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ARTICLE

Synergistic Activity of a Short Lipidated Antimicrobial Peptide (LipoAMP) and Colistin or Tobramycin against *Pseudomonas Aeruginosa* from Cystic Fibrosis Patients

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Declining pulmonary function, ultimately culminating in respiratory failure, is mainly caused by chronic *Pseudomonas aeruginosa* (*P. aeruginosa*) infections in patients with Cystic Fibrosis (CF). Due to its hypermutability, allowing to rapidly adapt to the selective constrain in a CF lung, and ability to form biofilms, *P. aeruginosa* is able to colonize and damage the lung by chronic infection. Exacerbations are being treated with a combination of common anti-pseudomonal antibiotics but (pan)resistance is increasingly reported. Antimicrobial peptides (AMPs) have a broad spectrum of antibacterial activity and their effectiveness is, still, less affected by induction of resistance. Here, we explore the *in vitro* applicability of the RWRWRWK(C_{10}) synthetic lipoAMP (named BA250-C10), a lipidated peptide with a C_{10}-lipid chain attached to the C terminus, as novel antibacterial agent against *P. aeruginosa*; and in particular its ability to inhibit biofilm formation. BA250-C10 was tested for the *in vitro* antibacterial activity against 20 clinical *P. aeruginosa* isolates of CF patients, each having a different resistance profile and ability to form biofilms. The modest antibacterial activity of the peptide against most *P. aeruginosa* strains (16–256 μg/mL) was significantly increased in the presence of colistin and less in the presence of tobramycin, supported by checkerboard assay and growth curves. In three biofilm forming strains, a synergistic effect was observed for BA250-C10 with colistin, but less with tobramycin. This indicates that combinations of lipidated AMPs and colistin may be further pursued as a potential novel treatment strategy against *P. aeruginosa* infections in CF patients.

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is the most prevalent and significant pulmonary pathogen in patients with cystic fibrosis (CF). Colonization with *P. aeruginosa* is associated with a faster decline of pulmonary function and overall worsening prognosis.[1] A crucial obstacle in antibiotic treatment is the ability of *P. aeruginosa* to form biofilms and its ability to rapidly adapt[2] to the ever-changing physiology within the CF airway.[3] Anti-pseudomonal therapies are used in three distinct clinical settings: (i) to delay onset of chronic colonization, (ii) in chronic maintenance therapy, and (iii) in periodic administration of intensive antibiotic regimens.[4] Standard treatment for an exacerbation of CF is intravenous therapy with two antibiotics, mainly aimed at decreasing the risk of resistance, but also to decrease dose-related toxicity, to treat polymicrobial infection, and to promote antimicrobial synergism.[5] Unfortunately, current antibiotics are becoming less effective in treating chronic *Pseudomonas* infections due to increasing antibiotic resistance and highly antibiotic-refractory biofilms.[6] In the last decade, no new antibiotics have been developed,[7] and there are only minor improvements in inhaled anti-pseudomonal antibiotics. New therapeutic options for patients with CF are designed to correct the function of the defective CF transmembrane conductance regulator (CFTR)-modulating protein,[8] and clinical effects of this treatment have been shown in different randomized clinical trials.[9] However, these treatments will be available only for a selection of CF patients, depending on the type of their genetic defect.[8] Therefore, CF patients will continue to suffer from pulmonary infection and new anti-bacterial therapies and treatment strategies are on continuous demand.[10]

A relatively new class of antibacterial compounds is the large family of host defense peptides (HDPs) and antimicrobial peptides (AMPs).[11] Many of these occur naturally as part of the host-defense system; whereas HDPs combine direct broad spectrum antibiotic activities with modulation of immune responses,[12] AMPs have only direct anti-bacterial activity.[13] Whereas naturally occurring HPDs and AMPs hold great promise when it comes to the antimicrobial activity and ability to

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inhibit biofilm formation,[14] their applicability in a clinical setting is limited due to poor PK/PD profiles.[12] In addition, their intricate structure hampers large-scale production and severely encumbers the modulation of their therapeutic profile. Nevertheless, the emergence of resistance is considered to be less of a problem compared to conventional antibiotics since many AMPs target the bacterial membrane rather than a specific single biomolecule.[12,15] Therefore, AMPs are considered as relevant new candidate treatment options in diseases such as CF in which multidrug-resistant organisms cause infections in a hyperinflammatory environment.[12,16]

Anti-pseudomonal synthetic AMPs (synAMPs) have been developed in recent years.[17] In addition, AMPs with specific anti-biofilm properties have been discovered.[11b,17a,b,d,18] For example, the dodecameric peptide with the sequence VRLIVAVRIWRR-NH_2 was shown to potently inhibit biofilm formation of CF pathogens by blocking a widespread stress response that contributes to biofilm development.[19] Short synAMPs can be prepared on a large scale, and can easily be modified to improve proteolytic stability, circulation lifetime, and bacterial specificity or to decrease general toxicity. This makes them attractive candidates for clinical applications. En route to that goal, the mode of action of one specific family of short synAMPs, i.e. those that contain the Arg-Trp sequence,[20] has been determined.[21] The activities against methicillin resistant *Staphylococcus aureus* (MRSA) of organometallic derivatives of such peptides are now identical to,[22] or even better than, vancomycin without inducing substantial hemolysis and displaying high toxicity *in vitro*.[23] These last two effects are usually problematic for lipidated AMPs. Their effect on planktonic growth and biofilm formation of *Escherichia coli* was also determined, showing promising results for the former, but limited results for the latter.[24] Similarly, N- or C-terminal lipidation of an Arg-Trp hexapeptide, resulting in so-called lipoAMPs, led to high activity against a broad spectrum of bacterial pathogens, including *P. aeruginosa* and *A. baumannii*.[25] Even more, their hemolytic activity could be reduced from ~16% to less than 1% when human red blood cells were treated with 250 µg/mL of the peptide.[26] Moreover, only a few examples have emerged in which the synergy of AMPs with existing antibiotics[27] as well as AMPs with anti-pseudomonal antibiotics have been described,[17b,18b,28] but a detailed study with a large panel of clinically isolated *P. aeruginosa* strains and lipoAMPs has not been performed yet.

Here, we now determined the activity of lipoAMPs against CF-related *P. aeruginosa* strains,[29,30] the synergistic activity of the most active lipoAMP with conventional antibiotics,[31,32,33] and their ability to inhibit biofilm formation. We assessed the activity of 12 different lipidated short peptides (i.e. the lipoAMPs) against three CF-related *P. aeruginosa* isolates. The peptide with the lowest MIC-value was used for further evaluation against a wider panel of clinical *P. aeruginosa* isolates. Growth curves and checkerboard assays were applied to probe for synergy between the lipoAMP and two commonly applied antibiotics, i.e. colistin and tobramycin, and biofilm interfering capacity was tested with in polystyrene assays.

Experimental

All experimental details and procedures are provided in the Supporting Information.

Results

The peptides that were used in this study have been described before.[25] Apart from the lipidated peptides, which are identified by the position and length of their lipid-chain (i.e. C8 refers to the $C(O)C_7H_{15}$ lipid attached to a C-terminally positioned lysine residue; N8 refers to the same lipid when attached to an N-terminally positioned lysine residue), two ferrocenoyl-derivatized peptides (indicated by 'Fc'), and one dye-labelled peptide, i.e. BA250-DEC, were also included.

Table 1. Pre-selection of lipoAMPs for their activity against three clinical isolates of *P. aeruginosa*. Minimal Inhibitory Concentrations (MIC) values are given, the activities of two common anti-pseudomonal antibiotics and DMSO are included as references.

	clinical isolate		
	VW1633	LES431	KD491
lipoAMP[a]	MIC (µg/mL)	MIC (µg/mL)	MIC (µg/mL)
BA250-CFc	32	>128	32
BA250-C6	16	>128	32
BA250-C8	16	128	16
BA250-C10	**16**	**32**	**16**
BA250-C12	32	64	>128
BA250-C14	>128	128	>128
BA250-NFc	32	>128	128
BA250-N6	32	>128	64
BA250-N8	32	64	16
BA250-N10	64	32	32
BA250-N12	>128	64	>128
BA250-N14	>128	>128	>128
BA250-DEC	64	>128	64
ciprofloxacin	6.4	6.4	1.6
polymyxin B	1.6	0.8	1.6
DMSO	>128	>128	>128

All peptides were obtained in high purity (>95%) after preparative HPLC and in acceptable yields of 21–46%; HR-MS spectrometry confirmed the identity of the peptide.[25]

Initially, MIC-values of 12 lipoAMPs against three clinical isolates of *P. aeruginosa* were determined (Table 1). The three isolates were chosen for their different susceptibility profile to standard applied anti-pseudomonal antibiotics; very resistant KD491, and intermediate resistant LES431 and VW1633. LipoAMPs containing either a C- and N-terminally positioned lipidated lysine residue were tested, as well as the two commonly applied antibiotics ciprofloxacin and polymyxin B. The general activity of these lipoAMPs against the very resistant KD491 was higher than against the less resistant strain LES431.

LipoAMP BA250-C10 was the most promising candidate for our study, with MIC-values of 16–32 µg/mL (i.e. 9–18 µM) (Table 1), and the activity of this peptide was further studied against a larger panel of clinical isolates of *P. aeruginosa* (Table 2). The

redox-active Fc-labelled lipoAMP did not display enhanced activity; in fact, the activity of this lipophilic peptide, of which the lipophilicity resembles that of a peptide containing a seven C-atom long lipid, is more or less within the expected range of lipidated AMPs. This indicates that this moiety mostly acts as a lipophilic moiety, potentially a membrane-anchor.

Table 2. Susceptibility of various *P. aeruginosa* strains for the commonly applied antibiotics: ciprofloxacin, colistin (polymyxin E), tobramycin, ceftazidim, tazocin, and meropenem, and lipoAMP BA250-C10.

Entry	Strain	ciprofloxacin	colistin	tobramycin	ceftazidim	tazocin	meropenem	BA250-C10	Resistance	Biofilm
1*	D599	0.25	4	0.5	1	4	0.5	128	0	+
2[34]	**Pa01**	**0.5–0.25**	**4**	**2**	**1**	**8**	**2**	**256**	**0**	**++**
3[35]	**Clone C**	**0.25**	**4**	**1**	**2**	**16**	**2**	**128**	**0**	**++**
4*	VW1501	16 (R)	2	4	4	4	0.25	128	1	–
5*	kl 1.1	4 (R)	4	4	2	4	4 (I)	128	1–2	–
6*	KD557	0.5	8 (R)	1	2	16–32 (R)	1–2	256	2	+
7*	VW1540	2–4 (R)	4	0.125	16 (R)	8	1–2	64	2	–
8*	VW178	1 (R)	4	32 (R)	2	8	2	32	2	+
9*	VW1633	1 (R)	2	0.125	>256 (R)	>512 (R)	1	32	3	–
10*	VW1485	8 (R)	>128 (R)	16 (R)	8	4	0.25	256	3	–
11*	VW0247	16 (R)	2	4	16 (R)	32 (R)	0.25	64	3	–
12*	kl 3.2	2–4 (R)	8 (R)	16 (R)	2	4–8	4	128	3	–
13[36]	LES431	4 (R)	2	2–4	256 (R)	512 (R)	8 (I)	64	3–4	–
14*	VW1538	8 (R)	2	8 (R)	8	64 (R)	64 (R)	64	4	–
15[37]	MIDLANDS	4 (R)	4	2	64 (R)	128 (R)	16 (R)	64	4	–
16[37]	LES400	2 (R)	4	8 (R)	32 (R)	32 (R)	2	64	4	–
17*	VW1471	16 (R)	4	8 (R)	128 (R)	256 (R)	32 (R)	64	5	–
18*	VW313	2 (R)	4	16 (R)	>256 (R)	64 (R)	16 (R)	32	5	–
19*	**KD491**	**8 (R)**	**2**	**8–16 (R)**	**>256 (R)**	**>512 (R)**	**16 (R)**	**32**	**5**	**++**
20[38]	**LESB58**	**8–16 (R)**	**32 (R)**	**8 (R)**	**256 (R)**	**512 (R)**	**2**	**128**	**5**	**+**

Notes: Minimal Inhibitory Concentrations (MIC) values are given in µg/mL; CLSI breakpoints for susceptibility of various strains for specific antibiotics are given in brackets behind the MIC-values: I = Intermediate, R = resistant, S = susceptible (S is left out for clarity); cut-off limits for the respective antibiotics are given below. Resistance is based on the number of antibiotics against which resistance is observed. The origins of the strains are indicated when known: entry-numbers that are marked with an asterisk (*) indicate that these strains were obtained from CF patients treated in the University Medical Center Utrecht; 'KD' refers to child, 'VW' to adult. Entries marked in bold indicate the strains that were used in subsequent studies. Cut-off limits for the CSLI breakpoints for susceptibility: ciprofloxacin: S ≤ 0.5 µg/mL and R > 1 µg/mL – colistin: S ≤ 4 µg/mL and R > 4 µg/mL – tobramycin: S ≤ 4 µg/mL and R > 4 µg/mL – ceftazidim: S ≤ 8 µg/mL and R > 8 µg/mL – tazocin: S ≤ 16 µg/mL and R > 16 µg/mL – meropenem: S ≤ 2 µg/mL and R > 8 µg/mL. [a] The ability to form biofilms is measured by the crystal violet assay where '++' indicates high, '+' indicates intermediate, and '–' indicates low tendency for biofilm formation.

Of the 20 clinically isolated *P. aeruginosa* strains against which activity was determined (Table 2), 6 were international *P. aeruginosa*-isolates and 14 were obtained from the University Medical Center Utrecht (UMCU).

The results demonstrated an inverse correlation between the resistance of the *P. aeruginosa* strains against a number of antibiotics and the MIC-value for BA250-C10; with lower MIC-values against BA250-C10 found in strains that are more resistant to more commonly applied antibiotics. For two biofilm forming *P. aeruginosa* strains, the MIC-value is 32 µg/mL (entries 8 and 19), for the other biofilm forming *P. aeruginosa* strains it is 128 or 256 µg/mL (entries 2 and 6, respectively). It should be noted that the results displayed in Table 1 were obtained in a different laboratory than those displayed in Table 2; this explains the 2-fold difference between the MIC-values of BA250-C10 against VW1633, LES431, and KD491.

Next, synergistic activity of the lipoAMP and colistin and tobramycin was mapped using a checkerboard assay. For this, strains KD491, LESB58, Pa01, and clone C were selected due to their strong tendency to form biofilms. Also, since the activity of BA250-C10 was low when that of colistin and/or tobramycin was high (entries 2 and 3), or when the activity of BA250-C10 was high and that of tobramycin was low (entry 19), synergism in both directions, i.e. of the antibiotics on the activity of lipoAMP or of the lipoAMP on the activity of both antibiotics, was studied. In addition, we determined if synergism could enhance the combined activity of compounds that are poorly active against the multi-resistance strain LESB58 (entry 20).

This study revealed that lipoAMP BA250-C10 showed synergy with colistin in three out of four tested strains, and with tobramycin in two out of four tested strains (Table 3). Strong synergy is found for BA250-C10 and colistin against strain KD491 with FIC<0.5. Whereas the MIC-value of colistin itself is 4 µg/mL, in the presence of 8 µg/mL of BA250-C10, the MIC of colistin drops to 1 µg/mL. Similarly, the MIC-value of BA250-C10 is 32 µg/mL, but in the presence of 2 µg/mL of colistin it drops to 2 µg/mL. Interestingly, the required amount of the second component is below the MIC-value of that compound. In addition, two strains that generally display higher levels of resistance, KD491 and LESB58, show very low FIC-indices (i.e. <0.5), which is indicative if a synergistic effect, whereas the two strains that are almost not-resistant against any of the

commonly applied antibiotics (see entries 2 and 3 of Table 2), Pa01 and clone C, have higher FIC-indices, i.e. lower synergy.

Table 3. Results of the checkerboard assays in which synergism between BA250-C10 and either colistin or tobramycin was assessed. The results are shown as the FIC-Index, and the effect is indicated.

	KD491		LESB58		Pa01		clone C	
	colistin	tobramycin	colistin	tobramycin	colistin	tobramycin	colistin	tobramycin
FIC-index #1	0.2625	0.5	0.375	0.5	0.3125	1	0.5	0.5
FIC-index #2	0.375	0.375	0.625	0.5	0.5	0.75	1	1
Effect[a]	**S**	**S**	**S / I**	**S**	**S**	**I**	**S / I**	**S / I**

Note: [a] Synergism (**S**) is defined as FIC ≤ 0.5, and indifference (**I**) as FIC = 0.5–4.

Subsequently, growth-curves of the four strains in the presence of the individual components and of sub-MIC concentrations of the mixtures were measured. The growth curve of KD491 shows normal growth in the presence of 4 µg/mL BA250-C10; a prolonged lag-phase of 4 hours, but normal growth rates are observed at exponential phase in the presence of 0.25 µg/mL colistin (Fig. 1, panel A). However, the combination of 4 µg/mL BA250-C10 and 0.25 µg/mL colistin almost completely inhibits growth. For the colistin-resistant strain LESB58 there is no growth of LESB58 in the presence of the combination of colistin (2 µg/mL) and BA250-C10 (4 µg/mL), even though there is normal growth of LESB58 with 4 µg/mL BA250-C10, and a prolonged lag phase but normal growth rate at exponential phase in the presence of 2 µg/mL colistin (Fig. 1 panel B).

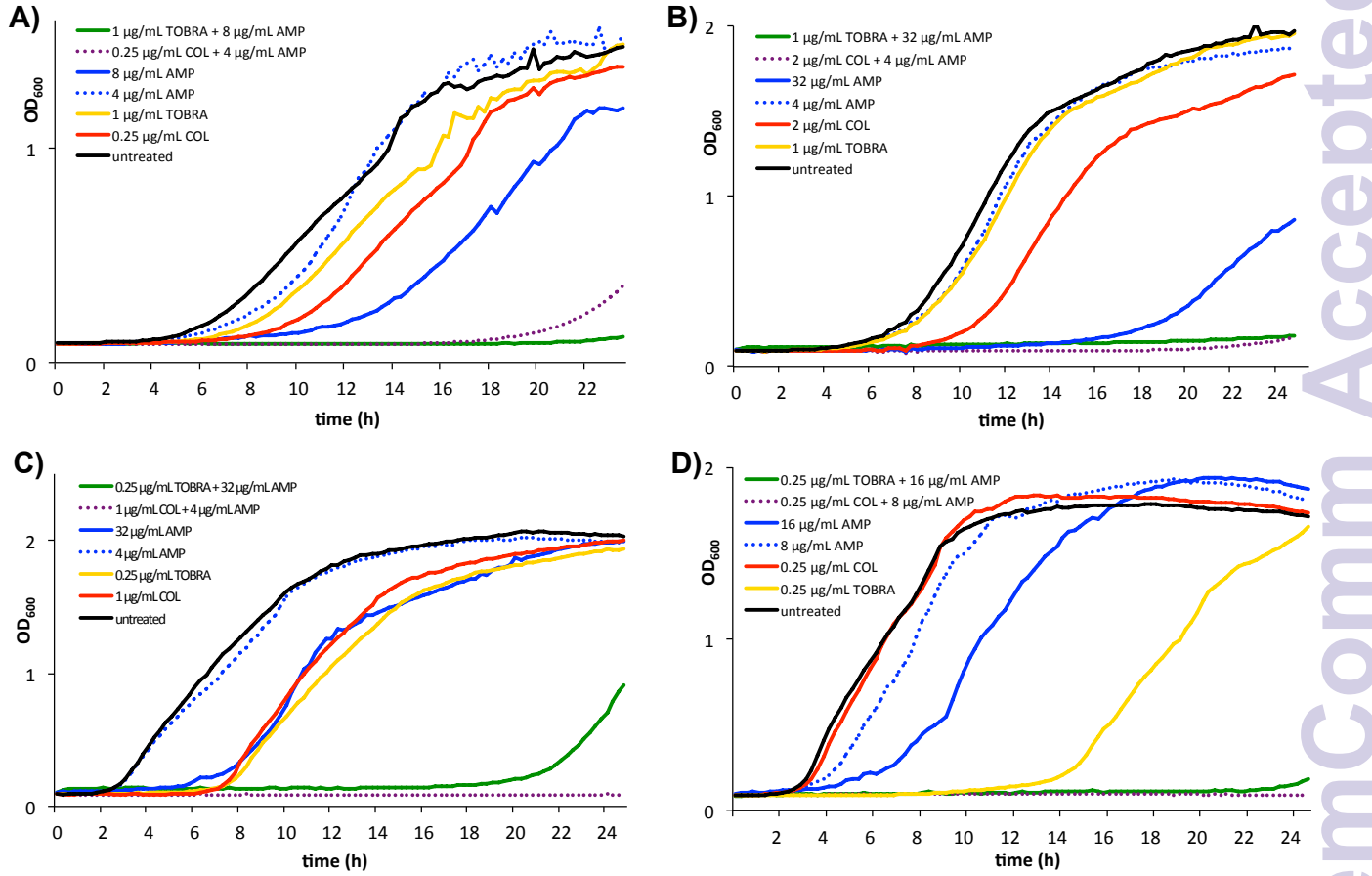

Figure 1. Growth curves of the clinical isolates *P. aeruginosa* KD491 (panel A), LESB58 (panel B), PA01 (panel C), and clone C (panel D) in the presence of BA250-C10 (blue curves), tobramycin (yellow curves), colistin (red curves), and a mixture of tobramycin with BA250-C10 (green curves) or colistin with BA250-C10 (purple dotted curves). The various amounts of antibacterial agents are indicated in the respective charts, with "AMP" = BA250-C10, "TOBRA" = tobramycin, "COL" = colistin.

These data confirmed the data of the checkerboard assay that indicated synergy between BA250-C10 and colistin. In Pa01 and clone C, a similar pattern is seen, suggesting synergy between colistin and lipoAMP BA250-C10 during the growth-phase of the bacteria (Fig. 1 panels C and D, respectively). In the presence of only BA250-C10 or tobramycin, growth of all strains is delayed and growth rates at exponential phase are lower, while the combination of BA250-C10 and tobramycin shows almost complete inhibition of growth.

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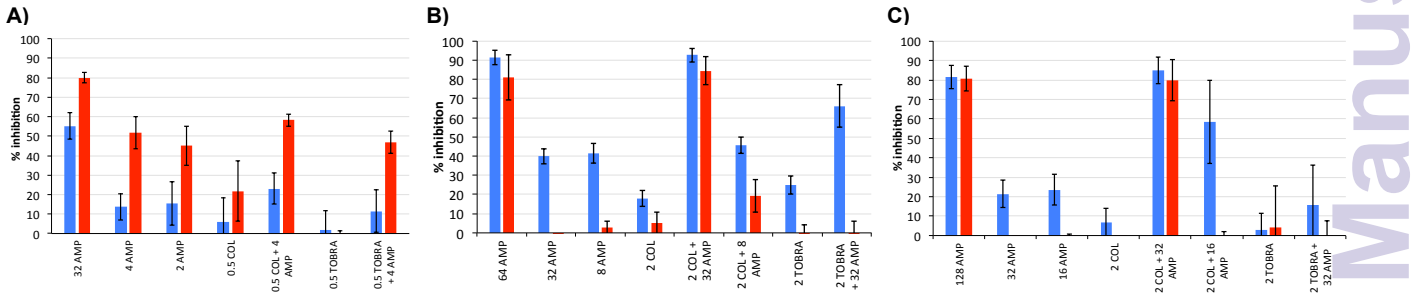

Figure 2. Inhibition of planktonic growth (blue bars) and biofilm formation (red bars) of *P. aeruginosa* strains KD491 (A), Pa01 (B), and clone C (C), by the application of lipoAMP BA250-C10, colistin or tobramycin, and by the simultaneous administration of the lipoAMP BA250-C10 ("AMP") with either colistin ("COL") or tobramycin ("TOBRA").

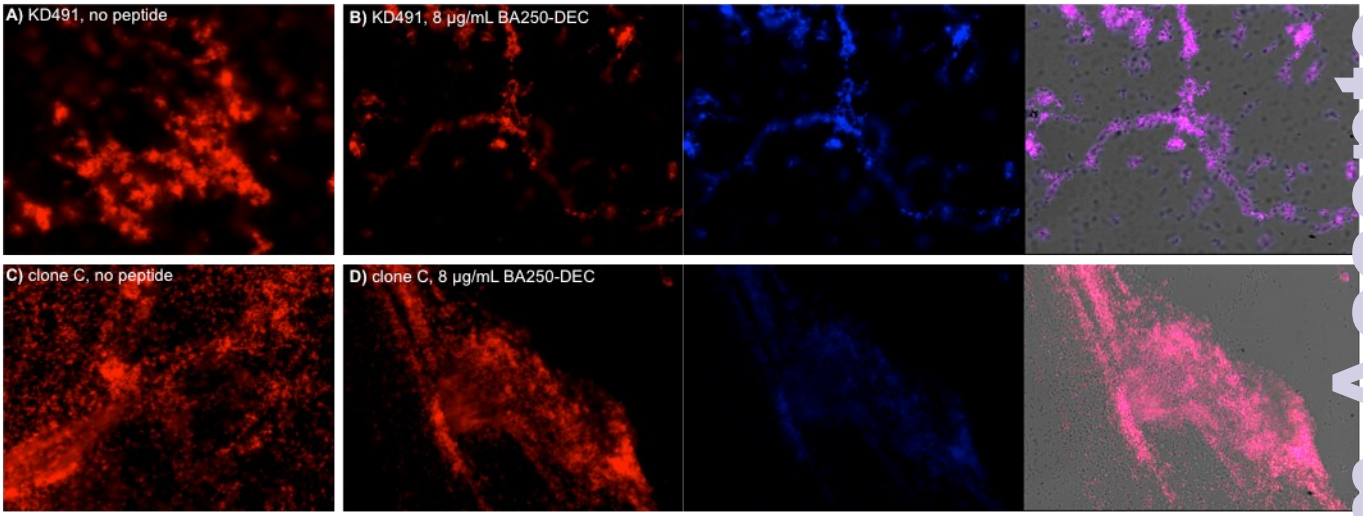

Figure 3. Visualization of the inhibition of biofilm formation by the lipoAMP BA250-DEC. Confocal images of KD491 (A, B) or clone C (C, D) biofilms in the absence (A, C) or presence of 8 µg/mL of BA250-DEC (B, D). Bacterial DNA is stained red with propidium iodide. For panels B and D, from left-to-right: biofilms identified with the propidium iodide DNA-staining (red), fluorescent peptide localization (blue), and combination of channels showing co-localization of the BA250-DEC lipoAMP and propidium iodide (purple/pink colour).

Next, we tested the ability of the isolated lipoAMP with combinations with colistin and tobramycin to inhibit biofilm formation in the polystyrene assay of KD491, Pa01, and clone C (Fig. 2). For KD491, high concentrations of BA250-C10 were needed to almost fully inhibit biofilm formation: 32 µg/mL for 80 ± 3% inhibition (Fig. 2, panel A). However, already at 2 and 4 µg/mL of BA250-C10 significant inhibition of biofilm formation of KD491 was observed, i.e 45 ± 11% and 52 ± 9%, respectively. At these concentrations, weak inhibition of planktonic growth was observed. No synergistic activity against biofilm formation between the lipoAMP and colistin or tobramycin was observed against KD491.

For the other two strains, significantly higher concentrations of BA250-C10 were needed in order to achieve substantial inhibition of biofilm formation, i.e 64 µg/mL for 81 ± 11% inhibition of Pa01 and 128 µg/mL for 82 ± 6% inhibition of clone C, respectively (Fig. 3, panels B and C, respectively). These concentrations could be lowered to 32 µg/mL in the presence of 2 µg/mL of colistin to achieve a similar level of inhibition of biofilm formation, i.e. 84 ± 8% and 70 ± 11% for Pa01 and clone C respectively. A concentration of 2 µg/mL colistin without additional compound poorly inhibited biofilm formation, up to 20%. Also, only 32 µg/mL of BA250-C10 was not able to inhibit biofilm formation in these two strains. Replacing colistin with equal weight of tobramycin did not lead to biofilm formation inhibition, showing that synergism is strictly limited to colistin. The observation that sub-MIC concentrations of AMPs already lead to observable inhibition of biofilm formation has been described before.[18a,39] The differences between the levels of inhibition of planktonic growth and biofilm formation suggest that the AMPs interfere with biofilm formation in a different manner than interference with planktonic growth.

In order to visualize the effect of the lipoAMP on the biofilms that were formed, we performed confocal microscopy studies on the biofilms of KD491 and clone C in the presence or absence of the lipoAMP. These two strains were selected since

the activity of BA250-C10 against KD491 was distinctly better than against clone C (Table 2, entries 20 and 3, respectively); LESB58 was excluded based on lower tendency to form biofilms in our assay, and Pa01 was excluded due to its high resistance against the lipoAMPs. Since BA250-C10 cannot be visualized directly with confocal microscopy, we applied the fluorescent peptide BA250-DEC, which contains a fluorescent diethylaminocoumarine moiety (λ_{ex} = 409 nm, λ_{em} = 473 nm) instead of the C_{10}-lipid. The retention time of this dye-labelled peptide is comparable to that of the C_{10}-lipidated peptide, i.e. 19.9 min *vs* 20.2 min, respectively (see Fig. 4 for structures), and the antibacterial activity is 4-fold lower, i.e. 64 μg/mL against KD491 (Table 1).

Incubation of *P. aeruginosa* strain KD491 and clone C revealed that also the dye-containing BA250-DEC is able to inhibit biofilm formation (Fig. 3). Clear difference in biofilm texture is apparent: the biofilm that is formed by KD491 is more dense and thicker, whereas that of clone C is more spread-out, containing more isolated cells. For KD491, there is a clear distinction between the biofilms that are formed in the presence or absence of the peptide, confirming the inhibition of biofilm formation by the lipoAMP BA250-C10 that was measured in the polystyrene. The lipoAMP more effectively inhibits biofilm formation of KD491 than that of clone C (panels A and B, and panels C and D, Fig. 3, respectively): upon treatment with the lipoAMP, KD491 forms a much thinner biofilm whereas that of clone C was much less altered, which corroborates our results obtained by the polystyrene biofilm assay.

Co-localization studies reveal a high degree of overlap between the parts of the biofilm that are stained with propidium iodide and those parts that are stained with the peptide. Our results show that the peptide has a high tendency to bind to those areas in the biofilm where bacteria are residing.

Discussion

Patients with cystic fibrosis (CF) are highly dependent of antibiotic treatment since most of these patients endure chronic respiratory infections, causing (slow) degradation of the respiratory tract, which leads to respiratory failure eventually. This accounts for the majority of mortality in CF patients. The main pathogens in the CF lung are *Pseudomonas aeruginosa* (> 80 % of the adult patients), *Staphylococcus aureus* (30–50 %), *Haemophilus influenzae*, *Xenotrophomonas maltophilia* (~8 %), and *Burkholderia cepacia*.[40] Recently, short Arg-Trp based peptides were discovered that showed broad-spectrum activity against various bacterial pathogens, including *P. aeruginosa*.[25] To explore if such short peptides have the potential to combat *P. aeruginosa* infections, we tested such lipoAMPs for their direct *in vitro* anti-pseudomonal activity. The most promising lead-compound, i.e. BA250-C10, was further tested for its potential synergy with conventional antibiotics colistin and tobramycin (see Fig. 4 for structures), and the potential in interfering with biofilm formation.

In this study, we demonstrated that the combination of BA250-C10 with one of the conventional anti-pseudomonal antibiotics colistin or tobramycin successfully inhibits planktonic growth in a synergetic way. Most synergy was seen in the combination of 2 μg/mL of BA250-C10 with 2 μg/mL of colistin. Colistin and tobramycin are frequently used in CF patients intravenously during exacerbations and chronically by nebulization. For both BA250-C10 and colistin it was shown that they delocalize peripheral membrane proteins,[41] hinting at a cooperative activity in weakening the membrane architecture. Such an effect was not observed before for this type of lipoAMP. In addition, for two of the three strains, biofilm formation was inhibited due to the synergistic effect between 2 μg/mL of colistin and 32 μg/mL of BA250-C10. With 50% hemolysis at 250 μg/mL of BA250-C10, which translates to <10% hemolysis at 32 μg/mL (assuming a linear correlation between concentration and hemolysis), these amounts are still problematic for systemic applications. However, in case of *P. aeruginosa* from KD491, only 4 μg/mL of BA250-C10 is needed to inhibit biofilm formation in the presence of 0.5 μg/mL of colistin. With this low concentration of lipoAMP, less than 1% hemolysis can be expected, a number that might even be lowered further by performing an L-to-D substitution of certain amino acid residues.[26] Although it is too early to investigate the clinical applicability of lipoAMPs like BA250-C10, the current studies reveals promising synergy between the lipoAMP and existing antibiotics, both at the level of bacterial growth as well as at the level of biofilm formation inhibition.

Further studies have to focus on the mechanism how BA250-C10 interferes with biofilm formation in KD491, even at low concentrations, and why it only interferes in biofilm formation in the other two strains at high concentrations. Tuning the lead compound or further testing of different configurations of the parent peptide can reveal a peptide with higher anti-biofilm and immunomodulatory activity. The class of lipoAMPs now under investigation is particularly interesting as add-on nebulization therapy in CF patients. Recently a high throughput screening has been developed for further optimizing peptides to generate novel sequences that possess a variety of biological properties.[42]

Conclusions

In conclusion, we have demonstrated that the 7-amino-acid residue long lipopeptide BA250-C10 has synergistic activity with two conventional anti-pseudomonal antibiotics in inhibiting planktonic growth of four *P. aeruginosa* strains. Synergism in the inhibition of biofilm formation was shown in three *P. aeruginosa* strains. For the most resistant biofilm forming strain, only 2 μg/mL of BA250-C10 was required to achieve ~50% biofilm formation inhibition; for the less resistant strains 32 μg/mL BA250-C10 and 2 μg/mL colistin was needed to obtain near quantitative inhibition. Localization of the lipoAMP at the bacteria was shown using a fluorescently labelled lipoAMP in confocal microscopy studies. Further studies have to reveal the working mechanism of biofilm interference. Amplification and tuning of the peptide lead compound is relatively easy and is a promising path to obtain peptides with more specific anti-pseudomonal and anti-biofilm properties.

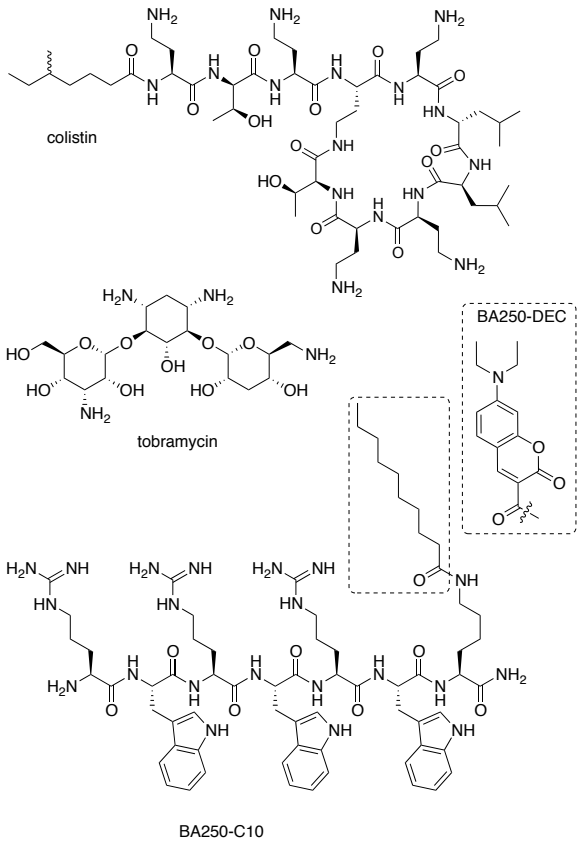

Figure 4. Structural formulas of colistin, tobramycin, and BA250-C10; the structure of the dye in BA250-DEC is shown in the dotted box, it replaced the lipid that is highlighted by the dotted square.

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Author contributions

MGdG and HBA designed research, analysed data, and wrote the paper; MJ and HGS conducted the experiments detailed in Table 1; MGdG conducted the synergism experiments (growth inhibition, growth curves, biofilm formation inhibition); RW, HL, JWJL, and BD designed and assisted in the synergistic activity studies; FLP conducted confocal microscopy studies; RvM selected the *Pseudomonas aeruginosa* strains and assisted in the MIC-studies described in Table 2.

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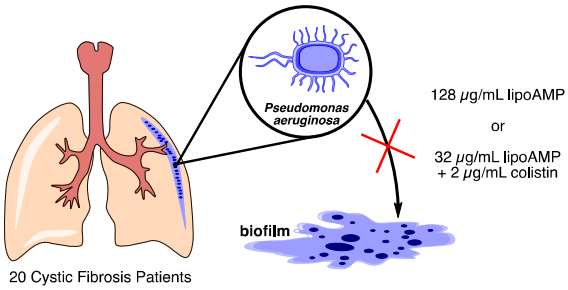

Synergistic effects between a lipoAMP and colistin against twenty clinical *P. aeruginosa* strains isolated from Cystic Fibrosis patients are described.