig. (2). Antibiofilm effects of AMPs and involved mechanisms. These effects include the inhibition of bacterial adhesion to a surface and thereby the prevention of biofilm formation at early stages and the disruption of preexisting biofilms. In contrast to many common antibiotics several AMPs have been additionally shown to rapidly penetrate biofilms and exert their killing actions even on slowly growing or non-growing biofilm cells. ↑ stimulation, ↓ inhibition, ROS: reactive oxygen species.

and directly released to their site of action. The size of mature cathelicidins mostly ranges from 12 to 80 amino acid residues yielding different secondary structures such as β-sheets and α-helices [53, 54].

LL-37, the major AMP in humans, is a 37 amino acid residue-containing, linear cationic α-helical peptide cleaved from cathelicidin hCAP18 [25]. It is expressed in a variety of different cell types and tissues, including neutrophils, bone marrow cells and epithelial cells of the respiratory tract, skin and gastrointestinal tissues [55]. Despite its modest antimicrobial activity against a broad range of Gram-negative and Gram-positive bacteria and its immunomodulatory properties, LL-37 has been shown to act as a potent inhibitor of bacterial biofilm formation [25, 56]. Overhage *et al*. demonstrated, that LL-37 at concentrations far below its minimal inhibitory concentration (MIC) against planktonic bacteria, is able to prevent *P. aeruginosa* biofilm formation and even to disrupt preformed *P. aeruginosa* biofilms [57]. Several studies from other groups confirmed this preventive antibiofilm effect for Gram-negative *P. aeruginosa* [58, 59], *E. coli* [60] and *F. novicidia* [61] and Gram-positive *Staphylococcus epidermidis* (*S. epidermidis)* [62] and *Staphylococcus aureus* (*S. aureus)* [63]. Gene expression studies of LL-37 treated *P. aeruginosa* cells revealed a downregulation of *rhl* and *las* quorum sensing and flagella genes, which are both required for biofilm formation, whereas twitching motility genes *pilT, pilI, pilJ, pilD, fimU, pilV, pilW and pilY1* were stimulated by LL-37 – suggesting that these alterations in gene expression may lead to the observed reduced attachment and biofilm formation of *P. aeruginosa* in response to sub-MIC levels of LL-37 [57, 61]. Kai-Larsen *et al*. demonstrated that LL-37 inhibits the polymerization of CsgA, a major subunit of *E. coli* curli, which are essential for *E. coli* adhesion to surfaces, by direct binding to CsgA and thereby prevents biofilm formation in uropathogenic *E. coli* [60]. Screening of a library of truncated LL-37 peptides revealed a preventive antibiofilm activity in *P. aeruginosa* of all peptide fragments containing the core α-helix structure. This finding indicated a key role of this secondary structure for its ability to inhibit bacterial adhesion. The most promising LL-37 fragment, peptide LL7-37, was furthermore able to kill *P. aeruginosa* biofilm cells and even reduce biomass of pregrown biofilms. In contrast to full length LL-37, viability of eukaryotic host cells was not affected by fragment LL7-37 [58]. Since LL-37, as well as other naturally occurring AMPs, is rapidly degraded by endogenous proteases such as neutrophil elastase, trypsin or cathepsin D [59, 63, 64] - a fact that strongly limits its therapeutic potential - many studies aim on the development of cathelicidin derivatives combining antimicrobial and antibiofilm activities of the natural peptide with an enhanced peptide stability. The D-enantiomer of LL-37, for example, showed a comparable biofilm inhibition in *P. aeruginosa* [59] and *S. aureus* [63] and was able to disperse preformed *P. aeruginosa* biofilms [59], while being inherent against protease fragmentation.

In addition to human LL-37, cathelicidins derived from other eukaryotes such as bovine BMAP-27 [65, 66], BMAP-28 [65-67] and tryptophan-rich indolicidin [57, 68], sheep SMAP-29 [65] and NA-CATH, an α-helical AMP of the Chinese cobra *Naja atra* [63] has been successfully demonstrated to prevent biofilm formation in clinically relevant pathogens. SMAP-29, BMAP-28 and BMAP-27 showed furthermore a potent killing of *P. aeruginosa*, *Stenotrophomonas maltophilia* (*S. maltophilia*) and *S. aureus* biofilm cells by rapid cell membrane permeabilization [65, 66] and were still active against various clinical isolates of *P. aeruginosa*, *S. maltophilia* and *S. aureus* under O_2-limiting, acidic, mucus-rich and high-salt conditions simulating the cystic fibrosis lung environment [66].

Novispirin-G10, a short α-helical synthetic cationic peptide, based on the sheep cathelicidin SMAP-29, has been considered as a promising local therapeutic for burn wound infections and cystic fibrosis pneumonia, since results of rat and porcine burn wound infection models [69, 70] and a porcine model of cystic fibrosis chronic lung infection [71] demonstrated a significant reduction of surviving *S. aureus* [70] or *P. aeruginosa* [69, 71] cells after topical peptide treatment. In addition, cytotoxicity against human lung epithelial cells and keratinocytes as well as hemolytic activity was considerably reduced for novispirin-G10 in comparison to the natural porcine AMP protegrin-1. Protegrin-1 has also been shown to eradicate bacterial biofilm cells, albeit with less efficiency than novispirin-G10 [69, 70].

NON-CATHELICIDIN AMPs OF HUMAN OR ANIMAL ORIGIN

In addition to natural cathelicidins and analogues, cationic peptides possessing a potent antibiofilm activity have been found in other classes of host defense peptides of human or animal origin, including defensins, defensin-like peptides, histatins and lactoferrin, a milk innate immune defense molecule, whose broad range antibiofilm properties were recently summarized in an excellent review by Ammons and Copié [72].

Among tested invertebrate peptides, the 43-mer coprisin was able to disperse biofilms of various Gram-negative and Gram-positive bacteria [73], whereas crab AMP tachyplesin III successfully killed *P. aeruginosa* cells within established *in vitro* and *in vivo* grown biofilms without exhibiting toxic effects on treated rats in the *in vivo* urinary catheter infection animal model [74]. Prevention of staphylococcal biofilm formation was achieved by application of sea urchin peptide 5-CC, which additionally destroyed pregrown *S. epidermidis* and *S. aureus* biofilms by a yet unknown mechanism [75].

Vertebrate AMPs citropin 1.1 and phylloseptin 1, both amphibian skin peptides, showed a considerable antimicrobial activity against sessile *S. aureus* cells [76, 77]; however, a biofilm killing effect of aurein 2.5, another frog peptide, has been reported only for *E. coli* and *Bacillus subtilis* (*B. subtilis)* [78]. Pleurocidin, a fish AMP, which exerts its antibacterial actions on various planktonic pathogens by NADH depletion and a subsequent massive increase in hydroxyl radical formation leading to membrane disruption and bacterial cell death, was furthermore able to effectively kill *P. aeruginosa*, *S. aureus*, *E. coli*, *Enterococcus faecium* (*E. faecium)* and *Propionibacterium acnes* (*P. acnes)* cells in an *in vitro* biofilm assay [79].

Human β-defensin-3 (HBD-3) is a member of the defensin class of mammalian AMPs, which are characterized as cationic, non-glycosylated 3.5 – 6 kDa peptides with a high arginine content and 6 cysteine residues exhibiting either α-helix (α-defensins) or β-sheet structures (β-defensins) [53]. In contrast to HBD-1 and HBD-2, whose direct antimicrobial activities are restricted to Gram-negative bacteria such as *E. coli* or *P. aeruginosa*, HBD-3 has been shown to affect both, Gram-negative and Gram-positive bacteria, including multi-drug resistant *S. aureus* and *E. faecium* strains [80]. Zhu *et al.* [81] and Huang *et al.* [82] additionally demonstrated, that HBD-3 is able to impair staphylococcal biofilm formation during the primary adhesion phase and furthermore kill and disperse pregrown *S. aureus* and *S. epidermidis* biofilms.

AMPs OF BACTERIAL ORIGIN

Bacterial AMPs include post-translationally modified class I bacteriocins (for example lantibiotic nisin), mostly unmodified class II bacteriocins (for example mersacin), which, in general, exhibit a strong antimicrobial activity against Gram-positive bacteria [83, 84], and cationic circular polymyxins (for example polymyxin B and colistin), targeting the cell membrane of Gram-negative bacteria [85]. The therapeutic potential of bacteriocins and derivatives and their prospective application in multi-drug resistant infections is extensively discussed in a recent review by Cotter *et al.* [83], whereas, with respect to bacterial AMPs, this review focuses on antibiofilm activities of polymyxins, which have been used as “last resort antibiotics” in clinical practice since the 1940s.

Polymyxins are amphiphatic circular polypeptide antibiotics, containing a cationic heptapeptide ring and a hydrophobic fatty acid chain, both connected via an additional tripeptide, which are produced by *Paenibacillus polymyxa* [85]. The antimicrobial activity of polymyxins against Gram-negative bacteria is mainly based on their ability to integrate into negatively charged bacterial membranes leading to a detergent-like membrane destabilization effect and lipopolysaccharide (LPS) neutralization by electrostatic interactions with antibiotics [86]. Adverse effects, such as a high neuro- and nephrotoxicity, hampered the widespread use of polymyxins soon after their discovery in 1947 [86]. The rise of infections caused by multi-drug resistant Gram-negative pathogens, led to a revival of polymyxins as "last hope antibiotics", primarily in the treatment of chronic lung infections with *P. aeruginosa*, local burn wound infections or device-related infections with multi-drug resistant *Acinetobacter baumanii* (*A. baumanii)* [85-87], since many recent studies report an antibiofilm activity of polymyxins in addition to their direct bactericidal functions. Different *in vitro* biofilm assays indicate a potent killing activity of colistin (polymyxin E) against *P. aeruginosa* [88, 89], *S. maltophilia* [90] and *A. baumanii* [91] biofilms; for example 24 – 48 hours treatment of 4-day old *P. aeruginosa* biofilms grown in a flow reactor with 10-fold MICs resulted in ~80 % reduction of viable cells compared to non-treated controls [88]. In a rat model mimicking cystic fibrosis chronic lung infections, animals were infected with *P. aeruginosa* cells which are embedded in alginate beads. Subsequent intratracheal application of colistin at 64-fold MIC concentrations for planktonic bacteria led to the survival of 80 % of infected rats during the 7 day experiment. In contrast, 92 % of infected animals died in the untreated control group. Additional administration of the antibiotic tobramycin enhanced the bacteriocidal effect of colistin, leading to a significant reduction of viability of biofilm cells, as confirmed by colony forming unit (CFU) counts after 7 days [88]. In a recent case report, a 33 year old man suffering from a persistent urinary infection with multi-resistant *A. baumanii* continuously received colistin through a urinary device over seven days, resulting in total elimination of intravesicular biofilm bacteria in urine, and without exhibiting any adverse effects of colistin administration [92].

Despite these promising reports regarding the clinical application of colistin, it has to be mentioned, that the antibiotic is not active against Gram-positive bacteria and therefore not suitable for the treatment of biofilm infections caused by MRSA or VRE [93].

SYNTHETIC AMPs AND PEPTIDOMIMETICS

Although innumerable studies affirm the potent antibiofilm activity of natural AMPs even in case of infections with multidrug resistant pathogens, an extensive clinical application is hindered due to several problems, such as high production costs, undesired immunomodulatory effects and rapid protease degradation. Therefore, much effort is made on the development of synthetic AMPs with improved properties.

SYNTHETIC AMPs

In a recent screen of a library containing short synthetic AMPs whose amino acid compositions are loosely based on bovine bactenicin-derivative Bac2A, a number of different peptides preventing biofilm formation in *P. aeruginosa* was identified. Since amino acids FRIRVRV were a common feature of all peptides exhibiting this antibiofilm activity, the sequence has been considered as antibiofilm consensus sequence [16]. The most potent agent, 1037, an amphiphilic 9-mer peptide, showed a significant inhibition of biofilm formation in *P. aeruginosa*, *Burkholderia cenocepacia* (*B. cenocepacia)* and *Listeria monocytogenes* (*L. monocytogenes)* at sub-MIC concentrations. For *P. aeruginosa* PAO1, half MIC concentrations of 1037 led to a 78 % reduction of biofilm biomass and concentrations of 10 µg/ml (~ 1/30 MIC) still inhibited biofilm development by 50 %. Microarray data and phenotypic analysis of *P. aeruginosa* cells treated with 1037 revealed a stimulation of twitching motility, which is associated with biofilm dispersal, and a downregulation of flagella-mediated swimming and swarming motility, which are both required for the primary adhesion step of biofilm formation. Further downregulated genes affecting attachment and biofilm formation, comprised genes *rhlB, lecB, nirS, norC, nosZ* and gene PA2204 encoding a probable ABC transporter binding protein, whereas chemotaxis genes were upregulated by 1037 [16]. It has been shown for *Pseudomonas fluorescens* that expression of ABC transporter genes *lap* is required for irreversible surface adhesion of bacteria [94]. In contrast, *L. monocytogenes* ABC transporter permease *lmG_1771* has been demonstrated to act as a negative regulator of biofilm development [95]. Thus, the relevance of ABC transporters for biofilm formation is still unclear.

A set of short tryptophan and arginine-rich linear and circular peptides, which only differ in chain length, exhibited considerable effects on *E. coli* planktonic and biofilm growth as well as on killing of persister cells in biofilms. In comparison to tetrameric peptides, longer hexameric $(RW)_3$-NH_2 and octameric peptides $(RW)_4$-NH_2 prevented biofilm growth and even dispersed pregrown biofilms, while swarming motility was impaired by the peptides. Circular dendrimeric peptide $(RW)_{4D}$ showed similar antibiofilm effects, but did not promote *E. coli* biofilm dispersal [96-98].

A further development in the area of biofilm control is the construction of selectively-targeted antimicrobial peptides (STAMPs) affecting only single species within biofilms in order to prevent undesired eradication of harmless probiotic bacteria. Examples are novispirin G10 derivative G10KHc, which affects *P. aeruginosa* cells [99] and β-6-20-G3K6 with respect to *S. epidermidis* biofilms [100]. DASamP1, a short cationic peptide selectively targeting *S. aureus* without promoting hemolysis has been tested in a murine model of catheter infection, where repeated peptide treatment (at the time of infection, after 24 hours and after 48 hours) clearly suppressed *S. aureus* biofilm formation in the inserted catheter [101].

PEPTIDOMIMETICS

Peptidomimetics are characterized as molecules, that mimic functions of AMPs, but do not only consist of α-amino acids, leading to an enhanced stability and improved therapeutic properties [102]. CSA-13 (ceragenin) is an amphiphilic steroid conjugate which exhibits antimicrobial activities against planktonic bacteria by membrane interaction even in human body fluids [103-106]. Additionally, it has been shown to prevent *P. aeruginosa, E. faecalis, S. aureus, H. pylori, M. catarrhalis* biofilm formation, when applied at sublethal concentrations [107, 108]. Nagant *et al*. demonstrated that CSA-13 was able to completely penetrate pregrown *P. aeruginosa* biofilms within 30 min, promoting an overall membrane permeabilization and the subsequent cell death of biofilm bacteria [109]. In a recent study, a series of protease-resistant peptoids (Oligo-*N*-substituted glycines = peptide isomers with side chains attached to the backbone nitrogen atom rather than the α-carbon atom) was synthesized, and antimicrobial and antibiofilm activities against *P. aeruginosa* PA14 were analyzed [110]. Among all tested compounds, peptoid-1 and its derivative 1-$C13_{mer}$ exhibited the strongest biofilm-killing and eradicating activities even at sub-MIC concentrations of 1 µM, leading to a biomass reduction of 40-70%, respectively. Initial steps of *P. aeruginosa* biofilm formation were furthermore blocked by sublethal concentrations of peptoids 1-$C13_{mer}$, 1-achiral and 1-Pro_9, at similar levels to human cathelicidin LL-37, validating them as promising therapeutic agents against chronic *P. aeruginosa* lung infections [110]. In a further study the combination of β-peptoids and peptides led to the development of a set of hybrid compounds with a considerable antibiofilm activity in *S. epidermidis*, affecting primary adhesion and promoting biofilm dispersal and cell death of bacteria within established biofilms [111]. Step-by-step replacement of single amino acid residues resulted in a significantly altered cytotoxicity against HeLa cells,

which was mainly dependent on the sequence length and the content of guanidinium side chains, allowing the identification of antibiofilm compounds with modest cytotoxic side effects [111].

CURRENT CHALLENGES OF THE THERAPEUTIC USE OF AMPs

Despite their promising ability to negatively affect bacterial biofilms at different stages of biofilm formation, the widespread use of AMPs in the fight against severe biofilm infections has been hindered due to various problems, including high production costs, unwanted side effects, an insufficient stability and activity under physiological conditions and upcoming adaptive bacterial resistances.

BACTERIAL RESISTANCE MECHANISMS AGAINST AMPs

Since natural AMPs represent one part of the first line host defense against invading pathogens, several resistance strategies have arisen during bacterial evolution [112]. These mechanisms comprise the inactivation or sequestration of peptides, active efflux and alterations of the main AMP target – the bacterial cell envelope [20, 112, 113]. Various bacteria have been shown to secrete proteases, which cleave AMPs, for example *S. aureus* aureolysin [114], *P. mirabilis* ZapA [115], *B. cenocepacia* ZmpA and ZmpB [116] and *P. aeruginosa* elastase LasB [117]. Another strategy to inactivate AMPs before reaching the bacterial membrane is the production of shielding compounds, such as *P. aeruginosa* exopolysaccharide alginate, *S. epidermidis* polysaccharide intercellular adhesion (PIA) and poly-γ-glutamic acid (PGA) [118, 119] or capsule polysaccharides of *K. pneumoniae* [120]. Chan and coworkers demonstrated that anionic alginate, in addition to its function as diffusion barrier for positively charged antibiotics [121], is able to induce self-aggregation of cationic peptides leading to inactivation [122, 123]. Since production of alginate, the major matrix component of mucoid *P. aeruginosa* biofilms, is strongly enhanced in chronic lung infections [124] and PIA/PGA excretion is also linked to *S. epidermidis* biofilm formation [125], the synthesis of AMP binding substances may represent an important resistance mechanism in biofilm infections. Furthermore it has been shown that alterations in cell envelope composition, affecting surface net charge and membrane fluidity, play a key role in bacterial resistance against host defense peptides in both, Gram-negative and Gram-positive bacteria. Neutralization of the surface net charge in Gram-positive bacteria can be achieved by addition of basic D-alanine residues to teichoic acid and by insertion of L-lysine into cell membranes, whereas alterations in Gram-negative bacteria mostly are directed to the modification of LPS [20, 112, 113]. In *Salmonella,* resistance to cationic antibiotic polymyxin B can be stimulated by binding of aminoarabinose moieties to lipid A, which is regulated by AMP-inducible two-component systems PhoPQ, PmrAB and RcsBCD [126-128]. In recent studies, similar mechanisms, leading to adaptive resistances against cationic antibiotics, including polymyxin B and colistin, have been identified in *P. aeruginosa*. A direct sensing of AMPs, however, could only be confirmed for the two-component systems ParRS and CprRS, but not for PhoPQ [129-134]. Furthermore, in a study performed by Cummins *et al.* [135], incubation of *P. aeruginosa* cells with sub-MIC concentrations of colistin led to an induction of quorum sensing and virulence genes, which would be also an undesired side effect of the use of colistin in the treatment of biofilm infections. Active expulsion of AMPs, including protegrin and LL-37 from the cytoplasm has been considered for *Neisseria* multi-drug efflux pump MtrCDE [136, 137] and for the RosA/RosB system in *Yersinia* [138], but not for main resistance-nodulation division efflux pumps of *P. aeruginosa*, *S. aureus* and *E. coli* so far [139]. However, since efflux pump expression has been shown to be upregulated in biofilm bacteria [140, 141], a possible AMP excretion may be implicated in resistance of biofilm cells to cationic peptides. In a recent publication, Berditsch *et al.* present evidence that exposure of *S. aureus*, *E. faecalis* and *P. aeruginosa* to sublethal concentrations of α-helical AMPs magainin-2, PGLa and MAP favours the formation of small adherent colonies, which exhibit an increased antibiotic resistance. This phenotype switch was not observed after incubation of bacteria with circular AMPs gramicidin S and polymyxin B, suggesting that the stimulation of adhesion is strongly dependent on peptide structure [142].

Due to the fact, that AMPs, in contrast to many conventional antibiotics which mainly inhibit specific cellular pathways, exert their antimicrobial activity by affecting multiple targets and additionally stimulate non-inflammatory host immune responses leading to elimination of invading pathogens, the rapid development of AMP resistant strains following clinical application is mostly considered as rather unlikely [24, 36]. Nevertheless, single colistin-resistant *P. aeruginosa* and *A. baumannii* strains have been isolated so far and it has to be noted that the development of adaptive cross-resistances against human host-defense peptides could be a possible risk of medical AMP treatment [29, 143].

BIOAVAILABILITY AND PRODUCTION COSTS

Since bioavailability of natural AMPs is very low and quantitative isolation from natural sources therefore difficult and expensive, much effort has been put into the optimization of chemical peptide synthesis [144]. Cost-minimizing strategies comprise the production of very short (< 25 amino acids), but still functional, cysteine-free peptides, which do not contain post-translational modifications [101, 144]. An array-based high-throughput method of peptide synthesis allowed the complete substitution of single amino acids of the 12-mer linear variant of bovine cathelicidin bactenecin following identification of peptides with a high antibacterial activity [145, 146]. Further modifications and deletions of amino acids then led to the development of a set of 9-mer peptides (for example 1037), that, in parts showed a considerable preventive antibiofilm activity against various bacteria [16, 147]. An alternative attempt to *in silico* synthesis is the large-scale biotechnological production of AMPs. This procedure has been successfully established for preparation of bacterial host defense peptides, such as lantibiotic nisin, which is commonly used in food industries [148, 149]. A low-cost method for recombinant expression of non-bacteria peptides, for example LL-37, in *E. coli* has been recently developed by Bommarius *et al*., yielding large amounts of purified functional peptides [150].

STABILITY AND ACTIVITY

Another challenge for the clinical use of AMPs refers to the high accessibility of many naturally derived peptides to proteolytic degradation by host enzymes and their rapid renal clearance. The half-life of nisin, for example, is only 0.9 hours in mice after systemic administration [84] and Afacan *et al.* reported even shorter half-lives of 2 min for different AMPs in the blood [24]. Strategies to improve the stability of AMPs comprise chemical modifications, such as the insertion of unusual chemical bonds (for example in β-peptoids [102, 110, 151]), additional branches [152] or functional groups (for example acyl-residues [153]) or the use of isomers of natural AMPs [59, 154, 155]. In a study performed by Dean *et al.,* the naturally occurring L-isomer of LL-37 was completely degraded by trypsin within one hour, whereas its D-enantiomer did not show any signs of degradation, while antibiofilm activity against *P. aeruginosa* and *S. aureus* was still present [59, 63]. Trypsin is a serine protease class enzyme which exhibits a substrate selectivity for L-form peptides containing L-lysine and L-arginine residues. D-enantiomeric peptides are usually not affected by trypsin-catalyzed peptide bond hydrolyzation [156]. Since direct antimicrobial activity of many AMPs is severely diminished under physiological conditions [24], *in vitro* findings with regard to probable antibiofilm activities have to be necessarily confirmed in experiments mimicking biofilm infections or in *in vivo* studies. However, some AMPs, for example CSA-13, but not LL-37, still

showed considerable antibacterial activity in human body fluids [105, 106, 108]. Animal models of burn wound, catheter or chronic lung infections furthermore approved antibiofilm properties of peptides LL7-31 [157], novispirin G10 [69-71], citropin 1.1 [76], BMAP-28 [67], tachyplesin III [74], DASamP1 [101] and bacitracin [158]. To what extent *in vivo* biofilm killing and dispersal are provoked by the AMP mediated recruitment of host immune cells, since immunomodulatory effects of AMPs are only marginally affected by physiological high salt concentrations [23], has not been elucidated so far and requires further investigation.

TOXICITY

Unfortunately, the killing actions of a large number of AMPs are not specifically directed against bacteria, but also target eukaryotic cells, causing severe tissue disruption or hemolysis in host organisms following systemic administration [24, 29]. The mechanisms of AMP-induced cytotoxicity remain unclear, since mammalian membranes are in general less susceptible to AMP-mediated disruption than bacterial membranes [24]. This is due to divergent membrane properties, primarily the high content in cholesterol, phosphatidylcholine, phosphatidyethanolamine and sphingomyelin and the lack of negatively charged lipids leading to an overall zwitterionic phospholipid bilayer. In contrast, bacterial cytoplasmic membranes contain high amounts of anionic phosphatidylglycerol and cardiolipin which facilitate the binding of cationic AMPs [34, 37, 159]. Other factors which could explain the lower affinity of AMPs to mammalian membranes are the lower transmembrane potential compared to bacteria and the asymmetric distribution of membrane components, for example the accumulation of anionic phospholipids on the cytoplasmic rather than on the exoplasmic membrane leaflet [37, 159]. However, examination of peptide toxicity in cell or tissue culture and animal experiments is a crucial factor in order to evaluate their prospective therapeutic potential [29]. In addition, several chemical modifications have been shown to significantly lower cytotoxicity and give further insights into the selectively directed actions of AMPs [144]. For example, decreasing the amphipathicity of AMP GS14 by enantiomeric substitutions led to a considerable drop in hemolytic activity, whereas direct antimicrobial effects were unaffected [47]. Liu *et al.* figured out, that cytotoxicity of β-peptide-peptoid oligomers clearly correlates with sequence length, the content of guanidinium side chains and the presence of α-chirality [111].

PROMISING THERAPEUTIC STRATEGIES FOR THE USE OF AMPs IN THE TREATMENT OF BIOFILM INFECTIONS

To overcome cost, stability and toxicity problems of high-dosage systemic AMP use, various studies aim on the combined administration of AMPs and conventional drugs (Table **3**) and thereby take advantage of potential synergistic activities or on the local application of tethered or unbound AMPs in the treatment of biofilm infections.

SYNERGISTIC EFFECTS BETWEEN AMPs AND CONVENTIONAL ANTIMICROBIAL AGENTS

Recently, the combination of AMPs and conventional antibiotics has been considered as a new promising strategy to prevent the formation of biofilms or to disperse mature biofilms since combined administration often results in a synergistic antibacterial effect, which enables the use of lower individual drug dosages. Thus, the development of drug resistances in bacteria and toxic side effects may be reduced [160]. Many synergy studies between AMPs and other antimicrobial compounds focus rather on planktonic growth of bacteria than on the prevention or dispersal of biofilms. Cirioni *et al.* studied the synergistic effect of citropin 1.1 and the hydrophobic antibiotics minocycline and rifampin in the prevention of *S. aureus* central venous catheter (CVC)-associated infections [76]. In the described animal model a silastic catheter, implanted into the rat superior vena cava, was filled for 30 min with 10 μg/ml citropin 1.1 24 hours after implantation. Subsequently, rats were challenged via the CVC with 1.0×10^6 CFU of *S. aureus,* and 24 hours later, catheters pre- or untreated with citropin 1.1 were filled for 1 hour with the antibiotics minocycline or rifampin at two concentrations: a low concentration which was equal to minimal bactericidal concentrations for adherent cells and a high concentration of 1024 μg/ml. After 9 days post-infection a significant reduction of biofilm load (ca. 9×10^7 to 2×10^3 CFU/ml) and bacteraemia (ca. 9×10^3 to 4×10^1 CFU/ml) could be observed for the citropin 1.1 treated catheters or the high doses of both antibiotics. Pre-treatment of CVCs with citropin 1.1 in combination with high doses of the antibiotics minocycline or rifampin further reduced bacterial biofilm load of catheters and venous tissues to 1.4×10^1 CFU/ml and 2.7×10^1 CFU/ml, respectively and bacteraemia was eliminated nearly completely (less than 10 CFU/ml) [76, 161]. Similar results were obtained for the bovine cathelicidin BMAP-28 in combination with antibiotics vancomycin, linezolid or quinupristin/dalfopristin in a rat CVC or ureteral stent model with Gram-positive bacteria *S. aureus* and *E. faecalis* [67, 162]. Additionally, the synthetic AMP protegrin IB-367 has been shown to positively affect the therapeutic efficacy of linezolid in the treatment of CVC-associated infections of both bacterial strains [163].

Several studies focus on the combined administration of polymyxins together with other commonly used antibiotics. The synergistic effect of the hydroquinone derivative 10‘(Z), 13‘(E)-heptadecadienylhydroquinone (HQ17-2) and polymyxin B, was analyzed with respect to biofilm-grown cells of *P. mirabilis* [164]. *P. mirabilis*, which is an important pathogen of the urinary tract [165-167] and which is highly resistant to polymyxin B [168] exhibited an increased polymyxin B susceptibility when co-treated with HQ17-2 [164]. Colistin was furthermore tested in combination with aminoglycoside tobramycin in a static and a dynamic *in vitro P. aeruginosa* biofilm model and in an *in vivo* rat lung infection model [88]. The authors could demonstrate that the colistin-tobramycin combination was more effective in killing of *P. aeruginosa* biofilm cells than treatment with single compounds. Moreover, flow cell analysis of 24 hours and 48 hours old mushroom-shaped biofilms revealed that colistin only killed the non-motile stalk bacterial population, which displayed a low metabolic activity, while the motile and high metabolically active cap subpopulation on top of the biofilm stalk [141, 169] was susceptible to tobramycin [88]. Thus, the results indicate that the co-administration of colistin and tobramycin exerts synergistic activities, leading to killing of almost all bacteria of the pre-formed *P. aeruginosa* biofilm [88]. Similar results were obtained for the co-treatment of colistin with ciprofloxacin or tetracycline, whereby metabolic active cells in the cap of biofilms were tolerant to colistin but not to the applied antibiotics, while low metabolic activity of cells, prevalent in the stalk-forming subpopulation led to a high colistin susceptibility [141]. Analogously, a variety of *S. maltophilia* biofilm-grown isolates from sputum and bronchoalveolar lavage of cystic fibrosis patients, have been shown to be highly susceptible to colistin-moxifloxacin, colistin-ceftazidim and colistin-levofloxacin combinations [90].

Besides the co-administration of AMPs and other antimicrobial agents, a combination of two AMPs or even the usage of chimeric peptides could represent a potential therapeutic strategy to combat bacterial biofilms. Eckert *et al.* designed the previously mentioned STAMP G10KHc which is a chimeric molecule consisting of a Novispirin G10 AMP domain and a KH targeting domain [99]. The combined treatment of a preformed *P. aeruginosa* biofilm with G10KHc (100 μg/ml) and standard aminoglycoside antibiotic tobramycin (100 μg/ml) resulted in massive reduction of culturable cells after 4 and 24 hours [99]. Moreover, a chimeric peptide composed of a 13-residue S4 derivative K_4-S4(1-13)$_a$ (DD_{13}) and a seven-amino acid peptide termed RNA III-inhibiting peptide (RIP) were tested against staphylococcal-associated infections [170].

Table 3. Synergistic antibiofilm activites of AMPs and other compounds.

Tested compounds		**Microorganisms**	
Compound A (AMP)	**Compound B (AMP or other)**	***In vitro* antibiofilm activity**	***In vivo* antibiofilm activity**
Bacitracin	Anprocide	*S. aureus*, *S. epidermidis* [224]	
BMAP-28	• Vancomycin • Q/D (Quinupristin/Dalfopristin) • Linezolid		*S. aureus* [67, 162] *E. faecalis* [162]
Cecropin (1-7)–Melittin A(2-9) amide	• Daptomycin • Linezolid • Teichoplanin • Ciprofloxacin • Azithromycin,	*S. aureus* [68]	
Citropin 1.1	• Rifampin • Monocycline	*S. aureus* [76]	*S. aureus* [76]
Colistin	• Tobramycin • Fosfomycin • Levofloxacin • Clarithromycin • Ciprofloxacin • Tetracycline	*P. aeruginosa* [88, 141] *S. maltophilia* [90] *A. baumannii* [91]	*P. aeruginosa* [88] *E. coli* [225]
DD_{13}-RIP • **DD_{13} [K_4-S4(1-13)$_a$** • **RIP**	Rifampin		*S. aureus*, *S. epidermidis* [170]
G10KHc	Tobramycin	*P. aeruginosa* [99]	
HBD-3	• DNAseI • Ultrasound-targeted Microbubble Destruction	*Nontypeable H. influenzae* [226] *S. aureus, S. epidermidis* [227]	
IB-367	Linezolid	*S. aureus*, *E. faecalis* [163]	*S. aureus*, *E. faecalis* [163]
Indolicidin	• Daptomycin • Linezolid • Teichoplanin • Ciprofloxacin • Azithromycin	*S. aureus* [68]	
Lactoferrin	• Rifampicin • Ceftazidim • Amikacim • Ciprofloxacin • Tobramycin	*B. cenocepacia, B. multivorans, B. dolosa* [214]	
	• Xylitol • Xylitol + Silver • Xylitol + Farnesol	*P. aeruginosa* [210] *P. aeruginosa*, *S. aureus* [228] *S. epidermidis, E. faecalis* [229]	

(Table 3) Contd….

Tested compounds		Microorganisms	
Nisin	• Daptomycin • Linezolid • Teichoplanin • Ciprofloxacin • Azithromycin	*S. aureus* [68]	
Polymyxin B	HQ17-2	*P. mirabilis* [164]	
Protamine sulfate	N-ethyl maleimide and analogs	*P. aeruginosa*, *S. epidermidis* [230]	
$(RW)_n$-NH_2, $(RW)_{4D}$ [*n* is 2, 3, or 4]	Ofloxacin	*E. coli* [98]	
Tachyplesin III	Piperacillin-tazobactam (TZP)	*P. aeruginosa* [74]	*P. aeruginosa* [74]

DD_{13} is a dermaseptin derivative of the frog skin peptide S4 [171] and displayed a low toxicity against human erythrocytes and a broad-spectrum antimicrobial activity *in vitro* and *in vivo* [172]. RIP has been identified as a probably non-self inhibitory autoinducing peptide of *S. warnerii* [173]. A graft infection rat model was used to analyze the efficacy of the chimeric peptide DD_{13}-RIP as well as the combination with the antibiotic rifampin, in preventing staphylococcal-associated infections. The results obtained demonstrated that treatment of grafts with DD_{13}-RIP further reduced bacterial colonization by MRSA and methicillin-resistant *S. epidermidis* in a dose-dependent manner compared to the untreated control and RIP or DD_{13} alone. Furthermore, rifampin presoaked grafts were tested in combination with DD_{13}, RIP and DD_{13}-RIP and a prevention of bacterial colonization for all examined combinations was observed, suggesting a synergistic effect for the combination of DD_{13} and RIP in terms of the chimeric peptide DD_{13}-RIP as well as for the co-treatment of rifampin with these peptides [161, 170]. However, the molecular mechanism of RIP activity in *S. aureus* is still unclear. In early studies it has been postulated that RIP inhibits the *agr* quorum sensing system via the repression of the target for RNAIII-activating protein (TRAP) phosphorylation [174, 175]. Since it has been published recently by three different research groups that a mutation in *traP* had no impact on *agr* expression, biofilm formation and virulence in *S. aureus*, this first explanation has become rather unlikely [176-178]. Furthermore, it has been shown in other studies that linear variants of autoinducing peptides including RIP were not able to inhibit *S. aureus agr* expression [173, 179]. Thus, Otto hypothesized that the observed antibiofilm activity of RIP at high peptide concentrations of 10 – 50 mg/l could be due to its detergent-like properties rather than to the inhibition of quorum sensing [180].

TETHERED AMPs

Bacterial colonization and subsequent biofilm formation on medical devices or implant surfaces, such as urinary and venous catheters, heart valves or stents [181] are one of the main reasons for the development of nosocomial infections [182, 183]. These infections cause the failure of indwelling devices, complex revision processes and implant removal, leading to a prolonged hospitalization or even death of the patient [181]. In order to prevent implant-associated infections, the impregnation of surfaces with antimicrobial agents or functionalized coatings may be a promising strategy [184, 185]. Particularly AMPs are potential antimicrobial agents for this purpose, due to their broad antimicrobial spectrum [29] and their high efficacy in killing bacteria and preventing bacterial biofilm formation [186]. AMPs can be immobilized onto solid surfaces either physically via adsorption or layer-by-layer assembly of polymeric films [187, 188] or chemically via covalent bonding (for example self-assembled monolayers (SAM) = functionalized polymer resins) [185]. Various immobilization methods as well as studies of tethered AMPs concerning their antibacterial activity have been reviewed elsewhere [181, 185, 189]. In contrast to the layer-by-layer technique, in which AMPs are directly embedded into polyelectrolyte multilayers [189], covalent binding of AMPs onto surfaces may increase long-term stability while decreasing toxicity [181] and thus representing another efficient approach in combating biofilms. Humblot *et al.* [190] immobilized magainin, a 23-residue antibacterial peptide of the African clawed frog *Xenopus laevis* [191] via a mixed 11-mercaptoundecanoïc and 6-mercaptohexanol SAM on a gold-surface. Adhesion studies with Gram-positive bacteria *E. faecalis* and *S. aureus* demonstrated a reduction of bacterial adhesion to the magainin-containing surface of more than 50 % in comparison to the substrate without peptide [190]. Also gramicidin A, a hydrophobic linear polypeptide antibiotic [192], was covalently bound to a cystamine SAM onto a gold surface and exhibits antimicrobial, but not anti-adhesive activity against *E. faecalis* and *S. aureus* [193]. In addition to the covalent immobilization of AMPs via SAMs, polymer brushes conjugated with peptides provide another method for the generation of infection-resistant coatings [194]. Gao *et al.* developed functionalized hydrophilic copolymer brushes tethered with AMPs Tet-20, Tet-26, Tet-123 and 1010cys on titanium surfaces. Tet-20 and Tet-26 immobilized titanium slides showed to be the most effective coatings in preventing biofilm formation of *P. aeruginosa*. After a long-term incubation of 7 days, 8.4 ± 6.6 or 175 ± 158 bacteria per 0.035 mm^2 have been observed for Tet-26 and Tet-20 conjugated brushes, compared to the uncoated titanium slide on which 1268 ± 695 bacteria per 0.035 mm^2 were adherent. An *in vivo* analysis with Tet-20 coated implants, incorporated subcutaneously on the dorsal side of rats, revealed a significantly decrease of adherent *S. aureus* cells to the implant in comparison to the control sample. Besides this, copolymer brushes did not result in platelet activation, adhesion and complement activation in human serum, thus indicating that they are not toxic [194].

Critical for the successful use of AMPs as coating material for implants or medical devices is the retention of its antimicrobial activity after immobilization to relevant surfaces. To this aim, it has to be considered that several factors such as surface concentration, spacer length and flexibility, peptide orientation, structure and sequence as well as the surrounding environment (for example pH, ionic strength) could have an influence on the activity of immobilized peptides [185]. Moreover, the formation of a conditioning layer composed of eukaryotic proteins such as fibronectin, fibrinogen, albumin and immunoglobulins and inorganic substances as

well as the accumulation of dead bacteria on the antimicrobial surface may mask the coating, favoring the microbial surface adhesion and biofilm formation [181, 185]. A common method to prevent the adsorption of a conditioning layer and consequently the bacterial colonization is the covalent immobilization of AMPs by functionalized polymer brushes such as polyethylene glycol (PEG) or other low-fouling polymers [185, 195]. Recently, various studies on PEG-based low-fouling coatings with incorporated AMPs have been reviewed by Salwiczek *et al.* [195]. In addition to its anti-adhesive properties, PEG has also been reported to enhance the activity of immobilized peptides [196, 197].

CONCLUSION

Due to the presence of shielding matrix components and an enhanced antibiotic resistance of biofilm bacteria, medical treatment of biofilm-associated infections using conventional drugs is still challenging and the fact that most antibiotics selectively target metabolically active cells, whereas many biofilm cells exhibit a lower metabolic activity, yet reduces the susceptibility to conventional antibiotics. Recently, different natural and synthetic AMPs and peptidomimetics have been assumed as one of the most promising agents in the fight against biofilm infections caused by multidrug resistant bacteria. In addition to their direct and indirect killing ability towards planktonic bacteria these compounds exert considerable effects regarding biofilm prevention, dispersal of preexisting biofilms and killing of biofilm cells. Despite current drawbacks postponing the widespread clinical use of AMPs such as the toxicity against host cells, a low bioavailability, high production costs, a decreased stability and activity under physiological conditions, several AMPs or AMP-related agents have been tested in clinical trials so far. Until now, medical use of AMPs is almost exclusively limited to topical applications, since development of systemic therapeutics is considerable more complex due to stability, delivery and toxicity issues [24, 31, 181]. An excellent overview about AMPs in clinical trials is given in recent reviews by Afacan *et al.* [24] and by Mok & Li [31]; for updated status also see http: //www.clinicaltrials.gov. Examples for clinically tested AMPs are indolicidin derivative omiganan in the topical treatment of venous catheter-related infections and the use of magainin-2 analog pexiganan in a cream against diabetic foot ulcers [24, 31]. Inhaled administration of AMP colistin in combination with antibiotic ciprofloxacin is successfully used for prevention or treatment of chronic *P. aeruginosa* lung infections in CF patients for more than 15 years now [87] and although there are a few reports about colistin-resistant *P. aeruginosa* CF isolates, occurrence of such strains seems to be sporadic [198, 199]. In contrast to the rapid expansion of MRSA strains only a couple of years after the discovery of methicillin [200, 201] no noteworthy spread of colistin-resistant *P. aeruginosa* strains has been observed so far [198, 199], indicating a lower risk of resistance development against AMPs in comparison to conventional antibiotics [24]. Moreover, the finding, that many AMPs prevent biofilm formation or even disperse preexisting biofilms at concentrations far below their MIC [16, 57, 61, 108, 110] (also see Table **2**), even diminishes the amount of AMPs, which is required for clinical application and thereby lowers the risk of potential toxic side effects against eukaryotic cells as well as treatment costs. A promising approach is the use of immobilized AMPs, for example as coating for implants in order to prevent implant-associated biofilm infections. In comparison to other local application strategies with uncontrolled AMP release, one main advantage of covalent AMP binding to the implant is the absence of a concentration gradient from the implant to other body compartments with locally low AMP concentrations which could promote the development of bacterial resistances [194, 202]. In addition, immobilized AMPs provide a long-lasting antimicrobial and antibiofilm activity and avoid harmful side effects such as the accumulation of peptides in liver, spleen and brain tissues [181, 203].

In conclusion, AMPs represent a promising class of antibiofilm agents which could facilitate medical treatment of persistent biofilm infections in the near future. Although much effort has been put into the improvement of AMPs with respect to antibiofilm properties during the last 5 years, the precise mode of antibiofilm action still remains unclear in large parts and thus essentially requires further investigation in order to enable a target-oriented design of stabile, effective and secure AMP based antibiofilm drugs. Moreover, since systemic use of AMPs is still challenging as mentioned before, appropriate administration strategies, permitting for example the controlled release of AMPs at sites of infections should be developed for future applications.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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