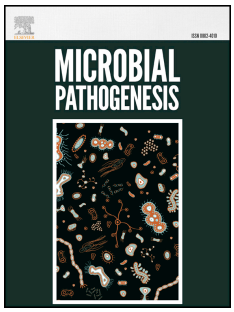

Antibacterial, anti-biofilm and *in vivo* activities of the antimicrobial peptides P5 and P6.2

Melina Martínez, Axel Polizzotto, Naiquen Flores, Liliana Semorile, Paulo César Maffía

PII: S0882-4010(19)30601-1

DOI: https://doi.org/10.1016/j.micpath.2019.103886

Reference: YMPAT 103886

To appear in: *Microbial Pathogenesis*

Received Date: 11 April 2019

Revised Date: 22 October 2019

Accepted Date: 21 November 2019

Please cite this article as: Martínez M, Polizzotto A, Flores N, Semorile L, Maffía PauloCé, Antibacterial, anti-biofilm and *in vivo* activities of the antimicrobial peptides P5 and P6.2, *Microbial Pathogenesis* (2019), doi: https://doi.org/10.1016/j.micpath.2019.103886.

Antibacterial, anti-biofilm and *in vivo* activities of the antimicrobial peptides P5 and P6.2

Melina Martínez[1,2], Axel Polizzotto[1], Naiquen Flores[1], Liliana Semorile[1], Paulo César Maffía[1,2]*

1. Laboratorio de Microbiología Molecular, Instituto de Microbiología Básica y Aplicada, Universidad Nacional de Quilmes, Buenos Aires, Argentina

2. Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.

*Corresponding author: PhD. Maffía, Paulo César. Roque Sáenz Peña 352, B1876BXD Bernal, Buenos Aires, Argentina. Tel: +54 11 4365-7017 ext. 5636. E-mail: paulo.maffia@unq.edu.ar

ABSTRACT

Cationic antimicrobial peptides (AMPs) are short linear amino acid sequences, which display antimicrobial activity against a wide range of bacterial species. They are promising novel antimicrobials since they have shown bactericidal effects against multiresistant bacteria. Their amphiphilic structure with hydrophobic and cationic regions drives their interaction with anionic bacterial cytoplasmic membranes, which leads to their disruption. In this work two synthetic designed AMPs, P5 and P6.2, which have been previously analyzed in their ability to interact with bacterial or eukaryotic membranes, were evaluated in their anti-biofilm and *in vivo* antibacterial activity. In a first step, a time-kill kinetic assay against *P. aeruginosa* and *S. aureus* and a curve for hemolytic activity were performed in order to determine the killing rate and the possible undesirable toxic effect, respectively, for both peptides. The biofilm inhibitory activity was quantified at sub MIC concentrations of the peptides and the results showed that P5 displayed antibiofilm activity on both strains while P6.2 only on *S. aureus*. Scanning electron microscopy (SEM) of bacteria treated with peptides at their MIC revealed protruding blisters on Gam-negative *P. aeruginosa* strain, but almost no visible surface alteration on Gram-positive *S. aureus*. These micrographs highlighted different manifestations of the membrane-disrupting activity that these kinds of peptides possess. Finally, both peptides were analyzed *in vivo*, in the lungs of neutropenic mice previously instilled with *P. aeruginosa*. Mice lungs were surgically extracted and bacteria and pro-inflammatory cytokines (IL-β, IL-6 and TNF-α) were quantified by colony forming units and ELISA, respectively. Results showed that instillation of the peptides produced a significant decrease in the number of living bacteria in the lungs, concomitant with a decrease in pro-inflammatory cytokines. Overall, the results

presented here suggest that these two new peptides could be good candidates for future drug development for anti-biofilm and anti-infective therapy.

Keywords: *antimicrobial peptides; Pseudomonas aeruginosa; anti-biofilm; anti-inflammatory; lung infection.*

1. INTRODUCTION

Since their discovery many decades ago, antimicrobial peptides (AMPs) have been proposed as potential new candidates for the development of novel antimicrobials [1–3]. Their broad spectrum antimicrobial activity and selectivity make them attractive candidates for novel drug compounds [4]. Although the potency of these AMPs against the most sensitive pathogens is normally not as strong as certain conventional antibiotics, one of its major strengths is their ability to kill multi-drug-resistant bacteria at relatively low concentrations. Compared with conventional antibiotics, the killing of bacteria by AMPs is extremely rapid and can involve multiple bacterial cellular targets [5].

Besides their direct antimicrobial activity, AMPs are also increasingly being considered as novel agents against bacterial biofilms and also as immune-modulators of the host immune system. Regarding their anti-biofilm activity, AMPs are believed to inhibit the biofilm formation or to eradicate established ones [6,7]. Bacterial susceptibility to AMPs in biofilms has been shown to be lower compared to the planktonic state [8],

however biofilm resistance to AMPs has not been extensively studied compared to other antimicrobial agents [9,10].

Finding new agents against bacterial biofilms has become an imperative task, since they cause chronic infections with increased tolerance to antibiotics as well as resisting phagocytosis. Particularly, biofilms produced by antibiotic resistant *P. aeruginosa* occur at an elevated frequency both in medical devices and in lungs of Cystic Fibrosis (CF) patients [9,11–13].

Besides their antibacterial activity, the immune-modulatory properties of AMPs have gained attention and many peptides are now known to modulate the innate immune response while suppressing potentially harmful inflammation [14]. It has been proposed that some cationic host defense peptides like human cathelicidin LL-37 [15] or bovine indolicidin [16] could induce a significant reduction of endotoxin-induced inflammatory responses [14]. The anti-inflammatory properties of AMPs may be relevant in diseases in which the exacerbated inflammation promotes cell damage and illness severity, like the lung infections of patients with CF, in which *Pseudomonas aeruginosa* is a prominent pathogen. This bacterial species activates epithelial and immune cells which results in the release of pro-inflammatory mediators [17,18].

In previous works we have evaluated two novel synthetic cationic AMPs, P5 and P6.2, designed as amphipathic short alpha helical molecules, with affinity toward the prokaryotic membranes rather than eukaryotic ones, and their structural characteristics and membrane interactions have been analyzed [19–21]. Both peptides have been designed using a combined rational and computer assisted approach. Cationic alpha helical peptides were designed identifying short putative active regions from AMPs databases. Then, these regions were combined or modified in order to have cationic

amphipathic sequences with different physicochemical parameters [19]. P5 and P6.2 were selected from two different families of related peptides, in accordance to their physicochemical performance and *in vitro* antimicrobial activity (Figure1 and Table 1).

In this work, the biofilm inhibition activity of both peptides was analyzed in Gram-negative (*P. aeruginosa* PAO1) and Gram-positive (*S. aureus* ATCC25923) bacteria. Scanning electron microscopy (SEM) was performed on *P. aeruginosa* and *S. aureus* after treatment with peptides in order to visualize the possible membrane disruptive activity these peptides have on Gram-negative and Gram-positive bacteria.

Both peptides were also analyzed *in vivo*, in the lungs of neutropenic mice previously instilled with PAO1 and viable bacteria from the lungs were quantified to determine the *in vivo* antimicrobial activity. Pro inflammatory cytokines (IL-1β, IL-6 and TNF-α) levels were also measured in the extracted lungs in order to evaluate the possible anti-inflammatory activity.

2 MATERIALS AND METHODS

2.1 Antimicrobial Peptides and bacterial strains

P5 and P6.2 are amphipathic cationic alpha helical antimicrobial peptides that were previously designed and analyzed [19,20,21]. P5 was selected from the related group of AMPs (P5, P8 and P8.1) [20] and P6.2 was selected from the P2, P6 and P6.2 group of related peptides [21]. Figure 1 depicts the helical wheel projection of the peptides, with their hydrophobic residues painted in yellow and their hydrophilic residues in blue. Table 1 shows the physicochemical parameters of each peptide, including the alpha helix content evaluated by circular dichroism in the presence of SDS micelles.

The bacterial strains used were *Pseudomonas aeruginosa* PAO1 *and Staphylococcus aureus* ATCC25923

2.2 Hemolytic activity

The hemolytic activity of peptides was assayed by a standard procedure [22,23]. In brief, fresh mice red blood cells (RBC) that were collected in the presence of heparin were washed three times in PBS. Peptides, dissolved in water, were added to the suspension of red blood cells (1% v/v in PBS) to a final volume of 200 µl in a U bottom 96-well microplate. Then, samples were gently mixed, incubated at 37 °C for 1h and centrifuged for 10 min at 2000 rpm. The supernatant (100 µl) from each well was transferred to a flat bottom 96-well plate, and the release of hemoglobin was measured by absorbance (Asample) at 540 nm. For negative and positive controls RBC in PBS (Ablank) or water (Awater) were used, respectively. The percentage of hemolysis was calculated according to the equation, Percentage of hemolysis = [(Asample - Ablank) / (Awater - Ablank)] x 100.

2.3 Time kill assay

Bactericidal activity of the peptides was evaluated using the time–kill assay. Growth-phase cultures in LB broth at 37°C were used for these experiments and the inoculum was adjusted by optical density. The assay was performed in 50 ml tubes, with a final volume of 10 ml of bacterial suspension in cation-adjusted Mueller–Hinton broth at a concentration of 5×10^5 CFU/mL; each peptide was added at its MIC. Aliquots were removed at different time points in a 3-hour interval, and ten-fold dilutions of the samples were plated in LB agar plates. The CFU number was determined after 24 h of

plaque incubation at 37°C. A culture without AMPs was used as bacterial growth control. The reduction of 3 logarithmic units was considered bactericidal activity.

2.4 Scanning electron microscopy

Sample preparation for SEM was performed according to [24] with some modifications. *P. aeruginosa* PAO1 and *S. aureus* ATCC25923 bacterial cells were grown to exponential phase in LB broth. After centrifugation at 1,000 g for 10 min, the cell pellets were harvested, washed twice with PBS, and re-suspended to an optical density (OD 600) of 0.2. Cells were incubated at 37°C for 1 h with AMPs at their MICs. After incubation, the cells were centrifuged at 5,000 g for 5 min. The cell pellets were harvested, washed twice with PBS, and subjected to fixation with 2.5% glutaraldehyde at 4°C overnight, and then washed twice with PBS. The cells were then dehydrated in a graded ethanol series (50%, 70%, 90%, and 100%) for 10 min and in 100% acetone. Finally, the specimens were coated with gold and examined using a Carl Zeiss NTS SUPRA 40 instrument.

2.5 Biofilm quantification

Inhibition of biofilm development was assayed in 96-wells flat-bottom polystyrene plates. Antimicrobials were two fold serially diluted in Mueller Hinton broth and then the bacterial inoculum was added to reach a final concentration of $5x10^5$ CFU/ml, with a final volume of 100 µl per well. Plates were incubated at 37°C for 24 h. Media alone or media with inoculum were used as negative and positive control, respectively. The inoculum was prepared from an ON culture. After incubation, optic density at 595 nm was measured to quantify bacterial growth. To determine the amount of biofilm, after incubation the supernatant was gently removed and the formed biofilms were washed

twice with 100 µl of saline solution to withdraw planktonic cells. The remaining biofilm was fixed with 100 µl of 100% methanol for 15 minutes, and then stained with 100 µl of crystal violet 1% (v/v) for 5 minutes. The dye was removed and washed twice with 200 µl of distilled water, and the plate was dried at 37°C for 30 minutes. Finally, 100 µl of 33% (v/v) acetic acid was added, samples were homogenized by gentle agitation and absorbance was measured in a microplate reader at 595nm.

2.5 Mice

Female BALB/c mice of 10-12 week of age were obtained from Bio Fucal S.A. (Argentina). All mice had access to food and water *ad libitum* and were used for studies of lung infection. Animals used in this study were followed for 1–5 days (4 animals per cage) to allow close observation from the beginning to end of each experiment. Feeding practices, light cycle, temperature, humidity, and cage and room cleaning procedures followed the regulations of this institution's central animal facility.

Ethic statement: All animal experiments complied with the Argentinian Government's animal experiment regulations and were approved by the Ethics Committee for Animal Experimentation of the National University of Quilmes (CICUAL), and were carried out in accordance with EU Directive 2010/63/EU for animal experiments.

2.6 Bacterial inoculum for infections

For mice infections, the *P. aeruginosa* PAO1 bacterial cells were grown ON in LB medium. Then, a fresh culture was prepared with 5% of the ON bacterial culture and incubated to reach an OD of 0.4. After centrifugation, the cell pellets were washed twice, re-suspended in saline solution and diluted to obtain a concentration of $8x10^6$

bacteria/20 µl, which is the inoculum used for instillation. Inoculum was checked by plating on LB agar plates.

2.7 Induction of neutropenia

To obtain the neutropenic mice model, the cyclophosphamide protocol previously described in [25] was followed. Briefly, neutropenia was induced by i.p. cyclophosphamide (CP; Laboratorios Filaxis S.A, Argentina) on days -4 (at 200 mg/kg of body weight) and -1 (at 100 mg/kg) before infection and evaluated the day after the last CP injection. In order to corroborate the neutropenia, a complete hematological analysis was done. Blood was drawn from mice under anesthesia into tubes containing heparin (Northia, Argentina), using the retro-orbital plexus technique. Hematologic parameters were determined using a hematology analyzer in a veterinary clinical laboratory (Laboratorio Equino S.R.L, Argentina).

2.8 *P. aeruginosa* lung infection

Neutropenic mice were anesthetized intraperitoneally with ketamine/xylazine (100mg/kg; Holliday Scott S.A. / 10 mg/kg; PRO-SER S.A., Argentina) and 20 µl of the bacterial solution were administrated directly into the nostrils. Thirty minutes after the infection, mice were treated with tobramycin, peptide or saline solution intranasally using an ultra-fine pipette tip. Mice were sacrificed 20 h after the infection and lungs surgically extracted.

2.9 Cytokine determination

Lung total weights were recorded and then they were disaggregated using a metal mesh. Lung homogenates were prepared in 2 ml of sterile saline solution. Ten-fold serial

dilutions of homogenates were plated on LB agar plates, and CFU were counted after 20 h incubation at 37°C. IL-1β, IL-6 and TNF-α concentration in lung homogenates were measured by ELISA (BD OptEIA Set; BD Biosciences) according to the manufacturer's instructions (with detection limit at 10 ng/ ml).

2.10 Statistical analysis

Statistical evaluation of differences between the experimental groups was determined by using One-way ANOVA followed by Dunnett post test, with Graphpad Prism 5 software (GraphPad Software Inc., San Diego, CA). All data are presented as mean ± standard error of the mean, and differences were considered to be statistically significant at a *P*-value <0.05.

3 RESULTS

3.1 Antimicrobial and hemolytic activity

Table 2 shows the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) for each peptide in *P. aeruginosa* PAO1 and *S. aureus* ATCC25923.

In order to analyze the bactericidal effects of both peptides at different times, a time-kill assay was performed (Figure 2A, B). The assay showed killing properties that are not possible to observe in the MIC determination, like the rate of killing and the possible bacteriostatic or bactericidal effect. The rate of killing of both peptides was remarkably stronger in *P. aeruginosa* than in *S. aureus*. For *P. aeruginosa*, in 30 minutes P6.2 reduced more than 2 logarithmic units of CFU/ml and P5 completely eradicated

bacterial cells. On the contrary, for *S. aureus* the reduction of almost three logarithmic units was reached after 180 minutes and only P5 demonstrated bactericidal activity (99.9% of bacteria killed). In an attempt to gain insight into the possible undesirable effect toward erythrocyte membranes at different concentrations, we analyzed the hemolytic activity of each peptide in the range of 0-1024 µg/ml (Figure 2C). P5 displayed less than 10% hemolysis in all the range tested and P6.2 exhibited a concentration dependent hemolytic activity, reaching values of 30% at the highest concentration, but remaining below 5% until 128µg/ml, which is fourfold or eightfold the MIC obtained for *S. aureus* or *P. aeruginosa*, respectively.

3.2 Bacterial membrane disturbance by peptide interaction analyzed by scanning electron microscopy (SEM)

In an attempt to visualize the possible effects these peptides may have on Gram-negative and Gram-positive bacteria, SEM was performed on *P. aeruginosa* and *S. aureus* treated with each peptide for 1 h at their MIC. The micrographs (Figure 3) showed that P5 and P6.2 produced surface alterations principally on *P. aeruginosa*, in which both peptides produced the formation of blisters or bubbles on the cell surface, revealing a possible direct interaction and destabilization of the bacterial membrane. On *S. aureus* the peptides at their MIC and for 1 hour incubation did not produced visible alterations on their surface, although a few bacterial cells began to shrink and some invaginations were visible.

3.3 Biofilm inhibition activity

Both peptides were tested on their biofilm inhibition activity *in vitro* (figure 4 C and D). Bacterial growth inhibition at sub-MIC of peptides was also performed prior to biofilm

evaluation in order to determine whether the possible biofilm inhibition could be due mainly to growth inhibition (figure 4 A and B).

P. aeruginosa and *S. aureus* were allowed to develop biofilm in the presence or absence of each peptide at sub-MIC concentrations. Peptide P5 displayed significant inhibition of biofilm at 0.5xMIC in both strains, but it did not show any activity at 0.25xMIC. On the other hand, P6.2 displayed no inhibition activity in *P. aeruginosa* but showed anti biofilm activity in *S. aureus* at both concentrations tested (figure 4C and 4D).

In *S. aureus*, the activity of P5 on biofilm inhibition could be related to the decrease in bacterial growth on both concentrations; but for P6.2, although this peptide displayed a similar growth inhibition than P5, the biofilm inhibition was higher, with statistical significance in 0.5xMIC and 0.25x MIC compared to control. On the other hand, for *P. aeruginosa*, P6.2 displayed growth inhibition but it did not inhibited biofilm formation at all, like P5 did at 0.5xMIC. In the light of these results, it seems evident that there are other mechanisms involved in biofilm inhibition, and that the growth inhibition is neither sufficient nor indicative of the anti-biofilm activity that the AMP may have.

These results also highlight the differential activity these peptides display, regarding Gram-negative or Gram-positive bacteria membrane and their biofilm characteristics.

3.4 *In vivo* analysis of peptides

In order to analyze the *in vivo* activity of both peptides, we tested whether instilled P5 and P6.2 could diminish the bacterial burden in the lungs of neutropenic infected mice.

In order to have a suitable model for PAO1 lung infection, neutropenia was induced in adult Balb/c mice.

3.4.1 Neutropenic mice

Cyclophosphamide (CP), a well-known cytostatic and immunosuppressant drug, was used to induce neutropenia. In order to confirm neutropenia, a group of three mice treated with CP and a control immune competent group of three mice were evaluated analyzing circulating neutrophils, monocytes and lymphocytes. Table 3 shows the results obtained for each animal, as a percentage of total circulating cells. A significant decrease in the circulating neutrophils and monocytes was observed in the three mice treated with CP. No significant changes of body-weight was observed (data not shown) suggesting the absence of major CP-induced toxicity. Both peptides were instilled at 10mg/kg in neutropenic mice and no significant side effect was detected.

3.4.2 *P. aeruginosa* PAO1 instillation and lung bacteria recovery

Neutropenic mice were instilled with an inoculum of *P. aeruginosa* PAO1, and afterwards P5 or P6.2 were instilled. Twenty hours later, mice were sacrificed and lungs surgically extracted, processed and plated on LB agar for *P. aeruginosa* count. At the bacterial load used, the instillation of P5 or P6.2 at 10mg/kg reduced the bacterial load in mice lungs (figure 5A). P6.2 showed better performance *in vivo* than P5 because the first produced a more pronounced decrease in the bacterial load than the latter, in contrast to what it was seen *in vitro* in the time-kill assay or for antibiofilm activity.

A group of mice were instilled with tobramycin as control, and accordingly no bacteria were recovered in the lungs.

3.4.3 Pro inflammatory cytokines quantification

Lungs of infected mice were processed and the pro inflammatory cytokines IL-1β, IL-6 and TNF-α were quantified. Figure 5 shows that both peptides significantly decreased lung IL-1β and IL-6 in neutropenic mice (figure 5 B and C) when administered

intranasally. TNF-α was also diminished (figure 5 D), although the differences between groups were less pronounced, probably because the peak time of this cytokine was reached earlier during this experiment. Control mice that received tobramycin after PAO1 instillation displayed the same cytokines´ values as control uninfected mice.

4 DISCUSSION

We have previously designed a group of related cationic amphipathic alpha helical AMPs, and analyzed their physicochemical properties and interactions with bacterial or eukaryotic membranes [19–21]. From these previous works, two peptides, P5 and P6.2 were selected for further anti biofilm and *in vivo* activity analysis.

The time-kill assay showed that P5 displays a fast killing activity at its MIC on *P. aeruginosa*, unlike P6.2 which possess a slower killing rate. However, as table 2 shows, on this strain the MBC for both peptides is only 1 dilution more concentrated than the MIC. On the other hand, for *S. aureus*, both AMPs displayed a similar killing activity at their MIC, and their MBC is two-fold more concentrated.

With the aim to visualize the effects of this kind of peptides on different bacterial membranes, SEM images were performed after incubating P5 and P6.2 at their MIC for 1h with both strains. Micrographs revealed a clear alteration of *P. aeruginosa* membrane integrity, evidenced by the presence of multiple bubbles or blisters on the cell surface. A similar phenomenon, although in a much less extent, has been reported for other AMPs that share some structural and physicochemical features. The appearance of blisters has been previously reported for the peptidyl-glycylleucine-carboxyamide (PGLa), which is a cationic amphipatic α-helical peptide of 21 amino acids from the magainin family [24] and also for isoforms of the HE2 peptide [27]. As a possible explanation for these blisters it has been suggested that the positively charged

AMPs can substitute the Mg^{2+} ions in the lipopolysaccharide layer on the outer membrane of Gram-negative bacteria and thereby destabilize the outer surface [28]. Such destabilization of the outer membrane would promote the penetration of AMPs and lead to a local disruption of the inner membrane, so that cytoplasmic material locally fills the periplasmic space, which induces the formation of blisters without disrupting the outer membrane.

On the other hand *S. aureus* did not show such visible membrane alteration, although some cells treated with P5 showed holes or invaginations and a visible shrinkage. These dents or holes seen on the surface of *S. aureus* cells are probably indicative of a mechanical rupture of the membrane and cell wall. In any case it is worth to notice that only a few Gram-positive cells treated with P5 displayed that evident membrane rupture, unlike *P. aeruginosa*, where 100% of the cells showed bubble-like structures on their surface. This phenomenon would probably be related to the different characteristics of the cell wall of both strains. Gram-positive bacteria has a thick layer of peptidoglycan on the surface that could mask the effects on the cell membrane.

Besides this evident difference between the strains, it is important to highlight that the time of incubation (1 hour) and the concentration of the peptides (1xMIC) are still not enough to see the complete lysis of the bacterial cells. For that reason what we are seeing here is the whole bacterium, previous to complete membrane disruption.

Further SEM studies with increasing concentrations of AMPs at different times are required in order to get a broader insight of the membranolytic activity of these peptides.

Besides AMPs antimicrobial activity, it was suggested that AMPs have the potential to act on multiple targets and stages of biofilm formation [29,30]. Some AMPs have been reported to prevent biofilm formation and/or to eradicate established ones, and in some

cases the mechanisms beyond these anti-biofilm effects have been hypothesized [29,31]. It has been proposed that some peptides can interfere with the early events of biofilm formation by preventing adhesion of bacterial cells to the substrate or to other cells, or by killing cells before they stably become part of the biofilm architecture [32].

An increasing number of peptides show activity against biofilms at concentrations much lower than their MIC, like LL-37 [29,33,34]. Particularly for this latter AMPs, that shares some common features with the two AMPs analyzed here, Overhage and coworkers [35] were able to demonstrate that LL-37 affects the development of biofilms in at least three ways. First, the initial attachment of *P. aeruginosa* cells to the surface was significantly reduced in the presence of LL-37; second, LL-37 promotes twitching, by stimulating the expression of genes related to type IV pilus biosynthesis and function; third, using microarray technology, they demonstrated that LL-37 affects the two major quorum-sensing systems of *P. aeruginosa*, namely the Las and the Rhl systems, by downregulating key components. Further detailed experiments would be necessary to elucidate if P5 and P6.2 shares also these mode of action.

Alternatively, as it was proposed for the cationic AMP hep-20 [29], P5 and P6.2 due to their cationic nature, could intercalate between the negatively charged bacterial cells interfering with the interactions of matrix of extracellular polymeric substances (EPS) components, thus reducing the amount of EPS that accumulates. In this work we report the anti-biofilm activity of P5 and P6.2, at sub MIC concentrations. Besides their partial growth inhibition, P5 displayed anti biofilm activity on both strains, *P. aeruginosa* and *S. aureus*, while P6.2 was only active on *S. aureus.* Although both peptides displayed some growth inhibiting activity at sub MIC concentration, inhibition of biofilm production was diminished in some conditions. It is worth to notice that the production of biofilm is not necessarily linked to the bacterial growth, at least in a certain growth

range. It can be seen that P6.2, for example, produced a 50% reduction in *P. aeruginosa* growth at 0.5MIC, but the production of biofilm was still the same as the control, which is an evidence of no anti-biofilm activity. On the other hand, also for *P. aeruginosa*, P5 displayed a 22% decrease in bacterial growth at 0.5xMIC, while it showed more than 42% biofilm inhibition at this concentration. Further experiments are required in order to determine the specific mechanism by which these peptides (and other similar cationic AMPs) affect, or not, biofilm formation beyond growth inhibition.

It is important to note that antibiotics at sub-optimal conditions promote biofilm formation, probably because bacteria produce biofilm in an attempt to protect itself from antimicrobial activity. For that reason, a major point for an antimicrobial agent is that it can exhibit anti-biofilm activity at sub-MIC concentrations, which is not always the case. Therefore, even though we are not able to elucidate the specific mechanism of antibiofilm activity, it is remarkable that both peptides displayed biofilm inhibition at sub-MIC concentrations.

P. aeruginosa is a cause of significant morbidity and mortality in hospitalized patients, particularly those with compromised immune systems like neutropenic patients [36].

In this work P5 and P6.2 were tested *in vivo*, in the lungs of neutropenic mice previosuly instilled with *P.aeruginosa* PAO1. In these experiments we analyzed the total bacterial burden and the local levels of three pivotal inflammatory cytokines in lung tissue homogenates upon *P. aeruginosa* PAO1 infection.

Upon instillation, P5 and P6.2 were capable to diminish the CFUs in lungs of neutropenic mice. These results demonstrated that the peptides were still active in the lungs of mice, and capable of killing bacteria in a pro inflammatory environment. It has been shown that most cathelicidins are less active or do not have activity over Gram-

negative bacteria in physiological conditions, some exceptions are chCATH-1, and -2, PMAP-36 and PR-39[37].

After peptides instillation, IL-1β and IL-6 were meaningfully diminished, while TNF-α was diminished to a less extent, this could be explained in part because of the time course of this cytokine in the lungs [38]. In fact TNF-α displays it peak at approximately 9 h post infection, and for the time the experiment was performed the cytokine level could be naturally diminishing, which is reflected in the mild increase in cytokine level between control uninfected mice and mice infected with PAO1. In that scenario, although it seems to be a mild reduction, actually the peptides reversed the cytokine concentration to the control levels.

The two novel designed AMPs, P5 and P6.2, besides reducing the bacterial load in the lungs, significantly reduced the production of three pivotal inflammatory cytokines upon acute lung infection in a neutropenic context. It is important to note that none of the peptides completely eliminated the bacterial load in the lungs, as tobramycin did; nevertheless the pro-inflammatory cytokines reduction was similar to this antibiotic. In the case of tobramycin, the decrease in pro-inflammatory cytokines levels should be expected as a result of the complete clearance of lung bacterial burden. In the case of P5 and P6.2 that did not completely eradicate bacteria from the lungs, the reduction in cytokine levels should have another explanation. Further work will be required in order to unravel the mechanism beyond this down-regulation, but these results lead us to believe that the peptides could be affecting the host cells, inducing the down regulation of cytokines.

Previous studies demonstrated that the cationic amphipathic AMP LL-37 has anti-inflammatory activity through various mechanisms, alter endotoxin aggregation through LPS interactions [39] and in peripheral blood mononuclear cells strongly suppressed

synergistic responses to TREM-1 and TLR4 stimulation, partly through the inhibition of TREM-1 expression on monocytes [40]. Another example is chCATH-2 (chicken cathelicidin-2), it has been shown that chCATH-2-mediated killing of *P. aeruginosa* inhibits pulmonary inflammation in a mouse lung model by reducing PMN recruitment and preventing the release of pro-inflammatory cytokines and chemokines [41–43].

Inflammation is essential for host defense, but a failure to tightly control immune responses to a pathogen can result in chronic inflammation and tissue destruction. It is worth to notice that the pathophysiological mechanisms of pneumonia-induced sepsis include a surge of pro-inflammatory cytokines. In fact IL-1β plays important roles in the up- and down-regulation of acute inflammation [44] but it can also functions as a mediator of chronic inflammation and promotes fibrosis. In this work we showed that these new alpha helical cationic peptides might be suitable candidates for the development of potential anti-biofilm and anti-infective drugs, to be used for instance for CF therapies, with both antimicrobial and anti-inflammatory functions.

5 COMPLIANCE WITH ETHICS GUIDELINES

Melina Martínez, Axel Polizzotto, Liliana Semorile and Paulo César Maffía declare that they have not conflict of interest.

6 ACKNOWLEDGEMENT

This work was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (PICT 0478 – 2016) and Universidad Nacional de Quilmes (Proyectos Orientados Práctica Profesional and Programa Microbiología Molecular Básica y Aplicada). PM is a member of the Research Career of CONICET (National Scientific and Technical Research Council, Argentina). LS is a member of the Research Career of

Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC-BA), MM is a doctoral fellow of CONICET.

We appreciate Dr. Elizabeth Tymczyszyn for her invaluable help with statistical analysis.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Peptide	MW*	Ip*	NC**	µH**	H**	AC
P 5	2356.98	11.26	7	0.58	0.455	88%
P 6.2	2515.09	11.75	7	0.793	0.328	46%

Table 1 – Physicochemical parameters analyzed *in silico* and experimentally for P5 and P6.2. MW: molecular weigth (daltons), Ip: isoelectric point, NC: net charge, µH: hydrophobic moment, H: hidrophbicity, AC: alpha helix content. The percent helix values were determined based on circular dichroism spectra calculated as the mean residue molar ellipticity at 222 nm, in SDS micelles.

* http://web.expasy.org/compute_pi/

** http://heliquest.ipmc.cnrs.fr/cgi-bin/ComputParamsV2.py

	P. aeruginosa		*S. aureus*	
	P5	**P6.2**	**P5**	**P6.2**
MIC	64	16	16	32
MBC	128	32	64	128

Table 2. MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) on *P. aeruginosa* and *S. aureus* for both peptides expressed in µg/ml.

Samples	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)
1) Neutropenic	4	96	0
2) Neutropenic	2	98	0
3) Neutropenic	3	97	0
4) Control	25	74	1
5) Control	30	69	1
6) Control	32	66	2

Table 3 - Effect of I.P injected cyclophosphamide on Balb/c mice

Figures

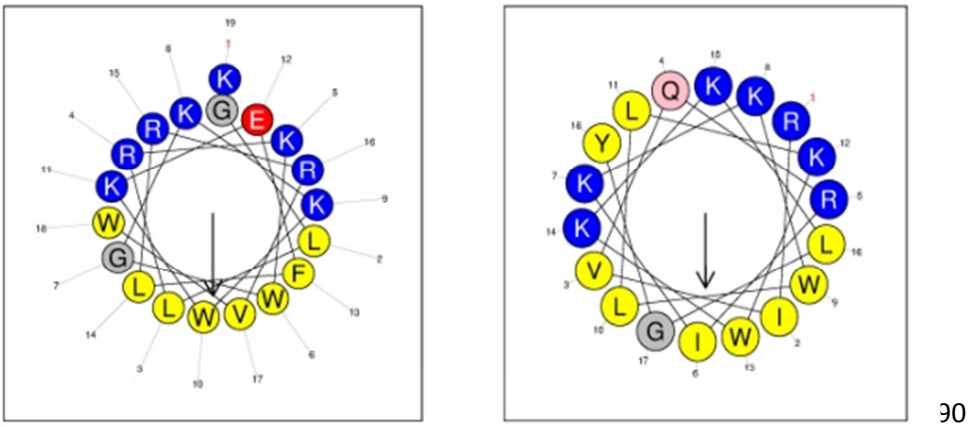

Figure 1. Helical wheel projection diagrams of the peptides, depicting the residues and their relative position in the alpha helix. Yellow circles represent hydrophobic residues and blue circles the positively-charged aminoacids, uncharged residues are painted in pink (Gln) and grey (Gly), and negatively charged residues (Glu) in red. The arrow depicts the hydrophobic moment. Left panel P6.2, right panel P5.

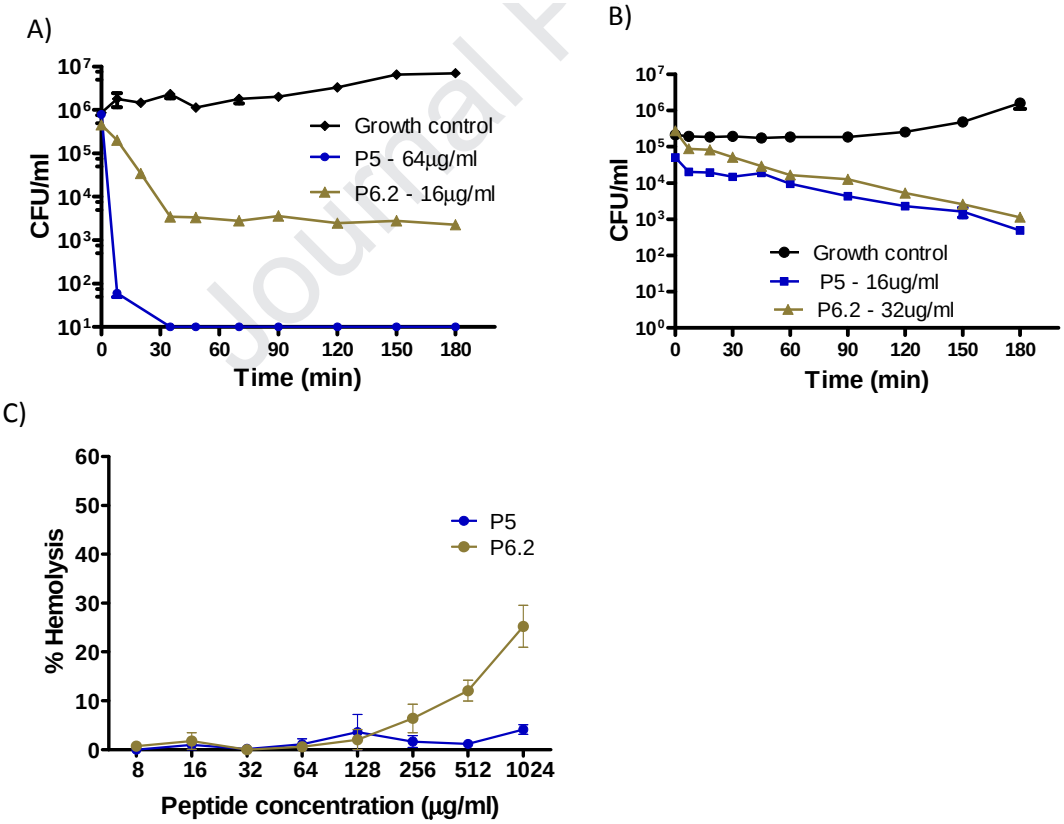

Figure 2. Killing kinetics with *P. aeruginosa* (A) and *S. aureus* (B). Each peptide was incubated at their MIC with approximately $5x10^5$ CFU/ml inoculum. Samples were taken at different intervals for three hours, plated and viable CFUs counted by triplicate. C) Hemolytic activity of P5 and P6.2 at different concentrations. Mouse erythrocytes were incubated for 1h with AMPs and trelease of hemoglobin was determined by measuring the absorbance at 540 nm.

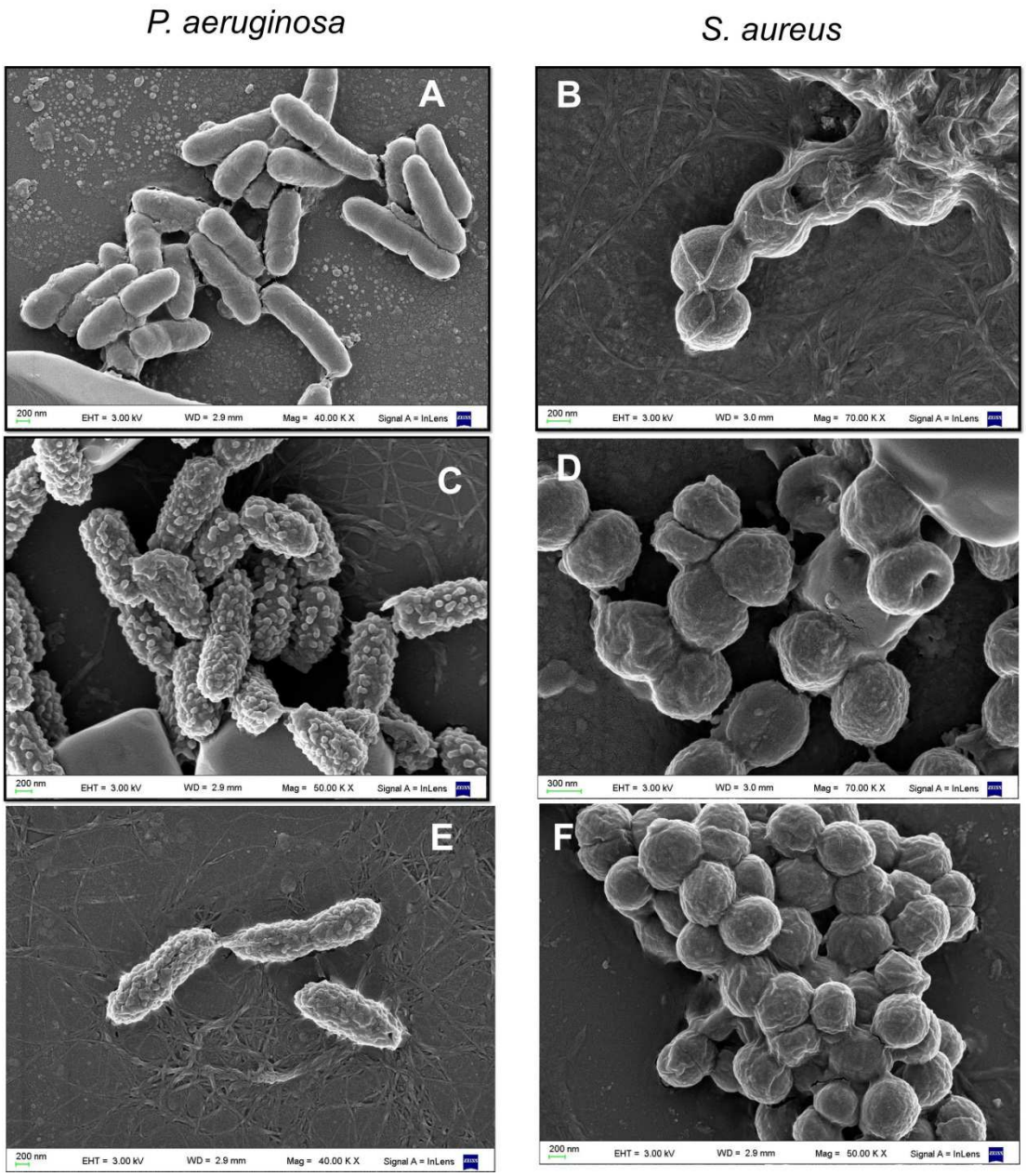

Figure 3. Scanning electron microscopy (SEM) of *P. aeruginosa* and *S. aureus*. Bacterial cells were incubated with P5 or P6.2 for 1 hour at 37ºC at their MIC. A) *P. aeruginosa* with no treatment. B) *S. aureus* with no treatment. C) *P. aeruginosa* treated with P5 D) *S. aureus* treated with P5 E) *P. aeruginosa* treated with P6.2 F) *S. aureus* treated with P6.2

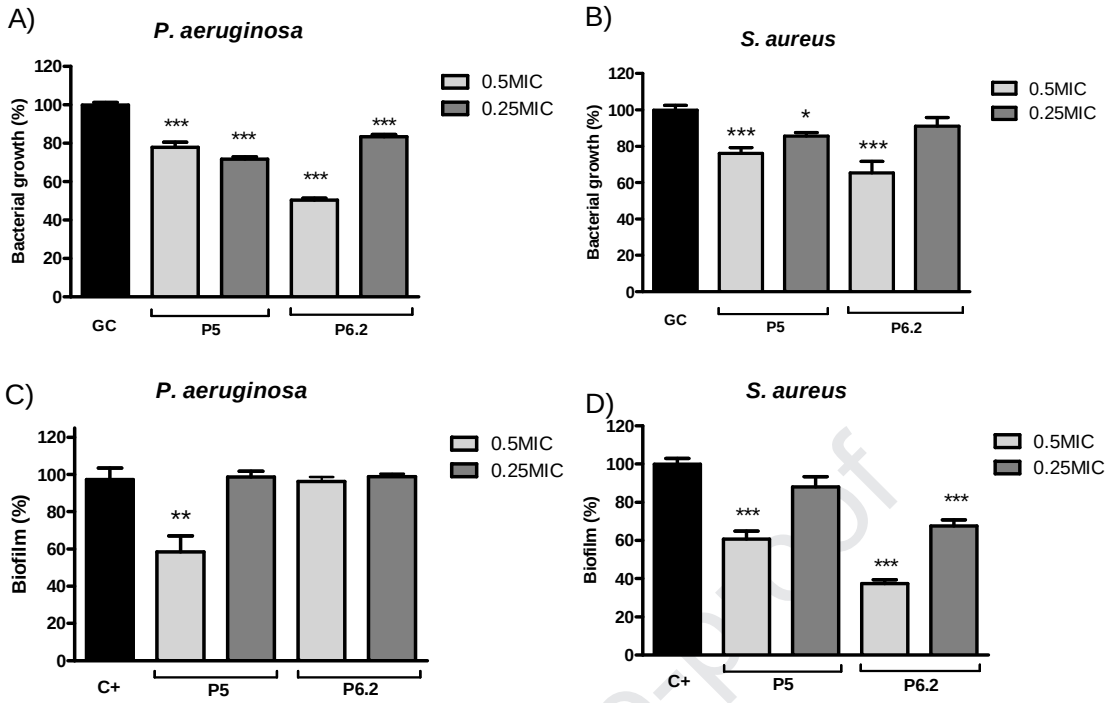

Figure 4. Bacterial growth and biofilm quantification. A, B) Bacterial growth of *P. aeruginosa* and *S. aureus* incubated with P5 or P6.2 at sub MIC concentrations. GC growth control with no antibiotic. C, D) Biofilm quantification. The total amount of biomass of *P. aeruginosa* and *S. aureus* incubated with P5 or P6.2 at sub MIC concentrations was quantified with crystal violet. One Way ANOVA - Dunnett's Multiple Comparison Test *p<0.05, **p<0.01, ***p<0.001 against GC (growth control) or C+ (positive control with no antibiotic)

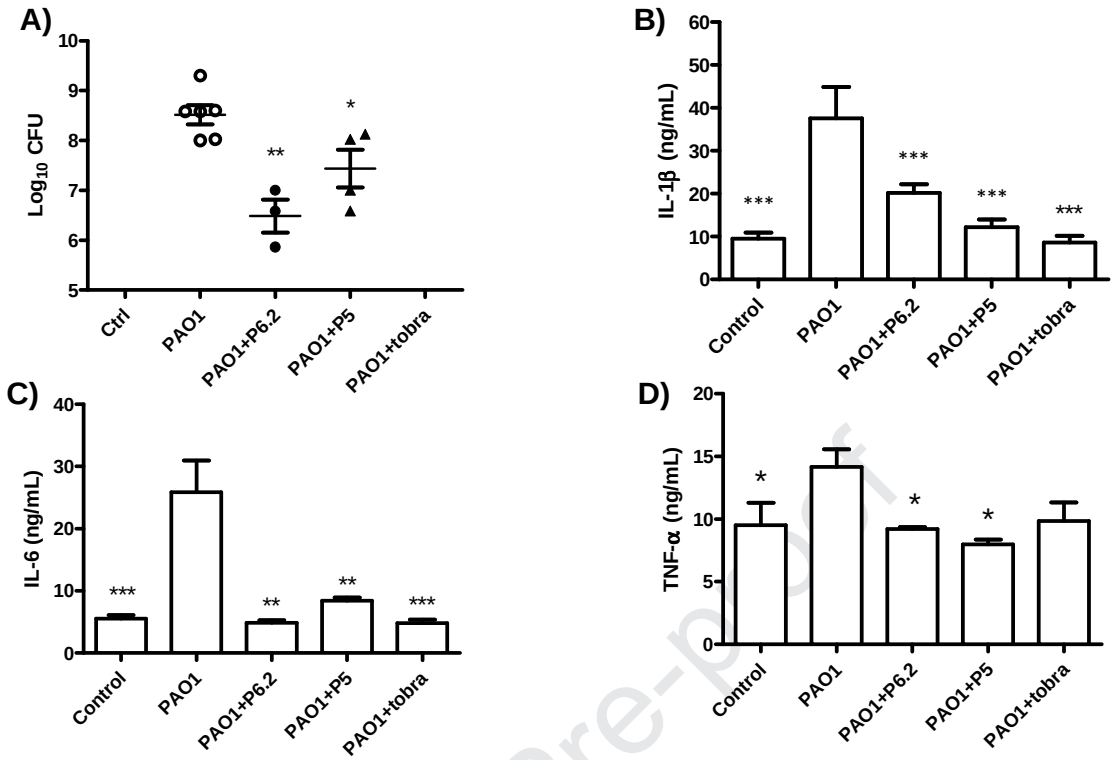

Figure 5. A) Total colony forming units (CFU) in the lung of infected neutropenic mice. Each mouse was intranassally infected with an inoculum of 10^6 CFU PAO1 and subsequently instilled with saline (PAO1), P6.2 (PAO1+P6.2), P5 (PAO1+P5) or tobramycin (PAO1+tobra). A group of mice were not infected and received saline (Control). After 20 h animals were sacrificed and their lungs surgically extracted and processed for viable bacteria counting. B, C, D) Cytokine quantification. ELISA was performed on the processed lungs samples to quantified pro inflammatory cytokines. Dunnett`s Multiple Comparison Test, *p<0.05, **p<0.01, ***p<0.001, compared to PAO1

References

[1] B. Gomes, M.T. Augusto, M.R. Felício, A. Hollmann, O.L. Franco, S. Gonçalves, N.C. Santos, Designing improved active peptides for therapeutic approaches against infectious diseases, Biotechnol. Adv. (2018). doi:10.1016/j.biotechadv.2018.01.004.

[2] M.-D. Seo, H.-S. Won, J.-H. Kim, T. Mishig-Ochir, B.-J. Lee, Antimicrobial Peptides for Therapeutic Applications: A Review, Molecules. (2012). doi:10.3390/molecules171012276.

[3] A.K. Marr, W.J. Gooderham, R.E. Hancock, Antibacterial peptides for therapeutic use: obstacles and realistic outlook, Curr. Opin. Pharmacol. (2006). doi:10.1016/j.coph.2006.04.006.

[4] A. Hollmann, M. Martinez, P. Maturana, L.C. Semorile, P.C. Maffia, Antimicrobial peptides: Interaction with model and biological membranes and synergism with chemical antibiotics, Front. Chem. 6 (2018). doi:10.3389/fchem.2018.00204.

[5] K.A. Brogden, Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria?, Nat. Rev. Microbiol. (2005). doi:10.1038/nrmicro1098.

[6] L. Grassi, G. Maisetta, G. Maccari, S. Esin, G. Batoni, Analogs of the Frog-skin Antimicrobial Peptide Temporin 1Tb Exhibit a Wider Spectrum of Activity and a Stronger Antibiofilm Potential as Compared to the Parental Peptide, Front. Chem. (2017). doi:10.3389/fchem.2017.00024.

[7] L.J. Bessa, P. Eaton, A. Dematei, A. Pĺacido, N. Vale, P. Gomes, C. Delerue-Matos, J.R.S.A. Leite, P. Gameiro, Synergistic and antibiofilm properties of ocellatin peptides against multidrug-resistant Pseudomonas aeruginosa, Future

Microbiol. (2018). doi:10.2217/fmb-2017-0175C.

[8] A. Folkesson, J.A.J. Haagensen, C. Zampaloni, C. Sternberg, S. Molin, Biofilm induced tolerance towards antimicrobial peptides, PLoS One. (2008). doi:10.1371/journal.pone.0001891.

[9] R. Nuri, T. Shprung, Y. Shai, Defensive remodeling: How bacterial surface properties and biofilm formation promote resistance to antimicrobial peptides, Biochim. Biophys. Acta - Biomembr. (2015). doi:10.1016/j.bbamem.2015.05.022.

[10] V. Luca, A. Stringaro, M. Colone, A. Pini, M.L. Mangoni, Esculentin(1-21), an amphibian skin membrane-active peptide with potent activity on both planktonic and biofilm cells of the bacterial pathogen Pseudomonas aeruginosa, Cell. Mol. Life Sci. (2013). doi:10.1007/s00018-013-1291-7.

[11] Y. Lequette, E.P. Greenberg, Timing and localization of rhamnolipid synthesis gene expression in Pseudomonas aeruginosa biofilms, in: J. Bacteriol., 2005. doi:10.1128/JB.187.1.37-44.2005.

[12] E. Drenkard, F.M. Ausubel, Pseudomonas biofilm formation and antibiotic resistance are linked to phenotypic variation, Nature. (2002). doi:10.1038/416740a.

[13] C. De la Fuente-Núñez, F. Reffuveille, L. Fernández, R.E.W. Hancock, Bacterial biofilm development as a multicellular adaptation: Antibiotic resistance and new therapeutic strategies, Curr. Opin. Microbiol. (2013). doi:10.1016/j.mib.2013.06.013.

[14] N. Mookherjee, R.E.W. Hancock, Cationic host defence peptides: Innate immune regulatory peptides as a novel approach for treating infections, Cell. Mol. Life Sci. (2007). doi:10.1007/s00018-007-6475-6.

[15] N. Mookherjee, L.M. Rehaume, R.E. Hancock, Cathelicidins and functional analogues as antisepsis molecules, Expert Opin. Ther. Targets. (2007). doi:10.1517/14728222.11.8.993.

[16] A. Giacometti, O. Cirioni, R. Ghiselli, F. Mocchegiani, M.S. Del Prete, C. Viticchi, W. Kamysz, E. Łempicka, V. Saba, G. Scalise, Potential therapeutic role of cationic peptides in three experimental models of septic shock, Antimicrob. Agents Chemother. (2002). doi:10.1128/AAC.46.7.2132-2136.2002.

[17] F. Seiler, C. Beisswenger, F. Langer, J. Hellberg, M. Bischoff, R. Bals, F. Lammert, A. Kamyschnikow, P.M. Lepper, H.-J. Schafers, M.D. Menger, C. Herr, FOXO Transcription Factors Regulate Innate Immune Mechanisms in Respiratory Epithelial Cells, J. Immunol. (2013). doi:10.4049/jimmunol.1200596.

[18] T.J. Bensman, J.G. Jayne, M. Sun, E. Kimura, J. Meinert, J.C. Wang, J.B. Schaal, D. Tran, A.P. Rao, O. Akbari, M.E. Selsted, P.M. Beringer, Efficacy of Rhesus Theta-Defensin-1 in Experimental Models of Pseudomonas aeruginosa Lung Infection and Inflammation, Antimicrob. Agents Chemother. 61 (2017) e00154-17. doi:10.1128/AAC.00154-17.

[19] D. Faccone, O. Veliz, A. Corso, M. Noguera, M. Martínez, C. Payes, L. Semorile, P.C. Maffía, Antimicrobial activity of de novo designed cationic peptides against multi-resistant clinical isolates, Eur. J. Med. Chem. 71 (2014). doi:10.1016/j.ejmech.2013.10.065.

[20] A. Hollmann, M. Martínez, M.E. Noguera, M.T. Augusto, A. Disalvo, N.C. Santos, L. Semorile, P.C. Maffía, Role of amphipathicity and hydrophobicity in

the balance between hemolysis and peptide-membrane interactions of three related antimicrobial peptides, Colloids Surfaces B Biointerfaces. 141 (2016). doi:10.1016/j.colsurfb.2016.02.003.

[21] P. Maturana, M. Martinez, M.E. Noguera, N.C. Santos, E.A. Disalvo, L. Semorile, P.C. Maffia, A. Hollmann, Lipid selectivity in novel antimicrobial peptides: Implication on antimicrobial and hemolytic activity, Colloids Surfaces B Biointerfaces. 153 (2017). doi:10.1016/j.colsurfb.2017.02.003.

[22] Z. Oren, Y. Shai, Selective lysis of bacteria but not mammalian cells by diastereomers of melittin: Structure-function study, Biochemistry. (1997). doi:10.1021/bi962507l.

[23] C. Subbalakshmi, R. Nagaraj, N. Sitaram, Biological activities of C-terminal 15-residue synthetic fragment of melittin: Design of an analog with improved antibacterial activity, FEBS Lett. (1999). doi:10.1016/S0014-5793(99)00328-2.

[24] W. Hong, X. Gao, P. Qiu, J. Yang, M. Qiao, H. Shi, D. Zhang, C. Tian, S. Niu, M. Liu, Synthesis, construction, and evaluation of self-assembled nano-bacitracin a as an efficient antibacterial agent in vitro and in vivo, Int. J. Nanomedicine. (2017). doi:10.2147/IJN.S136998.

[25] D. Jabés, C. Brunati, G.P. Candiani, S. Riva, G. Romanó, S. Donadio, Efficacy of the new lantibiotic NAI-107 in experimental infections induced by multidrug-resistant gram-positive pathogens, Antimicrob. Agents Chemother. (2011). doi:10.1128/AAC.01288-10.

[26] M. Hartmann, M. Berditsch, J. Hawecker, M.F. Ardakani, D. Gerthsen, A.S. Ulrich, Damage of the bacterial cell envelope by antimicrobial peptides gramicidin S and PGLa as revealed by transmission and scanning electron microscopy, Antimicrob. Agents Chemother. (2010). doi:10.1128/AAC.00124-

626 10.
627 [27] S. Yenugu, K.G. Hamil, F.S. French, S.H. Hall, Antimicrobial actions of the
628 human epididymis 2 (HE2) protein isoforms, HE2alpha, HE2beta1 and
629 HE2beta2, Reprod. Biol. Endocrinol. (2004). doi:10.1186/1477-7827-2-61.
630 [28] A. Da Silva, O. Teschke, Effects of the antimicrobial peptide PGLa on live
631 Escherichia coli, Biochim. Biophys. Acta - Mol. Cell Res. (2003).
632 doi:10.1016/j.bbamcr.2003.10.001.
633 [29] G. Batoni, G. Maisetta, S. Esin, Antimicrobial peptides and their interaction with
634 biofilms of medically relevant bacteria, Biochim. Biophys. Acta - Biomembr.
635 (2016). doi:10.1016/j.bbamem.2015.10.013.
636 [30] G. Batoni, F. Lisa Brancatisano, M. Campa, G. Maisetta, S. Esin, Use of
637 Antimicrobial Peptides Against Microbial Biofilms: Advantages and Limits,
638 Curr. Med. Chem. (2011). doi:10.2174/092986711794088399.
639 [31] M. di Luca, G. Maccari, R. Nifosí, Treatment of microbial biofilms in the post-
640 antibiotic era: Prophylactic and therapeutic use of antimicrobial peptides and
641 their design by bioinformatics tools, Pathog. Dis. (2014). doi:10.1111/2049-
642 632X.12151.
643 [32] L. Segev-Zarko, R. Saar-Dover, V. Brumfeld, M.L. Mangoni, Y. Shai,
644 Mechanisms of biofilm inhibition and degradation by antimicrobial peptides,
645 Biochem. J. (2015). doi:10.1042/bj20141251.
646 [33] J. Overhage, A. Campisano, M. Bains, E.C.W. Torfs, B.H.A. Rehm, R.E.W.
647 Hancock, Human host defense peptide LL-37 prevents bacterial biofilm
648 formation, Infect. Immun. (2008). doi:10.1128/IAI.00318-08.
649 [34] C. Nagant, B. Pitts, K. Nazmi, M. Vandenbranden, J.G. Bolscher, P.S. Stewart,
650 J.P. Dehaye, Identification of peptides derived from the human antimicrobial

peptide LL-37 active against biofilms formed by Pseudomonas aeruginosa using a library of truncated fragments, Antimicrob. Agents Chemother. (2012). doi:10.1128/AAC.00918-12.

[35] J. Overhage, A. Campisano, M. Bains, E.C.W. Torfs, B.H.A. Rehm, R.E.W. Hancock, Human host defense peptide LL-37 prevents bacterial biofilm formation, Infect. Immun. (2008). doi:10.1128/IAI.00318-08.

[36] D. Annane, P. Aegerter, M.C. Jars-Guincestre, B. Guidet, Current epidemiology of septic shock: The CUB-Réa network, Am. J. Respir. Crit. Care Med. (2003). doi:10.1164/rccm.2201087.

[37] M. Coorens, M.R. Scheenstra, E.J.A. Veldhuizen, H.P. Haagsman, Interspecies cathelicidin comparison reveals divergence in antimicrobial activity, TLR modulation, chemokine induction and regulation of phagocytosis, Sci. Rep. (2017). doi:10.1038/srep40874.

[38] M. Lange, Y. Nakano, D.L. Traber, A. Hamahata, L.D. Traber, P. Enkhbaatar, Time course of the inflammatory and oxidative stress response to pulmonary infection in mice, Exp. Lung Res. (2012). doi:10.3109/01902148.2012.663453.

[39] Y. Rosenfeld, N. Papo, Y. Shai, Endotoxin (lipopolysaccharide) neutralization by innate immunity host-defense peptides: Peptide properties and plausible modes of action, J. Biol. Chem. (2006). doi:10.1074/jbc.M504327200.

[40] G.D. Amatngalim, A. Nijnik, P.S. Hiemstra, R.E.W. Hancock, Cathelicidin peptide LL-37 modulates TREM-1 expression and inflammatory responses to microbial compounds, Inflammation. (2011). doi:10.1007/s10753-010-9248-6.

[41] M. Coorens, B.J.H. Banaschewski, B.J. Baer, C. Yamashita, A. van Dijk, H.P. Haagsman, R.A.W. Veldhuizen, E.J.A. Veldhuizen, Killing of Pseudomonas aeruginosa by chicken cathelicidin-2 is immunogenically silent, preventing lung

676 inflammation in vivo, Infect. Immun. (2017). doi:10.1128/IAI.00546-17.
677 [42] E.M. Molhoek, A. van Dijk, E.J.A. Veldhuizen, H. Dijk-Knijnenburg, R.H.
678 Mars-Groenendijk, L.C.L. Boele, W.E. Kaman-van Zanten, H.P. Haagsman, F.J.
679 Bikker, Chicken cathelicidin-2-derived peptides with enhanced
680 immunomodulatory and antibacterial activities against biological warfare agents,
681 Int. J. Antimicrob. Agents. (2010). doi:10.1016/j.ijantimicag.2010.06.001.
682 [43] Y. Xiao, Y. Cai, Y.R. Bommineni, S.C. Fernando, O. Prakash, S.E. Gilliland, G.
683 Zhang, Identification and functional characterization of three chicken
684 cathelicidins with potent antimicrobial activity, J. Biol. Chem. (2006).
685 doi:10.1074/jbc.M507180200.
686 [44] C. Gabay, C. Lamacchia, G. Palmer, IL-1 pathways in inflammation and human
687 diseases, Nat. Rev. Rheumatol. (2010). doi:10.1038/nrrheum.2010.4.
688
689
690
691
692

HIGHLIGHTS

- Two novel AMPs P5 and P6.2 were analyzed in *P.aeruginosa* and *S. aureus*
- Scanning electron microscopy revealed distinct membranolytic activity
- Both peptides display *in vitro* antibiofilm activity
- Peptides diminished *P.aeruginosa* burden in the lungs of neutropenic mice
- P5 and P6.2 diminished lung pro-inflammatory cytokines