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Articoli originali

Original articles

In vitro and *in vivo* effects of sub-MICs of pexiganan and imipenem on *Pseudomonas aeruginosa* adhesion and biofilm development

Effetti in vitro *e* in vivo *di sub-MIC di pexiganan e imipenem sull'adesione e sviluppo di biofilm di* Pseudomonas aeruginosa

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INTRODUCTION

Pseudomonas aeruginosa is an important opportunistic human pathogen responsible for nosocomial pneumonia, catheter and urinary tract infections, and sepsis in burn wound and immunocompromised patients [1-5]. It is the best described bacterium with regards to *in vitro* biofilm formation and development of antibiotic tolerance [3, 6-8]. *P. aeruginosa* biofilms are thought to be the underlying cause of many chronic infections and medical device infections, including the colonization of catheters and implants such as joints or stents [7, 9-11]. Biofilm is a structured community of microorganisms irreversibly encapsulated within a self-developed polymeric matrix and adherent to various biotic and abiotic surfaces [6, 12-15]. Once established, it constitutes a protected mode of growth that promotes survival in an hostile environment and it is difficult to treat due to its high inherent resistance to antimicrobial agents [6, 13, 14, 16]. For this reason, it is of primary importance to focus on the development of new strategies to prevent and to treat biofilm formation. Several studies showed that during antibiotic treatment there are periods of time when antimicrobials are below the minimal inhibitory concentration (sub-MIC), and this can occur in an intermittent way at the site of infection [7, 8, 17, 18]. In addition, the MIC of sessile or stationary phase bacteria is considerably higher compared with the MIC of exponential planktonic bacteria, therefore it is probable that antimicrobial compounds are at sub-MIC for sessile bacteria [7, 8]. Adherence and biofilm formation are some of the bacterial virulence parameters influenced by sub-MIC antibiotics [6-8, 17].

A growing body of research has revealed that antimicrobial peptides are an important com-

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ponent of the innate defences of all species of life used to effectively deal with microbes in their environment [19-22]. They are active against a wide spectrum of pathogens, including antibiotic-resistant bacteria. They kill very rapidly and are synergistic with conventional antibiotics [21, 23, 24].

Pexiganan is a 22-amino acid synthetic analogue of the peptide magainin II and it is engineered for potency against many bacteria. *In vitro*, pexiganan has activity against most clinical bacterial isolates cultured from infected diabetic foot ulcers [25-28]. In this study, we used sub-MICs of pexiganan and imipenem as a tool to assess the relevance of inhibiting adhesion as a way to prevent subsequent biofilm formation. Firstly, we studied the changes in both initial adhesion and in biofilm formation of a clinical isolate of slime-producer *P. aeruginosa* growing in the presence of sub-MICs of the above mentioned antibiotics. Later, we used the same strain previously treated with two antibiotics in an animal model of ureteral stent infection to verify the kind of impact that this treatment had also *in vivo* on the ability of the bacterial strain to produce biofilm.

MATERIALS AND METHODS

Antimicrobial agents and in vitro study

Pexiganan was synthesized by 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase chemistry according to the following procedure:

1) 2 and 20 min deprotection steps using 20% piperidine in dimethylformamide (DMF) in the presence of 1% Triton;
2) the coupling reactions carried out with the protected aminoacid diluted in DMF in the presence of 1% Triton using diisopropylcarbodiimide (DIC) as the coupling reagent in the presence of 1-hydroxybenzotriazole (HOBt) for 2 h.

The completeness of each coupling reaction was monitored by the chloranil test [26]. The protected peptidyl resin was treated with the mixture: 95% trifluoroacetic acid (TFA), 2.5% water and 2.5% triisopropylsilane (TIS) for 2 h. After cleavage the solid support was removed by filtration, and the filtrate was concentrated under reduced pressure. The cleaved peptide was precipitated with diethyl ether and lyophilized. Pexiganan was purified by high-pressure liquid chromatography (HPLC) on a Knauer K501 two-pump system. The resulting fractions with purity greater than 97-98% were tested by HPLC. The peptide was analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF).

Preparation of sterile stock solutions of imipenem (Merck Sharp and Dohme Italia S.p.A., Rome Italy) and pexiganan was performed according to the manufacturer's instructions. Determination of the MIC range for each strain was carried out according to CLSI standards [29]. The sub-MIC used was 0.125, 0.25, and 0.5 of the lowest MIC value, whenever just one antibiotic was added to the bacterial cell suspension. These concentrations were not high enough to inhibit bacterial growth.

Substrate preparation

Ureteral stents (Biosoft®Duo, Porges-Mentor, France) were cut into 10 × 10 mm squares and then immersed in a 0.2% solution of a commercial detergent overnight, after which they were transferred to a new solution of 0.2% of a commercial detergent and washed at 40°C with strong agitation for 5 min. The squares were then rinsed thoroughly with distilled water followed by rinsing with ultra-pure water and dried at 60 °C, overnight. For biofilm assays, surfaces were heat-sterilized by immersion in distilled water and autoclaving at 121°C for 15 min.

Preparation of the cells

A clinical isolate of slime-producer *P. aeruginosa* (AN207) was used in this study. Bacteria were stored at –70°C in brain-heart infusion medium (Merck Italia, Milano Italy) containing 20% glycerol. Tryptic soy broth (TSB) and tryptic soy agar (TSA) (Oxoid S.p.A., Milan, Italy) were prepared according to the manufacturer's instructions. The strain was inoculated into 15 ml of TSB from TSA plates not older than 2 days. Liquid cultures were grown for 24 (±2) h at 37°C in an orbital shaker at 130 rpm. The cells were harvested by centrifugation (for 5 min at 1000 *g* at 4°C), then washed and resuspended in a saline solution (0.9% NaCl prepared in distilled water) to an optical density equivalent to 1×10^9 cells ml^{-1}. This suspension was used in the biofilm assays. For adherence assays, 1 ml of this cell suspension was transferred to 30 ml of fresh TSB containing sub-MICs of antibiotics (0.125, 0.25, and 0.50↔MIC), and incubated for 18 (±2) h at 37°C with shaking at 130 rpm. After being harvested by centrifugation, cells were washed twice and resuspended in a saline solu-

tion (0.9% NaCl prepared in distilled water) and adjusted to an optical density equivalent to 1×10^9 cells ml^{-1} and used in the adherence assays [7,30].

Evaluation of adherence

The study was performed according to the procedure described by Cerca with some modification. Briefly, squares of ureteral stent were placed in 6-well tissue-culture plates containing 6 ml of a cell suspension grown in the presence of sub-MICs of antibiotics and adjusted to an optical density equivalent to 1×10^9 cells ml^{-1}. Initial adhesion to substrate was allowed to occur for 2 h at 37°C in a shaker at 120 rpm. Negative controls were obtained by placing acrilic squares in 6-well tissue-culture plates containing 6 ml of bacteria grown in fresh TSB without antibiotics. All squares were then carefully washed by immersion and dried at 37°C. All experiments were done in triplicate [7, 30]. For image observation and enumeration of adherent bacterial cells, the acrylic squares were stained with a 0.2% safranin solution, for contrast. Direct bacterial counts were done using a phase contrast microscope coupled to a 3CCD video camera [30]. For each surface analyzed, 20 images were taken. Cells were counted using automated enumeration software.

Evaluation of biofilm

Sterilized ureteral stent squares were placed in 6-well tissue culture plates containing 6 ml of TSB supplemented with 0.25% of glucose and the respective amount of antibiotic (0.125, 0.25, and 0.50↔MIC). Then 200 µl of a 0.9% NaCl solution containing 1×10^9 cells ml^{-1} were added and growth was allowed to occur for 48 h at 37°C in a shaker at 120 rpm. Every 8 h the TSB medium containing suspended bacterial cells was removed and an equal volume of fresh TSB with 0.25% glucose and antibiotic were added. Negative controls were obtained by incubating the surfaces in TSB supplemented with 0.25% glucose and antibiotics without adding any bacterial cells. All experiments were done in triplicate [30].

Biofilm was evaluated by dry-weight determinations. The colonized surfaces were removed from the plates and placed at 80°C overnight. The weight of them was determined on a digital scale. Surfaces were placed again at 80°C for 2 additional hours and weighed again, to check the stability of the dry weight. Then, the biofilm was mechanically removed from the surface, and the surfaces were thoroughly cleaned with 0.2% commercial detergent solution. Cleaned surfaces were kept overnight at 80°C prior to a third weight determination. The difference in the weight of the surface with and without the biomass attached is the biofilm dry-weight.

Evaluation of antibiotic resistance

The MICs for bacterial cells in biofilm formed in presence and absence of sub-inhibitory concentrations of imipenem and pexiganan were determined using the CLSI protocol [29]. Briefly, biofilm was scraped from the substratum surface, resuspended in TSB, and sonicated for 10 s at 20 W, and the preparations were adjusted to obtain a standard cell inoculum and incubated in 96-well microtiter plates with several twofold dilutions of imipenem and pexiganan for 24 h at 37°C in TSB. All experiments were done in triplicate.

Animal model

Adult female Wistar rats (weight range 170 to 230 g) (n=5) were used. Study included a control group (C_0) without bacterial challenge to evaluate the sterility of surgical procedure, a control group (C_1) infected with *P. aeruginosa* strain that was not previously treated and two groups (imipenem and pexiganan) infected with *P. aeruginosa* strain that was previously treated with imipenem or pexiganan. In the animal studies, for each antibiotic was chosen the concentration that was the most effective to inhibit adhesion and biofilm. Experiments were performed in duplicate (10 groups, each composed by 5 animals). For statistical analysis, the data were pooled and referred to all 10 animals from each pair of groups.

The rats were anesthetized by an intramuscular injection of ketamine and xylazine (30 mg/kg and 8 mg/kg respectively), the hair was shaved and the skin cleansed with a 10% povidone-iodine solution. The bladder was exposed through a suprapubic incision and opened at the dome [31]. After cystotomy, stents were inserted into the bladder. The bladder was sutured with 000 surgical silk. After the surgical intervention, a saline solution (1 ml) containing $2\leftrightarrow10^7$ CFU/ml of the clinical isolate was inoculated into the bladder using a tuberculin syringe. The animals were returned to individual cages and thoroughly examined daily. Twenty-four hours after ureteral stent placement, urine cultures were performed through a transvesical sample taken by an insuline syringe, to verify

the sterility or infection. Ureteral stents were explanted at day 5 following implantation and biofilm bacteria enumerated as described [31]. The study was approved by the animal research ethics committee of the I.N.R.C.A.-I.R.R.C.S., Ancona, Italy. The limit of detection for this method was approximately 10 CFU/ml.

Statistical analysis

MIC values are presented as the geometric mean of three separate experiments. Quantitative culture results regarding the *in vivo* experiments were presented as mean ± S.D. of the mean and the statistical comparisons between groups were made using analysis of variance (ANOVA) on the log-transformed data with Tukey-Kramer Honestly Significant Difference Test. Significance was accepted when the *P* value was ≤0.05.

RESULTS

In vitro studies

The clinical isolate was susceptible to both the antibiotics with MICs of 2 and 4 mg/L for imipenem and pexiganan, respectively. Results studying the effects of the growth with sub-MICs of antibiotics on bacterial adherence to ureteral stent showed that both the compounds prevented initial adherence at each MIC concentration used. Both for imipenem and pexiganan, 0.5×MIC was the most effective concentration able to inhibit adhesion (Figure 1). Imipenem was the highest effective antibiotic (average reduction of 42±5%). Pexiganan was slightly less effective reaching nearly 30% inhibition.

Data concerning biofilm formation on stent surface similarly showed a good activity of both antibiotics. Also in this case, imipenem was the compound with strongest activity. When using antibiotics at 0.5 of the MIC, imipenem and pexiganan prevent biofilm formation as average reduction of 34±8% and 27±4%, respectively (Figure 2).

In antibiotic resistance studies, a slight increase in resistance was observed for imipenem (median 1 mg/L, range 1-2 mg/L) in the cell grown in presence of imipenem, while no changes in the MIC of pexiganan for the colonies recovered from pexiganan-containing agar plates

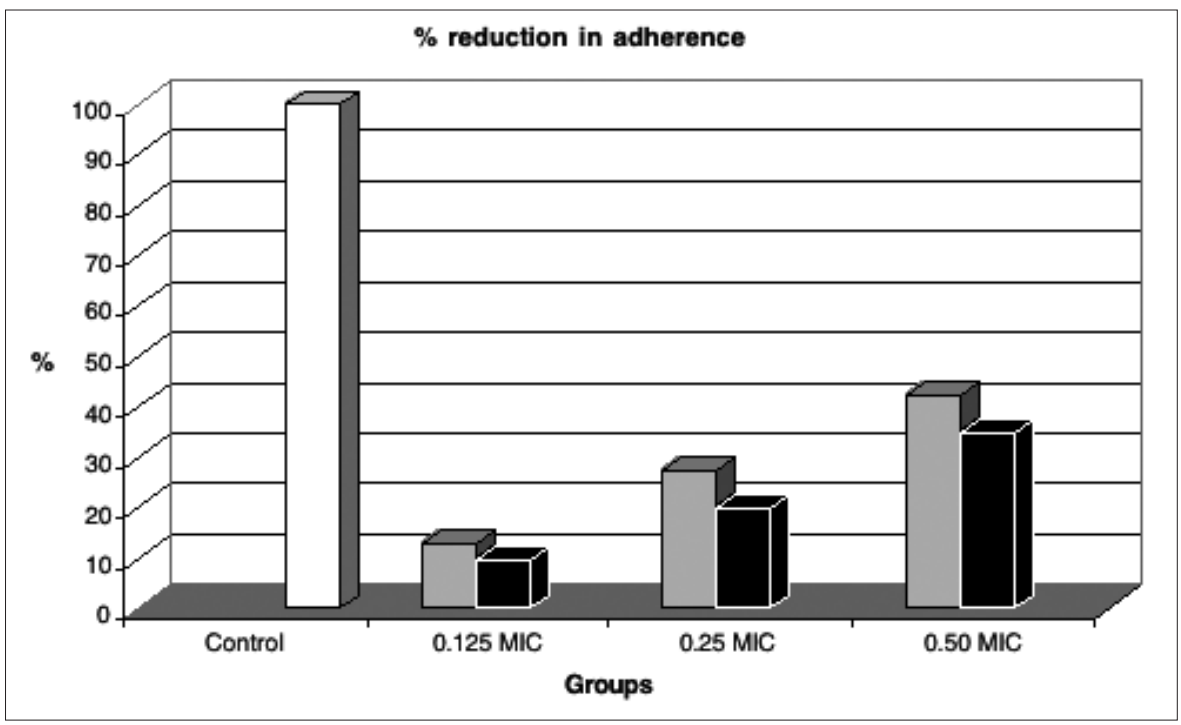

Figure 1 - Influence of sub-MICs concentrations of imipenem and pexiganan on adhesion of *Pseudomonas aeruginosa*. Bacteria were grown without antibiotics (white bar), with sub-inhibiting concentrations (0.125, 0.25, and 0.50↔MIC) of imipenem (grey bars), and with sub-inhibiting concentrations (0.125, 0.25, and 0.50↔MIC) of pexiganan (black bars).

were found compared with the MIC of the original inoculum. Finally, no cross-resistance was documented.

In vivo studies

Uncontaminated control group had not microbiological evidence of stent infection. Infected control group C_1 and the two imipenem and pexiganan groups demonstrated evidence of infection. Nevertheless, the groups treated with imipenem and pexiganan showed quantitative culture results significantly lower than C_1 ($3.3\leftrightarrow10^2\pm0.4\leftrightarrow10^2$, $5.0\leftrightarrow10^2 \pm 0.9\leftrightarrow10^2$ and $6.8\leftrightarrow10^6\pm1.2\leftrightarrow10^6$ CFU/ml, respectively). Differently, urine cultures showed the presence of infection without significant differences in all infected groups with a bacterial load of 10^5 for C_1, imipenem and pexiganan groups (Table 1).

DISCUSSION

Several studies showed that low concentrations of antimicrobial agents are potentially able to prevent bacterial adhesion to surfaces and the subsequent step of biofilm formation. Sub-

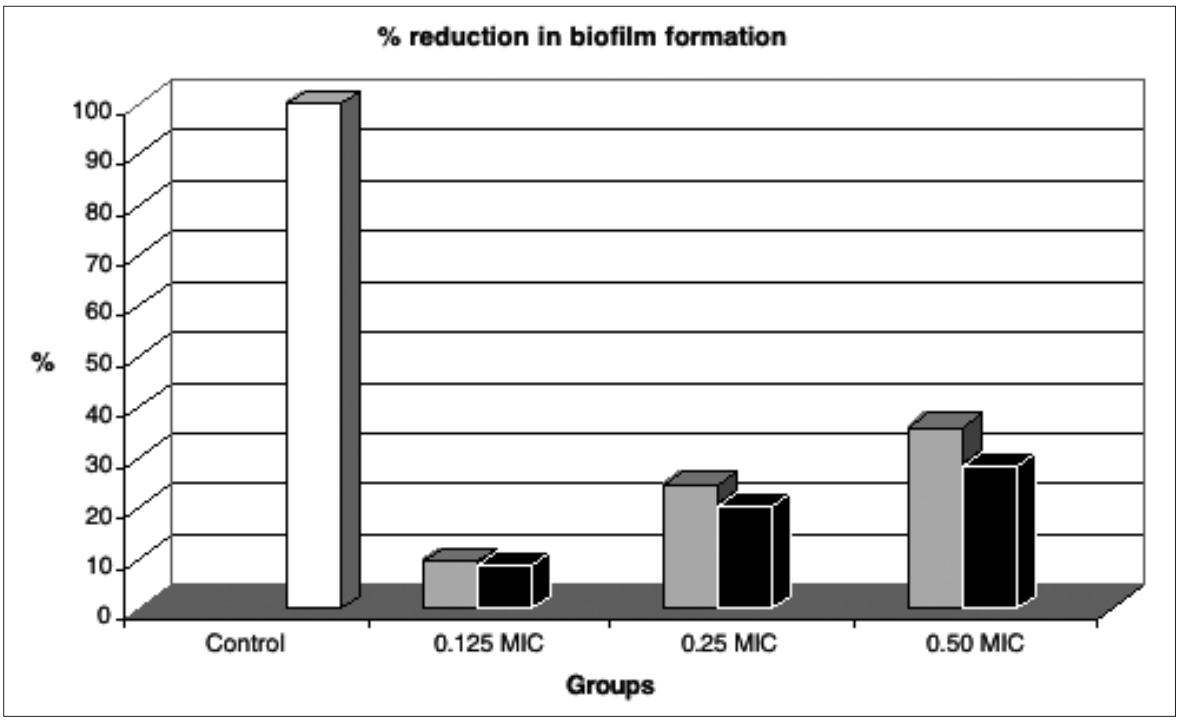

Figure 2 - Influence of sub-MICs concentrations of imipenem and pexiganan on biofilm formation of *Pseudomonas aeruginosa*. Bacteria were grown without antibiotics (white bar), with sub-inhibiting concentrations (0.125, 0.25, and 0.50↔MIC) of imipenem (grey bars), and with sub-inhibiting concentrations (0.125, 0.25, and 0.50↔MIC) of pexiganan (black bars

Table 1 - Activity of sub-MICs of imipenem and pexiganan on clinical isolate of Pseudomonas aeruginosa in a rat model of ureteral stent infection.

Group[a]	*Quantitative stent culture (CFU/ml)*[d]	*Urine culture (CFU/ml)*
Control C_0	<10	<10
Group C_1[a]	$6.8\leftrightarrow10^6\pm1.2\leftrightarrow10^6$	$7.5\leftrightarrow10^5\pm1.4\leftrightarrow10^5$
Imipenem Group[b]	$3.3\leftrightarrow10^2\pm0.4\leftrightarrow10^{2e}$	$4.3\leftrightarrow10^5\pm0.7\leftrightarrow10^5$
Pexiganan Group c	$5.0\leftrightarrow10^2\pm0.9\leftrightarrow10^{2e}$	$6.2\leftrightarrow10^5\pm1.1\leftrightarrow10^5$

[a]*P. aeruginosa* slime producer not pre-treated with sub-MICs of antibiotics; [b]*P. aeruginosa* slime producer pre-treated with 0.5 MIC of imipenem; [c]*P. aeruginosa* slime producer pre-treated with 0.5 MIC of pexiganan; [d]The limit of detection for the method was ≤10 CFU/ml; [e]Statistically significant when compared with the untreated control group and control group C_1.

MICs of antibiotics can affect bacterial adhesion abilities in different ways, primarily by the formation of altered adhesins, by increase in the surface proteins levels or by the potential release of adhesins (LPS) from the bacterial cells surface [7, 31-33].

Biofilm is an ancient and integral component of the prokaryotic life cycle being a key factor for survival in diverse environments [6, 34, 35]. In the human host, biofilm exists as a community of sessile bacteria embedded in a matrix of extracellular polymeric substances they have produced, which adhere to a foreign body or a mucosal surface with impaired host defense or ample roughness. Biofilm on medical devices is up to 1000 fold more tolerant to antibiotics [36]. The management of these materials with biofilm-based infection remains problematic and antibiotic therapy, although is effective in eliminating circulating bacteria it fails to protect the surfaces of the materials from colonisation with important risk of complications or recurrence [6].

This fact might be drawn for other possible interventions being considered to reduce the incidence of device-related infections, such as use of biomaterials with low intrinsic binding of microbes. It has previously been demonstrated that the initial adherence and subsequent biofilm formation by bacterial strains are two distinct phenomena [7, 8]. For this reason, the aim of this study was to increase the knowledge about the influence of antibiotic-induced changes of *P. aeruginosa* strains on its ability to adhere to surfaces and to form biofilm. In this study, we decided to determine if growth of *P. aeruginosa* in the presence of sub-MICs of imipenem and pexiganan was equally effective at preventing initial adherence and subsequent biofilm formation on ureteral stent surfaces. We evaluated their effects on either initial adhesion or biofilm formation using bacteria grown in low concentrations of them.

In our study, the clinical isolate was able to adhere in great extent to acrylic in the absence of antibiotics. Both the antibiotics prevented initial adhesion and biofilm formation but the most effective antibiotic was imipenem. Several studies showed that this decrease under the influence of sub-MIC could be due to the changes in hydrophobicity that decreases significantly in both growth phases, to the change in the bacteria shape, or finally to the changes in swimming and twitching abilities that decreases significantly in both growth phases [7, 8]. Other studies showed that, despite some similarities in data of adherence and biofilm inhibition assays, adherence inhibition assays cannot completely predict the outcome in terms of biofilm formation [7, 8, 33, 34, 37]. Nevertheless, it seems that the two antibiotics have a significant effect in preventing *P. aeruginosa* adhesion and also biofilm formation to surface. It is well known that standard bacterial susceptibility tests (with planktonic cells) often demonstrated higher susceptibility of bacteria to antibiotics, but these do not always show the same ability to prevent initial adhesion and biofilm formation. For this reason, standard bacterial susceptibility tests are not able to reveal the potential of an antibiotic to inhibit biofilm formation. Interestingly the antibiotic resistance studies showed that, although pexiganan was less effective in the prevention of adhesion than imipenem, no changes in its MIC for the colonies recovered from pexiganan-containing agar plates were found compared with the MIC of the original inoculum. In order to evaluate whether the *in vitro* data were corroborated by an *in vivo* study, we also realized a model of ureteral stent-associated pseudomonas infection. In this model, we used both the wild strain and the strain that has previously been treated with pexiganan or imipenem.

The results presented here show how the pre-treated strain had reduced its ability to adhere and then to form biofilm on medical device. This has been documented by the reduction in bacterial load on the ureteral stent tissue. Interestingly, the urine cultures showed no difference in bacterial growth for the wild and the pre-treated strain showing that the latter had reduced the ability to form biofilm but retained its ability to grow and cause infection. We can hypothesize that the pre-treatment of the strain with antibiotics can interfere with biofilm formation at different levels, but has less impact on the production of extracellular virulence factors. Concerning pexiganan, the interaction of antimicrobial peptides with bacterial cells is not yet fully understood, although they are known to have various targets within the bacterial cell, including cell membranes, DNA, RNA, and cellular proteins, among others [22, 38]. Particularly, recent studies showed that LL-37 affects the development of biofilms in at least three ways. First, the initial attachment of bacterial cells to the surface was significantly reduced in the presence of LL-37 with a smaller number of bacteria actually involved in the initial steps of

biofilm development. Second, LL-37 promotes twitching, a specialized form of surface motility mediated by the type IV pili. This increase of motility would cause bacteria to wander across the surface instead of forming biofilms. Third, LL-37 affects the two major quorum-sensing systems of *P. aeruginosa* by down regulating key components [22]. Other studies showed that antimicrobial peptides, as lactoferrin, were able to inhibit bacterial biofilm formation due to their iron-chelating properties, which also resulted in increased twitching motility [39]. Similarly to these studies, our data showed that the antibiofilm properties of pexiganan alone and in combination with imipenem could provide the basis to determine the best approach targeted at inhibiting bacterial adherence and preventing pseudomonas biofilm-related infection.

Keywords: *Pseudomonas aeruginosa*, biofilm, pexiganan, animal model, antimicrobial peptides, imipenem.

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SUMMARY

An *in vitro* and *in vivo* study was performed to quantify adhesion and biofilm formation ability of *Pseudomonas aeruginosa* slime producer under the effect of sub-minimal inhibitory concentrations (MICs) of pexiganan and imipenem. To evaluate adherence, squares of ureteral stents were placed in six-well tissue-culture plates containing 6 ml of a cell suspension grown in the presence of sub-MICs of study antibiotics. To evaluate biofilm formation sterilized squares were placed in six-well tissue culture plates containing 6 ml of triptic soy broth (TSB) supplemented with 0.25% of glucose and the respective amount of antibiotic. For *in vivo* study a biofilm infection rat model was performed. The study included an uninfected control group to evaluate the sterility of surgical procedure, a group infected with a slime-producer *P. aeruginosa* strain not previously treated with antibiotics and two groups infected with the strain previously treated with imipenem or pexiganan. Adherence and biofilm *in vitro* formation was strongly affected by pre-treatment with pexiganan and imipenem, with the latter being the more effective antibiotic. The *in vivo* results showed a reduction in bacterial load on the ureteral stent tissue of the pre-treated strain. Differently, urine cultures showed no differences in bacterial growth for the pre-treated strain showing that it retained its ability to cause infection. This study suggests that sub-MIC imipenem and pexiganan could be a good strategy to target the adhesion process during the infection cycle.

RIASSUNTO

Uno studio in vitro *e* in vivo *è stato svolto per quantificare la capacità di adesione e formazione di biofilm di* Pseudomonas aeruginosa *produttore di biofilm sotto l' effetto di sub-concentrazioni minime inibenti (sub-MICs) di pexiganan e imipenem. Per valutare l'adesione, parti di stent ureterali sono stati posti in piastre per coltura tessutale a sei pozzetti contenenti 6 ml di una sospensione di cellule cresciute in presenza di sub-MIC degli antibiotici in studio. Per valutare la formazione di biofilm, parti di stent ureterali sterilizzati sono stati posti in piastre per coltura tessutale a sei pozzetti contenenti 6 ml di brodo di soia triptico (TSB) addizionato con 0,25% di glucosio e la rispettiva quantità di antibiotico. Lo studio* in vivo *è stato eseguito su ratti con un modello di infezione- biofilm. Lo studio ha incluso un gruppo di controllo non infetto per valutare la sterilità della procedura chirurgica, un gruppo infettato da un ceppo di* P. aeruginosa *produttore di biofilm non precedentemente trattato con antibiotici e due gruppi infettati con il ceppo precedentemente esposto a imipenem o pexiganan. L'adesione e la formazione di biofilm* in vitro *è stata fortemente influenzata dal pretrattamento con pexiganan e imipenem e quest'ultimo ha evidenziato la maggiore efficacia. I risultati* in vivo *hanno mostrato una riduzione della carica batterica del ceppo pre-trattato sulla superficie dello stent ureterale. Diversamente, la coltura delle urine non ha evidenziato differenze nella crescita batterica per il ceppo pre-trattato, evidenziando quindi che questo ha mantenuto la sua capacità di infettività.*
Questo studio suggerisce come l'utilizzo di sub-MICs di imipenem e pexiganan potrebbe essere una buona strategia per controllare il processo di adesione durante il ciclo di infezione.

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