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Novel ways of treating bacterial skin infections: the antimicrobial peptides REI-26 and CD4-PP

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Master Degree Project in Infection Biology, 45 credits

Spring 2022

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Abstract

Severe skin and soft-tissue infections can lead to septic shock and organ failure. Antibiotic treatment is turning ineffective due to the rise of antimicrobial resistance. This calls for the need of novel antimicrobial therapies such as antimicrobial peptides. These peptides are small molecules that show antimicrobial activity and immune-related functions. The aim of this study was to determine the possible activity of the newly synthetized collagen-based REI-26 and cyclic CD4-PP in wound infections.

REI-26 and CD4-PP antimicrobial activity was determined by measurement of MIC values against *S. aureus*, MRSA, *P. aeruginosa* and Group A Streptococci. AMP treatment *in vitro* of infected keratinocytes and macrophages was also performed. The impact of treatment in immune-related and proliferation markers was assessed by quantitative PCR and confocal microscopy.

Both studied synthetic AMPs showed antimicrobial activity at low concentrations when MIC values were measured. Infected keratinocytes and macrophages with common skin bacterial pathogens showed decrease in bacterial load only when treated with the cyclic CD4-PP. In contrast, REI-26 treatment showed either no reduction or an increase in bacterial load. Upregulation of immune chemokines and AMPs and downregulation of proliferation markers was observed when keratinocytes were treated with CD4-PP.

The novel cyclic antimicrobial peptide, CD4-PP, presented promising results treating skin bacterial infections, with optimal antimicrobial activity in MIC values and survival assays. Further research is required in order to adapt CD4-PP treatment to a clinical setting.

Keywords: Antimicrobial peptides, skin infections, wound healing.

Popular Scientific Summary

A novel bacterial skin infection treatment

Skin and soft tissue infections can develop into septic shock and subsequently organ failure if they are not effectively treated. This type of infections entails 14 million outpatient visits and 900,000 admissions each year in the United States alone. Due to the increase of antimicrobial resistance, novel ways of treatment are required in order to prevent the life-threatening results of this type of infections.

One of the main alternatives to antibiotics are antimicrobial peptides. These peptides are naturally produced small molecules which can inhibit bacterial growth as well as they will promote the defense mechanisms that our body presents against microbes. CD4-PP is a novel synthetic cyclic peptide that has been produced using LL-37 as a base. This parental peptide is naturally produced and has showed antimicrobial activity against a wide range of bacteria. Another collagen-based linear peptide, REI-26, has also been produced as a possible alternative to treat bacterial infections. The aim of this study is to describe the possible clinical applications in wound context of both novel compounds, CD4-PP and REI-26.

CD4-PP and REI-26 showed antimicrobial activity towards the common skin bacterial pathogens *Staphylococcus aureus*, MRSA, *Pseudomonas aeruginosa* and Group A Streptococci at low concentrations. Therefore, treatment *in vitro* for keratinocytes and macrophages was analyzed. No reduction in bacterial load was observed when REI-26 treatment was applied to infected studied cells. In contrast, CD4-PP was able to significantly reduce the bacterial load of infected keratinocytes and macrophages.

Once treatment *in vitro* was studied, the production of short response immune related molecules was also analyzed. Keratinocytes treated for 2 hours with CD4-PP presented and increase in other antimicrobial peptides and alarm molecules which will trigger the response of other cells towards the microbial pathogen. This indicates that CD4-PP will be able to help our bodies to eliminate the bacterial pathogens. When observing the effect of this novel peptide in keratinocyte proliferation markers, a decreasing trend was observed.

REI-26 showed promising results in the case of antimicrobial activity but was not able to inhibit bacterial growth when treating keratinocytes and macrophages. This could be due to the possible inactivation or degradation when exposed to other cell components as it has been reported for other collagen-based peptides. Moreover, its linear structure may not have been as effective as if it presented a cyclic structure.

CD4-PP presents a high clinical potential due to its ability of reducing bacterial load when treating infected keratinocytes and macrophages. It also promotes immune response. Further investigation regarding the impact on wound healing properties and on other skin-present cells such as fibroblasts should be explored.

As a summary, this is the first report analyzing the antimicrobial effect of CD4-PP and REI-26 in a wound context. Collagen-based REI-26 requires further optimization while CD4-PP's effects needs to be explored in order to be able to apply it in a clinical setting. However, CD4-PP is a strong candidate as a novel treatment for skin and soft-tissue infections.

Introduction

Skin and soft-tissue bacterial infections (SSTIs) can present from mild to complicated symptoms. This type of bacterial infections can affect the upper layer of the skin as well as the underneath layers such as the subcutaneous tissue, the fascia and the muscle (1). The bacterial invasion of these layers can consequently result in the presence of bacteria in the blood which can cause a septic shock (2). This fatal medical condition can be one of the most life-threatening clinical outcomes since it can rapidly develop into multiple organ dysfunction and conclude in patient death (3,4).

Patient visits due to SSTIs are estimated in 900,000 admissions and 14 million outpatients' visits in the US alone (5). The development of a superficial infection to severe necrotizing infections presents high mortality and morbidity (6,7). Approximately, 20-30% of the patients admitted due to necrotizing infections die during hospital treatment. Moreover, the patient's quality of life can worsen after infection treatment (8).

Control and prevention of severe bacterial infection begins with early identification of the symptoms as well as appropriate antibiotic therapy. SSTIs can developed from different clinical situations such as cutaneous abscess, cellulitis, wound infection or surgical-site infection (9). The main clinical symptoms are caused due to inflammation as well as erythema, edema, and pain (4). Fever and hypotension can be symptoms derived from the activation of the immune system. Regarding treatment of this type of bacterial infections, conventional antibiotic therapy has been the most recommended prescription.

The most common bacterial pathogens involve in SSTIs are *Staphylococcus aureus* and *Streptococcus pyogenes* (10). The identification of these pathogens leads to the treatment with the β-lactam cloxacillin and the cephalosporins, cefazolin and cephalexin (11). Nonetheless, both of these bacteria can present virulence and resistance mechanisms which will lead to the non-clearance by antibiotic treatment (12,13).

During recent years, an increase in antimicrobial resistance has been described because of antibiotic misuse. This resistance has been observed in different common bacterial pathogens. In the context of SSTIs, incidence of methicillin-resistant *S. aureus* (MRSA) was described as 35.9% in North America, followed by Latin America with a percentage of 29.4% and Europe with 22.8%. Calculated incidence rate varied between different countries in Europe, being the southern ones the most affected (10,14).

Resistance in Group A Streptococci and *P. aeruginosa* has also been reported as well as different resistance mechanisms, such as biofilm formation, have been observed (13,15,16). Therefore, there is a need for novel treatments in order to improve the treatment of SSTIs.

Novel ways of treatment: the AMP alternative

Antimicrobial peptides (AMPs) are one of the main candidates as alternative treatment to bacterial infections. They are naturally produced small molecules that possess antimicrobial activity since they are one of the first-line defense mechanisms of the immune system. They present broad antimicrobial activity towards different bacteria, viruses, and fungi (17,18).

The majority of the discovered AMPs present a short amino acid sequence where positively charged and hydrophobic amino acids are present (19). This composition allows them to interact with the negatively charged microbial membrane as well as they are flexible molecules. They can present a broad range of secondary structure such as α-helix or β-sheets allowing them to adapt to the interaction with different pathogens (20). Hence, it has been described that AMPs are able of interacting with different components of the pathogenic microbe such as DNA, RNA and proteins (21,22).

Their specific mechanisms of action differs from different peptides depending on their target, but most of them are based in the negative charge interaction with the microbial membrane leading to posterior insertion and disruption (23). Due to the base of this interaction, the development of resistance from the bacteria is more difficult that in the case of conventional antibiotics (24).

AMPs can be produced in skin by keratinocytes, neutrophils and macrophages (25). Moreover, it has been reported that AMPs can play a role in wound healing, indicating that they can become a more suitable treatment for wound infections than the overused antibiotics (26,27).

Novel synthetic AMPs: CD4-PP and REI-26

CD4-PP is derived from the studied human cathelicidin LL-37. This parent peptide contains 37 amino acids in an α-helical structure (28). The mechanism of action of this antimicrobial peptide is based in its positive charge and structure that allows this peptide to aggregate and insert in the bacterial surface leading to pore formation and consequently microbial elimination (29). With the focus on developing a more potent synthetic AMP, LL-37 has been previously converted into a smaller linear peptide called KR-12 which only presents the residues from positions 18 to 29 (30). Nonetheless, KR-12 was not as suitable as expected due to proteolysis (31). Therefore, the Department of Pharmacognosy at Uppsala University developed a new derivative including the cyclization of two KR-12 peptides to form CD4-PP (31). CD4-PP has showed before antimicrobial activity towards uropathogens as well as it has been described as proteolytically stable (unpublished data).

The Department of Pharmacognosy at Uppsala University also studied the possibility of developing synthetic AMPs derived from collagen. This is due to the report of antimicrobial activity against Gram-positive and Gram-negative bacteria of collagen VI (32,33). Due to collagen VI being a component of the extracellular matrix in most of the connective tissues, host cytotoxicity of this compound and its derivatives is expected to be low (34). Furthermore, it is hypothesized that it can also present beneficial impact on wound healing mechanisms. Thus, a linear 26 amino acids peptide was synthetized and named as REI-26.

Study significance

Antimicrobial resistance supposes a threat towards the effectiveness of conventional antibiotics in community and hospital settings. Therefore, patients with bacterial skin infections risk to present complications if not treated effectively as well as the situation can lead to life-threatening consequences. It is believed that antimicrobial peptides can be a valid alternative to this type of bacterial infections and possible applications should

be explored. Moreover, they can also present beneficial properties such as the promotion of the immune innate response and wound healing properties.

Aim

The main aim of this study is to describe the antimicrobial properties of the AMPs REI-26 and CD4-PP in a wound context. Antimicrobial activity will be analyzed towards the common skin bacterial pathogens *S. aureus*, MRSA, *P. aeruginosa* and Group A Streptococci. Type strains of these bacteria will be analyzed as well as clinical isolates collected at the Karolinska University Hospital, in order to bring a perspective closer to a clinical setting. Effect of *in vitro* treatment will as well be evaluated in infected keratinocytes and macrophages, two cell lines that are present in the skin.

As a secondary aim, the impact of these two novel peptides in host immune response and wound healing factors will also be described.

Materials and Methods

Antimicrobial peptides (AMPs)

Antimicrobial peptides collagen-derived linear REI-26 and cathelicidin-derived cyclic CD4-PP were provided by Dr. Taj Muhammad, Dr. Adam Strömstedt and Prof. Ulf Göransson. They were synthetized at the Department of Pharmacognosy, Uppsala University and diluted in micellar water.

Cell lines

All cell lines were grown at 37°C and 5% CO_2. Human keratinocytes HaCaT (RRID: CVCL_0038) were used in this study. Two different macrophages cell lines were also used: murine RAW 264.7 macrophages (American Type Culture Collection [ATCC]; TIB-71) and differentiated THP-1s (ATCC; TIB-202). HaCaT and RAW 264.7 cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM; ref. 41966-029, Gibco). The media that was used for the dTHP1s was RPMI-1640 (ref. 11879-020, Gibco). Both cell culture medias used were supplemented with 10% of heat inactivated fetal bovine serum (FBS) (ref. 10270106, Gibco, country of origin Brazil). No antibiotics were used during the growth of these cell lines. The THP-1 monocytes were differentiated to inactivated macrophages by using phorbol myristate acetate (PMA) for 24 hours at a 150 ng/ml concentration. This differentiation step was followed by RPMI 1640 media replacement without PMA for another 24 hours.

Bacterial strains

ATCC and CCUG type strains are indicated in Table 1. Clinical isolates used for the description of minimal inhibitory concentrations (MIC) were isolated at the Department of Clinical Microbiology at the Karolinska University Hospital, Stockholm.

***Table 1*. Bacterial type strains**. Table 1 indicates the bacterial type strains used in this study and their respective ATCC and CCUG reference number.

Bacterial species	Type strain	Additional information
Staphylococcus aureus	ATCC 29213	Susceptible to oxacillin
Methicillin-resistant *S. aureus* (MRSA)	CCUG 31966	Resistant to methicillin, penicillin G, sulfonamide and tetracycline
Pseudomonas aeruginosa	ATCC 27853	
Streptococcus pyogenes Group A	ATCC 19615	Lancefield's group A
Listeria monocytogenes	ATCC 19111	

Minimal inhibitory concentration (MIC)

Type strains and clinical isolates from *S. aureus*, *P. aeruginosa* and Group A Streptococci were grown in blood agar (BA) at 37°C overnight. Single colonies were inoculated in 3% trypticase soy broth (TSB) and grown from 2-4 hours at 37°C shaking. These growth conditions were maintained until log phase was reached.

After 5 minutes of centrifugation at 2500 rpm, the bacterial pellet was resuspended in 3 ml of 10 mM Tris-buffer. This washing procedure was repeated twice. A densitometer (DensiCHEK^{TM} Plus, Biomérieux) was used to obtain a bacterial suspension with 0.5 McFarland turbidity. This suspension was later diluted in 10 mM Tris in a ratio of 1:150 to achieve bacterial suspension with 10^6 colony forming units per milliliter (CFU/ml). With the purpose of allowing the interaction of the AMP and the bacteria of interest, 50 µl of the performed bacterial suspension were added to a U-bottomed 96-well plate made of polystyrene (Costar) containing the different dilutions of AMPs.

AMPs two-fold dilutions from 6.25 µM to positive control growth with no peptide were prepared in 10 mM Tris-buffer with AMPs stock concentration of 1 mM. 50 µl of these serial dilutions were added to 7 wells of the 96-well plate. The final volume of the reaction was 100 µl and was incubated for 1 hour at 37°C. After 1 hour, 5 µl of 20% TSB were added to each well and the plate was further incubated for another 16 (± 2 hours) at 37°C. Measurement of the optical density (OD) at 598nm using the EZ read 400 microplate reader (Biochrom) indicated the MIC of each studied bacterial isolate.

Cell cytotoxicity assay

HaCaT and RAW 264.7 cells were grown in a flat-bottomed 96-well plate (Costar) until 80% of confluency was reached. Two-fold dilutions starting at 100 µM of REI-26 or CD4-PP were added to the confluent cells and incubated for 24 hours at 37°C. Washing of the cells was performed twice using 1XPBS. Fresh DMEM media with 10% FBS supplemented with 2.15 µl menadione (Sigma; 172.18g/ml) per ml of XTT (Sigma) was prepared. XTT was added in a relation of 20% to the total volume of media with menadione. Stock concentration of XTT in the media was of 1 mg/ml diluted in 1XPBS. Blank wells contained only prepared media with XTT-menadione. Cells were incubated for 3 hours at 37°C with 5% CO_2. OD was measured at 450 nm and 690 nm with EZ read

400 microplate reader (Biochrom). The OD value of 690 nm was subtracted from the OD value at 450 nm. The assays were performed with three natural replicates.

Bacterial survival assay in HaCaT and dTHP-1 cell lines

HaCaT cell line was seeded in a 24-well flat-bottomed plate (Costar) with DMEM media with 10% FBS until 80% confluency was reached. THP-1 cell line required differentiation step first, as it is indicated in the cell line section of methods. All bacterial strains of interest (*Table 1*) were plated on BA and incubated at 37°C overnight.

Three different conditions were set for this experiment performed in duplicates: untreated infected control, infected treated cells pre-infection (AMPs were added at the initial infection time-point), infected treated cells post-infection (AMPs were added 2 hours after infection was stablished). Cells were treated with the indicated REI-26 and CD4-PP's concentrations and their respective media without 10% FBS supplementation.

Bacterial suspension was prepared by adding few colonies to 3 ml of 1XPBS. This was followed by a centrifugation of 3 minutes at 1000 rpm. The stock bacterial solution presenting 10^8 CFU/ml was determined by measuring absorbance at 600 nm. The stock bacterial suspension was then serial diluted to 10^7 CFU/ml in 1XPBS and 100 µl were inoculated to the specified cells to achieve a multiplicity of infection (MOI) of 5. Plate was centrifuged for 1 min at 1000 g. Cells were then incubated for 2 hours at 37°C and 5% CO_2. Pre-inoculum density (PID) was calculated by plating 50 µl of the dilutions 10^4 and 10^3 CFU/ml in BA and grown overnight at 37°C. This was used as a comparison of the initial number of bacteria added to the cells and the remaining bacteria at the end of the experiment. After 2 hours of pre-infection treatment or no treatment, cells were washed thrice with lukewarm 1XPBS. Cell culture media with the respective AMPs was later added, and cells were then incubated for further 2 hours at 37°C and 5% CO_2. Cells were then washed again three times with 1XPBS and lysed and scraped with 200 µl ice-cold 1X PBS with 0.1% Triton X-100.

Lysate was serially diluted and 50 µl were plated in BA plates. Plates were incubated overnight at 37°C, to compare the survival bacteria for different treatment conditions. Lysate from cells infected with Group A Streptococci were plated in Brilliant Green agar (BG) and grown overnight anaerobically at 37°C.

Phagocytosis assay in RAW 264.7 and dTHP-1 cell lines

Phagocytosis assay was mainly conducted following the same protocol as bacterial survival assay. The main difference between these two methods was the addition of gentamicin for 30 minutes in a concentration of 10 µg/ml to the macrophages. This step was performed after 2 hours of incubation at 37°C with the bacterial type strain indicated. It assured the elimination of only extracellular bacteria due to the inability of gentamicin of surpassing the eukaryotic cell membrane (35). Treatment conditions pre-infection and post-infection were followed as in the bacterial survival description.

Plating of PID, and intracellular bacterial survival was achieved in the same manner as in bacterial survival assay. As indicated in the section of cell lines, THP-1s were first seeded and differentiated with exposure to 150 ng/µl of PMA for 24 hours. This cell line was ready to be infected once PMA-free media was added for other 24 hours.

Host response: measurement of mRNA levels

HaCaT cells were used for measuring gene expression of *S100A7, DEFB4, IL6, CXCL8, MKI67* and *VCL*. Cells were seeded in a flat-bottomed 24-well plate and exposed to the different indicated conditions (treatment with AMPs, infection with *S. aureus* ATCC 29213) once they reached 80% confluency. The different conditions were done in triplicates. The control reference were untreated cells. When infecting the cells, *S. aureus* ATCC 29213 was grown overnight at 37°C on BA plate and working bacterial solution was performed as indicated in the bacterial survival assay.

After total incubation of 2 hours at 37°C and 5% CO_2, media was removed, and cells were lysed by using 350µl RLT lysis buffer (Qiagen). RNA extraction was achieved as indicated in the protocol for RNeasy Midi Kit instructions (Qiagen). RNA quality was assessed by using nanodrop (ThermoFisher).

Total RNA was transcribed to cDNA using 100-300 ng of RNA with cDNA reverse transcription kit (Applied Biosystems). SYBR Green expression assay protocol (Applied Biosystems) was used to measure the gene expression of indicated genes of interest and compared to the expression of the housekeeping gene *ACTB*. qPCR was performed on a Rotor Gene RG3000 (Applied Biosciences). Forward and reverse primers used for each gene are shown in *Table 2* and were provided by Integrated DNA Technologies.

***Table 2*. Primers used for gene expression measurement**. Table 2 shows the forward and reverse primers used for the gene expression measurement of HaCaT cell line exposed to different conditions (treatment with CD4-PP, REI-26 and infection with *S. aureus* ATCC 29213).

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
ACTB	AAGAGAGGCATCCTCACCCT	TACATCGCTGGGGTGTTG
S100A7	CACCAGACGTGATGACAA	GGCTATGTCTCCCAGCAA
DEFB4	CCCTTTCTGAATCCGC	GAGGGTCTTGTATCTCCT
CXCL8	AAGAGAGCTCTGTCTGGACC	GATATTCTCTTGGCCCTTGG
IL6	TGGCATTTGTGGTTGGGTCA	TTCGGTCCAGTTGCCTCTC
MKI67	CTCTCCAAGGATGATGATGCTTTA	TCCCGCCTGTTTTCTTTCTGAC
VCL	CCAAAACATGTCTCCTATATCCTGG	GAAGTGTCCTTCAGACAGGG

Host response: protein levels measurement by confocal microscopy

HaCaT cells were seeded on coverslips in DMEM media with 10% FBS in a 24-well plate. Four different conditions were utilized: control where only media was added to the cells, 20 µM of CD4-PP, 1.6 µM of psoriasin (S100A7) and combination treatment of both AMPs. Treatment conditions were 20 µM of CD4-PP, 1.6 µM of psoriasin and combination treatment of both AMPs.

Once 24-hour treatment, old media was removed, and cells were fixed with 4% paraformaldehyde (PFA) for 15 minutes. After, cells were washed with 150 µl of 1XPBS and permeabilized by adding 50 µl of freshly prepared 0.1% Triton-X 100 for 5 minutes.

Blocking for unspecific interaction was done by adding 50 µl of 5% bovine serum albumin (BSA) to each coverslip for 30 minutes. Primary antibody staining was performed by adding the respective primary antibodies in a 1:200 dilution in equal volume of 1XPBST (1X PBS with 0.1% Tween) and 5% BSA and left at 4°C overnight. Primary antibodies were used against Ki67 (rabbit; AB15580-Abcam) and vinculin (mouse; V9131-Sigma). The following day cells were washed thrice with 150 µl of 1XPBST and stained with 1:1000 dilution of secondary antibody staining. Anti-rabbit Alexa fluor dye 488 (A-21467, Life Technologies Cooperation) was used for the staining of Ki67 while anti-mouse Alexa Fluor dye 647 (ref. A-21235, Life Technologies Cooperation) was used for the staining of vinculin. Binding step of primary and secondary antibody was performed for 1 hour at room temperature in the dark. Cells were washed again thrice with 1XPBST and 50 µl of 1:1000 dilution of DAPI staining solution (ref. D3571, Invitrogen) for 15 minutes at room temperature. Washing with 1XPBST was performed thrice. Coverslips were then mounted with 3 µl of Fluromount G (ref. 01001, Southern Biotech) in glass slides and observed under Zeiss LSM 700 confocal microscope with 63X oil immersion objective. Imaging was performed by Dr. John Kerr White.

Nitric oxide production

When phagocytosis assays were performed with RAW 264.7 and dTHP-1s, 150 µl of supernatant after 1 hour or 4 hours of incubation at 37°C and 5% CO_2 were removed and mixed with 120 µl of distilled water in a flat-bottomed 96-well. 20 µl of freshly prepared Griess reagent (ref. G7921, Invitrogen). 150 µl water was used as reaction blank and 150 µl of respective cell culture media were used as blank for the media. Plate was incubated for 30 minutes in the dark at room temperature, and then OD was measured at 562 nm using the EZ read 400 microplate reader (Biochrom).

Statistical analysis

Statistical analysis was performed by using GraphPad Prism 8. Statistical tests used are indicated in the figure legends for each result. Presence of outliers was checked using Grubb's test.

Results

REI-26 and CD4-PP show antimicrobial activity towards skin bacterial pathogens

While most skin infections heal even without other treatment than keeping the wound clean, others need antibiotic treatment and some infections may develop into severe infections and even septicaemia (2). Given this fact, antimicrobial activity of the novel AMPs REI-26 and CD4-PP was assessed by performing MIC assays against these skin bacterial pathogens. MICs were measured for the type stains and clinical isolates. The range of antimicrobial peptide concentrations tested started at 6.25 µM and were followed by decreasing 2-fold dilutions. A positive control for bacterial growth with no peptide was also performed. 6.25 µM was chosen as a starting concentration due to susceptibility of other bacterial pathogens to CD4-PP at 1.56 µM (unpublished data).

REI-26's MIC value for all type strains from the different skin bacterial pathogens was 1.56 µM (*Table 3*). Regarding the clinical isolates, 15 out of 20 clinical isolates of *S. aureus* presented an equal MIC value or lower than the type strain. In the case of *P. aeruginosa,* 17 clinical isolates out of 20 showed an equal or a lower MIC value while

for Group A Streptococci (GAS) was 18 clinical isolates out of 20. Thus, REI-26 showed antimicrobial activity against the type strains and also against most of the isolated clinical strains. Furthermore, only one clinical isolate of *S. aureus* presented an MIC of 6,25 μM. The growth of rest of the clinical isolates from the studied bacteria was inhibited at a concentration of 3,25 μM of MIC.

***Table 3*. REI-26 MIC values for skin bacterial pathogens.** Table 3 indicates the REI-26 MIC values for the type strain bacterial pathogen and how many of the tested clinical isolates (N=20) presented equal or lower MIC.

Bacterial species (ATCC number)	REI-26 MIC type strain	Percentage of clinical isolates with equal or lower MIC (N=20)
S. aureus (ATCC 29213)	1.56 μM	75% (n=15)
P. aeruginosa (ATCC 27853)	1.56 μM	85% (n=17)
Group A Streptococci (ATCC 19615)	1.56 μM	90% (n=18)

On the other hand, the MIC values for CD4-PP varied depending on the species that was analyzed. CD4-PP was able to inhibit growth of the type strains of *S. aureus* and Group A Streptococci at a concentration of 0.78 μM, and the type strain of *P. aeruginosa* at 1.56 μM. As for the clinical isolates, all clinical isolates from *P. aeruginosa* and Group A Streptococci showed equal or lower MIC than the type strain. In the case of *S. aureus*, growth of 18 out of 20 clinical isolates was inhibited when they were exposed to a concentration equal or lower than 0.78 μM.

***Table 4.* CD4-PP MIC values for skin bacterial pathogens**. Table 4 shows the CD4-PP MIC values for the type strain bacterial pathogen and how many of the tested clinical isolates (N=20) presented equal or lower MIC.

Bacterial species (ATCC number)	CD4-PP MIC type strain	Percentage of clinical isolates with equal or lower MIC (N=20)
S. aureus (ATCC 29213)	0.78 μM	90% (n=18)
P. aeruginosa (ATCC 27853)	1.56 μM	100% (n=20)
Group A Streptococci (ATCC 19615)	0.78 μM	100% (n=20)

Both novel AMPs were able to inhibit bacterial growth of common skin pathogens at low concentrations, below 2 μM. When comparing results, CD4-PP showed a lower MIC than REI-26 as well as it was able to prevent the growth of the different clinical isolates with the same MIC concentration or lower. However, both showed promising results towards a possible treatment for bacterial skin infection.

Treatment of infected keratinocytes and macrophages with novel AMPs

Keratinocytes are the major cell type present in the outer layer of the skin, the epidermidis. These cells are epithelial cells which are highly specialized and present structural function (36). Since the skin represents one of the main immune barriers of our body, immune cells such as macrophages are also present in the basal layers of the skin (37). This deeper layer will be exposed to microbial pathogens in a wound context. Therefore, in order to obtain a broader picture of a possible treatment of skin bacterial infections, *in vitro* assessment with these two cell types should be analyzed. Due to the favorable MIC results for both novel AMPs, treatment of keratinocytes (HaCaT cells) and macrophages (RAW 264.7 and differentiated THP-1s) was performed.

Prior to the possible effect of novel AMP treatment, the impact of both AMPs on cell viability was assessed using the XTT-menadione method in the different used cell lines. REI-26 showed no cytotoxic effect in cell viability in concentrations up till 100 µM. CD4-PP showed cytotoxicity at a concentration of 50 µM in RAW 264.7 and HaCaT cells. (*Figure S1*)

HaCaT and dTHP-1s cells were treated with a range of REI-26 concentrations to assess which one was the most effective. The chosen trial concentrations were 2, 4, 6 and 16 µM. All HaCaT cells were infected with *S. aureus* type strain ATCC 29213, which showed a MIC of 1.56 µM previously. REI-26 was added whether at the same time as the bacteria or 2 hours after infection with bacteria (2 h). No bacterial growth inhibition was observed when performing these survival assays (*Figure 1, 2*). Furthermore, an increasing trend in bacterial growth was observed when the cells were treated from the beginning of the infection and at higher REI-26 concentrations. No statistically significant difference was found between the different conditions due to the spread between the different sets.

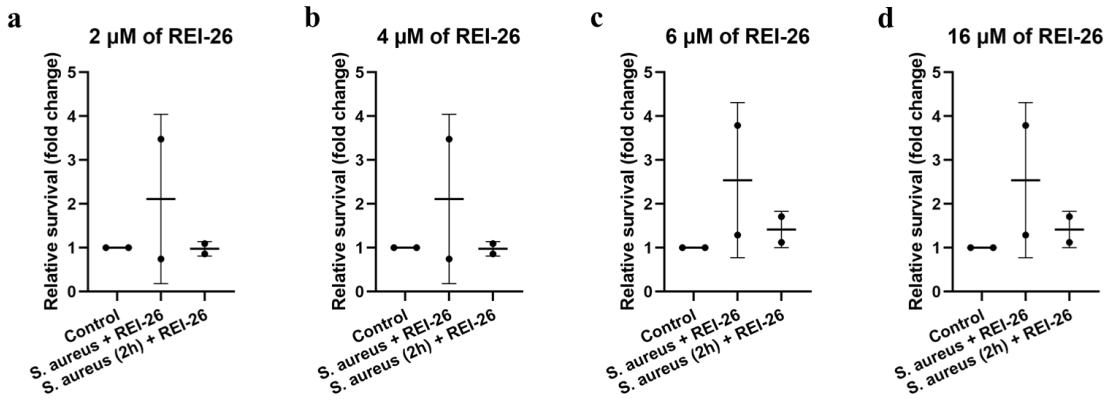

Figure 1. **Infection assay for *S. aureus* in HaCaT cells treated with REI-26.** A comparison between the untreated control infection and the different treatments was performed. The different used concentrations of REI-26 are indicated in each figure. The graphs comprehend the mean value of each set and the total mean value with the standard deviation. Statistical test applied was ANOVA Dunnett's multiple comparisons.

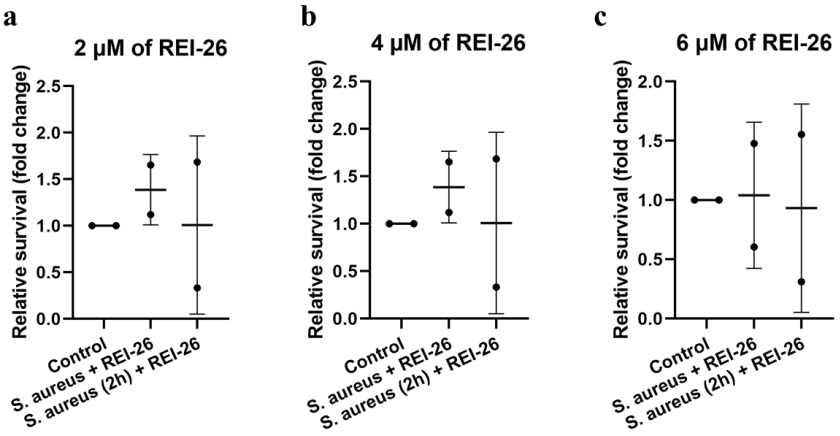

Figure 2. **Infection assay for *S. aureus* in cell line dTHP-1 treated with REI**. A comparison between the untreated control infection and the different concentration treatments was performed. The different used concentrations or REI-26 are indicated on top of each figure. Total mean value for fold change of each is described as well as the standard deviation. Statistical test applied was ANOVA Dunnett's multiple comparisons.

Due to CD4-PP's ability of inhibiting bacterial growth at low concentrations, it was also considered a potential candidate for possible *in vitro* skin and immune cell treatment. Previous to this study, HaCaT cells were infected with type strains of *S. aureus* ATCC 29213, MRSA CCUG 31966, *P. aeruginosa* ATCC 27853 and GAS ATCC 19615 and treated with 2 µM of CD4-PP. As indicated before, a treatment pre-infection and post-infection was performed. In the case of *S. aureus*, MRSA and Group A Streptococci, bacterial load was significantly decreased for both treatments, initial and two hours post-infection with a p-value ≤ 0.001 when considering the mean values of each set. When infecting with *P. aeruginosa*, treatment given after 2 hours of infection was unable to reduce the bacterial burden in infected HaCaT cells. (*Figure 3*)

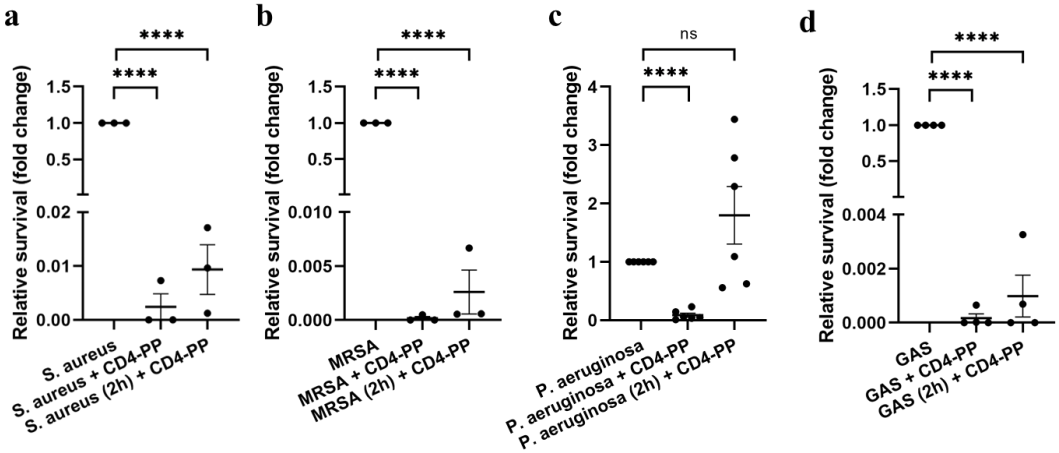

Figure 3. **Bacterial survival assay for different skin bacterial pathogens in HaCaT cell line treated with CD4-PP.** This was studied prior to this study by Dr. John Kerr White. Relative bacterial survival of the untreated control was compared to 2 µM CD4-PP treated replicates. Treatment was administered to HaCaT cells at the initial infection or 2 hours post-infection. Relative survival of type strains of S. aureus ATCC 29213 (a), MRSA CCUG 31966 (b), P. aeruginosa ATCC 27853 (c) and GAS ATCC 19615 (d) is indicated. 4 stars indicate a p-value ≤ 0.001 when ANOVA Dunnett's multiple comparisons were applied.

Due to the significant increase in bacterial clearance observed in infected HaCaT cell line with CD4-PP treatment, the effect of this novel AMP in the phagocytosis rate of immune cells was the next step to be considered. A trial phagocytosis assay experiment was

performed in mouse macrophages (RAW 264.7) since it was the first time that this AMP's effect was measured in immune cells. RAW 264.7 were infected with the intracellular bacteria *L. monocytogenes* due to its intracellular cycle as a way of measuring if CD4-PP enhanced the phagocytic capacity of murine macrophages (38).

MIC value for *L. monocytogenes* was 0.78 μM when exposed to CD4-PP. Due to some inactivation of the novel peptide when added to cell culture media, the concentrations tested were 2 and 4 μM. RAW 264.7 macrophages were treated and infected for a total of 1 hour and 4 hours. After one hour of infection and two hours of infection, gentamicin was added to eliminate extracellular bacteria (35). A significant decrease in intracellular bacteria was observed when treated control was compared to untreated control. This reduction was present in both time-period treatments and concentrations (*Figure S2*).

Once the effect of CD4-PP was also proven in intracellular bacteria and the minimal optimal CD4-PP concentration was described, phagocytosis assays in dTHP-1s was performed. As in the case of the keratinocytes, dTHP-1s were infected with skin common pathogens (*S. aureus* ATCC 29213, MRSA CCUG 31966, *P. aeruginosa* ATCC 27853 and GAS ATCC 19615) and treated at pre-infection time or 2 hours post-infection with 2 μM of CD4-PP. After 2 hours of infection, gentamicin was used with the same purpose as in RAW 264.7 phagocytic assay, to kill extracellular bacteria.

Lower intracellular bacterial load of all skin common bacterial pathogens was observed when comparing the treated replicates to the untreated controls. When treating pre-infection, all bacterial species tested showed a significant decreased with a p-value ≤ 0.001. However, when the treatment was performed 2 hours post-infection, significant reduction was observed.

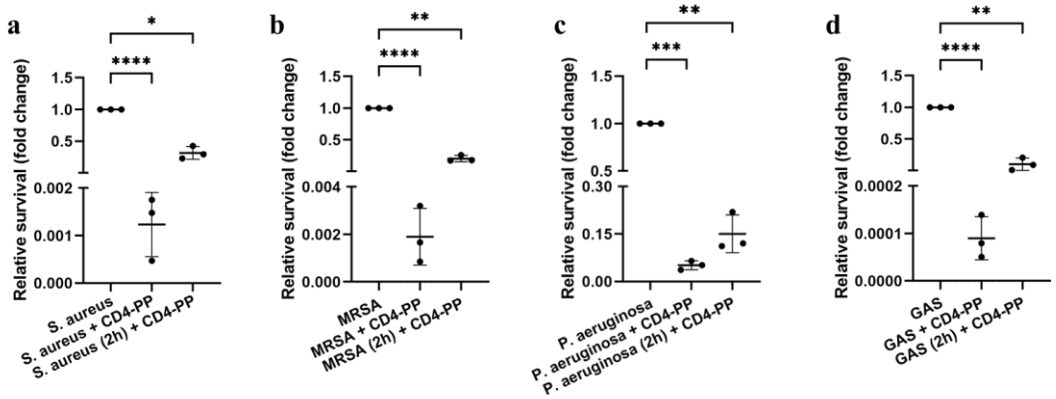

Figure 4. **Phagocytosis assay of infected dTHP-1s treated with CD4-PP.** Relative survival of type strains of *S. aureus* ATCC 29213 (a), MRSA CCUG 31966 (b), *P. aeruginosa* ATCC 27853 (c) and GAS ATCC 19615 (d) is indicated. 4 stars indicate a p-value ≤ 0.001, 2 stars, p-value ≤ 0.01 and 1 star, p-value ≤ 0.1. Statistical analysis was performed by using ANOVA Dunnett's multiple comparisons test.

REI-26 showed no decrease in survival of bacteria when treating infected keratinocytes and human macrophages. In contrast, CD4-PP showed significant bacterial reduction when treating infected keratinocytes and macrophages. Moreover, a reduction was observed in both type of treatments: pre-infection and post-infection.

Immune innate response of keratinocytes when exposed to novel AMPs

During infection, stressed keratinocytes release chemokines, antimicrobial peptides, and oxidative species as part of the innate immune response. AMPs themselves are also potent immunomodulators, capable of regulating different immune pathways and functions (39). Therefore, it was assessed whether CD4-PP would alter the expression of these immune-related compounds.

Firstly, even if REI-26 did not show any reduction in bacterial load when added to infected keratinocytes, it was hypothesized that it may present other beneficial properties for the host cells in a skin infection context. HaCaT cells were then treated during 2 hours with a range of REI-26 concentrations. The expression of the antimicrobial peptides beta-defensin 2 (*DEFB4)* and psoriasin (*S100A7)* were analyzed due to their described constant presence in the skin (40,41). mRNA levels of the chemokine *CXCL8* were also analyzed due to its chemoattractant properties. This studied chemokine is able to attract neutrophils to the site of infection as well as it has been described that is has an effect on epithelial cells and macrophages (42).

When measuring the mRNA levels of the indicated immune components in REI-26 treated HaCaT cells, only a decreasing trend could be observed in the case of *DEFB4* (*Figure 5).* No statistically significant differences in mRNA expression levels were found between untreated and treated cells nor the different concentrations tested.

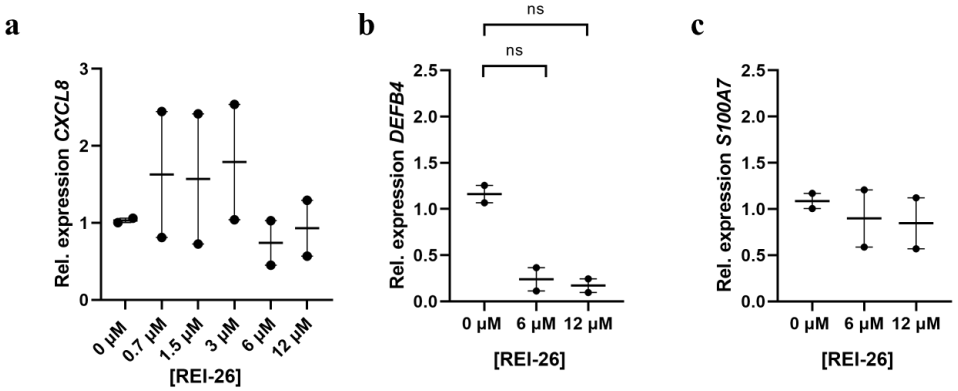

Figure 5. **mRNA levels of *CXCL8, DEFB4* and *S100A7* in REI-26 treated HaCaT cell line.** HaCaT cells were treated for 2 hours with the indicated concentration of REI-26. mRNA levels of *CXCL8* (a), *DEFB4* (b), *S100A7* (c), did not show any statistically significant differences when unpaired t-test was performed.

Secondly, the effect in the host innate immune response by CD4-PP was also analyzed. Prior to this study, a significant increase in the mRNA levels of antimicrobial peptide *S100A7* was observed when HaCaT cells were treated with 20 µM of CD4-PP for 2 hours.

Chemokine characterization had not yet been performed. Therefore, mRNA expression of the aforementioned chemoattractant CXCL8 was measured as well as for the inflammatory marker IL6. This last marker was of interest is it has been described that it also plays a key role in the modulation of skin reparative processes (43).

HaCaT cells were infected with *S. aureus* ATCC 29213 and treated with 20 µM of CD4-PP for 2 hours. mRNA levels of the different indicated chemokines were measured. An increasing trend was found in the case of *IL6* expression. When comparing the means

between treated and untreated groups, only *CXCL8* showed statistically significant increase with a p-value≤ 0.1. (*Figure 6)*

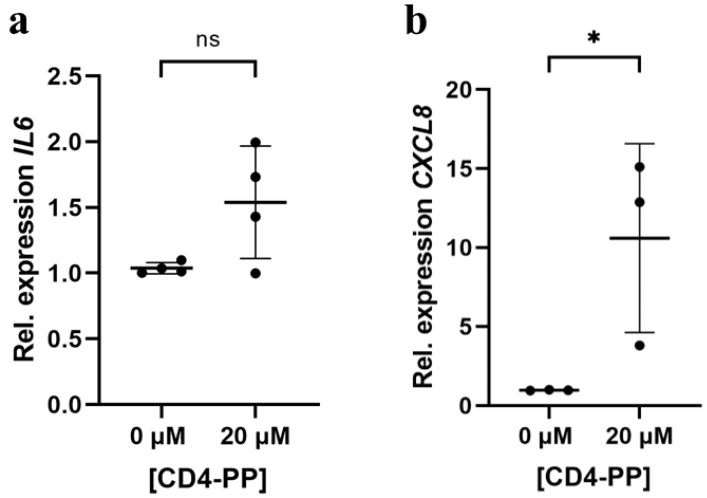

Figure 6. **mRNA levels of *CXCL8* and *IL6* in REI-26 treated keratinocytes infected with *S. aureus*.** mRNA level of *IL6* (a) showed no significant increase. *CXCL8* (b) showed significant increase with a p-value≤ 0.1. Statistical analysis was obtained by using unpaired t-tests.

Nitric oxide production of infected and treated with CD4-PP macrophages

Besides presenting phagocytic activity, macrophages are also able to eliminate intracellular bacteria by converting the amino acid arginine to nitric oxide which is highly toxic for microbes (44). Therefore, nitric oxide production was studied in RAW 264.7 cells infected with *Listeria monocytogenes* type strain ATCC 19111. RAW 264.7 macrophages were treated with 4 µM CD4-PP. Nitric oxide production was measured after 1 hour and 4 hours of infection. A significant decrease in nitric oxide production was observed when infected RAW 264.7 macrophages were treated with CD4-PP after 1 hour of infection (*Figure 7a*). Tris decreasing trend when adding CD4-PP treatment wanted to be confirmed as well in human macrophages. When analyzed, the previously described decreasing trend was not observed when dTHP-1s were infected and treated with 2 µM for 1 hour (*Figure 7b*).

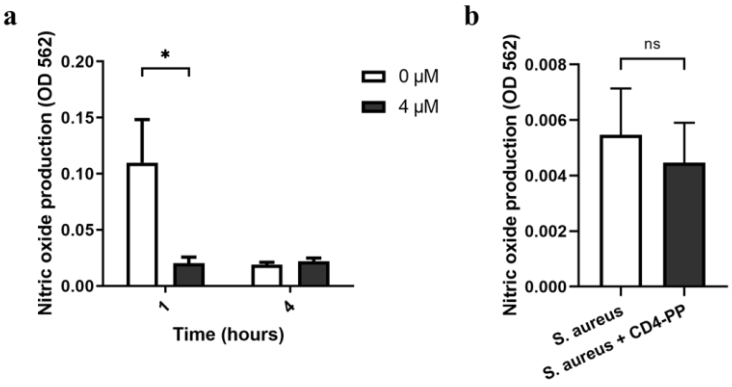

Figure 7. **Nitric oxide production in infected RAW 264.7 and dTHP-1s macrophages.** Nitric oxide production levels were measured at an optical density of 562 nm. Infected RAW 264.7 macrophages (a) showed a decrease in nitric oxide production when treated with 4 µM of CD4-PP for 1 hour, p-value≤ 0.1. This result was not observed when the experiment was performed in infected dTHP-1s macrophages (b).

CD4-PP treatment effect in proliferation markers of keratinocytes

This study is focused on beneficial effects of antimicrobial peptides in a wound context. Besides presenting antimicrobial activity, CD4-PP's beneficial impact on proliferation of keratinocytes and wound closure was also explored.

mRNA levels of keratinocyte proliferation markers *MKI67* and *VCL* were analyzed in uninfected HaCaT cells when exposed to 20 μM of CD4-PP for 6 hours. A significant decrease was observed in the case of *MKI67* with a p-value≤0.1, while no significant difference was found in the mRNA expression of *VCL*. (*Figure 8*)

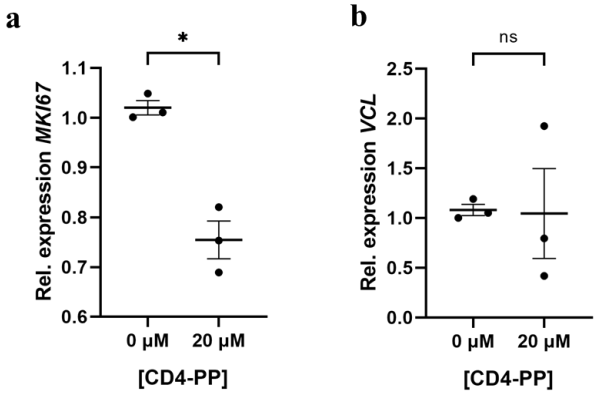

Figure 8. **mRNA levels of *MKI67* and *VCL* in treated HaCaT cells.** HaCaT cells were treated with 20 μM of CD4-PP for 6 hours. mRNA expression was compared to untreated control. mRNA levels of *MKI67* (a) decreased with p-value≤ 0.1. mRNA levels of *VCL* (b) showed a non-significant decreasing trend. The statistical test applied was unpaired t-test.

The described downregulation of the mRNA levels of these two proliferation markers, led to suggest the possibility of reduced protein levels of Ki-67 and vinculin. This was analyzed through confocal microscopy. Psoriasin (*S100A7*) has been described as an antimicrobial peptide involved in cell proliferation and keratinocyte differentiation (45,46). HaCaT cells were treated with 20 μM of CD4-PP, 1.6 μM of psoriasin and a combination of both AMPs for 24 hours. (*Figure 9*)

Cell observation through microscopy seemed to indicate a decrease in fluorescence intensity once CD4-PP was applied for both proteins, Ki-67 (green) and vinculin (magenta; *Figure 9b*). Moreover, a difference in the location of expression was also noticed for Ki-67. In the untreated control, fluorescence for this protein was mainly situated in the cell nucleus while when treated with CD4-PP the expression was displaced to the cytoplasm. In contrast to CD4-PP treatment, the fluorescence intensity of both proteins, seemed higher when using only psoriasin as treatment (*Figure 9c*). An increase in the protein expression of Ki-67 was observed when combinational treatment was used (*Figure 9d*). All visual comparisons were compared to the untreated control. Densitometry analysis of the fluorescence intensity presented the same trend as the one described in the visual analysis. Levels of nuclear Ki-67 appear to lower when treatment with CD4-PP was applied to HaCaT cells. Psoriasin seemed to upregulate the protein expression of both proteins. The recovery in protein expression was not observed when combined treatment was used. No statistically significant differences were described when comparing mean values. Significant decrease in nuclear Ki-67 and vinculin protein expression levels was observed when individual values were compared.

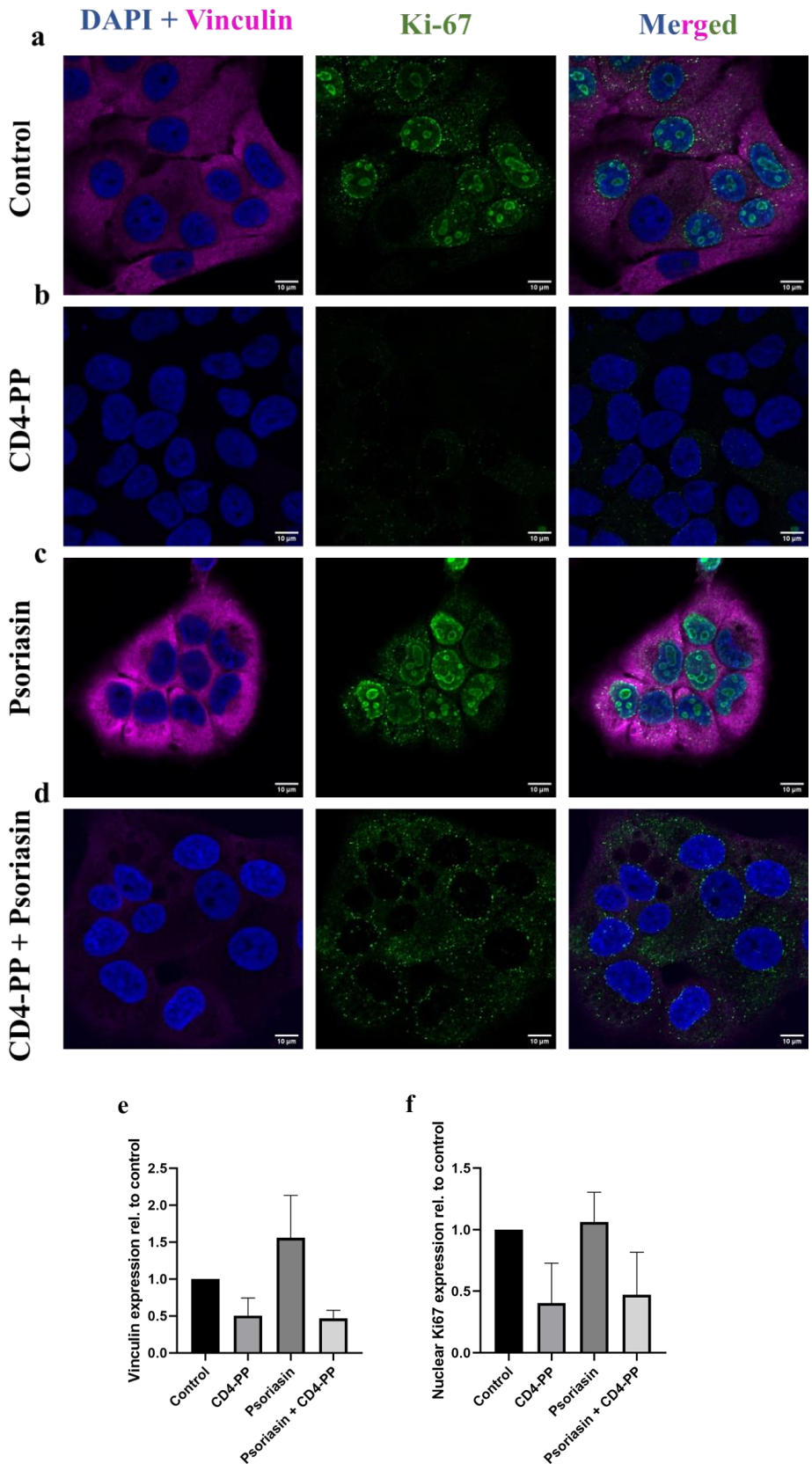

Figure 9. **Immunofluorescence of vinculin and Ki-67 in HaCaT cell line.** Representative cell images obtained from confocal microscopy with primary antibodies targeting the proteins Ki-67 and vinculin are shown. Alexa Fluor secondary antibodies were used for staining. Images obtained from untreated control (a). Decrease in Ki-67 and vinculin protein expression was observed HaCaT cells were treated with 20 µM of CD4-PP (b). Treatment with 1.6 µM of psoriasin (c) showed upregulation of the studied proteins. Faint

recovery was observed when combination treatment was used (d). Densitometry showed the same trend as observed in the representative cell images (e, f). Statistical analysis was performed by comparing the different mean groups by ANOVA Dunnett's multiple comparison test.

Discussion

Both novel AMPs showed antimicrobial activity towards common skin bacterial pathogens when MIC values measurements were performed. Treatment with AMPs of infected keratinocytes and macrophages showed decrease in bacterial load only when CD4-PP was applied. Regarding the regulation of immune factors such as AMPs and chemokines, treatment with REI-26 did not show any changes while CD4-PP presented an upregulation. The effect of CD4-PP in known proliferation markers was analyzed showing downregulation.

Here it is shown that CD4-PP is active against skin bacterial pathogens and can be considered a novel treatment towards bacterial skin infections. CD4-PP presents promising characteristics that can lead to the development of a novel way to treat bacterial skin infections. This is highly needed in the actual context where antimicrobial resistance is one of the main burdens of hospital care when treating bacterial skin infections (47,48).

Treatment with REI-26 did not show bacterial load reduction in infected cells

REI-26 did not present antimicrobial activity when it was used as treatment for infected keratinocytes and macrophages. It was then suggested that the peptide was no longer active due to the interaction with the cell culture media. This presumption was sustained by evidence of other studied AMPs which did not show antimicrobial activity once standard antimicrobial susceptibility tests where applied (49,50). The presence of negatively charged aminoacids and pH changes in the standard media used could lead to modifications in the AMPs charge. This alteration can subsequently lead to precipitation and AMP inactivation (51–53).

Moreover, REI-26 presents a linear structure while CD4-PP is cyclic. Cyclic peptides have been described to present an enhanced antimicrobial activity due to their persisting conformation. This maintained conformation improves the amphipathic interactions with the targeted negative-charged bacterial membrane resulting in a deeper insertion and the lysis of the bacterial pathogen (54,55). On the other hand, linear peptides present a less advantageous conformation towards interacting with the bacterial membrane since their structures are more flexible. No restriction in conformation leads to a lower charge density and subsequently the no disruption of the bacterial membrane (56).

CD4-PP treatment showed inhibited bacterial growth *in vitro*

Treatment with CD4-PP showed a reduction in bacterial growth when keratinocytes and macrophages where infected with all the strains. Nonetheless, HaCaT cells infected with *P. aeruginosa* and treated post-infection with CD4-PP did not show a clear reduction in bacterial load. *P. aeruginosa* has been described as a pathogen that contains multiple resistance mechanisms such as biofilm formation (16). This mechanism was considered a possible explanation towards the no effect of CD4-PP treatment. Hence, biofilm production was assessed after two hours infection and early biofilm formation was observed. This will then explain the no effective treatment by CD4-PP when added 2 hours after infection (unpublished data).

Towards a possible CD4-PP treatment, it would be important to consider the development of resistance since this has been already described for its parental peptide LL-37 in MRSA (57). Possible inactivation of CD4-PP from bacteria should be explored.

CD4-PP treatment increases mRNA levels of *IL6* and *CXCL8* in keratinocytes

IL-6 and IL-8 are inflammatory chemokines present in acute inflammatory response which lead to the differentiation of T cells and chemoattraction of neutrophils in the wound site (58,59). IL-6 can also interact with M2 macrophages and fibroblasts resulting in the activation of a fibrotic environment where proliferation and wound healing signalling is promoted in order to initiate wound closure (60). Besides interacting with neutrophils, CXCL8 can also attract fibroblasts to the wound site as well as it presents antibacterial activity (61). Therefore, the observed increase of IL-6 and CXCL8 when CD4-PP treatment was applied is coherent with what is expected in a wound context since this increase was observed after two hours of treatment. Moreover, upregulated expression levels of IL-6 and CXCL8 have been described in keratinocytes when exposed to other AMPs such as the parental LL-37 and human defensins (62).

It would be important to consider that the dysregulation of these chemokines can lead to a chronic fibrotic context causing diseases such as psoriasis (63). Thus, a longer exposure of keratinocytes to CD4-PP should be assessed as a way of describing a possible chronic effect.

Nitric oxide production is not affected by CD4-PP treatment in macrophages

A decrease in nitric oxide production was observed when CD4-PP treatment was applied to RAW 264.7 macrophages after 1 hour of infection with *L. monocytogenes*. This reduction was not observed in treated dTHP1s infected with *S. aureus*. It has been reported that nitric oxide presents antimicrobial function as well as it promotes vasodilatation and wound healing (64). Furthermore, novel therapies for wound healing involving this molecule are currently under study (65). Therefore, the no difference in nitric oxide production observed in treated dTHP1s is a positive result towards subsequent wound closure and healing.

CD4-PP treatment downregulated protein expression of Ki-67 and vinculin

Ki-67 has been described as one of the main cell proliferation markers due to its constant presence in cell nucleus throughout the cell cycle while vinculin is associated with cell adhesion and its absence indicates a decrease in cell spreading (66,67). The downregulation of both markers in keratinocytes when CD4-PP was applied is not positive towards possible wound treatment. This indicates that this novel peptide may not be suitable for the skin unless an increase of other keratinocyte-proliferative molecules is verified.

However, it is important to consider that this decrease was observed in keratinocytes exposed to treatment during 24 hours and it has been described that the migration of fibrotic cells does not occur until 2 days after injury (68,69). Moreover, a broader picture will be required to not consider this peptide as a suitable one for wound infections. Studies regarding the effect of this novel peptide in other regenerative cells such as fibroblasts and M2 macrophages should not be dismissed (70).

Future aspects and applications

In relation to collagen-based REI-26, the effect that cell culture media could have on its antimicrobial activity should be confirmed by performing survival assays with refined media (71). However, further investigations should also recognize the possible effect that this antimicrobial peptide may have in an *in vivo* context where inhibitory molecules might be present.

Regarding the promising CD4-PP peptide, the impact of this peptide in short response immune mechanisms should be assessed in other cells such as dTHP1s, T-cells and fibroblasts which play a major role in bacterial infections in wounds. Moreover, longer exposures to this peptide should also be described considering the regulation of different proliferation markers and the migration of different cells.

In order to observe the whole picture behind the CD4-PP treatment in skin bacterial infections, macrophages could be exposed to conditioned media obtained from treated infected keratinocytes (72). These macrophages will later be infected, and bacterial load would be analyzed. Another method that can be applied in this case for analyzing the induction of T cells is flow cytometry. The presence of activation markers in the cell surface may differ from treated to untreated cells (73).

Furthermore, as an intermediate step from *in vitro* to *in vivo*, the application of this peptide could be described in skin 3D bio model where migration of the different cells could be observed as well as treatment exposure could be prolonged (74).

The application of CD4-PP could be developed as a polymerized hydrogel since gel advantages comprehend an effortless application in skin, present strong adherence and can prevent quorum sensing and biofilm formation (75). In addition, hydrogels diminish the inflammation sensation by providing a cooling relief when they dissipate the heat related to inflammation (76). This gel based-form of application has already been adapted in other contexts such as implanted-devices and the use of parental LL-37, which is in Phase II of clinical trials (23,77).

Conclusions

The novel cyclic antimicrobial peptide CD4-PP presented promising results treating skin bacterial infections, with optimal antimicrobial activity in MIC values and survival assays. The expression of antimicrobial peptides and chemokine was upregulated when keratinocytes were treated with this novel AMP as expected. Further studies regarding the effect of skin application are required to assess the impact and safety of this novel molecule.

In contrast, the collagen-derived REI-26 showed limited clinical potential. No antimicrobial activity was described when REI-26 was applied as treatment to infected keratinocytes or macrophages. Recovery of its antimicrobial activity should be further analyzed.

Acknowledgements

I would like to thank Prof. Annelie Brauner for the warm welcome and for all the scientific discussions held during the progress of this project. Thank you to Dr. Soumitra Mohanty for your support and your optimism. I would also like to thank Dr. John Kerr White whose help, work and baking skills were crucial to this project. Furthermore, thank you the Giske group and the master students Natasha and Daniela, who were a big support to me.

Thank you to Prof. Ulf Göransson, Dr. Taj Muhammad and Dr. Adam Strömstedt of Uppsala University for producing both novel peptides and for giving scientific assistance when needed.

Lastly, thank you to my girls and my family who have been supporting me throughout this project and to the Vattholma people, who allowed me to disconnect when needed.

References

1. Eron LJ. Managing skin and soft tissue infections: expert panel recommendations on key decision points. Journal of Antimicrobial Chemotherapy. 2003 Nov 1;52(90001):3i–17.

2. Stieferman AE, Mazi P, Burnham JP. Severe Skin and Soft-Tissue Infections. Semin Respir Crit Care Med. 2022 Feb;43(01):003–9.

3. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, et al. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). JAMA. 2016 Feb 23;315(8):801.

4. Kumar V, Robbins SL, editors. Robbins basic pathology. 8th ed. Philadelphia, PA: Saunders/Elsevier; 2007. 946 p.

5. Hersh AL. National Trends in Ambulatory Visits and Antibiotic Prescribing for Skin and Soft-Tissue Infections. Arch Intern Med. 2008 Jul 28;168(14):1585.

6. Duane TM, Huston JM, Collom M, Beyer A, Parli S, Buckman S, et al. Surgical Infection Society 2020 Updated Guidelines on the Management of Complicated Skin and Soft Tissue Infections. Surgical Infections. 2021 May 1;22(4):383–99.

7. Endorf FW, Supple KG, Gamelli RL. The evolving characteristics and care of necrotizing soft-tissue infections. Burns. 2005 May;31(3):269–73.

8. Sartelli M, Coccolini F, Kluger Y, Agastra E, Abu-Zidan FM, Abbas AES, et al. WSES/GAIS/WSIS/SIS-E/AAST global clinical pathways for patients with skin and soft tissue infections. World J Emerg Surg. 2022 Dec;17(1):3.

9. Stevens DL, Bisno AL, Chambers HF, Dellinger EP, Goldstein EJC, Gorbach SL, et al. Practice Guidelines for the Diagnosis and Management of Skin and Soft Tissue Infections: 2014 Update by the Infectious Diseases Society of America. Clinical Infectious Diseases. 2014 Jul 15;59(2):e10–52.

10. Moet GJ, Jones RN, Biedenbach DJ, Stilwell MG, Fritsche TR. Contemporary causes of skin and soft tissue infections in North America, Latin America, and Europe: Report from the SENTRY Antimicrobial Surveillance Program (1998–2004). Diagnostic Microbiology and Infectious Disease. 2007 Jan;57(1):7–13.

11. Fung HB, Chang JY, Kuczynski S. A Practical Guide to the Treatment of Complicated Skin and Soft Tissue Infections: Drugs. 2003;63(14):1459–80.

12. Vestergaard M, Frees D, Ingmer H. Antibiotic Resistance and the MRSA Problem. Fischetti VA, Novick RP, Ferretti JJ, Portnoy DA, Braunstein M, Rood JI, editors. Microbiol Spectr. 2019 Apr 12;7(2):7.2.18.

13. Arvand M, Hoeck M, Hahn H, Wagner J. Antimicrobial resistance in Streptococcus pyogenes isolates in Berlin. Journal of Antimicrobial Chemotherapy. 2000 Oct 1;46(4):621–4.

14. Bassetti M, Baguneid M, Bouza E, Dryden M, Nathwani D, Wilcox M. European perspective and update on the management of complicated skin and soft tissue infections due to methicillin-resistant Staphylococcus aureus after more than 10 years of experience with linezolid. Clinical Microbiology and Infection. 2014 Apr;20:3–18.

15. Bowen AC, Lilliebridge RA, Tong SYC, Baird RW, Ward P, McDonald MI, et al. Is Streptococcus pyogenes Resistant or Susceptible to Trimethoprim-Sulfamethoxazole? J Clin Microbiol. 2012 Dec;50(12):4067–72.

16. Poole K. Pseudomonas Aeruginosa: Resistance to the Max. Front Microbio [Internet]. 2011 [cited 2022 May 20];2. Available from: http://journal.frontiersin.org/article/10.3389/fmicb.2011.00065/abstract

17. Lei J, Sun L, Huang S, Zhu C, Li P, He J, et al. The antimicrobial peptides and their potential clinical applications. Am J Transl Res. 2019;11(7):3919–31.

18. Magana M, Pushpanathan M, Santos AL, Leanse L, Fernandez M, Ioannidis A, et al. The value of antimicrobial peptides in the age of resistance. The Lancet Infectious Diseases. 2020 Sep;20(9):e216–30.

19. Haney EF, Mansour SC, Hancock REW. Antimicrobial Peptides: An Introduction. In: Hansen PR, editor. Antimicrobial Peptides [Internet]. New York, NY: Springer New York; 2017 [cited 2022 May 21]. p. 3–22. (Methods in Molecular Biology; vol. 1548). Available from: http://link.springer.com/10.1007/978-1-4939-6737-7_1

20. Boparai JK, Sharma PK. Mini Review on Antimicrobial Peptides, Sources, Mechanism and Recent Applications. PPL. 2019 Dec 10;27(1):4–16.

21. Ghosh A, Kar RK, Jana J, Saha A, Jana B, Krishnamoorthy J, et al. Indolicidin Targets Duplex DNA: Structural and Mechanistic Insight through a Combination of Spectroscopy and Microscopy. ChemMedChem. 2014 Sep;9(9):2052–8.

22. Hao G, Shi YH, Tang YL, Le GW. The intracellular mechanism of action on Escherichia coli of BF2-A/C, two analogues of the antimicrobial peptide Buforin 2. J Microbiol. 2013 Apr;51(2):200–6.

23. Moretta A, Scieuzo C, Petrone AM, Salvia R, Manniello MD, Franco A, et al. Antimicrobial Peptides: A New Hope in Biomedical and Pharmaceutical Fields. Front Cell Infect Microbiol. 2021 Jun 14;11:668632.

24. Wu Q, Patočka J, Kuča K. Insect Antimicrobial Peptides, a Mini Review. Toxins. 2018 Nov 8;10(11):461.

25. Piipponen M, Li D, Landén NX. The Immune Functions of Keratinocytes in Skin Wound Healing. IJMS. 2020 Nov 20;21(22):8790.

26. Petkovic M, Mouritzen MV, Mojsoska B, Jenssen H. Immunomodulatory Properties of Host Defence Peptides in Skin Wound Healing. Biomolecules. 2021 Jun 28;11(7):952.

27. de Souza GS, de Jesus Sonego L, Santos Mundim AC, de Miranda Moraes J, Sales-Campos H, Lorenzón EN. Antimicrobial-wound healing peptides: Dual-function molecules for the treatment of skin injuries. Peptides. 2022 Feb;148:170707.

28. Dürr UHN, Sudheendra US, Ramamoorthy A. LL-37, the only human member of the cathelicidin family of antimicrobial peptides. Biochimica et Biophysica Acta (BBA) - Biomembranes. 2006 Sep;1758(9):1408–25.

29. Henzler Wildman KA, Lee DK, Ramamoorthy A. Mechanism of Lipid Bilayer Disruption by the Human Antimicrobial Peptide, LL-37. Biochemistry. 2003 Jun 1;42(21):6545–58.

30. Gunasekera S, Muhammad T, Strömstedt AA, Rosengren KJ, Göransson U. Alanine and Lysine Scans of the LL-37-Derived Peptide Fragment KR-12 Reveal Key Residues for Antimicrobial Activity. ChemBioChem. 2018 May 4;19(9):931–9.

31. Gunasekera S, Muhammad T, Strömstedt AA, Rosengren KJ, Göransson U. Backbone Cyclization and Dimerization of LL-37-Derived Peptides Enhance Antimicrobial Activity and Proteolytic Stability. Front Microbiol. 2020 Feb 21;11:168.

32. Abdillahi SM, Maaß T, Kasetty G, Strömstedt AA, Baumgarten M, Tati R, et al. Collagen VI Contains Multiple Host Defense Peptides with Potent In Vivo Activity. JI. 2018 Aug 1;201(3):1007–20.

33. Abdillahi SM, Balvanovic S, Baumgarten M, Mörgelin M. Collagen VI Encodes Antimicrobial Activity: Novel Innate Host Defense Properties of the Extracellular Matrix. J Innate Immun. 2012;4(4):371–6.

34. Gara SK, Grumati P, Urciuolo A, Bonaldo P, Kobbe B, Koch M, et al. Three Novel Collagen VI Chains with High Homology to the α3 Chain. Journal of Biological Chemistry. 2008 Apr;283(16):10658–70.

35. Hamrick TS, Diaz AH, Havell EA, Horton JR, Orndorff PE. Influence of Extracellular Bactericidal Agents on Bacteria within Macrophages. Infect Immun. 2003 Feb;71(2):1016–9.

36. Eckert RL, Rorke EA. Molecular biology of keratinocyte differentiation. Environmental Health Perspectives. 1989 Mar;80:109–16.

37. Rosenblum D, Naik S. Epithelial–immune crosstalk in health and disease. Current Opinion in Genetics & Development. 2022 Jun;74:101910.

38. Pizarro-Cerdá J, Cossart P. Microbe Profile: Listeria monocytogenes: a paradigm among intracellular bacterial pathogens. Microbiology. 2019 Jul 1;165(7):719–21.

39. Roby KD, Di Nardo A. Innate immunity and the role of the antimicrobial peptide cathelicidin in inflammatory skin disease. Drug Discovery Today: Disease Mechanisms. 2013 Dec;10(3–4):e79–82.

40. Jia J, Duan Q, Guo J, Zheng Y. Psoriasin, A Multifunctional Player in Different Diseases. CPPS. 2014 Nov 28;15(8):836–42.

41. Falconer A, Ikram M, Bissett CE, Cerio R, Quinn AG, Ali RS. Expression of the Peptide Antibiotics Human β Defensin-1 and Human β Defensin-2 in Normal Human Skin. Journal of Investigative Dermatology. 2001 Jul;117(1):106–11.

42. Köhidai L, Csaba G. Chemotaxis and chemotactic selection induced with cytokines (IL-8, RANTES and TNF-alpha) in the unicellular Tetrahymena pyriformis. Cytokine. 1998 Jul;10(7):481–6.

43. Johnson BZ, Stevenson AW, Prêle CM, Fear MW, Wood FM. The Role of IL-6 in Skin Fibrosis and Cutaneous Wound Healing. Biomedicines. 2020 Apr 30;8(5):101.

44. Herb M, Schramm M. Functions of ROS in Macrophages and Antimicrobial Immunity. Antioxidants. 2021 Feb 19;10(2):313.

45. Hattori F, Kiatsurayanon C, Okumura K, Ogawa H, Ikeda S, Okamoto K, et al. The antimicrobial protein S100A7/psoriasin enhances the expression of keratinocyte differentiation markers and strengthens the skin's tight junction barrier. Br J Dermatol. 2014 Oct;171(4):742–53.

46. Nguyen HLT, Trujillo-Paez JV, Umehara Y, Yue H, Peng G, Kiatsurayanon C, et al. Role of Antimicrobial Peptides in Skin Barrier Repair in Individuals with Atopic Dermatitis. IJMS. 2020 Oct 14;21(20):7607.

47. Perera G, Hay R. A guide to antibiotic resistance in bacterial skin infections. J Eur Acad Dermatol Venerol. 2005 Sep;19(5):531–45.

48. European Food Safety Authority, European Centre for Disease Prevention and Control. The European Union Summary Report on Antimicrobial Resistance in zoonotic and indicator bacteria from humans, animals and food in 2019–2020. EFS2 [Internet]. 2022 Mar [cited 2022 May 18];20(3). Available from: https://data.europa.eu/doi/10.2903/j.efsa.2022.7209

49. Mercer DK, Torres MDT, Duay SS, Lovie E, Simpson L, von Köckritz-Blickwede M, et al. Antimicrobial Susceptibility Testing of Antimicrobial Peptides to Better Predict Efficacy. Front Cell Infect Microbiol. 2020 Jul 7;10:326.

50. Strömstedt AA, Park S, Burman R, Göransson U. Bactericidal activity of cyclotides where phosphatidylethanolamine-lipid selectivity determines antimicrobial spectra. Biochimica et Biophysica Acta (BBA) - Biomembranes. 2017 Oct;1859(10):1986–2000.

51. Alvares DS, Viegas TG, Ruggiero Neto J. Lipid-packing perturbation of model membranes by pH-responsive antimicrobial peptides. Biophys Rev. 2017 Oct;9(5):669–82.

52. Schwab U, Gilligan P, Jaynes J, Henke D. In Vitro Activities of Designed Antimicrobial Peptides against Multidrug-Resistant Cystic Fibrosis Pathogens. Antimicrob Agents Chemother. 1999 Jun;43(6):1435–40.

53. Tucker AT, Leonard SP, DuBois CD, Knauf GA, Cunningham AL, Wilke CO, et al. Discovery of Next-Generation Antimicrobials through Bacterial Self-Screening of Surface-Displayed Peptide Libraries. Cell. 2018 Jan;172(3):618-628.e13.

54. Mika JT, Moiset G, Cirac AD, Feliu L, Bardají E, Planas M, et al. Structural basis for the enhanced activity of cyclic antimicrobial peptides: The case of BPC194. Biochimica et Biophysica Acta (BBA) - Biomembranes. 2011 Sep;1808(9):2197–205.

55. Falanga A, Nigro E, De Biasi M, Daniele A, Morelli G, Galdiero S, et al. Cyclic Peptides as Novel Therapeutic Microbicides: Engineering of Human Defensin Mimetics. Molecules. 2017 Jul 20;22(7):1217.

56. Shai Y. Mode of action of membrane active antimicrobial peptides. Biopolymers. 2002;66(4):236–48.

57. Sakoulas G, Guram K, Reyes K, Nizet V, Zervos M. Human Cathelicidin LL-37 Resistance and Increased Daptomycin MIC in Methicillin-Resistant Staphylococcus aureus Strain USA600 (ST45) Are Associated with Increased Mortality in a Hospital Setting. Diekema DJ, editor. J Clin Microbiol. 2014 Jun;52(6):2172–4.

58. Yang R, Masters AR, Fortner KA, Champagne DP, Yanguas-Casás N, Silberger DJ, et al. IL-6 promotes the differentiation of a subset of naive CD8+ T cells into IL-21–producing B helper CD8+ T cells. Journal of Experimental Medicine. 2016 Oct 17;213(11):2281–91.

59. Qazi BS, Tang K, Qazi A. Recent Advances in Underlying Pathologies Provide Insight into Interleukin-8 Expression-Mediated Inflammation and Angiogenesis. International Journal of Inflammation. 2011;2011:1–13.

60. Prame Kumar K, Nicholls AJ, Wong CHY. Partners in crime: neutrophils and monocytes/macrophages in inflammation and disease. Cell Tissue Res. 2018 Mar;371(3):551–65.

61. Björstad Å, Fu H, Karlsson A, Dahlgren C, Bylund J. Interleukin-8-Derived Peptide Has Antibacterial Activity. Antimicrob Agents Chemother. 2005 Sep;49(9):3889–95.

62. Niyonsaba F, Ushio H, Nakano N, Ng W, Sayama K, Hashimoto K, et al. Antimicrobial Peptides Human β-Defensins Stimulate Epidermal Keratinocyte Migration, Proliferation and Production of Proinflammatory Cytokines and Chemokines. Journal of Investigative Dermatology. 2007 Mar;127(3):594–604.

63. Gabay C. Interleukin-6 and chronic inflammation. Arthritis Res Ther. 2006;8 Suppl 2:S3.

64. Luo J dong, Chen AF. Nitric oxide: a newly discovered function on wound healing. Acta Pharmacologica Sinica. 2005 Mar;26(3):259–64.

65. Pinto RV, Carvalho S, Antunes F, Pires J, Pinto ML. Emerging Nitric Oxide and Hydrogen Sulfide Releasing Carriers for Skin Wound Healing Therapy. ChemMedChem [Internet]. 2022 Jan 5 [cited 2022 May 20];17(1). Available from: https://onlinelibrary.wiley.com/doi/10.1002/cmdc.202100429

66. Goldmann WH, Ingber DE. Intact Vinculin Protein Is Required for Control of Cell Shape, Cell Mechanics, and rac-Dependent Lamellipodia Formation. Biochemical and Biophysical Research Communications. 2002 Jan;290(2):749–55.

67. Scholzen T, Gerdes J. The Ki-67 protein: From the known and the unknown. J Cell Phys. 2000 Mar;182(3):311–22.

68. Martin P, Leibovich SJ. Inflammatory cells during wound repair: the good, the bad and the ugly. Trends in Cell Biology. 2005 Nov;15(11):599–607.

69. Gurtner GC, Werner S, Barrandon Y, Longaker MT. Wound repair and regeneration. Nature. 2008 May 15;453(7193):314–21.

70. Opalenik SR, Davidson JM. Fibroblast differentiation of bone marrow-derived cells during wound repair. FASEB j. 2005 Sep;19(11):1561–3.

71. Turner J, Cho Y, Dinh NN, Waring AJ, Lehrer RI. Activities of LL-37, a Cathelin-Associated Antimicrobial Peptide of Human Neutrophils. Antimicrob Agents Chemother. 1998 Sep;42(9):2206–14.

72. Villarreal-Ponce A, Tiruneh MW, Lee J, Guerrero-Juarez CF, Kuhn J, David JA, et al. Keratinocyte-Macrophage Crosstalk by the Nrf2/Ccl2/EGF Signaling Axis Orchestrates Tissue Repair. Cell Reports. 2020 Nov;33(8):108417.

73. Horn S, Ahmed MIM, Geldmacher C, Marandu TF, Osei-Mensah J, Debrah A, et al. Flow cytometric analysis of cell lineage and immune activation markers using minimal amounts of human whole blood—Field method for remote settings. Journal of Immunological Methods. 2021 Apr;491:112989.

74. Randall MJ, Jüngel A, Rimann M, Wuertz-Kozak K. Advances in the Biofabrication of 3D Skin in vitro: Healthy and Pathological Models. Front Bioeng Biotechnol. 2018 Oct 31;6:154.

75. Manna S, Ghosh AK, Mandal SM. Curd-Peptide Based Novel Hydrogel Inhibits Biofilm Formation, Quorum Sensing, Swimming Mortility of Multi-Antibiotic

Resistant Clinical Isolates and Accelerates Wound Healing Activity. Front Microbiol. 2019 May 14;10:951.

76. Goodwin NS, Spinks A, Wasiak J. The efficacy of hydrogel dressings as a first aid measure for burn wound management in the pre-hospital setting: a systematic review of the literature: The efficacy of hydrogel dressings as a first aid measure for burns in the pre-hospital setting. Int Wound J. 2016 Aug;13(4):519–25.

77. Mateescu M, Baixe S, Garnier T, Jierry L, Ball V, Haikel Y, et al. Antibacterial Peptide-Based Gel for Prevention of Medical Implanted-Device Infection. Shih C, editor. PLoS ONE. 2015 Dec 14;10(12):e0145143.

Supplementary

Figure S1. **Cytotoxicity of cell line dTHP-1 for REI-26 and cell lines HaCaT and RAW 264.7 macrophages for CD4-PP**

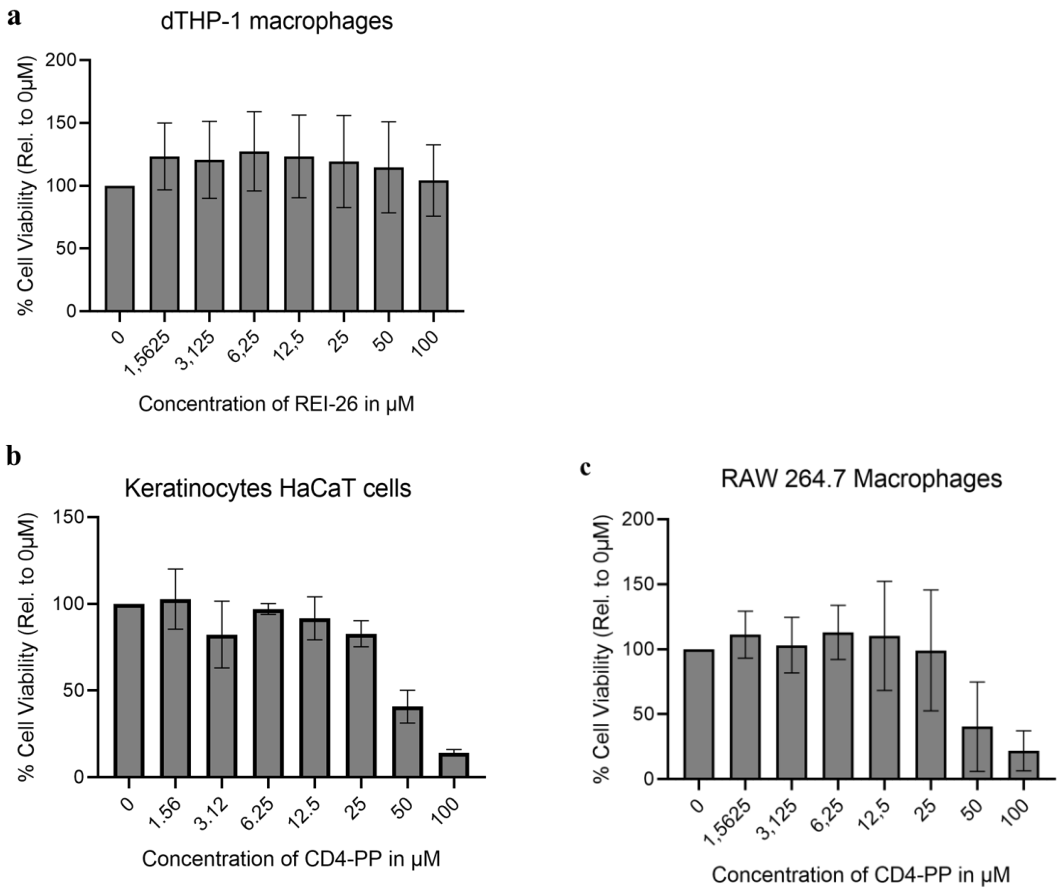

Figure S1a depicts the cytotoxicity of differentiated macrophages cells line THP-1 when treated with 2-fold dilutions of REI-26. No cytotoxicity was observed. Figure S1b and c show the cytotoxicity of HaCaT cells and RAW 264.7 macrophages when treated with 2-fold dilutions of CD4-PP. Effect in cell viability was observed at a concentration of 50 µM.

***Figure S2*. Phagocytosis assay of infected RAW 264.7 macrophages treated with CD4-PP**

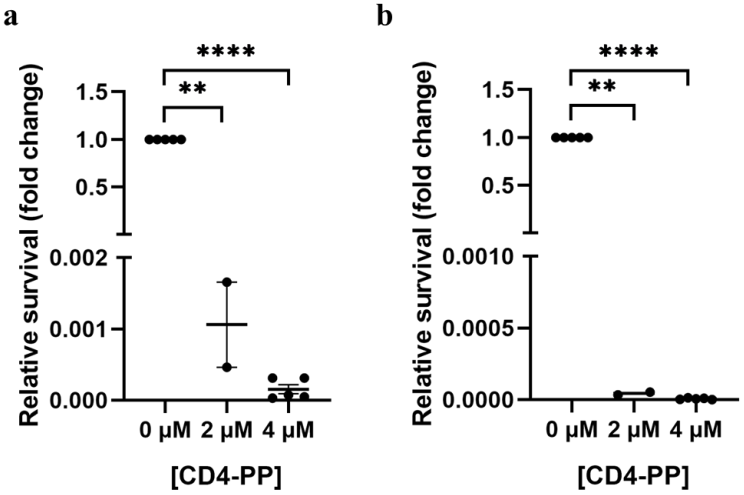

Figure S2 depicts the phagocytosis assay performed in infected RAW 264.7 macrophages with *L. monocytogenes* type strain. Figure 4a shows the effect of treatment for 1 hour and the effect of the different concentrations. 2 µM treatment presented a p-value ≤ 0.01 while 4 µM treatment presented a p-value ≤ 0.001. Figure 4b portrays the 4-hour CD4PP treatment effect in intracellular bacteria. A p-value ≤ 0.01 was observed in the case of 2 µM treatment and 4 µM treatment showed a p-value ≤ 0.001.