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Potential novel therapeutic strategies in cystic fibrosis: antimicrobial and anti-biofilm activity of natural and designed α-helical peptides against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Stenotrophomonas maltophilia*

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Abstract

Background: Treatment of cystic fibrosis-associated lung infections is hampered by the presence of multi-drug resistant pathogens, many of which are also strong biofilm producers. Antimicrobial peptides, essential components of innate immunity in humans and animals, exhibit relevant in vitro antimicrobial activity although they tend not to select for resistant strains.

Results: Three α-helical antimicrobial peptides, BMAP-27 and BMAP-28 of bovine origin, and the artificial P19(9/B) peptide were tested, comparatively to Tobramycin, for their *in vitro* antibacterial and anti-biofilm activity against 15 *Staphylococcus aureus*, 25 *Pseudomonas aeruginosa*, and 27 *Stenotrophomonas maltophilia* strains from cystic fibrosis patients. All assays were carried out in physical-chemical experimental conditions simulating a cystic fibrosis lung. All peptides showed a potent and rapid bactericidal activity against most *P. aeruginosa, S. maltophilia* and *S. aureus* strains tested, at levels generally higher than those exhibited by Tobramycin and significantly reduced biofilm formation of all the bacterial species tested, although less effectively than Tobramycin did. On the contrary, the viability-reducing activity of antimicrobial peptides against preformed *P. aeruginosa* biofilms was comparable to and, in some cases, higher than that showed by Tobramycin.

Conclusions: The activity shown by α-helical peptides against planktonic and biofilm cells makes them promising "lead compounds" for future development of novel drugs for therapeutic treatment of cystic fibrosis lung disease.

Keywords: Cystic fibrosis, Antimicrobial peptides, Biofilm

Background

Physicians treating patients with cystic fibrosis (CF) are increasingly faced with infections caused by multidrug-resistant strains. *Pseudomonas aeruginosa* and *Staphylococcus aureus* are the most common bacterial pathogens isolated from the CF respiratory tract where they cause persistent infections associated with a more rapid decline in lung function and survival [1,2]. In recent years, however, there has been an increasing number of reports on potentially emerging and challenging pathogens, probably due to improved laboratory detection strategies and to selective pressure exerted on bacterial populations by the antipseudomonal antibiotic therapy [2]. In this respect, both the overall prevalence and incidence of intrinsically antibiotic-resistant *Stenotrophomonas maltophilia* isolations from CF respiratory tract secretions have been recently reported [3-5].

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Efforts to treat CF infections are also hampered by the high microbial adaptation to the CF pulmonary environment, resulting in an increased ability to form biofilms intrinsically resistant to therapeutically important antibiotics such as aminoglycosides, fluoroquinolones, and tetracycline [6-10].

Novel antimicrobial agents that could replace or complement current therapies are consequently needed to fight chronic infections in CF patients.

Antimicrobial peptides (AMPs) are naturally occurring molecules of the innate immune system that play an important role in the host defence of animals and plants [11-13]. Over the last years, natural AMPs have attracted considerable interest for the development of novel antibiotics for several reasons [14,15]: i) the broad activity spectrum, comprised multiply antibiotic-resistant bacteria; ii) the relative selectivity towards their targets (microbial membranes); iii) the rapid mechanism of action; and, above all, iv) the low frequency in selecting resistant strains. Although the antimicrobial activity of AMPs has been extensively reported in literature [13-17], only few studies have been reported with respect to CF pathogens [18-21].

Hence, in an attempt to evaluate the therapeutic potential of AMPs in the management of CF lung infections, for the first time in the present study three cationic α-helical AMPs - two cathelicidins of bovine origin (BMAP-27, BMAP-28) and the artificial peptide P19(9/B) - were tested for their in vitro antibacterial effectiveness, as well as their *in vitro* anti-biofilm activity, against selected *S. aureus*, *P. aeruginosa*, and *S. maltophilia* strains collected from CF patients. The efficacy of the AMPs was compared to that of Tobramycin, selected as the antibiotic of choice used for chronic suppressive therapy in CF patients.

Since the conditions present in the CF patients' airway surface liquid could counteract the potency of antibiotics such as Tobramycin [22,23], in the present study all *in vitro* antimicrobial assays were carried out under experimental conditions simulating the physical-chemical properties observed in CF lung environment [24-26].

Results

Phenotypic features and clonal relatedness of CF strains

A total of 9 out of 25 *P. aeruginosa* strains tested showed mucoid phenotype on MHA, while 3 exhibited SCV phenotype. Among 15 *S. aureus* isolates tested, 7 were methicillin-resistant.

PFGE analysis showed 8, 21, and 12 different pulsotypes among *S. aureus*, *S. maltophilia*, and *P. aeruginosa* isolates, respectively. Among *S. aureus* isolates, only the PFGE type 1 was shared by multiple strains, which comprised 8 isolates and 7 PFGE subtypes. Among *S. maltophilia* isolates, 2 multiple-strains PFGE types were observed: PFGE type 23 (5 isolates, 2 PFGE subtypes), and PFGE type 73 (2 isolates with identical PFGE profile). Among *P. aeruginosa* isolates, 5 multiple-strains PFGE types were observed: PFGE type 5 (6 isolates, 2 PFGE subtypes), PFGE type 1 (4 isolates with indistinguishable PFGE profile), PFGE types 9 and 11 (3 isolates each, with identical PFGE pattern), and PFGE type 8 (2 isolates, one PFGE subtype) (data not shown).

In vitro activity of AMPs and Tobramycin against planktonic cells: MIC, MBC

In order to determine the efficacy of AMPs, the antimicrobial activity was measured against 67 CF clinical isolates, and results are summarized in Table 1. Overall, BMAP-28 showed the widest activity spectrum among AMPs tested, as suggested by MIC_{90} and MBC_{90} values (16 μg/ml, for both), although all of them exhibited a species-specific activity. In fact, although AMPs showed comparable activity against *P. aeruginosa*, BMAP-28 was found to be more active than P19(9/B) against *S. maltophilia*, and resulted the best active AMP against *S. aureus* (MIC_{90}: 32 μg/ml; MBC_{90}: 32 μg/ml). Compared to AMPs, Tobramycin exhibited a lower activity (MIC_{90} and MBC_{90}: >64 μg/ml) regardless of the species considered. Killing quotient values, calculated as MBC/MIC ratio, were < 4 for all AMPs**,** as well as for Tobramycin, clearly suggesting a bactericidal activity. No differences in susceptibility levels to AMPs were found with regard to phenotype (mucoid, SCV, MRSA), pulsotype, or susceptibility to Tobramycin (data not shown).

MIC and MBC values obtained under CLSI-recommended or "CF-like" experimental conditions (see Materials and Methods section) are shown in Table 2. Comparative evaluation of these values showed that mean $MIC_{CF\text{-}like}/MIC_{CLSI}$ and $MBC_{CF\text{-}like}/MBC_{CLSI}$ values obtained for Tobramycin (23.9 and 15.6, respectively) were significantly higher than those observed for BMAP-27 (1.5 and 1.2, respectively; $p < 0.001$), BMAP-28 (0.5 and 0.5, respectively; $p < 0.001$), and P19(9/B) (2.8 and 2.9, respectively; $p < 0.001$), regardless of species tested, indicating a reduced antibiotic activity of Tobramycin in CF-like conditions.

Bactericidal kinetics

Time-killing results have been summarized in Figure 1. BMAP-27, BMAP-28, and P19(9/B) exerted a rapid bactericidal activity against *P. aeruginosa*, reducing the number of viable bacterial cells of at least 3 logs within 60 min of exposure. However, the bactericidal effect of BMAP-28 against *P. aeruginosa* was incomplete for two (Pa6 and Pa22) of the three strains tested, allowing bacterial regrowth after 24-h incubation, although at levels lower than those observed for untreated control. In parallel experiments, Tobramycin showed only a bacteriostatic

Table 1 *In vitro* activity of BMAP-27, BMAP-28, P19(9/B), and Tobramycin against *P. aeruginosa, S. maltophilia* and *S. aureus* CF strains

Bacterial strains (n)	Test agent:			
	BMAP-27	BMAP-28	P19(9/B)	TOBRAMYCIN
***P. aeruginosa* (25)**				
MIC_{50}^{a}	8	16	8	16
MIC_{90}^{b}	16	32	32	>64
MIC_{range}	4-16	4–32	4–32	2- > 64
MBC_{50}^{c}	8	16	16	32
MBC_{90}^{d}	16	32	64	>64
MBC_{range}	4–16	4–64	4- > 64	2- > 64
MBC/MIC	1.3	1.2	1.9[e]	1.5[f]
***S. maltophilia* (27)**				
MIC_{50}^{a}	4	4	4	>64
MIC_{90}^{b}	8	4	16	>64
MIC_{range}	4-8	2–8	4–32	4- > 64
MBC_{50}^{c}	8	4	8	>64
MBC_{90}^{d}	16	8	32	>64
MBC_{range}	4–32	2–16	4–64	8- > 64
MBC/MIC	1.9	1.3	1.7	1.3[g]
***S. aureus* (15)**				
MIC_{50}^{a}	64	8	64	>64
MIC_{90}^{b}	>64	32	>64	>64
MIC_{range}	32- > 64	4–32	32- > 64	4- > 64
MBC_{50}^{c}	>64	8	>64	>64
MBC_{90}^{d}	>64	32	>64	>64
MBC_{range}	64- > 64	4–32	32- > 64	4- > 64
MBC/MIC	1.2[h]	1.2	1.2[i]	1.0[l]
Total (67)				
MIC_{50}^{a}	8	4	8	>64
MIC_{90}^{b}	>64	16	64	>64
MIC_{range}	4->64	2–32	4- > 64	2- > 64
MBC_{50}^{c}	8	8	16	>64
MBC_{90}^{d}	>64	16	>64	>64
MBC_{range}	4- > 64	2–64	4- > 64	2- > 64
MBC/MIC	1.5[m]	1.2	1.7[n]	1.4[o]

[a, b] MIC_{50} and MIC_{90}: MIC (μg/ml) inhibiting 50 and 90% of the strains tested, respectively.
[c, d] MBC_{50} and MBC_{90}: MBC (μg/ml) eradicating 50 and 90% of the strains tested, respectively.
Only isolates exhibiting in range MIC values were considered for killing quotient calculation (MBC/MIC): [e]n = 24; [f]n = 12; [g]n = 3; [h]n = 6; [i]n = 2; [m]n = 58; [n]n = 57;[o]n = 17.

Table 2 Antimicrobial activity of BMAP-27, BMAP-28, P19 (9/B) and Tobramycin evaluated under different experimental conditions: "CF-like" (5% CO_2, pH 6.8, SCFM) and "standard CLSI-recommended" (aerobiosis, pH 7.2, CAMHB)

	Susceptibility ($MIC_{CF\text{-}like}/MIC_{CLSI}$) to:			
Bacterial strains	**BMAP-27**	**BMAP-28**	**P19(9/B)**	**TOBRAMYCIN**
P. aeruginosa				
Pa1	8/4	8/8	4/16	4/0.25
Pa5	8/4	16/16	8/8	16/2
Pa6	8/8	16/16	16/8	8/8
Pa9	8/4	16/16	16/8	64/1
S. maltophilia				
Sm109	4/8	4/16	4/8	128/64
Sm126	8/16	8/32	4/32	256/64
Sm143	8/8	4/8	4/4	8/2
S. aureus				
Sa1	128/64	8/16	128/16	256/64
Sa3	64/64	4/32	64/16	256/16
Sa4	64/64	4/16	32/8	32/2
Sa7	64/16	4/16	64/8	256/2
Mean $MIC_{CF\text{-}like}/MIC_{CLSI}$	1.5	0.5	2.8	23.9
P. aeruginosa				
Pa1	8/8	8/16	16/32	4/1
Pa5	16/8	16/32	16/16	16/4
Pa6	16/8	16/16	16/32	8/8
Pa9	8/8	16/32	64/16	128/2
S. maltophilia				
Sm109	8/16	8/16	8/8	256/128
Sm126	8/32	16/32	8/32	256/64
Sm143	16/8	8/8	4/4	8/8
S. aureus				
Sa1	128/64	8/16	128/16	256/64
Sa3	64/64	4/32	64/16	256/32
Sa4	64/64	8/32	32/8	32/2
Sa7	64/ND[a]	8/16	64/8	256/4
Mean $MBC_{CF\text{-}like}/MBC_{CLSI}$	1.2	0.5	2.9	15.6

[a] ND, not determined.

effect against *P. aeruginosa,* causing no more than 1-log reduction in viable count after 24 h.

BMAP-27, BMAP-28 and P19(9/B) exerted bactericidal activity also against *S. maltophilia*, although with streaking strain-specific differences. Particularly, BMAP-28 exhibited only bacteriostatic effect against Sm192 strain, while P19(9/B) showed a rapid bactericidal effect against Sm138 strain, causing more than a 4-log reduction in viable count after 10 min-exposure. Tobramycin exhibited a late (after 24-h exposure) bactericidal effect only against Sm138 strain.

AMPs activity against *S. aureus* was significantly strain-specific, ranging from the rapid bactericidal activity of BMAP-28 against Sa10 strain, to the bacteriostatic effect of P19(9/B) and BMAP-28 against Sa4 strain. Tobramycin

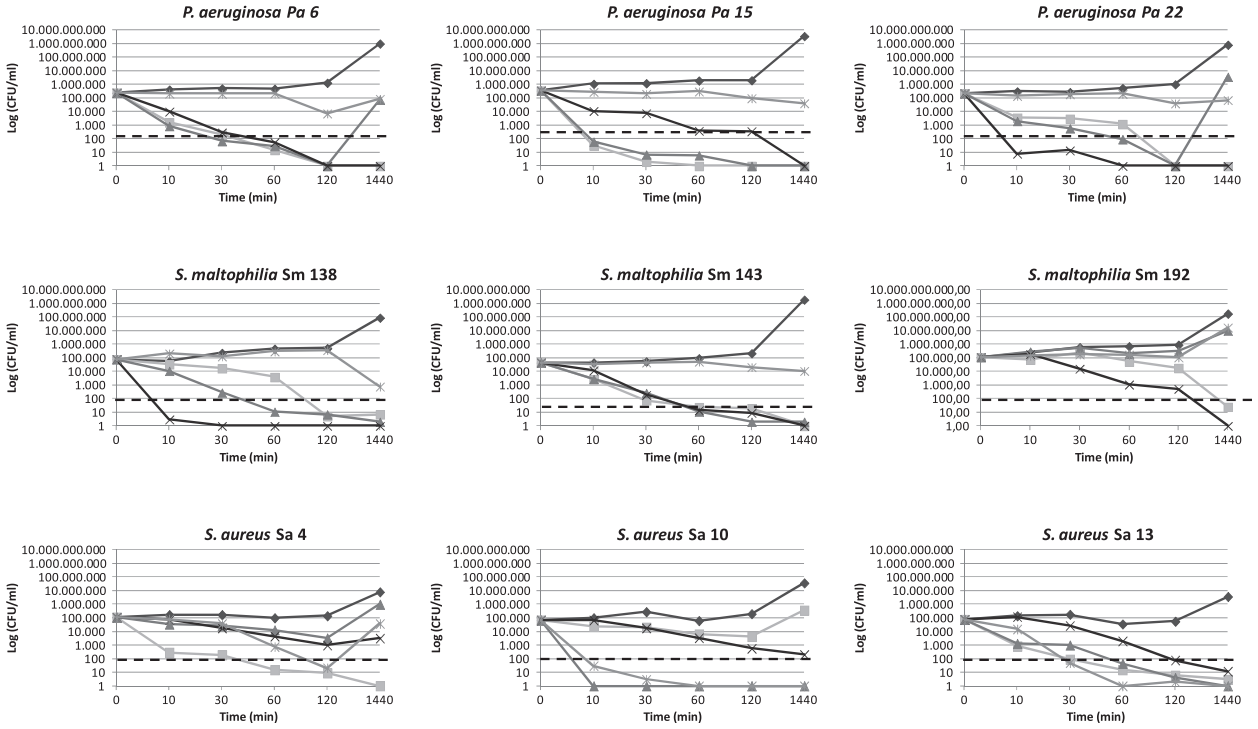

Figure 1 Time-killing kinetic of AMPs against CF strains. BMAP-27 (■), BMAP-28 (▲), P19(9/B) (×), and Tobramycin (●) were tested at MIC value against representative *P. aeruginosa* (Pa6, Pa15, and Pa22), *S. maltophilia* (Sm138, Sm143, and Sm192), and *S. aureus* (Sa4, Sa10, and Sa13) CF strains. Controls (♦) were not exposed to drugs. Values are the mean of two independent experiments performed in triplicate. The dotted line indicates a 3-log reduction in viability.

showed a bactericidal effect against all *S. aureus* strains tested, although allowing bacterial regrowth of Sa4 strain after 2-h exposure.

In vitro activity of Tobramycin-AMP combinations against planktonic cells

Results from checkerboard assays are summarized in Table 3. FICI values showed that all AMP + Tobramycin combinations tested showed an indifferent effect against *P. aeruginosa* and *S. maltophilia* strains. Conversely, BMAP-27 + Tobramycin (tested at 16 + 8, 16 + 4, and 16 + 2 μg/ml, respectively) combination exhibited synergic effect against Sa4 strain (the only one tested, 100% synergy), while P19(9/B) + Tobramycin (tested at 4 + 2, 4 + 1, and 8 + 1 μg/ml, respectively) combination exhibited synergic effect against *S. aureus* Sa10 strain (1 out of 3 strains tested, 33.3% synergy).

In vitro activity of AMPs and Tobramycin against biofilm

All CF strains were screened for biofilm forming ability on polystyrene. A significantly higher proportion of biofilm producer strains was found in *P. aeruginosa* and *S. aureus*, compared to *S. maltophilia* (96 and 80% vs 55%, respectively; $p < 0.01$) (data not shown). However, efficiency in biofilm formation was significantly higher in *P. aeruginosa* than in *S. aureus*, as suggested by median biofilm amounts produced (0.162 vs 0.109, respectively; $p < 0.01$) (data not shown).

Table 3 In vitro effect of AMP + Tobramycin (TOB) combinations against *P. aeruginosa*, *S. maltophilia*, and *S. aureus* CF strains

Drug combinations	*P. aeruginosa*			*S. maltophilia*			*S. aureus*		
	Synergy	Indifference	Antagonism	Synergy	Indifference	Antagonism	Synergy	Indifference	Antagonism
	FICI[a] ≤ 0.5	0.5 < FICI ≤ 4	FICI > 4	FICI ≤ 0.5	0.5 < FICI ≤ 4	FICI > 4	FICI ≤ 0.5	0.5 < FICI ≤ 4	FICI > 4
BMAP-27 + TOB	0 (0%)	12 (100%)	0 (0%)	0 (0%)	8 (100%)	0 (0%)	1 (100%)[b]	0 (0%)[b]	0 (0%)[b]
BMAP-28 + TOB	0 (0%)	12 (100%)	0 (0%)	0 (0%)	8 (100%)	0 (0%)	0 (0%)[c]	1 (100%)[c]	0 (0%)[c]
P19(9/B) + TOB	0 (0%)	12 (100%)	0 (0%)	0 (0%)	8 (100%)	0 (0%)	1 (33.3%)[d]	2 (66.7%)[d]	0 (0%)[d]

[a] Fractional Inhibitory Concentration Index (FICI).
Only isolates exhibiting in-range MIC values were considered for checkerboard titration method: *P. aeruginosa* (n = 12), *S. maltophilia* (n = 8), and *S. aureus* ([b] n = 1; [c] n = 1; [d] n = 3).

To determine if AMPs could be prophylactically used to prevent biofilm formation, we tested the effect of AMPs and Tobramycin at sub-inhibitory concentrations (1/2x, 1/4x, and 1/8xMIC) against biofilm formation (Figure 2). Tobramycin at 1/2x and 1/4xMIC caused a significantly higher reduction in biofilm-forming ability of *S. maltophilia* and *S. aureus*, in comparison with the three AMPs. This effect was more relevant with *S. aureus*, being observed also at 1/8xMIC. Tobramycin showed to be more effective than BMAP-27 against *P. aeruginosa* at concentrations equal to 1/4x and 1/8xMIC. The activity of Tobramycin in reducing biofilm formation was not related to drug susceptibility (data not shown). Among AMPs, BMAP-28 and P19(9/B) at 1/2xMIC were significantly more active compared to BMAP-27, and BMAP-28 at 1/4xMIC was significantly more active than other AMPs against *S. aureus*.

We further evaluated AMPs as potential therapeutics for CF by testing their efficacy against preformed biofilms. To this, BMAP-27, BMAP-28, P19(9/B), and Tobramycin at 1xMIC and at bactericidal concentrations (5x, and 10xMIC) were assayed against preformed (24 h) biofilms by six representative *P. aeruginosa* strains selected for high biofilm formation ability (Figure 3).

The activity of AMPs and Tobramycin against preformed biofilms resulted to be similar in 5 out of 6 strains tested, causing a highly significant reduction of biofilm viability compared to the controls (biofilm not exposed; $p < 0.0001$), regardless of the concentrations tested (Figure 3). AMPs showed to be active at all concentrations, also against biofilms formed by *P. aeruginosa* Pa32, against which Tobramycin was effective only at the highest concentration used (10xMIC). The activity of Tobramycin against preformed biofilms was not related to drug susceptibility (data not shown).

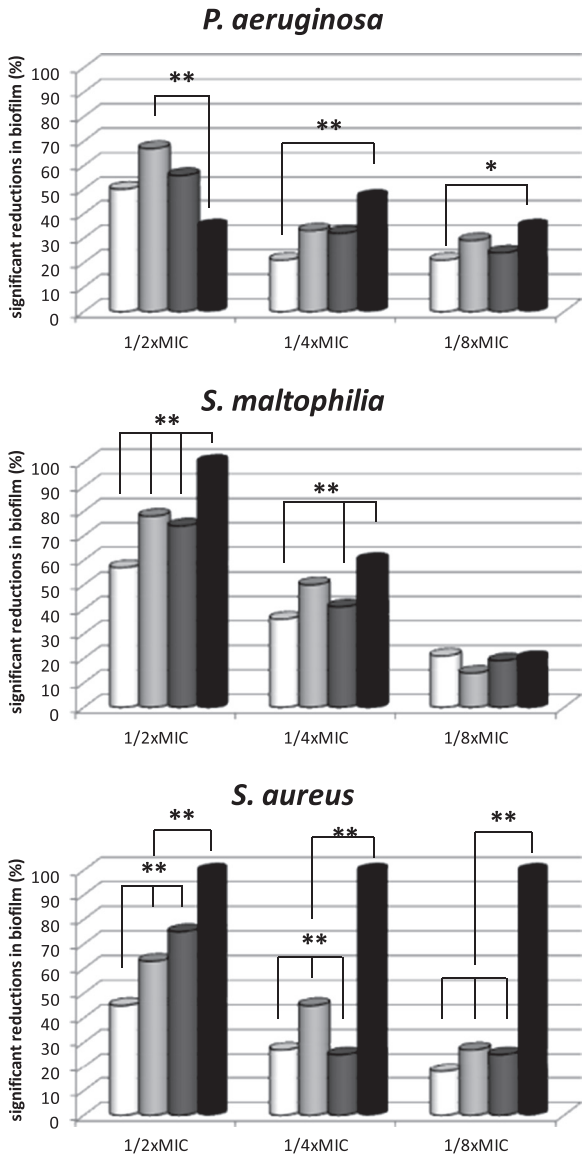

Figure 2 Effect of AMPs at sub-inhibitory concentrations against biofilm formation by CF strains. BMAP-27 (white bars), BMAP-28 (light gray bars), P19(9/B) (dark gray bars), and Tobramycin (black bars) were tested at 1/2x, 1/4x, and 1/8xMIC against biofilm formation by *P. aeruginosa* (n = 24, 24, 25, and 17, for BMAP-27, BMAP-28, P19(9/B) and Tobramycin, respectively), *S. maltophilia* (n = 14, 14, 27, and 5, for BMAP-27, BMAP-28, P19(9/B) and Tobramycin, respectively), and *S. aureus* (n = 11, 11, 8, and 3, for BMAP-27, BMAP-28, P19(9/B) and Tobramycin, respectively) CF strains. Prevention of biofilm formation was plotted as percentage of strains whose ability in forming biofilm was significantly decreased (of at least 25%) compared to controls (not exposed), as analyzed by a crystal violet staining assay.* $p < 0.05$; ** $p < 0.0001$, Fisher's exact test.

Discussion

This study was aimed at verifying the potential of some α-helical AMPs as lead compounds for the development of novel antimicrobials to treat lung disease in CF patients. To this, we tested the *in vitro* susceptibility of *P. aeruginosa*, *S. maltophilia* and *S. aureus* CF isolates to the naturally occurring AMPs BMAP-27 and BMAP-28, as well as the rationally designed P19(B/9), and we compared their effectiveness with that of Tobramycin, the antibiotic of choice for the inhalation therapy of chronic airway infections in CF patients.

BMAP-27 and BMAP-28 are two cathelicidin-derived peptides of bovine origin that have a role in innate defence [27,28]. The hallmark of cathelicidins is the presence of a conserved N-terminal proregion associated with C-terminal antimicrobial sequences showing a remarkable diversity and considerable inter-species differences [13]. BMAP-27 and BMAP-28 are cationic (charge: +11 and +8, respectively) and both adopt an α-helical structure on interaction with the negatively charged bacterial surface [28]. Recent results have suggested that AMPs with these characteristics

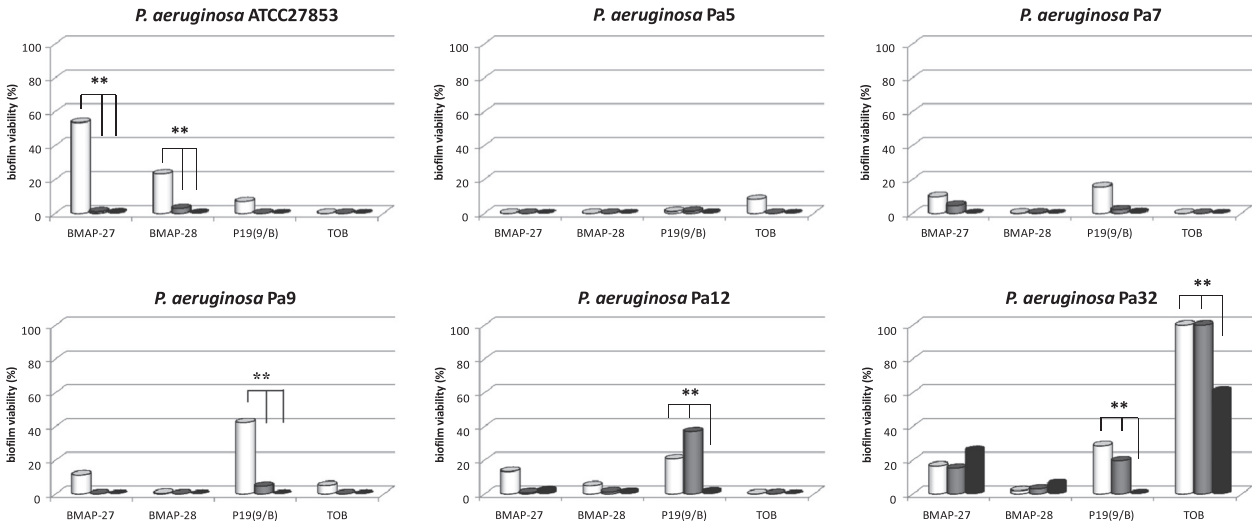

Figure 3 Activity of AMPs at bactericidal concentrations against preformed *P. aeruginosa* biofilms. BMAP-27, BMAP-28, P19(9/B), and Tobramycin were tested at 1x (white bars), 5x (gray bars), and 10xMIC (black bars) against preformed biofilm by 6 *P. aeruginosa* CF strains. Results are expressed as percentage of biofilm' viability compared to control (not exposed, 100% viability). ** $p < 0.0001$, Fisher's exact test.

may be the most effective against strains producing exogenous polysaccharides that are known to inhibit the activity of other types of AMPs [19,29]. For this reason, we added to our study also a third peptide from this class which has been rationally designed, making use also of non-proteinogenic aminoacids, to optimize its propensity to assume α-helical conformation [30].

Effort to treat CF are also hampered by the conditions present in patients' airway surface liquid where the accumulation of large volumes of viscous sputum (mucus) providing bacteria with a nutritionally rich growth environment composed of host- and bacterial-derived factors which deeply change their phenotype and possibly their susceptibility against AMPs [31]. Therefore, to accurately judge the feasibility of these peptides as potential anti-infectives in the context of CF, in this study we investigated the activity of AMPs under some CF-like experimental conditions, including acidic pH, reduced O_2 tension, and a chemically defined medium mimicking the nutritional composition of CF sputum [24-26].

These conditions allow pathogens to assume a physiology similar to that shown *in vivo* in the CF lung [24] and constitute a more realistic model to assay their sensitivity to AMPs.

Evaluation of MIC and MBC values, as well as time-killing assays against planktonic forms of different CF isolates of *P. aeruginosa, S. maltophilia,* and *S. aureus*, have shown that all three AMPs are highly active *in vitro* against most tested strains, although BMAP-28 showed the widest spectrum of activity. It is noteworthy that all the three peptides exhibited an activity higher than Tobramycin. This observation is even more evident when considering the molar concentration (μM) of each compound rather than that by weight (μg/ml), given that the peptides tested are at least six folds heavier than Tobramycin.

The poor activity showed by Tobramycin is probably due to the experimental conditions used in this study, as suggested by comparative evaluation of MIC values observed in both "CF-like" and CLSI-recommended conditions. On the contrary, the activity of AMPs tested resulted to be slightly enhanced (BMAP-28), unaffected (BMAP-27), or slightly reduced [P19(9/B)] in "CF-like" conditions, compared to CLSI-recommended ones, so they can be considered to be quite robust and medium insensitive.

MBC/MIC ratio clearly indicated that all AMPs exert a bactericidal effect against the CF isolates, in agreement with the known capability of BMAP-27, BMAP-28 and P19(B/9) to kill target cells by rapid permeabilization of their membranes [28]. Results of killing kinetic assays confirmed this mode of action, although bactericidal activity against *S. aureus* and *S. maltophilia* was strain-dependent. Again, the potency of AMPs was overall comparable or higher than that showed by Tobramycin.

Due to the different mechanism of action showed by AMPs and Tobramycin, we investigated the potential synergy between them. Interestingly, Tobramycin exhibited synergy with both BMAP-27 and P19(9/B) against planktonic *S. aureus* Sa4 and Sa10 strains, both resistant to Tobramycin, thus suggesting that at least in these cases both AMPs may overcome resistance to Tobramycin by facilitating the internalization of the aminoglycoside into the bacterial cells. Further studies on a more representative number of *S. aureus* strains will be mandatory to understand the mechanism of this synergy and the

feasibility to use these AMPs in association with traditional antibiotic treatments.

Within the CF lung, pathogens cells grow as biofilms, which are inherently recalcitrant to antimicrobial treatment and host response [32]. Even worse, it has recently been reported that some antibiotics may even stimulate biofilm formation at subinhibitory concentrations [7]. Biofilm resistance is mainly due to the slow growth rate and low metabolic activity of bacteria in such community. For these reasons, AMPs whose mechanism of action makes them active also on non-growing bacteria, should be able to efficiently inhibit or prevent biofilm formation.

Our results in fact indicate that the three α-helical peptides were all able to reduce biofilm formation, although generally at a less extent than Tobramycin. In particular, all peptides reduced the capacity of *P. aeruginosa, S. maltophilia* and *S. aureus* to form biofilms when used at sub-inhibitory concentrations, with the strongest effects at about 1/2xMIC values, while Tobramycin was efficacious also at lower concentrations (1/4x, and 1/8x MIC). This effect was particular evident with the isolates of *S. aureus*. Interestingly, no planktonic growth inhibition was observed at concentrations able to reduce biofilm formation, and also AMPs with poor killing capacity against some planktonic cells showed antibiofilm effects. These observations suggest that BMAP-27, BMAP-28 and P19(9/B) may interfere with biofilm formation by different mechanisms other than direct antimicrobial activity similarly to what observed with the human cathelicidin LL-37 [33], and recently reviewed by Batoni et al. [34].

Most CF patients are infected by *P. aeruginosa* whose persistence is due to the formation of antibiotic resistant biofilms in the lung [35]. Our results showed that BMAP-27, BMAP-28, and P19(9/B) were also as effective as Tobramycin in reducing cell viability of preformed biofilms formed by selected strains of *P. aeruginosa.* At MIC concentrations, and even more at 5xMIC values, the two cathelicidins caused highly significant reduction of biofilm viability of all six strains of *P. aeruginosa* whereas Tobramycin showed comparable results only for five isolates. It has previously been reported that extracellular DNA is an important biofilm component [36], and that in *P. aeruginosa* it is involved in cell-cell attachment and biofilm development [37]. Due to the high affinity of cationic AMPs for DNA [38], it may be presumed that this binding might facilitate the detachment or disruption of otherwise-stable biofilm structures.

Conclusions

The overall results of this study shed new insights on the antibacterial properties of α-helical peptides, allowing the selection of those with the best properties to cope with lung pathogens associated to CF. BMAP-27, BMAP-28 and also the rationally designed P19(9/B) may thus be considered useful not only as lead compounds for the development of novel antibiotics but also for compounds that may counteract bacterial biofilm formation and eradicate preformed biofilms, reflecting the modern understanding of the role of biofilm formation in chronic CF infections. However, before applying these molecules in the future for early prophylactic and therapeutic treatment of CF lung disease, further *in vitro* studies (against other CF pathogens, such as *Burkholderia cepacia*, and fungi), as well as in vivo studies are needed to evaluate their therapeutic potential.

Methods

Bacterial strains

Overall, 67 antibiotic-resistant bacterial strains were tested in the present study: 15 *S. aureus*, 25 *P. aeruginosa*, and 27 *S. maltophilia*. Strains were collected from respiratory specimens obtained from patients admitted to the CF Operative Unit, "Bambino Gesù" Children's Hospital and Research Institute of Rome. Identification to species level was carried out by both manual (API System; bioMérieux, Marcy-L'Etoile, France) and automated (BD Phoenix; Becton, Dickinson and Company, Buccinasco, Milan, Italy) biochemical test-based systems. Each isolate was collected from a single patient and resistant to at least three of the following groups of antibiotics: β-lactams with or without β-lactamase inhibitor, aminoglycosides, fluoroquinolones, folate-pathway inhibitors (trimethoprim-sulphamethoxazole), tetracyclines, and macrolides. Strains were stored at −80°C in a Microbank system (Biolife Italiana S.r.l., Milan, Italy) and subcultured in Trypticase Soya broth (Oxoid S.p.A., Milan, Italy), then twice on Mueller-Hinton agar (MHA; Oxoid S.p.A) prior to the use in this study.

Phenotypic and genotypic characterization of CF strains

All strains grown on MHA were checked for mucoid phenotype and the emergence of small-colony variants (SCVs). Further, they were screened for their susceptibility to antibiotics by agar-based disk diffusion assay, according to the CLSI criteria [39], and by the Etest following the manufacturer's instructions assays (Biolife Italiana S.r.l.; Milan, Italy).

All CF strains tested in this study were genotyped by Pulsed-Field Gel Electrophoresis (PFGE) analysis in order to gain clue on genetic relatedness of strains. DNA was prepared in agarose plugs for chromosomal macrorestriction analysis as previously described [40,41]. For *S. aureus* isolates, agarose plugs were digested with enzyme *SmaI* (40U). DNA from *P. aeruginosa* and *S. maltophilia* isolates was digested using *XbaI* (30U). PFGE profiles were visually interpreted following the

interpretative criteria previously described [27,40]: in particular, isolates with indistinguishable PFGE patterns were assigned to the same PFGE subtype; for *S. aureus*, isolates differing by 1 to 4 bands were assigned to different PFGE subtypes within the same PFGE type; for *S. maltophilia* and *P. aeruginosa*, isolates were assigned to the same PFGE type with different PFGE subtypes when they differed by 1 to 3 bands.

Peptide Synthesis, purification and characterization

P19(9/B) (GZZOOZBOOBOOBZOOZGY; where Z = Norleucine; O = Ornithine; B = 2-Aminoisobutyric acid) was a kind gift of Prof. A. Tossi and was prepared as described previously [30]. BMAP-27 (GRFKRFRKKFKKLFKKLSPVIPLLHL-am) and BMAP-28 (GGLRSLGRKILRAWKKYGPIIVPIIRI-am) were synthesised as C-terminal amides by solid-phase peptide Fmoc strategy on a Microwave-enhanced CEM Liberty Synthesizer on a Pal-PEG Rink Amide resin LL (substitution 0.18-0.22 mmol/g). The peptides were purified by RP-HPLC on a Phenomenex preparative column (Jupiter™, C18, 10 μm, 90 Å, 250 × 21.20 mm) using a 20-50% CH_3CN in 60-min gradient with an 8 ml/min flow. Their quality and purity were verified by ESI-MS (API 150 EX Applied Biosystems). Concentrations of their stock solutions, were confirmed by spectrophotometric determination of tryptophan ($\epsilon_{280} = 5500\ M^{-1}\ cm^{-1}$), by measuring the differential absorbance at 215 nm and 225 nm [42] and by spectrophotometric determination of peptide bonds (ϵ_{214} calculated as described by Kuipers and Gruppen [43]).

"CF-like" experimental conditions

In order to simulate the physical-chemical properties observed in CF lung environment [24-26], all *in vitro* antimicrobial assays against planktonic (MIC, MBC, time-kill kinetics, synergy testing) and sessile (biofilm formation, preformed biofilms) cells were performed in "CF-like" conditions: i) under reduced oxygen concentration (5% CO_2); ii) at acidic pH (6.8); and iii) in a chemically defined "synthetic CF sputum medium" (SCFM), that mimics the nutritional composition of CF sputum [24]. SCFM was prepared by using Casamino Acids Vitamin Assay (BD Difco) mixture containing each amino acid at concentration not significantly different from that originally described by Palmer and coworkers [24], except for a reduced amount of glycine and ornithine, which were therefore added from ad hoc prepared stock solutions to reach their required concentration.

Susceptibility testing

MICs and MBCs were determined by microdilution technique, in accordance with CLSI M100-S20 protocol [39], with some modifications. Briefly, serial two-fold dilutions (64 to 0.12 μg/ml) of each AMP and Tobramycin (Sigma-Aldrich S.r.l.; Milan; Italy) were prepared in SCFM at a volume of 100 μl/well in 96-well microtiter plates (Bibby-Sterilin Italia S.r.l.; Milan, Italy). Each well was then inoculated with 5 μl of a standardized inoculum, corresponding to a final test concentration of about $0.5\text{-}1 \times 10^5$ CFU/well. After incubation at 37°C for 24 h, the MIC was read as the lowest concentration of the test agent that completely inhibited visible growth. To measure the MBC, 100 μl of broth from clear wells were plated on MHA plates, and incubated at 37°C for 24 h. MBC was defined as the lowest concentration of the test agent killing of at least 99.99% of the original inoculum.

To evaluate the impact of "CF-like" experimental conditions on the antimicrobial activity of AMPs and Tobramycin, a set of PFGE-unrelated isolates representative for different levels of susceptibility to Tobramycin (4 *P. aeruginosa*, 3 *S. maltophilia*, and 4 *S. aureus*) was also tested for MIC and MBC values determined under standard CLSI-recommended conditions (i.e., aerobic atmosphere, cation-adjusted Mueller-Hinton broth, and pH 7.2).

Time-killing assay

Kinetics of AMPs' and Tobramycin' activity was evaluated by using the broth macrodilution method against three representative isolates within each tested species. Briefly, the standardized inoculum ($1x10^5$ CFU/mL) was exposed to the test agent at 1xMIC in SCFM, and incubated at 37°C. After 10 min, 30 min and 1, 2, and 24-h of incubation, aliquots of each sample were diluted and plated onto MHA, then the viable counts determined after 24-h of incubation at 37°C. Killing curves were constructed by plotting the log CFU/mL versus time.

Synergy testing

The activity of each AMP combined to Tobramycin against CF strains was evaluated by checkerboard technique by using 96-well polystyrene microplate (Kartell S. p.A., Noviglio, Milan, Italy). Briefly, concentrations of multiple compounds (range: 64–0.12 μg/ml) were combined in standard MIC format along with 5×10^5 CFU/ml of tested. Inoculated microplates were incubated at 37°C for 24 h under 5% CO_2. At the end of the incubation, for each combination interaction a Fractional Inhibitory Concentration (FIC) index was calculated as follows: FIC index = Σ ($FIC_A + FIC_B$), where FIC_A is the MIC of drug A in the combination/MIC of drug A alone, and FIC_B is the MIC of drug B in the combination/MIC of drug B alone. Synergy was defined as a FIC index of ≤0.5, indifference as a FIC index of >0.5 to ≤ 4, and antagonism as a FIC index of > 4.

In vitro activity against biofilm formation

In each well of a 96-well flat-bottom polystyrene tissue-culture microtiter plate (Iwaki; Bibby-Sterilin Italia S.r.l.), 5 μl of a standardized inoculum ($1–5 \times 10^7$ CFU/ml) were added to 100 μl of SCFM containing test agent at 1/2x, 1/4x, and 1/8xMIC. After incubation at 37°C for 24 h, non-adherent bacteria were removed by washing twice with 100 μl sterile PBS (pH 7.2; Sigma-Aldrich S.r.l.). Slime and adherent cells were fixed by incubating for 1 h at 60°C, and stained for 5 min at room temperature with 100 μl of 1% crystal violet solution. The wells were then rinsed with distilled water and dried at 37°C for 30 min. Biofilms were destained by treatment with 100 μl of 33% glacial acetic acid for 15 min, and the OD_{492} was then measured. The low cut-off was represented by approximately 3 standard deviations above the mean OD_{492} of control wells (containing medium alone without bacteria). The percentage of inhibition was calculated as follows: (1 – OD_{492} of the test/OD_{492} of non-treated control) x 100.

In vitro activity against preformed *P. aeruginosa* biofilms

In vitro activity of AMPs and Tobramycin was evaluated against biofilms formed by 6 *P. aeruginosa* strains, selected because strong biofilm-producers. Biofilms were allowed to form in each well of a 96-well flat-bottom polystyrene tissue-treated microtiter plate (Iwaki), as described above. Biofilms samples were then exposed to 100 μl of drug-containing SCFM (prepared at 1x, 5x, and 10x MIC). After incubation at 37°C for 24 h, non-adherent bacteria were removed by washing twice with 100 μl sterile PBS (pH 7.2), and biofilm samples were scraped with a pipette tip following 5-min exposure to 100 μl trypsin-EDTA 0.25% (Sigma-Aldrich S.r.l.). Cell suspension was then vortexed for 1 min to break up bacterial clumps. Bacterial counts were assessed by plating serial 10-fold dilutions of the biofilm cell suspension on MHA plates.

Statistical analysis

All experiments were performed at least in triplicate and repeated on two different occasions. Differences between frequencies were assessed by Fisher's exact test. Statistical analysis of results was conducted with GraphPad Prism version 4.00 (GraphPad software Inc.; San Diego, CA, USA), considering as statistically significant a p value of < 0.05.

Abbreviations

CF: Cystic Fibrosis; AMPs: Antimicrobial Peptides; MHA: Mueller-Hinton agar; SCVs: Small-Colony Variants; CLSI: Clinical Laboratory Standards Institute; PFGE: Pulsed-Field Gel Electrophoresis; SCFM: Synthetic CF sputum medium; MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration; CFU: Colony-Forming Unit; FICI: Fractionary Inhibitory Concentration Index.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

AP, VC, SP and VDV performed susceptibility assay, time-killing assay, synergy testing, and *in vitro* testing against biofilm formation and preformed biofilms. MS, MM, and RG took care of peptide synthesis, purification and characterization, and of SCFM preparation. GG and GD performed PFGE assay. EF collected clinical strains and also took care of their phenotypic characterization. GDB and MS drafted the manuscript, in collaboration with AP, GG, and RG. GDB also carried out the statistical analysis. All authors read and approved the final version.

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