In Vitro Activities of Dermaseptins K4S4 and K4K20S4 against Escherichia coli, Staphylococcus aureus, and Pseudomonas aeruginosa Planktonic Growth and Biofilm Formation

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ABSTRACT

The rising number of infections caused by biofilm formation and the difficulties associated with their treatment by conventional antimicrobial therapies have led to an intensive search for novel antibiofilm agents. Dermaseptins are antimicrobial peptides with a number of attractive properties that might offer alternative therapeutics against resistant microorganisms. In this study, we synthesized a set of dermaseptin-derivative peptides and evaluated their activity against Gram-positive and Gram-negative bacterial biofilm formation. All dermaseptin-derivative peptides elicited concentration-dependant antibiofilm activity at microgram concentration and their activity was dependent on the nature of the peptide, with the highest levels of activity being exhibited by highly charged molecules. Fluorescent binding and confocal microscopy demonstrated that dermaseptin K_4S_4, a substituted derivative from the native molecule S_4, significantly decreased the viability of planktonic and surface attached bacteria and stopped biofilm formation under dynamic flow conditions. Cytotoxicity assays on HeLa cells showed that some of the tested peptides were less cytotoxic than current antibiotics. Overall, these findings indicate that dermaseptin derivatives might constitute a new lead structure for the development of potent antibiofilm agents.

Keywords: Biofilm, antimicrobial peptides, dermaseptins, *Pseudomonas aeruginosa*, *Escherichia coli*, S*taphylococcus aureus*.

INTRODUCTION

A biofilm is a microbial community of sessile microorganisms composed of cells that are embedded in a matrix of extracellular polymeric substances, attached to a substratum or interface. Biofilm bacteria are phenotypically and physiologically different from planktonic or suspended cells (1, 2). Bacterial biofilms have been linked to a wide range of infections, particularly in patients requiring indwelling medical devices, such as catheters and prostheses. They have recently been associated with about 80% of all chronic human infections (3) and described as important mediators of healthcare-associated infections (4).

Osteomyelitis, infective endocarditis, chronic wounds, and infections related to indwelling devices are examples of infections that are often caused by biofilm-producing strains. Staphylococci and enterobacteriaceae account for a large proportion of these infections, with *Staphylococcus aureus* and *Escherichia coli* pathogens representing more than 50 % of the species isolated from patients with medical device-associated infections (6, 7). Such infections typically exhibit increased tolerance to antimicrobial, biocidal and immunological challenges, which makes their treatment with conventional chemotherapeutic agents difficult, and sometimes impossible. These concerns have prompted a persistent search for alternative therapies against planktonic microorganisms and biofilms. Of particular interest, dermaseptins, antimicrobial peptides produced by the immune system of frogs, offer a number of properties and attributes that might open promising opportunities for the development of new antibiofilm agents.

Antimicrobial peptides are produced by both prokaryotic and eukaryotic cells and have been extensively investigated in recent research because they are involved in several host defense mechanisms and lend themselves to various applications as therapeutic agents. (8–13). Dermaseptins are a family of eight closely related antimicrobial peptides that have originally been isolated from the skin of a tree-dwelling, South American frog (*Phyllomedusa sauvagei*). They are linear polycationic peptides composed of 28–34 amino acids that are structured in amphipathic α-helices in apolar solvents (14, 15). They all have a conserved Trp residue at position 3, an AG(A)KAAL(V/G)G(N/K)AV(A) consensus motif in the mid-region, and a positive charge attributable to the presence of Lys residues that punctuate an alternating hydrophobic and hydrophilic sequence (14). New members of the dermaseptin S family (S_9–S_{11}) that do not resemble any of the so far characterized natural antimicrobial peptides have recently been identified and cloned from a skin secretion-derived cDNA library (16). In fact, some dermaseptins show marked abilities to inhibit microbial cells efficiently,

rapidly, and irreversibly without toxic effects on mammalian cells. They also display cytolytic activity *in vitro* against a broad spectrum of host-free microorganisms, including bacteria (Gram-positive and Gram-negative) (17–21), protozoa (22–24), yeasts and filamentous fungi (19, 25) and viruses (26-28).

Dermaseptin S_1, from the skin secretion of *Phyllomedusa hypochondrialis* frogs, has recently been reported to exhibit electroanalytical activity to dopamine (DA) oxidation. The selectivity in the detection of DA is, in fact, a fundamental aspect for the development of electrochemical sensors with potential applications in the biomedical and pharmaceutical industries (29). Although the precise mechanism of action of antimicrobial peptides is not yet fully understood, the antimicrobial action of dermaseptin is thought to be mediated by the interaction of the amphipathic α-helix with the membrane phospholipids, resulting in the permeation of the target cell by destabilizing the plasma membrane via either a 'barrel-stave' mechanism or a 'non-pore carpet-like' mechanism (30,31).

Structure-activity relationship studies performed on native dermaseptin S_4 have recently led to the identification of synthetic derivatives with improved antimicrobial properties (32-34). When comparing the resistance emergence rates by propagating bacteria under selective antibiotic pressure, both Gram-positive and Gram-negative bacteria were noted to exhibit resistance to commercial antibiotics but not to the L-or D-isomers of the dermaseptin derivatives that were tested (33). Overall, the data obtained from *in vitro* and *in vivo* experiments indicate that some dermaseptin derivatives have a variety of potential medical and antimicrobial applications (32). Nevertheless, no previous work has so far been performed on the antibiofilm activity of dermaseptins. Accordingly, the present study was undertaken to investigate the feasibility and gain effects of using dermaseptins against biofilms formed by important biofilm-forming pathogens, including *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. It particularly focused on the evaluation of the antibiofilm potential of dermaseptin S_4 derivatives. A number of chemically-modified peptides were synthesized and investigated for antibiofilm activity. HeLa cultures were used to evaluate their safety and toxicity, and fluorescent binding and confocal microscopy assays were performed to assess the speed and irreversibility of their antibiofilm activity.

MATERIALS AND METHODS

Synthesis, purification and preparation of peptides

Peptides were prepared by stepwise solid phase synthesis using Fmoc polyamide-active ester chemistry on Milligen 9050 pepsynthesizer. All Fmoc-amino acids were from Milligen-Waters (France). 4-(Hydroxymethyl) phenoacetic acid-linked polyamide/kieselguhr resin (pepsin kA), Fmoc-aminoacid pentafluorophenyl (Pfp), and 3-hydroxy-2, 3-dehydro-4-oxo-benzotriazine (Dhbt) esters were from Milligen/Bioresearch. Cleavage of peptidyl-resin and side chain deprotection were carried out using 5 mg of peptidyl-resin in 1 ml mixture composed of trifluoroacetic acid, para-cresol, thioanisol, water, and ethyl methyl sulfide (82.5; 5.5; and 2.5% v/v) for 2 h at room temperature. After filtering to remove the resin and ether extraction, the crude peptides were purified by a combination of Sephadex gel filtration, ion exchange chromatography, and preparative high performance liquid chromatography (HPLC). The homogeneity of the synthetic peptides was assessed by analytical HPLC, amino acid analysis, solid phase sequence analysis, and mass spectrometry (35). All peptides were stored frozen as stock solutions at 1 mg/ml and -20°C in double-distilled water. Prior to experimentation, fresh solutions were diluted in the appropriate medium. For fluorescein-labeled peptides, fluorescein was introduced at the N-terminus of the peptide using fluorescein N-hydroxysuccinimide ester prior to Trifluoroacetic acid (TFA) treatment.

Bacterial strains and growth media

The strains used in this study are listed in Table 1. They were maintained as follows: *Pseudomonas aeruginosa* was grown in Lysogeny Broth (LB) medium, *Escherichia coli* was grown in Mueller Hinton (MH) broth (for antimicrobial assays) or in M63B1 medium containing 0.4% glucose medium (for biofilm assay), and *Staphylococcus aureus* was grown in Tryptic Soy Broth (TSB) containing 0.25 % Glucose.

Antimicrobial activities against planktonic cultures

To investigate the antimicrobial activities of dermaseptins (Table 2), we evaluated the planktonic growth of different strains in the absence or presence of each peptide at different concentrations. Accordingly, an overnight culture of each bacteria grown in the appropriate medium was used to inoculate 100 µl of sterile MH medium in a 96-well plate to an optical density at 600 nm (OD_{600}) of 0.0001. The peptides were added to final concentrations ranging from 0 to 100 µg/ml. Polymyxin used at 6.25 µg/ml served as a positive control. Three

replicates were made for each condition. The cultures were incubated at 37°C, with shaking for 24 h. The minimum inhibitory concentration (MIC) was determined from the lowest concentration that induced 100% inhibition.

***In vitro* biofilm susceptibility**

To quantify the biofilm formation of *E. coli* in the absence and presence of peptides, a microplate-based assay was performed as follows. Strains were grown in M63 media containing 0.4 % glucose for 24 h at 37°C in a shaking bath and then diluted to obtain a suspension with an optical density (OD) at 600 of 0.01. 100 µl of the diluted suspension was added into the wells of a Polyvinyl Chloride (PVC) microtiter plate and incubated for 24 h without shaking to allow biofilm formation. Planktonic cells were carefully removed by pipetting, and biofilms were then treated with 100 µl of different concentrations of each peptide at indicated concentrations and incubated for 24 h at 37°C. Biofilm formation was measured using crystal violet (CV) staining. After treatment, planktonic cells were gently removed; each well was washed three times with water and pat dried with a piece of paper towel in an inverted position. To quantify biofilm formation, each well was stained with 100 µl of 1 % crystal violet and incubated for 15 min at room temperature. The plates were then washed three times with water again to remove extra dye. After that, 125 µl of mixed 80% ethanol and 20% acetone were added to each well to dissolve all the absorbed dye. After 30 min of incubation at room temperature, OD_{570} was measured to quantify the total biomass of biofilm formed in each well. The viability of biofilm cells was evaluated using the XTT quantification method. After treatment, planktonic cells were removed by careful pipetting, and the biofilms were washed with 125 µl of PBS 1X. Each well was supplemented with 125 µl of 0.05 mg/ml XTT/ 10 mM menadione and incubated at 37°C for 4 h in the dark. The metabolic activity of biofilm cells reflecting their viability was assessed by determining the amount of product resulting from the degradation of XTT into formazan, through OD_{492} measurement.

Confocal microscopy: Peptides effect on a biofilm in continuous flow

Growth of biofilm

The bacterial strain used for this experiment was *E. coli* MG1655 F'Tet Δ*traD*:*apra* λ ATT km-mars. Biofilms were grown at 37°C in M63B1 supplemented with 0.4% glucose as a carbon source, in flow chambers with individual channel dimensions of 1 by 4 by 40 mm. The

198 flow system was assembled and prepared as described previously (36). Individual flow cells
199 were inoculated with fluorescent *E. coli* (see Table 1 and legend of Fig. 2) and adjusted to an
200 optical density at 620 nm of 0.1. To allow the adherence of bacterial cells to the substratum,
201 flow cells were left without flow for 1 h after inoculation at 37°C. A laminar flow was then
202 activated with a flow rate of 2µl/sec. (7,2ml/h calculated to eliminate unattached bacteria but
203 in planktonic phase in the channel).

204

205 *Exposure of biofilm to fluorescent dermaseptins*

206 After 5 h of biofilm growth in the flow cell, the flow was stopped, and 300 µl of each
207 fluorescein-tagged peptide sample at the following concentrations (0.78 µg/ml for K_4S_4 and
208 25 µg/ml for $D_4D_{20}S_4$) was added into the chamber by syringe injection. After 20 min, the
209 flow was restarted with a flow rate of 0.4µl/sec per section. Normal flow rate (2µl/sec) per
210 section was set up again 20 min later. Z-stack images were obtained every 1 h for at least 6 h.
211 Flow cells were imaged under confocal microscopy Leica SP5 using the following
212 parameters: Objective 63X oil, 512x512 pixels, line average 2, 1 µm z steps, 60 µm per stack,
213 excitation Filter Wavelengths: 475-490 nanometers and 540-565 nanometers, emission
214 wavenlengths: 500-535 nanometers and 570-620 nanometers. Side-view images of the biofilm
215 were converted into 3D structures using the Software Imaris. All microscopy observations
216 and image acquisitions were performed using methods described above. For quantification of
217 biofilm development with or without peptide, images from each sample were analyzed using
218 the computer program (Imaris MeasurementPro).

219

220 **Assay for eukaryotic cell viability**

221 The potential cytotoxicity of dermaseptin S_4 and its derivatives was measured in
222 normal human cervical HeLa cells using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl
223 tetrazolium bromide)-based assay. Briefly, cells were seeded into 96-well plates at a density
224 of 2 x 10^4 cells per well and incubated for 24 h at 37°C prior to drug exposure. On the day of
225 treatment, the culture medium was aspirated from the wells and replaced by the fresh medium
226 containing a drug concentration ranging from 0 to 500 µg/ml. Triplicate wells were used for
227 each treatment. Culture plates were then incubated for 30 min, 3 h or 6 h before the addition
228 of 10 µl of MTT solution (5 µg/ml in PBS) to each well. The wells containing only the
229 medium and MTT were used as controls for each plate. The tetrazolium/formazan reaction
230 was allowed to proceed for 4 h at 37°C, and then 100 µl of the solubilization buffer (10%

sodium dodecyl sulfate in 0.1% (v/v) HCL) was added to all wells and mixed thoroughly to dissolve the dark-blue formazan crystals. After overnight incubation at 37°C, the optical density at 540 nm was measured by a 96-well multiscanner auto-reader with the solubilization buffer serving as a blank. To translate the OD_{540} values into the number of live cells in each well, the OD_{540} values were compared with those of standard OD_{540}-versus-cell number curves generated for each cell line. The percentage of cell survival was expressed as live cell number in test group/live cell number in control group x 100.

Statistical analysis

Normality in this study was assessed using Proc Univariate in SAS software (37). All data were statistically analyzed using the least-squares method with the procedure GLM for General Linear Model. Effects were treatment, bacteria and the interaction treatment-bacteria. The SNK multivariate test was used to compare means for bacteria and Dunnett's test to compare each experimental mean of treatment (peptide) with the control mean. The following equation was used for statistical calculations:

$$Yijk= \mu+ T_i+ B_j+ T*B_k+ e_{ijk}$$

Where:

Y_{ijk} is the response variable (peptides), μ is the overall mean, T_i is the fixed effects of treatment, B_j is the fixed effect of bacteria, $T*B_k$ is the effect of treatment within bacteria and e_{ijk} is the residual error

RESULTS

In vitro toxicity of dermaseptin and derivatives against human cells

We tested the potential *in vitro* cytotoxicity of dermaseptin S_4 and its derivatives (described in Table 2) in confluent monolayers of human HeLa cells using the MTT cell viability assay. Cells were exposed to increasing concentrations of peptides ranging from 0 to 128 µg/ml. Cytotoxicity of dermaseptins was concentration-dependent (data not shown). Peptides concentrations that caused 50% cytotoxixity (CC_{50}) were determined (Table 3). The highest cytotoxicity rates observed for all S_4 derivatives were recorded at concentrations higher than 24.12 µg/ml (CC_{50}), except for S_4(5-28) whose toxicity was slightly lower than the other peptides (CC_{50} > 100 µg/mL). Interestingly, results showed that shortening the peptide in N- or C-terminal region (S_4(5–28) or K_4S_4(1–16)) and increasing its positive charge by different substitutions ($K_4K_{20}S_4$ or K_4S_4) leads to peptides with low toxicity.

Antimicrobial activity of dermaseptin S_4 derivatives on planktonic bacteria

We next evaluated the ability of dermaseptin S_4 and its derivatives to inhibit proliferation of Gram-positive *S. aureus*, and Gram-negative *P. aeruginosa* and *E. coli* planktonic cells (Table 3). All peptides under investigation inhibited antimicrobial growth. Their range of activity was, however, dependent on the nature of the peptide and the highly positive charged molecules. In fact, the derivatives in which positive charge was increased without shortening the length of the peptide, *i.e.* $K_4K_{20}S_4$ and K_4S_4, were the most potent inhibitors, and the derivatives in which positive charge was reduced, such as $D_4D_{20}S_4$, were less potent. Likewise, the substituted and deleted peptide K_4S_4(1–16) and the peptide with N-terminal deletions S_4(5-28) displayed a nearly homogenous potency in all assays, with a progressive loss of potency as compared to K_4S_4 particularly against *S. aureus*. However, the truncation of the N-terminal carboxyl S_4(5-28), which potentially resulted in a less toxic peptide (33), did not affect its potency.

Effect of dermaseptin S_4 derivatives on *in vitro* static biofilm

We first assessed the activity of the dermaseptin S_4 and its derivatives against mature bacterial biofilms using biofilms formed in microtiter PVC plates. While exposure of bacterial biofilms to the peptides at concentrations of 2 x MIC did not dissolve biofilms as measured by crystal violet staining (data not shown), survival of biofilm cells was reduced (Fig. 1). K_4S_4

and $K_4K_{20}S_4$ were the most active peptides against all the strains tested, whereas $D_4D_{20}S_4$ was the least active one. However, K_4S_4(1-16) and S_4(5-28), which strongly affected *E. coli* and *P. aeruginosa* biofilm, had less activity against *S. aureus* biofilm.

Effect of dermaseptin S_4 derivatives on *in vitro E. coli* biofilm formed in continuous flow conditions.

To further investigate the inhibiting effect of dermaseptin S_4 derivatives on pre-formed biofilm, we evaluated their activity on *E. coli* biofilm developed under continuous flow conditions using confocal imaging to visualize both bacterial cells labeled in red (red fluorescent protein, Rfp) and fluorescent dermaseptins.

As mentioned earlier, since K_4S_4 and $K_4K_{20}S_4$ exhibited a similar activity against *E. coli* biofilm and due to the lack of a fluorescent $K_4K_{20}S_4$ peptide, this experiment was performed with fluorescein tagged K_4S_4 and $D_4D_{20}S_4$. Confocal images (Fig 2A and B) showed that, under continuous culture conditions in flow cells for 6 h in minimal media with glucose as carbon source, the fluorescent peptide K_4S_4 used at 2x MIC (0.78 µg/ml, in green), targeted all biofilm cells, both in the interior part and in the upper layer of the biofilm. The presence of K_4S_4 appeared to completely stop the growth of the biofilm with just a little detachment, suggesting that this peptide has the ability to inhibit biofilm proliferation (Fig 2A). This was verified by quantitative analysis of bacterial biomass that showed that biofilm proliferation was stopped right after injection of K_4S_4 (Fig. 3). Co-localization measurements indicated that the peptide penetrated bacterial cells, which confirmed its bacterial cell permeation ability (data not shown). The fact that dermaseptin K_4S_4 activity was also discernable in the presence of the continuous flow suggests a strong effect of K_4S_4 despite the dilution factor. In contrast, when the biofilm was exposed to 2x MIC of dermaseptin $D_4D_{20}S_4$ (25 µg/ml), the established biofilm continued to grow steadily, and red cells were still visible and continued to multiply (Fig 2B). Interestingly, although $D_4D_{20}S_4$ showed both binding cell capacity and penetration, it was not as effective as K_4S_4 (co-location measures), and appeared to be unable to stop the growth of *E. coli* biofilm (Fig 3).

Among all peptides tested, we found that $K_4K_{20}S_4$ and K_4S_4 were the most potent to inhibit biofilm formation with non toxic concentrations comparing to the others peptides. We also showed that this inhibition of growth was dependent on the nature of the peptide, and the highly charged molecules were the most active.

DISCUSSION

Infections in which bacteria are either slow growing, dormant, or within a biofilm, pose a serious clinical challenge for therapy because the cells in these states exhibit tolerance to the activity of antimicrobial agents. Many of these infections, including otitis media, sinusitis, cholesteatoma, and tonsillitis, are caused by biofilm-forming mucosal pathogens, such as *Staphylococcus aureus*, *Escherichia col* and *Pseudomonas aeruginosa.* Accordingly, the discovery of anti-infective agents that are active against both planktonic microorganisms and biofilms is of great public health significance. Antimicrobial peptides represent a potential promising source for the development of new alternative agents to combat resistant bacteria. These molecules are rapidly bactericidal, and since their action relates to physical properties, it is all the more difficult for bacteria to develop resistance to such peptides. Moreover, their structure can be engineered with relative ease, because peptide chemistry allows a multitude of modifications that are relatively time and cost-effective. Nevertheless, most of the agents undergoing clinical trials lack specificity and are examined only for topical uses (31).

Dermaseptins are a specific class of antimicrobial peptides that have a broad range of antimicrobial activity. Despite their promising properties and attributes, little work has so far been performed to evaluate their effects on bacterial biofilm formation. The present study aims to investigate the antimicrobial activity of five related synthetic peptides derived from the natural peptide dermaseptin S_4. It evaluates the effects of dermaseptins and some of their derivatives against a series of Gram-negative and Gram-positive strains, including *S. aureus*, *E. coli* and *P. aeruginosa*, in planktonic and biofilm cells and describes their structure-function relationship.

Our findings revealed that when used at high concentrations, this natural antimicrobial peptide is cytotoxic *in vitro* for host cells. This toxicity can, however, be reduced through introducing a number of modifications to the native sequence without altering its activity. To increase the antimicrobial activity of S_4, four deletions and substitutions were tested based on previous studies performed with *Escherichia coli* and human red blood cells. Our results revealed that dermaseptins K_4S_4 and $K_4K_{20}S_4$ displayed a hundred times higher increase in antibiofilm potency than the other derivatives. The results also showed that a positive charge

substitution led to a lower level of cytotoxicity without altering antibiofilm activity of the peptides.

Bisubstituted derived peptide $K_4K_{20}S_4$ exhibited the best results. This dermaseptin derivative combined two substitutions, the first substitution of methionine by lysine in position 4 and the second asparagine by lysine in position 20. It was previously shown that increasing the number of positive charges (6 versus 4 for dermaseptin S_4) and reducing its hydrophobicity index (22.7 versus dermaseptin S_4 28.9) resulted in a reduction of hemolytic activity (34). Other studies demonstrated that dermaseptin $K_4K_{20}S_4$ had a good antimicrobial activity *in vivo* with no toxicity in mice (32). The combination of the deletion of the 12 C-terminal residues with the substitution of methionine by lysine in position 4 K_4S_4(1-16), the deletion of 5 N-terminal residues in dermaseptins S_4(5-28), or the reduction of positive charges by substituting methionine in position 4 and 20 by aspartic acid were reported to induce a marked decrease in its antimicrobial activity as compared to $K_4K_{20}S_4$. It can be concluded from these observations that increasing the net positive charges of the peptide without shortening its sequence would result in analogs that display potent antibiofilm activity and low cytotoxicity. Besides, the selective activity of antimicrobial dermaseptins depends on the membrane lipid composition of the microbe versus the host cell and its electrical potential (33). For example, although the lipid compositions of host cell and *P. falciparum* membrane are similar, the potential of the parasite membrane is higher than that of the host cell membrane, leading to the discriminating effect of S_4 (34).

Furthermore, the literature shows that several other peptides isolated from different sources have been investigated for their antibiofilm activity against several bacterial strains, including cathelicidin (38, 39), histatin, mucins (40, 41), magainins (42, 445), pleurocidin (43), and lactoferrin (44). Like dermaseptins, these peptides are cationic and unstructured and fold into amphipathic α-helices upon contact with membranes. The data presented in this work suggest that the antibiofilm activity of dermaseptins is significantly higher than that of these peptides. For example, magainins II, which are 23-residue α-helical peptides isolated from the skin of the frog *Xenopus laevis*, display antibiofilm activity against *P. aeruginosa* and *E. coli* at 246.9 µg/ml and 15.4 µg/ml, respectively (40), whereas 0.38 µg/ml of dermaseptin $K_4K_{20}S_4$ was enough to achieve the same effect. In addition, a novel peptide mimetic, meta-phenylene ethylene (mPE), modeled after magainin was tested for its efficacy against several biofilm forming strains, including *S. aureus*, and the results showed that the

eradication of biofilm was observed at 10xMIC (42, 45). Used at 2xMIC, on the other hand, dermaseptin was sufficient to prevent the formation of *S. aureus* biofilm. Like the archetypal dermaseptins, pyllospetins (PSN), a major family of phyllomedusine skin antimicrobials (45, 46), have recently been described to occur in some species in multiple isomeric forms. The evaluation of the activity of these peptides' (PSN-1) against biofilms is, however, very limited; only few strains have so far been evaluated, and no data have shown their ability to inactivate bacterial strains (46). Comparatively, dermaseptins, with their broad range of antibacterial activity, may certainly offer a new promising candidate for the inhibition of biofilm formation. Taken together, the data indicate that the dermaseptin presented in this work has a number of advantages over other antibiofilm cationic peptides tested.

Biofilms are known to constitute a diverse and complex aggregate of bacteria that exhibit over 100-fold resistance to conventional antibiotics (47). Once a biofilm is established, the live cells are typically buried beneath the surface or between layers of dead cells and encased by a glycocalyx, an extracellular matrix of carbohydrates, proteoglycans, DNA, and other cellular constituents. Not only does this complex of biological molecules inhibit diffusion due to steric hindrance, but its constituents are believed to carry charges that have been known to interfere with the diffusion of others antibiotics (42, 45). The confocal microscopy experiments of this study showed that some dermaseptins such as K_4S_4 penetrate well into the biofilm structure and inhibit biofilm formation.

The main question that remains is how these dermaseptins can cross a thick layer established by exopolysaccharides, DNA, and proteins, to be able to reach their target cells. In fact, Donlan and his coworkers showed that the matrix of exopolysaccharides also plays a minor role in the antibioresistance properties of the biofilm by being directly bound to the antimicrobial agents and by preventing them from penetrating within the biofilm (1). This suggests that even if the matrix stops certain molecules of antibiotics, other molecules, such as dermaseptins, can cross this barrier and penetrate within the biofilm. The penetration of dermaseptins can stimulate exopolysaccharides production in the matrix and contribute to the increase of its thickness. This increase does not seem to inhibit the peptide which remains active against all the tested biofilm even at low concentrations, suggesting that the matrix may not hold these peptides which are positively charged and that they can reach the target cells. Once in contact with these cells, dermaseptins establish electrostatic interactions with the

bacterial membranes, leading to the formation of ionic channels in the lipid bilayers inducing the death of the cell.

Finally, dermaseptin S_4 derivatives present a new lead structure for potent antibiofilm agents. Nevertheless, new studies are needed to correlate the activity of S_4 in the *in vivo* situation. The *in vitro* biofilm assays employed in this study are essentially preliminary screens. They provide evidence that dermaseptins have a strong effect against biofilm strains, and further studies on their mechanisms of action and activity against other biofilm sources are warranted.

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585

Table 1. Different stains used in this study**.**

Strains	**Relevant characteristics**	**Reference**
E. coli		
MG1655_λatt_Km_*mars*_F'tetΔ*traD*	Red fluorescent biofilm forming *E. coli* strain producing the F pilus, Km^R, Tet^R	Laboratory collection
P. aeruginosa		
PAO1	Wild type, prototroph, *chl*-2	B. Holloway
S. aureus		
15981	Biofilm forming strain	(48)

Table 2 Dermaseptin S_4 derivatives used in this study

.

Dermaseptins	**Sequence**
K_4S_4	ALW**K**TLLKKVLKAAAKAALNAVLVGANA
$K_4K_{20}S_4$	ALW**K**TLLKKVLKAAAKAAL**K**AVLVGANA
$D_4D_{20}S_4$	ALW**D**TLLKKVLKAAAKAAL**D**AVLVGANA
K_4S_4 (1-16)	ALW**K**TLLKKVLKAAAK- - - - - - - - - - - - -
S_4 (5-28)	TLLKKVLKAAAKAALNAVLVGANA

Table 3. Antimicrobial activity and dose-dependent effect of different derivatives of dermaseptins

		MIC[b]		
Peptides	CC^{a}_{50}	*S. aureus*	*P. aeruginosa*	*E. coli*
K_4S_4	28.11**	12.5**	12.5***	0.39*
$K_4K_{20}S_4$	27.92**	0.19*	0.39*	0.19*
$D_4D_{20}S_4$	24.12**	25***	12.5***	12.5***
K_4S_4 (1-16)	28.51**	25***	12.5***	3.125**
S_4 (5-28)	103.11***	25***	12.5***	6.25***
Polymyxin	ND	25***	6.25**	6.25***

[a] Peptide concentration (µg/ml) that causes 50% cytotoxicity of dermaseptin S_4 and derivatives on HeLa cells,

[b] Minimal inhibitory concentration (MIC). Values *in vitro* expressed in µg/ml for all strains tested. ND: not determined. According to SNK multivariate test, we noticed a significantly difference ($p < 0.001$) between peptides, with the best activity, especially against *E. coli*, was reported in $K_4K_{20}S_4$ and K_4S_4 (0.39 µg/ml and 0.19 µg/ml respectively) compared to other peptides with a means activity (12.5 µg/ml, 6.25 µg/ml and 3.12 µg/ml), respectively for $D_4D_{20}S_4$, S_4(5-28) and K_4S_4 (1-16).

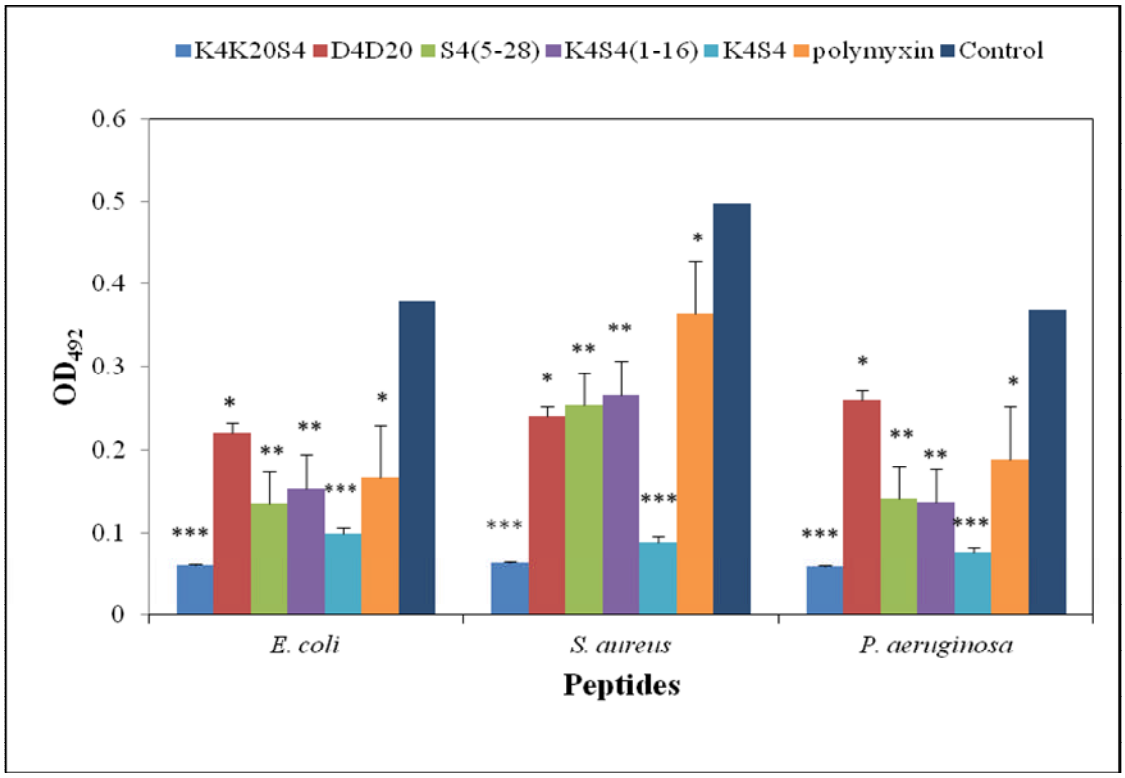
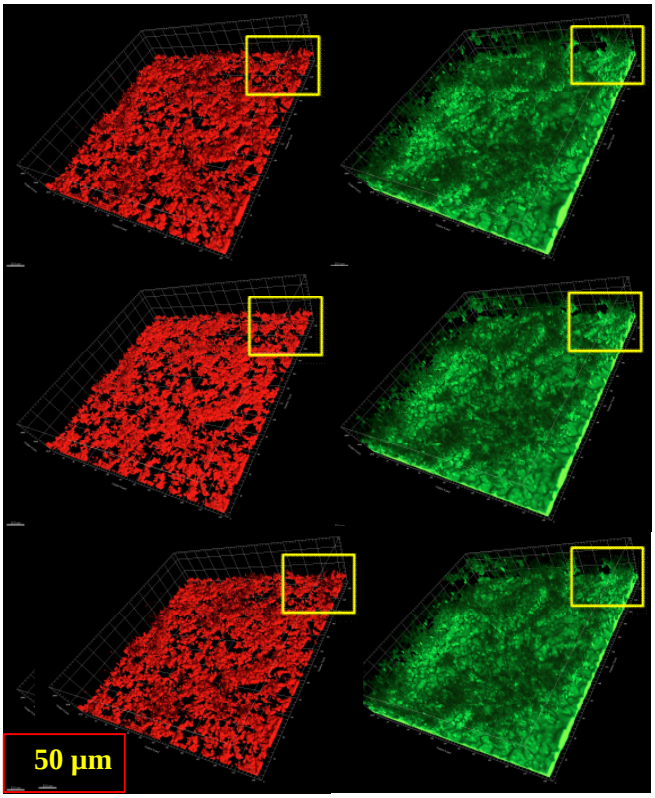
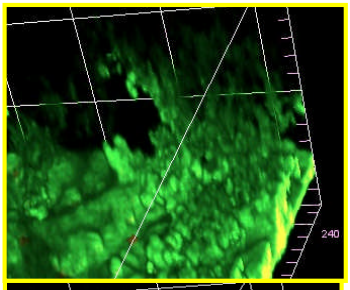
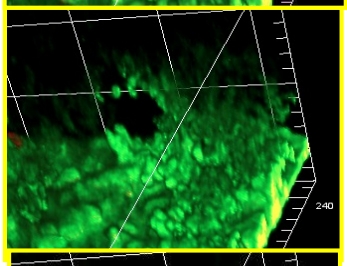
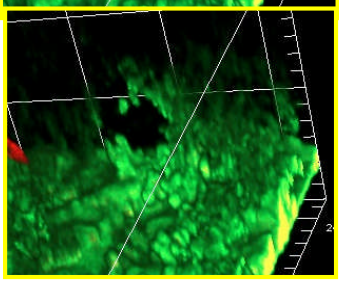


Figure 1. **Effects of dermaseptins S_4 derivatives on biofilm formation**. The viability of biofilm cells was evaluated using the XTT quantification method: Biofilm-forming *S. aureus*, *P. aeruginosa* and *E. coli* strains were grown in 96-well polystyrene plates and biofilms formed exposed to 2 x MIC concentrations of peptides during 24h. Control (negative control wells containing an appropriate medium for each strain only), Polymyxin (positive control wells). Statistical analysis was performed by using SNK multivariate test and Dunnett's test. $K_4K_{20}S_4$ and K_4S_4 were significantly different from control $p<0.001$, S_4(5-28) and K_4S_4(1-16) were different from control ($p<0.01$), and polymyxin is different from control ($p< 0.05$) ($^{***}p<0.001$; $^{**}p<0.01$; $^{*}p<0.05$)

A

Time post-Injection	Bacterial Biofilm	K_4S_4	MERGE X20
2h			
4h			
6h			

B

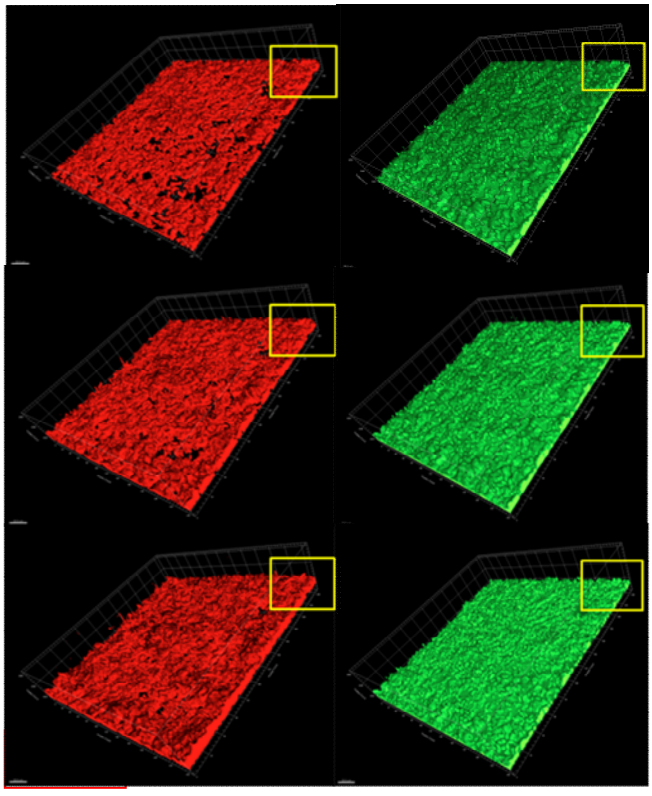
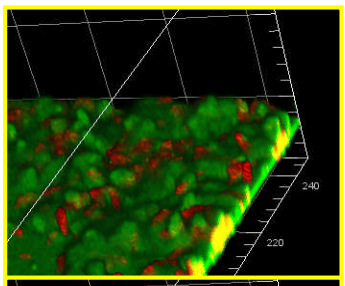
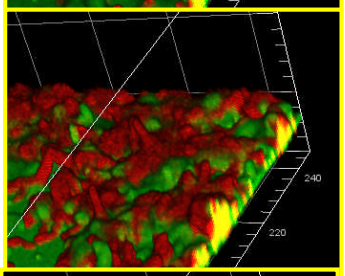
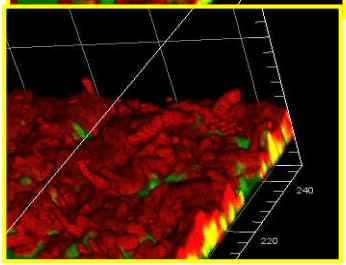
Time post-Injection	Bacterial Biofilm	$D_4D_{20}S_4$	MERGE X20
2h			
4h			
6h			

Figure 2 Effect of K_4S_4 on biofilms. To allow attachment of the bacterial cell to the substratum, flow cells were left without flow for 1h after inoculation at 37°C. Afterwards, a laminar flow with a flowrate of 2µl/sec was activated. After 5h of biofilm growth, the flow was stopped and 300 µl (0.78 µg/ml for K_4S_4 and 25 µg/ml for $D_4D_{20}S_4$) of each peptide sample was added. Confocal images acquisition showed that the fluorescent peptide **(A)**: K_4S_4 (green) has an instant effect on biofilm and stops its formation under dynamic flow conditions. Whereas, $D_4D_{20}S_4$, at 25µg/ml, (**B**), shows a less activity, biofilm cells exhibit high fluorescent signals (red) and survived the dermaseptin treatment.

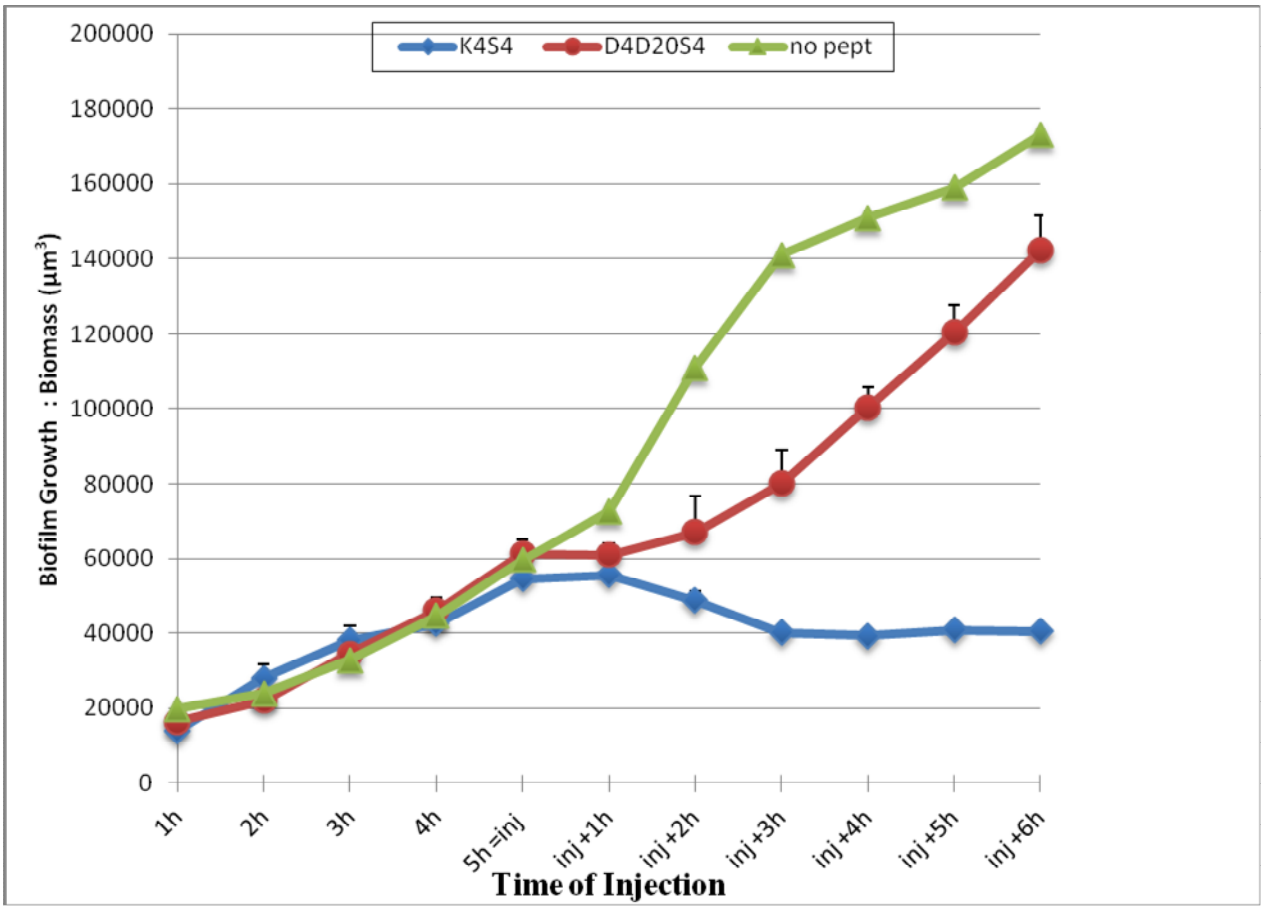


Figure 3. Confocal assays were confirmed by the quantification of biofilms. Differences in bacterial survival in the biofilms were quantified using the computer program Imaris MeasurementPro. Statistical analysis was performed by using SNK multivariate test and Dunnett's test. K_4S_4 was significantly different from control ($p<0.001$).