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Abstract	Patients with cystic fibrosis require pharmacological treatment against chronic lung infections. The alpha-helical antimicrobial peptides BMAP-27 and BMAP-28 have shown to be highly active in vitro against planktonic and sessile forms of multidrug-resistant *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Stenotrophomonas maltophilia* cystic fibrosis strains. To develop small antibacterial peptides for therapeutic use, we tested shortened/modified BMAP fragments, and selected the one with the highest in vitro antibacterial activity and lowest in vivo acute pulmonary toxicity. All the new peptides have shown to roughly maintain their antibacterial activity in vitro. The 1–18 N-terminal fragment of BMAP-27, showing MIC_{90} = 16 μg/ml against *P. aeruginosa* isolates and strain-dependent anti-biofilm effects, showed the lowest pulmonary toxicity in mice. However, when tested in a murine model of acute lung infection by *P. aeruginosa*, BMAP-27(1–18) did not show any curative effect. If exposed to murine broncho-alveolar lavage fluid BMAP-27(1–18) was degraded within 10 min, suggesting it is not stable in pulmonary environment, probably due to murine proteases. Our results indicate that shortened BMAP peptides could represent a starting point for antibacterial drugs, but they also indicate that they need a further optimization for effective in vivo use.

Keywords (separated by '-')	Antimicrobial peptide - Cathelicidin - BMAP - Cystic fibrosis - Biofilm - Multidrug-resistance - In vivo degradation
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ORIGINAL ARTICLE

In vitro and in vivo evaluation of BMAP-derived peptides for the treatment of cystic fibrosis-related pulmonary infections

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Abstract Patients with cystic fibrosis require pharmacological treatment against chronic lung infections. The alpha-helical antimicrobial peptides BMAP-27 and BMAP-28 have shown to be highly active in vitro against planktonic and sessile forms of multidrug-resistant *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Stenotrophomonas maltophilia* cystic fibrosis strains. To develop small antibacterial peptides for therapeutic use, we tested shortened/modified BMAP fragments, and selected the one with the highest in vitro antibacterial activity and lowest in vivo acute pulmonary toxicity. All the new peptides have shown to roughly maintain their antibacterial activity in vitro. The 1–18 N-terminal fragment of BMAP-27, showing $MIC_{90} = 16$ µg/ml against *P. aeruginosa* isolates and strain-dependent anti-biofilm effects, showed the lowest pulmonary toxicity in mice. However, when tested in a murine model of acute lung infection by *P. aeruginosa*, BMAP-27(1–18) did not show any curative effect. If exposed to murine broncho-alveolar lavage fluid BMAP-27(1–18) was degraded within 10 min, suggesting it is not stable in pulmonary environment, probably due to murine proteases. Our results indicate that shortened BMAP peptides could represent a starting point for antibacterial drugs, but they also indicate that they need a further optimization for effective in vivo use.

Keywords Antimicrobial peptide · Cathelicidin · BMAP · Cystic fibrosis · Biofilm · Multidrug-resistance · In vivo degradation

Introduction

Although understanding of the pathophysiology of cystic fibrosis (CF) has been increased in the recent years, pulmonary infections remain the major cause of morbidity and mortality in CF patients. In most cases, *Pseudomonas aeruginosa* and *Staphylococcus aureus* are the pathogens responsible of these complications (Dasenbrook et al. 2010; Emerson et al. 2002), although other pathogens, such as *Stenotrophomonas maltophilia*, are increasingly isolated from CF airways (Emerson et al. 2010; Millar et al. 2009), probably as a result of the selective effect due to the antipseudomonal therapy (Emerson et al. 2002). Another detrimental consequence of repeated antimicrobial treatments is the spreading of multidrug-resistant (MDR) pathogens. Moreover, even though bacteria do not acquire specific resistance to therapeutically important antibiotics, the microbial adaptation to the CF pulmonary environment results in an increased ability to form biofilms, sessile communities intrinsically resistant to many antimicrobial drugs, such as aminoglycosides, fluoroquinolones, and

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M. Mardirossian and A. Pompilio equally contributed to the work.

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Table 1 In vitro activity of BMAPs analogues vs strains from CF patients

Species (n)[b]	BMAP-27(1–18)		BMAP-28(1–18)		mBMAP-28	
	MIC_{90}[a]	MIC_{range}	MIC_{90}	MIC_{range}	MIC_{90}	MIC_{range}
P. aeruginosa (14)	16	2 to >32	16	2 to >32	16	2 to 16
S. aureus (15)	>32	16 to >32	>32	4 to >32	32	8 to >32
S. maltophilia (15)	>32	4 to >32	2	1 to 4	4	1 to 16
TOTAL (44)	>32	2 to >32	32	1 to >32	32	2 to >32

[a] MIC values are expressed as μg/ml. MIC_{90}, Minimum Inhibitory Concentration required to inhibit the growth of 90 % of the strains tested

[b] Number of strains tested

tetracyclines (Di Bonaventura et al. 2007; Hoffman et al. 2005; Linares et al. 2006; Molina et al. 2008; Singh et al. 2000). The sum of these intrinsic and acquired resistances depicts an alarming picture for the treatment of CF pulmonary infections. Novel antimicrobial agents are, therefore, needed to flank or replace current antibiotic therapies to overcome chronic infections in CF patients.

Antimicrobial peptides (AMPs) could represent a promising answer to this request. These natural molecules are important component of the innate immunity of animals and plants, representing a first line-defense against infections (Lai and Gallo 2009; Yang et al. 2002; Zanetti 2004; Zanetti et al. 2000, 2002). The eligibility of AMPs as starting point to develop new antibiotics is based on their broad spectrum of activity, on the efficacy against bacteria resistant to commonly used antibiotics, and on their poor ability to select for antibiotic resistance (Hancock and Sahl 2006; Zanetti 2004). The pulmonary infections related to CF represent, therefore, a suitable field of application for these molecules. The therapeutic potential of some AMPs in the management of CF lung infections started to be explored (Zhang et al. 2005). To this aim, among the galaxy of known AMPs, some peptides belonging to the cathelicidins family are interesting candidates given their good antimicrobial activity also against CF-related pathogens (Pompilio et al. 2011). The mammalian α-helical cathelicidins BMAP-27 and BMAP-28, and the artificial peptide P19(9/B), show a potent and rapid bactericidal and antibiofilm activity against MDR *S. aureus*, *P. aeruginosa*, and *S. maltophilia* strains collected from CF patients (Pompilio et al. 2011). Under physico-chemical conditions simulating those observed in CF lung environment (Palmer et al. 2007; Worlitzsch et al. 2002) the activity of these AMPs is comparable, or even higher, to Tobramycin (Pompilio et al. 2012). It has also been shown, that shortened fragments or derivatives of BMAPs peptides maintain a good antimicrobial potency in spite of decreasing their cytotoxicity and their cost of synthesis (Benincasa et al. 2003; Skerlavaj et al. 1996).

Aim of this study was to assess the acute pulmonary toxicity of BMAPs shortened forms in mice and to characterize their in vitro and in vivo activity to make them applicable in the future for early prophylactic and therapeutic treatment of CF lung disease.

Results

Antimicrobial activity of BMAPs fragments/analogues against MDR bacterial strains

Some synthetic peptides—comprising the N-terminal 18 residues of the α-helical BMAP-27 and BMAP-28 peptides, and a less hydrophobic BMAP-28 analogue—had indeed been shown to have reduced cytotoxicity against human neutrophils and erythrocytes when compared to their natural longer forms (Benincasa et al. 2003). On these bases, we evaluated the antimicrobial activity of these shortened peptides against a panel of previously characterized *S. aureus*, *P. aeruginosa*, and *S. maltophilia* strains isolated from CF patients (Pompilio et al. 2012). All peptides showed a good antimicrobial activity against *P. aeruginosa* and *S. maltophilia*, while a reduced activity was observed against *S. aureus*. Overall, these shortened or modified forms of cathelicidins substantially retained antimicrobial activity (Table 1), even compared with their parent forms (Pompilio et al. 2012).

In vivo acute toxicity of BMAPs fragments/analogues

Once assessed that shortened forms maintained a relevant antimicrobial potential, in vivo toxicity of BMAP-27(1–18), BMAP-28(1–18) and mBMAP-28 was comparatively evaluated in C57BL/6NCrl mice. Exposure to BMAP-27(1–18) caused the death of one mouse when administered at 4 mg/kg, a mortality rate significantly lower than that observed for the other two peptides, regardless of doses used (Table 2). Changes in body weight of mice treated with BMAP-27(1–18) were always less than those observed in mice treated with the other two peptides (see supplementary Fig. S1). Macroscopic C57BL/6NCrl mouse lung pathology was assessed on day 5 post-exposure (p.e.)

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Table 2 Mortality rate observed in C57BL/6NCrl mice ($n = 5$/group) following a single administration of each AMP tested at different doses

Doses	Mortality (%)		
	BMAP-27(1–18)	BMAP-28(1–18)	mBMAP-28
Ctrl	0	0	0
1 mg/kg	0	0	0
2 mg/kg	0	0	20*
4 mg/kg	20*	40	60
8 mg/kg	0*	100	80

Control mice (Ctrl) received vehicle (SALF water) only

* $p < 0.05$ vs other groups, Chi square test

Table 3 Macroscopic damage of C57BL/6NCrl mouse lungs following a single exposure to BMAP-27(1–18), BMAP-28(1–18) and mBMAP-28, each tested at different doses

Doses	BMAP-27(1–18)	BMAP-28(1–18)	mBMAP-28
Ctrl	1	1	1
1 mg/kg	2	3	2
2 mg/kg	3	3	4
4 mg/kg	2	3	4
8 mg/kg	2	4	4

Control mice (Ctrl) received vehicle (SALF water) only. Macroscopic lung pathology was assessed on day 5 p.e. using a "four-point scoring system" (Johansen et al. 1993): 1, normal; 2, swollen lungs, hyperemia, and small atelectasis; 3, pleural adhesion, atelectasis, and multiple small abscesses; and 4, large abscesses, large atelectasis, and hemorrhages

using a "four-point scoring" system. BMAP-27(1–18) at 1, 4 and 8 mg/kg caused a macroscopic pulmonary damage comparable to that observed in control mice (macroscopic score: 2 vs 1, respectively; $p > 0.05$) (Table 3). Furthermore, lung injury caused by BMAP-27(1–18) was generally less than those observed for the other peptides tested at the same concentrations. These results clearly indicated BMAP-27(1–18) as the less toxic peptide among those tested. Since BMAP-27(1–18) exhibited the best antibacterial activity/pulmonary cytotoxicity ratio among the peptides tested, it was selected for subsequent studies.

Bactericidal and anti-biofilm activities of BMAP-27(1–18)

We evaluated the minimal bactericidal concentration (MBC) of BMAP-27(1–18) against the previously tested *P. aeruginosa* and *S. maltophilia* strains. *S. aureus* strains were not further tested because, despite the MIC_{90} for this species was identical to that of *S. maltophilia*, overall the MIC values for the single strains were higher (see Table S1). BMAP-27(1–18) showed a MBC_{50} of 16 μg/ml for more than one-half of *P. aeruginosa* and *S. maltophilia* strains tested, thus confirming its bactericidal mechanism of action. We also assayed the capability of BMAP-27(1–18) to affect the biofilm formation by *S. maltophilia* (Fig. 1a) and *P. aeruginosa* (Fig. 1b) CF strains, and its potential to re-start an infection when tested at sub-inhibitory concentrations. To this aim, we evaluated the viability of sessile cells by the tetrazolium salt assay (MTT), rather than evaluating the biofilm biomass by crystal violet assay. Six out of 15 (40 %) *S. maltophilia* strains were significantly affected in new biofilm viability in the presence of the peptide. This effect was not concentration-dependent, except for Sm120 strain. The remaining nine *S. maltophilia* strains did not show significant variations in biofilm viability. Higher variability was observed for *P. aeruginosa* biofilms: BMAP-27(1–18) showed a significant anti-biofilm activity against 4 out of 14 (28 %) strains, while no effect was observed for 7 (43 %) strains. On the contrary, exposure to peptide even stimulated biofilm production in 3 (21 %) strains. In the case of PA08 strain both inhibiting and enhancing activity were observed, depending on the concentration considered. Taken together, our results indicate that the effect of sub-lethal concentrations of peptide on biofilm formation is strain-specific.

In vivo activity of BMAP-27(1–18) in a murine model of *P. aeruginosa* acute lung infection

The protective role of BMAP-27(1–18) was then evaluated in a murine model of acute pulmonary infection by *P. aeruginosa* RP73. C57BL/6NCr mice were intratracheally challenged with 10^6 CFU, immediately followed by the administration of the peptide at different doses via the same route. No significant differences in CFU/lung values were observed between treated and control mice, regardless of doses used (Fig. 2). Overall, our results suggest that BMAP-27(1–18), despite the antimicrobial activity exhibited in vitro, does not show a protective in vivo activity at safe concentrations and under our experimental conditions.

In vitro degradation of BMAP-27(1–18) by murine bronchoalveolar lavage fluid

To assess whether the absence of in vivo activity would be explained by the scarce stability of the peptide in the pulmonary environment, we incubated BMAP-27(1–18) with bronchoalveolar lavage (BAL) fluid collected from healthy mice. The peptide was rapidly degraded, already after 5 min of exposition to BAL, and the corresponding band disappeared within 20 min of incubation (Fig. 3). Following exposure of an identical amount of peptide to 0.9 % NaCl instead of BAL fluid, no degradation was observed

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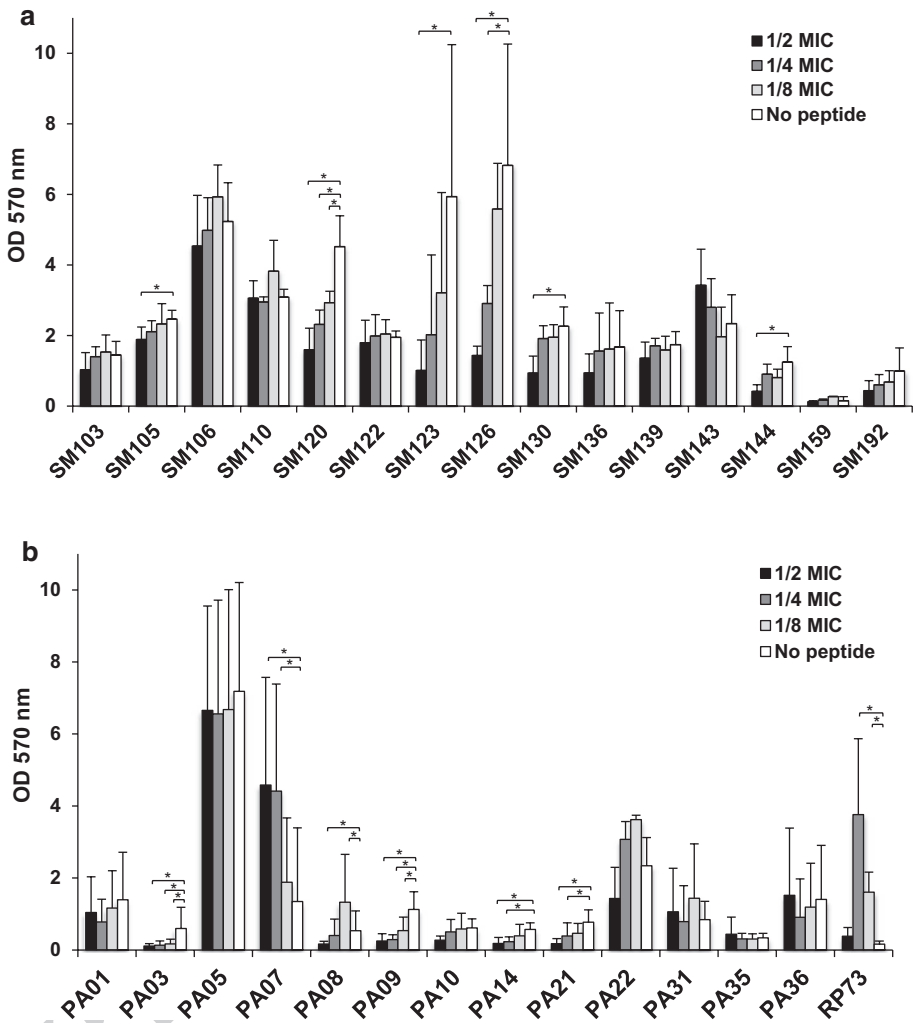

Fig. 1 Effects of sub-inhibitory concentrations of BMAP-27(1–18) on biofilm formation by **a** *S. maltophilia* (SM; $n = 15$) and **b** *P. aeruginosa* (PA; $n = 14$) strains from CF patients. Biofilm viability was evaluated by MTT assay. Results are mean (of three independent experiments and performed as internal duplicate) + SD ($n = 6$). *$p < 0.05$, unpaired t test. BMAP-27(1–18) affected the biofilm viability of SM 105, SM 120, SM 123, SM 126, SM 130, SM 144, PA03, PA09, PA14, and PA21 strains, but also stimulated biofilm growth of RP73, PA07 and PA08 strains

(Fig. S2), thus excluding instability per se of the molecule. These results strongly suggest that BMAP-27(1–18) is cleaved by mice pulmonary proteases into inactive fragments before exerting measurable antibacterial activity in the lungs, therefore, providing an explanation for the poor activity observed in vivo.

Discussion

In this study, we evaluated the potential of some rationally designed AMPs as compounds for the development of novel antibacterial to treat lung disease in CF patients. We looked for peptides active against multidrug-resistant bacterial strains and, at the same time, showing reduced in vivo toxicity. To this aim two peptides, BMAP-27(1–18) and BMAP-28(1–18)—corresponding to the N-terminal fragments of their natural peptides lacking the hydrophobic C-terminal tail—and mBMAP-28—a modified hydrophilic analogue of BMAP-28—have been assayed. All these α-helical peptides have a reduced hydrophobicity in comparison to the natural peptides, a key factor to increase the selectivity towards prokaryotic cells and, therefore, to decrease their toxicity. Previous in vitro cytotoxicity assays performed on these molecules confirmed this hypothesis (Skerlavaj et al. 1996).

Our results showed that all these shortened or modified peptides have a good antimicrobial activity against most of the isolates of *P. aeruginosa* and *S. maltophilia,* despite differences in specificity among the BMAPs, and in general a lower activity against *S. aureus* strains (Table S1). In spite of the low in vitro toxicity showed by BMAPs fragments, the acute pulmonary toxicity tests indicated that all the peptide analogues begin to be toxic already at a dose of 2 mg/kg. This quite surprising result is in contrast with our previous studies where no toxicity was observed when the same peptides were intraperitoneally administered up to 32 mg/kg (Benincasa et al. 2003). To explain this apparent discrepancy, we hypothesized that the higher toxicity reported in this study may be dependent on the pulmonary

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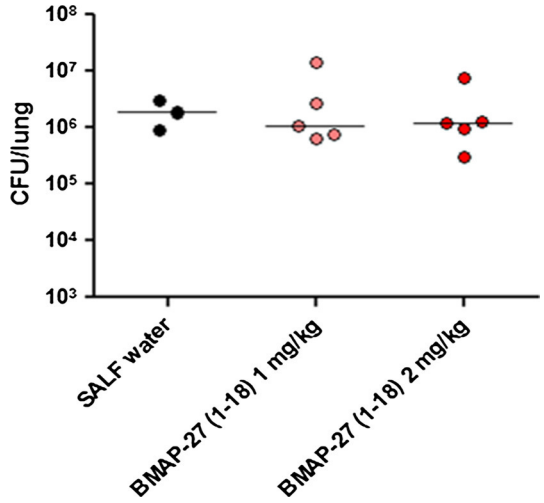

Fig. 2 *In vivo* protection assay. C57BL/6NCr mice were intratracheally challenged with 1×10^7 CFU of *P. aeruginosa* RP73, then 5 min after infection mice were intratracheally administered with SALF water, as a negative control, or with BMAP-27(1–18) at 1 and 2 mg/kg dissolved in SALF water. Following 24 h from exposure, control (infected but not treated) mice displayed classical clinical signs observed during an acute infection by *P. aeruginosa* RP73 strain

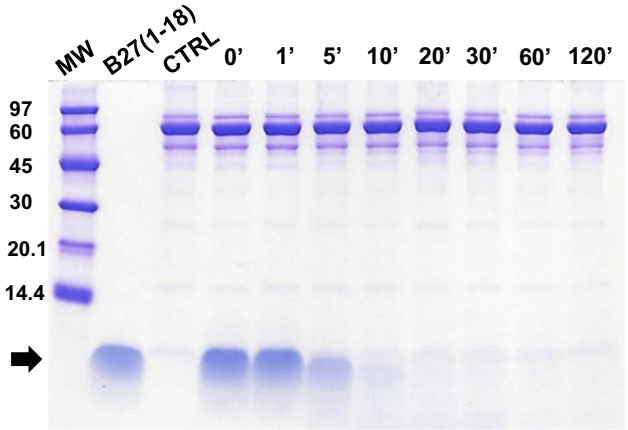

Fig. 3 Evaluation of the stability of BMAP-27(1–18) in the presence of murine bronchoalveolar lavage (BAL) fluid. The peptide is indicated by the *arrow*. Samples were collected at indicated times and analyzed by SDS-PAGE (gel 16 %, tricine) following staining with Coomassie Brilliant Blue. As controls, 2.4 μg of BMAP-27(1–18) [referred as B27(1–18)] and BAL alone [referred as CTRL] were loaded, corresponding to the original respective concentration of both compounds at the beginning of the time-course

administration route. Cytotoxicity could be due to a lytic effect on epithelial cells performed by the very concentrated peptide solution in the first minutes immediately after its intra-tracheal instillation before its spread into the lungs. Further studies will be necessary to explain these differences and, if possible, to reduce toxicity of these peptides in pulmonary applications. Anyhow, among the peptides, BMAP-27(1–18) was selected as the best candidate for its lower cytotoxicity and also for its good bactericidal activity against both *S. maltophilia* and *P. aeruginosa* (MBC_{50}, 16 μg/ml), suitable features to develop a peptide antibiotic that could be used to eradicate, and not only to contain, infections.

The prophylactic use of BMAP-27(1–18) at doses lower than MIC was investigated by the MTT assay on new biofilm formation. The rationale of this approach was to evaluate the potential of newly formed biofilm to trigger a new infection, and not simply to estimate the biomass production (e.g. by crystal violet assay). The peptide did not exert impressive results on *S. maltophilia* strains, significantly inhibiting the biofilm viability in 40 % of the tested strains. Regarding *P. aeruginosa*, markedly strain-specific data were collected. BMAP-27(1–18) reduced the viability of the biofilm in 28 % of the strains tested, did not exert any effect on the 43 % of the strains, and surprisingly enhanced the viability on the biofilm in 21 % of the isolates. The enhancement of biofilm viability in the presence of sub-inhibiting concentrations of BMAP27(1–18) might be due to the up-regulation of specific pathways using antimicrobial compound as activating signals, or to the triggering of a bacterial stress response inducing the bacteria to develop biofilm as a resistance form as had been previously observed also for antibiotics and bacterial species (Kaplan 2011) (Hsu et al. 2011; Wu et al. 2014) and for the unique human cathelicidin LL-37 (Limoli et al. 2014). This pathogens' behavior indicates the need to finely modulate the amount of antibiotic compound to be administered in therapy, to avoid detrimental side effect during antimicrobial therapy.

Unfortunately, BMAP-27(1–18) did not significantly reduce the bacterial load in the mice lungs infected with *P. aeruginosa* RP73. This result can be explained by the scarce stability of the peptide in murine BAL fluid, as suggested by the results of degradation assays performed in vitro. We showed that BMAP-27(1–18) is prone to a rapid degradation by host proteases, a problem already reported in CF sputum for the histatin derivative AMP P-113, (Sajjan et al. 2001). Despite no strict sequence similarity exists between P-113 and BMAP-27(1–18), both AMPs underwent a rapid and non-specific degradation in mice or human pulmonary environments.

The use of an enantiomeric D-BMAP-27(1–18) could represent a good strategy to avoid degradation as already suggested (Sajjan et al. 2001) and, as a consequence, to enhance its antibacterial in vivo activity. Moreover, a peptide form resistant to enzymatic digestion and potentially more active could allow its administration at lower doses, possibly reducing its toxicity. For these reasons, a D-form

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of BMAP-27(1–18) has already been synthesized and its characterization is in progress (manuscript in preparation).

In conclusion, this study shed new insights on the in vitro and in vivo antibacterial properties of BMAP α-helical peptides, allowed the selection of that with the best properties to cope with lung pathogens associated to CF and highlighted the impact that pulmonary proteases can have on AMPs in the treatment of lung infections. Peptide resistance to pulmonary proteases is a key factor that should be evaluated in the design of peptides for pulmonary applications. Further work is, therefore, needed allowing BMAP-27(1–18) application also in vivo and paving the way for its use in the future for early prophylactic and therapeutic treatment of CF-related lung infections.

Materials and methods

Bacterial strains

Previously characterized *S. aureus*, *P. aeruginosa*, and *S. maltophilia* strains were tested (Pompilio et al. 2012). All strains were previously isolated from respiratory specimens of CF patients admitted to the "Bambino Gesù" Children Hospital of Rome. *P. aeruginosa* RP73, and PAO1 reference strains were also tested. Isolates were stored at −80 °C in a Microbank System (Biolife Italiana srl, Milan, Italy) until use, when each isolate was subcultured in Trypticase Soy broth for 24 h at 37 °C, followed by two passages on Mueller–Hinton agar (MHA; Oxoid S.p.A., Milan, Italy).

Design and synthesis of BMAP-derived antimicrobial peptides

Peptides (BMAP-27_{1-18}:GRFKRFRKKFKKLFKKLS-am, BMAP-28_{1-18}: GGLRSLGRKILRAWKKYG-am, mBMAP-28: GGLRSLGRKILRAWKKYGPQAWPAWRQ-am) were synthesized using solid-phase Fmoc chemistry method on a CEM Liberty automated microwave peptide synthesizer (USA) as described in (Benincasa et al. 2006). The peptides have been purified by reversed phase HPLC and their quality and purity verified by ESI–MS (API 150 EX Applied Biosystems). Peptide concentrations of stock solutions, have been confirmed independently by three methods: by the determination of tryptophan absorbance (ε_{280} = 5500/M/cm), by measuring the 215/225 absorbance and by spectrophotometric determination of peptide bonds (ε_{214}) and then lyophilized. For the in vivo toxicity experiments, which required high amounts of peptides, the peptides were purchased (JPT Peptide Technologies, Germany) and checked for their quality, purity and concentrations as described above.

Evaluation of antimicrobial activity of optimized AMP analogues against MDR bacterial strains

MIC values were determined by microdilution technique. Briefly, serial two-fold dilutions of each peptide were prepared in Mueller–Hinton broth (MH; Oxoid S.p.A., Milan, Italy) using a 96-well U-bottom microtiter plates (Bibby-Sterilin Italia srl; Milan, Italy). Each well was inoculated with a standardized inoculum to achieve a final test concentration of about 5×10^5 CFU/ml. After incubation at 37 °C for 24 h, the MIC was measured as the lowest concentration of the peptide that completely inhibited visible bacterial growth. For MBC evaluation, following 24 h-incubation, 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 peptide killing at least 99.99 % of the original inoculum.

Biofilm formation assay

Serial two-fold dilutions of BMAP-27(1–18) were, respectively prepared in MH broth at a volume of 50 µl per well in 96-well U-bottom microtiter plates (Bibby- Sterilin Italia srl; Milan, Italy). Each well was inoculated with 50 µl of the standardized inoculum, corresponding to a final test concentration of about 5×10^5 CFU/ml. After incubation at 37 °C for 24 h, medium and non-adherent bacteria were discarded. Wells were washed three times using 150 µl of fresh MH broth, then 100 µl of MH broth containing 1 mM MTT (Sigma) were added to the wells. The plate was incubated for 4 h in the dark, then the MTT-containing medium was discarded and the wells washed with 150 µl PBS. Subsequently, 100 µl of a re-suspending solution (20 % wt/v SDS in 50 % v/v H_2O and 50 % v/v *N*,*N*-dimethylformamide) (Hansen et al. 1989) were added to the wells. The plate was incubated overnight for 16 h in the dark, and then the optical density at 570 nm was measured using a multi-well plate reader (Tecan Trading AG, Switzerland). The signal was directly proportional to the viability of the biofilm under the tested conditions.

In vivo toxicity of optimized AMPs in a murine model

C57BL/6NCrl mice (n = 5/group) (male; 22 g; 6 ± 2 week-old) were obtained from Charles River Laboratories Italia srl (Calco, Milan, Italy). The in vivo toxicity of each AMP was investigated following intratracheal administration of increasing doses (1, 2, 4 and 8 mg/kg) prepared in sterile distilled water. The control mice received vehicle only (sterile distilled water). Animal behavior, general health (ruffled coats, huddled position, lack of retreat in handler's presence), weight loss, and survival were monitored daily

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over a 5-day period, with respect to control mice. On day 5 post-administration, mice were sacrificed by intraperitoneal injection of Avertin (Sigma-Aldrich S.r.l), then lungs underwent to in situ for macroscopic analysis for assessing damage using "four-point scoring system" (Johansen et al. 1993).

In vivo activity of BMAP-27(1–18) in a mouse model of *P. aeruginosa* acute lung infection

C57/Bl6NCrl mice ($n = 5$/group) were intratracheally challenged with 1×10^7 cells *P. aeruginosa* RP73 clinical strain and, 5 min later, a single dose of BMAP-27(1–18) at different concentrations (1, 2, and 4 mg/kg) was intratracheally administrated. The vehicle (SALF water) alone was used as negative control. The concentrations of the peptide have been selected on the basis of previously performed in vivo toxicity assays. Following 24 h p.e. mice were sacrificed by CO_2 inhalation, then lungs were observed in situ for macroscopic analysis and finally removed en bloc from the chest via sterile excision. Lungs were homogenized (24,000 rpm) on ice in 2 ml of sterile PBS by use of an Ultra-Turrax T25-Basic homogenizer (IKA-Werke GmbH & Co. KG, Germany). Tenfold serial dilutions of lung homogenates were plated in triplicate on MHA (Oxoid SpA), and the number of colony-forming units (CFUs) was counted 24 h after incubation at 37 °C. Bacterial colony counts from each mouse were expressed as CFU/lungs, averaged, and compared between groups. These experiments were performed as an external service made available by the CF Animal Core Facility of the San Raffaele Hospital, Milan, Italy.

BMAP-27(1–18) degradation in bronchoalveolar lavage (BAL) fluids

BAL was collected from 3 C57/Bl6NCrl male healthy mice. Briefly, mice were sacrificed by dislocation, then 1 ml of sterile 0.9 % NaCl at 37 °C was instilled, by inserting a probe through mouth and trachea, in the lungs. Two washes were performed for each mouse. Identical volumes of the first wash from each mouse were pooled together, splitted in aliquots and stored at −20 °C until degradation experiments on BMAP-27(1–18). The total protein concentration of BAL fluids was determined to be 300 µg/ml by BCA assay (Pierce, BCA Protein Assay Kit).

To evaluate BMAP-27(1–18) degradation a concentrated solution of peptide was diluted in pooled BAL (see above) to achieve the final concentration of 300 µg/ml. In this manner, the ratio Peptide/BAL total proteins was 1:1 (wt/wt). Samples were then incubated at 37 °C and 30 µl of the mixture were taken at different times, cooled on ice and frozen at −20 °C. For results visualization, samples were denatured 5 min at 90 °C in the presence of 1× Laemli Sample Buffer A, 10 µl of each sample were separated by SDS-PAGE on a 16 % tricine gel [according to (Schagger 2006)] that was stained with Coomassie Brilliant Blue.

Statistical analysis

Statistical analysis was done on in vivo experiments performed at least in triplicate, and repeated on two different occasions. Differences between groups were evaluated using paired Student's t test (in vivo protection assays), or Chi-square test for percentages (survival). Statistical analysis of results were performed with GraphPad Prism 4.0 software (GraphPad Software Inc., San Diego, CA, USA), considering as statistically significant a p value less than 0.05. Statistic on biofilm was performed by Student t test, considering as statistically significant a p value less than 0.05.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All the procedures performed in studies involving animals were in accordance with the ethical standards of the Animal Care Committee of "G. d'Annunzio" University of Chieti-Pescara, and were carried out according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. This article does not contain any studies with human participants performed by any of the authors.

References

Benincasa M, Skerlavaj B, Gennaro R, Pellegrini A, Zanetti M (2003) In vitro and in vivo antimicrobial activity of two alpha-helical cathelicidin peptides and of their synthetic analogs. Peptides 24:1723–1731. doi:10.1016/j.peptides.2003.07.025

Benincasa M et al (2006) Fungicidal activity of five cathelicidin peptides against clinically isolated yeasts. J Antimicrob Chemother 58:950–959. doi:10.1093/jac/dkl382

Dasenbrook EC, Checkley W, Merlo CA, Konstan MW, Lechtzin N, Boyle MP (2010) Association between respiratory tract methicillin-resistant Staphylococcus aureus and survival in cystic fibrosis. JAMA 303:2386–2392. doi:10.1001/jama.2010.791

Di Bonaventura G et al (2007) Molecular characterization of virulence determinants of Stenotrophomonas maltophilia strains isolated from patients affected by cystic fibrosis. Int J Immunopathol Pharmacol 20:529–537

Emerson J, Rosenfeld M, McNamara S, Ramsey B, Gibson RL (2002) *Pseudomonas aeruginosa* and other predictors of mortality and morbidity in young children with cystic fibrosis. Pediatr Pulmonol 34:91–100. doi:10.1002/ppul.10127

Emerson J, McNamara S, Buccat AM, Worrell K, Burns JL (2010) Changes in cystic fibrosis sputum microbiology in the United

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States between 1995 and 2008. Pediatr Pulmonol 45:363–370. doi:10.1002/ppul.21198

Hancock RE, Sahl HG (2006) Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat Biotechnol 24:1551–1557. doi:10.1038/nbt1267

Hansen MB, Nielsen SE, Berg K (1989) Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J Immunol Methods 119:203–210

Hoffman LR, D'Argenio DA, MacCoss MJ, Zhang Z, Jones RA, Miller SI (2005) Aminoglycoside antibiotics induce bacterial biofilm formation. Nature 436:1171–1175. doi:10.1038/nature03912

Hsu CY, Lin MH, Chen CC, Chien SC, Cheng YH, Su IN, Shu JC (2011) Vancomycin promotes the bacterial autolysis, release of extracellular DNA, and biofilm formation in vancomycin-nonsusceptible *Staphylococcus aureus*. FEMS Immunol Med Microbiol 63:236–247. doi:10.1111/j.1574-695X.2011.00846.x

Johansen HK, Espersen F, Pedersen SS, Hougen HP, Rygaard J, Hoiby N (1993) Chronic *Pseudomonas aeruginosa* lung infection in normal and athymic rats. APMIS 101:207–225

Kaplan JB (2011) Antibiotic-induced biofilm formation. Int J Artif Organs 34:737–751. doi:10.5301/ijao.5000027

Lai Y, Gallo RL (2009) AMPed up immunity: how antimicrobial peptides have multiple roles in immune defense. Trends Immunol 30:131–141. doi:10.1016/j.it.2008.12.003

Limoli DH, Rockel AB, Host KM, Jha A, Kopp BT, Hollis T, Wozniak DJ (2014) Cationic antimicrobial peptides promote microbial mutagenesis and pathoadaptation in chronic infections. PLoS Pathog 10:e1004083. doi:10.1371/journal.ppat.1004083

Linares JF, Gustafsson I, Baquero F, Martinez JL (2006) Antibiotics as intermicrobial signaling agents instead of weapons. Proc Natl Acad Sci USA 103:19484–19489. doi:10.1073/pnas.0608949103

Millar FA, Simmonds NJ, Hodson ME (2009) Trends in pathogens colonising the respiratory tract of adult patients with cystic fibrosis, 1985–2005. J Cyst Fibros 8:386–391. doi:10.1016/j.jcf.2009.08.003

Molina A, Del Campo R, Maiz L, Morosini MI, Lamas A, Baquero F, Canton R (2008) High prevalence in cystic fibrosis patients of multiresistant hospital-acquired methicillin-resistant *Staphylococcus aureus* ST228-SCCmecI capable of biofilm formation. J Antimicrob Chemother 62:961–967. doi:10.1093/jac/dkn302

Palmer KL, Aye LM, Whiteley M (2007) Nutritional cues control *Pseudomonas aeruginosa* multicellular behavior in cystic fibrosis sputum. J Bacteriol 189:8079–8087. doi:10.1128/JB.01138-07

Pompilio A et al (2011) Antibacterial and anti-biofilm effects of cathelicidin peptides against pathogens isolated from cystic fibrosis patients. Peptides 32:1807–1814. doi:10.1016/j.peptides.2011.08.002

Pompilio A et al (2012) Potential novel therapeutic strategies in cystic fibrosis: antimicrobial and anti-biofilm activity of natural and designed alpha-helical peptides against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Stenotrophomonas maltophilia*. BMC Microbiol 12:145. doi:10.1186/1471-2180-12-145

Sajjan US et al (2001) P-113D, an antimicrobial peptide active against Pseudomonas aeruginosa, retains activity in the presence of sputum from cystic fibrosis patients. Antimicrob Agents Chemother 45:3437–3444. doi:10.1128/AAC.45.12.3437-3444.2001

Schagger H (2006) Tricine-SDS-PAGE Nat Protoc 1:16–22. doi:10.1038/nprot.2006.4

Singh PK, Schaefer AL, Parsek MR, Moninger TO, Welsh MJ, Greenberg EP (2000) Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature 407:762–764. doi:10.1038/35037627

Skerlavaj B, Gennaro R, Bagella L, Merluzzi L, Risso A, Zanetti M (1996) Biological characterization of two novel cathelicidin-derived peptides and identification of structural requirements for their antimicrobial and cell lytic activities. J Biol Chem 271:28375–28381

Worlitzsch D et al (2002) Effects of reduced mucus oxygen concentration in airway Pseudomonas infections of cystic fibrosis patients. J Clin Invest 109:317–325. doi:10.1172/JCI13870

Wu S et al (2014) Beta- lactam antibiotics stimulate biofilm formation in non-typeable haemophilus influenzae by up-regulating carbohydrate metabolism. PLoS One 9:e99204. doi:10.1371/journal.pone.0099204

Yang D, Biragyn A, Kwak LW, Oppenheim JJ (2002) Mammalian defensins in immunity: more than just microbicidal. Trends Immunol 23:291–296

Zanetti M (2004) Cathelicidins, multifunctional peptides of the innate immunity. J Leukoc Biol 75:39–48. doi:10.1189/jlb.0403147

Zanetti M, Gennaro R, Scocchi M, Skerlavaj B (2000) Structure and biology of cathelicidins. Adv Exp Med Biol 479:203–218. doi:10.1007/0-306-46831-X_17

Zanetti M, Gennaro R, Skerlavaj B, Tomasinsig L, Circo R (2002) Cathelicidin peptides as candidates for a novel class of antimicrobials. Curr Pharm Des 8:779–793

Zhang L, Parente J, Harris SM, Woods DE, Hancock RE, Falla TJ (2005) Antimicrobial peptide therapeutics for cystic fibrosis. Antimicrob Agents Chemother 49:2921–2927. doi:10.1128/AAC.49.7.2921-2927.2005

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