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XXXI CICLO DEL DOTTORATO DI RICERCA IN

BIOMEDICINA MOLECOLARE

Optimization of BMAP18 - an anti-infective peptide for the treatment of pulmonary infections

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ABSTRACT

Patients with lung infections require pharmacological treatment against multi drug resistant pathogens. The antimicrobial peptide BMAP27(1-18), a truncated form of BMAP-27, is active *in vitro* against planktonic and sessile forms of multidrug-resistant *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* strains isolated from cystic fibrosis (CF) patients. Despite the good antimicrobial activity *in vitro*, it has no protective activity *in vivo* in a murine model of acute pulmonary infection by *P. aeruginosa* and has a residual *in vivo* toxicity. Its inefficiency is caused by its scarce stability in the pulmonary environment, and this is confirmed by the observation of its degradation in presence of murine bronchoalveolar lavage fluid.

To overcome these drawbacks we operated in two directions. At first, we synthesized the enantiomer (*D*-BMAP18) to avoid its degradation. It has a good antimicrobial activity *in vitro* against sessile and planktonic form of *P. aeruginosa* strains. It is stable in the pulmonary environment and it is active in media mimicking the CF lung environment. Under these conditions its antimicrobial activity is enhanced in combination with mucolytic compounds already approved in CF-therapy. However, D-BMAP-18 showed a non-negligible toxicity both *in vitro* and *in vivo.*

To partially solve this weakness point, its pro-form (Pro-*D*-BMAP18) was synthesized in order to obtain an inactive peptide which could be converted to its active form only in the site of infection/inflammation thanks to the endogenous elastase, enabling a slowly release of the pharmacologically active peptide. Pro-*D*-BMAP18 reduces the side effects linked to the *D*-BMAP18, having reduced cytotoxicity and conserving its antimicrobial activity. The pro-form is correctly processes into *D*-BMAP18 in pulmonary environment and, under this condition, shows a good antimicrobial activity.

Pro-*D*-BMAP18 seems to be a promising compound for the *in vivo* treatment of pulmonary infection but only the *in vivo* assay will enable us to understand the real potential of Pro-*D*-BMAP18 in therapy.

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1 INTRODUCTION

1.1 Respiratory infection

1.1.1 General aspects

Respiratory tract infections (RTIs) are among the most common problems in clinical medicine.

The Respiratory Society highlights that acute respiratory infections (ARIs) are one of major player in the global burden of respiratory disease (Ferkol and Schraufnagel 2014). In developed countries they account for the majority of antibiotic prescriptions and the situation is more dramatic in developing countries where nearly 20% of mortality in children under the age of 5 years can be attributed to RTIs (Kurt 2015)

RTIs are divided in Upper Respiratory Infections and Lower Respiratory infections according to the anatomic district (**Fig 1.1**) (Baron 1996). The Upper Respiratory Infections include common cold, sinusitis, pharyngitis, epiglottitis and laryngotracheitis. Usually they are caused by viral infection but sometimes also by bacteria. They are usually transient and self-limited infections. The Lower Respiratory Infections are prevalently localized in lung and including bronchitis, bronchiolitis (infection and inflammation of the bronchial tree) and pneumonia (infection and inflammation of lung). They are predominantly due to bacterial pathogens but also virus could be found.

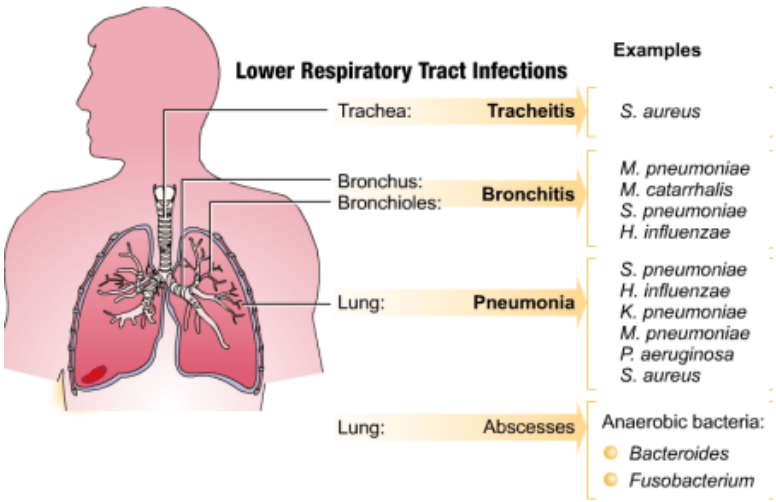


Fig. 1.1: Graphic representation of the Lower Respiratory infections and their etiological agents. (https://elearning.sgtuniversity.ac.in/2018/03/07/epidemiology-of-respiratory-infections-and-allergies-the-indian-context/)

Therefore, lungs are the most affected organs of the respiratory tract. Lung's infection represents one of the major causes of mortality among the RTIs. In 2015 the Burden of Disease Project at the World Health Organization (WHO) reported that lung infections accounted for more than 10% of the total global burden of disease.

Pneumonias occurring in healthy persons are classified as community-acquired pneumonias while the infection that arises in hospitalized patient are classified as nosocomial pneumonias. Etiologic pathogens associated with community-acquired and hospital-acquired pneumonias are different (Dasaraju and Liu 1996). A common feature of both is perturbations of the lung microbiota which undergoes to a significant decrease in the diversity (Gollwitzer et al., 2014, Cui et al., 2014). Usually a shift of bacterial community composition away from the *Bacteroidetes* phylum (which dominates the healthy lung microbiota) towards Gammaproteobacteria (consisting in gram-negative pathogens) is observed. These changes are associated with important clinical features of chronic lung disease such as exacerbation and mortality.

Community-acquired pneumonias are usually caused by aspiration of bacteria that normally live in the oropharyngeal region or stomach (Farver, 2018) or by environmental bacteria. Although bacteria are the main pathogens involved in this type of pneumonia; an important role is played by viruses either directly or as part of a co-infection (Galván, Rajas, and Aspa 2015). These infections are sustained predominantly by *Streptococcus pneumonia* which causes the 95% of cases; but other bacteria are involved such as *Haemophilus influenzae*, *Staphylococcus aureus*, *Moraxella catarrhalis*, *Pseudomonas aeruginosa* and other gram-negative bacilli. Once the community-acquired pneumonias are diagnosed, it is fundamental to determine the causative microorganism for a specific therapy (Musher and Thorner 2014)

Nosocomial infections are usually associated with ventilators and the most frequently are pulmonary infections in cystic fibrosis patients. Generally bacteria are the most common pathogens but the microorganisms involved vary depending upon different patient populations and medical devices (Steven and Koening 2006). Among them could be identified: *Acinetobacter* accounting for 80% of reported infections, *Bacteroides fragilis, Clostridium difficile, Enterobacteriaceae* and methicillin-resistant *S. aureus* (MRSA). An important role in nosocomial infection is also played by viruses such as *Hepatitis B* and C and *herpes-simplex virus* (Aitken and Jeffries 2001) and fungi such as *Aspergillus spp.* and *Candida albicans* (Khan, Baig, and Mehboob 2017).

The switch of pneumonia from an acute form to a chronic form is a multifactorial phenomenon which involves the development of bacterial persistence, antibiotics resistance (Levin-Reisman et al. 2017) and biofilms formation (Costerton, Stewart, and Greenberg 1999). Bacterial persistence could be induced by the environmental conditions of the surroundings and consists in a slow growth

coupled with an ability to survive to antibiotic treatment (Harms, Maisonneuve, and Gerdes 2016; Kussell et al. 2005). This is particularly relevant in lung. Indeed, variable levels of oxygen, nitrogen, carbon dioxide, and water vapour characterize pulmonary environment. Moreover the pH and temperature conditions could be stressful for the pathogens promoting a stress response involved in the development of a persistence phenotype (Trastoy et al. 2018).

Antibiotics resistance is usually acquired through genetic mutations which are preferred in presence of a selective pressure such as the overuse of antibiotics (Brauner et al. 2016). Resistance to antimicrobials may occurs in different ways like: inactivation of the drug, modification of the target, uptake reduction and increased efflux (Denyer, Hodges, and Gorman 2007).

Other than the bacterial resistance and persistence, biofilm formation is implied in chronic lung infection (Costerton, Stewart, and Greenberg 1999). This is particularly important because bacteria in the biofilm can resist to an antibiotic's concentration 1000 fold higher than the planktonic form (Arciola et al. 2015).

1.1.2 Biofilm and its role in the persistence

Biofilm-grown cells express properties different from planktonic cells, one of them is an increased resistance to antimicrobial agents (Mah and O'Toole 2001) and for this reason biofilm are one of the major cause of chronicity of lung infection and pulmonary damages. A biofilm is an assemblage of bacterial cells adherent to an abiotic or a biotic surface and enclosed in an extracellular polymeric substance matrix (EPS) (Flemming et al. 2016). They are composed for the 25% by bacteria (Raghupathi et al. 2018) and for the 70%-90% by EPS consisting in polysaccharides and eDNA (Jamal et al. 2015; Tang et al. 2015). The EPS plays an important role for the bacterial community because it is a virulent factor and a protection against antibiotic and immune-system (Limoli 2014).

Biofilm formation is a multistep process (**Fig. 1.2**), which required the initial attachment, the maturation and the final dispersion (Rabin et al. 2015). The first attachment is reversible, mediated by electrostatic interactions and/ or by weak interactions (Renner and Weibel 2011). This stage is followed by the formation of microcolonies (Stanley and Lazazzera 2004) and by the irreversible adhesion enabled by the production of EPS (Vu et al. 2009) and the released of eDNA (Gloag et al. 2013). The maturation is due to a massive release of matrix (Garrett, Bhakoob, and Zhanga 2008) and the biofilm assumes its mature form (Dunne 2002; Wilking et al. 2013). In the mature biofilm there is both the development of resistant cells and the formation of the persister cells, which comprise about the 1% of the stationary cells and are characterized by the high tolerability to the antibiotics (Lewis 2007; Wood, Knabel, and Kwan 2013). The last stage of biofilms' life is the

dispersion which is mediated by passive mechanisms and/or by the production of exo-enzymes and surfactants which degrade the matrix (Kaplan 2010; Kostakioti, Hadjifrangiskou, and Hultgren 2013; Soo- Kyoung and Joon- Hee 2016).

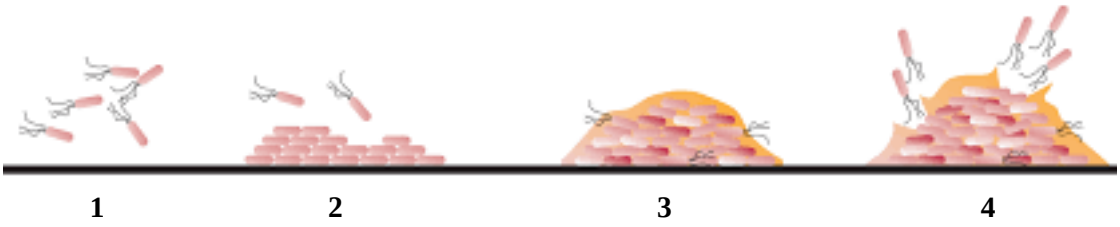


Fig. 1.2: Graphic representation of biofilm formation. (1) Reversible adhesion of bacteria to a surface, (2) Irreversible adhesion, (3) Biofilm growth and maturation, (4) Biofilm dispersion (http://www.4inno.com/wp-content/uploads/2014/01/diagram.png)

Biofilms resistance to antibiotics is a multifactorial phenomenon which involves different mechanisms (Lebeaux, Ghigo, and Beloin 2014). The major limit to the antibiotics activity is their slow or incomplete penetration in the biofilm, caused by the presence of the EPS which protects the bacteria both physically and chemically (Stewart 2015). Also the "persisters" play a fundamental role in resistance, they are metabolic inactive bacteria in the internal area of the biofilm, characterized by a slow rate of growing and a hyper resistance to antibiotics (Costerton 2001; Grassi et al. 2017). Additionally, biofilms are characterized by an accelerated rates of conjugation which lead to the acquisition of antibiotic resistance, virulence factors, and environmental survival capabilities (Kolter 2000).

Different examples of pathologies induced by biofilm infections have been reported regarding the respiratory tracts. It is estimated that biofilm formation is involved in at least 60% of all chronic and/or recurrent infections with considerable morbidity and life-threatening complications (Morris 2007; Pintucci, Corno, and Garotta 2010). Some examples are: chronic otitis media caused by the presence of *Staphylococcal* biofilm (Wessman et al. 2015), chronic rhinosinusitis due to the presence of *P. aeruginosa*, *S. pneumonia* e *S aureus* biofilms (Morris 2007) and chronic lungs biofilm infection in cystic fibrosis by *P. aeruginosa*.

1.1.3 Lung infection: focus on Cystic Fibrosis

As mentioned before, cystic fibrosis lungs infections are one of the most common chronic infections of lower respiratory tract. Cystic fibrosis is an autosomic recessive disorder caused by mutations in the *CFTR* gene (cystic fibrosis transmembrane regulator) (Cuthbert 2011) and it affects

approximately 1 in 2500 new births among Caucasian population (Rowntree and Harris 2003). The symptomatology is due to the formation of dense mucus that obstructs many organs and it is highly detrimental in the airway. In this environment are eased chronic bacterial colonization and hyper-inflammation which are the most likely cause of death for most patients (Cutting 2015).

The *CFTR* gene encodes a member of the ATP-binding cassette (ABC) transporter family. Generally it is located in the apical membrane of the epithelial cells and provides a pathway for Cl^- movement across epithelia (Farinha et al. 2004). Its role is fundamental in maintaining the surface liquid layer in lungs. Indeed lack of functional CFTR results in dense secretions (Riordan 2008). This aspect is particularly dangerous in the airway where the airway surface liquid (ASL) has a fundamental role in mucociliary clearance and in maintaining sterility (Verkman, Song, and Thiagarajah 2003). The ASL (thickness≈ 30 μm) is composed by two layers: the periciliary liquid which cover the cilia and the overlying viscous gel layer which entrapped the pathogens (Tarran 2004) and is transported out by the beating of the cilia for the effective clearance (Tarran et al. 2001). The excessive mucus production, typical of this pathology, inhibits the mucociliary clearance and allows chronic bacterial infections.

Two hypotheses were proposed for explaining how the deregulated ion transport is linked with the pathogenesis (**Fig. 1.3**). The compositional hypothesis is based on the role of CFTR's as an anion channel. Its dysfunction should cause an exceed of sodium chloride which inhibits the antimicrobial action of defensins and set the framework for chronic infection (Pezzulo et al. 2012). The low volume hypothesis is based the impairment of mucociliary clearance which allows pathogen invasion. This environment promotes a state of inflammation and onsets CF (Boucher 2004).

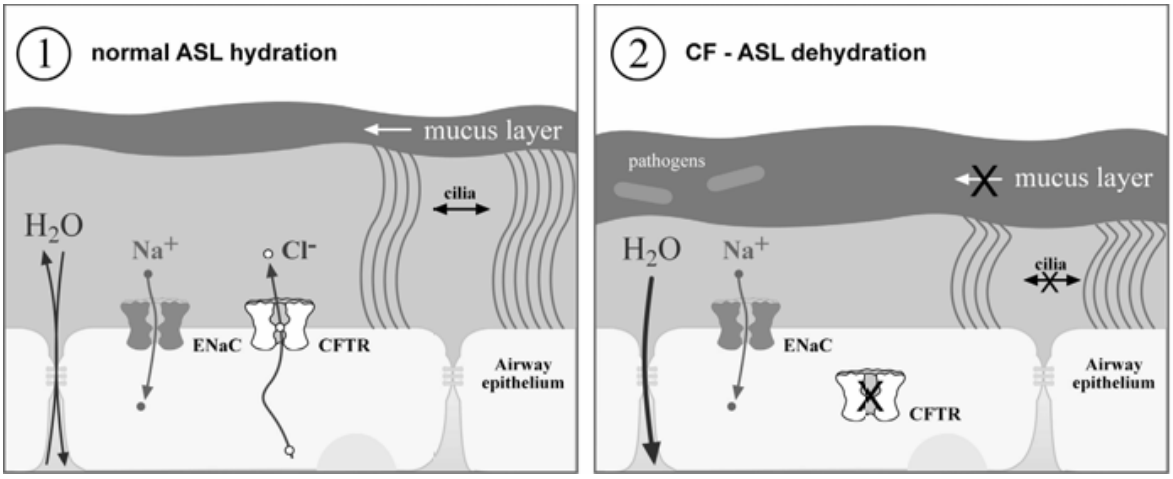


Fig. 1.3: Representation of the low-volume hypothesis. (1) The correct ion transport ensures a fluid mucus layer and the mucociliary clearance. (2) Mutations on the CFTR channel cause a hyper absorption of water. The mucus layer become thin and the micociliary clearance is prevent.

CF patients were born with normal lungs, followed by the acquisition of chronic, unrelenting bacterial infections of the airways during the first few years of life due to the failure of the innate immune defence. Next to bacterial colonization, biofilm formation is implied in lung infections. This represents an advantage for the bacteria because inside the biofilm they are protected from the antibiotics and the immune-system, indeed both antibacterials and neutrophils fail in penetrating into this structure. This pulmonary alteration leads to a persistent infection which is very difficult to eradicate (Bhagirath 2016) and enables the formation of a dynamic and varied bacterial community with a complex web of relationship among the pathogens (Surette 2014).

From early childhood to adulthood in patients there is a shift of pathogens composition. *H. influenzae* is an early colonizer followed by *S. aureus* and *P. aeruginosa* (Yonker et al. 2015). In older patients, *P. aeruginosa* is the most common pathogen but other bacteria such as *S. aureus*, *Burkholderia cepacia* and *Stenotrophomonas maltophilia* may be also be present. In addition to the bacterial community, in CF airway also fungi, such as *Aspergillus* spp. and *Candida* spp., and viruses are present. Moreover, it has been reported that a decrease in microbiota diversity is correlated with patients' age and/or disease severity (Lynch and Bruce 2013). Additionally, a multispecies environment is an opportunity for diversification because in polymicrobial communities the horizontal gene transfer is favoured. This mechanism enable the generation of novel strains which could evade host defences (Boon et al. 2014).

From an ecological point of view the development of the infection depends upon two classes of CF microbial community (**Fig. 1.4**). The attack community sustains the early stage of colonization. It is involved in lung remodelling and enables the establishment of the chronic climax community. The climax community is implicated in the chronicity of the infections. It is well adapted to CF environment and resistant to standard antibiotic therapy (Conrad et al. 2013).

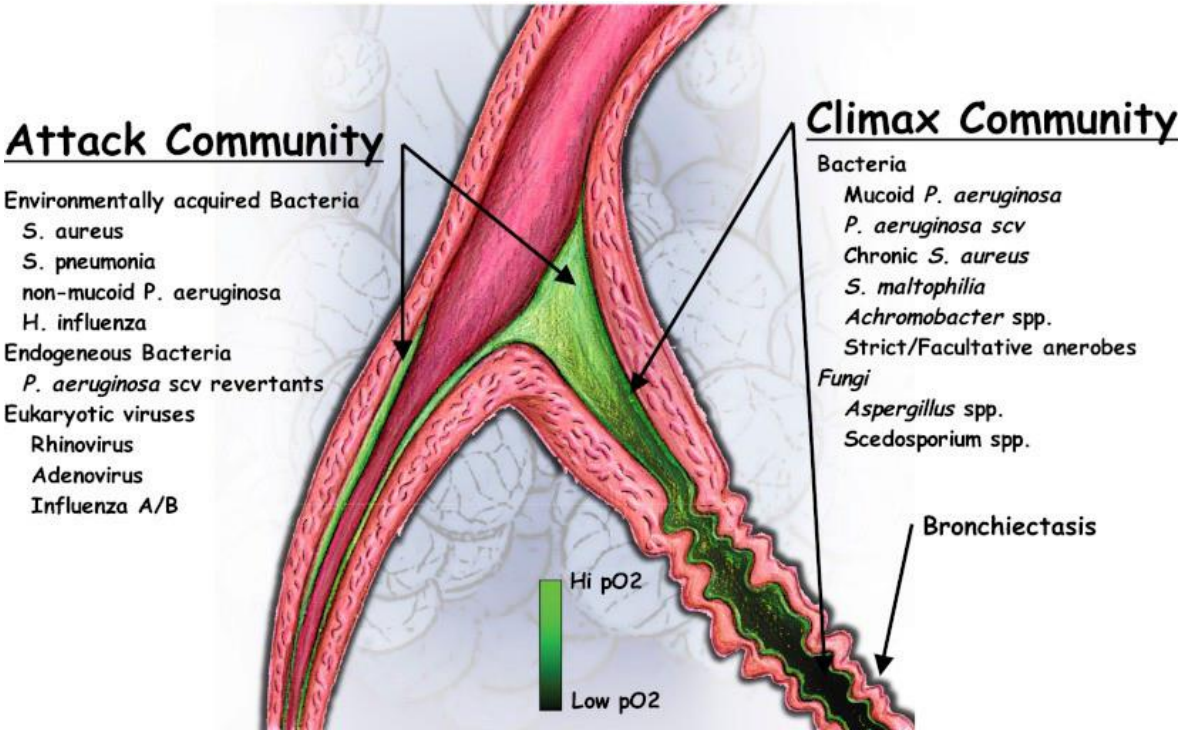


Fig. 1.4: Description of the communities in CF lungs. The initial colonization is due to the attack community, which is followed by the climax community (Conrad et al., 2013).

1.1.4 Focus on *P. aeruginosa*

P. aeruginosa plays the most detrimental role in lung infections where switches from acute infection to chronic infection because of its adaptability to this environment (Gellatly and Hancock 2013).

A multitude of virulence factors (**Fig 1.5**) are produced by *P. aeruginosa* such as a single flagellum and a variety of pili for the adhesion to the host epithelia, proteases and lipase for bacterial dissemination, and other which are mostly linked to the *quorum sensing* (QS) regulation (Streeter and Katouli 2016). QS enables bacteria to regulate their behaviour according to population density. In particular, *P. aeruginosa* uses QS to control the production of secreted virulence factors including elastase, alkaline protease, exotoxin A, rhamnolipids, pyocyanin, lectins and superoxide dismuatses (Davenport, Griffin, and Welch 2015; Venturi 2006). These virulence properties play a significant role in the persistence of the organism during infection, contribute to the formation of biofilms and to the evasion the host's immune system (Willcox et al. 2008). Moreover this bacterium is able to adapt to the surrounding environment and when subjected to antibiotic selective pressure, it develops antibiotic resistance.

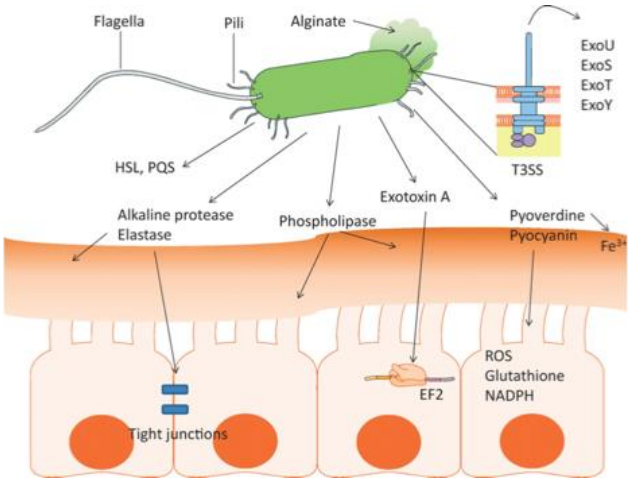


Fig. 1.5: Representation of *P. aeruginosa* virulence factors (Gellatly and Hancock 2013)

Its behaviour has largely been reported during host colonization of CF patients. Once entering in CF lungs, it gives rise to a chronic infection that can persist from years to decades due to its ability to switch to a CF adapted pathogen. One of the major reasons of its persistence is clonal expansion and diversification into specialized phenotype as a response to the selective pressure of CF lungs (*i.e*: the conversion from non- to mucoid phenotype or the formation of small colony variants which are particularly resistant to antibiotics, **Fig. 1.6**) (Sousa and Pereira 2014).

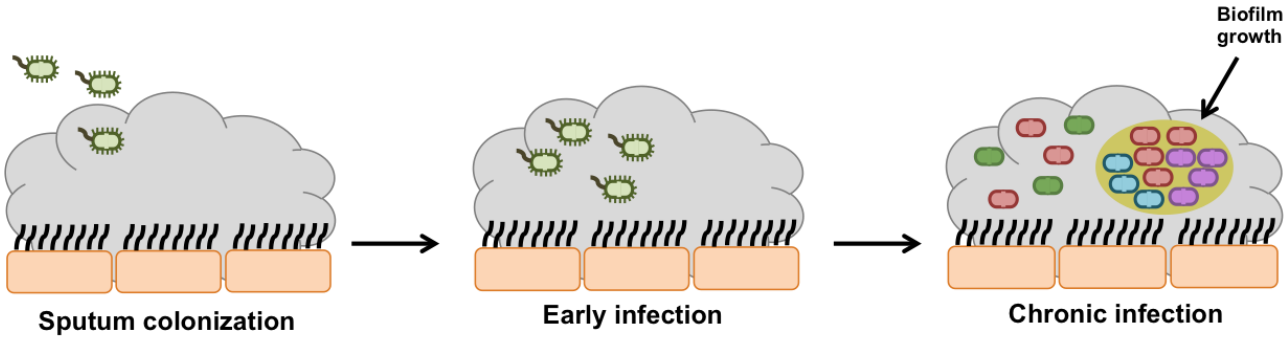


Fig. 1.6: Time course of *P. aeruginosa* infection. (1) *P. aeruginosa* enter in CF sputum; (2) *P. aeruginosa* adapt in CF environment; (3) Full adaptation and biofilm formation (Sousa et al., 2014).

The airway of >80% of CF patients is dominated by *P. aeruginosa* (O'Toole 2018). Oxygen restriction and the net of mucin within CF lung environment promotes the persistence of *P. aeruginosa* (**Fig. 1.7**) due to increased antibiotic tolerance, alginate production and biofilm formation whose dispersion gives to the bacteria the opportunity to colonize new zones of the lungs (Guttenplan 2013; Hogardt and Heesemann 2013). Evidences suggest that *P. aeruginosa* have the genetic capacity to synthesize alginate when ecological conditions are unfavourable for their survival (Kobayashi 2005). In *P. aeruginosa* biofilm the EPS is composed prevalently by alginate

which gives different advantages in the survival (Mann and Wozniak 2013) such as higher physical protection and a higher water and nutrient retention, which guarantee survival in stressing environment (Martha et al. 2010). Alginate is fundamental in persistence and in immune evasion. It confers resistance to the antimicrobial compounds and to the phagocytosis (Lambert 2002) and its production is strongly linked with the decline in clinical status (Hurley, Cámara, and Smyth 2012) . Moreover in the internal part of biofilms there is the generation of subclonal variant ("mutator") which achieves more quickly CF adaptations (Pereira 2014) and favour the intraclonal diversification. These lead to the formation of a multitude of variants which represent a biological advantage because preparing the *P. aeruginosa* population to unpredictable stress (insurance hypothesis) (Ciofu et al. 2012; Hogardt and Heesemann 2013).

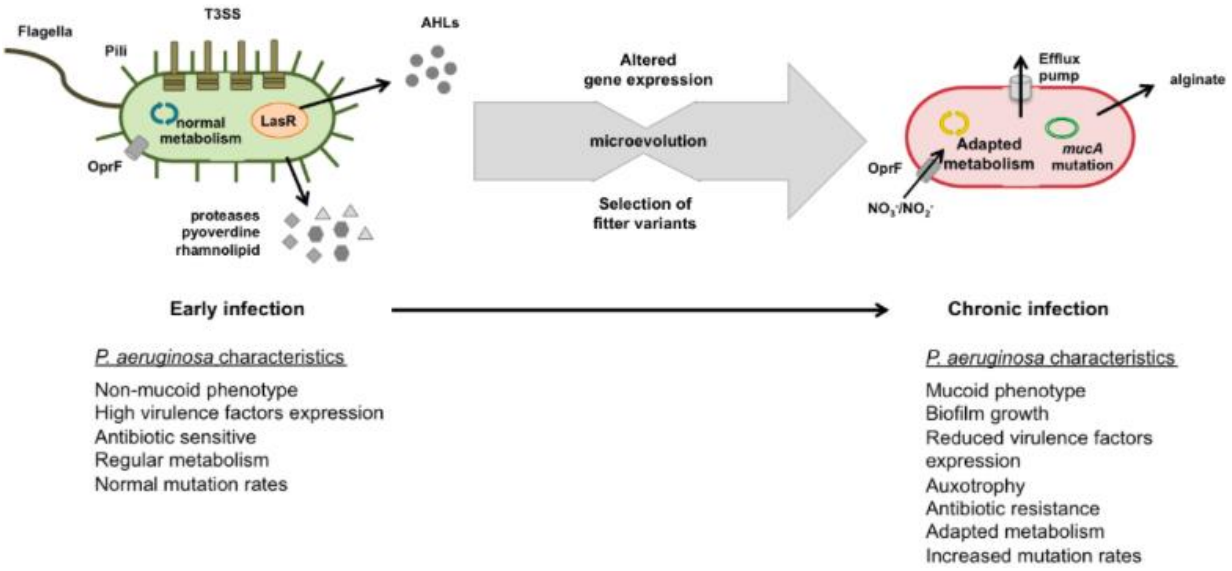


Fig. 1.7: Representation of *P. aeruginosa* adaptation in CF lung. In the early stage of infection it produces abundant virulence factors, later on *P. aeruginosa* loses them and starts to overproduce alginate (Sousa et al., 2014).

1.1.5 Therapy of lung infections and future perspective

Traditionally the therapeutic approach to bacterial lung infection was based on empirical observation such as: patient's age, severity of disease and past experience. When the susceptibility patterns were used for the clinical treatment, several problems became evident: multiple bacteria strains with different antibiotic susceptibility pattern are found, the treatment cannot be compared with the *in vitro* analysis and the presence of biofilm makes the treatment less effective

Guidelines for empirical antimicrobial therapy of lung infection have contributed to a greater uniformity of treatment: the use of a beta-lactam such as penicillin or amoxicilin and the use of

tetracycline is an empirical therapy for pneumonia and this treatment can be followed by the use of macrolides azithromycin, clarithromycin, erythromycin or roxithromycin or of doxycycline (Musher and Thorner 2014; Woodhead et al. 2011).

In these decades, one of the most important changes in the treatment of lung infections is the used of inhaled antibiotics. The advantages of this therapy are the direct administration in the site of infection, resulting in a reduction of the side effects and the bronchial-delivery of a high concentration of compound (Kuhn 2001). Numerous antibiotics have been tested for this delivery but different factors have concurred in the choice of the suitable compounds such as the pharmacokinetic, pharmacodynamics, bioavability and the stability in aqueous solution. Indeed the therapeutic effects depend on these features (Water and Smyth 2015). Since *P. aeruginosa* is the dominant infecting organism, different antipseudomonal treatments have been tested such as: azetreonam (reformulated as a lysine salt for inhalation), levofloxacin (a fluoroquinolone that causes cell death by inhibiting DNA synthesis) and fosfomycin (can act both against Gram- positive and Gram- negative bacteria) (Hewer 2012; Khan, Baig, and Mehboob 2017; Rogers, Hoffman, and Döring 2011).

Nowadays, nebulized colistin and tobramycin are the major inhaled antibiotics against *P. aeruginosa* infections. Colistin is usually the drug of first choice for the eradication therapy of early and chronic MDR *Pseudomonas* infection. It is a cationic polypeptide with activity against Gram-negative bacteria, targeting the lipopolysaccharide (LPS) in the outer bacterial membrane. After the permeabilization of the outer membrane, it promotes itself uptake and disrupts the inner membrane leading to bacterial death. The inhaled therapy is promising but the overused could be linked to the development of resistance due to LPS modifications (Gurjar 2015; Velkov et al. 2013).

Tobramycin is an aminoglycoside with bactericidal activity against Gram- negative bacteria and it binds the ribosome, inhibiting proteins synthesis. The use of aerosolized tobramycin was first described in 1983 with good results. It has also some criticality: the sputum inhibits the biological activity of inhaled tobramycin and in a long-term period *P. aeruginosa* is able to develop antimicrobial resistance (Vendrell, Muñoz, and de Gracia 2015).

Development of resistance is particularly damaging in case of long-term therapy. For this reason, the searching of new types of non-canonical antibiotic treatments is in progress. Different compounds can be pointed out for the treatment of lung infection such as: the use of gallium which is structurally similar to iron and can substitute it in the Fe-dependent pathways, inhibiting bacterial growth, Bupranolol which is an antagonist of β-Adrenergic receptor and shows antimicrobial activity against *E. coli* and *S. aureus* and N-acetylcysteine, a mucolytic agents actives against *S. aureus*, *P. aeruginosa* and *K. pneumonia* (Nigam, Gupta, and Sharma 2014). Promising compounds

to mention are also the antimicrobial peptides. They are part of the innate immune system of eukaryotes and their potential depend on their broad-spectrum of activity and the low incident of resistant strains (Zhang and Gallo 2016)

1.2 Antimicrobial peptides

1.2.1 General aspects and mechanism of action

The antimicrobial peptides (AMPs) are found as component of the innate immune-system both in vertebrates and invertebrates. At the end of '900, Hultmark et al. reported the presence of three bactericidal components in the immune-system of insects (Hultmark et al. 1980) and some years later Lehrer and Selsted purified two AMPs from rabbit lung macrophages and called them defensin (Selsted et al. 1983). These discoveries opened the studies of the antimicrobial peptides and their anti-infective potential. These molecules could be constitutively expressed or could be induced by pathogens. They are released by different cell types such as the epithelial cells in gastrointestinal and genitourinary tract, phagocytes and lymphocytes (Bahar and Ren 2013). They show a broad-spectrum of activities against Gram- positive and – negative bacteria, fungi and viruses (Diamond 2009).

The AMPs are a complex class of molecule but they share some common aspects (Zhang and Gallo 2016). They are short peptides, usually between 10 and 50 aa with a overall positive charge (among +2 and +12). Generally they are amphipathic with a substantial proportion (≥30%) of hydrophobic residues (Hancock, Brown, & Mookherjee, 2006). These characteristics are strongly linked to their antimicrobial activity enabling the interaction with the bacterial membranes (Yeaman 2003). AMPs do not show a conserved structure but usually they fold exposing the hydrophobic and charged regions, which are important for the interaction and the insertion in bacterial membrane. AMPs can be divided into four major structural groups according to their secondary structures. α- helical peptides, which are characterized by a disorder arrangement in solution and adopt an α- helical secondary structure while in contact with membranes. They usually show a selective action against Gram- positive bacteria and fungi. To this group belong e.g. magainin and LL-37. β- sheet AMPs usually contain cysteine residues that are conserved and form disulfide bonds. They are more structured in solution and do not undergo major structural changes when going from an aqueous environment to a membrane environment. The β- sheet peptides act preferentially against Gram-negative bacteria (Kumar, Kizhakkedathu, and Straus 2018). Mixed helical/sheet peptides and extended non-helical/sheet peptides (Lee, N. Hall, and Aguilar 2015) which are usually classified

thanks to the enrichment in specific amino acids such as arginine, proline or triptophane (Diamond 2009; Hancock, Brown, and Mookherjee 2006; Yeaman and Yount 2003; Zhang and Gallo 2016).

The ability of AMPs to kill bacteria usually depends upon their ability to interact with bacterial membranes or cell walls (Zhang and Gallo 2016). Moreover, some AMPs have also immunomodulatory functions, for example by acting like chemotactic factors for the immune-cells. Currently, 35 AMPs have showed chemotactic properties (Wang 2014). An examples is the human cathelicidin LL-37 which acts as a chemoattractant for neutrophils, monocytes, and mast cells (Duplantier and van Hoek 2013).

In human and other mammalians the two major families of antimicrobial peptides are defensins and cathelicidins. The defensins are usually cyclic peptides. They are divided in three subfamilies on the basis of the disulfide pairings between their six conserved cysteine residues (α- and β-defensins) or their macrocyclic nature (θ-defensins) (Ganz 2003; Jenssen, Hamill, and Hancock 2006). Cathelicidins are a group of AMPs precursors containing a wide variety of antimicrobial peptides, depending upon the amino acid sequence, the structure and the size. Their common feature is the presence of two functional domains: a more conserved N-terminal region called cathelin-like domain and a less conserved C-terminal region which is the antimicrobial part (Kościuczuk et al. 2012). Other mammalian antimicrobial peptides such as histatins and dermcidin are restricted to a few animal species and tissues (Ganz 2003).

AMPs activity depends on in their positive charge, it has generally accepted that the AMPs killing activity occurs through membrane permeation. This is also demonstrated by the observation that some antimicrobial peptides with lytic action maintain their antibacterial activity both if form by L-amino acids or D- amino acids. The interaction with the bacterial membrane occurs differently between Gram- negative and Gram- positive bacteria because of the different membrane's composition. Concerning Gram- negative bacteria, firstly the AMPs interact with lipopolysaccharides of the outer membrane and then interact with the inner membrane (Epand et al. 2016). In case of Gram- positive bacteria, the target are usually the peptidoglycans or the teichoic acids (Malanovic and Lohner 2016; Omardien, Brul, and Zaat 2016). The interaction is only the first step; after the reaching of a threshold concentration the peptide enters in the lipid bilayer and undergoes to conformational transition. In this way it promotes it self-aggregation which induces pore formation (Jenssen, Hamill, and Hancock 2006).

The lytic mechanism of AMPs can be classified in two major groups: pore formation and membrane solubilization (Chang et al. 2009; Sato and Feix 2006). Three models to explain their lytic mechanisms were proposed, depending on the orientation of the AMPs during the initial interaction with the membrane (**Fig. 1.8**). The Barrel stave model was the first proposed to describe the pore

formation by Baumann and Mueller; the AMPs interact with the bacterial membrane and then associate themselves to form a bundle with a central lumen (Yang et al. 2001). Concerning the carpet model, the peptide is oriented parallel to the membrane and it is active only when it reaches an high P:L ratio, enabling the formation of a carpet. This action seems similar to the action of a detergent (Wimley 2010). The toroidal model differs from the barrel-stave model because peptides are associated with the lipid headgroups even if they are perpendicularly inserted in the lipid bilayer (Huang 2006).

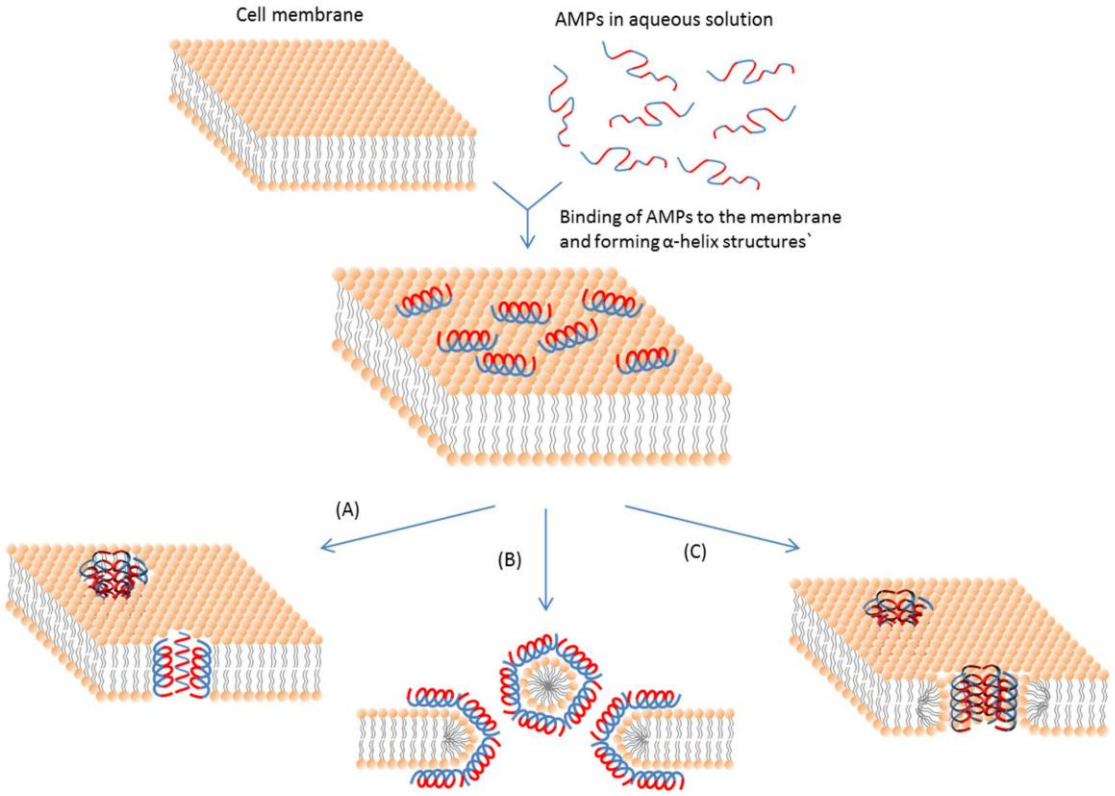


Fig. 1.8: Schematic representation of AMPs mode of action. (A) Barrel-Stave model. (B) Carpet model. (C) Toroidal pore model. In blue are represented the hydrophobic portions, in red the hydrophilic parts (Bahar et Ren 2013)

Probably different peptides utilize different mechanisms to lyse the microbial membrane. Moreover, these mechanisms are not mutually exclusive: one process may represent an initial or intermediate step and another may be its consequence. Additional factors such as lipid-to-peptide ratio and target membrane composition may also be involved in the type of mechanism (Sato and Feix 2006).

Some AMPs acting on bacterial internal targets (such as ribosomes, DNA, etc..) are known (Omardien, Brul, and Zaat 2016). Also in this case the interaction of these AMPs with the membrane is an essential step for the subsequent translocation. After the internalization they accumulate intracellular and mediate cell killing targeting different essential processes (Park and

Hahm 2005). An example is buforin II which enters in the cell and binds both DNA and RNA causing the inhibition of DNA and RNA synthesis (Brogden 2005) or numerous proline-rich peptides such as the mammalian Bac7(1-35) which binds the ribosome inhibiting protein synthesis (Mardirossian et al. 2014; Nicolas 2009; Shai 2002). Recently, it has been demonstrated that some AMPs increase lipid movement through the membrane in a concentration and time dependent way inducing membrane re-arrangements such as lipid flip-flop and peptide translocation across the bilayer coupled with pore formation and peptide translocation (Matsuzaki et al. 1996; Zhang, Rozek, and Hancock 2001).

Because of AMPs variety of targets, their activity is conceivable a multi-hit process. In this view a peptide could act against different target, inhibiting the activity of a number of anionic molecules including enzymes and nucleic acids (Zhang, Rozek, and Hancock 2001). Some examples are maganin and indolicin; they have multiple modes of action and through the multiple-hit strategy they can increase their efficiency and evade potential resistance mechanisms (Nguyen, Haney, and Vogel 2011). In this way, is also possible to explain the difficult of bacteria to evolve high-level resistance to antimicrobial peptides.

Some different characteristics between prokaryotic and eukaryotic cells are accountable for AMPs selectivity. Prokaryotic membranes are rich in phospholipids, phosphatidylglycerol and cardiolipin which confer a negative charged while the eukaryotic cells have a zwitterionic membrane. The cationic AMPs are preferentially attracted by the negative charges in the bacterial membrane. In addition, the absence of cholesterol makes the membrane more flexible and more suitable for the insertion of the AMPs (Glukhov et al. 2005). Also difference in transmembrane potential plays an important role in promoting or delaying AMPs interaction with the membrane. Selectivity may arise also by the different membrane composition and asymmetry. In eukaryotic cells transmembrane potential is usually between -90 mV and -110 mV while in prokaryotic it is between -130mV and -150 mV. Additionally, also AMPs structure and AMPs localization play a fundamental role in their selectivity. This is supported by the observation that AMPs could have a structure with antimicrobial activity different from the one with cytotoxic activity and they can undergo to conformational changes only in presence of the target membrane (Yeaman 2003).

AMPs selectivity and the slow bacterial rate in developing resistance against them suggest their possible therapeutic application, especially against the multi-drug resistant strains. However, despite a good *in vitro* activity, some critical points must be considered concerning the *in vivo* application. Indeed this compounds loss part of their antimicrobial activity in present of high salt concentration (Aoki and Ueda 2013; Mohanram and Bhattecharjya 2016) and in serum due to their

enzymatic degradation or to the binding to serum component (Böttger, Hoffmann, and Knappe 2017).

Moreover, although the selective activity against bacteria, several AMPs often show toxicity referred as their tendency to induce lysis of erythrocytes both *in vitro* and *in vivo* or as their effect on mammalian cells. *In vitro* AMPs cytotoxicity is usually evaluated as their hemolytic activity and reduction of mammalian cells viability and it is mostly caused by pore- formation or by the induction of apoptosis and/or necrosis (Bacalum and Radu 2015; Yeaman and Yount 2003).

To reduce the proteolytic degradations, the antimicrobial peptides could be optimized in different ways such as by cyclization of the AMPs or incorporation of D-amino acids (Fjell et al. 2011). Another important strategy to improve the properties of AMPs is the use of delivery systems, which improve the stability, toxicity, half-life and release profile of AMPs (Kumar, Kizhakkedathu, and Straus 2018). However, it has been reported that different AMPs naturally active in their native environment seem not able to kill the bacteria *in vitro* at the same concentration; this discrepancy could be linked to the immunomodulatory effects of the antimicrobial peptide (Mahlapuu et al. 2016; Marr, Gooderham, and Hancock 2006). For their therapeutic use is also important to consider that, the AMPs rarely induce resistance development but during the evolutionary process there is the selection of some pathogens, which are able to resist to antimicrobial peptides.

1.2.2 Therapeutic potential of AMPs

Antimicrobial peptides are currently among the most promising agents for the treatment of infections because of the widespread resistance of microorganisms to the common antibiotics. Their strengths are: limited capacity of the bacteria to develop resistance, wide spectrum of activity and high possibility of chemical modifications and optimization (Lazarev and Govorun 2010).

Nowadays only a few AMPs are approved for clinical use (including derivatives of polymyxin) but numerous AMPs are under clinical development for the treatment of various bacterial pathogens both with a topical and systemic administration. Some example of AMPs already approved for the clinic are: the bacterial gramicidin which was the first peptide antibiotic used to treat wound infections, daptomycin is another FDA-approved AMP for treating Gram- positive bacterial infections and nisin which is widely utilized as a food preservative in over 50 countries (Mishra et al. 2017).

Even if there is a large number of antimicrobial peptide in clinical development, there is a strong discrepancy between the patented AMPs and the real outcomes of the clinical trials (Kosikowska and Lesner 2016). This result is caused by the poor correlation between the *in vitro* antimicrobial

activity and the *in vivo* efficacy linked to the highly sensitivity to the environmental conditions (i.e.: high salt concentration, serum, etc..) and the low bioavability (Vlieghe et al. 2010).

Another weakness point is the possible development of bacterial resistance to AMPs. The constitutive resistance is the first line of bacterial defence and it is represented by secreted proteins (Andersson, Hughes, and Kubicek-Sutherland 2016), transient molecular modifications of the bacterial membrane leading to a change of its net charge and permeability (Moskowitz, Ernst, and Miller 2004) or the presence of efflux pumps which externalize toxic compound in an active-manner (Band and Weiss 2015). In addition to the intrinsic resistance, mechanisms of acquired resistance are known. The antimicrobial peptides are characterized by a slow rate in development of resistance but recent studies have showed that AMPs resistance can occurred, at least *in vitro*. An example is *S. aureus* in which the redundant treatment with the antimicrobial peptide LL-37 induces the formation of resistant small colony variant (SCV) (Hoffman et al. 2006). Also Perron et al. had showed that high-level bacterial resistance to an AMP could be induced in the lab using a *Pseudomonas fluorescens* strain within 600-700 generations of growth after the treatment with increasing concentrations of the synthetic magainin-analogue and pexiganan (Perron, Zasloff, and Bell 2006).

1.2.3 Focus on Cathelicidins and BMAP-18

As mentioned above, cathelicidins are one of the major AMPs families in vertebrates. They are cationic peptides with amphipathic properties and stored in neutrophil and macrophage granules (Bals and Wilson 2003). Structurally they are characterized by the presence of a N-terminal conserved proregion (cathelin-domain) and a more variable C-terminal active part (**Fig. 1.9**). They are stored in their inactive form in the secretory granules and they are released extracellularly upon activation by elastase (Treffers et al. 2005). The mammalian cathelicidin peptides display a wide repertoire of structures: α-helical, linear, cyclical, etc.. and they kill bacteria through membrane permeabilization, with some exceptions such as the proline-rich peptides (Gennaro and Zanetti 2000; Tomasinsig and Zanetti 2005; Zanetti 2005; Zanetti, Gennaro, and Romeo 1995).

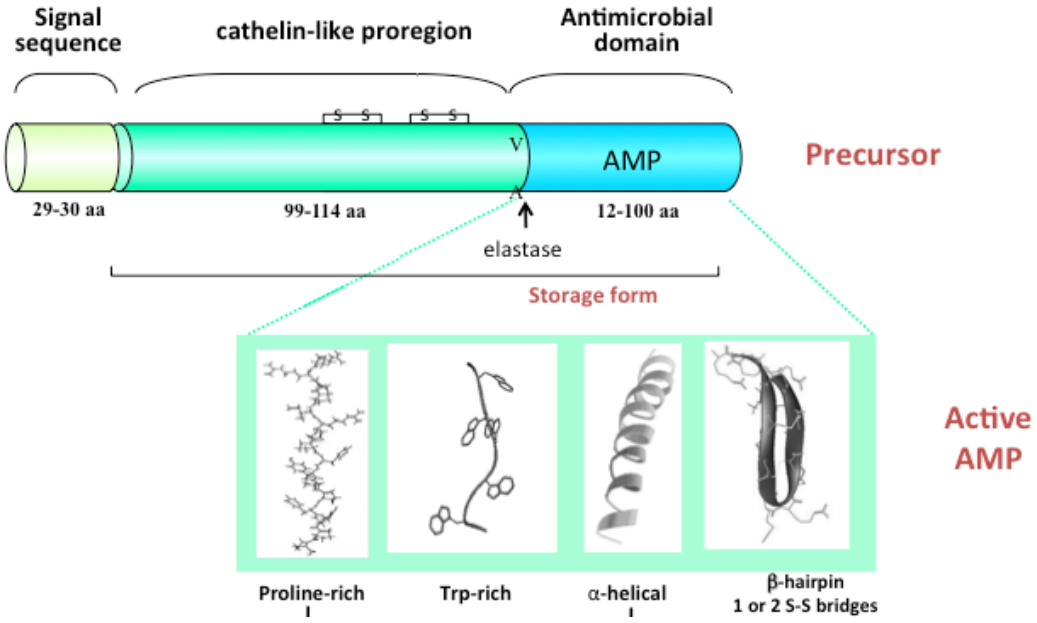


Fig. 1.9: Representation of the cathelicidin structure and of its varied C-terminal antimicrobial domains

They are found in humans and other species, including farm animals such as cattle, horses, pigs, sheep, goats, chickens, rabbits and in some species of fish (Kościuczuk et al. 2012). About 30 cathelicidin family members have been identified in mammalian species with different structures and characteristics (Kościuczuk et al. 2012).

The α-helical cathelicidins include the human LL-37 and the bovine BMAP-27 and BMAP-28. They act in a lytic way and are expressed both in neutrophils and in epithelial cells. Additionally, LL-37 acts in promoting wound healing, as direct and indirect antimicrobial factors and can also modulate the adaptive immunity (Bals et al. 1998; Gudmundsson et al. 1995; Joo, Fu, and Otto 2016). The proline- rich cathelicidins (PrAMPs) include Bac5, 6, and 7.5 isolated in sheep (Shamova et al. 1999) and the bovine Bac5 and Bac7. These peptides, act preferentially against Gram- negative bacteria (that express a specific transporter) targeting the ribosome and blocking protein synthesis (Gennaro, Skerlavaj, & Romeo, 1989; Mardirossian et al., 2014). Other important cathelicidins members are the tryptophan- rich peptides as the bovine indolicidin. This peptide acts on intracellular targets mostly like DNA and RNA (Kościuczuk et al. 2012).

In our lab we started to characterize BMAP-27 and BMAP-28. They showed a potent and broad spectrum antimicrobial activity *in vitro* against Gram- positive, Gram- negative bacteria and fungi. Their antimicrobial activity was tested also in the fields of pulmonary infections. In particular these peptides showed to be highly effective against the planktonic form of *P. aeruginosa* and *S.*

maltophilia CF isolates with a MIC_{50} 4-8 μg/ml and exerted a rapid bactericidal activity by membrane permeation. In addition, both the peptide showed to inhibit the biofilm formation of several *P. aeruginosa*, *S. malthopilia* and *S. aureus* strains. Overall their antimicrobial activity was comparable and in some cases higher than that of tobramycin (Pompilio et al. 2011, 2012). These results were a first good indication of the potential use of these cathelicidin-derived peptides as lead compounds for future development as therapeutic agents. However, since they shown to be quite cytotoxic to human erythrocytes and neutrophils (Skerlavaj et al. 1996) some truncated forms lacking the 9-10 C-terminal hydrophobic residues have been synthesized.

These truncated forms called BMAP-27(1–18) and BMAP-28(1–18) and an analogue with a modified C-terminal region (mBMAP-28) showed to possess an antimicrobial activity comparable to that of the parental peptides, and among them, BMAP-27(1-18) (from now: L-BMAP18) was that with the better ratio between *in vitro* antibacterial activity and acute lung toxicity in mice (Benincasa et al. 2003).

L-BMAP18 is an amphiphilic α-helical peptide, acting by membrane permeabilization against both Gram- positive and Gram- negative bacteria (**Fig. 1.10**)

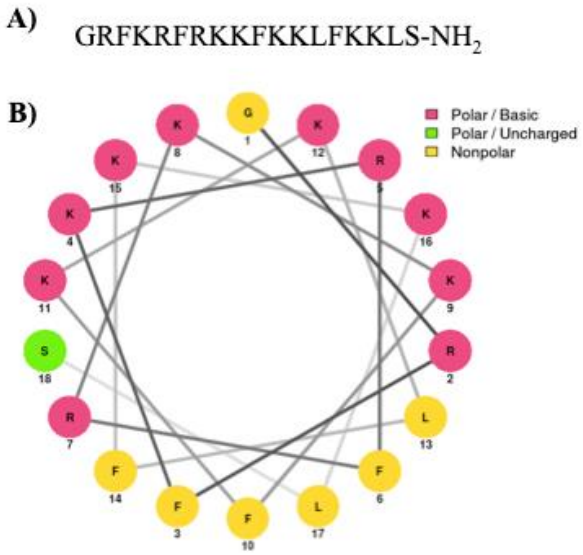


Fig: 1.10: Sequence and helical wheel projection of L-BMAP18. (A) Amino acids sequence of the peptide. (B) Helical Wheel Projections of the peptide.

Subsequently L-BMAP18 shown to be active against 14 different *P. aeruginosa* CF-isolates (MIC_{90} =16 μg/ml) and to inhibit *P. aerugionsa* biofilm formation at sub-MIC concentrations in a strain-dependent manner (Mardirossian et al. 2016). It showed a negligible acute pulmonary toxicity when intratracheally administered in lungs of a group of mice. L-BMAP18 caused the death of the 20% of the tested mice only at the higher tested concentration of 4 mg/Kg while at the other

tested concentrations it showed non-toxicity. However, when tested in a murine model of acute lung infection sustained by *P. aeruginosa*, the peptide did not reduce the bacterial load in lungs. Later, we demonstrated that lost of activity was due at least in part to its degradation by pulmonary proteases (Mardirossian et al. 2016).

2 AIM

The aim of my PhD project was to characterize the antibacterial properties of the AMP BMAP18 and to optimize its activity in order to obtain an effective antibacterial peptide suitable for the *in vivo* treatment of pulmonary infections.

Previous results have highlighted the scarce stability of L-BMAP18 in the pulmonary environment and its residual *in vivo* toxicity. To overcome these drawbacks in this study I operated in parallel in two directions.

The first was to find the best conditions to make L-BMAP18 more stable and more active in the physiological and pathological pulmonary environment. To reach this goal I took into account the use of the enantiomeric form of the peptide (*D*-BMAP18) which has been tested in different media resembling the lung habitat also in combination with different substances with enhancing activity, and against biofilms of respiratory pathogens.

In parallel I studied the possibility to overcome the problem of any residual toxicity by designing and testing a pro-drug derived from BMAP18 able to slowly release the pharmacologically active peptide into the target organ. The results contributed to understand the potentiality of *in vivo* application of this class of molecules for the treatment of infective diseases of the lungs.

3 MATERIAL and METHODS

3.1 Antibiotics and enzymes

The antibiotics used are Tobramycin (Sigma-Aldrich), N-Acetyl-L-cysteine (Sigma-Aldrich), Alginate Lyase (Sigma-Aldrich) and DNase I (Sigma-Aldrich) from bovine pancreas solved in sterile water and Amphotericin B (Sigma-Aldrich) solved in DMSO

3.2 Bacterial and fungal strains and culture conditions

P. aeruginosa PA03, PA05, PA07, PA08, PA09, PA10, PA14, PA21, PA22, PA31, PA35 strains were obtained by CF patients as previously reported by Pompilio et al. 2012. *P. aeruginosa* RP73, and PA01 were used as reference strains. All strains were stored at −80 °C until used and plated on Mueller–Hinton agar (MHA; Oxoid S.p.A., Milan, Italy). The fungal strains *C. albicans* SC5314 and ATCC 90092 were stored at −80 °C until use and plated on Sabouraud agar

3.3 Peptides design and synthesis

The peptides *D*-BMAP18 (GRFKRFRKKFKKLFKKLS-am) and Pro-*D*-BMAP18 were synthesized using the solid-phase Fmoc chemistry on a microwave peptide synthesizer Initiator +, Biotage (USA). Protected amino acids and Fmoc-linker-AM champion resins were purchased from Advanced Biotech Italia or Novabiochem. The double-coupling synthesis was performed at 75°C. For the coupling steps, the Fmoc-protected amino acid and coupling reagents were added in a 5-fold molar excess with respect to resin substitution. Couplings (30–60 min) were performed with N-hydroxybenzotriazole (HOBt) and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluroniumtetrafluoro-borate (TBTU). Cleavage from the resin and deprotection of the synthesized peptides were done with a solution of 85% trifluoroacetic acid, 2% water, 2% triisopropylsilane and 8% of phenol, 1,2-ethanedithiol and 3% of thioanisole. The peptides were both purified by reversed phase HPLC on a C18 column (19×300 mm; Waters) using an acetronitrile-water 0–40% linear gradients added with 0.05% trifluoroacetic acid with a 1ml/min flux. Peptides' quality and purity was verified by ESI–MS (Esquire 4000, Burker. Flux=180 μl/h, Voltage=5000 V). The peptides were lyophilized three times in a standardized volume of 10mM HCl (CT60e Heto, Technology of Scandinavia) and the concentration of the stock solution has been evaluated by spectrophotometric determination of peptide bonds (ε214, buffer composed by 80%H_2O, 20% AcCN and 1% TFA) and, when possible, using Waddell Spectrometric Method

(ε215 and ε225, buffer composed by 50% H_2O and 50% 1/150 N NaOH) with the instrument Ultraspec 2100 pro, Amersham Bioscience.

3.4 Degradation of *D*-BMAP18 in BAL

Bronchoalveolar fluid was collected from six C57/Bl6NCrl healthy male mice (2-3 months old, from the animal facility of the University of Trieste). Mice were killed by cervical dislocation and then lungs were washed with 1ml of sterile 0.9% NaCl three times. Equal volume of BAL samples from each mouse were pooled and stored in disposable aliquots at −20 °C, in order to reduce the variability of composition. The total protein concentration of BAL fluid (300 μg/ml) was determined by BCA assay (Pierce, BCA Protein Assay Kit). To evaluate *D*-BMAP18 resistance to proteases, the peptide was diluted in pooled BAL to a final concentration of 300 μg/ml, obtaining a 1:1 (wt/wt) peptide/BAL total proteins ratio. *D*-BMAP18 in BAL was then incubated at 37 °C. 30μl of the samples were taken at indicated times, immediately added to 10μl of *Laemly sample-buffer* 4× (SDS 12%, 0,4 M DTT, Glycerol 30%, Bromphenol blue 0,05%, 0,5 M TRIS HCl pH 8,8), cooled down and frozen at −20 °C. Heating for 5 min at 90°C denatured the samples and 10μl of each sample were loaded on a 16% tricine gel for SDS-PAGE. The gel was stained over-night with Coomassie Brilliant Blue (Coomassie Blue 0,05%, acetic acid 10%, methanol 50%) and de-stained with 10% acetic acid in water (v/v).

3.5 Activation of Pro-D-BMAP18 by human elastase in HEPES

Pro-*D*-BMAP18 (500 ng) was mixed with human neutrophil elastase (Human Neutrophil Elastase, Abcam) in a molar ratio 100:1 and incubated at 37°C in 50 μL HEPES 100 mM.15μl of the mixture were taken at different times and the reaction was stopped by dilution in 15μl of H_2O + 0,1% TFA, cooled on ice and frozen at −20°C for HPLC-MS analysis. Each sample was injected for an HPLC analysis using an analytic column (Symmetry® C18, 100Å, 3 μm, 3 mm X 100 mm, Waters). The elution was done by a linear gradient of solvent B (0,05 % TFA in AcCN) in A (0,05 % TFA in water) starting from 20% B in A to 35% B in A in 15' with a flux of about 15 μl/min. The HPLC was connected to the mass analyser HCT ultra (Burker Daltonics) using an electrospray ionization (ESI-MS) and a capillary voltage about 200 V.

3.6 Preparation of the conditioned medium from neutrophil-like cells

The human cell line HL-60 (ATCC CCL-240) grown in RPMI medium (Sigma) with 20% (v/v) fetal bovine serum (FBS, EuroClone), 1% pen/strept, 2 μM L-glutamine (EuroClone) at 37°C in 5% CO_2 in 24-wells flatted bottom microtiter plates (Sarstedt). Differentiation to neutrophils-like cells were induced by culturing cells in the presence of 100 nM Phorbol myristate acetate (PMA) (Sigma- Aldrich) for 6 days. The differentiation was demonstrated trough cell cycle's analysis. 10^6 cells were fixed in 1 ml of ethanol and washed twice in 1 ml of PBS (Sigma-Aldrich. Final concentration: phosphate buffer 0.01 M and NaCl 0.154 M; pH=7,4), then centrifuged 5 min at 1303 ×g. Cells were stained with a solution composed by 10% Propidium iodide (PI, Sigma-Aldrich, 0,1 mg/ml in PBS 1×), 0,5% FITC (Sigma-Aldrich), 0,4% RNasi A (Sigma-Aldrich) in PBS. After o/n incubation at 4°C the analysis were done by the flux cytofluorimeter Cytomics FC 500 (Beckman-Coulter, Inc., Fullerton, CA). After the differentiation, the exhausted medium was discarded and 1 ml of fresh medium RPMI containing 100 μM PMA was added to the wells for 2h. The medium and the cells were collected and centrifuged 10' 300×g. The supernatant was collected and then used for MIC assay.

3.7 Evaluation of the antimicrobial and antifungal activity of the peptide against selected strains

Minimum inhibiting concentration (MIC) values were determined by microdilution susceptibility test. Two-fold serial dilutions of *D*-BMAP18, Pro-*D*-BMAP18 (128 μg/ml) and Pro-*D*-BMAP18 (128 μg/ml) in presence of human neutrophil elastase (12,8 μg/ml, molar ratio100:1) were prepared in Mueller–Hinton broth (MH, Difco) using a 96 well U-bottom microtiter plates (Sarstedt). Bacteria were grown o/n in MH at 37°C with shacking (140 rpm). After the o/n incubation 350 μl of bacterial were added to 10 ml of fresh MH and they were incubated in the agitator (140 rpm) at 37°C until the bacteria reached an optical density (OD) of 0,300 measured at a wavelength of 600_{nm} (Ultraspec 2100 pro, Amersham Bioscience). Each well was inoculated with a standardize inoculum of 50 μl to achieve a final test concentration of 5×10^5 CFU/ml bacteria. After the incubation of plate at 37°C for 24h, the MIC was measured as the lower concentration of peptides that totally inhibits bacterial growth. The Minimal Bactericidal Concentration (MBC) was evaluated by plating 25μl on MH-agar (MHA) plates of the samples taken from the clear wells of the MIC assay, and by incubation at 37°C overnight. A definition of MBC as the lowest concentration of drug killing at

least 99.9 % of the original inoculums were considered.

Against the two *C. albicans* strains ATCC90029 and SC 5314 the protocol was performed as above but the final fungal concentration in the plate was about 5×10^4 CFU/ml and the plate was incubated at 30°C for 48h. The media used were Sabouraud broth (Difco) and Sabouraud agar.

3.8 Evaluation of the antimicrobial activity of Pro-*D*-BMAP18 in HL-60 exausted medium

The human cell line HL-60 was induced to release elastase in the cell medium as described above. Pro-*D*-BMAP18 (final concentration: 128 µg/ml) was incubated in the exhausted medium for 4h, 18h and 24h. For each time 100 µl of the incubated Pro-D-BMAP18 was plated and two-fold serial dilutions were performed in a 96 well U-bottom microtiter plates (Sarstedt) for a MIC assay against the *P. aeruginosa* strains PA01 and RP73.

3.9 Biofilm inhibition and eradication against bacterial and fungal cells

Biofilm inhibition assay was performed against the *P. aeruginosa* strains PA01, PA03, PA05, PA07, PA08, PA09, PA10, PA14, PA21, PA22, PA31, PA35 and RP73. Two-fold serial dilutions of *D*-BMAP18, Pro-*D*-BMAP18 and Pro-*D*-BMAP18 incubated with human neutrophil elastase (100:1 molar ratio) were prepared in 50 µl MH broth in 96-wells flat-bottom microtiter plates (Sarstedt). Each well was inoculated with 50 µl of *P. aeruginosa* strains to a final test concentration of about 10^6 CFU/mL. After a 24h-incubation at 37°C, wells corresponding to 1/2xMIC, 1/4xMIC, 1/8xMIC were washed three times using 150 µl of MH. 100 µl of MH containing 1 mM MTT (5 mg/ml, Thiazolyl Blue Tetrazolium Bromide, Sigma-Aldrich) were added, and the plate was incubated 4h in the dark at 37°C. The medium was discarded and the wells were washed with 150 µl of Phosphate buffered saline (150 mM NaCl in 10 mM sodium phosphate buffer pH 7, PBS). Subsequently 100 µl of re-suspending solution (20% SDS, Fulka wt/v in 50% DMF, Romil v/v in H_2O) were added to the wells. After incubation at 37°C for 16h in the dark, the viability of the biofilm was evaluated measuring the absorbance at 570_{nm} using the multi-well plate reader Nanoquant infinite M200pro (Tecan). For the fungal *C. albicans* strains ATCC 90029 and SC5314 the final test concentration was of about 10^5 CFU/mL in Sabouraud broth and the plate was incubated at 30°C for 48h.

Biofilm eradication activity was tested against the *P.aeruginosa* strains PA01, PA08, PA09 and RP73. 100 µl of a final test concentration of about $2,5\times10^5$ CFU/ml of *P. aeruginosa* cells grown in MH broth for 18h in 96-wells flat-bottom microtiter plates (Sarstedt) at 37°C. After incubation the medium was discarded and wells were washed once using 150 µl of MH broth, and 100 µl of MH broth containing different concentrations of the peptides were added to the wells. After a 24h-incubation at 37°C, the medium and non-adherent bacteria were discarded. 100 µl of MH broth containing 1 mM MTT (5 mg/ml) were added and the plate treated as reported above for MTT assay. In presence of DNase I (Sigma-Aldrich), N-Acetyl-L-cysteine (Sigma-Aldrich) and Alginate lyase (Sigma-Aldrich): after 18h-incubation the medium was discarded and wells were washed once using 150 µl of MH broth, and 50 µl of MH broth containing respectively 128 µg/ml of bovine DNase I, 10 mg/ml of N-Acetyl-L-cysteine and 1 mg/ml of Alginate lyase. The plate was incubated for 1h and then 50 µl of MH broth containing different concentrations of *D*-BMAP18 were added to the wells. After a 24h-incubation an MTT assay was performed as above.
Concerning the *C. albicans* ATCC 90029 and SC5314 was used a final test concentration of about 10^5 CFU/ml Sabouraud broth.

3.10 *In vitro* toxicity against human cell lines

The cytotoxicity was evaluated as cell's vitality by the MTT assay against the lung cell line A-549 and the keratinocytes HaCat cells, the blood cells MEC-1 and the macrophage differentiated THP-1 cells. A-549 and HaCat cells grown in Dulbecco's MEM (Sigma) 10% FBS + 2 mM Glu + 1% Pen/Strep. The MEC-1 cells and the THP-1 cells grown in RPMI (Sigma) 10% FBS + 2 mM Glu + 1% Pen/Strep.
20.000 A-549 or HaCat cells/50 µl were seeded in each well of a 96-wells flat-bottom microtiter plate (Sarstedt) and were incubated o/n at 37°C. Serial two-fold dilutions of the of *D*-BMAP18, Pro-*D*-BMAP18 and Pro-*D*-BMAP18 incubated with human neutrophil elastase (100:1 molar ratio) were prepared in eppendorf in the same cell growth medium and 50 µl of the samples were added to the cells. After a 20h-incubation at 37°C, 20 µl of MTT (5 mg/ml) were added to each well. Following 4h of incubation at 37°C, 100 µl of igepal (Sigma) 10% in 0,01N HCl were added and the plates was incubated o/n at 37°C. After the incubation the cytotoxicity was evaluated by analysis of the OD_{620nm} with the reader Nanoquant infinite M200pro (Tecan). Considering the MEC-1 and the THP-1 cells, the MTT was performed as reported above but seeding 100.000 cells/well in 50 µl. Using the HaCat cells the previous protocol was performed also in presence of pasteurized 25% CF-sputum. The protocol is performed as above but the dilutions of peptides were prepared in 25% CF-sputum in cell media.

3.11 Biological activity of the peptides in BAL and in CF-sputum

The assays were performed in murine BAL (see above) for testing the antimicrobial activity of *D*-BMAP18 against *P. aeruginosa* RP73 strain. Sterile 0.9% w/v NaCl alone was used as a control. Different concentrations of the peptide were prepared in 100 µl of BAL, in BAL added to 300 mM NaCl and in 0.9% w/v NaCl. Each concentration was then added with 100 µl of bacteria in MH broth at a concentration of 2×10^6 CFU/ml. Samples were then incubated at 37°C for 1h in a thermostatically-controlled water bath, serially ten-folds dilution of the samples were prepared in MH broth and 25 µl of each dilution were plated on MHA. Colonies were counted after the overnight incubation at 37°C. The antimicrobial activity of *D*-BMAP18 and Pro-*D*-BMAP18 was evaluated in 25% sputum of CF- patients by a viable cells count assay against *P. aeruginosa* RP73 and *C. albicans* SC5314. Samples were collected from 10 different patients in the frame of collaboration with the Childern Hospital Burlo Garofolo in Trieste and pooled. The pool was subdivided in aliquots and stored at -20°C. The assay was performed in 25% CF- sputum in SCFM (synthetic CF sputum prepared by using Casamino Acids Vitamin Assay (BD Difco) mixture as described by (Pompilio et al. 2012) with an addition of 300 mM NaCl and/ or 128 µg/ml of DNase I. *D*-BMAP18 and Pro-*D*-BMAP18 were added to 50 µl of diluted sputum and incubated for 4h at 37°C in a thermostatically-controlled water bath with 50 µl of bacterial cells (final concentration: 1×10^6 CFU/ml). After incubation, ten-folds dilutions of each sample were prepared in MH and 25 µl of each solution were plated on MHA medium and o/n incubated for colony count. Against *C. albicans* SC5314 the assay was performed as described above but the final test concentration for the incubation was about 5×10^5 CFU/ml and the medium used were Sabouraud and Sabouraud agar. A plate containing serial dilution of the 25% CF-sputum alone without the addition of bacteria/ fungi was used as a control for the presence of the endogenous flora. No endogenous bacterial growth was observed in the control plate after the incubation.

3.12 *In vitro* anti-inflammatory activity of D-BMAP18

600,000 THP-1 cells were seeded in 1 ml of RMPI medium added in 10 µg/ml of Phosphomolybdic acid (PMA, Sigma-Aldrich) in a 24-wells flat-bottom plate (Costar) and were incubated 2 days at 37°C in 5% CO_2. After the incubation the medium was discarded, each well was washed once using 1 ml of fresh medium and 500 µl of fresh medium were added. After an o/n incubation at 37°C the pro-inflammatory stimuli and/or the peptide were added and the plate was incubated for 24h. The

pro-inflammatory stimuli used were 10 ng/ml of lipopolisaccaride (LPS) and 100 ng/ml of Interferon-γ. After incubation the RNA extraction, cDNA synthesis and the Real-time quantitative PCR (RT-qPCR) were performed. Cells RNA was purified with euroGOLD Total RNA Kit (Euroclone, Milan, Italy) according to the supplier's instructions. The extracted total RNA was reverse-transcribed with iScript cDNA Synthesis Kit (Bio-Rad, Milan, Italy). Real-time quantitative PCR (qPCR) was carried out on a Rotor-Gene 6000 (Corbett, Explera, Ancona, Italy) using iQ SYBR Green Supermix (Thermo Scientific Fynnzymes, Milan, Italy). The primers used for qPCR evaluated TNF α and IL1-β expression (Thermo Scientific). The relative amount of gene production in each sample was determined by the Comparative Quantification (CQ) method supplied as part of the Rotor Gene 1.7 software (Corbett Research)

4 RESULTS

4.1 Synthesis of *D*-BMAP18

Previously it has been reported that L-BMAP18 has a good *in vitro* antimicrobial activity against CF isolates of *P. aeruginosa* and *S. maltophilia* and a low acute cytotoxicity when injected in mice lungs. Despite these results, it was scarcely protective against murine pulmonary acute infections by *P. aeruginosa* RP73. This effect was likely due to its *in vivo* degradation (Mardirossian et al. 2016). To overcome this drawback we designed its enantiomeric form composed by *D*- amino acids (GRFKRFRKKFKKLFKKLS-am, MW=2341,8 Da). *D*-BMAP18 was synthesized, purified at 95% and its identity was verified by MS analysis. Stability of *D*-BMAP18 was then verified in bronchoalveolar lavage (BAL) of healthy mice, mimicking the biological pulmonary environment. The all-*D*-peptide was incubated in BAL for different intervals of time and products analysed by tricine-SDS PAGE. *D*-BMAP18 was stable in BAL without any sign of degradation even after 7 days of incubation **(Fig. 4.1)**.

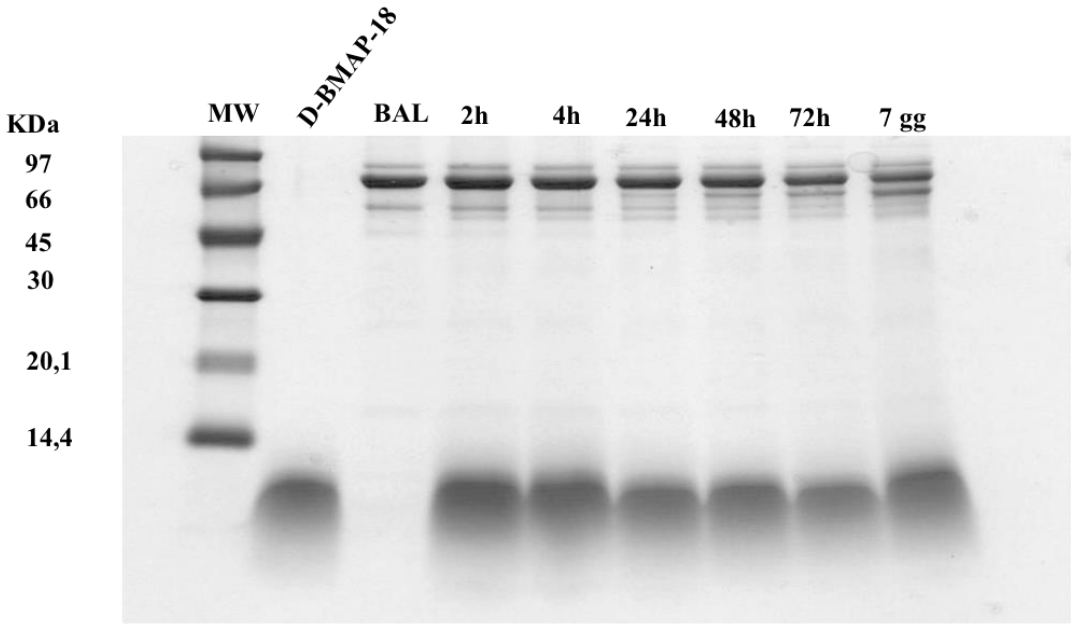


Fig. 4.1: Tricine-SDS analysis showing D-BMAP18 incubated in BAL for different times (from 2 h to 7 gg).

4.2 Biological features of *D*-BMAP18

4.2.1 Antimicrobial and antibiofilm activity of *D*-BMAP18

D-BMAP18's antibacterial activity was evaluated against a selected panel of *P. aeruginosa* and *S. maltophilia* strains CF isolated and susceptible to the L-peptide. The *D*-peptide showed to have a similar antibacterial activity to the parental L-form against *P. aeruginosa* and *S. malthopilia* unless for specific bacterial strains such as RP73, SM106 and SM110 (**Tab. 4.1**). This different activity could be likely due to its resistance to the bacterial secreted proteases.

Table 4.1: MIC (μg/ml) values of L-BMAP18 vs *D*-BMAP18 in MH medium.

Strains	**L-BMAP18**	**D-BMAP18**
P. aeruginosa		
PA01	4	4
RP73	16	4
PA03	8	8
PA05	4	4
PA07	8	8
PA08	8	8
S. maltophilia		
SM103	4	4
SM105	8	8
SM106	64	16
SM110	16	8

* CF isolated strains. The results are the average of three independent experiments in internal duplicate

The presence of *P. aeruginosa* biofilm is one of the major causes of the persistence of the infection and it is linked with a higher mortality in CF patients (Bjarnsholt et al. 2009). For these reasons we tested the activity of the *D*-peptide against the synthesis of new biofilm at sub-MIC concentrations and against preformed biofilm. Inhibition of biofilm formation was tested against a panel of *P. aeruginosa* CF strains and PA01, used as a reference strain. At sub-inhibitory concentrations *D*-BMAP18 generally inhibited biofilm formation in a concentration-dependent and strain-specific manner. Biofilm formation of PA08, PA09, PA10, PA21 and PA35 strains were significant inhibited by *D*-BMAP18 used at 1/2 MIC concentration (**Fig. 4.2**). Considering some bacterial

strains, sub-inhibitory concentrations of the peptide seem to induce biofilm formation. This observation have already been reported for macrolides which alter the formation of the biofilm matrix (Wozniak and Keyser 2004) or for imipenem which induces the production of alginate and peptidoglycan (Davies, Spiegelman, and Yim 2006).

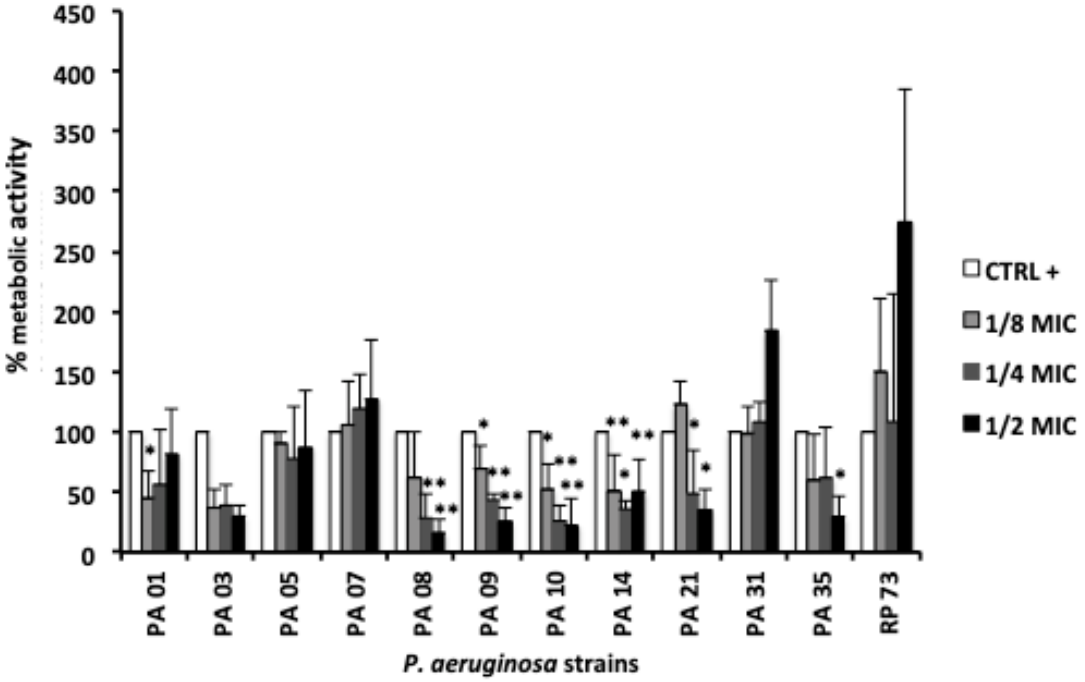


Fig. 4.2: Inhibition of *P. aeruginosa* biofilm formation. MTT assay evaluating peptide inhibition of biofilm formation against CF isolates of *P. aeruginosa* strains after 24h-treatment. Percentages of metabolic active biofilm in comparison with the untreated control of growth are shown. CTRL+ = untreated samples. The results are the average of three independent experiments in internal triplicate (n=9). *=p< 0,05 **=p< 0,01

The biofilm eradicating activity of *D*-BMAP18 was also evaluated, treating the preformed biofilms of *P. aeruginosa* PA01, PA08, PA09 and RP73 strains. PA01 and RP 73 were used as the reference strains while PA08 and PA09 were good biofilm producers and had low variability in this assay. The eradicating activity was tested incubating the biofilm with the peptide alone or after 1h-pretreatment with bovine DNase I, an enzyme used to destabilize biofilm matrix by the degradation of the eDNA or with alginate lyase, to degrade the *P. aeruginosa* alginate envelope. Usually the treatment with DNase alone did not affect biofilm viability. This was observed also testing alginate lyase, unless considering RP73 and PA01. Anyway, their presence enhanced the eradication activity of *D*-BMAP18. This is particularly evident for of PA08 and PA09 where the pre-treatments with DNase I cause a decreased to 20% of biofilm viability using the peptide at 32µg/ml (**Fig. 4.3**). In presence of N-acetilcysteine, a mucolytic agent approved for therapy, no increase of the antibiofilm

activity of *D*-BMAP18 was detected, probably because of its inactivity in degrading the alginate or the eDNA of *P. aeruginosa* biofilm (data not shown).

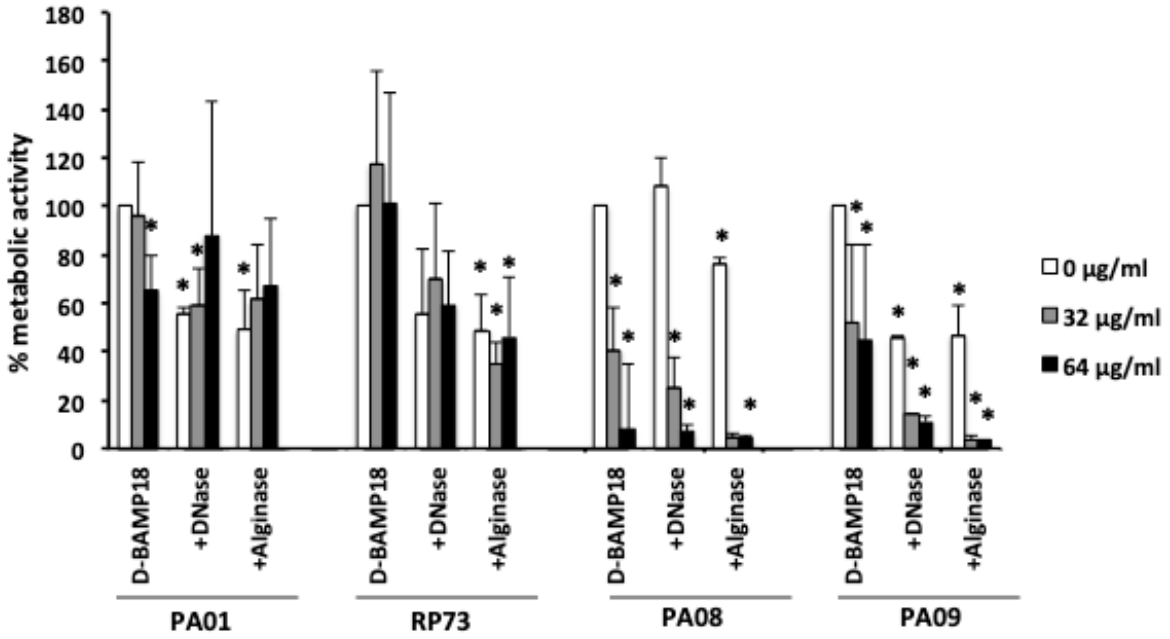


Fig. 4.3: Biofilm eradication of D-BMAP18. MTT viability test on preformed *P. aeruginosa* biofilms. After 1h-pretreatment with DNase I (128 µg/ml) or Alginase (1 mg/ml). Eradication activity has been reported as percentages of metabolic active biofilm in comparison with the untreated control of growth (0 µg/ml *D*-BMAP18) . The results are the average of three independent experiments in internal triplicate (n=9). *=p< 0.05. Test t-student

The activity of *D*-BMAP18 was tested against *C. albicans* ATCC 90029 and SC 5314 strains. *D*-BMAP18 has MIC values of 2 µg/ml against ATCC 90029 and 16 µg/ml against SC 5314 (data not shown). Anti-candidal activity of *D*-BMAP18 was tested at first as inhibition of biofilm formation (**Fig. 4.4 A**) and as eradication of preformed biofilm (**Fig. 4.4 B**).

The peptide reduced biofilm production of the ATCC 90029 strain in a concentration dependent manner: at 1/2 MIC the biofilm viability was reduced by the 30%. *D*-BMAP18 was highly active also against the preformed biofilm of ATCC 90029 and already at 32 µg/ml the cell viability of the biofilm was halved. Different results were obtained using the SC5314 strain. *D*-BMAP18 usually seemed to increase biofilm formation in the presence of sub-inhibitory concentration and had eradicating activity only at 128µg/ml reducing biofilm viability by the 40%.

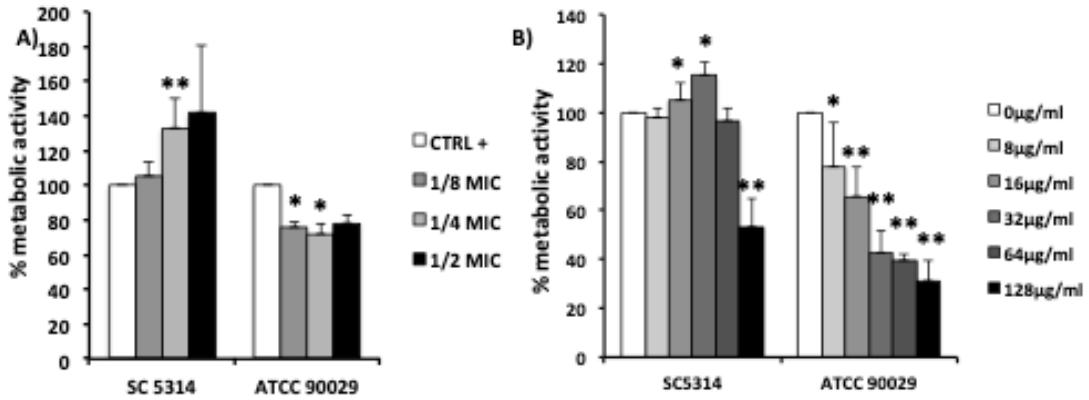


Fig. 4.4: Antibiofilm activities of *D*-BMAP18 against the C. albicans strains SC 5314 and ATCC 90029. MTT assay evaluating peptide inhibition of biofilm formation (A) and eradication activity (B) against *C. albicans* strains after 24h of treatment. Eradication activity has been reported as percentages of metabolic active biofilm in comparison with the untreated control of growth. The results are the average of three independent experiments in internal triplicate (n=9). *=p< 0,05 **=p< 0,01

4.2.2Biological activity of *D*-BMAP18 in BAL and CF-Sputum

The biological activity of *D*-BMAP18 was tested in media resembling the pulmonary environment so as to predict its *in vivo* effectiveness. At first the killing activity of the peptide was evaluated in mouse BAL or in a solution of 0,9% NaCl (150mM) which was used to perform the bronchoalveolar lavage in mice.

The peptide was very active against *P. aeruginosa* RP73 in 0,9% NaCl after 1h incubation but had a significant reduction of activity (> 2 logs) in presence of BAL. Since we previously showed that the peptide was not degraded, we supposed that *D*-BMAP18 maybe electrostatically bound and eventually inhibited by some BAL components. To verify this hypothesis we repeated the assay by increasing salt concentration (+300 mM NaCl) to weaken any electrostatic interactions between molecules. As summarized in **Fig. 4.5** the killing activity of *D*-BMAP18 is reduced in BAL but it is partially rescued in presence of high salt concentrations (total=450 mM derived from 150mM NaCl of BAL+ 300 mM NaCl added).

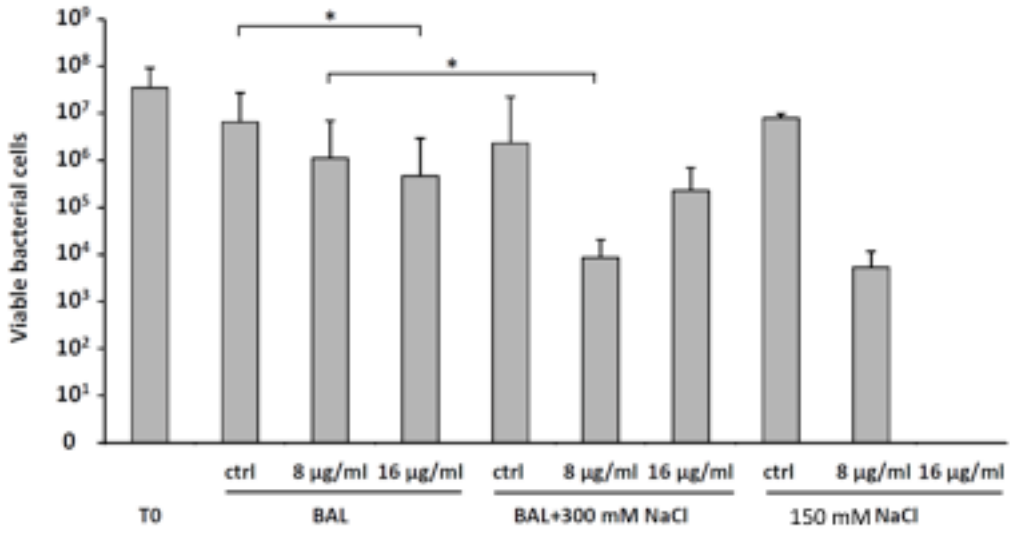


Fig. 4.5: Antimicrobial activity of *D*-BMAP18 in different media. Viable cell count of *P. aerugionsa* RP73 after 1h-incubation with *D*-peptide in BAL and BAL added with 300 mM NaCl. 150 mM NaCl was used as a control of activity. *=p< 0,05

This unexpected result indicates that *D*-BMAP18 may be partially inhibited in the lungs but at the same time, and differently to most AMPs, its activity is fully retained under high salts conditions.

The antimicrobial activity of *D*-BMAP18 against *P. aeruginosa* was also assessed in CF-sputum which resembled the lung environment of CF- patients. The peptide was incubated 4h with *P. aeruginosa* RP73 cells alone or in combination with 300 mM NaCl and/or DNaseI (128 µg/ml), which are commonly used in therapy for mucus thinning (Klodzińska et al. 2016). The result showed that the peptide lost its activity in 25% CF sputum while in SCFM (synthetic CF sputum medium; (Pompilio et al. 2012) it totally broke down the bacterial load. However the addition of DNase I or NaCl partially rescued its antibacterial activity. It is worth nothing that the combination of both NaCl and DNase I significantly improved the antipseudomonal action of *D*-BMAP18 reducing the bacterial load of more than 2 log (**Fig. 4.6**).

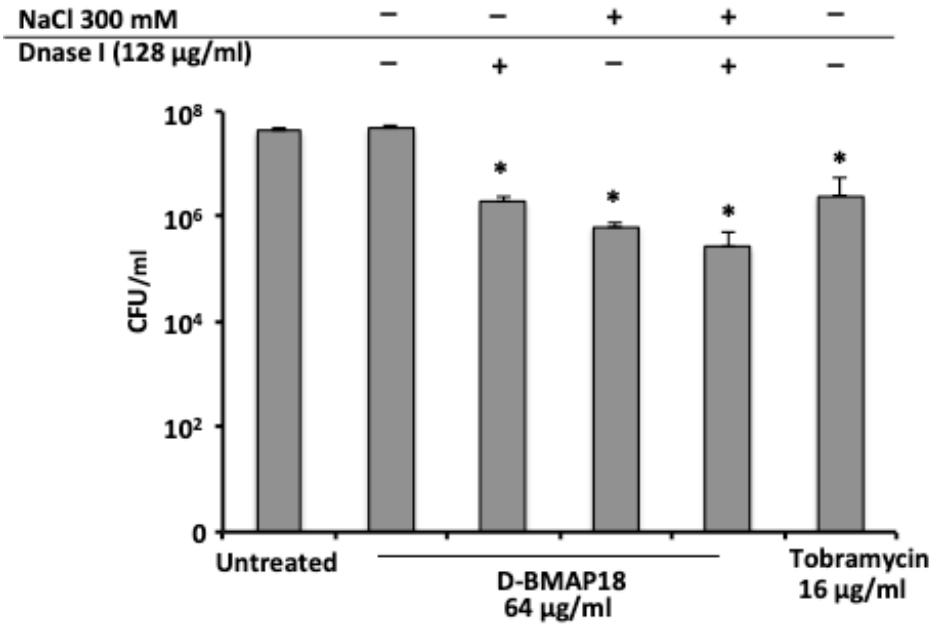


Fig. 4.6: Killing activity of *D*-BMAP18 in 25% CF- sputum. Viable cells count of *P. aeruginosa* RP73 after 4h-incubation with *D*-peptide in 25% CF sputum added with DNase I (128 µg/ml) and/or NaCl 300 mM. Tobramycin was used as a positive control of activity. *=p< 0,05

The anti-candidal activity of *D*-BMAP18 was also tested in 25% CF-sputum using the same conditions used for *P. aeruginosa.* It was tested against the SC5314 strain in order to test it against the stronger strain. In this case the peptide reduced by about 1 log the viability of *C. albicans* SC 5314 cells, but only if salt concentration had been increased to 300 mM NaCl. Under these conditions, 64 µg/ml D-BMAP18 had antifungal activity comparable to that of 2µg/ml amphotericin B, a common antifungal compound (**Fig. 4.7**).

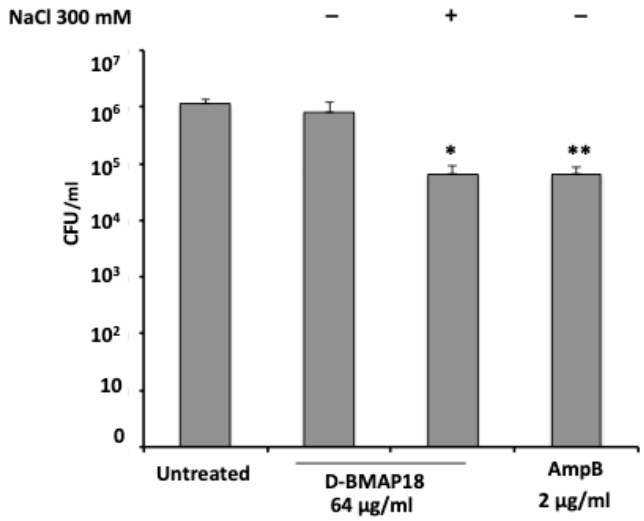


Fig. 4.7: *D*-BMAP18 activity against C. albicans SC 5314 in CF sputum. Viable fungal cells count of *C. albicans* SC5314 after 4h-incubation with D-BMAP18 and 300 mM NaCl in 25% CF sputum.

Amphotericin B (AmpB) was used as a positive control of activity under the same conditions. All compounds were tested at 4×MIC concentration in order to have a comparable condition of activity. The results are the average of three independent experiments in internal duplicate (n=6). *=p< 0.05, **p<0.01. Test t-student

4.2.3 *In vitro* anti-inflammatory activity of *D*-BMAP18

Hyper inflammation is one of the major causes of mortality of CF-patients, together with pulmonary damages. Since some AMPs have also shown to have anti-inflammatory activity, we investigated whether *D*-BMAP18 has also this function. The macrophage differentiated THP-1 cells were stimulated using LPS or IFN-γ as inflammatory stimulus and contemporary treated with low concentrations of *D*-BMAP18.

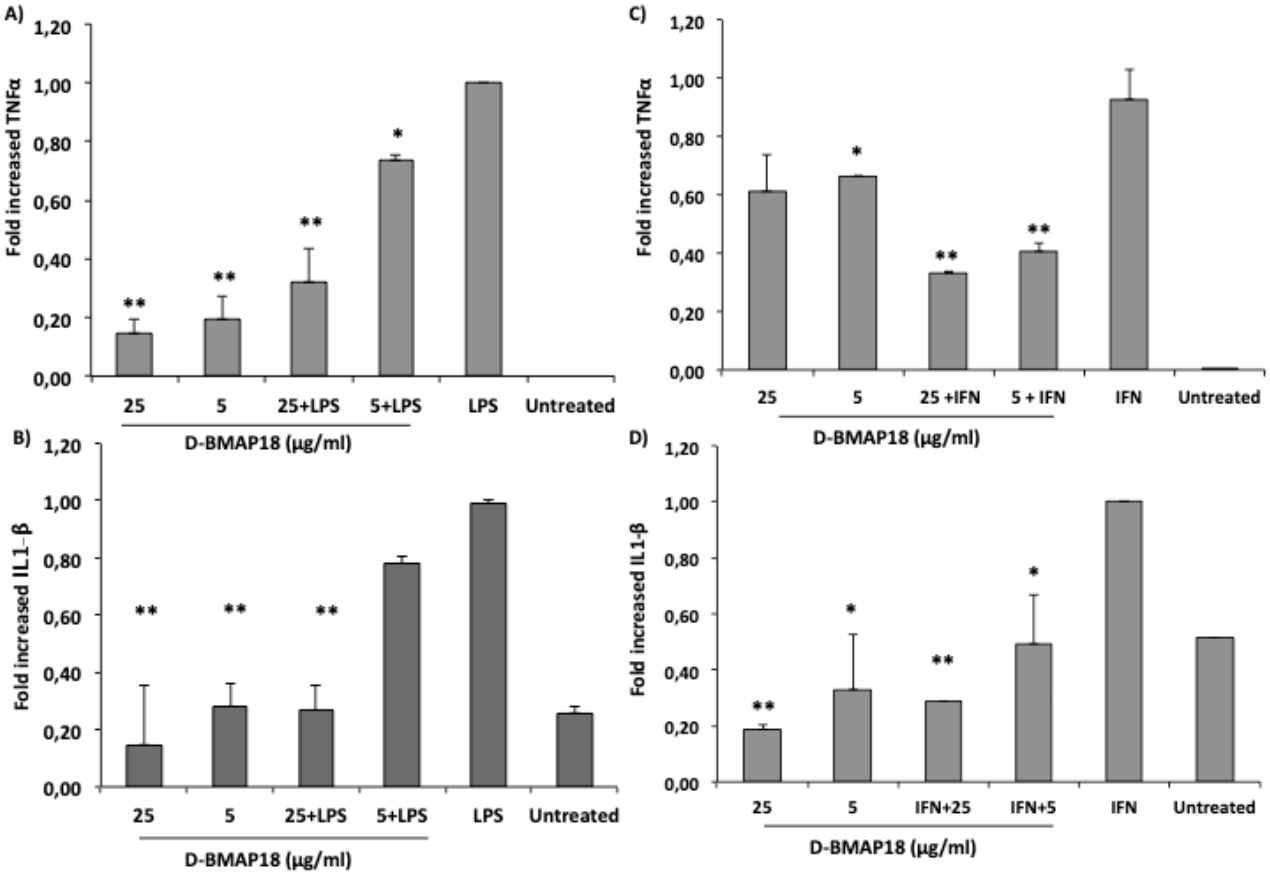


Fig. 4.8: *D*-BMAP18 anti-inflammatory activity on the macrophage differentiated THP-1 cells. RT-qPCR analysis of the pro-inflammatory cytokines A) TNF-α and B) IL1-β after a 24h-treatment with LPS and C) TNF-α and D) IL1-β after a 24h-treatment with IFN- γ and/or D-BMP18 at 5 and 25 µg/ml. The expression in presence of LPS and D-BMAP18 was evaluated as fold increased compared to the LPS alone. The results are the average of two independent experiments in internal duplicates (n=4). *=p< 0.05, **p<0.01. test t-student

The anti-inflammatory activity was evaluated measuring the expression level of the cytokines TNF-α and IL1-β by the comparison of the cells treated with LPS or IFN-γ + D-BMAP18 and the cells treated with LPS or IFN-γ alone (100% expression). Preliminary results by RT-qPCR analysis indicated that LPS-stimulated cells and incubated with *D*-BMAP18 down-regulated both TNF-α and IL1- β expression in a concentration dependent manner (**Fig. 4.8)**. The peptide itself determined a weak increase in the expression of the two cytokines but overall it reduced the intense signal obtained by using LPS of the IFN-γ as stimulus.

4.2.4 *In vitro* cytotoxicity of *D*-BMAP18

Potential toxic effects of *D*-BMAP18 against eukaryotic cells were evaluated on different human cells including the epithelial pulmonary A-549 cells, the HaCaT keratinocytes, the MEC-1 leukemia blood cells and the monocytes THP-1 differentiated in macrophages. The peptide exerted toxic effects on three out four different cell lines at 50 μg/ml concentrations. At lower concentrations, effects of D-BMAP18 were dependent on the cell type. The HaCat cells were the most susceptible ones and were slightly affected already at 1-5 μg/ml. Viability of MEC-1 and A-549 cells started to decrease at around 25-50 μg/ml, while the THP-1 cells showed to be more resistant and the treatment with 50 μg/ml did not reduced statistically cells viability (**Fig. 4.9**).

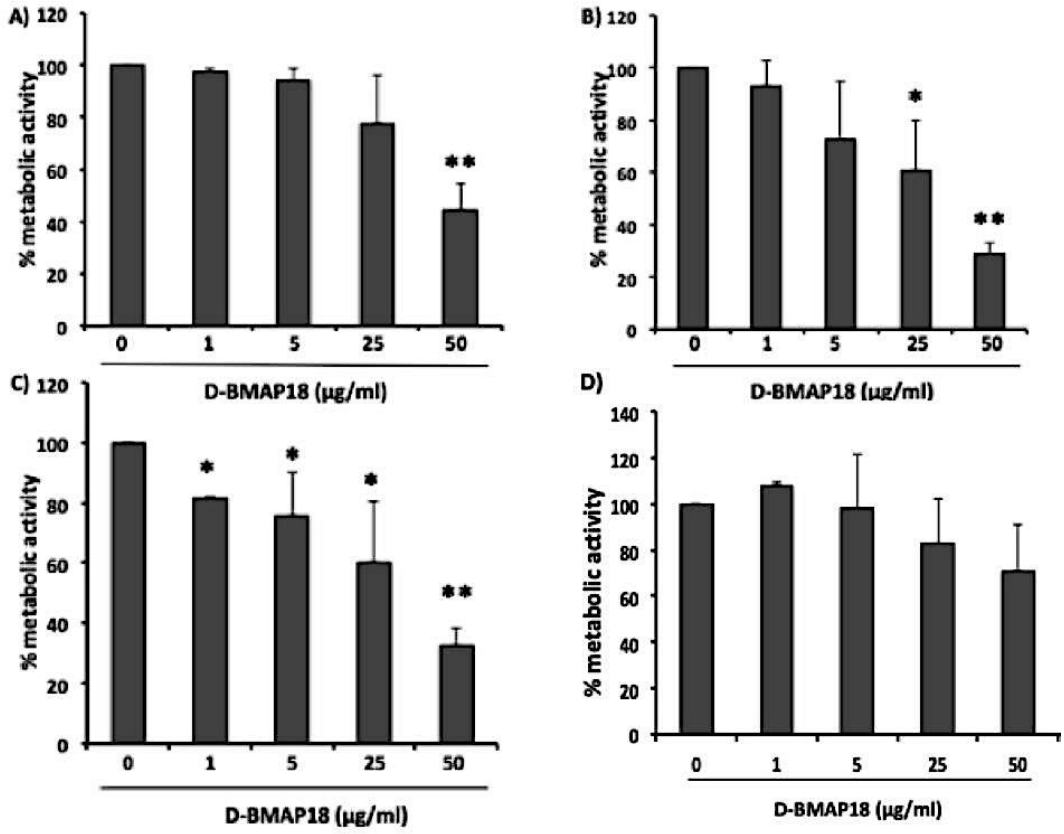


Fig. 4.9: *D*-BMAP18 effects on cell viability of four different human cell lines. MTT assay of A)

pulmonary A-549 cell line B) blood MEC-1 cells C) keratinocytes HaCaT cells D) the macrophages differentiated from THP-1 monocytes. Percentages of viable cells after 24h of treatment with different concentrations of *D*-BMAP18 were shown. The results are the average of three independent experiments in internal triplicate (n=9). *=p< 0.05, **p<0.01. test t-student

Since we previously observed that biological fluids such as BAL or CF-sputum significantly reduced the antimicrobial activity of *D*-BMAP18, we also investigated this effect on the cells. Cytotoxicity tests were repeated on the HaCat cells in presence of 25% CF-sputum, to understand whether this medium influenced the effects of *D*-BMAP18 on these cells. Interestingly, the toxic activity of the peptide was significantly decreased in diluted CF- sputum if compared to that in cell media alone. Under these conditions, 50 μg/ml *D*-BMAP18 caused a not significant reduction of cell viability while in the absence of the biological fluid it determined 40% decrease of viability (**Fig. 4.10**). Unexpectedly, when tested at low concentrations in CF-sputum, *D*-BMAP18 weakly affected cells' viability. These preliminary results must be further analyzed to understand these data. Overall, this result suggests that in CF- environment the toxic concentration of the peptide would be strongly higher than that estimated in cell medium.

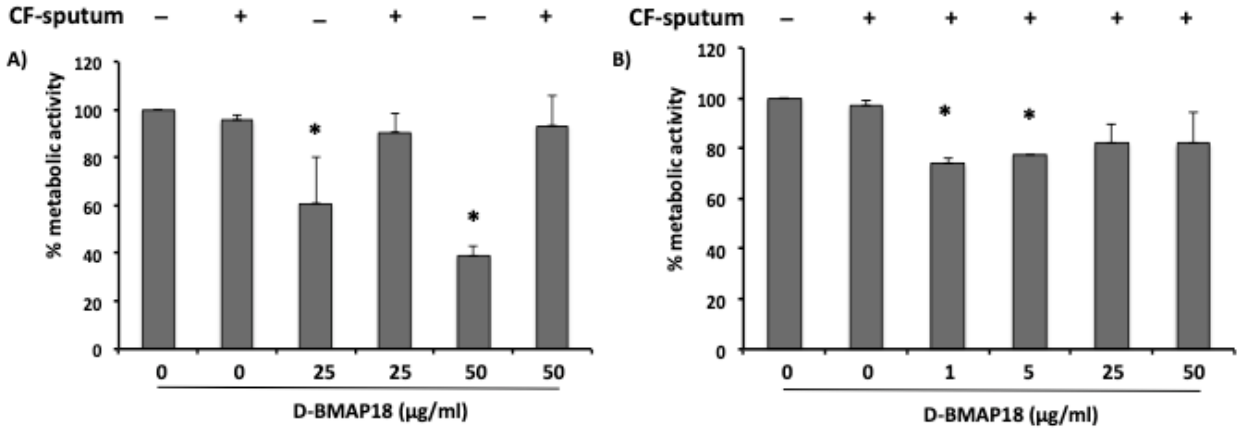


Fig. 4.10: *D*-BMAP18 cytotoxicity in 25% CF-sputum. MTT assay in A) cell medium in presence or absence of 25% CF- sputum and in B) 25%CF-sputum. Percentage of viable cell comparing to the untreated control of growth after 24h of treatment with different concentrations of *D*-BMAP18. The results are the average of three independent experiments in internal triplicate (n=9). *=p< 0.05. test t-student

4.2.5 *In vivo* toxicity and activity of *D*-BMAP18

The research group of Prof. Di Bonaventura at the University of Chieti assessed the *in vivo* assays on mice. *In vivo* toxicity and activity of the *D*-peptide were assessed in parallel to tobramycin a drug of election in the treatment for CF pulmonary infection. Acute toxicity was quantitatively evaluated using an index indicating the levels of macroscopic pulmonary damages. The results

showed the *D*-BMAP18 at a dose of 0,5-1 mg/kg was not toxic but that exposure to 2- 4 mg/Kg had non-negligible toxicity when intratrachally administered in a group of mice (**Fig. 4.11 A)**. The *in vivo* antimicrobial potential of *D*-BMAP18 on an acute pulmonary infection by *P. aeruginosa* RP73 was also assessed. A positive trend between the increasing concentration of *D*-BMAP18 and the reduction of the bacterial load could be seen (excluding the dose corresponding to 4 mg/Kg which damages the lung and creates a suitable environment for bacterial growth) but unfortunately this result was not statistically significant due to the relevant data dispersion (**Fig. 4.11 B)**.

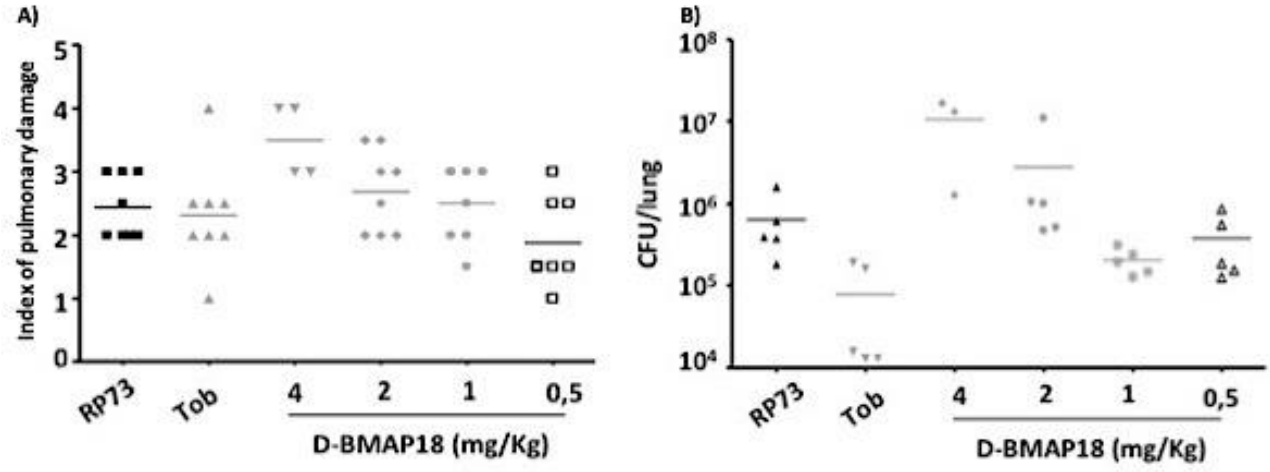


Fig. 4.11: *In vivo* toxicity and activity of *D*-BMAP18 in mice. A) Macroscopic damage of C57BL/6NCrl mouse lungs after a single intratracheal administration of the compound. Inoculum of *P. aeruginosa* RP73 was used as a control. The macroscopic damage was evaluated using the "four-point scoring system":1, normal; 2, swollen lungs, hyperemia, and small atelectasis; 3, pleural adhesion, atelectasis, and multiple small abscesses; and 4, large abscesses, large atelectasis, and hemorrhages. B) CFU count in lung after the treatment with *D*-BMAP-18 at different concentrations and tobramycin (10 mg/kg). An inoculum of only RP 73 was used as a control of growth while tobramycin was used as a reference control of activity.

In order to reduce *D*-BMAP18's toxicity and with a view of a future *in vivo* therapy, we formulated a strategy consisting in the construction of an activable proform of *D*-BMAP18 (Pro-*D*-BMAP18), a prodrug that should be inactivate and activable only at the site of interest. Since the site on infection/inflammation is rich in elastase released by neutrophils, we designed this pro-drug within the site of cleavage by elastase. This construct was expected to be processed only at site of infection/inflammation due to the presence of neutrophils and releasing slowly the active *D*-BMAP18 form to reduce its side effects.

4.3 Design and synthesis of Pro-*D*-BMAP18

Pro-*D*-BMAP18 was designed by adding to the N-terminal region of *D*-BMAP18 a negatively charged extrasequence using L-amino acids and containing the canonical cleavage site of elastase. It was expected that the anionic extra-region interacts with the positive charges of *D*-BMAP18 making it inactive and that *D*-peptide activation through the release of the pro-sequence is guaranteed by the presence of elastase at the site of infection.

The structure of the pro-peptide and a cartoon of the hypothetical activation mechanism are shown in **Fig. 4.12**.

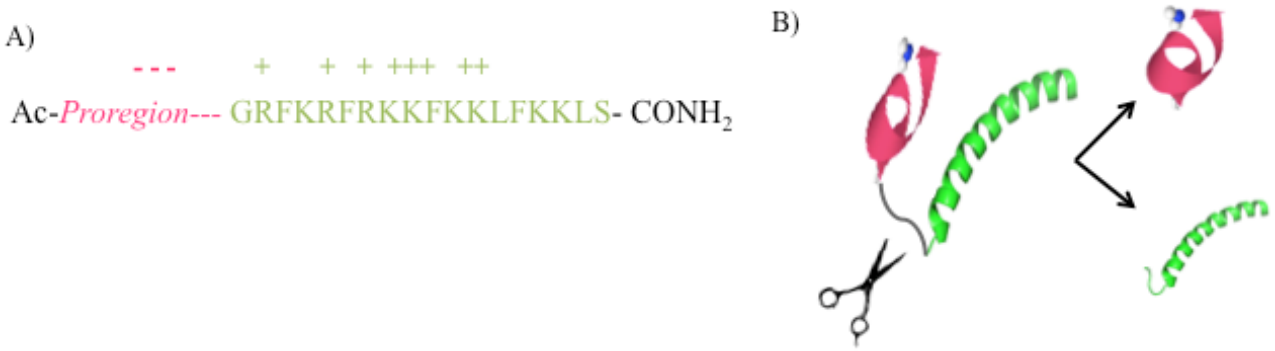
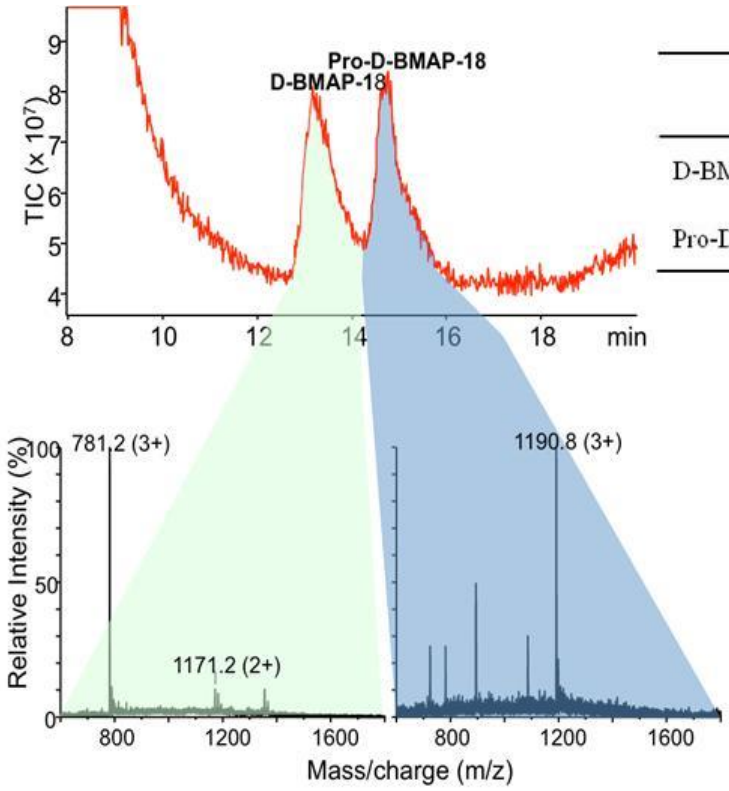


Fig. 4.12 Pro-*D*-BMAP18 structure and its expected processing A) sequence of Pro-*D*-BMAP18. The pink part represents the negatively charged extrasequence formed by L-amino acids, sequence not shown (pending patent). The green part represents the *D*-BMAP18. The site of cleavage of elastase is indicated by the lines B) a cartoon mimicking the structure and the activation of the pro-peptide.

The pro-peptide was synthesised on solid-phase After the detachment from the resin the peptide was purified by RP-HPLC and its identity verified by MS analysis showing the presence of a more abundant species with a MW= 3571 Da corresponding to that of Pro-*D*-BMAP18.

4.3.1 Conversion of Pro-D-BMAP18 into D-BMAP18

The *in vitro* cleavage of Pro-*D*-BMAP18 into *D*-BMAP18 in presence of human neutrophil elastase was confirmed by HPLC-MS analysis. Specific processing of the peptide occurred in presence of elastase. Pro-*D*-BMAP18 was 50% converted into *D*-BMAP18 in 1h and after 4h the reaction was completed (**Fig. 4.13**). No undesired products were observed.



	Expected Mw (Da)	Observed Mw (Da)
D-BMAP18	2342	2341,9
Pro-D-BMAP18	3571	2571,1

Fig. 4.13: HPLC-MS spectra of Pro-*D*-BMAP18 after 1h-incubation with elastase (molar ratio1:100). Table reports the expected and observed MW of *D*-BMAP18 and Pro-*D*-BMAP18 obtained by MS analysis.

4.3.2 Antimicrobial activity of Pro-*D*-BMAP18

The antimicrobial activity of the pro-peptide was tested against the selected panel of *P. aeruginosa* strains and *C. albicans* strains used in the characterization of *D*-BMAP18. MIC assay confirmed that Pro-*D*-BMAP18 lose activity (**Tab. 4.2**). Its activity was then assessed on *P. aeruginosa* cells after proteolytic activation by neutrophil elastase (1:100 molar ratio). The cleavage by elastase incompletely restored the antibacterial activity of the released peptide. The complete restoration of activity required the addition of 0,22% NaCl to the medium, likely promoting the release of the anionic prosequence from the cationic active part (**Tab. 4.2**). The release of *D*-BMAP18 was also evaluated by incubating the Pro-*D*-BAMP18 with exhausted medium of degranulated neutrophil-like cells differentiated from HL-60 cells. Even under this condition the pro-peptide recovered its antimicrobial activity.

Table 4.2: MIC values (μg/ml) of *D*-BMAP18 and Pro-*D*-BMAP18 incubated with elastase or elastase-containing conditioned medium.

	P. aeruginosa		***C. albicans***	
compound	PA01	RP73	ATCC 90029	SC 5314
D-BMAP18	16	8	2	16
Pro-D-BMAP18	128	128	16	>128
Pro-D-BMAP18+ elastase	32	32	4	32
Elastase	>128	>128	>128	>128
Pro-D-BMAP18+ elastase+ 0,22% NaCl	16	8	2	16
Pro-D-BMAP18+ 4h in cell supernatant	64	64	nd	nd
Pro-D-BMAP18+ 18h in cell supernatant	32	32	nd	nd
Pro-D-BMAP18+ 24h in cell supernatant	32	32	nd	nd

*The results are the average of three indipendent experiments in internal duplicate

The killing activity of Pro-*D*-BMAP18 was then tested in CF-sputum. Since this medium should contain elastase, we tested whether the elastinolytic activity present in sputum of CF patients was sufficient to process the Pro-*D*-BMAP18 and to unmask its antibacterial activity. Viable cells count of *P. aeruginosa* strains RP73 and *C. albicans* strain SC5314 were performed incubating the pathogens with Pro-*D*-BMAP18 in same experimental conditions described above for testing *D*-BMAP18. The results demonstrated that Pro-*D*-BMAP18 acquired activity in CF-sputum. After 4-h incubation the measured activity of Pro-*D*-BMAP18 was comparable to that of the *D*-BMAP18, with 3-log reduction of viable *P. aeruginosa* RP73 cells and about 1-log reduction of *C. albicans* cells (**Fig. 4.14**). Moreover, when the pro-drug was incubated with CF-sputum sample that had been heated to inactivate any enzymatic activity, it was not active against pathogens, confirming the role of the elastase or of other proteolytic enzymes present in CF sputum in its processing. This result suggested that physio(patho)logical samples derived from CF patients contain sufficient amount of active proteases (elastase) to convert the Pro-*D*-BMAP18 into a compound (*D*-BMAP18) that exert antimicrobial activity in that medium.

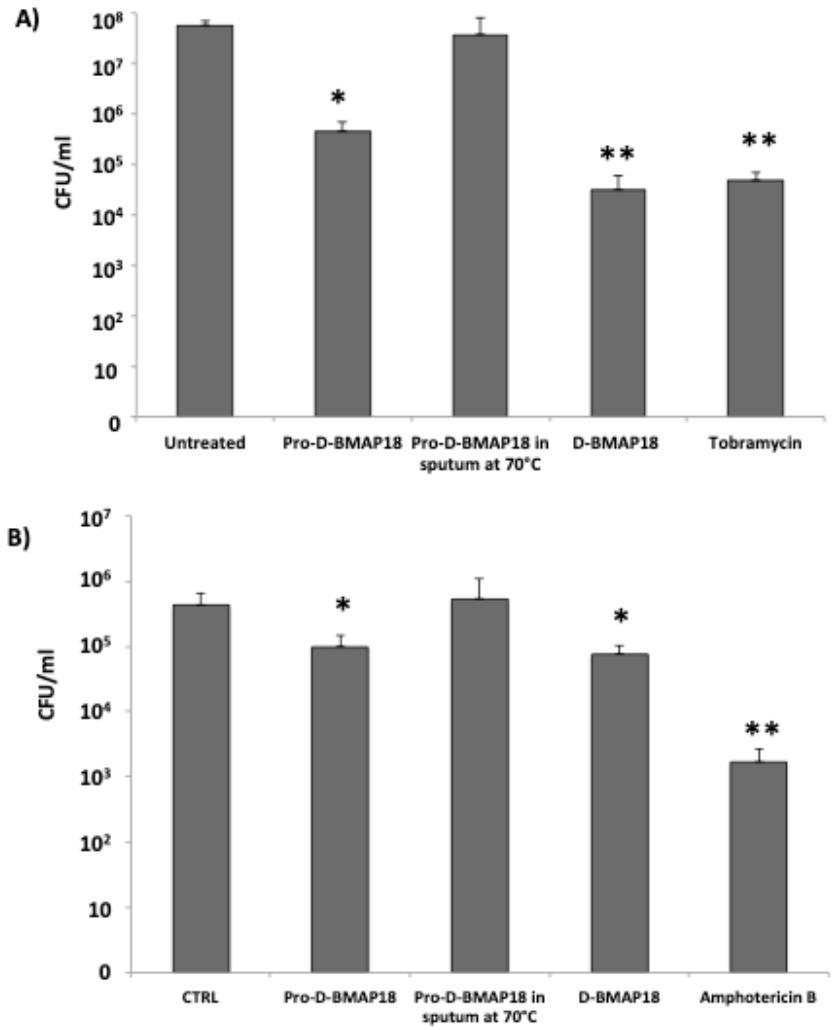


Fig. 4.14: Antimicrobial activity in 25% CF- sputum added in 300 mM NaCl. CFU/ml of A) *P. aeruginosa* RP73 and B) *C. albicans* SC5314 count after 4h-incubation with Pro-*D*-BMAP18 (64 μg/ml), *D*-BMAP18 (64 μg/ml), tobramycin (8 μg/ml) and amphotericin B (1 μg/ml) in 25% CF sputum. The Pro-*D*-BMAP18 (64 μg/ml) in the previously denaturized sputum was considered as a control. The results were obtained by three different experiments in internal duplicate (n=6).*=p< 0,05 **=p< 0,01

4.3.3 Antibiofilm activity of Pro-D-BMAP18 in presence of elastase

The assays reported above for *D*-BMAP18 were reproduced with the pro-peptide incubated with human neutrophil elastase in order to understand if the cleavage by elastase enable the fully restoration of the antibiofilm activity. The pro-peptide incubated in presence of human neutrophil elastase inhibited formation of new biofilm with activity similar to that observed with the original form. There was a significant difference in the potency of the Pro-*D*-BMAP18 and of the released

D-BMAP18 in presence of elastase (**Fig. 4.15** A). The same results were obtained testing the activity of Pro-*D*-BMAP18 against preformed biofilm of *P. aeruginosa* strains (**Fig. 4.15 B**).

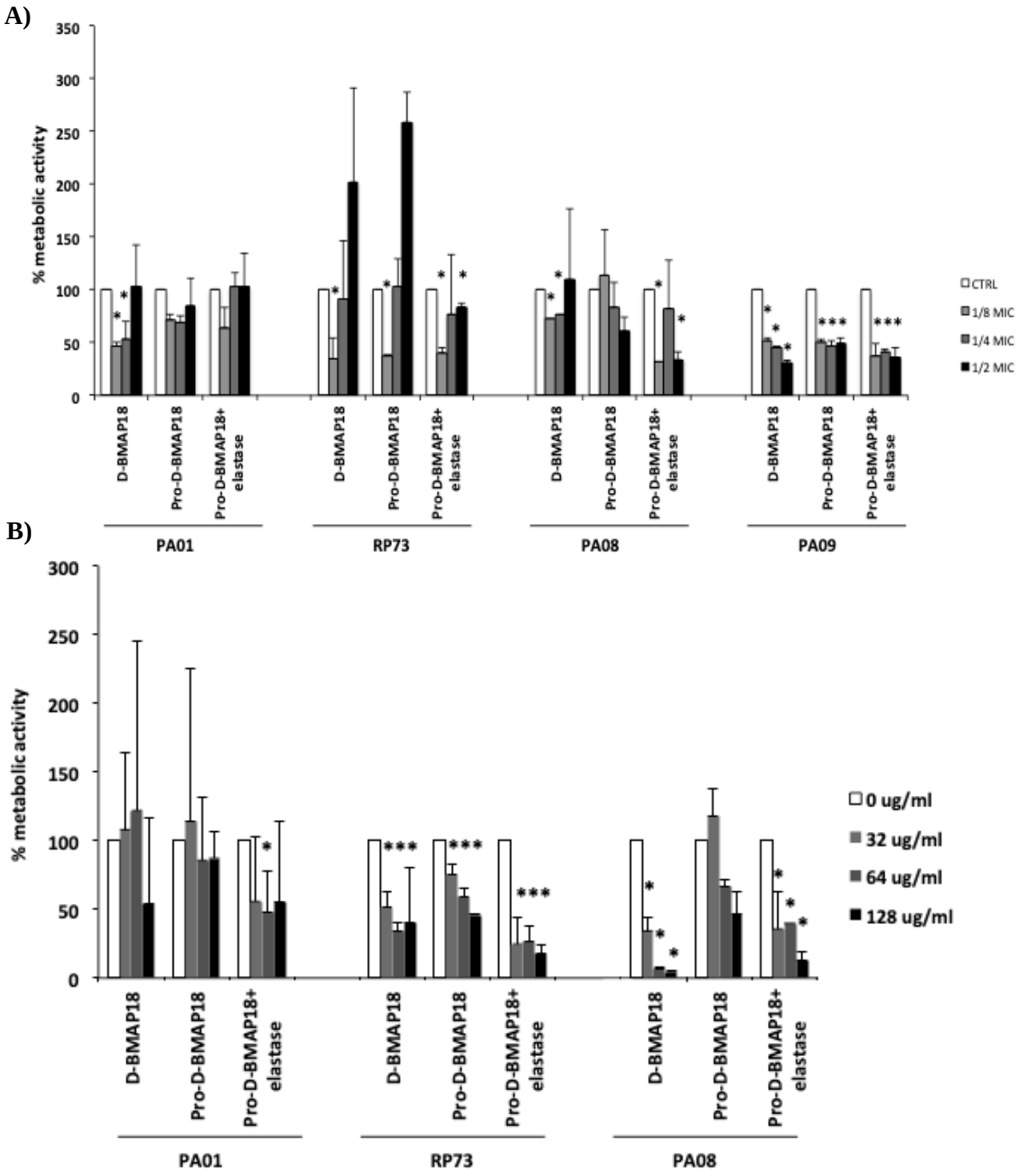


Fig. 4.15: Antibiofilm activity of Pro-*D*-BMAP18 incubated with elastase. Antibiofilm formation of *P. aeruginosa* strains evaluated by MTT assay A) inhibition of biofilm formation B) activity against preformed biofilm. Percentages of metabolic activity of the biofilm after 24h of treatment at sub-inhibitory concentrations of Pro-, *D*- and Pro-*D*-BMAP18 incubated with elastase. The results are the average of three independent experiments in internal triplicate (n=9). *=p< 0,01

4.3.4 *In vitro* cytotoxicity of Pro-*D*-BMAP18 in presence of elastase

In parallel to the assay on the pathogens, cytotoxicity assays using the *D*-BMAP18 prodrug were also performed. We were waiting a reduction of its cytotoxic activity and we tested it against the A-549 pulmonary cell line and against the blood MEC-1 cells. We tested the cytotoxicity of Pro-*D*-BMAP18 in presence of human neutrophil elastase after 24h-treatment versus the cytotoxicity of *D*-BMAP18. All the tested peptides showed scarce toxicity up to 25 μg/ml as expected. The Pro-*D*-BMAP18+elastase clearly showed a reduced cytotoxicity in comparison to the *D*-BMAP18 at higher concentrations, suggesting that the gradual activation of the pro-form resulted in a reduction of the toxicity (**Fig. 4.16**).

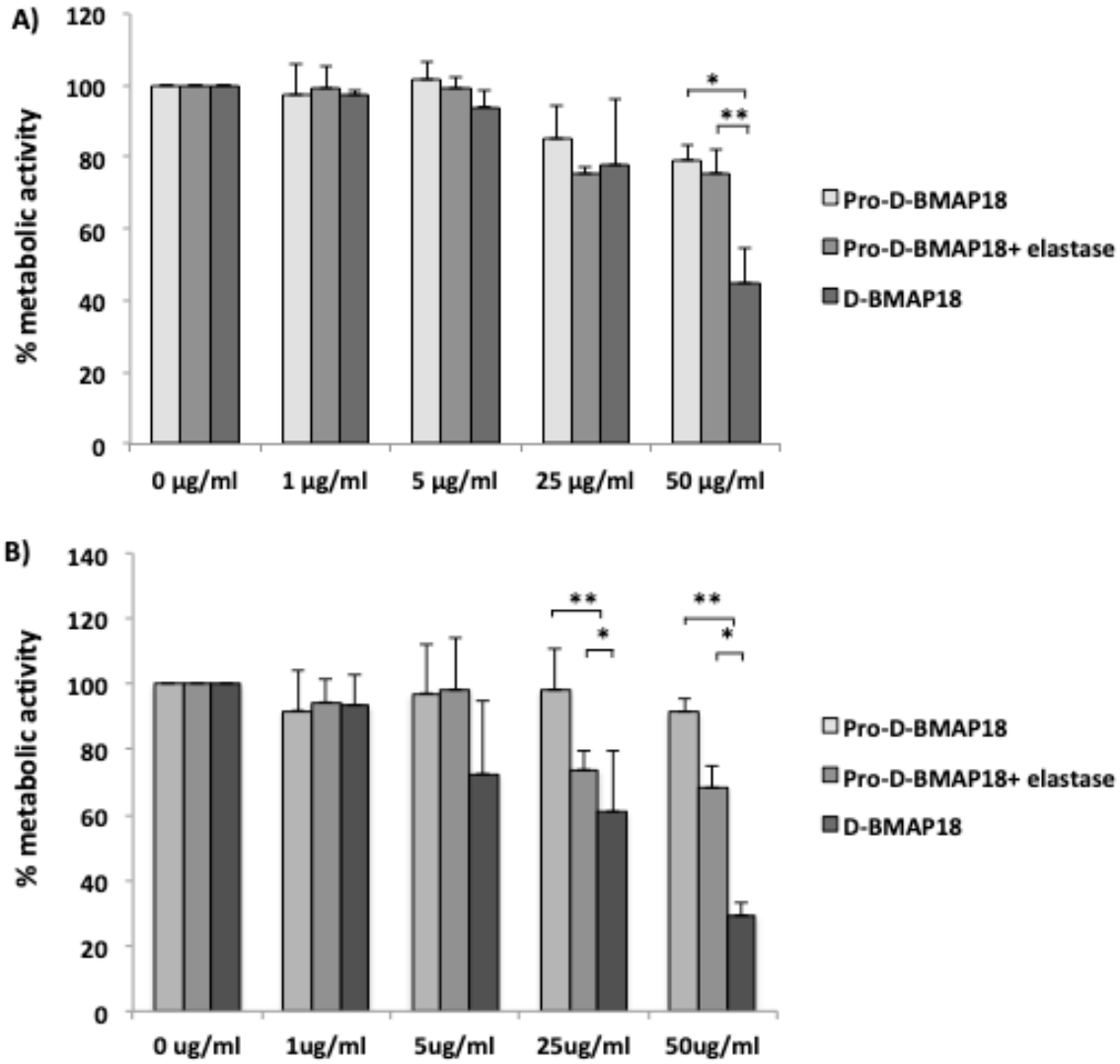


Fig. 4.16. Cytotoxicity of Pro-*D*-BMAP18 in presence of elastase vs *D*-BMAP18. MTT assay against A) the epithelial pulmonary cell line A-549 and B) the MEC-1 cell line. Percentage of metabolic active cells compared to the untreated control of growth after 24h of treatment at different concentration of Pro-, *D*- and Pro-*D*-BMAP18 incubated with elastase. The results were obtained by three different experiments in internal triplicate (n=9). *=p<0,05 **=p< 0,01

5 DISCUSSION & CONCLUSIONS

Patients with lung infections require pharmacological treatment against several pathogens. Unfortunately, the rapid emergence of resistant bacteria is occurring worldwide, endangering the efficacy of most antibiotics. For this reason new and more effective antimicrobial agents are urgently needed. In this study we focused our attention on the cathelicidin-derived antimicrobial peptide BMAP18 and investigated its potential as novel lead anti-infective agent. We demonstrated that the all-*D* peptide BMAP18 has potent *in vitro* antimicrobial activity against several clinical isolates of *P. aeruginosa* and *S. maltophilia* and that its activity is comparable to its L-form. Moreover, we proved that the peptide inhibits the formation of new biofilm and eradicates pre-formed biofilm for half of the tested *P. aeruginosa* isolates. This result is particularly interesting because the presence of *P. aeruginosa* biofilm is one of the major causes of persistence of lung infections and of pulmonary damages (Guttenplan and Kearns 2013; Hogardt and Heesemann 2013) and indicates that the use of the all-*D*-enantiomer of BMAP18 has some advantages for the treatment of persistent infections. *D*-BMAP18, in fact, other than to be resistant to proteolytic degradation, in some cases, revealed to be more active than the L-form. Its higher activity may be also linked to its higher stability. This hypothesis is supported by the fact that bacterial CF isolated strains produce a higher amount of proteases (Jagger, Bahner, and Warren 1983). Interestingly, we observed that antibiofilm activity of *D*-BMAP18 is enhanced by the presence of DNase I and alginase. The eDNA and alginate are the major components of *P. aeruginosa* biofilm. They probably mask the peptide and prevent its internalization into the biofilm. The addition of DNase I and alginase possibly degrades these two components and enhances *D*-BMAP18 activity. This result opens the possibility to use this compound in combination with DNase I, which has been already approved for the pharmacological therapy (with the name of dornase alfa) for reducing mucus viscosity (Pressler 2008).

The activity of *D*-BMAP18 against the fungal pathogen *C. albicans* ATCC 90092 and SC 5314, both in planktonic and sessile form, is also an interesting aspect. In fact, isolation of fungal pathogens such as *Candida* spp. and *Aspergillus* spp. (Schwarz et al. 2018; Williams, Ranjendran, and Ramage 2016) from CF patients is becoming more frequent, even if their role in lung disease is variable and not fully understood (Schwarz et al. 2018; Williams, Ranjendran, and Ramage 2016). *D*-BMAP18 demonstrates to have an anti-candidal activity against sessile and planktonic form of fungi comparable to that observed against bacterial pathogens. Overall these results, if confirmed on a larger panel of fungal pathogens, suggest that *D*-BMAP18 may be use both as an antibacterial and an antifungal compound.

D-BMAP18 lacks most of its antipseudomonal activity when assayed in BAL of healthy mice or in CF- sputum, both mimicking the pulmonary environment. Moreover, also the antifungal activity of *D*-BMAP18 is reduced in CF-sputum. These observations are not surprising, because of the complexity and heterogeneous composition of these biological fluids. We hypothesized that the inhibition of the activity in both cases might be due to an unspecific binding by one or more components of the media, which sequester the peptide preventing its action. Since the antibacterial activity is partially restored by the addition of 300 mM sodium chloride it may be that electrostatic interactions play an important role in peptide inhibition. It has been reported that some proteins and lipoproteins contained in BAL could inhibit the activity of cationic molecules (Rosenecker et al. 2002). It is worth noting that reduced antimicrobial activity of *D*-BMAP18 in CF- sputum has been partially restored by the addition of DNase I or/and NaCl. As reported above, increasing salts concentration probably reduces the electrostatic interactions between the peptide and sputum components. In addition, DNase I is expected to degrade the negatively charged eDNA released in lung (Ratjen et al. 2005) that could also have a role in inhibiting peptide activity. These results highlighted the possibility to use *D*-BMAP18 in combination with hypertonic solutions and/or dornase alfa (DNase I) that have already approved for the mucolytic therapy of CF patients (Reeves et al. 2012).

Notably, the peptide is highly active also in presence of 450 mM NaCl which are salt concentrations that usually inhibit most AMPs and not only salt sensitive peptides such as β- defensin 1(Aoki and Ueda 2013) or LL-37 (Turner et al. 1998). This low sensitivity to high salt concentrations enlarges the number of different media and environments in which BMAP18 could be used.

Preliminary data indicated that *D*-BMAP18 has anti-inflammatory activity on LPS- or IFN-γ-stimulated macrophages (THP-1 derived), leading to a significant reduction of the expression of pro-inflammatory cytokines TNF-α and IL1-β. This observation on macrophages had already been reported for other AMPs, such as the chensinin-1 and temporin derived-peptides, that reduce the expression of pro-inflammatory cytokines such as TNF-α on macrophages isolated from blood and stimulated using LPS (Dong et al. 2017; Popovic et al. 2012) and its corroboration on other cytokines could be promising because the state of hyper-inflammation is one of the major causes of mortality in CF patients (Bruscia and Bonfield 2016) leading to permanent structural damage of the CF airways and impairing lung function (Cantin et al. 2015). Moreover, the *D*-BMAP18 showed to downregulate the expression of TGF- β in macrophage differentiated THP-1 cells, which plays an important role in inducing pulmonary fibrosis linked to CF (Arribillaga et al. 2011). Nowadays, most CF therapies focus on treating secondary pulmonary complications and the importance of targeting TGF-β as a cytokine involved in fibrosis, inflammation, and injury response has already

been reported in literature (Kramer and Clancy 2018). For example, TGF-β activation at the site of injury has been inhibited by a monoclonal antibody preventing the development of pulmonary fibrosis (Fernandez and Eickelberg 2012). The confirmation of this preliminary result of D-BMAP18 on TGF- β expression may suggest a possible use of this peptide in contrasting this complication in CF patients.

Since previous studies indicated that *D*-BMAP18 was a quite cytotoxic compound when used at concentrations a little above the bactericidal ones (Mardirossian et al. 2017), here we further tested the peptides against four different human cell lines including immune and epithelial cells. Results indicated that *D*-BMAP18 started to affect cell viability at 25-50 μg/ml and that this value depended upon the cell line used. The THP-1 cells were the most resistant to the treatment and the higher tested concentration did not affect cells viability while the HaCat cells were the most susceptible ones and were slightly affected already at the lower concentrations used. The cytotoxicity for the HaCat cells is comparable with that observed for other antimicrobial peptides such as temporin A and protegrin tested under similar experimental conditions (Barańska-Rybak et al. 2013). The different susceptibility among different cell lines could be linked to differences in membrane composition, an hypothesis also reported for the lytic antimicrobial peptide Bovicin HC5 (Paiva et al. 2012). Importantly, we demonstrated that the addition of CF-sputum to the cells protect them from the eventual damaging effects of *D*-BMAP18. This result indicated that in the presence of biological fluids not only the antibacterial activity but also the cytotoxic effects of the peptide are inhibited, and suggests that 50 μg/ml *D*-BMAP18 could be able to kill *P. aeruginosa* cells in CF-medium without detrimental effects on host cells. This aspect need to be further investigated; the confirmation of the protective effect of CF-sputum on the host cells from the *D*-BMAP18 activity would result in a widen *in vivo* therapeutic window compared to that already hypothesized..

It is also true that *D*-BMAP18 has a non-negligible toxicity when intratracheally tested *in vivo* in murine lung. We hypothesize this effect could at least partially be linked to the way of administration in the mice. In fact the intratracheal instillation implies the release of a highly concentrated peptide (due to the small volume usable) in a small area of lungs (Bisav-Benita, Ziwier, Junginger, & Borachard, 2005). Despite this drawback, a positive trend between increasing concentrations of *D*-BMAP18 and reduction of the bacterial load has been observed treating mice with acute pulmonary infection by *P. aeruginosa*. D-BMAP18 appeared to reduce bacterial load at 1 mg/Kg, which is a non-toxic concentration for murine lungs. This is a preliminary encouraging result for further experiments in the next future using the pro-drug form of *D*-BMAP18.

The Pro-*D*-BMAP18, the inactive form of *D*-BMAP18 has been obtained by the addition of an inactivating extrasequence containing the cleavage site for elastase. This construct has been inspired

by a previous study on a different molecule (Forde et al. 2014) knowing that neutrophils represent the major cell population among inflammatory cells recruited in the airways of CF patients and they are involved in the release of this enzyme (Laval, Ralhan, and Hartl 2016). The presence of this specific site of cleavage should enable the gradual release of the active form only at the lung site of infection/inflammation in which neutrophils elastase is present.

We showed that the pro-peptide is correctly converted into *D*-BMAP18 by elastase. After the conversion, the product fully recovers its antimicrobial activity in presence of 0,22% NaCl both against planktonic and sessile form of bacteria. The electrostatic interaction between the anionic prosequence and the cationic active peptide likely maintains the two parts linked each other also after cleavage. Salts addition may completely release the pro-sequence from the active *D*-BMAP18 unmasking its fully activity.

We showed that Pro-*D*-BMAP18 is processed in CF-sputum pooled from different patients, resulting in a compound able to kill fungi and bacteria. This demonstrates that the amount of neutrophil elastases, or other proteolytic enzymes present in this CF-pool are sufficient to convert the Pro-*D*-BMAP18 into *D*-BMAP18 in few hours. In addition, this result suggests that the release of *D*-BMAP18 from the pro-drug could correctly occur also in the lungs of patients thanks to the endogenous enzymes that are always present in the inflamed lungs (Döring 1994). In parallel we also proved that the slow elastase-dependent release of *D*-BMAP18 from Pro-*D*-BMAP18 decreased any detrimental effects on host cells. All these observations suggest that Pro-*D*-BMAP18 could solve some side effect of *D*-BMAP18, having a lower *in vitro* cytotoxicity but maintaining the antimicrobial properties of the parental form.

Future *in vivo* assays will be necessary to establish the effectiveness of this antimicrobial compound and to confirm the activation process observed in the *in vitro* studies. As a first *in vivo* model we tested the toxicity and the activity of the Pro-*D*-BMAP18 in the larvae of the mouth *Galleria mellonella*. Under this condition the pro-drug as well as the *D*-BMAP18 were not toxic and also ineffective in protecting *G. mellonella* from *P. aeruginosa* infections. The same results have been obtained also testing other differently structured peptides such as proline-rich Bac7 and colistin, suggesting that *G. mellonella* could not be an optimal model for testing the antimicrobial activity of peptides may be because of unspecific binding and sequestration of the peptide drugs by heamolymph components. Indeed in literature is reported the preferential use of *G. mellonella* as a model of infection but not as a model for studying the protective activity against infection of peptide drugs (Desbois and Coote 2012; Tsai, Loh, and Proft 2016).

In conclusion this project let to obtain new insights on the antibacterial and anti-inflammatory properties of the all-D host defense peptide *D*-BMAP18 that overall suggests that it is a lead

compound for the development of a new antibacterial drug in the treatment of CF infection/inflammation. Moreover, we showed that the use of its inactive pro-drug, Pro-*D*-BMAP18 may represent a further advantage due to its in localized situ activation that may abolish any residual cytoxicity.

Further *in vivo* studies on protective activity and toxicity on different animal models and pharmacokinetics studies on administration/distribution parameters will necessary to enable us to understand the real potential of Pro-*D*-BMAP18 in the treatment of bacterial lung infections.

7 REFERENCES

Aitken, Carl, and David John Jeffries. 2001. “Nosocomial Spread of Viral Disease.” *Clin Microbiol Rev* 14(3): 528–46.

Andersson, D.I., D. Hughes, and J.Z. Kubicek-Sutherland. 2016. “Mechanisms and Consequences of Bacterial Resistance to Antimicrobial Peptides.” *Drug Resistance Updates* 26: 43–57.

Aoki, Wataru, and Mitsuyoshi Ueda. 2013. “Characterization of Antimicrobial Peptides toward the Development of Novel Antibiotics.” *Pharmaceuticals (Basel, Switzerland)* 6(8): 1055–81. http://www.ncbi.nlm.nih.gov/pubmed/24276381.

Arciola, Carla Renata, Davide Campoccia, Stefano Ravaioli, and Lucio Montanaro. 2015. “Polysaccharide Intercellular Adhesin in Biofilm: Structural and Regulatory Aspects.” *Frontiers in Cellular and Infection Microbiology* 5(February): 1–10. http://journal.frontiersin.org/Article/10.3389/fcimb.2015.00007/abstract.

Arribillaga, Laura et al. 2011. “Therapeutic Effect of a Peptide Inhibitor of TGF-β on Pulmonary Fibrosis.” *Cytokine* 53(3): 327–33. http://www.ncbi.nlm.nih.gov/pubmed/21185199.

Bacalum, M, and M Radu. 2015. “Cationic Antimicrobial Peptides Cytotoxicity on Mammalian Cells: An Analysis Using Therapeutic Index Integrative Concept.” *Internationla Journal of Peptide Research and Therapeutics* 21(1): 47–55.

Bahar, AA, and D Ren. 2013. “Antimicrobial Peptides.” *Pharmaceuticals* 6(12): 1543–75.

Bals, R, X Wang, M Zasloff, and J M Wilson. 1998. “The Peptide Antibiotic LL-37/HCAP-18 Is Expressed in Epithelia of the Human Lung Where It Has Broad Antimicrobial Activity at the Airway Surface.” *Proceedings of the National Academy of Sciences of the United States of America* 95(16): 9541–46. http://www.ncbi.nlm.nih.gov/pubmed/9689116.

Bals, R, and JM Wilson. 2003. “Cathelicidins: A Family of Multifunctional Antimicrobial Peptides.” *Cellular and Molecular Life Science* 60: 711–20.

Band, VI, and DS Weiss. 2015. “Mechanisms of Antimicrobial Peptide Resistance in Gram-Negative Bacteria.” *Antibiotics* 4(1): 18–41.

Barańska-Rybak, Wioletta et al. 2013. “Safety Profile of Antimicrobial Peptides: Camel, Citropin, Protegrin, Temporin a and Lipopeptide on HaCaT Keratinocytes.” *Acta poloniae pharmaceutica* 70(5): 795–801. http://www.ncbi.nlm.nih.gov/pubmed/24147357.

Baron, S. 1996. *Medical Microbiology, 4th Edition*.

Benincasa, Monica et al. 2003. “In Vitro and in Vivo Antimicrobial Activity of Two α-Helical Cathelicidin Peptides and of Their Synthetic Analogs.” *Peptides* 24(11): 1723–31.

Bhagirath, AY. 2016. “Cystic Fibrosis Lung Environment and Pseudomonas Aeruginosa Infection.” *BMC Pulmonary Medicine* 16: 174.

Bisav-Benita, M, R Ziwier, H E Junginger, and G. Borachard. 2005. “Non-Invasive Pulmonary Aerosol Delivery in Mice by the Endotracheal Route.” *European Journal of Pharmaceutics and Biopharmaceutics* 61(3): 214–18.

Bjarnsholt, Thomas et al. 2009. “Pseudomonas Aeruginosa Biofilms in the Respiratory Tract of Cystic Fibrosis Patients.” *Pediatric pulmonology* 44(6): 547–58. http://www.ncbi.nlm.nih.gov/pubmed/19418571.

Boon, Eva et al. 2014. “Interactions in the Microbiome: Communities of Organisms and Communities of Genes.” *FEMS microbiology reviews* 38(1): 90–118. http://www.ncbi.nlm.nih.gov/pubmed/23909933.

Böttger, Roland, Ralf Hoffmann, and Daniel Knappe. 2017. “Differential Stability of Therapeutic Peptides with Different Proteolytic Cleavage Sites in Blood, Plasma and Serum.” *PloS one* 12(6): e0178943. http://www.ncbi.nlm.nih.gov/pubmed/28575099.

Boucher, R. C. 2004. “New Concepts of the Pathogenesis of Cystic Fibrosis Lung Disease.” *European Respiratory Journal* 23(1): 146–58.

Brauner, Asher, Ofer Fridman, Orit Gefen, and Nathalie Q Balaban. 2016. “Distinguishing between Resistance, Tolerance and Persistence to Antibiotic Treatment.” *Nature Reviews Microbiology* 14: 320. https://doi.org/10.1038/nrmicro.2016.34.

Brogden, KA. 2005. “Antimicrobial Peptides: Pore Formers or Metabolic Inhibitors in Bacteria?” *Nature Reviews Microbiology* 3: 238–50.

Bruscia, Emanuela M, and Tracey L Bonfield. 2016. “Cystic Fibrosis Lung Immunity: The Role of the Macrophage.” *Journal of Innate Immunity* 8(6): 550–63. https://www.karger.com/Article/FullText/446825.

Cantin, André M., Dominik Hartl, Michael W. Konstan, and James F. Chmiel. 2015. “Inflammation in Cystic Fibrosis Lung Disease: Pathogenesis and Therapy.” *Journal of Cystic Fibrosis* 14(4): 419–30. https://linkinghub.elsevier.com/retrieve/pii/S1569199315000582.

Chang, William K, William C Wimley, Peter C Searson, and Kalina Hristova. 2009. “NIH Public Access.” *October* 1778(10): 2430–36.

Ciofu, Oana, Lotte F Mandsberg, Hengzhuang Wang, and Niels Høiby. 2012. “Phenotypes Selected during Chronic Lung Infection in Cystic Fibrosis Patients: Implications for the Treatment of Pseudomonas Aeruginosa Biofilm Infections.” *FEMS immunology and medical microbiology*

65(2): 215–25. http://www.ncbi.nlm.nih.gov/pubmed/22540844.

Conrad, Douglas et al. 2013. “Cystic Fibrosis Therapy: A Community Ecology Perspective.” *American Journal of Respiratory Cell and Molecular Biology* 48(2): 150–56.

Costerton, JW. 2001. “Antibiotic Resistance of Bacteria in Biofilms.” *Lancet* 358: 135–38.

Costerton, JW, PS Stewart, and EP Greenberg. 1999. “Bacterial Biofilms: A Common Cause of Persistent Infections.” *Science* (284): 1318–1322.

Cui, Lijia et al. 2014. “The Microbiome and the Lung.” *Annals of the American Thoracic Society* 11(8): S227–32.

Cuthbert, A. W. 2011. “New Horizons in the Treatment of Cystic Fibrosis.” *British Journal of Pharmacology* 163(1): 173–83.

Cutting, Garry R. 2015. “Cystic Fibrosis Genetics: From Molecular Understanding to Clinical Application.” *Nat Rev Genet* 16(1): 237–60.

Dasaraju, Purushothama V., and Chien Liu. 1996. “Infections of the Respiratory System.” In *Medical Microbiology*,.

Davenport, Peter W, Julian L Griffin, and Martin Welch. 2015. “Quorum Sensing Is Accompanied by Global Metabolic Changes in the Opportunistic Human Pathogen <Span Class="Named-Content Genus-Species" Id="Named-Content-1">Pseudomonas Aeruginosa</Span>” ed. G A O'Toole. *Journal of Bacteriology* 197(12): 2072 LP-2082. http://jb.asm.org/content/197/12/2072.abstract.

Davies, Julian, George B. Spiegelman, and Grace Yim. 2006. “The World of Subinhibitory Antibiotic Concentrations.” *Current Opinion in Microbiology* 9(5): 445–53.

Denyer, Stephen P., Norman A. Hodges, and Sean P. Gorman. 2007. Hugo and Russell’s Pharmaceutical Microbiology: Seventh Edition *Hugo and Russell’s Pharmaceutical Microbiology: Seventh Edition*.

Desbois, Andrew P, and Peter J Coote. 2012. “Utility of Greater Wax Moth Larva (Galleria Mellonella) for Evaluating the Toxicity and Efficacy of New Antimicrobial Agents.” *Advances in applied microbiology* 78: 25–53. http://www.ncbi.nlm.nih.gov/pubmed/22305092.

Diamond, G. 2009. “The Role of Antimicrobial Peptides in Innate Host Defence.” *Current Pharmaceutical Design* 15(21): 2377–92.

Dong, Weibing et al. 2017. “Antimicrobial and Anti-Inflammatory Activities of Three Chensinin-1 Peptides Containing Mutation of Glycine and Histidine Residues.” *Scientific reports* 7: 40228. http://www.ncbi.nlm.nih.gov/pubmed/28054660.

Döring, G. 1994. “The Role of Neutrophil Elastase in Chronic Inflammation.” *American journal of respiratory and critical care medicine* 150(6 Pt 2): S114-7.

http://www.ncbi.nlm.nih.gov/pubmed/7952645.

Dunne, Wm. 2002. "Bacterial Adhesion: Seen Any Good Biofilms Lately?" *Clin Microbiol Rev* 15(2): 155–66.

Duplantier, Allen J., and Monique L. van Hoek. 2013. "The Human Cathelicidin Antimicrobial Peptide LL-37 as a Potential Treatment for Polymicrobial Infected Wounds." *Frontiers in Immunology* 4(JUL): 1–14.

Epand, Richard M, Chelsea Walker, Raquel F Epand, and Nathan A Magarvey. 2016. "Molecular Mechanisms of Membrane Targeting Antibiotics." *Biochimica et biophysica acta* 1858(5): 980–87. http://www.ncbi.nlm.nih.gov/pubmed/26514603.

Farinha, CM et al. 2004. "Biochemical Methods to Assess CFTR Expression and Membrane Localization." *Journal of Cystic Fibrosis* 2: 73–77.

Ferkol, Thomas, and Dean Schraufnagel. 2014. "The Global Burden of Respiratory Disease." *Annals of the American Thoracic Society* 11(3): 404–6.

Fernandez, Isis E, and Oliver Eickelberg. 2012. "The Impact of TGF-β on Lung Fibrosis: From Targeting to Biomarkers." *Proceedings of the American Thoracic Society* 9(3): 111–16. http://www.ncbi.nlm.nih.gov/pubmed/22802283.

Fjell, Christopher D, Jan A Hiss, Robert E W Hancock, and Gisbert Schneider. 2011. "Designing Antimicrobial Peptides: Form Follows Function." *Nature reviews. Drug discovery* 11(1): 37–51. http://www.ncbi.nlm.nih.gov/pubmed/22173434.

Flemming, HC et al. 2016. "Biofilms: An Emergent Form of Bacterial Life." *Nature Reviews Microbiology* (14): 563–75.

Forde, Eanna et al. 2014. "Potential of Host Defense Peptide Prodrugs as Neutrophil Elastase-Dependent Anti-Infective Agents for Cystic Fibrosis." *Antimicrobial agents and chemotherapy* 58(2): 978–85. http://www.ncbi.nlm.nih.gov/pubmed/24277028.

Galván, JuanMaria, Osvald Rajas, and Julia Aspa. 2015. "Review of Non-Bacterial Infections in Respiratory Medicine: Viral Pneumonia." *Arch Bronconeumol* 51(11): 590–97.

Ganz, Tomas. 2003. "Defensins: Antimicrobial Peptides of Innate Immunity." *Nature Reviews Immunology* 3: 710. https://doi.org/10.1038/nri1180.

Garrett, TR, Manmohan Bhakoob, and Zhibing Zhanga. 2008. "Bacterial Adhesion and Biofilms on Surfaces." *Progress in Natural Science* 18(9): 1049–56.

Gellatly, SL, and REW Hancock. 2013. "Pseudomonas Aeruginosa: New Insignts into Pathogenesis and Host Defenses." *Phatogens and Disease* 67(3): 159–73.

Gennaro, R, and M Zanetti. 2000. "Structural Features and Biological Activities of the Cathelicidin-Derived Antimicrobial Peptides." *Biopolymers* 55(1): 31–49.

Gloag, Erin S. et al. 2013. “Self-Organization of Bacterial Biofilms Is Facilitated by Extracellular DNA.” *PNAS* 110(28): 11541–46.

Glukhov, Evgenia, Margareta Stark, Lori L Burrows, and Charles M Deber. 2005. “Basis for Selectivity of Cationic Antimicrobial Peptides for Bacterial versus Mammalian Membranes.” *The Journal of biological chemistry* 280(40): 33960–67. http://www.ncbi.nlm.nih.gov/pubmed/16043484.

Gollwitzer, ES et al. 2014. “Lung Microbiota Promotes Tolerance to Allergens in Neonates via PD-L1.” *Nat Med* 20: 642–47.

Grassi, Lucia et al. 2017. “Generation of Persister Cells of Pseudomonas Aeruginosa and Staphylococcus Aureus by Chemical Treatment and Evaluation of Their Susceptibility to Membrane-Targeting Agents.” *Frontiers in microbiology* 8: 1917. http://www.ncbi.nlm.nih.gov/pubmed/29046671.

Gudmundsson, G H et al. 1995. “Structure of the Gene for Porcine Peptide Antibiotic PR-39, a Cathelin Gene Family Member: Comparative Mapping of the Locus for the Human Peptide Antibiotic FALL-39.” *Proceedings of the National Academy of Sciences of the United States of America* 92(15): 7085–89. http://www.ncbi.nlm.nih.gov/pubmed/7624374.

Gurjar, Mohan. 2015. “Colistin for Lung Infection: An Update.” *Journal of Intensive Care* 3(1).

Guttenplan, SB. 2013. “Regulation of Flagellar Motility during Biofilm Formation.” *FEMS Microbiological Reviews* 37(6): 849–71.

Guttenplan, SB, and DB Kearns. 2013. “Regulaion of Flagellar Motility during Biofilm Formation.” *FEMS Microbiological Reviews* 37(6): 849–4871.

Hancock, Robert E.W., Kelly L. Brown, and Neeloffer Mookherjee. 2006. “Host Defence Peptides from Invertebrates - Emerging Antimicrobial Strategies.” *Immunobiology* 211(4): 315–22.

Harms, Alexander, Etienne Maisonneuve, and Kenn Gerdes. 2016. “Mechanisms of Bacterial Persistence during Stress and Antibiotic Exposure.” *Science* 354(6318): aaf4268. http://www.sciencemag.org/lookup/doi/10.1126/science.aaf4268.

Hewer, SL. 2012. “Inhaled Antibiotics in Cystic Fibrosis: What’s New?” *Journal of the royal sociaty of medicine* 105(2): S19–24.

Hoffman, Lucas R et al. 2006. “Selection for Staphylococcus Aureus Small-Colony Variants Due to Growth in the Presence of Pseudomonas Aeruginosa.” *Proceedings of the National Academy of Sciences of the United States of America* 103(52): 19890–95. http://www.ncbi.nlm.nih.gov/pubmed/17172450.

Hogardt, Michael, and Jürgen Heesemann. 2013. “Microevolution of Pseudomonas Aeruginosa to a Chronic Pathogen of the Cystic Fibrosis Lung.” *Current topics in microbiology and*

immunology 358: 91–118. http://www.ncbi.nlm.nih.gov/pubmed/22311171.

Huang, H W. 2006. "Molecular Mechanism of Antimicrobial Peptides: The Origin of Cooperativity." *Biochimica et Biophysica Acta (BBA)- Biomembranes* 1785(9): 1292–1302.

Hultmark, D, H Steiner, T Rasmuson, and H G Boman. 1980. "Insect Immunity. Purification and Properties of Three Inducible Bactericidal Proteins from Hemolymph of Immunized Pupae of Hyalophora Cecropia." *European journal of biochemistry* 106(1): 7–16. http://www.ncbi.nlm.nih.gov/pubmed/7341234.

Hurley, Matthew N, Miguel Cámara, and Alan R Smyth. 2012. "Novel Approaches to the Treatment of Pseudomonas Aeruginosa Infections in Cystic Fibrosis." *The European respiratory journal* 40(4): 1014–23. http://www.ncbi.nlm.nih.gov/pubmed/22743672.

Jagger, K S, D R Bahner, and R L Warren. 1983. "Protease Phenotypes of Pseudomonas Aeruginosa Isolated from Patients with Cystic Fibrosis." *Journal of clinical microbiology* 17(1): 55–59. http://www.ncbi.nlm.nih.gov/pubmed/6402520.

Jamal, Muhsin et al. 2015. "Bacterial Biofilm: Its Composition, Formation and Role in Human Infection." *Research & Reviews: Journal of Microbiology and Biotechnology* 4(3): 1–14.

Jenssen, Håvard, Pamela Hamill, and Robert E W Hancock. 2006. "Peptide Antimicrobial Agents." *Clinical microbiology reviews* 19(3): 491–511. http://www.ncbi.nlm.nih.gov/pubmed/16847082.

Joo, Hwang-Soo, Chih-Iung Fu, and Michael Otto. 2016. "Bacterial Strategies of Resistance to Antimicrobial Peptides." *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* 371(1695): pii: 20150292. http://www.ncbi.nlm.nih.gov/pubmed/27160595.

Kaplan, JB. 2010. "Biofilm Dispersal: Mechanisms, Clinical Implication and Potential Therapeutic Uses." *Journal of Dental Research* 89(2): 205–18.

Khan, Hassan Ahmed, Fatima Kanwal Baig, and Riffat Mehboob. 2017. "Nosocomial Infections: Epidemiology, Prevention, Control and Surveillance." *Asian Pacific Journal of Tropical Biomedicine* 7(5): 478–82. http://dx.doi.org/10.1016/j.apjtb.2017.01.019.

Klodzińska, Sylvia Natalie, Petra Alexandra Priemel, Thomas Rades, and Hanne Mørck Nielsen. 2016. "Inhalable Antimicrobials for Treatment of Bacterial Biofilm-Associated Sinusitis in Cystic Fibrosis Patients: Challenges and Drug Delivery Approaches." *International Journal of Molecular Sciences* 17(10).

Kobayashi, Hiroyuki. 2005. "Airway Biofilms: Implications for Pathogenesis and Therapy of Respiratory Tract Infections." *Treatments in respiratory medicine* 4(4): 241–53. http://www.ncbi.nlm.nih.gov/pubmed/16086598.

Kolter, R. 2000. “Biofilm, City of Microbes.” *Journal of Bacteriology* 182(10): 2675–79.

Kościuczuk, Ewa M et al. 2012. “Cathelicidins: Family of Antimicrobial Peptides. A Review.” *Molecular biology reports* 39(12): 10957–70. http://www.ncbi.nlm.nih.gov/pubmed/23065264.

Kosikowska, Paulina, and Adam Lesner. 2016. “Antimicrobial Peptides (AMPs) as Drug Candidates: A Patent Review (2003-2015).” *Expert opinion on therapeutic patents* 26(6): 689–702. http://www.ncbi.nlm.nih.gov/pubmed/27063450.

Kostakioti, M, M Hadjifrangiskou, and SJ Hultgren. 2013. “Bacterial Biofilms: Development, Dispersal, and Therapeutic Strategies in the Dawn of the Postantibiotic Era.” *Cold Spring Harb Perspect Med.* 3(4): a010306.

Kramer, Elizabeth L, and John P Clancy. 2018. “TGFβ as a Therapeutic Target in Cystic Fibrosis.” *Expert opinion on therapeutic targets* 22(2): 177–89. http://www.ncbi.nlm.nih.gov/pubmed/29168406.

Kuhn, RJ. 2001. “Formulation of Aerosol Therapeutics.” *CHEST Journal* 120(3): 94S–98S.

Kumar, Prashant, Jayachandran Kizhakkedathu, and Suzana Straus. 2018. “Antimicrobial Peptides: Diversity, Mechanism of Action and Strategies to Improve the Activity and Biocompatibility In Vivo.” *Biomolecules* 8(1): 4. http://www.mdpi.com/2218-273X/8/1/4.

Kussell, Edo, Roy Kishony, Nathalie Q Balaban, and Stanislas Leibler. 2005. “Bacterial Persistence: A Model of Survival in Changing Environments.” *Genetics* 169(4): 1807–14. http://www.ncbi.nlm.nih.gov/pubmed/15687275.

Lambert, PA. 2002. “Mechanisms of Antibiotic Resistance in Pseudomonas Aeruginosa.” *Journal of the Royal Society of Medicine* 95(41): 22–26.

Laval, Julie, Anjali Ralhan, and Dominik Hartl. 2016. “Neutrophils in Cystic Fibrosis.” *Biological chemistry* 397(6): 485–96. http://www.ncbi.nlm.nih.gov/pubmed/26854289.

Lazarev, V. N., and V. M. Govorun. 2010. “Antimicrobial Peptides and Their Use in Medicine.” *Applied Biochemistry and Microbiology* 46(9): 803–14. http://link.springer.com/10.1134/S0003683810090012.

Lebeaux, D, JM Ghigo, and C Beloin. 2014. “Biofilm-Related Infections: Bridging the Gap between Clinical Management and Fundamental Aspects of Recalcitrance toward Antibiotics.” *Microbiology and Molecular Biology Reviews* 78(3): 510–43. http://mmbr.asm.org/cgi/doi/10.1128/MMBR.00013-14.

Lee, Tzong-Hsien, Kristopher N. Hall, and Marie-Isabel Aguilar. 2015. “Antimicrobial Peptide Structure and Mechanism of Action: A Focus on the Role of Membrane Structure.” *Current Topics in Medicinal Chemistry* 16(1): 25–39.

http://www.eurekaselect.com/openurl/content.php?genre=article&issn=1568-0266&volume=16&issue=1&spage=25.

Levin-Reisman, Irit et al. 2017. "Antibiotic Tolerance Facilitates the Evolution of Resistance." *Science* 355(6327): 826–30. http://www.sciencemag.org/lookup/doi/10.1126/science.aaj2191.

Lewis, K. 2007. "Persister Cells, Dormancy and Infectious Disease." *Nature Reviews Microbiology* 5: 48–56.

Limoli, DH. 2014. "Bacterial Extracellular Polysaccharides in Biofilm Formation and Function." *Microbiol Spectr*.

Lynch, SV, and KD Bruce. 2013. "The Cystic Fibrosis Airway Microbiome." *Cold Spring Harbor Perspective in Medicine* 3(3): a009738.

Mah, G. A., and T F O'Toole. 2001. "Mechanisms of Biofilm Resistance to Antimicrobial Agents TT - 14.07.2011." *Trends in microbiology* 9(1): 34–39. https://www.bibliogo.com/#biblio:search&query=id:3090027.

Mahlapuu, Margit, Joakim Håkansson, Lovisa Ringstad, and Camilla Björn. 2016. "Antimicrobial Peptides: An Emerging Category of Therapeutic Agents." *Frontiers in Cellular and Infection Microbiology* 6(December): 1–12. http://journal.frontiersin.org/article/10.3389/fcimb.2016.00194/full.

Malanovic, N, and K Lohner. 2016. "Antimicrobial Peptides Targeting Gram-Positive Bacteria." *Pharmaceuticals* 9(3): 59.

Mann, EE, and DJ Wozniak. 2013. "Pseudomonas Biofilm Matrix Composition and Niche Biology." *FEMS Microbiological reviews* 36(4): 893–916.

Mardirossian, Mario et al. 2014. "The Host Antimicrobial Peptide Bac71-35 Binds to Bacterial Ribosomal Proteins and Inhibits Protein Synthesis." *Chemistry and Biology* 21(12): 1639–47.

———. 2016. "In Vitro and in Vivo Evaluation of BMAP-Derived Peptides for the Treatment of Cystic Fibrosis-Related Pulmonary Infections." *Amino acids* 48(9): 2253–60. http://www.ncbi.nlm.nih.gov/pubmed/27270571.

———. 2017. "D-BMAP18 Antimicrobial Peptide Is Active In Vitro, Resists to Pulmonary Proteases but Loses Its Activity in a Murine Model of Pseudomonas Aeruginosa Lung Infection." *Frontiers in chemistry* 5: 40. http://www.ncbi.nlm.nih.gov/pubmed/28674688.

Marr, Alexandra K, William J Gooderham, and Robert Ew Hancock. 2006. "Antibacterial Peptides for Therapeutic Use: Obstacles and Realistic Outlook." *Current opinion in pharmacology* 6(5): 468–72. http://www.ncbi.nlm.nih.gov/pubmed/16890021.

Martha, B et al. 2010. "Factors Associated with Mucoid Transition of Pseudomonas Aeruginosa in Cystic Fibrosis Patients." *Clinical microbiology and infection : the official publication of the*

European Society of Clinical Microbiology and Infectious Diseases 16(6): 617–23. http://www.ncbi.nlm.nih.gov/pubmed/20002106.

Matsuzaki, Katsumi, Osamu Murase, Nobutaka Fujii, and Koichiro Miyajima. 1996. "An Antimicrobial Peptide, Magainin 2, Induced Rapid Flip-Flop of Phospholipids Coupled with Pore Formation and Peptide Translocation †." *Biochemistry* 35(35): 11361–68. http://pubs.acs.org/doi/abs/10.1021/bi960016v.

Mishra, Biswajit, Scott Reiling, D Zarena, and Guangshun Wang. 2017. "Host Defense Antimicrobial Peptides as Antibiotics: Design and Application Strategies." *Current opinion in chemical biology* 38: 87–96. http://www.ncbi.nlm.nih.gov/pubmed/28399505.

Mohanram, H, and S Bhattecharjya. 2016. "Salt-Resistant Short Antimicorbial Peptides." *Biopolymers* 106(3): 345–56.

Morris, David P. 2007. "Bacterial Biofilm in Upper Respiratory Tract Infections." *Current Infectious Disease Reports* 9(3): 186–92. https://doi.org/10.1007/s11908-007-0030-3.

Moskowitz, Samuel M, Robert K Ernst, and Samuel I Miller. 2004. "PmrAB, a Two-Component Regulatory System of Pseudomonas Aeruginosa That Modulates Resistance to Cationic Antimicrobial Peptides and Addition of Aminoarabinose to Lipid A." *Journal of bacteriology* 186(2): 575–79. http://www.ncbi.nlm.nih.gov/pubmed/14702327.

Musher, Daniel M., and Anna R. Thorner. 2014. "Community-Acquired Pneumonia." *New England Journal of Medicine* 371(17): 1619–28. http://www.nejm.org/doi/10.1056/NEJMra1312885.

Nguyen, LT, EF Haney, and HJ Vogel. 2011. "The Expanding Scope of Antimicrobial Peptide Structures and Their Modes of Action." *Trends in Biotechnology* 29(9): 464–72.

Nicolas, Pierre. 2009. "Multifunctional Host Defense Peptides: Intracellular-Targeting Antimicrobial Peptides." *FEBS Journal* 276(22): 6483–96.

Nigam, Anshul, Divya Gupta, and Ashwani Sharma. 2014. "Treatment of Infectious Disease: Beyond Antibiotics." *Microbiological research* 169(9–10): 643–51. http://www.ncbi.nlm.nih.gov/pubmed/24661689.

O'Toole, George. 2018. "Cystic Fibrosis Airway Microbiome: Overturning the Old, Opening the Way for the New" ed. William Margolin. *Journal of Bacteriology* 200(4). http://jb.asm.org/content/200/4/e00561-17.abstract.

Omardien, Soraya, Stanley Brul, and Sebastian A J Zaat. 2016. "Antimicrobial Activity of Cationic Antimicrobial Peptides against Gram-Positives: Current Progress Made in Understanding the Mode of Action and the Response of Bacteria." *Frontiers in cell and developmental biology* 4: 111. http://www.ncbi.nlm.nih.gov/pubmed/27790614.

Paiva, A. D. et al. 2012. "Toxicity of Bovicin HC5 against Mammalian Cell Lines and the Role of

Cholesterol in Bacteriocin Activity." *Microbiology* 158(Pt_11): 2851–58. http://mic.microbiologyresearch.org/content/journal/micro/10.1099/mic.0.062190-0.

Park, Y, and Kyung Soo Hahm. 2005. "Antimicrobial Peptides (AMPs): Peptide Structure and Mode of Action." *Journal of Biochemical and Molecular Biology* 38(5): 507–16.

Pereira, MO. 2014. "Pseudomonas Aeruginosa Diversification during Infection Development in Cystic Fibrosis Lungs—A Review." *Pathogens* 3: 680–703.

Perron, Gabriel G, Michael Zasloff, and Graham Bell. 2006. "Experimental Evolution of Resistance to an Antimicrobial Peptide." *Proceedings. Biological sciences* 273(1583): 251–56. http://www.ncbi.nlm.nih.gov/pubmed/16555795.

Pezzulo, AA et al. 2012. "Reduced Airway Surface PH Impairs Bacterial Killing in the Porcine Cystic Fibrosis Lung." *Nature* 487(7405): 109–13.

Pintucci, J P, S Corno, and M Garotta. 2010. "Biofilms and Infections of the Upper Respiratory Tract." *European review for medical and pharmacological sciences* 14(8): 683–90. http://www.ncbi.nlm.nih.gov/pubmed/20707288.

Pompilio, Arianna et al. 2011. "Antibacterial and Anti-Biofilm Effects of Cathelicidin Peptides against Pathogens Isolated from Cystic Fibrosis Patients." *Peptides* 32(9): 1807–14. http://dx.doi.org/10.1016/j.peptides.2011.08.002.

———. 2012. "Potential Novel Therapeutic Strategies in Cystic Fibrosis: Antimicrobial and Anti-Biofilm Activity of Natural and Designed -Helical Peptides against Staphylococcus Aureus, Pseudomonas Aeruginosa, and Stenotrophomonas Maltophilia." *BMC Microbiology* 12(1): 1. ???

Popovic, Suzana, Edit Urbán, J. Miodrag Lukica, and Michael Conlon. 2012. "Peptides with Antimicrobial and Anti-Inflammatory Activities That Have Therapeutic Potential for Treatment of Acne Vulgaris." *Peptides* 34(2): 275–82.

Pressler, Tacjana. 2008. "Review of Ecombinant Human Deoxyribonuclease (RhDNase) in the Management of Patients with Cystic Fibrosis." *Biologics* 2(4): 611–17.

Rabin, N et al. 2015. "Biofilm Formation Mechanisms and Targets for Developing Antibiofilm Agents." *Future med chem* 7(4): 493–512.

Raghupathi, PK et al. 2018. "Synergistic Interaction within a Multispecies Biofilm Enhance Individual Specied Protection against Grazing by Pelagic Protozoan." *Frontiers in Microbiology* 8: 2649.

Ratjen, F et al. 2005. "DNA Concentrations in BAL Fluid of Cystic Fibrosis Patients with Early Lung Disease: Influence of Treatment with Dornase Alpha." *Pediatric pulmonology* 39(1): 1–4. http://www.ncbi.nlm.nih.gov/pubmed/15532079.

Reeves, Emer P, Kevin Molloy, Kerstin Pohl, and Noel G McElvaney. 2012. "Hypertonic Saline in Treatment of Pulmonary Disease in Cystic Fibrosis." *TheScientificWorldJournal* 2012: 465230. http://www.ncbi.nlm.nih.gov/pubmed/22645424.

Renner, LD, and DB Weibel. 2011. "Physicochemical Regulation of Biofilm Formation." *MRS Bull* 36(5): 347–55.

Riordan, JR. 2008. "CFTR Function and Prospect for Therapy." *Annual Review* 77: 701–26.

Rogers, Geraint B, Lucas R Hoffman, and Gerd Döring. 2011. "Novel Concepts in Evaluating Antimicrobial Therapy for Bacterial Lung Infections in Patients with Cystic Fibrosis." *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society* 10(6): 387–400. http://www.ncbi.nlm.nih.gov/pubmed/21775220.

Rosenecker, J et al. 2002. "Interaction of Bronchoalveolar Lavage Fluid with Polyplexes and Lipoplexes: Analysing the Role of Proteins and Glycoproteins." *the journal of gene medicone* 5(1): 49–60.

Rowntree, RK, and A Harris. 2003. "The Phenotypic Consequences of CFTR Mutations." *Annals of Human Genetics* 67: 471–85.

Sato, H, and JB Feix. 2006. "Peptide-Membrane Interactions and Mechanisms of Membrane Destruction by Amphipathic α-Helical Antimicrobial Peptides." *Biochimica et Biophysica Acta (BBA)- Biomembranes* 1758(9): 1245–56.

Schwarz, Carsten et al. 2018. "Progress in Definition, Prevention and Treatment of Fungal Infections in Cystic Fibrosis." *Mycopathologia* 183(1): 21–32.

Selsted, M E, D M Brown, R J DeLange, and R I Lehrer. 1983. "Primary Structures of MCP-1 and MCP-2, Natural Peptide Antibiotics of Rabbit Lung Macrophages." *The Journal of biological chemistry* 258(23): 14485–89. http://www.ncbi.nlm.nih.gov/pubmed/6643497.

Shai, Y. 2002. "Mode of Action of Membrane Active Antimicrobial Peptides." *Biopolymers* 66(4): 236–48.

Shamova, O et al. 1999. "Purification and Properties of Proline-Rich Antimicrobial Peptides from Sheep and Goat Leukocytes." *Infection and immunity* 67(8): 4106–11. http://www.ncbi.nlm.nih.gov/pubmed/10417180.

Skerlavaj, Barbara et al. 1996. "Biological Characterization of Two Novel Cathelicidin-Derived Peptides and Identification of Structural Requirements for Their Antimicrobial and Cell Lytic Activities." *Journal of Biological Chemistry* 271(45): 28375–81.

Soo- Kyoung, K, and L Joon- Hee. 2016. "Biofilm Dispersion in Pseudomonas Aeruginosa." *Journal of Microbiology* 54(2): 71–85.

Sousa, Ana, and Maria Pereira. 2014. "Pseudomonas Aeruginosa Diversification during Infection

Development in Cystic Fibrosis Lungs—A Review." *Pathogens* 3(3): 680–703. http://www.mdpi.com/2076-0817/3/3/680/.

Stanley, NR, and BA Lazazzera. 2004. "Environmental Signals and Regulatory Pathways That Influence Biofilm Formation." *Molecular Biology* 52(4): 917–24.

Steven, Marc, and Jhon David Thomas Koening. 2006. "Ventilator-Associated Pneumonia: Diagnosis, Treatment, and Prevention." *Clin Microbiol Rev* 19(4): 637–57.

Stewart, PS. 2015. "Antimicrobial Tolerance in Biofilms." *Microbiology Spectrum* 3(3).

Streeter, K, and M Katouli. 2016. "Pseudomonas Aeruginosa: A Review of Their Pathogenesis and Prevalence in Clinical Setting and Environment." *Infection, epidemiology and medicine* 2(1): 25–32.

Surette, Michael G. 2014. "The Cystic Fibrosis Lung Microbiome." *Annals of the American Thoracic Society* 11(SUPPL. 1): 61–65.

Tang, L et al. 2015. "Extracellular DNA in Adhesion and Biofilm Formation of Four Environmental Isolates: A Quantitative Study." *FEMS Microbiology Ecology* 86(3): 394–403.

Tarran, Robert et al. 2001. "The Relative Roles of Passive Surface Forces and Active Ion Transport in the Modulation of Airway Surface Liquid Volume and Composition." *The Journal of General Physiology* 118(2): 223–36. http://www.jgp.org/lookup/doi/10.1085/jgp.118.2.223.

———. 2004. "Regulation of Airway Surface Liquid Volume and Mucus Transport by Active Ion Transport." *Annals of the American Thoracic Society* 1(1): 42–46.

Tomasinsig, L, and M Zanetti. 2005. "The Cathelicidins-Structure, Function and Evolution." *Current protein & peptide science* 6: 23–34.

Trastoy, R et al. 2018. "Mechanisms of Bacterial Tolerance and Persistence in the Gastrointestinal and Respiratory Environments." *Clin Microbiol Rev* 31(4): 1–46.

Treffers, Charlotte, Liying Chen, Rachel C Anderson, and Pak-Lam Yu. 2005. "Isolation and Characterisation of Antimicrobial Peptides from Deer Neutrophils." *International journal of antimicrobial agents* 26(2): 165–69. http://www.ncbi.nlm.nih.gov/pubmed/16011891.

Tsai, Catherine Jia-Yun, Jacelyn Mei San Loh, and Thomas Proft. 2016. "Galleria Mellonella Infection Models for the Study of Bacterial Diseases and for Antimicrobial Drug Testing." *Virulence* 7(3): 214–29. http://www.ncbi.nlm.nih.gov/pubmed/26730990.

Turner, Jeffrey et al. 1998. "Activities of LL-37, a Cathelin-Associated Antimicrobial Peptide of Human Neutrophils." *Antimicrobial Agents and Chemotherapy* 42(9): 2206–14. http://aac.asm.org/lookup/doi/10.1128/AAC.42.9.2206.

Velkov, Tony et al. 2013. "Pharmacology of Polymyxins: New Insights into an 'old' Class of Antibiotics." *Future microbiology* 8(6): 711–24.

http://www.ncbi.nlm.nih.gov/pubmed/23701329.

Vendrell, Montserrat, Gerard Muñoz, and Javier de Gracia. 2015. "Evidence of Inhaled Tobramycin in Non-Cystic Fibrosis Bronchiectasis." *The open respiratory medicine journal* 9: 30–36. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4397829&tool=pmcentrez&rendertype=abstract.

Venturi, Vittorio. 2006. "Regulation of Quorum Sensing in Pseudomonas." *FEMS microbiology reviews* 30(2): 274–91. http://www.ncbi.nlm.nih.gov/pubmed/16472307.

Verkman, A. S., Y. Song, and J. R. Thiagarajah. 2003. "Role of Airway Surface Liquid and Submucosal Glands in Cystic Fibrosis Lung Disease." *AJP: Cell Physiology* 284(1): C2–15. http://ajpcell.physiology.org/cgi/doi/10.1152/ajpcell.00417.2002.

Vlieghe, P, V Lisowski, J Martinez, and M Khrestchatisky. 2010. "Synthetic Therapeutic Peptides: Science and Market." *Drug Discover Today* 15(1–2): 40–56.

Vu, Barbara, Miao Chen, Russell Crawford, and Elena Ivanova. 2009. "Bacterial Extracellular Polysaccharides Involved in Biofilm Formation." *Molecules* 14(7): 2535–54. http://www.mdpi.com/1420-3049/14/7/2535.

Wang, G. 2014. "Human Antimicrobial Peptides and Proteins." *Pharmaceuticals* 7(5): 545–94.

Water, V, and A Smyth. 2015. "Cystic Fibrosis Microbiology: Advances in Antimicrobial Therapy." *Journal of Cystic Fibrosis* 14(5): 551–60.

Wessman, Marcus et al. 2015. "Mucosal Biofilm Detection in Chronic Otitis Media: A Study of Middle Ear Biopsies from Greenlandic Patients." *European Archives of Oto-Rhino-Laryngology* 272(5): 1079–85. http://link.springer.com/10.1007/s00405-014-2886-9.

Wilking, J. N. et al. 2013. "Liquid Transport Facilitated by Channels in Bacillus Subtilis Biofilms." *Proceedings of the National Academy of Sciences* 110(3): 848–52. http://www.pnas.org/cgi/doi/10.1073/pnas.1216376110.

Willcox, M D P et al. 2008. "Role of Quorum Sensing by Pseudomonas Aeruginosa in Microbial Keratitis and Cystic Fibrosis." *Microbiology (Reading, England)* 154(Pt 8): 2184–94. http://www.ncbi.nlm.nih.gov/pubmed/18667552.

Williams, Craig, Ranjith Ranjendran, and Gordon Ramage. 2016. "Pathogenesis of Fungal Infections in Cystic Fibrosis." *Current fungal infection reports* 10(4): 163–69. http://www.ncbi.nlm.nih.gov/pubmed/28035247.

Wimley, WC. 2010. "Describing the Mechanism of Antimicrobial Peptide Action with the Interfacial Activity Model." *ACS Chemical Biology* 5(10): 905–17.

Wood, Thomas K., Stephen J. Knabel, and Brian W. Kwan. 2013. "Bacterial Persister Cell Formation and Dormancy." *Applied and Environmental Microbiology* 79(23): 7116–21.

http://aem.asm.org/lookup/doi/10.1128/AEM.02636-13.

Woodhead, M. et al. 2011. “Guidelines for the Management of Adult Lower Respiratory Tract Infections - Full Version.” *Clinical Microbiology and Infection* 17: E1–59. http://linkinghub.elsevier.com/retrieve/pii/S1198743X1461404X.

Wozniak, Daniel J., and Rebecca BS Keyser. 2004. “Effects of Subinhibitory Concentrations of Macrolide Antibiotics on Pseudomonas Aeruginosa.” *CHEST Journal* 125(2): 62S–69S.

Yang, L et al. 2001. “Barrel-Stave Model or Toroidal Model? A Case Study on Melittin Pores.” *Biophysical journal* 81(3): 1475–85. http://www.ncbi.nlm.nih.gov/pubmed/11509361.

Yeaman, MR. 2003. “Mechanisms of Antimicrobial Peptide Action and Resistance.” *Pharmacological Reviews* 55(1): 27–55. http://pharmrev.aspetjournals.org/cgi/doi/10.1124/pr.55.1.2.

Yeaman, MR, and NT Yount. 2003. “Mechanisms of Antimicrobial Peptide Action and Resistance.” *Pharmacological Reviews* 55(1): 27–55.

Yonker, LM, C Cigana, BP Hurley, and A Bragonzi. 2015. “Host-Pathogen Interplay in the Respiratory Environment of Cystic Fibrosis.” *Journal of Cystic Fibrosis* 14(4): 431–39.

Zanetti, M. 2005. “The Role of Cathelicidins in the Innate Host Defenses of Mammals.” *Current Issues in Moleclular Biology* 7: 179–96.

Zanetti, M, R Gennaro, and D Romeo. 1995. “Cathelicidins: A Novel Protein Family with a Common Proregion and a Variable C-Terminal Antimicrobial Domain.” *FEBS letters* 374(1): 1–5. http://www.ncbi.nlm.nih.gov/pubmed/7589491.

Zhang, Lijuan, Annett Rozek, and Robert E W Hancock. 2001. “Interaction of Cationic Antimicrobial Peptides with Model Membranes.” *Journal of Biological Chemistry* 276(38): 35714–22.

Zhang, Ling J., and Richard L. Gallo. 2016. “Antimicrobial Peptides.” *Current Biology* 26(1): R14–19. http://dx.doi.org/10.1016/j.cub.2015.11.017.

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